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CELLULAR RESPONSE TO INFECTION

Prior Dengue Virus Exposure Shapes T Cell Immunity to Zika Virus in Humans

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ABSTRACT While progress has been made in characterizing humoral immunity to Zika virus (ZIKV) in humans, little is known regarding the corresponding T cell responses to ZIKV. Here, we investigate the kinetics and viral epitopes targeted by T cells responding to ZIKV and address the critical question of whether preexisting dengue virus (DENV) T cell immunity modulates these responses. We find that memory T cell responses elicited by prior infection with DENV or vaccination with tetravalent dengue attenuated vaccines (TDLAV) recognize ZIKV-derived peptides. This cross-reactivity is explained by the sequence similarity of the two viruses, as the ZIKV peptides recognized by DENV-elicited memory T cells are identical or highly conserved in DENV and ZIKV. DENV exposure prior to ZIKV infection also influences the timing and magnitude of the T cell response. ZIKV-reactive T cells in the acute phase of infection are detected earlier and in greater magnitude in DENV-immune patients. Conversely, the frequency of ZIKV-reactive T cells continues to rise in the convalescent phase in DENV-naive donors but declines in DENV-preexposed donors, compatible with more efficient control of ZIKV replication and/or clearance of ZIKV antigen. The quality of responses is also influenced by previous DENV exposure, and ZIKV-specific CD8 T cells from DENV-preexposed donors selectively upregulated granzyme B and PD1, unlike DENV-naive donors. Finally, we discovered that ZIKV

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structural proteins (E, prM, and C) are major targets of both the CD4 and CD8 T cell responses, whereas DENV T cell epitopes are found primarily in nonstructural proteins.

IMPORTANCE The issue of potential ZIKV and DENV cross-reactivity and how preexisting DENV T cell immunity modulates Zika T cell responses is of great relevance, as the two viruses often cocirculate and Zika virus has been spreading in geographical regions where DENV is endemic or hyperendemic. Our data show that memory T cell responses elicited by prior infection with DENV recognize ZIKV-derived peptides and that DENV exposure prior to ZIKV infection influences the timing, magnitude, and quality of the T cell response. Additionally, we show that ZIKV-specific responses target different proteins than DENV-specific responses, pointing toward important implications for vaccine design against this global threat.

KEYWORDS ZIKV, DENV, T cells, heterologous immunity, cross-reactivity, immunodominance

The pandemic rise of Zika virus (ZIKV) has recently commanded the attention of the general public and medical research community alike (1–4).

ZIKV is a flavivirus most closely related to dengue virus (DENV) (5, 6) but also related with Japanese encephalitis virus (JEV), West Nile virus (WNV), and yellow fever virus (YF), all of which are transmitted primarily by mosquitoes (7). Understanding host protective immunity to this virus is critical for the design of optimal vaccines, but little is currently known about the immune responses to ZIKV in humans since infections with ZIKV have not been frequent in the past (8, 9). This is in contrast to a substantial wealth of information related to T cell immunity against the closely related DENV (10–12).

In the case of DENV, CD8 T cell responses mostly target nonstructural (NS) proteins, such as NS3, NS4B, and NS5, while CD4 T cell responses are focused on the C, E, and NS5 proteins, even though serotype-specific differences have been noted (10, 13–16). The main protein targets of CD4 and CD8 T cell immunity are presently unknown for ZIKV. This dearth of information is a severe knowledge gap, as robust T cell responses may be required for optimal ZIKV vaccine efficacy (9).

The issue of potential ZIKV and DENV cross-reactivity is of relevance for development of both diagnostic tests and vaccines. ZIKV and DENV have significant sequence similarity and share the same arthropod host, and the geographic range of ZIKV overlaps largely with areas where DENV is endemic or hyperendemic (6, 17). The concomitant cocirculation of DENV and ZIKV represents yet another biomedical challenge, since this phenomenon of common dual exposure increases the potential for cross-reaction. Serological cross-reactivity has been addressed by several reports (18–23). However, it is currently unknown to what extent ZIKV and DENV cross-react with each other at the level of T cell immunity.

According to the well-established phenomenon of heterologous immunity (24, 25), it is possible that preexisting DENV immunity will affect T cell responses to ZIKV and hence influence the dynamics and severity of ZIKV epidemics. Importantly, previous DENV infection can in some instances increase severity of a second DENV infection with a heterologous serotype, likely through antibody-dependent enhancement (ADE) of infection and disease (26). In the phase IIb/III clinical trials of the first licensed tetravalent dengue vaccine, increased vaccine efficacy in DENV-preexposed as opposed to DENV-naive vaccinees was observed, suggesting a protective role of preexisting cross-reactive DENV-specific T cells that are boosted upon vaccination (9). Thus, it is also possible that preexposure to either ZIKV or DENV infection will influence the disease course following infection with the other virus in either a favorable or detrimental fashion. For all these reasons, it is necessary to gain insight into human T cell responses to ZIKV.

RESULTS

DENV T cell responses are cross-reactive with ZIKV peptides. To address the interplay between DENV- and ZIKV-specific T cell responses, we studied HLA-typed peripheral blood mononuclear cell (PBMC) donations from Sri Lanka obtained between 2010 and 2016. We also studied PBMC from Nicaraguan donors obtained between 2010 and 2014, thus preceding the current ZIKV epidemic (10, 27, 28). To study CD8 responses, we selected nine DENV-positive donors who had been infected by DENV multiple times (secondary infections) based on serum neutralization titers and whose samples showed appreciable *ex vivo* responses to a pool of previously defined CD8 DENV epitopes (CD8-megapool) (28). A similar approach was used for CD4 responses, retrieving 5 DENV-positive donors with *ex vivo* responses to a previously defined DENV CD4-megapool (11). As negative controls, we used PBMC from donors who were DENV negative from the same sites.

We tested PBMC from these groups for reactivity against ZIKV peptides in *ex vivo* IFN-γ enzyme-linked immunosorbent sport (ELISPOT) assays. In the case of CD8 T cell responses (HLA class I), we tested panels of ZIKV-derived peptides predicted to bind each donor's HLA molecules (10). HLA restrictions were assigned based on testing short 9mers and 10mers that are predicted to bind with high affinity to the HLA allotypes of the responding donors. In the case of CD4 T cell responses (HLA class II), we tested a panel of 684 overlapping peptides spanning the entire ZIKV proteome. CD8-depleted PBMCs were used in these experiments to avoid inadvertently identifying CD8 epitopes nested in the 15mer peptide tested. In both cases, peptide pools were tested and the total reactivity observed in each donor was recorded. The peptide sets used in this study are summarized in Table 1.

As expected for CD8, T cells from the DENV-negative donors did not respond to either the previously defined DENV epitopes or to the ZIKV peptides. The cells were viable and responsive to stimulation, as shown by vigorous responses to phytohemagglutinin (PHA) mitogen stimulation. Interestingly, CD8 T cells from one-third of the DENV-positive donors recognized ZIKV-derived peptides (Fig. 1A). Higher levels of cross-reactivity emerged from the study of the CD4 T cells, as ZIKV-derived peptides were recognized by CD4 T cells from 4 out of 5 DENV-positive individuals (Fig. 1B).

In a further series of experiments, we analyzed responses from two additional cohorts of donors, a cohort of donors recently vaccinated with a tetravalent dengue attenuated vaccine (TDLAV) and a control cohort of donors negative for responses to DENV and other flaviviruses provided to the University of Vermont clinical site. Responses against the DENV CD8-megapool and pools of ZIKV predicted peptides matching the HLA A and B alleles expressed in each donor were tested in IFN-gamma (IFN-γ) intracellular cytokine staining (ICS) assays (Fig. 1C). CD8 T cells from the flavivirus-negative donors did not respond to either the previously defined DENV epitopes or the ZIKV peptides. In contrast, CD8 T cells from TDLAV donors recognized, as expected, the DENV CD8-megapool as well as more than 50% of the cases of the ZIKV-derived peptides. In conclusion, analysis of *ex vivo* responses of ZIKV-naive and DENV-positive donors revealed substantial cross-reactivity to ZIKV-derived peptides.

Identification of ZIKV epitopes cross-reactive with DENV responses. Individual epitopes were mapped in representative cases. Where sufficient cell numbers were available, positive pools were deconvoluted to identify ZIKV-specific epitopes across the ZIKV genome, including all structural and nonstructural (NS) proteins. The mapping of CD4 and CD8 responses was performed by sequential testing of pools and deconvolution to identify the positive peptides (Fig. 2A). The HLA-B*35:01 CD8 epitope encoded by ZIKV $NS3_{2867–2876}$ was recognized by PBMC from a DENV-positive Nicaraguan donor (Fig. 2B). This epitope was found to be highly similar (a single Y-to-F substitution) in DENV1 to -4 serotype consensus sequences obtained as previously described (10). A Sri Lankan donor recognized the B*07:02 ZIKV $NS3_{1725–1734}$ epitope (Fig. 2C). The same epitope was also recognized by a different DENV-positive Sri Lankan donor (Fig. 2D). The identical sequence was found in DENV2, -3, and -4.

