REVIEW

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Future MICROBIOLOGY

Elucidating the role of T cells in protection against and pathogenesis of dengue virus infections

Anuja Mathew*[,1], Elizabeth Townsley[1] & Francis A Ennis[1]

ABSTRACT: Dengue viruses (DENV) cause significantly more human disease than any other arbovirus, with hundreds of thousands of cases leading to severe disease in thousands annually. Antibodies and T cells induced by primary infection with DENV have the potential for both positive (protective) and negative (pathological) effects during subsequent DENV infections. In this review, we summarize studies that have examined T-cell responses in humans following natural infection and vaccination. We discuss studies that support a role for T cells in protection against and those that support a role for the involvement of T cells in the pathogenesis of severe disease. The mechanisms that lead to severe disease are complex, and T-cell responses are an important component that needs to be further evaluated for the development of safe and efficacious DENV vaccines.

KEYWORDS
• dengue
• immune response
• immunopathology
• nonstructural proteins
• primary infection
• secondary infection
• T lymphocyte • vaccine

Dengue viruses (DENV) have four closely-related serotypes that co-circulate in endemic regions. Infection results in a spectrum of clinical manifestations. Plasma leakage, a hallmark of severe DENV disease, occurs after several days of infection and coincides with viral clearance. However, pathological studies indicate that vascular endothelial cells are relatively structurally intact in patients with severe disease [1,2]. Since these patients typically experience a rapid recovery, it is thought that plasma leakage occurs due to endothelial cell malfunction, rather than lysis of infected endothelial cells [2]. This, together with evidence that increased disease severity is associated with secondary heterologous DENV infections, suggests a role for pre-existing adaptive immune responses in contributing to an immunopathologically mediated clinical outcome in severe dengue infections [3].

The pathogenesis of DENV disease is multifactorial

The primary human target cells for DENV infection *in vitro* include monocytes, macrophages and mature dendritic cells **(Figure 1)**. The precise targets for DENV infection *in vivo* are less clear and have been challenging to identify [4], but include alveolar macrophages, phagocytes and other hematopoietic cells [5]. DENV have also been shown to replicate in B cells, a central source of antibodies [6,7]; however, some recent data suggest that B cells are not natural targets for DENV infection [8]. Antibodies to DENV can mediate a number of activities *in vitro* [9]. Some antibodies are able to neutralize the virus but enhance virus uptake at higher dilutions, while other antibodies do not neutralize the virus but are also able to bind to the virus and Fcγ I and II receptors, and mediate more efficient entry into the host cell [10,11]. These non-neutralizing antibodies can result in higher production of infectious particles through a process known as antibody-dependent enhancement (ADE) [12]. DENV-specific antibodies of the appropriate subclasses bound to dengue antigens on the infected cell membrane can bind to complement proteins and promote complement-dependent lysis (CDL) of infected cells and

[1]Division of Infectious Diseases & Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01655, USA
*Author for correspondence: Tel.: +1 508 856 4182; Fax: +1 508 856 4890; anuja.mathew@umassmed.edu

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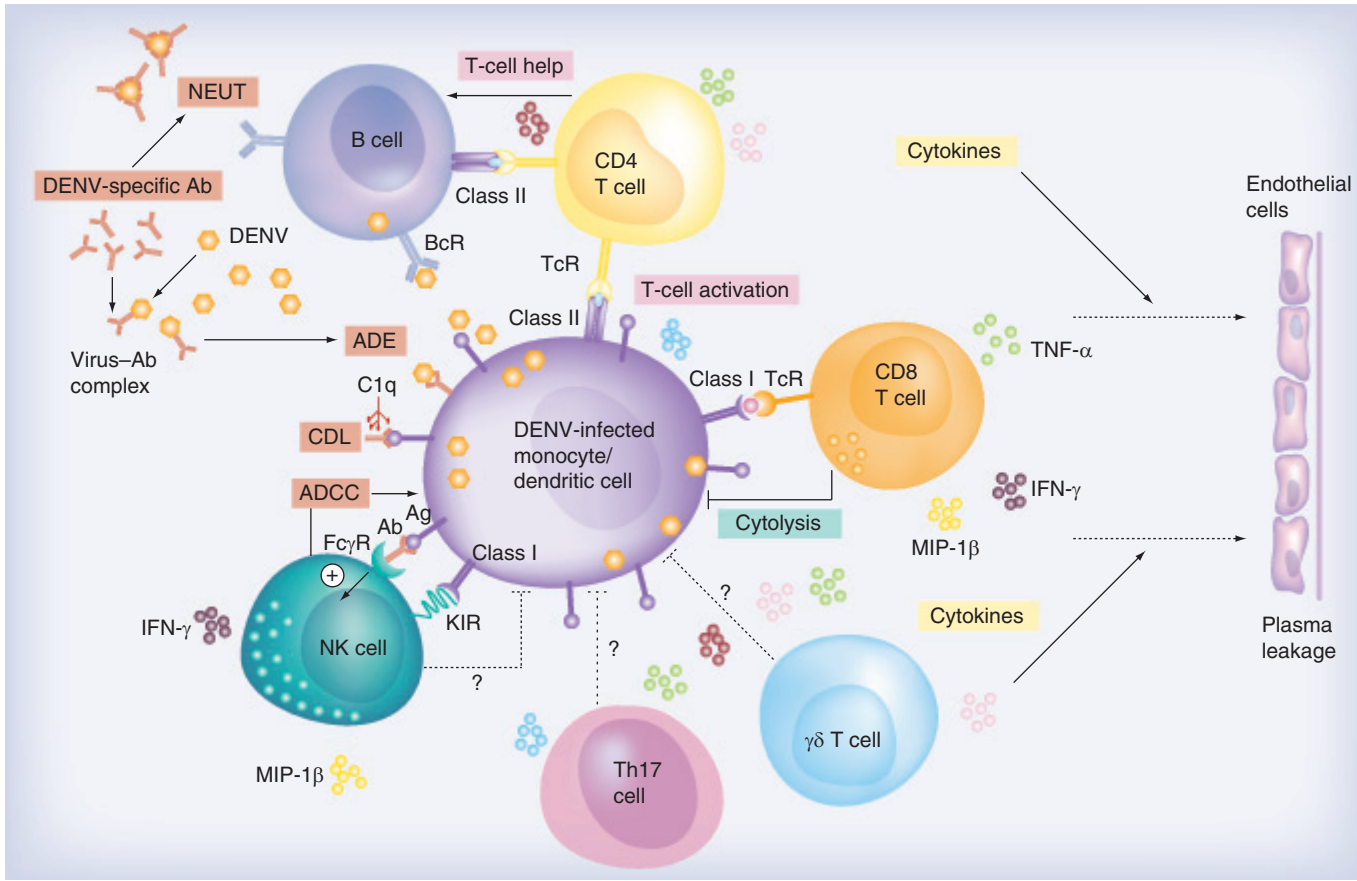

