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Review

Molecular Insight into Dengue Virus Pathogenesis and Its Implications for Disease Control

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Dengue virus (DENV) is a mosquito-transmitted RNA virus that infects an estimated 390 million humans each year. Here, we review recent advances in our understanding of the biology of DENV and describe knowledge gaps that have impacted the development of effective vaccines and therapeutics.

DENV Epidemiology and Clinical Disease

Dengue virus (DENV) is a member of the Flavivirus genus of single-stranded positive-sense RNA viruses that cause visceral and central nervous system disease in humans. DENV cycles in nature between its two principal mosquito vectors (*Aedes albopictus* or *Aedes aegypti)* and humans. Four DENV serotypes (1, 2, 3, and 4) circulate in tropical and sub-tropical regions of the globe, each capable of causing severe disease. DENV serotypes differ from one another by 25%–40% at the amino acid level and are separated further into genotypes that vary by up to ~3%. Over the past few decades, the number of people infected with DENV has risen steadily due to expansion of urban populations, global travel and commerce, and a paucity of mosquito control programs. It is now the leading cause of arthropod-borne viral disease in the world. DENV infects humans in more than 100 countries each year, with roughly 3.6 billion people at risk (Bhatt et al., 2013). DENV epidemics occur annually in the Americas, Asia, Africa, and Australia and affect travelers from endemic regions. Beyond their effects on public health, these epidemics have a massive economic impact in affected countries: the annual economic costs of DENV to the countries of Southeast Asia alone are estimated at 1 billion US dollars (Shepard et al., 2013).

DENV infections result in either inapparent disease (up to 75% of infections) or a spectrum of clinical illnesses ranging from self-limited dengue fever (DF) to severe dengue, a potentially lethal hemorrhagic and capillary leak syndrome previously termed dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF presents with an abrupt onset febrile illness characterized by headache, severe muscle and joint pain, and rash that typically lasts for 7–14 days. Severe dengue is characterized by the rapid onset of capillary leakage and is accompanied by significant thrombocytopenia and mild-to-moderate liver injury (Halstead, 2007). Hemorrhagic manifestations include bleeding in the skin and gastrointestinal tract. Rapid fluid loss into tissue spaces causes the hemoconcentration and hypotension that can result in mortality.

DENV Pathogenesis

Although primary infection confers durable if not life-long protection against re-infection by a homologous DENV serotype, secondary infection by viruses of a heterologous DENV serotype occurs frequently in endemic areas and is the single most important risk factor for severe disease (Halstead, 2007). Even during secondary infection, severe dengue is relatively rare, with only 0.5%–1% of infections progressing to DHF and DSS. In contrast, in infants born to dengue-immune mothers, primary infection may cause severe disease (termed "infant DHF"). This age-dependent epidemiology is attributed to the unique pathogenesis and immune enhancement associated with DENV.

The mechanism by which the immune response to DENV protects against or contributes to severe disease remains controversial. Antibodies neutralize infection when bound to virus particles in sufficiently large numbers. In contrast, at concentrations that fall below the threshold for neutralization, antibodies can promote entry of DENV into cells expressing Fcγ receptors (FcγR) via a process called "antibody-dependent enhancement" (ADE) (Halstead, 2007). Thus, low-affinity or poorly neutralizing cross-reactive antibodies against DENV structural proteins that were generated during primary infection can facilitate ADE in vivo during secondary infection, resulting in increased viral burden and more severe disease (Halstead, 2007). Indeed, passive administration of DENV-reactive monoclonal or polyclonal antibodies increases viral burden in interferon (IFN) receptor-deficient mice (Zellweger et al., 2010) and also in non-human primates (Goncalvez et al., 2007). In mice, transfer of enhancing concentrations of antibody promotes "cytokine storm" and vascular leakage; this disease phenotype requires interactions of the Fc region of antibody with FcγR (Balsitis et al., 2010). In infant DHF in humans, it is thought that DENV-specific maternal IgG antibodies transferred across the placenta wane to levels that facilitate enhancement of a newly acquired primary DENV infection. Thus, the quantity and quality of the cross-reactive antibody response that either is pre-existing in serum or rapidly induced by the memory B cell pool is believed to influence the severity of DENV infection.

