

Article

# Improved immune responses against Zika virus after sequential dengue and Zika virus infection in human

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**Abstract:** The high level of dengue virus (DENV) seroprevalence in areas where Zika virus (ZIKV) is circulating and the cross-reactivity between these two viruses have raised concerns on the risk of increased ZIKV disease severity for patients with a history of previous DENV infection. To determine the role of DENV pre-immunity in ZIKV infection, we analysed the T and B cell responses against ZIKV in donors with or without previous DENV infection. Using PBMCs from donors living in an endemic area in Colombia, we have identified, by interferon (IFN)- $\gamma$  enzyme-linked immunospot (ELISPOT) assay, most of the immunodominant ZIKV T-cell epitopes in the non-structural proteins NS1, NS3 and NS5. Analyses of the T and B-cell responses in the same donors revealed a stronger T-cell response against peptides conserved between DENV and ZIKV, with a higher level of ZIKV-neutralizing antibodies in DENV-immune donors, in comparison with DENV-naïve donors. Strikingly, the potential for antibody mediated enhancement of ZIKV infection was reduced in donors with sequential DENV and ZIKV infection in comparison with donors with DENV infection only. Altogether, these data suggest that individuals with DENV immunity present improved immune responses against ZIKV.

**Keywords:** *Dengue virus; Zika virus; T-cell epitopes; cross-reactive T cells; immunodominance; neutralizing antibodies; antibody-dependent-enhancement (ADE)*

## 1. Introduction

Zika virus (ZIKV) is a flavivirus transmitted by *Aedes* species mosquitoes. It is a single positive-stranded RNA virus closely related to yellow fever virus, dengue virus (DENV) and West Nile virus [1]. Initially isolated in the Zika forest in Uganda in 1947 [2], it caused an explosive outbreak for the first time in Yap Island, Federated States of Micronesia in 2007 [3]. Subsequent outbreaks with higher number of cases occurred in 2013–2014 in French Polynesia and other South Pacific Islands and more recently in the Americas [4–9]. Although initially believed to only cause mild, self-limiting disease, a causal relationship between ZIKV and neurological complications, such as Guillain-Barré syndrome or congenital malformations was established during the 2013 and 2015 outbreaks in French Polynesia and Brazil [10–13].

While mutations in ZIKV genome might have contributed to its increased pathogenicity or explosive spread [9, 14, 15], one of the most important concerns today is related to the high level of DENV seroprevalence in areas where ZIKV is circulating [16]. Indeed, recent studies have shown that anti-DENV antibodies may enhance ZIKV infection and increase disease severity [17–21]. Given these constraints, and the lack of appropriate treatment for ZIKV infection, there is an urgent need to develop a vaccine against this infectious disease.

While antibodies against the E protein of DENV or ZIKV were shown to be highly cross-reactive, T cells can be cross-reactive or not, depending on the targeted peptides. A low degree of CD4 T-cell cross-reactivity between DENV and ZIKV was indeed observed in human donors immune to one of these viruses [18], whereas DENV/ZIKV cross-reactive T cells were identified in humans and in DENV-immune mice after challenge with ZIKV [22–24]. Considering the sequence identity between DENV and ZIKV for the structural proteins capsid and envelope, and the non-structural proteins NS3 and NS5, that represent the main targets of DENV-specific CD4 and CD8 T cells, respectively, and the protective role of DENV-specific T cells [25, 26], efforts are currently directed towards the mapping of T-cell epitopes to design new and more effective vaccines against ZIKV [27]. Predictions of T-cell antigens have been conducted by modelling potential epitopes from the ZIKV proteome that could bind to different HLA class I or class II alleles [23, 28–30], or by analysing *ex vivo* T-cell responses in transgenic mice expressing human HLA-B\*0702 and HLA-A\*0101 molecules [23]. More recently, ZIKV epitopes targeted by CD4 and CD8 T cells have also been identified from human donors living in ZIKV and DENV endemic regions [22]. Quite unexpectedly, while the majority of T cell responses observed upon infection with DENV were directed against the non-structural proteins NS3, NS4B and NS5, ZIKV-specific T cells preferentially recognize structural proteins E, prM and C, with conserved epitopes between DENV and ZIKV representing the main targets for cross-reactive T cells [22, 23]. Furthermore, in the light of the recent identification of DENV/ZIKV cross-reactive T cells in the human and in different animal models [22, 24, 31–34], the precise identification of ZIKV T-cell epitopes in the human that activate these cross-reactive T cells is essential to assess the role of these T cells in ZIKV infection and disease. In the present study, we have identified these epitopes from blood donors with a history of either ZIKV-only infection or both DENV and ZIKV infection.

Using PBMCs from Colombian blood donors with previous ZIKV infection, we have first established a detailed map of the distribution of ZIKV T-cell epitopes, by quantifying *ex vivo* IFN- $\gamma$  responses against peptides covering the whole ZIKV proteomic sequence by enzyme-linked immunosorbent spot (ELISPOT) assay. Measurement of the magnitude of T-cell responses (mediated by CD4 and/or CD8 T cells) against these peptides allowed us to identify immunodominant epitopes that induce strong responses in donors carrying specific HLA alleles. More specifically, we show that the non-structural proteins NS1, NS3 and NS5 contain most of the immunodominant epitopes that induce a strong T-cell response. In donors with a history of DENV infection, the strongest T-cell responses were directed against peptides of the NS5 protein with a high level of amino acid identity with the four serotypes of DENV, and some matched previously described DENV CD8<sup>+</sup> T-cell epitopes, suggesting the activation of cross-reactive T cells.

The neutralizing or enhancing activity of ZIKV-induced antibodies from the same donors were also analysed using a flow cytometry-based assay. Results show that ZIKV infection in DENV-immune individuals resulted in increased levels of neutralizing antibodies against ZIKV, in comparison with DENV-naïve individuals, and in reduced potential for antibody enhancement of ZIKV infection, in comparison with DENV-immune ZIKV-naïve individuals. Altogether, these data strongly suggest that a sequential DENV and ZIKV infection may improve the immune protection against ZIKV infection but not against DENV infection.

**2. Materials and Methods**

*2.1. Ethics Statement*

Human blood samples were obtained from healthy adult donors from the Fundación Hematológica Colombia (Bogotá D.C., Colombia) in an anonymous manner. All protocols described in this study were approved by the institutional review board (IRB) of the EL Bosque University (Colombia). Donors were of both sexes and between 20 and 60 years of age. A total of 82 samples were obtained from different ZIKV-endemic areas near Bogotá D.C. (mainly from Villavicencio, Meta) between September and November 2016.

*2.2. Human blood samples*

PBMCs were purified by density gradient centrifugation (Lymphoprep™; Stemcell technologies), resuspended in FBS (Gibco) containing 10% dimethyl sulfoxide and cryopreserved in liquid nitrogen. Eleven of the 82 blood samples obtained had to be excluded from the study due to poor viability of cells.

*2.3. Viruses and Cell Lines*

The *in vitro* assays were conducted using the DENV1 KDH0026A strain (provided by Dr L. Lambrechts, Institut Pasteur, Paris), DENV2 R0712259 strain (provided by Dr. A-B. Failloux, Institut Pasteur, Paris), DENV3 KDH0010A strain (provided by Dr. L. Lambrechts, Institut Pasteur, Paris), DENV4 VIMFH4 (from the Institut Pasteur Collection) and ZIKV FG15 strain(provided by Dr. D. Rousset, Institut Pasteur, Cayenne). All viruses were grown using the Aedes Albopictus mosquito cell line C6/36 cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum containing 0.1mM non-essential amino acids and 1X tryptose phosphate broth. Vero-E6 and DC-SIGN-expressing U937 cells were kindly provided by Dr M. Flamand and Dr B. Jacquelin (Institut Pasteur, Paris), respectively.

*2.4. HLA Typing*

Genomic DNA isolated from PBMCs of the study subjects by standard techniques (QIAmp; Qiagen) was used for HLA typing. High resolution Luminex-based typing for HLA class I and class II molecules (alleles A, B, C and DRB1, respectively) was used according to the manufacturer's protocol (Sequence-Specific Oligonucleotides (SSO) typing; Immucor, Lifecodes).

