Chapter

# Dengue Immunopathogenesis: A Crosstalk between Host and Viral Factors Leading to Disease: PART II - DENV Infection, Adaptive Immune Responses, and NS1 Pathogenesis

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## Abstract

Severe disease is associated with serial infection with DENV of different serotypes. Thus, primary DENV infections normally cause asymptomatic infections, and secondary heterotypic infections with a new DENV serotype potentially increase the risks of developing severe disease. Despite many proposed hypotheses trying to explain it, the exact immunological mechanism leading to severe dengue disease is unknown. In turn, severe manifestations are believed to be a consequence of the combinations of many immunopathogenic mechanisms involving viral and host factors leading to increased pathogenesis and disease. Of these mechanisms, the adaptive immune response has been proposed to play a critical role in the development of severe dengue manifestations. This includes the effect of non-neutralizing but enhancing antibodies produced during primary infections, which results in enhanced-DENV infection of Fc-γ-receptor-expressing cells (e.g. monocytes and macrophages) during DENV heterotypic exposure in a phenomenon called *antibody-dependent enhancement* (ADE); the increased activation of memory T cells during secondary infections, which has low affinity for the current infecting serotype and high affinity for a past infection with a different serotype known as *the original antigenic sin*; the unbalanced production of pro-inflammatory cytokines that have a direct effect on vascular endothelial cells resulting in plasma leak in a phenomenon known as *cytokine storm*; and the excessive activation of the *complement system* that causes exacerbated inflammatory responses, increasing disease severity. In addition to the adaptive immune responses, a secreted viral factor known as the *nonstructural protein 1* (NS1) has been recently proposed as the missing corner piece of the DENV pathogenesis influencing disease. This *Part II* of the chapter will discuss the interplay between the distinct host adaptive immune responses and viral factors that together contribute to the development of DENV pathogenesis and severe disease.

**Keywords:** dengue, immunopathogenesis, dengue shock syndrome, severe dengue, adaptive immune response, antibody response, ADE, *cytokine storm*, T cells, complement system, *viral toxin*, NS1, endothelial dysfunction, vascular leak

## 1. Dengue immunopathogenesis and severe disease: host and viral factors

As discussed in *Part I* of this chapter, severe dengue is mainly characterized by the altered endothelial function in blood vessels and the disruption of the coagulation cascade that results in hypotension, shock, and severe hemorrhage manifestations [1, 2]. As the epidemiology of dengue indicates that appearance of severe manifestations occurs when the peak of viremia has passed, the key biological mechanisms leading to the pathogenesis of clinical complications during DENV infection, are believed to involve the activity of short-lived biological mediators closely linked to host innate and adaptive immune responses [3–6].

This *Part II* of the dengue immunopathogenesis section will address the multifactorial immunopathogenic process of DENV infection from the perspective of the pre-existing serotype cross-reactive antibodies, the hyperactivation of DENV-infected immune cells (e.g. monocytes, mast cells) leading to increased cytokine production, the role of T cell responses, the activation of complement pathways, and the new pathogenic roles of the secreted NS1 of DENV that may act together to occasionally cause severe dengue manifestations followed by shock and potentially death [1, 6–21].

### 1.1 Antibody response to DENV infection

The DENV complex refers to a group of four evolutionarily distinct, but antigenically and genetically related DENV serotypes (DENV-1 to DENV-4) [22]. During dengue disease, the humoral immune response is vital for controlling DENV virus infection and for the development of acquired immunity [10]. Neutralizing antibodies against the four serotypes are critical components of the protective immune response [23]. In this sense, during primary DENV infection, it is expected that a neutralizing type-specific antibody response should provide long-term protection against the primary DENV infecting serotype, but only transient protection against other DENV serotypes (cross-reactive antibodies) (See Figure 1 *Part I*). Infection with a primary serotype is thought to induce lifelong immunity that protects against re-infection with the same serotype (homotypic) [7, 23]. However, homotypic DENV infections have been found in symptomatic dengue cases in a community-based prospective cohort study suggesting that recurrent DENV infections, particularly in endemic areas can occurs in patients over time [24]. After subsequent infection with a different serotype (secondary infection), the neutralizing antibody response becomes broadly neutralizing and is thought to reduce the incidence of severe disease [25] (See Figure 1C, *Part I*). In fact, individuals with higher cross-reactive neutralizing antibody titers originated from pre-infection correlates with reduced likelihood of symptomatic secondary infection [23]. However, numerous studies worldwide involving human infections during dengue epidemics (e.g. hospitalized cases) or multiple epidemiological studies from prospective cohorts strongly support the heterotypic secondary DENV infection, defined as two or more sequential infections by different serotypes, as the epidemiologic greatest risk factor for developing severe dengue disease [17, 26–29].

Antibody responses during DENV infection are mainly directed against the envelope protein (E), the major structural protein of the virion, and the dominant

antigenic target for DENV neutralizing antibodies during natural infection, thus the focus of vaccine candidates design [30–32]. Antibodies specific for DENV proteins can mediate a wide range of functions *in vitro* [10]. They can neutralize DENV infection by direct hindering of virus-receptor interactions, blocking viral fusion with the endosomal membrane within host cells, viral clearance in a Fc-receptor dependent manner, lysis of virus infected cells via complement activation, and antibody dependent cell cytotoxicity (ADCC) of infected cells [33] (**Figure 1**). The E glycoprotein is composed of three structural domains (DI, DII, DIII), and the most extensive characterization of B cell epitopes has been conducted against them [34]. Neutralizing antibodies against the E protein of DENV include antibodies to nearly all the epitopes [35]. However, during the natural course of infection, the serological response to the E glycoprotein is highly serotype cross-reactive and predominantly targets epitopes containing highly conserved residues, for instance, the fusion loop of the domain II [31]. In addition, high-avidity and highly neutralizing antibodies against DENV, bind to domain III (DIII) of the E glycoprotein, which is implicated in DENV binding to its cognate receptor [36, 37]. These antibodies appear to be most effective at providing protection from infection and/or disease [38–40]. However, with an ~60% amino acid divergence between the E glycoproteins of all four DENV serotypes, immunity to one serotype usually does not confer long-lasting cross-protective immunity to the other serotypes [41]. A mature DENV particle contains 180 copies of the E protein covering the surface of the virion in either dimeric or trimeric (pre-fusion) conformations [42]. Neutralization is estimated to require a minimum occupancy of ~30 epitope sites per virion [34]. This may be attributed to the dense arrangement of E glycoproteins on the virion surface which has shown to be important for antigenicity, as many potently neutralizing human antibodies against flaviviruses that target either hidden cryptic or quaternary epitopes extent through multiple E proteins [43–47].

On the other hand, the adaptive immune response during DENV infection can also generate antibodies against pre-M proteins which are highly serotype cross-reactive [48]. Despite this high cross-reactivity, anti-pre-M antibodies rarely neutralize DENV infection even at high concentrations [48]. The pre-M protein forms a heterodimer with the E protein, and it gets cleaved by host cell-expressed furin during the final stage of virion maturation before egress [42]. The cleavage of pre-M is required for the activation of flavivirus infectivity, including DENV [49]. In the mature virion, the remaining M protein fragment is completely hidden by the E protein dimers which makes it inaccessible to antibody binding [34]. Therefore, maturation state of the virion matters and may influence the interaction between immature virus particles and anti-pre-M antibodies leading to neutralization or enhancement of the infection (**Figure 1**). The potential role of anti-E or pre-M antibodies in increasing DENV infection and pathogenesis will be further discussed later in this chapter.

