Platelet Activation and Apoptosis Modulate Monocyte Inflammatory Responses in Dengue

Eugenio D. Hottz, Isabel M. Medeiros-de-Moraes, Adriana Vieira-de-Abreu, Edson F. de Assis, Rogério Vals-de-Souza, Hugo C. Castro-Faria-Neto, Andrew S. Weyrich, Guy A. Zimmerman, Fernando A. Bozza and Patrícia T. Bozza

J Immunol published online 11 July 2014
http://www.jimmunol.org/content/early/2014/07/11/jimmunol.1400091
Dengue is the most prevalent human arbovirus disease in the world. Dengue infection has a large spectrum of clinical manifestations, from self-limited febrile illness to severe syndromes accompanied by bleeding and shock. Thrombocytopenia and vascular leak with altered cytokine profiles in plasma are features of severe dengue. Although monocytes have been recognized as important sources of cytokines in dengue, the contributions of platelet–monocyte interactions to inflammatory responses in dengue have not been addressed. Patients with dengue were investigated for platelet–monocyte aggregate formation. Platelet-induced cytokine responses by monocytes and underlying mechanisms were also investigated in vitro. We observed increased levels of platelet–monocyte aggregates in blood samples from patients with dengue, especially patients with thrombocytopenia and increased vascular permeability. Moreover, the exposure of monocytes from healthy volunteers to platelets from patients with dengue induced the secretion of the cytokines IL-1β, IL-8, IL-10 and MCP-1, whereas exposure to platelets from healthy volunteers only induced the secretion of MCP-1. In addition to the well-established modulation of monocyte cytokine responses by activated platelets through P-selectin binding, we found that interaction of monocytes with apoptotic platelets mediate IL-10 secretion through phosphatidylserine recognition in platelet–monocyte aggregates. Moreover, IL-10 secretion required platelet–monocyte contact but not phagocytosis. Together, our results demonstrate that activated and apoptotic platelets aggregate with monocytes during dengue infection and signal specific cytokine responses that may contribute to the pathogenesis of dengue. The Journal of Immunology, 2014, 193: 000–000.
and apoptotic platelets from dengue patients induced the secretion of IL-1β, IL-8, IL-10, and MCP-1 in monocytes. Interactions of monocytes with platelets from heterologous healthy volunteers induced the secretion of MCP-1, but not IL-1β, IL-8, and IL-10. In exploring the mechanisms involved, we evaluated the monocyte responses to agonist-stimulated platelets that showed features of activation and apoptosis. We observed that the release of cytokines depended on the P-selectin–mediated adhesion (11, 13), and, in addition, on the phosphatidylserine-mediated recognition of apoptotic platelets, which induced IL-10 secretion. Our findings provide new insights regarding inflammatory mechanisms in dengue infection and the biology of platelet–monocyte interactions.

Materials and Methods

**Human subjects**

Peripheral vein blood samples were obtained from 25 serologically and molecularly confirmed DENV-infected patients from the Instituto de Pesquisa Clínica Evandro Chagas–Fiocruz, Rio de Janeiro, Brazil; their characteristics are presented in Table I. The average day of sample collection after the onset of illness was 3.8 ± 1.5, and the average day of defervescence was 4.6 ± 1.3. Peripheral vein blood also was collected from 19 sexually active, nonpregnant healthy subjects. The study protocol was approved by the Institutional Review Board (Instituto de Pesquisa Clínica Evandro Chagas #016/2010 and University of Utah), and the experiments were performed in compliance with these protocols. Written informed consent was obtained from all volunteers prior to any study-related procedure.

The cohort consisted of mild dengue patients, of which 12 (48%) presented warning signs that were diagnosed according to World Health Organization guidelines (3). Levels of IgM and IgG specific for DENV-2 protein were measured in plasma from dengue patients using a standard capture ELISA Kit, according to the manufacturer’s instructions (E-Den01M and E-Den01G; PanBio). DENV NS1 protein was detected in patient plasma using the NS1 Detection Kit, according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Primary and secondary infections were distinguished using the IgM/IgG Ab ratio: values < 1.2 were considered secondary infection, as previously reported (18–20). Ninety-six percent of the patients were found to have secondary DENV infection.

