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Original Article

Dengue Virus neither Directly Mediates Hyperpermeability nor Enhances Tumor Necrosis Factor-α-Induced Permeability In Vitro

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SUMMARY: The mechanisms of endothelial barrier dysfunction in dengue disease remain poorly understood. Endothelial cell (EC) death due to virus infection or in combination with an infection-induced cytokine storm is deemed as one of the major causes of plasma leakage. Using an in vitro model of human endothelia and several dengue virus (DENV) strains (including a clinical isolate), the direct consequence of infection on endothelial permeability was investigated throughout the course of the infection. All employed DENV-2 strains were able to infect and replicate in ECs. Rather than increase endothelial permeability, DENV infection alone enhanced cell barrier integrity up to 7 days postinfection. Improved cell barrier function was mediated by type I interferon activation at the early phase of infection and by the survival advantage of the infected cells at the late phase of infection. Consistent with this phenomenon, DENV infection did not augment tumor necrosis factor-α-induced permeability. Our results prove that DENV infection does not directly account for vascular permeability; DENV neither induces hyperpermeability nor exacerbates the permeabilizing effect of cytokines. The contributory role of other factors on plasma leakage during dengue disease warrants further investigation.

INTRODUCTION

Dengue viruses (DENVs; serotypes 1–4) are mosquito-borne viruses of global health importance. An estimated 2.5 billion people in more than 100 countries are at risk of acquiring DENV infections, with more than 50 million new infections projected annually (1). DENV is a single-stranded, positive-sense RNA-containing enveloped virus that belongs to the *Flaviviridae* family (2).

DENV most commonly causes a mild febrile illness, called dengue fever, and less frequently, a life-threatening illness known as dengue hemorrhagic fever (DHF). Some cases of DHF are accompanied by hypovolemic shock, which is also known as dengue shock syndrome (DSS) (3). Plasma leakage is a hallmark of DHF/DSS that implies pathology to the vascular endothelium (4). However, until now, a defined mechanism leading to plasma leakage has remained poorly understood.

Many hypotheses have been offered to explain vascular leakage in DHF/DSS; the most common of which is that a cytokine storm led by immune responses to DENV infection creates a transient disruption of endothelial cell (EC) barrier function (4). However, a cytokine storm does not exclusively occur in DHF/DSS but is also present in other inflammatory conditions (5,6), which, in contrast, do not have the characteristics of vascular leakage in DHF/DSS.

ECs are permissive to DENV infection in vitro (7–9) as well as in human (10) and murine models (11), indicating that DENV infection of ECs may also contribute to its pathogenesis. DENV-induced apoptosis of infected ECs may lead to increased permeability (12,13). However, several reports have demonstrated that DENVs are not cytopathic to ECs in vitro (14,15), thereby contradicting this hypothesis. DENV antigens have been detected in human ECs but with minimal or no cellular destruction (10). The role of direct DENV infection of ECs in vascular leakage is not well understood, and thus remains an important issue to be addressed.

To shed light on the mechanism of vascular leakage, we assessed whether DENV infection is primarily responsible for EC hyperpermeability. Here we investigated the direct modulation of EC barrier function by different DENV-2 strains as well as possible synergistic effects with the proinflammatory cytokine tumor necrosis factor (TNF)-α on increased cellular permeability. It is worth to note that clinically, vascular leakage syndrome does not develop for several days after infection despite a robust innate immune response that produces proinflammatory and proangiogenic cytokines. The onset of increased vascular permeability in severe cases of dengue disease appears only around the time of defervescence and clearance of viremia (16), which indicates that the determinant of disease related EC permeability occurs particularly during the late infection period. To address this issue, we assessed the permeability of DENV-infected ECs throughout the course of infection in vitro.

MATERIALS AND METHODS

Cells: The C6/36 *Aedes albopictus* mosquito cell line was cultured at 28°C in minimum essential medium

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(MEM). Baby hamster kidney (BHK)-21 cells were purchased from ATCC and cultured at 37°C in MEM. Primary human umbilical vein ECs (HUVECs) were purchased from Clonetics (San Diego, Calif., USA) and propagated and maintained in supplemented EC basal medium-2 (EBM-2) at 37°C in humidified air containing 5% CO_2. HUVECs used for all experimental purposes were grown to confluence in fibrinogen-coated wells. Because membrane receptor expression can be reduced by repeated passages (17), HUVECs used in this study were limited to 3–5 passages.

Virus propagation: DENV2–16681 was obtained from an infectious clone (18), from two established DENV-2 strains (ThNH7-93 and D2-00st isolated from dengue patients in Thailand and the Philippines, respectively), and from clinical strain D2/BD/29/2009 (less than 5 passages). The viruses were propagated in C6/36 cells at 28°C for 7 days. Therefore, the culture supernatant was harvested, centrifuged at 3,000 rpm for 5 min, and filtered through the syringe-driven Millex GV 0.22-μm filter unit (Millipore Co., Bedford, Mass., USA). Virus stocks were stored at −70°C for future use.

