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Role of T and B cells in long lasting immunity against dengue and Zika virus infections

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Résumé

Le virus Zika est un virus émergent, appartenant à la famille des Flaviviridae, qui est transmis par le moustique de l'espèce *Aedes*. La forte séroprévalence de la dengue dans les régions où le virus Zika circule et la présence d'une immunité croisée entre ces deux virus, soulèvent des inquiétudes sur l'augmentation du risque des formes cliniques sévères pouvant apparaître chez les individus préalablement infectés par le virus de la dengue. Des études récentes ont notamment montré que la présence d'anticorps contre le virus de la dengue peut augmenter l'infection et la sévérité de la maladie induite par le virus Zika. Néanmoins, peu de données sont disponibles sur la capacité de ces anticorps préexistants à neutraliser et/ou potentialiser l'infection par le virus Zika chez ces individus. De plus, il n'existe à ce jour que très peu de données sur le rôle des cellules T dans le contrôle de l'infection par le virus Zika. En dépit de nombreux efforts pour prédire les épitopes potentiels pouvant se lier aux molécules d'histocompatibilité de classe I ou de classe II chez l'homme, l'identification précise de ces épitopes T, soit spécifiques du virus Zika, soit partagés par les virus le la dengue et Zika est encore incomplète. Pour déterminer le rôle de l'immunité préexistante contre la dengue dans l'infection par le virus Zika, notre objectif a consisté à analyser la spécificité et l'intensité des réponses des cellules T et B contre le virus Zika. En utilisant des cellules mononuclées du sang périphérique de donneurs ayant été infectés soit par les virus de la dengue et Zika, soit par le virus Zika seulement, nous avons identifié les épitopes du virus Zika en analysant les réponses cellulaires T en production d'interféron gamma (IFN-γ) contre des peptides chevauchants couvrant l'ensemble du virus Zika, par la technique dite de « Enzyme-linked Immunospot » (ELISpot). Nos résultats révèlent que les protéines non-structurales NS1, NS3 et NS5 contiennent la plupart des épitopes immunodominants capables d'induire une forte réponse des cellules T. De façon intéressante, chez les donneurs ayant préalablement été infectés par le virus de la dengue, certains peptides identifiés correspondent à des peptides dérivés du virus de la dengue, reconnus par les cellules T CD8+, et les plus fortes réponses ont été obtenues contre des séquences de peptides présentant une forte identité de séquences avec les quatre sérotypes du virus de la dengue. Ces résultats suggèrent fortement l'activation de cellules T cross-réactives chez ces donneurs. Dans cette étude, nous avons aussi quantifié par cytométrie de flux les activités neutralisantes et facilitantes des anticorps contre ces deux virus, chez ces mêmes individus. Nous avons pu montrer ainsi *in vitro* que les anticorps induits lors de l'infection par le virus de la dengue peuvent faciliter l'infection par le virus Zika, et que tous les échantillons provenant de l'infection par le virus Zika ne peuvent que neutraliser le virus Zika, à l'exception d'un échantillon provenant d'un donneur infecté par Zika, qui facilite l'infection par le virus de la dengue de sérotype 4. De plus, la plus forte activité neutralisante contre l'infection par le virus Zika a été observée chez les donneurs préalablement infectés par les deux virus de la dengue et Zika, ce qui suggère fortement l'induction d'anticorps cross-réactifs chez les individus infectés séquentiellement par ces deux virus. Ces résultats, qui présentent un intérêt majeur pour de futures stratégies vaccinales, devraient permettre en outre d'envisager l'analyse du rôle des sous-populations de cellules T spécifiques de chacun de ces virus dans l'induction des anticorps à large spectre neutralisant suite à des infections séquentielles par les flavivirus, pouvant moduler les formes sévères de la maladie.

Mots clefs : Virus Zika, virus de la dengue, épitopes T, cellules T cross-réactives, anticorps neutralisants, ADE, anticorps cross-réactifs.

Abstract

Zika virus (ZIKV) is an emerging virus of the Flaviviridae family, transmitted by *Aedes* species mosquitoes. The high level of dengue virus (DENV) seroprevalence in areas where ZIKV is circulating and the immunological cross reactivity between both viruses have raised concerns on the risk of increased disease severity for patients with a history of previous DENV infection. Indeed, recent studies have shown that anti-DENV pre-existing antibodies may enhance ZIKV infection and increase disease severity. However, little has been shown about the ability of these antibodies to neutralize or to facilitate ZIKV infection in the same context. In addition, little is known regarding the role of T cells in the control of ZIKV infection. Despite important efforts in predicting potential epitopes that could bind to different HLA class I or class II alleles, the precise identification of human T-cell epitopes that are either unique to ZIKV or shared with DENV is still lacking. Given these facts, to determine the role of DENV pre-immunity in ZIKV infection and disease outcome, the aim of this project was to analyze the specificity and strengths of T and B cell responses against ZIKV. Using PBMC from blood donors with previous history of DENV/ZIKV or ZIKV infection, we have identified ZIKV epitopes by screening T-cell responses against 15-mer overlapping peptides spanning the ZIKV proteome by IFN-gamma enzyme-linked immunospot (ELISPOT) analysis. Our results show that the non-structural proteins NS1, NS3 and NS5 contain most of the immunodominant peptides that induce a strong T-cell response. Interestingly, in donors with a history of DENV infection, specific peptides were also identified as DENV CD8+ T-cell epitopes and the strongest T-cell responses observed in these donors corresponded to sequences with a high level of amino acid identity with the four serotypes of DENV. These results strongly support the activation of cross-reactive T-cells in this context. We also developed a flow cytometry-based assay to quantify the neutralizing and enhancing activities of antibodies against DENV and ZIKV infections. Using plasma samples from the same donors, we have shown that DENV-induced antibodies can enhance ZIKV infection. In addition, all samples from ZIKV-infected donors exhibited neutralizing activity only against ZIKV, and one donor showed enhancing activity for DENV4 infection. The highest neutralizing activity against ZIKV infection was observed in samples from donors with previous DENV and ZIKV infection, strongly suggesting the induction of cross-reacting antibodies induced upon sequential DENV and ZIKV infection. These data have crucial implications for future ZIKV and DENV vaccines and provide new opportunities to study the role of subsets of DENV- or ZIKV-specific T cells in the induction of broadly neutralizing antibodies in the context of sequential flavivirus infections, which could modulate disease severity.

Keywords: Zika virus, dengue virus, T-cell epitopes, cross-reactive T cells, neutralizing antibodies, ADE, cross-reactive antibodies.

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Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is my own work and contains nothing which is the outcome of work done in collaboration with others, except as specified in the text and Acknowledgements.

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INTRODUCTION

ARBOVIRUSES: NOMENCLATURE AND DESCRIPTION

The term arbovirus, which results from a contraction of arthropod-borne virus, does not refer to a taxonomic classification per se, but to a large number of viruses that are transmitted to a susceptible vertebrate host by a hematophagous arthropod such as a mosquito. There are more than 500 viruses forming this group, of which approximately 150 members can cause diseases to the human being (1). First classified according to serological criteria (2, 3), they are now classified following a new molecular taxonomy (4, 5). The three most representative families in the arbovirus group are Bunyaviridae, Togaviridae and Flaviviridae. The genus *Flavivirus*, which is the most important genus in the Flaviviridae family, is composed of 53 virus species, placed in three clusters: mosquito-borne viruses, tick-borne viruses, and viruses with no known vectors (6-8). Within the *Flavivirus* genus, Zika virus (ZIKV) is a mosquito-borne virus that is phylogenetically related to other mosquito-borne flaviviruses medically important, such as Japanese encephalitis (JEV), West Nile (WNV), dengue (DENV) and yellow fever (YFV) viruses (9, 10). For ZIKV, two distinct transmission cycles have been identified (9): a sylvatic cycle which allows the maintenance of ZIKV between non-human primates and arboreal mosquitoes in forests, and an urban cycle involved in the transmission of ZIKV between humans and urban mosquitoes in towns. In some cases, however, the virus can be transmitted to humans by arboreal mosquitoes, when they are in close proximity. Concerning the vector, this virus was isolated from different species of mosquitoes of the genus *Aedes*, such as the *A. aegypti, A. africanus, A. albopictus*, and more rarely *A. hensilli* and *A. polynesiensis* (11, 12).

Flaviviruses, which are single positive-stranded RNA viruses, represent more than 70 species of virus, many of them being pathogenic for humans, inducing encephalitis, such as Japanese encephalitis, West Nile encephalitis, Murray Valley encephalitis, tick-borne encephalitis, Zika fever or dengue fever (13). Most infections are asymptomatic and are only detected by serology or using molecular tools. When symptoms occur, they start with an acute febrile

syndrome, which could be confused with a cold and usually resolves itself in a few days. When the virus induces a disease with clinical symptoms, four types of diseases can be identified depending on the infectious virus, the physiological and genetic features of the host and the environment: diseases of the central nervous system, mild febrile diseases, arthritis and hemorrhagic fever (1).

ZIKA VIRUS INFECTION

In its natural environment in the forests of Africa and Asia, the ZIKV circulates among mosquitoes of the genus *Aedes* and non-human primates (14). In Uganda, the virus was isolated for the first time in a monkey host (*Macaca mulata*), and in a mosquito vector (*Ae. Africanus*) (15). Subsequently, the virus was detected through viral isolation and serological studies in infected humans, mosquitoes and animals in Africa and Asia (16-19). Initially, the ZIKV was isolated from various species of mosquitoes collected in diverse ecosystems or natural landscapes, suggesting a wide viral circulation through mosquitos, even if there was no clear demonstration that these vectors were the main vectors of virus transmission in this region. This is only several years later, when the virus was isolated in Latin America, that the mosquito *Aedes aegypti*, was shown to be the main vector for urban transmission (20, 21).

Epidemiology

The epidemiology of Flaviviruses is directed by a complex interplay of climatic, entomologic, viral, and host factors that are not completely understood (22). ZIKV has been identified as a single serotype with 2 different lineages (African and Asian) having >95% amino acid identity. Initially isolated in the Zika forest in Uganda in 1947 (23), it caused an explosive outbreak for the first time in Yap Island, Federated States of Micronesia in 2007 (24). 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 (12, 25-28). Brazil was the most affected country of the Latin American region with approximately 440,000 to 1,300,000 ZIKV cases since March 2015 (29). Afterward, numerous outbreaks occurred in different American countries to reach more recently the USA (30). Phylogenetic analyses revealed that the ZIKV strains circulating in Latin America are homologous and constitute a new American clade within the Asian lineage (31).

During the recent outbreak, several alternative modes of virus transmission have been reported. For instance, the ZIKV can be transmitted by blood transfusion, as already shown in the case of dengue transmission through this pathway, with the identification of DENV viral RNA in platelets and red blood cells or other flaviviruses in the blood of human donors (32-34). This mode of transmission by transfusion in the case of Zika virus has been observed in French Polynesia during the 2013-2014 outbreak, where 2.8% of asymptomatic blood donors were tested positive for Zika virus RNA, and in Brazil with two possible cases of transfusion-transmitted Zika virus infections (35). To prevent this way of transmission, several actions have been undertaken by the World Health Organization, which include temporary deferral of blood donors in epidemic zones, donor self-reporting of symptoms after donation and pathogen inactivation of blood products, among other recommendations (36).

The transmission of Zika through sexual contact has been also evidenced (37). During the latest outbreak in the Americas, the ability for Zika to be transmitted sexually has attracted much more attention, and an increasing number of reports of sexual transmission have been documented (38-42). The reported cases range usually from individuals who have exhibited clinical symptoms, however, sexual transmission has also been shown from individuals that are asymptomatic at the time of sexual contact (43). The increasing number of cases of sexual transmission have led to a recommendation for abstinence or barrier protection during sexual contact for an extended period of time after infection with Zika virus.

Finally, the route of transmission that received the greatest attention, due to the high impact in safe motherhood and child health is vertical transmission during pregnancy. Even though the infection at any time point during pregnancy has been associated with adverse fetal outcomes, the first-trimester infection appears to be the highest risk for fetal injury (44, 45) when transmission across the developing placenta and into the amniotic or yolk sacs may occur (46). Despite the recent progress on the identification of different target organs and cells for ZIKV infection of the neuronal system, (47, 48), little is known regarding the mechanism of ZIKV vertical transmission.

Pathogenesis and disease

Both dengue and Zika viruses are transmitted by female mosquitoes of the genus *Aedes* (species *aegypti* and *albopictus*), currently distributed in all the tropical and subtropical countries around the world, allowing both virus and mosquito to circulate with less ecological restrictions. Flavivirus circulation between humans and mosquitoes occurs when the mosquito feeds on the blood of a viremic individual. Thus, the mosquito, by ingesting infected human blood, allows infection of the epithelial cells of its intestine; then, the viral particles produced in these cells are released into the hemocele and infect other mosquito´s organs such as the salivary glands, which become reservoir organs for the virus. The infection in the human occurs when an infected mosquito bites again to feed itself, releasing saliva and virus in a healthy individual (49). For Zika infection, the clinical signs typically include a combination of fever, headache, retro-orbital pain, conjunctivitis, a maculopapular rash, myalgia (muscle pain), and/or arthralgia (joint pain). Symptoms generally last 2-7 days, but the rash and arthralgias may last two weeks or longer (27, 50) and the virus can be detected in blood until 10 days after infection, or in other biological fluids (urine, semen, saliva) and organs (prostate) several weeks after the infection (51-53).

Colombia, the second Latin American country with the highest numbers of cases of Zika disease, is also endemic for dengue virus. In 2010, a dengue epidemic was described and 157,152 dengue cases were reported. 94% (147,670) of cases were classical dengue fever and the remaining 6% (9,482) were severe dengue, with a mortality of 2.28 % (217 fatal cases). During this time, the four DENV serotypes were identified with DENV1 and DENV2 being the most frequent viruses identified (54). In 2016, the national health authorities reported a total of 103,822 cases of dengue, of which 1,047 were severe dengue. Between August 9, 2015, to April 2, 2016 (during the Zika outbreak) a total of 65,726 cases of Zika virus disease (defined by the presence of clinical symptoms) were reported in Colombia, of which 2,485 (4%) were confirmed by means of reverse-transcriptase–polymerase-chain reaction (RT-PCR) assay. Among the total number of patients who were reported with symptomatic disease, only 4% were hospitalized at the time of report, including 938 of the 11,944 pregnant women (28).

Although initially believed to only cause mild, self-limiting disease, a causal relationship between ZIKV infection and neurological complications has been now established (55-58). The more serious effects of ZIKV infection in humans include Guillain–Barré syndrome (GBS) in adults and congenital malformations in fetuses. In a case–control study, among 42 patients diagnosed with GBS, 41 (98%) had anti-ZIKV immunoglobulin M (IgM) or immunoglobulin G (IgG), and all patients had ZIKV neutralizing antibodies. None of the patients died, but about 50% were still unable to walk without assistance at 3 months after discharge. No association was observed between the occurrence of GBS and a history of DENV infection in these patients, thus suggesting that a previous DENV infection probably did not provide cross-protection nor pose a risk of immune enhancement of ZIKV infection (56). In addition, vertical transmission associated with several sequelae, including microcephaly and other neurologic abnormalities, ocular abnormalities, fetal growth restriction, hydrops, and fetal loss has been also described (59-62).

The human placenta has several lines of defense against viral infections, ranging from a physical barrier of multinucleated syncytia to an innate and adaptive immune response. The association between Zika and fetal abnormalities has required rigorous studies and there is now evidence suggesting that, such as cytomegalovirus (CMV), ZIKV may have developed different ways to overcome the trophoblastic defense, with a unique predilection to attack the neural tissue of the fetus, causing abnormalities in neuro-development (63). The activation of the maternal immune system by infections, toxic and environmental factors can affect pregnancy and increase the risk of developing fetal problems (64).

Different studies have found that Zika virus would not affect the fetus at the end of pregnancy, due to the production of type III interferons (INF-λ) by the human trophoblast, which plays a protective role against infection by ZIKV (65, 66). Based on these observations, infection of the fetus must occur during the first trimester of pregnancy when the trophoblast is more permissive to ZIKV infection, due to the immaturity of the trophoblast villi. The reason why fetal neuronal cells are targeted by the virus may be related to the presence of receptors that allow the virus to enter the cell. Based on the knowledge of CMV infection, whose neuroinvasion is mediated by integrin and the EGFR (Endothelial Growth

Factor Receptor) in radial glial cells (67), it has been hypothesized that these same cells could selectively express proteins that promote the entry of ZIKV and infection during neurogenesis (68). As with DENV, it has been found that DC-SIGN (dendritic cell- specific intercellular adhesion molecule-3 grabbing non- integrin) receptors, TAM (Tyro3, Axl, and Mer) receptors and, to a lesser extent, TIM-1 (T cell immunoglobulin and mucin domain 1) receptors, could also mediate ZIKV viral entry (68).

Studies in mice have demonstrated an association between neurologic outcomes and ZIKV infection. Intracerebral inoculation of MP 1751 strain (isolated from *A. africanus*) of ZIKV in newborn and 5-week old Webster Swiss white mice resulted in enlargement of astroglial cells and destruction of pyriform cells of Ammon's horn (69). Other studies in mice have shown a correlation between neurologic disease signs in the form of hindlimb paralysis, and high levels of ZIKV RNA in brain and spinal cord tissue (69-71). When pregnant Swiss Jim Lambert (SJL) mice were infected intravenously with a Brazilian strain of the virus between days 10 and 13 of gestation, ZIKV RNA was detected in pup brain tissue. Though microcephaly could not be reproduced, cortical malformation with reduced cell numbers and thickness and morphology suggestive of cellular death, was reported (72).

STRUCTURE AND GENOME ORGANIZATION

As a member of the flavivirus genus, genome organization of ZIKV is similar to those of other members of this genus (73, 74). In general, flaviviruses are small (40-60 nm approximately), spherical particles containing an electron-dense core surrounded by a lipid envelope. Their genome consists of a single-stranded, positive sense RNA of approximately 11 Kb in length with a single open reading frame encoding 10 viral proteins (Figure 1A) that are cleaved co- and post-translationally from a polyprotein (75). Concerning the structural features of the viral particles, several conformational changes have been identified, which take place during life cycle in the cell, defining important features in the viral infectivity. Such structural changes have classified the viral particle in mature or immature viral forms, which are similar between flavivirus (76, 77). In this regard, the cryo-EM (cryo-electron microscopy) structure of the mature ZIKV particle revealed that the ZIKV structure is quite similar to the DENV structure (78, 79).

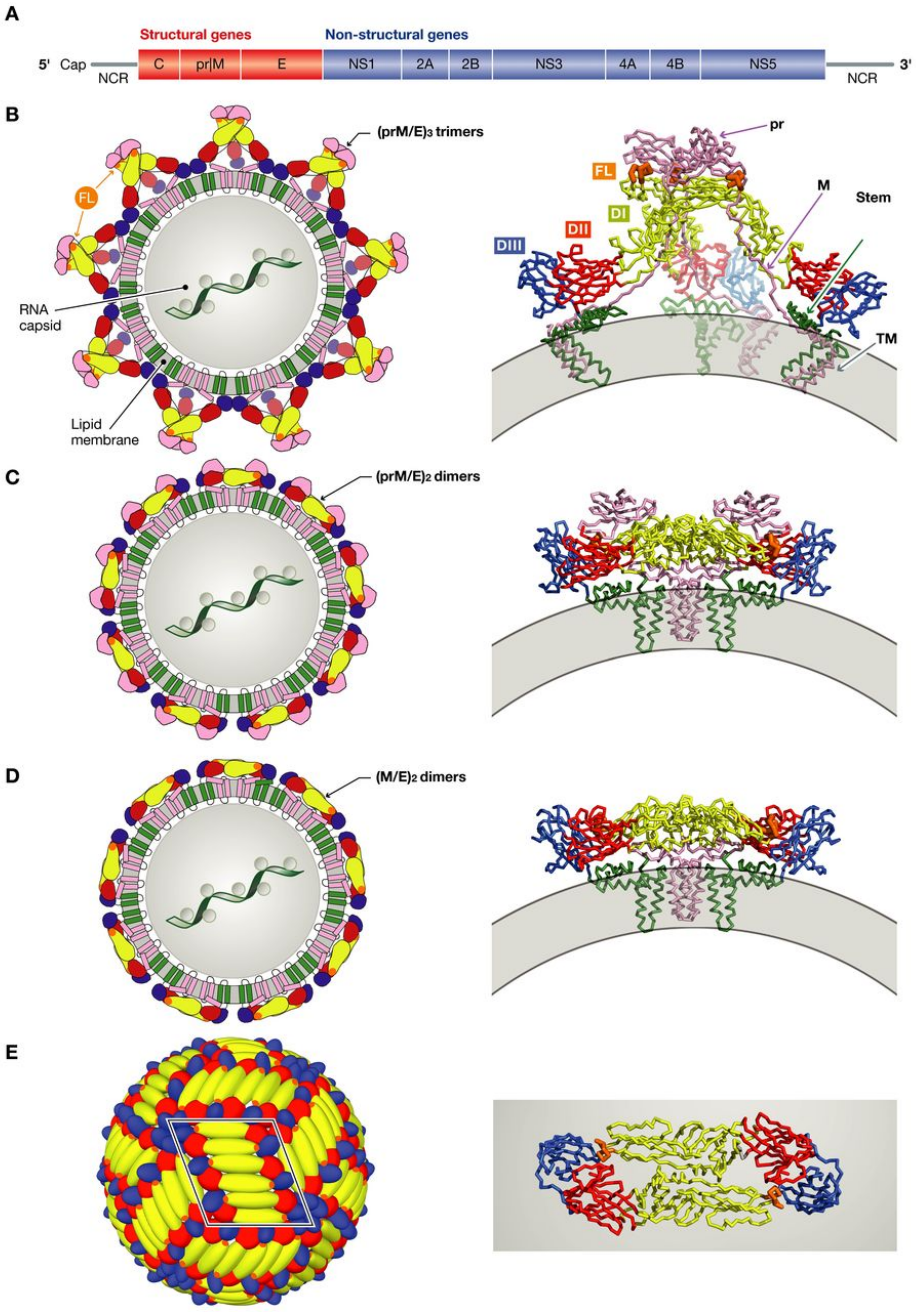

FIGURE 1 (modified from Rey et al., 2018) (80). Flavivirus particle assembly. (A) The flavivirus open-reading frame coding for a single precursor polyprotein. (B–E) Sketches representing the flavivirus particle at different maturation stages.

Structural proteins

The virion consists of three structural proteins, the capsid protein (C), the envelope glycoprotein (E) and the membrane protein (M). The C protein is relatively small compared

to other flavivirus proteins, containing about 114 amino acids. It is cleaved at the C-terminal hydrophobic sequence by the viral NS2B-NS3 protease, in a mature form containing approximately 100 residues (81, 82). An important feature of this structural protein is related to its physico-chemical properties, which are well conserved among flaviviruses despite the low amino acid homology between members of this genus (83). The main function of this protein during viral assembly is the conformation of the nucleoprotein core of the virion (Figure 1B left panel). This structure called nucleocapsid is formed by an array of C protein subunits complexed with the single-stranded RNA, packaging and protecting the viral genome (77).

The glycoprotein prM is the precursor of the M protein, and it is present only in the immature virion. Upon assembly of virions in the endoplasmic reticulum (ER), all virus particles are transported to the Golgi apparatus (84). Within the acidic environment of Golgi, the virion undergoes a surface conformational change where the trimeric spikes are dissociated and the 180 prM/E heterodimers re-associate into 90 prM/E dimers (Figure 1B and C). This change allows Furin, a calcium-dependent serine endoprotease, to cleave the prM protein into the M protein and a “pr” peptide forming the mature virion (Figure 1D and E) (85). This furin-mediated proteolytic transformation of prM has been described as an inefficient process, as viral particles maintaining uncleaved prM proteins at the viral surface can be detected after viral release and can thereafter reorganize again to conform E/prM heterodimers and remain as an immature virus in the extracellular milieu. This structural modification has a great influence in the virus ability to infect the target cell, as only mature virions can infect cells; however, some studies have shown that the complete cleavage of the prM protein is not a substantial prerequisite for viral infectivity (86, 87). The entire structural requirements for flavivirus infectivity in relation to the surface features of infectious virions are not well understood yet.

In addition, even though the M and E proteins are integral part of the lipid envelope of flavivirus, it has been suggested that the E protein plays a crucial role in the viral-host cell interaction during viral entry into the human cells (88). In this sense, the E protein is considered as the most important structural viral protein from a functional point of view.

Although it remains to identify the specific receptor(s) for Flaviviruses on target cells, several reports have identified ubiquitous cell surface molecules as potential candidate receptors expressed in various cell types (89). The flavivirus E proteins are class II viral fusion proteins which have three structurally distinct domains, and are distributed on the surface of the virus, forming prefusion head-to-tail homodimers (75). These domains are described as a central β-barrel domain I (DI), an elongated finger-like domain II (DII), and a C-terminal immunoglobulin-like domain III (DIII) (Figure 1B right panel). The domains II and III of each of the homodimer proteins are determinants for the interactions between the virus and the receptors of the susceptible cells. In addition, upon entry into target cells, the acidic endosomal environment triggers an irreversible conformational change in this protein inducing the formation of E protein trimers, which mediate membrane fusion events, allowing the viral genome to have access to the cellular machinery and to initiate virus replication (90). Importantly, the E glycoprotein is a strong antigen, which can trigger humoral immune responses, leading to either the production of neutralizing and cross-reactive antibodies or to sub-neutralizing antibodies with enhancing activity, facilitating virus infection (91, 92).

Non-structural proteins

This group of proteins, which are not part of the viral particle, includes the non-structural protein 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B and NS5. After virus replication, the NS1 glycoprotein is expressed at the cell membrane of infected cells as head-to-head dimers and it is also secreted into the extracellular milieu as hexamers (93). It has been shown that the intracellular NS1 along with other non-structural proteins and viral RNA are targeted towards ER, forming a replication complex (84), which plays an important role in viral replication. In addition, intracellular NS1 interacts with various host proteins to assist the viral translation and virion production (94-96); Strikingly, NS1 was also shown to induce the activation of TLRs, leading to endothelial permeability and vascular leak, but also to elicit a strong antibody response that could mediate protection against these deleterious effects (97-99). Importantly, the secreted NS1 protein in serum has been used as a diagnostic marker in flavivirus infections and an NS1 antigen-based ELISA has been used as a diagnostic

tool in JEV, WNV and DENV infections (100-102). For ZIKV diagnosis, different approaches are currently being evaluated (103, 104).

The NS3 protein is highly conserved among flaviviruses. This viral protease has also helicase and triphosphatase functions. NS3 is a multidomain protein, with an N-terminal domain with serine protease activity (NS3Pro), and a C-terminal portion containing the RNA triphosphatase (NS3RTPase) and RNA helicase (NS3Hel) activities involved in capping and viral RNA synthesis, respectively (105, 106). The activity of NS3Pro is strongly dependent on the association of a 40-mer region of NS2B acting as a cofactor, resulting in the formation of a heterodimeric complex. Additionally, this protein contains an important number of T-cell epitopes of importance in the protection and pathogenesis of flavivirus infections (107-109).

The NS5 protein is the largest and the most conserved protein among the flaviviruses. It consists of an N-terminal methyltransferase (NS5MTase) domain and an C-terminal RNA-dependent RNA polymerase (NS5RdRp) domain (110, 111). The NS5MTase activity is involved in the RNA capping process, transferring a methyl group from the cofactor S-adenosyl-l-methionine (AdoMet) onto the N7 atom of the cap guanine and onto the 2'OH group of the ribose moiety of the first RNA nucleotide (112). The NS5RdRp domain is responsible for the replication of the positive-strand RNA genome in an asymmetric and semi-conservative process, in which the antigenome is only present in a double-stranded RNA replication intermediate (113). As NS3, this protein contains an important number of T-cell epitopes (107-109). Additionally, ZIKV NS5 was identified as an antagonist of type I interferon signaling. It has been shown that the NS5 protein of ZIKV or DENV binds and degrades human STAT2 (Signal transducer and activator of transcription 2), but contrarily to DENV NS5, it does not use UBR4 (ubiquitin protein ligase E3 component n-recognin 4), following an unknown mechanism to degrade STAT2 (114).

Little is known about the functions of the NS2A, NS2B, NS4A and NS4B proteins. NS2A is a small protein (22 kDa), which was shown to promote viral assembly and replication *in vitro*. It appears that NS2A coordinates the usage of viral RNA produced in each cycle of replication as a new template to generate replicative intermediates or its association with the

nucleocapsid core during viral assembly (115). NS2B protein (14 kDa) has a hydrophobic region that anchors the NS2B/NS3 complex to the ER membrane and then, by proteolytic processing, a newly released hydrophilic domain of NS2B interacts with the protease domain of the NS3 protein to act as a cofactor (105). The data on the roles of NS4A and NS4B proteins on the viral life cycle are very limited, mainly due to their physico-chemical features related with a high hydrophobicity (116). These proteins seem to be involved in the host's immune system evasion and immune response, affecting several pathways mediating viral clearance (89, 117). In particular, DENV NS2A, NS4A, NS4B, NS2B3, and the noncoding subgenomic flavivirus RNA (sfRNA) have been shown to target distinct intermediates of the RIG-I (retinoic acid-inducible gene I)/MDA5 (melanoma differentiation-associated gene 5) signaling cascade responsible of inhibiting type I interferon (IFN) production (118, 119).

