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Characterization of recombinant yellow fever-dengue vaccine viruses with human monoclonal antibodies targeting key conformational epitopes

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ABSTRACT

The recombinant yellow fever-17D–dengue virus, live, attenuated, tetravalent dengue vaccine (CYD-TDV) is licensed in several dengue-endemic countries. Although the vaccine provides protection against dengue, the level of protection differs by serotype and warrants further investigation. We characterized the antigenic properties of each vaccine virus serotype using highly neutralizing human monoclonal antibodies (hmAbs) that bind quaternary structure-dependent epitopes. Specifically, we monitored the binding of dengue virus-1 (DENV-1; 1F4), DENV-2 (2D22) or DENV-3 (5J7) serotype-specific or DENV-1–4 cross-reactive (1C19) hmAbs to the four chimeric yellow fever-dengue vaccine viruses (CYD-1–4) included in phase III vaccine formulations using a range of biochemical and functional assays (dot blot, ELISA, surface plasmon resonance and plaque reduction neutralization assays). In addition, we used the "classic" live, attenuated DENV-2 vaccine serotype, immature CYD-2 viruses and DENV-2 virus-like particles as control antigens for anti-serotype-2 reactivity. The CYD vaccine serotypes were recognized by each hmAbs with the expected specificity, moreover, surface plasmon resonance indicated a high functional affinity interaction with the CYD serotypes. In addition, the hmAbs provided similar protection against CYD and wild-type dengue viruses in the *in vitro* neutralization assay. Overall, these findings demonstrate that the four CYD viruses used in clinical trials display key conformational and functional epitopes targeted by serotype-specific and/or cross-reactive neutralizing human antibodies. More specifically, we showed that CYD-2 displays serotype- specific epitopes present only on the mature virus. This indicates that the CYD-TDV has the ability to elicit antibody specificities which are similar to those induced by the wild type DENV. Future investigations will be needed to address the nature of CYD-TDV-induced responses after vaccine administration, and how these laboratory markers relate to vaccine efficacy and safety.

1. Introduction

Dengue is a mosquito-borne disease caused by one of four closely related but antigenically distinct virus serotypes of the genus Flavivirus in the family *Flaviviridae*. An estimated 390 million dengue infections occur annually, with 96 million experiencing clinically apparent disease [1]. The efficacy of Sanofi Pasteur live, tetravalent, recombinant Yellow fever 17D–dengue vaccine (CYD-TDV) was demonstrated against virologically-confirmed dengue in two pivotal phase III studies [2–4]. The World Health Organization (WHO) then recommended that countries with a high burden of dengue disease consider the introduction of CYD-TDV, which has since been licensed in several countries as Dengvaxia™. Vaccine efficacy, however, varies by serotype with higher efficacy rates against dengue virus-3 (DENV-3) and DENV-4 than against DENV-1 and DENV-2, despite similar neutralization antibody geometric mean titers induced across all four serotypes [2–4], as observed in an earlier phase IIb trial [5].

In order to further address potential differences between serotypes, we sought to determine (a) if key conformational epitopes, that could be the target of the neutralizing antibody responses

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were present in vaccine preparations; (b) whether the variable CYD-TDV efficacy by serotype was linked to serotype-specific responses and differing epitope presentation; and (c) whether conserved epitopes targeted by broadly cross-neutralizing antibodies are present on each CYD serotype. We used human monoclonal antibodies (hmAbs) targeting key serotype-specific quaternary structure-dependent epitopes (designated hmAbs 1F4, 2D22, and 5J7) or a conserved key epitope targeted by a broadly neutralizing hmAb (1C19) [6], and assessed whether these epitopes were present and accessible on the chimeric yellow fever-dengue (CYD-1–3) vaccine viruses. At the time of our study, hmAbs against DENV-4 were not available and as such specific interactions with this serotype could not be assessed. Live attenuated dengue vaccine and immature viruses or virus-like particles (VLPs) for serotype 2 were also used as controls for this serotype, as efficacy against serotype 2 appears lowest with CYD-TDV.

2. Material and methods

2.1. Tetravalent dengue vaccine (CYD-TDV; Dengvaxia™)

The dengue vaccine utilizes the yellow fever vaccine 17D (YFV-17D) backbone in which the structural preMembrane (prM) and envelope (E) genes were exchanged with the corresponding genes of each dengue serotype (DENV-1–4). The parental strains of the four chimeric dengue vaccines are as follows: Thai strain PUO-359/TVP-1140 for CYD-1, Thai strain PUO-218 for CYD-2, Thai strain PaH881/88 for CYD-3, and Indonesian strain 1228 (TVP-980) for CYD-4 (for reviews see [12,13]).

2.2. Cells and viruses

CYD-1–4 viruses were produced on Vero cells (African green monkey kidney cells) adapted to serum-free culture and purified at Sanofi Pasteur (Marcy-L'Etoile and Neuville-sur-Saône, France). These virus batches were prepared according to Phase III process, and some used in the CYD-TDV formulations assessed in large-scale phase III efficacy trials (CYD14 and CYD15) (Table 1) [2–4,14–16]. Immature CYD-2 were produced on Vero cells in the presence of 20 mM NH_4Cl as previously described [17], modified as follows. The Vero cells were inoculated with CYD-2 at a multiplicity of infection (MOI) of 0.01 $CCID_{50}$/cell, and 24 h after infection, culture medium was replaced by NH_4Cl 20 mM supplemented medium. The DENV2 live-attenuated derivative of the wild-type Mahidol strain was adapted for growth in Vero cells to yield Vero dengue vaccine-2 (VDV-2), as described elsewhere [18].

