Viral genetic diversity and protective efficacy of a tetravalent dengue vaccine in two phase 3 trials

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Two phase 3 placebo-controlled trials of the CYD-TDV vaccine, evaluated in children aged 2–14 y (CYD14) and 9–16 y (CYD15), demonstrated vaccine efficacy (VE) of 56.5% and 60.8%, respectively, against symptomatic virologically confirmed dengue (VCD). Sieve analyses were conducted to evaluate whether and how VE varied with amino acid sequence features of dengue viruses (DENVs). DENV premembrane/envelope amino acid sequences from VCD endpoint cases were aligned with the vaccine insert sequences, and extensions of the proportional hazards model were applied to assess variation in VE with amino acid mismatch proportion distances from vaccine strains, individual amino acid residues, and phylogenetic genotypes. In CYD14, VE against VCD of any serotype (DENV-Any) decreased significantly with increasing amino acid distance from the vaccine, whereas in CYD15, VE against DENV-Any was distance-invariant. Restricting to the common age range and amino acid distance range between the trials and accounting for differential VE by serotype, however, showed no evidence of VE variation with distance in either trial. In serotype-specific analyses, VE against DENV4 decreased significantly with increasing amino acid distance from the DENV4 vaccine insert and was significantly greater against residue-matched DENV4 at eight signature positions. These effects were restricted to 2- to 8-y-olds, potentially because greater seropositivity of older children at baseline might facilitate a broader protective immune response. The relevance of an antigenic match between vaccine strains and circulating DENVs was also supported by greater estimated VE against serotypes and genotypes for which the circulating DENVs had shorter amino acid sequence distances from the vaccine.

amino acid position signatures | CYD-TDV | dengue virus | sieve analysis | vaccine efficacy

T he four dengue virus (DENV) serotypes (DENV1, DENV2, DENV3, and DENV4) cause the most rapidly expanding infectious disease globally, with up to 390 million infections annually (1). Nearly half the world's population is estimated to be at risk for dengue infection (2), and there is no effective treatment for dengue disease, in particular for severe dengue fever. The licensed recombinant, live, attenuated, tetravalent dengue vaccine (Dengvaxia/CYD-TDV), developed and manufactured by Sanofi Pasteur, was evaluated in two phase 3 efficacy trials with harmonized designs. The first trial, CYD14, enrolled 10,275 healthy children aged 2–14 y in the Asian-Pacific region (3), and the second trial, CYD15, enrolled 20,869 healthy children aged 9–16 y in Latin America (4). In both trials, the CYD-TDV vaccine was administered at 0, 6, and 12 mo, and participants were followed with active surveillance for 25 mo. Vaccine efficacy (VE) in preventing symptomatic virologically confirmed dengue (VCD) of any serotype after first vaccination was estimated as 54.8% (95% CI, 46.8–61.7%) in CYD14 and 64.7% (95% CI, 58.7–69.8%) in CYD15. For prevention of VCD after 28 d following the third dose, VE was estimated as 56.5% (95% CI, 43.8–66.4%) in CYD14 and 60.8% (95% CI, 52.0–68.0%) in CYD15. In CYD14, VE after 28 d following the

third dose was estimated to be greater in older children [68.1% (95% CI, 58.3–75.5%) in 9- to 14-y-olds vs. 44.2% (95% CI, 31.5–54.5%) in 2- to 8-y-olds] (3). In addition, both trials found greater efficacy in participants with preexisting dengue-neutralizing antibodies (3, 4). CYD-TDV is approved for use in more than a dozen countries in Asia and Latin America and is indicated for individuals aged 9–45 y, with the exceptions of Paraguay (9–60 y) and Singapore (12–45 y). As dengue-seronegative vaccine recipients were found to have an elevated rate of hospitalization with dengue and severe dengue compared with placebo recipients over 5 y after first vaccination, the Strategic Advisory Group of Experts on Immunization has recommended that "a pre-vaccination screening strategy would be the preferred option, in which only dengue-seropositive persons are vaccinated" (5).

The four DENV serotypes exhibit only ∼62–67% amino acid homology (6) and are each classified into multiple genotypes (7–10).

Significance

Dengue virus (DENV) vaccine development is complicated by the existence of four genetically diverse DENV serotypes. A high degree of antigenic match between vaccine strains and circulating DENVs may be important to achieve high vaccine efficacy (VE). Using data from two phase 3 trials of the CYD-TDV vaccine, we assessed whether and how VE against virologically confirmed dengue varied with amino acid sequence characteristics and genotypes of the disease-causing DENVs. VE decreased with the degree of amino acid dissimilarity between the vaccine insert and disease-causing DENVs. After accounting for differential VE by serotype, this effect seemed to occur only for younger children, who also had lower baseline seropositivity and potentially a less broadly protective immune response.

While the dominant genotype (or clade) can sometimes be quickly displaced by a newly introduced genotype (or newly circulating strains) with a fitness advantage, dengue lineages can remain quite stable in some areas (10). The four recombinant CYD-TDV viruses, one per serotype, were constructed by replacing the premembrane/envelope (prM/E) sequences of the yellow fever virus 17D strain with the corresponding DENV sequences (DENV1-I PUO359, DENV2-Asian I PUO218, DENV3-II PaH881, and DENV4-II 1228) (11).

The reported estimates for serotype-specific VE against DENV1, -2, -3, and -4 in CYD14 and CYD15 were 50.0% and 50.3%, 35.0% and 42.3%, 78.4% and 4.0%, and 75.3% and 77.7%, respectively (3, 4). To better understand whether the limited VE for some serotypes could be partly due to antigenic mismatch of the vaccine inserts to the circulating viruses in the CYD14 and CYD15 trials, Rabaa et al. (12) conducted an extensive descriptive analysis of the sequences of the disease-causing DENVs in the CYD14 and CYD15 trials. Through phylogenetic analyses of sequences from VCD endpoint cases, they identified the following circulating genotypes: DENV1-I/IV, DENV2-Cosmopolitan/Asian I, DENV3-I/II/III, and DENV4-I/II in CYD14 and DENV1-V, DENV2-American-Asian, DENV3-III, and DENV4-II in CYD15 (12). Rabaa et al. also reported that only 27.2, 5.0, 0, and 29.6% of DENV1, -2, -3, and -4 VCD endpoint cases, respectively, in CYD14 and 0, 0, 0, and 100% DENV1, -2, -3, and -4 VCD endpoint cases, respectively, in CYD15 were caused by a DENV with a vaccine-matched genotype. Here we conducted a sieve analysis for CYD14 and for CYD15 to assess whether and how VE in preventing VCD varied with amino acid sequence characteristics and genotypes of disease-causing viruses.

Sieve analysis can provide insight into protective proteomic features of immunogens and thereby help improve vaccine design (13, 14). Rabaa et al. (12) also reported results showing how VE depends on circulating genotypes. Our analysis recapitulates these results with alternative statistical methods tailored to sieve analysis and provides several complementary results showing how VE depends on other amino acid sequence features (and, equally importantly, how VE is stable across certain amino acid sequence features). We also examined the extent to which the variability in VE across the 11 circulating genotypes can be explained by their similarity to the vaccine and identified the amino acid positions with greatest influence on VE. Our results inform future complementary studies that could evaluate vaccine-induced immune responses targeting these amino acid sequence features as immunological correlates of VE.