**Platelet and monocyte isolation**

Peripheral blood samples were drawn into acid citrate–dextrose and centrifuged at 200 × g for 20 min to obtain platelet-rich plasma (PRP). Platelets were isolated from PRP, and CD45− leukocytes were depleted from platelet preparations, as previously described (21, 22). The platelet preparation was resuspended in medium 199 (M199; Lonza Biologics, Basel, Switzerland), and its purity (≈99% CD41+) was confirmed by flow cytometry. PBMCs were isolated from whole blood after PRP was removed (bottom cell layer after the first centrifugation described above) by Ficoll-Paque (GE Healthcare) gradient centrifugation. The monocyte fraction was isolated by CD14+ selection (Human CD14+ Selection Beads, EasySep, STEMCELL Technologies, London, U.K. or AutoMACS Technology, Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. Cell viability (>95%) was assessed by a trypan blue exclusion test, and the purity of the preparations (>90% CD14+) was confirmed by flow cytometry.

**Flow cytometric analyses**

Platelet–monocyte aggregates were analyzed as previously described (10). Briefly, whole blood was incubated for 10 min with FACs Lysing Solution (BD Biosciences, San Jose, CA) and then centrifuged at 500 × g for 15 min. The supernatant was discarded, and the cells were resuspended in HBSS–0.05% Tween-20 and three times with HBSS. A total of 105 monocytes, resuspended in 300 μl M199 containing 10 mg/ml polymyxin B, was added to the covered surfaces and maintained at 37°C for 4 h. Adherent cells were fixed, stained with Giemsa, and counted by light microscopy.

**Cytokine measurement**

The levels of the cytokines FGF-β, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1α, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α, and VEGF in the supernatants from platelet–monocyte interactions were measured using a Multiplex cytokine immunoassay (Bio-Plex Human Cytokine Assay). Levels of IL-8 and IL-10 also were determined using a standard capture ELISA Kit (R&D Systems).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism, version 5.0 (GraphPad, San Diego, CA). The numerical demographic and clinical
variables are expressed as the median and the interquartile range (25–75%) or as a number and percentage (%). All of the numerical variables were tested for a normal distribution using the Kolmogorov–Smirnov test. We compared the continuous variables using the *t* test (parametric distribution) or the Mann–Whitney *U* test (nonparametric distribution). Correlations were assessed using the Pearson test.

**Results**

**Increased platelet–monocyte aggregates in patients with dengue**

We showed previously that platelets in the blood of patients with dengue are activated (8). Consistent with this, platelets in samples from dengue patients in the current study also had increased P-selectin surface expression (data not shown). P-selectin is the primary adhesion molecule on activated platelets that binds leukocytes (11, 13, 24). To investigate whether activated platelets can interact with monocytes during active dengue infection, we analyzed platelet–monocyte aggregates in peripheral whole blood samples by flow cytometry. As shown in Fig. 1A, dengue patients had increased platelet–monocyte aggregates compared with healthy volunteers (26.1 ± 14.1% versus 8.1 ± 1.7%, *p < 0.001).

Moreover, platelet P-selectin surface expression positively correlated with the levels of circulating platelet–monocyte aggregates in samples from patients with dengue and healthy volunteers (*r* = +0.69, *p < 0.01) (Fig. 1B).

**Platelet–monocyte aggregates are associated with thrombocytopenia and increased vascular permeability during dengue disease**

