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Emerging Concepts in Dengue Pathogenesis: Interplay between Plasmablasts, Platelets, and Complement in Triggering Vasculopathy

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ABSTRACT: Dengue is a mosquito-borne disease caused by infection with dengue virus (DENV) that represents a serious and expanding global health threat. Most DENV infections are inapparent or produce mild and self-limiting illness; however a significant proportion results in severe disease characterized by vasculopathy and plasma leakage that may culminate in shock and death. The cause of dengue-associated vasculopathy is likely to be multifactorial but remains essentially unknown. Severe disease is manifest during a critical phase from 4 to 7 days after onset of symptoms, once the virus has disappeared from the circulation but before the peak of T-cell activation, suggesting that other factors mediate vasculopathy. Here, we present evidence for a combined role of plasmablasts, complement, and platelets in driving severe disease in DENV infection. Massive expansion of virus-specific plasmablasts peaks during the critical phase of infection, coincident with activation of complement and activation and depletion of platelets. We propose a step-wise model in which virus-specific antibodies produced by plasmablasts form immune complexes, leading to activation of complement and release of vasoactive anaphylatoxins. Platelets become activated through binding of complement- and antibody-coated virus, as well as direct binding of virus to DC-SIGN, leading to the release of inflammatory microparticles and cytokines and sequestration of platelets in the microvasculature. We suggest that the combined effects of anaphylatoxins, inflammatory microparticles, and platelet sequestration serve as triggers of vasculopathy in severe dengue.

KEY WORDS: Cell activation, immune dysregulation, immune complexes, inflammation

ABBREVIATIONS: CR: complement receptor; **DENV**: dengue virus; **FcγR**: Fcγ receptor; **MBL**: mannose-binding lectin; **NS1**: non-structural protein-1; **PAF**: platelet-activating factor

I. INTRODUCTION

Dengue is the most important mosquito-borne viral disease of humans worldwide, with more than two billion people living in endemic areas and 50 to 100 million new infections occurring each year.[1] Dengue is caused by infection with dengue virus (DENV), a positive-strand RNA virus of the family Flaviviridae with four distinct serotypes: DENV-1 -2, -3 and -4. DENV infections can be asymptomatic or produce a spectrum of clinical disease from mild, self-limiting illness to severe dengue, characterized by systemic plasma leakage that may culminate in shock and death.[2] Many factors have been associated with disease severity in dengue, including age, female sex, host immunity, and virus fitness,[3,4] and the etiology of

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the laboratory and clinical changes that characterize severe disease is likely to be complex. A key clinical feature of dengue is the occurrence of vasculopathy during a critical phase between 4 to 7 days after the onset of symptoms. Epidemiologic data indicate that immunity to one DENV serotype increases the risk of developing severe dengue upon infection with a second serotype to which the individual is immunologically naïve.[5,6] The leading hypothesis to explain this finding is antibody-dependent enhancement, in which sub-neutralizing concentrations of DENV-specific antibodies bind virus and facilitate infection of Fc receptor-bearing cells.[7] This phenomenon may contribute to increased virus production early in the course of infection, but it is not likely to be a direct driver of vasculopathy, as virus burden wanes several days before the critical phase.[8] Systemic T-cell activation with subsequent production of proinflammatory cytokines is a well-described event in severe dengue;[9] however, this too is temporally dissociated from disease, generally peaking after hemoconcentration and other signs of plasma leakage have occurred.[10] In this review, we consider three different components of the human response to DENV infection that occur coincident with the critical phase of infection: the acute B-cell response, activation and dysregulation of complement, and activation and depletion of platelets. Additionally, we discuss how the interplay between these responses may trigger the vasculopathy that defines severe dengue.

