New insights into the protective power of platelets in malaria infection

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Platelets, as well as regulating blood hemostasis, are an important component of the body's defense against invading microbial pathogens. We previously reported that platelets protect during malaria infection by binding Plasmodium-infected erythrocytes (IE) and killing the parasite within. More recent studies have now revealed the platelet plasmocidal factor, platelet factor 4 (PF4) and the red cell-expressed Duffy-antigen molecule as the central players in the parasite killing activity of platelets.

Host Defenses Determine Disease Severity and Outcome in Malaria

Malaria remains one of world's major infectious diseases and an impediment to economic development. One third of the world's population are at risk of infection, around 250 million people develop clinical infections annually, and at least half a million die each year; most are children aged under five years.[1] The disease is caused by infection of circulating red blood cells by one of five species of the protozoan parasite, *Plasmodium*; *P. falciparum* causes most morbidity and mortality. The majority of antiplasmodial drugs are now severely compromised due to acquired resistance by the parasite, and the development of efficacious vaccines remains a challenge.[2] Novel approaches to treatment and a greater understanding of disease pathogenesis and the host response to infection are desperately needed.

Outcome to malaria infection is determined largely by the increasing parasite mass and the response of the host to the infection. A range of protective immunological responses are involved that modulate disease severity and survival. Importantly, less than 1% of infections progress to life-threatening stages, underscoring the efficacy of host protective mechanisms. Our current understanding of this involves the classical innate and adaptive responses. The innate protective response limits parasite growth

early in an infection in a non antigen-specific manner. It also allows time for the subsequent development of an adaptive response, which is capable of clearing the infection and protecting against clinically symptomatic malaria. The latter response is antibody-mediated and provides memory-based protection. It is also antigen-specific, and several years and many exposures are needed to build an effective immunity against the multitudinous array of parasite antigens in any given endemic region. Innate immune mechanisms are therefore crucial in all malarial infections to buffer against the early growth of blood-stage parasites. We recently reported that platelets are an important component of the host innate immune response against malaria infection.[3]

The Protective Role for Platelets in Malaria Infection

In addition to their well-defined role in hemostasis, platelets are increasingly implicated in immunological processes, including direct pathogen-killing functions (reviewed by Yeaman and colleagues, ref. 4). Platelets share many properties with classical immune cells. They express receptors that bind host immune response modulators (e.g., antibodies and cytokines) and Toll-like receptors that bind microbial products. They also express the CD154 co-stimulatory molecule and influence the development of adaptive immune responses. They also produce microbicidal products such as oxygen free-radicals and peptides. Importantly, their location in the circulation makes them ideal "sentinels" against any nascent infection. Platelet number and mass exceed that of all leukocytes in the circulation. Platelets respond to a variety of microbial cells by releasing immunomodulatory molecules and by directly killing microbial pathogens.

Malaria infections are commonly accompanied by a thrombocytopenia or loss of platelets, the severity of which closely mirrors the increasing parasite mass.[3,5] It is now clear from our study[3] and others[6,7] that platelets protect the host during erythrocytic infection. Mice with pre-existing platelet deficiencies are more susceptible to infection and exhibit higher loads of viable parasites. Treatment of normal mice with aspirin, a platelet activation and aggregation inhibitor, also reduces survival to infection.[3] Purified human platelets, when added to cultured *P. falciparum*, inhibit parasite growth.[3,6] Pre-treatment of these platelets with inhibitors (including aspirin) blocks the parasite killing effect. Platelets bind preferentially to *P. falciparum* IE, mainly through

interactions between the platelet-expressed scavenger receptor protein, CD36 and the *P. falciparum* erythrocyte membrane protein (PfEMP1), produced by the parasite and trafficked to the erythrocyte surface.[8] Importantly, platelet binding to IE is associated with parasite death.[3] We believe that platelets are active early in infection to slow the initial growth of malaria parasites in the bloodstream, providing greater opportunity for other defense mechanisms to control the infection and ensure survival.

Platelet Factor 4 is the Parasite-Killing Effector Molecule

More recent studies by our group[9] and others[10] have provided additional mechanistic and molecular insight to how platelets kill the intraerythrocytic parasite. Central to these findings is a platelet derived CXC-type chemokine called platelet factor 4 (PF4/CXCL4), which is released by activated platelets and kills the parasite. Approximately 25% of the protein released by platelets comprises of PF4, and concentrations surrounding activated platelets reach high micromolar ranges.[11,12] The parasite-killing activity of platelets appears to be entirely due to PF4. Neutralizing anti-PF4 antibodies completely block the activity of human platelet lysate[9] and PF4-deficient platelets from mice fail to kill parasites.[10] Platelets release PF4 when exposed to *P. falciparum* infected cells.[13] We found the released PF4 binds to and is internalized by the infected cell. Deposition of PF4 is associated with intraerythrocytic parasite death. Love and colleagues elegantly demonstrated that upon entering the cell, the protein relocates to the parasite digestive vacuole (DV, site of hemoglobin digestion), resulting in specific lysis of the organelle and death of the parasite.[10] Therefore PF4 is a unique example of a host-derived molecule with direct plasmocidal activity.

