

Activation of dengue virus-specific T cells modulates vascular endothelial growth factor receptor 2 expression

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Abstract

Background: The pathogenic mechanisms underlying the increased vascular permeability in dengue hemorrhagic fever (DHF) are not well understood. Enhanced cellular immune activation, especially activation of serotype-cross reactive T cells, has been implicated in plasma leakage in DHF. Changes in several biological markers and mediators including cytokines, chemokines, angiogenic factors and their receptors have been shown to correlate with disease severity. A decline in plasma levels of a soluble form of vascular endothelial growth factor receptor 2 (VEGFR2), a receptor of vascular endothelial growth factor (VEGF), has been associated with plasma leakage in dengue patients.

Objective: We aimed to investigate the effect of dengue virus (DV)-specific CD8⁺ T cells on the expression of VEGFR2 on endothelial cells.

Method: An *in vitro* model was developed in which dengue virus-specific CD8⁺ T cells generated from peripheral blood mononuclear cells (PBMCs) of DHF patients were co-cultured with antigen-presenting cells, human umbilical vein endothelial cells (HUVECs) and activated with DV non-structural protein 3 (NS3) peptides. The expression of VEGFR2 by endothelial cells was measured.

Results: DV-specific CD8⁺ T cells were serotype cross-reactive. Activation of DV-specific CD8⁺ T cells resulted in down-regulation of soluble VEGFR2 production and an up-regulation of cell-associated VEGFR2.

Conclusions: Our findings indicate that activation of DV-specific T cell is associated with modulation of VEGFR2 expression that may contribute to increased VEGF responsiveness and vascular permeability.

Keywords: Dengue hemorrhagic fever, vascular leakage, vascular permeability, vascular endothelial growth factor receptor 2, T cells.

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Introduction

Dengue virus (DV) is a positive single stranded RNA virus belonging to genus *Flavivirus* in the *Flaviviridae* family. DV comprises 4 distinct serotypes, DV1, DV2, DV3, and DV4. DV infection is an important public health problem especially in tropical and subtropical countries. A recent estimate indicates that every year 390 million people are infected with DV worldwide with 96 million cases of symptomatic infections.¹

Currently there are no effective agents for the treatment of DV infection.

Individuals infected with DV may be asymptomatic or develop mild symptoms of undifferentiated fever, or dengue fever (DF). However, some may develop a severe form of disease called dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).² Primary infection with DV provides

a lifelong immunity to the infecting serotype but temporary and partial protection against other serotypes. A subsequent heterotypic dengue infection has been shown to be associated with an increased risk of DHF/DSS.³ Plasma leakage is a major clinical manifestation of DHF and usually occurs in pleural and peritoneal spaces accompanied by increasing hematocrit levels, thrombocytopenia and bleeding, and may lead to shock and death.² Plasma leakage in DHF typically occurs rapidly during the time of adaptive immune activation and viral clearance at the defervescence phase of the disease.⁴ The underlying mechanisms of vascular leakage in DHF remain poorly understood. Both humoral and cell-mediated immunity have been implicated in the pathogenesis of dengue.⁴ A secondary DV infection with a heterotypic viral serotype has been shown to be associated with activation of cross-reactive DV-specific T cell responses.⁵⁻⁸ These T cells exhibited poor cytolytic activity and an exaggerated cytokine response which may contribute to vascular leakage in DHF.^{6,7} Several cytokines, chemokines and biological mediators are produced by virus infected cells *in vitro* and by activated T cells, and their levels have been found to be elevated in DHF patients.⁹ Some of these cytokines are mediators that relate to the increase in paracellular permeability in vascular endothelium.¹⁰ We have previously reported elevated levels of vascular endothelial growth factor-A (VEGF-A) during the period of plasma leakage in patients with DHF.¹¹ VEGF-A belongs a family of cytokines involved in vasculogenesis, angiogenesis, and lymphangiogenesis. In mammals, VEGF family consists of 5 related members, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PLGF).¹² Among these, VEGF-A is the most potent vascular permeability factor and has been shown to be required for vessel formation during embryogenesis and also involved in the formation of new blood vessels in tumors.^{12,13} VEGF-A binds to 2 major tyrosine kinase receptors (TKRs) namely VEGFR1 (Fms-like tyrosine kinase-1 or flt-1) and VEGFR2 (fetal liver kinase 1 or flk1 in mouse or kinase insert domain containing receptor or KDR in human).¹² Both VEGFR1 and VEGFR2 are expressed as membrane associated form and soluble molecules.¹⁴⁻¹⁷ VEGFR1 is expressed on many cell types such as monocytes, macrophages, endothelial cells. VEGFR2 is primarily expressed by endothelial cells and by few others cells.^{18,19} Signaling via VEGFR2 results in changes in cell morphology, cell proliferation, and increased membrane permeability.²⁰ Elevated levels of free VEGF have been reported in DHF patients at the time of plasma leakage in DHF patients and occurred simultaneously with a decline in soluble VEGFR2 levels suggesting that perturbation of the levels of VEGF and its receptors may play a key role in the mechanism of plasma leakage in dengue.¹¹ The timing of the decline in plasma soluble VEGFR2 levels is contemporaneous with the kinetics of the emerging antiviral T cell immune responses.¹¹

