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Platelets activate a pathogenic response to blood-stage *Plasmodium* infection but not a protective immune response.

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1. Platelets are not killer cells of blood-stage *Plasmodium* parasites.

2. Platelets are not required to activate the protective immune response to blood-stage *Plasmodium* infection in mice.

Abstract

Clinical studies indicate that thrombocytopenia correlates with the development of severe falciparum malaria, suggesting that platelets either contribute to control of parasite replication possibly as innate parasite killer cells or function in eliciting pathogenesis. Removal of platelets by anti-CD41 mAb treatment, platelet inhibition by aspirin, and adoptive transfer of WT platelets to CD40-KO mice, which do not control parasite replication, resulted in similar parasitemia compared with control mice. Human platelets at a physiological ratio of 1 platelet to 9 RBCs did not inhibit the *in vitro* development or replication of blood-stage *P. falciparum*. The percentage of iRBCs with bound platelets during the ascending parasitemia in *P. chabaudi* and *P. berghei* infected mice and the 48 hour *in vitro* cycle of *P. falciparum* was <10%. *P. chabaudi* and *P. berghei* iRBCs with apoptotic parasites (TdT+) exhibited minimal platelet binding (<5%), which was similar to non-apoptotic iRBCs. These findings collectively indicate platelets do not kill bloodstage *Plasmodium* at physiologically relevant effector to target ratios. *P. chabaudi* primary and secondary parasitemia was similar in mice depleted of platelets by mAb-injection just prior to infection, indicating that activation of the protective immune response does not require platelets. In contrast to the lack of an effect on parasite replication, adoptive transfer of WT platelets to CD40-KO mice, which are resistant to experimental cerebral malaria, partially restored experimental cerebral malaria mortality and symptoms in CD40-KO recipients, indicating platelets elicit pathogenesis and platelet CD40 is a key molecule.

Introduction:

A hallmark of blood-stage *Plasmodium* (P) infection in humans is the development of thrombocytopenia and a pro-coagulant state that is most pronounced in *P. falciparum* (Pf) infections, the most virulent of the 5 species of *Plasmodium* infecting humans[1,2]. Levels of thrombocytopenia and pro-coagulant state are elevated in severe <u>Pf</u> malaria compared with uncomplicated malaria patients and uninfected controls[1,2], suggesting that thrombocytopenia contributes to disease. However, there is still considerable uncertainty about platelet's role in malaria. Thrombocytopenia could lead to increased parasite replication by: (i) decreased killing of parasites, or (ii) decreased activation of the protective immune response controlling parasite replication.

In support of increased parasite replication contributing to severe malaria, two groups reported that platelets function as a critical component of innate immunity controlling parasite replication by binding to and directly killing parasites within red blood cells (RBCs)[3-5]. Based on increased mortality of aspirin-treated, *Plasmodium chabaudi* (Pc)-infected mice and inhibition of *in vitro* killing of Pf-infected RBCs (iRBCs) by aspirin[4], Greenbaum[6] proposed to change clinical practice by contra-indicating widely used non-steroidal anti-inflammatory agents with anti-platelet activity, such as aspirin, in malaria patients.

In contrast to reported iRBC killing by platelets[3-5], we reported that platelet-depletion by antibody injection does not affect *Plasmodium berghei* ANKA (PbA) parasitemia[7,8], which elicits experimental cerebral malaria (eCM) in mice. Early but not late platelet depletion protects against eCM pathogenesis by ameliorating the inflammatory response[7,8]. Here, we determine whether platelets are innate killers of blood-stage *Plasmodium* and/or regulate either the protective or pathogenic immune response during malaria and report that there is no evidence to

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support physiological platelet killing of blood-stage *Plasmodium.* Moreover, platelets do not lead

to a protective immune response that clears blood-stage *Plasmodium* infection, but do activate a

pathogenic response to infection.

Methods

Ethics statement. The Institutional Animal Care and Use Committee of La Jolla Infectious

Disease Institute approved all protocols and procedures.

Mouse studies. There are differences between humans and mice and between *Plasmodium*

species infecting humans and those infecting mice, indicating caution is needed in extrapolating

results to humans[9]. Nevertheless, key hallmarks of malarial thrombocytopenia, immunity, and

CM pathogenesis appear to be conserved[10]. We used three different strains of *Plasmodium* that

infect mice using standard protocols[11,12]. The iRBC inoculum details (0.2ml in PBS injected i.v)

and logic behind the selection of the three strains are summarized in Table 1. Day 0PI are

uninfected control mice. Between 200 and 1000 RBCs were counted in Giemsa-stained thin

blood films in experimental animals to assess parasitemia.

To assess the extent of eCM, *Pb*A-infected mice were given neurological tests daily from day

6PI onwards. These tests comprised the sum of the righting reflex and gripping reflex each on a

scale of 1-5 with 5 exhibiting no impairment[13,14]. Animals with a score of <4 were moribund and

euthanized.

Treatments. Aspirin (acetylsalicylic acid; Sigma, St. Louis, MO) was injected i.p. in 0.2ml saline

(25mg/Kg). Platelets were depleted by i.p. injection with 0.1mg platelet depleting anti-CD41

mAb in 0.1ml PBS (MWReg40); control mice were injected with rat IgG mAb (Affymetrix, San

Diego, CA). Immunodeficient CD40-KO mice and C57BL/6 WT controls were cured of

Plasmodium chabaudi adami (*Pc*a) infection with Trimethoprim (0.4 mg/ml) and Sulfamethoxazole (1.2 mg/ml) *ad libitum* for 1 week in drinking water after WT controls had suppressed their parasitemia to undetectable levels (from >day 28PI). Cure was confirmed by 3 negative thin blood films.

Platelet analysis and isolation. Platelets and RBCs were counted by flow cytometry in 1µl of tail vein blood as described previously[8]; the number of platelets/ml blood, platelet activation, and response was similar in tail vein blood compared with blood obtained by intra-cardiac puncture or via retro-orbital plexus[8].

Purified platelets for adoptive transfer were obtained by centrifugation of whole blood at 50×g for 20 min to generate platelet rich plasma (PRP). PRP was centrifuged at 800×g for 10 min and platelets resuspended in Tyrode's buffer at 0.2×10^9 in 0.2ml saline for injection.

Flow cytometry. All antibody incubations were performed at room temperature using antibodies purchased from Affymetrix (San Diego, CA). Anti-CD41-phycoerythrin (PE) fluorescence labeled platelets, and anti-Ter119-PE-cyanin7 (PE-Cy7) labeled erythrocytes. Cells were resuspended in 0.5ml PBS with 2.5×10^4 counting beads (Spherotech, Lake Forest, IL), and fluorescence intensities of the cells were acquired on an Accuri™ (Becton Dickinson, San Diego, CA) as described[12].

The *Pc*a high virulence (HV) and human platelet interaction with *P. falciparum* iRBCs were performed on an ImageStream Mk[II] flow cytometer (EMD Millipore, Seattle, WA), which distinguishes coincident events from close proximity/adhesion.

Fluorescence labeling of thin blood films. Thin blood films made on glass slides were fixed for 1min in methanol (Sigma). TUNEL assay was performed following the manufacturer's instructions (EMD Millipore, Temecula, CA). Platelets were labeled with anti-CD41-APC mAb.

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Parasite DNA and RNA were fluorescence labeled with 12µM ethidium bromide (Fisher

Scientific, Fair Lawn, NJ) in PBS for 15 minutes. Slides were mounted with Vectashield

Mounting Media (Vector Laboratories, Burlingame, CA).

