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Platelet Induction of the Acute-Phase Response Is Protective in Murine Experimental Cerebral Malaria

Angela A. Aggrey,*† Kalyan Srivastava,* Sara Ture,* David J. Field,* and Craig N. Morrell*

Platelets are most recognized as the cellular mediator of thrombosis, but they are increasingly appreciated for their immunomodulatory roles, including responses to Plasmodium infection. Platelet interactions with endothelial cells and leukocytes contribute significantly to the pathogenesis of experimental cerebral malaria (ECM). Recently, it has been suggested that platelets not only have an adverse role in cerebral malaria, but platelets may also be protective in animal models of uncomplicated malaria. We now demonstrate that these diverse and seemingly contradictory roles for platelets extend to cerebral malaria models and are dependent on the timing of platelet activation during infection. Our data show that platelets are activated very early in ECM and have a central role in initiation of the acute-phase response to blood-stage infection. Unlike platelet depletion or inhibition postinfection, preinfection platelet depletion or treatment with a platelet inhibitor is not protective. Additionally, we show that platelet-driven acute-phase responses have a major role in protecting mice from ECM by limiting parasite growth. Our data now suggest that platelets have a complex role in ECM pathogenesis: platelets help limit parasite growth early postinfection, but with continued platelet activation as the disease progresses, platelets contribute to ECM-associated inflammation. The Journal of Immunology, 2013, 190: 4685–4691.

Malaria is a leading cause of child morbidity and mortality worldwide. In 2011, the World Health Organization estimated there were 216 million cases of malaria with 655,000 deaths, 86% of which were children <5 y of age (1). Cerebral malaria (CM) is a severe complication of malaria with 655,000 deaths, 86% of which were children <5 y of age (1). Cerebral malaria (CM) is a severe complication of Plasmodium falciparum infection characterized by neurocognitive deficits, stroke-like cerebral vascular lesions, and often death. CM has a predilection for children <5 y of age and accounts for a significant percentage of malaria-related mortality. CM is typified by sequestration of infected RBCs (iRBCs) in cerebral microvessels, with postmortem brains showing petechial hemorrhage and vascular lesions with iRBCs, leukocytes, and platelets (2). Despite its potentially devastating outcome, the pathogenesis of CM is still not well understood.

Platelets have important roles in many vascular inflammatory diseases, including CM (3). Platelets are best known for their role as the cellular mediator of hemostasis, but platelets also have important immunomodulatory effects (4). Platelets interact with an intact, inflamed endothelial cell layer, leading to platelet activation and further vascular inflammation. An activated endothelium expresses selectins and adhesion molecules (ICAM, VCAM) that mediate platelet tethering, rolling, and eventually adhesion. Platelet activation leads to degranulation and the release of cytokines and chemokines, as well as the secretion of newly synthesized immune mediators, such as thromboxane and IL-1β, leading to more vascular inflammation and leukocyte recruitment. This contributes to the obstruction of vessels in CM by platelets and infiltrating leukocytes. Platelet-derived immune mediators also exert effects distant from the site of vascular injury. Because platelets are so numerous and packed with large numbers of immune mediators, platelet activation leads to high plasma concentrations of chemokines and cytokines such as PF4, RANTES, CXCL7, and IL-1β (5).

Vascular inflammation, immune stimulation, and vascular obstruction that typify CM are driven in large part by platelets (3). We have previously described a mechanism in which the platelet-derived chemokine PF4 contributes to CM pathogenesis by recruiting and activating monocytes and T cells at sites distant from cerebral vascular inflammation, helping to drive CM pathology (6). There are numerous reports demonstrating a mechanistic role for platelets in driving the pathology associated with CM (2, 3, 7, 8). There are recent reports indicating that platelets may also have a protective role in uncomplicated malaria by killing Plasmodium in iRBCs (9). This coupled with other conflicting reports on the benefits of antiplatelet therapy in humans (10, 11) prompted us to explore the complex role of platelets in CM development.

The acute-phase response (APR) is the earliest stage of the innate immune responses to injury or infection and results in the production of acute-phase proteins (APPs) from the liver. Major APPs include C-reactive protein (CRP), serum amyloids A (SAA) and P (SAP), and complement proteins. Proinflammatory cytokines produced early in inflammation activate APP production from hepatocytes (12). Major stimulators of APP production include IL-6, IL-1β, and TGF-β. Although IL-6 is not found in platelets,
IL-1β and TGF-β are abundant platelet-derived immune mediators. Activated platelets express adhesion molecules (e.g., P-selectin), and hepatic sinusoids have lectin receptors that may interact with the activated platelets to induce the production of APPs, demonstrating the potential for platelets to have a major role in the induction of the APR.

