DENGUE IMMUNOPATHOGENESIS: A CROSSTALK BETWEEN HOST AND VIRAL FACTORS LEADING TO DISEASE: PART I - DENGUE VIRUS TROPISM, HOST INNATE IMMUNE RESPONSES, AND SUBVERSION OF ANTIVIRAL RESPONSES

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Chapter

Dengue Immunopathogenesis: A Cross Talk between Host and Viral Factors Leading to Disease: Part I - Dengue Virus Tropism, Host Innate Immune Responses, and Subversion of Antiviral Responses

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Abstract

Dengue is the most prevalent emerging mosquito-borne viral disease, affecting more than 40% of the human population worldwide. Many symptomatic dengue virus (DENV) infections result in a relatively benign disease course known as dengue fever (DF). However, a small proportion of patients develop severe clinical manifestations, englobed in two main categories known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Secondary infection with any of the four dengue virus serotypes (DENV1, -2, -3, and -4) is a risk factor to develop severe forms of dengue disease. DSS is primarily characterized by sudden and abrupt endothelial dysfunction, resulting in vascular leak and organ impairment, which may progress to hypovolemic shock and death. Severe DENV disease (DHF/DSS) is thought to follow a complex relationship between distinct immunopathogenic processes involving host and viral factors, such as the serotype cross-reactive antibody-dependent enhancement (ADE), the activation of T cells and complement pathways, the phenomenon of the *cytokine storm*, and the newly described viral toxin activity of the nonstructural protein 1 (NS1), which together play critical roles in inducing vascular leak and virus pathogenesis. In this chapter that is divided in two parts, we will outline the recent advances in our understanding of DENV pathogenesis, highlighting key viral-host interactions and discussing how these interactions may contribute to DENV immunopathology and the development of vascular leak, a hallmark of severe dengue. *Part I* will address the general features of the DENV complex, including the virus structure and genome, epidemiology, and clinical outcomes, followed by an updated review of the literature describing the host innate immune strategies as well as the viral mechanisms acting against and in favor of the DENV replication cycle and infection.

Keywords: dengue, immunopathogenesis, dengue shock syndrome, severe dengue, virus replication, cell tropism, innate immune response, antiviral response, immune evasion, complement, endothelial dysfunction, vascular leak

1. Introduction

Dengue is still considered the most prevalent viral disease transmitted by arthropod mosquitoes (e.g., *Aedes* mosquitoes), with 50–100 million dengue infections occurring annually, and a global incidence of 30-fold increase observed over the past 50 years [1–3]. Most of the dengue infections with any of the four dengue virus (DENV) serotypes [1–4] result in inapparent, subclinical illness, or mild disease symptoms known as dengue fever (DF). However, some DENV infections can potentially evolve into more severe and fatal disease outcomes known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. DHF and DSS are mainly characterized by low numbers of circulating platelets (thrombocytopenia) associated with hemorrhagic manifestations and increased vascular permeability associated with endothelium hyperpermeability, resulting in plasma leakage, low blood pressure, and shock that can lead to death [2]. DENV infections frequently occur in the context of preexisting immunity, where the immune responses to prior DENV infection play an important role in determining the outcome of dengue epidemics and disease severity via antibody-dependent enhancement (ADE) and potentially harmful T cell responses in an *original antigenic sin*-dependent manner [4–8]. Both mechanisms lead to increased activation of immune cells, resulting in exacerbated immune responses or *cytokine storms* that cause endothelium dysfunction and vascular leak [9, 10]. Collectively, these host immunological responses are thought to create a physiological environment that promotes vascular permeability. However, the exact mechanisms underlying the capillary leak are probably more complex than a *cytokine storm*, and the risk of severe disease upon DENV infection cannot be explained completely by a misdirected host immune response to a prior infecting serotype; rather, disease severity appears to be determined by a combination of multiple host and viral factors leading to favorable and unfavorable interactions that regulate viral pathogenesis.

Despite considerable advances in understanding the immunological mechanisms activated during DENV infection, the pathogenic mechanisms underlying the alterations in permeability of the microvasculature remain unclear. The absence of a good animal model faithful to human disease and the limited knowledge of the factors regulating the intrinsic microvascular permeability in health have seriously hampered the research progress in this area. However, in the last decades, significant progress has been made regarding viral and host cellular components involved in DENV infection and disease [8]. The nonstructural protein 1 (NS1) protein of DENV and other related flaviviruses has been described as an essential cofactor in virus replication and assembly [11, 12]. Interestingly, the secreted form of NS1 is also implicated in immune evasion strategies via interaction with several proteins of the complement pathways that protect the virus-infected cells from the immune system processing [12–14]. Contrary, NS1 and anti-NS1 antibodies can also mediate complement activation that may alter capillary permeability [15]. Additionally, the soluble NS1 from DENV can interact with the surface of endothelial cells, immune cells, and platelets to cause endothelial barrier dysfunction and vascular leakage, and potentially hampers the coagulation cascades leading to hemorrhagic manifestations during DENV infection. These phenomena occur via activation of endothelial-intrinsic mechanisms leading to the disruption of the EGL and the integrity of the cell-to-cell contacts and/or induction of pro-inflammatory cytokines, chemokines,

and proteases via the TLR4 activation of monocytes/macrophages that may act also on endothelial cells leading to endothelial hyperpermeability and vascular leak [16–22]. Furthermore, NS1 is highly immunogenic and conserved between the *Flavivirus* genus; thus, NS1 from other flaviviruses have been also reported to activate endothelial-intrinsic mechanisms causing vascular leakage in a tissue-dependent manner that mimics each flavivirus disease pathophysiology [17, 23–25]. Additionally, NS1 immunization using mouse models and DENV vaccination or natural DENV infection in humans can elicit antibodies' responses that have been implicated in the contradictory roles of protection and pathogenesis in the infected host [25–42]. Today, no specific and effective vaccine candidate, antiviral therapy, or anti-inflammatory therapeutics have been licensed to combat dengue disease. An effective dengue vaccine is surely needed to avert the millions of dengue cases that occur around the world, continuously threatening with fatal outcomes. For decades, numerous experts in infectious diseases including clinicians, epidemiologists, basic scientists, and vaccine and drug developers have been trying to elucidate the ultimate mechanism of DENV pathogenesis leading to severe dengue disease. The NS1 protein of flaviviruses constitutes a unique "viral toxin" that seems to connect many of the already described DENV immunopathogenic mechanisms leading to severe dengue disease; thus, NS1 might represent the corner piece that completes the elusive dengue pathogenesis puzzle. Therapeutic approaches and vaccine development targeting NS1 may provide different opportunities for the future defeating of the global dengue disease. A better understanding of DENV immunopathogenesis will assist not only in the development of therapeutic interventions but also in the understanding of dengue vaccine efficacy or vaccine adverse events. This chapter briefly summaries the key clinical, virological, and epidemiological facts about DENV innate and humoral responses and gives an extensive update of insights about the viral and host factors that contribute to DENV pathogenesis leading to the development of severe dengue manifestations during DENV infection.

