Endothelial Cells Elicit Immune-Enhancing Responses to Dengue Virus Infection

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Dengue viruses cause two severe diseases that alter vascular fluid barrier functions, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Preexisting antibodies to dengue virus disposes patients to immune-enhanced edema (DSS) or hemorrhagic (DHF) disease following infection by a discrete dengue virus serotype. Although the endothelium is the primary vascular fluid barrier, direct effects of dengue virus on endothelial cells (ECs) have not been considered primary factors in pathogenesis. Here, we show that dengue virus infection of human ECs elicits immune-enhancing EC responses. Our results suggest that rapid early dengue virus proliferation within ECs is permitted by dengue virus regulation of early, but not late, beta interferon (IFN- β) responses. The analysis of EC responses following synchronous dengue virus infection revealed the high-level induction and secretion of immune cells (T cells, B cells, and mast cells) as well as activating and recruiting cytokines BAFF (119-fold), IL-6/8 (4- to 7-fold), CXCL9/10/11 (45- to 338-fold), RANTES (724-fold), and interleukin-7 (IL-7; 128-fold). Moreover, we found that properdin factor B, an alternative pathway complement activator that directs chemotactic anaphylatoxin C3a and C5a production, was induced 34-fold. Thus, dengue virus-infected ECs evoke key inflammatory responses observed in dengue virus patients which are linked to DHF and DSS. Our findings suggest that dengue virus-infected ECs directly contribute to immune enhancement, capillary permeability, viremia, and immune targeting of the endothelium. These data implicate EC responses in dengue virus pathogenesis and further rationalize therapeutic targeting of the endothelium as a means of reducing the severity of dengue virus disease.

Dengue viruses are transmitted by mosquitoes and infect ~50 million people annually (27, 29, 30). Expanding mosquito habitats are increasing the range of dengue virus outbreaks and the occurrence of two severe diseases with 5 to 30% mortality rates: dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (27, 29, 30). Patients with DHF and DSS display symptoms of increased edema, hemorrhage, shock, fever, and viremia (27, 29, 30). Although patient progression to DHF and DSS is not fully understood (13, 30), an antibody-dependent enhancement of infection (ADE) increases the potential for DSS and DHF (28, 30). The ability of dengue virus to infect immune and dendritic cells fosters a role for immune responses to act on the endothelium and increase capillary permeability (6, 10, 13, 26, 40, 60, 61). However, dengue virus-infected endothelial cells (EC) are also reported in DHF/DSS patient autopsy samples and in murine dengue virus disease models (11, 35, 74). This suggests that dengue virus-infected ECs also contribute to pathogenesis by increasing viremia, secreting cytokines, activating complement, or by transforming the endothelium into an immunologic target of cellular and humoral immune responses (27, 29, 30).

As the primary fluid barrier of the vasculature, the endothelium plays a central role in regulating fluid and cellular efflux from capillaries (1, 13, 18, 71). The fundamental importance of this is demonstrated by the redundant multifactorial regulation of vascular permeability. Unique EC receptors, adherens junctions, and signaling pathways respond to cytokines, permeability factors, immune complexes, clotting factors, and platelets, which normally act in concert to control vascular leakage (1, 13, 18, 23, 71). ECs also elicit immune-enhancing cytokine responses that recruit immune cells to the endothelium and at times direct fluid and immune cell efflux into tissues (1, 71). Virally induced changes in endothelial or immune cell responses have the potential to alter

this orchestrated balance with pathological consequences (1, 13, 18, 23, 71).

Although the mechanism of dengue virus pathogenesis remains to be determined, viremia, cytokines, complement, mast cell activation, and lymphocyte recruitment are associated with DHF and DSS (6, 10, 13, 29, 60). Dengue patients have notably high levels of cytokines, chemotactic complement anaphylatoxins C3a and C5a, and histamine, which have the potential to induce vascular permeability (5, 14, 24, 29, 30, 39, 62, 68, 75). A growing body of evidence indicates that the endothelium itself plays a prominent role in immune-enhanced pathology and that virally elicited EC responses contribute to increased vascular permeability in DHF and DSS patients (20, 29, 30, 65, 71, 74).