Figure 1. Interactions between multiple components of the immune system during dengue virus infection. The primary targets of DENV replication are monocytes, macrophages and dendritic cells, but B cells may also be infected with DENV. Antibodies secreted by B cells can mediate a wide range of functions including neutralization, ADE, ADCC and CDL. Virus-infected target cells secrete cytokines and chemokines and attract T cells. Viral peptides are presented on MHC class I and class II presentation pathways to $CD8^+$ and $CD4^+$ T cells, respectively. $CD4^+$ T cells predominantly produce cytokines but are capable of lysing virus-infected cells, and $CD8^+$ T cells lyse virus-infected cells and produce cytokines. The role of γδ T cells, Th17 and NK cell participation in the antiviral immune defense mechanisms requires further investigation. Question marks in the figure indicate that the evidence is not clear. The result of the cascade of immune activation leads to endothelial cell permeability and plasma leakage.
Ab: Antibody; ADCC: Antibody-dependent cell-mediated cytotoxicity; ADE: Antibody-dependent enhancement; Ag: Dengue antigen; BcR: B-cell receptor; C1q: Subcomponent of complement pathway; CDL: Complement-dependent lysis; DENV: Dengue viruses; FcγR: Fc gamma receptor; KIR: Killer-like immunoglobulin receptor; NEUT: Neutralization; NK: Natural killer; TcR: T-cell receptor; Th17: T helper 17.

contribute to antibody-dependent cellular cytotoxicity (ADCC) of infected cells [13,14]. Much less is known about the role of γδ T cells, Th17 and NK cells in anti-DENV defense mechanisms **(Figure 1)**. HLA restricted $CD4^+$ and $CD8^+$ T cells are activated upon viral infection and several epitopes have been identified in humans after natural infection. T-cell-produced cytokines have the ability to influence vascular permeability leading to plasma leakage, a hallmark of severe disease [15–17]. The outcome of DENV infection likely depends on the balance between favorable and unfavorable immune responses, host genetics, viral factors, the sequence of DENV infections and factors specific to the individual patient [3,18]. In this review, we will discuss efforts to evaluate T-cell responses to DENV infection in humans and mice and assess the contribution of T lymphocytes to protection against or pathogenesis of severe DENV disease.

T-cell responses to DENV after natural infection

In order to begin to understand the contribution of DENV-specific T cells in protection or enhanced immunopathology, significant effort has been spent over the last two decades to define T-cell epitopes to DENV **(Table 1)**. $CD4^+$ and

$CD8^+$ T-cell epitopes have been identified on multiple proteins of DENV [19–38]. MHC class I and II restricted minimal T-cell epitopes were characterized in a subset of T cells. While T-cell epitopes have been identified on the structural proteins, the vast majority of T-cell epitopes have been found on nonstructural proteins. Our early studies, using samples from donors who received experimental live-attenuated monovalent DENV vaccines and a smaller set of samples from donors with natural infection in Thailand, demonstrated that the NS3 protein is an immunodominant protein with multiple epitopes throughout the protein [21–24,39–43]. More recently, three studies have used overlapping peptide pools or strong binding peptides to the most common HLA alleles to identify several additional T-cell epitopes in different populations around the world. Duangchinda *et al.* set out to study T-cell responses across the entire DENV proteome in a cohort of DENV-infected children from Khon Kaen and Songkhla hospitals in Thailand [29]. While T-cell responses to NS3 were dominant, responses to multiple proteins were observed in most infected individuals. Rivino *et al.* assessed $CD4^+$ and $CD8^+$ T cell reactivity using an overlapping 15mer peptide library spanning the DENV 2 proteome using the peripheral blood mononuclear cells(PBMCs) of adult patients from Singapore experiencing secondary DENV infection [26]. They observed that $CD8^+$ T-cell epitopes preferentially targeted nonstructural proteins (NS3 and NS5), but $CD4^+$ T-cell epitopes were skewed toward recognition of viral components that were also targeted by B lymphocytes (envelope, capsid and NS1). Weiskopf *et al.* performed a comprehensive analysis of $CD8^+$ T-cell responses in the general population from a DENV hyperendemic area in Sri Lanka, measuring *ex vivo* IFNγ responses. NS3, NS4B and NS5 were the most vigorously and frequently recognized proteins and accounted for more than two-thirds of the total T-cell response [27].