VLPs from DENV-2 were produced in human embryonic kidney 293 cells (The Native Antigen Company Ltd, UK). These VLPs were expressed from prM and E dengue virus sequences in which the C terminal stem region of dengue E sequence was replaced with the corresponding sequence from the Japanese encephalitis virus (DENV-2 VLP). SDS-PAGE and cryo-electron microscopy (cryo-EM) showed that these DENV-2 VLPs were mainly of immature form (Supplementary Fig. S1).

2.3. Virus purification for surface plasmon resonance (SPR) experiments

SPR experiments required concentrated purified CYD viruses. In brief, monovalent vaccine bulk preparations stored at −80 °C were thawed at +4 °C. Sample concentration was adjusted at 11 $\log_{10}$ Geq/mL by dilution when necessary. PEG 8000/NaCl was added at 7% (v/v)/2.4% (w/v) final concentration. Samples were stirred for 2 h at +4 °C before centrifugation for 60 min at 16,800*g*. The pellet obtained was suspended in 50 mM Tris, NaCl 75 mM, 20 mM $CaCl_2$, 20 mM $MgCl_2$, pH8.0 (buffer A). The suspended samples were dialyzed against buffer A (Slide-A-Lyzer, 10 kDa MWCO, ThermoFisher Scientific, Walthman, USA) and concentrated by ultrafiltration (Amicon Ultracel, 100 kDa MWCO, Millipore, Billerica, USA). Glycerol 3% (v/v) was added to the concentrated samples. Total protein and Geq concentrations were determined by Bradford assay [19] and qRT-PCR, respectively (see below).

2.4. Viral genomic titration

CYD and VDV-2 viruses were quantified using YFV or DENV NS5 qRT-PCR assay, respectively. Viral RNAs were extracted with QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany). YFV NS5 qRT-PCR was performed as previously described [20]. DENV NS5 qRT-PCR reaction was performed with one-step QuantiTect® Probe RT-PCR Kit (Qiagen) on 5 μL of 10-fold serial dilution of RNA extracts, 0.5 μM of each primer (Forward 5′-AATGACAGACAC GACTCC-3′ and reverse 5′-CCCAAAACCTACTATCTTCAA-3′) and 0.2 μM of probe (5′-6-FAM-TGGAAGTCGGCACGTGA-MGB-3′) using a Stratagene thermocycler, analysis was performed with the MxPro

Table 1
Vaccine lots and assays used for each lot.

Serotype/vaccine	Batch	Phase III clinical studies[a]	Dot blot	Biacore®	ELISA	$PRNT_{50}$
CYD-1	FDV01131	CYD14/15/17/32	x	x	x	–
	FDV01151	CYD15/17	x	x	x	–
	FDV01168	CYD17	x	x	x	x
CYD-2	FDV01181	CYD14/17/32	x	x	x	x
	FDV01198	CYD15/17	x	x	x	–
	FDV01219	CYD17	x	x	x	–
	FDV01393	–	x	–	x	–
CYD-3	FDV01228	CYD14	x	x	x	–
	FDV01252	CYD15	x	x	x	–
	FDV01319	CYD15/17	x	x	x	–
	FDV01376	–	x	–	x	–
	FDV01868	–	–	–	–	x
CYD-4	FDV01254	CYD14	x	x	x	–
	FDV01286	CYD15	x	–	x	–
	FDV01318	CYD17	x	x	x	–
VDV-2	03LST026	–	x	–	x	–

[a] when applicable: CYD14: NCT01373281 ([2,4,14]; CYD15: NCT01374516 [3,4,14]; CYD17 NCT01134263 [15]; CYD32 NCT01254422 [16]; VDV-2 (Vero dengue vaccine, [18].

software (Agilent Technologies) and calculations of genomic titers were based on known concentrations of NS5 RNA transcripts.

2.5. Antibodies

Dengue-specific hmAbs, 1F4, 2D22, 5J7 and 1C19 were produced at Vanderbilt University Medical Center [6–11]. Human hybridoma cell lines secreting hmAbs were cultured in serum-free medium and supernatants collected. Mouse mAbs were produced from mouse ascites and biotinylated by Biotem (Apprieu, France). Table 2 provides a summary of the mAbs used in the study and of their specificity.

2.6. Neutralization assay

Neutralization of CYD viruses was assessed using a micro-$PRNT_{50}$ assay adapted from Timiryasova et al. [21]. Serial dilutions (1:2) of mAbs (1F4, 2D22, 5J7 or 4G2) were incubated with a viral suspension of each CYD serotype at a concentration of 200 PFU/50 μL. After incubation, 6×10^4 Vero cells/well were added. Cells were fixed, stained with serotype specific anti-E murine mAbs and revealed by colorimetric reaction with the addition of 1:5000 goat anti-mouse IgG-HRP and True Blue peroxidase substrate (KPL) (Jackson Immuno Research, West Grove, PA 19390, USA), as recommended by the manufacturer. Infectious events were counted using image analysis software (SPOT, Microvision®). Neutralizing end-point titers were expressed as the reciprocal dilution inducing 50% plaque reduction ($PRNT_{50}$).

2.7. Dot blot immuno-detection

Approximately 8–9 $\log_{10}$ of Geq/mL of each CYD bulk samples (Table 1) were adsorbed on 0.45 μm nitrocellulose membranes (Biorad, Hercules, CA 94547, USA). Negative control was undiluted Sanofi Pasteur rabies vaccine bulk. Membranes were hybridized with hmAb (1:500) at room temperature. As controls, membranes were incubated with the anti-E 3F11 and 4G2 murine mAbs, or with human anti-dengue polyclonal serum (1:100) (reference # 9,242,914 from SeraCare Life Sciences (Milford, MA 01757, USA)). Membranes were incubated with anti-species secondary antibodies conjugated to IRDye® 800CW (Rockland Immunochemicals Inc. Pottstown, PA 19464, USA). Fluorescence emission was acquired on an Odyssey® Fc Imaging System (LI-COR Biosciences, Lincoln, NE 68504, USA), after 1 min exposure at 800 nm.

