DENGUE VIRUS-SPECIFIC T CELL IMMUNITY

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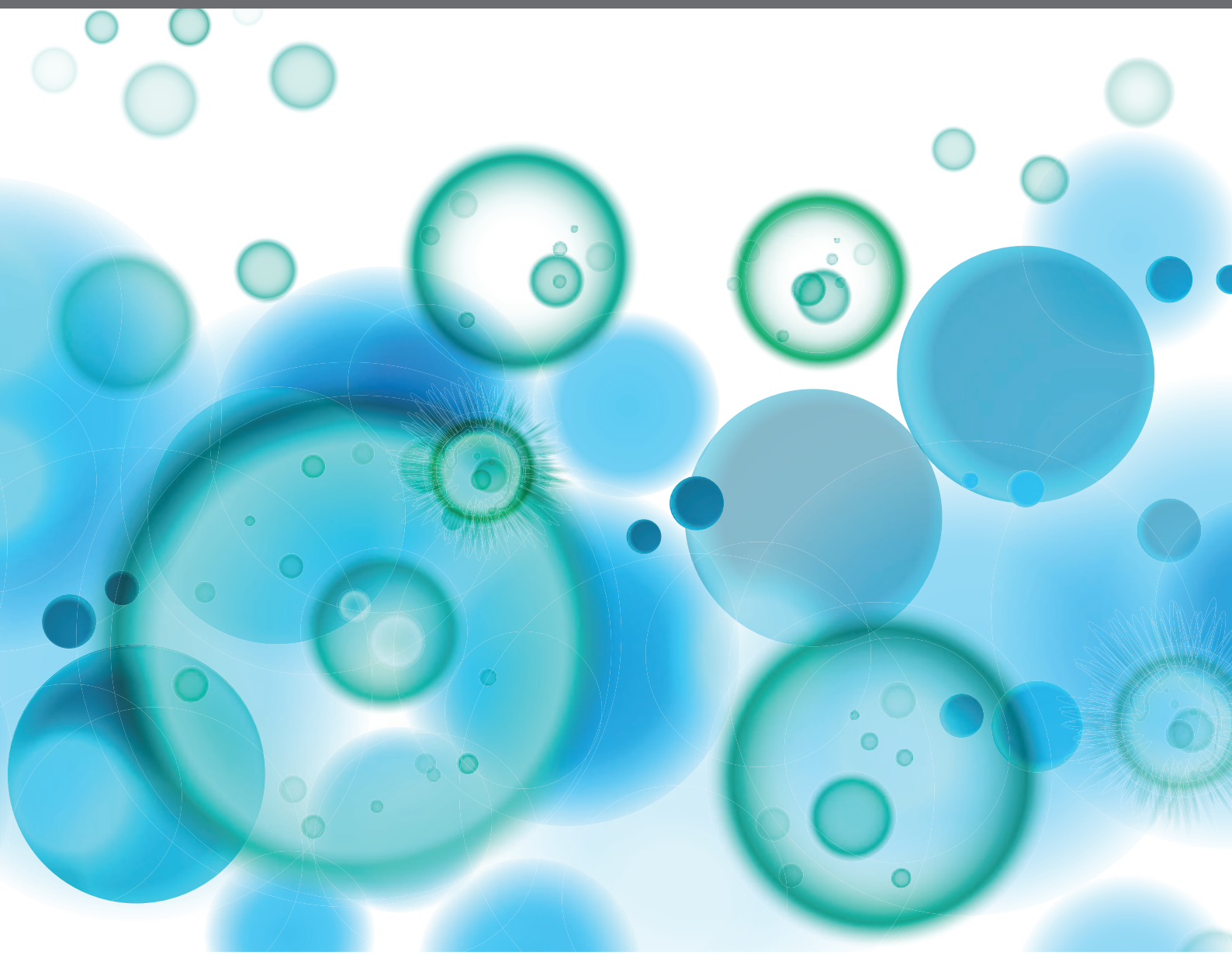

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DENGUE VIRUS-SPECIFIC T CELL IMMUNITY

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REVIEW
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Mouse Models of Heterologous Flavivirus Immunity: A Role for Cross-Reactive T Cells

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Most of the world is at risk of being infected with a flavivirus such as dengue virus, West Nile virus, yellow fever virus, Japanese encephalitis virus, tick-borne encephalitis virus, and Zika virus, significantly impacting millions of lives. Importantly, many of these genetically similar viruses co-circulate within the same geographic regions, making it likely for individuals living in areas of high flavivirus endemicity to be infected with multiple flaviviruses during their lifetime. Following a flavivirus infection, a robust *virus-specific* T cell response is generated and the memory recall of this response has been demonstrated to provide long-lasting immunity, protecting against reinfection with the same pathogen. However, multiple studies have shown that this flavivirus specific T cell response can be cross-reactive and active during heterologous flavivirus infection, leading to the question: *How does immunity to one flavivirus shape immunity to the next, and how does this impact disease?* It has been proposed that in some cases unfavorable disease outcomes may be caused by lower avidity cross-reactive memory T cells generated during a primary flavivirus infection that preferentially expand during a secondary heterologous infection and function sub optimally against the new pathogen. While in other cases, these cross-reactive cells still have the potential to facilitate cross-protection. In this review, we focus on cross-reactive T cell responses to flaviviruses and the concepts and consequences of T cell cross-reactivity, with particular emphasis linking data generated using murine models to our new understanding of disease outcomes following heterologous flavivirus infection.

Keywords: flavivirus, T cell cross-reactivity, heterologous immunity, original antigenic sin, Zika, dengue

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INTRODUCTION

Both historically and currently, flaviviruses have had a huge global impact on human health. The four serotypes of dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis (TBEV), Murray Valley encephalitis virus (MVEV), and Zika virus (ZIKV) are among the most notorious members of this group. Globalization, climate changes, and vector overlap have caused more flaviviruses to co-circulate in the same geographic regions, increasing the likelihood that a person will be exposed to multiple flaviviruses throughout their lifetime. How exposure to multiple flaviviruses impacts the pathogen-specific immune response and alters the efficacy of flaviviral vaccines has been an area of intense research, combining both studies of human infections and animal models of heterologous flavivirus challenges. We know from decades of research that prior immunity to a flavivirus impacts the

disease outcome (1–3). Considering this, and the established importance of the T cell response in disease outcomes of flavivirus infection (4), it is of the utmost importance to understand the effects of multiple flavivirus exposures on the development of protective immune responses against them. This review will provide an overview of the concepts and consequences of T cell cross-reactivity and what mouse models of flaviviral infection have told us and can teach us about heterologous T cell immunity.

Animal models of infection have provided important mechanistic insight into the role of T cells during flavivirus infection (5–13). Through the use of murine models, researchers have established a critical importance for T cells in protection from flaviviruses disease [reviewed in (4)]. The necessity of T cells is most clearly demonstrated through depletion or deletion studies, which show that in the absence of CD8+ T cells, uncontrolled viral replication and enhanced disease and mortality can occur in mouse models for JEV, ZIKV, WNV, YFV, and DENV infection (14–18). We have recently shown that a robust and polyfunctional CD4+ T cell response is elicited during ZIKV infection in mice (6). These cells infiltrate the CNS during infection, where they are able to restrict viral replication, resulting in limited disease and mortality (6). Importantly, they are absolutely critical for the prevention of ZIKV-induced paralysis in this model (6, 19). However, it is important to consider that, activities of antiviral T cells have also been reported to cause immune-mediated damage in the process of combating infection (20, 21). Paralysis and other neurological impairments seen in mouse models of neurotropic flaviviruses have been attributed to an aggressive neuroinvasive cytotoxic CD8+ T cell response in WNV, JEV, and more recently ZIKV (16, 19, 22). Due to the dual protective and immunopathogenic role of T cells during flavivirus infection, how the functional responses of these cells can change and impact disease outcomes in a heterologous infection environment requires further mechanistic investigation.

It is perhaps not surprising that T cell cross-reactivity exists between flaviviruses, as flaviviruses share between 30 and 70% amino acid identity across their coding region (23), and have a common genomic structure (**Figure 1**). The flavivirus genome is composed of a single positive stranded RNA, with a 5′ methylguanosine cap and a 3′ untranslated region with multiple variable stem loop structures (29). During replication, the genome is directly translated by host ribosomes into a single polyprotein that is subsequently cleaved by both viral and host proteases. Ten protein products are formed in total from these reactions including three structural proteins: capsid (C), membrane (prM/M), and envelope (E) and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. All of these have the potential to be targets of the antigen-specific T cell response (29, 30).

The critical need to study immunological interactions that occur as a result of multiple flavivirus exposures is best exemplified by sequential DENV infection. DENV co-circulates in mosquito populations as four distinct serotypes. Immunity generated to one serotype does not confer protection against the heterologous serotype and instead, often results in enhanced disease (3, 31–33). How this phenomenon occurs is not fully understood, though there is evidence for both antibody-mediated and T cell-mediated mechanisms [reviewed in (20, 34)]. As we cannot control when we are exposed to pathogens in our lifetime, we have used animal models to address questions about how heterologous pathogen exposures shape the immune response and the consequences of T cell cross-reactivity. Murine models have provided an important tractable model for understanding the enhanced disease phenotype observed in heterologous DENV infection (35–37). More recently, these models are being adapted to explore the impact of prior flavivirus exposure on ZIKV immunity and pathogenesis (26, 38–41). Similar to the case of heterologous DENV immunity, cross-reactive T cell responses between DENV and ZIKV have become important to understand with the recent expansion of the geographic range of ZIKV infection (42–44).

THE GENERATION OF T CELL CROSS-REACTIVITY

The T cell compartment is an arm of the adaptive immune system, which has the capacity to keep a record of past infections through immunological memory. Following T cell receptor (TCR) recognition of pathogen specific peptide epitopes presented on Major Histocompatability Complex (MHC) class I or II (45, 46) on antigen presenting cells (APCs), T cells expand and combat infection through various effector functions. However, of the considerable number of potential peptide sequences present during a given infection, only a relatively small fraction will be presented to and recognized by T cells to induce proliferation and effector function, which results in a numerical hierarchy of antigen-specific T cells termed "immunodominance" (47). With every immunological insult comes the potential for alterations to the T cell repertoire and the immunodominance hierarchy within the host.

The Theoretical Necessity of TCR Cross-Reactivity

The enormous theoretical potential of the T cell repertoire is vastly larger than the number of T cells that can occupy a single mouse or human at a given time (10^8 T cells in mice and up to 10^{12} in humans) (48, 49). It is also known that multiple T cells can express the same TCR, which can occur through homeostatic expansion of naïve T cells, proliferative maintenance of memory cells, and infection or vaccination-mediated boosting of T cells (50, 51). Taken together, calculations of TCR diversity have yielded estimates of 2×10^7 TCRs in humans (49) and 2×10^6 TCRs present in mice (52). Which presents a dilemma—in order to mount an adequate response to any theoretical pathogen, it would require the presence of many more T cell clones than are actually present in the body. It has been proposed that one way that the immune system deals with this is through TCR cross-reactivity (53). It has been calculated that given the maximal number of amino acid combinations for example an 11-mer peptide and controlling for restrictions in specific amino acids allowed at certain residues for presentation, that a given TCR may

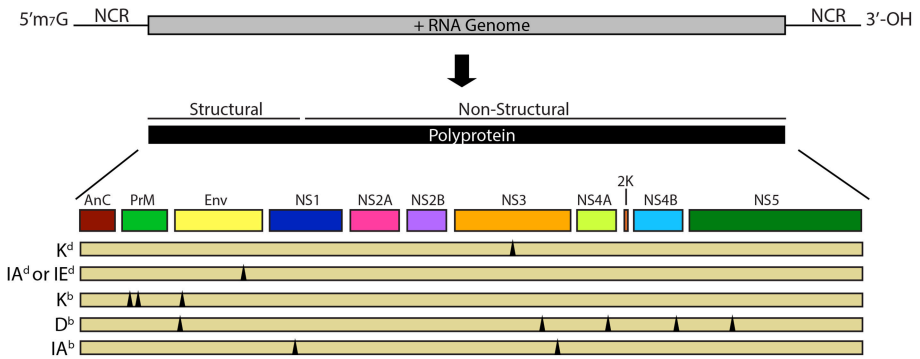

FIGURE 1 | Flavivirus genome and proteins. The flavivirus genome consists of a single positive-stranded RNA molecule with a 5′ methylguanosine cap followed by an untranslated region (UTR), open reading frame (ORF) and a 3′ UTR with multiple variable stem loop structures. The genome is translated from a single ORF into a polyprotein that is proteolytically cleaved by both viral and host proteases. The genome codes for three structural proteins (capsid, membrane, and envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Theoretically, peptides of any of these structural or non-structural proteins have the potential to be targets of the virus-specific T cell response. Multiple flavivirus cross-reactive T cell epitopes with murine MHC restriction have been demonstrated in various murine models, the breadth of which are indicated by the triangles below the polyprotein. For detailed information on these identified cross-reactive epitopes see **Table 2**.

be capable of recognizing between 10^6 and 10^8 p-MHC ligands (53). Based on these calculations and experimental mouse and human data, we now know that T cell cross-reactivity between related and even un-related pathogens is not an extraordinary occurrence, but an inevitability.

As we now know that the specificity described by clonal selection theory, which suggests one TCR for every one peptide, is a mathematical impossibility, we can presume that TCRs are not strictly epitope specific. Experimentally, it has been shown that there is a level of promiscuity in peptide recognition (54–57) and that one TCR can recognize a number of different peptides, each having variable levels of amino acid homology. The ability of one TCR to recognize multiple peptide epitopes is the basis for T cell cross-reactivity [reviewed in (58, 59)]. T cell cross-reactivity can occur if T cells with TCRs primed against epitopes elicited during infection with one pathogen cross-react with peptide sequences presented during infection by a different pathogen [reviewed in (60)]. Because these memory cells are present at a higher frequency and lower activation threshold than naïve cells specific to the second pathogen, they have the potential to be preferentially "boosted" over responses to epitopes specific to the secondary pathogen.

Original Antigenic Sin

Each infection can induce lasting changes to the individual's T cell repertoire because a portion of the antigen-specific T cells generated in response to infection will be retained into memory. These antigen specific cells remain at a higher frequency and lower activation threshold, so that they can be recalled and mount a more rapid and effective response if the same antigen were to be encountered again. The presence of a highly functional antigen specific memory lymphocyte population at a higher frequency than that of the naïve population is the fundamental basis for vaccination (61). However, in instances of T cell cross-reactivity between two pathogens, during the secondary heterologous infection cross-reactive memory T cells can preferentially expand over more pathogen specific naïve ones precisely due to elevated frequencies and reduced threshold for activation, in a phenomenon termed "Original Antigenic Sin" (OAS) (**Figure 2**) (5, 62–66). As discussed in greater detail below, altered effector functions of these cross-reactive T cells that are primed to rapidly respond to cross-reactive antigens can have profound impacts on the balance between the protection and pathogenesis.

IMMUNE RESPONSE TO DENV IN HUMANS

Of the studies on cross-reactive immune interactions between flaviviruses, DENV has the longest history of investigation. The term "DENV" refers not to a single virus, but to a group of four (DENV1-4) genetically similar serotypes transmitted primarily by Aedes spp. mosquitos (67). Many DENV infections cause a range of symptoms from inapparent to mild, characterized by chills, fever, general malaise, retro orbital pain with presentation of leukocytopenia and thrombocytopenia, lasting for 4–7 days (68). However, in a small subset of patients, dengue hemorrhagic fever (DHF) will occur and is characterized by increased vascular permeability, loss of plasma volume and the characteristic "cytokine storm" which can lead to shock. The basis for this pathological progression is thought to be multifactorial; involving elements of host genetics and immunological background, as well as viral-intrinsic factors, though the immunological background of the patient has been shown to be one of the main predictors of disease outcome (69).

Cross-Reactive Responses to DENV

Immunity to one serotype of DENV confers apparent lifelong protection from the same serotype and a brief period of heterotypic immunity to the other serotypes. However, following

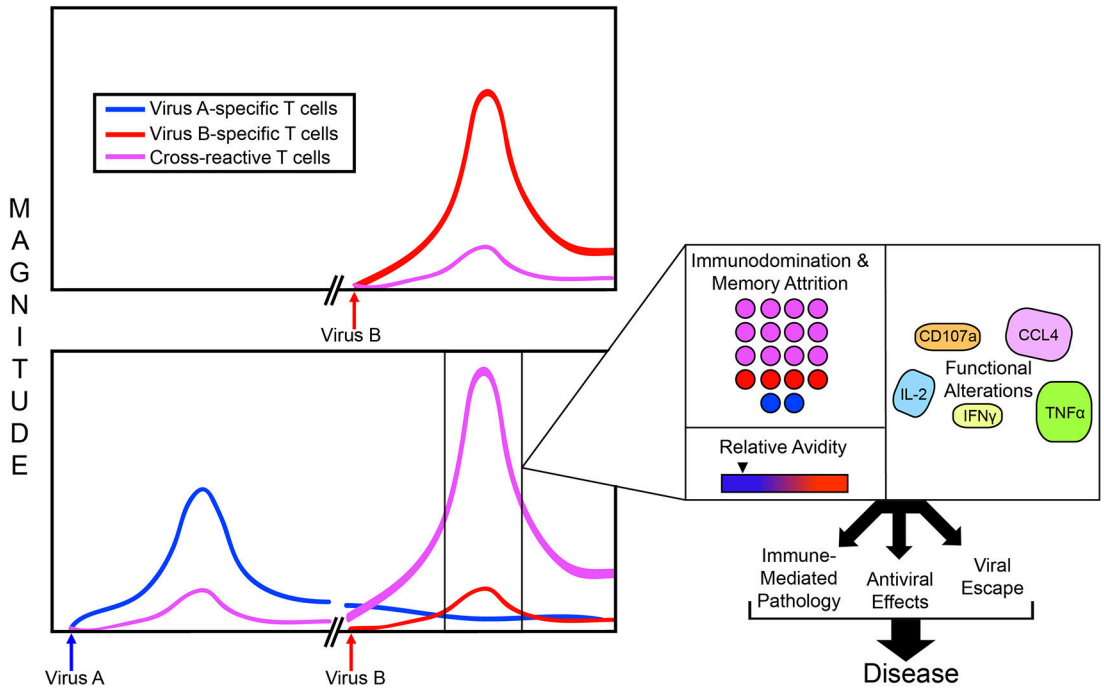

FIGURE 2 | Consequences of T cell cross-reactivity during heterologous infection. During a primary infection, (for example with Virus B), a diverse T cell response may be generated against multiple Virus-B-specific epitopes (Red) possibly in addition to some cross-reactive epitopes (Purple); both of which will contract to some degree following viral clearance. However, if an infection with Virus B is preceded by Virus A, and the two viruses share responses to the same cross-reactive epitopes, an altered T cell immunodominance hierarchy may occur during the heterologous infection. In this case, at the point of infection with Virus B, cross-reactive memory T cells generated during infection with Virus A are already present at a higher frequency and lower activation threshold than naïve T cells specific for Virus A. This can lead to a preferential expansion of the cross-reactive T cells often at the expense of the virus specific ones, or "immunodomination." During this process, memory cells specific to Virus A can even be lost from memory attrition, potentially impacting protection from future infections with Virus A. Sometimes, T cell cross-reactivity can occur in the absence of neutralizing antibody cross-reactivity, resulting in higher antigen loads than what would normally be present in a homologous boosted infection (Virus B followed by Virus B) which can lead to profound T cell activation of a higher magnitude. In the case of some flaviviruses cross-reactive antibody can even increase antigen load via ADE. The preferentially expanded, cross-reactive T cells can display different avidity compared to those that would have been generated during an infection with Virus A in the absence of prior heterologous exposure. During a primary infection with Virus A, the cross-reactive population would normally have a stronger avidity to the peptide variant of Virus A. However, during a heterologous infection, they have a stronger avidity to the peptide variant of the prior infection, Virus B. T cell cross-reactivity during heterologous infection can even have functional implications for cross-reactive T cells, though the alterations to cytokine profiles and their consequences is often virus-specific. All of these alterations to T cell populations and their functional capacities will dictate the balance between cross-protection and immunopathology, and can even result in viral escape; The sum of these, ultimately defining the disease outcome.

this window of cross-protective immunity, the cross-reactive adaptive immune response has the potential to enhance disease in infection with a heterologous serotype, increasing the risk of developing DHF by 15 to 80-fold (3, 69). One mechanism for enhanced dengue disease, first proposed by Halstead, is antibody-dependent enhancement (ADE) (34). It has been shown in *in vitro* and *in vivo* models that cross-reactive antibodies present at sub-neutralizing concentrations can promote DENV uptake into Fcγ-bearing cells leading to enhanced viral loads (37, 70–73). However, owing to the fact that DHF occurs *after* the peak of DENV viremia and closer to the peak in the T cell response, cross-reactive T cells have also been proposed to play a role in the pathology observed (20). It is important to consider that during a homologous secondary infection, the type-specific neutralizing antibody response functions to restrict the replication of virus, in effect lowering the antigenic load during T cell priming. Consequently, the boosted memory T cell response elicited may only be of modest size as this is dependent upon antigenic load. However, in a heterologous infection, the second infection may not be constrained by cross-reactive neutralizing antibody responses, and in the case of DENV, cross-reactive antibodies may even enhance the viral load (74). The large antigen load could drive a massive expansion of cross-reactive memory T cells, potentially leading to immune-mediated pathology, which is one hypothesis for the pathology observed during DHF (20).

In humans, DHF correlates with the magnitude of the T cell response and production of several cytokines, such as TNF-α, further providing a means for T cell cross-reactivity to play a role in disease severity (75). In addition to altered cytokine profiles during DHF, altered TCR avidities as a consequence prior DENV exposure have also been reported in humans. For example, in an analysis of a Thai cohort of DHF patients, it has been shown that the humans expressing HLA-A*11 possessed CD8+ T cells reactive to the NS3 epitope ($NS3_{133}$) present in multiple DENV serotypes (75). While those T cells could bind tetramers containing peptide variants from multiple DENV

serotypes, the avidity with which they did so varied based on the individual's serotype infection history, specifically with the lowest avidity attributed to the currently infecting serotype (76, 77). This observation supports the OAS hypothesis that cross-reactive cells of lower avidity are preserved in memory from a prior infection, then expand upon heterologous challenge, which yields T cell populations of lower avidity to the newly infecting serotype (76, 77). This was similarly demonstrated in an HLA-A*11 Vietnamese cohort of DENV-infected patients. In addition to these altered avidities, altered cytokine profiles in responses to the same cross-reactive variant peptide ligand as a consequence of secondary heterologous infection were also observed (78). In this case, the result of heterologous secondary infection was a skewing to the production of inflammatory cytokines TNF-α and CCL4 with decreased production of IFN-γ and IL-2 (78–80). This data supports the idea that T cell function can be impacted as a result of cross-reactive DENV infection in humans.

ANIMAL MODELS OF T CELL CROSS-REACTIVITY

T cell cross-reactivity reshapes the pathogen specific T cell population. Exposure to a heterologous challenge alters the functional profile of a cross-reactive T cell relative to T cells that had not seen a heterologous challenge by: (1) altering functional avidity (27, 65, 76, 77), (2) skewing the immunodominance hierarchy (5, 62–66), (3) deviation of cytokine profiles (81–83), and (4) altering memory populations (64, 76, 84, 85). Cross-reactive T cells can drive the generation of viral escape mutants, which would not be observed in the absence of heterologous challenge (62, 86, 87). As T cell cross-reactivity can have a profound impact on protection and disease (20, 35, 36, 88, 89), it is critically important to understand how and when T cell cross-reactivity can occur and the implications of a cross-reactive T cell response.

Lessons From Non-flaviviral Pathogens

Much of what we know about T cell cross-reactivity comes from the lymphocytic choriomeningitis virus (LCMV), with studies involving T cell cross-reactivity between flaviviruses coming to the forefront more recently. This has been eloquently shown in mouse models of T cell cross-reactivity between LCMV and Pichinde virus (PV). The immunodominance hierarchy of the T cell response to LCMV in C57BL/6 mice is predictable and stable, with most of the $CD8^+$T cells targeting GP_{33}, NP_{396}, and GP_{276} with responses to the epitope NP_{205} being largely subdominant (90). Importantly, the NP_{205} subdominant epitope is cross-reactive in mice infected with PV (64). When mice are sequentially infected with PV followed by LCMV, the immunodominance hierarchy completely shifts and even alters the population of T cells preserved in memory (memory attrition). Specifically, the responses to the normally subdominant but cross-reactive NP_{205} dominate the T cell response in a heterologous challenge (64, 84) (**Figure 2**). This demonstrates that immunodominance patterns can be influenced by an individual's infection history.

In LCMV as well as other pathogens, cross-reactive T cells can display altered functional profiles during heterologous secondary infection, leading to altered pathogen control and potentially immune-mediate pathology. Functional avidity is thought to correlate with enhanced *in vivo* effector capacity (91). T cells with higher functional avidity achieve effector functions with a lower concentration of peptide and would therefore be thought to be better at controlling pathogens in low antigen environments. Functional avidity can be determined by exposing T cells to increasing concentrations of cognate peptide *ex vivo* and measuring changes in effector responses. As cross-reactive T cells are initially primed to a different peptide ligand, they can display suboptimal avidity for the new ligand during a heterologous infection (**Figure 2**) (20).

Alterations to T cell polyfunctionality have also been observed in some instances of heterologous immunity. One study investigating Epstein-Barr virus (EBV) and influenza A virus (IAV) cross-reactive T cell responses compared individuals experiencing mild vs. severe acute infectious mononucleosis (AIM). During EBV infection, patients with IAV-EBV cross-reactive T cell responses, had altered cytokine profiles and experienced severe AIM (92). As these studies were conducted with human volunteers, a distinct correlation between less severe disease and specific cytokine profiles could not be made, however this study did support much of the scientific concepts developed using murine models demonstrating that heterologous immunity can impact T cell functionality, which can play a key role in determining pathogenesis vs. protection.

It is now being appreciated that a more diverse T cell repertoire correlates with protection from viral infection, and it is thought that one reason for this involves a decreased likelihood of the generation of pathogen escape mutants (62, 93, 94). RNA viruses, including flaviviruses, have a highly error prone RNA polymerase that introduces mutations to the viral genome at a rate of $\sim 1{:}10^4$ nucleotides (95). A consequence of a highly error prone RNA polymerase and a rapid replication rate is a virus with the potential to rapidly adapt to the constraints of immune restriction. In the context of heterologous immunity, focusing the T cell response to cross-reactive epitopes as opposed to promoting a highly diverse T cell repertoire drives the potential to select for viral mutants that escape this focused selection pressure (62, 86, 87). In the example above, heterologous infection with PV followed by LCMV results in extreme clonal dominance of the T cell repertoire to the cross-reactive epitope NP_{205}. In a related study, the same authors found that this did indeed results in the generation of NP_{205} epitope escape variants (62), suggesting that alterations to viral intra-host population dynamics, as a result of skewed immunodominance hierarchies may be an important consequence of heterologous immunity that needs to be investigated further.

Predicting disease outcomes based on the occurrence of cross-reactivity between two pathogens, does not appear to be a "one-size fits all" interaction. The consequences of these complex interactions can culminate in enhanced disease in one combination of infections and cross-protection in another or even have no effect on overall disease outcomes (**Table 1**) [reviewed in (60)]. Again, using the example of LCMV, infection

TABLE 1 | Select references for examples of the impact of flavivirus T cell cross-reactivity and murine models of pathogenesis.

Mouse strain	Priming virus	Challenging virus	Overall effect	Details of pathology	References
AG129	DENV4	DENV2	Protective	Cross-reactive T cells mediate reduction in viral titers and enhance survival	(36)
AG129	DENV3	DENV2	Protective	Cross-reactive T cells mediate reduction in viral titers and enhance survival	(36)
AG129	DENV1	DENV2	Protective	T cells contribute to protection during heterologous infection but are not necessary nor sufficient for protection from mortality	(96)
Ifnar1$^{-/-}$	DENV4	DENV2	Protective	Cross-reactive T cells mediate reduction in viral load and are required for reduction in morbidity	(36)
WT C57BL/6	DENV1	DENV2	Pathogenic	Elevated liver enzymes, low platelet counts, increased megakaryocytes in the spleen, more hematopoietic centers in the liver and increased vascular permeability. Observed phenotype requires TNF-α producing CD8+ T cells	(35)
WT C57BL/6	DENV2	DENV1	No effect		(35)
Ifnar1$^{-/-}$	DENV2	ZIKV	Protective	Cross-reactive CD8+ T cells mediate some protection from ZIKV-induced morbidity and mortality	(26)
Ifnar1$^{-/-}$ HLA-B*0702	DENV2	ZIKV	Protective	Enhanced viremia in mice deplete of CD8+ T cells during heterologous ZIKV challenge	(97)
Ifnar1$^{+/-}$ pregnancy model	DENV2	ZIKV	Protective	Reduced fetal resorption and reduced viral burden	(38)

of LCMV immune mice with PV results in a 10X reduction in PV titers (88) which was shown to be mediated by cross-reactive T cells via adoptive transfer experiments. Similarly, it was shown that CD8+ T cell cross-reactivity occurs between LCMV and vaccinia virus (VV), and that CD8+ T cells from LCMV immune mice can provide protection from VV (88). However, in sequential challenge experiments with LCMV followed by VV infection, while VV was cleared much faster due to cross-reactive T cells, the mice suffered from IFN-γ mediated acute fatty necrosis as a result of cross-reactive immune-mediated pathology (88). This observation emphasizes the need to include context and thoroughly understand the mechanism of pathogenesis in specific viral infections to determine how immune cross-reactivity can walk the fine line between protection and immune-mediated pathology.

DENV

Mouse models of DENV infection have been a valuable tool for answering specific questions regarding the potential contributions of T cells to pathogenesis and protection in heterologous infection [reviewed in (98)]. Some specific advantages to these models in studying this phenomenon include the conserved MHC haplotype and documented infection history in the laboratory setting, which allow for robust epitope mapping studies and reduced variability. WT C57BL/6 or BALB/c mice develop minimal DENV viremia and disease due to the restriction of replication by the type I IFN system, but still mount B and T cell responses that can be functionally evaluated in response to heterologous infection (24, 99). However, mice deficient in IFN-α/β or α/β/γ have been used to more closely mimic DENV disease (98). Depending upon the exact IFN deficiency, dose, route, age, and strain, these mice can display early viremia, elevated hematocrit, TNF-mediated plasma leakage, and elevated liver enzymes more similar to what is seen in severe DENV disease (37, 98, 100, 101).

Multiple strains of mice with various MHC restrictions have been used to study the consequences of flavivirus T cell cross-reactivity in heterologous infection on T cell populations and their functions (25, 35, 65, 102, 103). The cross-reactive epitopes identified in these studies are shown in **Table 2** and **Figure 1**. In WT BALB/c mice, ($H2^d$ restricted), cross-reactive CD8+ T cell responses to a peptide of the NS3 protein ($NS3_{298}$) have been observed for the four DENV serotypes (102). In sequential challenge experiments in these mice, Rothman et al. observed that while the overall kinetics of the antigen-specific T cell response were similar in a primary vs. heterologous secondary challenge, a preferential expansion of responses to this cross-reactive NS3 epitope during secondary heterologous challenge was observed, leading to a shifted immunodominance hierarchy toward the cross-reactive epitope (65). Moreover, this boosted NS3 cross-reactive response was characterized by enhanced TNF-α production compared to primary infection, which is potentially significant given the link between TNF-α levels and disease severity in humans (104). Most mouse models of DENV pathogenesis utilize the C57BL/6 ($H2^b$ restricted) background, warranting investigations of DENV T cell cross-reactivity within this MHC haplotype as well. On this background, cross-reactive CD8+ T cell responses have been described to be directed against a peptide of the NS4a protein ($NS4a_{249}$) (25). Similar to cross-reactive CD8+ T cell responses seen in the WT BALB/c model of heterologous DENV infection, T cell responses to these $H2^b$ restricted cross-reactive epitopes are preferentially boosted upon secondary challenge and drive a more TNF-α dominant cytokine phenotype (25, 35). These studies highlight the relevancy of the murine model to identify correlates of protection as well as the possible causative agents of immune mediated pathology.

TABLE 2 | Flavivirus cross-reactive T cell epitopes with murine MHC restriction identified using various murine models, named in the same manner as the papers they were identified in.

Epitope	Amino acid sequence	Viruses reported in	Mouse Strain	MHC restriction	References
$NS3_{298}$	ARGYISTRVGM ARGYISTRVEM	DENV1/3 DENV2/4	WT BALB/c	K^d	(24)
$NS4a_{249}$	YSQVNPLTL YSQVNPTTL	DENV1/3 DENV2/4	WT C57BL/6	D^b	(25)
PrM_{20}	ISFATTLGV LLFKTEDGV	ZIKV DENV	Ifnar1$^{-/-}$	K^b	(15, 26)
PrM_{44}	ATMSYECPM DTITYKCPL	ZIKV DENV2	Ifnar1$^{-/-}$	K^b	(15, 26)
E_4	IGVSNRDFV IGISNRDFV	ZIKV DENV2	Ifnar1$^{-/-}$	D^b	(15, 26)
E_7	SNRDFVEGM SNRDFVEGV	ZIKV DENV2	Ifnar1$^{-/-}$	K^b	(15, 26)
$NS3_{347}$	PSVRNGNEI PSIKAGNDI	ZIKV DENV2	Ifnar1$^{-/-}$	D^b	(15, 26)
$NS5_{18}$	CAEAPNMKII ESEVPNLDII	ZIKV DENV2	Ifnar1$^{-/-}$	D^b	(15, 26)
$NS4b_{209}$	GASSVWNATTAIGL GASAVWNSTTATGL	WNV JEV	C57BL/6	D^b	(27)
$NS1_{132}$	TFVVDGPETKECPT TFVVDGPETKECPD	WNV JEV	C57BL/6	IA^b	(27)
$NS3_{563}$	WCFDGPRTNTIL WCFDGPRTNAIL	WNV JEV	C57BL/6	IA^b	(27)
E-pep_1	SIGKAVHQVF	JEV WNV DENV	BALB/c	IA^d or IE^d	(28)

Through genetic manipulation of MHC haplotype, mouse models can be used to understand heterologous DENV T cell responses in humans in a more controlled environment. Experiments with Ifnar1$^{-/-}$ HLA B*0702 transgenic mice have been used in DENV sequential challenge and peptide vaccination experiments to understand the impact of DENV immune cross-reactivity in the context of human HLA (**Table 3**) (103). In one study, T cells from HLA B*0702 mice were infected with one of the four DENV serotypes, then stimulated with predicted peptide epitopes from both the homologous and heterologous DENV serotypes. Similar to studies completed using human PBMCs, the authors of this study observed alterations to cytokine profiles and functional avidity when the cells were stimulated with variant serotype peptides compared to the infecting serotype (103). Specifically, the cross-reactive cells displayed higher avidity to the peptide of the infecting serotype as opposed to the variant peptides, and when stimulated with a variant serotype peptide, the cells produced less IFN-γ, were less polyfunctional, and expressed less CD107a (a marker of cytotoxic degranulation). This intriguing study hints at possible differences in the effector functions of cross-reactive flavivirus specific T cell, and points to the need for further studies to explore the potential immune-pathologic or protective outcomes of T cells in the murine model.

Interestingly, when measuring disease outcomes as a result of DENV heterologous T cell responses, it appears that the genotype of the mouse, strain of virus used, and order of infection are all variables that dictate enhancement of disease vs. protection. In sequential challenge experiments of Ifnar1$^{-/-}$ or AG129 mice (which are globally deficient in type 1 interferon receptor or type 1 and type 2 interferon receptor, respectively), cross-reactive CD8+ T cells appear to play an important role in cross-protection, with a potentially minor role for CD4+ T cells (96). These mice, (similar to C57BL/6) are $H2^b$ restricted—an MHC restriction in which DENV CD8+ T cell cross-reactive responses have been described against $NS4a_{249}$ (25). Sequential challenge experiments in both AG129 and Ifnar1$^{-/-}$ mice show that prior exposure to a heterologous serotype of DENV confers cross-protection from DENV2 challenge (36, 96). Depletion of various immune subsets following primary DENV infection have shown that B cells, CD8+ and CD4+ T cells are all important for mediating protection during DENV challenge (105). Cross-reactive CD8+ T cells, however play a particularly important role in mediating cross-protection, where antibody-mediated protection dominates in protection from a homologous serotype challenge (96).

However, when similar experiments are done in WT C57BL/6 mice, cross-reactive CD8+ T cells have been reported to enhance immune-mediated pathology (35). As explained above, while IFNα/β sufficient mice only support transient replication of DENV and do not suffer from disease as measured by weight loss or mortality, it appears that these mice can still suffer from immune-mediated pathology driven by heterologous secondary infection. In a sequential heterologous challenge experiment, one group found that infection with DENV1 (PR/94 strain) followed with DENV2 (Tonga/74 strain) resulted in elevated liver enzymes, low platelet counts, increased megakaryocytes in the spleen, more hematopoietic centers in the liver, prolonged bleeding times, and increased vascular permeability (35). Interestingly, if the order of infections was reversed, or if different strains of virus were used, this enhancement phenomenon did not occur. In support of this observation, is the notion that pathology and protection induced by T cell cross-reactivity during heterologous infection are not always reciprocal, which has been shown at length in LCMV mouse models of T cell cross-reactivity (60). Nonetheless, the authors of this study were able to show through adoptive transfer experiments that TNF-α producing CD8+ T cells were crucial for this enhanced disease phenotype. This supports the idea that altered cytokine profiles as a result of T cell cross-reactivity (which have been observed extensively in human DENV infection) can drive immunopathology (25, 65, 78–80).

It is clear that while progress has been made in understanding the functional consequences of heterotypic DENV immunity on T cell expansion and function, we still have a long way to go in understanding the consequences for disease outcomes. Discrepancies in disease outcome during heterologous challenge between different genotypes of mice with the same MHC type may in fact be the unintentional result of the methods by which disease is measured in the two models. This represents a limitation with the animal models of DENV disease, which requires careful consideration when drawing conclusions from the data regarding heterologous immunity.

TABLE 3 | Flavivirus cross-reactive T cell epitopes identified using HLA transgenic murine models, named in the same manner as the papers they were identified in with the exception of (*)$NS5_{2695}$.

Epitope	Amino acid sequence	Viruses reported in	Mouse Strain	References
$NS3_{1682}$	LPAIVREAI LPSIVREAL	DENV2/1/3 DENV4	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(103)
$NS3_{1700}$	APTRVVAAEM APTRVVASEM	DENV2/3/4 DENV1	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(103)
$NS3_{2070}$	KPRWLDARI RPKWLDARV	DENV2 DENV3	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(103)
$NS4b_{2280}$	RPASAWTLYA HPASAWTLYA	DENV2/1/4 DENV1/3	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(103)
$NS5_{2885}$	TPRMCTREEF KPRLCTREEF	DENV2 DENV3	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(103)
$NS2a_{75}$	RPALLVSFIF	ZIKV DENV2	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(97)
$NS3_{206}$	APTRVVAAEM	ZIKV DENV2	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(97)
$NS3_{574}$	KPRWMDARV	ZIKV DENV2	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(97)
*$NS5_{2695}$	RPGAFCIKVL	ZIKV DENV2	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(97)
$NS5_{539}$	VPTGRTTW	ZIKV DENV2	Ifnar1$^{-/-}$ HLA-B*0702 transgenic	(97)
Env P1	IRCIGVSNRDFVEGMSGGTW FNCLGMSNRDFLEGVSGATW AHCIGITDRDFIEGVHGGTW MRCVGIGNRDFVEGLSGATW MRCIGISNRDFVEGVSGGSW MRCVGVGNRDFVEGLSGATW MRCVGVGNRDFVEGVSGGAW	ZIKV WNV YFV DENV1 DENV2 DENV3 DENV4	AG129 HLA-DR1, DR15, DQ8 transgenic	(40)
Env P25	ALVEFKDAHAKRQTVVVLGS HLVEFEPPHAATIKVLALGN LLVTFKTAHAKKQEVVVLGS RMVTFKVPHAKRQDVTVLGS	ZIKV YFV DENV1 DENV4	AG129 HLA-DR15 transgenic	(40)
Env P41	HRSGSTIGKAFEATVRGAKR FKKGSSIGKMFEATARGARR YKKGSSIGKMFEATARGARR	ZIKV DENV1 DENV3	AG129 HLA-DR15 transgenic	(40)
Env P7	YEASISDMASDSRCPTQGEA IEAKISNTTTDSRCPTQGEA IEAKLTNTTTESRCPTQGEP IEGKITNITTDSRCPTQGEA IEALISNITTATRCPTQGEP	ZIKV DENV1 DENV2 DENV3 DENV4	AG129 HLA-DQ8 transgenic	(40)
Env P8	DSRCPTQGEAYLDKQSDTQY KAACPTMGEAHNDKRADPAF DSRCPTQGEATLVEEQDANF ESRCPTQGEPSLNEEQDKRF DSRCPTQGEAVLPEEQDQNY	ZIKV WNV DENV1 DENV2 DENV3	AG129 HLA-DQ8 transgenic	(40)

In the original research publication the amino acid sequence was presumed to be ZIKV NS4b (NS4b$_{426}$). However our sequence alignment studies show the peptide to be within the NS5 region and therefore the peptide was renamed in this review for the sake of clarity with the first amino acid being position 2695 of the polyprotein.

DENV and ZIKV

Following the introduction of ZIKV to the Americas sometime in 2013, a devastating outbreak ensued. While generally ZIKV infection is asymptomatic in ∼80% of infected adults, the spread of ZIKV throughout the Americas was followed by severe disease outcomes including congenital Zika syndrome (CZS) in some fetuses born to infected mothers, Guillain-Barrè syndrome (GBS), encephalitis, uveitis, and severe thrombocytopenia (106). One hypothesis put forth to explain these unique observations of ZIKV pathogenicity was that prior heterologous flavivirus exposure of individuals in the flavivirus endemic areas of South and Central America perpetuated ZIKV pathogenesis (1). Similar to the cross-reactivity between DENV serotypes, DENV and ZIKV cross-reactive antibodies have been reported in humans, non-human primates (NHPs), and mice, indicating a theoretical potential for ADE of ZIKV by pre-existing DENV immunity

(5, 107, 108). Moreover, it has been observed in multiple studies in humans, NHPs, and mice that DENV immunity does modulate the cellular immune response to ZIKV—specifically that prior immunity to DENV leads to more robust CD8+ and CD4+ T cell responses during ZIKV infection (5, 43, 103, 109–111). These findings have provided a potential link between prior flavivirus exposure and ZIKV disease outcome. As we gather more epidemiological and experimental data using animal models, we are finding that the consequences of immune cross-reactivity between DENV and ZIKV may be different than that of immune cross-reactivity between the DENV serotypes. For example, in controlled NHP sequential challenge experiments, Pantoja et al. were unable to show that prior DENV immunity enhanced ZIKV disease (5). And longitudinal studies in a cohort of Nicaraguan children have even suggested a protective effect of existing DENV immunity on the outcome of ZIKV infection (2). As effective T cell responses remain a defined correlate of protection from flavivirus infection, the impact of prior flavivirus exposure on T cell responses during ZIKV infection is an area that has generated serious interest.

Mouse models of ZIKV infection have been extensively used to understand viral tropism, factors influencing neurological disease, transmission, adverse fetal outcomes, and the immune correlates of protection (6, 15, 112–119). However, due to its only recent re-emergence to the global spotlight, most of these ZIKV studies are done in immunologically naïve animals experiencing their first flavivirus infection. While this offers a deconvoluted analysis, it does not take into account immunological interactions that may take place as a result of immune cross-reactivity during a heterologous infection. It is clear that given the history of DENV and reports of ZIKV immune modulation by prior flavivirus exposure in humans and NHPs, that these studies are warranted.

Similar to murine models of ADE in heterologous DENV infection, ADE can be modeled in murine models of ZIKV infection through administration of specific subneutralizing concentrations of flavivirus cross-reactive antibody (120). However, it is unclear if this phenomenon can similarly enhance ZIKV disease in humans, particularly given the apparent protective effect of prior DENV exposure on ZIKV pathogenesis both in humans and NHPs (2, 5). It appears from the murine studies evaluating the impact of DENV immunity on ZIKV pathogenesis, that cross-reactive T cells play a protective role. In studies using Ifnar1$^{-/-}$ mice cross-reactive CD8+ T cell epitopes have been identified between DENV2 and ZIKV that target both structural and non-structural epitopes (PrM_{20}, PrM_{44}, E_4, E_7, $NS3_{347}$, and $NS5_{18}$) (26). Responses to these cross-reactive epitopes are boosted during heterologous infection as measured by IFN-γ production in response to peptide stimulation (26). It was found that these cross-reactive cells contribute to protection from ZIKV in this model by adoptive transfer of CD8+ T cells from DENV immune mice into naïve mice prior to ZIKV challenge. It was reported that these cells have the potential to mediate reduced ZIKV viremia and moreover can mediate protection from ZIKV-induced fetal destruction in this established mouse model (26). While these are exciting developments, work still needs to be done to functionally characterize these cross-reactive T cell responses, how they can facilitate protection, and determine under what conditions, if any, these cells may drive more severe disease.

Heterologous Immunity Between Other Flaviviruses

While much of the flavivirus cross-reactive T cell research focuses on the four serotypes of DENV as well as the newly emerged ZIKV, there have been studies addressing cross-reactivity between other flaviviruses. In humans, flavivirus cross-reactive T cell responses have been reported in both CD4+ and CD8+ T cells between DENV and WNV, DENV and YFV, and JEV and DENV, although functional studies have been limited (121, 122). Evidence of HLA-restricted T cell cross-reactivity between these other flaviviruses has also been corroborated using HLA transgenic mice (**Table 3**) (40). Some of the most exciting functional studies done to address cross-reactivity between other flaviviruses has been completed by Saron et al. who demonstrated that cross-reactive CD4+ T cell immunity to JEV altered the development of the immune response to DENV (105). In these studies, the authors demonstrated that a prior JEV infection specifically alter the follicular T cells leading to a change in the serocomplex immune response. Relative to DENV mouse models of heterologous T cell immunity, investigations into these cross-reactive responses are fewer in number, though an important contribution to the literature.

In WT BALB/c mice, vaccination with inactivated JEV results in cross-protection from lethal WNV challenge, whereas interestingly, the inverse challenge scenario only led to reduced disease severity, but equal mortality (123). The particular mechanism for this cross-protection remains unclear but, given that in this particular study the vaccine was an inactivated viral particle, it is likely that this cross-protection was mediated primarily through cross-reactive antibody. The specific contributions of JEV/WNV cross-reactive T cells have been more thoroughly investigated in $H2^b$ restricted C57BL/6 mice (27, 28). Within this model a single cross-reactive CD8+ T cell epitope was identified as $NS4b_{209}$ and two cross-reactive CD4+ T cell epitopes were identified as $NS1_{132}$ and $NS3_{563}$ (27). The authors of this study were able to investigate functional differences of these cross-reactive T cells and found that following vaccination with the live attenuated JEV-SA14-14-2, CD8+ T cells specific to the $NS4b_{209}$ cross-reactive epitope perhaps unsurprisingly displayed a higher functional avidity to the JEV peptide rather than the WNV peptide variant (27). Interestingly, altered cytokine profiles were also observed, with CD8+ cross-reactive T cells from WNV infected mice being more polyfunctional than JEV-SA14-14-2 infected mice. While this provides an interesting lead in yet another potential incident of flavivirus cross-reactivity, further studies are needed to understand how these functional alterations could contribute to protection from disease.

CONCLUSION

Exposure to a pathogen has the potential to shape the functional immune response to the next—with each infection leaving an

immunological signature housed within the memory of the adaptive immune system. This signature has the potential to alter immune responses to subsequent homologous and heterologous pathogen exposures. For flaviviruses, cross-reactive T cells can both limit infection and disease (4) and cause substantial immune-mediated pathology (20). This apparent dichotomous role of cross-reactive T cells in protection and pathogenesis makes it crucial to understand what factors drive this delicate balance. Mouse models of heterologous infection have been used to study flavivirus cross-reactive T cell responses, providing strong mechanistic insight into the impact of both pathogenic and protective T cell cross-reactivity. Based on these studies, many laboratories, including ours, are looking toward the future and using animal models to define the main drivers of immune-mediated disease enhancement and cross-protection mediated by flavivirus cross-reactive T cells. We are using animal models to test the feasibility of pan-flavivirus vaccines and broadening our study of flavivirus cross-reactivity to include overlooked endemic flaviviruses including Rocio and Powassan. Within such a diverse pathogenic and immunogenic family of viruses, prior studies of flavivirus cross-reactivity provide a strong foundation to both understand fundamental concepts in immune mediated cross-reactivity and developed the next generation of flavivirus vaccines and therapeutics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Cross-Reactive T Cell Immunity to Dengue and Zika Viruses: New Insights Into Vaccine Development

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Dengue virus (DENV) is a member of the Flavivirus family that includes Zika virus (ZIKV), West Nile virus, Japanese encephalitis virus, and yellow fever virus. As the most prevalent of the flaviviruses, DENV is responsible for tens of millions of infections each year. The clinical manifestations of infection with one of the four DENV serotypes (DENV1–4) range from no symptoms to hemorrhagic fever and shock ("severe dengue"), which is fatal in ∼25,000 patients annually. Many factors contribute to the development of severe dengue, including the DENV serotype and host expression of certain HLA alleles; however, it now seems clear that pre-existing immunity to DENV—and possibly other flaviviruses—is a major precipitating factor. While primary infection with one DENV serotype elicits strong cellular and humoral immune responses that likely confer long-lived protection against the same serotype, subsequent infection with a different serotype carries an increased risk of developing severe dengue. Thus, primary DENV infection elicits cross-reactive immunity that may be protective or pathogenic, depending on the context of the subsequent infection. Many flaviviruses share high sequence homology, raising the possibility that cross-reactive immunity to one virus may contribute to protection against or pathogenesis of a second virus in a similar manner. In addition, several flaviviruses are now endemic in overlapping geographic regions, underscoring the need to gain more knowledge about the mechanisms underlying cross-reactive immunity to different DENV serotypes and flaviviruses. Here, we review our current understanding of T cell immunity to DENV, focusing on cross-reactivity with other serotypes and flaviviruses such as ZIKV, and the role of DENV-elicited $CD4^+$ and $CD8^+$ T cells in protection. Recent work in this area supports a beneficial role for cross-reactive T cells and provides new insights into the design of safe and efficient flavivirus/pan-flavivirus vaccines.

Keywords: cross-reactive T cell response, Dengue, Zika, Epitope Mapping, vaccine development

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INTRODUCTION

Dengue virus (DENV) belongs to the flavivirus genus of the Flaviviridae family, which includes Zika virus (ZIKV), yellow fever virus (YFV), West Nile virus (WNV), and Japanese encephalitis (1). The flaviviruses are transmitted mainly through the bite of *Aedes* genus mosquitoes (namely, *Aedes aegypti* and *Aedes albopictus*), which have expanded to tropical and subtropical areas throughout

the globe (2). DENV is currently endemic in more than 128 countries, most of which are developing nations where it imposes major public health and economic burdens. DENV exists as four serotypes (DENV1-4) that share 60–75% amino acid homology (3), and infection with any serotype can be asymptomatic or cause a spectrum of symptoms ranging from mild aches and pains to life-threatening dengue fever/hemorrhagic fever (DF/DHF) leading to shock; this syndrome is now referred to as "severe dengue." Approximately 100 million new symptomatic cases are reported annually, of which about 2% result in severe dengue, with 25,000 fatalities (4). This number is probably an underestimate considering the challenges in public health surveillance faced by many DENV-endemic countries. In the past few decades, the incidence of DENV has increased and expanded, and half of the global population (∼3.6 billion people) is now estimated to be at risk for infection.

Efforts to understand why DENV infection causes such a range of symptoms have been ongoing for more than 60 years. Epidemiological studies showed that severe dengue was more prevalent in children and adults experiencing a secondary infection with a different DENV serotype (heterotypic) compared with the same serotype (homotypic infection) (5, 6). This and other observations suggested that DENV infection may elicit long-term protection against the same serotype but only short-term protection or even enhanced infection with a different serotype. Although multiple factors, such as genetic variation, age, and sex contribute to the development of severe dengue (6), the mechanisms underlying the role played by the immune response in dictating whether DENV infection is protective or pathogenic are the dominant subject of continued research.

The research centers around two mutually non-exclusive hypotheses in which both humoral and cellular immunity to DENV contribute to disease pathogenesis. The first hypothesis stems from the process known as antibody (Ab)-dependent enhancement (ADE), whereby pre-existing cross-reactive Abs enhance the viral burden during subsequent heterotypic infection by promoting Fcγ receptor-mediated cellular uptake. Increasing evidence from humans and mouse models support a direct role for DENV serotype-cross-reactive Abs in severe dengue (6–9). The second hypothesis for the enhanced disease risk during secondary DENV infection is built on studies suggesting a potential role for cross-reactive T cells in DENV pathogenesis (10, 11). According to the original T cell antigenic sin hypothesis, most activated T cells during acute DENV infection are cross-reactive with a previously encountered serotype(s) and have low affinity for the currently infecting serotype, leading to suboptimal control of infection and disease pathogenesis (10). However, at present, the evidence is stronger for a protective rather than a pathogenic role for cross-reactive T cells in DENV infection (8). The relative contribution of host humoral *vs.* cellular immunity to the control of DENV infection is of great relevance for the development of safe and effective DENV vaccines. The only currently licensed DENV vaccine, Dengvaxia® (CYD-TDV, Sanofi Pasteur), expresses the DENV E from YFV vector backbone which include the YF NS proteins and elicits Ab responses to DENV E protein (the major target of anti-flavivirus Ab responses) but not T cell responses to DENV NS proteins (the major targets of anti-DENV T cell response, as discussed below). Multiple clinical trials have revealed that Dengvaxia® elicits suboptimal Ab responses against all four DENV serotypes, and the vaccine-induced Ab responses wane within 3–4 years after vaccination (12, 13). In particular, analysis of pooled data from two phase 3 clinical trials of Dengvaxia® (CYD14 and CYD15) of Asian-Pacific and Latin American cohorts, respectively, revealed that young children (2–5 years of age) who had not previously been exposed to DENV were at increased risk for developing severe dengue upon vaccination (13, 14). This was ascribed to unequal Ab-mediated protection and low T cell responses elicited by the vaccine against the four DENV serotypes (15, 16), which may set the scene for ADE during subsequent infections. Thus, these Dengvaxia® findings support our hypothesis that the beneficial components of T cell responses should be harnessed in the design of optimal DENV vaccines (8).

A second flavivirus that has risen to the forefront of basic and clinical research in recent years is ZIKV. This virus was discovered in 1947 in Uganda, but the more recent outbreaks have brought worldwide attention to its potentially devastating effects, which include congenital ZIKV syndrome in infants born to ZIKV-infected mothers, and neurological disorders such as Guillain-Barre syndrome in adults (17). DENV and ZIKV share approximately 44% sequence identity at the amino acid level, are both widely distributed throughout the globe, and have overlapping areas of endemicity. These aspects, together with the studies of immunity to heterotypic DENV serotypes, raised the possibility that primary infection with DENV or ZIKV may induce an immune response cross-reactive with the reciprocal virus. Current work suggests that the mechanisms underlying cross-reactive humoral and cellular immunity resulting from ZIKV or DENV primary infection are as complex as those underlying heterotypic DENV infections (8). Here, we review recent developments in our understanding of the protective vs. pathogenic roles of DENV-elicited T cells in the context of subsequent infections with other DENV serotypes or ZIKV. The evidence presented here provides new insights into the need to consider both Ab and T cell immunity in the development of safe and effective flavivirus vaccines.

IMMUNODOMINANCE OF DENV SEROTYPE-SPECIFIC T CELL RESPONSES IN HUMANS AND ANIMAL MODELS

DENV is a positive-sense single-stranded RNA virus with an ∼11 Kb genome. As is the case for all flaviviruses, the DENV genome encodes three structural proteins (capsid [C], precursor membrane [prM], envelope [E]) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). A comprehensive understanding of the immunodominant T cell epitopes in each flaviviral proteome, the parameters that influence their immunodominance, and the extent to which each epitope elicits T cell cross-reactivity to heterotypic serotypes/viruses, are of crucial importance for vaccine design.

Substantial effort using patient samples and mouse models has resulted in the identification of many $CD4^+$ and $CD8^+$

T cell immunodominant epitopes during DENV and ZIKV primary and secondary infections. Some studies have taken the approach of testing isolated T cell responses to overlapping peptide libraries encompassing a viral protein or the entire viral proteome. Others have used predictive computational algorithms to identify the peptides most likely to interact with a given set of HLA or MHC molecules, which narrows the spectrum of epitopes to be screened. Studies using overlapping peptides spanning the DENV2 proteome identified NS3 as the most frequent target of the T cell response in DENV-infected patients in Thailand and India (18, 19). Using a similar approach with T cells isolated from DENV-infected Singaporean adults, Rivino et al. showed that $CD8^+$ T cells targeted mostly NS3 and NS5 proteins, whereas the $CD4^+$ T cell response was directed largely against C, E, and NS1 proteins (20). In contrast to the overlapping peptide approach, Weiskopf et al. used a T cell epitope prediction program to identify over 200 new $CD8^+$ T cell epitopes in DENV using peripheral blood mononuclear cells (PBMC) obtained from almost 200 healthy blood donors from Sri Lanka, and their results revealed that NS3, NS4B, and NS5 are the major $CD8^+$ T cell targets (21). Using a similar comprehensive approach of identifying immunogenic epitopes with broad coverage of HLA types expressed worldwide, Weiskopf and colleagues demonstrated that NS3, NS5, and the structural protein C are the major target proteins of the anti-DENV $CD4^+$ T cell response in humans (22–24). Collectively, these studies indicate that NS3 and NS5 are the major targets of the anti-DENV $CD4^+$ and $CD8^+$ T cell responses in humans.

However, a challenge of examining T cell immunity in humans is that an accurate infection history is not always available. Non-Human Primates (NHP) do not develop severe dengue disease manifestations, including vascular leakage, and need high maintenance and cost for each study. The majority of mouse models are genetically modified to allow effective replication and development of clinical outcomes. Although, many of these mouse models lack one or more components of the IFN system, as DENV cannot inhibit the IFN signaling in mouse cells (unlike in human cells) to establish robust replication, they are still important as the first step to study protective/pathogenesis effects and test vaccine or antiviral drug candidates. In the context of investigating T cell responses, the antigenic load dictates the level of T cell responses to viruses (25), and thus WT mice, which do not support robust DENV replication, are not ideal for investigating the contribution of T cells in modulating viral replication and disease manifestations. Despite their limitations, these immunodeficient mouse models have therefore proven to be invaluable in identifying virus-specific and cross-reactive T cell epitopes. In particular, type I interferon (IFN) receptor (Ifnar1)-deficient mice, which are more susceptible than wild-type strains to DENV infection (26), backcrossed to HLA transgenic mice, which enable investigation of epitopes likely to be immunodominant in humans (27), have provided key insights into immunodominance patterns during primary and secondary DENV infections. Using a model of primary DENV2 infection in HLA-A*0201, A*0101, A*1101, B*0702, and DRB1*0101 transgenic *Ifnar1*$^{-/-}$ mice, we first reported that the anti-DENV2 $CD8^+$ T cells recognize predominantly NS3 and NS5 epitopes, and $CD4^+$ T cells recognize C, NS3, and NS5 epitopes (27). Moreover, the mice expressing known protective HLA molecules, such as HLA-B*0702, elicited broader and higher magnitude responses than an HLA associated with susceptibility to DENV infection (HLA-A*0101), in agreement with the human data (21). In contrast with DENV2, our model of primary DENV3 infection in HLA transgenic *Ifnar1*$^{-/-}$ mice revealed that DENV3 induced a T cell response directed mainly (33%) against C, prM, and E epitopes, whereas only 3% of the T cell response in DENV2-infected mice was against these proteins (28). Similar results were observed in humans, where the $CD8^+$ T cell response in DENV3-infected individuals was directed more toward the structural proteins than the nonstructural proteins (29). We next modeled secondary homotypic and heterotypic DENV infections in HLA transgenic *Ifnar1*$^{-/-}$ mice and observed that $CD8^+$ T cell responses were broad (targeting both structural and NS proteins) following primary and homotypic secondary infection, whereas $CD8^+$ T cell responses following heterotypic secondary infection focused toward the conserved NS proteins (28), as observed in humans with natural DENV reinfections (19, 21, 30). Collectively, the HLA transgenic mouse model and human data indicate that (i) the $CD8^+$ T cell responses are mostly directed against nonstructural proteins NS3, NS4B, and NS5 for DENV1, 2, and 4 and both structural proteins (C, M, and E) and nonstructural proteins (NS3, NS4B, and NS5) for DENV3, (ii) $CD4^+$ T cells preferentially recognize epitopes in C, NS3 and NS5 proteins, and (iii) primary infection is dominated by serotype-specific T cells, whereas memory T cells that recognize conserved epitopes are expanded together with naïve serotype-specific T cells during secondary infection (**Figure 1**). Thus, while epitopes from all DENV proteins can potentially be recognized by $CD4^+$ and $CD8^+$ T cells, the precise pattern of immunodominance depends on the T cell type, the infecting serotype, and the individual's history of infection (**Table 1**).

IMMUNODOMINANCE OF DENV-ELICITED ZIKV CROSS-REACTIVE T CELL RESPONSES

Recent evidence indicate that DENV-elicited T cells are cross-reactive also with ZIKV. Our study using a sequential model of DENV2 followed by ZIKV infection in *Ifnar1*$^{-/-}$ HLA transgenic B*0702 and A*0101 mice showed that DENV2-elicited $CD8^+$ T cells recognized epitopes in ZIKV NS proteins, of which ∼40% were located in NS3 and ∼20% each in NS2A, NS4B, and NS5 (37). We also defined DENV2-elicited ZIKV cross-reactive epitopes in WT and congenic *Ifnar1*$^{-/-}$ mice in the C57BL/6 genetic background (38). We observed that DENV2-elicited $CD8^+$ T cells mainly recognized epitopes in ZIKV NS3, NS5, prM and E, and that these cross-reactive CD8 T cell responses dominated during ZIKV infection in *Ifnar1*$^{-/-}$and WT C57Bl/6 mice with Ifnar1 blockade (via pre-treatment with blocking anti-Ifnar1 Ab). These mouse data have been validated by a human study that compared ZIKV cross-reactive T cells from naïve *vs.* DENV-exposed individuals from two DENV-endemic countries (Sri Lanka and Nicaragua)

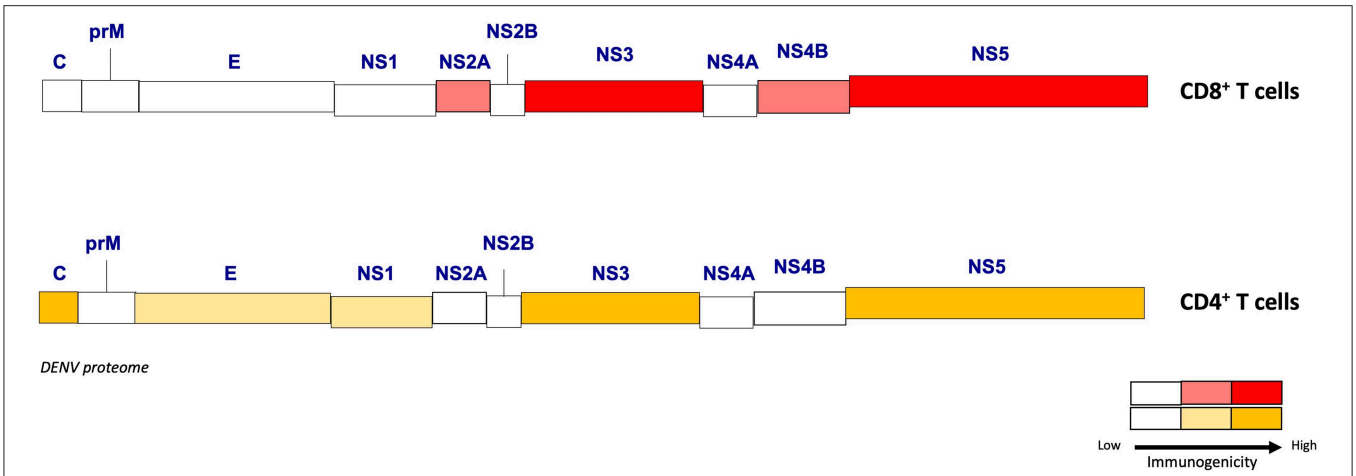

FIGURE 1 | Immunogenicity of the DENV proteome for $CD4^+$ and $CD8^+$ T cells. Different levels of immunogenicity (from low to high) are represented for $CD8^+$ and $CD4^+$ T cells among structural (C, M, E) and non-structural (NS1, 2A, 2B, 3, 4A, 4B, 5) DENV proteins. This schematic highlights the DENV proteins that are able to induce strong T cell responses.

TABLE 1 | Parameters that influence the immunodominance of the T cell responses to DENV and ZIKV.

Parameters	References	Conclusions
Cell types	(27, 30)	Distinct immunodominance patterns between $CD4^+$ and $CD8^+$ T cells
Serotypes	(23, 28)	DENV3 showed a distinct immunodominance compared to DENV1,2, 4 serotypes
Sequence of infection	(28)	The immunodominance patterns of T cells are shaped by the serotype infecting during the primary vs. secondary infection
Primary *vs*. secondary infections	(21, 28, 31, 32)	During primary infection, serotype-specific epitopes are targeted by $CD4^+$ and $CD8^+$ T cells, although conserved epitopes are mostly targeted during secondary infections
HLA alleles	(21, 23, 27, 33–35)	Magnitude, frequency, and breadth of T cell responses are associated with particular HLA alleles (protective or susceptible HLA restriction)
Pre-existing immunity to DENV	(36–39)	DENV-immunity modulated ZIKV immunodominance patterns

(36). Specifically, Grifoni and colleagues observed not only that DENV-specific $CD8^+$ T cells recognized ZIKV epitopes but also that prior exposure to DENV altered the pattern of epitope immunodominance (36). Thus, *in vitro* screening of $CD8^+$ T cells from DENV-immune individuals showed a dominant response to epitopes in ZIKV non-structural proteins (mainly NS3 and NS5), whereas cells from DENV-naive individuals targeted C, E, and prM. In line with this finding, a study with West African patients exposed to ZIKV and/or DENV showed that T cell cross-reactivity was more strongly directed against epitopes from the DENV and ZIKV NS3 helicase region (71% sequence homology) than the protease region (53% sequence homology) (40). Similarly, in another study of DENV-immune individuals, several epitopes in ZIKV NS3 were recognized by cross-reactive DENV-elicited $CD4^+$ and $CD8^+$ T cells, whereas fewer cross-reactive epitopes were located in ZIKV C protein (41). The high level of sequence conservation among flaviviral NS3 proteins most likely explains the immunodominant response to NS3. Collectively, these mouse and human studies have demonstrated that DENV-elicited $CD8^+$ and $CD4^+$ T cells are highly cross-reactive with ZIKV. Additionally, in the context of reciprocal infection, mouse studies have already shown that ZIKV-elicited $CD8^+$ T cells are cross-reactive with DENV. Further studies with animal models and humans in particular are now necessary to define the precise features of the cross-reactive ZIKV-elicited T cells against DENV and vice-versa.

PATHOGENIC VS. PROTECTIVE FUNCTIONS OF DENV-ELICITED CROSS-REACTIVE T CELLS

Earlier studies with DENV-infected humans suggested that T cells may be playing a pathogenic role during secondary infection with heterotypic DENV. In particular, Green et al. reported that activated T cells ($CD69^+$) were more abundant in patients with severe dengue compared with mild disease or no symptoms (42). In addition, Mongkolsapaya et al. observed a higher frequency of DENV-reactive $CD8^+$ T cells with low affinity in patients experiencing severe dengue compared with mild disease (10). These results were in agreement with other studies demonstrating different immune profiles (cytokine production and cytotoxicity) for $CD8^+$ and $CD4^+$ T cells from severe dengue patients compared with mild dengue patients (19, 43). For instance, NS3-specific $CD8^+$ T cells from donors with severe dengue had a higher production of tumor necrosis factor (TNF) vs. IFNγ compared with children with mild dengue (19). Along the same line, $CD4^+$ T cells from Thai school

children with secondary DENV infection produced more TNF when stimulated with heterotypic DENV antigens compared with homotypic antigens *in vitro* (43). In support of these human studies implicating a pathogenic role for cross-reactive T cells during DENV infections, a study with wildtype C57BL/6 mice demonstrated that adoptive transfer of DENV1-elicited $CD8^+$ T cells into naïve mice triggered some signs of disease following DENV2 challenge (44). However, wildtype C57BL/6 mice are highly resistant to DENV infection, do not develop vascular leakage, a hallmark of severe dengue, and the T cell response in wildtype mice may be limited due to a small antigenic load (8). Thus, at present, direct evidence linking cross-reactive T cells to severe dengue pathogenesis is lacking.

On the other side of the protective *vs*. pathogenic immunity debate, increasing evidence supports a protective role for DENV serotype-cross-reactive $CD4^+$ and $CD8^+$ T cells. In particular, comprehensive epitope identification studies have revealed that $CD4^+$ and $CD8^+$ T cell responses restricted to HLA molecules associated with a "low risk" of severe dengue display more robust and polyfunctional responses than cells restricted to "high-susceptibility" HLA molecules (21, 23). Weiskopf et al. used healthy blood donors from a DENV hyperendemic country, Sri Lanka, with 80% of the general population being DENV seropositive. Detailed analysis of DENV epitope-specific $CD8^+$ T cell responses in healthy DENV-immune individuals showed no differences in the magnitude, phenotype, functionality, and avidity of the responses to serotype-specific and conserved epitopes (21). Notably, the magnitude, frequency, and breadth of the memory T cell responses were dictated by the restricting HLA, in that presentation by protective HLA molecules (B*3501, B*0702, and B*5801) elicited strong and broad responses compared with susceptible molecules (HLA-A*2402 and A*0101) (21). Consistent with these findings, examination of the transcriptional signatures of PBMC from asymptomatic and symptomatic DENV-infected individuals revealed higher expression of genes related to adaptive immunity and T cell activation in the asymptomatic subjects compared with the patients with severe dengue (45). More recently, a higher frequency of DENV-specific T cells was detected in patients with mild dengue relative to those with severe dengue (46). Collectively, these human data implicate a protective role of T cells in dengue disease control. However, more studies evaluating the precise phenotype and epitope specificity of $CD4^+$ and $CD8^+$ T cells during various phases of DENV infection (acute and convalescence) are needed to better understand the contribution of T cells in protective vs. pathogenic immunity.

In line with the human data, studies using models of DENV infection in gene-deficient mice lacking type I IFN receptor or both type I and II IFN receptors, which can support robust DENV replication and manifest severe dengue-like disease, have provided direct evidence in support of a protective role for serotype-cross-reactive T cells (47–49). Higher mortality and viral tissue burden were observed in mice that had been primed with DENV4, depleted of $CD8^+$ T cells, and then challenged with DENV2 compared with similarly treated $CD8^+$ T cell-sufficient mice (48). Transfer of DENV4-elicited $CD8^+$ T cells to naive mice reduced the viral burden upon challenge with DENV2, whereas $CD8^+$ T cells were not required for protection after challenge with homotypic DENV4 (48). Moreover, DENV2-cross-reactive $CD8^+$ T cells contributed to the control of viral burden in HLA-B*07 transgenic mice after vaccination with variant peptides from other DENV serotypes (49). Taken together, the human and mouse data indicate that serotype-cross-reactive $CD8^+$ T cells are important mediators of protection against DENV infection.

Recent human studies have also begun to identify potential T cell-based correlates of protection against DENV in the context of both natural infections (21, 50, 51) and vaccination (TV003/TV005) (24, 50). A protective $CD8^+$ T cell response appears to be driven by DENV-specific effector memory T_{EM} ($CCR7^-$ $CD45RA^-$) and T_{EM} expressing CD45RA, known as T_{EMRA} ($CCR7^-$ $CD45RA^+$). Both $CD8^+$ T_{EM} and T_{EMRA} subsets produce IFNγ and TNF, express cytotoxic molecules such as granzyme B or CD107a, and upregulate PD1. A positive correlation between the expression of PD-1 and the magnitude of the $CD8^+$ T cell response restricted by HLA*B3501, associated with a low risk of developing severe dengue, has been observed (51). $CD8^+$ T_{EM}/T_{EMRA} cells in blood from dengue-infected adults during acute phase (52) expressed high levels of CLA (cutaneous lymphocyte-associated antigen), a skin homing receptor, and chemokine receptors CXCR3, CCR5, and CXCR6, which support T cell migration to inflamed tissues (52). In this study, CLA-expressing DENV-specific $CD8^+$ and $CD4^+$ T cells were able to home to the skin during acute DENV infection, suggesting that these cells may be retained in the skin as first line of defense during DENV reinfection. Taken together, these observations indicate that the phenotypes of DENV-specific $CD8^+$ T cells that are associated with protection include T_{EM} and T_{EMRA} subsets expressing IFNγ, TNF, cytotoxicity markers, PD1, CLA, and chemokine receptors CXCR3, CCR5, and CXCR6, and they suggest that the anti-DENV $CD8^+$ T cells may exert antiviral effects both through production of cytokines such as IFNγ and TNF and cytotoxicity.

The phenotype of DENV-specific $CD4^+$ T cells that are associated with protection have also begun to be investigated. In a study involving healthy donors, a higher frequency of DENV-specific $CD4^+$ T_{EMRA} that produce more IFNγ than $CD4^+$ T_{EM} or T_{CM} cells and express CXCR3 and cytolytic molecules (e.g., granzyme B and perforin) was found in the blood of individuals classified as primary or secondary infections compared with dengue-negative healthy individuals (53). There was no significant difference between primary vs. secondary infection groups. In this study, secondary infection referred to seropositive healthy donors with neutralizing titers to more than one serotype and primary infection corresponds to donors with neutralizing titers to only one serotype. These IFNγ-producing cytotoxic $CD4^+$ T cells (CTL) were more abundant in donors expressing HLA associated with protection (HLA DRB1*0401) than those associated with susceptibility to DENV infection (HLA DRB1*0802) (53). Recently, single-cell RNA sequencing identified DENV-specific $CD4^+$ T CTL as a distinct population (54). In addition to $CD4^+$ CTLS, $CD4^+$ follicular T helper cells (Tfh) that express $CXCR5^+$ have been observed in PBMCs from convalescent DENV-infected patients after stimulation with

DENV peptides (30). A recent study of DENV-infected children in Thailand showed that peripheral Tfh cells were expanded and activated (PD-1^{hi}, CD38^{+}) during acute infection, and a higher frequency of activated Tfh cells were observed in patients with severe disease relative to those with mild disease (55). Thus, the magnitude and precise phenotype of CD4^{+} T cells that are polarized to cytotoxic Th1 and Tfh cells may serve as important correlates of protection against DENV infections.

Since the recent emergence of ZIKV in the Americas, studies have begun to examine how prior infection with DENV influences T cell immunity and the pathogenesis of ZIKV. Similar to the observations in patients with heterotypic DENV secondary infection, CD4^{+} and CD8^{+} T cells from DENV-immune humans (naturally infected or vaccinated) are mostly T_{EM}/T_{EMRA}, express more polyfunctional cytokines, and display higher levels of cytotoxicity and activation markers (granzyme B, PD-1) than cells from ZIKV-infected DENV-naïve individuals (36, 40, 56). Importantly, studies modeling sequential DENV followed by ZIKV infection in a variety of mouse strains, including *Ifnar1*$^{-/-}$ HLA transgenic, wildtype, and *Ifnar1*$^{-/-}$, have demonstrated that DENV-elicited CD8^{+} T cells are required for protection against ZIKV infection in both non-pregnant and pregnant mice (37–39, 57). Thus, these mouse model studies suggest that prior DENV immunity can afford cross-protection against ZIKV via CD8^{+} T cells.

Altogether, evidence to date supports an important role for DENV-elicited CD8^{+} T cells in mediating protection against heterotypic DENV and ZIKV infections. In comparison with CD8^{+} T cells, the function of cross-reactive CD4^{+} T cells during sequential infection with heterotypic DENV serotypes or with DENV and ZIKV has been explored to a lesser extent. Mouse models are thus poised to reveal the precise roles of the cross-reactive CD4^{+} T cell responses and the interplay between the pre-existing DENV-elicited CD8^{+} and CD4^{+} T cell responses during various reinfection settings.

DESIGNING ANTI-FLAVIVIRAL VACCINES TO INDUCE BOTH T CELL AND HUMORAL IMMUNITY

The quest for a safe and effective DENV vaccine has been ongoing for nearly 70 years. However, one of the most successful human vaccines designed to date is the live-attenuated yellow fever virus vaccine (YFV-17D), which has significantly reduced the incidence of this important flaviviral disease worldwide. YFV-17D induces strong Ab and T cell responses that mediate long-term protection in humans (58), and the YFV-17D-elicited CD8^{+} T cell response includes terminally differentiated CD8^{+} T_{EMRA} cells that express PD-1 and are highly proliferative and polyfunctional (59). To produce Dengvaxia®, Sanofi Pasteur generated chimeric viruses with prM and E proteins from DENV1-4 and NS proteins from YFV-17D. This is currently the only licensed DENV vaccine and it has several drawbacks. The vaccine elicits suboptimal Ab responses with differential neutralizing activity against the four DENV serotypes; for example, titers of anti-DENV2 neutralizing Ab are particularly low (15), which could explain the elevated risk of severe dengue observed in vaccinated 2- to 5-year-olds (14). In addition, Dengvaxia® failed to protect naïve individuals (14, 60). Based on these outcomes, Dengvaxia® is recommended only for DENV-immune individuals older than 9 years of age (13). The failure of Dengvaxia® to fully protect may well be due to the absence of DENV NS proteins, which, as noted here, are the predominant targets of DENV-specific and cross-reactive T cell responses. In contrast, the vaccine's beneficial effect in DENV-immune individuals could result from a combination of the Ab response and stimulation of pre-existing DENV-elicited, YFV NS3-cross-reactive T cells (61). This possibility is supported by studies showing that Dengvaxia® induced a strong anti-YFV NS3 CD8^{+} T cell response in DENV-naïve individuals but a weak anti-DENV CD8^{+} T cell response in DENV-immune individuals (16, 62). This observation substantiates the need to include immunodominant DENV-specific proteins in vaccines against DENV.

Two different DENV vaccine candidates (from Takeda and NIH/Butantan) that also represent live-attenuated chimeric viruses are now in phase III trials and have shown promising results in eliciting both cellular and humoral immune responses. TV003 is a tetravalent formulation composed of DENV1–3 and a chimeric DENV2 that includes prM and E from DENV2 and other proteins from DENV4. A single dose of TV003 has been shown to induce neutralizing Abs in up to 90% of vaccinees (63) and polyfunctional T cell responses that mainly target the most conserved epitopes in NS3 and NS5 (50). Another live-attenuated DENV vaccine candidate, TAK-003 from Takeda, is a tetravalent formulation of DENV2 and prM and E from DENV1, DENV3, and DENV3 within the same DENV2 backbone. This vaccine candidate induces a polyfunctional CD8^{+} T cell response against DENV2 NS1, NS3, and NS5 proteins that is cross-reactive on the same proteins from DENV1, 3, and 4 (64). However, the absence of nonstructural proteins from DENV1, 3, or 4 in this vaccine may be limiting, given that the pattern of immunodominant epitopes is influenced not only by the serotype but also by the vaccinee's infection history (28). In addition, it is important to note that the cross-reactive T cell response to heterologous flaviviral infection confers only short-term protection (39). Long-term monitoring of the vaccinated individuals is necessary to determine whether vaccines can induce a sustained cross-reactive T cell response, which is the ideal outcome.

