Maturation of Dengue Virus Nonstructural Protein 4B in Monocytes Induces Dengue Hemorrhagic Fever-Associated Immunomediators that Modulate Microvascular Endothelial Cell Adhesion Molecules and Vascular Permeability

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ABSTRACT

Dengue virus (DENV) belongs to the Flaviviridae family and exists as four distinct serotypes, DENV-1, -2, -3, and -4. Of the 50-100 million DENV infections worldwide each year, approximately 80% present as mild and self-limiting dengue fever (DF). A limited number of infected patients progress to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), leading to a half-million hospitalizations annually. Mechanisms explaining why some individuals progress to severe DHF/DSS are unclear. Of the ten DENV proteins, several nonstructural proteins (NS), including NS4B and NS5, are capable of inhibiting interferon signaling. DENV preferentially infects peripheral blood monocytes, which secrete elevated levels of chemokines and cytokines in patients progressing to severe disease.

The focus of this dissertation was to study the role of DENV in the induction of chemokines and cytokines and the effects these immunomediators have on endothelial cell adhesion molecules expression and permeability. For the first time, we report that NS4B is a potent inducer of immunomediators associated with severe disease. We demonstrate that sequential processing of the NS4AB polyprotein by the viral protease NS2B3(pro) and via the intermediate 2KNS4B, is significantly more potent than NS4B alone, inducing immunomediators to levels similar to DENV infection. Further, the 2K-signal peptide is not required for the induction of immunomediators yet it plays a synergistic role with NS4B.

A classic clinical sign associated with the progression of DHF/DSS is plasma leakage, which involves vascular endothelial cell changes such as increased
expression of adhesion molecules and permeability. DHF/DSS patients present with elevated levels of tumor necrosis factor-alpha (TNFα) and interleukin (IL)-8, which can modulate vascular endothelial cell changes. Based on our initial finding, we hypothesized that levels of immunomediators induced by maturation of NS4B would be sufficient to modulate human microvascular endothelial cell (HMVEC) phenotypic changes associated with severe dengue disease. We employ a HMVEC monolayer and transwell permeability model in-vitro and demonstrate that the immunomediator milieu present in the supernatants collected from DENV-infected THP-1 cells increase HMVEC permeability and expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (E-sel), while direct DENV infection of HMVEC does not. Furthermore, we demonstrate that the supernatants from monocytes expressing 2KNS4B are sufficient to increase vascular permeability and expression of adhesion molecules, which appear to be synergistically induced by TNFα and IL-8.

Our data demonstrate that maturation of NS4B is primarily responsible for the induction of immunomediators associated with severe dengue disease. Based on clinical data and our findings, therapies to prevent the progression of DHF/DSS-associated phenotypic changes may include antivirals targeting the maturation of NS4B or TNFα inhibitors to neutralize damaging effects caused by their high levels. Given that NS4B topologies are conserved across flaviviruses, NS4B may be an attractive target for the development of Flavivirus-wide therapeutic interventions.
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<td>Ae.</td>
<td><em>Aedes</em></td>
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<tr>
<td>ADE</td>
<td>antibody-dependent enhancement</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>c/EPB</td>
<td>CCAAT/enhancer binding protein</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DENV</td>
<td>dengue virus</td>
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<td>DENV-2</td>
<td>dengue virus serotype 2</td>
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<td>DF</td>
<td>dengue fever</td>
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<td>DHF</td>
<td>dengue hemorrhagic fever</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DSS</td>
<td>dengue shock syndrome</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGM</td>
<td>endothelial cell growth medium</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>ELAM-1</td>
<td>endothelial leukocyte adhesion molecule 1</td>
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<td>E-sel</td>
<td>E-selectin</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
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<td>HCV</td>
<td>hepatitis C virus</td>
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<td>HEK293</td>
<td>human embryonic kidney cell line</td>
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<td>HMVEC</td>
<td>human microvascular endothelial cells</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<td>IFN</td>
<td>interferon</td>
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IL       interleukin
IMPDH    inosine monophosphate dehydrogenase
IP-10    interferon gamma-induced protein 10
LPS      lipopolysaccharide
MEME     minimum essential medium Eagle
MFI      mean fluorescent intensity
MOI      multiplicity of infection
NFκB     nuclear factor kappa-B
NGC      New Guinea C
NS       nonstructural protein
OMPDC    orotidine monophosphate decarboxylase
PAF      platelet-activating factor
pDNA     plasmid deoxyribonucleic acid
PET      polyethylene terephthalate
PFU      plaque forming unit
PMO      phosphorodiamidate morpholino oligomers
qRT-PCR  quantitative real-time polymerase chain reaction
RI       relative intensities
STAT     signal transducers and activators of transcription
TEER     trans-endothelial electrical resistance
THP-1    human acute monocytic leukemia cell line
TNF      tumor necrosis factor
VCAM-1   vascular cell adhesion molecule 1
VEGF     vascular endothelial growth factor
VEGFR2   vascular endothelial growth factor receptor 2
WNV      West Nile virus
YFV      yellow fever virus
CHAPTER 1

BACKGROUND
DENGUE VIRUS

Dengue viruses belong to the family Flaviviridae, genus *Flavivirus*. The uninterrupted open reading frame of the RNA encodes three structural proteins, including the capsid (C) protein, pre-membrane (prM), the precursor to the membrane protein, and the envelope (E) protein, as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [1] (Fig. 1). The dengue virion has a spherical shape, measuring 40–50 nm in diameter [2].

RNA genome

The four genetically distinct DENV serotypes have a positive-sense, single-stranded RNA genome of approximately 11 kilobases (kb) in length. Members of the Flavivirus genus have a type I cap structure (m7GpppAmp) at the 5' end of the genome and a highly structured 3' untranslated region (UTR) [3]. The flavivirus genomic RNA is distinguished by the absence of a 3'-terminal poly(A) tail [4]. There is evidence that the 5' and 3' ends interact, causing cyclization of the genome which may be an important event during RNA replication [5, 6]. The viral genome is translated in the cytoplasm as a polyprotein. The translocation of the polyprotein back and forth across the endoplasmic reticulum (ER) membrane is directed by signal and stop-transfer sequences. The polyprotein is subsequently co- and post-translationally modified by viral and host-encoded proteases to produce the three structural and seven nonstructural proteins (NS) (Fig. 2).
Figure 1. Schematic of the flavivirus genome. Untranslated regions of the genome include the 5' NH$_2$ and 3' COOH terminal structures. The single open reading frame encodes a polyprotein (C to NS5) that is processed by the viral NS2B3 protease (shaded gray and cleavage sites shown with open arrows) and host proteases (solid arrows). The genome is not drawn to scale. Figure reprinted from reference [7] with permission.

Structural proteins

The C protein is highly basic and forms a structural component of the nucleocapsid [8]. The terminal hydrophobic signal sequence is cleaved by the viral NS2B3 protease before virion assembly and maturation [9]. The DENV C protein functions to encapsulate the viral genome, although the mechanism is not known. Infected cells release subviral particles that do not contain either C or the viral genomic RNA, suggesting an important role for C in proper packaging of the infectious virion [10]. DENV-2 C protein contains a hydrophobic cleft on one side where it is proposed to interact with the viral lipid bilayer, and a positively charged region on the opposite side that is thought to interact with the viral RNA [11].
Figure 2. Putative membrane topology of flavivirus proteins. The orientation of the polyprotein and individual proteins with respect to the ER membrane are shown. The proteins are not drawn to scale (left to right). Structural proteins are colored red and nonstructural proteins are colored yellow. The polyprotein is co- and post-translationally processed by viral proteases (blue arrow), host proteases (scissors) and furin (orange triangle). Figure adapted from reference [1].

The prM protein is a glycoprotein that forms heterodimers with E on the intracellular immature virion surface. The dimerization of prM and E is thought to prevent exposure of the fusion peptide within the cell [12]. The prM protein contains two transmembrane helices a signal sequence and a stop transfer sequence, which localizes both prM and E to the lumen of the ER (Fig. 2). prM and E dimerize within the lumen of the ER upon cleavage by a host signalase. Upon maturation and release of the virion from the cell, prM is further cleaved by the host enzyme, furin, to produce M. This process releases E from prM [13]. The mature virion has a smooth appearance compared to the immature virion, which contains spikes on the surface.

The E glycoprotein contains three domains. The amino terminus is located in the center of the folded protein (domain I). Domain II contains the dimerization domain and the fusion peptide. On the other side of domain I is domain III, which contains
the immunoglobulin-like domain. This domain is thought to bind to the cellular receptor [14]. The E protein is glycosylated and responsible for attachment to the cellular receptors and fusion with cell membrane; it also contains the main epitopes recognized by neutralizing antibodies. These epitopes are DENV-specific and cross-reactive for other flaviviruses. It is understood that neutralizing antibody plays a key role in protective immunity against DENV. The role of cellular immunity in the protection against DENV is not completely understood.

**Nonstructural proteins (NS)**

The seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, are encoded in the 3’ region of the viral genome (Fig. 1). The NS1 protein contains a signal sequence that is located in the carboxy terminus of the E protein and is inserted into the ER [15]. NS1 is post-translationally cleaved from NS2A by an unknown ER-resident host protease [16]. NS1 is a glycoprotein that forms homodimers and interacts with membranes; it is also secreted from infected cells or localized to sites of RNA replication within the cell [17-19]. Small amounts of the NS1 associate with the cell surface and are capable of signaling through a glycosyl-phosphatidylinositol (GPI) anchor in response to cross-linking by NS1-specific antibodies [20-22]. In addition, NS1 is an essential component of the viral replication complex [21].

The remaining NS are localized on the cytoplasmic side of the ER membrane as shown in Fig. 2 [16, 23]. NS2A was found to be involved in proteolytic cleavage of NS1 at its carboxy terminus [15]. NS2B associates with NS3 to form the viral protease. The NS2B3 protease mediates highly regulated cleavage events of the
viral polyprotein at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4A/NS4B and NS4B/NS5 junctions [24-26]. NS3 is part of the replication complex and has several enzymatic functions. The serine protease domain of NS3 is in the amino terminus of the protein [4, 27, 28] whereas the helicase function [29], the nucleoside 5′-triphosphatase (NTPase) [29] and the RNA 5′ triphosphatase (RTPase) [30] of the protein is in the carboxy terminus. The small proteins NS4A and NS4B have recently been shown to block type I interferon (IFN) signaling [31-33]. Moreover, others have shown that NS4B interacts with NS3 and dissociates it from ss-RNA [34]. Regulated cleavage at the NS4A-2K-NS4B junction plays a major role in the rearrangement of cytoplasmic membranes involved in replication. One study demonstrated that NS4B could translocate to the nucleus [35].

The NS5 protein is the largest of the DENV proteins (104 kDa) and is highly conserved among the flaviviruses [36]. NS5 consists of a well-characterized RNA dependent RNA polymerase (RdRP) in the carboxy terminus [37-39]. Additionally, the first 296 amino acids of the DENV-2 NS5 amino terminus exhibit methyltransferase activity on capped RNA [40]. Also, NS5 interacts with NS3 as part of the replication complex [41] wherein NS5 can stimulate the NTPase activity of NS3, which is necessary for unwinding of dsRNA substrates by helicase activity during viral replication [42].

**VIRUS LIFE CYCLE AND TRANSMISSION**

Infection of the host cell with flaviviruses occurs by receptor-mediated endocytosis into clathrin-coated pits [43-45] (Fig. 3). Acidification of endosomes causes E protein rearrangement to form homotrimeric on the surface of the virion [46, 47]. It has been
proposed that flaviviruses follow class II membrane fusion [14]. Receptor binding and low pH expose the fusion peptide within domain II (Fig. 3). The hinge between domains I and II presumably allow E to insert the fusion peptide into the lipid bilayer; domain III is thought to fold back onto itself bringing the host bilayer and the viral bilayer into contact allowing hemifusion. The E proteins then form trimers on the virion surface [14]. After the virion has fused with the host cell membrane, the nucleocapsid core is released into the cytoplasm of the cell [45, 48]. The C protein disassociates from the viral RNA and translation of the viral RNA is initiated. Following translation and processing of the viral proteins, NS form a viral replicase and bind to the viral RNA, initiating replication of the viral genome [49].

Cytoplasmic membranes in the perinuclear region of the cell undergo proliferation of the ER and subsequent appearance of smooth membrane vesicles during flavivirus infection [50, 51]. These vesicles are sites of polyprotein processing and viral RNA replication. Once the viral proteins are translated and the genome is replicated, the virion begins to assemble within the lumen of the ER. Immature viral particles are shuttled through the trans-golgi network. During exocytosis, furin protease cleaves prM to M releasing E [52]. E then forms homodimers on the surface of the virion. Mature viral particles are exocytosed and released via secretory vesicles.
**Figure 3. Flavivirus life cycle.**

A. Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis.

B. In the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of RNA into the cytoplasm.

C. Viral RNA is translated into a polyprotein that is processed by viral and cellular proteases.

D. Viral non-structural proteins replicate the genome RNA.

E. Virus assembly occurs at the ER membrane, where capsid protein and viral RNA are enveloped by the ER membrane and glycoproteins to form immature virus particles.

F. Immature virus particles are transported through the secretory pathway. In the low pH of the trans-Golgi network (TGN) furin-mediated cleavage of prM drives maturation of the virus.

G. Mature virus is released into the cytoplasm. Numbers shown in colored boxes refer to the pH of the respective compartments. Figure was reprinted from reference [48] with permission.
CLINICAL DISEASE IN HUMANS

Dengue disease

Over 90% of DENV infections are asymptomatic or result in a self-limiting disease, known as dengue fever (DF), which resolves without complications [53, 54]. A subset of infected patients progresses to a more severe sequel, known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which result in more than a half-million hospitalizations worldwide each year [55, 56]. After an incubation period of 2–7 days, there is a sudden onset of biphasic fever, usually accompanied by retro-orbital or frontal headache and myalgia and bone pain [57] (Fig. 4). A transient macular rash that turns pale under pressure, nausea, vomiting, lymphadenopathy, and taste aberrations can develop [58, 59]. Dengue symptoms are accompanied by leucopenia, variable degrees of thrombocytopenia and possibly seizures. Patients usually recover without complications 3-4 days after the onset of DF, unless they progress to DHF/DSS.

Patients who progress to DHF initially present with similar symptoms as DF patients, including fever, malaise, vomiting, headache, anorexia, and cough [59]. Rapid clinical deterioration occurs shortly after viral clearance, when fever subsides and proinflammatory cytokines are elevated [60]. In the second phase, patients demonstrate cold clammy extremities, warm trunk, flushed faces, irritability, and may progress to a rapid weak pulse and hypotension [57, 61, 62]. These clinical signs last approximately 24 - 36 h. Bleeding and plasma leakage, the classical sign of severe dengue disease, begin when the patient’s temperature returns to normal.
Evidence of plasma leakage—typically manifested as a rise in hematocrit equal to or greater than 20% above average for age, sex and population—differentiates DHF/DSS from DF [57]. Severe dengue diseases are most commonly observed among children less than 15 years of age and who have experienced secondary infection, but DHF/DSS also occurs in adults and in children experiencing primary DENV infections [63].
Pathological findings

Clinical evidence suggests that plasma leakage progresses rapidly shortly after peak viremia and defervescence when chemokines and cytokines circulate at elevated levels [64-72]. High levels of chemokines and cytokines are associated with DHF/DSS [60]. Interestingly, as observed in autopsy tissues, there is little evidence that the vascular endothelium is damaged during DENV infection [73] even though DENV replicates efficiently in human vascular endothelial cells in vitro [74-82]. In spite of these in-vitro findings, data demonstrating DENV infection of endothelial cells in humans are very limited, unlike patients with other viral hemorrhagic fevers, such as Ebola [83] or hantavirus cardiopulmonary syndrome [84]. In one autopsy study, DENV antigen was detected in sinusoidal endothelial cells of the liver and vascular endothelium in the lungs; however, viral RNA was not detected and the authors suggested that their findings represented pinocytosis rather than true infection of endothelial cells in-vivo [85]. None-the-less, plasma leakage is thought to be due to altered vascular permeability rather than structural damage to the endothelial cells. Although the mechanism explaining DENV-induced plasma leakage is still undefined, in-vitro studies suggest that increased plasma leakage is mediated by pro-inflammatory cytokines and chemokines such as interleukin (IL)-6, IL-8 and TNF-α [74, 86]. Apart from increased plasma leakage, moderate degrees of abnormal hepatic function manifested as symptoms resembling acute hepatitis are associated with DHF/DSS. Most liver alterations are mild with fatty changes and degeneration of hepatocytes and Kupffer cells [87, 88]. As a result, aspartate transaminase (AST) and alanine transaminase (ALT) levels are significantly higher as severity of dengue disease progresses.
**EPIDEMIOLOGY**

DENV causes substantial human morbidity and mortality worldwide, primarily in tropical and subtropical areas. Approximately 2.5 billion people, or nearly half of the world’s population in over 100 countries, are at risk of infection [56]. Of the estimated 50-100 million DENV infections occurring annually worldwide, approximately 25,000 deaths occur depending on the epidemic activity and the presence of the *Aedes* (Ae.) mosquito vector [56, 89] (Fig. 5). Moreover, DENV causes explosive outbreaks with infection rates as high as 80-90% among individuals previously unexposed to the virus [89]. Based only on a small number of prospective cohort studies, severe disease has increased dramatically around the globe during the past two decades as the result of an expanding geographical distribution of the *Ae.* mosquito and increased human travel [57, 62, 89, 90]. Also, the resurgence of global dengue epidemics may be due to unprecedented population growth, unplanned and uncontrolled urbanization, inadequate waste management and water supply, lack of effective mosquito control [53, 63].

**PATHOGENESIS**

Approximately 10% of DHF cases occur during a primary DENV infection, while the vast majority of DHF patients are infected more than once [91, 92]. Moreover, DHF commonly occurs in areas where multiple DENV serotypes co-circulate, increasing the chance of a secondary infection with a different DENV serotype and leaving the patient at risk for enhanced infection (see below section on antibody-enhanced infection).
Figure 5. Global distribution of dengue epidemics and presence of *Ae. aegypti*. Black: areas where dengue epidemics are reported. Grey: confirmed presence of *Ae. aegypti*. Figure used with permission from reference [57].

Moreover, clinical studies indicate that patients who progress to DHF/DSS demonstrate elevated viremia and high levels of chemokines and cytokines, which are thought to alter vascular endothelial cells and increase permeability and plasma leakage [60, 64-67, 70, 81, 93, 94]. Given that increased plasma leakage following DENV infection is generally short-lived and without endothelium damage, the general dogma remains that pathogenesis is due to an immunological response wherein DENV infection of monocytes, macrophages and dendritic cells (DCs) either via direct infection or antibody-dependent enhancement (ADE) causes an overproduction of chemokines and cytokines that cause phenotypic changes to the endothelium leading to increased vascular permeability (Fig. 6).
**Figure 6. Model of DHF immunopathogenesis.** After primary infection with a virulent DENV strain or secondary heterologous DENV strain in monocytes or macrophages, increased viremia and elevated levels of chemokines and cytokines, such as IL-6, IL-8 or TNFα, can alter vascular endothelial cells leading to increased expression of adhesion molecules and increased vascular permeability and plasma leakage. Other factors influencing elevated levels of chemokines and cytokines include the complement system and activated T cells. Several risk factors associated with severe dengue disease include virulence of viral strains and levels of viremia, primary or secondary infection, chemokines and cytokines, and immune cells (particularly monocyte, DCs and T cells).

Other potential factors leading to increased chemokine and cytokine production include the complement system and altered T-lymphocyte reactivity.
**Virulence of viral strains**

Virus virulence can be defined as the capacity of a virus to produce disease. The different manifestations of DF, DHF, and DSS may be caused by variants of DENV with different degrees of virulence. Historically, the risk of DHF/DSS is higher in infections with DENV serotype 2 and genotypes from the Asian lineage compared to the other serotypes (DENV-1, DENV-3 and DENV-4) and genotypes from the Americas [95, 96]. Phylogenetic analyses suggest that increased pathogenicity in the Americas by DENV-2 strains originated from Southeast Asian DENV-2 strains that displaced the American genotypes [96]. Also, Southeast Asian genotype viruses demonstrated a greater production of viral progeny from DCs than viruses from the American genotype [97], which may indicate a virus’ ability to induce elevated levels of immunomediators. It is known that high viremia is associated with the development of DHF/DSS. Studies demonstrate that peak viral infectivity titers are 100- to 1,000-fold higher in patients with DSS than those in DENV-infected Thai children with DF [94, 98]. Whether viral load or rapid growth rate in vivo accurately reflects the virulence of specific genotypes remains unclear. Moreover, genetic differences have also been found among various isolates of DF and DHF patients, suggesting that expression of viral proteins may be more important for virulence than originally suspected [99].

