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## A View of Platelets in Dengue

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/intechopen.73084>

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### Abstract

Platelets were mainly associated with coagulation and hemostasis; however, other biological effects have been attributed to platelets, including angiogenesis, extracellular matrix synthesis, inflammation, and immune response. Dengue virus infection causes 200 million cases of severe flu-like illness annually, escalating to life-threatening hemorrhagic fever or shock syndrome. Some hypotheses are postulated for immunopathogenesis of dengue, including antibody enhancement theory, T-cell activation of cross-reactive memory, and original antigenic sin. All hypotheses, to some extent, induce an overproduction or a skewed profile of cytokine release, giving rise to the term cytokine storm/cytokine tsunami. Although thrombocytopenia is typical of both mild and severe diseases, the mechanism triggering platelet reduction is incompletely understood. In dengue, platelets are one of the major cell populations affected by direct and/or indirect mechanisms of infection. It is common to observe both thrombocytopenia and platelet dysfunction in dengue, both strongly related to the clinical outcome. Thus, platelets are frequently affected in dengue, either for alteration of their own functionality, for “silent transport” of virus, or as an anti-viral immune cell. In this way, we describe some of functional aspects of platelets on dengue, observing circulating mediators, intraplatelet proteins contents, morphology, activation markers, and ability to interact with dengue virus.

**Keywords:** dengue, platelets, thrombocytopenia, dysfunction of platelets, immunopathogenesis

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### 1. Introduction

As the first cellular components accumulate at sites where there is vascular wall damage, platelets rapidly initiate events such as aggregation, exocytosis of granule constituents, adhesion protein expression, cytokine, and others inflammatory mediator's secretion and directly interact with endothelial cells and immune cells. In addition, they can perform the synthesis

of new proteins through their complex post-transcription repertoire for post-activation translation, corroborating the existence of potential biological functions of platelets. In dengue, platelets are one of the major cell populations affected by direct and/or indirect mechanisms of infection.

### 1.1. An overview of immunopathogenesis of dengue

Dengue is one of the arboviruses transmitted by mosquitoes of the genus *Aedes* in a human-mosquito-human cycle. It is endemic in more than 120 countries, where 50 to 100 million infections occur each year, which correspond to 55% of the world population live at risk of infection. Therefore, dengue has the greatest impact on public health worldwide with higher morbidity, albeit fortunately with low mortality rate [1]. The etiologic agent is the dengue virus (DENV), which has four antigenically distinct viral serotypes, the DENV 1 to 4. DENVs share between 65 and 75% homology in their RNA sequences. As a member of the *Flaviviridae* family, the DENV consists of an envelope formed by a lipid bilayer derived from the endoplasmic reticulum of the host cell into which the envelope (E) and membrane (M) proteins are inserted. The viral particle is spherical in shape and approximately 50 nm in diameter. Below the viral envelope is a nucleocapsid composed of an icosahedral viral capsid formed by the capsid protein (C) and complexed to a single-stranded RNA molecule with positive polarity [2, 3]. Viral RNA DENV is approximately 10.7 Kb and is modified at its 5' end by the addition of the cap structure but is devoid of the poly-A tail at the 3' end and has a single open reading frame encoding a protein precursor polyprotein viral infection. This precursor protein is cleaved by both host cell proteases and viral protease, yielding 10 proteins: structural C, pre-Membrane (prM)/ M, E, and nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The translated structural proteins are incorporated into the viral particles during their maturation, while the nonstructural proteins are involved in the replication and/ or assembly of the virions. The 3' and 5' noncoding regions are also important for viral replication [4–6].

Dengue fever is generally an acute disease, with a broad spectrum of clinical manifestations ranging from a clinically inapparent infection, an undifferentiated acute febrile illness, dengue fever (DF), to more severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The first symptoms of DF and DHF are indistinguishable, but DHF is associated with hemorrhagic manifestations, plasma extravasation, and thrombocytopenia (counts below 150,000 platelets/mm<sup>3</sup>). Thrombocytopenia is not necessarily restricted to severe forms of dengue, since it is possible to see small bleeding in mild infections. DSS is distinguished from DHF by the presence of cardiovascular or other organs impairment, which occurs when loss of plasma to interstitial spaces results in shock. In general, DSS is a serious disease, with mortality rates of up to 20%, but may also be less than 1% in places with sufficient resources and with clinical experience [7, 8]. Symptomatic disease typically follows three phases: an initial febrile phase lasting 3 to 7 days; a critical phase around the defervescence during which complications may appear in some patients; and a spontaneous recovery phase [9].