Several ZIKV vaccine candidates have been tested in animal models (65). Of these, prM/E DNA vaccines have shown the most promising results, but they appear to elicit poor T cell responses, despite high neutralizing anti-E protein Ab titers, in both mice (66) and rhesus monkeys (67). Moreover, the monkey study demonstrated protection against ZIKV infection that correlated with the Ab titer, but transfer of Abs from vaccinated to naïve monkeys conferred only partial protection against subsequent ZIKV infection (67), suggesting that Ab responses alone may be insufficient for full protection. Importantly, ZIKV-elicited Abs are able to induce ADE of DENV infection *in vitro* and *in vivo* (68–70). This finding underscores the potential dangers of vaccinating DENV-immune individuals with a ZIKV

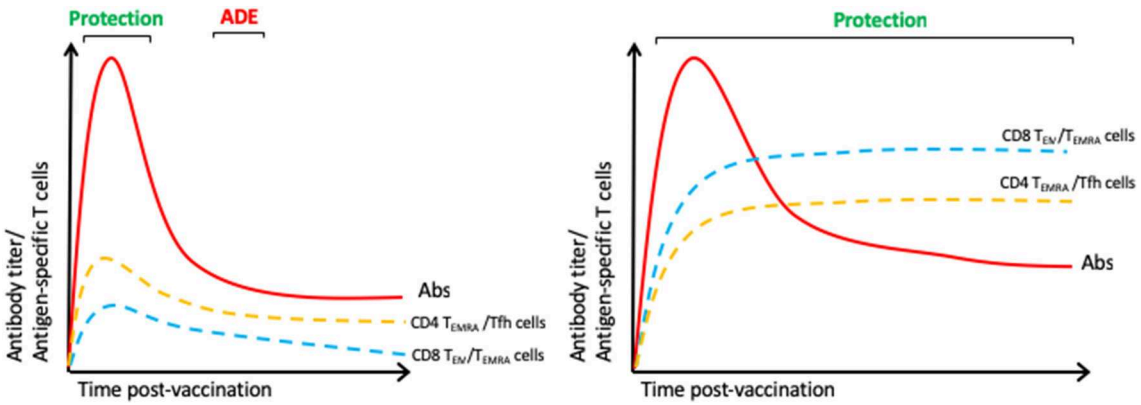

FIGURE 2 | Immune responses of an ideal DENV/ZIKV vaccine. We hypothesize that the licensed DENV vaccine elicits suboptimal antibody responses and inefficient $CD4^+$ and $CD8^+$ T cell responses **(Left)** and that an ideal vaccine should induce balanced Ab, $CD8^+$ (T_{EM}/T_{EMRA}) and $CD4^+$ (Th1, CTL, and Tfh) T cell responses **(Right)**.

vaccine that induces only an Ab response. In contrast, DENV-elicited cross-reactive $CD8^+$ T cells were able to protect against ZIKV in virgin and pregnant mice (37–39). There are direct evidences showing that DENV or ZIKV protein/epitopes induced protection in mice via T cells. Costa et al. showed that Balb/c mice vaccinated with DNA vaccines based on full-length or helicase domain NS3 of DENV2 are protected against lethal challenge (71). Similarly, we showed protection mediated by $CD8^+$ T cells in AG129 and wild-type mice vaccinated with VRP expressing the DENV2-E protein ectodomain (DENV2 E85-VRP) (72). In this study, $CD8^+$ T cells from vaccinated mice significantly contribute to the reduction of viral RNA in tissues (72). Vaccination of human relevant peptides HLA-restricted, identified as immunodominant epitopes, contribute to the reduction of DENV/ZIKV viral burden (37, 49). We demonstrated that peptide immunization of HLAB*0702 and HLAA*0101-restricted epitopes contribute to protection. Based on studies implicating an important role for cross-reactive T cells in mediating protection against DENV and ZIKV, a DENV/ZIKV vaccine could be designed to induce balanced Ab, $CD4^+$ Tfh, $CD4^+$ Th1, and $CD8^+$ T cell responses in order to confer long-lived protection that is mediated by both humoral and cellular immunity (**Figure 2**).

In conclusion, the studies reviewed here strongly support a key role for T cells in protecting against DENV and ZIKV infections. Comprehensive studies that examine the T cell responses in humans at several time points during acute DENV/ZIKV infections and include patients exhibiting a wide spectrum of clinical disease manifestations (including no symptoms), infection history, ethnicity, and geographic location now need to be conducted. Knowledge gained from these studies will provide insights into the design of safe and effective DENV and ZIKV vaccines that elicit balanced Ab and T cell responses. Several studies have already shown that DENV/ZIKV proteomes contain cross-reactive immunogenic epitopes that can elicit polyfunctional effector T cell responses. Thus, we propose that pan-flavivirus vaccine candidates that take into consideration of such epitopes should be designed to solve the global problem associated with genetic and antigenic similarity and co-circulation of DENV and ZIKV.

AUTHOR CONTRIBUTIONS

AEN wrote the review, and SS edited the review drafts.

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Longitudinal Analysis of Memory B and T Cell Responses to Dengue Virus in a 5-Year Prospective Cohort Study in Thailand

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Prior exposure to dengue virus (DENV) has a profound impact on the outcome of infection, which varies according to the interval between infections. Antibodies secreted by B cells and cytokines secreted by T cells are thought to contribute both to protective immunity against DENV and the pathogenesis of dengue disease. We analyzed peripheral blood mononuclear cells (PBMC) collected from Thai children over a 5-year prospective cohort study to define the dynamics of DENV-specific memory B and T cell responses and the impact of symptomatic or subclinical DENV infections. To measure B cell responses, PBMC were stimulated with IL-2 plus R848 and culture supernatants were tested for DENV-binding antibodies by ELISA. To measure T cell responses, PBMC were stimulated in dual-color ELISPOT assays with overlapping peptide pools of structural and non-structural proteins from the four DENV types. B cell responses were low to one or more DENV types prior to symptomatic infection and increased with reactivity to all four types after infection. Subjects who had a subclinical infection or who did not experience a DENV infection during the study period showed strong memory B cell responses to all four DENV types. T cell responses to DENV peptides demonstrated a cytokine hierarchy of IFN-γ > IL-2 > IFN-γ/IL-2. T cell responses were low or absent prior to secondary infections. The trends in T cell responses to DENV peptides over 3 year post-infection were highly variable, but subjects who had experienced a secondary DENV1 infection showed higher cytokine responses compared to subjects who had experienced a secondary DENV2 or subclinical infection. The longitudinal nature of our study demonstrates persistent memory B cell responses over years and a lasting but variable impact of secondary DENV infection on DENV-specific T cell responses.

Keywords: dengue, T cells, cytokines, IFN-γ, IL-2, B cells, antibodies, ELISPOT

INTRODUCTION

Immune memory is the hallmark of the adaptive immune response. The generation and persistence of memory B and T cells are key to protection against a subsequent infection with a pathogen and are the principles behind effective vaccination. Most of our understanding of durable adaptive immune responses to viruses comes from mouse models where pathogens are administered in germ-free environments under controlled conditions. Studies in mice, however, do not recapitulate the exposure in humans to multiple pathogens or even different strains of the same pathogen over time.

Dengue is the most important arthropod-borne viral disease caused by infection with any of the four dengue virus serotypes (DENV1-4) (1, 2). Most infections are subclinical but in some cases lead to clinical symptoms ranging from mild febrile illness (dengue fever, DF) to the life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (3). A primary infection with any of the four DENV serotypes elicits long-lasting homotypic immunity (4, 5), although there are a few reported cases of homotypic reinfections (6–9). In endemic areas, periodic inapparent exposures to DENV may boost this DENV-specific immunity (10–12). However, infection with a different DENV serotype (heterotypic secondary infection) is associated with an increased risk for DHF/DSS (4, 5, 13). Both antibodies and T cells are hypothesized to contribute to this increased risk of severe disease through a variety of mechanisms, although other data point to their ability to protect against severe DENV disease (14–20). Furthermore, evidence for a lower risk of severe disease during third or fourth DENV infections suggests that the cumulative effects of cross-reactive memory immune responses are beneficial (21–23).

The degree to which B and T cell immune responses remain stable or fluctuate over time after DENV infection may influence the balance between protective and detrimental effects of DENV-specific immune responses, but remains relatively poorly characterized. In a prospective cohort study conducted in Kamphaeng Phet province in north-central Thailand, we collected peripheral blood mononuclear cells (PBMC) over a 5-year period from children who were followed for the occurrence of DENV infections. These samples provided the opportunity to analyze the dynamics, magnitude, and durability of memory B and T cell responses to DENV over an extended period. We found that both B and T cell responses were low before symptomatic infection and increased after infection, albeit with different kinetics. In contrast, PBMC from subjects who did not experience a DENV infection during the 5-year study period had higher antibody titers at baseline and maintained broadly reactive memory B cell responses over the 5-year period. Our data demonstrate persistent memory B cell responses over years and a lasting but variable impact of secondary DENV infection on DENV-specific T cell responses.

MATERIALS AND METHODS

Study Subjects

Subjects were selected from a 5-year prospective cohort study in Thailand that has been described elsewhere (24). In brief, blood samples from schoolchildren were collected during January of every year from 1998 to 2002. PBMC were cryopreserved in liquid nitrogen until used. In addition, sera were collected in June, August, and November of each year. Surveillance for school absences and febrile illnesses was conducted between June and November each year; acute and convalescent blood samples were collected from children with fever or history of fever. Symptomatic DENV infection was identified by febrile illness with laboratory confirmation of acute DENV infection defined by virus isolation and/or reverse transcriptase polymerase chain reaction (RT-PCR) in acute serum samples or seroconversion between acute and convalescent serum samples by IgM/IgG ELISA or hemagglutination inhibition (HAI) assay. Subclinical infection was defined by the absence of symptoms and $\geq$4-fold increase in HAI antibody titer from June–August or August–November. Acute primary or secondary DENV infection were distinguished by IgM/IgG ratio and HAI titers as previously described (24, 25). Subjects who consistently had no detectable antibody or stable (<4-fold change) antibody titer by HAI assay over the 5-year period were defined as having had no DENV infection during the study. Written informed consent was obtained from each subject or his/her parent or guardian. The study protocol was approved by the Institutional Review Board of the Thailand Ministry of Public Health, the Human Use Review and Regulatory Agency of the Office of the U.S Army Surgeon General and the Institutional Review Board of the University of Massachusetts School of Medicine.

Detection of Memory B Cell-Derived Total IgG, DENV-Specific IgG, and DENV-Specific IgG Subclasses

Approximately 1–2 $\times$ 10^6 PBMC were stimulated with 2.5 μg/ml of R848 (Invitrogen, San Diego, CA, USA) and 1,000 U/ml of IL-2 (Peprotech, Rocky Hill, NJ, USA) in a 48 well plate for 7 days at 37°C and 5% CO_2. Culture supernatants were tested for total IgG by Human IgG ELISA Quantitation Set according to the manufacturer's instructions (No. E80-104, Bethyl Laboratories, Inc., Montgomery, TX, USA). Total IgG levels in the supernatants from stimulated PBMC ranged from 4.21 to 32.73 μg/ml (mean 16.92 μg/ml). Samples with a concentration below the limit of detection (1.56 μg/ml) were excluded. DENV-specific IgG and the four IgG subclasses (IgG1-4) were detected in the supernatant by ELISA. Briefly, 96 well plates were coated overnight with 10 ng/well of DENV1-4 virus-like particles (VLPs) (Native Antigen Company). The plates were blocked for 90 min with 1% BSA. The diluted culture supernatant was added to the wells for 60 min. Plates were washed and goat anti-human IgG coupled to HRP (A80-104P; Bethyl Laboratories Inc., Montgomery, TX, USA) was added for total DENV-specific IgG detection. HRP conjugated secondary antibodies for IgG subclasses detection include goat anti-human IgG1 FC (9054-05), IgG1 hinge (9052-05), IgG2 FC (9060-05), IgG3 hinge (9210-05), and IgG4 FC (9200-05); all were from Southern Biotechnology Associates, Birmingham, AL, USA. The assay was developed with TMB substrate (34021; Thermo Scientific, MA, USA), stopped with 1M HCL, and read at 450 nm.

Peptide Pools

To evaluate memory T cell responses, we used four peptide pools spanning the prM and E proteins from DENV1-4, four peptide pools spanning NS1, NS3, and NS5 proteins (NSA pools) from DENV1-4, and one peptide pool spanning NS2a/b, NS4a/b, and C protein from DENV2 (NSB pool). Peptide pools contained peptides that ranged in length from 12 to 20 amino acids (aa), in overlap from 10 to 14 aa. The total number of peptides per pool ranged from 88 to 323 aa (**Table S1**). Peptides were obtained from NIH Biodefense & Emerging Infections Research Resources Repository (BEI Resources, Bethesda, MD, USA) and Peptide technologies (JPT, Acton, MA, USA). Further details of the peptides are available at http://www.beiresources.org/.

Ex vivo Dual Color IL-2 and IFN-γ Enzymatic ELISPOT Assay

The ELISPOT assay was performed according to the manufacturer's instructions (CTL, Cleveland, OH, USA). Cryopreserved PBMC were thawed and plated at a density of 1–2 $\times$ 10^5 cells/well. Peptide pools were added at a final concentration of 2 μg/ml/peptide. As a positive control, PBMC were incubated with anti-CD3 and anti-CD28 antibodies at final concentrations of 1 and 0.1 μg/ml, respectively. As a negative control, PBMC were incubated with medium. PBMC were stimulated for 45 h at 37°C with 5% CO_2. As a positive control, every plate had PBMC from a well-characterized DENV-immune subject tested with the same conditions. The number of spots per well was determined using an automated ELISPOT reader (S5UV analyzer, CTL, Cleveland, OH, USA) with the double color software. Determinations from duplicate wells were averaged. Data were analyzed by subtracting the mean number of spots in the wells with cells and medium-only from the mean counts of spots in wells with cells and antigen and expressed as spot-forming cells (SFC) per 10^6 PBMC. If the response to anti-CD3/CD28 antibodies was below 500 IFN-γ SFC per million PBMC, the sample was excluded.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software V 8.00 (GraphPad Software Inc., La Jolla, CA). The non-parametric Mann-Whitney U or Wilcoxon signed rank test was used to compare two groups as appropriate. Statistical significance was set at $P < 0.05$.

RESULTS

Characteristics of the Study Population

PBMC from 27 subjects were selected for study based on available data from 5-years of follow-up (**Table S2**). Sixteen subjects were selected for B cell assays: six who had symptomatic DENV infection, four who had a subclinical infection and six subjects with no DENV infection. Twenty-five subjects were selected for T cell assays: 13 who had a symptomatic secondary DENV infection (six with DENV1, six with DENV2, and one without a serotype determined), six who had a subclinical infection, and six with no DENV infection. For the symptomatic and subclinical infection groups, we selected subjects who experienced an infection in the first or second year of the study to allow us to test PBMC collected 1–2 years before and 3–4 years after secondary DENV infection. HAI antibody titers to the four DENV serotypes at baseline (start of the study) were higher in the subclinical and no DENV infection groups compared to the symptomatic DENV1 and DENV2 groups ($p < 0.05$) (**Figure S1**).

Memory B Cell Responses to DENV

To study the dynamics, magnitude, and durability of the memory B cell (MBC) responses to DENV, PBMC from 16 subjects were stimulated with IL-2 plus R848. Dengue-specific IgG antibodies were assessed in supernatants of stimulated PBMC by ELISA using VLPs from each of the four DENV serotypes (**Figures 1A–C**). A total of 74 PBMC samples were analyzed. DENV-specific MBC responses were low to the infecting serotype prior to secondary DENV infection in hospitalized subjects 7, 13, 14, and 15 while they were high to all four serotypes in two subjects who were not hospitalized (**Figure 1A**, **Figure S2A**). For some subjects, MBC responses were low to one or more additional DENV serotypes. MBC responses increased in the first year after infection, with cross-reactivity to all four serotypes suggesting an expansion of DENV-cross-reactive B cells (**Figure 1A**, **Figure S2**). The increased MBC response was maintained in PBMC from two of four hospitalized individuals with symptomatic infection (subjects 13 and 14) for at least 3–4 years after infection (**Figure 1A**). In contrast, MBC responses to all four DENV serotypes were high and persisted over the 5 years in 2 of 4 subjects with subclinical infection and 5 of 6 subjects studied who did not experience a DENV infection over the 5-year period of observation (**Figures 1B,C**). Serial dilution of supernatants did not reveal preferential binding to a specific DENV serotype (**Figure 1D**, **Figure S2**). DENV-specific antibodies detected in MBC culture supernatants were predominantly of the IgG1 subclass (**Figures 1E,F**). IgG subclass-specific responses did not differ between subjects with symptomatic vs. no DENV infection.

Memory T Cell Responses to DENV

To assess the dynamics, magnitude, and durability of the memory T cell responses to DENV, 106 PBMC samples from 25 subjects were stimulated with peptide pools of structural and non-structural proteins. DENV-specific memory T cells producing IFN-γ, IL-2, or IFN-γ/IL-2 were determined by dual color ELISPOT. Because of the limited number of cells available, PBMC from subjects who experienced a symptomatic DENV1 or DENV2 infection were stimulated with DENV1 or DENV2 prM/E peptides, respectively, in addition to all non-structural peptide pools. PBMC from subjects with a subclinical or no DENV infection were stimulated with prM/E peptides from all four DENV serotypes in addition to the non-structural peptide pools (**Table S1**).

Overall, T cell responses to DENV peptide pools demonstrated a hierarchy of cytokine production of IFN-γ > IL-2 > IFN-γ/IL-2 in all subjects (**Figure S3**). DENV-specific IFN-γ responses were low or absent prior to symptomatic DENV

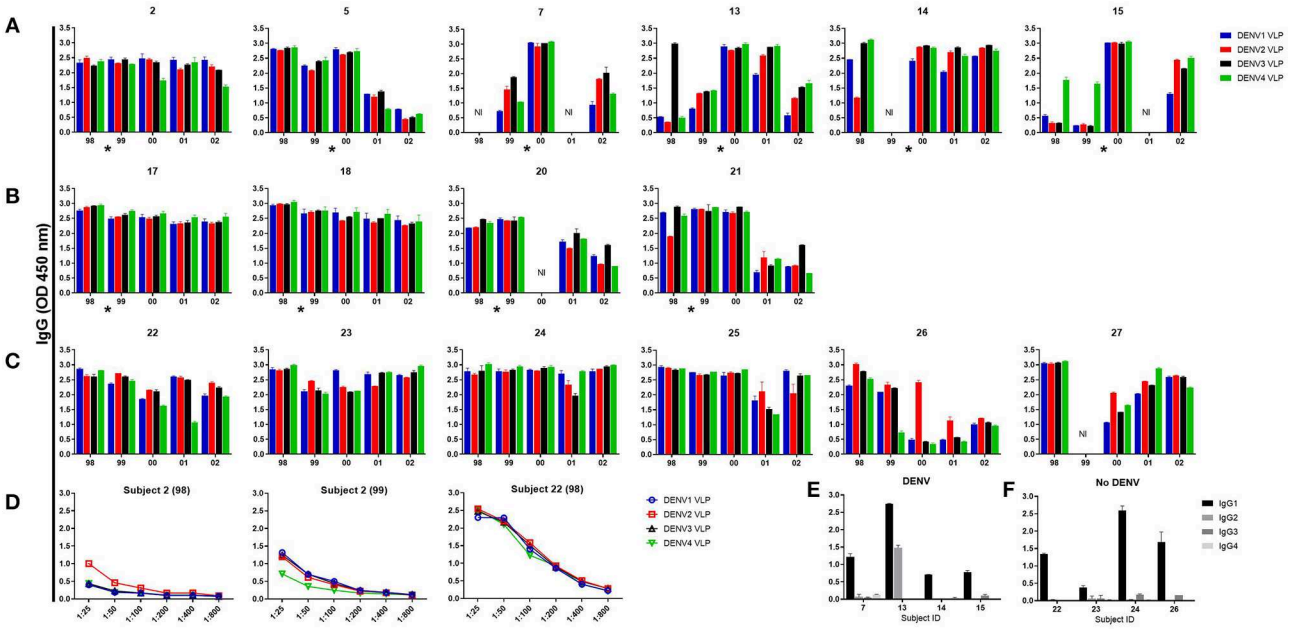

FIGURE 1 | Memory B cell responses to DENV. Peripheral blood mononuclear cells from 6 subjects with symptomatic secondary DENV infection (subjects 2 and 5 were not hospitalized, subjects 7, 13, 14, and 15 were hospitalized) **(A)**, 4 subjects with subclinical infection **(B)**, and 6 subjects with no DENV infection **(C)** were cultured with R848 plus IL-2 for 7 days and supernatants were tested for DENV-specific IgG Abs. Representative data of supernatant dilution from a subject with symptomatic and no DENV infection **(D)**. DENV-specific IgG isotypes in supernatants from the year 2000 in 4 subjects with DENV and 4 subjects with no DENV, respectively **(E,F)**. Bars represent mean with standard error of the mean of the OD values. The asterisk indicates the approximate time when DENV infection occurred. NI, not included.

infection. Responses increased after infection in most of the subjects (**Figure 2**, **Figure S4**). However, the peak frequency of DENV-specific IFN-γ-producing memory T cells was not uniformly detected in the first sample collected after infection. Overall, the difference in magnitude of DENV-specific IFN-γ responses before vs. after infection did not reach statistical significance. DENV-specific T cells were somewhat more stable over the 5-year period in subjects who did not experience a DENV infection (**Figure 2**).

DENV-specific IFN-γ responses in PBMC collected in the first year of the study (baseline responses) reflect immune responses potentially predictive of outcome. There were no significant differences in baseline DENV-specific IFN-γ responses between subjects who subsequently had a symptomatic infection vs. those who had a subclinical infection. However, baseline responses tended to be higher in subjects who did not experience a DENV infection compared to the other groups (symptomatic or subclinical DENV infection; **Figure S5**).

DENV-specific IFN-γ responses in PBMC collected in later years of the study assess the effects of secondary DENV infection on memory T cell responses. There was a trend toward higher IFN-γ responses following symptomatic DENV1 infection compared to the other groups (symptomatic DENV2, subclinical or no DENV infection), but this did not reach statistical significance (**Figure 2**, **Figure S6**). As expected, PBMC collected post-infection showed IFN-γ responses to more than one DENV serotype (**Figure 2**).

DISCUSSION

We conducted a longitudinal analysis of memory B and T cell responses to DENV over a 5-year period in primary schoolchildren living in a dengue-endemic area. Subjects were chosen to elucidate the impacts of incident DENV infections, both clinical and subclinical, on DENV-specific immunological memory, to follow changes in DENV-specific immunological memory over 3+ years after infection, and to compare findings in subjects who had symptomatic, subclinical, or no DENV infections during the study period. Other studies have measured memory B and T cell responses to DENV, however, these have largely been cross-sectional studies of individuals following natural DENV infection or vaccination (19, 26–33). The longitudinal nature of our study, availability of PBMC samples both before and after DENV infection, and the inclusion of groups with different DENV experience led to several novel observations.

Our assay of memory B cell responses to DENV used ELISA to evaluate binding to virions by antibodies secreted from circulating memory B cells after *in vitro* stimulation. Responses were detected in PBMC collected prior to an incident DENV infection. This finding is consistent with the known high baseline seroprevalence in this population and supports the secondary nature of most infections in our cohort even when titers of HAI and neutralizing antibody were undetectable (15, 34, 35). These samples also showed lower responses to one or more serotype(s),

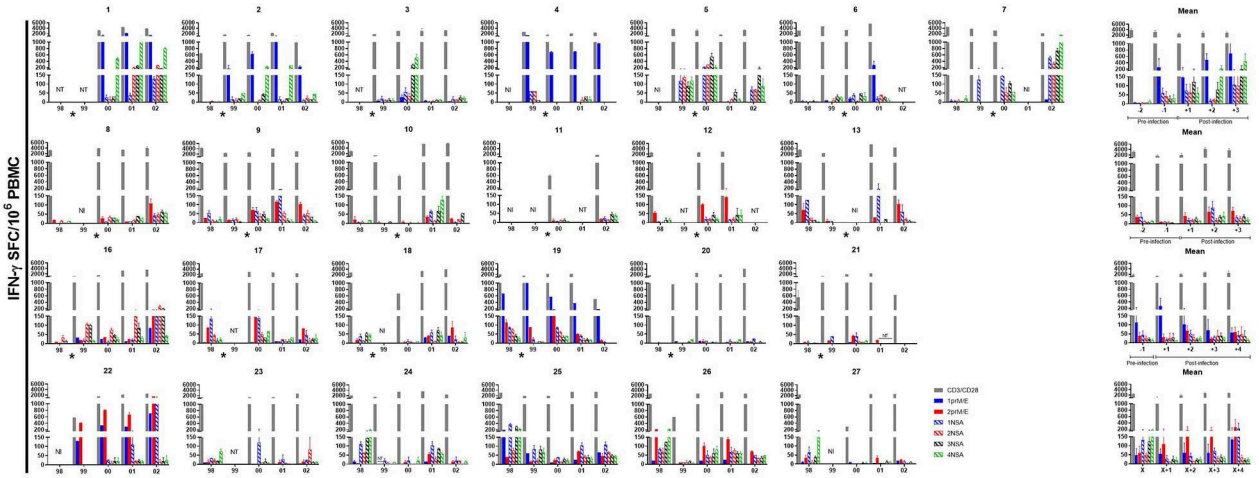

FIGURE 2 | IFN-γ ELISPOT responses in individual subjects. DENV peptide-specific interferon γ (IFN-γ) spot-forming cells (SFC) were determined by dual color ELISPOT in PBMC collected at the indicated time points. PBMC were collected at the beginning of each year. Subjects who experienced a **(A)** DENV1 infection **(B)** DENV2 infection **(C)** subclinical dengue infection and **(D)** no dengue infection over the 5-year period. Bars represent mean with standard error of the mean for duplicate wells. The asterisk indicates the approximate time when DENV infection occurred. NT, not tested; NI, not included.

which in each case included the serotype that caused the next infection. This finding suggests that assays of memory B cells might help to assess susceptibility to infection among DENV-experienced individuals, which has been difficult to define based on serum assays (14, 36). The high responses to all four serotypes in study subjects who did not experience a DENV infection may therefore reflect protective immunity in those subjects (37).

Following an incident DENV infection, memory B cell responses increased in magnitude and breadth, consistent with a model wherein serotype-cross-reactive memory B cells are preferentially expanded by sequential DENV infections (32, 33, 38). However, while memory B cell responses increased in the earliest blood sample collected after infection, there was substantial heterogeneity in the durability of this increase between study subjects and, for each subject, across viral serotypes. Human memory B cells have been detected decades after infection or vaccination, but are less durable for some vaccines and pathogens (39–44). Our data imply an important role of subject-specific and cell-intrinsic factors in the durability of DENV-specific memory B cells (44). Further studies are needed to define these factors and their relationship to susceptibility or resistance to subsequent DENV infection or disease.

We measured memory T cell responses to DENV using dual IFN-γ/IL-2 ELISPOT assays and stimulation with overlapping peptide pools. In contrast to memory B cell responses, we did not detect memory T cell responses in all subjects. Whereas, some studies of adult volunteers after natural DENV infection or administration of live virus vaccine candidates have reported DENV-specific memory T cell responses in nearly all subjects (26, 45, 46), our results are in line with other cross-sectional studies of dengue-endemic populations (15, 16, 19, 27, 29, 47–50). Other factors that may contribute to lower memory T cell responses in our study include the young age of our subjects, the number of prior exposures to DENV, and other endemic infections (51, 52). Memory T cell responses were generally lower in those subjects who experienced a symptomatic DENV infection, as we observed for memory B cells. There have been very few prospective studies of DENV-specific memory T cell responses in PBMC collected prior to secondary DENV infection (15, 49, 53). An inverse relationship was previously found in our cohort between DENV-specific T cell responses measured by intracellular staining for IFN-γ or IL-2 and symptomatic DENV infection (15). Others reported an inverse correlation between DENV-specific IFN-γ ELISPOT responses and HLA class I-related susceptibility to dengue (16).

Memory T cell responses to DENV were higher in the PBMC collected after an incident DENV infection, but these responses were quite variable over a 3-year period, and responses were not consistently highest in the samples collected closest in time to the infection. Previous studies have either been cross-sectional in nature (26, 27, 29, 30) or covered a short time period (46, 47, 54), and therefore are not directly comparable to our findings. It is possible that measurement of other T cell functions would have reflected different trends in cell frequency (55–57). Given ongoing DENV transmission in the study area throughout the period of observation, it is also possible that some subjects experienced additional exposures to DENV that modulated T cell frequency but were not detected as significant increases in antibody titer.

Our findings must be interpreted in the context of several limitations of our study design. The quantity of PBMC available from the small blood volumes collected from young children was a major consideration for assay design. As a result, we could not test all antigen specificities or effector functions of memory B and T lymphocytes. Antibody responses to NS1 (58), T cell responses to other structural and non-structural antigens (59, 60), and multiparameter single-cell analyses (46, 61) would

be of interest and might modify some of our conclusions. We used pools containing a large number of overlapping peptides in T cell ELISPOT assays, which has been reported to be suboptimal for detection of responses (19, 46, 62–64). We studied memory T and B cell responses in PBMC; lymphocyte repertoires in blood and tissue have been shown to differ in some infections, such as influenza (65). As DENV causes systemic infection, the most relevant tissues have not been defined, and most other studies of the immune repertoire have utilized PBMC. The narrow criteria we used for selection of cohort subjects limited the number available for these studies and reduced the statistical power for comparisons between groups. Our cohort study design along with these criteria also restricted the age range, host genetics, and geographic distribution of study subjects, which is likely to have limited the diversity of prior DENV infection history (66). Comparable studies in other populations will be needed to determine the generalizability of our findings.

Overall, this longitudinal analysis of memory B and T cell responses to DENV and the impacts of intervening symptomatic and subclinical DENV infections supports the paradigm of selective expansion of DENV-specific immunological memory by sequential exposures, but reveals additional heterogeneity between and within individual subjects than could be ascertained from cross-sectional studies. This heterogeneity should be considered in the interpretation of data from cohort studies of natural DENV infection and clinical trials of dengue vaccines. We suggest that further studies are warranted to evaluate the associations with susceptibility vs. resistance to DENV infection and disease.

ETHICS STATEMENT

Written informed consent was obtained from each subject or his/her parent or guardian. The study protocol was approved by the Institutional Review Board of the Thailand Ministry of Public Health, the Human Use Review and Regulatory Agency of the Office of the U.S Army Surgeon General and the Institutional Review Board of the University of Massachusetts School of Medicine.

AUTHOR CONTRIBUTIONS

LS-V, AS, AM, and AR conceived and designed the experiments and wrote the manuscript text. LS-V, SK, and MS performed experiments and prepared figures. LS-V conducted statistical analyses. AS, KA, DE, and TE supervised the clinical study, subject enrollment, and collection of clinical data and blood samples. AS, KA, and DE contributed to the analysis of clinical, virologic, and serologic data. JC contributed to the analysis of immunological data. All authors contributed to the final manuscript and agree with the results and conclusions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01359/full#supplementary-material

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A Modified mRNA Vaccine Targeting Immunodominant NS Epitopes Protects Against Dengue Virus Infection in HLA Class I Transgenic Mice

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Dengue virus (DENV) induces strong T and B cell responses upon infection. Hence, it is difficult to determine the contribution of cell-mediated immunity alone in the long lasting protection against DENV infection and disease. Numerous CD4+ and CD8+ T cell epitopes have been identified, mainly in the non-structural proteins of DENV. Taking into account the immunogenicity and peptide sequence conservation among the different DENV serotypes, a minimal DENV antigen, called DENV1-NS, has been designed. This antigen is enriched in conserved and highly antigenic epitopes located in the NS3, NS4B, and NS5 regions of DENV1. To evaluate the ability of the DENV1-NS poly-epitope to express the antigenic peptides in the context of different HLA class I molecules, we established its *in vivo* immunogenicity by measuring, after DNA immunization and electroporation, the activation of DENV-specific CD8 T cells in transgenic mice expressing the human HLA-A*0201, -A*2402, -B*0702, and -B*3502 class I alleles. We then engineered a lipid nanoparticle (LNP) encapsulated modified mRNA vaccine encoding DENV1-NS and tested immunogenicity and protection in these human HLA class I transgenic mice, after transient blockade of the interferon (IFN) type I receptor. Significant protection was observed, after two injections of the mRNA vaccine. Collectively, these data strongly support the development of T cell-based vaccines targeting immunodominant T cell epitopes that generate potent virus-specific T cell responses conferring immunity against DENV infection.

Keywords: dengue virus (DENV), T cells, vaccine, chimeric vaccine, DNA vaccine, NS epitopes, human HLA transgenic mice

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Dengue virus (DENV) is an arthropod-borne virus transmitted to humans by the mosquito spp., *Aedes aegypti,* and *Aedes albopictus*. Dengue has become one of the most important global public health threats in recent decades, with a global estimate of 3.6 billion people at risk of infection (1). Four DENV (DENV1-4) serotypes co-circulate and are endemic to many tropical and sub-tropical countries, causing 400 million new infections every year, of which 100 million cases are symptomatic, ranging from a self-limiting febrile illness named dengue fever (DF) to more severe life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Over 2 million DHF/DSS cases, 500,000 hospitalizations and 25,000 deaths are estimated to occur every year, primarily among children (2). Due to uncontrolled urbanization, increased human migration and the spread of DENV-transmitting mosquitoes, the frequency of epidemics has been constantly growing since the 1960s and the global distribution of dengue has extensively spread.

Dengue fever is characterized by fever with headache, retro-orbital pain, myalgia, arthralgia, rash, abdominal pain, and a viremia that begins 3–4 days following a bite by an infectious mosquito. As the fever subsides at days 4–6 of illness, a fraction of patients develop DHF/DSS characterized by thrombocytopenia, hemorrhagic manifestations, and signs of plasma leakage, which can lead to hypovolemic shock, organ failure and, without appropriate treatment, death (3). Strikingly, while a primary infection with one DENV serotype can induce long-term immunity to homotypic DENV, a subsequent infection by heterotypic serotypes increase the risk of developing severe dengue, a phenomenon postulated to be due to non-neutralizing or sub-neutralizing antibodies. Here, in a process called antibody-dependent enhancement (ADE) serotype-crossreactive immune complexes lead to enhanced infection of FcγR bearing immune cells and/or an increased inflammatory response (4, 5). While type-specific neutralizing responses were initially thought to elicit long-lasting immunity to the primary infecting DENV serotype (6, 7), several cases of symptomatic diseases have been observed following reinfections with the same serotype, which depend on the concentration of pre-existing anti-DENV antibodies (8, 9). Moreover, a strong correlation has been established between the risk to develop severe dengue disease and a specific range of pre-existing anti-DENV antibody titers (10, 11). Thus, both the quality and the quantity of neutralizing anti-DENV antibodies play a crucial role in neutralizing and protecting against dengue infection and disease.

Similarly to the ADE phenomenon, it was proposed, in a scenario termed "original antigenic sin," that T cells which are primed after a primary DENV infection, can nevertheless undergo clonal expansion upon secondary heterotypic infection, and hinder the specific T cell response against the infecting serotype, making the elimination of DENV-infected cells less efficient (12, 13). These cross-reactive T cells, when stimulated by secondary and heterotypic DENV infection, display an altered cytokine profile, with higher ratios of Tumor Necrosis Factor (TNF) to Interferon gamma (IFN-γ) producing CD4 T cells (14), and high cytokine production but suboptimal degranulation or impaired IFN-γ production (15, 16). However, in spite of these studies, direct evidence for a role of cross-reactive T cells in the pathogenesis of severe dengue disease is lacking, and in fact, recent data strongly support a protective role for serotype-specific and cross-reactive T cells against DENV infection (17). Among the arguments supporting a beneficial role of T cells, a strong correlation was established between the protection against severe dengue, the expression of certain Human Leukocyte Antigen (HLA) alleles and a polyclonal memory $CD8^+$ T cell response with a high magnitude in healthy dengue-immune individuals (18–24). Likewise, in a recent study from Cambodian children, a role for T cells in protection from clinical dengue was further supported, with a higher activation of Natural Killer (NK) cells and T cells observed in strictly asymptomatic dengue-infected individuals, compared to clinical dengue patients, whereas the up-regulation of gene expression pathways leading to plasmablast development and the secretion of DENV-specific antibodies correlated with the development of clinical dengue (25).

In humans, both CD4 and CD8 T cells contribute to protection against DENV, with CD4 T cells mainly targeting structural proteins Capsid (C) and Envelope (E) and the non-structural protein NS1, and CD8 T cells preferentially targeting non-structural proteins NS3, NS4B, and NS5 (24, 26–29). Given the expression of sub-neutralizing or cross-reactive antibodies and the enhanced risk of severe dengue following vaccination among seronegative vaccine recipients (30) or after multiple natural infection episodes, a vaccine candidate has been designed on its ability to induce a strong T cell response. This vaccine, called DENV1-NS, is composed of the most immunogenic regions of NS3, NS4B, and NS5. DNA immunization with a plasmid encoding DENV1-NS in mice expressing different human HLA class I molecules confirmed the induction of a strong CD8 T cell response against peptides derived from these NS regions. Using this strategy, and with the intention to develop an effective T cell-based vaccine, we report here that a prime-boost immunization of human HLA class I transgenic mice with low dose of a modified mRNA encoding DENV1-NS induces a strong T cell immunity, with a significant protection against DENV1 infection, after transient blockade of the IFN type I receptor (31–33), in the absence of neutralizing or sub-neutralizing anti-DENV antibodies. These data clearly demonstrate the validity of the approach using minimal NS-derived T cell epitopes in the induction of a protective immunity against dengue infection and disease.

MATERIALS AND METHODS

Mice and Infections

HLA-A*0201, -A*2402, -B*0702, and -B*3501 monochain transgenic/H-2 null mice on the C57BL/6 background (34–36) were bred in the Institut Pasteur facility and used between 6 and 10 week of age. All animals were intraperitoneally inoculated with 2 mg of anti-ifnar1 antibody (clone MAR1-5A3, Interchim, France) (33) 24 h prior to DENV inoculation. For all experiments, mice were infected by retro-orbital injection of 10^6 PFU DENV1 KDH0026A, in 200 µl PBS, then bled on days 1, 2, 3, and 6 after virus inoculation, for measurement of viremia and sacrificed on day 7 post-infection. All mouse experiments were performed

following Institutional animal care and use committee-approved animal protocols.

Design of the Nucleotide Sequence Encoding DENV1-NS Poly-Epitope

Given the identification of CD8+ T cell epitopes from previously infected donors (24, 28, 37), 4 regions have been selected in the non-structural proteins NS3, NS4B, and NS5, for a total of 540 amino acids: 2 regions in NS3 (185 and 134 amino acids, respectively), 1 region in NS4B (86 amino acids) and 1 region in NS5 (135 amino acids) (**Figure 1**). Based on the sampled genetic diversity of the 4 serotypes of DENV, and with the idea that T cell epitopes are either conserved among different serotypes, or are serotype-specific with nevertheless the ability to induce cross-reactive CD8+ T cell responses, a prototype consensus sequence based on epidemic strains of DENV1 has been selected, the DENV1-NS T cell poly-epitope (patent WO2015/197565, initially filled on June 23, 2014) (**Figure 1**). A total of 2,033 full-length DENV genome sequences (865 for serotype 1, 1,663 for serotype 2, 427 for serotype 3 and 63 for serotype 4) were aligned using MAFFT (38), with manual adjustments according to the amino acid sequence. Mean pairwise sequence identity was evaluated at each position intra- and inter-serotypes at the nucleic and the amino-acid levels. Analysis of the concatenated regions of interest revealed strong intra-serotype conservation, and generally a higher degree of sequence identity compared to the genome as a whole, including for inter-subtypes comparisons. A serotype 1 consensus sequence was selected, as it presented the highest average sequence identity with the 4 serotypes (vs. serotype 1: 99.48%; serotype 2: 83.48%; serotype 3: 89.39%; serotype 4: 76.95%). For DNA immunization, the optimized nucleotide sequence encoding DENV1-NS poly-epitope was cloned in the pcDNA3.1 plasmid, under the CMV promoter. For mRNA vaccination, a lipid nanoparticle (LNP) encapsulated modified mRNA vaccine encoding DENV1-NS was prepared as described previously (39).

Generation of Modified mRNA and LNP

Chemically modified mRNA was synthesized *in vitro* by T7 RNA polymerase-mediated transcription from a linearized DNA template containing an open reading frame flanked by a 5′ and 3′UTR and a polyA tail. Uridine was fully replaced with N1-methylpseudouridine, and Cap1 was utilized to increase mRNA translation efficiency.

Lipid nanoparticle formulations were generated as described previously (40). Briefly, lipid stocks were dissolved in ethanol at a molar ratio of 50:10:38.5:1.5 (ionizable lipid: helper lipid: structural lipid: PEG-lipid) and mixed with mRNA at a ratio of 3:1 (mRNA:lipid). mRNA-loaded nanoparticles were exchanged into final storage buffer and had final particle sizes of 80–100 nm, >80% encapsulation, and <10 EU/mL endotoxin.

Immunizations

Before immunization, mice were first anesthetized with a mix solution of xylazine 2% (Rompun, Bayer santé, Loos, France) and ketamine 8% (Imalgen 1000, Merial, Lyon, France) in PBS through the intraperitoneal (I.P.) route according to individual animal weight and duration of anesthesia. DNA immunization was performed following a prime boost administration regimen at 3-week interval by intradermal (I.D.) injection of plasmid DNA pcDNA3.1 encoding the DENV1-NS sequence (each injection consists of 2 simultaneous I.D. injections of 50 μg plasmid DNA in the lower back followed by *in vivo* electroporation) and spleen cells were harvested 10 days after the boost. The electroporation settings, using the AgilePulse apparatus (BTX, Harvard apparatus, MA, USA) consist of 3 Voltages groups: 450 V, with a pulse length of 50 microseconds, a pulse interval of 0.2 microseconds and 1 pulse; 450 V, with a pulse length of 50 microseconds, a pulse interval of 50 milliseconds and 1 pulse; and 110 V, with a pulse length of 10 milliseconds, a pulse interval of 20 milliseconds and 8 pulses. The immunization with mRNA was performed following a prime boost regimen at 3-week interval or 4-week interval, by intramuscular (I.M.) injection of LNP-based mRNA encoding the DENV1-NS sequence or irrelevant mRNA as a negative control (each injection consists of one I.M. injection of 10 or 2 μg mRNA vaccine encoding the DENV1-NS sequence or 10 μg irrelevant mRNA). For quantification of the T cell responses following mRNA immunization, the prime boost regimen was performed at 3-week interval, and spleen cells were harvested 7 days after the boost. For the evaluation of the vaccine efficacy, the prime boost regimen was performed at 4-week interval, and mice were challenged with the virus 4 weeks after the boost.

Viruses and Cell Lines

The *in vivo* and the *in vitro* assays were conducted using the DENV1 KDH0026A strain (provided by Dr. L. Lambrechts, Institut Pasteur, Paris). The DENV1 virus was grown using the Aedes Albopictus mosquito cells line C6/36 cultured in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum containing 0.1 mM non-essential amino acids and 1x tryptose phosphate broth. Vero-E6 cells were provided by Dr. M. Flamand (Institut Pasteur, Paris).

Quantification of Viral Loads

For assessment of viremia, blood was collected by retro-mandibular puncture in EDTA coated-microvette tubes (Fisher Scientific, Illkirch, France). After centrifugation, plasma samples from DENV-infected mice were extracted with the RNeasy Mini Kit (Qiagen). DENV RNA levels were determined by TaqMan one-step quantitative reverse transcriptase PCR (qRT-PCR) on a QuantStudio 12K Flex Real-time PCR system (Life Technologies) using standard cycling conditions. Viral burden is expressed on a $\log_{10}$ scale as viral RNA equivalents per milliliter after comparison with a standard curve produced using serial 10-fold dilutions of DENV1 RNA from known quantities of infectious virus. The following primer sets were used:

Forward: 5′-GGAAGGAGAAGGACTCCACA-3′;
Reverse: 5′-ATCCTTGTATCCCATCCGGCT-3′;
Probe: 5′-(FAM) CTCAGAGACATATCAAAGATTCCAGGG-3′ (MGB).

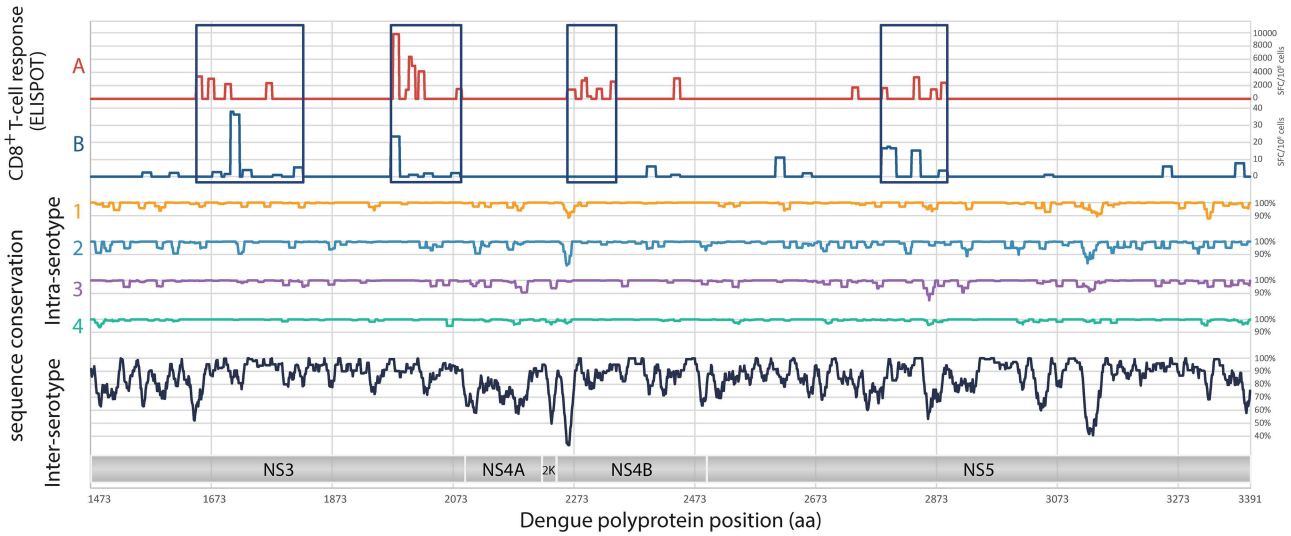

FIGURE 1 | Analysis of target regions for T cells and sequence conservation of non-structural proteins of DENV. Analyses of the magnitude of T cell responses, in SFC/10^6 cells **(A)**, from Weiskopf et al. (24), and in SFC/10^6 cells **(B)**, from Rivino et al. (28). Intra-serotypes and inter-serotypes sequence conservation are represented for DENV1 (yellow line), DENV2 (blue line), DENV3 (purple line), DENV4 (green line), and for all DENV serotypes (black lines), respectively.

Synthetic Peptides

All peptides were synthesized by Proimmune Ltd (Oxford, UK), as crude material, and were tested individually in Elispot assay and in intracellular cytokine staining for IFN-γ and TNF-α secretion. The peptides used in this study and corresponding to DENV1 sequences or to their DENV2, 3 or 4 variants are listed in **Table 1**.

IFN-γ ELISPOT Assay

Mouse spleen cells were collected and depleted of red blood cells and IFN-γ-producing splenocytes were quantified by ELISPOT assays after a 24 h period of stimulation with peptides, as already described (43). Briefly, 96-well nitrocellulose-backed plates (Multiscreen, Merck-Millipore, Molsheim, France) were coated with anti-mouse IFN-γ mAb at 5 μg/ml (BD Pharmingen, France) in 50 μl of 50 mM carbonate buffer (pH 9.6) overnight at 4°C. Wells were blocked with 200 μl of complete medium at room temperature for 2 h and then washed three times with serum-free medium. The coated wells were filled in quadruplicate with splenocytes from immunized mice in complete α-MEM medium with individual peptides at 2 μg/ml and incubated at 37°C for 20 h. After incubation, the wells were washed three times with PBS-0.05% Tween 20, and then incubated with 50 μl biotinylated anti-mouse IFN-γ (BD bioscience, France) at 1 μg/ml in PBS Tween for 1 h at room temperature. The spots were developed using streptavidin-alkaline phosphatase (Mabtech, Stockholm, Sweden) and BCIP/NTB substrate (Promega, Madison, MI, USA) and counted using an automated ELISPOT reader (Immunospot, Cellular Technology Limited, Cleveland, OH, USA). The number of IFN-γ-producing cells was expressed as spot-forming cells (SFC) relative to 1×10^6 spleen cells. Values were calculated by subtracting the number of spots detected in non-stimulated control wells. Values were considered positive if they were equal to or >20 spots and at least three times above the means of the unstimulated control wells. As positive wells, cells were stimulated with ConA at 10 μg/ml (Sigma Aldrich, Lyon, France).

Intracellular Cytokine Staining

Spleen cells from immunized mice (100 μl/well in 96 U-bottom plates) were incubated with individual peptides (adding 50 μl at 4 μg/ml for 2 h, and 50 μl Golgi Stop at 4 μl/ml for 4 h), in complete medium containing 10% FCS. Cells were then harvested, membrane stained using anti-mouse CD4-V500 (BD Biosciences), CD3-alexa488 (BD Biosciences), and CD8-PerCP (eBioscience) antibodies, and intracellular staining was performed using the intracellular staining kit (BD Biosciences) and anti-mouse IFN-γ-APC and TNF-α-eFluor 450 antibodies (eBioscience).

Virus Neutralization Assay

The quantification of neutralizing activity of antibodies against DENV was determined using a flow cytometry-based assay, as described previously (44). Briefly, 2-fold serial dilutions of plasma samples were incubated at 37°C for 1 h with a 1/200 dilution of virus inducing 15–20% infection. Virus - antibody mixture was then added to 5×10^4 Vero cells for 2 h at 37°C, after which cells were washed with fresh medium and incubated for 24 h at 37°C. Cells were then fixed with 4% paraformaldehyde, stained using the intracellular staining kit (BD Biosciences) with 4G2 antibody conjugated to Alexa Fluor™ 488, and the percentage of infected cells was measured by flow cytometry. Plasma samples from mice immunized with mRNA CT vaccine and from *ifnar*$^{-/-}$ mice injected

TABLE 1 | DENV Peptides and serotypes variants used in this study.

Peptide[a]	Sequence[b]	Serotype	NS region[c]	HLA restriction[d]	References[e]
p30	**RYLPAIVREAI**	DENV1, 2, 3	NS3.1	A*3101/**B*0702**/**A*0201**	(28) / (41)
p30 (DV4)	RILPSIVREAL	DENV4		B*0702/B*3501	IEDB
p36	**APTRVVASEM**	DENV1	NS3.1	**B*0702**	(41)
P36 (DV2, 3, 4)	APTRVVAAEM	DENV2, 3, 4		B*0702/B*3501	(28, 41) / (24)
p49	**TPEGIIPAL**	DENV1, 3	NS3.2	**B*3501**/B*0702	(24, 36) / (24)
p49 (DV2)	**TPEGIIPSM**	DENV2		**B*3501**/B*0702	(24)
p49 (DV4)	**TPEGIIPTL**	DENV4		**B*3501**/B*0702	(24)
p50	**LPVWLSYKVA**	DENV1, 4	NS3.2	**B*5301**/B*5101	(24)
p50 (DV2)	**LPVWLAYRVA**	DENV2		B*5101	(24)
p50 (DV3)	**LPVWLAHKVA**	DENV3		**B*3501**	(24)
p32	**QYSDRRWCF**	DENV1	NS3.2	**A*2402**	IEDB
p32 (DV2.1)	**NYADRRWCF**	DENV2		**A*2402**	(28)
p32 (DV2.2)	NYADRKWCF	DENV2		A*2402	IEDB
p32 (DV3)	KYTDRKWCF	DENV3		A*2402	IEDB
p32 (DV4)	SYKDREWCF	DENV4		A*2402	IEDB
p17	**LDARTYSDPLALREFKEF**	DENV1	NS3.2	B*3501	IEDB
p17 (DV2)	LDARIYSDPLALKEFKEF	DENV2		A*2402	(28, 29)
p17 (DV3)	LDARTYSDPLALKEFKEF	DENV3		B*3501	IEDB
p17 (DV4)	LDARVYADPMALKDFKEF	DENV4		B*3501	(24)
p21	**HPASAWTLY**	DENV1, 3	NS4B	**B*3501**	IEDB
p21 (DV2, 4)	RPASAWTLY	DENV2, 4		B*0702/B*3501	(41)
p51	**TLYAVATTI**	DENV1, 4	NS4B	A*0201	IEDB
p51 (DV2)	**TLYAVATTF**	DENV2		A*2402	IEDB
p51 (DV3)	**TLYAVATTV**	DENV3		A*0201	(42)
p33	**ITPMMRHTI**	DENV1	NS4B	**A*2402**	IEDB
p33 (DV2)	VTPMLRHSI	DENV2		B*0702	(41)
p33 (DV3)	ITPMLRHTI	DENV3		A*2402	IEDB
p33 (DV4)	LTPMLRHTI	DENV4		–	–
p56	**SMVNGVVKL**	DENV1, 4	NS5	**A*0201**	IEDB
p56 (DV2)	SMGNGVVRL	DENV2		A*0201	(42)
p56 (DV3)	SMINGVVKLL	DENV3		A*0201	IEDB
p15	**KPRICTREEF**	DENV1	NS5	**B*0702**	(24)
p15 (DV2)	TPRMCTREEF	DENV2		B*0702/B*3501	(24, 41) / (24)
p15 (DV3)	KPRLCTREEF	DENV3		B*0702	(24)
p15 (DV4)	NPRLCTREEF	DENV4		B*0702	(24)

[a] *peptides in bold are derived from the Poly DENV1-NS sequence used for immunization, and tested by Elispot.*

[b] *sequences in red are peptides variants corresponding to DENV2, 3, or 4 sequences, and sequences in bold are peptides tested in this study.*

[c] *The NS3.1, NS3.2, NS4B, and NS5 regions are derived from DENV serotype 1, serotype 2, serotype 3 and serotype 4 (Genbank accession number NP_059433.1, NP_056776.2, YP_001621843.1 and NP_073286.1, respectively).*

[d] *The HLA restriction was determined from responding human donors or human HLA transgenic mice, or predicted from the Immune Epitope Database and Analysis Resource (www.iedb.org), with a percentile rank <0.2. HLA restrictions are shown in bold when they were also identified in this study.*

[e] *Except a few peptides predicted from the Immune Epitope Database and Analysis Resource (www.iedb.org), all peptides used in this study were previously identified in humans or in HLA class I transgenic mice, using ELISpot assay.*

intravenously with 10^6 pfu DENV1 were used as negative and positive controls, respectively.

Statistical Analyses

Differences between 2 groups were evaluated using non-parametric Mann-Whitney U-test in the assessment of the immunogenicity and the immune protection or unpaired *t*-test in the evaluation of the expansion and the contraction phases of the T cell response. All computer analyses were performed using GraphPad Prism 7 (GraphPad Software Inc. La Jolla, CA, USA). $P < 0.05$ were considered statistically significant.

RESULTS

Immunogenicity of the DENV1-NS Poly-Epitope

Given the identification of $CD8^+$ T cell epitopes from human donors and to minimize the dilution of the immune response

observed following DENV infection, a minimal DENV antigen has been designed from conserved and highly antigenic T cell epitopes. Using compiled DENV T cell epitope distribution and strength (24, 28, 37, 41) and the consensus prediction method available through the IEDB Analysis Resource (available at: www.iedb.org), we have selected four regions from a DENV serotype 1 consensus that are enriched in CD8 epitopes: two regions in NS3, one in NS4B, and one in NS5 (**Figure 1**). The resulting DENV1-NS poly-epitope corresponds to the concatenation of amino acid positions 1650-1829, 1959-2092, 2262-2347, and 2766-2899 based on the reference sequence NP_059433. Within serotypes, pairwise identity along the genome ranged between 0.878 and 1 for DENV1, 0.816 and 1 for DENV2, 0.847 and 1 for DENV3 and 0.94 and 1 for DENV4.

To determine whether the DENV1-NS poly-epitope can be produced endogenously and then processed and presented in the context of human HLA class I molecules, we performed DNA-based immunization combined with electroporation (EP) in HLA-A*0201, -A*2402, -B*0702, and B*3501 monochain transgenic/H-2 null mice (34, 36), which are associated with low T cell responses (for the HLA-A*0201 and -A*2402 alleles) and high T cell responses (for the HLA-B*0702 and -B*3501 alleles) (24), and represent the most frequent alleles in Caucasoid, Oriental and Amerindian ethnic groups, from the allele frequency Net Database website (available at: *www.allelefrequencies.net*). The animals were immunized by intradermal (I.D.) injection of plasmid DNA expressing the DENV1-NS under a CMV promoter and the interferon (IFN)-γ response of spleen cells was quantified by Enzyme Linked Immunospot (ELISpot) assay against individual peptides. Among the potential epitopes expressed by DENV1-NS, and predicted from the Immune Epitope Database and Analysis Resource (www.iedb.org) to bind to HLA-A*0201, or shown in literature to induce a significant T cell response in the context of HLA-A*0201 molecules (41, 42, 45), 2 antigenic peptides were identified, which elicit a strong T cell response in the HLA-A*0201 transgenic mice: p30 and p56 located in the NS3.1 and the NS5 regions of the DENV1-NS, respectively (**Figure 2** and **Table 1**). In the immunized HLA-A*2402 transgenic mice, 3 peptides were also identified: p17 and p32 located in the NS3.2 region and p33 located in the NS4B region (**Figure 2**). These results confirm previous identification of HLA-A*0201- or HLA-A*2402-restricted epitopes, as targets for T cell responses in humans (16, 28, 29, 37, 46), or predicted to bind strongly these alleles (www.iedb.org). Finally, 3 and 4 peptides have been identified in HLA-B*0702 and HLA-B*3501 transgenic mice, respectively, with a higher number of antigenic peptides inducing a stronger T cell response in the HLA-B*3501 mice, in comparison with HLA-B*0702 transgenic mice (**Figure 2**). These data clearly show that the DENV1-NS poly-epitope is expressed and processed correctly, resulting in the presentation of antigenic peptides, which elicit a strong T cell response in the context of different HLA molecules. They also show that all the antigenic peptides identified in DENV1-NS correspond to T cell epitopes previously identified from human donors or from HLA transgenic mice infected with DENV.

An mRNA Vaccine Encoding DENV1-NS Poly-Epitope Induces Strong CD8 T Cell Activation

Intramuscular delivery of LNP with encapsulated modified mRNA revealed strong immunogenicity *in vivo* (39). Therefore, we inoculated HLA transgenic mice with the LNP-based mRNA vaccine encoding DENV1-NS. Eight to 10-week old HLA-B*3501 transgenic mice were divided into 3 groups, which received an intramuscular inoculation of 10 or 2 μg of the mRNA encoding DENV1-NS or 10 μg of an irrelevant mRNA as a negative control. After a booster immunization at day 28, spleen cells from immunized animals were harvested 8 days later, at day 36, and the frequency of CD8 T cells secreting IFN-γ and TNF-α and spleen cells producing IFN-γ was evaluated by intracellular staining and by ELISpot assay, respectively. Both assays were used in parallel, as they provide complementary information, in particular by allowing the identification of responding cells with intracellular cytokine measurement by flow cytometry, and the detection of small number of IFN-γ-producing cells in response to poorly immunogenic peptides with the ELISpot assay which is more sensitive. Flow cytometry analyses of responding CD8 T cells after 6 h *in vitro* stimulation with the HLA-B*3501-restricted peptides p49, p50, or p51 revealed different capacity of these peptides to stimulate CD8 T cells, with 26% of CD8 T cells producing IFN-γ and TNF-α- against p49, whereas only 0.85 and 0.06% of CD8 T cells produce both cytokines in response to p50 and p51, respectively (**Figure 3A**). As the amino acid sequence of the antigenic peptides may vary between the 4 DENV serotypes, with a few amino acid substitutions located in the HLA binding sites (anchor residues) (**Table 1**), we asked whether mice immunized with the DENV1-NS vaccine could recognize peptide variants, which are derived from heterotypic DENV serotypes. As shown in **Figures 3A,B**, both the p49 peptide (TPEGIIPAL for DENV1, 3) and its 2 serotype variants (TPEGIIPSM and TPEGIIPTL for DENV2 and DENV4, respectively) stimulate CD8 T cells to produce IFN-γ and TNF-α. We then compared the frequency of T cells responding to the DENV1-specific p50 and p51 peptides, with the frequency of cells responding to the serotype variants of these peptides, using the ELISpot assay, which is more sensitive than flow cytometry. Results show that while p50 and p51 induce a significant T cell response, none of the 2 DENV serotype variants of p50 (LPVWLAYRVA for DENV2 or LPVWLAHKVA for DENV3) stimulate T cells, and only 1 serotype variant of p51 (TLYAVATTF for DENV2) can stimulate T cells. Interestingly, the DENV2 variant of p51 is more potent in stimulating T cells than the DENV1-specific p51 peptide, both for the 10 μg and the 2 μg dose of DENV1-NS vaccine (**Figure 3B**). Altogether, these data show that immunization with mRNA vaccine encoding DENV1-NS induces a strong stimulation of CD8 T cells, which in some cases, could be highly cross-reactive against heterotypic peptides.

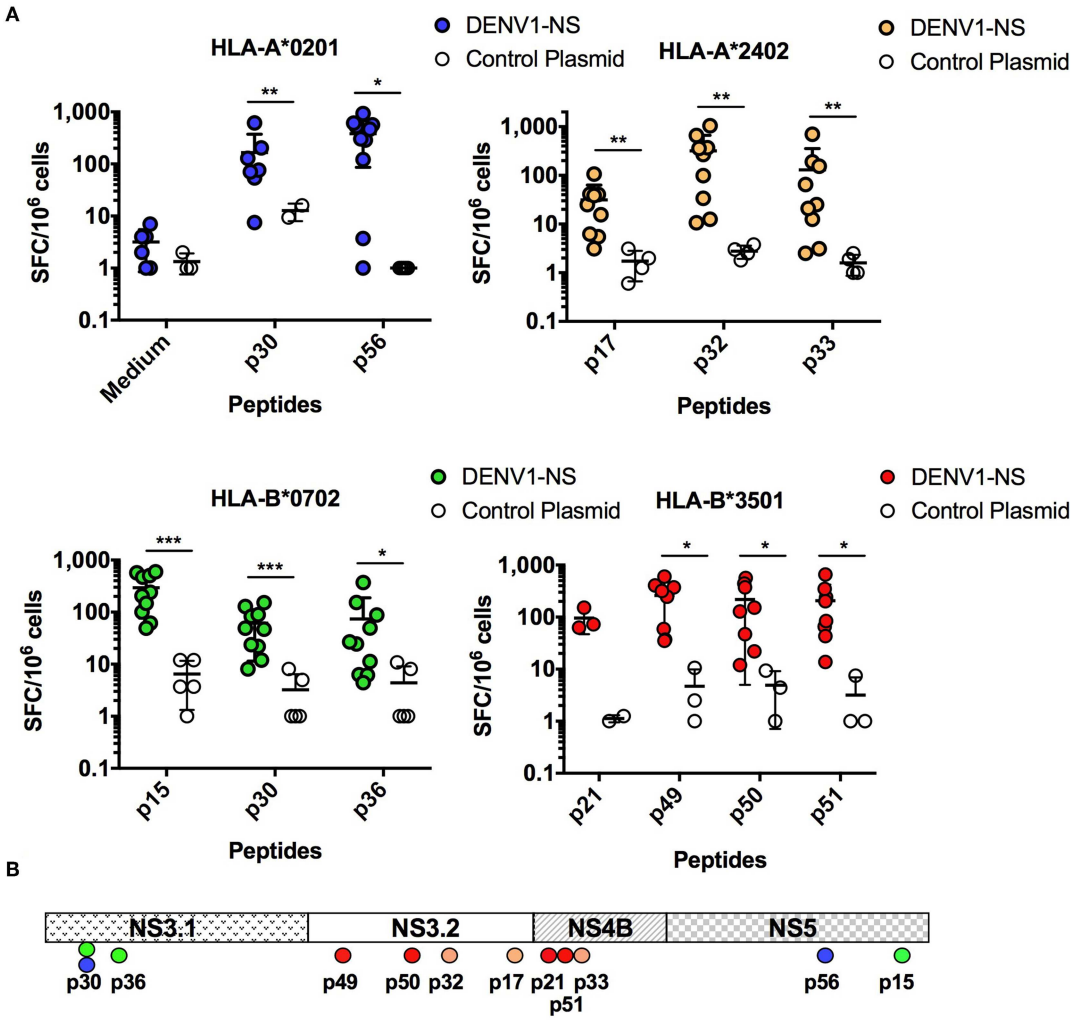

FIGURE 2 | Quantification of the T cell responses in HLA-A*0201, -A*2402, -B*0702, and -B*3501 transgenic mice by ELISpot assay. **(A)** Three independent experiments were performed, in which a total of 10 and 4 HLA-A*0201 transgenic mice received the DENV1-NS construct and the control plasmid (CT), respectively, 9 and 4 HLA-A*2402 transgenic mice received the DENV1-NS construct and the control plasmid, respectively, 10 and 5 HLA-B*0702 transgenic mice received the DENV1-NS construct and the control plasmid, respectively, and 8 and 3 HLA-B*3501 transgenic mice received the DENV1-NS construct and the control plasmid, respectively. All the animals were immunized by intradermic injection (100 μg DENV1-NS or control plasmid) followed by *in vivo* electroporation. Two immunizations were performed at 3-week interval, and spleen cells were tested for IFN-γ secretion by ELISpot 10 days after the second injection. Individual mice were tested in parallel with different peptides at 2 μg/ml and with concanavalin A (ConA) at 5 μg/ml, final concentration. Lines represent mean and SEM. Differences between mice immunized with the DENV1-NS construct and the control plasmid were evaluated using non-parametric Mann-Whitney U-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). **(B)** Schematic representation of the T cell epitopes from the DENV1-NS poly-epitope, which induce a significant T cell response in HLA transgenic mice. NS3.1, NS3.2, NS4B, and NS5 represent the 4 antigenic regions selected in the DENV1-NS poly-epitope.

Efficient Maturation of CD8+ T Cells After Late Transient Blockade of Type I IFN Signaling

Most mouse models for dengue infection and disease make use of mice lacking type I or type I and type II IFN receptors, rendering them highly susceptible to DENV infection (47). However, most of these models are not relevant to study the role of memory and effector memory T cells in the immune protection against DENV infection. For instance, type I IFN plays a major role in controlling the CD8 T cell response to viral infection, notably by blocking the expansion of pre-existing non-specific memory T cells (out-of-sequence signaling), while promoting the proliferation of antigen-specific CD8 T cells at the beginning of the response (in-sequence signaling) (48). Importantly, this regulation depends essentially on the timing of type I IFN exposure relative to T cell receptor signaling. In this context, we developed a mouse model, which allows the clonal expansion of antigen-specific T cells, while retaining DENV susceptibility. This was achieved by injecting, 1 day before the challenge with DENV, the anti-IFNAR antibody to block transiently the type I IFN signaling, as already reported for WNV and ZIKV infection in wild type mice (32, 49). The benefit of this

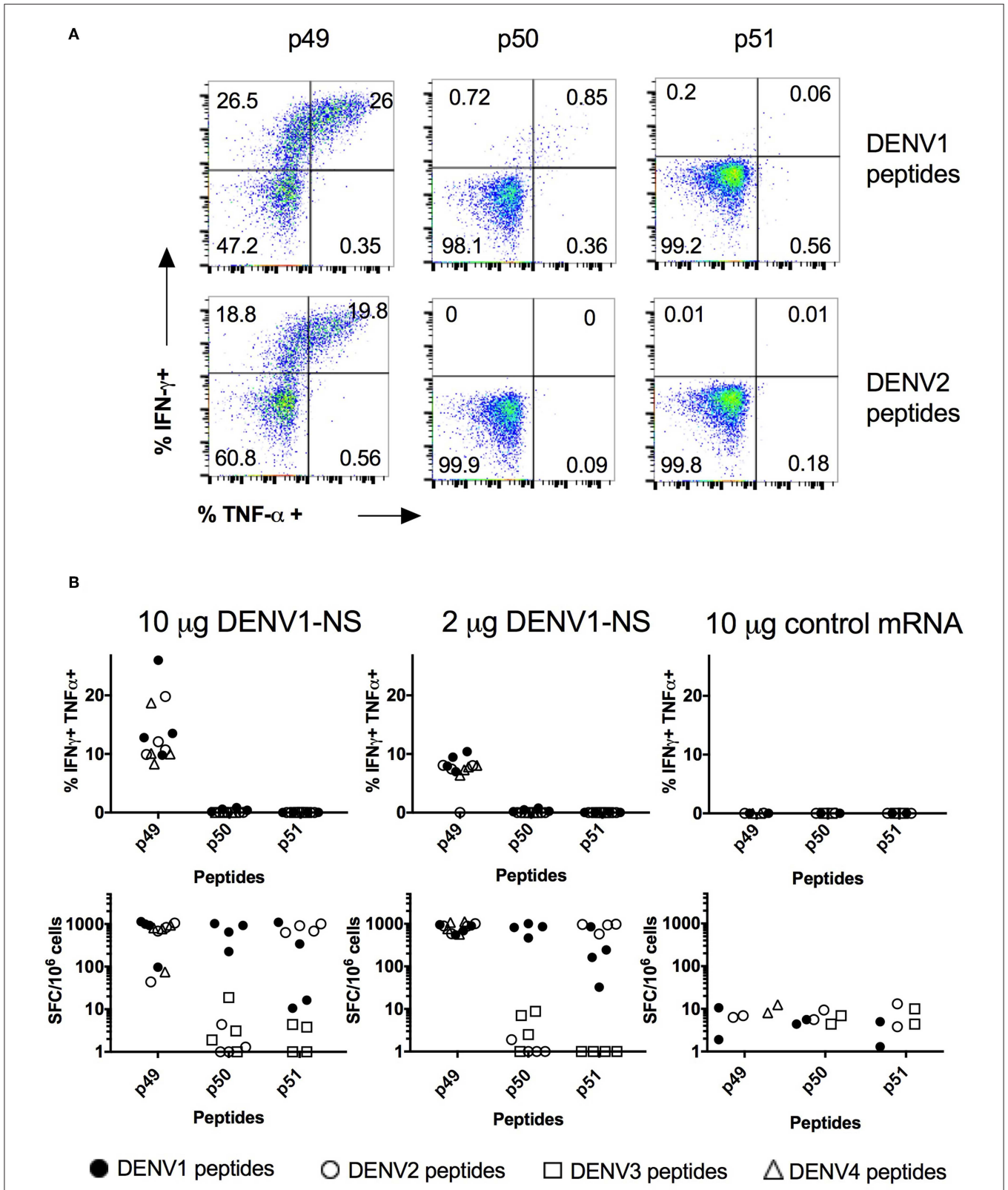

FIGURE 3 | Quantification of the T cell responses in HLA-B*3501 transgenic mice by intracellular cytokine staining and ELISpot assay. Two groups of 4 HLA-B*3501 transgenic mice received an intramuscular inoculation of 10 or 2 μg of the LNP-based mRNA encoding DENV1-NS and 1 group of 2 HLA-B*3501 transgenic received

(Continued)

FIGURE 3 | 10 μg of the LNP-based irrelevant mRNA as a negative control. Two immunizations were performed at 3-week interval and spleen cells were tested 8 days after the second immunization for IFN-γ and TNF-α secretion by intracellular staining, after 6 h *in vitro* stimulation with peptides, in the presence of Golgi stop or by ELISpot assay after 20 h stimulation with peptides. **(A)** Flow cytometry analyses of gated CD3+, CD8+, for IFN-γ and TNF-α secretion. Plots show CD8 T cell responses from one representative animal against p49, p50, and p51 derived from DENV1 sequence (upper panels) or from their DENV2 variants (lower panels) after immunization with 10 μg of the LNP-based mRNA encoding DENV1-NS. **(B)** Comparison of the T cell responses analyzed by intracellular cytokine staining (upper panels) and by ELISpot assays (lower panels). Closed circles: peptides derived from DENV1. Open circles: peptide variant derived from DENV2. Open squares: peptide variant from DENV3. Open triangles: peptide variant from DENV4.

approach is that it ensures the normal development of memory T cell responses, because blockade of type I IFN signaling is performed just before the challenge, once the immune response is established.

Thus, to verify that the transient blockade of type I IFN signaling, performed at a late stage after the prime with the antigen (at day 27 after the immunization), does not prevent the establishment of a memory T cell response, we quantified T cell responses before and after the boost, with and without blocking IFN response (**Figure 4A**). Quantification of the T cell response in HLA-A*0201 and HLA-A*2402 transgenic mice revealed a 7-fold and a 50-fold increase in the frequency of p30- and p32-specific T cells, respectively, between days 27 and 36, confirming the expansion of antigen-specific T cells after the boost at day 28. The same T cell proliferation was observed in response to p30 (DENV4) or p32 (DENV2) peptides corresponding to serotype variants (**Figure 4A**). More importantly, the same proliferation was observed in these transgenic mice, with or without anti-IFNAR treatment, showing that depletion of type I IFN signaling at a late stage does not prevent the proliferation of antigen-specific T cells, which expand after the boost (**Figure 4A**). To assess also the effect of anti-IFNAR treatment on the contraction phase of effector T cells, the antibody was administered at a late stage after the boost (at day 55), and peptide-specific T cell responses were measured in HLA-A*0201 and -A*2402 treated or not with the antibody.

As shown in **Figure 4B**, in HLA-A*0201 transgenic mice, the anti-IFNAR treatment does not prevent but rather increases the proliferation of p30-specific T cells, whereas it does not modify the proliferation of p32 or p32 (DENV2.1)-specific T cells in HLA-A*2402 mice at day 56, after the contraction phase. This result shows that, while a transient blockade of type I IFN signals in HLA-A*0201 can increase the proliferation of peptide-specific T cells, it does not prevent the contraction phase of peptide-specific T cells in HLA-A*2402 mice, which is required for the development of memory T cell responses (**Figure 4B**).

Vaccine Efficacy in Human HLA Transgenic Mice

Given the potential to induce strong T cell responses against several NS epitopes, we wanted to assess efficacy of the mRNA vaccine encoding DENV1-NS, in immunocompetent mice expressing different HLA class I molecules. The ability to elicit immunity and protection against DENV infection was tested in the HLA-A*0201, -A*2402, and B*3501 transgenic mice, which developed a strong T cell response against different peptides located in the NS3, NS4B and NS5 proteins. The animals were vaccinated following a prime-boost immunization, with a boost at day 28 after the prime, followed by a treatment with anti-IFNAR antibody at day 55 and a challenge at day 56 with the DENV1 strain KDH0026A, derived from a clinical isolate.

In the HLA-A*0201 mice, 5 out of 6 mice immunized with the control mRNA vaccine developed a significant viremia, at day 2 after the challenge, whereas 3 out of 6 mice vaccinated with the mRNA encoding DENV1-NS revealed a detectable viremia, of which 2 mice had viremia values close to the lower limit of quantitative detection (LLOQ) (**Figure 5A**). More strikingly, 5 out of 6 vaccinated HLA-A*2402 mice revealed no viremia at days 1, 2, and 3 after the challenge, whereas 4 out of 5 HLA-A*2402 mice immunized with the control mRNA developed a significant viremia until day 3 (**Figure 5B**). Finally, 8 out of 10 vaccinated HLA-B*3501 mice revealed no detectable viremia at day 2 after the challenge, whereas 9 out of 9 HLA-B*3501 mice immunized with the control mRNA vaccine developed a significant viremia at day 2 (**Figure 5C**). At day 3, no viremia was detected in vaccinated HLA-B*3501 animals, whereas 5 out of 8 HLA-B*3501 mice immunized with the control mRNA vaccine still had significant viremia, indicating that even in case an initial viremia is developed in vaccinated mice, it is resolved faster than in non-vaccinated animals (**Figure 5C**). Finally, it should be also noted that none of the vaccinated or control mice died after DENV infection.

To rule out the possibility that the immune protection induced by DENV1-NS vaccination could be mediated in part by neutralizing antibodies, plasma samples from vaccinated mice were tested for their neutralizing potential against DENV infection *in vitro*. Using a flow cytometry-based assay, as described previously (50), we thus analyzed the ability of immune serum from mice vaccinated with DENV1-NS to neutralize DENV1 infection of Vero cells. While serum from immunocompromised mice lacking type I IFN signaling (*ifanr*$^{-/-}$ mice) and infected with DENV1 can efficiently neutralize DENV1 infection, plasma samples from HLA-B*3501 mice immunized either with mRNA DENV1-NS vaccine or with CT mRNA did not reveal any neutralizing activity against DENV1 infection, even at a high concentration (**Supplementary Table 1**). These results confirm the absence of neutralizing antibodies induced after DENV1-NS vaccination, which could mediate protection against DENV1 infection. Taken together, these data demonstrate the efficacy of a T-cell based vaccine, which targets the immunodominant T cell epitopes from NS proteins, in the induction of a protective immunity against DENV infection and disease.

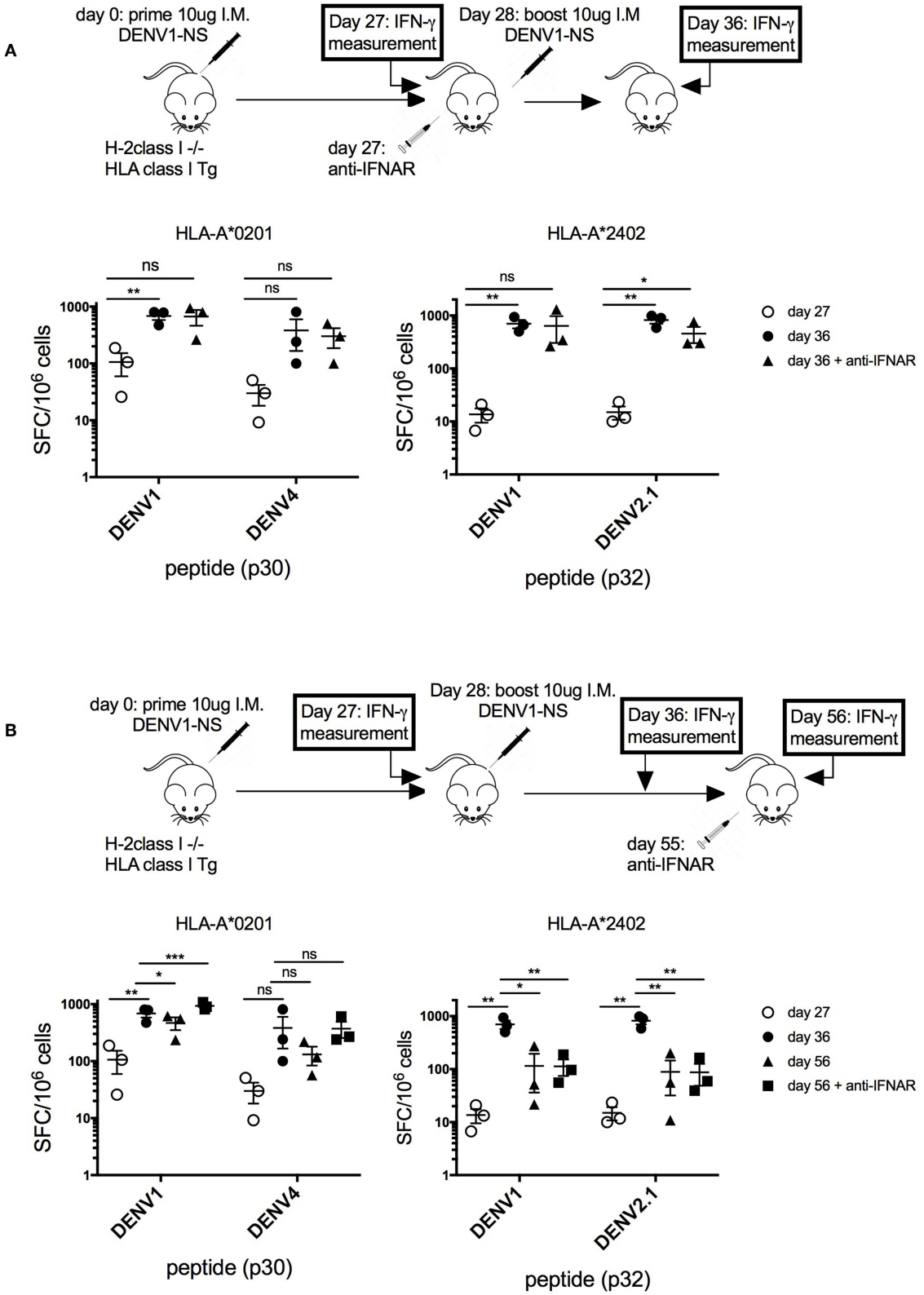

FIGURE 4 | Effect of transient blockade of type I IFN signals on the expansion and contraction phases of antigen-specific T cells. **(A)** Three groups of 3 HLA-A*02:01 transgenic mice and 3 groups of 3 HLA-A*24:02 transgenic mice received 2 intramuscular inoculations of 10 μg of the LNP-based mRNA encoding DENV1-NS at

(Continued)

FIGURE 4 | 4-week interval. One group of mice received in addition 1 intraperitoneal inoculation of 2 mg anti-IFNAR antibody at day 27, just before the boost. Spleen cells were tested by ELISpot assay for IFN-γ secretion at days 27 and 36, after *in vitro* stimulation with the p30 peptide from DENV1 or its peptide variant from DENV4 for HLA-A*0201 transgenic mice or the p32 peptide from DENV1 or its peptide variant from DENV2.1 for HLA-A*2402 transgenic mice. Mean and SEM are shown. Differences in the ELISpot responses between 2 groups were evaluated using unpaired *t*-test (ns, non-significant, $*p < 0.05$, $**p < 0.01$). **(B)** Three groups of 3 HLA-A*0201 transgenic mice and 3 groups of 3 HLA-A*2402 transgenic mice received 2 intramuscular inoculation of 10 μg of the LNP-based mRNA encoding DENV1-NS at 3-week interval. One group received in addition 1 intraperitoneal inoculation of 2 mg anti-IFNAR antibody at day 55. Spleen cells were tested by ELISpot assay for IFN-γ secretion at days 27, 36, or 56. Mean and SEM are shown. Differences in the ELISpot responses between 2 groups were evaluated using unpaired *t*-test (ns, non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

DISCUSSION

In this study, we have shown that vaccination of HLA transgenic mice with a LNP-encapsulated mRNA encoding a minimal antigen, enriched in conserved and highly antigenic epitopes from NS3, NS4B, and NS5 regions of DENV1 induces a potent T cell response and a protection against DENV1 infection. Lipid nanoparticles (LNP) have been be already used for the delivery of small interfering RNA (siRNA) and they are currently being evaluated in late-stage clinical trials via intravenous administration (51). Moreover, the LNP-based, modified-mRNA vaccine platform generates robust and protective immune responses in different animal models, including mice and monkeys (52). In the light of these results, we took advantage of this technology to elicit a potent T cell response against several DENV peptides and, to assess CD8 T cell-induced protection in the context of different HLA class I backgrounds.