Antibody responses in DENV infection can be also directed against several non-structural proteins such as NS1, NS3, and NS5 as found in sera collected from DENV infected patients [50, 51]. These antibody responses have been mainly detected during secondary cases which open the possibility of implementing it in early diagnostic assays of DENV infection [51, 52]. NS3 (viral helicase/protease) and NS5 (virus RNA dependent polymerase) localize exclusively within virus-infected cells, but cell lysis owing to viral cytopathic effect or immune cell-mediated lysis may make these proteins accessible for binding to B cell receptors, inducing an antibody response [40, 52]. In this same line of interest, T cells may play an important role in the immune response against DENV nonstructural proteins. This topic will be later discussed in this chapter. On the other hand, the NS1 protein is the only flavivirus glycoprotein secreted by infected cells [53]. NS1 forms a multimeric structures either expressed on the surface of infected cells (dimer) or released as a soluble

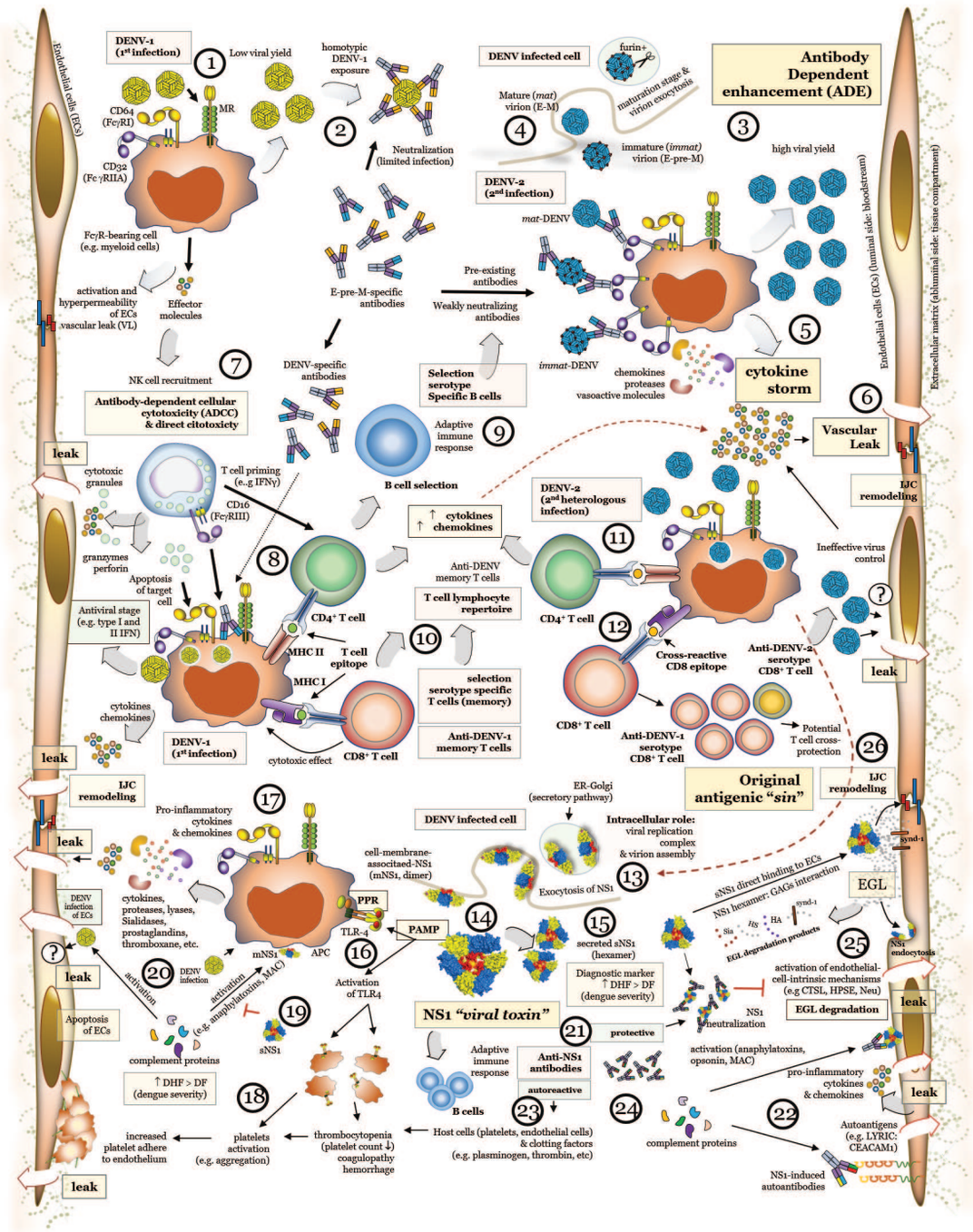


**Figure 1.**

*The supercomplex interplay of the immunopathogenic mechanisms triggered by systemic DENV infection leading to disease. The DENV complex is composed by four serotypes [1–4], and a primary infection with any of these serotypes triggers an adaptive immune response that results in the generation of neutralizing antibodies mainly directed against the infecting serotype, for instance, DENV-1 (here in yellow) [1, 2] (see also Figure 1c, Part I). When the host is re-exposed to the same DENV serotype as the one from the primary infection (homotypic infection), the neutralizing antibodies generated during the first encounter, prevent APCs to be infected [2]. However, during a sequential DENV infection with a different serotype, here DENV-2 (in blue) [3], the pre-existing antibody response (1st DENV infection) do not neutralize but increases the infection of APCs via a mechanism called antibody-dependent-enhancement (ADE). This ADE phenomenon relies on the cell surface expression of Fcγ-receptors (FcγRs) including FcγRI (CD64) and FcγRII (CD32) mainly found in monocytes/macrophages and dendritic cells [1, 3], also Fc εR found in mast cells resident of the skin (see also Figure 2, Part I). Importantly, ADE can be also modulated by the structural heterogeneity of the DENV particles (maturation state) [4]. During the DENV replication cycle, right before virus exocytosis of the infected cell, the newly assembled virus particles suffer a final processing step known as the 'maturation state' of the virion, in which the pre-M protein is cleaved by the cellular protease, furin. Inefficient furin activity leads to the release of viral particles containing a wide variety of pre-M/E protein complex known as partially mature virions [4]. Although immature and partially mature DENV viral particles are unable to infect cells via its cognate receptor, they can use the ADE mechanisms as a way to infect FcR-bearing cells which results in increased viral production (high virus yield) and increased secretion of cytokines and chemokines and other soluble components with vasoactive and pro-inflammatory activities in a process known as the 'cytokine storm'. [5] The term "cytokine storm" is referred to the*