*2.5. Serology*

ZIKV seropositivity was determined using a recombinant antigen-based (ZEDIII antigen) indirect ELISA, as previously described [35]. Briefly, 96-well plates (Nunc, Life Technologies, Rochester, NY) were coated overnight at 4°C with 50 ng of antigen in PBS. After washing, 200µl PBS containing 3% skimmed milk and 0.1% Tween-20 were added for 1hr at 37°. The blocking solution was replaced by 100µl of plasma diluted 1:500 in PBS containing 1.5% BSA and 0.1% Tween-20, and plates were incubated at 37°C for 60 min. After three washes, bound antibodies were detected with a horseradish peroxidase-conjugated goat anti-human IgG immunoglobulin (Rockland). Following incubation at 37°C for 1hr and three washes, 100µl of a substrate solution containing TMB (KPL, Eurobio) were added. After 15 min incubation, the optical density (OD) was determined at 650 nm with an automated plate reader (Tecan infinite 200 pro). Each plasma sample was tested in duplicate. Plasma samples obtained from individuals with positive DENV IgG serology collected before the ZIKV outbreak were used as negative controls. The cut-off was calculated from the negative controls and was 0.196. DENV seropositivity was determined by indirect ELISA for IgGs (Panbio; Alere) and by capture ELISA for IgM (Tecnosuma) following the manufacturer's instructions. The quantification of neutralizing and enhancing activities of antibodies against DENV and ZIKV infections was determined using a flow cytometry-based assay, as described previously [36, 37]. Briefly, 10-fold serial dilutions of plasma samples were incubated at 37°C for 1 hour with

either a dilution of virus inducing 7-15% of infection (for neutralization assay) or a dilution of virus inducing between 0.5 and 2% infection (for Antibody Dependent Enhancement (ADE) Assay). Virus-antibody mixture was then added to  $5 \times 10^4$  cells (U937-DC-SIGN cells for neutralization of DENV1-4 infection, or Vero cells for neutralization of ZIKV infection, or K562 cells for the ADE assay, for 2 hours at 37°C after which cells were washed 2 times with fresh medium and then incubated for 24h. The cells were then fixed with 4% paraformaldehyde, stained with 4G2 antibody conjugated to Alexa-488, and the percentage of infected cells was measured by flow cytometry. The neutralization titer of antibodies was expressed as the reciprocal dilution of plasma at which 50% of the virus was inhibited. Plasma samples from donors collected before ZIKV outbreak or from negative samples provided from the Kits to detect anti-DENV antibodies did not reveal any neutralization activity against ZIKV or DENV infection, respectively. For the ADE assay, the peak titer was expressed as the logarithm of reciprocal dilution of plasma at which the percentage of infection was maximal. Following the ELISA and neutralization assays, from the 71 plasma samples selected for this study, a total of 9 samples from ZIKV-seropositive and DENV-seronegative individuals (ZIKV donors) and 11 samples from DENV/ZIKV-seropositive individuals (DENV/ZIKV donors) were further selected for ELISPOT analysis. The full list of the 20 blood donors included in the study is listed in Table S1.

## 2.6. RT-PCR assays for detection of DENV and ZIKV

RNA was extracted from plasma using the QIAamp Viral RNA Mini kit (Qiagen) according to the manufacturer's instructions. Samples were tested for DENV and ZIKV using a specific nested-PCR assay, as previously described [38]. Detection of ZIKV was confirmed in 3 out of the 9 plasma samples from ZIKV donors and in 6 out of the 11 plasma samples from DENV/ZIKV donors, whereas DENV was detected in 2 out of the 11 plasma samples from DENV/ZIKV donors (Table S1).

## 2.7. Viral sequences

The identical amino acid sequence of 2 Zika virus from Colombia (KX087102 and KU820897) was used as a reference for the set of overlapping 15-mer peptides. A total of 50 full-length protein coding DENV sequences from Colombia (serotype 1: 14 sequences; serotype 2: 16 sequences; serotype 3: 13 sequences; serotype 4: 7 sequences) were retrieved from GenBank and used for pairwise sequence identity comparisons.

## 2.8. Peptides

All peptides were synthesized by Mimotopes (Victoria, Australia). A total of 853 15-mer peptides overlapping by 11 amino acids and 197 9-mer peptides overlapping by eight amino acids were tested by ELISPOT assay. For the identification of T-cell epitopes, 15-mer peptides were combined into pools of 12 peptides, and individual peptides from the positive pools were tested in a second ELISPOT assay. Following the identification of the positive 15-mer peptides, and according to their HLA class I or class II restriction potential (predicted or shared between at least two donors), 9-mer peptides were synthesized and tested individually.

## 2.9. Ex Vivo IFN- $\gamma$ ELISPOT assay

PBMCs ( $2 \times 10^5$ ) were incubated in 96-well flat bottom plates (MSIPS 4510, Millipore, Bedford, MA) coated with anti-IFN- $\gamma$  mAb (clone 1-D1K, Mabtech, Sweden) with 0.2ml of complete RPMI containing 10% human AB serum with pools of 12 peptides (2 $\mu$ g/ml, final concentration) or individual peptides (1 $\mu$ g/ml final concentration) for 20 hours. Following a 20h-incubation at 37°C, the wells were washed with PBS/0.05% Tween 20 and then incubated with biotinylated anti-IFN- $\gamma$  mAb (clone 7-B6-1, Mabtech) for 1h 30mn. The spots were developed using Streptavidin-alkaline phosphatase (Mabtech) and BCIP/NBT substrate (Promega, France) and counted using an automated ELISPOT reader (Immunospot, Cellular Technology Limited, Germany). The number of IFN- $\gamma$ -producing cells was expressed as spot forming cells (SFC) relative to  $1 \times 10^6$  PBMCs. Values



were calculated by subtracting the number of spots detected in the non-stimulated control wells. Values were considered positive if they were equal to or greater than 20 spots and at least three times above the means of the unstimulated control wells. As a positive control, cells were stimulated with CEF peptide pool (Mabtech).

*2.10. Immunogenicity and HLA restrictions prediction*

The evaluation of binding possibilities of peptides to MHC class I and class II alleles was analyzed using the NetMHCpan3.0 and NetMHCIIpan3.1 servers, respectively [39, 40].