Results

VE Against VCD by Amino Acid Sequence Distance of the Disease-Causing DENV from the Vaccine Insert. *VE against VCD of any serotype decreases with increasing residue mismatch proportion to the vaccine insert while controlling for serotype in CYD14.* We calculated the residue mismatch proportion of each VCD of any serotype (DENV-Any) case's amino acid sequence to the vaccine sequence of the same serotype in all 661 aligned positions (659 for DENV3) as well as in 65 human neutralizing antibody (NAb) contact sites and a by-serotype range of 225–236 surface-exposed sites on the mature virion. We first conducted a marginal VE analysis which assessed variation in VE with the residue mismatch distance, not accounting for serotype. Marginal VE decreased significantly with the residue mismatch proportion in all sites ($P = 0.0012$) and in the surface-exposed sites ($P = 0.00063$) in CYD14 and in all sites ($P = 0.00027$) in CYD15 (Fig. 1). To understand these results better, we also conducted an analysis that assessed variation in VE jointly with the residue mismatch distance and serotype to address whether and how the variation in VE with the distance might be partially explained by differences in VE against individual serotypes. The steepest decline in serotype-adjusted VE was detected for the residue mismatch proportion in the surface-exposed sites in CYD14
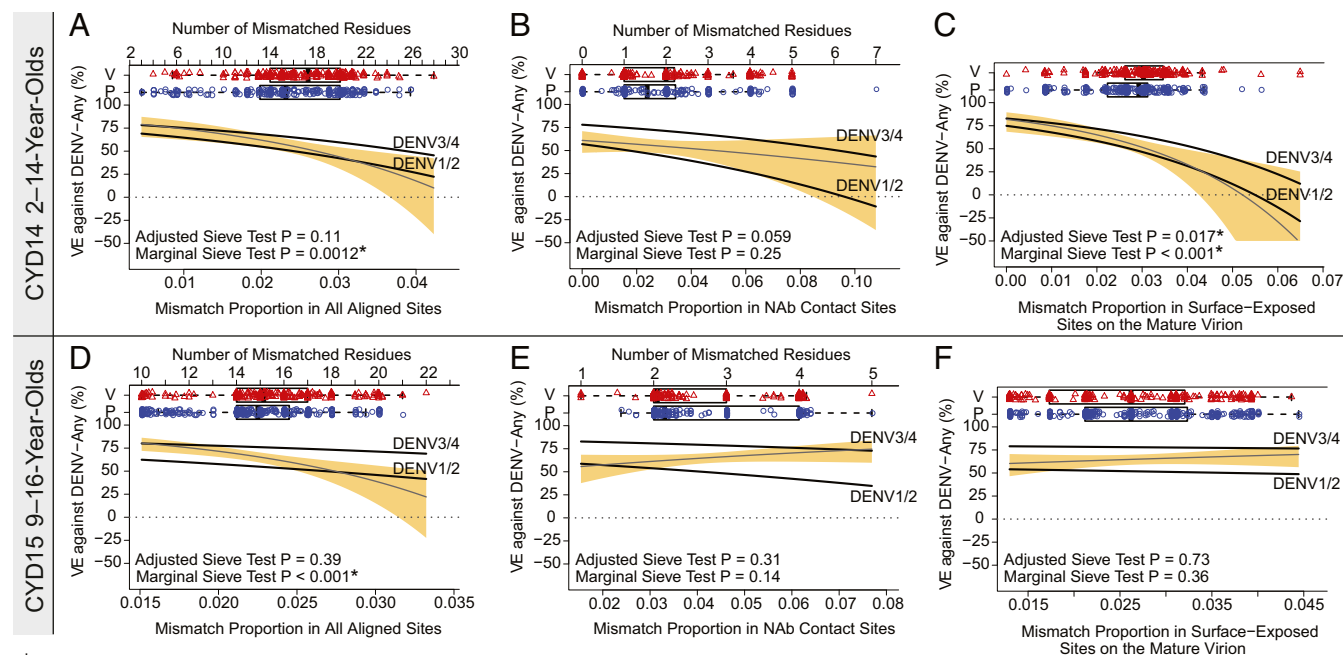
* Unadjusted P-value ≤ 0.05 and Q-value ≤ 0.2
† The only alignment gap reflects that DENV3 sequences are characteristically missing AA at alignment positions 322 and 323.
‡ The number of surface-exposed sites on the mature virion varies by serotype (229, 231, 225, and 236 for DENV1, DENV2, DENV3, and DENV4, respectively), hence no common top axis is displayed in panels C and F.

Fig. 1. Marginal and serotype-adjusted VE against DENV-Any by residue mismatch proportion in all aligned sites (*A* and *D*; see footnote "†" in the figure), NAb contact sites (*B* and *E*), and surface-exposed sites on the mature virion in the ITT cohort (*C* and *F*; see footnote "‡" in the figure) of CYD14 2- to 14-y-olds (*A–C*) and CYD15 9- to 16-y-olds (*D–F*). The saffron-shaded area represents the 95% pointwise CI for the marginal VE. P, placebo; V, vaccine.

($P = 0.017$ for decreasing VE after accounting for serotype), with VE of 82.8% (95% CI, 70.1–90.1%) and 74.9% (95% CI, 51.4–87.0%) against a perfectly vaccine-matched DENV3/4 and DENV1/2 sequence, respectively, and VE of 31.8% (95% CI, −42.1 to 67.3%) and 0.3% (95% CI, −70.7 to 41.8%) against DENV3/4 and DENV1/2 sequences mismatched to the vaccine at 13 residues (5.5% of residues in this site set). However, the magnitude of decline in serotype-adjusted VE with the residue mismatch distance was smaller than with marginal VE, indicating the gradient in the marginal VE curve was partially due to differential VE by serotype. The age-subgroup analysis in CYD14 was not supportive of a restriction of the observed VE gradient to 2- to 8-y-olds (*SI Appendix*, Fig. S1); however, restricting the serotype-adjusted VE analyses in CYD14 and CYD15 to their common age range (9–14 y) and mismatch distance ranges (*SI Appendix*, Fig. S2) yielded a consistent result across trials of invariant VE to residue mismatch when controlling for serotype in this older cohort.

VE against DENV4 decreases with increasing residue mismatch proportion to the vaccine insert in 2- to 8-y-olds, but there is no evidence of variation in VE with residue mismatch proportion for other serotypes or for 9- to 14-y-olds. For the serotype-specific endpoints, the analysis was conducted as in the DENV-Any marginal analysis. We found significant sieve effects only for DENV4, with decreasing VE with residue mismatch proportion in all sites ($P = 0.010$) and the surface-exposed sites ($P = 0.029$) in CYD14 (*SI Appendix*, Fig. S3). The DENV4 sieve effects in CYD14 were restricted to 2- to 8-y-olds ($P = 0.016$ for decreasing VE with distance), with the steepest decline in VE in this age category detected for the residue mismatch proportion in all 661 aligned sites with VE 83.4% (95% CI, 54.0–94.0%) against DENV4 sequences with three mismatched residues (0.5% of all residues) and VE 25.8% (95% CI, −39.8 to 60.6%) against DENV4 sequences with 13 mismatched residues (2.0% of all residues) (Fig. 2). The DENV4 results in 9- to 14-y-olds in CYD14 were remarkably consistent with those in 9- to 16-y-olds in CYD15 (*SI Appendix*, Fig. S3), suggesting an invariant VE to residue mismatch in this age category. The presented evidence for decreasing VE against DENV-Any and DENV4 with residue mismatch proportion to the vaccine warranted a systematic approach to studying differential VE by various amino acid sequence and genotype features, with the hope of gaining insight into the distance-based sieve effect results.

VE Against VCD by Serotype and by Genotype of the Disease-Causing DENV.
VE is greater against DENV3 and DENV4 than against DENV1 and DENV2. We first compared serotype-specific VE between all pairs of serotypes using a proportional hazards statistical method designed for this purpose (15) which has not been previously applied in analyses of CYD14 or CYD15 and found significantly greater VE

against DENV3 and DENV4 than against DENV1 and DENV2 in both trials (Fig. 3 *A* and *B*).

VE is similar across genotypes for DENV-Any, DENV1, and DENV2 in CYD14. Given the evidence for a decline in VE with residue mismatch proportion, we hypothesized that the vaccine may provide differential protection against disease-causing DENVs that matched vs. mismatched the genotype of the vaccine strain. This analysis was only possible in CYD14, as the disease-causing DENVs in CYD15 all belonged to a single genotype for each serotype. There was no evidence for differential VE against DENV-Any with a genotype-matched vs. mismatched vaccine strain in CYD14 (Fig. 3*B*). Estimated VE was notably similar against the vaccine-matched DENV1-I genotype [55.6% (95% CI, 27.9–72.7%)] and the vaccine-mismatched DENV1-IV genotype [54.4% (95% CI, 38.7–66.0%)] (Fig. 3*C*). For DENV2, there was also no evidence for differential VE by matched vs. mismatched genotype [21.2% (95% CI, −26.5 to 51.0%)] against the vaccine-matched DENV2-Asian I genotype and 42.7% (95% CI, 14.9–61.4%) against the vaccine-mismatched DENV2-Cosmopolitan genotype (Fig. 3*C*).

