High dengue virus load differentially modulates human microvascular endothelial barrier function during early infection

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Abstract
Plasma leakage is the main pathophysiological feature in severe dengue, resulting from altered vascular barrier function associated with an inappropriate immune response triggered upon infection. The present study investigated functional changes using an electric cell-substrate impedance sensing system in four (brain, dermal, pulmonary and retinal) human microvascular endothelial cell (MEC) lines infected with purified dengue virus, followed by assessment of cytokine profiles and the expression of inter-endothelial junctional proteins. Modelling of changes in electrical impedance suggests that vascular leakage in dengue-infected MECs is mostly due to the modulation of cell-to-cell interactions, while this loss of vascular barrier function observed in the infected MECs varied between cell lines and DENV serotypes. High levels of inflammatory cytokines (IL-6 and TNF-α), chemokines (CXCL1, CXCL5, CXCL11, CX3CL1, CCL2 and CCL20) and adhesion molecules (VCAM-1) were differentially produced in the four infected MECs. Further, the tight junctional protein, ZO-1, was down-regulated in both the DENV-1-infected brain and pulmonary MECs, while claudin-1, PECAM-1 and VE-cadherin were differentially expressed in these two MECs after infection. Non-purified virus stock was also studied to investigate the impact of virus stock purity on dengue-specific immune responses, and the results suggest that virus stock propagated through cell culture may include factors that mask or alter the DENV-specific immune responses of the MECs. The findings of the present study show that high DENV load differentially modulates human microvascular endothelial barrier function and disrupts the function of inter-endothelial junctional proteins during early infection with organ-specific cytokine production.

INTRODUCTION
Dengue is a mosquito-borne viral disease caused by infection with dengue virus (DENV), which circulates in nature as four antigenically distinct serotypes. The clinical manifestations range from asymptomatic, mild dengue fever, with or without warning signs, through to severe dengue, characterized by plasma leakage, haemorrhage or organ dysfunction [1]. Although the mortality rate of dengue fever is lower than 1 % with early diagnosis and proper medical care, the case-fatality rate for severe dengue remains high at 2.5 % due to the development of complications, such as bleeding, fluid accumulation, shock and organ impairment [2].

Plasma leakage is the main pathophysiological feature in severe dengue, resulting from altered vascular barrier function associated with inappropriate immune response triggered by the infection [3–5]. Exaggerated cytokine responses from antibody-dependent enhancement [6] and complement activation by virus-antibody complexes [7] appear to be responsible for the immunopathology of vascular leakage. Different immune response genes have been shown to be up-regulated during the progressive stages of infection.
DENV infection [8, 9]. The increase in vascular permeability leads to loss of fluid into the extravascular compartments, specifically the peritoneal and pleural surfaces [5]. Vascular damage caused by endothelial cell (EC) apoptosis may also result in haemorrhagic manifestations in the skin [10], the retina [11], the gums, the gastrointestinal tract and the urogenital system [12].

DENV targets and infects the ECs [13], lymph nodes [13], lung [13], liver [13], spleen [13], kidney [13], heart [14], skeletal muscles [14] and immune cells [15, 16], such as macrophages, dendritic cells and leukocytes. ECs function as the regulator of vascular homeostasis, forming a selective barrier that from time to time experiences modulations at the cell-to-cell junctional complexes, cell-to-matrix interactions and the adaptation of the cytoskeleton to tissue needs under specific conditions [17]. The permeability of the endothelial barrier is not constant, but is regulated physiologically by factors such as neurotransmitters, electrolyte balance and immune or inflammatory mediators, including cytokines and chemokines that participate in immune activation and inflammation [18]. Given these characteristics, ECs have been implicated as having a possible role in the immunopathogenesis of severe expression of dengue haemorrhagic manifestations. Dengue-infected ECs enhance viraemia through rapid viral replication and induce immune responses that augment the production of inflammatory cytokines [19, 20]. These responses imply a direct contribution of the infected ECs to capillary leakage and immune enhancement, thus justifying studies focusing on the infected endothelium in the pathogenesis of severe dengue [3].

Earlier studies investigating changes corresponding to the loss of vascular barrier function focused on the examination of ante- and post-mortem biopsies [13, 21, 22], the role of endothelial activation [20, 23, 24], the effects of inflammatory mediators on vascular permeability [25–27] and the expression of endothelial junctional proteins [28, 29]. The structural and functional changes in the vasculature, however, appear to be controversial. On the one hand, the factors affecting vascular permeability have been attributed to functional rather than structural damage, as no marked inflammation is obvious in the leaking surfaces and the shock caused by the infection is recoverable [5, 30]. On the other hand, studies have proposed that the infection of endothelial cells cause structural damage by inducing apoptosis [31–34]. Furthermore, the vascular changes in early infection remain relatively indeterminate, although there are some case reports indicating that plasma leakage starts as early as 2 days after fever onset in dengue patients [35, 36]. Early immune and endothelial activation also correlate with disease severity and the development of vascular leakage [37, 38]. Therefore, studies on the vascular changes and its correlation to immune responses immediately upon dengue infection are essential for a better mechanistic understanding of the immune pathogenesis of dengue virus leading to vascular leakage.