II. B CELLS IN IMMUNITY TO DENGUE

A. Differentiation of B Cells in Viral Infection

During a primary virus infection, naïve B cells located in the extrafollicular foci of secondary lymphoid organs can become activated without T-cell help, differentiating into antibody-secreting cells that contribute to the early immune response. Generally, these cells have not undergone affinity maturation and produce "natural" antibodies of the IgM isotype.[11,12] Alternatively, naïve B cells engage antigen and migrate to germinal centers where, with CD4+ T-cell help, they undergo somatic hypermutation, affinity maturation, and class switch recombination, becoming either plasmablasts or long-lived memory B cells. It is still unknown whether naïve B cells have the capacity to become long-lived plasma cells or whether only germinal center-derived plasmablasts or memory cells have this capability.[13,14] Long-lived plasma cells reside in bone marrow, where they are responsible for the maintenance of antibodies in serum. Memory B cells remain in secondary lymphoid organs and are able to react quickly after secondary antigenic challenge, becoming short-lived plasmablasts. Circulating human plasmablasts can be identified by flow cytometry as CD19+CD10– mature B cells that are CD27+CD21–CD38+CD20–, distinguishing them from activated memory B cells, which have low or absent expression of CD38 and continue to express CD20.[15,16]

B. B Cell Response to DENV

1. Humoral Immunity

The serum antibody response to DENV has been studied in depth. DENV-specific antibodies in immune individuals generally are broadly cross-reactive for all DENV serotypes,[17,18] although antibody specificities appear to be different during primary and secondary DENV infections and throughout the course of disease.[17–19] Other reports have characterized immortalized memory B cells isolated from immune individuals and have shown that the majority of B cells has specificity against envelope or precursor-membrane proteins that are reactive across serotypes and can induce antibody-dependent enhancement.[20–23] Antibodies specific to non-structural protein-1 (NS1), which is not incorporated into virions but is found in the cellular membrane or secreted into the extracellular environment,[24–26] are highly cross-reactive with very poor neutralizing capacity,[22] and studies have suggested that NS1-specific antibodies may contribute to pathogenesis.[27–29] The disparity between antibody half-life (several weeks) and the period of disease (several days) indicates that it is unlikely that NS1-specific antibodies are directly involved in increased capillary permeability. It is more plausible that

immune complexes with NS1 and virus particles serve to activate complement in DENV infection which impacts disease, as discussed in the following section.

2. Plasmablast Response during Acute DENV Infection

a. Kinetics of Plasmablast Expansion

Recent studies have shown that acute DENV infection results in a profound surge in the frequency of plasmablasts in the circulation peaking within 4 to 7 days after the onset of symptoms, coincident with the critical phase of disease.[30–33] Plasmablasts dominate the B-cell compartment during this expansion, frequently constituting 70% or more of circulating B cells, and the vast majority of these plasmablasts has specificity for DENV.[31,32] By comparison, plasmablast frequencies in healthy individuals are approximately 0.14% of circulating B cells,[34] and following vaccination against influenza or yellow fever viruses, this proportion increases to 2–3%.[35,36] In primary infection with HIV, which is characterized by hyperactivity and terminal differentiation of B cells and hypergammaglobulinemia, the proportion of B cells that is comprised of plasmablasts may reach 5%,[15] but only a small fraction of these plasmablasts is HIV-specific.[37] Hence, while a direct comparison remains to be conducted, the current data suggest that the frequency of virus-specific plasmablasts in individuals with acute DENV infection may be substantially higher than that induced by other virus infections. The degree of B-cell activation, proliferation, and apoptosis in acutely infected individuals approaches that of T cells,[31] although whether activated B cells themselves produce proinflammatory cytokines and contribute to immune activation in dengue remains to be determined.