VE is potentially greater against DENV3-II than against DENV3-I/III in CYD14, but with low precision. While there was no significant sieve effect by genotype for DENV3 in CYD14, the point estimates of VE showed a difference similar to that for DENV4 (87.6% vs. 61.2%, $P = 0.34$ for the difference) (Fig. 3*C*). The lack of statistical significance could be due to low statistical power to detect a difference given the 90.7% fewer matched endpoints for DENV3 than for DENV4.

VE is greater against DENV4-II than against DENV4-I in CYD14, with the sieve effect restricted to 2- to 8-y-olds. DENV4 was the only serotype for which VE was significantly greater against the vaccine-matched (DENV4-II) [83.9% (95% CI, 69.3–91.5%)] vs. -mismatched genotype (DENV4-I) [58.7% (95% CI, 31.1–75.2%)] in CYD14, with differential VE $P = 0.027$ for all ages of 2- to 14-y-olds (Fig. 3*C*). The difference in VE estimates for DENV4-II vs. DENV4-I was similar in magnitude to that for DENV3-II vs. DENV3-I/III (26.4% vs. 25.2%, respectively). The DENV4 sieve effect by genotype in CYD14 appeared to be restricted to participants aged 2−8 y, for whom VE against the vaccine-matched DENV4-II genotype was 76.3% (95% CI, 44.9–89.8%) and VE against the vaccine-mismatched DENV4-I genotype was 23.9% (95% CI, −48.7 to 61.1%), with differential VE $P = 0.031$ (Fig. 3*D*). In contrast, in 9- to 14-y-olds in CYD14, the VE against the DENV4-II and DENV4-I genotypes was similar (89.8% and 85.5%, respectively) and highlighted the difference in VE against DENV4-I between the age categories. The age-by-genotype interaction P value was 0.35.
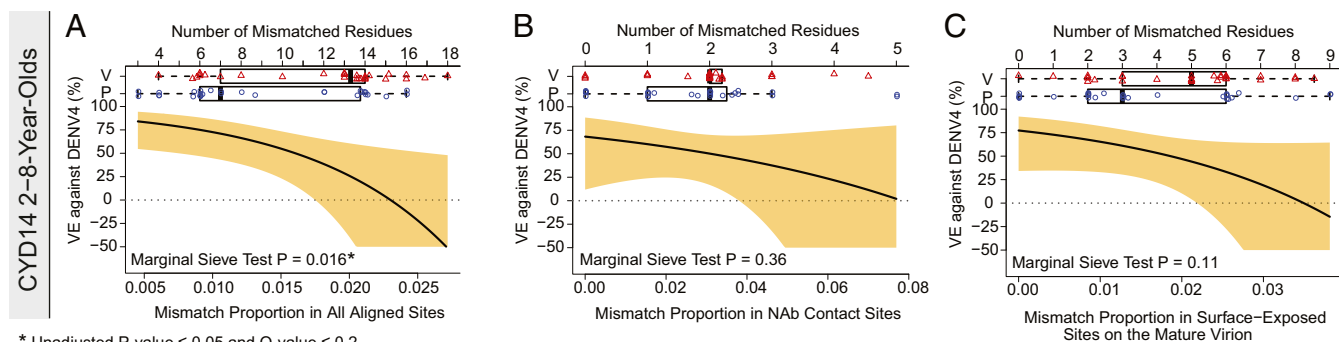
Fig. 2. VE against DENV4 by residue mismatch proportion of various amino acid sets in the ITT cohort of 2- to 8-y-olds in CYD14. (*A*) All aligned sites (see footnote "†" in the figure). (*B*) NAb contact sites. (*C*) Surface-exposed sites on the mature virion. The saffron-shaded area represents the 95% pointwise CI. P, placebo; V, vaccine.

* Unadjusted P-value ≤ 0.05 and Q-value ≤ 0.2
† The only alignment gap reflects that DENV3 sequences are characteristically missing AA at alignment positions 322 and 323.
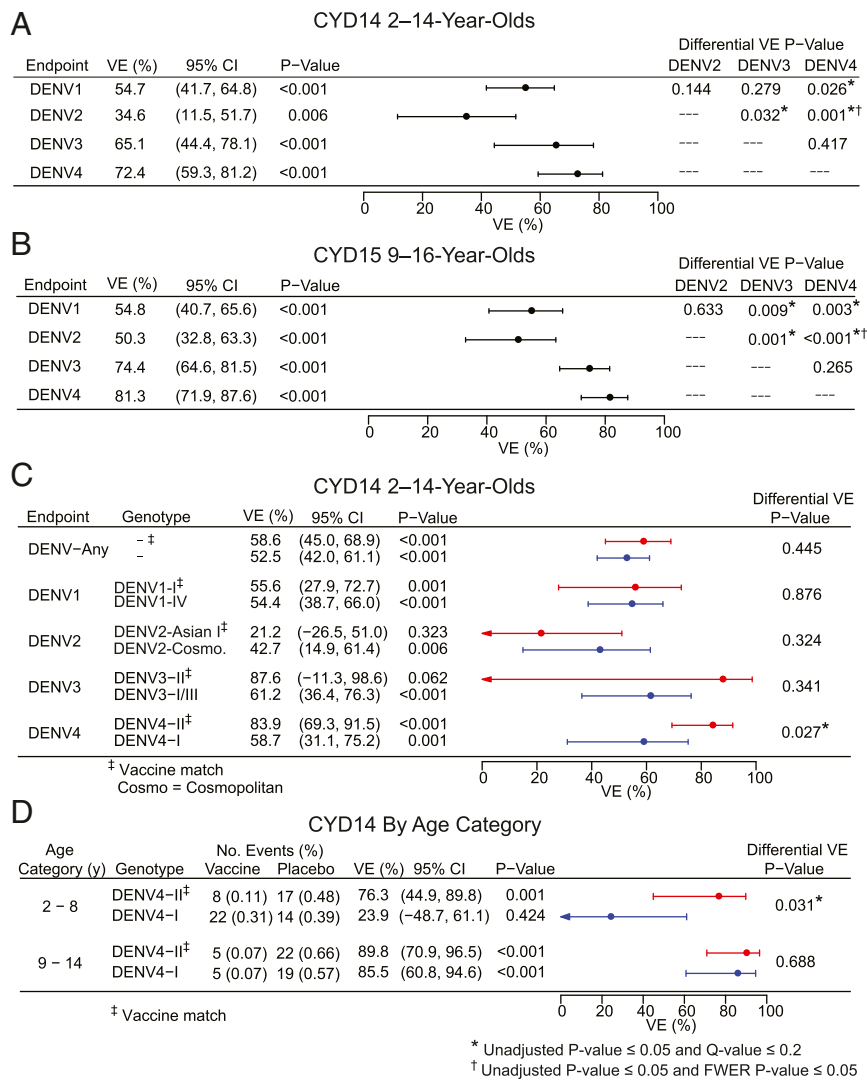
Fig. 3.   VE by serotype and genotype in the ITT cohorts of CYD14 and CYD15. (*A*) VE against the serotype-specific dengue endpoints and evidence for differential VE between serotypes in CYD14. (*B*) VE against the serotype-specific dengue endpoints and evidence for differential VE between serotypes in CYD15. (*C*) VE against the primary dengue endpoint of any serotype (DENV-Any) and the serotype-specific dengue endpoints with a matched and mismatched genotype to the vaccine strain of the same serotype in CYD14. (*D*) VE against DENV4 with the vaccine-matched DENV4-II and vaccine-mismatched DENV4-I genotype in 2- to 8-year-olds and 9- to 14-y-olds in CYD14.

VE Against VCD by Match vs. Mismatch at Individual Amino Acid Positions for the Disease-Causing DENVs Compared with the Vaccine Inserts.

VE against DENV-Any and DENV4 was greater with amino acid positions matched vs. mismatched to the vaccine in CYD14, with the DENV4 sieve effect restricted to 2- to 8-y-olds, but there was no evidence for VE variation with residue positions in CYD15. We next set out to understand local determinants of the observed sieve effects with the aim of discovering individual amino acid residues that may be important for vaccine-induced protection and informing epitope-mapping experiments. We assessed VE against disease-causing DENVs that were matched vs. mismatched to the serotype-matched vaccine strain at given individual amino acid positions, repeating this analysis across all eligible amino acid positions. For the DENV-Any, DENV1, DENV2, DENV3, and DENV4 endpoints, 133, 48, 42, 25, and 28 amino acid positions in CYD14 and 89, 25, 11, 6, and 8 amino acid positions in CYD15 were eligible for the intention-to-treat (ITT) cohort sieve analysis based on sufficient amino acid residue diversity as described in *Methods* (*SI Appendix,* Table S1).

