View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Carolina Digital Repository

Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8$^+$ T cells

Daniela Weiskopf[a], Michael A. Angelo[a], Elzinandes L. de Azeredo[a], John Sidney[a], Jason A. Greenbaum[a], Anira N. Fernando[b], Anne Broadwater[c], Ravi V. Kolla[a], Aruna D. De Silva[a,b], Aravinda M. de Silva[c], Kimberly A. Mattia[d], Benjamin J. Doranz[d], Howard M. Grey[a,1], Sujan Shresta[a], Bjoern Peters[a], and Alessandro Sette[a,1]

[a]Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037; [b]Genetech Research Institute, Colombo 00800, Sri Lanka; [c]Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC 27599; and [d]Integral Molecular, Philadelphia, PA 19104

Contributed by Howard M. Grey, March 19, 2013 (sent for review January 22, 2013)

The role of CD8$^+$ T cells in dengue virus infection and subsequent disease manifestations is not fully understood. According to the original antigenic sin theory, skewing of T-cell responses induced by primary infection with one serotype causes less effective response upon secondary infection with a different serotype, predisposing individuals to severe disease. A comprehensive analysis of CD8$^+$ responses in the general population from the Sri Lankan hyperendemic area, involving the measurement of ex vivo IFNγ responses associated with more than 400 epitopes, challenges the original antigenic sin theory. Although skewing of responses toward primary infecting viruses was detected, this was not associated with impairment of responses either qualitatively or quantitatively. Furthermore, we demonstrate higher magnitude and more polyfunctional responses for HLA alleles associated with decreased susceptibility to severe disease, suggesting that a vigorous response by multifunctional CD8$^+$ T cells is associated with protection from dengue virus disease.

Dengue virus (DENV) is the etiologic agent of dengue fever (DF), the most significant mosquito-borne viral disease in humans. Disease can be caused by any of the four DENV serotypes (DENV1 to -4), which share 67–75% sequence homology with one another (1). DENV transmission occurs in more than 100 countries and is an increasing public health problem in tropical and subtropical regions (2). Demographic changes, urbanization, and international travel contribute to the expansion of geographical areas where transmission occurs, and all four DENV serotypes are now circulating in Asia, Africa, and the Americas (3, 4). Up to 100 million DENV infections occur every year (5), and severity can range from asymptomatic to an acute self-limiting febrile illness (DF). In a small proportion of patients, the disease can exacerbate and progress to dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS), characterized by severe vascular leakage, thrombocytopenia, and hemorrhagic manifestations (4).

Although natural infection by any of the four DENV serotypes (primary infection) produces a lasting protective immunity against reinfection by the same serotype, it does not protect against infections with other serotypes (secondary infections) (6, 7). Epidemiologic studies have shown that the majority of individuals that develop DHF/DSS had been previously infected with a different serotype (8). Consequently, the development of DENV vaccines has been hampered by the potential risk of vaccine-related adverse events and the requirement to induce long-lasting protective immune responses against all four DENV serotypes simultaneously (9). The cause for the increased frequency of DHF following secondary infections has not been fully elucidated. One hypothesis is that serotype cross-reactive antibodies exacerbate disease by increasing infection of cells bearing Fcγ receptors, resulting in higher viral loads and more severe disease via this antibody-dependent enhancement (ADE) of infection (10, 11). Indeed, nonhuman primate and murine models have demonstrated that antibodies can lead to enhancement of DENV infection and disease in vivo (12–15).

Another hypothesis postulates that T cells raised against the first infecting serotype dominate during a secondary heterologous infection in a phenomenon termed "original antigenic sin" (16, 17). This term was first applied to the humoral response to influenza epidemics (18) but has also been observed in CD8$^+$ T-cell responses against lymphocytic choriomeningitis virus (19). This hypothesis postulates that, during secondary infection, expansion of preexisting lower avidity cross-reactive memory T cells dominate the responses over that of naïve T cells that are of higher avidity for the new DENV serotype. This expansion of low avidity T cells results in less efficient elimination of DENV-infected cells.

A role for T cells in control of DENV infection is suggested by several studies that implicate HLA associations as a genetic component to variable susceptibility to DENV disease (20–26). However, it has not been addressed whether these associations might indicate a positive or detrimental role for T-cell responses. One major obstacle to the elucidation of the function of T cells is the lack of a comprehensive characterization of HLA-restricted DENV responses in the context of natural infection.

Herein, we present a comprehensive analysis of functional T-cell memory against DENV and are able to correlate this with HLA alleles expressed in the very same donors. Collectively, the data suggest an HLA-linked protective role of CD8$^+$ T-cell responses and

Significance

Dengue virus is the etiologic agent of dengue fever, the most significant mosquito-borne viral disease in humans, affecting over 100 million individuals each year. Currently there is no licensed vaccine or effective antiviral therapy available, and treatment is largely supportive in nature. This study presents a comprehensive analysis of functional T-cell memory against dengue viruses and suggests an HLA-linked protective role for CD8$^+$ T cells. This demonstration of the protective role of T-cell responses points the way forward to identifying robust correlates of protection in natural immunity and vaccination against dengue virus.

Author contributions: D.W., R.V.K., H.M.G., S.S., B.P., and A.S. designed research; D.W., M.A.A., E.L.d.A., J.S., J.A.G., A.N.F., A.B., A.D.D.S., A.M.d.S., K.A.M., B.J.D., and B.P. performed research; A.N.F. and A.D.D.S. contributed new reagents/analytic tools; D.W., M.A.A., E.L.d.A., J.S., J.A.G., A.B., A.M.d.S., K.A.M., B.J.D., H.M.G., B.P., and A.S. analyzed data; and D.W., H.M.G., and A.S. wrote the paper.

The authors declare no conflict of interest.

Data deposition: A complete list of epitopes is available from the Immune Epitope Database, www.iedb.org (submission ID 1000504).

See Commentary on page 8761.

[1]To whom correspondence may be addressed. E-mail: hgrey@liai.org or alex@liai.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305227110/-/DCSupplemental.

do not support a causative role for $CD8^+$ T cells in the induction of severe disease following secondary heterologous infection.

Results

Effective Coverage of the General Population from the Colombo Endemic Area. We aimed to define the T-cell memory response against DENV in a donor population derived from an endemic area. For a broad coverage, we selected a panel of HLA A and HLA B alleles that includes any allele present with a frequency of 4.5% (HLA A) or 5% (HLA B) in both the general population and that of Sri Lanka. Thus, we have collected and subsequently HLA typed peripheral blood mononuclear cells (PBMCs) from 250 healthy adult blood donors from the National Blood Center, Ministry of Health, Colombo, Sri Lanka. The 27 selected alleles allowed matching at least 3 out of 4 possible HLA A and B alleles expressed per donor in 90% of our cohort (3 out of 4 in 41% and 4 out of 4 in 49%, respectively, Fig. 1*A*, black bars and solid line). Considering closely related HLA alleles within the same supertype (27), the allelic coverage of at least 3 of 4 MHC class I alleles increased to 99% of the donors (Fig. 1*A*, white bars).