2.8. Capture ELISA

Microtitre plates were coated with anti-E 3F11 or anti-pr 2H2 mAb at 0.5 μg/mL and 4 μg/mL in PBS 1X (Eurobio, Courtaboeuf, France), respectively, and incubated overnight at 4 °C. After a blocking step at 37 °C with PBS-Tween-milk, two-fold serial dilutions of viruses or VLPs were added to the wells and incubated 1 h at 37 °C. The plates were then washed 3 times with PBS-Tween before addition of biotinylated anti-E 5C12 murine mAb or 1F4, 2D22, 5J7 or 1C19 hmAbs at 1–4 μg/mL and incubation for 1 h at 37 °C. The plates were again washed before addition of horseradish peroxidase-streptavidin (HRP) (Sigma-Aldrich, St. Louis, MO 63118, USA) (125 ng/mL in PBS-Tween) or goat anti-human IgG conjugated with HRP (Sigma-Aldrich) (diluted 1:1000 in PBS-Tween-milk) into the wells exposed to biotinylated 5C12 mAb or the orther hmAbs, respectively, and followed by another 1.5 h incubation at 37 °C. The plates were finally washed before the addition of tetramethylbenzidine (TMB) (Tebu-bio laboratories, Le Perray-en-Yvelines, France) for 30 min in the dark at room temperature. The colorimetric reaction was stopped with HCl 1 N (VWR Prolabo Fontenay-sous-Bois, France) and optical densities (OD) measured at 450 and 650 nm on a plate reader Versamax (Molecular Devices, Sunnyvale, CA 94089, USA). Blank values (mean negative control values) were subtracted from sample raw data (OD read at 450–650 nm).

2.9. Surface plasmon resonance (SPR)

The Biacore T200™ instrument (GE Healthcare, UK) with the CM5 sensor chip (BR-1005–30, GE Healthcare, UK) activated (NHS/EDC) was used to assess antibody binding experiments. Anti-human IgG capture antibodies at 25 μg/mL in 10 mM acetate buffer pH 4.5 (BR-1003–50) were injected onto the sensor chip at a flow rate of 5 μL/min, providing a signal increase of ~10,000 resonance units (RU). Subsequently, virus-type specific antibodies were diluted to 7 μg/mL in HBS-EP buffer and injected into different flow cells at 7 μL/min, resulting in signal increase of 700–1800 RU. Antibodies specific to the four viral types were captured with similar efficiencies. After a washing with HBS-EP, diluted viral samples (3–5 μg/mL) were injected at 10 μL/min; the increase in RU was measured over 300 s and dissociation for 300 s. Virus/antibody complexes formed on the surface of the sensor chip were dissociated by injection of 3 M $MgCl_2$ at 30 μL/min.

For kinetics experiments, anti-human IgG capture antibodies, at a concentration of 25 μg/mL in 10 mM acetate buffer pH 4.5 (BR-1003–50), were injected leading to covalent antibody immobilization (8000–13,000 RU) by an amine coupling mechanism. Subsequently, virus-type specific antibodies were diluted 0.1–0.3 μg/mL in HBS-EP buffer and injected through the flow cells at a rate of 30 μL/min, in order to avoid mass transport limitations, for 5–10 s causing an increase of 8 RU. Following washing, 5 concentrations of each virus type (0.125–2 nM) were injected at 30 μL/min during 300 s and dissociation was followed for 1400 s. The virus/antibody complexes formed were dissociated by 3 M $MgCl_2$ at 30 μL/min between viral sample injections. Kinetics parameters analysis was performed using multi-cycle kinetic (MCK).

Table 2
Monoclonal antibodies used in the study.

Antibody	Origin	Specificity	Targeted proteins/E domains	Reference
1F4	Human	DENV-1	One E protein within a homodimer: DI, and DI-DII hinge (quaternary structure dependent)	[8,9]
2D22	Human	DENV-2	E homodimer: DIII + glycan loop (serotype specificity) on one E protein and DII around fusion loop (FL) on the other E protein.	[6,11]
5J7	Human	DENV-3	3 different E proteins within 2 homodimers and 3 domains, one on each E: DI/II hinge, DIII, DII including the FL	[6,10]
1C19	Human	Cross-reactive	E / DII bc loop (quaternary structure dependent)	[7]
3F11	Mouse	Cross-reactive	E	In-house
5C12	Mouse	Cross-reactive	E	
2H2	Mouse	Cross-reactive	pr peptide	[34]
4G2	Mouse	Cross-reactive	E/FL	[35]

3. Results

3.1. Human monoclonal antibodies recognize CYD viruses in dot blot assays

Human mAbs 1F4, 2D22, 5J7 and 1C19 were tested for their binding capacities to four CYD vaccine viruses. For each serotype, three to four CYD vaccine batches (Table 1) were adsorbed directly on a membrane. Two cross-reactive anti-E murine mAbs and DENV-convalescent human serum were shown to recognize all batches of the four different CYD serotypes, indicating proper adsorption of the vaccine viruses to the membrane (Fig. 1). Serotype-specific hmAb, 1F4, 2D22 and 5J7, bound exclusively to the corresponding CYD serotype batches, *i.e.*, CYD-1, CYD-2 and CYD-3, respectively, with no cross-reactivity observed. For DENV serotype cross-reactive 1C19 hmAb, the four CYD serotypes were detected equally. All batches tested per CYD serotype showed similar reactivity with the corresponding hmAb, indicating consistency across vaccine batches (Fig. 1). These results demonstrate that key serotype-specific and cross-reactive epitopes recognized by neutralizing hmAbs are present on CYD viruses.