Pf culture and analysis. Blood-stage Pf strains DD2, FCR3, and NK54 were cultured *in vitro*

as described[15,16]. Blood was obtained from 3 volunteers who had not taken anti-inflammatory

medications for > 2 weeks before venipuncture. The blood was collected into 5ml citrate

vacutainers using a 21G needle (Becton Dickinson, San Jose, CA), processed into PRP, washed

in Tyrode's buffer, resuspended in complete media and added at a ratio of 1 platelet per 9 RBCs

to the Pf cultures. Parasitemia of cultures with and without platelets and the percentage of RBCs

with an adherent platelet was assessed at 0, 2, 4, 6, 24, and 48h using imaging flow cytometry.

The thrombin (Fisher Scientific, Chronolog) dose response (CD62P levels) was assessed for each

donor at the above selected time points.

Statistical analysis. Analysis of variance with the Prism program (GraphPad) with Tukey's

post-hoc test was performed to statistically compare all measurements with a *P* value cut-off of

0.05. The mean and standard error of the mean of the results are reported in text and figures.

Survival curves are compared with non-parametric Logrank test with a *P* value cut-off of 0.05

with the "neurological period" for the development of eCM occurring day 6 to 12 in C57BL/6

mice.

Results

Platelet killing of *Plasmodium*. Malaria in both humans and mice results in profound

thrombocytopenia, occurring during the period of ascending patent parasitemia day 4 to 7PI for

*Pc*a in mice. Non-lethal *Pc*a, low (LV) and high virulence (HV), elicit exponential ascending

parasitemia until close to peak parasitemia with slopes varying depending on parasite virulence

(Table 1); after peak, parasitemia declines to undetectable levels (<0.1%) as a result of the activation of adaptive immune responses[11,17,18]. When plotted linearly, thrombocytopenia coincides with patent parasitemia and its exponential rise, suggesting that parasite replication may be increased when circulating platelet numbers are reduced[4].

Onset of thrombocytopenia exhibits no detectable effect on parasite replication. To test whether the rate of change of parasitemia is altered when thrombocytopenia occurs (and hence decreased platelet killing), we infected mice with Pca LV. The increase in parasitemia is concurrent with the onset of thrombocytopenia when plotted on a linear scale (Figure 1A).

Thrombocytopenia starts on day 4PI and becomes marked during ascending and peak parasitemia (Figure 1A). Further, *in vitro* cultured human platelets exhibit >80% killing of Pf-iRBC with platelet to iRBC target ratios >160:1[4,5] and <10% killing at 10:1[5]; platelets should exhibit similar levels of killing *in vivo*. Based on these platelet-iRBC ratios, the period of platelet killing of *Pc*a-iRBCs was between day 0 and 4 PI (Figure 1); beyond day 4PI, minimal platelet killing of *Pc*a-iRBCs occurs.

The log(parasitemia) at each day of infection from day 0 to day 7PI (ascending parasitemia) increases in a straight line with slope of 0.45 ± 0.03 ($R^2=0.99$) (Figure 1B). This slope determines the multiplicity of infection (MOI) for Pca, which replicates nightly to produce new iRBCs. Each cycle (i.e., day) ~3 merozoites ($10^{0.45}=2.8$) invade a new RBC and develop into viable circulating iRBCs. During ascending parasitemia, there was no detectable change in slope in the period of platelet killing of Pca-iRBCs (day 0-4PI) and the period of no killing (>day 4PI). Hence, the MOIs were identical at 2.8.

Models indicate that thrombocytopenia should affect slope of log(parasitemia) when platelet killing diminishes. To predict the effects of platelet killing of iRBCs on *Pc*a parasitemia, we

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modelled its potential effects on parasitemia. There are two possible scenarios for parasitemia: (i) iRBC sequestration does not markedly affect parasitemia, and (ii) iRBC sequestration decreases parasitemia. We model two levels of platelet killing of iRBCs: 80% above platelet-iRBC threshold of 160 (day 0-4PI)[4,5] or a conservative estimate of 40%.

With minimal iRBC sequestration in organs, parasitemia reflects the total number of iRBCs. During the period of platelet killing, only 20%, 60%, or 100% of iRBCs survive for 80%, 40%, and 0% killing, respectively, where MOIs are 0.6, 1.7, and 2.8, respectively. The parasitemia on day 0PI is calculated (Table 1) and the above MOIs are used to calculate the daily parasitemia until day 4PI (Figure 1C). Because the period of platelet killing ends on day 4PI, the MOI for each platelet killing scenario now equals 2.8 (0% killing) that is used to calculate the subsequent daily parasitemia until peak (Figure 1C).

With marked iRBC sequestration, parasitemia does not reflect the total parasite burden because sequestered iRBCs are not circulating and counted. If we assume similar levels of sequestration throughout ascending parasitemia, then the slope of log(%parasitemia) between day 0 and 4PI provides the "effective" rate of parasite replication in blood with sequestration and platelet killing. The slope was measured at 0.45, so the "effective MOI" during platelet killing is $10^{0.45}$=2.8. The 80%, 40%, or 0% iRBC killing by platelets has stopped from day 4-7PI, so the "effective MOI is now: 14, 7, and 2.8, respectively. The calculated parasitemia (Figure 1D) then results in markedly different parasitemia with 40 and 80% killing by platelets compared with 0% (which fits the observed parasitemia). The slope of log(%parasitemia) did not change markedly after day 4PI as predicted by modeling of parasitemia with sequestration, indicating that platelet killing of iRBCs is minimal, if any.

Platelet inhibition by aspirin has no detectable effect on parasite replication in blood. To determine whether inhibition of platelet activation increases parasite replication, we injected high dose aspirin or vehicle control starting on day-1PI and ending at peak parasitemia (day 10PI); this aspirin regimen was chosen because it was used for the conclusion that aspirin is detrimental in malaria patients based on this aspirin regimen's elicitation of mortality in *Pc*a DS strain[4,6]. Ascending parasitemia was similar in high dose aspirin and vehicle control mice (Figure 2A). The slope of log(%parasitemia) during ascending parasitemia (day 0 to 6PI) was similar (P=0.49) in both aspirin-treated (0.45 ± 0.03; R^2=0.99) and vehicle control mice (0.48 ± 0.03; R^2=0.99). No mortality was observed in either group. Aspirin did not prevent thrombocytopenia during the course of *Pc*a LV infection (Figure 2A).

Platelet depletion exhibits no detectable effect on parasite replication in blood. To determine whether depletion of platelets early in infection decreases early killing of iRBCs and/or affects the protective immune response, we injected C57BL/6 mice on day –1PI and every 2-3 days with either anti-CD41 or isotype control mAb. Few platelets were detected by flow cytometry during the course of *Pc*a infection in platelet-depleted mice during ascending parasitemia whereas larger numbers of platelets were detected in isotype controls (Figure 2B). Despite >92% platelet depletion from day 0-4, there was no significant (P>0.05) difference in parasitemia between platelet-depleted and control groups during the course of ascending or descending parasitemia and suppression of the infection (Figure 2B). No mortality was observed in either group.