IMMUNE RESPONSE TO ZIKV INFECTION

While in dengue, it has been well established that a secondary heterotypic infection is the main risk factor for severe disease, through antibody-dependent enhancement (ADE) of virus infection, the exact role of cross-reactive T cells induced after heterotypic DENV infections is still a question of debate. In this scenario, termed "original antigenic sin" (120-122), there is an expansion of cross-reactive T cells with higher avidity for the previous infecting serotype that would mask the specific T cell response against secondary infection with a different serotype, resulting in less efficient elimination of DENV-infected cells. However, in the case of ZIKV, the exact mechanisms that drive severe outcomes of ZIKV infection remain largely unknown. In this respect, due to the strong amino acid sequence identity between several DENV and ZIKV proteins, and the co-circulation of these two viruses in the same endemic areas, many studies have focused on the potential impact of the immunological cross-reactivity between ZIKV and other flaviviruses on infection and disease outcome (123-125).

The Innate response

The initial events following ZIKV infection in humans are not well understood. However, concerning the early phases of viral infection and host immune responses, it was shown that human dermal fibroblasts, epidermal keratinocytes, and monocyte-derived dendritic cells

are targets for ZIKV (126, 127). The antiviral gene expression profile in infected primary human fibroblasts revealed an upregulation of transcripts coding for TLR3 (Toll-like receptor 3), RIG-I and MDA5 (127). Furthermore, several *in vitro* studies using both primary human cells and human-derived cell lines infected with ZIKV have highlighted the role of IFN response during ZIKV infection. The substantial production of type I (α, β), type II (γ), and type III (λ) IFNs as well as the activation of IFN-stimulated genes following ZIKV infection was clearly demonstrated on various cell types (65, 127, 128). In one recent study, Zika virus infection was shown to trigger the RIG-I and IRF3 (Interferon regulatory factor 3) pathways, resulting in type I IFN secretion, and MHC class I upregulation and inhibition of natural killer (NK) cell killing of infected cells (129). In addition to IFN secretion, other cytokines have been detected during human ZIKV infection. Indeed, human ZIKV infection was correlated with the induction of pro-inflammatory cytokines (IL-1β, IL-6, MIP1α), chemokines (IP-10 and RANTES) and cytokines that promote polyfunctional T cell responses (IL-2, IL-4, IL-9 and IL-17) (130). Finally, the IL-1β secretion in human PBMCs and macrophages from infected individuals, as well as from mouse bone marrow dendritic cells (BMDCs), was shown be induced by the NLRP3 inflammasome through a mechanism that depends on the synthesis of NS5 (131).

The B cell response

While ZIKV-infected individuals with no previous flavivirus exposure generally display ZIKV-specific serum antibodies, sera from subjects with prior flavivirus infection were shown to cross-react with other flaviviruses besides ZIKV (123, 132). This cross-reactivity has been shown to decrease over time after the initial infection. Indeed, while sera from patients in acute or early convalescent phase of dengue disease can potently neutralize ZIKV infection (124, 133), sera from patients in late convalescent phase after DENV infection, revealed a limited cross-reactivity to ZIKV and a decreased neutralizing potential (134, 135). Thus, while it is clear that DENV infections can induce the production of cross-reactive antibodies that recognize the four DENV serotypes, and to a lesser extent ZIKV, in most cases, they do not induce durable, high level ZIKV cross-neutralizing antibodies (Figure 2) (135).

In addition to the cross-neutralization activity of DENV-specific antibodies, anti-DENV pre-existing antibodies were shown also to enhance ZIKV infection and increase disease severity (Figure 2) (123, 124, 133, 136, 137). Depending of the recognition of a specific antigen, cross-reactive antibodies have been shown to have different effects on ZIKV or DENV infections *in vitro*. For instance, plasma samples immune to DENV and human monoclonal antibodies specific for DENV envelope, especially those binding to epitopes in the fusion loop epitope, were shown to cross-react with ZIKV and to induce enhancement of ZIKV infection *in vitro* (124, 133). The same ADE effect has been also observed with sub-neutralizing antibodies against the EDI/EDII domains of ZIKV E protein, these antibodies inducing enhancement of DENV infection. In both situations, the strong antigenic similarity between these viruses, with amino acid sequences differing by 30-35% between the different DENV serotypes and by 41-46% between DENV and ZIKV, provides an explanation for the induction of cross-reactive or sub-neutralizing antibodies (123).

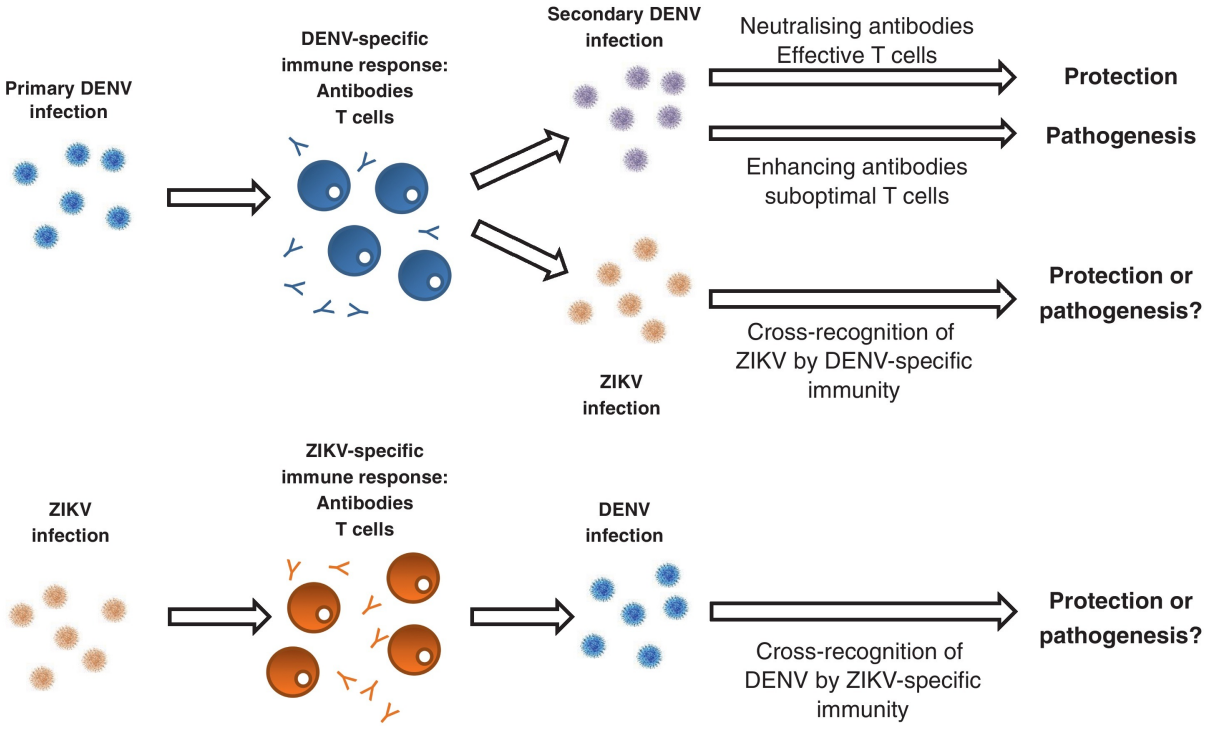

FIGURE 2 (modified from Culshaw et al., 2017) (138). Cross-reactive DENV/ZIKV-specific immune responses.

Contrarily to these EDI/EDII-specific and sub-neutralizing antibodies, monoclonal antibodies have been characterized that potently neutralize ZIKV infection *in vitro* and *in vivo*, and induce protection of susceptible mice from lethal ZIKV infection (123, 134, 139, 140). These neutralizing antibodies target epitopes located in the EDIII domain of ZIKV E protein and quaternary epitopes displayed on infectious virions (123, 140). Finally, different monoclonal antibodies derived from blood samples of DENV patients that target quaternary epitopes at the interface between two subunits of the E protein dimer were also shown to potently cross-neutralize ZIKV and to be protective against lethal infection in mice (134, 139). Altogether, these findings highlight the possibility of inducing broadly neutralizing antibodies against DENV and ZIKV, in future vaccine strategies against these closely related viruses.

The T cell response

Although the exact role of T cells during DENV infection and disease is still a matter of debate (Figure 2) (138), there is currently a growing body of evidence supporting a protective role for T cells in DENV infection, both in mice and in humans (141, 142). It has been demonstrated notably that CD8+ T cells can control viral infection through different mechanisms, including direct cytotoxicity of infected cells and production of proinflammatory cytokines such as IFNγ and TNFα (143). Similarly, CD4+ T cells were shown also to control viral infection through the production of cytokines with proinflammatory or antiviral activity and through the activation of antigen-specific B cell responses (144).

Concerning dengue virus infection, both CD4+ and CD8+ T cells contribute to the protection of individuals against infection. The first studies on dengue-specific CD4+ and CD8+ T cells responses highlighted the ability of these cells to proliferate, to produce IFNγ and induce the death of infected cells, in response to serotype-specific DENV antigens, suggesting that these cells are functional in individuals who have undergone primary dengue infections (145-147). More recently, DENV-specific CD4+ and CD8+ T cells were analyzed in a group of children who suffered after secondary infections with varying clinical outcomes. In this study, the frequencies of polyfunctional DENV-specific T cells were higher in children who developed subclinical secondary infections, in comparison with children who had symptomatic secondary infections (148). This report was the first to establish a significant correlation

between the cellular immune response and subclinical or non-apparent forms of disease. Additionally, studies using murine models have also shown that both CD4+ T cells and CD8+ T cells may contribute to protection against infection with dengue virus (149, 150). More recently, a higher activation of NK cells and T cells, with a higher proliferation and cytotoxic activity was detected in asymptomatic dengue viral infections, compared to individuals with symptoms during DENV infection (151).

Likewise, several HLA alleles were shown to be associated with a lower or weaker CD8+ T cell response and a higher risk of developing severe dengue disease, whereas an efficient T cell activation against DENV immunodominant epitopes was shown to be associated with specific HLA alleles and a better protection against severe disease (108). Finally, in addition to their protective role against severe dengue disease, T cells were also shown to prevent the ADE induced by sub-neutralizing antibodies (152, 153). Altogether, these arguments strongly argue for a beneficial role of T cells in inducing an immune protection against severe dengue disease.

AIM OF THE THESIS

As noted above, DENV and ZIKV are two flavivirus antigenically related to various degrees, and immunological cross-reactions have been identified. Recent evidence has shown that pre-existing antibodies from a prior DENV infection may enhance ZIKV infection and may increase disease severity. In addition, a high level of DENV seroprevalence has been observed in areas where ZIKV is circulating and more clinical cases of disease severity, including neurological complications, have been also reported during the most recent ZIKV outbreaks across the Americas.

Given this situation, and knowing the very limited number of reports on the role of ZIKV-specific T cells in ZIKV infection and disease, a new research opportunity has emerged and the study of the impact of previous DENV infection on the specificity and magnitude of T and B cell responses against ZIKV is therefore urgently needed. The objective of this study was to analyze the humoral and cellular immune responses against ZIKV. Knowing that we have

selected human blood donors from endemic areas where DENV and ZIKV are circulating, this study offered a good opportunity to analyze in the same donors both the T cell and the B cell responses against these closely related viruses, in order to determine the impact of previous DENV infection on the strength of T and B cell responses against ZIKV.

In this context, the first goal was to identify the immunodominant regions and epitopes of ZIKV polyprotein recognized by T cells, that can be used in future research to explore the protective or pathological role of T cell responses; the second goal was to quantify in the same human samples, the neutralizing and enhancing activities of antibodies induced after ZIKV infection in a context of previous DENV infection. This overall approach should provide new opportunities to study the respective role of different subsets of DENV- or ZIKV-specific T cells in the induction of broadly neutralizing antibodies following sequential flavivirus infections, and should help to define correlates of protection in natural immunity and vaccination.

MATERIALS AND METHODS

Human blood samples

A total of 92 blood samples were obtained from different DENV- and ZIKV-endemic areas of two departments of Colombia (Cundinamarca and Meta). Eleven of the 92 samples had to be excluded from the study due to poor cell viability. The characteristics of all samples used are shown in the Table 1. The first 71 samples listed were collected over a time course of three months between September and November 2016 from healthy adult blood bank donors from the Fundación Hematológica Colombia (Bogotá D.C., Colombia) in an anonymous manner. The last 10 samples (identified with the letter R before identification number) were obtained in February 2014, before ZIKV was introduced in Colombia, from individuals who were enrolled in a cohort study from Anapoima and Apulo, two Cundinamarca's municipalities. From these 81 donors, 38 plasma samples were analyzed for their neutralizing or enhancing activity against DENV and ZIKV infection, which include 20 blood samples tested for T cell reactivity against ZIKV-derived peptides (Table 1). All protocols were approved by the institutional review board of the EL Bosque University, Colombia (IRB-00010617; FWA-00024353). PBMCs were purified by density gradient centrifugation (Lymphoprep™; STEMCELL technologies) and resuspended in FBS (Gibco) containing 10% dimethyl sulfoxide and cryopreserved in liquid nitrogen. Plasma samples were heat-inactivated at 56°C for 30 minutes and stored at -80°C thereafter.

DENV and ZIKV serology

ZIKV seropositivity was determined using a recombinant antigen-based (EDIII antigen) indirect ELISA, as previously described (154). Briefly, 96-well plates (Nunc, Life Technologies, Rochester, NY) were coated overnight at 4°C with 50 ng of EDIII-antigen in PBS. After three washes, the wells were saturated with 200µl PBS containing 3% skimmed milk and 0.1% Tween-20 for 1hr at 37ºC. 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 37ºC for 60 min. After three washes, bound antibodies were detected using horseradish peroxidase-

conjugated goat anti-human IgG immunoglobulin (ROCKLAND). Following incubation at 37ºC 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. Anti-DENV-EDIII antibodies were also quantified by ELISA using 96-well plates coated with recombinant EDIII proteins from DENV1, DENV2, DENV3 or DENV4 as previously described (155), with some modifications: bound antibodies were detected using horseradish peroxidase-conjugated goat anti-human IgG immunoglobulin (ROCKLAND) and plasma samples from DENV- and ZIKV-seronegative donors were used as negative controls. A total of 9 samples from ZIKV-seropositive individuals and 11 samples from DENV/ZIKV-seropositive individuals were selected for RT-PCR assay, HLA typing and ELISPOT analysis (Table 1 and Table 2).

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 ZIKV and/or DENV using a specific nested-PCR assay, as previously described (156). Detection of ZIKV was confirmed in 3 samples from the 9 ZIKV-seropositive individuals and in 6 samples from the 11 DENV/ZIKV-seropositive individuals, whereas DENV was detected in 2 samples from the 11 DENV/ZIKV-seropositive individuals (Table 2).

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 (alleles A, B and C) and HLA class II (allele DRB1) was used according to the manufacturer's protocol (Sequence-Specific Oligonucleotides (SSO) typing; INMUCOR, Lifecodes). The full list of the 20 HLA-typed blood donors is listed in Table 2.

Viruses

For the identification of ZIKV T cell epitopes, the identical amino acid sequence of 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 identified from GenBank and used for pairwise sequence identity comparisons. The *in vitro* assays were conducted using the DENV1 KDH0026A (provided by Dr L. Lambrecht, Institut Pasteur, Paris), DENV2 R0712259 (provided by Dr. A. Failloux, Institut Pasteur, Paris), DENV3 KDH0010A (provided by Dr. L. Lambrecht, Institut Pasteur, Paris), DENV4 CRBIP10.4VIMFH4 (from the Institut Pasteur Collection) and ZIKV KU312312 (provided by Dr. Dominique Rousset, Institut Pasteur, Cayenne). All viruses were grown using the *Aedes Albopictus* mosquito cell line C6/36.

Cell lines

U937 cells expressing dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN; U937-DC-SIGN) and K562 cells, were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Vero cells (clone E6) were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO) supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. C6/36 cells were cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids and 1X tryptose phosphate broth. Cell cultures were incubated in a humidified atmosphere of 5% CO2 at 37°C (for U937-DC-SIGN, K562 and Vero) or 28°C (for C6/36). Vero-E6 cells and DC-SIGN-expressing U937 were kindly provided by Dr M. Flamand and Dr B. Jacquelin (Institut Pasteur, Paris), respectively. The K562 cell line was kindly provided by F. Porrot (Institut Pasteur, Paris).

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 8 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.

Ex vivo IFN-γ ELISPOT assay

PBMCs ($2x10^5$) were incubated in 96-well flat bottom plates (MSIPS 4510, Millipore, Bedford, MA) coated with anti-IFN-γ mAb (clone 1-D1K, Mabtech, Sweden) with 0.2ml of complete RPMI containing 10% human AB serum with pools of 12 peptides (2μg/ml, final concentration) or individual peptides (1μg/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-γ mAb (clone 7-B6-1, Mabtech) for 1h 30mn. The spots were revealed 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-γ-producing cells was expressed as spot forming cells (SFC) relative to 1 x 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).

Immunogenicity and HLA restrictions prediction

The evaluation of the binding potential of peptides to MHC class I and class II alleles was analyzed using the NetMHCpan3.0 and NetMHCIIpan3.1 servers, respectively (157, 158).

Titration of viral infectivity

For the neutralization and antibody-dependent enhancement (ADE) assays, DENV1-4 and ZIKV were titrated on Vero cells and on U937-DC-SIGN and K562 cells, as previously

described (92, 159). Briefly, 2-fold serial dilutions of virus stock solutions (from 1:2 to 1:2048) were incubated with an equivalent volume of media at 37°C for 1h before mixing with $5x10^4$ cells (Vero, U937-DC-SIGN or K562). After incubation of the virus–cell mixture at 37°C for 2h, the medium was replaced with fresh medium and cells were incubated at 37°C for 24h. The cells were then fixed/permeabilized and stained with Alexa Fluor 488-coupled anti-Flavivirus E protein (4G2) antibody to detect infected cells by flow cytometry (MACSQuant Analyzer 10). The percentage of infected cells was measured using the FlowJo X software. The infectivity plots for each virus (in each different cell line) were used to define the dilutions inducing 7-15% infection and 0.5-2% infection, in the neutralization and the antibody-dependent enhancement (ADE) assays, respectively.

Virus neutralization and antibody-dependent enhancement (ADE) assay

The quantification of neutralizing and enhancing activities of antibodies against DENV and ZIKV infections was determined using a flow cytometry-based assay, as already described (92, 159). Briefly, 10-fold serial dilutions of heat-inactivated plasma samples were incubated at 37°C 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% of infection (ADE assay) as defined in the titration assay. One hour later, the virus-antibody mixture was incubated with $5x10^4$ cells (Vero or U937-DC-SIGN for neutralization assay and K562 for ADE assay) at 37°C for 2h and the media from the wells were replaced with fresh media. Twenty four hours later, the cells were fixed/permeabilized and stained with Alexa Fluor 488-coupled anti-Flavivirus E protein (4G2) antibody to detect infected cells by flow cytometry (MACSQuant Analyzer 10). The percentage of infected cells was defined using the FlowJo X software. The neutralizing antibody titer was expressed as the sample dilution giving 50% reduction of infection ($Neut_{50}$), as previously described (159). For the ADE assay, two different parameters were measured (160): the peak titer (or the sample dilution at which the percentage of infection is maximal) and the percentage of infection in the peak (or the maximum percentage of infection obtained in the assay).

Flow cytometry

For intracellular detection of DENV and ZIKV, the cells were washed with PBS then fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) for 20 min at 4°C. The cells were then washed once with Perm/Wash 1X (Pharmingen) and incubated with Alexa Fluor 488-coupled anti-Flavivirus E protein (4G2) antibody (diluted 1/800) for 30 min at 4°C and finally washed twice with Perm/Wash 1X and suspended in 150 µl of PBS. The analysis of stained cells was performed using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec). 10,000 gated events were acquired and the percentage of infected cells was defined using the FlowJo X software.

Statistics

All data were analysed 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. Correlations between the neutralizing and ADE results were calculated using the non-parametric Spearman correlation test. Differences were considered significant at $p < 0.05$.

RESULTS

In order to gain a better understanding of the role of DENV pre-immunity in ZIKV infection, we first analyzed the specificity and strength of T and B cell responses against ZIKV in blood samples obtained of individuals from different DENV- and ZIKV-endemic areas in Colombia. Samples from all study participants were tested for the presence of Zika virus IgG and dengue virus IgM and IgG and 20 samples from ZIKV-seropositive individuals were selected for the analysis of ZIKV-specific T cell responses (Table 1). In addition to these 20 samples, 14 samples from ZIKV-naive DENV-immune donors and 4 samples from DENV/ZIKV-naive donors were further selected for the evaluation of the neutralizing/enhancing activity of antibodies on DENV and ZIKV infection (Table 1). The results obtained are presented in two different chapters: In the first chapter, we report on the analysis of T cell responses against ZIKV to determine the most immunogenic regions of the ZIKV polyprotein with potential ZIKV-specific or ZIKV/DENV-shared epitopes; In the second chapter, we study the antibody response against ZIKV and evaluate the potential cross-reactivity of these antibodies against ZIKV and the four DENV serotypes in the context of a DENV/ZIKV sequential infection.

Table 1. Characteristics of the donor cohort

Donor[a]	Age (yr)	Gender	Origin	Serological test			Immune response analysis	
				DENV		ZIKV	T-cell	Antibodies
				IgM	IgG	IgG		
01	41	Male	Cundinamarca	-	-	+	X	X
02	37	Male	Cundinamarca	-	-	-		
03	19	Male	Meta	-	+	-		
06	21	Female	Cundinamarca	-	-	-		
07	23	Male	Meta	-	+	-		X
08	41	Female	Meta	+	+	-		X
09	20	Female	Cundinamarca	-	+	-		
10	33	Female	Meta	+	+	-		
12	30	Male	Cundinamarca	-	+	-		
14	41	Male	Meta	-	+	-		X
15	26	Female	Cundinamarca	-	+	-		X
16	20	Female	Meta	-	+	+	X	X
17	56	Male	Meta	+	+	+		
18	22	Male	Cundinamarca	-	+	+		
20	28	Female	Meta	+	+	+	X	X
21	26	Male	Cundinamarca	-	-	+	X	X
22	36	Male	Cundinamarca	+	+	-		
23	38	Female	Cundinamarca	-	-	-		X

Continued

Table 1. Characteristics of the donor cohort – cont'd

Donor[a]	Age (yr)	Gender	Origin	Serological test			Immune response analysis	
				DENV		ZIKV	T-cell	Antibodies
				IgM	IgG	IgG		
24	18	Female	Cundinamarca	-	-	-		
25	20	Male	Meta	-	+	-		
26	29	Male	Meta	-	+	+	X	X
28	35	Female	Meta	-	+	+	X	X
29	25	Male	Cundinamarca	-	+	-		
30	22	Male	Meta	+	+	-		
33	32	Female	Cundinamarca	-	+	+	X	X
34	19	Female	Cundinamarca	-	-	-		
35	39	Female	Cundinamarca	-	-	+	X	X
36	55	Male	Cundinamarca	-	-	-		X
37	34	Male	Cundinamarca	-	-	+	X	X
38	22	Male	Cundinamarca	-	-	-		
39	34	Male	Cundinamarca	+	-	-		
40	36	Male	Cundinamarca	-	-	-		
41	22	Male	Cundinamarca	-	+	-		
42	40	Male	Cundinamarca	-	-	+	X	X
43	21	Male	Cundinamarca	-	-	-		
44	19	Male	Cundinamarca	-	-	-		
45	24	Male	Cundinamarca	-	-	-		X
46	25	Male	Cundinamarca	-	-	+	X	X
47	28	Male	Cundinamarca	-	-	-		
48	23	Female	Cundinamarca	-	-	-		
49	21	Male	Meta	-	+	+		
50	28	Male	Meta	-	+	-		
52	42	Male	Meta	-	+	-		
53	54	Female	Meta	-	+	+	X	X
54	21	Male	Meta	-	+	-		
55	23	Male	Meta	-	+	+	X	X
56	28	Female	Meta	-	+	+	X	X
57	33	Female	Meta	-	+	-		
58	57	Female	Meta	-	-	-		X
59	26	Male	Meta	-	-	+	X	X
60	20	Female	Meta	-	-	+	X	X
61	18	Female	Meta	-	+	-		
62	31	Female	Meta	-	+	-		
63	24	Female	Meta	-	+	+	X	X
64	21	Male	Meta	-	+	-		
65	28	Male	Meta	-	+	-		
66	21	Female	Meta	-	+	+	X	X
67	35	Male	Meta	-	+	-		
68	26	Female	Meta	-	+	-		
69	25	Male	Meta	+	+	+	X	X
70	32	Male	Meta	+	+	-		
71	26	Male	Meta	-	+	-		
72	27	Male	Meta	-	+	+		
73	22	Female	Meta	-	+	-		
74	26	Female	Meta	-	+	+		
75	32	Male	Meta	+	+	-		
76	25	Male	Meta	-	+	-		

Continued

Table 1. Characteristics of the donor cohort – cont'd

Donor[a]	Age (yr)	Gender	Origin	Serological test			Immune response analysis	
				DENV		ZIKV	T-cell	Antibodies
				IgM	IgG	IgG		
77	18	Female	Meta	-	-	+	X	X
78	28	Female	Meta	-	+	+		
79	19	Female	Meta	-	+	+		
80	29	Female	Meta	-	+	+		
R19	10	Female	Cundinamarca	-	+	-		X
R51	13	Female	Cundinamarca	-	+	-		X
R64	13	Male	Cundinamarca	-	+	-		X
R80	14	Female	Cundinamarca	-	+	-		X
R106	8	Male	Cundinamarca	-	+	-		X
R152	8	Male	Cundinamarca	-	+	-		X
R176	10	Female	Cundinamarca	-	+	-		X
R206	9	Female	Cundinamarca	-	+	-		X
R213	9	Female	Cundinamarca	-	+	-		X
R221	12	Female	Cundinamarca	-	+	-		X

[a] The shaded rows show donors selected for immune response analysis

CHAPTER 1. ANALYSIS OF T CELL RESPONSES AGAINST ZIKV

Characteristics of blood sample donors

Among ZIKV-seropositive individuals selected in this study, 45% were male and 55% female with an average age of 29 years, between 18 and 54 years of age. All samples were collected between September and November 2016, one year after the beginning of Zika virus outbreak in Colombia in October 2015. All donors were asymptomatic for ZIKV disease. For 2 out of 11 DENV/ZIKV-seropositive subjects, serological evidence of a recent secondary DENV infection before blood sampling was detected (positive for IgM and IgG test; donors 20 and 69, Table 1). As with the other donors, these two individuals remained asymptomatic one week before and after blood drawn. The three DENV-seropositive donors 16, 26 and 33 (3 of out 11 DENV/ZIKV-seropositive donors) were the only donors who exhibited mild symptoms of dengue disease 1.5, 2 and 25 years ago, respectively. The 20 samples from ZIKV-seropositive donors were further HLA-typed and analyzed for the presence of DENV- and ZIKV-specific neutralizing antibodies and viral RNA in their plasma (Table 2).