In CYD15, no eligible amino acid positions had evidence of differential VE by residue match vs. mismatch according to the prespecified significance threshold (see *Methods* for definition). In contrast, in CYD14, VE against DENV-Any differed significantly by residue match vs. mismatch at a single position, E226 (E224 for DENV3), with VE 60.5% (95% CI, 52.5–67.2%) against residue-matched DENV-Any (residue T for each serotype) and 24.3% (95% CI, −9.0 to 47.3%) against residue-mismatched DENV-Any (residue K for DENV2; no mismatches for DENV1, 3, or 4), with differential VE $P = 0.0041$. As only DENV2 cases had any E226 mismatched residues, this sieve effect is entirely attributed to DENV2, and analysis of the DENV2 endpoint suggested its potential restriction to 2- to 8-y-olds with VE of 66.0% vs. 1.7% against an E226 matched vs. mismatched residue, respectively, compared with VE of −35.5% vs. 43.5% for 9- to 14-y-olds (*SI Appendix,* Table S2).

For the serotype-specific endpoints, all evidence of individual position sieve effects in CYD14 was restricted to the DENV4 endpoint, with VE significantly greater against residue-matched than mismatched DENV4 at amino acid positions pr73, M65, E46, E120, E160, E203, E461, and E478 (henceforth "signature positions") (Fig. 4 and *SI Appendix,* Fig. S5). Age-subgroup

AA Position	Residue	No. Events (%) Vaccine	No. Events (%) Placebo	% DENV4−I Events	VE (%)	95% CI	P−Value	Differential VE P−Value
pr73	T‡	9 (0.13)	19 (0.54)	1.0	76.4	(47.8, 89.3)	<0.001	0.016
	A	21 (0.30)	12 (0.34)	100.0	13.8	(−76.7, 58.0)	0.343	
M65	V‡	10 (0.14)	20 (0.56)	10.0	75.0	(46.5, 88.3)	<0.001	0.017
	I	20 (0.28)	11 (0.31)	100.0	11.5	(−84.8, 57.6)	0.372	
E46	T‡	9 (0.13)	19 (0.54)	0.0	76.1	(47.1, 89.2)	<0.001	0.018
	I	21 (0.30)	12 (0.34)	100.0	15.6	(−71.5, 58.5)	0.320	
E120	L‡	10 (0.14)	19 (0.54)	3.0	73.5	(42.8, 87.7)	<0.001	0.035
	S	20 (0.28)	12 (0.34)	100.0	19.7	(−64.3, 60.7)	0.274	
E160	V‡	13 (0.18)	26 (0.73)	29.0	74.1	(49.3, 86.8)	<0.001	0.004
	E	17 (0.24)	5 (0.14)	100.0	−50.4	(−312.9, 45.2)	0.786	
E203	K‡	11 (0.15)	20 (0.56)	10.0	72.5	(42.6, 86.8)	<0.001	0.032
	T,A	19 (0.27)	11 (0.31)	100.0	16.0	(−76.6, 60.0)	0.323	
E461	F‡	10 (0.14)	19 (0.54)	10.0	73.6	(43.3, 87.7)	<0.001	0.031
	L	20 (0.28)	12 (0.34)	100.0	18.8	(−66.1, 60.3)	0.284	
E478	T‡	11 (0.15)	20 (0.56)	10.0	72.5	(42.6, 86.8)	<0.001	0.032
	S	19 (0.27)	11 (0.31)	100.0	16.0	(−76.6, 60.0)	0.323	

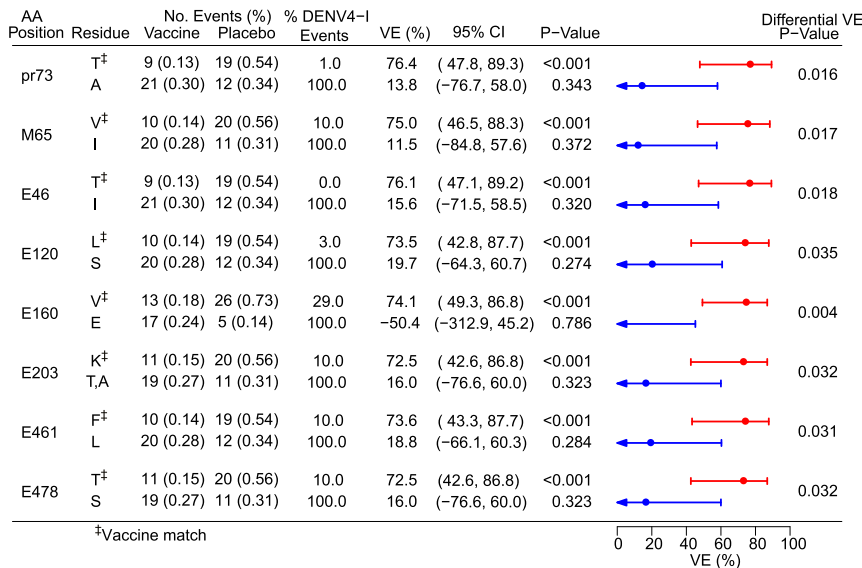‡Vaccine match

VE (%): 0 20 40 60 80 100

Fig. 4. VE against DENV4 with a vaccine-matched and -mismatched residue at signature amino acid positions in the ITT cohort of 2- to 8-y-olds in CYD14.

analyses showed these results were restricted to 2- to 8-y-olds (Fig. 4 and *SI Appendix*, Fig. S5). Residues at all signature positions except E160 highly covaried (i.e., exhibited linkage disequilibrium) (*SI Appendix*, Fig. S4) and were highly associated with the DENV4 genotypes (with no residue variation for DENV4-II and ≤ two DENV4-I cases with a minority variant) (Fig. 5), indicating that the sieve effects observed at these positions echoed the DENV4 genotype-specific sieve effect described above. In contrast, residues at position E160 were less associated with DENV4 genotypes, where 10 (30.3%) DENV4-I sequences had a minority variant. VE results by residue match vs. mismatch for these signature positions pooling over both age categories and for 9- to 14-y-olds in CYD14 as well as for 9- to 16-y-olds in CYD15 are shown in *SI Appendix*, Fig. S5.

VE is greater against serotypes and genotypes with disease-causing circulating viruses with amino acid sequences more similar to the vaccine inserts. To further understand why VE varied by serotype and by DENV4 genotype, we examined the level of amino acid sequence similarity of disease-causing circulating viruses to the amino acid sequence of the vaccine-encoded protein, as first measured by the placebo group cases' (including all ages) amino acid sequence distances to the vaccine reference sequences (Fig. 6). DENV3 and DENV4 placebo case sequences were closer to the vaccine than DENV1 and DENV2 placebo case sequences in both trials; e.g., in CYD14, DENV1 placebo case sequences had on average 2.0-fold (95% CI, 1.8- to 2.3-fold) more vaccine-mismatched residues in all aligned positions than DENV4 placebo case sequences, and DENV2 placebo case sequences also had on average 2.0-fold (95% CI, 1.8- to 2.2-fold) more vaccine-mismatched residues than DENV4 placebo case sequences (*SI Appendix*, Table S3). When considering only positions with ≥75% difference in the mismatch rate between the placebo sequence and the corresponding vaccine insert, more such positions were seen for DENV1 vs. DENV4 and for DENV2 vs. DENV4 in CYD15 (12 and 13 positions, respectively) than in CYD14 (seven and three positions, respectively) (*SI Appendix*, Fig. S6). Furthermore, the greater dissimilarity of DENV1 and DENV2 placebo case sequences from the vaccine was associated with smaller VE against these two serotypes in both trials, and the two genotypes with the greatest VE, DENV3-II and DENV4-II, were closest to the vaccine (Fig. 6).