PF4 belongs to growing list of chemokine molecules called kinocidins, which have a remarkable capacity to function as both chemotactic and antimicrobial molecules.[14] The different functions are localized to structurally distinct domains, and the latter at least is separable. Chemotactic activity resides in the N-terminal portion of the protein where the classical CXC chemokine motif exists. This domain interacts with chemokine receptors on various target cells, leading to receptor-mediated signaling events. The C-terminal domain, rich in positive charged amino acids, mediates the antimicrobial effects of PF4 and other kinocidins. Peptide scanning analyses have mapped the bactericidal and fungicidal activity of the rabbit PF4 ortholog, platelet microbiocidal protein 1 to the carboxyl terminal 15 residues, 5 of which are lysines.[15] Notably, Love and colleagues showed that a peptide encompassing the carboxyl terminal 12 residues of human PF4 (C12) had plasmocidal activity. This function was abrogated when three out of the four lysine residues were substituted to alanines.[10] The charged, amphipathic nature of kinocidin C-terminal domains gives them propensity to interact with cell membranes. In bacteria, this has been variously shown to result in disruption of membrane potential, cell permeabilization and inhibition of protein and DNA synthesis.[16-18] Consistent with these former effects, PF4 and C12 treatment of *P. falciparum*

results in lysis of the DV. However, exactly how and why the DV is specifically targeted by PF4 is unclear, especially given that the molecule must cross three separate membranes in order to reach the DV (red cell plasma membrane (PM), parasitophorous vacuole membrane and parasite PM).

The Duffy Antigen and the Parasite Killing Function of Platelets

PF4 binds to Duffy, a red cell antigen receptor for chemokines (DARC/Fy)[19,20] and our results show that platelet and PF4-mediated killing of intraerythrocytic parasites requires DARC.[9] The likely mechanism involves the binding of PF4 to DARC on the infected cell surface, following by internalization of the PF4-DARC complex. Binding occurs via a domain on DARC known to bind a number of other chemokines. Antibodies to this domain block PF4-mediated killing. Also, other chemokines with a high affinity for DARC out-compete the PF4 parasite binding and killing of parasites. In addition, PF4 binding and internalization are reduced in cells that lack DARC.[9] The molecular details of how the complex may translocate into the parasite (as well as the existence of the complex within the parasite) remain to be demonstrated. But interestingly the uptake of host membrane proteins by intraerythrocytic Plasmodium, including DARC, has been documented.[21,22] Uptake of DARC appears to be an active process and requires the tubovesicular membrane (TVM) network that derives from the parasitophorous vacuole membrane and connects the parasite with the erythrocyte PM. The TVM develops within the first 24 h following merozoite invasion and host proteins accumulate in conjunction with parasite maturation.[21] Uptake is also selective for certain host proteins, especially those that reside in cholesterol-rich detergent-resistant host membranes; more abundant cytoskeletal molecules such as glycophorin-A, band-3 and Glut1 are excluded.[23] Interestingly, establishment of the TVM and maximal accumulation of DARC coincide with the parasite stage most sensitive to PF4 (trophozoites).[9,10] The intracellular fate (and function) of these imported host proteins remains to be determined. In the case of DARC, details of parasite intracellular localization are not known. As well as establishing if PF4-directed lysis of the DV requires DARC, it will be of interest to determine if DARC translocates to the parasite DV membrane, and thereby provide a plausible mechanism for the delivery of PF4 from the activated platelet to this organelle.

Platelet-Mediated Protection in Human Malaria

The protective role played by platelets in human malarial infections has yet to be directly answered, but there are a number of lines of evidence that support the affirmative. Depletion of platelets (or inhibition of platelet function) in murine models of malaria where outcome is determined by bloodstream parasitemia (as opposed to the inflammatory response-based cerebral malaria syndrome) results in reduced survival.[3,7] Although a similar platelet depletion study is difficult to perform in humans, we do know that thrombocytopenia, which is a common clinical

accompaniment of all malarial infections, has been correlated with a poor outcome in falciparum malaria.[24-27] A loss in the protective function afforded by platelets could explain this.

