Platelets Kill Intraerythrocytic Malarial Parasites and Mediate Survival to Infection.

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Platelets play a critical role in the pathogenesis of malarial infections by encouraging the sequestration of infected red blood cells within the cerebral vasculature. But platelets also have well-established roles in innate protection against microbial infections. We found that purified human platelets killed *Plasmodium falciparum* parasites cultured in red blood cells. Inhibition of platelet function by aspirin and other platelet inhibitors abrogated the lethal effect human platelets exert on *P. falciparum* parasites. Likewise, platelet-deficient and aspirin-treated mice were more susceptible to death during erythrocytic infection with *Plasmodium chabaudi*. Both mouse and human platelets bind malarial-infected red cells and kill the parasite within. These results indicate a protective function for platelets in the early stages of erythrocytic infection distinct from their role in cerebral malaria.

Adaptive immunity to malarial infection accumulates slowly over the lifetime of an individual living in an endemic region. Cross-immunity between isolates is low, and every new infection requires the development of a virtually novel immune response. Therefore, innate mechanisms that limit parasite growth within the red blood cell (RBC) are extremely important in determining survival, especially during the first few years of life, because clinical severity correlates closely with parasite mass. Several known mutations affect the RBC and decrease the severity of infection through either impediment of parasite entry or development within the cell (1). However, we know little about other non-RBC mechanisms that may influence the course of infection before the onset of effective adaptive immune responses.

Thrombocytopenia is a common clinical accompaniment of *Plasmodium falciparum* (*2–4*), *Plasmodium vivax* (*5, 6*), and *Plasmodium chabaudi* (*7*) malarial infections. Low platelet concentrations correlate with increased parasite density (*8, 9*) and poor outcome (*10*). Platelets bind preferentially to infected RBC**s** (*11, 12*) and have been postulated to play a role in the pathogenesis of malarial infection, either positively (*13, 14*) or negatively (*15, 16*). Given the role platelets play in other infectious diseases (*17*), we explored their influence on the course of a malarial infection in the mouse model, *P. chabaudi*, and in vitro using the cultured human malarial parasite, *P. falciparum*.

The megakaryocyte growth and differentiation factor, C-mpl, is the receptor for thrombopoietin and is encoded by the *Mpl* gene. Homozygous disruption of the *Mpl* gene on a C57BL/6 background (Mpl$^{-/-}$) results in mice with only one-tenth as many circulating platelets as their undisrupted counterparts have (*18*). Platelet-deficient Mpl$^{-/-}$ mice were significantly more susceptible to death from *P. chabaudi* than were isogenic C57BL/6 mice ($P < 0.0001$, Mantel-Cox log-rank test on survival data), irrespective of well-known sex differences. In females, 5% of C57BL/6 versus 50% of Mpl$^{-/-}$ mice died. Twenty percent of the male C57BL/6 mice and all the male Mpl$^{-/-}$ mice died (Fig. 1, A and B).

Cohort analysis of groups of mice showed that platelet numbers decreased concomitantly with the appearance of parasites in the peripheral circulation (Fig. 1, E and F). Platelet numbers dropped to 10 to 20% of normal values in the C57BL/6 mice, with a smaller percentage drop in the Mpl$^{-/-}$ mice. Platelet numbers reached a nadir before the onset of severe disease symptoms and anemia. No evidence of excess bleeding was seen in the Mpl$^{-/-}$ mice during autopsy. Similar erythropoietic kinetics occurred between Mpl$^{-/-}$ and C57BL/6 mice during both malarial challenge and experimentally–induced hemolysis (figs. S1 and S2), excluding differential erythropoiesis as a cause for the different susceptibilities. Nor were differences in immunological responses, including production of lymphocytes and inflammatory cytokines, observed between the mouse strains during infection (fig. S3).

Acetyl salicylic acid (aspirin) inhibits cyclooxygenases I and II, resulting in a decrease in thromboxane A2 and decreased platelet activation as seen by lower platelet aggregation (*19*). Mice were given 25 mg of aspirin per kilogram of body weight intra-peritoneally daily from the day before *P. chabaudi* infection until day 10 of infection and monitored for survival. C57BL/6 and Mpl$^{-/-}$ mice of both sexes treated with aspirin were significantly more susceptible than placebo-treated–mice (Fig. 1, C and D) (Cox proportional hazard analysis, $P = 0.0009$). Thus, pharmacological inhibition of platelet function increases susceptibility to malarial infection in mice, although other indirect effects of aspirin cannot be excluded.