A simplified categorization for the classification of dengue severity was proposed by the World Health Organization (WHO) in 2009 in which DHF and DSS were grouped as severe

dengue [1]. This classification was based on a multicenter study that treats the illness as a dynamic and systemic event. The new classification describes three sets of clinical signs and symptoms: (1) Dengue fever without signs of alarm (DF) characterized by nausea, vomiting, rash, myalgia, headache, arthralgia, and positive tourniquet test with no signs bleeding and leukopenia; (2) Dengue fever with warning signals (DFwWS) includes abdominal pain or tenderness, persistent vomiting, fluid accumulation, lethargy, agitation, hepatomegaly (increase > 2 cm), elevated serum transaminases, and decreased platelet count; and (3) Severe Dengue Fever characterized by severe plasma extravasation, leading to shock and fluid accumulation, accompanied by respiratory discomfort, severe bleeding, and involvement of organs such as the liver, central nervous system (with altered consciousness), heart, and other organs. This new classification proved to be more sensitive to the identification of severe forms, reducing the proportion of patients previously unclassifiable, which facilitated the clinical management of patients [10].

Briefly, immunopathogenesis of dengue is postulated by some hypotheses, including antibody enhancement theory [11, 12], T-cell activation of cross-reactive memory, and original antigenic sin [13]. All hypotheses, to some extent, induce an overproduction or a skewed profile of cytokine release, giving rise to the term cytokine storm/cytokine tsunami [14].

The humoral response is mainly directed to the prM, E, and NS1 proteins, whereas in the cases of secondary infection, the response against NS3 and NS5 is observed [15, 16]. It is believed that a primary infection can create effective, lasting protection against reinfection by the same serotype, but triggers short-term cross-protection against the other serotypes [17]. Neutralization of infection by specific antibodies may occur by inhibiting the entry of the virus through its specific receptors into the target cell [18] or by inhibiting viral fusion into the target cell cytoplasm [19]. On the other hand, epidemiological studies suggest that homologous immunity may increase the severity of the disease during a subsequent infection by a heterologous serotype [11]. It is believed that low neutralizing antibodies, those that induce cross-reaction, produced in response to a previous serotype, contribute to the pathogenesis of dengue by promoting the entry of the virus through Fc $\gamma$  receptors into myeloid cells, a phenomenon known as antibody-dependent infection (ADE) [20].

The role of T cells during dengue infection is still controversial, with studies supporting either an immunoprotective or immunopathological role [21]. Pioneer studies proposed that T cells have a detrimental role during secondary dengue infections in a process termed “original antigenic sin.” Based on this theory, cross-reactive T cells generated during primary infection, which recognize secondary-infected DENV serotype with low affinity, are poorly functional but prone to inducing immunopathology [13]. Thus, as cross-reactive memory, T cells are present in increased numbers and have a low activation threshold. They may outcompete their naïve subsets that have high affinity for secondary-infected serotype with an overall detrimental outcome for protective immunity [22]. Collectively, studies showed that dengue infection elicits a broad-specific T cell response that peaks around day 8–10 from fever onset [23, 24]. Dengue-specific CD8<sup>+</sup> T cells are present at higher frequencies compared to their CD4<sup>+</sup> counterparts and preferentially target nonstructural proteins NS3, NS4b, and NS5, while CD4<sup>+</sup> T cells are mainly directed toward the capsid envelope and the secreted protein NS1 [23].

Moreover, studies have demonstrated that high concentrations of circulating cytokines, mainly released by T cells, monocytes, macrophages, and endothelial cells from patients, would be involved in the pathogenesis of dengue [24]. Initially, antiviral mechanisms of innate immune response mediated by interferons (IFNs), mainly produced by dendritic cells (DCs), monocytes, macrophages, and natural killer (NKs) cells, are involved in initial infection control. The antiviral activity of type I IFNs (IFN- $\alpha/\beta$ ) is initiated hours after infection and promotes inhibition of viral replication of infected cells, activation of the antiviral state by uninfected cells, and stimulation of the antiviral activity of the cells NK and CD8<sup>+</sup> T lymphocytes [25, 26]. DENV proteins such as NS4B and NS5 have been shown to inhibit IFN- $\alpha/\beta$  signaling [27–29]. However, *in vitro* and *in vivo* studies have demonstrated that DENV is capable of activating the production of IFN- $\alpha$  by human plasmacytoid dendritic cells (pDC) [30]. The IFN- $\gamma$  (or IFN-type II), a cytokine involved with Th1 profile, is produced primarily by T lymphocytes, NK cells, and to a lesser extent by macrophages. The IFN- $\gamma$ , like other IFNs, has an antiviral effect and promotes increased expression of human leukocyte antigen (HLA) class I and II molecules and stimulates antigen presenting and cytokine production by antigen-presenting cells (APCs) [31]. Kurane et al. reported higher levels of IFN- $\gamma$  in the serum of patients with DHF and DF compared to healthy subjects, but IFN- $\gamma$  levels were still higher after defervescence in patients with DHF. According to the authors, these results suggest that IFN- $\gamma$  would play an important role in infection control; however, high levels of this cytokine after defervescence, together with increased T cell activation, would contribute to the pathogenesis of DHF [32]. TNF- $\alpha$  is another cytokine that appears to play an important role in dengue. TNF- $\alpha$  is produced by mononuclear phagocytes, neutrophils, lymphocytes, and NK cells. The interaction of TNF- $\alpha$  and endothelial cells promotes induction of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin (E-CD62), being strongly involved in vascular damage, septic shock, and anti-tumor immunity [31]. In dengue, TNF- $\alpha$  appears to be involved in vascular damage, and authors observed an increased permeability and morphological changes in endothelial cells treated *in vitro* with TNF- $\alpha$  [33]. Studies have shown elevated plasmatic cytokines in dengue, such as IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, and IL-18, transforming growth factor-beta-receptor (TGF- $\beta$ ) [34–40]. Chaturvedi et al. reported that DF patients had higher levels of IFN- $\gamma$  and IL-2, whereas the majority of DHF patients had IL-4, IL-6, and IL-10 elevation, on the 4th and 8th days of the disease, coinciding with the defervescence phase [41]. Pretreatment of monocytes/macrophages with Th2 profile cytokines (IL-4 or IL-13) increased the susceptibility of these cells to DENV infection [42]. Plasma levels of IL-10 were correlated with thrombocytopenia in dengue patients [34, 43]. High production of TNF- $\alpha$ , IL-1 $\beta$ , IL-12, IL-17, soluble IL-1 receptor type 1 protein (sST2), and tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL), as well as apoptosis in DENV-infected monocyte/macrophages cultures, was also observed. It has been shown, therefore, that beneficial or deleterious biomarkers may be present in dengue, regardless of the severity of the disease [44].

