Epidemiology and Immunopathogenesis of Ebola and Flaviviruses

Epidemiology and Immunopathogenesis of Ebola and Flaviviruses

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ABSTRACT

The spillover of emerging viruses into human populations continuously threatens public health. From 2013-2016, Ebola virus (EBOV) caused an explosive outbreak in West Africa with more than 28,000 cases, 11,000 of which were fatal. Simultaneously, from 2015-2016, Asian Zika virus (ZIKV) caused an unprecedented epidemic wave with an estimated 1.5 million infections and associated neuropathology throughout dengue virus (DENV) endemic regions of the Americas and the Caribbean. In response to these outbreaks, we employed seroepidemiological techniques and utilized a modified anthrax toxin delivery system to study the antibody and T cell responses in individuals infected or exposed to EBOV, African and Asian ZIKV, and DENV.

Prior to the 2015-2016 outbreak, the incidence of ZIKV in Africa had not been evaluated and the prevalence estimates for DENV were scarce. We showed continued human transmission of ZIKV and DENV and phylogenetic analysis revealed evidence for distinct African ZIKV strains circulating in West Africa for decades. T cell analysis demonstrated that individuals mount sustained specific and cross-reactive responses to nonstructural protein 3 (NS3) during the acute and late convalescent phases. Development of a T cell diagnostic based on responses to NS3 distinguishes human infections by African ZIKV and DENV. We confirmed this finding in human immunodeficiency virus (HIV)-infected and uninfected individuals from Salvador, Brazil. We also showed that HIV-infection is associated with T cell responses that are lower in

magnitude to ZIKV and DENV proteins. These findings fill a critical knowledge gap in the epidemiological research on ZIKV/DENV in Africa and have important implications for vaccine and diagnostic development.

Serological surveys conducted after the 2013-2016 EBOV outbreak revealed that a significant portion of transmission events went undetected because some individuals contracted infection with few if any symptoms. While several hypotheses could explain this phenomenon, including properties of the infecting virus, low inoculum, route of transmission, or various host factors, a robust immune response is also a potential explanation. We identified EBOV antibody-positive individuals from Lagos, Nigeria and the Democratic Republic of Congo who had not experienced Ebola virus disease (EVD)-like illness. We showed that the seropositive asymptomatic individuals from Lagos had virus-specific T cell responses to the nucleoprotein, matrix protein, and glycoprotein that were greater in magnitude when compared to survivors of severe EVD. These findings suggest that T cell immunity may protect against severe EVD, which has important implications for understanding the immunopathogenesis of EVD and EBOV vaccine development.

CHAPTER 1

Introduction

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CHAPTER 1

Introduction

Filoviridae

Overview

Filoviridae is divided into three genera including *Ebolavirus*, *Marburgvirus*, and *Cuevavirus* (1). There is great divergence within the *Ebolavirus* genus including five recognized species: *Zaire ebolavirus* (EBOV), *Sudan ebolavirus* (SEBOV), *Reston ebolavirus* (REBOV), *Tai Forest ebolavirus* (TAFV - formerly *Cote d'Ivoire ebolavirus*, ICEBOV), and *Bundibugyo ebolavirus* (BEBOV). There is less divergence within the *Marburgvirus* genus with two species, *Lake Victoria marburgvirus* (MARV) and Ravn virus (RAVV). A distinct filovirus sequence was recently obtained from bats in Spain; however, a virus has yet to be isolated. This "putative virus," designated Lloviu virus, represents the single species *Lloviu cuevavirus* within the *Cuevavirus* genus.

Due to the high the mortality rate associated with infection, potential for person-to-person transmission and bioterroism, and lack of approved vaccines or immunotherapeutics, *Filoviridae* are classified as biosafety level 4 (BSL-4) pathogens, for which maximum containment facilities are required when handling infectious agents. There is an urgent need for improved understanding of the immunobiology of *Filoviridae.* The focus of this thesis is on the epidemiology and human immunology of EBOV.

Genome structure and function

Filoviridae contain a single-stranded, negative-sense, ~19,000-base-long RNA genome with seven sequentially arranged genes including the nucleoprotein (NP), virion protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, and the RNA-dependent RNA polymerase (RdRp), L (1). Each gene is flanked by highly conserved start and stop signals and are separated by short intergenic regions spanning 4-7 nonconserved nucleotides. The genome 5' and 3' ends consist of short nontranscribed regions containing cis-acting signals important for replication, transcription initiation, and RNA genome encapsidation (2-5).

Filoviridae encode at least 7 structural proteins including NP, VP35, VP40, GP, VP30, VP24, and L. In the case of Ebola virus species, an additional secreted glycoprotein (sGP) is produced by transcriptional editing of the GP mRNA. As the only surface protein on the virion, GP mediates receptor binding and fusion during virus entry into cells (6-8). VP40, similar to the matrix protein of other viruses, is a peripheral membrane protein and plays a role in the budding of viral particles (7, 9). VP24 is involved in nucleocapsid formation and assembly, and also counteracts the type I interferon response (10-12). NP, the polymerase cofactor, VP35 (also a type I interferon antagonist), the transcription activator VP30, and the RdRp L are associated with the viral RNA genome (13, 14). These proteins form the nucleocapsid and are involved in viral morphogenesis and catalyze the replication and transcription of the RNA genome (15-18). The function of the EBOV sGP is not entirely understood. It is thought that sGP acts as an antibody decoy and/or an anti-inflammatory factor by protecting the endothelial cell barrier function during infection (19, 20).

Life cycle

Filoviruses infect a variety of mammals, which has complicated the identification of cellular proteins required for viral entry (1). However, studies have shown that the filovirus replication process begins when virion attachment is mediated by the binding of GP to a diverse group of cell surface factors (1). To date, several factors have been reported as EBOV receptors or co-receptors. The C-type lectin family contains carbohydrate recognition domains (CRDs) that bind to the highly glycosylated glycan cap on GP (21). Additionally, asialoglycoprotein receptor (ASGP-R), dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN), human macrophage galactose and acetylgalactosamine-specific C-type lectin (hMGL), and lymph node sinusoidal endothelial cell C-type lectin (LSECtin/CLEC4G), have all been shown to facilitate viral attachment (22-25). Once filoviruses are macropinocytosed into cells and internalized into endosomes, host cellular proteases cleave the GP causing fusion between the virion and vesicle, mediated by Niemann-Pick C1 (NPC1), and resulting in the release of the viral genome into the cell (26-28). Further steps of filoviral replication are believed to occur in the cytoplasm. The viral particle uncoats and its anti-genome is transcribed into mRNA resulting in the production of at least 7 proteins. Transcription of the genome is mediated via a complex of VP30, VP35, and L bound to a NP-coated genome (18, 29). Disassociation of VP30 from the complex signals a switch from transcription to replication (30). During assembly, L associates with ribonucleoproteins and VP40, and viral particles extrude through the plasma membrane (31).

Epidemiology and pathology

In mid-August 1967, an epidemic of MARV started in Marburg, Germany with three laboratory workers who contracted Marburg hemorrhagic fever (MHF) after processing organs from African green monkeys imported from Uganda. There was a total of 31 cases during this outbreak with seven deaths (23% case fatality rate, CFR) (32). MARV remained obscure until 1975 when three cases were reported in Johannesburg, South Africa (33% CFR). Further outbreaks of MHF have been reported in Kenya in 1980 and 1987 (50% and 100% CFR, respectively), Kosovo, Russia in 1988 and 1990 (100% and 0% CFR, respectively), the Democratic Republic of Congo (DRC) in 1998 (83% CFR), Angola in 2004 (90% CFR), and Uganda in 2007 (25% CFR) (33). The acute clinical presentation of MHF is generic flu-like symptoms, a characteristic high fever, severe headache, chills, myalgia, prostration, and malaise (34). In most cases, this follows with gastrointestinal symptoms including abdominal pain, severe nausea, vomiting, and watery diarrhea. By day 5 to 13, infected individuals display neurological symptoms including encephalitis, confusion, delirium, and irritability with clear hemorrhagic manifestation such as mucosal bleeding, bloody diarrhea, hematemesis, and ecchymosis. At this stage, multiple organs are negatively impacted including the pancreas, kidney, and liver, and death often ensues.

Ebola virus disease (EVD) was first reported in 1976 during two simultaneous epidemics in the DRC (88% CFR) and Sudan (53% CFR) (35, 36). These epidemics were determined to have been caused by two distinct species (EBOV and SEVOB). Since then, more than 30 outbreaks have been recorded mostly in central Africa with CFRs ranging from 25-90% (37). In 1994, TAFV was isolated from an ethnologist who

had become infected while performing a necropsy on a dead chimpanzee (38). In 2007, BEBOV was responsible for an outbreak in the Bundibugyo district in western Uganda (39). This outbreak has the lowest reported CFR among all Ebola viruses that have caused outbreaks in central Africa to date. In 1989, REBOV was recognized in a shipment of cynomologus monkeys housed at a quarantine facility in Reston, Virginia (40). These monkeys were imported from the Philippines. During import, an unusually high mortality was observed in animals; however, no human pathogenicity by REBOV has been reported. In 2013, an outbreak of EVD was detected in Guinea, which spread rapidly throughout the West Africa (41). Approximately, 28,000 symptomatic cases were reported with more than 11,000 deaths. In a majority of cases, the clinical presentation of EVD resembles that of MHF with hematological, lymphatic, and immunological manifestations (42). There is also increasing evidence to suggest that, in a minority of cases, EVD may be associated with mildly symptomatic or asymptomatic infection (43-46). The immunopathogenesis of asymptomatic EVD has not been well investigated.

Humoral responses to Ebola virus

While innate immunity may play an important role in controlling EBOV early during infection in humans, there is limited data in the current literature. Largely based on animal model data, the current thinking is that adaptive immune responses are critical for viral clearance and recovery. Various mouse, guinea pig, and nonhuman primate models have been exploited to better understand EBOV pathogenesis. Passive immunotherapy with convalescent sera or monoclonal antibodies has prevented

disease in mice and guinea pigs and delayed viremia and death in nonhuman primates (47, 48). Additional studies have shown a correlation between early IgM responses and subsequent IgG isotype switching with protection (49). These observations were further supported by individuals evacuated to the United States and Europe for medical treatment during the West African EVD outbreak. EVD survivors mounted early IgM responses and had increasing levels of serum IgG over the course of infection (50, 51). In contrast, limited IgM and IgG responses have been shown to be associated with fatal EVD and MHF (52, 53).

Neutralizing (N_{Ab}) and non-neutralizing (non-N_{Ab}) antibodies are important for protection against many viral infections; however, their role during EVD has not been fully elucidated and, thus far, studies have reported conflicting results. Early during EBOV infection, individuals elicit little to no N_{Ab} and non-N_{Ab} responses, suggesting a minimal role in the acute phase of infection (54, 55). Nevertheless, long-term survivors develop functional and cross-protective N_{Ab}, mainly targeting the Ebola virus and MARV GPs (56-59). Non-N_{Ab} with antibody-dependent cellular cytotoxicity (ADCC) activity have also been detected in long-recovered EVD survivors (58, 60). These findings suggest that antibody-mediated immune memory may provide long-term protection against secondary filovirus infections. Additionally, recent studies in individuals who experienced asymptomatic EVD have also detected EBOV-specific antibodies (43, 44, 46). The epitopes in EBOV for which these antibodies engage and their antiviral mechanisms have yet to be investigated.

T cell responses to Ebola virus

T cells are important for viral clearance especially during the acute phase of infection (61). Naïve T cells recognize pathogen-specific peptides bound to major histocompatibility complex (MHC) molecules expressed on the surface of other immune cells such as dendritic cells (DC) (62). Recognition of a pathogen-specific peptides triggers T cell expansion and differentiation into effector cells that migrate to peripheral sites of infection for removal of infected cells.

It was originally hypothesized that T cells would not be important for protection against EBOV since infection results in DC inactivation, resulting in poor T cell priming, and subsequent T cell apoptosis (53, 63). While these findings are still valid, studies during the West Africa outbreak revealed dynamic and potentially protective CD4+ and CD8+ T cell activity against EBOV (50, 51). Limited evidence has shown that the EBOV NP drives most of the T cell response; however, responses have been detected against other proteins such as the GP and VP40 (64). Additionally, co-expression and activation markers such as CD38 and HLA-DR, as well as the proliferation marker Ki-67, were detected in a significant percentage of CD4+ and CD8+ T cells in EVD patients (64, 65).

An important question, therefore, is why T cell activation does not lead to EBOV clearance. Studies have shown that T cell ineffectiveness may be related to defects in negative immune checkpoints, namely the molecular mechanisms that control the transition from activation to homeostasis (66). An early review hypothesized that expression of immune checkpoint markers PD-1 and CTLA-4 in T cells could be associated with dysfunction resulting in a nonfunctional but reversible status termed T

cell exhaustion (67). Indeed, a recent study found that T cells from EBOV-infected individuals express high levels of PD-1 and CTLA-4, which were significantly higher among the fatal cases (65). For this thesis, I developed a T cell-based enzyme-linked immunospot (ELISPOT) assay to detect and characterize *ex vivo* T cell responses against EBOV. Utilizing this assay, I identified EBOV antibody-positive individuals who never had symptoms, but have EBOV-specific T cell responses that were greater in magnitude when compared to individuals who experienced symptomatic EVD, which implicates the important role of the immune response in the development of severe EVD (Chapter 2).

Ebola virus diagnostics and surveillance

The ability to detect EBOV is an essential component of effective outbreak management; however, establishing safe and efficient diagnostic platforms for this BSL-4 pathogen in resource-poor environments remains challenging. During the 2013-2016 West African EVD outbreak, diagnosis relied primarily on testing venipuncture blood samples from symptomatic individuals in biocontainment settings, leading to delayed results and proper treatment and care. Subsequently, there has been an unprecedented surge in the development of rapid and point-of-care diagnostics for EBOV.

Historically, the presence of EBOV has been confirmed by virus isolation in cell culture, typically using Vero E6 African Green monkey kidney cells, combined with electron microscopy or immunofluorescence for a visual readout (1). While detection of EBOV is often accurate, these methods are generally restricted to BSL-4 laboratories

with high skilled laboratory technicians. Serological assays, such as the enzyme-linked immunosorbent assay (ELISA), have been used to detect EBOV-specific IgG and IgM (49, 68). However, limited data exist assessing the sensitivities or specificities and the exact diagnostic parameters of many of these ELISAs have not been well validated in the clinic further hindering diagnostic interpretation. A recent study of acutely ill EVD patients infected during the West African outbreak showed the onset of IgM and IgG responses between 6 and 11 days and 9 and 11 days after symptom onset, respectively (69). In many cases, EBOV-infected individuals become deathly ill by day 5-13 rendering ELISAs often ineffective. Notably, however, ELISAs continue to serve as useful tools for population-level seroprevalence studies (43, 44, 46). Detection of EBOV particles via protein antigen capture or nucleic acids via (real-time)-RT-PCR have also been extensively used, often with many of the same challenges described above (70, 71). Finally, promising strategies relying on CRISPR and biosensor technologies are in development to offer field-applicable, cost-effective, and point-of-care EBOV detection (72).

These diverse diagnostic methods have proven useful in diagnosing EVD, as well as providing critical information during outbreak situations. Over a 7-month period during the 2013-2016 West African outbreak, 232 full-length EBOV genomes were assembled by coupling high-throughput Illumina sequencing with bioinformatics pipelines (37, 41). In doing so, the movement of EBOV throughout Sierra Leone was closely tracked and these data demonstrated that the enhanced EBOV human-to-human transmission was due to a single amino acid mutation in the virus. These studies, enabled by genome sequencing technologies, were not only important for

detecting the virus and understanding the genetics driving enhanced EBOV transmission in humans, but they also emphasized the need for strict national border protocols including fever-screening as well as the need for community outreach interventions.

Ebola virus vaccines and therapeutics

Despite the significant development of products for anti-EBOV use, a licensed treatment for EVD remains elusive. A number of nucleic acid-based inhibitors had shown promise in nonclinical studies prior to the West African outbreak including antisense phosphorodiamidate morpholino oligomers (PMOs) and small-interfering RNAs (siRNAs) (73-75). Concern continues to revolve around these types of antivirals because they tend to be sequence specific and may be ineffective against new outbreak variants. A combination PMO therapeutic, which targets EBOV VP24 and VP35, demonstrated efficacy in NHP and safety Phase I studies; despite these promising results, the product was withdrawn from development, which is a common phenomenon seen in the pharmaceutical industry (76, 77). An anti-EBOV siRNA product had shown efficacy NHPs, but because this siRNA was based on the 1995 EBOV-Kikwit variant, it contained mismatch siRNA targets when tested against the West African Makona variant (78, 79). This led to the development of siEbola3, which demonstrated efficacy against EBOV-Makona; however, when tested in Sierra Leone during the outbreak on 14 individuals, the pre-specified futility boundary was reached, indicating a low probability of survival, and the trial was terminated (80). Immunotherapeutics including convalescent plasma, ZMapp, and Interferon-β (IFN- β)

were also tested during the West African outbreak. Convalescent plasma was tested in three separate clinical trials. Only one of the trials has reported results, which demonstrated no significant difference in protection between those that received the plasma and those that did not (81). ZMapp, a cocktail comprised of three murine monoclonal antibodies targeting EBOV GP, had shown promise in NHPs, however, when tested in the PREVAILII study, it did not meet the pre-specified threshold for efficacy (82, 83). A possible explanation is that none of the monoclonal antibodies target the EBOV receptor binding site, and thus, EBOV is still capable of entering cells and causing disease. Finally, IFN-β had shown suppressed EBOV infection in NHPs and was tested in humans in Guinea (84, 85). Human data for this trial is currently not available.

Conclusion

As described above, the unprecedented magnitude of the West African EVD epidemic allowed, for the first time, in-depth evaluation of clinical, epidemiological, and immunological aspects of human EBOV infection. These studies helped accelerate the development of rapid diagnostics, promising vaccines and immunotherapeutics, and highlighted the importance of a unified global public health system. Despite these advances, our basic understanding of the basic immunobiology of EBOV infection in humans remains limited. There is a need for continued investigations by combining innovative experimental strategies with epidemiology. Thus, this thesis focused, in part, on the seroepidemiology and immunopathogenesis of symptomatic and asymptomatic EVD in Africa.

Flaviviridae

Overview

Flavivirdae is divided into three genera including *Flavivirus*, *Pestivirus*, and *Hepacivirus*. A fourth genus, *Pegivirus*, has been proposed to encompass the previously unclassified GB viruses. As detailed below, the *Flaviviridae* share similarities in virion morphology, genome organization, and replication strategy, but exhibit diverse biological properties and are the cause of a variety of human pathologies. The increasing re-emergence of *Flaviviridae*, and particularly viruses of the genus *Flavivirus*, emphasizes the need for continued research. The focus of this thesis is on the epidemiology and human immunology of Zika (ZIKV) and Dengue (DENV) viruses.

Genome structure and function

Flaviviridae contain a single, positive-strand, ~11,000-base-long RNA genome with a 5' type 1 cap, m^7GpppAmN (1). The cap serves to stabilize the viral RNA, initiate translation, and subvert innate antiviral defenses. The genome encodes a large open reading frame (ORF, ~3,400 codons) flanked by 5' and 3' noncoding regions (NCRs) of ~100 nucleotides (nts) and 400 to 700 nts, respectively. The ORF produces a single polyprotein that is co- and post-translationally cleaved into 10 proteins. The N-terminal region of the polyprotein encodes the structural proteins (Capsid, C; precursor membrane, prM; and the Envelope, E), followed by seven nonstructural (NS) proteins (NS1, NS2, NS2B, NS3, NS4A, NS4B, and the RNA-dependent RNA polymerase, NS5).

Host and viral proteases are responsible for cleavage between C and prM, prM and E, E and NS1, NS2A and NS2B, NS2B and NS3, NS3 and NS4A, NS4A and NS4B, and NS4B and NS5. The C protein is involved in packaging the viral genome and forming the nucleocapsid core (86). The glycoproteins prM and E each contain two transmembrane helices. The prM protein is thought to act as a chaperone to promote folding and assembly of the E proteins (87). The E protein contains one or more cellular receptor-binding sites and a fusion peptide important for the initial attachment of the viral particle to the host cell (87-89). The nonstructural proteins NS1 (involved in RNA replication and particle assembly), NS2A (involved in RNA replication and immune evasion), NS2B (cofactor of NS3), NS3 (major viral protease), NS4A (cofactor of NS3), NS4B (involved in RNA replication and immune evasion) and NS5 (RNA-dependent RNA polymerase, involved RNA synthesis and modification) are essential for viral replication (90-92).

Life cycle

The flavivirus replication process begins when a virion attaches to a diverse group of host cell receptors or when the Fc portion of a flavivirus-containing immune complex attaches to a Fc receptor on the target cell and subsequently enters by receptor-mediated endocytosis (1). The initial targets for DENV and West Nile virus (WNV) are immature dendritic cells and Langerhans cells (93, 94). A likely receptor for ZIKV is Gas6-AXL tyrosine kinase receptor complex found in the brain, muscle, and male reproductive tissues (95-97). Once the virion is in the endosome, acidification triggers the irreversible trimerization of E, mediating fusion between the viral and the

endosomal membranes, allowing the release of the nucleocapsid into the cytoplasm (98). The viral RNA is subsequently released into the cytoplasm and translated into a single polyprotein that is processed by viral and host proteases. The viral replication complex is synthesized at which point RNA translation switches off and RNA synthesis begins by the transcription of an antisense viral RNA followed by the amplification of viral RNA. The viral RNA is packaged by C, forming a nucleocapsid. The nucleocapsid buds into the endoplasmic reticulum (ER) lumen where assembly occurs of non-infectious, immature virions containing E, prM, the lipid membrane, and the nucleocapsid. Immature virions transit from the ER through the trans-Golgi network, where acidification induces conformational changes of the virion and exposes the furin cleavage sites (99-102). After cleavage of prM, the pr peptides dissociate from virions readying them for the low-pH-triggered fusion event during cell entry.

Dengue and Zika virus epidemiology and pathology

Flaviviruses, including DENV, Japanese encephalitis virus (JEV), WNV, Yellow Fever virus (YFV), and ZIKV, are transmitted to humans by mosquitoes of the genus *Aedes*, notably *A. aegypti* and *A. albopictus* (1). The global distribution of *Aedes* species mosquitoes, particularly in the tropical and subtropical regions of the world, explains the heavy burden of flavivirus transmission and presents a constant risk for the spread of flaviviruses into non-endemic areas (103).

DENV refers to a group of genetically and antigenically related viruses that are known as serotypes (DENV1-4); all four serotypes co-circulate in most regions in which the mosquito is endemic. DENVs are responsible for an estimated 390 million

infections annually (104). Most DENV infections, known as dengue fever (DF), are inapparent and pass with minimal or no symptoms. The clinical presentation of DF is typically an acute illness lasting approximately 4-7 days accompanied with fever, chills, and malaise (105). Symptoms and signs of infection resolve in the majority of cases (106). However, spontaneous bleeding, plasma leakage, or both may occur in the course of infection; these infections are known as dengue hemorrhagic fever (DHF). Plasma leakage in DHF results in decreased plasma volume, haemoconcentration, and pleural and peritoneal effusions, and life-threatening shock and death often ensues (107).

ZIKV, named after the Ugandan forest from which it was isolated, is genetically similar to DENV. It was first isolated in 1947 in a macaque monkey and shortly thereafter recognized to cause a mild febrile illness including fever and rash in humans (108). For decades, ZIKV was considered an obscure virus and thought to be confined to Africa and parts of Asia (109). However, in mid-2015, an outbreak of ZIKV erupted in northeast Brazil with associations between ZIKV infection and fetal microcephaly and other neurological pathologies such as Guillain-Barré syndrome in adults (108, 110, 111). New modes of ZIKV transmission were also revealed including maternal-fetal routes and sexual transmission. By February 2016, ZIKV had spread throughout the Americas and the Caribbean and the World Health Organization officially declared it a Public Health Emergency of International Concern, which ended in November 2016 (112).

Humoral responses to dengue and Zika viruses

Upon a primary DENV infection, serotype-specific and cross-reactive antibodies are found in human serum (113). Serotype-specific antibodies strongly neutralize homologous DENV serotypes and are postulated to confer life-long immunity against disease caused by the homologous serotype. On the other hand, during a secondary DENV infection, cross-reactive antibodies that are weakly neutralizing are thought to contribute to antibody-dependent enhancement (ADE) (114-116). ADE occurs when low avidity antibodies opsonize instead of neutralize a virus and promote viral uptake into Fc receptor-bearing cells leading to enhanced viral replication. Individuals with tertiary or quaternary DENV infections are presumed to have only cross-reactive antibodies. In the context of secondary ZIKV with primary DENV infections, it is thought that the DENV-specific antibodies could cross-protect against ZIKV.

Antibodies from RT-PCR confirmed acute DENV-infected individuals were found to bind and neutralize ZIKV, demonstrating the capacity to protect against heterologous infection after secondary flavivirus infections (117). However, late convalescent DENV-immune sera exhibit limited cross-neutralization against ZIKV, resembling the serotype-specific phenotype. In another study, long-lasting cross-reactive antibodies from DENV neutralized ZIKV, but to a lower extent than they neutralized DENV (118, 119). Similarly, convalescent ZIKV-immune sera neutralized ZIKV to a greater extent than they neutralized DENV. In a different study, the monoclonal antibody, K562, isolated from a ZIKV-infected individual was shown to enhance DENV infection, consistent with the ADE theory (120). To evaluate the potential for ADE *in vivo*, two DENV or ZIKV monoclonal antibodies specific for E were

administered to AG129 mice prior to infection with DENV (120). Both monoclonal antibodies lethally enhanced DENV infection in this mouse model of DENV-induced vascular leakage.

T cell responses to dengue and Zika viruses

The role of HLA-linked T cells in DENV infection has been extensively studied. These studies have implicated T cells in both protection and pathogenesis and have demonstrated preferential T cell targeting of the NS proteins, namely NS3 and NS5 (121-125). T cell responses to ZIKV have been less investigated. One study showed that CD8+ T cell depletion results in higher ZIKV viremia in mice (126). Further, at the peak of ZIKV infection, CD4+ T cells polarized to a Th1 profile and produced interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin two (IL-2) (126). As with antibodies, pre-existing DENV immunity may impact the T cell response against ZIKV. To investigate this hypothesis, a study in DENV-immune mice infected with ZIKV, demonstrated an expansion of ZIKV/DENV cross-reactive CD8+ T cells, whereas DENV-naïve mice challenged with ZIKV developed ZIKV-specific CD8+ T cells (127). Moreover, ZIKV-infected mice immunized with ZIKV-specific or ZIKV/DENV cross-reactive peptides elicited CD8+ T cell responses that were protective, as depletion of CD8+ T cells resulted in increased ZIKV infection (127). These studies demonstrate differential T cell responses between DENV- naïve and DENV-immune mice and demonstrate a protective role for CD8+ T cells against ZIKV infection.

Few studies exist to date on the role of T cells in human ZIKV infections. A study in Asian lineage ZIKV-infected donors from Puerto Rico, Brazil, Nicaragua, and Mexico

demonstrated ZIKV/DENV cross-reactive T cell responses against several proteins of the ZIKV proteome (128). The cross-reactive T cell responses were immunologically consequential as DENV-immune donors mounted CD4+ and CD8+ T cell responses against ZIKV more rapidly and of greater magnitude. This study also demonstrated patterns of differential immunodominance between DENV and ZIKV. While T cell responses target the NS proteins during DENV infection, CD8+ T cells were shown to predominantly target the structural proteins E, prM, and C (128). For this thesis, I developed a T cell-based enzyme-linked immunospot (ELISPOT) assay to detect and characterize *ex vivo* T cell responses against ZIKV and DENV. Utilizing this assay, I demonstrated sustained ZIKV- and DENV-specific and ZIKV/DENV cross-reactive T cell responses to human infections by the African and Asian viral strains in individuals from Senegal, West Africa and Bahia, Brazil (Chapters 4 and 5). Consistent with previous reports, my data revealed enhanced T cell responses in individuals with previous flavivirus exposure. The clinical relevance of these enhanced T cell responses remains to be determined.

Dengue and Zika virus diagnostics

Flaviviral diagnosis is complicated by three major factors (129). First, flavivirus-infected individuals exhibit similar signs and symptoms to other bacterial, parasitic, and viral infections; therefore, syndromic diagnosis is often inaccurate. Second, the limited 4-7-day viremia phase of most flaviviral infections greatly reduces the ability of detecting virus by nucleic-based assays. Third, the antibody-based diagnostic methods are challenged by flavivirus antibody cross-reactivity such that most

serodiagnostic screening requires additional validation by multiple high-cost assays and/or laboratory-intensive methods such as plaque reduction neutralization tests (PRNT). There remains an urgent need for cost-effective, virus-specific diagnostic tests.

The flavivirus NS1 protein has been shown to be a useful marker of infection based on its abundant release from infected cells into the bloodstream (130). A number of commercially available DENV NS1 diagnostics have been developed and analyzed for efficacy (130-133). Recently, the development of a flaviviral NS1 antigen-based rapid test was shown to detect and distinguish the four DENV serotypes, as well as ZIKV, without cross-reactivity (134). Another study, utilizing CRISPR-based diagnostics, demonstrated specific detection of DENV and ZIKV strains (135). However, the detection window for these diagnostics are still limited to the viremic phase. In order to detect DENV and ZIKV infections outside of that viremic phase, enzyme-linked immunosorbent assays (ELISAs) were developed based on DENV and ZIKV NS1 IgM and IgG (136). In a majority of cases, these ELISAs were capable of distinguishing DENV from ZIKV, as well as determining the order in which infections occurred in the cases of multiple flavivirus infections. For this thesis, I utilized the flavivirus ELISPOT assay, mentioned above, to distinguish human infections by DENV and ZIKV strains based on T cell responses to NS3 (Chapter 4 and 5).

Flavivirus vaccines and therapeutics

Despite the significant advances in anti-flaviviral drug discovery, few therapeutic agents for flavivirus infections have been approved for human use. The live attenuated

YFV 17D vaccine is derived from a wild-type YFV isolated in Ghana in 1927 (137). Two substrains of the 17D vaccine virus are currently used for vaccine production, namely 17D-204 and 17DD, which are given as a single subcutaneous or intramuscular injection and are highly protective against YFV infection (138-140).

In contrast, the development of safe and effective dengue vaccines has faced many challenges, complicated by the ADE phenomenon. In the last decade, live attenuated virus, chimeric virus, inactivated virus, subunit, DNA, and vectored vaccines have been developed with suboptimal efficacy (141-147). Vaccine development has therefore focused on the generation of a tetravalent vaccine aimed at providing long-term protection against all four DENV serotypes. Recently, a live-attenuated tetravalent vaccine based on chimeric YFV-DENV completed two Phase III clinical trials and provided ~80% protection from risk of hospitalization (148, 149). The vaccine, however, showed weak to moderate efficacy against the widely prevalent DENV2.

There are approximately forty-five ZIKV vaccine candidates currently under consideration and at various stages of development (150). Five candidate vaccines, including DNA, synthetic peptide, mRNA, and inactivated whole organisms, are in Phase I clinical trials, with plans for larger Phase II and III studies pending the results of the Phase I trials. In addition to vaccines, other antiviral approaches are being considered for ZIKV and other flaviviruses including interferons, nucleic acid-based inhibitors, and small molecule inhibitors (151-155). Despite these advances, and due to lack of FDA-approved DENV and ZIKV vaccines and therapeutics, infected patients generally receive supportive care, and vector control through the use of insecticides and bed nets are highly encouraged for combatting transmission (156).

Conclusion

The global burden of flaviviruses continues to increase and, despite promising advances in vector control and an increasingly active search for antivirals that target specific viruses, research on flaviviruses should remain a top priority for the global public health community. Many questions specific to DENV and ZIKV still need to be addressed, particularly by combining *in vitro*, *ex vivo*, and *in vivo* findings in the context of epidemiology. Thus, this thesis focused, in part, on the epidemiology and human immunology of DENV and ZIKV in West Africa and Brazil, with the hope that the findings generated during these investigations would shed light on the basic immunobiology of these re-emerging pathogens.

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CHAPTER 2

A modified anthrax toxin-based ELISPOT reveals robust T cell responses in symptomatic and asymptomatic Ebola virus exposed individuals

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Chapter 2: A modified anthrax toxin-based ELISPOT reveals robust T cell responses in symptomatic and asymptomatic Ebola virus exposed individuals

Abstract

The 2013-2016 West African Ebola virus (EBOV) outbreak is the largest on record with over 28,000 reported symptomatic cases and more than 11,000 deaths. Despite its high lethality in some individuals, EBOV infection can produce little to no symptoms in others. A better understanding of the immune responses in individuals who experienced asymptomatic infection could aid the development of more effective vaccines and antivirals against EBOV and related filoviruses. In this study, we collected blood samples from 19 study participants in Lagos, Nigeria, including 3 Ebola virus disease (EVD) survivors, 10 individuals with documented close contact with symptomatic EVD patients, and 6 control healthcare workers. The Lagos samples, as well as archived serum collected from healthy individuals living in surrounding areas of the 1976 Democratic Republic of Congo (DRC) epidemic, were tested for EBOV IgG using commercial enzyme-linked immunosorbent assays (ELISAs) and Western blots. We detected antibodies in 3 out of 3 Lagos survivors and identified 2 seropositive individuals not known to have ever been infected. Of the DRC samples tested, we detected antibodies in 9 out of 71 (12.7%). To characterize the T cell responses in the Lagos samples, we developed an anthrax toxin-based enzyme-linked immunospot (ELISPOT) assay. The seropositive asymptomatic individuals had T cell responses against EBOV nucleoprotein, matrix protein, and glycoprotein 1 that were stronger in magnitude compared to the survivors. Our data provide further evidence of EBOV exposure in individuals without EVD-like illness and, for the first time, demonstrate that these individuals have T cell responses that are stronger in magnitude compared to

severe cases. These findings suggest that T cell immunity may protect against severe EVD, which has important implications for vaccine development.

