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Review

T-cell Responses to Dengue Virus in Humans

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Abstract: Dengue virus (DENV) is a leading cause of morbidity and mortality in most tropical and subtropical areas of the world. Dengue virus infection induces specific CD4+CD8– and CD8+CD4– T cells in humans. In primary infection, T-cell responses to DENV are serotype cross-reactive, but the highest response is to the serotype that caused the infection. The epitopes recognized by DENV-specific T cells are located in most of the structural and non-structural proteins, but NS3 is the protein that is most dominantly recognized. In patients with dengue hemorrhagic fever (DHF) caused by secondary DENV infection, T cells are highly activated in vivo. These highly activated T cells are DENV-specific and oligoclonal. Multiple kinds of lymphokines are produced by the activated T cells, and it has been hypothesized that these lymphokines are responsible for induction of plasma leakage, one of the most characteristic features of DHF. Thus, T-cells play important roles in the pathogenesis of DHF and in the recovery from DENV infection.
Key words: Dengue virus, T-cell response, T-cell receptor, Dengue fever, Dengue hemorrhagic fever

1. INTRODUCTION

Dengue fever (DF)/dengue hemorrhagic fever (DHF) is one of the most important infectious diseases in tropical and subtropical areas of the world. The areas where DF/DHF is a serious health concern have been expanding, and the number of DHF patients has been increasing. The immune responses to DENV have been analyzed in humans. The antibody responses are well known and the roles of specific antibodies in the prevention of DENV infection and in the pathogenesis of DHF have been established. On the other hand, T-cell responses to DENV is not as well understood as that of antibodies.

2. DENGUE VIRUS (DENV)

DENV belongs to the family *Flaviviridae*, the genus *Flavivirus*. The genome of dengue virus is a single, plus-stranded RNA nearly 11 Kb in length. The open reading frame codes three structural proteins (capsid (C) protein, preM protein, and envelope (E) protein) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). Dengue virion has a spherical shape and measures 40–50 nm in diameter. The envelope is a lipid bilayer containing two envelope-associated proteins: the E and M proteins.

DENV is transmitted to humans by infected mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus*. Humans are natural hosts of DENV, and DENV is maintained between mosquitoes and humans in nature. There are four serotypes: DENV types 1, 2, 3 and 4. Although DENV types 1, 2, 3 and 4 are referred to as serotypes, it is generally accepted that the four serotypes are different albeit antigenically related species.

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3. DENGUE FEVER (DF) AND DENGUE HEMORRHAGIC FEVER (DHF); TWO CLINICAL MANIFESTATIONS OF DENGUE VIRUS INFECTION

Dengue virus infection can be asymptomatic or cause two forms of illness, DF and DHF, although the majority of DENV infections are asymptomatic. New diagnostic names have recently been proposed [1], but the terms DF and DHF are still widely used.

DF is a self-limiting febrile illness. After an incubation period of 2–7 days, a sudden onset of fever occurs. The fever is usually accompanied by retro-orbital or frontal headache. Myalgia and bone pain occur soon after the onset of fever. A transient macular rash that blanches under pressure, nausea, vomiting, lymphadenopathy and taste aberrations can develop. These symptoms are accompanied by leucopenia and variable degrees of thrombocytopenia. One to two days after defervescence, a generalized morbilliform maculopapular rash appears. The rash spares palms and soles. Patients usually recover from the symptoms without complications about a week after the onset of disease.

Some patients infected with dengue virus demonstrate plasma leakage into interstitial spaces, thrombocytopenia and also hemorrhagic manifestation. This severe life-threatening syndrome is called DHF. The incubation period of DHF is similar to that of DF. Illness starts with fever, malaise, vomiting, headache, anorexia, and cough. Rapid clinical deterioration and collapse follow after 2–5 days. In the second phase, the patients demonstrate cold clammy extremities, warm trunk, flushed face, restlessness, irritability, middle gastric pain, and may progress to a rapid weak pulse, hypotention and narrow pulse pressure. The crisis lasts for 24–36 hours, and the patients may require fluid therapy, but usually recover rapidly once convalescence starts. The hematological manifestations include an increase in hematocrit, thrombocytopenia, a prolonged bleeding time, and an increased prothrombin time. The temperature returns to normal when capillary leakage occurs. The occurrence of capillary leakage differentiates DHF from DF. When plasma leakage is so profound that shock occurs, it is also referred to as dengue shock syndrome (DSS).

