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The Good, the Bad, and the Shocking: The Multiple Roles of Dengue Virus Nonstructural Protein 1 in Protection and Pathogenesis

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Abstract

Dengue virus (DENV) is the most prevalent medically important mosquito-borne virus in the world. Upon DENV infection of a host cell, dengue nonstructural protein 1 (NS1) can be found intracellularly as a monomer, associated with the cell surface as a dimer, and secreted as a hexamer into the bloodstream. NS1 plays a variety of roles in the viral life cycle, particularly in RNA replication and immune evasion of the complement pathway. Over the past several years, key roles for NS1 in the pathogenesis of severe dengue disease have emerged, including direct action of the protein on the vascular endothelium and triggering release of vasoactive cytokines from immune cells, both of which result in endothelial hyperpermeability and vascular leak. Importantly, the adaptive immune response generates a robust response against NS1, and its potential contribution to dengue vaccines is also discussed.

Keywords

dengue; NS1; endothelial permeability; vascular leak; immune evasion; vaccines

BACKGROUND

Epidemiology

Dengue is the most prevalent mosquito-borne viral disease of humans worldwide, with up to an estimated 396 million dengue virus (DENV) infections and 96 million cases annually (1). Approximately half of the world's population is at risk of infection, with the majority of cases occurring in tropical and subtropical regions, including Latin America, Southeast Asia, and India (1). The four serotypes of DENV (DENV1–4) are transmitted by infected female *Aedes aegypti* and *Aedes albopictus* mosquitoes and are members of the *Flavivirus* genus in the *Flaviviridae* family, which also contains the medically important Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis, yellow fever (YFV), and tick-borne encephalitis viruses (2).

DISCLOSURE STATEMENT

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Dengue is a major public health problem and causes substantial economic burden (3). The geographic distribution of dengue has increased greatly over the past 50 years, with a 30-fold increase in global disease incidence (4). In 2010, it was estimated that the direct and indirect costs of dengue, including mosquito control, surveillance, and medical care, were $39 billion (5).

Life Cycle

DENV is a positive-sense RNA virus whose ~10.7-kb genome encodes for three structural proteins [capsid, premembrane/membrane (prM/M), envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (6). DENV primarily infects myeloid lineage immune cells in humans, including monocytes, macrophages, and dendritic cells (DCs) (7–9). Upon receptor-mediated entry into host cells, a conformational change in the E protein is triggered by acidification of the endosome, releasing the viral capsid into the cytoplasm, where uncoating of the genome occurs (6, 10). The genome is translated into a single polyprotein and cleaved into ten proteins by viral and host proteases. Following synthesis of the NS5 RNA-dependent RNA polymerase and the NS3 helicase, RNA replication begins, and viral particle assembly occurs in the endoplasmic reticulum (ER). Particles pass through the ER and Golgi, where posttranslational modifications and maturation occur before particles are secreted from the host cell (6, 10).

CLINICAL CHARACTERISTICS OF DENGUE DISEASE

Following an incubation period of 2–7 days, DENV infection can result in a wide range of outcomes, with ~75% of infections leading to asymptomatic or inapparent disease (1). Most patients who are symptomatic suffer from classical dengue fever (DF), a self-limiting but debilitating disease characterized by high fever; muscle, joint, and bone pain; and rash (11). However, some patients progress to severe disease, initially designated dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), characterized by vascular leakage, thrombocytopenia, and bleeding, potentially resulting in shock and organ failure (11). Viremia peaks in the early acute phase (days 1–4), whereas vascular leak and shock occur during the critical phase (days 4—6). In 2009, to facilitate clinical management, the World Health Organization introduced a new classification scheme of disease severity consisting of dengue without warning signs, dengue with warning signs, and severe dengue (12).

Immunopathogenesis of Dengue Disease

One of the primary hypotheses for severe dengue disease is immunopathogenesis, where secondary infection with a different DENV serotype from the first can lead to more severe disease (13, 14). This is thought to occur in part because of antibody-dependent enhancement (ADE), whereby cross-reactive but subneutralizing antibodies facilitate viral uptake and infection of Fcγ receptor–bearing cells, leading to increased viremia and activation of target immune cells (15–17). In vitro experiments have shown that subneutralizing antibodies increase infection of Fcγ receptor–bearing cells, and passive transfer of heterologous, poorly neutralizing antibodies results in antibody-enhanced lethal disease in mouse models (16, 18, 19). A recent study in a large, well-characterized pediatric cohort in Nicaragua demonstrated that a narrow range of preexisting low antibody titers

predicted increased disease severity while high titers were protective, demonstrating that ADE occurs in humans and contributes to DHF/DSS (20).

In addition to antibodies, serotype cross-reactive T cells may play a role in both the pathogenesis of severe dengue disease and protection (21,22), and activation of $CD4^+$ and $CD8^+$ T cells occurs in human patients (23, 24). During secondary infection, memory T cells are activated, leading to production of interferon-γ and tumor necrosis factor (TNF)-α, further activating macrophages and monocytes, which in turn produce inflammatory cytokines [e.g., TNF-α, interleukin (IL)-6, and IL-8] that can increase vascular permeability (23, 25, 26). This dysregulated production of cytokines by T cells, macrophages, and monocytes, sometimes referred to as a "cytokine storm," is thought to contribute to severe dengue disease (27, 28). In addition to cytokines, other inflammatory mediators such as histamine, platelet-activating factor, and leukotrienes are produced by mast cells, platelets, and other cell types during DENV infection and are associated with DHF (29–31).

Determinants of Endothelial Barrier Function

The two primary determinants of endothelial barrier function are the endothelial glycocalyx and intercellular junctional complexes, including tight and adherens junctions (32, 33). When the endothelial barrier is disrupted, hyperpermeability occurs, and fluids and molecules leak across the endothelium, extravasating from the vasculature and accumulating in tissues (34, 35). The endothelial glycocalyx is a complex, membrane-bound structure of carbohydrates, proteoglycans, and glycoproteins (GPs) that lines the luminal surface of the endothelium. This dense, forest-like matrix protects the underlying endothelial cells from shear forces generated by blood flow and creates a physical barrier that blocks molecules and fluids from reaching the endothelial surface. The glycocalyx contributes to hemostasis, molecular signaling, and interactions of blood cells with endothelial cells (36). Sialic acid residues and glycosaminoglycans, such as heparan sulfate, chondroitin sulfate, and hyaluronic acid, are the major constituents of the glycocalyx that contribute to the maintenance of the endothelial barrier. Intercellular junctions are key complexes involved in endothelial cell adhesion, communication, and barrier function (33). Specifically, tight and adherens junctions regulate paracellular permeability, or the passage of molecules between cells (37). Occludin, claudin, and cadherin proteins, anchored by zona occludens (ZO) and catenin proteins, connect neighboring endothelial cells to one another and prevent molecules and cells in the blood from accessing the underlying tissue (38). Upon disruption of the glycocalyx and intercellular junctions, fluid and molecules are able to extravasate from the bloodstream more freely, leading to the hallmark characteristics of vascular leak observed in severe dengue disease (39).

NS1 BIOLOGY

NS1 is synthesized by DENV-infected cells as a monomer, which then dimerizes in the ER. NS1 is glycosylated in both the ER and the trans-Golgi network. NS1 forms part of the replication complex and can be found on intracellular membranes and the cell surface. NS1 is also secreted into the extracellular milieu as a hexamer and circulates in the blood during acute illness, where its presence is useful as a diagnostic biomarker. NS1 is a key factor

during infection, with roles in viral RNA replication, virion production, immune evasion, and multiple aspects of pathogenesis.

Structure and Morphology of a Multimeric Viral Protein

The NS1 gene in all flaviviruses is approximately 1,056 nucleotides in length and encodes a 352-amino acid (aa) protein with a molecular weight of 46–55 kDa, depending on its glycosylation status (40, 41). Phylogenetic analyses of NS1 amino acid sequences from different flaviviruses, including DENV1–4, ZIKV, WNV, JEV, and YFV as well as St. Louis encephalitis, Murray Valley encephalitis, and tick-borne encephalitis viruses, revealed variable conservation that ranges from 50% to ~80% (42, 43). This analysis suggests that different flavivirus NS1 proteins have conserved functions as well as unique features leading to group-specific NS1 clusters (42).