In this study, we investigated the effect of virus-specific CD8⁺ T cell activation on the expression of endothelial cell VEGFR2. This was carried out using a coculture model in which dengue specific T cells were activated with DV peptides in the presence of antigen presenting cells and human umbilical endothelial cells (HUVECs).

Methods

Generation and characterization of dengue-specific T cell lines and clones

T cell lines were generated from peripheral blood mononuclear cells (PBMCs) collected from DV infected patient at early convalescence (approximately 5-10 days after hospital discharge). The details of the study have been previously described.²¹ The study has been approved by the Ethical Review Committee for Research in Human Subjects, The Ministry of Public Health, Thailand, and by The Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Written informed consent was obtained from the legal guardian of each participant. Assent was obtained from children ≥ 7 years old. Dengue was confirmed by paired plasma sample serology to detect DV-specific IgM and IgG. Infecting viruses were identified by type-specific reverse transcription-polymerase chain reaction technique (RT-PCR). Patients with HLA-A11 were identified by HLA typing using genomic DNA samples.

Primary T cell lines were generated by stimulating PBMCs with autologous monocyte-derived dendritic cells and DV-NS3 (amino acids 134-143) peptide from DV2 or DV3/4 (5-10 $\mu\text{g/ml}$, GenScript, USA) in AIM-V medium (Gibco, USA) supplemented with 10% heat inactivated fetal bovine serum (HIFBS, Gibco, USA) and 25 U/ml of recombinant human interleukin-2 (rhIL-2, R&D System, USA). Cells were cultured at 37 °C in humidified incubator with 5% CO₂. One hundred to two hundred microliters of fresh AIM-V medium contained 10% HIFBS and rhIL-2 (final concentration 25 U/ml) were added every 2-3 days of the cultivation. After 7-14 days post stimulation, DV-specific T cells lines were analyzed in functional assays by peptide stimulation using DV1, DV2, and DV3/4 NS3 peptides at the indicated concentration in the presence of a HLA-A11 expressing Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines (BLCLs). Culture supernatants were collected at the indicated time points and assayed for interferon-gamma (IFN- γ).

DV-specific T cell clones were generated from primary T cell lines by limiting dilution method. DV-specific T cell lines were harvested and washed 2 times with AIM-V medium (Gibco, USA) supplemented with 20% HIFBS (Gibco, USA). CD8⁺ T cells were isolated using anti-CD8⁺ antibody conjugated magnetic beads (MACS[®], Miltenyi Biotec, USA). Positively selected cells were then serially diluted in AIM-V medium (Gibco, USA) supplemented with 10% HIFBS (Gibco, USA) and seeded at 10, 5, and 2.5 cells/well in 96-well-plates. HLA-restricted DV NS3 peptide from the same serotype of current infection (10 $\mu\text{g/ml}$), HLA-matched monocyte-derived dendritic cells and rhIL-2 (25 U/ml) were added to each well. The cells were cultured by adding fresh media contained 25 U/ml of rhIL-2 every 2-3 days. Growth of T cells clones was observed daily under inverted light microscope and proliferating cells were selected for further expansion. Successfully expanded DV-specific T cell clones were tested for their specificity and cross-reactivity to DV by measuring IFN- γ production in response to DV peptide stimulation.

T cell - human umbilical vein endothelial cells (HUVECs) co-culture

Primary human umbilical vein endothelial cells (HUVECs, Lonza, USA) were grown and maintained in endothelial cell growth medium containing growth supplements (Clonetics EGM-2 BulletKit, Lonza, USA) at 37 °C with 5% CO₂. HUVECs monolayers were grown in 96-well-plate at a density of 1.5×10⁴ cells/well for 48 hours and then co-cultivated with DV-specific T cell lines (5×10³ cells/well), HLA-matched BLCLs (5×10³ cells/well) and HLA-A11 restricted DV NS3 peptides from DV1, DV2, or DV3/4 at the indicated concentrations, or DMSO as control. Forty eight hours post co-cultivation supernatants were collected. T cells were removed and wells were washed twice with phosphate buffer saline (PBS) and HUVECs monolayers were lysed and cell lysate samples were prepared in sample buffer solution containing protease inhibitors. Supernatants were analyzed for the levels of IFN-γ and soluble VEGFR2 (sVEGFR2), and cell lysate samples were analyzed for cell-associated VEGFR2 (cVEGFR2) by enzyme-linked immunosorbent assay (ELISA). All experiments were performed in triplicates or quadruplicates.