**Antibody-dependent enhancement (ADE)**

The presence of cross-reactive, sub-neutralizing anti-DENV antibodies upon infection is a risk factor for developing DHF/DSS. Sub-neutralizing antibodies can form virus-antibody complexes that bind to target cell Fcγ receptors, resulting in
enhancement of infection (Fig. 6). Enhanced infection of Fcγ receptor-positive cells by sub-neutralizing antibody-virus complexes is known as ADE. Sera obtained before infection from children who later developed DHF/DSS were much more likely to demonstrate ADE in-vitro than those who had DF [100]. Additionally, infants less than one year of age who acquire maternal anti-DENV antibody are also susceptible to ADE, developing DHF/DSS following primary infection; the levels of maternal anti-DENV antibodies in the infants had to decline to levels that would enhance DENV infection and lead to DHF [101]. These observations are consistent with the idea that enhancing antibodies increase the number of DENV-infected cells and viremia, leading to DHF. The antibodies that mediate ADE in DHF are primarily directed against the E protein [102].

**Complement activation**

Activation of complement is another important clinical manifestations in DHF. Levels of C3a and C5a, complement activation products, are correlated with the severity of DHF, both of which peak at defervescence when plasma leakage is most apparent [103]. Both C3a and C5a are proinflammatory molecules involved in activation and chemotaxis of myeloid cells expressing anaphylaxtin receptors which further instigate an altered state of innate immunity upon activation [104]. C5a is a potent chemoattractant of neutrophils and monocytes while C3a is known to induce histamine release from mast cells, which enhances vascular permeability [104]. Additionally, high levels of secreted NS1 and pre-existing cross-reactive antibody may also mediate complement activation by depositing C3 and C5b-9 on DENV-infected endothelial cells [77]. These mechanisms have been proposed to contribute to increased vascular leakage in DHF [77].
Immune cells: monocytes and T lymphocytes

Monocytes have been reported to be the most permissive cell type for *in-vitro* DENV infection [105]. Similarly, in PBMC obtained from dengue patients, infectious DENV was detected more frequently and at higher levels in the adherent PBMC population than in the nonadherent cell population [106, 107]. Based on these findings, monocytes and tissue macrophages are thought to be the predominant cell target for DENV infection *in vivo*. Moreover, DENV-infected monocytes increase chemokine and cytokine production [74, 108, 109], demonstrating that DENV infection of human monocytes induces the production of vasoactive factors and play a significant role during DHF immunopathogenesis.

It has been proposed that T cells play an important role in the immunopathogenesis of DENV diseases [110, 111]. Cytotoxic T lymphocytes (CTLs) have been demonstrated to play an important role in viral clearance from the host [112] as virus-specific CTLs directly lyse DENV-infected antigen-presenting cells. Although very important during the host defenses against virus establishment, these CTLs can release chemokines and cytokines, such as TNFα and interferon-gamma (IFNγ), upon activation contributing to vascular phenotypic changes. IFNγ increases the expression of Fcγ receptors on monocytes which can increase antibody-virus complex uptake and production of endothelial cell altering chemokines and cytokines [113]. Additionally, others found that some DENV-specific CD8+ T cells were cross reactive with heterotypic DENV serotypes; this cross reactivity indicates their potential to be activated during secondary DENV infection, not necessarily having killing effects of DENV-infected cells but secreting high amounts of chemokines and cytokines [114, 115]. This immunological response is known as original antigenic sin.
DENV AND HUMAN INNATE IMMUNITY

Interferon (IFN) α and β responses

The innate immune system is the first line of defense against invading viral pathogens, including DENV. Once the host recognizes the invading virus, signaling pathways are activated to induce expression of multiple transcription factors that bind to the interferon α and β promoters [116, 117]. As with other viruses, the innate immune response is activated by DENV infection and can inhibit DENV replication. Knockout mice that lack IFNα/β and IFNγ receptors develop paralysis and die after challenge with DENV [118, 119]. Pretreatment of cultured cells with IFNα/β dramatically reduced DENV replication through inhibition of translation of DENV RNA; by contrast, addition of IFNα/β after DENV replication has little effect on DENV replication [120, 121]. These data suggest that IFN signaling is not able to properly function in cells that are infected with DENV. In addition, DENV replicates in humans to high titers, up to 10 infectious doses per mL, even though there are high levels of circulating IFNα [71, 122]. These results suggest that there are mechanisms that DENV uses to counter the IFN response, as seen with other viruses [123].

Chemokines and cytokines

DENV-infected monocytes and other immune cells such as macrophages and DCs contribute to the overproduction of chemokines and cytokines found during severe disease [106]. Moreover, DENV-infected primary human monocytes in-vitro secrete high levels of DHF/DSS-associated chemokines and cytokines [124, 125].

Chemokines are small molecules whose primary role during the immune response is
to attract leukocytes to sites of inflammation. Chemokines have been implicated in many aspects of immunity including hemopoietic cell migration, Th1/Th2 development, angiogenesis/angiostasis, cell recruitment, lymphoid trafficking and lymphoid organ development [126]. An important chemokine associated with severe DHF/DSS is interleukin (IL)-8 (also known as CXCL8). IL-8 is a member of the CXC chemokine family and is produced by many cell types, including monocytes/macrophages, T cells, neutrophils, fibroblasts, endothelial cells, keratinocytes, hepatocytes, astrocytes and chondrocytes. Expression of IL-8 is induced not only by viral infection, but also as a result of other stimuli such as LPS, TNFα, and IL-1β [127]. Its chemoattractant effects on neutrophils and T cells [128] has been shown to promote adhesion of neutrophils to endothelial monolayers [129] and stimulate neutrophils migration across endothelium [130]. Also, recombinant IL-8 alone has been shown to promote vascular endothelial cell permeability [131].

Differential expression of chemokine receptors and the expression of specific chemokines such as IL-8, determine the migration of particular cells during inflammation [132]. Moreover, chemokines regulate the migration of leukocytes into extravascular tissues [133]. For example, leukocytes stop active rolling along the endothelium at sites of infection once specific vascular endothelial cell ligands, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) bind to their leukocyte integrin receptors [134]. As chemokines expressed at the sites of infection produce a gradient of proteins, an increase in the avidity of leukocyte receptor binding to endothelial ligands results, thus creating a tight adhesion of the leukocyte to the vascular endothelium. This adhesion allows the leukocyte to transmigrate, or undergo diapedesis, at intercellular borders or junctions.
found within the endothelium [134]. Leukocytes then move along the chemokine concentration gradient toward the site of infection. Therefore, chemokines are an important defense against virus infections and ultimately play an important role influencing the disease outcome.

Cytokines are involved in intracellular communication and are also important during innate immunity. TNFα, undoubtedly one of the most prolifically studied cytokines, is involved in acute phase systemic inflammation. A potent regulator of immune cells, TNFα serves several functions during innate immunity including induction of apoptotic cell death, induction of inflammation, and inhibition of tumorigenesis and in some cases, virus replication [135]. Moreover, TNFα induces the expression of endothelial cell adhesion molecules, migration of inflammatory cells to sites of infection or injury and activates the production of inflammatory mediators from immune cells such as macrophages and neutrophils [136]. TNFα is not usually detected in healthy individuals; however increased serum and tissue levels are found during acute inflammation and DENV infection, corresponding to the severity of dengue disease [57, 64, 137, 138]. During DENV infection, the innate immune system is often altered, leaving immunomediator production unchecked. High levels of chemokine and cytokines may have a detrimental effect on disease outcome by promoting over migration of leukocytes, over production of other immunomediators and further promotion of permeability leading to increased plasma leakage.

**Virus-cell interactions governing chemokine and cytokine production**

Immediately following virus infection, a strong host response and production of chemokines and cytokines may be initiated via a number of pathways (Fig. 7).
Figure 7. Virus-cell interactions influencing chemokine and cytokine expression. Viruses induce cells to produce cytokines by a number of mechanisms. First, virus-receptor interactions can induce intercellular signal transduction leading to cytokine expression. Second, virion-associated proteins released into the infected cell upon entry may stimulate immunomediator production. Third, infected cells detect accumulation or viral dsRNA through several mechanisms that support production of proinflammatory mediators. Fourth, protein overload in the ER, due to massive production of viral proteins, activates cellular stress signaling and can trigger immunomediator production. Finally, virus encoded proteins produced during the productive replication cycle can initiate a number of cellular signal transduction pathways and hence support expression of chemokines and cytokines. For each mechanisms a few examples are given. EMCV, encephalomyocarditis virus. Figure was reprinted from reference [139] with permission.

The mere interaction of viral surface proteins with cellular surface proteins can initiate intracellular signaling events that cause chemokine and cytokine production [139]. Also, accumulation of viral RNA and overload of the cellular protein synthesis machinery induces signals that are able to trigger an early host response and
influence immunomediator production [139]. Moreover, several viral proteins not present in the infectious particle but produced during the course of the viral life cycle can modulate cellular signaling and cause chemokine and cytokine production. Intracellular viral proteins can interfere with cellular signal transduction and transcription factor activity, thus promoting viral replication and expression of immunomediators. Among the signaling pathways activated by the viruses, NF-kB seems to play a particularly important role [140].

**DENV-INDUCED VASCULAR PERMEABILITY**

**Vascular endothelium and its dysfunction**

The vascular endothelium lines the inner surface of blood vessels and capillaries and serves as the first line of interface for circulating blood components interacting with the cell wall. A major function of the vascular endothelium, especially microvascular capillaries, is to provide a semi-permeable barrier that controls blood–tissue exchange of fluids, nutrients, and metabolic wastes while preventing pathogens or harmful materials in circulation from entering into tissues [141]. Appropriate regulation of microvascular fluid hydrodynamics and endothelial barrier function is vital to support normal tissue viability and organ function [141]. During host defense against virus, or other pathogen infections, or tissue injury, endothelial barrier dysfunction results in an inflammatory response. Inflammation and endothelial barrier dysfunction are characterized by leakage of fluids, proteins, or small molecules and clinically manifests as accumulation of plasma fluid in the extravascular space. Plasma leakage is often accompanied by leukocyte migration and subsequent infiltration into the surrounding tissues. Moreover, excessive leakage can disturb fluid
homeostasis and can lead to hemorrhagic manifestations, multiple organ dysfunction, shock and death [141]. Although endothelial barrier dysfunction is a common and key process underlying DHF/DSS, it is exceptionally difficult to treat clinically due to unavailable therapeutic interventions.

**DENV infection and dysfunction of the vascular endothelium**

Although perivascular edema is obvious in DHF/DSS patients, little or no destruction of the vascular endothelium is evident. It is thought that release of immunomediators in DENV-infected patients probably causes the functional alteration in endothelial cells. DENV infection can induce apoptosis of endothelial cells *in vitro* but this effect is dependent on the DENV isolate employed for infection [77].

**In-vitro permeability models**

In-vitro DENV-induced permeability changes can be measured by several methods (Table 1). The most common method is measuring the migration of a specific molecule from the apical to basolateral side of an endothelial cell permeability model. This can be accomplished by using molecules of physiologically relevant size, such as horseradish peroxidase, FITC-conjugated dextran, or albumin. A more sensitive method measures transendothelial electrical resistance (TEER) changes across the endothelial cell permeability model. TEER utilizes concentric electrodes to measure the differential voltage across endothelial cells where even very small changes in permeability can be determined. A number of other studies examining permeability changes or DHF/DSS utilize both of these methods [131, 134, 142-151].
Table 1. Select studies that investigate DENV-initiated permeability.

<table>
<thead>
<tr>
<th>Model</th>
<th>Cell Type</th>
<th>Permeability assay</th>
<th>Pathogen/exposure</th>
<th>Results</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transwell cell culture inserts</td>
<td>HUVEC</td>
<td>Trans-endothelial albumin flux (good for &gt;21 days)</td>
<td>TNF, developed to measure DHF</td>
<td>TNF induced a reversible increase in permeability in a dose depend manner</td>
<td>[145]</td>
</tr>
<tr>
<td>Transwell cell culture inserts</td>
<td>HUVEC</td>
<td>Horseradish peroxidase (HP) after exposure to thrombin and TNF</td>
<td>Supernatant from DENV-2 infected monocytes</td>
<td>Induction of permeability did not coincide with release of TNF</td>
<td>[86]</td>
</tr>
<tr>
<td>Transwell cell culture inserts</td>
<td>ECV304, endothelial cell line</td>
<td>Trypan blue labeled albumin (TB-BSA)</td>
<td>DENV-2</td>
<td>ECV cell line monolayers more stable</td>
<td>[78] [152] [152] [152] [152] [143]</td>
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<tr>
<td>Miillacell filter inserts</td>
<td>Human dermal microvascular endothelial (HMEC1)</td>
<td>Paracellular passage of dextran and [3H]mannitol from apical to basolateral</td>
<td>DENV-2</td>
<td>Alterations of permeability at 48 h and actin cytoskeleton rearrangements</td>
<td>[149]</td>
</tr>
<tr>
<td>Transwell membrane plate</td>
<td>HUVEC</td>
<td>TEER (Endohm chamber)</td>
<td>DENV-2</td>
<td>Cytokines and DENV-2 had different effects on TEER and cell morphology</td>
<td>[143]</td>
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HYPOTHESIS AND SPECIFIC AIMS

The long-term goal of the proposed research is to understand the basic molecular events involved in DHF immunopathogenesis as a prerequisite to developing therapeutic interventions and mitigating the progression of severe dengue diseases. The objective is to elucidate the role of DENV proteins—as expressed in THP-1 monocytic cells—on immunomediator production and the downstream effects of secreted immunomediators on primary human microvascular endothelial cells (HMVEC), including adhesion molecule expression and permeability modulation. The
central hypothesis of this study is that DENV NS expressed sequentially in the monocytic cell line, THP-1, induce immunomediators that modulate endothelial cell adhesion molecules and permeability similar to the effects caused by the wild-type DENV infection.

To address the hypothesis, the following specific aims are proposed:

1. To characterize chemokine and cytokine induction potential of DENV and DENV NS in monocytes.
2. To establish the role of DENV-induced, monocyte-derived chemokines and cytokines on the modulation of endothelial cell adhesion molecules and permeability.

The rationale is based on data demonstrating that expression of all DENV NS or NS5 alone induces IL-8 in HEK293 cells and that DENV-infected monocytes induce immunomediators that activate the expression of adhesion molecules on HMVEC. The proposed research is innovative as it focuses on a neglected area of DENV research, specifically viral protein induction of host immunomediators from monocytes, cells thought to be a primary target during DENV infection. Also, we will employ primary HMVEC, a clinically relevant cell type, to study phenotypic alterations, including expression of adhesion molecules and permeability. Our expectations at the end of this work are that we will characterize and identify one or more DENV NS responsible for initiating the production of immunomediators, which modulate endothelial phenotypic changes associated with severe DHF/DSS. These fundamental data will have significant impact on understanding DHF immunopathogenesis and will add to our understanding of viral-host interactions that
may be exploited to develop therapeutic interventions and mitigate DHF/DSS.

**SIGNIFICANCE**

DENV infects 50 to 100 million people worldwide each year with approximately 500,000 cases progressing to DHF. Trends show that the incidence of DHF is on the rise yet no antiviral drugs or vaccines have been developed to mitigate the expanding problem. Circulating high levels of secreted immunomediators from DENV-infected monocytes, DCs and other cell types are thought to increase vascular permeability by altering endothelial cell adhesion molecules or tight junction proteins. However, a neglected area of DENV research involves understanding the ability of DENV proteins to induce host immunomediators that alter vascular permeability. Given that no ideal animal model exists to study DHF immunopathogenesis, the proposed research will utilize *in-vitro* model systems to delineate cellular and molecular events involved in DENV induction of immunomediators. The proposed research is significant, as we will discover essential DENV proteins capable of initiating altered innate immune response, thereby eventually exploiting critical cellular-host interactions and developing therapeutic interventions to mitigate the severity of DENV diseases.
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CHAPTER 2

MATURATION OF DENGUE VIRUS NONSTRUCTURAL PROTEIN 4B IN MONOCYTES ENHANCES PRODUCTION OF DENGUE HEMORRHAGIC FEVER-ASSOCIATED CHEMOKINES AND CYTOKINES

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Dengue virus, DENV; dengue hemorrhagic fever, DHF; chemokines and cytokines; nonstructural protein NS5, NS4B; 2K-signal sequence; THP-1 monocytes
ABSTRACT

High levels of viremia and chemokines and cytokines underlie the progression of severe dengue disease. Dengue virus (DENV) preferentially infects peripheral blood monocytes, which secrete elevated levels of immunomediators in patients with severe disease. Further, DENV nonstructural proteins (NS) are capable of modifying intracellular signaling, including interferon inhibition. We demonstrate that peak secretions of immunomediators such as IL-6, IL-8, IP-10, TNFα or IFNγ in DENV-infected monocytes correlate with maximum virus production and NS4B and NS5 are primarily responsible for the induction of immunomediators. Furthermore, we demonstrate that sequential NS4AB processing initiated by the viral protease NS2B3(pro) and via the intermediate 2KNS4B significantly enhances immunomediator induction. While the 2K-signal peptide is not essential for immunomediator induction, it plays a synergistic role with NS4B. These data suggest that NS4B maturation is important during innate immune signaling in DENV-infected monocytes. Given similar NS4B topologies and polyprotein processing across flaviviruses, NS4B may be an attractive target for developing Flavivirus-wide therapeutic interventions.
INTRODUCTION

Dengue virus (DENV) causes considerable risk to human health worldwide infecting an estimated 50-100 million people annually and causing explosive outbreaks with infection rates as high as 80-90% among individuals previously unexposed to the virus [1]. The incidence of dengue diseases has dramatically increased during the past two decades as the result of an expanding geographical distribution of the Aedes mosquito vector and increased human travel [1-3]. Over 90% of DENV infections are asymptomatic or result in self-limiting dengue fever (DF) cases that resolve without complications [4, 5]. However, a subset of infected patients progresses to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), resulting in over a half-million hospitalizations each year worldwide [6, 7]. No definitive mechanisms explain the progression of DHF/DSS, which can be defined in part as bleeding and increased plasma leakage into the pleural cavities and peripheral tissue without morphological damage to the capillary endothelium [8, 9]. Clinical studies indicate that patients who progress to severe disease demonstrate elevated viremia [10-12] and high levels of interleukin (IL)-6, IL-8 and tumor necrosis factor alpha (TNFα) in the bloodstream [13-18]. Similarly, peripheral blood monocytes from patients with DHF/DSS display elevated DENV antigen and increased expression of activation markers and production of immunomediators [19-21], implicating monocytes as important cells during infection and severe disease pathogenesis. Moreover, DENV-infected primary monocytes secrete DHF/DSS-associated immunomediators [22, 23].
DENV belongs to the family *Flaviviridae* and consists of four genetically distinct serotypes having a positive-sense, single-stranded RNA genome of approximately 11 kilobases (kb) in length. The RNA encodes for a polyprotein precursor that is co- and post-translationally processed into three structural (C, PrM and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [24, 25]. The NS are responsible for various enzymatic activities during replication, including the NS5 RNA-dependent RNA polymerase (RdRP) and methyltransferase activity required for viral RNA capping [26-30], the NS3 helicase and the NS2B3 protease (NS2B3pro) [31-33]. Several NS, such as NS3, NS4B and NS5, interact as part of the viral replication complex facilitating transcription and translation of the viral genome [34-40]. Further, accumulating evidence suggests that intrinsic DENV genetic characteristics within NS4B and NS5 are associated with severe disease outcomes [41].

DENV NS5 induces IL-8 transcription and protein secretion in human embryonic kidney cells, [42] and inhibits the interferon alpha (IFNα) response through binding and degradation of STAT2 [43, 44]. Also, NS4B strongly inhibits the IFN transduction cascade by interfering with STAT1 phosphorylation [45]; and processing of NS4AB by viral and host proteases is required to initiate an IFN-antagonistic function [46]. Nonstructural protein-induced subversion of the host IFN response and induction of immunomediators may simultaneously promote DENV survival while increasing the risk of severe disease outcomes. Based on the aforementioned data, to further understand the role of NS in dengue immunopathogenesis, we hypothesized that NS5 and maturation of NS4B expressed in monocytes would induce DHF-associated immunomediators. In this
we demonstrate that both NS5 and NS4B induce immunomediators and that NS4B maturation via cleavage of the NS4AB polypeptide, in a 2KNS4B-dependent manner, significantly enhanced immunomediator production in monocytes.

**MATERIALS AND METHODS**

*Virus and cell culture*

DENV-2 New Guinea C (NGC) strain was obtained from Dr. Duane Gubler. A virus stock was produced by passaging virus twice in C6/36 cells. Given the extensive use of the THP-1 cell line by others examining DENV pathogenesis and due to their permissibility to DENV infection [47-51], we chose to work with THP-1 cells in this study. The THP-1 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, Cat. No. TIB-202) and were cultured in RPMI-1640 (ATCC) supplemented with 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS) (Gibco Labs, Grand Island, NY) and 2-mercaptoethanol to a final concentration of 0.05 mM; cells were incubated at 37°C in a 5% CO₂ atmosphere. The Vero cells (monkey kidney epithelial cells) (ATCC, Cat. No. CCL-81) were maintained in M199 and supplemented with 1% penicillin/streptomycin and 10% FBS.