Synchronized cultures of *P. falciparum* trophozoites were grown in human RBCs and incubated with increasing concentrations of purified human platelets. A concentration-dependent increase in the inhibition of parasite growth was observed (Fig. 2A). Growth inhibition (measured after 44 hours of incubation) peaked at 65% with 25– to 75 million platelets/ml. Most viable parasites observed at this time were mature trophozoites and shizonts. Reductions in mature shizonts at 5 hours and new rings at 21– to 29 hours (fig. S4) suggest that platelets were inhibiting developmental maturation of the parasites.

Using the *P. falciparum* model, we investigated the dependence of parasite growth inhibition on platelet function by first incubating platelets with specific platelet antagonists. Maintenance of elevated intracellular adenosine $3',5'$-monophosphate and guanosine $3',5'$- monophosphate concentrations are key mechanisms in suppressing platelet activation (*20*, *21*). Addition of the adenylyl cyclase activator prostaglandin E1 completely abrogated the platelet growth- inhibitory activity. Exposure of platelets to the guanylyl cyclase activator, NO (donated by sodium nitroprusside), also suppressed platelet-mediated effects on parasite growth (Fig. 2B). To test for the requirement of adenosine $5'$-diphosphate (ADP), an important physiological regulator of platelet activation [reviewed in (*20*)], we incubated the platelet—*P. falciparum* cultures with adenosine diphosphatase (ADPase) (*22*) to remove extracellular ADP from the culture medium. Unimpeded parasite growth in the presence of platelets and ADPase was observed. ADP-dependent platelet activation is mediated via two metabotrophic puronergic receptors, $P2Y_1$ and $P2Y_{12}$. Antagonism of $P2Y_1$, but not $P2Y_{12}$, abolished the parasite killing activity (Fig. 2B). Thus, *P. falciparum* growth is inhibited by platelets in a concentration-dependent and activation-dependent manner that requires ADP and activation through the $P2Y_1$ receptor.

As with *P. chabaudi* in the mouse, inhibition of cyclooxygenase by aspirin also prevented the inhibition of parasite growth by platelets (Fig. 2B). To approximate a clinical effect, a volunteer platelet-donor took aspirin orally twice daily (4.2 mg/kg) for a week before donating blood for platelet purification. These platelets were unable to inhibit growth of *P. falciparum* parasites (Fig. 2C), implying that aspirin is potentially harmful in malarial infections.

In mice infected with *P. chabaudi* for 8 days, with parasitemias at ~10 to 15%, platelets were preferentially bound to infected RBCs. As shown with Giemsa-stained thin smears and light microscopy (Figs. 3A, i and ii), the percentage parasitemia was twice as high when only platelet-bound RBCs were counted compared with all RBCs (Fig. 3B). CD41 (a platelet surface antigen) on the surface of RBCs served as a marker for platelet binding. Three times as many infected RBCs stained for CD41 as did uninfected RBCs (Fig. 3C). Similar results were obtained from cultured *P. falciparum*, where twice the numbers of infected RBCs were bound by human platelets (counted on Giemsa-stained thin smears) (Fig. 3A, iii and iv, and 3D) and twice the number of infected RBCs were also CD41 positive (Fig. 3E). Therefore, platelets selectively bind infected RBCs.

The presence of dead parasites in the peripheral circulation was investigated by adapting the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique to thin blood smears. TUNEL stains nuclei containing sheared or degraded DNA, an accompaniment of apoptosis or necrosis. As a positive control, all extra-erythrocytic parasites, which commonly occur in late *P. chabaudi* infections, stained positive for TUNEL (fig. S5). In mice with lower parasitemias (day 7), TUNEL staining of intra-erythrocytic parasites was evident (Fig.

4A), indicative of intraerythrocytic parasite death.

Twice as many dead intra-erythrocytic parasites were seen in C57BL/6 mice as in Mpl$^{-/-}$ mice (Fig. 4C). Although no differences in total parasitemia between Mpl$^{-/-}$ and C57BL/6 were detected at this time point (most TUNEL-stained parasites are morphologically indistinguishable from live parasites), parasitemias measured at later time points were significantly higher in Mpl$^{-/-}$ than in C57BL/6 mice (fig. S6). This suggests that platelet-mediated parasite killing influences parasite numbers past the platelet nadir, resulting in higher parasitemias in Mpl$^{-/-}$ mice, and explains their increased susceptibility.