Although a targeted analysis of the dengue virus-infected endothelium has yet to be reported, we have shown that $\geq 80\%$ of primary human ECs are infected with dengue virus *in vitro*, resulting in the rapid production and release of virions at 12 to 24 h postinfection (hpi) (20). Here, we kinetically analyzed responses of ECs resulting from synchronous dengue virus infection. Using microarrays, we determined that more than 59 human genes are induced ≥ 30 -fold in dengue virus-infected ECs compared to mock-infected ECs. Supporting the early replication of dengue virus in ECs (20), we found that beta interferon (IFN- β) and several IFN-stimulated genes (ISGs) were highly induced, but only at late times postinfection. Moreover, ECs responded to dengue virus

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FIG 1 Dengue virus infection of primary ECs. EC monolayers were infected with dengue virus (ST4; MOI of 6) and methanol fixed at the times indicated. Dengue virus proteins within infected cells were detected with anti-DV4 HMAF and immunoperoxidase staining as described previously (20).

infection by inducing the immune response-enhancing cytokines BAFF, CXCL9/10/11, RANTES, and interleukin-6/7/8 (IL-6/7/8) (4- to 724-fold) and the alternative pathway (AP) complement activator properdin factor B (34-fold). As a result, dengue virus-infected ECs secrete cytokines and an activator of C3a and C5a production that are consistent with responses observed in DHF and DSS patients (6, 29, 30, 62). These results demonstrate that dengue virus-infected ECs actively participate in enhancing immune responses that increase vascular permeability and are associated with severe dengue virus disease (13, 29, 30, 62).

MATERIALS AND METHODS

Cells and virus. C6/36 cells (*Aedes albopictus*) were grown in M199 (with 5% fetal bovine serum [FBS]) at 32°C (20). Human umbilical vein endothelial cells (ECs) (passages 3 to 8) (Cambrex) were grown in EBM2 medium (10% FBS, 50 µg/ml gentamicin, 50 µg/ml amphotericin B) at 37°C and 5% CO₂. Dengue virus serotype 4 (ST4) was provided by C.-J. Lai (NIH, NIAID, LID) and propagated and titered on C6/36 monolayers as described previously (20).

Dengue virus infection of human endothelial cells. Dengue virus infection of primary human endothelial cells was described previously (20). Briefly, dengue virus (ST4) was adsorbed to ~70% confluent primary human endothelial cell monolayers for 1 to 1.5 h. Following adsorption, cell monolayers were washed to remove unbound virus and the medium was replaced with supplemented EBM2 (with 10% FBS). For interferon studies, medium was replaced and supplemented with 1,000 U/ml blocking antibodies to IFN-α or IFN-β (R&D Systems) or pretreated with IFN-α (200 U/ml) (Sigma) for 24 h prior to infection.

Detection of dengue virus-infected cells. The immunoperoxidase staining of dengue virus-infected cells was previously described (20). Briefly, cells were methanol fixed at the desired times, blocked in phosphate-buffered saline (PBS)-1% bovine serum albumin (BSA), incubated sequentially with anti-DV4 hyperimmune mouse ascitic fluid (HMAF) (1:4,000 in PBS-1% BSA) and anti-mouse horseradish peroxidase (HRP)-labeled antibody (Amersham) (1:4,000 in PBS-1% BSA), and immunoperoxidase stained with 3-amino-9-ethylcarbazole (AEC) (20). The number of infected cells was quantitated by light microscopy on a Olympus IX51 microscope.

Cytokine ELISAs. Levels of RANTES, IL-6, CXCL10, and CXCL11 in the supernatant of mock- or dengue virus-infected ECs were measured using a Multianalyte ELISArray kit (Qiagen), while IL-7 and BAFF were measured using Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). Samples were analyzed using manufacturer protocols and concentrations determined from standard curves of serially diluted antigen standards.

qRT-PCR analysis. Quantitative real-time PCR (qRT-PCR) was performed as described previously (53). Briefly, total RNAs were extracted from mock- and dengue virus-infected ECs and purified using RNeasy kits (Qiagen). cDNA synthesis was performed using the Transcriptor First-Strand cDNA synthesis kit (Roche). Primers for qRT-PCR were obtained from Applied Biosystems or designed and ordered from Operon.