b. Potential Contribution of Plasmablasts to Complement and Platelet Activation

Plasmablast expansion closely mirrors the onset of severe disease in DENV-infected individuals, and evidence in adult patients suggests that the magnitude of the response is greatest in severe secondary infections;[31] hence, it is tempting to speculate that plasmablasts contribute in some way to disease. Whether plasmablasts comply with the concept of original antigenic sin[38] by producing antibodies that preferentially neutralize the previously encountered virus rather than the infecting virus, facilitating antibody-dependent enhancement, is a key question, and data available to date are somewhat contradictory.[30–33] Recent studies suggest that plasmablasts that are expanded during acute secondary DENV infection produce antibodies with significantly different specificities from those made by long-lived plasma cells and secreted into serum.[39] The fact that virus production in plasma peaks several days before detectable expansion of plasmablasts makes it unlikely that plasmablast-derived antibodies play a significant role in promoting virus replication per se, although the possibility exists that tissue reservoirs of virus persist and are impacted by plasmablast-derived antibodies. Here, we consider an alternative contribution of plasmablasts to dengue pathogenesis—the production of virus-specific antibodies that leads to activation of complement and aids in the activation of platelets. In the following sections, we discuss the findings relating to complement and platelet activation in dengue and how this activation may ultimately trigger vasculopathy (Fig. 1).

III. COMPLEMENT ACTIVATION IN DENGUE

A. Complement Cascade

The complement system is a component of the innate immune response that can be activated through the classical, alternative, and lectin-dependent pathways, resulting in inflammation and lysis of pathogens and infected cells. The classical pathway is activated mostly by antibody-antigen complexes[40] that interact with C1 complex (C1q, C1r, and C1s). The lectin pathway is initiated by mannose-binding lectin (MBL) or ficolin in complex with MBL-associated serine proteases after interaction with carbohydrate residues on the surface of pathogens. The activation of the classical and lectin pathways leads to the activation of the C3 convertase (C4b2b). The alternative pathway is activated by the binding of C3b,