Chapter 2: A modified anthrax toxin-based ELISPOT reveals robust T cell responses in symptomatic and asymptomatic Ebola virus exposed individuals

Background

Ebola viruses including the five antigenically distinct species, *Bundibugyo ebolavirus* (BDBV), *Reston ebolavirus* (RESTV), *Tai Forest ebolavirus* (TAFV), *Sudan ebolavirus* (SUDV), and *Zaire ebolavirus* (EBOV), are enveloped filamentous viruses belonging to the family *Filoviridae* (1-3). These viruses carry negative strand RNA genomes approximately 19 kb in length that code for 7 structural proteins: the nucleoprotein (NP), VP35, the matrix protein (VP40), the glycoprotein (GP), VP30, VP24, and the RNA-dependent RNA polymerase (1).

Since the discovery of EBOV in 1976 in the Democratic Republic of Congo (DRC), BDBV, SUDV, and EBOV have caused sporadic epidemics of lethal hemorrhagic fever or Ebola virus disease (EVD), largely in Central Africa, with case fatality rates of 23-90% (4-7). In December 2013, an outbreak of EBOV was detected in Guinea, which led to the largest ever recorded epidemic spanning seven West African countries. By 2016, more than 28,000 symptomatic cases of EVD were reported with a case fatality rate of 40% (8). Although there are several in the pipeline, there are currently no licensed vaccines against EBOV and treatment remains largely supportive.

Richardson et al. recently conducted a survey of close household contacts of individuals who had severe EVD and showed that a significant portion of EBOV transmission events went undetected during the West African outbreak because some individuals contracted infection but had mild illness or were asymptomatic (9). This work adds to a growing body of evidence suggesting that despite its high lethality in some individuals, EBOV infection can produce little to no symptoms in others (10).

Seroprevalence surveys conducted in Africa have historically described asymptomatic EBOV infection, however, due to uncertainty in serologic assays, consensus on the significance of these findings has not been reached. Nonetheless, while several hypotheses might explain minimally symptomatic and asymptomatic EBOV infection, including properties of the infecting virus (e.g. less virulent isolate), low inoculum, route of transmission, host factors (e.g. resistance through viral cell receptor polymorphism), or a robust innate and/or adaptive immune response are potential explanations.

Antibody therapy is considered an effective and powerful treatment strategy against many infectious pathogens. While studies of whole-blood transfusion or serum as passive immunity for EBOV treatment has demonstrated limited efficacy, monoclonal antibodies have shown promise in animal models and have been tested in human clinical trials (11-15). The most successful of these is the antibody cocktail ZMapp, which comprises three humanized murine monoclonal antibodies that target the EBOV GP. ZMapp reversed advanced disease and rescued 100% of rhesus macaques up to 5 days post-viral challenge; however, it did not meet a pre-specified threshold for efficacy in humans when tested during the 2013-2016 outbreak (13, 16).

Immune depletion studies in the macaque EBOV model demonstrated that humoral responses were beneficial in containing virus, but CD8+ T cells were essential for vaccine-induced protection. These findings suggest that humoral immunity alone may not account for full recovery or secondary protection. T cell responses of appropriate quality and magnitude were shown to be important for human protection against EBOV (17-19). During the West African outbreak, Ruibal et al. demonstrated

unique mechanisms that regulate T cell homeostasis in fatal and non-fatal EVD cases, suggesting that EBOV infections can trigger T cell responses that may be more effective in some individuals than others (20). More studies are needed to better understand the immunopathogenesis of EVD, especially in mildly symptomatic or asymptomatic cases.

In this study, we adapted the modified anthrax lethal factor (LFn) delivery system to enable the detection and characterization of T cell responses in previously EBOV exposed individuals from Lagos, Nigeria, three years after the outbreak. We fused the EBOV (Makona variant) NP, VP40, and the receptor binding subunit of GP, known as GP1, to LFn. The LFn-EBOV recombinant proteins were expressed and used as antigens to stimulate peripheral blood mononuclear cells (PBMCs) in an ELISPOT assay. We report robust T cell responses in EVD survivors and in EBOV seropositive asymptomatic individuals.

Table 1 List and characteristics of participants used in this study.

Study participants	Sex	Age	Exposure Setting	EBOV exposure type	Notable symptoms during the 2014 EVD outbreak in Lagos, Nigeria
EVD survivors					
EV01	M	34	Containment hospital	Body fluid	Fever, Red eyes, Diarrhea, Vomiting, Headache, Irrational talk,
EV02	F	55	Containment hospital	Body fluid	Fever, Red eyes, Diarrhea, Vomiting, Headache, Muscle/Joint pain
EV03	F	31	Containment hospital	Body fluid	Fever, Sunken eyes, Diarrhea, Vomiting, Headache Muscle/Joint pain
Documented EVD contacts					
EV04	M	43	Household contact	Contact with EVD patient without PPE	-
EV05	M	37	Community clinic	Contact with febrile patients	-
EV06	F	47	Containment hospital	Contact with EVD patient with PPE	-
EV07	M	34	Household contact	Contact with EVD patient without PPE	-
EV08	M	31	Containment hospital	Contact with EVD patient with PPE	-
EV09	M	34	Containment hospital	Contact with EVD patient with PPE	-
EV10	M	47	Containment hospital	Contact with EVD patient with PPE	-
EV11	F	55	Household contact	Contact with EVD patient without PPE	-
EV12	M	47	Household contact	Contact with EVD patient without PPE	-
EV13	M	65	Containment hospital	Contact with EVD patient with PPE	Fever, Diarrhea, Vomiting, Headache, Sore throat, Chest pain, Anxiety
Control healthcare workers					
EV14	F	30	University teaching hospital	-	-
EV15	M	32	University teaching hospital	-	-
EV16	F	32	University teaching hospital	-	-
EV17	M	25	University teaching hospital	-	-
EV18	M	24	University teaching hospital	-	-
EV19	F	24	University teaching hospital	-	-

Results

Antigen Capture, qRT-PCR, and Serology Data

At the time of sample collection, all Lagos study participants recruited were healthy. The median age was 34 years (range, 24-65 years), and 37% were female. Study participant characteristics are summarized in Table 1.

We assessed Lagos plasma samples for the presence of EBOV nucleic acid, EBOV antigen, and EBOV-specific antibodies (Table 2). All study participants tested negative for EBOV by qRT-PCR and antigen capture ELISA. Using the Zalgen ReEBOV® IgG ELISA, which assesses antibodies specific to EBOV VP40, 3 of 3 EVD survivors and 2 of 10 documented EVD contacts tested positive, and all 6 control HCWs were negative. Using the MyBioSource EV-IgG ELISA, which uses inactivated and homogenized EBOV, 2 of 3 EVD survivors and the same 2 out of 10 EVD contacts tested positive while the control HCWs remained negative. Consistent with the Zalgen ReEBOV® IgG ELISA, Western blot analysis using the LFn-EBOV-GP1 and -sGP fusion proteins revealed GP1- and sGP-specific IgG antibodies in the 3 EVD survivors and the same 2 out of 10 documented EVD contacts, with no detectable antibodies in the control HCWs (Fig. 1).

Of the serum samples collected during the 1976 EVD epidemic in the DRC, 9 of 71 (12.7%) contained IgG antibodies that were reactive to LFn-EBOV-GP1 and/or -sGP as demonstrated by Western blot (Table 3, Fig. 2). Antibodies specific to LFn-EBOV-GP1 were found in 1.4% (1/71), 9.6% (7/71) to LFn-EBOV-sGP and 1.4% (1/71) to both LFn-EBOV-GP1 and -sGP.

Table 2 qRT-PCR, antigen ELISA, and serology results, Lagos, Nigeria.

Study participants	qRT-PCR	ReEBOV® Antigen ELISA	ReEBOV® IgG ELISA	EV-IgG ELISA	LFn-EBOV-GP1 Western blot	LFn-EBOV-sGP Western blot
EVD survivors	0/3	0/3	3/3	2/3	3/3	3/3
Documented EVD contacts	0/10	0/10	2/10	2/10	2/10	2/10
Control healthcare workers	0/6	0/6	0/6	0/6	0/6	0/6

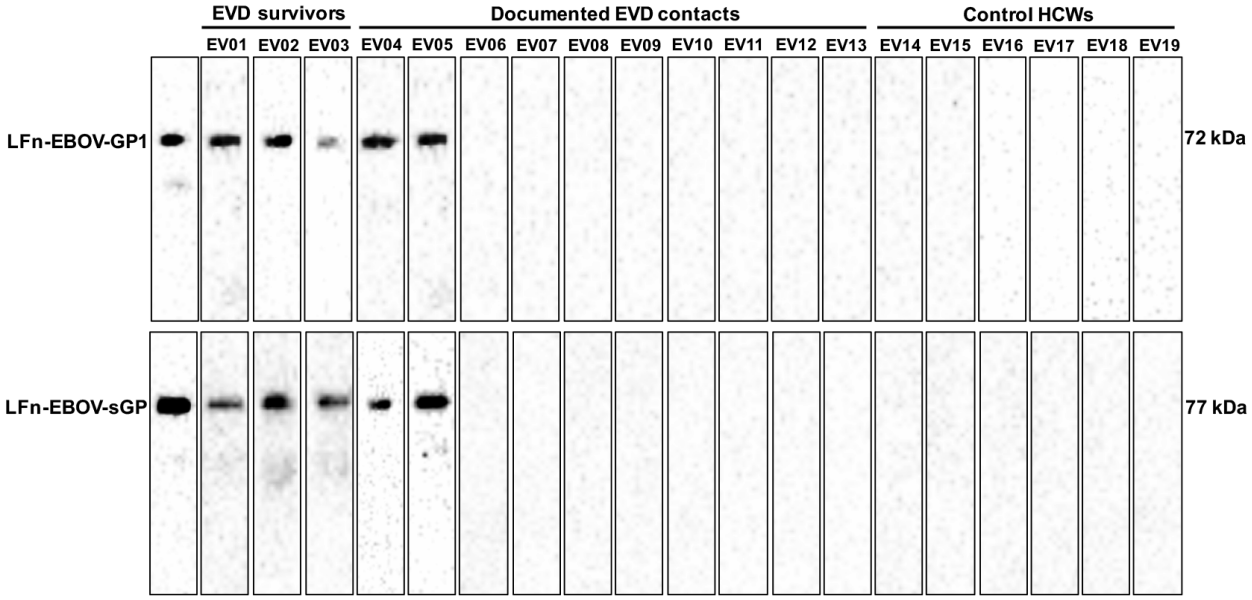

Figure 1. Detection of EBOV antibodies, Lagos, Nigeria. Sera samples from 3 EVD survivors, 10 documented EVD contacts, and 6 control HCWs were subjected to LFn-EBOV-GP1 and LFn-EBOV-sGP Western blot analysis. +, positive control. Molecular size marker units are kDa. EVD, Ebola virus disease. HCWs, health care workers.

Table 3 Serology results, 1976 Democratic Republic of Congo.

DRC sera (N=71)	LFn-EBOV-GP1 Western blot	LFn-EBOV-sGP Western blot	LFn-EBOV-GP1 + -sGP Western blot
No. positive/Total number tested (%)	1/71 (1.4)	1/71 (1.4)	9/71 (12.7)

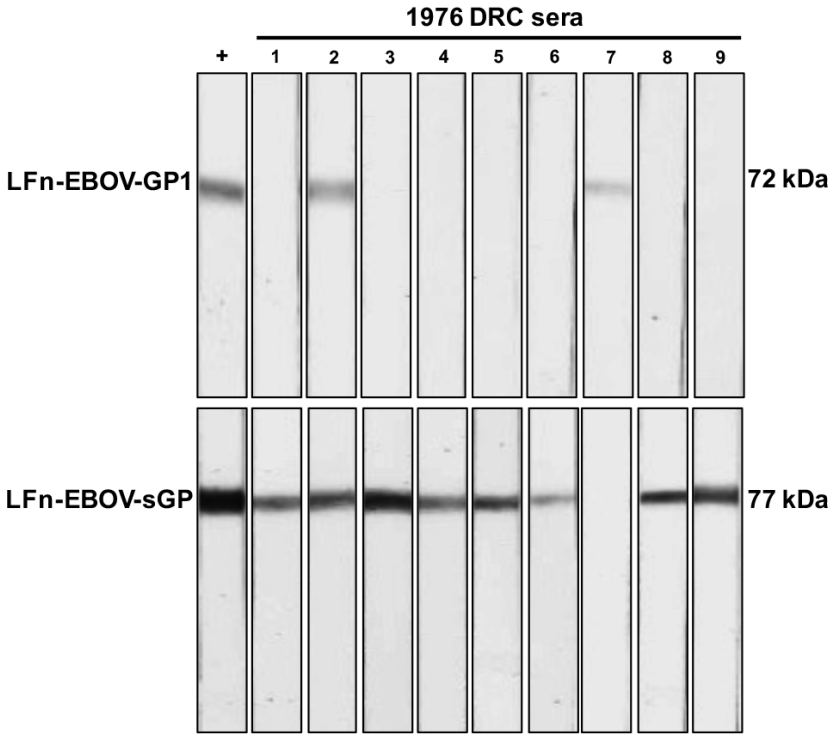

Figure 2. Detection of EBOV antibodies, 1976 Democratic Republic of Congo. (A) Representative image of reactive LFn-EBOV-GP and LFn-EBOV-sGP Western blot analysis in 9 sera collected in surrounding areas of the 1976 Ebola virus outbreak in the Democratic Republic of Congo. +, positive control. Molecular size marker units are kDa. EVD, Ebola virus disease. HCWs, health care workers.

Ex vivo ELISPOT Data

We then assessed the post-infection cellular responses in all Lagos study participants by LFn fusion protein stimulation of PBMCs in IFN-γ and TNF-α ELISPOTs. All 3 EVD survivors and the 2 seropositive EVD contacts mounted detectable IFN-γ and TNF-α cellular responses to LFn-EBOV-NP, -VP40, and/or -GP1 (Table 4, Fig. 3). The remaining 8 documented EVD contacts and all 6 control HCWs mounted cellular responses below the positive threshold (Table 4).

In most cases, the EVD survivors and the seropositive contacts mounted the strongest IFN-γ and TNF-α cellular against LFn-EBOV-NP (Fig. 4). IFN-γ and TNF-α cellular responses against LFn-EBOV-GP1 were stronger compared to responses against LFn-EBOV-VP40. Additionally, the seropositive EVD contacts mounted IFN-γ and TNF-α cellular responses against LFn-EBOV-NP, -VP40, and -GP1 that were stronger in magnitude compared to survivors; similarly, IFN-γ responses against LFn-EBOV-NP and -GP1 and for TNF-α responses against LFn-EBOV-NP and -VP40 were stronger in contacts compared to survivors.

We further evaluated the post-infection CD8+ and CD4+ T cell responses in the EVD survivors and the seropositive EVD contacts. Due to sample availability, we assessed IFN-γ and TNF-α responses against LFn-EBOV-NP and -GP1. In nearly all cases, mean IFN-γ and TNF-α CD8+ and CD4+ responses were stronger among the seropositive EVD contacts compared to the survivors, though not statistically significant (Fig. 5). As the only exception, mean IFN-γ CD4+ response against LFn-

EBOV-NP was stronger in the EVD survivors compared to the seropositive contacts, though not statistically significant (Fig. 5C).

Table 4 Ex vivo ELISPOT results using the LFn-EBOV fusion proteins, Lagos, Nigeria.

Study participants	EBOV seropositive	IFN-γ$^+$ SFC / 10^6 PBMC			TNF-α$^+$ SFC / 10^6 PBMC		
		LFn EBOV NP	LFn EBOV VP40	LFn EBOV GP1	LFn EBOV NP	LFn EBOV VP40	LFn EBOV GP1
EVD survivors							
EV01	Y	572	187	400	522	84	440
EV02	Y	418	30	366	455	29	120
EV03	Y	508	75	313	337	120	383
Documented EVD contacts							
EV04	Y	705	135	552	605	165	498
EV05	Y	770	250	590	670	214	562
EV06	N	12	9	14	5	12	13
EV07	N	23	17	18	10	15	16
EV08	N	7	9	13	7	9	8
EV09	N	33	20	18	8	4	7
EV10	N	6	10	8	8	9	11
EV11	N	2	12	7	15	14	18
EV12	N	14	9	13	4	6	12
EV13	N	10	12	6	8	12	11
Controls healthcare workers							
EV14	N	8	3	12	5	11	7
EV15	N	16	13	23	9	10	6
EV16	N	27	14	19	12	6	17
EV17	N	16	7	9	5	10	11
EV18	N	3	8	13	4	7	12
EV19	N	12	21	16	14	9	8

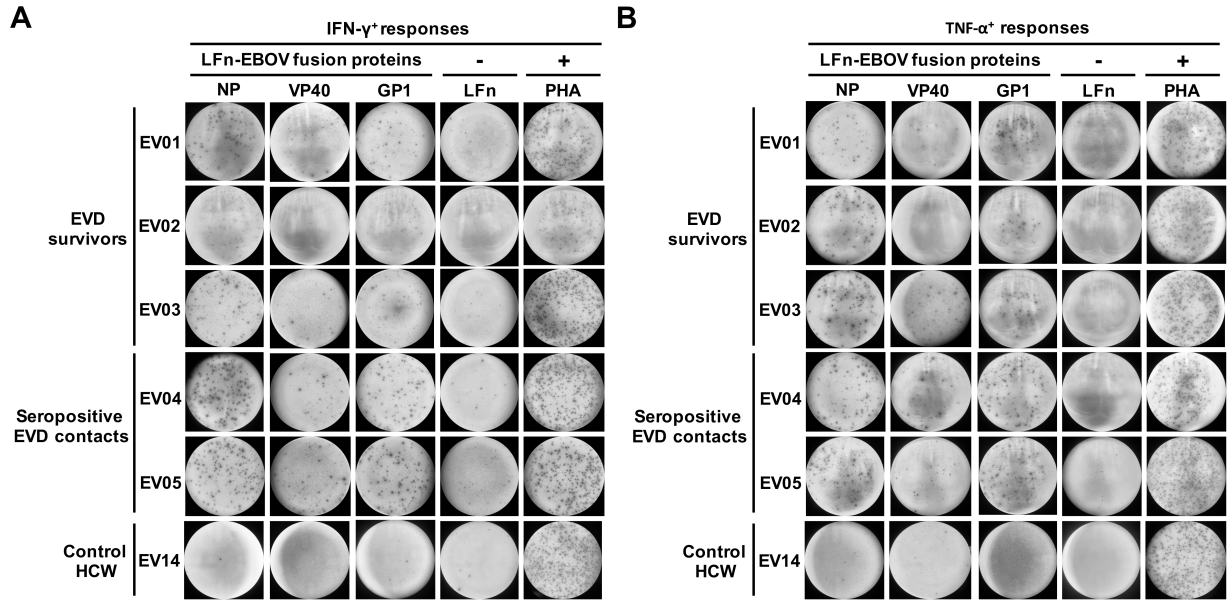

Figure 3. Cellular immune responses, Lagos, Nigeria. PBMC samples from the EVD survivors, documented EVD contacts, and control HCWs were treated with the LFn-EBOV fusion proteins and the IFN-γ+ and TNF-α+ cellular responses were detected by LFn ELISPOT ex vivo experiments. Representative image of IFN-γ (A) and TNF-α (B) cellular responses when stimulated with the LFn-Ebola virus fusion proteins. NP, LFn-EBOV-NP. VP40, LFn-EBOV-VP40. GP1, LFn-EBOV-GP1. LFn, negative control. PHA, phytohemaglutanin, positive control.

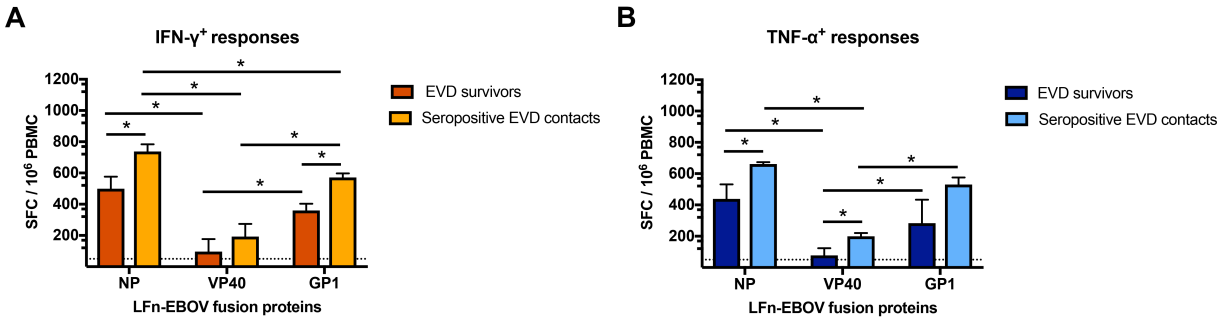

Figure 4. Ex vivo cellular reactivity to EBOV LFn fusion proteins, Lagos, Nigeria.

PBMC samples from the EVD survivors and documented EVD contacts were treated with the LFn-EBOV fusion proteins and the IFN-γ+ and TNF-α+ cellular responses were detected by LFn ELISPOT ex vivo experiments. Average magnitude of convalescent IFN-γ (A) and TNF-α (B) responses are shown. Dotted lines represent the cut-off value. *, $p < 0.05$.

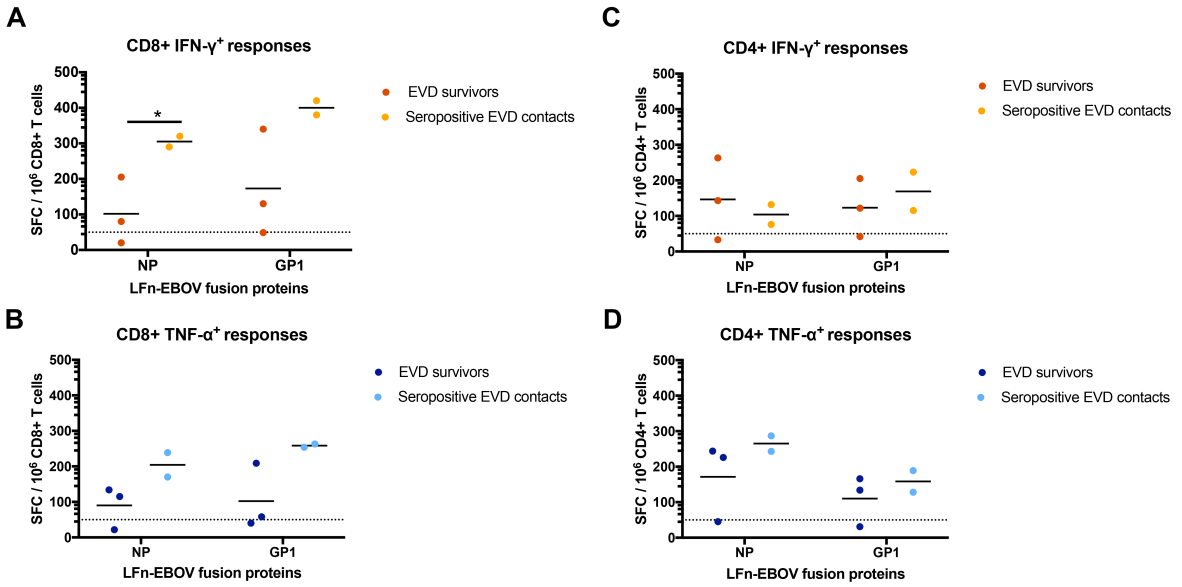

Figure 5. Ex vivo CD8+ and CD4+ cellular reactivity to EBOV LFn fusion proteins, Lagos, Nigeria. CD8+ and CD4+ cells from the EVD survivors and documented EVD contacts were treated with the LFn-EBOV fusion proteins and the IFN-γ+ and TNF-α+ responses were detected by LFn ELISPOT ex vivo experiments. Individual and mean CD8+ IFN-γ+ (A) and TNF-α+ (B) and CD4+ IFN-γ+ (C) and TNF-α+ (D) responses are shown. Dotted lines represent the cut-off value. *, $p<0.05$.

Discussion

The characterization of the immune response in mild and asymptomatic EBOV infection may explain how certain individuals infected with this otherwise highly lethal virus avoid severe disease. This could contribute to efforts in the development of more effective vaccines and immunotherapeutics against EBOV and related filoviruses. Therefore, we adapted the modified anthrax toxin delivery system to design LFn-EBOV fusion proteins for antibody and/or T cell analyses in EVD survivors, documented close EVD contacts, and in presumed healthy individuals living in nearby areas of the 1976 EVD outbreak in the DRC.

T cell responses in survivors of EVD epidemics has been relatively well studied. Four patients with acute EVD demonstrated CD8+ and CD4+ T cell activation against several viral proteins, and this activation persisted for up to one month after infection (17). Consistent with this study, prolonged T cell activation was observed in a single patient who cleared EBOV infection without the use of experimental drugs (21). Another study evaluating patients during the acute phase of infection, demonstrated that survivors have T cells that express lower levels of the inhibitory molecules CTLA-4 and PD-1, compared to fatal EVD cases with high viral load (20). Additionally, a study of SUDV survivors, 12 years post infection, demonstrated strong memory CD4+, but not CD8+, T cell activation and neutralizing humoral immunity (22). Whole irradiated SUDV was used to stimulate PBMC samples in these long-recovered survivors, likely contributing to the limited CD8+ activation.

Moreover, minimally symptomatic and asymptomatic EBOV infections are not new phenomena. Using an immunofluorescence assay, World Health Organization researchers identified EBOV-infected individuals who had symptoms that ranged in severity, from mild to rapidly fatal, during the first outbreaks of EBOV in Zaire and Sudan in 1976 and 1979, respectively (4, 5, 23). Since then, a number of additional studies utilizing diverse methods have identified EBOV-infected individuals who nonetheless remained asymptomatic (24-28). However, studies examining the cellular immune responses during mild or asymptomatic infection are scarce. In one study of Gabonese individuals with asymptomatic EVD infection, high concentrations of pro-inflammatory cytokines were detected in plasma samples; yet, no T cell-derived cytokines were observed (27). A follow-up study in the same individuals demonstrated mRNA expression of T cell cytokines and cytotoxic activation markers, suggesting cytotoxic T cell activation; however, EBOV-specific activation was not studied (29).

In this study, we demonstrated for the first time, to our knowledge, EBOV-specific cellular responses in seropositive asymptomatic individuals as well as EVD survivors. The seropositive asymptomatic individuals mounted stronger IFN-γ and TNF-α responses to all three LFn-EBOV fusion proteins (LFn-EBOV-NP, -VP40, and -GP1) compared to the EVD survivors. Cellular responses were significantly stronger for IFN-γ responses to LFn-EBOV-NP and LFn-EBOV-GP1 and for TNF-α responses LFn-EBOV-NP and LFn-EBOV-VP40. Consistent with previous studies, cellular responses directed to the EBOV NP were strongest compared to other EBOV antigens, in both the EVD survivors and the seropositive asymptomatic individuals. We also showed that

seropositive asymptomatic individuals have IFN-γ and TNF-α cellular responses that were stronger when compared to the survivors. These results suggest that T cell immunity may play a protective role against severe EVD. Additionally, we detected EBOV-specific antibodies in serum collected from individuals living in surrounding areas of the 1976 EVD epidemic in the DRC. These results further support undiagnosed EBOV infection in individuals living in EBOV endemic regions.

While the study of long recovered SUDV survivors was unable to elicit CD8+ T cells, our study demonstrates robust CD8+ T cells against the LFn-EBOV fusion proteins in 3-year post-infection EVD survivors and 2 contacts not known to have ever been infected by EBOV. The LFn-EBOV fusion proteins elicit specific and sensitive T cell responses with low background signals in ELISPOT. Thus, LFn-EBOV priming systems may offer utility as an alternative and inexpensive technology for ex vivo screening of EBOV-specific T cell responses in future vaccine clinical trials. Additionally, our detection of both EBOV-specific CD8+ and CD4+ T cell responses suggests that the LFn delivery system is capable of efficiently presenting exogenous EBOV proteins to the MHC class I and II pathways; therefore, the use of LFn-EBOV fusion constructs presents an attractive technology for EBOV antigen delivery in vaccine design.

Our study has several limitations. Our study population is small and we recruited only a limited number of individuals with documented close EVD contact. It is likely that there are more individuals who were infected with EBOV and remained healthy during the outbreak in Nigeria. While we used different tests to identify individuals with

markers of EBOV infection, we did not perform microneutralization assays to confirm infection specificity or define antiviral function of the antibodies detected in either the EVD survivors or the seropositive asymptomatic individuals. Future studies are expected to examine the functional characteristics of antibodies in minimally symptomatic and asymptomatic EBOV infections. We also demonstrated that the seropositive asymptomatic individuals have stronger T cell responses compared to the EVD survivors; however, additional studies with more study participants are needed to validate these results. We cannot rule out the possibility that the asymptomatic individuals were exposed to a low inoculum or dead antigen, precluding the development of EVD, and generated EBOV-specific T cell responses. Whether these CD8+ and CD4+ cells remain functional during secondary exposure to EBOV remains to be elucidated.

In conclusion, our findings raise new questions and highlight the need for further investigation to better understand the immune responses associated with minimally symptomatic and asymptomatic EBOV infections. By development of the LFn-EBOV ELISPOT assay, we detected cellular immune responses in EVD survivors and individuals with documented close contact with EVD patients, approximately three years after the outbreak in Lagos, Nigeria. These results suggest the importance of T cell responses in disease progression and have important implications for vaccine development, as well as for potential EBOV diagnostics based on T cell responses.

Materials and methods

Ethical statement

The protocols used in this study were approved by the Health Research Ethics Committee at the College of Medicine, University of Lagos, Nigeria (CM/HREC/09/16/055) and by the Internal Review Board (IRB) at the Harvard T.H. Chan School of Public Health (Harvard Chan), Boston, USA (Protocol number IRB16-1321). All Lagos study participants provided written informed consent for the collection of samples and corresponding data were banked and de-identified prior to analyses. The DRC samples were collected under surveillance in 1976. The samples were anonymized by the US CDC prior to shipment to Harvard Chan in 1985.

Study populations

On 20 July 2014 an individual who travelled from Liberia presenting with fever was transported to a private hospital in Lagos; he denied contact with known EVD cases and was treated with antimalarial drugs (30). The individual's condition worsened over the next three days, at which point EVD was suspected. Filovirus and EBOV-specific PCR testing was performed by Lagos University Teaching Hospital (LUTH) and Redeemer's University (RUN) African Centre of Excellence for Genomics of Infectious Diseases (ACEGID), respectively, and the patient was confirmed EVD positive on 25 July 2014; he died on that day. During this period, many were exposed to the virus and all contacts were traced, placed under surveillance, and monitored for clinical features

of EVD. The chain of EVD transmission from this single individual resulted in 19 laboratory-confirmed cases, 8 of whom succumbed to infection. Nigeria was declared EBOV free by the World Health Organization on 20 October 2014.

We recruited 3 EVD survivors with RT-PCR positive results for EBOV, 10 individuals with documented close contact with symptomatic EVD patients, who remained healthy during the outbreak, and 6 healthcare workers (HCWs) from a different hospital in Lagos with a low likelihood of previous EBOV exposure based on proximity to the outbreak epicenter and subsequent questionnaires. All methods described subsequently were performed on the Lagos samples from the EVD survivors, documented EVD contacts, and controls.

Additionally, an epidemic of EVD erupted in Yambuku, a small village near Yandongi, Democratic Republic of Congo (formerly Zaire) in 1976 (4). In the course of epidemiological investigations of this outbreak, hundreds of serum samples were collected from residents of the surrounding areas. In 1985, these serum samples were screened for antibodies to human immunodeficiency virus (HIV) (31), and in 1988 additional screening was conducted at Harvard Chan, with excess samples stored. The availability of 71 archived samples from the first known EBOV outbreak offered an opportunity to assess EBOV-specific antibodies in serum collected from potentially EBOV exposed but presumed uninfected individuals. Only Western blot testing to EBOV fusion antigens was performed on the DRC samples for EBOV serostatus.

PBMC and Plasma Isolation performed at LUTH

PBMCs were separated from plasma and whole blood in EDTA tubes by Ficoll-Paque gradient density (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and cryopreserved in freezing media (10% dimethyl sulfoxide [DMSO, Sigma-Aldrich, St. Louis, MO, USA], 90% fetal bovine serum [FBS, ThermoFisher Scientific, Rockford, IL, USA) at -80°C prior to transfer to liquid nitrogen. Plasma was separately aliquoted and immediately transferred to -80°C.