Immunodominant Regions of the DENV Polyprotein. PBMCs from all donors were screened with pools of predicted HLA matched class I binding peptides covering all four serotypes in IFNγ enzyme-linked immunosorbent spot (ELISPOT) assays. Ex vivo reactivity was detected for 22% of primary and 43% of secondary donors (Fig. 1*B*). By definition, primary cases showed antibody neutralization for only one serotype whereas secondary cases are individuals with antibodies capable of neutralizing more than one serotype. The average response per donor, as well as the average number of epitopes detected, was higher in donors who had experienced secondary infection compared with primary infection. In total, 753 donor/peptide responses were identified, corresponding to 408 unique $CD8^+$ T-cell epitopes. A complete list of these epitopes is available on the Immune Epitope Database (www.iedb.org; submission ID 1000504). To investigate the relative immunodominance of different parts of the DENV proteome, we plotted response magnitude [as spot forming cells (SFCs) per 10^6 PBMC values] and frequency of responding donors (as a heat map) as a function of the genomic position of DENV proteins (Fig. 2*A*). Nonstructural (NS) proteins 3, 4B, and 5 were the most vigorously and frequently recognized proteins and accounted for more than two thirds of the total response. Conversely, proteins known to be main antibody targets NS protein 1 and Envelope (E) were less prominently recognized by $CD8^+$ T cells.

Clustering of these epitopes based on sequence identity greater than 80% resulted in the definition of 267 antigenic regions. The 25 most antigenic regions accounted for half of the total response (Fig. 2*B*). Several of these regions contained epitopes derived from multiple serotypes and restricted by a variety of different alleles. The sequences, proteome location, serotype affiliation, HLA restriction, frequency, and magnitude of responses of these 25 immunodominant antigenic regions are shown in Table 1.

Differences Between Serotype-Specific Responses. The data were next analyzed as a function of different serotype reactivity. At the same time, we 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). Conserved sequences accounted for 37% of the overall responses in our cohort. With respect to serotype-specific responses, responses against DENV2-derived epitopes were by far most prevalent (42%), followed by DENV3 (12%), DENV4 (6%), and DENV1 (3%).

Next, for each of the four serotypes, we compiled responses from donors exhibiting responses against serotype-specific epitopes, used as an indicator of previous infection with that specific serotype. The responses of donors who responded to serotype-specific and/or conserved epitopes are shown in Table 2. No significant difference in total magnitude of responses was noted between the groups ($P > 0.108$ for any pairwise comparison). On average, donors recognizing DENV2-specific epitopes elicited a T-cell response of 1,987 SFCs per 10^6 PBMCs against serotype-specific and 822 SFCs per 10^6 PBMCs against conserved epitopes, corresponding to a ratio of 2.4, showing that the majority of responses in these donors were directed against serotype-specific regions (Table 2, see "Secondary infection" column). The knowledge of the origin of epitopes allowed interpreting the data in context of the epidemiological history of Sri Lanka. The known historical prevalence of DENV serotypes circulating in Sri Lanka indicates that, whereas DENV2 has historically always been prevalent in Sri Lanka, recent years have seen the appearance of new substrains of DENV3 Lanka (28, 29), and DENV1 has only

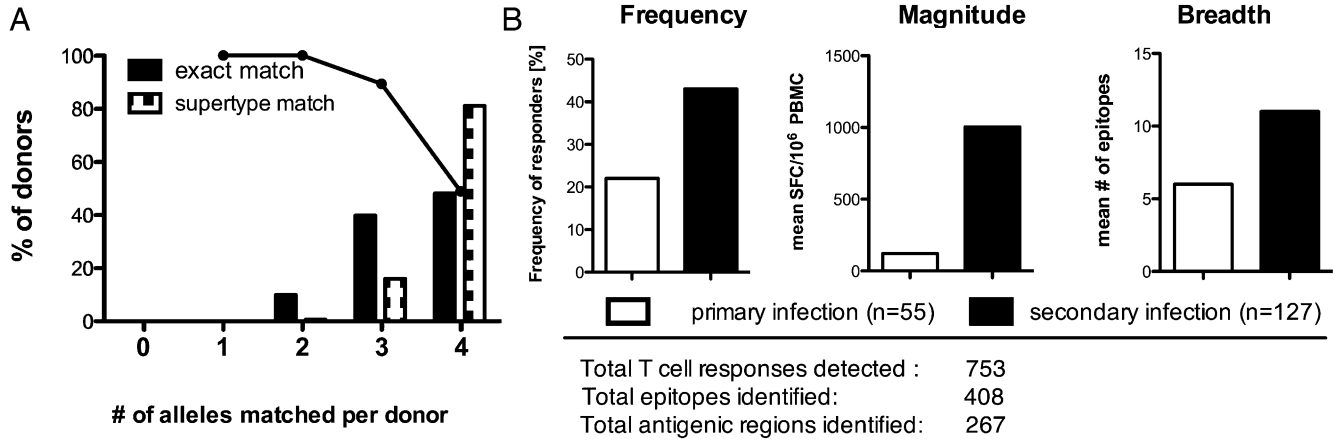

Fig. 1. HLA coverage and T-cell reactivity of the study population. (*A*) HLA allele coverage in the Sri Lankan cohort is shown. Bars represent the relative number of donors in which the donor-specific HLA alleles have been exactly matched (filled bars) or matched within the same supertype (open bars) with the 27 alleles selected for our study. The black line represents the cumulative number of donors in which 1–4 alleles have been matched exactly. (*B*) T-cell reactivity in blood donors after primary ($n = 55$; open bars) and secondary infection ($n = 127$; filled bars) with DENV. Shown are the relative response frequency (*Left*), the average magnitude of responses per donor (*Center*), and the average number of epitopes per donor (*Right*).