The present study focused on investigating the early effects of DENV infection on the barrier function, cell integrity and membrane permeability of four human microvascular endothelial cells (MECs) derived from the brain, dermal, pulmonary and retinal vasculature, which are typically or occasionally involved in the clinical manifestations associated with severe dengue. Cytokine profiles and the expression of junctional proteins were also studied to correlate immune responses with vascular leakage. The differences associated with using purified and non-purified virus stock (crude supernatant harvested from Vero cells infected with DENV) were also investigated to allay concerns that the degree of purity of the virus stock used for in vitro viral infection of ECs may constitute a confounding factor in the investigation of the early effects of DENV infection [39, 40].

**RESULTS**

**Permeability of purified DENV-infected MECs varies between cell lines and serotypes**

Changes in the impedance (resistance and capacitance) of the MECs (dermal, pulmonary, retina and brain) infected with purified DENV (1–4) at a multiplicity of infection (m.o.i.) of 5 were measured using an electrical cell-substrate impedance sensing (ECIS) system for 24 h post-infection (p.i.). All of the MECs, sans brain, responded in a similar manner, starting with an increase in resistance upon the introduction of the virus, followed by a resistance reduction 1 to 2 h p.i., which persisted for the remaining hours of study (Fig. 1). Although the modulation found in the dermal MEC was not significantly different for any one of the serotypes compared to the uninfected cells (P<0.05), the small changes may still be biologically significant. The resistance of brain MEC was compromised immediately after DENV infection, which then recovered at approximately 2 h p.i., and fluctuated over the remaining hours. The resistance changes induced by the different dengue serotypes generally varied between the MECs, with this happening the most for DENV-1 and DENV-2, and the least for DENV-4.

In general, the capacitance of the infected MECs, with the exception of the brain MEC, decreased upon infection, which then immediately increased within the first 2 h, followed by a fluctuating but progressive reduction leading to recovery (Fig. 2). This was observed for at least one of the serotypes, with it happening the most for DENV-1 in the pulmonary, and the least for DENV-4. Although the modulation in the dermal MEC was not significantly different for any one of the serotypes compared to the uninfected cells (P<0.05), the small changes may be biologically relevant. The pattern was generally the opposite in the brain MEC: the capacitance increased upon infection for each of the four serotypes, and then decreased before increasing again between 8 to 10 h p.i., and eventually decreasing again for the remaining hours (with the exception of DENV-3).

The measurement of impedance (resistance and capacitance) was modelled into barrier function (Rb), cell–substrate interaction (a) and cell membrane capacitance (Cm), which
represents the cell-to-cell interactions, cell-to-matrix interactions and membrane permeability, respectively (Fig. 3a–d). The modulation observed in purified DENV-infected dermal MEC appears to be mainly due to the cell-to-cell interactions (Rb) (Fig. 3a), likely through the modulation of a paracellular pathway. Upon DENV infection, the dermal endothelium responded by restricting the paracellular path, and the barrier function slowly compromised after 1 h p.i. DENV-3 induced a prolonged leakage in dermal MEC over 24 h, while the cells infected with DENV-1, DENV-2 and DENV-4 recovered gradually over time. DENV also altered the paracellular path of pulmonary MEC, with a larger vascular leakage being observed in DENV-1-infected cells (Fig. 3b). The barrier function (Rb) of the pulmonary MEC was compromised 1 h after DENV-1 infection, with slight alterations in the cell-to-matrix interactions (α) and cell membrane permeability (Cm); the function of the paracellular pathway gradually recovered after 4 h p.i., however at 24 h p.i., the level of leakage remained greater than that for cells infected with other serotypes. The modulation in the retinal MEC infected by DENV appeared to be caused by the functional loss of cell-to-cell interactions and increased cell membrane permeability (Fig. 3c). Although DENV-1 altered the barrier function in a similar manner to the other three serotypes, it modulated the membrane permeability differentially, where a reduction was first observed upon infection before the increased permeability, while the other serotypes increased the membrane permeability of the retinal MEC immediately after infection. DENV modulated brain MEC in three respects (Rb, Cm and α), including the functional loss of the paracellular pathway immediately upon DENV infection, followed by an increase in membrane permeability from 12 h p.i. (Fig. 3d).