4. T-CELL RESPONSES TO DENV

T-cells are known to play an important role in recovery from virus infections in general. Analysis of T-cell responses to DENV is essential to understand the mechanism of recovery from DENV infections and also the pathogenesis of DHF. There are not very satisfactory animal models to analyze T-cell responses to dengue virus and pathogenesis of DHF, although many groups have attempted to do so [2]. Analysis of human peripheral blood mononuclear cells (PBMC) is still the best way to elucidate the DENV-specific T-cell responses.

4-1. Analysis of memory T-cells

T-cell responses to DENV have been analyzed mainly using memory T-cells in PBMCs. DENV-specific memory CD4+CD8– T-cells and CD4–CD8+ T cells were detected in PBMCs from human subjects after natural infection with dengue virus, or after immunization with experimental, live-attenuated dengue vaccines.

4-2. Analysis of DENV-specific CD4+ memory T-cells

Specific CD4+CD8– memory T-cells induced by primary DENV infection were serotype-cross-reactive in bulk culture analysis, but the highest response was to the same serotype that caused the infection [3]. In some subjects, however, CD4+ T-cell memory responses after primary infection were serotype-specific [4]. The presence of serotype-cross-reactive and serotype-specific CD4+ T-cells was also demonstrated in precursor frequency assays [5]. In PBMC from DENV-1-immunizued individuals, the frequency of DENV-1 responsive T-cells was nearly 10 times higher than the frequency of other three serotypes [5].

DENV-specific CD4+ T-cell clones were established and analyzed for serotype specificities and functions. Most of the T-cell clones demonstrated specific cytotoxic activities. Consistent with the results of bulk culture analyses, DENV-specific CD4+ T-cell clones established from respective subjects have at least two types of serotype specificities: dengue serotype-specific and serotype-cross-reactive [6, 7]. The patterns of serotype cross-reactivities, and the proportion of serotype-specific and cross-reactive clones varied among individuals.

4-3. Analyses of DENV-specific CD8+ memory T-cells

DENV-specific CD8+CD4– memory T-cells induced by primary DENV infections were serotype cross-reactive in bulk culture analysis, and the highest response was to the same serotype that caused the primary infection [8]. DENV-specific CD8+ T-cell clones were established. The clones also contained serotype-specific and serotype cross-reactive clones [9]. Thus, similar to CD4+ T-cell responses, DENV infection with one serotype of DENV induces both serotype-specific and serotype cross-reactive CD8+ memory T-cells.

5. PROTEINS RECOGNIZED BY DENV-SPECIFIC T-CELLS

Proteins recognized by DENV-specific T-cells were determined in bulk culture and T-cell clonal analyses. In

these analyses, the non-structural proteins NS3 and NS1,2a were determined to be the proteins mainly recognized by both CD4+T cells and CD8+T cells [10–13]. However, other proteins such as C, E and NS4b also contained T cell epitopes [14–16]. The recognition pattern of the proteins differs according to the type of HLA.

6. Activation of T cells in vivo in patients with DF/DHF

Activation of DENV-specific T cells in vivo has been analyzed using PBMC samples obtained in early stages of DF and DHF by some groups. Green et al. reported that absolute counts of CD4+ T-cells, CD8+ T-cells, NK cells, and γδT-cells were decreased in children with DHF compared to those with DF early in the course of illness. The percentage of cells expressing CD69, an activation marker, was more increased in CD8+ T-cell and NK cell populations in children who developed DHF than in those with DF [17]. Mongkolsapaya et al. reported that many CD8+ T-cells possessed an activation phenotype and were undergoing programmed cell death [18]. These studies provided direct evidence of T-cell activation in vivo in the acute phase of DENV infections.