Nucleic Acid Testing

RNA was extracted from all plasma samples using the QIAamp RNA Viral Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was quantified using quantitative RT-PCR (qRT-PCR) for both EBOV and human ribosomal RNA (18S), as previously described (32). Briefly, the assay mix included 3ul of RNA, 0.03umol/L sense and anti-sense primers, 5ul of X2 Power ZYBR Green RT-PCR Mix and 0.08ul of RT Enzyme Mix (ThermoFisher Scientific, Rockford, IL, USA). The cycling conditions were 48°C for 30 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 30 sec with a melt curve of 95°C for 15 sec, 55°C for 15 sec, 95°C for 15 sec. qRT-PCR was performed on the LightCycler 96 (Roche, Indianapolis, IN, USA). The amplicons were cleaned using AMPure XP beads (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions and amplicon concentrations were converted to EBOV copies per microliter for quantification.

Antigen and Antibody ELISAs

Plasma was screened for the presence of EBOV specific IgG antibodies as well as viral antigens by commercial ELISAs. For antibody studies, plasma samples were screened by the ReEBOV® IgG ELISA kit (Zalgen Lab, Germantown, MD, USA) and the EV-IgG ELISA kit (MyBioSource, San Diego, CA, USA) according to the manufacturers' instructions. For antigen capture, plasma was screened by the ReEBOV® Antigen ELISA kit (Zalgen Lab, Germantown, MD, USA) according to the manufacturer's instructions. Measurement of OD was performed within 5 min of stopping the reaction at 450nm (antibody) and 620nm (antigen) with the MiltiskanTM GO Plate Reader (ThermoFisher Scientific, Rockford, IL, USA).

Construction and Expression of LFn Fusion Proteins

Commercially synthesized amino acid fragments corresponding to the EBOV (Makona variant) NP, VP40, GP1, and sGP were cloned into the LFn expression plasmid (pET15bLFn), which contains a T7 promoter, histidine tag, and the terminal domain of the anthrax lethal factor. The EBOV NP, VP40, GP1, and sGP sequences were derived consensus sequences generated from the initial 99 genomes initially published during the 2013-2016 outbreak (33). The pET15bLFn containing the coding sequences of EBOV NP, VP40, and GP1 were transformed into E. coli BLR (DE3) (Millipore, Medford, MA, USA) for expression. Clones containing the correct reading frame as verified by sequencing were used for protein expression, as previously

described (34). The pET15bLFn was expressed and purified as described above for use as a negative control.

Western Blot

All plasma was additionally screened for the presence of IgG antibodies to bind to the recombinant LFn-EBOV-GP1. Briefly, 50ug LFn-EBOV-GP1 was added to reducing buffer (2% SDS, 0.5 M Tris [pH 6.8], 20% glycerol, 0.001% bromophenol blue, and 5% 2-Mercaptoethanol) and subjected to 12% polyacrylamide gel electrophoresis (PAGE). Western Blot analysis was conducted using plasma samples (1:100) as primary antibody and anti-human IgG HRP (ThermoFisher Scientific, Rockford, IL, USA) as secondary antibody. Visualization was performed using SuperSignal Femto Chemiluminescent Substrate (ThermoFisher Scientific, Rockford, IL, USA) and with the Chemi Doc XRS+ Imaging System (Bio Rad Technologies, Hercules, CA, USA).

Ex Vivo ELISPOT Assay

ELISPOT assays were performed as previously described (35). Briefly, a total of $2x10^5$ PBMCs were incubated with 0.1ml complete RPMI 1640 medium (ThermoFisher Scientific, Rockford, IL, USA) in the presence of 2.5ug/ml final concentration LFn fusion proteins in antibody coated 96-well polyvinylidene difluoride (PVDF)-backed $MultiScreen_{HTS}$ (MSIP) microtiter plates (Millipore, Medford, MA, USA). After 24 hr of incubation at 37°C, the plates were washed and incubated with secondary antibodies,

followed by an overnight incubation at 4°C. Plates were washed, then incubated for 2 hr at room temperature with the enzymatic conjugate. Spots were developed using Vector Blue substrate solution (Vector Laboratories, Burlingame, CA, USA) and counted manually using a stereozoom microscope (20X magnification). In CD8 and CD4 experiments, CD8+ and CD4+ T cells were isolated from PBMCs using the CD8+ and CD4+ T cell Isolation kits (Miltenyi Biotec, Auburn, CA, USA). EBOV-specific spots were calculated by subtracting the mean of the negative control values of the replicates from the mean values of the specific stimulation. Positive responses were greater than four times the mean background, three standard deviations above the background, and ≥55 spot-forming cells per (SFC)/10^6 PBMCs.

Statistical Analysis

All statistical analyses were performed using Prism 7 (GraphPad, San Diego, CA, USA). Data are expressed as geometric positive means ± standard deviation. Non-parametric Mann-Whitney test was used to compare data and determine statistical significance. $P < 0.05$ was considered significant.

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CHAPTER 3

Continued human transmission of Zika virus in West Africa, 1992-2016

Adapted from:

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Chapter 3: Continued human transmission of Zika virus in West Africa, 1992-2016

Abstract

First identified in 1947 in Uganda, Zika virus (ZIKV) has remained largely unstudied until the recent outbreak in Latin America. This study aimed to measure the prevalence of ZIKV in febrile patients in Senegal and Nigeria in samples collected from 1992-2016. The seroprevalence of ZIKV was 6.2% based on ZIKV IgM and negative for dengue reactivity. ZIKV envelope (E) was amplified from 4 samples. Phylogenetic analysis showed that the ZIKVs belonged to the African lineage, grouping with either the Nigerian or MR766 sub-lineages. This study provides evidence that ZIKV has been silently circulating in West Africa for at least two decades.

Background

The recent unprecedented outbreak of Zika virus (ZIKV) in the Americas has established it as a globally relevant human pathogen. A flavivirus closely related to dengue virus (DENV), ZIKV is transmitted by mosquitoes of the family *Aedes* and infection in humans can lead to a wide range of clinical manifestations, from mild fever and rash to severe neurological disorders including transmitted congenital microcephaly and Guillain-Barré syndrome in adults (1, 2). Further, ZIKV has recently been reported to be transmitted via maternal-fetal and sexual routes. No effective antiviral agent or vaccine yet exists to treat or control ZIKV infection and, therefore, treatment remains supportive.

Despite considerable global efforts to control or eliminate the mosquito vector in response to other viral and parasitic pathogens, ZIKV has expanded in its geographical distribution over the past 70 years, with limited published research until recently. ZIKV was first isolated in 1947 from a sentinel rhesus monkey stationed in the Zika forest in Uganda (2). In 1948, the virus was isolated from *A. africanus* mosquitoes, and in 1954 human disease related to ZIKV was reported in Nigeria (3). ZIKV remained obscure with only 14 documented human cases confined to Africa and Asia, until a 2007 ZIKV fever epidemic in Yap island in Micronesia (4). Larger epidemics followed in Oceania, including the 2013-14 French Polynesian outbreak, and subsequently, in Latin America and Cape Verde Archipelago in Africa (2, 5). In May 2015, ZIKV was first reported in Brazil, where nearly 1.5 million people were subsequently infected (2). By June 2016, autochthonous transmission of ZIKV was confirmed in 40 countries and territories

throughout Latin America (6). Simultaneously, over 7,500 suspected cases were reported in Cape Verde between October 2015-May 2016 (5).

While ZIKV was responsible for significant neuropathology throughout French Polynesia, Latin America, and Cape Verde, human infections prior to 2015 were not associated with neurological disease. Recent hypotheses suggest that the genetic diversification of ZIKV may be responsible for its emergence, neurotropism, and expansion. Phylogenetic analyses using partial and complete genome regions of ZIKV revealed the evolution of the virus into two distinct groups, designated African and Asian lineages, with the recent outbreak in Latin America traced to the Asian lineage (4, 7). Additionally, disease severity in humans may be correlated with enhanced ZIKV infection caused by pre-existing DENV antibodies, a phenomenon known as antibody-dependent enhancement (8).

The explosive transcontinental spread of ZIKV highlights the need to quantify the prevalence of ZIKV in regions endemic for the insect vector. Understanding the geographical distribution and burden of disease attributable to ZIKV, in the context of other flaviviruses, across endemic and high-risk regions is also critical. Minimal data exist on the prevalence of ZIKV in humans in West Africa, a region with approximately 450 million people inhabiting areas environmentally suitable for the transmission of multiple mosquito-borne pathogens (9). In this study, we evaluated previously collected samples from febrile patients from 3 different study cohorts in Senegal and Nigeria for potential ZIKV infection.

Results

A total of 387 samples collected over a 24-year period were included in the study (Table 1). The age of study participants ranged from 1-70 with an overall median of 35 years (IQR: 27-41) and 70% were female. The average fever temperature was highest in the Senegalese malaria cohort (average: 39.1°C), followed by the Nigerian HIV cohort (average: 38.3°C) and the Senegalese FSW cohort (average: 37.8°C).

Of the total number of samples tested for ZIKV IgM antibodies, 24 tested positive, resulting in an overall ZIKV seroprevalence of 6.2%. The highest ZIKV seroprevalence was observed in the Senegalese malaria cohort (6/80, 7.5%), followed by the Nigerian HIV cohort (24/188, 6.3%), and the Senegalese FSW cohort (6/119, 5%). All 24 ZIKV IgM positive samples tested negative for DENV IgM.

ZIKV IgM positive samples were found throughout a two-decade span between 1992 and 2016 (Figure 1A). While we tested samples from 1992-2004 in the Senegalese FSW cohort, ZIKV was detected in samples collected between 1996 and 2001. Positive samples were detected in both the 2005 and 2013 groups in the Senegalese malaria cohort. While we tested samples from 7 years between 2004-2016 in the Nigerian HIV cohort, positive samples were detected in 2005, 2011, 2013, and 2015. A majority of ZIKV-IgM-positive patients did not report signs or symptoms corresponding to ZIKV infection. Additionally, logistic regression using decade (1992-1999, 2000-2009, 2010-2016), rainy season (April-October: Nigeria, June-October: Senegal), age, sex, and temperature as predictors, clustering by cohort, found a lower odds of ZIKV IgM positivity after 2009 compared to before 2000 (odds ratio = 0.80, $p = 0.003$) (data not shown).

Table 1. Cohort Characteristics and Zika virus (ZIKV)/Dengue virus Serology and ZIKV-Reverse-Transcription Polymerase Chain Reaction

Characteristic	Senegal FSW Cohort (n = 119)	Senegal Malaria Cohort (n = 80)	Nigeria HIV Cohort (n = 188)	Total (N = 387)
Demographics				
Sample year range	1992–2004	2005 and 2013	2004–2016	…
Age, y, median (range)	37 (21–58)	15 (1–50)	37 (20–70)	35 (1–70)
% Female	100	33	66	70
Temperature, average, °C	37.8	39.1	38.3	…
Malaria status, No. (%)				
Malaria positive	NT	80 (100)	NT	80 (20.7)
Malaria negative	NT	…	NT	…
HIV status, No. (%)				
HIV negative	80 (67.2)	NT	…	80 (20.7)
HIV-1	23 (19.3)	NT	188 (100)	211 (54.5)
HIV-2	12 (10.1)	NT	…	12 (3.1)
HIV-dual	4 (3.4)	NT	…	4 (1.0)
Serology and ZIKV RT-PCR, No. (%)				
ZIKV IgM	6 (5.0)	6 (7.5)	12 (6.4)	24 (6.2)
DENV IgM[a]	0	0	0	0
ZIKV RT-PCR[a]	1	1	2	4

Abbreviations: DENV, dengue virus; FSW, female sex worker; HIV, human immunodeficiency virus; IgM, immunoglobulin M; NT, not tested; RT-PCR, reverse-transcription polymerase chain reaction; ZIKV, Zika virus.

[a]DENV IgM and ZIKV RT-PCR were performed on 24 ZIKV IgM-positive samples.

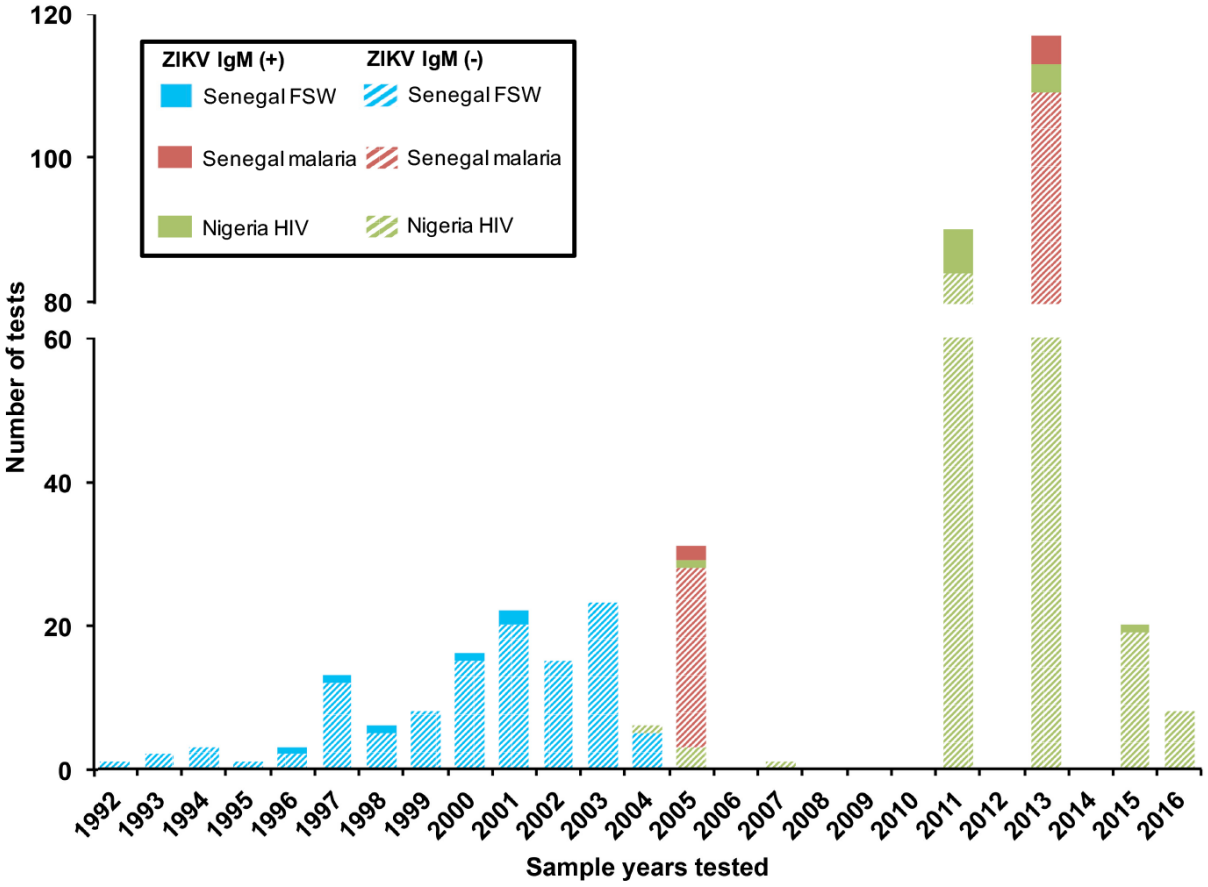

Figure 1A. Frequency of Zika virus (ZIKV) results between 1992 and 2016. Blue, Senegalese female sex worker (FSW) cohort; red, Senegalese malaria cohort; green, Nigerian human immunodeficiency virus (HIV) cohort. The solid bars represent the number of immunoglobulin M (IgM)-positive samples and the hashed bars represent the number of negative samples.

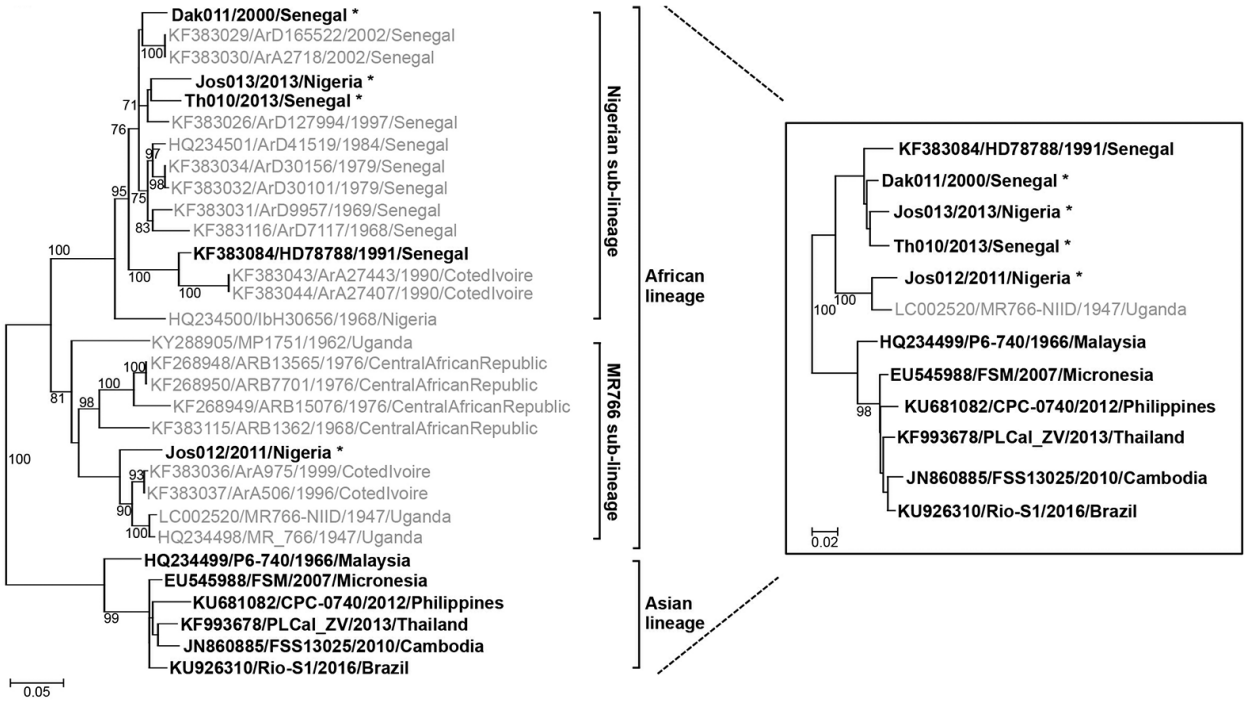

Figure 1B. Phylogenetic analysis of ZIKV envelope (E) genes of 4 ZIKVs from Senegal and Nigeria using reference ZIKV sequences isolated from humans, monkeys, and mosquitoes. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model with 1000 boot resampling. Spondweni virus was used to root the tree. The boxed maximum-likelihood tree was produced as above using ZIKVs isolated from only humans and the MR766 monkey ZIKV. The 4 ZIKVs identified in this study are indicated with an asterisk (*).

Amplicons corresponding to a specific 364-nucleotide region of the ZIKV E gene were amplified by RT-PCR in 4 out of 24 ZIKV IgM positive samples (Table 1). Of the RT-PCR positive samples, one was from the Senegalese FSW cohort (Dak011) collected in 2000, one from the Senegalese malaria cohort (Th010) collected in 2013, and two from the Nigerian HIV cohort (Jos012, Jos013), collected in 2011 and 2013, respectively. ZIKV RT-PCR positive samples from the Senegalese FSW and Nigerian HIV cohorts were HIV-1 positive, while the positive sample from the Senegalese malaria cohort screened positive for malaria.

Full-length ZIKV E was sequenced for the 4 ZIKV RT-PCR positive samples. Using reference E genes from human, monkey and mosquito ZIKVs, nearly identical phylogenetic trees were generated by several methods (maximum-likelihood, neighbor-joining, minimum-evolution). A final maximum-likelihood tree was generated and analyzed with 1,000 replicates for bootstrap testing (Figure 1B). Dak011, Jos013, and Th010 clustered with the Nigerian ZIKV sub-lineage, while Jos012 clustered with the MR766 sub-lineage. Within the MR766 sub-lineage, Jos012 was most closely related by nucleotide sequence to reference mosquito ZIKVs from Cote d'Ivoire.

Discussion

Although these three study cohorts were distinct, we achieved a relatively homogenous high-risk study population by focusing on febrile patients with temperature ≥37.5°C at the time of sample collection. This study revealed a previously unmeasured ZIKV IgM seroprevalence among febrile patients in Senegal and Nigeria of 6.2%, demonstrating the continued transmission of ZIKV in these countries over two

decades (10). It is noteworthy that while our study utilized the ZV-IgM ELISA (MyBioSource), a recent FDA alert suggested higher false positives than expected via the FDA EUA approved ZIKV Detect™ IgM Capture ELISA (InBios). Additionally, uncertainty revolves around the persistence of ZIKV IgM after infection and whether ZIKV IgM seropositivity indicates recent infection. For other flaviviruses such as DENV and West Nile virus, IgM may persist up to 6 to 7 months for each virus, respectively (11).

DENV is thought to circulate throughout West Africa, and due to extensive cross-reactivity between the flaviviruses, we tested the ZIKV IgM positive samples for DENV IgM, a CDC-recommended protocol. All of the ZIKV IgM positive samples tested negative for DENV IgM, indicating that DENV cross-reactivity is unlikely; however, these results do not exclude cross-reactivity between other flaviviruses that may circulate in Senegal and Nigeria.

Of the ZIKV IgM positive samples, 70% were from females. Since our study population was comprised of 70% females, this is proportionate to our study population. Our logistic regression analysis revealed a 20% lower odds of a positive ZIKV IgM during 2010-2016 compared to 1992-1999; however, this correlation could be an artifact of the small number of ZIKV IgM positive samples (n=24) or the small number of samples tested between 1992-1999 (n=38).

Prior to this study, there was published ZIKV sequence data from only one human infection in Africa, isolated in 1991 in Senegal (KF383084/HD78788) (4). Our study identified 4 additional human African ZIKVs from both Senegal and Nigeria. These newly characterized ZIKVs grouped with the African lineage, as expected.

Interestingly, Jos012 clustered with the MR766 sub-lineage, comprised of mostly East African ZIKVs, and was most closely related to mosquito ZIKVs from Cote d'Ivoire, while the other three ZIKVs clustered within the Nigerian sub-lineage, comprised of mostly West African mosquito ZIKVs. Similar results were observed when a maximum-likelihood tree was generated using E genes of human ZIKVs and the 1947 MR766 monkey ZIKV. These results corroborate the presence of two African ZIKV sub-lineages circulating throughout West Africa (4).

Although ZIKV appears to be endemic in Senegal and Nigeria, data regarding the prevalence of the virus in humans in West Africa has been lacking. Though not population-based, our study found a 6.2% prevalence of ZIKV in febrile patients presenting to clinics, providing evidence that ZIKV, though largely unreported, has been circulating in West Africa for the past two decades. Furthermore, we have shown that concurrent infection of HIV/malaria and ZIKV does occur. Our study emphasizes the need for improved detection methods for ZIKV, in order to distinguish among fever-causing pathogens. Further studies will help elucidate the impact of ZIKV on patient outcomes, leading to improved understanding of the pathogenesis of ZIKV disease in Africa.

Material and methods

Study population and study sites

This retrospective study used samples and data from three cohorts: 1) A Senegalese HIV-1/-2 female sex workers (FSW) cohort; 2) a Senegalese malaria cohort; and, 3) a Nigerian HIV treatment cohort.

The Senegalese FSW cohort, described previously (12), included 119 samples prospectively collected between 1992 to 2004 from self-identified FSW regularly visiting a health care clinic in Dakar. Participants underwent annual blood tests for various sexually-transmitted infections, including HIV-1 and HIV-2. This HIV-1/-2 natural history study helped define some of the in vivo characteristics of HIV-2 infection in West Africa. The Senegalese malaria cohort, also described previously (13, 14), included 80 samples originally collected under passive surveillance from febrile patients who had positive malaria smear/rapid diagnostic test results during various malaria transmission seasons (August-December). 27 samples were collected from a health facility in Velingara (2005), a highly malaria rural endemic area in southern Senegal, and 53 samples from a facility in Thiès (2013), a hypo-endemic urban area 70 km from Dakar. The Nigerian HIV cohort included 188 samples collected between 2004 and 2016 from adult patients (≥15 years of age) receiving HIV care and treatment at Jos University Teaching Hospital, Jos, Nigeria, in the Harvard T.H. Chan School of Public Health/AIDS Prevention Initiative in Nigeria (APIN) HIV treatment program, as previously described (15). Clinical visit records from all three studies were queried for fever ≥37.5°C, and of these, excess stored blood specimens that were collected within 7 days of the fever visit were included in the study.

All patients from each of the three cohorts provided informed consent for the original collection of samples. The primary studies under which the samples and data were collected received ethical clearance from the Harvard Institutional Review Board (IRB) and the local research ethics committees at Cheikh Anta Diop University, Dakar, Senegal or Jos University Teaching Hospital, Jos, Nigeria. All excess samples and

corresponding data were banked, and de-identified prior to the analyses. This study received an exemption determination from the Harvard IRB and from the Senegal Ministry of Health IRB committee.

Serologic testing

Serum or plasma were screened for the presence of IgM antibodies by ZV-IgM ELISA (MyBioSource, San Diego, CA, USA), according to the manufacturer's instructions. Samples that tested ZIKV IgM positive were subsequently screened by DENV Detect™ IgM Capture ELISA (InBios, Seattle, WA, U.S.A), according to the manufacturer's instructions. Assay performances were monitored by using internal controls, and cutoffs were determined as specified by the manufacturer for individual kits.

Nucleic acid testing and sequencing

RNA was extracted from all ZIKV IgM positive samples using the QIAamp RNA Viral Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse transcribed using ZIKV-specific primers for a 364-nucleotide region of the ZIKV envelope (E) gene as previously described (4). RT-PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). PCR products of expected size were purified from agarose gels and the complete nucleotide sequence of ZIKV E was determined by Sanger sequencing (GENEWIZ, Cambridge, MA, U.S.A.).

Phylogenetic analysis

Sequences were aligned with full-length or partial reference ZIKV E genes from GenBank using MEGA6 (16). Phylogenetic trees were constructed using the maximum-likelihood-method logarithm based on the Tamura-Nei model with 1,000 bootstrap resampling.

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CHAPTER 4

Sustained specific and cross-reactive T cell responses to Zika and Dengue virus nonstructural protein 3 in West Africa

Adapted from:

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Chapter 4: Sustained specific and cross-reactive T cell responses to Zika and Dengue virus nonstructural protein 3 in West Africa

Abstract

Recent studies on the role of T cells in Zika virus (ZIKV) infection have shown that T cell responses to Asian ZIKV infection are important for protection, and that previous Dengue virus (DENV) exposure amplifies the protective T cell response to Asian ZIKV. Human T cell responses to African ZIKV infection, however, remain unexplored. Here, we utilized the modified anthrax toxin delivery system to develop a flavivirus ELISPOT. Using human ZIKV and DENV samples from Senegal, West Africa, our results demonstrate specific and cross-reactive T cell responses to nonstructural protein 3 (NS3). Specifically, we found that T cell responses to NS3 protease are ZIKV and DENV specific, but responses to NS3 helicase are cross-reactive. Sequential sample analyses revealed immune responses sustained many years after infection. These results have important implications for African ZIKV/DENV vaccine development, as well for potential flavivirus diagnostics based on T cell responses.

Background

Dengue virus (DENV), serotypes 1-4, and Zika virus (ZIKV) belong to the family *Flaviviridae* (1). These viruses contain a single-stranded, positive-sense RNA genome encoding a polyprotein that is cleaved into 10 polypeptides: three structural proteins – capsid (C), premembrane (prM), and envelope (E) – and seven nonstructural proteins (NS) proteins – NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (2, 3).

More recently, DENV endemic regions have been experiencing ZIKV outbreaks. Discovered in Uganda in 1947, ZIKV had caused sporadic disease throughout Africa and Asia with limited clinical consequences (4-6). ZIKV remained obscure until the 2007 Micronesia outbreak, with subsequent larger epidemics in 2013-14 and 2015-16 in French Polynesia and Latin America, respectively (7-9). In April 2015, ZIKV was detected for the first time in the Western Hemisphere in Brazil, and by January 2016 reports of the virus had spread throughout the Americas and the Caribbean (10-12).

Phylogenetic analyses have distinguished African and Asian ZIKV lineages, with Asian ZIKV responsible for the recent outbreaks (5, 13, 14). As with DENV, both Asian and African ZIKV are transmitted to humans by *Aedes* mosquitoes (15). Additional modes of transmission for Asian ZIKV including sexual and maternal-fetal routes have been shown (16, 17). Recent evidence also points to the capacity of Asian ZIKV to cause severe neuropathology, including disorders of fetal brain development and Guillain-Barré syndrome (11, 18, 19). Given the recent epidemic in Latin America with the previously unrecognized neuropathology in Africa, the distinctions between the pathogenesis of African and Asian ZIKV have taken on new significance.

The role of the immune system in DENV infection has been extensively investigated (20, 21). A number of recent studies have sought to understand the consequences of DENV and ZIKV interactions, with conflicting results. Some studies have suggested that previous DENV infection may protects against ZIKV through DENV-induced cross-reactive neutralizing antibodies (22-25); in contrast, other studies have shown that cross-reactive antibodies enhance infection of ZIKV, supporting the antibody-dependent enhancement phenomenon (23, 26-28). There is a need to better understand the evolution and maintenance of immune responses to these related viruses in people.

While the role of T cells continues to be debated in DENV infection, recent work has found evidence of a protective role in ZIKV infection. *In silico* analysis has identified T cell reactivity conserved across all flaviviruses including the ZIKV nonstructural proteins, namely NS3 and NS5 (29). Studies in mice have identified immunodominant and protective roles for T cells that were either ZIKV-specific or cross-reactive for DENV and ZIKV (30). One study involving ZIKV-infected humans found poorly cross-reactive memory T cells, even in individuals that were previously exposed to DENV (24). Another study demonstrated more rapid and stronger T cell responses to the structural proteins of Asian ZIKV in ZIKV-positive DENV-negative individuals (31). To date, no study has examined the role human T cells play in African ZIKV and DENV infections.

The aforementioned T cell studies examined the role of T cells in ZIKV infection by peptide stimulation or a T cell library method. Bacterial toxins represent an

alternative strategy for the delivery of antigen into the cytosol of host cells for MHC processing, an essential step in T cell activation (32). A modified, non-toxic form of the *Bacillus anthracis* (anthrax) toxin has been shown to translocate protein antigens to both the MHC class I and class II pathways (33). This antigen delivery system exploits the exotoxin properties of anthrax. The toxin produced by anthrax is tripartite, composed of protective antigen (PA), edema factor (EF), and lethal factor (LF). Terminally-truncated LF (LFn; lacking 255 amino acids) has been shown to be non-toxic and contain the information necessary to translocate into the cytosol any heterologous protein that can be stably fused to it (34). Proteins delivered by this system will be processed by either the MHC class I or class II pathways for T cell presentation.

Various studies have successfully employed the modified anthrax toxin delivery system to stimulate specific T cell responses. One study demonstrated *in vitro* generation of T cell epitopes against HIV-1 V3 by fusion of LFn to the Gp-120 portion of the HIV-1 envelope (35). The utility of LFn and its large coding capacity was demonstrated with its fusion to HIV-1 p24 and nef and their use in the induction of strong T cell responses in mice and humans (36). LFn-HIV-1-p24C and -gag vaccine candidates demonstrated capacity to elicit cell-mediated immune responses in Chinese rhesus macaques (37). We previously developed a LFn ELISPOT assay to assess HIV-2 specific T cell responses using peripheral blood mononuclear cells (PBMCs) from HIV-2 infected subjects to demonstrate increased anti-HIV-2 gag cellular responses compared to other T cell assay methodologies (38).

In this study, we applied the LFn ELISPOT assay to analyze T cell responses in African ZIKV and DENV infections. To assess the modified anthrax toxin antigen delivery system, we fused the subdomains, protease and helicase, of African ZIKV and DENV NS3 to LFn. The LFn-ZIKV and -DENV protease and helicase recombinant proteins were expressed and used as antigens to PBMCs isolated from human West African ZIKV and DENV infections in an ELISPOT. We report, for the first time, both specific and cross-reactive, long-term responses to African ZIKV and DENV.

Results

Generation of LFn-ZIKV-protease and -helicase and LFn-DENV-protease and -helicase

We aimed to define the T cell response to African ZIKV and DENV in samples previously collected from female sex workers (FSW) reporting to a local clinic in Dakar, Senegal, West Africa. We focused the design of the LFn fusion antigens on NS3, the conserved and preferential T cell target demonstrated in multiple flavivirus studies (39-41). Prior to the 2007 and 2009 DENV3 outbreaks, human infections in Senegal were primarily caused by DENV2 (42, 43). The PBMC samples used in this study were collected between 1992 and 2004; therefore, we fused the coding sequences corresponding to the NS3 subdomains, protease and helicase, of African ZIKV and DENV2 to LFn (Fig. 1 A).