Apart from ADE, the ligation of FcγR receptors on myeloid or mast cells by DENV immune complexes may modulate host immunity and disease pathogenesis by increasing IL-10 production, skewing $CD4^+$ T cell responses, or promoting degranulation of vasoactive molecules that enhance capillary leakage

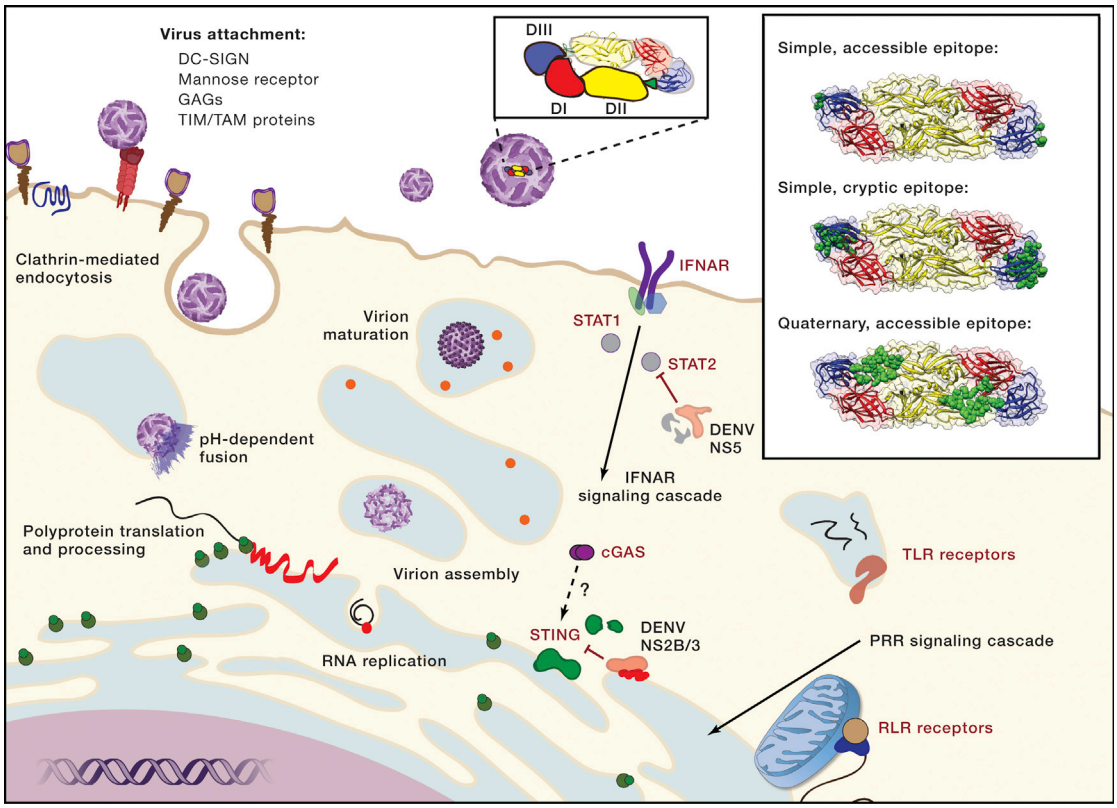

Figure 1. Cell Biology of DENV Infection

DENV interacts with target cells via one or more host factors that enhance attachment. DENV subsequently is internalized by endocytosis and fuses with membranes of the late endosome in a pH-dependent manner. Virus binding and membrane fusion are orchestrated by the envelope (E) protein, which is composed of three domains (top center, DI, shown in red; DII, shown in yellow; and DIII, shown in blue). Viral RNA translation and replication occur in association with membranes of the endoplasmic reticulum (ER). Newly synthesized viral RNA is packaged into immature virions, which bud into the ER lumen. During egress, the virion undergoes a maturation step defined by cleavage of the prM protein by furin proteases (orange spheres). DENV infection is detected by host pattern recognition receptors that selectively bind viral RNA molecules, including TLRs and RIG-I-like receptors (RLRs). Additionally, DENV infection activates the DNA sensor cGAS and STING pathway via unknown mechanisms. These cell-intrinsic host defense systems stimulate the production of IFNs to promote expression of antiviral ISGs. DENV has evolved mechanisms, including the NS2B/3-mediated cleavage of STING and NS5-mediated degradation of STAT2, to antagonize antiviral pathways. (Inset) An increasingly detailed understanding of the structural basis of antibody recognition has emerged. Antibodies have been characterized that bind epitopes (in green) on the virion comprised of a single or multiple E proteins. For example, the DIII lateral ridge epitope is contained within a single E protein (top), whereas the E-dimer-dependent quaternary epitope is composed of residues of both E proteins in the anti-parallel dimer (bottom). In contrast, other mAbs bind epitopes not predicted to be accessible using existing models of virion structure (middle).