VEGFR2 ELISA

The levels of soluble VEGFR2 in culture supernatant and cell-associated VEGFR2 from HUVECs lysate were determined with human VEGFR2 ELISA (human VEGFR2/KDR DuoSet ELISA, R&D Systems®, USA). Co-cultured supernatants and HUVEC cell lysates were collected at 48 hours from the co-culture system. HUVEC cell lysate was prepared by adding 50 μl of lysate solution to each well (R&D Systems®, USA) and let incubate at 4 °C, rocking for 15 minutes. Lysate samples were spun at 350g for 5 minutes at 4 °C and the supernatants were collected for ELISA.

Confocal microscopic analysis of VEGFR expression by HUVECs

Confluent monolayers of HUVECs were prepared into 8-well chamber-glass slides (Lab-Tek II CC² Chamber Slide system, Nunc, USA) at a density of 5.6×10⁴ cells/well and incubated for 48 hours before used in the experiment. DV-specific CD8⁺ T cell lines (2×10⁴ cells/well), HLA-matched BLCLs (2×10⁴ cells/well), and HLA-A11 restricted DV NS3 peptides (5 μg/ml of DV1, DV2, or DV3/4), or DMSO (0.5%) were added to HUVECs monolayers cultures. After 48 hours supernatants and T cells were removed and HUVECs monolayers were stained with fluorescence conjugated antibodies to CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) 1:25 in 1% BSA in PBS (Anti-CD31-FITC, eBioscience, USA), and VEGFR2 1:25 in 1% BSA in PBS (anti-human VEGFR-2/KDR-APC, R&D System, USA) at 4 °C for 30 minutes, washed twice with 1% BSA in PBS. Then, the HUVECs were incubated with DAPI (0.5 μg/ml, Sigma-Aldrich, USA) for 5 minutes at room temperature, and fixed with 1% paraformaldehyde. The slides were mounted with VectorShield® mounting solution and inspected with confocal fluorescence microscopy (AxioVision LE software, Zeiss, Germany).

Statistical analysis

Variables are reported as mean (SD). Continuous variables were compared using Student's T-test or Mann-Whitney test. A p value less than 0.05 was considered significant. All analyses were performed using SPSS (version 12) statistical program.

Results

Generation and characterization of DV-specific T cell lines

Primary T cell lines were generated from peripheral blood mononuclear cells (PBMCs) collected at early convalescence from 2 dengue cases with a secondary dengue infection: 1) line 170-08 was generated from a DHF case with a secondary DV serotype 4 infection, 2) line 287-07 was generated from a DHF case with a secondary DV serotype 2 infection. The cases were selected based on their HLA-A11 haplotype. Cells were stimulated with autologous dendritic cells generated from CD14⁺ cells cultured in rhGM-CSF and rhIL-4, and DV NS3 derived peptide of the infecting serotype at 10 μg/ml and rhIL-2 at 25 U/ml. After 1-2 weeks cells were stained for surface expression of CD4⁺ and CD8⁺ by flow cytometry and found to consist primarily (more than 80%) of CD8⁺ T cells, and tested for antigen specificity by co-culturing with HLA-A11 expressing BLCLs in the presence of NS3 peptides from DV1, 2, 3/4, or DMSO as control. The amino acid sequences of these peptides are shown in **Table 1**. IFN-γ in culture supernatants collected at 48 hours after stimulation was measured by ELISA. These T cell lines produced IFN-γ in response to peptide stimulation in different patterns (**Figure 1A**). 287-07 showed reactivity to all DV-derived peptides with the strongest response to DV3/4 peptide while 170-08 T cell line produced IFN-γ almost exclusively to DV3/4 peptide.

Table 1. Amino acid sequences of NS3-derived peptides of DV1, DV2, and DV3/4

| Sequence | Serotype variant |
|------------|------------------|
| GTSGSPIVNR | DV1 |
| GTSGSPIVDR | DV2 |
| GTSGSPIINR | DV3/4 |

To investigate the effect of peptide concentrations on the patterns of cross reactivity, T cell lines were stimulated with various concentrations of DV1, 2, or 3/4 NS3 peptides at a range of concentrations in the presence of BLCLs antigen presenting cells and the secretion of IFN-γ was measured. T cell line 170-08 showed narrow specificity to D3/4 peptide at lower peptide concentrations and showed some degrees of cross-reactivity to DV1 and DV2 peptide at a high peptide concentration (10 μg/ml). A similar pattern was observed for T cell line 287-07 albeit a broader cross reactivity in comparison to 170-08 cell line (**Figure 1B**).