**Cytokine production in purified DENV-1 infected MECs varied between cell lines**

The cytokine profile of the MECs upon DENV infection was studied over six time points for a duration of 24 h p.i.; DENV-1 was used for this as it induced the most significant change in the four cell lines. A total of 10 to 14 cytokines, from a selection of 21 commonly studied human cytokines, were differentially modulated in purified DENV-infected MECs (Fig. 4): inflammatory cytokines (IL-6 and TNF-alpha), chemokines (CXCL1, CXCL5, CXCL11, IL-8, IP-10, CX3CL1, CCL2, CCL5 and CCL20), growth factor (VEGF)
and adhesion molecules (ICAM-1 and VCAM-1). Overall, the concentrations of the studied cytokines, expressed as area under the curve (AUC), were higher in DENV-1-infected MECs compared to uninfected cells over the 24 h. The cytokine production was only slightly elevated in the infected dermal and brain MECs. Conversely, cytokine production in the infected pulmonary and retinal MECs was highly elevated compared to that in the uninfected MECs. The most produced cytokines were CCL2 in the dermal MEC, ICAM-1 in the pulmonary MEC and CCL5 in both the retinal and brain MECs. The infected pulmonary MEC exhibited a higher level of cytokine production than the uninfected MECs.

**DENV infection alters the junctional integrity of brain and lung MECs**

DENV-infected pulmonary and brain MECs showed differential modulation of barrier function from the ECIS model, hence Western blots were carried out to investigate the differences in the expression of junctional proteins in these two MECs infected by DENV-1. Western blots revealed that the expression of (i) tight junctional proteins – zona occludens-1 (ZO-1), occludin, claudin-1 and endothelial cell-selective adhesion molecules (ESAM); (ii) adherent proteins – vascular endothelial (VE)-cadherin; (iii) gap proteins – connexin 43 (CX43); and (iv) adhesive molecules – platelet endothelial cell adhesion molecule-1 (PECAM-1) was organ-specific, where differential expression was found in DENV-infected pulmonary and brain MECs (Fig. S1, available with the online version of this article).

ZO-1, occludin, VE-cadherin and PECAM-1 were detected in both the infected pulmonary and brain MECs, while ESAM and CX43 were only detected only in the infected pulmonary MEC, and claudin-1 was only detected only in the infected brain MEC. The relative percentage of protein expression in the infected MECs was calculated by comparison against the uninfected MECs. VE-cadherin was highly expressed in the infected pulmonary MEC upon infection, at a level of more than 200% compared to the uninfected lung MEC, with its highest expression being seen at 3 h p.i. (Fig. 5a). The expression of occludin, ESAM and CX43 was reduced in the infected pulmonary MEC immediately upon infection and increased gradually across time. The expression of ZO-1 was also reduced in the infected pulmonary MEC starting from 1 h p.i. and the level increased gradually but remained lower at ~50% up to 24 h. In the infected brain MEC, claudin-1 was most highly expressed from 3 h p.i. onwards, while the level of ZO-1 and PECAM-1 was reduced upon infection and remained lower up to 24 h p.i. (Fig. 5b).

**Purified and non-purified DENV-1 modulate the permeability of MECs differentially**

MECs were infected with non-purified DENV-1 (crude supernatant collected from Vero cells after virus
propagation) and the changes in vascular barrier function and cytokine profile were compared with the results obtained previously with purified DENV-1 infected MECs. This was done to assess the effect of factors in the crude supernatant of non-purified virus preparations. DENV activates immune responses and induces the production of inflammatory cytokines – not only in human cell lines, but also in Vero and C6/36 cell lines. Vero and C6/36 cells, which are commonly used in DENV propagation, produce inflammatory molecules that may alter or mask the immune responses of ECs challenged with DENV directly from the crude supernatant of Vero or C6/36 cells [39]. The infection kinetics shown when using the ECIS model suggest that both purified and non-purified DENV-1 induced a similar pattern of response in each MEC. The non-purified DENV-1 also caused an immediate modulation of Rb, alpha (α)
and Cm in the four cell lines, with major modulation to the para-cellular pathway and minor alterations in the cell substrate interactions and membrane capacitance (Fig. 6). However, non-purified DENV-1 showed reduced barrier function loss in pulmonary and brain MECs compared to the purified DENV-1-infected MECs. Furthermore, pulmonary MEC infected with non-purified DENV showed a delay in leakage (5 h p.i.) as compared to purified virus, where the leakage began at 1.5 h p.i.

**Purified and non-purified DENV-1 modulate cytokine production differentially**

The effect of non-purified DENV on the cytokine profile was also assessed by using DENV-1 for the same time points post-infection as purified DENV-1. The cytokine profiles between purified and non-purified DENV-1-infected MECs varied between the cell lines. For example, pulmonary MEC infected by purified DENV-1, on average, produced significantly higher levels of inflammatory cytokines (IL-6 and TNF-α), chemokines (CXCL1, CXCL5, CXCL11, CX3CL1, CCL2 and CCL20) and adhesion molecules (VCAM-1) compared to non-purified DENV-1 (Figs 7 and S2), for which large amounts of cytokines, such as IP-10 (each of the four MECs in general, Fig. 8a), CCL5 (dermal and retinal MECs, Fig. 8b) and VEGF (dermal and retinal MECs, Fig. 8c) were observed.

