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Platelet power: sticky problems for sticky parasites?

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Abstract

Platelets might have a crucial role in the pathogenesis of both human and rodent malarias by assisting in the sequestration of infected erythrocytes within the cerebral vasculature. However, recent elegant work by McMorran *et al.* suggests that they are also involved in innate protection during the early stages of infection. Here, we discuss the implications of their important findings in the context of immunity to malaria.

Platelet: friend or foe?

The platelet, traditionally known for its role in blood clotting, is also known for its putative involvement in malaria pathology [1,2]. However, a recent study using C57BL6 mice genetically deficient in the megakaryocyte growth and differentiation factor C-mpl (encoded by the *Mpl* gene), and resulting in mice with 90% fewer platelets, showed that these animals were significantly more susceptible to death when infected with *Plasmodium chabaudi* [3]. Furthermore, the authors went on to show that purified human platelets killed *Plasmodium falciparum* parasites within red blood cells when added to *in vitro* cultures and that various platelet antagonists, including aspirin, reversed this antiparasitic activity both *in vitro* and *in vivo*. This result raises concerns over the use of aspirin as an antipyretic in patients with malaria. Using specific receptor antagonists, the authors demonstrated that killing and control of parasite growth in *P. falciparum* cultures was dependent on platelet activation via P2Y1, an ADP-dependent metabotropic purinergic receptor (Figure 1).

These results in animals seem counterintuitive, in that platelets are believed to be involved in pathological disease states that hasten death, such as cerebral malaria [1,2]. For example, mice with significantly compromised platelet function (CXCL4 or CXCR3 deficient) have been shown to survive longer than their wild-type counterparts [4]. By contrast, platelet depletion by anti-CD41 monoclonal antibody injection early, but not late, in the course of disease is known to protect C57BL6 mice from *Plasmodium berghei* ANKA-induced severe experimental cerebral malaria (ECM) by altering levels of pathogenic cytokines [5]. Unfortunately, the study by McMorran *et al.* used *P. chabaudi*, a rodent malaria that – although capable of sequestering to a number of organs – is not known to develop ECM. It should also be noted that non-sequestering *Plasmodium* species also give rise to ECM in some inbred mouse strains, *Plasmodium yoelii* 17XL in BALB/c mice being a good example [6].

It is also important to consider issues of mouse genetic background. All knockout studies to date, including those reported by McMorran *et al.* for the *Mpl* gene, have been done in the C57BL6 mouse (susceptible to ECM). These experiments now need to be repeated in animals backcrossed onto different genetic backgrounds, such as BALB/c and DBA/2 mice (resistant to ECM), to determine whether other contributory genetic factors are at play. A

great deal of caution is also required in extrapolating these mouse models of ECM to the involvement of platelets in human disease.

Although these findings are clearly important, the authors did not address three other, equally sticky issues. First, how do platelets bind to infected erythrocytes? Second, what is or are the mechanism(s) by which platelets induce apoptosis and death for parasites hidden within the confines of the parasitophorous vacuole? And third, given the known importance of the common γ -chain in platelet activation and function, what part might Fc receptors (FcRs) and antibodies play in this process (Figure 1)?

A cornucopia of receptors

The first of these questions is easier to explain for *P. falciparum* than for *Plasmodium vivax* or the murine malaras. Platelet-mediated clumping is common in *P. falciparum* field isolates, is distinctive from other adhesive phenotypes and involves the host receptors CD36 [7] and gC1qR/HABP1/p32 [8]. Whether these are the only platelet receptors involved is debatable and worth exploring. Although GPIIb/IIIa (CD41/CD61) and GPIb/IX (CD42a/CD42b)-deficient platelets still clump to infected erythrocytes [7], the role of other key platelet adhesion or aggregation receptors – including GPVI, $\alpha_2\beta_1$, $\alpha_{11}\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$, PSGL-1, and platelet-endothelial cell adhesion molecule-1 (PECAM-1) – have not been explored (Figure 1). Furthermore, although not primary receptors involved in binding, the recruitment of other receptors after initial tethering could nonetheless be important for stabilization of the platelet-infected erythrocyte complex or for triggering functions from them, as is known for other immunological synapses. PECAM-1 is particularly interesting in this respect because it is known to be a ligand for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of variant surface antigens [9], binds glycosaminoglycans [10] and has been shown to inhibit platelet responses [11,12], suggesting that PECAM-1 triggering might be advantageous to the parasite.