Activation of DV-specific T cells modulated soluble and cell-associated VEGFR2 expression

To study the effects of T cell activation on VEGFR2 expression of endothelial cells, DV-specific primary T cell

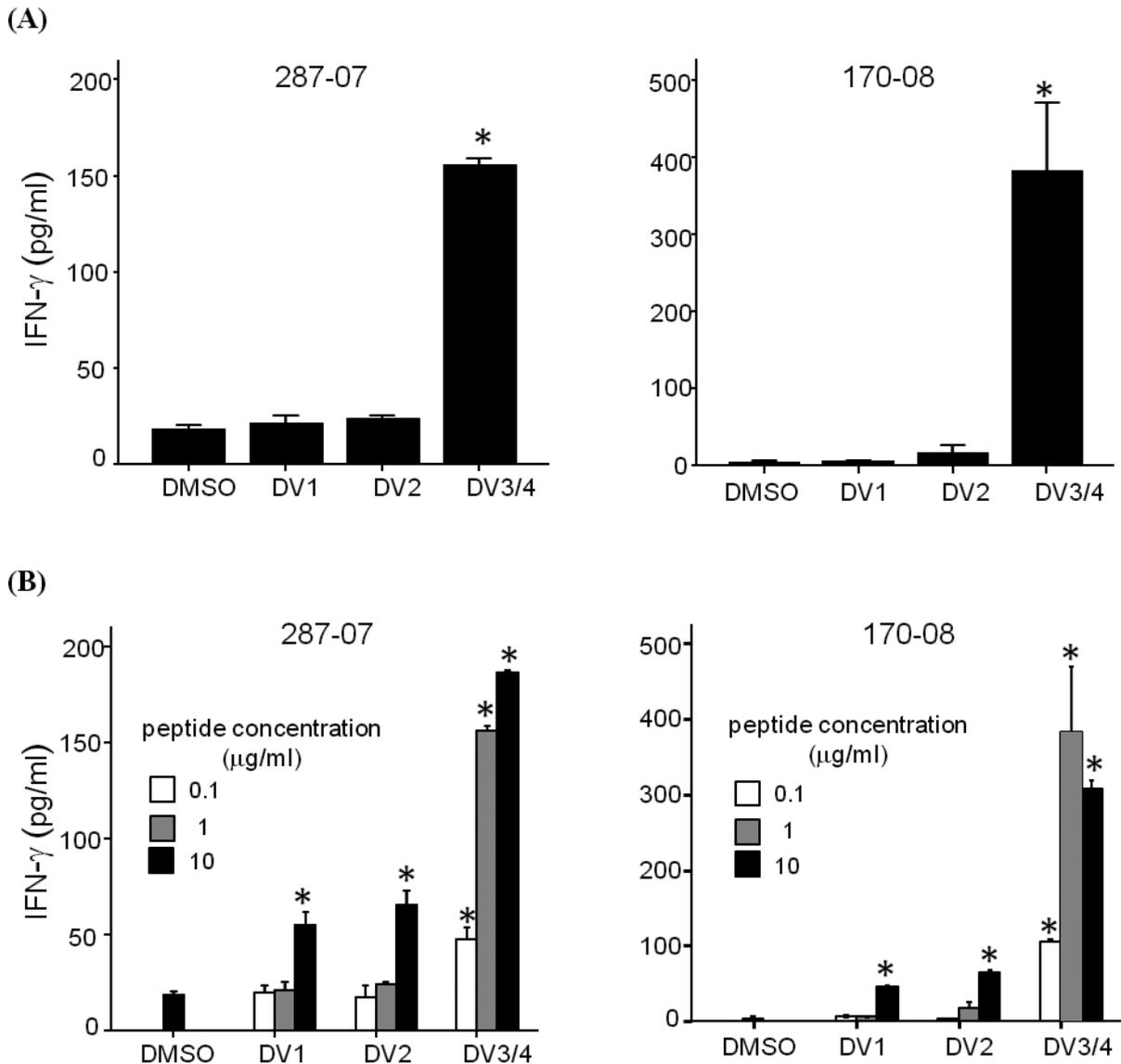


Figure 1. Peptide specificity of DV-specific primary T cell lines. Primary T cell lines generated from PBMCs were stimulated with the indicated DV NS3 derived peptides corresponding to amino acid sequences of DV1, DV2, or DV3/4, or DMSO as a control. Peptides were added at 1 μ g/ml. Supernatants collected 48 hours after stimulation were assayed for IFN- γ by ELISA (A). To examine the peptide reactivity at different peptide concentrations, T cell lines were stimulated with peptides at the indicated concentrations and IFN- γ levels in culture supernatants were measured 48 hours later by ELISA (B). * indicates difference from DMSO control ($p < 0.05$).