3.2. Human monoclonal antibodies recognize CYD vaccine viruses in ELISA

3.2.1. Serotype-specific antibodies

ELISA curves indicated a serotype-specific binding of the three human mAbs to their corresponding CYD viruses (Fig 2a A–C). Cross-reactivity between CYD serotypes was not observed (Supplementary Fig. S2A–D).

Due to the propensity for DENV-2 to adopt temperature-dependent conformational changes [22], and the relatively low efficacy against DENV-2 observed with CYD-TDV in clinical trials [4], we focused on serotype 2; comparing the binding of 2D22 hmAb to CYD-2 and DENV-2 antigens. Immature CYD-2 or DENV-2 VLPs were also used as negative controls. CYD-2 and live attenuated VDV-2 viruses were detected with 2D22 hmAb (Fig 2b A, B). In contrast, immature DENV-2 VLP and immature CYD-2 were not recognized by 2D22 (Fig 2b A), but both antigens were detected with murine cross-reactive anti-E mAb 5C12 (Fig 2b B). Thus, although specific epitopes recognized by the hmAbs were present on CYD-1, CYD-2, VDV-2 and CYD-3 vaccine viruses, this was not the case with immature DENV-2 VLP or immature CYD-2.

3.2.2. Cross-reactive antibodies

Cross-reactive antibodies induced after secondary heterologous infection have a higher persistence and cross-neutralizing activity than those induced after primary infection, [23,24], and may correlate with protection conferred against subsequent DENV infections. When CYD viruses were captured by murine cross-reactive anti-E 3F11 mAb, binding with 1C19 was observed for the CYD-1–4 viruses (Fig 2c A, B) at the same level as murine anti-E cross-reactive mAb 5C12 (Supplementary Fig. S2D), indicating that the 1C19 epitope was present on all four CYD vaccine viruses.

3.3. Human monoclonal antibodies recognize CYD viruses in SPR analyses

The recognition of the hmAbs for CYD viruses was assessed by binding experiments to their respective monovalent clinical CYD virus preparations (Fig 3a A–D). The functional affinity analysis to immobilized hmAbs was done over a concentration range of 0.125–2 nM of purified viral samples (Fig 3b A–D). The respective calculations of association and dissociation rate constants (k_a and k_d, respectively), and equilibrium dissociation constant (K_D) are summarized in Table S1. Although the experiments were not designed to test monovalent interactions in a classic 1:1 interaction model, the Langmuir model provided the best fit. Calculated K_D values were in the lower pM range, corresponding to the lower affinity limit of the SPR instrument. It could therefore be concluded that all four hmAbs displayed similar functional affinities, in the pM range indicative of very tight binding.

3.4. Human monoclonal antibodies neutralize CYD viruses

To evaluate if the hmAbs tested were able to neutralize CYD vaccine viruses, a Vero cell-based neutralization assay was conducted. The 1F4, 2D22 and 5J7 hmAbs neutralized the homologous CYD viruses in a serotype-specific manner. Cross neutralization of the heterologous CYD serotypes was not observed (data not shown). The half maximal neutralizing inhibitory concentration (IC_{50}) values obtained with hmAbs were 10 to 100-fold lower than those obtained with the pan-flavivirus murine anti-E mAb 4G2 (Table 3). Although different assays were used, the low IC_{50} values obtained in the Vero cells-based $PRNT_{50}$ assay were the same order of magnitude as those previously obtained in U937/DC-SIGN cells by flow cytometry [6].

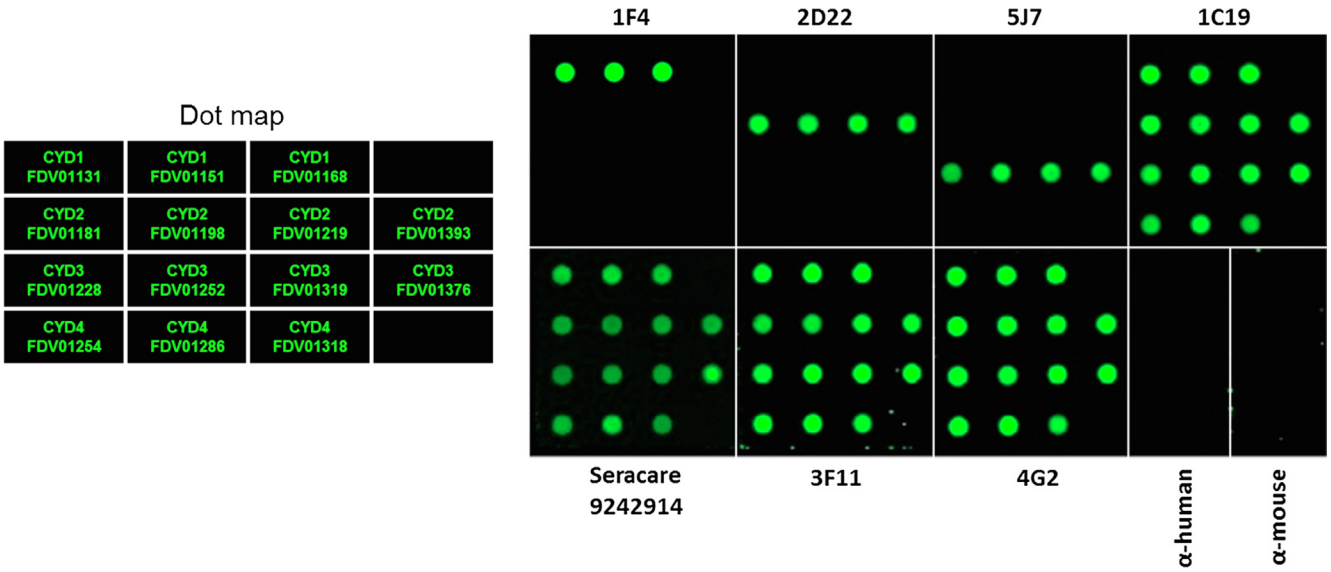

Fig. 1. Human mAb reactivity to CYD monovalent vaccine lots.

