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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 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-naive 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.

Importance

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.

103 **Introduction**

104 The pandemic rise of Zika virus (ZIKV) has recently commanded the attention of the
105 general public and medical research community alike
106 (13, 15, 31, 33).
107 ZIKV is a flavivirus most closely related to dengue virus (DENV)(24, 53) but also
108 related with Japanese encephalitis virus (JEV), West Nile virus (WNV), and yellow
109 fever virus (YF), all of which are transmitted primarily by mosquitoes (54). .
110 Understanding host protective immunity to this virus is critical for the design of
111 optimal vaccines, but little is currently known about the immune responses to ZIKV
112 in humans since infections with ZIKV have not been frequent in the past (27, 29).
113 This is in contrast to a substantial wealth of information related to T cell immunity
114 against the closely related DENV(44, 45, 49).
115
116 In the case of DENV, CD8 T cell responses target mostly non-structural (NS) proteins
117 such as NS3, NS4B and NS5, while CD4 T cell responses are focused on the C, E and
118 NS5 proteins, even though serotype specific differences have been noted (1, 2, 43,
119 44, 46). The main protein targets of CD4 and CD8 T cell immunity are presently
120 unknown for ZIKV. This dearth of information is a severe knowledge gap as robust T
121 cell responses may be required for optimal ZIKV vaccine efficacy (29).
122
123 The issue of potential ZIKV and DENV cross-reactivity is of relevance for
124 development of both diagnostic tests and vaccines. ZIKV and DENV have significant
125 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) (52). 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.

147 **Material and Methods**

148

149 **Human blood samples**

150 All samples have been collected after informed consent and the study has
151 been approved by the LJI IRB committee (IRB#: VD-154). An overview of the clinical
152 and serological characteristics of all ZIKA samples is provided in **Table 1.** The
153 sample allocation was provided by collaborators that collected the samples. The
154 investigators were aware of the group allocation during the experiment and when
155 assessing the outcome. In addition **Supplementary Table 1** provides a summary of
156 the HLA typing data of the PBMC donor and DENV infection history were available
157 of all the donors analyzed in this study.

158

159 *Samples from flavivirus naive controls*

160 Healthy adult male and non-pregnant female volunteers 18–50 years of age were
161 enrolled from Baltimore, Maryland, Washington, DC, and Burlington, Vermont and
162 tested for the presence of serum antibodies to all DENV serotypes, yellow fever
163 virus, West Nile virus, and St. Louis encephalitis virus, as previously described (11).

164

165 *Samples from DENV endemic areas*

166 Blood donations from healthy adult blood donors of both sexes between the age of
167 18 and 65 were collected by the National Blood Center, Ministry of Health, Colombo,
168 Sri Lanka collected in anonymous fashion between the years of 2010 and 2016 and
169 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

193 Evaluation Study-III (REDSIII) were collected approximately 3 months following
194 ZIKV RNA pos. blood donation.
195
196 *Samples from the Nicaraguan Pediatric Dengue Cohort Study (PDCS*)
197 A total of 14 children RT-PCR-pos. for ZIKV who experienced signs and symptoms of
198 Zika, from the Nicaraguan Pediatric Dengue Cohort Study (PDCS) were included.
199 The PDCS is a community-based prospective study of children 2 to 14 years of age
200 that has been ongoing since August 2004 in Managua, Nicaragua (19). Participants
201 present at the first sign of illness to the Health Center Sócrates Flores Vivas and are
202 followed daily during the acute phase of illness. Acute and convalescent (~14-21
203 days after onset of symptoms) blood samples are drawn for dengue, chikungunya
204 and Zika diagnostic testing from patients meeting the case definition for dengue or
205 Zika (starting in January 2016) or presenting with undifferentiated febrile illness. In
206 the PDCS, a healthy blood sample is collected annually from participants; anti-DENV
207 antibody titers are measured in paired annual samples using an Inhibition ELISA
208 (EI)(4, 14), and infections are defined by seroconversion or a ≥4-fold rise in anti-
209 DENV titers. In this study, confirmed ZIKV cases were classified as DENV-naïve if
210 they entered the cohort study with no detectable anti-DENV antibodies (as
211 measured by EI) and had no documented DENV infections (symptomatic or
212 inapparent) during their time in the cohort or were classified as DENV-immune if
213 they either entered the cohort with detectable anti-DENV EI antibodies or entered
214 the cohort study with no detectable anti-DENV antibodies and had one or more
215 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 Trioplexor 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

239 inactivated fetal bovine serum, depending on the processing laboratory, (FBS;
240 Hyclone Laboratories, Logan UT) and stored in liquid nitrogen until used in the
241 assays. PBMC collected in Sri Lanka were stored in Synth-a-Freeze
242 Cryopreservation medium (Cat A1254201 Thermo Fisher Scientific, USA).
243 Volunteers from the National Institutes of health were enrolled into protocol
244 VRC200 (NCT00067054) and leukapheresed. PBMC were processed and
245 cryopreserved as described previously (22).
246
247 **Serology**
248 In general, DENV seropositivity was determined by DENV IgG or an
249 Inhibition ELISA, as previously described(14, 16). Flow cytometry-based or Vero
250 cell-based focus reduction neutralization assays were performed for further
251 characterization of Pos. donors, as previously described (18, 38).
252
253 **rRT-PCR assays for ZIKV determination**
254 RNA was extracted from serum or urine using the QIAamp Viral RNA Mini kit
255 (Qiagen). Samples were tested for ZIKV, and/or DENV using the ZCD assay, as
256 previously described(42). DENV-pos. samples were serotyped, using a serotype-
257 specific DENV multiplex assay(40, 42). In some laboratories samples were tested by
258 RT-PCR for ZIKV as previously described(20). At BSRI Blood donors were identified
259 as ZIKV RNA pos. through routine donor screening using the cobas Zika test (Roche
260 Molecular Systems, Inc., Pleasanton, CA (RMS) under IND.
261 **HLA typing**