*2.11. Statistics*

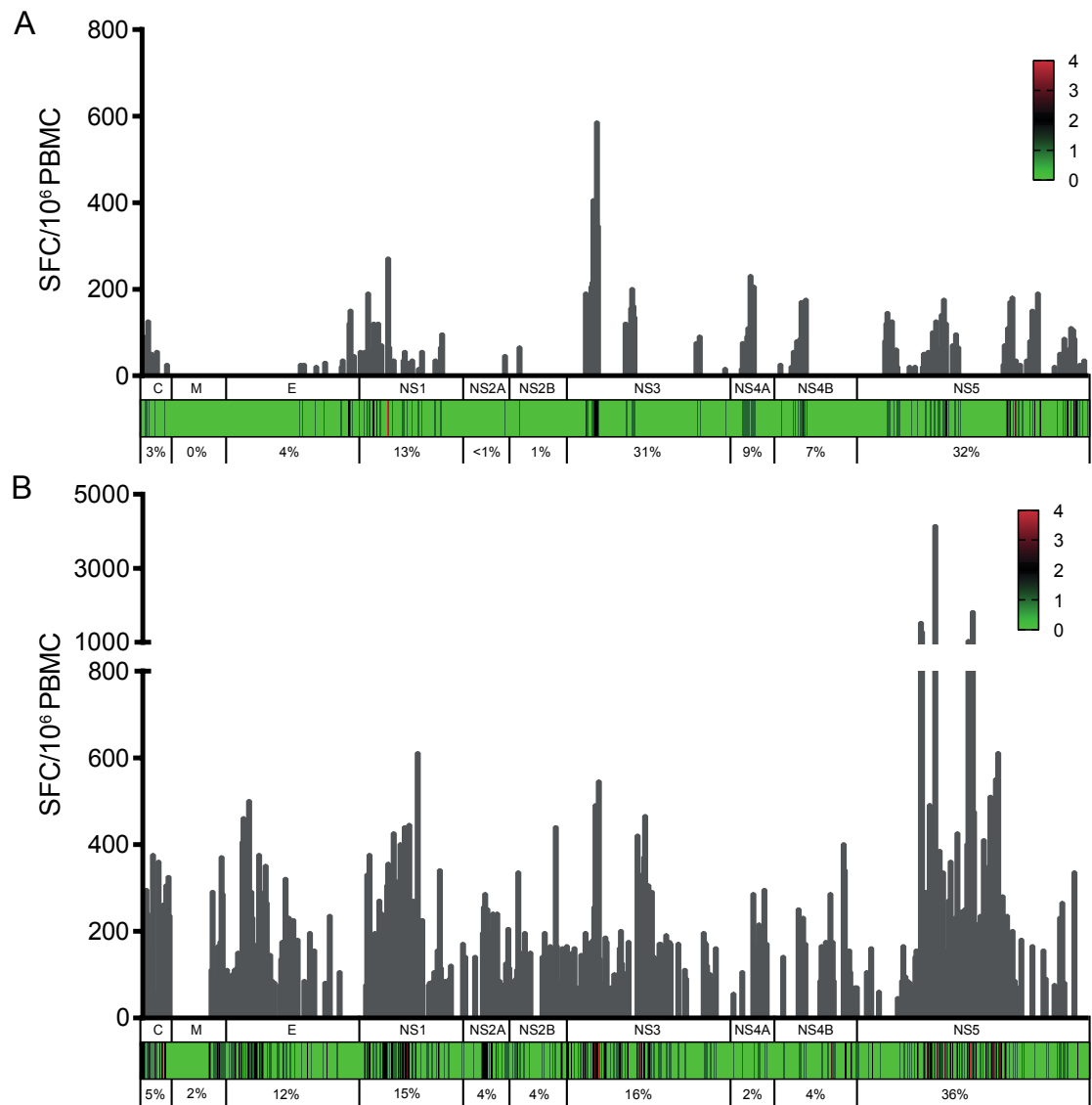
All data were analyzed with Prism software version 7.0 (GraphPad Software). Statistical significance was determined using the nonparametric two-tailed Mann-Whitney test to compare two independent groups. Differences were considered significant at  $P < 0.05$ .

**3. Results**

*3.1. Identification of immunodominant regions of the ZIKV proteome*

To investigate T-cell immunity induced after ZIKV infection, we examined responses from blood donors living in a ZIKV endemic area in gamma interferon (IFN- $\gamma$ )-specific enzyme-linked immunosorbent spot (ELISPOT) assays. Blood samples from all study participants were first tested for the presence of Zika virus IgG and dengue virus IgM and IgG by ELISA, and for the presence of virus-specific antibodies by flow cytometry-based neutralization assay against ZIKV and the 4 DENV serotypes. Recent infection with ZIKV was confirmed by RT-PCR in 3 out of 9 plasma samples from ZIKV donors, and in 6 out of 11 plasma samples from DENV/ZIKV donors, whereas recent infection with DENV was detected in 2 out of 11 plasma samples from DENV/ZIKV donors (Table S1). To confirm previous DENV or ZIKV infection in the PCR negative samples, all samples were screened for specific neutralizing antibodies against the 4 DENV serotypes and against ZIKV. While all samples from ZIKV donors revealed significant levels of neutralizing antibodies against ZIKV, they did not neutralize DENV1-4 infection, confirming the absence of DENV-specific antibodies in these donors (Figure S1). On the contrary, all samples from DENV/ZIKV donors revealed high neutralization titers against both ZIKV and DENV (Table S1 and Figure S1). Because anti-DENV antibodies have been shown to cross-react with ZIKV [18, 20], blood samples collected before the ZIKV outbreak were used as negative controls for ZIKV neutralization. In these samples from DENV donors, a strong neutralization activity was measured against at least one DENV serotype whereas a low neutralization activity was detected against ZIKV, in contrast to samples from DENV/ZIKV showing a high neutralization activity against both DENV and ZIKV (Figure S1). Based on these data, two groups of donors were selected for further studies, donors having only high levels of ZIKV-neutralizing antibodies (called “ZIKV”) and donors having high levels of DENV- and ZIKV-neutralizing antibodies (called “DENV/ZIKV”). Details of the HLA-typed blood donors included in this study are listed in Table S1. PBMCs from 20 ZIKV-seropositive donors were screened for T-cell reactivity against pools of 15-mer peptides (overlapping by 11 amino acids) spanning the entire ZIKV proteome. Analysis of the response magnitude (as spot forming cells (SFC) per  $10^6$  cells) and frequency of responding donors revealed that the non-structural (NS) proteins NS1, NS3 and NS5 were the most vigorously and frequently recognized proteins, and accounted for more than 65% of the total response (Figure 1). As these donors were selected in DENV- and ZIKV-endemic areas, and as these viruses share an overall 43% protein sequence identity (with up to 68% for the non-structural proteins), we sought to distinguish between the ZIKV-specific epitopes and those shared by both viruses. Among the 20 ZIKV-seropositive blood donors, 11 individuals had both anti-DENV and anti-ZIKV IgG antibodies (DENV/ZIKV donors) and 9 individuals did not reveal any detectable anti-DENV antibodies (ZIKV donors) (Table S1 and Figure S1). Strikingly, NS1, NS3 and NS5 proteins represented 13%, 31% and 32% of the total response, respectively, in ZIKV donors (Figure 1A) and 15%, 16% and 36% of the responses in DENV/ZIKV donors (Figure

1B), whereas the NS3, NS4B and NS5 proteins have been reported to account for 31%, 15% and 22% of the DENV-specific T-cell response, respectively [25, 41-43].



**Figure 1.** Cumulative IFN- $\gamma$  responses (as spot-forming cells (SFCs) per million cells) for each overlapping peptide spanning the ZIKV proteome. (A) SFCs per million cells are shown for ZIKV donors; (B) SFCs per million cells are shown for DENV/ZIKV donors. The heat map indicates the number of donors with a positive IFN- $\gamma$  response to each peptide within each protein (C, capsid; M, membrane; E, envelope, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The numbers below each graph represent percentages of the total response for each protein.

The analysis of the T-cell responses in these two groups of donors confirmed that NS1, NS3 and NS5 are the main targets for T cells in ZIKV-infected donors, regardless of a previous infection with DENV. From the 853 peptides spanning the entire ZIKV proteome, 410 peptides elicited a significant T-cell response, some of which being recognized by multiple donors. For most antigenic peptides, the HLA class I and class II alleles of the responding donors coincide with the alleles predicted to bind to this epitope [39, 40]. Among the epitopes inducing a strong response in ZIKV and DENV/ZIKV donors, several 15-mer peptides contained sequences predicted to bind strongly to at least one allele expressed by the responding donors (Table 1 and Table S2).