(Halstead et al., 2010; Syenina et al., 2015). Although cross-reactive, low-affinity T cells that produce high levels of vasoactive cytokines and do not clear DENV-infected cells historically have been implicated in disease pathogenesis during secondary infection (Mongkolsapaya et al., 2003), recent studies suggest that cross-reactive $CD8^+$ T cells may protect against severe DENV infection (Weiskopf et al., 2013).

DENV Biology

DENV is a spherical 50 nm virion comprised of three structural proteins (capsid [C], pre-membrane/membrane [prM/M], and envelope [E]), a lipid envelope, and a 10.7 kilobase capped RNA. The E protein is a class II viral membrane fusion protein that has an elongated three-domain (domains [D] I–III) structure; it directs several critical steps of the viral replication cycle, including engagement with cellular attachment and entry factors, membrane fusion, and virion assembly (Figure 1). DENV binds to target cells via a structurally diverse group of attachment factors, including glycosaminoglycans (heparan sulfate), C-type lectins (DC-SIGN [CD209] and the mannose receptor [CD206]), and immunomodulatory proteins (TIM/TAM receptors). As a definitive receptor for virus entry has not been identified, the molecular basis of DENV tropism remains uncertain. Targets for DENV infection in vivo include monocytes, macrophages, dendritic cells, mast cells, and possibly hepatocytes and endothelial cells.

Internalization of bound virions is mediated primarily by clathrin and is followed by the trafficking of virus particles to the acidified late endosome, where structural rearrangements of the E protein promote fusion of viral and host membranes. After nucleocapsid penetration into the cytoplasm, the viral genomic RNA is translated into a single polyprotein that is subsequently cleaved into three structural and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by viral (NS3) and as yet undetermined host proteases. Negative-strand viral RNA is synthesized and directs positive-strand RNA synthesis in association with a virus-induced network of membranes.

Positive-strand RNA is packaged in progeny virions, which bud into the ER to form enveloped immature virions. The conformation and organization of E on the virion change considerably during virion assembly and release (Figure 1). The E proteins of newly synthesized immature virions exist as dimers with prM and are incorporated into the virion as heterotrimeric spikes that project off its surface. Virion maturation occurs through the actions of furin proteases in the *trans*-Golgi network during viral egress, and virion secretion into the extracellular space occurs by exocytosis. On mature virions, E proteins lie relatively flat against the virus membrane in a pseudo-icosahedral pattern, resulting in a relatively smooth appearance (Lindenbach et al., 2013).

DENV Structure(s): A Moving Target for Antibody Neutralization

The first cryoelectron microscopy reconstructions of DENV identified relatively smooth spherical virus particles on which anti-parallel E protein dimers were arranged in a dense herringbone pattern (Kuhn et al., 2002). Our understanding of flavivirus structure has since expanded and is complicated by newly revealed physical properties of the virus particle. (1) Virion maturity. While cleavage of the structural protein prM on newly synthesized immature virions is required for infectivity, many virus particles are processed inefficiently during egress from infected cells. These partially mature virions may still be infectious, although the precise stoichiometry of prM cleavage required for infectivity is unknown. Uncleaved prM impacts the functional properties and antigenic features of the virion (reviewed in Pierson and Diamond, 2012). Indeed, monoclonal and polyclonal antibodies differentially neutralize DENV infection as a function of the prM content of virions. Uncleaved prM is commonly recognized by poorly neutralizing antibodies present in human sera that readily support ADE in vitro, raising the possibility that anti-prM antibodies contribute to pathogenesis (Dejnirattisai et al., 2010); (2) Structural heterogeneity. Exposure of DENV-2 (but not DENV-1 or -4) to normal human body temperature results in an increase in the structural heterogeneity of virus populations. Analysis of the average structural state of a subset of these virions revealed that incubation at 37°C triggered an expanded "bumpy" conformation of the virion on which E protein dimers are rotated to expose a portion of the viral membrane. This arrangement may expose unique epitopes and influence how DENV virions engage target cells in a manner distinct from the herringbone model derived from viruses produced at 30°C in insect cells (reviewed in Rey, 2013); (3) Viral "breathing." Flaviviruses exist in an ensemble of structures at equilibrium due to the conformational flexibility of their E proteins (reviewed in Kuhn et al., 2015). Although not fully understood, changes in the conformation or configuration of E proteins on the virion can modulate epitope accessibility as the virion samples different structural states. In support of this concept, reversible time- and temperature-dependent patterns of neutralization have been observed with anti-DENV antibodies.