After translation of the flavivirus polyprotein in the ER, newly synthesized NS1 monomers undergo posttranslational modifications in the *trans*-Golgi network, such as glycosylation at asparagine (Asn)130 (complex glycan), Asn207 (high-mannose glycan), and, in some flaviviruses, Asnl75 (complex glycan) (41, 44, 45). Following glycosylation, NS1 monomers dimerize, facilitating membrane association of the protein. The dimeric form associates with intracellular membranes as well as the plasma membrane at the cell surface. Subsequent oligomerization of flavivirus NS1 dimers leads to formation of different multimeric species (e.g., tetramers, hexamers) (41, 46). The hexameric form is secreted from infected cells, and glycosylation at Asnl30 and Asn207 stabilizes the hexamer and facilitates its secretion (47) (Figure 1).

DENV NS1 is secreted at high levels into the extracellular environment during DENV infection, predominantly as a soluble hexamer that is a barrel-shaped, high-density lipoprotein containing a hydrophobic core that interacts tightly with lipids such as triglycerides, cholesteryl esters, and phospholipids (41, 48, 49) (Figure 1). Recently, the macromolecular organization of NS1 was finally solved at high resolution (3.0 Å) (50, 51). The crystal structure of NS1 reveals three distinct domains that constitute each NS1 monomer. A small dimerization domain known as the central β-roll domain (aa 1–29) forms the inner-facing hydrophobic face that interacts with lipids. A second, protruding domain designated the wing domain (aa 30–180) contains two glycosylation sites (Asnl30 and Asnl75), an internal disulfide bond (Cys55-Cysl43), and two discrete subdomains: the α/β subdomain (aa 38–151) and a discontinuous connector subdomain (aa 30–37 and 152–180) that links the wing domain to the central β-roll domain. The hydrophobic protrusion (greasy finger) between the β-roll and the wing domains facilitates NS1 interaction with the membrane of the ER and viral proteins, such as NS4A and NS4B, that are essential for viral replication. Finally, a third domain, known as the β-ladder (aa 181–352), is formed by a continuous β-sheet that forms half of the C terminus of NS1. As the extracellular NS1 hexamer is a target of humoral immune system recognition, the C-terminal tip of the β-ladder along with the wing domain are the most antigenic regions and contain the most frequently identified epitopes of the NS1 hexamer.

Viral Cofactor in Flavivirus Replication

Intracellular expression of NS1 in the lumen of the ER colocalizes with double-stranded RNA and other components of the viral replication complex in membranous compartments known as vesicle packets (52, 53). This finding suggests that NS1 plays a role in viral replication and negative-strand viral RNA synthesis (54–56), and deletion of NS1 prevents viral replication and infection (55). Substitution of DENV sequences into WNV NS1 at RQ10NK, a site previously identified as playing a role in cell membrane association, enhanced WNV NS1 secretion but also reduced viral replication (52). NS1 interacts with multiple host proteins and the transmembrane viral replicase proteins NS4A and NS4B (52, 56). More recently, physical interactions between NS1 and the structural proteins E and prM were reported (57). Together, these data suggest that NS1, along with other viral proteins, may fulfill a structural role, helping to anchor the replication complex to membranes and inducing formation of membrane compartments that facilitate viral RNA replication, virus assembly, and release.

Another important feature of secreted NS1 that may influence its role in viral replication is constituted by the two N-glycosylation sites at Asn130 and Asn207. Removal of either one or both glycosylation sites in DENV, WNV, or YFV results in decreased NS1 secretion and virus yield as well as reduced invasion of the central nervous system and neuropathology in mice (40, 44, 47, 58). Recently, a global proteomic analysis of human host factors interacting with NS1 combined with a functional RNA-interference screen identified many host cellular pathways, including those related to glycosylation of NS1 and NS4B, that may facilitate or restrict DENV infection (59). Additionally, the flavivirus replication cycle induces intracellular lipid redistribution that results in extensive membrane rearrangements (60, 61). Given that NS1 dimers localize with cholesterol-associated molecules on cell membranes (62) and present a markedly hydrophobic surface that promotes interactions with the ER membrane (50), NS1 may contribute to the modulation of cellular lipid dynamics during flavivirus infection and/or lead to recruitment of cholesterol and triglycerides to the replication complex. Together, these results indicate that NS1 glycosylation as well as the lipid composition of the hydrophobic core of NS1 may represent important factors not only for protein secretion, but also for viral RNA replication and possibly membrane rearrangement.

Diagnostic Antigen and Potential Biomarker for Clinical Prognosis

Laboratory diagnosis methods for confirming DENV infection involve different molecular and serological approaches, such as RT-PCR (reverse transcription-polymerase chain reaction), virus isolation, and detection of increased levels of IgM and IgG antibodies, especially against E. Currently, the detection of NS1 circulating in the serum/plasma of infected patients constitutes a commonly used approach for the early diagnosis of flavivirus infection, particularly DENV, although sensitivity varies by serotype and is greater in primary than secondary infection (63, 64). Further, the duration of NS1 antigenemia is significantly longer in primary than in secondary infections (65). Different generations of commercially available kits and in-house enzyme-linked immunosorbent assays as well as rapid immunochromatographic assays are widely available (64). NS1 antigenemia in DENV-infected patients correlates with disease severity (65–67). Circulating levels of NS1 vary

from 0.1 to 1 μg/mL, with reports of up to 50 μg/mL, and persist for up to 14 days from the onset of fever (66, 68–72). These data are based on NS1 levels measured in the bloodstream of infected patients. However, NS1 may exit the bloodstream into tissues, leading to an apparent clearance of NS1 from the blood and increased presence in tissues.

With the recent emergence of new flaviviruses, such as ZIKV (73), and the potential reemergence of flaviviruses such as YFV (74), the differential diagnosis of flavivirus infection is challenging, especially in regions of the world where multiple flaviviruses cocirculate (75, 76). The limited window to detect viral RNA in body fluids as well as the highly cross-reactive antibody responses generated during different flavivirus infections such as DENV and ZIKV, especially during the acute phase, represent a significant obstacle for the efficient, accurate, and specific diagnosis of these infections using either molecular techniques or serological assays. Recently, two studies reported NS1-based assays for differential diagnosis and surveillance of ZIKV and DENV infections (77, 78). One assay detects the circulation of anti-NS1 antibodies, the other detects soluble NS1, and both distinguish between DENV and ZIKV infections with high sensitivity and specificity (77–79). An NS1-based assay was also recently used to distinguish natural infection from vaccination with Sanofi Pasteur's Dengvaxia, a chimeric dengue vaccine in a YFV backbone, enabling determination of preimmune status (80). Thus, NS1 can serve as a diagnostic and prognostic biomarker and can differentiate between flavivirus infections.

DENGUE VIRUS NS1-MEDIATED PATHOGENESIS

Because NS1 antigenemia levels correlate with disease severity, NS1 may directly influence pathogenesis. However, until recently, NS1 had not been shown to play a direct pathogenic role during DENV infection. Researchers from two different groups showed that DENV NS1 alone is capable of inducing endothelial hyperpermeability of human pulmonary, dermal, and umbilical vein endothelial cells in vitro (81, 82). DENV NS1 also induced lethality in a mouse model when administered with a sublethal dose of virus, and NS1 alone triggered vascular leak in the lung, liver, and small intestine of mice (81). The specific mechanisms of NS1-mediated pathogenesis identified to date are described below.

ENDOTHELIAL-INTRINSIC PATHOGENESIS

Endothelial Glycocalyx

After being secreted by infected cells, DENV NS1 circulates in the blood of patients and can bind to the surface of microvascular endothelial cells in capillary beds (83). Recent studies have shown that NS1 from all four DENV serotypes induces endothelial hyperpermeability in multiple human endothelial cell lines as measured by *trans*-endothelial electrical resistance (84). NS1 up-regulates expression of the sialidases Neu1, Neu2, and Neu3, leading to cleavage of sialic acid on the surface of endothelial cells. NS1 also triggers increased activation of cathepsin L, which leads to increased expression and activation of heparanase that then cleaves heparan sulfate and heparan sulfate proteoglycans, such as syndecan-1, from the surface of the endothelium. Altogether, the ensuing degradation of the endothelial glycocalyx-like layer results in barrier dysfunction. Cathepsin L activation, heparanase expression, sialic acid shedding, and hyperpermeability can all be blocked in

vitro using inhibitors of sialidases (Zanamivir), heparanase (OGT 2115), and cathepsin L (Cathepsin L Inhibitor) (84, 85). These results were recently supported in an animal model of localized vascular leak, where NS1-induced vascular leak in the dorsal dermis of wild-type mice was prevented using a cocktail of Zanamivir, OGT 2115, and Cathepsin L Inhibitor, suggesting that the glycocalyx and its components are key mediators of NS1-induced pathogenesis in vivo (85) (Figure 2a).

