Prior Dengue virus exposure shapes T cell immunity to Zika virus in humans

Alba Grifoni 1), John Pham 1, John Sidney 1), Patrick H. O’Rourke 3), Sinu Paul 1), Bjoern Peters 1), Sheridan R. Martini 1), Aruna D. de Silva 1), 2) Michael J. Ricciardi 3), Diogo M. Magnani 3), Cassia G. T. Silveira 4), Alvino Maestri 4), Priscilla R. Costa 4), Luzia Maria de-Oliveira-Pinto 5), Elzinandes Leal de Azeredo 5), Paulo Vieira Damasco 6), Elizabeth Phillips 7), Simon Mallal 7), Aravinda M. de Silva 8), Matthew Collins 8), Anna Durbin 9), Sean A. Diehl 10), Cristhiam Cerpas 11), Angel Balmaseda 11), Guillermina Kuan 12), Josefina Coloma 13), Eva Harris 13), James E. Crowe, Jr. 14), Mars Stone 15), Phillip J. Norris 15), Michael Busch 15), Hector Vivanco-Cid 16), Josephine Cox 17), Barney S. Graham 17), Julie E. Ledgerwood 17), Lance Turtle 18), 19), 20), Tom Solomon 19), 21), 22), Esper G. Kallas 4), David I. Watkins 3), Daniela Weiskopf 1) and Alessandro Sette 1)#

1) Division of Vaccine Discovery, La Jolla Institute for Allergy and Immunology, La Jolla, CA
2) Genetech Research Institute, Colombo, Sri Lanka
3) Department of Pathology, University of Miami Miller School of Medicine, Miami, FL
4) Division of Clinical Immunology and Allergy, School of Medicine, University of São Paulo, Brazil
5) Fundação Oswaldo Cruz, Rio de Janeiro, Brazil
6) Federal University of the State of Rio de Janeiro (UNIRIO)
7) Institute for Immunology and Infectious Diseases, Murdoch University, Perth, Western Australia, Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN
8) Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC
9) Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD
10) University of Vermont, School of Medicine, Burlington, VT
11) National Virology Laboratory, National Center for Diagnosis and Reference, Ministry of Health, Managua, Nicaragua
12) Health Center Sócrates Flores Vivas, Ministry of Health, Managua, Nicaragua
13) Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, USA
14) Vanderbilt Vaccine Center, Vanderbilt University Medical Center, Nashville, TN
15) Blood Systems Research Institute, San Francisco, CA
16) Instituto de Investigaciones Medico-Biologicas, Universidad Veracruzana, Veracruz, Mexico
17) Vaccine Research Center, NIAID, NIH, Bethesda, MD
18) Centre for Global Vaccine Research, Institute of Infection and Global Health, University of Liverpool, 8 West Derby Street, Liverpool, L69 7BE, UK.
19) NIHR Health Protection Research Unit for Emerging and Zoonotic Infections, University of Liverpool, 8 West Derby Street, Liverpool, L69 7BE, UK.
20) Tropical & Infectious Disease Unit, Royal Liverpool University Hospital, Liverpool, L7 8XP, UK.
Institute of Infection and Global Health, University of Liverpool, 8 West Derby Street, Liverpool, L69 7BE, UK.

Walton Centre NHS Foundation Trust, Liverpool, L9 7LJ, UK

#corresponding author: Dr. Alessandro Sette
e-mail: alex@lji.org, phone: (858)-752 6916, 9420 Athena Circle, La Jolla, CA 92037

Running title: DENV pre-exposure influences ZIKA T cell responses.

Abstract word count: 365

Text word count: 6475

Keywords: ZIKV, DENV, T cells, heterologous immunity, cross-reactivity, immunodominance.
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 pre-existing 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-naïve donors, but declines in DENV pre-exposed 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 form DENV pre-exposed donors selectively up-regulated granzyme B and PD1, as compared to DENV-naïve donors. Finally, we discovered that ZIKV 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.
The issue of potential ZIKV and DENV cross-reactivity and how pre-existing DENV T cell immunity modulates ZIKA T cell responses is of great relevance as the two viruses often co-circulate and ZIKA virus has been spreading in geographical regions where DENV is endemic or hyper-endemic. 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 towards important implications for vaccine design against this global threat.
Introduction

The pandemic rise of Zika virus (ZIKV) has recently commanded the attention of the general public and medical research community alike (13, 15, 31, 33).

ZIKV is a flavivirus most closely related to dengue virus (DENV) (24, 53) 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 (54).

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 (27, 29).

This is in contrast to a substantial wealth of information related to T cell immunity against the closely related DENV (44, 45, 49).

In the case of DENV, CD8 T cell responses target mostly non-structural (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 (1, 2, 43, 44, 46). 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 (29).

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, share the same arthropod host and the geographic range of
ZIKV overlaps largely with areas where DENV is endemic or hyper-endemic (53). The concomitant co-circulation 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 (5, 9, 20, 28, 36, 37). However, it is currently unknown as to what extent ZIKV and DENV may cross-react with each other at the level of T cell immunity.

According to the well established phenomenon of heterologous immunity (32, 50), it is possible that pre-existing 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 (30). In the Phase IIb/III clinical trials of the first licensed tetravalent dengue vaccine, increased vaccine efficacy in DENV pre-exposed as opposed to DENV-naive vaccinees was observed, suggesting a possible protective role of pre-existing cross-reactive DENV-specific T cells that are boosted upon vaccination (29). Thus, it is also possible that pre-exposure 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.
Material and Methods

Human blood samples

All samples have been collected after informed consent and the study has been approved by the LJI IRB committee (IRB#: VD-154). An overview of the clinical and serological characteristics of all ZIKA samples is provided in Table 1. 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 Supplementary Table 1 provides a summary of the HLA typing data of the PBMC donor and DENV infection history were available of all the donors analyzed in this study.

Samples from flavivirus naive controls

Healthy adult male and non-pregnant female volunteers 18–50 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 (11).