With the limitation that CD8 T cells were only identified by intracellular staining, the strong T cell responses detected by ELISpot, supports a role for these CD8 T cells in the immune protection against DENV infection. This is also in agreement with previous reports showing the induction of peptide-specific responses in HLA transgenic mice that provided protection against DENV challenge (53). Four different HLA class I transgenic strains were selected for measuring the T cell response against DENV peptides, and for the evaluation of the immune protection against a challenge with DENV: the HLA-A*0201, -A*2402, and HLA-B*0702 and -B*3501 which represent the most frequent alleles in Caucasoid, Oriental and Amerindian ethnic groups, and are associated with low and high response frequency and magnitude, respectively (24). Depending on the antigenic peptides recognized by T cells, and the positions of amino acid substitutions expressed in the different DENV serotypes, serotype-specific T cells or cross-reactive T cells were stimulated, which target peptides from both homotypic as well as heterotypic DENV strains. Contrary to previous observations showing that cross-reactive T cells could display an altered cytokine profile upon stimulation with peptide from serotypes variants, T cells from the HLA-B*3501 transgenic mice immunized with the mRNA vaccine did not reveal any difference in the cytokine profile following *in vitro* stimulation with the DENV2 and 4 serotype variants of p49. Further analyses of the cytokine profile and the ratio of TNF to IFN-γ producing CD8 T cells in response to more peptides and their serotype variants, at different concentrations, should confirm the unaltered T cell response to peptides from serotypes variants, in comparison with peptides derived from DENV1. Taking into account this limitation, quantification by ELISpot of the T cell response to the p30 and p32 peptides or to their serotype variants revealed the same percentage of IFN-γ-producing cells, strongly suggesting the activation of cross-reactive T cells that recognize different DENV serotypes. Further analyses of the T cell responses against other heterologous peptides, such as p56 DENV2, 3 in HLA-A*0201, p17 DENV2, 3 and 4 in HLA-A*2402 and p15 DENV2, 3 and 4 in HLA-B*0702 transgenic mice, should confirm the activation of cross-reactive T cells induced following DENV1-NS immunization, which target conserved epitopes, in accordance with the high genetic identity between the 4 DENV serotypes in the selected DENV1-NS sequence. Importantly, such cross-reactive T cells were also identified in DENV-immune donors infected with ZIKV, after *in vitro* stimulation with the APTRVVAAEM peptide from DENV2, 3, 4, or ZIKV or the APTRVVASEM peptide from DENV1, which corresponds to the p36 peptide derived from the DENV1-NS poly-epitope, and restricted by the HLA-B*0702 molecule (54). Further experiments in HLA class I transgenic mice are required to determine whether vaccination with the DENV1-NS poly-epitope induces significant protection against other DENV serotypes in the context of different HLA class I molecules.

In a recent study from naturally infected human donors it was shown that the pattern of the immunodominant CD8 T cell epitopes differs according to the DENV serotype, with DENV3-specific responses predominantly targeting structural proteins, whereas DENV1-, DENV2- and DENV4-specific responses are mainly directed against non-structural proteins (55). In this context, it would be important to determine whether the DENV1-NS poly-epitope, which contains only immunodominant epitopes from non-structural proteins, can nevertheless induce the activation of effector CD8+ T cells which can mediate immune protection against the other DENV serotypes, including more specifically DENV3.

Since immunization with the DENV1-NS mRNA vaccine induces protective immunity at least 1 month after the boost, phenotypic analyses of tetramer-positive CD8+ T cells in different HLA transgenic mice should confirm the induction of memory T cells after DENV1-NS vaccination. A more precise identification of the different peptides from DENV1 or from the other DENV serotypes and a comparison in their capacity to induce and to maintain such memory T cells would be helpful to refine the sequence of the DENV1-NS poly-epitope

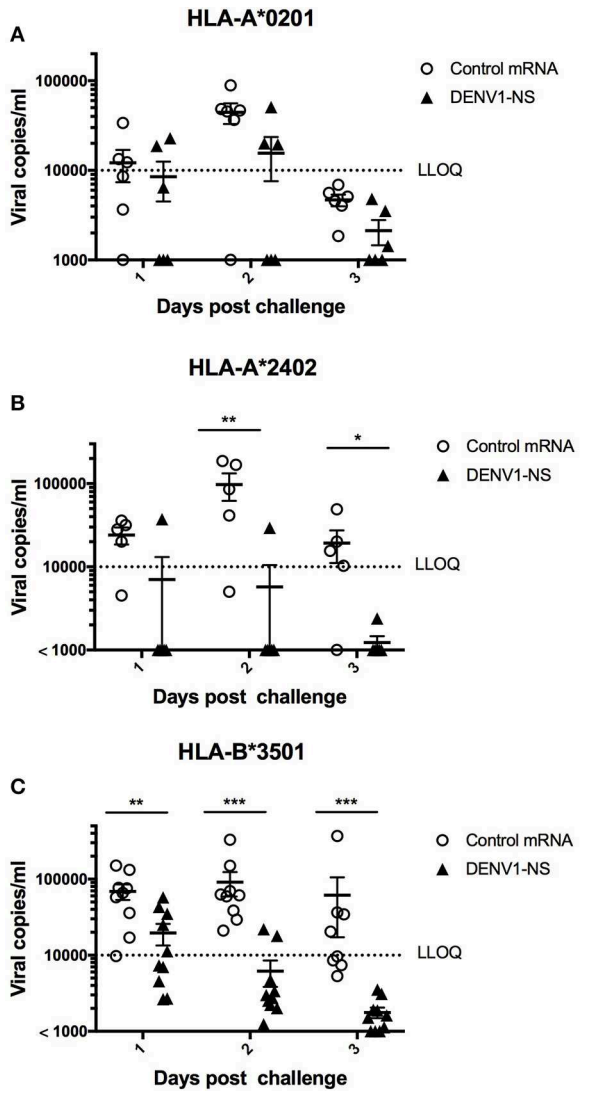

FIGURE 5 | Immune protection induced by immunization with the LNP-based mRNA encoding DENV1-NS. **(A)** Two groups of 6 HLA-A*0201 transgenic mice were immunized with the LNP-based mRNA encoding DENV1-NS or control mRNA, **(B)** Five and six HLA-A*2402 transgenic mice received the LNP-based mRNA encoding DENV1-NS and control mRNA, respectively, **(C)** Nine and ten HLA-B*3501 transgenic mice received the LNP-based mRNA encoding DENV1-NS and control mRNA, respectively. Mice were immunized by intramuscular injection of 10 μg LNP-based mRNA encoding DENV1-NS or control mRNA, at day 0 and at day 28. One day prior challenge with the virus, mice received 1 intraperitoneal inoculation of 2 mg anti-IFNAR antibody (MAR1-5A3) and they were challenged at day 56 by retro-orbital injection of 10^6 pfu DENV1 (KDH0026A strain). Quantification of virus replication in plasma samples was performed by qRT-PCR at days 1, 2, and 3 after the challenge. LLOQ: Lower limit of quantitative detection. Lines represent mean and SEM. Differences between mice immunized with the DENV1-NS vaccine and the CT vaccine were evaluated using non-parametric Mann-Whitney U-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

and thus to improve the immune protection in the context of multiple DENV infections and in different HLA backgrounds. In this sense, a more detailed map of the distribution and strength of the CD4 and CD8 T cell epitopes from DENV1-NS, by quantifying the IFN-γ responses with overlapping peptides, should confirm a hierarchy in the strength of the T cell responses in different HLA backgrounds, which should coincide with the immune protection, as observed in humans and transgenic mice expressing the protective HLA-DRB1*0401 and the HLA-B*0702 or -B*3501 class II and class I alleles, respectively (24, 56).

In addition to inducing strong T cell responses to non-structural proteins, with an immune memory, the advantage of our strategy resides in the absence of neutralizing antibody induction against E and/or prM-protein, hence the absence of possible vaccine induced antibody-dependent enhancement. More specifically, the aim of this study was not to compare the efficiency of a vaccine targeting T cells vs. a vaccine targeting B cells and inducing neutralizing antibodies, either alone or in combination with this T cell vaccine on protection against infection, but rather to show that the activation of T cells, in the absence of neutralizing antibodies is efficient in inducing an immune protection against DENV infection. These results are consistent with previous studies showing a protective role for T cell-mediated immunity against DENV infection, in the presence or in the absence of anti-DENV neutralizing antibodies (53, 57). Similar protection was also observed in the absence of neutralizing anti-DENV antibodies after immunization of IFN-α/βR$^{-/-}$ mice with antigenic peptides from DENV or BALB/c mice or African green monkeys with recombinant capsid protein (58, 59). Although we did not observe any enhanced DENV infection in vaccinated animals, infection experiments *in vitro* with FcγR bearing target cells in the presence of different concentrations of immune serum should allow us to confirm the absence of enhancing antibodies induced after vaccination with the DENV1-NS poly-epitope.

In summary, our results highlight the fact that a minimal poly-epitope, containing immunodominant T cell epitopes from NS3, NS4B, and NS5 non-structural proteins of DENV1 is able to induce a potent CD8 T cell response against DENV1 peptides, resulting in a protective immunity against DENV1 infection. Future studies will be directed to confirm the immunogenicity and protective efficacy of the DENV1-NS or other DENV T cell poly-epitope vaccines against infection with different DENV serotypes in transgenic mice expressing different HLA class I and class II molecules and in other animal models.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethical committee: CETEA under number 2014-0001, and the CHSCT committee under number 12.454 at the Institut Pasteur, Paris, France.

AUTHOR CONTRIBUTIONS

CR and ES-L designed the DENV1-NS poly-epitope. CR and KB designed the research. CR, CC, MP, IC, and LL performed the experiments. ES-L and AS provided valuable scientific discussion

in the field of flavivirus virology. PL-D, JT, KB, CG, TC, GC, and AS provided valuable scientific discussion and expertise in the field of vaccinology. CR wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01424/full#supplementary-material

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T Cell Responses Induced by DNA Vaccines Based on the DENV2 E and NS1 Proteins in Mice: Importance in Protection and Immunodominant Epitope Identification

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The importance of the cellular immune response against DENV has been increasingly highlighted in the past few years, in particular for vaccine development. We have previously constructed two plasmids, pE1D2, and pcTPANS1, encoding the envelope (E) ectodomain (domains I, II, and III) and the non-structural 1 (NS1) protein of dengue virus serotype 2 (DENV2), respectively. In the present work, we analyzed the induction of the cellular response in mice immunized with these DNA vaccines and identified the immunogenic peptides. Vaccinated BALB/c mice became protected against a lethal challenge of DENV2. Depletion of $CD4^+$ cells in vaccinated animals almost completely abolished protection elicited by both vaccines. In contrast, a significant number of pE1D2- and pcTPANS1-immunized mice survived virus challenge after depletion of $CD8^+$ cells, although some animals presented morbidity. To identify immunogenic peptides recognized by T cells, we stimulated splenocytes with overlapping peptide libraries covering the E and NS1 proteins and evaluated the production of IFN-γ by ELISPOT. We detected two and three immunodominant epitopes in the E and NS1 proteins, respectively, and four additional NS1-derived peptides after virus challenge. Characterization by intracellular cytokine staining (ICS) revealed that both $CD4^+$ and $CD8^+$ T cells were involved in IFN-γ and TNF-α production. The IFN-γ ICS confirmed reaction of almost all E-derived peptides before challenge and identified other epitopes after infection. All NS1-derived peptides were able to elicit IFN-γ production in $CD4^+$ cells, while only a few peptides induced expression of this cytokine in $CD8^+$ T lymphocytes. Interestingly, we observed an increase in the frequency of either $CD4^+$ or $CD8^+$ T cells producing TNF-α after immunization with the pE1D2 and challenge with DENV2, while lymphocytes from pcTPANS1-vaccinated animals maintained ordinary TNF-α production after virus infection. We also assessed the recognition of E and NS1 immunogenic peptides in C57BL/6 mice due to the difference in MHC haplotype

expression. Two NS1-derived epitopes featured prominently in the IFN-γ response with cells from both animal strains. Overall, our results emphasize the importance of the T cell response involved in protection against dengue induced by E and NS1 based DNA vaccines.

Keywords: dengue, DNA vaccines, T cell response, NS1, envelope protein, mice

INTRODUCTION

Dengue is one of the most important mosquito-borne viral diseases, with an overall estimation of 390 million people infected worldwide per year (1). This disease can manifest as a broad range of symptoms varying from the self-limiting dengue fever to potentially lethal severe forms, the dengue hemorrhagic fever, and dengue shock syndrome (2, 3).

Currently, different vaccines against dengue virus (DENV) are undergoing clinical trials and one is commercially available (4–6). This tetravalent vaccine, developed by Sanofi-Pasteur, is based on the backbone of the yellow fever 17D vaccine with the replacement of membrane and envelope proteins of each dengue serotype (7). Results from phase III clinical trials revealed that although individuals, especially children, presented high levels of neutralizing antibodies toward DENV2 after vaccination, they were not protected against this virus (8–10). Furthermore, recent reports showed that children who were dengue naïve at vaccination time were more susceptible to develop severe dengue after virus exposure (11–13). Such results point out that the induction of neutralizing antibodies is not the only arm of the immune response involved in protection against DENV. In fact, the importance of the cellular immune response against DENV has been increasingly highlighted in the past few years, concerning both protection and/or disease enhancement (14–20).

Most vaccine strategies against dengue are based on the envelope (E) glycoprotein. The E protein is organized in 90 homodimers in the virus surface and is reassembled into trimers at the fusion state. Each monomer is composed of domains I, II, and III, a membrane-proximal stem and a transmembrane anchor (21). Since the E protein is the major component of the virion surface and interacts with receptors present on host cells mediating virus internalization, this protein is the primary target for induction of neutralizing antibodies (22–24). However, after virus infection a T cell response that may be involved in protection is also elicited toward this protein (25–30).

Another highly immunogenic dengue protein is the non-structural 1 (NS1) glycoprotein, which is also considered an antigen for vaccine development (31–34). The NS1 is found in infected mammalian cells associated with plasma membrane as well as secreted into the circulation as soluble multimers (35–37). The secreted form seems to be implicated in immune evasion strategies (38). Moreover, convalescent dengue patients present high levels of antibodies against NS1. Recent studies also showed that this protein can induce T cell responses either in experimental animals or in humans (27, 29, 30, 39).

Based on this evidence, in the present work, we analyzed the induction of the cellular immune response in mice immunized with two previously constructed DNA vaccines (pE1D2 and pcTPANS1) against DENV2. The plasmid pE1D2 encodes the ectodomain of the E protein (domains I, II and III) (40, 41), and the plasmid pcTPANS1 contains the *ns1* gene (33, 42). BALB/c mice immunized with these DNA vaccines became protected against a lethal challenge of DENV2 and we evaluated the role of T cells in protection. Depletion of $CD4^+$ T cells in vaccinated animals completely abolished protection elicited by both vaccines, while a significant number of pE1D2- and pcTPANS1-immunized mice survived virus challenge after depletion of $CD8^+$ T cells. However, near half of the vaccinated animals depleted from $CD8^+$ T cells presented clinical signs of infection.

We then identified the immunogenic peptides recognized by T cells from vaccinated animals, before and after virus challenge. We evaluated IFN-γ production by stimulating splenocytes with overlapping peptide libraries covering the E and NS1 proteins in enzyme-linked immunospot (ELISPOT) assays. Intracellular cytokine staining (ICS) assays revealed the involvement of both $CD4^+$ and $CD8^+$ T cells in IFN-γ production by splenocytes reacting mainly to the selected E-derived peptides, before and after virus challenge. In contrast, almost all the selected NS1-derived peptides were recognized by $CD4^+$ T lymphocytes, while $CD8^+$ cells reacted only to half of these peptides. TNF-α production was also evaluated by ICS assays, revealing expression of this cytokine by $CD4^+$ and $CD8^+$ T cells upon stimulation with almost all E-derived peptides, especially after virus challenge. On the other hand, only two NS1-derived peptides were able to significantly induce TNF-α production. To assess the recognition of immunogenic E- and NS1-derived peptides in a different MHC haplotype, we investigated IFN-γ production in C57BL/6 mouse cells by ELISPOT. Distinct epitopes were recognized by C57BL/6 splenocytes using the same peptide libraries, although two NS1-derived epitopes were positive in the assays performed with cells from both BALB/c and C57BL/6 animals. Overall, our results emphasize the importance of the T cell response involved in dengue protection and may contribute for the development of more effective vaccines against dengue, in particular for DNA vaccines based on the E and NS1 proteins.

MATERIALS AND METHODS

Virus and DNA Vaccines

The dengue 2 virus (DENV2), strain New Guinea C (NGC, GenBank M29095), was used for cloning the NS1 and E

sequences as well as for mice challenge assays. The DNA vaccines pcTPANS1, pE1D2, and pcTPA were previously described (33, 40). Briefly, the pE1D2 plasmid encodes the ectodomain (domains I, II, and III) of the E protein while pcTPANS1 encodes the full-length *ns1* gene. In both constructions, genes were fused to the sequence encoding the human tissue plasminogen activator (t-PA) signal peptide, under the control of the cytomegalovirus (CMV) promoter region. The negative control, pcTPA, was derived from the commercial vector pcDNA3 (Invitrogen) and contains only the t-PA signal peptide sequence.

For DNA vaccine preparations, *Escherichia coli* DH5-α strain was transformed with the different plasmids, which were then extracted by alkaline lysis and purified by Qiagen Endofree Plasmid Giga Kit (Qiagen), according to manufacturer's instructions. Plasmids were suspended in sterile water and stored at −20°C until use. The DNAs were quantified by measuring absorbance at 260 nm in spectrophotometer (Bio Photometer, Eppendorf). Concentration and integrity of all plasmids were confirmed by 1% agarose gel electrophoresis, stained with Nancy-520 and visualized in ultraviolet transilluminator.

Animal Immunization

Four-week-old BALB/c or C57BL/6 mice, specific pathogen free (SPF), were purchased from the Multidisciplinary Center for Biological Investigations (CEMIB, UNICAMP-SP, Brazil). Animals were inoculated by the intramuscular route (i.m.) with 50 μg of DNA vaccines diluted in 50 μL of phosphate buffer saline (PBS) in each tibialis posterior muscles (100 μg/mice), using 30-gauge needles. Each animal group received two doses of pE1D2 or pcTPANS1, administered 2 weeks apart, and mice were euthanized or challenged 4 weeks after the first dose. Negative control groups included naïve or pcTPA-inoculated mice. For euthanasia, animals were overexposed with a mixture of ketamine-xylazine (43) and bled by cardiac puncture.

Virus Challenge

BALB/c mice were anesthetized with a mixture of ketamine-xylazine and inoculated by the intracerebral (i.c.) route with 30 μL of a neuroadapted NGC DENV2 diluted in E199 medium, corresponding to 40 LD_{50}. Animals were euthanized either 7 or 21 days after infection (dpi) as described on figures legends. To ascertain vaccine protection, mice were followed up to 21 dpi, and morbidity was recorded. Clinical signs of infection were noted according to an arbitrary scale ranging from 0 to 4: 0 = no clinical signs; 1 = paralysis in one leg or alteration of the spinal column; 2 = severe paralysis in one leg and alterations of the spinal column or severe paralysis on both hind legs; 3 = severe paralysis in the hind legs and alteration of the spinal column; 4 = death. Moribund animals were submitted to euthanasia.

In vivo Depletion of $CD4^+$ and $CD8^+$ Cells

BALB/c mice (n = 8–10) immunized with pE1D2 or pcTPANS1 were depleted from $CD4^+$ or $CD8^+$ cells upon inoculation of in-house produced ascitic fluids containing anti-CD4 (clone GK 1.5) or anti-CD8 antibodies (clone 56-3.7) (39). Animals were inoculated by the intraperitoneal route (i.p.) with 20 μL of ascitic fluids on days 4 and 2 before DENV2 challenge. T cell depletion was monitored by flow cytometry in blood cells stained with anti-CD3 FITC (clone 145-2C11), anti-CD8 PerCP (clone 33-6.7) and anti-CD4 PE (clone RM4-5) antibodies (BD Biosciences), and evaluated on FlowJo software.

E and NS1 Peptide Libraries

The T cell epitope mapping and characterization were performed using overlapping peptide libraries spanning the E and NS1 protein sequences of DENV2 NGC strain, consisting of 15-mer peptides overlapping each other by 11 amino acid residues. Lyophilized synthetic peptides (Mimotopes Pty Ltd) were suspended in ultrapure water at a final concentration of 2 μg/μL, with an average purity of ∼80%. After the suspension, peptides were stored at −20°C until use. Initial studies were carried out with peptides grouped in pools of 8–10 peptides according to each protein (E protein: 98 peptides, 10 pools; NS1 protein: 86 peptides, 9 pools).

Cell Isolation

Spleens and peripherical blood were harvested from previously pE1D2-, pcTPANS1- or pcTPA-inoculated animals, challenged or not with DENV2, as well as from naïve mice. For ELISPOT and ICS assays, splenocytes were isolated, and erythrocytes were lysed with BD Pharm Lyse™ (BD Biosciences), according to the manufacturer's instructions. After red cell lysis, splenocytes were washed with PBS and suspended in RPMI-1640 medium (Sigma), in 10% fetal calf serum (FCS), 100 IU/mL penicillin, and 100 ug/mL streptomycin. Blood samples from T cell-depleted mice, collected at the same day of virus challenge in the other animal groups, were treated with FACS Lysing Solution (BD Biosciences) for erythrocytes lysis and cell fixation, prior to staining and flow cytometry analysis.

Interferon Gamma ELISPOT Assays

The assays were performed using the IFN-γ ELISPOT mouse set (BD Biosciences) upon splenocyte stimulation with E and NS1 peptide libraries, according to the manufacturer's instruction. The assays were initially performed using pooled peptides. Afterwards, positive pools were selected for screening of individual peptides. Briefly, 96-well ELISPOT plates were coated overnight at 4°C with anti-IFN-γ capture monoclonal antibody (5 μg/mL). On the next day, plates were blocked and 5 x 10^5 splenocytes (n = 3, pooled cells) from naïve, pE1D2- or pcTPANS1-vaccinated or pcTPA-inoculated mice, challenged or not with DENV2, were incubated in triplicate with 2 μg of E or NS1 peptides (pooled or individually). Non-stimulated and concanavalin A (Con A, 5 μg/mL) stimulated cells were used as negative and non-specific positive controls, respectively. After an 18-h stimulation period at 37°C in 5% CO_2, cells were discarded, and plates were incubated for 2 h at 37°C with 2 μg/mL anti-IFN-γ biotinylated detection antibody. Plates were then incubated with streptavidin-horseradish peroxidase conjugate for 1 h at room temperature (diluted 1:100). Finally, plates were washed

and the spots were revealed by adding the AEC substrate set (BD Biosciences) at room temperature. The reaction was stopped by washing plates with distilled water. Spots were counted in the automated immunospot reader (AMBRIEX, Cellular Technology Ltd) at the ELISPOT Multi-User Platform (Fiocruz). Positivity was established by using a low stringent approach, in which $\geq$ 5 spot-forming cells (SFC) per 5×10^5 cells were considered positive after subtraction of the number of spots detected in the respective non-stimulated cells, and as long as above the number observed in controls (detected in cells from pcTPA-inoculated or naïve mice).

Intracellular Cytokine Staining (ICS) Assays

Splenocytes isolated from immunized or naïve BALB/c mice ($n = 5$), infected or not with DENV2, were tested by IFN-γ and TNF-α ICS assays, using the peptides previously identified as positive by the ELISPOT analysis. A total of 2×10^6 cells/well were plated in 96-well U-bottom plates. Splenocytes were stimulated with 2 μg of E- or NS1-derived peptides or with Con A, and incubated at 37°C in 5% CO_2 for 6 h. Brefeldin A (1 μL/mL, GolgiPlug BD Biosciences) was added to the cultures after an initial stimulation period of 1 h and 30 min. Cells were then collected and washed in staining buffer (PBS, 2% FCS, 2 mM EDTA, 55 μM β-mercaptoethanol), blocked with 10% inactivated murine serum in PBS for 30 min, fixed with 4.0% paraformaldehyde in PBS for 20 min at 4°C, washed in PBS, and then maintained at 4°C. On the next day, cells were stained with pre-titrated anti-CD3 PE (clone 145-2C11, BD Biosciences), anti-CD4 PerCP or APC (clone RM4-5, BD Biosciences) and anti-CD8 FITC or PerCP (clone 33-6.7, BD Biosciences), fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences), according to the manufacturer's instructions. Cells were then stained for intracellular cytokine detection with anti-IFN-γ Alexa Fluor 488 (clone XMG12, BioLegend) and anti-TNF-α Alexa Fluor 647 (clone MP6-XT22, BD Biosciences). All antibodies were diluted in staining buffer. Cells were incubated for 30 min at 4°C, washed and maintained in staining buffer at 4°C until the next day. Fifteen thousand cells on the lymphocyte gate were acquired in a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo software v.10 (TreeStar).

Statistical Analysis

All statistical differences were assessed using GraphPad Prism software v6.0, applying a minimum level of significance of 95%. Statistical significance was evaluated by the non-parametric Mann-Whitney test for ELISPOT and ICS assays. Morbidity comparisons were made using one-way ANOVA with Bonferroni correction. Survival rates were evaluated using the Log-Rank statistical test.

RESULTS

Protective Efficacy of pE1D2 and pcTPANS1 DNA Vaccines in BALB/c Mice

Protection conferred by the DNA vaccines based on the ectodomain of the envelope (pE1D2) and the NS1 (pcTPANS1) proteins was evaluated in BALB/c mice, after a lethal DENV2 challenge, inoculated by the i.c. route. Mice were followed for 21 days after challenge and compared to animals inoculated with the control plasmid pcTPA or to non-immunized mice, both also challenged with DENV2 (**Table 1**). Ninety-five percent of pE1D2-immunized mice survived the challenge, and only 15% presented clinical signs of infection. Immunization with pcTPANS1 also generated a high protection level of 85% survival, and only 20% of mice displayed morbidity. On the other hand, both pcTPA-inoculated and non-immunized mice (DENV2 group) presented low survival (30 and 20%, respectively) and high morbidity rates (90%; **Table 1**).

The protection elicited by the DNA vaccines was also measured by analyzing the signs of infection in the different experimental groups through a clinical score scale from 0 to 4. Following such analysis, both pE1D2 and pcTPANS1-immunized mouse groups presented the median clinical score of 0. In contrast, animals inoculated with the control pcTPA or non-immunized mice and challenged with DENV2 presented morbidity degrees of 3 and 4, respectively (**Table 1**). Overall, taking together survival and morbidity rates, our results highlight the protective potential of these DNA vaccines.

The Impact of $CD4^+$ and $CD8^+$ T Lymphocytes on the Survival of Immunized Mice After Lethal Virus Challenge

We evaluated the contribution of T cell populations on the vaccine-induced protective immunity against DENV2 by treating pE1D2- and pcTPANS1-immunized BALB/c mice with anti-CD4 or anti-CD8 antibodies. On the challenge day, the success of the depletion protocol was confirmed (**Supplementary Figure 1**). Survival rates and signs of infection in immunized and treated mice were daily monitored for 21 days after DENV2 challenge. The schematic timeline with immunization, depletion and virus challenge is presented in **Figure 1A**.

In the pE1D2-immunized group, depletion of $CD8^+$ cells resulted in a small reduction of survival rate, from 100 to 80%, with no statistical significance comparing to non-depleted animals (**Figure 1B**). However, $CD8^+$ T cell depletion had an impact on the clinical signs of infection. In addition to mice that succumbed to infection (degree 4),

TABLE 1 | Survival and morbidity of BALB/c mice immunized with the DNA vaccines pE1D2 and pcTPANS1 after lethal challenge with DENV2.

Experimental group	Survival	Morbidity	Degree of morbidity
pE1D2	19 (95%)	3 (15%)	0 ± 0.9
pcTPANS1	17 (85%)	4 (20%)	0 ± 1.5
pcTPA	6 (30%)	18 (90%)	3 ± 1.5
DENV2 (non-immunized)	4 (20%)	18 (90%)	4 ± 1.3

Survival and morbidity, followed 21 days after virus challenge, were represented as the absolute number of individual events in the experimental group (n = 20) and the percentage it represents (in parentheses). The degree of morbidity was represented as the median ± standard deviation of the mean.

other animals presented morbidity degrees ranging from 2 to 3 (**Figure 1C**). On the other hand, protection provided by pE1D2 was completely abolished after $CD4^+$ T cells depletion. Similar to the control of non-immunized mice (DENV2 group), only 10% of pE1D2-vaccinated and anti-CD4-treated mice survived the virus challenge, which was statistically different from non-depleted or $CD8^+$ T-cell depleted groups (**Figure 1B**). Furthermore, the only animal that survived virus infection presented morbidity degree of 2 (**Figure 1C**).

Similar results were observed in animals immunized with pcTPANS1 and depleted from $CD4^+$ or $CD8^+$ T lymphocytes. Although not statistically significant, the survival rate of animals immunized with pcTPANS1 decreased from 80 to 50% when depleted from $CD8^+$ T cells, while the impact of $CD4^+$ T cell depletion was more significant, abolishing protection almost completely (only 10% survival; **Figure 1B**). Depletion of $CD8^+$ T cells also increased morbidity, as more animals showed clinical signs of DENV infection, even though this difference was not statistically significant when compared to the pcTPANS1 non-depleted group (**Figure 1C**).

T Cell Epitope Map of E and NS1 Antigens by ELISPOT Assays in BALB/c Vaccinated Animals

After we observed that protection induced in BALB/c mice immunized with the pE1D2 and pcTPANS1 DNA vaccines depends on $CD4^+$ and $CD8^+$ T cells, we decided to identify the peptides contained in the E and NS1 proteins which were immunogenic in vaccinated animals. For this purpose, we used two synthetic peptide libraries spanning the ectodomain of the E protein and the whole sequence of NS1 protein from the dengue serotype 2, strain New Guinea C. Each peptide is 15 amino acids in length with an overlap of 11 amino acid residues. Immunogenicity of E and NS1-derived epitopes was assessed by IFN-γ ELISPOT assays, performed initially with peptide pools (8–10 peptides each) used to stimulate splenocytes from vaccinated and control animals, with cells also pooled in the different groups. After detection of positive pools, peptides were individually tested on splenocytes pooled from the different experimental groups and then evaluated with cells from individual animals. When two positive detected peptides were adjacent, we chose the peptide that generated the highest magnitude of the response. The initial ELISPOT analyses were performed using a low stringency parameter in which the response magnitude of $\geq$ 5 SFC per 5×10^5 cells were considered positive, after subtraction of the number of spots detected in non-stimulated cells and as long as above the observed in pcTPA-inoculated or naïve control groups. Concanavalin A was used as a non-specific positive control.

In order to investigate whether the immunodominant pattern of the E- and NS1-derived peptides could be altered after virus infection, we also evaluated the IFN-γ response with cells collected from animals immunized with the pE1D2 or pcTPANS1 and challenged with DENV2. The schematic timeline with immunization, virus challenge and euthanasia is presented in **Figure 2A**.

The screening of E-derived peptide pools recognized by splenocytes from pE1D2-immunized BALB/c mice revealed 3 positive sets without the DENV2 challenge (pools 4, 8, and 9) and 4 more sets after virus infection (pools 2, 5, 7, and 10) for IFN-γ production (**Figure 2B**). Positive pools were then selected for individual peptide screening. Peptides E40, E71, E75, E82, and E83 were considered positive without the virus challenge (**Supplementary Figure 2**) by applying the same selection criteria as before and comparing to control (cells from pcTPA-inoculated animals). Given the adjacent localization of peptides E82 and E83 and the higher magnitude of response induced by the peptide E82, this peptide was selected for further analysis. The screening of individual peptides after the DENV2 challenge revealed that not all positive pools presented peptides able to stimulate IFN-γ production. Results reinforced peptides E40 and E82 as the most immunogenic, with the further addition of peptides E75, E84, E86, and E98 (**Supplementary Figure 2**). To further confirm the immune relevance of these peptides, we performed *in vitro* stimulation tests with cells from individual BALB/c mice. Results showed that peptides E40 and E82 significantly stimulated IFN-γ production in splenocytes collected from pE1D2-immunized mice when comparing to cell spots of pcTPA-inoculated or naïve animals stimulated with the same peptides (**Figure 2C**). These peptides were immunogenic in vaccinated-only mice as well as in immunized animals challenged with DENV2, although the response was higher after virus infection. Besides, both peptides were also able to elicit IFN-γ production in splenocytes collected from non-immunized mice infected with DENV2, although in a lower magnitude when compared to the pE1D2-immunized groups (**Figure 2C**). In contrast, the responses elicited by peptides E71, E75, E84, E86, and E98 were not significantly different from the negative controls. As expected, stimulation with Con A in all ELISPOT assays performed with cells from pE1D2-vaccinated or control animals induced high IFN-γ response.

The same screening analysis was used to evaluate the immunogenicity of NS1-derived peptides in the context of the pcTPANS1 DNA vaccine. Four peptide pools (pools 2, 5, 6, and 7) were able to elicit an IFN-γ response with magnitudes higher than or equal to 5 SFC per 5×10^5 cells in splenocytes collected from pcTPANS1-immunized mice without virus challenge, and three other pools (pools 4, 8, and 9) were identified as positive after dengue infection (**Figure 3A**). Individual peptide screening tests revealed 4 immunogenic peptides: N17, N46, N66, and N67 (**Supplementary Figure 3**). Considering the position of each immunogenic region, we selected peptides N17, N46, and N67 for the assays performed with cells from individual BALB/c mice without virus challenge. The IFN-γ responses elicited by all these three NS1-derived peptides were statistically higher when comparing cells from pcTPANS1-vaccinated and pcTPA-inoculated mice (**Figures 3B,C**). The response

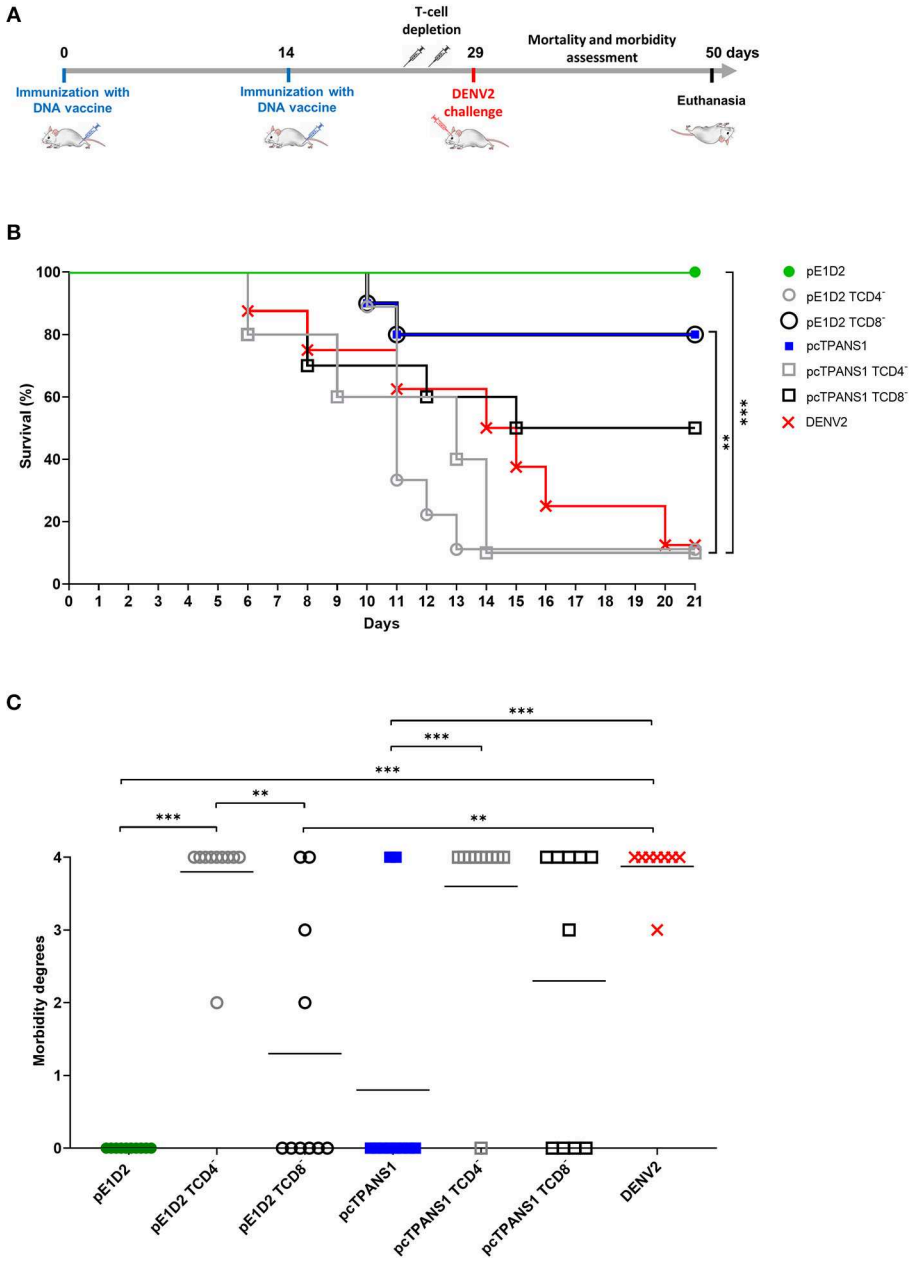

FIGURE 1 | Effects of $CD4^+$ and $CD8^+$ T lymphocyte depletion on survival and morbidity in animals immunized with pE1D2 or pcTPANS1 and challenged with DENV2. Schematic timeline representation of the experiment **(A)**. BALB/c mice intramuscularly immunized with pE1D2 or pcTPANS1 ($n = 10$) were intraperitoneally inoculated with anti-CD4 or anti-CD8 antibodies at days 4 and 2 prior to intracerebral virus challenge (40 x LD_{50} of a neuroadapted DENV2). The control group (DENV) consisted of non-immunized mice ($n = 8$) inoculated with DENV2. All experimental groups were monitored for 21 days after challenge to record the survival rates **(B)** and degree of morbidity **(C)**. Asterisks indicate significant differences using Log-Rank (Mantel-Cox) test in **(B)** or one-way ANOVA test in **(C)** (*$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$). Lines in **(C)** represent the median of each group.

against these NS1-derived peptides also increased after virus challenge, mainly regarding peptides N17 and N67. Besides, we observed that the number of immunogenic peptides increased after virus challenge. Peptides N12, N14-20, N35, N41, N43, N46-50, N64, N66, N67, N71, N72, N76, and N69 were considered positive by applying our selection criteria (**Supplementary Figure 3**). Nevertheless, the ELISPOT assays performed with cells from individual BALB/c mice and the selected NS1-derived peptides revealed that only 7 peptides (N12, N17, N18, N35, N41, N46, and N67) were able to stimulate an IFN-γ response significantly higher in splenocytes collected from mice immunized with pcTPANS1

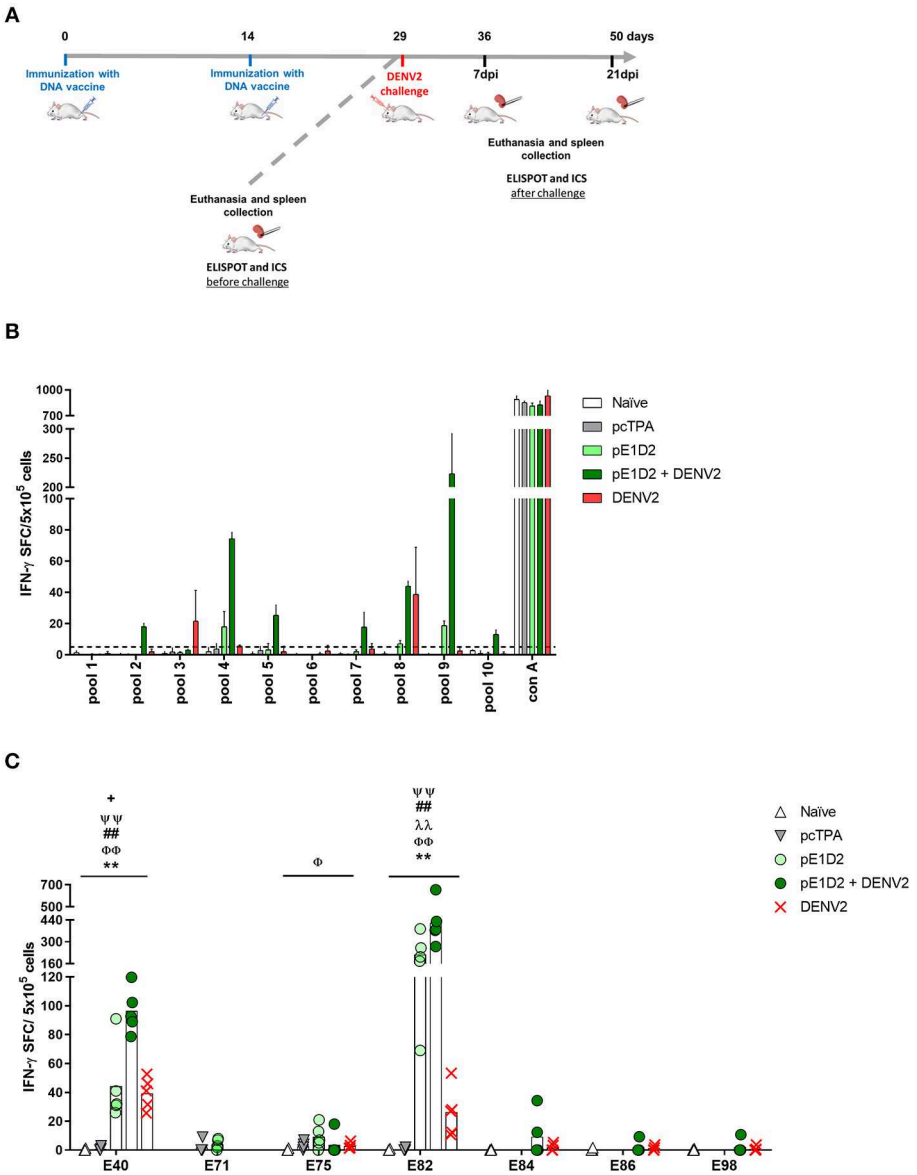

FIGURE 2 | T cell E-derived peptide identification in pE1D2-immunized BALB/c mice by IFN-γ ELISPOT assays. Schematic timeline representation of the experiment **(A)**. Splenocytes isolated from pE1D2-immunized BALB/c mice ($n = 3$, pooled), challenged or not with a neuroadapted DENV2 strain (NGC), were stimulated with E-derived peptides for 18 h, and the number of cells producing IFN-γ was measured by ELISPOT assay. Peptides and cells were evaluated in pools **(B)** and positive peptides were confirmed by tests performed with cells from individual mice ($n = 5$) **(C)**. ELISPOT assays were performed using splenocytes collected 15 days following DNA inoculation (gray and light green bars/dots) or 21 **(B)** and 7 days post-infection **(C)** (dark green bars/dots). Cells from naïve or pcTPA-inoculated mice were used as negative controls **(B,C)**. The horizontal dotted lines represent the cut-off selection point (≥5 SFC/5 × 10^5 cells) **(B)**. Bars represent the mean plus standard deviation of triplicate data in **(B)** and the means of each group in **(C)**. Symbols represent significant differences between groups, using non-parametric two-tailed Mann-Whitney statistical tests: * pcTPA- vs. pE1D2-inoculated mice; # naïve animals vs. pE1D2-immunized mice after DENV2 challenge; + pE1D2-immunized mice vs. pE1D2-immunized animals after DENV2 challenge; *Φ* naïve animals vs. DENV2 challenged mice; λ pE1D2-immunized mice vs. DENV2 challenged animals; Ψ pE1D2-immunized animals challenged with DENV2 vs. DENV2 challenged mice. One symbol: $p < 0.05$; two symbols: $p < 0.001$; three symbols: $p < 0.0001$.

and challenged with DENV2, when comparing to cells obtained from naïve or only DENV2 infected animals (**Figures 3B,C**). Interestingly, only the N17 and N67 peptides were able to induce production of IFN-γ in cells collected from non-immunized animals challenged with DENV2. As expected, stimulation with Con A in all ELISPOT assays performed with cells from pcTPANS1-vaccinated or control animals induced high IFN-γ response.

Phenotyping T Cells That Recognized the Immunogenic E- and NS1-Derived Peptides

We next characterized the peptide-specific responses by investigating IFN-γ and TNF-α production in ICS assays performed with splenocytes collected from BALB/c mice immunized with the pE1D2 or pcTPANS1 DNA vaccines, before

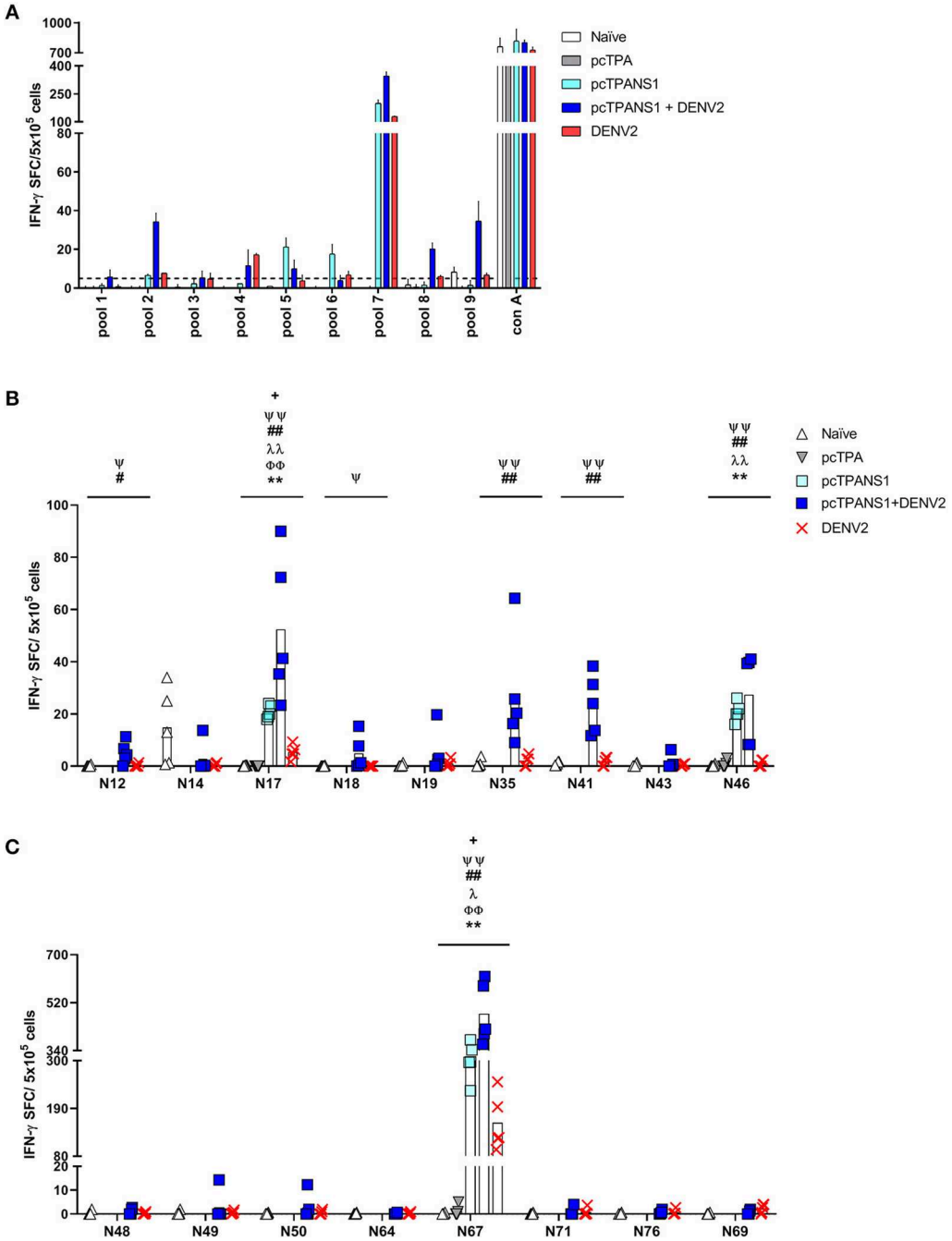

FIGURE 3 | T cell NS1-derived peptide identification in pcTPANS1-immunized BALB/c mice by IFN-γ ELISPOT assays. Immunizations, virus challenge and euthanasia were outlined in **Figure 2A**. Splenocytes isolated from pcTPANS1-immunized BALB/c mice ($n = 3$, pooled), challenged or not with DENV2, were stimulated with NS1-derived peptides for 18 h, and the number of cells producing IFN-γ was measured by ELISPOT assay. Peptides and cells were evaluated in pools **(A)** and positive peptides were confirmed by tests performed with cells from individual mice ($n = 5$) **(B,C)**. ELISPOT assays were performed using splenocytes collected 15 days following DNA inoculation (gray and light blue bars/dots) or 21 **(A)** and 7 days post-infection **(B,C)** (dark blue bars/dots). Cells from naïve or pcTPA-inoculated mice were used as negative controls. The horizontal dotted lines represent the cut-off selection point (≥5 SFC/5 × 10^5 cells) **(B,C)**. Bars represent the mean plus standard deviation of triplicate data in **(A)** and the means of each group in **(B,C)**. Symbols represent significant differences between groups, using non-parametric two-tailed Mann-Whitney statistical tests: ***** pcTPA- vs. pcTPANS1-inoculated mice; # naïve animals vs. pcTPANS1-immunized mice after DENV2 challenge; **+** pcTPANS1-immunized mice vs. pcTPANS1-immunized animals after DENV2 challenge; *Φ* naïve animals vs. DENV2 challenged mice; λ pcTPANS1-immunized mice vs. DENV2 challenged animals; *Ψ* pcTPANS1-immunized animals challenged with DENV2 vs. DENV2 challenged mice. One symbol: $p < 0.05$; two symbols: $p < 0.001$; three symbols: $p < 0.0001$.

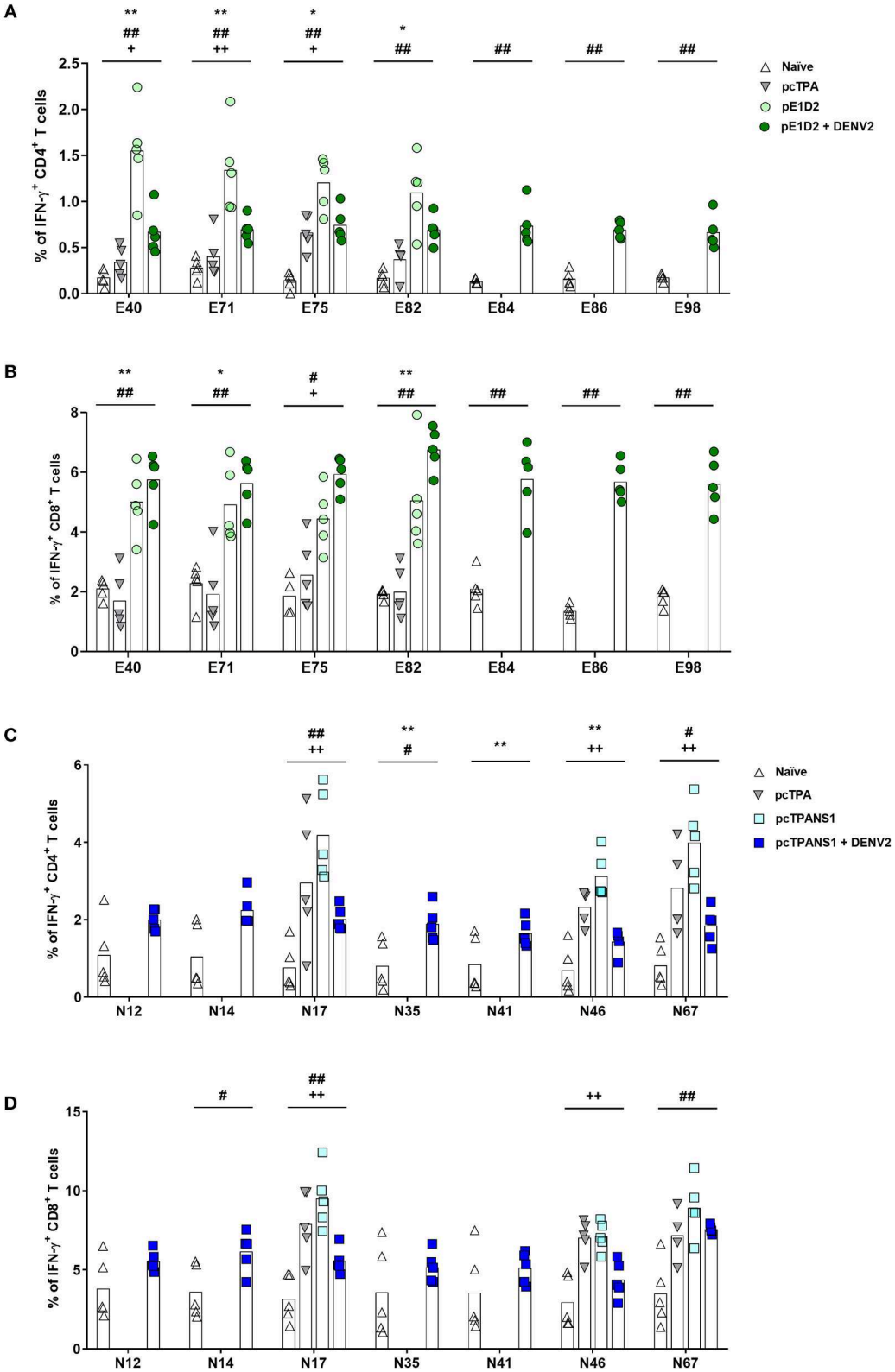

FIGURE 4 | Frequencies of IFN-γ-producing T cells after stimulation with E- or NS1-derived peptides by ICS assays. Immunizations, virus challenge, and euthanasia were outlined in **Figure 2A**. Splenocytes isolated from BALB/c mice immunized with pE1D2 or pcTPANS1 ($n = 5$), challenged or not with DENV2, were stimulated

(Continued)

FIGURE 4 | with E- **(A,B)** or NS1-derived peptides **(C,D)** for 6 h and INF-γ production was detected by ICS assay. Peptides were selected according to previous ELISPOT results, and splenocytes were harvested 15 days following DNA inoculation or 7 days post-infection. Numbers indicate $CD4^+$ **(A,C)** or $CD8^+$ T cells **(B,D)** producing INF-γ as a percentage of total splenocytes. Cells from naïve or pcTPA-inoculated mice were used as negative controls. Bars represent the means of each group. Symbols represent significant differences between groups, using non-parametric two-tailed Mann-Whitney statistical tests: * pcTPA- vs. pE1D2- or pcTPANS1-inoculated mice; # naïve animals vs. pE1D2- or pcTPANS1-immunized mice after DENV2 challenge; + pE1D2- or pcTPANS1-immunized mice vs. pE1D2- or pcTPANS1-immunized animals after DENV2 challenge. One symbol: $p < 0.05$; two symbols: $p < 0.001$; three symbols: $p < 0.0001$.

and after virus challenge. Following a brief stimulation with the peptides selected by our ELISPOT assays, we examined the intracellular cytokine production by $CD4^+$ or $CD8^+$ T cell populations. The flow cytometry gate strategy applied to this analysis is described in **Supplementary Figures 4**, **5**.

The ICS performed with E-derived peptides revealed the involvement of both $CD4^+$ and $CD8^+$ T cells in IFN-γ production by splenocytes collected from pE1D2-immunized mice (**Figures 4A,B**). Almost all tested peptides induced IFN-γ responses in cells obtained from vaccinated animals significantly higher when compared to the response observed in lymphocytes from pcTPA-inoculated mice. The peptide E40 elicited the highest number of $CD4^+$ T cells producing IFN-γ (1.5%), an increase of 4.5 times compared to the control group (**Figure 4A**). This peptide also led to high frequencies of IFN-γ-producing $CD8^+$ T cells collected from immunized mice, although peptide E82 was the most immunogenic, with a 5% IFN-γ^+ $CD8^+$ T cell frequency (2.5-fold increase compared to cells from pcTPA-inoculated animals; **Figure 4B**). In general, the IFN-γ response in $CD4^+$ T cells upon stimulation with all the selected peptides decreased after virus infection (average frequency from 1.5 to 0.7%, with a 2.1-fold decrease), while the frequencies of IFN-γ^+ $CD8^+$ T cells increased (average frequency of 4.7–6.0%, with a 1.3-fold increase; **Figures 4A,B**).

We further investigated the immune response elicited by the NS1-derived peptides by characterizing IFN-γ production by ICS assays performed with splenocytes from pcTPANS1-vaccinated mice. We also observed the production of this cytokine by $CD4^+$ and $CD8^+$ T cells. Although $CD4^+$ T lymphocytes responded to almost all tested peptides, $CD8^+$ T cells responded only to some of them (peptides N14, N17, and N67) after the dengue infection (**Figures 4C,D**). In terms of statistical significance, the peptide N67 was more immunogenic leading to a 4% IFN-γ^+ $CD4^+$ T cell frequency (1.4-fold increase compared to the control pcTPA; **Figure 4C**). Peptide N17 also induced high frequencies of $CD4^+$ T cells expressing IFN-γ, although not statistically significant compared to the pcTPA group. Overall, in the context of the pcTPANS1 DNA vaccine, the percentage of T cells expressing IFN-γ seemed to have been reduced after the infection with DENV2 (**Figures 4C,D**).

We also evaluated TNF-α production upon stimulation with the same E- and NS1-derived peptides. After the infection with DENV2, all E-derived peptides induced a TNF-α response in $CD4^+$ and $CD8^+$ T cells collected from pE1D2-vaccinated animals, except for $CD8^+$ T lymphocytes stimulated with the peptide E98 (**Figures 5A,B**). Without the virus challenge, on the other hand, an increase in the TNF-α production was not statistically detected, except for $CD8^+$ T cells upon stimulation with peptide E71. Concerning the pcTPANS1 vaccine, a remarkable difference in levels of TNF-α production was not detected by the ICS assays using cells from vaccinated animals, challenged or not with DENV2 (**Figures 5C,D**). The only peptides able to induce TNF-α expression were N17 in $CD4^+$ T cells and N67 in $CD8^+$ T cells.

Results from the screening of E- and NS1-derived peptides and production of IFN-γ and TNF-α are summarized in **Table 2**, as well as their amino acid sequences and location on the E and NS1 proteins.

T Cell Epitope Map of E and NS1 Proteins in C57BL/6 Mice

In addition to studies with BALB/c animals, we also investigated the immunodominance of E- and NS1-derived peptides in the context of a different MHC haplotype expression. For this purpose, we immunized C57BL/6 mice with pE1D2 and pcTPANS1 DNA vaccines and evaluated the IFN-γ response by ELISPOT assays using the same E and NS1 peptide libraries. Following the low stringent analysis performed before, peptide pools that elicited a magnitude of response higher or equal to 5 SFC per 5×10^5 cells were considered positive.

A total of 4 E-derived peptide pools were identified as positive for stimulating IFN-γ production (pools 1, 6, 7, and 9) and were selected for individual peptide screenings (**Figure 6A**). Peptides E01, E02, E59, E60, E65-67, E87, and E88 were able to induce an IFN-γ response in splenocytes collected from animals vaccinated with pE1D2 (**Figure 6B**).

Similarly, 5 NS1-derived peptide pools (pools 1, 2, 4, 6, and 7) were able to stimulate IFN-γ production in cells collected from C57BL/6 mice immunized with the pcTPANS1 DNA vaccine (**Figure 7A**). Individual peptide screenings revealed that peptides N08, N16-18, N20, N33-36, N56, and N62 elicited IFN-γ expression in splenocytes obtained from pcTPANS1-immunized C57BL/6 mice (**Figure 7B**). These results revealed that peptides N17 and N35 were immunogenic in the context of the two different evaluated MHC haplotypes, i.e., in BALB/c and in C57BL/6 mice.

DISCUSSION

In this study, we evaluated the contribution of $CD4^+$ and $CD8^+$ T cells for the protection induced in mice by DNA vaccines encoding the ectodomain of the envelope (pE1D2) and the non-structural 1 (pcTPANS1) proteins from DENV2. We also identified the T cell immunogenic peptides in vaccinated animals by the production of IFN-γ and TNF-α. We have described the DNA vaccines construction elsewhere (33, 40) and in the present work, we confirmed protection elicited in immunocompetent BALB/c mice challenged with a

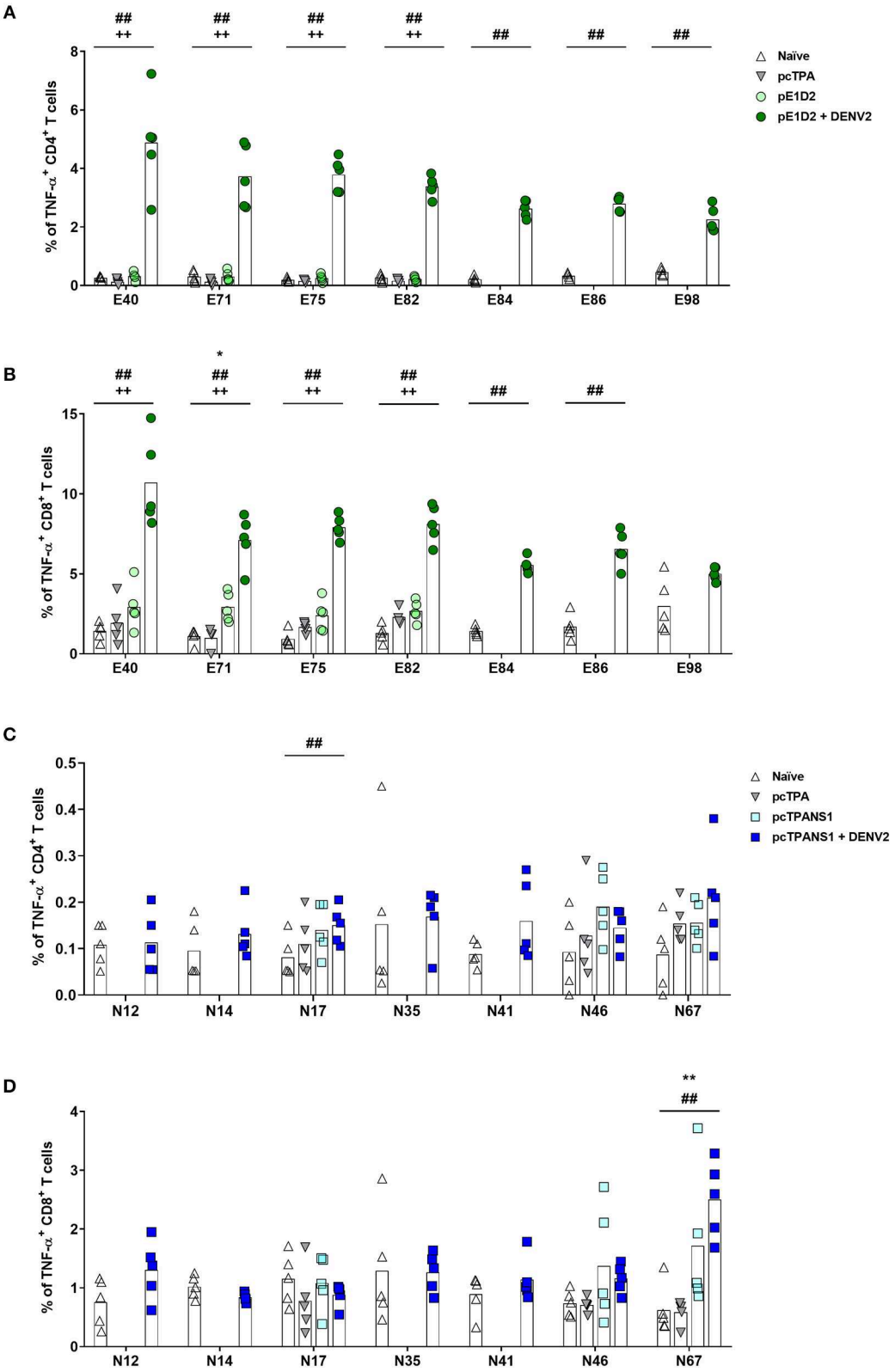

FIGURE 5 | Frequencies of TNF-α-producing T cells after stimulation with E- or NS1-derived peptides by ICS assays. Immunizations, virus challenge, and euthanasia were outlined in **Figure 2A**. Splenocytes isolated from BALB/c mice immunized with pE1D2 or pcTPANS1 ($n = 5$), challenged or not with DENV2, were stimulated

(Continued)

FIGURE 5 | with E- **(A,B)** or NS1-derived peptides **(C,D)** for 6 h and TNF-α production was detected by ICS assay. Peptides were selected according to previous ELISPOT results, and splenocytes were harvested following DNA inoculation or 7 days post infection. Numbers indicate $CD4^+$ **(A,C)** or $CD8^+$ T cells **(B,D)** producing TNF-α as a percentage of total splenocytes. Cells from naïve or pcTPA-inoculated mice were used as negative controls. Bars represent the means of each group. Symbols represent significant differences between groups, using non-parametric two-tailed Mann-Whitney statistical tests: * pcTPA- vs. pE1D2- or pcTPANS1-inoculated mice; # naïve animals vs. pE1D2- or pcTPANS1-immunized mice after DENV2 challenge; + pE1D2- or pcTPANS1-immunized mice vs. pE1D2- or pcTPANS1-immunized animals after DENV2 challenge. One symbol: $p < 0.05$; two symbols: $p < 0.001$; three symbols: $p < 0.0001$.

TABLE 2 | Characteristics and summary results of E and NS1 positive peptides.

			IFN-γ ELISPOT Score			% of IFN-γ^+ T cells				% of TNF-α^+ T cells			
Envelope peptides	**Sequence**	**Location**	**pE1D2**	**pE1D2 +DENV2**	**DENV2**	**pE1D2**		**pE1D2 +DENV2**		**pE1D2**		**pE1D2 + DENV2**	
						$CD4^+$	**$CD8^+$**	**$CD4^+$**	**$CD8^+$**	**$CD4^+$**	**$CD8^+$**	**$CD4^+$**	**$CD8^+$**
E40	KHGKEIKITPQSSIT	157–171	++	+++	++	1.55	5.01	0.67	5.76	0.32	2.94	4.89	10.7
E71	GHLKCRLRMDKLQLK	281–295	NS	—	—	1.34	4.92	0.69	5.63	0.31	2.92	3.73	7.11
E75	MSYSMCTGKFKVVKE	297–311	NS	NS	NS	1.21	4.45	0.75	5.94	0.25	2.4	3.79	7.91
E82	QYEGDGSPCKIPFEI	325–339	++++	++++	++	1.1	5.06	0.69	6.76	0.22	2.67	3.39	8.12
E84	CKIPFEIMDLEKRHV	333–347	—	NS	NS	—	—	0.74	5.77	—	—	2.62	5.55
E86	DLEKRHVLGRLITVN	341–355	—	NS	NS	—	—	0.69	5.68	—	—	2.8	6.55
E98	QLKLNWFKKGSSIVI	386–400	—	NS	NS	—	—	0.66	5.6	—	—	2.25	5.01
NS1 peptides	**Sequence**	**Location**	**pcTPANS1**	**pcTPANS1 + DENV2**	**DENV2**	**pcTPANS1**		**pcTPANS1 + DENV2**		**pcTPANS1**		**pcTPANS1 + DENV2**	
						$CD4^+$	**$CD8^+$**	**$CD4^+$**	**$CD8^+$**	**$CD4^+$**	**$CD8^+$**	**$CD4^+$**	**$CD8^+$**
N12	AIQKAHEEGICGIRS	45–59	—	+	NS	—	—	2.0	5.55	—	—	0.11	1.3
N14	GICGIRSVTRLENLM	53–67	—	NS	NS	—	—	2.24	6.15	—	—	0.13	0.84
N17*	NLMWKQITPELNHIL	65–79	++	+++	+	4.19	9.5	2.02	5.56	0.14	1.08	0.15	0.88
N35*	GPETAECPNTNRAWN	137–151	—	++	NS	—	—	1.89	5.17	—	—	0.17	1.26
N41	GVFTTNIWLKLREKQ	161–175	—	++	NS	—	—	1.65	5.13	—	—	0.16	1.14
N46	SKLMSAAIKDNRAVH	181–195	++	++	NS	3.13	7.12	1.43	4.38	0.19	1.37	0.14	1.16
N67	AGPWHLGKLEMDFDF	265–279	++++	++++	++++	3.99	8.92	1.85	7.56	0.15	1.71	0.21	2.5

The location of positive peptides was determined according to the amino acid sequence of E and NS1 proteins from NGC DENV2. Results from IFN-γ ELISPOT assays performed with cells from individual mice are displayed according to the following arbitrary scale: (+): ≥ 5 to 20; (++): 21 to 50, (+++): 51 to 100, (++++): ≥100 SFC/5 × 10^5 cells. % of IFN-γ^+ and TNF-α^+ T cells are represented as mean ICS frequencies. (*) Peptides also positive in IFN-γ ELISPOT with cells from C57BL/6 mice. (—): means non-existent data; (NS): non-statistical.

neuroadapted NGC DENV2. Almost all immunized animals survived virus infection with none or low morbidity degrees. The murine model of BALB/c mice inoculated with DENV2 by the i.c. route is well-established in our laboratory and provides a straightforward readout parameter for vaccine testing. Therefore, in order to expand the knowledge about the different arms of the immune system that may act on the protection against DENV, we investigated in BALB/c mice the role of the T cell response elicited by the DNA vaccines we have constructed. We observed that the $CD4^+$ T lymphocytes response was essential for protection generated by both vaccines, although depletion of $CD8^+$ T cells also impacted in survival and morbidity after virus challenge. In keeping with these results, we have previously shown that pcTPANS1-vaccinated animals exhibited a significant increase of activated $CD4^+$ and $CD8^+$ T cells in spleen and blood circulation after the DENV2 challenge (44).

Our group has also reported the importance of $CD4^+$ T cells in pcTPANS1-immunized mice in a study showing that these lymphocytes in association with anti-NS1 antibodies are fundamental for protection (39). In addition, the $CD4^+$ T cells induced by the pcTPANS1 vaccine seem to act by a different mechanism than helping B-cell antibody production. In fact, our previous study with adoptive transfer of CD4+ T cells combined with NS1 antiserum, both obtained from vaccinated mice, led to the protection of recipient BALB/c animals after challenge with DENV2, with survival rates not significantly different from those observed in pcTPANS1-immunized mice (39). Since recipient animals did not receive B cells, only $CD4^+$ T cells and antibodies, the mechanism involved in the protection mediated by these T cells must be other than helping B lymphocytes.

On the other hand, several studies have pointed out the importance of $CD8^+$ T cells in controlling DENV infection, either in mouse models or in humans (16, 17, 19, 45–47). Yet, other reports suggest the involvement of $CD4^+$ T lymphocytes in the protection provided by experimental vaccines (25, 28). Yauch and colleagues showed that $CD8^+$ T cells played an essential role

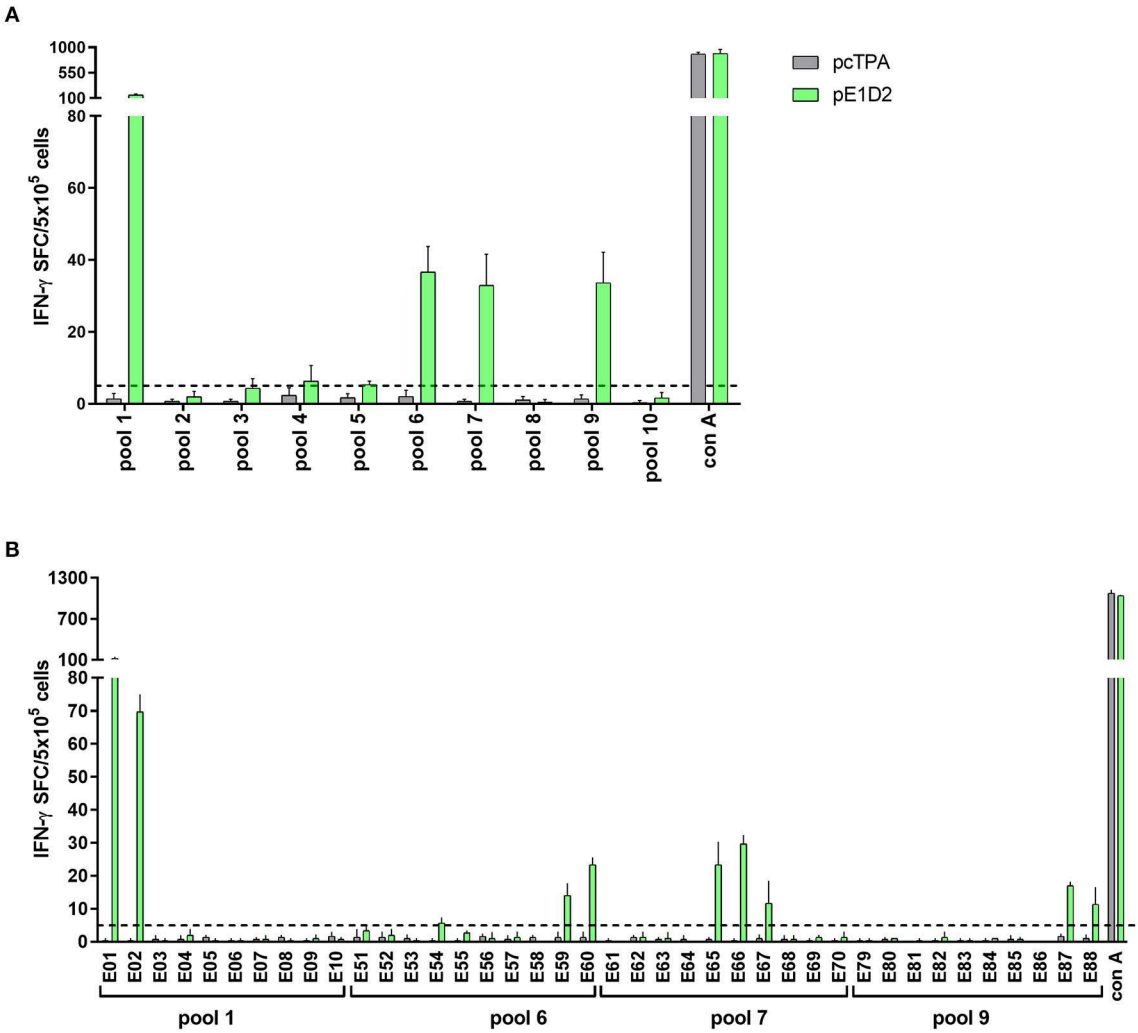

FIGURE 6 | T cell E-derived peptide identification in pE1D2-immunized C57BL/6 mice by IFN-γ ELISPOT assays. Splenocytes from pE1D2-immunized C57BL/6 mice ($n = 3$, pooled) were harvested 15 days following DNA inoculation, stimulated with E-derived peptides for 18 h, and the number of cells producing IFN-γ was measured by ELISPOT assay. Peptides and cells were evaluated in pools **(A)** and positive pools were selected for individual peptide screening **(B)**. Cells from pcTPA-inoculated mice were used as a negative control. The horizontal dotted lines represent the cut-off selection point (≥ 5 SFC/5 × 10^5 cells). Bars represent the mean plus standard deviation of triplicate data.

in viral clearance, while $CD4^+$ T lymphocytes were not required to control primary DENV2 infection in IFN-α/β$R^{-/-}$ C57BL/6 mice. However, immunization with $CD4^+$ T cell epitopes conferred protection by reducing viral load after challenge, thus supporting the importance of $CD4^+$ T cell induction by vaccination (25). Besides, activation of cytotoxic $CD4^+$ T cells has been reported after DENV infection, which seems to be associated with protective immunity (18, 48). Additionally, $CD4^+$ and $CD8^+$ T cell epitope reactivities against DENV2 was investigated in patients experiencing secondary DENV infection (27). Authors observed that $CD8^+$ T cells preferentially targeted epitopes contained in the NS3 and NS5 proteins, while $CD4^+$ T lymphocytes recognized mainly epitopes derived from the virus envelope, capsid, and NS1 proteins. Nevertheless, another study suggested that the hierarchy of immunodominance between the different DENV proteins depends on the virus serotype (47).

Mouse models have been essential in determining the T cell epitopes involved in the protection and/or the pathogenesis of dengue infections (46). Our study conducted in an immunocompetent mouse model revealed that the pE1D2 and pcTPANS1 DNA vaccines were able to induce T cell responses targeting both E and NS1 proteins. The T cell epitopes were screened by ELISPOT and ICS assays for IFN-γ production in splenocytes from vaccinated mice, using 15-mer synthetic peptide libraries with 11 amino acid residues overlapping, spanning the E and NS1 proteins. The sets of peptides of 15 amino acids length were chosen since they can efficiently stimulate both $CD4^+$ and $CD8^+$ T cells (49). The assays to measure IFN-γ response were selected since several reports

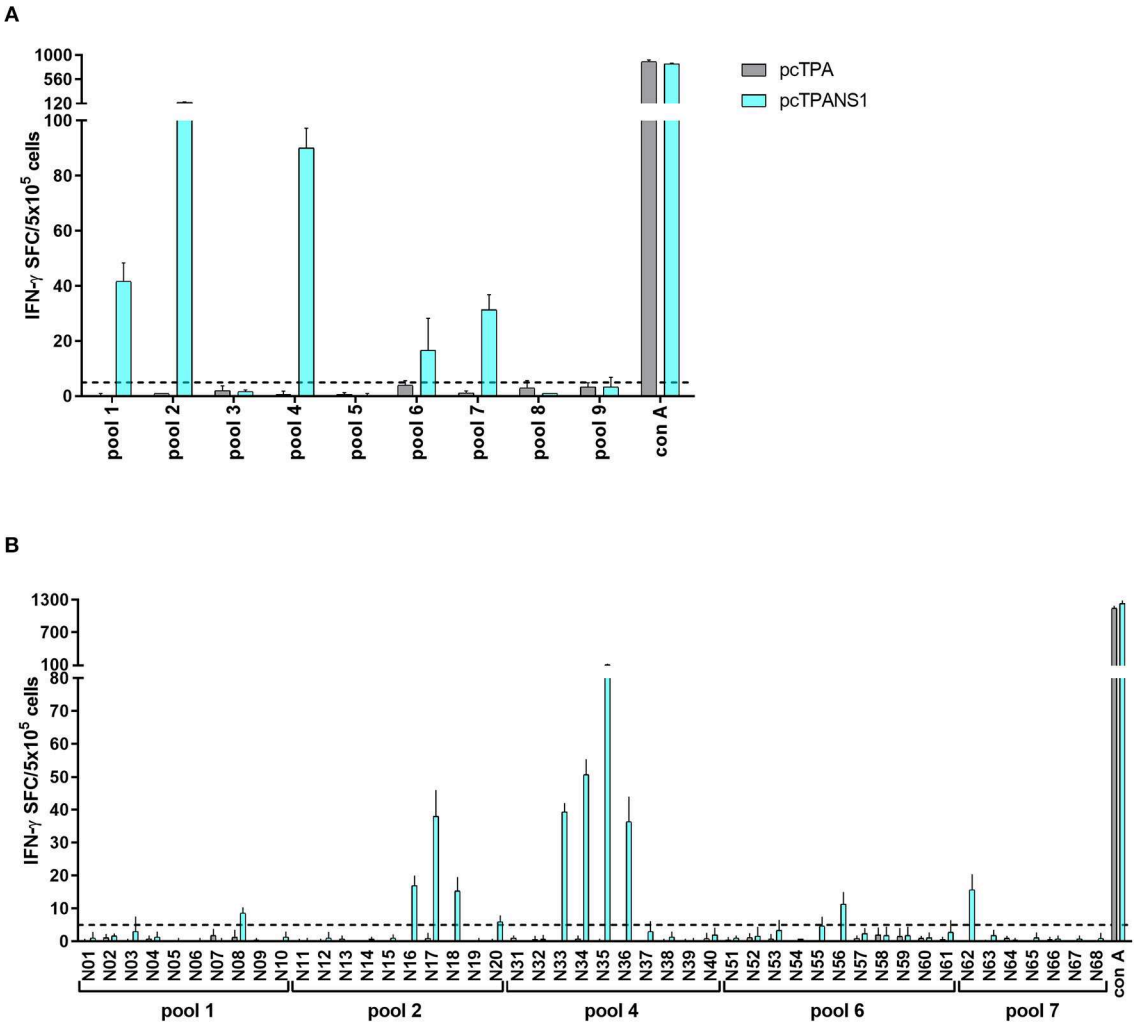

FIGURE 7 | T cell NS1-derived peptide identification in pcTPANS1-immunized C57BL/6 mice by IFN-γ ELISPOT assays. Splenocytes from pcTPANS1-immunized C57BL/6 mice ($n = 3$, pooled) were harvested 15 days following DNA inoculation, stimulated with NS1-derived peptides for 18 h, and the number of cells producing IFN-γ was measured by ELISPOT assay. Peptides and cells were evaluated in pools **(A)** and positive pools were selected for individual peptide screening **(B)**. Cells from pcTPA-inoculated mice were used as a negative control. The horizontal dotted lines represent the cut-off selection point (≥5 SFC/5 × 10^5 cells). Bars represent the mean plus standard deviation of triplicate data.

indicate that the production of this cytokine contributes to protection against DENV in animal models as well as in humans (25, 50, 51). The screening of T cell epitopes was also assessed in C56BL/6 mice immunized with the pE1D2 and pcTPANS1 vaccines in order to investigate the T cell response in the context of a different MHC haplotype expression. However, this mouse strain is not susceptible to our neuroadapted DENV2 sample. Therefore, experiments were not performed after virus challenge.