Virus infection and titration: HUVECs were infected with DENV-2 at 80–90% confluence. In brief, the medium was removed from the culture dish and the cells washed with phosphate-buffered saline (PBS) (−). Then, either the viruses or C6/36 supernatant was added to the cells and adsorption was induced at 37°C for 2 h in 5% CO_2. After washing with PBS, fresh medium was added. Virus titers in BHK-21 cells were determined using a focus formation assay. In brief, 10-fold dilutions of virus stock were used for infection, and then an overlay of MEM supplemented with 2% fetal calf serum and 1.25% methylcellulose was added after virus adsorption. Therefore, the cells were incubated at 37°C for 4 days in 5% CO_2 until focus staining, which was performed as previously described (19).

Indirect immunofluorescence assay (IFA): Next, DENV-2 antigens were stained and then detected using an indirect IFA. In brief, infected HUVECs on the slide chamber were washed in PBS (−), fixed with 4% paraformaldehyde (WAKO, Osaka, Japan), permeabilized with 1% NP-40 for 20 min at room temperature, and then incubated in Blockace (DS Pharma Biomedical, Osaka, Japan) at 37°C for 60 min. After washing with PBS (−), the cells were incubated with 12D11/7E8 anti-flavivirus antibody (19) at 37°C for 60 min. For the secondary antibody, cells were treated with 1:50 dilution of fluorescein isothionyanate (FITC)-goat anti-mouse IgG at 37°C for 60 min (Bethyl Laboratories, Inc., Montgomey, Tex., USA). The slides were covered with Syva microtech mounting fluid (Vector Laboratories, Inc., Burlingame, Calif., USA) and observed under a fluorescence microscope.

Flow cytometry: Confluent HUVECs were infected with DENV-2 in 24-well plates and harvested at the indicated times. After washing with PBS, the cells were fixed with 2% paraformaldehyde and permeabilized with IC Perm™ buffer (Biosouce, Camarillo, Calif., USA), according to the manufacturer's instructions. The 12D11/7E8 anti-flavivirus antibody was used to detect DENV-2 E glycoprotein using flow cytometry as previously described (19), and the cells were analyzed using a FACSCalibur™ and CellQuest software (Beckton Dickinson, Spark, Md., USA) with a 488 nm laser to detect Alexa Fluor 488 fluorescence.

EC permeability assay: EC permeability assays were performed using a previously described protocol (20) with minor modifications. In brief, approximately 90% confluent HUVECs were harvested using 0.25% trypsin (Sigma, St. Louis, Mo., USA), washed twice with HBSS (Gibco, Gaithberg, Md., USA), and seeded into fibronectin (25 μg/ml; Sigma)-coated 24-well transwell polycarbonate membrane tissue culture dishes (6.5-mm diameter, 3.0-μm pore size; Corning Costar, Cambridge, Mass., USA) at a density of 1×10^5 cells per well, which were placed into a lower chamber containing 600 μl of EBM-2 complete medium. After 1–2 days, confluent HUVECs were infected with different DENV-2 strains, mock medium (C6/36 supernatant), or left uninfected. At 2 or 6 days postinfection, the cells were starved overnight with EBM-2 0.5% bovine serum albumin without growth factors. To determine macromolecule paracellular permeability, 0.5 mg/ml of FITC-conjugated 70-kDa dextran (FITC-dextran) (Sigma) was added to the upper chamber. After incubation for 2 h at 37°C, FITC-dextran present in the lower chamber was assayed using a Perkin-Elmer fluorimeter (490-nm excitation, 530-nm emission). Fluorescence intensity measurements were expressed directly or as the increase in percentage over that of an uninfected control.

For neutralization experiments, HUVECs were infected for 12 h with DENV2-16681 at a multiplicity of infection (MOI) of 0.1 or left uninfected and then treated with different concentrations of an anti-interferon (IFN)-β antibody cocktail containing anti-human IFN-β (PBL Interferon Source, Piscataway, N.J., USA) and anti-human IFN-α/βR2 (CD118) (PBL Interferon Source). Cell permeability was measured on day 3 or 7 postinfection. In another experiment, uninfected or infected HUVECs were treated 24 h postinfection with different concentrations of rIFN-α and rIFN-β (PBL Interferon Source), and permeability was measured on day 3 and 7 postinfection. For TNF-α analysis, the infected and uninfected cells as described above were treated with TNF-α (10 ng/ml; PeproTech, Inc., Rocky Hill, Conn., USA), and permeability was measured 18 h post-treatment.

HUVEC morphology and survival: HUVECs were grown to confluence in 12-well plates (Nunc, Roskilde, Denmark), infected with DENV2-16681 at an MOI of 1 or mock infected, and then starved by substituting normal EC growth medium for basal medium (EBM-2 with 5% serum minus growth factors) 24 h postinfection. On day 7 postinfection, the medium was removed and the cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and then observed for morphological changes under a light microscope.