Activation of T-cells has also been suggested by increased levels of lymphokines in serum or plasma samples from patients with DF/DHF. It has been reported that levels of IL-2, IFNγ and other lymphokines are highly elevated in patients with DHF compared to those with DF, and healthy subjects [19]. Serum levels of soluble tumor necrosis factor receptors (sTNFRs), soluble CD8 and soluble IL-2 receptor were higher in children who developed DHF than in those with DF or non-dengue febrile illness [20]. These studies indicated that T-cells are highly activated in patients with DF and DHF, and the levels of T cell activation are higher in patients with DHF than in those with DF. The results also suggested that activation of CD8+ T-cells is more profound than that of CD4+ T-cells. The results suggesting massive T-cell activation in DHF are summarized in Table 1.

Table 1. Characteristics of T-cell responses in patients with DF and DHF.

1. T-cell responses to DENV in primary infection are serotype cross-reactive, but the highest response is to the serotype that caused the primary infection. Most of the memory T-cells after secondary infection are serotype cross-reactive.
2. Massive T-cell activation in DHF.
   * High percentage of CD69+ T-cells.
   * The levels of T-cell activation: Higher in CD8+ T-cell poputation than in CD4+ T-cell population.
   * High levels of lymphokines: IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-γ
   * High levels of cellular proteins: sIL-2R, sCD4, sCD8, sTNFR

7. Determination of the clonality of T-cells activated in vivo in patients with DF/DHF

As stated above, T-cells are highly activated in patients with DF and DHF, and there is a positive relationship between the levels of T-cell activation and severity of dengue illness [21]. One of the important questions is: Are the majority of activated T-cells DENV-specific, or are most of the activated T cells non-specific for DENV and activated as bystanders? This question was addressed by analyzing T-cell receptors.

The usage of T-cell receptor α-chain variable region (TCRAV) and β-chain variable region (TCRBV) repertoires was analyzed in patients with DF or DHF. This analysis can determine whether T-cell activation in the patients is antigen-specific. If common antigens are responsible for proliferation of the T-cells, complementarity-determining region 3 (CDR3) diversity should be constrained, and homologous CDR3 sequences might be found in the T cells [22, 23]. We then determined the nucleotide sequences of CDR3 of TCR α-chain and β-chain gene from the T-cells. The delta score was used as an indicator to evaluate the extent of skew of TCR repertoires [24]. The delta score indicates the sum of absolute differences between the frequencies of respective V segments.

Peripheral blood mononuclear cells (PBMC) were collected from patients with DF or DHF on study days –4, –2, 2 and 180, where day 0 is defined as the day of defervescence. These donors developed dengue illness after secondary infections. TCR analysis was performed by microplate hybridization assay, size spectratyping and sequencing of CDR3 region. We determined whether T-cells are oligoclonally expanded in dengue patients, and whether there is a unique T-cell receptor usage in patients with DHF or DF.

The patterns of T-cell receptor usage changed in individuals during the course of illness. The pattern of TCR usage on day 180 was considered the control for each of the patients. Representative data for TCR VB usage in PBMC from a donor is shown in Figure 1. The percentages of T-cells with VB051 and VB091 increased significantly. The TCRs that demonstrated an increase in percentage differ from donor to donor. In order to estimate the levels of skew in TCR usage, delta scores were calculated (Figure 2). The delta scores changed between acute and convalescent stages, suggesting the skew in TCR usage. There was a positive relationship between delta scores and the severity of dengue illness; the levels of skew of TCR usage were greater in

