Supplementary Material

Methods

Virus Strains used in the study. The DENV strains used for the neutralization assay, preparing antigens for depletions and ELISA binding assays are DENV1 WestPac-74, DENV2 S16803, DENV3 CH53489 and DENV4 TVP-376. The DENV envelopes in Dengvaxia were derived from DENV1 Thailand PUO-359, DENV2 Thailand PUO-218, DENV3 Thailand PaH881/88 and DENV4 Indonesia 1228.

Serum Sample Collection Dengvaxia phase III clinical trials in Asia (CYD14) and Latin America (CYD15) were previously described(1, 2). In brief, CYD14 was conducted in five countries in Asia where 2-14 years old healthy children were enrolled, while CYD15 was conducted in four countries in Latin America and Puerto Rico, enrolling 9-16 years old healthy children. Both studies were randomized 2:1 where participants received three doses of either the vaccine or placebo at months 0, 6 and 12 of the study. Sera was drawn in month 13 of clinical trial, one month after completion of three dose Dengvaxia regimen. Baseline blood samples were collected from approximately 10% of children before vaccination. The serostatus of these children before vaccination was determined by testing the baseline blood samples for DENV neutralizing antibodies. For the remaining children with no baseline blood samples, a DENV NS1 antibody test was used to determine serostatus. As Dengvaxia does not contain the DENV NS1 protein, any child with DENV NS1 antibodies at the termination of vaccination was designated as baseline DENV-seropositive. The development, validation and use of this assay to determine

serostatus has already been described (3). Supplementary Table 1 indicates how baseline serostatus was determined for the subjects analyzed for the current study.
The natural infection serum samples were collected from US residing healthy adults, who experienced a dengue infection while traveling to a dengue endemic country.

Antibody Depletion Depletion studies were performed as previously described.(4) Dynabeads were covalently linked to anti-E MAb 1M7 for 18 hrs at 37°C. Complex was subsequently blocked with 1% BSA in PBS solution at 37°C and washed with 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer. Beads were incubated with purified virus serotypes separately. Virion/bead/MAb complex was then washed with PBS. DENV specific antibodies were depleted from sera by incubating virus/bead complex with sera diluted at 1:10 in PBS for 1 hr at 37°C with end over end mixing for three sequential rounds of depletion. Removal of all antibodies binding to the depleting viral antigen was confirmed by ELISA. All Ab depletion experiments to characterize NAbs to a particular serotype included the following experimental groups: A) a control depletion group (sera incubated with beads containing no DENV antigen) to measure total level of NAb to the serotype; B) a complete Ab depletion group (sera incubated with beads containing the homologous DENV serotype) to measure loss of NAb following removal of all virus binding (TS and CR) Abs; C) a CR Ab depletion group (sera incubated with beads containing one or more heterologous DENV serotype) to measure levels of NAb after removal of CR Ab only. DENV do not grow to very high titers in cell culture and purification viral antigen for depletion is a laborious and expensive process. We did not have sufficient viral antigen to consistently use all three heterologous serotypes for removing CR Ab.

The heterologous serotypes selected to deplete CR Abs for each study was based on the availability of purified viral antigens in the laboratory. As CR Abs bind to epitopes that are conserved across all 4 DENV serotypes, efficient depletion is likely to mainly depend on the quantity of heterologous DENV antigen used and not the number of heterologous serotypes. To directly evaluate the impact of using one versus three heterologous serotypes for removing CR Abs, we measured levels of DENV4 TS NAbs in 13 vaccinated subjects after removing CR Abs with DENV2 alone or a mix of DENV1, 2 and 3. Both approaches led to similar estimates of DENV4 TS NAbs in each subject (Supplementary Figure 5), validating the use of one or more than one heterologous serotype for removing CR Abs.