Samples from DENV endemic areas

Blood donations from healthy adult blood donors of both sexes between the age of 18 and 65 were collected by the National Blood Center, Ministry of Health, Colombo, Sri Lanka collected in anonymous fashion between the years of 2010 and 2016 and processed at the Genetech Research Institute as previously described(45). Similarly,
National Blood Center (NBC) of the Nicaraguan Red Cross in Managua, Nicaragua has provided anonymous blood samples collected between 2010 and 2014 prior to the introduction of ZIKV to the country.\(^{(46)}\)

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 a phase I clinical trials (registration numbers NCT01506570 and NCT01436422) at the Johns Hopkins Bloomberg School of Public Health (JHSPH) and at the University of Vermont (UVM) Vaccine Testing Center and the Center for Immunization as previously reported.\(^{(3, 17, 43)}\)

Samples from ZIKV virus endemic areas

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 documented history of DENV infection. Depending on the time of sample collection after onset of symptoms, samples were either classified as acute (2-14 days post onset of symptoms or hospitalization) or convalescent (more than 15 days post onset of symptoms). Blood samples collected within the Recipient Epidemiology and Donor
Evaluation Study-III (REDSIII) were collected approximately 3 months following ZIKV RNA pos. blood donation.

Samples from the Nicaraguan Pediatric Dengue Cohort Study (PDCS)

A total of 14 children RT-PCR-pos. for ZIKV who experienced signs and symptoms of Zika, 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 (19). Participants present at the first sign of illness to the Health Center Sócrates Flores Vivas and are followed daily during the acute phase of illness. Acute and convalescent (~14-21 days after onset of symptoms) blood samples are drawn for dengue, chikungunya and Zika diagnostic testing from patients meeting the case definition for dengue or Zika (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 an Inhibition ELISA (EI)(4, 14), 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-naïve 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 or 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 Zika suspected
cases were confirmed by RT-PCR in serum and/or urine using triplex assays that simultaneously screen for DENV and CHIKV infections (ZCD assay (42), CDC Trioplex) in some cases the CDC ZIKV monoplex assay(20) in parallel with a DENV-CHIKV multiplex assay(41)). The PDCS was approved by the Institutional Review Boards 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 virus infected US travellers

Blood samples were collected at the University of North Carolina, University of Miami, Vanderbilt University and the National Institute of Health, from patients displaying symptoms of a suspected ZIKV infection following return to the US from ZIKV endemic areas. One donor had not traveled outside of the US 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 post onset of symptoms, samples were either classified as acute or convalescent as described above.

PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient sedimentation using Ficoll-Paque (Lymphoprep, Nycomed Pharma, Oslo, Norway) as previously described (44). Isolated PBMC were cryopreserved in cell recovery media containing 10% DMSO (Gibco), supplemented with 10-50% heat
inactivated fetal bovine serum, depending on the processing laboratory, (FBS; Hyclone Laboratories, Logan UT) and stored in liquid nitrogen until used in the assays. PBMC collected in Sri Lanka were stored in Synth-a-Freeze Cryopreservation medium (Cat A1254201 Thermo Fisher Scientific, USA).

Volunteers from the National Institutes of health were enrolled into protocol VRC200 (NCT00067054) and leukapheresed. PBMC were processed and cryopreserved as described previously (22).

Serology

In general, DENV seropositivity was determined by DENV IgG or an Inhibition ELISA, as previously described (14, 16). Flow cytometry-based or Vero cell-based focus reduction neutralization assays were performed for further characterization of Pos. donors, as previously described (18, 38).

rRT-PCR assays for ZIKV determination

RNA was extracted from serum or urine using the QIAamp Viral RNA Mini kit (Qiagen). Samples were tested for ZIKV, and/or DENV using the ZCD assay, as previously described (42). DENV-pos. samples were serotyped, using a serotype-specific DENV multiplex assay (40, 42). In some laboratories samples were tested by RT-PCR for ZIKV as previously described (20). At BSRI Blood donors were identified as ZIKV RNA pos. through routine donor screening using the cobas Zika test (Roche Molecular Systems, Inc., Pleasanton, CA (RMS) under IND.

HLA typing
Donors were HLA typed by an ASHI-accredited laboratory at Murdoch University (Western Australia) as previously described (45). HLA typing was performed for Class I (HLA A; B; C) and Class II (DQA1; DQB1; DRB1; 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 was quantitated using kappa universal qPCR library quantification kits. Sequencing was performed using either a Roche 454 FLX+ sequencer with titanium chemistry or an Illumina MiSeq using a 2 x 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.

MHC 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 9- and 10-mers ZIKV peptides predicted to bind one or more of 27 HLA class I A and B allelic variants chosen because of their high prevalence in the general population, as previously described (44). Class I binding predictions were done with Tepitool using the consensus method (26) (23). For each allele, and considering 9- and 10-mers separately, the top 2% scoring peptides (n=68) based on predicted percentile rank were selected; the final set synthesized had 1836 (68 X 27) 9-mers and 10-mers each, for a total of 3672 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 2 lists the number of peptides synthetized for each allele as a function of protein of provenance. In addition, we synthetized a panel of 15-mer 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 3. For screening studies, these peptides were combined into 10 megapools of 25-180 peptides according to the ZIKV protein from which they were derived (C, prM, E, NS1, NS2A, NS2B, NS3, NS4A+2k, NS4B, NS5). For deconvolution studies, pos. 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, see references(45, 48)). Epitopes identified in this study have been submitted to the Immune Epitope Database (IEDB; Submission ID_1000720).

IFNγ ELISPOT assay

A total of 20 x 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 a 20 h incubation at 37°C, the plates were incubated with biotinylated IFNγ mAb (mAb 7-B6-1 Mabtech, Stockholm, Sweden) for 2h and developed as previously described (44, 47). In CD4 experiments, CD8 cells were depleted before incubation using magnetic beads and pos. selection (MACS Miltenyi
Cells from donors with PHA values below 250 SFC / 10^6 PBMC have been excluded from the analysis.

