The Human CD8⁺ T Cell Responses Induced by a Live Attenuated Tetravalent Dengue Vaccine Are Directed against Highly Conserved Epitopes

Daniela Weiskopf,[a] Michael A. Angelo,[a] Derek J. Bangs,[a] John Sidney,[a] Sinu Paul,[a] Bjoern Peters,[a] Aruna D. de Silva,[a,e] Janet C. Lindow,[b] Sean A. Diehl,[b] Stephen Whitehead,[c] Anna Durbin,[d] Beth Kirkpatrick,[b] Alessandro Sette[a]

Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, California, USA[a]; University of Vermont College of Medicine and Vaccine Testing Center, Burlington, Vermont, USA[b]; National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA[c]; Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA[d]; Genetech Research Institute, Colombo, Sri Lanka[e]

ABSTRACT

The incidence of infection with any of the four dengue virus serotypes (DENV1 to -4) has increased dramatically in the last few decades, and the lack of a treatment or vaccine has contributed to significant morbidity and mortality worldwide. A recent comprehensive analysis of the human T cell response against wild-type DENV suggested an human lymphocyte antigen (HLA)-linked protective role for CD8⁺ T cells. We have collected one-unit blood donations from study participants receiving the monovalent or tetravalent live attenuated DENV vaccine (DLAV), developed by the U.S. National Institutes of Health. Peripheral blood mononuclear cells from these donors were screened in gamma interferon enzyme-linked immunosorbent spot assays with pools of predicted, HLA-matched, class I binding peptides covering the entire DENV proteome. Here, we characterize for the first time CD8⁺ T cell responses after live attenuated dengue vaccination and show that CD8⁺ T cell responses in vaccinees were readily detectable and comparable to natural dengue infection. Interestingly, whereas broad responses to structural and nonstructural (NS) proteins were observed after monovalent vaccination, T cell responses following tetravalent vaccination were, dramatically, focused toward the highly conserved NS proteins. Epitopes were highly conserved in a vast variety of field isolates and able to elicit multifunctional T cell responses. Detailed knowledge of the T cell response will contribute to the identification of robust correlates of protection in natural immunity and following vaccination against DENV.

IMPORTANCE

The development of effective vaccination strategies against dengue virus (DENV) infection and clinically significant disease is a task of high global public health value and significance, while also being a challenge of significant complexity. A recent efficacy trial of the most advanced dengue vaccine candidate, demonstrated only partial protection against all four DENV serotypes, despite three subsequent immunizations and detection of measurable neutralizing antibodies to each serotype in most subjects. These results challenge the hypothesis that seroconversion is the only reliable correlate of protection. Here, we show that CD8⁺ T cell responses in vaccinees were readily detectable and comparable to natural dengue virus infection. Detailed knowledge of the T cell response may further contribute to the identification of robust correlates of protection in natural immunity and vaccination against DENV.

Infections with dengue virus (DENV) occur with high incidence in more than 100 countries around the world. Recent reports estimate the number of annual infections with any of the four DENV serotypes (DENV1 to -4) to be as high as 390 million, of which 96 million manifest as clinically significant diseases, including life-threatening conditions such as dengue hemorrhagic fever and dengue shock syndrome (1). This constitutes an increasing public health problem in tropical and subtropical regions and underscores the urgent need for a vaccine against DENV (2).

Exposure to one serotype confers long-term immunity to that serotype (homotypic immunity) but only short-term protection against the other three serotypes (heterotypic immunity), creating a unique challenge for vaccine developers (3). Indeed, suboptimal immune heterotypic responses have been associated with severe disease, which is most often associated with exposure to a secondary infection with a heterologous serotype (4–6). Thus, it is essential that vaccination induces a balanced and long-lasting protection against all four serotypes simultaneously. To date, correlates of protection are unknown, and proof of vaccine efficacy has to rely on large field-based phase III clinical trials. A recent efficacy trial of the most advanced dengue vaccine candidate, a live-atten-

uated tetravalent chimeric yellow-fever dengue vaccine in which all nonstructural proteins are derived from yellow fever 17D vaccine, demonstrated only partial protection against three of the DENV serotypes, despite three subsequent immunizations and high mean neutralizing antibody titers against all four serotypes in

most subjects. These results challenge the hypothesis that seroconversion is the only reliable correlate of protection (7).

A hallmark of live attenuated vaccines (LAV) is their ability to induce both humoral and cellular immune memory. It has been extensively shown that several DENV live attenuated vaccine (DLAV) candidates are able to induce neutralizing antibody responses against all serotypes (7, 8). However, whether these vaccines can also induce meaningful T cell responses against DENV has not been investigated in detail. Recent data have suggested an HLA-restricted protective role for CD8$^+$ T cells in natural infection, stressing the need to investigate the T cell immunity elicited by a DLAV (9). Here, we characterize immune responses induced by both monovalent and tetravalent DLAVs encoding a full complement of both structural and nonstructural (NS) DENV proteins. The responses induced are comparable to those seen in natural DENV infection in terms of specificity, breadth, magnitude, and functionality. We further report that tetravalent vaccination is associated with a response remarkably focused on T cell epitopes conserved among all four serotypes and among a vast variety of field isolates. These results are encouraging in the context of further evaluation of DLAVs in clinical trials.

MATERIALS AND METHODS

Ethics statement. The clinical data and serum samples for the present study were derived from separate phase I clinical trials performed at the University of Vermont (UVM) Vaccine Testing Center and the Center for Immunization Research at the Johns Hopkins School of Public Health (JHSPH). Clinical trials are described at Clincaltrials.gov under numbers NCT01084291, NCT01073306, NCT00831012, NCT00473135, NCT00920517, NCT00831012, and NCT01072786. Study design and clinical protocols were approved by the Committees for Human Research Protection (UVM) and the Western Institutional Review Board (JHSPH). Ethics approvals in Sri Lanka were obtained from the Ethics Review Committee, University of Colombo, Sri Lanka.

Study populations. Healthy adult male and nonpregnant female volunteers 18 to 50 years of age were enrolled and vaccinated with either one of the four monovalent vaccine components or a tetravalent vaccine formulation. Table S1 in the supplemental material lists HLA types and demographic information for all donors. All enrolled subjects were seronegative for all DENV serotypes, yellow fever virus, West Nile virus, St. Louis encephalitis virus, hepatitis B and C viruses, and human immunodeficiency virus. Study participants were recalled after vaccination (monovalent mean of 47 months [range, 33 to 90 months]; tetravalent mean, of 12 months [range, 11 to 13 months]) to donate a unit of blood. Blood samples from donors experiencing natural infection were obtained from healthy adult blood donors from the National Blood Center, Ministry of Health, Colombo, Sri Lanka, in an anonymous fashion. Donors were of both sexes and between 18 and 60 years of age (9). Blood processing and HLA typing of both study populations was performed as previously described (9).