HUVEC viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay, according to the manufacturer's protocol (Promega, Madison, Wis., USA). HUVECs were seeded into 96-well plates at a density of 1×10^4 cells/well in 0.1 ml of medium. Confluent cells were treated as described above. Cell viability was monitored at several time points. Next, 15 ul of the dye solution was added to the cells and incubated at 37°C in a

humidified atmosphere of 5% CO_2. After 4 h, 100 μl of stop solution was added, incubated for 1 h, and then mixed. The absorbance was recorded at an optical density of 570 nm, using a 96-well plate reader. The experiment was performed in quadruplicate.

Quantitative real-time RT-PCR: To measure mRNA levels of type-1 IFN and tight junction proteins, total RNA was isolated from DENV-infected HUVECs at different time points using the RNeasy Mini Kit (Qiagen, Valencia, Calif., USA). RNA (500 ng–1.0 μg) in a total volume of 20 μl was reverse transcribed using the Superscript III (Invitrogen, Carlsbad, Calif., USA) with an oligo(dT) 12–18 primer (Invitrogen). Three microliters of the product was used for SYBR green real-time PCR (Applied Biosystems, Foster City, Calif., USA), which was performed in triplicate. Primer sequences for the target genes are listed in Table 1. For type-1 IFN, cellular mRNA expression was measured through relative quantification using the Pfaffl method (21) and normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The fold change of tight junction gene expression was calculated relative to control cells using the $2^{-\Delta\Delta CT}$ method (22) and normalized to GAPDH gene expression. DENV RNA copy numbers were calculated using the TaqMan real-time RT-PCR with specific probes, as previously described (23). The number of DENV-2 RNA copies was calculated using absolute quantification that was based on in vitro-transcribed DENV-2 RNA standards.

Table 1. Primers employed for real-time RT-PCR analysis

Gene	Primer sequence (5′ to 3′)
Primate IFN-β	
Forward	TGCCTCAAGGACAGG ATGAAC
Reverse	GCGTCCTCCTTCTGGAACTG
Primate IFN-α	
Forward	GCCTGAAGGACAGACATGACTTT
Reverse	GGATGGTTTCAGCCTTTTGGAAC
Primate GAPDH	
Forward	AAATCAAGTGGGGCGATGCTG
Reverse	CAAATGAGCCCCAGCCTTCTC
VE-cadherin	
Forward	TGGGCACCTCTGTGGGCT
Reverse	GTCGCCCCGCAAGATGCT
ZO-1	
Forward	AGCCCACCCCAGCTCCTT
Reverse	GCTGCCTCAGTACTTGGTGTG

Vascular endothelial growth factor (VEGF) ELISA: VEGF protein levels were measured in the supernatant of DENV-infected HUVECs using the Human VEGF Quantikine ELISA Kit (R&D, Minneapolis, Minn., USA), according to the manufacturer's instructions.

Statistical analysis: For statistical analysis, SPSS 16.0 was used. T-test was used to compare the means of 2 sample populations and one-way ANOVA was used to compare the means of more than 2 sample populations. P value < 0.05 was considered statistically significant. Data are presented as the means ± SD for at least 2 separate experiments.

RESULTS

ECs support productive DENV replication: The reported permissiveness of ECs to DENV infection varies among several studies. In the present study, a more in-depth examination of EC permissiveness was conducted by using 3 complementary detection methods: (i) IFA to identify cells that express the DENV envelope protein; (ii) focus assay to confirm productive viral replication; and (iii) FACS to determine the infec-

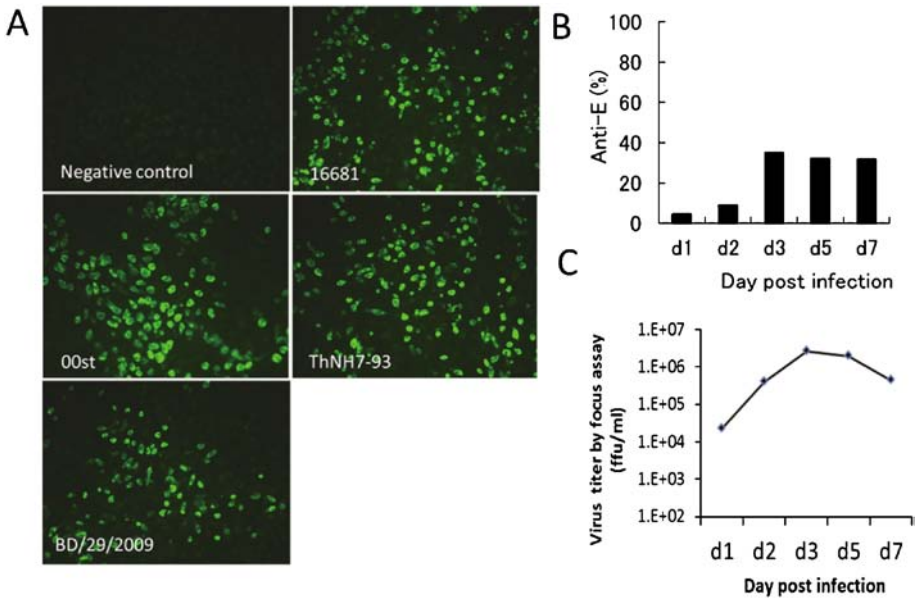

Fig. 1. ECs support productive DENV replication. (A) HUVECs were infected with different strains of DENV-2 at MOI 0.5. At day 3 postinfection, cells were stained with 12D11/7E8 anti-flavivirus antibody plus FITC conjugated goat anti-mouse IgG and observed using fluorescence microscope. (B) Cells were harvested at different times postinfection, stained with Alexa-conjugated mouse anti-DENV E antibody, and analyzed using FACS. Numbers indicate the percentage of positively stained cells. (C) Culture supernatants from infected cells were collected at the indicated days postinfection and virus titers were determined by focus assay in BHK-21 cells.