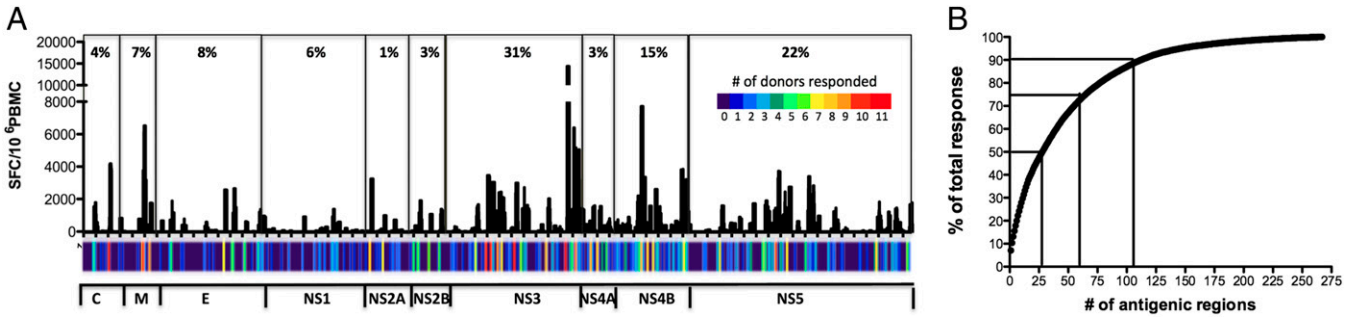

Fig. 2. Immunodominant regions of the DENV polyprotein. (*A*) The genomic position of DENV-encoded proteins (C, capsid; prM, premembrane; M, membrane; E, envelope, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and the total observed response magnitude for every amino acid along the proteome is shown (filled bars). The data are expressed as total number of IFNγ SFCs per 10^6 PBMCs. The heat map indicates the number of donors that showed a positive cytokine response to peptides within these regions. (*B*) Identified antigenic regions were plotted as a function of the percentage of the total response. Lines indicate the number of regions needed to account for 50%, 75%, and 90% of the total response, respectively.

recently appeared in this population (30). Accordingly, we hypothesized that original antigenic sin should dictate that the ratio of serotype specific to conserved responses should be decreased when comparing DENV2 (the "original" antigen) with other serotypes.

As predicted by this reasoning, the overall response of donors recognizing serotype-specific regions of either DENV1 or DENV3 was significantly skewed toward conserved regions, reflected by the ratio of specific vs. conserved responses of 0.3 and 0.6 for DENV1 and DENV3, respectively ($P = 0.037$ for DENV1 and $P = 0.028$ for DENV3 in a Mann–Whitney test). The same trend, albeit not statistically significant ($P = 0.079$), for a difference in the ratio of serotype-specific/conserved epitopes was observed in the case of DENV4.

Analysis of the primary infection donors who showed T-cell reactivity against serotype-specific and/or conserved epitopes, although not significant due to low numbers, further supports the notion that, following primary infection, serotype-specific responses dominate over conserved ones and that the shift to a focus on conserved epitopes is a result of secondary infection (Table 2, see "Primary infection" column). Accordingly, the dominance of conserved epitopes in DENV3 and DENV1 responses likely reflects previous DENV2 infection, and thus expansion of T cells recognizing conserved epitopes during secondary infection (original antigenic sin).

Original Antigenic Sin Is Not Associated with Differences in Avidity or Multifunctionality of T-Cell Responses. These observations allowed us to test whether original antigenic sin was associated with differential quality or quantity of response (differences in magnitude, avidity, and types of cytokines made). Accordingly, pools of peptides corresponding to either serotype-specific or conserved epitopes were tested in intracellular cytokine staining (ICS) assays with several representative donors (Fig. 3). No appreciable difference in the magnitude, phenotype, pattern of multifunctionality, or avidity of the T-cell responses between serotype-specific and conserved responses was detected (Fig. 3 *A*, *B*, *D*, and *F*, respectively). In both groups, the majority of the DENV-specific T cells displayed an effector memory phenotype ($CD45RA^-CCR7^-$; 42% and 44% for conserved and specific, respectively), followed by central memory ($CD45RA^-CCR7^+$; 24%) and T cells: Effector Memory re-expressing CD45RA (T_{EMRA}) ($CD45RA^+$, $CCR7^-$; 26% and 25%) (Fig. 3*C*, *Left*). The expression levels of CD27 and the marker for cytotoxicity CD107a were also comparable (Fig. 3*C*, *Center* and *Left*). In terms of cytokine expression patterns, no difference between serotype-specific and conserved responses was detected (Fig. 3*D*). Seventeen percent of cells responding to serotype-specific pools were positive for all three cytokines tested (IFNγ, TNFα, and IL2), compared with 15% of cells responding to conserved pools. The majority of responding cells were double positive for two cytokines (50% and 54%, respectively), followed by cells positive for only one cytokine (33% and 21%) (Fig. 3*E*).

Low Magnitude T-Cell Responses Are HLA-Linked and Associated with Disease Susceptibility. Previous studies have highlighted that certain HLA alleles are associated with either increased or decreased risk of clinical manifestations. To investigate this issue further, we correlated HLA types expressed in our cohort with T-cell responses. Fig. 4*A* shows the frequency (black bars) and the magnitude (white bars) of T-cell responses sorted according to their HLA restriction element. A wide variation in terms of frequency and magnitude was detected as a function of the different HLA alleles. Interestingly, certain alleles were associated with low response frequency and magnitude (e.g., A*0101, A*2402), whereas others were associated with high response frequency and magnitude (B*0702, B*3501). Still other alleles were associated with low frequency and high magnitude response (B*4001), and conversely others were associated with high frequency and low magnitude response (A*2601). Overall, HLA B restricted responses were of significantly higher magnitude, but not frequency, compared with HLA A restricted responses ($P = 0.036$ and $P = 0.113$, respectively in an unpaired *t* test). To test whether T-cell responses correlated with HLA-associated disease susceptibility, we compiled all data available in the literature (20–26) and performed a metaanalysis as described in *Materials and Methods*. When we compared average magnitudes of HLA restricted responses with disease susceptibility, it was found that weak T-cell responses correlated with disease susceptibility (Fig. 4*B*, *Left*; $P = 0.05$). This correlation was accounted for by the response magnitude rather than frequency (Fig. 4*B*, *Center*; $P = 0.04$). Further analysis revealed that the magnitude per epitope, rather than the breadth of responses, correlated best with disease susceptibility (Fig. 4*B*, *Right*; $P = 0.02$), such that low T-cell responses were associated with disease susceptibility.

A possible explanation for these observations is that alleles associated with higher magnitude responses may in turn be associated with higher degrees of multifunctionality, most beneficial in protecting from disease. A detailed analysis of cytokines produced by DENV-specific T cells revealed that, indeed, high magnitude responses were associated with a greater proportion of multifunctional T-cell responses. The response hierarchy was IFNγ > TNFα > IL2 in all experiments (Fig. 4*C*).