Vaccines. Attenuation of the different dengue viruses was achieved by deleting one (rDEN1Δ30 and rDENV4Δ30) or two (DEN3Δ30/31) regions from the 3′ untranslated region (UTR), as previously described (10). DEN2/4Δ30 is a chimeric virus in which the DENV2 prM and E genes replaced those of the DEN4Δ30 vaccine candidate (10). For the tetravalent vaccination used in the present study (TV003), the four monovalent vaccines were combined into a tetravalent admixture prior to vaccination (8). Subjects received either one (DEN2/4Δ30, DEN3Δ30/31, and DEN4Δ30) or two (DEN1Δ30 and DEN2/4Δ30) doses of a monovalent vaccine or two doses of the tetravalent vaccine 6 months apart. Each monovalent vaccine was given at a dose of 10^3 PFU with the exception of five recipients of the DEN1Δ30 vaccine who received only 10^1 PFU. Each dose of the tetravalent vaccine contained 10^3 PFU of each of the monovalent components.

MHC class I binding predictions and peptide selection. Sets of 9- and 10-mer peptides encoded by the proteome of the vaccine strains were predicted for their binding affinity to 27 major histocompatibility complex (MHC) class I molecules. A panel of 16 HLA A alleles (A*01:01, A*26:01, A*32:01, A*02:01, A*02:03, A*02:06, A*68:02, A*2301, A*24:02, A*03:01, A*11:01, A*30:01, A*31:01, A*33:01, and A*68:01) and 11 HLA B alleles (B*40:01, B*44:02, B*44:03, B*57:01, B*58:01, B*15:01 B*07:02, B*35:01, B*51:01, B*53:01, and B*08:01) were selected; these alleles account for 97% of HLA A and B allelic variants in most ethnicities (11). Binding predictions were performed using the Immune Epitope Database MHC class I binding prediction tool available on the web site (http://tools .immuneepitope.org/) (12). For each allele and length combination, peptides from each included polyprotein were selected if they were in the top 1% of binders in a given strain. Homologous peptides from two or more serotypes that were predicted to bind to one HLA molecule were placed in the "conserved peptides" group. This resulted in the synthesis of 6,083 peptides (Mimotopes, Victoria, Australia), which were subdivided into pools of 10 individual peptides according to their HLA restriction and the vaccine serotype they are derived from (see Table S2 in the supplemental material).

Conservancy analysis. Full-length DENV polyprotein sequences were retrieved for each serotype from the National Center for Biotechnology Information (NCBI) protein database using the following query: txid11053 AND 3000:5000[slen] with the corresponding NCBI taxonomy identification substituted for each serotype. To eliminate geographical bias, the number of isolates from any one country was limited to 10. Sequences were considered unique if they varied by at least 1 amino acid from all other sequences. As a result, 162 DENV1, 171 DENV2, 169 DENV3, and 53 DENV4 sequences (a total of 555) were retrieved from the NCBI protein database and utilized to evaluate the conservancy of the identified epitopes within the sequences of the respective serotypes (13).

***Ex vivo* gamma interferon (IFN-γ) enzyme-linked immunosorbent spot (ELISPOT) assay.** We coated 96-well flat-bottom plates (Immobilon-P; Millipore) overnight with anti-IFN-γ monoclonal antibody (MAb; AN18; Mabtech). A sample of 2 × 10^5 peripheral blood mononuclear cells (PBMC) was incubated in triplicate cultures with 0.1 ml of complete RPMI 1640 in the presence of HLA-matched peptide pools (2 µg/ml). After 20 h of incubation at 37°C, the cells were incubated with biotinylated-IFN-γ MAb (7-B6-1; Mabtech) for 2 h and developed as previously described (9). Responses against peptides were considered positive if the net spot-forming cells (SFC) per 10^6 were ≥20, had a stimulation index of ≥2, and a $P < 0.05$ in a t test comparing replicates with those from the negative control. Pools positive in two individual experiments were subsequently deconvoluted to identify the individual epitopes eliciting the IFN-γ response.

Flow cytometry and intracellular cytokine staining. The following MAbs were used in this study: anti-CD8a V500 (RPA-T8) and anti-CD3 Alexa Flour 700 (UCHT1) (both from BD Biosciences), anti-CD45RA eFluor 450 (H100), anti-IFN-γ FITC (4S.B3), anti-IL-2 PE (MQ1-17H12), anti-TNF-α APC (MAb11), and anti-CD107a PE (clone ebioH4A3) (all from eBioscience), and anti-CCR7 PerCP-CY5.5 (G043H7; Biolegend). PBMC were cultured in the presence of HLA-matched peptide pools (10 µg/ml) and GolgiPlug containing brefeldin A (BD Biosciences) for 6 h and subsequently permeabilized, stained, and analyzed as previously described (9).

RESULTS

DENV-specific T cell responses are readily detected *ex vivo* after vaccination with monovalent live attenuated DENV vaccine candidates. To perform a comprehensive analysis of T cell responses after monovalent vaccination we examined responses from 42 recipients of four different monovalent DLAV candidates ($n = 11$ DENV1; $n = 10$ DENV2; $n = 11$ DENV3; $n = 10$ DENV4). PBMC samples from all study participants were screened in IFN-γ ELISPOT assays with pools of predicted class I
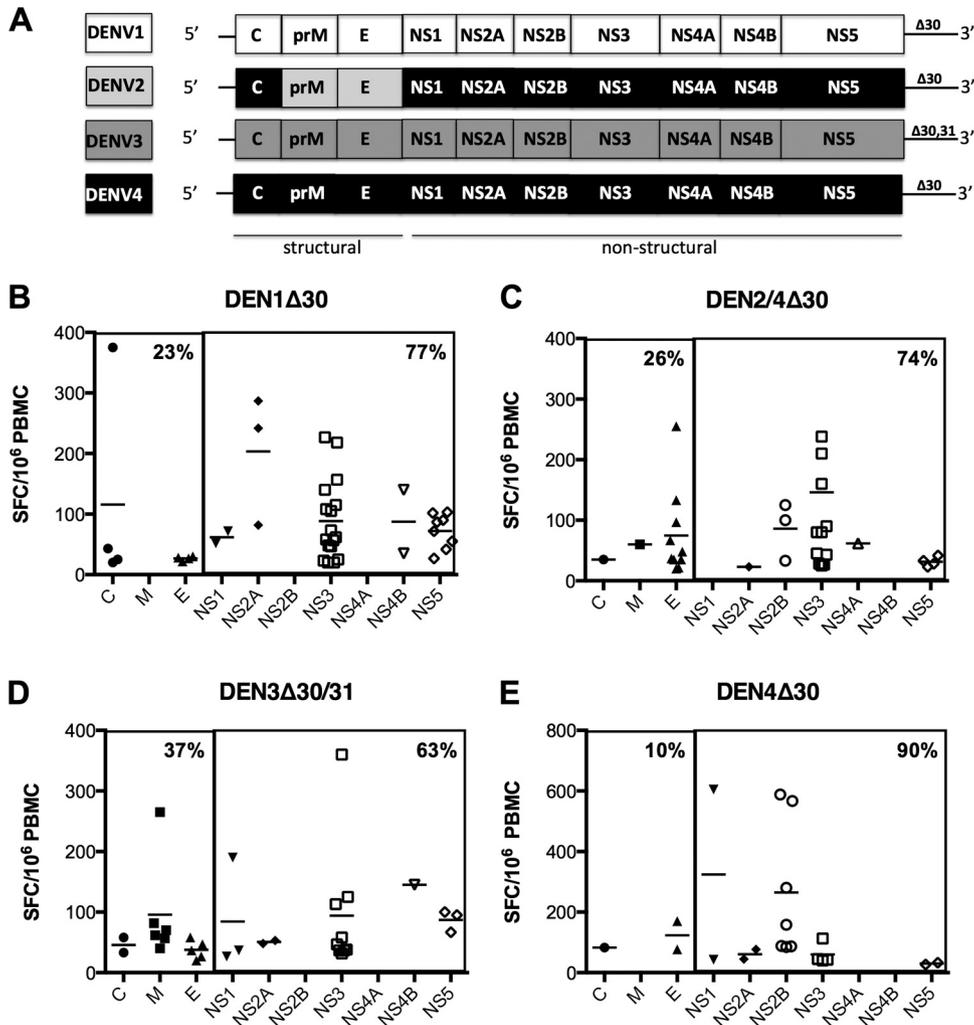
FIG 1 Protein location of epitopes varies as a function of the DENV serotype. (A) Study participants were vaccinated with one of the four live attenuated dengue vaccine candidates. PBMC from each donor were tested against peptides predicted for the exact strain with which they were vaccinated. Responses detected in study participants vaccinated with DEN1Δ30 (B), DEN2/4Δ30 (C), DEN3Δ30/31 (D), or DEN4Δ30 (E) live attenuated monovalent dengue vaccine candidates are shown. Our stringent criteria of positivity require consistent responses in two out of two independent experiments. Responses are expressed as the number of IFN-γ-secreting cells per 10^6 PBMC and considered positive if the magnitude of response to the test peptide was significantly different compared to a negative-control peptide ($P < 0.05$, Student t test) and the stimulation index (SI; SI = ratio test SFC/control SFC) was >2.0.