Table 2 | Characteristics of the Zika virus patient cohort used for the epitope reactivity study

Donor[a]	HLA Genotyping				Serological test			Neutralizing activity (Neut50)[c]					PCR	
					DENV		ZIKV							
	HLA-A	HLA-B	HLA-C	DRB1	IgM	IgG	IgG	DENV1	DENV2	DENV3	DENV4	ZIKV	DENV	ZIKV
1	02:01:01	35:43:01	01:02:01	04:07:01	-	-	+	17	23	16	25	**311**	-	-
	24:02:01	51:01:01	01:02:01	12:01:01										
16	01:01:01	15:17:01	07:01:01	13:01:01	-	+	+	158	79	47	**357**	**2270**	-	+
	03:01:01	38:01:01	12:03:01	13:02:01										
20	01:01:01	07:02:01	07:02:01	04:11:01	+	+	+	**2049**	**452**	**214**	**438**	**2470**	+	+
	31:01:02	39:05:01	07:02:01	15:01:01										
21	31[b]	35:01	01[b]	04:01:01	-	-	+	13	13	19	14	**566**	-	+
	03:01	18:01	17:02	03:01										
26	02:01:01	07:02:01	01:02:01	14:02:01	-	+	+	**5397**	**601**	**735**	**235**	**2476**	-	+
	24:02:01	48:01:01	07:02:57	15:01:01										
28	02:17:01	40:02:01	03:05	04:11:01	-	+	+	**75**	39	24	**67**	**5587**	-	-
	29:02:01	44:03:01	16:01:01	07:01:01										
33	24:02:01	15:46	01:02:01	04:07:01	-	+	+	**306**	114	63	51	**829**	-	-
	24:02:01	35:31	03:05	04:07:01										
35	24:02:01	14:01:01	03:05	01:03	-	-	+	11	13	10	<10	**340**	-	-
	68:01:02	40:02:01	05 :129	08:02:01										
37	02:45	35:01:01	04:01:01	01:03	-	-	+	18	12	35	12	**280**	-	-
	11:01:01	50:01:01	06:02:01	13:01:01										
42	26:01:01	35:01:01	04:01:01	04:02:01	-	-	+	11	12	25	12	**1689**	-	-
	26:01:01	38:01:01	06:76:02	11:04:01										
46	32:01:01	39:01:01	06:02:01	04:07:01	-	-	+	14	11	12	11	**903**	-	-
	68:01:02	50:01:01	07:02:01	07:01:01										
53	23:01:01	40:02:01	01:10	07:01:01	-	+	+	**464**	116	29	**543**	**3081**	-	+
	31:01:02	44:03:01	04:01:01	08:02:01										
55	03:01:01	35:01:01	04:11:01	01:01:01	-	+	+	**2395**	**612**	**222**	**301**	**3196**	-	-
	11:01:01	51:01:01	15:02:01	07:01:01										

Continued

Table 2 | Characteristics of the Zika virus patient cohort used for the epitope reactivity study – cont'd

Donor[a]	HLA Genotyping				Serological test			Neutralizing activity (Neut50)[c]					PCR	
					DENV		ZIKV							
	HLA-A	HLA-B	HLA-C	DRB1	IgM	IgG	IgG	DENV1	DENV2	DENV3	DENV4	ZIKV	DENV	ZIKV
56	02:01:01	15:17:01	05:01:01	03:01:01	-	+	+	**215**	55	<10	156	**110**	-	+
	02:01:01	18:01:01	07:01:01	11:01:01										
59	03:01:01	35:43:01	01:02:01	04:01:01	-	-	+	31	30	16	24	**194**	-	+
	24:02:01	40:01:01	03:04:01	04:07:01										
60	02:05:01	55:01:01	01:02:01	11:01:01	-	-	+	11	<10	10	10	**514**	-	+
	69:01	58:01:01	07:01:01	13:03:01										
63	02:01:01	07:02:01	07:02:01	15:01:01	-	+	+	**999**	187	126	144	**4057**	-	-
	23:01:01	51:08:01	17:02:01	15:03:01										
66	02:01:01	39:01:01	03:02:01	08:02:01	-	+	+	**2572**	**471**	**1386**	167	**2905**	-	-
	03:01:01	40:02:01	07:29:01	15:01:01										
69	01:01:01	35:01:01	01:02:01	04:07:01	+	+	+	**749**	**463**	**961**	92	**1205**	+	+
	24:02:01	35:43:01	04:01:01	13:05:01										
77	02:01:01	40:02:01	04:01:01	13:01:01	-	-	+	17	12	15	33	**110**	-	-
	02:01:01	51:01:01	07:01:01	14:02:01										

[a] The shaded rows show donors with previous DENV infection
[b] Allelic variant was not determined
[c] The values in each cell are the 50% neutralization titers determined from two replicates of one experiment. The highest titers for each sample is indicated in boldface

As shown in Table 2, all ZIKV-immune plasma samples revealed the presence of ZIKV-specific neutralizing antibodies whereas DENV-specific neutralizing antibodies were detected only in DENV-seropositive donors (more detailed information is provided in chapter 2). In addition, recent ZIKV infection was confirmed by PCR in 3 out of 9 plasma samples from ZIKV-seropositive individuals, and in 6 out of 11 plasma samples from DENV/ZIKV-seropositive individuals, whereas recent DENV infection was confirmed by PCR in 2 out of 11 plasma samples from DENV/ZIKV-seropositive individuals (Table 2). The HLA typing for these donors revealed that the most frequent alleles were the HLA-A*02 (27.5%) and -A*24 (17.5%) for the HLA-A locus; the HLA-B*35 (22.5%) and -B*40 (15%) for the HLA-B locus; the HLA-C*07 (25%) and -C*01 (22.5%) for the HLA-C locus; and the HLA-DRB1*04 (27.5%) and DRB1*13 (15%) for the HLA-DRB1 locus (Table 2), which corresponds to the allele frequency expected in this country (161).

Identification of immunodominant regions of the ZIKV proteome

Following the recent identification of ZIKV epitopes targeted by CD4 and CD8 T cells (162), to examine further the immune response induced after ZIKV infection, we analyzed for each of these donors the T cell response against ZIKV using gamma interferon (IFN-γ)-specific enzyme-linked immunosorbent spot (ELISPOT) assay. PBMCs from 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 69% of the total response (Figure 1A).

Strikingly, these NS1, NS3 and NS5 proteins represent 15%, 19% and 35% of the total response, respectively, in ZIKV donors, 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 (108, 121, 163, 164). 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.

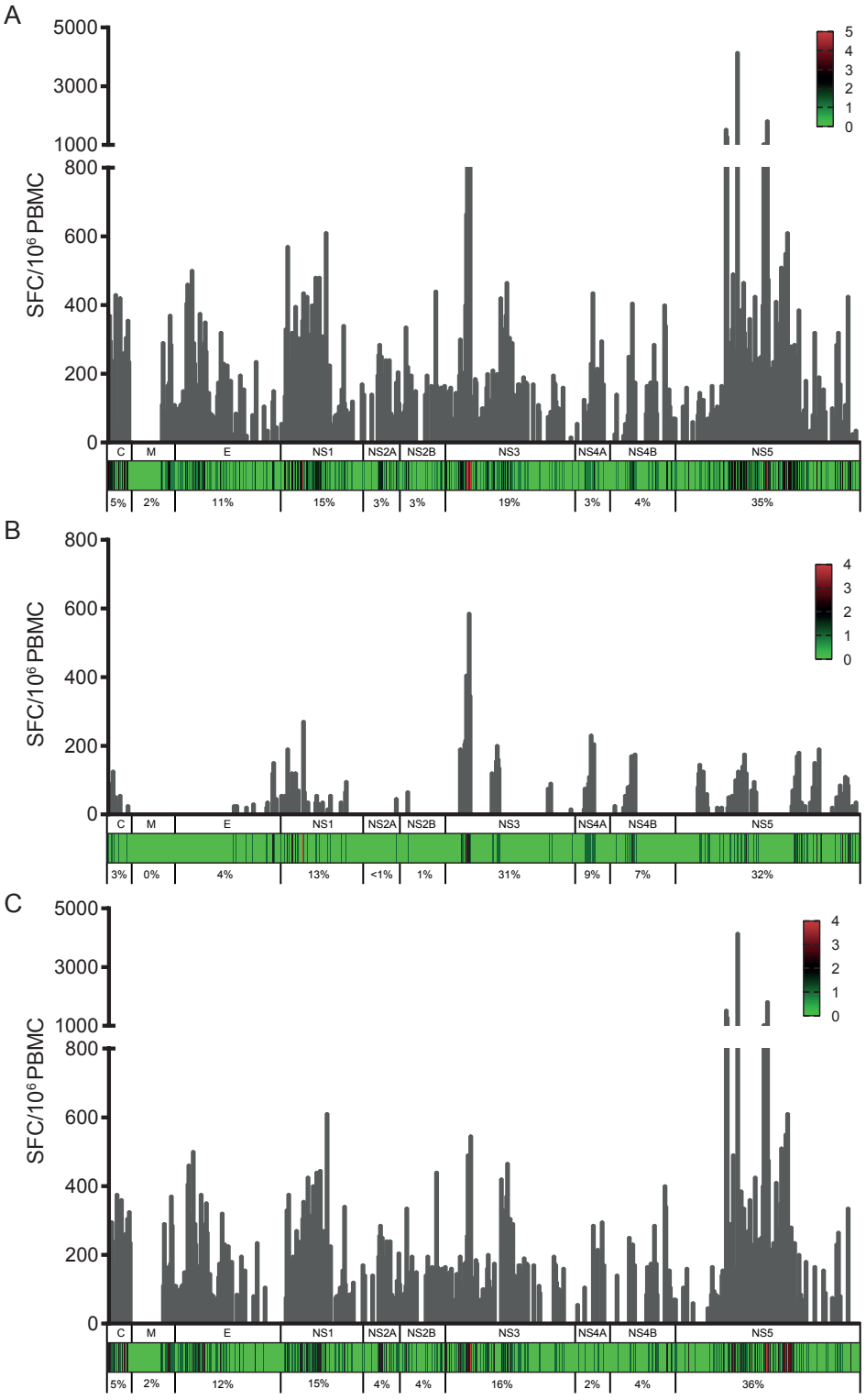

FIGURE 1 | ZIKV-specific response magnitude and frequency of responding donors. Cumulative IFN-γ responses (as spot-forming cells (SFCs) per million cells) for each overlapping peptide spanning the ZIKV proteome is shown for **(A)** all donors, **(B)** ZIKV donors or **(C)** DENV/ZIKV donors. The heat map indicates the number of donors with a positive IFN-γ 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.

Among the 20 ZIKV-seropositive blood donors, 11 individuals had both anti-DENV and anti-ZIKV IgG antibodies whereas 9 individuals did not reveal any detectable anti-DENV antibodies (Table 2). The presence of virus-specific antibodies detected by ELISA was further confirmed by the analysis of the neutralization activity of each plasma sample on DENV or ZIKV infection of U937-DC-SIGN or VERO cells, respectively (Table 2). We thus analyzed separately T cell responses from donors having only a history of ZIKV infection (ZIKV donors) and those from donors having a history of DENV and ZIKV infection (DENV/ZIKV donors). The NS1, NS3 and NS5 proteins accounted for 13%, 31% and 32% of the responses in ZIKV donors, respectively, whereas they accounted for 15%, 16% and 36% of the responses in DENV/ZIKV donors (Figure 1B, C). These results confirm that NS1, NS3 and NS5 are the main targets for T cells in ZIKV-infected donors, regardless of a previous infection with DENV. Interestingly, these data do not coincide with the recent identification of structural proteins E, prM and C as the main targets for CD4 and CD8 T cell responses in ZIKV donors (162).

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 (157, 158). Among the epitopes inducing a strong response in ZIKV and DENV/ZIKV donors, several 15-mer peptides contained short sequences predicted to bind strongly to at least one allele expressed by the responding donors (Table 3). For instance, the $NS2B_{117-131}$ peptide contains a 10-mer sequence predicted to bind strongly to the HLA-A*0301 and HLA-A*1101 molecules expressed by the responding donor 55. 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 $E_{455-469}$ 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. This also applies to the $NS5_{13-27}$ 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. 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 (165).

Table 3. Characteristics of antigenic peptides from ZIKV identified in this study

Peptide[a]	Sequence[b]	Donors	HLA[c]	SFC/million PBMC[d]		Predicted epitope	Predicted HLA	Score (rank)[e]
				15-mer	9-mer			
C_{13-27}	IVNM**LKRGVARVS**PF	28	A**02**,29; B40,44; C03,16; DRB104,07	170	<20	MLKRGVARV	A0217	1.9
		60	A**02**,69; B55,58; C01,07; DRB111,13	50	60		A0205	1.3
C_{85-99}	KKDL**AAMLRIINA**RK	26	A**02**,24; B07,48; C01,07; DRB114,15	100	<20	AAMLRIINA	A0201	4.5
		60	A**02**,69; B55,58; C01,07; DRB111,13	<20	75	KDLAAMLRI	B5501	1.4
	K**KDLAAMLRI**INARK	28	A**02**,29; B40,44; C03,16; DRB104,07	230	65		B4002	2.0
$E_{455-469}$	GMSWFSQILIGTLLM	1	A**02**,24; B35,**51**; C01,01; DRB104,12	120	NT	GMSWFSQILI	A0201	0.9
		77	A**02,02**; B40,**51**; C04,07; DRB113,14	35	NT	MSWFSQILI	B5101	0.12
$NS1_{63-77}$	MEN**IMWRSVEGE**LNA	21	A31,03; B35,18; C01,17; DRB1**04**,03	65	50	MENIMWRSVEGELNA	DRB10405	50
	MENIM**WRSVEGELN**A	28	A**02**,29; B40,44; C03,16; DRB1**04**,07	245	58	IMWRSVEGEL	A0217	0.5
		56	A**02,02**; B15,18; C05,07; DRB103,11	70	148		A0201	1.2
$NS1_{83-97}$	G**VQLTVVVGS**VKNPM	26	A**02**,24; B07,48; C01,07; DRB114,15	145	23	VQLTVVVGSV	A0201	1.7
		28	A**02**,29; B40,44; C03,16; DRB104,07	165	35		A0217	3
$NS1_{163-177}$	FHTS**VWLKVREDY**SL	28	A02,29; B40,44; C03,16; DRB1**04**,07	230	75	HTSVWLKVREDY	A0101	0.4
	FHTSV**WLKVREDYS**L	55	A03,11; B35,51; C04,15; DRB101,07	110	125	HTSVWLKVR	A3101	0.4
		46	A32,68; B39,50; C06,07; DRB1**04**,07	50	32		A6801	0.6
		20	A01,31; B07,39; C07,07; DRB1**04**,15	310	105	VWLKVREDY	A2902	1.3
						FHTSVWLKV	B3905	0.4
							B3901	0.4
$NS1_{275-289}$	IRFEE**CPGTKVHVE**E	33	A24,24; B15,**35**; C01,03; DRB104,04	215	55	CPGTKVHVE	B3501	8.5
		55	A03,11; B**35**,51; C04,15; DRB101,07	130	115		B3531	6.5

Continued

Table 3. Characteristics of antigenic peptides from ZIKV identified in this study – cont'd

Peptide[a]	Sequence[b]	Donors	HLA[c]	SFC/million PBMC[d]		Predicted epitope	Predicted HLA	Score (rank)[e]
				15-mer	9-mer			
$NS2B_{117-131}$	AAGAWYVYVKTGKRS	55	A03,11; B35,51; C04,15; DRB101,07	445	NT	AAGAWYVYVK	A0301	0.6
							A1101	0.12
						YVYVKTGKR	A0301	1.8
$NS3_{219-233}$	TVILAPTRVVAAEME	53	A23,31; B**40**,44; C01,04; DRB107,**08**	100	NT	TVILAPTRVVAAEME	DRB10802	1.5
		66	A02,03; B39,**40**; C03,07; DRB1**08**,15	65	NT	ILAPTRVVAA	A0201	1.6
$NS3_{271-285}$	LQPIRVPNYNLYIMD	42	A26,26; B35,38; C05,06; DRB104,11	165	NT	VPNYNLYIM	B3501	0.06
$NS3_{311-325}$	AAI**FMTATPPGT**RDA	28	A02,29; B40,44; C03,16; DRB1**04**,07	255	85	AAIFMTATPPGTRDA	DRB10401	4
						FMTATPPGT	A0217	5.5
	AA**IFMTATPPG**TRDA	33	A24,24; B15,35; C01,03; DRB1**04**,04	215	30	IFMTATPPG	A2402	5
$NS4A_{86-100}$	VTLGASAWLMWLSEI	55	A03,11; B35,51; C04,15; DRB101,07	178	NT	SAWLMWLSEI	B5101	0.9
		60	A02,69; B55,58; C01,07; DRB111,13	125	NT	VTLGASAWL	A6901	1.3
						LGASAWLMW	B5801	0.07
$NS4B_{112-126}$	**AIILLVAHY**MYLIPG	28	A**02**,29; B40,44; C03,16; DRB104,07	60	58	AIILLVAHY	A2902	0.6
		37	A**02**,11; B35,50; C04,06; DRB101,13	75	30		A1101	3.5
	AII**LLVAHYMYLI**PG	60	A**02**,69; B55,58; C01,07; DRB111,13	100	68	LLVAHYMYL	A0205	0.3
	AIIL**LVAHYMYL**IPG	60	A**02**,69; B55,58; C01,07; DRB111,13	100	35	LVAHYMYLI	A6901	0.15
							A0205	0.2
$NS5_{13-27}$	KARLNQMSALEFYSY	55	A03,11; B**35**,51; C04,15; DRB101,07	260	NT	MSALEFYSY	B3501	0.15
		69	A01,24; B**35,35**; C01,04; DRB104,13	145	NT		A0101	0.09

Continued

Table 3. Characteristics of antigenic peptides from ZIKV identified in this study – cont'd

Peptide[a]	Sequence[b]	Donors	HLA[c]	SFC/million PBMC[d]		Predicted epitope	Predicted HLA	Score (rank)[e]
				15-mer	9-mer			
$NS5_{293-307}$	WFFDEN**HPYRTWAYH**	55	A03,11; B**35**,51; C04,15; DRB101,07	1580	308	HPYRTWAYH	B3501	0.4
	W**FFDENHPYR**TWAYH	69	A01,24; B**35,35**; C01,04; DRB104,13	40	218	FFDENHPY	A0101	1.6
$NS5_{297-311}$	E**NHPYRTWAY**HGSYE	55	A03,11; B**35**,51; C04,15; DRB101,07	1280	358	NHPYRTWAY	B3501	3
	ENHP**YRTWAYHGS**YE	69	A01,24; B**35,35**; C01,04; DRB104,13	75	188	YRTWAYHGSY	B3501	1.7
							A0101	0.3
	ENHPY**RTWAYHGSY**E	69	A01,24; B**35,35**; C01,04; DRB104,13	75	205	RTWAYHGSY	A0101	0.5
$NS5_{345-359}$	TDT**TPYGQQRVF**KEK	33	A24,24; B15,**35**; C01,03; DRB104,04	1315	395	TPYGQQRVF	B3531	0.7
		55	A03,11; B**35**,51; C04,15; DRB101,07	2095	523		B3501	0.3
		69	A01,24; B**35**,35; C01,04; DRB104,13	785	763			
$NS5_{425-439}$	**EAVNDPRFW**ALVDKE	28	A02,29; B40,44; C03,16;DRB104,07	150	100	AVNDPRFWALVDK	A0301	1.1
		55	A03,11; B35,51; C04,15; DRB101,07	120	125		A1101	0.6
		56	A02,02; B15,18; C05,07; DRB103,11	90	240			
$NS5_{461-475}$	KKQGEFGKAKGSRAI	28	A02,29; B**40**,44; C03,16;DRB104,**07**	300	NT	KKQGEFGKAKGSRAI	DRB10701	32
		53	A23,31; B**40**,44; C01,04; DRB1**07**,08	105	NT	GEFGKAKGSRAI	B4002	0.7
$NS5_{473-487}$	RAIWYMWLGARFLEF	28	A02,29; B40,44; C03,16; DRB104,**07**	210	NT	YMWLGARFL	A0217	0.03
		55	A03,11; B35,51; C04,15; DRB101,**07**	295	NT	AIWYMWLGAR	A0301	1.3
						RAIWYMWLGARFLEF	DRB10701	16

Continued

Table 3. Characteristics of antigenic peptides from ZIKV identified in this study – cont'd

Peptide[a]	Sequence[b]	Donors	HLA[c]	SFC/million PBMC[d]		Predicted epitope	Predicted HLA	Score (rank)[e]
				15-mer	9-mer			
$NS5_{546-560}$	RFDLENEALITNQME	28	A02,29; B**40**,44; C03,16; DRB104,07	245	NT	NEALITNQM	B4002	0.8
		53	A23,31; B**40**,44; C01,04; DRB107,08	190	NT		B4403	0.6
		66	A02,03; B39,**40**; C03,07; DRB108,15	80	NT		B3901	1.8
$NS5_{565-579}$	LALAIIKYTYQNKVV	28	A02,29; B**40**,44; C03,16; DRB104,07	240	NT	LALAIIKYTY	A2902	0.5
		53	A23,31; B**40**,44; C01,04; DRB107,08	120	NT	ALAIIKYTY	A2902	0.25
		56	A02,02; B15,18; C05,07; DRB103,11	150	NT	LALAIIKYTY	B1517	1.2
$NS5_{605-619}$	QVVT**YALNTFTNL**VV	33	A**24,24**; B15,35; C01,03; DRB104,04	240	NT	TYALNTFTNL	A24:02	0.09
		59	A03,**24**; B35,40; C01,03; DRB104,04	50	42	YALNTFTNL	B35:43	0.4
							B35:31	0.25

[a] The position of peptides were determined according to NCBI Reference Sequence YP_002790881.1; [b] The underlined and in bold sequence correspond to the 9-mer peptide tested; [c] The common alleles between donors are underlined and in bold; [d] Cumulative SFC/million PBMC; NT, not tested; [e] Calculated using NetMHCpan 3.0 Server: strong binders <0.5, weak binders <2.

Similarly, a strong T cell response was observed against the $NS5_{546-560}$ peptide in donors 28, 53, and 66 that express the HLA-B*4002 and -B*4403 alleles and against the $NS5_{605-619}$ peptide in donors 33 and 59 that share the predicted HLA-A*2402 allele (Table 3).

Finally, we have also identified several 9-mer immunodominant epitopes in the NS4B and NS5 proteins, included in the $NS4B_{112-126}$, the $NS5_{293-307}$, $NS5_{297-311}$ and $NS5_{345-359}$ peptides, which induced substantial T-cell responses in donors that share one or several alleles with a strong potential for binding to these peptides. Remarkably, among the NS3 and NS5 proteins, several epitopes have been already 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 (165-169). Indeed, among the 9-mer peptides identified in DENV/ZIKV donors, the $NS5_{293-307}$, $NS5_{297-311}$ and $NS5_{345-359}$ 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 $NS5_{297-311}$ and $NS5_{345-359}$ peptides from ZIKV, respectively (164, 166, 170) (Table 3). 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 $NS3_{219-233}$ peptide, which contains the APTRVVAAEM 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 DLAV or in *ifnar-/-* HLA-B*0702 transgenic mice after ZIKV infection (165, 166). This suggests that the $NS3_{219-233}$ peptide contains another epitope or a promiscuous epitope that binds to other HLA alleles, besides HLA-B*0702 or B*3501.

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 (123), 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 1B, C). 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 (p=0.0410, 0.0052 and 0.0061 respectively), and a higher magnitude of response in donors with previous DENV infection (p=0.0189; Figure 2A, B).

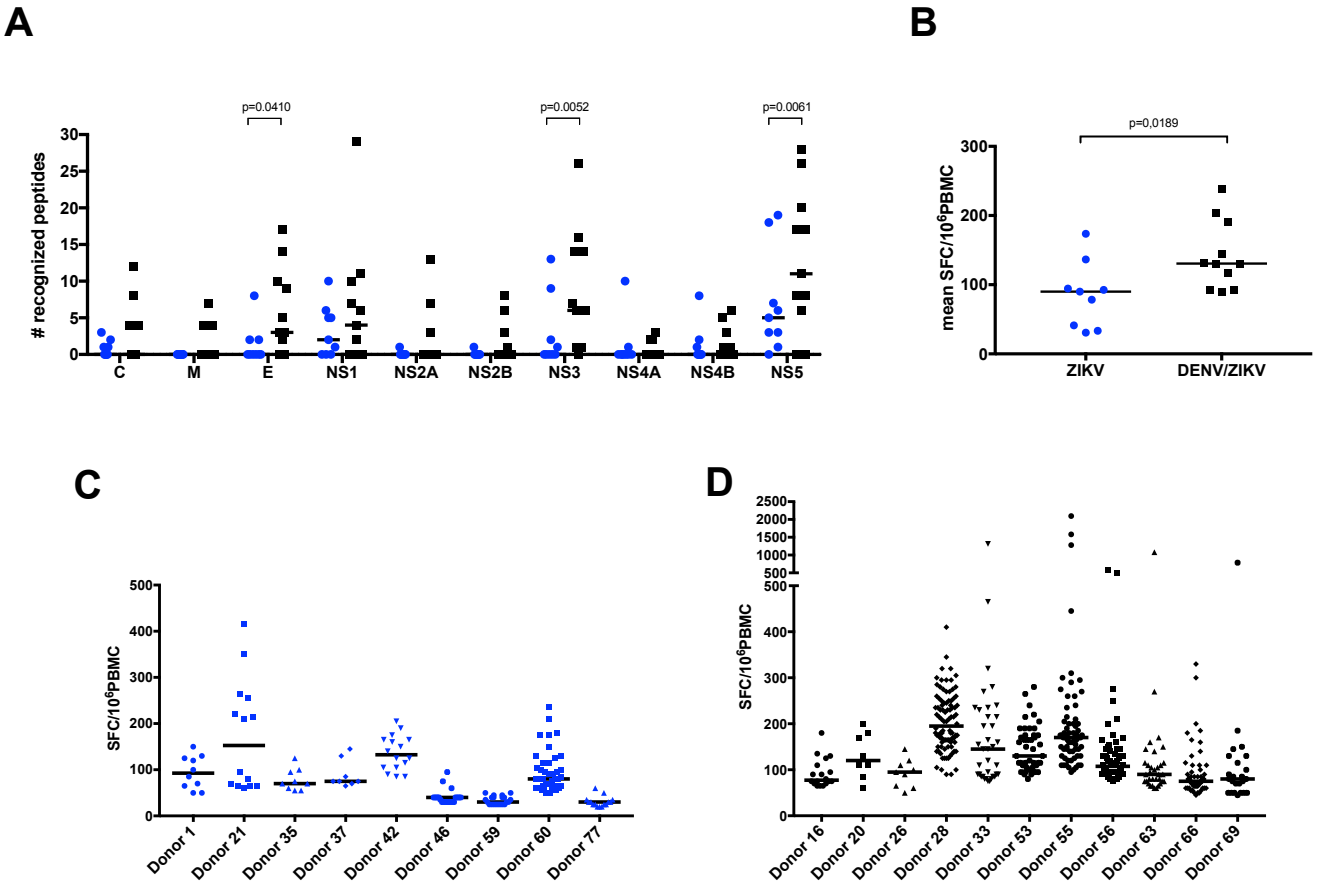

FIGURE 2 | ZIKV donors with previous DENV infection reveal a broader T cell response with a higher magnitude. **(A)** Breadth and **(B)** magnitude of responses in ZIKV and DENV/ZIKV donors. Each dot represents one donor and the bars represent the median value for each group of donors (blue circles, ZIKV donors; black squares, DENV/ZIKV 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 nine individuals among the ZIKV donors revealed a median response higher than 100 SFC/million cells, whereas six out of eleven DENV/ZIKV donors developed this strong response, which was also directed against a higher number of peptides (Figure 2C, D). These data confirm recent results showing a higher magnitude of T cell responses in ZIKV patients following previous DENV infection (169), and reveal the activation of a significantly higher frequency of T cells against peptides from E, NS3 and NS5 ZIKV, 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 (165, 169).

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 4). 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 4 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 4 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 4).