2. Dengue virus features: genomic organization, structure, and life cycle

Dengue virus (DENV) belongs to the genus *Flavivirus* (family *Flaviviridae*), a group of small (50-nm virion diameter) viruses containing a single-positive-stranded RNA genome [5′ capped, not 3′ poly(A) tail] which encodes for three structural proteins: capsid (C), membrane (M), and envelope (E) and seven nonstructural (NS) proteins named NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 **(Figure 1A,B)** [43]. The DENV group is comprised of four evolutionary distinct but antigenically and genetically related viruses better known as DENV serotypes –1, –2, –3, and –4 (DENV-1-4), [44, 45]. These four established serotypes (DENV 1–4) share a high degree of sequence similarity between the genomes (~65–70%) [46], with average sequence identity between proteomes of 39–79% [47]. DENV is transmitted to humans by *Aedes* mosquitoes, mainly *Aedes aegypti*. However, the global distribution of *Aedes albopictus* (the Asian tiger mosquito), considered a secondary vector for DENV transmission, is changing rapidly and it is now becoming an increasingly important vector and a common cause of epidemics in *Aedes aegypti*-free countries [48–50]. Human-to-mosquito transmission occurs once the mosquito takes a blood meal from DENV-infected people who are viremic, which is normally up to 2 days before someone shows symptoms of the illness or up to 2 days after the fever has resolved. High viremia and high fever in patients are positively associated with a high rate of DENV transmission from humans to mosquitoes [51]. After feeding on a DENV-infected person, depending mainly on temperature, the virus rapidly replicates in the mosquito's midgut, and within an average of 5.9 days,

it disseminates to secondary tissues, including the salivary glands where the virus can be transmitted to the new host (extrinsic incubation period) [52]. Once infectious, the mosquito is capable of transmitting the virus for the rest of its life [53].

After a mosquito bites a human, DENV is delivered into the dermis where it can infect/replicate in dendritic cells (DCs) (Langerhans cells) and keratinocytes residing in the basal and suprabasal layers of the epidermis [54–56] **(Figure 2)**. Virus dissemination to the local lymph nodes occurs in association with infected migratory dendritic cells or as free viruses of the lymphatic fluid leading to viremia [57]. At this stage, mosquito saliva has shown to enhance the replication and

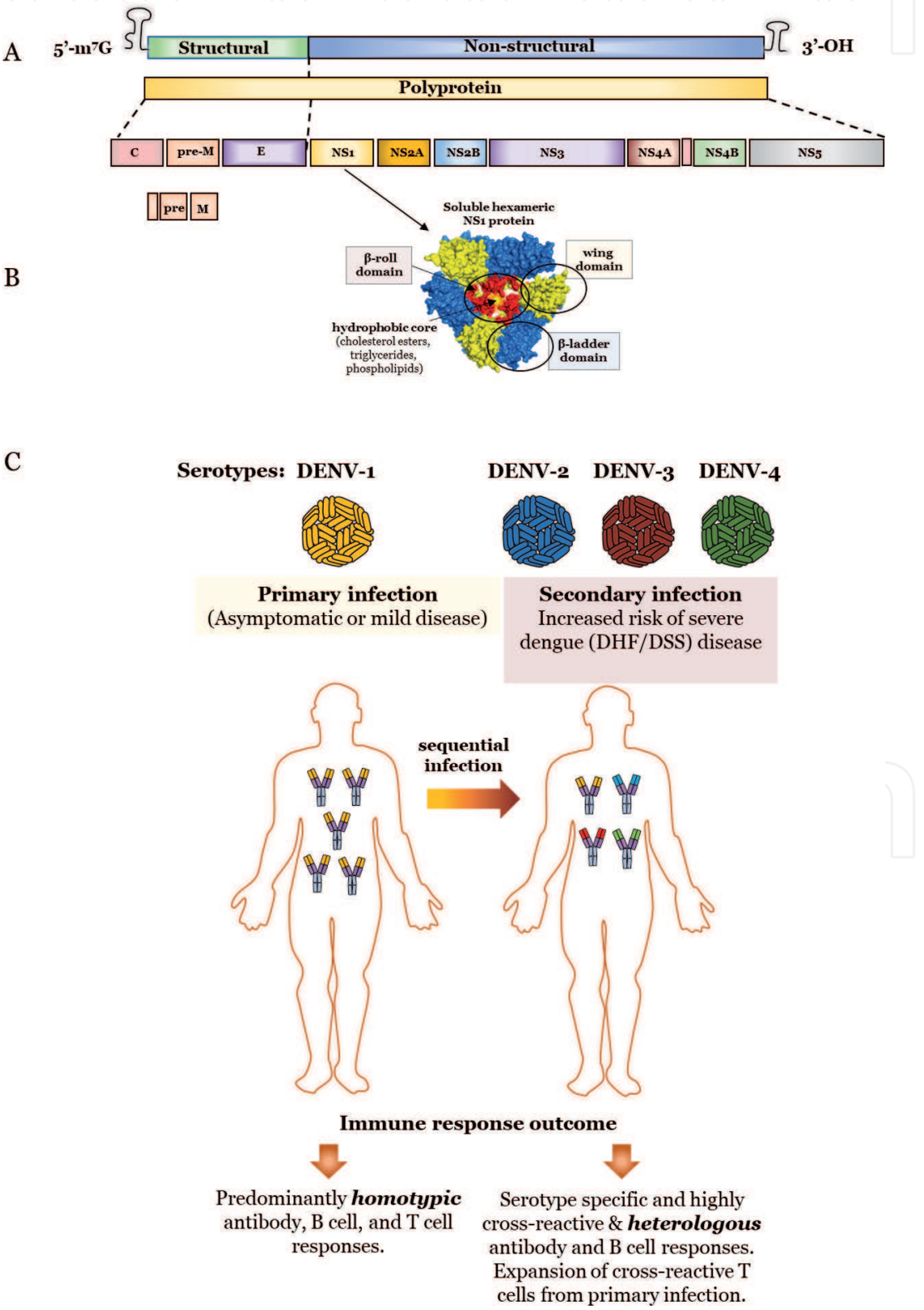

Figure 1.
Dengue virus genome organization, NS1 structure, and dengue epidemiology and disease outcome. (A) Schematic representation of the DENV genome and polyprotein. Dengue virus (DENV; genus Flavivirus, family Flaviviridae) is a positive-sense, single-stranded (~11-kb length), and RNA-enveloped virus with an icosahedral capsid protecting the virus genome, which is transmitted by mosquitoes of the Aedes genus (Aedes aegypti, Ae. albopictus) and affects more than 40% of the human population worldwide living in tropical and subtropical areas. The viral RNA genome poses one single open reading frame encoding for one single polyprotein, which after being processed by cellular and viral proteases generates three structural proteins known as the capsid (C), the membrane (M), and the envelope (E) and seven nonstructural (NS) proteins known as NS1, NS2A, NS2B, NS3, NS4A, NS4B, and N5. The viral RNA contains a cap in the 5′-end, and it has no poly-a tail in the 3′-end. Several secondary structures or UTRs (untranslated regions) are found in both ends which have been shown to participate in viral replication as well as host adaptation. Of the structural proteins, the envelope (E) is the major protein on the virion, which participates in cellular receptor recognition to infect the host cells and the main target for adaptive immune responses in humans. On the other hand, the NS proteins play critical roles in the virus replication cycle and the subversion of the host antiviral responses, particularly those triggered by the innate immune response against DENV infection. (B) Of these NS proteins, NS1 is the only viral protein secreted by DENV-infected cells in which the plasma circulating levels are increased during the acute phase of DENV-infected patients undergoing severe disease. The NS1 protein circulates as a lipoprotein-like particle with a hexameric conformational structure containing three domains termed as the wing domain (here in yellow), the β-ladder domain (in blue), and the β-roll domain (in red) (NS1 hexamer is depicted in this figure). NS1 has been demonstrated to play critical roles in the formation of new viral particles, the evasion of the immune system, and very recently, it was implicated in modulating the virus pathogenesis of DENV that is mainly associated to its potential role in acting as a pathogen-associated pattern molecule (PAMP) which activates the production of pro-inflammatory host soluble factors such as cytokines, chemokines, proteases, etc. from the immune cells and directly triggers the barrier dysfunction of endothelial cell cultures in vitro and vascular leak in the mouse models in vivo. Taking these together, NS1 is now considered as a viral toxin not only of the DENV complex but of many of the closely related flaviviruses, including ZIKV, WNV, and YFV among others. (C) The DENV complex is composed of four serologically but antigenically related types of viruses, known as DENV serotypes (1, 2, 3, and 4). The primary infection with any of the four serotypes often cause inapparent, asymptomatic, or mild diseases that prime the immune system for a long-life immunity against the infecting DENV serotype (here DENV-1, in yellow) which is dominated by homotypic immune responses at mostly all levels, including antibody-B cells' and T cells' responses. During secondary infections, things get more complicated as infection with a different DENV serotype as the one from the primary infection; here DENV-2 (blue), DENV-3 (red), and DENV-4 (green) results in cross-reactive and heterologous immune responses that are considered the main risk factor to develop severe manifestations of DENV infections, including dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), which may lead to life-threatening health complications and sometimes, death. In the course of this chapter, we will explain how these epidemiological factors may be associated with the development of severe dengue disease that may be a consequence of a combined interplay set of immunopathogenic mechanisms triggered by the host immune response having DENV infection as the main trigger. The model of NS1 hexamer was built based on the crystal structure deposited in the Protein Data Bank (PDB4O6B). Molecular graphics were performed using the PyMOL molecular graphics.