Using platelet counts determined on the day of sample collection, patients were classified as thrombocytopenic (<150,000/mm³) or nonthrombocytopenic (Table I). Based on this grouping, 45% of the patients were thrombocytopenic, whereas 55% were not. Platelet–monocyte aggregates were higher in thrombocytopenic dengue patients compared with nonthrombocytopenic dengue patients (34.2 ± 18.4% versus 20.3 ± 6.8%, *p = 0.0151) (Fig. 2A). The breakdown was similar in patients who were positive or negative for signs of increased vascular permeability. Increased vascular permeability was evidenced by one or more of the following signs: increase >20% in hematocrit, hypoalbuminemia, postural hypotension, ascites, and/or oliguria (Table I). According to the presence or absence of these signs, 48% of patients were...
FIGURE 2. Platelet–monocyte aggregates correlate with thrombocytopenia and increased vascular permeability in dengue. The percentage of CD14+CD41+ monocytes was assessed in healthy volunteers (control) and dengue patients that were positive or negative for thrombocytopenia (A) or signs of increased vascular permeability (B). The boxes indicate the median and interquartile ranges, and the whiskers indicate the 5–95 percentiles. The percentage of CD14+CD41+ monocytes was plotted against the platelet counts obtained on the same day that platelet–monocyte aggregates were analyzed (C) and the lowest plasma albumin level for each patient (D). Linear regressions were traced according to the distribution of points. *p < 0.01, versus control; #p < 0.05, positive versus negative.

Platelets from dengue-infected patients aggregate with control monocytes and induce cytokine release in vitro

Next, we investigated the ability of platelets isolated from patients with dengue to aggregate with monocytes from healthy volunteers and modulate monocyte responses. Increased platelet–monocyte aggregate formation was observed when monocytes from healthy volunteers were exposed to platelets from DENV-infected patients in comparison with platelets from heterologous healthy volunteers (62.5 ± 9.1% versus 30.7 ± 11.6%, p = 0.006). Incubation of platelets from healthy volunteers with monocytes from dengue patients did not promote any increment in platelet–monocyte aggregates compared with control platelets plus control monocytes (41.8 ± 19.5%, p = 0.2234) (Fig. 3).

We showed previously that signaling by activated adherent platelets enhances cytokine and chemokine production by monocytes, including TNF-α, IL-1β, IL-8, and MCP-1 (10, 11, 25). In this study, the levels of IL-1β and IL-8 were significantly (p < 0.05) elevated in the supernatant of monocytes exposed to platelets from dengue-infected patients compared with control platelets (Fig. 4A, 4B). The levels of MCP-1 were elevated in all platelet–monocyte interactions compared with monocytes alone, regardless of patient or control source of the cells (Fig. 5C). Also, increased RANTES secretion was observed in platelets from healthy volunteers compared with dengue-infected patients (Fig. 4D).

The secretion of TNF-α was not different between monocytes exposed to platelets from control or dengue subjects or in platelet–monocyte interactions compared with monocytes alone (Fig. 4E). Interestingly, we observed that monocytes exposed to platelets from dengue-infected subjects secreted increased levels of IL-10 (Fig. 4F), a cytokine not previously demonstrated to be directly modulated by platelet–monocyte binding. Other cytokines measured in the multiplex assay were either below the detection limit or were not different among platelets, monocytes, or platelet–monocyte interactions from patients and controls (data not shown).

P-selectin and phosphatidylserine are required for IL-10 secretion in platelet–monocyte aggregates

Previously, we showed increased platelet apoptosis, in addition to platelet activation, in patients with dengue (8). Platelets from patients in the current study similarly showed increased phosphatidylserine exposure (26.9 ± 7.9% versus 4.9 ± 3.2%, for dengue patients and healthy volunteers, respectively). We hypothesized that monocytes secrete IL-10 (Fig. 4F) in response to the recognition of apoptotic platelets in platelet–monocyte interaction.
aggregates. Thus, we evaluated monocyte responses after exposure to activated platelets or to activated and apoptotic platelets. Platelet activation and apoptosis were induced by specific agonist stimulation, as previously described (26). Platelets stimulated with thrombin or convulxin alone became activated but not apoptotic; in contrast, platelets stimulated with thrombin plus convulxin became activated and apoptotic, as demonstrated by P-selectin surface expression, phosphatidylserine exposure, and loss of $\Delta \Psi_m$ (Fig. 5A). As shown in Fig. 5B, monocytes incubated with thrombin-activated platelets secreted IL-8 but not IL-10. In contrast, the exposure of monocytes to platelets stimulated with thrombin plus convulxin induced both IL-8 and IL-10. Importantly, the agonists alone did not induce significant cytokine secretion in monocytes.