tion rate. All virus strains were employed for IFA and only 1 (strain 16681) was used for the other 2 assays.

HUVECs exposed to all DENV-2 strains, including clinical isolate D2/BD/29/2009, at a relatively low MOI of 0.5 were positively stained with anti-envelope antibody (Fig. 1A). Infectivity of the clinical isolate was comparable with that of the established strains, which strongly demonstrated the permissiveness of HUVECs to DENV infection.

The temporal replication kinetics of DENV-2 in HUVECs were examined using a time-course experiment. Confluent HUVECS were infected with strain 16681 at an MOI of 0.5, harvested at different times postinfection, and then analyzed for intracellular DENV envelope expression using the FACS assay (Fig. 1B). In parallel, titers of virus particles secreted in the supernatant of BHK-21 cells were measured (Fig. 1C). DENV infection of the HUVECs rapidly resulted in a titer of 10^4 FFU/ml in cell supernatant 1 day postinfection (Fig. 1C). Both the virus titer and the rate of infection peaked at about day 3 postinfection (Figs. 1B and 1C). Up to 35% of the infected cells were positively stained for the DENV envelope antigen (Fig. 1B). These findings demonstrated that DENV-2 can efficiently infect HUVECs, thereby resulting in productive replication and release of infectious virions.

DENV infection does not increase EC permeability, but rather improves cell barrier integrity: Because DENV infection of ECs was suggested to increase permeability through cell apoptosis, we examined the direct consequences of DENV-2 infection on cell barrier function. Confluent HUVECS grown on fibrinogen-coated transwell membranes were infected with different DENV-2 strains and macromolecule cell permeability was measured at 3 and 7 days postinfection (the early and late infection phases, respectively). DENV infection alone did not induce endothelial permeability but rather improved cell barrier function, as indicated by the decreased permeability of FITC-dextran (Fig. 2). This effect occurred in a dose-dependent fashion and was more pronounced during the early infection phase. Similar results were observed for different DENV-2 strains infected at the same MOI, in which permeability decreased up to approximately 60% compared with that of the uninfected control cells.

Cell junction molecules are responsible for maintaining the integrity of ECs. To confirm the observed enhancement of barrier function in DENV-infected ECs, mRNA expression levels of the junction molecules ZO-1 and VE-cadherin were monitored throughout the course of infection by real time RT-PCR. ZO-1 transcript levels increased at day 3 postinfection, and then decreased (Fig. 3A). Although not statistically significant, VE-cadherin mRNA levels increased in response to DENV 48 h postinfection (Fig. 3B). The up-regulation of early-phase junction protein expression in the present study during DENV-2 infection appears to support the phenomenon of enhanced EC barrier integrity.

DENV-2 activates type–1 IFN at the early infection phase to regulate EC barrier: Type-1 IFN enhances EC barrier function (24,25), and IFN induction has been observed in HUVECs in response to DENV infection (26). Therefore, we initially verified IFN activation by DENV-2 infection in our HUVEC model by measuring

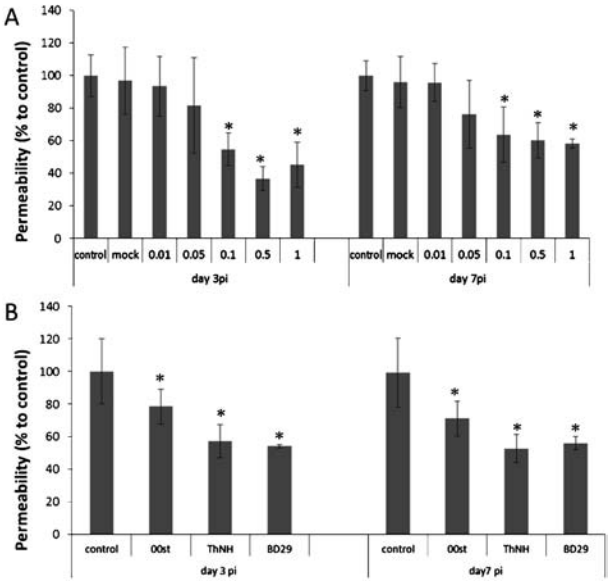

Fig. 2. DENV improves cell barrier function in a dose-dependent manner. (A) Confluent HUVECs on collagen-coated transwell membranes were infected with DENV-2 strain 16681 at various MOI. FITC-dextran macromolecule permeability was determined at 3 and 7 days postinfection. (B) ECs were infected with different strains of DENV-2 at MOI 0.1 and macromolecule permeability was measured at the indicated times. Results were derived from at least 2 different experiments with 4 replicates each and permeability was calculated as percentage relative to control. Data are means ± SD; $^*P < 0.05$ vs. untreated control.