pathogenesis of numerous arthropod-borne viruses, including DENV [58–62]. DENV infection cycle initiates with the virus attachment to the target cells [63]. The current model suggests that DENV uses both attachment factors and primary receptor(s) that facilitate virus recruitment on the cell surface, and later, internalization inside host cells via receptor-mediated endocytosis including clathrin-mediated and nonclassical clathrin-independent endocytosis [64, 65]. Despite this, the single definitive receptor mediating this critical step in the DENV replication cycle continues to be elusive. So far, numerous candidates have been described in the mammalian and mosquito cells, including glycosaminoglycans such as heparan sulfate and lectins, the adhesion molecule of dendritic cells (DC-SIGN), the mannose receptor (MR) of macrophages, the lipopolysaccharide (LPS) receptor CD14, and stress-induced proteins such as the heat-shock proteins 70 and 90 and the endoplasmic reticulum (ER) chaperonin GRP78 [64–68]. This suggests that DENV may not use a unique, specific receptor to enter cells, but recognizes diverse molecules, both in the vertebrate and mosquito hosts, which can potentially explain the broad tissue range that defines DENV tropism and infection.

After the internalization of the virion, a fusion between the viral E protein and the endosomal membrane mediates the access of the viral genome into the cytoplasm [43, 65]. The E protein is a glycosylated viral protein and a member of class II viral membrane fusion protein family [43, 69]. The crystal structure of

E glycoprotein ectodomain revealed three domains contributing to the β-barrel central structure of the protein (domain I, DI), permitting the fusion of viral and cellular membranes during virus entry (domain II, DII, and *fusion loop*), and a structural basis for immune recognition and cellular receptor binding (domain III, DIII) [43, 70]. The low pH of the endosomal compartment induces conformational changes in the E glycoprotein, which allows the fusion of the viral and host membranes [43, 69]. This results in the release of the viral RNA genome into the cytoplasm. The single-stranded positive-sense RNA immediately acts as a messenger RNA, which can be subsequently translated by cellular machinery to generate viral polyproteins, subsequently processed by both cellular and viral proteases to generate mature viral proteins [69, 70]. In this stage, the nonstructural proteins have been shown to induce massive remodeling of ER membranes, manifesting as convoluted membranes and vesicle packets (VPs) to form a dynamic and membrane-bound multi-protein assembly, named the replication complex (RC) where the genome is replicated, and new viral RNA copies are incorporated into nascent particles [71, 72]. Viral RNA synthesis relies on NS5, the RNA-dependent RNA polymerase as well as on critical RNA secondary and tertiary structures [73–75]. NS3 is a