TABLE 1 ZIKV peptides used in this study

Allele	No. of peptides positive for:												
	C	pr	M	E	NS1	NS2A	NS2B	NS3	NS4A	2K	NS4B	NS5	Total
ZIKV predicted peptide set composed of 9- and 10mer peptides													
HLA-A*01:01	0	10	5	21	6	8	6	21	4	0	17	38	136
HLA-A*02:01	7	0	6	20	3	23	5	17	10	3	26	16	136
HLA-A*02:03	9	0	6	16	3	23	8	20	9	4	23	15	136
HLA-A*02:06	4	2	2	14	6	25	5	17	17	6	25	13	136
HLA-A*03:01	12	4	4	11	10	17	4	22	5	0	8	39	136
HLA-A*11:01	14	6	2	11	9	6	7	23	6	0	11	41	136
HLA-A*23:01	5	2	4	20	7	7	1	21	7	0	21	41	136
HLA-A*24:02	4	3	4	16	5	9	2	16	7	0	24	46	136
HLA-A*26:01	6	5	1	15	6	10	15	16	9	3	17	33	136
HLA-A*30:01	9	3	1	18	16	8	3	26	3	0	10	39	136
HLA-A*30:02	1	10	5	17	11	2	8	24	1	0	21	36	136
HLA-A*31:01	10	3	8	8	18	11	2	25	1	0	5	45	136
HLA-A*32:01	6	3	6	21	9	18	6	16	7	1	11	32	136
HLA-A*33:01	9	1	5	6	15	12	3	22	2	0	5	56	136
HLA-A*68:01	9	4	5	12	13	8	3	35	3	0	7	37	136
HLA-A*68:02	7	5	5	17	6	11	7	18	8	5	22	25	136
HLA-B*07:02	4	2	6	12	15	16	5	35	6	2	11	22	136
HLA-B*08:01	11	4	2	13	13	16	0	24	10	0	7	36	136
HLA-B*15:01	4	7	7	18	6	12	7	17	6	1	23	28	136
HLA-B*35:01	4	5	3	14	5	12	9	23	7	2	26	26	136
HLA-B*40:01	2	4	4	17	17	4	8	25	10	0	6	39	136
HLA-B*44:02	1	4	1	15	18	3	7	32	7	0	5	43	136
HLA-B*44:03	3	3	2	14	20	3	7	33	7	0	4	40	136
HLA-B*51:01	4	0	8	13	6	19	9	17	9	5	17	29	136
HLA-B*53:01	6	3	2	18	13	12	6	18	8	2	17	31	136
HLA-B*57:01	3	5	4	15	16	12	3	13	4	0	13	48	136
HLA-B*58:01	7	1	5	17	16	14	3	11	5	0	11	46	136
Total	161	99	113	409	288	321	149	587	178	34	393	940	3672
15mer peptides spanning the ZIKV polyprotein HLA class II	25	18	15	100	70	46	26	123	25	5	50	180	683

In the case of CD4 (Fig. 2E), the ZIKV $NS5_{2986-3000}$ epitope, 100% conserved in DENV1 to -4 sequences, was recognized by PBMC from a DENV-positive Sri Lankan donor. PBMC from a Nicaraguan donor recognized the ZIKV $NS1_{986-1000}$ epitope (Fig. 2F). Here, the recognized 15mer contained a core $NS1_{989-998}$ sequence that was also highly conserved in all DENV serotypes, with A-to-S and M-to-L conservative substitutions. A different pattern was observed for the ZIKV $E_{486-500}$ epitope, which was recognized by PBMC from a different DENV-positive Nicaraguan donor (Fig. 2G). In this case the most homologous 9mer (sequence LYYLTMNNK) shared only 4 identities with DENV1 sequences and had 2 conservative (L to M and N to E) and 3 semiconservative (Y to V, Y to L, and K to N) substitutions. Additional sequence homology analysis using GenBank sequences did not reveal any sequences with higher homology from other common flaviviruses, such as JEV, WNV, DENV, and YFV.

In conclusion, in 5 out of 6 instances the cross-reactivity from the DENV-positive (and presumably ZIKV-negative) donors was directed to ZIKV sequences found to be identical to or highly conserved with sequences in DENV serotypes.

Recruitment of donor cohorts differing in ZIKV and DENV preexposure status. To address the effect of preexisting immunity on T cell responses to secondary flavivirus infection, we investigated six donor groups, namely, ZIKV acute, convalescent, and negative, and for each of these cohorts we further subdivided our cohorts into DENV-positive and -negative individuals. For the purpose of classification in the various cohorts, the following criteria were used. Infection with ZIKV was confirmed using reverse transcription-PCR (RT-PCR) performed on acute infection samples, as described in more detail below. Depending on the time of sample collection after the onset of

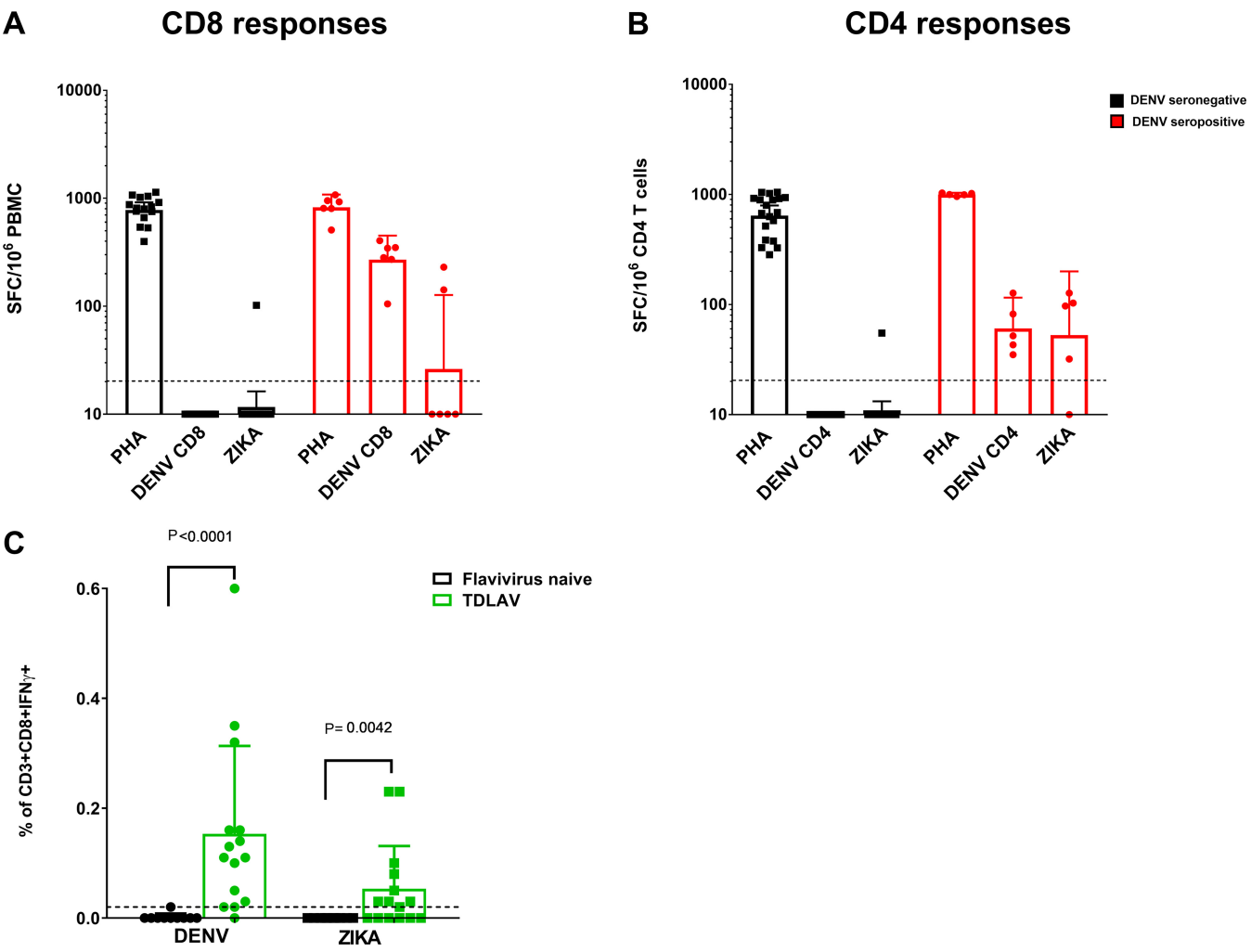

FIG 1 *Ex vivo* reactivity to ZIKV-derived peptides and previously defined DENV epitopes in DENV-positive and -negative donors and DENV vaccines. CD8 (A) and CD4 (B) T cell reactivity to DENV epitopes and ZIKV peptides in ELISPOT *ex vivo* experiments are shown for DENV-positive (red) or -negative (black) donors. Responses were expressed as the number of IFN-γ-secreting cells per 10^6 PBMC and were considered positive if the net SFC/10^6 PBMC was ≥20, had a stimulation index of ≥2, and had a *P* value of <0.05 in a *t* test or in a Poisson test comparing replicates with those from the negative control. Donors with PHA values of <250 SFC per 10^6 PBMC have been excluded from the analysis. Data are expressed as geometric means with 95% CI. (C) CD8 T cell reactivity to a DENV megapool and ZIKV HLA-restricted pools in ICS experiments are shown in DENV vaccinees (green) and compared with flavivirus-naive donors (black). Data are expressed as averages ± standard deviations (SD) of the percentages from $CD3^+$ $CD8^+$ IFN-γ^+ cells.

symptoms or ZIKV RNA-positive blood donation, samples were classified as either acute or convalescent, as described in more detail in Materials and Methods. ZIKV negativity was inferred based on donations being obtained before or outside the area affected by the epidemic. DENV-positive or -negative status was determined on the basis of IgG status at the time of clinical presentation or blood donation or, in the case of the Nicaraguan samples, from documented history of DENV infection in the longitudinal cohort study. The subjects studied spanned a very diverse breadth of ethnicities and clinical sites, including Brazil (Rio de Janeiro and Sao Paulo), Nicaragua, Puerto Rico, Mexico, returned U.S. travelers, and a U.S. flavivirus-negative cohort. The general features of the subjects are detailed in Table 2. The relative proportion of females across all cohorts was 60%, and the average age was 34 years (range, 3 to 70 years).