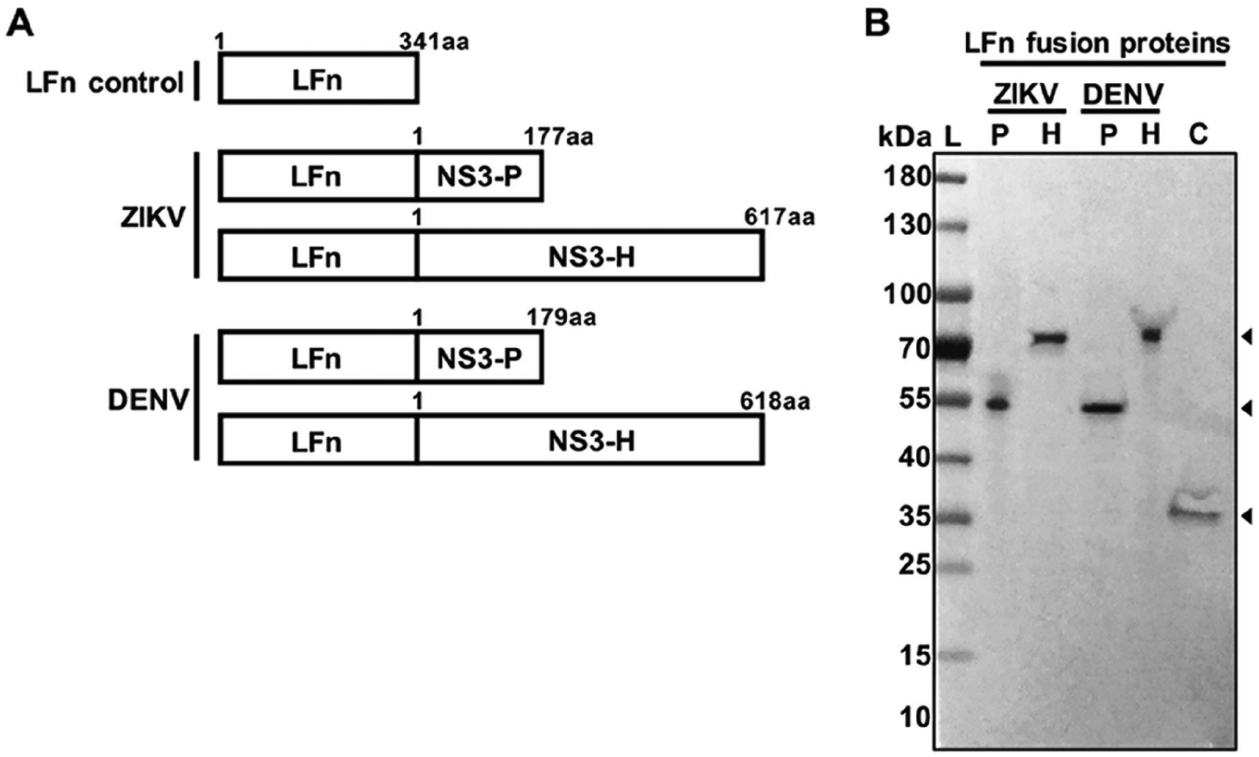

Figure 1. (A) Schematic representation of the LFn control and LFn-ZIKV and -DENV fusion proteins. aa, amino acid. (B) Western blot analysis of the purified proteins LFn-ZIKV-NS3-protease (ZIKV P), LFn-ZIKV-NS3-helicase (ZIKV H), LFn-DENV-NS3-protease (DENV P), and LFn-DENV-NS3-helicase (DENV H) C, LFn control. Arrowheads indicate the size of the proteins. Molecular size marker units are kilodaltons.

Table 1. Cohort characteristics, Zika virus (ZIKV) and dengue virus (DENV) serology, and reverse-transcription polymerase chain reaction (RT-PCR) results.

Characteristic(s) and serology	Value(s) for Senegal FSW cohort (n = 224)
Sample date range	1992–2004
Age, yr, median (range)	38 (21–66)
Avg temp, °C	37.8
HIV status [no. (%)]	
HIV negative	148 (66.1)
HIV-1 positive	40 (17.9)
HIV-2 positive	25 (11.2)
HIV dual positive	11 (4.8)
Serology and RT-PCR, no. positive/total no. tested (%)	
ZIKV	
ZIKV IgM	14/224 (6.3)
ZIKV RT-PCR	2/14 (14.3)
Prior flavivirus exposure	6/14 (42.8)
DENV	
DENV IgM	11/118 (9.3)
DENV RT-PCR	0/11 (0)
Prior flavivirus exposure	5/11 (45.5)

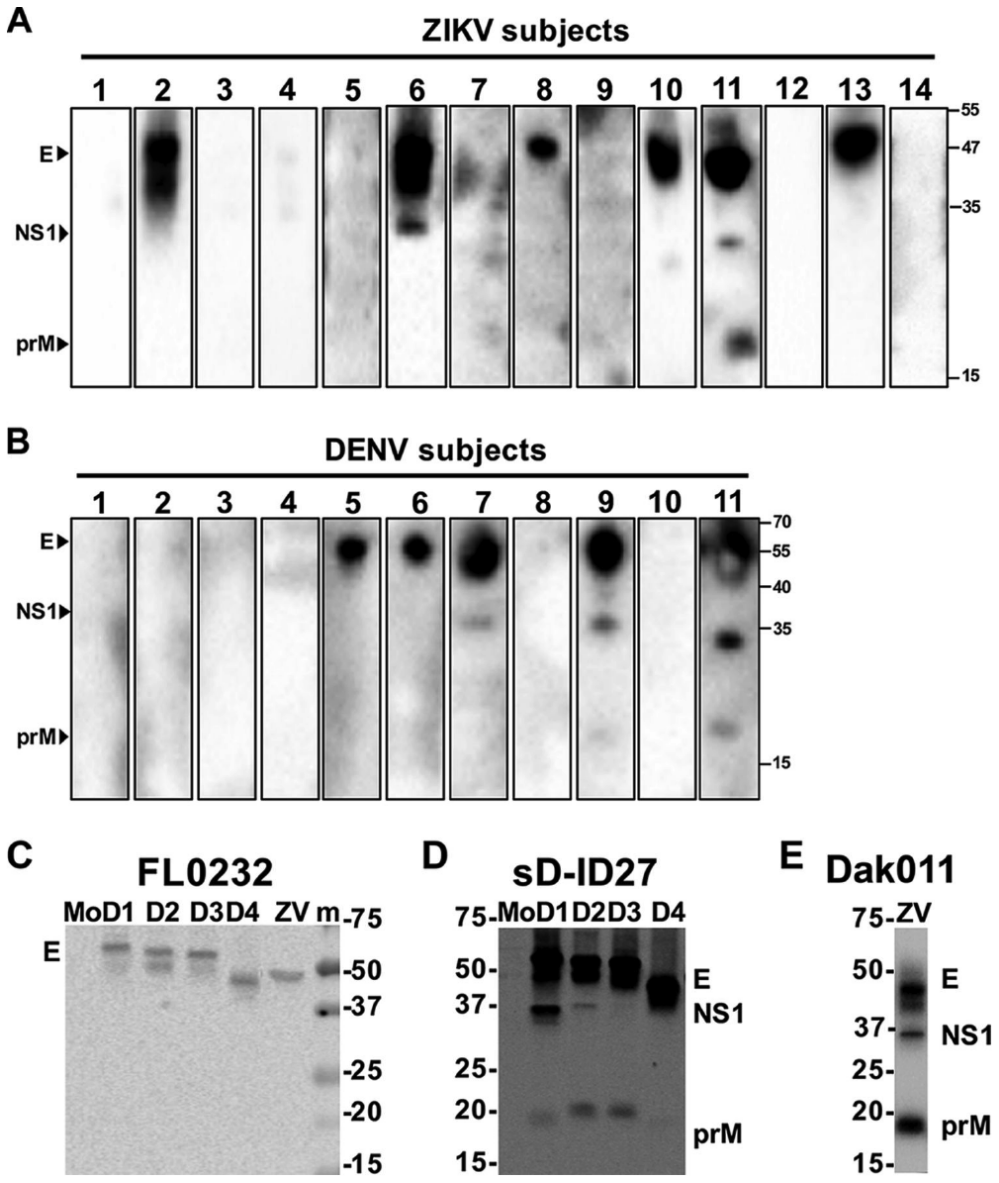

Figure 2. Antibody responses to different ZIKV or DENV proteins. Preinfection sera from ZIKV (A) or DENV (B) IgM-positive subjects were subjected to Western blot analysis using lysates derived from African ZIKV (unpublished strain)- or DENV2 (NGC strain)-infected Vero cells. (C) FL0232, a mouse anti-E monoclonal antibody recognizing similar flaviviral E protein levels, was used to standardize comparable amounts of E proteins from different virus-infected Vero cell lysates. (D) sD-ID27 is a

Figure 2. (continued)

confirmed case of secondary DENV1 infection at 3 months postinfection run on DENV1-4 Vero cell lysates. (E) Dak010 is a confirmed case of African ZIKV infection run on an African ZIKV Vero cell lysate. Arrowheads indicate PrM, E, and NS1 proteins recognized. Molecular size marker units are kilodaltons. Mo, mock-infected; m, molecular mass marker; D1, DENV1; D2, DENV2; D3, DENV3; D4, DENV4; ZV, ZIKV.

Table 2 Association between ZIKV or DENV IgM-positive results and patient characteristics

Parameter	Frequency[a]	Odds ratio (95% CI)	*P* value
ZIKV IgM			
Sample year			
1988–1999	4/51 (7.8)	Reference	
2000–2004	10/173 (5.8)	0.72 (0.22–2.40)	0.594
Season			
Dry (Nov-May)	8/137 (5.8)	Reference	
Rainy (Jun-Oct)	6/87 (6.9)	1.19 (0.40–3.57)	0.750
Temp, °C			
37.5–37.9	10/171 (5.9)	Reference	
38.0–39.4	4/53 (7.6)	1.31 (0.39–4.38)	0.656
Age, yr (range, 21–66)		0.95 (0.88–1.02)	0.143
HIV serology			
Negative	12/148 (8.1)	Reference	
Positive	2/76 (2.6)	0.31 (0.07–1.41)	0.128
DENV IgM			
Sample yr			
1988–1999	5/51 (9.8)	Reference	
2000–2004	7/173 (4.1)	0.39 (0.12–1.28)	0.120
Season			
Dry (Nov-May)	9/137 (6.6)	Reference	
Rainy (Jun-Oct)	3/87 (3.5)	0.51 (0.13–1.93)	0.320
Temp, °C			
37.5–37.9	3/171 (1.8)	Reference	
38.0–39.4	9/53 (17.0)	11.45 (2.98–44.10)	<.001
Age, yr (range, 21–66)		0.99 (0.92–1.07)	0.845
HIV serology			
Negative	6/148 (4.1)	Reference	
Positive	69/76 (7.9)	2.03 (0.63–6.52)	0.235

[a]Frequency data are number of cases positive for Zika virus (ZIKV) by ZIKV-specific IgM or for dengue virus (DENV) by DENV-specific IgM divided by total number of patients. Values in parentheses are percentages.

One-step purification by affinity chromatography of LFn-ZIKV and -DENV-NS3-protease (LFn-ZIKV and -DENV-NS3-P; 56-kDa), LFn-ZIKV and -DENV-NS3-helicase (LFn-ZIKV and -DENV-NS3-H; 77-kDa), and the LFn control (37-kDa) produced soluble and stable proteins. The purified LFn fusion proteins were recognized by rabbit polyclonal antibodies to LFn (Fig. 1B).

Cohort characteristics

We recently reported on the continued human transmission of African ZIKV in Senegal and Nigeria using samples collected from three different cohorts over a 25 year period; the presence of ZIKV in these locales had previously not been appreciated (44). 224 plasma samples from the Senegalese HIV-1/2 female sex worker cohort were available for ZIKV serology, and of them, 118 samples were also available for DENV serology.

Characteristics associated with the samples included year of sample collection, patient's age, temperature, and HIV serology (Table 1). Of 224 total plasma samples tested for ZIKV IgM antibodies, 14 tested positive (6.3%); all 14 ZIKV IgM-positive samples tested negative for DENV IgM. 118 out of the 224 plasma samples were available for DENV serology. Of the plasma tested for DENV IgM antibodies, 11 tested positive (9.3); all 11 DENV IgM-positive samples tested negative for ZIKV IgM. In order to determine previous flavivirus exposure in the IgM-positive subjects, we assessed IgG antibody responses to prM, E, and/or NS1 by Western blot in the most recent available plasma sample that preceded the ZIKV or DENV IgM-positive sample. 6 out of 14 (42.8%) subjects who tested positive for ZIKV IgM and 5 out of 11 (45.5%)

subjects who tested positive for DENV IgM demonstrated previous flavivirus exposure (Fig. 2A-B). Control Western blot with a monoclonal antibody or with confirmed ZIKV and DENV immune sera were performed on the ZIKV and DENV1-4 Vero cell lysates (Fig. 2C-E).

Logistic regression using sample year (1988-1999 and 2000-2004), rainy season (June-October), age, HIV serology, and body temperature as potential predictors found no association with ZIKV IgM positivity, and increased odds of DENV IgM positivity with temperature ≥38°C only (odds ratio, 11.45 (95% CI: 2.98-44.10); $P < 0.001$) (Table 2). ZIKV or DENV nucleic acid could not be successfully amplified by RT-PCR for any of the new samples that tested positive for ZIKV or DENV IgM.

LFn-ZIKV-protease and -helicase and LFn-DENV-protease and -helicase elicit virus-specific T cells responses

To assess the magnitude of T cell responses during ZIKV and DENV infection, we first measured the response by homologous LFn fusion antigen stimulation of PBMCs in an IFN-γ and TNF-α ELISPOT from IgM-positive subjects (presumed acute or recent phase of infection). PBMC samples collected during the recent phase of infection were available for analysis from 11 out of 14 ZIKV and 8 out of 11 DENV IgM-positive subjects. Responses to LFn-ZIKV-NS3-P and LFn-ZIKV-NS3-H as compared to the LFn control were demonstrated in 11 out of 11 ZIKV IgM-positive samples (100%) (Fig.3A-B, Table 3); the frequency of IFN-γ^+ and TNF-α^+ T cells responsive to LFn-ZIKV-NS3-P ranged from 57 to 218 spot forming cells (SFC)/10^6 PBMCs and 55 to 152 SFC/10^6 PBMCs, respectively, while the frequency responsive to LFn-ZIKV-NS3-H

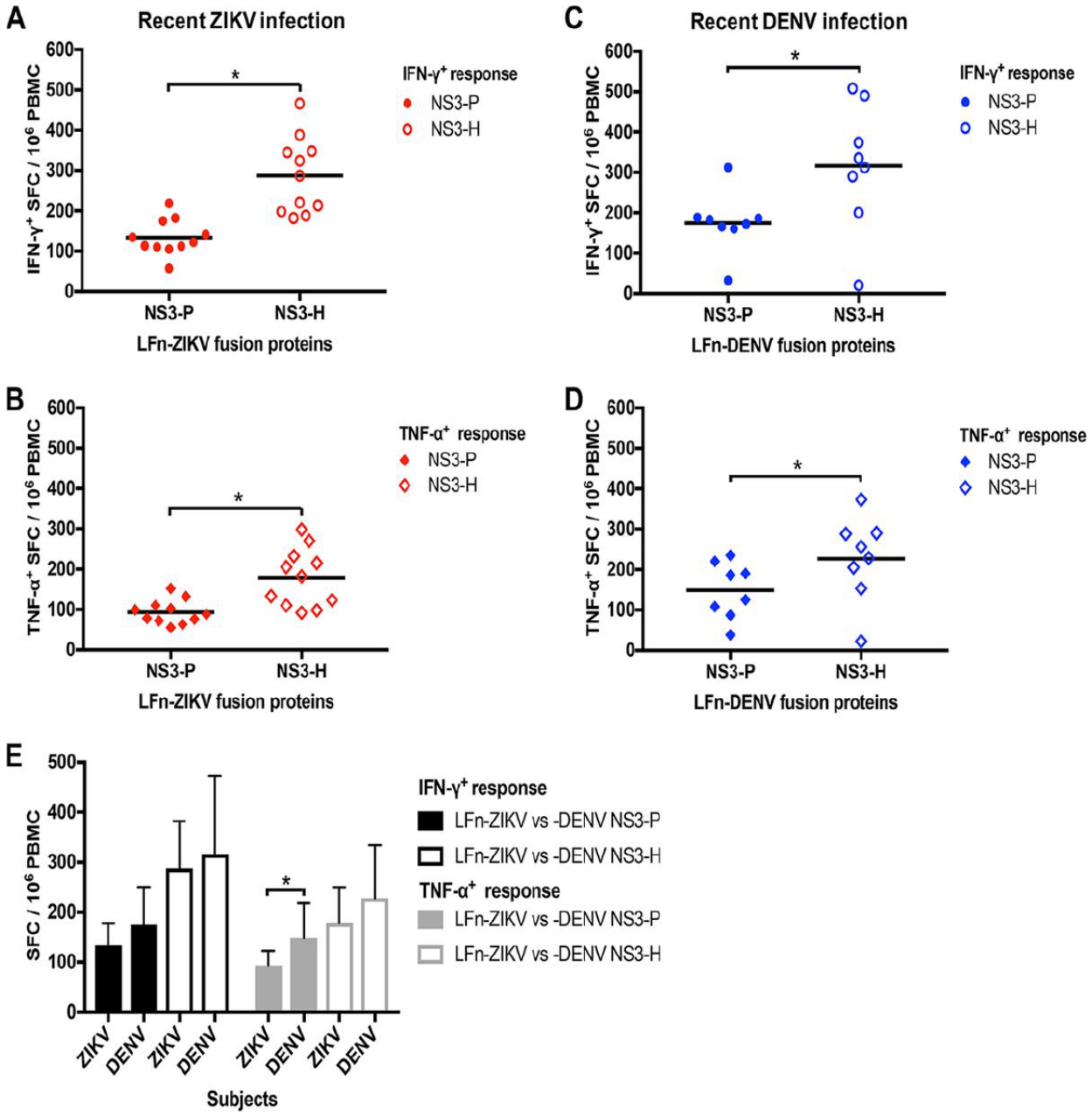

Figure 3. *Ex vivo* T cell reactivity to ZIKV or DENV LFn fusion proteins in the recent phase of ZIKV or DENV infection. PBMC samples from ZIKV IgM-positive subjects collected during the recent phase of infection were subjected to homologous LFn NS3 protease and helicase protein stimulation in IFN-γ (A) and TNF-α (B) LFn

Figure 3. (continued)

ELISPOT *ex vivo* experiments. LFn-ZIKV-NS3-P IFN-γ, red-shaded circle; LFn-ZIKV-NS3-H IFN-γ, red-outlined circle; LFn-ZIKV-NS3-P TNF-α, red-shaded diamond; LFn-ZIKV-NS3-H TNF-α, red-outlined diamond. PBMC samples from DENV IgM-positive subjects collected during the recent phase of infection were subjected to homologous LFn NS3 protease and helicase protein stimulation in IFN-γ (C) and TNF-α (D) LFn ELISPOT *ex vivo* experiments. LFn-DENV-NS3-P IFN-γ, blue-shaded circle; LFn-DENV-NS3-H IFN-γ, blue-outlined circle; LFn-DENV-NS3-P TNF-α, blue-shaded diamond; LFn-DENV-NS3-H TNF-α, blue-outlined diamond. Responses are expressed as the number of secreting cells per 10^6 PBMCs and are considered positive if the net numbers of spot-forming cells (SFC) per 10^6 are ≥55, are greater than four times the mean background, and are three standard deviations above the background. (E) Comparison of IFN-γ ZIKV versus DENV NS3 protease (black-shaded bars) and helicase (black-outlined bars) and TNF-α ZIKV versus DENV NS3 protease (gray-shaded bars) and helicase (gray-outlined bars) T cell responses in ZIKV and DENV subjects. Magnitude of responses is expressed as geometric positive mean, and statistical analysis was performed with Mann-Whitney U test. *, $P < 0.05$.

Table 3 Characteristics of ZIKV and DENV LFn fusion proteins during recent phase of infection

Subject	HIV status	Flavivirus immune status	Infection PBMC collection yr[a]	No. of SFC/10^6 PBMC			
				IFN-γ^+		TNF-α^+	
				LFn ZIKV or DENV NS3-P	LFn ZIKV or DENV NS3-H	LFn ZIKV or DENV NS3-P	LFn ZIKV or DENV NS3-H
ZIKV							
1	Negative	No	1998	57	188	55	92
2	Negative	Yes	1999	182	466	63	205
3	HIV dual	No	2000	105	220	78	182
4	Negative	No	2000	122	213	99	123
5	HIV-1	No	2000	141	182	132	110
6	Negative	Yes	2001	112	345	102	298
7	Negative	No	2001	134	287	152	133
8	Negative	Yes	2002	175	324	110	270
9	Negative	No	2003	113	198	72	98
10	Negative	Yes	2003	218	348	76	215
11	Negative	Yes	2003	110	388	88	232
12	Negative	No	NA				
13	HIV dual	Yes	NA				
14	Negative	No	NA				
DENV							
1	Negative	No	1999	172	335	186	205
2	HIV-2	No	1999	165	290	87	153
3	Negative	No	2000	32	20	38	22
4	Negative	No	2000	186	312	235	228
5	Negative	Yes	2000	183	374	125	266
6	Negative	Yes	2001	188	490	190	288
7	Negative	Yes	2002	312	508	220	373
8	HIV-1	No	2002	160	200	108	290
9	HIV-1	Yes	NA				
10	Negative	No	NA				
11	Negative	Yes	NA				

[a]NA, not applicable.

ranged from 182 to 466 SFC/10^6 PBMCs and 92 to 298 SFC/10^6 PBMCs, respectively. T cell responses to LFn-DENV-NS3-P and LFn-DENV-NS3-H as compared to the LFn control were demonstrated in 7 out 8 DENV IgM-positive samples (87.5%) (Fig. 3C-D, Table 3); the frequency of IFN-γ^+ and TNF-α^+ T cells responsive to LFn-DENV-NS3-P ranged from 32 to 312 SFC/10^6 PBMCs and 38 to 235 SFC/10^6 PBMCs, respectively, while the frequency responsive to LFn-DENV-NS3-H ranged from 20 to 508 SFC/10^6 PBMCs and 22 to 373 SFC/10^6 PBMCs, respectively. DENV subject 3 demonstrated IFN-γ^+ and TNF-α^+ T cell responses that were well below the positive threshold. Overall, IFN-γ^+ and TNF-α^+ responses to LFn-ZIKV/DENV-NS3-H were significantly stronger than responses to LFn-ZIKV/DENV-NS3-P. Both IFN-γ^+ and TNF-α^+ T cell responses were greater in subjects who tested positive for DENV IgM compared to those who tested positive for ZIKV IgM; this difference was statistically significant for TNF-α^+ responses to LFn-DENV-NS3-P (Fig. 3E).

IgG antibody responses are sustained after ZIKV or DENV infection

We then assessed sequential antibody responses in available plasma samples by IgG Western blot. Sequential date-matched plasma and PBMC samples were available for analysis in 6 ZIKV and 4 DENV IgM-positive subjects.

Western blot analysis using ZIKV- or DENV-infected Vero cell lysates was carried out to test for IgG antibody responses to several ZIKV or DENV proteins including prM, E, and NS1. 2 out of 6 ZIKV subjects demonstrated previous flavivirus exposure prior to testing positive for ZIKV IgM (ZIKV patients 2 and 6; 33.3) (Fig. 4A). During the recent phase of infection, ZIKV patients 2, 4, 6, and 12 had detectable IgG

antibodies to ZIKV prM, E, and/or NS1, but ZIKV patients 1 and 5 did not. Probing plasma collected months up to almost 5 years after infection (convalescent phase to later time points post-infection), antibody responses to prM, E, and/or NS1 were detectable in all cases, with some responses waning over time (ZIKV subject 1: prM and NS1; ZIKV subject 4: NS1; ZIKV subject 12: prM and E). With the exception of ZIKV subject 6, who tested ZIKV IgM-positive at the first convalescent time point (5 months after infection), all convalescent phase sera tested negative for ZIKV and DENV IgM.

Among the DENV cases, 1 out of 4 subjects demonstrated previous flavivirus exposure prior to testing positive for DENV IgM (DENV subject 7; 25%); this subject was also the only one to demonstrate detectable IgG in the recent phase of infection (Fig. 4B). In all cases, IgG antibody responses to DENV prM, E, and/or NS1 were sustained months up to over 4.5 years after infection. As above, all convalescent phase sera from the DENV subjects tested negative for DENV and ZIKV IgM.

T cell responses are sustained after ZIKV or DENV infection

We further examined the T cell responses by IFN-γ ELISPOT in sequential date-matched PBMC samples. In all ZIKV cases, robust T cell responses to LFn-ZIKV-NS3-P or LFn-ZIKV-NS3-H were detected at dynamic levels throughout the time points tested (Fig. 5A). Peak responses were observed in the recent phase of infection for ZIKV subjects 1 (NS3-P and -H) 2 (NS3-P), 4 (NS3-P and -H), 5 (NS3-P and -H), and 6 (NS3-H). T cell responses waned over time, but were sustained well above the negative cutoff for subject 2 (NS3-P and -H), 4 (NS3-P and -H) 5 (NS3-H), and 6 (NS3-P). For

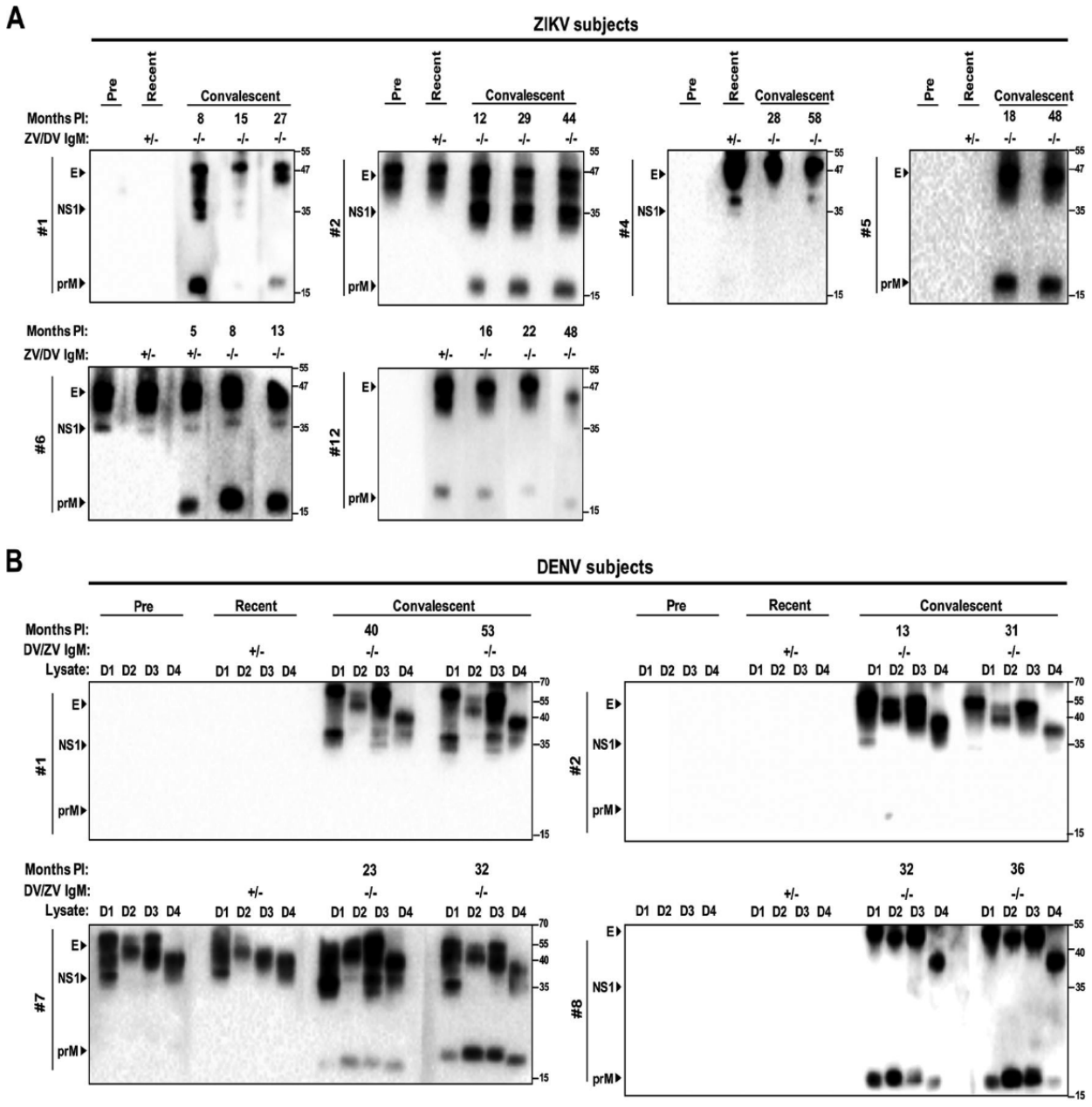

Figure 4. Sequential antibody responses to different ZIKV or DENV proteins in ZIKV or DENV subjects. Preinfection, recent-, and convalescent-phase sera from ZIKV (A) or DENV (B) IgM-positive subjects were subjected to Western blot analysis using lysates derived from ZIKV- or DENV-infected Vero cells. Arrowheads indicate

Figure 4. (continued)

PrM, E, and NS1 proteins recognized. Molecular size marker units are kilodaltons. Dates are given in month/year format. Lysates include ZIKV African strain (unpublished), DENV1 Hawaii strain (D1), DENV2 NGC strain (D2), DENV3 H87 strain (D3), and DENV4 H241 strain (D4). Months PI, months postinfection. ZV/DV IgM, results for ZIKV (ZV) or DENV (DV) IgM serology expressed as positive (+) or negative (–).

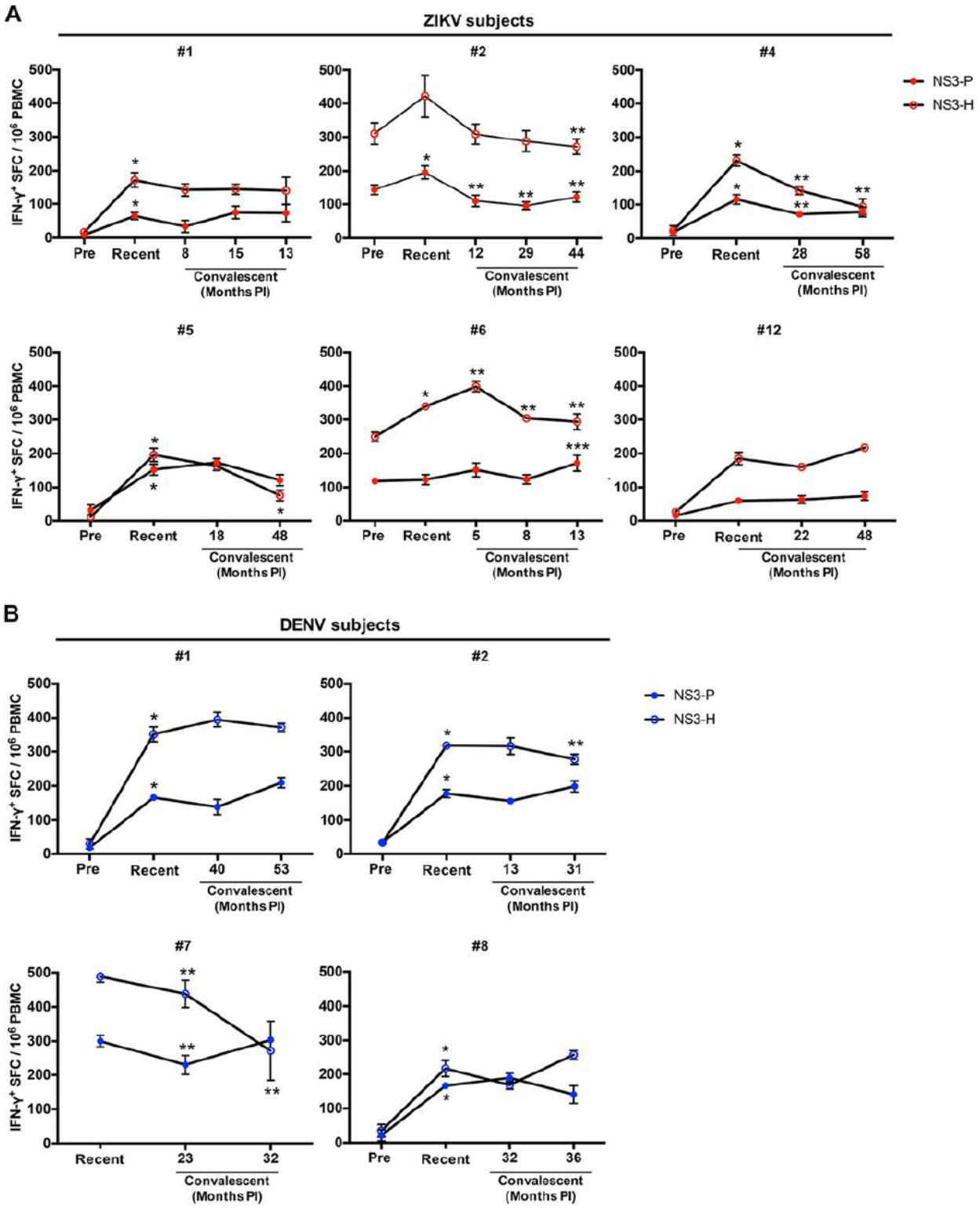

Figure 5. Sequential IFN-γ T cell reactivity to ZIKV or DENV LFn fusion proteins in ZIKV or DENV subjects. Preinfection, recent-, and convalescent-phase PBMC

Figure 5. (continued)

samples from ZIKV (A) or DENV (B) IgM-positive subjects were subjected to homologous LFn NS3 protease (ZIKV, red-shaded circle; DENV, blue-shaded circle) and helicase (ZIKV, red-outlined circle; DENV, blue-outlined circle) protein stimulation in IFN-γ LFn ELISPOT *ex vivo* experiments. Responses for each time point are expressed as the number of secreting cells per 10^6 PBMCs. Months PI, months postinfection. *, preinfection versus recent, $P < 0.05$; **, recent versus convalescent time point 1, $P < 0.05$; ***, convalescent time point 2 versus time point 3, $P < 0.05$.

subject 6, a peak response was observed to 13 months post infection. PBMCs were not available at the time of IgM-positivity for ZIKV subject 12; however, this subject demonstrated trends of responses during the recent phase of infection that were sustained throughout the convalescent time points tested. Only the two subjects with antibody responses demonstrating previous flavivirus exposure (ZIKV subjects 2 and 6) demonstrated pre-infection T cell responses, and their T cell responses were generally stronger compared to responses in the other subjects.