*exacerbated and unbalanced production of cytokines and chemokines which exert many effector functions including antiviral response and inflammation. Many of these components pose some vasoactive activities which alter the homeostasis of mostly all biological barriers including the microvascular endothelium, leading to a phenomenon called vascular leak (hereafter, 'leak'), a consequence of the increased endothelial cell (EC) permeability, and a hallmark of severe dengue disease [6]. The homeostasis of the endothelial barrier is mainly maintained by two structures: a) the endothelial glycocalyx layer (EGL), a network of membrane-bound glycosaminoglycans (e.g heparan sulfate [HS], hyaluronic acid [HA], chondroitin sulfate [CS]) and proteoglycans (e.g syndecan-1, perlecan, glypicans), covering the endothelium luminally and playing critical roles in vascular physiology and pathology, including mechanotransduction, hemostasis, signaling, and blood cell-vessel wall interactions; and b) the intercellular junction complex (IJC), mainly composed of the tight (e.g. ZO-1, occluding, claudins) and adherens (e.g. VE-cadherin, beta-catenin) junction proteins that maintain the cell-to-cell contacts to control fluids and small molecules exchange between the luminal side (bloodstream) and the abluminal side (tissues compartment) of blood vessels. The disruption of any of these two main components under pathological conditions leads to increased inflammatory responses with pathogenic consequences involving barrier dysfunction and vascular disorders that result in excessive extravasation of fluids and proteins into the tissues, hypotension, shock, and sometimes, death. In addition to modulating the endothelial barrier function, cytokine and chemokines secreted by DENV infected cells pose important effector functions that promote the recruitment and activation of other immune cells such as NK cells with the ability to induce apoptosis of susceptible target cells (e.g. DENV infected cells) via NK cell-mediated cytotoxicity, and antibody (Ab)-dependent NK cell-mediated cytotoxicity (ADCC) [7]. Activation of NK cells also lead to the secretion of an array of immunoregulatory cytokines which conditioned a bidirectional crosstalk between NK cells and other immune cells such as dendritic cells (DC), macrophages, mast cells, and T cells. The release of cytotoxic granules and cytokines by NK cells may also contribute to a cytokine storm, and the tissue damage associated with infection clearance may exacerbate the pro-inflammatory environment. On the other hand, during DENV infection of immunes cells, viral clearance of DENV results from a coordinated action of multiple cell types and mechanisms including innate immune responses such as the production of type I and type II interferons, cell killing by cytotoxic lymphocytes and production of neutralizing antibodies by B cells [7, 8, 9]. Intracellular expression of newly synthesized DENV proteins (e.g. NS3, NS1, NS5 proteins) enter the MHC class I and II presentation pathways in which viral peptide epitopes are presented on the cell surface either through MHC class II molecules for CD4+ T cells, and MHC class I molecules for CD8+ T cells, which principally lyse infected cells but also produce cytokines with effector functions on the endothelial biology. Like with the antibody response, after primary DENV infection, T cell responses are mainly characterized by higher homotypic than heterotypic responses [8, 10]. However, during DENV secondary infections, this pattern breaks down as B and T cells induced by the prior exposure to a different DENV serotype rapidly turn into highly serotype cross-reactive responses mainly directed against the previously encountered DENV serotype [11]. This phenomenon results in the consistent preferential expansion of pre-existing cross-reactive heterologous memory T cells with higher avidity for the previous DENV serotype and weak-affinity for the new infecting DENV, that may lead to an inefficient in clearing DENV infection, while producing excessive cytokines, leading to the onset of immune pathology such as vascular leak which results in severe disease. This alteration in the T cell immune responses, skewed by the 'memory' of the previous infection, is referred to as 'original antigenic sin' [12]. In addition to these host factors, the non-structural protein 1 (NS1) of DENV is a multifaceted viral factor that has been also demonstrated to play many roles in DENV pathogenesis. In DENV infected cells, NS1 can be found intracellularly expressed as a monomer in the ER where it forms dimers that play critical roles in the viral replication cycle, particularly the assembly of new viral particles [13]. Secreted NS1 protein from DENV infected cells is a hexameric protein with a barrel-like shape that contains a hydrophobic lipid core (cholesterol and triglycerides), and three domains known as the "wing domain"(here in yellow), the β-ladder domain (in blue), and the β-roll domain (in red) ([14], see also Figure 1B). Plasma circulating levels of soluble NS1 (sNS1) have been described to correlate with the appearance of severe disease manifestations such as dengue hemorrhagic fever (DHF) in DENV-infected patients [15]. sNS1 from DENV has been shown to act as a potential pathogen associated-molecular pattern (PAMP) that on immune cells and platelets interacts with Toll-like receptor 4 (TLR4), a pattern-recognition-receptor (PPR) [16] resulting in the production of pro-inflammatory cytokines from human PBMCs and monocytes [17] and the activation of human platelets which results in their aggregation and increase adherent onto the ECs lining the blood vessels, which hampers the homeostasis of the EC-barrier and the microvasculature [18]. Due to its ability to trigger the production of cytokines via TLR4, the term 'viral toxin' is now used to describe this pathogenic function of sNS1 during systemic DENV infection [14]. Furthermore, sNS1 can activate and block the antiviral activity of the complement pathway, an important component of the innate immune response against many human pathogen infections [19]. NS1 can either lead to pathogenesis by increasing the deposition of active complement components on the surface of ECs and human tissues [20], or by directly interacting with the component itself blocking its function or getting rid of some of the critical components involved in complement activation cascade [19]. An additional route for NS1 to cause virus pathogenesis or protection is related to the adaptive immune response generated against the secreted NS1 protein during DENV infection [21]. First, NS1-induced autoantibodies (anti-NS1 antibodies that recognize antigens on the host components) increase the activation of ECs causing barrier dysfunction by increasing the production of proinflammatory cytokines and the action of the complement pathway or binds to autoantigens encoded in proteins of the clotting cascade such as plasminogen, thrombin, and fibrinogen inducing coagulation disorders that along with the thrombocytopenia leads to bleeding manifestations [22, 23]. Second, anti-NS1 antibodies have been reported to prevent the direct interaction of sNS1 with the surface of ECs which blocks the NS1-induced activation of endothelial-intrinsic pathways leading to the disruption of the EGL and the remodeling the cell-to-cell contacts which disrupts the integrity of the intercellular junction complex formed by tight and adherens junction proteins (TJ/AJ) [24–26]. Overall, this summary figure represents the complex crosstalk between distinct host and viral factors that together trigger a diverse cascade of effector functions leading to protection or pathogenesis, the latter sometimes associated to the development of life-threatening disease manifestations that can result in death.*

hexameric during DENV infection *in vitro* and *in vivo* (See Figure 1B, *Part I*) [12, 54–56]. Specific antibodies for NS1 proteins have been also found circulating in DENV infected patients, particularly in secondary cases and are highly serotype cross-reactive [57–59].

*In vivo* experiments using mouse models for DENV infection, the adoptive transfer of immune serum or monoclonal antibodies specific for pre-M, E or NS1 proteins prevented mortality from lethal challenge with DENV [9, 38, 60–63]. Similarly, passive transfer of anti-E antibodies can protect against infection with DENV in nonhuman primate models [64, 65]. Dengue virus-specific antibodies of the appropriate subclasses can also bind to complement proteins and promote their activation. Fixation of complement to virions by antibodies specific for the pre-M and/or E proteins can inhibit viral infection [66]. NS1-specific antibodies mediate complement-dependent lysis of infected cells; however, this may not fully explain their protective effects *in vivo* [67]. Additionally, NS1-specific antibodies may also contribute to antibody-dependent cellular cytotoxicity [67, 68]. Recently, the role of the antibody immune response against the soluble DENV NS1 has become more relevant in the development of future dengue vaccines [9, 62, 69] as NS1 was described to play a key role in the development of DENV pathogenesis. Further evidences from a candidate dengue vaccine has demonstrated the functionality of anti-NS1-specific IgG responses against NS1 pathogenesis *in vitro* [70]. The phenomenon of NS1 being directly involved in modulating DENV pathogenesis will be discussed in more detail in a different section of this chapter.