Enzyme-Linked Immunosorbent Assay (ELISA) To confirm successful depletion of a certain DENV specific population of Abs, IgG binding ELISA was conducted as previously described. (5) Purified DENV was either directly coated or captured using a DENV specific monoclonal Ab on a 96-well ELISA plate, the plate was then blocked using either 3% Normal Goat Serum (NGS) or 3% Non-Fat Dry Milk (NFDM) respectively to eliminate any nonspecific binding. The depleted serum sample is then added at 1:20 dilution and incubated at 37°C for an hour, then washed off. Binding was evaluated by 1 hour incubation with secondary anti-human-alkaline phosphatase conjugated Ab (Sigma A9544) at 37°C, which is then washed off. *P*-nitrophenyl phosphate substrate is then added and the Optical Density (OD) is measured at 405nm. In a successful depletion of a dengue experienced serum sample, the OD in the control depleted sample should be high (≥1) and the OD in the homologous depleted sample should be close to background

or Normal Human Serum (NHS) level. If that sample has Abs that are specific to a particular serotype, the OD in the heterologous depleted sample should be higher than the NHS and background level. Limit of detection was defined as average of normal human sera + 3x standard deviation.

Focus Reduction Neutralization Test (FRNT) The neutralization tests were conducted in 96-well format on Vero-81 cells (ATCC CCL-81). $2x10^4$ cells were seeded overnight. On the day of the assay, virus stocks were diluted in dilution media (Dulbecco modified Eagle medium (DMEM) with 2% Fetal Bovine Serum (FBS)) to achieve 60-80 foci/well in the virus + cells only wells. Separately the serum samples to be tested were serially diluted three folds starting at 1:10 in the same dilution media. The diluted virus is then added to the serum in a 1:1 ratio making the final starting dilution of serum at 1:20 and the complex is then incubated at 37°C for 1 hour before being added to the cells and incubated for another hour at 37°C. The cells were then washed with the dilution media and Opti-MEM (Gibco) supplemented with 2% FBS, 1% Anti-Anti (Gibco) and 1% Carboxymethylcellulose is added and cells are incubated at 37°C for 45-52 hours before fixing using 4% PFA. The reported EC_{50} values were calculated using variable slope-sigmoidal dose response equation using GraphPad Prism 8. All reported results were subjected to our quality control parameters of $R^2 \geq 0.75$, a hill slope of $|\geq 5|$ and the calculated EC_{50} value should be within the range of the assay. All values that did not meet these standards were assigned the baseline value.

Supplementary Tables

Supplementary Table 1. Wild type DENV1 and DENV3 infection specimens					
Sample	**Country of Infection**	**Infecting Virus**	**Year of Infection**	**Collection Year**	**Interval between infection and sample collection (Years)**
WT DV1-1	India	DENV1	2007	2009	2
WT DV1-2	Ecuador		2006	2014	8
WT DV1-3	Bolivia		2012	2014	2
WT DV1-4	India		1991	2015	24
WT DV1-5	Unknown		Unknown	Unknown	Unknown
WT DV1-6	Unknown		Unknown	Unknown	Unknown
WT DV1-7	Virgin Islands		1982-1995	2005	13
WT DV1-8	Brazil		1998	2005	7
WT DV1-9	Dominican Republic		2004	2005	1
WT DV1-10	Guyana		2010	2014	4
WT DV1-11	Malaysia		2008	2016	8
WT DV3-1	Unknown	DENV3	Unknown	Unknown	Unknown
WT DV3-2	Unknown		Unknown	Unknown	Unknown
WT DV3-3	Nicaragua		1995	2009	14
WT DV3-4	Thailand		2002	2009	7
WT DV3-5	Sri Lanka		2008	2009	1
WT DV3-6	Nicaragua		2009	2010	1
WT DV3-7	Sri Lanka		2011	2012	1
WT DV3-8	Nicaragua		1998	2016	18