**Flow Cytometry**

Detailed information of all monoclonal antibodies used in this study is listed in Table 4. 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 hours and subsequently permeabilized, stained and analyzed with the same monoclonal antibody panel as described previously (44). Cells from donors have been excluded from the analysis if the IFNγ response to PMA 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 mean with 95% CI or percent of frequency and data comparison has been performed with Mann-Whitney or Fisher test respectively.
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 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 (8, 44, 48). To study CD8 responses, we selected nine DENV-Pos. donors who had been infected by DENV multiple times (secondary infections) based on serum neutralization titers and whose samples showed appreciable ex vivo response to a pool of previously defined CD8 DENV epitopes (CD8-megapool)(48). A similar approach was used for CD4 responses, retrieving 5 DENV Pos. donors with ex vivo responses to a previously defined DENV CD4-megapool(45). As neg. controls, we used PBMC from donors who were DENV neg. from the same sites.

We tested PBMC from these groups for reactivity against ZIKV peptides in ex vivo IFNγ ELISPOT assays. In the case of CD8 T cell responses (HLA class I), we tested panels of ZIKV-derived peptides predicted to bind each donors HLA molecules(44). HLA restrictions were assigned based on testing short 9-10 mers 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 2.

As expected for CD8, T cells from the DENV neg. donors did not respond to either the previously defined DENV epitopes, nor to the ZIKV peptides. The cells were viable and responsive to stimulation, as shown by vigorous responses to PHA mitogen stimulation. Interestingly, CD8 T cells from one third of the DENV-Pos. donors recognized ZIKV-derived peptides (Figure 1A). Higher level 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-Pos. individuals (Figure 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 for 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 ICS assays (Figure 1C). CD8 T cells from the Flavivirus neg. donors did not respond to either the previously defined DENV epitopes, nor to the ZIKV peptides. By contrast CD8 T cells from TDLAV donors recognized, as expected the DENV CD8 megapool, but also in more than 50% of the cases the ZIKV-derived peptides. In conclusion, analysis of ex vivo responses of ZIKV naive and DENV Pos. 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, pos. 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 response was performed by sequential testing of pools and deconvolution to identify the positive peptides (Figure 2A). The HLA-B*35:01 CD8 epitope encoded by ZIKV NS3\textsubscript{2867-2876} was recognized by PBMC from a DENV-Pos. Nicaraguan donor (Figure 2B). This epitope was found to be highly similar (a single Y>F substitution) in DENV\textsubscript{1-4} serotypes consensus sequences obtained as previously described(44). A Sri Lankan donor recognized the B*07:02 ZIKV NS3\textsubscript{1725-1734} epitope (Figure 2C). The same epitope was also recognized by a different DENV-Pos. Sri Lankan donor (Figure 2D). The identical sequence was found in DENV\textsubscript{2, 3 and 4}.

In the case of CD4 (Figure 2E), the ZIKV NS5\textsubscript{2986-3000} epitope, 100% conserved in DENV\textsubscript{1-4} sequences, was recognized by PBMC from a DENV-Pos. Sri Lankan donor. PBMC from a Nicaraguan donor recognized the ZIKV NS1\textsubscript{986-1000} epitope (Figure 2F). Here, the recognized 15 mer contained a core NS1\textsubscript{989-998} sequence that was also highly conserved in all DENV serotypes, with A>S and M>L conservative substitutions. A different pattern was observed for the ZIKV E\textsubscript{486-500} epitope, which was recognized by PBMC from a different DENV-Pos. Nicaraguan donor (Figure 2G). In this case the most homologous 9-mer (sequence LYYLTMNNK), shared only 4 identities, with DENV\textsubscript{1} sequences, 2 are conservative (L>M and N>E) and 3 semiconservative (Y>V, Y>L and K>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-pos. (and presumably ZIKV-neg.) donors was directed to ZIKV sequences found to be identical or highly conserved with sequences in DENV serotypes.

Recruitment of donor cohorts differing in ZIKV and DENV pre-exposure status

To address the effect of pre-existing immunity on T cell responses to secondary flavivirus infection, we investigated six donor groups, namely ZIKV acute, convalescent or neg., and for each of these cohorts we further subdivided our cohorts into DENV-Pos. or -neg. individuals. For the purpose of classification in the various cohorts, the following criteria were used. Infection with ZIKV was confirmed using RT-PCR performed on acute infection samples as described in more detail below. Depending on the time of sample collection after the onset of symptoms or ZIKV RNA-pos. blood donation, samples were either classified as acute or convalescent as described in more detail in Materials and Methods. ZIKV negativity was inferred based on donations being obtained before- or outside of the area affected by the epidemic. DENV pos. or neg. 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 US travelers, and a US flavivirus-neg. cohort. The general features
of the subjects are detailed in Table 1. The relative proportion of females across all cohorts was 60% and the average age was 34 (range 3-70).

**ZIKV-specific responses are modulated by previous DENV exposure**

Next, we compared ZIKV T cell reactivity in the subjects described above as a function of ZIKV status (i.e. neg., 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 overlapping 15-mers spanning the entire ZIKV proteome were divided into ten pools corresponding to the ten encoded ZIKV proteins. Intracellular cytokine staining (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 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, pos. pools were deconvoluted, and specific epitopes identified. Overall, PBMC from 17-33 donors/patients were tested for each of the different categories (Table 5).