Discussion

The role of $CD8^+$ T cells in DENV infection is not yet fully understood. Although in animal models $CD8^+$ T cells are associated with protection from infection and disease (31, 32), previous studies in humans suggest that T cells are associated with pathogenesis according to the original antigenic sin hypothesis (17, 33). Although definitions vary somewhat, original antigenic

Table 1. Immunodominant regions of the DENV polyprotein

Antigenic region	Epitope sequence	Proteome location	Sero type	HLA allele	No. of responders	T-cell response (SFC)	
						No.	Total
1	TPEGIIPAL	1978	1,4	B*0702	1	183	10,671
	TPEGIIPALF	1978	1,4	B*3501	9	2,213	
	TPEGIIPSM	1978	2	B*3501, B*5301	5	2,717	
	TPEGIIPSMF	1978	2	B*0702, B*3501	7	1,772	
	TPEGIIPTLF	1978	4	B*3501,B*0702, B*5301	11	2,914	
	YTPEGIIPTL	1977	4	A*0206	1	872	
2	GEARKTFVDL	2005	2	B*4001	3	1,405	5,005
	GEARKTFVEL	2005	1	B*4001	3	1,330	
	GEQRKTFVEL	2005	4	B*4001	2	1,050	
	GESRKTFVEL	2005	3	B*4001	3	1,220	
3	LPVWLAHKVA	2020	3	B*3501	1	23	4,157
	LPVWLAYKV	2020	2	B*5301, B*5101	2	923	
	LPVWLAYKVA	2020	2	B*3501	7	2,427	
	LPVWLAYRVA	2020	2	B*5101	1	470	
	LPVWLSYKV	2020	1	B*5101	1	313	
4	HPGAGKTKRY	1672	2	B*3501	10	3,047	3,047
5	NPEIEDDIF	1653	2	B*3501	10	3,390	3,390
6	DTTPFGQQR	2840	1,2,3,4	A*6801, A*3301	8	3,260	3,260
7	MSFRDLGRVM	1176	2	B*3501	8	3,250	3,250
8	ATGPILTLW	2444	4	B*5801	2	938	3,089
	ATGPISTLW	2444	2	B*5801	2	236	
	ATGPITTLW	2444	3	B*5801	1	505	
	ATGPLTTLW	2444	1	B*5801	1	275	
	ATGPVLTLW	2444	4	B*5801	2	390	
	LATGPVLTLW	2443	4	B*5301	1	745	
9	CLIPTAMAF	108	4	B*1501	1	67	2,815
	MLIPTAMAF	108	2	B*3501	7	2,748	
10	VATTFVTPM	2290	2	B*3501	8	2,777	2,777
11	KPRICTREEF	2885	1	B*0702	1	340	2,756
	KPRLCTREEF	2885	3	B*0702	1	350	
	NPRLCTREEF	2885	4	B*0702	1	365	
	RPRLCTREEF	2885	3	B*0702	4	501	
	TPRMCTREEF	2885	2	B*0702, B*3501	5	1,200	
12	VPLLAIGCY	2338	2	B*3501	7	1,520	2,603
	VPLLAMGCY	2338	4	B*3501	1	1,083	
13	MSYSMCTGKF	578	2	B*3501	6	2,553	2,553
14	DPASIAARGY	1768	1,2,3	B*3501	9	2,383	2,383
15	IANQATVLM	2315	2	B*3501	7	1,518	1,518
16	APTRVVAAEM	1700	2,3,4	B*0702, B*3501	8	1,623	2,230
	APTRVVASEM	1700	1	B*0702	5	607	
17	TPMLRHTIEN	2296	3,4	B*0702	2	388	2,123
18	MLVTPSMTM	274	3	B*3501	8	1,735	1,735
19	FTMRHKKATY	2738	2	B*3501	4	1,723	1,723
20	FTILALFLAH	248	3	B*3501	8	1,715	1,715
21	WHYDQDHPY	2787	2	B*3501	5	1,628	1,628
22	MALKDFKEF	2083	4	B*3501	7	1,497	1,497
23	LMKITAEWLW	2868	2	B*5301	1	423	1,417
	MEITAEWLW	2869	3	B*5801	1	583	
	VMGITAEWLW	2868	3	B*5301	1	410	
24	TETTILDVDL	2266	4	B*4001	2	1,413	1,413
25	GEFRLRGEQR	1999	4	B*4001	3	1,373	1,373

sin in the context of DENV infection is usually defined as the imprint of responses associated with primary infection of a given serotype, which shapes and biases responses following a secondary infection with a different serotype toward epitopes shared by the two serotypes (34). Herein, we show that, whereas the predicted outcome of original antigenic sin can be detected in the general population from Sri Lanka, its consequence is not to generate a less functional response, but rather it results in honing responses toward recognition of conserved viral sequences. We further show that different HLA alleles are associated with differential magnitude of anti-DENV responses, and that HLA alleles known to be associated with increased risk of severe DENV disease (22, 24) are associated with weaker $CD8^+$ responses. Furthermore, the higher magnitude responses are associated with more polyfunctional $CD8^+$ T cells. Taken together, these data support a protective role for $CD8^+$ T cells and challenge the notion that DENV-associated pathogenicity is a result of original antigenic sin.

Table 2. Differences between serotype specific responses in primary and secondary infections

Infection	Serotype	*n*	Average T-cell response/donor			
			Total	Specific	Conserved	Ratio
Secondary infection	DENV1	18	957 ± 310	206	751	0.3
	DENV2	30	2,819 ± 1,203	1,987	822	2.4
	DENV3	22	2,032 ± 619	766	1,266	0.6
	DENV4	20	2,399 ± 789	1,124	1,275	0.9
Primary infection	DENV1	5	167 ± 58	111	56	1.9
	DENV2	0	0	0	0	0
	DENV3	2	443 ± 37	389	54	7.2
	DENV4	1	27	27	0	—

Before this study, only a few $CD8^+$ T-cell epitopes have been defined, and lack of knowledge of T-cell epitopes presented by common MHC alleles expressed by populations in endemic areas allowed only an episodic evaluation of responses. In this study, we report a comprehensive ex vivo characterization of HLA restricted DENV-specific T-cell memory in the general population of Sri Lanka. The impact of these results can be appreciated by noting that previous studies, as compiled by the Immune Epitope Database (IEDB; www.iedb.org), identified a total of 82 unique $CD8^+$ T-cell epitopes. This study expands this number by almost fivefold. Twenty-five main antigenic regions could account for about 50% of the responses. This relatively small number provides a future tool to further investigate the $CD8^+$ responses in small sample volumes typically available from acute fever patients and/or children.

Consistent with the known epidemiologic history of DENV infection in Sri Lanka (28–30) and the notion of original antigenic sin, we see that, whereas serotype-specific responses predominate for DENV2, there is a shift toward conserved epitopes for DENV3 and DENV1 responses, compatible with the epidemiologic data that indicate that initial infection is most likely caused by DENV2 and thus expansion of T cells recognizing conserved epitopes following infection with the more recently prevalent serotypes DENV1 and -3. In the case of DENV4, the same trend, albeit not statistically significant, was observed. Also, DENV4 is the most divergent serotype (35) and thus shares less conserved regions with other DENV serotypes, which might further contribute to the somewhat increased response to serotype-specific epitopes.