Platelets are immune modulators in experimental CM (ECM), but the mechanisms and cell interactions have not been fully elucidated. Because the timing and phase of the response is key in immune pathogenesis, this immune stimulatory role for platelets may account for the seemingly conflicting reports on the roles of platelets in Plasmodium-related disease pathogenesis. We now demonstrate that platelets are activated very early during blood-stage infection with *P. berghei* and platelets initiate the APR. Furthermore, we demonstrate that adverse versus protective roles for platelets depend on the timing during infection and the activation of the APR.

**Materials and Methods**

**Reagents**

ELISAs for mouse IL-1β, mouse PF4, and human CRP were purchased from R&D Systems. Mouse SAA and SAP ELISAs were purchased from Invitrogen and Immunology Consultants Lab, respectively. Platelet-depleting as well as fluorescent platelet-labeling Abs were purchased from Emfret Analytics. SYBR Green DNA labeling dye was purchased from Invitrogen. Clopidogrel was obtained from the Bristol-Myers Squibb/Sanofi Partnership. Murine recombinant SAP (rSAP) and murine recombinant IL-1β were purchased from R&D Systems.

**Animals**

Wild-type (WT) mice on a C57BL6/J background between 6 and 8 wk of age were purchased from The Jackson Laboratory. Mice transgenic for rabbit CRP on a C57BL6/J background for >10 generations are described elsewhere (13) and were a kind gift from Philip Shaul (University of Texas Southwestern, Dallas, TX). All experimental procedures using mice were approved by the University of Rochester School of Medicine and Dentistry Institutional Animal Care and Use Committee.

**Platelet depletion**

Mice were platelet depleted by an i.p. injection with 4 μg/g platelet-depleting Ab (directed against GPIbα; CD42b) or control IgG (isotype control).

**Murine ECM model**

Mice were injected i.p. with 0.5–0.7 × 10⁶ *P. berghei* ANKA (PbA) iRBCs (clone 2.34 provided by Dr. Fidel Zavala, The Johns Hopkins University School of Public Health). Survival was determined within 10 d post-PbA inoculation, and mice were monitored for ECM symptoms: cachexia, deviation of the head, prostanation, and coma. Mice exhibiting two or more of these symptoms were deemed ECM+ and sacrificed if unable to be aroused from a prostrate position. Parasitemia was determined by SYBR Green (Invitrogen) labeling of iRBCs (14) and confirmed by Diff Quik staining of thin blood smears.

**Cytokine and chemokine analyses**

Mouse blood was collected by retro-orbital sinus into EDTA-coated tubes and plasma obtained by subsequent centrifugation at 3200 rpm.
for 10 min. The plasma was then used in ELISAs per the manufacturer’s instructions.

**Platelet isolation**

Murine blood was obtained through the retro-orbital plexus into heparinized tyrodes buffer (134 mM NaCl, 0.34 mM Na₂HPO₄, 2.9 mM KCl, 12 mM NaHCO₃, 20 mM HEPES [pH 7], 5 mM glucose, and 0.35% [w/v] BSA) in Eppendorf tubes. The blood was then centrifuged at 1000 rpm for 5 min at room temperature to obtain platelet-rich plasma, which was then transferred into new tubes containing tyrodes buffer (with 1% PGE₂ to prevent platelet activation). Platelets were pelleted by a subsequent 2600 rpm centrifugation for 5 min at room temperature, resuspended in tyrodes buffer, and used for further experiments within 2 h.

**Platelet trafficking by fluorescent microscopy**

Mice were infected, and 24 h postinfection, mice were injected i.v. with fluorescently labeled anti-platelet Ab (X488; Emfret). Mice were then euthanized, livers thinly sliced (<1-mm sections) and imaged with a Nikon Ti microscope (Nikon), and still images captured using Elements software. Platelet foci from 10 fields were analyzed and quantified using ImageJ software (National Institutes of Health).

**Transmission electron microscopy**

Mice were infected with *P. berghei*, euthanized 24 h later, and livers excised into PBS/BSA buffer without perfusion. The excised livers were then thinly sectioned and immersion fixed in a combination fixative containing 4.0% paraformaldehyde/2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After 24 h of primary fixation, the tissue was rinsed in the same buffer and postfixed for 1 h in buffered 1.0% osmium tetroxide, dehydrated in a graded series of ethanol to 100%, transitioned into propylene oxide, infiltrated with EPON/Araldite resin overnight, embedded into molds, and polymerized for 48 h at 60°C. Using a glass knife, epoxy-embedded liver blocks were sectioned at 1 μm and stained with toluidine blue to determine areas for thin sectioning. A diamond knife inserted into the ultramicrotome was used to cut 70-nm thin sections, which were placed onto carbon-coated nickel grids. The grids were stained with aqueous uranyl acetate and lead citrate and examined using a Hitachi 7650 TEM (Hitachi) with an attached Gatan 11 megapixel Erlangshen digital camera and Digitalmicrograph software.