The glycocalyx was originally hypothesized to play a role in severe dengue after increased levels of heparan sulfate were detected in the urine of children with DSS (39, 86). A later study investigating the molecular sieving properties of the microvasculature in Vietnamese dengue patients further implicated the glycocalyx (87). Because dextran fractional clearance studies showed no difference between dengue patients with evidence of vascular leak and healthy controls, administration of dextran may have contributed to stabilization of the glycocalyx after loss of plasma proteins during infection, leading to restoration of endothelial glycocalyx integrity and normal barrier function (87). Further, recent studies have evaluated patient sera during acute DENV infection and found that levels of key endothelial glycocalyx molecules, such as hyaluronic acid, heparan sulfate, chondroitin sulfate, and syndecan-1, are all elevated to a greater degree in patients with severe disease (88, 89). This suggests that disruption of the glycocalyx occurs during DENV infection, potentially as a result of NS1 interactions with the vascular endothelium, and is associated with disease severity in humans.

Intercellular Junction Complexes

No lasting structural damage to the endothelium has been observed in human DHF/DSS cases (9, 90). Therefore, it is likely that transient disruption of intercellular junctions that, along with the glycocalyx, control vascular homeostasis may be one of the mechanisms responsible for vascular leak. DENV NS1 stimulates production of macrophage migration inhibitory factor (MIF) from dermal endothelial cells in vitro, and MIF can induce endothelial hyperpermeability through autophagy of intercellular junction proteins in dermal endothelial cells (91). Both NS1-induced endothelial hyperpermeability and autophagy were prevented using a specific inhibitor of MIF, and hyperpermeability was prevented by blocking autophagy. Further, loss of the normal distribution of the adherens junction protein VE-cadherin (vascular endothelial cadherin) was blocked by MIF and autophagy inhibitors, suggesting its involvement in increased permeability (91). Additionally, studies using human endothelial cells from different tissues including lung, brain, and umbilical vein also showed that NS1 induces disruption of both tight and adherens junction proteins in a cytokine-independent manner. Using confocal microscopy analyses, investigators found that NS1 induces mobilization of intercellular junction proteins (e.g., ZO-1, VE-cadherin, and β-catenin) via clathrin-mediated internalization and/or phosphorylation, which may compromise the integrity of cell-to-cell contacts (H. Puerta-Guardo & E. Harris, unpublished data). Further, in a study of dengue patients in Indonesia, increased levels of the tight junction protein claudin-5 circulating in the blood were associated with vascular leak during DHF, suggesting a role for the disruption of intercellular junctions during severe dengue (88). Together, these data suggest an additional mechanism for NS1-mediated vascular leak via disruption of endothelial intercellular junctions (Figure 2b).

CYTOKINE-MEDIATED PATHOGENESIS

Inflammatory cytokines are thought to play an important role in the pathogenesis of severe dengue. Dysregulated production of cytokines has been hypothesized as a mechanism for vascular leak in DHF/DSS, and several cytokines, including TNF-α, IL-10, IL-6, and IFN-γ, have been proposed as potential predictors of disease severity (92–96). In human studies, levels of TNF-α were elevated in DHF/DSS cases (93), and levels of IL-6 were elevated in patients with fatal dengue compared with survivors of severe or mild disease (97, 98). In a mouse model, intravenous administration of DENV2 NS1 resulted in significantly higher levels of both TNF-α and IL-6 in the blood 3 days postinjection compared with injection of the control protein ovalbumin, supporting a role for NS1 in inducing secretion of inflammatory cytokines that may contribute to vascular leak during severe disease (81) (Figure 2c,d).

DENV NS1 activates both murine bone marrow-derived macrophages and human peripheral blood mononuclear cells via Toll-like receptor 4 (TLR4), leading to secretion of the inflammatory cytokine IL-6 and transcription of the TNF-α, IL-1β, and IL-8 genes (82). Further, vascular leak in the small intestine and liver of mice infected with DENV2 was reduced in the presence of lipopolysaccharide from *Rhodobacter sphaeroides* (LPS-RS), a TLR4 antagonist (82). Treatment of human pulmonary endothelial cells with LPS-RS also partially reduced loss of sialic acid on the cell surface following treatment with NS1, an effect that may be mediated by TLR4-dependent translocation of Neu1 to the cell surface (84). Another group has reported that NS1 may induce secretion of inflammatory cytokines from peripheral blood mononuclear cells via TLR2 and TLR6 (99); however, these data are controversial (100).

Monocyte-derived dendritic cells (mo-DCs) are one of the primary targets of DENV infection, and they take up NS1 in vitro (101). Internalization of NS1 increased infection and early viral replication in mo-DCs by DENV1, and this led to an increase of IL-6 production during infection; however, NS1 did not affect expression of DC maturation markers, such as DC-SIGN, HLA-DR, CD80, or CD86 in uninfected mo-DCs (101). Though it is unclear if NS1, virus infection, or a synergistic effect leads to the increase of IL-6 production, these data suggest that NS1 may mediate the production of IL-6 by DCs, contributing to the cytokine storm observed in DHF.

In addition to inflammatory cytokines, levels of IL-10, an immunoregulatory cytokine, are elevated in patients with severe dengue disease (102, 103). NS1 stimulates high levels of IL-10 production from monocytes, and this effect was blocked with NS1-specific monoclonal antibodies (MAbs) (104). Though the precise role of IL-10 in dengue disease has yet to be defined, these results support a role for NS1 in increasing the levels of IL-10 in DHF patients.

Collectively, these studies demonstrate that NS1 can interact with innate immune cells, potentially through TLR4, leading to the secretion of cytokines and other mediators that contribute to vascular leak and viral pathogenesis. Though it has yet to be demonstrated, NS1 may also interact with other immune cells that have been described to actively

participate in DENV pathogenesis, including mast cells and platelets (31, 105–108), leading to their activation and secretion of noncytokine mediators such as eicosanoids, leukotrienes, or proteases that also may contribute to inflammation and vascular leak during DHF/DSS (Figure 2c,d).

NS1 PATHOGENESIS: A COMPLEX INTERPLAY

Research to explore the relative contributions of the endothelial glycocalyx and inflammatory cytokines to NS1-mediated pathogenesis has shown that the two mechanisms are separable (85). In vitro, NS1 did not induce secretion of TNF-α or IL-6 from human dermal or pulmonary endothelial cells, and blocking these cytokines with the corresponding MAbs did not affect NS1-induced endothelial hyperpermeability. Further, when comparing TLR4- and TNE-α receptor-deficient mice with wild-type mice, no significant differences were observed in NS1-mediated vascular leak in a model of local vascular leak in the mouse dorsal dermis. In contrast, a cocktail of sialidase, cathepsin L, and heparanase inhibitors prevented NS1-induced hyperpermeability as well as abrogated sialic acid and heparan sulfate degradation and the increase of cathepsin L activity in vitro. The same cocktail also prevented NS1-mediated local vascular leak in wild-type mice. Interestingly, when *Tlr4*$^{-/-}$ *x Ifnar*$^{-/-}$ mice were infected with a lethal dose of DENV2, there was no significant difference in morbidity or mortality, though both were slightly delayed in TLR4-deficient mice. Additionally, levels of vascular leak in the lung and liver of mice injected with NS1 were similar in both *Tlr4*$^{-/-}$ and wild-type animals, with slightly lower levels observed in *Tlr4*$^{-/-}$ mice (85). These data suggest that TLR4 is not a critical driver of NS1-mediated pathogenesis in these in vivo models; however, Modhiran et al. (82) observed that administration of LPS-RS reduces vascular leak in a mouse model of DENV infection, supporting a role for TLR4 as a mediator of disease during infection. Taken together, these results demonstrate the complexity of dengue pathogenesis, particularly regarding the role for NS1 and the mechanisms behind its induction of endothelial dysfunction.