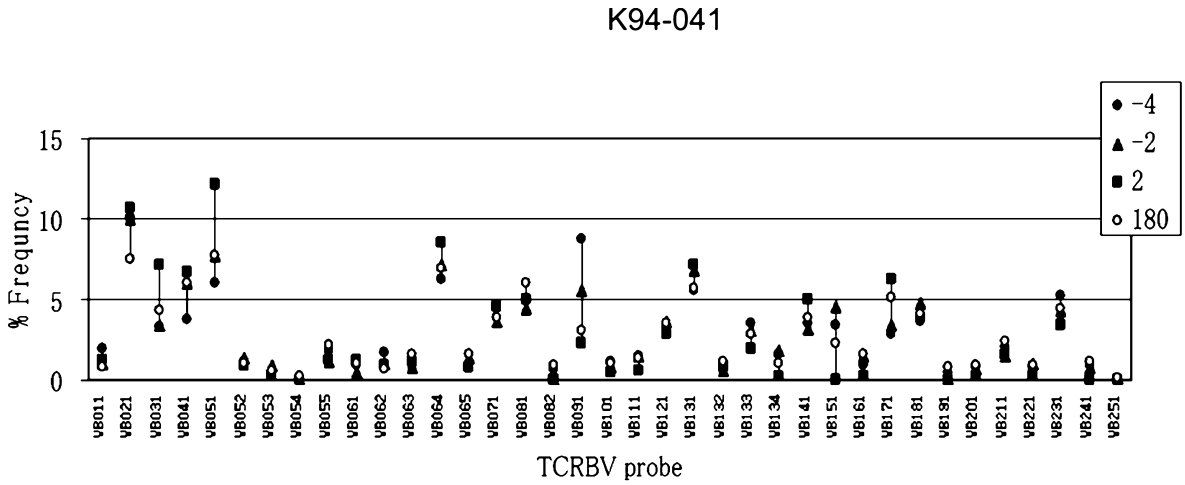

Fig. 1. T-cell receptor usage in PBMCs collected from a patient with DHF.
TCR VB usage in PBMC from a patient with DHF. PBMCs were collected on days –4, –2, 2 and 180, where day 0 is defined as the day of defervescence.

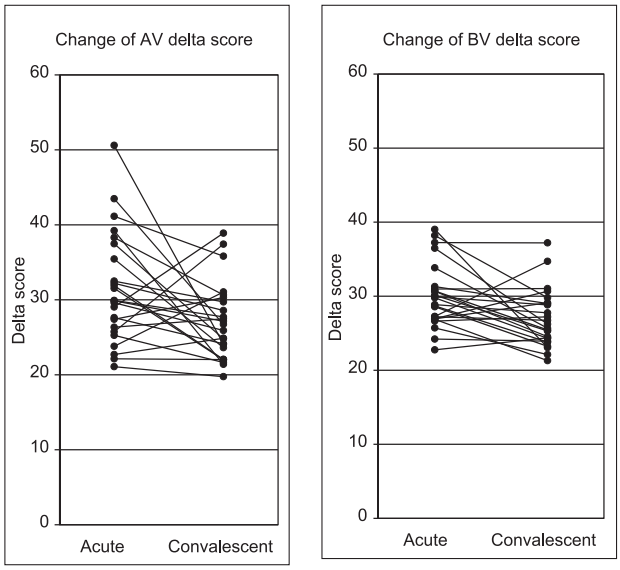

Fig. 2. Delta scores of TCR AV and BV.
Delta scores were compared between acute and convalescent stages. $P < 0.01$ between acute and convalescent stages in TCR AV and BV.

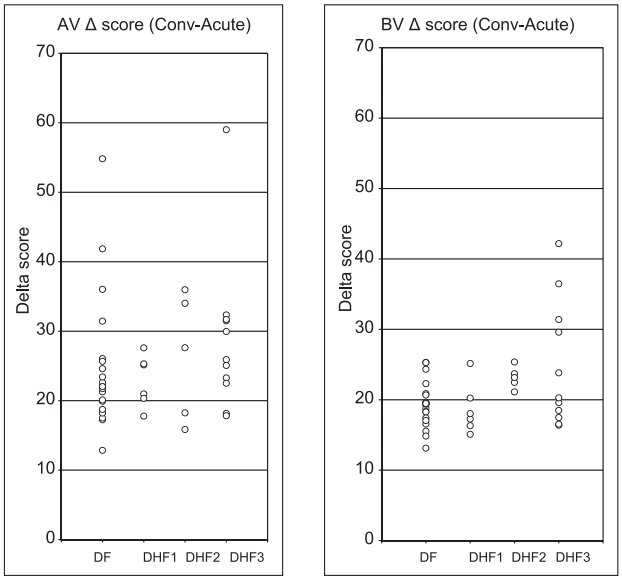

Fig. 3. Relationship between TCR delta scores and the severity of dengue illness.
There was a positive relationship between delta scores and severity of dengue illness in TCR BV usage.