The frequency of *ex vivo* responses in ZIKV-infected patients was 30-40% 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 (*Figure 3A and D left panels*). Marginal CD8 responses to the ZIKV peptides were noted in the
case of the ZIKV-neg. DENV-neg. donors (Figure 3A). However, ZIKV-neg. DENV-
Pos. donors showed appreciable reactivity both in terms of increased frequency and
magnitude of responses, confirming a degree of T cell cross-reactivity between
DENV-ZIKV responses observed above (Figures 1 and 2). In the acute ZIKV-
pos./DENV-Pos. donors, CD8 responses to ZIKV peptides were of significantly higher
magnitude compared to those acute ZIKV subjects who were DENV neg. (Figure 3B
and C). After ZIKV convalescence, the CD8 responses to ZIKV-restricted peptides
were still elevated as compared to ZIKV-neg. donors, but were not significantly
different by DENV serostatus (Figure 3B and C). The pattern of CD4 responses to
ZIKV-restricted class II peptides was remarkably similar with regard to ZIKV acute
and convalescence phase and impact of DENV seropositivity, with trends for ex vivo
ZIKV T cell responses being delayed in DENV neg. donors and lower frequency and
magnitude of responses observed in respect to the CD8 counterpart. (Figure 3D-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 pos. donors (combined in this plot) as a
function of the antigen targeted is presented in Figure 4. In the case of ZIKV-specific
CD8 responses in DENV-neg. donors 57% of the response was directed against
structural proteins (Figure 4A). In the context of a previous DENV-exposure,
however, only 30% of the ZIKV-specific responses were directed against structural
proteins (Figure 4B). This can be compared to historical data regarding DENV
responses from presumably ZIKV-neg. donors (since samples were collected prior to the 2015-2016 ZIKV epidemic) where only 14.9% of the response was directed against structural proteins (44). Thus, the CD8 response to ZIKV is more focused on structural proteins compared to the focus on nonstructural proteins by DENV-specific T cells. Nonetheless, DENV pre-exposure modulates the ZIKV-reactive immunodominance pattern for CD8 cells, resulting in a broad recognition across the ZIKV proteome.

In the context of CD4, responses were directed in approximately equal proportions against structural and non-structural proteins (Figure 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 non-structural proteins (45). In the present study, the fraction of ZIKV-specific responses directed against structural proteins was 58% or 67% for DENV-neg. subjects and DENV-Pos. ZIKV-pos. donors, respectively (Figure 4C-D).

As above, whenever possible, peptides pools were deconvoluted and specific epitopes mapped using same mapping approach previously shown in Figure 2A. Two ZIKV NS5 epitopes (NS5\textsubscript{2819-2828} and NS5\textsubscript{2868-2887}) both predicted to be restricted by HLA B*35:01, were recognized in an HLA matched DENV Pos. donor (Figure 5A-B). One of these epitopes was independently identified in a DENV-Pos., ZIKV-neg. donor (Figure 2B). In both cases, the ZIKV epitope differed from DENV sequences by a single conservative substitution. A second DENV pos. donor responded to the ZIKV ENV\textsubscript{719-728} epitope (predicted B*40:01 restriction), which
differs from DENV3 sequences by one single conservative substitution (Figure 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 (Figure 5D).

Independent experiments showed that the very same ZIKV E_{485-493} HLA A*01:01 epitope also was recognized in a DENV-neg. subject (Figure 5E; Ricciardi et al. manuscript submitted). Interestingly longer version of this peptide were not recognized. It is possible that both 9 mer and 10 mer bind with high affinity, but in somewhat different registers. Additional epitopes recognized in DENV-neg. 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 B*0801 and B*41:02 (Figure 5F-H). Additionally, we selected two ZIKV peptides TPYGQQRVF and APTRVVAAEM that were recognized by DENV seropositive donors (Figures 2A-C), and synthetized the corresponding DENV peptides. These peptides were then tested in parallel with the original ZIKV peptides with PBMCs from the donor originally utilized to map the responses in standard IFN-γ Elispot assays. Likewise we also tested the ZIKV ENV GLDFSDLYY epitope defined in a DENV seronegative donor (Figure 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 TPFGQQRVF and APTRVVASEM 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
GLDFNEMVL and GIDFNEMVL were recognized by the DENV seronegative donor response (Table 6).

Phenotype analysis of CD8 T cell 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 IFNγ in response to the ZIKV peptide pools. As expected (Figure 6A), these cells were predominantly TEM (CCR7+CD45RA−; approximately 60% on average) and TEMRA (CCR7+CD45RA+; approximately 30% on average). Approximately 50% of the IFNγ+ CD8 T cells were TNFα+ as compared to less than 1% of the IFNγ− cells (Figure 6B), thus establishing that a large fraction of the responding cells are polyfunctional. Similar patterns were observed for ZIKA+DENV− and ZIKA+DENV+ donors in terms of both memory phenotypes and polyfunctionality.

By contrast, significant differences were seen between ZIKA+DENV− and ZIKA+DENV+ donors when the granzyme B and PD1 markers were considered. The expression of granzyme B in CD8 T cells from ZIKA+DENV− was not significantly increased in IFNγ+ cells as compared to the background level of approximately 30% seen in IFNγ− cells, while in the case of ZIKA+DENV+ approximately 80% of the IFNγ+ cells were also granzyme positive (Figure 6C). Similarly, PD1 was only mildly expressed in IFNγ+ cells from ZIKA+DENV−, while 60% on average of the ZIKA+DENV+ IFNγ+ cells also upregulated PD1 (Figure 6D). These data indicates
that DENV pre-exposure affect 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, pre-existing T cell responses against DENV recognize peptide sequences encoded in the ZIKV proteome. Second, cross-reactivity is immunologically consequential, as DENV-Pos. 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 mainland USA (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 compared the sequences in terms of the percentage of similarity/differences to the peptide libraries used. Thus, it is possible that intra ZIKV sequence variation might influence some of the results, which should be interpreted with this caveat in mind.