binding peptides (HLA-matched) corresponding to the specific DLAV serotypes of the administered vaccine. In the case of the DEN2/4Δ30 vaccine, DENV2 specific peptides were predicted for the prM and E proteins, while DENV4 specific peptides were predicted for the remaining proteins (Fig. 1A). As shown in Tables 1 and 2, *ex vivo* reactivity was detected in 50 to 70% of all monovalent vaccine recipients studied. Blood samples prior vaccinations were not available. However, a large number of DENV negative donors ($n = 45$) have been tested in *ex vivo* IFN-γ ELISPOT assays in a previous study with response rates under >2% (9). In total, 191 donor/peptide responses were identified, corresponding to 94 unique CD8$^+$ T cell epitopes. Table 3 shows a complete list of the identified epitopes. Responses to each different monovalent DLAV were comparable in terms of average magnitude (mean of 95 SFC/10^6 PBMC, range 76 to 123) and repertoire breadth (mean of six epitopes/vaccine [range, three to eight epitopes/vaccine]). The observation that the induced responses are strong enough to be readily detected *ex vivo* is remarkable and allows for evaluation

of vaccine-induced responses while avoiding any potential artifacts introduced by *in vitro* restimulation of the cells.

We also investigated the specificity of the vaccine-elicited immune response at the antigen level and analyzed the relative

TABLE 1 T cell reactivity after receipt of a monovalent or tetravalent DLAV

Vaccine	n	Frequency of responders (%)	Avg response per donor[a]	Avg no. of epitopes per donor
DEN1Δ30	11	50	83	8
DEN2/4Δ30	10	70	76	5
DEN3Δ30,31	11	55	98	6
DEN4Δ30	10	60	123	3
TV003[b]	10	73	235	8

[a] Expressed as IFN-γ SFC/10^6 PBMC.
[b] TV003 is a tetravalent vaccine admixture containing all four monovalent serotypes.

TABLE 2 Comparison of T cell reactivity after vaccination and natural infection

Parameter	Vaccine recipients (NIH)		Naturally infected donors (Sri Lanka)[c]	
	Monovalent[b]	Tetravalent	Primary	Secondary
Frequency of responders (%)	59	73	22	43
Avg no. of epitopes per donor	6	8	6	11
Avg response per donor[a]	95	235	96	220
n	41	11	55	127

[a] Expressed as IFN-γ SFC/10^6 PBMC.
[b] Expressed as the mean response observed in vaccinees with any of the four monovalent vaccines.
[c] Previously reported by Weiskopf et al. (20). Secondary donors are defined as having neutralization titers against at least two of the four serotypes.

strength of recognition of the three structural proteins (capsid [C], membrane [M], and envelope [E]), and the seven NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). As shown in Fig. 1, epitopes were identified from all 10 DENV proteins. Interestingly, the antigenic dominance seemed to differ depending on the DENV serotype used in the vaccine. In the case of the DENV4 vaccine the majority of the responses were derived from the seven NS proteins, which accounted for 90% of the total IFN-γ response, although responses to NS3 and NS5 were lower than for the other serotypes (Fig. 1E). In the case of DENV3, all three structural proteins were targeted and accounted for 37% of the total response (Fig. 1D). Among the seven NS proteins, the responses to NS3 were dominant, regardless of the vaccination serotype (Fig. 1B to E). In contrast, only vaccination with DENV4 elicited a strong response against NS2B. It has been previously shown in natural infections with DENV and after vaccination with attenuated yellow fever virus (9, 14) that the size and breadth of an antiviral T cell response varies as a function of the HLA phenotype, suggesting a strong genetic determinant of these antiviral T cell responses. Although it is possible that differences in the HLA types of individual vaccinees may contribute to the different response patterns observed, these results might also point to a differential immunodominance hierarchy as a function of the infecting serotype. It cannot be excluded that that some of the results related to protein specific responses may be due to the differences in the prediction algorithms utilized rather than utilizing peptides spanning the entire genome for each vaccine.

The immune response induced by tetravalent vaccination is targeted against highly conserved epitopes and displays a multifunctional effector memory phenotype. Next, we examined the T cell response in 10 recipients of a tetravalent DLAV (TV003), which consists of a mixture of all of the individual monovalent vaccines, each at the same potency used for monovalent vaccination (8). Using the methodology described above, PBMC from all study participants were screened in IFN-γ ELISPOT assays with pools of HLA-matched predicted class I binding peptides covering all four serotypes. *Ex vivo* reactivity was detected in 73% of vaccine recipients, with an average magnitude of 235 SFC/PBMC and an average repertoire breadth of eight epitopes per donor (Table 1).

Previous studies detailed CD8^{+} responses in Sri Lankan individuals naturally exposed to DENV of different serotypes (9). Interest-

ingly, comparable levels of CD8^{+} reactivity were noted between responses following DLAV monovalent and tetravalent vaccination and those elicited by natural immunity following primary and secondary infection (Table 2). Further analysis of the response specificity observed in tetravalent vaccinees revealed a remarkably sharp focus on the nonstructural proteins, accounting for 99.8% of the response (Fig. 2A). Although responses to six of the seven NS proteins were detected, NS3 and NS5 were the most dominantly targeted, together accounting for 97% of the response.

