Delineating the serotype-specific neutralizing antibody response to a live attenuated tetravalent dengue vaccine

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ABSTRACT

The dengue virus (DENV) vaccines that are licensed or in clinical development consist of DENV serotype 1–4 tetravalent formulations given simultaneously and are not acquired sequentially like natural infections. It is unclear what effect this has on development of protective levels of immunity to all four serotypes. Serotype-specific neutralizing antibody (NAb) is considered the most relevant correlate of protection from dengue disease. Here we assessed levels of serotype-specific and cross-reactive NAb in immune sera from 10 subjects vaccinated with a live attenuated tetravalent DENV vaccine developed at the Walter Reed Army Institute of Research. The majority of subjects NAb responses to DENV-2 and DENV-4 were type-specific, while their NAb responses to DENV-1 and DENV-3 were primarily cross-reactive. Vaccine virus RNAemia has been most frequently detected for DENV-2 and DENV-4 in vaccinated subjects, strongly suggesting that replication is important for eliciting serotype-specific immunity.

1. Introduction

Dengue virus (DENV) serotypes 1–4 are of significant importance to public health in endemic countries, which are widely distributed in tropical and subtropical areas of the world. It has been estimated that 390 million DENV infections occur annually [1]. Dengue vaccine development has been complicated by co-circulation of all four DENV serotypes in endemic countries and the possibility of sequential infections with different serotypes causing more severe disease, which necessitates a tetravalent vaccine for simultaneous protection against all 4 serotypes. Historical evidence suggests that infection with any one DENV serotype will provide life-long immunity to that serotype and short-term cross-protection against the heterologous DENV serotypes that lasts only a few months [2]. This paradigm has been challenged by more recent studies indicating that homologous re-infections, although rare, can occur in some people [3]. The immune responses to post-primary DENV infections display an anamnestic response that is broadly cross-reactive among DENV serotypes and longer periods of cross-protection are observed [4–7].

The DENV vaccines that are currently licensed or in clinical development are tetravalent formulations given simultaneously

and DENV 1–4 are not acquired sequentially like they are in endemic areas. It is unclear what effect this has on development of protective levels of immunity in humans. Furthermore, neutralizing antibody (NAb) measured by the plaque reduction neutralization test (PRNT) is considered the most relevant correlate of protection and is routinely used to evaluate vaccine efficacy. However, only moderate clinical efficacy in phase III trials was observed for recipients of the CYD-TDV vaccine (Sanofi Pasteur) who had balanced tetravalent NAb responses [8–10]. This indicates that our understanding of NABs as a correlate of protection needs to be refined.

The DENV envelope protein is the major target of neutralizing antibodies [11]. Potently neutralizing human NABs have been mapped to epitopes on the E protein that link multiple E protein monomers [12–18]. The presence of serotype-specific NAb is evidence of previous exposure to a specific DENV serotype and if it were elicited by natural or experimental infection then it would be expected to correlate with protection against homologous reinfection, as previous human studies have demonstrated [2,19]. Cross-reactive NAb is not necessarily an indicator of prior exposure to any specific serotypes but has been shown to reduce the risk of disease from heterologous DENV infection [20]. Recently, virus-specific serum antibody depletion methods have been developed to determine the contribution made by DENV type-specific and cross-reactive antibodies to virus neutralization in vitro after primary and secondary infections or immunizations [7,12,19,21].

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After a primary DENV infection, serotype-specific antibodies contribute most to neutralizing activity against the infecting serotype, while cross-reactive antibodies contribute less to neutralization and can mediate enhancement of heterologous DENV infection in cell culture and in mice [22]. The proportion of cross-reactive antibodies that contribute to virus neutralization appeared to increase after secondary DENV infection or immunization [7,21]. After immunization with the CYD-TDV vaccine, a combination of all four serotypes delivered simultaneously, subjects had type-specific NAb primarily to DENV-4 and cross-reactive NAb to DENV 1–3 [19]. This bias was hypothesized to be the result of better replication of the DENV-4 vaccine component in most subjects.

The live attenuated CYD-TDV vaccine has been licensed for use in several endemic countries (trademarked as Dengvaxia) including Brazil, El Salvador, Mexico, Paraguay, and the Philippines [23–26]. However, the CYD-TDV vaccine had only moderate efficacy in DENV seronegative individuals, limiting its utility in these populations. Two other live attenuated vaccines are in clinical development. The live attenuated TV003/TV005 vaccine (US National Institutes of Health [NIH]) is in phase III development and has been licensed to Merck, and companies in Brazil (Instituto Butantan), India (Panacea Biotech and Serum Institute of India), and Vietnam (Vabiotech) [27–31]. The TDV vaccine (Takeda) is currently undergoing phase III studies [32–35].