Supplementary Table 2. Dengvaxia Breakthrough Infection Specimens

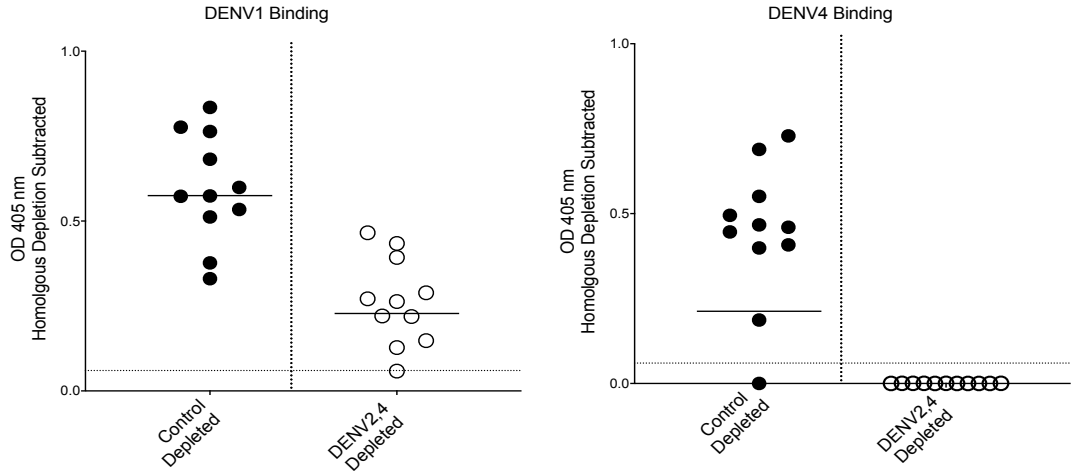
Sample ID	Country of Origin	Assay to determine DENV serostatus before vaccination	Days between vaccination and DENV infection	Infecting DENV serotype
DV1-1	Colombia	NS1 Ab ELISA	157	DENV1
DV1-2	Colombia	NS1 Ab ELISA	112	
DV1-3	Philippines	NS1 Ab ELISA	1108	
DV1-4	Philippines	NS1 Ab ELISA	130	
DV1-5	Philippines	NS1 Ab ELISA	308	
DV1-6	Philippines	NS1 Ab ELISA	336	
DV1-7	Thailand	Neutralization test	972	
DV1-8	Thailand	NS1 Ab ELISA	994	
DV1-9	Mexico	NS1 Ab ELISA	46	
DV1-10	Mexico	NS1 Ab ELISA	714	
DV1-11	Mexico	NS1 Ab ELISA	263	
DV1-12	Mexico	NS1 Ab ELISA	243	
DV1-13	Vietnam	NS1 Ab ELISA	502	
DV1-14	Vietnam	NS1 Ab ELISA	264	
DV1-15	Vietnam	NS1 Ab ELISA	1003	
DV3-1	Colombia	NS1 Ab ELISA	213	DENV3
DV3-2	Colombia	NS1 Ab ELISA	150	
DV3-3	Colombia	NS1 Ab ELISA	74	
DV3-4	Colombia	NS1 Ab ELISA	58	
DV3-5	Colombia	NS1 Ab ELISA	339	
DV3-6	Colombia	NS1 Ab ELISA	93	
DV3-7	Colombia	NS1 Ab ELISA	155	
DV3-8	Colombia	NS1 Ab ELISA	359	
DV3-9	Colombia	NS1 Ab ELISA	662	
DV3-10	Colombia	NS1 Ab ELISA	47	
DV3-11	Honduras	NS1 Ab ELISA	411	
DV3-12	Honduras	NS1 Ab ELISA	337	
DV3-13	Honduras	NS1 Ab ELISA	359	
DV3-14	Philippines	NS1 Ab ELISA	1103	
DV3-15	Thailand	NS1 Ab ELISA	316	
DV3-16	Thailand	NS1 Ab ELISA	960	
DV3-17	Thailand	Neutralization test	1047	
DV3-18	Vietnam	Neutralization test	398	

Supplementary Table 3. Percentage of DENV serotype specific antibodies in Dengvaxia recipients