Specific genes were analyzed using Sybr green methods on an ABI 7300 (Applied Biosystems) (53). Responses were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, and the fold induction was calculated as the difference between dengue virus- and mock-infected ECs at each time point using the $2^{-\Delta\Delta CT}$ method (53).

Affymetrix array analysis. Total RNAs were extracted from mock- and dengue virus-infected ECs and purified using RNeasy kits (Qiagen). cDNA synthesis and labeling were performed at the Stony Brook University DNA Microarray Facility and used to probe >47,000 genes displayed on the human genome U133 Plus 2.0 GeneChip according to Affymetrix protocols. mRNA levels of dengue virus- and mock-infected ECs at each time point were compared and are presented as the fold increase (>3-fold) of dengue virus- over mock-infected levels.

Microarray data accession number. Microarray data obtained from these studies were deposited in NCBI's Gene Expression Omnibus database (GEO) under accession number [GSE34628](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34628).

RESULTS

ECs elicit immune-enhancing responses to dengue virus infection. The importance of ECs in regulating vascular permeability and immune cell chemotaxis (1, 13, 23, 65) and reports of dengue virus-infected ECs in patients and murine models (11, 35, 74) suggest that ECs are critical targets of dengue virus infection that can contribute to viremia and pathogenesis (13, 30). In contrast to prior studies, where dengue virus was reported to infect only 2 to 10% of ECs or an ECV304 cell line (6, 9, 10, 34, 72), we found that the dengue virus infection of primary human ECs was highly efficient (20). Using a multiplicity of infection (MOI) of 6, we observed that >80% of ECs were dengue virus infected at 24 hpi, followed by a decline in dengue virus-infected ECs at 48 to 72 hpi (Fig. 1). These findings correlate with the early production and release of dengue virus progeny at 12 to 24 hpi (~10⁵ FFU/ml) (20) and suggest that dengue virus elicits discrete early- and late-phase EC responses that initially permit and subsequently restrict viral replication.

We analyzed human EC transcriptional responses to synchronous dengue virus infection at 12, 24, and 48 hpi using Affymetrix microarrays (human genome U133 Plus 2.0 GeneChip; >47,000 genes). We found that 281 genes were induced ≥5-fold by dengue virus infection at 24 hpi, while <30 genes were significantly down-regulated (NCBI GEO accession no. [GSE34628](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34628)) (Tables 1 and 2). High-level gene induction was observed 24 to 48 hpi, with 59 genes induced 30- to 12,417-fold and 75 genes induced ≥20-fold. Interestingly, dengue virus infection of ECs resulted in a dramatic induction of immune-enhancing cytokines, complement-activating factors, and immune cell-recruiting chemokines 24 to 48 hpi (Table 1). Dengue virus-induced cytokines include BAFF, a B cell-activating factor (44), IL-7, a T cell expansion and cytokine-enhancing factor (2), and several immune cell chemotactic factors,

TABLE 1 Induction of selected genes from microarray analysis of dengue virus-infected ECs

Gene and category	Fold induction at:		
	12 h	24 h	48 h
Cytokines/chemokines			
BAFF		119	79
CXCL2/GRO beta		12	
CXCL3/GRO3		14	
CXCL9/MIG		60	
CXCL10/IP-10	16	338	315
CXCL11/ITAC		45	45
IL-6		7	3
IL-7		128	37
IL-8		4	3
RANTES		84	724
Complement factors			
Properdin factor B (CFB)		21	34
SERPING1		15	194
C1s		15	294
IFN inducible			
IFN β		1024	223
RIG-I		18	13
MDA5		39	23
TLR3		24	21
ISG54		181	32
ISG56	832	12417	478
OAS2		209	294
OAS3		21	34
MxA	13	294	223
MxB		104	3327
Viperin	18	512	2048
IFIT4	6	104	91
IFIT5		16	14
IFITM1	5	74	64
ISG20	5	52	42
ISG15		15	17
IFRG28		891	724
IFI75		15	11
IRF7		13	15
APOBEC3G		17	11