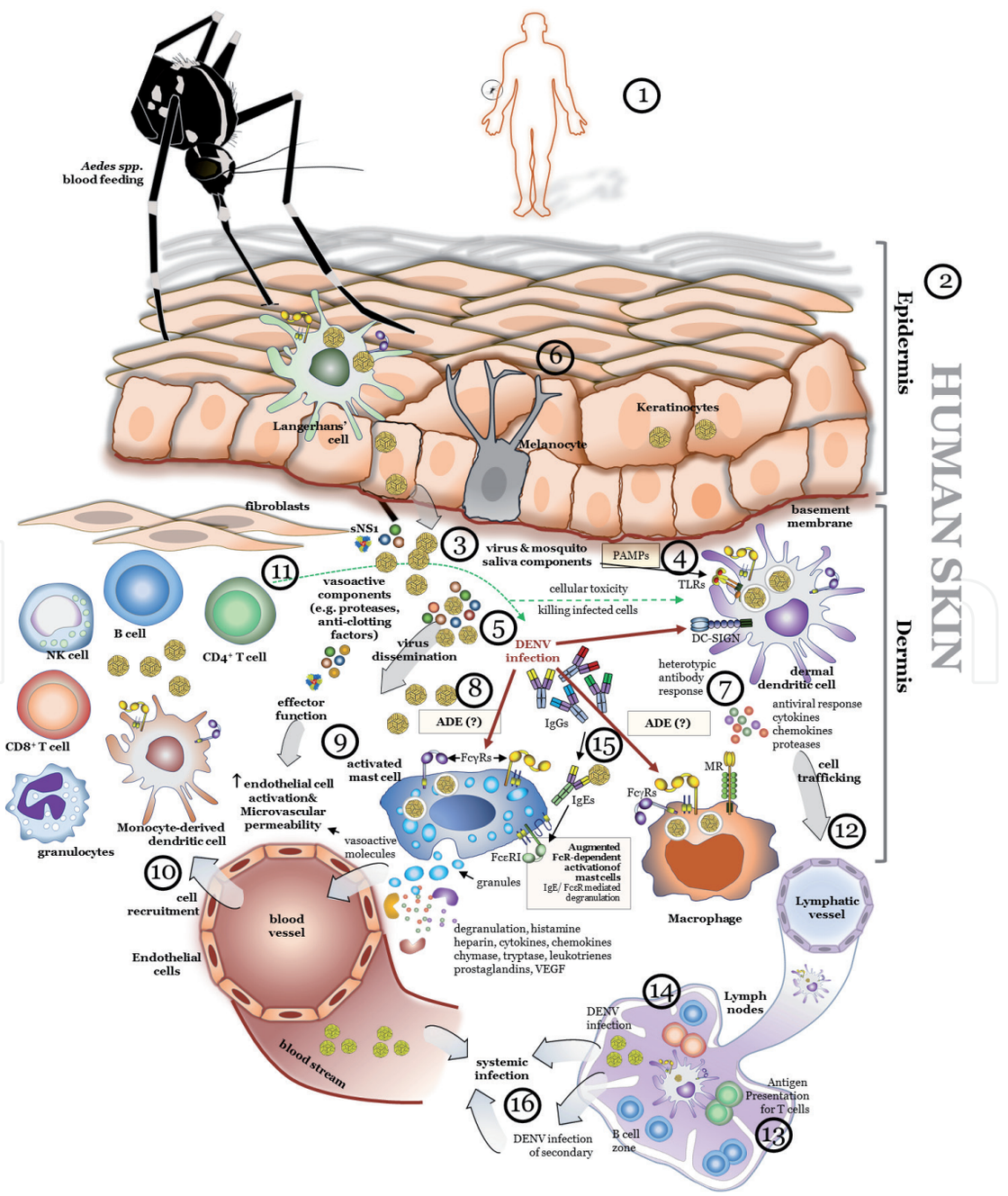

Figure 2.
Dengue virus infection, pathogenesis, and immune responses in the skin. The skin represents the first line of defense of the human body against pathogens such as viruses, as it generates early immune responses, aiming to protect humans against cutaneous and systemic infection (1). The human skin constitutes a complex organ nicely structured in three escalated layers known as the epidermis, which is composed of closely packed epithelial cells including keratinocytes, melanocytes, and Langerhans cells (LCs), a specialized type of dendritic cell (DC) that constantly probes for antigen in the most exposed, superficial layer of the skin; the dermis, which is made of dense, irregular connective tissue, blood vessels, and other structures; and the inner hypodermis, which is composed mainly of loose connective and fatty tissues (2). Upon disturbance of the epidermal barrier by mosquito blood-feeding, DENV-infected mosquitoes inoculate newly generated infectious virus particles along with mosquito saliva in which a complex mixture of proteins that exerts profound effects in the human immune system allows the acquisition of the mosquito blood meal from its host by circumventing vasoconstriction, platelet aggregation, coagulation, and inflammation or hemostasis (3). In the skin, major constituents of the innate immune system include phagocytic cells such as macrophages, neutrophils, and DCs as well as innate leukocytes such as natural killer (NK) cells, mast cells (MCs), basophils, and eosinophils. Also, epidermal keratinocytes act as active innate immune cells. In response to sensing pathogen-associated molecular patterns (PAMPs) expressed by microbes and host danger molecules, innate immune receptors present on keratinocytes and APCs become activated, causing the release of inflammatory cytokines and antimicrobial peptides (4). At the site of inoculation in the skin, the key targets of DENV infection are immune cells of the myeloid lineage, including various subsets of DCs, monocytes/macrophages, and MCs (5). Despite this, limited virus particles are thought to be deposited in the epidermis during mosquito blood-feeding (3), and in that location, Langerhans cells as well as keratinocytes are considered target cells (6). In the dermis, DCs and monocytes/macrophages are also prime infection targets (7). MCs are not substantially infected in the skin (8). However, exposure to DENV triggers an augmented activation of MCs leading to degranulation and release of de novo-synthesized inflammatory and vasoactive mediators, including proteases, leukotrienes, and histamine that, along with some vasoactive molecules and maybe the secreted NS1 originated from infected mosquito cells in the salivary glands, promote edema within the site of infection as a consequence of the increased microvascular permeability (9). Activation of MCs also induces the secretion of cytokines and chemokines that leads to the recruitment of NK cells, neutrophils, and monocyte-derived dendritic cells (mDCs) to the site of infection (10). Already in the skin, mDCs can serve as targets of infection, allowing the amplification of the virus in the skin, while natural killer (NK) cells, natural killer T (NKT) cells, and CD8+ T cells can kill DENV-infected cells and promote virus clearance in a cellular cytotoxic-dependent manner (11). Once human skin is infected, DENV-infected DCs take virus into the draining lymph nodes using afferent lymphatic vessels where they spread DENV infection and most importantly activate antigen-specific $CD4^+$ and $CD8^+$ T cells which initiates the adaptive immune response (12, 13). In the T cell zones, activated T cells become effectors cells to promote the development of DENV-specific memory B cells and plasma cells in the germinal center of LN. Activated T cells can reenter circulation and potentially return to the skin for virus clearance during subsequent DENV infections, playing an important role in protecting against DENV (14). In the skin, in addition to the mosquito saliva, DENV infection of target cells such as DCs, monocytes/macrophages, and MCs can be also modulated by the presence of preexisting antibody responses against previous infections with distinct DENV serotypes or other closely related flaviviruses, in an antibody-dependent enhancement (ADE) manner (See DENV-ADE in Part II for more details). For MCs and DCs, DENV-ADE is possible through Fc-γ-receptors (FcγRs). Besides, MCs degranulation can be enhanced through cross-linking of FcεRs when bound to DENV-specific IgE, leading to augmented MC activation and presumably immune-mediated vascular injury (15). After skin infection, DENV must achieve systemic infection to complete its transmission cycle by infecting new mosquito hosts. Infection of secondary LNs following infection of the draining LN are considered the amplification centers for DENV that contributes to the systemic infection and virus transmission (16).

protease-helicase which together with its cofactor NS2B, participates in the processing, efficient RNA synthesis, and capping of the viral polyprotein [72, 76, 77]. NS2A recruits nascent RNA as well as C-pre-M-E [78]. NS1 interacts with structural proteins and NS4A-2 K-4B to facilitate the production of infectious virus particles [11, 79]. Because of their critical roles in the DENV replication cycle, NS2B, NS3, and NS5 along with NS4B are the main focus to design new inhibitors for antiviral therapy against DENV and other related flaviviruses [80–83]. Assembled viruses are transported through the trans-Golgi network (TGN) where under acidic conditions, a cellular protease, *furin*, cleaves pre-M, allowing full maturation of infectious virions that will be finally released via exocytosis [84].

3. Dengue virus infection, epidemiology, and clinical features

Dengue is the arboviral infection with the highest disease incidence worldwide, with 2.5 billion people living in dengue-endemic tropical and subtropical regions [1, 85, 86]. In the last four decades, the geographical spread and intensity of dengue

have grown dramatically around the world accompanied by the wide distribution of the two main vector mosquitoes, *Aedes aegypti* and *Aedes albopictus*, which today are fully adapted to human dwellings creating new opportunities not only for DENV but also for other arthropod-transmitted viruses (arboviruses) transmission, such as Zika virus (ZIKV) and chikungunya virus (CHIKV), within human populations. These features along with the continuous growing of urbanization, globalization, and the lack of effective mosquito control represent some of the critical factors that have contributed to the emergence and reemergence of mosquito-transmitted viruses around the world [48, 87, 88].

Infection with any of the four DENV serotypes results in a diverse range of symptoms going from mild undifferentiated fever to life-threatening manifestations, which are characterized by increased vascular permeability, hemorrhage, and shock [89] **(Figure 1C)**. In 1997, the World Health Organization (WHO) classified symptomatic DENV infections into three categories and subcategories known as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). After an incubation period of 3–7 days, symptoms start suddenly and follow three phases: an initial febrile phase, a critical phase around the time of defervescence, and a spontaneous recovery phase [89]. Classical DF is an incapacitating disease that affects older children, adolescents, and adults, mainly characterized by the abrupt onset of fever (up to 40°C) and severe headache, accompanied by retro-orbital pain, myalgia, arthralgia, gastrointestinal discomfort, and transient rash [89]. In turn, DHF and DSS can rapidly deteriorate, progressing to hemorrhage with or without vascular leak after an early acute-onset febrile period, particularly during defervescence, where the symptoms are similar to those presented during classical DF. DHF and DSS are classified into four subcategories or grades (I–IV), where grades I and II (DHF) are represented by mild cases presenting some bleeding manifestations without shock (petechiae, purpura, ecchymosis, bruising, epistaxis, etc.), whereas III and IV (DSS) are more severe and accompanied by severe hemorrhagic manifestations and thrombocytopenia (platelets counts: <100,000 platelets/μL) and evidence of increased vascular permeability (ascites, pleural effusion, increased hematocrit concentrations, and severe abdominal pain) during a critical period, sometimes accompanied with a profound and prolonged shock that potentially leads to death [90]. In this critical stage, liver failure, myocarditis, and encephalopathy often occur with minimal associated plasma leakage [89]. In 2009, the WHO revised the classification system for dengue and established new guidelines that replaced the more complicated dengue fever/dengue hemorrhagic fever (DF/DHF) system to separate patients enduring severe disease from those with non-severe manifestations. This new guideline defined two new major entities—dengue and severe dengue—which encompasses a set of "warning signs" intended to help clinicians identify the patients likely to develop complications during the critical phase of the illness [89].