262 Donors were HLA typed by an ASHI-accredited laboratory at Murdoch
263 University (Western Australia) as previously described(45). HLA typing was
264 performed for Class I (HLA A; B; C) and Class II (DQA1; DQB1, DRB1; DPB1) using
265 locus-specific PCR amplification on genomic DNA. Primers used for amplification
266 employed patient-specific barcoded primers. Amplified products were quantitated
267 and pooled by subject, and up to 48 subjects were pooled. An unindexed (454
268 technique 8-lane runs) or indexed (8 indexed MiSeq technique runs) library then
269 was quantitated using kappa universal qPCR library quantification kits. Sequencing
270 was performed using either a Roche 454 FLX+ sequencer with titanium chemistry or
271 an Illumina MiSeq using a 2 x 300 paired-end chemistry. Reads were quality-filtered
272 and passed through a proprietary allele-calling algorithm and analysis pipeline
273 using the latest IMGT HLA allele database as a reference.
274
275 **MHC class I binding predictions and peptide selection**
276 The BeH818995 ZIKV isolate (GenBank accession no. AMA12084.1) was used
277 to perform ZIKV peptide selection. We selected a set of 9- and 10-mers ZIKV
278 peptides predicted to bind one or more of 27 HLA class I A and B allelic variants
279 chosen because of their high prevalence in the general population, as previously
280 described(44). Class I binding predictions were done with Tepitool using the
281 consensus method(26) (23). For each allele, and considering 9- and 10-mers
282 separately, the top 2% scoring peptides (n=68) based on predicted percentile rank
283 were selected; the final set synthetized had 1836 (68 X 27) 9-mers and 10-mers
284 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

Biotec, Auburn, CA). 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.

326 **Results**

327

328 **DENV T cell responses are cross-reactive with ZIKV peptides**

329 To address the interplay between DENV- and ZIKV-specific T cell responses,
330 we studied HLA-typed PBMC donations from Sri Lanka obtained between 2010 and
331 2016. We also studied PBMC from Nicaraguan donors obtained between 2010 and
332 2014, thus preceding the current ZIKV epidemic(8, 44, 48). To study CD8 responses,
333 we selected nine DENV-Pos. donors who had been infected by DENV multiple times
334 (secondary infections) based on serum neutralization titers and whose samples
335 showed appreciable *ex vivo* response to a pool of previously defined CD8 DENV
336 epitopes (CD8-megapool)(48). A similar approach was used for CD4 responses,
337 retrieving 5 DENV Pos. donors with *ex vivo* responses to a previously defined DENV
338 CD4-megapool(45). As neg. controls, we used PBMC from donors who were DENV
339 neg. from the same sites.

340 We tested PBMC from these groups for reactivity against ZIKV peptides in *ex*
341 *vivo* IFNγ ELISPOT assays. In the case of CD8 T cell responses (HLA class I), we
342 tested panels of ZIKV-derived peptides predicted to bind each donors HLA
343 molecules(44). HLA restrictions were assigned based on testing short 9-10 mers
344 that are predicted to bind with high affinity to the HLA allotypes of the responding
345 donors. In the case of CD4 T cell responses (HLA class II), we tested a panel of 684
346 overlapping peptides spanning the entire ZIKV proteome. CD8-depleted PBMCs
347 were used in these experiments to avoid inadvertently identifying CD8 epitopes
348 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

372 Individual epitopes were mapped in representative cases. Where sufficient
373 cell numbers were available, pos. pools were deconvoluted to identify ZIKV-specific
374 epitopes across the ZIKV genome including all structural and nonstructural (NS)
375 proteins. The mapping of CD4 and CD8 response was performed by sequential
376 testing of pools and deconvolution to identify the positive peptides (**Figure 2A**). The
377 HLA-B*35:01 CD8 epitope encoded by ZIKV $NS3_{2867-2876}$ was recognized by PBMC
378 from a DENV-Pos. Nicaraguan donor (**Figure 2B).** This epitope was found to be
379 highly similar (a single Y>F substitution) in DENV1-4 serotypes consensus
380 sequences obtained as previously described(44). A Sri Lankan donor recognized the
381 B*07:02 ZIKV $NS3_{1725-1734}$ epitope **(Figure 2C).** The same epitope was also
382 recognized by a different DENV-Pos. Sri Lankan donor **(Figure 2D).** The identical
383 sequence was found in DENV2, 3 and 4.