We established that DENV-specific memory T cells recognize peptide sequences encoded in the ZIKV proteome. This point was established with a separate set of PBMC donations obtained either in Sri Lanka, where ZIKV has not been reported, as well as 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 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(53), and by a recent study utilizing HLA transgenic mice (51). 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-Pos. donors), as compared with an overall 56% level of homology detected when the overall sequences of ZIKV and DENV proteomes were compared (Table 3). 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...
amongst different DENV serotypes that showed that secondary DENV infections are associated with preferential recognition of epitopes conserved amongst different DENV serotypes (44). Also, sequential exposure to different DENV serotypes in animal DENV models results in expansion of T cells recognizing cross-reactive epitopes (12, 46). It would have been interesting to examine if primary versus secondary DENV infection or the time interval between DENV and ZIKV infection 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 eleven 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 may be 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, ZIKA E_485-493 with 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 PBMCs 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 wane afterwards (7).
We also find that DENV pre-exposure influences ZIKV responses. This could be understood in the context of the well recognized phenomenon of heterologous immunity (32, 50). Specifically, ZIKV-specific T cells responses for both CD4 and CD8 T cells responses develop more rapidly in DENV-Pos. 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-neg. individuals. The percentage of subjects with confirmed ZIKV infection who showed a positive T cell response (Figures 3A and 3D) is relatively low, consistent with a primary infection and with ZIKV being in most cases associated with a milder clinical presentation than DENV (46). This pattern is reflective and characteristic of the differences in a primary compared to a classic secondary response (55). 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 ZIKA virus by mounting a cross-reactive memory T cell response against ZIKA virus. 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 (51). In addition a recent paper has shown that Zika virus pathogenesis in rhesus macaques is unaffected by pre-existing immunity to dengue virus (25). 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 case of heterologous infection with DENV pointing to a protective effect of these cross-reactive responses (44). 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 indicates that DENV pre-exposure also alters the quality of responses. While no difference was seen between DENV pre-exposed and DENV-naïve donors at the level of composition of memory subsets in the responding cells or the degree of multifunctionality, DENV specific CD8 responses from DENV pre-exposed donors significantly upregulated granzyme B and PD1, suggesting a more differentiated phenotype, similar to what detected in secondary DENV infection (6, 8).

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 ZIKA virus. 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 ZIKA virus as this study does is an important step, toward understanding potential immunopathology in ZIKA virus infection.
An unexpected result of our analysis is that almost 60% of the ZIKV-specific CD8 responses in ZIKA-pos. but DENV-neg. 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 (44), even though serotype specific differences have been noted (1, 2, 43, 44, 46). Interestingly, the percentage of CD8 T cell responses directed against structural proteins in DENV-Pos. ZIKV patients is 30%, thus suggesting that previous DENV exposure may alter the patterns of immunodominance, skewing it towards a pattern more similar (but still not identical) to that observed in DENV Pos. donors in absence of ZIKV infection.

The degree of homology (conservation) between NS proteins of DENV and ZIKV is on average 51%, 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 non-structural proteins requires the caveat that is based on comparing data generated in separate studies, which have used different methods (e.g., ELISPOT versus flow cytometry). In addition, it cannot be excluded that the strong magnitude of one donor may have an substantial effect on the percent of the total response directed towards 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 pre-existing 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 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 Japan Encephalitis (21, 39). These two flaviviruses are both associated with neurological complications (34). Further, we previously shown in an HLA-transgenic model a trend towards higher recognition of structural proteins for DENV3 (as compared to other DENV strains) (46), which previously also was reported to be associated with neurological symptoms (10, 35). Similarly, we have previously shown that human DENV3-serotype specific CD8+ T cell responses preferentially recognize structural proteins. Conversely, DENV 1 and DENV4 serotypes preferentially recognized non-structural proteins. Finally DENV2 serotype showed a broader recognition of all proteins but still elicited the strongest CD8+ T cell response against non-structural proteins (48). As no higher level of homology is observed between ZIKV and DENV3 respect to the other DENV serotypes that could explain the preferential recognition of structural proteins (Table 3), we could hypothesize that common processing pathways or similar CD8+ T cell elicitation might occur that differs from the other DENV serotypes and will need further investigation.

Mapping of over ten different ZIKV epitopes suggest that DENV-Pos. donors tend to recognize DENV/ZIKV highly conserved epitopes, while DENV neg. subjects may recognize more divergent targets. An average 76% level of homology existed
between DENV and ZIKV sequences among cross-reactive epitopes (defined as ZIKV sequences recognized in DENV-Pos. donors), as compared with an average 55% level of homology between DENV and ZIKV sequences at the level of ZIKV epitopes recognized in DENV-neg. 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 can also 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 may exist 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 pre-exposure (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 immune-compromised patients) advantages; however, these vaccines may not comprise the full set of antigens required to induce protective immunity. Our results that approximately 55-60% of the ZIKV-specific CD4 and CD8 response is directed against structural proteins is encouraging that cellular responses necessary to directly limit ZIKV infection and...
support T-dependent antibody responses may be achievable with vaccine approaches being pursued.

Acknowledgments

This work was supported by National Institutes of Health contracts and grants Nr. HHSN272200900042C, HHSN27220140045C, U19 AI118626-01 to A.S, HHSN268201100001I to BSRI as well as by the 1P01AI106695-01A1 to E.H, and RO1 AI127828 to J.E.C. Further support was provided by ZikaPLAN that has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no 734584 to A.S. and by BMGF grant 457 OPP1104710 to A.P.D, and an internal grant from the University of Miami Clinical and Translational Research Institute to DIW and grants from Consejo Nacional de Ciencia y Tecnología (CONACyT) Fonteras de la Ciencia 2015-02-1192, Ciencia básica SEP-CONACyT 256235 and Fosiss-CONACyT 233697 to HVC. Blood donor samples from Puerto Rico and Florida were collected as part of the National Heart, Lung and Blood Institute REDS III (Recipient Epidemiology and Donor Evaluation Study-III).

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.

References


38  


Figure Legends

Figure 1. Ex-vivo reactivity to ZIKV derived peptides and previously defined DENV epitopes in DENV-Pos., -neg. donors and DENV vaccines. CD8 (A) and CD4 (B) T cell reactivity to DENV epitopes and ZIKA peptides in ELISPOT ex-vivo experiments are shown for donors DENV Pos. (red) or neg. (black). Responses were expressed as the number of IFNγ secreting cells per 10^6 PBMC and were considered pos. if the net spot-forming cells (SFC) per 10^6 were ≥20, had a stimulation index of ≥2, and a p<0.05 in a t test or in Poisson test comparing replicates with those from the neg. control. Donors with PHA values <250 SFC per 10^6 PBMC have been excluded from the analysis. Data are expressed as geomean with 95% CI. CD8 (C) T cell reactivity to DENV megapool and ZIKA HLA-restricted pools in ICS experiments are shown in DENV vaccinees (green) in comparison with flavivirus naïve donors (black). Data are expressed as average ± SD of the percentage of CD3+CD8+IFNγ+ cells.