A live attenuated tetravalent dengue vaccine was developed at Walter Reed Army Institute of Research (WRAIR) in collaboration with GlaxoSmithKline (GSK) [36–38]. The DENV 1–4 live attenuated vaccine (TDENV) viruses developed at WRAIR were attenuated by serial passage in primary dog kidney (PDK) cells [38–50]. A safe and immunogenic formulation of TDENV was selected based on a phase II clinical study in flavivirus naïve subjects [46]. This formulation demonstrated good safety and efficacy in infants and children [47,48]. The TDENV vaccine viruses were re-derived and tested in a phase II study in flavivirus naïve adults, demonstrating adequate safety and immunogenicity after two doses [49]. The TDENV vaccine, in combination with a tetravalent DENV purified inactivated vaccine [51] is currently undergoing clinical evaluation using a heterologous prime-boost approach. In the current study, sera from 10 flavivirus naïve subjects that received two doses of TDENV were evaluated for levels of serotype-specific and cross-reactive NAb.

2. Material and methods

2.1. Viruses and cells

The DENV strains used for in vitro assays are DENV-1 West Pac 74, DENV-2 S16803, DENV-3 CH53489, and DENV-4 TVP-360. Virus stocks were produced by passage in *Aedes albopictus* mosquito C6/36 cells as described previously [52].

2.2. Vaccine study sera

The vaccine study design is described in detail in Ref. [49] and was approved by the US Army Human Subjects Research Review Board and the Office of the Surgeon General (www.clinicaltrials.gov identifier: NCT00350337). A total of 10 sera from study day 210 (30 days post dose two) were selected from subjects that were flavivirus naïve, had received two doses of vaccine, and had consented to use of their sera.

2.3. Purification of dengue viruses

The DENV 1–4 were amplified in Vero cells, concentrated by tangential flow ultrafiltration, and purified by sucrose gradient ultracentrifugation as described previously [53].

2.4. Depletion of DENV-specific antibodies from human sera

The antibody depletion method was carried out as described previously [12]. Purified DENV or BSA control protein was adsorbed onto 4 µm Polybead polystyrene microspheres following the manufacturer's instructions (Polysciences, Inc.). The polystyrene microspheres were washed three times with 0.1 M borate buffer (pH 8.5) and were incubated with purified DENV or BSA control protein in borate buffer overnight at room temperature. The control and DENV-adsorbed beads are blocked three times with BSA (10 mg/ml) in borate buffer for 30 min at room temperature and washed six times with PBS. Human immune sera were depleted of DENV-specific antibody by incubation with virus-adsorbed beads for 2 h at 37 °C with gentle mixing. Sequential rounds of antibody depletion were performed and the depleted sera were tested for successful removal of the desired DENV-specific antibodies by ELISA.

2.5. ELISA

Binding of human immune sera to purified DENV 1–4 was measured as described previously [12]. ELISA plates were coated with 50 ng of purified virus. Bound antibody was detected with an alkaline phosphatase conjugated goat anti-human IgG antibody and p-nitrophenyl phosphate substrate and was read at an absorbance of 405 nm on a spectrophotometer. End-point titration of serum samples was initially performed to assess the relative levels of DENV-reactive antibody. To confirm successful removal of antibody by the depletion method the virus antigen-depleted or control-depleted sera were tested for binding at a 1:100 dilution. The DENV antigens used for depletion of a given serum sample matched those used for testing that depleted sample by ELISA.

2.6. Flow cytometry-based 50% neutralization (FlowNT50) assay

Neutralizing antibody titers in heat-inactivated sera were determined using a flow cytometry-based neutralization assay in U937 cells expressing DC-SIGN as previously described [12,54]. Data were analyzed by nonlinear regression to determine 50% neutralization titers in GraphPad Prism 6. Some sera had low NAb titers near the 1:40 limit of quantification of the assay and made it more difficult to fully resolve the type-specific and cross-reactive NAb populations. Also, in some cases, the control depleted sera had marked reductions in NAb titer compared to undepleted sera, which is likely due to non-specific removal of DENV antibody and neutralization assay variability.