These issues certainly need to be explored, and the availability of increasing numbers of mice deficient in various platelet-adhesion receptors and ligands might provide novel insights into the role of platelets in protection from malaria, especially under hydrodynamic shear flow in the bloodstream [13]. In addition, many of these receptors (including CD36 and gC1qR/HABP1/p32) are expressed by other important immune cells, including dendritic cells, neutrophils and B cells. Are these cells also found within platelet clumps *in vivo*, and if not, why not? Equally weighty issues concern the affinity and avidity of such interactions and whether binding occurs in the presence or absence of the native ligands for these receptors. This is particularly interesting for gC1qR/HABP1/p32 that binds the globular head domains of C1q and hyaluronic acid [8,14]. Do these host ligands share overlapping binding sites on gC1qR/HABP1/p32 with the parasite molecules expressed on the infected erythrocytes? And what is the hierarchy of binding in terms of affinity? What role complement and/or immune complexes (abundant in malaria-infected individuals) containing C1q might play in the observations made by McMorran *et al.* clearly need to be investigated in future studies because these also correlate with the severity of severe pathology. The *in vitro* platelet assays set up by the authors will be particularly useful for addressing these thorny problems.

The receptors on the infected erythrocyte responsible for the binding to platelets also remain a mystery for both human and rodent malaras. Although no direct evidence exists to show that PfEMP1 is directly involved in platelet binding via either CD36 or gC1qR/HABP1/p32, the platelet plasma membrane does contact knob-like structures on the infected erythrocyte, where PfEMP1 resides [7]. If PfEMP1 does turn out to be responsible for platelet binding,

which DBL domains are involved? And what happens *in vivo* when PfEMP1 is saturated with other known soluble ligands that might influence this interaction?

To die upon a platelet's kiss

The second question concerning the mechanism of killing, especially for an intracellular parasite residing in the red blood cell, is less easy to explain. Perhaps the recruited platelets release toxic mediators or cytokines. Platelets secrete dense granules (containing ADP, ATP, Ca²⁺ and serotonin) and alpha granules (containing CXCL4, PDGF, fibronectin, von Willebrand factor, fibrinogen and coagulation factors V and XIII), as well as microparticles (Figure 1). Platelet-derived microparticles account for 90% of the plasma microparticles from healthy individuals and recent proteomic analysis has shown them to contain chemokines CXCL4, CXCL7 and RANTES (CCL5) [15]. It is not known whether these secreted factors directly affect intracellular developing parasites or whether they indirectly recruit other mechanisms of killing, perhaps via pro-inflammatory mediators including cytokines.

The Duffy antigen receptor for chemokines (DARC), which is needed for invasion of erythrocytes by *P. vivax*, is characterized by its ability to bind a wide array of pro-inflammatory chemokines (especially RANTES, which is highly effective in suppressing HIV-1 replication) [16]. Whether these platelet-derived chemokines have a similar role in malaria, perhaps by blocking access of *P. vivax* merozoites to DARC, is unknown. Another possibility is that platelets clumped around an infected erythrocyte might starve the parasite of key metabolites by blocking nutrient uptake through parasite or host transporter proteins located within the erythrocyte plasma membrane (Figure 1). Given that serotonin is found in platelet dense granules, the observation that serotonin receptor agonists and other products of tryptophan catabolism inhibit *P. falciparum* by blocking membrane channels [17] and modulating the parasite cell cycle [18] is very exciting. It will now be important to throw some of these platelet mediators directly into parasite cultures to determine what effect they have on inducing apoptosis in the authors' TUNEL assays. Unfortunately, these assays were only carried out over a 24-h period, so the effect of platelet clumping on merozoite egress or invasion of fresh erythrocytes was not investigated.

A spoonful of antibodies makes the platelets go down?

Close examination of the *in vivo* data presented by McMorran *et al.* suggests that the impact of thrombocytopenia on mouse survival occurs ten days after parasite injection (when platelet levels are low), a time point that will coincide with the production of antibodies. Are antibodies involved in the observed malaria-induced thrombocytopenia? Most malaria patients with thrombocytopenia possess platelet-associated antibodies that bind to platelet-bound malaria antigens [19], and the extent of thrombocytopenia correlates with disease severity and immunoglobulin E (IgE) levels [20]. One hypothesis is that platelet autoantibodies and subsequent phagocytosis by splenic macrophages are responsible for the observed thrombocytopenia, although in experiments with *P. berghei* in μ MT mice (which are considered devoid of functional B cells and IgG antibodies), the mice still develop thrombocytopenia [21]. However, this work needs reappraisal in the light of more recent work showing mice deficient in μ - or δ -chain expression, on numerous genetic backgrounds, generate robust and functional IgA and IgE responses (human platelets express FcRs for both these antibody classes), although these studies are yet to be extended to malaria [22,23].