**Table 1.** HLA class I- or class II-restricted T cell epitopes identified in ZIKV proteins

Peptide <sup>1</sup>	Sequence	SFC/million PBMC <sup>2</sup>	HLA	Score <sup>3</sup>
C <sub>86-94</sub>	KDLAAMLRI	65	B*55:01	1.4
			B*40:02	2.0
C <sub>89-97</sub>	AAMLRIINA	75	A*02:01	4.5
NS1 <sub>63-77</sub>	MENIMWRSVEGELNA	310	DRB1*04:05	50
NS1 <sub>68-76</sub>	WRSVEGELN	206	B*40:02	48
			B*18:01	48
NS1 <sub>167-175</sub>	VWLKVREDY	75	A*29:02	1.3
NS1 <sub>280-288</sub>	CPGTKVHVE	170	B*35:01	8.5
			B*35:31	6.5
NS3 <sub>219-233</sub>	TVILAPTRVVAEME	165	DRB1*08:02	1.5
NS3 <sub>311-325</sub>	AAIFMTATPPGTRDA	470	DRB1*04:01	4.0
NS3 <sub>313-321</sub>	IFMTATPPG	30	A*24:02	5.0
NS3 <sub>314-322</sub>	FMTATPPGT	85	A*02:17	5.5
NS4B <sub>112-120</sub>	AIILLVAHY	88	A*29:02	0.6
			A*11:01	3.5
NS4B <sub>115-123</sub>	LLVAHYMYL	68	A*02:05	0.3
NS4B <sub>116-124</sub>	LVAHYMYLI	35	A*69:01	0.15
			A*02:05	0.2
NS5 <sub>298-306</sub>	NHPYRTWAY <sup>4</sup>	358	B*35:01	3.0
NS5 <sub>299-307</sub>	HPYRTWAYH <sup>4</sup>	308	B*35:01	0.4
NS5 <sub>302-310</sub>	RTWAYHGSY	205	A*01:01	0.5
NS5 <sub>348-356</sub>	TPYGQQRVF <sup>4</sup>	1681	B*35:31	0.7
			B*35:01	0.3
NS5 <sub>425-433</sub>	EAVNDPRFW	465	B*44:03	5.0
			B*15:17	7.0
			B*35:01	5.0
NS5 <sub>461-475</sub>	KKQGEFGKAKGSRAI	405	DRB1*07:01	32
NS5 <sub>473-487</sub>	RAIWYMWLGARFLEF	505	DRB1*07:01	16
NS5 <sub>609-617</sub>	YALNTFTNL	42	B*35:43	0.4
			B*35:31	0.25

<sup>1</sup> The position of peptides were determined according to NCBI reference sequence YP\_002790881.1;

<sup>2</sup> Cumulative SFC/million PBMC; <sup>3</sup> Calculated using NetMHCpan 3.0 or NetMHCIIpan3.1 server;

<sup>4</sup> Peptides identified previously in human individuals infected with DENV and ZIKV [22]

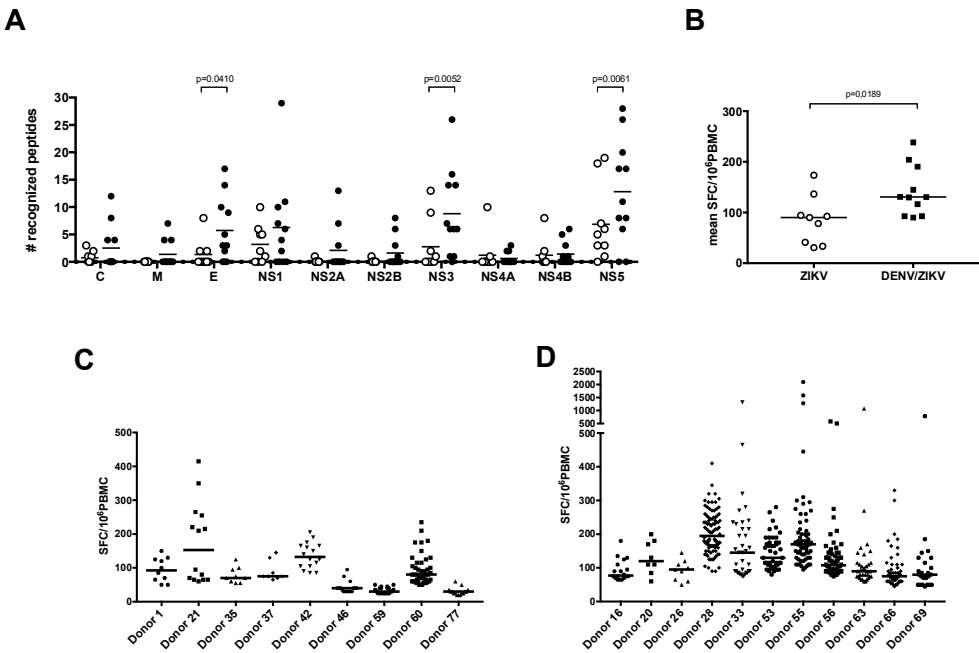
For instance, the NS2B<sub>117-131</sub> peptide contains a 10-mer sequence predicted to bind strongly to the HLA-A\*0301 and -A\*1101 molecules expressed by the responding donor 55 (Table S2). In other cases, multiple responding donors express at least one common allele with strong potential for binding to the stimulating peptide. This holds for the E<sub>455-469</sub> peptide in the envelope that contains the 9-mer and the 10-mer sequences predicted to bind to the HLA-B\*5101 and HLA-A\*0201 alleles, both alleles being expressed by the responding donors 1 and 77 (Table S2). This also applies to the NS5<sub>13-27</sub> peptide, which induced a strong response in donors 55 and 69 that share the HLA-B\*3501 allele, this allele being predicted to bind to the 9-mer peptide MSALEFYSY with a high affinity (Table S2). Interestingly, this epitope was also shown to induce a significant response in transgenic mice carrying the HLA-A\*0101 molecule, which is expressed by donor 69 [23]. Similarly, a strong T-cell response was observed against the NS5<sub>546-560</sub> peptide in donors 28, 53, and 66 that express the HLA-B\*4002 and -B\*4403 alleles and against the NS5<sub>605-619</sub> peptide in donors 33 and 59 that share the predicted HLA-A\*2402 allele (Table S2). Finally, we have also identified several 9-mer and 15-mer

immunodominant epitopes in the Capsid, NS1, NS3, NS4B and NS5 proteins, which induced substantial T-cell responses in donors that share one or several alleles with a strong potential for binding to these peptides (Table 1).

Within the NS3 and NS5 proteins, several epitopes were previously described as immunodominant epitopes, either predicted or validated experimentally after DENV infection or vaccination in humans or after ZIKV infection in mice and in humans [22, 23, 29, 44, 45]. Indeed, among the 9-mer peptides identified in DENV/ZIKV donors, the NS5<sub>293-307</sub>, NS5<sub>297-311</sub> and NS5<sub>345-359</sub> have been already detected in PBMCs from HLA-B\*3501 individuals, after infection with DENV1, DENV2, or vaccination with DENV live attenuated vaccine (DLAV), with a lysine-to-arginine and a phenylalanine-to-tyrosine amino acid substitution at residues 302 and 350 in the NS5<sub>297-311</sub> and NS5<sub>345-359</sub> peptides from ZIKV, respectively [22, 43, 44, 46] (Table 1). These results obtained from DENV/ZIKV donors thus confirm that these NS5 peptides contain nested epitopes restricted by the HLA-B\*3501 molecule. Yet the 15-mer NS3<sub>219-233</sub> peptide, which contains the APTRVVAEM epitope, induced a substantial response in 2 DENV/ZIKV donors that express neither HLA-B\*0702 nor B\*3501, although these alleles were expressed in responding donors vaccinated with the live attenuated DENV vaccine DLAV or in *ifnar*<sup>-/-</sup> HLA-B\*0702 transgenic mice or in humans after ZIKV infection [22, 23, 44]. This suggests that the NS3<sub>219-233</sub> peptide contains another epitope or a promiscuous epitope that binds to other HLA alleles, besides HLA-B\*0702 or B\*3501 (Table 1).

3.2. Broader responses with a higher magnitude in donors with previous DENV infection

Given the ZIKV-specific antibody response against NS1 and the low level of CD4 T-cell cross-reactivity between DENV and ZIKV against the E and NS1 proteins [18], we wished to compare, among the immunodominant epitopes, the T-cell responses in PBMCs from ZIKV donors with those from DENV/ZIKV donors. First, comparison of the frequency of responding T cells in ZIKV and DENV/ZIKV donors underlined the higher magnitude of response in DENV/ZIKV donors, relative to ZIKV donors (Figure 1A and B). The number of stimulating peptides per donor for each viral protein, as well as the average response per donor differed in these two groups, with a significantly broader response against the E, NS3 and NS5 proteins and a higher magnitude of response in donors with previous DENV infection (Figure 2A and B).



**Figure 2.** ZIKV donors with previous DENV infection reveal a broader T-cell response with a higher magnitude. (A) Breadth and (B) magnitude of responses in ZIKV and DENV/ZIKV donors. Each dot represents one donor (open circles, ZIKV donors; filled circles, DENV/ZIKV donors) and the bars represent the median value for each group of donors. The P values were calculated using the nonparametric two-tailed



Mann-Whitney test. Frequency of responses against individual peptides, per donor, in ZIKV (C) and DENV/ZIKV (D) donors. Each dot represents one peptide. The bars represent the median response for each donor.