including CXCL2/3/9/10/11, IL-6, IL-8, and RANTES (4- to 724-fold; [Table 1](#)). Dengue virus infection of ECs also induced the transcription of complement factors SERPING1, C1S, and properdin factor B (complement factor B [CFB]) (34- to 294-fold; [Table 1](#)). Factor B activates the alternative complement pathway by binding C3b to form C3 convertase, ultimately stimulating the production of chemotactic anaphylatoxins C3a and C5a, which direct histamine release from mast cells and induce vascular permeability ([16, 31, 38, 51, 59, 62, 66, 73, 75](#)). This is of fundamental importance to dengue virus pathogenesis, since elevated levels of C3a and histamine are associated with increased vascular permeability in severe DHF and DSS diseases ([6, 29, 51, 62, 68, 73](#)). The specific high-level induction of selected cytokines and complement factors following dengue virus infection was substantiated by qRT-PCR ([Table 3](#)). Our findings indicate that dengue virus-infected ECs elicit chemotactic cytokine and complement transcriptional responses that are known to enhance inflammatory immune responses.

TABLE 2 Genes downregulated 3-fold or more in dengue virus-infected ECs (determined by microarray)

Gene and category	Downregulation at:		
	12 h	24 h	48 h
Transcription/translation			
EIF4B			-3
Transcription factor DP-2			-4
c-maf			-6
Translation initiator factor 3, sub 5			-4
Signaling/trafficking			
Syntrophin, gamma 1	-7		
Activator of G protein signaling 3		-3	
TPD52		-21	
Cyclic AMP phosphodiesterase PDE7			-4
Fatty acid binding protein 4			-5
Calcineurin binding protein 1		-13	
Development			
p45			-4
p57 (cyclin-dependent kinase)		-3	-3
MOX2		-3	
Periostin		-4	-6
MMP-1			-3
Retinoic acid-induced protein 2			-7
Metabolism			
Glycine amidinotransferase		-12	
Cytochrome P450			-9
Aconitase 1			-8
Glutaminase			-5
Retinol binding protein 1			-4
Miscellaneous			
C-type lectin, s9	-6		
Killer cell lectin receptor, sA	-7		
SERPINF2 (protease inhibitor)		-3	
HSPC133 (methyltransferase)	-4		
Collagen type 1			-10
Thymosin beta			-4
Serum amyloid A	-11		

Biphasic innate immune responses of ECs to dengue virus infection. The ability of dengue virus to replicate in ECs at early, but not late, times after infection suggests the unique regulation of innate immune responses. Microarray and RT-PCR analysis reveal little or no IFN or ISG response induced at 12 hpi compared to responses of mock-infected ECs, while innate IFN and ISG responses are highly induced at 24 to 48 hpi ([Tables 1 and 3](#)). In fact, the only highly induced ISG observed at 12 hpi is ISG56 (832-fold), a protein that blocks IFN- β induction and IRF3 activation ([42](#)). In contrast, IFN- β is induced 1,024-fold at 24 to 48 hpi, along with $\sim$ 20 ISGs (13- to $>3,000$ -fold). Late innate immune responses include the induction of RIG-I and MDA5, which are upstream IFN- β pathway activators, and IRF7, a master regulator of type I IFN-dependent immune responses ([33](#)). Late EC responses to dengue virus also resulted in the induction of antiviral ISGs, including MxA/B and OAS2/3 ([Table 1](#)) ([57](#)). IFN- β and ISG responses elicited by ECs are consistent with the high-level induction of IFN and ISGs observed in the serum of DHF and DSS patients ([27, 29, 30](#)). Although our data suggest that dengue virus