lines were cocultivated with confluent monolayers of human umbilical vein endothelial cells (HUVECs) and activated with DV NS3 peptides at concentrations ranging from 0.1-10 μ g/ml. The levels of soluble VEGFR2 (sVEGFR2) were measured in culture supernatants collected at 48 hours. The levels of cell-associated VEGFR2 (cVEGFR2) were measured in the cell lysate of HUVECs. As shown in **Figure 2**, the levels of sVEGFR2 in the culture supernatants of HUVECs cocultivated with T cells activated with NS3 peptides of DV3/4 decreased when compared to the levels in supernatants from HUVECs cultured with T cells that were activated either with DV1 NS3, DV2 NS3 peptides or DMSO carrier control. The modulatory effect was concentration dependent as it was observed only with peptide concentration 1 and 10 μ g/ml. Both primary T cell lines showed a similar pattern of down-regulation of sVEGFR2 when

stimulated with DV3/4 NS3 peptides (**Figure 2A**).

Analysis of the levels of VEGFR2 in cell lysate revealed a pattern distinct to that of sVEGFR2; activation of T cells with either DV2, or DV3/4 peptides was associated with increased VEGFR2 expression by HUVECs (**Figure 2A-B**). In addition, the up-regulation of cVEGFR2 was observed at the lowest concentration (0.1 μ g/ml) of DV2 and DV3/4 NS3 peptides. In contrast, stimulation with DV1 NS3 peptide did not result in up-regulation cVEGFR2 expression (**Figure 2B**).

Clones derived from T cell lines regulated VEGFR2 expression by HUVECs

To further define peptide specificity and pattern of VEGFR2 modulated by peptide-specific T cells we derived clones from the two primary T cell lines by limit dilution. Several T cell