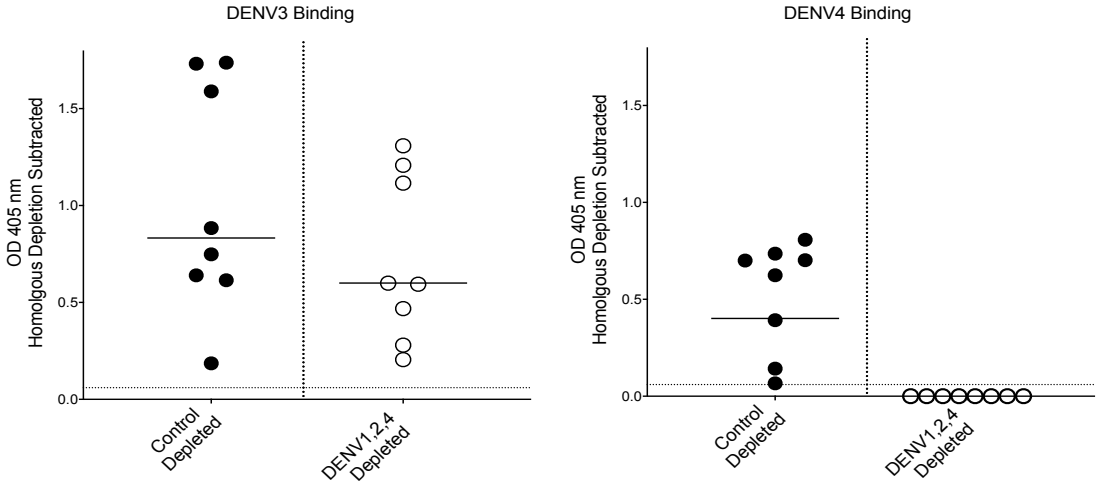
Subject	Breakthrough Infection	Dep Strategy	% DV1 TS-Ab	% DV3 TS-Ab	% DV4 TS-Ab
C-1	None	BSA, DV1/2, DV3/4	0	**25**	0
C-2		BSA, DV1/2, DV3/4	0	0	**35**
C-3		BSA, DV1/2, DV3/4	0	0	0
C-4		BSA, DV1/2, DV3/4	0	0	**46**
C-5		BSA, DV1/2, DV3/4	0	0	0
C-6		BSA, DV1/2, DV3/4	0	0	**37**
C-7		BSA, DV1/2, DV3/4	0	0	**51**
C-8		BSA, DV1/2, DV3/4	0	**17**	0
C-9		BSA, DV1/2, DV3/4	**14**	0	**48**
C-10		BSA, DV1/2, DV3/4	0	**52**	0
C-11		BSA, DV1/2, DV3/4	**81**	0	0
DV3-1	DENV3	BSA, DV3, DV1/2/4	**34**	**61**	**35**
DV3-2		BSA, DV3, DV1/2/4	**44**	0	**11**
DV3-3		BSA, DV3, DV1/2/4	0	0	**42**
DV3-4		BSA, DV3, DV1/2/4	**10**	0	0
DV3-5		BSA, DV3, DV1/2/4	0	0	**100**
DV3-6		BSA, DV3, DV1/2/4	0	0	**56**
DV3-7		BSA, DV3, DV1/2/4	0	0	**30**
DV3-8		BSA, DV3, DV1/2/4	**16**	**42**	**23**
DV3-9		BSA, DV3, DV1/2/4	0	0	**64**
DV3-10		BSA, DV3, DV1/2/4	0	0	**30**
DV3-11		BSA, DV3, DV1/2/4	0	0	0
DV3-12		BSA, DV3, DV1/2/4	0	0	**15**
DV3-13		BSA, DV3, DV1/2/4	0	**35**	**20**
DV3-14		BSA, DV3, DV1/2/4	0	0	**56**
DV3-15		BSA, DV3, DV1/2/4	**20**	0	**22**
DV3-16		BSA, DV3, DV1/2/4	0	0	0
DV3-17		BSA, DV3, DV1/2/4	**49**	0	0
DV3-18		BSA, DV3, DV1/2/4	0	**33**	**22**
DV1-1	DENV1	BSA, DV1, DV2/4	0	NT	0
DV1-2		BSA, DV1, DV2/4	0	NT	0
DV1-3		BSA, DV1, DV2/4	0	NT	0
DV1-4		BSA, DV1, DV2/4	0	NT	**38**
DV1-5		BSA, DV1, DV2/4	0	NT	0
DV1-6		BSA, DV1, DV2/4	0	NT	**100**
DV1-7		BSA, DV1, DV2/4	0	NT	**22**
DV1-8		BSA, DV1, DV2/4	**29**	NT	**15**
DV1-9		BSA, DV1, DV2/4	0	NT	0
DV1-10		BSA, DV1, DV2/4	0	NT	**22**
DV1-11		BSA, DV1, DV2/4	0	NT	**71**
DV1-12		BSA, DV1, DV2/4	0	NT	**55**
DV1-13		BSA, DV1, DV2/4	**29**	NT	**52**
DV1-14		BSA, DV1, DV2/4	**34**	NT	**100**
DV1-15		BSA, DV1, DV2/4	**42**	NT	0