patients with DHF grades 3 and 4 (Figure 3). Clonality of activated T-cells was then evaluated by size spectratyping of the CDR3 of TCRs that demonstrated increased usage. The CDR3 of these TCRs showed only a few peaks, suggesting that T-cells with these TCRs proliferated in an oligoclonal fashion. The nucleotide sequences of the CDR3 regions of these TCRs showed that a high percentage of the sequenced CDR3 clones had identical amino acid sequences (Table 2). These results are consistent with those of the spectratyping analyses and confirmed that activation of T-cells is oligoclonal in some of the DHF patients (Figure 4). The results suggest that the majority of T-cells highly activated in vivo are DENV-specific in patients with DHF.

Table 2. Alignment of amino acid sequences of CDR3 regions in TCRs that demonstrated increased levels of usage

Patient	Fever Days	Occurrence	AV/BV segment	Amino acid sequences in CDR3 region			AJ/BJ segment
				AV/BV seq.	N (N-D-N)	AJ/BJ seq.	
K94-017	0	2/7	AV1S4	YFCAVS	P	NRDDKIIFG	AJ30
K94-029	1	3/8	AV1S4	YFCAVS	AS	NSGGSNYKLTFG	AJ53
K94-029	1	5/7	AV21S1	YFCAAS	F	SGGYQKVTFG	AJ13
K94-041	–4 & –2	6/7	AV31S1	YLCA	AEY	TGGFKTIFG	AJ9
K94-007	1	3/11	AV14S1	YFCAYRS	GG	YKLSFG	AJ20
C94-138	0	5/7	AV20S1	YYCLVGD	D	DYKLSFG	AJ20
K94-041	–4	6/6	BV9S1	YFCASS	FS	NEQFFG	BJ2S1
	–2	3/5	BV9S1	YFCASSQ	DRD	SGANVLTFG	BJ2S6
K94-004	–1	8/11	BV6S5	YLCASS	RGG	EQYFG	BJ2S7
C94-081	–2	2/7	BV8S1	YFCASS	YG	NEQFFG	BJ2S1