Beyond its demonstrated functions during human infection, NS1 also enhances mosquito acquisition of flaviviruses, including DENV. When mosquitoes feed on infected humans, they acquire virus along with soluble NS1 in the blood meal. NS1 helps the virus overcome the midgut barrier in mosquitoes—a key step in the flavivirus life cycle in the vector—through the inhibition of reactive oxygen species production and the JAK-STAT pathway, thus helping the virus establish infection in the mosquito (109). Additionally, soluble NS1 has been detected in the saliva of infected *A. aegypti* mosquitoes, suggesting that NS1 may be inoculated along with virus during blood feeding (110). Combined with proteins in mosquito saliva that impact viral replication and pathogenesis (111–113), soluble NS1 may play a role in early immune evasion, viral infection, and induction of endothelial hyperpermeability in the human dermis, possibly contributing to further virus dissemination (Figure 2).

Taken together, NS1 plays a variety of roles in DENV pathogenesis, and the relative contributions of each role are difficult to dissect from each other. Most likely, disruption of the endothelial glycocalyx and production of inflammatory cytokines occur throughout the course of severe dengue disease, but with varied kinetics and magnitude, though peaking

during the critical period, leading to a complex synergy of viral- and host-specific factors that contribute to endothelial barrier dysfunction and vascular leak. Beyond its involvement in vascular leak, NS1 also helps facilitate the spread of DENV from humans into the mosquito vector, and it may also play an important role locally immediately after inoculation from the mosquito into the host dermis.

The role of NS1 in virus acquisition by mosquitoes and in the dermal microenvironment may be potential targets for vaccines and therapeutics designed to decrease transmission or halt the establishment of infection in hosts. Additionally, because of the effect of NS1 on the glycocalyx (84, 85), currently licensed sialidase inhibitors for treatment of influenza (114) and heparanase inhibitors used in cancer therapy (115, 116) may prevent NS1-induced degradation of the glycocalyx and subsequent vascular leak. Together, these pathways offer potential therapeutic targets for the treatment of severe dengue disease.

NS1 PATHOGENESIS IN OTHER FLAVIVIRUS INFECTIONS

Different flavivirus NS1 proteins share conserved sequences as well as diverse features that may lead to differential interactions with the host and subsequent pathogenesis (42, 43). Although NS1 plays a substantial role in pathogenesis in DENV infection, its role in other flavivirus infections has yet to be determined. NS1 from encephalitic WNV has no effect on the permeability of human pulmonary microvascular endothelial cells, and it does not induce vascular leak in the dermis of mice in an animal model of localized vascular leak (84, 85); however, it does increase the permeability of human brain microvascular endothelial cells in vitro (H. Puerta-Guardo, D.R. Glasner & E. Harris, unpublished data). Similarly, JEV NS1 also induces hyperpermeability in brain endothelial cells, whereas NS1 from ZIKV, which targets the brain and placenta in humans, increases the permeability of brain and umbilical vein endothelial cells. In contrast, and as expected from the liver disease characteristic of yellow fever, YFV NS1 induces hyperpermeability only of liver endothelial cells in vitro. These effects are mediated through the same endothelial-intrinsic mechanisms as of DENV NS1, including activation of endothelial sialidases, cathepsin L, and heparanase, regardless of the tissue origin of the endothelial cells (H. Puerta-Guardo, D.R. Glasner & E. Harris, unpublished data). This overall pattern was recapitulated upon inoculation of different NS1 proteins into mice, with WNV, JEV, and ZIKV NS1 resulting in increased leakage in the brain and YFV NS1 causing leakage in the liver, suggesting that flavivirus NS1 species- and tissue-specific mechanisms drive induction of endothelial barrier dysfunction, which may impact viral tropism, dissemination, and pathogenesis.

NS1 AND COMPLEMENT

Early literature identified DENV NS1 as a soluble complement-fixing protein (117–119). Complement proteins are primarily serine proteases found throughout the blood and in tissues and when activated can lead to inflammation, opsonization, or cell lysis via formation of the C5b-9 membrane attack complex (Figure 2e).

Anti-NS1 Antibodies and Complement Activation

Anti-NS1 antibodies activate complement (C'), presumably to lyse virus-infected cells (120), and recent work has identified an anti-NS1 MAb that triggers complement-mediated lysis of DENV-infected cells (121). Soluble NS1 activates serum C', but cell surface-associated NS1 may cause antibody-dependent C' lysis, which could be a mechanism to eliminate DENV-infected cells (122). However, if soluble NS1 binds nonspecifically to cells, it could trigger antibody-mediated C' activation and lysis of uninfected cells and, thus, potentially enhance viral pathogenesis (83).

Immune Evasion

DENV NS1 binds to the complement protein C4 and to recruit and activate the protease C1s, leading to cleavage of C4 to C4b and reducing both the deposition of C4b on the surface of cells and C3 convertase activity, thereby protecting DENV from complement-mediated neutralization and protecting DENV-infected cells from lysis (123) (Figure 2e). A second mechanism was identified wherein NS1 binds to C4b binding protein, a protein that attenuates the classical and lectin pathways of complement, leading to inactivation of C4b in solution and on the cell surface (124). More recently, NS1 has been shown to bind to vitronectin, a complement regulator, and this interaction was demonstrated in vitro and in DENV-infected patients (125). In the same study, NS1 inhibited the membrane attack complex and polymerization of the complement protein C9. NS1 also binds to mannose-binding lectin, a key component in complement activation, thereby protecting DENV from the lectin pathway of complement (110). Taken together, evidence supports a role for NS1 in binding complement components, thus protecting the virus from complement-mediated neutralization and preventing the killing of infected cells.

Pathogenesis

Clinically dysregulated complement activation has been demonstrated in DHF patients, with higher levels of complement Factor D and lower levels of Factor H observed in DHF versus DF patients, suggesting formation of the C3 convertase and activation of complement (126). Further, NS1, C3a, C5a, and soluble C5b-9 were present at higher levels in the plasma of DHF patients starting 3 days before signs of plasma leakage, suggesting a role for complement activation in severe dengue disease (122). Additionally, NS1 may lead to generation of higher levels of C5a, resulting in increased vascular leak and pathogenesis (Figure 2e).

IMMUNE-MEDIATED PROTECTION

Responses to NS1 During Natural Infection

A substantial proportion of the antibody response to DENV after infection is directed against NS1, and anti-NS1 antibodies have been found in convalescent sera after primary DENV infection, during the acute and convalescent phases of secondary DENV infection, and in DF and DHF/DSS patients (127–131). Although no differences have been found in the levels of total anti-NS1 antibodies (127–130), antibodies to particular NS1 epitopes were present at

higher levels in DF versus DHF patients (132) (see below). It is not clear yet what precise role these antibodies play in resolving infection or preventing reinfection.

NS1 as Part of a Dengue Vaccine

Overall, candidate NS1 recombinant protein and DNA vaccines are highly immunogenic and protective in mouse models (120,133–135). In one study, immunization with adjuvanted recombinant NS1 as well as passive transfer of polyclonal antibodies from NS1-immunized mice or of certain anti-NS1 MAbs to naive mice protected against vascular leak disease, presumably by triggering lysis of infected cells and/or blocking the pathogenic effects of secreted NS1 (81, 136, 137). Inclusion of NS1 in a DENV vaccine or other flavivirus vaccines remains under discussion. The approved CYD vaccine (Dengvaxia) for dengue from Sanofi Pasteur does not include DENV NS1 (138), whereas other live-attenuated DENV vaccines in development [e.g., from the National Institutes of Health (NIH) and Takeda] include expression of DENV NS1 and should elicit strong antibody responses to NS1 (139). In human trials, the NIH TV-003 DENV vaccine elicited NS1-specific $CD4^+$ and $CD8^+$ T cell responses similar in magnitude and breadth to those found after natural infection (140, 141). An NS1 DNA vaccine study in mice used depletion and transfer studies to demonstrate that $CD4^+$ T cells were critical for NS1-specific immune protection from virus challenge (142). Thus, immune responses to NS1 may well be important to elicit in dengue vaccines.