We next categorized T cell reactivity on the basis of whether it was directed against serotype-specific sequences (found only in one serotype) or against conserved/homologous sequences (sequences found in two or more serotypes, allowing a single residue substitution to account for potential cross-reactivity of highly homologous sequences). Strikingly, conserved sequences accounted for 93% of the overall response (Fig. 2B), suggesting that the responses induced by the tetravalent vaccine should be able to recognize epitopes from all four serotypes. In comparison, conserved sequences accounted for 46% DEN1Δ30-, 16% DEN2/4Δ30-, 54% DEN3Δ30/31-, and 7% DEN4Δ30-induced responses when administered as a monovalent vaccine. The low number of conserved sequences elicited by the DEN2/4Δ30 vaccine consists mostly of the DEN4Δ30 backbone and the DEN4Δ30 vaccine itself could reflect the fact that DENV4 is the most genetically distinct serotype compared to DENV1 or -3 and thus responses reflect more serotype specific responses.

Figure 2C shows the gating strategy for phenotype and cytokine profile of responding cells. As shown in Fig. 2D, the majority of the responses elicited by the tetravalent vaccine were produced by T cells displaying an effector memory phenotype (mean values of 49% CD45RA^{+} CCR7^{-} T$_{EMRA}$ [effector memory T cells reexpressing CD45RA] and 28% CD45RA^{-} CCR7^{-} T$_{EM}$ [effector memory T cells]). In terms of cytokine expression patterns, on average 0.3% of CD8^{+} T cells responding to vaccine-specific pools were double positive for IFN-γ and tumor necrosis factor alpha (TNF-α), followed by cells positive for a single cytokine (<0.1% of CD8^{+} T cells for IFN-γ or TNF-α, respectively; Fig. 2E). The expression frequency of the marker for cytotoxicity, CD107a, was >95% of IFN-γ-producing cells among all donors tested, indicating that the responding cells exhibit a phenotype capable of eliminating virus-infected cells.

Epitopes induced by DLAV are highly conserved in field isolates of DENV and recognized by donors exposed to natural infection with DENV. To further assess the relevance of vaccine-induced epitopes in the context of natural infections, we investigated the conservancy of the vaccine-derived epitopes from all four DENV serotypes, compared to the sequences from 555 field isolates (Fig. 3A). Vaccine-specific epitopes among monovalent vaccinees were conserved in 85 to 88% of the corresponding field isolate serotypes (Fig. 3A, left panel). We further found that the epitopes recognized after tetravalent vaccination were 98% conserved within DENV1, -2, and -4 field isolates and 84% conserved among all DENV3 field isolates (Fig. 3A, middle panel). When all epitope reactivity was combined and monovalent and tetravalent vaccinations were compared, we found that administration of tetravalent vaccination shifted the response toward even more intraserotype conserved regions (Mann-Whitney, $P = 0.002$ [two tailed]; Fig. 3A, right panel), indicating that vaccine-induced responses should be able to recognize a vast variety of natural occurring DENV strains occurring in areas of endemicity around

TABLE 3 Epitopes identified in this study

Protein	Vaccine(s)	HLA	Length (aa)[a]	Sequence
C_{14-23}	DEN1Δ30	A*3301	10	FNMLKRARNR
C_{16-24}	DEN1Δ30	B*0801	9	MLKRERNRV
C_{43-52}	DEN1Δ30, DEN3Δ30,31	B*0702	10	GPMKLVMAFI
C_{45-53}	DEN4Δ30	A*0201	9	MVLALITFL
C_{87-96}	DEN1Δ30	B*4001	10	KEISSMLNIM
$C_{104-113}$	DENV2/4Δ30	A*0201	10	ITLLCLIPTV
$M_{164-172}$	DENV2/4Δ30	A*0201	9	VTYECPLLV
$M_{245-254}$	DEN3Δ30,31	B*3501	10	HPGFTILALF
$M_{274-282}$	DEN3Δ30,31	B*3501	9	MLVTPSMTM
$E_{329-337}$	DEN1Δ30	B*4001	9	TEVTNPAVL
$E_{330-338}$	DEN2/4Δ30	A*3301, A*6801	9	EAKQPATLR
$E_{450-459}$	DEN1Δ30	A*0101	10	PTSEIQLTDY
$E_{456-464}$	DEN1Δ30	A*0101	9	LTDYGALTL
$E_{456-465}$	DEN3Δ30,31	B*3501	10	LPEYGTLGLE
$E_{499-507}$	DEN3Δ30,31	B*3501	9	LPWTSGATT
$E_{513-521}$	DENV2/4Δ30	A*2301, B*1501	9	IQKETLVTF
$E_{513-521}$	DEN4Δ30	A*2402	9	NYKERMVTF
$E_{551-560}$	DENV2/4Δ30	A*2301	10	IQMSSGNLLF
$E_{578-587}$	DEN3Δ30,31	B*3501	10	MSYAMCTNTF
$E_{579-587}$	DENV2/4Δ30	A*2301	9	SYSMCTGKF
$E_{716-725}$	TV003, DEN3Δ30,31	B*1501, B*3501	10	MVHQIFGSAY
$E_{726-734}$	DENV2/4Δ30	B*5801	9	GAAFSGVSW
$E_{738-746}$	DEN2/4Δ30	B*5701, B*5801	9	ILIGVIITW
$NS1_{802-810}$	TV003	B*5701	9	HTWTEQYKF
$NS1_{880-890}$	DEN4Δ30	B*5301	10	PPASDLKYSW
$NS1_{988-996}$	DEN4Δ30	B*4001	9	IEKASLIEV
$NS1_{1033-1041}$	DEN1Δ30	B*0702	9	RPGYHTQTA
$NS1_{1042-1051}$	DEN1Δ30, DEN3Δ30,31	B*0702	10	GPWHLGKLEL
$NS1_{1090-1099}$	DEN3Δ30,31	A*0101	10	RSCTLPPLRY
$NS1_{1112-1121}$	DEN3Δ30,31	B*3501	10	RPINEKEENM
$NS2A_{1143-1151}$	DEN3Δ30,31	B*3501	9	LAILFEEVM
$NS2A_{1160-1168}$	DEN3Δ30,31	B*3501	9	MIAGVFFTF
$NS2A_{1221-1230}$	DEN1Δ30	B*0801	10	FRRLTSREVL
$NS2A_{1244-1252}$	DEN4Δ30	B*5301	9	IPHDLMELI
$NS2A_{1265-1273}$	DEN1Δ30	A*0101	9	LTDFQSHQL
$NS2A_{1332-1340}$	DENV2/4Δ30, DEN4Δ30	A*0201	9	ALPVYLMTL
$NS2A_{1333-1342}$	TV003	B*3501	10	LPVYLMTLMK
$NS4B_{1377-1385}$	DEN1Δ30	B*0702	9	GPLVAGGLL
$NS2B_{1437-1445}$	DENV2/4Δ30, DEN4Δ30	B*4001, B*4403	9	EETNMITLL
$NS2B_{1457-1465}$	DEN4Δ30	B*5301	9	YPLAIPVTM
$NS2B_{14631472}$	TV003	A*0301	9	ALWYVWQVK
$NS3_{1494-1502}$	TV003	A*0201	9	VLDDGIYRI
$NS3_{1494-1502}$	DENV2/4Δ30, DEN4Δ30	A*0201	9	ALSEGVYRI
$NS3_{1499-1508}$	DEN1Δ30	B*0801	10	IYRILQRGLL
$NS3_{1506-1515}$	DENV2/4Δ30, DEN4Δ30	A*0201	10	GLFGKTQVGV
$NS3_{1519-1527}$	DEN2/4Δ30	B*4403	9	MEGVFHTMW
$NS3_{1598-1607}$	DEN2/4Δ30	B*4001, B*4403	10	GEIGAVTLDF
$NS3_{160-1617}$	TV003	A*0301, A*1101	10	GTSGSPIINK
$NS3_{1608-1616}$	DEN1Δ30, DEN3Δ30,31	B*0702	9	KPGTSGSPI
$NS3_{1625-1634}$	DEN2/4Δ30, DEN4Δ30, TV003	A*0301	10	GLYGNGVVTK
$NS3_{1653-1661}$	DEN1Δ30	B*3501	9	NPEIEDDIF
$NS3_{1689-1697}$	DEN1Δ30	B*0801	9	AIKRKLRTL
$NS3_{1700-1709}$	TV003	B*0702, B*3501	10	APTRVVAAEM
$NS3_{1753-1761}$	TV003	B*3501	9	VPNYNLIVM
$NS3_{1768-1777}$	DEN3Δ30,31	B*3501	10	DPASIAARGY
$NS3_{1813-1822}$	TV003	B*4402	10	EERDIPERSW
$NS3_{1887-1895}$	TV003	A*2601	9	DISEMGANF
$NS3_{1899-1908}$	TV003	A*0301	10	RVIDPRRCLK
$NS3_{1899-1907}$	DEN1Δ30, DEN3Δ30,31	B*0702	9	RVIDPRRCL
$NS3_{1902-1910}$	DEN1Δ30, DEN3Δ30,31	B*0702	9	DPRRCLKPV
$NS3_{1978-1986}$	DEN1Δ30	B*070, B*3501	9	TPEGIIPSM
$NS3_{1978-1987}$	DEN1Δ30, DEN3Δ30,31	B*0702, B*3501, B*5301	10	TPEGIIPALF