**SAP reconstitution in vivo**

Mice were platelet depleted or treated with isotype control as above. Platelet-depleted mice were given murine rSAP (1 mg/kg; i.p.) from days 1–3 post-*P. berghei* infection and survival monitored.

**Data analysis**

Data are expressed as mean ± SDs. Statistical significance of values was determined by using unpaired Student two-tailed *t* tests; *p* values ≤0.05 were deemed significant. Survival data were analyzed by log-rank tests using Prism GraphPad software (GraphPad).

**Results**

The pathogenic effect of platelets in ECM is time dependent

Several reports have shown that platelets have an adverse role in ECM pathogenesis (7, 8) and that platelet depletion postinfection is protective (6, 15). However, there is also a report of increased parasitemia in platelet-deficient mice and platelet-mediated killing of intraerythrocytic *Plasmodium* parasites in a mouse model of

**FIGURE 2.** Platelets drive the APR to *P. berghei* infection. (A) Mice were platelet depleted or treated with control IgG 24 h preinfection and plasma SAP measured by ELISA (n = 5; mean ± SD, *p* < 0.05). (B) Mice were platelet-depleted or treated with IgG 24 h preinfection and plasma SAA measured by ELISA (n = 5; mean ± SD, *p* < 0.05). (C) Mice were platelet-depleted or treated with IgG 24 h postinfection and plasma SAP measured by ELISA (n = 5; mean ± SD). (D) Mice were platelet depleted or treated with IgG 24 h postinfection and plasma SAA measured by ELISA (n = 5; mean ± SD). UC, Uninfected control.
uncomplicated malaria (9). We therefore sought to determine whether the timing of platelet activation influences the platelet-dependent outcome in ECM. C57BL6/J mice were infected with \(0.5 \times 10^6\) PbA iRBCs. As a marker of platelet activation, plasma concentrations of the platelet-derived chemokine PF4 were measured daily. Platelets were activated within 48 h of infection (Fig. 1A), and platelet activation increased over time independent of blood collection (Supplemental Fig. 1). Platelet activation correlated with parasitemia within the first 3 d postinfection before mice presented with symptoms of ECM (Fig. 1A). These data indicate that platelet activation may be a direct consequence of parasite burden.

To determine whether platelet-mediated ECM outcome is dependent on the timing of blood-stage infection, we platelet-depleted mice using an anti-GPIb Ab either 24 h before or 24 h after PbA infection, and survival was monitored. Platelet depletion preinfection did not protect mice from ECM (Fig. 1B). However, as we previously reported (6), mice platelet depleted postinfection had increased survival (Fig 1B). Platelet-depleted mice also had higher parasitemia compared with isotype control IgG-treated mice on day 5 postinfection (Fig. 1D). Similarly, pretreatment of \(P.\) \(berghei\)-infected mice with the platelet inhibitor clopidogrel beginning 24 h before infection failed to protect mice from ECM (Fig. 1C), but mice treated with clopidogrel beginning 24 h postinfection had increased survival compared with controls (Fig. 1C). These data demonstrate that platelet activation begins early post-\(P.\) \(berghei\) infection, and this early platelet activation may be protective, in part, by reducing parasite burden. However, platelet activation beyond this very early protective phase may be deleterious.

**Platelets drive the APR to \(P.\) \(berghei\) infection**

The APR is the rapidly induced first stage of response to infection or tissue injury. In contrast to mice platelet depleted postinfection, mice platelet depleted preinfection had a loss of protection from ECM. To determine whether platelet-dependent induction of the APR may have a protective role, and account in part for the loss of protection from ECM when mice are platelet depleted preinfection, we first measured the plasma concentrations of SAA and SAP as markers of the murine APR. SAP and SAA were significantly decreased in mice platelet depleted preinfection compared with IgG control mice (Fig. 2A, 2B). Plasma concentrations of SAP and SAA in postinfection platelet-depleted mice were not significantly different from IgG control-infected mice, indicating that by 24 h postinfection, platelets have contributed greatly to induction of the APR to blood-stage \(P.\) \(berghei\) infection (Fig. 2C, 2D, respectively).