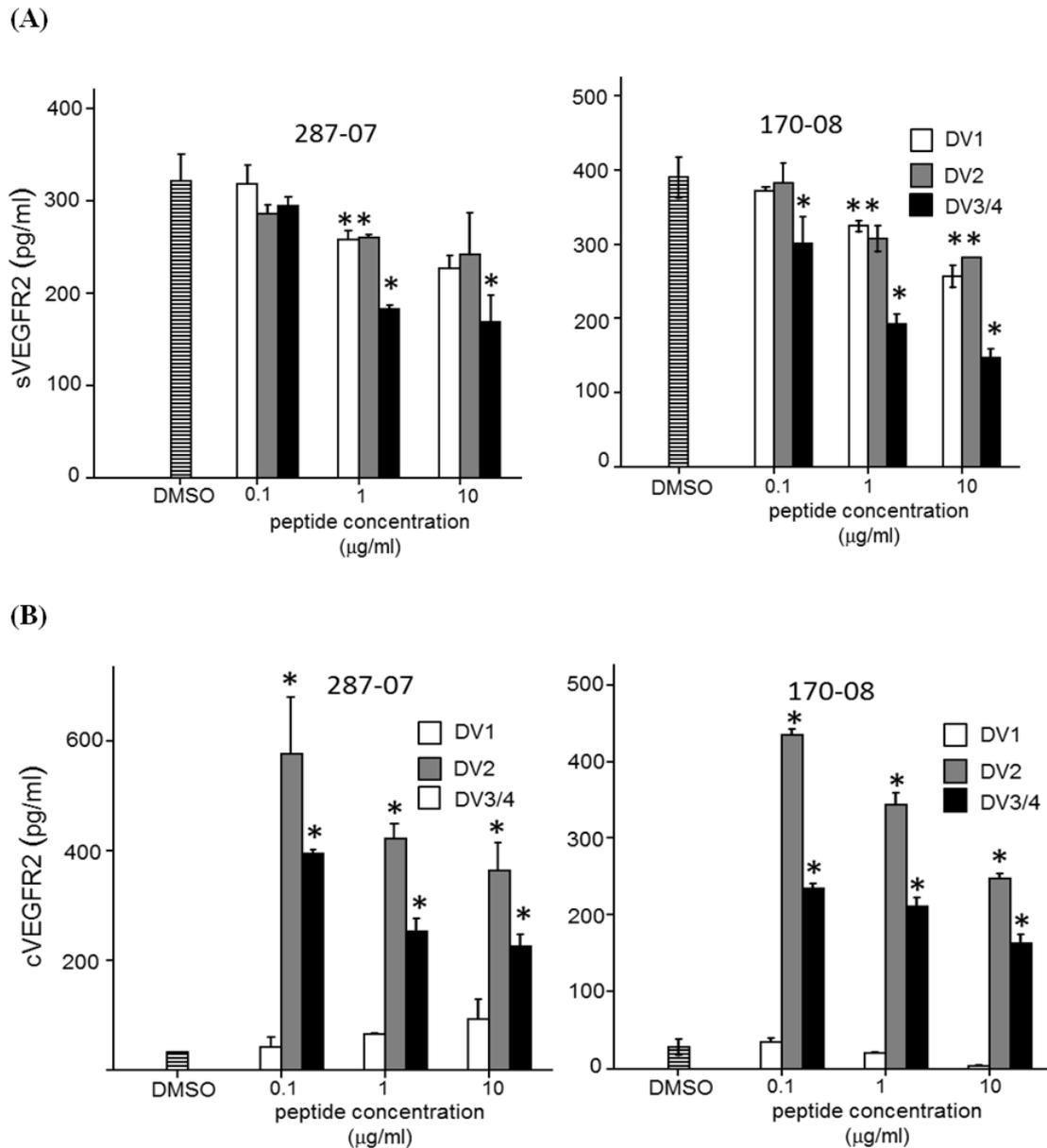


Figure 2. Modulation of soluble and cell-associated VEGFR2 by DV-specific T cell lines. T cell lines were cocultivated with confluent monolayers of HUVECs and stimulated with the indicated peptides at various concentrations or with DMSO. The levels of VEGFR2 in the supernatants (A) or cell lysate (B) at 48 hours after T cell activation were determined by ELISA. * indicates difference from DMSO control ($p < 0.05$).

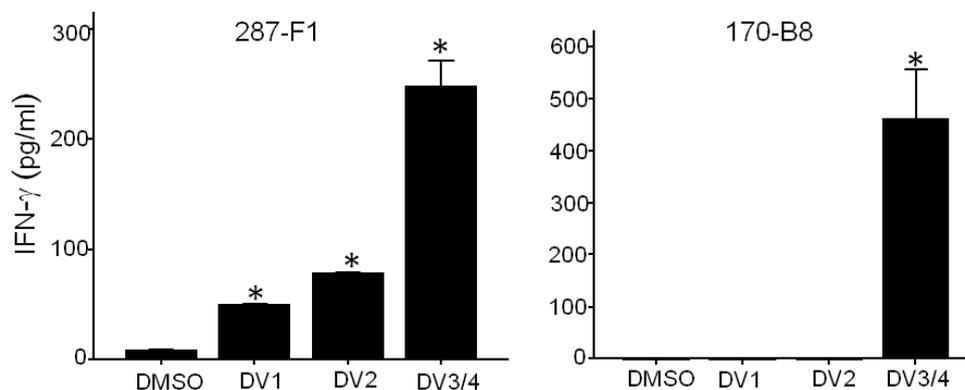


Figure 3. Peptide specificity of DV-specific T cell clones. T cell clones derived from primary T cell lines were stimulated with DV peptides at 10 µg/ml and the levels of IFN-γ in culture supernatants were measured 48 hours later by ELISA. * indicates difference from DMSO control ($p < 0.05$).