Similarly, in all DENV cases, robust and dynamic T cell responses to LFn-DENV-NS3-P or LFn-DENV-NS3-H were detected (Fig. 5B). For DENV subject 1, 2, and 8, peak responses to LFn-DENV-NS3-P and -H were observed during the recent phase of infection. T cell responses significantly waned over time for subjects 2 (NS3-H) and 7 (NS3-P and -H), while T cell responses in subjects 1 and 8 were sustained throughout the time points tested. DENV subject 7, the only of the 4 tested who demonstrated previous flavivirus exposure, had generally the strongest T cell responses.

While sequential analysis was not possible for a majority of the subjects, we examined the IFN-γ and TNF-α T cell response using available PBMC samples collected during the convalescent phase of infection for 13 ZIKV and 11 DENV subjects. T cell responses to LFn-ZIKV-NS3-P and LFn-ZIKV-NS3-H as compared to the LFn control were demonstrated in 12 out of 13 samples (92.3%) (Fig.6A-B, Table 4). ZIKV subject 14 demonstrated IFN-γ^+ and TNF-α^+ T cell responses that were well below the positive threshold. T cell responses to LFn-DENV-NS3-P and LFn-DENV-NS3-H as compared to the LFn control were demonstrated in 9 out of 11 samples from

the DENV subjects (81.8%) (Fig. 6C-D). DENV subjects 3 and 10 demonstrated IFN-γ$^+$ and TNF-α$^+$ T cell responses that were well below the positive threshold. Overall, IFN-γ and TNF-α convalescent-phase T cell responses to LFn-ZIKV/DENV-NS3-H were significantly stronger than responses to LFn-ZIKV/DENV-NS3-P. Both IFN-γ and TNF-α T cell responses were greater in subjects who tested positive for DENV IgM compared to those who tested positive for ZIKV IgM; this difference was statistically significant for IFN-γ responses to LFn-DENV-NS3-P (Fig. 6E). Additionally, in both the ZIKV and DENV cases, IFN-γ and TNF-α T cell responses to LFn-ZIKV/DENV-NS3-P and LFn-ZIKV/DENV-NS3-H showed a trend towards stronger responses in the recent phase of infection compared to during convalescence, though not statistically significant (Fig. 6F-G). Importantly, all ZIKV and DENV date-matched convalescent sera samples tested negative for ZIKV and DENV IgM (Table 4).

ZIKV or DENV specific and cross-reactive T cell responses

To assess specific and/or cross-reactive T cell responses between ZIKV and DENV among the subjects, PBMCs collected during the recent and convalescent phases of infection were stimulated with homologous and heterologous LFn fusion antigens in an IFN-γ ELISPOT.

PBMC samples were individually stimulated with LFn-ZIKV-NS3-P, LFn-DENV-NS3-P, LFn-ZIKV-NS3-H, and LFn-DENV-NS3-H. At the time of ZIKV IgM-positivity, T cell responses to NS3 protease were largely ZIKV specific, even in cases where individuals had previous flavivirus exposure (Fig. 7A-B), while responses to NS3 helicase were largely cross-reactive between ZIKV and DENV (91% specific for

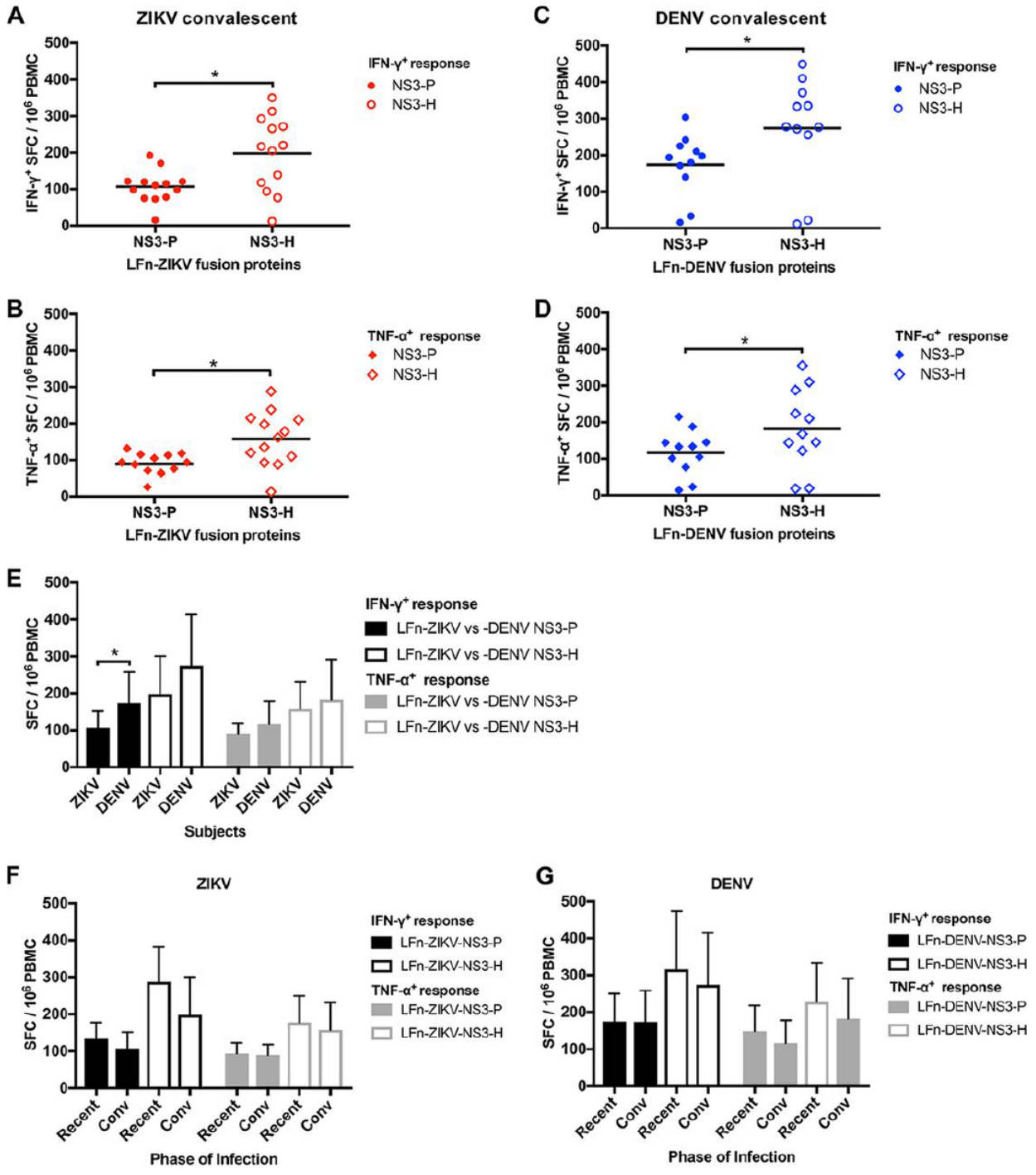

Figure 6. *Ex vivo* T cell reactivity to ZIKV or DENV LFn fusion proteins in ZIKV or DENV convalescence. PBMC samples from ZIKV IgM-positive subjects collected

Figure 6. (continued)

during the convalescent phase of infection were subjected to homologous LFn NS3 protease and helicase protein stimulation in IFN-γ (A) and TNF-α (B) LFn ELISPOT *ex vivo* experiments. LFn-ZIKV-NS3-P IFN-γ, red-shaded circle; LFn-ZIKV-NS3-H IFN-γ, red-outlined circle; LFn-ZIKV-NS3-P TNF-α, red-shaded diamond; LFn-ZIKV-NS3-H TNF-α, red-outlined diamond. PBMC samples from DENV IgM-positive subjects collected during the convalescent phase of infection were subjected to homologous LFn NS3 protease and helicase protein stimulation in IFN-γ (C) and TNF-α (D) LFn ELISPOT *ex vivo* experiments. LFn-DENV-NS3-P IFN-γ, blue-shaded circle; LFn-DENV-NS3-H IFN-γ, blue-outlined circle; LFn-DENV-NS3-P TNF-α, blue-shaded diamond; LFn-DENV-NS3-H TNF-α, blue-outlined diamond. Responses are expressed as the number of secreting cells per 10^6 PBMCs and are considered positive if the net numbers of spot-forming cells (SFC) per 10^6 are ≥55, are greater than four times the mean background, and are three standard deviations above the background. Comparison of IFN-γ^+ ZIKV/DENV NS3 protease (black-shaded bars) and helicase (black-outlined bars) and TNF-α^+ ZIKV/DENV NS3 protease (gray-shaded bars) and helicase (gray-outlined bars) T cell responses in ZIKV and DENV subjects (E) and between recent and convalescent (Conv) phases of ZIKV (F) or DENV (G) infection. The magnitudes of responses are expressed as geometric positive means ± standard deviations, and statistical analyses were performed with Mann-Whitney U test. *, $P < 0.05$.

Table 4 Characteristics of ZIKV and DENV LFn fusion proteins during convalescence

Subject	HIV status	Flavivirus immune status	Infection PBMC collection yr	No. of SFC/10^6 PBMC			
				IFN-γ^+		TNF-α^+	
				LFn ZIKV or DENV NS3-P	LFn ZIKV or DENV NS3-H	LFn ZIKV or DENV NS3-P	LFn ZIKV or DENV NS3-H
ZIKV							
1	Negative	No	1998	57	188	55	92
2	Negative	Yes	1999	182	466	63	205
3	HIV dual	No	2000	105	220	78	182
4	Negative	No	2000	122	213	99	123
5	HIV-1	No	2000	141	182	132	110
6	Negative	Yes	2001	112	345	102	298
7	Negative	No	2001	134	287	152	133
8	Negative	Yes	2002	175	324	110	270
9	Negative	No	2003	113	198	72	98
10	Negative	Yes	2003	218	348	76	215
11	Negative	Yes	2003	110	388	88	232
12	Negative	No	NA				
13	HIV dual	Yes	NA				
14	Negative	No	NA				
DENV							
1	Negative	No	1999	172	335	186	205
2	HIV-2	No	1999	165	290	87	153
3	Negative	No	2000	32	20	38	22
4	Negative	No	2000	186	312	235	228
5	Negative	Yes	2000	183	374	125	266
6	Negative	Yes	2001	188	490	190	288
7	Negative	Yes	2002	312	508	220	373
8	HIV-1	No	2002	160	200	108	290
9	HIV-1	Yes	NA				
10	Negative	No	NA				
11	Negative	Yes	NA				

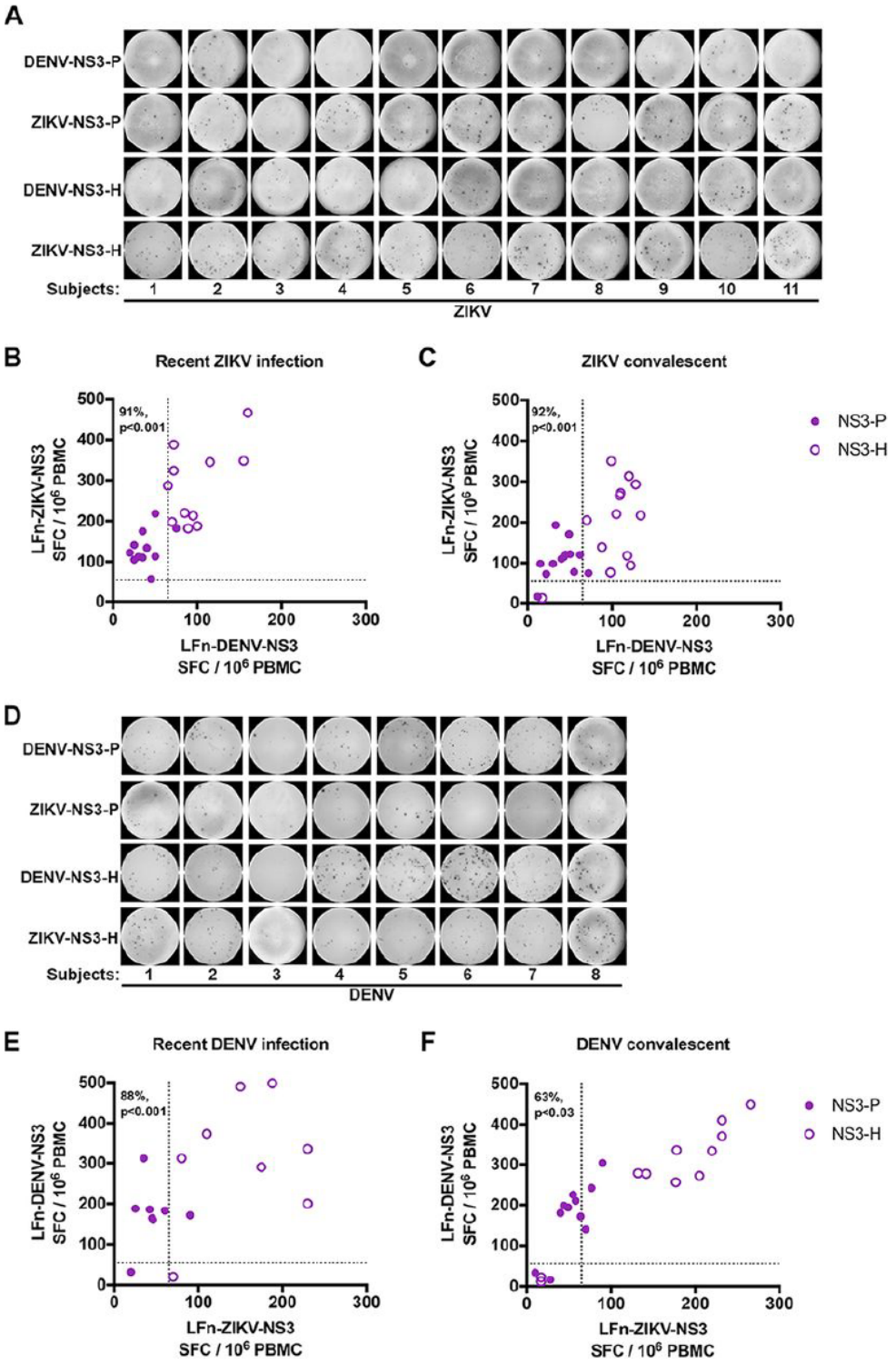

Figure 7. T cell-specific and cross-reactive NS3 responses in the recent and convalescent phases of ZIKV or DENV infections. Recent- and convalescent-phase

Figure 7. (continued)

PBMC samples from ZIKV or DENV subjects were treated with homologous and heterologous LFn-ZIKV and -DENV NS3 protease and helicase proteins, and the specific IFN-γ^+ and TNF-α^+ T cells were detected by LFn ELISPOT *ex vivo* experiments. (A to C) Representative image and magnitude of recent ZIKV/DENV infection-phase responses (A and B) and magnitude of convalescent-phase responses (C) from ZIKV subjects. (D to F) Representative image and magnitude of recent DENV/ZIKV infection-phase responses (D and E) and magnitude of convalescent responses from DENV subjects (F). Shown is the average magnitude of response of individual subjects to either LFn-ZIKV or -DENV NS3 protease (purple-shaded circle) and helicase (purple-outlined circle) proteins. Dotted lines represent the cutoff value. *, $P < 0.05$.

protease vs. 0% specific for helicase; $p<0.001$); both LFn-ZIKV-NS3-H and LFn-DENV-NS3-H elicited significant responses. Similarly, ZIKV convalescent T cell responses to NS3 protease were specific for ZIKV, but cross-reactive to NS3 helicase (Fig. 7C; 92% specific for protease vs. 0% specific for helicase; $p<0.001$). Although PBMCs were not available for ZIKV subject 14 and could not be evaluated, convalescent responses for this subject were not observed by ELISPOT; this same subject also demonstrated no TNF-α response to LFn-ZIKV-NS3-P or LFn-DENV-NS3-H (Table 3), suggesting false ZIKV IgM-positivity.

Consistent with these results, at the time of DENV IgM-positivity and at the convalescent time point, T cell responses to NS3 protease were DENV specific, but were largely cross-reactive between DENV and ZIKV to NS3 helicase (Fig. 7D-F; recent: 88% specific for protease vs. 0% specific for helicase, $p<0.001$; convalescent: 63% specific for protease vs. 0% specific for helicase, $p<0.03$). There were a few exceptions in which NS3 protease responses were cross-reactive between DENV and ZIKV, in particular during the convalescent-phase. Responses for DENV subjects 3 and 10 were not observed by ELISPOT at the recent and convalescent phases of infection (PBMCs were not available for DENV subject 10 at the acute recent of infection and could not be evaluated); these results corroborate the unobserved TNF-α response to LFn-ZIKV/DENV-NS3P or LFn-ZIKV/DENV-NS3-H for these subjects (Tables 3 and 4), suggesting false DENV IgM-positivity.

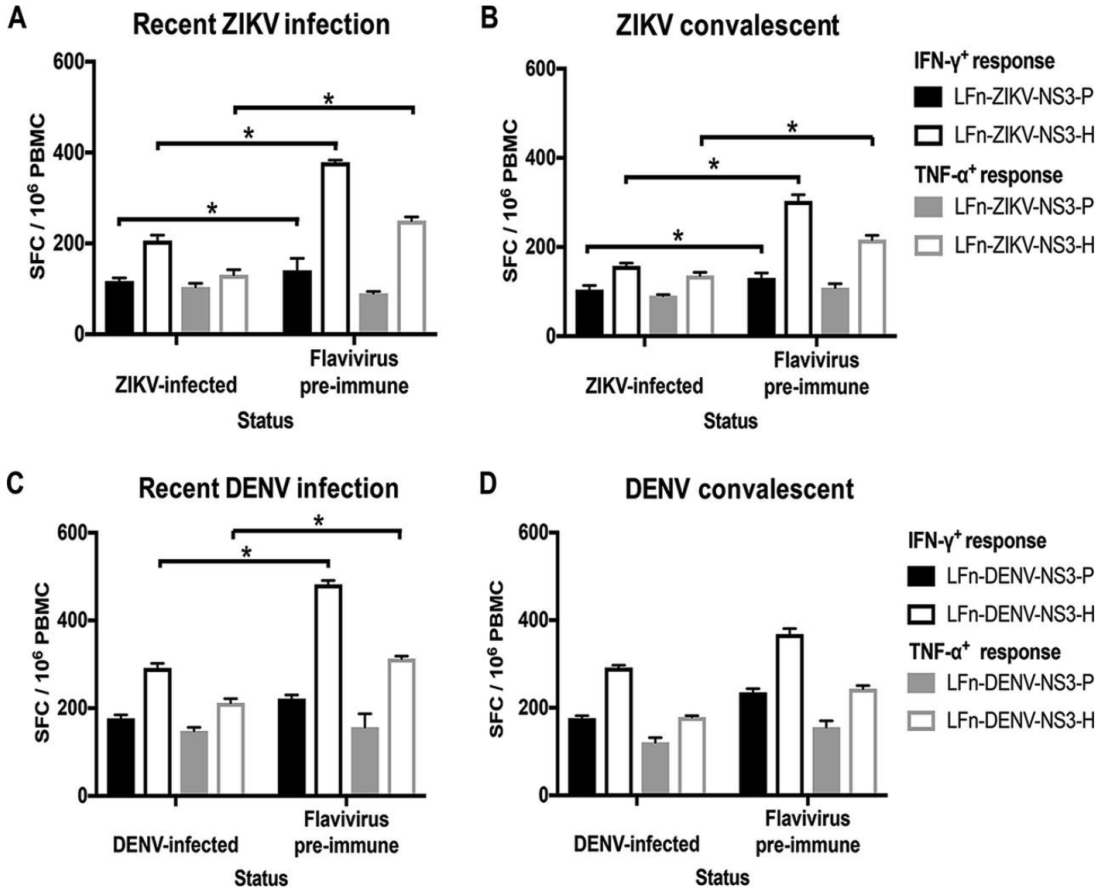

Figure 8. Impact of prior flavivirus infection on the ZIKV or DENV T cell response.

Comparison of recent (A)- and convalescent (B)-phase IFN-γ^+ ZIKV NS3 protease (black shaded bars) and helicase (black unshaded bars) and TNF-α^+ ZIKV NS3 protease (gray shaded bars) and helicase (gray unshaded bars) T cell responses from presumed primary ZIKV-infected and flavivirus preimmune groups of the ZIKV subjects. Comparison of recent (C)- and convalescent (D)-phase IFN-γ^+ DENV NS3 protease (black shaded bars) and helicase (black unshaded bars) and TNF-α^+ DENV NS3 protease (gray shaded bars) and helicase (gray unshaded bars) T cell responses from presumed primary DENV-infected and flavivirus preimmune groups of the DENV subjects. Magnitude of response for each group is expressed as geometric positive

Figure 8. (continued)

means ± standard deviations, and statistical analyses were performed with Mann-Whitney U test. *, $P < 0.05$.

Previous flavivirus exposure is associated with enhanced ZIKV or DENV T cell responses

We further evaluated the impact of previous flavivirus exposure on the frequency and magnitude of the T cell response in PBMC samples that were collected during both the recent and convalescent phases of infection. At the time the subjects tested positive for ZIKV IgM, responses for 6 subjects who demonstrated flavivirus pre-immunity (flavivirus pre-immune) and 5 who did not (primary ZIKV-infected) were included in the analysis. Additionally, responses corresponding to the convalescent phase of infection were available for analysis for 5 subjects with flavivirus pre-immunity and 8 primary ZIKV-infected. ZIKV subject 14 was excluded from the analysis due to the suspected ZIKV IgM-false-positivity. During both recent and convalescent phases of infection, LFn-ZIKV-NS3-P and LFn-ZIKV-NS3-H elicited significantly stronger IFN-γ T cell responses in subjects with flavivirus pre-immunity (Fig. 8A). LFn-ZIKV-NS3-P elicited slightly increased TNF-α T cell responses in subjects who experienced a primary ZIKV infection at the IgM-positive date, though the difference was not statistically significant. LFn-ZIKV-NS3-H also elicited significantly stronger TNF-α T cell responses in subjects with flavivirus pre-immunity during both the recent and convalescent phases of infection (Fig. 8B).

At the time the subjects tested positive for DENV IgM, responses for 3 flavivirus pre-immune subjects and 4 primary DENV-infected were included in the analysis; DENV subject 3 was excluded due to the suspected DENV IgM-false-positivity. Additionally, responses corresponding to the convalescent phase of infection were

available for analysis for 4 subjects with flavivirus pre-immunity and 5 primary DENV-infected; both DENV subjects 3 and 10 were excluded from the analysis due to suspected DENV IgM-false-positivity. During both the recent and convalescent phases of infection, LFn-ZIKV-NS3-P and LFn-ZIKV-NS3-H elicited stronger IFN-γ and TNF-α T cell responses in subjects with flavivirus pre-immunity; the difference was statistically significant for the responses to LFn-DENV-NS3-H at the time the DENV-infected subjects tested DENV IgM-positive (Fig 8C-D).

Discussion

For decades, human ZIKV infection has remained geographically limited to Africa and Asia, and has gone largely unnoticed due to its mild clinical outcomes with symptoms similar to that of other acute febrile diseases endemic in the same regions (5). However, the recent rapid emergence of ZIKV in the Pacific and the Americas and Caribbean have greatly heightened awareness of the virus. A retrospective analysis of the 2014 French Polynesian ZIKV outbreak identified associations between infection and adult Guillain-Barré syndrome (GBS), an autoimmune disease causing acute or subacute flaccid paralysis (18, 45). The 2015 ZIKV outbreak, which started in Brazil, highlighted the association of infection with other severe neurologic manifestations including microcephaly and congenital malformations in infants born to infected mothers that had previously not been recognized (19).

The mechanisms that cause these severe ZIKV disease outcomes remain unknown; however, associations between infection and neurologic symptoms are

becoming elucidated. Asian ZIKV strains demonstrated neurotropism, causing cellular death in human neural progenitor cells (NPC) and immature cortical neurons, thereby affecting neurogenesis (16, 46). Despite the absence of reported associations between ZIKV infection and neuropathology in Africa, several African strains were also shown to infect and impair growth of human NPC (47). Efforts are needed to examine similarities and differences in infection and outcomes between the African and Asian ZIKV lineages.

The human immunologic response to ZIKV and DENV infections represents a critical research gap that may potentially shed light on the observance of severe ZIKV-associated disease outcomes in some individuals but not in others. Identifying immune responses with a protective profile is also useful for vaccine development. Therefore, the goal of the present study was to develop a novel modified anthrax-based ELISPOT assay utilizing the LFn-ZIKV-NS3-P/H and LFn-DENV-NS3-P/H priming systems to measure the T cell response to African ZIKV and DENV infections. There are 4 important findings from our study: 1) robust IFN-γ and TNF-α T cell responses against African ZIKV and DENV NS3, 2) ZIKV- or DENV-specific T cell responses to NS3 protease, but cross-reactive responses to NS3 helicase, 3) long-term dynamic antibody and T cell responses, and 4) stronger T cell responses in patients with flavivirus pre-immunity.

We initially screened plasma collected from febrile patients between 1992 and 2004 from a natural history HIV Senegalese FSW cohort for the presence of ZIKV and DENV IgM antibodies. Consistent with our previous findings, the overall seroprevalence

of ZIKV IgM in this study remained at approximately 6.3% (44). Our serology testing also revealed a DENV IgM seroprevalence of 9.3%. These results provide evidence that both African ZIKV and DENV are endemic to Senegal and cause infection in humans.

Activation of CD4+ and CD8+ T cells has been extensively examined in several flavivirus infections, with responses preferentially targeting epitopes within the non-structural proteins, namely NS3, NS4b, and NS5 (39-41). These studies have also shown that CD8+ and CD4+ T cell reactivity in Sri Lankan Nicaraguan populations were HLA-linked and associated with increased or decreased disease susceptibility and were also associated with responses of lower quality, breadth, and magnitude (40, 48, 49). A recent study, however, demonstrated that a significant number of CD4+ and CD8+ T cell responses in ZIKV-positive individuals were directed against the structural proteins, and that previous DENV exposure amplifies these responses (31). A study using HLA transgenic mice infected with ZIKV revealed immunodominant and protective roles for DENV cross-reactive CD8+ T cells (30). Where adaptive HLA-linked T cell responses are clinically beneficial or detrimental during human ZIKV infections is currently not known.

We report for the first time significant IFN-γ and TNF-α T cell responses against African ZIKV and DENV NS3 during both the recent and convalescent phases of infection. We found that for both ZIKV and DENV, responses against NS3 helicase were significantly stronger than against NS3 protease during both the recent and convalescent phases. In general, T cell responses against DENV NS3 were stronger than responses against ZIKV NS3. For both subjects with ZIKV and those with DENV

infection, T cell responses were generally stronger during the recent phase of infection compared to responses measured during the convalescence.

A study on secondary DENV infection in Thai children demonstrated NS3-specific T cell responses that peaked during early convalescence (50). In another study on Vietnamese children, NS3-specific T cell responses were undetectable until after the development of plasma leakage that occurs in dengue hemorrhagic fever in the first few days of illness (51). In most cases, our sequential T cell analysis of ZIKV and DENV infections demonstrated peak frequencies of NS3-specific T cells during the recent phase, and several responses to either NS3 protease and helicase that were sustained throughout the time points tested. A possible explanation for these differences is that NS3-specific T cell responses might differ between African and Thai or Vietnamese patients. Interestingly, in 2 ZIKV (subjects 1 and 5) and 3 DENV (subjects 1, 2, and 8) cases, IgG was not detectable during the recent phase of infection; however, strong T cell responses were mounted.

In flavivirus-endemic regions of West Africa, where different viruses including ZIKV, DENV, West Nile virus, and Yellow Fever virus co-circulate, multiple sequential infections are common. It is therefore possible that low levels of endemic infection by these viruses may lead to pre-existing flavivirus immunity that impacts the subsequent immune response. Our IgG serology demonstrates significant flavivirus exposure. Among the subjects who tested ZIKV IgM-positive, 42.8% (6/14) had experienced a previous flavivirus infection. Similarly, among the subjects who tested DENV IgM-positive, 41.6% (5/12) experienced a previous flavivirus infection. Due to the high

number of cross-reactive flaviviruses circulating in Senegal, determining the previous flavivirus infections in these subjects is outside the scope of the present study. These results however suggest that humans residing in Senegal may experience multiple infections by flaviviruses throughout their lives.

Studies examining the impact of T cell responses to prior DENV infections on the outcome of the current infection have been inconclusive with respect to the pathogenic or protective consequences (39, 40). Our results demonstrate that pre-existing flavivirus immunity is associated with enhanced T cell responses. For both ZIKV and DENV infections, subjects with flavivirus pre-immunity demonstrated stronger responses than those experiencing a primary infection. Whether the enhanced responses result in protection or more severe disease remains to be determined.

Our analysis of T cell cross-reactivity between ZIKV and DENV, demonstrates ZIKV- or DENV-specific responses to NS3 protease in particular during the recent stage, but cross-reactive responses to NS3 helicase during both the recent and convalescent phases of infection. While we did not define the epitopes targeted by T cells during ZIKV or DENV infections, the higher amino acid percent identity between the ZIKV and DENV helicases (71%) versus the ZIKV and DENV proteases (53%), might help explain the ZIKV/DENV cross-reactive T cell responses to the helicase. These results have several important implications. NS3 helicase cross-reactivity between ZIKV and DENV suggests that the T cell response may be protective in individuals experiencing a secondary ZIKV infection with pre-existing DENV immunity or vice versa. Because cross-reactive responses were observed during the convalescent

phase of infection, the T cell response may be protective even if the secondary infection occurs months to years after the primary infection. Finally, the combination of specific and cross-reactive responses to NS3 elicited by the LFn priming system has diagnostic potential. Diagnosis of flaviviruses is complicated by two major factors: 1) the endemicity of related flaviviruses which cross-react in typical serologic tests, and 2) the diagnostic usefulness of nucleic acid tests are limited to the recent phase of infection. Our novel modified anthrax-based flavivirus ELISPOT has the potential to be developed into a specific T cell-based diagnostic with detection capabilities well beyond the recent phase of infection.

This study has a number of limitations. First, our study population is comprised entirely of women, and is not necessarily generalizable to men, and the ZIKV and DENV sample sizes are small. Second, it was not within the scope of our study to identify the virus that caused the primary infection, or if there were multiple prior infections. Given the low level of endemic infection by ZIKV and DENV in West Africa, prospective studies using specific diagnostic tools to actively screen for these infections are needed. Third, the plasma and PBMC samples used in this study were cryopreserved for an extended period of time – 12 to almost 20 years. Future studies are needed to validate these observations using fresh samples. Fourth, the focus of the T cell response was on ZIKV and DENV NS3. While we observed strong responses to NS3, there are likely other proteins that are being targeted by T cells, which may correlate with disease severity. Finally, due to the limited amount of PBMC available for each patient, we were unable to define the CD8+ and CD4+ specific T cell response.

In summary, while this is the first characterization of human T cell responses to African ZIKV and DENV, it raises new questions and highlights the need for further investigation to better understand the immune mechanisms and consequences related to the high cross-reactivity among these related viruses and strains. This study has identified responses to NS3 with specific and cross-reactive phenotypes that could be detected during both the recent and convalescent phases of infection. These results suggest the potential for development of a simple and low-cost African ZIKV and DENV diagnostic assay based on T cell responses. Our analysis of prior flavivirus exposure on the T cell response to ZIKV and DENV demonstrates enhanced responses in those with flavivirus pre-immunity. This finding has critical implications for development of vaccines against ZIKV and DENV, which should include the induction of ZIKV- and DENV-specific and ZIKV/DENV cross-reactive T cell responses, and should also consider specific responses to between the African and Asian viral lineages.

Materials and methods

Clinical Samples and Ethics Statement

Plasma and peripheral blood mononuclear cell (PBMC) samples were obtained from a female sex worker (FSW) cohort in Dakar, Senegal. Self-identified FSW visiting a healthcare clinic in Dakar underwent annual blood tests for multiple sexually-transmitted infections, including HIV-1/2. This natural history HIV-1/2 cohort helped define some of the first *in vivo* characteristics of HIV-2 infection in West Africa; epidemiological and clinical details of this cohort have been reported elsewhere (52).