## 2. Thrombocytopenia and dysfunction of platelets in dengue

Reduced proliferative capacity of hematopoietic cells in bone marrow and/or increased destruction of platelets from peripheral blood are two main events associated with thrombocytopenia



[45–47]. Thrombocytopenia occurs when platelet formation (thrombopoiesis) is insufficient to balance physiological or pathological platelet consumption. Thrombocytopenia may occur in patients with either mild or severe cases of dengue infection and are associated in the early days of dengue infection [1]. The WHO guidelines for 2009 reaffirmed that a rapid decline or platelet count below  $150,000/\text{mm}^3$  of blood are one of the indicators of clinical dengue worsening. Together, the functional disturbance associated with deregulation of the plasma quinine system is related with the immunopathogenesis of dengue [1, 48, 49].

### **2.1. Thrombocytopenia induced by bone marrow suppression, lysis of megakaryocytes and/or peripheral destruction of platelets**

The kinetic observation of platelet counts in dengue patients showed a mild to moderate decrease in the 3rd to the 7th day, a significant decrease on day 4, reaching normal levels in the 8th or 9th day of the disease [50, 51]. Profound thrombocytopenia (nadir platelet count  $\leq 20,000/\text{mm}^3$ ) was significantly more likely to detect early warning signs and longer hospital stays, but profound thrombocytopenia was not affected by DENV serotypes, coinfections, and secondary DENV infections [52]. However, a study involving 245 dengue patients showed no relationship between bleeding and platelet count, while 81 nonbleeding patients had a score below  $20,000/\text{mm}^3$  [53]. In contrast, another study involving 225 dengue patients suggested that bleeding occurred more frequently in patients with PT [54]. Most clinical guidelines recommend platelet transfusion in patients with dengue who develop severe bleeding or platelet counts below  $10\text{--}20,000/\text{mm}^3$ . However, another study confirms that platelet transfusion does not prevent the development of severe bleeding or shorten coagulation time [55], and in severe dengue disease with hemorrhagic manifestations, the need for intensive care was not significantly associated with PT [52].

Previous published data indicated that DENV can induce thrombocytopenia through bone marrow suppression, lysis of megakaryocytes, and/or peripheral destruction of platelets [56]. Three main mechanisms seem to be involved, although partially explained, such as a direct lesion of progenitor cells by DENV, infected stromal cells, and modification of bone marrow regulation [51]. In fact, studies have shown a hypocellularity in bone marrow and inhibition of megakaryocyte maturation [51, 57]. *In vitro* studies using an adventitious reticular cell line, which are bone marrow stromal cells, incubated with DENV found DENV antigens in the perinuclear region of these cells [58]. These interactions lead to a modification in the cytokine profile produced in the bone marrow, as in the case of TGF- $\beta$  capable of inhibiting the differentiation of multipotent stem cells into megakaryocyte precursor cells, leading to inhibition of the cell differentiation process [59, 60]. Another cytokine, the thrombopoietin (TPO), regulates megakaryocytopoiesis and platelet production specifically through the activation of myeloproliferative leukemia virus oncogene (c-MPL), the TPO receptor [61, 62]. When platelet counts fall, circulating levels of TPO increase and may function as a useful indicator of megakaryocytopoiesis in dengue [63, 64]. Recently, authors showed that mice inoculated with recombinant DENV-envelope protein domain III (DENV-EIII)-suppressed megakaryopoiesis of progenitor cells from murine bone marrow and human cord blood *in vitro*, similarly to those observed with DENV infection. Additional analyses suggested that autophagy impairment and apoptosis are involved in DENV-EIII-mediated suppression of megakaryopoiesis. Thus, these data suggest that, even without viral replication, the binding of DENV-EIII to the cell surface is sufficient to suppress megakaryopoiesis [65].