To determine whether the platelet depletion affects protective immune responses after a second infection, we treated the two infected groups, with Trimethoprim-Sulfamethoxazole for 1 week starting on day 32PI and allowed 1 week for drug clearance before injecting them and an uninfected group i.v. with 1×10^7 *Pc*a iRBCs. The secondary parasitemia was similar and below

0.1% in anti-CD41 and isotype control-injected groups whereas the primary infection controls parasitemia peaked at 14±1% on day 10PI (Figure 2C). A maximum of 1000 RBCs were counted resulting in a lower detection limit of 0.1% parasitemia. At least 10 pRBCs/1000 RBCs or 1% parasitemia likely provides a reliable estimate of parasitemia. Most animals exhibited 0 pRBCs/1000 RBCs, resulting in an average parasitemia below 0.1%. Removal of platelets by antibody depletion does not affect parasitemia at any point during primary or secondary infections, indicating that platelets are not killing parasites and are not required for activation of the protective immune response.

Intact platelets adoptively transferred to CD40KO recipients do not affect parasitemia or elicit a protective immune response. To determine whether wild-type (WT) CD40^{+} platelets adoptively transferred to CD40KO mice (which do not control *Pca* parasite replication) elicit a reduction in the initial inoculum and/or activate a protective adaptive immune response, we injected CD40-KO mice with 0.2×10^9 platelets from uninfected C57BL/6 mice or saline alone on day –1 and 0PI and infected both groups plus infection control mice group (C57BL/6) with *Pc*a. All groups of mice exhibited similar ascending parasitemia; both groups of CD40KO mice exhibited similarly high levels of unremitting parasitemia whereas the C57BL/6 mice controlled their parasitemia to low levels (Figure 2D).

We tested the secondary protective immune response in the CD40-KO mice receiving (i) platelets or (ii) saline and in C57BL/6 mice by drug curing the 3 groups of animals and then infecting them plus an infection control group with *Pc*a. The secondary parasitemia was similar in both CD40KO groups. Both groups exhibited some initial control and then high levels of parasitemia (Figure 2E). CD40-KO mice develop anti-parasite IgM but do not isotype switch to IgG (unpublished results), which may account for the initial control of parasitemia in these mice.

*Increased platelet binding to iRBCs and terminal deoxynucleotidyl transferase dUTP nick end labeled (TUNEL+) platelets is not detected in thin blood films during ascending Pc*a. To analyze direct platelet cytotoxicity during ascending Pca parasitemia[4,5], we counted apoptotic/dying parasites and iRBC-platelet conjugates in thin blood films. The platelet to target ratio was 1,070±55:1 and declined rapidly to about 1 as parasites replicated exponentially during ascending parasitemia and circulating platelet numbers declined (Figure 3A). During ascending parasitemia (day 0-7PI), the %parasitemia using all RBCs is similar to that seen in conjugates of platelets and iRBCs (Figure 3B). Most platelet:RBC conjugates comprise platelets with uRBCs rather than iRBCs (Figure 3C). Less than 10% of iRBCs exhibit an attached platelet and this percentage does not change markedly during the course of *Pc*a infection (Figure 3D). The %iRBCs that are TUNEL+ is significantly greater during peak and descending parasitemia and most apoptotic parasites do not have a platelet attached to the iRBC (Figure 3E, F). Collectively, these findings indicate that platelet:RBC adhesion is low and few if any TUNEL+ Pca parasites exhibit a bound platelet, indicating that direct platelet adhesion is unlikely to kill significant numbers of parasites.

Neither platelet inhibition by aspirin nor platelet depletion affects the replication of high virulence Pca parasites. To increase the likelihood of detecting platelet-killing of iRBCs, we increased the initial platelet to iRBC target ratio by 100-fold and increased the time for platelets to kill parasites by injecting 100-fold fewer Pca HV parasites into mice. We injected Pca HV-infected mice with i) high dose aspirin, ii) saline; iii) anti-CD41 mAb or iv) isotype control until peak parasitemia to detect changes in platelet killing of iRBCs and changes in immune response controlling parasite replication. The parasitemia was similar at each time point during ascending parasitemia in aspirin-treated and vehicle controls (Figure S1A) and anti-CD41 mAb-injected

and isotype controls (Figure S1E). No mortality was observed in either aspirin or vehicle control group. Mortality was similar in anti-CD41 mAb-injected and controls and occurred after ascending parasitemia (Figure S1E). The parasitemia in RBC-platelet conjugates was greater than in all RBCs (Figure S1B) but aspirin did not inhibit this. The percentage of platelet:RBC conjugates was low (<0.1% of RBCs). The number of iRBC-platelet conjugates was low from day 4 to day 8PI and similar to background (day 0PI), but the number of uRBC-platelet conjugates declined (Figure S1C). The percentage of iRBCs with a bound platelet was <10% throughout course of infection (Figure S1D). These findings indicate that neither platelet inhibition by aspirin nor platelet depletion affects parasite replication or the activation of the protective immune response that controls parasitemia.

Human platelets do not inhibit in vitro replication of several strains of Pf. To confirm that human platelets do not kill Pf-iRBCs *in vitro* at physiological concentrations, we incubated platelets with RBCs at a 1:9 ratio with time 0 parasitemia of Pf at trophozoite stage ranging from 4-8%. Parasitemia was similar over an entire cycle between cultures containing platelets and those without (Figure 4A). The percent decline in parasitemia in platelet-containing cultures to no platelet controls was minimal; at 24 and 48 hrs, the parasitemia was higher in platelet-containing cultures, providing a negative percent decline (Figure 4B). One donor was tested against FCR3 and DD4 strains of Pf; the parasitemia was similar at each time point in platelet-containing cultures to no platelet controls. After excluding coincident events, the percentage of iRBCs with bound platelet remained constant and <10%; the percentage of uRBCs with bound platelets was lower and < 3% (Figure 4C). Platelets in the co-cultures were primarily (~90%) unbound (Figure 4D). Platelets cultured in media alone exhibited similar thrombin dose responses at early time points, but impaired response at 48h (Figure 4E,F).

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Platelet function in experimental cerebral malaria.

Because Pf that elicits human CM is proposed to be susceptible to platelet killing[3,4], therefore, the eCM-eliciting parasite, PbA, should also be susceptible to platelet killing. This however appears to contradict our earlier report that platelet depletion actually protects against eCM without affecting parasitemia[7,8]. We therefore reexamined platelet killing in PbA infections of mice.

Neither increased platelet binding to iRBCs and dying (TUNEL+) platelets nor activated, circulating platelets are detected during eCM. To determine whether platelets function in parasite killing when eCM is elicited, we repeated the Pca analysis with PbA. PbA parasitemia increased exponentially up to and including day 5 (R^2=0.99 with slope of 0.66) when the rate of increase slows (Figure 5A). Thrombocytopenia starts on day 4, is marked by day 5 (Figure 5A), and occurs during the period of exponential parasite replication. PbA parasitemia in RBCs with bound platelets is significantly higher than all RBCs ($P<0.05$) on day 6PI (Figure 5B). The percentage of platelet bound iRBCs increased significantly ($p<0.05$) on day 6PI (Figure 5C). However, the percentage of iRBCs at risk for platelet killing declined from day 4 to day 6PI and was <10% (Figure 5D) with no preferential binding of platelets to apoptotic parasites (Figure 5E,F). Platelet depletion by anti-CD41 mAb does not significantly alter PbA parasitemia[7,8].