We identified 4 and 7 E-derived epitopes in pE1D2-vaccinated BALB/c animals before and after virus challenge, respectively (**Table 2**). Five of these peptides (E75, E82, E84, E86, and E98) are located in domain III of the E protein. This domain is also an important target for induction of neutralizing antibodies during virus infection (23), which is another fundamental arm of the immune response against DENV and therefore an essential region for vaccine designs. Besides, peptides E40 and E82 were immunodominant in our study, leading to a significantly higher IFN-γ production in vaccinated animals, challenged or not with DENV2, as well as in mice only infected with the virus. Peptide E82 contains the sequence SPCKIPFEI, which was the first immunodominant epitope described for H-2^d-restricted $CD8^+$ T cells in DENV2 infected mice (BALB/c) (52). As far as we know, our study is the first to identify the region present in the E40 peptide as immunodominant for T cell response.

Studies using different murine models have also identified an immunogenic T-cell epitope contained in peptides E86/E87 (amino acids 345–359) (28, 53). Chen and colleagues described a $CD4^+$ T cell epitope between amino acids 349–363 in BALB/c mice immunized with a tetravalent DNA vaccine based on the domain III of the DENV envelope protein (28). On the other

hand, Li and colleagues observed that one epitope present in the peptide E87 (between amino acids 345–359) can stimulate IFN-γ production in cells obtained from C57BL/6j mice infected with DENV2 (53). In our study, analyzing peptides recognized by T cells from C57BL/6 mice immunized with the pE1D2 DNA vaccine, the peptide E87 was also immunogenic for induction of IFN-γ production, although the response to this peptide was not immunodominant. Thus, the region comprising E86/E87 peptides is able to stimulate lymphocytes from mice with the distinct haplotypes H-2^d and H-2^b.

Further reports, using transgenic mice with human leucocyte antigen (HLA) and infected with DENV, also identified a $CD8^+$ T cell epitope contained in E86/E87 sequence, in addition to another epitope present in the peptide E75, likewise immunogenic in pE1D2-immunized BALB/c animals (26, 54). Regarding human T cell recognized epitopes, most of the E epitopes mapped in pE1D2-vaccinated animals have also been identified in DENV-infected patients (E71, E75, E82, E84, E86, and E98) and in volunteers who received a live attenuated tetravalent vaccine (E75) (17, 55). Therefore, our results together with published data reveal the potential of these E-derived regions to induce a T cell response that may be involved in protection against DENV.

On the other hand, studies on NS1 are less abundant. Our mapping of NS1 epitopes identified 3 and 7 immunogenic peptides in pcTPANS1-vaccinated mice, before and after DENV2 infection, respectively (**Table 2**). The N67 peptide was the immunodominant epitope, inducing high IFN-γ production, in agreement with another report that evaluated the response induced in BALB/c mice immunized with an adenovirus-based vaccine containing the DENV2 NS1 gene (56). Authors identified the $CD8^+$ T cell immunodominant epitope AGPWHLGKL, which is contained in the N67 peptide and is highly conserved among strains of the four DENV serotypes. In addition, a screening of T cell epitopes derived from structural and non-structural proteins of DENV3 in HLA-transgenic mice and validated using T cells from human DENV3 immune volunteers detected an epitope contained in N17/N18 peptides that activated T-cell memory (57). In our studies, the N17 peptide was immunogenic in BALB/c animals before and after virus infection, as well as in C57BL/6 mice. Another evaluation using DENV2-infected HLA-transgenic mice revealed one peptide able to induce high levels of IFN-γ, which sequence is contained in peptide N41 (54). Moreover, human studies in Nicaragua with subjects previously infected with DENV, and in volunteers who received a live attenuated tetravalent vaccine, mapped some NS1-derived T cell epitopes, including sequences contained in the peptides N17, N41, and N67 (17, 55). Besides, one report with adult patients experiencing secondary DENV infection identified $CD8^+$ T cell epitopes by ELISPOT assay and ICS contained in N41 and N48 peptide sequences (27).

Apart from evaluating the IFN-γ response induced by the screened E- and NS1-derived peptides, we also investigated TNF-α production in splenocytes from immunized animals stimulated with the previously selected peptides. Only one peptide (E71) was able to induce TNF-α production in pE1D2-vaccinated BALB/c mice without virus challenge. In contrast, almost all tested E-derived peptides led to a TNF-α response after the DENV infection either in $CD4^+$ or $CD8^+$ T cells. Elevated levels of TNF-α and soluble TNF-α receptors have been reported in severe cases of dengue (58, 59). Since this cytokine is involved in cellular apoptosis and increased vascular permeability, its association with dengue severity has been extensively studied, reviewed by Pang et al. (60), Srikiatkhachorn et al. (61), and Kuczera et al. (62). However, the correlation between TNF-α production and the outcome of dengue disease is still uncertain. Some reports analyzing dengue-infected patients have not observed a significant difference between TNF-α levels from dengue fever and severe dengue (63–65).

Regarding the NS1 protein, we only observed a significant increase in TNF-α^+CD8^+ T cells from pcTPANS1-immunized mice after stimulation with the peptide N67. Interestingly, $CD4^+$ and $CD8^+$ T lymphocytes from pcTPANS1-vaccinated animals after virus challenge maintained ordinary TNF-α production by stimulation with almost all NS1-derived peptides. Considering the increase of TNF-α as a deleterious effect of DENV infection, our results regarding the pcTPANS1 vaccine suggest a protective effect by inhibiting the production of such cytokine. On the other hand, analysis of polyfunctional T cell responses has drawn attention for vaccine development against flavivirus, suggesting that T lymphocytes producing multiple cytokines including IFN-γ, TNF-α, CD107a, and IL-2 are the most effective in controlling the virus (17, 18, 48, 66, 67).

In the present work, we only investigated protection against homo-typic infection. It is well-known that the severe form of the dengue disease is usually associated with hetero-typic infection, and it is a consensus that a dengue vaccine must be protective against the four DENV serotypes. Therefore, other studies will be necessary in order to evaluate the cross-reaction of the identified peptides against other DENV serotypes and its association with protection or pathogenesis. Besides, it will be likewise important to investigate whether the T cell responses induced by our DNA vaccines based on the envelope and NS1 proteins are multifunctional, as well as to examine the involvement of the identified peptides in the protection conferred by these vaccines. Our previous studies with the pE1D2 and pcTPANS1 vaccines also pointed out the importance of antibodies in protection, including the production of neutralizing antibodies against the E protein. Taking together with the results shown in the present work, we believe that the efficiency of these DNA vaccines is the combination of both arms of the immune system, the humoral and cellular immune response. Consequently, the knowledge of dominant epitopes targeted by $CD4^+$ and $CD8^+$ T cells might be essential for elucidating the mechanism involved in protection, resulting in more effective vaccines against dengue as well as against other flaviviruses.

ETHICS STATEMENT

This study was carried out under ethical principles in animal experimentation stated by the Brazilian College of Animal Experimentation and approved by the Ethical Committee of Animal Use of Oswaldo Cruz Institute in Fiocruz (CEUA-IOC approval ID: L039/2015).

AUTHOR CONTRIBUTIONS

PP, MA, SC, and AA conceived and designed the experiments. PP, MA, AP, LL, KQ, BP, and SC performed the experiments. PP, MA, AV, SC, and AA analyzed the data. PP, MA, SC, and AA wrote the paper. AV revised the paper. All authors approved the final copy of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01522/full#supplementary-material

Supplementary Figure 1 | $CD4^+$ and $CD8^+$ T cell depletion in BALB/c mice. Representative flow cytometry dot plots displaying $CD4^+$ and $CD8^+$ T cells frequencies in blood samples from T-cell depleted mice. Animals treated intraperitoneally with anti-CD4 or anti-CD8 antibodies were evaluated on the challenge day to verify the success of the depletion protocol. Blood samples were stained with anti-CD3 PE, anti-CD4 APC, and anti-CD8 FITC. The $CD3^+$ population was backgated on FSS x SSC dot plot to orient the lymphocyte gate. On lymphocyte gate, CD4 x CD8 dot plots were evaluated. Cells from non-depleted naïve mice were stained or incubated with isotype antibodies and were used as positive and negative control samples.

Supplementary Figure 2 | Peptide screening by evaluation of IFN-γ production in splenocytes isolated from pE1D2-immunized BALB/c mice, challenged or not with DENV2. Positive E-derived peptide pools, previously evaluated by ELISPOT assay, were selected for individual peptide screening. Screening of peptides contained in pools 2, 4 and 5 **(A)**, and in pools 7, 8, 9 and 10 **(B)**. Splenocytes were isolated from BALB/c mice 15 days following the DNA inoculation (gray and light green bars) or 21 days post-infection (dark green bars), stimulated with E-derived peptides for 18 h, and the number of cells producing IFN-γ was measured by ELISPOT assay. Cells from naïve or pcTPA-inoculated mice were used as negative control. The horizontal dotted lines represent the cut-off selection point (≥5 SFC/5 × 10^5 cells). Bars represent the mean plus standard deviation of triplicate data.

Supplementary Figure 3 | Peptide screening by evaluation of IFN-γ production in splenocytes isolated from pcTPANS1-immunized BALB/c mice, challenged or not with DENV2. Positive NS1-derived peptide pools, previously evaluated by ELISPOT assay, were selected for individual peptide screening. Screening of peptides contained in pools 2, 4, 5 and 6 **(A)**, and in pools 7, 8 and 9 **(B)**. Splenocytes were isolated from BALB/c mice 15 days after the DNA inoculation (gray and light blue bars) or 21 days post-infection (dark blue bars), stimulated with NS1-derived peptides for 18 h, and the number of cells producing IFN-γ was measured by ELISPOT assay. Cells from naïve or pcTPA-inoculated mice were used as negative control. The horizontal dotted lines represent the cut-off selection point (≥5 SFC/5 × 10^5 cells). Bars represent the mean plus standard deviation of triplicate data.

Supplementary Figure 4 | IFN-γ ICS flow cytometry analysis to evaluate $CD4^+$ and $CD8^+$ T cell populations from pE1D2 and pcTPANS1-immunized mice, challenged or not with DENV2. Splenocytes previously stimulated with E or NS1-derived peptides were stained with anti-CD3 PE, anti-CD4 APC, and anti-CD8 PerCP followed by intracellular staining with anti-IFN-γ Alexa Fluor 488. We backgated the $CD3^+$ population on an FSS x SSC dot plot to construct the lymphocyte gate. The IFN-γ-producing $CD4^+$ or $CD8^+$ T cells were analyzed on $CD4^+CD3^+$ gate. Staining example of $CD4^+$ or $CD8^+$ T cells for IFN-γ from a pE1D2-immunized mouse.

Supplementary Figure 5 | TNF-α ICS flow cytometry analysis to evaluate $CD4^+$ and $CD8^+$ T cell populations from pE1D2 and pcTPANS1-immunized mice, challenged or not with DENV2. Splenocytes previously stimulated with E or NS1-derived peptides were stained with anti-CD3 PE, anti-CD4 PerCP, and anti-CD8 FITC followed by intracellular staining with anti-TNF-α Alexa Fluor 647. We backgated the $CD3^+$ population on an FSS x SSC dot plot to construct the lymphocyte gate. The TNF-α-producing $CD4^+$ or $CD8^+$ T cells were analyzed on $CD4^+CD3^+$ gate. Staining example of $CD4^+$ or $CD8^+$ T cells for TNF-α from a pE1D2-immunized and challenged mouse.

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Characterization of Magnitude and Antigen Specificity of HLA-DP, DQ, and DRB3/4/5 Restricted DENV-Specific CD4+ T Cell Responses

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Background: Dengue Virus (DENV) associated disease is a major public health problem. Assessment of HLA class II restricted DENV-specific responses is relevant for immunopathology and definition of correlates of protection. While previous studies characterized responses restricted by the HLA-DRB1 locus, the responses associated with other class II loci have not been characterized to date. Accordingly, we mapped HLA-DP, DQ, and DRB3/4/5 restricted DENV-specific CD4 T cell epitopes in PBMCs derived from the DENV endemic region Sri Lanka.

Methods: We studied 12 DP, DQ, and DRB3/4/5 alleles that are commonly expressed and provide worldwide coverage >82% for each of the loci analyzed and >99% when combined. CD4+ T cells purified by negative selection were stimulated with pools of HLA-predicted binders for 2 weeks with autologous APC. Epitope reactive T cells were enumerated using IFNγ ELISPOT assay. This strategy was previously applied to identify DRB1 restricted epitopes. In parallel, membrane expression levels of HLA-DR, DP, and DQ proteins was assessed using flow cytometry.

Results: Epitopes were identified for all DP, DQ, and DRB3/4/5 allelic variants albeit with magnitudes significantly lower than the ones previously observed for the DRB1 locus. This was in line with lower membrane expression of HLA-DP and DQ molecules on the PBMCs tested, as compared to HLA-DR. Significant differences between loci were observed in antigen immunodominance. Capsid responses were dominant for DRB1/3/4/5 and DP alleles but negligible for the DQ alleles. NS3 responses were dominant in the case of DRB1/3/4/5 and DQ but absent in the case of DP. NS1 responses were prominent in the case of the DP alleles, but negligible in the case of DR and DQ. In terms of epitope specificity, repertoire was largely overlapping between DRB1 and DRB3/4/5, while DP and DQ loci recognized largely distinct epitope sets.

Conclusion: The HLA-DP, DQ, and DRB3/4/5 loci mediate DENV-CD4 specific immune responses of lower magnitude as compared to HLA-DRB1, consistent with their lower levels of expression. The responses are associated with distinct and characteristic patterns of immunodominance, and variable epitope overlap across loci.

Keywords: DENV, CD4+T cells, HLA-DP, HLA-DQ, HLA-DRB3/4/5, adaptive immunity

INTRODUCTION

The burden of DENV disease has dramatically increased worldwide in the past decades. Recent epidemiological data estimate that almost 400 million DENV infections occurs per year, of which 25% are symptomatic and associated with clinical presentations of various severity (1). These numbers clearly highlight the health threat that DENV represents worldwide (2, 3).

DENV-specific immune responses have been extensively studied in past years, highlighting key roles of both B and T cell responses during infection. Whether T cells play a greater role in disease protection or pathogenesis has been debated. Several recent studies have shown that both CD4 and CD8 T cells can exert a protective effect in the context of DENV infections (4–10). This is in contrast to an earlier hypothesis that suboptimal cross-reactive memory T cells may impair viral control upon secondary heterologous infections (11–13).

To enable comprehensive worldwide assessment of the magnitude, antigen specificity, and epitope repertoire associated with DENV-specific T cells, several studies have been carried out in the general populations of endemic areas and following vaccination. At the level of CD8 T cell responses, responses restricted by more than 30 different HLA–A and B allelic variants have been defined (6, 14–18). Several studies defined HLA class II-restricted responses both in the endemic areas of Sri Lanka and Nicaragua, and following vaccination (7, 8, 15, 17, 19–23).

HLA class II molecules are heterodimers composed of an alpha and beta chain. DRB1 is a highly polymorphic locus which encodes a beta chain which pairs with an essentially monomorphic alpha chain. The same alpha chain also pairs with the beta chains produced by the DRB3/4/5 locus. Similarly, additional alpha and beta chains are encoded by the DP and DQ loci (24, 25). Thus, in a heterozygote individual, a total of eight different HLA class II heterodimers can be expressed (two DRB1/DRA, two DRB3/4/5/DRA, two DP, and two DQs).

Our previous study of class II restricted DENV epitopes focused on responses associated with HLA DRB1 allelic variants since DRB1 is the most commonly studied human class II molecule, and DRB1 restricts the majority of defined epitopes in the literature. However, this initial focus was by definition incomplete, since it did not address the contribution of the DRB3/4/5, DP, and DQ loci. A general bias toward DRB1-restriction may be observed throughout the scientific literature. For instance, querying the Immune Epitope Database (IEDB) (https://www.iedb.org) that collects a list of experimental data on B and T cell epitopes, reveals that 1,027, 0, 8, and 0 instances of DENV epitopes restricted by HLA-DRB1, -DRB3/4/5, -DP, and -DQ loci, respectively. Thus, data available regarding DENV-epitopes restricted for DRB3/4/5, DP, and DQ loci are scarce and this represents an important gap of knowledge. In this study, we sought to characterize the contribution of DRB3/4/5, DP, and DQ and loci in DENV-specific CD4 T cell immune responses and compare it to the previously defined DRB1 locus.

MATERIALS AND METHODS

Human Blood Samples

A total of 120 peripheral blood samples were obtained from healthy adult blood donors from the National Blood Center, Ministry of Health, Colombo, Sri Lanka in an anonymous fashion as previously reported (14). Both sexes were represented and donors ranged from 18 to 60 years of age. All protocols described herein, were approved by the institutional review boards of both LJI and Medical Faculty, University of Colombo (serving as NIH approved IRB for Genetech). Blood collection and processing was performed, as previously described (14). The blood samples were obtained before DENV serology screening. Serology screening (described in depth in the section below) identified 96 samples collected and used for this study with broad neutralization profile, suggesting that the donors had experienced one or more DENV infections prior to blood donation. Donor serology informations are shown in **Supplementary Table S1**.

Serology

Serum neutralization assays were performed in Vero cells in all the donors of this study as previously reported (26). Briefly, Vero-81 cells (2×10^4 cells/well, ATCC no. CCL-81) were seeded on 96-well flat-bottom tissue culture-treated plates (Greiner Sigma Aldrich), and incubated at 37°C overnight. Equal volumes of DENV viruses (DENV-1 West Pac 74, DENV-2 S-16803, DENV-3 CH54389, and DENV-4 TVP-360) and eight 4-fold serial dilutions of heat inactivated human sera were mixed and incubated for 1 h at 37°C and then transferred to 96 well-plate with complete cell monolayer. Plates were incubated for 1 h at 37°C with 5% CO_2 for virus adsorption. After washing, an overlay medium (1%carboxymethylcellulose) (200 µl/well) was added in the wells and incubated for 48 h. Carboxymethyl cellulose solution was discarded, plates were washed and cells were fixed with 4% para formaldehyde. Plates were blocked primary antibody was added (crude mAb 4G2 pan-DENV anti-E protein and $2H_2$ PrM binding antibody in 1:300 dilutions) followed by wash and incubation with goat anti mouse IgG –HPR conjugated (1:1,000 in blocking buffer). After washing, True blue substrate was added (True Blue HRP substrate, VWR). The number of foci were visualized and quantified

by automatic counting using a CTL ImmunoSpot (Cellular Technology Limited). The $\log_{10}$ of the reciprocal serum dilution was plotted against relative infection, calculated as [(# spots sample–# spots non-infected control)/(# spots (virus + normal human serum)–# spots non-infected control)], and fitted with a sigmoidal dose-response curve using GraphPad Prism software version 8.0 (La Jolla, CA). The titer of antibody (serum dilution) that achieved a 50% reduction in infection (50% neutralization titer, NT50), is expressed as the reciprocal of the serum dilution. Maximum infection was calculated from monolayers infected in the presence of normal human serum. Stringent QC rules, including the absolute sum of squares <0.2 and the coefficient of determination (R2) of the non-linear regression >0.9, were required to ensure the reliability of results. Serology results are shown in **Supplementary Table S1**.

HLA Typing and Phenotype Frequency Calculations

HLA typing was performed by an ASHI-accredited laboratory at Murdoch University (Western Australia) for Class I (HLA A; B; C) and Class II (DRB1, DRB3/4/5, DQA1/DQB1, DPB1) as previously described (15, 22, 23). Allele and phenotype frequencies for individual DP/DQ alleles in Sri Lanka population have been previously described (27). Worldwide phenotype frequency were calculated as previously described and based on data available at DbMHC and allelefrequencies.net (15, 28–32). Population combined coverage for DP, DQ, and DRB345 loci was calculated as follows:

$$\textit{Population coverage} = \mathrm{A} + [(100 - \mathrm{A}) * \frac{\mathrm{DQ\ coverage}}{100}]$$

$$\text{Where A} = \textit{DRB345 coverage} + [(100 - \mathrm{DRB345\ coverage}) * \frac{\mathrm{DP\ coverage}}{100}]$$

MHC Class II Binding Predictions and Peptide Selection

A set of DENV peptides predicted to bind various DRB3/4/5 (DRB3*0202, DRB4*0101, DRB5*0101), DP (DPA1*01/DPB1*0401, DPA1*0103/DPB1*0201, DPA1*0201/DPB1*0101, DPA1*0301/DPB1*0402), and DQ (DQA1*0301/DQB1*0302, DQA1*0101/DQB1*0501, DQA1*0102/DQB1*0602, DQA1*0501/DQB1*0201, DQA1*0501/DQB1*0301) alleles was chosen based on criteria further described in the results section. Fifteen-mer peptides from all serotypes were predicted for their binding affinity to the selected HLA class II molecules as previously described (15, 33, 34). This resulted in the synthesis of 432, 448, and 562 predicted binding peptides for HLA-DRB3/4/5, DP, and DQ and loci, respectively, (Mimotopes, Victoria, Australia) as shown in **Supplementary Table S1**. For screening purposes, pools of peptides predicted to bind each of the HLA class II allelic variants were used for stimulation and deconvoluted to the single peptide level as previously reported (8, 15).

In vitro Expansion of Denv-Specific T Cells and IFNγ Elispot Assay

CD4+T cells stimulation was performed as previously described (8, 15). Briefly, frozen Peripheral Blood Mononuclear Cell (PBMCs) were thawed and $CD4^+$ T cells were isolated by magnetic bead negative selection and co-cultured with autologous APC derived from the positive selection in a 2:1 ratio. Cells were stimulated with DENV-specific pools kept at 37°C in 5% CO_2, IL-2 (10 U/mL; eBioscience) was added at 4, 7, and 11 days after initial antigenic stimulation and harvested on day 14. Harvested cells were counted and plated 5×10^4 $CD4^+$ T cells in triplicates in the presence of the HLA-matched peptide pools used for stimulation [1 μg/ml] and the individual peptide contained in the pool used for stimulation [10 μg/ml]. When the number of cells were not sufficient to test all the peptides contained in the pool used for stimulation a factorial approach has been followed, alternatively, all the peptides were directly deconvoluted after 14 days restimulation. On day 14, no additional APC were added to the culture. After 20 h of incubation at 37°C, cells were incubated with biotinylated IFNγ mAb (mAb 7-B6-1 Mabtech, Stockholm, Sweden) for 2 h and developed as previously described (7, 14).

Flow Cytometry

PBMCs from 10 randomly selected Sri Lankan donors were evaluated for Class II MHC expression by flow cytometry using anti-DR, -DP, or -DQ antibodies (LB3.1, B7/21, and SPV-L3 clones, respectively). Briefly, thawed PBMCs were counted and the cell suspension adjusted to 1×10^6 cells/ml. Cell suspensions were washed by centrifugation in ice-cold FACS Buffer (PBS, 10% FBS) followed by incubation with unconjugated primary antibodies for 30 min at 4°C. After subsequent wash steps, cells were incubated with FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Inc. West Grove, PA) for 30 min at 4°C. Additionally, control cells were incubated with isotype antibody only. Data were acquired on a BD FACSCanto™ II analyzer and Median Fluorescence Intensities (MFI) were determined using FlowJo v10 (FlowJo, LLC).

Cluster Analysis

A List of epitopes positive in more than one donor and restricted to different HLA class II loci (DP, DQ,DRB3/4/5, and DRB1 for reference) has been clustered using identity threshold of 70% and the recommended Cluster-break method implemented in cluster tool 2.0 (35) available in IEDB (http://tools.iedb.org/cluster2/). The resultant clusters and singlets overlap across the different HLA class II loci were then graphically shown in a Venn diagram format using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

RESULTS

Selection of a Set of HLA, DP, DQ, and DRB3/4/5 Alleles Enabling High Population Phenotypic Coverage

To assess the contribution of DRB3/4/5, DP, and DQ loci in restricting DENV-specific responses, we selected three to

five of the most frequent alleles worldwide for each locus (**Figure 1**, white bars). In the case of the DRB3/4/5, these allelic variants were DRB3*0202, DRB4*0101, and DRB5*0101. These alleles are found at frequencies in the 25–52% range, and combined afford coverage of 87% (**Figure 1A**, white bars). In the case of DP, we selected DPB1*0401, DPB1*0201, DPB1*0101, and DPB1*0402. While the alpha chain of DP molecule is also variable, haplotype, and typing data is not generally available, the alpha chain is not very polymorphic, and the polymorphisms are generally conservative. These alleles are found at frequencies in the 16–41.6% range, and combined afford a coverage of 85% (**Figure 1B**, white bars). In the case of DQ we selected DQA1*0301/DQB1*0302, DQA1*0101/DQB1*0501, DQA1*0102/DQB1*0602, DQA1*0501/DQB1*0201, and DQA1*0501/DQB1*0301. These alleles are found at frequencies in the 11.3–35.1% range, and combined afford coverage of 81.6% (**Figure 1C**, white bars). The phenotypic coverage provided when the three loci are combined (assuming no linkage disequilibrium as a first approximation) was projected at >99%.

In our studies, we utilized PBMC from the general population of Colombo, Sri Lanka. Accordingly we compared worldwide frequencies with the ones observed in the Sri Lankan population (**Figure 1**, black bars) based on HLA typing studies we previously reported (27). In general, the selected alleles afforded good population coverage also in the Sri Lankan population in the 48.2–83.4% range across loci. The phenotypic coverage provided when the three loci are combined was projected at >98%.

For each allele, we then selected five to seven PBMC samples for an immunogenicity screen as described in the following section.

Identification of DENV-Derived Putative DRB3/4/5, DP, and DQ Restricted Epitopes

We synthesized and screened sets of epitopes candidates restricted for each of the selected HLA alleles derived from each of the main four DENV serotypes, based on their predicted capacity to bind each HLA allelic variant, as previously described (8, 15). The number of predicted epitopes for each allele combinations studied in Sri Lanka are shown in **Supplementary Table S2**. Each of the peptide sets was screened in 5–7 HLA matched donors, serologically screened, and confirmed to be exposed to one or more DENV serotype prior to blood drawn (**Supplementary Table S1**). A complete list of all epitopes identified in this study, including HLA restriction, response frequency, and DENV protein composition has been submitted to the IEDB (http://www.iedb.org/subid/1000789) and is available for reference in **Supplementary Table S3**.

We identified 111, 123, and 106 epitopes restricted by HLA-DRB3/4/5, -DP, and –DQ, respectively. From the 340 epitopes identified, 250 were newly identified and not previously reported in the IEDB. For each HLA class II allele belonging to DRB3/4/5, DP, or DQ loci, **Table 1** shows the breadth and the magnitude of the responses normalized by the number of instances each allele-specific peptide set combination has been tested. On average, we identified a repertoire breadth of five epitopes per allele tested, ranging from 0 to 34 epitopes recognized in a single donor and with an average response per epitope of 891 SFC/10^6 PBMCs (**Table 1**). The percentage of epitopes as a function of the DENV serotype is also shown; we considered conserved epitopes when sequences shared more than 70% of homology after cluster analysis (35). We found that the majority of the epitopes recognized are conserved across the DENV serotype when 70% homology cutoff was used with an average of 60% of the epitope recognized being conserved ranging between 55 and 70% across the DRB3/4/5, DP, and DQ loci. Overall, a similar DENV serotype recognition was observed across the loci analyzed (**Table 1**).

In terms of magnitude of responses, we calculated the average magnitude of response per donor (or multiple donors used to test the complete set of predicted peptide per HLA allele) by summing the total responses for each of the various HLA class II alleles studied and dividing the sum by the number of tested donors, using the same methodology as previously reported for DRB1 alleles (15). As we sought to compare the DP, DQ, DRB3/4/5 data with the ones previously published on DRB1 (8, 15), we kept consistent both experimental and analysis strategies.

Figure 2A shows the average magnitude of responses, where each allele represent a data point. Despite a rather substantial range of responses the median magnitude for responses associated with the DRB3/4/5, DP, and DQ loci were similar, with values of ∼4,000 SFC/donor. In particular, the range was highest for DRB3/4/5, with the DRB3*02:02 allele having the highest and DRB4*01:01 the lowest reactivity. These results represent the first description of DENV-derived putative DRB3/4/5, DP, and DQ restricted epitopes.

Comparison of Magnitude of HLA DRB3/4/5, DP, and DQ vs. HLA DRB1 Responses

Next we compared the responses in the case of the DRB3/4/5, DP, and DQ loci with responses previously defined for different DRB1 alleles (15), using the exact same methodology. For each allele, the sum of magnitude of responses was added and divided by the number of donors tested to calculate the average magnitude/donor response. **Figure 2A** shows the average magnitude of responses, for the DRB3/4/5, DP, and DQ loci, compared to data from 16 DRB1 alleles previously published (15) which is shown here for reference purposes. HLA-DRB1 was associated with higher magnitude of responses (about 2-fold) compared to the other HLA class II loci combined (Mann Whitney, $p = 0.0473$; **Figure 2A**).

We hypothesized that this increased magnitude of responses could be due to a difference in cells surface expression of the three different HLA class II loci. Indeed, several reports indicate that DRB1 gene products are expressed at levels 5–10 higher than those encoded by the DRB3/4/5, DP, and DQ loci (36). To verify that this was also the case in our experimental system, PBMCs derived from 10 randomly selected Sri Lanka blood bank donors, were stained for the expression of HLA-DRA (the antibody used could not therefore, discriminate between DRB1 and DRB3/4/5 loci) (37), DP, and DQ (38). As expected, we did observe significantly higher (about 5-fold) expression of DR in

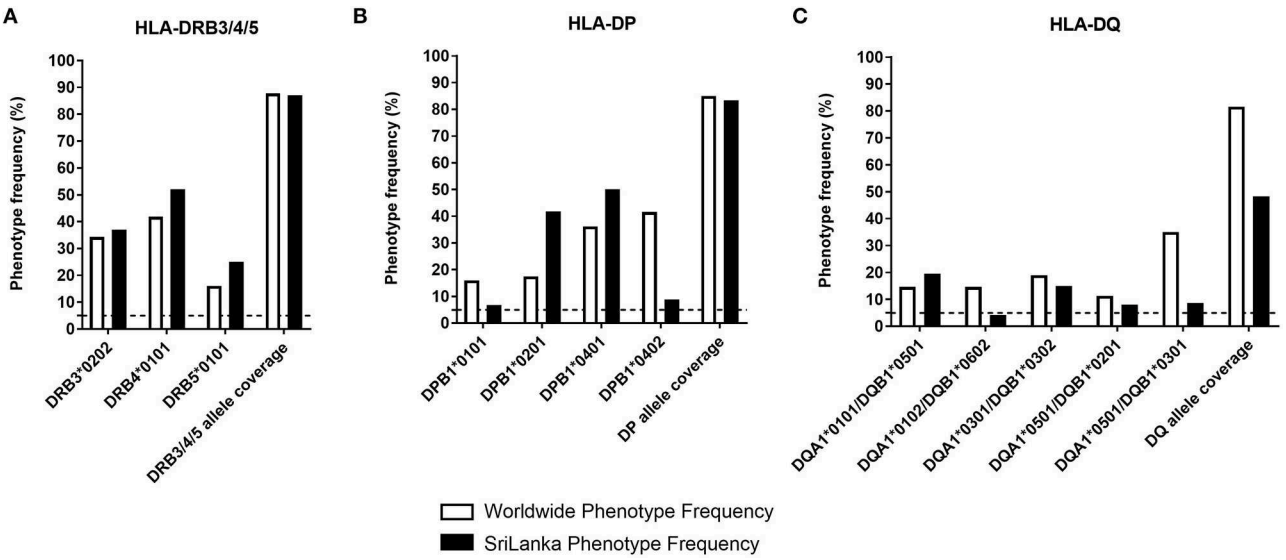

FIGURE 1 | HLA class II phenotype frequencies in DP, DQ, and DRB3/4/5 loci. The HLA-DRB3/4/5 **(A)**, -DP **(B)**, and -DQ **(C)** phenotypic frequency and allele coverage worldwide (white bars), is compared to the one observed in Sri Lanka donors ($n = 714$, black bars) and based on previously published data (27).

comparison with DP and DQ (Wilcoxon test, DP vs. DR $p = 0.020$; DQ vs. DR $p = 0.002$) (**Figures 2B,C**). Overall, the HLA-DRB1 locus is associated with higher magnitude of responses, which correlates with higher expression level on cell surfaces among the various HLA class II loci.

Differences and Similarities in Immunodominance Patterns of DENV-Specific CD4+T Cells Responses Protein as a Function of HLA-Class II Loci

We next analyzed the pattern of immunodominance of responses as a function of the different HLA class II loci. Specifically, for each HLA allelic variant we calculated the fraction of the total response directed against each of the 10 antigens encoded by the DENV polyprotein. We next investigated whether locus-specific differences were present (**Figure 3**). For the sake of comparison, we also included previously reported data, related to 16 alleles of the DRB1 locus which showed a preferential response toward capsid, NS3 and NS5 proteins (**Figure 3**). We emphasize that the DRB1 data was previously published (15) and is shown here only for reference purposes. This analysis revealed difference in the pattern of immunodominance for some of the DENV antigens (**Figure 3**). Pie charts showing the fraction of the total response corresponding to each DENV protein are also shown to highlight these differences (**Supplementary Figure S1**). Specifically, while Capsid was dominantly recognized by DRB1 and DRB3/4/5 (accounting for about 25% of the response), it was only marginally recognized by DQ alleles ($p = 0.0442$ in a Kruskal-Wallis test). The NS1 antigen was dominantly recognized by DP alleles, but only marginally by DRB1, DRB3/4/5, and DQ loci ($p = 0.0028$ in a Kruskal-Wallis test). When looking at the NS1- specific immunodominance observed in the context of DP locus, we also identified an immunodominant NS1 region ($NS1_{933-949}$ DYGFGVFTTNIWLKLRE) recognized across the four DP alleles (**Supplementary Table S3**). Conversely the NS3 antigen was not recognized by DP responses, but otherwise dominantly recognized by DRB1, DRB3/4/5, and DQ loci ($p = 0.0279$ in a Kruskal-Wallis test).

DENV HLA Class II-Restricted Epitopes Overlap Across the Different Loci

As a final analysis of similarity and differences in the CD4 responses to DENV as a function of different HLA loci, we examined the responses for overlap at the level of the epitope repertoires associated with each locus. For this purpose, we considered epitopes that were positive in at least two of the donors tested for each allele, also including those previously identified in the context of DRB1 locus. As such we considered 22, 32, and 39 epitopes restricted to DP, DQ, and DRB3/4/5 restriction, respectively, for a total of 93 epitopes, and 183 epitopes restricted to DRB1 and previously identified in the same population (15). To account for minor sequence variations and difference in the peptide frame, these epitopes were clustered using the cluster break-method at 70% of homology using cluster 2.0 tool available in IEDB (35). The results of this analysis identified 112 epitope clusters shown as a Venn diagram in **Figure 4**. Of those 112 clusters, 78 were recognized by DRB1 (70%). Of those 78, 11 were shared with DRB3/4/5 (14%), 6 with DP (8%), while only 4 were shared with DQ (5%). In the case of DRB3/4/5, 11 clusters were recognized (10%); all were shared with DRB1 (100%), while only two with DP (18%), and two with DQ (18%). In the case of DP, 11 clusters were recognized (10%); of those six were shared with DRB1 (54%), while only two with DRB3/4/5 (18%), and three with DQ (27%). Finally, in the case of DQ, 24 clusters were recognized (21%); of those four were

TABLE 1 | Characteristics of DENV-specific responses in DRB3/4/5, DP, and DQ loci.

Locus	Allele	No. of times tested*	Avg no. of epitopes per HLA allele#	Avg response per epitope#	Avg response per HLA allele#	% of epitopes per HLA allele per serotype				
						DENV1 (%)	DENV2 (%)	DENV3 (%)	DENV4 (%)	Conserved (70% homology)
DRB3/4/5	DRB3*0202	6	2	162	271	10	10	10	0	70
	DRB4*0101	6	5	591	2,661	11	11	4	0	74
	DRB5*0101	7	11	1,921	20,309	3	11	8	12	66
DP	DPB1*0101	6	4	622	2,694	8	4	8	8	73
	DPB1*0201	6	3	308	873	6	6	18	6	65
	DPB1*0401	5	8	892	7,166	10	0	20	10	61
	DPB1*0402	5	8	1,165	9,087	23	10	18	8	41
DQ	DQA1*0101/ DQB1*0501	5	6	1,514	9,525	12	18	3	9	59
	DQA1*0102/ DQB1*0602	7	5	1,093	5,911	13	20	5	13	50
	DQA1*0301/ DQB1*0302	6	3	1,375	4,082	11	21	0	26	42
	DQA1*0501/ DQB1*0201	6	1	364	395	14	29	0	0	57
	DQA1*0501/ DQB1*0301	6	1	680	635	17	0	0	17	67
	Avg	**6**	**5**	**891**	**5,301**	**11**	**12**	**8**	**9**	**60**

*Factorial approach has been used for this study. Responses have been normalized based on the number of instances a full HLA allele-specific peptide set has been tested.
#Data calculated as (SFC/10^6 CD4).

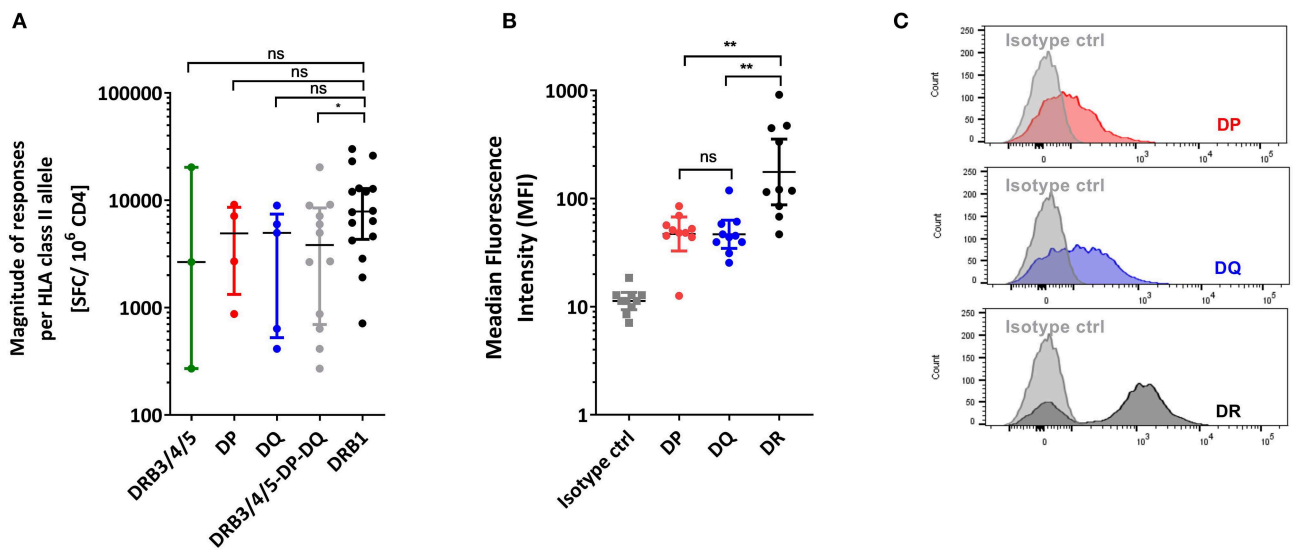

FIGURE 2 | HLA class II loci comparison. **(A)** Magnitude of CD4+T cell responses per HLA class II allele expressed as SFC/10^6 CD4 cells/donor after *in vitro* stimulation. **(B)** Membrane expression of HLA-DR, DP, DQ molecules in PBMCs expressed as Median Fluorescence Intensity (MFI). All statistical analysis were performed using unpaired non-parametric Mann-Whitney test. *$p < 0.05$, **$p < 0.01$, ns, not significant. **(C)** Representative overlay histograms derived from one donor PBMCs staining showing the membrane expression of isotype control (gray) overlayed with HLA-DR (black), DP (blue) or DQ (red) molecules, respectively.

shared with DRB1 (17%), two with DRB3/4/5 (8%), and three with DP (12%).

Overall, these results suggest that there is large overlap between DRB1 and DRB3/4/5 loci, with DP showing an intermediate pattern of repertoire overlap, and DQ being associated with a mostly unique repertoire.

DISCUSSION

Dengue Virus-specific CD4+T cells responses are important in the control of infection and strong responses were previously associated with control of DENV infection in asymptomatic individuals (39). We previously studied in the context of DENV-specific CD4+T cell responses the role of DRB1 locus in

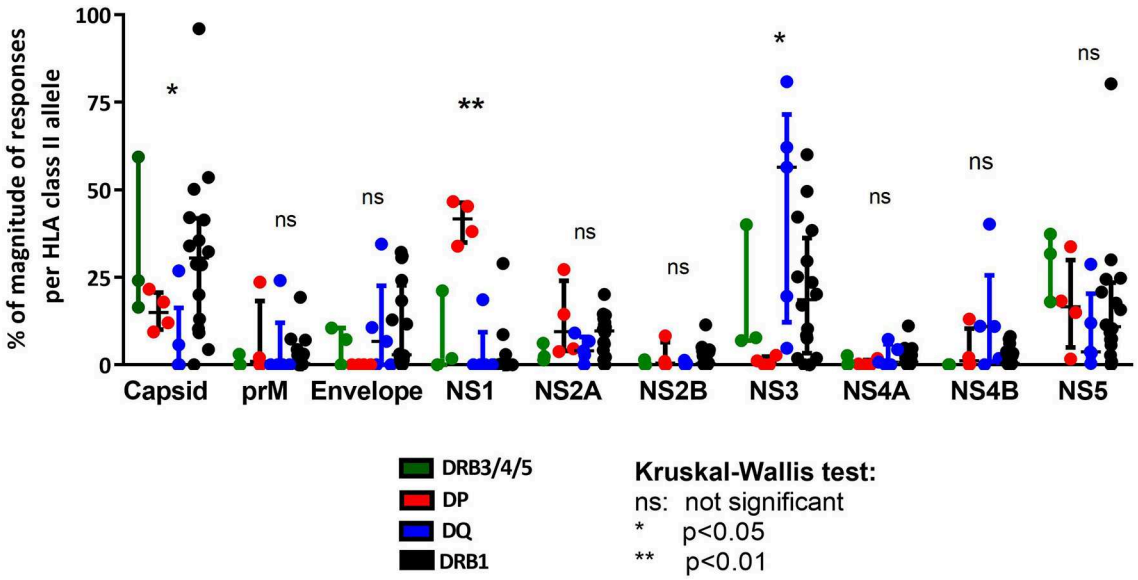

FIGURE 3 | Immunodominance protein pattern of HLA class II loci comparison. Protein immunodominance observed in the previously published HLA-DRB1 locus as reference (black) (15) is compared with HLA-DP (red), HLA-DQ (blue), and HLA-DRB3/4/5 loci (green). Each dot represents the percentage of magnitude of responses per allele in the respective locus divided for DENV protein composition. Statistical analysis per protein have been performed using one-way ANOVA non-parametric Kruskal-Wallis test.

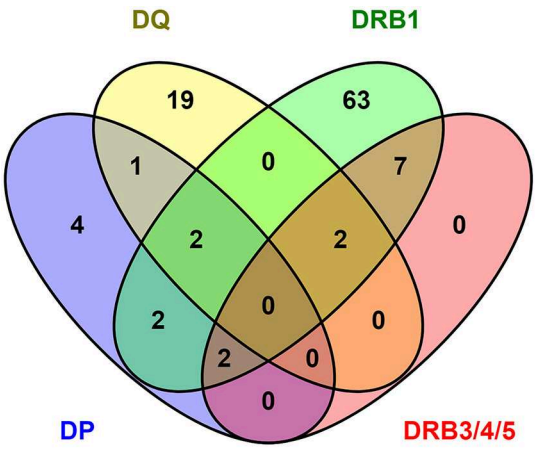

FIGURE 4 | DENV epitope overlap across HLA class II loci. The number of epitopes positive in more than one donor have been clustered using cluster 2.0 (35) (http://tools.iedb.org/cluster2/) and shown as Venn diagram using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

different DENV endemic populations (8, 15). In this study, we extended our analysis to DP, DQ, and DRB3/4/5 loci using the same strategy and same DENV population previously reported for DRB1 locus.

In general, in humans most defined restrictions are associated with the DRB1 locus. By querying the IEDB (www.IEDB.org) for T cell responses in human hosts that have known MHC class II restriction, we found that 4,010 instances of epitopes restricted by DRB1 molecules, in contrast to 348, 246, and 584 instances of DRB3/4/5, DP, and DQ restrictions. It is however not clear based on this analysis alone, whether this data preponderance is to be ascribed to the fact that these responses are simply less frequently studied, or whether this reflect a lower magnitude of responses/epitope numbers associated with these loci.

Significantly weaker responses and lower number epitopes were identified for DP, DQ, and DRB3/4/5 loci combined when compared to DRB1 locus. These data are also consistent with other studies which reported lower number of epitopes found in DQ and DP loci compared to DR locus in both allergy, autoimmune diseases and viral contexts (40–42). In the allergy context, we have previously shown that the frequency of locus distribution for HLA class II antigen-specific responses was the highest for DR (61 and 49% of total SFC), followed by DP (21 and 35% of total SFC), and DQ (18 and 16% of total SFC) (42).

Given the 2 log variability in the magnitude of the CD4 response depending on the MHC II allele considered, it is not obvious that DRB3/4/5 alleles induce responses of lower magnitude compared to DRB1. However, when DRB3/4/5, DP, and DQ loci are combined together we do observe a significant lower magnitude of response respect to DRB1 locus. This is consistent with the previously reported lower expression of heterodimers encoded by DP, DQ, and DRB3/4/5 (43, 44), also confirmed in our study. Specifically, we showed that HLA-DR protein (which combines expression of both DRB1 and DRB3/4/5 loci) is significantly more highly expressed on cell surface when compared to DP and DQ loci, suggesting a higher probability that epitopes DR-restricted are recognized by CD4+T cells respect to DP and DQ. This evidence is consistent with other studies showing the same pattern of protein expression in several

Antigen Presenting Cells (APC) derived from PBMCs or cell lines (43, 44).

As this study evaluated only IFNγ-specific T cell responses, it cannot be excluded that epitope recognition in the context of DP, DQ, and DRB3/4/5 could induce effector T cell responses differing from IFNγ release. However, lower general reactivity of DP and DQ compared with DR was noted as well by Kwok group using tetramer reagents (personal communication), thus arguing against this possibility.

Our results also show some qualitative differences between the immunodominance of the responses restricted by the various loci. Capsid was dominantly restricted by DR but marginally by DP and DQ. NS1 is preferentially presented by DP, and NS3 responses are restricted by all loci except DP. These differences are at least in part accounted by how frequent the peptides carrying specific motifs are found in the various proteins. Further studies should be focused on explaining why NS1 is more efficiently recognized when restricted by DP compared with other loci. In any case, these data reemphasize the fact that MHC class II allele polymorphism is a key determinant of immune responsiveness. Indeed, for this exact reason MHC class II genes were originally labeled in the 1970s as "IR" or "Immune Response" genes (45).

Finally, we considered the epitope repertoire overlap across loci. When comparing the DRB1-restricted epitopes with the ones identified in this study, a strong overlap is shown with DRB3/4/5 loci. In contrast, less overlap was found between DR and DP, and the least minimal overlap of DQ with the other loci. The complete overlap of DRB3/4/5 locus with DRB1 is consistent with the shared used of the alpha chain encoded by the DRA gene. Previous data have shown similarity in binding repertoire between DP and DRB1 loci based on similarities in the peptide-binding motifs (30, 46). In contrast, DQ molecules are associated with more loose peptide-binding motifs and its peptide-binding capability appears more dependent on the main backbone than the specific anchor position giving more importance to the alpha and beta chain combination being both polymorphic and both important in the characterization of the peptide-binding capability (31). Overall the pattern of epitope repertoire overlap is consistent with previous studies by Greenbaum (47) utilizing large collections of peptide binding assays and a collection of purified HLA molecules from different alleles of the DP, DQ, DRB3/4/5, and DRB1 loci. Based on the available knowledge, predictions for the HLA class II molecules in each loci will cover ∼50% of the total response, with DRB1-restrictred responses found to be dominant and an appreciable fraction of the responses found instead to be restricted by DP, DQ, and DRB3/4/5 loci (30, 31, 48–50). Our current dataset could not alone evaluate the efficiency of epitope prediction but will provide additional experimental data for future benchmarking of DRB3/4/5, DP, and DQ epitope prediction. Additionally, the present study by defining epitopes restricted by this loci enables future studies to address the relevance of this loci in DENV.

In conclusion, our data highlights that DP, DQ, and DRB3/4/5 restricted are a minor but significant component (as compared to DRB1 restrictions) in DENV-CD4 specific T cell response. This is consistent with lower expression of DP and DQ in APCs, and what has been reported in the general literature for other immunogenic systems highlighting the major role of HLA-DR in triggering CD4+ DENV-specific T cell responses.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**. All the epitopes generated in this study have been also submitted to IEDB (www.IEDB.org: submission ID:1000789).

ETHICS STATEMENT

Blood samples were obtained from healthy adult blood donors from the National Blood Center, Ministry of Health, Colombo, Sri Lanka in an anonymous fashion. All protocols described in this manuscript were approved by the institutional review boards of both LJI and Medical Faculty, University of Colombo (serving as NIH approved IRB for Genetech).

AUTHOR CONTRIBUTIONS

AG, EM, and HV performed experiments, reviewed data, and planned the experimental strategy. JS and BP performed bioinformatics analyses. EP and SM performed and coordinated HLA typing and related analysis. RJ and AMD performed and coordinated serology analysis. ADD collected samples and provided clinical information. AG, DW, and AS conceived and directed the study, and wrote the manuscript. All authors have critically read and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01568/full#supplementary-material

Supplementary Table S1 | List of the peptides tested in Sri Lanka population for HLA class II studies. The number of peptides is shown per each allele belonging to –DRB3/4/5,-DP, and -DQ, according to DENV protein composition.

Supplementary Table S2 | List of the epitopes recognized in Sri Lanka population for HLA class II studies. For each epitope, DRB3/4/5, DP, or DQ allele restriction, DENV deriving protein and response frequency details are shown. All those epitopes have been submitted to IEDB (www.IEDB.org: submission ID: 1000789).

Supplementary Figure S1 | Pie chart showing the percentage of the average magnitude per HLA allele calculated in DRB3/4/5 (green), DP (red), DQ (blue), and DRB1 (black) loci.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Identification of Protective CD8 T Cell Responses in a Mouse Model of Zika Virus Infection

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Many flaviviruses including dengue (DENV), and Zika (ZIKV) have attracted significant attention in the past few years. As many flaviviruses are spread by arthropods, most of the world's population is at risk of encountering a flavivirus, and infection with these viruses has created a significant disease burden worldwide. Vaccination against flaviviruses is thought to be one of the most promising avenues for reducing the disease burden associated with these viruses. The optimism surrounding a vaccine approach is supported by the highly successful vaccines for yellow fever and Japanese encephalitis. Central to the development of new successful vaccines is the understanding of the correlates of protection that will be necessary to engineer into new vaccines. To aid in this endeavor we have directed our efforts to identify correlates of protection that will reduce the disease burden associated with ZIKV and DENV. Within this study we have identified a novel murine ZIKV specific $CD8^+$ T cell epitope, and shown that the ZIKV epitope specific $CD8^+$ T cell response has a distinct immunodominance hierarchy present during acute infection and is detectible as part of the memory T cell responses. Our studies confirm that ZIKV-specific $CD8^+$ T cells are an important correlate of protection for ZIKV and demonstrate that both naïve and ZIKV immune $CD8^+$ T cells are sufficient for protection against a lethal ZIKV infection. Overall this study adds to the body of literature demonstrating a role for $CD8^+$ T cells in controlling flavivirus infection.

Keywords: Zika, dengue, CD8+ T cells, epitope, correlates of protection, immunology and infectious diseases, immunodominance

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INTRODUCTION

The possibility of becoming infected with an arbovirus has increased dramatically over the past 40 years (WHO). Some of the most prominent emerging arboviruses are members of the *Flaviviridae* family. The *Flavivirus* genus consists of ∼70 arthropod-borne viruses with approximately half causing human disease, including Zika virus (ZIKV), West Nile virus (WNV), Dengue virus (DENV), Japanese Encephalitis Virus (JEV), and Yellow fever virus (YFV). The majority of flaviviruses replicate in ticks or mosquitoes and transmit virus to vertebrates by biting. Flaviviruses have also shown their capacity for rapid and explosive spread, as seen in the cases of WNV in 1999 (1), ZIKV in 2015 (2), and YFV in 2016/2017 (3, 4). In all cases, and particularly notable with YFV, diagnosis of the outbreak lagged behind the emergence and spread of the virus. The need for a vaccine to provide protection from emerging flaviviruses is evident and understanding the T cell epitopes responsible for flavivirus protection will aid in identifying the immune protective responses and directly inform vaccine design.

That a ZIKV infection could cause disease was first noted in 1964 (5). Excluding laboratory acquired infections, disease was noted again in febrile children in 1975 (6) and in at least seven patients in Central Java between 1977 and 1978 (7). Prior to the globalization, ZIKV has also been routinely detected by serological assays, when screening for arboviruses in Africa (6, 8–14). In 2017, there were over 1,000 cases of ZIKV disease reported to the CDC in the United States, including US territories. As of September of 2018, that number had dropped to roughly 150; worldwide the numbers have also decreased, likely due to multiple factors including vector control, public awareness and screening, and herd immunity. However, this precipitous drop in ZIKV disease does not mean we are done with ZIKV. While epidemiological surveillance of ZIKV endemic areas is incomplete, they do highlight a common pattern reoccurring disease outbreaks associated with seasonal or environmental changes, similar to what has been seen for DENV and YFV (3, 4). The re-emergence of outbreaks for many pathogens throughout history points to a future where rates of ZIKV infection and disease will be cyclical. Knowing this, we can assume that the incidence of disease associated with ZIKV will re-emerge, and without vaccines or therapeutics to treat infection ZIKV will again become a global health concern.

Diagnosing a ZIKV infection is complicated for a number of reasons, including the high prevalence of asymptomatic infection or generalized symptoms. Indeed, 80–90% of those infected with ZIKV will be asymptomatic or have mild symptoms. A patient presenting with symptoms of acute Zika infection often has generalized symptoms including a mild fever, rashes, and joint pain, which is indicative of a number of infections including DENV(CDC) and the mild symptoms usually resolve within a week and do not often necessitate a visit to a doctor, therefore most ZIKV infections are undiagnosed (15). While the symptoms of disease are relatively short, if present at all, infected individuals can shed virus for several months (16–19) and there are the numerous recent reports demonstrating that ZIKV can be transmitted through contact with bodily fluids (18, 20–22). These factors combined with the evidence that ZIKV infection is linked with congenital malformations and abortions by mother-to-fetus transmission during pregnancy (23–25) makes identifying correlates of protection to design effective treatments and to reduce the risk of disease and viral spread a significant public health priority.

As a members of the family *Flaviviridae,* ZIKV and DENV share many common features in both their structure and genome. ZIKV and DENV are small enveloped viruses that contains a single, positive-sense ∼11-kb RNA genome with a 5′ and 3′ untranslated regions flanking a polyprotein (26) (**Figure 1A**). The polyprotein encodes three structural (C, prM/M, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (27). The E protein is comprised of three domains (I (E-DI), II (E-DII), and III (E-DIII), with E-DII and E-DIII containing the fusion peptide and putative viral receptor binding site(s), respectively [reviewed in (28, 29)]. Among the structural proteins, prM and E proteins are primary antigenic targets of the humoral immune response in humans for flaviviruses (30–33). As prM and E drive a strong humoral immune response, most vaccines being designed against ZIKV and DENV have tried to incorporate these two key humoral targets.

T cells have been demonstrated to play an important role in protection from a number of flaviviruses by the production of antiviral cytokines and through the killing of infected cells (34–38). In the Ifnar1-/- murine model of DENV infection polyfunctional cytokine producing and cytolytic $CD8^+$ T cells prevent uncontrolled replication in peripheral tissues and this protective function can even be elicited through peptide vaccination targeting the immunodominant $CD8^+$ T cell epitopes, presenting a point about the importance of eliciting a robust vaccine-mediated $CD8^+$ T cell response in protection from DENV (38, 39). This point is also well illustrated in the vaccination of mice with the vaccine strain of YFV (YF-17D), which also elicits a robust $CD8^+$ T cell response which has been shown to be important in its vaccine-mediated protection particularly in concert with antibody-mediated protection (37). In the case of neurotropic flaviviruses like WNV and JEV, CD8+ T cells are critical for controlling the infection in neurons and subsequently for protection from disease (40–43). Indeed, it has been shown for both JEV and WNV that vaccination induces robust CD8+ T cell responses and that those cells are an important contribution to the protective capacity of the vaccine (34, 44). Virus-specific and even cross-reactive $CD8^+$ T cell peptide epitopes have been identified in mouse models for a number of these viruses and have been an absolutely critical tool in finely dissecting the functions of these cells and the correlates of protection from these viruses (35, 38, 45–47).

Recent studies have identified some human and mouse ZIKV $CD4^+$ and $CD8^+$ T cell epitopes (35, 48–54) and have begun to identify the role of these T cells in protection against ZIKV. Our group has contributed to the identification of ZIKV specific $CD4^+$T cell epitopes in mice noting a novel role for $CD4^+$ T cells in the protection against ZIKV neuroinvasive disease in mice (49). In this study we are adding to the current literature for the identification and functional importance of $CD8^+$ T cell responses to ZIKV infection, noting that virus-specific $CD8^+$ T cells are both necessary and sufficient for survival. We have identified a novel murine ZIKV-specific $CD8^+$ T cell epitope, and shown that the ZIKV epitope-specific $CD8^+$ T cell response has a distinct immunodominance hierarchy present during acute infection and is detectible as part of the memory T cell responses. These studies confirm the importance of $CD8^+$ T cells in ZIKV infection and uniquely noting that naïve $CD8^+$ T cells can protect against a lethal viral challenge. As with DENV, understanding the role $CD8^+$ T cells will play in protection against severe disease will aid in future vaccine design.

MATERIALS AND METHODS

Ethics Statement

The animal studies were approved by the Saint Louis University Animal Care and Use Committee and done in accordance with the Guide for Care and Use of Laboratory Animals.

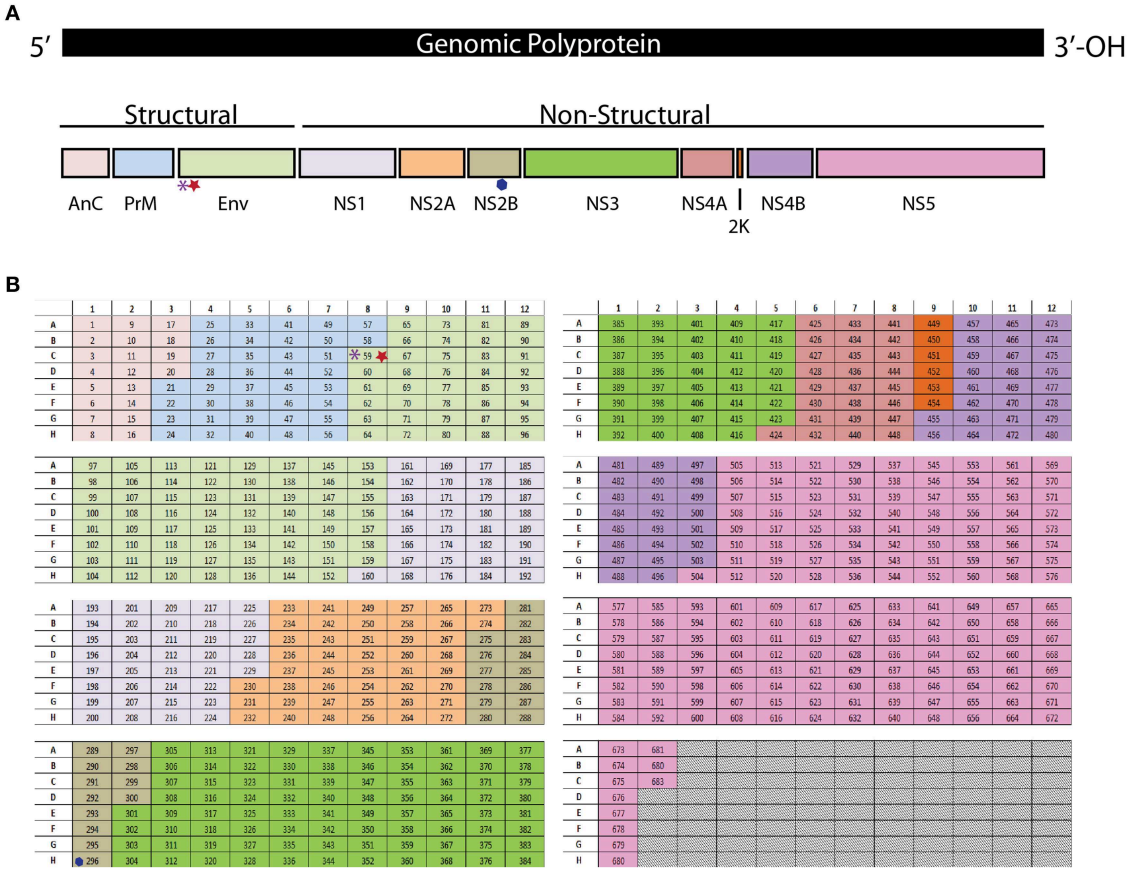

FIGURE 1 | (A) The polyprotein encodes three structural (C, prM/M, and E) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. **(B)** Each peptide from the library is in an individual well in a 96 well plate. The purple asterisk denotes the E294 epitope, The red star denotes the E297 epitope. The blue hexagon denotes the NS2B1478 epitope.

Viruses and Cells

ZIKV (strain PRVABC59) was obtained from BEI (catalog No.: NR-50240) and passaged once in Vero cells (African green monkey kidney epithelial cells) purchased from American Type Culture Collection (ATCC CCL-81). All viruses were titered using a standard focus forming assay (FFA) on Vero cells as previously described (55).

Mice and Infections

Wild type C57BL/6J and interferon αβ receptor 1 knockout (Ifnar1$^{-/-}$) mice (strain: B6.129S2-Ifnar1^{tm1Agt}/Mmjax), commercially purchased from Jackson Laboratories were housed in a pathogen-free mouse facility at the Saint Louis University School of Medicine. For CD8+ T cell depletion studies, 8–12-week-old Ifnar1$^{-/-}$ mice were infected subcutaneously (SC) via footpad injection with 10^5 FFU of ZIKV. For epitope identification, wild type C57BL/6J mice were infected intravenously (IV) with 10^5 FFU of virus and boosted 30 days later with 10^5 FFU of virus. Wild type C57BL/6J mice were used for epitope identification as opposed to Ifnar1-/- mice due to the established persistence of ZIKV that has been observed in Ifnar1-/- mice which we anticipated would impact effective T cell responses due to continuous antigen exposure (49). As we had done previously for the CD4+ T cell studies, (49), for CD8+ T cell adoptive transfer studies, 8–12-week-old Ifnar1$^{-/-}$ mice were infected IV with 10^5 FFU of ZIKV 1 day after adoptive transfer of cells. During the course of infection mice were assessed for weight loss, signs of neurological disease, and mortality daily. Signs of disease range and in the most severe cases accelerate in the following manner from no apparent disease, limp tail, hind limb weakness, hind limb paralysis, complete paralysis, and death. Occasionally mice will display multiple signs of disease at once, such as limp tail accompanied by hind limb weakness. In such instances, mice are scored as the more severe sign of disease (e.g., hind limb weakness).

Measurement of Viral Burden

On the indicated days post infection (DPI), intracardiac perfusion (20 ml of PBS) was performed and organs were recovered. EDTA coated tubes were used to collect blood. For organ harvests, the organs were snap frozen and weighed before homogenization with a BeadMill 24 (Fisher scientific). TriReagent RT or RNAzol BD was used to extract viral RNA from the organ lysates or blood, respectively. The following sequences were used to quantify viral RNA by qRT-PCR: Forward- CCGCT GCCCAACACAAG, Reverse- CCACTAACGTTCTTTTGCAG

ACAT, Probe- AGCCTACCTTGACAAGCAGTCAGACACTCAA.

Peptide Library

The ZIKV peptide library was constructed using the amino acid sequence from ZIKV-PRVABC59. The library spans the polyprotein and consists of 683 15-mer peptides, overlapping by 10 amino acids. Peptides were reconstituted to 10 mg/ml in 90% DMSO and stored at −80°C. We did not identify any peptides that appeared to be completely insoluble. A final concentration of ∼2 μM for each peptide was used for epitope identification. For the peptide stimulation and intracellular cytokine assays the optimal 9-mer peptides (E_{294}: IGVSNRDFV, E_{297}: SNRDFVEGM, and $NS2b_{1478}$: ICGMNPIAI) were purchase from 21st Century Biochemicals.

Peptide Stimulation

Splenocytes were harvested from mice 4, 5, or 8 DPI for acute experiments or >30 DPI for assessment of memory responses. Spleens were ground over a 100 μm cell strainer and suspended in RPMI with 10% FBS and HEPES. 10^6 cells were plated per well in a round-bottom 96-well plate and stimulated with peptide for 6 h at 37°C, 5% CO_2 in the presence of 10 μg/ml brefeldin A (BFA), and α-CD3 (clone 2C11) was used as a positive control.

Flow Cytometry

For intracellular cytokine assays, assays were done as described previously (49). Following the peptide stimulation, cells were washed and stained with the cell surface markers: α-CD4-APC-Cy7 (clone RM4-5), α-CD8- PerCP-Cy 5.5 (clone 53–6.7), α-CD3-AF700 (clone 500A2), and α-CD19- AF488 (clone 1D3). After surface staining the cells were fixed, permeabilized, and stained for the intracellular markers: α-IFNγ- APC (clone B27) and α-TNFα- PE (clone Mab11). The cells were analyzed with either an Attune NxT or a BD LSRII.

Adoptive Transfer of CD8± T Cells

WT C57BL/6J mice (8–10 weeks old) were injected IV with 10^5 FFU of ZIKV or PBS. Splenocytes were harvested 30 DPI and CD8+ T cells were purified to >97% purity using a Miltenyi negative selection kit. Approximately 3 × 10^6 cells were administered via IV route to Ifnar1$^{-/-}$ mice 1 day prior to a lethal challenge with ZIKV.

Statistical Analysis

All statistical analyses were performed using Graph Pad Prism. Statistical differences in survival were determined using a Mantel-Cox test. Differences in disease burden by weight loss were determined using an unpaired *t*-test with Welch's correction. Statistical differences in viral burden were determined by Mann-Whitney test.

RESULTS

To identify the ZIKV-specific CD4+ (49) and CD8+ T cell responses we have used a peptide library screening method. The peptide library was generated from the amino acid sequence of the ZIKV strain PRVABC59 (Accession #U501215.1). The peptide library is comprised of 683 peptides, each peptide is 15 amino acids (aa) long and the peptide sequences overlap by 10aa. Each peptide from the library is in an individual well in a 96 well plate (**Figure 1B**). The resulting peptide library is spread across eight plates. The peptides are reconstituted in 90% DMSO to make a stock of peptides at 10 mg/ml which was used for all the studies detailed below.

To identify the antigen specific CD8+ T cells in our primary screen we infected wild type C57BL/6 mice with 10^5 FFU of

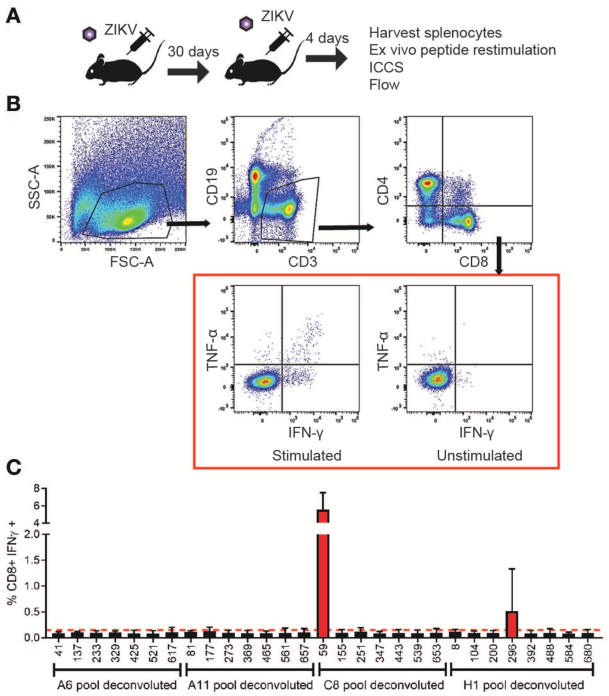

FIGURE 2 | Identification and functional analysis of ZIKV-specific CD8+ T cell epitopes. **(A)** Diagram of C57BL/6J mice infected 10^5FFU of ZIKV IV, rested for 30 days then boosted with a second ZIKV infection again with 10^5 FFU of virus IV. Spleens were harvested 4 days after the boost. The splenocytes isolated from the ZIKV boosted animals were then used in the intracellular cytokine assay. Peptide pools were then used to identify the antigen experienced CD8$^+$ T cells using an intracellular cytokine assay. **(B)** Representative gating strategy used to analyze intracellularly with antibodies specific for the mouse cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Splenocytes were harvested and stimulated with peptide pools in the presence of brefeldin A. Cells were stained for surface markers (CD3, CD19, CD4, and CD8), stained intracellularly for IFNγ and TNFα and analyzed by flow cytometry. Cells were gated using a lymphocyte gate, CD19$^-$, CD4$^-$, CD8$^+$, and were functionally analyzed by expression of IFNγ. Data is presented as the percent of CD8+ T cells that produced IFNγ in response to pooled peptide stimulation. **(C)** Peptide pools from wells C8 and H11 from the individual were deconvoluted and the cytokine responses detected from the individual 15mer peptides from the library allowed us to identify 2 possible CD8$^+$ T cell epitopes from the deconvoluted peptide pools. The 15mer peptide epitope was considered positive if the results were two times over background unstimulated samples. The 15mer peptide found in well 59 of the peptide library mapped to and area overlapping between PrM and E was identified from the C8 pool, and a 15mer peptide found in well 196 of the peptide library mapped to NS2b was identified from the H1 pool.

TABLE 1 | Epitope identities.

Name	Amino acid sequence
E_{294}	IGVSNRDFV
E_{297}	SNRDFVEGM
$NS4b_{1478}$	ICGMNPIAI

virus IV. It was confirmed that the dose and route of virus infection would result in transient replication sufficient for effective antigen presentation by harvesting the spleen and lymph nodes of a subset of these mice on days 3 and 6 post infection and virus detection via qPCR (**Supplementary Figure 1**). The mice were then rested for at least 30 days then boosted with a second ZIKV infection again with 10^5 FFU of virus IV to allow for the most robust response driven by anamnestic recall (**Figure 2A**). The splenocytes were isolated 4 days after the boost and were plated into 96 well plates and stimulated for 6 h with Brefeldin A (BFA) and a pool of 6–8 peptides. To generate these pools we combined the same well from each of the eight plates from the aliquoted library shown in **Figure 1B**. Unstimulated cells were setup as a negative control and as a positive control, anti-CD3 (45-2C11) was used to stimulate antigen experienced $CD8^+$ T cells from the ZIKV boosted animals. After the stimulation, splenocytes were stained with the cell surface antibodies, α-CD3, α-CD8, α-CD4, and α-CD19 then stained intracellularly with antibodies against the mouse cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (**Figure 2B**). The cytokine responses detected from the pooled peptide wells allowed us to identify 2 possible $CD8^+$ T cell epitopes within the peptide pools. We detected responses to peptides pools located in wells C8 and H1 in our original screen. The individual peptides within the peptide pools were identified by repeating the ZIKV boosting strategy in the C57BL/6J mice. The individual 15mer peptides that induced cytokine responses in our ZIKV boosted CD8+ T cells were identified by expanding the positive pooled wells (**Figure 2C**). With this approach we identified two wells that contained peptides which induced a cytokine response suggesting that we had one epitope in well 59 and one epitope in well 296. For both of these wells the amount of IFN-γ produced following peptide stimulation was at least two times higher than the background unstimulated cell control well. Shown in **Figure 2C**, the peptide identified in well 59 is the most dominant CD8+ T cell epitope we identified. The epitope in well 59 mapped to the a very proximal region of the Envelope protein and the epitope in well 296 mapped to the NS2b protein (**Figures 1A,B**).

Using the information from the 15mers in the peptide screen we set out to identify the optimal peptide sequence for each of the epitopes (**Table 1**). We named the ZIKV peptide epitopes using the same nomenclature as used previously for WNV (46), with the abbreviated name of the viral protein followed by the number of the amino acid based upon the flavivirus open reading frame, for example E_{294} would mean the epitope began at the 294th amino acid in the open reading frame and is present in the E protein. For well 59 we were surprised to discover that the sequence analysis suggested that there were two possible epitopes within this region E_{294} and E_{297} which were determined to be a H2-D^b and H2-K^b epitopes, respectively. Analysis of the literature confirmed this observation that there were two epitopes identified within this region (56). The NS2b epitope in well 296 was identified as $NS2B_{1478}$. To determine the avidity of the T cell responses to each identified epitope we performed peptide dose response assays on day eight after intravenous (IV) ZIKV infection (10^5 FFU) (**Figure 3A**). E_{294} and $NS2b_{1478}$ both had similar T cell peptide functional avidities with Log_{EC50} of −9.2 for E_{294} and Log_{EC50} of −8.4 for $NS2b_{1478,}$ which is line with other identified T cell epitopes. For E_{297} the T cell response dropped off rapidly, with T cell peptide functional avidities with Log_{EC50} of −4.8 suggesting a much lower functional avidity than the two other epitopes identified in the screen. For E_{294}, E_{297}, and $NS2b_{1478}$, we also had tetramers made by the NIH Tetramer facility. We then used splenocytes from day eight ZIKV infected C57BL/6J mice to determine the tetramer binding frequency compared to the intracellular cytokine IFN-γ response at the same time point (**Figure 3B**). For E_{294} and $NS2b_{1478}$, the tetramer analysis demonstrated a similar percentage and number of responding CD8+ T cells as we had seen with the intracellular cytokine staining for IFN-γ. This finding emphasizes validity of our cytokine-based screening approach and the likelihood that we have identified the optimal 9-mer peptide sequence for these two epitopes. However, we were unable to detect any staining with the E_{297} tetramer although we did see responses above background by intracellular cytokine staining. We are continuing to investigate this observation and our current interpretation is that we have not yet identified the optimal E_{297} epitope.

To establish the expression hierarchy for the peptide epitopes we identified, C57BL/6J mice were infected with 1×10^5 FFU of ZIKV IV and after 8 days the mice were sacrificed and splenocytes isolated. The individual peptides were used to stimulate the splenocytes in the presence of BFA for 6 h then cells are stained with cell surface and intracellular cytokines antibodies to identify the responding antigen specific CD8+ T cells (**Figure 3C**). The immunodominance hierarchy was determined by identifying the proportion of the acute CD8+ T cell response dedicated to each of the ZIKV-specific epitopes. The expression hierarchy of the immunodominant epitopes was determined to be E_{294}, $NS2B_{1478}$, and E_{297}. We next wanted to demonstrate that the E_{294} epitope specific CD8+ T cell response was preserved into memory. We repeated our infection of C57BL/6J mice with 1×10^5 FFU of ZIKV IV bleeding sequentially on days 5, 8, 14, and 45 DPI, performing an intracellular cytokine stain for each time point (**Figure 3D**). An E_{294} polyfunctional response was detectable in all mice out to day 45 suggesting that the ZIKV specific CD8+ T cell response is preserved. Similarly, $NS2B_{1478}$ polyfunctional memory responses were also detected in splenocytes from ZIKV infected C57BL/6J mice at least 45 days post infection (**Supplementary Figure 2A**). While we were able to observe a polyfunctional memory response in splenocytes stimulated with E_{297} from ZIKV infected C57BL/6J mice at least 45 days post infection relative to naïve animals (**Supplementary Figure 2B**), we were unable to show that these responses were statistically significantly different due to limited n.

Previous studies have established an important role for CD8+ T cells in protection against a lethal ZIKV challenge in type 1

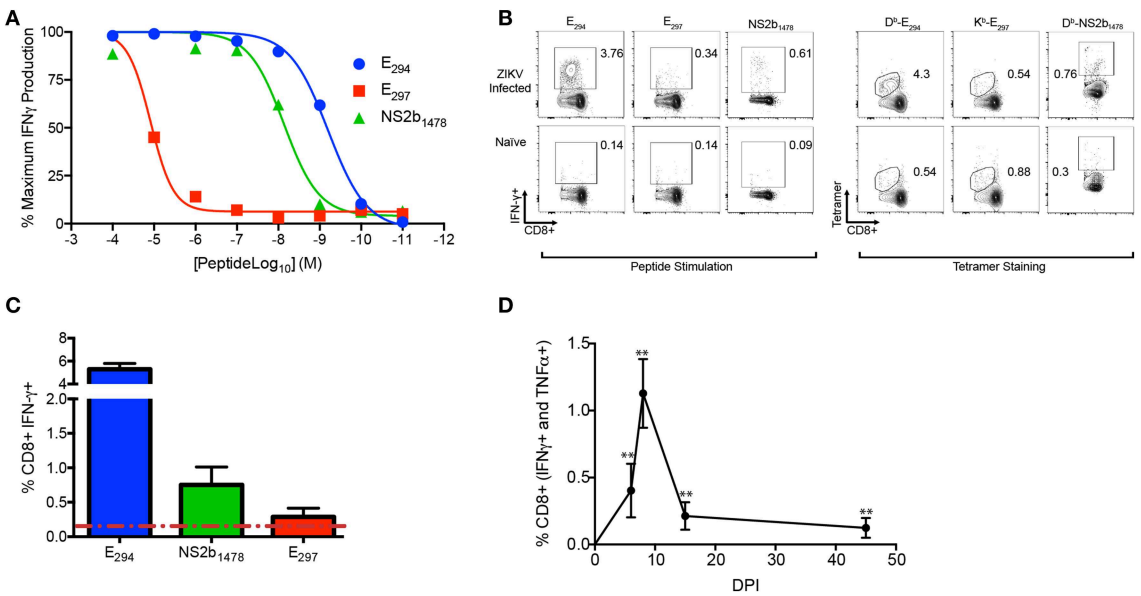

FIGURE 3 | ZIKV-specific CD8+ T cell epitope identification in the acute and memory phases of infection. **(A–D)** C57BL/6J mice (*n* between 3 and 5) were injected IV with 10^5 FFU of ZIKV bled or harvested at the timepoints indicated **(A)** The functional avidity of the CD8 T cell epitopes identified. On day 8 post infection splenocytes were harvested and stimulated with the indicated peptides (E_{294}, E_{297}, and $NS2b_{1478}$) with the concentrations listed on the y-axis in the presence of brefeldin A. Cells were gated using a lymphocyte gate, $CD19^-$, $CD4^-$, $CD8^+$, and were functionally analyzed by expression of IFNγ. Data is presented as the normalized maximal percent of CD8+ T cells that produced IFNγ in response to stimulation). **(B)** Representative flow plot comparing tetramer binding frequency to IFN-γ response for E_{294}, E_{297}, and $NS2b_{1478}$. On day 8 post infection splenocytes were harvested and either stimulated with the E_{294}, and E_{297} peptides or stained with peptide specific tetramers. **(C)** Expression hierarchy of E_{294}, $NS2B_{1478}$, and E_{297} during the acute infection. On day 8 post infection splenocytes were stimulated with E_{294}, $NS2B_{1478}$, or E_{297} peptides in the presence of BFA and the proportion of the CD8+ T cell response dedicated to each of epitope was determined. **(D)** Functional response to E_{294} is preserved in memory. C57BL/6J mice were injected IV with 10^5 FFU of ZIKV and on day 0, 5, 8 15, and 45 mice were bled and were stimulated and stained as described above. Data is presented as the percent of CD8+ T cells that produced IFNγ and TNFα in response to stimulation. Data is from a single experiment ($n = 6$). Asterisks indicate values that are statistically significant (**$p < 0.005$) as determined by Mann-Whitney test.

interferon insufficient mice (35, 54, 56–58). To confirm the role of CD8+ T cells in our hands we depleted CD8+ T cell from 8–12-week-old type I interferon receptor deficient ($Ifnar1^{-/-}$) mice, which are the same MHC haplotype (H2-b) as the C57BL/6 mice used in our epitope mapping experiments (**Figure 4A**). The $Ifnar1^{-/-}$ mice received the CD8+ T cell depleting antibody 3 days prior to infection and a second dose on the day of subcutaneous (SC) infection with 1×10^5 focus forming units (FFU) of ZIKV. Following ZIKV infection we monitored the mice daily, recording: mortality, weight, and clinical signs of disease (**Figures 4A–C**). In both the CD8 depleted and control mice we saw evidence of ZIKV infection and disease, which included weight loss, and temporary hind limb paralysis. There was a significant difference in the mortality between the CD8 depleted and control mice, as 100% of the CD8 depleted mice succumb to ZIKV, compared to a 25% mortality of the $Ifnar1^{-/-}$ control mice (**Figure 4A**). Unlike what we had previously observed with $CD4^+$T cell depletions (49), we did not observe any differences in weight loss between the control and depleted mice prior to the mice succumbing to infection (**Figure 4B**) and the disease scores between the two groups were similar with the onset of disease occurring 6 days post infection (**Figure 4C**). The results of these studies confirmed the necessity of CD8+ T cells for the control of ZIKV infection in $Ifnar1^{-/-}$ mice, but point to a different role for CD8+ T cells in the control of ZIKV as compared to what we had previously observed with $CD4^+$T cells (49).