384 In the case of CD4 (**Figure 2E),** the ZIKV $NS5_{2986-3000}$ epitope, 100%
385 conserved in DENV1-4 sequences, was recognized by PBMC from a DENV-Pos. Sri
386 Lankan donor. PBMC from a Nicaraguan donor recognized the ZIKV $NS1_{986-1000}$
387 epitope **(Figure 2F).** Here, the recognized 15 mer contained a core $NS1_{989-998}$
388 sequence that was also highly conserved in all DENV serotypes, with A>S and M>L
389 conservative substitutions. A different pattern was observed for the ZIKV $E_{486-500}$
390 epitope, which was recognized by PBMC from a different DENV-Pos. Nicaraguan
391 donor **(Figure 2G).** In this case the most homologous 9-mer (sequence
392 LYYLTMNNK), shared only 4 identities, with DENV1 sequences, 2 are conservative
393 (L>M and N>E) and 3 semiconservative (Y>V, Y>L and K>N) substitutions.
394 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

441 case of the ZIKV-neg. DENV-neg. donors **(Figure 3A)**. However, ZIKV-neg. DENV-
442 Pos. donors showed appreciable reactivity both in terms of increased frequency and
443 magnitude of responses, confirming a degree of T cell cross-reactivity between
444 DENV-ZIKV responses observed above **(Figures 1 and 2).** In the acute ZIKV-
445 pos./DENV-Pos. donors, CD8 responses to ZIKV peptides were of significantly higher
446 magnitude compared to those acute ZIKV subjects who were DENV neg. (**Figure 3B**
447 **and C)**. After ZIKV convalescence, the CD8 responses to ZIKV-restricted peptides
448 were still elevated as compared to ZIKV-neg. donors, but were not significantly
449 different by DENV serostatus **(Figure 3B and C).** The pattern of CD4 responses to
450 ZIKV-restricted class II peptides was remarkably similar with regard to ZIKV acute
451 and convalescence phase and impact of DENV seropositivity, with trends for *ex vivo*
452 ZIKV T cell responses being delayed in DENV neg. donors and lower frequency and
453 magnitude of responses observed in respect to the CD8 counterpart. **(Figure 3D-F)**.
454
455 **Different proteins are targeted by ZIKV versus DENV immunity**
456 We next determined whether DENV serostatus affected the antigenic targets
457 of ZIKV-reactive T cells across the ZIKV polyprotein. A breakdown of ZIKV CD8
458 responses in acute and convalescent ZIKV pos. donors (combined in this plot) as a
459 function of the antigen targeted is presented in **Figure 4**. In the case of ZIKV-specific
460 CD8 responses in DENV-neg. donors 57% of the response was directed against
461 structural proteins (**Figure 4A).** In the context of a previous DENV-exposure,
462 however, only 30% of the ZIKV-specific responses were directed against structural
463 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 ($NS5_{2819-2828}$ and $NS5_{2868-28876}$) 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 $ENV_{719-728}$ 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 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

GLDF**NEMVL** and G**I**DF**NEMVL** 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

532 that DENV pre-exposure affect not only the quantity but also the quality of
533 responses observed following ZIKV infection.
534

535 **Discussion**

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

554 We established that DENV-specific memory T cells recognize peptide
555 sequences encoded in the ZIKV proteome. This point was established with a
556 separate set of PBMC donations obtained either in Sri Lanka, where ZIKV has not
557 been reported, as well as from Nicaragua collected between 2010 and 2014 before

558 the introduction of ZIKV into the country. In this study we did not test recognition of
559 the DENV peptides corresponding to the ZIKV epitopes. We note this limitation in
560 our interpretation, as for example, recognition of the corresponding DENV peptide
561 could be much higher than for the ZIKV peptide. The molecular basis of this cross-
562 reactivity was established by mapping several different CD4 and CD8 epitopes.
563 These epitopes represent the first mapping of DENV/ZIKV cross-reactive epitopes in
564 humans, and in most cases the cross-reactivity could be explained by identity or
565 high similarity to sequences previously identified in one or more DENV serotypes.
566 This finding was predicted by previous analysis conducted by the IEDB analysis
567 resource(53), and by a recent study utilizing HLA transgenic mice (51). Nonetheless,
568 identification of specific sequences here allows for a comprehensive assessment of
569 whether the cross-reactivity is focused on regions that are highly conserved. Most
570 importantly, we demonstrate that DENV-specific CD8 responses induced by TDLAV
571 vaccination recognize ZIKV derived peptides. This cross-reactivity indicates a
572 potential for the TDLAV to provide some degree of protection against ZIKV infection.
573 An average homology level of 77% was observed between the sequences of
574 DENV and ZIKV cross-reactive epitopes (defined as ZIKV sequences recognized in
575 DENV-Pos. donors), as compared with an overall 56% level of homology detected
576 when the overall sequences of ZIKV and DENV proteomes were compared **(Table**
577 **3)**. We conclude that sequential exposure to DENV and ZIKV sequences
578 preferentially expands responses against conserved sites between the viruses.
579 Similar observations were made in previous studies that showed that secondary
580 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.

650 An unexpected result of our analysis is that almost 60% of the ZIKV-specific CD8
651 responses in ZIKA-pos. but DENV-neg. individuals are directed against structural
652 proteins. This is in contrast to the relative paucity of structural protein-directed T
653 cell responses observed in DENV infection where only 15% of CD8 T cell responses
654 are directed against structural proteins (44), even though serotype specific
655 differences have been noted (1, 2, 43, 44, 46). Interestingly, the percentage of CD8 T
656 cell responses directed against structural proteins in DENV-Pos. ZIKV patients is
657 30%, thus suggesting that previous DENV exposure may alter the patterns of
658 immunodominance, skewing it towards a pattern more similar (but still not
659 identical) to that observed in DENV Pos. donors in absence of ZIKV infection.
660 The degree of homology (conservation) between NS proteins of DENV and ZIKV is
661 on average 51%, compared to 49% for structural proteins and 58% compared to
662 51% when accounting for size difference, so a higher degree of homology does not
663 itself drive or focus cross-reactive responses on these antigens. The conclusion that
664 T cell epitopes for ZIKV and DENV differ in their distribution between structural and
665 non-structural proteins requires the caveat that is based on comparing data
666 generated in separate studies, which have used different methods (e.g., ELISPOT
667 versus flow cytometry). In addition, It can not be excluded that the strong
668 magnitude of one donor may have an substantial effect on the percent of the total
669 response directed towards nonstructural proteins.
670 It would have been of interest to determine the number of epitopes detected in the
671 structural and nonstructural regions on a per donor basis. This analysis could
672 provide additional support for the notion that pre-existing immunity to DENV