IP-10 was detected at a high concentration at 0 h p.i. for the non-purified DENV-1; however, the level did not increase, but instead decreased gradually over the 24 h in the non-purified DENV-1-infected MECs (Fig. 8a). Nonetheless, the level was still higher for non-purified DENV-1 compared to purified DENV-1 for all time points for each of the cell lines, with the exception of the pulmonary, where the production of IP-10 in purified DENV-1-infected pulmonary MEC increased gradually after 6 h p.i. (Fig. 8a).

**DISCUSSION**

The measurement of change in impedance (resistance and capacitance) using an ECIS system allows modelling of the barrier function of cells and cell-to-matrix interactions, as well as the membrane permeability of DENV-infected MECs, in real time [41]. The sensitivity of the ECIS machine in detecting low viral effects is well described, with resistance changes being observed at an m.o.i. range of as low as 0.001 in endothelial or epithelial cells infected with different viruses, including West Nile virus, mouse hepatitis virus type-3 and human bocavirus-1 [42–44]. The present findings indicate that DENV alone with a high concentration (m.o.i. of 5) can induce damage in the vascular beds, most likely through modulation of the paracellular pathway. Severe clinical symptoms have been reported to be...
associated with a high level of viral load in dengue-infected patients, with cohort studies across the decade showing that a higher viral load is detected in patients with haemorrhagic manifestations during early infection, while the level of plasma dengue viral load remains high in these patients even during defervescence [45–47].

The loss of vascular barrier function observed in the infected MECs varied between cell lines. The clinical symptoms observed in patients with severe dengue typically involve dysfunction of the ECs of multiple organ origins, such as the skin, retina, lungs and brain, among others [10, 29, 48]. ECs from various organs exhibit distinct cell structures, express different surface markers and do not contain the same intracellular enzymes, therefore maintaining tissue-specific features of the specialized endothelial function [49, 50]. EC-mediated immune response is influenced by the tissue microenvironment through tissue-specific EC–leukocyte interactions and inflammation activation [18]. This heterogeneity between the ECs may explain the differential modulations observed in the present study for dermal, pulmonary, retinal and brain MECs after DENV infection, where pulmonary MEC were shown to be most susceptible to DENV infection, while dermal MEC were less susceptible to DENV infection compared to other MECs (Fig. S3a).

The loss of vascular barrier function also varied between the DENV serotypes. Purified DENV-1 induced the most significant change in permeability among the four cell lines. This could be due to the higher virulence of the DENV-1 isolate used in the study (Fig. S3b) resulting in larger vascular leakage that may contribute to the severe manifestations observed clinically [51, 52]. The alterations triggered by the purified DENV-4 serotype were the least pronounced among the four human EC lines, supporting the notion that it is the least virulent of the four serotypes (Fig. S3b), a
conclusion that is in line with the clinical observations that symptomatic infections with DENV-4 are observed mainly in secondary rather than primary infections [53]. However, the relative virulence of each DENV serotype is difficult to evaluate, as there are three to five genotypes (genetic groups) within each serotype. Furthermore, the available DENV genomic studies indicate a limited contribution from DENV genetic variation to the progression of severe dengue [54]. Although evolutionary and epidemiology studies reported virulence differences with each DENV serotype, the resulting variations in pathogenesis are still poorly understood. Therefore, new methods and models are required to investigate the importance of viral factors in causing severe epidemics and the mechanism behind the progression of severe dengue.

The degree of vascular permeability in dermal MEC infected with the purified virus was smaller than that in the other MECs studied. The ECIS model of the dermal microvascular endothelium response suggests that the disruption of vascular permeability was via cell-to-cell interactions, which may have a role in the skin manifestations of DENV infection that can not only be observed in life-threatening severe dengue, but also in mild dengue fever. Rash appears in early DENV infection as one of the skin manifestations, and is often used as an indicator in the diagnosis of the disease. It is commonly observed as the first sign of endothelial dysfunction, regardless of the severity of the infection [2].