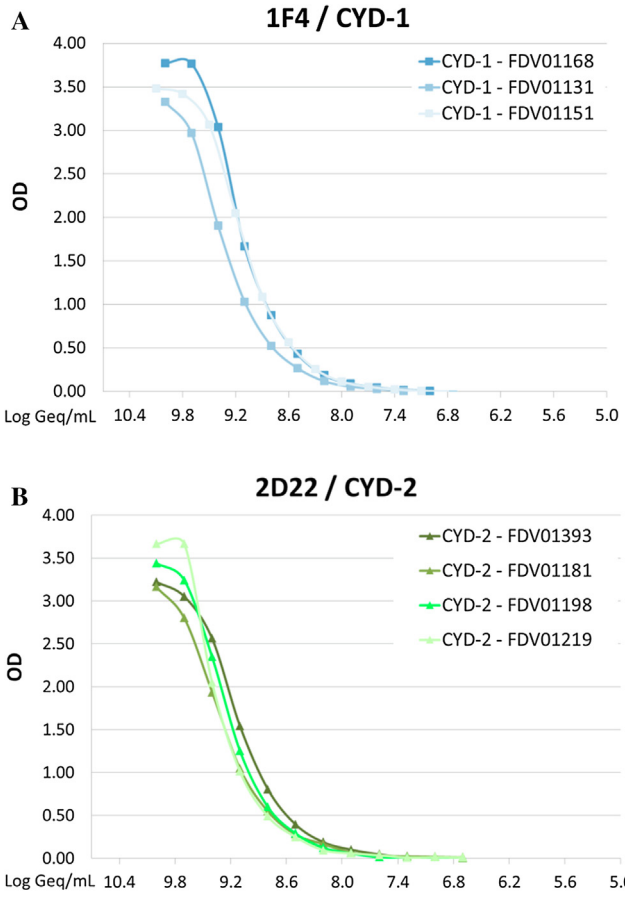

Fig. 2a. (A) ELISA 1F4/CYD-1; (B) 2D22/CYD-2; (C) 5J7/CYD-3.

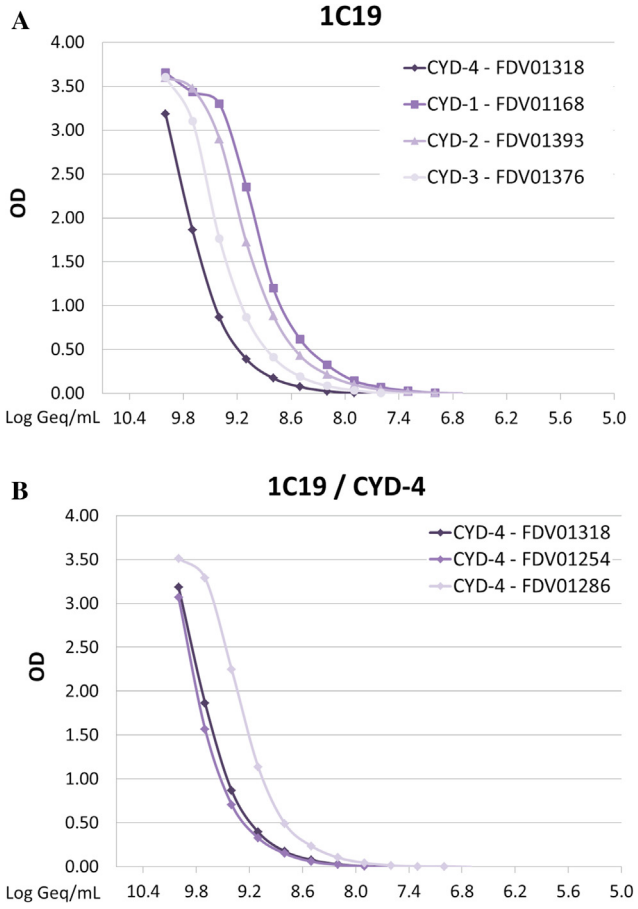

Fig. 2c. ELISA results: 1C19/ CYD-1-4 virus.

4. Discussion

Our study confirms the presence of key conformational epitopes on the surface of CYD viruses through the use of a panel of hmAbs that target specific quaternary structures on DENV viruses as the hmAbs binds the CYD viruses with the expected specificity and high functional affinity. Epitopes recognized by the three serotype-specific hmAbs have been mapped by cryo-EM structural studies of Fab-virus complexes [9–11] (Table 2), and residues potentially involved in 1C19 binding identified. These residues were conserved between the published epitope and DENV E consensus or CYD sequences with only a few exceptions, consistent with the observed CYD recognition [25]. In addition, the hmAbs