To better understand the mechanisms by which monocytes secrete cytokines in response to apoptotic and/or activated platelets, monocytes were exposed to platelets in the presence of anti-P-selectin or anti-phosphatidylserine Abs. As previously reported (11), blocking of P-selectin damped the secretion of IL-8 in monocytes interacted with activated platelets, independently if stimulated with thrombin or thrombin plus convulxin (Fig. 5C). Interestingly, the secretion of IL-10 in monocytes exposed to platelets stimulated with thrombin plus convulxin also was dampened by P-selectin blocking (Fig. 5D). The secretion of IL-8 by platelet–monocyte aggregates was not affected by anti-phosphatidylserine Abs (Fig. 5E). Nevertheless, blocking of phosphatidylserine on apoptotic platelets significantly reduced the secretion of IL-10 (Fig. 5F). These data indicate that IL-10 secretion by platelet–monocyte aggregation depends on both P-selectin–mediated binding and phosphatidylserine recognition on activated and apoptotic platelets.

Phosphatidylserine recognition is sufficient to induce IL-10 secretion by monocytes

Beyond its immunomodulatory activities, phosphatidylserine recognition is the main signal for apoptotic cell phagocytosis. During platelet–monocyte aggregation, the levels of platelet phagocytosis were higher in monocytes interacting with thrombin plus...
convulxin–stimulated platelets compared with unstimulated platelets (Fig. 6A, 6B). Treatment of platelets with anti–P-selectin or anti-phosphatidylserine (PhSer) Abs significantly reduced the phagocytosis of apoptotic platelets (Fig. 6A, 6B). To better understand the role played by platelet phagocytosis in the regulation of IL-10 secretion, monocytes were pretreated (30 min) with the cytoskeleton assembly inhibitors cytochalasin D (10 μg/ml) and cytochalasin B (10 μg/ml), which significantly impaired uptake of apoptotic platelets (Fig. 6A, 6B). Interestingly, platelet phagocytosis was not required for the secretion of IL-8 or IL-10 (Fig. 6C, 6D). We next investigated whether a synergistic signaling of P-selectin and phosphatidylserine is required to induce IL-10 synthesis. We observed increased adhesion of monocytes plated on P-selectin and/or phosphatidylserine compared with HSA (Fig. 6E, 6F). Monocytes plated on P-selectin and/or phosphatidylserine also secreted increased levels of IL-8. However, the secretion of IL-10 was preferentially found in monocytes adherent to phosphatidylserine or to P-selectin plus phosphatidylserine compared with HAS-coated plates (Fig. 6G). These results indicate that phosphatidylserine recognition is sufficient to induce IL-10 secretion by monocytes, suggesting that phagocytosis of apoptotic platelets or synergistic signaling by P-selectin plus phosphatidylserine is not required (Fig. 7).

Discussion

Thrombocytopenia and increased vascular permeability are hallmarks of dengue illness. Although high concentration of pro- and

**FIGURE 5.** Apoptotic platelet recognition mediates IL-10 secretion by platelet–monocyte aggregates. (A) Representative density plots showing P-selectin surface expression, Δψm, and phosphatidylserine (PhSer) exposure in platelets activated with thrombin (Thr) and/or convulxin (Cvx). (B–F) Monocytes were exposed to resting platelets or platelets activated with Thr or Thr+Cvx in the presence or absence of neutralizing Abs against CD62-P or PhSer. Concentrations of IL-8 (B, C, and E) and IL-10 (B, D, and F) in the supernatants of cells incubated in each condition. The bars represent mean ± SEM of four to eight independent experiments. *p < 0.05, versus unstimulated platelets; *p < 0.05, versus IgG.
anti-inflammatory cytokines have been extensively reported in dengue patients (4, 27–29), the sources and determinants for cytokine secretion are not fully elucidated. Our results demonstrate a role for platelet–monocyte interactions in the activation of monocytes during dengue infection. We observed increased levels of platelet–monocyte aggregates in patients with dengue, especially in samples from patients who exhibited thrombocytopenia and signs of increased vascular permeability. We found evidence that platelet binding modulates cytokine responses by monocytes in dengue. Interaction with platelets from patients with dengue enabled monocytes from healthy volunteers to synthesize and secrete IL-1β, IL-8, and IL-10. Experiments with in vitro–stimulated platelets showed that the secretion of cytokines is regulated by P-selectin–mediated adhesion and, in addition, recognition of apoptotic platelets through phosphatidylserine (Fig. 7). Induction of immunomodulatory gene expression in platelet–monocyte aggregates by phosphatidylserine signaling has not been reported previously.