After demonstrating the necessity of CD8+ T cells for protection from ZIKV in Ifnar1-/- mice, we next examined if CD8+ T cells from a ZIKV immune mouse were sufficient for protection. We isolated CD8+ T cells from naïve or ZIKV immune C57BL/6J Ly5.1 mice. The Ly5.1. mice were infected with ZIKV 30 days prior to adoptive transfer. On the day of the transfer, spleens were harvested from naïve or ZIKV immune Ly5.1. mice and CD8+ T cells were isolated by negative selection with magnetic beads. The CD8+ T cell isolation resulted in a purity of approximately 97% as determined by flow cytometry (data not shown). 4×10^6 naïve or ZIKV CD8+ T cells were then adoptively transferred into 8-week-old $Ifnar1^{-/-}$ mice 1 day prior to 10^5 FFU ZIKV IV lethal challenge. The mortality, weight loss and clinical scores of the ZIKV infected $Ifnar1^{-/-}$ mice were monitored for 14 days (**Figures 5A–C**). All of the $Ifnar1^{-/-}$ mice that received the CD8+ T cells from the ZIKV immunized mouse survived, and surprisingly 80% of the mice that received the naive CD8+ T cells survived the lethal challenge (**Figure 5A**). The mice that received the CD8+ T cells from the ZIKV immune mice lost significantly less weight than the mice that received the naïve T cells on days 8–14 (**Figure 5B**). The

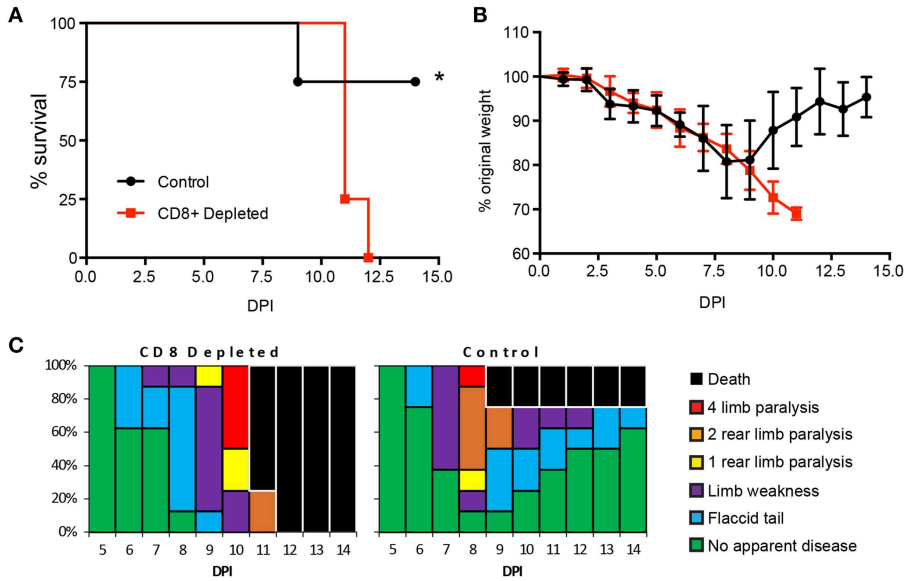

FIGURE 4 | CD8+ T cells are necessary for protection from ZIKV challenge. **(A)** Survival of 10–12-week-old Ifnar1$^{-/-}$ mice following CD8+ T cell depletion and inoculation with 10^5 FFU of ZIKV by footpad injection ($n = 9$ control, $n = 12$ depleted). On day −3 and day 0, mice were administered 100 μg of depleting antibody anti-CD8 or isotype control intraperitoneally ($n = 9$ or 12 mice per group, respectively). Survival differences were statistically significant (*$p = 0.018$) as determined using a Mantel-Cox test. **(B)** Weight loss during acute ZIKV infection of 10–12-week-old mice. As a measure of disease, mice were weighed daily for 15 days. Prior to depleted mice succumbing to infection there were no significant differences between the CD8$^+$ depleted group and control group as determined using an unpaired *t*-test with Welch's correction. **(C)** Clinical scoring associated with acute ZIKV infection. Mice were evaluated for signs of neurological disease daily and graphed on each day as a percentage of mice displaying that disease indicator. Signs of disease range and in the most severe cases accelerate in the following manner from no apparent disease, limp tail, hind limb weakness, hind limb paralysis, complete paralysis and death. All data is a compellation of 2 independent experiments.

ZIKV infected Ifnar1$^{-/-}$ mice that received naïve T cells had an earlier detection of clinical scores than the mice that receive the ZIKV immune CD8+ T cells, day 5 compared to day 6, and the duration of detectible clinical scores in the naïve mice last longer with 20% of the mice still showing evidence of disease at day 14 post infection (**Figure 5C**). While all mice that received the adoptively transferred CD8+ T cells showed some signs of diseases as detected by the clinical scores the proportion of mice with elevated disease scores also was higher in the Ifnar1$^{-/-}$ mice that received the naïve CD8+ T cells with as many as 60% of the mice showing signs of 2 rear limb paralysis on day 7 as compared to 10% in the mice that had received the ZIKV immune CD8+ T cells.

Finally, we were interested to see if the CD8+ T cells transferred from the ZIKV immune mice were better able to prevent ZIKV persistence compared the transferred naïve CD8+ T cells (**Figure 5D**). We have previously shown that ZIKV could persist in Ifnar1$^{-/-}$ mice (49). To determine if the transferred CD8+ T cells from the ZIKV immune mice could clear the ZIKV infection we harvested organs from five of the remaining ZIKV infected mice that received the naïve and ZIKV immune CD8+ T cells 14 days post infection. We titered the virus in the spleens, livers, kidneys, brains, spinal cords, and from whole blood using quantitative real-time PCR. For all mice in both groups we were able to detect virus in all the organs analyzed suggesting that the ZIKV immune CD8+ T cells alone were not able to clear virus from the Ifnar1$^{-/-}$ mice. However, in multiple organs, (kidney, brain, and spinal cord as well as whole blood) we did note a significantly lower viral titer in the mice that had received CD8+ T cells from ZIKV immune mice compared to the mice that had received naïve CD8+ T cells, suggesting that the virus specific T cells were better at controlling the virus in the Ifnar1$^{-/-}$ mice.

The results of our study suggest that there is a strong detectible CD8+ T cell response to ZIKV following infection and that ZIKV-specific CD8+ T cells are able to reduce the signs of disease and protect against a lethal ZIKV infection. Surprisingly, we saw a similar level of protection against mortality when we adoptively transferred naïve CD8+ T cells into the lethally challenged mice. The mechanisms responsible for this protection are currently being investigated. These results indicate that ZIKV specific CD8+ T cells are both necessary and sufficient to protect against lethal ZIKV infection.

DISCUSSION

Achieving a protective humoral immune response has driven much of the focus for effective vaccine design. However, we now understand that most of the highly effective vaccines incorporate both humoral and T cell responses. For flaviviruses, T cell responses in particular have proven to be highly relevant for controlling viral infection and reducing disease severity (35, 59–63). Yellow fever virus vaccine (YFV-17D) provides one of the best examples for the need to consider the T cell response for the development of an effective vaccine [reviewed in (64)].

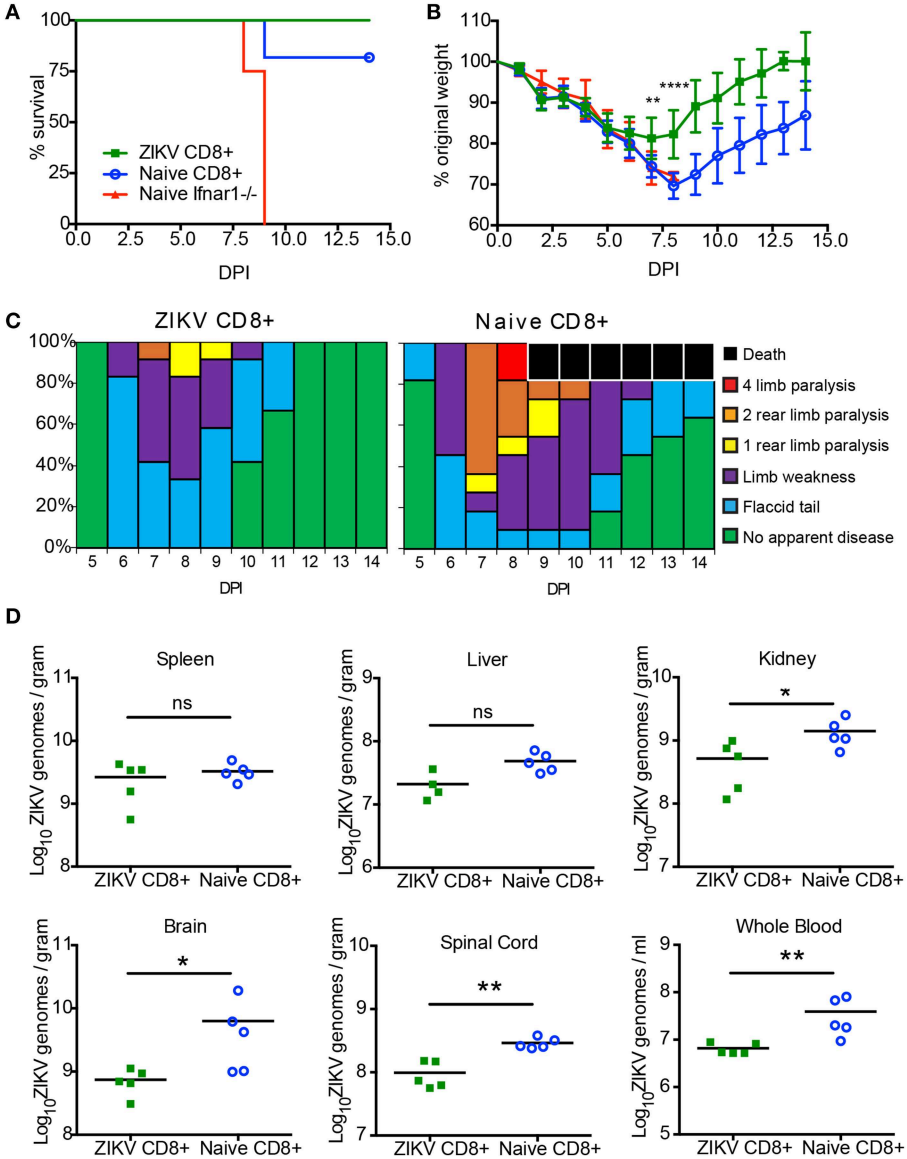

FIGURE 5 | CD8+ T cells are sufficient to protect against a lethal ZIKV challenge. **(A)** Survival of 10–12-week-old mice following adoptive transfer of CD8+ T cells and IV route ZIKV challenge. At 30 days post infection, CD8+ T cells were isolated to >97% purity from ZIKV infected or naïve C57BL/6J mice and transferred IV into 10- to 12-week-old Ifnar1$^{-/-}$ mice (~3 × 10^6 /mouse) 1 day prior to IV infection with 10^5 FFU of ZIKV (n = 9–11 per group). Survival differences were not statistically significant between the two groups that received CD8 T cells but were statistically different than mice that received PBS as determined by Mantel-Cox test. **(B)** Weight loss during IV ZIKV infection of 10–12-week-old mice following adoptive transfer. As a measure of disease, mice were weighed daily for 14 days. There were significant differences between the mice that receive ZIKV immune CD8$^+$ T cells from group compared to the mice that received naïve CD8 T cells on day 7 (**p = 0.001) and day 8 (****p < 0.0001) determined using an unpaired t-test with Welch's correction. **(C)** The clinical scores associated with IV ZIKV challenge following adoptive transfer. Mice were evaluated for signs of neurological disease daily and graphed on each day as a percentage of mice displaying that disease indicator. Signs of disease range and in the most severe cases accelerate in the following manner from no apparent disease, limp tail, hind limb weakness, hind limb paralysis, complete paralysis, and death. **(D)** Viral burden in the peripheral and CNS tissues after CD8$^+$ adoptive transfer and ZIKV infection. Ifnar1-/- mice that received CD8$^+$ T cells from naïve or day 30 ZIKV immune C57BL/6J Ly5.1 mice were infected with 10^5 FFU ZIKV via IV route. On day 14 (n = 5 per group) post-infection, organs were harvested, snap frozen, weighed, and homogenized. Levels of viral RNA were quantified by qPCR in whole blood, liver, spleen, kidney, spinal cord, and brain. Data are shown as Log$_{10}$ focus-forming unit equivalents (eq.) (as determined by standard curve) per gram or ml of tissue or blood, respectively. Asterisks indicate values that are statistically significant (*p < 0.05, **p < 0.001) as determined by Mann-Whitney test.

More recently studies have demonstrated that strong T cell responses is important for controlling DENV infections and for reducing the possible effects of antibody dependent enhancement (ADE) (65, 66). These studies point to the important role for the cellular immune responses during flavivirus infection and vaccine-mediated protection and begin to highlight the shift

in focus for vaccine development to designing vaccines that stimulate both the cellular and humoral immune responses. There are multiple requirements for the development of a vaccine that incorporates the cellular immune response including (1) the identification of immune-stimulatory antigenic epitopes of the virus, (2) confirmation of the protective capacity of the cellular response, and (3) evidence that the epitope specific T cell responses are maintained into memory. While ultimately these studies must be carried out with human vaccine trials, animal models have been critical in establishing the correlates of protection that should be incorporated into informing an effective vaccine strategy. Epitope identification has long been established as the fundamental foundational work that needs to be done to begin to understand the immune correlates of T cell protection. ZIKV-specific CD8+ T cell epitope identification using animal models allows for the initial study of immune correlates of protection and opens up new models assessing vaccine efficacy.

We set out to identify H2-K^b-and H2-D^b restricted ZIKV CD8+ T cell epitopes using the production of intracellular cytokine responses following peptide stimulation from a full length overlapping peptide library (**Figure 1**). Traditionally the production of IFN-y and TNF-α has been correlated with potent effector function with flaviviruses (67), so we hypothesized that $CD8^+$ T cell responses we identified using this approach would correlate with potent protective responses. We show here that ZIKV mounts a potent CD8+ T cell response in mice that persists into memory. Using a whole genome peptide library approach, we were able to identify three MHCI-restricted CD8+ T cell epitopes; two corresponding to the Envelope (E_{294}) (E_{297}) and one corresponding to the non-structural protein NS2b ($NS2b_{1478}$) (**Figure 3**). Notably this is the first reported study that identified $NS2b_{1478}$ as a ZIKV-specific epitope in mice on a C57BL/6J background. Using this approach, we were not able to detect responses to some of the previously identified CD8+ T cell epitopes from C57BL/6J mice (35). This may be due to our use of IFN-y and TNF-α as a means to identify ZIKV-specific CD8+ T cell epitopes or possibly the use of a full15-mer peptide library as opposed to using epitope prediction software. By requiring the T cells to produce cytokine as a means of identification we biased our results toward these effector cell populations potentially missing ZIKV specific CD8+ T cell populations that do not make these cytokine responses. As such, it should be noted that studies have demonstrated that the T cells that don't produce IFN-y may be important for control of some viruses (68). Additionally, the use of 15mer amino acids to screen for epitopes requires some level of processing to the optimal 9mer for CD8+ T cell stimulation and identification, therefore we could miss epitopes that could not be optimally processed. To gain the most accurate picture of the ZIKV-specific CD8+ T cell response we and others will need to conduct further studies.

It is worth noting that in our initial prime-boost based screening assays, we did not detect strong ZIKV-specific CD8+ T cell responses in the Ifnar$1^{-/-}$ mice compared to the immune competent C57BL/6J (data not shown). Because we have previously demonstrated Ifnar$1^{-/-}$ mice had persistent viral titers for >30 days after infection with ZIKV (49), we hypothesized that the virus specific T cell population was more exhausted in the Ifnar$1^{-/-}$ mice due to persistent antigenic stimulation. As we relied on a functional assay with cytokine production to map the T cell response in our animals we used C57BL6/J mice, which are of the same MHC haplotype as Ifnar1-/- mice (H2-b). Based on the epitopes we identified in this study and the CD4+ T cell epitopes we identified previously (49) we are currently conducting studies using CD4+ and CD8+ T cell tetramers to further investigate this observation of potential exhaustion.

After the identification of the virus specific CD8+ T cell responses in our model we next wanted to determine if CD8+ T cells were necessary for protection against a sublethal ZIKV challenge. Through depletion studies we demonstrated that the loss of CD8+ T cells lead to a significantly higher mortality in susceptible Ifnar1-/- mice as compared to mice that had received an isotype control antibody (**Figure 4A**). However, unlike what we had observed for the CD4+ T cell depletion studies, we noted that there were no significant differences in weight loss between the CD8+ depleted and control mice prior to the depleted mice succumbing to infection (**Figure 4B**). Based on our clinical scoring observations the onset of disease was similar between the groups with some mice in both groups showing signs of a flaccid tail on day 6 post infection (**Figure 4C**). Notably more of the isotype control mice showed signs of neurological disease and limb paralysis earlier (Day 8) than the CD8+ depleted mice (Day 9). These results are in line with the previously published observations by Jurado et al that suggest that CD8+ T cells may cause some of the neuropathology seen in ZIKV disease in mice (57). However, ultimately all of the CD8+ T cell depleted mice succumb to infection where the most of the isotype control treated mice recovered demonstrating that CD8 T cells are necessary for protection against ZIKV mortality in Ifnar1-/- mice.

Multiple studies have suggested a dominant role of CD8+ T cells in controlling ZIKV infection (35, 50, 51, 56, 57, 69–71) although at least one study highlights the potential for ZIKV specific CD8+ T cells to play an immunopathological role in neuroinvasive disease (19). We sought to determine if ZIKV specific CD8+ T cells were sufficient for protection against a lethal ZIKV challenge in our model (**Figure 5**). Similar to what has previously been observed we noted that CD8+ T cells transferred from ZIKV immune mice were protective against a lethal ZIKV challenge. However, we also observed a similar level of protection from mortality when we adoptively transferred naïve C57BL6/J CD8+ T cells into the Ifnar1-/- mice prior to lethal challenge. The mice that received the naïve CD8+ T cells did lose significantly more weight than the mice that received CD8+ T cells from ZIKV immune mice. Additionally, 75% of the mice that received naïve T cells showed some level of limb paralysis as compared to 20% of the mice that received CD8+ T cells from immune mice. Moreover, mice that received CD8+ T cells from ZIKV immune mice had reduced viral burden in multiple organs. Taken together, these results indicate that CD8+ T cells from ZIKV immune mice were sufficient to reduce the viral burden, morbidity, and clinical signs of ZIKV disease, while both naïve and ZIKV immune CD8+ T cells from C57BL/6J mice were sufficient to protect Ifnar1-/- mice from a lethal challenge relative to un-manipulated infected Ifnar1-/- mice.

In summary, our current study characterizes the protective capacity of CD8+ T cells during a ZIKV infection in a susceptible mouse model. We confirmed the identification of the ZIKV specific epitopes E_{294} and E_{297} and identified a novel ZIKV CD8+ T cell epitope $NS2B_{1478}$. In this study we also demonstrated that CD8+ T cells were necessary for protection against ZIKV lethality and that while CD8+ T cells from ZIKV immune C57BL/6J mice contributed to reduced viral burden and ZIKV induced morbidity, both naïve or T cells from ZIKV immune mice were sufficient for protection from lethality. These results highlight a need for further studies looking into the role of the virus specific CD8+ T cells directed against our identified epitopes in protection from ZIKV infection.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Saint Louis University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MH and MGH performed the experiments and contributed to the manuscript. JB and AP wrote the manuscript and directed the research.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01678/full#supplementary-material

Supplemental Figure 1 | qPCR to measure viral load in lymphoid organs of mice used in epitope mapping. Wild type C57BL/6J mice were infected with 10^5 FFU of ZIKV via IV route. On days 3 and 6 post infection, the spleens and lymph nodes were harvested, weighed, and homogenized. qRT-PCR was used to quantify the viral load in each organ.

Supplemental Figure 2 | *In vivo* expansion of $NS2b_{1478}$ and E_{297} specific cells. Wild type C57BL/6J mice were infected with 10^5 FFU of ZIKV via IV route. At days 0, 5, 8, or 45 post-infection, splenocytes were harvested and stimulated with $NS2b_{1478}$ peptide **(A)** or E_{297} peptide **(B)** for 6 h in the presence of brefeldin A. Cells were stained for surface markers (CD3, CD19, CD4, and CD8), stained intracellularly for IFNγ and TNFα and analyzed by flow cytometry. Cells were gated using a lymphocyte gate, $CD19^-$, $CD4^-$, $CD8^+$, and were functionally analyzed by expression of IFNγ and TNFα. Data is presented as the percent of CD8+ T cells that produced both IFNγ and TNFα in response to peptide stimulation. Asterisks indicate values that are statistically significant ($^*p < 0.05$) as determined by Mann-Whitney test.

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Assessing the Diversity and Stability of Cellular Immunity Generated in Response to the Candidate Live-Attenuated Dengue Virus Vaccine TAK-003

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The development of an efficacious DENV vaccine has been a long-standing public health priority. However, this effort has been complicated significantly due to the hazard presented by incomplete humoral immunity in mediating immune enhancement of infection and disease severity. Therefore, there is a significant need for DENV vaccine platforms capable of generating broad immune responses including durable cellular immunity, as well as novel analytical tools to assess the magnitude, diversity, and persistence of vaccine-elicited immunity. In this study, we demonstrate that a single dose of the recombinant, tetravalent, live-attenuated DENV vaccine TAK-003 elicits potent and durable cellular immunity against both the structural and non-structural proteins of all four DENV serotypes, which is maintained for at least 4 months post-immunization. Although not contained within the vaccine formulation, significant reactivity against the non-structural (NS) proteins of DENV-1,-3, and-4 is observed following vaccination, to an extent directly proportional to the magnitude of responses to the corresponding vaccine (DENV-2) components. Distinct, quantifiable, and durable patterns of DENV antigen reactivity can be observed in individuals following vaccination. Detailed epitope mapping of T cell reactivity against the DENV-2 proteome using a matrix of overlapping peptide pools demonstrated that TAK-003 elicits a broad response directed across the DENV-2 proteome, with focused reactivity against NS1 and NS3. We conclude that, as measured by an IFN-γ ELISPOT assay, a single dose of TAK-003 generates potent T cell-mediated immunity which is durable in magnitude and breadth through 4 months post-vaccination.

Keywords: dengue, vaccine, T cell, cellular immunity, epitope discovery

INTRODUCTION

Dengue virus (DENV) is the causative agent of an acute febrile illness in humans and is a significant source of global morbidity (1). It is an arthropod-borne virus that is transmitted predominantly by the mosquito vectors of the genus *Aedes* (2). DENV is a single-stranded positive sense RNA virus that belongs to the family Flaviviridae, genus *Flavivirus*, and consists of four genetically and immunologically distinct serotypes: DENV-1; DENV-2; DENV-3; and DENV-4. DENV infects between 280 and 550 million people worldwide every year with as many as 100 million infection resulting in clinical presentation (any severity of disease) (1, 3). Infection with DENV is subclinical in the majority of cases, but it may also cause dengue fever, a debilitating flu-like illness that lasts for up to 2 weeks. Approximately 500,000 cases per year develop into severe dengue, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which has a mortality rate of up to 20% (4–7). Dengue is endemic in south and south-east Asia, the western Pacific, sub-Saharan Africa, and Central and South America, and hence at least 40% of the world's population is at risk of infection (1). Extensive globalization of DENV and increases in regional endemic serotype make-up and complexity continue to occur (1). As a result, developing a protective, durable, and safe vaccine product to counter the global threat of DENV infection is a public health priority. However, the difficulty in inducing immunity to all four DENV serotypes, the lack of a validated animal model for vaccine testing, and the lack of defined immune correlates of protective immunity represent significant obstacles to this effort. These challenges are further highlighted by the recent revelation that the only currently available DENV vaccine (Dengvaxia®) not only fails to protect previously DENV naïve individuals from infection, but can increase the risk of hospitalization with virologically confirmed dengue (8, 9). Therefore, there is significant need for new DENV vaccine candidates, as well as new tools and approaches to assess DENV vaccine safety, efficacy, durability, and immunogenicity.

To date, much focus has been placed on measuring neutralizing antibodies (NAb) as an immune correlate of protection against subsequent dengue infection (10–14). However, data from Phase IIb and Phase III trials of the Sanofi-Pasteur vaccine product (Dengvaxia®) demonstrated a discordance between vaccine efficacy and NAb titers (15–17). In addition, while DENV-elicited humoral immunity undoubtedly contributes to protection against homotypic and heterotypic reinfection, the same humoral milieu may play a more pathologic role upon heterotypic infection. While the pathogenesis of DHF/DSS is mechanistically complex and may involve some degree of genetic predisposition (18, 19), waning humoral immunity following infection and partial antibody-mediated cross-recognition of heterotypic DENV is one potential explanation for the increased incidence of severe disease in the setting of secondary infection (5, 20, 21). Antibody-dependent enhancement (ADE) of DENV infection has been demonstrated in numerous *in vitro* experimental models and *in vivo* adoptive transfer animal models (5, 10, 22–24), although these models have not translated to human disease, and definitive *in vivo* evidence of ADE in humans has been elusive.

In light of gaps in knowledge about the relationship between humoral immunity and outcome of DENV infection, there is the need to investigate the contribution of other immune response parameters, particularly cell mediated immunity (CMI)—especially T cell-mediated immunity—to the outcome of DENV infection (25–27). Recent studies suggest that the expression of certain HLA alleles—and the nature/magnitude of the T cell responses they facilitate—correspond to susceptibility or resistance to disease, and potential DENV vaccine efficacy (28, 29). Human T cell responses to DENV were first characterized over 30 years ago, and many of the general principles originally described have remained consistent (25, 27). Infection with one DENV induces both $CD4^+$ and $CD8^+$ memory T cells specific for DENV epitopes, with a small number of epitopes dominating the response in each individual (28, 29). Epitopes are located throughout the DENV polyprotein, although several regions, especially non-structural protein 3 (NS3) and the capsid protein, appear to have a concentration of immunodominant epitopes targeted by $CD8^+$ T cells and $CD4^+$ T cells, respectively (30–32). The amino acid homology across the four DENV serotypes varies for each epitope; however, most epitopes are well-conserved among strains within the same serotype and differ at relatively few positions (1–3 of 9 residues) from the corresponding epitopes of other DENV serotypes (and other flaviviruses) (33, 34). As is observed in DENV-elicited humoral immunity, the overall T cell response induced by a primary DENV infection is strongest to the serotype to which the subject was exposed, but variable degrees of cross-reactivity are usually observed to one or more of the other serotypes (35, 36).

TAK-003 is a tetravalent, recombinant DENV vaccine candidate based on the attenuated PDK-53 DENV-2 virus strain that is currently undergoing phase III testing with a two-dose vaccine schedule (37). The PDK-53 strain was initially derived from the WT DENV-2 16,681 isolate, and attenuated by serial passage in primary dog kidney (PDK) cells. Key attenuation mutations have been identified in the 5′ UTR, NS1, and NS3 regions of the viral genome (38). This DENV-2 backbone virus was previously shown to be safe, immunogenic, and capable of stimulating durable cellular and humoral immunity (39–43). To create a vaccine capable of eliciting an immune response against all four DENV serotypes, recombinant viruses were created using the PDK-53 DENV-2 genetic backbone and the prM and E genes from DENV-1,-3, and-4 (44). The tetravalent TAK-003 formulation was also shown to be safe, immunogenic, and protective against lethal DENV challenge in both rodent models and non-human primates (37, 44, 45). In clinical trials, TAK-003 is well-tolerated and capable of generating significant humoral immunity against all four DENV serotypes in both children and adults, regardless of previous dengue serostatus (46–51). Previous analysis of the T cell cytokine production profile generated by TAK-003 administration in flavivirus-naïve recipients demonstrated that this product generates a pool of NS1, NS3, and NS5 reactive $CD8^+$ T cells capable of producing IFN-γ, TNF-α, and to a lesser extent IL-2 upon *ex vivo* restimulation (52). However, the magnitude, stability,

and antigen specificity of cell-mediated immunity generated in response to a TAK-003 vaccination has not previously been described in detail.

In this study, we demonstrate that a single dose of TAK-003 elicits a potent T cell response as assessed by IFN-γ ELISPOT 28- and 120-days post vaccination. Reactivity against the structural genes of DENV-1,-2,-3, and-4 contained within the vaccine formulation was observed to be significantly elevated over pre-vaccination levels 28 days post vaccination, with reactivity against the structural proteins of DENV-2 and-4 maintained for at least 120 days. However, reactivity against the structural regions of DENV represented only 13–20% of the total T cell response observed, with the rest of the T cell response directed against the non-structural DENV proteins. As the PDK-53-derived DENV-2 backbone is the only source of non-structural DENV antigen in TAK-003, a significant amount of reactivity against these gene products is observed, with reactivity against DENV-2 NS1, NS3, and NS5 dominating. While there are no DENV-1,-3, and-4 non-structural antigens present in TAK-003, a significant amount of T cell cross-reactivity against these antigens was observed 28- and 120-days post vaccination. The magnitude of cross-reactivity observed was directly proportional to the strength of the response directed against the DENV-2 non-structural antigens contained within TAK-003. While the overall response rate to vaccination as measured by IFN-γ ELISPOT was very high in this study (85% at day 120), significant individual-to-individual variability was observed in antigen immunodominance hierarchy. Dimensional-reduction visualization (tSNE projection) and hierarchal cluster analysis of ELISPOT reactivity data revealed the presence of 7 distinct patterns of DENV-2 reactivity following TAK-003 vaccination. Individuals falling within these distinct clusters of reactivity maintained their relative cluster localization between 28- and 120-days post vaccination, suggesting that these designations represent stable outcomes of vaccination. Finally, detailed epitope mapping of T cell reactivity against the DENV-2 proteome using a matrix of overlapping peptide pools demonstrated that TAK-003 elicits a broad response directed across the DENV-2 proteome.

MATERIALS AND METHODS

Cells/Samples

The samples used in this study were collected during a Phase 1 trial in US adults of a tetravalent, live-attenuated dengue virus vaccine candidate, TAK-003 ("Impact of Subcutaneous vs. Intramuscular Administration of Inviragen's Live Attenuated Dengue Vaccine on Safety and Immunogenicity" NCT01728792; WRAIR #1987). Subjects received one dose of TAK-003 either subcutaneously or intramuscularly and whole blood was collected at day of vaccination (day 0) and at days 14, 28, and 120 post-vaccination. Individuals were not stratified by route of vaccination in subsequent analysis. Whole blood was collected in Cell Preparation Tubes (BD Vacutainer) for isolation of PBMC. Cells were cryopreserved at $\sim 10^7$ per mL and stored in vapor-phase liquid nitrogen until use. Vaccine administration and PBMC collection were performed after written informed consent in accordance with the Declaration of Helsinki. The studies and protocols were approved by the institutional review boards at the State University of New York Upstate Medical University and the Human Subjects Research Review Board for the Commanding General of the U.S. Army Medical Research and Material Command. Exclusion criteria for participation in this study include history of dengue fever, Japanese Encephalitis, West Nile or Yellow Fever disease, and history of travel to dengue endemic areas including the Caribbean, Mexico, Central America, South America or Southeast Asia during the month prior to screening, or planned travel to a dengue endemic area during the study period. All subjects were screened to be seronegative to dengue or West Nile at the time of study initiation.

T Cell ELISPOT Assay

Cryopreserved PBMC were thawed and placed in RPMI 1640 medium supplemented with 10% heat-inactivated normal human serum (100–318, Gemini Bio-Products), L-glutamine, penicillin, and streptomycin. After an overnight rest at 37°C, the PBMC were washed, resuspended in serum free medium (SFM; X-VIVO 15, Lonza), cellular viability assessed by trypan blue exclusion, and $1–2 \times 10^5$ viable cells were plated per well of a 96-well Millipore MAIPSWU plate coated with anti-IFN-γ antibody according to the manufacturer's instructions (3420-2HW-Plus, Mabtech Inc.). Peptide pools were added to the cells at a final concentration of 1 μg/mL/peptide prior to incubation at 37°C overnight. Controls included SFM plus 0.5% DMSO (negative) and anti-CD3 (positive). The ELISPOT plates were developed using TMB substrate and read using a CTL-ImmunoSpot® S6 Ultimate-V Analyzer (Cellular Technology Limited). All peptide pools were tested in duplicate, and the mean value of the duplicate wells utilized as the reported value. Individuals were considered reactive to a peptide pool when the response was >50 SFC/10^6 PBMC and was 4-fold over the corresponding negative control. For matrix ELISPOT analysis, positive wells were defined as those with a signal 5-fold over the negative (no stimulation) control, and >5 spots per well after subtracting the negative (no stimulation) control. All data were normalized based on the number of cells plated per well and are presented herein as SFC/10^6 PBMC values.

Peptides

Overlapping peptide pools corresponding to the full-length envelop (E), non-structural 1 (NS1), NS3, and NS5 proteins for DENV-1-4 and NS2a/b and NS4a/b proteins for DENV-2 were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (**Supplemental Table 4**). Additional overlapping peptide pools covering the capsid (C) and precursor membrane (prM) proteins of DENV-1-4, were purchased from JPT Peptide Technologies (**Supplemental Table 4**). Peptide pool stocks were reconstituted in DMSO at a concentration of 200 μg/mL/peptide and stored at −80°C.

Statistical Analysis

TSNE projection, hierarchical clustering, and visualization of multidimensional IFN-γ ELISPOT data was performed in R (v3.5.2). tSNE projection and hierarchical clustering

was calculated using the package rtsne utilizing background-subtracted data, a perplexity value of 30 and theta value of 0.5. Hierarchical clustering was performed using a cluster value of 7. The resulting two-dimensional dataset was visualized using the package ggplot2. All code is available upon request from the corresponding author. All other statistical analyses were performed using GraphPad Prism v6 Software (GraphPad Software, La Jolla, CA). A p-value <0.05 was considered significant.

RESULTS

TAK-003 Elicits Significant Cellular Immunity Against the Structural Proteins of DENV-1,-2,-3, and -4

TAK-003 is a recombinant, tetravalent, live-attenuated dengue vaccine candidate derived from the attenuated PDK-53 DENV-2 strain. To generate a vaccine product which contains the structural proteins from all four DENV serotypes, the structural genes (CprM/E) from the parental DENV-2 backbone were sequentially replaced with those from DENV-1, DENV-3, or DENV-4 to create three additional recombinant DENV strains (**Figure 1A**). These four live-attenuated viruses were co-formulated for simultaneous vaccine administration.

Vaccination with TAK-003 resulted in a significant increase in the number of circulating IFN-γ producing cells at day 28 post vaccination responding to the structural proteins (CprM/E) from DENV 1 (**Figure 1B**, **Table 1**), DENV-2 (**Supplemental Figure 1**, **Figure 1C**, **Table 1**), DENV-3 (**Figure 1D**, **Table 1**), and DENV-4 (**Figure 1E**, **Table 1**) as assessed by IFN-γ ELISPOT. However, while the number of circulating IFN-γ producing cells reacting to the structural proteins (CprM/E) of DENV-2 and DENV-4 remained significantly elevated on day 120 post vaccination relative to pre-vaccination (**Figures 1C,E**), the number of circulating DENV-1 CprM/E and DENV-3 CprM/E reactive cells on day 120 post vaccination was returning to baseline (pre-vaccination) levels when assessed as a group (**Figures 1B,D**). Therefore, despite significant reactivity against CprM/E derived from all serotypes at 28-days post vaccination, there were significantly more DENV-2 and DENV-4 reactive T cells on day 120 than DENV-1 and DENV-3 reactive cells (**Figure 1F**).

TAK-003 Elicits Significant and Broad Cellular Immunity Against the Non-structural Proteins of DENV-2

While the majority of the protective humoral immunity generated by DENV infection or vaccination is canonically thought to be directed against the DENV envelope protein (53–56), a significant fraction of the cell-mediated immune response to DENV infection is directed against the non-structural proteins (NS1, NS2a/b, NS3, NS4a/b, and NS5) (33, 34). As the only non-structural DENV proteins contained within the TAK-003 formulation originate from the DENV-2 backbone (**Figure 1A**), we assessed the magnitude and breadth of the T cell response generated against all five DENV-2 NS proteins on days 28 and 120 post vaccination.

Immunization with TAK-003 resulted in a significant increase in the number of circulating IFN-γ producing T cells at day 28 post vaccination responding to DENV-2 NS1 (**Supplemental Figure 1**, **Figure 2A**, **Table 2**), DENV-2 NS2a/b (**Supplemental Figure 1**, **Figure 2B**, **Table 2**), DENV-2 NS3 (**Supplemental Figure 1**, **Figure 2C**, **Table 2**), DENV-2 NS4a/b (**Supplemental Figure 1**, **Figure 2D**, **Table 2**), and DENV-2 NS5 (**Supplemental Figure 1**, **Figure 2E**, **Table 2**). These responses persisted for at least 120 days post vaccination, some of which increased moderately from day 28 to day 120 (**Figures 2A–E**). Responses to non-structural proteins accounted for ∼85% of the total reactivity against DENV-2, with NS1, NS3, and NS5 contributing a combined ∼75% of the overall DENV-2 response (**Figure 2F**). While the total number of IFN-γ producing DENV-2 reactive T cells increased from day 28 to day 120 post vaccination, the relative distribution of the responses to individual DENV-2 structural and non-structural proteins remained consistent.

TAK-003 Elicits DENV Serotype Cross-Reactive Responses Against the Non-structural Proteins of DENV-1,-3, and -4

While the only non-structural DENV genes contained within TAK-003 are DENV-2 in origin, a significant cellular immune response to the non-structural proteins (NS1, NS3, NS5) of DENV-1,-3, and-4 was observed 28- and 120-days post TAK-003 administration as assessed by IFN-γ ELISPOT (**Supplemental Figure 2**, **Table 3**). The only exception to this trend was the cross-reactive response observed against DENV-1 NS1. While some individuals did have a detectible DENV-1 NS1 response (**Table 3**) the overall magnitude of all subjects failed to reach significance at either 28- or 120-days post vaccination (**Supplemental Figure 1**). While the overall magnitude of the observed cross-reactive response against most non-structural proteins of DENV-1,-3, and-4 was significantly elevated relative to baseline following TAK-003 administration, the relative responses to NS1 (**Figure 3A**), NS3 (**Figure 3B**), and NS5 (**Figure 3C**) from DENV-1,-3, and-4 were significantly lower than the responses observed against the corresponding proteins from DENV-2. These data suggest that any reactivity against the non-structural proteins of DENV-1, -3, and-4 is dependent on a strong response against the non-structural proteins of DENV-2. This hypothesis is supported by the observation that the magnitude of TAK-003-stimulated DENV-1 (**Figure 3D**), DENV-3 (**Figure 3E**) and DENV-4 (**Figure 3F**) non-structural protein cross-reactivity is directly proportional to the magnitude of the corresponding DENV-2 non-structural protein response. Therefore, while multi-serotype cellular immunity against DENV non-structural proteins can be generated in response to what is essentially a monovalent exposure, the magnitude of cross-reactivity is significantly lower and directly proportional to the magnitude of the vaccine-directed response.

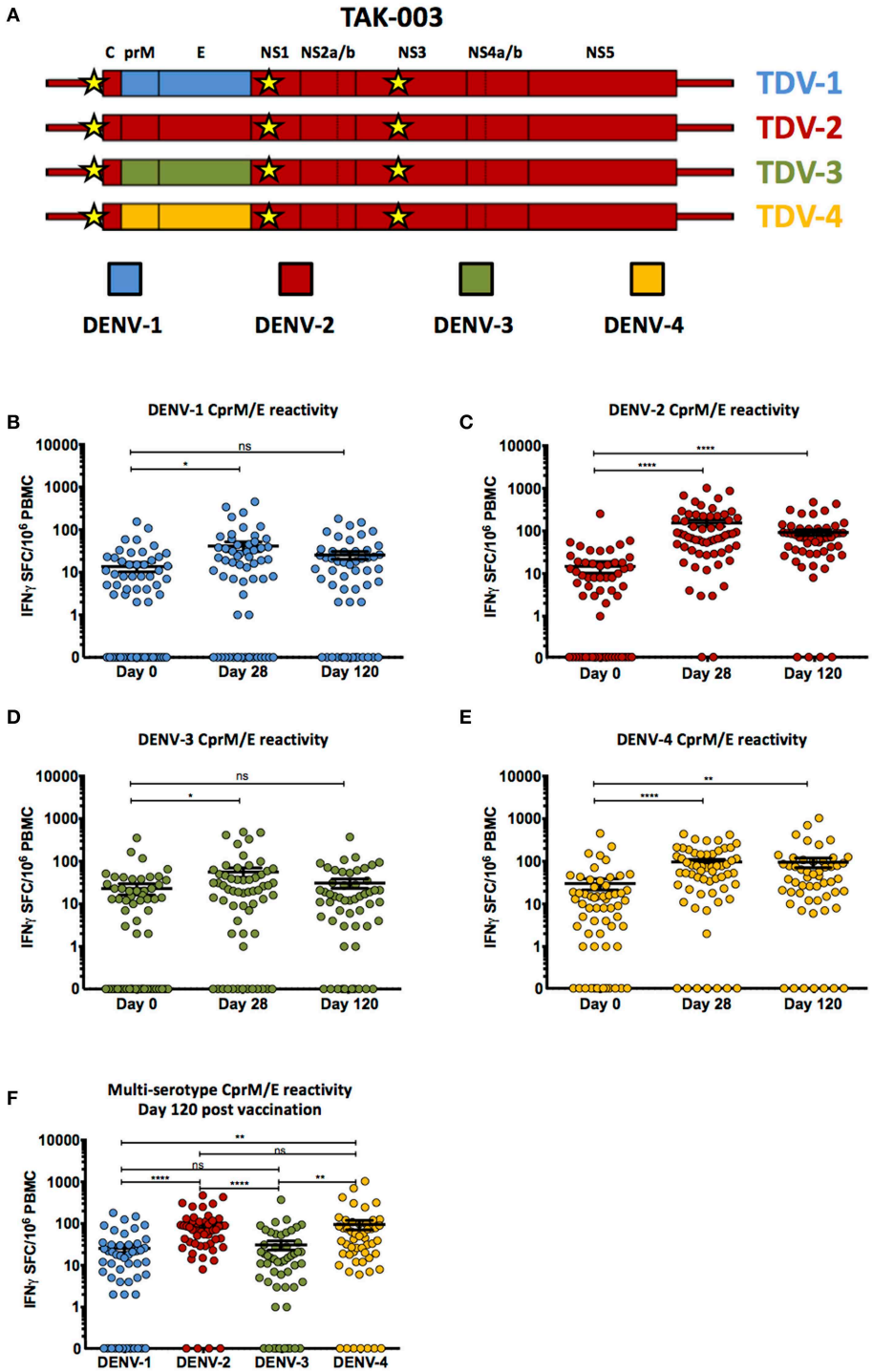


FIGURE 1 | Cell-mediated reactivity against DENV-1,-2,-3, and-4 structural proteins following TAK-003 administration. **(A)** Schematic representation of the genetic structure of the tetravalent TAK-003 vaccine platform. Key attenuating mutations in the 5′ UTR, NS1, and NS3 are indicated by the yellow stars. **(B)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-1 CprM/E peptide stimulation at the indicated time points following vaccination. **(C)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-2 CprM/E peptide stimulation at the indicated time points following vaccination. **(D)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-3 CprM/E peptide stimulation at the indicated time points following vaccination. **(E)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-4 CprM/E peptide stimulation at the indicated time points following vaccination. **(F)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-1,-2,-3, and-4 CprM/E peptide stimulation at day 120 post vaccination. $n = 60$ for days 0 and 28, $n = 54$ for day 120. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$, ns, not significant Paired two-tailed t-test. Bars indicate group mean ± SEM. Zero values were superimposed on the logarithmic graph.

TABLE 1 | Percent of subjects reactive to DENV structural proteins.

		Percent of subjects reactive to DENV structural proteins							
		C/prM				*E*			
	N	DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4
Day 0 (Pre-vaccination)	60	0%	0.0%	0%	5%	0%	0.0%	1.7%	1.7%
Day 28 (Post-vaccination)	54	1.7%	16.7%	1.7%	16.7%	6.7%	26.7%	6.7%	6.7%
Day 120 (Post-vaccination)	54	0%	9.3%	1.9%	18.5%	3.7%	22.2%	3.7%	9.3%

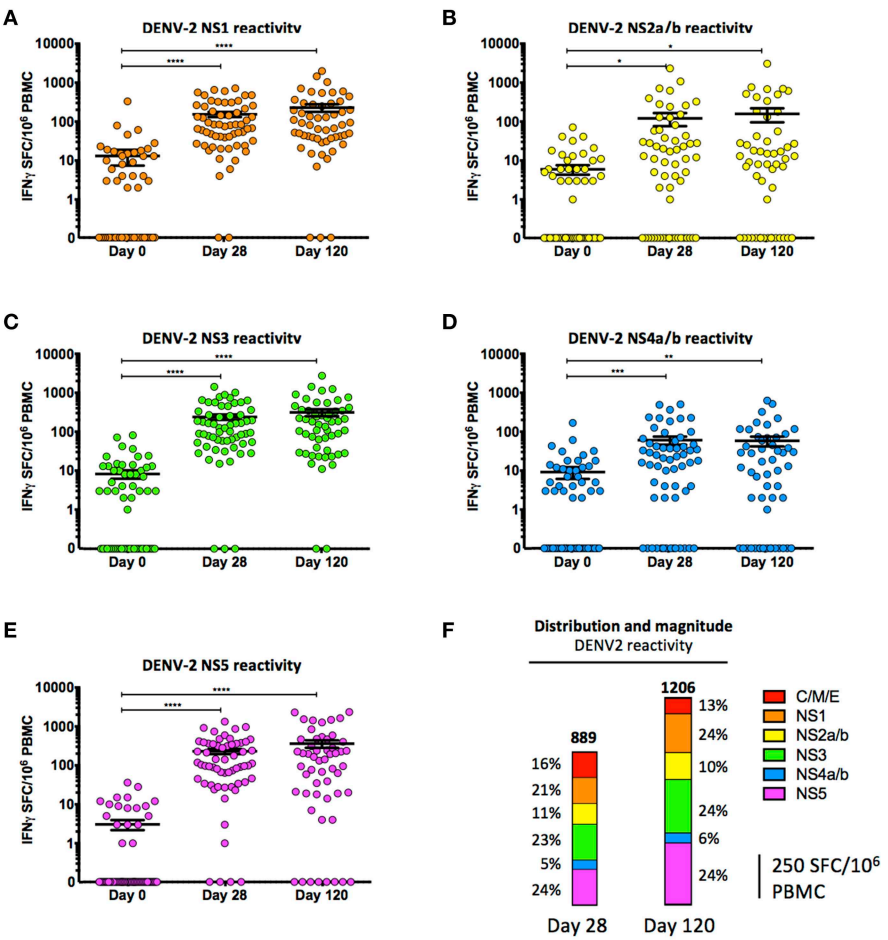


FIGURE 2 | Cell-mediated reactivity against DENV2 non-structural proteins following TAK-003 administration. **(A)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-2 NS1 peptide stimulation at the indicated time points following vaccination. **(B)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-2 NS2a/b peptide stimulation at the indicated time points following vaccination. **(C)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-2 NS3 peptide stimulation at the indicated time points following vaccination. **(D)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-2 NS4a/b peptide stimulation at the indicated time points following vaccination. **(E)** Frequency of IFN-γ producing cells as quantified by ELISPOT following DENV-2 NS5 peptide stimulation at the indicated time points following vaccination. **(F)** Relative distribution and magnitude of DENV2 reactivity at day 28 and 120 post vaccination. Numbers at the top of each bar indicate average number of Spot Forming Cells (SFCs) per million PBMCs observed in response to DENV-2 stimulation in all subjects at the relevant time points. $n = 60$ for days 0 and 28, $n = 54$ for day 120. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ Paired two-tailed *t*-test. Bars indicate group mean ± SEM. Zero values were superimposed on the logarithmic graph.

Distinct Patterns of Antigen Reactivity Following TAK-003 Administration

Although administration of TAK-003 resulted in a significant and fairly uniform overall increase in total DENV-2 reactivity 28- and 120-days post vaccination (**Table 2**), there was significant individual-to-individual variation in the pattern of DENV-2 protein reactivity. As has been observed following natural DENV infection or vaccination with other DENV vaccine candidates, individuals often exhibit differences in antigen immunodominance hierarchy (33, 34). This variation in

TABLE 2 | Percent of subjects reactive to the DENV-2 proteome.

		Percent of subjects DENV-2 proteome reactive							
	N	Total proteome	C/prM	*E*	NS1	NS2a/b	NS3	NS4a/b	NS5
Day 0 (Pre-vaccination)	60	1.7%	0.0%	0.0%	1.7%	0.0%	0.0%	0.0%	0.0%
Day 28 (Post-vaccination)	54	85.0%	16.7%	26.7%	41.7%	20.0%	51.7%	16.7%	53.3%
Day 120 (Post-vaccination)	54	81.5%	9.3%	22.2%	42.6%	18.5%	61.1%	20.4%	55.6%

TABLE 3 | Percent of subjects reactive to DENV non-structural proteins.

		Percent of subjects reactive to DENV non-structural proteins											
		NS1				NS3				NS5			
	N	DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4
Day 0 (Pre-vaccination)	60	3.3%	1.7%	0%	1.7%	1.7%	0.0%	0%	0%	1.7%	0.0%	0%	3.3%
Day 28 (Post-vaccination)	54	8.5%	41.7%	10.2%	6.8%	26.7%	51.7%	26.7%	21.7%	15%	53.3%	18.3%	11.7%
Day 120 (Post-vaccination)	54	5.6%	42.6%	9.3%	6.7%	37%	61.1%	25.9%	22.2%	20.4%	55.6%	20.4%	13%

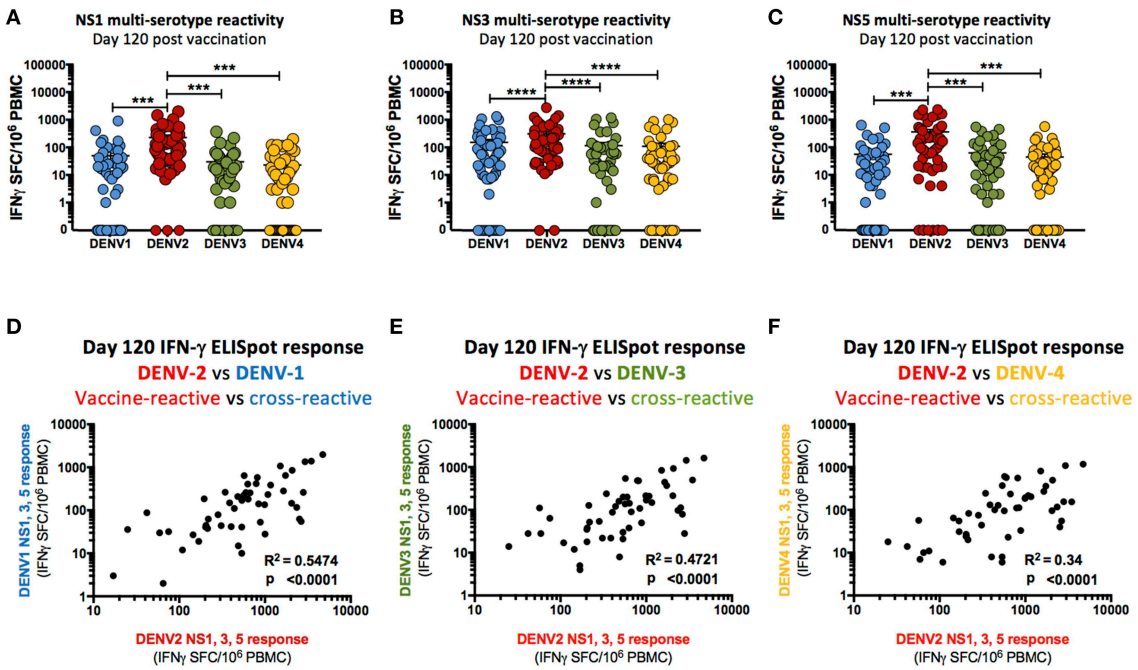


FIGURE 3 | Cross-reactivity of T cell responses following TAK-003 administration. **(A)** Frequency of IFN-γ producing cells as quantified by ELISPOT upon DENV-1,-2,-3, and-4 NS1 peptide stimulation at day 120 post vaccination. **(B)** Frequency of IFN-γ producing cells as quantified by ELISPOT upon DENV-1,-2,-3, and-4 NS3 peptide stimulation at day 120 post vaccination. **(C)** Frequency of IFN-γ producing cells as quantified by ELISPOT upon DENV-1,-2,-3, and-4 NS5 peptide stimulation at day 120 post vaccination. **(D)** Relative frequency of IFN-γ producing cells activated in response to DENV-1 NS1, NS3, and NS5 vs. DENV-2 NS1, NS3, and NS5 peptide stimulation at day 120 post vaccination. **(E)** Relative frequency of IFN-γ producing cells activated in response to DENV-3 NS1, NS3, and NS5 vs. DENV-2 NS1, NS3, and NS5 peptide stimulation at day 120 post vaccination. **(F)** Relative frequency of IFN-γ producing cells activated in response to DENV-4 NS1, NS3, and NS5 vs. DENV-2 NS1, NS3, and NS5 peptide stimulation at day 120 post vaccination. $n = 54$. $^{***}p < 0.001$, $^{****}p < 0.0001$ Paired two-tailed *t*-test. R^2 calculated by linear regression. Bars indicate group mean ± SEM. Zero values were superimposed on the logarithmic graph.

individual DENV epitope reactivity can be partially attributed to differences in host HLA genotype, which dictates which DENV-derived epitopes can be efficiently presented by infected cells or professional antigen presenting cells to putative DENV-reactive T cells (28).

In an attempt to capture and quantify the unique individual patterns of DENV reactivity elicited by TAK-003 administration in an unsupervised fashion—and to potentially define statistically unique patterns of reactivity that might eventually correlate with vaccine efficacy or durability—we utilized dimensional

compression analysis to display the magnitude and specificity of the IFN-γ ELISPOT response directed against the DENV-2 proteome (CprM, E, NS1, NS2a/b, NS3, NS4a/b, and NS5) on days 0, 28, and 120 post vaccination in 2-dimensional space. We used t-Distributed Stochastic Neighbor Embedding (tSNE) visualization and hierarchical cluster analysis to visualize and group 174 data points generated during this study. Each data point corresponds to one time point for one subject, and reflects the cumulative reactivity against both the structural and non-structural proteins of DENV-2.

Using these tools, we were able to identity 7 statistically distinct clusters of DENV-2 reactivity in TAK-003 recipients on days 0, 28, or 120 post vaccination (**Figures 4A,B**). Data points falling in cluster 1 possess little-or-no DENV reactivity, and overwhelmingly correspond to pre-vaccination samples (**Figure 4C**). Cluster 2 contains a mix of day 0, 28, and 120 post vaccination samples. While exhibiting slightly higher DENV-2 reactivity than cluster 1, cluster 2 does not exhibit a distinct pattern of reactivity and contains those individuals who responded poorly to vaccination or whose response to vaccination did not persist (**Figure 4D**). Data points falling in clusters 3 and 4 are from primarily day 28 and day 120 post vaccination samples and exhibit a strong NS1 or NS2a/b biased response, respectively (**Figures 4E,F**). Cluster 5 is characterized by a dominant NS5 response (**Figure 4G**), while clusters 6 and 7 have dual NS3 and NS5 reactivity of differing magnitudes (**Figures 4H,I**).

Since our analysis contained multiple time points for each individual post vaccination, we were able to assess the stability of our statistically defined clusters as TAK-003-elicted immunity matured over time, and how localization in a given cluster corresponded to an individual's long-term DENV reactivity. Only two individuals fell within cluster 1 at day 28 post vaccination and either remained in cluster 1 on day 120 post vaccination or moved to the poorly-responsive cluster 2 (**Supplemental Figure 3**, **Supplemental Table 1**), suggesting that these individuals are true non-responders to vaccination from the perspective of T cell immunity. As predicted by the low-level and relatively non-specific DENV-2 reactivity that characterizes cluster 2, this cluster is highly unstable between days 28 and 120 post vaccination and likely represents a "transition" state between more stable outcomes of vaccination (**Supplemental Figure 3**, **Supplemental Table 1**). Individuals falling within cluster 3 and cluster 4 on day 28 post vaccination (NS1 or NS2a/b reactive) are highly stable, with 82% of individuals staying within the same cluster between days 28 and 120 post vaccination (**Supplemental Figure 3**, **Supplemental Table 1**). There is significant movement of individuals between clusters 5, 6 and 7 between days 28 and 120, but little movement of individuals out of this "super-group." As clusters 5, 6, and 7 are characterized by different magnitudes of reactivity against the same antigens (NS3 and/or NS5), movement between these clusters might be predicted as immunity stabilizes after vaccination. No information is currently available on how these clusters may correspond to protection against subsequent challenge or infection, but these data demonstrate a novel method to assess the diversity and stability of vaccine-elicited cellular immunity.

Epitope Mapping of DENV-2 Reactivity Day-120 Post Vaccination

In light of the complex—yet restricted—pattern of DENV-2 reactivity observed following TAK-003 administration, we endeavored to define the exact DENV-2 derived epitopes recognized by the immune system following vaccination. To this end, we performed a matrix ELISPOT analysis of PBMC samples from 40 individuals obtained 120 days post TAK-003 vaccination using a library of 532 overlapping peptides spanning the entire DENV-2 proteome. The peptides used were 15-20 AA in length, overlapping by 10–14 AA, and were derived from the DENV-2 NGC strain.

A total of 221 peptides from the library of 532 possible candidates were recognized as reactive across all 40 individuals screened in this assay (**Figure 5A**, **Supplemental Table 2**). Of these immunogenic peptides, 109 were recognized by 5% or more of subjects analyzed (**Supplemental Table 3**), while 17 peptides were recognized by more than 10% of subjects (**Figure 5B**). The frequency of observed reactivity was highest for peptides derived from NS3, with 79.5% of all peptides derived from this protein eliciting a positive response in at least one subject, and with each peptide being recognized by 4.7% of subjects on average (**Supplemental Table 2**). In contrast, only 13.4% of peptides derived from E were recognized as immunogenic in TAK-003 immunized individuals, with each peptide being recognized by 0.48% of subjects on average. While proportionally the majority of the immunogenic peptides identified in this analysis were derived from NS3 (18.5% of all positive peptides), the most commonly recognized peptide screened in this analysis was derived from NS1, with 30% of subjects exhibiting reactivity against this single peptide. Notably, 90% of subjects falling into the previously described NS1-reactive "cluster 3" (**Figure 4A**) exhibited reactivity against this single dominant NS1-derived peptide or its adjacent neighbor (with which it shares 10 of 16 AA), suggesting that responsiveness to this epitope may be a significant driving factor in determining NS1- biased reactivity following vaccination.

On average, amino acid level sequence conservation (identity) is 70% between all DENV serotypes (57). However, there is variation in the extent of sequence conservation in the various DENV protein subunits, including regions compatible with HLA presentation (33, 34). This fact is reflected in the relative amino acid level conservation of the 17 dominant immunogenic peptides identified in our screen. For those peptides derived from DENV-2 NS1 identified as immunogenic in this analysis, the average amino-acid level conservation (identity) between DENV-2 and the other 3 DENV serotypes is only 58%. In contrast, the immunogenic peptides derived from DENV-2 NS3 shared on-average 76% amino-acid level identity with NS3 from the other 3 DENV serotypes. These values offer a potential mechanistic explanation for the weak DENV-1 NS1 cross-reactivity observed in TAK-003 recipients, as well as the correspondingly robust NS3 cross-reactivity (**Supplemental Figure 2**).

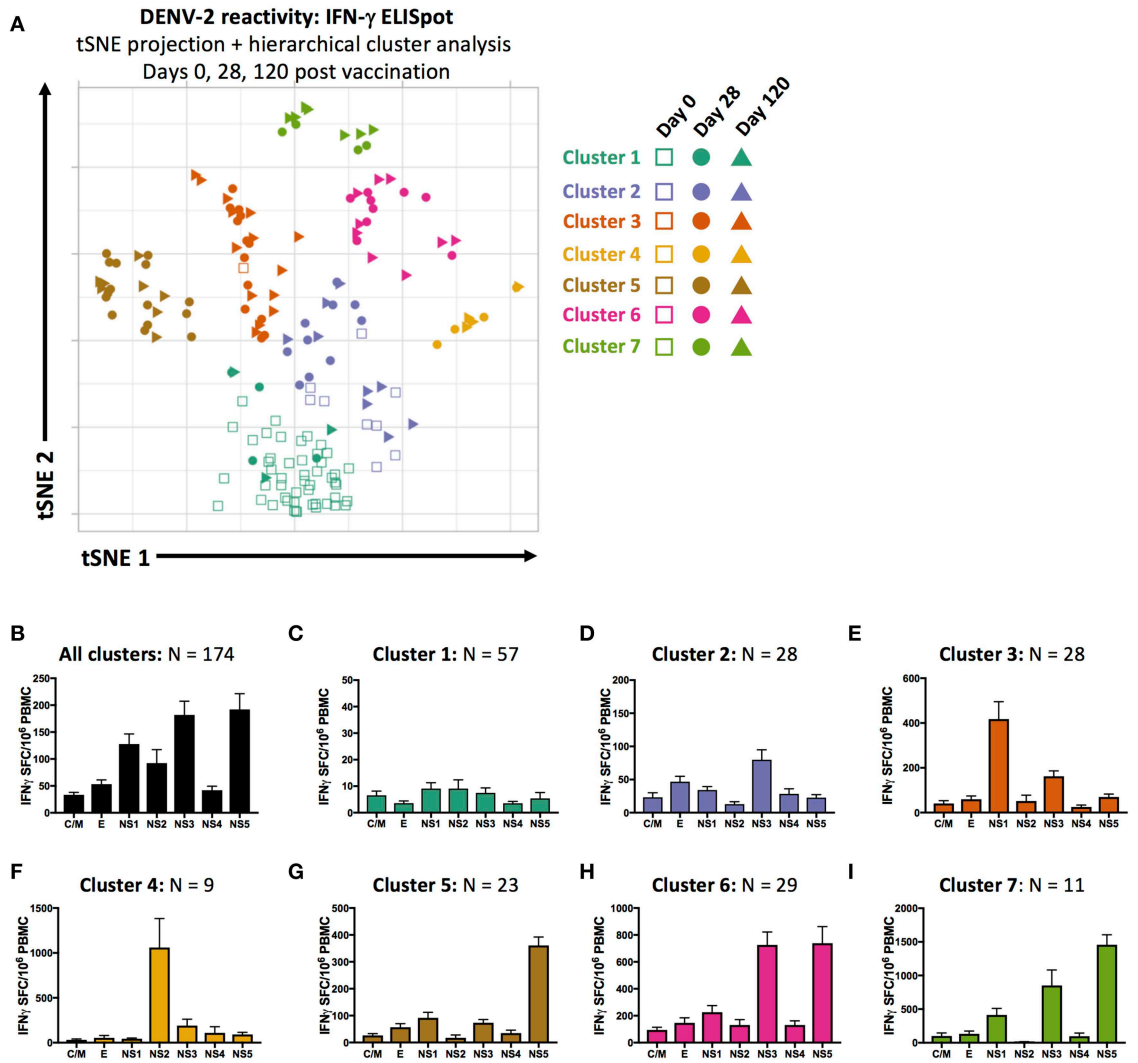
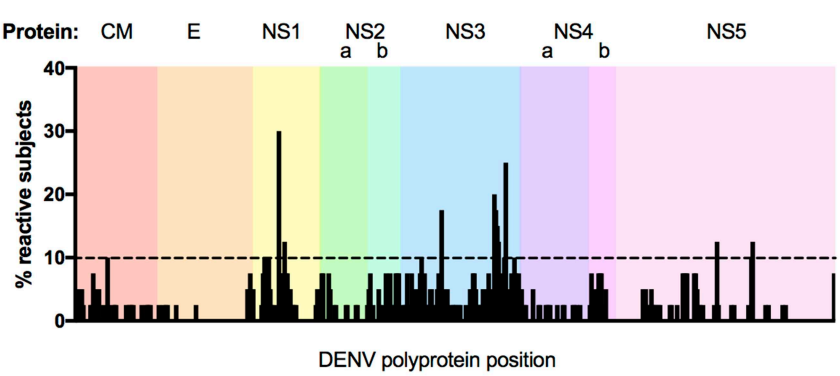


FIGURE 4 | Unsupervised dimensional reduction and hierarchal clustering of DENV-2 antigen reactivity. **(A)** tSNE projection and hierarchal clustering of 7-dimentional DENV-2 reactivity data (C/M, E, NS1, NS2a/b, NS3, NS4a/b, and NS5 stimulation) as assessed by IFN-γ ELISPOT in all samples from days 0, 28, and 120 post TAK-003 administration. Average frequency of IFN-γ producing cells as assessed by ELISPOT in response to stimulation with the indicated peptide pools in **(B)** all data points, **(C)** cluster 1 data points, **(D)** cluster 2 data points, **(E)** cluster 3 data points, **(F)** cluster 4 data points, **(G)** cluster 5 data points, **(H)** cluster 6 data points, or **(I)** cluster 7 data points. Bars indicate group mean ± SEM. tSNE clustering was performed with a perplexity value of 30 and theta of 0.5. Hierarchical clustering analysis was performed using a cluster value of 7.

DISCUSSION

In this study, we demonstrate that a single dose of the candidate tetravalent DENV vaccine TAK-003 elicits DENV-specific T cell responses in humans as assessed by IFN-γ ELISPOT at both 28- and 120-days post immunization. These responses were directed against all the antigens contained within the formulation, including the structural regions of DENV-1 to-4, as well as the non-structural regions of the DENV-2 derived vaccine backbone. In addition, significant cross-reactivity against the non-structural gene products (NS1, NS3, and NS5) of DENV-1,-3, and-4 was observed, with the magnitude of the cross-reactivity dictated by the magnitude of the response generated by the non-structural gene products of the vaccine's DENV-2 backbone. While the fraction of individuals responding to TAK-003 vaccination as assessed by IFN-γ ELISPOT was very high (85% at day 28), significant individual-to-individual variation in the pattern of antigenic reactivity was observed. We were able to categorize individuals into statistically distinct clusters based on their pattern of antigen reactivity using unsupervised dimensional reduction projection/visualization and hierarchical clustering of the IFN-γ ELISPOT data. Finally, we performed fine-epitope mapping of the DENV-2 T cell responses generated by TAK-003 vaccination using a matrix of 532 overlapping peptides representing the entire DENV-2 proteome. This approach identified 221 potential T cell epitopes spread across the DENV-2 proteome, of which 109 were found to be immunogenic in 5% or more study-subjects following

A



B

			Epitope Conservation (percent identity)		
Peptide ID	Reactive individuals	Peptide sequence (DENV-2)	DENV1	DENV3	DENV4
CM-23	10%	FLKQNEPEDIDCWCNSTSTW	55.0%	70.0%	75.0%
NS1-10	10%	KQITPELNHILSENEVKL	66.7%	66.7%	55.6%
NS1-13	10%	IKGIMQAGKRSLQPQPTEL	42.1%	58.0%	42.1%
NS1-20	30%	TAECPNTNRAWNSLEV	62.5%	62.5%	75.0%
NS1-24	12.5%	LKLREKQDVFCDSKLMSA	55.6%	61.1%	50.0%
NS3-16	10%	TKPGLFKTNAGTIGAVSL	55.6%	55.6%	77.8%
NS3-30	17.5%	LRTLILAPTRVVAAEM	87.5%	100.0%	100.0%
NS3-67	20%	LDNINTPEGIIPSMF	86.7%	86.7%	80.0%
NS3-68	17.5%	TPEGIIPSMFEPEREKV	82.4%	82.4%	76.5%
NS3-69	15%	SMFEPEREKVDAIDGEYR	77.8%	77.8%	66.7%
NS3-70	12.5%	KVDAIDGEYRLRGEARK	88.2%	76.5%	76.5%
NS3-74	10%	LAYRVAAEGINYADRRW	64.7%	64.7%	58.8%
NS3-75	25%	EGINYADRRWCFDGIK	68.8%	68.8%	56.3%
NS3-81	10%	KPRWLDAKIYSDPLALK	76.5%	82.4%	64.7%
NS5-74	12.5%	KWKSAREAVEDSRFWEL	70.6%	64.7%	76.5%
NS5-98	10%	NHMEGEHKKLAEAIFKL	58.8%	58.8%	58.8%
NS5-99	12.5%	HKKLAEAIFKLTYQNKV	76.5%	82.4%	88.2%

FIGURE 5 | Fine epitope mapping of DENV-2 T cell responses following TAK-003 administration. Magnitude and distribution of DENV-2 reactivity in TAK-003 recipients 120 days post vaccination. **(A)** Schematic representation of DENV-2 epitope reactivity following TAK-003 vaccination across the DENV-2 proteome. Bar height indicates the percentage of subjects responding to the indicated peptide at the corresponding genomic location. **(B)** Immunodominant DENV-2 epitopes identified in this analysis, defined as peptides eliciting a response in 10% or more of study participants following vaccination. Conservation of identified immunodominant DENV-2 peptides shown relative to DENV-1 (Singapore/S275/1990), DENV-3 (Philippines/H87/1956), and DENV-4 (Singapore/8976/1995). Conservation calculated as percent identify at the amino acid level. $n = 40$ subjects.

vaccination, while 17 peptides were found to be immunogenic in 10% or more study-subjects.

Assessing and leveraging cell-mediated immunity in the setting of dengue vaccine design is increasing in relevance and urgency in light of the weak correlation observed between NAb titers and protective immunity following vaccination with Dengvaxia®) (15–17). While neutralizing antibodies are necessary and sufficient to provide sterile immunity to viral infection in animal models (58), growing evidence suggests that they are not sufficient to provide complete protection from infection in humans (15–17). Vaccine-directed cell-mediated immunity can in theory restrict viral replication and dissemination following DENV infection without risking the development of severe dengue via antibody-mediated immune enhancement during waning immunity. Furthermore, the development of stable, diverse, and mature humoral immunity is dependent on T cell help during B cell germinal center maturation (59). All vaccine products can reasonably be expected to induce some form of cell-mediated immunity—whether it is direct effector cells such as classical CTL, or NK cells, helper T cells which modulate the antibody and CTL responses, or more likely some combination of these. However, ensuring and confirming that the antigen-specific cellular immune response (T cell mediated) is directed against antigens that can enable lysis of infected cells or facilitate maturation of nascently activated B cells has not always been a focus in immunogenicity studies of candidate dengue vaccines.

In addition to producing durable and functional immunity, a key consideration for DENV vaccine design is generating immunity against four immunologically distinct viruses in a balanced and simultaneous manner. There is significant amino acid level homology between the four DENV serotypes (∼70%), meaning that immunological cross-reactivity is readily achieved for both humoral and cell-mediated immunity, albeit with varying degrees of balance. However, the role that cross-reactive T cells play in facilitating DENV immunity and/or pathology is complex and incompletely understood. Severe dengue is often associated with significant peripheral T cell activation and expansion, including a significant number of serotype cross-reactive T cells (35, 60–62). Prior DENV exposure appears to have little impact on the number and frequency of DENV cross-reactive cells activated in response to acute infection, although the timing of their expansion and circulation differs between primary and secondary infection. However, as clinical symptoms of severe dengue (plasma leakage, etc) occur with or after defervescence and viral clearance, severe dengue is a consequence of dysregulated immunity following symptomatic viral infection (63). Therefore, categorizing cross-reactive T cells as either pathogenic or protective based on their presence during severe dengue is potentially problematic, and may misconstrue cause-and-effect. As there are currently no data available on the relationship between the generation of cross-reactive T cells following vaccination and protection from infection or severe disease, the precise role these cells play in protective immunity—especially in previously flavivirus-naïve individuals—remains unclear.

In addition to quantifying the cellular immunogenicity of the candidate DENV vaccine TAK-003, this study highlights two important features of DENV cellular immunity which can help refine approaches for functionally assessing future vaccines. Firstly, caution should be exercised when down-selecting epitopes or antigens to assess cellular immunity following vaccination or natural exposure to DENV. While NS3-derived peptides dominate the list of immunogenic epitopes identified in this analysis and NS3 responses exhibit the most cross-reactivity (presumably due to NS3 sequence conservation between serotypes), a significant fraction of vaccinated individuals in this trial exhibited little-or-no reactivity against NS3, and instead exhibited monotypic reactivity against NS1, NS2a/b, or NS4a/b. These distinct and stable patterns of individual reactivity are most likely attributable to differences in host HLA genotype. Whether or not these unique patterns of reactivity correlate with protection following vaccination is still unclear, but the observation underlines the prudence of assessing the immunogenicity of a broad panel of vaccine-derived antigens. Secondly, due to the unique design of the TAK-003 vaccine product, this study is among the first to directly assess the magnitude and persistence of vaccine-elicited DENV serotype-cross-reactivity in previously flavivirus-naïve individuals. We observed that the magnitude of serotype cross-reactivity for any given antigen is directly proportional to the magnitude of the response elicited by the corresponding vaccine-component. This observation provides mechanistic insight into the pattern of vaccine-driven immunity, and can also guide future vaccine design. Notably, while reactivity against the structural regions of DENV-1 and DENV-3 (antigens contained within the vaccine formulation) wanes by 120-days post vaccination, significant reactivity against the non-structural regions of DENV-1,-3, and-4 can still be observed at 120-days, despite the fact that these antigens are not contained within the vaccine formulation. The persistence of this cross-reactive response is attributable to the fact that the non-structural regions of the TAK-003 DENV-2 backbone accounts for the majority of the cellular immune response observed following vaccination. While the cross-reactive immune response directed against the non-structural regions of DENV-1,-3, and-4 is significantly lower than the vaccine-elicited response to the same regions of DENV-2, the relative magnitude of these responses still means that cross-reactive cellular immunity can persist even when responsiveness to other vaccine components wanes.

ETHICS STATEMENT

The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70–25. The studies were approved by the institutional review boards at the State University of New York Upstate Medical University and the Human Subjects Research Review Board for the Commanding General of the U.S. Army Medical Research and Material Command.

AUTHOR CONTRIBUTIONS

MG and AK generated the data. JC conceived of the analysis, designed the experiments, and analyzed the data. RJ provided project oversite and secured funding. TE and ST conceived and designed the clinical trial. MP executed and oversaw the clinical trial and collected samples. HF designed and executed experiments and analyzed the data. AW analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01778/full#supplementary-material

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REVIEW
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Human T Cell Response to Dengue Virus Infection

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DENV is a major public health problem worldwide, thus underlining the overall significance of the proposed Program. The four dengue virus (DENV) serotypes (1–4) cause the most common mosquito-borne viral disease of humans, with 3 billion people at risk for infection and up to 100 million cases each year, most often affecting children. The protective role of T cells during viral infection is well-established. Generally, CD8 T cells can control viral infection through several mechanisms, including direct cytotoxicity, and production of pro-inflammatory cytokines such as IFN-γ and TNF-α. Similarly, CD4 T cells are thought to control viral infection through multiple mechanisms, including enhancement of B and CD8 T cell responses, production of inflammatory and anti-viral cytokines, cytotoxicity, and promotion of memory responses. To probe the phenotype of virus-specific T cells, epitopes derived from viral sequences need to be known. Here we discuss the identification of CD4 and CD8 T cell epitopes derived from DENV and how these epitopes have been used by researchers to interrogate the phenotype and function of DENV-specific T cell populations.

Keywords: dengue, CD4 T cell, CD8 T cell, T cell epitope, vaccine

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DENV INFECTION AND THE COMPLEX ROLES OF T CELLS

Dengue virus (DENV) belongs to the genus *Flavivirus* and is closely related to several other flaviviruses including Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and West Nile virus (WNV) (1). DENV is a serious public health issue especially in tropical and subtropical areas, and it is estimated that ∼390 million people are infected yearly with DENV (2). DENV infection is associated with a range of clinical manifestations, from asymptomatic to more severe presentations including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). There is currently no specific therapy available for the treatment of dengue diseases other than supportive care. Furthermore, Dengvaxia® (Sanofi Pasteur), the first licensed DENV vaccine, is associated with efficacy and safety concerns (3–7). Sridhar et al. integratively analyzed data from three clinical trials and reported that Dengvaxia® increases the risk of severe dengue and hospitalization among vaccinees who have not been exposed to DENV before the vaccination (8). In order to develop effective DENV therapeutics and vaccines, it is important to define immunological correlates of protection against DENV infection as well as biomarkers that can be used to access their safety and efficacy.

Although T cells have important functions in combating viral pathogens, both pathological and protective effects of T cells have been reported in the context of DENV infection (9–14). According to T cell original antigenic sin, cross-reactive T cells that are specific for a primary DENV serotype become predominant during a secondary heterologous infection (9–16). Consequently,

the expansion of preexisting cross-reactive and low-affinity memory T cells results in ineffective viral control and contributes to immunopathology and severe dengue disease through excessive production of inflammatory cytokines (9–16). In contrast to the implications of original antigenic sin, several lines of evidence indicate that T cells contribute to the control of DENV infection. Murine studies demonstrate that CD4 T cells and especially CD8 T cells can play a protective role against DENV challenge (17–24). Furthermore, HLA alleles associated with protection from severe dengue disease are also associated with strong and multifunctional T cell responses, supporting the notion that T cells have protective functions during DENV infection (25–28). The main characteristic of an efficient vaccine is the prophylactic effect provided by protective neutralizing antibodies. Therefore, it is possible that in Dengvaxia® vaccines, native conserved masked conformational DENV (1–4) epitopes are not unmasked and therefore not accessible for highly neutralizing and broadly protective antibodies. Nevertheless, Dengvaxia® is a yellow fever dengue chimeric vaccine and lacks DENV non-structural (NS) proteins that contain a large proportion of T cell epitopes (25, 28, 29). Therefore, the suboptimal efficacy of Dengvaxia® may partially due to its defective ability to induce T cell responses (30). Indeed, a single dose of the live attenuated tetravalent DENV vaccine TV003 provides complete protection against infection with a DENV-2 challenge virus (31), potentially highlighting the importance of harnessing the protective functions of both humoral and cellular antiviral immunity.

METADATA ANALYSIS OF DENV-DERIVED CD4 AND CD8 T CELL EPITOPES

Human antigen-specific T cell immune responses are driven by two factors that are host specific. First the capability of antigen-derived peptides to be bound and presented in the context of HLA class I and II molecules. Second, the immunogenicity of those peptides that depends on the capability of T cells to recognize through T cell receptor (TCR) the HLA-peptide complex and trigger T-cell specific immune responses. Several studies have identified the DENV epitopes able to induce CD8 and/or CD4 T cells specific-response and consecutively the immunodominance of DENV proteins for DENV-specific T cell response. In this review, we summarize previous published data of all the DENV-epitopes experimentally identified by us and others by performing an overall analysis of data available in Immune Epitope Database (www.IEDB.org).

The IEDB database was queried on July 8th 2019 using the following search parameters: Positive assays only, Organism: Dengue virus (ID:12637), No B cell assays, No MHC ligand assays, Host: Homo sapiens (Human). This query retrieved a total of 57 different publications (**Table 1**). Most of the studies were focused on NS3 protein or multiple DENV proteins defined as immunodominant region based on previous studies (**Table 1**). Additional studies evaluated the full DENV polyprotein targeting either HLA-Transgenic DENV infected mice (32, 72) or human samples. Specifically, we considered human samples derived from different geographical locations and collected from either healthy DENV seropositive blood donors DENV (25, 26, 28, 73, 77, 80) or patients during acute dengue infection (29, 74, 75) or healthy donor after experimental vaccination with dengue virus (76, 78, 81) (**Table 1**). A total of 2191 epitopes were described in the 57 references retrieved by the query (**Table 1**); of those, 825 were restricted by HLA class I molecules, and 1345 epitopes were restricted by HLA class II. To define the most dominant epitopes, we considered epitopes that were reported positive in multiple donors and/or multiple studies. For example, the $NS3_{1608-1618}$ is the most dominant HLA class I epitope, being independently identified by several studies also incorporating different amino acid variants (16, 28, 33–35, 53, 73) (**Supplementary Table 1A**). Conversely, in the case of HLA class II molecules the C_{50-64} and C_{72-86} epitopes are examples of dominant epitopes, recognized in the majority of donors tested (12 positive out of 17 and 9 positive out of 11, respectively) in several independent studies (25, 77, 78). **Supplementary Tables 1A,B** list the most dominant class I and class II epitopes. Specifically, to identify the top ∼80 epitopes we selected class I epitopes recognized in 5 donors or more (84 epitopes), and class II epitopes recognized in 8 donors of more (77 epitopes).

TABLE 1 | List of references for DENV-specific T cell epitopes.

Protein target	#of studies	HLA restriction	References
NS3 protein	13	Class I	(16, 32–43)
	7	Class II	(44–49)
	3	Class I and II	(50–52)
Multiple DENV proteins or epitopes	12	Class I	(32, 53–63)
	3	Class II	(64–66)
	5	Class I and II	(67–71)
Polyprotein	6	Class I	(28, 72–76)
	5	Class II	(25, 26, 77–79)
	3	Class I and II	(29, 80, 81)
Total	57		

DENV epitopes studies divided per protein target and HLA restriction and available in the IEDB (www.IEDB.org).