TABLE 3 qRT-PCR analysis of dengue virus-infected ECs

Gene	Fold induction at:		
	12 h	24 h	48 h
Cytokines			
CXCL9/MIG		108	26
CXCL10/IP-10	30	52,745	20,013
CXCL11/ITAC	3	1,892	827
IL-6		19	4
IL-7		58	91
IL-8		11	3
RANTES	11	968	1,275
BAFF		307	254
Complement factors			
Properdin factor B (CFB)		12	53
C1s		18	141
IFN inducible			
IFN β		49	12
MDA5	3	94	59
RIG-I		31	25
Viperin	24	23,978	15,451
ISG56	7	711	809
ISG20	5	82	99
IRF7		20	37
MxA	24	2,600	1,526

regulates the early, but not late, induction of type I IFN in ECs, dengue virus is reported to continuously block dendritic cell IFN and ISG induction (56). This suggests that dengue virus regulation of interferon induction is cell type specific and uniquely biphasic in ECs.

Interferon secretion blocks dengue virus spread in ECs. Secreted IFN may protect neighboring ECs from dengue virus infection (21, 25). To determine if IFN responses regulate dengue virus spread, ECs were infected at a low MOI and assayed for viral proliferation at 48 to 72 hpi in the presence or absence of blocking antibodies to IFN- α or IFN- β . Only the addition of antibodies to IFN- β permitted the cell-to-cell spread of dengue virus (Fig. 2A and B). Antibodies to IFN- β also dramatically reduced the induction of ISG56 and viperin in dengue virus-infected ECs. In contrast, the induction of CXCL10 and CXCL11, which are induced independent of IFN- β secretion, was unaltered by the addition of IFN- β antibodies (Fig. 3). These data are consistent with the selective production of IFN- β , but not IFN- α , by ECs and the ability of added type I IFN to block dengue virus replication (>4 log) (Fig. 2C). These results demonstrate that IFN- β secreted by dengue virus-infected ECs limits viral spread and contributes to the late induction of antiviral ISGs.

Dengue virus-infected ECs secrete chemotactic cytokines. ECs secrete factors that can enhance immune cell activation, chemotaxis, and inflammation observed in dengue patients (29). Mock- and dengue virus-infected EC supernatants were analyzed for the secretion of selected cytokines. Consistent with transcriptional responses, we observed a dramatic increase in the secretion of chemotactic cytokines CXCL10, CXCL11, IL-6, and RANTES in the supernatants of dengue virus-infected ECs at 24 to 48 hpi, as well as IL-7 and BAFF secretion from infected ECs at late time points (Fig. 4). These findings provide the first evidence that den-

gue virus-infected ECs secrete factors linked to the severity of dengue disease (14, 24, 29, 39, 62).

DISCUSSION

DHF and DSS are severe manifestations of dengue virus infection that result in increased vascular permeability, hemorrhage, and shock (30). The presence of preexisting antibodies to dengue virus predisposes patients to severe disease following infection by a sec-

FIG 2 IFN- β restricts dengue virus spread within ECs. (A) ECs were infected with dengue virus (MOI of 0.5) in the presence or absence of neutralizing antibodies to IFN- α or IFN- β (1,000 U/ml). At the indicated times, EC monolayers were immunoperoxidase stained as described for Fig. 1 (20). (B) The percentages of dengue virus-infected ECs from panel A were quantitated (6 fields) and presented as a percentage of untreated ECs. (C) Supernatants from dengue virus-infected human EC monolayers treated as described for panel A or pretreated with IFN- α (200 U/ml) were collected at 24 h postinfection to quantitate progeny virus. Diluted supernatants were adsorbed to C6/36 cells, which were fixed at 20 hpi and immunoperoxidase stained as described for Fig. 1. Titers were determined based on the number of dengue virus antigen-containing cells and are reported as focus-forming units per ml (FFU/ml).