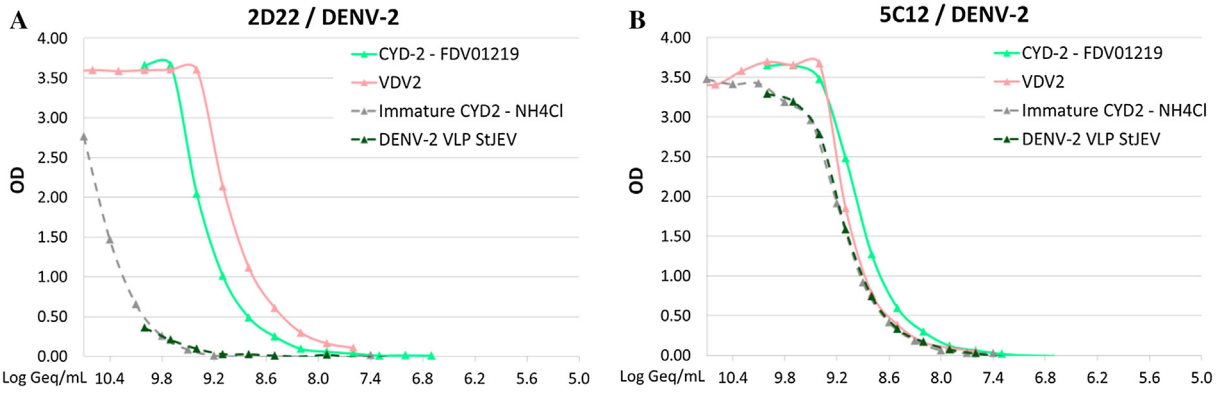

Fig. 2b. ELISA results: (A) 2D22 or (B) 5C12/DENV-2 immature CYD-2 virus and live attenuated DENV-2.

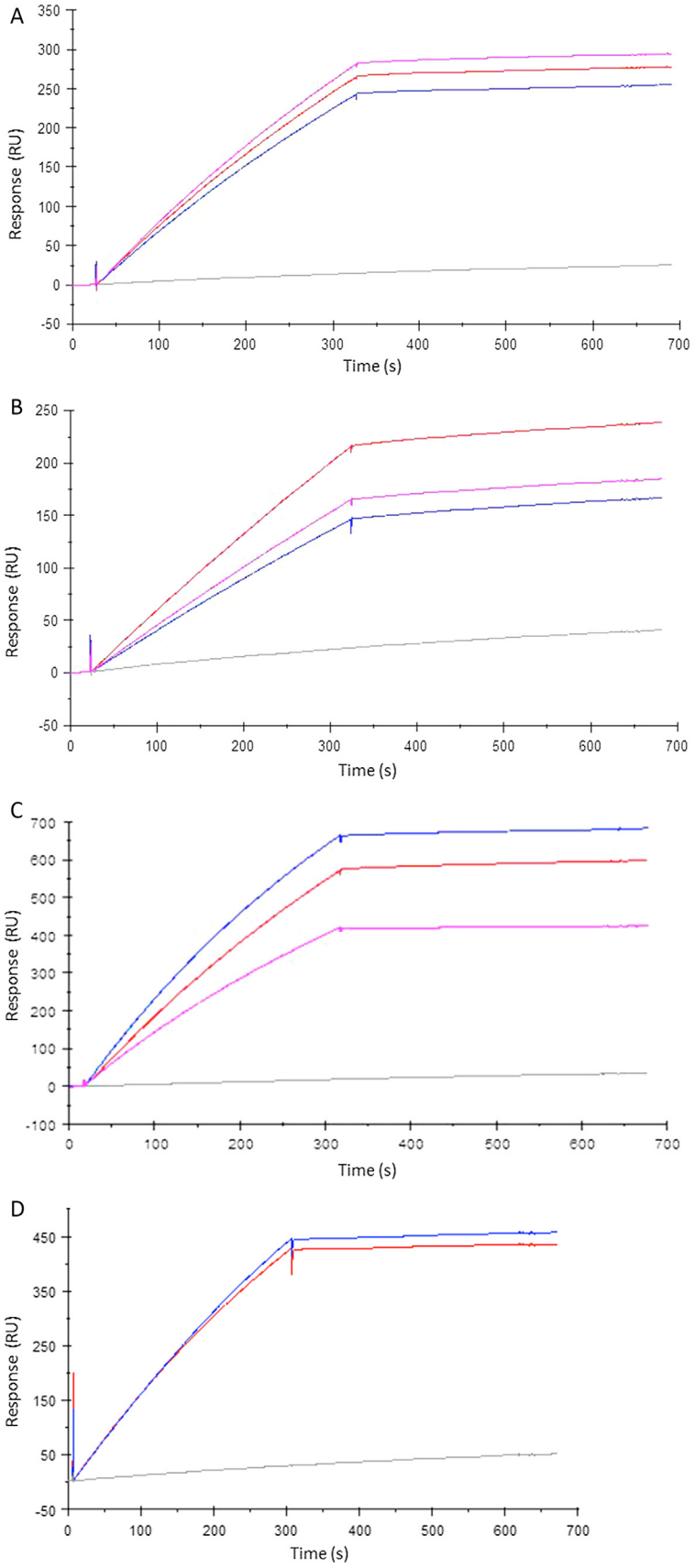

Fig. 3a. Human mAbs - CYD interaction assessed using surface plasmon resonance. (A) CYD-1 with hmAb 1F4, FDV01131 (red line), FDV01151 (blue line), FDV01168 (pink line), (B) CYD-2: hmAb 2D22/FDV01181 (red line), FDV01198 (blue line), FDV01219 (pink line), (C) CYD-3: hmAb 5J7/FDV01319 (red line), FDV01252 (blue line), FDV01228 (pink line), (D) CYD-4: hmAb 1C19/FDV01254 (blue line), FDV01318 (red line); buffer alone for all figures: grey line.