ZIKV-specific responses are modulated by previous DENV exposure. We next compared ZIKV T cell reactivity in the subjects described above as a function of ZIKV status (i.e., negative, acute infection, or convalescent status) and also considering prior DENV infection as a variable. To assess T cell reactivity, we devised a strategy to account for the fact that in most cases the amount of PBMC was limiting. Accordingly, the

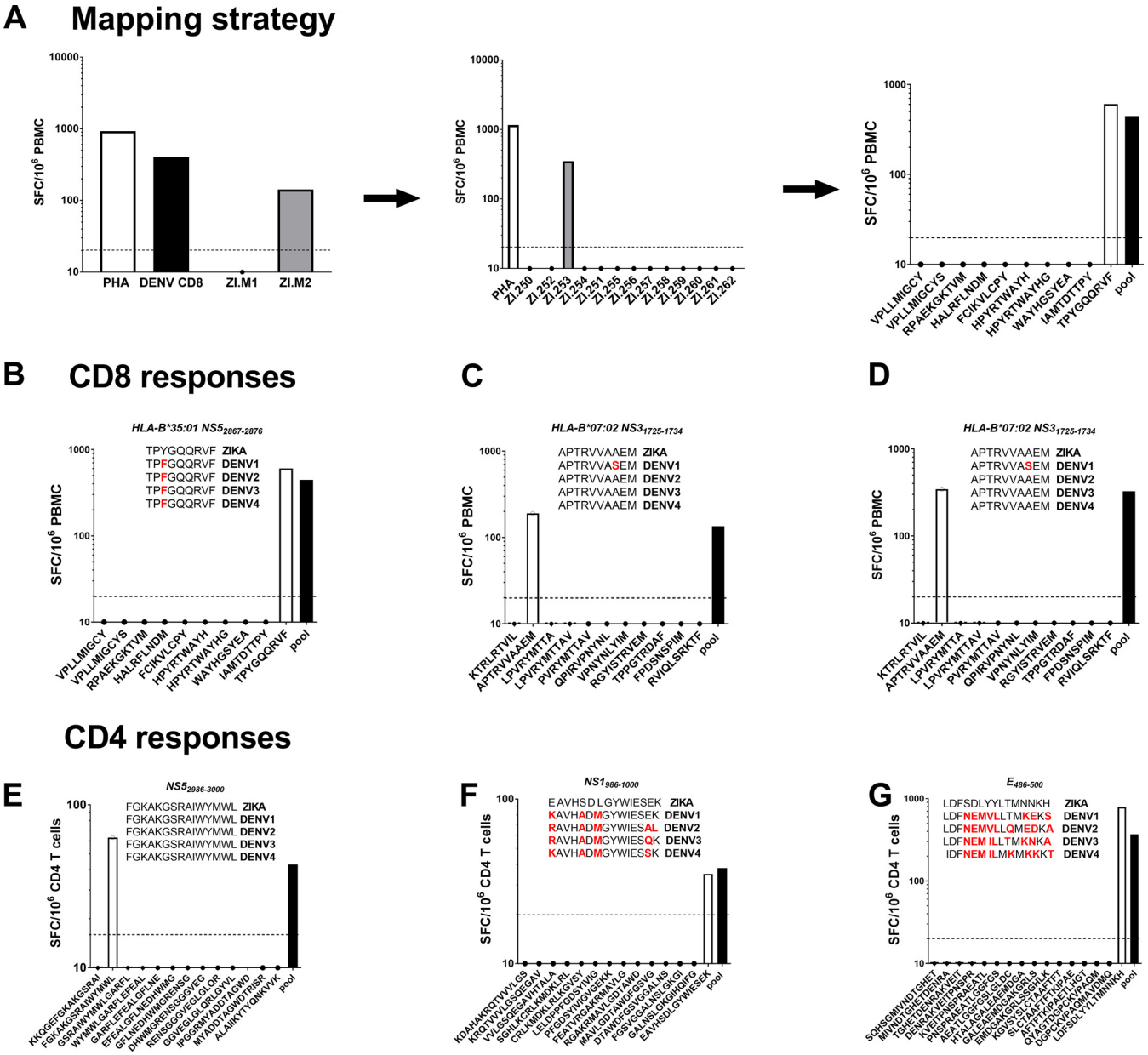

FIG 2 Mapping of CD8 and CD4 cross-reactive DENV-ZIKV T cell epitopes. (A) Example of the mapping strategy. CD8 (B to D)- and CD4 (E to G)-restricted epitopes were mapped by peptide deconvolution in ELISPOT *ex vivo* experiments in individual donors. ZIKV epitope sequences were aligned with consensus sequences of DENV1, DENV2, DENV3, and DENV4 serotypes. Amino acid mismatches between the ZIKV sequence and the DENV consensus sequences are shown in red.

overlapping 15mers spanning the entire ZIKV proteome were divided into 10 pools corresponding to the 10 encoded ZIKV proteins. ICS assays and CD8/CD4 gating allowed assessment of CD8 and CD4 responses in parallel without the need to know the HLA phenotype of the donor. All of the ZIKV CD8 responses in ZIKV samples have been assessed using these pools of overlapping peptides and gating on $CD8^+$ responding T cells in the ICS assay. In a few instances where the number of PBMC available from each donor did not allow testing of all pools, a factorial design was utilized: while not all pools were tested in all donors, all pools were tested in the same number of donors. Whenever sufficient cell numbers were available, positive pools were deconvoluted and specific epitopes identified. Overall, PBMC from 17 to 33 donors/patients were tested for each of the different categories (Table 3).

TABLE 2 General features of ZIKV-infected cohorts

Source	Country	No. of patients	Age[a] (yr)	Sex (% female)	% DENV$^+$
University of São Paulo	Brazil	7	45 (25–61)	85	85
Fundação Oswaldo Cruz	Brazil	12	35 (22–60)	20	100
PDCS	Nicaragua	14	7 (3–14)	78	14
REDSIII	Puerto Rico/USA	20	46 (21–70)	35	85
Universidad Veracruzana	Mexico	19	38 (6–69)	63	26
University of North Carolina	Unites States	8	37 (18–53)	71	50
University of Miami	United States	2	29 (26–32)	100	50
Vanderbilt University	United States	9	42 (19–62)	56	11
National Institutes of Health	United States	7	29 (26–40)	42	71
Overall		98	34 (3–70)	60	54

[a]Expressed as the average age of the cohort (range).

The frequency of *ex vivo* responses in ZIKV-infected patients was 30 to 40% for both CD4 and CD8 responses, with the exception of CD8 responses in acutely infected donors, which were detected in approximately 90% of the cases (Fig. 3A and D, left). Marginal CD8 responses to the ZIKV peptides were noted in the case of the ZIKV-negative, DENV-negative donors (Fig. 3A). However, ZIKV-negative, DENV-positive donors showed appreciable reactivity both in terms of increased frequency and magnitude of responses, confirming a degree of T cell cross-reactivity between the DENV-ZIKV responses observed above (Fig. 1 and 2). In the acute ZIKV-positive/DENV-positive donors, CD8 responses to ZIKV peptides were of significantly higher magnitude than those of acute ZIKV subjects who were DENV negative (Fig. 3B and C). After ZIKV convalescence, the CD8 responses to ZIKV-restricted peptides were still elevated compared to those of ZIKV-negative donors but were not significantly different by DENV serostatus (Fig. 3B and C). The pattern of CD4 responses to ZIKV-restricted class II peptides was remarkably similar with regard to ZIKV acute and convalescence phases and impact of DENV seropositivity, with trends for *ex vivo* ZIKV T cell responses being delayed in DENV-negative donors and lower frequency and magnitude of responses observed with respect to the CD8 counterpart (Fig. 3D to F).

Different proteins are targeted by ZIKV versus DENV immunity. We next determined whether DENV serostatus affected the antigenic targets of ZIKV-reactive T cells across the ZIKV polyprotein. A breakdown of ZIKV CD8 responses in acute and convalescent ZIKV-positive donors (combined in this plot) as a function of the antigen targeted is presented in Fig. 4. In the case of ZIKV-specific CD8 responses in DENV-negative donors, 57% of the response was directed against structural proteins (Fig. 4A). In the context of a previous DENV exposure, however, only 30% of the ZIKV-specific responses were directed against structural proteins (Fig. 4B). This can be compared to historical data regarding DENV responses from presumably ZIKV-negative donors (since samples were collected prior to the 2015-2016 ZIKV epidemic), where only 14.9% of the response was directed against structural proteins (10). Thus, the CD8 response to ZIKV is more focused on structural proteins than on nonstructural proteins by DENV-specific

TABLE 3 Donors tested in each category

No. of samples	ZIKV status[a]	DENV status[b]	Country of origin
18	Acute	Pos.	Brazil/Mexico
17	Acute	Neg.	Nicaragua/Mexico
33	Convalescent	Pos.	Brazil/U.S. travelers/blood bank donors
30	Convalescent	Neg.	U.S. travelers/blood bank donors
20	Neg.	Pos.	Nicaragua/Sri Lanka
20	Neg.	Neg.	USA

[a]Infection with ZIKV was confirmed by RT-PCR. ZIKV-negative (Neg.) samples were collected before the onset of the ZIKV epidemic.

[b]Previous exposure to DENV was determined by the presence of detectable DENV-specific IgG titers. Pos., positive.