*Infection of THP-1 cells*

For infection experiments, 1 x 10⁶ THP-1 cells were infected with DENV at MOI-0.1 or -1. After 1.5 h at 37°C and 5% CO₂, the cells were washed and further cultured with fresh growth media. UV-inactivated DENV was generated using
previously published protocols [52, 53]. Briefly, DENV was diluted in 500 µL PBS in a 35-mm culture plate and exposed to UV radiation using a Stratalinker 2400 device (Stratagene) for 10 min. Inactivation of virus infectivity was verified by plaque assay using Vero cells. Mock-infected control THP-1 cells were infected with UV-inactivated DENV and for positive controls, THP-1 cells were incubated with 1 µg/mL of lipopolysaccharide (LPS) for 1 h, washed and incubated with fresh growth media until collection at 24 h. Every 24 h, cells and supernatants were collected while remaining cells were replenished with fresh growth media. Infectious virus released from infected cells was confirmed by plaque assay on Vero cells, as described below.

Plaque assay

To determine the amount of infectious virus released from DENV-infected THP-1 cells, plaque assay was conducted using Vero cell monolayers as described previously [54]. Briefly, 2.5 x 10⁵ Vero cells per well were seeded in 6-well culture plates (Corning, Lowell, MA) and incubated for 2 to 3 days until confluent. Supernatants from DENV-infected THP-1 cells were serially diluted 10-fold in Dulbecco’s Modified Eagles Medium (DMEM) (Gibco Labs) supplemented with 10% FBS and 100 µL of each dilution was added to each well of the Vero cells followed by incubation at 37°C and 5% CO₂ for 1 h with rocking every 15 min. Three mL of primary nutrient agar containing 1% SeaKem® LE agarose (Lonza, Walkersville, MD) was added to each well and the plates were incubated for 5 days. Three mL of secondary nutrient agar (primary agar containing 1% neutral red) was added to each well and the plates were incubated for an additional two
days before counting plaques and calculating viral titers [54]. Titers were expressed as plaque forming units (PFU) per mL.

Construction of plasmids

We employed standard molecular biology techniques to clone the DENV genes [55]. Each primer listed in Table 2 was designed from the DENV-2 NGC reference genome, NCBI accession number M29095. All forward primers contain the Kozac sequence and ATG start codon (Table 2). PCR was conducted using AmpliTaq Gold® DNA polymerase (Applied Biosystems, Carlsbad, CA) with either the first-strand cDNA template (iScript® cDNA synthesis kit, BioRad Inc., Hercules, CA) synthesized from the wild-type DENV-2 NGC RNA extracted from stock viral supernatant using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) or the DENV-2 NGC DNA infectious clone (kindly provided by Barry Falgout, Food and Drug Administration) [56]. Following the manufacturer’s protocol, PCR products were cloned into either the pcDNA3.1/V5-His-TOPO® or the pcDNA3.1/CT-GFP-TOPO® vector (Invitrogen, Carlsbad, CA) having the CMV promoter for mammalian expression and detection. Proper gene orientation and identity were confirmed by DNA sequencing (Greenwood Molecular Biology Facility, University of Hawaii) and sequence analysis was conducted using Sequencer® 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). Selected plasmids were isolated using cesium chloride ethidium bromide equilibrium centrifugation, as previously described [55].

Transfections
In order to express the DENV-V5 fusion proteins, we transfected each construct into THP-1 cells using the Neon™ Transfection System (Invitrogen) after performing a series of optimization protocols as specified by the manufacturer. Briefly, 5 x 10^5 cells were electroporated with 1 µg of plasmid DNA using 1,250 volts and 40 msec for 1 pulse. Cells were directly added to growth media without penicillin/streptomycin and immediately incubated at 37°C and 5% CO₂. The supernatants and cells were harvested at 40 h after electroporation and used for the assays described below.

**Flow cytometry**

Intracellular expression of DENV-V5 or DENV-green fluorescent protein (GFP) fusion proteins was detected at 40 h after transfection using a Guava EasyCyte flow cytometer (Guava, Hayward, CA). Cells expressing DENV-V5 proteins were washed, fixed and permeabilized (Fix & Perm reagents, Invitrogen) for intracellular labeling with a mouse monoclonal V5 antibody (Invitrogen) diluted 1:500 in PBS and a secondary antibody against mouse IgG coupled with Alexa Fluor® 594 antibody (Invitrogen). Similarly, cells expressing DENV-GFP were washed, fixed and resuspended in PBS for flow cytometry. Data were analyzed using FlowJo software version 4.3 and expressed as percent cells expressing DENV protein.

**Quantitative real-time RT-PCR (qRT-PCR)**

Total cellular RNA was extracted from DENV-infected, pDNA-transfected and control THP-1 cells harvested at various time points using the RNeasy® Plus kit with RNase-Free DNase (Qiagen) as per manufacturer protocol. cDNA was
synthesized using 1 µg of RNA using the BioRad iScript® kit in a 20 µL reaction volume. BioRad iCycler iQ™ Multicolor Real-Time PCR Detection System was employed to conduct qRT-PCR for quantitation of DENV and V5 copy numbers using Bio-Rad iQ™ SYBR® Green Supermix, 2 µL of 1:3 diluted cDNA, and 10 pmol each of forward and reverse primers (Table 1) in a final reaction volume of 20 µL. Thermal cycling reactions for both DENV and V5 amplifications were initiated with a denaturing step of 4 min at 95°C, followed by 40 cycles of 95°C (30 sec) and 55°C (30 sec for DENV and 15 sec for V5).

Table 1. Primers employed for the analysis of DENV and host genes by qRT-PCR.

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<tr>
<th>Gene and Primer Names</th>
<th>Primer Sequence (5' - 3')</th>
<th>Tm (°C)</th>
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<td>TNFα-R</td>
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*a* Forward, sense sequence; reverse, antisense sequence

*b* Real-time PCR primers used to determine DENV2 NGC and V5 copy numbers

*c* Real-time RT-PCR primers to amplify immunomediator genes
A standard curve was developed from 10-fold serial dilutions of linear DENV or V5 gene having known concentrations to quantitate the dynamic range of detection of $10^1$ to $10^8$ copies per µg of RNA. Host cellular gene changes relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene were determined as previously described [57]. Primers used to measure cellular gene changes are listed in Table 1.

*Cytokine quantitation*

Interleukin (IL)-6, IL-8, IFN-gamma-inducible protein (IP)-10, tumor necrosis factor alpha (TNFα), vascular endothelial growth factor (VEGF) and interferon gamma (IFNγ) levels were measured in the supernatants of DENV-infected and pDNA transfected THP-1 cells using a Milliplex human cytokine and chemokine 6-plex immunoassay kit (Millipore Corp., Billerica, MA) together with the Luminex® 100™ System (Luminex, Austin, TX) to determine mean fluorescent intensities (MFI) as recommended by the manufacturer. Protein concentrations were calculated from MFI data using 10-fold serially diluted standards and Bead View analysis software version 1.0.4 (Millipore). The minimum detectable concentrations were 0.4 pg/mL for IL-6, 0.3 pg/mL for IL-8, 1.3 pg/mL for IP-10, 0.2 pg/mL for TNFα, 10.1 pg/mL for VEGF and 0.4 pg/mL for IFNγ.

*Western blot*

Total cellular protein extracts were prepared from THP-1 cells at 40 h after electroporation with DENV-V5 fusion plasmids. Cells were washed once with cold PBS and extracted with 200 µL of M-PER mammalian protein extraction buffer (Thermo Scientific, Rockford, IL) or NP-40 detergent buffer containing
EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN). Either 20 µL or 50 µg of total protein was fractionated on a 4-12% gradient SDS polyacrylamide gel using the Mini-Protean II (Bio-Rad, Hercules, CA) and then transferred onto a 0.2 µm nitrocellulose filter (Bio-Rad Laboratories) as previously described [57]. Nonspecific binding sites were blocked using 5% FBS in 1x Tris buffered saline with 0.1% Tween and membranes were incubated at 4°C overnight with primary V5 or β-actin antibodies (dilution 1:1,000) followed by incubation with secondary antibodies conjugated to HRP (dilution 1:5,000) at room temperature for 1 h. Protein was detected with enhanced chemiluminescence (Amersham ECL, GE Healthcare Limited, Buckinghamshire, UK) using Amersham ECL Hyperfilm (Kodak, Rochester, NY). To determine the relative intensity (RI) of protein bands, the absolute intensity of the DENV-V5 protein band was divided by the absolute intensity of its corresponding β-actin band. Absolute intensities were calculated using Photoshop by multiplying the given pixel value and mean intensity of selected bands as previously described [58].

Cell viability assay

To determine the cell viability, 1 x 10⁶ THP-1 cells were transfected with plasmid DNA or incubated with 1 µg/mL of LPS for 1 h, washed and incubated with fresh growth media until collection at 24 h. Transfected cells were collected at 40 h for measurement of cell viability. Cell viability was measured using the CellTiter96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) kit as per the manufacturers’ protocol [59].
Statistical Analysis

Statistical tests, including paired and unpaired Student’s t tests, were conducted for qRT-PCR and cytokine and chemokine immunoassay using GraphPad InStat version 5.0 (GraphPad software, San Diego, CA). Values were expressed as mean ± SD of three independent observations. P values of <0.05 were considered significant.

RESULTS

_Elevated secretion of immunomediators from DENV-infected THP-1 monocytes corresponds with peak viral titers and copy numbers_

To establish whether DENV-infected monocytes secrete DHF-associated immunomediators, we infected THP-1 cells with DENV-2 New Guinea (NGC) strain and collected cells and culture supernatants each day for five consecutive days. Plaque assay and qRT-PCR data demonstrated that peak viral titers and copy numbers for both MOI-0.1 and -1 occurred on day 3 after infection; however, infection with MOI 1 resulted in approximately log 1.4 higher titer and log 0.5 higher viral RNA transcripts than infection with an MOI 0.1 on day 3 after infection (Fig. 1A). We determined by qRT-PCR that the peak induction of IL-8 and TNFα transcripts occurred at day 3 after infection (Fig. 2); therefore, we assayed the THP-1 supernatants for immunomediators at days 1 and 3 after infection.
**Fig. 1.** Kinetics of chemokines and cytokines secretion in DENV-infected THP-1 cells. THP-1 cells were infected with DENV-2 NGC strain at MOI-0.1 and -1 and cells and supernatants were collected every 24 h after infection for five consecutive days. (A) Viral titers and RNA copy numbers were determined by plaque assay and qRT-PCR, respectively, on samples collected each day after infection (MOI-1, solid line; MOI-0.1, dotted line). (B) Chemokine and cytokine levels in mock- and DENV-infected THP-1 cell culture supernatants collected at days 1 and 3 after infection (mock-infected, white bars; DENV-infected MOI-0.1, light gray bars; DENV-infected MOI-1, dark gray bars; and LPS treatment, striped bars). Data points and bars represent the mean ± SD of three independent experiments conducted in duplicate. (*) indicates statistical significance at $p < 0.05$ as compared to the same day controls; N.D., not detected.
The Luminex® multiplex data corresponded with transcript data in that the THP-1 cells infected with an MOI-1 induced peak secretion of IL-6, IL-8, IP-10 and TNFα at day 3 after infection (Fig. 1B). Levels of IL-6, IL-8, IP-10, and TNFα peaked at 52, 2,000, 40 and 65 pg/mL, respectively. Also slight overall increases were observed in VEGF and IFNγ levels at day 3 after infection. THP-1 cells incubated with LPS significantly produced all of the immunomediators tested both at the transcript and protein levels (Fig. 1B and Fig. 2) whereas THP-1 cells mock-infected with UV-treated virus produced low expression levels similar to those observed in untreated THP-1 cells (Fig. 1B).

Overall, these results demonstrated that DENV-2 NGC-infected THP-1 cells produced DHF-associated immunomediators and that maximum transcripts expression and protein secretion levels corresponded with peak viral titers and copy numbers. These data are consistent with other in-vitro data demonstrating that infected THP-1 cells secrete elevated chemokines and cytokines, which correspond with increased viral titers [47, 48, 51].
Fig. 2. Induction of immunomediator transcripts in DENV-infected THP-1 cells. Cells were infected at MOI-0.1, MOI-1 or incubated with LPS for 1 h and collected each day for four consecutive days. qRT-PCR was conducted to determine A) IL-8 and B) TNFα mRNA fold-changes as compared to same day controls. GAPDH was used as the endogenous control gene. Bars represent the mean ± SD of four samples and (*) indicates statistical significance at $p < 0.05$ compared to same day levels induced by MOI-0.1.
NS4B or NS5 expressed in THP-1 cells induces IL-6, IL-8 and IP-10

To evaluate the role of DENV NS on the observed induction of immunomediators, we constructed plasmids (Fig. 3A) with a commercially available V5 expression vector using primers designed from the wild-type NGC genome (Table 2). Following the manufacturer’s optimization instructions for the Neon™ system, we determined that maximum expression levels of the V5 control plasmid or NS5-V5 plasmid and THP-1 cell viability (approximately 70% for all plasmids) occurred at 40 h after transfection (Fig. 4). Therefore, successful expression of each DENV plasmid was confirmed at 40 h after transfection by immunofluorescence assay (data not shown), qRT-PCR and western blot (Fig. 3B-C).
<table>
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<tr>
<th>Construct and Primer Names(^a)</th>
<th>Nuclide Position(^b)</th>
<th>Primer Sequence (5' - 3')(^c)</th>
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<th>Amplicon Size (bp)</th>
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\(^a\)Forward, sense sequence; reverse, antisense sequence  
\(^b\)Determined from NCBI accession number M29095 (DENV NGC strain)  
\(^c\)Kozac sequence shown as bold lower-case letters, fill-in nucleotides for in-frame translation as lower case letters and DENV sequence as upper case letters
Fig. 3. Expression of nonstructural DENV-V5 fusion protein constructs in THP-1 cells. THP-1 cells were transfected with each DENV nonstructural gene plasmid and cell lysates were collected at 40 h after transfection for expression analysis by qRT-PCR and western blot. (A) Constructs and approximate nucleotide number (kb) of cloned viral genes. Each gene was constructed using a V5 reporter vector having the CMV promoter for expression in mammalian cells. (B) V5 copy numbers were determined for each expressing plasmid using qRT-PCR and reported as log of V5 mRNA transcripts per µg of RNA. A standard curve was prepared by serially diluting known amount of V5 transcripts. (C) Western blot analysis of DENV-V5 proteins expression. Twenty µL (approximately 50 µg) of each cell lysate was loaded and electrophoresed on SDS-PAGE followed by immunoblotting with V5 antibody. Protein loading amounts were confirmed using β-actin and a relative intensity (RI) was calculated by dividing the absolute intensity of the DENV-V5 protein band by the absolute intensity of its corresponding β-actin band. The cell lysate loaded in each well is specified at the top and its corresponding molecular mass (kDa) is depicted at the left or right. Bars represent the mean ± SD of three independent experiments conducted in duplicate. No significant differences (p < 0.05) were found across transcripts.
Fig. 4. Cell viability of THP-1 cells transfected with plasmid DNA or treated with LPS for 1 h. THP-1 cells were grown to confluence and 1 x 10^6 cells were treated as indicated in the graph. Viability of treated cells was determined by cell proliferation assay. Results are presented as percent viable cells. Data are expressed as mean ± SD of two independent experiments performed in duplicate.

We conducted qRT-PCR and demonstrated similar mRNA expression levels, wherein approximately log 4.25 copies of transcripts were expressed by all DENV-V5 fusion plasmids with no significant difference between the minimum (NS4B) and maximum (NS5) expression levels (p=0.32) (Fig. 3B).
Also, we conducted western blot and demonstrated similar relative intensities (RIs), calculated as described in the materials and methods section, for all DENV proteins, wherein NS1 reached a maximum RI of 1.11 and NS2B3(pro) reached a minimum RI of 0.92 (Fig. 3C). Having established similar transcript and protein expression levels for all DENV plasmids at 40 h after electroporation, we screened transfected cells for immunomediator induction using qRT-PCR. We demonstrated that NS4B significantly increased IL-6 transcripts approximately 4-fold and IL-8 almost 9-fold relative to NS4AB (Fig. 5). Moreover, NS5 significantly increased IL-6 transcripts approximately 8-fold and IL-8 over 11-fold relative to NS4AB (Fig. 5). However, relative to NS4AB expression NS4B (p=0.07) or NS5 (p=0.09) did not significantly increase TNFα transcripts levels (Fig. 5).
Fig. 5. DENV NS4B and NS5 induce IL-6, IL-8, and TNFα transcripts in THP-1 cells. THP-1 cells expressing individual DENV nonstructural genes were harvested at 40 h after transfection and total mRNA was isolated for immunomediator transcript analysis by qRT-PCR. The fold-change induction of IL-6, IL-8, and TNFα transcripts was determined for each DENV gene relative to V5 controls. LPS was used as an positive control and GAPDH was used as the endogenous control. Bars represent the mean ± SD of three independent experiments conducted in duplicate. (*) indicates statistical significance at $p < 0.05$ as compared to transcript levels induced by the NS4AB-V5 fusion protein.
Given that NS4B and NS5 induced DHF-associated immunomediator transcripts, we examined the culture supernatant for secreted immunomediators induced by NS4B, NS5 and the viral replication complex-associated proteins NS2B3(pro), NS3 and NS4AB. As an alternative method to confirm plasmid expression efficiencies, we conducted flow cytometry using a V5 antibody to detect each DENV-V5 fusion protein (Fig. 6A). We observed similar protein levels for all plasmids tested wherein a range of 12-18% of THP-1 cells expressed DENV-V5 fusion proteins (Fig. 6A).
Fig. 6. DENV NS4B and NS5 induce DHF-associated immunomediators in THP-1 cells. THP-1 cells were electroporated with DENV NS and after 40 h, cells and supernatants were collected to measure expression efficiencies and immunomediator protein levels, respectively. (A) Percent of THP-1 cells expressing DENV-V5 fusion proteins as determined by flow cytometry. Cells were fixed, permeabilized and V5-expression proteins were detected using an anti-V5 primary antibody and Alexa Fluor® 594 secondary antibody. (B) Chemokine and cytokine levels (pg/mL) in the supernatants of THP-1 cells expressing NS at 40 h after electroporation and as determined by the Luminex® multiplex technology. Bars represent the mean ± SD of three independent experiments conducted in duplicate and (*) indicates statistical significance at $p < 0.05$ as compared to vector controls.
Having established similar NS-V5 transcript and protein expression levels by qRT-PCR, western blot and flow cytometry, we quantitated secreted immunomediator levels in the culture supernatants using the Luminex® technology. As expected, NS4B or NS5 significantly induced IL-6, IL-8, IP-10 and IFNγ when compared to cells expressing only the V5 epitope (Fig. 6B). Cells expressing NS4B secreted 57, 1,100 and 48 pg/mL of IL-6, IL-8 and IP-10, respectively, while cells expressing NS5 secreted slightly higher levels of these immunomediators (Fig. 6B). NS4B and NS5 increased TNFα production but the difference was not significant, p=0.11 and 0.08, respectively. However, DENV-infected or LPS-treated cells significantly induced TNFα (Fig. 1B) indicating an alternative route of induction. When we electroporated the THP-1 cells without plasmid DNA (pDNA) or the V5 vector control, we observed chemokine and cytokine expression levels similar to endogenous THP-1 levels (Fig. 4B and data not shown). Cell viability reached approximately 70% at 40 h after treatment, including the no-plasmid transfected and LPS-treated control cells (Fig. 4), further indicating that the differences observed were due to the expression of NS4B and NS5.
Maturation of NS4B via cleavage of NS4AB enhances IL-6 and IL-8 production

During replication, the DENV protease NS2B3(pro) interacts with the NS4AB polyprotein presumably as part of the viral replication complex. Previous work has demonstrated sequential processing of NS4AB wherein NS2B3(pro) cleaves NS4AB, releasing NS4A from NS4B [33, 60]. We proposed that expressing both NS4AB and NS2B3(pro) in THP-1 cells would best mimic natural processing and maturation of NS4B and possibly affect the immunomediator induction pattern observed during the expression of individual viral proteins. To detect THP-1 cells expressing both NS4AB and NS2B3(pro), we constructed the NS4AB-GFP fusion protein plasmid (Fig. 7A) for co-expression with the NS2B3(pro)-V5 fusion protein. We demonstrated by flow cytometry that approximately 9% of electroporated THP-1 cells co-expressed NS4AB-GFP and NS2B3(pro)-V5, 23% expressed NS4AB-GFP and 12, 14 and 17% expressed NS4B, NS2B3(pro) and NS5, respectively, 40 h after electroporation (Fig. 7B).
Fig. 7. Maturation of NS4B via processing of the polyprotein, NS4AB, enhances the production of IL-6 and IL-8. (A and B) The NS4AB gene was cloned into a GFP reporter vector and co-expressed with NS2B3(pro)-V5 fusion protein in THP-1 cells for detection by flow cytometry. The expression efficiencies were measured at 40 h after transfection and expressed as percent of THP-1 cells expressing DENV-V5-GFP, V5 or GFP fusion proteins. (C) Western blot analysis of THP-1 cells expressing DENV-V5 fusion proteins NS 4AB, 2B3(pro), 4B and both 4AB and 2B3(pro). β-actin was used as the endogenous control and estimated molecular weight markers (kDa) are shown on the left. (D) Induction of immunomediator transcripts in THP-1 cells relative to vector controls as determined by qRT-PCR. Bars represent mean ± SD of six experimental samples and (*) indicates statistical significance at p < 0.05.
To confirm the flow cytometry data and examine functional cleavage events, we conducted western blot using cells co-expressing NS4AB and the viral protease NS2B3(pro). As expected, we observed that the co-expression resulted in the cleavage of NS4AB which produced four distinct bands: NS4AB, NS2B3(pro), 2KNS4B and NS4B (Fig. 7C). Co-expression of NS4AB and NS2B3(pro) significantly enhanced the induction of IL-6 and IL-8 transcripts when compared to NS4B or NS5 alone, approximately doubling the relative fold change of IL-6 transcripts from 4- to 8-fold and IL-8 transcripts from 9- to 18-fold (Fig. 7D). Given that NS4B co-localizes with NS3 and NS5 during IFN antagonistic function and as part of the viral replication complex [60-62], we tested the induction potential of NS4B with NS3 or NS5 and demonstrated that the induction potential was unaltered relative to NS4B or NS5 alone for IL-6 or IL-8 (data not shown), further supporting our results that enhancement occurs primarily during NS4B maturation and polyprotein cleavage events. Interestingly, the cleavage event did not significantly enhance the induction of IP-10, suggesting that multiple pathways may be responsible for inducing immunomediators. Although this result is unexpected, signal transduction crosstalk is regulated in a dynamic manner and may differ under homeostatic and pathologic conditions making it difficult to determine the exact mechanisms responsible for induction outcomes [63]. However, taken together these results suggest that NS4AB expressed with NS2B3(pro) initiates sequential processing of NS4AB and maturation of NS4B [60, 64], thereby enhancing the induction of immunomediators.
Expression of the 2K-signal peptide with NS4B is sufficient to enhance IL-6 and IL-8 induction