### 1.2 Antibody-dependent enhancement of DENV infection and the cytokine storm

DENV has four distinct serotypes, and infection with one serotype results in the development of homotypic immunity which has been suggested to confer a durable and possible life-long protection against the infecting DENV serotype, but only short-term cross-reactive protection against other serotypes (heterotypic immunity) [13]. This cross-serotype–reactive antibody response is thought to wane to subneutralizing levels, where antibodies still bind, but do not neutralize the infecting virion. In turn, these antibodies contribute to enhanced infection of Fc receptors (FcRs) bearing cells during heterologous DENV encounters (**Figure 1**) [71, 72]. This phenomenon called "*antibody dependent enhancement*" or ADE, potentially increases the risk of developing severe disease by virtue of increasing the number of virus infected cells and therefore the viral biomass *in vivo* accompanied by hyperactivation of infected-immune cells and increased release of vasoactive mediators that resembles some pathologic features of what occurs in patients suffering severe dengue disease including capillary permeability and vascular leak (**Figure 1**) [73–78].

Multiple prospective cohort studies in Asia and Latin America have identified secondary infection as an epidemiological risk factor for severe dengue [17, 28, 79, 80]. Classical epidemiologic and observational studies have suggested that pre-existing sub-neutralizing antibodies in closely association with immunologic markers and clinical events supports the hypothesis of ADE and the risk of severe dengue during secondary infections [16, 26, 27, 78, 81–83]. Maternally derived subneutralizing levels of DENV-reactive IgGs have been also postulated to be a critical risk factor for severe dengue during infancy [26, 27, 84–86]. Numerous studies performed in animal models have reiterated that ADE results in higher viral load in patients at specific concentration of antibodies (*a peak of enhancement*), especially at early stages of infection, thereby increasing the risk of developing DHF/DSS [14, 87]. Studies performed in rhesus monkeys confirmed that passive transfer of immune serum or monoclonal antibodies resulted in increased viremia; however, no apparent signs of

severe disease was observed, indicating that severe dengue manifestations may not only be a main consequence of increased viremia [65, 88].

Despite all these evidences, yet no conclusive evidence exist that a risk of severe dengue disease and ADE occur in humans. A recent study using samples from a well-characterized DENV cohort study in children showed that the risk of developing severe dengue disease during secondary dengue infections existed within a narrow range of preexisting anti-DENV antibody titers, *a peak of enhancement*, detected in humans [29]. Recently, a phase 3 clinical trial of the only dengue vaccine licensed, Dengvaxia [89] showed an increased risk of hospitalization for severe dengue in children not exposed to DENV before vaccination [90], raising concerns about the need to assess dengue vaccine safety at the earliest development stages prior to human vaccination, and confirming that vaccination of DENV-naïve individuals may induce poorly neutralizing anti-DENV antibodies that increase the risk of severe dengue disease [91]. All these observations indicate that ADE may occur in humans and should be an obligate consideration for future designing, implementation, and evaluation of vaccine trials, especially those in the flavivirus field.

DENV-ADE can be mediated by E protein-specific antibodies either at low antibody concentrations or low antibody avidity, when the number of antibody molecules bound per virion is below the threshold necessary for neutralization of the virus (*a peak of enhancement*) [92]. As DENV E protein binds to cellular receptors and mediates viral fusion during entry, it is thought to be the major target of neutralizing antibodies [33, 41, 93]. However, a substantial proportion of antibodies generated in response to natural DENV infection are directed toward the pre-M protein which represents an important part of the adaptive immune response in DENV infected patients [40, 94].

During DENV infection, the cleavage of pre-M protein represents a critical step for the virus maturation process [49, 95, 96]. As the cleavage of pre-M is not complete in all dengue virions, a proportion of secreted viral particles from infected cells are partially mature dengue virions that contain a varying amount of cleaved and uncleaved pre-M proteins [42, 96, 97]. Immature dengue viral particles contains regular trimeric E-pre-M protein complexes and are noninfectious [49, 98]. In contrast, some partially mature forms, containing some pre-M protein–E protein trimers, are partially infectious. In both cases, uncleaved pre-M protein on immature or partially mature virions can be targeted by the host anti-pre-M antibody response which despite being highly cross-reactive among all four DENV serotypes, can rarely neutralize virus infection even at high concentrations, but promote ADE (**Figure 1**) [48, 75, 76, 98, 99].

Historically, the DENV-ADE phenomenon has been recapitulated by numerous *in vitro* studies where FcR-bearing immunes cells including human monocyte/macrophages-like cell lines [48, 73, 74, 77, 98–103], human pre-basophil-like and immature mast cell-like cells [104, 105], human primary derived-monocytes/macrophages, dendritic cells, mast cells [75, 76, 106–110], and human derived-PBMCs [111, 112] have been infected with distinct DENV serotype strains in the presence of monoclonal antibodies or human derived-serum/plasma obtained from DENV infected cases [59, 72, 75, 76, 104, 105, 113–115]. Controversially, DENV-ADE for other considered immune cells such as endothelial cells [116, 117] have been also described *in vitro* and *in vivo* [118–121]. However, histochemistry of autopsy samples from fatal dengue cases and *in vitro* assays using peripheral blood cells revealed that macrophages/monocytes are primary targets for DENV infection and not endothelial cells [122–127].

*In vitro*, the increased infection of these target cells results in augmented expression/production of cytokines and vasoactive mediators and their release into the extracellular milieu where provoke an exacerbated activation of neighboring

immune cells and endothelial cells which leads to another critical phenomenon that may frequently occur during severe dengue infections known as the "*cytokine storm*" [10, 11, 73, 107, 128–130]. This phenomenon has been described for several other virus infections (such as influenza viruses, hantaviruses, and potentially coronaviruses), where the scenario envisioned is that this excessive immune activation creates a cascade of cytokine production or "*cytokine storm*" resulting in increased vascular permeability [131–134]. *In vivo*, experimental DENV infection in AG129 [135], showed that sub-neutralizing concentrations of anti-E or anti-pre-M antibodies increased DENV pathogenesis and mortality in mice mainly associated with increased circulation of pro-inflammatory cytokines and increased vascular leakage [40, 48, 87, 118, 136].