We also evaluated the vaccine's 9-mer coverage of placebo case sequences, i.e., the proportion of 9-mers in placebo case sequences that are perfectly matched to the corresponding vaccine sequence 9-mer of the same serotype (*SI Appendix*, Fig. S7). The DENV3 and DENV4 vaccine strains had greater mean 9-mer coverage (82.3% and 89.0%, respectively, in CYD14 and 82.6% and 88.3%, respectively, in CYD15) than the DENV1 and
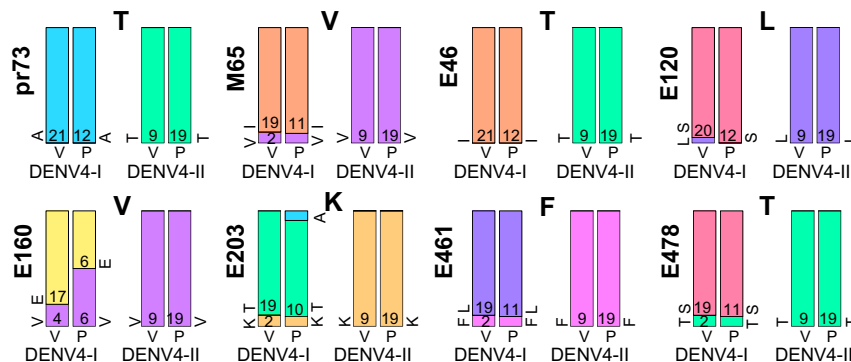
Fig. 5. The amino acid residue distribution by genotype and study group at DENV4 signature positions in the ITT cohort of 2- to 8-y-olds in CYD14. Counts show numbers of VCD DENV4 endpoints; residues in the DENV4-II vaccine sequence are shown at the top. Each color represents a different residue. P, placebo; V, vaccine.

DENV2 vaccine strains (77.2% and 79.1%, respectively, in CYD14 and 78.3% and 81.2%, respectively, in CYD15). For example, in CYD14 the DENV4 vaccine strain covered on average 1.15-fold (95% CI, 1.13- to 1.17-fold) more 9-mers than the DENV1 vaccine strain and 1.13-fold (95% CI, 1.11- to 1.15-fold) more 9-mers than the DENV2 vaccine strain (*SI Appendix*, Table S3). The coverage patterns convey a message similar to that of the residue mismatch proportion patterns in both trials, with both the DENV3 and DENV4 placebo case sequences being more closely matched and more completely covered by 9-mers in the vaccine reference sequences than DENV1 and DENV2 placebo case sequences.

The amino acid sequence similarity between genotypes is greater for DENV1 and DENV2 than for DENV3 and DENV4 in CYD14. We assessed whether differences in amino acid sequence distances between vaccine-matched vs. mismatched genotypes within each serotype could partially explain why in CYD14 there was evidence of differential VE by genotype for DENV3 and DENV4 but not for DENV1 and DENV2. Affirmatively, there was greater amino acid sequence similarity between the vaccine-matched and mismatched genotypes for DENV1 and DENV2 than for DENV3 and DENV4 (Fig. 6). For all four serotypes, placebo case sequences of the vaccine-matched genotype had on average significantly fewer mismatched residues in all aligned sites than placebo case sequences of a vaccine-mismatched genotype (*SI Appendix*, Table S3). Moreover, for CYD14, based on the calculations for Figs. 1 and 2, we estimated a slope for each serotype defined by change in log relative risk (vaccine vs. placebo) per unit change in amino acid sequence distance for all aligned sites and found steep and similar slopes for DENV3 and DENV4

and flat and similar slopes for DENV1 and DENV2 (*SI Appendix*, Fig. S8).

Discussion

We conducted a sieve analysis of data from the active phase of surveillance of CYD14 and CYD15. The sieve analyses supported the finding that the VE of the CYD-TDV vaccine was invariant across DENV viruses with different prM/E protein amino acid sequences in children/adolescents aged 9 y and older in both trials. While there was a nonsignificant trend in CYD15 for lower VE against DENV variants with very large amino acid mismatch distances to the vaccine strains, our main interpretation is that VE is stable across DENV amino acid variants in the older age cohort.

The sieve analysis demonstrated statistically significantly greater VE against DENV3 and DENV4 than against DENV1 and DENV2, again in both trials. In CYD14, VE against DENV-Any declined significantly with an increasing number of vaccine-mismatched residues, both marginally and controlling for serotype, and these analyses are causal analyses based on the randomization. The marginal decline in VE with the mismatch distance was partially associated with an observed gradient in VE across serotypes, in that the DENV3 and DENV4 vaccine strain amino acid sequences were closer to the circulating DENV3 and DENV4 viruses (in amino acid mismatch distances and in 9-mer coverage) than were the DENV1 and DENV2 vaccine strain amino acid sequences to the circulating DENV1 and DENV2 viruses. This finding could explain why the VE was greater against DENV3 and DENV4 than against DENV1 and DENV2 in both trials. The DENV2 circulating viruses were particularly far from
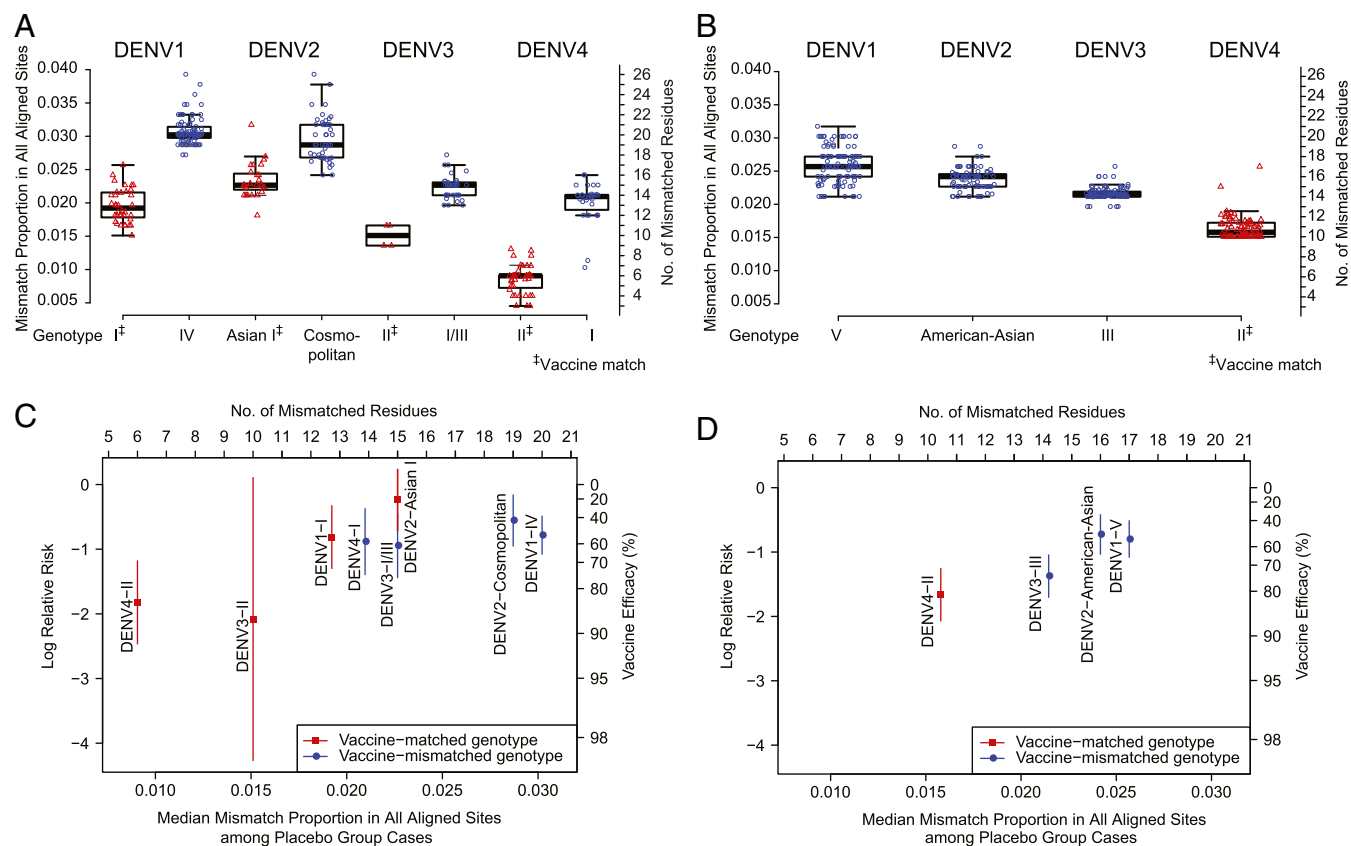