Studies on immune responses to a related flavivirus, yellow fever virus (YFV), also found many T-cell epitopes on NS3 [44]. In the murine system, NS3 is the major target for DENV-specific H-2k cytotoxic lymphocytes (CTL) [45], as well as Murray Valley encephalitis (MVE), Kunjun and West Nile virus-immune CTLs [46–48].

NS3 is a dominant target of CD8 T-cell epitopes

The predominant protein recognized by $CD8^+$ CTLs in the PBMC of DENV immune individuals from different parts of the world is the nonstructural protein NS3. Although there is a predominance of recognition of determinants on the NS3 protein, there is no single immunodominant epitope. NS3 is the second largest viral protein and is one of the most highly conserved proteins among flaviviruses [49]. The crossreactive nature of CTLs against NS3 may be due to the high level of amino acid conservation among the NS3 proteins of the different serotypes. NS3 has both a protease (N terminus) and a nucleotide triphosphatase helicase (C terminus) activity. Unlike other viruses where different antigens are produced early and late during the virus lifecycle, the DENV genome encodes for a single polyprotein that gets co- and post-translationally cleaved to the individual proteins by host- and virus-specific proteases that include NS3 [50,51]. This processing means that the individual proteins are produced in equimolar concentrations. The structural proteins (C, prM and E), NSI and the N terminus of NS2a are translocated into the lumen of the endoplasmic reticulum via a series of signal and stop-transfer sequences. On the other hand, the NS3 and NS5 proteins, which are the largest proteins encoded by the DENV genome, have a cytoplasmic localization. Although speculative, possible differences in intracellular targeting and stability may lead to preferential processing and presentation of NS3 peptides.

HLA associations with disease

A number of studies have linked specific HLA class I, II, and III alleles with different DENV disease manifestations. *HLA-A*0203* correlated with mild DENV disease (DF) as compared with *HLA-A*0207*, which correlated with severe disease (DHF) [52]. This report and others identified additional HLA class I and II correlations with likelihood for decreased (*HLA-A29, A31, A33, B13, B14, B44, B52, B62, B76, B77, DRB1*04, DRB1*07, DRB1*09*) or increased (*HLA-A1, A24, A31, B15, B46, B51, DQ1*) risk for severe DENV [53,54].

Separate studies reported that a single nucleotide polymorphism (SNP) at position 308 in the gene for tumor necrosis factor (TNF-α) is associated with DHF, and particular SNP alleles of the gene for IL-10 are associated with low levels of IL-10 protein production, and correlated with DHF [55,56]. These groups suggested that high TNF-α/low IL-10 production helped mediate severe DENV disease. Another study reported

Table 1. T-cell epitopes recognized by virus-specific T cells.

Protein	Amino acids[†]	Sequence[‡]	CD4/CD8	MHC[§]	Ref.
C	22–31	RVSTVQQLTK	CD8	A03/11	[31]
	40–60	LFMALVAFLRFLTIP	CD4		[26]
	71–85	TIKSKAINVLRGFR	CD4		[26]
	47–55	VLAFITFLR	CD4	DPw4	[19]
	62–81	TAGILKRWGTIKKSKAINVL	CD4		[28]
	83–92	GFRKEIGRML	CD4	DR1,DPw4	[19,28]
	107–115	CLIPTAMAF	CD8	B15	[27]
	107–115	MLIPTAMAF	CD8	B35	[27]
prM	41–60	LGELCEDTITYKCPLLRQNE	CD4		[28]
	121–135	QRIETWILRHPGFTM	CD4		[26]
	133–141	FTILAFLAH	CD8	B35	[18]
E	46–55	LKTEVTNPAV			[33]
	41–55	LDFELIKTEAKQPAT	CD4		[26]
	206–220	WLVHRQWFLDLPLPW	CD4		[26]
	236–250	TLVTFKNPHAKKQDV	CD4		[26]
	241–255	KNPHAKKQDVVVLGS	CD4		[26]
	246–160	KKQDVVVLGSQEGAM	CD4		[26]
	211–219	FFDLPLPWT	CD8	A02	[73]
	297–306	MSYSMCTGKF	CD8	B35	[27]
	340–359	RDVNKEKVVGRVISSTPLAE	CD4		[33]
	414–422	ILGDTAWDF	CD8	B07	[28]
NS1	26–34	HTWTYQEF	CD8	B57	[61]
	88–99	IMTGDIKGIMQA	CD4		[33]
	111–125	LKYSWKTWGKAKMLS	CD4		[26]
	326–350	EDGCWYGMEIRPLKEKEENLNSLV	CD4		[26]
	206–220	LNDTWKIEKASFIEV	CD4		[26]
NS2a	99–118	RENLLLGVGLAMATTLQLPE	CD4		[33]
	108–127	AMTTTLSIPHDLMELIDGIS	CD4		[33]
	109–133	SLVASVELPNSLEELGDGLAMGIMI	CD4		[33]
	135–148	IVTQFDNTQVGTLA	CD4		[33]
	184–203	SSQQKTDWIPLALTIKGLNP	CD4		[33]
	196–216	GSLGCKPLTMFLIAENKIWG	CD4		[33]
	198–206	ATGPILTLW	CD8	B58	[27]
NS2b	52–60	ELERAADVK	CD8	A03/11	[31]
	63–82	DQAEISGSSPILSITISEDG			[33]
	83–102	TMRIKDDETENILTVLLKTA			[33]
	97–106	ILIRTGLLVI	CD8	A0201/24	[31]
NS3	25–32	RIKQKGIL	CD8	B08	[31]
	64–74	RIEPSWADVK	CD8	A03/11	[31]
	71–79	SVKKDLISY	CD8	B62	[37]
	112–120	AIKRGLRTL	CD8	A02/24	[31]
	130–144	GTSGSPIIDKK	CD8	A11.1	[25]
	146–154	VIGLYGNGV	CD4	DR15	[22]
	157–173	TSGTYVSAIAQAKASQE			[33]
	176–184	NPEIEDDIF	CD8	B35	[27]

[†]Sequence positions vary slightly between strains.
[‡]Sequence as reported by the cited reference. These sequences do not necessarily reflect the minimal epitope. As sequences vary between serotypes and strains these epitopes may not represent the sequence found in prevalent circulating strains.
[§]HLA restriction was not confirmed in all studies and some were based on peptide-binding predictions.
Data taken from [18].