Mechanistically, it is thought that ADE-DENV infection of Fc receptor-bearing cells, particularly through the Fc-gamma-receptor IIA (FcγRIIA or CD32) [75, 102, 137, 138], not only results in a large virus-infected cell mass, rather the activation of intracellular signaling pathways [109, 139–142] which leads to the increased secretion of vasoactive immune products by infected cells, such as TNF-α, a cytokine produced by activated monocytes, which in elevated levels has been found in serum of DHF/DSS patients [71, 86, 143–145]. In addition to TNF- α, an important number of immune modulators have been described to be implicated in DENV pathogenesis acting upon a complicated network of events to provoke the severe dengue outcomes mainly related to increased vascular leakage. In DENV-infected primary monocytes or monocytic cell lines, the production of IL-6, IL-8, and interferon gamma-induced protein 10 (IP-10), IL-12p70, IL-1β, IL-10 and the prostaglandin E2 (PGE2) have been found upregulated [73, 74, 76, 77, 109, 112, 137, 142, 146]. In DENV-infected dendritic cells, the production of inflammatory cytokines TNF-α, IFN-α, IL-6, IL-10; the chemokines IFN-γ-inducible chemokines CXCL9, CXCL10, and CXCL11, PGE2, and also matrix metalloproteinases (MMPs) such as MMP-2 and MMP-9 were found increasingly produced [75, 76, 147–149]. On the other hand, DENV infection of mast cells has showed to elicit the release of potent vasoactive cytokines such as IL-1β and IL-6; chemokines, such as CCL3, CCL4, CCL5 and CXCL10, and other mast cell-derived mediators including proteases such as chymase and tryptase, leukotrienes, prostaglandins, histamine, and vascular endothelial growth factor (VEGF) which shows the significant influence of mast cells in immunity and pathogenesis during DENV infection [104, 107, 150, 151].

In humans, increased levels of many of these soluble factors including IFN-γ, TNF-α, IL-1β, IL-4, IL-6, IL-7, IL-10, IL-13, IL-15, IL-17, IL-18, macrophage migration inhibitory factor (MIF), chemokines such as IL-8, CCL2, CCL4, CCL5, CXCL10 (IP-10) and the monocyte chemoattractant protein-1 (MCP-1) have been reported in patients with DHF when compared to DF [1, 10, 152–154]. Studies show that elevated levels of IL-6, IL-10, IFN-γ, MIF, and CCL-4 could be used as potential biomarkers of severe dengue [155, 156]. Additional biological markers such as serum lipids [157, 158], prostaglandins, leukotrienes and thromboxane [151, 159], free radical compounds such as reactive oxygen and nitrogen oxide species [160, 161], MPPs [162–164], and several components of the endothelium in the microvasculature such as the vascular cell adhesion molecule 1 (VCAM-1), Angiopoietin-1 and -2 (Ang-1, -2), endothelial-1, [5, 163, 165–167] or carbohydrates including glycosaminoglycans (GAGs) and proteoglycans (e.g. HSPG) [168–171] have been suggested to play important roles in the pathogenesis of different viral infections, including DENV. Changes in the plasma or urine levels of these molecules have been shown to act as potential predictors for clinical outcome between patients with different stages of DHF disease severity and predictors of disease severity in animal models *in vivo* [168, 170–174].

### 1.3 T cell responses and "the antigenic sin" of DENV infection

During DENV replication cycle, the viral genomic RNA encodes for a single polyprotein that after being cleaved by cellular and viral proteases yield three structural proteins and seven nonstructural proteins [42, 175] (See Figure 1A, *Part I*). Numerous studies have demonstrated that DENV infection leads to potent T cell responses, and many DENV-T cell epitopes have been found throughout the DENV polyprotein *ex vivo* using human leukocytes and *in vivo* using murine models [176–179]. These T cell epitopes appear to follow the general principles of T cell epitope immunogenicity, as they show similar MHC molecule binding kinetics to those of other immunodominant viral epitopes. Several studies indicate that nonstructural proteins are more frequently recognized by CD8+ T cells, while structural proteins are better recognized by CD4+ T cells. [180, 181]. However, most of the identified CD8+ and/or CD4+ T cells epitopes predominantly reside in the nonstructural proteins 3 (NS3) suggesting that NS3 protein is immunodominantly recognized by T cell epitopes in humans infected with DENV [8, 177, 182–184]. On the other hand, CD8+ and CD4+ T cell responses have been also identified, to a lesser extent, against other viral proteins such as the viral capsid, Ns1, NS2A/B, NS4A/B, and NS5 proteins [177, 178, 185, 186]. The recognition pattern of T cell receptors (TCRs) to these proteins expressed in the context of MHC differs according to the type of HLA which confers either susceptibility or protection to severe dengue infections [187, 188]. HLA class I and class II alleles have been shown to be associated with the development of DHF/DSS in different populations [182, 187, 189–192]. However, some specific HLA alleles are found to be more significantly common among patients with dengue fever than those undergoing severe dengue manifestations suggesting a protective effect of DENV-specific T cells [191]. Overall, the T cell immunodominance of DENV is quite complex and widely focused on different epitopes identified across the whole virus proteome [177, 183, 192]. Given that around 70% of amino acid identity exist between all four DENV serotypes, T cell epitopes are also highly cross-reactive and this has been suggested to play important roles in protective immunity not only against DENV but also other related flavivirus such as Zika virus (ZIKV) [193–195].

The protective role of T cells in viral infections is well established [196]. Dengue virus-specific T cells recognize virus-infected cells and respond with a diverse set of effector functions, including proliferation, target cell lysis and the production of a range of cytokines [197]. *In vivo*, CD8+ T cells control viral infection via direct cytotoxicity, and production of pro-inflammatory cytokines such as IFN-γ and TNF-α; in turn, CD4+ T cells induce enhancement of B and CD8+ T cell responses, production of inflammatory and anti-viral cytokines, cytotoxicity, and promotion of memory responses to defeat viral infections [178, 179, 198–200]. T cell activity requires the presentation of viral peptides on the surface of infected cells in the context of MHC molecules and, unlike B cells, T cells do not recognize intact virions [196]. *In vitro*, DENV specific CD8+ memory T-cells can lyse MHC-matched virus-infected cells as an antiviral mechanism leading to protection [199, 201]. Activation of cytotoxic CD8+ T cells after presentation of viral peptides by infected antigen presenting cells (APCs) can generate immediate effector functions by expressing cytotoxic molecules such as granzyme B and perforin to kill virus-infected cells via MHC I- and MHC II-dependent mechanisms [10]. *In vivo* studies have shown that adoptive transfer of DENV-specific CD8+ T cell can partially protect mice from lethal challenge with DENV [202]. Other studies involving immunization with antigens that induce DENV-specific T cells but not neutralizing antibodies, have also shown that T cells are enough to protect mice from lethal infection [179, 203, 204]. These studies suggest that CD4+ or CD8+ T cells may have beneficial roles in controlling virus replication during DENV infections.

Although T cells have important functions in combating viral pathogens, both pathological and protective effects of T cells have been reported in the context of DENV infection [177, 178]. The association of severe dengue symptoms with a rapid decline in viral loads and a peak of pro-inflammatory cytokine secretion have led to the proposal of a role for a T cell mediated immune response in driving immunopathology in severe dengue [205]. T cell responses after primary DENV infections are characterized by higher homotypic than heterotypic responses [194]. However, in secondary DENV infections, T cell responses are highly serotype cross-reactive [206] with higher responses maintained to the previously encountered DENV serotype [207]. This alteration in the T cell immune responses, skewed by the 'memory' of the previous infection, is referred to as '*original antigenic sin*" [10, 207] (**Figure 1**). According to this hypothesis, secondary DENV infection is dominated by the expansion of pre-existing nonprotective, cross-reactive and low affinity T cells to the new infecting serotype that results in ineffective viral control and elicit an aberrant immune response that contribute to immunopathology and severe dengue disease through an excessive production of inflammatory cytokines [10, 177, 178, 205, 207, 208]. Distinct studies *in vitro* using tetramers containing peptides from either the primary or secondary infecting viruses, have provided evidences for the original antigenic sin occurring in secondary T cell responses to DENV [207, 209].