FIG 3 Neutralization of IFN- β reduces ISG but not cytokine mRNA induction within dengue virus-infected ECs. Total RNA was extracted from mock and dengue virus-infected ECs treated as described for Fig. 2A and analyzed by qRT-PCR for the induction of ISG56, viperin, CXCL10, and CXCL11 mRNAs. For each time point, mRNA levels were standardized to GAPDH and the fold mRNA induction in dengue virus- versus mock-infected ECs was determined (53). Grey bars, anti-IFN- α ; black bars, anti-IFN- β .

and dengue serotype (30). Many hypotheses have been offered to explain dengue disease, and a myriad of responses have been associated with dengue virus infection that may contribute to disease, but the pathogenic mechanisms that result in DHF and DSS remain ambiguous (6, 27, 29, 30). One common element of the dengue disease process is that enhanced immune responses increase vascular permeability by acting on the endothelium, the primary fluid barrier of the vasculature. Although it is clear that immune cells and their responses contribute to pathogenesis, the endothelium, which regulates vascular leakage, has not been considered a significant component of DHF and DSS (1, 13, 29, 71). This may in part stem from the relative ease of assessing blood components and the inability to kinetically study the endothelium in dengue patients. However, factors secreted from ECs are also plasma constituents, and autopsy samples and murine dengue disease models clearly demonstrate that vascular ECs are infected (11, 12, 35, 74). In fact, a murine antibody enhancement model of dengue disease finds a dramatic increase in infected hepatic ECs that coincides with severe disease (74).

Here, we evaluated responses of primary human ECs to synchronous dengue virus infection *in vitro*. Our findings demonstrate that dengue virus-infected ECs elicit high-level transcriptional and secretory responses that are consistent with immune-enhancing, permeability-inducing responses observed in dengue patients (30). We found that dengue virus-infected ECs highly induce and secrete chemotactic cytokines (CXCL9/10/11 and RANTES), immune cell response-modulating cytokines (IL-7 and BAFF), and a key complement-activating factor (factor B) that contribute to both immune cell chemotaxis and mast cell degranulation (31, 38, 51, 59, 66, 73). Thus, dengue virus-infected ECs, along with infected immune cells, serve as sources of various key

FIG 4 Analysis of secreted factors from dengue virus-infected ECs. Supernatants from mock- and dengue virus (DV)-infected ECs were collected (12, 18, 24, 36, 48, and 72 hpi) and analyzed for secreted CXCL10, CXCL11, RANTES, IL-7, BAFF, and IL-6 by ELISA. Results are reported in pg/ml using antigen standard curves.

immune-enhancing factors. These findings uniquely associate responses of infected ECs with immune enhancement and increased vascular permeability following dengue virus infection.

Dengue virus-infected ECs rapidly release infectious virus at early, but not late, times after infection (20). These findings are inversely correlated with the late, but not early, induction of IFN- β and antiviral ISGs (57). This biphasic response also results in the late induction of IRF7, which amplifies IFN- β and ISG responses (33). Prior reports analyzing transcriptional responses of other dengue virus-infected cell types, including human liver cells, also highlight the induction of similar ISGs and type 1 IFN at 48 or 72 hpi (14, 19, 24, 36, 69). Importantly, we show that dengue virus-infected ECs secrete IFN- β and that dengue virus spread within ECs is restricted by IFN- β secretion (Fig. 2). However, the late induction of IFN responses in ECs is distinct from the sustained regulation of IFN reported in dengue virus-infected den-

dritic cells and suggests discrete IFN pathway regulation by dengue virus proteins in ECs (50, 56). Dengue virus proteins reportedly regulate various steps of the IFN signaling pathway (3, 48, 49, 55, 56), and future studies are required to determine how these proteins regulate EC IFN responses to permit early dengue virus replication and spread in ECs. Since IFN reportedly stimulates the proliferation of primary human ECs (25), IFN secretion by dengue virus-infected ECs is also likely to contribute to vascular repair following dengue virus infection. This may in part explain the enhanced pathogenesis of dengue virus infections in IFN receptor knockout mice (12, 37, 64).