Platelet adhesion to leukocytes is mediated by platelet P-selectin surface expression (11, 13, 24), which is increased in platelets from patients with dengue (8). Onlamoon et al. (30) found that DENV elicits platelet–monocyte and platelet–neutrophil aggregates in a primate model for severe dengue. Platelet–monocyte aggregates also were observed in mild dengue in humans (14). In these interactions, the binding of P-selectin on activated platelets to P-selectin glycoprotein ligand (PSGL)-1 on monocytes not only tethers the cells together but also triggers functional responses in the monocytes (24), among them cytokine synthesis and secretion (10, 11, 25). Of importance, the cytokines IL-1β, IL-8, and IL-10, which were released by monocytes in response to interactions with platelets from dengue patients, are frequently increased in plasma from patients with severe dengue (4, 27–29).

It is known that signals delivered to monocytes by binding of platelet P-selectin to PSGL-1 are integrated and amplified by factors secreted from platelets (11, 31), including the chemokine RANTES (11). In this study, platelets from dengue-infected patients secreted lower levels of RANTES in vitro than did platelets from healthy volunteers. This may be explained by extensive release of platelet granule contents in vivo before platelet isolation, because platelets from patients with dengue were shown to be activated. Furthermore, we showed previously that platelets release RANTES in response to DENV exposure (21). Because
**FIGURE 7.** Schematic representation of platelet-induced cytokine secretion by monocytes. (A) Interaction of activated and apoptotic platelets with monocytes during DENV infection promotes secretion of IL-1β, IL-8, IL-10, and MCP-1. (B) Surface binding of P-selectin to PSGL-1 and recognition of phosphatidylserine (PhSer) by PhSer receptors, in parallel with the secretion of chemokines from platelet α-granules, are the main mechanisms for immunomodulation of monocytes by activated and apoptotic platelets after agonist stimulation. Even though we show phosphatidylserine-mediated signaling in platelet–monocyte aggregates in vitro and during dengue infection, it potentially occurs in any disease condition where platelet activation and platelet apoptosis take place (i.e. dengue, sepsis).

MCP-1 synthesis by monocytes in response to platelet adhesion depends on concomitant RANTES signaling (11), the exhaustion of RANTES in platelets from dengue-infected patients may explain why these platelets failed to induce higher MCP-1 secretion compared with control platelets, even though they express more P-selectin. Platelet–monocyte aggregate formation probably contributes to MCP-1 release in vivo, because both platelet–monocyte aggregates and RANTES are increased in patients with dengue (29, 32).

In previous studies from our group (4) and other investigators (33, 34), the levels of MCP-1, IL-1β, IL-8, and IL-10 were associated with severe thrombocytopenia in dengue. These associations are in agreement with the formation of platelet–monocyte aggregates as a mechanism for both thrombocytopenia and cytokine release, as suggested by the correlation of platelet–monocyte aggregates with platelet counts in the present work. Platelet–monocyte aggregates also were associated with increased vascular permeability in dengue patients. The endothelial effects of the cytokines released in response to platelet–monocyte binding might explain this association. The cytokines IL-1β, IL-8, and MCP-1 are reported to be mediators in DENV-triggered endothelial leak based on in vitro assays (21, 35, 36). Furthermore, IL-1β is linked to enhanced vascular permeability, hypotension, and hemococoncentration in dengue-infected patients (4, 21). We showed previously that activated platelets are important sources of IL-1β during dengue infection, and that platelet-released IL-1β contributes to increased endothelial permeability in dengue (21). We now show that activated platelets also contribute to IL-1β levels in dengue by inducing IL-1β synthesis in monocytes. However, we cannot exclude that IL-1β secreted by platelets from patients with dengue may have contributed to the higher levels of IL-1β in platelet–monocyte interactions (Fig. 4A). Relevant to the importance of platelets and platelet–monocyte aggregates as sources of IL-1β in systemic infections, depletion of platelets completely reduced the circulating levels of IL-1β in mice with experimental cerebral malaria (37).