The impedance measurements suggested that DENV infection of pulmonary MEC caused an immediate reduced vascular permeability that lasted approximately for 1 to 2 h before a sudden increase in permeability, indicating vascular leakage. The clinical symptoms that present in the lung, such as pleural effusion and respiratory distress, are categorized by the 2009 WHO classification as dengue with warning signs and severe dengue [2]. The pulmonary MEC were most susceptible to purified DENV-1 compared to the other three serotypes tested, whereby DENV-1 induced the largest modulation. The ECIS model suggests that this modulation was due largely to barrier function (Rb; cell-to-cell interactions, such as vascular junctions) and cell membrane capacitance (Cm; membrane permeability), and, to an extent, cell–substrate interaction (α; cell-to-matrix interactions, such as adherent junctions). The prolonged junctional leakage in DENV-1-infected pulmonary MEC may play a role in the fluid accumulation observed in the lungs, which is the most common sign of plasma leakage observed in dengue patients [35]. No statistically significant association between the serotype of infection with the presence of pleural effusion has been reported, although DENV-2 caused a greater level of pleural effusion in secondary infections, while DENV-1 was associated with more severe manifestations in primary infections [47, 55]. By infecting the pulmonary MEC with purified virus, the present experiments mimicked primary DENV infection in lung ECs. The results corroborate previous findings that DENV-1 induced a more severe
pulmonary leakage that contributed to pleural effusion in primary infections.

The impedance data showed that the infection of both retinal and brain MECs by purified DENV induced an immediate loss of vascular barrier integrity, which was sustained over 24 h.p.i. DENV mainly modulated the vascular barrier function of retinal MEC by affecting cell-to-cell interactions, likely through paracellular pathways, and to a lesser extent by affecting cell membrane permeability. In contrast, brain MEC infected with purified DENV not only showed an alteration of cell-to-cell interactions, but also possibly involved the transcellular pathway by increasing cell membrane permeability, and inducing apoptotic cell death.

![Fig. 7. Percentage differences in the level of (a) inflammatory cytokines, (b) chemokines and (c) adhesion molecules in HPMEC infected with purified and non-purified DENV-1 at 0, 1, 3, 6, 12 and 24 h.p.i. The differences in the cytokine production between non-purified and purified DENV-1-infected MECs were statistically significant, as noted by *P<0.05 or **P<0.01.](image-url)
(decreased cell-to-matrix interaction). Cases have been reported in which retinal haemorrhage and encephalitis were present from day 2 of illness during DENV infection [11]. The detection of apoptotic ECs after DENV infection in several in vivo and in vitro studies [31–33, 56] further supports the possible cytopathic effects of DENV in MECs. In vitro, DENV led to 50% ATP reduction and 25% cell death in infected human umbilical cord vein ECs after 24 h and to 73% reduction and 60% cell death by 72 h p.i. [31].

A case study on fatal cases of severe dengue showed that apoptotic cells were found in intestinal and pulmonary MECs, as well as in white cells and cerebral cells [32]. Although clinical signs such as retinal haemorrhages and encephalopathy are rare and seen mostly at the later stages of the disease, this study suggests that high viral load (m.o.i. 5) during the early course of infection might obscure the prognosis of severe dengue when the virus infects these relatively secured MECs and causes life-threatening symptoms [34, 57, 58].

Notably, a number of the cytokines were highly expressed over the 24 h p.i. of the MECs by purified DENV: IL-6, TNF-α, CXCL1, CCL2, CCL5, CCL20, VEGF, ICAM-1 and VCAM-1. The high expression of IL-6 was probably amplified by TNF-α [59]. Both of these inflammatory cytokines are highly expressed in patients with severe dengue from day 1 after the onset of symptoms [60]. However, in this study, TNF-α was not highly expressed as IL-6 in the case of DENV-infected dermal MEC; the possible suppression of TNF-α production merits further investigation [61]. Chemokines such as CXCL1, CCL2, CCL5 and CCL20, produced by all the infected MECs studied, mediate inflammatory responses through the recruitment and infiltration of immune cells and increase vascular permeability [26, 27, 46]. VEGF, the growth factor produced mainly in ECs, was elevated in all of the infected MECs studied, and may contribute to increases in microvascular permeability by promoting vasodilatation [62]. Adhesion molecules such as ICAM-1, which were elevated in all of the infected MECs, and VCAM-1, which was only elevated in the case of infected pulmonary MEC, may play a role in modifying the cytoskeleton proteins or tight junctions (TJs) [63], leading to enhanced vascular leakage.