Currently, there is no effective and safe vaccine or FDA-approved specific antiviral drug options to combat dengue disease, with treatment being purely supportive [91]. Prevention or reduction of DENV transmission by implementing combined effective control strategies remains as the primary approach to be used to prevent DENV transmission within human populations [92]. With the majority of DENV infections being asymptomatic (70–80%), and most symptomatic infections not progressing to severe disease [3], the global distribution of dengue remains highly uncertain as the actual numbers of dengue cases are underreported and many cases are misclassified. One recent study estimate indicates that 390 million DENV infections occur annually with more than 500,000 cases of hospitalizations and more than 25,000 deaths (2.5% case fatality, annually) [1]. A different study estimated that 3.9 billion people living in 128 countries are at risk of being infected with dengue viruses [85]. These studies

demonstrate the worldwide expansion of the dengue disease and the establishment of an increasingly important infectious disease of global public health significance.

4. Dengue immunopathogenesis and severe disease: host and viral factors

The hallmark of severe dengue is the transient perturbation in the integrity of the endothelium lining the inner side of blood vessels as well as the alteration in the coagulation cascade leading to shock and severe hemorrhage manifestations [9, 89]. Increased vascular permeability in severe dengue results in decreased circulating plasma volume, haemoconcentration, and pleural and peritoneal effusions that result in severe life-threatening shock [93–96]. Numerous epidemiological pieces of evidence indicate that appearance of the life-threatening manifestations during severe dengue occurs shortly after the defervescence stage of dengue disease, when the peak of viremia passed, meaning that host innate and adaptive immune responses have cleared the virus from host tissues [97, 98]. At this time, a transient vascular leakage pathology is observed followed by a rapid recovery in association with the late febrile phase. This association led to the suggestion that the key biological mechanisms such as alterations on the vasculature that leads to the pathogenesis of clinical complications during DENV infection are rather functional than the structural changes in the endothelium and are primarily a consequence of short-lived biological mediators closely linked to the host immune responses [93–96].

Although many severe infections occur upon secondary encounters with heterologous DENV serotypes [9, 99], suggesting an immune-mediated process is involved, the multifactorial immunopathogenic process of DENV infection implies a complex interaction between distinct viral and host processes that sometimes leads to increased virus infection, exacerbated immune responses, and the appearance of life-threatening severe manifestations such as severe plasma leakage, hemorrhage, and organ failure. Higher virus pathogenicity (virulence), preexisting serotype cross-reactive antibodies, activation of DENV-infected immune cells [e.g., monocytes and mast cells (MCs)], T cell responses, activation of complement pathways, the potential infection of endothelial cells, and the new pathogenic roles of the secreted NS1 of DENV may work synergistically to induce the release of vasoactive cytokines which results in increased endothelial permeability causing vascular leakage and pleural effusion, which are still considered pathognomonic features of severe dengue that leads occasionally to shock and death [8, 9, 35, , 96, 99–110]. In this section, we highlight in two parts I and II, the immunological events elicited by DENV infection, which have been suggested to play a key role in the development of severe dengue manifestations.

4.1 Dengue virus tropism and infection of immune cells

Numerous *in vitro* studies have shown that DENV is able to infect a variety of cell types including epithelial cells, endothelial cells, hepatocytes, muscle cells, dendritic cells, monocytes, B cells, and mast cells [65, 66, 111–117]. Several autopsies and *ex vivo* studies have found the presence of DENV antigens (e.g., envelope protein, NS3) in some tissues such as the skin, liver, spleen, lymph node, kidney, bone marrow, lung, thymus, and brain [56, 67, 68, 118–122]. However, infectious virus particles have not always been isolated from all these organs but only from the liver and peripheral blood mononuclear cells (PBMCs), suggesting that: (a) the presence of DENV antigens such as the structural proteins E, pre-M, and C in several organs may not always be associated with the evidence of productive viral infection and severe organ pathology and (b) the immune cells and liver may be the main targets for DENV replication during the dengue disease

[67]. In animal models such as the alpha/beta (IFN-α/β)-deficient mice (*Ifnar*$^{-/-}$) and nonhuman primates, DENV has been recovered from the spleen, liver, peripheral lymph nodes, and the central nervous system [123–127]. However, the absence of an appropriate animal disease model has largely hampered with the understanding of the role played by DENV tropism *in vivo*. Sustained viral replication and severe manifestations have been observed in *Ifnar*$^{-/-}$ mice after infection with DENV, which gives a clear advantage to study DENV pathogenesis *in vivo*, but the absence of intact IFN signaling is a limitation that must be considered when interpreting data [128].

The fact that DENV can infect many mammalian and insect cell types *in vitro* and *in vivo* suggest there are different molecules or cellular routes that might be controlling virus attachment and internalization, resulting in productive infection [63]. Numerous studies have shown that C-type lectins including DC-SIGN (CD209) and C-type lectin domain family 5, member A (CLEC5A) expressed on dendritic cells and macrophages act as cellular receptors for DENV [129–131]. Other extensively studied DC receptors are the mannose receptor (MR), Langerins, Fc-receptors, TIM3, TIM4, and AXL [63, 65, 132, 133]. Contrary to the DC-SIGN that may primarily function as a viral attachment factor, DENV binding to CLEC5A (C-type lectin domain family 5, member A), highly expressed by monocytes, macrophages, neutrophils, and dendritic cells, has been shown to induce the production of antiviral and pro-inflammatory cytokines suggesting that this C-type lectin may act as a cognate receptor for dengue virion [131, 134]. These cytokines include type I IFNs and chemotactic factors such as migration inhibition factor (MIF), monocyte chemotactic factor (MCP), and IL-8 [102, 134]. DENV infection of DCs also induces the production of matrix metalloproteinases (MPPs), MMP-2 and MMP-9, which induces migration of DCs to lymph nodes where virus further replicates before it disseminates into the blood circulation [135]. In the skin, DENV also infects mast cells that can be activated leading to degranulation and increased secretion of various inflammatory cytokines (IL-1, IL-6, TNF-α, and IFN-α), chemokines (CCL5, CXCL12, and CX3CL1), and chymase, the latter being a protease found circulating at high levels in the blood of dengue patients, suggesting a potential role in the development of severe dengue that contributes to vascular leakage [115, 136–140]. All these innate immune processes together lead to an antiviral state in nearby cells, generating an inflammatory response and recruitment of natural killer (NK) cells to combat DENV infection [54, 141].

Along with DCs, monocytes and macrophages are also the primary targets of DENV infection [142, 143]. In lymphoid and nonlymphoid tissues, macrophages are considered the primary reservoirs of DENV after its dissemination from the skin [144]. Macrophages susceptible to DENV have been found in different organs in the mouse models or human autopsies, namely, Kupfer cells in the liver, alveolar macrophages in the lungs, dermal macrophages, microglial cells (brain and spinal cord), and monocytes in the peripheral blood [118, 120, 122, 145–147]. Comparable to DENV infection of DCs, DENV can use an array of cell surface receptors to infect monocytes and macrophages, including mannose receptor (CD205), CD14-associated protein, heat shock proteins (HSP70/HSP90), DC-SIGN (CD209), CD300a, AXL, TIM4, PD1, and the Fc receptors, particularly FcγRI (CD64) and FcγRII (CD32, 63). These two Fc-Rs play major roles in enhancing DENV infection of monocytes and macrophages, particularly during secondary infections [148–151].