Table 4. Immunodominant epitopes in ZIKV and DENV/ZIKV donors

Peptide[a]	Sequence	ZIKV		DENV/ZIKV		% Identity			
		Donors	SFC/million PBMC[b]	Donors	SFC/million PBMC[b]	DENV1	DENV2	DENV3	DENV4
C_{49-63}	AILAFLRFTAIKPSL	60	60	28,63	365	60,0%	53,3%	60,0%	40,0%
E_{67-81}	DMASDSRCPTQGEAY			33	465	66,7%	53,3%	66,7%	53,3%
E_{87-101}	DTQYVCKRTLVDRGW			56	505	66,7%	53,8%	73,3%	66,7%
$NS1_{19-33}$	VFVYNDVEAWRDRYK	21,46,60	195	28,56	380	46,7%	33,3%	46,7%	40,0%
$NS1_{55-69}$	CGISSVSRMENIMWR	35,46	125	56	275	67,1%	66,3%	60,0%	60,0%
$NS1_{91-105}$	GSVKNPMWRGPQRLP	21,35,46,60	275	28	165	13,3%	33,3%	20,0%	33,3%
$NS1_{107-121}$	PVNELPHGWKAWGKS			28,53	430	40,0%	46,7%	46,7%	50,5%
$NS1_{147-161}$	HRAWNSFLVEDHGFG	46	40	33,53	445	66,7%	73,3%	66,7%	76,2%
$NS1_{163-177}$	FHTSVWLKVREDYSL	46	35	20,28,55	450	46,7%	46,3%	53,3%	46,7%
$NS1_{195-209}$	HSDLGYWIESEKNDT			28,33	615	80,0%	73,3%	66,2%	73,3%
$NS2B_{117-131}$	AAGAWYVYVKTGKRS			55	445	33,3%	33,3%	26,7%	26,7%
$NS3_{131-145}$	PAGTSGSPILDKCGR	21,42	405	26,55,63	495	53,3%	60,8%	53,3%	54,3%
$NS3_{143-157}$	CGRVIGLYGNGVVIK	21	350	20,55,63,66	550	60,0%	66,7%	72,3%	80,0%
$NS3_{311-325}$	AAIFMTATPPGTRDA			28,33	470	80,0%	80,0%	93,3%	80,0%
$NS5_{13-27}$	KARLNQMSALEFYSY			55,69	405	53,3%	46,7%	53,3%	40,0%
$NS5_{293-307}$	WFFDENHPYRTWAYH			55, 69	1620	66,7%	66,7%	60,0%	66,7%
$NS5_{297-311}$	ENHPYRTWAYHGSYE			55,69	1330	80,0%	80,0%	73,3%	80,0%
$NS5_{325-339}$	VVRLLSKPWDVVTGV			28, 55, 66	495	73,3%	80,0%	73,3%	66,7%
$NS5_{345-359}$	TDTTPYGQQRVFKEK			33,55,69	4195	93,3%	93,3%	93,3%	93,3%
$NS5_{373-387}$	QVMSMVSSWLWKELG	60	130	55,66,69	340	40,0%	53,3%	46,7%	46,7%
$NS5_{461-475}$	KKQGEFGKAKGSRAI			28,53	405	93,3%	93,3%	93,3%	86,7%
$NS5_{465-479}$	EFGKAKGSRAIWYMW			28,53,55,56	1085	100,0%	100,0%	100,0%	93,3%
$NS5_{473-487}$	RAIWYMWLGARFLEF			28,55	505	100,0%	100,0%	93,3%	100,0%
$NS5_{481-495}$	GARFLEFEALGFLNE			28,53,56,63	1870	93,3%	100,0%	93,3%	100,0%
$NS5_{546-560}$	RFDLENEALITNQME			28,53,66	515	60,0%	47,1%	53,3%	60,0%
$NS5_{573-586}$	TYQNKVVKVLRPAEK			28,53,56	615	72,9%	66,7%	73,3%	80,0%
$NS5_{849-863}$	CGSLIGHRPRTTWAE	60	90	33,55	340	66,7%	66,7%	66,7%	66,7%

[a] The position of peptides were determined according to NCBI Reference Sequence YP_002790881.1
[b] Cumulative SFC/million PBMC

CHAPTER 2. ANALYSIS OF ANTIBODY RESPONSES AGAINST ZIKV

Sample selection and development of the *in vitro* assays

To determine whether plasma samples from donors infected with DENV or ZIKV contain cross-reactive antibodies (with neutralizing or enhancing activity), we developed a flow cytometry-based assay to quantify the neutralizing and enhancing potential of the different plasma samples against both DENV and ZIKV. We first compared different cell lines for their ability to be infected and neutralized by ZIKV-specific or DENV-specific antibodies, and we also compared the results obtained using plasma or serum samples for neutralization using the two cell lines U937-DC-SIGN and Vero cells. As shown in Figure 4, a significant correlation was observed using plasma or serum samples tested on either Vero or U937-DC-SIGN cells (Figure 4A, B) for DENV neutralization assay; similarly, we observed a significant correlation using either plasma or serum samples, tested on VERO or U937-DC-SIGN cells (Figure 4C, D). Following these results and considering the ease of manipulating cells in suspension for flow cytometry, the U937-DC-SIGN cells were used to quantify neutralization of DENV infection. To quantify neutralization of ZIKV infection, the Vero cells were used, as a higher correlation was observed with these cells using either plasma or serum, in comparison with U937-DC-SIGN cells (data not shown). For the analysis of ADE activity, the K562 cells were used for both DENV and ZIKV infections.

For the subsequent analysis of the antibody response against ZIKV and DENV infection, four groups of donors were selected depending on the expression of DENV- and/or ZIKV-specific IgG antibodies, measured by ELISA.

1- Samples from anti-DENV IgG / anti-ZIKV IgG negative donors **(naïve donors)**

2- Samples from anti-DENV IgG positive / anti-ZIKV IgG negative donors **(DENV donors)**

3- Samples from anti-DENV IgG negative / anti-ZIKV IgG positive donors **(ZIKV donors)**

4- Samples from anti-DENV IgG / anti-ZIKV IgG positive donors **(DENV/ZIKV donors)**

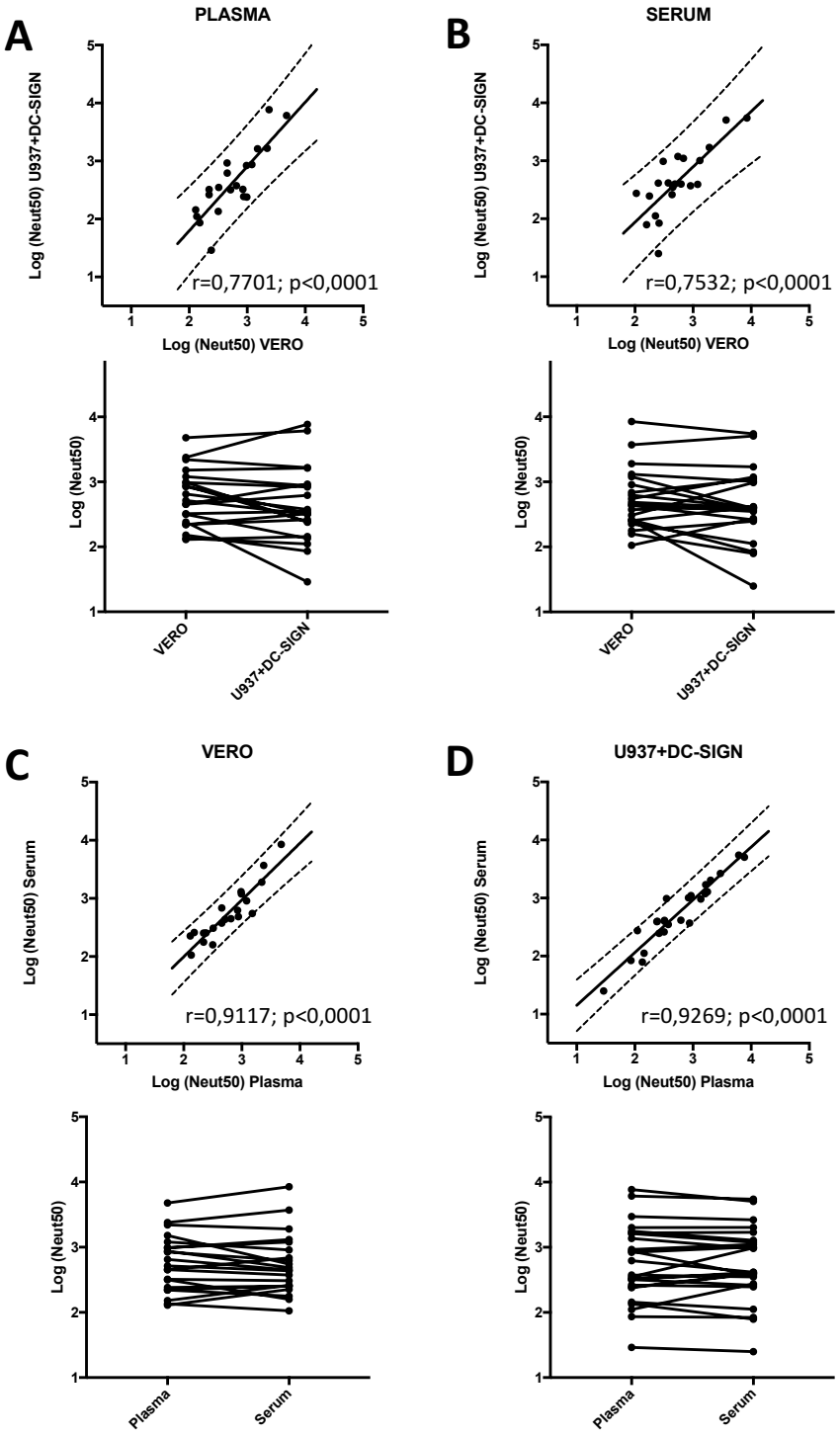

FIGURE 4 | Correlation between the DENV neutralization titers ($Neut_{50}$) obtained with plasma or serum samples using two different cell lines. The neutralizing activity of EDTA containing plasma **(A)** or serum **(B)** samples from nine DENV-seropositive donors were analyzed using Vero **(C)** or U937-DC-SIGN **(D)** cells and the $Neut_{50}$ values obtained were sample-based or cell line-based correlated. Each dot represents the individual $Neut_{50}$ value obtained for at least one DENV serotype which was neutralized during the *in vitro* infection of either Vero or U937-DC-SIGN cells. The Spearman's correlation coefficient and p value are shown. The dashed lines represent the 95% confidence intervals of the best-fit line after linear regression.

The plasma samples included in the groups 3 and 4 correspond to donors analyzed for T cell responses in chapter 1. The group 2 includes 10 samples from DENV-seropositive donors, (identified with a R letter, Table 1) collected before ZIKV outbreak and 4 samples (numbered 07, 08, 14 and 15) from DENV-seropositive donors collected after ZIKV outbreak. In order to avoid any inter-assay variability between cells and viruses, each infection experiment (for either the neutralization or ADE assay) was performed using one virus combined with different plasma samples at the same time.

Neutralizing activity against ZIKV infection is higher in donors with previous DENV infection

To identify donors with a history of ZIKV infection, we first developed an ELISA assay for quantifying the binding of IgG antibodies to the ZIKV EDIII (ZEDIII) domain. The specificity of the assay was confirmed using plasma from DENV-seropositive donors collected before ZIKV outbreak, with no ZEDIII-specific antibodies, or plasma from naive donors. In these conditions, measurement of the binding of different plasma samples to recombinant ZEDIII revealed a significant higher binding activity of plasma samples from DENV/ZIKV donors, in comparison with plasma from ZIKV donors (O.D. median values of 0.7426 and 0.3341 in DENV/ZIKV and ZIKV donors, respectively) (Figure 5A). This result shows that a primary infection with DENV followed by a secondary ZIKV infection induces a higher production of antibodies recognizing the EDIII domain of ZIKV, such as the antibody response induced upon a secondary antigenic stimulation. This result strongly suggests the existence of cross-reactive antibodies recognizing B cell epitopes shared by EDIII of DENV and ZIKV, which are induced after sequential DENV and ZIKV infection.

To confirm this cross-reactivity and to determine whether this difference in the level of ZEDIII binding capacity was associated with a different potential to neutralize ZIKV infection, we compared the neutralizing activity of plasma samples from ZIKV with plasma samples from DENV/ZIKV donors, on ZIKV infection *in vitro*. Remarkably, a significantly higher ZIKV $Neut_{50}$ median value was measured in plasma samples from ZIKV-seropositive donors with previous DENV infection ($Neut_{50}$ median value 2476), in comparison with plasma samples from donors infected only with ZIKV ($Neut_{50}$ median value 340) (Figure 5B).

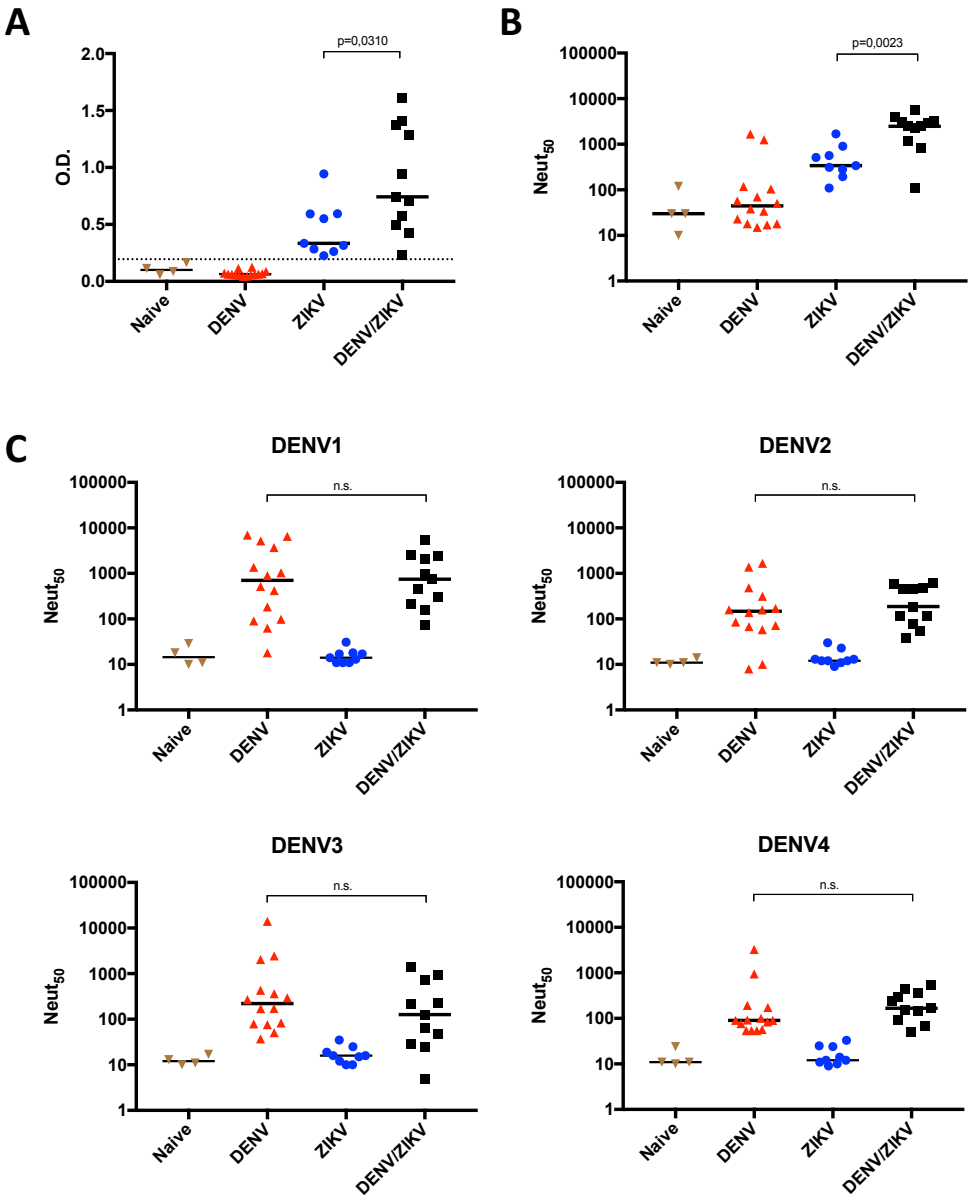

FIGURE 5 | Analysis of ZEDIII binding and ZIKV- or DENV- neutralizing activity. Plasma samples from blood donors with serological evidence of either previous DENV and/or ZIKV infection were analyzed for the detection of ZEDIII-binding antibodies by ELISA **(A)** or for the neutralizing activity against ZIKV **(B)** or the four DENV serotypes **(C)** infection. 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.

This shows that the higher binding activity of samples from DENV/ZIKV donors is accompanied by a higher potential to neutralize ZIKV. However, no significant correlation could be established between the level of ZIKV binding antibodies and their $Neut_{50}$ values (Spearman r=0.3731; p=0.1052). These results confirm that at least a fraction of the antibodies induced following a primary DENV infection can recognize and neutralize ZIKV; however, although the level and/or the affinity of these neutralizing antibodies increases

after a secondary infection with ZIKV, not all these cross-reactive antibodies can neutralize ZIKV infection. These results also reveal that different donors have different levels of cross-reactive Abs, a result that could be explained by the different kinetics of sequential DENV and ZIKV infection in different donors, although we do not know exactly when these donors have been infected with DENV or ZIKV.

To confirm the specificity of ZIKV neutralization, plasma samples from DENV-seropositive /ZIKV-seronegative donors (DENV donors) were used as negative controls. Using these samples, the neutralizing activity was shown to be similar to the neutralization obtained with naïve donors, except for 2 donors, donor 15 and R64 (Figure 5B). Plasma samples from these donors revealed high ZIKV $Neut_{50}$ median values above 1000, (Figure 5B), and revealed multiple DENV reactivity detected by their neutralization potential against at least 2 DENV serotypes (Table 5). As these donors were collected before ZIKV outbreak, these results confirm other reports showing the induction, in a fraction of DENV donors, of cross-reactive antibodies recognizing ZIKV, in DENV-immune ZIKV naïve donors (123, 133, 171).

Interestingly, plasma samples from ZIKV donors did not neutralize DENV infection, and revealed the same DENV $Neut_{50}$ median values that those obtained with naïve donors (Figure 5C, Table 5). When we compared the DENV $Neut_{50}$ median values of the different groups of donors regarding each DENV serotype, we did not observe any significant difference between DENV- and DENV/ZIKV-seropositive donors in their neutralizing activity against the four DENV serotypes (Figure 5C). These results suggest that contrarily to samples from DENV donors, antibodies from ZIKV-exposed donors have less cross-neutralizing reactivity towards the different DENV serotypes. In other words, these data show that ZIKV infection in DENV immune donors does not increase the neutralizing potential against any of the 4 DENV serotypes.

Since all samples from ZIKV donors did not cross-neutralize any of the 4 DENV serotypes (Table 5), we wondered whether these samples could nevertheless bind to the EDIII domain of DENV1-4.

Table 5. Neutralization activity ($Neut_{50}$) of plasma samples against all DENV serotypes and ZIKV[a]

Group	Donor	DENV1	DENV2	DENV3	DENV4	ZIKV
Naïve	**23**	10	14	10	11	30
	36	11	11	11	11	30
	45	29	10	17	24	10
	58	18	11	13	10	119
DENV	**07**	**1372**	**483**	172	92	18
	08	99	**157**	51	84	23
	14	18	<10	37	**90**	18
	15	**1036**	85	**269**	54	**1673**
	R19	63	68	**433**	54	118
	R51	**6519**	138	**365**	90	57
	R64	**5181**	**1671**	**14004**	**3267**	**1250**
	R80	**6974**	**1377**	**2446**	**953**	104
	R106	90	72	76	**100**	15
	R152	**896**	10	79	54	38
	R176	**420**	171	170	77	34
	R206	**3694**	**316**	**294**	193	70
	R213	**184**	58	83	57	17
	R221	**517**	160	**2048**	173	51
ZIKV	**01**	17	23	16	25	**311**
	21	13	13	19	14	**566**
	35	11	13	10	<10	**340**
	37	18	12	35	12	**280**
	42	11	12	25	12	**1689**
	46	14	11	12	11	**903**
	59	31	30	16	24	**194**
	60	11	<10	10	10	**514**
	77	17	12	15	33	110
DENV/ZIKV	**16**	158	79	47	**357**	**2270**
	20	**2049**	**452**	**214**	**438**	**2470**
	26	**5397**	**601**	**735**	**235**	**2476**
	28	**75**	39	24	**67**	**5587**
	33	**306**	114	63	51	**829**
	53	**464**	116	29	**543**	**3081**
	55	**2395**	**612**	**222**	**301**	**3196**
	56	**215**	55	<10	156	110
	63	**999**	187	126	144	**4057**
	66	**2572**	**471**	**1386**	167	**2905**
	69	**749**	**463**	**961**	92	**1205**

[a] The values in each cell are the 50% neutralization titers determined from two replicates of one experiment. The highest titers for each sample is indicated in boldface.

Surprisingly, DENV cross-reactive antibodies against the EDIII domain of DENV serotypes 1 and 4 were detected in 2 DENV naive ZIKV immune donors (Figure 6). In addition, no significant difference was observed in the titer of EDIII binding activity between DENV immune and DENV/ZIKV immune samples (*p* values of 0.8652, 0.8072, 0.5870 and 0.8165 for DENV1 to 4 respectively). Altogether, using a limited number of plasma samples, these

results show that ZIKV infection in DENV naïve individuals induces mainly ZIKV-specific antibodies that do not neutralize DENV1-4, with only a small fraction of individuals producing low levels of cross-reactive antibodies against DENV1 and DENV4.

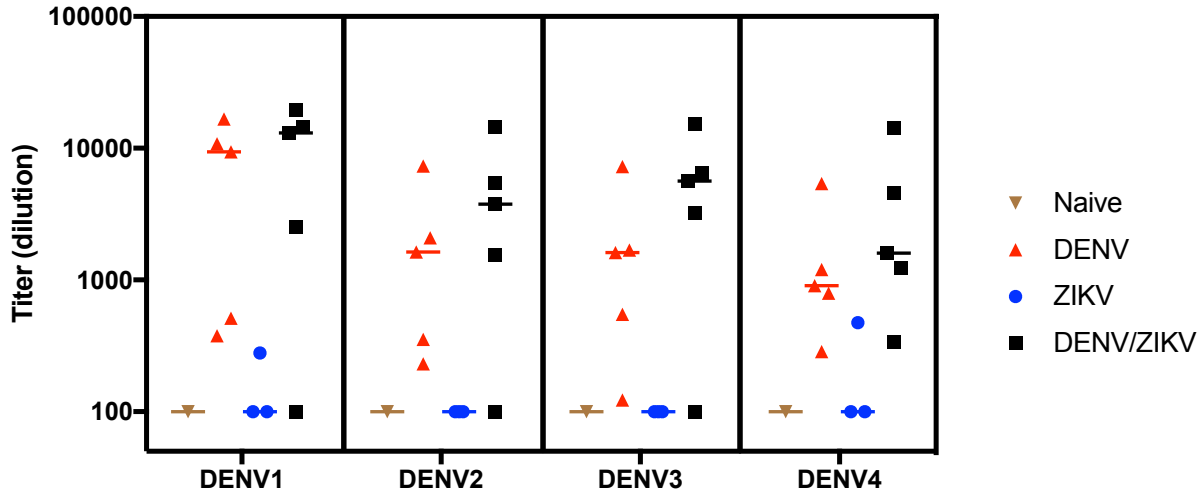

FIGURE 6 | Quantification of DENV anti-EDIII antibodies level. Plasma samples from blood donors with serological evidence of either previous DENV and/or ZIKV infection were analyzed for the detection of DENV anti-EDIII antibodies by ELISA. Each dot represents one donor and the bars represent the median value for each group of donors.

Conversely, ZIKV infection in DENV-immune individuals induces mainly cross-reactive antibodies that can neutralize ZIKV infection, with an increase in the neutralization titer against ZIKV, but not against DENV. In addition, comparison of the neutralizing activity against the four DENV serotypes, among the different samples from DENV or DENV/ZIKV immune donors revealed that most donors have a higher neutralization activity against DENV1, in comparison with the three other DENV serotype, a result which confirms the higher prevalence of this DENV serotype in areas where these donors are living (172) (Table 5). In conclusion, we have shown that while a primary DENV infection induces DENV-specific antibodies that cross-react and cross-neutralize ZIKV infection *in vitro,* a ZIKV infection induces mainly ZIKV-specific antibodies that do not cross-neutralize DENV infection *in vitro*. However, while a primary ZIKV infection does not induce DENV-cross neutralizing antibodies, a secondary ZIKV infection, in DENV immune individuals, induces an increase in the level of ZIKV-neutralizing antibodies but not DENV-neutralizing antibodies, in comparison with DENV naïve individuals (Table 6). These results strongly suggest that a sequential DENV/ZIKV

infection induces the activation of memory B cell responses against common antigenic determinants shared between DENV and ZIKV.

Table 6. Cross-neutralizing activity of polyclonal antibodies induced after DENV and ZIKV infection

Primary infection	Secondary infection	Neutralizing activity	
		DENV	ZIKV
DENV	-	++	+
ZIKV	-	-	++
DENV	ZIKV	++	+++

Modulation of the ADE activity against ZIKV following sequential DENV and ZIKV infection

A widely shared concept is that a primary DENV infection by one DENV serotype can induce a lifelong immunity against re-infection by the same DENV serotype, whereas subsequent infections by heterologous serotypes increase the risk of developing severe dengue. This phenomenon due to non-neutralizing or sub-neutralizing antibodies is called Antibody-Dependent Enhancement (ADE) (92, 173, 174). In support of the ADE hypothesis, it was proposed that the low level of serotype cross-reactive antibodies produced following a primary infection, could enhance a secondary infection through the formation of DENV-antibody complexes that bind to the Fc-γ receptors of monocytes/macrophages, resulting in an increased viral load and production of inflammatory mediators responsible of vascular permeability (175, 176).

Similarly to the ADE phenomenon induced following infection by different DENV serotypes, it was also recently shown that cross-reactive anti-DENV antibodies could enhance ZIKV infection, both *in vitro* and *in vivo* (124, 137). However, while such cross-reactive antibodies against both viruses have been identified in humans living in endemic areas, the exact role of these cross-reactive antibodies in the outcome of dengue or Zika disease in human remains to be determined. In this study, we have quantified the ADE activity of plasma samples against DENV and ZIKV, from all subjects previously defined, i.e. naïve donors, DENV donors, ZIKV donors and DENV/ZIKV donors, knowing that all ZIKV and DENV/ZIKV donors did not

report any apparent clinical symptoms associated with ZIKV infection, one week before and after blood sampling.

To measure the ADE activity of antibodies from blood samples, serial dilutions of plasma samples were tested for their potential to increase DENV or ZIKV infection of K562 cells. A schematic representation of the typical Gaussian curve showing an ADE activity *in vitro* is shown in figure 7. To quantify the infection enhancement, two different parameters can be taken into account: the percentage of infection at the maximum of infection (% of infection at the peak) and the dilution of the plasma sample (titer) for which the percentage of infection is maximal (Peak titer).

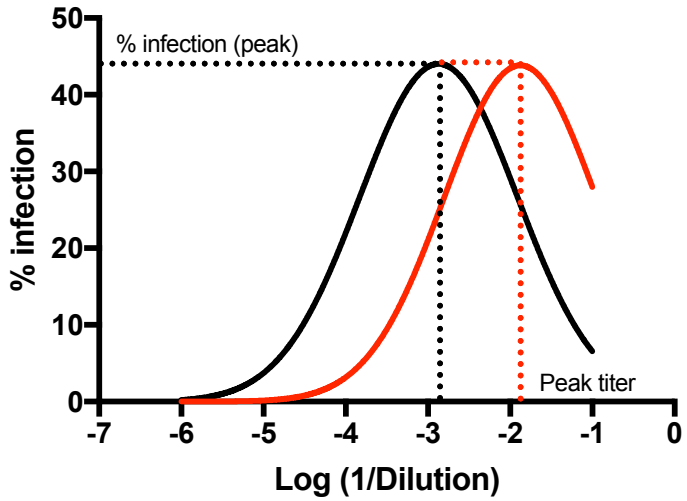

FIGURE 7 | Schematic graph of percentage of infection vs. dilution of plasma sample (Log (1/dilution)) in cultures of K562 cells. The analysis of the ADE activity of each plasma sample is based in the measurement of two parameters: the percentage of infection in the peak of the infectivity curve (% infection (peak)) and the titer of plasma sample at which the percentage of infection is maximal (Peak titer).

To facilitate the quantification and interpretation of the ADE activity when peak titers are compared, this parameter was expressed as the logarithm of the inverse of dilution factor (Log (1/Dilution). For example, in the schematic graph shown in figure 7, the sample denoted by the black line (sample 1) shows a lower level of ADE activity, in comparison with the sample denoted by the red line (sample 2). This means that the sample 1 needs a ten-fold higher dilution to reach the sub-neutralizing antibody concentration to obtain the maximal enhancement of infection, in comparison with the sample 2. In other words, in the same

biological sample, a high dilution of the sample required to induce the peak of ADE activity corresponds to a low ADE activity and coincides with a strong neutralization potential, and vice versa. In these conditions, the background (or the activity measured without enhancing antibodies) defined in the absence of plasma, gave a percentage of infection on K562 cells of 2.49% (represented as a dotted line in Figure 8A-D). Likewise, In the presence of plasma samples from naïve donors, no ADE activity was detected (Figure 8A).