673 broadens recognition across the ZIKV proteome. Due to the small volume of blood
674 samples collected we were not able to deconvolute all positive pools to identify the
675 exact epitope. Future studies where larger amounts of blood are collected will allow
676 to comprehensively address this point. It is also worth noting that significant $CD8^+$
677 responses directed against structural proteins were reported in the case of West
678 Nile and Japan Encephalitis (21, 39). These two flaviviruses are both associated with
679 neurological complications(34). Further, we previously shown in an HLA-transgenic
680 model a trend towards higher recognition of structural proteins for DENV3 (as
681 compared to other DENV strains)(46), which previously also was reported to be
682 associated with neurological symptoms(10, 35). Similarly, we have previously
683 shown that human DENV3-serotype specific CD8+ T cell responses preferentially
684 recognize structural proteins. Conversely, DENV 1 and DENV4 serotypes
685 preferentially recognized non-structural proteins. Finally DENV2 serotype showed a
686 broader recognition of all proteins but still elicited the strongest CD8+ T cell
687 response against non-structural proteins(48). As no higher level of homology is
688 observed between ZIKV and DENV3 respect to the other DENV serotypes that could
689 explain the preferential recognition of structural proteins (**Table 3**), we could
690 hypothesize that common processing pathways or similar CD8+ T cell elicitation
691 might occur that differs from the other DENV serotypes and will need further
692 investigation.

693 Mapping of over ten different ZIKV epitopes suggest that DENV-Pos. donors
694 tend to recognize DENV/ZIKV highly conserved epitopes, while DENV neg. subjects
695 may recognize more divergent targets. An average 76% level of homology existed

696 between DENV and ZIKV sequences among cross-reactive epitopes (defined as ZIKV
697 sequences recognized in DENV-Pos. donors), as compared with an average 55%
698 level of homology between DENV and ZIKV sequences at the level of ZIKV epitopes
699 recognized in DENV-neg. donors, and an overall 56% level of homology detected
700 when the overall sequences of ZIKV and DENV proteomes were compared. These
701 results emphasize that previous exposure to DENV influences the fine repertoire of
702 epitopes being recognized. It remains to be seen if cross-reactivity of T cells can also
703 be detected between ZIKV and other related flaviviruses. In the present study we
704 have not characterized WNV or JEV exposure. It is possible that cross reactivity at
705 the T cell level may exist between ZIKV and other more distantly related
706 flaviviruses, and this point will be address in future studies.

707 In the majority of cases, the degree of homology between ZIKV and DENV
708 was very high, suggesting that a ZIKV diagnostic assay based on T cell responses is
709 not immediately practical, and conversely reemphasizing that DENV pre-exposure
710 (or vaccination) might influence ZIKV immunity. Vaccines against ZIKV that are
711 currently under development and focus on structural protein antigens rather than
712 live virus may have logistical (no need for cold chain) and safety (no risk of virulent
713 reversion and safe to administer to pregnant and immune-compromised patients)
714 advantages; however, these vaccines may not comprise the full set of antigens
715 required to induce protective immunity. Our results that approximately 55-60% of
716 the ZIKV-specific CD4 and CD8 response is directed against structural proteins is
717 encouraging that cellular responses necessary to directly limit ZIKV infection and

support T-dependent antibody responses may be achievable with vaccine approaches being pursued.

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741 clinical information and interpretation of the data. A.G., D.W., and A.S. conceived and
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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.

994 **Figure 3. *Ex-vivo* reactivity of ZIKV donors to ZIKV peptides**. CD8 (A-C) and CD4
995 (D-F) ZIKV-restricted responses in ZIKV-neg., acute and convalescent donors are
996 shown in intra cellular cytokine experiments. Each group is further divided in
997 DENV-Pos. (red) or -neg. (black). Each donor has been tested with at least 5 protein
998 pools (C-NS2A or NS2B-NS5) or the full set of protein pools depending on the
999 availability of cells (**A-B; D-E**). Each data points represents the response of a single
1000 donor response if all 10 protein have been tested or the combined response of two
1001 donors tested with the two different sets of 5 protein pools. **Panels C** and **F** show all
1002 the responses against individual pools regardless of the donor it has been tested.
1003 Statistical significance for differences in frequency of responders (left panels) was
1004 performed using a Fisher test. Magnitude of responses (central and right panels) is
1005 expressed as geometric means with 95% CI, and statistical analyses were performed
1006 with Mann-Whitney U test.
1007
1008 **Figure 4. Immunodominance pattern of CD8 and CD4 responses against ZIKV-**
1009 **derived peptides.** ZIKV CD8 (A and B) and CD4 (C and D) responses to 10 ZIKV
1010 proteins are shown in ZIKV-pos. DENV-neg. subjects (left panels, A and C), or DENV-
1011 Pos. subjects (right panels, B and D). Structural (C, prM, E) and non-structural (NS1,
1012 NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins are divided by a dotted line, and their
1013 magnitude in percentage shown in each graph. The total magnitude of the responses
1014 has been calculated and the resulting percentage of responses for structural and non
1015 structural proteins shown respectively in the upper left and right of each figure
1016 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.