Fig. 6. (*A* and *B*) Amino acid sequence distances to the vaccine insert sequences among placebo group cases in the ITT cohort of CYD14 (2- to 14-y-olds) (*A*) and CYD15 (9- to 16-y-olds) (*B*). (*C* and *D*) VE (on the log relative risk scale) versus median amino acid sequence distance from the vaccine insert sequence among placebo group cases in the ITT cohort of CYD14 (2- to 14-y-olds) (*C*) and CYD15 (9- to 16-y-olds) (*D*). The vertical bars show 95% CIs.

the vaccine in CYD14, with 47% of DENV2 placebo cases having amino acid sequence mismatch proportion distances in all aligned sites greater than all corresponding amino acid distances of DENV3 and DENV4 placebo cases; this may help explain why VE was lowest against DENV2 in the CYD14 trial and was estimated to be near zero in CYD23 (16). However, an interpretation that VE would be expected to increase if the DENV vaccine strains were more closely matched to the circulating DENV viruses has the caveat that the "coverage" analyses of Fig. 6 detect associations that do not imply causation—other factors besides amino acid sequence distance could cause the associations. One example of a possible alternative cause comes from the analysis of DENV2, where the point estimate of VE in CYD14 against the vaccine-matched genotype was smaller than that against the vaccine-mismatched genotype (although not significantly so; $P = 0.32$) (Figs. 3C and 6). Thus, future studies will be important for testing the hypothesis that closer vaccine match leads to greater VE.

The sieve analysis found that VE against DENV-Any was significantly greater against disease-causing DENVs with a vaccine-matched residue at position E226 (or E224 for DENV3), which was attributed entirely to the DENV2 endpoint and seemed to be restricted to 2- to 8-y-olds. Residue E226 lies in the footprint of the human monoclonal antibody 2D22 (17), which specifically neutralizes DENV2 (18). However, residue E226 was not in the set of residues hypothesized to make important contacts between the DENV virion and the 2D22 antibody (17). Considering the complex structure of the quaternary epitope recognized by 2D22 (19), the significance of variation at position E226 with respect to NAb recognition must be addressed experimentally. We also note that the energetics of antibody–antigen interactions are generally influenced by only a small number of hot-spot residues at the paratope–epitope interface (20, 21), and thus it appears unlikely that the E226 sieve effect is related to 2D22-mediated neutralization. Alternatively, E226 may be involved in "zippering" of adjacent envelope molecules during viral membrane fusion. This possibility is supported by the crystal structure of late-stage fusion DENV1 envelope intermediates, in which residue E226T interacts with residue E403E of the adjacent DENV virion (22).

The sieve analysis also identified several amino acid sequence features and a genotype feature associated with variation in VE against DENV4, all of which were restricted to 2- to 8-y-olds. In this age category, VE was significantly greater against the vaccine-matched DENV4-II genotype and against DENV4 sequences with a vaccine-matched residue at positions pr73, M65, E46, E120, E160, E203, E461, and E478. This finding raises the hypothesis that these amino acid residues may be part of protective epitopes; experiments are currently underway to test this hypothesis. Of the identified signature positions, all except E160 covaried strongly with each other and with DENV4-I and DENV4-II, indicating that these results recapitulated the result of greater VE against DENV4-II than against DENV4-I. Position E160 had considerable residue variability within the DENV4-I genotype, suggesting that VE may have depended on this position in ways not fully explained by the two genotypes. Additionally, in 2- to 8-y-olds, VE against DENV4 declined significantly with an increasing number of vaccine-mismatched residues, again partially associated with differential VE against DENV4-II and DENV4-I in that circulating DENV4-II viruses were closer to the DENV4-II vaccine strain than circulating DENV4-I viruses. In contrast, in 9- to 14-y-olds, VE remained greater than 80% even against DENV4 sequences with the largest number of vaccine-mismatched residues in both trials. While no significant viral signatures of VE were found for DENV3, the point estimates of VE by genotype were similar to those for DENV4 in CYD14, such that the absence of evidence could be explained by the lower statistical power for DENV3 than for DENV4; this precludes the conclusion that DENV4 is distinctive compared with DENV3 in its amino acid sequence association with VE. Moreover, we note that some of the identified

differential VE signature positions may be false-positive findings, given that we used false-discovery rate adjustment and not the more stringent familywise error rate (FWER) adjustment. As such, these results advance hypotheses requiring further validation in immunology experiments and in efficacy trials. We suggest that future efficacy trials conduct sieve analyses of the same amino acid sequence features to help confirm or reject the results.

Analysis of the influence of amino acid positions on the decrease in VE against DENV4 with increasing percent residue mismatch to the vaccine in 2- to 8-y-olds suggests that differential VEs against vaccine-matched vs. mismatched sequences at all eight DENV4 signature positions, together with nonsignature positions E329 and E429, have the greatest influence in explaining the result (*SI Appendix*, Fig. S9). Four influential positions reside in mapped epitope regions of two recently isolated DENV4-specific human monoclonal NAbs DV4-126 and DV4-131, and, of those, three positions (E46, E120, and E203) are proximal to the residues K51, V53, K124, K200, and K202 in the E protein's DI/II hinge and domain DII identified as critical for DV4-126 and DV4-131 binding to DENV4 (the critical residues were perfectly matched in all DENV4 breakthrough sequences) (23). Fig. 7 displays the mapped epitopes of DV4-126, DV4-131, and 5H2 [a chimpanzee DENV4-specific monoclonal NAb (24)], in addition to the amino acids in the five DENV4 signature sites, in the crystal structure of the envelope ectodomain. All five ectodomain-resident DENV4 signature sites are located in the same hinge region and are located close to, or within, these MAb epitopes. High-resolution structures of the Fab fragments of DV4-126 and DV4-131 bound to DENV4 remain to be solved and will allow a focused sieve analysis using amino acid sequence distances based on the entire antibody footprints. Epitope-mapping studies of additional human DENV4-specific NAbs may provide further insight into the results.

Analysis of the CYD14 data suggested that age modified VE against the vaccine-mismatched genotype DENV4-I, estimated as 86% for 9- to 14-y-olds but only 24% for 2- to 8-y-olds (DENV4-I cases were not observed in CYD15). This finding is consistent with the corresponding VE estimates reported by Rabaa et al. (86.2% and 16.9%, respectively) (12). In contrast, age did not appear to impact VE against the vaccine-matched DENV-II genotype: Our estimates were 89.8% and 76.3%, and Rabaa et al. (12) reported 89.4% and 75.1% for the two age groups, respectively. As a potential explanation of this apparent moderating impact of age on VE against the vaccine-mismatched genotype, we note that the older age category had greater prior exposure to DENV, with 80% (68%) of 9- to 14-y-olds seropositive at baseline to any serotype (DENV4) compared with 58% (37%) of 2- to 8-y-olds and also had higher Month 13 titers in vaccine recipients, with geometric mean average titers to the four serotypes of 287 for 9- to
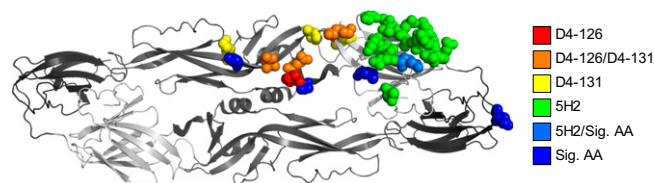

Fig. 7. Locations of DENV4 signature amino acid sites and the epitopes of DENV4-neutralizing monoclonal antibodies. The amino acids at the five DENV4 signature sites located in the ectodomain and the residues in the footprint of one or more DENV4-specific monoclonal antibodies (D4-126, D4-131, and 5H2) (23, 24) are shown on one monomer of the DENV E protein dimer (Protein Databank ID code 1OAN). Residues common to both the D4-126 and D4-131 epitopes are shown in orange, and the amino acids at signature sites present in the 5H2 epitope are shown in light blue. This figure was generated using MacPyMOL.