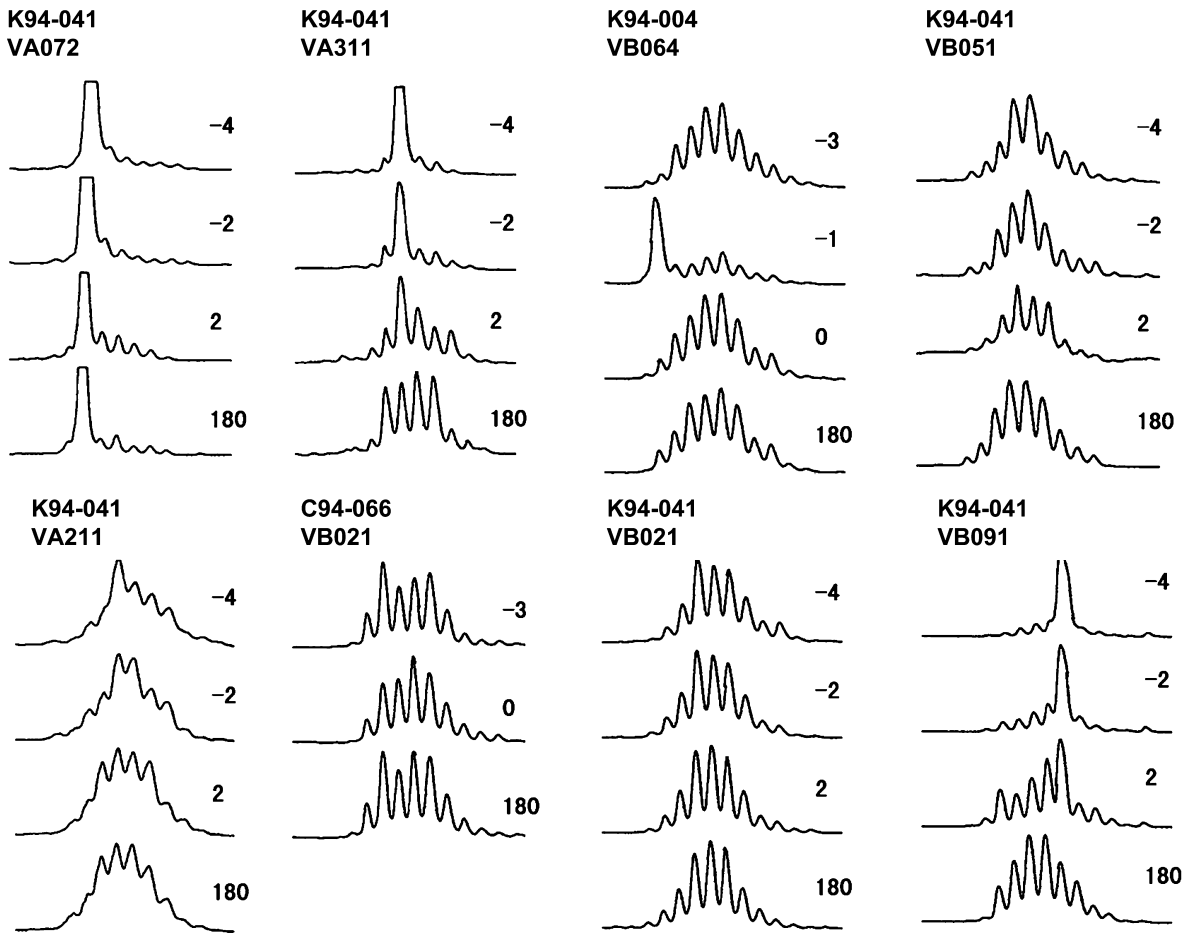

Fig. 4. Size spectratyping of CDR3 of selected TCRs in PBMCs obtained from patients with DF and DHF. PBMCs were collected on days –4, –2, 2 and 180, where day 0 is defined as the day of defervescence.

8. Role of T-cells in the pathogenesis of DHF

The role of the activated T-cells in the pathogenesis of DHF has been hypothesized [25]. A series of studies have suggested that plasma leakage, which differentiates DHF from DF, is caused by malfunction of vascular endothelial cells induced by cytokines or chemical mediators rather than by destruction of the small vessels [26, 27]. Plasma levels of various lymphokines are significantly higher in DHF than in DF. The lymphokines elevated in patients with

DHF include TNF-α, IL-2, IL-6, IL-8, IL-10, IL-12 and IFN-γ. However, it is not clearly understood how these lymphokines cause malfunction of vascular endothelial cells and lead to plasma leakage.

Some of the cytokines, i.e. IFN-γ, IL-2 and TNF-α were mainly produced by DENV-specific T-cells upon activation [28]. It is likely, therefore, that activated specific T-cells are responsible for increased levels of cytokines that play a key role in the pathogenesis of DHF. Consistent with the assumption, there was a correlation between the levels T-cell activation and dengue disease severity. It should, however, be understood that these activated T-cells may also contribute to the recovery from DENV infection, as has been reported in other virus infections.

9. CONCLUSIONS

New findings regarding T-cell responses to DENV have been accumulating. However, a complete understanding of the T-cell activation in patients with DF and DHF and the roles of activated T-cells in recovery and in the pathogenesis of DHF remains a challenge. A better understanding of T-cell responses as well as function and role in DENV infection will facilitate the development of dengue vaccines, new treatment programs and measures to prevent DENV infection.

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