In light of the recent controversy surrounding the Sanofi Pasteur DENV vaccine, particularly the potential to trigger ADE during secondary DENV infection (143, 144), it may be prudent to explore alternative vaccine designs and strategies. NS1 vaccination does not induce ADE and is protective in animal models of DENV infection (81). Vaccination also induces cross-reactive antibodies against NS1 that can protect against lethal challenge (81, 132). As such, inclusion of NS1 in future vaccine designs would be pertinent.

Modified NS1 Vaccine

To reduce potential immune-mediated pathogenesis, NS1 can be modified to alter or remove regions of the protein that are thought to be detrimental. In one example, a recombinant protein vaccine was created by substituting the C terminus of DENV NS1 (aa 271–352) with a JEV NS1 sequence to remove the region postulated to cross react with circulating plasma proteins or cell membrane proteins expressed on the surface of endothelial cells (136). Passive transfer of polyclonal sera from mice immunized with the chimeric DENV/JEV NS1 reduced viral load and hemorrhagic manifestations in a mouse model (145). Anti-DENV/JEV NS1 antibodies also blocked mast cell degranulation, chemokine production, and tryptase expression (146). Likewise, NS1 proteins containing site-specific mutations that eliminate the ability to trigger endothelial hyperpermeability in vitro are being investigated as potential vaccine immunogens (C. Wang & E. Harris, data not shown). Thus, a modified NS1 could be integrated into current vaccines as a recombinant protein in combination or in a prime-boost strategy with a live vaccine.

NS1 Antibody Epitopes

Many groups have identified immunodominant regions of NS1 in mice (128, 147, 148), some with reactivity in both NS1-immunized and virus-infected mice as well as naturally infected humans (128) (Figure 3; Table 1). Akey et al. (50) mapped immunodominant epitopes to the most accessible parts of the NS1 hexamer. An important immunodominant region first identified in mouse sera by Falconar et al. (147) and later in human sera (128) was recently shown to be protective in vivo (132); this region was mapped to the surface-exposed disordered distal tip of the NS1 wing domain (50, 128, 149) (Figure 3; Table 1). A modified peptide of this NS1 region was used as an immunogen in a mouse model to demonstrate protection against DENV-induced disease as well as reduced binding to human umbilical vein endothelial cells by the antibodies generated. Further, antibodies to this peptide were found at higher levels in acute sera from DF versus DHF patients (132). Together, these data suggest the wing region of NS1 as a key component of a DENV vaccine. Other regions of NS1 elicit antibody responses that may block a pathogenic function or attachment of NS1 to the cell surface (128) or may provide protection through triggering complement-mediated lysis of infected cells (121). Passive transfer of an NS1-specific MAb directed to aa 25–33 (1H7.4) (147) protected against pathogenic DENV infection in a mouse model (81). A key advantage of using a recombinant NS1 protein vaccine would be generation of antibodies to the protein that are found only with vaccination. At least five immunoreactive regions of NS1 were identified in natural DENV infection, and an additional five immunoreactive regions were elicited only after vaccination with recombinant NS1 (128, 148). The majority of these epitopes are recognized across at least three DENV serotypes (Table 1).

Anti-NS1 Antibody Cross-Reactivity Across Four Serotypes

DENV infection in humans results in the production of serotype-specific NS1 antibodies as well as cross-reactive antibodies to at least one if not all three other serotypes (127, 128, 150). In addition, vaccination with recombinant DENV1, −3, or −4 NS1 combined with adjuvant provided partial protection against lethal DENV2 infection in mice. Furthermore, investigators identified several immunodominant B cell NS1 epitopes in naturally infected mice that are conserved across all four DENV serotypes (148). Therefore, vaccination with a single NS1 or live vaccine could potentially provide protection against other DENV serotypes.

CROSS-REACTIVE PATHOGENIC ANTI-NS1 ANTIBODIES

Murine anti-NS1 antibodies bind to human platelets, thrombin, plasminogen, and endothelial cells in vitro (151–153), and data from mouse models suggested that cross-reactive antibodies to NS1 may contribute to pathogenesis (144, 154–157). Initial studies tested the cross-reactivity of MAbs and polyclonal antibodies from mice (151, 158); subsequent studies examined human anti-DENV sera for binding to endothelial cells (159) and plasminogen (160). Another study showed that anti-NS1 antibodies could trigger endothelial cell apoptosis via nitric oxide production by glycogen synthase kinase-3b-induced NF-kB activation and inducible nitric oxide synthase expression (158, 161); however, whether DENV infection leads to endothelial cell apoptosis in humans has not been established. The

ability of anti-NS1 antibodies to bind to self-molecules and trigger increased production of nitric oxide provides evidence of indirect pathogenesis triggered by NS1 (Figure 2f). Host cross-reactive NS1 epitopes have been mapped to various regions of NS1, with the crossreactivity to endothelial cells thought to target epitopes in the C terminus of NS1 (aa 300–352) (Figure 3; Table 1). In a vaccine mouse model, deleting this region of NS1 reduced the production of host cross-reactive antibodies (136, 145, 162, 163). Though a role for NS1-derived autoantibodies has been implicated in DENV infection and NS1 immunization models, it has been difficult to demonstrate a direct role for these antibodies in human dengue disease, and there is no indication of autoimmune manifestations during DENV infections or post-infection sequelae.

VIRAL TOXINS IN THE PATHOGENESIS OF OTHER DISEASES

Hepatitis C Virus p7

DENV and other viruses in the *Flavivirus* genus all produce structurally similar secreted NS1 hexamers from infected cells. In contrast, the *Hepacivirus* genus of *Flaviviridae* does not produce NS1; however, hepatitis C virus (HCV) does express the protein p7, which is not required for replication but is crucial for the release of viral particles. p7 forms hexameric or heptameric complexes inside the cell and is thought to act as a viroporin, enabling release of virions from infected cells (164–166). HCV p7, like DENV NS1, is located in the genome and polyprotein after E2 and before NS2a, but in contrast, p7 is only 63 aa in length, is expressed intracellularly only, and is thought to act as an ion channel (167). Though p7 can form oligomers of four to seven subunits, the hexamer is thought to provide the cylindrical structure needed for pore formation (168). The analogous hexameric structure may suggest evolutionary similarity between DENV NS1 and HCV p7 or perhaps indicate that DENV NS1 plays an additional role in virion release and/or modulation of ions in the ER that may be cofactors for viral replication. However, the larger size and the secretory characteristic of DENV NS1 suggest a far greater role for this protein.

Ebola Virus Glycolipid

Ebola virus (EBOV) belongs to a distinct family of RNA viruses, *Filoviridae*, but EBOV, like DENV, infection can lead to vascular leak and hemorrhagic manifestations. Interestingly, EBOV also expresses a structural GP that is a trimeric viral surface protein formed by two subunits: GP1 and GP2. GP1 mediates virus attachment to host cells, whereas GP2 is involved in membrane fusion (169, 170). A unique feature of EBOV is that following infection, GP is released from cells in two soluble forms: a soluble GP, secreted via the classical secretory pathway, and a truncated surface (or shed) GP, which is cleaved from the cell surface by a cellular metalloprotease. Both soluble and shed GPs can be detected in the blood of patients and experimentally infected animals (171, 172). EBOV shed GP has been implicated in triggering vascular leak by activating immune cells to produce inflammatory cytokines and by acting directly on endothelial cells (173). When added directly to human umbilical vein endothelial cells, shed GP triggers changes in relative permeability, and the same effects were observed after treatment with supernatants collected from shed GP-treated macrophages. The effects of shed GP were diminished with anti-TLR4 antibodies or the deglycosylation of shed GP (173). These results seem

remarkably parallel to the DENV NS1 results, showing direct and indirect effects of NS1 on endothelial cells as well as a potential role for TLR4 signaling (81, 82).

Rotavirus NSP4

Rotavirus expresses the enterotoxin NSP4, which acts as an ER-localized viroporin that can form ion channels, disrupt cellular calcium homeostasis, and inhibit sodium adsorption, leading to the characteristic diarrhea during rotavirus infection (174). NSP4 is actively secreted in oligomeric forms by infected epithelial cells and binds to glycosaminoglycans on uninfected cells (175). NSP4 is often considered the first viral protein shown to mimic the effects of classic bacterial enterotoxins, and a role for NSP4 as a pathogen-associated molecular pattern during rotavirus infection has been proposed (176). However, NSP4 is more similar to cholera toxin, with its effects on triggering fluid release in the intestine. In contrast, DENV NS1 is more similar to LPS and other toxins that directly trigger endothelial cell effects leading to vascular leak and that activate a cascade of inflammatory cytokines, potentially resulting in severe systemic shock.