The DENV 2K-signal sequence is a 17-amino acid peptide linking NS4A with NS4B and has little known function. Having observed the cleavage product 2KNS4B in the co-transfected cells, we questioned whether the 2K-signal sequence was responsible for the observed enhanced induction of immunomediator transcripts. To address this question, we constructed the plasmids 2KNS4B and NS4A without 2K (-2K) (NS4A-2K) (Fig. 8A) and expressed them alone or in combination with other NS, particularly NS2B3(pro) due to its intrinsic enzymatic properties and involvement in the replication complex. By using qRT-PCR and western blot, we demonstrated the production of approximately log 4 copies of V5 mRNA transcripts by each plasmid or combination of plasmids (Fig. 8B) and cleavage of 2KNS4B, respectively (Fig. 8C). Importantly, 2KNS4B expression and cleavage, presumably by host proteases, significantly enhanced the induction of IL-6 and IL-8 transcripts (Fig. 8D). Expression of NS4A(-2K) with or without NS2B3(pro) did not induce transcripts of any of the immunomediators tested while NS4B expressing with NS2B3(pro) showed slightly reduced induction potential, suggesting that the viral protease may interfere with NS4B during the induction process (Fig. 8D).
Fig. 8. NS4B with the 2K-signal sequence is sufficient to enhance the induction of immunomediators. (A) The DENV genes 2KNS4B and NS4A without the 2K-signal peptide, NS4A(-2K), were cloned in the V5 reporter vector. (B) Log of V5 mRNA transcripts in THP-1 cells expressing nonstructural plasmids at 40 h after transfection as determined by qRT-PCR. (C) Western blot analysis of THP-1 cells expressing DENV-V5 fusion proteins NS 2B3(pro) and 2K4B, 2K4B, 4AB and 2B3(pro), 2B3(pro) and 4B, 4B, 2B3(pro) and 4A(-2K), and 4A(-2K). Estimated molecular weights (kDa) are depicted on the left and β-actin was detected as the endogenous control. (D) Immunomediators mRNA fold-changes as compared to corresponding V5 controls; GAPDH was used as the endogenous control gene. Bars represent mean ± SD of six independent experimental samples and (*) indicates statistical significance at p < 0.05.

Overall, these data suggest that the induction of DHF-associated chemokines and cytokines by NS4B is enhanced when NS4AB is cleaved by NS2B3(pro), specifically during 2KNS4B processing by host signalases and possibly during NS4B localization into the membrane structures [36, 40, 60, 62, 64].
DISCUSSION

It is well established that elevated levels of immunomediators detected in DHF/DSS patients can adversely alter endothelial cell integrity leading to plasma leakage and unresolved vascular permeability [65-68]. However, mechanisms underlying severe DHF immunopathogenesis remain elusive. The lack of an animal model for DHF immunopathogenesis has lead to extensive clinical and in-vitro studies suggesting that both host and viral factors influence elevated circulating immunomediators and severe disease outcomes [69, 70]. DENV is capable of modulating intracellular signaling, such as the IFN response, or host inflammatory responses such as activation of the transcription factor NF-κB [71, 72]. DHF/DSS is presumed to occur when the virus outcompetes host antiviral responses and establishes infection while initiating a robust inflammatory response, which is critical for the progression of severe dengue disease. Although other investigators have examined DENV-initiated IFN inhibition, very little is known regarding the mechanism by which the DENV proteins initiate DHF/DSS-associated immunomediators. In this report, we demonstrate that maximum production of immunomediators from DENV-infected THP-1 cells corresponds with peak levels of secreted infectious virus and cellular viral RNA. Moreover, using a plasmid construct system that consistently expresses similar levels of viral gene transcripts and viral-V5 fusion proteins, we demonstrate that DENV NS4B and NS5 are potent inducers of DHF/DSS-associated immunomediators in monocytes and that maturation of NS4B via host protease cleavage of 2KNS4B is a critical step during the observed induction.
Peak levels of chemokines and cytokines in DENV-infected THP-1 cells correspond with maximum virus yield

We demonstrate that DENV-infected THP-1 monocytes induce transcription and secretion of immunomediators such as IL-6, IL-8, IP-10, TNFα and IFNγ and that peak levels of these chemokine and cytokines correspond with an increased generation of infectious virus and viral RNA copy numbers. These data are consistent with previous reports demonstrating that secreted immunomediator levels directly correspond with the amount of virus production in THP-1 cells [47, 48]. Other investigators have demonstrated no infectious virus production from DENV-infected THP-1 cells using low passage clinical isolates and in the absence of enhancing antibodies [73]. This inconsistency may be due to differences in virus passage, as high-passage virus isolates in-vitro may acquire dominant mutations that exhibit aberrant phenotypic characteristics not found in low-passage isolates [74]. Our data suggest that immunomediator induction occurs during virus replication when viral RNA copies are elevated. Moreover, others have demonstrated that the antiviral drug ribavirin inhibits DENV replication and IL-6 and IL-8 production in a dose dependent manner [18]. Regardless, it remains difficult to pinpoint the precise immunomediator initiation step due to the dynamic characteristics of virus replication. It is possible that immunomediator induction corresponds with the initial translation of input positive-strand viral RNA, negative-strand viral RNA synthesis, viral protein production or packaging, and/or secretion of virions. Functional studies demonstrate that the expression of all NS in THP-1 cells induces the activation of NFκB, the ubiquitous transcription factor involved in the rapid production of many
chemokines and cytokines [50]. Similarly, expression of NS or NS5 alone in human embryonic kidney cells induces IL-8 transcription and protein secretion [42, 75], further suggesting that NS effectively modulate intracellular signaling and initiate immunomediator induction.

NS5 induces DHF-associated immunomediators in THP-1 monocytes

We demonstrate that NS5 induces high levels of IL-8 as well as IL-6, IP-10 and IFNγ transcripts and protein secretion in THP-1 cells. Moreover, it appears that NS5 triggers a rapid and sustained production of immunomediators as demonstrated by increased transcript and protein secretion at 24 and 72 h after DENV infection, and 20 and 40 h after transfection with NS5 constructs, respectively. NS5 has a potent induction potential as demonstrated by consistent and equivalent expression levels of all tested viral gene transcripts and V5 fusion proteins. Although we observed that NS5 localized to the nucleus and the cytoplasm at 20 and 40 h after transfection, most NS5 localized to the nucleus (data not shown) consistent with other reports [75, 76].

The largest and most highly conserved protein among the flaviviruses, NS5 serves as the RdRP and methyltransferase [26, 27, 30, 34, 77, 78]. Other investigators have reported that DENV NS5 induces IL-8 via the activation of the CAAT/enhancer binding protein (c/EBP) and NFκB [42] as it shuttles between nuclear and cytoplasmic compartments [75, 76]. Upon positive-sense RNA translation and polyprotein processing in the cytoplasm, soluble NS5 is thought to undergo phosphorylation allowing the host proteins importin-α/β to bind at two distinct nuclear localization sites (NLS) and import NS5 into the nucleus [75].
While hyperphosphorylated NS5 localizes to the nucleus, its dephosphorylation elicits a conformational change, allowing exportin-1 to bind and translocate NS5 to the cytoplasm where it interacts with NS3 as part of the viral replication complex [35, 76, 79]. Although the function of nuclear NS5 is unclear, greater nuclear accumulation of NS5 results in decreased IL-8 production and vice versa [75, 76], suggesting that the observed induction of immunomediators occurs when NS5 is localized in the cytoplasm. Moreover, because the majority of NS5 localizes to the nucleus and only a relatively small amount of NS5 is required for virus replication in the cytoplasm [80, 81], it is probable that very low levels of cytoplasmic NS5 is required to initiate immunomediator induction. Although the induction mechanisms by NS5 have not been defined, it is possible that NS5 triggers c/EBP and NFκB signaling events during NS5/importin interactions in the cytoplasm. However, this theory remains to be tested. Recent studies demonstrate that NS5 interacts with NS4B in the cytoplasm, and inhibits IFN signaling by binding and preventing STAT2 phosphorylation [43, 44, 61], further suggesting that viral/host protein interactions are important for modulating host signaling.

**NS4B induces DHF-associated immunomediators in THP-1 monocytes**

In screening the induction potential of DENV NS in monocytes, we demonstrate for the first time that NS4B induces the secretion of IL-6, IL-8, IP-10 and IFNγ. The potent NS4B induction pattern is a surprise given its highly hydrophobic nature and intimate integration within the ER membrane. Yet, we demonstrate that NS4B has an early and sustained induction potential as suggestive of its ability to induce IL-8 at 20 and 40 h after transfection. A highly hydrophobic
protein, NS4B consists of three endoplasmic reticulum (ER) membrane-spanning segments and may serve as an anchor for the viral replication complex [60, 64, 82]. NS4B can act as a potent IFN antagonist via interference and degradation of cellular STAT1 [45] which is synchronized with NS4B maturation via NS4AB cleavage by the viral protease, NS2B3(pro) [46, 83]. Interestingly, IFN-β-mediated inhibition of IL-8 expression requires the presence of STAT1 and STAT2 [84]. Given that NS4B maturation inhibits IFN signaling via STAT1 degradation, we hypothesized that NS4B maturation via NS4AB proteolytic modification(s), including host signalase cleavage of 2KNS4B, would lead to increased levels of IL-8 and possibly other immunomediators. As such, we tested the induction potential of the processing events involved in NS4B maturation.

*Maturation of NS4B via processing of 2KNS4B enhances the induction of immunomediators*

During polyprotein processing, NS4AB is cleaved by the viral protease NS2B3(pro), releasing NS4A from NS4B having the 2K-signal peptide (2KNS4B) before a host signalase cleaves the 2KNS4B junction in the lumen of the ER to release mature NS4B and allowing for its integration into the ER membrane [33, 60, 85]. To mimic natural NS4B maturation via polyprotein processing, we co-expressed NS4AB with NS2B3(pro) and observed that polyprotein cleavage events initiated a more potent induction of IL-6, IL-8 and IP-10 transcripts than NS4B or NS5 alone. The enhancement appears to be due to NS4AB processing or maturation and localization of NS4B in the ER, not increased levels of NS expression as demonstrated by similar expression levels of viral protein and gene transcripts. Moreover, our data suggest that the 2K-signal peptide is not
required for NS4B induction, yet post-translational proteolytic cleavage events including host signalase cleavage of the 2K-peptide from NS4B is primarily responsible for the enhancement.

The 2K-signal peptide appears to play an important role during a variety of intracellular alterations, including anchoring of NS4B to the ER membrane [85]. Proteolytic removal of the 2K-peptide from NS4A during DENV replication induces membrane alterations that may harbor the viral replication complex [64] and the West Nile virus (WNV) 2K-peptide is important for cytoplasmic rearrangements, foci development and Golgi trafficking [36]. Moreover, IFN inhibition by NS4B appears to require the presence of the 2K-signal peptide for at least three flaviviruses, DENV, WNV and yellow fever virus (YFV) [46]. Interestingly, the initial 125 amino acid residues of DENV 2KNS4B, predicted to be located in the cytoplasm between the first and second transmembrane domains, are required for IFN antagonism [46], while the YFV 2K-peptide may be important for the translocation of cleaved NS4B to the lumen side of the ER [86].

Given that IFN-β-mediated inhibition of IL-8 production requires the presence of STAT1 and STAT2 [84], and NS5 or processing of 2KNS4B by host proteases is synchronized with IFN inhibition via STAT1 and STAT2 degradation, it will be interesting to determine whether both IFN inhibition and immunomediator induction occur in the presence of mature NS4B as intracellular STAT1 and STAT2 diminish. Overall, we demonstrate that the viral protease has no intrinsic induction potential alone, yet it cleaves NS4AB and generates NS4A(-2K) and 2KNS4B, which is then cleaved by a host protease, perhaps allowing NS4B to reorient across the ER and initiate IFN inhibition and immunomediator induction.
Conclusions

Effective host defenses against DENV infection include a robust IFN response and a rapid production of chemokines and cytokines important for immune signaling. Importantly, DENV and other flaviviruses are capable of inhibiting host IFN signaling to establish productive infections and concurrently promote an overproduction of immunomediators associated with severe disease. Throughout this chapter, I have described DENV-associated immunomediator induction patterns in the monocytic cell line, THP-1. We have demonstrated that THP-1 cells sustain excellent viability after electroporation, are permissive to DENV infection, and thus are an ideal model to examine early events after infection or expression of DENV NS proteins. We also demonstrated that NS4B maturation events, specifically cleavage of 2KNS4B by host proteases, induce significantly higher levels of immunomediators than NS4B or NS5 alone. Therefore, it appears that maturation of NS4B is important for both IFN inhibition and immunomediator induction. Given the shared phenotype of IFN antagonism across the virus family, it is quite possible that IFN inhibition and immunomediator induction patterns induced during NS4B maturation are similar for other flaviviruses. Even though Flavivirus-wide NS4B sequences show divergent and sometimes negligible genotypic resemblance [87, 88], predicted flavivirus NS4B structural topologies, including the multiple transmembrane and cytoplasmic regions, are strikingly similar as well as NS4AB cleavage events. Reduced flavivirus protein expression or polyprotein processing during infection may dampen virus-induced IFN inhibition and the overproduction of potentially deleterious immunomediators, minimizing the risk of severe disease. As such,
NS4B may very well be an attractive target for the development of Flavivirus-wide therapeutic interventions.

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CHAPTER 3

DENGUE HEMORRHAGIC FEVER-ASSOCIATED IMMUNOMEDIATORS INDUCED VIA MATURATION OF DENGUE VIRUS NONSTRUCTURAL PROTEIN 4B IN MONOCYTES MODULATE ENDOTHELIAL CELL ADHESION MOLECULES AND HUMAN MICROVASCULAR ENDOTHELIAL CELL PERMEABILITY
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KEY WORDS:

Dengue virus, DENV; chemokines and cytokines; nonstructural protein 2K-NS4B; THP-1 monocytes; human microvascular endothelial cells, HMVEC; adhesion molecules, ICAM-1, VCAM-1, E-selectin; transwell permeability model; TEER, FITC-dextran
ABSTRACT

Dengue virus (DENV) infection often results in asymptomatic or mild febrile cases, however, some patients progress to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). A classic clinical sign associated with the progression of DHF/DSS is increased plasma leakage, which involves vascular endothelial cell alterations such as, elevated expression of adhesion molecules and increased vascular endothelial cell permeability. Also, increased levels of tumor necrosis factor-alpha (TNFα) and interleukin (IL)-8, observed during DHF/DSS can modulate vascular endothelial cell changes singly or synergistically. We previously demonstrated that maturation of DENV nonstructural protein (NS) 4B, via cleavage of 2KNS4B, significantly increased DHF/DSS-associated immunomediators to levels found after DENV infection in monocytes. In this report using a primary human microvascular endothelial cell (HMVEC) monolayer and a HMVEC transwell permeability model, we demonstrate that the immunomediator milieu present in the supernatants collected from DENV-infected monocytes increases HMVEC permeability and expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (E-sel). We also demonstrate that the supernatants from monocytes expressing 2KNS4B are sufficient to increase HMVEC permeability and expression of adhesion molecules, which appear to be synergistically induced by TNFα and IL-8. Moreover, we demonstrate significant improvement in HMVEC permeability using TNFα and IL-8 neutralizing antibodies. We have identified a threshold level of immunomediators required to increase vascular endothelial cell adhesion molecules and HMVEC permeability
in our model system. Taken together, our data suggest that maturation of NS4B, via processing of 2KNS4B, in monocytes is a critical step in the induction of TNFα and IL-8, which are able to increase endothelial cell permeability. As such, therapies targeting the processing of 2KNS4B may be ideal for reducing overall DHF-associated immunomediator levels. Also, anti-TNFα antibodies may be a valid intervention for DHF progression. Moreover, clinical correlates could be identified to approximate suppression requirements for therapeutic interventions.

INTRODUCTION

Dengue virus (DENV) causes substantial human morbidity and mortality worldwide infecting an estimated 50-100 million people annually and causing over 500,000 hospitalizations [1]. Approximately 10% of patients presenting with classic dengue fever (DF) progress to severe dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), which may lead to death [2, 3]. DHF/DSS mortality rates average 5%, with approximately 25,000 deaths each year [4]. Although mechanisms leading to DHF/DSS are unclear, an indisputable characteristic of severe disease is plasma leakage resulting from vascular endothelial cell activation, as measured by adhesion molecules expression and increased vascular cell permeability [5-9]. Clinical evidence suggests that plasma leakage progresses rapidly after peak viremia and defervescence when levels of chemokines and cytokines in the periphery are elevated [10-15].

There is little evidence that vascular endothelial cells are infected with DENV in patients progressing to DHF/DSS [16]. Fluorescent antibody, immunohistochemistry and in situ hybridization studies were conducted to find
DENV antigens in lung endothelial cells collected post-mortem (Jessie, 2004). However, due to the scarcities of human autopsy studies and the timing of sample collection, whether DENV infects endothelial cells during DHF pathogenesis remains inconclusive. Although DENV replicates efficiently in human endothelial cells in-vitro [17-25], the significance of this observation during in-vivo pathogenesis remains unclear. Endothelial cells treated in-vitro with the supernatants from DENV-infected monocytes or monocyte-derived macrophages increase expression of adhesion molecules and show increased permeability, respectively, while direct infection of endothelial cells does not alter either phenotype [17, 21, 26, 27], suggesting that the elevated immunomediators in DHF/DSS patients are responsible for modulating endothelial cell adhesion molecules and thereby cell permeability.

DENV preferentially infects peripheral blood monocytes which contribute to the overproduction of immunomediators found during severe disease [28]. Moreover, DENV-infected primary human monocytes secrete high levels of DHF/DSS-associated immunomediators [27, 29]. We previously demonstrated that DENV nonstructural protein (NS) 5, NS4B or maturation of NS4B via cleavage of 2KNS4B increased the secretion of IL-6, IL-8, TNFα, and IP-10 in THP-1 monocytic cell line (Kelley et al., submitted, 2011). Other researchers report that IL-6, IL-8 or TNFα are primarily responsible for initiating and sustaining increased vascular endothelial cell permeability [12, 26, 30] whereas TNFα and IL-1β are the primary stimulatory molecules for intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (E-sel) [17, 31-33]. Currently, animal models are insufficient to study DENV immunopathogenesis
and *in-vitro* studies examining adhesion molecule expression and permeability vary widely due to these experiments utilizing macro-and micro-vascular endothelial cells.

In this report we demonstrate that TNFα or IL-8 in the supernatants of DENV-infected THP-1 monocytic cells and cells expressing 2KNS4B are sufficient to increase primary human microvascular endothelial cell (HMVEC) adhesion molecules and cell permeability. Also, the data suggest that TNFα and IL-8 synergistically induce the observed phenotypic changes with TNFα being the stronger of the two inducers. We have identified a threshold level of immunomediators required for the observed phenotypic changes. These findings provide, in part a mechanistic explanation for the source of immunomediator-induced HMVEC alterations and confirm that increased HMVEC adhesion molecule expression and permeability are primarily an indirect route, requiring the participation of virus-infected monocytes, specifically expression of 2KNS4B, rather than virus-infected endothelial cells.