Although several aspects of the pathogenesis of thrombocytopenia are still not clearly understood, La Russa and Innis in 1995 demonstrated that DENV-induced bone marrow suppression depressed platelet synthesis [58]. In the same year, Wang et al. found that DENV-2 can bind to human platelets in the presence of virus-specific antibody, proposing an immune-mediated clearance of platelets [66]. No infectious model that mimics DHF/DSS has yet been reported until Huang et al. described that the immunocompetent mice intravenously inoculated with DENV-2 developed transient thrombocytopenia and generate IgG class anti-platelet antibody. This was the first evidence of an association between anti-DENV immune response with cross-reactivity to platelets [67]. Falconar gave a strong contribution when identified a highly avid subclone monoclonal antibody MAb 1G5.4-A1-C3 from DENV-2 NS1 and others anti-NS1 MAbs, which in addition of producing hemorrhage in mice, cross-reacted with human fibrinogen, thrombocytes, and endothelial cells, with known epitopes or active sites on human clotting factors and integrin/adhesin proteins present on these cells [68].

Previous study described a strong association between activation status of platelets and their destruction/depletion from circulation in febrile dengue patients [69]. Peripheral destruction of platelets can occur through the direct interaction of the virus in the platelet, as well as indirectly, since the infection leads to the formation of aggregates platelet-endothelial cells and platelets leukocytes or still to the secretion of anti-platelet antibodies and production of factors detrimental to platelets [56]. During dengue infection, cross-reactivity autoantibodies, including antiplatelet antibodies, are generated. In addition, anti-NS1 antibodies belong to the IgM class cross-react with platelets. This last one has the potential for activation of the cascade complement system, leading to the induction of cell lysis and inhibition of platelet aggregation [70, 71]. Notably, high anti-platelet IgM titers were detected in patients with DHF/DSS compared to DF. In accordance with high titers of IgM, serum from DHF/DSS patients causes more platelet lysis than the DF patient serum [71]. Autoantibodies against endothelial cells and blood coagulation molecules have also been described [72]. In fact, molecular mimicry between platelets, endothelial cells, or blood clotting molecules and NS1, prM, and E may explain the cross-reactivity of anti-NS1, anti-prM, or anti-E antibodies between host proteins and proteins. Cross-reactivity antibodies can cause platelet dysfunction, endothelial cell damage, coagulation deficiencies, and activation of macrophages [73]. In addition, it has been recognized that platelet surface P-Selectin (P-CD62) activates integrins and mediates adhesion, aggregation, and secretion of mediators [74].

Among the soluble factors that play a role in the peripheral destruction of platelets, they include platelet-activating factor (PAF) [75], von Willebrand factor (vWF) [76], TNF- $\alpha$ , IL-1 $\beta$  [35], and IL-10 [34].

Platelet apoptosis and phagocytosis associated with high-serum TPO levels were significantly increased in dengue patients during the early stages of convalescence when compared to the late convalescence phase and in healthy volunteers. These results suggest that the abrupt drop in the number of platelets at the beginning of infection is outweighed by TPO-mediated thrombopoiesis [77]. Another study confirmed that platelets from patients exhibited classic signs of the apoptosis intrinsic pathway that include increased phosphatidylserine exposure, mitochondrial depolarization, and activation of caspase-9 and -3 [78].

## 2.2. Dysfunction of platelets in dengue

Platelet activation is a phenomenon common during physiological dysbalance, such as damage to blood vessels when in contact with components of the subendothelial matrix (collagen and vWF) [79], virus infections such as DENV and human immunodeficiency virus [80], hypothermia [81], diabetes mellitus [82], and arterial thrombosis [83]. Moreover, some agonists involved in platelet activation include adenosine diphosphate (ADP), thromboxane A<sub>2</sub> (TXA<sub>2</sub>), collagen, serotonin, epinephrine, and thrombin [84], in addition to pathogens and toxins [85].