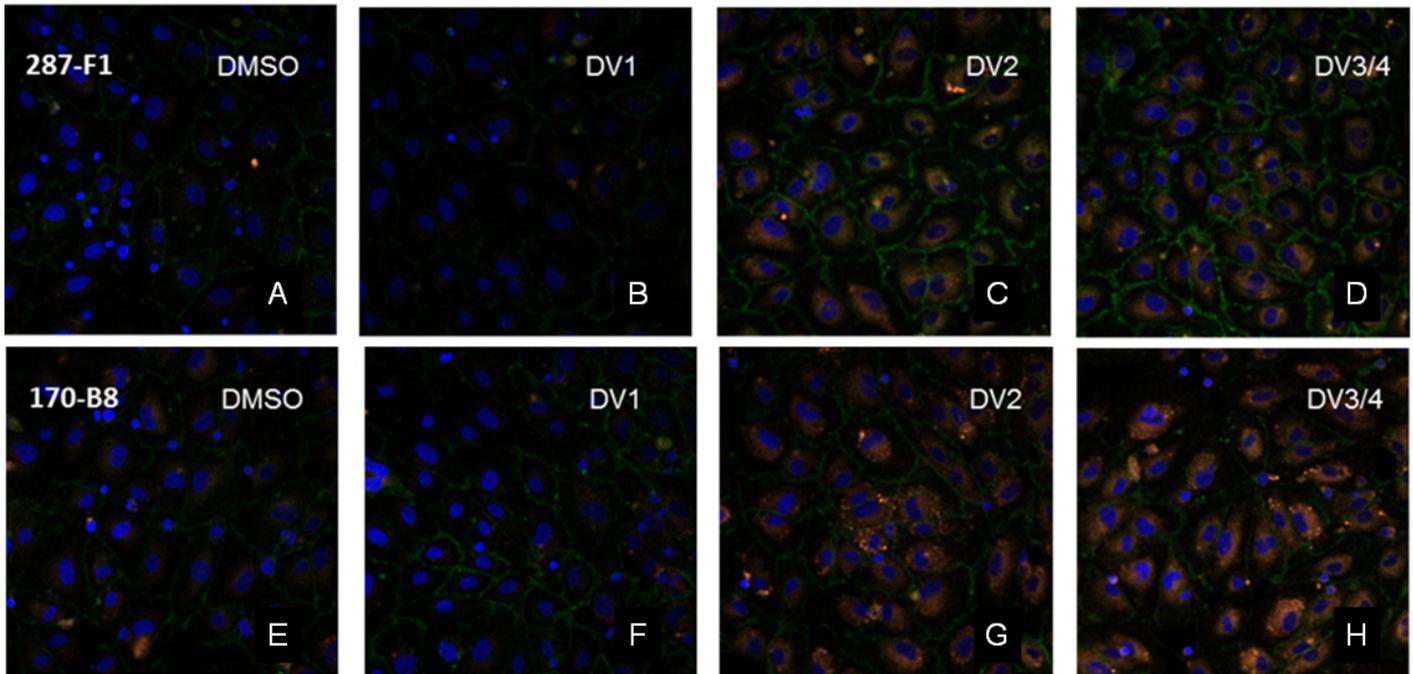


Figure 4. Modulation of endothelial cell VEGFR2 expression by activated DV-specific T cells. Confocal microscopy of HUVECs monolayers cocultivated with either 287-F1 T cell clone (A-D), or 170-B8 T cell clone (E-H). T cells were activated with DV1, DV2, DV3/4 peptide, or DMSO control. T cells were removed after 48 hours of cocultivation and the endothelial monolayers were stained with anti-CD31 (green), anti-VEGFR2 (orange), and DAPI (blue) and examined by confocal microscopy.

clones were derived. Two cell clones that proliferated well were chosen for further expansion: 1) 170-B8 T cell clone derived from 170-08 T cell line, 2) 287-F1 T cell clone derived from 287-07 T cell line. We analyzed peptide specificity of these clones and found that they exhibited specificity that reflected the specificity of the parental cell lines; 170-B8 T cell clone showed reactivity restricted to DV3/4 NS3 peptide while 287-F1 T cell clone which were derived from 287-07 primary T cell line exhibited broader reactivity with a clear response to DV1 and DV2 NS3 peptide albeit much weaker than the response to DV3/4 NS3 peptide (**Figure 3**). To examine the effects of these T cell clones, these T cell clones were cocultivated with HUVECs and activated with DV1, DV2, or DV3/4 NS3 peptides, or with peptide solvent control (DMSO). At 48 hours after T cell stimulation, HUVECs monolayers were stained for VEGFR2 and examined with confocal microscopy.

Activation of T cell clones resulted in a clear up-regulation of VEGFR2 expression by HUVECs (**Figure 4**). The effect was peptide-specific as only DV2 and DV3/4 NS3 peptides were able to exert this effect. The patterns of peptide specific up-regulation of VEGFR2 were different in the two T cell clones examined; DV2 and DV3/4 appeared to upregulate VEGFR2 expression to a similar extent when used to activate 287-F1 T cell clone while DV3/4 peptide was more effective than DV2 peptide in the upregulation VEGFR2 mediated by 170-B8 T cell clone.

Discussion

Increased vascular permeability is a pivotal event in dengue that leads to plasma leakage, volume depletion, shock, and organ failure in severe cases. Hemorrhage is another manifestation contributing to severity in dengue.⁴ The

mechanisms of plasma leakage and bleeding disorder in dengue are not well understood but likely involve the activation of endothelial cells leading to changes in vascular integrity and activation of the coagulation system.

Histological studies in fatal human cases have demonstrated mild endothelial cell swelling accompanied by tissue edema.²² Endothelial cell apoptosis has been demonstrated in some studies although the extent of cell death seemed inconsistent with plasma leakage in DHF.^{23,24} These findings in conjunction with the rapid recovery of DHF cases after the critical phase of the illness suggests that transient disturbance of vascular integrity rather than structural damage is the underlying mechanism. In connection with this notion, recent studies have demonstrated changes in the levels of angiogenic factors and their receptors that may contribute to enhanced permeability in dengue.^{11,25,26} We have previously reported the elevated levels of plasma VEGF that were associated with the suppressed levels of sVEGFR2 at the time of plasma leakage in DHF cases and correlated with the extent of plasma leakage.¹¹ In addition, the levels of angiotensin-1 a functional antagonist of VEGF-A have been shown to be suppressed in DHF.²⁶ The combined effect of elevated VEGF, and decreased sVEGFR2 and angiotensin-1 may be an increase in permeability and activation of the coagulation system, two critical processes in the pathogenesis of DHF and other viral hemorrhagic fevers.