Irrespective of their controversial role in inducing thrombocytopenia, could antibodies recruit platelet killing during a secondary adaptive immune response? Human platelets express FcRs for IgG, IgA and IgE (Figure 1) [24]. As the only IgG FcR expressed by

human platelets, Fc γ RIIA contributes to the pathophysiology of diseases such as heparin-induced and antibody-mediated thrombocytopenias and to antiphospholipid antibody syndrome-mediated arterial thrombosis. In addition, Fc γ RIIA has been shown to stimulate platelets through interaction with other molecules and receptors, including α 2b β 3, GP1b-IX-V and von Willebrand factor [25–27]. Furthermore, Fc γ RIIA and PECAM-1 are physically and functionally associated on the surface of human platelets [28]. Human platelets have been shown to internalize IgG-containing immune complexes, abundant during malaria infection, and polymorphisms in Fc γ RIIA have been implicated in susceptibility to severe malaria [24,29]. Whether these polymorphic variants of Fc γ RIIA have functional consequences for killing of malaria parasites by human platelets, in either the presence or the absence of specific antibody, is unknown. Mice transgenic for human Fc γ RIIA and whose platelets express this receptor are available and might provide interesting models with which to explore the role of both mouse and human antibodies in platelet killing of infected erythrocytes during the adaptive immune response [24].

Concluding remarks

Exciting times lie ahead for the humble platelet.

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Figure 1.

Hypotheses on the interaction of platelets with infected erythrocytes (IEs). (1) Platelets are activated by unknown molecules released from IEs through the metabotropic purinergic receptor P2Y₁. It is unclear whether activation requires prior binding and tethering of IEs via platelet-expressed CD36 and gC1qR (also known as HABP1/p32) [7,8]. The potential roles of other platelet receptors in tethering and triggering are unclear, as are the identities of the parasite ligands interacting with them. (2) Activation of platelets results in the release of both α-granules and dense granules, loaded with numerous potent pharmacological and immunological mediators. Serotonin results in increased vascular permeability and smooth muscle contraction and has been shown to activate dendritic cells (DCs). It might also influence the IE directly; serotonin receptor agonists and tryptophan catabolites are known to modulate the parasite life cycle and inhibit parasite growth in culture [17,18]. (3) Recent analysis of the secreted platelet proteome have detected numerous chemokines including CXCL4, CXCL7 and regulated upon activation normal T-cell expressed and secreted (RANTES, or CCL5) that have important roles in the phased arrival of leukocytes and natural killer (NK) cells and granulocytes (eosinophils, or Eos), polymorphonuclear neutrophils (PMNs) and mast cells (Mast) [15]. CXCL4 and its cognate receptor CXCR3 expressed on T helper (Th) cells have been shown to impact directly on the severity of experimental cerebral malaria in rodents [4]. CXCL4 stimulates monocyte release of tumour necrosis factor (TNF-α) and reactive oxygen intermediates (ROIs) and has been shown to induce apoptosis of endothelial cells (ECs) that, together, might compromise the integrity of the blood–brain barrier. Soluble factors released by IEs are known to induce apoptosis in human brain ECs. CXCL7 recruits PMNs in particular that release large quantities of platelet-activating factor (PAF). RANTES is a potent pro-inflammatory chemokine and inhibitor of HIV replication *in vitro* and is known to bind the Duffy antigen receptor for chemokines (DARC), coincidentally required for invasion of erythrocytes by *P. vivax* [16]. Whether RANTES can inhibit growth of malaria parasites when administered to growing cultures is unknown. (4) How all these molecules eventually lead to apoptosis in the parasite and the pathways leading to death have also yet to be worked out. Although parasites are known to possess two metacaspase proteins, whether their expression is increased after culture in the presence of platelets now needs to be determined. (5) What role antibodies and/or immune complexes (ICs) have in thrombocytopenia is still unclear. Given that

platelets express numerous Fc receptors for antibody, what part these play in the function of platelets should be investigated. What role platelets might have in subsequent adaptive immune responses to malaria is unclear. Whether *Mpl*-deficient mice can be immunized successfully or whether passive transfer experiments with antibody can be done in the absence of platelets could be usefully explored.