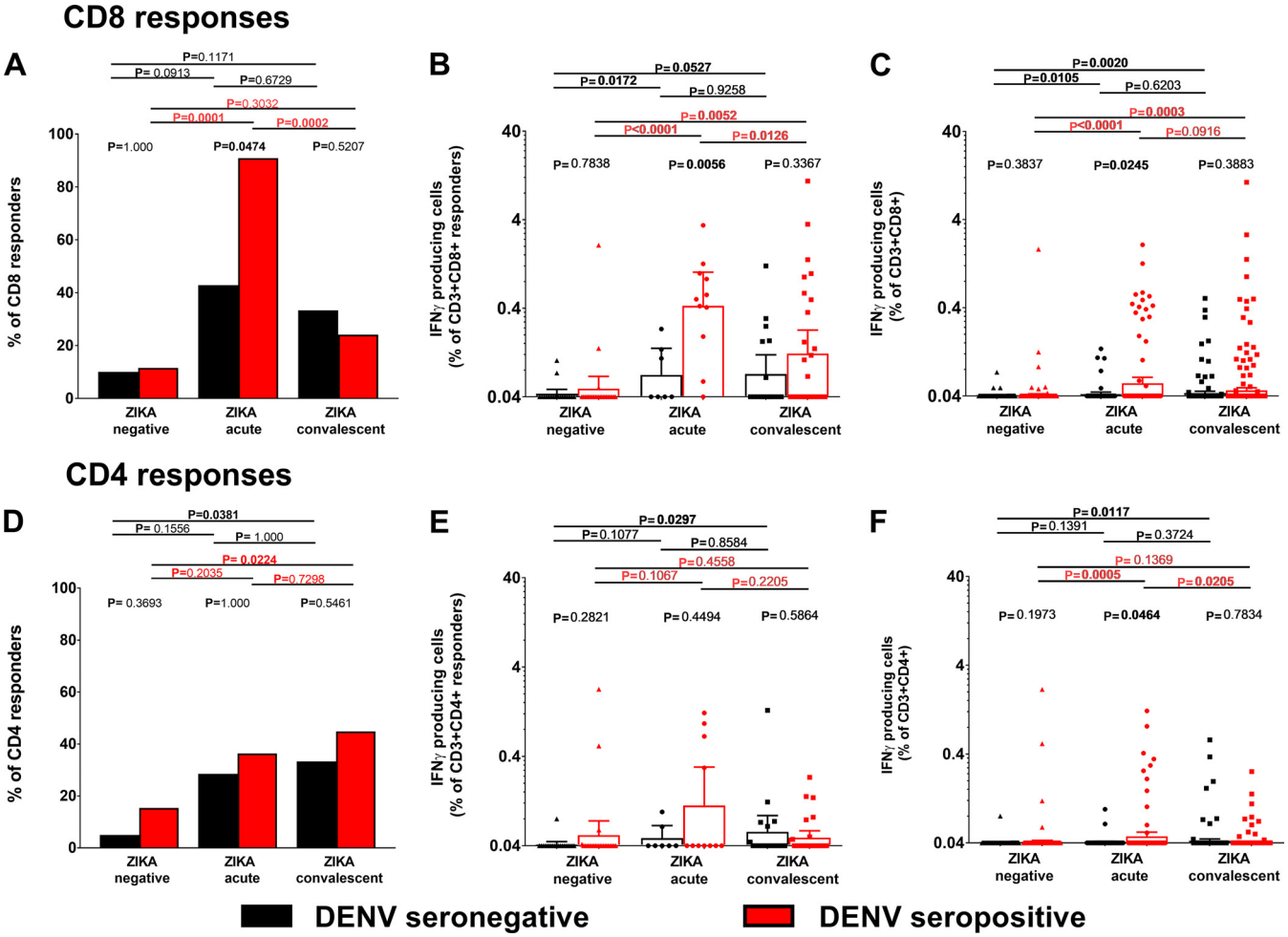

FIG 3 *Ex vivo* reactivity of ZIKV donors to ZIKV peptides. CD8 (A to C) and CD4 (D to F) ZIKV-restricted responses in ZIKV-negative, acute, and convalescent donors are shown in intracellular cytokine experiments. Each group is further divided in DENV positive (red) or negative (black). Each donor has been tested with at least 5 protein pools (C-NS2A or NS2B-NS5) or the full set of protein pools depending on the availability of cells (A, B, D, and E). Each data point represents the response of a single donor if all 10 proteins were tested or the combined response of two donors tested with the two different sets of 5 protein pools. Panels C and F show all of the responses against individual pools regardless of the donor tested. Statistical significance for differences in frequency of responders (left) was performed using a Fisher test. The magnitude of responses (center and right) is expressed as geometric means with 95% CI, and statistical analyses were performed with Mann-Whitney U test.

T cells. Nonetheless, DENV preexposure modulates the ZIKV-reactive immunodominance pattern for CD8 cells, resulting in broad recognition across the ZIKV proteome.

In the context of CD4, responses were directed in approximately equal proportions against structural and nonstructural proteins (Fig. 4B). Differences between DENV and ZIKV patterns of immunodominance were not prominent, which was not surprising, since, according to published data, the DENV-specific response is already focused almost equally (50%) on structural and nonstructural proteins (11). In the present study, the fraction of ZIKV-specific responses directed against structural proteins was 58% or 67% for DENV-negative subjects and DENV-positive, ZIKV-positive donors, respectively (Fig. 4C and D).

As described above, whenever possible, peptide pools were deconvoluted and specific epitopes mapped using the same mapping approach as that previously shown in Fig. 2A. Two ZIKV NS5 epitopes ($NS5_{2819-2828}$ and $NS5_{2868-28876}$), both predicted to be restricted by HLA B*35:01, were recognized in an HLA-matched DENV-positive donor (Fig. 5A and B). One of these epitopes was independently identified in a DENV-positive, ZIKV-negative donor (Fig. 2B). In both cases, the ZIKV epitope differed from DENV sequences by a single conservative substitution. A second DENV-positive donor re-

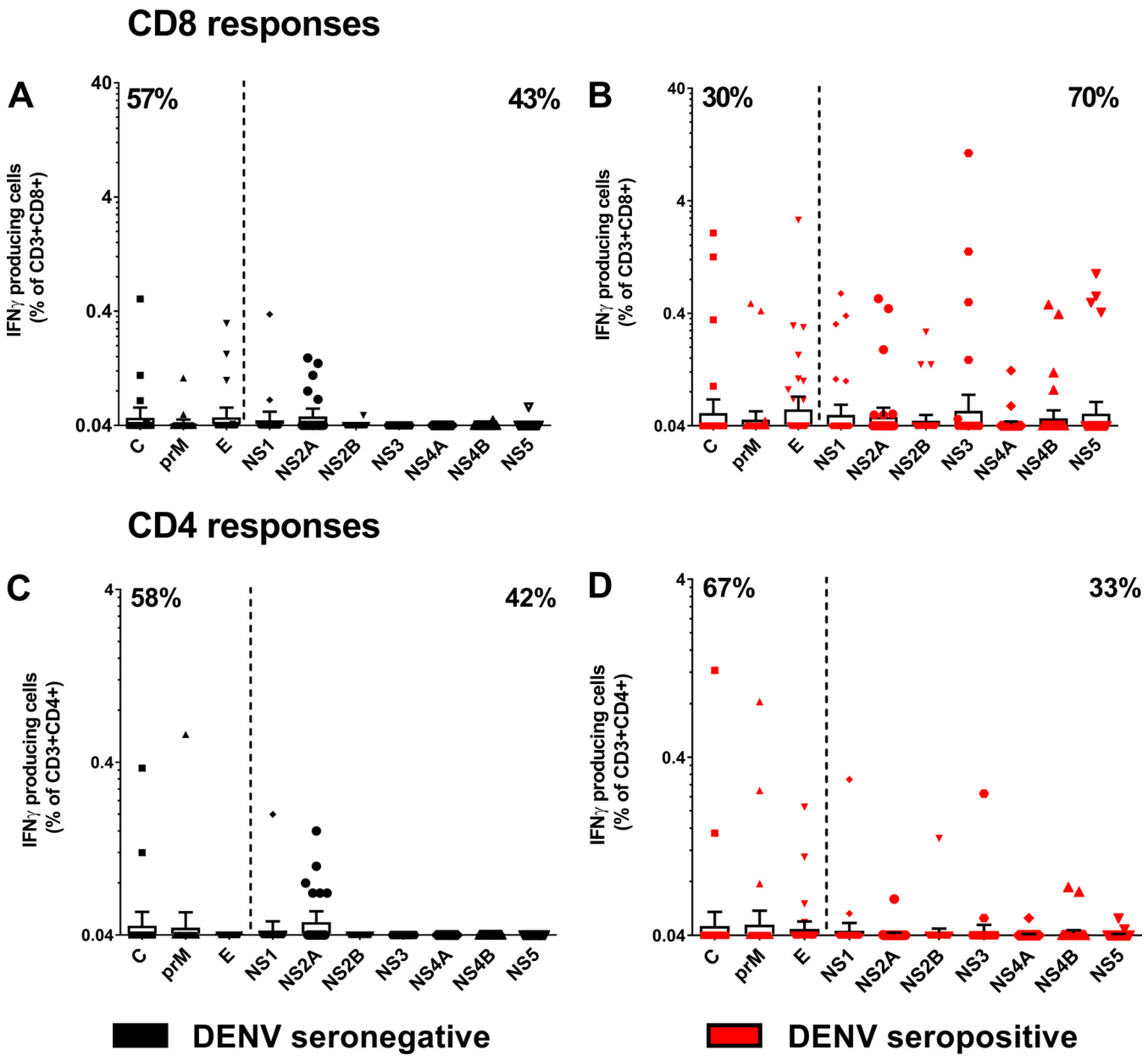

FIG 4 Immunodominance pattern of CD8 and CD4 responses against ZIKV-derived peptides. ZIKV CD8 (A and B) and CD4 (C and D) responses to 10 ZIKV proteins are shown in ZIKV-positive and DENV-negative subjects (A and C, left) or DENV-positive subjects (B and D, right). Structural (C, prM, and E) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins are divided by a dotted line, and their magnitudes in percentages are shown in each graph. The total magnitude of the responses has been calculated and the resulting percentage of responses for structural and nonstructural proteins shown in the upper left and right, respectively, of each panel. Data are expressed as geometric means with 95% CI.

sponded to the ZIKV $ENV_{719-728}$ epitope (predicted B*40:01 restriction), which differs from DENV3 sequences by a single conservative substitution (Fig. 5C). Another E protein epitope was identified in the same donor ($E_{481-495}$; restricted by HLA A*01:01), which in this case had more limited homology to DENV sequences (Fig. 5D).