Other populations of immune cells including NK cells can also be activated during DENV infection, particularly in patients with DHF compared to those with DF [141, 152, 153]. Additionally, B cells and T cells have been studied to test permissiveness to DENV, but these studies have resulted in contradictory results [154–156]. *In vitro* studies using B cell and T cell lines (e.g., *Raji* cells, Daudi, and Jurkat) and primary B cells derived from healthy human peripheral blood mononuclear cells

(PBMCs) have revealed the potential role of these cells in DENV replication, both in presence and absence of heterologous antibodies [67, 155, 157–159]. Additional studies using a humanized mouse model found that DENV infected both B and T cells accompanied by an important production of pro-inflammatory cytokines such as IL-6 and TNF-α, like monocytes and macrophages [160]. Despite this evidence, the role of lymphoid cells such as B and T cells in DENV tropism and replication needs further exploration.

4.2 DENV infection and the host innate immune responses

Although plasma leakage in severe dengue occurs at the end of the acute illness, there is substantial evidence that the pathophysiologic processes start at the earliest stages of DENV infection [95, 96]. Introduction of DENV particles along with mosquito saliva triggers a variety of host innate immune responses leading to the production of antiviral and pro-inflammatory cytokines mostly from the immune cells exposed to DENV [57, 62, 138]. At this stage, innate immune cells are the first to respond to infection through stimulation of patterns recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs) as well as endogenous molecules released from damaged cells, termed damage-associated molecular patterns (DAMPs) [161, 162]. PRR recognition triggers the production of cytokines and chemokines, which induces a local antiviral state [54, 55]. This local innate response could potentially play an important role in modulating local viremia and virus dissemination by recruiting susceptible target cells for DENV infection at the inoculation site [57, 62, 144].

PRRs include transmembrane proteins such as the Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as well as cytoplasmic proteins such as the retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) [161]. These are an essential part of the innate immune response against the virus, sensing viral replication in the cytoplasm [161, 163]. The PRRs that are associated with DENV recognition after infecting target cells are the cytoplasmic retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated protein 5 (MDA5) and the endosomal Toll-like receptor 3 (TLR3) and TLR7 [164–166]. Recognition of DENV RNA by TLR-3 results in the production of type I IFN and chemokines such as IL-8 via sensing of phosphate-containing RNA and long double-stranded RNA (dsRNA) in the cytoplasm or inside endosomal compartments [167, 168]. DENV infection in nonhuman primates demonstrated that the administration of TLR-3 and TLR7 agonists resulted in significantly decreased viral replication and increased production of pro-inflammatory chemokines as well as increased production of antibodies targeting DENV [169], indicating a protective role for TLRs during DENV infection.

Additional pathways such as the cyclic GMP-AM synthase (cGAS), a DNA-sensor pathway which triggers the simulator of IFN genes (STING) pathway are also activated during DENV infection leading to the production of type I IFN and activation of TLRs (TLR9), an endosomal PRR that recognizes cytoplasmic DNA originated from mitochondrial damage [170–172]. In addition to type I IFN production, small RNAs such as micro RNAs (miRNA) and the complement system are important components of the innate immune response against viral infections [173, 174]. miRNAs are processed by and interact with the proteins in the RNA interference (RNAi) pathway, such as Dicer, Drosha, Argo1, and Argo2 [175]. RNA interference (RNAi) is an important antiviral defense response in plants and invertebrates [176]. In DENV infection, knockdown of these components resulted in increased DENV replication in mammalian cells, suggesting that the RNAi pathways may play important roles in the cellular anti-DENV responses [177, 178]. Additional evidence showed that DENV can interfere with RNAi pathways in human hepatocytes cells via NS4B and subgenomic flavivirus RNA (sfRNA) interactions with Dicer's ability to process small

RNA *in vitro* [179]. sfRANs are abundant noncoding RNA sequences derived from the stalling of the host 5′-3′ exoribonuclease XRN1/Pacman in the 3′-untranslated regions (3′UTRs) of the viral genomic RNA [180]. sfRNAs have been shown to block exonuclease XRN1, increasing the overall messenger RNA stability within the host cell which may also benefit the viral RNA [181–183]. However, evidence for RNAi contribution to mammalian antiviral defense are few and still controversial [179]. miRNAs have been shown to regulate TLRs and IL-1 signaling pathways in response to viral infection, which provides control of host innate immune responses [184]. So far, there have been reported several cellular miRNAs (miRNAome) that are modulated during DENV infection of mammalian cells, mainly related to the regulation of IFN-β signaling pathways [185–188]. Some of these miRNAs have been proposed to be used as biomarkers in dengue-infected patients [189, 190]. Interestingly, modulation of microRNA expressions have been also described upon DENV infection of insect cells (e.g., C6/36 cells) as well as adult vector mosquitoes such as *Aedes albopictus* [191–193], suggesting that DENV might also regulate the activation of these antiviral RNAi pathways in vector mosquitoes, potentially avoiding viral clearance that promotes viral replication and transmission [194]. Despite RNAi constituting an evolutionarily conserved phenomenon of the mosquito-innate immune response to virus infections [195], viruses have found ways to subvert the RNAi-mediated antiviral responses in vector mosquitoes to manipulate miRNA profiles to their own benefit [196].

Regarding the complement system, this multifaceted pathway has been shown to limit DENV replication; however, excessively activated complement components have been also associated with disease severity [197]. The complement cascade constitutes an integral component of the immune system, composed of many plasma proteins that once activated can initiate a proteolytic cascade, resulting in the release of chemokines, facilitation of particle phagocytosis via opsonization, and deposition of the cell-killing membrane attack complex (MAC) designed to target and destroy foreign pathogens such as viruses [174]. Activation of the complement system occurs via three convergent pathways: the classical, the lectin, and the alternative pathways [174]. *In vitro* experiments showed that DENV replication enhances complement activation [197–199]. Additionally, clinical and *in vivo* studies have shown that excessive consumption of some complement components (e.g., C3, C4, and factor B) contributed to severe manifestations by increasing the levels of complement-activated products which enhance vascular permeability to cause severe dengue disease [9, 108, 109, 200]. In fact, increased circulation of anaphylatoxins (C3a, C4a, and C5a) in the blood of severe patients correlated with symptoms of vascular leakage [109, 200]. In autopsy studies from children who died of acute severe dengue manifestations (DHF/DSS), augmented deposition of complement components from both classical and alternative pathways were found on hepatocytes which results in severe liver damage and death [120]. Altogether, these data support the hypothesis that exacerbated complement activation influences dengue disease immunopathogenesis leading to disease severity [197].

4.3 DENV subversion of antiviral responses

The first barrier to overcome for successful viral infection is the rapid innate immune responses of the host, including type I IFNs, inflammatory cytokines, complement responses, NK cells, apoptosis, and autophagy [201, 202]. These innate immune responses are meant to defeat viral infections by engaging specific viral components (e.g., RNA and DNA) leading to activation of immediate protective defense mechanisms such as the rapid recognition of PAMP in nonimmune and innate immune cells [161]. IFN production is a key goal of PRR activation for viral pathogens, and DENV is highly susceptible to effective induction of both type

I (IFN α/β) and type II (IFN γ) interferons [124, 203, 204]. Accordingly, *in vivo* DENV infection of wild-type mice causes little disease; in contrast, in mice lacking of type I IFN receptors (IFANR), DENV infection causes mortality [126].