APRs are primarily liver derived and induced by cytokines such as IL-6, IL-1\(\beta\), and TGF-\(\beta\). Platelets synthesize and secrete IL-1\(\beta\) upon activation (16). To determine whether the platelet-dependent APR to infection involves IL-1\(\beta\), we measured IL-1\(\beta\) in the plasma of mice platelet depleted before and postinfection. Mice platelet depleted preinfection had decreased plasma IL-1\(\beta\) compared with nondepleted controls (Fig. 3A). In contrast, mice platelet depleted postinfection had similar levels of circulating plasma IL-1\(\beta\) as non-platelet-depleted IgG controls (Fig. 3B). As noted in Fig. 2, plasma SAP was also decreased by preinfection depletion, but plasma SAP levels were restored with murine recombinant IL-1\(\beta\) treatment within the first 72 h of infection (Fig. 3C). These data indicate that platelets are central to IL-1\(\beta\) response to infection. To demonstrate
that platelets are a direct source of IL-1β, washed murine platelets were permeabilized and intracellular IL-1β measured by flow cytometry. Intracellular IL-1β was significantly increased in infected mice 24 h postinfection compared with uninfected controls (Fig. 3D).

Because the liver is the major site of APP production, we determined whether platelets are recruited to the liver early in P. berghei blood-stage infection. At 24 h postinfection, mice were injected with a fluorescent platelet-labeling Ab, livers were removed, cut into thin sections, and imaged on a fluorescent microscope (Fig. 4A). Mice infected with P. berghei had ∼3-fold more platelet foci in liver sections compared with uninfected controls, indicating an increase in platelet trafficking to the liver during APR induction (Fig. 4B). We further examined platelet localization to the liver using electron microscopy. Transmission electron micrographs (TEMs) of murine livers 24 h postinfection also showed increased platelet-rich foci within the liver microvasculature of infected mice compared with uninfected controls (Fig. 4C, top panel). In addition, there were multiple platelets with extended lamellipodia and platelet interactions with liver sinusoidal endothelial cells in infected mice that were not seen in control mice (liver sinusoidal endothelial cells [LSECs], Fig. 4C, bottom right panel). Platelets present in the lumen of hepatic microvessels of uninfected control mice appeared quiescent and were not interacting with the sinusoid endothelium (Fig. 4C, bottom left panel).

Taken together, these results indicate that platelets induce the APR and that platelet contact–dependent mechanisms as well as platelet-derived or -induced IL-1β have central roles in eliciting the APR.

**The APR is protective in ECM**

Based on our findings that platelets initiate the APR and production of APPs, we determined whether the main mouse and human APPs, SAP, and CRP, respectively, are protective in the ECM model. Mice were platelet depleted preinfection and given exogenous rSAP or control buffer (PBS) on days 1–3 postinfection. Platelet-depleted mice treated with rSAP had a delay in death compared with platelet-depleted mice (Fig. 5A). The survival of the rSAP-treated mice was similar to control mice, indicating that loss in platelet induction of SAP may in part account for the preinfection platelet-depletion phenotype (Fig. 5A).

Mice do not use CRP as an APP. We therefore used CRP-transgenic (CRP-Tg) mice (13) in the ECM model to determine the effects of the dominant human APP in ECM pathogenesis. CRP-Tg mice were infected and found to be completely protected from ECM death (Fig. 5B) and had significantly reduced parasitemia compared with WT control mice (Fig. 5C). CRP-Tg mice also had a significantly reduced parasite burden in mice infected with a nonlethal P. yoelii strain (Fig. 5D). These data indicate a protective role for APP in ECM, in part by reducing parasitemia (Fig. 6).

**Discussion**

Our data demonstrate a novel time-dependent role for platelets in the pathogenesis of ECM. Our prior studies (6, 17), and those of many other investigators (2, 3, 7, 8), have demonstrated that platelets have a central role in cerebral vascular injury leading to ECM. When platelets were depleted, or mice treated with platelet inhibitors postinfection, ECM survival was greatly improved (6, 15, 18). In this study, we show that platelets have a more complex role in ECM than previously thought. Very early in infection, platelets are activated and initiate the APR that is protective by limiting the development of and death due to ECM. Depletion of platelets or the use of platelet inhibitors preinfection was not survival protective and increased parasitemia. The APR is initiated in the liver, and we demonstrated that platelets are recruited to the