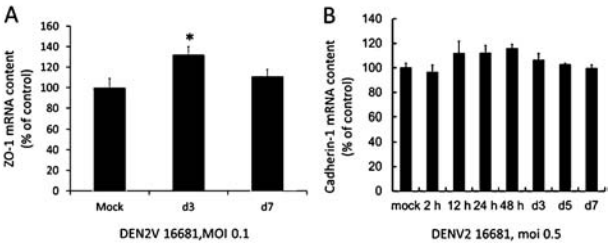

Fig. 3. DENV modulates cell junction adhesion molecules. Time course quantitative real-time RT-PCR analysis of mRNA expression of (A) ZO-1 and (B) cadherin-1 was performed on HUVECs infected with DENV-2 16681. Results were normalized to internal control GAPDH mRNA and expressed as percentage increase in levels relative to control.

IFN mRNA expression. Total RNA was harvested from cells infected at an MOI of 0.1, and IFN-α and -β mRNA levels were quantified. All strains of DENV-2 activated IFN-β, whereas strain ThNH7-93 activated both IFN-α and -β. IFN activation peaked at 24 h postinfection (Figs. 4A and 4B). Beyond 48 h postinfection, IFN inductions diminished, and UV-inactivated viruses were unable to induce IFN expression (data not shown), indicating that active infection is a requirement for IFN activation. Because virus RNA acts as a pathogen-associated molecular pattern that triggers the IFN activation, DENV RNA expression was also monitored. High intracellular viral RNA levels of all DENV-2 strains (Fig. 4C) were detected, which correlated well with IFN induction in the infected cells (Figs. 4A and 4B).

To validate the role of IFN in the enhancement of

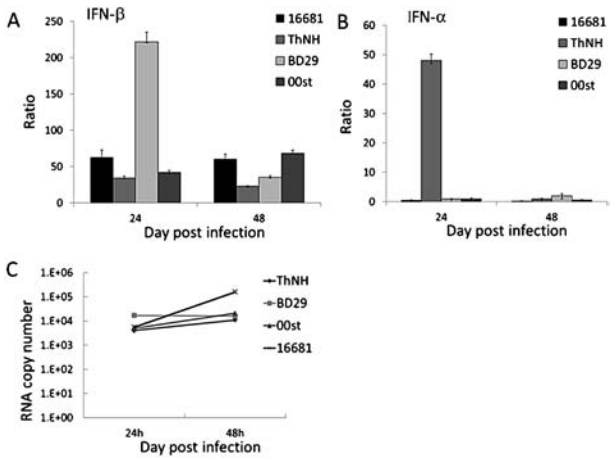

Fig. 4. DENV-2 induces type-1 IFN activation. HUVECs were infected with different DENV-2 strains at MOI 0.1 and total RNA was harvested from cells at the indicated times. RNA levels were quantified via quantitative real-time RT-PCR. (A, B) IFN-β and -α mRNA expression levels were expressed as fold increase over mock treated cells and normalized to GAPDH. (C) The number of copies of DENV-2 RNA was calculated using absolute quantification based on in vitro-transcribed DENV-2 RNA standards.

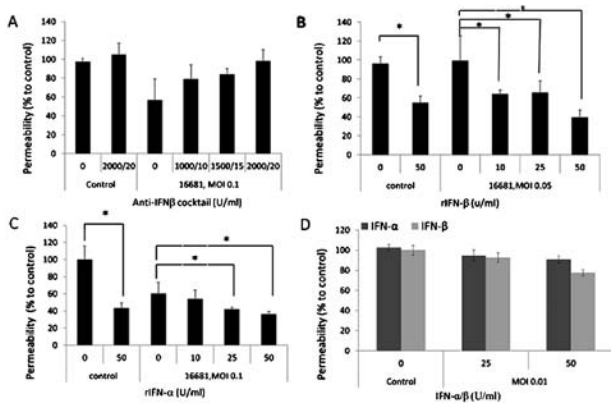

Fig. 5. Type-1 IFNs regulate endothelial barrier function at early phase of infection. (A) Infected cells at low MOI of DENV-2 16681 were treated at 12 h postinfection with anti-IFN-β antibody cocktail or left untreated. Each cocktail contains anti-human IFN-β mixed with anti-human IFN-α/βR2 at specific concentrations. Anti-human IFN-β at concentrations of 1,000, 1,500, and 2,000 units/ml was mixed with anti-human IFN-α/βR2 at concentrations of 10, 15, and 20 U/ml, respectively (labeled in the figure as 1,000/10, 1,500/15, and 2,000/20). Permeability was determined on day 3 postinfection. Untreated cells and uninfected cells treated with a concentration designated as 2,000/20 of anti-IFN-β cocktail were used as controls. (B, C) HUVECs infected with DENV-2 16681 at MOI of 0.05 and 0.1 were treated with IFN-β and IFN-α, respectively. Uninfected cells treated with 50 U/ml of IFN-β or IFN-α were used as positive controls. Permeability was assessed on day 3 postinfection. (D) HUVECs infected with DENV-2 16681 at MOI 0.01 were exposed to different concentrations of IFN-β and IFN-α and permeability was determined on day 7 postinfection. Uninfected cells were used as controls.