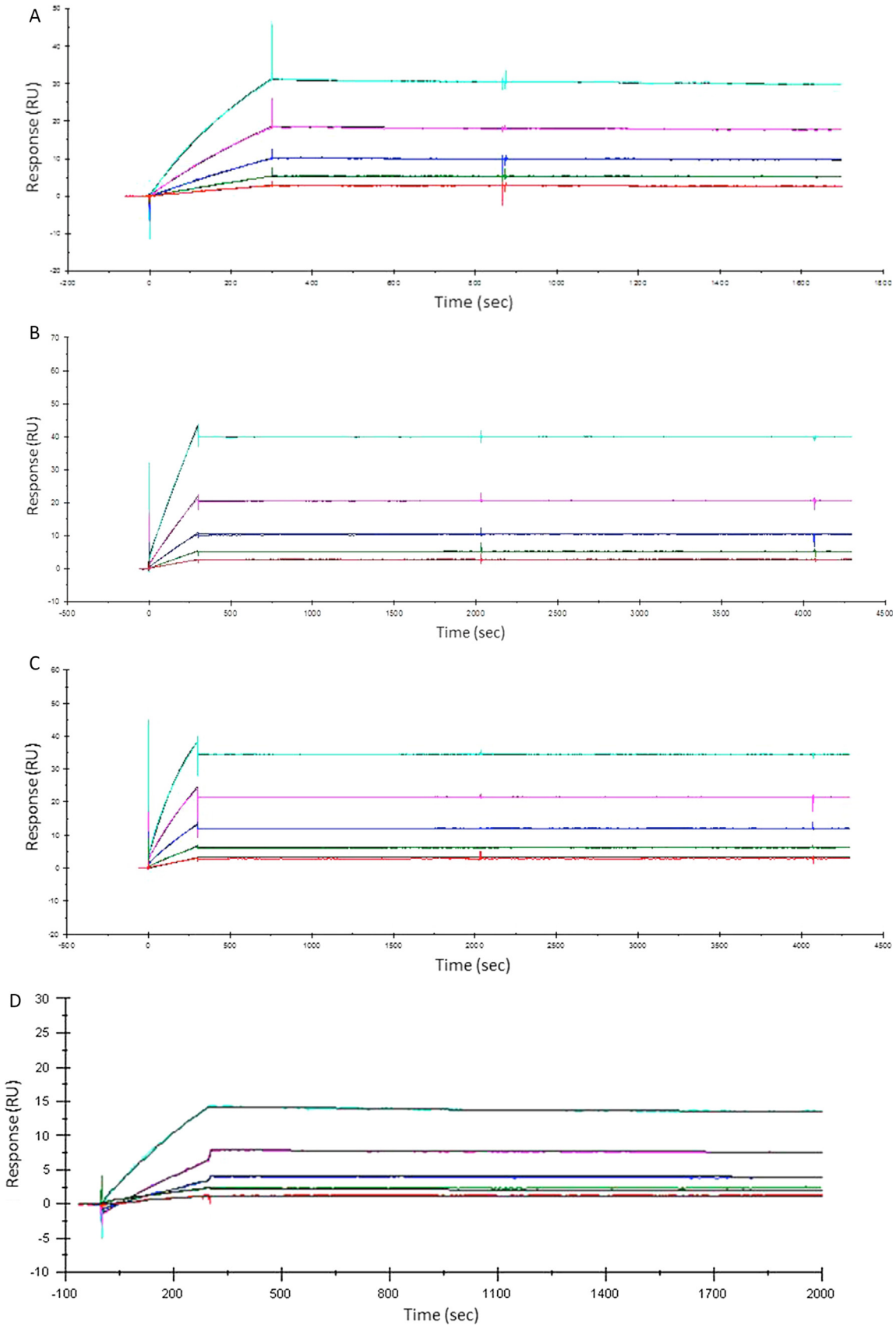

Fig. 3b. Determination of binding constants [36]. CYD-1 with hmAb 1F4 (A), CYD-2 with hmAb 2D22 (B), CYD-3 with hmAb 5J7 (C) and CYD-4 with hmAb 1C19 (D). Shown are SPR sensorgrams derived from the binding of different viral concentrations to the DENV-specific hmAbs. The following viral concentrations were used (from top to bottom): 0.125, 0.25, 0.5, 1, 2 nM. The derived kinetic parameters are provided in Table S1. The black line corresponds to the best fit of the experimental data with a Langmuir binding model of the BIAevaluation 4.1. software for the interaction of an antibody of 150kDa with a viral particle of 22,000 kDa.

Table 3
Neutralization properties of hmAbs against CYD viruses. Neutralization values in the Vero cells $PRNT_{50}$ neutralization of CYDs (IC_{50} μg/mL) assay. Examples from representative phase III CYD lots.

	Human mAbs			Murine mAb
	1F4	2D22	5 J7	4G2
CYD-1	0.03	–	–	5
CYD-2	–	0.05	–	0.37
CYD-3	–	–	0.09	0.83

tested in our study were highly neutralizing against the CYD viruses, in line with previously reported neutralization of wild-type viruses, again supporting similar conformations between vaccine and wild-type viruses. Our findings suggest that CYD viruses display, at least in part, a similar conformation to wild-type DENV strains. Although we were unable to test DENV-4 serotype-specific hmAbs, the highest homotypic responses in seronegative individuals are induced by CYD-4 [26], which suggests that conformational epitopes which target neutralizing responses are also present on this vaccine virus.