2.7. Viremia determination by RT-qPCR (RNAemia)

A DENV serotype-specific RT-qPCR assay (described in Ref. [49]) to assess vaccine virus RNAemia in all study subjects on two separate study visits in a 14 day window after both doses of vaccine. Depending on the subject, the first sampling time-point was day 2, 5, 8 or 12 and the second sampling time-point was day 8, 12, or 14. The limit of detection of the assay was 6500 genome equivalents (GEQ)/ml for DENV-1, 160,000 GEQ/ml for DENV-2, 1250 GEQ/ml for DENV-3, and 33,000 GEQ/ml for DENV-4.

2.8. Statistical analysis

All statistical analyses were performed with GraphPad Prism version 6.02. The levels of seroconversion to DENV 1–4 were compared among and within groups by Fisher's exact test with $p < 0.05$ required for significance. The NAb responses to DENV 1–4 were compared among and within groups using log10 transformed titers

in an ANOVA with Tukey's multiple comparisons test and $p < 0.05$ required for significance.

3. Results

3.1. Selection of sera for antibody depletions

Ten sera from subjects that received two doses of live attenuated tetravalent DENV vaccine in any of three vaccination groups (PRE-F17, TDEN F17, and TDEN F19) [49] were selected for DENV-specific antibody depletion to estimate levels of type-specific and cross-reactive NAb. The PRE-F17 vaccine formulation was optimized in a previous phase I clinical study [38]. The F17 vaccine formulation matched that of PRE-F17 but contained vaccine viruses that were re-derived by transfection of purified viral RNA into fetal rhesus lung cells. The F19 vaccine formulation matched F17 except for a moderate 4-fold lower dose of the DENV-4 component. Sera from all subjects that had consented to use from the original study were ranked based on DENV 1–4 NAb valency and then by magnitude of NAb titers. The data in Table 1 indicate that the selected sera are representative of the overall NAb response to all three vaccine formulations with similar seroconversion and geometric mean neutralizing antibody titers (GMTs), except for DENV-2 that had a significantly higher GMT compared to groups TDEN F17 and F19. A microneutralization (MN50) assay was used to determine 50% titers previously [49]. Here we used a flow cytometry-based neutralization assay that gives comparable 50% neutralization titers. The FlowNT50 titers against DENV 1–4 are shown in Table 2. There were 5 tetravalent

(50%), 1 trivalent (10%), 3 bivalent (30%), and 1 monovalent (10%) NAb responders. This closely matched NAb valency for all subjects and groups from the original study [49].

3.2. DENV-specific antibody depletions

The DENV-specific antibodies were depleted from immune sera of all 10 subjects in order to estimate the relative contribution of serotype-specific and cross-reactive antibody to neutralization of DENV 1–4. To accomplish this, polystyrene beads were coated with purified DENV virions to selectively remove antibody to each serotype or a combination of serotypes and removal of antibody was confirmed by ELISA (Fig. 1). All single and combination serotype depletions reduced antibody binding against the respective DENV serotype(s) to background levels, while control depleted sera retained their binding capacity. Subject 2 had some DENV-reactive antibody that was not completely depleted by this method. It is possible that this subject had a sub-population of high concentration but low avidity antibody that was inefficiently removed from serum by the virus-adsorbed beads. Importantly, the residual DENV-reactive antibody in this sample did not appear to contribute to neutralization.

3.3. DENV type-specific and cross-reactive NAb

The NAb titers were determined for DENV-specific antibody depleted and control depleted sera by FlowNT50 assay (Table 3). Homologous DENV antigen depletions reduced NAb titers to the respective DENV serotype to undetectable levels, except for sera

Table 1

Geometric mean neutralizing antibody titers and seroconversion for selected subjects compared to all DENV naïve subjects that received two doses of TDENV vaccine.

Group	N=	NAb GMT ^{a,b}				Seroconversion ^c			
		D1	D2	D3	D4	D1	D2	D3	D4
PRE-F17 ^d	14	198	666	75	279	93%	100%	71%	79%
TDEN F17 ^d	15	50	156	31	70	73%	80%	67%	73%
TDEN F19 ^d	12	118	226	52	46	83%	100%	83%	67%
Selected sera ^{e,f,g}	10	144	2133	37	193	80%	100%	60%	60%

^a The GMT values were calculated for all subjects in each group. The NAb titers that were below the limit of detection for FlowNT50 (<40) or MN50 (<10) were given a value of 5.