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TABLE 3 (Continued)

Protein	Vaccine(s)	HLA	Length (aa)[a]	Sequence
NS3$_{2000-2008}$	TV003	A*3301	9	EFRLRGEQR
NS3$_{2051-2060}$	DEN2/4Δ30	B*4403, B*4402	10	LEENMEVEIW
NS3$_{2064-2073}$	DEN2/4Δ30	B*4403	10	GERKKLKPRW
NS3$_{2070-2078}$	DEN1Δ30	B*0702	9	RPRWLDART
NS3$_{2079-2087}$	DEN4Δ30	A*0101	9	YSDPLALRE
NS3$_{2093-2102}$	DEN1Δ30	B*0801	10	AGRRSVSGDL
NS4A$_{2184-2192}$	DEN2/4Δ30	B*5701	9	IAVASGLLW
NS4B$_{2350-2358}$	DEN1Δ30	B*0702	9	NPLTLTAAV
NS4B$_{2455-2464}$	DEN1Δ30	B*0702	10	SPGKFWNTTI
NS4B$_{2464-2472}$	DEN3Δ30,31/Tetra	B*3501	9	IAVSMANIF
NS4B$_{2468-2476}$	TV003	B*3501	9	MANIFRGSY
NS4B$_{2486-2494}$	TV003	A*0301	9	IMKSVGTGK
NS5$_{2612-2620}$	TV003	A*0301	9	ATYGWNLVK
NS5$_{2812-2821}$	TV003	B*5701	10	ASSMVNGVVR
NS5$_{2830-2838}$	DEN1Δ30	B*0702	9	IPMVTQIAM
NS5$_{2842-2850}$	TV003	B*3501	9	TPFGQQRVF
NS5$_{2885-2894}$	DEN1Δ30/DEN2/4Δ30, TV003	B*0702, B*5301	10	TPRMCTREEF
NS5$_{2899-2908}$	DEN2/4Δ30	B*1501	10	RSNAAIGAVF
NS5$_{2921-2929}$	DEN1Δ30	B*4001	9	VEDERFWDL
NS5$_{2956-2964}$	TV003	A*0301	9	KLGEFGRAK
NS5$_{2962-2970}$	TV003	B*5701	9	KAKGSRAIW
NS5$_{2977-2986}$	TV003	A*2301	10	RFLEFEALGF
NS5$_{3083-3091}$	DEN1Δ30	A*6801	9	TVMDVISRR
NS5$_{3173-3182}$	TV003	A*0301	10	ALLALNDMGK
NS5$_{3182-3190}$	DEN4Δ30, TV003	B*5701, B*5801	9	KVRKDIPQW
NS5$_{3218-3227}$	DEN1Δ30	B*4001	10	REIVVPCRNQ
NS5$_{3246-3254}$	Tetravalent	A*2601	9	ETACLGKSY
NS5$_{3250-3258}$	DEN4Δ30	B*5701	9	LGKSYAQMW
NS5$_{3254-3262}$	DEN2/4Δ30	B*1501, B*5301	9	YAQMWSLMY
NS5$_{3290-3299}$	DEN1Δ30	A*2301	10	TWSIHAHHQW
NS5$_{3291-3299}$	TV003	B*5701	9	WSIHAHHQW
NS5$_{3327-3338}$	DEN3Δ30,31, TV003	A*0201	9	TTWEDVPYL

[a] aa, amino acids.

the world. To examine this possibility, we evaluated recognition of the vaccine-specific epitopes in blood donors from the Sri Lanka DENV area of endemicity, i.e., donors who have previously experienced natural DENV infection. Responses were readily detected in naturally exposed donors and were associated with phenotype and cytokine profiles similar to those observed in DLAV recipients (Fig. 3B and C). To enable an unbiased comparison, donors expressing HLA B*0702 were selected from both cohorts. These data underline the relevance of vaccine-induced T cell immunity in the context of natural infection.

DISCUSSION

The development of effective vaccination strategies against DENV infection and clinically significant disease is a task of high public health value and significance but also a challenge of significant complexity. The immune correlates of DENV vaccine efficacy are poorly understood. Recent data suggest that neutralizing antibody titers, previously thought to be a correlate of protection in dengue infection, may be insufficient to predict vaccine efficacy and that CD8^{+} responses are an important component of natural protection (7, 9). The results of a recent phase 3 trial of the most advanced DENV vaccine will allow a more detailed evaluation of the role of antibodies in protection against disease (15). Here, we report a comprehensive *ex vivo* characterization of HLA-restricted T cell memory responses in recipients of a monovalent or a tetrava-

lent DLAV. CD8^{+} T cell responses have been identified testing vaccine-specific peptide pools in PBMC from MHC class I matched donors. Although it is possible that CD4 T cells can recognize 9- and 10-mers, the vast preponderance of known MHC class II-restricted epitopes are 12 to 20 residues in length, which also comports with known binding MHC class II binding characteristics (16–18). Using fluorescence-activated cell sorting analysis we demonstrated that vaccine-specific CD8^{+} T cell responses are similar in magnitude and frequency to those observed in naturally infected populations, as detected *ex vivo* in subjects exposed to either experimental or natural infection with DENV.