To determine whether WT platelets transferred to CD40KO mice, which are eCM resistant[19], affects parasitemia and/or elicits a pathogenic response, we injected 0.2×10^9 platelets from uninfected mice or vehicle control into eCM-protected CD40-KO mice[19] on day –1 and day 1PI. Significantly ($p<0.05$) decreased clinical scores and increased mortality in CD40-KO mice receiving platelets compared with vehicle controls (Figure 6A,B) indicate that CD40^{+} platelets elicit pathogenesis in a resistant CD40KO mouse; consequently, platelet CD40 is a key molecule

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in triggering pathogenesis and platelets do not affect PbA replication *in vivo* (Figure 6C). Together with *Pc*a findings, they also indicate that the pathways leading to control of parasite replication and pathogenesis are separate and distinct.

Discussion

The explanations for thrombocytopenia correlating with severe malaria include (i) platelets kill iRBCs, (ii) platelets activate the protective immune response, and/or (iii) absence of platelets contributes to pathogenesis. As a consequence of (i) and (ii), thrombocytopenia would result in increased parasitemia and disease. Two groups[3-5] have proposed platelet killing of *Plasmodium* in iRBCs. The first line of evidence supporting platelet killing of iRBCs is the concurrence of thrombocytopenia during *Pca* infection in mice with the development of patent parasitemia and the exponential rise in parasitemia until peak parasitemia[4]. However, the parasite is replicating exponentially in the blood compartment from the time of initial infection to about peak parasitemia; thus, the log(%parasitemia) fits with a straight line. Our data indicate that the slope of this line does not change with the onset of thrombocytopenia, which is predicted if platelets are controlling parasite replication. Thus, the coincidence of patent parasitemia and thrombocytopenia is likely due to the host response the parasite has elicited rather than the direct killing of iRBCs by platelets.

Second, depletion of platelets by mAb leads to increased parasitemia in PbA-infected mice[20]. Significantly increased parasitemia occurs at a single time point (day 5PI) in PbA-infected mice depleted of platelets with anti-CD42b mAb on day -1PI compared with isotype Ig controls[20]. Parasitemia measured at a single time point by conventional flow cytometry may yield incorrect results because some uRBCs contain nucleic acids that are then interpreted as iRBCs. In the present study, we used a different mAb to deplete platelets (anti-CD41 mAb MWreg40) and

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verified our depletion throughout ascending parasitemia, measured parasitemia at multiple timepoints, and tested 2 different species of *Plasmodium* without detecting any effect of platelet depletion[7,8]. Both McMorran's[4] and our analyses of inhibition of platelet activity by high dose aspirin indicate that this regimen does not affect *Pc*a or PbA parasitemia. Our data therefore indicate that neither inhibition of platelets nor depletion of platelets with mAb affects parasitemia.

McMorran et al.[4] use death rather than parasitemia to detect platelet killing of parasites because iRBC sequestration in organs may result in parasitemia not reflecting total numbers of parasites, resulting in an inability to detect parasite killing by platelets. However, our data (Figure 1) indicate that the slope of log(%parasitemia) during ascending parasitemia is linear and dependent on parasite virulence. Parasitemia and the slope of log(%parasitemia), therefore, should be different in the presence and absence of platelets given the 80% killing observed *in vitro*[4,5].

The use of the animal's death as a biomarker for parasite replication maybe problematic. Death is complex, multi-factorial, and occurs at low and high parasitemia, so death may not be directly linked to parasite burden. Indeed, the majority of deaths reported by McMorran et al.[4] occur after ascending parasitemia at time points when parasite burden is being suppressed by the adaptive immunity[11,17,18] and thrombocytopenia has been marked for a while. Thus, there is little evidence to indicate that an elevated parasite burden is the cause of the animal's death.

The platelet-mediated killing of iRBCs *in vivo* is proposed to be contact-dependent based on: (i) increased *Pc*a parasitemia when platelet-RBC conjugates are counted compared with all RBCs and (ii) significantly (p<0.05) increased % of parasites that are TUNEL+ in platelet-deficient MPL$^{-/-}$ mice compared with C57BL/6 controls[4]. However, our analysis of platelet:xRBC (x=u or i) conjugates during the course of *Pc*a infection supports the conclusion that platelets are not

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cytotoxic for parasites. Our findings that most platelets adhere to uRBCs, <10% of iRBCs have a bound platelet, and few apoptotic parasites occur in platelet:iRBC conjugates, collectively indicate that it is unlikely that platelets are adhering to iRBCs early during the infection prior to the onset of thrombocytopenia in order to kill the parasite within the RBC.

Others reported contact-dependent intra-Pf-RBC killing by human platelets *in vitro*[3-5]. Peyron et al.[5] reported at a platelet:Pf-iRBC ratio of 160:1 (corresponding to a platelet:RBC ratio ~1:1, which is not physiological) results in >80% inhibition of parasite development. If this finding is valid, then platelet inhibition should reduce this killing. However, in this study, *in vitro* platelet killing of Pf-iRBC is not inhibited by 1mM aspirin or 2mM histidine. In contrast, McMorran et al.[3,4] reported that this platelet killing is abrogated by known platelet inhibitors. However, their observation that the total parasitemia at 29h (trophozoite) and 44h (schizont) of platelet-Pf-iRBC cocultures are similar suggests that 15h of culture have passed with no killing or inhibition of development detected in the presence of platelets[4]. In the present study, we also did not detect any change in parasitemia with Pf ring-stages maturing to schizonts in the presence or absence of platelets. We replicated our *in vivo* condition as much as possible with human cells at 1:9 platelet:RBC ratio of normal blood and ~ 2:1 platelet:iRBC ratio. We tested multiple human donors and parasite strains, confirmed platelet thrombin responses throughout the co-culture, but did not detect inhibition of parasite replication or development by platelets. The percentage of platelets bound to a Pf-iRBC was greater than uRBC, but these percentages were <10% and remained constant throughout the coculture. Clinical studies report either an association of elevated numbers of platelet-RBCs conjugates (rosettes) with severe human malaria or no association[1,2]. Platelet-Pf-iRBC rosettes associate with high parasitemia and cerebral malaria[21]. If platelets killed Pf-iRBCs within rosettes, then elevated rosette numbers should reduce

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parasitemia and be associated with lower parasitemia and likely with protection from severe malaria. Collectively, these observations suggest that under physiological conditions platelets do not inhibit the replication or intra-RBC development of Pf. Thus, there is no rationale for avoiding the use of NSAIDs in the treatment of malaria[6].

Besides their putative role as innate killer cells, platelets function in the activation of immune responses. Indeed, CD40L+ WT platelets adoptively transferred to CD40LKO mice activate the protective immune response in virus- or bacteria-infected mice[22-24]. However, neither high dose aspirin nor platelet depletion starting at day -1PI affected the primary parasitemia. Platelet depletion during the primary infection also did not alter the secondary parasitemia. Moreover, adoptive transfer of CD40+ WT platelets into CD40KO mice that do not control their Pca parasitemia had no detectable effect on the primary and secondary immune responses controlling parasitemia. These results indicate that (i) adoptive transfer of WT CD40+ platelets to CD40KO mice does not restore immune control of primary or secondary parasitemias, as reported in some other infections[22-24], and (ii) platelets are not required for the development of a protective primary and secondary immune responses to resolve Pca.

The observations that C57BL/6 mice depleted of platelets by anti-CD41 mAb injection early (day 1PI) but not late (day 4 onwards) exhibit decreased inflammatory responses, decreased acute phase proteins, and are protected from eCM[7,8] indicate that the activation of the pathogenic eCM response is impaired in the absence of platelets. Their requirement for the onset of the pathogenic response has however not been tested. Our observation that adoptive transfer of WT CD40+ platelets to eCM-resistant CD40KO mice partially restores mortality and clinical symptoms of eCM supports the role of platelet CD40 in activating a pathogenic response.