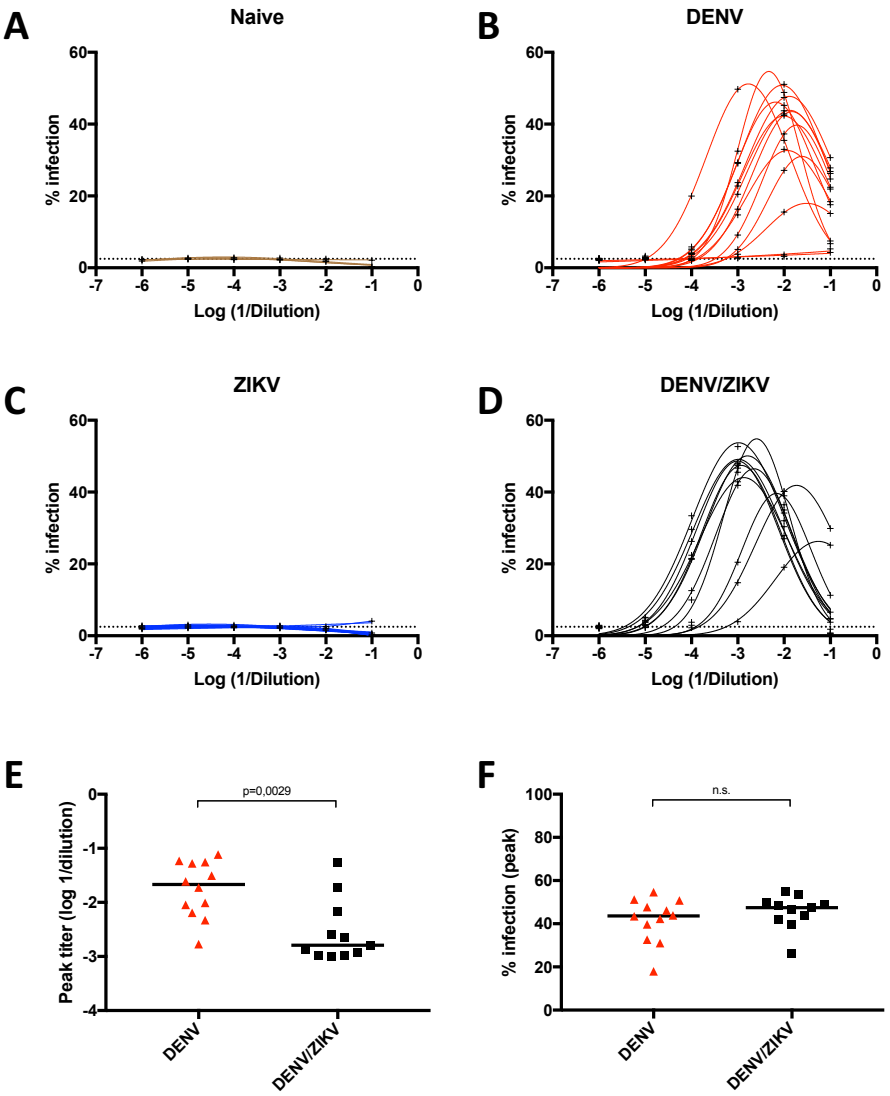

FIGURE 8 | ADE activity of plasma samples in ZIKV infection. Plasma samples from blood donors in the naïve **(A)**, DENV **(B)**, ZIKV **(C)** or DENV/ZIKV **(D)** groups were analyzed for ADE activity in ZIKV infection. Infectivity curves for each donor is shown. The dotted line represents the percentage of infection in the control (only virus). Comparison of the peak titer **(E)** or the percentage of infection in the peak **(F)** between DENV and DENV/ZIKV donors was done. 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.

On the contrary, an ADE activity on ZIKV infection was clearly detected in almost all plasma samples from DENV donors, with different titers of enhancing potential (Figure 8B). The peak titer value for each of these samples is shown in Table 7.

Table 7. ADE activity of plasma samples for dengue and Zika virus infection[a]

Group	Donor	DENV1	DENV2	DENV3	DENV4	ZIKV
Naïve	23	-	-	-	-	-
	36	-	-	-	-	-
	45	-	-	-	-	-
	58	-	-	-	-	-
DENV	07	1409 (-3.1)	354 (-2.5)	388 (-2.6)	345 (-2.5)	67 (-1.8)
	08	274 (-2.4)	308 (-2.5)	251 (-2.4)	442 (-2.6)	15 (-1.2)
	14	213 (-2.3)	290 (-2.5)	137 (-2.1)	285 (-2.5)	85 (-1.9)
	15	863 (-2.9)	252 (-2.4)	294 (-2.5)	244 (-2.4)	77 (-1.9)
	R19	62 (-1.8)	46 (-1.7)	318 (-2.5)	78 (-1.9)	32 (-1.5)
	R51	3311 (-3.5)	279 (-2.4)	419 (-2.6)	278 (-2.4)	102 (-2.0)
	R64	6067 (-3.8)	1256 (-3.1)	11561 (-4.1)	1552 (-3.2)	592 (-2.8)
	R80	2812 (-3.4)	1189 (-3.1)	1932 (-3.3)	1026 (-3.0)	213 (-2.3)
	R106	68 (-1.8)	73 (-1.9)	119 (-2.1)	244 (-2.4)	-
	R152	394 (-2.6)	75 (-1.9)	138 (-2.1)	135 (-2.1)	41 (-1.6)
	R176	326 (-2.5)	215 (-2.3)	278 (-2.4)	207 (-2.3)	53 (-1.7)
	R206	2128 (-3.3)	337 (-2.5)	401 (-2.6)	521 (-2.7)	155 (-2.2)
	R213	153 (-2.2)	19 (-1.3)	63 (-1.8)	39 (-1.6)	-
	R221	407 (-2.6)	335 (-2.5)	1535 (-3.2)	419 (-2.6)	111 (-2.0)
ZIKV	01	-	-	-	-	-
	21	-	-	-	-	-
	35	-	-	-	-	-
	37	-	-	-	-	-
	42	-	-	-	-	-
	46	-	-	-	-	-
	59	-	-	-	-	-
	60	-	-	-	-	-
	77	-	-	-	75 (-1.9)	-
DENV/ZIKV	16	675 (-2.8)	601 (-2.8)	374 (-2.6)	2080 (-3.3)	971 (-3.0)
	20	2891 (-3.5)	687 (-2.8)	451 (-2.7)	1884 (-3.3)	959 (-3.0)
	26	2917 (-3.5)	1230 (-3.1)	1074 (-3.0)	1146 (-3.1)	617 (-2.8)
	28	230 (-2.4)	191 (-2.3)	163 (-2.2)	455 (-2.7)	1012 (-3.0)
	33	324 (-2.5)	289 (-2.5)	230 (-2.4)	280 (-2.4)	146 (-2.2)
	53	277 (-2.4)	118 (-2.1)	87 (-1.9)	594 (-2.8)	18 (-1.3)
	55	2080 (-3.3)	378 (-2.6)	318 (-2.5)	671 (-2.8)	445 (-2.6)
	56	313 (-2.5)	223 (-2.3)	201 (-2.3)	492 (-2.7)	54 (-1.7)
	63	1517 (-3.3)	662 (-2.8)	489 (-2.7)	1253 (-3.1)	741 (-2.9)
	66	2228 (-3.3)	711 (-2.9)	2371 (-3.4)	713 (-2.9)	836 (-2.9)
	69	530 (-2.7)	318 (-2.5)	807 (-2.9)	294 (-2.5)	393 (-2.6)

[a] The values in each cell are the peak titers expressed as sample dilution determined from two replicates of one experiment. The values in parenthesis are the log 1/dilution. When the sample does not have ADE activity, it is represented by a dash.

Strikingly, with the exception of the 2 DENV donors R106 and R213 that did not induce any ADE activity, all DENV donors have antibodies with ADE activity against both ZIKV and the four DENV serotypes. In these samples, the ADE activity against DENV follows the ADE

activity against ZIKV infection, meaning that a high ADE activity against DENV is also accompanied by a high ADE activity against ZIKV. This is what is observed with samples 07, 08, 14, 15, R19, R152 and R176, with a high ADE activity against both DENV and ZIKV, whereas the other samples (R51, R64, R80, R206 and R221) have a lower ADE activity against DENV and ZIKV. In addition, as observed in samples 08, 14, R152 and R176, a lower level of neutralizing antibodies against DENV1-4 is associated with a high level of ADE activity (or sub-neutralizing activity) against heterotypic DENV serotypes and ZIKV (Table 5 and Table 7). These results confirm other studies showing that cross-reactive antibodies against DENV with low neutralizing activity can mediate ADE activity *in vitro* (177, 178).

Contrarily to these plasma samples from DENV donors, none of the plasma samples from ZIKV donors enhanced ZIKV infection of K562 cells (Figure 8C). Strikingly, ZIKV ADE activity was detected in ZIKV-immune plasma samples from donors with previous DENV infection (Figure 8D; Table 7), with significant lower median peak titer values than for DENV donors (-2.8 vs -1.7, respectively; p=0.0029) (Figure 8E) and similar percentages of infection at the peak (Figure 8F). This result clearly shows that a primary DENV infection can enhance DENV and ZIKV infection *in vitro*, through ADE, whereas a secondary ZIKV infection in DENV donors decreases the ADE activity against ZIKV infection. This is also accompanied by an increase in the titer of ZIKV-specific neutralizing antibodies (Figure 5A).

Representation of both ZIKV neutralizing and ADE activities in the same plot from three representative donors of each group reveals that DENV/ZIKV donors have higher levels of neutralizing antibodies and lower levels of ADE activity against ZIKV, than DENV or ZIKV donors (black plots, in comparison with red and blue plots, Figure 9). Conversely, samples from DENV donors reveal a higher ADE potential against ZIKV, than samples from DENV/ZIKV donors, and no ADE activity was detected in samples from ZIKV donors (Figure 9). This result suggests that a primary (or multiple) DENV infection can elicit the production of cross-reactive antibodies with enhancing activity against heterotypic DENV or ZIKV, whereas a secondary infection with ZIKV increases the titer of neutralizing antibodies against ZIKV and decreases the amount of cross-reactive antibodies with ADE activity against ZIKV infection.

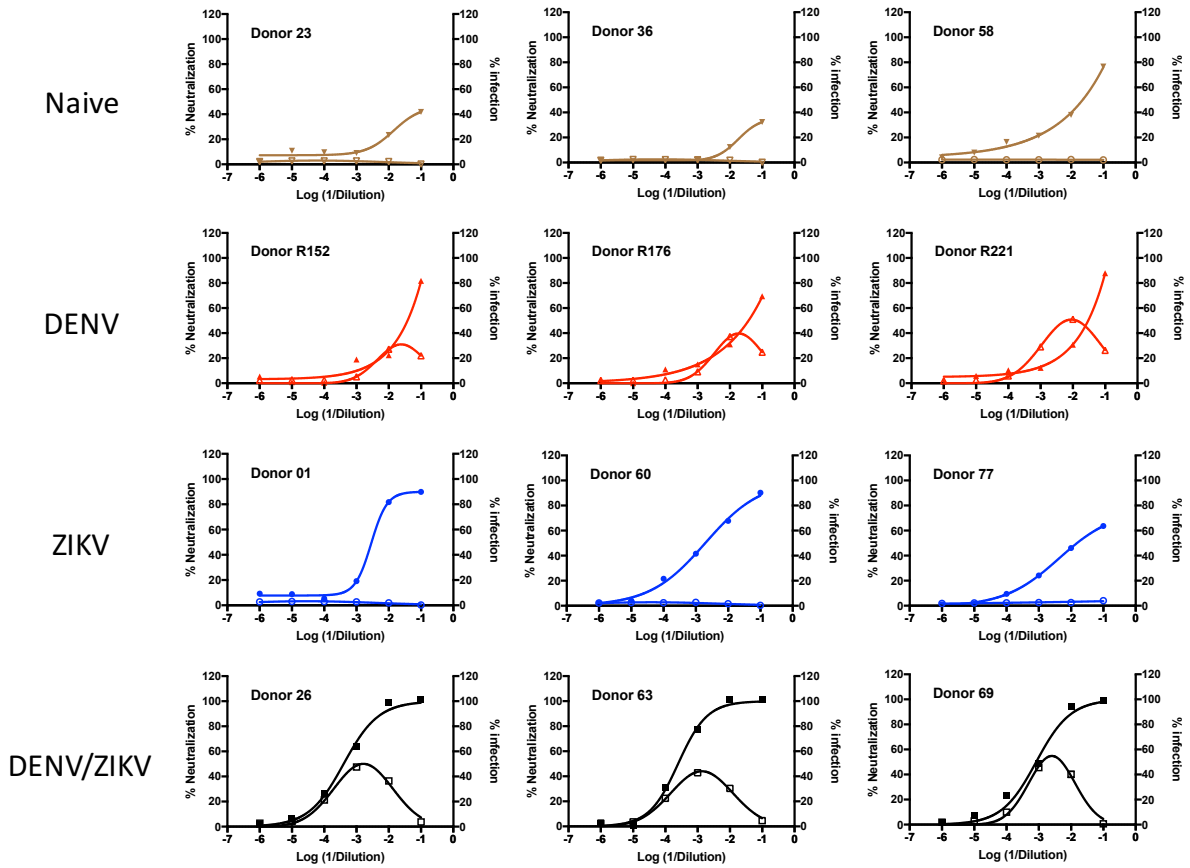

FIGURE 9 | Comparison of the neutralizing and ADE activities of plasma samples in ZIKV infection. Results obtained for three representative blood donors of each group are shown. Open symbols represent the % of infection for ADE activity; closed symbols represent the % of neutralization for neutralizing activity. Each dot represents the mean value of two replicates of one experiment.

The specific case of ZIKV-reactive antibodies with ADE activity against DENV4

To determine whether ZIKV-immune donors produce cross-reactive antibodies with enhancing activity against a specific DENV infection, ADE assays where performed using the four DENV serotypes. In the assay, the mean percentages of infection of K562 cells for the ADE negative control (cells infected with DENV without plasma sample) were 0.33%, 0.49%, 0.43% and 0.58% for DENV1, DENV2, DENV3 and DENV4, respectively. ADE activity with the four DENV serotypes was clearly detected in all plasma samples from DENV and DENV/ZIKV donors, with various peak titers values and percentages of infection in the different samples (Table 7, Figure 10). On the contrary, in the presence of plasma samples from naïve donors, no ADE activity for any DENV serotype was detected (Table 7, Figure 11A).

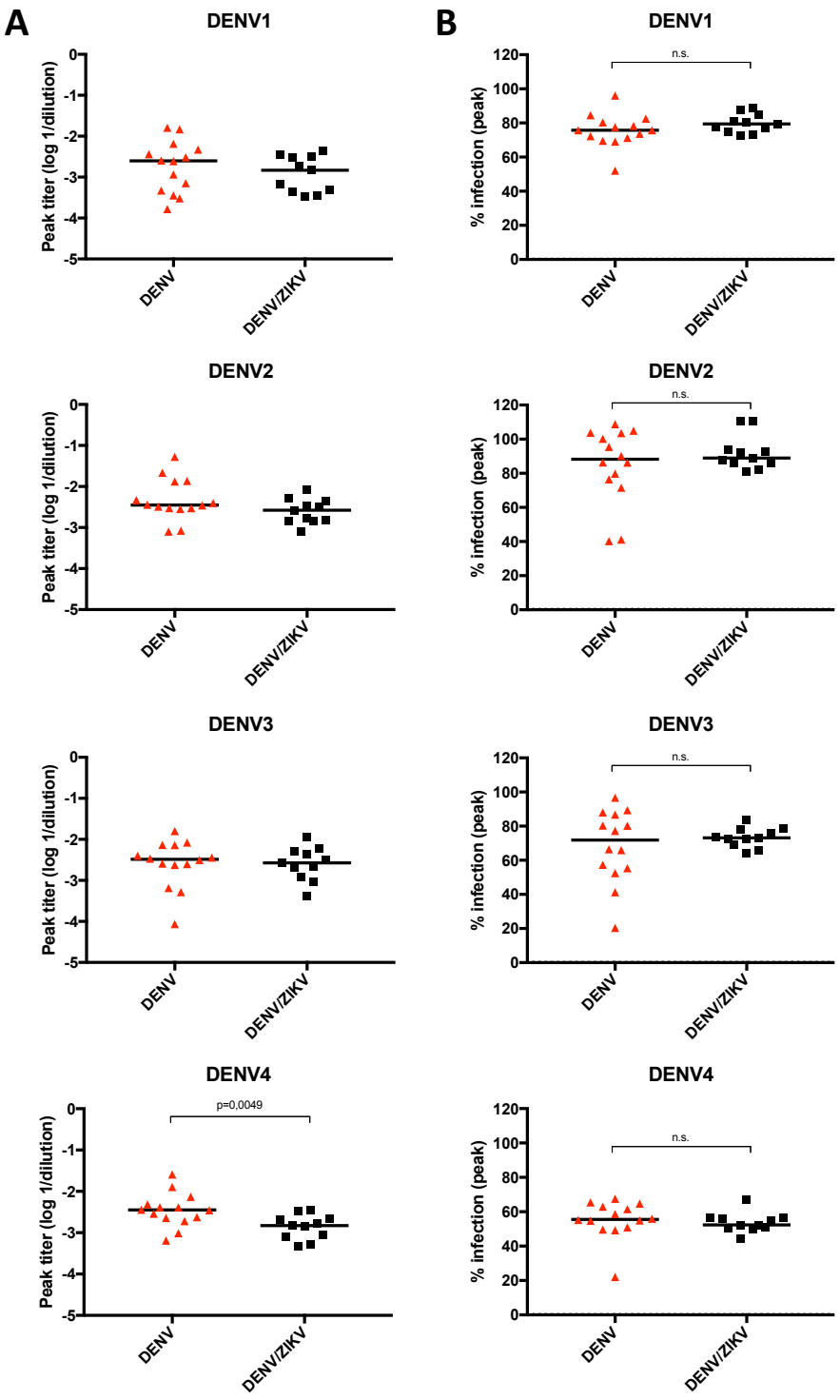

FIGURE 10 | Comparison of the ADE activity parameters in DENV infection between DENV and DENV/ZIKV donors. The peak titer **(A)** or the percentage of infection in the peak **(B)** values obtained for all DENV serotypes in DENV and DENV/ZIKV donors were compared. Each dot represents one donor and the bars represent the median value for each group of donors. The P values were calculated using the nonparametric two-tailed Mann-Whitney test.

While the ADE activity against ZIKV infection was higher in plasma samples from DENV than from DENV/ZIKV donors (Figure 8E), we did not observe a significant difference in the level of ADE activity against DENV infection between these two groups of donors, except for DENV4 (Figure 10A). Interestingly, a significant difference in the median peak titer value was observed with this DENV4 serotype, with a lower ADE activity for DENV/ZIKV donors, in comparison with DENV donors (Figure 10A and Figure 11B and D). This result shows that a sequential DENV and ZIKV infection does not increase the risk to develop a severe dengue disease, but it rather decreases this risk, in comparison with heterotypic DENV infection.

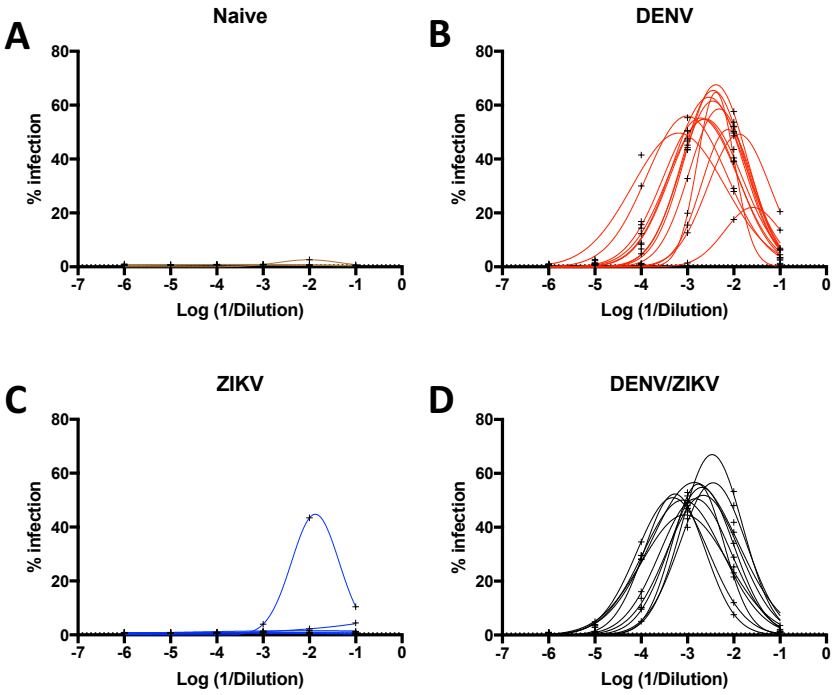

FIGURE 11 | ADE activity of polyclonal antibodies on DENV4 infection of K562 cells. Plasma samples from blood donors in the naïve **(A)**, DENV **(B)**, ZIKV **(C)** or DENV/ZIKV **(D)** groups were analyzed for ADE activity in DENV4 infection. Infectivity curves for each donor is shown. The dotted line represents the percentage of infection in the control (virus only).

We also analyzed the individual ADE activity of plasma samples from ZIKV donors against the different DENV serotypes (Table 7). Results show that while 8 out of the 9 samples from ZIKV donors did not reveal any DENV ADE activity, a strong ADE activity against DENV4 was observed with 1 sample (donor 77), this sample having a low level of neutralizing antibodies against ZIKV and DENV1-4 (Table 5, Table 7, Figure 11C). Altogether, these results clearly

show that during a primary ZIKV infection, DENV4 cross-reactive antibodies with enhancing but not neutralizing activity can be induced. Further experiments with more blood samples are needed to confirm the ADE potential for DENV4 infection in ZIKV immune donors. Interestingly, although ZIKV infection in DENV naïve individuals can elicit antibodies with ADE activity against DENV4, it seems that a ZIKV infection in DENV immune individuals boosts the ZIKV-specific antibody response with a lower ADE activity against DENV4.

Recently, cross-reactivity between DENV1 and ZIKV has been described using monoclonal human antibodies (179), suggesting that a previous exposure to DENV1 induces cross-reactive antibodies targeting the ZEDIII domain, which after ZIKV infection, boosted ZIKV neutralization activity. In order to evaluate whether such cross-reactive antibodies were present in the plasma samples of donors included in our cohort, we analyzed the neutralizing and ADE activity in samples from DENV and DENV/ZIKV donors previously infected only with DENV1 (based on the neutralization results in table 5). As shown in table 5, none of these donors in the DENV group (donors R152, R176, R213) showed a significant ZIKV neutralizing activity, and, the only two donors that showed a strong ZIKV neutralizing activity (donors 15 and R64) were exposed additionally to DENV3 or DENV2, DENV3 and DENV4, respectively. With the limit of the low number of donors, we did not observe any clear correlation between the infection with one DENV serotype and the induction of cross-reactive antibodies between DENV and ZIKV. Furthermore, there was also no clear correlation between samples showing multiple DENV infections and cross-neutralizing activity against ZIKV.

Impact of a secondary ZIKV infection on the level of neutralizing and enhancing activity against DENV

Considering that DENV and DENV/ZIKV donors exhibited neutralizing and ADE activity against DENV and ZIKV *in vitro* infections (Tables 5 and 7), we compared the level of neutralizing antibodies and the ADE activity in these two groups of donors. As shown in Figure 12A, no significant correlation was observed between the level of neutralizing antibodies and enhancing activity on ZIKV infection neither in DENV samples nor in DENV/ZIKV samples. Most plasma samples from DENV/ZIKV donors revealed a high

neutralizing/low ADE activity against ZIKV infection, and, conversely, most of plasma samples from DENV donors had no neutralizing/high ADE activity against ZIKV infection. Comparison of the neutralizing and ADE activities against DENV infection between samples from these 2 donor populations revealed that a secondary infection with ZIKV does not modify the level of neutralizing or enhancing antibodies against DENV1, 2 and 3, whereas it affects the neutralizing and enhancing activity against DENV4, by increasing the titer of neutralizing antibodies and decreasing their ADE activity (Figure 10 and Figure 12B). In all cases, a significant inverse correlation between the level of neutralizing antibodies and the ADE activity against DENV was observed.

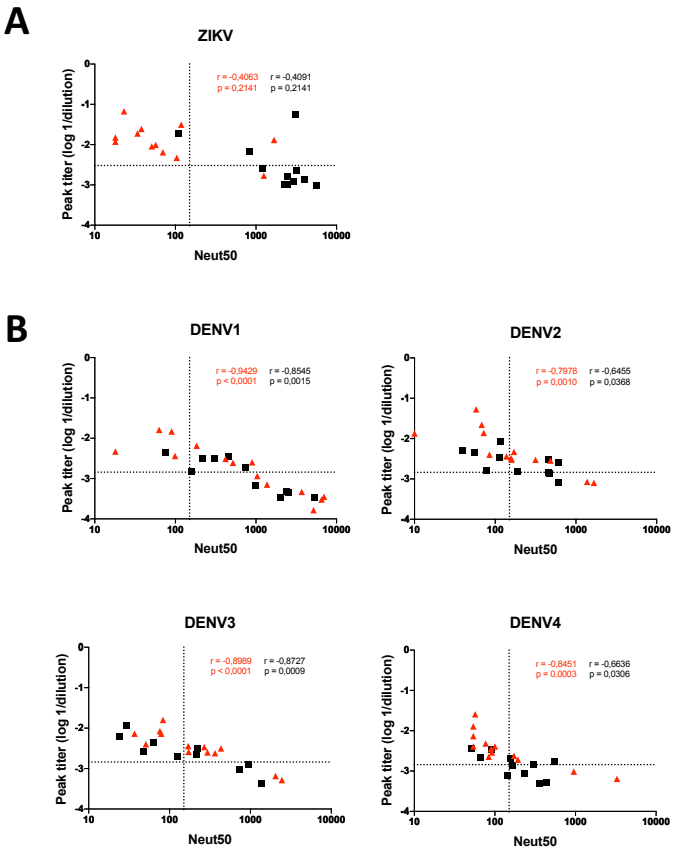

FIGURE 12 | Correlation between the neutralizing and ADE activities in DENV and DENV/ZIKV donors. The neutralizing ($Neut_{50}$) and ADE (peak titer) activities to ZIKV **(A)** and DENV **(B)** infection of plasma samples from DENV (red triangles) and DENV/ZIKV (black squares) donors, were correlated. Each dot represents one donor. The Spearman's correlation coefficient and p value are shown. The dotted lines in each axis represent the cut-offs to identified a sample with neutralizing ($Neut_{50}$ > 150) or ADE (peak titer value > -2.52 or > -2.84 for ZIKV or DENV serotypes, respectively) activities.

To facilitate a deeper understanding of this correlation, we established a cut-off value for each parameter to identify donors with specific characteristics. For instance, based on their capacity to neutralize or enhance DENV1 infection, we were able to identify plasma samples with high neutralizing potential ($Neut_{50}$ values > 1000) and low ADE activity (Peak titer values < -2.84). Likewise, we also identified plasma samples with a low DENV1 neutralizing activity ($Neut_{50}$ values between 150 and 1000) and having a moderate ADE activity (Peak titer values slightly above the cut-off). Finally, we identified samples from donors with no DENV1 neutralizing activity ($Neut_{50}$ values < 150) and a high ADE activity (Peak titer values considerably above the cut-off) (Figure 12B).

When this analysis was applied to others DENV serotypes, we also observed a similar distribution in the number of samples in these 3 groups of responders between DENV and DENV/ZIKV donors; however, for DENV4 the number of samples having no neutralizing/high ADE activity was higher in DENV donors. Most DENV/ZIKV donors were located in the region of low neutralizing /low-moderate ADE activity (Figure 12B). This difference clearly reflects the results shown above since the difference between these group of donors in the ADE activity in DENV4 was statistically significant (Figure 10A; p=0.0049) unlike the difference observed in the neutralizing activity (Figure 5C; p=0.1694).

ZIKV donors with previous DENV infection with high ZIKV neutralizing antibodies have strong T cell responses against ZIKV.

To determine whether there is a positive correlation between the T cell response and the neutralizing antibody activity against ZIKV, we compared the T and B cell responses in samples from ZIKV and DENV/ZIKV donors. Considering that E protein is the main target of ZIKV neutralizing antibodies, we initially focused our analysis against this structural protein. As we have already shown (Figures 1 and 2), the ZIKV-specific T cell responses differ between ZIKV donors and DENV/ZIKV donors. First, a significant difference was observed in the number of donors recognizing peptides from the E protein. Indeed, E specific-T cells responses were detected in 3 out of 9 (33.3%) ZIKV donors and in 8 out of 11 (72.7%) DENV/ZIKV donors, with the sum of T cells responses detected for each group of donors being 4% and 12%, respectively. In addition, the number of stimulating peptides per donor,

as well as the mean response per donor differed between these two groups, with a significantly broader response and a higher magnitude of response in donors with previous DENV infection (Figures 1B, C and 2A).

Based on these data and considering that DENV/ZIKV donors showed a higher ZEDIII-binding capacity and higher titers of neutralizing antibodies, we wondered whether a significant correlation between the level of neutralizing antibodies and the strength of T cell response could be established. While no statistically 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 donors having both a high antibody response with strong neutralization potential and a strong cross-reactive T cell responses against ZIKV (Figure 13). For instance, 6 out of the 8 DENV/ZIKV donors with ZIKV $Neut_{50}$ values higher that 1500, revealed a moderate/low ZIKV ADE activity (peak titer values > 450) and a moderate/high T cell responses (> 100 mean SFC/10^6 PBMC) against either E, NS1, NS3 or NS5 ZIKV proteins. In contrast, donors with a moderate/low ZIKV neutralizing activity ($Neut_{50}$ values lower that 1500) revealed a moderate/high ZIKV ADE activity (peak titer values < 450) and variable T cell responses against E, NS1, NS3 or NS5 proteins (Figure 13).