The kinetics of IFN-γ and TNF-α production by CD4^{+} T cells have been previously investigated up to 6 weeks postvaccination with the monovalent DENV1 vaccine (19). Our data show that *ex vivo* T cell responses are still robust months postvaccination (monovalent mean of 47 months and tetravalent mean of 12 months), suggesting the development of long-lasting memory responses, a desirable attribute for a vaccine candidate.

In vaccinees receiving the monovalent vaccine we have detected broad responses against the majority of the 10 proteins regardless if the vaccinees have received one or two doses. Likewise, we did not see a difference in magnitude and breadth between these two groups. We have previously shown in a mouse model of DENV infection that secondary infection with the same serotype did not change the repertoire toward conserved epitopes,
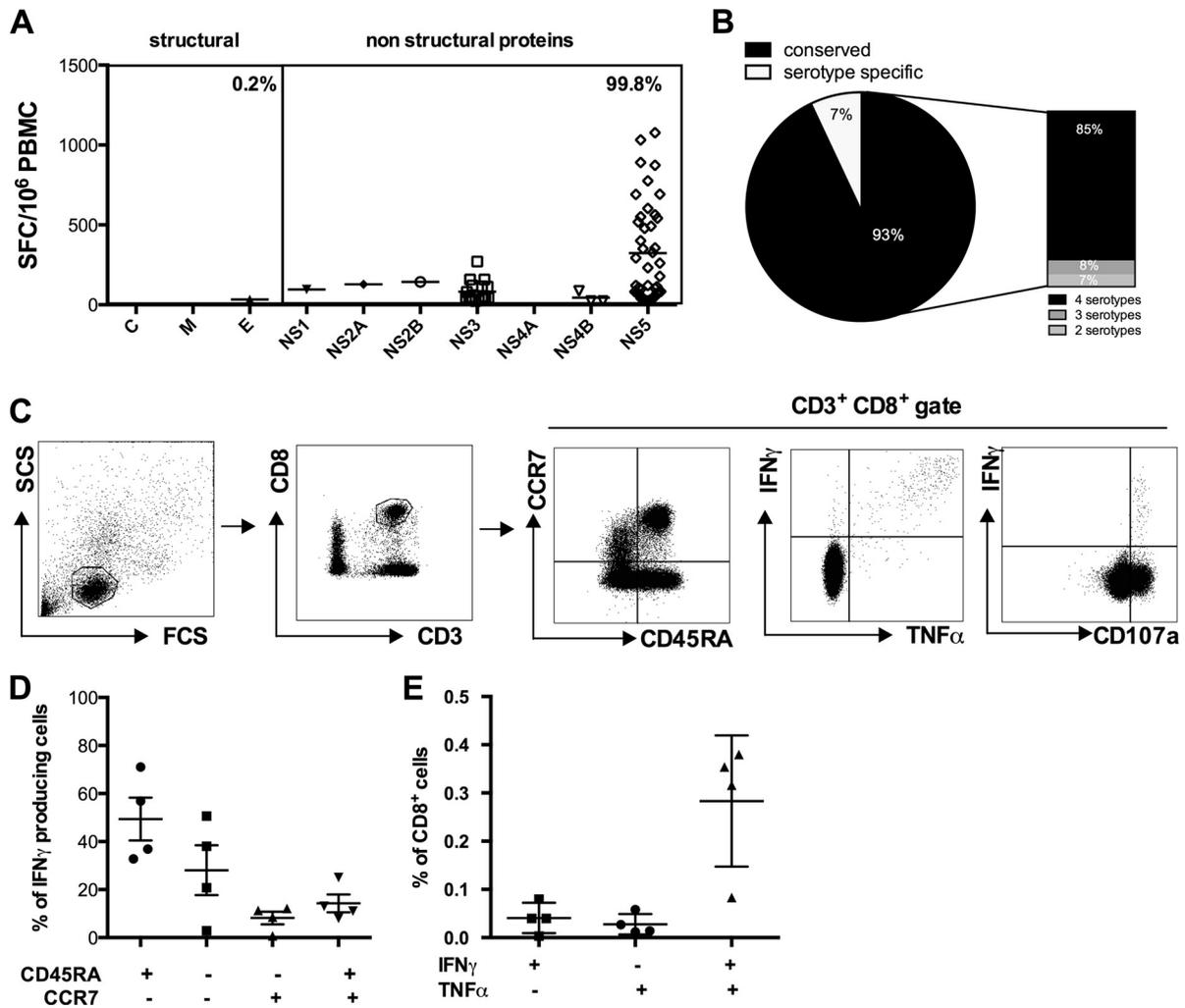
FIG 2 The immune response induced by tetravalent vaccination is targeted against highly conserved proteins and displays a multifunctional effector memory phenotype. (A) Responses detected in study participants vaccinated with tetravalent DLAV (TV003) are shown. Responses are expressed as the number of IFN-γ-secreting cells per 10^6 PBMC. (B) Analysis of conservancy of the epitopes identified. The relative response by epitopes derived from serotype-specific regions (white pie chart) or regions conserved between serotypes (black pie chart) is shown. The right panel shows the number of serotypes in which the responses are conserved. (C) Gating strategy for phenotype and cytokine profiling of responding cells. (D and E) PBMC from tetravalent vaccinees ($n = 4$) were incubated with HLA-matched epitope pools and assayed for the production of IFN-γ and TNF-α as described in Materials and Methods. Each sample was stained additionally with antibodies against CD45RA and CCR7 to determine the proportion of cells in the following subsets: CCR7$^-$ CD45RA$^-$ T$_{EM}$ (effector memory T cells), CCR7$^+$ CD45RA$^-$ T$_{CM}$ (central memory T cells), CCR7$^+$ CD45RA$^+$ T$_N$ (naive T cells), and CCR7$^-$ CD45RA$^+$ T$_{EMRA}$ (effector memory T cells reexpressing CD45RA). The relative distribution of T cell subsets within the IFN-γ positive cells is shown (D), as well as CD8$^+$ T cells positive for one of the cytokines (E; IFN-γ, ●; TNF-α, ■) or double positive for both cytokines (E; ▲). Stimulated frequencies are shown as net frequencies with the baseline levels subtracted (cytokine production of stimulated CD8$^+$ T cells minus that of unstimulated CD8$^+$ T cells).

supporting this observation (20). We observed that after tetravalent vaccination the induction of responses predominantly targeting the nonstructural proteins NS3 and NS5, which are also preferentially targeted in natural DENV infection (9, 21, 22). This has potential relevance in the context of the insufficient protection against all four serotypes by the most advanced tetravalent dengue-yellow fever chimeric virus vaccine, in which DENV nonstructural proteins are absent (23).

The focus of responses toward conserved epitopes was somewhat unexpected. In the tetravalent vaccination setting, viruses of all four serotypes are administered simultaneously and thus differ from natural secondary infections, where the different serotypes are usually encountered sequentially. It is possible that the 4-fold greater representation of epitopes with conserved sequences over

serotype-specific ones also influences immune dominance. It has been reported previously that natural secondary infection is associated with the immunodominance of responses toward NS proteins, especially NS3, with conserved epitopes in those regions (21, 22). We previously reported a protective role for CD8$^+$ T cells focusing on conserved regions upon secondary heterologous infection (9). Although primary infection with one serotype induces mostly serotype specific T cell responses and only short-term cross-protection against other serotypes, the evolution of secondary T cell responses toward conserved regions likely contributes to the general protection against tertiary DENV infections (24, 25).