CONCLUSIONS

Taken together, recent research advances have further demonstrated the multifactorial role of NS1 during dengue disease. Beyond its described functions in immune evasion and viral replication, recent literature supports a key role for NS1 in the pathogenesis of severe dengue disease and in inducing protective immune responses. Researchers have identified multiple pathways that contribute to NS1-mediated vascular leak, including degradation of the endothelial glycocalyx and secretion of inflammatory cytokines following NS1 stimulation of innate immune cells. NS1 is also an important target of the humoral immune response, and antibodies against NS1 protect mice from lethal DENV infection. There remain several significant unanswered questions, including the molecular determinants of pathogenesis, the binding partner(s) for NS1 on endothelial and immune cells, and the full mechanism of action of NS1 in its various functions. NS1 offers a potential target for both therapeutics and vaccine design, and further research into the mechanisms that drive pathogenesis and the nuances of the immune response to the protein may lead to substantial advances in the treatment and prevention of severe dengue disease.

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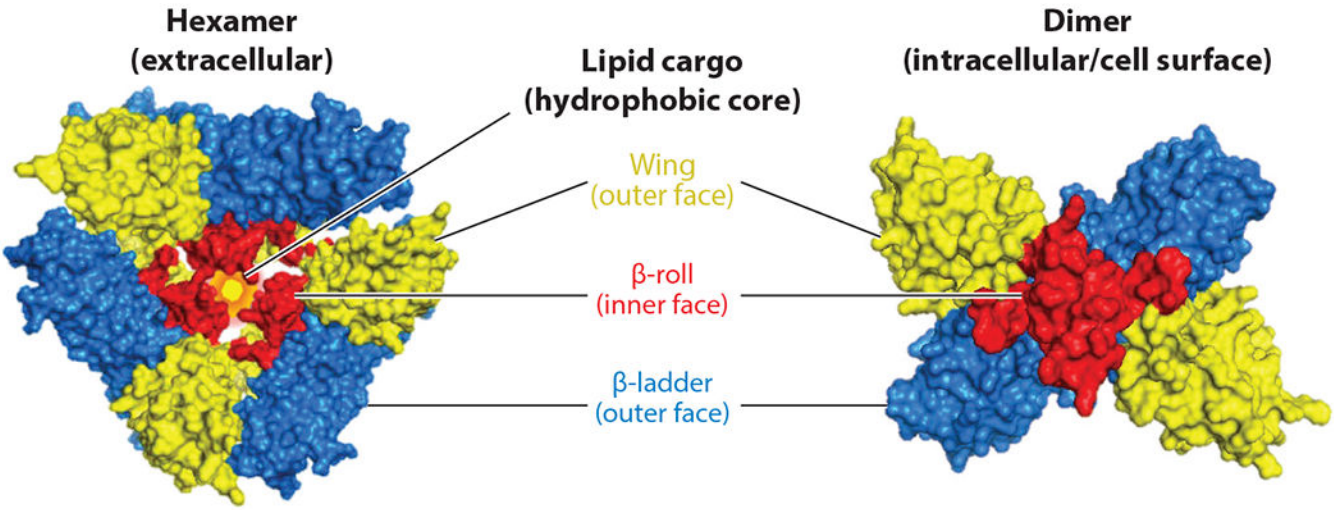
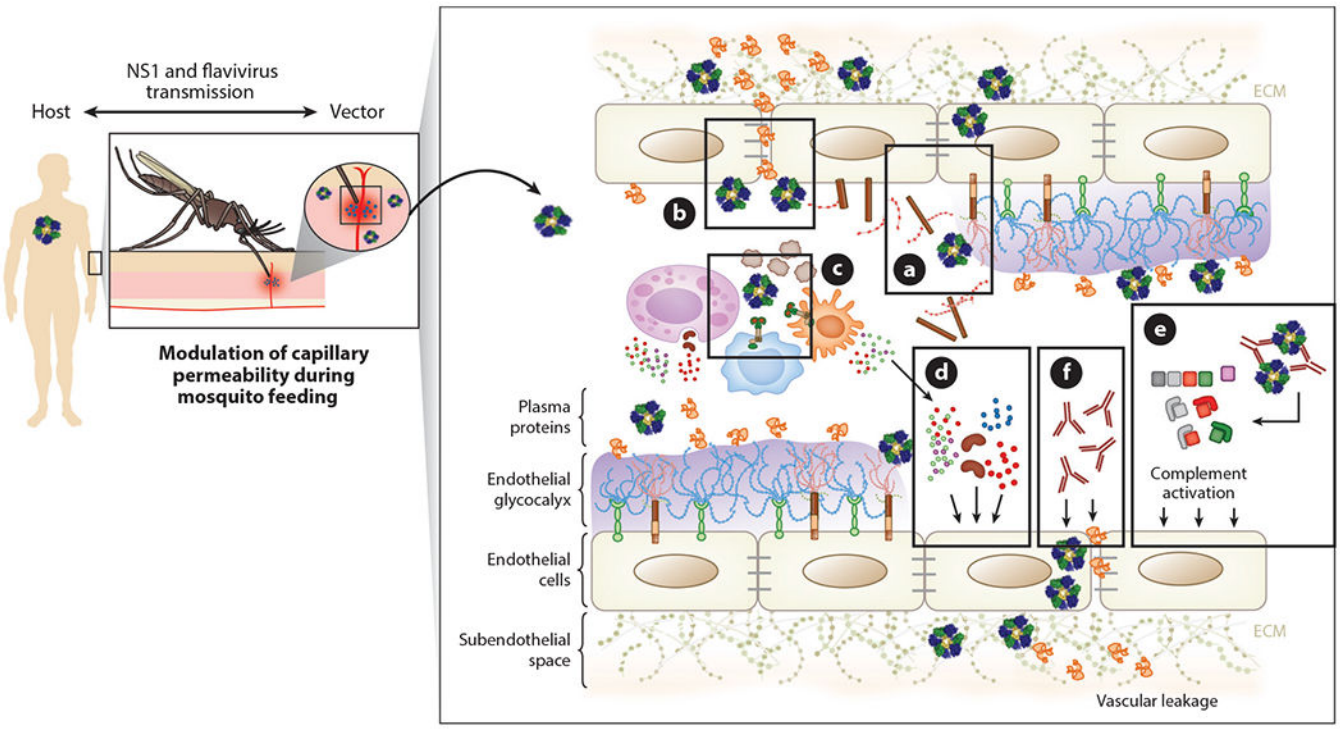
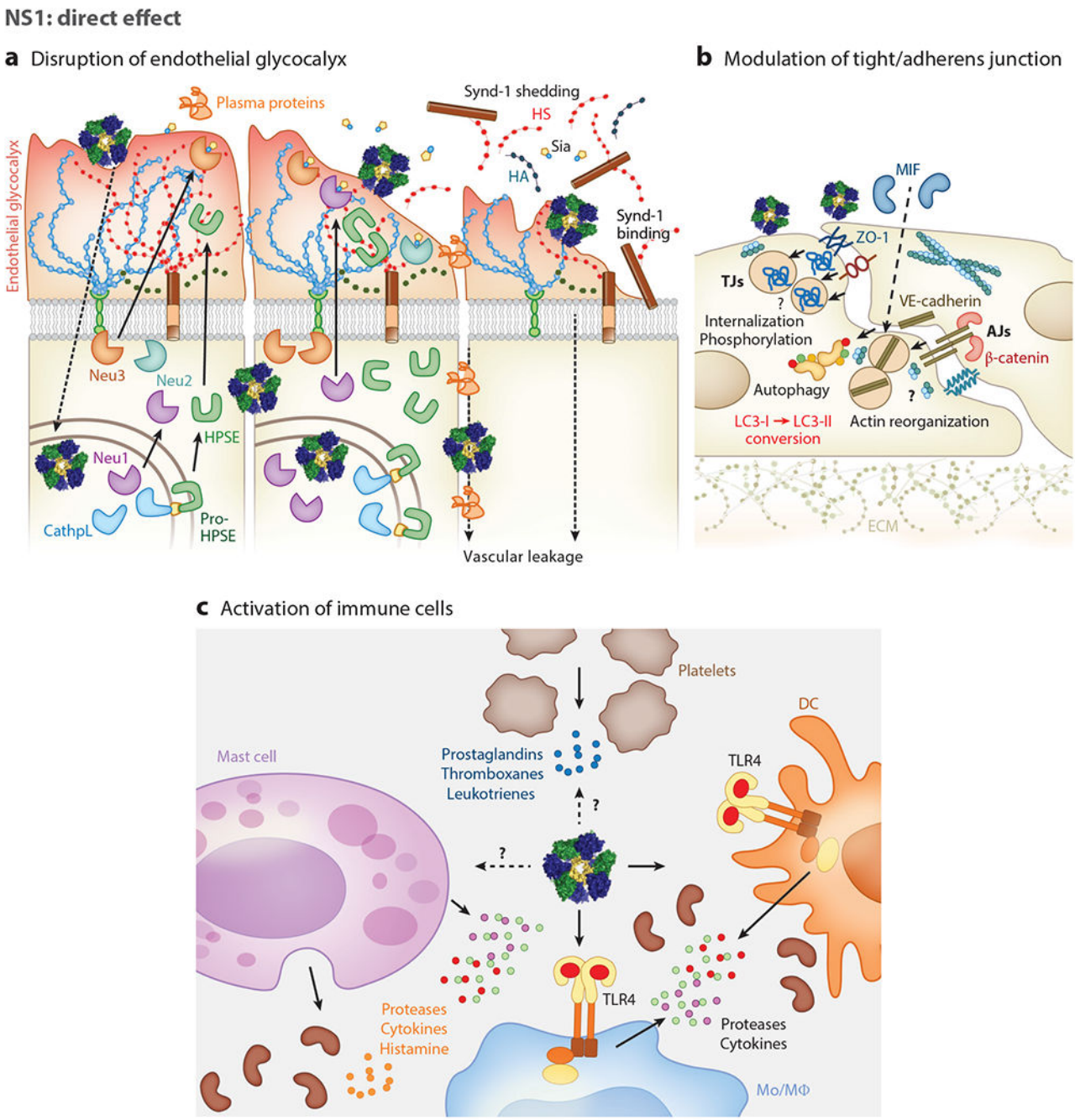