To determine whether this difference concerned only a small number of peptides that elicit a stronger response in each donor, or if it concerned the majority of the peptides, we plotted the frequency of responses against the different peptides, per donor, in the two different groups. Two out of 9 individuals among the ZIKV donors revealed a median response higher than 100 SFC/million cells, whereas 6 out of 11 DENV/ZIKV donors developed this strong response, which was also directed against a higher number of peptides (Figure 2C and D). This result reveals the activation of a higher frequency of T cells against ZIKV peptides, with a higher magnitude of response, in donors previously infected with DENV, in comparison with naïve donors. This strongly argues for the existence of cross-reactive T cells, these T cells being primed during the initial infection with DENV and expanded thereafter during the following infection with ZIKV, as shown recently in mice and in humans, after sequential infection with DENV and ZIKV [22, 23].

3.3. DENV/ZIKV-cross-reactive T cells mainly target the NS5 protein

To identify more specifically ZIKV-specific peptides and DENV/ZIKV cross-reactive peptides, we compared the sequences of the most immunodominant epitopes recognized by both types of donors. The NS1 and NS3 proteins contain a high proportion of peptides that elicit strong responses in both ZIKV and DENV/ZIKV donors, whereas the E protein and to a higher extent the NS5 protein contain a majority of peptides inducing a strong response only in DENV/ZIKV donors (Figure 2A and Table 2).

Table 2. Immunodominant epitopes in ZIKV and DENV/ZIKV donors

Peptide <sup>a</sup>	Sequence	ZIKV		DENV/ZIKV		% Identity			
		Donors	SFC/million PBMC <sup>b</sup>	Donors	SFC/million PBMC <sup>b</sup>	DENV1	DENV2	DENV3	DENV4
C <sub>49-63</sub>	AILAFRLRTAIKPSL	60	60	28,63	365	60,0%	53,3%	60,0%	40,0%
E <sub>67-81</sub>	DMASDSRCPTQGEAY			33	465	66,7%	53,3%	66,7%	53,3%
E <sub>87-101</sub>	DTQYVCKRTLVDGRGW			56	505	66,7%	53,8%	73,3%	66,7%
NS <sub>119-33</sub>	VFVYNDVEAWRDYK	21,46,60	195	28,56	380	46,7%	33,3%	46,7%	40,0%
NS <sub>155-69</sub>	CGISSVSRMENIMWR	35,46	125	56	275	67,1%	66,3%	60,0%	60,0%
NS <sub>191-105</sub>	GSVKNPMPWRGPQLRP	21,35,46,60	275	28	165	13,3%	33,3%	20,0%	33,3%
NS <sub>1107-121</sub>	PVNELPHGWKAWGKS			28,53	430	40,0%	46,7%	46,7%	50,5%
NS <sub>1147-161</sub>	HRAWNSFLVEDHGF	46	40	33,53	445	66,7%	73,3%	66,7%	76,2%
NS <sub>1163-177</sub>	FHTSVWLKVREDYSL	46	35	20,28,55	450	46,7%	46,3%	53,3%	46,7%
NS <sub>1195-209</sub>	HSDLGYWIESEKNDT			28,33	615	80,0%	73,3%	66,2%	73,3%
NS <sub>2117-131</sub>	AAGAWYVYVKTGKRS			55	445	33,3%	33,3%	26,7%	26,7%
NS <sub>3131-145</sub>	PAGTSGSPILDKCGR	21,42	405	26,55,63	495	53,3%	60,8%	53,3%	54,3%
NS <sub>3143-157</sub>	CGRVIGLYGNGVVIK	21	350	20,55,63,66	550	60,0%	66,7%	72,3%	80,0%
NS <sub>3311-325</sub>	AAIFMTATPPGTRDA			28,33	470	80,0%	80,0%	93,3%	80,0%
NS <sub>513-27</sub>	KARLNQMSALEFYSS			55,69	405	53,3%	46,7%	53,3%	40,0%
NS <sub>5293-307</sub>	WFFDENHPYRTWAYH			55,69	1620	66,7%	66,7%	60,0%	66,7%
NS <sub>5297-311</sub>	ENHPYRTWAYHGSYE			55,69	1330	80,0%	80,0%	73,3%	80,0%
NS <sub>5325-339</sub>	VVRLLSKPWDVVTGV			28,55,66	495	73,3%	80,0%	73,3%	66,7%
NS <sub>5345-359</sub>	TDTPYGGQQRVFKEK			33,55,69	4195	93,3%	93,3%	93,3%	93,3%
NS <sub>5373-387</sub>	QVMSMVSSWLWKELG	60	130	55,66,69	340	40,0%	53,3%	46,7%	46,7%
NS <sub>5461-475</sub>	KKQGEFGKAKGSRAI			28,53	405	93,3%	93,3%	93,3%	86,7%
NS <sub>5465-479</sub>	EFGKAKGSRAIWMW			28,53,55,56	1085	100,0%	100,0%	100,0%	93,3%
NS <sub>5473-487</sub>	RAIWMWLGARFLEF			28,55	505	100,0%	100,0%	93,3%	100,0%
NS <sub>5481-495</sub>	GARFLEFEALGFLNE			28,53,56,63	1870	93,3%	100,0%	93,3%	100,0%
NS <sub>5546-560</sub>	RFDLENEALITNQME			28,53,66	515	60,0%	47,1%	53,3%	60,0%
NS <sub>5573-586</sub>	TYQNKVVVKVLRPAEK			28,53,56	615	72,9%	66,7%	73,3%	80,0%
NS <sub>5849-863</sub>	CGSLIGHRPRTTWAE	60	90	33,55	340	66,7%	66,7%	66,7%	66,7%

<sup>1</sup> The position of peptides was determined according to NCBI Reference Sequence YP\_002790881.1;

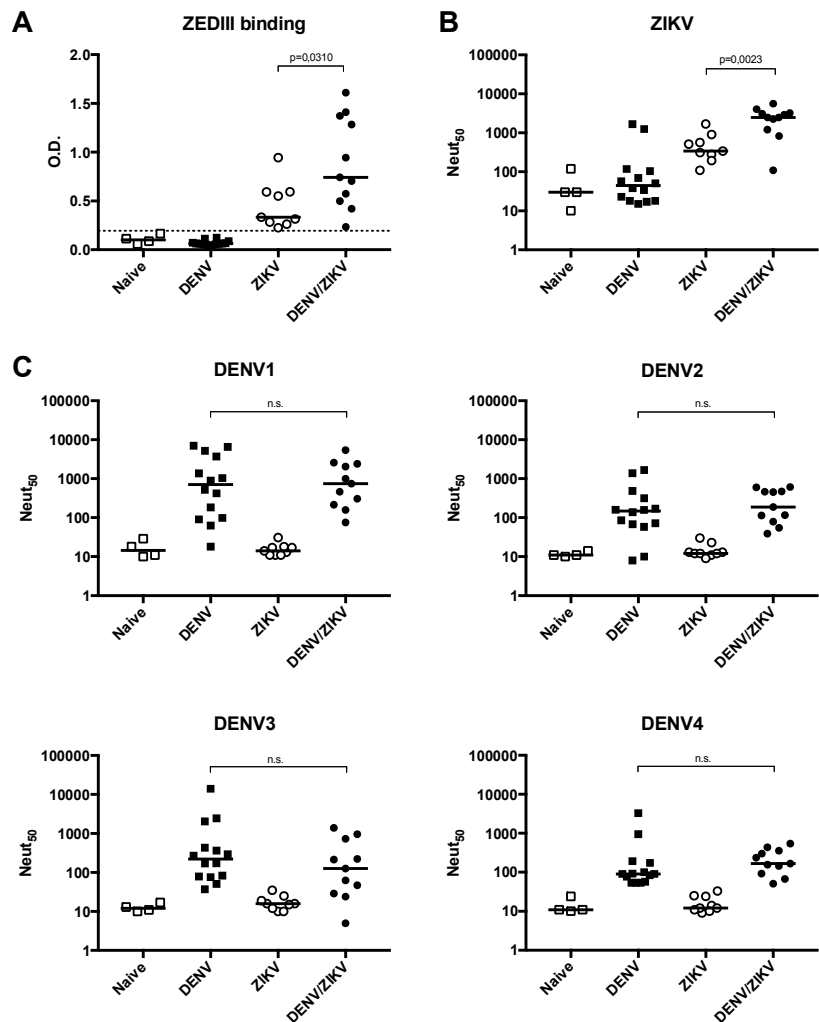
<sup>2</sup> Cumulative SFC/million PBMC