Influenza virus is another RNA virus associated with a high degree of sequence variation. It has been shown that cross-reactive cytotoxic T cells recognizing conserved epitopes across viral sub-
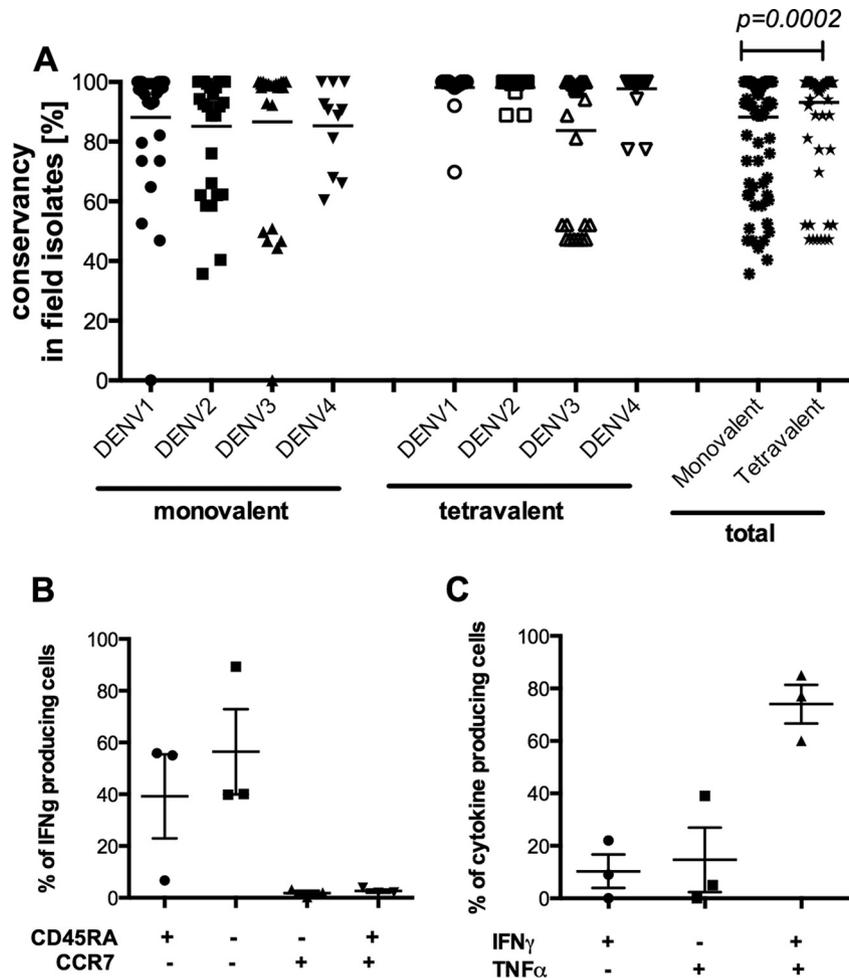
FIG 3 Epitopes induced by DLAV are highly conserved in field isolates of DENV and recognized by donors exposed to natural infection with DENV. (A) Conservancy of epitopes identified within field isolates of DENV. Totals of 162 DENV1, 171 DENV2, 169 DENV3, and 53 DENV4 sequences were retrieved from the NCBI protein database. The relative conservancy within the sequences of the respective serotypes for all epitopes identified after monovalent (left panel) or tetravalent vaccination (middle panel) is shown. The right panel shows all epitope reactivity to either the monovalent or tetravalent vaccination. (B and C) Phenotype (B) and cytokine (C) profiles of T cell responses in blood donors previously exposed to natural secondary infection with DENV epitopes. PBMC samples ($n = 4$) were incubated with HLA matched vaccine-specific epitope pools and assayed for production of IFN-γ and TNF-α as described in Materials and Methods. Each sample was also stained with antibodies against CD45RA and CCR7 to determine the proportion of cells in the following subsets: CCR7$^-$ CD45RA$^-$ T$_{EM}$ (effector memory T cells), CCR7$^+$ CD45RA$^-$ T$_{CM}$ (central memory T cells), CCR7$^+$ CD45RA$^+$ T$_N$ (naive T cells), and CCR7$^-$ CD45RA$^+$ T$_{EMRA}$ (effector memory T cells reexpressing CD45RA). Responses were detected in three out of four donors tested. Shown is the relative distribution of T cell subsets within the IFN-γ-positive cells (B) and the relative distribution of cells positive for one of the cytokines (C; IFN-γ, ●; TNF-α, ■) or double positive for both cytokines (C; ▲).

types contribute to heterotypic immunity against different strains of influenza virus (26). Furthermore, a CD8$^+$ T cell subset specific for highly conserved epitopes from core proteins has been recently reported as a correlate of protection against symptomatic infection with influenza virus (27). As shown in our work, the epitopes elicited by the tetravalent vaccine are highly conserved across a huge variety of field isolates, supporting the notion that T cells will also respond to infection with a virus different from the vaccine strain. This is highly relevant for a global vaccine since the circulating viruses can evolve and gain mutations over time and vary as a function of geographic location (28).

Finally, the phenotype of the cells induced by tetravalent vaccination is also of note. These cells were found to be multispecific, expressing the cytotoxicity marker CD107, and are mostly contained in T$_{EM}$ and T$_{EMRA}$ subsets. These features have been associated with protective capacity from viral infections in different

systems (14, 27). In conclusion, our results highlight the fact that DLAVs are able to induce a multifunctional CD8$^+$ T cell response in the small number of donors tested. More donors will have to be tested in future studies to establish the generality of the conclusions. We also report here the first comprehensive characterization of responses after monovalent and tetravalent vaccination. Detailed knowledge of the T cell response may further contribute to the identification of robust correlates of protection in natural immunity and vaccination against DENV.

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REFERENCES

1. **Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI.** 2013. The global distribution and burden of dengue. Nature **496**:504–507. http://dx.doi.org/10.1038/nature12060.

2. **Stahl HC, Butenschoen VM, Tran HT, Gozzer E, Skewes R, Mahendradhata Y, Runge-Ranzinger S, Kroeger A, Farlow A.** 2013. Cost of dengue outbreaks: literature review and country case studies. BMC Public Health **13**:1048. http://dx.doi.org/10.1186/1471-2458-13-1048.

3. **Sabin AB.** 1952. Research on dengue during World War II. Am J Trop Med Hyg **1**:30–50.

4. **Zellweger RM, Prestwood TR, Shresta S.** 2010. Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe **7**:128–139. http://dx.doi.org/10.1016/j.chom.2010.01.004.

5. **Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S, Duangchinda T, Dong T, Rowland-Jones S, Yenchitsomanus PT, McMichael A, Malasit P, Screaton G.** 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med **9**:921–927. http://dx.doi.org/10.1038/nm887.

6. **Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E.** 2010. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog **6**:e1000790. http://dx.doi.org/10.1371/journal.ppat.1000790.

7. **Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chantavanich P, Suvannadabba S, Jiwariyavej V, Dulyachai W, Pengsaa K, Wartel TA, Moureau A, Saville M, Bouckenooghe A, Viviani S, Tornieporth NG, Lang J.** 2012. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. Lancet **380**:1559–1567. http://dx.doi.org/10.1016/S0140-6736(12)61428-7.