Figure 2. Mapping of CD8 and CD4 cross-reactive DENV-ZIKV T cell epitopes. Panel A shows an example of the mapping strategy. CD8 (B-D) and CD4 (E-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, 2, 3 and 4 serotypes. Amino acid mismatches between the ZIKV sequence and the DENV consensus sequences are shown in red.
Figure 3. *Ex-vivo reactivity of ZIKV donors to ZIKV peptides.* CD8 (A-C) and CD4 (D-F) ZIKV-restricted responses in ZIKV-neg., acute and convalescent donors are shown in intra cellular cytokine experiments. Each group is further divided in DENV-Pos. (red) or -neg. (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-E). Each data points represents the response of a single donor response if all 10 protein have been tested or the combined response of two donors tested with the two different sets of 5 protein pools. Panels C and F show all the responses against individual pools regardless of the donor it has been tested. Statistical significance for differences in frequency of responders (left panels) was performed using a Fisher test. Magnitude of responses (central and right panels) is expressed as geometric means with 95% CI, and statistical analyses were performed with Mann-Whitney U test.

Figure 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-pos. DENV-neg. subjects (left panels, A and C), or DENV-Pos. subjects (right panels, B and D). Structural (C, prM, E) and non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins are divided by a dotted line, and their magnitude in percentage shown in each graph. The total magnitude of the responses has been calculated and the resulting percentage of responses for structural and non structural proteins shown respectively in the upper left and right of each figure panel. Data are expressed as geometric means with 95% CI.
Figure 5. Mapping of CD8 ZIKV epitopes in ZIKV-pos. donors.

ZIKV-restricted epitopes mapped by peptide deconvolution in ELISPOT *ex-vivo* experiments in DENV-Pos. (A-D) or DENV-neg. (E-H) individuals. ZIKV epitope sequences were aligned with consensus sequences of DENV1, 2, 3 and 4 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.

Figure 6. Phenotype characterization of CD8- ZIKV specific immune responses in ZIKV-pos. donors.

Memory phenotype (A) and polyfunctionality (B-D) of ZIKV CD8 T cells were compared in donors ZIKV-pos. DENV-neg (black) and ZIKV-pos. DENV-pos (red). A) Average of percentage of memory phenotype populations (naïve: CD45RA+CCR7+, central memory: CD45RA-CCR7+, effector memory: CD45RA-CCR7- and Temra: CD45RA+CCR7-) in CD8-ZIKV specific IFNγ producing cells. IFNγ- (oblique lines) and IFNγ+ (blank pattern) CD8 T cells were analyzed for the co-expression of TNFα (B), Granzyme B (C) and PD1 (D). Data were expressed as average ± 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.
**Table 1: General features of the ZIKV infected cohorts**

<table>
<thead>
<tr>
<th>Site</th>
<th>Country</th>
<th>#</th>
<th>Age (^a)</th>
<th>Sex (^b)</th>
<th>DENV+ (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of São Paulo</td>
<td>Brazil</td>
<td>7</td>
<td>45 (25-61)</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Fundação Oswaldo Cruz</td>
<td>Brazil</td>
<td>12</td>
<td>35 (22-60)</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>PDCS (^d)</td>
<td>Nicaragua</td>
<td>14</td>
<td>7 (3-14)</td>
<td>78</td>
<td>14</td>
</tr>
<tr>
<td>REDSIII (^e)</td>
<td>Puerto Rico/US</td>
<td>20</td>
<td>46 (21-70)</td>
<td>35</td>
<td>85</td>
</tr>
<tr>
<td>Universidad Veracruzana</td>
<td>Mexico</td>
<td>19</td>
<td>38 (6-69)</td>
<td>63</td>
<td>26</td>
</tr>
<tr>
<td>University of North Carolina</td>
<td>United States</td>
<td>8</td>
<td>37 (18-53)</td>
<td>71</td>
<td>50</td>
</tr>
<tr>
<td>University of Miami</td>
<td>United States</td>
<td>2</td>
<td>29 (26-32)</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Vanderbilt University</td>
<td>United States</td>
<td>9</td>
<td>42 (19-62)</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>National Institutes of Health</td>
<td>United States</td>
<td>7</td>
<td>29 (26-40)</td>
<td>42</td>
<td>71</td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td>98</td>
<td>34 (3-70)</td>
<td>60</td>
<td>54</td>
</tr>
</tbody>
</table>

\(^a\) expressed as the average age of the cohort (range)
\(^b\) expressed as the relative proportion of females in the cohort (%)
\(^c\) expressed as percentage of DENV Pos. individuals in the cohort
\(^d\) Pediatric Dengue Cohort Study
\(^e\) Recipient Epidemiology and Donor Evaluation Study-III
Table 2: ZIKV peptides used in this study

a) ZIKV predicted peptide set composed by 9- and 10-mer peptides.