During its activation, the platelets undergo a structural change process, in which the discoid cells undergo modifications in the cytoskeleton, disassembly of a ring of microtubules, resulting in an intermediate spherical shape. Next, actin polymerization and filopodia extension occur, causing the cell to acquire lamellar or dendritic morphology [86]. The major activated platelet receptors on interactions with other cells are glycoprotein GP IIb/IIIa (CD41/CD61) and P-CD62. The CD41/CD61 binds to adhesion proteins that contain the Arginine-Glycine-Asparagine peptide sequence (RGD sequence), thus allowing the pooling and binding of activated platelets to leukocytes and endothelial cells via “bridge molecules,” such as fibrinogen [87]. P-selectin is a glycoprotein stored in platelet  $\alpha$ -granules that is translocated to the surface and released in suspension during platelet activation [85]. It is the main adhesion molecule responsible for platelet interaction with monocytes [85, 88–90], and circulating platelet-monocyte aggregates have been detected in dengue patients [89, 91]. As for the morphological and physiological profile of the platelets exposed to DENV-2, it was observed that there is platelet activation with increased expression of P-CD62 and fibrinogen binding. For morphological changes related to activation, the authors cited membrane architecture alterations, degranulation, the presence of filopodia, and dilation of the open canalicular system in platelets exposed to DENV-2 [92].

The events related to activation are not restricted to changes in morphology, having consequences in several biological functions developed by the platelets. It is also observed exocytosis of constituents of platelet granules, expression of adhesion proteins, and secretion of cytokines and other immunological mediators [93]. Activated platelets secrete mediators stored or synthesized in their granules, which act on several functions. In addition, during plaquetogenesis, megakaryocytes transfer platelet pre-mRNAs, such as tissue factor (TF, inflammatory mediator, and coagulation regulator) pre-mRNA to platelet, are processed to mature mRNA and translated into biologically active TF [94]. In this way, platelets have a complex post-transcriptional repertoire able to translate new proteins, a phenomenon evidenced in response to activation [95, 96]. Multiple pathways lead to platelet activation, including agonists such as collagen, ADP, TXA<sub>2</sub>, epinephrine, serotonin, and thrombin, through interaction with receptors on platelet surface, leading to release of its granular content, increase of intracellular Ca<sup>2+</sup> levels, and activation of the fibrinogen receptor,  $\alpha$ IIb $\beta$ 3 integrin [97–100]. Studies have been reporting platelet dysfunction in dengue infections. In this context, suppression of platelet aggregation has been shown to occur along with an increased release of beta thromboglobulin ( $\beta$ TG) and Platelet Factor 4 (PF4/CXCL4) during the acute phase of DHF [101]. Assays using mononuclear leukocytes (MNLs) from healthy donors exposed to DENV-1 and 2 release significantly greater amounts of PAF, TXB<sub>2</sub>, and Prostaglandin D2 (PGD2) than the donor not exposed to any DENV serotypes [102]. Previous data showed that TXB<sub>2</sub> plasma



levels of DHF patients with shock decreased significantly than those of normal controls and DHF patients without shock patients, supposing that failure or inadequate TXB<sub>2</sub> production may eventually lead to shock [103].

In addition to exerting an effector role, platelets influence the production of cytokines by peripheral mononuclear cells. Activated platelets exhibit anti-inflammatory properties related to the CD40 and CD40L interaction, leading to increased IL-10 production and inhibition of TNF- $\alpha$  by monocytes [104]. The authors also verified that the interaction of apoptotic monocytes and platelets regulates the secretion of IL-10 through the recognition of platelet phosphatidylserine. It appears that IL-10 secretion requires only monocyte-platelet contact, but not phagocytosis, indicating that activated and apoptotic platelets aggregate to monocytes during infection [86]. Azeredo et al. found that IL-10 levels were correlated with low platelet counts [34]. Platelets are the major source of TGF- $\beta$ 1 in the human body [105]. One study has shown that circulating levels of TGF- $\beta$ 1 are significantly lower in patients with DHF than in controls [106]. Patients with immune thrombocytopenia have low levels of TGF- $\beta$ 1 in the circulation. However, after therapy to restore normal platelet count, their TGF- $\beta$ 1 levels return to levels found in healthy controls [107].

The innate immune system recognizes infection and changes in cellular homeostasis to initiate responses to clear pathogens and repair tissue damage. Toll-like receptors (TLRs) are part of the innate immune system, key players that modulate the inflammatory response and tumor dynamics. Many investigators have confirmed the expression of TLR1-9 both human and murine platelets [108]. Other major complex involved in these processes is the inflammasome, a multimeric protein complex that activates pro-caspase-1, which then proceeds to cleave multiple substrates including the pro-inflammatory cytokines IL-1 $\beta$  and IL-18 [109]. The presence of the nucleotide-binding domain leucine rich repeat containing protein (NLRP3) inflammasome in platelets activated after infection by DENV has been described, inducing the production of IL-1 $\beta$  by platelets and platelet-derived microparticles of dengue patients [89].

### **3. Deregulation of the platelets' role in dengue: Hemostasis, site of virus, and immune cells**

#### **3.1. Deregulation between pro-and anticoagulant mechanisms in dengue**

Hemostasis is a dynamic process in which physical and biochemical mechanisms promote blood clotting in a fast and regulated way [110]. Platelet adhesion and activation are mediated through the interactions between GP Ib-IX-V receptors with vWF and GP VI with subendothelial collagen [111]. Alternatively, when the vascular endothelium is damaged, collagen sites are exposed, facilitating platelet adhesion at these specific sites. Platelets adhere to molecules in the subendothelial tissues at the lesion site, where they aggregate and interact with leukocytes and endothelial cells, thus initiating the coagulation cascade [112]. The coagulation factors present in the bloodstream are involved in a tightly controlled activation sequence, resulting in the formation of thrombin and subsequently fibrin. These factors circulate as zymogens, which require processing by proteolysis to be activated. According to the new model of blood clotting, it occurs in three overlapping stages: initiation phase, amplification phase, and propagation phase [113].