The magnitude of the T cell response positively correlates with disease severity [183, 207]. Studies of the function of dengue-specific T cells has revealed an interesting difference between mild and severe infections [183]. Profound activation of naïve T cells into effector T cells and increased cytokine production have been reported in patients with DHF during both primary and secondary DENV infections [1]. T cell responses in severe dengue patients mainly produce IFN-γ and TNF-α [183]. Additionally, a broad spectrum of cytokines has been shown to be produced by DENV-specific T cells in responses to the recognition of peptide–MHC complexes on target cells. This array of cytokines follows a T helper 1 (TH1)- or TH0-like profile including the production of IFN-γ, TNF-α, IL-2, and MIP1β, also known as CCL4, and less commonly, the TH2-type cytokines, IL-4 [15, 193]. Many of these T cell-derived cytokines have pleiotropic effects, including the induction or enhancement of inflammation and the alteration of vascular permeability that may contribute to the systemic disturbances leading to DHF [1, 10, 208].

Despite all this evidence, the relative contribution of DENV activated T cells in dengue pathogenesis and control of viral infection is still controversial. Severe dengue can occur during a primary dengue infection in which cross-reactive T cells and original antigenic sin would not be operative [210]. In addition, the relatively low numbers of circulating T cells seen during acute DENV infection and the temporal mismatch between the appearance of DENV NS3-specific CD8+ T cells, and the appearance of vascular leakage manifestations, suggests that other mechanisms independent of CD8+ T cells are responsible for early triggering of capillary leakage in children with DHF [207, 211]. A recent study found that numbers of DENV-specific T cells were augmented in the skin of DENV-infected patients compared to those circulating in the peripheral blood, suggesting that during the acute phase, DENV-specific T cells may migrate to the skin and return the bloodstream upon viral clearance [212, 213]. During secondary infections, the expansion of the low-avidity T cells specific for the primary DENV infection may delay viral clearance and thereby lead to higher viral loads. These results may explain the asynchronies timing between T cells circulating in the blood and the beginning of capillary permeability that occurs in DENV infection. However, in the absence of a good animal model of disease, it remains controversial whether the expansion of low-avidity cross-reactive T cells in secondary dengue infection contributes to disease pathogenesis. In summary, during DENV infection, the generation of an early T cell response may be

protective, whereas the late generation of T cell populations that have a proportion of low-avidity T cells and are skewed to inflammatory cytokine production in the absence of degranulation may predispose to immunopathology in the presence of high viral antigen loads that contributes to the cytokine storm and vascular leak.

### 1.4 DENV infection, NS1 pathogenesis, and vascular leak

The hallmark and critical feature of severe dengue disease is the increased capillary permeability, causing plasma leakage, which can lead to hemodynamic compromise and dengue shock syndrome (DSS) [2–4]. The plasma leakage syndrome, is defined as the extensive extravasation and accumulation of fluids out of blood vessels into the surrounding tissues and serous cavities, causing serositis which includes pleural effusion, and pericardial and peritoneal ascites, leading to hemoconcentration, hypotension, organ disfunction, and life-threatening shock [6, 214]. In DENV infection, the transient nature of plasma leakage, its association with the late febrile phase and the paucity of structural damage to the vasculature identified by autopsy studies initially suggested that circulating factors were primarily responsible for this phenomenon [5, 125, 126, 205].

Although a major risk factor for developing severe dengue disease is related to the DENV-ADE phenomenon that correlates with increased plasma levels of pro-inflammatory cytokines found in the acute phase of patients undergoing severe dengue manifestations [1, 83], ADE alone seems not to be sufficient to explain the vascular pathology associated with DHF since not all secondary heterotypic infections results in severe disease, and many individuals also experience DHF during primary infection [16]. The association of severe dengue symptoms with a rapid decline in viral loads and a peak of pro-inflammatory cytokine secretion suggests that although subneutralizing antibodies can increase dengue disease severity via ADE, other factors may also influence disease outcome, driving the immunopathology in severe dengue [10, 205].

Very recently, a new piece in DENV pathogenesis puzzle, known as the nonstructural protein 1 or NS1, was reported to directly be involved in inducing endothelial dysfunction *in vitro* and vascular leakage *in vivo* via alteration of the endothelial barrier function and activation of immune cells and platelets, the latter resulting in induction of pro-inflammatory signaling pathways leading to increased secretion of vasoactive molecules and vascular leakage [9, 215, 216]. Initially recognized as a soluble complement-fixing (SCF) antigen detected in the blood of DENV-infected patients [217], NS1 of the flavivirus genus including DENV, is the only flaviviral protein described to be secreted by infected mammalian and insect cells [218–221], which circulates in the bloodstream of DENV-infected patients for up to 14 days since the onset of symptoms [222]. This bioavailability feature of NS1 launched it as a diagnostic marker for acute primary and secondary DENV infections and potentially other flavivirus diseases [54, 223–225]. NS1 antigenemia can reach as high as 50 μg/mL during the acute phase of dengue illness correlating with the development of DHF and sometimes fatality cases [12, 14, 54, 170, 223, 226–228]. These observations suggest that circulating levels of NS1 in the bloodstream of patients during the clinical phase of the disease may contribute to DENV pathogenesis.

In infected cells, NS1 is found as a membrane-associated dimer in both cellular compartments and on the cell surface. NS1 is intracellularly generated as a monomeric glycoprotein in the ER of DENV infected cells, where it has been demonstrated to play essential roles as cofactor in virus replication and virus assembly by recruiting cellular proteins as well as viral proteins such as the envelope protein during virus morphogenesis which results in the biogenesis of the membranous DENV RC organelle [229–233]. NS1 is also secreted by infected cells and recent structural

analyses showed that secreted NS1 circulates as a soluble hexamer glycoprotein with an atypical open barrel-shape that contains a prominent central lipid-enriched core of triglycerides, cholesteryl esters, and phospholipids that evocates a plasma high-density lipoprotein [53, 234] (**Figure 1**). Elucidation of the crystal structures of the NS1 hexamers reveal an amphipathic molecule with a hydrophobic inner face and a hydrophilic outer face containing three structural domains known as the hydrophobic "β-roll", the "wing" domain, and the c-terminal "β-ladder" domain that likely have distinct roles in membrane association, replication complex assembly, and interactions with the immune system and are the basis for elucidating the molecular mechanism of NS1 function [235, 236] (See Figure 1B, *Part I*). These same structural domains were also identified by cryo-EM reconstruction studies of other related flavivirus NS1 proteins such as West Nile virus (WNV), Zika virus (ZIKV), and yellow fever virus (YFV) [237–239].