^b The D2 NAb GMT for the selected sera group was significantly higher than for groups TDEN F17 and F19, but there were no other significant differences among groups by ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

^c The levels of seroconversion for D1–D4 were not significantly different among groups by Fisher's exact test ($p < 0.05$).

^d The data for PRE-F17, TDEN F17, and TDEN F19 were generated previously by MN50 assay [49].

^e Sera used in current study to evaluate type-specific and cross-reactive NAb. Data were generated in this study by FlowNT50 assay.

^f For the selected sera group, the D2 NAb GMT was significantly higher than the D3 NAb GMT, but there were no other significant differences for this group by ANOVA with Tukey's multiple comparisons test ($p < 0.05$).

^g For the selected sera group, the levels of seroconversion for D1–D4 were not significantly different by Fisher's exact test ($p < 0.05$).

Table 2

Neutralizing antibody titers and valency of selected sera from DENV naïve subjects that received two doses of TDENV vaccine.

Subject number	Group	Study day ^a	NAb titer for:				NAb valency
			DENV-1	DENV-2	DENV-3	DENV-4	
17	PRE-F17	210	863	3500	168	19,290	Tetravalent
37	PRE-F17	210	58	319	<40	354	Trivalent
91	PRE-F17	210	2462	7231	209	4350	Tetravalent
96	PRE-F17	210	202	2489	60	6325	Tetravalent
2	TDEN F17	210	<40	565	<40	<40	Monovalent
14	TDEN F17	210	185	1107	55	463	Tetravalent
85	TDEN F17	210	311	5297	<40	<40	Bivalent
40	TDEN F19	210	<40	2991	170	<40	Bivalent
69	TDEN F19	210	99	1050	<40	<40	Bivalent
72	TDEN F19	210	1056	9302	383	1327	Tetravalent

^a One month post-dose two of TDENV.

Fig. 1. Antibody depletion of immune sera. The DENV-specific antibodies were depleted from immune sera of all 10 subjects (panels a-j). Antibodies to a single serotype or combination of serotypes were selectively depleted with polystyrene beads coated with the indicated purified DENV virions or BSA control and this was confirmed by ELISA against the matched antigens. The mean background absorbance was 0.1 and virus antigen-depleted sera had absorbance readings at or near background levels. The control-depleted sera retained high levels of antibody binding and had absorbance readings that were >10-fold mean background levels. Subject 2 (panel a) had some DENV-reactive antibody that was not completely depleted by this method. In each graph the corresponding control-depleted and antibody-depleted pairs of sera are separated by a dashed, vertical line. The mean absorbance and SEM from replicate measurements are shown.

from subject 17, which had residual neutralizing activity against DENV-1. The relative levels of type-specific and cross-reactive NAb were interpreted based on the reduction in NAb titer following heterologous DENV antigen depletion and accounted for up

to 3-fold assay-to-assay variability, which is common for neutralization assays. A reduction of ≤ 3 -fold was interpreted as predominantly type-specific NAb, >3-fold reduction interpreted as a mixture of type-specific and cross-reactive NAb, and a complete

Table 3

Neutralizing antibody titers after depletion of immune serum with homologous and heterologous purified dengue virions.

Subject number	Depletion antigen(s)	NAb titer for:			
		DENV-1	DENV-2	DENV-3	DENV-4
2	Control	<40	644	56	<40
	D2	<40	<40	<40	<40
	D1 + D3 + D4	<40	698	<40	<40
14	Control	104	782	64	568
	D2	<40	<40	<40	374
	D4	<40	242	<40	<40
	D1 + D3	<40	233	<40	224
17	Control	1072	680	314	>3240
	D4	638	107	<40	<40
	D1 + D2 + D3	294	<40	<40	>3240
37	Control	<40	170	<40	364
	D4	<40	75	<40	<40
	D1 + D2 + D3	<40	<40	<40	258
40	Control	<40	740	245	<40
	D2	<40	<40	159	<40
	D1 + D3 + D4	<40	763	<40	<40
69	Control	<40	63	<40	<40
	D2	<40	<40	<40	<40
	D1 + D3 + D4	<40	<40	<40	<40
72	Control	187	310	234	575
	D4	<40	<40	<40	<40
	D1 + D2 + D3	<40	<40	<40	77
85	Control	158	219	<40	<40
	D2	<40	<40	<40	<40
	D1 + D3 + D4	<40	<40	<40	<40
91	Control	133	855	331	4596
	D4	<40	176	<40	<40
	D1 + D2 + D3	<40	<40	<40	6931
96	Control	104	407	204	1203
	D4	<40	133	<40	<40
	D1 + D2 + D3	<40	<40	<40	3185

Table 4

Characteristics of neutralizing antibody responses to DENV 1–4 and serotype-specific valency.