Table 1. T-cell epitopes recognized by virus-specific T cells (cont.).

Protein	Amino acids[†]	Sequence[‡]	CD4/CD8	MHC[§]	Ref.
NS3 (cont.)	186–200	RKLTIMDLHPGSGKT	CD4	ND	[38]
	194–203	HPGAGKTKRY	CD8	B35	[27]
	202–211	RKYLPAIVRE	CD4	DR15	[36]
	222–230	APTRVVAAE	CD8	B07	[24]
	224–234	TRVVAAEMEEA	CD4	DR15	[41]
	235–243	AMKGLPIRY	CD8	B62	[37]
	241–249	IRYQTTATK	CD4	DR15	[36]
	255–264	EIVDLMCHAT	CD4	DPw2	[34]
	276–290	PNYNLIIMDEAHFTD	CD4		[26]
	291–300	DPASIAARGY	CD8	B35	[27]
	352–362	WITDFVGKTVW	CD4	DR15	[36]
	351–365	VTDFKGKTVWFVPSI	CD4		[26]
	422–431	RVIDPRRCMK	CD8	A03/11	[31]
	500–508	TPEGIIPTL	CD8	B35	[23]
	521–530	GEFRLRGEQR	CD8	B40	[27]
	526–540	LRGEARKTFVELMRR			[32]
	528–537	GEARKTFVEL	CD8	B40	[27]
	555–564	INYADRRWCF	CD8	A24	[28]
	584–598	KEGERKKLRPRWLDA	CD4	ND	[38]
	606–614	MALKDFKEF	CD8	B35	[27]
NS4a	2–21	LTLNLITEMGRLPTFMTQKA			[33]
	56–64	LLLGLMILL	CD8	A2	[73]
	55–64	LLLLTLLATV	CD8	A02/24	[31]
	6–13	LEKTKKDL	CD8	B08	[31]
NS4b	23–32	TETTILDVDL	CD8	B53	[27]
	40–48	TLYAVATTI	CD8	A02	[35]
	49–58	TPMLRHTIEN	CD8	B07	[27]
	69–77	IANQATVLM	CD8	B35	[27]
	92–100	VPLLAIGCY	CD8	B35	[27]
	111–119	VLLLVTHYA	CD8	A2	[73]
	119–128	AIIGPGLQAK	CD8	A03/11	[31]
	181–189	LLLMRTSWA	CD8	A02	[73]
	198–206	ATGPILTLW	CD8	B58	[27]
	15–27	SRLNALGKSEFQI	CD4		[33]
NS5	182–190	VLNPYMPSV	CD8	A02/24	[31]
	263–282	HVNAEPETPNMDVIGERIKR	CD4		[33]
	291–299	WHYDQDHPY	CD8	B35	[27]
	291–310	WHYDEDNPYKTWAYHGSYEV			[32]
	301–315	KTWAYHGSYETKQTG	CD4		[26]
	329–337	KPWDVIPMVT	CD8	B55	[32]
	343–351	DTTPFGQQR	CD8	A68	[27]
	373–382	VMGITAEWLW	CD8	B53	[27]
	375–383	KITAEWLWK	CD8	A03/11	[31]
	389–398	KPRICTREEF	CD8	B07	[27]
	393–402	TPRMCTREEF	CD8	B07/35	[27]
	563–571	KLAEAIFKL	CD8	A02/24	[31]

[†]Sequence positions vary slightly between strains.
[‡]Sequence as reported by the cited reference. These sequences do not necessarily reflect the minimal epitope. As sequences vary between serotypes and strains these epitopes may not represent the sequence found in prevalent circulating strains.
[§]HLA restriction was not confirmed in all studies and some were based on peptide-binding predictions.
Data taken from [18].

a strong genetic linkage between the SNP allele -238A in the TNF-α gene and the lymphotoxin-α (LTA)-3 haplotype, which is associated with high TNF-α and LTA-α production during acute viremia in DENV-infected patients [57,58]. Patients with *TNF-238A* and *LTA-3* were at greater risk for developing DHF compared with DF, and DHF patients with these SNP profiles were nearly all shown to have *HLA-B48* and/or *HLA-B57* [58], in the Thai population [52]. Most studies have been performed in small cohorts of individuals. Such analyses underscore the need to assess extended haplotypes in larger prospective genetic studies of DENV infections from multiple ethnic populations in order to better understand potential mechanisms for the immunopathology observed in DENV infection.