Independent experiments showed that the very same ZIKV $E_{485-493}$ HLA A*01:01 epitope also was recognized in a DENV-negative subject (Fig. 5E and M. J. Ricciardi et al., submitted for publication). Interestingly, longer versions of this peptide were not recognized. It is possible that both 9mers and 10mers bind with high affinity but in somewhat different registers. Additional epitopes recognized in DENV-negative donors were mapped to a ZIKV C_{23-32} epitope restricted by HLA A*03:01, showing again limited homology to DENV sequences, and two additional ZIKV NS3 epitopes restricted by HLA

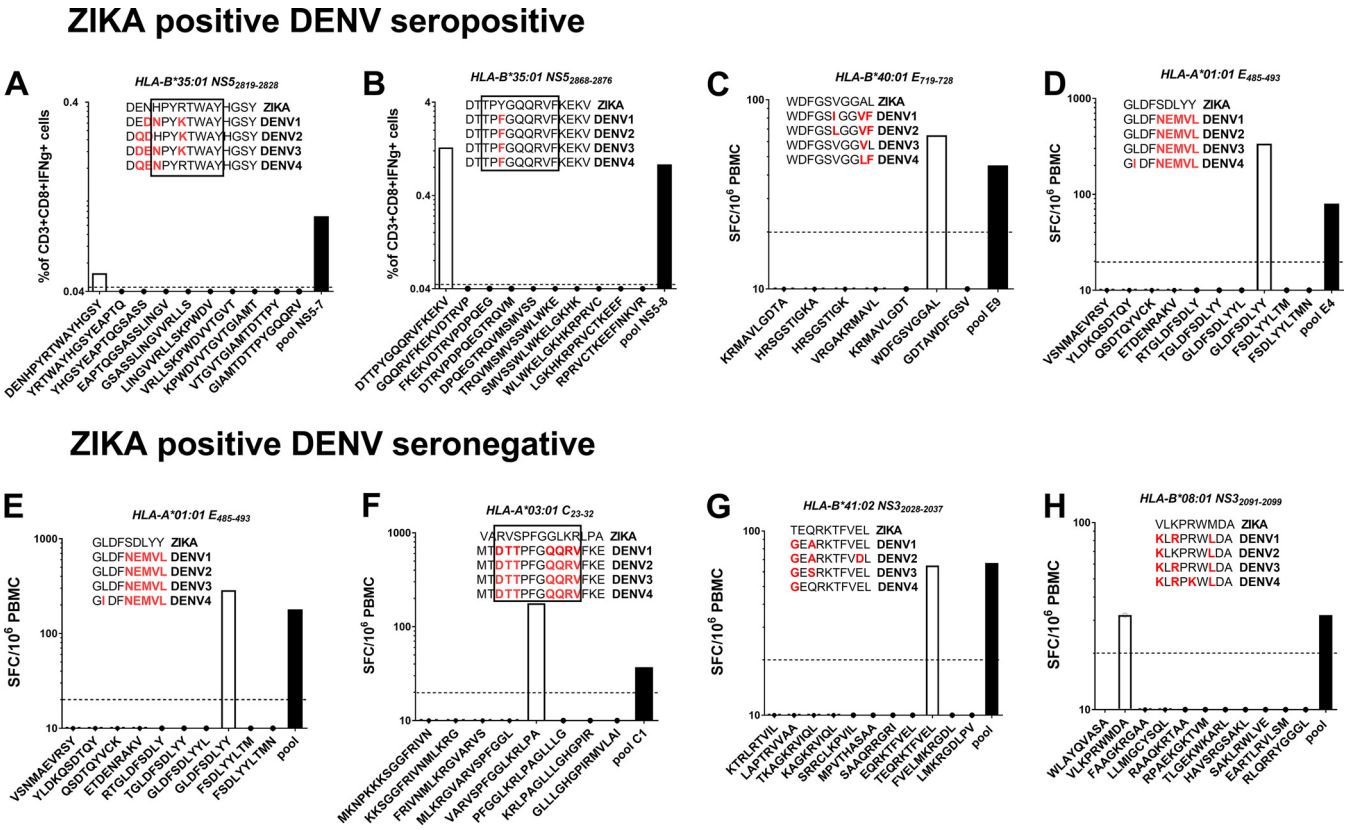

FIG 5 Mapping of CD8 ZIKV epitopes in ZIKV-positive donors. ZIKV-restricted epitopes mapped by peptide deconvolution in ELISPOT *ex vivo* experiments in DENV-positive (A to D) or DENV-negative (E to H) individuals. ZIKV epitope sequences were aligned with consensus sequences of DENV1, DENV2, DENV3 and DENV4 serotypes. Amino acid mismatches between the ZIKV sequence and the DENV consensus sequences are shown in red. Boxes indicate the optimal epitope restricted by the specific HLA phenotype present in this donor.

B*0801 and B*41:02 (Fig. 5F to H). Additionally, we selected two ZIKV peptides, TPYGQQRVF and APTRVVAAEM, which were recognized by DENV-seropositive donors (Fig. 2A to C), and synthesized the corresponding DENV peptides. These peptides then were tested in parallel with the original ZIKV peptides with PBMC from the donor originally utilized to map the responses in standard IFN-γ ELISPOT assays. We also tested the ZIKV ENV GLDFSDLYY epitope, defined in a DENV-seronegative donor (Fig. 5E), and tested the corresponding DENV peptides in parallel with the originally identified ZIKV peptide. The ZIKV TPYGQQRVF and APTRVVAAEM peptides, as well as the corresponding highly homologous DENV TP**F**GQQRVF and APTRVVA**S**EM peptides, were recognized by the DENV-seropositive donor with comparable magnitude. In contrast, the ZIKV Env GLDFSDLYY, but not the fairly discordant corresponding DENV epitopes GLDFN**EMVL** and GI**D**F**NEMVL** (amino acid residues that differ from those of the ZIKV original epitope are in boldface), were recognized by the DENV-seronegative donor response (Table 4).

Phenotype analysis of CD8 T cells responsive to ZIKV peptides. To gain further insights into the potential biological significance of these patterns of reactivity, we determined cell surface phenotypes of the CD8 T cells producing IFN-γ in response to the ZIKV peptide pools. As expected (Fig. 6A), these cells were predominantly T effector memory (TEM) (CCR7$^+$ CD45RA$^-$; approximately 60% on average) and terminally differentiated effector memory (TEMRA) (CCR7$^+$ CD45RA$^+$; approximately 30% on average). Approximately 50% of the IFN-γ^+ CD8 T cells were tumor necrosis factor alpha positive (TNF-α^+) compared to less than 1% of the IFN-γ^- cells (Fig. 6B), thus establishing that a large fraction of the responding cells are polyfunctional. Similar patterns were observed for ZIKV$^+$ DENV$^-$ and ZIKV$^+$ DENV$^+$ donors in terms of both memory phenotypes and polyfunctionality.

TABLE 4 Testing of DENV peptides for ZIKV $NS5_{2868-2876}$, $NS3_{1725-1734}$, and $E_{485-493}$ peptides

Donor	DENV status	ZIKV status	Protein	Source	Peptide sequence	SFC/10^6 PBMC[a]
GN0101	Pos.	Neg.	$NS5_{2868-2876}$	ZIKV	TPYGQQRVF	353 ± 240
				DENV1–4	TPFGQQRVF	366 ± 120
GS0157	Pos.	Neg.	$NS3_{1725-1734}$	ZIKV	APTRVVAAEM	330 ± 75
				DENV1	APTRVVASEM	219 ± 64
2894	Neg.	Pos.	$E_{485-493}$	ZIKV	GLDFSDLYY	287 ± 50
				DENV1–3	GLDFNEMVL	0
				DENV4	GIDFNEMVL	0

[a]Averages and standard deviations of net responses from 6 to 9 independent wells for donors GN0101 and GS0157 and 3 independent wells for donor 2894.

In contrast, significant differences were seen between $ZIKV^+$ $DENV^-$ and $ZIKV^+$ $DENV^+$ donors when the granzyme B and PD1 markers were considered. The expression of granzyme B in CD8 T cells from $ZIKV^+$ $DENV^-$ was not significantly increased in IFN-γ^+ cells compared to the background level of approximately 30% seen in IFN-γ^- cells, while in the case of $ZIKV^+$ $DENV^+$ donors approximately 80% of the IFN-γ^+ cells