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Collectively, our findings indicate that platelets (i) do not function in the killing of blood-stage *Plasmodium* parasite, (ii) are not required for the activation of the protective immune response, but (iii) are required to elicit pathogenesis. Platelets may function in part by activating the liver to produce acute phase proteins or by activating the pathogenic immune response[8,20].

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Authorship.

IG, and HH developed hypotheses, designed and performed the experiments, analyzed data, and wrote the manuscript. JV and MW performed experiments, and analyzed data. VC and GG developed hypotheses, reviewed data, and wrote the manuscript. There are no non-author contributions and disclosures. The authors have no conflict of interest to declare.

Figure Legends.

Figure 1. No change in slope of log(%parasitemia) after onset of thrombocytopenia that would suggest platelets affecting parasite replication in resolving, non-eCM *Pc*a LV infection. Parasitemia denoted by filled symbols (left y-axis) in A-D with corresponding platelet counts/ml in open symbols (right y-axis) for A, B. (A): Parasitemia of *Pc*a-infected mice (n=10) (circle) plotted on a linear scale with latent parasitemia coinciding with thrombocytopenia. (B): Parasitemia in (A) plotted on logarithmic scale and no change in slope occurs at the onset of thrombocytopenia on day 4 PI. This experiment was replicated 3 times. (C): Modeling of log of %(parasitemia) at selected rates of parasite killing by platelets between day 0 and 4 PI assuming sequestration does not affect parasitemia. We model the Pca LV parasitemia in a 20g mouse, which calculates the initial parasitemia (Table 1). Platelet killing is of Pf-iRBCs is ~80%[4,5], and we compare this to a more conservative 40% killing and no killing. At initiation of LV infection, the 20g mouse has: 1×10^6 iRBCs; 1.6×10^{10} RBCs; 1.6×10^9 platelets; and a platelet effector to iRBC target ratio > 1,000, resulting in ~80% killing of iRBCs by platelets based on in vitro killing of Pf-iRBCs[4,5]. At the onset of thrombocytopenia (day 4PI: ~0.5% parasitemia; 1.6×10^{10} RBCs, and 1.6×10^9 platelets), the platelet effector to target ratio is 20:1; theoretically, platelet killing is minimal below 160:1[5]. The period of platelet killing of iRBCs is therefore between day 0 and 4 PI (shaded, labeled "Period of platelet killing"). During ascending parasitemia (day 7PI: ~6% parasitemia; 1.6×10^{10} RBCs; and ~1.6×10^8 platelets), the ratio is 1:600. The parasite replicates each night producing new progeny, and the MOI during period of no killing and determined from Figure 1B is 2.8. If each day during platelet killing period, 80% or 40% of iRBCs are killed by platelets, then MOI declines to 0.6 (20% of 2.8) and 1.7 (60% of 2.8) respectively. The measured parasitemia (Figure 1B) fits 0% line rather than 40% or 80% killing.

(D): Modeling of log % parasitemia assuming similar degrees of iRBC sequestration throughout ascending parasitemia and platelet killing until day 4 PI as described above in C. Because the slope of log(%parasitemia) is linear, parasitemia likely reflects overall parasite load. The slope of log(%parasitemia) during period of platelet killing from day 0 to 4PI is 2.8 (Panel B); this estimates effective rate of parasitemia increase with sequestration. Because platelet killing is minimal beyond day 4 PI, this rate of parasitemia increase should increase markedly with an MOI from 2.8 (0% killing) to 14 (80% killing) and 7 (40% killing) after day 4 PI. The measured parasitemia (Panel B) clearly fits 0% platelet killing best. The modeling indicates that the slope of log(%parasitemia) should change markedly if platelet killing occurs.

Figure 2. Platelet removal or inhibition does not affect parasitemia in resolving, non eCM *Pc*a LV infection and does not affect activation of protective immunity. Parasitemia for each group denoted by filled symbols (left y-axis) with corresponding platelet counts/ml in open symbols (right y-axis). (A): Aspirin- (triangle) and saline-injected (circle) groups (n=5) of *Pc*a-infected mice. (B): Platelet-depleting anti-CD41 mAb (triangle) or isotype control (circle) injected on day –1, 1, 3, and 6PI. (C): Secondary parasitemia after injection of 1×10^7 *Pc*a on day 0PI into the anti-CD41 mAb and isotype control groups of mice that had resolved their primary infection; a group of uninfected mice with primary parasitemia is infection control. (D): Primary parasitemia in CD40KO mice that do not resolve *Pc*a infection after iv injection of WT platelets on day -1 and 0PI and in intact mice that resolve their *Pc*a infection. (E): Secondary parasitemia after injection of 1×10^7 *Pc*a into 3 groups of mice in Panel D; a group of uninfected mice with primary parasitemia is infection control. Average value ± SEM are reported. *: *P*<0.05. The experiment in Panel A was repeated twice, and in B was repeated once.

Figure 3. Platelets are not associated with dying parasites during resolving, non-eCM *Pc*a infection in thin blood films. The analyses were performed in thin blood films fluorescence labeled with anti-CD41 (blue; platelets), ethidium bromide (red: parasites), and TUNEL (green). (A): Platelet effector to target (iRBC) ratios (■), platelet: uRBC ratios (□), and parasitemia (●). Platelet:RBC is an abbreviation for a platelet RBC conjugate; the RBC maybe uninfected (uRBC) or infected (iRBC). (B): Percent PbA parasitemia for all RBCs (□) $\left(\frac{\#\ iRBC}{\#\ RBC}\ \%\ \right)$ and considering only RBCs with bound platelets (■) $\left(\frac{\#\ platelet:iRBC}{\#\ platelet:RBC}\ \%\ \right)$. (C): Percentage of RBCs with bound platelets with uninfected (□) $\left(\frac{\#\ platelet:uRBC}{\#\ platelet:RBC}\ \%\ \right)$ or infected (■) RBCs: ($\frac{\#platelet:iRBC}{\#\ platelet:RBC}$ %). (D): The percentage of iRBCs with an adherent platelet $\left(\frac{\#\ platelet:iRBC}{\#\ iRBCs}\ \%\ \right)$. (E): Representative thin blood film made on day 6PI and fluorescently labeled; blue arrows indicate platelets, red arrows indicate parasites without TUNEL, and yellow arrows indicate parasites with TUNEL (green+red). (F): Percentage on day 6, 8, and 12PI of iRBCs exhibiting TUNEL+ labeling (gray-filled bar, x-axis) $\left(\frac{\#\ TUNEL+\ iRBCs}{\#\ iRBCs}\ \%\ \right)$ and TUNEL– (open bar, x-axis) $\left(\frac{\#\ TUNEL-\ iRBCs}{\#\ iRBCs}\ \%\ \right)$ with the percentage of each iRBC+, TUNEL+ or iRBC+, TUNEL– $\left(\frac{\#platelet:\ TUNEL-\ iRBCs}{\#\ iRBCs}\ \%\ \right)$ exhibiting an adherent platelet shown as an inset (filled black bar). This experiment was repeated twice (n=5) and verified by flow cytometry. Values are average ± SEM.