In the clinical studies, CYD-TDV efficacy against DENV-2 was lower than for other serotypes (DENV-3 and DENV-4, in particular) [4], which necessitated a more detailed examination of the CYD-2 virus. The 2D22 hmAb was unable to recognize immature DENV-2 VLPs or immature CYD-2 viruses, confirming that this antibody was specific to the mature-dimeric conformation of DENV-2, as suggested by the cryo-EM structure of DENV-2-2D22 complex [11]. As CYD-2 is recognized by the 2D22 hmAb, this indicates that it is mature to a large extent; this has been confirmed by mass spectrometry and cryo-EM (M. Trauchessec et al., unpublished results). In addition, the 2D22 hmAb reactivity against the "classic" live attenuated DENV-2 virus (VDV-2, "full-dengue") produced similar results to CYD-2, in agreement with the vaccine virus displaying a conformation at least partially similar to the "full-dengue" serotype 2 virus. Of notice, a recent study characterized the polyclonal antibody responses against DENV2 using two new DENV-2-specific hmAbs [28]; further work using these new mAbs could bring relevant additional information on the epitopes present on CYD2.The four CYD serotypes were also recognized by the cross-reactive 1C19 hmAb, isolated from a patient with a heterologous second dengue infection shown to compete with low-neutralizing pan-flavivirus antibodies targeting the fusion loop, as well as with the 1F4 hmAb [7]. Induction of cross-neutralizing antibodies may be involved in broad protection conferred after multiple DENV infections; this follows the discovery of antibodies such as 1C19, and those targeting envelope dimer epitope (EDE) [27]. Binding of the 1C19 hmAb to the four CYD viruses suggests that the CYD-TDV has the potential to induce such antibodies. Our data suggests that differing serotype-specific efficacies with CYD-TDV observed in clinical trials are not associated with inappropriate conformations for the less efficacious CYD-1 and CYD-2 serotypes, but other parameters may be involved [12]. Although the conformation of the viral antigens appears to be correct in the CYD-TDV preparations, further exploration in vaccine recipients is needed to determine the ability of the CYD viruses to induce significant levels of antibodies targeting the selected epitopes *in vivo*.

In this regard, the main conclusions of a recent analysis performed by Sanofi Pasteur was released in November 2017, which led not to recommend vaccination in individuals who have not been previously infected by the dengue virus ("seronegative individuals"). The analysis did, however, confirm that vaccination provides persistent protective benefit against dengue infections in those who have had a prior dengue infection. The importance of baseline serostatus was already identified and discussed, and was the basis of the cluster or "accelerated secondary infection" hypotheses raised by Sanofi Pasteur [29,30], meaning that the first wild type infection in seronegative vaccinees may correspond to a secondary one from an immunological standpoint, then more likely to be symptomatic/severe. However, this hypothesis also meant that this risk would be temporary, which was confirmed by additional data obtained after several years in the phase III efficacy trials [31].

Overall, it appears that the observed higher risk in seronegatives could be related to what has been observed in the field following primary wild type infection. In this regard, antibody dependent enhancement (ADE) has been proposed as one of the leading explanations for severe disease upon secondary infection. We did study carefully ADE activity after vaccination, and studies performed in FcγR+ cells with sera from vaccine recipients demonstrated no link between the level of protection and *in vitro* ADE activity [32]. Furthermore, no differences in the cytokine profile were observed between placebos and vaccines upon breakthrough dengue leading to hospitalization [33]. Taken together, these data do not support increased ADE activity in vaccine versus placebo recipients.

One can nevertheless discuss the results of our present study in light of this hypothesis: could the level and nature of the antibody response induced in seronegative vaccinees induce ADE activity *in vivo* and explain the observations?

First, while efficacy afforded by CYD1 and CYD2 in the efficacy trials was significantly lower than the one afforded by CYD3 and CYD4, it does not seem that CYD1 and CYD2 display unexpected structures based on our limited analysis, and also based on maturation levels (Trauchessec et al., unpublished results). Therefore, while these structural data are positive regarding the conformation of the injected vaccine, it is difficult to link them with short and long-term protection/safety. Nevertheless, ongoing experiments are currently investigating the nature and location of epitopes recognized by vaccine-induced responses in vaccines *in vivo*.

Second, regarding qualitative aspects of the immunogenicity of vaccine lots, when dissecting further serotype specificity using depletion assays, responses against DENV-4 in seronegatives are dominant, while responses against the three other serotypes are in the majority cross-reactive [26]. Our results suggest that the lower serotype-specific response against CYD1, CYD2 and CYD3 is not due to differences in viral structure, and that the imbalanced response towards CYD4 might rather be explained by *in vivo* CYD4 dominance at the replication and/or immunological levels. The cross-reactive responses against the other serotypes, afforded by this dominant CYD4 response, might not be potent enough, in contrast to the one elicited by prior wild type infection (s) in previously exposed individuals, for whom the vaccine may act in part as a booster of such previously induced responses [26]. In the context of such a broadly cross-neutralizing response, the impact of vaccine structure might also be more difficult and complex to assess. Overall, the causes of severe dengue are multifactorial, including the potential virulence of the infecting serotype, age, host genetic determinants and pre-immune status involving some types and profiles of humoral and cellular responses, and ADE may only play a partial role in this regard. Therefore, the present data, while important to consider, cannot predict the long term safety and efficacy of the vaccine after administration in the field.

In conclusion, the CYD-TDV preparations used in clinical trials, including the efficacy studies, display key conformational and functional epitopes. The use of additional monoclonal antibodies, such as those targeting EDE and the recently reported DENV-2 hmAbs may also provide important information [27,28]. Future investigations will need to address the capacity of CYD-TDV to elicit neutralizing antibodies against these critical epitopes, and how the observed immunogenicity relates to vaccine efficacy and safety, considering also serostatus of vaccines at baseline.

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Conflicts of interest

VL, CB, AS, SN, CM, YG-C, and NJ are employees of Sanofi Pasteur. BG was an employee of Sanofi Pasteur at the time of the study. J.E.C. has served as a consultant for Takeda Vaccines, Sanofi Pasteur, Pfizer, and Novavax, is on the Scientific Advisory Boards of CompuVax, GigaGen, Meissa Vaccines, PaxVax, and is Founder of IDBiologics, Inc.

Author contribution

NJ BG VL AS CM conceived and designed the experiments, CB SN AS performed the experiments, AS CB VL SN CM BG NJ analyzed the data, JC contributed reagents, YGC contributed analytical tools, BG VL CB CM AS JC YGC wrote the paper, all authors approved the final version of the paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.04.065.

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