1037 **Table 1: General features of the ZIKV infected cohorts**

Site	Country	#	Age [a]	Sex [b]	DENV+ [c]
University of São Paulo	Brazil	7	45 (25-61)	85	85
Fundação Oswaldo Cruz	Brazil	12	35 (22-60)	20	100
PDCS [d]	Nicaragua	14	7 (3-14)	78	14
REDSIII [e]	Puerto Rico/US	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

1038

1039 [a] expressed as the average age of the cohort (range)

1040 [b] expressed as the relative proportion of females in the cohort (%)

1041 [c] expressed as percentage of DENV Pos. individuals in the cohort

1042 [d] Pediatric Dengue Cohort Study

1043 [d] Recipient Epidemiology and Donor Evaluation Study-III

1044

1045 **Table 2: ZIKV peptides used in this study**

1046

1047 **a) ZIKV predicted peptide set composed by 9-and 10-mer peptides.**

Allele	C	pr	M	E	NS1	NS2A	NS2B	NS3	NS4A	2K	NS4B	NS5	Total
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**

1048

1049 **b) 15-mer peptides spanning the ZIKV polyprotein**

Allele	C	pr	M	E	NS1	NS2A	NS2B	NS3	NS4A	2K	NS4B	NS5	Total
HLA class II	25	18	15	100	70	46	26	123	25	5	50	180	**683**

1050

1051 **Table 3: Sequence homology between ZIKV and DENV .** Homology analysis

1052 between BeH818995 ZIKV isolate (GenBank accession no. AMA12084.1) and DENV1, 2, 3, 4

1053 consensus sequences obtained as previously reported(44, 45).

		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%
Average	**56%**	**47%**	**43%**	**58%**	**55%**	**31%**	**39%**	**67%**	**44%**	**51%**	**67%**
Average of structural proteins [a]				**49%**	**Average of non-structural proteins [a]**						**51%**
Average of structural proteins accounting for size [b]				**51%**	**Average of non-structural proteins accounting for size [b]**						**58%**

1054 [a] Average of structural and non-structural proteins based on average of the different

1055 homology values in the four DENV serotypes for each protein.

1056 [b] Average conservation on a per-residue based of structural and non-structural proteins

1057 accounting for size.

1058

1059 **Table 4: Monoclonal antibodies used in this study.**

Target	Color	Clone	Company
CD3	AlexaFluor700	UCHT1	eBioscience
CD4	APC-eFluor780	RPA-T4	eBioscience
CD8	BV650	RPA-T8	Biolegend
CD14	V500	M5E2	BD Biosciences
CD19	V500	HIB19	BD Biosciences
Live/Dead	ef506		eBioscience
IFNγ	FITC	4S.B3	eBioscience
CD45RA	eFlour450	HI100	eBioscience
CCR7	PerCPCy5.5	G043H7	Biolegend
TNFα	PE-Cy7	Mab11	EBioscience
PD1	PE-CF594	EH12.1	BD Biosciences
Granzyme B	PE	GB11	EBioscience

1062 **Table 5: Donors tested in each category**

# of samples	ZIKV status [a)]	DENV status [c)]	Country of origin
18	Acute	Pos.	Brazil /Mexico
17	Acute	Neg.	Nicaragua/Mexico
33	Convalescent	Pos.	Brazil/US travelers/ blood bank donors
30	Convalescent	Neg.	US travelers/ blood bank donors
20	Neg. [b)]	Pos.	Nicaragua/ Sri Lanka
20	Neg.	Neg.	US

1063 **a)** Infection with ZIKV was confirmed by RT-PCR

1064 **b)** ZIKV-neg. samples were collected before the onset of the ZIKV epidemic

1065 **c)** Previous exposure to DENV was determined by the presence of detectable DENV-

1066 specific IgG titers.

1067

1068 **Table 6. Testing of DENV corresponding peptides for ZIKV $NS5_{2868-2876}$ $NS3_{1725-1734}$, and $E_{485-493}$ peptides.**