Supplementary Figures

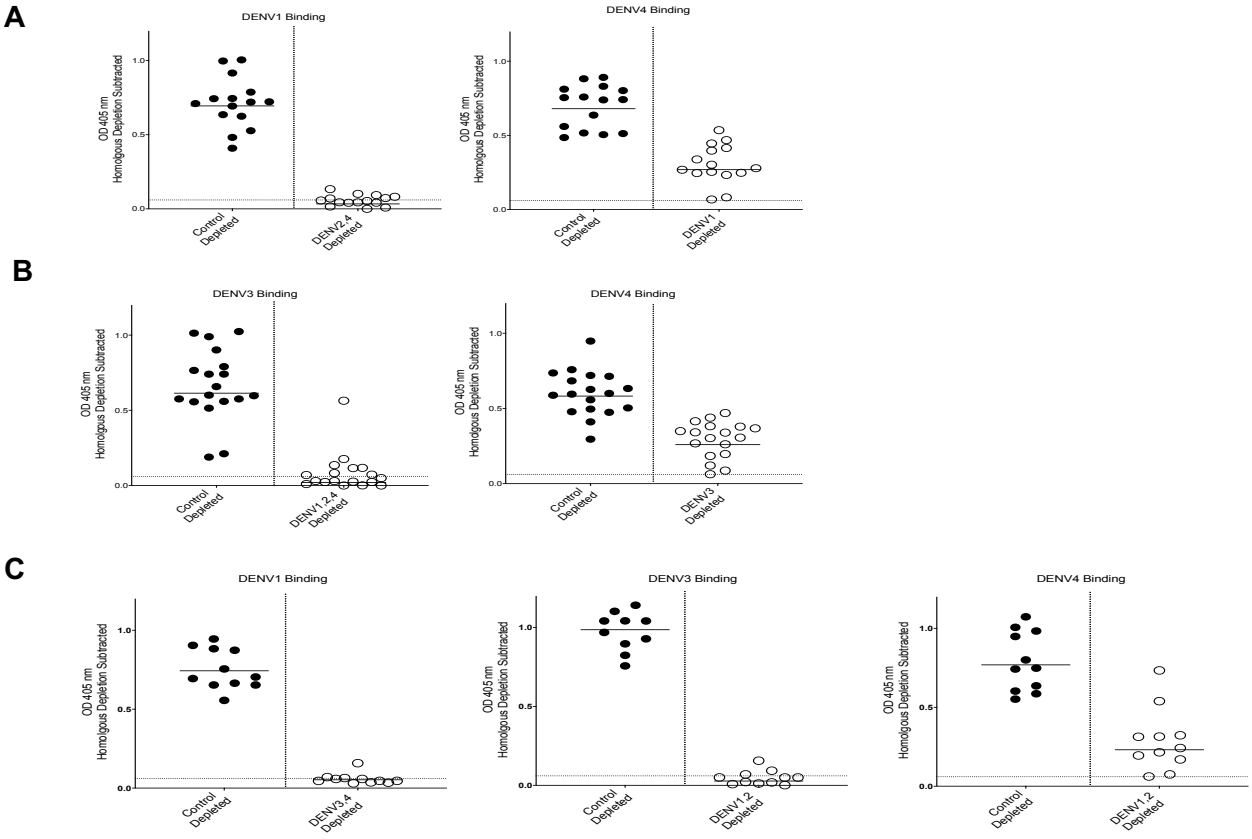
A) Primary DENV1 Natural Infection



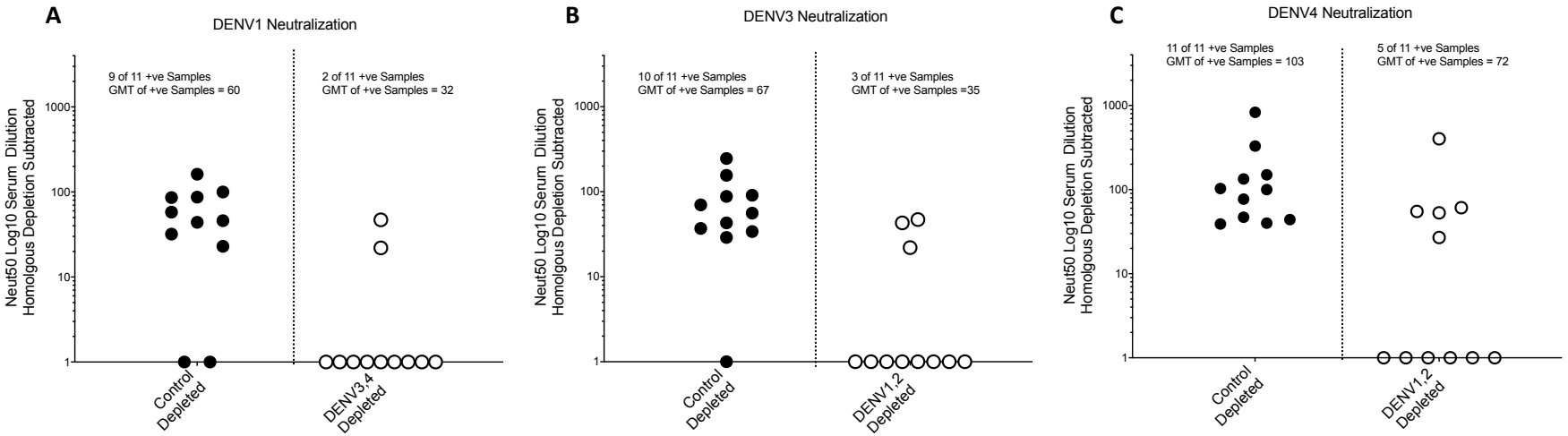
B) Primary DENV3 Natural Infection



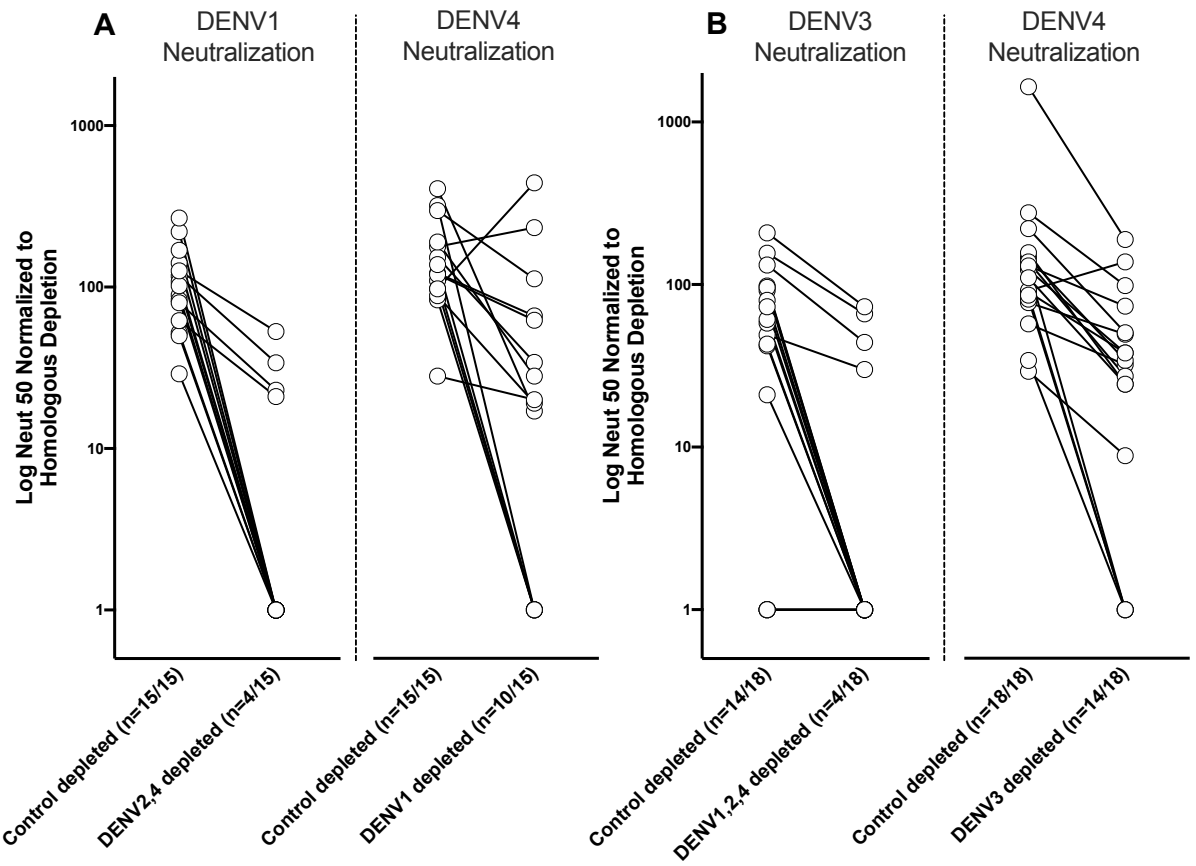
<u>Supplementary Figure 1: Depletion of DENV serotype cross-reactive antibodies in sera from people exposed to primary DENV1 or DENV3 infections.</u> Convalescent sera from people exposed to primary **(A)** DENV1 or **(B)** DENV3 infections were incubated with beads coated with heterologous DENV serotypes to remove serotype CR Abs. A mix of DENV 2 and 4 antigens were used to remove CR Abs in DENV1 immune sera and a mix of DENV1, 2, and 4 antigens was used to remove CR Abs in DENV3 immune sera. The depleted sera were tested by ELISA for Abs binding to a heterologous serotype (DENV4) to confirm removal of CR Abs and to the homologous serotype (DENV1 or DENV3) to estimate levels of TS binding Abs. Limit of detection (dashed dotted line) was defined as average of normal human sera + standard deviation multiplied by 3.