Figure 1.

Model of the three-dimensional organization of the DENV NS1 hexamer and dimer. Three NS1 dimers bind together to form a barrel-shaped hexamer structure. Each dimer contains three domains: β-roll (*red*), wing (*yellow*), and β-ladder (*blue*). The lipid cargo-containing hydrophobic core (*orange*) inside the hexamer is also shown.





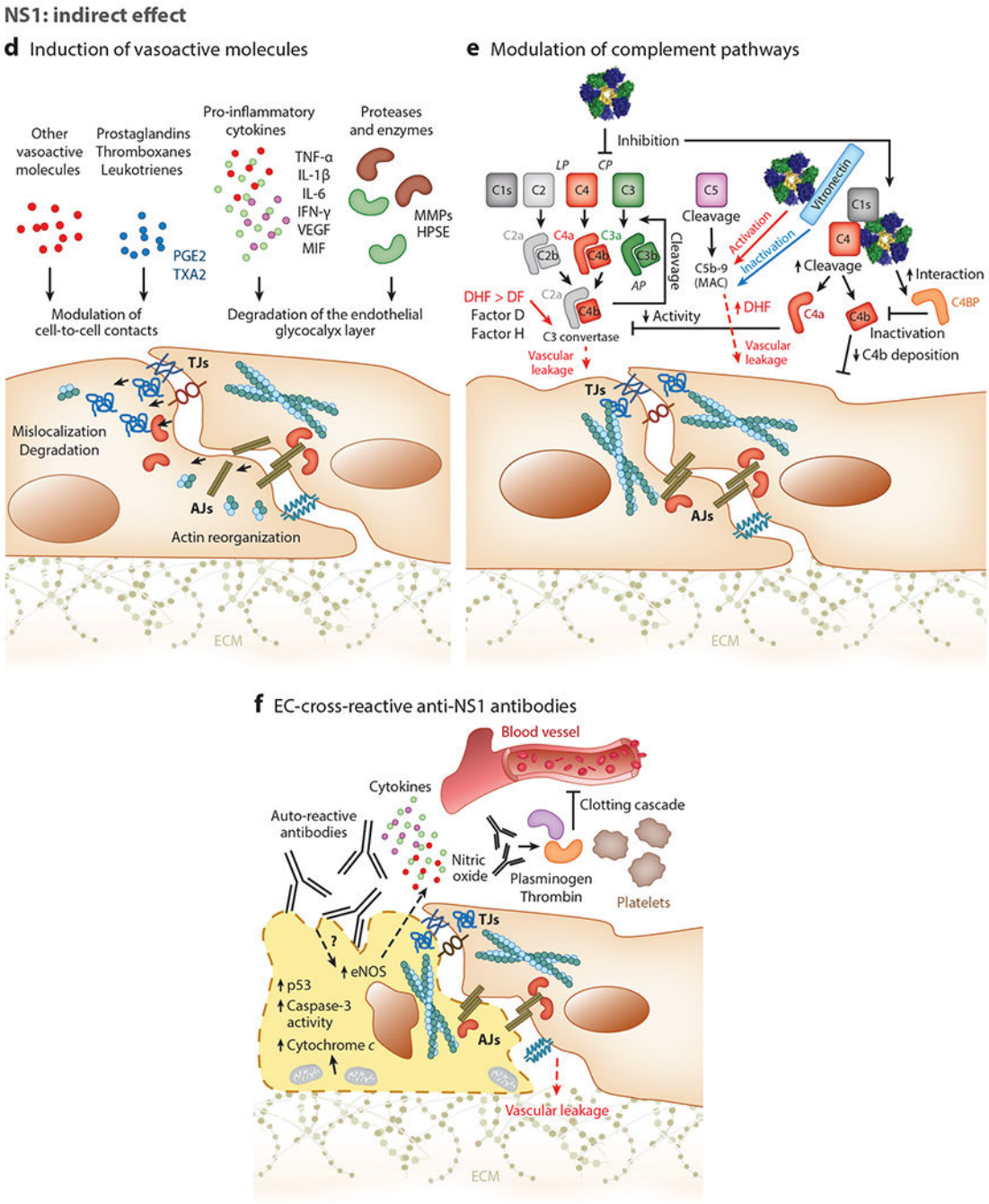


Figure 2.

Mechanisms of DENV NS1 pathogenesis leading to disease during DENV infection. DENV NS1 mediates pathogenesis through multiple pathways in the host. Secreted DENV NS1 circulating in the blood of infected humans influences virus transmission from human to mosquito by helping DENV to overcome the midgut barrier in the vector, and NS1 in the saliva of infected mosquitoes may modulate capillary permeability in the dermis of the host and impact viral dissemination in humans, (*a*) Direct DENV NS1 interaction with endothelial cells leads to increased expression and/or activation of cathepsin L; heparanase;

and the endothelial sialidases Neu1, Neu2, and Neu3, leading to disruption and shedding of key glycocalyx components (HS, Synd-1, Sia). (*b*) NS1 also modulates intercellular junction proteins, resulting in endothelial hyperpermeability. (*c,d*) NS1 can directly activate TLR4-expressing immune cells to trigger the secretion of proinflammatory cytokines that cause endothelial dysfunction. NS1 may also stimulate secretion of other soluble molecules with vasoactive and proteolytic activities that can affect endothelial barrier integrity, (*e*) NS1 contributes to immune evasion via interaction with components of the complement pathway, leading to their activation (e.g., C3 convertase; Factor D, Factor H, C5b-9) or degradation (e.g., C4b, C5b-9). Hence, NS1 protects DENV from complement-mediated clearance and DENV-infected cells from complement-mediated lysis, leading to more viral replication and potentially contributing to endothelial injury resulting in vascular leakage. (*f*) Cross-reactive anti-NS1 antibodies may also contribute to DENV pathogenesis by binding to platelets and components of the clotting cascade (e.g., plasminogen, thrombin) and may recognize autoreactive epitopes expressed on the surface of endothelial cells, potentially leading to endothelial cell damage via apoptosis. Mosquito graphic modified with permission from Chisenhall et al. (182). Abbreviations: AJ, adherens junctions; AP, alternative pathway; CathpL, cathepsin L; CP, classical pathway; DC, dendritic cell; DENV, dengue virus; DHF, dengue hemorrhagic fever; DF, dengue fever; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; HPSE, heparanase; HS, heparan sulfate; IFN, interferon; IL, interleukin; LC3, light chain 3; LP, lectin pathway; MAC, membrane attack complex; MIF, migration inhibitory factor; MMP, matrix metalloproteinases; Mo, monocyte; MΦ, macrophage; Neu, neuraminidase; NS1, nonstructural protein 1; PGE, prostaglandin; Sia, sialic acid; Synd-1, syndecan-1; TJ, tight junction; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TXA, thromboxane; VE, vascular endothelial; VEGF, vascular endothelial growth factor; ZO, zonula occludens.