The paracellular pathway mediates the movement of solutes through inter-endothelial junctions while the transcellular route transports plasma proteins via caveolae-mediated vesicle carriers [64, 65]. The regulation of these pathways in response to extracellular stimulus is crucial for ECs to maintain vascular permeability upon infection [66]. ECIS modeling suggested that DENV-1 modulates ECs’ barrier function through the paracellular pathway regulated by inter-endothelial junctional proteins. In the infected pulmonary MECs, the expression of occludin, ESAM and CX43 was reduced upon infection. Occludin is a major membrane protein of the endothelial TJs and is required for the cytokine-induced regulation of TJ barriers [67]. The roles of ESAM and CX43 in vascular leakage have not been well studied, but their reduced expression and involvement in the adherent (AJs) and gap junctions (GJs) might contributes to the loss of barrier function in the infected pulmonary MECs. The expression of the tight junctional protein, ZO-1, was also reduced upon infection. Occludin is a major membrane protein of the endothelial TJs and is required for the cytokine-induced regulation of TJ barriers [67]. The roles of ESAM and CX43 in vascular leakage have not been well studied, but their reduced expression and involvement in the adherent (AJs) and gap junctions (GJs) might contributes to the loss of barrier function in the infected pulmonary MECs. The expression of the tight junctional protein, ZO-1, was also reduced upon infection. Occludin is a major membrane protein of the endothelial TJs and is required for the cytokine-induced regulation of TJ barriers [67]. The roles of ESAM and CX43 in vascular leakage have not been well studied, but their reduced expression and involvement in the adherent (AJs) and gap junctions (GJs) might contributes to the loss of barrier function in the infected pulmonary MECs. The expression of the tight junctional protein, ZO-1, was also reduced upon infection. Occludin is a major membrane protein of the endothelial TJs and is required for the cytokine-induced regulation of TJ barriers [67].
involved in restoring vascular barrier function during DENV infection. VE-cadherin is a cell–cell adhesion molecule that is specifically located in the AJs of ECs for the control and maintenance of ECs’ homotypic adhesion [69]. Although studies have shown that VE-cadherin was mostly down-regulated in DENV-infected ECs after 24 h of infection, some in the presence of peripheral blood mononuclear cells or NS1 protein [70–72], the current study showed that VE-cadherin was up-regulated within the first few hours of infection in MECs infected with a high level of DENV alone, while the expression slowly reduced from 12 to 24 h p.i. This might be a protective mechanism that is used to counter the first DENV challenge, probably by reinforcing the integrity of inter-endothelial junctions through increased anchorage of VE-cadherin to the actin cytoskeleton [73], as well as by up-regulating the expression of tight junctional proteins [74].

Western blots of junctional proteins in the infected brain MEC showed that the expression of ZO-1 and PECAM-1 was reduced right upon infection, while claudin-1 was strongly expressed from 3 h p.i. onwards. ZO-1 and claudin-1 are transmembrane proteins that are present in the TJs and are particularly prominent and abundant in the brain ECs to form the blood–brain barrier (BBB), where strict regulation of permeability is required [75]. PECAM-1 is an inter-endothelial junctional protein that is mainly expressed in the surface of leukocytes and platelets, in the lateral borders between ECs [76] involved in maintaining junctional stability and to restore the integrity of disrupted endothelial barriers [77]. The virus-induced down-regulation of ZO-1 could reduce the recruitment of signalling molecules to the TJs and disrupt the linking of tight junctional proteins with actin cytoskeleton [78]. This rearrangement of the actin cytoskeleton might allow the entry of DENV during the early phase of infection, and subsequently destabilize inter-cellular junctional proteins and increase endothelial permeability [79]. At low concentrations, PECAM-1 might also fail to localize into inter-cellular junctions through homophilic interaction and therefore be unable to strengthen the adhesion between ECs and the cytoskeleton [80]. However, another integral membrane protein in the TJs, claudin-1, was found to be highly expressed along the cell-to-cell barrier in the infected brain MEC. The sealing claudins, such as claudin-1, are the barrier-forming components of TJs and are primarily expressed in the brain MEC, where they are integrated stably into the TJs to maintain the strength of the intercellular binding in the BBB [81]. DENV infection of the brain MEC up-regulated claudin-1 expression, possibly to enhance the integrity of the paracellular barrier through the reconstitution of TJ strands in response to the infection [82].

Purified and non-purified DENV-1 responses across the four MECs were studied to assess the effect of factors in the crude supernatant of non-purified virus preparations. A differential response was observed between the non-purified and purified DENV-1, and this was most noticeable in the case of pulmonary MECs. Purified DENV-1 enhanced vascular leakage and induced a higher production of cytokines, including IL-6, TNF-α, CXCL1, CXCL5, CXCL11, CX3CL1, CCL2, CCL20 and VCAM-1. The production of these cytokines was lower in the case of non-purified DENV-1-infected pulmonary MEC, probably due to the confounding influences of any possible pre-existing cytokines presented in the crude supernatant. Additionally, vascular leakage was reduced in the case of non-purified DENV-infected MECs. These data suggest the presence of protective cellular factors against vascular leakage in the non-purified virus stock. This is in agreement with previous studies, where C6/36 or Vero cells were found to produce factors that could delay or reduce the cytopathology in cells newly infected with DENV [39, 40]. Notably, the non-purified DENV-1 strongly expressed three cytokines compared to the purified virus, namely IP-10, CCL5 and VEGF. The purified DENV did not elevate the production of these cytokines in MECs over the 24 h p.i., except for a gradual increase in IP-10 for pulmonary MECs starting at 6 h p.i. and a fluctuating increase in the brain MECs at 6 h p.i., suggesting that the expression of these cytokines may be suppressed by DENV as a strategy for survival to avoid the recruitment of immune cells such as monocytes, leukocytes and T cells [83, 84]. The high concentrations of CCL5, IP-10 and VEGF detected upon infection suggest that these three cytokines were probably introduced to the MECs from the non-purified virus stock and exerted their chemotactic effects during early infection to reduce the virulence of the dengue virus itself.