Both type I and type II interferons are prominently observed in the circulation of dengue virus patients (29, 30). These innate immune responses may contribute to increased immune cell activation, inflammation, and viral clearance, as well as protecting ECs from infection and NK cell lysis and modulating dendritic cell responses (8, 14, 40). These findings point out the delicate balance of EC functions which recruit, bind, and extravasate immune cells across the endothelium while normally preventing vascular permeability and immune cell targeting of the endothelium itself (23). Dengue infection of ECs may both alter the regulation of vascular permeability and contribute to the immune cell targeting of viral antigens within the endothelium.

Our studies are the first to analyze the synchronous infection of a high percentage of ECs ($\geq 80\%$ infected) and demonstrate that ECs secrete immune-enhancing factors. Prior reports found that dengue virus infection of 2 to 10% of ECs or ECV304 cell lines resulted primarily in a low-level ISG transcriptional response with little or no cytokine induction (6, 34, 54). However, these studies were performed on 90 to 98% uninfected cells at late time points, and they were complicated by the use of ECV304 cell lines, which are reportedly bladder carcinoma rather than endothelial in nature (6, 72). In retrospect, prior low infection rates and induced responses are explained by data in Fig. 2 and 3, which indicate that IFN secreted by a small percentage of initially infected cells protects surrounding ECs from dengue virus spread. Some dengue virus-induced EC responses were also analyzed in LSEC-1, HMEC-1, or HPMEC-ST1.6R cell lines (9, 10, 41, 63). Dengue virus infection of the HPMEC-ST1.6R cell line increased IL-6 and IL-8 (6 to 8 days pi) as well as vascular endothelial cell growth factor (VEGF) levels (9, 10). In contrast, we found no induction of capillary permeabilizing VEGF (9, 23) in primary human ECs, and 1 to 2 days after infection we detected IL-6 and IL-8 induction.

Little is known about EC responses following natural dengue virus infection or how immune responses that target or alter EC functions contribute to dengue virus pathogenesis (30). Murine models further complicate our understanding of the dengue disease process, since they do not fully mimic human responses to infection, lack interferon receptors, or require mouse-adapted viruses (4, 12, 58, 64, 74). In support of a role for infected ECs in mediating severe dengue disease, Zellweger et al. recently reported that, in the presence of subneutralizing levels of dengue-specific antibodies (ADE mediated), a large percentage of infected murine hepatic ECs were detected and correlated directly with disease severity (74). Since evidence for infected ECs is difficult to obtain during human infections, mouse models provide the only current resource for studying the kinetics of dengue virus infection of the endothelium during the course of infection. The early infection of ECs likely plays a pivotal role by increasing viral loads and secreting cytokines and chemokines into the blood. At late times in

infection, responses elicited by dengue virus-infected immune and endothelial cells, as well as circulating dengue antibodies, converge on the endothelium and contribute to vascular permeability. Our findings foster this link between enhanced infection of ECs and viremia, secreted inflammatory factors, and pathogenic vascular permeability (29, 30).

A hallmark of severe dengue disease is the presence of elevated levels of cytokines and chemokines, including IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), CXCL10, CXCL11, IL-10, IFN- γ , tumor necrosis factor alpha (TNF- α), and complement C3a and C5a within the blood of dengue virus-infected patients (7, 24, 27, 29, 30, 62). However, the source of these factors and their role in disease progression are not fully understood. Chemokines CXCL9/10/11, IL-6/8, and RANTES are associated with the recruitment of circulating immune cells to sites of inflammation (39, 43) and are secreted by infected immune cells, such as macrophages, T cells, dendritic cells, and mast cells (15, 17, 22, 24, 32, 56). Our findings, however, provide the first direct evidence that primary human ECs induce properdin factor B and secrete CXCL10, CXCL11, RANTES, BAFF, and IL-7. In contrast, TNF- α and IL-1 β , which were previously suggested to be induced at singular time points (8 or 48 hpi) (72), were unchanged in our arrays. Our data uniquely identified BAFF, a B cell-activating factor that promotes antibody production (44), and IL-7, which stimulates T cell differentiation and survival (2), as factors secreted by dengue virus-infected ECs. Complementing these immune-enhancing responses, CXCL10 promotes viral clearance and induces chemotaxis and the transendothelial migration of activated T cells, while IL-7 also amplifies cytokine production and promotes cytoprotective responses (2). Although these factors may contribute to enhanced immune responses and altered T cell repertoires, BAFF and IL-7 have yet to be evaluated in DHF or DSS patients. These findings suggest that responses of dengue virus-infected ECs are likely to play a significant role in immune cell recruitment, activation, and ADE-directed dengue virus pathogenesis.