Figure 4. Human platelets at physiological ratios do not inhibit *in vitro* replication of human strains of *Plasmodium falciparum*. (A): Representative percent parasitemia measured by imaging flow cytometry over the course of 48hours in cultures with platelets (□) at 1:9 RBC ratio and without platelets (■). (B): Calculated replication inhibition of *P. falciparum* replication in

platelet cultures compared with no platelet controls (n=6) at selected time points during the

development of *P. falciparum* parasites. (C): The percentage of uRBCs (□) $\left(\frac{\#\,platelet:uRBC}{\#\,uRBCs}\,\%\right)$

or iRBCs (■) $\left(\frac{\#\,platelet:iRBC}{\#\,iRBCs}\,\%\right)$ with attached platelet over course of *P. falciparum*

development. (D): Percentage of all platelets that are unbound (■) $\left(\frac{\#\,unbound\,platelets}{\#\,platelets}\,\%\right)$,

bound to uRBCs (□) $\left(\frac{\#\,platelet:uRBC}{\#\,platelets}\,\%\right)$, or bound to iRBCs (■) $\left(\frac{\#\,platelet:iRBC}{\#\,platelets}\,\%\right)$ during the

course of *P. falciparum* development. (E): Intensity of CD62P fluorescence on the surface of

platelets after stimulation with selected doses of thrombin at selected time points of culture. (F):

Calculated thrombin EC50 for platelet response at the selected time points of culture.

Figure 5. Platelets are not associated with dying parasites during non resolving, eCM-eliciting

*Pb*A in C57BL/6 mice. The analyses were performed in thin blood films fluorescence labeled

with anti-CD41 (blue; platelets) and ethidium bromide (red: parasites) and TUNEL (green). (A):

Platelet effector to target (iRBC) ratios (■), platelet: uRBC (□) ratios and parasitemia (●) on left

axis and platelet counts/ml (○) on right axis. (B): Percent PbA parasitemia for all RBCs (□)

$\left(\frac{\#\,iRBC}{\#\,RBC}\,\%\right)$ and considering only RBCs with bound platelets (■) $\left(\frac{\#\,platelet:iRBC}{\#\,platelet:RBC}\,\%\right)$.

Platelet:RBC is an abbreviation for a platelet RBC conjugate; the RBC maybe uninfected

(uRBC) or infected (iRBC). (C): Percentage of RBCs with bound platelets with uninfected (□)

$\left(\frac{\#\,platelet:uRBC}{\#\,platelet:RBC}\,\%\right)$ or infected (■) RBCs: $(\frac{\#platelet:iRBC}{\#\,platelet:RBC}\,\%)$. (D): The percentage of iRBCs

with an adherent platelet $\left(\frac{\#\,platelet:iRBC}{\#\,iRBCs}\,\%\right)$. (E): Representative thin blood film made on day

6PI and fluorescently labeled; blue arrows indicate platelets, red arrows indicate parasites

without TUNEL, and yellow arrows indicate parasites with TUNEL (green+red). (F):

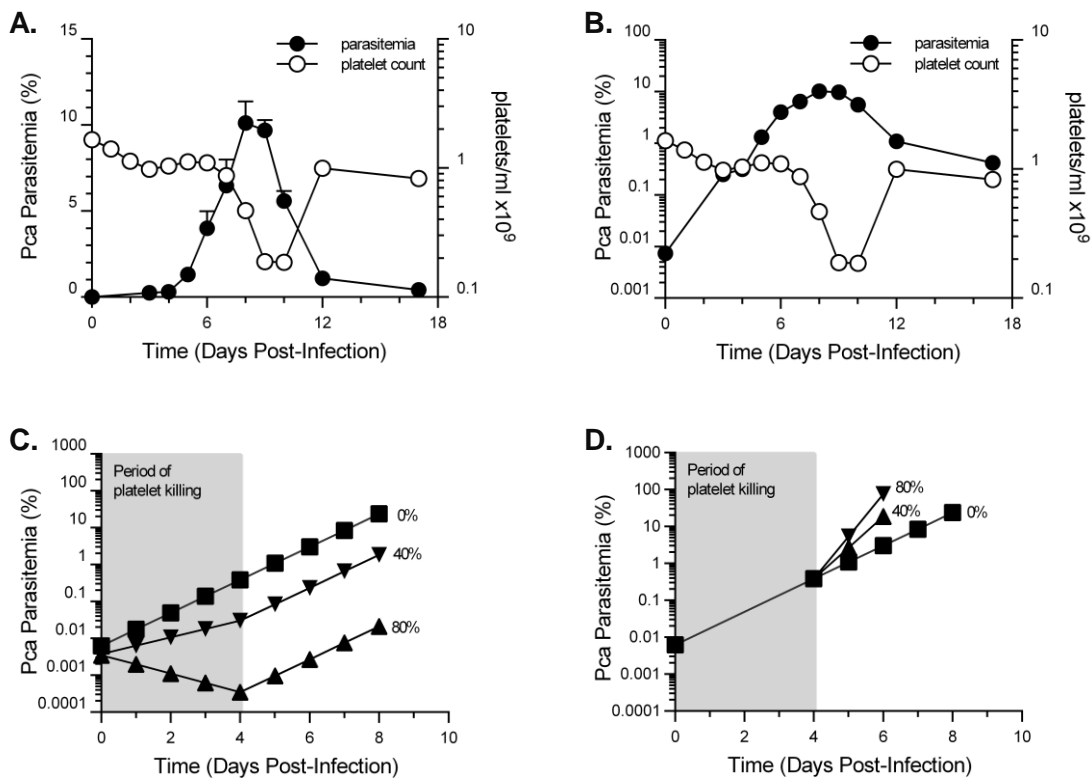Percentage on day 4 and 6PI of iRBCs exhibiting TUNEL+ labeling (gray-filled bar, x-axis)

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$\left(\frac{\# \ TUNEL+ \ iRBCs}{\# \ iRBCs} \ \% \right)$ and TUNEL– (open bar, x-axis) $\left(\frac{\# \ TUNEL- \ iRBCs}{\# \ iRBCs} \ \% \right)$ with the percentage of

each iRBC+, TUNEL+ or iRBC+, TUNEL– $\left(\frac{\# platelet: \ TUNEL- \ iRBCs}{\# \ iRBCs} \ \% \right)$ exhibiting an adherent

platelet shown as an inset (filled black bar). This experiment was repeated twice (n=5) and

verified by flow cytometry. Values are average ± SEM. This experiment was repeated twice

(n=5).

Figure 6. Platelets partially restore pathogenesis to eCM-protected CD40-KO mice. (A):

Clinical scores, (B): percent survival, and (C): parasitemia for groups (n=5) CD40-KO

reconstituted with WT platelets (■), CD40-KO injected with saline (□) or C57BL/6 controls (▲).