Several caveats need to be considered in a study such as this. A major limitation of ECIS is the fact that the measurement does not provide direct information at the molecular level. Thus, ECIS measurements are usually most informative at the beginning of an experimental series to help associate a scientific problem with cellular structures or properties and provide significant input for the generation of a testable hypothesis [85]. Another major disadvantage of ECIS migration studies is the diversity of the cell behaviours that change impedance. Changes in adhesion and cell density will alter the impedance. These changes are indistinguishable from changes due to migration [86]. However, the above limitations are not applicable here, because this study focuses on the cellular level and is not related to migration. One of the principal caveats for this study is the type of MEC lines used, with dermal, pulmonary and retinal MECs being the primary cell lines, while the brain MEC was transfected to ease the growth of the cells, and may have lost certain properties during the transfection. Another limitation of the study was the fact that cytokines were detected at 0 h p.i. even for MECs infected with purified DENV. This may be a result of the inefficiency of the virus purification through density gradient purification [87, 88], where the purity of the virus is in strict correlation to the procedures employed and impurities such as small proteins might still remain.
In summary, the findings of the present study show that high DENV load modulates the human microvascular endothelial barrier function differentially during early infection with organ-specific cytokine production. The ECs studied were activated differentially as soon as the virus was introduced, exhibiting either temporarily enhanced permeability (dermal MECs) or long-term barrier loss (pulmonary, retinal and brain MECs). The activated ECs produced inflammatory cytokines, chemokines and adhesion molecules that could further augment the disruption of the endothelial barrier. Inter-endothelial junctional proteins have been differentially expressed in pulmonary and brain MECs, either as a response to DENV infection leading to vascular leakage, or (when up-regulated) to restore the barrier function. As the endothelium dynamically elicits responses that may be responsible for vascular permeability, the MECs of the various vascular beds showed differential responses to DENV and the effect may be aggravated in secondary infections due to the involvement of antibody-dependent enhancement, cross-reactive T cell responses and cytokine storms. The viral purity and the choice of host for virus propagation should also be taken into consideration, as they may be factors masking or altering the DENV-specific immune responses in MECs.

**METHODS**

**In vitro models of microvascular endothelial cells (MECs)**

Four MEC lines, including primary human dermal microvascular endothelial cells (HDMEC) (ATCC, Manassas, VA, USA), primary human pulmonary microvascular endothelial cells (HPMEC) (Sciencell, Carlsbad, CA, USA), primary human retinal microvascular endothelial cells (HRtMEC) (CSC, Tysons, VA, USA) and transfected human brain microvascular endothelial cells (THBMEC) (ATCC) were cultured using vascular cell basal medium (ATCC), endothelial cell growth medium (ECM) (Sciencell) [89], CSC complete serum-free medium (CSC) [90] and medium 199 (Thermo Fisher, Waltham, MA, USA) [91], respectively. The four cell lines were maintained at 37 °C in humidified air containing 5% CO₂.

**DENV propagation, purification and titration**

Four serotypes of dengue viruses, DENV-1 (WP), DENV-2 (NGC), DENV-3 (S78) and DENV-4 (VP) were propagated in Vero C1008 cells (ATCC) with DMEM (Thermo Fisher) supplemented with 2% foetal bovine serum (FBS). The harvested viruses were purified using 20% sucrose through gradient centrifugation at 28 000 r.p.m. for 3.5 h. Both the non-purified and purified virus stocks were titrated with a focus reduction neutralizing assay using immunostaining as described [92]. Anti-flavivirus mouse monoclonal antibodies (Merck Millipore, Billerica, MA, USA) and peroxidase-conjugated goat anti-mouse IgG (Merck Millipore) were used for immunostaining. The number of plaques were counted and the virus titre was obtained with the formula: dilution factor (FD) × conversion factor (FC) × average number of plaques = p.f.u. ml⁻¹ [93].

**DENV infection of MECs**

HDMEC, HPMEC, THBMEC and HRtMEC were grown to confluent monolayers prior to the infection with DENV, either in 24-well plates in duplicates for the assessment of infection kinetics, an eight-well electrode array (8W10E+) (Applied Biophysics, Troy, NY, USA) in triplicates for ECIS measurement or in the eight-well Lab-Tek II chamber slide system (Thermo Fisher) in duplicates for cytokine profiling. A real-time quantitative polymerase chain reaction was performed on harvested supernatant and cell lysates at 0, 3, 6, 12, 24 and 72 h p.i. according to Yong et al. to evaluate the DENV infection kinetics in four MECs [94]. For ECIS measurement, MECs were infected with the four DENV serotypes at an m.o.i. of 5.0, while for cytokine profiling, MECs were infected with DENV-1 only at an m.o.i. of 5.0.