Secreted type I IFNs trigger autocrine and paracrine induction of cellular antiviral responses and warning signals to noninfected adjacent cells, such as the expression of the interferon stimulated genes (ISGs) [205, 206]. ISGs have been shown to exert numerous antiviral effector functions, many of which are still not fully described [207]. Upon DENV infection, RLRs are activated to trigger antiviral responses based on the induction of type I IFN and pro-inflammatory cytokines [208]. The binding of type I IFN with its receptor activates multi-subsets of ISGs through JAK-STAT signaling which amplifies and sustains the initial antiviral responses [207, 209, 210]. However, ISGs can also be activated in IFN-independent pathways during DENV infection [211]. DENV infection has been shown to trigger the transcriptional activation of ISGs *in vivo* and *in vitro* [208, 212–215]. For instance, a tripartite motif (TRIM) protein encoding gene, *TRIM69*, is induced during DENV infection as an ISG. TRIM69 restricts DENV replication by direct interaction with DENV NS3, which mediates its polyubiquitination and degradation in a process called ISGylation [216]. In addition to ISGs, activation of the transcription factors IRF-3, IRF-7, and NF-κB through either the TLR or RIG-I/MDA5 pathways results in the production of type I IFN which contributes to anti-DENV immunity [217, 218]. IRF-3 and IRF-7 are part of the interferon regulatory factors (IRFs) considered the master regulators of the type I IFN production that contribute to the suppression of viruses [219]. Due to the central importance in viral defense, many pathogenic viruses, including DENV, have evolved mechanisms to suppress IRF signaling. In the case of DENV, the nonstructural proteins restrict IRF3 and IFN response which facilitate DENV replication and virulence [220].

In recent years, considerable advances have been made toward understanding of the specific IFN antagonistic mechanisms evolved by DENV to subvert these intracellular antiviral mechanisms and directly inhibiting these cellular signaling cascades, which results in enhanced virus infection, pathogenesis, and disease [167, 221]. This is supported by the increased susceptibility of mice deficient in IFN-α/β and IFN-γ receptors (AG129) to DENV infection as compared to wild-type mice [124, 126, 127]. Although IFN response is antagonized in mouse, human cells still induce high levels of IFN production in response to DENV, so this pathway is not entirely abrogated in humans during infection [203, 222]. Accordingly, humans infected with DENV have high levels of circulating of type I and type II IFNs [223–225]. Strong IFN-α responses have shown to correlate with milder dengue clinical conditions [226]. Similarly, the levels of the dengue-related gene expression of ISGs have been reported to be lower in patients with more severe disease [227–229] suggesting that DENV may abrogate IFN responses to facilitate viral infection which results in severe manifestations.

From the viral perspective, DENV uses its nonstructural (NS) proteins to block and inhibit the antiviral sensing pathways in infected cells. NS2a, NS3, NS4a, NS4b, and NS5 prevent the virus from being sensed by RIG-I, inhibiting IFNβ induction [230–233]. NS2a, NS4a, and NS4b complex inhibits STAT1 signaling after IFNAR activation *in vitro* [233, 234]. NS5 induces proteasomal degradation of STAT2 which inhibits IFN-mediated response [230]. NS2b induces degradation of cGAS, which prevents DNA sensing resulting from mitochondrial damage [170, 171, 235]. The NS2b/3 protease complex cleaves STING which inhibits IFN production [236]. This phenomenon has been shown to occur in human but not nonhuman primates, suggesting that DENV may have evolved to increase viral titers in human populations, while maintaining decreased titers and pathogenicity in rare animals would serve as a sustainable reservoir in nature [237].

In addition to NS proteins, flavivirus sfRNAs have been described to regulate the innate immune responses via binding and inactivating RNA-binding proteins which are crucial for innate immunity [180, 238]. DENV 3′UTRs possess RNA structures

necessary for viral genome cyclization, viral RNA synthesis, translation, and replication [239]. sfRNAs regulate the pathogenicity in both mammalian and mosquito cells after interacting with proteins such as TRIM25 to inhibit RIG-I signaling and translation of ISGs [73, 240, 241]. Interestingly, reduced IFN responses have been found during DENV outbreaks where the infecting DENV serotype produced greater levels of sfRNA than the less pathogenic strains [100, 240]. Thus, high levels of sfRNAs may cause an epidemiological fitness of DENV, which results in lower stimulation of RIG-IMDA5 RNA sensors and reduced production of IFN, causing higher viremia levels that could be translated in more infections and severe diseases.

On the other hand, DENV utilizes the endoplasmic reticulum (ER) of host cells for replication and assembly. In this process, the ER undergoes extensive rearrangements and expansion that requires *de novo* synthesis of viral proteins [71]. Accumulation of unfolded proteins in the ER lumen leads to an unfolded protein response (UPR), a pro-survival cellular reaction induced in response to DENV-mediated ER stress [242, 243]. DENV has evolved to manipulate the UPR to cope with ER stress which hijacks the host cell machinery to evade the host immunity, facilitating viral replication [112]. Distinct *in vitro* and *in vivo* studies have shown that DENV induced ER stress and manipulates the host metabolism and protein production by increasing the autophagy (lipophagy) activity, viral replication, and pathogenesis through UPR signaling pathways [244–247]. Autophagy is the lysosomal degradation of cytoplasmic contents, which results in the recycling of cellular macromolecules as well as the activation of cellular host responses to starvation or stress [248]. Autophagy has been implicated as an innate immune response that would engulf and destroy pathogens by degrading cytosolic contents [249, 250]. In DENV infection, functional autophagy components have been shown to either promote or restrict viral RNA replication and virus production [251–253]. However, DENV has found ways of preventing autophagic processing and degradation of viral components [254, 255]. Several studies have linked DENV induction of autophagy to the regulation of lipid metabolism, leading to increased degradation of lipid droplets that produces more fatty acid material important for viral replication [247]. In this process, the NS3, NS1, and C proteins of DENV have been found to increase fatty acid biosynthesis and recruitment of lipid droplets to the DENV replication complex, facilitating viral particle assembly [256, 257].

Furthermore, several studies have shown that lipids and lipoproteins play a role in modifying DENV infectivity in both mammal and insect cells *in vitro* [258, 259]. Modulation of cholesterol levels in the host cells facilitates viral entry, replication, virus assembly, and control type I IFN response [260, 261]. This modulation involves the regulation of cholesterol levels, expression of cholesterol receptors as well as changes in cholesterol synthesis related to important modifications in the cellular metabolism [114, 262, 263]. Interestingly, clinical studies have found that levels of total serum cholesterol and LDL-C levels are modulated over the course of dengue illness, with generally lower levels associated with increased dengue severity [264–266]. In general, low cholesterol levels have been associated with critical illness related to sepsis and vascular disorders [267]. Thus, the association of cholesterol with severe dengue outcome may be an important indicator of the pathophysiology of DHF/DSS.

About the complement pathway, DENV has evolved strategies to limit recognition and activation of the complement cascade [108, 165]. NS1 is the only flavivirus protein that is secreted by infected cells and has been shown to modulate the complement pathway [14, 268]. NS1 promotes efficient degradation of C4 to C4b to protect DENV from complement-dependent neutralization [13, 269]. The NS1 protein of DENV and other flaviviruses such as WNV NS1 interacts with some components of the alternative complement pathway such as the C3bBb convertase, which limits the formation of C5b-9 membrane attack complex (MAC) [268, 270]. Additional studies have found that NS1 proteins from DENV, WNV, and YFV all attenuate classical and

lectin pathway activation by directly interacting with C4, which reduces C4b deposition and C3 convertase (C4b2a) activity [13, 271]. Also, anti-NS1 antibodies have been shown to induce complement consumption and C5b-9 generation [272]. Overall, through protein-to-protein interactions between the viral and host factors involved in antiviral responses and careful manipulation of cellular processes, such as ER expansion, autophagy and lipid metabolism, and complement pathways, DENV hijacks many host antiviral responses to facilitate virus replication leading to pathogenesis.