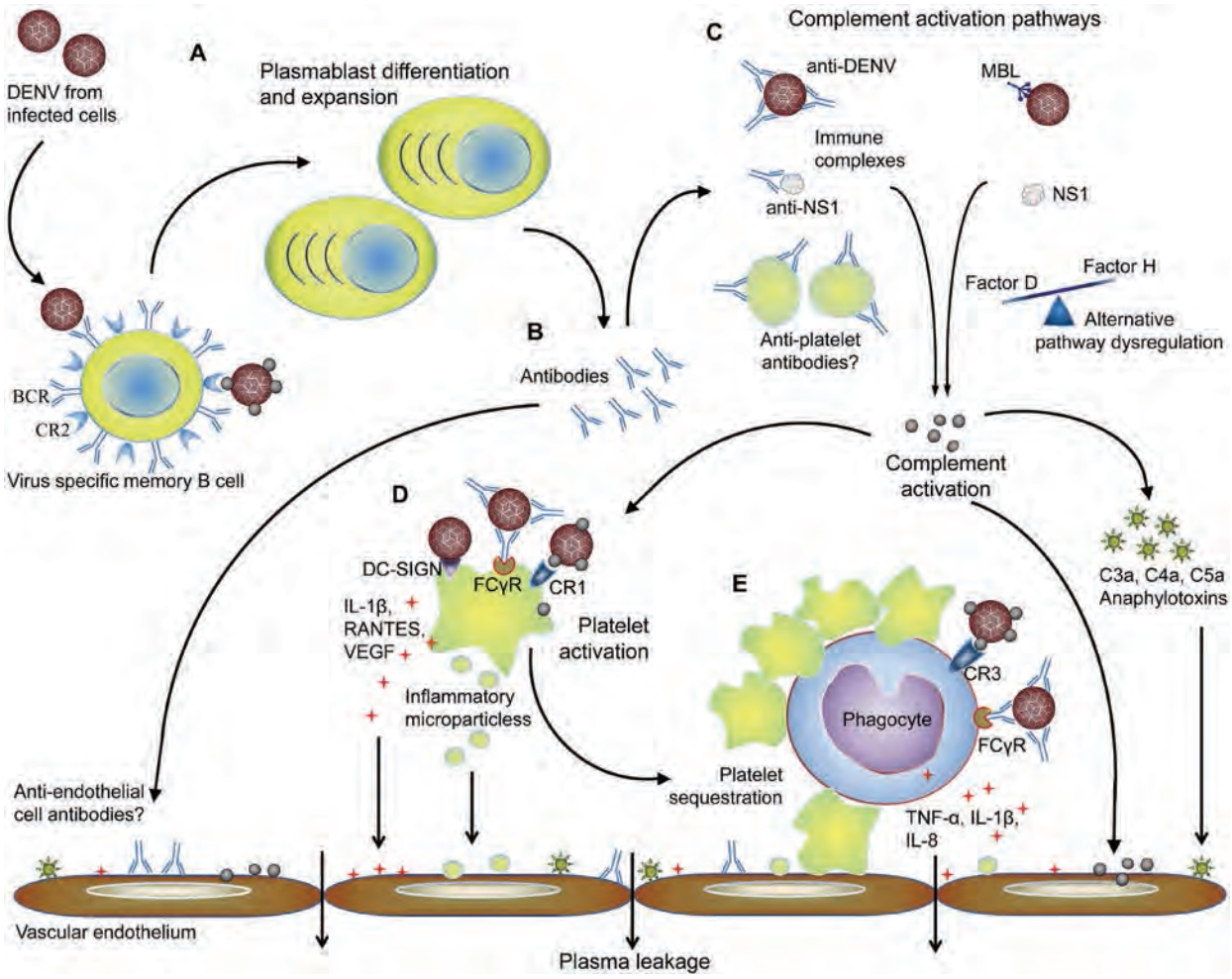

FIG. 1: Model of dengue vasculopathy: Interplay between plasmablasts, platelets and complement in the critical phase of infection. (A) DENV released from infected cells during a secondary infection binds to virus-specific memory B cells which differentiate and proliferate into plasmablasts, peaking at days 4 to 7 following the onset of symptoms. **(B)** Antibodies produced by plasmablasts form immune complexes with virus particles and with NS1, activating the classical pathway of complement, while antibodies targeting endothelium could potentially damage vascular endothelium directly. **(C)** Free NS1 and MBL bound to virus particles also lead to complement activation, which is amplified further by regulation imbalances through increases in factor D and decreases in factor H, which favor production of alternative pathway C3 convertase. Antibody coating of platelets may also lead to complement activation. Complement factors including vasoactive anaphylatoxins bind to endothelium and mediate plasma leakage. **(D)** Platelets become activated through binding of complement- and antibody-coated virus particles, by binding of DENV to DC-SIGN, and by direct binding of complement to the platelet surface. Activated platelets produce inflammatory cytokines and microparticles that act on vascular endothelium to promote plasma leakage. **(E)** Activated platelets are sequestered by phagocytes in the microvasculature, promoting thrombocytopenia and phagocyte activation, which is enhanced by binding of complement- and antibody-coated virus. Activated phagocytes release proinflammatory cytokines that may directly damage endothelium.

which is spontaneously generated by the hydrolysis of C3, to targeted surfaces. This surface-bound C3b interacts with factor B forming the complex C3bB.[41] The C3bB complex, in turn, is cleaved by factor D to yield the active form of the C3 convertase of the alternative pathway (C3bBb) that is further stabilized by properdin. C3 convertases cleave C3 into C3b, which is covalently bound to the targeted surface, and C3a, which is released. C3b contributes to the formation of C3 convertase of the alternative pathway and C5 convertase (C3bBbC3b or C4bC2bC3b). These proteases in turn cleave C5 into C5b that initiates formation of the membrane attack complex and C5a, a small released fragment. C3a, C5a, and C4a are anaphylatoxins, promoting inflammation through chemotaxis of leukocytes and fluid leakage.

B. Complement Regulation

The complement cascade is tightly controlled by a range of regulators present on cell surfaces and in plasma. The cell-surface regulators include complement receptor-1 (CR1/CD35), decay accelerating factor (CD55), membrane cofactor protein (MCP/CD46), and protectin (CD59), whereas the soluble regulators include C4b binding protein (C4bBP), factor H, factor I, clusterin, and vitronectin. These regulators work either by destabilizing and inactivating the convertases, by inactivating and degrading C3b into iC3b, C3c, and C3dg, or by inhibiting the formation of the membrane attack complex. Recognition of products of degradation of C3b through CR1, CR2/CD21, CR3/[CD11b/CD18] and CR4/[CD11c/CD18] mediates several important leucocyte functions.[42] CR1 engagement of C3b promotes phagocytosis and processing of immune complex-bound C3b. In addition, erythrocyte- and platelet-mediated immune complex clearance is also associated with CR1 function. CR2 recognizes iC3b/C3dg and reduces the activation threshold of B cells. CR3 and CR4 recognize iC3b, inducing synthesis of reactive oxygen metabolites and promoting phagocytosis (monocytes and neutrophils) and degranulation (neutrophils). Additionally, immune complexes can mediate cross talk between CR3 and Fcγ receptors (FcγR) in monocytes, inhibiting antiviral responses and producing anti-inflammatory cytokines, thereby altering the ability to combat infection.[43]