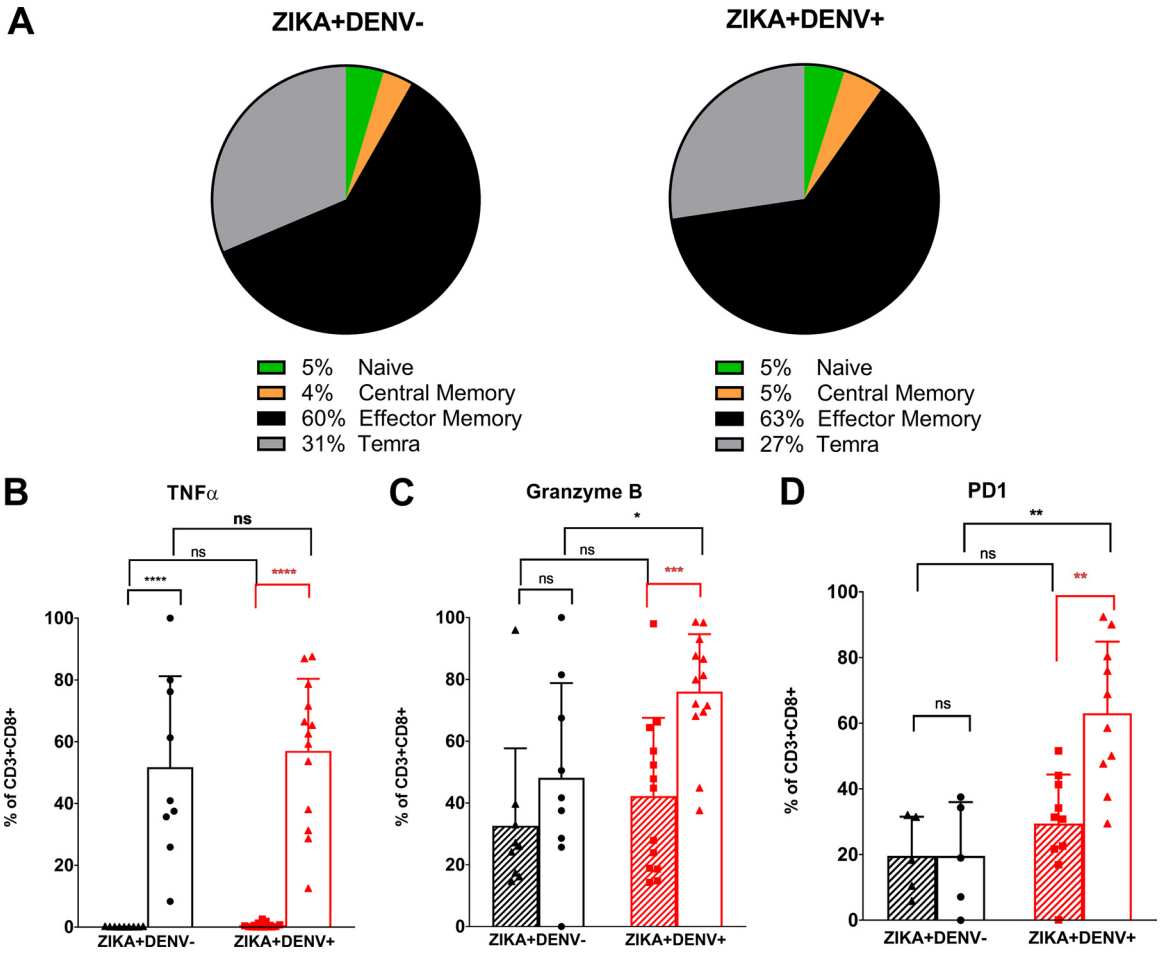

FIG 6 Phenotype characterization of $CD8^-$ ZIKV-specific immune responses in ZIKV-positive donors. Memory phenotype (A) and polyfunctionality (B to D) of ZIKV CD8 T cells were compared in ZIKV-positive, DENV-negative (black) donors and ZIKV-positive, DENV-positive (red) donors. (A) Average percentage of memory phenotype populations (naïve, $CD45RA^+$ $CCR7^+$; central memory, $CD45RA^-$ $CCR7^+$; effector memory, $CD45RA^-$ $CCR7^-$; and TEMRA, $CD45RA^+$ $CCR7^-$) in $CD8^-$ ZIKV-specific IFN-γ-producing cells. IFN-γ^- (oblique lines) and IFN-γ^+ (blank pattern) CD8 T cells were analyzed for the coexpression of TNF-α (B), granzyme B (C), and PD1 (D). Data were expressed as averages ± SD of the percentage of $CD3^+$ $CD8^+$ cells. Statistical analysis was performed with Mann-Whitney U test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$; ****, $P < 0.001$.

were also granzyme positive (Fig. 6C). Similarly, PD1 was only mildly expressed in IFN-γ^+ cells from ZIKV$^+$ DENV$^-$ donors, while on average 60% of the ZIKV$^+$ DENV$^+$ IFN-γ^+ cells also upregulated PD1 (Fig. 6D). These data indicate that DENV preexposure affects not only the quantity but also the quality of responses observed following ZIKV infection.

DISCUSSION

We report the first characterization in humans of both ZIKV-specific and ZIKV/DENV cross-reactive T cell responses and the influence of DENV serostatus on T cell immunity to ZIKV. Our study established three main points. First, preexisting T cell responses against DENV recognize peptide sequences located in the ZIKV proteome. Second, cross-reactivity is immunologically consequential, as DENV-positive individuals at the time of ZIKV infection respond more strongly to ZIKV both in terms of CD4 and CD8 T cell responses. Third, patterns of immunodominance are different in the case of DENV and ZIKV infection, with ZIKV-specific CD8 T cell responses predominantly targeting structural proteins such as E, prM, and C. Our study involves samples form ZIKV-infected donors derived from a variety of different geographical locations, including the U.S. mainland (travelers returning from affected areas), Puerto Rico, Brazil, Nicaragua, and Mexico. As such, we believe that the pattern of responses we observed is of general relevance and not limited to a specific population or clinical context. In the present study, we did not isolate representative viruses from the different cohorts and compare the sequences in terms of the percentage of similarity/differences to the peptide libraries used. Thus, it is possible that intra-ZIKV sequence variation influences some of the results, which should be interpreted with this caveat in mind.

We established that DENV-specific memory T cells recognize peptide sequences located in the ZIKV proteome. This point was established with a separate set of PBMC donations obtained in Sri Lanka, where ZIKV has not been reported, and from Nicaragua, collected between 2010 and 2014, before the introduction of ZIKV into the country. In this study, we did not test recognition of the DENV peptides corresponding to the ZIKV epitopes. We note this limitation in our interpretation, as, for example, recognition of the corresponding DENV peptide could be much higher than that for the ZIKV peptide. The molecular basis of this cross-reactivity was established by mapping several different CD4 and CD8 epitopes. These epitopes represent the first mapping of DENV/ZIKV cross-reactive epitopes in humans, and in most cases the cross-reactivity could be explained by identity or high similarity to sequences previously identified in one or more DENV serotypes. This finding was predicted by previous analysis conducted by the IEDB analysis resource (6) and by a recent study utilizing HLA transgenic mice (29). Nonetheless, identification of specific sequences here allows for a comprehensive assessment of whether the cross-reactivity is focused on regions that are highly conserved. Most importantly, we demonstrate that DENV-specific CD8 responses induced by TDLAV vaccination recognize ZIKV-derived peptides. This cross-reactivity indicates a potential for the TDLAV to provide some degree of protection against ZIKV infection.

An average homology level of 77% was observed between the sequences of DENV and ZIKV cross-reactive epitopes (defined as ZIKV sequences recognized in DENV-positive donors) compared with an overall 56% level of homology detected when the overall sequences of ZIKV and DENV proteomes were compared (Table 5). We conclude that sequential exposure to DENV and ZIKV sequences preferentially expands responses against conserved sites between the viruses. Similar observations were made in previous studies that showed that secondary DENV infections are associated with preferential recognition of epitopes conserved among different DENV serotypes, showing that secondary DENV infections are associated with preferential recognition of epitopes conserved among different DENV serotypes (10). Also, sequential exposure to different DENV serotypes in animal DENV models results in expansion of T cells recognizing cross-reactive epitopes (16, 30). It would have been interesting to examine if primary versus secondary DENV infection or the time interval between DENV and ZIKV infection

TABLE 5 Sequence homology between ZIKV and DENV[a]

	Homology (%) to ZIKV										
Serotype	Polyprotein	C	prM	E	NS1	NS2A	NS2B	NS3	NS4A + 2k	NS4B	NS5
DENV1	55	50	43	57	54	30	35	66	43	51	67
DENV2	56	41	41	55	54	27	41	67	52	53	67
DENV3	57	50	42	58	55	29	38	67	39	52	67
DENV4	57	49	47	56	54	34	41	67	44	49	68
Avg	56	47	43	58	55	31	39	67	44	51	67
Avg % of structural proteins				49[b]		Avg % of nonstructural proteins					51[b]
Avg % of structural proteins accounting for size				51[c]		Avg % of nonstructural proteins accounting for size					58[c]

[a]Homology analysis between the BeH818995 ZIKV isolate (GenBank accession no. AMA12084.1) and DENV1, -2, -3, and -4 consensus sequences obtained as previously reported (10, 11).
[b]Averages of structural and nonstructural proteins were based on averages of the different homology values in the four DENV serotypes for each protein.
[c]Average conservation was determined on a per-residue basis of structural and nonstructural proteins accounting for size.

influences T cell responses to ZIKV peptides. However, this information is not available to us from all different sites, and an analysis of this variable could be addressed in future studies specifically designed to examine this issue.

It is also noteworthy that three out of 11 of the identified epitopes were identified in multiple independent donors (ZIKV $NS3_{1725-1734}$, $NS5_{2868-2876}$, and $E_{485-493}$). Albeit based on a limited number of subjects, these results indicate that ZIKV responses are associated with strong immunodominance of particular epitopes.

In addition, $NS5_{2868-2876}$ was identified in $DENV^+$ $ZIKV^+$ and $DENV^+$ $ZIKV^-$ individuals, but no reactivity was detected in pools containing this peptide in $DENV^-$ $ZIKV^+$ donors. Conversely, ZIKV $E_{485-493}$, with a lower homology level with DENV, was identified in $DENV^+$ $ZIKV^+$ and $DENV^-$ $ZIKV^+$ individuals but not in $DENV^+$ $ZIKV^-$ donors.