T-cell immune responses in primary versus secondary DENV infections

Individuals who have been infected with a DENV serotype for the first time (primary DENV infection) have long-term protective immunity against re-infection with the same serotype. Individuals can be infected for a second time with another DENV serotype and are experiencing a secondary DENV infection. In endemic countries, most individuals have been exposed very early in life to DENV and it is challenging to obtain samples to evaluate immune responses to primary infections. Memory T cells that are re-activated during a second infection may not have optimal avidity for the corresponding epitopes of the new infecting virus because of sequence diversity between the DENV serotypes [3,59]. Therefore, it would be reasonable to predict skewed secondary T-cell responses since peptide variants found in different DENV serotypes could act as altered peptide ligands impacting the responsiveness of the T cell. Higher frequencies of epitope-specific T cells in donors with natural secondary infections might be expected since memory T cells from the first infection would be more readily activated compared with naïve T cells. We have sought to assess the frequencies of epitope-specific T cells during and after primary and secondary DENV infection to validate these predictions. For A11-$NS3_{133–142}$-specific T cells, we found similar frequencies of serotype-cross-reactive T cells *ex vivo* in naturally infected patients with primary and secondary DENV infections [60]. For B57-$NS1_{26–34}$-specific T cells, since the sequence in a secondary DENV infection was identical to the sequence from an earlier primary DENV infection, we predicted that PBMC from donors with secondary infection would have particularly strong responses to the B57-$NS1_{26–34}$ epitope [61]. However, our findings in all but one individual with a secondary infection (n = 8/9) indicated that frequencies were similar to individuals with primary infections (n = 2), to frequencies of A11-$NS3_{133–142}$ and A2-$E_{213–221}$-specific T cells in the same subjects, and to the frequencies of A11-$NS3_{133–142}$ T cells reported elsewhere [25,60]. In our hands, the magnitude of A11 and B57 epitope-specific T cells were not significantly different between primary and secondary infections. Owing to the limitations of sample size we did not extensively test effector functions of T cells in PBMC from these children. It is possible that the function of T cells might differ in individuals with primary versus secondary infection. Furthermore, during acute infection, trafficking of antigen-specific T cells to tissues may not allow an accurate assessment in the peripheral blood. Ideally, frequencies and functional responses need to be assessed against multiple epitopes in large prospective cohort studies to conclusively demonstrate that T-cell responses are skewed during a second infection with DENV. It has also been challenging to assess the extent to which the order of the infecting serotype affects subsequent T-cell responses.

Studies that have examined a role for DENV-specific T cells with pathogenesis of DENV infections

T-cell associated cytokines have been found to be elevated in the sera of patients with mild disease, and some were increased to higher levels in severe DENV disease [18,57,62–67]. While the precise cellular sources of various cytokines and chemokines found in the circulation of dengue patients are currently unknown, several studies have found that DENV-specific T cells stimulated with homologous and heterologous variant peptides *in vitro* also secrete these cytokines [32,38,68]. To have a better understanding of how T-cell responses relate to the onset of clinical symptoms, recent studies have used peptide MHC tetramer technology to investigate the kinetics of expansion and activation of DENV-specific T cells during acute infection and convalescence. The magnitude of A11-$NS3_{133–142}$-specific T cell expansion did not correlate with disease severity in a study by Friberg *et al.* [60]. We compared PBMC from patients with mild and severe disease (DF versus DHF) as well as

other clinical measures of disease severity such as pleural effusion index, hemoconcentration or platelet counts. A similar lack of association between the frequency of A11-NS3$_{133–142}$-specific T cells and disease severity was reported in two studies in Vietnam [69,70]. A strength of these studies is the information on clinical profile, viral isolation and HLA typing in individuals with primary and secondary DENV infection [60,69,70]. Furthermore, samples were obtained during and up to 3 years following the critical phase of illness with mild and severe disease from all four serotypes [60]. Simmons *et al.* found T-cell IFN-γ ELISPOT responses were weakly correlated with the extent of hemoconcentration in individual patients, but not with overall disease severity [28]. Other studies had reported higher frequencies of DENV-specific T cells in patients with DHF, but these associations were found at 2 weeks [29,71] or 6 months [72] post-infection **(Table 2)**. Differences in timing or differences in infection history (e.g., serotype of primary and secondary infection) may explain the differences in results between these studies. However, the lack of a correlation with disease severity, and the timing of peak tetramer-positive T-cell frequencies in early convalescence rather than at the time of plasma leakage, suggest that the frequency of A11-NS3$_{133–142}$ tetramer-positive T cells may not be the principal determinant of disease. On the other hand, the number of samples tested during capillary leakage is very small and it is likely that highly activated T cells may be localized at the sites of infection, lost in processing or by apoptosis, and that the later memory T-cell progeny reflects the importance of those T cells in the acute phase. T-cell responses to other DENV epitopes are also likely to contribute to disease, as is suggested by our limited data on B7-NS3$_{222–230}$-specific T cells in *HLA-A11*$^{-}$*B7*$^{+}$ subjects during the acute phase. Alternatively, characteristics of the DENV-specific T-cell response other than the quantity detected in samples of acute PBMC, for example effector responses [73], may also be more important.

Phenotypic markers on T cells have been used to characterize effector and memory T cells in acute human viral infections [74,75]. We have used CD38, CD69 and CD71 to phenotype DENV-specific T cells in our clinical cohort. There were no significant correlations between the expression of CD38 (a marker of activation) and disease severity on A11-NS3$_{133–142}$-specific T cells. This stands in contrast to the results of other studies focused on CD69 (an early activation marker) on total CD8^{+} T cells [62,70]. We recently assessed CD71 (transferrin receptor) expression on HLA B57-NS1$_{26–34}$, on A11-NS3$_{133–142}$ and A2-E$_{213–221}$-specific CD8^{+} T cells over the course of DENV infection. We observed upregulation of CD71 predominantly on DENV-specific CD8^{+} T cells and not on total CD8^{+} T cells [61]. However, CD69 and CD38 expression was similar between epitope-specific T cells and total CD8^{+} T cells during acute DENV infection. The finding of a novel and distinct phenotype (CD71^{+}) in these epitope-specific T cells suggests differential activation that merits further investigation. Current data are limited and have been obtained from a small number of subjects; however, they suggest that the frequency of select epitope-specific T cells may not be the principal determinant in the association between T lymphocyte responses and disease.