Secondary infection is a well-recognized risk factor for DHF. Activation of cross-reactive T cells that have been primed during a previous infection is thought to contribute to disease severity during a secondary DV infection.²⁷ In this study we demonstrated that T cells generated from patients with a secondary dengue infection can recognize peptide epitopes

derived from other dengue viral serotypes. The activation of predominantly DV3/4 peptide specific T cells in patients with a secondary infection by either DV2 or DV4 suggests that these responses represent activation of memory T cells specific to primary infecting DV serotypes. Activation of CD8⁺ T cells resulted in modulation of surface expression of a receptor of VEGF, namely VEGFR2. Up-regulation of cell expression of VEGFR2 was observed while the production of soluble form of VEGFR2 by endothelial cells decreased in the presence of T cell activation. This is consistent with the decreased levels of soluble VEGFR2 observed during dengue infections and the relatively lower levels of soluble VEGFR2 in DHF cases in comparison to those of DF cases.¹¹ Increased expression of cell-associated VEGFR2 may increase the sensitivity to VEGF stimulation and lead to endothelial activation and plasma leakage, and coagulopathy. We have previously shown that DV induced up-regulation of VEGFR2 expression enhanced VEGF responsiveness as measured by receptor tyrosine phosphorylation. It remains to be directly demonstrated whether this occurs in endothelial cells co-cultured with activated T cells. Elevated levels of VEGF have also been reported in lung edema fluid and in serum from Hantavirus-infected patients.²⁸ Furthermore, decreased levels of soluble VEGFR2 have also been reported in this disease.²⁹ Since plasma leakage and hemorrhage are two common features of viral hemorrhagic fevers, these findings suggest that alterations in VEGF and its receptors secondary to CD8⁺ T cell activation may be a common pathogenic mechanism in viral hemorrhagic fevers.

Our study demonstrated that endothelial cells can be affected as a by-stander to T cell activation. However, endothelial may also be directly affected by T cells as antigen presenting cells presenting viral peptide epitopes in the context of HLA class I or class II. Studies have demonstrated the presence of DV envelope protein in endothelial cells in various anatomical sites including lung capillaries and hepatic sinusoidal cells.^{24,30} However, antigen presentation via HLA class I pathway is most effective when antigens reside in cytosol which occurs within virally infected cells. Literature regarding direct infection of endothelial cells by DV is conflicting. Some, but not other, studies have demonstrated the presence of DV genome or expression of nonstructural proteins of DV within endothelial cells indicating direct viral infection.^{24,30,31} Alternatively, presentation in the context of HLA class I molecules may occur through antigens taken by endothelial cells into the endosomal pathway and processed by cross-presentation to HLA class I pathway.³²

Expression of VEGFR2 by endothelial cells may be regulated by transcription and post-transcriptional process. The balance between soluble and cell-associated isoforms of VEGFR2 may be regulated by several processes. The soluble isoform of VEGFR2 has been reported to be generated by differential splicing of mRNA as well as by posttranslational enzymatic cleavage of cell surface molecules.^{17,33} Activated CD8⁺ T cells may affect both processes by altering the preferentially expressed isoform or by regulating the enzymatic activity involved in the generation of soluble VEGFR2. The potential enzymes involved in this process include a disintegrin and

metalloproteinase-10 (ADAM-10) and ADAM-17.^{33,34} Further studies will be required to identify molecular mechanisms by which CD8⁺ T cells regulate the expression of VEGFR2 by endothelial cells.

Activation of T cells is associated with changes in cell surface markers and functions. Studies have demonstrated that cross-recognition of DV-specific CD8⁺ T cells is associated distinct patterns of cytokine production.³⁵ We have shown in this study that there was no correlation between the levels of IFN- γ produced in response to peptide stimulation with the magnitudes of VEGFR2 expression modulated by T cells; all peptides were able to modulate VEGFR2 expression regardless of their ability to induce IFN- γ production. This indicates that the activation threshold required to induce this effect by CD8⁺ T cells is different than the intensity of activation required for IFN- γ production. Future studies will be needed to understand the differences in signaling requirements for these T cell to mediate these distinct effects. Nevertheless the broad recognition of cross reactive T cells that leads to modulation of VEGFR2 expression may allow for the development of plasma leakage during a secondary infection with various serotypes. This is consistent with the observation that DHF can occur with any combinations of sequences of infection.

The limited numbers of T cell lines and clones which recognized only one DV NS3 epitope examined in this study likely do not represent the whole repertoire of antigen specificity and functional spectrum of dengue-specific T cells *in vivo*. However, this *in vitro* co-culture model demonstrates a novel mechanism that may be relevant to T cell mediated plasma leakage. Further studies to investigate the frequencies of effector T cells with this functional phenotype in primary and secondary DV infection will be needed to evaluate the significance of this mechanism.

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