C. Increased Complement Activation in Dengue

1. Complement Activation and the Antibody Response

Several reports have demonstrated increased complement activation in DENV infection, with complement proteins across both classical and alternative activation pathways, including C4, factor B, and C3, being consumed as disease severity increases.[44–46] In addition, increased levels of the vasoactive products of complement activation, including C3a, C4a and C5a, are elevated in patients with severe dengue, and these anaphylatoxins could contribute directly to vascular leakage (Fig. 1).[46–50] As with plasmablasts, complement activation coincides temporally with the critical phase of DENV infection and the manifestations of vasculopathy.[44,46] Mechanistically, antibody responses against DENV are likely to be intimately linked to complement activation through formation of immune complexes that trigger the classical pathway. Increased levels of both virus and soluble NS1 protein are seen in severe dengue,[47] and immune complexes with either virus or NS1 are seen in secondary DENV infections and have been associated with severe disease.[45,50–52] The increase in immune complexes in severe dengue may be the result of exhaustion of the clearance capacity.[50] In some individuals, decreased immune complex clearance may result from a polymorphism in the FcγRIIa gene that is associated with reduced affinity to IgG1 and accumulation of serum DENV-specific IgG and immune complexes.[53] Immune complexes are found in skin rashes as well as kidney and brain in fatal cases of dengue, co-localizing with products of complement activation.[54,55] In addition, greater levels of DENV-specific IgG1 and IgG3, with greater ability to fix and activate complement then the other IgG isotypes, are found in severe dengue during the febrile acute phase of infection.[56,57] Interestingly, after natural infection, most circulating DENV-specific

memory B cells in peripheral blood produce IgG1,[58] and if this property extends to plasmablasts, it would greatly promote complement activation.

2. Antibody-Independent Mechanisms of Complement Activation in Dengue

Dysregulation of the alternative pathway contributes to overall complement activation in dengue. In patients with severe dengue relative to those with mild disease, serum levels of factor D that drive alternative pathway C3 convertase are increased, whereas levels of the regulatory protein factor H are reduced. This effect is not a result of preferential loss of small molecules through fluid leakage, as factor H is a large complex molecule of 155 kDa, six times the size of factor D, yet it is factor H that is reduced in severe disease. The imbalance favors complement activation and is consistent with increased levels of C3 convertase and the corresponding consumption of C3 seen in severe dengue (Fig. 1).[46] A genetic polymorphism in factor H gene is associated with increased basal protein levels and is protective against severe dengue,[59] consistent with a role for loss of this regulatory pathway in disease pathogenesis. In addition to alternative pathway activation, MBL can bind to envelope protein of DENV[60] and thus could trigger the lectin pathway of complement activation during infection (Fig. 1). During severe dengue, levels of functional (oligomerized) MBL are increased relative to mild disease.[46] Moreover, individuals with a MBL genotype associated with reduced levels of protein are less likely to develop thrombocytopenia during DENV infection than patients with the wild-type genotype,[61] suggesting an association between complement activation and platelet consumption. Robust genetic association studies using larger numbers of individuals are warranted to better determine the association between specific complement factors and disease severity in dengue. Finally, viral NS1 protein can directly activate complement, and increased levels of NS1 are associated with increased disease severity.[47] NS1 is not likely to drive endothelial dysfunction directly, as primary DENV infections often are associated with high circulating levels of NS1 in the absence of severe disease.