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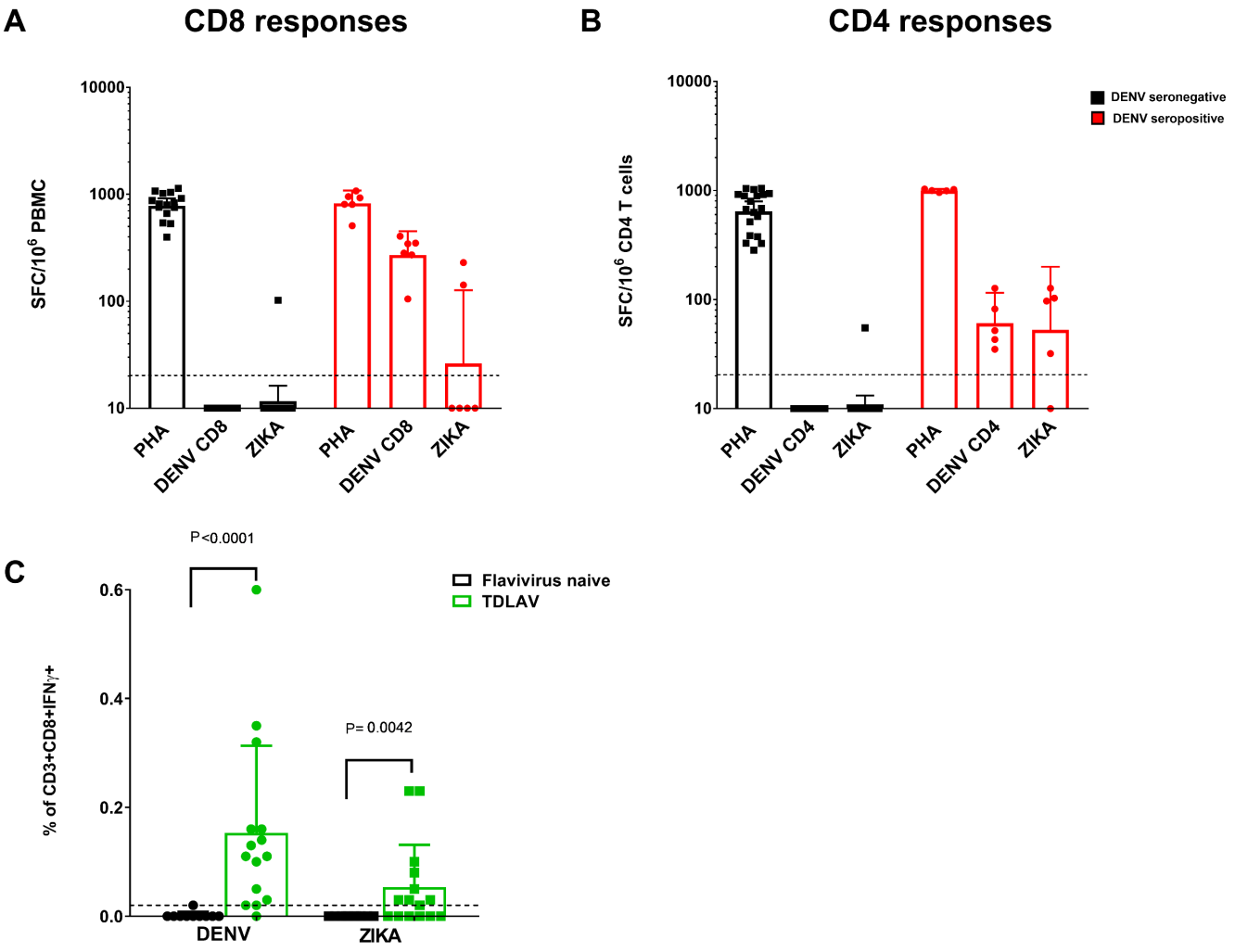
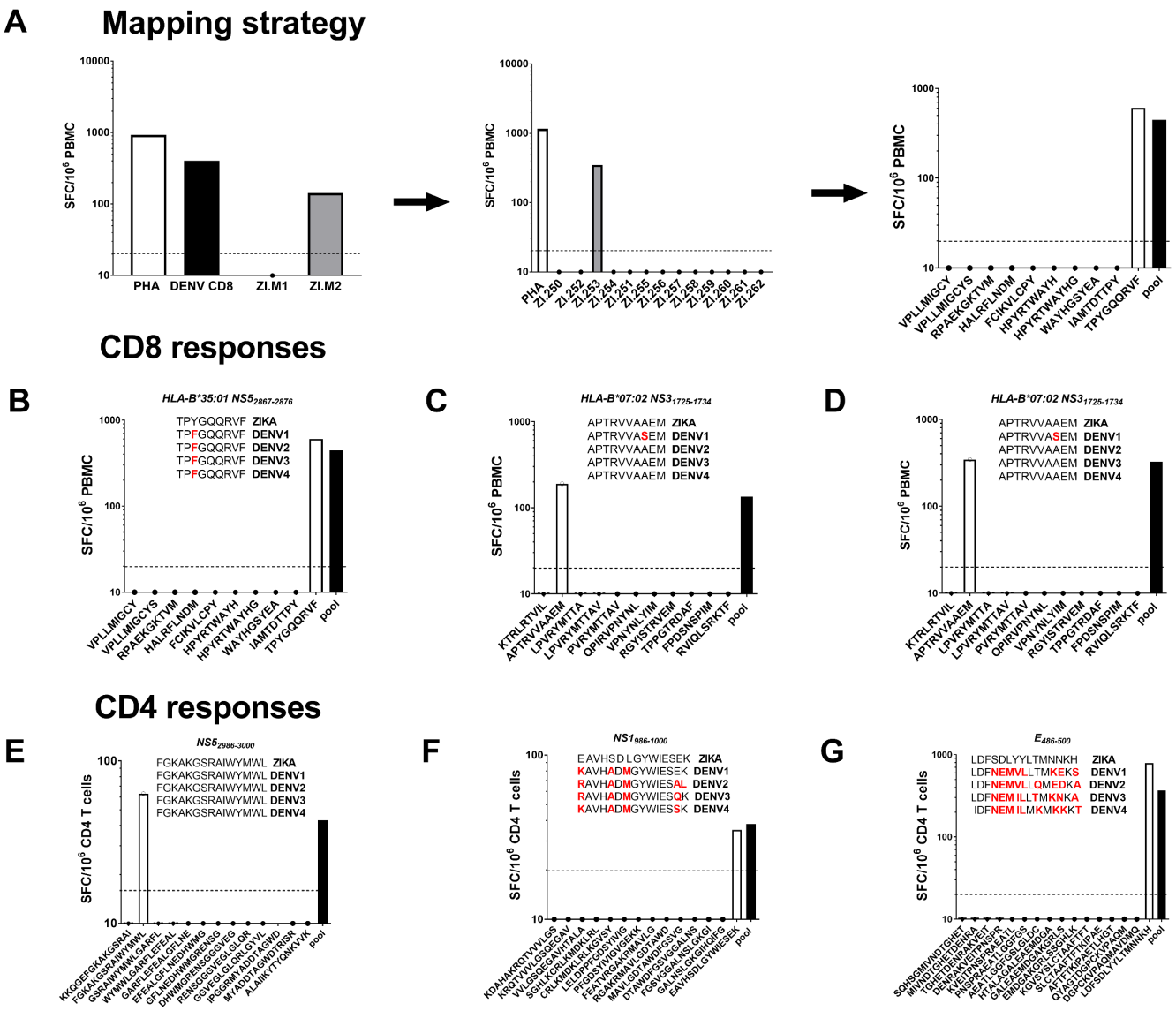
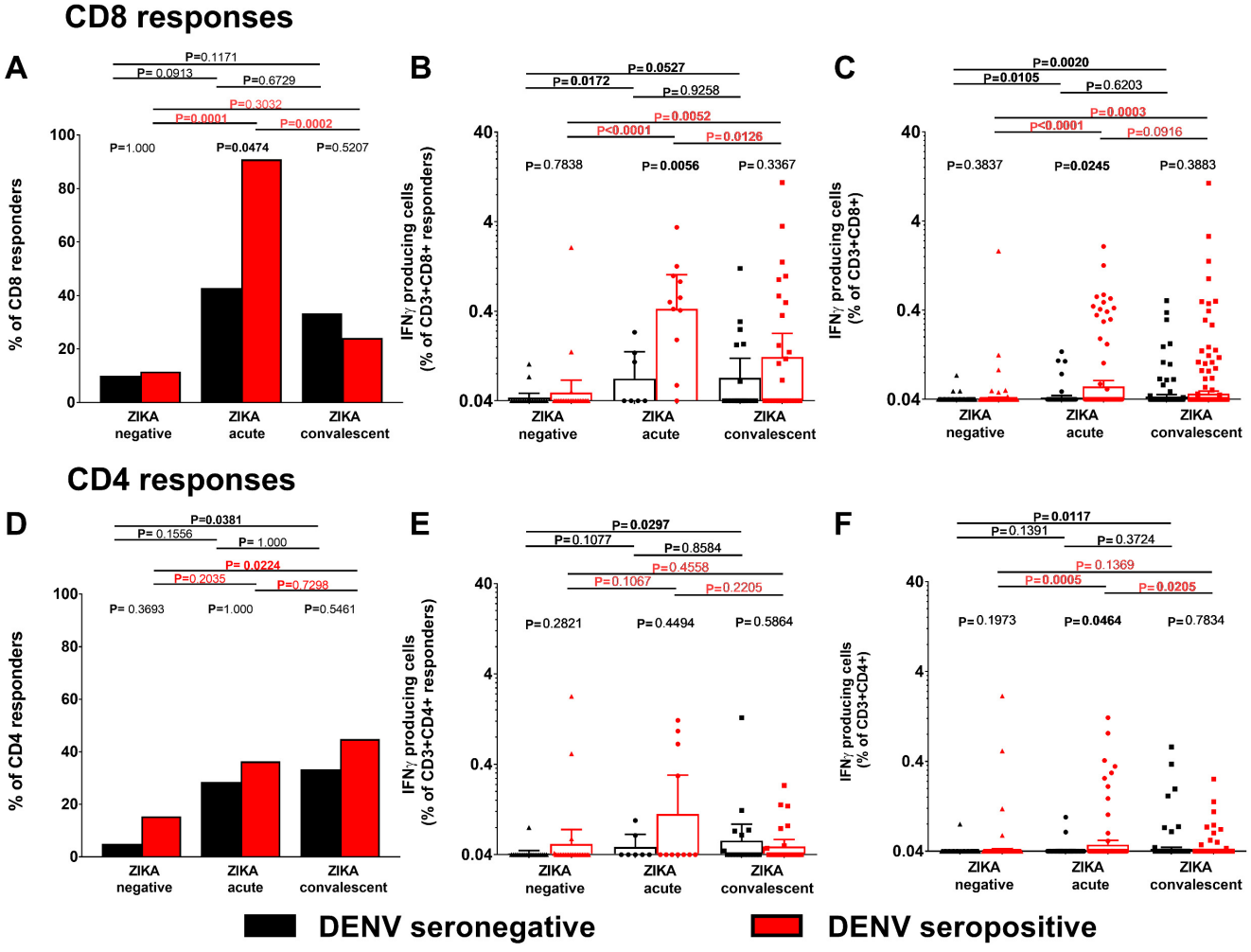
Donor	DENV Status	ZIKV Status	Protein	Source	Peptide Sequence	SFC/10^6 [a)]
GN0101	pos	neg	$NS5_{2868-2876}$	ZIKV	TPYGQQRVF	353 ± 240
				DENV1-4	TPFGQQRVF	366 ± 120
GS0157	pos	neg	$NS3_{1725-1734}$	ZIKV	APTRVVAAEM	330 ± 75
				DENV1	APTRVVASEM	219 ± 64
2894	neg	pos	$E_{485-493}$	ZIKV	GLDFSDLYY	287 ±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