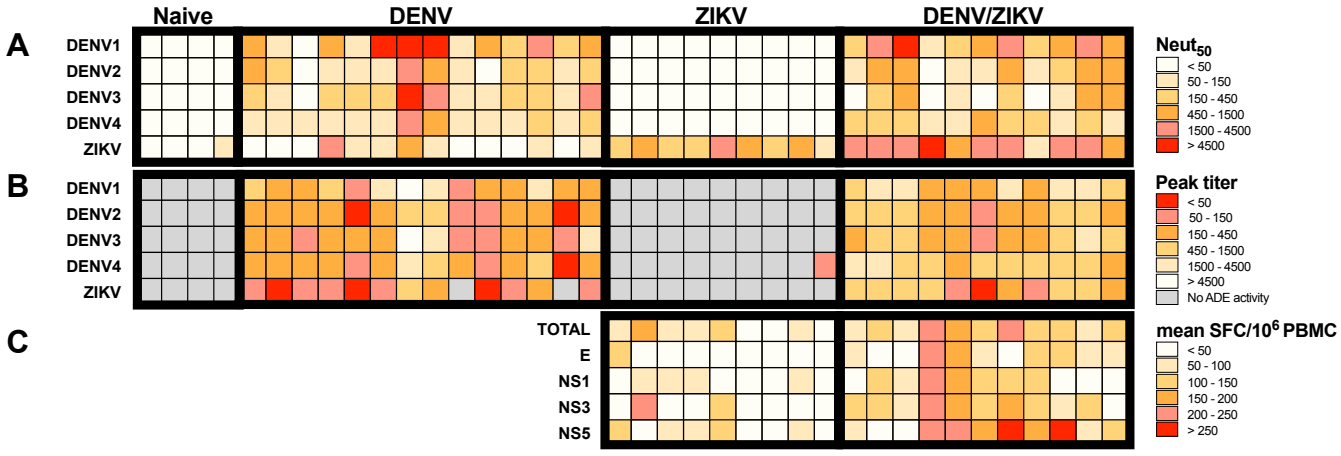

FIGURE 13 | Specificity and cross-reactivity of antibody and T cell responses from blood donors with serological evidence of previous DENV and/or ZIKV infection. Heat map of the reactivity of plasma samples for the analysis of neutralizing **(A)** or ADE **(B)** activities for each of donors included in this study. **(C)** Heat map of the mean T-cell responses against peptides from the whole proteome (TOTAL) or against the most immunogenic proteins in ZIKV (E, NS1, NS3 and NS5). The peak titer values are shown as the plasma dilution factor. The $Neut_{50}$ and Peak titer values are shown in tables 4 and 5.

Conversely, only 1 out of 9 ZIKV donors showed a high ZIKV neutralizing activity. ZIKV-specific T cell response in this donor was predominantly moderate against NS3 and NS5 and low against E and NS1 proteins. The others 8 out 9 ZIKV donors showed a moderate /low neutralizing activity and the ZIKV-specific T cell responses were predominantly low, with the exception of 1 donor exhibiting a high response against NS3 (Figure 13). Together, these results suggest that cross-reactive T cells from ZIKV-seropositive donors with previous DENV infections, which could have a strong reactivity against specific epitopes of ZIKV, could play a role in the production of antibodies with high ZIKV neutralizing activity. Additional studies on the phenotype of these cross-reactive T cells would be helpful to determine and to confirm the potential role of these T cells, in the induction and development of B cells producing antibodies with high neutralizing activity against ZIKV.

DISCUSSION AND CONCLUSION

In the first part of this study, using PBMCs from ZIKV-infected human blood donors living in an endemic region in Colombia, we have identified numerous T cell epitopes that are specific to ZIKV or shared between DENV and ZIKV. Contrary to what has been shown for DENV-specific T cell responses directed mainly against the NS3, NS4B and NS5 non-structural proteins, our results reveal that ZIKV-specific T cells target epitopes in the NS1, NS3 and NS5 proteins. We have also shown that the strongest T cell responses are observed in donors with previous DENV infection, and against epitopes with high level of amino acid identity with the 4 DENV serotypes. These data strongly support the activation of cross-reactive T cells induced upon sequential DENV and ZIKV infection. Notably, these results confirm recent studies showing the activation of cross-reactive T cells in DENV- and ZIKV-immune donors (162). However, the antigenic regions identified in our study do not match the regions identified by Grifoni et al. (162), with ZIKV-specific T cell responses mainly targeting structural proteins C, prM and E in ZIKV donors, and non-structural proteins in DENV/ZIKV donors (162).

Several hypotheses might be considered to explain these discrepancies: first, ZIKV-specific T cell epitopes identified in our cohort only partially reflects the high number of ZIKV donors reported in the study of Grifoni et al., which have been collected from different geographical locations. A higher number of ZIKV donors collected from various regions in Colombia would be helpful in confirming the immunodominant regions targeted by ZIKV-immune/DENV-naïve individuals. Second, the study of Grifoni et al. identified the most antigenic regions recognized by CD4 and CD8 T cells, using pools of peptides from different proteins, and quantifying IFN-γ producing T cells by intracellular staining and Flow Cytometry. In our study, knowing that we quantified the frequency of IFN-γ producing cells (including CD4, CD8 T cells and NK cells) by ELISpot, and considering the higher sensitivity and the lack of discrimination between IFN-γ producing cells in this assay, it is possible that the frequency of cytokine producing cells measured in our study is overestimated. A more precise quantification of the

frequency of responding CD4 and CD8 T cells, using pools of peptides and intracellular cytokine staining would allow us to define more precisely the immunodominant proteins recognized by CD4 and CD8 T cells from ZIKV and DENV/ZIKV immune individuals. Finally, the clinical status of ZIKV donors differs significantly between these 2 studies, with only asymptomatic patients in our cohort whereas symptomatic donors were analyzed in the study of Grifoni et al., (17 out of 47 donors were in acute phase of disease), which makes a comparison between the two studies difficult.

The stronger and broader IFN-γ 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. This result is also consistent with the higher percentage of identity observed between ZIKV and DENV peptide sequences in the NS5 protein, in comparison with the NS1 protein (Table 4). For instance, the peptides $NS5_{297-311}$, $NS5_{345-359}$ and $NS5_{465-479}$ identified in our study, contain the cross-reactive T cell epitopes HPYRTWAY, TPYGQQRVF and FGKAKGSRAIWYMWL, respectively, which have been recently identified from DENV/ZIKV-seropositive donors (162). Likewise, the two epitopes EAVHSDLGYWIESEK and APTRVVAAEM previously identified in the NS1 and NS3 proteins, respectively (162, 165), and having a high amino acid identity with the four dengue serotypes, were also identified as $NS1_{195-209}$ and $NS3_{219-233}$ immunodominant epitopes targeted by T cells from DENV/ZIKV donors (Table 3). Besides these epitopes, our study allowed us to identify additional cross-reactive epitopes in the NS5 protein, which are immunodominant in several DENV/ZIKV donors (Table 4). Interestingly, in addition to its lower sequence identity between DENV and ZIKV, the high NS1 secretability observed with the Asian lineages of ZIKV found in South America (180) could also explain the higher frequency of NS1-specific T cells induced in ZIKV-infected donors, in comparison with the frequency of NS1-specific T cells observed in DENV-infected donors (108).

For several epitopes, the 15-mer or 9-mer peptides matched epitopes recently identified in human samples or in transgenic mice expressing human HLA molecules, thus confirming the class I allele restriction observed for these peptides. This is the case for the 9-mer peptides

TPYGQQRVF and NHPYRTWAY, which induce a strong response in donors expressing the HLA-B*3501 allele (donors 55 and 69) or the HLA-B*3531 allele (donor 33) (Table 3) (162). The same correlation has been established with the NS5 peptide KARLNQMSALEFYSY ($NS5_{13-27}$) which contains the immunodominant epitope MSALEFYSY identified in a human donor expressing the HLA-A*0101 allele (Table 3), and in transgenic mice expressing the same allele (165). In other cases, several epitopes previously identified from transgenic mice or human samples were also identified in responding donors that nevertheless do not express this allele. This is the case for the $NS3_{219-233}$ peptide (Table 3) or the $NS1_{19-33}$ and the $NS5_{13-27}$ peptides (Table 4), which elicit a significant response in donors that express neither of the two alleles, HLA-B*0702 or HLA-A*0101 previously identified in transgenic mice (165). For these donors, one possibility could be that the epitope identified in transgenic mice has an higher affinity for a human HLA allele distinct from the allele expressed by the transgenic mice, or that the 15-mer peptide contains another epitope that binds to a different allele. For these CD8 epitopes, 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 $NS5_{293-307}$ and $NS5_{297-311}$ peptides contain the amino acid sequence HPYRTWAYH that shares seven amino acids with an epitope previously identified in Pacific Islanders infected with DENV1 (DNPYKTWAYH) (170). Similarly, the $NS5_{325-339}$ peptide contains the amino acid sequence KPWDVVTGV, which is also 66.7% identical to the epitope KPWDVIPMV identified in these individuals infected with DENV1 (170). Finally, the $NS5_{345-359}$, $NS5_{465-479}$ and $NS5_{481-495}$ peptides inducing the strongest response in DENV/ZIKV donors (Table 4) also contained 9-mer epitopes that were previously identified in DENV-infected individuals (166). 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 (181).

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 2). This is what we observed for the $NS5_{13-27}$, $NS5_{293-307}$, $NS5_{345-359}$ and $NS5_{546-560}$ epitopes, predicted to be strong binders to the HLA-B*3501 and HLA-B*4002 alleles, respectively, that are frequently expressed by ZIKV donors (Table 3). Altogether, these results show that, in the case of primary 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 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 (108). As all blood samples were obtained from donors with asymptomatic ZIKV infection history, we cannot link the strength of the ZIKV-specific T cell response 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 be also 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 (165, 182, 183). 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+ T cells in ZIKV infection and disease protection as it was shown for CD8+ T cells (183-185). 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 in specific HLA alleles and are associated with multifunctionality (108).

A major concern today about DENV and ZIKV is due to the co-circulation of these viruses in the same areas, which can lead to an exacerbation of the immune response, and in an increased disease severity in individuals living in endemic zones. Studying the different parameters of the immune response through the various stages of the disease, from the asymptomatic cases to the most severe life-threatening clinical stages would help to define the role of immunity in disease protection or progression. In this line, a strong peptide identity between ZIKV and altered human myelin proteins was suggested to induce an autoimmune reaction resulting in neurological complications, such as microcephaly, brain calcifications and GBS (186-188). Strikingly, a large number of 5- or 6-mer peptides have been identified by Lucchese et al. (187), and among them, several match the 15-mer peptides identified in our study. For instance, peptides E_{67-81} and $NS3_{219-233}$ contains sequences shared with human microcephaly-related proteins (tRNA-splicing endonuclease subunit Sen54 protein and Piccolo presynaptic protein) and peptides C_{85-99}, E_{67-81}, $E_{455-469}$, $NS1_{55-69}$, $NS1_{147-161}$, $NS1_{195-209}$, $NS3_{219-233}$, $NS5_{481-495}$ and $NS5_{546-560}$ contain sequences shared with human proteins related to myelin, (de)myelination and/or axonal neuropathies (Aspartoacylase, Adenylate cyclase type 6, Alpha-N-acetylglucosaminidase, Choline transporter-like protein 1, Gap junction beta-1 protein, Dihydropyrimidinase-related protein 2, Ezrin, Guanine nucleotide-binding protein G(o) subunit alpha, G-protein coupled receptor 6, HS71A and Myelin-associated glycoprotein precursor). While these short amino acid peptides derived from human proteins revealed a high amino acid identity with ZIKV sequence, the differences in the flanking sides of the 5 amino acid peptides makes it unlikely that these peptides are targeted by ZIKV-specific CD8 T cells, resulting in autoimmunity. However, knowing that penta- and hexapeptides can be recognized by specific antibodies (189-191), future studies of the complex interaction between ZIKV-specific CD4 T cells and B

lymphocytes in the production of antibodies against these short peptides, should help to clarify the role of the immune response against ZIKV in disease severity.

To study further the different parameters of the immune response in these donors, we have also analyzed their antibody response against DENV and ZIKV, with the objective of determining the impact of previous DENV infection in ZIKV neutralization or enhancement. Having quantified T cell responses in these donors, we have also looked for a potential correlation between the strength of the T cell response and the quality of the antibody response against ZIKV, with the objective to determine the immune parameters leading to an efficient neutralizing antibody response. To determine the quality of the antibody response against ZIKV, two different parameters were measured: first, the global amount of antibodies recognizing the EDIII domain of DENV or ZIKV, using an ELISA assay, and second, the titer of neutralizing antibody response against DENV or ZIKV, using a flow cytometry-based neutralization assay (159). Our results show that polyclonal antibodies from ZIKV immune donors with previous DENV exposure (DENV/ZIKV samples) have a high level of ZEDIII binding antibodies and a high neutralizing activity, in comparison with ZIKV immune/DENV naïve donors. While these data strongly support the induction of cross-reactive antibodies induced by previous DENV infection, no clear correlation could be established between the level of ZEDIII biding antibodies and the neutralization potential of these antibodies; several samples exhibiting a high ZEDIII-binding activity and a low/medium neutralizing activity against ZIKV infection and vice versa were observed. This lack of correlation suggests a strong diversity among cross-reactive antibodies from DENV/ZIKV samples.

One reason for the diverse specificity these cross-reactive antibodies could be linked to the different kinetics in the sequential DENV and ZIKV infection, with a higher cross-reactivity when the two infections were chronologically close to each other. Indeed, although the exact dates of infections in these donors were not registered because of the absence of symptoms, studies with sera obtained at different time points after primary DENV infection have suggested that the magnitude of cross-reactive responses depend on the length of time separating the two different infections and the frequency of the previous DENV exposure

(124, 133, 134). In this sense, and despite the high level of cross-reactivity among DENV and ZIKV and their co-circulation in the same endemic areas, which have complicated the serology-based diagnosis of ZIKV infections (104, 192), it is now clear that prior flavivirus exposures may boost cross-reactive titers to ZIKV (124, 133), and that these cross-reactive B cell responses depend on the length of time between the two infections and the frequency of previous flavivirus exposure (123, 132, 193).

In addition, the lack of significant correlation between the level of ZEDIII binding antibodies and the titer of neutralizing antibodies could also result from the expression of strongly neutralizing antibodies that target quaternary epitopes on ZIKV (123), as shown after DENV infection (194). Although this type of antibodies remains to be identified in our study, it is possible that such antibodies with high neutralizing activity against ZIKV and no binding to ZEDIII might exist among a low number of DENV donors, such as donors 15 and R64 (Table 5). Alternatively, these two plasma samples could bind to an epitope shared by DENV and ZIKV, exactly as mentioned for cross-reactive antibodies in sera of acute or early convalescent DENV-infected individuals (123, 133, 139).

In summary, we have shown that most samples from DENV donors reveal a significant and heterogeneous cross-reactivity against different DENV serotypes, whereas a fraction of DENV-infected individuals produce antibodies with cross-neutralizing activity against ZIKV. Conversely, ZIKV infection mainly elicits ZIKV-specific antibodies without any cross-reactivity against DENV. In addition, the higher neutralization titer of antibodies in samples from DENV/ZIKV donors, in comparison with samples from ZIKV donors confirms the re-activation of memory B cell responses, which are boosted upon secondary ZIKV infection, and produce cross-reactive antibodies neutralizing DENV and ZIKV, as shown recently (179).

A clear enhancing activity on DENV or ZIKV infection was also detected in plasma samples from DENV donors (Figure 8, Table 7) confirming the findings of several groups (124, 133, 137). On the contrary, not only there was no detectable enhancing activity on ZIKV infection in plasma samples from ZIKV donors, but also a secondary ZIKV infection in DENV-immune individuals decreases the ADE potential of DENV-specific antibodies against ZIKV infection

(Figure 8D and E). Interestingly, no ADE activity on DENV infection was detected in samples from ZIKV, except on the DENV4 serotype, and in only 1 out of 9 ZIKV samples (Table 7 and Figure 11C), a result that does not coincide with other reports showing that antibodies from ZIKV-immune individuals can enhance DENV infection (123). These data show that ZIKV infection in DENV-naïve individuals may induce antibodies with strong ADE activity against DENV4, whereas ZIKV infection in DENV-immune individuals increases the production of ZIKV-specific antibodies with lower enhancing potential against DENV4. Interestingly, the existence of such cross-reactive antibodies against ZIKV and DENV4 is consistent with the higher amino acid identity between ZIKV and DENV4, in comparison with DENV1, 2 and 3 (125, 139).

The inverse correlation between the titer of neutralizing antibodies and the titer at the peak of ADE activity has been already documented in the study on the role of maternal dengue-specific antibodies in the development of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) induced by DENV2 infection in infants (195). This study reported that infants born to DENV2 antibody-positive mothers are more susceptible to develop DHF/DSS upon primary DENV2 infection, when the anti-DENV2 titer wanes to sub-neutralizing levels. Similarly, it was also shown that the severity of dengue occurring after a secondary heterotypic infection is inversely correlated with the neutralizing antibody titer against the heterologous serotype, which depends on the length of time between the two sequential DENV infections (with a decrease in neutralizing antibodies over time) (196-198). In our study, we also observed an inverse correlation between the neutralizing and ADE activities of plasma samples from DENV and DENV/ZIKV donors, against infection with all four DENV serotypes. However, we did not find this correlation on ZIKV infection (Figure 12). This phenomenon could be explained by the existence of antibodies recognizing EDE epitope in the plasma samples of DENV/ZIKV donors, with strong neutralizing potential against DENV and ZIKV, that inhibit the ADE activity of DENV-specific antibodies in these donors, as shown in other studies (199) (124, 139).

Finally, to highlight a possible correlation between the activation of T cells and the production of neutralizing antibody against ZIKV, we compared in different donors the

strength of the T cell response against ZIKV E protein with the $Neut_{50}$ values against ZIKV. First, we observed that ZIKV donors with previous DENV infection exhibited a broader T cell response with a higher magnitude against ZIKV E protein (Figure 1 and 2A). However, no significant correlation between the T cell response and the level of neutralizing antibodies in these donors could be drawn. When the analysis was performed donor by donor and taking into account the T cell response against the NS1, NS3 or NS5 proteins. ZIKV donors with previous DENV infection revealed both a high ZIKV neutralizing activity and a high T cell response against these ZIKV proteins (Figure 13). Knowing that *in vitro* stimulation of T cells was performed using 15-mer peptides, it is possible that a robust activation of CD4+ T cells can provide a sufficient activation of B cells which can thus produce high levels of neutralizing antibodies against structural or non-structural proteins. This type of helper activity provided by CD4+ T cells was already described in several viral infections, even when T and B cells recognize distant epitopes on the same protein, such as Gag- and gp41-specific CD4+ T cells that help Env-specific B cells in producing Env-specific antibodies with neutralizing activity against HIV (200). In this respect, it is possible that cross-reactive T cells which are strongly activated following sequential DENV and ZIKV infection, and which target shared epitopes between DENV and ZIKV, contribute to the expansion of memory B cells producing highly neutralizing antibodies.

In addition to the magnitude of the T cell response, the quality of T cell help largely conditions the development and maturation of memory B cells to produce broadly neutralizing antibodies (201). Importantly, while the follicular helper T cells (T_{FH}) were initially identified in the Germinal Centers (GC), where they actively help adjacent GC B cells via cognate interaction, a recent report highlighted the identification of rare CD4+ T_{FH} cells in the blood (202). In this sense, it would be thus informative to identify these T_{FH} cells in the blood of ZIKV-infected individuals, to determine whether they could play a role in the induction of antibodies with strong neutralizing potential. These cells, which have been associated with the development of germinal centers, were also shown to provide the optimal signals required for B cell maturation and differentiation resulting in the production of antibodies with high affinity (203).

One of the major characteristics of this subset of CD4+ T cells is their role in the development of memory B cells in Germinal Centers (GC) which produce broadly neutralizing antibodies (bNAbs), as shown for bNAbs against $SHIV_{AD8}$ in rhesus macaques (204). In this model, the frequency of Env-responsive T_{FH} cells expressing IL-4 and CD154 correlated positively with the number of IgG+ Env-specific GC B cells and the strong antibody response (204). Although this correlation remains to be established for DENV or ZIKV infection, the identification, in DENV-infected individuals, of circulating CD4+ T cells having the phenotype of T_{FH} cells strongly suggests that these cells are interacting with DENV-specific B cells *in vivo*, and participate in the process of memory B cell development and maturation (164). In our study, it could be thus helpful to explore whether the frequency and/or the quality of ZIKV-specific T_{FH} cells could be correlated with the level of neutralizing antibodies against ZIKV upon a sequential ZIKV and DENV infection.

In conclusion, this study shows that the non-structural proteins NS1, NS3 and NS5 contain most of the immunodominant peptides that induce a strong T cell response against ZIKV. Interestingly, donors with a history of DENV infection revealed a substantial response against peptides previously identified as DENV CD8+ T-cell epitopes. The strongest T cell responses observed in these donors corresponded to sequences with a high level of amino acid identity with the four DENV serotypes, suggesting the activation of cross-reactive T cells. In addition, we have shown that samples from ZIKV-infected donors exhibited neutralizing activity only against ZIKV, with one donor exhibiting enhancing activity for DENV4 infection. The highest neutralizing activity against ZIKV infection was observed in samples from donors with previous DENV and ZIKV infection, strongly suggesting the induction of cross-reacting ZIKV neutralizing antibodies induced upon sequential DENV and ZIKV infection. While we could not establish a correlation between the high T cell response and the strong neutralizing antibody response against ZIKV in the context of a prior DENV infection, our results strongly suggest the induction of a cross-immunity with potential protective features upon sequential dengue and Zika virus infection.

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Appendix 1

Submitted manuscript to *The Journal of Infectious Diseases*

Activation of cross-reactive T cells upon sequential dengue and Zika virus infection in human

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Running title: Human T-cell response to Zika virus

Background. While many studies have focused on the antibody response against Zika virus (ZIKV), more specifically the identification of B cell epitopes shared between ZIKV and dengue virus (DENV), little is known regarding the T cell epitopes that are specific or shared between both of these viruses.

Methods. Using peripheral blood mononuclear cells (PBMC) from blood donors living in an endemic area in Colombia, with recent history of ZIKV infection, and seropositive or not for DENV, we have screened T-cell responses against 15-mer overlapping peptides spanning the ZIKV proteome by interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assay.

Results. Specific regions among the different non-structural (NS) proteins were identified as primary targets for ZIKV-specific or DENV/ZIKV-specific T cells, and the strongest responses were observed in donors with a previous history of DENV infection against peptides previously identified as DENV $CD8^+$ T-cell epitopes.

Conclusions. The stronger and broader IFN-γ 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. These studies provide new opportunities to study the role of ZIKV-specific and DENV/ZIKV shared T-cell epitopes in the induction of long-term immunity against these viruses.

Topic. Zika virus; dengue virus; T-cell epitopes; Cross-reactive T cells; vaccination.

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 [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 cause mild disease, a causal relationship between ZIKV and neurological complications, such as Guillain-Barré syndrome or congenital malformations was established only recently, with the 2013 and 2015 outbreaks in French Polynesia and Brazil [10-13].

Today, one of the most important concerns is related to the high level of DENV seroprevalence in areas where ZIKV is circulating [14]. Indeed, recent studies have shown that anti-DENV pre-existing antibodies may enhance ZIKV infection and disease [15-19]. Considering the sequence identity between DENV and ZIKV, and the protective role of DENV-specific T cells [20, 21] or DENV/ZIKV cross-reactive T cells [22], efforts are currently directed towards the mapping of T-cell epitopes to design effective vaccines against ZIKV [23]. Predictions of T-cell antigens have been conducted by modelling potential ZIKV epitopes that could bind to different HLA class I or class II alleles [22, 24-26], or by analysing *ex vivo* T-cell responses in transgenic mice expressing human HLA molecules [22]. 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 [27]. 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, 27]. Furthermore, since cross-reactive $CD8^+$ T cells were shown to control ZIKV infection in different animal models [28-32], the precise identification of ZIKV T-cell epitopes in the human that activate 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 only ZIKV infection or both DENV and ZIKV infection. More specifically, we have shown that the non-structural proteins NS1, NS3 and NS5 of ZIKV 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 with a high level of amino acid identity with the four serotypes of DENV, and some matched previously described DENV $CD8^+$ T-cell epitopes, suggesting the activation of cross-reactive T cells.

Our results provide new insights into T-cell responses to ZIKV and identify in immune individuals, novel T-cell epitopes that could be used for future ZIKV and DENV vaccine candidates.

MATERIALS AND METHODS

Ethics Statement

Human blood samples have been collected from healthy adult donors of both sexes and between 20 and 60 years of age from the Fundación Hematológica Colombia (Bogotá D.C., Colombia) in an anonymous manner. All protocols were approved by the Institutional Review Board (IRB) of El Bosque University (Colombia). A total of 82 samples were obtained from different ZIKV-endemic areas near Bogotá D.C. (Villavicencio, Meta) between September 2016 and November 2016.

Human blood samples

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

Viruses and Cell Lines

The *in vitro* assays were conducted using the DENV1 KDH0026A (provided by Dr L. Lambrecht, Institut Pasteur, Paris), DENV2 R0712259 (provided by Dr. A. Failloux, Institut Pasteur, Paris), DENV3 KDH0010A (provided by Dr. L. Lambrecht, Institut Pasteur, Paris), DENV4 CRBIP10.4VIMFH4 (from the Institut Pasteur Collection) and ZIKV KU312312 (provided by Dr. Dominique Rousset, Institut Pasteur, Cayenne). All viruses were grown using the C6/36 cells cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum, 0.1mM non-essential amino acids and 1X tryptose phosphate broth. Vero-E6 cells and U937-DC-SIGN cells were provided by Dr M. Flamand and Dr B. Jacquelin (Institut Pasteur, Paris), respectively.

HLA Typing

Genomic DNA extracted from PBMCs 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 performed according to the manufacturer's protocol (Sequence-Specific Oligonucleotides (SSO) typing; Inmucor, Lifecodes).

Serology

ZIKV-specific antibodies were detected using a recombinant antigen-based (EDIII antigen) indirect ELISA, as previously described [33]. Briefly, after coating overnight with 50ng recombinant antigen and saturation at 37°C for 1h with PBS, 3% skimmed milk, 0.1% Tween-20, plates were incubated at 37°C for 1h with 100µl of diluted plasma (1:500 in PBS 1.5% BSA, 0.1% Tween-20). Bound antibodies were detected using horseradish peroxidase-conjugated goat anti-human IgG (ROCKLAND) and TMB substrate (KPL, Eurobio). The optical density (OD) was measured at 650 nm with an automated plate reader (Tecan infinite 200 pro). Plasma samples collected before ZIKV outbreak were used as negative controls and gave a cut-off value of 0.196. The titration of DENV-specific antibodies was performed using indirect ELISA for IgGs (Panbio; Alere) and capture ELISA for IgM (Tecnosuma) following the manufacturer's instructions. The level of cross-reactivity of plasma samples against ZIKV and DENV was determined using a flow cytometry-based neutralization assay as already described [34, 35]. Briefly, 10-fold serial dilutions of plasma samples were incubated for 1 h at 37°C with a dilution of virus giving 7-15% infection. Virus-antibody mixture was then added to U937-DC-SIGN cells for DENV1-4 neutralization, or to Vero cells for ZIKV neutralization, and the percentage of infected cells was measured after 24h incubation at 37° using 4G2 antibody conjugated to Alexa-488. The neutralization titer of antibodies was expressed as the reciprocal dilution of plasma giving 50% inhibition of infection. Plasma samples collected before ZIKV outbreak, or negative samples provided from the Kits to detect anti-DENV antibodies, did not reveal any neutralization against ZIKV or DENV infection, respectively. From the 71 plasma samples selected for this study, 9 samples from ZIKV-seropositive/DENV-seronegative individuals (ZIKV donors) and 11 samples from DENV/ZIKV-seropositive individuals (DENV/ZIKV donors) were further selected for ELISPOT analysis.

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 the specific nested-PCR assay, as previously described [36]. 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 1).

Viral sequences

The identical amino acid sequence of 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.

Peptides

All peptides were synthesized by Mimotopes (Victoria, Austtralia). For the identification of T-cell epitopes, 15-mer peptides overlapping by 11 amino acids 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 overlapping by 8 amino acids were tested individually.

Ex Vivo IFN-γ ELISPOT assay

PBMCs ($2x10^5$) were incubated in 96-well plates (MSIPS 4510, Millipore, Bedford, MA) coated with anti-IFN-γ mAb (clone 1-D1K, Mabtech, Sweden) with 0.2ml of complete medium containing 10% human AB serum with pools of 12 peptides (2μg/ml, final concentration) or individual peptides (1μg/ml final concentration) for 20 hours. After three washes with PBS 0.05% Tween 20 and incubation with biotinylated anti- IFN-γ 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). Responses were expressed as number of IFN-γ spot forming cells (SFC) per 1 X 10^6 PBMCs. Values were calculated by subtracting the number of spots detected in the non-stimulated control wells, and were considered positive if the magnitude of response was > 20 SFCs and had a ratio of test SFCs to control SFCs >3.