TUNEL--stained intracellular parasites were also observed in the *P. falciparum* cultures (Fig. 4B). The frequency of stained parasites was more than three times as high in platelet-treated cultures (Fig. 4D). Significantly higher proportions of TUNEL-stained than non-stained infected RBCs were bound by human platelets (by staining with antibodies to CD41) (Fig. 4E). The greater numbers of dead intra-erythrocytic parasites in the presence of normal numbers of platelet in vivo and in vitro implies that platelets directly kill malarial parasites.

Increased mortality in platelet-deficient mice and direct killing of *P. falciparum* by human platelets indicate that platelets are important in controlling malarial infection. Thrombocytopenia occurs early in infection in both humans and mice, before severe forms of the disease develop (*3*, *6*). Therefore, the major protective effect of platelets will be early in infection. Modulation of parasite growth by platelets will buffer the parasite growth rate and allow the engagement of the adaptive immune response. This finding is in addition to the well-established role for platelets in cerebral disease (*15*, *23*, *24*). We have also shown that inhibition of platelet activation abrogates the protective effect, which could explain the deleterious effect aspirin may have on malarial outcome (*25*).

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Supporting online material contents

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Figs. S1 to S6 References

Fig. 1. Survival data and parasitological and hematological kinetics of *P. chabaudi* infection in C57BL/6 and Mpl$^{-/-}$ mice. (A and B) Survival curves for Mpl$^{-/-}$ (red) and C57BL/6 (black) mice infected with *P. chabaudi* and monitored for survival. (C and D) Effect of aspirin or vehicle-only treatment on survival of Mpl$^{-/-}$ and C57BL/6 mice. Given that gender contributes a significant effect, the females (A and C) and males (B and D) have been separated. (E and F) Parasite and hematological parameters in C57BL/6 (E) and Mpl$^{-/-}$(F) mice. Parasitemias (percentage of infected RBCs), RBC counts ($\times 10^{12}$/liter), and platelet counts ($\times 10^9$/liter).