Significant differences in frequency or magnitude of T cell responses to ZIKV peptides in PBMC from $ZIKV^-$ $DENV^+$ donors compared with $ZIKV^-$ $DENV^-$ donors were detected in the acute phase of infection with ZIKV. This parallels similar observations made in terms of antibody responses that showed that ZIKV/DENV cross-reactivity is most readily detected close to infection and wanes afterwards (31). We also find that DENV preexposure influences ZIKV responses. This could be understood in the context of the well-recognized phenomenon of heterologous immunity (24, 25). Specifically, ZIKV-specific T cell responses for both CD4 and CD8 T cells develop more rapidly in DENV-positive individuals and are already apparent in the acute phase of the disease. These responses subside at convalescence but remain elevated compared to those in ZIKV-negative individuals. The percentage of subjects with confirmed ZIKV infection who showed a positive T cell response (Fig. 3A and D) is relatively low, consistent with a primary infection and with ZIKV being associated, in most cases, with a milder clinical presentation than DENV (16). This pattern is reflective and characteristic of the differences in a primary compared to a classic secondary response (32). Here, we demonstrate how prior DENV infection alters ZIKV-specific immune responses, and we provide the first evidence that prior DENV infection leads to stronger and faster responses, thus providing evidence of a biological outcome. This is the first evidence in humans that previous exposure to dengue virus can influence subsequent infection with ZIKV by mounting a cross-reactive memory T cell response against ZIKV. Recent data in HLA transgenic mice demonstrated that ZIKV challenge following immunization of mice with ZIKV-specific and ZIKV/DENV cross-reactive epitopes elicited $CD8^+$ T cell responses that reduced infectious ZIKV levels, and $CD8^+$ T cell depletions confirmed that $CD8^+$ T cells mediated this protection (29). In addition, a recent paper has shown that Zika virus pathogenesis in rhesus macaques is unaffected by preexisting immunity

to dengue virus (33). Together, these data underline important implications for ZIKV vaccine development.

We have previously shown that stronger T cell responses are associated with certain HLA alleles associated with protection in cases of heterologous infection with DENV, pointing to a protective effect of these cross-reactive responses (10). Given that the groups were drawn from different study populations (age and genetic background), which could influence the magnitude of the T cell responses, further studies will provide more evidence on the generality of our findings. It remains to be seen whether this effect will be mimicked by DENV or ZIKV vaccination. Importantly, our data indicate that DENV preexposure also alters the quality of responses. While no difference was seen between DENV-preexposed and DENV-naive donors at the level of composition of memory subsets in the responding cells or the degree of multifunctionality, DENV-specific CD8 responses from DENV-preexposed donors significantly upregulated granzyme B and PD1, suggesting a more differentiated phenotype, similar to what was detected in secondary DENV infection (27, 34).

Our data provide an example of adaptive heterologous immunity, where cross-reactive memory dengue-specific CD8 T cells are enhancing the T cell responses to ZIKV. At this time, these studies do not yet address whether this will be beneficial in the majority of cases while at other times it could be detrimental based on the specific cross-reactive pattern of each patient. However, identifying key cross-reactive epitopes in humans and demonstrating that they influence the characteristics of the subsequent T cell response to ZIKV, as this study does, is an important step toward understanding potential immunopathology in ZIKV infection.

An unexpected result of our analysis is that almost 60% of the ZIKV-specific CD8 responses in ZIKV-positive but DENV-negative individuals are directed against structural proteins. This is in contrast to the relative paucity of structural protein-directed T cell responses observed in DENV infection, where only 15% of CD8 T cell responses are directed against structural proteins (10), even though serotype-specific differences have been noted (10, 13–16). Interestingly, the percentage of CD8 T cell responses directed against structural proteins in DENV-positive ZIKV patients is 30%, suggesting that previous DENV exposure alters the patterns of immunodominance, skewing it toward a pattern more similar (but still not identical) to that observed in DENV-positive donors in the absence of ZIKV infection.

The degree of homology (conservation) between NS proteins of DENV and ZIKV is 51%, on average, compared to 49% for structural proteins and 58% compared to 51% when accounting for size difference, so a higher degree of homology does not itself drive or focus cross-reactive responses on these antigens. The conclusion that T cell epitopes for ZIKV and DENV differ in their distribution between structural and nonstructural proteins requires the caveat that this is based on comparing data generated in separate studies, which have used different methods (e.g., ELISPOT assay versus flow cytometry). In addition, it cannot be excluded that the strong magnitude of one donor may have an substantial effect on the percentage of the total response directed toward nonstructural proteins.

It would have been of interest to determine the number of epitopes detected in the structural and nonstructural regions on a per-donor basis. This analysis could provide additional support for the notion that preexisting immunity to DENV broadens recognition across the ZIKV proteome. Due to the small volume of blood samples collected, we were not able to deconvolute all positive pools to identify the exact epitope. Future studies where larger amounts of blood are collected will allow us to comprehensively address this point. It is also worth noting that significant $CD8^+$ responses directed against structural proteins were reported in the case of West Nile and Japanese encephalitis viruses (35, 36). These two flaviviruses both are associated with neurological complications (37). Further, we previously showed in an HLA-transgenic model a trend toward higher recognition of structural proteins for DENV3 (compared to other DENV strains) (16), which also was reported previously to be associated with neurological symptoms (38, 39). Similarly, we have previously shown that human DENV3

serotype-specific $CD8^+$ T cell responses preferentially recognize structural proteins. Conversely, DENV1 and DENV4 serotypes preferentially recognized nonstructural proteins. Finally, the DENV2 serotype showed a broader recognition of all proteins but still elicited the strongest $CD8^+$ T cell response against nonstructural proteins (28). As no higher level of homology is observed between ZIKV and DENV3 with respect to the other DENV serotypes that could explain the preferential recognition of structural proteins (Table 5), we hypothesize that common processing pathways or similar $CD8^+$ T cell elicitation occurs that differs from that of the other DENV serotypes and will need further investigation.

Mapping of over 10 different ZIKV epitopes suggests that DENV-positive donors tend to recognize DENV/ZIKV highly conserved epitopes, while DENV-negative subjects may recognize more divergent targets. On average, 76% homology existed between DENV and ZIKV sequences among cross-reactive epitopes (defined as ZIKV sequences recognized in DENV-positive donors), compared with a 55% average level of homology between DENV and ZIKV sequences at the level of ZIKV epitopes recognized in DENV-negative donors and an overall 56% level of homology detected when the overall sequences of ZIKV and DENV proteomes were compared. These results emphasize that previous exposure to DENV influences the fine repertoire of epitopes being recognized. It remains to be seen if cross-reactivity of T cells also can be detected between ZIKV and other related flaviviruses. In the present study, we have not characterized WNV or JEV exposure. It is possible that cross-reactivity at the T cell level exists between ZIKV and other more distantly related flaviviruses, and this point will be address in future studies.

In the majority of cases, the degree of homology between ZIKV and DENV was very high, suggesting that a ZIKV diagnostic assay based on T cell responses is not immediately practical and conversely reemphasizing that DENV preexposure (or vaccination) might influence ZIKV immunity. Vaccines against ZIKV that are currently under development and focus on structural protein antigens rather than live virus may have logistical (no need for cold chain) and safety (no risk of virulent reversion and safe to administer to pregnant and immunocompromised patients) advantages; however, these vaccines may not comprise the full set of antigens required to induce protective immunity. Our results that approximately 55 to 60% of the ZIKV-specific CD4 and CD8 response is directed against structural proteins is encouraging, in that cellular responses necessary to directly limit ZIKV infection and support T cell-dependent antibody responses may be achievable with vaccine approaches being pursued.

MATERIALS AND METHODS

Human blood samples. All samples were collected after informed consent, and the study was approved by the La Jolla Institute institutional review board (IRB) committee (IRB number VD-154). An overview of the clinical and serological characteristics of all ZIKV samples is provided in Table 2. The sample allocation was provided by collaborators that collected the samples. The investigators were aware of the group allocation during the experiment and when assessing the outcome. In addition, Table S1 in the supplemental material provides a summary of the HLA typing data of the PBMC donor, and DENV infection history was available for all donors analyzed in this study.

Samples from flavivirus-naive controls. Healthy adult male and nonpregnant female volunteers, 18 to 50 years of age, were enrolled from Baltimore, Maryland, Washington, DC, and Burlington, Vermont, and tested for the presence of serum antibodies to all DENV serotypes, yellow fever virus, West Nile virus, and St. Louis encephalitis virus, as previously described (40).

Samples from areas where DENV is endemic. Blood donations from healthy adult blood donors of both sexes between the ages of 18 and 65 years were collected by the National Blood Center, Ministry of Health, Colombo, Sri Lanka, in anonymous fashion between the years of 2010 and 2016 and processed at the Genetech Research Institute as previously described (11). Similarly, the National Blood Center (NBC) of the Nicaraguan Red Cross in Managua, Nicaragua, provided anonymous blood samples collected between 2010 and 2014, prior to the introduction of ZIKV into the country (16).

Samples from DENV tetravalent vaccination. Healthy donors were enrolled and vaccinated with TV005, a tetravalent DENV vaccine formulation. Blood samples were collected as a part of phase I clinical trials (ClinicalTrials registration numbers NCT01506570 and NCT01436422) at the Johns Hopkins Bloomberg School of Public Health (JHSPH) and at the University of Vermont Vaccine Testing Center and Center for Immunization, as previously reported (15, 41, 42).

Samples from area where ZIKV is endemic. Blood samples were collected from patients displaying symptoms of a suspected ZIKV infection in Brazil, Nicaragua, and Mexico. Samples were also collected from blood donors identified through routine donor screening in Puerto Rico and Florida. Infection with

ZIKV was confirmed using RT-PCR, as described in more detail below. All samples were screened for previous DENV exposure by measuring DENV-specific IgG titers and/or neutralizing antibodies or from a documented history of DENV infection. Depending on the time of sample collection after onset of symptoms, samples were classified as acute (2 to 14 days postonset of symptoms or hospitalization) or convalescent (more than 15 days postonset of symptoms). Blood samples collected within the Recipient Epidemiology and Donor Evaluation Study III (REDSIII) were collected approximately 3 months following ZIKV RNA-positive blood donation.