During acute DENV infection, coagulation and fibrinolysis are activated, leading to coagulation changes and fibrinolytic parameters that may lead to disseminated intravascular coagulation (DIC) [114–116]. Funahara et al. reported that dengue patients with DIC had decreased platelet counts, transient prolongations of partial thromboplastin time (PTT) and prothrombin time (PT), and decreased levels of fibrinogen, prothrombin activity, factor VIII, antithrombin III, and plasminogen [117]. DIC leads to platelet activation, formation of fibrin, and deposition of small clots in the microcirculation, possibly contributing to organ failure. Notably, the consumption of clotting factors usually leads to paradoxical hemorrhagic disorders due to their consumption [118]. Later, it has been demonstrated that acute DIC that occurs in patients with DHF is associated with increased vascular permeability [117]. Thus, parameters such as platelet count, PTT, and PT present predictive value in the diagnosis of severe dengue [119].

The mechanisms that trigger DIC are mainly related to endothelial lesions and increased circulating TF levels [118]. Several studies have suggested that increased TF expression plays an important role in the pathogenesis of dengue. Huerta-Zepeda et al. showed that DENV regulates levels of protease-activated receptor type 1 (PAR-1) and TF in the activated endothelium [120]. These data are reinforced by the evidence of increased plasma levels of TF in dengue patients, and the expression of TF in monocytes was inversely correlated with platelet counts [121, 122]. Our previous data found that dengue patients with a good outcome showed decreased circulating levels of TF than those with a poor outcome (Severe). Similarly to TF, tissue factor pathway inhibitor (TFPI) levels were significantly lower in patients with a good outcome, but increased TFPI plasma levels were observed in severe patients. We also demonstrated that TF and TFPI levels were significantly higher among patients with hemorrhagic manifestations. In addition, DENV-1 or -2 patients were more likely to have increased levels of TF than DENV-4 patients [123]. Activation of PAR-1 is accompanied by positive regulation of adhesion molecules and production of proinflammatory cytokines [124]. The coagulation enzymes generated in DENV infection can activate PAR-1 receptors, thus increasing the increase of pro-inflammatory cytokines and leukocyte migration. These cytokines, along with coagulation enzymes (and *vice versa*), perpetuate the inflammatory response, which promotes increased interaction between activated monocytes, activated endothelial cells, and platelets. The result is a convergence of signals that lead to exacerbated expression of TF. Therefore, the coagulation and inflammation processes are closely related and establish a bidirectional relationship mediated by the activation of PARs [125].

Since hemostasis depends on the balance between coagulation and fibrinolysis, Huang et al. evaluated some coagulation parameters (platelet count and PTT) as well as fibrinolytic parameters (tPA and PAI-1) in patients with DHF and DF. Patients with DF show thrombocytopenia, PTT prolongation, and increased tPA levels, indicating coagulation activation and fibrinolysis. However, the parameters used indicated much more severe activation of coagulation and fibrinolysis in patients with DHF. In the convalescent phase, there is an increase in PAI-1 and platelet counts with concomitant decline in tPA levels and normalization of PTT, both in patients with DHF and in DF. According to the authors, the activation of coagulation and fibrinolysis during the acute phase of DENV infection is compensated by the increase of platelets and PAI-1 during the convalescence phase. These results suggest that the degree of activation of coagulation and fibrinolysis induced during dengue infection is associated with the severity of the disease [114].