This suggests that the NS1 and NS3 proteins contain more ZIKV-specific epitopes, whereas the NS5 protein contains more epitopes shared by DENV and ZIKV and recognized by cross-reactive T cells. Strikingly, most of the peptides recognized only by DENV/ZIKV donors exhibit high degree of identity with the four DENV serotypes. For instance, in the NS1 protein, 2 out of the 5 epitopes that induced a response in ZIKV donors reveal a sequence identity higher than 60% with the four DENV serotypes, whereas 8 out of the 11 epitopes in the NS5 protein that induced a strong response in

DENV/ZIKV donors show a sequence identity higher than 66.7% with the four DENV serotypes (Table 2). Altogether, these data strongly support the activation of cross-reactive T cells induced after DENV and ZIKV infection, which recognize common epitopes between DENV and ZIKV mainly located in the NS5 protein, and which dominate the T-cell response against ZIKV.

3.4. Increased neutralizing antibody titer against ZIKV in donors with previous DENV infection

To determine whether the high T-cell response in DENV/ZIKV donors is also accompanied by a strong antibody response against ZIKV in these donors, we analyzed the ability of ZIKV-specific antibodies to bind and to neutralize ZIKV and DENV1-4. Plasmas samples from ZIKV donors, DENV/ZIKV donors as well as DENV-immune ZIKV-naïve (DENV) donors were used to analyze the ability of antibodies to bind to ZIKV EDIII (ZEDIII) and to neutralize ZIKV infection. Results revealed an increased level of antibodies that bind to ZEDIII in plasma samples from ZIKV-immune DENV-immune donors, in comparison with plasma samples from ZIKV-immune DENV-naïve donors (Figure 3A). On the contrary, plasma samples from DENV donors did not reveal any binding activity on ZEDIII.

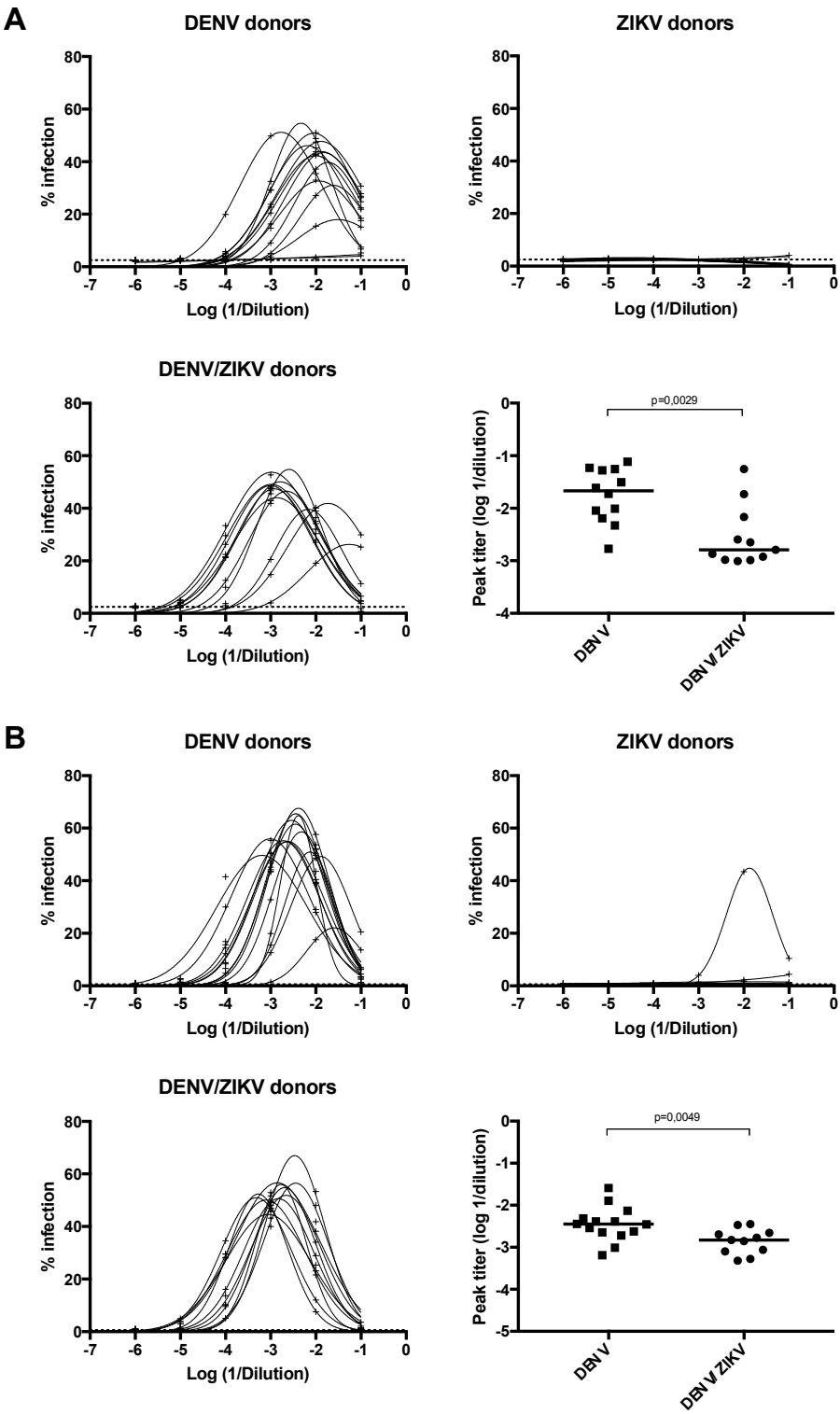


**Figure 3.** Analysis of ZEDIII binding and ZIKV- and DENV-neutralizing activity of plasma samples from DENV- and/or ZIKV-immune donors. (A) Detection of ZEDIII-binding antibodies by ELISA; (B) Quantification of neutralizing activity against ZIKV; (C) Quantification of neutralizing activity against DENV1, 2, 3 and 4. Each dot represents one donor and the bars represent the median value for each group of donors. The P values were calculated using the nonparametric two-tailed Mann-Whitney test. The dotted line represents the ELISA cut-off value.

Knowing that EDIII-specific antibodies are more efficient in neutralizing ZIKV infection [47], we then asked whether the higher binding capacity to ZEDIII is also accompanied by a stronger neutralization against ZIKV infection. Comparison between the neutralization activity of antibodies between ZIKV and DENV/ZIKV donors revealed a significantly higher neutralization potential against ZIKV in donors with a history of DENV infection (Figure 3B). Interestingly, while most DENV-immune ZIKV-naïve donors did not show any neutralization against ZIKV infection, 2 out of 14 DENV donors in this group could also neutralize ZIKV (Figure 3B), even though these 2 plasma samples were collected before the ZIKV outbreak, confirming the induction of ZIKV-cross-neutralizing and non-EDIII binding antibodies in DENV-immune donors [18, 20, 48]. To determine whether these neutralizing antibodies against ZIKV infection could also cross-neutralize DENV, we evaluated the ability of these antibodies to neutralize the 4 DENV serotypes. Comparison of the neutralizing activity of samples from DENV and DENV/ZIKV donors did not reveal any increase in the neutralization titer against the 4 DENV serotypes, showing that a secondary ZIKV infection in DENV-immune donors only increases the level of ZIKV-neutralizing antibodies without affecting the level of DENV-neutralizing antibodies (Figure 3C). In addition, no DENV-neutralizing activity was detected in ZIKV donors. These data confirm previous studies on the induction of ZIKV-specific neutralizing antibodies induced after ZIKV infection independently of prior DENV immunity [48].