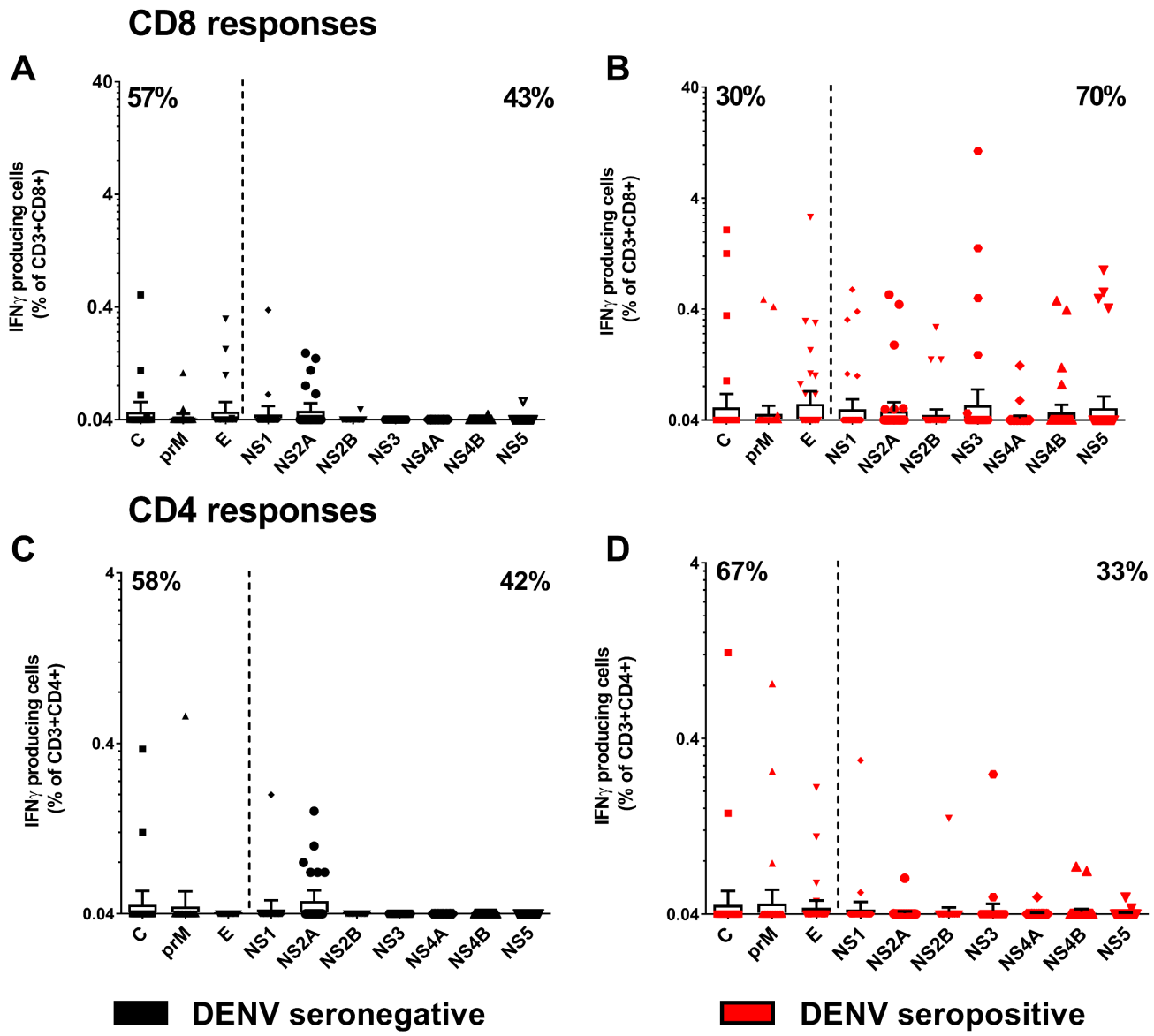
1071 [a)]Average and Standard deviation of net responses from 6-9 independent wells for donors GN0101 and GS0157,