5. Concluding remarks (Part I)

Dengue is the most prevalent arboviral disease transmitted by mosquitoes, which poses an enormous burden to the public health systems worldwide as more than 40% of the world population is at risk of infection. The infection with any of the four DENV serotypes (DENV1–4) can lead to a wide spectrum of clinical manifestations that range from the asymptomatic or inapparent to moderate flu-like symptoms, known as dengue fever (DF), and life-threatening manifestations identified by the WHO, known as the dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), also known as severe dengue, with or without warning signs. In endemic areas where multiple DENV serotypes can seasonally circulate, distinct epidemiological studies have demonstrated that an individual human being can be exposed to sequential infections with distinct DENV serotypes, which poses a risk of developing severe manifestations such as DHF/DSS. This phenomenon has been attributed to the potential enhancement activity that the preexisting antibody response elicited from a previous infection with one serotype (e.g., DENV-1) may have on the infection with a different serotype (e.g., DENV-2). This process leads to an increased viral burden that triggers a series of immunological and cellular events (e.g., ADE, cytokine storm, skewed T cell responses, and complement pathways), which despite being intended to prevent the invasion and infection of the infecting viral pathogens, can induce host tissue damage leading to pathology and disease. The cellular and molecular mechanisms involved in this phenomenon will be explained in more detail in the Part II of this chapter entitled "Adaptive immune response and NS1 pathogenesis."

As an arthropod-transmitted virus (arbovirus), DENV is initially transmitted by an infected vector mosquito in which the virus has already been amplified after replication in its distinct tissues, starting at the midgut to finalize in the salivary glands, where a new transmission cycle begins after blood feeding from a new host (**Figure 2**). Following inoculation from the bite of an infected mosquito, viruses undergo replication in the local tissues such as the skin. In the skin, infectious virus particles along with mosquito saliva components including proteases and immunomodulatory proteins among others are sown in the epidermis and dermis, leading to an activation of a cascade of events including the recruitment of skin resident cells (e.g., Langerhans cells, mast cells, and keratinocytes) and new cells (e.g., T cells and neutrophils) into the site of the infection that later serve as viral targets for viral replication. After infection of target cells, sensing of viral products (e.g., PAMPs and DAMPs) results in the activation of innate immune responses (e.g., type I IFN chemokines), the first line of defense, which establishes inflammatory and antiviral states intended to prevent the virus to colonize and to replicate in the skin; however, DENV has elaborated several pathogenic mechanisms to hijack these responses and escape from the normal immune system processing, which results in its dissemination and seeds into the lymph nodes. There, DENV further replicates in monocyte lineage cells, resulting in a primary viremia after its systemic dissemination through the circulatory bloodstream, which results in the subsequent infection of peripheral tissues such as the liver, spleen, and kidney. Overall, the skin represents not only the first line of

defense against arboviruses but also the main place where viruses have learned to evade the host immune responses leading to invasion and dissemination toward the establishment of systemic host infection, which will potentially assure subsequent virus transmission into a new host. In this *Part I* of the chapter entitled "Dengue virus tropism, host innate immune responses, and subversion of antiviral responses," we discussed the distinct features of DENV as well as the biological and molecular mechanisms that can tilt the balance to either a local viral infection and dissemination through the skin or to the control and prevention of viral infection by the innate and adaptive immune responses at the site of the infection. Thus, the immunopathogenesis of arboviruses such as DENV in the skin is a critical step and must be a focus of future studies intended to reduce/block arthropod-borne transmission into humans.

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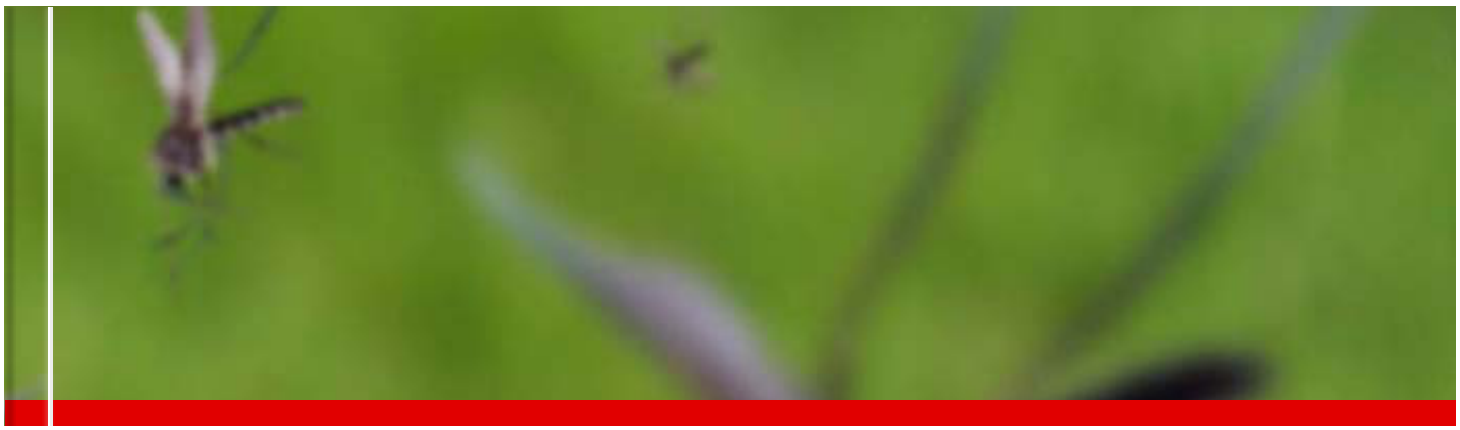

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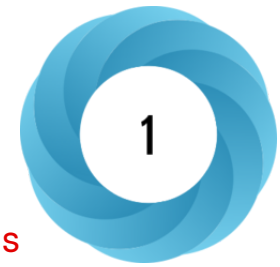

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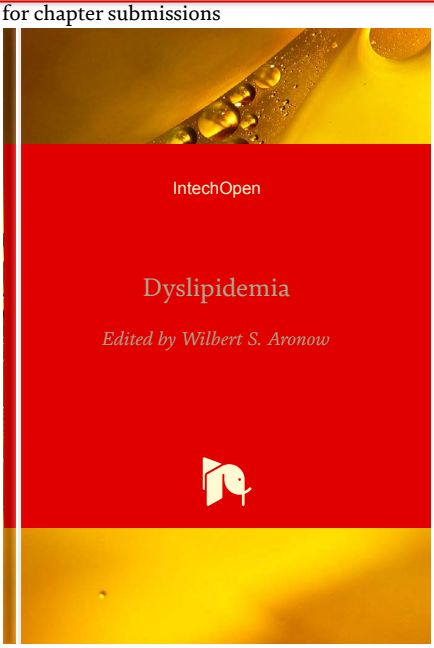

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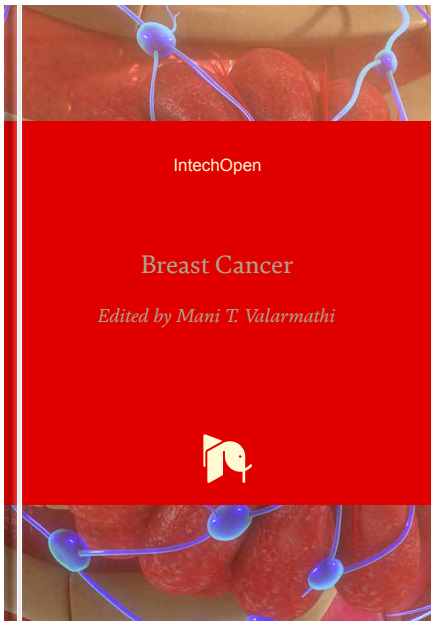

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