### 3.5. Decreased enhancing potential on ZIKV infection in donors with previous DENV infection

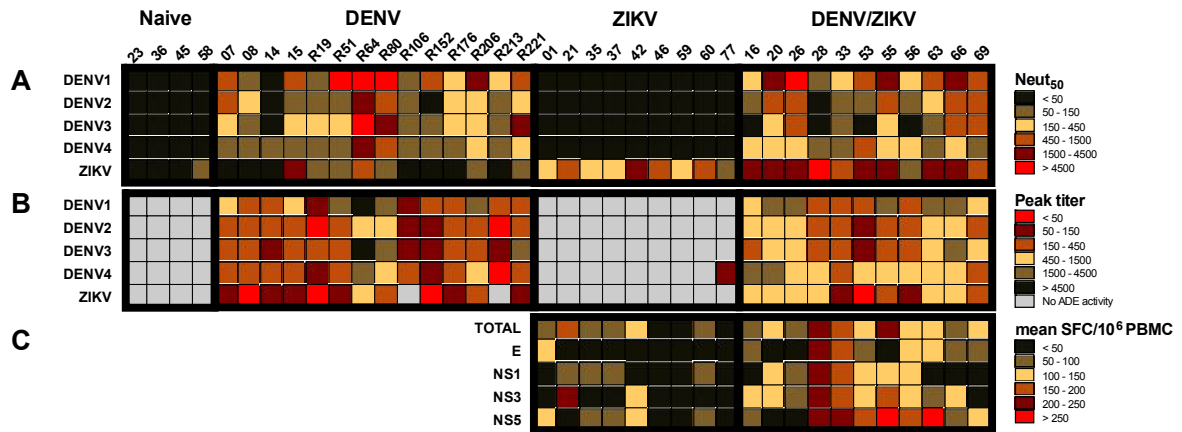
As DENV infection was previously shown to induce sub-neutralizing antibodies against DENV1-4 and ZIKV, which could mediate enhancement of DENV and ZIKV infection [17, 18, 20, 21, 49, 50], we measured the ADE activity of plasma samples from DENV donors, ZIKV donors and DENV-ZIKV donors on ZIKV infection and DENV1-4 infection *in vitro*. Results showed that plasma samples from DENV-immune ZIKV-naïve donors have an enhancing potential on ZIKV infection, whereas plasma samples from ZIKV-immune DENV-naïve donors do not have this ADE activity (Figure 4A). More strikingly, a significant decrease in the ADE activity for ZIKV infection was observed in DENV/ZIKV-immune samples, in comparison with ZIKV-immune samples (Figure 4A). These results clearly show that the ADE activity against ZIKV infection, induced after a primary DENV infection, decreases after a secondary infection with ZIKV. Interestingly, this decrease in the ADE activity was also observed, albeit to a lesser extent, against DENV4 infection, but not against DENV1, 2 and 3 (Figure 4B and data not shown). These results highlight the ability of a secondary ZIKV infection to reduce the enhancing activity against a DENV4 strain *in vitro* but not against the other DENV serotypes. Analyses of the ADE activity using a higher number of DENV and DENV/ZIKV donors should confirm these results. Interestingly, 1 out of 9 samples from ZIKV donors revealed a clear ADE activity on DENV4 infection but not on DENV1-3 infection (Figure 4B). These results show that ZIKV infection in DENV-naïve donors can induce antibodies with enhancing activity against DENV4 in some donors, thus confirming previous reports showing the ability of ZIKV-induced antibodies to enhance DENV infection *in vitro* [18]. They also show that the ADE activity against DENV4 is reduced following sequential DENV and ZIKV infection.



**Figure 4.** Analysis of ADE activity of plasma samples on ZIKV and DENV4 infection. (A) Detection of ADE activity on ZIKV infection from plasma samples of DENV (higher left panel), ZIKV (higher right panel) and DENV/ZIKV (lower left panel) donors. Comparison of the peak titer values of antibodies from DENV and DENV/ZIKV donors (lower right panel). Each dot represents one donor; (B) Detection of ADE activity on DENV4 infection from plasma samples of DENV (higher left panel), ZIKV (higher right panel) and DENV/ZIKV (lower left panel) donors. Infectivity curves for each donor are shown. The P values were calculated using the nonparametric two-tailed Mann-Whitney test. The dotted line represents the ELISA cut-off value.

3.6. High titer of neutralizing antibodies with low enhancing activity are associated with strong T-cell responses

Considering that DENV/ZIKV donors have a higher titer of neutralizing antibodies against ZIKV, in comparison with ZIKV donors, we wondered whether a significant correlation could be established between the level of neutralizing antibodies and the strength of the T cell response. Although no significant correlation could be drawn between these 2 different parameters of the immune response, the analysis of T- and B-cell responses in individual donors revealed a higher number of DENV/ZIKV donors having both a high neutralizing antibody response (and a low ADE activity) and a strong cross-reactive T-cell response against ZIKV than ZIKV donors (Figure 5).



**Figure 5.** Specificity and cross-reactivity of antibody and T-cell responses from donors with DENV and/or ZIKV infection. (A) Heat map of the neutralizing activity of plasma samples from DENV, ZIKV and DEN/ZIKV donors on DENV1-4 and ZIKV infection. (B) Heat map of the ADE activity of plasma samples from DENV, ZIKV and DENV/ZIKV donors on DENV1-4 and ZIKV infection; (C) Heat map of the mean T-cell response against peptides from the whole ZIKV proteome (TOTAL) or the immunodominant proteins of PBMCs from ZIKV and DENV/ZIKV donors; Each column represents one donor.

For instance, 6 out of 8 DENV/ZIKV donors with a Neut<sub>50</sub> value higher than 1500 revealed a moderate/low ADE activity (peak titer  $\geq 450$ ) on ZIKV infection and a moderate/high T-cell response (mean SFC/10<sup>6</sup> PBMC  $\geq 100$ ) against E, NS1, NS3 or NS5 (donors 16, 20, 28, 55, 63 and 66). On the contrary, donors with a moderate/low neutralizing activity against ZIKV (donors 33, 56 and 69, Neut<sub>50</sub>  $\leq 1500$ ) revealed a moderate/high ZIKV ADE activity (peak titer  $\leq 450$ ) and variable T cell responses against E, NS1, NS3 or NS5 proteins (Figure 5). Conversely, only 1 out of 9 ZIKV donors revealed a high neutralizing activity against ZIKV, with a moderate and a low T-cell activity against NS3 and NS5 and against E and NS1, respectively. With the exception of 1 donor (donor 21) showing a high T-cell activity against NS3 and a moderate/low level of neutralizing antibodies, all the other ZIKV donors exhibited a low T-cell activity and a moderate/low level of neutralizing antibodies. These results suggest that cross-reactive T cells, which are induced after a sequential DENV and ZIKV infection, and exhibiting a strong functional activity against specific ZIKV epitopes, could play a role in the induction of antibodies with high neutralizing potential against ZIKV.