Neutralizing antibodies are a critical component of protective immunity against DENV and therefore must bind efficiently to this dynamic and heterogeneous population of infectious virions. The pseudo-icosahedral symmetry arrangement of E proteins on the virion creates three distinct chemical environments with the potential to impact antibody engagement (Figure 1); thus, not all E proteins on the virion are equivalent with respect to epitope exposure and accessibility. Beyond this, many mouse and human mAbs bind epitopes predicted to be inaccessible in any symmetry environment using static models of virion structure. Antibodies that bind these "cryptic" epitopes often have limited neutralization capacity and are sensitive to changes in virion structure resulting from inefficient virion maturation or conditions that promote viral breathing (reviewed in Kuhn et al., 2015; Pierson and Diamond, 2012).

Recent studies with human mAbs against DENV derived from patients have yielded new insights into the varied ways in which antibodies bind to and neutralize the virion. Many inhibitory mAbs do not bind soluble monomeric forms of the E proteins but instead recognize complex conformational epitopes present on E proteins arrayed exclusively on the intact virion or epitopes that bridge more than one E protein monomer. Indeed, several modes of virion recognition by mAbs have been identified. For example, human mAbs have been characterized that bind an epitope unique to the E dimer interface (Rouvinski et al., 2015), epitopes composed of residues in the flexible DI-II hinge of one E protein and DIII of the adjacent E protein molecule (de Alwis et al., 2012), and residues found on three adjacent E proteins (Fibriansah et al., 2015) (Figure 1). The recognition of quaternary epitopes may confer unique mechanisms for neutralization that involve blocking of key structural changes required for entry or membrane fusion. Of interest, these mAbs neutralize the entire population of DENV released from cells even though incomplete virion maturation and viral breathing processes should reduce the number of intact epitopes on the virion. One explanatory hypothesis is that antibodies recognizing quaternary epitopes shift the distribution of DENV structures at equilibrium to those with the herringbone pattern associated with mature virions, even when prM is present. Antibodies against a recently characterized E-dimer-dependent epitope, which overlaps the footprint of prM on the E protein, have been suggested to bind partially mature virions by displacing prM from E protein trimers and trapping E as anti-parallel dimers (Rouvinski et al., 2015). A more detailed understanding of how antibodies bind a heterogeneous and dynamic population of DENV virions may allow for the development of immunogens that elicit predictably potent neutralizing antibodies to complex or infrequently displayed epitopes.

Innate Immune Evasion by DENV

The type I IFN system inhibits virus infections by establishing a non-permissive state in infected and uninfected cells. Type I IFN production is triggered by DENV infection after Toll-like (TLR) and RIG-I-like (RLR) receptors detect viral RNA and initiate a signaling cascade through adaptor molecules (e.g., MyD88, MAVS, or STING). Type I IFN binding to cells induces a JAK-STAT-dependent signaling cascade that culminates in expression of hundreds of IFN-stimulated genes (ISGs), some of which block specific steps in the DENV lifecycle (Schoggins et al., 2012). Nevertheless, DENV antagonizes type I IFN production and signaling in key myeloid cell targets. In addition to modulating DENV replication, antagonism of type I IFN signaling in antigen-presenting cells can attenuate or skew the adaptive immune response.

DENV inhibits type I IFN production through its NS2B-NS3 protease complex, which targets the adaptor molecule STING for degradation (Figure 1) (Aguirre et al., 2012). STING normally promotes phosphorylation of the transcription factor IRF-3, which activates the IFN-β promoter. As DENV NS2B-NS3 cleaves human, but not mouse, STING, this may contribute to the higher levels of DENV infection in mice lacking type I IFN signaling compared to immunocompetent mice. DENV also inhibits type I IFN signaling, which directly reduces expression of inhibitory ISGs. While the exact mechanism for DENV protein antagonism of IFN signaling remains uncertain, NS4B expression prevents STAT1 phosphorylation (Muñoz-Jordán et al., 2005), possibly by inhibiting phosphorylation of Tyk2, a molecule upstream of STAT1 activation. Expression of DENV NS5 is linked to a loss of STAT2 expression, which also is required for type I IFN signaling. STAT2 degradation by DENV is host specific, as NS5 promotes degradation of human, but not mouse, STAT2 (Ashour et al., 2010). Accordingly, DENV is more sensitive to the antiviral action of IFN in mouse compared to human cells.