1072 and 3 independent wells for donor 2894.









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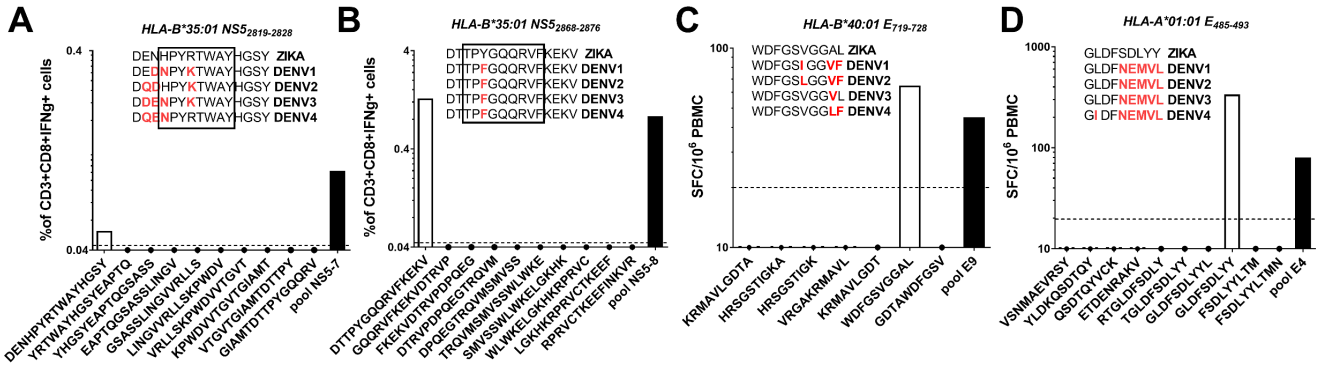
ZIKA positive DENV seropositive



ZIKA positive DENV seronegative