Studies that have examined a role for DENV-specific T cells with protection against DENV infections

• Human studies

A number of published T-cell studies have focused on the role of T cells in the pathogenesis of DENV infections. Very few studies have examined the role of T cells in protection from disease in humans. Collection of PBMC from individuals prior to infection in prospective clinical studies is key to measuring correlations between T-cell function in pre-infection PBMC and disease outcome. Therefore, whether T cells contribute to protection against DENV infection in humans remains unknown. Several prospective studies have assessed these associations. Mangada *et al.* compared the T-cell responses of the pre-secondary infection PBMC of patients who were hospitalized during their subsequent DENV infection to those of patients who were not hospitalized [76]. IFN-γ production in response to the infecting serotypes was significantly more common among patients who were not hospitalized. In a study performed by Hatch *et al.*, the level of T-cell activity in pre-illness PBMC was compared between subjects who subsequently developed a subclinical secondary DENV infection or had a symptomatic secondary infection [77]. They found higher frequencies of cytokine-producing (TNF-α, IFN-γ and IL-2) CD4^{+} T cells in patients who did not develop symptomatic infection. Gunther *et al.* studied cellular immune responses in recipients

Table 2. Studies that examined a role for T cells in dengue virus pathogenesis.

Study (year)	Target	Correlates	Time point	Ref.
Studies that support a role for T cells				
Green *et al.* (1999)	Total $CD8^+$	$CD69^+$ vs disease outcome	Acute	[62]
Zivna *et al.* (2002)	B7-$NS3_{222–231}$	IFN-γ vs disease outcome	≥6 months	[72]
Mongkolsapaya *et al.* (2003)	A11-$NS3_{133–142}$	TET^+ vs disease outcome	2 weeks	[25]
Simmons *et al.* (2005)	C,E,M,NS3 peptides	IFN-γ vs hemoconcentration	2 weeks	[28]
Mongkolsapaya *et al.* (2006)	A24-$NS3_{556–564}$	TET^+ vs disease outcome	2 weeks	[71]
Chau *et al.* (2008)	Total $CD8^+$	$CD69^+$ vs disease outcome	Acute	[70]
Duangchinda *et al.* (2010)	All prot., A11-$NS3_{133–142}$	TNF-α, IFN-γ vs disease outcome	2 weeks	[29]
Dung *et al.* (2010)	Total $CD8^+$	$CD38^+HLA\text{-}DR^+$, $HLA\text{-}DR^+Ki\text{-}67^+$, $CD38^+Ki\text{-}67^+$	Acute	[69]
Malagive *et al.* (2012)	NS3 specific T cells	Serum cytokines vs disease outcome	Acute	[33]
Mangada *et al.* (2002)	Total CD4	TNF-α, IFN-γ to those who subsequently were hospitalized	Pre-secondary infection PBMC	[76]
Hatch *et al.* (2011)	Total $CD4^+$ and $CD8^+$	TNF-α, IFN-γ to those who subsequently were hospitalized	Pre-secondary infection PBMC	[77]
Studies that do not support a role for T cells				
Simmons *et al.* (2005)	C,E,M,NS3 peptides	IFN-γ vs disease outcome	2 weeks	[28]
Chau *et al.* (2008)	A11-$NS3_{133–142}$	TET^+ vs time	Acute	[70]
Dung *et al.* (2010)	A11-$NS3_{133–142}$	TET^+ vs time	Acute	[69]
Friberg *et al.* (2011)	A11-$NS3_{133–142}$	TET^+ vs disease outcome	Acute and 2 weeks	[60]

PBMC: Peripheral blood mononuclear cells.

who received a candidate tetravalent vaccine and were subsequently challenged with infectious DENV. They found that *in vitro* IFN-γ responses mediated by DENV-specific T cells in the peripheral blood were associated with protection against fever or viremia [78]. Lindow *et al.* found a trend of more multifunctional $CD4^+$ T cells in nonviremic vaccinees relative to viremic vaccinees after administration of a low-dose DENV-1 vaccine [79]. These observations suggest that multifunctional $CD4^+$ T cells may be indicators of individuals who are more able to control DENV infection and therefore may have less severe clinical disease.

• Murine studies

DENV does not cause severe infections in immunocompetent mice. Since DENV does not block IFN signaling in murine cells, *type 1 IFN* knockout mice are highly susceptible to infection with laboratory strains of DENV, with paralysis commonly seen in infected mice [80]. *Type 1 IFN* knockout mice expressing HLA transgenes have been utilized to identify T-cell epitopes to dengue [81]. Under certain experimental conditions, when mice lacking type 1 IFNs are infected with select passaged strains of DENV, they have increased vascular leakage and TNF-α levels showing some characteristics of human DENV disease [82]. *IFN*α/β receptor knockout mice infected with a mouse-adapted DENV strain S221 have higher viral loads upon depletion of $CD8^+$ T cells [83], but not after depletion of $CD4^+$ T cells [84]. Additionally, immunization of the mice with $CD8^+$ or $CD4^+$ epitopes enhanced viral clearance upon subsequent DENV challenge, supporting a protective role for T cells against DENV infections.

$CD8^+$ T-cell depletion also negated the vaccine immunized protection against a very high dose challenge with a lethal strain of DENV-infected BALB/c mice [85,86]. However, a different mouse model (HepG2-grafted SCID) suggested that DENV-specific $CD8^+$ T cells have both protective and pathogenic roles [87]. Specifically, mice inoculated with DENV-specific $CD8^+$ T cells

and subsequently challenged with a lethal dose of DENV showed slightly reduced mortality compared with uninoculated mice (80% versus 100%); however, the mice that died did so much more quickly (day 12.8 versus day 17.4). The studies above examined mechanisms of protective immunity in mouse models during primary DENV infection or upon homologous virus re-challenge. A different study used adoptive transfer experiments to demonstrate that serotype-cross-reactive antibody was more protective against homologous and heterologous challenge with DENV-2 compared with cross-reactive cell-mediated immune responses [88].