<table>
<thead>
<tr>
<th>Allele</th>
<th>C</th>
<th>pr</th>
<th>M</th>
<th>E</th>
<th>NS1</th>
<th>NS2A</th>
<th>NS2B</th>
<th>NS3</th>
<th>NS4A</th>
<th>2K</th>
<th>NS4B</th>
<th>NS5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A*01:01</td>
<td>0</td>
<td>10</td>
<td>5</td>
<td>21</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>21</td>
<td>4</td>
<td>0</td>
<td>17</td>
<td>38</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*02:01</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>20</td>
<td>3</td>
<td>23</td>
<td>5</td>
<td>17</td>
<td>10</td>
<td>3</td>
<td>26</td>
<td>16</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*02:03</td>
<td>9</td>
<td>0</td>
<td>6</td>
<td>16</td>
<td>3</td>
<td>23</td>
<td>8</td>
<td>20</td>
<td>9</td>
<td>4</td>
<td>23</td>
<td>15</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*02:06</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>14</td>
<td>6</td>
<td>25</td>
<td>5</td>
<td>17</td>
<td>17</td>
<td>6</td>
<td>25</td>
<td>13</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*03:01</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>11</td>
<td>10</td>
<td>17</td>
<td>4</td>
<td>22</td>
<td>5</td>
<td>0</td>
<td>8</td>
<td>39</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*11:01</td>
<td>14</td>
<td>6</td>
<td>2</td>
<td>11</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>23</td>
<td>6</td>
<td>0</td>
<td>11</td>
<td>41</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*23:01</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>20</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>21</td>
<td>7</td>
<td>0</td>
<td>21</td>
<td>41</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*24:02</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>16</td>
<td>5</td>
<td>9</td>
<td>2</td>
<td>16</td>
<td>7</td>
<td>0</td>
<td>24</td>
<td>46</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*26:01</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>15</td>
<td>6</td>
<td>10</td>
<td>15</td>
<td>16</td>
<td>9</td>
<td>3</td>
<td>17</td>
<td>33</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*30:01</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>18</td>
<td>16</td>
<td>8</td>
<td>3</td>
<td>26</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>39</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*30:02</td>
<td>1</td>
<td>10</td>
<td>5</td>
<td>17</td>
<td>11</td>
<td>2</td>
<td>8</td>
<td>24</td>
<td>1</td>
<td>0</td>
<td>21</td>
<td>36</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*31:01</td>
<td>10</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>18</td>
<td>11</td>
<td>2</td>
<td>25</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>45</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*32:01</td>
<td>6</td>
<td>3</td>
<td>6</td>
<td>21</td>
<td>9</td>
<td>18</td>
<td>6</td>
<td>16</td>
<td>7</td>
<td>1</td>
<td>11</td>
<td>32</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*33:01</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>15</td>
<td>12</td>
<td>3</td>
<td>22</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>56</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*68:01</td>
<td>9</td>
<td>4</td>
<td>5</td>
<td>12</td>
<td>13</td>
<td>8</td>
<td>3</td>
<td>35</td>
<td>3</td>
<td>0</td>
<td>7</td>
<td>37</td>
<td>136</td>
</tr>
<tr>
<td>HLA-A*68:02</td>
<td>7</td>
<td>5</td>
<td>5</td>
<td>17</td>
<td>6</td>
<td>11</td>
<td>7</td>
<td>18</td>
<td>8</td>
<td>5</td>
<td>22</td>
<td>25</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*07:02</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>15</td>
<td>16</td>
<td>5</td>
<td>35</td>
<td>6</td>
<td>2</td>
<td>11</td>
<td>22</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*08:01</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>13</td>
<td>13</td>
<td>16</td>
<td>0</td>
<td>24</td>
<td>10</td>
<td>0</td>
<td>7</td>
<td>36</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*15:01</td>
<td>4</td>
<td>7</td>
<td>7</td>
<td>18</td>
<td>6</td>
<td>12</td>
<td>7</td>
<td>17</td>
<td>6</td>
<td>1</td>
<td>23</td>
<td>28</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*35:01</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>14</td>
<td>5</td>
<td>12</td>
<td>9</td>
<td>23</td>
<td>7</td>
<td>2</td>
<td>26</td>
<td>26</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*40:01</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>17</td>
<td>17</td>
<td>4</td>
<td>8</td>
<td>25</td>
<td>10</td>
<td>0</td>
<td>6</td>
<td>39</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*44:02</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>15</td>
<td>18</td>
<td>3</td>
<td>7</td>
<td>32</td>
<td>7</td>
<td>0</td>
<td>5</td>
<td>43</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*44:03</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>14</td>
<td>20</td>
<td>3</td>
<td>7</td>
<td>33</td>
<td>7</td>
<td>0</td>
<td>4</td>
<td>40</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*51:01</td>
<td>4</td>
<td>0</td>
<td>8</td>
<td>13</td>
<td>6</td>
<td>19</td>
<td>9</td>
<td>17</td>
<td>9</td>
<td>5</td>
<td>17</td>
<td>29</td>
<td>136</td>
</tr>
<tr>
<td>HLA-B*53:01</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>18</td>
<td>13</td>
<td>12</td>
<td>6</td>
<td>18</td>
<td>8</td>
<td>2</td>
<td>17</td>
<td>31</td>
<td>136</td>
</tr>
</tbody>
</table>
### b) 15-mer peptides spanning the ZIKV polyprotein

<table>
<thead>
<tr>
<th>Allele</th>
<th>C</th>
<th>pr</th>
<th>M</th>
<th>E</th>
<th>NS1</th>
<th>NS2A</th>
<th>NS2B</th>
<th>NS3</th>
<th>NS4A</th>
<th>2K</th>
<th>NS4B</th>
<th>NS5</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA class II</td>
<td>25</td>
<td>18</td>
<td>15</td>
<td>100</td>
<td>70</td>
<td>46</td>
<td>26</td>
<td>123</td>
<td>25</td>
<td>5</td>
<td>50</td>
<td>180</td>
<td>683</td>
</tr>
</tbody>
</table>

| 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 |
Table 3: Sequence homology between ZIKV and DENV. Homology analysis between BeH818995 ZIKV isolate (GenBank accession no. AMA12084.1) and DENV1, 2, 3, 4 consensus sequences obtained as previously reported (44, 45).