**MATERIALS AND METHODS**

*Virus, cell culture, supernatants from THP-1 cells and other reagents*

We used a low passage DENV-2 NGC strain (gift from Dr. Duane Gubler) propagated in C6/36 cells to infect primary HMVEC and Vero (monkey kidney epithelial) cells (Kelley et al: doi:10.1016/j.virol.2011.07.006, July 2011). For mock-infection control virus was inactivated by UV-light irradiation for 10 minutes as previously described [34]. For the endothelial cell activation and permeability
experiments, we used primary HMVEC purchased from the Lonza Group Ltd. (Basel, Switzerland). As per the manufacturer’s technical sheet, the HMVEC stain positive for acetylated LDL and von Willebrand’s (Factor VIII) antigen and stain negative for smooth muscle α-actin. The HMVEC were received at passage 3 and grown in endothelial cell growth medium EGM-2-MV and SingleQuot® growth supplements including, bovine brain extract with heparin, hEGF, hydrocortisone, gentamicin, amphotericin B, and 5% fetal bovine serum (FBS) (Lonza). All experiments were conducted using HMVEC passage 5. The Vero cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in minimum essential medium Eagle (MEME) supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin and 10 µg/mL gentamicin (Gibco Labs, Grand Island, NY). Both cell types were incubated at 37°C in a 5% CO₂ atmosphere and the growth media was changed every two or three days. The supernatants used to treat the HMVEC were collected from THP-1 cells of monocytic lineage (ATCC) either infected with DENV at MOI-1, treated with 1 µg/mL of LPS for 1 h, washed and incubated with fresh growth media until collection after 24 h, or cells expressing individual DENV nonstructural proteins (NS) as described previously (Chapter 2, Materials and Methods section). For positive controls we used recombinant TNFα, IL-8, or IL-1β proteins and to neutralize immunomediators, we used antibodies against TNFα and IL-8 (R&D Systems, Minneapolis, MN). For experiments involving neutralizing antibodies, cells were washed, and media was changed to 10% heat-inactivated FBS DMEM before antibody treatment.

Infection of HMVEC and Vero cells
The DENV2 NGC strain was used to infect $1 \times 10^6$ HMVEC or Vero cells in 12-well tissue culture plates or $1 \times 10^5$ HMVEC cells on fibronectin-coated polyethylene terephthalate (PET) 24-well insert membranes at MOI-1. After 1.5 h at 37°C and 5% CO$_2$, the cells were washed twice with fresh growth media and further cultured with growth medium. For the mock-infected controls, we inoculated HMVEC with UV-inactivated DENV, as described previously [34]. As a positive control for adhesion molecule experiments, $1 \times 10^6$ HMVEC were incubated with 200 pg of IL-1β for 1 h, washed and incubated with fresh growth media until collection at 8 h after incubation for detection of ICAM-1 and 16 h after incubation for detection of VCAM-1 and E-sel. Every 24 h, cells and supernatants were collected while remaining cells were replenished with fresh growth media.

*Plaque assay*

To determine the amount of infectious virus released from DENV-infected THP-1 cells, plaque assay was conducted using Vero cell monolayers as described in Chapter 2 Material and Methods section.

*Quantitative real-time RT-PCR (qRT-PCR)*

We extracted total cellular RNA, synthesized cDNA and conducted qRT-PCR as described in Chapter 2, Materials and Methods section. Primers used to measure cellular gene changes are listed in Table 1.
Table 1. Primers employed for the analysis of DENV and host genes by qRT-PCR.

<table>
<thead>
<tr>
<th>Gene (GenBank no.)</th>
<th>Primer Sequence (5' - 3')</th>
<th>Tm (°C)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DENV Capsid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAATATGCTGAAACGCGAGAG</td>
<td>60</td>
<td>167</td>
</tr>
<tr>
<td>Reverse</td>
<td>CATCTATTCAGAATCCCTGCT</td>
<td></td>
<td></td>
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<tr>
<td><strong>ICAM-1 (NM_002162)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>AGCGGCAGTTACCATGTTAGG</td>
<td>58</td>
<td>146</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTTTATTGGTGCGGAATCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>VCAM-1 (BC068490)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TGCTGTCAGATGAGAGACTC</td>
<td>55</td>
<td>113</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCCTCACCTTCCGCCTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E-selectin (NM_000450)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TGGACAGAGGGCGAGCTG</td>
<td>57</td>
<td>199</td>
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<tr>
<td>Reverse</td>
<td>CGTGGAGGTTGTTGAAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH (BC025925)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>AGTTAGCGCGCATCTTCTTTTGC</td>
<td>57</td>
<td>96</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAATAGCCCAATCCGTTGACT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Real-time PCR primers to determine DENV2 NGC copy numbers
2. Real-time RT-PCR primers to amplify adhesion molecule genes or GAPDH endogenous control

**In-vitro permeability model**

We constructed the *in-vitro* permeability model using Millipore Biocoat® cell culture inserts with fibronectin-coated PET membranes with 3 µm pores and 0.33 cm² diameter (BD Bioscience, Bedford, MA) as described previously [34]. Briefly, after rehydrating the inserts in warm culture medium, 1 x 10⁵ HMVEC in 250 µl fresh medium were seeded in the upper chamber and 1 mL medium was added to the lower chamber of the 24-well insert plate. Further, the inserts were incubated at 37°C in a 5% CO₂ atmosphere. The integrity of the membranes was measured from days 3 to 15 after seeding HMVEC by using transendothelial electrical resistance (TEER) and Fluorescein isothiocyanate (FITC) dextran (4 KDa mol. wt., Sigma) permeability assays. We choose 4 KDa FITC-dextran
based on previous research using primary human brain microvascular endothelial cells [34]. Similarly, TEER was measured using the Endohm chamber (World Precisions, Saratoga, FL) and permeability was calculated as per manufacturers’ protocol [34]. FITC-dextran crossing the membrane was measured by adding to the upper chamber 250 µl of 100 µg/mL FITC-dextran medium with or without either 0.5 ng/mL of TNFα or 2.5 ng/mL IL-8. Supernatants, 250 µL, from the THP-1 cells were added to the upper chamber and incubated with the HMVEC model at a 1:1 dilution of supernatant in EBM plus 100 µg/mL FITC-dextran for 1.5 h at 37°C in a 5% CO2. Duplicate samples of 150 µL of medium was aliquoted from the lower chamber into a 96-well plate to measure the fluorescence of transmigrated FITC-dextran on a Victor 1420 (PerkinElmer Life Sciences and Analytical Sciences, Boston, MA) with excitation at 485 nm and emission at 535 nm. The FITC-dextran migration across the membrane was calculated as a percentage of the total amount initially added in the upper chamber and data was calculated as a percent change to untreated control membranes. For DENV infection experiments, HMVEC in the upper chamber were infected with either UV-treated DENV or DENV at MOI-1 beginning at day 6 after seeding, and TEER and FITC-dextran permeability were measured as previously described [34].

Cytokine quantitation and conversion into dose treatment values

IL-6, IL-8, TNFα, and IFNγ were measured in the supernatants of DENV-infected HMVEC using a Milliplex human cytokine and chemokine 4-plex immunoassay kit (Millipore Corp., Billerica, MA) together with the Luminex® 100™ System
(Luminex, Austin, TX) to determine mean fluorescent intensities (MFI) as recommended by the manufacturers. Protein concentrations were calculated from MFI data using 10-fold serially-diluted standards and Bead View analysis software version 1.0.4 (Millipore). The minimum detectable concentrations were 0.4 pg/mL for IL-6, 0.3 pg/mL for IL-8, 0.2 pg/mL for TNFα and 0.4 pg/mL for IFNγ. Given that we diluted THP-1 supernatants 1:1 in EBM and we divided the given concentrations by half to obtain treatment concentration levels in pg/mL (Table 5). To test adhesion molecule changes, we seeded 500,000 cells in 12-well tissue culture plates and to measure permeability, we seeded 100,000 HMVEC in 24-well insets.

**Western blot**

Total cellular protein extracts were prepared from HMVEC cells either 8 h after treatment (ICAM-1), 16 h after treatment (VCAM-1 and E-sel) or at otherwise indicated time point. Cells were washed once with cold PBS and extracted with 200 µL of M-PER mammalian protein extraction buffer (Thermo Scientific, Rockford, IL) containing EDTA-free complete protease inhibitor cocktail (Roche, Indianapolis, IN). Fifty µg in 20 µL of total protein was fractionated on a 4-12% gradient SDS polyacrylamide gel using the Mini-Protean II (Bio-Rad, Hercules, CA) and then transferred onto a 0.2 µm nitrocellulose filter (Bio-Rad Laboratories) as previously described [35] and as described in Chapter 2, Materials and Methods section. Membranes were incubated with primary DENV envelope antibody (courtesy of Dr. Wei Kung Wang, band size ~52 KDa) or ICAM-1 mouse monoclonal (band size ~90 KDa), VCAM-1 rabbit polyclonal
(band size ~110 KDa), E-sel mouse monoclonal (band size ~150 KDa) or β-actin IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution and incubated at 4°C overnight followed by incubation with a secondary antibody conjugated to HRP (dilution 1:5000) at room temperature for 1 h. Proteins bands were detected with enhanced chemiluminescence (Amersham ECL, GE Healthcare Limited, Buckinghamshire, UK) on Amersham ECL Hyperfilm (Kodak, Rochester, NY). To determine the relative intensity (RI) of protein bands, the absolute intensity of the adhesion molecule protein band was divided by the absolute intensity of its corresponding β-actin band. Absolute intensities were calculated using Photoshop by multiplying the given pixel value and mean intensity of selected bands as previously described [36].

Cell viability assay

To determine the cell viability, control HMVEC or HMVEC were treated with TNFα (0.5 ng/mL), IL-8 (2.5 ng/mL), IL-1β (200 pg/mL) or THP-1 supernatant (1:1 dilution with EBM) or were infected with DENV MOI-1. The assay was competed as described in Chapter 2, Materials and Methods section. Briefly, 2 x 10^4 HMVEC were seeded on 96-well tissue culture plates and after 3 days, triplicate wells of confluent monolayer were incubated with 200 µl of each of the aforementioned treatments for 8, 16, 24 or 48 h, and cell proliferation was measured using the CellTiter96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) kit as per the manufacturers’ protocol [37].

Statistical analysis

Statistical tests, including paired and unpaired Student’s t-tests, were conducted
for qRT-PCR and cytokine and chemokine immunoassay using GraphPad InStat version 5.0 (GraphPad software, San Diego, CA) as described in Chapter 2, Materials and Methods section.

RESULTS

*Immunomediators secreted in the supernatants of DENV-infected THP-1 monocytes increase HMVEC adhesion molecules and permeability.*

We previously demonstrated that DENV-infected THP-1 monocytes significantly increased the secretion of IP-10, TNFα, IL-6 and IL-8 (Table 2).

**Table 2.** Immunomediators detected in the supernatants of DENV-infected THP-1 cells.

<table>
<thead>
<tr>
<th></th>
<th>IFNγ</th>
<th></th>
<th>TNFα</th>
<th></th>
<th>IL-6</th>
<th></th>
<th>IL-8</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>8 h control supernatant</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>--</td>
<td>61</td>
<td>8.9</td>
<td>571</td>
<td>113</td>
</tr>
<tr>
<td>8 h DENV supernatant</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>--</td>
<td>84</td>
<td>12</td>
<td>691</td>
<td>146</td>
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<td>Day 1 control supernatant</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>--</td>
<td>86</td>
<td>26</td>
<td>621</td>
<td>106</td>
</tr>
<tr>
<td>Day 1 DENV supernatant</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>--</td>
<td>104</td>
<td>35</td>
<td>893</td>
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<td>Day 3 control supernatant</td>
<td>ND</td>
<td>--</td>
<td>ND</td>
<td>--</td>
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<td>10</td>
<td>634</td>
<td>99</td>
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<tr>
<td>Day 3 DENV supernatant</td>
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<td>ND</td>
<td>--</td>
<td>115</td>
<td>19</td>
<td>852</td>
<td>116</td>
</tr>
</tbody>
</table>

*pg/mL; n ≥ 4 samples from at least 2 experiments  
**SD = standard deviation  
ND = not detected  
Supernatant from HMVEC cells infected with UV-treated DENV NGC at MOI 1  
Supernatant from HMVEC cells infected with DENV NGC at MOI 1

Before determining whether the immunomediators secreted in the aforementioned supernatants could increase HMVEC adhesion molecules or permeability, we optimized the *in-vitro* HMVEC models. The HMVEC transwell
permeability model was constructed with Transwell® 24-well inserts consisting of a PET/fibronectin membrane layered with primary HMVEC (Fig. 1A). To measure permeability, we implemented both trans-endothelial electrical resistance (TEER) and FITC-dextran permeability assays as previously described by us and other investigators [21, 26, 30, 34, 38]. We observed that HMVEC seeded on PET membranes pretreated with fibronectin provided more effective membrane integrity than the PET membranes without fibronectin and the ideal treatment window for future experiments was between days 6 and 10 after HMVEC seeding (Fig. 1B). An increased and sustained TEER reading of approximately 200 Ω/cm² corresponded with a decreased FITC-dextran reading of approximately 2 percent crossing for each time point from days 6 to 10 (Fig. 1B). Given that TNFα and IL-8 have been demonstrated to increase permeability [30, 39-41] and to determine optimal control and incubation times, we tested both mediators as positive controls. We observed that TNFα (0.05 ng/mL) and IL-8 (2.5 ng/mL) consistently decreased cell permeability. Maximum decrease in cell permeability occurred at 24 h after treatment as measured by TEER wherein TNFα and IL-8 decreased TEER by approximately 50% and 25% of controls, respectively (Fig. 1C). The same concentrations of TNFα and IL-8 increased FITC-dextran crossing the membrane to almost 400% and 150% of controls, respectively. As expected after washing the membranes with buffer for removal of TNFα and IL-8, a full recovery of membrane integrity was observed after an additional 24 h of incubation with only culture media (Fig. 1C).
Fig. 1. Optimization of the *in-vitro* HMVEC permeability model. (A) Transwell® 24-well inserts with PET membranes either with or without fibronectin coating were used for seeding 100,000 HMVEC cells and incubated with 250 µl and 2 mL of media in the upper and lower chambers, respectively. (B) To determine the optimal treatment window, TEER, presented as Ω/cm², and the percent FITC-dextran crossing the HMVEC/PET membranes were measured for every day for 15 days after seeding cells. The maximum membrane tightness was observed on day 6 after seeding when the TEER was above 200 Ω/cm² and less than 4% of FITC-dextran crossed the membrane. Therefore, (*) represents the time point, day 6 after seeding, chosen to start treatment of the HMVEC permeability model. (C) To determine the optimal incubation period and end point time to measure permeability, either 250 µL of 0.5 ng/mL TNFα (125 pg total) or 2.5 ng/mL of IL-8 (625 pg total) was incubated with the PET-fibronectin/HMVEC membrane beginning on day 6 after seeding and TEER and FITC-dextran assay was conducted every day. Data are presented as percents of non-treated control membranes. The optimal time point to measure permeability was found to be 24 h after treatment. Bars represent the mean ± SD of two independent experiments conducted in triplicate.
To better understand adhesion molecules expression levels and corresponding permeability increases, we tested the supernatants from DENV-infected THP-1 monocytes containing immunomediators for increase in ICAM-1, VCAM-1 and E-sel expression and permeability. The supernatants from DENV-infected THP-1 cells were treated with UV to inactivate infectious virus and incubated with HMVEC monolayers for 16 h as previously described [17]. We demonstrated by western blot that supernatants collected at days 1 and 3 after infection induced VCAM-1 and E-sel at 16 h compared to the supernatants from mock-infected THP-1 control cells. However, we did not observe any changes in ICAM-1 protein expression at 16 h after treatment (Fig 2A). The western blot data was confirmed by qRT-PCR and demonstrated that VCAM-1 and E-sel mRNA transcripts levels increased 10- and 30-fold, respectively, but ICAM-1 mRNA did not significantly increase at 16 h, similar to the protein expression (Fig. 2B). Studies have demonstrated that micro- or macro-vascular endothelial cell adhesion molecule expression kinetics vary by cell type and by the cytokine treatment conditions [42, 43].
Fig. 2. Supernatants from DENV-infected THP-1 cells increase HMVEC adhesion molecules and permeability. (A) Western blot analysis of ICAM-1, VCAM-1 and E-sel expression on HMVEC monolayers seeded on 12-well culture plates and treated for 16 h with supernatants from DENV-infected THP-1 cells. The control supernatants are from mock-infected (UV-treated DENV) THP-1 cells while the infected supernatants are from DENV-infected THP-1 cells both collected at day 1 and day 3 after infection. As a positive control, HMVEC were treated directly with 200 pg of IL-1β and β-actin was used as an endogenous control. (B) qRT-PCR analysis of ICAM-1, VCAM-1 and E-sel mRNA fold-change in HMVEC treated for 16 h with supernatants from control or infected THP-1 cells collected at day 1 (D1 Sup) and day 3 (D3 Sup) after infection or 24 h after LPS treatment. As positive controls, HMVEC were treated for 16 h with the supernatants from LPS treated THP-1 cells or incubated directly with 200 pg of IL-1β. GAPDH was used as the endogenous control. (C) To determine the permeability potential of DENV-infected THP-1 supernatants, TEER and FITC-dextran crossing the PET membrane were determined as described in Fig. 1 and in the materials and methods section. Before treatment, supernatants were UV-treated to inactivate viable virus. As positive controls, 0.5 ng/mL of TNFα or supernatants from LPS treated THP-1 cells were used. Data are presented as percent of non-treated control membranes and bars represent the mean ± SD of two independent experiments in triplicate. (*) indicates statistical significance at p < 0.05 as compared to controls.
Given that other investigators have clearly demonstrated ICAM-1 expression on HMVEC cells [32], we performed a time point experiment incubating HMVEC with 200 pg of IL-1β and demonstrated by western blot that expression of ICAM-1 peaked at 8 h after incubation (Fig. 3A). Therefore, we incubated HMVEC with the supernatants from DENV-infected THP-1 cells for 8 h, and observed increase in the expression of ICAM-1 (Fig. 3B).

**Fig. 3. Western blot time point analysis of HMVEC ICAM-1 expression.** (A) HMVEC were incubated with 200 pg of IL-1β in 12-well tissue culture plates and harvested at pre-determined time points. (B) Western blot analysis of HMVEC ICAM-1 expression after incubation for eight hours with supernatants from DENV-infected THP-1 cells as described in Fig. 1A. β-actin was used as an endogenous control.
We then examined the effects of the immunomediators in the UV-treated supernatants on HMVEC permeability and demonstrated that day 1 supernatants significantly increased HMVEC permeability at 24 h after incubation whereas day 3 supernatants increased permeability at 16 and 24 h after incubation as measured by TEER and FITC-dextran crossing the PET/HMVEC membrane (Fig. 2C). Moreover, we demonstrated no significant differences in HMVEC viability for up to 2 days after treatment with TNFα and IL-8 controls, DENV infection or supernatants from THP-1 cells incubated with LPS (Fig. 4).

**Fig. 4. Cell viability of HMVEC treated with cytokines and cell culture media.** HMVEC cells were grown to confluence in 96-well tissue culture plates and cell viability of treated cells was determined by cell proliferation assay. Results are presented as percent change compared to non-treated controls. Data are expressed as mean ± SD of three independent experiments performed in triplicate.
Taken together, these data suggest that the immunomediators present in supernatants of DENV-infected THP-1 cells increase HMVEC adhesion molecules and this induction corresponds with increased permeability at 16 and 24 h.

*DENV-infected HMVEC do not increase expression of ICAM-1, VCAM-1 or E-sel nor increase permeability levels.*

Human endothelial cells are susceptible to DENV infection [18, 19]. However, other investigators have demonstrated that direct infection of endothelial cells *in-vitro* does not alter cell permeability [21, 39] or expression of adhesion molecules [17]. In figure 2 we demonstrated that UV-treated supernatants from DENV-infected THP-1 cells increase HMVEC permeability, which is induced by the immunomediators. To ensure that DENV infection *per se* does not lead to the observed increase in adhesion molecule expression and HMVEC phenotype change, we first infected HMVEC monolayers with DENV at MOI-1 and measured DENV replication kinetics by plaque assay and qRT-PCR. On day 3 after infection of HMVEC monolayer, maximum viable virus released was log 5 PFU/mL and copy numbers reached approximately log 7 viral RNA transcripts/µg of RNA (Fig. 5A).
Fig 5. DENV-infected HMVEC do not increase expression of adhesion molecules or permeability. HMVEC cells were seeded on 12-well culture plates or 24-well Transwell inserts as described in Fig. 2 and were infected with DENV-2 NGC strain at an MOI-1 to evaluate replication kinetics, adhesion molecule expression and permeability changes. (A) HMVEC or Vero cells were infected with DENV with an MOI-1 and cells and supernatants were collected each day for four consecutive days. Viral titers and RNA copy numbers were determined by plaque assay and qRT-PCR, respectively, on samples collected each day after infection (HMVEC, solid line; Vero cells, dotted line). (B) Western blot analysis of DENV envelope, ICAM-1, VCAM-1 and E-sel expression levels on mock-infected and DENV-infected HMVEC harvested at 8, 16, and 24 h after infection. As a positive control, HMVEC were treated directly with 200 pg of IL-1β for 8 h (ICAM-1) or 16 h (VCAM-1 and E-sel) and β-actin was used as an endogenous control. (C) qRT-PCR analysis of ICAM-1, VCAM-1 and E-sel mRNA fold-change in DENV-infected HMVEC compared to same time point controls harvested at 8, 16, and 24 and 48 h after infection. HMVEC directly incubated with 200 pg of IL-1β for 8 h (ICAM-1) or 16 h (VCAM-1 and E-sel) are positive controls and GAPDH was used as the endogenous control. (D) HMVEC were seeded on PET-fibronectin membranes and infected with DENV. The percent change of TEER (solid line) and FITC-dextran crossing the membrane (dotted line) were measured for up to 72 h after infection. Bars represent the mean ± SD of at least six samples.
Given that other investigators have demonstrated that DENV-infected HUVEC increase IL-6 and IL-8 expression, which may regulate adhesion molecule expression via autocrine induction, we assessed using Luminex® multiplex technology whether DENV-infected HMVEC increased the expression of immunomediators. On or before day 3 after HMVEC infection, DENV did not significantly increase IL-6 (115 pg/mL, p=0.12) and IL-8 (852 pg/mL, p=0.09) levels compared to control cells (85 pg/mL and 634 pg/mL, respectively) (Table 3). Importantly, we did not detect TNFα or IFNγ in the supernatants from either mock-infected control or DENV-infected HMVEC. As demonstrated by western blot, the expression of DENV envelope gene was detected at 16 and 24 h after infection.