The presence of elevated C3a, C5a, and histamine has been associated with severe permeability deficits in dengue virus patients and the development of DHF and DSS (6, 29, 30, 45, 62). Curiously, we observed the high-level induction of properdin factor B in dengue virus-infected ECs. Factor B is the catalytic component of C3 convertase, which ultimately directs the cleavage of C3 and C5 to C3a and C5a effectors (31, 51, 59, 62, 66). These findings provide a direct means for dengue virus-infected ECs to enhance complement activation and C3a and C5a production through EC-elicited responses. C3a opsonizes viral pathogens, directs immune cell chemotaxis by binding C3a receptors on immune cells, primes T cell responses, and is a potent anaphylatoxin that directs mast cell degranulation, histamine release, and vascular permeability (38, 51, 62, 73, 75). Thus, EC-induced factor B has the potential to trigger complement-mediated immune responses by enhancing mast cell recruitment and anaphylatoxin production that result in localized histamine-directed permeability (31, 59, 61, 66).

The dengue virus NS1 protein has been shown to modulate classical complement activation by binding to the C4b binding protein (5). Interestingly, factor B activation stimulates the alternative complement pathway, which bypasses NS1 regulation of the classical complement pathway. In fact, factor B-induced complement pathway activation has the potential to enhance immune cell recruitment and localized vascular permeability (16, 31, 38,

51, 59, 66). Moreover, antibody targeting of factor D, which activates factor B through cleavage, inhibits complement and leukocyte activation in nonhuman primates, and several therapeutics have been developed that antagonize C3a and C5a receptor binding (46, 47, 67, 70). These advances suggest that the alternative pathway may be a new target for therapeutically reducing the severity of DHF and DSS diseases. Additional barrier-stabilizing effectors that target the endothelium also may be considered as a means of therapeutically reducing vascular leakage and inflammation, which contribute to dengue virus pathogenesis (52, 65).

Although we have defined transcriptional and secreted responses of dengue virus-infected ECs, our findings exclude responses of PBMCs that contribute to DHF and DSS (22, 27–30). *In vitro* studies show the transcriptional induction and secretion of cytokines, such as RANTES, CXCL10, CXCL11, IL-6, IL-8, TNF- α , IL-1 β , and/or type I IFN from macrophages, dendritic cells, and mast cells (15, 17, 24, 32, 56). However, the endothelium coordinates signals that normally regulate vascular leakage (1, 13, 71), and factors released by immune cells ultimately act on the endothelium to contribute to edema and hemorrhage (1, 71). ECs balance fluid barrier functions with roles for directing immune cell chemotaxis, extravasation, and activation that are likely to contribute to an antibody-enhanced dengue virus disease process. Our findings suggest that the dengue virus infection of ECs alters this delicate balance of functions that regulate potentially lethal vascular permeability deficits.

Clearly the endothelium is not a static conduit that simply separates tissue from vascular contents (1, 18, 71), and our findings provide a new perspective on the role of ECs in dengue virus pathogenesis. The endothelium dynamically elicits responses that may contribute to immune enhancement and vascular permeability during dengue virus infection, and these findings are fundamental to understanding pathogenic mechanisms of dengue disease. Our data uniquely demonstrate that dengue virus-infected ECs elicit chemotactic factors, immune cell effectors, and complement activators that are likely to potentiate professional immune cell responses and direct vascular permeability. These results make dengue virus-infected ECs a source of immune-enhancing responses and suggest that ECs play a pivotal role in DHF and DSS diseases.

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