barrier properties in DENV-infected ECs, a dose-dependent analysis of IFN effects on endothelial permeability was performed. Following treatment of DENV-infected cells with different concentrations of an anti-IFN antibody cocktail to neutralize IFN biological function, macromolecular permeability was assessed at 3 days postinfection. Addition of anti-IFN antibody cocktail negated the effect of DENV infection in a dose-dependent manner by raising the permeability of the infected cells to basal levels, as demonstrated by the untreated controls (Fig. 5A). In a follow-up study, the effects of type-1 IFNs on EC barrier function was evaluated in both the infected and uninfected cells. IFN-α and -β both stabilized barrier function in a dose-dependent manner (Figs. 5B and 5C). A 50 U/ml IFN treatment improved cell integrity by more than 2-fold (Figs. 5B and 5C), a rate quite similar to that of cells infected with an MOI of 0.5–1.0 (Fig. 2A). Taken together, these results strongly indicate the direct impact of type-1 IFN on EC barrier function enhancement.

In order to elucidate whether type-1 IFN retains its biological effects that promote EC barrier function beyond 3 days postinfection, cells exposed to a low MOI of 0.01 were treated with IFN-α and -β and permeability was measured at day 7 postinfection. The effect of IFN on cell barrier improvement diminished over time (Fig. 5D), thereby indicating that type-1 IFNs regulate cell barrier function at the early infection phase and to a lesser extent at the late phase.

DENV confers a survival advantage to ECs at the late infection phase: In order to explain the higher cell barrier integrity at the late phase of DENV-2 infection (Fig. 2), we examined the survival rate of DENV-infected ECs. Beyond day 3 postinfection, DENV-infected ECs displayed a higher rate of cell survival compared with the uninfected cells, as observed by changes in cell morphology (Fig. 6A) and quantified using the MTT assay (Fig. 6B). This effect was more pronounced in growth factor withdrawal conditions, when the cells were subjected to greater stress. DENV-infected HUVECs released low VEGF levels (Fig. 6C), which may account for the survival advantage of the infected HUVECs during the late infection phase and consequent improvement in EC barrier function.

DENV infection does not augment TNF-α-induced endothelial permeability: A number of cytokines induced during DENV infection can directly mediate endothelial permeability. TNF-α, one of the most potent permeabilizing agents, circulates at high concentrations during dengue diseases (27). We wondered whether the enhanced cell barrier function of DENV-infected ECs would have an effect on TNF-α-induced permeability. We treated mock- or DENV-2-infected ECs (MOI, 0.01–1 for 16681 and 0.1 for other DENV-2 strains) with TNF-α at a concentration of 10 ng/ml on days 2 and 6 postinfection (early and late infection phases, respectively). TNF-α alone had a strong permeabilizing effect on ECs (Figs. 7A and 7B). DENV infection partially inhibited this effect (Fig. 7A). Similar results were observed for other DENV-2 strains (Fig. 7B). These findings demonstrated that TNF-α-induced permeability was not augmented by a combined DENV infection.

DISCUSSION

DHF/DSS are characterized by increased vascular permeability, resulting in hemorrhagic and edematous diseases (1). DENV infection of ECs has been proposed to contribute to this pathophysiology; however, the mechanism by which infected ECs modulate vascular