These observations provided an opportunity to test whether antigenic sin was associated with lower quality of responses at the level of the general population from an endemic region. No significant difference in the magnitude, phenotype, pattern of multifunctionality, or avidity of the T-cell responses between serotype-specific and conserved/homologous responses was detected. It has been shown that $CD8^+$ T cells acquire activation markers, proliferate actively, and express cytotoxic molecules during infection, directly contributing to the killing of infected cells (36, 37). Additionally, strong responses were associated with multifunctionality,

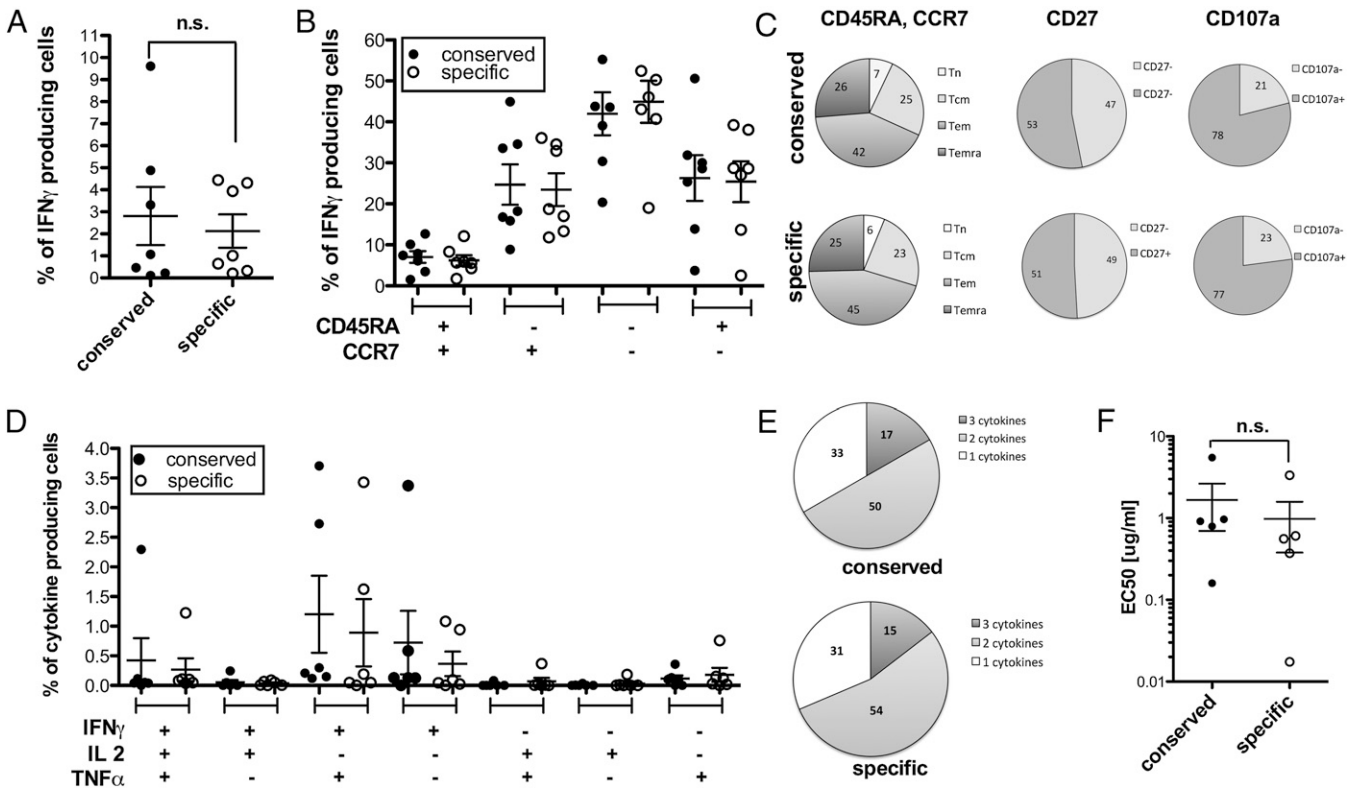

Fig. 3. Comparison between responses to conserved and serotype-specific epitopes. Representative donors who had been secondarily infected previously were incubated with donor-specific peptide pools (1μg/mL) originated either from regions serotype-specific for DENV3 (open circles) or regions conserved between two or more other serotypes (filled circles) for 6 h in the presence of brefeldin A. Cells were then stained with mAB against surface markers CD3, CD8, CD45RA, and CD27 and mAB against intracellular CD107a, IFNγ, TNFα, and IL2. Magnitude of response (*A*) and phenotype of responding cells (*B*) of the individual donors ($n = 7$) based on gating of the IFNγ producing cells are shown. The average CD45RA, CCR7, CD27, and CD107a expression for all responding cells is shown in *C*. Multifunctional responses are shown for individual donors (*D*) and as the average of all donors studied (*E*, $n = 6$). Avidity of responding T cells was determined by incubating PBMCs with ascending concentrations of peptide pools (0.001, 0.01, 0.1, 1, and 10 μg/mL). The peptide concentration necessary to induce 50% of the maximum responses (EC_{50}) was calculated, and the average EC_{50} was compared between the conserved and serotype-specific epitopes (*F*).