Clinical visit records from the cohort were queried for recorded fever of ≥37.5°C. Of these, excess stored plasma samples that were collected within 7 days of the fever visit were used in serological testing. PBMCs were separated from whole blood collected in EDTA tubes by Ficoll-Hypaque gradient density (Organon Technika, Durham, NC, U.S.A.) and cryopreserved in freezing media (10% dimethyl sulfoxide [DMSO, Sigma-Aldrich, St. Louis, MO, U.S.A.], 90% fetal bovine serum [FBS, Medicorp, Montreal, Quebec, Canada) at -80°C overnight prior to transfer to liquid nitrogen. The plasma and PBMC samples included in the study were prospectively collected between 1992 and 2004.

The Harvard T.H. Chan School of Public Health Institutional Review Board (IRB) and the local research ethics committees at Cheikh Anta Diop University, Dakar, Senegal approved the primary studies under which the samples and data were collected. All patients provided informed consent for the original collection of samples. Excess samples and corresponding data were banked and anonymized. This study received an exemption determination from both the Harvard Longwood Medical Area IRB.

Construction and Expression of LFn Fusion Antigens

Commercially synthesized amino acid fragments encoding the NS3 protease and helicase of African ZIKV(consensus sequence based on the GenBank accession numbers: KU955594.1, KF268948.1, KF268949.1, KF268950.1, KU955591.1, KU955592.1, KU955595.1) and DENV2 (consensus based on the GenBank accession

numbers: EF105384.1, EF105383.1, EF105389.1, EF105390.1, EF457904.1) were cloned into the LFn expression plasmid (pET15bLFn). The pET15bLFn plasmid contains a T7 promoter, histidine tag (His_6), and the terminal domain of the LF (LFn; 255aa). The pET15bLFn containing the coding sequence of African ZIKV and DENV2 were transformed into E. coli BLR (DE3) (Millipore, Medford, MA, U.S.A., U.S.A.) for expression. Selected clones were sequenced to verify the reading frame, and clones containing the correct sequence were used for protein expression.

The LFn-DENV and -ZIKV fusion antigens were expressed upon isopropylthiogalactoside (IPTG) (Thermo Fisher Scientific, Rockford, IL, U.S.A.) induction in Luria broth containing carbenicillin and chloramphenicol. Cells were pelleted by centrifugation and resuspended in imidazole (1 mM) biding buffer (Novagen, Madison, WI, U.S.A.), in the presence of protease inhibitor cocktail (Boehringer Mannheim, Framingham, MA, U.S.A.). Cell pellets were sonicated, centrifuged at 4°C, and the supernatants were loaded in an equilibrated nickel-charged column for affinity purification based on the His_6 tag present in the amino terminal of the fusion protein. The bound proteins were eluted in 125-200 mM imidazole, desalted with a Sephadex G-25 M column (Sigma-Aldrich, St. Louis, MO, U.S.A.), and eluted in PBS. The PBS-eluted proteins were passed through an endotoxin removing gel, Detoxi-Gel (Thermo Fisher Scientific, Rockford, IL, U.S.A.). Protein concentrations were determined and samples were stored at -80°C. The pET15bLFn was expressed and purified as described above for use as a control.

Serology and RT-PCR

Plasma were screened for the presence of IgM antibodies by ZV-IgM ELISA (MyBioSource, San Diego, CA, U.S.A) or DENV Detect™ IgM Capture ELISA (InBios, Seattle, WA, U.S.A), according the manufacturer's instructions. Using internal controls, assay performances were monitored, and cutoffs were determined as specified by the manufacturer for individual kits.

RNA was extracted from serum using the QIAamp Viral RNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were tested by RT-PCR for ZIKV as previously described (5). Samples were tested by RT-PCR for DENV as previously described (53).

Western Blot

Briefly, C6/36 or Vero cells infected with DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), DENV4 (H241 strain), or African ZIKV (strain unpublished) were lysed when cytopathic effects were observed in 20% of cells with 1% NP40 lysis buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 20 mM EDTA, 1% NP40, 0.5% Na deoxycholate) containing protease inhibitors (Roche Diagnostics, U.S.A.), followed by centrifugation at 20,000xg at 4°C for 30 minutes to obtain cell lysates, as previously described (54). Aliquots of cell lysates were added to non-reducing buffer (2% SDS, 0.5 M Tris [pH 6.8], 20% glycerol, 0.001% bromophenol blue [final concentration]) and subjected to 12% polyacrylamide gel electrophoresis (PAGE) and Western Blot analysis using patient serum (1:100) as primary antibody and anti-human IgG HRP

(Thermo Fisher Scientific, Rockford, IL, U.S.A.; 1:2500) as secondary antibody. Visualization was performed using SuperSignal Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, U.S.A.), as per the manufacturer's instructions, and with a molecular imager (Chemi Doc XRS+ Imaging System, Bio Rad Technologies, Hercules, CA, U.S.A.).

Ex vivo ELISPOT Assay

96-well polyvinylidene difluoride (PVDF)-backed MultiScreen$_{HTS}$ (MSIP) microtiter plates (Millipore, Medford, MA, U.S.A.) were treated with 100ul of 90% methanol for 30 seconds and washed 5 times with sterile phosphate buffer saline (PBS, Sigma-Aldrich, St. Louis, MO, U.S.A.). Plates were coated with 100ul of capture antibodies (Ab) in PBS at the following concentrations: 15ug/ml anti-human IFN-γ mAb 1-D1K and 3ug/ml anti-human TNF-α mAb TNF3/4 (Mabtech, Cincinnati, OH, U.S.A). Plates containing capture antibodies were incubated overnight at 4°C. Plates were blocked with 100ul/well of 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, U.S.A.) in PBS for 1 hour and then washed 6 times with PBS. Cryopreserved PBMCs were thawed in R10 medium (RPMI, 20% FBS) and incubated overnight at 37°C. PBMCs were washed two times with PBS and seeded in triplicate at 2 x 10^5 cells/well in a final volume of 100ul/well. Fusion LFn-ZIKV and -DENV proteins were added to each well at 2.5ug/ml final concentration. As a positive control, PBMCs were stimulated with 5ug/ml of phytohemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO, U.S.A.). As a negative control, wells received LFn with no fused protein at 2.5ug/ml final concentration.

After incubation for 28-32 hours at 37°C in 5% CO_2, the cells were discarded and plates were washed three times with PBS, and again three times with PBS with 0.05% Tween-20 (PBST, Bio Rad Technologies, Hercules, CA, U.S.A.) to remove cells. The detection Ab were added at 100ul/well in PBST, 1% BSA at the following concentrations: 1ug/ml anti-human IFN-γ mAb 7-B6-1 and 0.5ug/ml anti-human TNF-α mAb TNF5 (Mabtech, Cincinnati, OH, U.S.A). Plates were incubated overnight at 4°C. Plates were washed six times with PBST, then incubated for 2 hours at room temperature with 100ul/well of a mixture with the following enzymatic conjugate: 0.5ug/ml streptavidin alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.).

To develop the spots, plates were washed four times with PBST, three times with PBS, and one time with water. Vector Blue substrate solution (Vector Laboratories, Burlingame, CA, U.S.A.) was added at 100ul/well for 5-15 minutes before rinsing with water and air-drying. Digitized images were analyzed for spots using CTL ImmunoSpot reader (Cellular Technology Limited, Cleveland, OH, U.S.A.) or by counting spots using a stereozoom microscope (20X magnification). ZIKV- or DENV-specific spots were calculated by subtracting the mean of the negative control values of the replicates from the mean values of the specific stimulation. Positive responses had to be greater than four times the mean background, three standard deviations above the background, and ≥55 spot-forming cells per (SFC)/10^6 PBMCs.

Statistical Analysis

Statistical analyses were performed using the programs Prism 7 (GraphPad Software, San Diego, CA, U.S.A.) and Stata v. 13.1 (College Station, TX, U.S.A.). Data were expressed as geometric positive means ± standard deviation. Data comparisons were conducted using the Wilcoxon ranksum and Fisher's exact tests, as relevant. A threshold of $p \leq 0.05$ was considered statistically significant.

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CHAPTER 5

T cell responses to nonstructural protein 3 distinguish infections by Dengue and Zika viruses

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Chapter 5: T cell responses to nonstructural protein 3 distinguish infections by Dengue and Zika viruses

ABSTRACT

The 2015-16 Zika virus (ZIKV) epidemic in the Americas and the Caribbean demonstrates that clinical assays to detect, distinguish, and characterize immune responses to flaviviral infections are needed. ZIKV and dengue virus (DENV) are mosquito-transmitted flaviviruses sharing overlapping geographical distribution and have significant sequence similarity that can increase the potential for antibody and T cell cross-reaction. Using nonstructural protein 1-based enzyme-linked immunosorbent assays (ELISAs), we determine the serostatus of individuals living in a DENV- and ZIKV-endemic region in Brazil, identifying individuals with primary DENV (pDENV) and ZIKV (pZIKV), ZIKV with primary DENV (ZIKVwpDENV), and secondary DENV (sDENV) infections; pDENV and pZIKV were further confirmed by neutralization tests. Development of an enzyme-linked immunospot (ELISPOT) assay for DENV and ZIKV structural and nonstructural (NS) protein antigens enables us to distinguish infections by these viruses based on T cells and to characterize those responses. We find that IFN-γ and TNF-α T cell responses to NS3 differentiates DENV and ZIKV infections with 94% sensitivity and 92% specificity. In general, we also show that pDENV and sDENV cases and pZIKV and ZIKVwpDENV cases elicit similar T cell response patterns, and that HIV-infected individuals have T cell responses that are lower in magnitude compared to HIV-negative individuals. These results have important implications for DENV and ZIKV diagnostic and vaccine development and provide critical insights into the T cell response in individuals with multiple flaviviral infections.

Background

Aedes mosquitoes transmit globally relevant flaviviruses including dengue virus (DENV) and Zika virus (ZIKV). DENV exists as four antigenic serotypes, DENV1 to DENV4 (1). These viruses have a wide geographic distribution with approximately 390 million infections annually and more than a quarter of the world's population at risk (2). Prior to 2015, ZIKV was considered obscure and known to circulate in Africa and Southeast Asia as two separate viral lineages, African and Asian (3). While most asymptomatic, the clinical presentation of ZIKV infection resembles that of dengue including fever, rash, conjunctivitis, arthralgia, and myalgia (4). In early 2015, thousands of Asian ZIKV cases appeared in northeast Brazil, with accompanying reports of severe neuropathology including congenital microcephaly and Guillain-Barré syndrome (5, 6). In February 2016, the World Health Organization declared ZIKV a public health emergency of international concern (7). By June 2016, autochthonous transmission of ZIKV had been reported in 40 countries and territories throughout South and Central America and the Caribbean (8).

The emergence of ZIKV in DENV-endemic regions is of particular concern and relevant for diagnostic and vaccine development. The cocirculation of these genetically similar viruses can result in co-infection or sequential exposure, which has been shown to potentiate cross-reactive immunity at both the antibody and T cell levels (9-12). The envelope (E) protein is the major target of the antibody response in humans during flaviviral infection (1). Antibody-based assays were found to detect extensive cross-reactivity to ZIKV E protein with other flaviviruses, requiring confirmation by plaque

reduction neutralization tests (PRNTs) (11, 13-16). These tests, however, are challenged in their ability to confirm infection in individuals with multiple flaviviral infections especially during the acute and early convalescent phases. Several studies have also shown that most dengue-immune sera or DENV E monoclonal antibodies cross-react to ZIKV, but contain limited cross-neutralization activity and can instead enhance ZIKV infection, known as antibody-dependent enhancement (ADE) (17-22). In contrast, recent studies reported antibodies to ZIKV nonstructural protein 1 (NS1) were able to discriminate infections by these viruses (23, 24). We previously showed that combinations of DENV and ZIKV NS1-based enzyme-linked immunosorbent assays (ELISAs) were capable of distinguishing confirmed cases with past and present flaviviral infections including primary DENV (pDENV) and ZIKV (pZIKV), ZIKV with primary DENV (ZIKVwpDENV), and secondary DENV (sDENV) infections (12). These ELISAs are applicable for routine serological tests for DENV and ZIKV as well useful in retrospective studies to identify individuals with primary and multiple flaviviral infections.

Pre-existing T cell responses to DENV have also been shown to react to peptides encoded throughout the ZIKV proteome. DENV-naïve mice challenged with ZIKV developed ZIKV-specific $CD8^+$ T cells, whereas DENV-immune mice challenged with ZIKV elicited cross-reactive $CD8^+$ T cells that reduced infectious ZIKV (25). A study in humans infected with Asian ZIKV demonstrated that DENV serostatus influences the T cell response to ZIKV (10). DENV-immune individuals elicited $CD4^+$ and $CD8^+$ T cell responses to ZIKV more rapidly and of greater magnitude compared to DENV-naïve

ZIKV-infected individuals. In addition, different patterns of immunodominant T cell responses were observed in the case of DENV and ZIKV infections. While $CD8^+$ T cell responses against DENV target nonstructural (NS) proteins such as NS3, NS4B, and NS5, ZIKV-specific $CD8^+$ T cell responses target the structural proteins, capsid (C), premembrane (prM), and E (10, 26). We previously developed a modified anthrax toxin (LFn)-based enzyme-linked immunospot (ELISPOT) assay, which revealed long-term T cell responses that were ZIKV- and DENV-specific to NS3 protease but cross-reactive to NS3 helicase in individuals infected with DENV and African ZIKV (27). The impact of cross-reactive immune responses in protection or development of ZIKV-mediated neuropathology remains unclear.

In this study, we utilized our NS1-based ELISAs to determine the DENV and ZIKV serostatus of individuals from Salvador, Brazil, a DENV-hyperendemic region with one of the highest incidence rates of ZIKV during the 2015-16 epidemic (28). We then tested the ability of our LFn ELISPOT assay to distinguish infections by DENV and Asian ZIKV based on T cells and to characterize those responses.

Table 1 Cohort characteristics and results of serological tests

	Characteristics			Acute-phase sera			Late convalescent-phase sera						
ID	Sex	Age	HIV status	ZIKV-NS1 IgG[a]	DENV-E IgG[a]	PRNT test[b]	ZIKV-NS1 IgG[c]	DENV-NS1 IgG[c]	rOD ratio[c]	ZIKV-E IgG[d]	DENV-E IgG[d]	NT test[f] D1/D2/D3/D4/ZIKV	Interpretation
ZK0978	F	49	-	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0982	F	50	-	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0987	F	33	-	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0999	F	49	+	-	-	-	-	-	NA	-	-	<10/<10/<10/<10/<10	negative
ZK0979	F	28.9	-	+	+		+	-	NA	+[e]	+	<10/<10/<10/<10/>160	pZIKV
ZK0993	M	27	+	+	+		+	-	NA	+[e]	+	<40/<40/<10/<10/>160	pZIKV
ZK0998	M	55	+	+	+		+	-	NA	+[e]	+	<10/<10/<40/<10/>160	pZIKV
ZL1006	F	51	+	+	+		+	-	NA	+[e]	+	<160/<40<10/<40/>160	pZIKV
ZK0996	F	23	-	+	-	+	+	-	NA	+[e]	+	<10/<10/<10/<10/>160	pZIKV
ZK0966	M	34	+	-	+	-	-	-	NA	+	+[e]	<40/160/<10/<40/<10	pDENV
ZK0980	F	33	-	-	+		-	-	NA	+	+[e]	<40/<40/>160/<40/<10	pDENV
ZK0995	M	42	+	-	+		-	-	NA	+	+[e]	>640/160/<10/<10/<10	pDENV
ZK0997	F	37	-	ND	ND		-	-	NA	+	+[e]	<40/>160/<40/<40/<10	pDENV
ZK0972	F	41	+	+	+		+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK0975	F	67	+	+	+		+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK0989	M	42	+	+	+	+	+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK0991	F	24	-	+	+	+	+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK1000	M	56	+	+	+		+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK1009	M	52	+	+	+	+	+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK1011	M	72	+	+	+		+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK1012	F	40	+	+	+	+	+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK1014	F	41	-	+	+	+	+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK1015	M	54	+	+	+	+	+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK0968	M	46	+	+	ND		+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK0984	F	58	+	+	+		+	+	≥0.24	+	+	ND	ZIKVwpDENV
ZK0976	F	50	+	-	+		+	+	<0.24	+	+	ND	sDENV
ZK0986	F	53	+	-	+		+	+	<0.24	+	+	ND	sDENV
ZK0967	F	41	+	+	+		+	+	<0.24	+	+	ND	sDENV
ZK0969	M	32	+	+	+		+	+	<0.24	+	+	ND	sDENV
ZK0971	F	29	+	+	+		+	+	<0.24	+	+	ND	sDENV
ZK0977	M	52	+	+	+		+	+	<0.24	+	+	ND	sDENV

Table 1 (continued)

ZK0983	F	43	+	+	+	+	+	+	<0.24	+	+	ND	sDENV
ZL0985	F	33	+	+	+	+	+	+	<0.24	+	+	ND	sDENV
ZK0988	M	40	+	+	+		+	+	<0.24	+	+	ND	sDENV
ZK0992	M	53	+	+	+	+	+	+	<0.24	+	+	ND	sDENV
ZK0994	F	42	+	+	+	-	+	+	<0.24	+	+	ND	sDENV
ZK1001	M	40	+	+	+		+	+	<0.24	+	+	ND	sDENV
ZK1010	M	50	+	+	+		+	+	<0.24	+	+	ND	sDENV
ZK1013	F	31	-	+	ND		+	+	<0.24	+	+	ND	sDENV
ZK0973	M	62	+	-	ND		-	+	<0.24	+	+	ND	sDENV
ZK0974	M	39	+	-	+		-	+	<0.24	+	+	ND	sDENV
ZK1002	M	35	+	-	+	-	-	+	<0.24	+	+	ND	sDENV
ZK1003	F	57	+	-	+	-	-	+	<0.24	+	+	ND	sDENV
ZK1007	F	48	+	-	+		-	+	<0.24	+	+	ND	sDENV
ZK1016	M	46	+	-	+		-	+	<0.24	+	+	ND	sDENV
ZK0990	F	45	-	-	+	-	-	+	<0.24	+	+	ND	sDENV
ZK1004	M	28	-	+	+		-	+	NA	+	+	ND	undetermined
ZK1005	F	53	+	+	ND		-	+	NA	+	+	ND	undetermined
ZK1008	M	62	+	+	+		-	-	NA	+	+	ND	undetermined
ZK0981	F	47	-	-	ND		-	-	NA	-	-	ND	undetermined

[a]Euroimmun ZIKV-NS1 and DENV-E IgG ELISAs were performed on acute-phase sera (28).

[b]PRNT was performed on acute-phase sera to detect neutralization antibody to ZIKV (29).

[c]ZIKV-NS1 and DENV-NS IgG ELISAs were described previously (12). The rOD ratio (ZIKV-NS1/DENV-NS1) < or ≥0.24 was classified as sDENV or ZIKVwpDENV (12).

[d,e]ZIKV-E and DENV-E IgG ELISAs utilized ZIKV VLP and DENV virions, respectively (30). ΔrOD (rOD of ZIKV–rOD of DENV) ≥ 0.17 or < –0.17 was classified as pZIKV or pDENV infection, respectively.

Table 1 (continued)

[f]Micro-neutralization test (NT) were performed (with NT_{90} titers shown) to confirm none, pZIKV or pDENV infection (30, 31).

Results

NS1-based ELISAs and neutralization test determine DENV and ZIKV serostatus

During the ZIKV outbreak in Salvador, Brazil, acute-phase blood samples were collected from hundreds of suspected ZIKV-infected patients attending HIV outpatient clinics between November 2015 and May 2016. Serological testing for ZIKV-NS1 IgG and DENV-E IgG was performed, revealing a high incidence of ZIKV infection in presumed DENV-immune and -naïve individuals (28). Fifty of these patients were included in the present study. Their median age was 43 (range: 23-72), 49% female, and 76% were human immunodeficiency virus (HIV)-infected. All HIV-infected individuals were on antiretroviral therapy; more than 92% had undetectable viral loads and normal CD4 counts. Patient characteristics and acute serology data are summarized in Table 1.

In order to determine the DENV and ZIKV serostatus among the study participants who had potentially been dual exposed, we collected late convalescent-phase blood samples and employed our previously developed ZIKV-NS1 and DENV-NS1 IgG ELISAs (22). For samples positive for DENV-NS1, we calculated the ratio of relative optical density (rOD) of ZIKV-NS1 to that of DENV-NS1 and used the rOD ratio $< \text{or} \geq 0.24$ to determine sDENV or ZIKVwpDENV infection, respectively (22). Twelve ZIKVwpDENV and 21 sDENV infections were identified (Table 1, Fig. 1A to C). Since these samples were collected more than one year post-infection, some anti-NS1 antibodies may have declined to levels below detection, we further tested with ZIKV and DENV E protein-based ELISAs and identified four samples negative for both ZIKV

and DENV in all four ELISAs tested (Table 1). Based on the difference in rOD of ZIKV and DENV E proteins (ΔrOD=rOD of ZIKV – rOD of DENV), we identified five pZIKV (ΔrOD ≥0.17) and four pDENV (ΔrOD <–0.17) infections (Table 1, Fig. 1D to E). The negative, pZIKV and pDENV samples were further confirmed by micro-neutralization test; all four negative samples had NT_{90} titers <10 to DENV1-4 and ZIKV, and the five pZIKV and four pDENV samples showed monotypic neutralization pattern to ZIKV and to one of the four DENV serotypes, respectively (Table 1). An additional three samples (ZK1004, 1005, 1008), which had ΔrOD between –0.17 and 0.17, positive ZIKV-NS1 IgG at acute-phase sera but negative at late convalescent-phase, were classified as undetermined (Table 1). Another sample (ZK0981), for which DENV acute-phase serology was not performed, was negative for ELISAs using late convalescent-phase serum and was also classified as undetermined.

T cell responses to NS3 distinguish DENV and ZIKV infections

We recently reported the development of a LFn ELISPOT assay based on NS3 protease and helicase to distinguish DENV and African ZIKV human infections (27). To assess the ability of the assay to distinguish infection by DENV and Asian ZIKV, we performed DENV and ZIKV homologous and heterologous LFn-NS3 protease and helicase stimulation of date-matched late convalescent-phase PBMCs in an IFN-γ and TNF-α ELISPOT among the serological-validated pDENV, pZIKV, sDENV, and ZIKVwpDENV, and undetermined cases. Using a NS3 protease to helicase ratio cutoff of 1.05 for the IFN-γ ELISPOT, pDENV and sDENV cases and pZIKV, ZIKVwpDENV and the 3 out of the 4 serologically undetermined cases appeared to group together

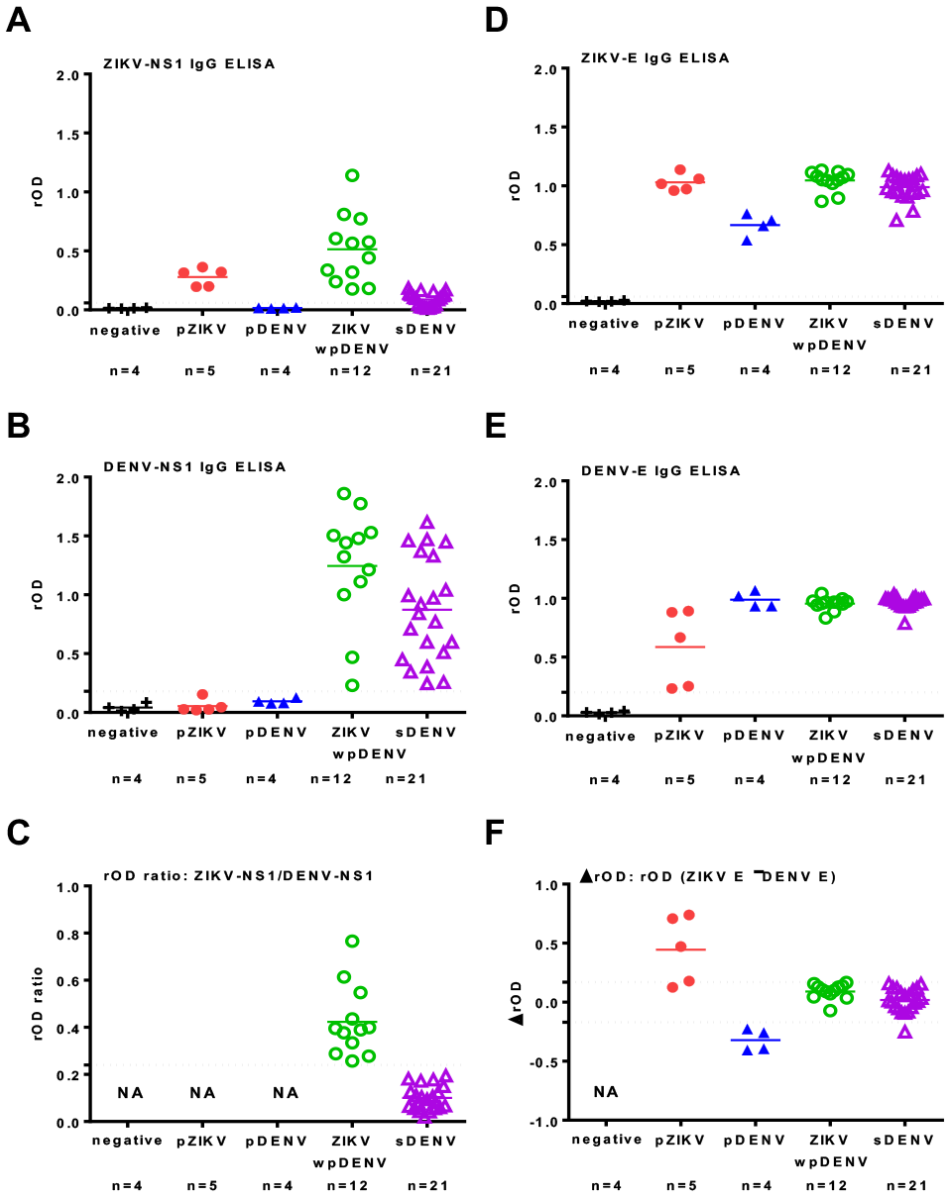

Figure 1. ZIKV and DENV NS1-based and E-based IgG ELISAs. (A) ZIKV-NS1, (B) DENV-NS1 IgG ELISAs and (C) rOD ratio. (D) ZIKV-E, (E) DENV-E and (F) ΔrOD=rOD of ZIKV–rOD of DENV. Dots lines indicate cut-off values, 0.24 for rOD ratio and 0.17 for ΔrOD. pZIKV: primary ZIKV infection; pDENV: primary DENV infection; sDENV: secondary DENV infection; ZIKVwpDENV: ZIKV infection with previous DENV infection. NA, not applicable.

(Fig. 2A). From the undetermined cases, 3 out of 4 (ZK1004, 1005, 1008) grouped with the ZIKV-exposed individuals, while ZK0981 grouped with the DENV-infected individuals. Using a ratio cutoff of 1.048 for the TNF-α ELISPOT, similar groupings were observed (Fig. 2B). We were unable to distinguish sequential infections based on T cell responses to NS3 protease and helicase.

Test data were further analyzed to define sensitivity (identifying true positives; individuals who had been infected by DENV versus ZIKV) and specificity (true negatives; DENV- or ZIKV-uninfected individuals). We evaluated sensitivity and specificity as functions of the IFN-γ and TNF-α cutoff values, above which a sample was considered positive and below which a sample was considered negative. We grouped pDENV and sDENV and pZIKV and ZIKVwpDENV cases together based on the clustering observed and excluded the 4 serologically undetermined cases from the analysis. Receiver Operating Characteristic (ROC) curves and corresponding numerical values illustrate the performance of the ELISPOTs as a function of the discrimination threshold, plotted as sensitivity versus 1 – specificity. The areas of the ROC curves represent test performance, where 1 represents a perfect test, and 0.5 represents a random predictor. We measured areas of 0.96 and 0.97 for the IFN-γ and TNF-α ELISPOTs, respectively (Table 2, Fig. 2 C-D). Using the cutoff values, the test sensitivity and specificity for both the IFN-γ and TNF-α ELISPOTs were 94% and 92%, respectively.

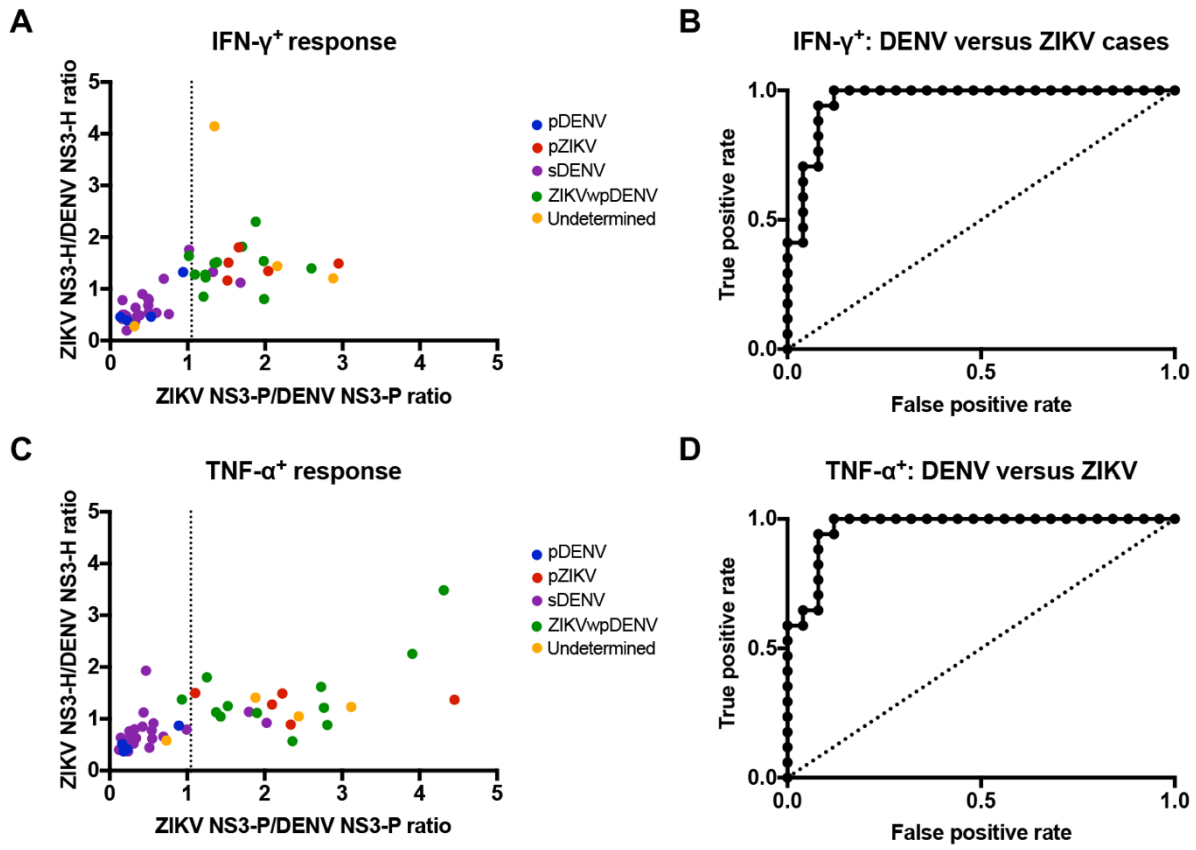

Figure 2. T cell responses to NS3 protease and helicase and ROC analysis of the ELISPOT test. Late convalescent-phase PBMCs from DENV- and/or ZIKV-infected individuals were treated with homologous and heterologous LFn-DENV and -ZIKV NS3 protease and the specific IFN-γ and TNF-α T cell responses were detected by *ex vivo* ELISPOTs. (A) Scatter plot of the ratios of ZIKV NS3 protease to DENV NS3 protease IFN-γ responses versus ratios of helicase. (B) ROC analysis for the IFN-γ ELISPOT. (C) Scatter plot of the ratios of ZIKV NS3 protease to DENV NS3 protease TNF-α T cell responses versus ratios of helicase. (D) ROC analysis for the TNF-α ELISPOT. The dashed line on (A) represents the optimal cutoff value of 1.05 and the dashed line on

Figure 2. (continued)

(C) represents the optimal cutoff value of 1.048. Individual colored dots represent serologically-validated DENV- and/or ZIKV-infected individuals and the undetermined cases.

Table 2 Numerical values of ROC analysis and sensitivity and specificity results

	IFN-γ⁺ DENV vs. ZIKV	TNF-α⁺ DENV vs. ZIKV
AUC[a]	0.96	0.97
95% CI[b]	0.91-1.02	0.92-1.01
Cutoff	1.055	1.048
Sensitivity	94%	94%
Specificity	92%	92%

[a]Area under the curve, [b]Confidence interval

LFn-DENV and -ZIKV structural and nonstructural proteins elicit robust T cell responses and prior DENV exposure does not affect the response

To assess the magnitude of T cell responses among the study participants, we stimulated late convalescent-phase PBMCs in IFN-γ and TNF-α ELISPOTs using the following six LFn fusion proteins: LFn-DENV-NS3-Protease (LFn-DV NS3-P), LFn-DENV-NS3-Helicase (LFn-DV NS3-H), LFn-ZIKV-Capsid (LFn-ZV C), LFn-ZIKV-premembrance (LFn-ZV prM), LFn-ZIKV-NS3-Protease (LFn-ZV NS3-P), LFn-ZIKV-NS3-Helicase (LFn-ZV NS3-H). Individuals with pDENV and sDENV infections elicited similar IFN-γ and TNF-α T cell response patterns (Fig. 3 A-B). These individuals had T cell responses to LFn-DV NS3-H and LFn-ZV NS3-H that were stronger in magnitude than to LFn-DV NS3-P and LFn-ZV NS3-P, respectively. Additionally, T cell responses to LFn-DV NS3-P and NS3-H were stronger compared to LFn-ZV NS3-P and NS3-H. The amount of T cell cross-reaction to the ZIKV structural proteins (LFn-ZV C and LFn-ZV prM) was limited, compared to high cross-reactivity to LFn-ZV NS3-P and NS3-H. Furthermore, individuals with pZIKV and ZIKVwpDENV infections elicited T cell responses to LFn-ZV NS3-H and LFn-DV NS3-H that were stronger in magnitude compared to LFn-ZV NS3-P and LFn-DV NS3-P, respectively (Fig. 3 C-D). While individuals with pZIKV and ZIKVwpDENV infections had stronger IFN-γ T cell responses to LFn-ZV NS3-H than to the ZIKV structural proteins, TNF-α responses to the ZIKV structural proteins were stronger than to LFn-ZV NS3-P.