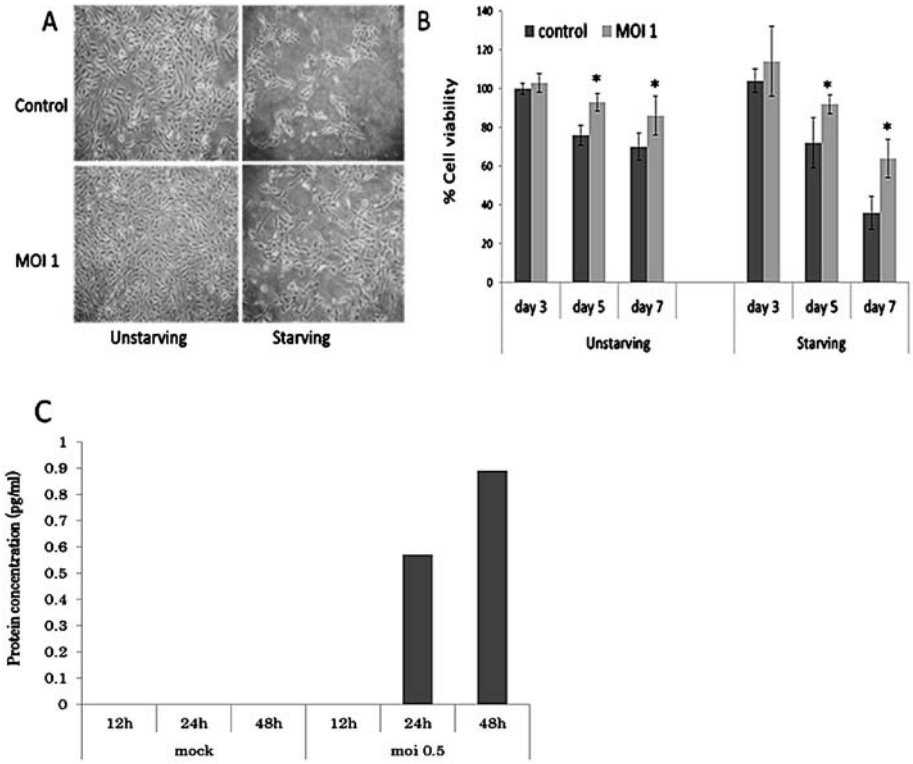

Fig. 6. DENV infection confers survival advantage to HUVECs at late phase of infection. (A) HUVECs were grown to confluence in normal EC growth medium and infected with DENV-2 16681 at MOI 1 or left uninfected. For starving cells, the medium was switched to basal medium without growth factor and 0.5% serum supplement 24 h after infection. Seven days postinfection, cell morphology was observed under light microscope. (B) Confluent HUVECs on 96-well plates were infected at MOI 1. Cell viability was determined by MTT assay at the indicated days. (C) VEGF levels in supernatants from DENV 16681-infected HUVECs were quantified using ELISA.

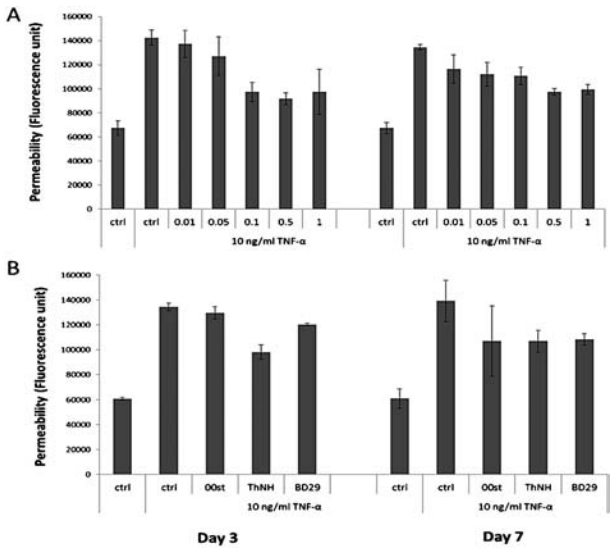

Fig. 7. DENV-infection does not augment TNF-α-mediated hyperpermeability. (A) HUVECs were infected with DENV-2 16681 at various MOI or (B) with different strains of DENV-2 at MOI 0.1. By day 3 or 7 postinfection, cells were treated with 10 ng/ml TNF-α and macromolecule permeability was measured 18 h later.

leakage remains unclear. The permissiveness of ECs to the consequences of DENV infection is also a subject of debate. Many studies have demonstrated that ECs are permissive to DENV infection, but the results are varied. Aside from the different methodologies employed, variation in the origin of ECs and virus strains chosen are also sources of inconsistency. For example, some studies employed an ECV304 cell line (12,28,29), which is reported to be a bladder carcinoma rather than endothelial in nature (30). In addition, ECs from different tissue origins, such as LSEC-1, HMEC-1, or HPMEC-ST1.6R, which may have different characteristics, responded differently to DENV infection (9,15,31,32). In the present study, primary HUVECs isolated from normal human umbilical vein were evaluated because they are more relevant to the pathophysiology of DHF/DSS and the most commonly used model to investigate macromolecule transport (33,34). Here we showed that all DENV-2 strains were able to infect ECs and replicate actively (Fig. 1). At a low MOI of 0.5, a relatively high percentage of ECs (approximately 35%) were infected. However, our findings are in contrast to the study of Arévalo et al., who reported that only a small portion (merely 2%) of ECs were infected by the same molecularly cloned DENV-2 16681 even at a high MOI of 20 (35). One possible explanation for this discrepancy is the history of viral passage. Instead, of using low passage, we employed a high passage of molecularly cloned DENV-2 16681, which may have acquired adaptive mutations in cell culture (36,37). Nevertheless, the permissiveness of ECs, as shown in the present study (more importantly to a clinical isolate and various strains of DENV-2) indicates that DENV infection of ECs may contribute to pathogenesis.

Previous studies have attempted to link apoptosis of ECs to the disruption of cell barrier function and its implication in plasma leakage in DHF/DSS (12,13). Consistent with reports by other groups (14,15), phase-contrast microscopy in this study neither demonstrated cytopathic effect nor any morphological changes regarding DENV-infected ECs even at an MOI of 10 (data not shown). Therefore, disruption of EC barrier function and permeability are not due to cell apoptosis or damage.