### 3.2. Platelets as target cells for DENV replication

Platelets contain receptors related to DENV entry, such as Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) [126]. *In vitro* infection by DENV induces activation, mitochondrial dysfunction, and platelet apoptosis through mechanisms dependent on the DC-SIGN receptor [127]. A study detected DENV RNA by conventional reverse transcription polymerase chain reaction (RT-PCR) and DENV-like particles by electron microscopy in patients' platelets [128] and later confirmed the presence of viral antigen in platelets by immunofluorescence and confocal microscopy [129]. It is still unclear whether platelets are involved in the spread of viral infection. Recent data from our group have confirmed the *in vitro* interaction of DENV with platelets, leading to subsequent morphological modifications characteristic of platelet activation, such as presence of membrane extensions (filopodia), loss of cytoplasmic content and dilation of the membrane system [Azamor and Oliveira submitted]. A study using blood cells from dengue patients and rhesus monkeys experimentally infected revealed DENV antigens present in vesicles of varying sizes and often in nuclear cells like platelets. The DENV RNA was detected in a highly enriched population of rhesus platelet-characteristic CD61+ cells in the acute phase of infection, indicating that the virus may be directly linked to dysfunction and low platelet counts [130]. More recent and for the first time, authors demonstrated in fact that platelets directly bind DENV saturably and produce infectious virus. Interestingly, at 37 and 25°C, platelets replicated the positive sense single-stranded RNA genome of DENV by up to ~4-fold over 7 days, with production of viral NS1 protein. The infectivity of DENV intrinsically decayed *in vitro*, which was moderated by platelet-mediated generation of viable progeny. DC-SIGN and heparan sulfate proteoglycan (HSP) were implicated as coreceptors because only the combination of anti-DC-SIGN and low-molecular-weight heparin prevented binding. Thus, expression of antigen encoded by DENV is a novel consideration in the pathogen-induced thrombocytopenia mechanism [131]. Finally, very recent analyze revealed that DENV works as the primary driver of platelet activation and also enters and replicates in platelets but does not result in a productive infection of platelets. Moreover, the DENV-exposed/DENV antigen-positive platelets associate with CD14+CD16+ monocytes may mediate platelet clearance from the circulation [132].

### 3.3. Platelets as immune cells

Platelets, when interacting physically with leukocytes and endothelial cells, act as a kind of signaling of inflammation and immune response. When stimulated and activated, these cells initiate events, such as aggregation, formation of microparticles and exosomes [133], expression of adhesion proteins and receptors, and exocytosis of constituents present in their granules [76]. Exocytosis of  $\alpha$ -dense granules and lysosomes releases cytokines and biological mediators with various immunological and inflammatory functions. In addition to secreting soluble mediators, platelets express receptors involved with the immune defense, such as Fc receptors that are able to recognize IgG, IgE, and IgA classes. These receptors may confer rudimentary antibacterial activities of platelets, such as the secretion of antimicrobial peptides and phagocytosis against direct interaction with bacteria, viruses, protozoa, or helminths. These immunoreceptors influence platelet adhesion activity through the modulation of integrin production [76]. Another set of receptors present on platelets with immunological

action is that of TLRs. Among the 10 TLRs identified in humans, six are expressed in platelets, the TLR-1, -2, -4, -6, -8, and -9. TLR-4 has been shown to modulate sepsis and inducer of TNF- $\alpha$  *in vivo* [134]. TLR-2 modulates IL-1 $\beta$  RNA processing, inducing the production of IFN type 1 and other inflammatory cytokines [135]. The set of inflammatory, hemostatic, angiogenic, and coagulators reactions are multicellular events that include chemotaxis, adhesion, interactions between leukocytes, endothelial cells, and platelets in the walls of blood vessels. Platelets contribute to these interactions through secretion of adhesion proteins and regulation of chemokine synthesis by leukocytes and endothelial cells [136]. Molecules such as IL-1 $\beta$ , PAF, and P-CD62 stand out as the main mediators derived from platelets, able of activating leukocytes [137]. Studies have shown that activated platelets induce increased IL-10 expression and decreased TNF- $\alpha$  by monocytes [101].

Several studies have indicated that DENV infection leads to the activation of endothelial cells, which increase the expression on the surface of the E-CD62 molecule. E-CD62, as well as P-CD62, are adhesion molecules responsible for platelet adhesion to endothelial cells [113, 138]. In addition to the endothelium, P-CD62 is expressed on the surface of activated platelets, promoting their interaction with leukocytes and formation of aggregates between platelets and monocytes and/ or neutrophils in primates [139]. Platelet-monocyte aggregates are also observed in DENV-infected patients, leading to the synthesis of IL-1 $\beta$ , CXCL8/ IL-8, CCL4/ MCP-1, and IL-10 by monocytes [107]. The formation of cell aggregates results in an increase in the inflammatory response in dengue, as well as contributing to the generation of thrombocytopenia both by the physical retention of cells, by lowering the number of circulating cells and by the induction of cell death [140]. In 1992, Butthep et al. showed that platelets, as well as neutrophils and lymphocytes, preferentially bind to endothelial cells exposed to DENV, compared to cells not exposed to DENV. It has been suggested that increased platelet endothelial cell binding may contribute to thrombocytopenia in dengue patients [141]. Protein disulfide isomerase (PDI), an endoplasmic reticular protein, is located on the surface of platelets [142] and is involved in the regulation of integrin-mediated platelet aggregation, since anti-PDI antibodies block platelet adhesion and aggregation [143]. Previous studies have demonstrated that platelet surface PDI can be recognized by anti-NS1 antibodies. Rachman and co-workers observed a similar kinetic profile between anti-NS1 and PDI antibodies [144]. PDI enzyme activity and platelet aggregation were reduced with anti-NS1 action. Amino acid residues 311–330 (P311–330) of NS1 represent an epitope that shares sequence homology with the PDI thiorredoxin domain [145]. In contrast, although the serum of dengue patients inhibits platelet aggregation, there is no correlation between anti-NS1 and PDI with platelet aggregation dysfunction, suggesting that other mechanisms may be involved in the inhibition of platelet aggregation [144, 145]. Platelets are responsible for the maintenance of vascular integrity due to the constitutive release of pro-angiogenic cytokines and growth factors. The  $\alpha$ -granule-derived molecules such as angiopoietin-1,  $\alpha$  and  $\beta$ -catenins, and PAF bind to specific receptors on the surface of endothelial cells, causing intracellular signaling that stabilizes the intercellular adhesion junctions [146]. Angiopoietins, key molecules of vascular integrity, are also stored in platelets. Both dengue-associated thrombocytopenia and endothelial activation are associated with an imbalance in the ratio of angiopoietin-2: angiopoietin-1 plasmatic. Studies have shown that there is an inverse correlation between angiopoietin-1 and plasma extravasation markers but a direct correlation between angiopoietin-2 and markers of plasma