14-y-olds compared with 141 for 2- to 8-y-olds and geometric mean titers to DENV4 of 194 and 110, respectively. The difference in baseline seropositive frequency between the two age groups is relevant because the DENV4 NAbs elicited by CYD-TDV vaccination have been shown to differ in quality between seronegative and seropositive participants. DENV4 NAbs elicited by CYD-TDV vaccination of DENV-seronegative participants have been shown to be mostly homotypic, whereas in DENV-seropositive participants they were mostly heterotypic (25). Combined with the result that the DENV4 in placebo-recipient cases had amino acid sequence distances similar to those of the CYD-TDV vaccine in both age categories (*SI Appendix*, Fig. S10), the observations of Henein et al. (25) provide a potential explanation for the sieve effect observed here: We conjecture that the greater (both natural and vaccine-induced) and more heterotypic antibody responses in 9- to 14-y-old vaccine recipients in CYD14, who were more likely to be seropositive at baseline, generated greater humoral cross-reactivity (supported by ref. 25), thereby maintaining VE against mismatched DENV4s at a level comparable to that against the most closely vaccine-matched DENV4s. In contrast, CYD-TDV vaccination of 2- to 8-y-old vaccine recipients in CYD14, who were more likely to be seronegative at baseline, did not elicit DENV4-neutralizing heterotypic antibodies that may have protected them against amino acid sequence-mismatched DENV4. Moreover, the finding that CYD-TDV vaccination elicited mostly heterotypic DENV1, DENV2, and DENV3 NAbs in seronegative participants (25) could explain why the sieve effect here was observed for DENV4 only in 2- to 8-y-olds. Cumulatively, these results highlight the importance of improving vaccine-induced antibody responses for young children. Follow-up studies of antigen-specific neutralization responses could provide further insight into how response breadth reduces variation in VE with amino acid sequence distance of the virus.

We conclude by discussing some limitations of this study. First, there is evidence indicating that baseline serostatus modifies the efficacy of the CYD-TDV vaccine (3, 4, 26). However, baseline serostatus data were available/measurable for only a small set of CYD14 and CYD15 study participants, precluding an analysis that would directly assess baseline serostatus as a modifier of differential VE, which would have allowed investigation of the hypothesis that baseline serostatus constitutes the underlying cause of the effect modification by age. Second, while the clinical spectrum of DENV infection is broad, our analysis assesses only differential VE for the study outcome, VCD, which aggregates different severities of disease, and thus our study does not assess differential VE for other relevant dengue outcomes such as infection, VCD requiring hospitalization, or other definitions of severe dengue disease. Third, the sieve analysis plan was designed after the primary study results were available and was not specified in the original protocols. Fourth, analyses focusing on individual serotypes or genotypes had limited numbers of VCD endpoints, resulting in wide CIs about feature-specific VE and differential VE parameters. Fifth, technical limitations made it difficult to obtain prM/E sequences from VCD cases with low DENV viremia, raising the possibility that prM/E sequences are more readily missing from less virulent DENVs, complicating the interpretation of the results. Finally, our conclusions may be specific to the CYD-TDV vaccine; other DENV vaccines in development are of different types and include different DENV vaccine strain inserts, such that VE of these vaccines could have a different relationship with DENV amino acid sequences. These limitations notwithstanding, this study represents an advance toward understanding how dengue VE depends on dengue amino acid features and generates hypotheses meriting testing in laboratory and clinical studies.

Methods

Ethics Statement. The CYD14 and CYD15 trials were conducted at >30 sites in 10 countries (3, 4). The trial protocols were reviewed and approved by all relevant ethics review boards, including site-specific institutional review boards in addition to local and/or national ethics committees, when applicable (Brazil, Thailand). In accordance with local regulations, parents/guardians gave written informed consent, and older children provided written informed assent before participation.

Analysis Cohorts and Dengue Endpoints. We conducted the sieve analysis separately in each trial and in two study cohorts within each trial: the ITT cohort of participants who received the first immunization, and the Month 13 cohort consisting of ITT participants who neither experienced the primary dengue endpoint nor were lost to follow-up between the start of the trial and 28 d after the third immunization [409 (4.0%) and 900 (4.3%) ITT participants were excluded from the Month 13 cohort in CYD14 and CYD15, respectively]. The cohorts were followed for VCD for 25 mo and 12 mo, respectively, and five VCD endpoints were analyzed separately: the first occurrence of VCD of any serotype (DENV-Any) and the first occurrence of VCD of each individual serotype. Table 1 and *SI Appendix*, Table S4 list the numbers of participants in the ITT cohort with a VCD endpoint (i.e., cases) by serotype and genotype in each trial. In the vaccine and placebo group, 147 (51.4%) and 165 (53.4%) DENV-Any ITT cases in CYD14 and 134 (48.4%) and 199 (51.7%) DENV-Any ITT cases in CYD15 had complete amino acid sequence information in the prM/E region (*SI Appendix*, Table S1). Point estimates of VE in analyses using the ITT cohort and those using the Month 13 cohort were similar in each trial. We focus on the results of the ITT analysis, in part because of its greater precision due to the 59% (CYD14) and 37% (CYD15) fewer DENV-Any cases in the Month 13 vs. ITT cohort.

DENV Sequencing and Sequence Selection. DENV nucleotide sequences were determined from ITT VCD cases occurring during the active surveillance phase (12). The prM/E portion of the DENV genome was sequenced, translating to 661 amino acids (659 for DENV3) and representing the complete antigen-coding region of each vaccine insert. The amino acid sequences were multiply aligned with the four vaccine sequences. We observed no deletions and only one insertion: a valine following position E485 in a DENV-4 sequence found in a CYD15 placebo recipient.

A single sequence was obtained from each case except for five placebo recipients in CYD14, six vaccine recipients in CYD15, and eight placebo recipients in CYD15 with a VCD endpoint with two serotypes at a single visit. For these participants, a single sequence, observed or imputed, was randomly selected for inclusion in the DENV-Any analysis. Additionally, four vaccine recipients and eight placebo recipients in CYD14 (two vaccine recipients and one placebo recipient in CYD15) had VCD endpoints with two distinct serotypes at different visits; only the sequence pertaining to the analysis endpoint was included. The observed sequence was assumed to be a sequence present at the time of exposure that led to the dengue infection associated with the VCD endpoint.

The Amino Acid Sequence Imputation Process. One hundred thirty-one (45.8%) vaccine and 134 (43.4%) placebo DENV-Any cases in CYD14 and 125 (45.1%) vaccine and 173 (44.9%) placebo DENV-Any cases in CYD15 had completely missing sequences due to a DENV titer below the lower limit of quantification, insufficient (<250 μL) volume of unthawed serum, or a lack of participant consent. The numbers of vaccine and placebo DENV-Any cases with partial sequence data (i.e., missing sequence data in the N- and/or C-terminal regions of prM/E) were 13 (4.7%) and 12 (4.0%), respectively, for CYD14 and 15 (4.4%) and 12 (3.5%), respectively, for CYD15. In both trials, the presence of missing data in the terminal regions was largely due to incomplete PCR amplification stemming from insufficient primer coverage. The number of missing amino acid residues in the terminal regions ranged from 2 to 508 (median = 34) in CYD14 and from 2 to 498 (median = 33.5) in CYD15. Each missing sequence was imputed as a full-length sequence by randomly selecting an observed sequence from a participant's geographically proximal cases infected with the same serotype. Three levels of geographical proximity—clinic, site, and country—were considered. Imputation was performed at a broader level only if there were no serotype-matched cases at the more constrained level. Imputation of partially missing sequences was performed separately for the N- and C-terminal regions following a similar random selection process from cases infected with the same serotype and circulating genotype. Fourteen cases in CYD14 and 27 cases in CYD15 lacked serotyping information, which precluded imputation of their missing sequences. All these cases were excluded from analyses involving serotype or sequence data. Additionally, no proximal cases were found for four cases in CYD14 and four cases in CYD15 at any of the geographic levels, and they were excluded from analyses involving sequence data. The numbers of cases with imputed sequence information are summarized by geographical proximity in *SI Appendix*, Table S1. Twenty imputed datasets were generated for analysis.