**ECIS measurement of DENV-infected MECs**

An ECIS Zθ with a 16-well array station (Applied Biophysics) was used to measure the changes in impedance (resistance and capacitance) induced in MECs before and after DENV infection. The real-time changes of MECs were monitored at multiple frequencies for 24 h after infection using simple ECIS measurement. Resistance changes were detected at a low alternative current (AC) frequency (2000 Hz), where the current flows between the cells through paracellular passages. Resistance increases during the proliferation stage of the attached cells, and plateaus when the cells are confluent. Reduced resistance after cell manipulation indicates an induced loss of barrier function mediated by changes of cytoskeleton, cell attachments or the regulation of cell-to-cell junction complexes [41]. Capacitance was detected at a higher AC frequency (40 000 Hz) by measuring the current flowing through the insulating cell membrane by transcellular paths. The spreading of the proliferating cells over the electrodes also restricts the flow of current and thus the capacitance decreases linearly with the electrode coverage by the cells [41]. The embedded ECIS mathematical model allows us to calculate cell morphological parameters, including barrier function (Rb), cell-to-substrate interactions (α) and cell membrane capacitance (Cm) from the resistance (R) and capacitance (C) measurements [95–97]. The raw data were first normalized to the value obtained at 0 h p.i. to eliminate background signals, followed by normalization to the uninfected MECs to compare the effects of DENV in the infected MECs. The standard error of mean between the triplicates was also determined and presented at regular intervals. Our preliminary work showed that the modulation of barrier function only occurred at a high viral load with an m.o.i. of 5.0 during infection, whereas no significant differences were observed between the infected and uninfected cells at low viral loads (m.o.i.s 0.1 and 1.0). Hence, this subsequent study focused on the modulation of MECs triggered by a high viral load of DENV (m.o.i. of 5) during the first 24 h of infection.
Cytokine profiling

Cell supernatants were collected at six time points (0, 1, 3, 6, 12 and 24 h p.i.) selected through ECIS real-time monitoring when major comparative changes occurred between purified/non purified DENV-infected and uninfected MECs. The level of the 21 most studied cytokines, chemokines, adhesion molecules and growth factors secreted by the MECs at these time points were determined using the Human Magnetic Luminex multiplex screening assay based on flow cytometric analysis of magnetic antibody-coated beads specific to the analytes of interest (R&D Systems, Minneapolis, MN, USA). The coefficient of variance of the replicates was calculated to certify the precision and repeatability of the assays. The changes of cytokine production in the infected MECs were normalized to the uninfected cells over time and the findings were presented as percentage differences.

Functional protein expression of DENV-infected MECs

The barrier integrity of the MECs was further examined via Western blot analysis at 0, 1, 3, 6, 12 and 24 h upon DENV infection. At the end of each time point, the infected/non-infected cells were lysed with lysis buffer on ice, resolubilized in rehydration solution and finally kept at –80 °C until further analysis. Prior to electrophoresis, the total protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Cell lysates from DENV-infected HPMEC and THBMEC were then resolved on 12% SDS-PAGE gels. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. Non-specific binding of the membrane was blocked with Tris-buffered saline (TBS) containing 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20 (TBST) for 2 h. The membranes were then washed with TBST three times for 10 min and incubated with an appropriate dilution of specific primary antibodies in TBST overnight at 4°C. The next day, the membranes were washed with TBST and incubated with the corresponding secondary antibodies for 2 h. The primary antibodies used were unconjugated mouse monoclonal IgG1 against ZO-1, occludin, claudin-1, CD31 (PECAM-1) and connexion-43; unconjugated mouse monoclonal IgG2a against VE-CADH; and unconjugated goat polyclonal IgG against ZO-1, occludin, claudin-1, CD31 (PECAM-1). The secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat polyclonal anti-mouse IgG and HRP-conjugated rabbit polyclonal anti-goat IgG (Invitrogen). The membranes were then washed with TBST thrice and chromogenic substrate containing 4-chloro-1-napthol and hydrogen peroxide was added to visualize the bands. The reaction was stopped by washing with distilled water. The membranes were then scanned and the band intensity was analysed with ImageJ software. Loading controls α-tubulin or β-actin were included in all membranes for normalization. The percentage of protein expression was calculated by dividing the band intensity of infected ECs by the uninfected cells.

Statistics

All of the data are presented as mean with error bars representing the standard error of the mean. Two-way ANOVA with Bonferroni’s post-test was used to evaluate the differences in the resistance and capacitance readings between the infected and uninfected MECs. Student’s t-test was used to evaluate significant differences in cytokine production between non-purified DENV-1 and purified DENV-1-infected MECs.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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