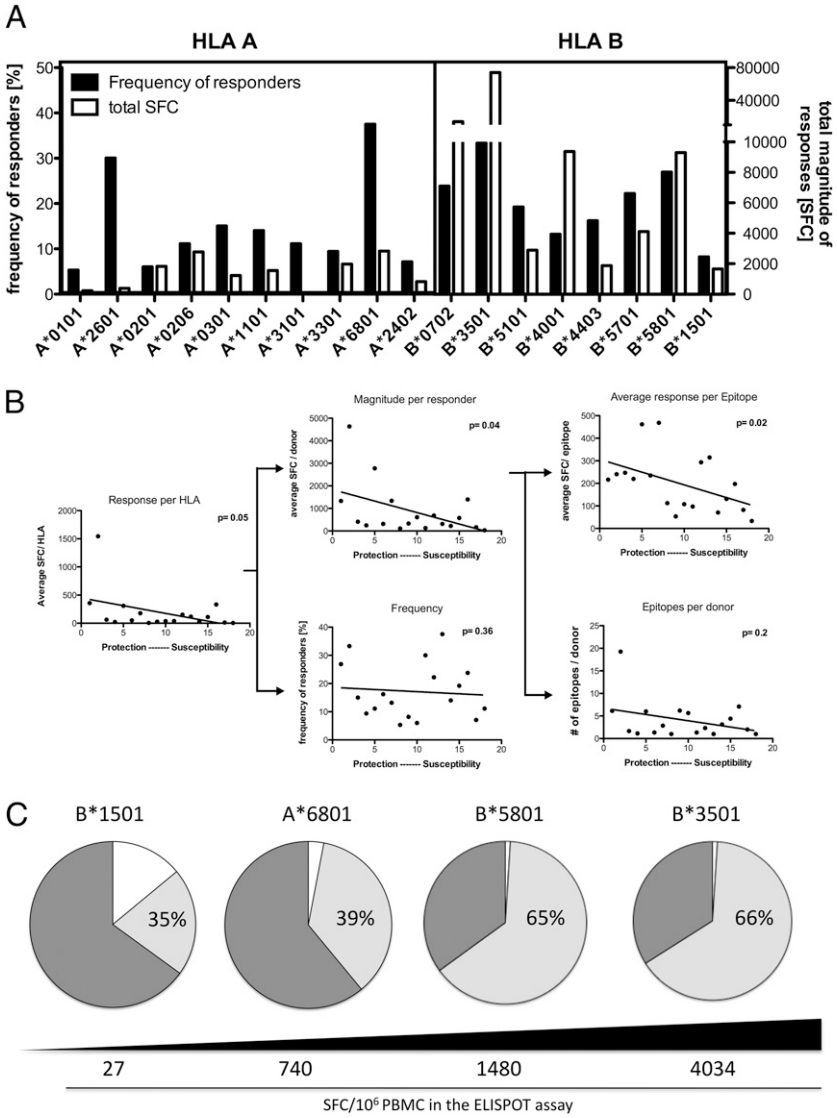

Fig. 4. HLA-linked T-cell responses. (*A*) Differential frequency and magnitude of HLA restricted responses in dengue-seropositive donors (n = 182). Frequency (filled bars) and the magnitude (open bars) of T-cell responses (as total SFCs per 10^6 PBMC values) sorted according to their restriction element are shown. (*B*) Association of HLA restricted T-cell responses with disease susceptibility. Disease susceptibility was correlated with the average magnitudes of HLA restricted responses (*Left*), with the frequency and the magnitude of T-cell responses per donor (*Center*) and with the magnitude per epitope as well as breadth of response (*Right*). One-tailed Spearman test was then used to calculate correlations. The best positive correlation was observed with the magnitude of response per epitope (P = 0.02). (*C*) Multifunctionality of HLA restricted responses. Representative donors were stimulated with HLA restricted donor-specific peptide pools for 6 h in the presence of BFA. Cells were then stained with mAB against surface markers CD3 and CD8 and mAB against intracellular IFNγ, TNFα, and IL2. Pie charts represent cytokine profiles of individual donors. The relative number of cells producing one (dark gray areas), two (light gray areas), or three (white areas) of the measured cytokines is shown. Percentages in the pie charts represent the relative number of cells that produce two or more cytokines. Numbers under the pie charts represent the T-cell responses (as IFNγ SFCs per 10^6 PBMCs) of this specific donor/allele combination in the ELISPOT assay (n = 4).

which has been shown to be a main predictor of immunity in other viral systems (38, 39).

The evidence revealed by the current study is more aptly described by maturation of the specificity of $CD8^+$T-cell responses, induced by repeated stimulation with partially or completely cross-reactive antigens. The protective role of T cells in humans was supported by this study and reverberates with renewed urgency and newsworthiness in light of a recent phase 2b proof-of-concept efficacy vaccine trial (40) that showed only 30% overall efficacy, demonstrating partial (60–80%) protection toward 3 of 4 DENV serotypes, with no protection against DENV2 infection despite three subsequent immunizations and high neutralization titers against all four serotypes. Our demonstration of the protective role of T-cell responses, dominantly targeting the nonstructural proteins NS3, NS4B, and NS5, which are absent in the recombinant live, attenuated tetravalent dengue-yellow fever chimeric virus vaccine (41), both provides an explanation for these disappointing results and underlines the broad relevance of our study.

Our observations are in contrast to other studies that reported that humans who contract a secondary dengue infection generate a $CD8^+$ T-cell response that recognizes a previously encountered dengue serotype with low avidity, which was hypothesized to contribute to pathogenesis (17). This hypothesis, however, is also in conflict with the observation that heterologous T-cell responses are not needed to produce DHF in infants, and the same severe vascular permeability clinical syndrome, and the same concentrations of cytokines in blood, are produced during primary dengue immune responses in infants, as they are in children with secondary

dengue infections (42). Indeed, a recent study has shown a temporal mismatch between the $CD8^+$ T-cell response and commencement of capillary leakage, suggesting that $CD8^+$ T cells are not responsible for early triggering of capillary leakage in children with DHF (37).

Multiple HLA class I alleles have been associated with either protection or susceptibility to dengue infection (22, 23, 25, 26). HLA A*24 is associated with susceptibility to disease not only in secondary, but also in primary infections with DENV (22, 24), and a recent report associated HLA A*01 with susceptibility to DHF in a Brazilian population (43). In contrast, B*3501 was negatively associated with symptomatic disease in Mexican dengue fever patients (25), and one study reported that HLA A and not HLA B genes are associated with DHF (22). However, until now, it was not clear whether this association reflected differences in the corresponding T-cell responses. In our experiments, A*0101 and A*2402 restricted responses were among the lowest observed in terms of frequencies as well as magnitude whereas B*3501 restricted responses were associated with high magnitude responses. Additionally, strong responses were associated with multifunctionality, which has been shown to be an important predictor of immunity in other viral systems (38, 39). Furthermore, higher frequencies of DENV-specific IFNγ-producing T cells are present in children who subsequently developed subclinical infection, compared with those who develop symptomatic secondary DENV infection (44).

We are aware that our data relate to the features of immunity present in the general population and do not directly address the features that may be present at the time of acute manifestations such as DHF and DSS. Thus, although our data demonstrate that secondary infection does not negatively affect the response at the level of the general population, one could speculate a pathogenic role for T cells in the relatively few individuals that develop DHF or DSS (17). For example, the presence of disease-enhancing antibodies together with the HLA-linked lack of a multifunctional T-cell response might be responsible for immune linked pathogenesis in these individuals. This hypothesis would explain why only a small subset of individuals, the ones who carry nonprotective HLA alleles, develop severe disease in secondary infection with DENV. This approach combines the two major hypotheses, ADE and T-cell–mediated pathogenesis in one model and points the way forward to identify robust correlates of protection in natural immunity and vaccination against DENV.