Immunogenicity and HLA restrictions prediction

The evaluation of peptide binding affinity to MHC class I and class II alleles was analysed using the NetMHCpan3.0 and NetMHCIIpan3.1 servers, respectively [37, 38].

Statistics

All data were analysed 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 $P<0.05$.

RESULTS

Identification of immunodominant regions of the ZIKV proteome

Blood samples from all study participants were first tested for the presence of Zika virus IgG and dengue virus IgM and IgG by ELISA. Recent ZIKV infection 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 DENV infection was detected in 2 out of 11 plasma samples from DENV/ZIKV donors (**Table 1**). To confirm previous DENV or ZIKV infection in the PCR negative samples, the level of specific neutralizing antibodies against DENV1-4 and against ZIKV was also measured. Results show that while all samples from ZIKV donors had significant level of neutralizing antibodies against ZIKV, they did not neutralize DENV1-4 infection, confirming the absence of DENV-specific antibodies in these ZIKV donors. On the contrary, all samples from DENV/ZIKV donors revealed high neutralization titers against both ZIKV and DENV (**Table 1** and Supplementary Figure 1). Knowing that anti-DENV antibodies can cross-react with ZIKV [16, 18], and to confirm the expression of ZIKV-specific antibodies in samples from DENV/ZIKV donors, blood samples from DENV donors, collected before ZIKV outbreak were used as negative control of ZIKV neutralization. Samples from these DENV donors revealed a high level of DENV neutralizing antibodies, and a low level of ZIKV-neutralizing antibodies, whereas samples from DENV/ZIKV donors revealed a high level of DENV and ZIKV neutralizing antibodies (**Table 1** and Supplementary Figure 1).

PBMCs from the 9 ZIKV and 10 DENV/ZIKV donors were then screened by IFN-γ ELISPOT assay for T-cell reactivity against pools of 15-mer overlapping peptides 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 69% of the total response (**Figure 1A**). Strikingly, these NS1, NS3 and NS5 proteins represent 15%, 19% and 35% of the total response, respectively, in ZIKV donors,

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 [20, 39-41]. As a fraction of these donors were previously infected with DENV, to distinguish between ZIKV-specific epitopes and those shared by both viruses, we analysed separately T-cell responses from ZIKV donors and those from DENV/ZIKV donors. The NS1, NS3 and NS5 proteins accounted for 13%, 31% and 32% of the responses in ZIKV donors, respectively, whereas they accounted for 15%, 16% and 36% of the responses in DENV/ZIKV donors (**Figure 1B and C**). These results confirm that NS1, NS3 and NS5 are the main targets for T cells in ZIKV-infected donors, regardless of a previous infection with DENV.

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 [37, 38]. For instance, the $NS2B_{117-131}$ 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 2**). Likewise, the $E_{455-469}$ peptide in the envelope contains a sequence predicted to bind to the HLA-B*5101 and HLA-A*0201, both alleles being expressed by donors 1 and 77. This also applies to the $NS5_{13-27}$ peptide, which induced a strong response in donors 55 and 69 that share the HLA-B*3501 allele, predicted to bind to the 9-mer peptide MSALEFYSY with a high affinity (**Table 2**). Similarly, a strong T-cell response was observed against the $NS5_{546-560}$ peptide in donors 28, 53, and 66 that express the HLA-B*4002 and -B*4403 alleles and against the $NS5_{605-619}$ peptide in donors 33 and 59 that share the predicted HLA-A*2402 allele. Finally, several 9-mer epitopes in the $NS4B_{112-126}$, the $NS5_{293-307}$, $NS5_{297-311}$ and $NS5_{345-359}$ peptides, induced substantial T-cell responses in donors that share one or several alleles with a strong potential for binding to these peptides.

Remarkably, among the NS3 and NS5 proteins, several peptides have been already identified as immunodominant epitopes in mice and in humans [22, 25, 27, 42, 43]. Thus, in DENV/ZIKV donors, the $NS5_{293-307}$, $NS5_{297-311}$ and $NS5_{345-359}$ epitopes have been detected in PBMCs from HLA-B*3501 individuals, after infection with DENV or vaccination with DENV live attenuated vaccine (DLAV), [27, 41, 42, 44] (**Table 2**). Yet the 15-mer $NS3_{219-233}$ peptide, which contains the APTRVVAAEM 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 donors vaccinated with DLAV or in *ifnar-/-* HLA-B*0702 transgenic mice or in humans after ZIKV infection [22, 27, 42]. This suggests that the $NS3_{219-233}$ peptide contains another epitope or a promiscuous epitope that binds to other HLA alleles, besides HLA-B*0702 or B*3501.

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

Given the potential T-cell cross-reactivity between DENV and ZIKV against the E, NS3 and NS5 proteins [27, 32, 45], 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 1B and C**). For each protein, the number of stimulating peptides and 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**). To determine whether this difference concerned only a small number of peptides in each donor, or if it concerns 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 nine individuals among the ZIKV donors revealed a median response higher than 100 SFC/million cells, whereas six out of eleven DENV/ZIKV donors developed this strong response, which was also directed against a higher number of peptides (**Figure 2C and D**). This result strongly argues for the existence of cross-reactive T cells, primed during the initial infection with DENV and expanded thereafter during the following infection with ZIKV, as shown recently in mice and humans [22, 27, 32, 45].

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 3**). This suggests that the NS1 and NS3 proteins contain more ZIKV-specific epitopes, whereas the NS5 protein contains more epitopes 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, two out of the five epitopes that induced a response in ZIKV donors reveal a sequence identity higher than 60% with the four DENV serotypes, whereas eight out of the eleven 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 3**).

DISCUSSION

In this study, we have shown that most ZIKV-specific epitopes are located in the NS1, NS3 and NS5 proteins, with NS5 being preferentially targeted by cross-reactive T cells induced following sequential DENV and ZIKV infection. Among these epitopes, several peptides matched the sequences recently identified from ZIKV-positive donors, such as the $NS5_{293-307}$, $NS5_{345-359}$ and $NS5_{465-479}$ peptides recognized by CD8 and CD4 T cells, in the context of HLA-B*3501 and HLA-DRB1*-0701, respectively [27].

Several peptides matched also epitopes identified recently in HLA transgenic mice, or in humans exposed to ZIKV. This is the case for the $NS5_{13-27}$ peptide inducing a significant response in a donor expressing the HLA-A*0101 allele (Table 2), which contains the MSALEFYSY sequence shown to elicit a strong response in HLA-A*0101 transgenic mice [22]. 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 $NS3_{219-233}$ peptide (**Table 2**) and the $NS1_{19-33}$ or the $NS5_{13-27}$ peptides (**Table 3**), 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 9-mer epitopes identified in transgenic mice are promiscuous, or that the 15-mer peptides contain several epitopes that bind to different alleles. 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 preferentially targeted by cross-reactive T cells. Among these peptides, the $NS5_{293-307}$ and $NS5_{297-311}$ peptides (Table 2) contain the HPYRTWAYH sequence that shares seven amino acids with an epitope identified in DENV1-positive or in DENV- and ZIKV-positive donors [27, 44]. Similarly, the $NS5_{325-339}$ peptide contains the amino acid sequence KPWDVVTGV (**Table 3**), which is also 66.7% identical to the epitope KPWDVIPMV identified in individuals infected with DENV1 [44]. Finally, the strongest T-cell responses in DENV/ZIKV donors were observed with the $NS5_{481-495}$ peptide or the $NS5_{345-359}$ and $NS5_{465-479}$ peptides (**Table 3**), which contain 9-mer epitopes identified previously in DENV-infected individuals [42] or more recently in ZIKV-positive donors, respectively [27]. Altogether, these data reveal the activation of DENV/ZIKV cross-reactive T cells that dominate the response following sequential DENV and ZIKV infection.

Notably, despite a high degree of sequence identity with DENV, these cross-reactive peptides 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 [46]. 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 1**). Altogether, these results show that, following initial ZIKV infection, there is a preferential recognition of ZIKV-specific epitopes, whereas more frequent and stronger T-cell responses are induced against cross-reactive epitopes after 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 [20]. 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 [22, 28]. 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.

Notes

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Potential conflicts of interest. The authors have no conflicts of interest to declare.

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Figure legends

FIGURE 1. ZIKV-specific response magnitude and frequency of responding donors. Cumulative IFN-γ responses (as spot-forming cells (SFCs) per million cells) for each overlapping peptide spanning the ZIKV proteome is shown for **(A)** all donors, **(B)** ZIKV donors or **(C)** DENV/ZIKV donors. The heat map indicates the number of donors with a positive IFN-γ 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.

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.

Figure

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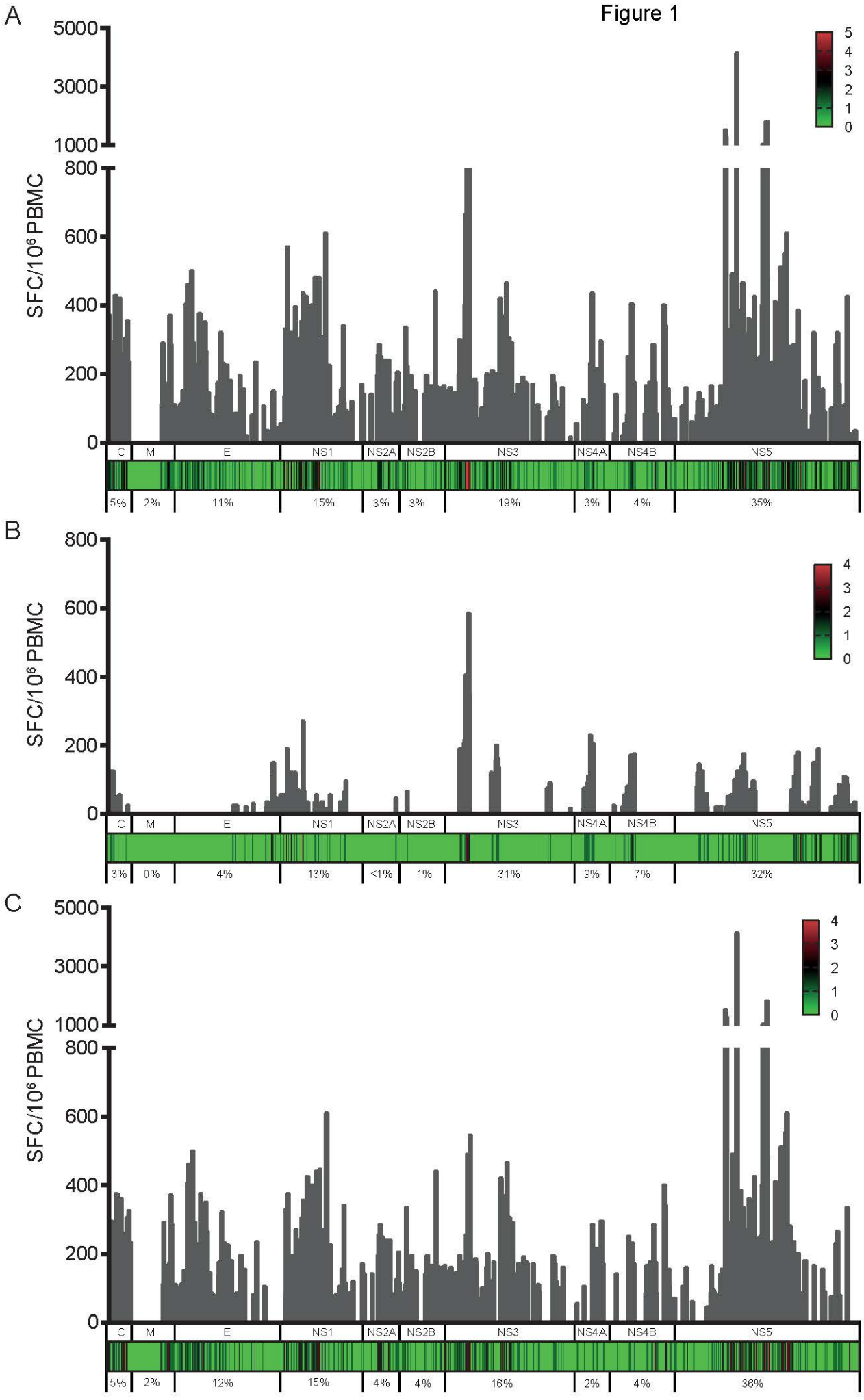

Figure

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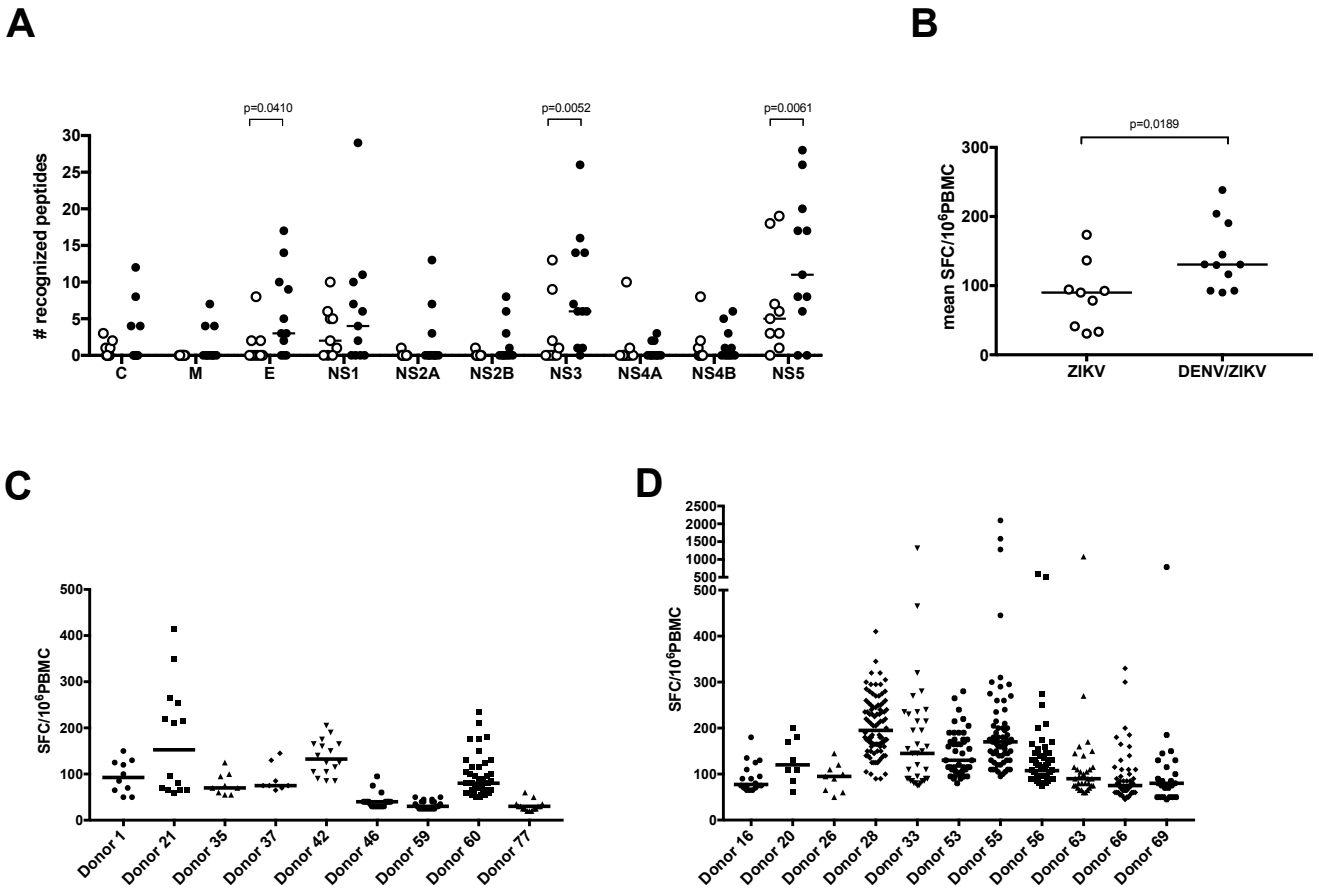
Figure 2



Table

Table 1 | Characteristics of the Zika virus patient cohort used for the epitope reactivity study

Donor[a]	Age (yr)	Gender	HLA Genotyping				Serological test			Neutralizing activity (Neut50)[c]					PCR	
							DENV		ZIKV							
			HLA-A	HLA-B	HLA-C	DRB1	IgM	IgG	IgG	DENV1	DENV2	DENV3	DENV4	ZIKV	DENV	ZIKV
1	41	Male	02:01:01	35:43:01	01:02:01	04:07:01	-	-	+	17	23	16	25	**311**	-	-
			24:02:01	51:01:01	01:02:01	12:01:01										
16	20	Female	01:01:01	15:17:01	07:01:01	13:01:01	-	+	+	158	79	47	**357**	**2270**	-	+
			03:01:01	38:01:01	12:03:01	13:02:01										
20	28	Female	01:01:01	07:02:01	07:02:01	04:11:01	+	+	+	**2049**	**452**	**214**	**438**	**2470**	+	+
			31:01:02	39:05:01	07:02:01	15:01:01										
21	26	Male	31[b]	35:01	01[b]	04:01:01	-	-	+	13	13	19	14	**566**	-	+
			03:01	18:01	17:02	03:01										
26	29	Male	02:01:01	07:02:01	01:02:01	14:02:01	-	+	+	**5397**	**601**	**735**	**235**	**2476**	-	+
			24:02:01	48:01:01	07:02:57	15:01:01										
28	35	Female	02:17:01	40:02:01	03:05	04:11:01	-	+	+	**75**	39	24	**67**	**5587**	-	-
			29:02:01	44:03:01	16:01:01	07:01:01										
33	32	Female	24:02:01	15:46	01:02:01	04:07:01	-	+	+	**306**	114	63	51	**829**	-	-
			24:02:01	35:31	03:05	04:07:01										
35	39	Female	24:02:01	14:01:01	03:05	01:03	-	-	+	11	13	10	<10	**340**	-	-
			68:01:02	40:02:01	05 :129	08:02:01										
37	34	Male	02:45	35:01:01	04:01:01	01:03	-	-	+	18	12	35	12	**280**	-	-
			11:01:01	50:01:01	06:02:01	13:01:01										
42	40	Male	26:01:01	35:01:01	04:01:01	04:02:01	-	-	+	11	12	25	12	**1689**	-	-
			26:01:01	38:01:01	06:76:02	11:04:01										
46	25	Male	32:01:01	39:01:01	06:02:01	04:07:01	-	-	+	14	11	12	11	**903**	-	-
			68:01:02	50:01:01	07:02:01	07:01:01										
53	54	Female	23:01:01	40:02:01	01:10	07:01:01	-	+	+	**464**	116	29	**543**	**3081**	-	+
			31:01:02	44:03:01	04:01:01	08:02:01										
55	23	Male	03:01:01	35:01:01	04:11:01	01:01:01	-	+	+	**2395**	**612**	**222**	**301**	**3196**	-	-
			11:01:01	51:01:01	15:02:01	07:01:01										
56	28	Female	02:01:01	15:17:01	05:01:01	03:01:01	-	+	+	**215**	55	<10	156	**110**	-	+

			02:01:01	18:01:01	07:01:01	11:01:01										
59	26	Male	03:01:01	35:43:01	01:02:01	04:01:01	-	-	+	31	30	16	24	**194**	-	+
			24:02:01	40:01:01	03:04:01	04:07:01										
60	20	Female	02:05:01	55:01:01	01:02:01	11:01:01	-	-	+	11	<10	10	10	**514**	-	+
			69:01	58:01:01	07:01:01	13:03:01										
63	24	Female	02:01:01	07:02:01	07:02:01	15:01:01	-	+	+	**999**	187	126	144	**4057**	-	-
			23:01:01	51:08:01	17:02:01	15:03:01										
66	21	Female	02:01:01	39:01:01	03:02:01	08:02:01	-	+	+	**2572**	**471**	**1386**	167	**2905**	-	-
			03:01:01	40:02:01	07:29:01	15:01:01										
69	25	Male	01:01:01	35:01:01	01:02:01	04:07:01	+	+	+	**749**	**463**	**961**	92	**1205**	+	+
			24:02:01	35:43:01	04:01:01	13:05:01										
77	18	Female	02:01:01	40:02:01	04:01:01	13:01:01	-	-	+	17	12	15	33	**110**	-	-
			02:01:01	51:01:01	07:01:01	14:02:01										

[a] The shaded rows show donors with previous DENV infection
[b] Allelic variant was not determined
[c] The values in each cell are the 50% neutralization titers determined from two replicates of one experiment. The highest titers for each sample is indicated in boldface

Table

Table 2 | Characteristics of antigenic peptides from ZIKV identified in this study

Peptide[a]	Sequence[b]	Donors	HLA[c]	SFC/million PBMC[d]		Predicted epitope	Predicted HLA	Score (rank)[e]
				15-mer	9-mer			
C_{13-27}	IVNM**LKRGVARVS**PF	28	A**02**,29; B40,44; C03,16; DRB104,07	170	<20	MLKRGVARV	A0217	1.9
		60	A**02**,69; B55,58; C01,07; DRB111,13	50	60		A0205	1.3
C_{85-99}	KKDL**AAMLRIINA**RK	26	A**02**,24; B07,48; C01,07; DRB114,15	100	<20	AAMLRIINA	A0201	4.5
		60	A**02**,69; B55,58; C01,07; DRB111,13	<20	75	KDLAAMLRI	B5501	1.4
	K**KDLAAMLRI**INARK	28	A**02**,29; B40,44; C03,16; DRB104,07	230	65		B4002	2.0
$E_{455-469}$	GMSWFSQILIGTLLM	1	A**02**,24; B35,**51**; C01,01; DRB104,12	120	NT	GMSWFSQILI	A0201	0.9
		77	A**02,02**; B40,**51**; C04,07; DRB113,14	35	NT	MSWFSQILI	B5101	0.12
$NS1_{63-77}$	MEN**IMWRSVEGE**LNA	21	A31,03; B35,18; C01,17; DRB104,03	65	50	MENIMWRSVEGELNA	DRB10405	50
	MENIM**WRSVEGELN**A	28	A**02**,29; B40,44; C03,16; DRB104,07	245	58	IMWRSVEGEL	A0217	0.5
		56	A**02,02**; B15,18; C05,07; DRB103,11	70	148		A0201	1.2
$NS1_{83-97}$	G**VQLTVVVGS**VKNPM	26	A**02**,24; B07,48; C01,07; DRB114,15	145	23	VQLTVVVGSV	A0201	1.7
		28	A**02**,29; B40,44; C03,16; DRB104,07	165	35		A0217	3
$NS1_{163-177}$	FHTS**VWLKVREDY**SL	28	A02,29;B40,44;C03,16; DRB104,07	230	75	HTSVWLKVREDY	A0101	0.4
	FHTSV**WLKVREDYS**L	55	A03,11;B35,51;C04,15; DRB101,07	110	125	HTSVWLKVR	A3101	0.4
		46	A32,68;B39,50;C06,07; DRB104,07	50	32		A6801	0.6
		20	A01,31;B07,39;C07,07; DRB104,15	310	105	VWLKVREDY	A2902	1.3
						FHTSVWLKV	B3905	0.4
							B3901	0.4
$NS1_{275-289}$	IRFEE**CPGTKVHVE**E	33	A24,24; B15,**35**; C01,03; DRB104,04	215	55	CPGTKVHVE	B3501	8.5
		55	A03,11; B**35**,51; C04,15; DRB101,07	130	115		B3531	6.5
$NS2B_{117-131}$	AAGAWYVYVKTGKRS	55	A03,11; B35,51; C04,15; DRB101,07	445	NT	AAGAWYVYVK	A0301	0.6
							A1101	0.12
						YVYVKTGKR	A0301	1.8

$NS3_{219-233}$	TVILAPTRVVAAEME	53	A23,31; B40,44; C01,04; DRB107,**08**	100	NT	TVILAPTRVVAAEME	DRB10802	1.5
		66	A02,03; B39,40; C03,07; DRB1**08**,15	65	NT	ILAPTRVVAA	A0201	1.6
$NS3_{271-285}$	LQPIRVPNYNLYIMD	42	A26,26; B35,38; C05,06; DRB104,11	165	NT	VPNYNLYIM	B3501	0.06
$NS3_{311-325}$	AAI**FMTATPPGT**RDA	28	A02,29; B40,44; C03,16; DRB104,07	255	85	AAIFMTATPPGTRDA	DRB10401	4
						FMTATPPGT	A0217	5.5
	AA**IFMTATPPG**TRDA	33	A24,24; B15,35; C01,03; DRB104,04	215	30	IFMTATPPG	A2402	5
$NS4A_{86-100}$	VTLGASAWLMWLSEI	55	A03,11; B35,51; C04,15; DRB101,07	178	NT	SAWLMWLSEI	B5101	0.9
		60	A02,69; B55,58; C01,07; DRB111,13	125	NT	VTLGASAWL	A6901	1.3
						LGASAWLMW	B5801	0.07
$NS4B_{112-126}$	**AIILLVAHY**MYLIPG	28	A02,29; B40,44; C03,16; DRB104,07	60	58	AIILLVAHY	A2902	0.6
		37	A02,11; B35,50; C04,06; DRB101,13	75	30		A1101	3.5
	AII**LLVAHYMYLI**PG	60	A02,69; B55,58; C01,07; DRB111,13	100	68	LLVAHYMYL	A0205	0.3
	AIIL**LVAHYMYL**IPG	60	A02,69; B55,58; C01,07; DRB111,13	100	35	LVAHYMYLI	A6901	0.15
							A0205	0.2
$NS5_{13-27}$	KARLNQMSALEFYSY	55	A03,11; B**35**,51; C04,15; DRB101,07	260	NT	MSALEFYSY	B3501	0.15
		69	A01,24; B**35,35**; C01,04; DRB104,13	145	NT		A0101	0.09
$NS5_{293-307}$	WFFDEN**HPYRTWAYH**	55	A03,11; B**35**,51; C04,15; DRB101,07	1580	308	HPYRTWAYH	B3501	0.4
	W**FFDENHPYR**TWAYH	69	A01,24; B**35,35**; C01,04; DRB104,13	40	218	FFDENHPY	A0101	1.6
$NS5_{297-311}$	E**NHPYRTWAY**HGSYE	55	A03,11; B**35**,51; C04,15; DRB101,07	1280	358	NHPYRTWAY	B3501	3
	ENHP**YRTWAYHGS**YE	69	A01,24; B**35,35**; C01,04; DRB104,13	75	188	YRTWAYHGSY	B3501	1.7
							A0101	0.3
	ENHPY**RTWAYHGSY**E	69	A01,24; B35,35; C01,04; DRB104,13	75	205	RTWAYHGSY	A0101	0.5
$NS5_{345-359}$	TDT**TPYGQQRVF**KEK	33	A24,24; B15,**35**; C01,03; DRB104,04	1315	395	TPYGQQRVF	B3531	0.7
		55	A03,11; B**35**,51; C04,15; DRB101,07	2095	523		B3501	0.3
		69	A01,24; B**35**,35; C01,04; DRB104,13	785	763			

$NS5_{425-439}$	**EAVNDPRFW**ALVDKE	28	A02,29; B40,44; C03,16;DRB104,07	150	100	AVNDPRFWALVDK	A0301	1.1
		55	A03,11; B35,51; C04,15; DRB101,07	120	125		A1101	0.6
		56	A02,02; B15,18; C05,07; DRB103,11	90	240			
$NS5_{461-475}$	KKQGEFGKAKGSRAI	28	A02,29; B**40**,44; C03,16;DRB104,**07**	300	NT	KKQGEFGKAKGSRAI	DRB10701	32
		53	A23,31; B**40**,44; C01,04; DRB1**07**,08	105	NT	GEFGKAKGSRAI	B4002	0.7
$NS5_{473-487}$	RAIWYMWLGARFLEF	28	A02,29; B40,44; C03,16; DRB104,**07**	210	NT	YMWLGARFL	A0217	0.03
		55	A03,11; B35,51; C04,15; DRB101,**07**	295	NT	AIWYMWLGAR	A0301	1.3
						RAIWYMWLGARFLEF	DRB10701	16
$NS5_{546-560}$	RFDLENEALITNQME	28	A02,29; B**40**,44; C03,16; DRB104,07	245	NT	NEALITNQM	B4002	0.8
		53	A23,31; B**40**,44; C01,04; DRB107,08	190	NT		B4403	0.6
		66	A02,03; B39,**40**; C03,07; DRB108,15	80	NT		B3901	1.8
$NS5_{565-579}$	LALAIIKYTYQNKVV	28	A02,29; B**40**,44; C03,16; DRB104,07	240	NT	LALAIIKYTY	A2902	0.5
		53	A23,31; B**40**,44; C01,04; DRB107,08	120	NT	ALAIIKYTY	A2902	0.25
		56	A02,02; B15,18; C05,07; DRB103,11	150	NT	LALAIIKYTY	B1517	1.2
$NS5_{605-619}$	QVVTYALNTFTNLVV	33	A**24,24**; B15,35; C01,03; DRB104,04	240	NT	TYALNTFTNL	A24:02	0.09
		59	A03,**24**; B35,40; C01,03; DRB104,04	50	42	YALNTFTNL	B35:43	0.4
							B35:31	0.25

a The position of peptides were determined according to NCBI Reference Sequence YP_002790881.1; **b** The underlined and in bold sequence correspond to the 9-mer peptide tested; **c** The common alleles between donors are underlined and in bold; **d** Cumulative SFC/million PBMC; NT, not tested; **e** Calculated using NetMHCpan 3.0 and NetMHCIIpan3.1 servers: for MHC class I, strong binders <0.5, weak binders <2.