We further evaluated the impact of DENV immunity on the magnitude of T cell responses. We compared the magnitude of the IFN-γ and TNF-α T cell responses

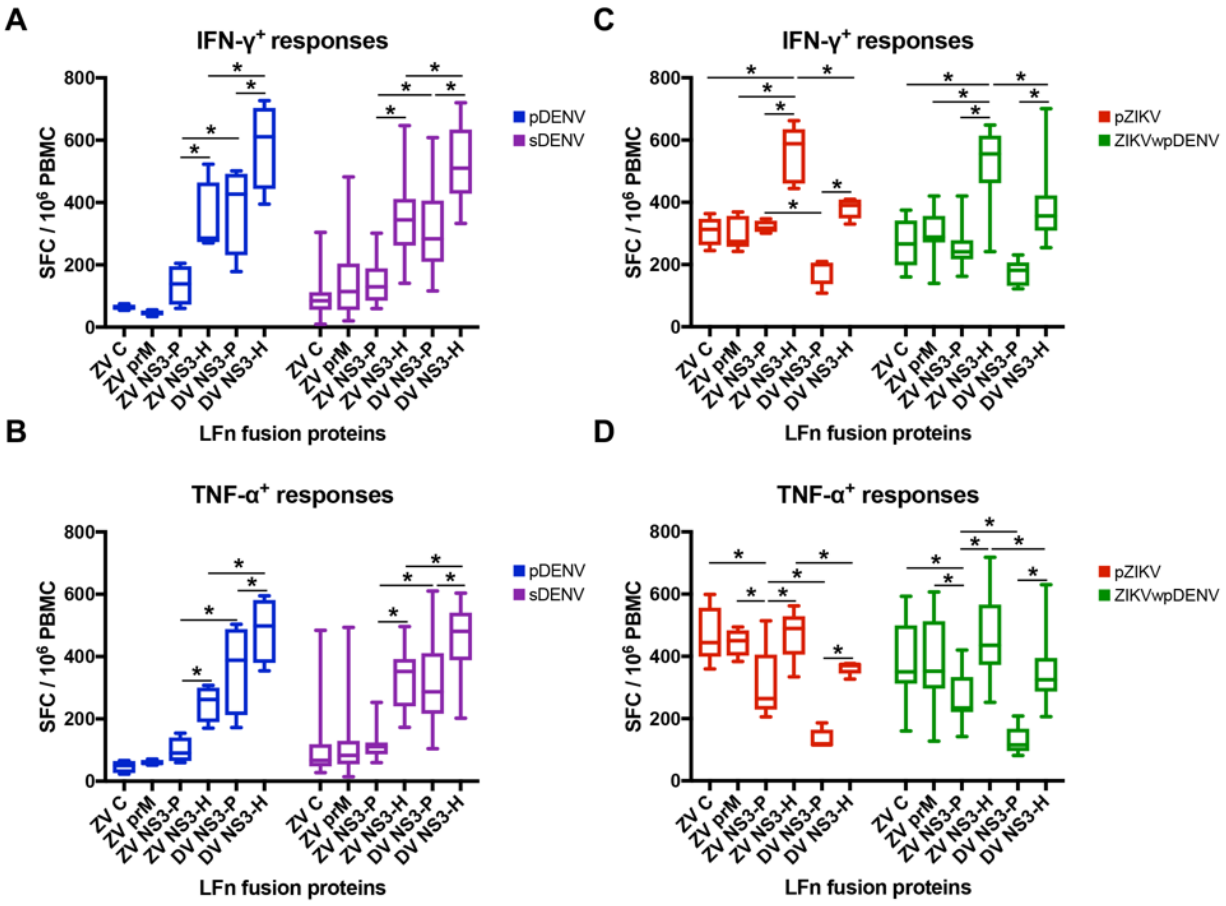

Figure 3. T cell responses to ZIKV and/or DENV structural or nonstructural proteins among subgroups with different DENV and ZIKV serostatus. Late convalescent-phase PBMCs from DENV- and/or ZIKV-infected individuals were treated with homologous and/or heterologous LFn-DENV and -ZIKV capsid (ZV C), premembrane (ZV prM), NS3 protease (DV or ZV NS3-P) and NS3 helicase (DV or ZV NS3-H) and the specific IFN-γ and TNF-α T cell responses were detected by *ex vivo* ELISPOTs. IFN-γ and TNF-α spot forming cells (SFC) were detected, counted, and expressed as box plots with mean and standard deviations. Comparison of late convalescent-phase (A) IFN-γ and (B) TNF-α T cell responses between individuals with pDENV and sDENV infections.

Figure 3. (continued)

Comparison of late convalescent-phase (C) IFN-γ and (D) TNF-α T cell responses between individuals with pZIKV and ZIKVwpDENV infections. Individual colored plots represent serologically-validated DENV- and/or ZIKV-infected individual. *, $p<0.05$.

between individuals with pDENV and sDENV infections and pZIKV and ZIKVwpDENV infections. In all cases, T cell responses in individuals with prior DENV exposure were not significantly higher compared to individuals with a primary DENV or ZIKV infection (Fig. 3 A-D). While IFN-γ and TNF-α responses appeared stronger to the ZIKV structural proteins in individuals with sDENV infections than to individuals with pDENV infections, these differences were not statistically significant. Similarly, individuals with ZIKVwpDENV infection had comparable IFN-γ and TNF-α T responses to those with pZIKV infections (Fig. 3 C-D).

HIV influences the T cell response in DENV-exposed individuals

We also compared the magnitude of the IFN-γ and TNF-α T cell responses in DENV-exposed (grouping individuals with pDENV and sDENV infections together), pZIKV, and ZIKVwpDENV individuals who were HIV-negative or HIV-infected. DENV-exposed HIV-negative individuals had stronger IFN-γ responses to LFn-ZV C, -ZV NS3-P, and -ZV NS3-H compared to HIV-infected individuals. IFN-γ responses to LFn-DV NS3-P and NS3-H appeared to be stronger in the HIV-infected individuals, although these differences were not statistically significant ($p=0.61$ and $p=0.13$, respectively) (Fig. 4 A). A similar pattern of responses was observed for TNF-α (Fig. 4 D). In general, ZIKVwpDENV HIV-negative individuals had stronger IFN-γ and TNF-α responses compared to individuals that were HIV-infected (Fig. 4 B and E). In contrast, there was largely no difference in the IFN-γ and TNF-α responses in pZIKV HIV-negative and HIV-infected individuals (Fig. 4 C and F). There was an exception where the TNF-α response to LFn-ZV NS3-H was stronger in individuals that were HIV-negative.

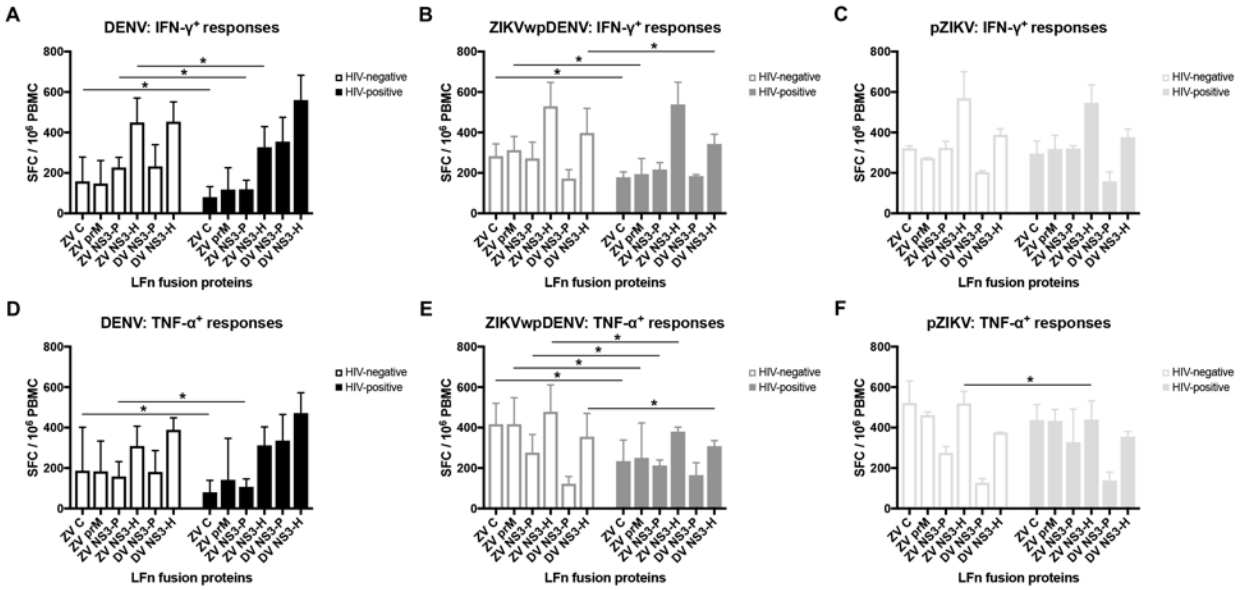

Figure 4. Impact of HIV status on the T cell response. Comparison of mean convalescent-phase IFN-γ T cell responses expressed as bars and standard deviation between (A) HIV-negative (open black bars) and HIV-infected (shaded black bars) individuals with pDENV and sDENV infections grouped together, (B) HIV-negative (open dark grey bars) and HIV-infected (shaded dark grey bars) individuals with ZIKVwpDENV infections, and (C) HIV-negative (open light grey bars) and HIV-infected (shaded light grey bars) individuals with pZIKV infections. Comparison of mean convalescent-phase TNF-α T cell responses expressed as bars and standard deviation between (D) HIV-negative (open black bars) and HIV-infected (shaded black bars) individuals with pDENV and sDENV infections grouped together, (E) HIV-negative (open dark grey bars) and HIV-infected (shaded dark grey bars) individuals with ZIKVwpDENV infections, and (F) HIV-negative (open light grey bars) and HIV-infected (shaded light grey bars) individuals with pZIKV infections. *, $p<0.05$.

Discussion

We report on the characterization of late convalescent-phase antibody and T cell responses in individuals from Salvador, Brazil, a DENV-hyperendemic region that was burdened by the 2015-2016 ZIKV outbreak. Our study presents three major findings in a serologically-validated group of DENV and/or ZIKV infected individuals. First, IFN-γ and TNF-α T cell response ratios of ZIKV NS3 protease to DENV NS3 protease can discriminate infections in individuals exposed to these viruses. Second, individuals with pDENV and sDENV infections have similar T cell response patterns, with extensive cross-reactivity to ZIKV NS3 helicase, whereas individuals with pZIKV and ZIKVwpDENV infections have strong responses to both ZIKV structural and nonstructural proteins, with high cross-reaction to DENV NS3 helicase. Third, HIV-infection is associated with responses that are lower in magnitude in DENV exposed individuals.

Our previous study of NS1-based ELISAs on convalescent-phase serum from RT-PCR confirmed cases with pZIKV, pDENV, sDENV and ZIKVwpDENV infections showed that sDENV infection panel cross-react to ZIKV-NS1 and the rOD ratio of ZIKV-NS1 to DENV-NS1 in IgG ELISA can distinguish sDENV and ZIKVwpDENV infections (22). Since anti-NS1 antibodies may decline over time and become undetectable especially for those with primary infection, we further tested these samples with E protein-based IgG ELISAs and identified four negative samples, five pZIKV and four pDENV infections. All these 14 samples have been verified by neutralization test using NT_{90} ≥10 as cutoff based on the CDC guidelines (16), suggesting that ΔrOD based on

Table 3 Sequence homology of DENV and Asian ZIKV NS3[a]

	% homology to ZIKV		
Serotype	NS3 protease	NS3 helicase	Full-length
DENV1	55%	71%	66%
DENV2	58%	72%	67%
DENV3	58%	72%	67%
DENV4	59%	71%	67%

[a]Homology analysis between Asian ZIKV (GenBank accession number: NC_035889.1) and DENV1 (ACO06157.1), DENV2 (JN819419.1), DENV3 (ACY70771.1), and DENV4 (AEW50183.1).

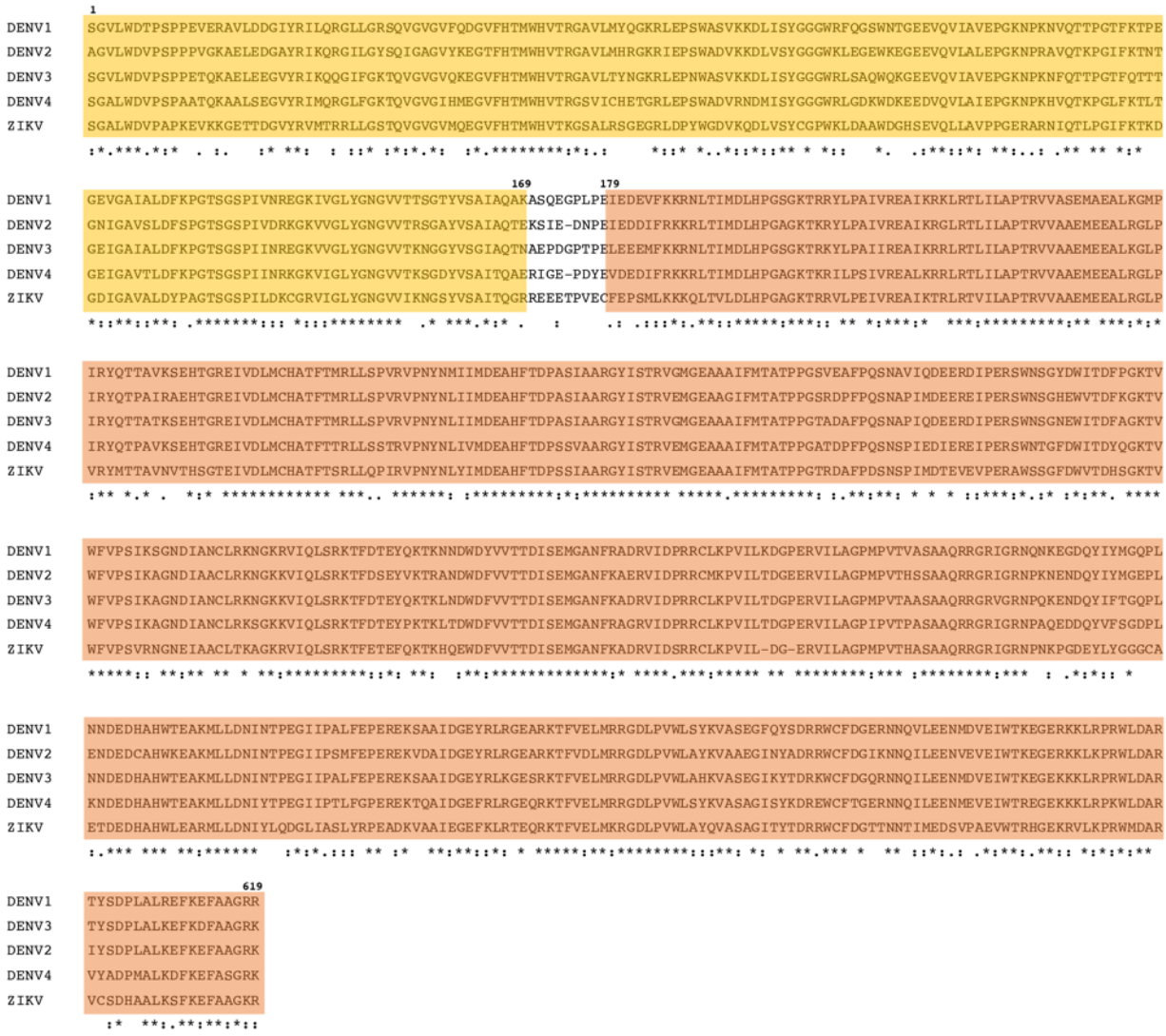

Figure 5. Clustal Omega generated amino acid sequence alignment of DENV serotypes 1 to 4 and Asian ZIKV. The residues in yellow represent the NS3 protease domain (amino acids 1-169) and the residues in orange represent the helicase domain (amino acids 179-619). *, single, fully conserved residue. :, conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix. ., conservation between groups of weakly similar properties – scoring =< 0.5 in the Gonnet PAM 250 matrix.

ZIKV-E and DENV-E IgG ELISAs can distinguish pZIKV and pDENV infections; this could potentially be a useful tool for epidemiology and pathogenesis study in endemic regions. However, the sample size is small and the ΔrOD of 0.17 was based on a single serum dilution of 1:800, future studies involving larger sample size and different dilutions or end-point titers are needed to further validate these observations.

The degree of amino acid sequence identity between DENV and ZIKV structural and nonstructural proteins is 49% and 51%, respectively (10). Multiple sequence alignment and homology determination of DENV and ZIKV NS3 demonstrates high amino acid sequence identity of 67%, with protease and helicase homology of 58% and 72%, respectively, consistent with the higher degree of DENV/ZIKV cross-reaction in NS3 helicase (Table 3, Fig. 5). Our recent characterization of acute- and convalescent-phase T cells collected from individuals infected with DENV and African ZIKV in Senegal, West Africa, revealed sustained DENV- and ZIKV-specific responses to NS3 protease and cross-reactive responses to NS3 helicase (27). Our findings in individuals infected with DENV and Asian ZIKV are in agreement with our previous observations. Although we were unable to distinguish sequential exposure, the LFn NS3 protease ELISPOT differentiates infections between DENV- and ZIKV-infected individuals with high sensitivity and specificity of 94% and 92%, respectively.

A relatively large body of epidemiological and laboratory-based evidence has suggested that severe and often fatal forms of dengue disease occurs most commonly during a secondary infection by a heterotypic DENV serotype (32, 33). Another phenomenon, known as original antigenic sin (OAS), has been observed in antibody as

well as T-cell responses, in which less-effective T cells generated in response to a primary DENV infection predominate during a subsequent infection with a different DENV serotype, resulting in an inappropriate response and predisposing individuals to severe disease (34, 35). The OAS hypothesis was challenged by a study in Sri Lankan individuals infected with DENV, which showed that the phenomenon does not generate less functional responses, but instead correlates with protective responses to conserved viral sequences (26). Unexpectedly, we did not observe T cell responses that were significantly higher in magnitude in individuals with prior DENV exposure. These results are in contrast to our data on African ZIKV infections, which showed that previous flavivirus exposure was associated with enhanced T cell responses (27). One possibility is that the proportion of HIV infection among those with prior DENV exposure was higher compared with DENV-naïve in this study (90.5% versus 50% comparing sDENV and pDENV cases; 83.3% versus 60% comparing ZIKVwpDENV and pZIKV cases). Nevertheless, as co-circulation of DENV, ZIKV, and other flaviviruses occurs throughout many parts of the world, it is critical to continue to develop tools to better understand T cell immunity in individuals exposed to multiple flaviviruses.

A recent study using human leukocyte antigen (HLA) transgenic mice infected with DENV2 and Asian and African ZIKV strains revealed cross-reactive T cell responses to HLA-restricted epitopes (25). Out of 8 ZIKV NS3 epitopes computationally predicted to bind HLA class I molecules, only 3 epitopes elicited DENV2/Asian ZIKV cross-reactive T cell responses. Of note, the cross-reactive

epitopes were all positioned within the helicase domain of NS3, further supporting our observations of high DENV/ZIKV NS3 helicase T cell cross-reaction. Another study demonstrated ZIKV-specific and ZIKV/DENV cross-reactive T cell responses in humans (10). T cell responses generated in response to prior DENV exposure recognized peptides sequences located throughout the ZIKV proteome. DENV serostatus also influenced T cell immunity to ZIKV. DENV-naïve ZIKV-positive individuals had predominant CD8 T cell responses directed against structural proteins. In contrast, a majority of CD8 T cells responses were directed against nonstructural proteins in DENV-immune ZIKV-positive individuals, suggesting that previous DENV exposure can alter the T cell response.

While the above studies used peptide stimulation to characterize the T cell response, there are concerns around this approach (36). Some of the HLA-predicted peptides may fail to stimulate strong T cell responses as expected. Longer and shorter peptides have also been shown to elicit different types of responses (37-39). An alternative to peptide stimulation is the anthrax LFn, which has the capability to deliver full length antigen into the cytosol for native processing via the MHC pathways, and to elicit better T cell responses compared to peptides in some cases (40-45). Our adaptation of the LFn ELISPOT, not only allowed detection of human DENV and ZIKV infections, but also characterization of the associated T cell responses to structural and nonstructural proteins. We demonstrated that individuals with pDENV and sDENV infections had similar IFN-γ and TNF-α T cell response patterns with high cross-reactivity to ZIKV NS3 helicase, but low cross-reactivity to the ZIKV structural proteins.

A small number of individuals with sDENV infections had cross-reactive T cell responses to ZIKV structural proteins. Interestingly, however, individuals with pZIKV and ZIKVwpDENV infections had similar IFN-γ and TNF-α T cell responses patterns, with strong responses to structural and nonstructural proteins. It is noteworthy that we observed comparably strong T cell responses to the structural proteins in pZIKV and ZIKVwpDENV cases, in contrast to Grifoni et al., suggesting that the most recent infection may dictate the T cell response (10). Another possibility that cannot be excluded is the differences in T cell stimulation strategies, which may be contributing to the observed differences. Additionally, due to the limited collection of blood samples from each patient, we were unable to characterize CD4- and CD8-specific responses. Future characterization studies using the LFn delivery system on CD4 and CD8 T cells will be important.

Our study of ZIKV seroprevalence in West Africa demonstrated continued human transmission of the virus in HIV- and malaria-infected individuals (46). The co-infection of flaviviruses with HIV or malaria could potentially impact pathophysiological mechanisms, induce different clinical and laboratory findings, and interfere with treatment. Previous studies have shown a suppression of HIV-1 replication during acute DENV infection (47, 48). In this study, we demonstrated that DENV-exposed individuals who are HIV-infected had T cell responses that were significantly lower in magnitude compared to HIV-negative individuals except in individuals with pZIKV infections. We also observed that DENV-exposed HIV-infected individuals have T cell responses that were lower in magnitude to ZIKV proteins compared to DENV-exposed

HIV-negative individuals. Whether HIV infection in DENV-exposed individuals reduces the ability to induce cross-reactive T cell responses has important implications. More studies with larger sample sizes are needed to increase our limited understanding of the epidemiological and immunopathogenesis interactions of flavivirus exposure in individuals with HIV and other comorbidities.

In summary, despite high sequence homology between DENV and ZIKV, diagnostic assays based on antibodies to NS1 and T cell responses to NS3 protease are effective at distinguishing human infections by these viruses. The LFn ELISPOT assay has enabled direct comparison of T cell characterization in DENV, Asian and Africa ZIKV human infections. As vaccines against DENV and ZIKV are currently being developed, the information generated from these characterization studies are of high relevance. The results of these characterization studies may contribute to the design and development of DENV and ZIKV vaccines and T cell based diagnostics.

Material and methods

Clinical samples and ethical statement

Fifty late convalescent-phase blood samples were obtained from patients at Professor Edgard Santos University Hospital, Federal University of Bahia, Salvador, Brazil. These individuals were suspected ZIKV-infected during the 2015-2016 ZIKV epidemic and their acute-phase sera were screened for ZIKV and DENV antibodies. Late convalescent-phase peripheral blood mononuclear cells (PBMCs) were separated from whole blood in EDTA tubes by Ficoll-Hypaque gradient density (Sigma-Aldrich,

St. Louis, MO, USA) and cryopreserved in freezing media (10% dimethyl sulfoxide [DMSO], Sigma-Aldrich, St. Louis, MO, USA) at -80°C overnight prior to transfer to liquid nitrogen. Convalescent-phase serum was aliquoted and immediately transferred to -80°C.

The Federal University of Bahia Institutional Review Board (IRB), the Harvard T.H. Chan School of Public Health IRB, and the University of Hawaii IRB approved the primary studies under which the samples and data were collected. All patients provided informed consent for the collection of samples. Excess samples and corresponding data were banked, coded prior to analyses, and stored at the Federal University of Bahia.

ELISAs

For acute-phase sera, commercial ZIKV-NS1 and DENV-E based IgG ELISAs (Euroimmun, Luebeck, Germany) were performed (27). For late convalescent-phase sera, ZIKV- and DENV1-NS1 IgG ELISAs were performed as described previously (22). Briefly, purified NS1 proteins (16 ng per well) were coated onto 96-well plates overnight, followed by blocking and incubation with primary (serum at 1:400 dilution) and secondary (anti-human IgG conjugated with HRP, Jackson) antibodies (22). The OD at 450 nm was read with a reference wavelength of 650 nm. Each ELISA plate included two positives (two confirmed-Zika or confirmed-dengue samples for ZIKV- and DENV-NS1 ELISAs, respectively), four negatives (4 flavivirus-naïve sera), and tested samples (all in duplicates). The OD values were divided by the mean OD value of

positive controls to calculate the rOD values. The cut-off was defined by the mean rOD value of negatives plus 12 standard deviations as described previously (22). For samples positive for both ZIKV- and DENV-NS1 ELISAs, the ratio of rOD (=rOD of ZIKV-NS1/rOD of DENV-NS1) was calculated; rOD ratio < or ≥ 0.24 indicated sDENV or ZIKVwpDENV infection, respectively (22).

E protein-based IgG ELISAs using DENV1 virion or ZIKV (MR766 strain) virus like particles (VLP) were also tested for late convalescent-phase sera (30). Briefly, DENV1 virions or ZIKV-VLP derived from ultracentrifugation of culture supernatants of virus-infected Vero cells or pENTR-ZIKV prME plasmid-transfected 293T cells, respectively, were UV inactivated (for virions) and coated on 96-well plates at 4°C overnight, followed by blocking and incubation with primary (serum at 1:800 dilution) and secondary antibodies as above. The rOD and cut-off rOD values were similarly calculated. The difference in rOD of ZIKV and DENV E proteins (ΔrOD=rOD of ZIKV – rOD of DENV) was determined; ΔrOD ≥ 0.17 or < –0.17 was classified as pZIKV or pDENV infection, respectively.

Neutralization test

PRNT was performed on acute-phase sera to detect neutralization antibody to ZIKV as reported previously (29). For late convalescent-phase sera, a previously described micro-neutralization test was performed (31). Briefly, flat-bottom 96-well plates were seeded with Vero cells (3×10^4 cells per well) 24 h prior to infection. Four-fold serial dilutions of serum (starting from 1:10) were mixed with 50 focus-forming

units of DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (CH53489), DENV4 (H241 strain) or ZIKV (PRVABC59 strain) at 37°C for 1 h. The mixtures were added to each well followed by incubation for 48 h (except 70 h for DENV1), removal of medium, and fixation as described previously (30). After adding murine mAb 4G2 and secondary antibody mixture (IRDye® 800CW-conjugated goat anti-mouse IgG at 1:10000 and DRAQ5™ Fluorescent Probe at 1:10000), the signal (800 nm/700 nm fluorescence) was detected by Li Cor Odyssey classic (LiCor Biosciences) and analyzed by Image Studio software to determine percent neutralization at different concentrations and NT_{90} as described previously (30, 31).

LFn fusion protein design

Commercially synthesized gene fragments encoding the NS3 protease and helicase of DENV2 and C, prM, and NS3 protease and helicase of Asian ZIKV and were cloned into the LFn expression vector (pET15bLFn). The pET15bLFn vector contains a T7 promoter, histidine tag (His_6), and the terminal domain of the anthrax lethal factor (LFn; 255 amino acids). The pET15bLFn containing the coding sequences of the DENV and Asian ZIKV proteins were transformed into *E. coli* BLR (DE3) (Millipore, Medford, MA, USA). Selected clones were sequences to verify the reading frame, and clones containing the correct sequence were used for protein expression.

The LFn-DENV and -ZIKV fusion proteins and the LFn control were expressed upon isopropylthiogalactoside ([IPTG], Sigma-Aldrich, St. Louis, MO, USA) induction in 5L Luria broth containing carbenicillin and chloramphenicol for 2-4 hours. Cells were

pelleted by centrifugation and resuspended in imidazole (1mM) binding buffer (Novagen, Madison, WI, USA) in the presence of a protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA). Cell pellets were sonicated, centrifuged at 4°C, and the supernatants were loaded in an equilibrated nickel-charged column for affinity purification. The bound proteins were eluted in 100-200 mM imidazole, desalted with a Sephadex G-25M column (Sigma-Aldrich, St. Louis, MO, USA), and eluted in PBS (Sigma-Aldrich, St. Louis, MO, USA). The PBS-eluted proteins were passed through Detoxi-Gel (Thermo Fisher Scientific, Rockford, IL, USA). Protein concentrations were determined samples were stored at -80°C.

ELISPOT assay

Ex vivo ELISPOTs were performed as previously described. Briefly, 96-well polyvinylidene difluoride (PVDF)-backed MultiScreen$_{HTS}$ (MSIP) microtiter plates (Millipore, Medford, MA, USA) were treated with 100ul of 90% ethanol for 30 seconds and washed 5 times with sterile PBS. Plates were coated with 100ul of capture antibodies (Abs) in PBS. Plates containing capture Abs were incubated overnight at 4°C. Plates were then blocked with 1% bovine serum albumin ([BSA], Sigma-Aldrich, St. Louis, MO, USA) in PBS and washed 6 times with PBS. Cryopreserved PBMCs were thawed in R10 medium and incubated overnight at 37°C. PBMCs were washed 2 times with PBS and seeded at 2 x 10^5 cells/well in a final volume of 100ul/well. LFn-DENV and -ZIKV proteins were added to each well. As a positive control, PBMCs were stimulated with phytohemagglutinin ([PHA], Sigma-Aldrich, St. Louis, MO, USA). As a

negative control, wells received LFn. After incubation for 24-28 hours at 37°C in 5% CO_2, the cells were discarded and plates were washed 3 times with PBS and 3 times with PBS with 0.05% Tween-20 ([PBST], Bio Rad Technologies, Hercules, CA, USA) to remove cells. The detection antibodies were added and plates were incubated overnight at 4°C. Plates were then washed 6 times with PBST, then incubated for 2 hours at room temperature with mixtures containing the enzymatic conjugates. To develop spots, plates were washed 4 times with PBST, three times with PBS, and 1 time with water. Vector Blue substrate solution (Vector Laboratories, Burlingame, CA, USA) was added for 5-15 mins before rinsing with water and air-drying. Digitized images were analyzed for spots using CTL ImmunoSpot reader (Cellular Technology Limited, Cleveland, OH, USA). DENV and ZIKV spots were calculated by subtracting the mean of the negative control value from the mean value of the specific stimulation. Positive responses had to be greater than 4 times the mean background, 3 standard deviations above the background, and ≥55 spot-forming cells per (SFC)/10^6 PBMCs.

ROC analysis

The ELISPOTs were validated using PBMCs from individuals that were confirmed DENV- and/or ZIIV-infected by ELISA and/or neutralization tests. DENV and ZIKV NS3 protease to helicase values were calculated, resulting in normalized test ratios (ZIKV NS3 protease divided by DENV NS3 protease) ranging from 0.15-2.95. On the basis of these data, we determined the optimal cutoffs between 0.15 and 2.95 by calculating the sensitivity (number of true positives divided by total confirmed positive

values) and specificity (number of true negatives divided by the total confirmed negatives) at increasing 0.05 to the theoretical cutoffs. After calculating the sensitivity and specificity values, the optimal cutoffs were defined as the highest sum of sensitivity and specificity, such that the optimal cutoff values reflected the optimal sensitivity and specificity. The optimal cutoffs obtained for the IFN-γ and TNF-α was 1.05 and 1.048, respectively (Prism 7, GraphPad Software, San Diego, CA, USA).

Multiple sequence alignment and percent homology analysis

Multiple sequence alignment of DENV1-4 and ZIKV NS3 was performed using the Clustal Omega program (EMBL-EB, Cambridgeshire, UK). Averages of DENV and ZIKV NS3 protease and helicase proteins were calculated using the ExPASy Bioinformatics Resource Portal (Swiss Institute of Bioinformatics, Lausanne, Switzerland) and based on averages of the different homology values in the four DENV serotypes and ZIKV. Average conservation was determined on a per-residue basis for NS3 protease, helicase, and full-length protein.

Statistical analysis

Statistical analysis was performed using Prism 7 (GraphPad Software, San Diego, CA, USA). Where appropriate, data were expressed as geometric positive means on box whisker and bar graphs ± standard deviation. Data comparisons were conducted using the Wilcoxon rank sum test. A threshold of $p<0.05$ was considered statistically significant.