Materials and Methods

Selection of DENV Sequences. We retrieved 2,376 (984 DENV1, 795 DENV2, 493 DENV3, and 101 DENV4) full-length DENV polyprotein sequences for each serotype from the National Center for Biotechnology Information Protein database using the following query: txid11053[orgn] AND 3000:5000[slen]. The number of sequences available varied drastically as a function of geographic locations. To ensure a balanced representation, the number of isolates by geographical region was limited to a maximum of 10. Sequences were considered "unique" if they varied by at least 1 amino acid from all other sequences. In total, 162 DENV1, 171 DENV2, 169 DENV3, and 53 DENV4 sequences were selected. Polyproteins were broken down into all possible 9- and 10-mer sequences for binding predictions as described below.

MHC Class I Binding Predictions and Peptide Selection. All 9- and 10-mer peptides were predicted for their binding affinity to 27 MHC class I molecules. A panel of 16 HLA A (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, which account for 97% of HLA A and B allelic variants in most ethnicities (45). Binding predictions were performed using the command-line version of the consensus prediction tool available on the IEDB web site (www.iedb.org) (46). 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 and they existed in at least 30% of the isolates for that serotype. Homologous peptides from two or more serotypes that were predicted to bind to one HLA molecule were placed in the "conserved peptides" group. This analysis resulted in the synthesis of 8,088 peptides (Mimotopes), which were subdivided into pools of 10 individual peptides.

Human Blood Samples. We obtained 250 peripheral blood samples from healthy adult blood donors from the National Blood Center, Ministry of Health (Colombo, Sri Lanka) in an anonymous manner. The institutional review boards (IRBs) of both the La Jolla Institute for Allergy and Immunology (LIAI) and the Medical Faculty, University of Colombo (serving as National Institutes of Health-approved IRB for Genetech) approved all protocols described in this study. Donors were of both sexes and between 18 and 60 y of age. Samples were collected over a time course of 19 mo between February 2010 and August 2011. PBMCs were purified by density gradient centrifugation (Ficoll-Paque Premium; GE Healthcare Biosciences) resuspended in FBS (Gemini Bio-Products) containing 10% dimethyl sulfoxide, and cryo-preserved in liquid nitrogen. Twenty-three of the 250 blood samples obtained from the National Blood Center had to be excluded from the study due to poor viability of cells after shipment to LIAI. Genomic DNA isolated from PBMCs of the study subjects by standard techniques (QIAmp; Qiagen) was used for HLA typing. High resolution Luminex-based typing for HLA class I was used according the manufacturer's protocol [Sequence-Specific Oligonucleotides (SSO) typing; One Lambda]. Where needed, PCR-based methods were used to provide high resolution subtyping [Sequence-Specific Primer (SSP) typing; One Lambda).

Serology. For the 227 samples included in the study, DENV seropositivity was determined by dengue IgG ELISA as previously described (28). In Sri Lanka, levels of seropositivity for DENV approach 70% by the age of 10 (47), and DENV-specific IgG ELISAs confirmed that 80% of the donors in our cohort were seropositive (182 out of 227). Flow cytometry-based neutralization assays were performed for further characterization of seropositve donors, as previously described (48). Neutralization assays determined that 127 donors have experienced infection with more than one serotype, further referred to as secondary infections. The primary cases demonstrated that all four serotypes circulate in Sri Lanka (14 DENV1, 18 DENV2, 20 DENV3, and 3 DENV4 donors), confirming previous reports (29).

Ex Vivo IFNγ ELISPOT Assay. PBMCs (2×10^5) were incubated in triplicates with 0.1 mL of complete RPMI 1640 in the presence of HLA-matched peptide pools (2 μg/mL) as previously described. Following a 20-h incubation at 37 °C, the cells were incubated with biotinylated IFNγ mAb (mAb 7-B6-1; Mabtech) for 2 h and developed as previously described (49).

Epitope Cluster Analysis. All epitopes identified were clustered using the Epitope Cluster Analysis tool from the IEDB Web site (www.iedb.org). This tool groups epitopes into clusters based on sequence identity. A cluster is defined as a group of sequences that have a sequence similarity greater than the minimum sequence identity threshold specified. The threshold of minimum sequence identity used in this study was set at 80%.

Meta Analysis for HLA Associations. To test whether T-cell responses correlated with HLA-associated disease susceptibility, we compiled all data available in the literature (20–26). For each of the studies, we ranked all investigated alleles according to their association with clinical manifestations (DF, DHF, and DSS; Dataset S1). We then calculated a percentile ranking across all studies for the 18 alleles detected in significant frequencies in our own cohort and correlated the rankings with T-cell responses.

Flow Cytometry and ICS. The following monoclonal antibodies from eBioscience were used in this study: anti-CD8a PerCP-Cy5.5 (clone RPA-T8), anti-CD3 efluor 450 (clone UCHT1), anti-CD107a FITC (clone ebioH4A3), anti-CD45RA PE-CY7 (clone H100), anti-CD27 PE (clone o323), anti-CD197 (CCR7) APC efluor 780 (clone 3D12), anti-IFNγ APC (clone 4SB3), anti-IL 2 PE (clone MQ1-17H12), and anti-TNFα (clone MAD 110). PBMCs were cultured in the presence of peptide pools (10 μg/mL) and GolgiPlug containing brefeldin A (1 μg/mL) (BD Biosciences). Cells were then washed and stained for 30 min on ice, fixed with 1% of paraformaldehyde (Sigma-Aldrich), and kept at 4 °C overnight. Samples were acquired on an LSR II flow cytometer (BD Immunocytometry Systems) and analyzed with FlowJo software (Tree Star).

ACKNOWLEDGMENTS. We thank the National Blood Center, Ministry of Health (Colombo, Sri Lanka) for providing buffy coat samples used in this study. This work was supported by National Institutes of Health Contract HHSN272200900042C (to A.S.).