IV. IMPACT OF DENV INFECTION ON PLATELETS

A. Thrombocytopenia

A key clinical correlate of disease severity in dengue is thrombocytopenia. Platelet-count decline is temporally coincident with the critical phase of infection and the onset of vasculopathy and plasma leakage.[8] Megakaryocytes are the main target for DENV in the bone marrow[62,63] and there is evidence for the presence of DENV in platelets from DENV-infected patients.[64,65] Bone-marrow suppression, impaired thrombopoiesis, and increased peripheral platelet destruction are potential mechanisms that lead to dengue-associated thrombocytopenia.[66] DENV propagation in bone marrow[67,68] may contribute to suppression of megakaryopoiesis through inhibition of proliferation and differentiation of hematopoietic progenitors[69–72] and alteration of cytokine production in bone marrow.[73,74] In addition, dengue thrombocytopenia has been correlated with complement activation and high levels of C3a and C5a. Antibody-mediated platelet destruction, complement-mediated platelet lysis and hemophagocytosis have all been proposed as mechanisms to drive thrombocytopenia,[75–78] as discussed below.

B. PLATELET ACTIVATION IN DENGUE

1. Antibody-Mediated Activation and Sequestration of Platelets

Recently, platelet activation and apoptosis, which increase platelet aggregation with phagocytes in the microvasculature and apoptotic platelet clearance, were shown to be major contributors for dengue-associated thrombocytopenia.[79,80] DENV directly activates platelets[64,80,81] through a mechanism that may involve DC-SIGN,[80,82] a C-type lectin that mediates DENV infection of dendritic cells.[83] In contrast to dendritic cells, it is likely that the effect of DENV on platelets is independent of productive infection because platelet activation, apoptosis, and thrombocytopenia peak during the non-viremic phase

of disease.[79,80] Host factors that are recognized by platelet signaling receptors resulting in platelet activation such as thrombin and platelet-activating factor (PAF) are also increased in DENV infection.[84–86] A potentially major factor for platelet activation in dengue is the presence of immune complexes, which stimulate platelet activation through FcγRIIa and sensitize platelets to further activation by thrombin (Fig. 1).[87,88] Because FcγRIIa can mediate DENV entry into macrophages,[89] ligation of DENV immune complexes to FcγRIIa on platelets probably also contributes to platelet activation in secondary DENV infection. DENV-specific antibodies can potentiate DENV binding to platelets.[90] Platelet FcγRIIa has also been demonstrated to have roles in *Staphylococcus aureus*–induced platelet activation,[91] and this is enhanced by complement factors such as C1q,[92,93] providing a further link between antibodies, complement and platelet activation in dengue.

2. Complement-Mediated Activation of Platelets

Complement likely plays a major role in activation and subsequent depletion of platelets in dengue, and consumption of C3 parallels thrombocytopenia in severe disease.[46] Complement could promote platelet sequestration during platelet-mediated clearance of immune complexes[90] or subsequent to virus deposition on the platelet surface, which would then be targeted by virus-specific antibody to activate complement.[94] Platelet activation could also occur following binding of uncleaved complement proteins to CR1[95] or potentially after binding of cross-reactive NS1-specific antibodies to the platelet surface, subsequently activating complement.[75]

3. Consequences of Platelet Activation in Dengue

a. Release of Mediators of Inflammation

Platelet activation results in expression of P-selectin,[80,96,97] a glycoprotein in platelet α-granules that translocates to the platelet surface during degranulation,[98] and surface expression of P-selectin is seen in platelets from individuals with DENV infection.[79,80] Platelet activation by DENV also results in release of the chemokine RANTES/CCL5, a preformed factor stored in platelet granules.[82,98] Interestingly, cytokines that are stored in platelet granules, including RANTES/CCL5, PF4/CXCL4, MIP-1α/CCL3, VEGF and PDGF,[98] are increased in plasma from patients with severe dengue, associated with decreasing platelet counts.[99–103] Considering that platelets are a major source of these mediators, the secretion of chemokines by platelets that become activated during DENV infection could be an important pathogenic mechanism (Fig. 1). These observations are in agreement with others demonstrating that thrombocytopenia and disease severity are reduced in mice lacking the receptor for PAF,[104] as well as CCR2 and CCR4, receptors for the chemokines MIP-1α/CCL3 and RANTES/CCL5, respectively.[105]