IMMUNODOMINANT DENV PROTEINS RECOGNIZED BY CD4 AND CD8 T CELL

Dissecting the immunodominance of T cell response has considerable implications in vaccine design as the first licensed Dengvaxia® vaccine (Sanofi Pasteur) is composed of DENV structural proteins (prM, E) in a yellow fever backbone while both TV003 (NIH) and TDV (Takeda) vaccines contains both DENV structural and non-structural proteins (31, 82, 83). To assess DENV immunodominance without the bias of adding multiple studies that take in consideration only a single or few DENV proteins, we focused on the 14 studies

performed on the full DENV polyprotein and summarized in **Table 1**.

Epitopes derived from those 14 studies have been extracted from IEDB, redundant epitopes independently defined in donors with different HLA restriction have been included to give prominence to promiscuous peptides able to be presented and recognized by different HLA molecules. Finally, epitopes have been divided based on class I or class II restriction and plotted on a DENV reference sequence (ID:12637) by using the Immunobrowser tool (http://tools.iedb.org/immunomebrowser/) (84) freely available on the IEDB. **Figure 1** shows the immunodominance of CD8 (**Figure 1A**, HLA class I restriction) and CD4 T cells (**Figure 1B**, HLA class II restriction), respectively.

CD8 T cell responses targets mainly NS3 protein, followed by Capsid, NS5 and NS4A/B proteins (**Figure 1A**). NS3 immunodominance is confirmed throughout all the different studies used for the analysis, representing the most frequent target of CD8 T cell response, disregarding geographical location, and HLA restriction.

Conversely, CD4 T cell responses targets mainly Capisd followed by Envelope, NS3, NS2A/B, and NS5 proteins (**Figure 1B**). Capsid immunodominance is consistently identified in all the different studies analyzed, representing the most frequent target of CD4 T cell response. Finally, protein immunodominance for both CD4 and CD8 T cells is also function of the multiple exposure of DENV infection, that tends to skew protein immunodominance toward epitopes highly conserved across the different DENV serotypes as previously reported (85). Overall, T cell protein immunodominance is quite complex and widely focused on different protein targets, suggesting that in order to trigger an efficient DENV-specific T cell response both Structural (C, prM, Envelope) and Non-Structural (NS1-5) proteins are required.

CHARACTERIZATION OF HUMAN DENV-SPECIFIC T CELL RESPONSES

Megapool Approach to Detect DENV-Specific T Cell Responses

Epitope identification studies have provided the basis for phenotyping DENV-specific T cell responses directly *ex vivo* without the need for *in vitro* stimulation that could potentially alter T cell phenotypes. Utilizing the knowledge of the epitopes recognized we developed the megapool approach, which allows for combining a large number of peptides into one peptide pool based on sequential lyophilization. This enables detection of DENV-specific T cell responses irrespective of HLA types and DENV serotypes in various immunological contexts where only small amounts of blood are available (78). DENV megapools have been generated for both CD4 and CD8 T cells, which consist of 180 and 268 peptides, respectively (25, 27, 28, 73, 77). These peptides are pooled, lyophilized, and resuspended to form a master mix, which is then used to stimulate T cell *ex vivo* (86). DENV CD4 and CD8 megapools account for 62 and 90% of the IFN-γ response in Sri Lankan and Nicaraguan cohorts, respectively, and have been validated

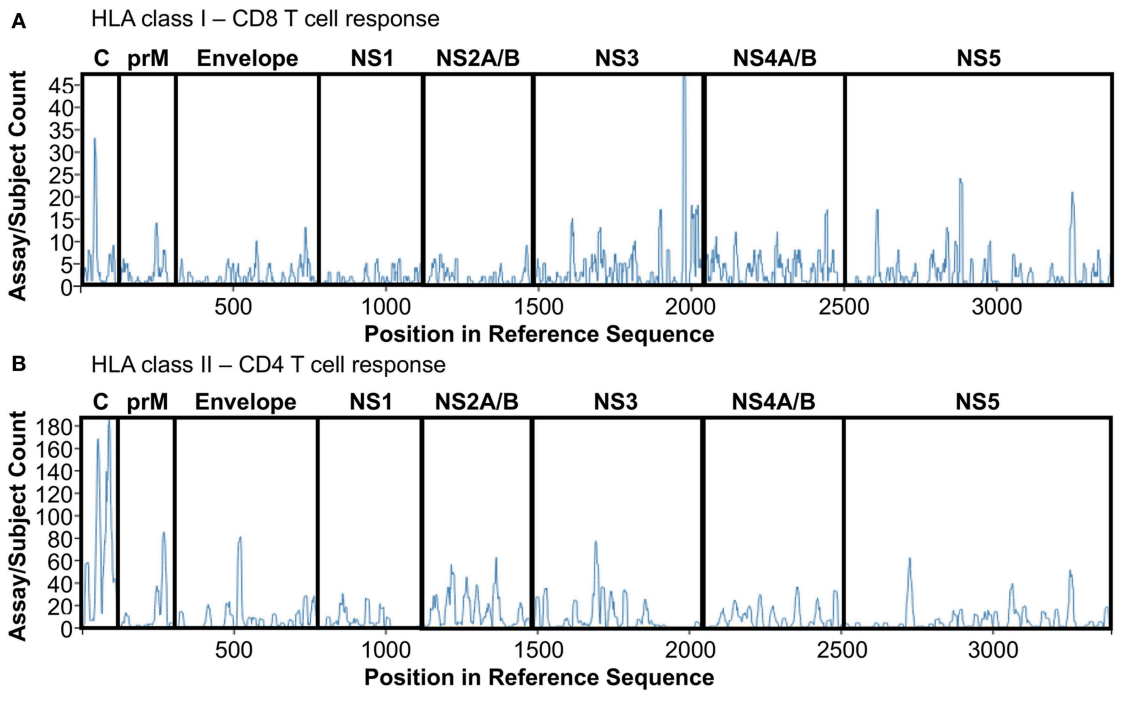

FIGURE 1 | DENV T cell immunodominance. List of epitopes derived from DENV polyprotein studies summarized in **Table 1** have been extracted and plotted on a reference DENV sequence (ID:12637) by using Immunobrowser. **(A)** HLA class I restriction. **(B)** HLA class II restriction.

in different geographical locations supporting their global applicability (25, 27, 28, 73, 77).

DENV-Specific CD8 T Cells: Activated, Skin-Homing, and Functional

Using tetramers incorporating three variants of the HLA-A*1101-restricted DENV $NS3_{133-142}$ epitope, Friberg et al. reported that cross-reactive CD8 T cells develop following both primary and secondary DENV infections and that the magnitude of tetramer$^+$ CD8 T cell response does not correlated with disease severity (35). Although tetramer$^+$ CD8 T cells upregulate the activation marker CD38 during the acute phase of infection (35), the phenotype of these DENV-specific CD8 T cells was not further assessed in this study. More recent studies using DENV peptide pools shows that higher magnitude and more polyfunctional CD8 T cell responses correlate with HLA alleles that are associated with reduced risk of severe dengue disease (27, 28), which is consistent with the report that the frequency of DENV-specific cytokine-producing CD8 T cells is higher among children who subsequently developed subclinical secondary infection than those who developed symptomatic secondary infection (87). Using a pool containing 268 CD8 T cell epitopes derived from DENV (termed megapool), de Alwis et al. demonstrated that the majority of DENV-specific IFN-γ^+ CD8 T cells have a CD45RA$^-$CCR7$^-$ effector memory (Tem) or CD45RA$^+$CCR7$^-$ effector memory re-expressing CD45RA (Temra) phenotype (27). Notably, DENV-specific CD8 T cells are also associated with increased PD-1 expression in donors expressing the immunodominant allele HLA-B*35:01. In contrast to classical exhausted CD8 T cells, these DENV-specific PD-1$^+$ CD8 T cells do not co-express other exhaustion makers and are apparently proliferative and functional (27), suggesting that PD-1 may serve as a marker of activated and highly functional antigen-specific CD8 T cells in the context of DENV infection.

Since DENV infection initiates at the site of the mosquito bite in the host skin, it is possible that CD8 T cells may migrate to the site of infection and mediate localized responses. Indeed, DENV NS3 27-specific CD8 T cells in the periphery blood upregulate the expression of several chemokine receptors including CCR5, CXCR3, and CXCR6 as well as the skin-homing molecule cutaneous lymphocyte-associated antigen (CLA) during acute DENV infection (67). Moreover, DENV-specific CD8 T cells are readily detectable in the skin of DENV-infected individuals at the acute stage (67), suggesting that these cells may exert effector functions at the site of infection. Tissue-resident memory T (Trm) cells reside in non-lymphoid tissues including the skin and can serve as a front line of defense against invading pathogens such as vaccinia virus (88). It would be interesting to investigate whether DENV-specific CD8 T cells could differentiate into Trm cells in the skin that may mount rapid and localized protective immunity upon reinfection.

Comprehensive transcriptomic profiling of DENV-specific CD8 T cells has also been carried out. Chandele et al. performed microarray analysis on HLA-DR$^+$CD38$^+$ activated CD8 T cells isolated from the PBMCs of DENV-infected patients and found that these cells upregulate genes involved in T cell proliferation, activation, migration and cytotoxicity (89). Interestingly, HLA-DR$^+$CD38$^+$ CD8 T cells also display increased expression of multiple inhibitory receptors and downregulate several genes that are involved in TCR signaling (89). A more recent study from our group characterized the transcriptomic profiles of DENV-specific CD8 Tem and Temra subsets identified by their production of IFN-γ following simulation with the megapool of DENV-derived epitopes (90). DENV-specific Tem and especially Temra cells display specialized gene expression profiles and upregulated genes that are associated with activation, co-stimulation, and effector functions (90), which is consistent with the previous study from Chandele et al. Since these DENV-specific Tem and Temra cells were isolated from healthy donors with secondary DENV infection, these studies suggest that DENV-specific CD8 T cell populations may maintain an activated phenotype in donors that have been infected multiple times with DENV. Interestingly, DENV-specific Temra cells may have higher expression of a few

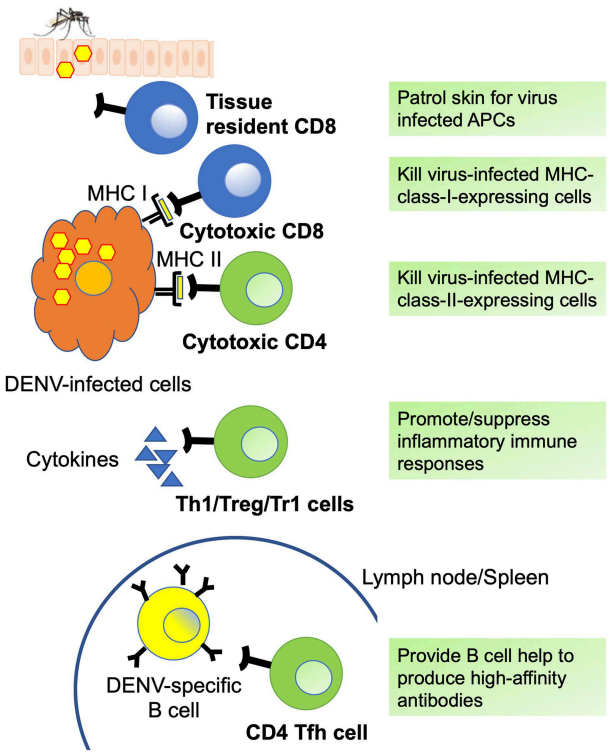

FIGURE 2 | DENV specific T cell phenotypes. Summary of DENV-specific CD8 (blue) and CD4 (green) T cell phenotypes and functions. Tissue resident CD8 T cells patrol skin for infected antigen presenting cells (APCs) and can generate immediate effector functions. Cytotoxic CD8 and CD4 T cells express cytotoxic molecules such as granzyme B and perforin and can kill virus-infected cells via MHC I- and MHC II-dependent mechanisms. Th1 cells mediate and promote antiviral immune responses via the production of inflammatory cytokines such as IFN-γ and TNF-α, whereas regulatory CD4 T cells including Treg and Tr1 cells suppress inflammatory immune responses by producing cytokines such as IL-10 and TGF-β. Tfh cells provide help to DENV-specific germinal center B cells (yellow) and are essential for optimal germinal center reactions, thus promoting the generation of high-affinity antibodies, memory B cells, and long-lived plasma cells.

killer cell immunoglobulin-like receptor (KIR) genes including *KIR2DL3* by comparison with DENV-specific Tem cells (90). In addition, DENV-specific CD8 T cells may show preferential usage of TCR beta-chain variable (TRBV) genes (90), which is in line with the report that DENV $NS3_{133}$-specific CD8 T cells targeting HLA-A*11:01-restricted epitope variants derived from DENV1, DENV3, and DENV4 but not DENV2 preferentially use a few TRBV segments including TRBV9, TRBV12-3/4, and especially TRBV11-2 (33).

DENV-Specific CD4 T Cells: A Tale of Cytotoxicity

The majority of antigen-specific CD4 T cells differentiate into T helper type 1 (Th1) and follicular helper T (Tfh) cells following viral infections and provide help to CD8 T cells and B cells (91–93). Indeed, DENV-specific CD4 T cells produce Th1 cell-associated cytokines including IFN-γ, TNF-α, and IL-2 following both infection and vaccination (87, 94, 95). In addition, DENV-specific CD4 T cells with cytotoxic activity have been reported by numerous studies (12) and their frequency may be lower in patients with more severe dengue disease (96). Interestingly, a subset human CD4 T cells, which is $CD45RA^{+}CCR7^{-}$ and termed effector memory re-expressing CD45RA T (Temra) cells, expands in individuals that have been infected with DENV multiple times, and the frequency of DENV-specific CD4 Temra cells is higher in donors expressing an HLA allele associated with protection from severe dengue disease (26). Despite the production of IFN-γ, these cells may not represent classical Th1 cells as they lack the expression of CXCR3 (26). CD4 Temra cells have increased expression of several cytotoxic molecules including CD107a, perforin, granzyme B as well as the CX3CL1 (fractalkine) receptor, CX3CR1 (26). Notably, CX3CR1 has recently been reported to be a member of a 20-gene set that can predict severe dengue disease (97). Subsequent transcriptomic profiling studies further revealed the gene expression patterns and heterogeneity of CD4 Temra cells and identified additional phenotypic markers such as GPR56 and CD244 that are specifically expressed by cytotoxic CD4 Temra cells and confirmed their expression on DENV-specific Temra cells (98, 99). Additionally, cytotoxic CD4 Temra cells may have undergone extensive clonal expansions based upon TCR analysis (98, 99), supporting the notion that these cells are induced by repeated DENV infections.

TABLE 2 | Markers expressed by DENV-specific T cell populations.

T cell population	Makers
Skin-homing CD8 T cells	CCR5, CXCR3, CXCR6, CLA
CD4/CD8 Tem	$CD45RA^{-}CCR7^{-}$
CD4/CD8 Temra	$CD45RA^{+}CCR7^{-}$
Cytotoxic CD8 T cells	Perforin, Granzyme B, PD-1
Cytotoxic CD4 T cells	Perforin, Granzyme B, CX3CR1, GPR56, CD244
Th1 cells	IFN-γ, TNF-α, and IL-2
Tfh cells	CXCR5, PD-1, OX40, PD-L1

Both $Foxp3^{+}$ regulatory T (Treg) cells and $Foxp3^{-}$ type 1 regulatory T (Tr1) cells can suppress inflammation and exert immunoregulatory effects (100, 101). However, their functional significance in the context of DENV infection is less well-defined (14). It has been reported that the frequency of Treg cells and the ratio of Treg cells to effector T cells are significantly higher during acute DENV infection than after recovery in patients with mild disease but not in those with severe disease (102). However, subsequent studies indicate that the frequency of Treg cells is not associated with viral load or disease severity (103). Therefore, whether and how Treg and Tr1 cells influence antiviral immune response and disease progression during DENV infection warrants further investigation.

T cells help to B cells is provided by a CD4 T cells subset termed follicular T helper cells (Tfh) (104). Tfh cells have been associated with protective roles in human infectious disease (105–107) and vaccinees (108–110). They provide several forms of T cell help to B cells such as signals that promote survival, proliferation, plasma cell differentiation, hypermutation, class-switch recombination, adhesion and chemoattraction (cell migration) (111). Tfh cells are essential for the generation of most isotype switched and affinity matured antibodies, and therefore they have an obvious role in protective immunity against pathogens. A recent breakthrough has been the ability to detect Tfh cells in peripheral blood (105, 112) thus allowing their assessment in PBMC samples based on surface markers. A central marker of Tfh cells is the CXC-chemokine receptor 5 (CXCR5) which is required for T and B cells to enter into follicles. OX40, and PD-L1 have further been identified as TCR activation-dependent markers of human Tfh cells (113, 114). Recent studies have reported an expansion of peripheral Tfh cells in DENV-infected children during the acute phase (115). Furthermore, Tfh cells are more abundant in patients with secondary DENV infection and in those who developed a more severe dengue disease (115). Although Tfh cells has been shown to promote DENV-specific antibody responses in mice (116), the differentiation and functional significance of DENV-specific Tfh cells in humans warrants further investigation.

CONCLUSION AND PERSPECTIVE

All DENV-specific T cell phenotypes discussed in this review as well as the markers they express are summarized in **Figure 2** and **Table 2**, respectively. Comprehensive epitope identification over the last few years has provided the tools that allow one to probe for DENV specific T cell responses in donors exposed to natural infection and vaccination. Global assessment of dengue virus-specific CD4 and CD8 T cell responses in dengue-endemic areas led to the development of new tools (megapools) that allow analysis of small samples typically available from pediatric and hospital cohorts. It has also been demonstrated that DENV-specific CD4 and CD8 responses are more complex than previously thought, with different subsets revealed by in depth phenotypic and transcriptomic analyses. The hypothesis that these different subsets have unique roles and dictate and shape

clinical outcomes and vaccine efficacy will have to be explored in the near future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02125/full#supplementary-material

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Seeking *Flavivirus* Cross-Protective Immunity

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The Flavivirus genus is composed by viral serocomplexes with relevant global epidemiological impact. Many areas of the world present both, vector fauna and geographical conditions compatible with co-circulation, importing, emergence, and epidemics of flaviviruses of different serocomplexes. In this study, we aimed to identify both, immunological determinants and patterns of immune response possibly involved in flavivirus serocomplex cross-protection. We searched B and T cells epitopes which were thoroughly shown to be involved in flavivirus immunological control. Such epitopes were analyzed regarding their conservation, population coverage, and location along flavivirus polyprotein. We found that epitopes capable of eliciting flavivirus cross-protective immunity to a wide range of human populations are concentrated in proteins E, NS3, and NS5. Such identification of both, immunological determinants and patterns of immune response involved in flavivirus cross-protective immunity should be considered in future vaccine development. Moreover, cross-reactive epitopes presented in this work may be involved in dynamics of diseases caused by flaviviruses worldwide.

Keywords: epitopes, flavivirus, serocomplex, cross-protection, immune response

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INTRODUCTION

The Flavivirus genus of the Flaviviridae family is composed by over than 70 viral species with relevant global epidemiological impact. Flaviviruses viral particles have icosahedral capsid, are enveloped and present a single stranded genomic RNA of positive sense (1). The entry of flaviviruses is mainly based on endocytosis mediated by clathrin-coated pits and transport by an endocytic compartment. The low-pH environment within endosomes triggers conformational changes in the envelope glycoprotein (E), leading to membrane fusion of the viral envelope with the endosomal membrane and subsequent release of the nucleocapsid into the cytosol. The genomic RNA is translated into a viral polyprotein, which is cleaved by viral and cellular proteases, to originate flavivirus proteins (1). Three of these proteins are structural components of viral particle, called structural proteins. The E protein is the major antigen of the viral envelope. In addition to E protein, flavivirus particles contains also another envelope protein (M) and the capsid protein (C) (1). The polyprotein also originates non-structural proteins, which are not present on viral particle, but have important roles in viral replication and pathogenesis. There are seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5. After protein synthesis, the assembly of viral particles takes place in the endoplasmic reticulum. Immature viral particles are then sent to Golgi complex to be maturated by furin activity. Mature viral particles are then transported through vesicles to the plasma membrane and released by exocytosis (1).

The *Flavivirus* genus is involved in important chapters of both, World and Brazilian epidemiology histories. *Yellow fever virus* (YFV), *Dengue virus* (DENV) and *Zika virus* (ZIKV) were sequentially imported to Brazil and South America. Cases of yellow fever, caused by YFV, were first reported in Brazil in 1685. Thousands of yellow fever cases were noticed until the beginning of the twentieth century, when the *Aedes aegypti* mosquitoe was identified as the main vector of the disease. Vector eradication together with vaccination contributed to elimination of yellow fever from Brazilian urban areas (2). In 1981, the first epidemic caused by DENV was confirmed in Brazil (3). Since then, many epidemics have reached the country, usually related to the introduction of a new serotype or the change of the dominant serotype in a given region (3, 4). Such flavivirus is responsible for approximately 390 million of infections annually by one of its four serotypes (DENV1-4) (5). Thus, DENV is considered as the flavivirus of highest epidemiologic relevance of the world. But recently, ZIKV which was a pathogen previously associated with mild infections in Africa and Asia, was associated to microcephaly and *Guillain-Barré* syndrome in the Americas (6). The first cases of ZIKV infection were reported in early 2015, in the Brazilian Northeast. The virus was very quickly spreaded and 141 suspected cases of microcephaly were reported in Northeastern states, in addition to many cases of spontaneous abortions and stillbirths (6–9). In 2016 yellow fever cases were reported again in Brazil, with a total of 1,127 cases and 331 confirmed deaths until April 2018 (10). There is a number of additional flaviviruses with worldwide epidemiological importance or which are highly pathogenic. Examples are *West Nile virus* (WNV), *Japanese encephalitis virus* (JEV), *Saint Louis encephalitis virus* (SLEV) and *Rocio virus* (ROCV) (11). Such pathogens are vector-borne viruses, which are capable of traveling over long distances, carried by both, the human and/or the arthropod hosts. The risk of importing, emergence and epidemics caused by additional flaviviruses is clear in both, Brazil and other tropical regions of the world.

Humoral immune response to flaviviruses is complex and is involved in both, viral clearance and pathogenesis. An exacerbation of the disease severity mediated by antibodies, known as antibody-dependent enhancement (ADE), is observed in some cases of DENV infection. Immunoglobulins produced in a first infection by a specific serotype cross-react with viral particles present in a second infection caused by a different serotype. Viral particles are targeted to Fc-γ receptors bearing cells by non-neutralizing antibodies, which facilitates penetration and replication of DENV (1, 11). Increased viral replication leads to increased viral loads, exacerbated inflammation, increased release of inflammatory cytokines and vasoactive amines, a phenomenon known as cytokine storm (11, 12). ADE has also been described between DENV and ZIKV *in vitro* (8, 13–15). This phenomenon does not explain by itself the occurrence of severe dengue, but contribute to it in a relevant way. Investigations on ADE between different species of flavivirus have been reported, both *in vitro* (8, 13–16) and *in vivo* (17, 18). Most of the residues exposed at the external surface of flaviviral envelope are not conserved and are specific to each virus. Antibodies directed against envelope proteins potently neutralize the autologous and closely related viruses only (16, 19–21). This criterion of cross-neutralization by polyclonal antibodies has led to the classification of flaviviruses into serocomplexes (22). At present, the literature does not show evidence that antibodies are capable of conferring anti-flavivirus cross-protective immunity by themselves.

On the other hand, cellular immune responses to flaviviruses were shown to be involved in cross-protective immunity (23–28). Non-structural proteins, especially NS3 and NS5, are the main targets of T cells involved in flavivirus cross-protection (27, 29). The use of such proteins as subunit vaccine antigens was well documented in the literature. However, such vaccines were not tested in a serocomplex cross-protection context. Recent reports showed the importance of non-structural proteins in autologous (27, 30–32) or heterologous protection (23), regarding immunization with live attenuated viruses or natural infections. In contrast to structural proteins, it seems there are conserved residues in non-structural proteins which are capable of eliciting flavivirus cross-protective T cell immunity.

The Americas, Africa, Middle East, South East Asia and Europe have at least one *Flavivirus* serocomplex in circulation (23). Many areas of the world present both, vector fauna and geographical conditions compatible with co-circulation, importing, emergence and epidemics of flaviviruses. The encounter with multiple flaviviruses in a lifetime is increasingly likely. However, flavivirus cross-protective immunity is not well understood. In this study we aimed to find both, immunological determinants, and patterns of immune response possibly involved in flavivirus cross-protective immunity. We searched B and T cells epitopes which were thoroughly shown to be involved in flavivirus immunological control. Such epitopes were analyzed regarding their conservation along with different flavivirus serocomplexes. The epitopes were also studied regarding their population coverage, considering HLA allele frequencies of different human populations. Finally, epitopes with highest conservancy and population coverage were studied regarding their position along flavivirus polyprotein. Results presented in this study indicate that epitopes capable of eliciting flavivirus cross-protective immunity to a wide range of human populations are concentrated in proteins E, NS3, and NS5. Such identification of both, immunological determinants and patterns of immune response involved in flavivirus cross-protective immunity should be considered in future vaccine development. In addition, cross-reactive epitopes presented in this work may be involved in dynamics of diseases caused by flaviviruses worldwide.

METHODS

Study Design

In this study we aimed to find both, immunological determinants and patterns of immune response possibly involved in flavivirus cross-protective immunity. As shown in **Figure 1**, we carried out a literature search of flavivirus epitopes involved in protective immunity. Such epitopes were analyzed with regard to their conservancy, population coverage and location in flavivirus polyprotein.

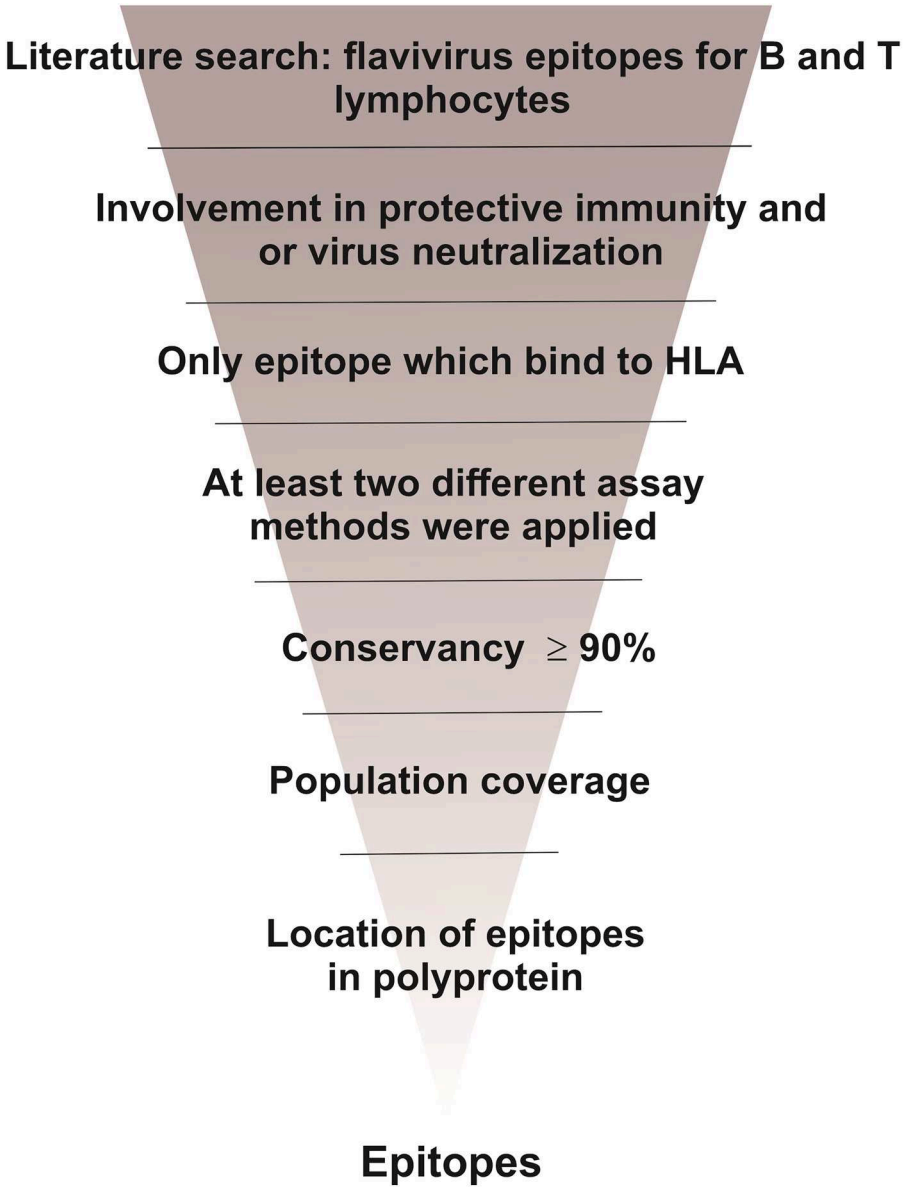

FIGURE 1 | Study design.

Flavivirus Polyprotein Database

A database was built with polyprotein sequences from flaviviruses of different serocomplexes isolated in different continents of the world. Viruses taken in consideration were those of highest epidemiological relevance in Brazil and worldwide: *Dengue virus* (DENV), *Zika virus* (ZIKV), *Yellow fever virus* (YFV), *West Nile virus* (WNV), *Japanese encephalitis virus* (JEV), *Saint Louis encephalitis virus* (SLEV), *Rocio virus* (ROCV), *Cacipocore virus* (CPCV), *Ilhéus virus* (ILHV), and *Iguape virus* (IGUV). Sequences in FASTA format were retrieved from the National Center for Biotechnology Information (NCBI) protein database from September-2017 to September-2018 (http://www.ncbi.nlm.nih.gov/protein/). We aimed to recover sequences from all listed flavivirus species, isolated in all continents, with a limit of three sequences per country of each viral species. We noticed that for some species there were few deposits. In these cases, all available sequences were selected. Additional criteria for selecting sequences were: (i) complete annotation of structural and non-structural proteins within deposited polyprotein sequence and (ii) absence of undefined amino acid into sequence. The database consisted of a total of 325 polyprotein amino acid sequences (**Supplemental Material 1**).

Search of T Cell Epitopes Involved in Flavivirus Protective Immunity

We carried out a literature search for reports of experimental characterization of protective epitopes. $CD4^+$ and $CD8^+$ T cell epitopes which were related to protective immune response to flavivirus were searched in reports available at Pubmed from September-2017 to September-2018 (https://www.ncbi.nlm.nih.gov/pubmed/). Criteria for selecting research articles were: (i) evaluation of epitope binding to HLA; (ii) determination of cytokine production pattern for both, $CD4^+$ and $CD8^+$ T cells; (iii) use of at least two different methods for determining cytokine production pattern and (iv), clear association with control of flavivirus infection. This last criterion involves validation of epitopes associated with survival under challenge experiments using humanized animals (transgenic mice expressing HLA), control of viral load under challenge experiments using humanized animals, infection of humans without disease and identification of important epitopes in immune responses elicited by well-known protective vaccines. Words used in the search were: $CD4^+$ T cell, $CD8^+$ T cell, epitopes, protection, immunity, *Dengue virus*, *Zika virus*, *Yellow Fever virus*, *West Nile virus*, *Japanese encephalitis virus*, *Saint Louis encephalitis virus*, *Rocio virus*, *Cacipocore virus*, *Ilheus virus,* and *Iguape virus*.

Search of B Cell Epitopes Targeted by Neutralizing Antibodies

B cell epitopes were searched at Immune Epitope Database and Analysis Resource-IEDB (http://www.iedb.org) (34). The search was carried out until September 30, 2018, using the following conditions: any epitope at the epitope field; flavivirus at the antigen field, humans in the host field; positive assays only, B cells assay, *in vitro* and/or *in vivo* neutralization and 3D structure at the assay field; any MHC restriction at the MHC restriction field and infectious disease at the disease field.

Conservancy Analysis of Selected Epitopes

The IEDB conservancy analysis tool (http://tools.iedb.org/conservancy) was used to determine the conservancy of the selected epitopes among the flavivirus sequences in the database previously constructed, as previously described (34, 35). Only epitopes at least 90% conserved among all sequences were selected.

Population Coverage Analyses

Epitopes selected after conservancy analysis were submitted to population coverage analysis, by using the IEDB population coverage calculation tool (http://tools.immuneepitope.org/tools/population/iedb_input), as previously described (35, 36).

Localization of Selected Epitopes Along Flavivirus Polyprotein

Epitopes with high conservancy were submitted to location analysis along flavivirus polyprotein. Preliminary localization was retrieved from NCBI annotations. Then, 3D protein models of E, NS3 and NS5, deposited at the Protein Data Bank- PDB (https://www.rcsb.org/), were used for fine localization. Pymol (http://www.pymol.org/) was used to highlight selected epitopes along the proteins 3D models, as previously described (33, 35).

TABLE 1 | Discontinuous B lymphocyte epitope selected from IEDB website and, posteriorly, chosen through conservancy analyzes using cut off ≥90%.

Epitope ID[a]	Virus[b]	Epitope sequence/location	Conservancy (≥90%)	100% identity[c]	Protein location
110534	WNV	W101, G104, G106	99.67%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
161539	DENV	W101, G106, L107, F108	99.34%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
178102	DENV	W101, L107,G109	99.67%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
191061	DENV	T76, W101, G106, L107, F108	90.43%	DENV1, DENV2, DENV3, DENV4, ZIKV, WNV	E protein
191066	DENV	W101, G106, F108	99.67%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
191060	DENV	T76, L107, F108	90.76%	DENV1, DENV2, DENV3, DENV4, ZIKV, WNV	E protein
191068	DENV	W101, L107, F108	99.67%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
191062	DENV	T76, W101, L107, F108	90.76%	DENV1, DENV2, DENV3, DENV4, ZIKV, WNV	E protein
191057	DENV	G78, W101, L107, F108	99.67%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
238073	DENV	R99, G102, G106, L107	99.34%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
504083	DENV	R73, G78, E79	91.42%	DENV1, DENV2, DENV3, DENV4, ZIKV, WNV	E protein
161528	DENV	K307, K310, L389	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
161534	DENV	T303, T329, G383	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
191056	DENV	G78, W101, F108	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
504090	DENV	W101, L107, G111	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
504074	DENV	N103, G104, G111	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
504080	DENV	G100, W101, F108	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein
530626	DENV	D378, R379, W381	100.00%	DENV1, DENV2, DENV3, DENV4, ZIKV, YFV, WNV	E protein

[a] *Number of epitope ID at IEDB website.*

[b] *Virus in which the epitope was described.*

[c] *Viruses in which epitopes have 100% identity.*

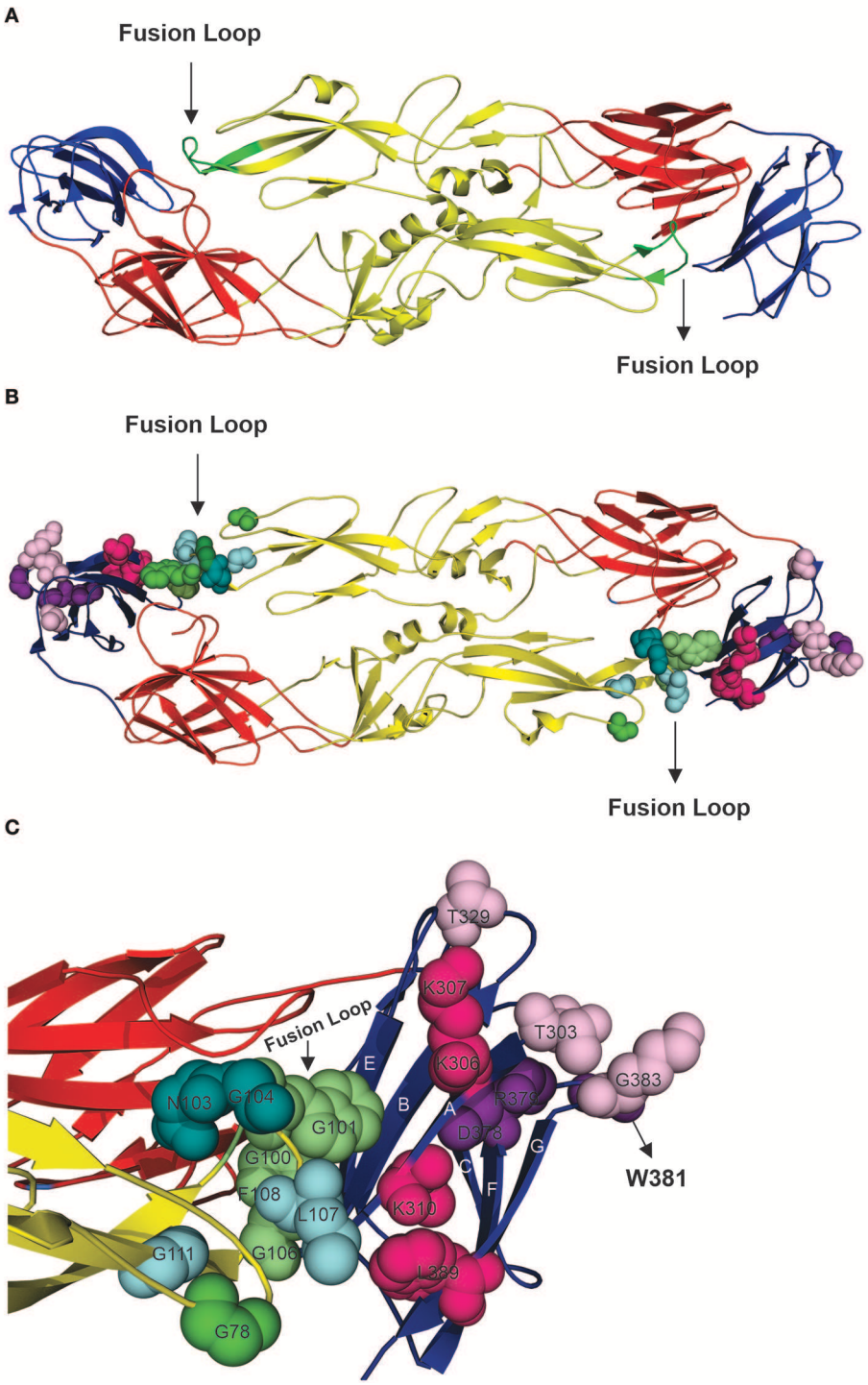

FIGURE 2 | Structural analysis of highly conserved neutralizing epitopes in flaviviruses. **(A)** Model of DENV envelope glycoprotein arranged as a dimer [(33), PDB number 1UZG]. Domain I is shown in red, domain II is shown in yellow, and domain III is shown in blue, and the fusion loop is shown in green. **(B)** Overview of locations of the neutralizing epitopes in E protein. **(C)** Approximate view of locations of epitopes which are targets for neutralizing antibodies. Epitopes shown to be located in domain II are: G78, W101, F108 (shown in green), W101, L107, G111 (shown in cyan), N103, G104, G111 (shown in deepteal cyan) and G100, W101, F108 (shown in lime green). Note that these epitopes are overlapping and that are they concentrated at the fusion loop and share W101 and G111. Domain III presents the epitopes: T303, T329, G383 (shown in light pink), K307, K310, L389 (shown in hot pink) and D378, R379, W381 (shown in violet purple).

RESULTS

Selection and Localization of Epitopes Which Are Targets for Neutralizing Antibodies

From over than 2,000 epitopes retrieved from search carried out as described in materials and methods, 19 were selected after conservancy analyses. One of the epitopes is contained inside a larger epitope as a consensus, in the same location. Thus, 18 epitopes are shown in **Table 1**. Such epitopes are discontinuous. From these, seven epitopes were shown to be 100% conserved and were selected for structural analyses. All selected epitopes are located in flavivirus envelope glycoprotein, mainly in domains II and III, as shown in **Figure 2**. Our results indicate that the seven neutralizing epitopes selected are highly conserved among flaviviruses and may be involved in cross-protection and restriction of virus circulation in some regions of the world.

Selection of T Cell Epitopes Which May Be Involved in Flavivirus Serocomplex Cross-Protective Immunity

Nineteen research articles were selected reporting 529 epitopes. Such antigen determinants were experimentally characterized and associated with protective immunity. From the 529 epitopes 15 were selected after conservancy analysis. All selected epitopes are class I HLA ligands. Their amino acid sequences, HLA ligands, locations and percent conservations are shown in **Table 2**. Three epitopes showed conservation higher than 99% among all flavivirus polyproteins analyzed. In addition, virus-specific identity was also analyzed and is shown in **Table 3**. Epitope identity was shown to be constant in most of cases for each flavivirus. Together, these results indicate that there are fifteen epitopes associated with protective immunity which are highly conserved among flavivirus serocomplexes.

Population Coverage Analyses of Conserved T Cell Epitopes

As shown in conservancy analyses, we selected fifteen epitopes with at least 90% identity among all flaviviruses. Such epitopes were shown to present relevant population coverages with regard to the most prevalent HLA alleles and main ethnicities in Brazil and United States, as shown in **Table 4**. In addition, population coverage for selected epitopes were shown to be also relevant for most of world regions, as shown in **Table 5**. Our results indicate that those highly conserved epitopes bind to HLA molecules of different human populations worldwide, with high population coverage.

Localization of Selected T Cell Epitopes Along Flavivirus Polyprotein

All of selected epitopes were concentrated in flavivirus non-structural proteins 3 and 5 (NS3 and NS5, respectively). Thus, we used NS5 and NS3 3D models to study the locations of the epitopes. From fifteen epitopes, six were located in Helicase domain of the NS3 protein (NS3H), and eight were located in the

TABLE 2 | T cell epitopes selected from literature review as contributing to flavivirus control and showing at least 90% conservation in the polyprotein dataset.

References[a]	Epitope	HLA allele[b]	Protein[c]	Conservancy[d]
Wen et al. (24)	APTRVVAAEM	B*07:02	NS3 1725–1734	91.08%
Weiskopf et al. (27), Weiskopf et al. (37), Elong Ngono et al. (38), Weiskopf et al. (39)	APTRVVAAEM	B*07:02, B*35:01	NS3 1700–1709	91.08%
Weiskopf et al. (27), Weiskopf et al. (37)	DPASIAARGY	B*35:01	NS3 1768–1777	91.69%
Weiskopf et al. (27)	DISEMGANF	A*26:01	NS3 1887–1895	93.85%
	KAKGSRAIW	B*57:01	NS5 2962–2970	92.00%
	RFLEFEALGF	A*23:01	NS5 2977–2986	100.00%
de Melo et al. (40)	TDTTPFGQQRVFKEK	B*39	NS5 345–359	100.00%
	EFGKAKGSRAIWYMW	B*15	NS5 465–479	99.69%
	AKGSRAIWYMWLGAR	B*15	NS5 469–483	99.69%
Weiskopf et al. (37)	LPVWLAYKVA	B*35:01	NS3 2005–2014	93.85%
Elong Ngono et al. (38)	APTRVVASEM	B*07:02	NS3 1700–1709	96.92%
Weiskopf et al. (39)	SRAIWYMWLGARFLE	DRB1*01:01	NS5 2966–2980	94.15%
Rivino et al. (41)	GEAAGIFMTA	B*40:06	NS3 301–315	92.92%
Duan et al. (42)	WYMWLGARFL	A*24:02	NS5 475–484	94.46%
Turtle et al. (43)	MTTEDMLQVW	B*58:01	NS5 3336–3345	96.00%

[a] *References of experimental validation of epitopes involved in the protective immune response against flavivirus that presented ≥ 90% of conservation in Flavivirus polyprotein sequences analyzed.*

[b] *HLA alleles which bind the selected epitopes.*

[c] *Location of selected epitopes in flavivirus polyprotein. Numbers refer to starting and ending positions of epitopes into flavivirus polyprotein sequences. Proteins in which epitopes are located were also indicated.*

[d] *Percent conservation of epitopes flavivirus polyprotein dataset. A homology cut off of 90% was applied with regard to flavivirus polyprotein dataset.*

TABLE 3 | Percent of identity of selected epitopes into species-specific polyprotein datasets.

Epitopes sequences	DENV1	DENV 2	DENV3	DENV4	ZIKV	YFV	WNV	SLEV	JEV	IGUV	ILHV	ROCV
APTRVVAAEM[a,b]	90%	100%	100%	100%	100%	–	100%	–	100%	100%	90%	90%
DPASIAARGY[a]	100%/90%	100%	100%	–	90%	90%	100%	100%	100%	90%	100%	100%
DISEMGANF	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
KAKGSRAIW	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
RFLEFEALGF	100%	100%	90%	100%	100%	90–100%	100%	100%	90%	100%	100%	100%
TDTTPFGQQRVFKEK	100%	100%	100%	100%	93.33%	100%	100%	100%	100%	100%	100%	100%
EFGKAKGSRAIWYMW	100%	100%	100%	93.33%	100%	100%	93.33%	100%	93.33%	100%	100%	100%
AKGSRAIWYMWLGAR	100%	100%	100%	100%	100%	100%	93.33%	100%	93.33%	100%	100%	100%
LPVWLAYKVA	90%	90–100%	90%	90%	90%	–	100%	100%	100%	90%	90%	90%
APTRVVASEM	100%	90%	90%	90%	90%	90%	90%	–	90%	90%	–	–
SRAIWYMWLGARFLE	100%	100%	93,33%	100%	100%	93.33–100%	93.33%	100%	–	100%	100%	100%
GEAAGIFMTA	90%	100%	90%	90%	90%	–	90%	90%	90%	–	90%	90%
WYMWLGARFL	100%	100%	100%/90%	100%	100%	100%/90%	90%	100%	–	100%	100%	100%
MTTEDMLQVW	90%	90%	90%	90%	90%	90%	90%	90%	100%	90%	100%	90%

Only percent identities ≥90% were indicated.

[a] *Epitopes experimentally validated by more than one research group. Repeated epitopes were removed from the table.*

[b] *Epitopes which share amino acid sequences, but are located in different positions of viral polyprotein.*

TABLE 4 | Population coverage by ethnicity in Brazil and United States of America.

Epitopes sequences	Brazil (62.30%)[a]				United States of America (88%)[a]							
	Amerindian	Caucasoid	Mixed	Mulatto	Amerindian	Asian	Austronesian	Black	Caucasoid	Hispanic	Mestizo	Polynesian
APTRVVAAEM	0.00%	12.18%	19.18%	0.00%	3.46%	5.14%	0.00%	14.27%	23.23%	10.44%	9.67%	2.77%
APTRVVAAEM	0.00%	19.86%	28.09%	0.00%	30.32%	13.15%	0.00%	25.85%	33.59%	21.69%	22.02%	31.55%
DPASIAARGY	0.00%	8.21%	9.94%	0.00%	27.37%	8.23%	0.00%	12.54%	11.87%	11.91%	13.02%	29.22%
DISEMGANF	0.00%	7.26%	4.84%	0.00%	1.65%	7.37%	0.00%	2.91%	5.79%	5.59%	4.72%	23.24%
KAKGSRAIW	0.00%	1.00%	1.40%	0.00%	1.27%	3.72%	0.00%	1.40%	7.21%	2.36%	3.93%	0.00%
RFLEFEALGF	0.00%	10.32%	7.94%	0.00%	1.24%	0.56%	0.00%	20.25%	3.04%	6.90%	4.53%	0.00%
TDTTPFGQQRVFKEK	35.68%	3.17%	9.75%	0.00%	21.89%	3.40%	0.00%	2.71%	3.34%	12.62%	16.68%	14.78%
EFGKAKGSRAIWYMW	22.91%	14.41%	17.55%	0.00%	9.99%	24.90%	0.00%	24.57%	15.22%	14.70%	14.86%	30.06%
AKGSRAIWYMWLGAR	22.91%	14.41%	17.55%	0.00%	9.99%	24.90%	0.00%	24.57%	15.22%	14.70%	14.86%	30.06%
LPVWLAYKVA	0.00%	8.21%	9.94%	0.00%	27.37%	8.23%	0.00%	12.54%	11.87%	11.91%	13.02%	29.22%
APTRVVASEM	0.00%	12.18%	19.18%	0.00%	3.46%	5.14%	0.00%	14.27%	23.23%	10.44%	9.67%	2.77%
SRAIWYMWLGARFLE	0.41%	10.76%	9.43%	11.64%	0.98%	5.50%	0.99%	4.92%	14.81%	7.12%	7.74%	0.00%
GEAAGIFMTA	0.00%	0.00%	0.00%	0.00%	0.00%	6.69%	0.00%	0.08%	0.00%	0.43%	0.40%	11.24%
WYMWLGARFL	14.44%	21.85%	16.16%	0.00%	53.94%	33.22%	0.00%	4.57%	18.26%	23.41%	26.97%	56.79%
MTTEDMLQVW	0.00%	4.15%	4.35%	0.00%	0.59%	11.70%	0.00%	7.01%	1.70%	2.59%	1.78%	0.00%
Total coverage[b]	60.60%	65.91%	69.96%	11.64%	81.09%	74.53%	0.99%	68.51%	71.45%	68.62%	71.52%	92.04%

[a] *Percent values between parentheses refer to total population coverage of all epitope combined in countries.*

[b] *Population coverage of the epitope set presented in table computed in combination for each ethnicity.*

RNA-dependent RNA polymerases domain of the NS5 protein (NS5p) (**Figure 3**). These results indicate that NS3H and NS5p concentrate most of relevant T cell epitopes in the context of flavivirus cross-protective immunity.

DISCUSSION

In this study, we aimed to identify both, immunological determinants and patterns of immune response possibly involved in flavivirus serocomplex cross-protection. Our premise was based on epidemiology of flaviviruses: most of them have both, vectoring and geographic conditions to co-circulate all together in the Americas, Africa, Asia and Oceania (44–49). However, it does not happen. In addition, recent studies showed evidences of cross-protective immunity induced by vaccines or sequential infections (23, 24, 26, 50–54). We searched B and T cells epitopes, which were thoroughly shown to be involved in flavivirus infection control. We found a relevant number of epitopes which are capable of eliciting flavivirus protective immunity and are also highly conserved among serocomplexes. In addition, such

TABLE 5 | Population coverage of selected epitopes for populations of different regions of the world.

Epitopes sequences	North America	Central America	South America	Europe	East Africa	West Africa	Central Africa	North Africa	South Africa	Northeast Asia	South Asia	East Asia	Southeast Asia	Southwest Asia	Oceania
APTRVVAAEM	12.70%	0.00%	4.75%	21.45%	6.15%	7.01%	10.54%	5.57%	0.03%	2.60%	2.74%	9.44%	1.20%	6.02%	3.17%
APTRVVAAEM	25.20%	0.00%	8.43%	30.73%	10.51%	24.53%	20.80%	16.02%	0.05%	6.29%	8.62%	22.01%	3.76%	11.28%	5.37%
DPASIAARGY	13.41%	0.00%	3.78%	10.50%	4.51%	18.20%	10.86%	10.77%	0.02%	3.74%	5.96%	13.23%	2.58%	5.44%	2.24%
DISEMGANF	5.35%	0.00%	1.95%	7.73%	1.91%	5.83%	2.20%	2.45%	0.01%	4.15%	8.55%	12.73%	5.68%	8.61%	2.31%
KAKGSRAIW	3.36%	0.00%	0.98%	6.24%	1.77%	1.65%	0.00%	2.78%	0.02%	1.42%	4.90%	0.37%	1.26%	1.25%	0.60%
RFLEFEALGF	8.21%	0.80%	4.42%	4.32%	14.89%	27.50%	18.14%	19.40%	0.07%	0.97%	1.15%	0.58%	0.11%	5.21%	1.35%
TDTTPFGQQRVFKEK	8.62%	0.00%	22.55%	4.12%	2.99%	2.55%	1.61%	7.22%	0.01%	3.63%	1.53%	7.06%	9.06%	7.36%	6.76%
EFGKAKGSRAIWYMW	19.66%	0.00%	25.68%	12.71%	25.37%	26.83%	17.15%	15.84%	0.15%	48.92%	18.16%	26.01%	31.42%	12.26%	28.69%
AKGSRAIWYMWLGAR	19.66%	0.00%	25.68%	12.71%	25.37%	26.83%	17.15%	15.84%	0.15%	48.92%	18.16%	26.01%	31.42%	12.26%	28.69%
LPVWLAYKVA	13.41%	0.00%	3.78%	10.50%	4.51%	18.20%	10.86%	10.77%	0.02%	3.74%	5.96%	13.23%	2.58%	5.44%	2.24%
APTRVVASEM	12.70%	0.00%	4.75%	21.45%	6.15%	7.01%	10.54%	5.57%	0.03%	2.60%	2.74%	9.44%	1.20%	6.02%	3.17%
SRAIWYMWLGARFLE	13.55%	1.34%	4.05%	15.19%	3.96%	4.47%	2.12%	2.33%	0.00%	2.77%	9.25%	10.21%	0.78%	2.67%	0.81%
GEAAGIFMTA	0.22%	0.00%	0.00%	0.11%	0.00%	0.41%	0.00%	0.72%	0.03%	3.11%	14.14%	8.33%	2.06%	5.32%	0.00%
WYMWLGARFL	22.87%	0.00%	23.76%	17.38%	2.38%	5.04%	2.00%	8.00%	0.04%	24.09%	16.66%	49.65%	40.08%	10.29%	52.33%
MTTEDMLQVW	4.66%	1.40%	1.40%	1.45%	11.14%	8.44%	6.51%	5.93%	0.02%	7.68%	9.08%	3.75%	13.27%	5.13%	2.59%
Total Coverage[a]	74.17%	3.50%	67.96%	69.60%	58.61%	73.78%	55.97%	60.88%	0.30%	74.77%	66.06%	84.70%	74.75%	54.27%	73.66%

[a]*Population coverage of the epitope set presented in table computed in combination for each region of the world.*

epitopes cover a wide range of human populations and are concentrated in proteins E, NS3, and NS5. For such epitopes, mechanisms involved in virus control targeting the E protein are all related to antibody-mediated virus neutralization. On the other hand, mechanisms involved in virus control by targeting NS3 and NS5 proteins are mainly related to cytotoxicity mediated by $CD8^+$ T limphocytes.

The glycoprotein E is involved in binding of the viral particle to host cell receptors. In addition, such protein is also involved in membrane fusion. The E glycoprotein is arranged in three domains (DI, DII, and DIII). Domains I (DI) is central, surrounded by DII and DIII (55). The hydrophobic fusion loop is located in DII. It is highly conserved in flaviviruses serocomplexes due to its important role in mediating membrane fusion (55). The fusion loop has the same conformation in both protein forms trimer and dimer. In trimer, three amino acids are conserved in all flaviviruses (Trp 101, Leu 107, and Phe 108) and are exposed on molecular surface. Such amino acids have an important role in holding the fusion loop structure (33). Replacement of the Trp101 by an alanine prevents membrane fusion (56). In addition, natural infection by DENV was hypothesized to generate anti-DENV antibodies with cross-neutralizing activity against the fusion loop (57). All of discontinuous epitopes found in our study are located in E protein and most of them present these three conserved amino acids. We hypothesize that B cell epitopes presented in our study remain highly conserved across flavivirus serocomplexes due to their location at fusion loop.

The role of DIII in the flaviviral cycle is still unclear, although frequently related to a receptor binding function (55, 58, 59). It was previously shown that antibodies targeting different regions in DIII are able to strongly neutralize WNV. The regions are the N-terminal linker region (residues 302 to 309) and three strand-connecting loops, namely, BC (residues 330 to 333), DE (residues 365 to 368), and FG (residues 389 to 391) (60). In our study we found three discontinuous epitopes which are located in those regions: T303 T329 G383, K307 K310 L389, and D378 R379 W381. These epitopes revealed 100% conservancy among all 325 sequences analyzed. All of the selected B cell epitopes were show to be important in DENV neutralization (57, 61–63). Of course, a physic validation regarding cross-neutralizing activity remains to be carried out. However, positions highlighted in **Figure 2** are located in the same structures of flaviviruses with known envelope glycoprotein structure. Our results indicate that such epitopes are important targets to be considered in flavivirus serocomplex cross-immunity due to their key roles in viral life cycle.

With regard to conservation of T cell-specific immunological determinants among flavivirus serocomplexes, the YVF lost the highest amount of 100% conserved T cell epitopes (see **Table 3**). This is in accordance with the highest genetic distance of YFV serocomplex with regard to other flavivirus serocomplexes (23). An important explanation for the epidemiology of flaviviruses may arise from this observation. For example, vaccination against YFV in Brazil is not currently related to protection against DENV and ZIKV epidemics. However, DENV and JEV serocomplexes share most of epitopes identified in this

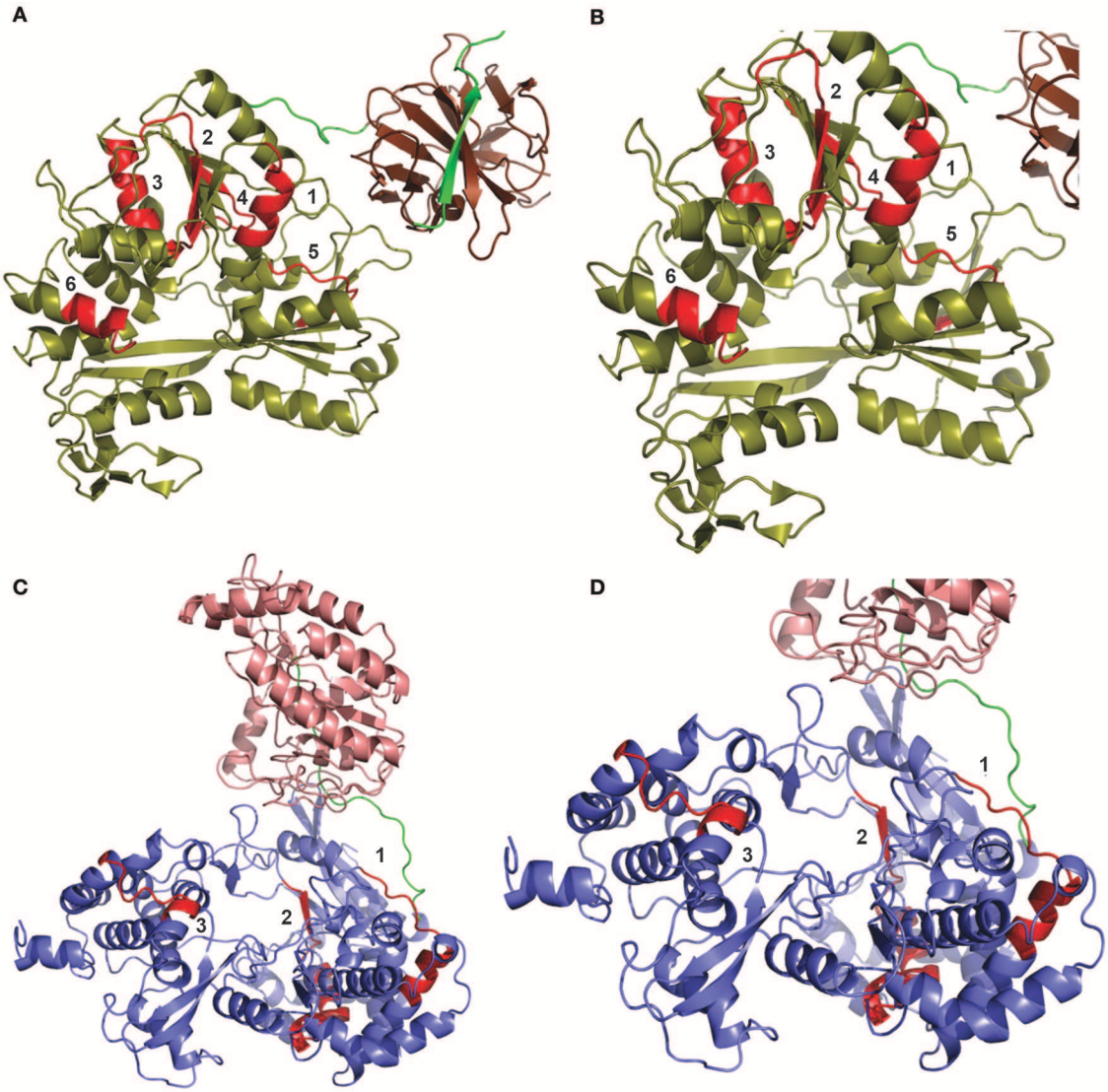

FIGURE 3 | Distribution of T cell epitopes ≥90% conserved among flavivirus polyproteins. The locations of epitopes are shown in two different models: DENV NS3 protein **(A,B)** and ZIKV NS5 protein **(C,D)**. **(A)** The helicase domain of the DENV NS3 protein is shown in olive, while its protease domain is shown in chocolate brown. Six epitopes (shown in red) were shown to be located in NS3, all of them located in the protease domain. **(B)** Zoomed view of NS3 protein to detail epitopes. The numbers refer to the following epitopes, respectively: (1) APTRVVAAEM or APTRVVASEM, (1) APTRVVAAEM, (2) DPASIAARGY, (3) GEAAGIFMTA, (4) DISEMGANF, and (5) LPVWLAYKVA. **(C)** The methyltransferase domain of the NS5 protein is shown in light salmon, while its RNA polymerase domain is shown in slate blue. Eight epitopes (shown in red) were shown to be located in the NS5 protein, all of them in the RNA polymerase domain. **(D)** Approximate view of NS5 protein to detail epitopes. Some epitopes present overlapping. Thus, we show three different regions: Region 1 is composed by one epitope TDTTPFGQQRVFKEK. Region 2 contain six epitopes: KAKGSRAIW, RFLEFEALGF, EFGKAKGSRAIWYMW, AKGSRAIWYMWLGAR, SRAIWYMWLGARFLE, and WYMWLGARFL. Region 3 is composed of only one epitope: MTTEDMLQVW.

work. It seems immunity to DENV precludes viruses of JEV serocomplex of circulating in South America. Recent studies showed evidences of cross-protective immunity between DENV and JEV serocomplexes (23, 54). The same does not occur in Central America, Africa and Asia. Interestingly, Central America and South Africa present a low population coverage of the selected T cell epitopes. Nevertheless, population coverage of class-I HLA specific epitopes presented in this work does not seem to explain the whole scenario. For example, the population of United States is 88% covered by the set of epitopes, but there is co-circulation of DENV and JEV serocomplexes in that country. In addition, most of regions in Africa and Asia, which have also a high population coverage of our set of epitopes, present co-circulation of DENV and JEV serocomplexes. The explanation for this is probably related to class-II HLA restricted epitopes, from which only two were selected according to our

criteria. Such epitopes did not present a high conservation among all serocomplexes and probably confer cross-protection in specific combinations of viruses and serocomplexes, as previously described (23). It was recently shown that cross-reactive epitopes can promote recall from a pool of flavivirus serocomplex cross-reactive memory $CD4^+$ T cells (23). However, such epitopes are not enough conserved in order to induce cross-protection among all serocomplexes.

Cross-protective immunity into a given serocomplex seems to be more easily achieved (27, 50–53, 64, 65). Such cross-protection depends on recall of serocomplex cross-reactive memory $CD4^+$ T cells. There is not a high number of highly conserved class-II HLA restricted epitopes among all serocomplexes. Nevertheless, we identified two peptides capable of binding class-II HLA, involved in anti-YFV protective immune response which are highly conserved among all flavivirus serocomplexes. Thus, it seems achieving multivalent protection among serocomplexes depends on overcoming immunodominance. Highly conserved class-II HLA restricted epitopes do not seem to be immunodominant in order to promote recall of serocomplex cross-reactive memory $CD4^+$ T cells in a multivalent way.

With regard to class-I HLA restricted epitopes, we found fifteen highly conserved peptides which were thoroughly shown to be involved in flavivirus infection control. The peptides APTRVVAAEM, DPASIAARGY, DISEMGANF, KAKGSRAIW, and RFLEFEALGF are located in the NS3 or NS5 proteins. They bind to a subset of multifunctional CD8+ T limphocytes capable of producing IFN-γ and TNF-α against DENV infection (24, 27). In addition, the peptide APTRVVAAEM is involved in cross-protection against DENV and ZIKV. The epitopes TDTTPFGQQRVFKEK, EFGKAKGSRAIWYMW, and AKGSRAIWYMWLGAR are concentrated in the NS5 protein and contain multiple HLA-I and -II binding motifs which are involved in elicitation of protective immune memory against YFV (40). The epitope MTTEDMLQVW is involved in protection against JEV (43). The remaining selected epitopes (see **Table 2**) were also related to protection against DENV. All of the selected epitopes are involved in induction of anti-flavivirus $CD8^+$ T cells. Some of the listed studies report generation os memory cells, which could also be recalled in order to favor cross-protective immunity.

Conserved epitopes could be enriched in artificial or natural immunizations. For example, sequential infection or administration of different anti-flavivirus vaccines would recall cross-reactive memory T $CD4^+$ and T $CD8^+$ lymphocytes. It is important to stress that approved vaccines represent three serocomplexes: DENV, JEV and YFV. However, most of those epitopes which are important for recalling cross-reactive T cells, are concentrated in non-structural proteins. The only anti-DENV vaccine currently approved for use in humans is a chimera between DENV and YFV. Its structural antigens are representative of DENV proteins, while non-structural antigens are representative of YFV proteins. In addition, the JEV vaccine is based on inactivated viruses. Enhancing the recall of cross-protective memory T cells would ideally happen with the use of live attenuated viruses or other vaccine approach capable of providing epitopes of non-structural proteins.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

AUTHOR CONTRIBUTIONS

JA conceived the study and wrote the paper. LS, LG, and WL analyzed data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02260/full#supplementary-material

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OPINION
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Wanted Dead or Alive: A Correlate of Protection Against Dengue Virus

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Keywords: dengue virus, vaccines, antibodies, cell-mediate immunity, protection, antibody-dependent enhancement of infection

The licensing of Dengvaxia® 4 years ago seemed to signal an end to the dry spell that has plagued the development of dengue vaccines for almost a century. Regrettably, Dengvaxia® turned out to partially mimic primary infection in dengue-naïve individuals, thus increasing the risk of severe dengue upon subsequent infections in a scenario not dissimilar to that occurring during natural secondary infections (1). On the flip side, Dengvaxia®'s failure has contributed to our understanding of what could constitute a protective immune response in the context of dengue, especially regarding the role of neutralizing antibodies, although it has also tempered expectations on the future outcome of ongoing dengue vaccine efficacy trials. Developing dengue vaccines is far from a trivial endeavor, due to the lack of suitable animal models, the need for a tetravalent formulation effective against all four viral serotypes -and the associated problem of waning immunity, which may render a vaccine recipient susceptible to severe dengue during natural infection- and, especially, due to the fact that a reliable correlate for protection has not been found. Until the latter hurdle is cleared, efficacy trials of dengue vaccines will need to be conducted based on clinical endpoints, following virologically-confirmed dengue cases of any severity due to any serotype (2).

Dengue is a mosquito-borne viral infection that affects tropical and subtropical areas of the world (3, 4). It is caused by four antigenically related but distinct dengue virus (DENV) serotypes belonging to the family *Flaviviridae*, genus *flavivirus* (5). These viruses produce around 390 million infections and 20,000 deaths annually worldwide (6). DENV are transmitted mainly by mosquitoes from *Aedes* genus. The infection results in different clinical outcomes: asymptomatic (most common) or mildly symptomatic illness, uncomplicated dengue fever, or more severe disease including plasma leakage, hemorrhage, and vascular collapse (1, 7).

A lot of studies have supported the protective role of the humoral immune response, specifically, neutralizing antibodies. The first evidence of the protective role of antibodies was described by Blanc and Caminopetros in 1929, when healthy volunteers were inoculated with sera from DENV-infected individuals and 10 days later were challenged with a wild-type strain of DENV (8), observing partial protection.

As early as 1969, Halstead and colleagues described, in our opinion, the most important epidemiological observation related with DENV infection (9). Babies born from DENV-immune mothers were protected during the first 3–4 months of their life and this protective response was undoubtedly mediated by neutralizing antibodies transferred from mothers to children. However, when the levels of neutralizing antibodies decreased due to the catabolism, children became susceptible to develop the severe form of the disease during the first natural infection, even with the same virus serotype that previously infected their mothers. Similar observations published by Kliks et al. (10), supported the previous one. These and other studies constituted the bases to propose the occurrence of a key phenomenon associated to the immunopathogenesis of DENV infection: the antibody-dependent enhancement (ADE) of infection. Several studies conducted *in vitro* and *in vivo* sustain the ADE phenomenon and its implication during DENV infection and vaccination.

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Several observations of the immunopathogenesis of DENV infection and the contribution of antibodies to this phenomenon have been exemplified by many authors (11–14). One of them was the experiment reported by Halstead and coworkers in 1979 using rhesus monkeys. Animals were inoculated with human cord blood serum from DENV-immune or non-immune individuals and later infected with DENV-2. As a result, all animals receiving DENV-immune sera showed an increased viremia in comparison with that detected in animals inoculated with DENV-negative sera (15).

Obviously, this phenomenon has important implications for vaccine developers. Nevertheless, all vaccine candidates developed up to date have as their main goal, the elicitation of neutralizing antibodies. The protective role of this type of antibodies is undoubtedly, but their protective capacity is limited by their affinity and most important by their concentration, which decreases with the time.

The potential risk of ADE is the main challenge associated with the development of a safe vaccine against DENV (16). A vaccine inducing sub-protective anti-DENV antibodies may be inefficient, and also may cause ADE-mediated severe disease. In addition, despite the induction of a protective antibody response, its levels could wane and get concentration after vaccination that can mediate the ADE of the infection (14, 17, 18). The measurement of neutralizing antibodies *in vitro* may not accurately correlate with protection *in vivo*, as it was demonstrated by results of the phase IIb clinical trial performed with Dengvaxia® (19). Dengvaxia® partially mimics primary infection and increases the risk of severe dengue during subsequent infection, similar to that observed during a natural secondary dengue infection (20). One of the most accepted hypotheses to explain the non-outstanding results obtained with Dengvaxia® is the absence of T-cell epitopes in the chimeric viruses and the occurrence of ADE of the infection (21).

Unlike Dengvaxia®, the National Institute of Health of US has developed a live-attenuated tetravalent vaccine candidate; using molecular attenuation and chimerization strategies (22). These viruses contain the capsid and non-structural proteins from three out of four DENV, which are the main targets of cytotoxic and antiviral cytokine-secreting $CD4^+$ and $CD8^+$ T cells (23, 24). The cellular immune response generated by this vaccine candidate is comparable to those observed after natural dengue infection: a broad response to structural and non-structural proteins after monovalent vaccination and a T-cell response against highly conserved epitopes from non-structural proteins after tetravalent immunization (25). Additionally, this vaccine candidate is able to protect humans after challenge with partial attenuated strains of DENV-2 or DENV-3 (26).

In the last years the protective role of the cell-mediate immune response against DENV has taken a crucial role. Several mice experiments have demonstrated that $CD8^+$ and even $CD4^+$ T cells contribute to protection, controlling the viral disease or reducing viral load in blood and different organs (27–29). Nevertheless, some researchers have associated this response with the development of severe dengue during heterologous infection (30, 31), a phenomenon known as original antigenic sin. This hypothesis postulates that during secondary infection, the expansion of pre-existing lower avidity cross-reactive memory T cells dominates the response over that of naïve T cells which have higher avidity for the new DENV serotype. It is further hypothesized that peptide variants derived from the second serotype (known as altered peptide ligands) can induce a response that is qualitatively different from that induced by the original antigen, producing a different pattern of cytokines (32). These altered T-cell responses may produce a "cytokine storm" during heterologous secondary infection and thus contribute to the immunopathogenesis of severe dengue disease (33). In 2006, Mongkolsapaya and colleague demonstrated this phenomenon, but only using lymphocytes from one individual (30), which limits, in our opinion, its generalization. Four years later, the same group tried to support their hypothesis analyzing a higher number of individuals who experienced primary or secondary infections. The results demonstrated the predominance of a functional cross-reactive T cells response during the secondary heterologous infection, which showed cytotoxic activity, avoiding the development of severe dengue (31) (please, see figure 3 of this paper).

The association of the original antigenic sin with the T-cell response is contradictory; because the heterologous T-cell response not always produces severe disease in infants. As it was previously mentioned, severe dengue in infants, from endemic areas, generally occurs between 6 and 12 months after delivery (34), despite the fact that they have never been infected with DENV and lack of DENV-specific memory T cells (35). Besides, an important study has shown that the $CD8^+$ T-cell response and the capillary leakage do not match in time, suggesting that $CD8^+$ T cells are not responsible for severe signs in children infected with DENV (36).

T cells may contribute to protection against homologous or heterologous DENV infection. Some studies suggest the occurrence of homologous reinfection, specifically with a different genotype of the same virus (37–39), but this infection is in the majority of cases, asymptomatic. We hypothesize that despite the role of the ADE phenomenon that always occurs during a secondary infection, the memory T-cell response generated during the primary infection is able to control the homologous secondary infection by a different virus genotype. To support our hypothesis, we can analyze recent results published by Juraska and coworkers (40). These authors observed a genotype-mediated protection induced by Dengvaxia® against DENV-4. This vaccine only protected individuals against the natural infection with same virus genotype that was used to obtain the chimeric yellow fever-dengue-4 virus. Nevertheless, this result is completely expected taking into account that Dengvaxia® does not induce DENV-specific T-cell responses (41) that can control the replication of any genotypes. Although, genotype-specific antibodies could be considered a correlate of protection, in practice the development of vaccine candidates inducing this kind of response is actually difficult. In that case, seasonal vaccines must be obtained, depending on the circulating genotypes, but for DENV this is impossible. That is why, genotype-specific antibodies must not be considered as a correlate of protection.

DENV-specific human $CD4^+$ T and $CD8^+$ T cells proliferate, produce IFNγ, and lyse infected cells in humans who suffer a primary infection (42, 43). In fact, high frequencies of DENV-specific IFNγ-producing T cells are present in children with subclinical infections, in comparison with children with symptomatic secondary DENV infection (44, 45).

A deep study conducted in 2013 demonstrated the correlation between a polyfunctional $CD8^+$ T-cell response and protection against the disease (23). This study proposes that the HLA haplotype defines the quality of the cellular immune response. There are certain alleles that during secondary infection inadequately stimulate the $CD8^+$ T cells that show a dysfunctional immune response (46). Therefore, in the context of ADE, during heterologous virus infections, a dysfunctional T-cell response cannot control the viral load and infected individual can develop severe dengue. This hypothesis could explain epidemiological data showing that only 3–5% of secondary infections develop the severe form of the disease, despite the existence of cross-reactive antibodies with the potential capacity to induce ADE of infection. On the contrary, remaining secondary infections do not develop alarm signs, neither severe dengue. With a very high probability, in this group of individuals, the memory T-cell response generated during the primary infection limits the viral replication, even in the context of ADE of infection, avoiding complications of the disease. Although, results obtained in animal models could not translate to humans, several studies conducted in mice during the last years have demonstrated the protective role of cross-reactive T cells and its capacity to control the disease in presence of sub-protective antibodies (47, 48).

An additional evidence to support the potential role of T cells as a correlate of protection against DENV could be the findings described by Weiskopf et al. (49). These researchers highlight that during DENV infection there are differences in the immune responses depending on the infecting serotype. These differences have important implications for vaccine design and development. The majority of the DENV-2-specific T cell responses in human are directed against non-structural proteins (23), whereas about one-third of the DENV-3-specific response is directed against the membrane and envelope proteins (49). This issue could explain the low protective efficacy of Dengvaxia® against DENV-2 and the higher protective rate observed against DENV-3 (19). Despite the presence of neutralizing antibodies against both serotypes, the protective efficacy against these viruses could be related with the unequal antigen-induced T-cell responses.

To our knowledge, only one vaccine candidate has been designed to induce only a cell-mediated immunity (CMI). This vaccine candidate is based on the recombinant capsid proteins of DENV expressed in the bacteria *Escherichia coli* and these proteins form nucleocapsid-like particles (NLP) after their incubation with a synthetic oligonucleotide, containing CpG motives to stimulate the immune response. NLP from DENV-2 elicited in mice and monkeys an IFNγ-secreting cell response with cytotoxic activity that successfully reduced the viral load after a homologous viral challenge (50, 51). In the year 2016, the same authors demonstrated that the tetravalent formulation of NLP elicited a cellular immune response that significantly reduces viral load in mice after challenge with each DENV and the viremia in monkeys after challenge with DENV-3 (52). It is important to highlight, that anti-capsid antibodies do not play any role in the protective capacity of this vaccine candidate. Anti-capsid antibodies do not recognize the virus neither neutralize the infection (50, 53). In accordance with these results, Weiskopf and coworkers demonstrated that the capsid proteins of DENV are the main target of cytolytic and IFNγ-producing $CD4^+$ T cells generated in humans during a natural infection (24, 54). This last finding supports the use of capsid proteins as a vaccine candidate.

The tetravalent formulation of NLP could reduce viral load modulating the clinical course of the disease, from severe dengue to mild dengue fever or from mild dengue fever to asymptomatic infection, thus preventing the appearance of severe signs of the disease. This CMI-based vaccine candidate has as the main advantage the absence of virus-binding antibodies, avoiding the ADE phenomenon. Therefore, the risk to develop a pathogenic response will be very low even if the antigen-induced immunity could not be as high as expected (55).

However, in the year 2014, Slifka published a review with the main aim to define a potential correlate of protection against DENV (56). The review analyzed the results published by Monath et al. (57) and Guirakhoo et al. (58), from clinical studies conducted to determine the role of pre-existing Yellow fever virus (YFV)-specific immunity in the replication of attenuated viruses based on the strain 17D of YFV. The authors evaluated the vaccine strain YFV-17D and chimeras, in which the envelope and PrM proteins of YFV-17D were changed by the envelope and PrM proteins of DENV-2 (58) or Japanese encephalitis virus (JEV) (57). The chimeric viruses, YFV-DENV-2 and YFV-JEV, have the non-structural proteins of YFV-17D and in consequence the same $CD4^+$ and $CD8^+$ T-cell epitopes. However, these chimeras cannot be neutralized by YFV-17D-specific antibodies. This scenario provided the opportunity to measure the role of T cells in the absence of neutralizing antibodies. The vaccination of naïve individuals with the three viruses (YFV-17D, YFV-DENV-2, or YFV-JEV) produced a detectable viremia in more than 80% of individuals. However, when YFV-17D-vaccinated individuals were inoculated with each virus, no viremia was observed after the inoculation with YFV-17D. On the contrary, the chimeric viruses (YFV-DENV-2 or YFV-JEV) produced viremias similar to those observed in YFV-17D-naïve subjects.

Taking into account these results, Slifka suggested that $CD8^+$ and $CD4^+$ memory T-cell responses against YFV, in absence of neutralizing antibodies, do not reduce viral load after flaviviruses infection. In our opinion, these results only demonstrate that the T-cell response does not control the viral load produced by YFV. Indeed, it has been demonstrated that protection induced by YFV-17D is mainly mediated by antibodies (59). A recent study conducted in mice to elucidate the immune mechanisms that underlie 17D-based vaccine efficacy demonstrated that antibodies and $CD4^+$ T cells, but not $CD8^+$ T cells contribute to protection after viral challenge (60). Therefore, we consider that the conclusion arose by Slifka is a complete misunderstanding of the results obtained in the clinical trials. The extension of

his conclusion to all flaviviruses is incorrect because it was only demonstrated for YFV.

On the other hand, properly designed protection experiments in monkeys may provide data quite relevant to humans. A recent study reported by Borges and colleagues support that the evaluation of vaccine candidates in non-human primates that measure viremia and RNAemia after viral challenge and also determine cytokines associated with the severe form of the disease could be crucial to translate the results from animals to humans (61).

We recognize the protective role of neutralizing antibodies against DENV, but their protection capacity is limited as it has been demonstrated for several researchers during more than 60 years. Neutralizing antibodies are correlates of protection for other flavivirus, like YFV or JEV, but unfortunately not for DENV. Dengue is an acute disease and ancient dogmas state that in this kind of infection, antibodies are the main arm to confer protection. However, DENV infection up-regulates the expression of MHC-I molecules, a common mechanism of many flaviviruses to avoid the action of the innate immune system (62). This mechanism facilitates the virus to reach the viral load necessary to be transmitted to a mosquito, guarantying its persistence in the nature. However, the up-regulation of the expression of MHC-I molecules constitutes another evidence supporting the protective role of the CMI.

The scientific community has not accepted T cells response as a feasible correlate of protection, despite all the experimental evidences accumulated during the last 10 years supporting that. T cells can only reduce viral load, but in DENV infection this is very important, due to viremia correlates with the severity of the disease. Finally, we think that a vaccine against DENV must not induce virus-binding antibodies due to their demonstrated risk. Vaccines based on the induction of cellular immune response could be the potential solution against this expanding and threatening human pathogen and T-cell response can be the so prized correlate of protection. Why not?

AUTHOR CONTRIBUTIONS

LG wrote and reviewed the manuscript. AM reviewed the manuscript. LL wrote and reviewed one part of the manuscript.

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Strong CD4 T Cell Responses to Zika Virus Antigens in a Cohort of Dengue Virus Immune Mothers of Congenital Zika Virus Syndrome Infants

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Background: There is an urgent need to understand the complex relationship between cross-reactive anti-viral immunity, disease susceptibility, and severity in the face of differential exposure to related, circulating Flaviviruses. Co-exposure to Dengue virus and Zika virus in Brazil is a case in point. A devastating aspect of the 2015–2016 South American Zika outbreak was the dramatic increase in numbers of infants born with microcephaly to mothers exposed to Zika virus during pregnancy. It has been proposed that this is more likely to ensue from Zika infection in women lacking cross-protective Dengue immunity. In this case series we measure the prevalence of Dengue immunity in a cohort of mothers exposed to Zika virus during pregnancy in the 2015–2016 Zika outbreak that gave birth to an infant affected by microcephaly and explore their adaptive immunity to Zika virus.