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Polyprotein</th>
<th>C</th>
<th>prM</th>
<th>E</th>
<th>NS1</th>
<th>NS2A</th>
<th>NS2B</th>
<th>NS3</th>
<th>NS4A+2k</th>
<th>NS4B</th>
<th>NS5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV1</td>
<td>55%</td>
<td>50%</td>
<td>43%</td>
<td>57%</td>
<td>54%</td>
<td>30%</td>
<td>35%</td>
<td>66%</td>
<td>43%</td>
<td>51%</td>
<td>67%</td>
</tr>
<tr>
<td>DENV2</td>
<td>56%</td>
<td>41%</td>
<td>41%</td>
<td>55%</td>
<td>54%</td>
<td>27%</td>
<td>41%</td>
<td>67%</td>
<td>52%</td>
<td>53%</td>
<td>67%</td>
</tr>
<tr>
<td>DENV3</td>
<td>57%</td>
<td>50%</td>
<td>42%</td>
<td>58%</td>
<td>55%</td>
<td>29%</td>
<td>38%</td>
<td>67%</td>
<td>39%</td>
<td>52%</td>
<td>67%</td>
</tr>
<tr>
<td>DENV4</td>
<td>57%</td>
<td>49%</td>
<td>47%</td>
<td>56%</td>
<td>54%</td>
<td>34%</td>
<td>41%</td>
<td>67%</td>
<td>44%</td>
<td>49%</td>
<td>68%</td>
</tr>
<tr>
<td>Average</td>
<td>56%</td>
<td>47%</td>
<td>43%</td>
<td>58%</td>
<td>55%</td>
<td>31%</td>
<td>39%</td>
<td>67%</td>
<td>44%</td>
<td>51%</td>
<td>67%</td>
</tr>
</tbody>
</table>

- **a)** Average of structural and non-structural proteins based on average of the different homology values in the four DENV serotypes for each protein.
- **b)** Average conservation on a per-residue based of structural and non-structural proteins accounting for size.
### Table 4: Monoclonal antibodies used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Color</th>
<th>Clone</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>AlexaFluor700</td>
<td>UCHT1</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD4</td>
<td>APC-eFluor780</td>
<td>RPA-T4</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD8</td>
<td>BV650</td>
<td>RPA-T8</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD14</td>
<td>V500</td>
<td>M5E2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD19</td>
<td>V500</td>
<td>HIB19</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Live/Dead</td>
<td>ef506</td>
<td></td>
<td>eBioscience</td>
</tr>
<tr>
<td>IFNγ</td>
<td>FITC</td>
<td>4S.B3</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD45RA</td>
<td>eFlour450</td>
<td>H100</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CCR7</td>
<td>PerCPCy5.5</td>
<td>G043H7</td>
<td>Biolegend</td>
</tr>
<tr>
<td>TNFα</td>
<td>PE-Cy7</td>
<td>Mab11</td>
<td>EBioscience</td>
</tr>
<tr>
<td>PD1</td>
<td>PE-CF594</td>
<td>EH12.1</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Granzyme B</td>
<td>PE</td>
<td>GB11</td>
<td>EBioscience</td>
</tr>
</tbody>
</table>
Table 5: Donors tested in each category

<table>
<thead>
<tr>
<th># of samples</th>
<th>ZIKV status a)</th>
<th>DENV status c)</th>
<th>Country of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>Acute</td>
<td>Pos.</td>
<td>Brazil/Mexico</td>
</tr>
<tr>
<td>17</td>
<td>Acute</td>
<td>Neg.</td>
<td>Nicaragua/Mexico</td>
</tr>
<tr>
<td>33</td>
<td>Convalescent</td>
<td>Pos.</td>
<td>Brazil/US travelers/ blood bank donors</td>
</tr>
<tr>
<td>30</td>
<td>Convalescent</td>
<td>Neg.</td>
<td>US travelers/ blood bank donors</td>
</tr>
<tr>
<td>20</td>
<td>Neg. b)</td>
<td>Pos.</td>
<td>Nicaragua/ Sri Lanka</td>
</tr>
<tr>
<td>20</td>
<td>Neg.</td>
<td>Neg.</td>
<td>US</td>
</tr>
</tbody>
</table>

a) Infection with ZIKV was confirmed by RT-PCR

b) ZIKV-neg. samples were collected before the onset of the ZIKV epidemic

c) Previous exposure to DENV was determined by the presence of detectable DENV-specific IgG titers.
Table 6. Testing of DENV corresponding peptides for ZIKV NS5<sub>2868-2876</sub> NS3<sub>1725-1734</sub>, and E<sub>485-493</sub> peptides.

<table>
<thead>
<tr>
<th>Donor</th>
<th>DENV Status</th>
<th>ZIKV Status</th>
<th>Protein</th>
<th>Source</th>
<th>Peptide Sequence</th>
<th>SFC/10&lt;sup&gt;6&lt;/sup&gt; &lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN0101</td>
<td>pos</td>
<td>neg</td>
<td>NS&lt;sub&gt;5&lt;/sub&gt;</td>
<td>ZIKV</td>
<td>TYPGQQRVF</td>
<td>353 ± 240</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2868-2876</td>
<td>DENV1-4</td>
<td>TPFQGQRVF</td>
<td>366 ± 120</td>
</tr>
<tr>
<td>GS0157</td>
<td>pos</td>
<td>neg</td>
<td>NS&lt;sub&gt;3&lt;/sub&gt;</td>
<td>ZIKV</td>
<td>APTRVAAEM</td>
<td>330 ± 75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1725-1734</td>
<td>DENV1</td>
<td>APTRVASEM</td>
<td>219 ± 64</td>
</tr>
<tr>
<td>2894</td>
<td>neg</td>
<td>pos</td>
<td>E&lt;sub&gt;485-493&lt;/sub&gt;</td>
<td>ZIKV</td>
<td>GLDFSLLY</td>
<td>287 ± 50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DENV1-3</td>
<td>GLDFNEMVL</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>DENV4</td>
<td>GIDFNEMVL</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>)Average and Standard deviation of net responses from 6-9 independent wells for donors GN0101 and GS0157, and 3 independent wells for donor 2894.
ZIKA positive DENV seropositive

A: HLA-B*22:01 N55_2010-2020

B: HLA-B*35:01 N55_2010-2018

C: HLA-B*14:01 E_Tyr119

D: HLA-A*01:01 E_Asp463

ZIKA positive DENV seronegative

E: HLA-A*24:01 E_Asp463

F: HLA-A*12:01 C_Tyr112

G: HLA-B*41:01 N55_2007-2008

H: HLA-B*08:01 N55_2007-2008