Table 3. Immunomediators detected in the supernatants of DENV-infected primary HMVEC cells.

<table>
<thead>
<tr>
<th></th>
<th>IFNγ</th>
<th>TNFα</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>8 h control supernatant&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>--</td>
<td>61</td>
<td>8.9</td>
</tr>
<tr>
<td>8 h DENV supernatant&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ND</td>
<td>--</td>
<td>84</td>
<td>12</td>
</tr>
<tr>
<td>Day 1 control supernatant&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>--</td>
<td>86</td>
<td>26</td>
</tr>
<tr>
<td>Day 1 DENV supernatant&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ND</td>
<td>--</td>
<td>104</td>
<td>35</td>
</tr>
<tr>
<td>Day 3 control supernatant&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>--</td>
<td>85</td>
<td>10</td>
</tr>
<tr>
<td>Day 3 DENV supernatant&lt;sup&gt;g&lt;/sup&gt;</td>
<td>ND</td>
<td>--</td>
<td>115</td>
<td>19</td>
</tr>
</tbody>
</table>

<sup>a</sup>pg/mL, n ≥ 4 samples from at least 2 experiments  
<sup>b</sup>SD = standard deviation  
<sup>c</sup>ND = not detected  
<sup>d</sup>Supernatant from HMVEC cells infected with UV-treated DENV NGC at MOI 1  
<sup>g</sup>Supernatant from HMVEC cells infected with DENV NGC at MOI 1
However, ICAM-1, VCAM-1 and E-sel protein expression did not increase at 8, 16 or 24 h after infection (Fig. 5B). These data were confirmed by qRT-PCR wherein ICAM-1, VCAM-1 and E-sel mRNA transcript changes in DENV-infected HMVEC were below the 2-fold cutoff relative to controls at 8, 16, 24, and 48 h after infection (Fig. 5C). To determine if DENV-infected HMVEC increased permeability, we infected HMVEC with DENV MOI-1 directly on the permeability model and measured TEER and FITC-dextran crossing the membrane. We observed no significant permeability changes for up to 72 h after infection (Fig. 5D) even though plaque assay data demonstrated a steady increase in viable virus release from the infected permeability model, reaching approximately log 4 PFU/mL at 72 h after infection (data not shown). Overall, these data suggest that DENV infection per se does not modulate HMVEC adhesion molecule expression or permeability but instead the immunomediators present in the supernatants from DENV-infected THP-1 monocytes are responsible for the observed changes.

*Maturation of NS4B in THP-1 monocytes induces immunomediators sufficient to alter HMVEC adhesion molecules and permeability.*

We previously demonstrated that maturation of NS4B, via cleavage of 2KNS4B by host proteases, significantly enhanced the production of immunomediators compared to levels induced by NS5, which also significantly induced immunomediators compared to the V5 vector control (Chapter 2, Fig 8D). Using the Luminex® multiplex technology, we quantitated immunomediators secreted in the supernatants of THP-1 cells expressing DENV NS (Table 4) and given that the levels found in the supernatants of THP-1 cells expressing 2KNS4B were
similar to the levels found in the supernatants of DENV-infected THP-1 cells (Table 2), we expected that the supernatants from THP-1 cells expressing 2KNS4B would modulate expression of adhesion molecules in HMVEC and their cell permeability.

Table 4. Immunomediators present in the supernatants of THP-1 cells expressing DENV NS.

<table>
<thead>
<tr>
<th>Immomediator</th>
<th>IFNγ Mean</th>
<th>VEGF Mean</th>
<th>IP-10 Mean</th>
<th>TNFα Mean</th>
<th>IL-6 Mean</th>
<th>IL-8 Mean</th>
</tr>
</thead>
<tbody>
<tr>
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<td>17</td>
<td>13</td>
<td>4.4</td>
<td>1.9</td>
<td>241</td>
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<tr>
<td>V5 supernatant</td>
<td>1.5</td>
<td>18</td>
<td>14</td>
<td>5.3</td>
<td>2.5</td>
<td>202</td>
</tr>
<tr>
<td>NS3B3(pro) supernatant</td>
<td>1.8</td>
<td>22</td>
<td>10</td>
<td>11</td>
<td>2.8</td>
<td>218</td>
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<td>20</td>
<td>12</td>
<td>12</td>
<td>2.3</td>
<td>229</td>
</tr>
<tr>
<td>NS4B supernatant</td>
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<td>19</td>
<td>44</td>
<td>27</td>
<td>57*</td>
<td>1,100*</td>
</tr>
<tr>
<td>2KNS4B supernatant</td>
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<td>46</td>
<td>65</td>
<td>30</td>
<td>185§</td>
<td>2,413§</td>
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<td>NS5 supernatant</td>
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<td>59</td>
<td>29</td>
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<td>LPS supernatant</td>
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<td>63</td>
<td>115</td>
<td>223</td>
<td>3,109</td>
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</table>

*pmil.: n ≥ 6 samples from at least 3 experiments

SD = standard deviation

Supernatant from THP-1 cells electroporated without pDNA

Supernatant from THP-1 cells electroporated with specified pDNA

Statistical significance (p < 0.05) compared to V5 pDNA control

Statistical significance (p < 0.05) compared to NSS pDNA sample
Using HMVEC model we demonstrate that the supernatant from THP-1 cells expressing NS4B, 2KNS4B or NS5 significantly induce ICAM-1 (8 h after treatment), and VCAM-1 and E-sel (16 h after treatment) mRNA transcripts relative to levels induced by the supernatants from THP-1 cells expressing the V5 control vector. Supernatants from monocytes expressing 2KNS4B induced maximum levels of ICAM-1, VCAM-1 and E-sel reaching 8-, 11- and 23-fold respectively relative to the V5 control, which were significantly higher than levels induced by the supernatant from THP-1 cells expressing NS4AB (p<0.05) (Fig. 6A). Western blot data confirmed that the supernatants from THP-1 cells expressing 2KNS4B or NS5 increased ICAM-1, VCAM-1 and E-sel protein expression compared to the supernatant from THP-1 cells expressing V5 or NS4AB proteins (Fig. 6B). When we tested for permeability changes, we observed that the supernatants from THP-1 cells expressing 2KNS4B significantly increased permeability compared to the supernatant from NS4AB at 16 and 24 h after treatment using both TEER and FITC-dextran assays, approximately 25% and 140% of controls, respectively (Fig. 6C). Overall, we demonstrate that maturation of 2KNS4B in THP-1 cells is capable of inducing a significant quantity of immunomediators, which induce the expression of HMVEC adhesion molecules and permeability, similar to the levels induced by DENV-infection.
Fig. 6. Immunomediators secreted by THP-1 cells expressing 2KNS4B or NS5 promote endothelial cell activation and permeability. (A) qRT-PCR analysis of ICAM-1, VCAM-1 and E-sel mRNA fold-change from HMVEC after treatment with supernatants from THP-1 cells expressing DENV NS2B3(pro), NS3, NS4AB, NS4B, 2KNS4B or NS5 or incubated with 200 pg of IL-1β. Values for ICAM-1 (8 h after treatment) and VCAM-1 and E-sel (16 h after treatment) are normalized to expression levels induced by supernatants from THP-1 cells expressing V5 control epitope. GAPDH is the endogenous control and (*) indicates statistical significance at p < 0.05 as compared to values determined for NS4AB. (B) Western blot analysis of ICAM-1, VCAM-1 and E-sel on HMVEC after treatment with supernatants from THP-1 cells expressing DENV NS4AB, 2KNS4B or NS5 or V5 control. HMVEC were harvested at 8 h (ICAM-1) or 16 h (VCAM-1 and E-sel) after treatment. As a positive control, HMVEC were treated directly with 200 pg of IL-1β and β-actin is shown as the endogenous control. (C) HMVEC were seeded on PET-fibronectin membranes and incubated with supernatants described in Fig. 3A. The percent change of TEER and FITC-dextran crossing the membrane were measured for supernatant from THP-1 cells expressing NS4AB, 2KNS4B and NS5. The supernatant from LPS treated THP-1 cells was used as a positive control (dotted grey line). Bars represent the mean ± SD of at least 4 samples. (*) indicates statistical significance at p < 0.05 as compared to controls.
TNFα and IL-8 synergistically modulate HMVEC adhesion molecules and permeability.

TNFα effectively increases the expression of ICAM-1, VCAM-1 and E-sel in HUVEC [17]. Moreover, TNFα at concentrations ranging from 10 pg/mL to 10 µg/mL [26, 39, 44-46] and IL-8 at concentrations ranging from 50 ng/mL to 1 µg/mL, [30, 47] can modulate endothelial cell permeability depending on the permeability model used for the assay. In contrast, Beyon and colleagues have demonstrated that low concentrations of immunomediators in combination synergistically alter adhesion molecules and permeability when compared to addition of individual recombinant chemokines or cytokines [48]. Given that the supernatants from THP-1 cells infected with DENV or expressing individual NS consist of an array of immunomediators, we hypothesized that TNFα and IL-8 synergistically modulate adhesion molecule changes or permeability. Therefore, we used neutralizing antibodies against these two immunomediators. Upon neutralizing IL-8 in the supernatants from THP-1 cells expressing 2KNS4B, we observed using western blot that there was no reduction of adhesion molecule expression levels compared to the effects caused by the supernatants without neutralizing IL-8 antibodies (Fig. 7A-B). However, we did observe a slight reduction in TEER or FITC-dextran crossing the permeability membrane but the changes were not significant (Fig. 7C).
Fig. 7. Neutralization of TNFα and IL-8 secreted by 2KNS4B expressing THP-1 cells synergistically inhibits HMVEC activation and permeability.

Neutralizing antibodies against TNFα or IL-8 were incubated with the supernatant from THP-1 cells expressing 2KNS4B and Western blot, and permeability assays were conducted using the neutralized supernatant. (A) Western blot analysis of ICAM-1, VCAM-1 and E-sel using HMVEC treated with supernatants from THP-1 cells expressing NS4AB, 2KNS4B, or supernatants from THP-1 cells expressing 2KNS4B incubated with neutralizing antibodies against TNFα or IL-8 or TNFα and IL-8. HMVEC were harvested at 8 h (ICAM-1) or 16 h (VCAM-1 and E-sel) after treatment. β-actin was employed as an endogenous marker. (B) Percent relative intensity of test samples compared to relative intensity of positive control IL-1β. Relative intensity was calculated by dividing the absolute intensity of the adhesion molecule protein band by the absolute intensity of its corresponding β-actin band. Relative intensities of the positive control were then divided by each sample. (*) indicates a statistically significant reduction in relative intensities. (C) HMVEC were seeded on PET-fibronectin membranes and incubated with supernatants from Fig. 4A and as described in Fig. 1C except without UV-irradiation. The percent change of TEER and FITC-dextran crossing the membrane were measured. The supernatant from LPS treated THP-1 cells was used as a positive control (dotted grey line). (*) indicates a statistically significant reduction compared to supernatants from THP-1 cells expressing 2KNS4B and (∞) indicates a statistically significant reduction in permeability compared to supernatants from THP-1 cells expressing 2KNS4B and treated with anti-TNFα neutralizing antibody, both at p < 0.05. Bars represent the mean ± SD of at least 4 samples.
When we neutralized TNFα, we observed a significant reduction of adhesion molecule expression levels by western blot (Fig. 7A-B). Moreover, membrane integrity was significantly compromised as demonstrated by a TEER reduction from approximately 12 to 25% of controls and FITC-dextran crossing from 115 to 150% of controls after TNFα neutralization (Fig. 7C). Interestingly, when we neutralized TNFα and IL-8 together and treated HMVEC, we observed an enhanced reduction in adhesion molecule expression by western blot (Fig. 7A-B) and a significantly compromised integrity than the samples treated with TNFα neutralizing antibodies alone as measured by TEER (Fig. 7C). Overall, these data suggest that TNFα and IL-8 synergistically increase ICAM-1, VCAM-1 and E-sel expression and HMVEC permeability.

A minimum dose of TNFα or IL-8 is required to modulate HMVEC adhesion molecules or permeability changes.

We utilized in-vitro models that mimic a natural infection environment in order to effectively control for and identify individual immunomediators associated with HMVEC adhesion molecule expression and permeability. Our previously data provided immunomediator concentrations, which allowed for initiation of DHF-associated phenotypes in our HMVEC models. DENV-infected or LPS-treated monocytes produced immunomediators sufficient to increase adhesion molecules and permeability. NS5 and maturation of NS4B via cleavage of 2KNS4B are primarily responsible for inducing high levels of immunomediators.
Table 5. Threshold dose levels of chemokines and cytokines that induce HMVEC adhesion molecules based on change in permeability.

<table>
<thead>
<tr>
<th></th>
<th>IFN</th>
<th>VEGF</th>
<th>IP-10</th>
<th>TNFα</th>
<th>IL-6</th>
<th>IL-8</th>
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<tr>
<td><strong>No change in permeability</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Day 1 supernatant: - mock</td>
<td>N.D</td>
<td>1.5</td>
<td>4.3</td>
<td>3.7</td>
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<td>21</td>
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<tr>
<td>Day 3 supernatant: - mock</td>
<td>ND</td>
<td>2.7</td>
<td>8.3</td>
<td>4.2</td>
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<td>&lt;1</td>
<td>9.2</td>
<td>6.8</td>
<td>2.6</td>
<td>1.2</td>
<td>101</td>
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<tr>
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<td>5.1</td>
<td>6.8</td>
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<td>4.8</td>
<td>8.1</td>
<td>1.2</td>
<td>114</td>
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<tr>
<td>2KNS4B supernatant + anti-IL-8 + anti-TNFα Abs</td>
<td>&lt;1</td>
<td>23</td>
<td>33</td>
<td>&lt;1</td>
<td>93</td>
<td>&lt;1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1 supernatant: - DENV infected</td>
<td>ND</td>
<td>2.5</td>
<td>19</td>
<td>14</td>
<td>1.6</td>
<td>483</td>
</tr>
<tr>
<td>Day 3 supernatant: - DENV infected</td>
<td>1.2</td>
<td>6.0</td>
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<td>31</td>
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<td>965</td>
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<tr>
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<td>23</td>
<td>33</td>
<td>15</td>
<td>93</td>
<td>1206</td>
</tr>
<tr>
<td>2KNS4B supernatant + anti-IL-8 Ab</td>
<td>&lt;1</td>
<td>23</td>
<td>33</td>
<td>15</td>
<td>93</td>
<td>&lt;1</td>
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<td>2KNS4B supernatant + anti-TNFα Ab</td>
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<td>33</td>
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<td>55</td>
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<td>51</td>
<td>114</td>
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During model optimization experiments, we observed that treatment of HMVEC models with 250 µl of supernatants from LPS-treated THP-1 cells diluted 1:1 with media caused significant induction of adhesion molecules and permeability (Fig. 2B-C). Hence, we were able to quantitate minimum functional mean concentrations of the immunomediators required to increase the expression of adhesion molecules or permeability (Table 5).

The various supernatants from transfected or infected HMVEC are categorized as being able to alter permeability or not alter permeability and are expressed as pg/mL. Minimum immunomediator levels present in the supernatants able to increase permeability are derived from supernatants collected at day 1 after DENV infection; 14 pg of TNFα and 483 pg of IL-8 (Table 5). Supernatants having < 9.5 pg of TNFα in combination with < 160 pg of IL-8 were unable to modulate permeability. Of note, VEGF was not significantly different for the two groups yet IP-10 and IL-6 levels were elevated in the supernatants that increased adhesion molecules or permeability even though they appeared to have no direct effect when TNFα and IL-8 were neutralized (Table 5). Given that clinical samples from DHF/DSS patients demonstrate elevated levels of TNFα and IL-8, these data may provide a foundation of which clinical samples can be tested for immunomediator levels and HMVEC phenotypic changes, which than can be used to better understand a threshold cutoff for severe disease.

DISCUSSION

Although the mechanisms by which DENV causes severe disease are not clearly defined, DHF/DSS is associated with elevated levels of chemokines and
cytokines, particularly TNFα, that over-stimulate vascular endothelial cells leading to expression of adhesion molecules, increased permeability and ultimately can cause irreversible endothelial cell dysfunction [48, 49]. We have previously demonstrated that DENV, primarily via maturation of NS4B and sequential processing of 2KNS4B, induces DHF-associated immunomediators, such as TNFα, in THP-1 cells (doi:10.1016/j.virol.2011.07.006). Herein we demonstrate that immunomediators levels induced by 2KNS4B are sufficient to initiate DHF-associated phenotypic changes, including HMVEC permeability and adhesion molecule expression changes. Also, we demonstrate using neutralization experiments that HMVEC phenotypic changes appear to be caused primarily by TNFα and IL-8 from THP-1 cells rather than direct DENV infection of HMVEC, which is consistent with other data [17, 26]. It appears that a minimum level of TNFα and IL-8 is required during HMVEC activation or permeability. Based on these data, antiviral therapies that block the maturation of NS4B or TNFα inhibitors that reduced elevated levels of TNF may prevent the progression of DHF/DSS.

*Immunomediators in the supernatants of DENV-infected THP-1 monocytes increase HMVEC adhesion molecules and permeability.*

Others demonstrate that DENV, in the presence of sub-neutralizing antibodies, infects monocytes and produces chemokines and cytokines sufficient to alter adhesion molecule expression on large vascular HUVEC; ICAM-1 and VCAM-1 had the greatest expression levels after 16 h of treatment whereas E-sel appeared to be more transient, as indicated by its detection at 3 h after treatment
In contrast, we demonstrate that immunomediators present in the supernatants from DENV-infected THP-1 cells increase VCAM-1 and E-sel at 16 h after treatment of small vascular HMVEC while ICAM-1 peaked at 8 h after treatment. The expression timing differences suggest unequal endothelial cell susceptibility to varying concentrations of immunomediators. Because vascular endothelial cells are highly organized and physiologically specialized, experimental differences observed between HMVEC and HUVEC are most likely due to differences in cell function. In fact, others demonstrate that differences in adhesion molecule expression levels depend on the vascular cell bed of origin [43, 50, 51], emphasizing the complexity of vascular endothelial cell function in-vivo.

Increased vascular permeability and plasma leakage in DHF/DSS cases appear to occur at defervescence when immunomediator levels are elevated. Others demonstrate that supernatants from DENV-infected monocytes or monocyte-derived macrophages increase HUVEC permeability [26, 52]. Moreover, other viruses that cause systemic hemorrhaging such as Marburg virus, can trigger HUVEC permeability after treatment with supernatants from infected monocytes/macrophages [53]. In this report, we demonstrate that DENV-infected THP-1 monocytes secrete immunomediators sufficient to increase HMVEC permeability, peaking at 24 h after treatment. The observed increase in permeability begins 8 h after treatment when ICAM-1 levels are highest. Interestingly, cytokine-induced permeability changes occur after adhesion molecule expression and possibly in part due to ICAM-1 mediated signaling [44, 54]. Moreover, persistent over-expression of endothelial cell adhesion molecules
can weaken tight junction proteins [41, 46, 55] and prolong vascular permeability via cytoskeleton reorganization and endothelial cell shape change [44, 54, 56]. Similarly, others demonstrate that increased HUVEC permeability begins 6 h after treatment with TNFα, and after maximum expression of another adhesion molecule, endothelial leukocyte adhesion molecule-1 (ELAM-1) at 4 h [44]. Also, TNFα alone, albeit 5 times the concentration as in the supernatant from LPS-treated THP-1 cells, appears to increase permeability much more effectively than the supernatants from DENV-infected THP-1 cells, suggesting a dose dependent effect on permeability. Together, these data establish that the mediators present in the supernatants from DENV-infected THP-1 cells increase HMVEC permeability, which corresponds with ICAM-1 expression at 8 h after treatment; and that TNFα, with other mediators, may be a potent inducer of HMVEC phenotypic changes.

DENV-infected HMVEC do not increase expression of ICAM-1, VCAM-1 or E-sel nor increase permeability levels.