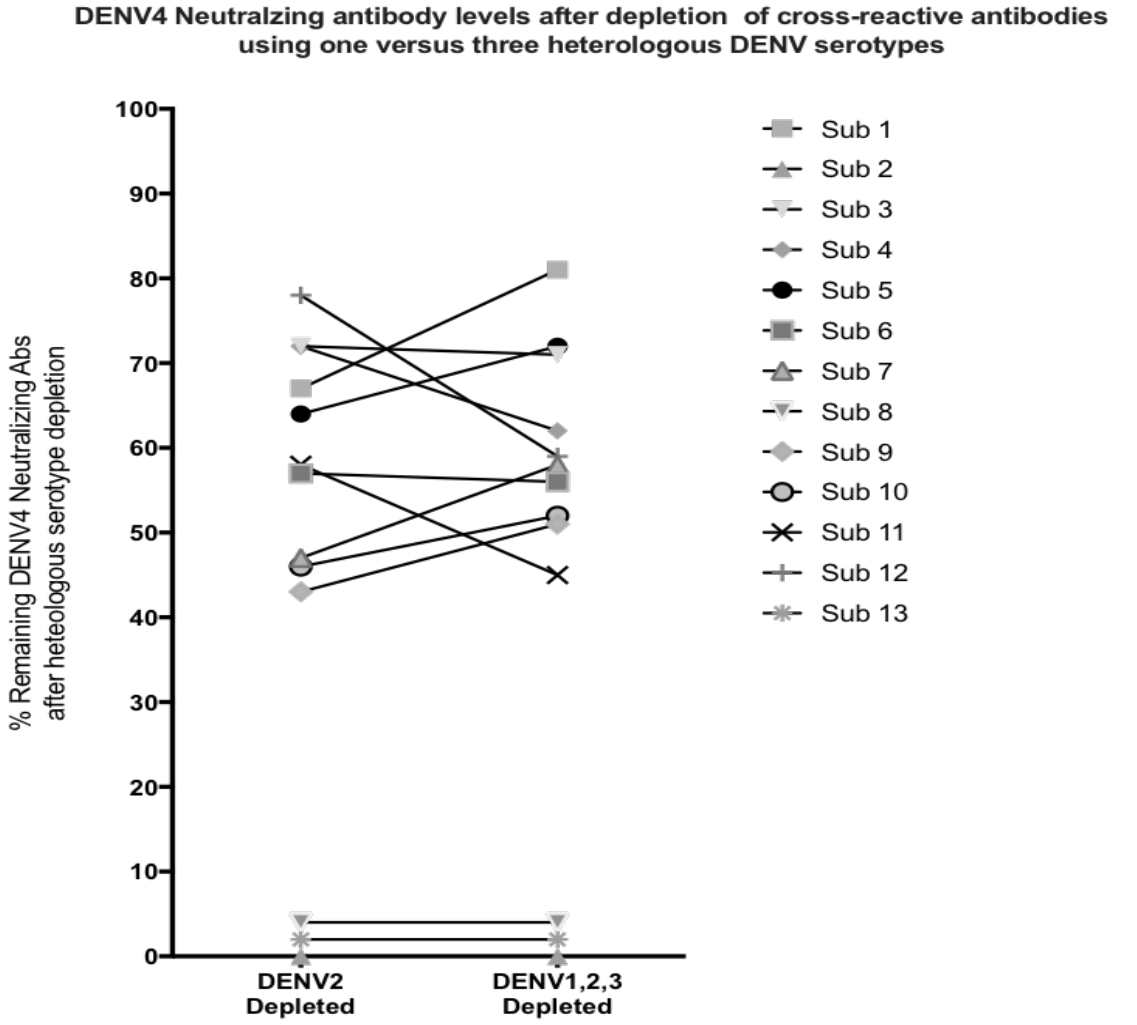
Supplementary Figure 2: Depletion of DENV-specific antibody populations from immune sera collected from dengue naïve children who received Dengvaxia. Vaccine immune sera from children who subsequently experienced DENV1 breakthrough infections **(A)**, DENV3 breakthrough infections **(B)** or no DENV infections **(C)** were incubated with beads coated with different DENV serotypes to deplete specific Ab populations. The depleted sera were tested by ELISA to estimate levels of TS binding antibodies to DENV1, DENV3 and DENV4. Limit of detection (dashed dotted line) was defined as average of normal human sera + standard deviation multiplied by 3.