The missing sequences do not bias the statistical inferences, because the imputation model was nonparametric and the imputations were highly accurate, given that geographical location and serotype were strongly predictive

Table 1. Numbers of VCD endpoint cases in the ITT cohorts of CYD14 and CYD15

Serotype	Genotype	DENV-Any endpoint		Serotype-specific endpoint	
		Vaccine, n (%)	Placebo, n (%)	Vaccine, n (%)	Placebo, n (%)
All participants in CYD14 (2- to 14-y-olds)					
DENV1		116 (40.6)	119 (38.5)	116	126
	I*	15 (12.9)	16 (13.4)	15 (12.9)	18 (14.3)
	IV	40 (34.5)	50 (42.0)	40 (34.5)	51 (40.5)
	Missing[†]	61 (52.6)	53 (44.5)	61 (52.6)	57 (45.2)
DENV2		94 (32.9)	70 (22.7)	97	74
	Asian I*	26 (27.7)	15 (21.4)	28 (28.9)	15 (20.3)
	Cosmopolitan	27 (28.7)	18 (25.7)	28 (28.9)	21 (28.4)
	Missing[†]	41 (43.6)	37 (52.9)	41 (42.3)	38 (51.4)
DENV3		30 (10.5)	43 (13.9)	30	43
	I	9 (30.0)	14 (32.6)	9 (30.0)	14 (32.6)
	II*	0 (0.0)	4 (9.3)	0 (0.0)	4 (9.3)
	III	4 (13.3)	7 (16.3)	4 (13.3)	7 (16.3)
	Missing[†]	17 (56.7)	18 (41.9)	17 (56.7)	18 (41.9)
DENV4		39 (13.6)	70 (22.7)	40	72
	I	19 (48.7)	18 (25.7)	19 (47.5)	18 (25.0)
	II*	7 (17.9)	23 (32.9)	8 (20.0)	24 (33.3)
	Missing[†]	13 (33.3)	29 (41.4)	13 (32.5)	30 (41.7)
Missing[‡]		7 (2.4)	7 (2.3)	—	—
Total		286	309	—	—
All participants in CYD15 (9- to 16-y-olds)					
DENV1		98 (35.4)	107 (27.8)	99	109
	V	52 (53.1)	74 (69.2)	53 (53.5)	76 (69.7)
	Missing[†]	46 (46.9)	33 (30.8)	46 (46.5)	33 (30.3)
DENV2		80 (28.9)	81 (21.0)	83	83
	American-Asian	48 (60.0)	49 (60.5)	48 (57.8)	50 (60.2)
	Missing[†]	32 (40.0)	32 (39.5)	35 (42.2)	33 (39.8)
DENV3		52 (18.8)	102 (26.5)	55	106
	III	23 (44.2)	45 (44.1)	23 (41.8)	47 (44.3)
	Missing[†]	29 (55.8)	57 (55.9)	32 (58.2)	59 (55.7)
DENV4		32 (11.6)	83 (21.6)	32	83
	II*	11 (34.4)	31 (37.3)	11 (34.4)	31 (37.3)
	Missing[†]	21 (65.6)	52 (62.7)	21 (65.6)	52 (62.7)
Missing[‡]		15 (5.4)	12 (3.1)	—	—
Total		277	385	—	—

*This genotype is contained in the vaccine.
[†]Sequences were unobtainable due to a DENV titer below the lower limit of quantification, insufficient volume of unthawed serum, or a lack of participant consent. Some participants with VCD were found to be positive for more than one serotype. These participants were included in the analysis for each appropriate endpoint. For the DENV-Any endpoint, the sequence from the earliest VCD event was included for analysis.
[‡]Participants were confirmed as VCD cases, but the disease-causing strains could not be positively serotyped. These participants were not included in the analysis, as their sequences could not be imputed without serotype information.

of sequence content. This accuracy was verified by testing the imputation procedure on cases with observed complete sequences (*SI Appendix*, Fig. S11).

VE by a Dengue Amino Acid Sequence Feature or Genotype. We define "feature-specific VE" as 1 minus the ratio (vaccine/placebo) of instantaneous incidences of a VCD endpoint with a specific amino acid sequence feature or genotype of the associated breakthrough DENV. Each amino acid sequence feature is defined as a contrast between a case's amino acid sequence and the vaccine immunogen sequence of the same serotype, henceforth referred to as the "reference sequence." Definitions of all analyzed amino acid sequence features were finalized before treatment-unblinded analysis. A sieve effect is defined as statistically significant evidence for differential VE across multiple levels of a given amino acid sequence feature. VE against each defined VCD endpoint of a specified serotype or amino acid sequence type was estimated as 1 minus the estimated hazard ratio (vaccine/placebo) of VCD with this type, using a competing risks Cox model stratified by protocol-specified age category and country (15). Wald tests were used to test for differential VE against a vaccine-matched vs. -mismatched amino acid sequence feature. In all analyses of amino acid sequence feature-specific VE, multiple imputation (27) was used to obtain point and 95% CI estimates of VE parameters and P values.

First, we assessed how VE varied with the amino acid sequence distance (residue mismatch proportion in a specified set of amino acid positions) of a case's virus to the vaccine construct reference sequence. For the DENV-Any endpoint, a "marginal" analysis estimated the VE-by-distance curve ignoring the serotype information, while a "serotype-adjusted" analysis estimated these curves separately for each serotype category (DENV1/2 and DENV3/4). These analyses did not consider separate curves for all four serotypes given limited numbers of serotype-specific VCD endpoints, and the choice to assume a common curve for DENV1 and DENV2 and for DENV3 and DENV4 was made based on similar VE estimates against DENV1 and DENV2 and against DENV3 and DENV4. A distance-specific hazard-ratio model was used to estimate the marginal and serotype-adjusted VE-by-distance curves (28), where the latter analysis assumed a constant shift in the log distance-specific hazard ratio across the serotype categories. Wald tests from this model were used to test for variation in VE with the distance.

Next, using phylogenetic genotypes for DENV1 (29), DENV2 (30), DENV3 (31), and DENV4 (32), we dichotomized cases according to whether a case's genotype matched or mismatched the genotype of the vaccine reference sequence. Thereafter, we conducted a site-scanning analysis restricted to amino acid positions that exhibited sufficient residue variability to allow

possible detection of a sieve effect. A position was declared sufficiently variable if four or more cases had a residue matching the residue in the vaccine construct reference sequence and four or more cases had a residue mismatching the residue in the reference sequence. In this position-specific analysis, we dichotomized cases according to whether a case's residue at a given position matched or mismatched the residue in the reference sequence. This analysis has the objective of generating hypotheses about amino acid positions included in protective epitopes whose protection depends partially on amino acid match.

We graphically assessed the association of the estimated VEs against each of the circulating matched and mismatched genotypes with the proximities of the vaccine strains to the circulating DENV sequences. This proximity for each genotype was quantified by the amino acid sequence distances of placebo-recipient cases to the vaccine strains, which represent the distribution of circulating viruses causing VCD during the trial.

Finally, the influence of each amino acid position on the observed decrease in VE with increasing amino acid sequence distance was quantified by the product of a differential VE term and an amino acid sequence diversity term, the former defined by the difference in estimated log hazard ratio (vaccine/placebo) for residue-matched vs. residue-mismatched dengue and the latter defined by the prevalence of the minority residue property (matched or mismatched) in placebo-recipient cases. The two product terms were individually scaled between 0 and 1 to give them the same influence, and then the product (the position influence) was scaled between 0 and 1.

Sets of Amino Acid Positions. The distance-specific and site-scanning VE analyses were conducted for all eligible amino acid positions (defined in *Results*) and for prespecified amino acid subsets based on biological knowledge [sets of amino acid positions modeled as highly solvent-accessible (further details are given in *SI Appendix, Supplementary Text*) and a set of human NAb contact sites (33)].

Covariability Analysis. Covariation between pairs of amino acid positions was calculated using the normalized mutual information statistic based on the Kullback–Leibler divergence between the observed joint distribution of amino acids at a given position pair and the expected joint distribution if the positions were independent (34). P values testing for covariation were obtained using a permutation test with 1,000 permutations.

Multiplicity Adjustment. Adjustments of sieve effect test P values for multiple testing were applied separately to the two trials and the two analysis cohorts within each trial. A multiple comparison procedure was applied to all serotype pairs within each trial in Fig. 3 *A* and *B*, to all five endpoints in Fig. 3*C*, and to the two age categories in Fig. 3*D*. The VE analysis by amino acid sequence distance treated all marginal and adjusted tests for all three amino acid site sets as multiple comparisons, separately for each endpoint. The site-scanning VE analysis treated all eligible amino acid positions as multiple comparisons, separately for each endpoint. In all analyses, adjustments to control the false-discovery rate (35) (Q values) and the FWER (36) were applied. Results with unadjusted P values ≤ 0.05 and either Q values ≤ 0.2 or FWER P values ≤ 0.05 were considered to indicate statistical significance. Tables and figures report unadjusted P values. All P values and Q values are two-sided.

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