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CHAPTER 6

Discussion and future directions

Ebola virus

Summary of findings

From 2013-2016, Ebola virus (EBOV) caused an explosive outbreak in West Africa. Despite the reported numbers – more than 28,000 cases, 11,000 of which were fatal – serological surveys done after the outbreak revealed that a significant portion of transmission events went undetected because some individuals contracted infection but had little to no symptoms (1, 2). While several hypotheses could explain this phenomenon, including properties of the infecting virus (e.g. less virulent isolate), low inoculum, route of transmission, or infected host (e.g. resistance through viral cell receptor polymorphism), another possibility is that a robust adaptive immune response played a major role. The dissertation work described in Chapter 2 was undertaken to better understand the immunopathogenesis of EBOV in survivors of severe disease and in individuals who experienced asymptomatic infection. Our long-term goal was to develop novel approaches to detect and compare EBOV-specific T cell responses in individuals who experienced EBOV infection with different outcomes.

Due to the logistic difficulties in conducting EBOV immunopathogenesis studies during an ongoing epidemic in low to middle income countries, most in depth studies have been focused on few patients that were transported to the United States and Europe. While early studies demonstrated EBOV-induced dendritic cell inactivation and T cell apoptosis, data gathered during the 2013-2016 West African outbreak have suggested that lymphocytes display dynamic kinetics, which may include early proliferation followed by lymphopenia (3-6). The application of multiparametric flow

cytometry during the recent outbreak allowed for the first collection of phenotypic and functional information from single cells in studies. These studies revealed extensive T cell activation during Ebola virus disease (EVD) in both surviving and fatal cases. In a significant percentage of antigen-specific CD4 and CD8 T cells, co-expression of activation markers such as CD38 and HLA-DR, as well as proliferation markers like Ki-67, were detected in EVD patients during the acute and convalescent phases of infection (7, 8). These studies were conducted on a total of five patients. Another study found that acute phase peripheral blood T cells from a significantly larger pool of EVD patients (n=204) expressed high levels of the T cell co-inhibitor molecules, PD-1 and CTLA-4 (9). The coexpression of PD-1 and CTLA-4 were higher in fatal cases, suggesting that T cell ineffectiveness may be related to defects in negative immune checkpoints.

Moreover, minimally symptomatic and asymptomatic EBOV infections are not new phenomena. During the first outbreaks of EBOC in Zaire and Sudan in 1976 and 1979, World Health Organization (WHO) researchers used an immunofluorescence assay to identify infected individuals who had symptoms that ranged in severity, from mild to rapidly fatal (10-12). Since then, a number of additional studies have identified EBOV-infected individuals who nonetheless remained asymptomatic. Two outbreaks of EVD occurred in northern Gabon in 1996, resulting in case fatality rates of 66% and 75% among 59 and 60 symptomatic cases (13). Antibody analyses by enzyme-linked immunosorbent assays (ELISAs) and Western blot demonstrated the development of both IgM and IgG responses to EBOV antigens in 11 of 24 Gabonese individuals with documented close contact with EVD patients (14). Other studies using ELISAs have

provided evidence of EBOV seropositivity in healthy individuals residing in areas without large outbreaks (15, 16). In a study of Efé pygmies living in the Watsa region of the Democratic Republic of Congo (DRC) with known exposure to potential risk factors of EBOV infection demonstrated an 18.7% IgG prevalence by ELISA (17). More recently, a serosurvey in a hotspot for EBOV transmission in Sierra Leone of documented close household contacts with symptomatic EVD patients identified 14 seropositive individuals not known to have had EBOV infection (1).

Given the potential role of the adaptive immune response in protection against severe EVD, we investigated the possibility of identifying EBOV seropositivity among survivors and individuals who did not experience severe disease but were potentially exposed to the virus. Our rationale was that individuals who experienced asymptomatic infection may have more robust immune responses that contributed to their protection against severe EVD and that the information gathered from immunopathogenesis studies could aid the development of more effective vaccines. Using ELISAs and Western blot approaches we identified seropositive EVD and individuals who never recalled experiencing EVD-like illness from the Democratic Republic of Congo (DRC) and Lagos, Nigeria. Specifically, we detected EBOV IgG in 9 out of 71 (12.9%) individuals residing in areas surrounding the 1976 EBOV outbreak in Yambuku, DRC. We also detected EBOV antibodies in the 3 survivors and 2 out of 10 individuals with documented close contact with EVD patients from Lagos, Nigeria.

We therefore established the ability to detect EBOV-specific T cell responses utilizing the modified anthrax toxin delivery system. T cell responses are typically studied using overlapping peptides; however, this approach has been associated with

inaccurate T cell response observations in some cases (18-21). Bacterial toxins represent an alternative strategy to delivery antigen into the cytosol for MHC processing and T cell presentation. A modified form of the anthrax toxin has been shown to translocate protein antigens to elicit CD4 and CD8 T cells in vitro and in vivo (22-27). The modified anthrax toxin delivery system has also been used in diagnostic and vaccine development for variety of bacteria, parasites, and viruses (27). We cloned and purified the anthrax toxin fused to EBOV glycoprotein 1 (GP1), the matrix protein (VP40), and the nucleoprotein (NP). We asked whether we could detect and compare T cell responses against these antigens in the seropositive survivors and individuals who experienced asymptomatic infection.

We stimulated bulk peripheral blood mononuclear cells (PBMCs) from EBOV seropositive survivors and asymptomatics from Lagos and, in combination with the enzyme-linked immunospot (ELISPOT) assay, we studied their cellular responses. We observed strong IFN-γ and TNF-α cellular responses to all antigens tested, with the asymptomatics displaying the strongest responses compared to survivors of severe EVD. We next asked whether we could define CD4+ and CD8+ T cell responses. In previous studies, EBOV-specific CD8+ T cell responses have been difficult to detect, likely as a result of either CD8+ T cell response waning and/or the methodology used to study the T cell response (e.g. peptide stimulation). Our analysis revealed strong CD4+ and CD8+ IFN-γ and TNF-α T cell responses to the EBOV antigens tested in 4 out of the 5 seropositive patients (2 out of 3 survivors and 2 out of 2 asymptomatics). As observed with bulk PBMCs, the asymptomatics appeared to have stronger T cell responses compared to the survivors. Notably, using the modified anthrax toxin

delivery system combined with the ELISPOT assay, we were able to detect robust CD4+ and CD8+ T cell responses.

In summary, our results provide further evidence that asymptomatic EBOV infections occur and suggest that T cell immunity may play a protective role against severe disease manifestations. Our study raises new questions and highlights the need for further investigation to better understand the immune responses associated with minimally symptomatic and asymptomatic EBOV infections. These results have important implications for vaccine and immunotherapeutic development, as well as for potential EBOV diagnostics based on T cell responses.

Future directions

In this section, we discuss potential research directions that may further our understanding of EBOV pathogenesis, with a focus on implications for therapeutic interventions. Currently there no approved vaccines or immunotherapeutics against EBOV and related filoviruses; treatment is therefore limited to supportive care.

Antibodies play many important roles during the immune response to pathogens, including neutralization and antibody-dependent killing of virus-infected cells. However, neutralizing antibodies likely play a minimal role in recovery from acute EVD, since in many survivors neutralizing antibodies are not detected until weeks or even months after recovery (28, 29). The reasons for this delayed production of neutralizing antibodies is still as of yet unknown. One possibility is that EBOV infection disrupts lymphoid architecture and compromises germinal center formation and B cell affinity maturation, an observation made during Lassa fever infection (30).

Nevertheless, long-term survivors eventually develop effective neutralizing antibodies that are directed against the EBOV GP and specifically the GP1,2 glycan cap and the region bridging GP1 and GP2, which has made antibody-based therapies against these epitopes promising targets (31-34). ZMapp, a cocktail of humanized murine mAbs that target the EBOV surface GP, was shown to cure advanced disease in non-human primates infected with EBOV (35). ZMapp, however, was not effective when tested in humans during the 2013-2016 West African outbreak (36). Therefore, there is an urgent need for improved immunotherapeutics against EBOV.

Several serological surveys, including our own, have revealed anti-EBOV antibodies in individuals throughout Africa who did not recall ever having EVD-like illness. A robust adaptive immune response may offer, at least in part, an explanation for asymptomatic EBOV infection. My work in Chapter 2 has revealed EBOV seropositive individuals who experienced asymptomatic infection and who have EBOV-specific T cell responses that are stronger in magnitude compared to severe cases. The potency of the antibody response during asymptomatic infection is as of yet unknown. In order to develop more effective antibody cocktails, it will be important to isolate and compare monoclonal antibodies from survivors and asymptomatics. It will also be important to determine the antiviral mechanisms and epitopes targeted by these antibodies, with the ultimate goal of developing antibody cocktails with a diverse range of antiviral mechanisms that are effective against EBOV and related filoviruses.

While we and others have demonstrated strong T cell responses in survivors, the important question still remains of why robust T cell activation does not lead to viral clearance during EVD in a majority of cases. The observation that during EVD T cells

express co-inhibitor molecules such as PD-1 and CTLA-4 and that higher levels of these molecules are detected in fatalities suggests that T cells may be nonfunctional (9). Determining the correlation between high expression of T cell inhibitory molecules and T cell function will be important. Due to the difficulty of studying EBOV immunopathogenesis in humans, determining this correlation will most likely require relevant in vivo models that can reproduce this T cell phenotype. Utilizing immunotherapeutic approaches to block PD-1 and CTLA-4 function during postexposure EBOV infection treatment to restore T cell function may provide a potential opportunity to aid in viral clearance. Another important and related questions is whether broad and polyfunctional T cell responses can lead to decreased susceptibility. To address this question, an extensive analysis of EBOV T cell immunodominance and HLA association studies in humans must be performed. Previous studies have shown that CD8 T cells predominantly target the EBOV NP (7). This finding has significant implications for vaccine development and may explain, in part, why most GP-based vaccines induce poor T cell immunity.

Dengue and Zika viruses

Summary of findings

From 2015-2016, an unprecedented epidemic wave of Zika virus (ZIKV) spread throughout South and Central America and the Caribbean (37, 38). An estimated 1.5 million people were infected by the virus and new associations of severe neurological complications including transmitted congenital microcephaly and Guillain Barré

syndrome were reported (39). While the cause of these severe ZIKV disease outcomes remains unknown, the links between infection and neurological symptoms are beginning to be elucidated. Asian ZIKV strains demonstrate neurotropism, causing cellular death in human neural progenitor cells (NPC) and immature cortical neurons, thereby affecting neurogenesis (40, 41). Despite the absence of reported ZIKV outbreaks in Africa, several African strains also infect and impair growth of human NPC (42). Efforts are needed to understand the immunopathogenesis of ZIKV infection in humans and whether these immunological events are beneficial or pathogenic in development of disease. The dissertation work described in Chapters 3-5 was conducted to analyze the epidemiology of ZIKV and Dengue virus (DENV) in Africa and the immune responses in individuals infected with either African or Asian ZIKV strains and DENV. Our long-term goal was to develop novel approaches to detect and compare antibody and T cell responses in individuals exposed to ZIKV and/or DENV.

Prior to the 2015-2016 outbreak, the incidence of ZIKV in Africa had not been evaluated and the prevalence estimates for DENV were scarce. In order to determine the incidence of ZIKV and DENV in West Africa, in Chapters 3 and 4, we conducted IgM seroprevalence surveys on archived febrile samples collected from malaria- and HIV-infected individuals living in Senegal and Nigeria. Of the samples tested, we demonstrated continued human transmission of both ZIKV and DENV over a two-decade period (43). Specifically, the IgM seroprevalence for ZIKV and DENV was 6.4% and 9.1%, respectively, suggesting that individuals residing in these countries are continually exposed to these endemic viruses at low levels. In Chapter 3, we also

provided some of the first ZIKV sequence data collected from human infections, which revealed clustering of our ZIKVs into either the MR766 or Nigerian sublineages.

Given the potential beneficial or pathogenic (either through antibody-dependent enhancement or T cell antigenic sin, as described in Chapter 1) role of the adaptive immune response against flavivirus infections, we established antibody and T cell-based assays to study the immunopathogenesis of African ZIKV and DENV human infections from individuals identified in Chapters 3 and 4. In Chapter 4, we reported long-term antibody and T cell responses detected in both our ZIKV and DENV patients. The ZIKV and DENV patients exhibited antibody responses to the envelope, capsid (C), and pre-membrane (prM) proteins for over two years in a majority of cases and in some cases for up to at least 5 years post-infection (44).

We cloned and purified the anthrax toxin fused to ZIKV and DENV nonstructural protein 3 protease (NS3-P) and helicase (NS3-H). We then asked whether we could detect and compare T cell responses against these antigens in individuals exposed to either ZIKV or DENV and how pre-existing flavivirus exposure might impact the T cell response. Using the LFn delivery system in combination with the ELISPOT assay, we similarly demonstrated sustained IFN-γ and TNF-α T cell responses to nonstructural protein 3 protease (NS3-P) and helicase (NS3-H) (44). Interestingly, ZIKV and DENV patients had specific responses to NS3-P, but cross-reactive responses to NS3-H. These T cell responses were used to distinguish ZIKV from DENV infections. This work was the first demonstration of a flavivirus diagnostic capability based on the T cell response. Additionally, our work showed that individuals with prior flavivirus exposure contain enhanced ZIKV and DENV T cell responses. Future studies in animal models or

well documented ZIKV cases will be important to determine whether an enhanced T cell responses protects against or contributes to neuropathology.

In order to validate our novel NS3 T cell diagnostic, in Chapter 5, we conducted a study on samples collected from DENV and Asian ZIKV exposed individuals in Salvador, Brazil. In addition to validate the diagnostic, we also were interested in characterizing the T cell responses generated in response to Asian ZIKV and also asked whether previous flavivirus exposure or HIV status had any effect on the T cell response. We first set out to establish a validated cohort of exposed individuals by screening antibodies using our new enzyme-linked immunosorbent assays (ELISAs) capable of distinguishing primary ZIKV (pZIKV) and DENV (pDENV) infections from secondary infections; these primary cases were also confirmed by plaque reduction neutralization tests. Using these ELISAs, we identified 4 individuals who had never been exposed, 5 individuals with pZIKV and 4 individuals with pDENV infections, 12 individuals with ZIKV with primary DENV (ZIKVwpDENV) infections, and 21 individuals with two DENV infections (sDENV).

Using reconfigured LFn Asian ZIKV NS3-P and NS3-H as well as ZIKV C, prM, and DENV NS3-P and NS3-H antigens, we stimulated bulk peripheral blood mononuclear cells (PBMCs) from the serologically-validated individuals in ELISPOT assays. We observed strong IFN-γ and TNF-α cellular responses to all antigens tested, with similar T cell responses patterns in individuals with pZIKV and ZIKVwpDENV infections and pDENV and sDENV infections. Individuals with pZIKV and ZIKVwpDENV infections had strong responses to both the ZIKV structural (C and prM) and nonstructural proteins (NS3-P and NS3-H) with high cross-reactivity to DENV NS3-H.

Interestingly, pre-existing DENV immunity had little impact on the T cell response; however, individuals that were human immunodeficiency virus (HIV)-infected had responses that were generally lower in magnitude. Along with our work in Chapters 3 and 4, this is some of the first data on T cell responses in individuals with other comorbidities such as HIV. Finally, we demonstrated that IFN-γ and TNF-α T cell response ratios of ZIKV NS3 to DENV NS3 is capable of discriminating infections in individuals exposed to these viruses with high sensitivities and specificities. This work supports our previous findings and has important implications for the development of flavivirus T cell-based diagnostics that may be able to circumvent pan-flavivirus cross-reactivity.

In summary, we provided the first evidence of ZIKV incidence in Africa and demonstrate that individuals are capable of being infected by multiple African strains. Our T cell work showed long-term responses in individuals infected with African and Asian ZIKV and/or DENV with diagnostic potential. Our results raise important questions and highlights the need for further investigation into the immunopathogenesis of these viruses in order to design the most effective vaccines and immunotherapeutics.

Future directions

In this section, we discuss potential research directions that may further our understanding of ZIKV immunopathogenesis with a particular emphasis on the mechanisms contributing to the development of neurological disease. It is currently not understood how ZIKV induces severe neuropathology and whether host factors and/or

genetics contributes to the process. Additionally, the important question of whether African ZIKV can cause severe neuropathology in humans remains to be elucidated.

The CRISPR-Cas system has recently been developed as a powerful gene editing tool in mammalian cells (45-48). This system allows for easy knockout of both alleles, enabling improved approaches for performing genome-wide screening. It will be important to design CRISPR-Cas screening strategies to identify host genes essential in ZIKV-induced cell death (49-51). A recent study with the related flavivirus, West Nile virus, demonstrated that deletion of seven host ER-associated protein degradation (ERAD)-associated genes conferred strong protection against WNV-induced cell death without blocking replication (52). Previously unrecognized, the ERAD pathway is now being considered as a novel therapeutic target to prevent WNV-induced mortality. The potential to identify new host pathways essential in blocking ZIKV-induced cell death of NPC should remain a top priority.

Host genetics and differences between ethnic groups has been shown to influence the DENV infection outcomes (53). Early epidemiological studies in Cuba reported that dark-skinned individuals have remarkable resistance against dengue disease compared to light-skinned individuals. These early observations were confirmed during the 1981 Cuban DENV hemorrhagic fever outbreak when ethnicity was recognized as a possible risk factor, and confirmed afterwards in another Cuban outbreak (54). In addition, specific single nucleotide polymorphisms (SNPs) in HLA genes are associated with protection against severe dengue disease in Asian and Latin American populations (55, 56). A recent human study identified oxysterol binding protein-like 10 (OSBPL10) as being expressed significantly lower in Africans compared

to Europeans, and that knockdown of the protein markedly reduced DENV replication in vitro, providing a possible explanation for the observed protection (57). Thus, it will be important to perform genetic studies such as HLA typing in well-documented ZIKV cases in combination with global ancestry studies of both African and South American ZIKV-infected individuals. The potential to identify candidate genes that confer protection against disease in some individuals versus others will be important and may offer additional and more specific therapeutic targets.

Finally, while we demonstrated low levels of continued human transmission of ZIKV in West Africa, the clinical relevance of infection remains to be determined (43). It is necessary to develop tools that will enable point-of-care surveillance of ZIKV infections in the hospital and clinics, especially among pregnant women. In order to determine whether African ZIKV infection causes congenital microcephaly, we must perform extensive serosurveys and/or antigenic tests for ZIKV in pregnant women reporting to the clinics with long-term follow-up through pregnancy to determine outcomes. In combination with immunological studies, we may be able to define more specific immune correlates of disease and/or protection.

Utility of the modified anthrax toxin (LFn) delivery system

T cell activation requires the presentation of viral peptides on the surface of infected cells in the context of MHC class I or class II molecules. Unlike B cells, T cells cannot recognize intact virions; therefore, it would not be possible for EBOV- or flaviviral-specific T cells to provide sterilizing immunity against infection by these pathogens. Nevertheless, lysis of infected cells and/or the antiviral effects of T cell

cytokines could, in principle, restrict viral replication, reduce viral reservoirs, and inhibit the development of disease.

T cell-inducing vaccines typically rely on naked DNA or utilize viral vectors to express antigen. Bacterial toxins represent an alternative strategy for the delivery of antigen to the cytosol of host cells for MHC processing and presentation. A genetically modified, nontoxic form of the *Bacillus anthracis* (anthrax) toxin has been shown to translocate protein antigens to both the MHC class I and class II pathways. The toxin produced by anthrax is tripartite and composed of protective antigen (PA), edema factor (EF), and lethal factor (LF) (58). PA (83 kDa) binds to its cellular receptors with subsequent cleavage by furin or furin-like proteases releasing an N-terminal 20 kDa fragment. The resulting 63 kDa PA (PA63) assembles into an oligomeric complex with binding sites for EF and LF. After assembly of PA63 bound to EF or LF, the toxin complex enters the cell through endocytosis and is targeted to endosomes. The acidic nature of endosomes triggers a conformational change in the complex, which in turn, allows EF/LF translocation into the cytosol of cells. LF blocks mitogen-activated protein kinase (MAPK) pathways eventually causing death by system shock in animals and humans. Studies have shown that a 255 amino acid N-terminal truncation of LF (LFn) in the absence of its catalytic domain lacks any toxic effect both in vitro and in vivo.

Delivery of protein antigen to the cytosol for MHC processing by LFn has been used previously to elicit significant T cell responses to Listeriolysin protein of *Listeria monocytogenes* (59). The utility of LFn, in the presence and/or absence of PA, was fused to HIV-1 p24 and nef genes to induce strong T cell responses in mice and

humans (22, 25). LFn-HIV-1 p24C and gag vaccine candidates, in the absence of PA, demonstrated capacity to elicit cell-mediated immunity in Chinese rhesus macaques (24). The Kanki laboratory also developed a simple and rapid LFn-ELISPOT assay to assess HIV-2-specific cellular responses in female sex workers from Senegal (27).

In Chapter 2, we fused the EBOV NP, VP40, and GP1 to LFn, in the absence of PA, to compare by ELISPOT T cell responses in EVD survivors and seropositive asymptomatic individuals. The seropositive asymptomatic individuals mounted T cell response against the LFn-EBOV antigens that were stronger in magnitude compared to survivors, suggesting that T cell immunity may contribute, in part, to the phenomenon of asymptomatic EVD. In Chapter 4, we fused ZIKV and DENV NS3 protease and helicase to LFn, in the absence of PA, and observed sustained ZIKV- and DENV-specific T cell responses to NS3-P and ZIKV/DENV cross-reactive responses to NS3-H. In Chapter 5, we confirmed the diagnostic capability of the NS3 LFn ELISPOT assay in a ZIKV and/or DENV serologically validated cohort from Salvador, Bahia, Brazil. The NS3 LFn ELISPOT performed with high sensitivity and specificity, further supporting the development of a flaviviral diagnostic based on T cell responses.

Given the adaptability of LFn in T cell analysis and diagnostic and vaccine development, an important question regarding the biological aspects of the delivery system is by what mechanism does LFn enter cells in the absence of PA? Previous studies have shown that LF and PA must act together as a toxin unit. While LF by itself inactivates MAPK in solution, such enzymatic activity in live cells is entirely PA-dependent. Furthermore, injection of rats with LF in the absence of PA did not result in disease, further supporting the idea that the pathogenic effect of LF is PA-dependent

(60). In contrast, another study demonstrated that LF could enter into cells in the absence of PA with colocalization of LFn-GFP with the proteasome (23). Altogether these findings suggest LF requires PA to function as a lethal toxin, but not necessarily to enter into cells. To further validate this hypothesis, one could truncate portions of LFn and stimulate PBMCs with control LFn (LFn-viral antigen), test LFn (truncated LFn-antigens), and viral antigen by itself and observe T cell responses by ELISPOT. If in tact LFn is required for translocation of antigen into cells, then LFn-viral antigen should induce stronger T cell responses compared to truncated LFn-viral antigens and/or antigen by itself. In addition, one could take a biochemical approach by stimulating PBMCs with LFn-viral antigen in a time series experiment, isolate proteasome and/or endosome complexes, and perform a Western blot to test whether proteasome and/or endosome fractions contain LFn-viral antigen. The findings from these experiments could further support the delivery of antigen into cells by LFn without PA. Alternatively, it is possible that during LFn-viral antigen stimulation of PBMCs, the cell culture media in which the reaction is taking place contains proteases that cut the antigen into peptides that are loaded onto empty MHC pockets on the surface of cells.

Regardless of the molecular mechanism of lethal toxin's function, we and others have demonstrated that LFn is capable of use in T cell analysis, T cell based diagnostics, and as a vaccine delivery vehicle. Further studies are warranted to completely understand LFn's entry mechanism and subsequent trafficking within cells.

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APPENDIX

Archeogenotyping of African ZIKV isolates

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Letter

Arthropodborne viruses are the causative agents of substantial morbidity and mortality among humans and have pronounced effects on public health globally. Zika virus (ZIKV), discovered in Uganda in 1947 (1), is a mosquito-transmitted flavivirus related to the following viruses: dengue (DENV), West Nile (WNV), Japanese encephalitis (JEV), and yellow fever (YFV) (2). In 2007, ZIKV emerged as a public health threat when it caused an epidemic in Micronesia (3). Since then, a number of ZIKV epidemics have occurred elsewhere including in Oceania and recently throughout the Americas and the Caribbean (2015-2016) (4, 5). Historically, while ZIKV had been thought to cause mild and self-limited infection, new associations of severe neuropathology including transmitted congenital microcephaly and Guillain-Barré syndrome were uncovered during the 2015-2016 outbreak (6).

Recent phylogenetic analyses have revealed two major ZIKV lineages, African and Asian (7). African strains isolated from *Aedes* mosquitoes in Senegal and Asian strains isolated from infected humans throughout Asia, the Americas and the Caribbean, have begun to clarify its genomic diversity. However, there remains a paucity of genetic data on African strains isolated from infected humans.

We conducted seroepidemiological and genetic studies on samples collected between 1992 and 2016 in Senegal and Nigeria, which demonstrated continued human transmission of ZIKV in these countries for decades (8, 9). Out of 492 samples tested for ZIKV immunoglobulin M (IgM), 32 tested positive (6.5% incidence rate), and of those 32, 4 (12.5%) were positive for ZIKV by reverse-transcription polymerase chain reaction (RT-PCR). Our phylogenetic analysis showed two sublineages within Africa,

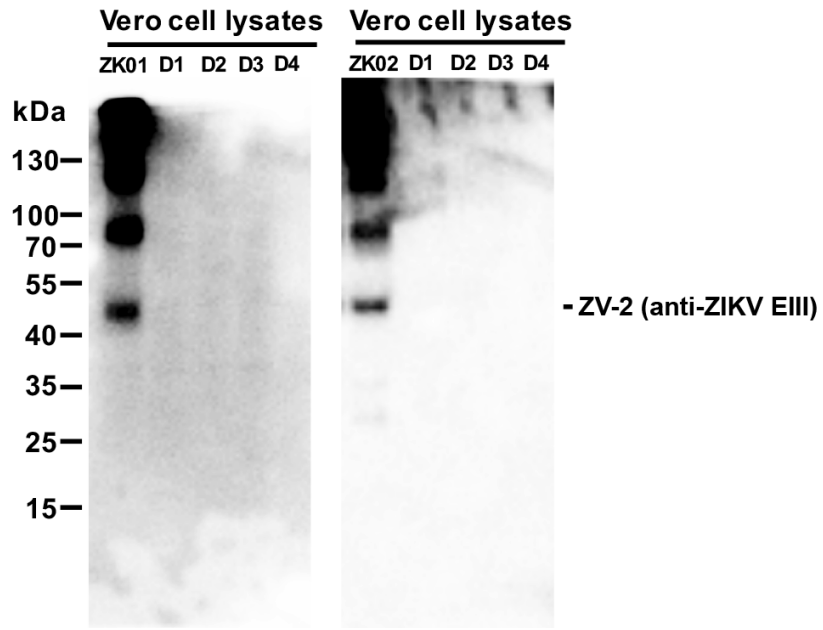

Figure 1. Representative image of Western blot analysis of the ZIKV and DENV Vero cell lysates. ZK01 and ZK02 were generated by infecting Vero cells with IgM positive serum or plasma for 7-10 days and harvesting with 1% NP40 lysis buffer. ZK01 and ZK02, ZIKV Vero cell lysates. D1, DENV1 Vero cell lysate; D2, DENV2 Vero cell lysate, D3, DENV3 Vero cell lysate, D4, DENV4 Vero cell lysate. ZV-2, anti-ZIKV envelope domain III.

with one ZIKV more closely related to east African strains or the MR766 ancestral (monkey) strain and the other three more closely related to west African strains.

Given the low success rate of ZIKV amplification by RT-PCR, we developed an in vitro strategy to capture sufficient ZIKV content for genetic and biochemical studies. We placed varying amounts of ZIKV IgM positive serum or plasma onto Vero cells and incubated at 37°C with 5% CO_2 for 7-10 days in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing low concentration (2%) fetal bovine serum (FBS). Upon observation of cytopathic effects (CPE), we harvested the Vero cell infection supernatant for cryopreservation and generated a ZIKV Vero cell lysate in 1% NP40 lysis buffer. We then transferred varying amounts of the Vero cell infection supernatant onto C6/36 cells in DMEM with low concentration FBS and allowed the infection to ensue at 37°C with 5% CO_2 until CPE was observed (7-10 days), at which point the C6/36 cell infection supernatant was collected for cryopreservation and/or subjected to RNA extraction (QIAamp® Viral RNA Kit, QIAGEN, Hamburg, Germany).

Western blot analysis demonstrates reactivity between the anti-ZIKV-envelope antibody (ZV-2; MilliporeSigma, Burlington, MA, USA) and the ZIKV (ZV01 and ZV02) but not DENV Vero cell lysates (Fig. 1). ZV-2 specifically recognizes ZIKV envelope domain III and has no cross-reactivity between the four DENV serotypes, WNV, JEV, and YFV. RNA that was extracted out of C6/36 cell infection supernatant was further analyzed by a Fragment Analyzer (Advanced Analytical Technologies, Inc., Ankeny, IA, USA), a capillary electrophoresis instrument designed to eliminate laboratory bottlenecks caused by slow and unreliable quality control analysis of nucleic acids. The

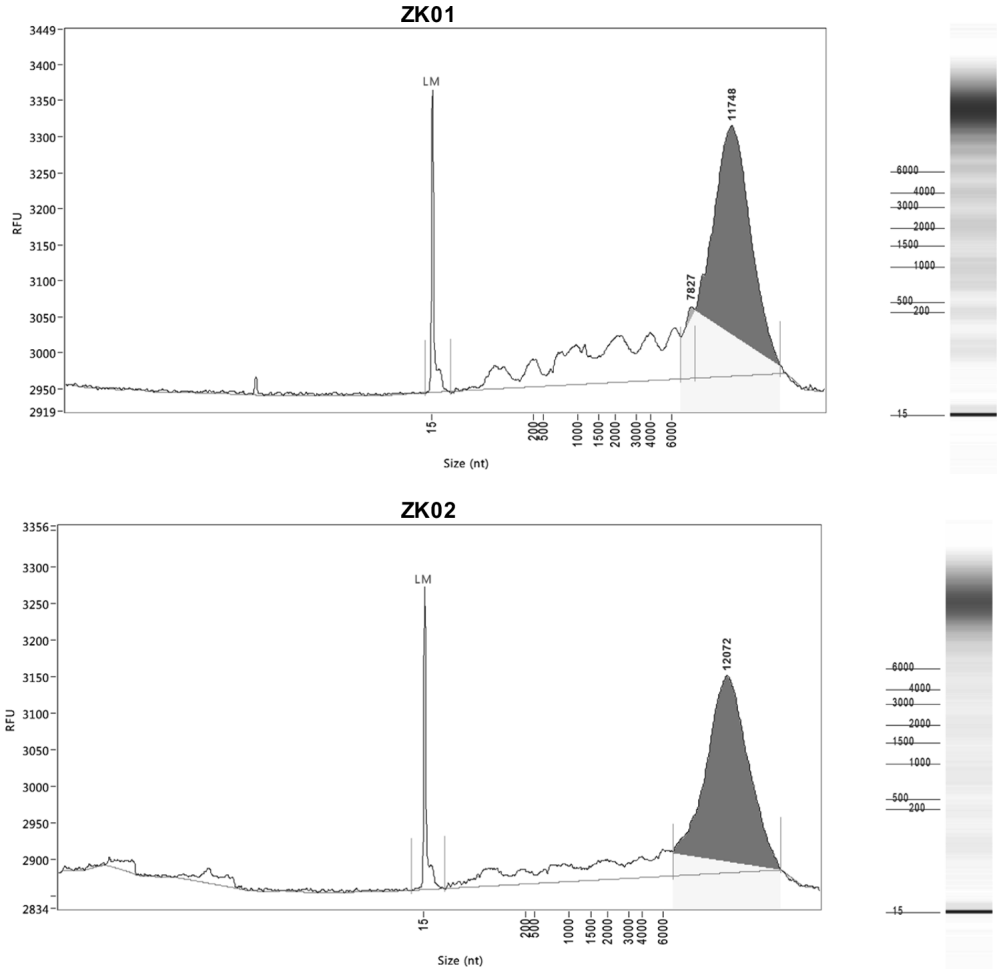

Figure 2. Fragment Analyzer representative results of RNA gel electrophoresis and trace files revealing the ~11,700-12,000 kilobase RNA fragments.

Fragment Analyzer resolved ZV01 and ZV02 nucleic acid fragments with high resolution and accuracy, demonstrating RNA traces of 11,748 and 12,072 base pairs, approximately the size of the ZIKV genome (~11 kilobase genome) (Fig. 2). These RNA samples were reverse transcribed and further subjected to ZIKV-specific RT-PCR and were positive. These results demonstrate a strategy to successfully isolate ZIKV out of archived human serum or plasma.

Although reported, human cases of ZIKV infection have been rare in Africa and only through retrospective studies have cases been identified. The isolation and characterization of ZIKV from archived human serum or plasma samples further support our findings of low levels of endemic infection in Africa. Future studies are expected to combine deep sequencing technologies to obtain full-length African ZIKV genomes from archived human samples. Additionally, the African ZIKV isolates are expected to be used in pathogenesis studies to investigate infection of various cell types including neural progenitor cells. These studies will shed light into the genetic diversity of the virus in Africa and allow comparative pathogenesis studies of Asian and modern African strains, which may allow a better understanding of why clinical disease has gone unrecognized in Africa.

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