Subject number	NAb responses for ^a				Type-specific NAb valency
	DENV-1	DENV-2	DENV-3	DENV-4	
2	none ^b	type ^c	cross ^d	none	Mono
14	cross	mix ^e	cross	type	Bi
17	type	mix	cross	type	Tri
37	none	type	none	type	Bi
40	none	type	type	none	Bi
69	none	cross	none	none	None
72	cross	cross	cross	mix	Mono
85	cross	cross	none	none	None
91	cross	mix	cross	type	Bi
96	cross	mix	cross	type	Bi

^a Relative levels of type-specific and cross-reactive NAb were interpreted based on the reduction in NAb titer following heterologous DENV antigen depletion, with ≤ 3 -fold reduction interpreted as predominantly type-specific NAb, > 3 -fold reduction interpreted as a mixture of type-specific and cross-reactive NAb, and a complete reduction (titer < 40) interpreted as predominantly cross-reactive NAb.

^b None = undetectable NAb titer to that serotype in the depletion experiment.

^c Type = DENV serotype-specific NAb.

^d Cross = DENV cross-reactive NAb.

^e Mix = Mixture of DENV serotype-specific and cross-reactive NAb.

reduction (titer < 40) interpreted as predominantly cross-reactive NAb. Subject 91, for example, neutralized DENV 1–4 with titers of 133, 855, 331, and 4596, respectively. Depletion of this subject's serum with DENV-4 antigen reduced the NAb titer to DENV-1 and DENV-3 to undetectable levels and indicated that these NAb responses were primarily cross-reactive. The NAb titer to DENV-2 remained detectable after DENV-4 antigen depletion, but was reduced by 4.9-fold, indicating a mixture of type-specific and cross-reactive DENV-2 NAb. Depletion of this subject's serum with

a combination of DENV-1, 2, and 3 had no apparent effect on the NAb titer to DENV-4 (≤ 3 -fold reduction) and indicated that the DENV-4 NAb is primarily type-specific. The NAb titer data for depleted sera from all 10 subjects were interpreted using these criteria.

The NAb response characteristics for DENV 1–4 and the type-specific NAb valency for each subject are indicated in Table 4. Subjects with a mixture of type-specific and cross-reactive NAb were included for type-specific NAb valency. Two subjects (69 and 85)

Fig. 2. Serotype specificity of neutralizing antibody responses. In panel a, the number of subjects with type-specific, mixture of type-specific and cross-reactive, cross-reactive only, or undetectable neutralizing antibody to DENV 1–4 are indicated. In panel b, the DENV 1–4 serotype-specific 50% neutralization (NT50) titers are shown. These are the NT50 titers reported after heterologous antibody depletion in Table 3. Sera with undetectable serotype-specific neutralizing antibody (<40) were given an NT50 value of 10. The geometric mean neutralizing antibody titers are indicated by a bar.

had undetectable levels of type-specific NAb to any serotype but both had cross-reactive NAb to DENV-2. Subjects 2 and 72 had monovalent type-specific NAb responses to DENV-2 and DENV-4, respectively, and cross-reactive NAb to some or all of the other serotypes. One subject, 17, had a trivalent type-specific NAb response to DENV-1, -2 and -4, and a cross-reactive NAb response to DENV-3. Half of the subjects that we tested had a bivalent type-specific NAb response. One subject, 40, had type-specific NAb to DENV-2 and DENV-3. The other four subjects (14, 37, 91 and 96) had type-specific NAb to DENV-2 and DENV-4 and undetectable or cross-reactive NAb to the other serotypes.

The characteristics of NAb responses to DENV 1–4 are summarized in Fig. 2a and the serotype-specific NAb titers and GMTs are shown in Fig. 2b. The majority of subjects had type-specific NAb responses to DENV-2 and DENV-4 and cross-reactive NAb responses to DENV-1 and DENV-3. The DENV 1–4 type-specific NAb GMTs for these subjects were <40, 87, <40, and 149, respectively.