Improvement of existing animal models

There are a number of animal models each with significant limitations [89,90]. The lack of an animal model for DHF severely limits the use of animal models to study the pathogenesis of disease. The generation of novel humanized mouse and non-human primate models presents opportunities to overcome deficiencies of other mouse models [91]. A major advantage of humanized models is the presence of human cells in a physiological setting. However, both humoral and cellular responses in humanized models need further improvement to match responses detected in humans and mechanistic studies may be challenging to perform in these models [92,93]. Infection of non-human primates results in viral replication accompanied by neutralizing antibodies and T-cell responses; however, there is only limited evidence of disease or hematologic abnormalities [94–96]. In addition, large-scale vaccine testing in non-human primate models involves significant cost and accessibility. Animal models need to be improved to reproduce the immunological response in humans in order to be reliably used to test vaccine strategies.

Induction of optimal T-cell responses

The specific definition of 'optimal' or 'sub-optimal' T-cell immune responses is unknown and there is no single metric to identify a protective T-cell response. The generation of multifunctional T cells with high-quality responses may be protective, while the generation of lesser-quality T cells is considered suboptimal [97]. Cross-reactive DENV-specific T cells have quantitative and qualitative differences in degranulation and cytokine responses to variant peptides [71,73]. Peptide variants that differ even by a single amino acid are able to elicit strikingly different cytokine and cytolytic responses in T-cell lines [68]. We believe individuals experiencing secondary DENV infections have the potential to generate both 'optimal' and 'suboptimal' T-cell responses to multiple epitopes **(Figure 2)**. If 'optimal' T-cell responses outweigh 'suboptimal' responses and these responses occur in the context of other 'protective' viral, immunological and genetic factors, the clinical outcome is positive, as is seen in >95% of secondary infections, and individuals are protected. If 'suboptimal' T-cell responses outweigh 'optimal' T-cell responses in individuals and the responses occur in the context of other associated 'pathogenic' risk factors, the balance is altered from a protective to pathogenic outcome.

T cell responses to multivalent DENV vaccination

Multiple DENV vaccines developed on diverse platforms are currently in clinical trials [9,98,99]. Owing to the immunopathology seen in natural secondary heterologous infections, a key consideration for vaccine manufacturers is to create a DENV vaccine that induces robust immunity to all four serotypes. The Sanofi Pasteur candidate vaccine contains four chimeric live YFV with the prM and E of each of the four DENV serotypes (DENV 1–4). Both DENV and YFV17D-204-specific $CD4^+$ and $CD8^+$ cellular responses induced by tetravalent chimeric DENV vaccines (CYD-TDV) were analyzed in flavivirus-naive or flavivirus-immune patients in Phase I clinical trials. Significant YFV 17D NS3-specific $CD8^+$ responses and DENV serotype-specific T helper responses were detected in PBMC of vaccinated subjects [100]. An IFN-γ/TNF-α ratio dominated by IFN-γ, for both $CD4^+$ and $CD8^+$ T-cell responses, was detected with an absence of a detectable Th2 response. Responses were impacted by the YFV and DENV immune status of an individual, and a booster vaccination broadened serotype-specific responses.

In a Phase II trial in Singapore, T-cell responses were assessed before and 28 days after a first and third injection of CYD-TDV and 1 year after the third injection in a subset of 80 subjects [101]. $CD4^+$ cytokine responses (IFN-γ/TNF-α) were detected to DENV NS3 prior to vaccination. Following vaccination, $CD8^+$ IFN-γ responses were detected to the YFV-17D-NS3 protein in addition to a Th1 cellular response in all participants to the tetravalent vaccine, characterized by IFN-γ secretion compared with