DENV and other viruses, such as Ebola virus, Crimean-Congo hemorrhagic fever virus and Hantavirus, are associated with hemorrhagic diseases yet the mechanisms defining their underlying pathology remain unclear. Ebola virus, a filovirus, replicates efficiently in endothelial cells and produces a destructive infection in-vitro which parallels endothelial cell lysis observed in infected humans [57, 58]. In contrast, DENV is able to efficiently replicate in HUVEC and HMVEC in-vitro but histopathological studies indicate low or nonexistent infection of endothelial cells as well as little structural damage to capillaries in tissue from DHF/DSS patients [16, 59-61]. It is possible that DENV does in fact infect
endothelial cells but given that DHF/DSS patients undergo severe symptoms several days after peak viremia and after viral clearance, autopsy studies may not detect DENV particles or antigens due to the timing of sample collection. Regardless, our data support clinical findings and what others have shown *in-vitro* demonstrating that DENV-infected HMVEC do not increased adhesion molecules or permeability [17, 21, 26, 27]. Rather, altered endothelial cell phenotypes appear to occur via indirect methods caused by the soluble immunomediators present in the supernatants. Interestingly, both DENV-infected endothelial cells and mock-infected controls produce fairly high endogenous levels of IL-8, however, TNFα was not secreted, further suggesting that TNFα is a potent inducer of adhesion molecules and permeability during DENV infections.

*Maturation of NS4B in THP-1 cells produces TNFα and IL-8, which synergistically increase HMVEC permeability.*

In our previous study we demonstrated that THP-1 cells expressing 2KNS4B significantly increase immunomediator production compared to levels induced by NS5, and to comparable levels if wt-DENV (doi:10.1016/j.virol.2011.07.006). As such, we examined the ability of supernatants from monocytes expressing NS to increase adhesion molecules and permeability. We demonstrate that expression of either NS5 or 2KNS4B induces sufficient mediators to increase adhesion molecules and permeability, while 2KNS4B has a slightly greater effect. TNFα is a well-known potent endothelial cell activating factor, increasing HUVEC adhesion molecule expression [17] and HMVEC permeability [62]. Others performing TNFα blocking experiments show a significant reduction of HMVEC
ICAM-1 expression as induced by dengue patient sera [31]. Moreover, two independent mouse models of dengue infections confirm the association of TNFα with disease severity, which was attenuated by antibodies to TNFα [63, 64]. Also, IL-8 alone can induce permeability at high concentrations [30, 41, 47] and as determined during our permeability model optimization experiments. Herein, we demonstrate that IL-8 acts synergistically with TNFα to increase permeability and adhesion molecules. The synergistic effect may be due to increased induction of intracellular signaling pathways but the exact mechanism remains unclear. Overall, these data suggest that TNFα is primarily responsible for increasing expression of adhesion molecules and permeability, while IL-8 may play a synergistic role with TNFα, confirming what others have found [26, 39, 65]. These data confirm that maturation of NS4B via cleavage of the 2K signal peptide is sufficient to induce mediators capable of inducing endothelial cell phenotypic changes associated with severe DHF/DSS.

A minimum level of TNFα or IL-8 is required to increase HMVEC adhesion molecules or permeability changes.

It has been postulated that elevated immunomediators are responsible for sustained endothelial cell changes during severe DHF/DSS. It is plausible that a minimum or threshold level of chemokines and cytokines, particularly TNFα and IL-8, is needed to modulate endothelial cell phenotypic changes during disease progression. Indeed, utilizing our models we demonstrate that a threshold level of TNFα and IL-8 is required to alter HMVEC adhesion molecule expression and permeability. Supernatants having at least a two-fold higher level of TNFα and a
four-fold higher level of IL-8, while maintaining similar levels of VEGF, IP-10 and IL-6, increase adhesion molecules expression and permeability. Moreover, we demonstrate that HMVEC treated with individual positive controls, TNFα or IL-8, increase permeability but require over 50 or 8 times higher levels respectively than supernatants having lower levels but combinations of several immunomediators. Others demonstrate that low levels of chemokines and cytokines more effectively increase permeability than individual mediators [48] and TNFα, IFNγ and IL-1 together induce a greater increase in permeability compared to when each cytokine acts alone [66]. Also, it is well known that even low levels of cytokines can induce the production of other cytokines, suggesting that a complex and interactive immunomediator induction network exists which may further facilitate increased mediators involved in DHF/DSS immunopathogenesis [67].

In the absence of an adequate animal model, human clinical studies have been important for understanding the immunopathogenesis of DHF/DSS. The important role of TNFα in increasing endothelial cell permeability during severe dengue disease is supported both in clinical studies and a mouse model [31, 68]. Even though clinical studies have identified physiologic levels of TNFα and IL-8 in dengue patients, chemokines and cytokines are produced from a number cells. IL-8 is produced from macrophages, endothelial cells, monocytes and T-cells and functions to assist in monocyte and neutrophil adherence to the vascular endothelium and extravasation towards sites of infection. A direct correlation of IL-8 levels and severity of dengue disease was found during an outbreak in India wherein IL-8 concentration levels ranged from 200 to 5,568 pg/mL with a mean
level of approximately 700 pg/mL in DHF patients [12]. In another study, patients with DF or other febrile illnesses demonstrate IL-8 levels reached up to 100 pg/mL wherein DHF/DSS patients demonstrated >300 pg/mL [24]. Transactivation of VEGF receptor 2 (VEGFR2) by IL-8 occurs in a dose-dependent manner and is required for IL-8-induced endothelial permeability [30]. As such, it is possible that a minimum level of IL-8 is required to initiate the transactivation of VEGFR2 during dengue infection, but this mechanism remains to be tested.

Other than the direct TNFα-mediated permeability, TNFα can activate the production of lipid mediators including platelet-activating factor (PAF), leukotrienes and prostglandins, all of which can act as potent activators of permeability [69]. While control patients’ sera demonstrate mean TNFα levels of 10 pg/mL, sera from DF and DHF patients reach 220 and 239 pg/mL respectively [70]. Another studied from Brazil demonstrates that sera from DF patients have a mean TNFα level of approximately 28 pg/mL and sera from DHF patients have 128 pg/mL [14]. Moreover, circulating soluble TNF receptors have been associated in children with DHF, correlating with subsequent shock [71]. Given that macrophages, mast cells, natural killer cells, T and B cells and other cells produce TNFα, we are unable to correlate clinical estimates. Moreover, excess levels of IL-6 and VEGF are associated with DHF/DSS [72-75] and with increased permeability in-vitro [30, 41, 47, 76, 77], yet we did not find IL-6 or VEGF to be associated with increased adhesion molecules or permeability at levels expressed in our models, consistent with other data [32, 39]. The inability for IL-6 or VEGF to induce endothelial cell phenotypic changes may be due to
the relatively low levels induced by DENV-infected monocytes or monocytes expressing 2KNS4B.

Summary and conclusions

The clinical presentations of DHF/DSS patients, such as pleural effusion, pericardial effusion and ascitis, indicate that severe dengue disease is due to alterations of endothelial cells and extensive increased permeability over the entire body [78]. Nearly all in-vitro models employed to characterize DENV-initiated adhesion molecule expression changes consist of HMVEC monolayers [32, 42, 79], whereas models examining permeability changes utilize large-vessel HUVEC [39, 40, 46-48, 52] even though permeability changes associated with DHF/DSS appear to be systemic and not limited to large-vessel endothelial cells. Moreover, phenotypic changes induced by immunomediators vary between these two cell types, micro- and macro-vascular endothelial cells [43], potentially leading to inconsistent interpretations of adhesion molecule expression and permeability data. Electron microscopy studies of microvascular capillaries on skin biopsies from DHF patients show slight swelling and distortion, while damage to larger vessels are not observed [59] suggesting that microvascular endothelial cells are more susceptible to damage during severe disease. Others examining endothelial cell damage and activation in DHF patients observed high levels of soluble ICAM-1 and VCAM-1 and circulating endothelial cells, further suggesting that endothelial cells increase expression of adhesion molecules and may undergo damage during severe disease [80]. Also, transendothelial barrier function of cultured large-vessel HUVEC monolayers shows great fluctuations depending on culture conditions, making them an inadequate system to assess
endothelial permeability alterations [21, 40].

Apart from having a direct effect on permeability, high levels of immunomediators increase expression of ICAM-1, VCAM-1 and E-sel on endothelial cells that act as receptors for complementary ligands on neutrophils and other mononuclear cells. The mononuclear cells may compound endothelial cell damage and permeability by secreting histamine, serotonin or bradykinin, which can increase permeability. Moreover, the sequestration of platelets on activated endothelial cells may contribute to thrombocytopenia, damaged endothelium and increased permeability. Overall, the result of this cascade of immune cell activation leads to changes in endothelium integrity, endothelial cell permeability and plasma leakage [81]. Taken together, these data support a current working model for DHF immunopathogenesis in which a high concentration of circulating TNFα and IL-8, in part secreted by DENV-infected monocytes and specifically associated with maturation of NS4B, may over stimulate HMVEC ICAM-1, VCAM-1, and E-sel and sustain vascular permeability. Given our results and clinical data indicating TNFα as a key mediator associated with DHF/DSS, it is quite possible that TNFα inhibitors approved for clinical use could be a useful therapy to mitigate severe dengue disease.

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Author Contributions

Conceived and designed the experiments: JFK, PHK and VRN. Performed the experiments JFK, PHK. Analyzed the data: JFK and VRN. Contributed reagents/materials/analysis tool: JFK, PHK and VRN. Wrote the manuscript: JFK and VRN.
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CHAPTER 4

THERAPEUTIC INTERVENTIONS:

ANTIVIRALS AND INHIBITORS OF CHEMOKINES AND CYTOKINES
INTRODUCTION

DENV vaccines, antiviral drugs and other therapeutic interventions are not yet available for clinical use. The development of tetravalent vaccine candidates, including live-attenuated, recombinant and chimeric, and non-replicating vaccines [1] is a top priority to prevent dengue infection. However, under certain circumstances, therapies to prevent or reduce viremia or control circulating chemokines and cytokines are more appropriate. DHF/DSS and other viral hemorrhagic fevers (VHF) have been an impetus for continued research and development of antiviral therapies, some of these efforts have been more successful than others. As such, it is useful to consider therapeutic strategies undertaken for VHF as clues for anti-DENV therapies. Many technical approaches are utilized to identify potential antiviral compounds, which target DENV entry and replication and host responses. Given that high levels of tumor necrosis factor alpha (TNF$\alpha$) is often associated with DHF and VHF, TNF$\alpha$ inhibitors used to treat chronic infections or possibly mechanical devices used to filter virus or immunomediators may be viable therapeutic options. Antiviral therapies and inhibitors of immunomediators should be closely considered alongside vaccines as options to prevent or mitigate severe dengue disease.

WHY ANTIVIRAL DRUGS AND IMMUNOMEDIATOR INHIBITORS?

Although vaccines are the most sought mechanism to prevent DENV infection and disease, effective anti-DENV therapies could play an important role in health management in certain situations. For example, non-immune infants from DENV endemic areas, visitors and temporary workers from non-endemic areas are
particularly vulnerable to DENV infections [2]. Also, those who are immunocompromised, elderly or malnourished also constitute high-risk groups. Antivirals could offer an effective alternative or adjunct to vaccination, both for prophylactic treatment and to reduce viremia.

DENV immunopathogenesis is associated with elevated viremia [3-5] and high levels of interleukin (IL)-6, IL-8 and TNFα in the bloodstream [6-11]. The presence of elevated immunomediators is thought to modulate vascular endothelial cells and increase vascular permeability, leading to increased plasma leakage and hemorrhaging. Importantly, others have demonstrated reduced DENV replication and reduced production of IL-6 and IL-8 from endothelial cells using the broad-spectrum antiviral nucleoside ribavirin [11]. Therefore, in theory, antiviral therapies, such as ribavirin, may be used to prevent progression to DHF/DSS. Although ribavirin is used in combination therapy in humans to treat chronic HCV infections, the drug has not been tested in humans against DENV infection. Also, DENV-infected mice treated with ribavirin alone did not reduce viremia [12]. Therefore, more research in the development of other anti-DENV therapies is required.

DENV immunopathogenesis also involves elevated chemokines and cytokines such as TNFα and IL-8, which peak as viremia wanes and initial fever subsides, approximately 4-6 days after onset of illness [13]. Elevated TNFα and IL-8 are associated with increased vascular permeability and with DHF/DSS. Neutralizing mAbs targeting these vasoactive molecules may mitigate the progression of DHF/DSS. Several TNFα inhibitors are approved by the United States Food and
Drug Administration (FDA) for use against chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel syndrome, but no documented evidence exists demonstrating their ability to prevent acute viral diseases.

ANTIVIRALS FOR VIRAL HEMORRHAGIC FEVERS (VHF)

Like DENV, other RNA viruses, including yellow fever virus (YFV), hantaviruses and filoviruses, can trigger elevated levels of chemokines and cytokines from infected host cells that can lead to increased vascular permeability, hemorrhage and shock [14-17]. Successful treatment against one of these RNA viruses would ideally have broad-spectrum activity against the others. Mechanisms describing virus-specific pathogenesis are not considered in detail throughout this chapter; however, information from select VHF is useful to consider for therapeutic development options and are briefly introduced below. As therapies to treat any of these diseases are developed and validated, their efficacy against other VHF should be considered and possibly tested against DHF/DSS.

Yellow fever

Even though a highly effective single-dose vaccine has been in use since the 1930s, up to 200,000 cases of yellow fever still occur each year, resulting in thousands of deaths [18]. The causative agent is maintained in wild primates in rain forests of South America and Africa, where mosquito transmission results in sporadic infections among local residents [18]. Epidemic disease is seen only in Africa, when heavy rainfall is followed by an increase in vector density. Ribavirin is protective in rodent models [19], but has not prevented illness in laboratory primates or been tested for efficacy in humans.
Hantavirus cardiopulmonary syndrome (HCPS)

HCPS is acquired by inhaling infectious material, but rather than representing an infection of the epithelial lining of the respiratory tract, HCPS is considered a variant of a VHF with an intense inflammatory response manifested as increased vascular permeability in the lungs [15]. Ribavirin inhibits hantavirus replication *in-vitro* and is beneficial for treating Old World forms of hantavirus disease known as hemorrhagic fever with renal syndrome. However, ribavirin did not show an effect against HCPS in a placebo-controlled trial, quite possibly because patients who received the drug were severely ill by the time their disease was recognized [20].

Marburg and Ebola hemorrhagic fever

Marburg and Ebola hemorrhagic fever present the greatest challenge to the development of new antiviral therapies for highly pathogenic RNA virus infections. Five different vaccine approaches have given solid protection; one is effective when administered after virus challenge [21]. Additionally, several treatment regimens have prevented fatal illness in macaques when administered early in the incubation period, suggesting beneficial effects for persons infected with a filovirus who have not yet become ill [22]. Unfortunately, limited resources at the sites of African outbreaks have precluded providing victims with any type of advanced medical care, often making it impossible even to administer intravenous fluids; case fatality rates continue to be extremely high, often exceeding 80% of infected patients [22].
DENV ANTIVIRALS: DEVELOPMENT AND TARGETS

Drug development strategies include rational design of compounds based on viral protein structures, screening compound libraries, and testing/modifying known inhibitors of other viruses for anti-DENV activity [23]. Each strategy attempts to identify successful anti-DENV therapies that target unique steps during the virus life cycle, such as virus entry, RNA synthesis, virus assembly and other replication stages [23]. Additionally, intravenous antibody- and nucleic acid-based therapies are potentially effective strategies. Antiviral therapies target either viral or host factors. Antiviral development strategies targeting flaviviruses and host processes, focusing primarily on DENV, are discussed below.

VIRUS TARGETS FOR ANTIVIRAL FUNCTION

Helicase and protease inhibitors

The flavivirus NS3 helicase and NS2B3 protease have long been attractive targets for antiviral therapies, since their enzymatic activities are essential for productive infection. Previous studies indicate that a nucleoside analogue (HMC-HO4) inhibits WNV helicase activity in-vitro and WNV replication in Vero cells [24]. More recently, other small flavivirus-specific helicase inhibitors have also been shown to inhibit WNV NS3-mediated unwinding of RNA substrates; importantly this effect is specific for viral helicase and does not affect host molecule interactions [25].

The flavivirus NS2B3 proteases, unlike host cellular proteases, are thought to recognize cleavage sites containing dibasic amino acid residues and a small
amino acid side chain [26]. Thus, cellular protease inhibitors such as benzamidine and PMSF are inactive against DENV and WNV proteases [27]. Recently, compounds capable of inhibiting viral protease activity have been identified. Peptides that mimic the protease cleavage site competitively inhibit NS2B3 by binding to its catalytic site [27, 28]. Although several compounds can effectively inhibit in-vitro helicase or protease activity of NS3, future research should aim to improve their specificity and potency in animal models.

**Nucleoside analogs to inhibit viral RNA synthesis**

Due to their essential role during the replication of RNA viral genomes, virally encoded RdRp is an attractive target for antiviral agents. One strategy, the use of nucleoside analogies, indirectly targets the productivity of the RdRp by reducing viral replication through RNA chain termination. Nucleoside analogues have been approved for treatment against HIV and hepatitis B virus infection [23]. Recent reports have indicated that treatment of Vero cells with adenosine substituted with methyl groups at the 2-carbon position can effectively inhibit DENV, WNV and YFV by chain termination of RNA synthesis [29, 30] (Table 1). When used as antiviral drugs, these nucleosides can be incorporated safely into cellular RNA [29], yet further optimizations may be required to increase their efficacy against flaviviruses and to limit the possibility of generating virus escape mutants.

**Virus entry inhibitors**

Several inhibitors of DENV entry into host cells have recently been reported. These antiviral compounds can be categorized as sulfated polysaccharides and polyoxotungstates [23]. Several polyoxotungstates were able to inhibit DENV-2 in
Vero cells without any apparent cytotoxic affects [31] but their specific mode of action has not been elucidated. In contrast, sulfated polysaccharides have been suggested to inhibit DENV-2 adsorption and internalization in human and monkey cells, but not in mosquito cells [32, 33]. Several DL-galactan hybrid compounds extracted from the red seaweed Gymnogongrus torulosus greatly inhibited DENV-2 and herpes simplex virus [33]. These compounds may have virucidal properties as well but full inhibitory activity was achieved when the galactans were present during virus adsorption, suggesting that their mode of action involves interference in the binding of the surface envelope glycoprotein with the cell receptor [33]. Further studies are required to clarify their modes of action.

**Intravenous immunoglobulin-based therapies**

Passive transfer of intravenous immunoglobulin (Ig) is being investigated for the treatment of flavivirus infection. Pre-administration of serum or pooled plasma from WNV-infected mice or polyclonal human IgG from WNV-infected humans protected mice from WNV infection, while post-administration of these treatments offered only partial protection [34, 35]. Interestingly, a humanized mAb (E16) against domain III of the E protein administered to mice 5 days after exposure to WNV improved survival to 90%; WNV was completely cleared from the brains of 68% of the surviving mice [36]. These experiments emphasize the potential of Ig therapy for blocking viral replication post-exposure, during the viremic stage. Importantly, antibody-dependent enhancement (ADE) during DENV infection may increase the chance of DHF progression; therefore, any Ig therapy would have to include antibodies sufficient to neutralize all 4 DENV serotypes. Determining
sufficient Ab titers to effectively neutralize DENV without enhancing infection in all cases may prove too difficult. Evidence is very limited for trials involving passive Ig therapy against DENV and has not shown significant benefit in terms of survival or improvement in clinical parameters [37]. It is known that the Fcγ receptor IIA cytoplasmic domain is essential for the ability of Fc receptor bearing cells to mediate ADE in vitro [38]. Therefore, one consideration, to prevent ADE upon Ig therapy, is to provide recombinant anti-DENV IgG Ab without Fcγ portion thereby eliminating Fcγ receptor uptake and ADE. As such, passive Ig transfer could be considered an option to control DHF progression. However, this therapeutic option requires future testing clinical evidence.

**Nucleic acid-based therapies**

Nucleic acid-based approaches have been explored for DENV and flavivirus therapy. RNA silencing (siRNA) was shown to suppress DENV-2 [39] and WNV [40] in tissue culture. Also, phosphorodiamidate morpholino oligomers (PMOs) are a novel approach to inhibit flavivirus replication [41]. PMOs are efficiently taken up by cells and are nuclease resistant; they function by binding to RNA and inhibiting translation. Recent WNV and DENV studies demonstrated that PMOs target RNA elements critical for flavivirus reproduction [42]. A recent study showed that treatment of AG129 mice with PMOs targeting 5′ terminal region (5'SL) or the 3′-cyclization sequence (3′CS) increase survival following DENV-2 challenge [43] (Table 1).
HOST TARGETS FOR ANTIVIRAL FUNCTION

Viral assembly inhibitors

Several compounds have been shown to inhibit DENV-2 and JEV through the inhibition of the cellular glycoprotein-processing enzyme, α-glucosidase [44]. These inhibitors strongly affect N-linked oligosaccharide trimming of prM, E, and NS1, leading to protein misfolding, reduction in glycoprotein secretion, and decreased intracellular levels of NS1 [44]. The former two defects contribute to an unproductive virion assembly pathway whereas the latter is thought to reduce virus replication. Others showed that the indolizine alkaloid, castanospermine, is a potent inhibitor of DENV infection in-vitro and in-vivo via inhibition of glucosidase enzymes and protein misfolding [45] (Table 1).