3.4. Vaccine virus RNAemia

All subjects were tested for DENV 1–4 vaccine virus RNAemia by RT-qPCR on two separate study visits in a 14 day window after both doses of vaccine. Two of 10 subjects with sera that were selected for the current study had detectable levels of vaccine virus RNAemia. Subject 17 had 4.6 log₁₀ GEQ/ml of DENV-4 at 14 days post-dose 2 of vaccine. Subject 96 had 5.2 log₁₀ GEQ/ml of DENV-4 at 8 days post-dose 2 of vaccine. The sera from these two subjects also had high titer DENV-4 type-specific NAb of >3240 and 3185, respectively. Approximately 214 subjects in four separate clinical studies have been vaccinated with TDENV [47–50]

and detectable RNAemia has been reported for DENV-2 (5 subjects), DENV-3 (1 subject), and DENV-4 (15 subjects). There were almost certainly more subjects with vaccine virus RNAemia that was not captured by the 1 or 2 days of sampling done in these studies. Based on these limited data, replication of the vaccine viruses in humans appears to follow the pattern DENV-4 > DENV-2 > DENV-3 > DENV-1. This is similar to the observed pattern of type-specific NAb responses of the 10 subjects in this study, which were predominantly elicited by DENV-2 and DENV-4.

4. Discussion

Primary DENV infections elicit both type-specific and cross-reactive NAb that correlate with protection against that serotype, but not durable cross-protection against other serotypes [2,12,22]. Post-primary DENV infections elicit more broadly cross-reactive NAb and correlate with a longer period of cross-protection [4–7]. The DENV vaccines in clinical development, including the WRAIR TDENV vaccine consists of all four serotypes delivered simultaneously and it was unclear what effect that would have on NAb responses. Therefore, we evaluated levels of type-specific and cross-reactive NAb in sera from flavivirus naive subjects that received two doses of the WRAIR TDENV vaccine.

Sera from subjects in a previous phase II clinical study were selected for testing based on high levels of neutralizing antibody to DENV 1–4 and consent for future testing. For the subset of study sera that we selected, seroconversion levels were not significantly different between DENV serotypes. Also, the only significant difference in NAb GMTs were between DENV-2 and DENV-3. In general, seroconversion and GMT were not good predictors of type-specific NAb responses. A much higher proportion of sera from the 10 subjects that we evaluated had DENV-2 and DENV-4 type-specific NAb than DENV-1 or DENV-3 type-specific NAb. Most subjects had cross-reactive NAb to DENV-1 and DENV-3, which may have actually been elicited by the better replicating DENV-2 and DENV-4 vaccine viruses. Thus, many of these subjects might not have specifically responded to DENV-1 or DENV-3 vaccine viruses, even though they were given a DENV 1–4 tetravalent mixture.

The DENV-2 and DENV-4 components of the WRAIR TDENV vaccine were most frequently detected replicating in vaccinated subjects in four separate clinical studies [47–50]. This observation correlates with the relatively high proportion of DENV-2 and DENV-4 type-specific NAb responses observed in the current study. Some subjects did have type-specific NAb to DENV-1 and DENV-3 after vaccination, indicating that these vaccine viruses probably replicated in some individuals. Unfortunately, only 1 or 2 samples were collected post-vaccination for evaluating RNAemia, which means that many RNAemia positive time-points were likely missed. Therefore, we could not determine if detectable levels of vaccine virus replication were required to elicit type-specific immunity. However, the two subjects with detectable levels of DENV-4 RNAemia in the current study also produced high titer DENV-4 type-specific NAb.

Natural human DENV infections elicit potentially neutralizing serotype-specific antibody, which is a better long-term correlate of protection than cross-reactive NAb [12,55]. DENV vaccine development has been complicated by the need to simultaneously vaccinate with all four serotypes, which differs from natural infections that are acquired sequentially. The DENV 1–4 components of live attenuated vaccines that are currently licensed or in clinical development each have some level of unbalanced replicative fitness in humans [31,33,56]. It is conceivable that tetravalent, type-specific immunity elicited by balanced replication of all four vaccine viruses is not required for protection, but a lack of type-specific immunity to one or more serotypes could result in poor

overall clinical efficacy. Evidence supporting this comes from phase III studies of the CYD-TDV vaccine [8–10]. Naïve subjects that received this vaccine had similar DENV 1–4 NAb titers but primarily had type-specific NAb to DENV-4, which was attributed to better replication of the DENV-4 vaccine virus [19,56], and resulted in better levels of protection compared to DENV-1 and DENV-2. This, in part, illustrates the importance of evaluating serotype-specific NAb responses elicited by tetravalent DENV vaccines.

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Conflict of interest

The authors report no conflict of interest.

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