8. **Durbin AP, Kirkpatrick BD, Pierce KK, Elwood D, Larsson CJ, Lindow JC, Tibery C, Sabundayo BP, Shaffer D, Talaat KR, Hynes NA, Wanionek K, Carmolli MP, Luke CJ, Murphy BR, Subbarao K, Whitehead SS.** 2013. A single dose of any of four different live attenuated tetravalent dengue vaccines is safe and immunogenic in flavivirus-naive adults: a randomized, double-blind clinical trial. J Infect Dis **207**:957–965. http://dx.doi.org/10.1093/infdis/jis936.

9. **Weiskopf D, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, Broadwater A, Kolla RV, De Silva AD, de Silva AM, Mattia KA, Doranz BJ, Grey HM, Shresta S, Peters B, Sette A.** 2013. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8$^+$ T cells. Proc Natl Acad Sci U S A **110**:E2046–E2053. http://dx.doi.org/10.1073/pnas.1305227110.

10. **Lindow JC, Durbin AP, Whitehead SS, Pierce KK, Carmolli MP, Kirkpatrick BD.** 2013. Vaccination of volunteers with low-dose, live-attenuated, dengue viruses leads to serotype-specific immunologic and virologic profiles. Vaccine **31**:3347–3352. http://dx.doi.org/10.1016/j.vaccine.2013.05.075.

11. **Sette A, Sidney J.** 1999. Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. Immunogenetics **50**:201–212. http://dx.doi.org/10.1007/s002510050594.

12. **Kim Y, Ponomarenko J, Zhu Z, Tamang D, Wang P, Greenbaum J, Lundegaard C, Sette A, Lund O, Bourne PE, Nielsen M, Peters B.** 2012.

13. **Bui HH, Sidney J, Li W, Fusseder N, Sette A.** 2007. Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. BMC Bioinformatics **8**:361. http://dx.doi.org/10.1186/1471-2105-8-361.

14. **Akondy RS, Monson ND, Miller JD, Edupuganti S, Teuwen D, Wu H, Quyyumi F, Garg S, Altman JD, Del Rio C, Keyserling HL, Ploss A, Rice CM, Orenstein WA, Mulligan MJ, Ahmed R.** 2009. The yellow fever virus vaccine induces a broad and polyfunctional human memory CD8$^+$ T cell response. J Immunol **183**:7919–7930. http://dx.doi.org/10.4049/jimmunol.0803903.

15. **Capeding MR, Tran NH, Hadinegoro SR, Ismail HI, Chotpitayasunondh T, Chua MN, Luong CQ, Rusmil K, Wirawan DN, Nallusamy R, Pitisuttithum P, Thisyakorn U, Yoon IK, van der Vliet D, Langevin E, Laot T, Hutagalung Y, Frago C, Boaz M, Wartel TA, Tornieporth NG, Saville M, Bouckenooghe A, CYD14 Study Group.** 2014. Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. Lancet **384**:1358–1365. http://dx.doi.org/10.1016/S0140-6736(14)61060-6.

16. **Madden DR.** 1995. The three-dimensional structure of peptide-MHC complexes. Annu Rev Immunol **13**:587–622. http://dx.doi.org/10.1146/annurev.iy.13.040195.003103.

17. **van Kasteren SI, Overkleeft H, Ovaa H, Neefjes J.** 2014. Chemical biology of antigen presentation by MHC molecules. Curr Opin Immunol **26**:21–31. http://dx.doi.org/10.1016/j.coi.2013.10.005.

18. **Neefjes J, Jongsma ML, Paul P, Bakke O.** 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol **11**:823–836. http://dx.doi.org/10.1038/nri3084.

19. **Lindow JC, Borochoff-Porte N, Durbin AP, Whitehead SS, Fimlaid KA, Bunn JY, Kirkpatrick BD.** 2012. Primary vaccination with low dose live dengue 1 virus generates a proinflammatory, multifunctional T cell response in humans. PLoS Negl Trop Dis **6**:e1742. http://dx.doi.org/10.1371/journal.pntd.0001742.

20. **Weiskopf D, Angelo MA, Sidney J, Peters B, Shresta S, Sette A.** 2014. Immunodominance changes as a function of the infecting dengue virus serotype and primary versus secondary infection. J Virol **88**:11383–11394. http://dx.doi.org/10.1128/JVI.01108-14.

21. **Duangchinda T, Dejnirattisai W, Vasanawathana S, Limpitikul W, Tangthawornchaikul N, Malasit P, Mongkolsapaya J, Screaton G.** 2010. Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. Proc Natl Acad Sci U S A **107**:16922–16927. http://dx.doi.org/10.1073/pnas.1010867107.

22. **Rivino L, Kumaran EA, Jovanovic V, Nadua K, Teo EW, Pang SW, Teo GH, Gan VC, Lye DC, Leo YS, Hanson BJ, Smith KG, Bertoletti A, Kemeny DM, MacAry PA.** 2013. Differential targeting of viral components by CD4$^+$ versus CD8$^+$ T lymphocytes in dengue virus infection. J Virol **87**:2693–2706. http://dx.doi.org/10.1128/JVI.02675-12.

23. **Guy B, Barrere B, Malinowski C, Saville M, Teyssou R, Lang J.** 2011. From research to phase III: preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. Vaccine **29**:7229–7241. http://dx.doi.org/10.1016/j.vaccine.2011.06.094.

24. **Gordon A, Kuan G, Mercado JC, Gresh L, Aviles W, Balmaseda A, Harris E.** 2013. The Nicaraguan pediatric dengue cohort study: incidence of inapparent and symptomatic dengue virus infections, 2004-2010. PLoS Negl Trop Dis **7**:e2462. http://dx.doi.org/10.1371/journal.pntd.0002462.

25. **Wikramaratna PS, Simmons CP, Gupta S, Recker M.** 2010. The effects of tertiary and quaternary infections on the epidemiology of dengue. PLoS One **5**:e12347. http://dx.doi.org/10.1371/journal.pone.0012347.

26. **Gras S, Kedzierski L, Valkenburg SA, Laurie K, Liu YC, Denholm JT, Richards MJ, Rimmelzwaan GF, Kelso A, Doherty PC, Turner SJ, Rossjohn J, Kedzierska K.** 2010. Cross-reactive CD8$^+$ T-cell immunity between the pandemic H1N1-2009 and H1N1-1918 influenza A viruses. Proc Natl Acad Sci U S A **107**:12599–12604. http://dx.doi.org/10.1073/pnas.1007270107.

27. **Sridhar S, Begom S, Bermingham A, Hoschler K, Adamson W, Carman W, Bean T, Barclay W, Deeks JJ, Lalvani A.** 2013. Cellular immune correlates of protection against symptomatic pandemic influenza. Nat Med **19**:1305–1312. http://dx.doi.org/10.1038/nm.3350.

28. **Holmes EC, Twiddy SS.** 2003. The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol **3**:19–28. http://dx.doi.org/10.1016/S1567-1348(03)00004-2.

Immune epitope database analysis resource. Nucleic Acids Res **40**:W525–W530. http://dx.doi.org/10.1093/nar/gks438.