Results: Fifty women from Sergipe, Brazil who gave birth to infants with microcephaly following Zika virus exposure during the 2015–16 outbreak were tested for serological evidence of Dengue exposure and IFNγ ELISpot spot forming cell (SFC) response to Zika virus. The majority (46/50) demonstrated Dengue immunity. IFNγ ELISpot responses to Zika virus antigens showed the following hierarchy: Env>NS1>NS3>C protein. Twenty T cell epitopes from Zika virus Env were identified. Responses to Zika virus antigens Env and NS1 were polyfunctional with cells making IFNγ, TNFα, IL-4, IL-13, and IL-10. In contrast, responses to NS5 only produced the immune regulatory TGFβ1 cytokine. There were SFC responses against Zika virus Env (1-20) and variant peptide sequences from West Nile virus, Dengue virus 1–4 and Yellow Fever virus.

Conclusion: Almost all the women in our study showed serological evidence of Dengue immunity, suggesting that microcephaly can occur in DENV immune mothers. T cell

immunity to Zika virus showed a multifunctional response to the antigens Env and NS1 and immune regulatory responses to NS5 and C protein. Our data support an argument that different viral products may skew the antiviral response to a more pro or anti-inflammatory outcome, with an associated impact on immunopathogenesis.

Keywords: zika virus, dengue virus, T cell epitope, flavivirus, cross-reactivity, microcephaly, adaptive immunity

INTRODUCTION

There was considerable alarm caused by the Zika virus (ZIKV) epidemic which spread across more than 70 countries, especially the Americas, during 2015–2016. ZIKV is one of several viruses that are spread by *Aedes aegypti* mosquitoes in temperate climates (1). In many of the affected countries they can spread ZIKV, different serotypes of Dengue virus (DENV), and Yellow Fever virus (YFV) (2). There is a need to understand the complex relationship between human immunity and disease susceptibility in the face of differential exposure and immune memory to these viruses for which the patterns of immune cross-reactivity are as yet poorly mapped. Overlaid on these unknowns are the additional issues of *Aedes* population dynamics and the variable impact of climate, posing multi-faceted, and unresolved dilemmas (3). Areas of uncertainty include the cyclical patterns of cases for any given virus from rainy season to rainy season, raising concerns for example over when wide-scale ZIKV will return and what measures should be in place (3).

The phylogenetic relationships of the Flaviviruses are such that there is a degree of antigenic cross-reactivity due to sequence conservation across the family: ZIKV is closely related to DENV 1-4, and more distantly to WNV and YFV. This antigenic cross-reactivity increases the complexity of developing specific serodiagnostics, and has also stimulated considerable speculation with respect to implications for either protection or pathogenesis (4–7). In settings where individuals may have been exposed to symptomatic or asymptomatic infection by any of the 4 DENV serotypes, those facing subsequent exposure to ZIKV might be expected either to benefit from cross-reactive protection, to suffer enhanced disease (or enhanced disease to any fetus developing during the infection) through a mechanism such as antibody dependent enhancement (ADE), or neither of these. The impact of recognition of cross-reactive epitopes has been studied in the context of the response by B cells, CD4 and CD8 T cells. The consequences of cross-reactivity have been evaluated with *in vitro* neutralization studies and using *in vivo* models in mice or non-human primates. Findings differ somewhat depending on the specific DENV epitopes targeted by the human mAbs studied. In general, it appears that while some *in vitro* studies find evidence for ADE (8) with respect to DENV-immune, ZIKV infected patients, the effect is not detectable *in vivo* (9).

The question of prior Flavivirus exposure has especially profound implications with respect to the risk of delivering a child affected by congenital Zika virus syndrome in mothers infected by ZIKV during pregnancy. An initial report based on a murine model made the case that this risk, put as high as about 1 in 10, might correlate with lack of cross-reactive CD8 immunity from prior DENV infection (10). However, a later study proposed that, prior DENV antibodies might act to enhance ZIKV pathogenesis through placental damage leading to an increase in infected trophoblasts (11).

A number of observations have been reported with respect to the extent and consequences of CD4 and CD8 T cell epitope Flavivirus cross-reactivity (5, 12–17). In murine models, several $CD8^+$ T cell epitopes from ZIKV were found to be cross-reactive with DENV and epitope responses stimulated by DENV2 were cross-reactive with ZIKV. This study suggested a $CD8^+$ T cell-dependent mechanism, reducing viral titers in multiple tissues, responsible for cross-protection against ZIKV infection when there is prior DENV immunity (5). Gifroni and colleagues studied ZIKV epitope CD4 and CD8 responses in infected donors, with or without prior DENV immunological memory (13). They found that DENV immune donors showed ZIKV T cell responses that were larger, more rapid, and more cytotoxic. Another study of the T cell response to NS3 in sequentially DENV and ZIKV exposed people in West Africa similarly showed that prior infection increases the magnitude of response (14).

We and others have mapped the $CD4^+$ T cell response to ZIKV, either in conventional, inbred mice, or in panels of HLA class II transgenic mice (15, 17). CD4 epitopes are found in all proteins. In particular, we found a response to an Env epitope that was shared across several different HLA class II restricting alleles. In several cases, ZIKV primed CD4 cells responded to the homologous epitope sequences from other viruses, including DENV1-4, WNV or YFV. Importantly, these cross-reactive responses could confer immune deviation, for example, the recall response of ZIKV-primed T cells by the Env DENV4 p1 epitope in HLA-DR1 transgenics resulted in a response skewed to IL-17A immunity. This suggested the possibility of a more complex relationship between sequential Flavivirus exposures, whereby responses may be pushed to become, not just different in terms of greater, or lesser magnitude, protection or pathogenicity, but of a differing effector phenotypic profile.

For the present study, we had the opportunity to study and map the ZIKV memory T cell responses (and cross-reactive epitopes from DENV) of a cohort of mothers from the Sergipe region of Brazil who had delivered affected children during the 2015–2016 ZIKV outbreak. This was an opportunity to study a clinical cohort of high specific interest; working with a cohort of symptomatically-infected mothers from the 2015 to 2016 ZIKV outbreak and living in an area of high DENV seroprevalence, we have regarded this observational study as one in the long and valuable clinical tradition of "uncontrolled case series," rather than as a laboratory-controlled experimental design.

RESULTS

Zika Virus Infected Cohort and Viral Serology

Characteristics of the clinical cohort of 50 individuals are shown in **Table 1**. All of the individuals in the cohort delivered an infant with microcephaly, the majority at full-term (37–52 weeks) of their pregnancy. For 15 individuals, the timing of onset of ZIKV infection was specified as during the 1st or 2nd trimester, one was infected during the 3rd trimester, and the remainder were unable to specify the exact timing of onset of ZIKV-related symptoms. The blood samples used in this study were collected at different time-points after delivery of the infant for each donor. The time interval between the birth of the infant and blood sample collection ranged from 1 to 24 months; for 25 out of 50 of the donors, the time between the birth of the infant and collection of the blood sample was up to 12 months.

Serum samples were tested using ZIKV-specific and DENV-specific IgG ELISAs. While there remain concerns about the genuine potential for serological cross-reactivity as a confounder in ELISA screening, these ELISAs have been standardized for use as recommended following trials by the Ministry of Health in Brazil. All the mothers in the cohort tested positive by ZIKV IgG ELISA. Of these, 4 tested negative by DENV-specific IgG by ELISA (**Table 1**). This commercial NS1 ELISA is validated for clinical use in Brazil under a working assumption that ZIKV IgG indicates ZIKV immunity and a result for DENV IgG, DENV immunity; however the authors acknowledge that there is uncertainty on this point in the field.

Elispot Responses to ZIKV Antigens

We initially tested 16 PBMC samples, selected at random from the donor pool and shown to have sufficient cell numbers and viability, and conducted IFNγ ELISpot assays to investigate responses to recombinant protein preparations of ZIKV Env, NS1, NS3, NS5, and C protein (**Figure 1**). Fifteen of 16 donors showed strong ZIKV antigen SFC responses. In terms of magnitude of response, the hierarchy of antigen recognition among responders was Env>NS1>NS3>C protein. Many donors displayed a T cell response to ZIKV ENV with mean SFC in excess of 200 SFC/10^6. We did not detect an IFNγ ELISpot response to NS5 antigen.

ELISpot Responses to Env (1-20) Epitope and Corresponding Variant Epitopes From Related Viruses

Our previous studies in murine models, either antigen-primed or ZIKV-infected, had highlighted an epitope within ZIKV Env (1-20), with amino acids capable of binding multiple HLAII alleles, six of the 7 common alleles tested, and recognized in the T cell response (17). The epitope within Env peptide 1–20 is one that overlies a protection-associated ZIKV CD8 epitope sometimes termed 294–302 (based on numbering of the full viral sequence (14). Furthermore, we showed in murine studies that ZIKV immune T cells could recognize the related Env (1-20) peptide sequence from several other Flaviviruses, but with the interesting caveat that this cross-reactive response could promote immune deviation, notably from IFNγ to IL-17 in response to DENV 3 and 4 variant peptides. We, therefore, tested responses of the same 16 donors to Env (1-20) and to the equivalent sequence variants from WNV, DENV1-4, and YFV (**Figure 1**). Nine out of 16 donors scored positive for Env (1-20) and the majority of individuals showed positive responses for the variant peptides. This analysis did not have the resolution to determine at the clonal level whether the DENV epitope responses constituted individual cells able to recognize both sequences cross-reactively, or distinct clones of ZIKV and DENV memory cells. All the individuals who showed a positive response to ZIKV and DENV1-4 peptide (1-20) were among those with evidence from serology of prior DENV immunity (**Table 1**: 007, 053, 044, 012, 026, 059, 060, 040, and 031). Individuals who responded to ZIKV Env (1-20), but not the variant DENV epitopes were donors 010 and 018. It can be seen from **Table 1** that donor 010 was DENV seropositive, while donor 018 was without serological evidence for prior DENV exposure. These findings are compatible with the notion of SFC responses to the DENV1-4 variant epitopes as representing either DENV1-4 memory or cross-reactive priming by ZIKV. It would have been desirable to confirm T cell responses using a complementary approach such as intracellular cytokine staining (ICS) flow cytometry, but with ethical approval allowing one vial of $<20 \times 10^6$ cells from each donor, this was not possible.

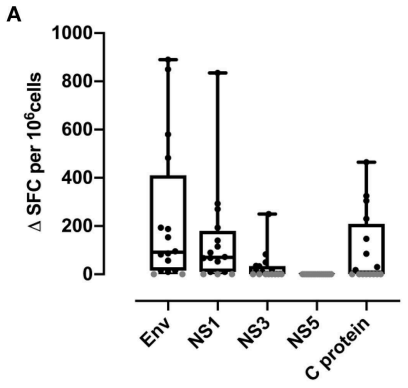
Differential Cytokine Profiles in ZIKV Antigen Responses

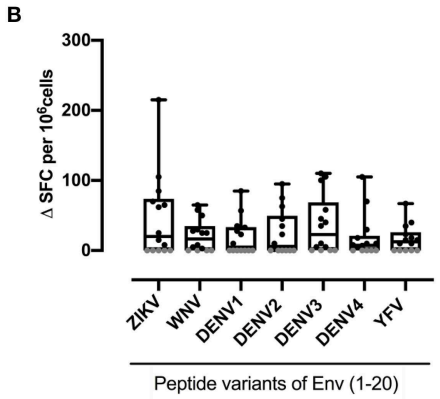
We next investigated the cytokine profiles of responses elicited by this panel of ZIKV antigens and Env (1-20) peptide and its variants (**Figure 2**). Our earlier data in Env primed or ZIKV infected mice had shown that specific epitopes, and especially, recall with variant epitopes from related viruses, could be associated with divergent cytokine profiles (17). None of the human donor T cell responses studied here produced detectable IL-17A or IL-5. Responses to recombinant Env and NS1 antigens in virtually all individuals were noteworthy for high TNFα, IL-10, IL-4, and IL-13, in addition to IFNγ as already demonstrated by ELISpot analysis. While it was not possible to repeat the analysis at the single-cell level by flow, the findings are best explained by a highly polyfunctional T cell response to ZIKV.

Interestingly, we identified highly differential cytokine responses between different viral antigens: the majority of individual donors showed a strong TGFβ response elicited by NS5 and C protein, but not by the other antigens tested. Responses to NS5 had been silent with respect to IFNγ ELISpot. In several individuals, the response elicited by the DENV 1-4 variants of Env (1-20) involved TGFβ, IL-10, IL-4, and/or IL-13, compatible with a component of the response that is mediated by Treg and Th2 subsets. We note that the vast majority of donors were seropositive in the DENV-specific ELISA, so that we are unable by this assay to distinguish between recall responses through DENV T cell memory, and cross-reactive responses of ZIKV-specific T cells.

TABLE 1 | Cohort of mothers exposed to Zika virus during pregnancy in the 2015–2016 South American Zika outbreak that gave birth to an infant affected by microcephaly.

Donor ID	Age (years)	City (Brazil)	Timing of ZIKV infection	ZIKV IgG test	DENV IgG test
ARB 001-01	20–24	Itabaiana	Unknown	Positive	Positive
ARB 003-01	30–34	Itabaiana	Unknown	Positive	Positive
ARB 004-01	35–39	Itabaiana	Unknown	Positive	Positive
ARB 005-01	25–29	Itabaiana	First and second trimester	Positive	Positive
ARB 006-01	25–29	Santa Luzia do Itanhi	Unknown	Positive	Positive
ARB 007-01	20–24	Graccho Cardoso	First trimester	Positive	Positive
ARB 008-01	30–34	Itabaiana	Second trimester	Positive	Positive
ARB 009-01	25–29	Poço Redondo	Unknown	Positive	Positive
ARB 010-01	20–24	Nossa Senhora do Socorro	Unknown	Positive	Positive
ARB 012-01	30–34	Tobias Barreto	Second trimester	Positive	Positive
ARB 013-01	40–44	Itaporanga D'ajuda	Unknown	Positive	Positive
ARB 015-01	40–44	Umbaúba	First trimester	Positive	Positive
ARB 017-01	15–19	Nossa Senhora da Glória	Unknown	Positive	Positive
ARB 018-01	30–34	Tomar do Geru	Unknown	Positive	**Negative**
ARB 019-01	20–24	Itabaianinha	Unknown	Positive	Positive
ARB 021-01	25–29	Ribeirópolis	Unknown	Positive	Positive
ARB 025-01	15–19	Umbaúba	Unknown	Positive	Positive
ARB 026-01	20–24	Itabaiana	Unknown	Positive	Positive
ARB 031-01	15–19	Ponta dos Mangues	Unknown	Positive	Positive
ARB 032-01	30–34	Paripiranga	Unknown	Positive	Positive
ARB 033-01	25–29	Siriri	Unknown	Positive	Positive
ARB 035-01	35–39	Lagarto	Unknown	Positive	Positive
ARB 037-01	15–19	Lagarto	Unknown	Positive	Positive
ARB 038-01	25–29	Siriri	Eighth month	Positive	Positive
ARB 040-01	15–19	São Miguel do Aleixo	Unknown	Positive	Positive
ARB 041-01	25–29	Nossa Senhora do Socorro	Unknown	Positive	Positive
ARB 044-01	15–19	Pirambu	Unknown	Positive	Positive
ARB 046-01	20–24	Frei Paulo	Unknown	Positive	Positive
ARB 047-01	20–24	Itabaianinha	Unknown	Positive	Positive
ARB 048-01	15–19	Malhador	Unknown	Positive	Positive
ARB 049-01	30–34	Santa Luzia do Itanhi	First trimester	Positive	**Negative**
ARB 050-01	20–24	Poço Redondo	Unknown	Positive	**Negative**
ARB 051-01	15–19	Nossa Senhora do Socorro	Unknown	Positive	Positive
ARB 052-01	30–34	Aracaju	Fourth month	Positive	Positive
ARB 053-01	35–39	Riachão do Dantas	Third month	Positive	Positive
ARB 054-01	40–44	Simão Dias	Third trimester	Positive	**Negative**
ARB 055-01	20–24	Tobias Barreto	Third month	Positive	Positive
ARB 056-01	25–29	Nossa Senhora do Socorro	Second month	Positive	Positive
ARB 057-01	15–19	Pacatuba	Unknown	Positive	Positive
ARB 058-01	25–29	Aracaju	Unknown	Positive	Positive
ARB 059-01	15–19	Maruim	Unknown	Positive	Positive
ARB 060-01	–	–	Sixth month	Positive	Positive
ARB 061-01	30–34	Nossa Senhora da Glória	Sixth month	Positive	Positive
ARB 062-01	20–24	Nossa Senhora do Socorro	First month	Positive	Positive
ARB 063-01	20–24	Nossa Senhora do Socorro	Unknown	Positive	Positive
ARB 064-01	35–39	Nossa Senhora da Glória	Unknown	Positive	Positive
ARB 065-01	35–39	Nossa Senhora do Socorro	Unknown	Positive	Positive
ARB 067-01	25–29	Cristinápolis	Second month	Positive	Positive
ARB 068-01	15–19	Capela	Unknown	Positive	Positive
ARB 069-01	35–39	Itabaianinha	Unknown	Positive	Positive





C

Peptide name	Peptide Sequence	Sequence Identity
Peptide variants of Env (1-20)		
ZIKV	IRCIGVSNRDFVEGMSGGTW	20/20
WNV	**FN**C**L**G**M**SNRDF**L**EG**V**SG**A**TW	13/20
YFV	**AH**CIG**ITD**RDF**I**EG**VH**GGTW	12/20
DENV1	**M**RC**V**G**IG**NRDFVEG**L**SG**A**TW	14/20
DENV2	**M**RCIG**I**SNRDFVEG**V**SGG**S**W	16/20
DENV3	**M**RC**V**GV**G**NRDFVEG**L**SG**A**TW	15/20
DENV4	**M**RC**V**GV**G**NRDFVEG**V**SGG**A**W	15/20

FIGURE 1 | Strong IFNγ T cell recognition to ZIKV antigens Env, NS1, and flavivirus variants of Env (1-20). PBMC from 16 patients with a history of ZIKV infection were cultured with **(A)** the recombinant ZIKV antigens envelope protein (Env), non-structural proteins 1, 3, or 5 (NS1, NS3, or NS5), capsid (C) protein or **(B)** with ZIKV Env peptide 1 variants from the flaviruses ZIKV, West Nile virus (WNV), Dengue virus serotypes 1, 2, 3, or 4 (DENV1, DENV2, DENV3, or DENV4) or Yellow Fever virus (YFV). IFNγ responses were determined by ELISpot assay. An IFNγ response was defined as positive for a given individual if the delta spot forming cell (Δ SFC) value (that is, antigen response minus medium control) was >2SD of the mean of the no antigen control (black filled circle). Responses not defined as positive are shown as 0 (gray filled circle). **(C)** Amino acid sequence of Env 1-20 flavivirus variant peptides. Error bars indicate the mean and SD.

T Cell Responses to Specific ZIKV Peptide Epitopes

In an effort to define individual T cell epitopes accounting for the response to ZIKV Env in the majority of infected individuals screened, we used samples from additional donors to map responses at the level of individual peptides. As the full overlapping peptide panel for this sequence contains 50 peptides and the PBMC sample cell numbers were limiting, we opted to screen donor ELISpot responses to a subset of the 20 peptides. To select which peptides to analyse we ranked the peptides using data derived from NetMHCIIpan 3.2 (www.cbs.dtu.dk/services/NetMHCIIpan) to predict the relative strength of CD4 binding for epitopes within ZIKV Env for the five most common MHC class II alleles in Sergipe Brazil (DRB1*13, *07, *11, *04, 15 as identified using AlleleFrequencies.net) and HLA-DRB1*01. We also included data from our published relative peptide binding data (17) and epitopes published by others (18). All twenty of the peptides tested were confirmed as containing a

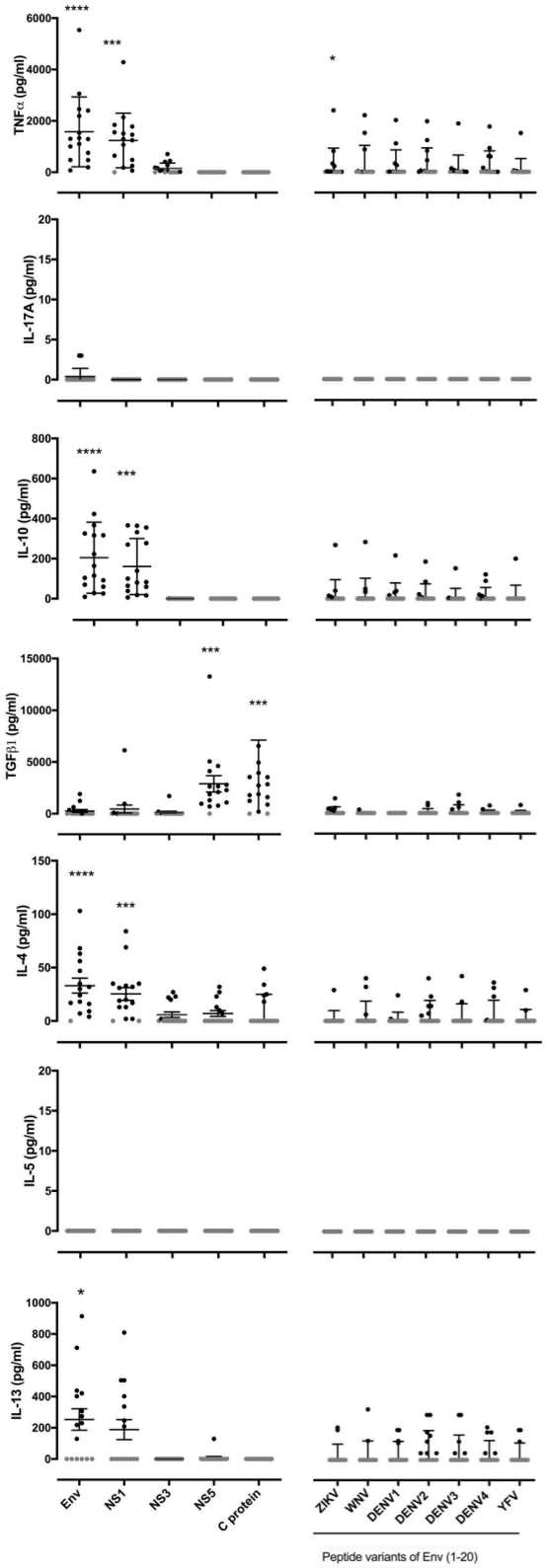


FIGURE 2 | Polyfunctional cytokine responses to ZIKV antigens Env and NS1 while NS5 and C protein antigen specific responses limited to TGFβ. PBMC *(Continued)*

FIGURE 2 | from 16 patients with a history of ZIKV infection were cultured with the recombinant ZIKV antigens envelope protein (Env), non-structural proteins 1, 3 or 5 (NS1, NS3, or NS5), capsid (C) protein or with ZIKV Env (1-20) variants from the flaviviruses ZIKV,West Nile virus (WNV), Dengue virus serotypes 1, 2, 3, or 4 (DENV1, DENV2, DENV3, or DENV4) or Yellow Fever virus (YFV). After 24 h of culture, cell culture supernatants were collected and levels of TNFα, IL-17A, IL-10, TGFβ, IL-4, IL-5, and IL-13 measured by Luminex® assay. Data are presented minus the cytokine concentrations measured for the no antigen control samples for each individual. Responses that were zero are indicated using a gray filled circle. Statistical significance between negative control samples and protein antigen or peptide stimulation was determined using a Wilcoxon matched-pairs signed rank test. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$, $^{****}p < 0.0001$.

T cell epitope recognized by several donors (**Figure 3**). The most prominent epitope in terms of percent responders and frequency of spot forming cells was Env 331-350. All six individuals tested responded to this peptide. We next investigated the extent to which the detected responses to Env peptides encompassed release of other cytokines (**Figure 4**). The response to Env 331-350 involves a polyfunctional release of TNFa, IL-17A, IL-10, TGFb, IL-4, and IL-13. In some donors the responses to epitopes Env 211-230, Env 281-300, Env 291-310, and Env 451-470 includes IL-4 and IL-13 while the response to epitope Env 131-150 involves TGFb, and Env 41-60 includes IL-13. These results indicate highly differential polyfunctional responses to different Env peptide epitopes.

DISCUSSION

In the present study we had the opportunity to analyze T cell responses to ZIKV antigens in a cohort of mothers who had suffered demonstrable infection during the recent ZIKV outbreak and delivered babies affected by congenital Zika virus syndrome. We currently lack additional, local, comparator groups needed to analyse implications of these immune responses comprehensively. For this reason, we regard the very real value of this study as being in the nature of an observational, "case series study" (19), rather than as a conventional, controlled study. One would ideally further analyse how these patterns of response differ from those in infected mothers who gave birth to unaffected babies and how might responses compare between definitively stratified groups for presence or absence of prior DENV infection? The latter comparison addresses an important mechanistic question generated by murine model studies of laboratory infection, but is extremely difficult to address in the "real-life" setting of this part of Brazil, where the majority of people may be unaware of asymptomatic DENV exposure.

Brazil is one of many countries in which Aedes mosquitoes can spread several Arboviruses, notably in this context, the phylogenetically and antigenically related species, ZIKV and DENV1-4. When the potential for contemporaneous spread of these different viruses is combined with the facts that many exposed individuals may suffer asymptomatic disease, be unaware of their exposure, and that, despite strenuous efforts, unequivocally differential clinical serodiagnostics remain

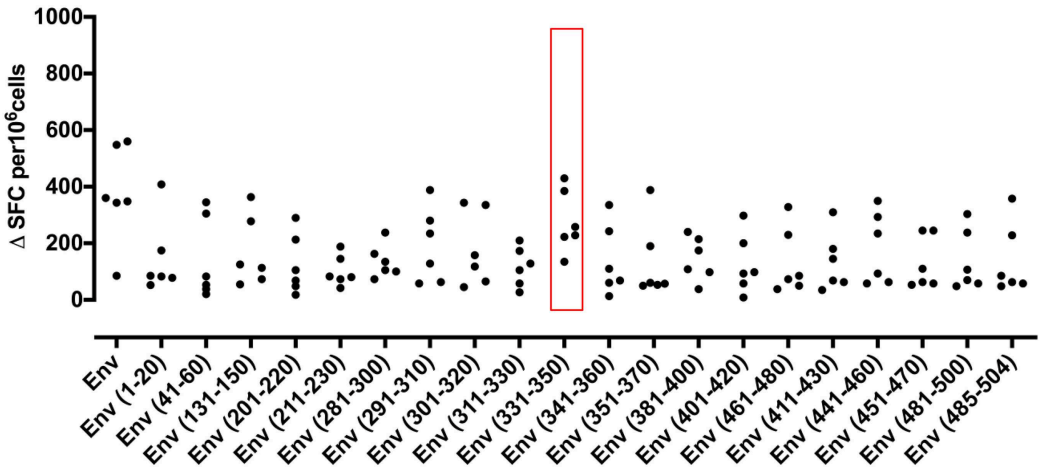


FIGURE 3 | IFNγ T cell responses to twenty peptides identify frequent epitopes from ZIKV Envelope protein. PBMC from 6 patients with a history of ZIKV infection were cultured with individual 20 mer peptides from ZIKV Envelope protein (Env). IFNγ responses were determined by ELISpot assay. An IFNγ response was defined as positive for a given individual if the delta spot forming cell (Δ SFC) value was >2SD of the mean of the no antigen control. Responses not defined as positive are shown as 0.

a challenge, unraveling the interactions between different components of virus-specific immunity is not trivial. At its simplest, the question of interaction between DENV and ZIKV immunity may be framed as one of whether the immune repertoires are additive and cross-reactively protective, pathogenic through a variant of ADE, or poorly cross-reactive and thus independent of each other. Conflicting datasets in this regard have emerged from *in vitro* and *in vivo* studies, including mouse and non-human primate challenge models. A specific context for consideration of this matter has been evaluation of why it is that a minority of mothers who are infected with ZIKV during pregnancy go on to deliver a child which is affected by congenital ZIKV syndrome, especially microcephaly. A hypothesis that has been put forward is that the affected births are more likely among those mothers with no prior cross-reactive immunity through DENV exposure (10). Almost all of the mothers in our cohort who delivered affected babies were seropositive for both ZIKV and DENV. We are, therefore, confident in our description of this cohort as one containing a majority of individuals with prior immunity to DENV. However, recent analysis of a Brazilian cohort of congenital Zika virus syndrome mothers reported reduced DENV seroprevalence with a lower number of neutralizing serotypes (20). While the overwhelming likelihood in our Sergipe cohort is that DENV exposure preceded ZIKV exposure and did not occur in the months between the birth and donation of the blood sample, we cannot formally exclude the possibility that DENV exposure could have taken place after the ZIKV illness and that may have skewed or altered the responses in some way.

We acknowledge that it cannot be proved unequivocally that affected births were due to ZIKV exposure, not least since many exposures were asymptomatic. The 2015–2015 ZIKV outbreak coincided with an unprecedented rise in microcephaly births, these occurring in others who had shown evidence of ZIKV infection during the pregnancy. However, in only a tiny minority of cases was it possible to take steps to investigate a causal relationship. All of the mothers in this cohort were seen through the pediatric Service at the University Hospital of the Federal University of Sergipe, followed up through the Congenital Zika Syndrome unit. This follow-up included full neurological assessment, cephalic perimeter, exclusion of other sources of neurological injury, and exclusion of infection by CMV, Toxoplasma, Rubella or HSV.

Although we had limited numbers of PBMC from each donor, we have sought to take some of the first steps to addressing questions about T cell immunity in symptomatic ZIKV infection. We have been able to look at the hierarchy of T cell recognition of viral antigens, the range of cytokine responses to these antigens, the epitopes within Env and, to a limited extent, the relationship to prior immunity to DENV1-4 serotypes. While many attempts to examine CD4 T cells responses in ZIKV-exposed donors have relied on screening large peptide pools, we here adopted the strategy of analyzing responses to individual recombinant protein antigens, and individual peptides. Koblischke and colleagues previously mapped responses to a C/PrM/E peptide pool using PBMC from 14 $ZIKV^{+}$ travelers returning to Europe following recovery from acute infection (21). This showed a diverse spread of epitope recognition, with some degree of focused recognition in the region of C protein amino acids 77–107. Analysis in an infected cohort from Senegal focused on analysing responses to NS3, showing high frequency ELISpot responses, including a component targeted to the NS3 helicase region and cross-reactive between DENV and ZIKV-immune donors (16).

The hierarchy of T cell antigen recognition observed in our study is different from previous published reports. Grifoni et al. showed that most of the NS responses in ZIKV exposed, DENV naïve subjects were directed against NS2a. In ZIKV/DENV exposed subjects, responses were found throughout the NS proteins, in roughly equal numbers (13). Earlier work, based

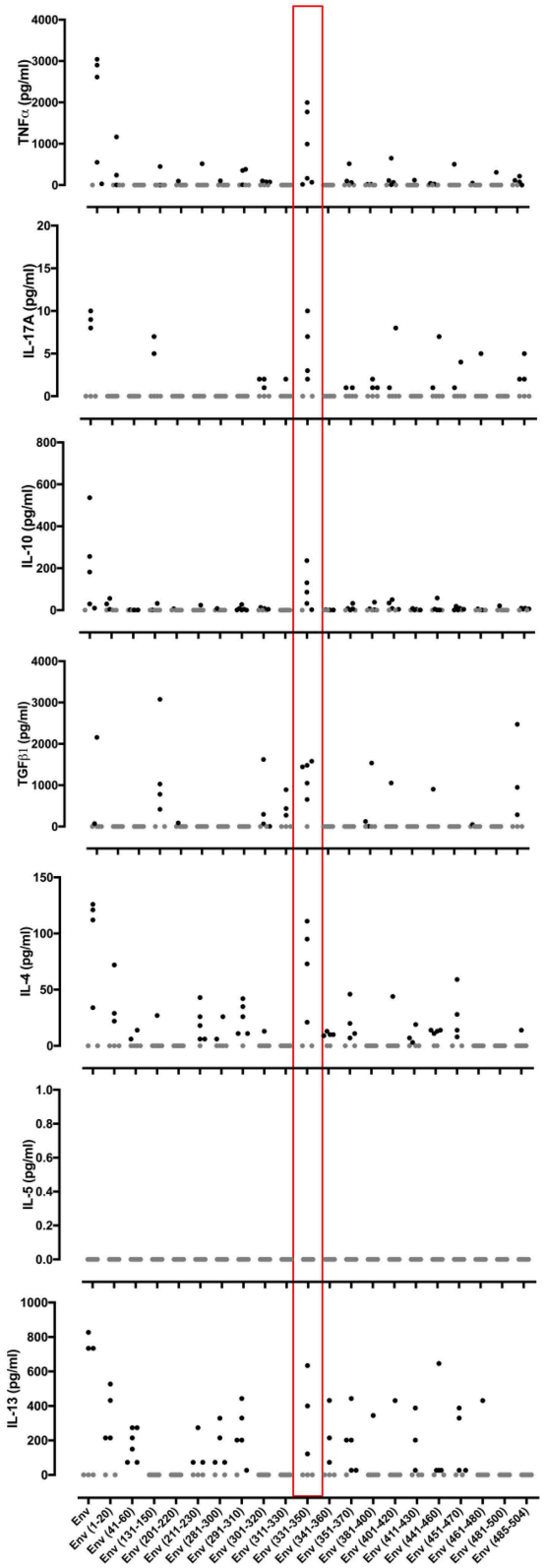


FIGURE 4 | Cytokine responses to different peptides from the ZIKV Envelope protein. PBMCs from 6 patients with a history of ZIKV infection were cultured *(Continued)*

FIGURE 4 | with individual 20 mer peptides from ZIKV Envelope protein (Env). After 24 h of culture, cell culture supernatants were collected and levels of TNFα, IL-17A, IL-10, TGFβ, IL-4, IL-5, and IL-13 measured by Luminex® assay. Data are presented minus the cytokine concentrations measured for the no antigen control samples for each individual. Responses that were zero are indicated using a gray filled circle.

on use of peptide pools, had found T cell recognition of ZIKV NS5 epitopes. We found a hierarchy of T antigen recognition whereby polyfunctional Env and NS1 recognition dominated. T cell recognition was associated with strong IFNγ, TNFα, IL-4, IL-13, and IL-10 responses. We could find no recognition of NS5 by IFNγ ELISpot. Rather, NS5 T cell recognition in our patient cohort was associated with a strong TGFβ1 response to NS5 and C protein. We did not have additional vials of cells to check the cellular origin of the TGFβ1 release by ICS, but assume that this profile may indicate preferential activation of ZIKV-specific Tregs by NS5 and C protein. Whether Treg activation is protective or detrimental in this context will require more detailed dissection of the underlying immunopathogenesis. As with DENV infections, the case can be made in ZIKV infections that the more severely symptomatic cases may be those in which there is a more exuberant, poorly regulated response (22–24). We cannot offer a definitive explanation for the observed differences between antigen hierarchies in the various published studies, but this may well be attributable that most previous work was reliant on decoding mixed peptide pools, whereas our study started from recombinant protein antigens.

The epitope mapping of individual peptides from ZIKV Env confirmed some previously identified epitopes as well as identifying several new ones. The epitope defined within Env (1-20) is noteworthy, since it overlaps a previously defined MHCI-restricted epitope previously described by us stimulating both CD4 and CD8 responses in HLA transgenic lines and shown to bind a number of different HLAII heterodimers. The epitope is relatively conserved within the Env sequence of several other Flaviruses, including DENV1-4. Our analysis of T cell responsiveness to ZIKV Env (1-20) and the variant sequences from DENV serves to underline the complexity of the immunological relationship between memory to these related sequences: while there has been previous conjecture as to the extent to which responses may be either additive in conferring protection, or pathogenic in terms of the potential to support ADE, one must also add the confounder of responses that become skewed to an alternate, regulatory programme.

Our initial epitope mapping of the T cell response to ZIKV Env identified epitopes within 20 different peptides, each recognized by several different donors. ZIKV Env can thus be regarded a relatively T cell epitope-rich antigen. Virtually all ZIKV immune donors whose T cell responses were analyzed at the level of responses to individual peptides showed an extremely strong response to Env (331-350). This common, high-frequency response was especially noteworthy for its breadth of polyfunctionality, showing induction of strong cytokine release with respect to IFNγ, TNFα, IL-17A, IL-10, IL-4, IL-13, and

TGFβ. Polyfunctionality of response is generally considered a correlate of protection in immunity and has previously been highlighted as a feature of ZIKV-specific T cells (25).

In summary, this study shows that a cohort of ZIKV-infected mothers who delivered infants with microcephaly during the 2015–2016 outbreak in Brazil comprised a group who showed evidence of what we presume to be prior DENV immunity, suggesting that microcephaly can occur in DENV immune mothers. We found a hierarchy of T cell recognition to recombinant ZIKV antigens in the order Env>NS1>NS3>C protein in terms of IFNγ responses and NS5, C protein in terms of TGFβ responses. It is noteworthy that some antigen responses were associated with an alternate cytokine profile, such as the more regulatory signal elicited by NS5 and C protein. Thus, different viral products may arguably skew the antiviral response to a more pro- or anti-inflammatory outcome, with associated impact on immunopathogenesis.

MATERIALS AND METHODS

Study Design

During 2017, peripheral blood samples were collected at the University Hospital of the Federal University of Sergipe, Brazil, from a cohort of 50 females who had given birth to a child affected by Congenital ZIKV syndrome, specifically, microcephaly. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation and frozen for later use. Serum was collected for DENV and ZIKV ELISAs at the University of Sergipe labs. Frozen cell vials were then shipped to the Imperial College London for ELISpot and cytokine assays.

Ethics Statement

This study was carried out in accordance with the recommendations of the Declaration of Helsinki. All blood donors gave written, informed consent. The study was approved by the Ethics and Research Committee of the Federal University of Sergipe (CAAE 54835916.2.0000.5546) and all patients provided informed consent for their participation in the study.

Clinical Cohort

Fifty female donors who had given birth to infants with microcephaly were recruited with full written, informed consent from the towns around the University Hospital of the Federal University of Sergipe. A clinical history was taken, including the date of onset of symptomatic ZIKV infection and infants' diagnosis. The maternal blood samples were collected at between 1 and 24 months after delivery of the infant affected by congenital Zika syndrome.

Cohort Serology

ELISAs were carried out to measure DENV IgG and ZIKV IgG (Euroimmun, Medizinische Labordiagnostika AG, Germany) (25, 26). For serological assays we used Anti-Zika Virus ELISA (IgG) and an Anti-Dengue Virus ELISA (IgG) and followed the manufacturer's instructions. These assays are validated for clinical use as non-cross-reactive insofar as they are the recommended test adopted by the Ministry of Health in Brazil. In brief, the samples were diluted 1:100 in sample buffer and incubated at 37°C (Anti-Zika Virus assay) or at room temperature (Anti-Dengue Virus assay) for 1 h. The optical density was measured at 450 and 630 nm using an Epoch spectrophotometer (BioTek®). Results were analyzed semi-quantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of the calibrator. The samples were categorized as negative (ratio < 0.8), borderline (ratio ≥ 0.8–< 1.1) or positive (ratio ≥ 1.1).

Recombinant Antigens and Peptide Panels

Sequences of the envelope protein (Env), capsid protein (C), and non-structural proteins NS1, NS3, and NS5 of ZIKV were taken from the Brazilian isolate, GenBank accession no. AMH87239.1. After codon optimisation, recombinant proteins were expressed in *Escherichia coli* and purified by His-Tag. The hydrophobic transmembrane domain (456–504) of Env was not included to improve protein expression, solubility and stability. (Biomatik, Cambridge, ON, Canada) (17). While generation of recombinant proteins in E. coli may arguably impact on correct folding and glycosylation, the assumption in the field is that any impact of this on the ability to process antigen and generate T cell epitopes is marginal. Synthetic peptides, 20 amino acids (aa) in length and overlapping by 10 aa were generated for each of the recombinant proteins (GL Biochem, Shanghai, China) (17). Flavivirus variants of Env (1-20) for WNV (accession no. AFJ05105.1), YFV (accession no. AIZ07887.1), DENV 1 (accession no. AKQ00039.1), DENV 2 (accession no. AKQ00040.1), DENV 3 (accession no. ACO06174.1), and DENV 4 (accession no. AKQ00037.1) were also synthesized (17).

ELISpot Analysis

T cell responses to antigen were inferred by IFNγ ELISpot spot forming cells (Mabtech, Sweden). One hundred and eighty thousand PBMC per well were assayed in duplicate or triplicate using pre-coated 96-well polyvinylidene difluoride (PVDF) plates. Protein or peptide were added to a final concentration of 25 μg/ml and plates were cultured at 37°C and 5% CO_2 for 24 h. Cells plus cell culture media alone were added to negative control wells; anti-CD3 to a final concentration of 1 μgml was added as a positive control for cell viability and responsiveness. Prior to assay development, supernatants were collected from the cell culture plates and stored at −40°C for additional cytokine analysis. Following plate washing, IFNγ secretion was detected by incubation with an HRP conjugated antihuman-IFNγ detection antibody at room temperature for 2 h. Spots were revealed using a BCIP/NBP-plus substrate solution. IFNγ spots were quantified using an AID ELISpot reader (Autoimmun Diagnostika GMBH, Germany). Results were calculated as spot forming cells (SFC)/million PBMC after subtraction of the number of spot forming cells observed following culture with cell culture media alone (that is, ΔSFC). Responses were defined as positive if SFC/million PBMC was >2SD of the mean of the negative control wells for each individual tested. We were

not able to analyse samples from all 50 donors side-by-side in every given set of assays; due to low cell numbers in many samples, we, therefore, analyzed randomly selected vials in different parts of the study, for example antigen responses vs. Env peptide mapping.

Cytokine Analysis

Supernatants from PBMC cultured with individual proteins or peptides were collected prior to ELISpot assay development and levels of TNFα, IL-17A, IL-10, IL-4, IL-5, IL-13, and TGFβ were measured using Luminex® assay kits (Bio-Techne, USA) on a Bio-Plex 200 instrument (Bio-Rad Laboratories, Ltd, UK). Cytokine concentrations in response to protein or peptide were calculated by subtracting values obtained for negative control cultures. IFNγ was not assayed in supernatants as findings would have been confounded by consumption of IFNγ in prior ELISpot assays.

Statistical Analysis

For cytokine data, statistical significance between negative control samples and protein antigen or peptide stimulation was determined using a Wilcoxon matched-pairs signed rank test and Graphpad Prism 7 software.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics and Research Committee of the Federal University of Sergipe (CAAE 54835916.2.0000.5546). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CR and PW developed and performed experiments, analyzed and interpreted data, and helped prepare the manuscript. KB helped prepare the introduction to the manuscript. AF, AB, and RP recruited the clinical cohort for the study. CS, DR, JA, AJ, and RA clinically characterized patients for the study including DENV IgG and ZIKV IgG ELISA. RA designed the study, clinically characterized the patient cohort, interpreted the data and helped prepare the manuscript. RB and DA conceived and designed the study, interpreted the data, and wrote the manuscript. RB and DA supervised the research and contributed equally to the study. All the authors discussed the results and commented on the manuscript.

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Rapid Induction and Maintenance of Virus-Specific CD8$^+$ T_{EMRA} and CD4$^+$ T_{EM} Cells Following Protective Vaccination Against Dengue Virus Challenge in Humans

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Dengue virus (DENV) is a mosquito-borne flavivirus that causes serious human disease. The current lack of an effective vaccine to simultaneously protect against the four serotypes of DENV in seronegative individuals is a major unmet medical need. Further, the immunological basis for protective immunity in the setting of DENV infection or vaccination is not fully understood. Our team has developed a live attenuated tetravalent dengue virus vaccine that provides complete protection in a human model of dengue virus challenge. The goal of this study was to define, in the context of protective human vaccination, the quality of vaccine-induced DENV-specific CD8$^+$ and CD4$^+$ T cells and the temporal dynamics associated with their formation and maintenance. Multifunctional, DENV-specific CD8$^+$ and CD4$^+$ T cells developed 8–14 days after vaccination and were maintained for at least 6 months. Virus-specific CD8 T$^+$ cells were a mixture of effector memory T cells (T_{EM}) and effector memory T cells re-expressing CD45RA (T_{EMRA}), with T_{EM} cells predominating until day 21 post-vaccination and T_{EMRA} cells thereafter. The majority of virus-specific CD4$^+$ T cells were T_{EM} with a small fraction being T_{EMRA}. The frequency of virus-specific CD8$^+$ and CD4$^+$ T cells were further skewed to the T_{EMRA} phenotype following either a second dose of the tetravalent vaccine or challenge with a single serotype of DENV. Collectively, our study has defined the phenotypic profile of antiviral CD8$^+$ and CD4$^+$ T cells associated with protective immunity to DENV infection and the kinetics of their formation and maintenance.

Keywords: dengue, vaccine, CD8, CD4, T_{EMRA}, protective immunity, memory, human challenge

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INTRODUCTION

Dengue virus (DENV), a mosquito-borne flavivirus, is the most prevalent cause of arboviral disease in humans. Nearly half of the world's population is at risk for DENV disease and each year there are ∼390 million cases in over 120 countries (1). There are four distinct serotypes of DENV (DENV1-4) and each is capable of causing the full range of clinical disease, from asymptomatic infection to death from DENV disease (2). While many individuals experience a relatively undifferentiated febrile illness, others develop severe clinical syndromes (dengue hemorrhagic fever and dengue shock syndrome) that are associated with severe thrombocytopenia and clotting disorders, as well as plasma leakage. These more severe disease syndromes are associated with increased risk of death, particularly in areas lacking sufficient medical care or in the very young or old (3). Although a vaccine for the prevention of DENV disease was recently approved by the United States Food and Drug Administration (FDA), its use is restricted to individuals 9–16 years of age with laboratory-confirmed previous dengue infection (4). Therefore, there remains a critical need for a broadly effective vaccine that protects dengue-naïve individuals.

A unique feature of DENV that complicates vaccine development is the observation that individuals, when infected with a second and different serotype of DENV, have a higher risk of severe disease and poor outcomes (5). Serotype-specific neutralizing antibodies raised following a first infection successfully protect against symptomatic infection with that serotype for life. However, non-neutralizing antibodies capable of binding other DENV serotypes can also be induced by a primary infection. These antibodies, when bound to a virus particle from a heterologous DENV serotype, are thought to predispose the heterologous virus for "enhanced" entry and replication in target cells when the individual is infected subsequently with this heterologous serotype (6). Thus, antibody-dependent enhancement (ADE) of infection is thought to be a key mechanism by which heterotypic, non-neutralizing antibodies may increase the risk of severe clinical disease and must be accounted for in vaccination strategies (7).

Because of the risk of developing ADE, the major global concern surrounding dengue vaccine development is that vaccination may create gaps in simultaneous coverage to all four serotypes. These gaps may emerge either when the initial vaccine series does not sufficiently prompt initial protective immunity against all four serotypes and/or due to waning coverage over time to one or more serotypes (8). In either scenario, partial protection from vaccination may expose a vaccinated individual to a risk of severe and/or life-threatening dengue disease if infected with the serotype for which there is a gap in coverage. It is possible that this risk may be higher due to vaccination than for those who were never vaccinated when the vaccine induces only partial protection. Indeed, this very concern has emerged following introduction of the Dengvaxia® vaccine into endemic areas (9). This tetravalent vaccine is constructed on the non-structural backbone of the 17D Yellow Fever vaccine. It contains the structural membrane (M) and envelope (E) proteins of DENV and the structural capsid (C) and non-structural proteins of Yellow Fever virus. Early studies of Dengvaxia® in humans and non-human primates suggested incomplete immunity (10) and imbalanced antibody responses across serotypes in early human trials (11–14). Field data now confirms that individuals who are dengue-naïve when they received Dengvaxia® have a higher risk of hospitalization with subsequent dengue infection compared with unvaccinated individuals (9, 15, 16). A possible contributing mechanism to poor protection may be the vaccine's lack of non-structural DENV proteins, which have been demonstrated to be the predominant target of dengue-specific $CD4^+$ and $CD8^+$ T cell responses (17–21).

Members of our team developed the NIH dengue live attenuated tetravalent vaccine (DLAV). Constructed via reverse genetics, this vaccine encodes wild-type dengue structural and non-structural proteins and one or more 30-nucleotide deletions in the 3′ untranslated region as its core attenuation strategy (22–25). Comprehensive development over 20 years (26–31) has led to two tetravalent formulations (TV003 and TV005) that are well-tolerated with no fever, and no liver function or clotting function abnormalities. This vaccine induces neutralizing antibodies to DENV1-4 with high frequency (31, 32) and also elicits multifunctional $CD8^+$ and $CD4^+$ T cells to each DENV serotype (19, 20). In an effort to evaluate the protective efficacy of DLAV, we developed a controlled human model of immunization and challenge in which individuals were immunized with DLAV and challenged 6 months later with under-attenuated strains of DENV. Notably, in the setting of this controlled human infection model, DLAV immunization resulted in complete protection against DENV2 or DENV3 infection (e.g., the vaccinees did not develop viremia, rash, or neutropenia) (32) (data not shown).

At present there is an incomplete understanding of what constitutes protective immunity in the setting of DENV infection. Further, it is unknown how quickly protective immunity is established following infection or vaccination. Neutralizing antibodies certainly contribute to protection, possibly by providing sterilizing immunity to a subsequent DENV exposure. There is also evidence to suggest that antiviral $CD8^+$ and $CD4^+$ T cells contribute to protective immunity and abrogation of severe disease. First, in the setting of murine infection, both cell types play a direct role in protection (33–40). Second, HLA alleles associated more severe disease correlate with weak $CD4^+$ and $CD8^+$ T cell responses while HLA alleles associated with less severe disease correlate with more robust and multifunctional T cell responses (18, 21, 41, 42). This data collectively suggests that anti-DENV T cells contribute to protective immunity.

In the current study, our goal was to evaluate $CD8^+$ and $CD4^+$ T cell phenotype and function following protective human vaccination with DLAV. In particular, we studied two cohorts: one that was vaccinated with DLAV and then boosted 180 days later (31) and the other that was vaccinated with DLAV and then challenged 180 days later with DENV2Δ30 (Tonga/74), an American genotype DENV2 strain that was isolated during an outbreak of DENV in the Kingdom of Tonga in 1974 and is heterotypic to the parent of the vaccine strain (DENV2 strain New Guinea C) (32, 43). Notably, all individuals in the latter study were completely protected from DENV challenge; DENV2

challenge virus was not detected in any vaccinated subject either by infectious virus isolation or by RT-PCR (32). Here, we describe the natural history of DLAV-induced $CD8^+$ and $CD4^+$ T cell formation and maintenance and the phenotypic attributes of these T cell subsets.

MATERIALS AND METHODS

Study Participants

Subjects in this study were participants of phase I studies to evaluate the safety and immunogenicity of the tetravalent live attenuated dengue vaccine TV003 trial CIR268 (Clinicaltrials.gov NCT01072786) (31) and trial CIR287 (Clinicaltrials.gov NCT02021968) (32). Based on the availability of high quality cryopreserved peripheral blood mononuclear cells (PBMC), we were able to evaluate T cell responses from 16 CIR 268 donors ($n = 6$ who were immunized with a single dose of TV003; $n = 10$ who were immunized with TV003 and then given a second dose 180 days later) and 8 CIR287 donors. All subjects were serologically confirmed as flavivirus-naïve at the time of immunization. Studies were approved by the Institutional Review Boards at the University of Vermont and Johns Hopkins University. Informed consent was obtained in accordance with federal and international regulations (21CFR50 and ICHE6). External monitoring was performed by National Institute of Allergy and Infectious Diseases Data Safety Monitoring board every 6 months.

Clinical Sample Procurement

At study visits, blood was collected by venipuncture into serum separator tubes for analyses of viremia and serology, and into EDTA tubes for isolation of peripheral blood mononuclear cells (PBMC). Serum was frozen at −20°C until use. PBMC were isolated by Ficoll-paque density gradient separation, counted, and frozen in cell culture medium with 10% dimethyl sulfoxide (DMSO) and 40% fetal bovine serum (FBS), and cryopreserved in liquid nitrogen vapor phase.

Vaccine (TV003) and Challenge Virus (rDEN2Δ30)

The TV003 formulation of DLAV is an admixture composed of three DENVs attenuated by deletion(s) in the 3′ untranslated region (3′UTR): rDENV1Δ30, rDENV3Δ30/31, and rDENV4Δ30, and a fourth component that is a chimeric virus with the prM and E proteins of DENV2 NGC (New Guinea C strain) exchanged for DENV4 in the rDENV4Δ30 genome (rDENV2/4Δ30) (illustrated in **Figure 1**) (31, 32). Each donor received 10^3 PFU of each DENV strain via subcutaneous inoculation. The challenge strain rDEN2Δ30 is a recombinant virus derived from the DENV2 Tonga/74 wild-type virus (43), a different genotype than DEN2 NGC. Study participants received 10^3 PFU of this challenge virus via subcutaneous injection.

DENV Epitopes

To facilitate detection of DENV-specific T cell responses irrespective of HLA types and DENV serotypes in various immunological contexts where only small amounts of blood are available, we combined previously identified DENV epitopes into a single peptide pool [megapool (MP)] that was used for T cell stimulation. DENV MPs were generated for both $CD4^+$ and $CD8^+$ T cells, and consisted of 180 and 268 peptides, respectively (see **Table S1** for a list of these peptides). Peptides were pooled, lyophilized, and resuspended in DMSO to form a master mix, which was then used to stimulate T cells *ex vivo*. DENV CD4 and CD8 MPs account for 62 and 90% of the IFN-γ response in Sri Lankan and Nicaraguan cohorts, respectively, and have been validated in different geographical locations supporting their global applicability (18, 21, 42, 44).

Ex vivo IFN-γ Enzyme-Linked Immunosorbent Spot (ELISPOT) Assay

Flat-bottom, 96-well nitrocellulose plates (Immobilon-P; Millipore) were pre-coated overnight with 50 μL of anti-human IFN-γ mAb 1-D1K (1 mg/mL) (3420-3-250; Mabtech). The next day, after washing the plates three times with PBS, 2×10^5 PBMC from each donor were plated in triplicate with either 0.5 μL of the DENV CD8 MP (4 μg/mL), 0.5 μL DMSO (negative control), 20 μL of phytohemagglutinin (PHA [1 mg/mL]) (positive control), or 1 μL each of PMA (100 μg/mL) and ionomycin (1 mg/mL) (positive control) for 16–20 h at 37°C. Plates were then washed six times with PBS/0.05% Tween 20 and incubated with 100 μL/well of biotinylated anti-IFN-γ mAb 7-B6-1 (1 mg/mL) (3420-6-250; Mabtech) for 2 h at 37°C. After six additional washes with PBS/0.05 Tween 20, IFN-γ spots were developed by sequential incubation with Vectastain ABC peroxidase (Vector Laboratories) and 3 amino-9-ethyl carbazole solution (Sigma-Aldrich) and counted by computer assisted image analysis (ZEISS KS ELISPOT Reader). Each patient sample was tested in three replicate wells and the experimental values were expressed as mean spots/10^6 PBMC. For each sample tested, responses to DMSO were measured (to establish background values) and subtracted from the response to the DENV CD8 MP.

Flow Cytometry and Intracellular Cytokine Staining (ICS) Assay

PBMCs (2×10^6) were cultured in the presence of DENV CD8 or CD4 MPs (1 μg/mL), DMSO (negative control), or PMA (100 ng/mL)/ionomycin (1 μg/mL) (positive control) for 2 h at 37°C. GolgiPlug (BD Biosciences) was then added and cells were incubated for an additional 4 h at 37°C. Cells were washed, and then stained with Live Dead Fixable Blue staining reagent (Thermo Fisher) at 4°C for 30 min, after which they were resuspended in staining buffer (PBS/1% human AB serum) and Brilliant Violet Staining buffer (BD Biosciences) containing surface staining antibodies and incubated at 4°C for 30 min. For intracellular staining, cells were fixed in ice-cold PBS/4% paraformaldehyde for 10 min, washed, and incubated in staining buffer at 4°C overnight. Cells were permeabilized with PBS/1% human AB serum/0.1% sodium azide/0.1% saponin, after which they were incubated with 10% human serum in permeabilization buffer, and then stained for intracellular cytokine expression

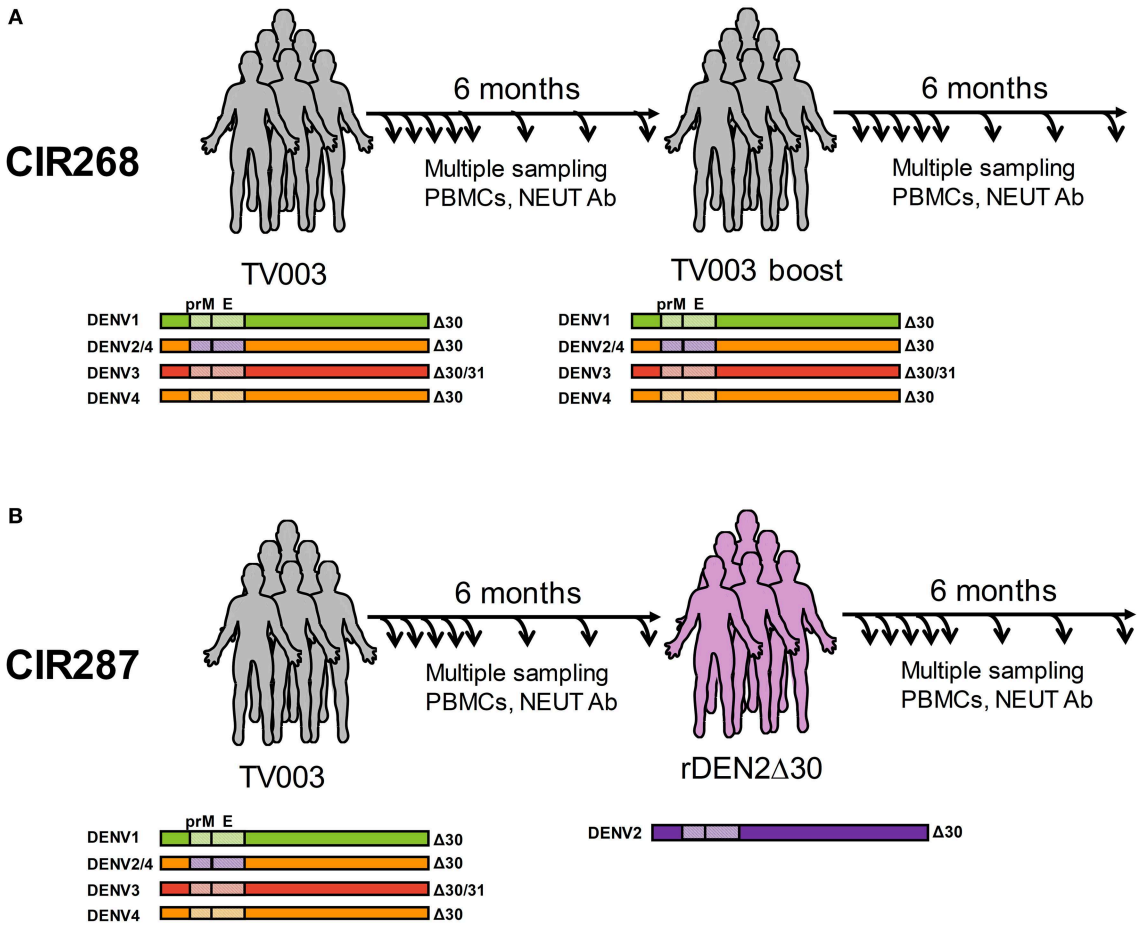

FIGURE 1 | Overview of human cohorts for measurement of anti-DENV T cells following vaccination and/or challenge. **(A)** Immunization schedule of the CIR268 study. Donors received the TV003 formulation of DLAV on day 0 and were given a second dose of TV003 on day 180 post-primary vaccination. **(B)** Immunization and challenge schedule of the CIR287 study. Donors were immunized with TV003 on day 0 and were challenged with rDENV2Δ30 (Tonga/74) on day 180 post-vaccination. For both studies, blood and PBMC were collected at multiple times post-vaccination or post-challenge for analysis by ELISPOT, ICS, or FRNT.

at 4°C for 30 min. Flow cytometry data were collected on a LSRII flow cytometer (BD Bioscience) and analyzed with FlowJo software (Treestar). For the $CD8^+$ and $CD4^+$ T cell analyses, the background signal from DMSO was subtracted from the signal elicited by the DENV CD8 MP or the DENV CD4 MP.

Antibodies used in these experiments were as follows: CD3 (UCHT1), CD19 (SJ25C1), CD14 (M0Pg) from BD Biosciences, CD4 (OKT4), CD8a (RPA-T8), and CD197 (G043H7) from Biolegend, CD45RA (HI100) from Thermo Fisher Scientific, and TNF-α (Mab11) and IFN-γ (4S.B3) from eBioscience.

Focus Reduction Neutralization (FRNT) Assay

Serum neutralizing antibody titers against DENV1-4 were determined by focus reduction neutralization test (FRNT), using the lowest serum dilution that yielded a 50% reduction in viral foci ($FRNT_{50}$) as previously described (32). The virus strains used were DENV1 (WestPac/74), DENV2 (New Guinea C), DENV3 (Slemen/78), and DENV4 (Dominica/81).

RESULTS

Human Cohorts for Measurement of Anti-DENV T Cells Following Vaccination and/or Challenge

We have previously reported on the ability of the NIH DENV tetravalent live-attenuated vaccine (DLAV) to induce DENV-specific T cells (19, 20) and neutralizing antibodies (31, 32), as well as its ability to protect against challenge with an under-attenuated strain of DENV (32). In the current study, our goal was to define the natural history of antiviral $CD8^+$ and $CD4^+$ T cells in the setting of protective vaccination. We leveraged T cells obtained from two vaccination studies. The first was study CIR268, where individuals were either (i) vaccinated with DLAV and followed for 180 days or (ii) vaccinated with

DLAV and given a boost of DLAV 180 days later (**Figure 1A**). We previously reported on the immunogenicity of vaccination in these individuals relating to the formation of anti-DENV neutralizing antibodies (31). The second study was CIR287, which followed individuals that received DLAV and 180 days later were protected from challenge with DENV2Δ30 (Tonga/74) (32) (**Figure 1B**). Herein, we report on the phenotypic and temporal properties of DENV-specific T cells in the context of these two studies, which collectively provide models of (i) single-dose vaccination, (ii) multi-dose vaccination, or (iii) single-dose vaccination and subsequent protection against challenge.

Natural History of DENV-Specific CD8$^+$ T Cell Formation and Maintenance Following Vaccination and a Subsequent Boost

We previously demonstrated that DENV-specific CD8$^+$ T cells can be detected 11–13 months after DLAV vaccination (19). Using the CIR268 cohort (described in **Figure 1A**), we wanted to determine (i) the timing of DENV-specific CD8$^+$ T cell formation and maintenance following DLAV vaccination and (ii) the impact of a second DLAV dose on the frequency and durability of these vaccine-induced CD8$^+$ T cells. To ensure maximal sensitivity, we initially employed the ELISPOT assay to detect CD8$^+$ T cells capable of secreting IFN-γ in response to the DENV CD8 MP, which contains the most frequently observed CD8$^+$ T cell epitopes from each of the four DENV serotypes, regardless of HLA background (for further description, see Materials and Methods). As shown in **Figure 2**, when all donors ($n = 6$ who were immunized with a single dose of DLAV; $n = 10$ who were immunized with DLAV and then given a second dose 180 days later) were examined, DENV-specific CD8$^+$ T cells first became detectable as early as 8 days post-vaccination, with most donors exhibiting their first measurable responses 14–21 days after vaccination. Peak frequencies were typically observed between 21 and 42 days post-vaccination, followed by declining responses through day 180 post-vaccination. However, responses remained detectable through this entire time period for most donors, with the exception of 268-003-067 and 268-003-068, who had undetectable responses at the day 180 post-vaccination time point. Additionally, responses were not detectable by ELISPOT at any time point for donors 268-003-057 and 268-003-083, despite the fact that both donors generated neutralizing antibodies (data not shown). This could reflect (i) a limit in the sensitivity of our ELISPOT assays to detect responses that may be present in these donors, (ii) an incompatibility between the HLA genotype of these donors and the CD8$^+$ T cell epitopes included in the megapool, or (iii) that these donors failed to make CD8$^+$ T cell responses to vaccination.

For the CIR268 donors who received a second dose of DLAV on day 180 after primary DLAV vaccination, we observed that DENV-specific CD8$^+$ T cell frequencies either increased ($n = 7$), or decreased ($n = 2$) when compared to the levels detectable at the day 180 post-vaccination time point (**Figure 2C**). For those with CD8$^+$ T cell expansion, maximal cell frequencies were observed between 14 and 42 days after the second dose, followed by a decline similar to what was observed following primary vaccination. The majority of vaccinees who received a second dose retained detectable antiviral CD8$^+$ T cell cells through day 360 post-primary vaccination (day 180 post-boost).

DLAV Vaccination Elicits Multifunctional CD8$^+$ T Cells

Having defined the kinetics and dynamics of DENV-specific CD8$^+$ T cell formation and maintenance following DLAV vaccination or subsequent boosting, we next wished to characterize the phenotypic properties of these cells using the intracellular cytokine staining (ICS) assay. Specifically, PBMC from CIR268 vaccinees were stimulated with the DENV CD8 MP and the virus-specific CD8$^+$ T cells within each PBMC population were assessed for their ability to produce cytokines (IFN-γ or TNF-α) as well as express memory markers (CCR7 and CD45RA). **Figure 3A** shows our gating scheme. As shown in **Figures 3B,C**, DENV-specific CD8$^+$ T cells secreting IFN-γ or TNF-α became detectable 14 days after vaccination and peaked at 21 days. In contrast, the appearance of multifunctional, DENV-specific CD8$^+$ T cells secreting both IFN-γ and TNF-α appeared later at day 21 post-vaccination and reached peak values 28–42 days following vaccination. Similar to the cohort-wide averaged ELISPOT results shown **Figures 2A,B**, the frequencies of all three populations of DENV-specific CD8$^+$ T cells (those making IFN-γ, TNF-α, or both) declined thereafter until day 180 post-vaccination (**Figures 3B,C**). Delivering a second dose of DLAV did not lead to a significant change in the cell frequencies through the remaining 180 days following the boost (**Figure 3C**).

DLAV Vaccination Elicits Dynamic DENV-Specific T_{EM} and T_{EMRA} CD8$^+$ T Cell Populations

Our team has previously reported that the majority of DENV-specific memory CD8$^+$ T cells induced by DLAV at 11–13 months after vaccination are of the T effector memory phenotype where some cells were re-expressing CD45RA (T_{EMRA}) (CD45RA$^+$, CCR7$^-$) (19). The frequent sampling points in the CIR268 study provided an opportunity to map the kinetics DENV-specific memory CD8$^+$ T cell formation following vaccination and boosting. **Figure 4A** shows our gating scheme while **Figures 4B,C** show memory marker expression on total CD8$^+$ T cells or DENV-specific, IFN-γ$^+$CD8$^+$ T cells, respectively. An examination of DENV-specific CD8$^+$ T cells expressing IFN-γ revealed a mixture of T effector memory (TEM) (CD45RA$^-$, CCR7$^-$) and T_{EMRA} on day 14 following vaccination, while no T central memory (TCM) (CD45RA$^-$, CCR7$^+$) cells were detected (**Figure 4C**). Strikingly, we noted a predominance of DENV-specific CD8$^+$ T_{EM} cells at days 14–21 post-vaccination, with a peak CD8$^+$ T_{EM} frequency at day 21 (∼83% of IFN-γ$^+$CD8$^+$ T cells). Thereafter, CD8$^+$ T_{EM} frequencies declined, while CD8$^+$ T_{EMRA} frequencies steadily increased until day 180 post-vaccination, where the proportion

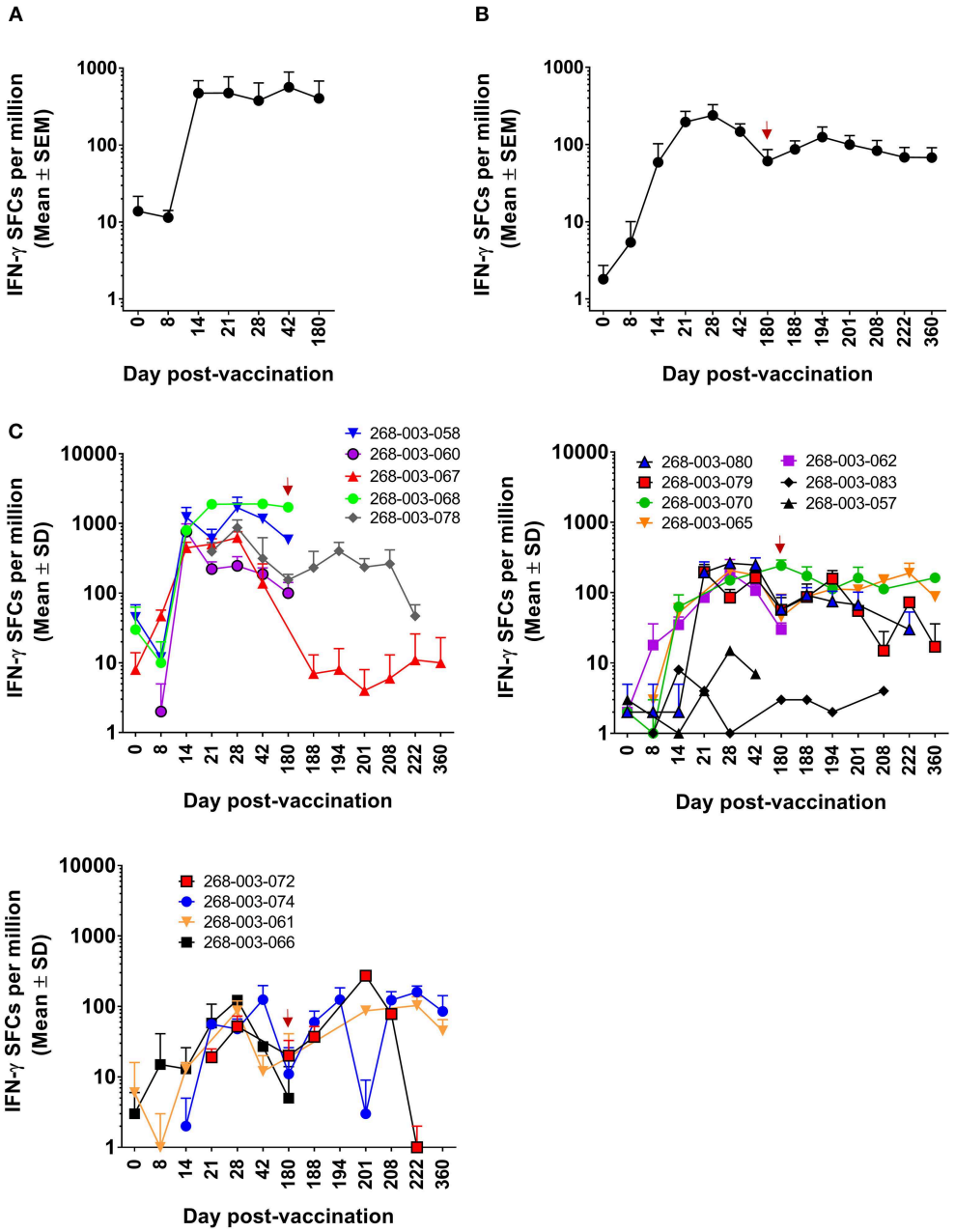

FIGURE 2 | ELISPOT measurement of DENV-specific IFN-γ^+CD8$^+$ T cells following DLAV vaccination and boosting in CIR268 individuals. ELISPOT analysis was used to identify the number of IFN-γ producing CD8$^+$ T cells that responded to the DENV CD8 MP following DLAV vaccination on day 0 and DLAV boosting on day 180 post-primary vaccination. Note that the time of DLAV boosting (day 180 post-primary vaccination) is shown in each graph in **(B,C)** with a red arrow. **(A)** Depicts the mean responses ± SEM for the donors that received only the primary DLAV vaccination and were followed to day 180 ($n = 6$) while **(B)** shows the mean responses ± SEM for donors that received DLAV vaccination on day 0 and a boost of DLAV on day 180 post-primary vaccination ($n = 10$). **(C)** Shows individual donor responses for all vaccinees.

of T_{EM} and T_{EMRA} IFN-γ^+CD8$^+$ T cells was similar (~50% of each). Interestingly, following the second dose of DLAV at day 180 post-vaccination, CD8$^+$ T_{EMRA} cells continued to increase while CD8$^+$ T_{EM} cells decreased. Specifically, at day 188 post-primary vaccination (8 days post-boost), the frequency of IFN-γ^+CD8$^+$ T cells that were T_{EMRA} was 57 vs. 40% that were T_{EM}. Together, these data indicate that DENV-specific CD8$^+$ T cell response to TV003 is characterized by an early T_{EM} response that gradually gives rise to a long-lasting T_{EMRA} response.

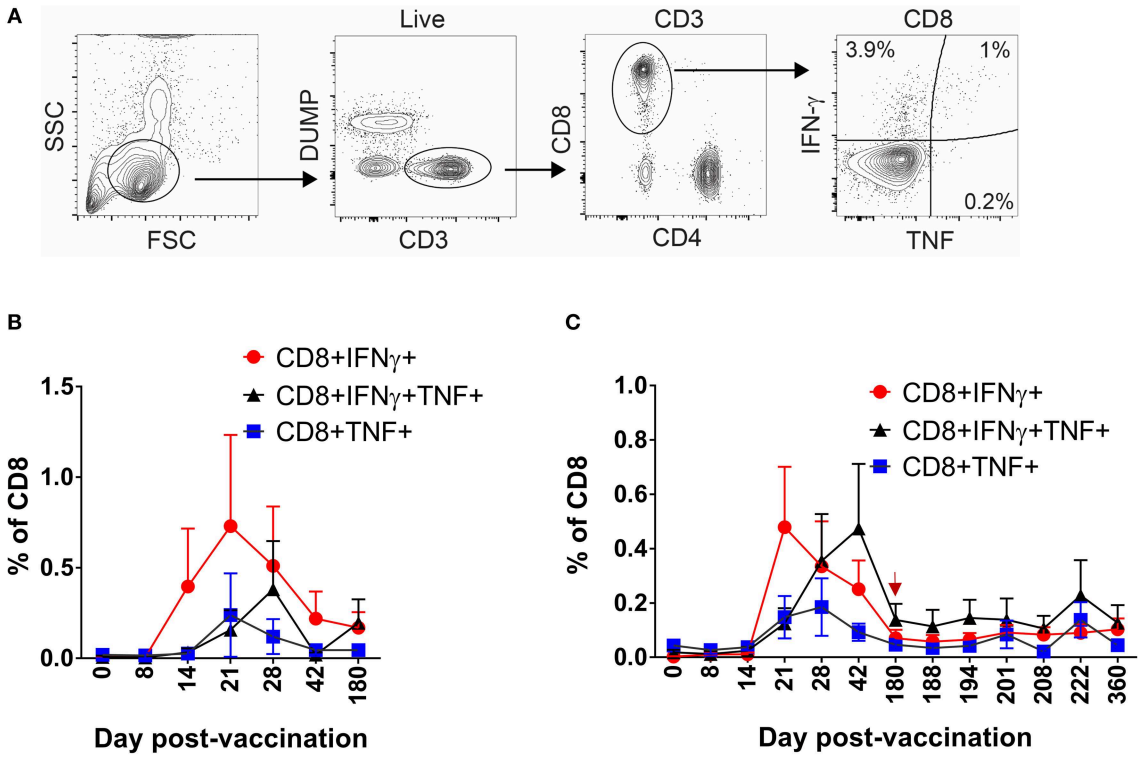

FIGURE 3 | Kinetics of multifunctional CD8$^+$ T cell formation and maintenance following DLAV vaccination or boost in CIR268 individuals. ICS was used to measure the frequency of CD8$^+$ T cells that made IFN-γ, TNF-α, or both in response to the DENV CD8 MP following DLAV vaccination on day 0 and DLAV boosting on day 180 post-primary vaccination. The time of DLAV boosting (day 180 post-primary vaccination) is shown with a red arrow in **(C)**. **(A)** Depicts the gating strategy used for these analyses. For each patient sample, the background signal to DMSO was subtracted from the signal to the DENV CD8 MP. **(B)** Depicts the mean ± SEM responses for the donors that received only the primary DLAV vaccination and were followed to day 180 ($n = 6$) while **(C)** shows the mean responses for donors that received DLAV vaccination on day 0 and a boost of DLAV on day 180 post-primary vaccination ($n = 10$).

Natural History of DENV-Specific CD8$^+$ and CD4$^+$ T Cell and Neutralizing Antibody Responses in the Setting of Protective Vaccination in Humans

Previous studies from our team have demonstrated that DLAV vaccination provides protection against a subsequent DENV challenge in humans. In particular, the CIR287 study summarized in **Figure 1B** demonstrated that DLAV-vaccinated individuals were fully protected against the development of DENV viremia and rash when challenged 180 days after vaccination with the under-attenuated DENV2Δ30 (Tonga/74) (32). Thus, the CIR287 study provided us with a unique opportunity to evaluate both DENV-specific CD8$^+$ and CD4$^+$ T cells, as well as neutralizing antibodies, in the context of protective vaccination.

We initially evaluated CD8$^+$ T cell responses to the DENV CD8 MP via ELISPOT. As shown in **Figures 5A,B**, in six CIR287 donors, the kinetics and magnitude of the DENV-specific CD8$^+$ T cells behaved similarly to that observed in the CIR268 cohort (**Figure 2**). Responses were detectable as early as day 8 post-vaccination, reached peak titers between 21 and 42 days after vaccination, and, for most donors, began to wane by day 180 post-vaccination. The magnitude of response varied between donors, but in all cases, responses were detectable throughout the first 180 days following vaccination. Similar to the CIR268 cohort, we observed accelerated kinetics of DENV-specific CD8$^+$ T cells making IFN-γ or TNF-α, followed by the appearance of multifunctional CD8$^+$ T cells making both IFN-γ and TNF-α (**Figure 6A**).

We next used ICS to measure the frequency and kinetics of DENV-specific CD4$^+$ T cells from eight CIR287 vaccinees that responded to the DENV CD4 MP. Following vaccination, DENV-specific CD4$^+$ T cells expressing IFN-γ, TNF-α, or both were detectable by day 14, peaked on day 21, decreased in frequency through day 56, and then remained relatively unchanged until day 180 (**Figure 6B**). Unlike the antiviral CD8$^+$ T cells, multifunctional IFN-γ$^+$TNF-α$^+$ CD4$^+$ T cells formed with the same kinetics as CD4$^+$ T cells expressing only IFN-γ$^+$ or TNF-α$^+$.

While we did not screen for anti-DENV2 NGC neutralizing antibodies until day 28 post-vaccination, the eight CIR287 vaccinees examined in **Figures 6A,B** began to exhibit low levels of neutralizing antibodies on day 28 post-vaccination (**Figure 6C**). Antibody titers then increased and reached peak levels on day 90 post-vaccination and remained stable until day 180 post-vaccination. We observed no correlation between the

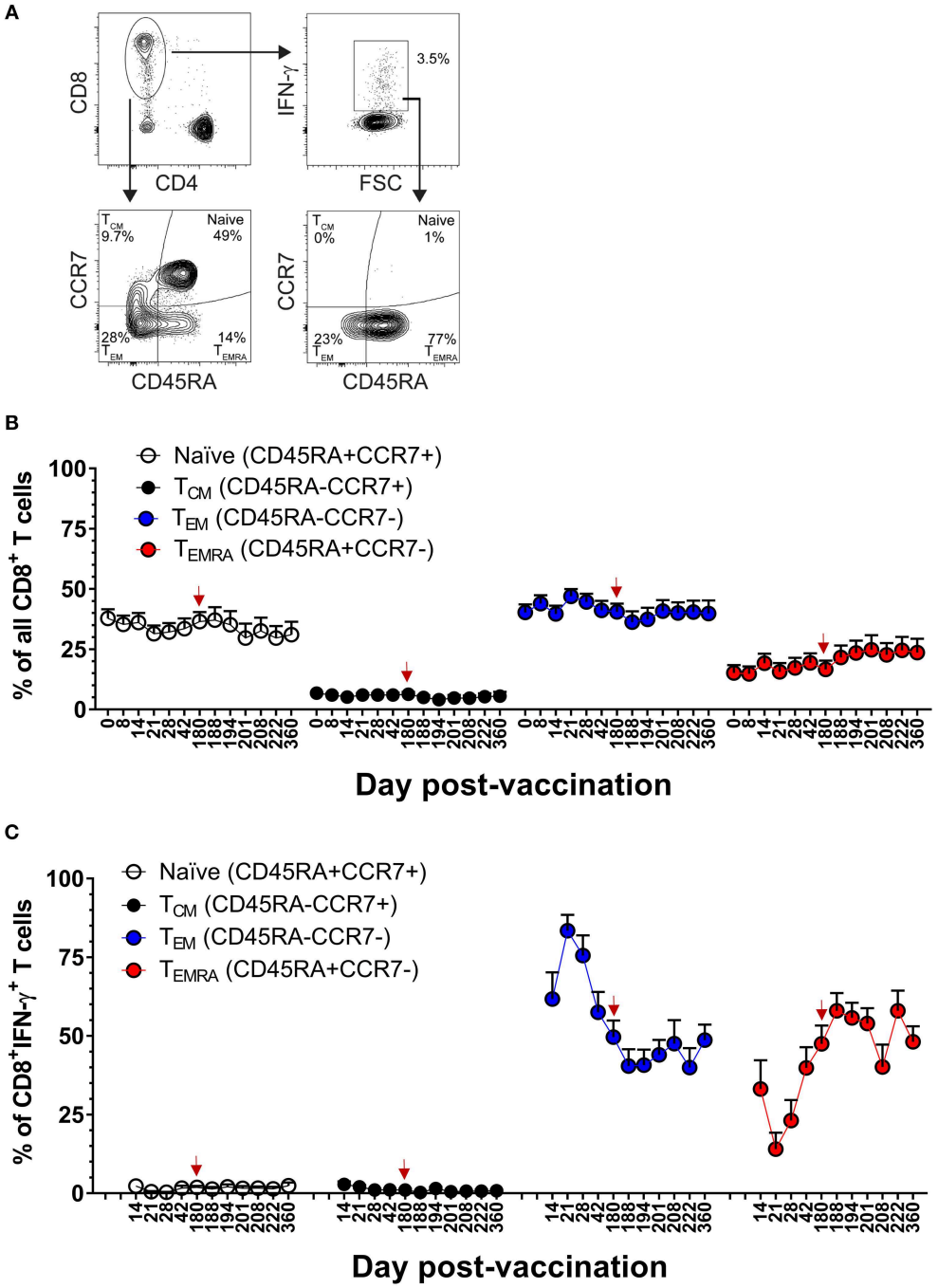

FIGURE 4 | DLAV vaccination and boosting elicits dynamic DENV-specific T_{EM} and T_{EMRA} $CD8^+$ T cell populations. **(A)** Depicts the gating strategy used for these analyses. **(B)** ICS was used to measure the frequency of all $CD8^+$ T cells exhibiting different memory T cell phenotypes (naïve, $CD45RA^+CCR7^+$; T_{CM}, $CD45RA^-CCR7^+$; T_{EM}, $CD45RA^-CCR7^-$; or T_{EMRA}, $CD45RA^+CCR7^-$) in CIR268 vaccinees following DLAV vaccination on day 0 and DLAV boosting on day 180 post-primary vaccination. **(C)** Shows the frequency of IFN-γ^+CD8^+ T cells that exhibited different memory phenotypes following stimulation with the DENV CD8 MP. Mean values ± SEM are shown ($n = 6$ that received only the primary DLAV vaccination and were only following this primary vaccination; $n = 11$ that received DLAV vaccination on day 0 and a boost of DLAV on day 180 post-primary vaccination). Note that the time of DLAV boosting (day 180 post-primary vaccination) is shown in each graph with a red arrow.

magnitude of multi-functional CD4 and CD8 T cell responses on day 180 and antibody neutralization titers (data not shown). Thus, multifunctional $CD4^+$ T cell formation and peak expansion (**Figure 6B**) occurs prior to the generation of antiviral neutralizing antibodies (**Figure 6C**) as well as multifunctional $CD8^+$ T cells (**Figure 6A**).