Platelets have also been implicated as susceptibility factors in the development of cerebral malaria (CM), a complex collection of syndromes specific to *P. falciparum* infections and a major cause of death. Central to the pathophysiology of CM is the accumulation or sequestration of IE in the cerebral microvasculature, causing the obstruction of blood flow, leukocyte accumulation, localized intravascular inflammation, activation and damage of the endothelium and disruption of the blood-brain barrier.[28] Platelets are often found at sites of IE sequestration in both human CM[29,30] and mouse CM models[31,32] where they are believed to mediate IE binding in the microvasculature, and release molecules that affect endothelial cell viability and promote leukocyte adhesion.[33] The parasite-killing activity of platelets may also contribute to the pathophysiology of CM. Platelet-directed killing may moderate the local inflammatory responses to live parasites, or dead parasite toxins and exudate could contribute to the inflammatory and cell-damaging milieu.

The effect of non-steroidal anti-inflammatory drugs, especially aspirin, may add an additional level of complexity in assigning the importance of platelets in human malaria. Aspirin irreversibly inhibits the cyclooxygenase enzyme and suppresses platelet activation and aggregation. We showed previously that aspirin prevented platelet-mediated killing of cultured *P. falciparum* and reduced survival in *P. chabaudi*-infected mice.[3] Aspirin is cheap and readily available, and its usage as an antipyretic remains widespread (although contraindications such as Dengue fever and Reyes syndrome have led to revisions on usage guidelines in many Westernized countries). A recent Cochrane Review found that the available data are insufficient to conclude if aspirin (as well as other antipyretics) are beneficial or detrimental in malaria.[34] Therefore studies to access the impact of aspirin on malaria are highly warranted.

Platelet-Mediated Protection in Duffy-Negative Individuals

The polymorphism responsible for the Duffy-negative blood group, a T to C substitution in a GATA1 promoter binding site, prevents erythrocytic expression of the Duffy gene. The C allele is very common in African populations; frequencies are close to 100% in most of Central and Western Africa.[35] Our findings on the requirement for DARC in killing of parasites by platelets have led us to speculate that platelet-mediated protection may be inadequate in Duffy-negative (DARC deficient) individuals.[9] Duffy-negativity provides protection against *P. vivax* infection by virtue of the requirement of the receptor for merozoite invasion[36] (although the simplicity of this assumption has been recently challenged[37,38]), and *P. vivax* is rarely seen in Africa.[39] However since erythrocytic expression of Duffy is also necessary for the platelet-mediated killing of *P. falciparum* parasites,

we might expect to see increased severity or a poorer outcome to falciparum malaria in Duffy-negative populations. At a broad level, this appears to be the case. Using the best available global estimates, rates of infection and death due to *P. falciparum* are much higher in Africa compared with other parts of world (Asia and South America), where Duffy-negativity is rare.[1,40] An ineffective protective response involving platelets could be one reason for this. But many other factors also contribute to this difference, such as the type of parasite and its level of virulence, vector control measures, rates of host immunity, access to healthcare and other socioeconomic factors. In Africa as much as half of malaria-associated deaths are attributable to CM.[1] The failure of platelets to kill sequestered parasites growing in Duffy-negative red cells may counter intuitively exacerbate this complication by allowing viable parasites to remain within the cerebral vasculature. Importantly, studies are needed to test the relationship between Duffy-antigen status and platelet protection where, ideally, *P. falciparum* disease severity, incidence and outcome would be compared within Duffy-negative and positive individuals living in the same area and affected by the same parasite strains and other environmental factors.

If a lack of platelet protection is indeed detrimental in Duffy-negative individuals, it is challenging to reconcile evolutionarily why the Duffy-negative polymorphism should remain common in Africa. However the events that led to, and maintained, the Duffy-negative allele in Africans are likely to be complicated. It is widely held that the polymorphism arose through positive selection at a distant time in human evolutionary history to protect against, what was presumably then, a lethal form of *P. vivax*.[41] Compared with *P. vivax*, the advent of *P. falciparum* infection in humans appears to have been a more recent event.[42,43] Therefore fixation of the Duffy-negative allele may have preceded *P. falciparum* in humans. There may also be other positive selective pressures for the polymorphism that we do not know about. Indeed the function of the DARC molecule on uninfected red blood cells remains unclear.[44]

Concluding Remarks

The protective power of the platelet in malaria infection is only beginning to be appreciated. While convincing evidence of their importance in clinical infection is still needed, advances in understanding the molecular detail of parasite killing process have led to some very interesting findings. Useful novel antimalarial molecules based on the PF4 platelet effector molecule are an exciting prospect.[10] The findings may also help to explain why *P. falciparum* is a particular problem in Africa. Every minute of every day, a child still dies of malaria in Africa. We must continue to direct our efforts to control this scourge of humankind, especially in this part of the world.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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