extravasation in patients with DHF/DSS [147]. Hottz et al. demonstrated that DENV-infected patients who showed signs of increased vascular permeability demonstrated a higher percentage of platelets and platelet-derived microparticles (MP) expressing IL-1 $\beta$  and caspase-1 activator compared to patients who had no evidence change in vascular permeability. These results were confirmed in experiments in which platelet-derived MPs exposed to DENV caused an increase in the permeability of endothelial cells that was blocked by IL-1Ra [127].

More recently, proteome analysis related to platelet activating signaling from platelets from dengue patients demonstrated an increase of PAR-4 (F2RL3), G protein subunits (GNA12 and GNA14), and p38 MAPK (MAPK14), in which potentially contributing to increased platelet activation during dengue infection. Moreover, dengue patients had increased P-CD62 surface expression on platelets from patients presenting dengue with warning signs and severe dengue syndromes compared to mild dengue. In agreement, they observed exhaustion of the granule-stored chemokine PF4/CXCL4 in platelets from patients with dengue [148], similarly to another study reported that patients with severe dengue have lower levels of PF4/CXCL4 in plasma when compared to mild dengue patients [149].

4. Conclusion

Platelets are cellular fragments derived from hematopoietic precursors megakaryocytes, primarily associated with coagulation and hemostasis and also with inflammation, immune response, angiogenesis, and extracellular matrix synthesis. In fact, platelets contain several preformed molecules, large amounts of mRNA, and the packaged translational process required to synthesize new biologically active proteins, including growth factors, cytokines, and chemokines. Platelets are one of the major cell populations affected in dengue, once both thrombocytopenia and platelet dysfunction are common manifestations of infection and strongly related to the patient’s clinical outcome. Dysfunction of platelets is implicated in prothrombotic complications associated with severe cases of dengue. Thus, platelets could be considered cells that are active against the anti-DENV immune response, and therefore, thrombocytopenia is a key prognostic factor in the immunopathogenesis of dengue.

Abbreviations

DENV	dengue virus
E	envelope
M	membrane
C	capsid
prM	pre-Membrane
NS	nonstructural

DF	dengue fever or dengue fever without signs of alarm
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
WHO	World Health Organization
DFwWS	dengue fever with warning signals
ADE	antibody-dependent infection
IFNs	interferons
NKs	natural killer cells
pDC	plasmacytoid dendritic cells
DCs	dendritic cells
IFN- $\alpha/\beta$	type I IFNs
IFN- $\gamma$	or IFN-type II
HLA	human leukocyte antigen
APCs	antigen-presenting cells
ICAM-1	intracellular adhesion molecule-1
VCAM-1	vascular cell adhesion molecule-1
E-CD62	E-selectin
TGF- $\beta$	transforming growth factor-beta-receptor
sST2	soluble IL-1 receptor type 1 protein
sTRAIL	tumor necrosis factor-related apoptosis-inducing ligand
PT	profound thrombocytopenia
TPO	thrombopoietin
DENV-EIII	DENV-envelope protein domain III
P-CD62	P-selectin
PAF	platelet-activating factor
vWF	von Willebrand factor
ADP	adenosine diphosphate
TXA <sub>2</sub>	thromboxane A <sub>2</sub>
GP	glycoprotein
RGD sequence	Arginine-Glycine-Asparagine peptide sequence

TF	tissue factor
βTG	beta thromboglobulin
PF4/CXCL4	platelet factor 4
PGD2	Prostaglandin D2
TLRs	toll-like receptors
NLRP3	nucleotide-binding domain leucine rich repeat containing protein
DIC	disseminated intravascular coagulation
PTT	partial thromboplastin time
PT	prothrombin time
PAR-1	protease-activated receptor type 1
TFPI	tissue factor pathway inhibitor
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
RT-PCR	reverse transcription polymerase chain reaction
HSP	heparan sulfate proteoglycan
PDI	protein disulfide isomerase
MP	microparticles

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