b. Platelet Activation and Increased Vascular Permeability

Beyond the release of preformed granule-stored factors, the ability of activated platelets to splice constitutive intron-containing RNA to produce mature tissue factor and IL-1β mRNA was recently demonstrated.[106–108] Platelet neo-synthesis of tissue factor and/or IL-1β has been implicated as a main mechanism in inflammation and dysregulated hemostasis in bacterial sepsis, malaria, and most recently, in dengue.[109–112] Importantly, analysis of platelets from patients with dengue together with *in vitro* models indicate that DENV-triggered IL-1β synthesis in platelets is a mechanism for endothelial activation and increased vascular permeability (Fig. 1).[112] Synthesis of IL-1β occurs in parallel with the assembly of the NLRP3-inflammasome, which mediates IL-1β processing and release into microparticles.[112] Platelet shedding of microparticles likely contributes to the pathogenesis of dengue not only by delivering IL-1β but also through release of tissue factor and RANTES. The precise contribution of circulating microparticles to vasculopathy and vascular instability in severe dengue syndromes is currently under investigation.

c. Platelet Interaction and Sequestration with Leukocytes

Platelet activation also supports the adhesion of

platelets to leukocytes. Onlamoon and colleagues[113] found that DENV elicits platelet-monocyte and platelet-neutrophil aggregates in a primate model of dengue, and platelet-monocyte aggregates have been also observed in mild dengue in humans.[114] In these interactions, the binding of P-selectin on activated platelets to P-selectin glycoprotein ligand-1(PSGL-1) on leukocytes is a dominant molecular event.[115,116] This molecular interaction not only tethers the cells together but also triggers gene expression pathways and induces functional responses by the leukocytes.[115,116] Signals delivered to the monocytes by ligated PSGL-1 are amplified by soluble factors such as PAF, RANTES, and IL-1β,[116–118] and at least two of these factors are known to be secreted by DENV-activated platelets.[112] Interestingly, cytokines that are synthesized as a result of platelet-dependent signaling of monocytes, such as TNF-α, IL-1β, IL-8, and MCP-1,[116–118] are also recognized as important pathogenic factors in severe dengue.[119–122] The aggregation of activated platelets with themselves or with monocytes and neutrophils could lead to their sequestration in the microvasculature (Fig.1), as has been described in sepsis,[123–125] a condition that has many parallels to severe dengue.[126] Platelet activation and thrombocytopenia are considered ubiquitous in human septic syndromes, and sequestration of platelets in microvascular beds can induce vascular damage in part through deposition of fibrin.[127,128] Whether aggregates of platelets and phagocytes become sequestered in the microvasculature in patients with severe dengue is a critical question that needs to be addressed.

V. CONCLUSION

In this review, we focused on three seemingly disparate aspects of the host response to DENV infection; each coincides temporally with the critical phase of infection and the development of the severe form of disease. We have emphasized the interplay between the acute virus-specific plasmablast response, the activation of complement, and the activation and sequestration of platelets, and we have proposed a model of how these interactions could provide multiple triggers of vasculopathy leading to plasma leakage and severe disease. Several questions remain to be addressed. The ineffectiveness of short-course oral corticosteroid therapy in preventing the development of severe disease in children with DENV infection has cast doubt on the contribution of immune dysregulation in dengue pathogenesis.[129,130] While this finding may be inconsistent with a mechanistic role of activated T cells and the ensuing cytokine storm in severe dengue,[9] the extent to which corticosteroids would suppress the key processes of complement and platelet activation described in our model is not clear. It is important to recognize that severe dengue is a syndrome with a complex pathogenesis, and it is possible that no single pathway or potentially even set of pathways entirely accounts for this disease. Future studies using clinical specimens from dengue patients with different disease outcomes will help determine the relative contributions of factors described in this review in mediating dengue pathogenesis.

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