In addition to the role played in viral replication, NS1 participates in dengue immunopathogenesis by inhibiting platelet aggregation and prothrombin activation, directing complement against endothelial cells, inducing endothelial cell apoptosis, and facilitating the evasion of DENV particles from complement system–dependent neutralization [229, 240]. Regarding the complement pathways, NS1 mediates complement inactivation through multiple interactions with the complement proteins including factor H, C1s and C4, and the C4 binding protein [241–244]. These interactions result in attenuation of complement classical, lectin, and alternative pathways suggesting that extracellular NS1 protein may function to minimize immune system responses by decreasing complement recognition of DENV infected cells [19]. However, in flavivirus infections, the complement system has been described to play an important role in protecting the host but also influencing disease pathogenesis [19]. In DENV infected patients with DSS, accelerated complement consumption and a marked reduction in plasma complement components has been observed, which led to the proposal that complement activation plays an important role in disease pathogenesis [20, 245]. Recent studies from human autopsies have identified more evidences of increased deposition of complement components from both classical and alternative pathways associated with increased liver damage [126].

In the context of DENV NS1, both soluble NS1 and cell membrane-associated NS1 have been identified to triggers complement activation and anaphylatoxin formation in the presence of polyclonal or monoclonal anti-NS1 antibodies [246] (**Figure 1**). *In vitro* and *in vivo* experiments using anti-NS1 specific antibodies as well as antiserum obtained from DENV immunized mice and rabbits have reported their cross-reactivity with various epitopes found on human plasma proteins involved in coagulation pathways such as fibrinogen, plasminogen, and thrombin as well as integrin/adhesion proteins, endothelial cells and platelets leading to inflammation, apoptosis, and dysfunction of endothelial cells and platelets which sometimes results in bleeding issues [247–252]. Based on these evidences, autoimmune mechanisms mediated by anti-NS1 antibodies have been also proposed to lead to symptoms of DHF related to increasing vascular permeability maybe in a complement dependent manner. However, numerous past and new growing evidence describing the role of anti-NS1 antibody responses in NS1-immunized mouse models, DENV infected patients or DENV vaccine trails suggest an important protective effect of anti-NS1 immune responses as prophylactic or therapeutic options against DENV infection and other related flavivirus infections [9, 57, 62, 63, 70, 186, 253–260]. Therefore, the dual role of anti-NS1 antibodies in protection and disease still represents a critical challenge that needs to be overcome to develop an effective and safe NS1-based vaccine against flavivirus infections.

As mentioned previously, endothelial barrier dysfunction leading to vascular leakage and shock are the major causes of death in patients with dengue

hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. The vascular alterations observed in dengue cases have been described to be a consequence of the imbalance of the host immune system, specially cytokine storm, cytotoxic T cell and complement activations [1, 10, 11], in addition to endothelium injuries caused by the direct infection of the virus of endothelial cells, as reported by several *in vitro* studies [261, 262]. Endothelial cells, lining the inner side of blood vessels, constitute a critical component of the vascular endothelium which are in direct contact with the plasma proteins and all cellular components circulating in the bloodstream [263]. Under homeostatic conditions, this layer of endothelial cells crucially conducts several essential processes such as maintenance of vessel integrity, supply of oxygen and nutrients to underlying tissues and patrolling immune cell trafficking [264, 265]. Thus, alterations on its integrity under pathologic circumstances result in malfunction which contributes to inflammation and disease [265] (**Figure 1**).

Secreted hexameric DENV NS1 protein has been described to attaches to the surface of uninfected cells, primarily human endothelial cells *in vitro* and *in vivo* via interactions with heparan sulfate and chondroitin sulfate E [266]. In 2015, a new role for soluble NS1 in eliciting direct pathogenic effects in DENV disease was described [9]. In this study, the barrier function of human pulmonary microvascular endothelial cells (HPMEC) cultured under polarized conditions on semipermeable membrane filters (*e.g.* Transwells inserts) was compromised after being exposed to physiological concentrations (0.5–5 ug/mL) of recombinant soluble and hexameric NS1 proteins from all four DENV serotypes (DENV 1–4) *in vitro*. Interestingly, this pathogenic effect was recapitulated *in vivo* when inoculation of mice (e.g. *Ifnar*$^{-/-}$) with only DENV NS1 in the absence of DENV infection results in increased mice morbidity. More interesting, a combination of DENV NS1 with a sublethal dose of DENV2 made mice succumbed. These NS1-induced morbidity/mortality effects *in vivo* was significantly related to the increased vascular leak observed in these mice, phenomenon that was shown to be prevented by NS1 immunization or prophylactic treatment of mice using NS1-derived mouse antiserum or anti-NS1 monoclonal antibodies that also blocked NS1-increased permeability of HPMEC cultures *in vitro*. This study demonstrate how NS1 alone was able to mediate DENV pathogenesis by triggering endothelial dysfunction *in vitro* which was linked to increased vascular leak and mortality *in vivo* [9]. Interestingly, an additional study showed that the secreted form of NS1 may act as a pathogen associated-molecular pattern (PAMP) as purified NS1 protein was able to directly activate mouse macrophages and human PBMCs via Toll-like receptor 4 (TLR4), which leads to the induction and release of pro-inflammatory cytokines and chemokines *in vitro* (*e.g.* TNF-α, IL-6, IFN-β, IL-1β, and IL-12); later, this effect was prevented by TLR4 antagonists and anti-TLR4 antibody treatment in a mouse model of DENV infection [215]. These evidences strongly support the important contribution of NS1 in modulating the endothelial cell biology and the inflammatory responses of immune cells as two of the main mechanisms described to influence DENV pathogenesis and therefore severe disease.

On the endothelium, two main structures work together to maintain the homeostasis of the microvasculature: a network of glycosaminoglycans, glycoproteins, and proteoglycans known as the endothelial glycocalyx layer (EGL) and an array of protein-to-protein interactions that integrates the intercellular junction complex (IJC), mainly composed by tight and adherens junction proteins, and other structures such as gaps and desmosomes [257–259]. Based on the first set of evidence showing a direct role of NS1 on the endothelial cell barrier, subsequent studies have identified distinct mechanisms triggered by DENV NS1 to cause endothelial hyperpermeability and vascular leak such as disruption of EGL (*e.g.* sialic acid,

heparan sulfate, syndecan-1) expressed on the surface of HPMEC and the microvasculature *in vivo* via activation/expression of endothelial enzymes including sialidases, heparanase, and cathepsin L, a lysosomal cysteine proteinase, all of these occurring in a cytokine-independent manner [267, 268] (**Figure 1**). An additional study corroborates these findings showing that NS1 induces the increased secretion of vasoactive molecules such as the macrophage migration inhibitory factor (MIF) and the angiopoietin-1 and 2 (Ang-1/Ang-2) from human endothelial cells (e.g. HMEC-1 from dermis) and DENV infected patients. These molecules were shown to activate autophagy pathways, phosphorylation cascades, and actin cytoskeleton rearrangements leading to disarrangement and internalization of VE-cadherin, an adherens junction protein of endothelial cell-to-cell contacts, inflammation, and also secretion of heparanase, shedding of sydencan-1 (CD138), and expression of MMP-9 from immune cells, resulting in degradation of EGL and hyperpermeability *in vitro* [269–272]. Follow up studies in human primary monocytes, monocytic cell lines, and human platelets stimulated with exogenous NS1 *in vitro* have additionally demonstrated the NS1-mediated activation and stimulation of pro-inflammatory cytokines and proteases (*e.g.* MMPs) via TLR4 signaling supporting previous reports of NS1 protein acting as a PAMP leading to inflammation, thrombocytopenia, hemorrhage and disease in DENV infection [273–275].