Gray shaded area indicates the time period for the development of neurological symptoms by

C57BL/6 mice. Values are average ± SEM. *: $p<0.05$ for comparison of CD40-KO + platelets

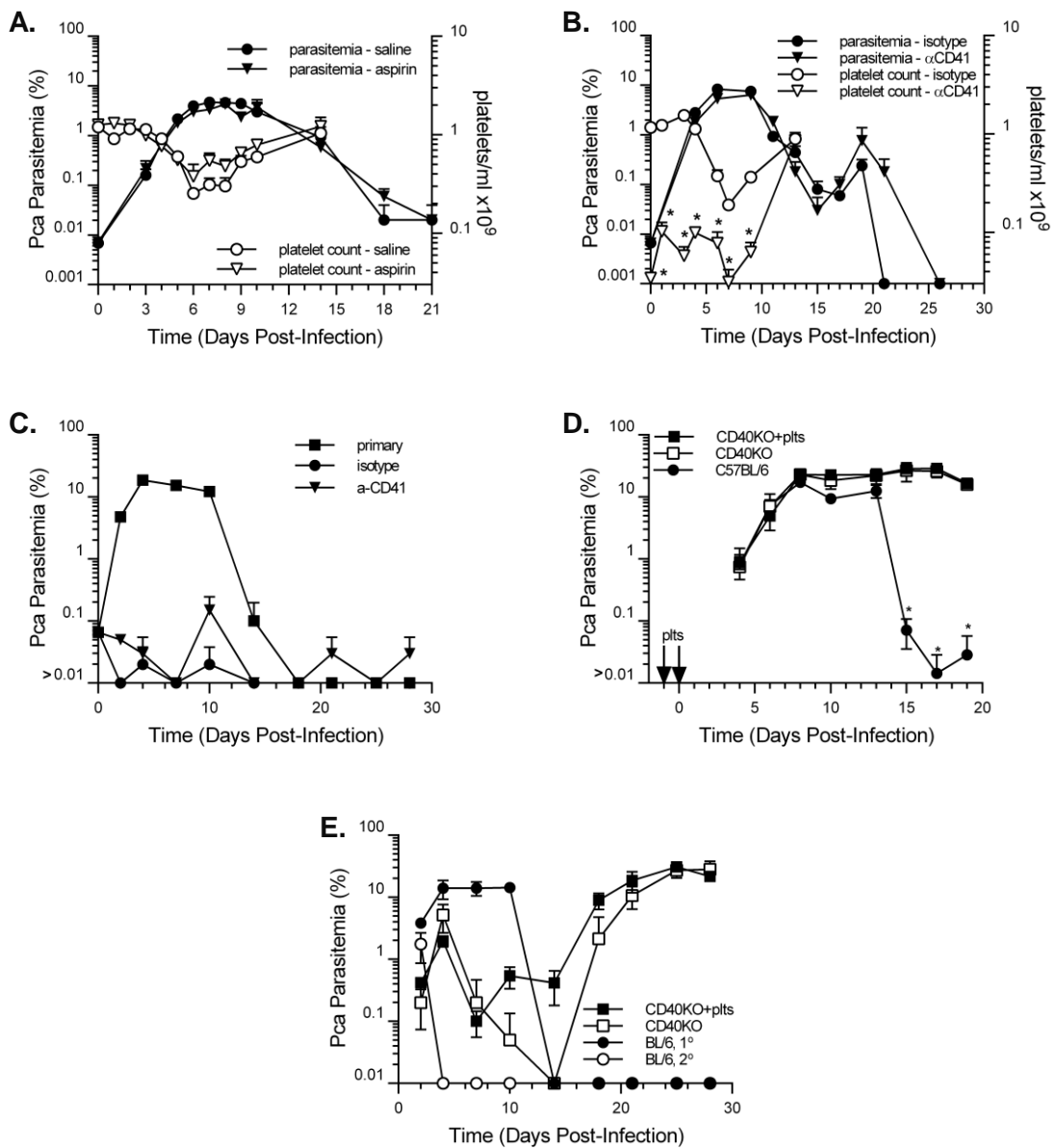groups with other 2 groups; #: $p<0.05$ for comparison of C57BL/6 with other 2 groups.