TABLE 3 | Immunodominant epitopes in ZIKV and DENV/ZIKV donors

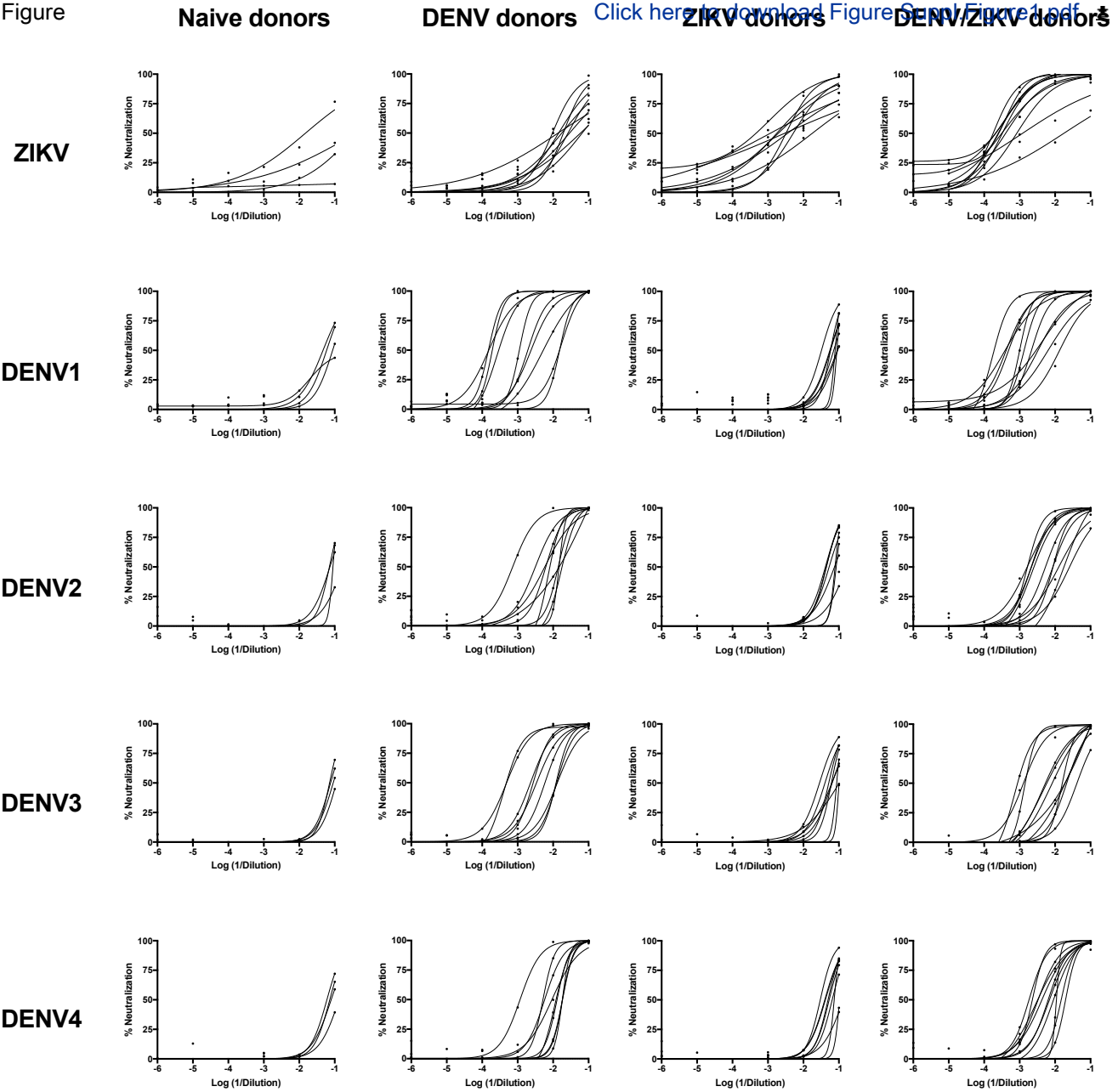
Peptide[a]	Sequence	ZIKV		DENV/ZIKV		% Identity			
		Donors	SFC/million PBMC[b]	Donors	SFC/million PBMC[b]	DENV 1	DENV 2	DENV 3	DENV 4
C_{49-63}	AILAFLRFTAIKPSL	60	60	28,63	365	60,0%	53,3%	60,0%	40,0%
E_{67-81}	DMASDSRCPTQGEAY			33	465	66,7%	53,3%	66,7%	53,3%
E_{87-101}	DTQYVCKRTLVDRGW			56	505	66,7%	53,8%	73,3%	66,7%
$NS1_{19-33}$	VFVYNDVEAWRDRYK	21,46,60	195	28,56	380	46,7%	33,3%	46,7%	40,0%
$NS1_{55-69}$	CGISSVSRMENIMWR	35,46	125	56	275	67,1%	66,3%	60,0%	60,0%
$NS1_{91-105}$	GSVKNPMWRGPQRLP	21,35,46,60	275	28	165	13,3%	33,3%	20,0%	33,3%
$NS1_{107-121}$	PVNELPHGWKAWGKS			28,53	430	40,0%	46,7%	46,7%	50,5%
$NS1_{147-161}$	HRAWNSFLVEDHGFG	46	40	33,53	445	66,7%	73,3%	66,7%	76,2%
$NS1_{163-177}$	FHTSVWLKVREDYSL	46	35	20,28,55	450	46,7%	46,3%	53,3%	46,7%
$NS1_{195-209}$	HSDLGYWIESEKNDT			28,33	615	80,0%	73,3%	66,2%	73,3%
$NS2B_{117-131}$	AAGAWYVYVKTGKRS			55	445	33,3%	33,3%	26,7%	26,7%
$NS3_{131-145}$	PAGTSGSPILDKCGR	21,42	405	26,55,63	495	53,3%	60,8%	53,3%	54,3%
$NS3_{143-157}$	CGRVIGLYGNGVVIK	21	350	20,55,63,66	550	60,0%	66,7%	72,3%	80,0%
$NS3_{311-325}$	AAIFMTATPPGTRDA			28,33	470	80,0%	80,0%	93,3%	80,0%
$NS5_{13-27}$	KARLNQMSALEFYSY			55,69	405	53,3%	46,7%	53,3%	40,0%
$NS5_{293-307}$	WFFDENHPYRTWAYH			55, 69	1620	66,7%	66,7%	60,0%	66,7%
$NS5_{297-311}$	ENHPYRTWAYHGSYE			55,69	1330	80,0%	80,0%	73,3%	80,0%
$NS5_{325-339}$	VVRLLSKPWDVVTGV			28, 55, 66	495	73,3%	80,0%	73,3%	66,7%
$NS5_{345-359}$	TDTTPYGQQRVFKEK			33,55,69	4195	93,3%	93,3%	93,3%	93,3%
$NS5_{373-387}$	QVMSMVSSWLWKELG	60	130	55,66,69	340	40,0%	53,3%	46,7%	46,7%
$NS5_{461-475}$	KKQGEFGKAKGSRAI			28,53	405	93,3%	93,3%	93,3%	86,7%
$NS5_{465-479}$	EFGKAKGSRAIWYMW			28,53,55,56	1085	100,0%	100,0%	100,0%	93,3%
$NS5_{473-487}$	RAIWYMWLGARFLEF			28,55	505	100,0%	100,0%	93,3%	100,0%
$NS5_{481-495}$	GARFLEFEALGFLNE			28,53,56,63	1870	93,3%	100,0%	93,3%	100,0%
$NS5_{546-560}$	RFDLENEALITNQME			28,53,66	515	60,0%	47,1%	53,3%	60,0%
$NS5_{573-586}$	TYQNKVVKVLRPAEK			28,53,56	615	72,9%	66,7%	73,3%	80,0%
$NS5_{849-863}$	CGSLIGHRPRTTWAE	60	90	33,55	340	66,7%	66,7%	66,7%	66,7%

a The position of peptides were determined according to NCBI Reference Sequence YP_002790881.1

b Cumulative SFC/million PBMC

Figure

Click here to download Figure Suppl Figure 1.pdf



Supplementary Figure 1: Neutralization activity against ZIKV and DENV infection using plasma samples from Naïve donors (left panels), DENV donors (middle left panels), ZIKV donors (middle right panels) and DENV/ZIKV donors (right panels). Comparison of the neutralization titer ($Neut_{50}$) against DENV1-4 infection *in vitro* between ZIKV donors with or without previous DENV infection.

Appendix 2

Published review in the *International Journal of Environmental Research and Public Health*

International Journal of
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MDPI

Review

Immune Responses to Dengue and Zika Viruses—Guidance for T Cell Vaccine Development

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Abstract: Despite numerous efforts to identify the molecular and cellular effectors of the adaptive immunity that induce a long-lasting immunity against dengue or Zika virus infection, the specific mechanisms underlying such protective immunity remain largely unknown. One of the major challenges lies in the high level of dengue virus (DENV) seroprevalence in areas where Zika virus (ZIKV) is circulating. In the context of such a pre-existing DENV immunity that can exacerbate ZIKV infection and disease, and given the lack of appropriate treatment for ZIKV infection, there is an urgent need to develop an efficient vaccine against DENV and ZIKV. Notably, whereas several ZIKV vaccine candidates are currently in clinical trials, all these vaccine candidates have been designed to induce neutralizing antibodies as the primary mechanism of immune protection. Given the difficulty to elicit simultaneously high levels of neutralizing antibodies against the different DENV serotypes, and the potential impact of pre-existing subneutralizing antibodies induced upon DENV infection or vaccination on ZIKV infection and disease, additional or alternative strategies to enhance vaccine efficacy, through T cell immunity, are now being considered. In this review, we summarize recent discoveries about cross-reactive B and T cell responses against DENV and ZIKV and propose guidelines for the development of safe and efficient T cell vaccines targeting both viruses.

Keywords: Zika virus; dengue virus; T cell epitopes; vaccination

1. History

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 (YFV), dengue virus (DENV) and West Nile virus (WNV) [1]. First isolated in Uganda in 1947 [2], it remained confined to several regions in Africa and Asia from that time until the early 2000s. In 2007, however, it caused an explosive outbreak for the first time outside of Africa and Asia, on Yap Island, Federated States of Micronesia [3,4], followed by subsequent outbreaks with higher numbers of cases in 2013–2014 in French Polynesia and other South Pacific Islands and more recently in the Americas [5–9]. Although initially believed to only cause mild disease, the 2013–2014 and 2015 outbreaks in French Polynesia and Brazil clearly revealed that ZIKV causes neurological complications, such as Guillain-Barré syndrome in adults and microcephaly in infants born to ZIKV-infected women [10–13]. Phylogenetic studies indicated the presence of two lineages of ZIKV, the African and Asian lineages, the latter being responsible for the recent major outbreaks in French Polynesia and South America [14,15]. Notably, it was suggested that the enhanced infectivity of the Asian lineage of ZIKV was due to a spontaneous mutation in the gene coding for Non-Structural Protein 1 (NS1) leading to its higher secretion in the serum and infectivity to

mosquitoes [16], which could explain its recent re-emergence in the Americas [14,15] despite its relative absence in South East Asia. More strikingly, several amino acid substitutions in the proteome or more specifically in the precursor membrane (prM) protein with possible functional implications for ZIKV biology and pathogenesis have been identified from ZIKV outbreak strains in South America [17,18].

In addition to the high infectivity of the Asian lineage in the Americas, one of the most important concerns today is related to the high level of DENV seroprevalence in areas where ZIKV is circulating [19]. This is particularly important given the structural similarities between ZIKV and DENV [20–22], and the existence of cross-reactive immune responses associated with disease pathogenesis [23–27]. Nevertheless, while it is now well established that a secondary infection with a heterologous DENV serotype represents a risk factor for the development of severe dengue disease, because of serotype cross-reactive or sub-neutralizing antibodies which can mediate antibody dependent enhancement (ADE) [28], it remains to be determined whether a previous DENV infection can also increase the risk of developing a more severe ZIKV disease in humans, as suggested by studies in mice [26]. Likewise, while ZIKV-immune plasma can enhance DENV infection in immune-deficient mice [24], the role of ZIKV immunity in protection or enhancement of dengue disease in humans is still unknown.

In this review, we address the most recent findings regarding the adaptive immune response against ZIKV, focusing on the effect of DENV pre-existing immunity on ZIKV infection, the underlying idea being to identify immunological parameters predictive of increased susceptibility or protection against ZIKV infection and disease. In this respect, we will review the current state of knowledge on the impact of anti-DENV antibodies on ZIKV infection and disease, and then summarize the recent data on the potential role of T cells in DENV and ZIKV immunity, with the aim to promote a long lasting immune protection against these two viruses.

2. Antibody Cross-Reactivity between Zika and Dengue Viruses

The high level of cross-reactivity among flaviviruses, especially DENV and ZIKV which share 54–59% sequence identity in the E protein [20,29,30], and their co-circulation in the same endemic regions have complicated serological approaches to discriminate between these two viral infections. In most cases, reverse transcription-polymerase chain reaction (RT-PCR)-based assays within a week post-infection, in combination with serological binding assays to recombinant proteins and functional neutralization assays in vitro, either by Plaque Reduction Neutralization Test (PRNT) or Flow-Cytometry-Based Neutralization Assay have been developed to distinguish between ZIKV and DENV infections [23,24,31–34].

Upon primary DENV infection, different types of antibodies have been found in polyclonal human sera: serotype-specific antibodies with strong neutralizing activity against the homologous DENV serotype, and cross-reactive antibodies with weak neutralizing activity and strong enhancing potential against heterologous DENV serotypes [35,36]. In secondary DENV infections, both type-specific and cross-reactive neutralizing antibodies, as well as cross-reactive enhancing antibodies to the other DENV serotypes, are elicited [37]. Following multiple DENV infections, the polyclonal response has been shown to contain mainly cross-reactive antibodies that recognize the different DENV serotypes, these antibodies being potentially associated with a more durable cross-protective immunity [37,38].

Upon ZIKV infection, it has been shown that the quality and type of antibody response depends on previous infection with DENV, with a more restricted specificity against ZIKV in DENV naïve individuals [24,25]. Using a panel of monoclonal antibodies (mAbs) derived from ZIKV-infected individuals with or without previous DENV immunity, it was shown notably that the antibody response against ZIKV includes envelope domain III (EDIII)-specific antibodies with strong neutralizing activity against ZIKV, as well as envelope domains I and II (EDI/II)-specific antibodies with high degree of cross-reactivity against the E protein of all four DENV serotypes and low neutralizing potential against ZIKV [24]. Thus, just like how EDI/II-specific and cross-reactive antibodies induced after DENV infection can enhance DENV and ZIKV infection [23,39–43], EDI/II

cross-reactive antibodies raised by ZIKV infection can also potently enhance DENV and ZIKV infection [24,27]. Interestingly, not all cross-reactive antibodies are enhancing, as shown in a recent study where memory B cell clones derived from different ZIKV-infected individuals could produce antibodies that neutralize both ZIKV and DENV serotype 1 (DENV1) [44]. In this case, cross-reactivity was shown to result from a previous DENV1 infection and the clonal expansion of memory B cells specific of an EDIII epitope of ZIKV and DENV1 and neutralize both types of viruses. This observation provides the first evidence for a ZIKV-neutralizing antibody response that derives from pre-existing immunity to DENV. However, the low frequency of these cross-reactive memory B cells within the polyclonal population of circulating memory B cells raises the question of the impact of such responses in the immune protection against ZIKV infection. In this sense, it would be helpful to determine whether multiple DENV infections could boost such memory B cells with a broad spectrum of neutralization, in the same manner as the DENV1-induced cross-neutralizing response [44].

At the polyclonal level, it was confirmed that while a primary ZIKV infection could induce predominantly ZIKV-specific antibodies that poorly cross-react with the four DENV serotypes, a ZIKV infection in individuals with previous DENV infections results in the production of both ZIKV-specific and DENV-cross-reactive antibodies [45]. Interestingly, depletion of these DENV-cross-reactive antibodies did not affect the level of anti-ZIKV neutralizing antibodies, suggesting that ZIKV-specific neutralizing antibodies are produced after ZIKV infection regardless of previous DENV immunity [45].

Thus, while it is clear that DENV infections can induce the production of cross-reactive antibodies that recognize the four DENV serotypes, and to a lesser extent ZIKV, in most cases, they do not induce durable, high-level ZIKV cross-neutralizing antibodies [45].

Similarly, upon primary or secondary DENV infections, it is now well established that the level of DENV-specific neutralizing antibodies decay rapidly in the absence of re-exposure, or more slowly in endemic settings [46–49], and individuals with high neutralizing antibody titers have lower probability of symptomatic infections, in comparison with individuals with low neutralizing antibody titers [49]. In this sense, a positive correlation was clearly established between the low level of pre-existing anti-DENV antibodies and the severity of secondary dengue disease in humans, with a higher risk of severe dengue with anti-DENV antibody titers of 1:21 to 1:80 and a stronger protection against symptomatic dengue disease observed with higher antibody levels [19,50]. More recently and finally, while type-specific neutralizing responses were initially thought to elicit life-long immunity against homologous reinfections [51,52], there is now clear evidence that homologous reinfection can occur in the presence of serotype-specific neutralizing antibodies, leading to symptomatic disease [53,54]. While in some cases these homotypic reinfections have been observed more than 10 years after the initial infection [53], in other cases, such reinfections were identified with a shorter time interval of 1 to 2 years between the successive infections [54]. In both situations, the specific level of pre-existing anti-DENV antibodies provides an explanation for the homologous serotype reinfection and symptomatic disease observed in individuals from different endemic regions [53,54]. Although it is too early to determine whether the same correlation applies to ZIKV infection, it seems likely that the decay in the level of ZIKV-specific neutralizing antibodies in the absence of re-exposure to this virus should affect the outcome of future ZIKV infection and disease.

3. T Cell Responses against DENV and Prospects for a Vaccine

Although the exact role of T cells during dengue virus infection and disease is still a matter of debate [55], there is currently a growing body of evidence supporting a protective role for T cells in dengue virus infection, both in human and mouse studies [56,57].

Briefly, similarly to the ADE phenomenon hypothesis associated to cross-reactive antibodies, it was proposed that DENV-specific T cells could play a detrimental role during secondary dengue infection. In this "original antigenic sin" scenario, an expansion of cross-reactive T cells with higher avidity to the previous infecting serotype would mask the specific T cell response against secondary infection, and would result in less efficient elimination of DENV-infected cells [58–60]. These cross-reactive

T cells, stimulated upon a secondary infection with a different serotype, also displayed quantitative and qualitative differences in their response to the cross-reactive epitope [61], with higher ratios of Tumor Necrosis Factor alpha (TNF-α) to Interferon gamma (IFN-γ)-producing CD4 T cells [62], suboptimal degranulation but high cytokine production [63], or more specifically impaired IFN-γ production [64].

However, in spite of these studies, the direct demonstration of a pathogenic role of DENV-specific T cells in patients experiencing natural secondary dengue infection is still missing, and recent reports do not support a causative role for cross-reactive T cells in the pathogenesis of dengue hemorrhagic fever during secondary infections [65,66]. Indeed, increased frequencies of DENV-specific $CD4^+$ and $CD8^+$ T cells were detected in school children who subsequently experienced subclinical infection, in comparison with symptomatic secondary DENV infections [67]. In addition, protection or susceptibility to severe dengue disease has been associated with the expression of certain Human Leukocyte Antigen (HLA) molecules [68–73] and a beneficial function of $CD8^+$ T cells against DENV infection was demonstrated after depletion of the CD8 T-cell compartment in interferon-α receptor knock-out mice (*ifnar*$^{-/-}$) [74]. More strikingly, a strong correlation was established between protection against severe dengue and a polyfunctional memory $CD8^+$ T cell response with a high magnitude in healthy dengue-immune individuals [75]. Finally and more recently, we observed a higher activation of Natural Killer (NK) cells and T cells in asymptomatic dengue viral infection; different T cell populations proliferate more and have an activated phenotype, with increased pathogen recognition, signal transduction and higher cytotoxic activity in asymptomatic DENV infected individuals compared to individuals with symptoms during DENV infection [76].

While $CD4^+$ T cell responses are mainly directed toward the structural proteins capsid (C) and envelope (E) and the non-structural protein NS1, similarly to DENV-specific B cells that target prM, E and NS1, most CD8 T cell epitopes reside in the non-structural proteins NS3, NS4B and NS5, and to a lesser extent in the structural protein E [60,75,77–80].

Besides the identification of DENV-specific T cell epitopes recognized by activated T cells, these studies contributed to identifying correlates of protection, as the result of an efficient T cell activation by immunodominant peptides restricted by certain HLA alleles, and a robust and polyfunctional CD4 or CD8 response [75,81–85]. This is true, for example, for the HLA-B*0702 and -B*3501 class I molecules, and the HLA-A*0101 and -A*2402 associated with a high and a low CD8 T-response frequency and magnitude, respectively [75]. The same goes for the HLA-DRB1*0401 or -DRB1*0802 class II alleles, which are associated with an increased resistance or susceptibility to severe dengue disease, respectively, and the phenotype of responding cytotoxic CD4 T cells [81].

4. Identification ZIKV-Specific and DENV/ZIKV Cross-Reactive T Cells

In contrast to a large number of studies on T cell responses to DENV infection, to date, relatively little is known regarding the analysis of T cell responses to ZIKV infection.

First predictions of ZIKV T cell antigens were conducted by modelling potential epitopes that could bind to different HLA class I or class II alleles, from the ZIKV proteome [86–88], and by identifying short peptides targeted by DENV-specific $CD8^+$ T cells with conserved sequences between DENV and ZIKV [89], with the underlying assumption that these epitopes should stimulate cross-reactive T cells after sequential DENV and ZIKV infection.

While such sequence conservation between several immunogenic $CD8^+$ T cell peptides from DENV and the corresponding ZIKV sequence was highlighted in the E, NS1, NS3 and NS5 proteins [89], a study revealed that memory T cells against NS1or E proteins were poorly cross-reactive, even in donors previously infected by DENV [24].

To identify first the dominant epitopes of ZIKV recognized by $CD8^+$ T cells in the context of human HLA class I molecules, and to clarify the protective role of $CD8^+$ T cells in ZIKV infection, different mouse models have been used. Type I interferon receptor-deficient mice expressing human HLA class I molecules (HLA-B*0702 or HLA-A*0101), which are susceptible to DENV and ZIKV

infection, enabled identification of ZIKV peptides that are targeted by $CD8^+$ T cells, and to show that dengue or Zika virus infections can induce the development of cross-reactive $CD8^+$ T cells that are protective against ZIKV infection [90]. In HLA class II transgenic mice, CD4 immunodominant epitopes have also been mapped from ZIKV Envelope, and from non-structural proteins NS1, NS3 and NS5, among which several peptides in the envelope revealed cross-reactivity with other flaviviruses [91] (Figure 1).

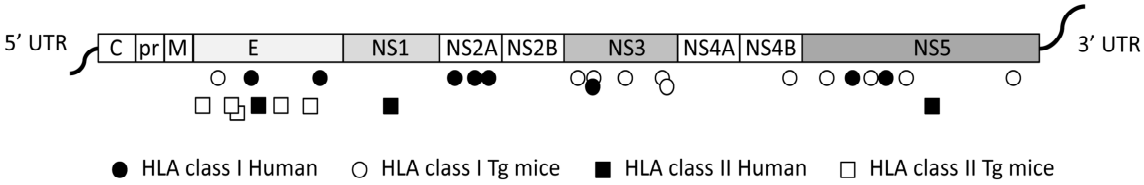

Figure 1. Schematic representation of class I- and class II-restricted cross-reactive Zika virus (ZIKV) epitopes identified in humans and transgenic mice. The ZIKV genome is not to scale. E: Envelope; HLA: Human Leukocyte Antigen; M: Protein M; NS: Non-Structural Proteins; pr: Precursor; Tg: Transgenic; UTR: Untranslated Region.

In another mouse model, which lacks the type I interferon receptor in a subset of myeloid cells [92], adaptive transfer of ZIKV-immune $CD8^+$ T cells was shown to inhibit primary ZIKV infection and replication [93]. Likewise, in *ifnar*$^{-/-}$ and in wild-type mice, adoptive transfer and depletion experiments have demonstrated that DENV-immune $CD8^+$ T cells, but not DENV-immune sera can mediate cross-protective responses against ZIKV infection [94]. Although a correlation was previously established in mice and rhesus monkeys between the level of neutralizing antibodies induced upon ZIKV vaccination and the immune protection against ZIKV challenge [95,96], these studies highlight the essential role of $CD8^+$ T cells in providing a protective immunity, in the presence or not of pre-existing DENV immunity, a finding that could help define future strategies of vaccine against DENV and ZIKV [97]. Finally, wild-type immunocompetent mice, *ifnar*$^{-/-}$ mice and Interferon Alpha Receptor (IFNAR)-depleted *rag1*$^{-/-}$ mice, have helped to characterize the phenotype of responding T cells after ZIKV infection and to demonstrate the protective effect of $CD8^+$ T cells against ZIKV infection in various organs, including the brain and testes [98–100]. However, while these studies clearly demonstrate a major role for $CD8^+$ T cells in controlling ZIKV infection, they all relied on experiments performed in mice with an altered immune system, a model that supports DENV or ZIKV infection but does not reflect the human situation. In addition, these mouse models do not take into account the human innate immune response against these flaviviruses, more specifically the NK cells that kill infected cells through the recognition of viral peptides in the context of HLA-C*0102 [101]. In summary, even though HLA transgenic mice expressing a single HLA allele do not mimic the human situation, the identification of immunodominant epitopes in these transgenic mice constitutes an important step towards preclinical evaluation of T-cell based vaccines.

For this purpose, to formally identify ZIKV-derived peptides in humans, and to determine whether a previous DENV infection can activate cross-reactive T cells, functional studies have been performed using human Peripheral Blood Mononuclear Cells (PBMC) collected from ZIKV endemic areas. From blood donors from different countries in Central and South America, the majority of the $CD8^+$ T cell responses in DENV seronegative individuals were shown to be directed against structural proteins, whereas in DENV seropositive donors, a large proportion of $CD8^+$ T cell responses were directed against the non-structural proteins with cross-reactivity for peptides in NS2A, NS3, and NS5 [102] (Figure 1). Strikingly, cross-reactive $CD4^+$ and $CD8^+$ T cells recognized peptides with identical or highly conserved sequences between DENV and ZIKV, with a higher magnitude of response, showing that DENV-specific memory $CD8^+$ T cells can enhance the T cell responses to ZIKV.

In light of the low protection observed in individuals DENV-seronegative at the time of vaccination with Dengvaxia, which lacks DENV non-structural proteins and fails to induce a competent T cell

response [103], and taking into account the role of $CD8^+$ T cells in preventing ADE [104,105] it is now becoming obvious that efficient vaccine candidates against DENV and ZIKV should be formulated to include CD4 and CD8 T cell epitopes, either alone, or in combination with B cell epitopes.

Based on the recent identification of these T cell epitopes derived from ZIKV, work is currently ongoing to define a minimal antigen, which includes the most immunodominant peptides recognized by cross-reactive T cells, and which can induce a long lasting immune protection against DENV and ZIKV infection and disease.

5. Conclusions

Our understanding of the immune response to ZIKV has dramatically increased in the last years through in vitro studies with polyclonal and monoclonal antibodies from ZIKV-infected individuals, and through the identification of the epitopes inducing a strong memory and cross-reactive T cell response. While several animal models that could mimic the human situation have been developed to clarify the beneficial or detrimental role of the different immune mediators in disease protection, further phenotypic analyses of ZIKV-specific T cells, in asymptomatic or symptomatic donors will help define correlates of protection in natural immunity and vaccination against ZIKV infection and disease.

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