Besides DENV, the flavivirus genus includes other human medically important mosquito-borne pathogens such as ZIKV, WNV, Japanese encephalitis virus (JEV), and YFV [276]. In humans, flaviviruses can cause a wide spectrum of systemic or neurotropic-encephalitic pathologies ranging from clinically inapparent infections to severe, sometimes fatal disease, characterized by hemorrhagic manifestations and vascular leakage with organ failure (DENV and YFV), encephalitic manifestations (JEV and WNV), and congenital Zika syndrome in pregnancy and Guillain-Barré syndrome in adults associated with ZIKV infection [277, 278]. In recent studies, NS1 proteins from other DENV-closely related flavivirus including ZIKV, WNV, JEV, and YFV also demonstrated to cause endothelial hyperpermeability and vascular leak [172, 173, 279]. Interestingly, NS1 proteins selectively bind to and alters permeability of human endothelial cells from distinct tissues including lung, dermis, umbilical vein, brain, and liver *in vitro* and causes tissue-specific vascular leakage in mice, reflecting the pathophysiology of each flavivirus. Mechanistically, flaviviruses NS1 trigger the disruption of EGL components to cause endothelial hyperpermeability [172, 173, 253, 279]. On the vascular endothelium, the EGL constitutes a network of GAGs such as heparan sulfate, chondroitin sulfate, and hyaluronic acid, and proteoglycans (e.g., syndecans, glypicans, and perlecan) that contributes to maintain the homeostasis of the endothelial barrier function [280]. Degradation of the EGL and the detection of its degradation products in cell supernatants and human plasma have been linked to virus pathogenesis and disease severity in several viral hemorrhagic fever diseases [281], including dengue, where increased levels of heparan sulfate, hyaluronic acid, sialic acid, and syndecan-1 have been found to correlate with severe dengue disease in humans and lethality in animal models [3, 168–170, 172].

This NS1 pathogenic effect on the endothelium requires the internalization of the soluble NS1 protein inside human endothelial cells [279] (**Figure 1**). This process occurs via clathrin-mediated endocytosis and relies on one of the glycosylation sites (Asparigine-207) located in the *Wing* domain of NS1 [279]. DENV NS1 contains two conserved N-linked glycans at the asparagine-130 (N130) and the asparagine-207 (N207) which have been implicated in NS1 hexamer secretion, stability, and function [234, 236, 282, 283]. Previous studies investigating the importance of the N-glycans on NS1 have found that deglycosylated flaviviral NS1 proteins at either site, exhibited significant attenuation of neurovirulence in mice

compared to the wild-type virus [284–286]. Additional *in vitro* studies have shown that endocytosis of DENV NS1 occur in human hepatocytes which may potentialize subsequent DENV infection [221].

Flavivirus infection has been shown to compromise the integrity of many biological barriers, including the lung microvascular endothelium and the blood–brain barrier, which are usually able to protect against virus infection [287, 288]. Numerous studies of flavivirus infection in different animal models as well as human autopsies have shown a selective tropism of distinct groups of flaviviruses that target different tissues, leading to systemic versus neurotropic-encephalitic pathology [277]. The fact that NS1 internalization is required to induce endothelial hyperpermeability and increased vascular leak through a flaviviral-conserved endothelial cell-intrinsic pathways, and the finding that the flaviviral virulence depends on the expression of N-glycans on soluble NS1, suggest the possibility that NS1 may favor virus propagation and pathogenesis *in vivo*. During the acute phase of DENV infection high NS1 antigenemia have been correlated with increased risk of developing severe dengue disease, including vascular leakage [12, 14]; however, little is known about circulating levels of NS1 from other flavivirus infections. Future studies intended to investigate the kinetics and dynamics of NS1 circulation in flavivirus-infected patients different than DENV, will help to better understand the role of NS1 in flavivirus pathogenesis and disease. NS1 is well conserved among flaviviruses (20–40% identity, 60–80% similarity) [289], therefore, these findings reveal the capacity of a secreted viral protein from related flaviviruses named as NS1 flaviviral toxin as critical for pan-flavivirus pathogenesis through modulation of the endothelial barrier function in a tissue-specific manner, potentially influencing virus dissemination and pathogenesis of target organs and representing a novel target for anti-flaviviral therapy and potential vaccine candidates against flavivirus infections.

## 2. Concluding remarks (*PART II*)

Systemic vascular leakage associated with DENV infection is the most serious complication and the most important contributor to severe clinical outcomes during severe dengue disease that result in life-threatening complications such as hypotension, organ failure, and shock. Epidemiological data strongly associate severe dengue disease with secondary heterotypic DENV infections occurring in the presence of pre-existing antibody responses, widely attributed to the phenomenon of *antibody-dependent enhancement* (ADE). Numerous studies *in vivo* and *in vitro* have tightened DENV-ADE to the increased activation of immune cells such as monocytes, macrophages, dendritic cells, and mast cells leading to the generation of the pro-inflammatory environment found in many patients undergoing severe dengue, known as "*cytokine storm*". Along with DENV-ADE, heterotypic immunity originated during primary DENV infection may also lead to alterations in immune responses of T cells, skewed by the 'memory' of the previous infection, referred to as '*original antigenic sin*'. These DENV cross-reactive T cell responses produce only inflammatory cytokines and might be inherently inefficient in killing DENV-infected cells, resulting in enhanced infection, which may predispose to the immunopathology of DENV.

Along with this evidence, in the last decades, several other immunologic mechanisms such as activation of complement pathways and autoimmune responses (e.g. mimetic anti-DENV antibodies) have been also linked to ensure severe dengue manifestations leading to the activation and apoptosis of immune cells and endothelial cells, aggregation of platelets, and inactivation of plasma proteins involved in coagulation cascades. On the other hand, viral biomarkers such as NS1, which

high circulating levels correlated with the appearance of severe dengue disease, was reported to modulate complement pathways, facilitating virus infection via immune evasion strategies. NS1 has demonstrated to exert an amazing array of different functions. More recently, NS1 was demonstrated to be a multitasking protein of the flavivirus genus which could directly cause disruption of the EGL and endothelial cell-to-cell contacts, two main components of the homeostasis balance in the microvasculature, and to induce the production of soluble immunoregulators resulting in increased endothelial barrier dysfunction and vascular leakage. This evidence provides new insights into the biology of the multifaceted NS1 protein of flavivirus that may improve the understanding of the flavivirus pathogenesis, strongly supporting the inclusion of NS1 protein in flavivirus vaccine development and the generation of new targets for future therapies against flavivirus infections.

In conclusion, the immunopathogenesis of DENV infection represents an extraordinarily complex interplay between several viral and host factors that together contribute intimately to the activation of distinct immunopathological processes that although were intended to control the viral infection and replication, instead unleash an unbalanced host immune response leading to increased endothelial dysfunction and vascular leakage, reflected in the appearance of dengue severe manifestations. As no effective vaccine or antiviral therapy are available to treat either prophylactically or therapeutically the DENV infection, the incidence of dengue disease is expanding globally and continues to threat the public health services worldwide, particularly in endemic areas. An increased understanding of DENV immunopathogenesis mechanisms involved in the development of severe disease, their components, biological triggers, and their potential connections will assist not only the development of potentially more effective novel therapeutic interventions but also the understanding of dengue vaccine efficacy or vaccine adverse events that can be considered during vaccine trial interventions.

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