Fig. 2. Platelet-mediated growth inhibition of *P. falciparum* cultured in human RBCs. (A) Parasite growth inhibition (relative to Tyrode's buffer–treated cultures) with increasing concentrations of purified platelets. Chloro, parasites incubated with 640 nM chloroquine. (B) Parasite growth inhibition with platelets (50 million/ml) pre-incubated in platelet activation inhibitors at the indicated concentrations. Control, untreated platelets; PGE1, prostaglandin E1; SNP, sodium nitroprusside; MRS2179, P2Y$_1$ receptor antagonist; MRS2395, P2Y$_{12}$ receptor antagonist. (C) Inhibition of parasite growth in the presence of platelets (50 million/ml) pre-incubated with aspirin before addition to culture (in vitro aspirin) or untreated (control) compared with platelets taken from the same donor on separate occasions after treatment with aspirin (4.2 mg/kg taken orally twice daily for 1 week before platelet collection) (in vivo aspirin). All experiments were performed with washed platelets. Drugs used alone did not affect parasite growth. Bars for graphs represent the means of at least three independent experiments (A and B) and two independent experiments (C). Error bars indicate ±1 SD.
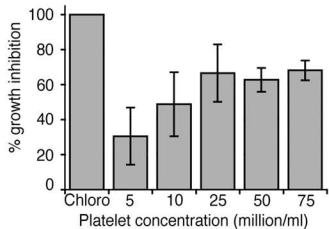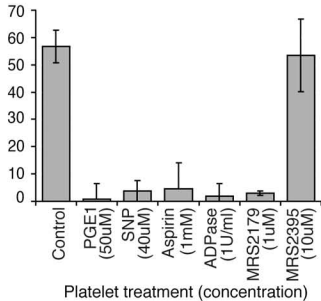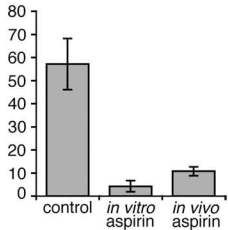
Fig. 3. Infected RBCs are preferentially bound by platelets. (A) Giemsa-stained thin blood smears showing individual RBC-bound platelets (black arrows) [(i) and (ii), mouse; (iii) and (iv), human]. Some parasites look dysmorphic (red arrows) compared with normal parasites (blue arrows) (B) The percentage parasitemia (counted on Giemsa-stained thin smears) of all mouse RBCs compared to only those with platelets bound ($P < 0.01$). (C) The percentage CD41 staining of thiazole orange (TO) positive (infected) versus TO-negative (uninfected) mouse RBCs ($P < 0.05$) (D) Percentage of *P. falciparum*–infected and uninfected human RBCs bound by platelets counted on Giemsa-stained thin smears ($P < 0.05$). (E) Percentage of infected (TO-positive) and uninfected (TO-negative) human RBCs that stained positively for CD41 ($P < 0.01$). (B) and (C) refer to the in vivo mouse model (day 8 of infection) and (D) and (E) to the in vitro human falciparum culture model (after 24 hours of incubation with platelets). Bars for graphs represent mean of three independent experiments. Error bars represent ±1 SD.
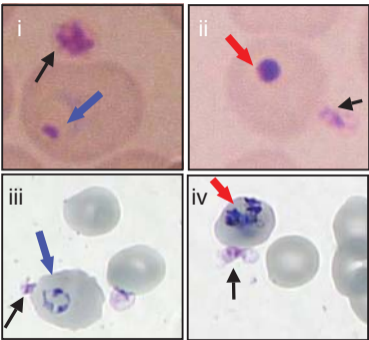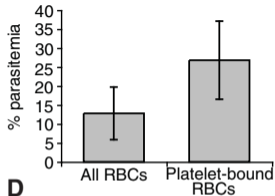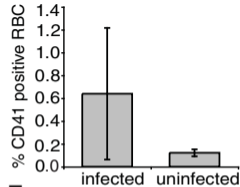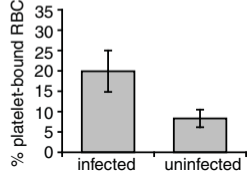
Fig. 4. Evidence that intra-erythrocytic parasites are killed by platelets. (A) TUNEL staining of a *P. chabaudi*–infected C57BL/6 thin blood smear (day 7 of infection). (i) Hoechst staining of parasite DNA. (ii) TUNEL staining of parasite DNA, and (iii) both TUNEL and Hoechst staining superimposed on dark-field microscopy showing intra-erythrocytic parasites. (B) Immunofluorescent images of *P. falciparum*-parasitized human RBCs cocultured with platelets for 24 hours. Separate images of cells stained
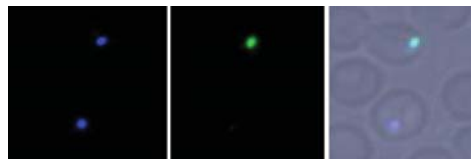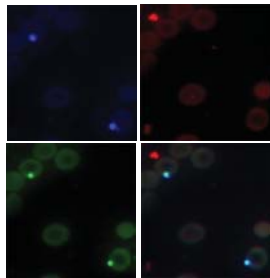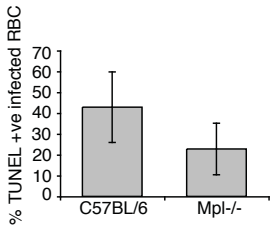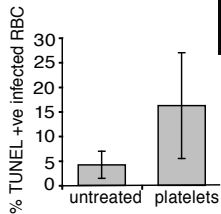
with (i), Hoechst —(all parasites), (ii) anti-CD41 -(platelets), and (iii) TUNEL —(dead parasites),; (iv) merged image of (i), (ii), and (iii) (anti-CD41, red; TUNEL, green; Hoechst, blue). (C) The percentage of all *P. chabaudi* parasites staining positive with TUNEL in C57BL/6 and Mpl$^{-/-}$ mice ($P < 0.05$). (D) Percentage of TUNEL- positive parasites in *P. falciparum* cultures incubated with platelets (50 million/ml) and without platelets for 24-hours ($P < 0.05$). (E) Percentage of *P. falciparum*—parasitized RBCs bound by platelets that stained positively or negatively with TUNEL stain ($P < 0.05$). Bars for graphs represent the mean of at least three independent experiments with error bars representing ±1 SD.

A

% growth inhibition (y-axis, 0–100)

Platelet concentration (million/ml): Chloro, 5, 10, 25, 50, 75

B

(y-axis, 0–70)

Platelet treatment (concentration): Control, PGE1 (50uM), SNP (40uM), Aspirin (1mM), ADPase (1U/ml), MRS2179 (1uM), MRS2395 (10uM)

C

(y-axis, 0–80)

control, in vitro aspirin, in vivo aspirin

A

B

C

% TUNEL +ve infected RBC

C57BL/6   Mpl-/-

D

% TUNEL +ve infected RBC

untreated   platelets

E

% platelet-bound infected RBC

TUNEL +ve   TUNEL -ve