Beyond proinflammatory cytokines, exposure to platelets from dengue-infected patients induced the secretion of IL-10 in monocytes. IL-10 is an anti-inflammatory and regulatory cytokine with many immunomodulatory properties. This cytokine inhibited DENV-specific T cell responses, as shown by ex vivo and in vitro models (38). Accordingly, patients with severe dengue exhibit increased levels of IL-10 compared with patients with mild dengue (39, 40), and higher levels of IL-10 are observed in nonsurvivors than in survivors among patients with severe dengue (41).

In addition to platelet activation, we showed previously that platelets from dengue patients have characteristics that are indicative of apoptosis, among them the exposure of phosphatidylserine (8). Similarly, platelets from dengue patients in the current study had increased phosphatidylserine exposure. Phosphatidylserine exposure is the main “eat-me” signaling for apoptotic cell clearance. Recognition of phosphatidylserine by mononuclear phagocytes promotes apoptotic cell uptake, as well as immunoregulatory responses, including IL-10 synthesis and secretion (16, 42). Alonzo et al. (15) showed that cultured macrophages phagocytized platelets from dengue-infected patients, depending on phosphatidylserine recognition. In our model for agonist-induced platelet apoptosis, the recognition of phosphatidylserine induced platelet phagocytosis, as well as IL-10 secretion. IL-10 secretion also was induced in monocytes exposed to platelets from patients with dengue. Guðbrandsdóttir et al. (43) recently reported higher IL-10 synthesis in platelet-attached monocytes after stimulation of PBMCs with LPS in the presence of platelets. In agreement, platelet phagocytosis and IL-10 secretion also were influenced by P-selectin–mediated binding in our platelet–monocyte interactions. Although blocking P-selectin reduced IL-10 secretion in platelet–monocyte aggregates, our results with isolated adherent monocytes indicate that the recognition of phosphatidylserine is sufficient to induce IL-10 secretion. P-selectin binding probably contributes to apoptotic platelet phagocytosis and IL-10 secretion by facilitating phosphatidylserine recognition while tethering the cells together. These results are in agreement with those from other investigators who showed that apoptotic platelet clearance by neutrophils is a biphasic event that depends on P-selectin–mediated adhesion, followed by phosphatidylserine-mediated internalization (44). Thus, P-selectin– and phosphatidylserine-mediated cell interactions may represent new targets for dengue treatment research, and Abs against P-selectin and phosphatidylserine were recently shown to be safe for human use in phase 1 clinical trials (45–47). Although our findings suggest that monocytes secrete IL-10 through recognition of apoptotic platelets in dengue, we cannot exclude that this also occurs in response to other apoptotic cell types. In a previous study, levels of IL-10 were associated with the presence of apoptotic lymphocytes in dengue-infected patients (39).

In summary, to our knowledge, we report for the first time the contributions of platelets to inflammatory and immunomodulatory responses of monocytes during dengue infection. We provide evidence for platelet–monocyte aggregate formation during dengue infection and platelet-dependent monocyte activation with IL-1β, IL-8, IL-10, and MCP-1 synthesis and secretion. We also provide new insights regarding the biology of platelet–monocyte interactions, with the recognition of phosphatidylserine on apoptotic platelets as a key immunoregulatory event. Each of these events and cellular interactions potentially contribute to the pathogenesis of dengue.

**Acknowledgments**

We thank Robert Campbell for technical assistance and the Programa de Desenvolvimento Tecnológico em Insumos para Saúde platform for multiplex analysis.

**Disclosures**

The authors have no financial conflicts of interest.
References


The Journal of Immunology