Nucleoside triphosphate synthesis inhibitors

Besides direct incorporation of nucleosides into RNA molecules during viral synthesis, some nucleoside analogs work by inhibiting host nucleoside triphosphate synthesis. Several inhibitors of nucleotide triphosphate synthesis have been reported to have antiviral properties against WNV production [49]. All of the compounds inhibit orotidine monophosphate decarboxylase (OMPDC) except for mycophenolic acid and ribavirin which inhibit inosine monophosphate dehydrogenase (IMPDH) [49]. In addition to the inhibition of IMPDH, ribavirin was shown to have other antiviral mechanisms such as causing error catastrophe and serving as a RNA cap analog [50]. Mycophenolic acid appears to be the most promising of these inhibitors [51]; however, successful in-vivo efficacy results have not yet been reported.
<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug target</th>
<th>Serotype tested</th>
<th>Model tested</th>
<th>Study size</th>
<th>Response</th>
<th>Refs</th>
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<tr>
<td>Castanospermine</td>
<td>Host α-glucosidase</td>
<td>1 x 10^5 pfu DEN2</td>
<td>A/J mice</td>
<td>30-45</td>
<td>90% survival</td>
<td>[45]</td>
</tr>
<tr>
<td>6-O-butanyol castanospermine</td>
<td>Host α-glucosidase</td>
<td>2 x 10^6 pfu TSV01</td>
<td>AG129 mice</td>
<td>8</td>
<td>88% survival</td>
<td>[30]</td>
</tr>
<tr>
<td>N-nonyl-deoxynojirimycin</td>
<td>Host α-glucosidase</td>
<td>2 x 10^6 pfu TSV01</td>
<td>AG129 mice</td>
<td>8</td>
<td>93% reduction in viremia; significant reduction in cytokines</td>
<td>[30]</td>
</tr>
<tr>
<td>7-deaza-2',C'-methyl-adenosine</td>
<td>RdRp</td>
<td>2 x 10^6 pfu TSV01</td>
<td>AG129 mice</td>
<td>8</td>
<td>70% reduction in viremia; significant reduction in cytokines</td>
<td>[30]</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>RNA metabolism</td>
<td>2 x 10^6 pfu TSV01</td>
<td>AG129 mice and Rhesus Macaque</td>
<td>8/10</td>
<td>None detectable</td>
<td>[30] [46]</td>
</tr>
<tr>
<td>Phosphorodiamidate morpholigo (PMO)</td>
<td>5’ terminal nucleotides and 3’ cyclization sequence</td>
<td>1 x 10^6 pfu DEN2 NGC (IP)</td>
<td>AG129 mice</td>
<td>4-6</td>
<td>None detectable</td>
<td>[47]</td>
</tr>
<tr>
<td>Peptide-conjugated PMO (PPMO)</td>
<td>5’ terminal nucleotides and 3’ cyclization sequence</td>
<td>1 x 10^6 pfu DEN2 NGC (IP)</td>
<td>AG129 mice</td>
<td>4-6</td>
<td>8 days longer than controls</td>
<td>[47]</td>
</tr>
<tr>
<td>PI-88</td>
<td>E protein/GAG interaction</td>
<td>1 x 10^6 pfu DEN2 NGC (IP)</td>
<td>AG129 mice</td>
<td>9, 24</td>
<td>Increased average survival from 15 to 22 days</td>
<td>[48]</td>
</tr>
</tbody>
</table>

Abbreviations: pfu, plaque forming units; IC, intracranial; IP, intraperitoneal; IM, intramuscular; RdRp, RNA-dependent RNA polymerase; GAG, glycosaminoglycan.

**Host protease inhibitors**

Cellular proteases may be a viable option for antiviral targets. The host protease furin is involved in the maturation of viral proteins via cleavage of the M protein from its precursor prM [52, 53]. Also, host signal peptidases, located in the lumen of the ER, are required for proper processing and maturation of nonstructural proteins, such as NS1 and NS4B from NS4AB [54-56]. It is worth noting that
compounds similar to those involved in inhibiting host glycoprotein processing $\alpha$-glucosidase enzymes and preventing flavivirus assembly, could be utilized to target host proteases involved specifically in viral polyprotein processing. To date, specific inhibitors serving this function have not been discovered.

Other host process targets

Genome-wide studies have allowed for comprehensive assessments of host factors involved in DENV, WNV, HCV and other RNA virus infections [57-59]. Sessions et al. employed a 22,632 strand RNA library and discovered 116 candidate host factors involved in viral replication; 42 were confirmed as functionally involved in DENV replication in human cells [59]. This work sets a precedent for discovering potential host targets as antiviral therapy and suggests new approaches to control infection via the host processes.

INHIBITION OF CHEMOKINES AND CYTOKINES

As discussed, DENV immunopathogenesis is associated with elevated viremia and high levels of chemokines and cytokines, such as TNF$\alpha$, that can initiate increased vascular permeability and plasma leakage. DENV infection is rarely diagnosed before the patient is severely ill due to the fact that the DENV transmission event via a mosquito bite usually passes unnoticed, the incubation period is measured in days to weeks, and the initial signs and symptoms are nonspecific. By illness onset, elevated chemokines and cytokines and increased plasma leakage may dominate the clinical picture. Attempts to block viral replication may have little impact on the course of illness. Therefore, it is important to consider alternative treatments to antivirals, such as TNF$\alpha$ inhibitors
or even mechanical devices that may dampen or control high levels of TNFα and potentially mitigate the progression of DHF/DSS.

**TNFα INHIBITORS**

Others have demonstrated that anti-TNF serum improved survival of mice without affecting serum virus titers, which points to TNFα being at least partially responsible for the lethal outcome in DENV-2 infection in mice [60]. In another study, 129/Sv mice doubly deficient in IFN-αβ and IFN-γ receptors (AG129) were infected with D2S10, an adapted mouse strain. D2S10 but not the parental DEN strain, induced significant levels of serum TNFα, and the neutralization of TNFα activity prevented early death of D2S10-infected mice [61]. In-vitro experiments demonstrated that when incubated in supernatants from DENV infected cells, TNFα specific antibodies prevent endothelial cell activation when incubated [62]. Moreover, sera from DENV infected patients when incubated on endothelial cells induced increased activation and apoptosis and showed elevated levels of TNFα; endothelial activating effect of the sera was inhibited up to 80% by pre-treatment with monoclonal antibodies against TNFα [63]. Also, evidence has shown that inhibiting TNFα in the supernatants from DENV-infected monocytes dampens the expression of endothelial cell adhesion molecules, ICAM-1, VCAM-1 and E-selectin [64]. The striking effect of anti-TNFα serum on mice survival without reducing serum virus titers supports the idea that TNFα inhibitors, already FDA approved for use in humans, such as Etanersept (TNFα receptor-Fc fusion protein) or Infliximab (anti-TNFα Ab), could be beneficial in mitigating TNFα-initiated DHF/DSS. Others have also suggested anti-TNFα as a therapeutic
approach [65], yet further studies need to be conducted to determine their efficacy in patients progressing to severe disease.

Pharmacological inhibitors of immunomediator production

Substantial efforts have been made to identify pharmacologic molecules that inhibit chemokine and cytokine production. Pharmacological agents are commonly used to help define signaling pathways induced by various stimuli; therefore, mechanisms of action for several compounds have been well characterized. As such, inhibition of inflammatory gene expression is accomplished through various mechanisms, including transcriptional control, particularly of NFKB and AP-1, as well as post-transcriptional and translational control [66]. Small molecule inhibitors can access signaling pathways within a cell and can have wide ranging anti-inflammatory therapeutic potential [67].

One such small molecule inhibitor is rolipram. Rolipram is a selective inhibitor of phosphodiesterase (PDE) IV, the predominant isoenzyme expressed in myeloid and lymphoid cells [68]. Rolipram has been used in clinical trials safely as an antidepressant [69] and has also been shown to suppress activation of inflammatory cells [70]. Production of TNFα was suppressed in LPS-stimulated monocytes after treatment of rolipram [71]. Also, it has also been shown that rolipram can significantly decrease IL-8 and TNFα production and migration of eosinophils and neutrophils into bronchoalveolar cavities of monkeys undergoing experimental antigen-induced asthma [72].

Another small molecule inhibitor of cytokine production is PDTC, a derivative of DDTC (diethyldithiocarbamate). PDTC is an antioxidant that inhibits reactive
oxygen species (ROS) [73], inducible nitric oxide synthase (iNOS) activity and nitric oxide production [74]. Studies have shown that PDTC can inhibit IL-8 production from human dermal microvascular endothelial cells [75], epithelial cells [76] and human PBMC [77] in parallel to its inhibition of NFκB. Given these data, pharmacological inhibitors may prove useful for mitigating production of immunomediators but more in-vivo work is required before any FDA approval process can proceed.

Mechanical devices

Blood filtering machines have been proposed as a means to prevent DHF/DSS. In 2006, Aethlon Medical, Inc. launched the Hemopurifier™, which was designed to isolate and capture DENV. The company claims that the Hemopurifier™ is the only proposed treatment for DHF/DSS, simultaneously targeting broad-strain clearance of DENV and assisting in the reduction of excessive cytokine activity [78]. It is not yet known if the device can effectively achieve a sufficient drop of virus titer and cytokines to prevent DHF/DSS; clinical testing is ongoing.

LIMITATIONS OF ANTIVIRALS THERAPIES

The scientific and drug-design communities have developed tools to synthesize, select, improve and evaluate drugs in the laboratory. However, the most significant bottleneck in the development of DENV vaccines and antiviral therapies is the lack of an appropriate animal model. There are two kinds of animal models to consider: a model for DENV infection and protective action of a given drug and a disease model that may yield interesting therapeutic avenues for tailored response-modifier drugs [79]. There have been several attempts to
 develop DENV infection and disease mouse models to test vaccines, antiviral drugs and investigate immunopathogenesis [30, 43, 80-86]; many of these models are summarized in the 2010 NIAID report on DENV animal models [79]. Although DENV mouse models are under development, they too are limited in their ability to sustain high and long-lived viremia. Monkey models exist but are expensive and still need to be optimized for proposed uses.

Another limitation to DENV drug design is that the incidence of severe DHF/DSS occurs primarily in children and infants while most drug design programs have involved adult patients [79]. This is not only important regarding drug-dosage but since genome-wide comparisons between infected children and adults have not been conducted, it is difficult to be certain of consistent results in the non-tested study group.

Early diagnosis of DENV infection before treating patients is critical. Recent efforts to assess the therapeutic efficacy of ribavirin in HCPS show the importance of early diagnosis. Ribavirin was found beneficial for Old World hantavirus infections only if treatment began early; however, because patients with New World hantavirus infections had respiratory failure by the time HCPS was diagnosed and ribavirin therapy began, the therapy regime had no effect [87]. Therefore, the success of antiviral therapy for DENV and other RNA virus infections appears to be linked to the development of rapid diagnostic techniques that can identify patients early enough for effective treatment.
SUMMARY

DENV infection leads to elevated viremia and levels of immunomediators associated with DHF/DSS. Inhibiting virus replication could reduce the overall levels of immunomediators, thereby mitigating increased vascular permeability and plasma leakage. Antiviral therapies may target a number of virus or host processes. Viral targets currently include NS3 helicase, NS3B3 proteases and RNA chain assembly with nucleoside analogues. Antivirals may also prevent virus entry into cells; to reduce viremia, intravenous Ig- or nucleic acid-based therapies may be ideal. Host processes to limit virus replication are also under consideration, which may target intracellular virus assembly, nucleoside triphosphate synthesis, or host proteases. Moreover, host-virus interactions are under investigation via genome-wide studies. These analyses are uncovering new potential host processes involved in DENV replication associated with increased (or decreased) virus production. Based on our reports, we propose that NS4B and maturation of NS4B via NS4AB cleavage by NS3B3(pro) and 2KNS4B cleavage by host proteases may prove attractive targets for antiviral drug development.

Apart from inhibiting viremia, certain circumstances such as late diagnosis or lack of antiviral administration may call for the use of other therapies to mitigate high levels of immunomediators. Some of these potential therapies include TNFα inhibitors, other pharmacological inhibitors and possibly mechanical devices to filter patients’ blood. No therapies have been approved for use in the United States and without further evidence as to efficacy and effectiveness at preventing severe DHF/DSS, antiviral drugs cannot be recommended for use until further
testing. Moreover, animal models are needed to support their testing. Therefore, much work for developing appropriate animal models and testing antiviral efficacy in vivo is required in order to manufacture an ideal therapy for DHF/DSS.
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CHAPTER 5

OVERVIEW, LIMITATIONS AND FUTURE PLANS
OVERVIEW

Dengue virus infection can cause DHF/DSS, which is associated with high viremia and elevated levels of chemokines and cytokines that are able to modulate vascular endothelial cells adhesion molecules, increased vascular permeability and possibly irreversible endothelial cell dysfunction [1, 2]. Due to a lack of animal models it is challenging to study DHF immunopathogenesis in vivo thereby creating a gap in our ability to develop effective antivirals and vaccines. Specifically, mechanisms underlying DENV NS induced immunomediators that alter endothelial cell adhesion molecules and vascular permeability remain unclear. The long-term goal of this dissertation was to understand the basic molecular events involved in DHF immunopathogenesis as a prerequisite to understand and develop optimal therapeutic interventions. We hypothesized that DENV NS, and sequential processing and maturation of NS4B in monocytes, would induce immunomediators capable of increasing HMVEC adhesion molecules and permeability similar to the effects caused by a wild-type DENV infection. The rationale for this hypothesis was based on data demonstrating that expression of DENV NS induces IL-8 in HEK293 cells, sequential processing and maturation of NS4B inhibits host IFN responses and DENV-infected monocytes induce immunomediators that activate endothelial cells [3-7]. We chose to employ an in-vitro model system consisting of THP-1 monocytes and primary HMVEC to best mimic a natural DENV infection. Data obtained from this study may have significant impact on understanding important viral factors involved in DHF immunopathogenesis, a prerequisite for developing potential therapeutic interventions. This dissertation consists of five chapters.
Chapter 1 includes a summary of the relevant literature associated with clinical dengue disease, immunopathogenesis, sequential processing of DENV proteins, and DENV-induced vascular permeability. The incidence of DHF/DSS has been progressively increasing with the spread of the Ae. aegypti mosquito and increased human travel [2, 8]. Circulating high levels of secreted immunomediators from DENV-infected monocytes, dendritic cells and other cell types are hypothesized to increase vascular permeability by altering endothelial cell adhesion molecules [9]. However, a neglected area of DENV research involves understanding the ability of DENV proteins to induce host immunomediators and the subsequent effect of immunomediators on vascular permeability changes. These data will be significant in order to better understand critical host-virus interactions potentially useful for the development of therapeutic interventions to mitigate the severity of DHF/DSS.

In Chapter 2, I discuss in detail our findings on the immunomediator induction potential of DENV NS in THP-1 monocytes. We developed our system using the DENV-2 NGC strain and cloned individual proteins for expression in the THP-1 monocytic cell line (see limitations section below for more discussion on NGC and THP-1 cells). It is well known that DENV and other flaviviruses are capable of inhibiting host IFN signaling to establish productive infection and at the same time promote an overproduction of immunomediators associated with DHF/DSS. We demonstrated that sequential processing and maturation of NS4B, specifically cleavage of 2KNS4B by host proteases, induce significantly higher levels of immunomediators than NS4B alone. Given the shared phenotype of IFN antagonism across Flaviviruses, it is quite possible that IFN inhibition and
immunomediator induction patterns induced during the sequential cleavage of NS4B are similar across the family. These data, which are published in the journal *Virology* (2011, doi:10.1016/j.virol.2011.07.006) provide a foundation for our subsequent experiments and chapters.

Throughout Chapter 3, I highlight our efforts to understand the role of DENV on vascular permeability. For this, we adopted a HMVEC monolayer and transwell permeability model system to investigate the effects of THP-1-derived immunomediators on adhesion molecule expression and permeability changes. Our data suggest that DENV-infected monocytes are sufficient to modulate HMVEC phenotypic changes associated with severe DHF/DSS and that sequential processing and maturation of NS4B are primarily responsible for the presence of high levels of immunomediators. Moreover, TNFα is primarily responsible for the observed phenotypic alterations while IL-8 plays a synergistic role in increasing permeability. It is well known that increased plasma leakage is the most identifiable clinical feature of DHF/DSS [10-13]. Our data support clinical and *in-vitro* data demonstrating that increased vascular permeability found during DHF/DSS is most likely caused indirectly by DENV-infected monocytes, or other cells, and their subsequent release of TNFα and IL-8 [14, 15]. Data derived from these studies is drafted into a manuscript for publication.

In Chapter 4, I discuss the current status of therapies under development to mitigate DHF/DSS. Currently, fluid replacement and management are the gold standard therapies for patients progressing to severe disease. No vaccines or antivirals exist. However, potential antiviral targets and inhibitors of various stages of DENV replication and host processes have been tested *in-vivo* and *in-
vitro and are discussed throughout the chapter. Limited mouse and primate DENV-infection or disease models are highlighted; however, the most significant bottleneck in the development of both vaccines and antiviral therapies is the lack of an appropriate animal model. Throughout the chapter, I highlight antiviral options currently under development and also propose that TNFα inhibitors, approved by the FDA for use against chronic inflammatory diseases, may be an appropriate option for patients progressing to severe DHF/DSS.

LIMITATIONS AND FUTURE PLANS

The scope of this dissertation is to determine phenotypic alterations caused by DENV NS. While we conclusively demonstrate the importance of sequential processing and maturation of NS4B for the induction of DHF-associated immunomediators, these data are derived solely from DENV-2 NGC strain. Given that numerous studies have documented altered intracellular states using the DENV-2 NGC strain [3, 16-23], NGC infectious clones [23, 24] and NGC replicons expressing only nonstructural proteins [3, 4, 25] and to remain consistent with the published data, we employed the DENV-2 NGC strain as an ideal model. We have yet to address endothelial cells permeability caused by divergent DENV strains within or across serotypes. Now that we have data for the DENV-2 NGC strain, we propose to clone DENV genes from other DENV serotypes to compare phenotypic alterations of the endothelial cells. Potentially, these data will not only validate our in vitro model system but will also provide clues, if differences are observed, as to genotypic differences that drive phenotypic changes.
We chose to employ THP-1 cells for several reasons. Other investigators have demonstrated that THP-1 cells are an ideal model to mimic both *in-vitro* DENV ADE infections [26-29] and direct infections [30-33]. Both types of infection experiments resulted in productive infection and immunomediator induction. Even though our THP-1 infection experiments demonstrated only moderate peak titers (10E4 pfu/mL), this level of virus load is similar to that reported by other investigators [26, 31]. Moreover, we are the first to demonstrate the expression of DENV proteins in monocytes (THP-1 cells). Therefore, our well characterized in vitro model using THP-1 cells and HMVEC is ideal to test for replication and immunomediator induction potential of other DENV serotypes.

We are planning to investigate DENV-induced phenotypic alterations using the two-cell culture system consisting of THP-1 cells and HMVEC. We aim to better understand the relevance of sequential processing and maturation of NS4B on immunomediator induction for other DENV strains and serotypes. Therefore, we will construct and employ NS plasmid clones from other DENV strains and serotypes to delineate immunomediators induction patterns in THP-1 cells, and will test their effects on HMVEC adhesion molecules and permeability models. It has been reported that NFκB and AP-1 transcription factors are involved in DENV-induced IL-8 and TNFα [3]. However, detailed mechanisms of induction are not understood; therefore, we will examine host proteins involved in the induction pathway. Also, we plan to employ mouse models to examine the effects DENV infection and TNFα inhibitors on disease immunopathogenesis.
CONCLUDING SUMMARY

The current working model for DHF immunopathogenesis involves high viremia and increased level of circulating immunomediators, in part secreted by DENV-infected monocytes. We have demonstrated that the elevated level of immunomediators produced by DENV-infected THP-1 cells is due to sequential processing of NS4AB and maturation of NS4B via 2KNS4B cleavage. These high levels of immunomediators, primarily TNFα, can stimulate vascular endothelial cell ICAM-1, VCAM-1, and E-sel and increase vascular permeability [34-37]. These endothelial cell changes increase overall vascular permeability and plasma leakage, signs of severe disease progression. To mitigate viremia or increased levels of immunomediators, one could theoretically block DENV replication or circulating virus using antivirals or Ig therapy, which are being developed but not yet available for human use. Conversely, one could reduce the level of circulating immunomediators, such as TNFα, by administering TNFα inhibitors later during infection as viremia wanes and patients show signs of progressing to severe disease. This proposed treatment is ideal as early DENV diagnosis is often difficult and administration of therapies later in infection are generally more appropriate. Therapies may be used in adjunct to DENV vaccines or under circumstances where vaccines are not available. Antivirals and TNFα inhibitors may be more appropriately used at different stages of infection, specific for patients’ disease progression and at varying times of infection, to prevent DHF/DSS. Based on our data, we propose that NS4B and maturation of NS4B via NS4AB cleavage by NS3B3(pro) and 2KNS4B cleavage by host proteases may prove attractive targets for antiviral drug development.
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