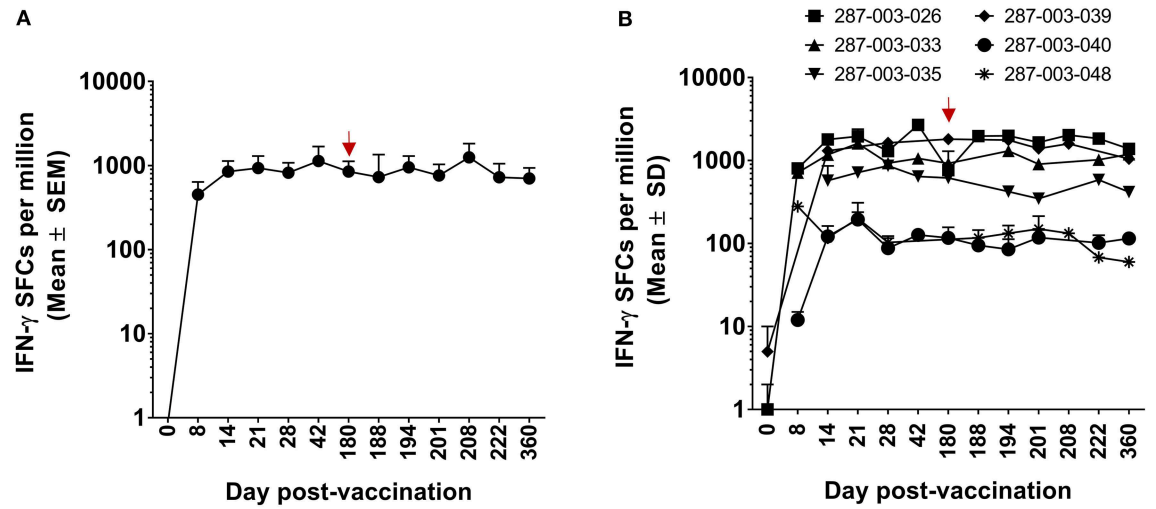

FIGURE 5 | ELISPOT measurement of DENV-specific IFN-γ^+CD8$^+$ T cells in the setting of protective vaccination in CIR287 individuals. ELISPOT analysis was used to identify the number of IFN-γ producing CD8$^+$ T cells that responded to the DENV CD8 MP following DLAV vaccination on day 0 and DENV2Δ30 (Tonga/74) challenge on day 180 post-vaccination. Note that the time of DENV2Δ30 (Tonga/74) challenge (day 180 post-vaccination) is shown in each graph with a red arrow. **(A)** Depicts the mean responses for selected individuals that were examined ($n = 6$) while **(B)** shows the individual response of these six donors.

Similar to the ELISPOT analysis of CD8$^+$ T cells from individual donors in **Figures 2C**, **5B**, there was heterogeneity in the frequency and kinetics of individual CD8$^+$ T cell, CD4$^+$ T cell, and DENV2-specific neutralizing antibody responses. To illustrate this, **Figure 7** shows the antiviral CD8$^+$ T cell, CD4$^+$ T cell, and DENV2 neutralizing antibody titers observed in representative donors (287-03-033, 287-03-035, 287-03-039, and 287-03-048). The findings from these individual donors are mostly consistent with the average trends seen when examining the mean values of the entire CIR287 cohort shown in **Figure 6**. There are several key observations from this analysis. First, there appears to be a relatively equal rate of IFN-γ^+, TNF-α^+, or IFN-γ^+TNF-α^+ CD4$^+$ T cell formation. Further, at the peak of expansion on day 21 post-vaccination, IFN-γ^+ CD4$^+$ T cells reach higher frequencies when compared to TNF-α^+ or IFN-γ^+TNF-α^+ CD4$^+$ T cells. Second, there is a staggered appearance of IFN-γ^+ and IFN-γ^+TNF-α^+ CD8$^+$ T cells, with the IFN-γ^+-only population forming earlier and the IFN-γ^+TNF-α^+ cells reaching peak levels later and remaining the highest frequency CD8$^+$ T cell subset through 180 days post-vaccination. Last, neutralizing antibodies to DENV2 appear (and peak) after the establishment of DENV-specific CD8$^+$ and CD4$^+$ T cells.

Impact of Virus Challenge on DENV-Specific CD8$^+$ and CD4$^+$ T Cells

Following challenge of CIR287 vaccinees with DENV2Δ30 (Tonga/74) at day 180 post-vaccination, virus-specific CD8$^+$ and CD4$^+$ T cell responses varied by donor. By CD8$^+$ T cell ELISPOT, two donors showed increased DENV CD8$^+$ T cells following challenge, while one showed a decline, and three remained unchanged (**Figure 5B**). By ICS, when mean values for the entire CIR287 cohort were examined, there appeared to be a trend of multifunctional IFN-γ^+TNF-α^+ CD4$^+$ and CD8$^+$ T cells increasing slightly on day 184 following vaccination (day 4 post-challenge) and then either maintaining at this frequency or decreasing through day 360 post-vaccination (d180 post-challenge) (**Figures 6A,B**). When examined at the individual level, the vaccinees shown in **Figure 7** did not show appreciable boosting of DENV-specific CD4$^+$ T cells by DENV2Δ30 (Tonga/74) challenge, with the possible exception of donor 287-03-035. However, this analysis is complicated by the fact that CD4$^+$ T cell reactivity was below the limit of detection for all three donors at most time points following challenge. With regard to DENV-specific CD8$^+$ T cells, two of the three donors (287-03-033 and 287-03-039) shown in **Figure 7** had increases in IFN-γ^+TNF-α^+ cells following challenge with DENV2Δ30 (Tonga/74) while donor 287-03-035 maintained even cell frequencies directly after challenge.

Memory CD8$^+$ and CD4$^+$ T Cell Populations in the Setting of Protective DENV Vaccination

We next examined the kinetics and phenotypic profile of DENV-specific memory CD8$^+$ and CD4$^+$ T cells that were elicited by DLAV vaccination in the CIR287 cohort and associated with complete protection against DENV2Δ30 (Tonga/74) challenge. CIR287 individuals exhibited a similar pattern of memory CD8$^+$ T cell formation to that seen for the CIR268 vaccinees in **Figure 4**. Specifically, the initial virus-specific CD8$^+$ T cell response on days 14–21 following vaccination was dominated by T_{EM} cells in CIR287 donors (**Figure 8B**) (Note that **Figure 8A** shows memory subset frequencies for CD8$^+$ T cells unable to elicit IFN-γ in response to CD8 megapool stimulation). Thereafter, the frequency of CD8$^+$ T_{EM} cells steadily declined and returned to baseline levels. Conversely, the frequency of CD8$^+$ T_{EMRA} cells steadily increased from day 21 after vaccination to day 180 where it represented 68% of virus-specific IFN-γ^+CD8$^+$ T cells. Following DENV2Δ30 (Tonga/74) challenge, CD8$^+$ T_{EMRA}

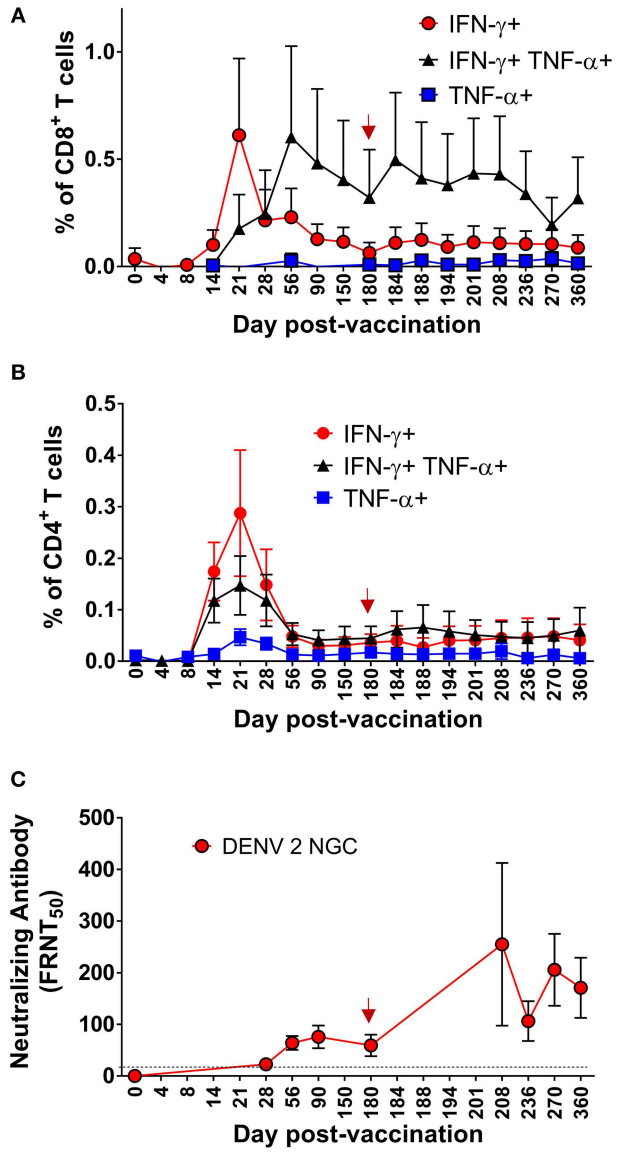

FIGURE 6 | Kinetics of multifunctional $CD8^+$ and $CD4^+$ T cell or neutralizing antibody formation and maintenance in the setting of protective DENV vaccination in CIR287 individuals. Following DLAV vaccination on day 0 and DENV2Δ30 (Tonga/74) challenge on day 180 post-vaccination, ICS was used to measure the frequency of $CD8^+$ T cells **(A)** or $CD4^+$ T cells **(B)** that made IFN-γ, TNF-α, or both in response to the DENV CD8 MP **(A)** or DENV CD4 MP **(B)** while FRNT was used to measure the titer of anti-DENV2 NGC neutralizing antibodies **(C)**. Note that the time of DENV2Δ30 (Tonga/74) challenge (day 180 post-vaccination) is shown in each graph with a red arrow and that the limit of detection in **(C)** is indicated with a dashed line. For each patient sample, the background signal to DMSO was subtracted from the signal to the DENV CD8 MP **(A)** or DENV CD4 MP **(B)**. Mean values ± SEM are shown for the eight CIR287 individuals examined **(A–C)**.

cells continued to increase for 8 days, then declined slightly and became stable whereas $CD8^+$ T_{EM} cells showed a slight increase on day 4 post-challenge (day 184 post-vaccination), and then remained fairly stable through day 180 post-challenge (day 360 post-vaccination).

We had previously reported that DLAV vaccination induces virus-specific $CD4^+$ T cells that are predominantly T_{EM} at 10–26 months after vaccination (20). Here, we confirm and extend this observation by demonstrating that while the majority of DENV-specific memory $CD4^+$ T cells were T_{EM} (range 78–98% of IFN-γ^+CD4^+ T cells), vaccination induces a progressive increase in the DENV-specific $CD4^+$ T_{EMRA} cells over 180 days, and that this frequency is maintained for at least 180 days after challenge with DENV (Tonga/74) (**Figure 9**). DENV-specific $CD4^+$ T_{EMRA} cells were initially very low between days 14 and 28 post-vaccination (range 1 to 2% of IFN-γ^+CD4^+ T cells) and then steadily increased to ∼7–19% of IFN-γ^+CD4^+ T cells. Following challenge, there appeared to be a slight trend of gradually increasing antiviral $CD4^+$ T_{EMRA}. Taken together, these data suggested that protective immunity against DENV in a human challenge model was associated with an early effector phase marked by the generation of multi-functional $CD4^+$ and $CD8^+$ T_{EM} cells, followed by late phase marked by a progressive increase in the frequency of $CD4^+$ and $CD8^+$ T_{EMRA} cells.

DISCUSSION

DENV is a serious threat to human health and the current lack of an FDA-approved vaccine for dengue-naïve individuals to safely prevent disease from all four DENV serotypes is a major unmet medical need. Further, the immunological basis for protective immunity to DENV infection is not fully understood. Certainly there is support that both arms of the adaptive immune response, T and B cells, play an important role (45, 46). In the current study, we had the opportunity to detail the natural history and functional attributes of DENV-specific $CD8^+$ and $CD4^+$ T cells in the setting of protective DENV vaccination and to view these T cell responses concurrently with antiviral neutralizing antibodies. There were several key findings. First, multifunctional (e.g., IFN-γ^+TNF-α^+-producing) $CD8^+$ and $CD4^+$ T cells specific for DENV form rapidly, typically within the first 8–14 days after vaccination and remain detectable for at least 6 months. Second, multifunctional $CD4^+$ T cells form prior to both multifunctional $CD8^+$ T cells and antiviral neutralizing antibodies and thus may contribute the establishment and quality of these $CD8^+$ T cell and antibody responses. Third, vaccine-induced $CD8^+$ T cells that are dominated by T_{EM} early after vaccination eventually give way to increased frequencies of T_{EMRA} cells that remain elevated 1 year after vaccination. Last, although the majority of DENV-specific $CD4^+$ T cells induced by DLAV vaccination are T_{EM}, and only a small proportion are T_{EMRA}, the frequency of virus-specific $CD4^+$ T_{EMRA}, is significantly increased after vaccination and challenge. Thus, our study details for the first time the formation, maintenance, and phenotypic profile of antiviral $CD8^+$ and $CD4^+$ T cells associated with protection against DENV infection.

There are several lines of evidence to suggest that DENV-specific $CD8^+$ and $CD4^+$ T cell responses play a protective role against DENV infection and/or disease severity (18, 21, 33–42). Indeed, data from the CIR287 study (DLAV vaccination followed by DENV2Δ30 (Tonga/74) challenge) adds support to this hypothesis. Specifically, of the 21 DLAV vaccinees who

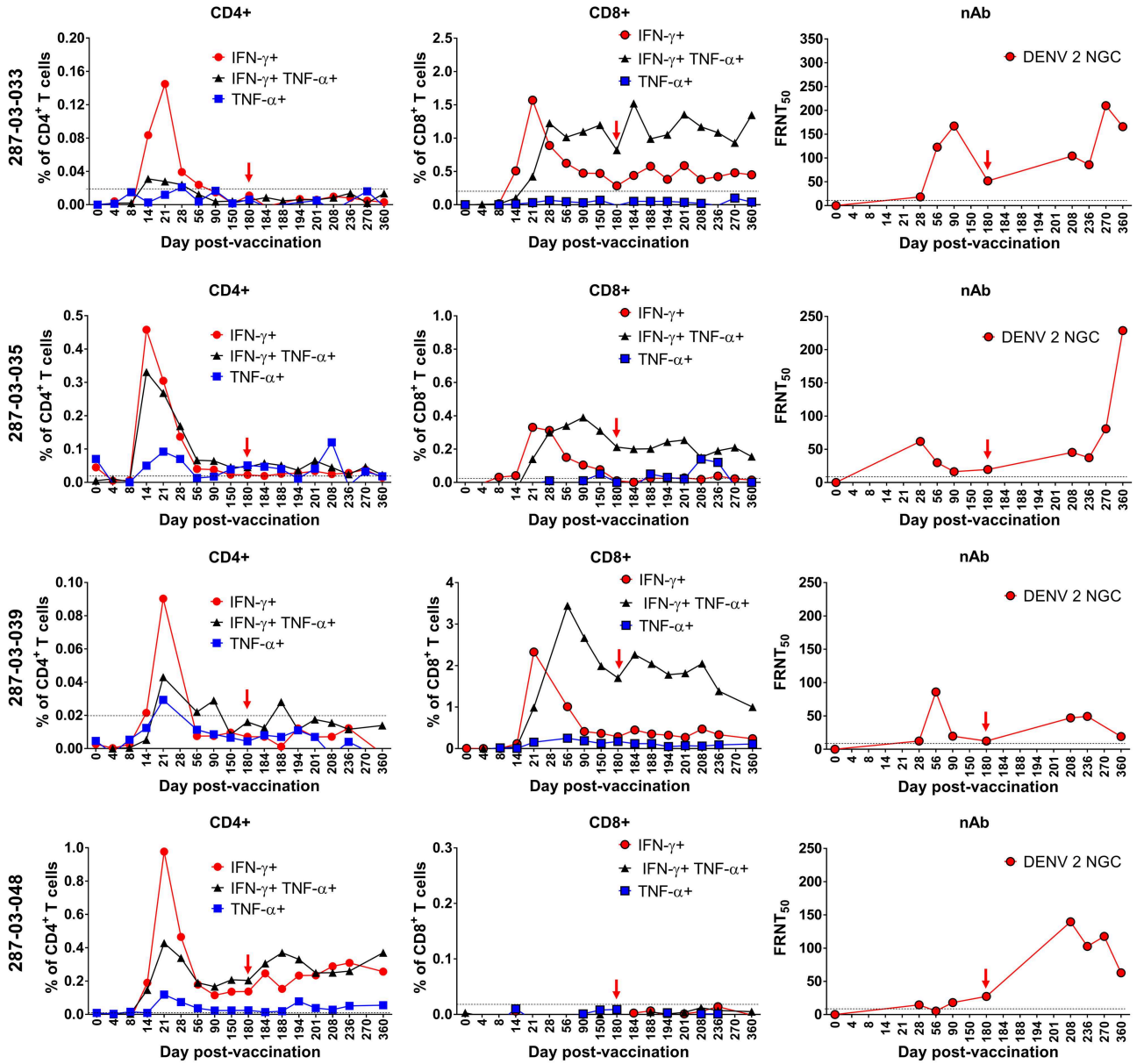

FIGURE 7 | Kinetics of neutralizing antibody and multifunctional $CD8^+$ and $CD4^+$ T cell formation and maintenance in the setting of protective DENV vaccination in CIR287 individuals. ICS was used to measure the frequency of $CD8^+$ T cells or $CD4^+$ T cells that made IFN-γ, TNF-α, or both in response to the DENV CD8 MP or DENV CD4 MP, respectively, following DLAV vaccination on day 0 and DENV2Δ30 (Tonga/74) challenge on day 180 post-vaccination. Neutralizing antibody titer against DENV2 strain New Guinea C (NGC) was measured in the same individuals via the focus reduction neutralization ($FRNT_{50}$) test. Note that in each graph the time of DENV2Δ30 (Tonga/74) challenge (day 180 post-vaccination) is shown with a red arrow and the limit of detection is indicated with a dashed line.

were protected from DENV2Δ30 (Tonga/74) challenge, nine exhibited a 4-fold or greater boost in their antiviral neutralizing antibodies following challenge (32). This result indicates that sterilizing immunity from neutralizing antibodies was not the sole mechanism of protection at work in these individuals. Rather, it is possible that the multifunctional $CD8^+$ and/or $CD4^+$ T cells detected in the CIR287 vaccinees contributed to the observed protection. However, formally demonstrating that DLAV-induced $CD8^+$ and/or $CD4^+$ T cells are sufficient to protect humans against DENV infection remains a challenge considering the high rate of neutralizing antibody induction typically observed in vaccinees (31, 32).

Our team previously demonstrated that DLAV vaccination elicits virus-specific $CD8^+$ and $CD4^+$ T cells recognizing all four DENV serotypes with the same antigen specificity and phenotypic attributes as those formed during natural DENV infection (19, 20). Further, these DLAV-induced $CD8^+$ and $CD4^+$ T cell responses remain detectable for at least 12 or 26

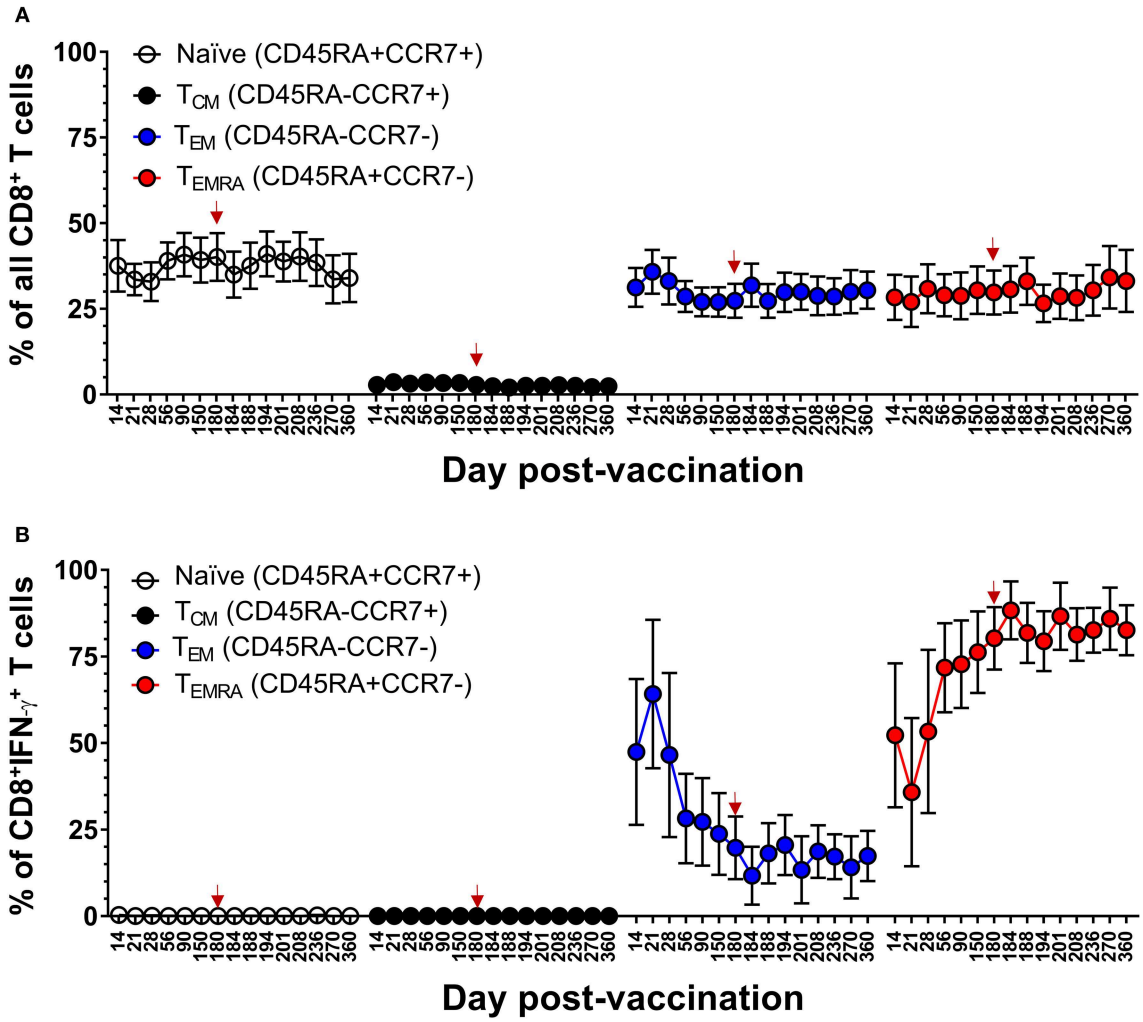

FIGURE 8 | Memory $CD8^+$ T cell populations in the setting of protective DENV vaccination in CIR287 individuals. **(A)** ICS was used to measure the frequency of all $CD8^+$ T cells exhibiting different memory T cell phenotypes (naïve, $CD45RA^+CCR7^+$; T_{CM}, $CD45RA^-CCR7^+$; T_{EM}, $CD45RA^-CCR7^-$; or T_{EMRA}, $CD45RA^+CCR7^-$) in CIR287 vaccinees following DLAV vaccination on day 0 and DENV2Δ30 (Tonga/74) challenge on day 180 post-vaccination. **(B)** Shows the frequency of IFN-γ^+CD8^+ T cells that exhibited different memory phenotypes following stimulation with the DENV CD8 MP. Mean values ± SEM are shown for the eight CIR287 individuals examined. Note that the time of DENV2Δ30 (Tonga/74) challenge (day 180 post-vaccination) is shown in each graph with a red arrow.

months, respectively (19, 20). In the current study, we were able to fine map the appearance of DENV-specific $CD8^+$ and $CD4^+$ T cells following vaccination. Thus, assuming that these T cells are protective, our studies collectively suggest that DLAV vaccination may provide protection within 8–14 days and that this protection could last for at least a year. The kinetics and phenotype of $CD8^+$ T cell induction and maintenance in humans has also been examined following vaccination with Takeda's live-attenuated tetravalent dengue vaccine (TDV) consisting of an attenuated DENV2 strain (TDV-2), and three chimeric viruses encoding the pre-membrane (prM) and E proteins of DENV1, 2, or 4 on the TDV-2 backbone. Chu et al. examined DENV-specific $CD8^+$ T cells on days 14 and 90 after primary vaccination and, by ICS, could detect multifunctional (IFN-γ^+TNF-α^+) $CD8^+$ T cells at the day 90 time point (47). Subjects received a boost at this same time point (day 90 post-primary vaccination) and retained DENV-specific $CD8^+$ T cells for another 90 days. More recently, Waickman et al. detected DENV-specific $CD8^+$ T cells by IFN-γ ELISPOT as early as 28 days following administration of this same live-attenuated tetravalent dengue virus vaccine candidate (48). Thus, there are similarities in $CD8^+$ T cell responses elicited by the DLAV and TDV platforms and our studies help to more precisely fill in the timing of anti-DENV T cell formation and maintenance following vaccination. Future human challenge studies will be required to define how quickly protective immunity is established following vaccination and the durability of this protective response.

A considerable challenge to the development of a safe DENV vaccine has been the requirement to simultaneously induce protective immunity to all four DENV serotypes. Failure to do

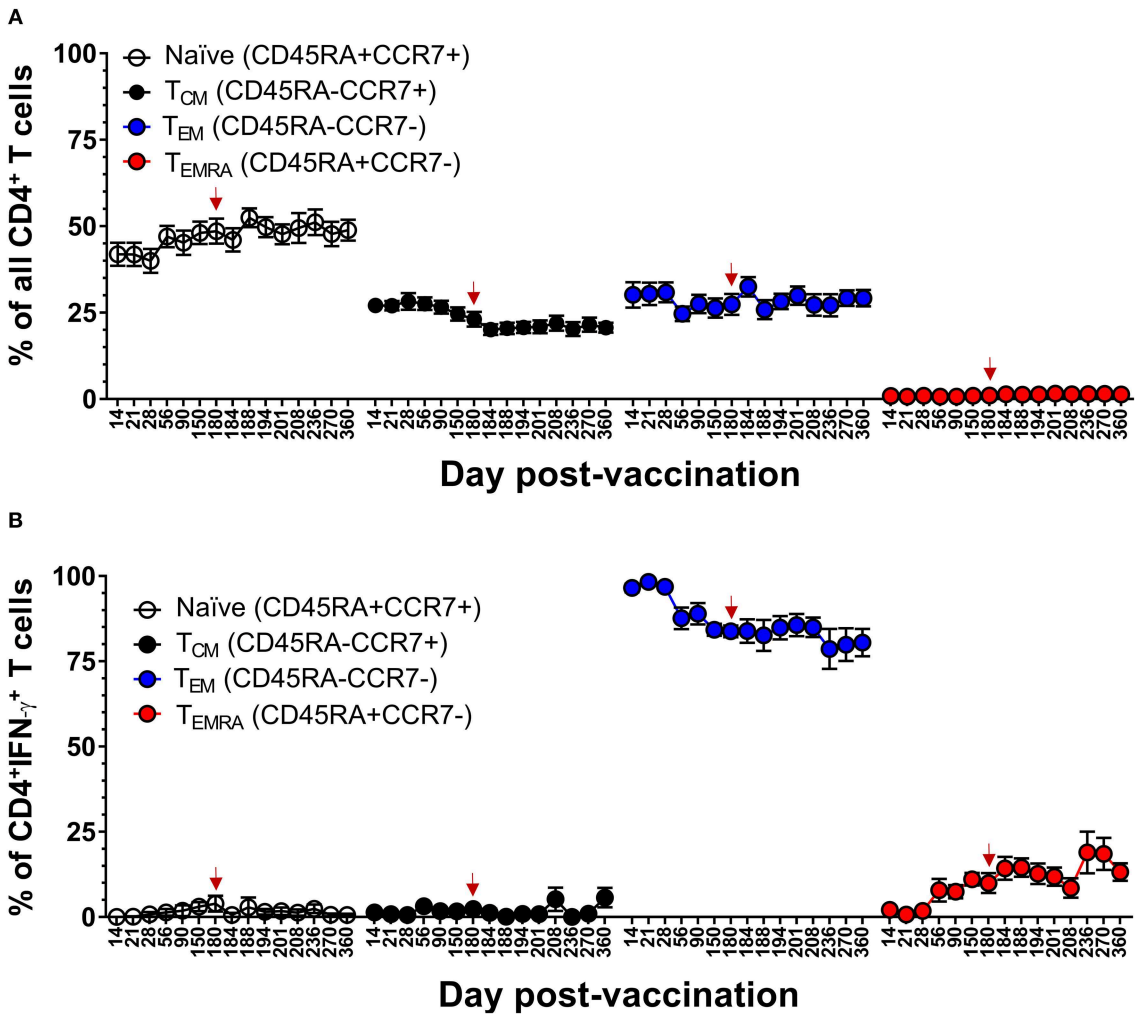

FIGURE 9 | Memory $CD4^+$ T cell populations in the setting of protective DENV vaccination in CIR287 individuals. **(A)** ICS was used to measure the frequency of all $CD4^+$ T cells exhibiting different memory T cell phenotypes (naïve, $CD45RA^+CCR7^+$; T_{CM}, $CD45RA^-CCR7^+$; T_{EM}, $CD45RA^-CCR7^-$; or T_{EMRA}, $CD45RA^+CCR7^-$) in CIR287 vaccinees following DLAV vaccination on day 0 and DENV2Δ30 (Tonga/74) challenge on day 180 post-vaccination. **(B)** Shows the frequency of IFN-γ^+CD4^+ T cells that exhibited different memory phenotypes following stimulation with the DENV CD4 MP. Mean values ± SEM are shown for the eight CIR287 individuals examined. Note that the time of DENV2Δ30 (Tonga/74) challenge (day 180 post-vaccination) is shown in each graph with a red arrow.

so theoretically puts vaccinees at risk of developing severe DENV disease due to antibody-dependent enhancement, a phenomenon whereby antiviral antibodies raised against one serotype (e.g., DENV1) can bind a second serotype (e.g., DENV3) and lead to enhanced entry of this virus into target cells (7). Indeed, the underperformance of Dengvaxia (9–14), a tetravalent DENV vaccine with the prM and E proteins of DENV and the backbone of yellow fever virus, illustrates the possible danger of a vaccine that primarily targets the generation of neutralizing antibodies, but not antiviral T cells. Not only has Dengvaxia failed to fully protect against DENV infection, it increases the risk of hospitalization in DENV-naïve individuals when compared to unvaccinated individuals (9, 15, 16). One possible advantage of a live-attenuated vaccine like DLAV is that it induces balanced $CD8^+$ and $CD4^+$ T cells responses to all four DENV serotypes after a single dose, with a particular focus on several of the DENV non-structural proteins that are missing from Dengvaxia (17–21). It is a possibility that the multifunctional $CD8^+$ and $CD4^+$ T cells induced by DLAV may not only provide protection against primary DENV infection, but could also counteract the more severe DENV disease caused by antibody-dependent enhancement.

The generation of $CD8^+$ T cell memory after vaccination is associated with progressive changes in the frequencies of virus-specific T_{EM} and T_{EMRA} cells (49, 50). Previous studies have demonstrated that long-term $CD8^+$ and $CD4^+$ T cell memory following both natural DENV infection and DLAV vaccination is associated with multi-functional T_{EM} and/or T_{EMRA} cells (19, 20, 51). Our results here reveal the dynamics of the formation and maintenance of these memory T cell populations in the

setting of a protective immune response to dengue virus (32). For both $CD4^+$ and $CD8^+$ T cells, the generation of multifunctional T cells in the first 2–4 weeks after vaccination is associated primarily with a T_{EM} phenotype, after which there is a steady increase in the frequency of virus-specific T_{EMRA} cells until 180 days after vaccination. These kinetics are similar to those previously observed after vaccination with both yellow fever and smallpox (52), indicating that these phenotypic changes are not restricted to specific pathogens. Rather, they are phenotypic features associated with the generation of virus-specific T cell memory. Indeed, previous reports that a high frequency of $CD8^+$ T_{EMRA} is associated with protection against symptomatic H1N1 influenza (53) and HSV-1 reactivation (52) underscore the relevance of using T_{EMRA} generation as a primary goal in the design of effective vaccines.

The mechanisms underlying the efficacy of T_{EMRA} in the memory response remain unclear. We found that although the initial response to DLAV is dominated by T_{EM} cells, the virus-specific response upon dengue challenge or DLAV boost is dominated by T_{EMRA} cells, indicating that it may be the T_{EMRA} subset that drives the memory T cell immune response. This finding is consistent with a recent report indicating that $CD8^+$ T_{EMRA} cells retain epigenetic marks that foster rapid effector function (50). Although comparatively less is known of $CD4^+$ T_{EMRA}, it was recently shown that DENV-specific $CD4^+$ T_{EMRA} cells are cytolytic and are associated with protective immunity (41, 54, 55).

In conclusion, these data provide a detailed map of the natural history of DENV-specific $CD4^+$ and $CD8^+$ T cell phenotype and function in a human challenge model of protective DLAV vaccination. Our data demonstrate that the protective DLAV vaccine elicits multi-functional $CD4^+$ and $CD8^+$ T_{EMRA} cells and suggest that these virus-specific T cells may play a role in protective immunity. Future studies will be needed to determine whether these DENV-specific T cell populations are a bona fide correlate of protection against DENV infection.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards at the University of Vermont and Johns Hopkins University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, DW, JEB, and JWB conceived and designed the experiments. NG, PE, and DW performed the experiments. KP, BK, AD, SD, and SW conducted the clinical trials at UVM and JHU, and provided the specimens. NG, PE, JEB, and JWB analyzed the data. BK, JEB, and JWB wrote the manuscript and all co-authors participated in the editorial process and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00479/full#supplementary-material

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REVIEW
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Two Is Better Than One: Evidence for T-Cell Cross-Protection Between Dengue and Zika and Implications on Vaccine Design

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Dengue virus (DENV, family *Flaviviridae*, genus *Flavivirus*) exists as four distinct serotypes. Generally, immunity after infection with one serotype is protective and lifelong, though exceptions have been described. However, secondary infection with a different serotype can result in more severe disease for a minority of patients. Host responses to the first DENV infection involve the development of both cross-reactive antibody and T cell responses, which, depending upon their precise balance, may mediate protection or enhance disease upon secondary infection with a different serotype. Abundant evidence now exists that responses elicited by DENV infection can cross-react with other members of the genus Flavivirus, particularly Zika virus (ZIKV). Cohort studies have shown that prior DENV immunity is associated with protection against Zika. Cross-reactive antibody responses may enhance infection with flaviviruses, which likely accounts for the cases of severe disease seen during secondary DENV infections. Data for T cell responses are contradictory, and even though cross-reactive T cell responses exist, their clinical significance is uncertain. Recent mouse experiments, however, show that cross-reactive T cells are capable of mediating protection against ZIKV. In this review, we summarize and discuss the evidence that T cell responses may, at least in part, explain the cross-protection seen against ZIKV from DENV infection, and that T cell antigens should therefore be included in putative Zika vaccines.

Keywords: dengue, Zika, T-cells, cross-reactivity, vaccine, epitope, animal models

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INTRODUCTION

During the last two decades, the rate of infections with flaviviruses, particularly dengue virus (DENV) and Zika virus (ZIKV), has risen significantly (**Figure 1**). At present, half of the world's population is considered at risk for DENV and cases of ZIKV continue to be reported globally, including the first local cases in southern Europe (1, 2). DENV and ZIKV are spread via the bite of infected mosquitoes, *Aedes spp.*, whose expanding ecological niches beyond the tropical and sub-tropical regions pose a major public health threat (3). Infection with DENV can present with a spectrum of clinical manifestations ranging from an asymptomatic illness to an acute

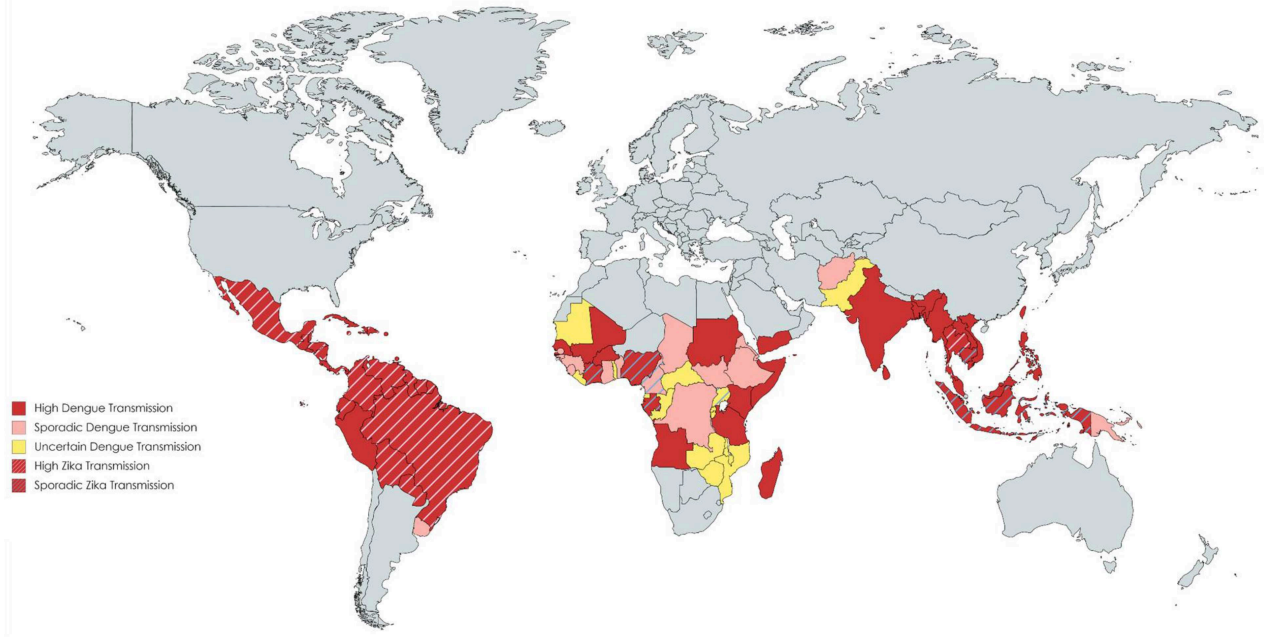

FIGURE 1 | Distribution of countries with Dengue and Zika transmission. Data compiled using the CDC yellow book and HealthMap©. Each color reflects a varying transmission rate from high dengue transmission (in red) to uncertain dengue transmission (in yellow). Countries with cross-hatched lines reflect those with high or sporadic Zika transmission.

fever/arthralgia/rash (dengue fever, DF) that usually is self-limiting, to more severe disease (dengue hemorrhagic fever/dengue shock syndrome, DHF/DSS) that is characterized by vascular leakage and/or hemorrhage (4). ZIKV causes a similar febrile illness that is often mild, with the exception of rare cases of neurological disease, such as Guillain-Barré syndrome (5). In pregnancy, ZIKV infection is associated with adverse fetal/neonatal outcomes such as congenital Zika syndrome (CZS) (6). Given that these viruses share similar geographic distributions and high sequence homology, immunological cross-reactivity between DENV and ZIKV is a well-recognized and unsurprising phenomenon. In this review, we will focus on the role of T cells during DENV and ZIKV infections in humans and in animal models, summarizing the major findings, discussing how cross-reactivity might impact immunity, and providing evidence why incorporating T cell epitopes into vaccine design is favorable.

VIROLOGY

There are four dengue viruses, DENV1-4, which are antigenically distinct (hence called serotypes) and possibly represent four distinct introductions into humans from the sylvatic cycle in non-human primates (7). On occasion several serotypes can circulate concomitantly within endemic areas, or as individual serotypes in sequence (8). A primary dengue infection generally results in lifelong immunity against the same serotype, although homotypic DENV re-infections have also been described (9). DENV infections can generate cross-reactive, poorly neutralizing antibodies that bind the other serotypes (10). Upon secondary infection with a heterologous DENV serotype, there is then a risk of severe disease, thought to be mediated via a mechanism called antibody-mediated enhancement (ADE) (11, 12). ADE arises when antibodies against one serotype can bind to, but not fully neutralize, another DENV serotype. These virus-antibody complexes can bind to the Fcγ receptors on the surface of mononuclear phagocytes enhancing viral entry and facilitating viral replication (13).

ZIKV has three genotypes, East African, West African and Asian (14). Recent Zika outbreaks have indicated a role for pre-existing immunity against DENV to modulate ZIKV infection (15). Data from a large pediatric cohort in Nicaragua found that prior DENV infection reduced the risk of symptomatic ZIKV infection by about one third (16) and in a Brazilian cohort high pre-existing antibody titers to DENV were associated with reduced risk of ZIKV infection and symptoms (17). Furthermore, protection against congenital Zika syndrome was shown to be associated with prior DENV immunity (18).

VIRAL STRUCTURE

The flavivirus virion is enveloped, and contains a single-stranded, positive-sense RNA that is ∼11 kb size. The viral genome encodes three structural proteins [capsid, precursor membrane (prM) and envelope (E)] involved in virion assembly and seven non-structural (NS) proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b,

and NS5) that function in the viral life cycle (19). The canonical role of NS proteins is in viral replication where with host factors they function in the assembly of the membrane-bound multi-protein replication complex (RC). NS proteins are also the target of most of the flavivirus CD8 T cell epitopes (20–22). A mature flavivirus particle has a well-organized outer glycoprotein shell with an icosahedral T = 3 symmetry, a host derived lipid bilayer membrane and a poorly defined inner nucelocapsid core (23). Flavivirus particles can assume various morphologies (immature, mosaic-like, and mature) that vary between flaviviruses and have important implications on antibody binding specifically regarding the availability and accessibility of epitopes (24) [reviewed in (25)].

ANTIBODY-MEDIATED IMMUNITY

Neutralizing antibody plays a crucial role in immunity to flaviviruses. Animal models show that robustly neutralizing monoclonal antibodies are sufficient for protection against many flaviviruses (24, 26, 27). However, antibody responses against flaviviruses can also be notoriously cross-reactive and the neutralization potential of these antibodies can vary considerably. The neutralizing antibody response is directed against the E protein, but responses against other proteins such as prM and NS1 also form a significant fraction of the response after both DENV and ZIKV infections (25, 28–30).

In recent years, much has been learned about anti-flavivirus antibody responses by cloning antibodies from infected or previously infected humans. These studies have demonstrated that, while many different classes of antibody are made, those which most potently neutralize frequently recognize quaternary epitopes on the viral surface and bind across multiple Envelope proteins (24, 31–33). The probable mechanism of neutralization by antibodies against quaternary epitopes is through interfering with viral fusion by locking the particle in a non-fusogenic form (24). Although some of these antibody classes can neutralize all four DENV serotypes and ZIKV (34, 35), they may not be durable in humans and their effectiveness may wane with time (36). In fact, a period of cross-protection exists even after a primary DENV infection, as observed by Sabin who found that subjects re-challenged with heterotypic DENV infection were protected if the re-challenge occurred 2–3 months after the original infection (37).

A major epitope recognized by the human antibody response is the viral fusion-loop of E domain II (25). Fusion loop antibodies are highly cross-reactive and strongly binding, but weakly neutralizing, not able to cross-neutralize other viruses, and can mediate ADE (38). During secondary flavivirus infections, the resulting antibody response can be predominantly focused upon the earlier infecting virus, a phenomenon known as "original antigenic sin (OAS);" and thus may be poorly neutralizing against the current infection (39). It may be challenging for new vaccines (such as those against ZIKV), when introduced in areas of intense flavivirus transmission, to protect if the balance of enhancing and neutralizing antibody is not optimal, or the development of new antibody responses is impaired (38). In addition, the development of congenital Zika syndrome has been linked to ADE (40) and could be due to the presence of cross-reactive fusion loop antibodies. Prior DENV immunity can protect against ZIKV, and, in the cases where inefficient antibody responses arise, possibly due to OAS, it might be that a cross-reactive CD8+ T cell response contributes to protection.

T CELL RESPONSES TO DENV IN HUMANS

Early work on cellular immunity to DENV demonstrated that T cell responses were readily detectable, and serotype cross-reactive responses of both CD4+ and CD8+ T cells were described (20–22, 41–46). The existence of serotype cross-reactivity at the level of individual T cell epitopes was found in both subjects given an experimental DENV vaccine (47, 48) and after natural exposure (49). In fact, a single DENV infection can elicit a cross-reactive T cell response against several serotypes (50), and the same T cell receptor (TCR) can recognize epitopes from multiple serotypes (51–53). Although variant epitopes may be recognized by the same TCR, the degree of overall serotype cross-reactivity is also likely to be influenced by the targeting of immunodominant responses, for example non-structural (NS) proteins are more highly conserved than structural proteins (**Table 1**). Responses biased toward sequences that are conserved between serotypes (possibly in NS proteins) give rise to higher potential for serotype cross-reactivity (55). The factors that determine whether responses are focused on conserved or variant epitopes are not known. However, interestingly, the pattern of conserved/serotype specific epitope recognition was remarkably similar in two different populations studied (**Figure 2**), implying

TABLE 1 | Percent homology across structural and non-structural proteins between Zika and DENV serotypes 1–4.

	ZIKA										
	Polyprotein%	**C%**	**prM%**	**E%**	**NS1%**	**NS2A%**	**NS2B%**	**NS3%**	**NS4A%**	**NS4B%**	**NS5%**
DENV1	55	50	43	57	54	46	35	66	43	51	67
DENV2	56	41	41	55	54	44	41	67	52	53	67
DENV3	57	50	42	58	55	46	38	67	39	52	67
DENV4	57	49	47	56	54	45	41	67	44	49	68

The results of this table was generated from data available in Grifoni et al. (54).

that the factors underlying the phenomenon are not constrained to specific populations (55). The immunodominant targets of the T-cell response can vary between CD4+ and CD8+ T cells (**Figure 3**), and also between DENV serotypes (22, 55), as well as with exposure to other flaviviruses. Interestingly, the stimulus for the most cross-reactive T cell responses of all appears to be the tetravalent live attenuated DENV vaccine TV003, which includes the NS protein from DENV serotypes 1, 3, and 4, where the vast majority of the response is directed against NS proteins (**Figure 4**) (56). CD8+ T cells from TV003 vaccines can also cross-recognize ZIKV peptides, suggesting that the tetravalent DENV vaccination can induce T cell cross-reactivity across DENV serotypes and the closely related ZIKV (54).

Demonstrating a clear role for DENV specific T cell responses in protection or disease has been more challenging. Initially, it was thought that the T cell response against DENV was pathological to the host. Some variant DENV epitopes may function as inefficient TCR agonists (58), and during acute disease CD8+ T cell responses can be more focused on variant epitopes from a previous infection (original antigenic sin) (51), possibly leading to less efficient responses. Moreover, many of these DENV specific T cells were found to be apoptotic (51). Some studies have demonstrated that *ex-vivo* T cell cytokine responses are greater with more severe disease, when tested shortly after disease onset (59, 60), suggesting a role for T cells in mediating excessive inflammation. In addition, degranulation of T cells [a surrogate marker for cytotoxicity (61)] was not greater in DHF (60, 62), implying that it is in fact the balance between cytokine production (pathological?) and killing (protective?) that may influence disease phenotype.

However, not all studies have shown a relationship between acute responses and disease phenotype, with some authors pointing out that the appearance of DENV specific T cells occurs after resolution of clinical outcomes in severe dengue (63). Furthermore, not all studies show a relationship between higher cytokine production and disease severity in DHF (64). In fact,

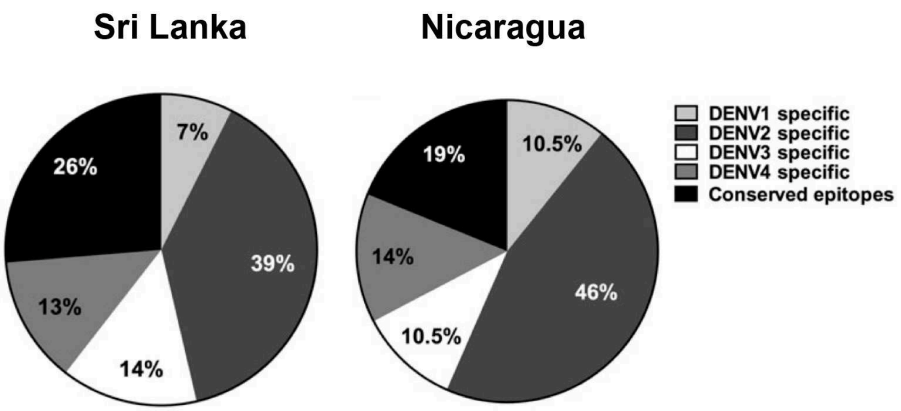

FIGURE 2 | Distribution of CD8+ T cell epitopes across the four DENV serotypes in two clinically characterized cohorts from Sri Lanka and Nicaragua. Adapted from results in Gordon et al. (16), Weiskopf et al. (55).

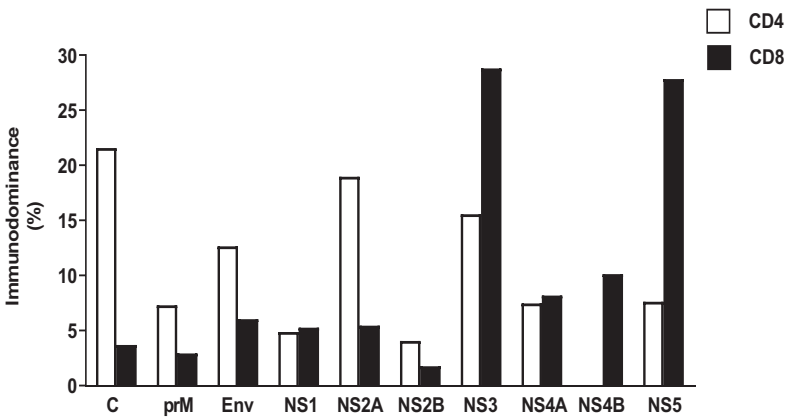

FIGURE 3 | Immunodominant protein pattern of DENV-specific CD4 and CD8 T cell response based on IEDB data. HLA class I and class II restricted epitopes for CD4 and CD8 DENV-specific T cell responses, respectively, have been derived querying IEDB database (www.IEDB.org) on 02-Aug-2019. Epitope list was filtered using Immunobrowser, selecting epitope lists tested in at least 10 donors. Protein immunodominance was calculated as percentage of epitopes per protein per HLA restriction.

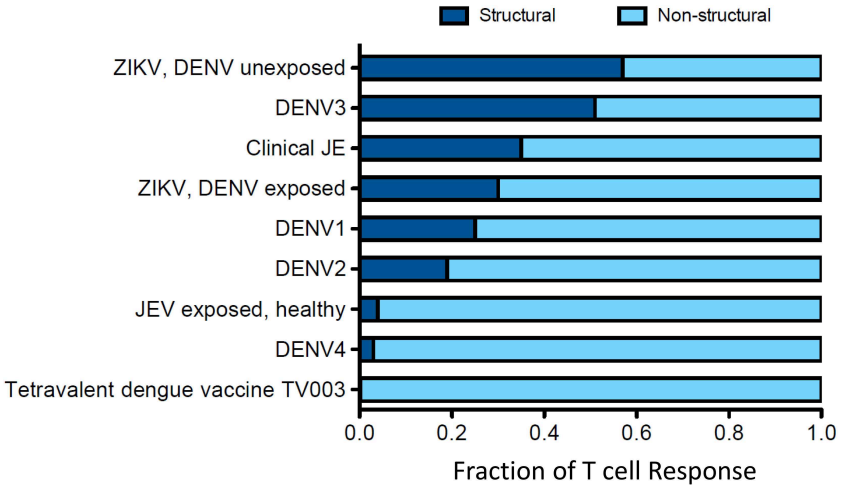

FIGURE 4 | Distribution of the T cell response to structural and non-structural components is influenced by prior clinical history. Data for this figure was compiled from the following studies: Turtle et al. (20), Weiskopf et al. (21, 56), Grifoni et al. (54), and Delgado et al. (57). T cell responses are from subjects with natural infections or vaccine recipients. Responses were identified by ELISPOT and intracellular cytokine staining (ICS) assays.

when sampled early in the disease course, or as in one prospective study before disease onset, T cell cytokine production correlated with lower viremia and less severe disease (65, 66), implying a protective role for T cells. One study of HLA association with disease severity of dengue showed certain alleles to be protective whilst others were detrimental, potentially explaining why studies of T cells and disease severity give discrepant results (21, 67, 68).

DENV/ZIKV T CELL CROSS-REACTIVITY

Given the potential for T cell responses to DENV to be protective, at least in some circumstances, it is therefore possible that T cells primed by DENV could recognize ZIKV and be protective. Grifoni et al. found that in DENV exposed individuals, the T cell response to ZIKV is earlier, larger and exhibits greater cytotoxic capacity (54). In the same study it was shown that in two large cohorts from Sri Lanka and Nicaragua (54), the imprint of previous DENV exposure is clearly detectable, and that the resulting T cell response to Asian ZIKV was biased toward the non-structural proteins (**Figure 4**). Similarly, evidence of pre-existing flavivirus immunity has been shown to result in enhanced T cell responses directed to NS3 of DENV and African ZIKV (69). Whether such responses are protective is unknown, but two studies have demonstrated that short-term T cell cultures of flavivirus specific T cells are capable of killing targets pulsed with peptides that are found in ZIKV, indicating that they likely have anti-viral function (**Figure 5**) (20, 70). With DENV infection appearing to confer partial protection against Zika illness (16, 18), cross-reactive T cell responses may be one such mechanism by which this protection is mediated. Additionally, transcriptomic profiles of ZIKV-specific CD8+ T cells in DENV naïve or pre-exposed patients showed no qualitative differences in ZIKV- specific CD8+ T cell responses

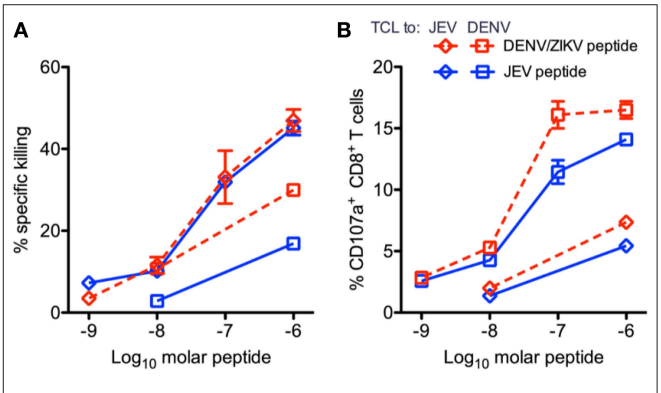

FIGURE 5 | Peptide-pulsed, CFSE-labeled, HLA-matched targets were incubated with CD8+ T cell line effector cells, and the percent specific killing was measured by flow cytometry in response to JEV and DENV/ZIKV peptides. Percent killing **(A)** and the percent of CD107+ CD8+ T cells **(B)** are shown. Diamonds indicate T cell lines expanded with JEV peptide, and squares indicate lines expanded with DENV/ZIKV peptide. Assays were performed in duplicate for each T cell line/peptide pair. Error bars represent standard error of the mean. This figure was adapted from Turtle et al. (20).

supporting the fact that cross-reactive T cell responses share the same protective phenotype observed after single flavivirus exposure (71). This phenomenon may also not be confined to ZIKV. There is some evidence that partial protection against Japanese Encephalitis (JE) appears to be conferred by prior DENV infection, with the number of JE cases lower than expected in areas with DENV outbreaks (72). Also it has been shown that the severity of JE is reduced by previous flavivirus infection (73). These effects may be mediated by T cell responses (20). The degree to which T cell responses are targeted to structural vs. non-structural flavivirus proteins may vary according to previous exposure. For example, in the case of ZIKV, being affected

by prior DENV infection biases the response toward the non-structural proteins (**Figure 4**). Responses against non-structural proteins tend to be more cross-reactive, meaning that previous dengue infection has the potential to bias the T cell response to ZIKV toward more cross-reactive epitopes. In an environment where multiple flavivirus exposure occurs, such epitopes, are likely to receive a great number of re-stimulations and may rise to the top of a "hierarchy of immunodominance." Including such epitopes in vaccines, therefore, has merit in that such a vaccine may be made more effective if there is a degree of pre-existing immunity in the population, lowering the threshold for vaccine responses to be generated. Although further studies are required to unequivocally show that DENV-primed T cell responses can mediate protection against ZIKV in humans, mouse studies provide convincing evidence that T cells can mediate cross-protection.

T CELL RESPONSES IN MICE AND NON-HUMAN PRIMATES (NHP)

Mice are not natural hosts for flaviviruses as the murine type I IFN system provides a very effective defense, which thwarts viral dissemination and thus prevents them being useful models of severe disease phenotypes (74). As such, to establish a model of productive viral infection, which can be used to examine T cell function and test potential vaccine candidates, multiple strains of immunocompromised mice have been generated (74). These strains include mice deficient in either type I or type II or both IFN receptors, mice with STAT2 knocked-out, mice with mouse STAT2 replaced by human STAT2, and more nuanced models where type I IFN receptors are absent in specific cells or tissues (74). In combination with either mouse-adapted or human viral strains, this has established an infection model that closely, but not perfectly, mimics human disease. For DENV and ZIKV, strains that are commonly used either lack type I IFN receptor (IFNAR$^{-/-}$ and A129) or both type I and II receptors (AG129 mice) (74). Human Leucocyte Antigen (HLA)-transgenic mice have also been used to model CD8+ and/or CD4+ T cell responses in flavivirus infection. Work in HLA-transgenic mice show a broad epitope repertoire, whilst some HLA variants, such as HLA-B*0702, have been specifically studied due to a known association with high T cell response frequency and magnitude in humans, as well as decreased susceptibility to severe dengue disease (75, 76).

Control of primary DENV infection in mice requires CD8+ T cells to a greater extent than CD4+ cells. In IFNAR$^{-/-}$ mice, depletion of CD8+ cells was directly associated with increased viral burden in tissues that was not ameliorated by the transfer of serum or B cells (77). Protection in the study was mediated by increased cytotoxic activity in DENV-specific CD8+ T cells; activity which was further enhanced when mice received a peptide vaccination (77). However, challenge experiments in other mouse models (HEPG2-grafted SCID) find that DENV-specific CD8+ T cell responses were associated with reduced mortality, which suggests that T cells may contribute to disease severity in some instances, and prevent mortality in others (78). Crossing IFNAR$^{-/-}$ mice with HLA transgenic mice showed that protective CD8+ T cell responses tended to be polyfunctional and principally targeted non-structural proteins such as NS3 and NS5, similar to that in humans (75). Responses targeted against NS proteins have also shown their protective potential in homotypic secondary DENV infections where wildtype mice primed with a non-lethal DENV2 strain ACS46 were challenged with a lethal encephalitic homotypic strain JHA1 (79). In this model, protective immunity was reduced when both CD4+ and CD8+ T cells were depleted. In most challenge models CD4+ T cells play an accessory and non-essential role in which they contribute to viral clearance when induced by immunization (80).

Models of heterologous DENV infection also demonstrate the importance of T cells during the anti-DENV response. Collectively these studies show that CD8+ responses can protect against heterologous DENV challenge in non-lethal (81) and lethal models (82). CD8+ T cell responses contribute to protection during heterotypic reinfection, whereas homotypic reinfection can be contained by neutralizing antibodies against the infecting serotype (81), as is believed to be the case in humans. Comparison of specific and cross-reactive T cell responses in IFNAR$^{-/-}$ mice reveal that, despite their relatively low magnitude and avidity, cross-reactive CD8+ T cells from prior DENV exposure reduce viral load and exhibit a polyfunctional response in a manner comparable to that of serotype-specific cells (46). However, in a model of secondary DENV infection resulting in severe disease, cross-reactive CD4+ and CD8+ T cells were found to be pathogenic in wildtype mice infected with a non-mouse adapted DENV strain (83). In summary, the contribution of T cells to disease and protection in dengue mouse models is still not fully understood. The variability in current data are likely shaped by factors such as differences in mouse immune function, infection methods, strain difference, and experimental end-points.

Similar to observations for DENV, experiments in mice in which T cell responses are lacking, or on the other hand are enhanced (e.g., through peptide immunization or adoptive cell transfer), demonstrate a protective role for CD8+ T cells against ZIKV (84, 85). CD8+ T cell responses in primary ZIKV infection appear to be essential for immunity. In LysMCre^{+} IFNAR$^{fl/fl}$ (type I IFN receptor absent only in myeloid cells) and IFNAR$^{-/-}$ mice, ZIKV-immune CD8+ T cells protect against infection through cytotoxic, polyfunctional cellular responses (46, 86). However, in some instances, the resulting cytotoxicity may damage the host, in a tissue specific manner. For example, in IFNAR$^{-/-}$ mice ZIKV infection of astrocytes results in a breakdown of the blood-brain barrier, allowing an influx of CD8+ T cells into the central nervous system (CNS) where they mediate apoptosis of ZIKV-infected neurons, but also results in severe neuropathology (87). Similarly, CD8+ cellular infiltration was also found in the CNS following ZIKV infection in C57/BL6 neonatal mice who developed hind limb collapse, cerebellar degeneration (88) and in the case of adult wildtype C57BL/6 mice, encephalitis (89). Whilst the CD8+ T cell response may be detrimental in the CNS, in IFNAR$^{-/-}$ pregnant mice cross-reactive DENV-specific CD8+ cells are protective

against ZIKV infection of the fetus, including the fetal central nervous system, and are associated with increased fetal growth and viability (90). The CNS may represent a special case, where infection in the absence of CD8+ T cells results in severe viral pathology, and in the presence of CD8+ T cells in immunopathology, with little difference in survival in either case, as is seen in Japanese encephalitis (91).

Responses to sequential DENV-ZIKV infection (summarized in **Figure 6**) share similarities with secondary heterotypic DENV infection. Firstly, DENV-immune CD8+ T cell responses—either from prior exposure, peptide immunization or transfer of DENV-immune CD8+ T cells—can protect against ZIKV infection (90, 92, 93). This is an important result, which corroborates human studies that demonstrate prior DENV immunity can reduce the risk of Zika infection (16). Likewise, prior exposure to DENV provided IFNAR$^{-/-}$ mice protection against maternal and fetal ZIKV infection as compared with non-immune controls (90). As in heterotypic DENV, in mice as well as humans—the immunodominance pattern of the CD8+ T cell response to ZIKV infection was altered by prior DENV immunity and focused on conserved cross-reactive epitopes (93). ZIKV/DENV cross-reactive T cells performed comparably to ZIKV-specific T cells in viral load reduction in the serum and brain of knockout mice, which have key implications for ZIKV vaccine development.

Contrary to mice, non-human primates (NHP) are natural hosts for DENV and ZIKV. Several different primate models have been employed, including rhesus and cynomolgus macaques. In line with epidemiological observations in humans, experiments in NHP demonstrate that dengue immunity may curb Zika replication and potentially symptoms (94). Performing the order of viral challenge the other way around gave a similar result, where macaques with prior ZIKV immunity mounted strong humoral and cellular responses against DENV (95). Furthermore, this model found that a longer convalescence between ZIKV and DENV challenge was associated with higher and more durable antibody and T cell responses suggesting that ZIKV immune memory can contribute to protection against DENV (95).

DENGUE AND ZIKA VACCINES IN DEVELOPMENT

There are currently three dengue vaccines that have either been tested in, or are currently in, phase III trials. At the time of writing the only licensed dengue vaccine is Sanofi-Pasteur's Dengvaxia® (CYD-TDV), a live-attenuated, chimeric, tetravalent vaccine, in which the genes encoding prM and E of the four DENV serotypes have been inserted into YFV-17D (**Figure 7**) (96). The vaccine was developed to produce DENV neutralizing antibodies in human subjects, but protection against disease is incomplete despite high levels of seroconversion (97, 98), and one trial found no efficacy at all against DENV2 (99). During longer follow up of clinical trial participants, it was observed that young children in the vaccinated group had excess hospital admissions due to dengue compared with the placebo group (100). Dengvaxia is

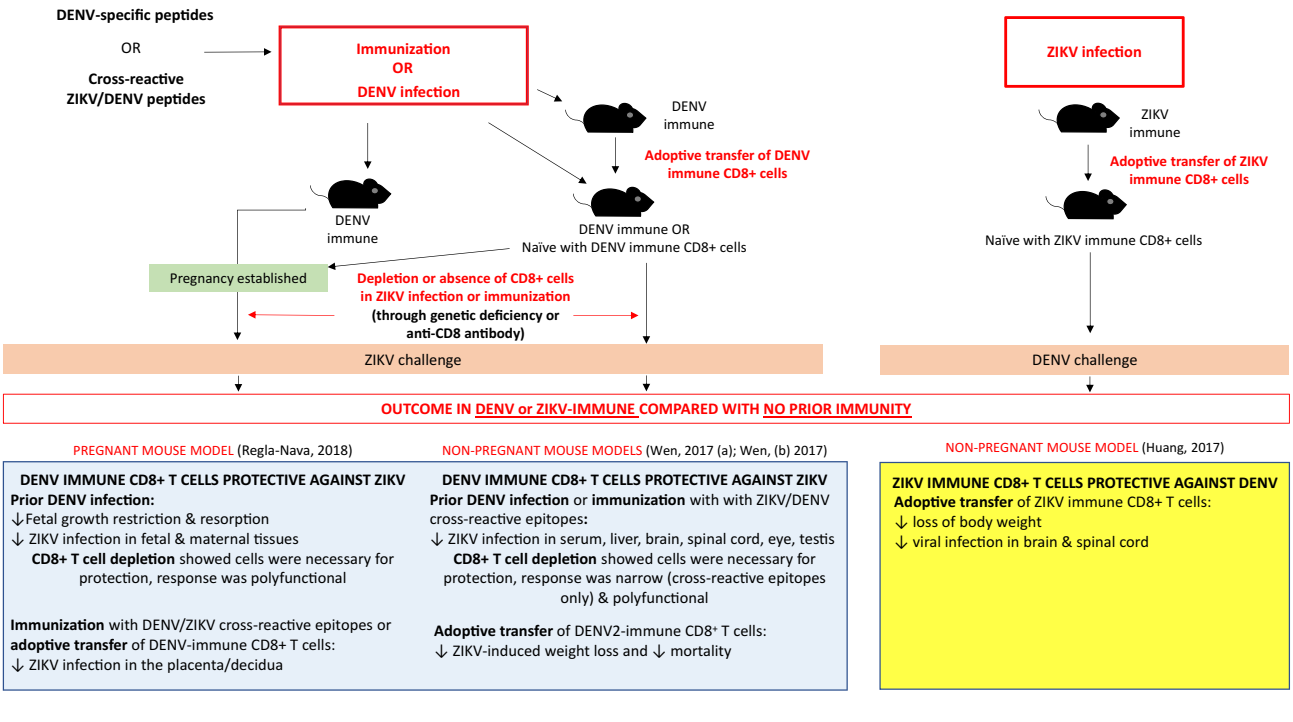

FIGURE 6 | The CD8+ T cell response and impact of prior flavivirus immunity in secondary flavivirus infection in mice. Summary of experiments (infections, immunizations, adoptive cell transfer, and depletions) demonstrating the protective effect of prior DENV immunity on ZIKV challenge and prior ZIKV immunity on DENV challenge in pregnant and non-pregnant mouse models.

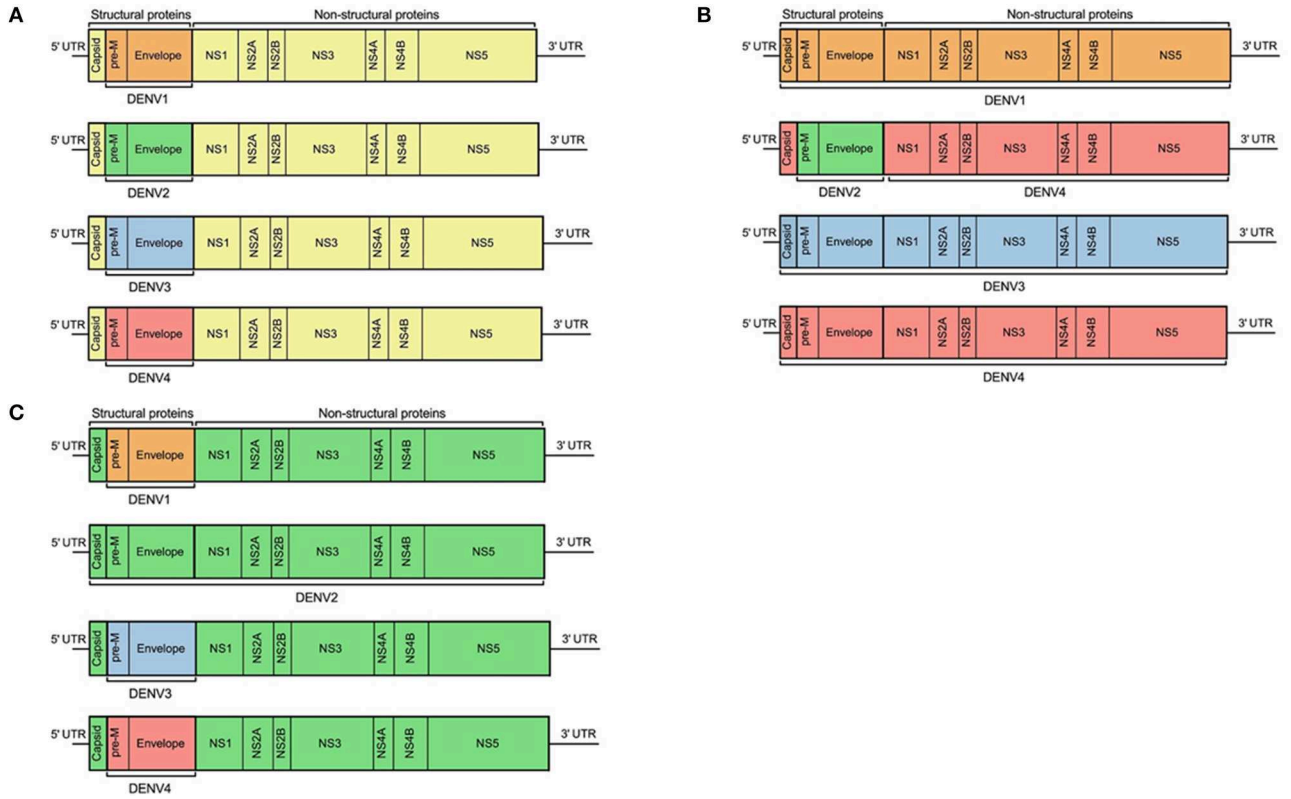

FIGURE 7 | Design schematic of three dengue vaccines. **(A)** represents Sanofi's licensed vaccine, Dengvaxia®, **(B)** represents the NIH live attenuated tetravalent dengue vaccine candidate (TV003), and **(C)** represents Takeda's tetravalent dengue vaccine (TDV). Each color represents regions from different flaviviruses: yellow, YFV; orange, DENV1; green, DENV2; blue, DENV3; and red, DENV4.

most effective in individuals who are DENV-seropositive at the time of immunization, while in seronegative subjects the vaccine is not protective and increases the risk of severe disease (101). One hypothesis for failure to protect DENV-naïve subjects may be that the T cell response generated was directed against the yellow fever NS proteins present within the vaccine, rather than DENV NS proteins (68, 102). These observations suggest the need to determine the optimal T cell antigens and incorporate them into new vaccines. The other two dengue vaccines in development are based on full length DENV. One [Takeda, tetravalent dengue vaccine (TAK-003)] uses an attenuated strain of DENV2, with the prM and E genes of the other serotypes inserted into it (103). Regardless of dengue serostatus, TAK-003 elicited strong humoral responses against all four DENV serotypes (104), and generated a polyfunctional CD8+ T cell response to the non-structural proteins of DENV2, which cross-reacted against DENV1, DENV3, and DENV4 (105). Preliminary findings of a phase III trial show TAK-003 to have ∼81% efficacy against symptomatic dengue (106), though protection against DENV3 was slightly lower. Therefore, a vaccine that induces better—and potentially more cross-reactive T cell responses also seems to have higher efficacy. However, these vaccines have not been compared directly and TAK-003 may still enhance disease in DENV naïve people, and could protect through a mechanism not involving CD8+ T cells. The other dengue vaccine in phase III trials is TV003, which is a tetravalent formulation of DENV1-3 with an additional chimeric DENV4 with the DENV2 prM and E genes inserted (**Figure 7**). Administration of TV003 induced a T cell response which predominantly targeted conserved epitopes of NS3 and NS5 (55) and was found to be immunogenic in subjects with prior flavivirus exposure (107). Field efficacy data are not yet available for TV003, but the vaccine protects against rash and viremia in a dengue human challenge model (108).

The majority of Zika vaccines are still in phase I/II trials (**Table 2**). These vaccines include DNA/mRNA, purified inactivated ZIKV, and recombinant virus-vectored vaccines; with most vaccine constructs containing the prM and E antigens as the main immunogen, as these proteins are targets for neutralizing antibodies (109). Three DNA-based vaccines (GLS-5700, VRC5283, and VRC5288) that have entered human testing show promising results, with one, VRC5283 advancing into phase II. VRC5283 and VRC5288 are both chimeric vaccines that utilize a JE virus (JEV) prM signal sequence followed by either the full-length E protein from wildtype ZIKV (VRC5283) or a modified E region in which the terminal 98 amino acids are exchanged with the analogous JEV sequence (VRC5288) (110). Both vaccines were shown to be immunogenic in mouse and NHP models (110) and a phase I trial found that vaccination with VRC5283

TABLE 2 | Current Zika vaccines in clinical trial.

Vaccine platform	Name	Immunogen	Adjuvant	Dose*	Sponsor	Phase I	Phase II
DNA	VRC5283	prM-E	None	4 mg IM (Phase I) and 4 mg vs. 8 mg IM (Phase II)	NIAID/VRC	NCT02996461	NTC03110770
	VRC5288	prM-E	None	4 mg IM	NIAID/VRC	NCT02840487	–
	GLS5700	prM-E	None	1,2 mg ID	GeneOne Life Science Inovio Pharmaceuticals	NCT02809443 NCT02887482	–
mRNA	mRNA-1325	prM-E	None	–	Moderna Therapeutics	NCT03010489	–
Inactivated Virus	ZPIV	virion	Alum	5 μg IM	NIAID/WRAIR/BIDMC	NCT02963909 NCT02952833 NCT02937233	–
	BBV121	virion	Alum	2.5 μg vs. 5 μg vs. 10 μg IM	Bharat Biotech	CTRI/2017/05/008539	–
	PIZV	virion	Alum	2 μg vs. 5 μg vs. 10 μg IM	Takeda	NCT03343626	–
	VLA1601	virion	Alum	2.5 μg vs. 5 μg vs. 10 μg IM	Valneva Emergent Biosolutions	NCT03425149	–
Viral Vectored	MV-ZIKV	prM-E	None	Low dose vs. High dose IM	Themis Biosciences	NCT02996890	–
	Ad26.ZIKV.001	M-E	None	–	Janssen Vaccines	NCT03356561	–

**IM, intramuscular; ID, intradermal.*

elicited neutralizing antibodies and cellular responses in all of the participants (111). A randomized placebo-controlled phase II study of VRC5283 is currently underway. A limitation of VRC5283 is whether the incorporation of a sequence from a different flavivirus (JEV prM) will provide protection against congenital Zika infection (111). To address this point, the vaccine was tested in a non-human primate pregnancy model. Vaccinated animals displayed fewer fetal losses and had reduced placental and fetal pathology; vaccine protection correlated with serum neutralizing antibody and antiviral T cell responses (112). However, these results may have to be considered cautiously as the model does not reflect early gestational exposure to ZIKV (112).

In addition to DNA-based vaccines, a ZIKV purified inactivated vaccine (ZPIV) based on the Puerto Rican strain PRVABC59 was found to be immunogenic at phase I with an acceptable safety profile (113). Vaccination with ZPIV elicited neutralizing antibody responses in the majority of tested individuals (113). One vaccine recipient from the trial produced cross-neutralizing antibodies to both ZIKV and DENV; responses which were linked to the individual's prior flavivirus exposure (114). These antibodies were shown to target the E domain I/III linker and could protect IFNAR$^{-/-}$ mice challenged either with ZIKV or DENV-2 (114). The durability of these protective responses were only evaluated up to 8 weeks post-vaccination (114) and longer follow up is still needed to fully demonstrate the longevity of these responses.

Enhanced immunogenicity associated with viral vectors (115) make these attractive candidates for Zika antigen delivery. Of the vaccines in clinical testing, two use viral vectors: one an attenuated measles strain (116) and the other a replication-incompetent human adenovirus serotype 26 (Ad26) (117). A single immunization with the Ad26 construct containing the Zika prM and E (Ad26.ZIKV.M-Env) antigens was able to elicit protective humoral and cellular responses in mice and NHP (117). Ad26.ZIKV.M-Env also protected IFNAR$^{-/-}$ dams and fetuses from ZIKV in a pregnancy model (118).

As discussed previously, all of vaccine constructs in clinical testing target structural proteins and may therefore not be optimal in their ability to induce cellular responses or boost pre-existing responses. Therefore, vaccines that include non-structural proteins should be considered, given that vaccination with non-structural proteins can induce cytotoxic T cell and polyfunctional helper T cell responses (119). Furthermore, given that non-structural proteins can elicit cross-reactive T cell responses, vaccines that incorporate these proteins may provide suitable priming for the development of memory responses during secondary flavivirus challenge (74, 94, 95, 109, 110, 115, 120–140).

CONCLUSION

There still remains a need to develop Zika vaccines, and also to better understand the cross-reactivity of flavivirus immune responses so that this can be harnessed for the emergent flaviviruses of the future. Dengue infection appears to be protective against ZIKV through mechanisms mediated by cross-reactive T cell responses against NS proteins. Given that in general, non-structural proteins elicit the most cross-reactive responses, and that these responses are likely to be protective against other viruses beyond dengue, there is a strong argument for including non-structural proteins to act as CD8 T cell antigens in novel flavivirus vaccines. As the majority of the population in flavivirus endemic areas are repeatedly exposed, vaccines

incorporating non-structural antigens may be more efficient and may require fewer doses due to the constant boosting of an existing T cell response. Finally, the degree of cross-reactivity seen with human CD8+ T cell responses to flaviviruses raises the possibility of engineering a single component containing T cell antigens that could be used in multiple vaccines, or even in a multivalent vaccine, provided suitable B cell antigens can be found.

AUTHOR CONTRIBUTIONS

KS contributed to the conceptualization of the article, wrote the first draft, and reviewed the manuscript. SL and LG contributed to conceptualization of the article and wrote the first draft of sections of the article. AG contributed viral homology measures and also to conceptualization of the article. DW and LT contributed to conceptualization of the article, contributed to early drafts, and reviewed the article. All authors reviewed the final draft.

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