**FIGURE 4.** Platelets are recruited to the liver early post–P. berghei infection. (A) Platelets (white arrows) are recruited to the liver postinfection. At 24 h postinfection, mice were injected with anti–platelet fluorescent Ab and sacrificed. Livers were imaged with a fluorescent microscope (representative images, original magnification ×20). (B) Platelet foci were quantified from 10 different fields (n = 5, mean ± SD, *p < 0.01, unpaired Student t test). (C) Platelets (P) interact with liver sinusoidal endothelial cells (LSEC) during infection (representative TEM images; top panels, original magnification ×12,000 and bottom panels, original magnification ×30,000). Ultrathin liver sections from uninfected controls (left panels) and infected mice (right panels) 24 h post–P. berghei infection.
liver upon *P. berghei* infection and that platelets are needed to increase the plasma concentration of a major acute-phase initiator IL-1β. This may in part be a direct platelet effect, as we noted increased intracellular platelet IL-1β in *P. berghei*-infected mice, or the effect of platelet interaction with other IL-1β-producing cells such as monocytes. The APR is protective, as CRP-Tg mice and mice treated with exogenous SAP had greatly improved survival and delayed onset of death, respectively. Platelets are thus a critical mediator of protection in ECM through initiation of the APR.

The adverse role for platelets in ECM is largely due to platelet inflammatory functions, the capacity for platelets to mediate iRBC sequestration, as well as platelet secretion of chemokines that lead to leukocyte recruitment. As infection progresses, platelets therefore establish an inflammatory environment that drives and exacerbates severe disease. But evidence of a more complex role for platelets in malaria, particularly in uncomplicated malaria, came with the discovery that platelets may bind to and kill intraerythrocytic parasites through yet to be understood mechanisms. Our new findings for a time-dependent protective role for platelets indicate that this paradigm may extend to severe malaria in the very early stages of infection. The APR, an ancient defense mechanism, is the earliest phase of the innate immune response to infection and trauma and occurs within 24–72 h after stimulus. There is a robust APR in human malaria infection both to the liver (19) and erythrocytic stages (20). We have found an early platelet-activation response to infection and a decrease in APPs when platelets are depleted or inhibited, indicating an important role for platelets in activating the APR. Together, this work and our past studies establish a new important paradigm: platelets are activated by *Plasmodium* infection, leading to a protective APR. However, with continued platelet activation, there is an adverse innate and acquired immune response as well as thrombosis (21) that accelerates ECM.

Upon activation, platelets undergo numerous changes, including the release of inflammatory molecules that have effector functions both locally and at sites distant from their site of release. IL-1β is a major platelet-derived cytokine (16, 22) with important roles in fever and APRs to infection (23). IL-1β knockout mice have a severely attenuated APR (24), and low doses of exogenous IL-1β have been shown to protect mice against lethal CM (25). Human studies have demonstrated that low levels of IL-1β are associated with increased severe malaria anemia (26). Our studies indicate a potentially important role for platelet IL-1β in protec-

**FIGURE 5.** The APR is protective in ECM. (A) Exogenous SAP administration of platelet-depleted *P. berghei*-infected mice returns them to control survival levels. Mice were platelet depleted 24 h preinfection or platelet depleted and treated with rSAP for the first 3 d postinfection. Survival (*n* = 5, *p* < 0.05, log-rank test versus platelet depleted + SAP). (B) CRP-Tg mice are protected from ECM. CRP-Tg or WT mice were infected with *P. berghei* and survival monitored (*n* = 5, *p* < 0.05, log-rank test). (C) CRP reduces the parasite burden in ECM. Mice were infected with *P. berghei* and parasitemia determined by flow cytometry analysis of SYBR Green-labeled iRBCs (*n* = 5, *p* < 0.05, unpaired Student *t* test). (D) CRP reduces the parasite burden in *P. yoelii* infections. Mice were infected with *P. yoelii* and parasitemia determined by flow cytometry analysis of SYBR Green-stained iRBCs (*n* = 8, mean ± SD; *p* < 0.05, unpaired Student *t* test).
tive immune responses; however, as platelet depletion did not completely abrogate plasma IL-1β in infected mice, other cells likely also contribute IL-1β or are stimulated by activated platelets to contribute to the total circulating IL-1β.

APPs are commonly used as markers of infection, injury, or trauma, but also have immune effector functions. For example, during *Staphylococcus pneumoniae* infection, SAP has been found to be protective by aiding complement activation (27). In patients presenting with *P. falciparum* infection in Papua, New Guinea, acute uncomplicated malaria infection was positively correlated to high serum CRP levels, whereas patients with severe or fatal malaria during acute uncomplicated malaria infection was positively correlated to high serum CRP levels, whereas patients with severe or fatal malaria disease protects against Plasmodium berghei pathogenesis by altering the levels of pathogenic cytokines. Blood 105: 1956–1963.