DENV Vaccines

A requirement to elicit protection against four different viruses is a significant challenge, as incomplete vaccine-induced immunity against any single serotype theoretically could predispose an individual to severe disease during subsequent natural infection. Numerous strategies to create DENV vaccines have been explored, ranging from the attenuation of DENV through serial passage to those exploiting an increasingly sophisticated understanding of the molecular and cell biology of flaviviruses. Three live-attenuated tetravalent DENV vaccine candidates are currently being evaluated in large clinical trials. The first to complete phase III study is the Sanofi Pasteur CYD-TDV candidate, which contains four chimeric viruses in which the structural genes of the yellow fever vaccine virus (YFV-17D) were replaced with those of each DENV serotype. This vaccine showed moderate efficacy in the field, as its protective effects varied considerably among different DENV serotypes (35%–78%) (Villar et al., 2015 and references within). Unexpectedly, the vaccine benefited only those with prior DENV exposure, suggesting that its primary utility is in boosting natural cross-protective immunity. Moreover, the mean neutralization titer of serum samples from vaccine recipients did not correlate with protection in the clinical trials. Given the complexity of the findings, discussions have been renewed as to appropriate immune correlates of DENV protection. For example, because the CYD-TDV vaccine is composed largely of sequence derived from YFV-17D, its lack of T cell epitopes present in the non-structural proteins could limit induction of an optimally protective T cell response. Also of potential concern, the CYD-TDV vaccine lacks DENV NS1, an immunogenic viral protein secreted at high levels in infected individuals that has been hypothesized to contribute directly to disease pathogenesis (Muller and Young, 2013).

Two additional vaccine candidates have advanced to phase II trials. DENVax is a live-attenuated tetravalent vaccine candidate being developed by Takeda Pharmaceuticals that is composed of an infectious clone-derived attenuated DENV-2 strain and three chimeric viruses that incorporate the structural genes of the other three serotypes. A recent clinical study revealed that administration of two doses of this vaccine candidate elicited a tetravalent neutralizing antibody response in 44%–80% of recipients depending on the route of immunization and dose (George et al., 2015). The National Institute of Allergy and Infectious Diseases (NIAID) vaccine candidate (TV005) is composed of a mixture of modified full-length and chimeric DENV strains. Two recent clinical studies revealed that a single dose of TV005 was sufficient to elicit a neutralizing antibody response against all four DENV serotypes in 90% of recipients (Kirkpatrick et al., 2015).

Treatment and Control of DENV Infection

Beyond vaccine development, other strategies have been pursued to control DENV infections. A renewed effort has been made in evaluating inhibitors of specific steps in the DENV lifecycle. High-throughput antiviral drug discovery screens have been performed to identify inhibitors of the fusogenic viral envelope protein (E), the protease and helicase proteins (NS3), integral membrane proteins required for replication (NS2A and NS4B), and the RNA-dependent RNA polymerase and methyltransferase (NS5), with further pre-clinical development ongoing (Whitehorn et al., 2014). Nonetheless, because drugs against viral proteins could select for resistant variants, the concept of targeting host molecules required for DENV infectivity has emerged as an alternative strategy (reviewed in Krishnan and Garcia-Blanco, 2014). Drugs that target steps in DENV pathogenesis in vivo or cell-intrinsic immunity rather than infectivity also are under consideration; this class includes modulators of autophagy, platelet activation, and mast cell degranulation.

Exciting progress also has been made on reducing DENV transmission by limiting infection in the mosquito host. For example, the *trans*-infection of *Aedes aegypti* mosquitoes with the endosymbiotic bacteria *Wolbachia* resulted in invasion of mosquito populations and interference with DENV replication (Moreira et al., 2009). Modeling studies suggest that the establishment of *Aedes aegypti* strains with *Wolbachia* infection in a DENV-endemic setting could abolish DENV transmission (Ferguson et al., 2015). *Wolbachia*-infected *Aedes aegypti* mosquitoes have been released in Australia where outbreaks of DF occur and have been stable over several years (Hoffmann et al., 2014). Other groups have created genetically engineered *Aedes aegypti* mosquitoes that inherently are resistant to DENV infection through the induction of antiviral RNA interference (Franz et al., 2014).

Concluding Remarks

The unique complexity of DENV pathogenesis and its relationship to immune enhancement has delayed the generation of a safe and effective DENV vaccine, although licensing of live-attenuated tetravalent vaccines may come soon. Despite remaining fundamental knowledge gaps, recent advances in DENV biology in the areas of host-pathogen-vector interactions, commensal biology, genetic variability, structural biology, and immunobiology have fostered new therapeutic and ecologic strategies for control of DENV disease burden. Complementary approaches to control infection and dissemination are needed given the social, political, and economic impact of the relentless expansion of DENV disease worldwide. Hopefully, implementation of such measures will occur in the near future.

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