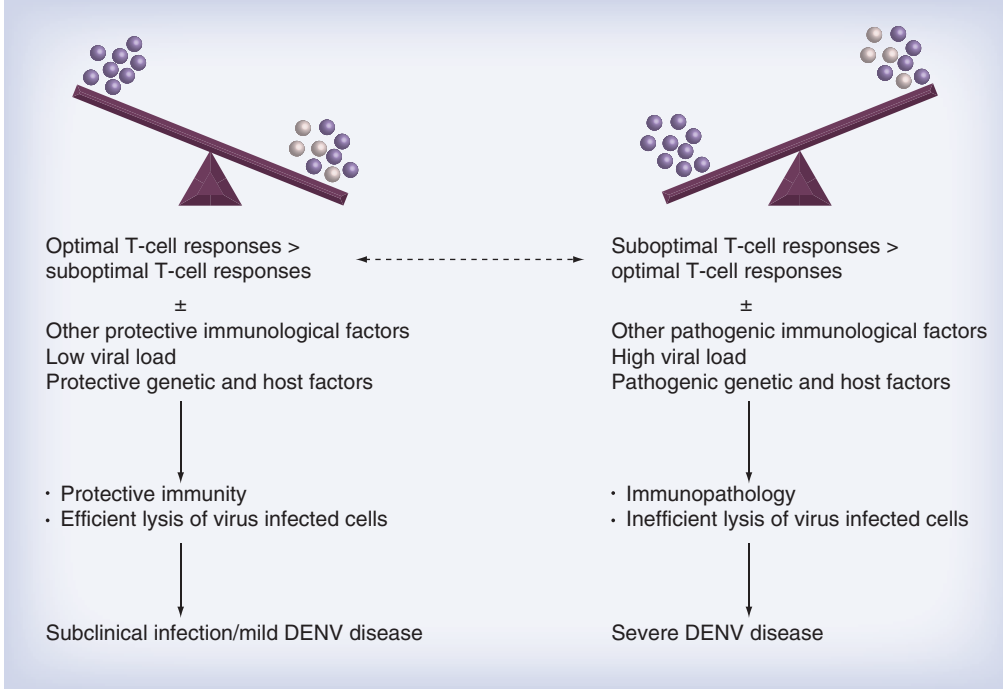

Figure 2. Disease outcome is multifactorial. Every person can generate 'optimal' or 'suboptimal' responses to individual DENV-specific T-cell epitopes. The total T-cell response to DENV in an individual is the cumulative response to multiple epitopes. A favorable clinical outcome occurs when overall 'optimal' T-cell responses occur in the context of protective host, genetic and other immunological factors. An unfavorable clinical outcome occurs when 'suboptimal' T-cell responses occur in the context of pathogenic host, genetic and other immunological factors.
DENV: Dengue viruses.

TNF-α. A booster vaccination induced more balanced responses against all four serotypes. Unfortunately, early results from a Phase II trial of this vaccine showed little protection [102].

The National Institute of Allergy and Infectious Diseases (NIAID) Division of Intramural Research has developed live, attenuated vaccines to each of the four DENV serotypes (DENV-1, 2, 3 and 4). These vaccines were designed by introducing deletions into the genomes of each virus in the 3′ untranslated region. Subjects were vaccinated with a low-dose (10 PFU) of a DENV-1 vaccine and a kinetic T-cell study was performed. Using multiparametric flow cytometry, DENV-1 specific $CD4^+$ T cells were found to secrete IFN-γ, TNF-α and IL-2 at 21 days post vaccination [79]. Little T-cell cross-reactivity was detected to the other three DENV serotypes.

It is important to assess the characteristics (serotype-crossreactivity and effector responses) of the T lymphocyte responses to monovalent and tetravalent vaccines currently in clinical trials. Many of these trials are being conducted in populations where there is pre-existing immunity to DENV and other related flaviviruses. Immunization with chimeric flaviviruses does not seem to skew the specificity of the T lymphocyte response significantly, although certain serotypes elicited stronger responses [100]. Development of immunocompetent animal models to determine whether the immunization method (dose, location, timing and sequence) affects vaccine-mediated T-lymphocyte responses will further our understanding of T-cell responses in humans. Strategies to 'sculpt' vaccine-induced immune response to achieve a balanced and robust response to all four serotypes must be considered.

Conclusion & future perspective

With a number of DENV vaccines in preclinical, Phase I–III trials, there is an opportunity to further our understanding of immune responses to DENV vaccination. It will be important to determine whether the same correlates apply for vaccine-induced T-cell responses as for natural infection. T-cell responses elicited to different

vaccines (DNA vs chimeric attenuated vs inactivated) are likely distinct and need to be evaluated. Some groups have attempted to determine if there are patterns of T-lymphocyte responses that are most strongly correlated with protection from or enhanced risk of severe DENV disease. Prospective cohorts where PBMC are collected prior to primary and secondary natural DENV infections are critical to identify the immunological predictors of protective versus pathogenic outcomes.

While substantial progress has been made in recent years, clinical and animal studies have revealed that immune responses to DENV are complicated. The presence of four closely related serotypes, interplay between multiple cellular subsets and lack of an authentic animal model has hindered progress. Gaps still remain in our understanding of DENV-specific T-lymphocyte responses and their associations with protection against or pathogenesis of severe disease. Well-designed prospective clinical studies are needed to provide the best insights in order to reduce the burden of this pathogen.

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EXECUTIVE SUMMARY

The pathogenesis of dengue viruses disease is multifactorial

- Many factors including host genetics, viral factors and pre-existing immunity likely contribute to dengue virus (DENV) pathogenesis.

T-cell responses to DENV after natural infection

- The majority of T-cell responses following natural infection with DENV are directed against nonstructural proteins, with responses to NS3 being dominant.
- T-cell-associated cytokines are elevated in patients with severe disease.
- In a limited number of studies, the frequency of epitope-specific T cells was not significantly different between primary and secondary DENV infections. Frequencies and functional responses need to be assessed against multiple epitopes in large prospective cohort studies to conclusively demonstrate that T-cell responses are skewed during a second infection with DENV.

Studies that have examined a role for T cells with pathogenesis of DENV virus infections

- T-cell-derived cytokines have been found to be elevated in patients with mild and severe DENV disease.
- Association between certain Class I HLA alleles and risk for disease severity supports a role for CD8 T cells in DENV pathogenesis.
- The frequency and activation phenotype of epitope-specific T cells have been examined in PBMC from patients with mild and severe disease during and after the critical time period of capillary leakage.

Studies that have examined a role for T cells with protection against DENV virus infection

- Prospective clinical studies are critical to clearly define a role for T cells in protection from disease in humans.
- Multifunctional $CD4^+$ T cells may be indicators of individuals who are better able to control DENV infection.
- Murine studies support a protective role for T cells against DENV infections, albeit in immunocompromised animals.
- An appropriate animal model for severe DENV infections is not available; however, groups are actively working to develop better models that recapitulate human disease.

T-cell responses to multivalent DENV vaccination

- T-cell responses to DENV vaccines are currently being evaluated in human vaccine trials.
- Multifunctional T cells may contribute to protection.
- Strategies to 'sculpt' vaccine-induced immune response to achieve a balanced and robust response to all four serotypes must be considered.

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