<u>Supplementary Figure 3: Vaccine induced neutralizing antibody responses in children with no documented DENV breakthrough infections</u>. Vaccine responses were analyzed in baseline seronegative children (n = 11) who received Dengvaxia and did not experience breakthrough infections during the clinical trial. The sera were tested to measure levels of total and TS NAbs to DENV1 **(A)**, DENV3 **(B)** and DENV4 **(C).**



Supplementary Figure 4. Individual subject level analysis of vaccine stimulated total and DENV serotype-specific NAbs in children who subsequently experienced breakthrough infections. Vaccine responses were analyzed in children who received Dengvaxia and subsequently experienced DENV1 **(A)** or DENV3 **(B)** breakthrough infections. **(A)** In children who experienced DENV1 breakthrough infections (N=15), DENV1 NAb responses after vaccination were measured without depleting any antibody (Control Depleted) and after removal of CR Ab (DENV2,4 depleted). DENV4 NAb responses after vaccination were measured without depleting any antibody (Control Depleted) and after removal of CR Ab (DENV1 depleted). **(B)** In children who experienced DENV3 breakthrough infections (N=18), DENV3 NAb responses after vaccination were measured without depleting any antibody (Control Depleted) and after removal of CR Ab (DENV1, 2, 4 depleted). DENV4 NAb responses after vaccination were measured without depleting any antibody (Control Depleted) and after removal of CR Ab (DENV3 depleted). n= x/x denotes number with neutralizing antibody over total number of subjects.



Supplementary Figure 5: Impact of depleting vaccine induced antibodies using one or three heterologous DENV serotypes on DENV4 neutralization. Immune serum was collected from 13 baseline seronegative subjects one month after receiving the final dose of Dengvaxia (Clinical trial CYD 17). The sera were depleted of heterologous antibodies (in relation to DENV4) using DENV2 alone or a mixture of DENV1,2 and 3 antigens. Control, and antibody depleted sera were tested for neutralization of DENV4. Ten of the 13 subjects retained >40% of DENV4 neutralizing antibodies, confirming the high frequency of DENV4 type-specific responses after vaccination. Removal of cross-reactive antibodies using one or all three heterologous serotypes yielded similar estimates for levels of DENV4 type-specific neutralizing antibodies in each subject.

1. Capeding MR, Tran NH, Hadinegoro SR, Ismail HI, Chotpitayasunondh T, Chua MN, et al. Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. *Lancet.* 2014;384(9951):1358-65.
2. Hadinegoro SR, Arredondo-Garcia JL, Capeding MR, Deseda C, Chotpitayasunondh T, Dietze R, et al. Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. *N Engl J Med.* 2015;373(13):1195-206.
3. Sridhar S, Luedtke A, Langevin E, Zhu M, Bonaparte M, Machabert T, et al. Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. *N Engl J Med.* 2018;379(4):327-40.
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5. de Alwis R, Beltramello M, Messer WB, Sukupolvi-Petty S, Wahala WM, Kraus A, et al. In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection. *PLoS Negl Trop Dis.* 2011;5(6):e1188.