Samples from the PDCS. A total of 14 children, RT-PCR positive for ZIKV and who experienced signs and symptoms of Zika virus, from the Nicaraguan Pediatric Dengue Cohort Study (PDCS) were included. The PDCS is a community-based prospective study of children 2 to 14 years of age that has been ongoing since August 2004 in Managua, Nicaragua (43). Participants present at the first sign of illness to the Health Center Sócrates Flores Vivas and are monitored daily during the acute phase of illness. Acute and convalescent (~14 to 21 days after onset of symptoms) blood samples are drawn for dengue, chikungunya (CHIKV), and Zika virus diagnostic testing from patients meeting the case definition for dengue or Zika virus (starting in January 2016) or presenting with undifferentiated febrile illness. In the PDCS, a healthy blood sample is collected annually from participants; anti-DENV antibody titers are measured in paired annual samples using enzyme-linked immunosorbent assay inhibition (EI) (44, 45), and infections are defined by seroconversion or a ≥4-fold rise in anti-DENV titers. In this study, confirmed ZIKV cases were classified as DENV naive if they entered the cohort study with no detectable anti-DENV antibodies (as measured by EI) and had no documented DENV infections (symptomatic or inapparent) during their time in the cohort. They were classified as DENV immune if they either entered the cohort with detectable anti-DENV EI antibodies or entered the cohort study with no detectable anti-DENV antibodies and had one or more documented DENV infections during their time in the cohort. All suspected ZIKV cases were confirmed by RT-PCR in serum and/or urine using triplex assays that simultaneously screen for DENV and CHIKV infections (ZCD assay [46], CDC Trioplexor and, in some cases, the CDC ZIKV monoplex assay [20], in parallel with a DENV-CHIKV multiplex assay [47]). The PDCS was approved by the IRB of the Nicaraguan Ministry of Health and the University of California, Berkeley. Parents or legal guardians of all subjects provided written informed consent, and subjects ≥6 years old provided assent.

Samples from ZIKV-infected U.S. travelers. Blood samples of travelers were collected at the University of North Carolina, University of Miami, Vanderbilt University, and the National Institutes of Health from patients displaying symptoms of a suspected ZIKV infection following return to the United States from areas where ZIKV is endemic. One donor had not traveled outside the United States and thus locally acquired ZIKV infection in Miami, FL. All samples were screened for previous DENV exposure by measuring DENV-specific serum IgG titers and/or neutralizing antibodies. Depending on the time of sample collection postonset of symptoms, samples were classified as acute or convalescent as described above.

PBMC isolation. PBMC were isolated by density-gradient sedimentation using Ficoll-Paque (Lymphoprep; Nycomed Pharma, Oslo, Norway) as previously described (10). Isolated PBMC were cryopreserved in cell recovery media containing 10% dimethyl sulfoxide (DMSO) (Gibco), supplemented with 10 to 50% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan UT), depending on the processing laboratory, and stored in liquid nitrogen until use in the assays. PBMC collected in Sri Lanka were stored in Synth-a-Freeze cryopreservation medium (A1254201; Thermo Fisher Scientific, USA).

Volunteers from the National Institutes of Health were enrolled into protocol VRC200 (ClinicalTrials registration no. NCT00067054) and leukapheresed. PBMC were processed and cryopreserved as described previously (48).

Serology. In general, DENV seropositivity was determined by DENV IgG or an inhibition ELISA, as previously described (45, 49). Flow cytometry-based or Vero cell-based focus reduction neutralization assays were performed for further characterization of positive donors, as previously described (50, 51).

RT-PCR assays for ZIKV determination. RNA was extracted from serum or urine using the QIAamp viral RNA minikit (Qiagen). Samples were tested for ZIKV and/or DENV using the ZCD assay, as previously described (46). DENV-positive samples were serotyped using a serotype-specific DENV multiplex assay (46, 52). In some laboratories, samples were tested by RT-PCR for ZIKV as previously described (20). At the Blood Systems Research Institute (BSRI), donors were identified as ZIKV RNA positive through routine donor screening using the cobas Zika test (Roche Molecular Systems, Inc., Pleasanton, CA).

HLA typing. Donors were HLA typed by an ASHI-accredited laboratory at Murdoch University (Western Australia) as previously described (11). HLA typing was performed for class I (HLA A, B, and C) and class II (DQA1, DQB1, DRB1, and DPB1) using locus-specific PCR amplification on genomic DNA. Primers used for amplification employed patient-specific barcoded primers. Amplified products were quantitated and pooled by subject, and up to 48 subjects were pooled. An unindexed (454 technique, 8-lane runs) or indexed (8 indexed MiSeq technique runs) library then was quantitated using kappa universal quantitative PCR library quantification kits. Sequencing was performed using either a Roche 454 FLX+ sequencer with titanium chemistry or an Illumina MiSeq using a 2-by-300 paired-end chemistry. Reads were quality filtered and passed through a proprietary allele-calling algorithm and analysis pipeline using the latest IMGT HLA allele database as a reference.

Major histocompatibility complex class I binding predictions and peptide selection. The BeH818995 ZIKV isolate (GenBank accession no. AMA12084.1) was used to perform ZIKV peptide selection. We selected a set of 9mer and 10mer ZIKV peptides predicted to bind one or more of 27 HLA class I A and B allelic variants, which were chosen because of their high prevalence in the general population, as previously described (10). Class I binding predictions were done with Tepitool using the consensus method (53, 54). For each allele, and considering 9mers and 10mers separately, the top 2%

TABLE 6 Monoclonal antibodies used in this study

Target	Dye[a]	Clone	Company
CD3	Alexa Fluor 700	UCHT1	eBioscience
CD4	APC-eFluor 780	RPA-T4	eBioscience
CD8	BV650	RPA-T8	BioLegend
CD14	V500	M5E2	BD Biosciences
CD19	V500	HIB19	BD Biosciences
Live/Dead	ef506		eBioscience
IFN-γ	FITC	4S.B3	eBioscience
CD45RA	eFlour450	HI100	eBioscience
CCR7	PerCPCy5.5	G043H7	BioLegend
TNF-α	PE-Cy7	Mab11	eBioscience
PD1	PE-CF594	EH12.1	BD Biosciences
Granzyme B	PE	GB11	eBioscience

[a]APC, allophycocyanin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein; PE, phycoerythrin.

scoring peptides ($n = 68$) based on predicted percentile rank were selected; the final set synthesized had 1,836 (68×27) 9mers and 10mers each, for a total of 3,672 peptides (A&A, San Diego, CA). For screening studies, the class I peptides were combined into pools of approximately 10 to 11 individual peptides according to their predicted HLA restriction, resulting in approximately 13 pools per HLA allele. Table 1 lists the number of peptides synthesized for each allele as a function of protein of provenance. In addition, we synthesized a panel of 15mer peptides, overlapping by 10 residues, spanning the entire sequence of the ZIKV BeH818995 isolate. The sequence homology between ZIKV and DENV for each protein is listed in Table 5. For screening studies, these peptides were combined into 10 megapools of 25 to 180 peptides according to the ZIKV protein from which they were derived (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A + 2k, NS4B, and NS5). For deconvolution studies, positive peptide pools were deconvoluted to identify individual epitopes, often going to an intermediate step of screening smaller pools before the individual peptide tests. To assess DENV reactivity, pools of previously identified and described DENV epitopes were utilized (i.e., DENV megapools [11, 28]).

IFN-γ ELISPOT assay. A total of 20×10^4 PBMC were incubated in triplicate with 0.1 ml complete RPMI 1640 medium in the presence of peptide pools (1 μg/ml) or individual peptides (10 μg/ml). Following 20 h of incubation at 37°C, the plates were incubated with biotinylated IFN-γ monoclonal antibody (MAb 7-B6-1; Mabtech, Stockholm, Sweden) for 2 h and developed as previously described (10, 55). In CD4 experiments, CD8 cells were depleted before incubation using magnetic beads and positive selection (MACS; Miltenyi Biotec, Auburn, CA). Cells from donors with PHA values below 250 spot-forming cells (SFC)/10^6 PBMC have been excluded from the analysis.

Flow cytometry. Detailed information of all monoclonal antibodies used in this study are listed in Table 6. For the intracellular cytokine staining, PBMC were cultured in the presence of HLA-matched peptide pools (1 μg/ml) and Golgi-Plug containing brefeldin A (BD Biosciences, San Diego, CA) for 6 h and subsequently permeabilized, stained, and analyzed with the same monoclonal antibody panel as that described previously (10). Cells from donors have been excluded from the analysis if the IFN-γ response to phorbol myristate acetate and ionomycin stimulation was lower than 1% in the $CD3^+$ cells. All data shown are background subtracted.

Statistical analysis. All statistical analyses were performed using the program Prism 7 (Graph-Pad Software, San Diego, CA). Data are expressed as geometric means with 95% confidence intervals (CI) or percentage of frequency, and data comparison was performed with Mann-Whitney or Fisher test, respectively.

Accession number(s). Epitopes identified in this study have been submitted to the Immune Epitope Database (IEDB; submission ID_1000720).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.01469-17.

SUPPLEMENTARY FILE 1, XLSX file, 0.1 MB.

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A.G., J.P., P.H.O., C.G.T.S., A.M., P.R.C., L.M.D-O.-P., and E.L.D.A. performed experiments, reviewed data, and planned the experimental strategy. J.S., S.P., and B.P. performed bioinformatics analyses. E.P. and S.M. performed and coordinated HLA typing and related analysis. S.R.M., M.S., J.C., A.D.D.S., M.J.R., D.M.M., E.G.K., P.V.D., A.M.D.S., M.C., A.D., S.A.D., C.C., A.B., G.K., J.C., E.H., J.E.C., M.S., P.J.N., M.B., H.V.-C., J.C., B.S.G., J.E.L., L.T., T.S., and D.I.W. collected samples and provided analysis of clinical information and interpretation of the data. A.G., D.W., and A.S. conceived and directed the study and wrote the manuscript. All authors have critically read and edited the manuscript.

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