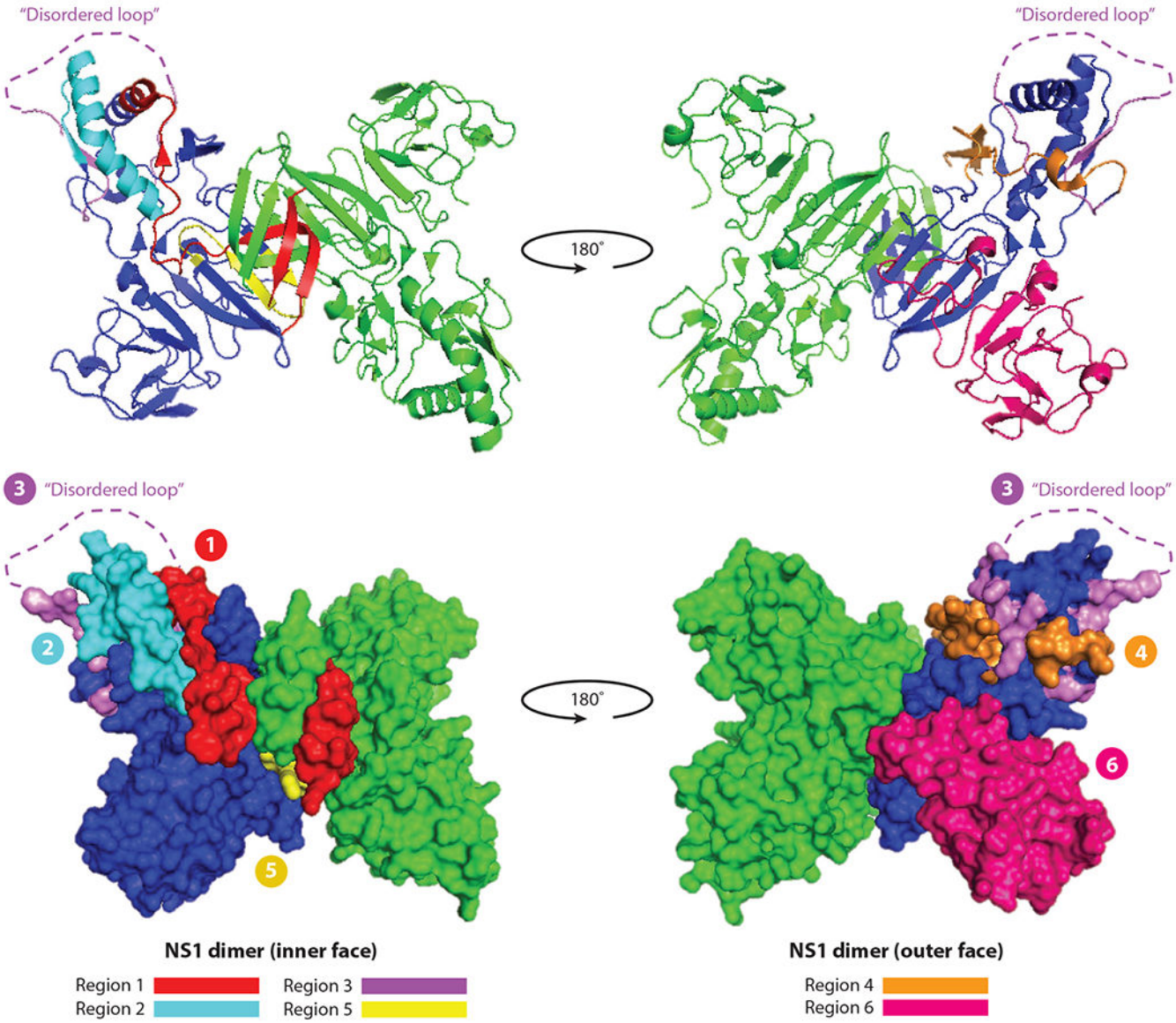


Figure 3.

DENV NS1 regions targeted by antibodies, showing a molecular representation of six regions that are targets of anti-NS1 antibodies on the NS1 dimer. Three-dimensional crystal structure of DENV NS1 dimers represented as ribbon (*top*) or solid surface (*bottom*) diagrams, indicating the six defined regions of the NS1 antibody response. Each region is highlighted in different numbers and colors as indicated in panel *a*, located in either the inner (*left*) or outer (*right*) face of the NS1 dimer. Analyses of the structural factors and coordinates were performed using the DENV NS1 protein crystal structure deposited in the Protein Data Bank (PDB4O6B) (50). Molecular graphics were performed using the PyMOL Molecular Graphics system v1.3r1. Abbreviations: DENV, dengue virus; NS1, nonstructural protein 1.

Table 1

Dengue NS1 antibody epitopes

Amino acid	Region/domain	Antibodies tested and putative function	Serotype reactivity	Reference(s)
1–15	Region 1 [β-roll wing (connector subdomain)]	Human PAb	DENV1, -2, -3, -4	177
1–15		Mouse MAb	DENV1	148
1–40		Mouse MAb	DENV1, -2, -3	178
21–35		Mouse MAb	DENV1, -2, -3, -4	148
21–40		Mouse PAb	DENV1, -3	128
25–33		Mouse MAb cross reacts with human endothelial cells	DENV2	147, 151
36–45		Rabbit poly	DENV2	179
61–69	Region 2 (wing)	Mouse MAb	DENV2, -4	147
71–85		Mouse MAb	DENV1	148
80–89		Rabbit PAb	DENV2	179
101–128	Region 3 (wing)	Mouse/human PAb	DENV1, -2, -3	128
103–112		Rabbit poly	DENV2	179
110–122		Mouse MAb binds human endothelial cells LYRIC protein	DENV1, -2, -3, -4	180
111–121		Mouse MAb cross reacts with human endothelial cells	DENV1, -2, -3, -4	147
111–125		Mouse MAb	DENV1, -2, -3, -4	148
112–120		Mouse MAb and NS1 peptide protect in vivo	DENV1, -2, -3, -4	132
121–130		Rabbit poly	DENV2	179
141–168	Region 4 (wing)	Mouse MAb	DENV1, -2, -3, -4	178
156–176		Mouse PAb	DENV2	128
187–196	Region 5 (β-ladder)	Rabbit PAb	DENV2	179
191–205		Mouse MAb	DENV1, -2, -3, -4,	148
190–200		Mouse MAb protective in vivo	DENV1, -2, -3, -4	121
231–250	Region 6 (β-ladder)	Mouse PAb	DENV3	128
261–275		Mouse MAb	DENV1, -2, -3, -4	148
267–312		Mouse MAb	DENV2, -3, -4	178
276–296		Mouse MAb	DENV1, -2, -3, -4	181
291–305		Mouse MAb	DENV1, -2, -3, -4	148
295–304		Rabbit PAb	DENV2	179
296–330		Mouse/human PAb	DENV1, -2, -3	128
299–309		Mouse MAb cross reacts with human clotting factors	DENV2, -4	147, 151
311–330		Human PAb binds HMEC-1 cells	DENV2	163
315–324		Rabbit PAb	DENV2	179
338–352		Mouse MAb	DENV1	148

DENV, dengue virus; HMEC-1, human microvascular endothelial cell; MAb, monoclonal antibody; NS1, nonstructural protein 1; PAb, polyclonal antibody.