4. Discussion

In this study, using PBMCs from ZIKV-infected human blood donors, we have identified numerous T-cell epitopes specific to ZIKV or shared between DENV and ZIKV. While the DENV-specific T-cell responses are predominantly directed against NS3, NS4B and NS5, the response against ZIKV mainly targets epitopes in the NS1, NS3 and NS5 proteins. The stronger and broader IFN- $\gamma$  response against peptides from the NS5 protein observed in donors previously



infected with DENV, led us to postulate that this region contains more peptides recognized by cross-reactive T cells, whereas the NS1 protein is preferentially targeted by ZIKV-specific T cells, which is consistent with the higher percentage of identity observed between ZIKV and DENV sequences in the NS5 protein, in comparison with the NS1 protein. Among the epitopes activating cross-reactive T cells, several peptides in the NS5 protein matched the T-cell epitopes recently identified from ZIKV-positive donors, such as the NS5<sup>298-306</sup>, NS5<sup>348-356</sup> and NS5<sup>461-475</sup> peptides recognized by CD8 and CD4 T cells, in the context of HLA-B\*3501 and HLA-DRB1\*-0701, respectively [22]. For several epitopes, the 15-mer or 9-mer peptides matched epitopes recently identified in transgenic mice expressing human HLA molecules, or in humans exposed to ZIKV, thus confirming the class I allele restriction for these peptides. This is the case for 15-mer peptide VARVSPFGGLKRLPA inducing a response in a donor expressing the HLA-B\*0702 allele (data not shown), which contains the C<sub>25-35</sub> peptide SPFGGLKRLPA shown to elicit a significant response in HLA-B\*0702 transgenic mice infected with ZIKV [23]. The same correlations have been established with NS3 (FPDSNSPIM), NS4B (RGSYLAGASLIYTVT) and NS5 (NQMSALEFYYSY) peptides that induced a strong response in human donors expressing the HLA-B\*0702 and HLA-A\*0101 alleles, respectively (data not shown and Table S2), and in transgenic mice expressing these alleles [23]. In other cases, the epitopes identified in HLA-B\*0702 and HLA-A\*0101 transgenic mice were also identified in responding donors that nevertheless do not express these alleles, such as the NS3<sup>219-233</sup> peptide (Table 1) and the NS1<sup>19-33</sup> or the NS5<sup>13-27</sup> peptides (Table 2), which elicit a response in donors that express neither of the two alleles, HLA-B\*0702 or HLA-A\*0101. For these donors, one possibility could be that the epitope identified in transgenic mice has a higher affinity for a human HLA allele different from the allele expressed by the transgenic mice, or that the 15-mer peptide contains another epitope that binds to a different allele. Binding studies with 9-mer epitopes and HLA class I stabilization assays using TAP-deficient cells should discriminate between these possibilities. We also report the identification of several peptides that share common sequences with DENV and are preferentially targeted by cross-reactive T cells, after DENV and ZIKV infection. Among these peptides, the NS5<sup>293-307</sup> and NS5<sup>297-311</sup> peptides contain the amino acid sequence HPYRTWAYH that shares seven amino acids with an epitope identified in DENV1-positive or in DENV-positive and ZIKV-positive donors [22, 46]. Similarly, the NS5<sup>325-339</sup> peptide contains the amino acid sequence KPWDVVTGV, which is 66.7% identical to the epitope KPWDVIPMV identified in individuals infected with DENV1 [46]. Finally, the strongest T cell responses in DENV/ZIKV donors were observed with the NS5<sup>481-495</sup> peptide or the NS5<sup>345-359</sup> and the NS5<sup>465-479</sup> peptides (Table 2), which contain 9-mer epitopes identified previously in DENV-infected individuals [44] or more recently in ZIKV-positive donors, respectively [22]. Altogether, these data reveal the activation of DENV/ZIKV cross-reactive T cells that dominate the response following sequential DENV and ZIKV infection. Notably, although these cross-reactive peptides exhibit a high degree of sequence identity with DENV and can stimulate a T-cell response after DENV infection, they do not induce a response after primary infection with ZIKV, suggesting that they are immunodominant in the context of DENV but not in the context of ZIKV infection. This result is expected, as the immunodominance of an epitope or its relative abundance depends on the other epitopes expressed by the protein. This is also in agreement with previous observations showing that epitope production correlates with cleavability of flanking residues expressed in the protein sequence [51]. Importantly, for these cross-reactive epitopes, the absence of a T-cell response in ZIKV-infected donors is not simply due to the absence of the presenting HLA allele in this population, as most of the alleles expressed in responding DENV/ZIKV donors were also expressed in ZIKV donors (Table S1). This is what we observed for the NS5<sup>13-27</sup>, NS5<sup>293-307</sup>, NS5<sup>345-359</sup> and NS5<sup>546-560</sup> epitopes, predicted to be strong binders to the HLA-B\*3501 and HLA-B\*4002 alleles, respectively, that were frequently expressed by our ZIKV donors (Table S2). Altogether, these results show that, in the case of initial ZIKV infection, there is a preferential recognition of ZIKV-specific epitopes, whereas there is a more frequent and stronger T-cell response against cross-reactive epitopes after sequential heterologous DENV/ZIKV infection. Interestingly, the strong T-cell response observed in DENV/ZIKV donors against these NS5 epitopes relies primarily on donors that express the HLA-B\*3501 allele, an allele associated with high

magnitude responses against DENV, and a stronger protection against DENV infection and disease [25]. As all blood samples were obtained from donors with asymptomatic ZIKV infection history, we cannot relate the strength of the ZIKV-specific T-cell response obtained in HLA-B\*3501 donors to the protection against the disease. Further studies with more subjects with a higher susceptibility to disease following primary ZIKV infection are required to determine whether, as for DENV, there is an HLA-linked protective role for T cells in ZIKV infection. Likewise, it would also be important to compare disease severity in donors having or not experienced a previous DENV infection, to determine whether cross-reactive T cells induced after DENV infection could mediate a better protection against ZIKV infection and disease, as recently suggested in mice [23, 31]. As both CD4+ and CD8+ T cells were shown to contribute to protection against DENV infection, a comprehensive analysis of MHC class II-restricted response is needed to determine the role of CD4 in ZIKV infection and disease protection. Finally, further phenotypic analyses of ZIKV-specific T cells, in asymptomatic or symptomatic donors will help in defining correlates of protection in natural immunity and vaccination against ZIKV infection and disease. It will be particularly important to determine whether, as for DENV-specific T cells, strong responses against ZIKV-specific peptides are more frequent for specific HLA alleles and are associated with multifunctionality [25].

From the same individuals, we have also shown that while a primary DENV infection induces cross-reactive antibodies with enhancing activity against the heterologous DENV serotypes and against ZIKV, a primary ZIKV infection in naïve donors mainly elicits ZIKV-specific antibodies with no enhancing activity against DENV. Strikingly, ZIKV infection in DENV-immune donors was shown to increase the level of neutralizing antibodies against ZIKV, in comparison with DENV-naïve donors, and to decrease the enhancing activity of antibodies against ZIKV, in comparison with donors infected only with DENV. Taken together, these results strongly support the activation and clonal expansion of memory B cells induced after sequential DENV and ZIKV infection, as shown recently in DENV1-infected individuals [52, 53]. These data also show that while a sequential DENV and ZIKV infection results in an increased neutralization potential and decreased enhancing activity against ZIKV, it does not affect the level of neutralizing or enhancing antibodies against DENV, suggesting that ZIKV infection in DENV-immune individuals should not have a significant impact on dengue disease following subsequent DENV infection. However, as a small fraction of individuals infected with ZIKV only were shown to express antibodies with enhancing activity against DENV4, it will be important in the future to analyze in these individuals the impact of ZIKV infection on the outcome of dengue disease following DENV4 infection.

Finally, in light of the stronger T cell response and the higher titer in neutralizing antibodies against ZIKV observed in individuals sequentially infected with DENV and ZIKV, it would be important to determine whether antigen-specific T follicular helper (Tfh) cells could be detected in these individuals, which could account for the activation of memory B cells, as shown recently in PBMC of DENV-infected patients [54]. In this sense, our study provides a framework for detailed analyses of cell populations potentially linked to the activation of memory B cells with high neutralization potential against DENV and ZIKV.

## 6. Patents

C.R., E.S.-L., A.S. F.G.D. are inventors of a patent filing related to this work.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/journal/viruses/special\\_issues/Dengue\\_Research\\_Network\\_Meeting](http://www.mdpi.com/journal/viruses/special_issues/Dengue_Research_Network_Meeting), Figure S1: Comparison of the neutralization activity against ZIKV and DENV1-4 infection between plasma samples from DENV, ZIKV or DENV/ZIKV donors, Table S1: Characteristics of the Zika virus patient cohort used for the epitope reactivity study, Table S2: Characteristics of antigenic peptides from ZIKV identified in this study.

**Author contributions:** Conceptualization: C.R., F.G.D. E.S.L. and A.S. Methodology, design and supervision: F.G.D. and C.R. Collection and genotyping of human blood samples: C. R-S. Data treatment, interpretation and statistical analysis: C.R. and F.G.D. Writing of the manuscript: C.R.

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**Conflicts of interest:** The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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