1. Fu J, Tan BH, Yap EH, Chan YC, Tan YH (1992) Full-length cDNA sequence of dengue type 1 virus (Singapore strain S275/90). *Virology* 188(2):953–958.
2. Suaya JA, et al. (2009) Cost of dengue cases in eight countries in the Americas and Asia: A prospective study. *Am J Trop Med Hyg* 80(5):846–855.
3. Guzman MG, et al. (2010) Dengue: A continuing global threat. *Nat Rev Microbiol* 8(12, Suppl):S7–S16.
4. Kyle JL, Harris E (2008) Global spread and persistence of dengue. *Annu Rev Microbiol* 62:71–92.
5. Guzman A, Istúriz RE (2010) Update on the global spread of dengue. *Int J Antimicrob Agents* 36(Suppl 1):S40–S42.
6. Burke DS, Nisalak A, Johnson DE, Scott RM (1988) A prospective study of dengue infections in Bangkok. *Am J Trop Med Hyg* 38(1):172–180.
7. Sangkawibha N, et al. (1984) Risk factors in dengue shock syndrome: A prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. *Am J Epidemiol* 120(5):653–669.
8. Halstead SB (1988) Pathogenesis of dengue: Challenges to molecular biology. *Science* 239(4839):476–481.
9. Heinz FX, Stiasny K (2012) Flaviviruses and flavivirus vaccines. *Vaccine* 30(29): 4301–4306.
10. Morens DM (1994) Antibody-dependent enhancement of infection and the pathogenesis of viral disease. *Clin Infect Dis* 19(3):500–512.
11. Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM (2010) Intrinsic antibody-dependent enhancement of microbial infection in macrophages: Disease regulation by immune complexes. *Lancet Infect Dis* 10(10):712–722.
12. 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(2):128–139.
13. Balsitis SJ, et al. (2010) Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. *PLoS Pathog* 6(2):e1000790.
14. Halstead SB (1979) In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. *J Infect Dis* 140(4):527–533.
15. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ (2007) Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. *Proc Natl Acad Sci USA* 104(22):9422–9427.
16. Halstead SB, Rojanasuphot S, Sangkawibha N (1983) Original antigenic sin in dengue. *Am J Trop Med Hyg* 32(1):154–156.
17. Mongkolsapaya J, et al. (2003) Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med* 9(7):921–927.
18. Davenport FM, Hennessy AV, Francis T, Jr. (1953) Epidemiologic and immunologic significance of age distribution of antibody to antigenic variants of influenza virus. *J Exp Med* 98(6):641–656.
19. Klenerman P, Zinkernagel RM (1998) Original antigenic sin impairs cytotoxic T lymphocyte responses to viruses bearing variant epitopes. *Nature* 394(6692):482–485.
20. Malavige GN, et al. (2011) HLA class I and class II associations in dengue viral infections in a Sri Lankan population. *PLoS ONE* 6(6):e20581.
21. Stephens HA, et al. (2002) HLA-A and -B allele associations with secondary dengue virus infections correlate with disease severity and the infecting viral serotype in ethnic Thais. *Tissue Antigens* 60(4):309–318.
22. Loke H, et al. (2001) Strong HLA class I-restricted T cell responses in dengue hemorrhagic fever: A double-edged sword? *J Infect Dis* 184(11):1369–1373.
23. Appanna R, Ponnampalavanar S, Lum Chai See L, Sekaran SD (2010) Susceptible and protective HLA class 1 alleles against dengue fever and dengue hemorrhagic fever patients in a Malaysian population. *PLoS ONE* 5(9).
24. Nguyen TP, et al. (2008) Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. *PLoS Negl Trop Dis* 2(10):e304.
25. Falcón-Lezama JA, et al. (2009) HLA class I and II polymorphisms in Mexican Mestizo patients with dengue fever. *Acta Trop* 112(2):193–197.
26. Sierra B, et al. (2007) HLA-A, -B, -C, and -DRB1 allele frequencies in Cuban individuals with antecedents of dengue 2 disease: Advantages of the Cuban population for HLA studies of dengue virus infection. *Hum Immunol* 68(6):531–540.
27. Sidney J, Peters B, Frahm N, Brander C, Sette A (2008) HLA class I supertypes: A revised and updated classification. *BMC Immunol* 9:1.
28. Kanakaratne N, et al. (2009) Severe dengue epidemics in Sri Lanka, 2003-2006. *Emerg Infect Dis* 15(2):192–199.
29. Messer WB, et al. (2002) Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever. *Am J Trop Med Hyg* 66(6): 765–773.
30. Tissera HA, et al. (2011) New dengue virus type 1 genotype in Colombo, Sri Lanka. *Emerg Infect Dis* 17(11):2053–2055.
31. Yauch LE, et al. (2009) A protective role for dengue virus-specific CD8+ T cells. *J Immunol* 182(8):4865–4873.
32. Zompi S, Santich BH, Beatty PR, Harris E (2012) Protection from secondary dengue virus infection in a mouse model reveals the role of serotype cross-reactive B and T cells. *J Immunol* 188(1):404–416.
33. Duangchinda T, et al. (2010) Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. *Proc Natl Acad Sci USA* 107(39):16922–16927.
34. Rothman AL (2011) Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat Rev Immunol* 11(8):532–543.
35. Zanotto PM, Gould EA, Gao GF, Harvey PH, Holmes EC (1996) Population dynamics of flaviviruses revealed by molecular phylogenies. *Proc Natl Acad Sci USA* 93(2):548–553.
36. Rivino L, et al. (2013) Differential targeting of viral components by CD4+ versus CD8+ T lymphocytes in dengue virus infection. *J Virol* 87(5):2693–2706.
37. Dung NT, et al. (2010) Timing of CD8+ T cell responses in relation to commencement of capillary leakage in children with dengue. *J Immunol* 184(12):7281–7287.
38. Almeida JR, et al. (2009) Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. *Blood* 113(25):6351–6360.
39. Park SH, et al. (2012) Successful vaccination induces multifunctional memory T-cell precursors associated with early control of hepatitis C virus. *Gastroenterology* 143(4): 1048–1060.
40. Sabchareon A, et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: A randomised, controlled phase 2b trial. *Lancet* 380(9853):1559–1567.
41. Guy B, et al. (2011) From research to phase III: Preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. *Vaccine* 29(42): 7229–7241.
42. Nguyen TH, et al. (2004) Dengue hemorrhagic fever in infants: A study of clinical and cytokine profiles. *J Infect Dis* 189(2):221–232.
43. Monteiro SP, et al. (2012) HLA-A*01 allele: A risk factor for dengue haemorrhagic fever in Brazil's population. *Mem Inst Oswaldo Cruz* 107(2):224–230.
44. Hatch S, et al. (2011) Intracellular cytokine production by dengue virus-specific T cells correlates with subclinical secondary infection. *J Infect Dis* 203(9):1282–1291.
45. Sette A, Sidney J (1999) Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* 50(3-4):201–212.
46. Kim Y, et al. (2012) Immune epitope database analysis resource. *Nucleic Acids Res* 40 (Web Server issue):W525–30.
47. Tissera HA, et al. (2010) Dengue Surveillance in Colombo, Sri Lanka: Baseline seroprevalence among children. *Procedia in Vaccinology* 2:109–112.
48. Kraus AA, Messer W, Haymore LB, de Silva AM (2007) Comparison of plaque- and flow cytometry-based methods for measuring dengue virus neutralization. *J Clin Microbiol* 45(11):3777–3780.
49. Weiskopf D, et al. (2011) Insights into HLA-restricted T cell responses in a novel mouse model of dengue virus infection point toward new implications for vaccine design. *J Immunol* 187(8):4268–4279.