In line with this observation, instead of increased permeability, DENV-infected ECs exhibited enhanced barrier integrity, which was more pronounced at the early phase of infection (Fig. 2).

ECs induced type 1 IFN activation as an early response to DENV infection (Fig. 4), and neutralization of IFN activity increased permeability (Fig. 5A), strongly suggesting that IFN modulates EC barrier function during DENV infection. This finding was further confirmed by an exogenous treatment with recombinant IFNs (Figs. 5B and 5C). Indeed, type-I IFNs enhance barrier properties using both in vitro (24,25,38) and in vivo models of the endothelium (39,40). This effect appears to be mediated by up-regulation of junctional adhesion molecules (41), directly resulting in the increased integrity of intracellular junctions. Up-regulation of tight junction ZO-1, as observed during the early phase of DENV infection (Fig. 3A), appears to contribute to the augmentation of EC barrier tightness. However, other possible mechanism(s) (e.g., signaling pathways that lead to the assembly of other junctional proteins) may also be involved. In addition, it has been reported that IFN is involved in the maintenance of EC barrier function and contributes to vascular repair through CD73 activation. The primary function of CD73 is to convert adenosine monophosphate (5′-AMP) to adenosine to strengthen adherent junctions, cell surface adhesion molecule expression, and actin cytoskeletal reorganization, which, in turn, augments endothelial barrier function (42). In addition, IFN stimulates proliferation of primary human ECs in a concentration-dependent manner (43). In the present study, no significant increase in cell proliferation upon exposure to DENV at day 3 postinfection (Fig. 6B) was observed, indicating that IFN secretion by DENV-infected ECs may be sufficient to stimulate a physiological response to EC infection, such as the induction of barrier function alterations, but not enough to stimulate cell proliferation.

Beyond 3 days of infection, IFN treatment no longer offered significant improvement to barrier integrity (Fig. 5D). Nevertheless, DENV-infected cells retained enhanced barrier function (Fig. 2). We suggest that this phenomenon is, in part, linked to a survival advantage of the infected cells (Figs. 6A and 6B), most likely through growth factor(s) induced by DENV infection. Low-level VEGF expression was detected at an early point of infection (Fig. 6C). In physiological concentrations, VEGF is a key regulator of angiogenesis, which drives EC survival as well as proliferation and capillary repair (44). In addition to VEGF, other host and/or viral factors responsible for EC survival could not be excluded.

Enhanced barrier properties induced by IFN activity could potentially protect the endothelium from external permeabilizing factors through resistance to their down regulation (38,45). In the present study, TNF-α-mediated permeability was partially inhibited at the early DENV infection phase (Fig. 7), presumably due to IFN activation. A similar phenomenon was observed in another study (46). At a late phase of infection, wherein IFN production and activity are diminished (Figs. 4A, 4B, and 5D), ECs are expected to exhibit a more sensitive response to permeability, which should be augmented further by DENV infection. However, DENV infection reduced the permeabilizing effect of TNF-α (Fig. 7), most likely through a cell survival advantage conferred at the late stage of infection. Contrary to the previous study (46), our results demonstrated that DENV infection does not synergize with TNF-α to increase permeability. We propose that this phenomenon is not only unique to TNF-α but also to other vasoactive substances or cytokines induced by DENV infection. This study has clarified one aspect of dengue; DENV neither directly induces endothelial permeability nor augments the permeability action of cytokines, thereby leading us to postulate that other factors may contribute to disease development.

A defined mechanism for vascular leakage during DENV infection is currently being investigated. Although we have reason to believe that recruitment of effector cells may play a role in this phenomenon, DENV infection directly activated ECs by up-regulation of the intercellular adhesion molecule in a dose-dependent manner (data not shown), in accordance with that reported elsewhere (47). It is well known that activated ECs facilitate leukocyte adherence and, in turn, activate cell adherence (48). Adherence of DENV-infected ECs to a peripheral blood mononuclear cell has been demonstrated (49). Furthermore, inflammatory cytokines interleukin (IL)-6 and IL-8 are upregulated in DENV-infected ECs (15,31,50) and have been detected at elevated serum levels among DHF/DSS patients (51). IL-6 and IL-8 are associated with the recruitment of circulating immune cells to inflammation sites (52,53). A comprehensive cytokine study on DENV infection revealed that ECs secrete chemotactic factors, immune cell effectors, and complement activators that have been linked to immune-enhancing responses (54). Taken together, these findings suggest that DENV-infected ECs may recruit effector cells to sites of intravascular infection and modulate vascular permeability. In addition, other factors that may promote immune-enhancing responses are currently being investigated. Platelet activating factor receptor, which expressed in the plasma and nuclear membranes of leukocytes, platelets, and ECs (55), plays a major role in the pathogenesis of experimental dengue infection (56). The interaction between infected ECs and immune cells is a promising concept to unravel the mechanisms of plasma leakage in dengue disease.

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Conflict of interest None to declare.

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