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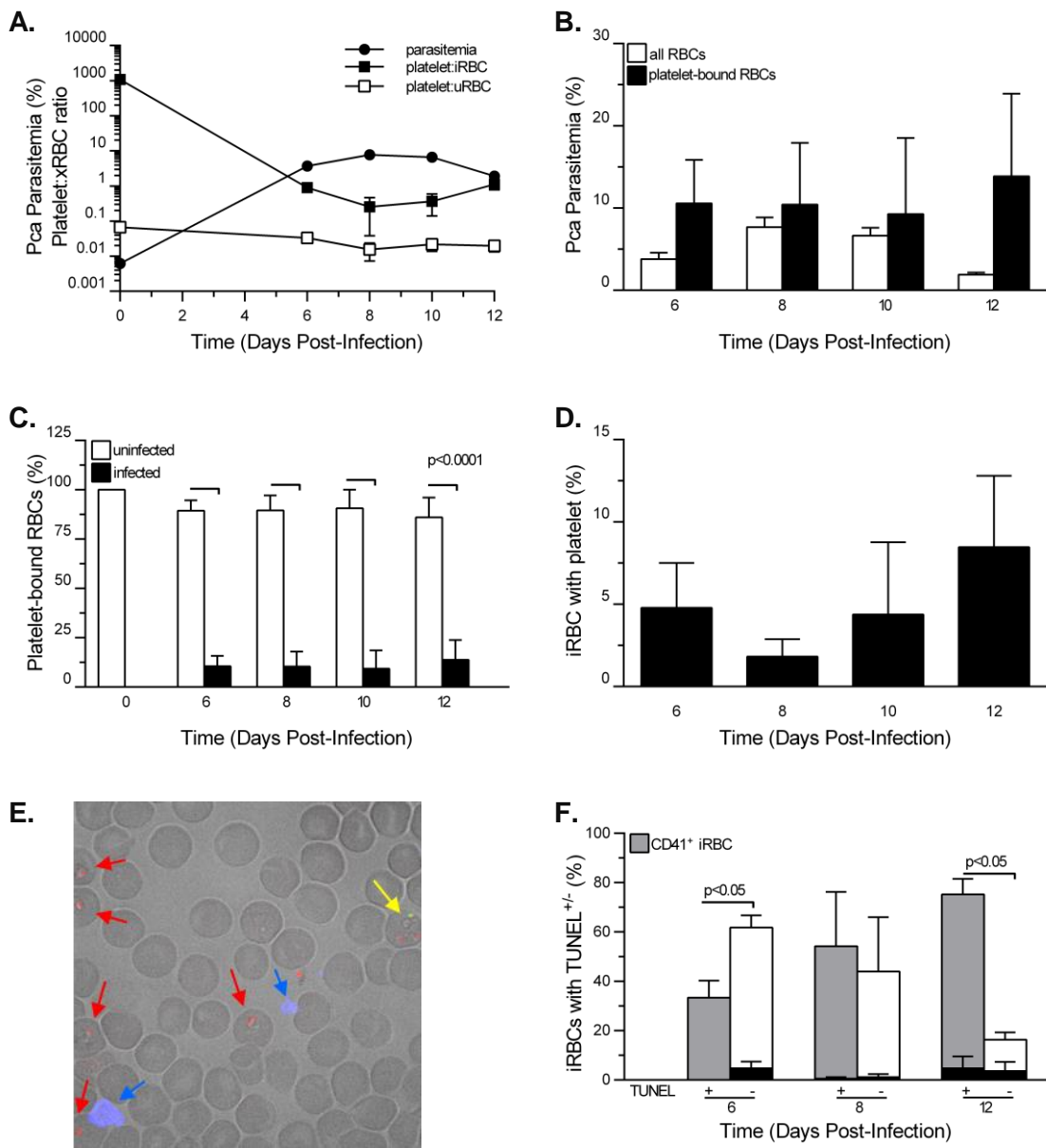
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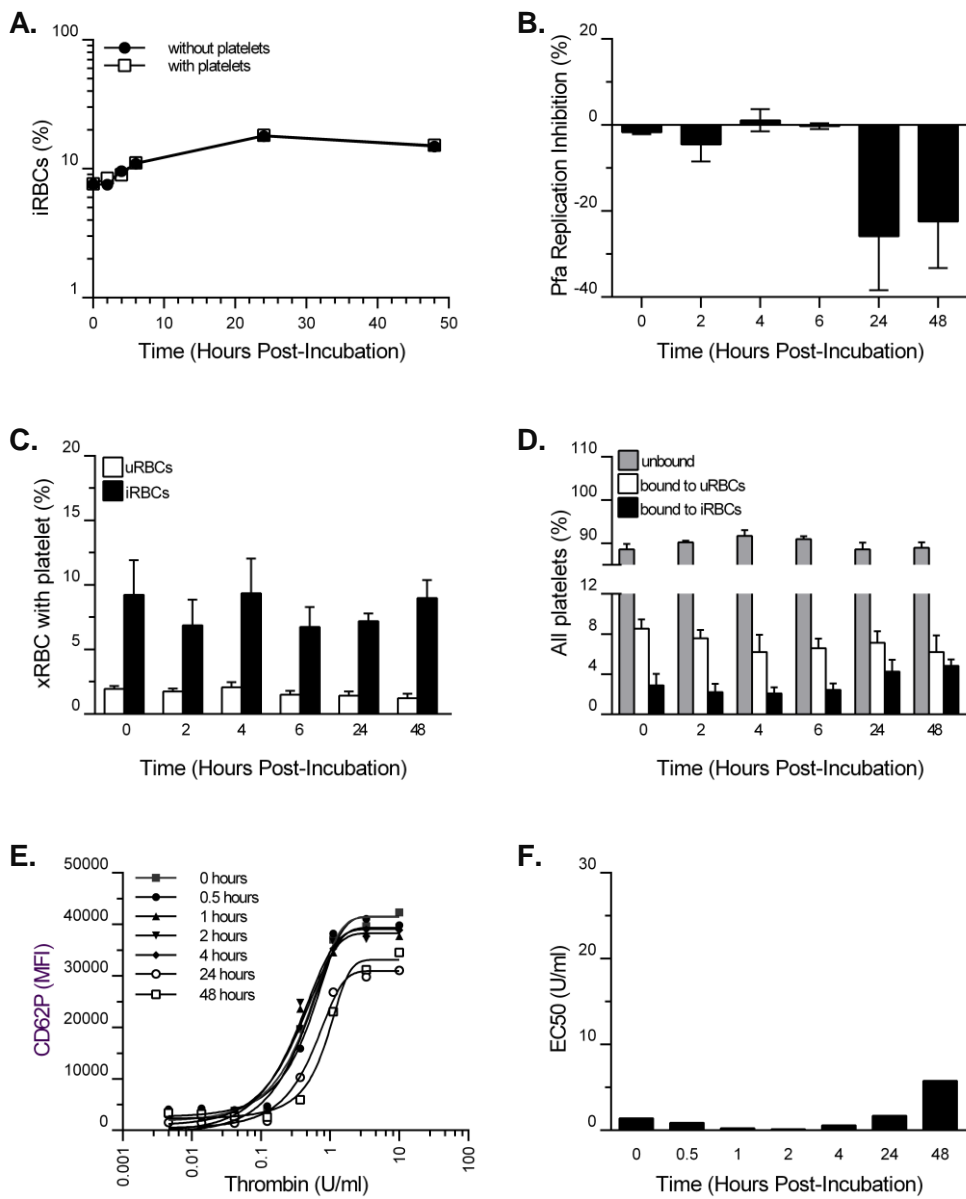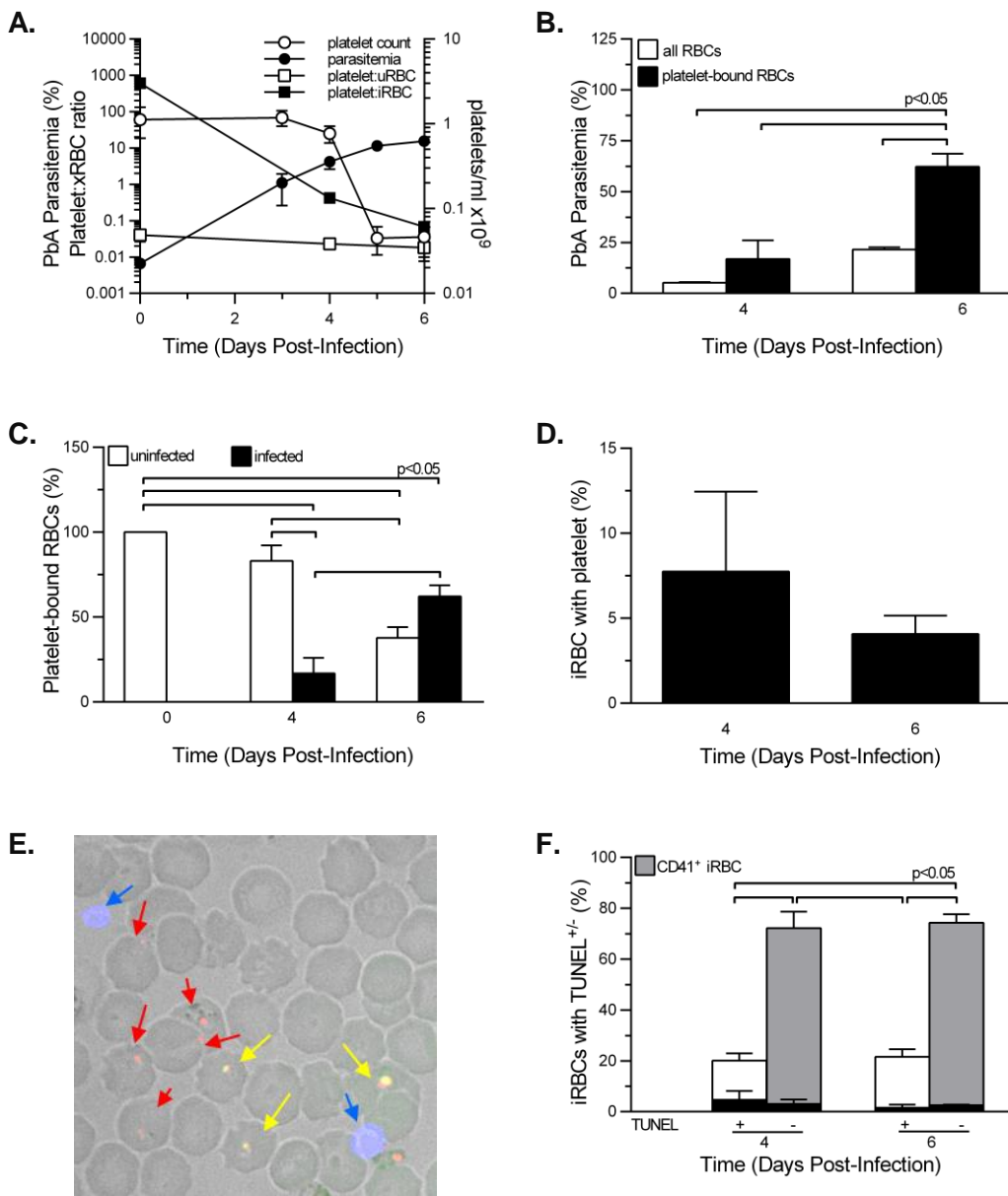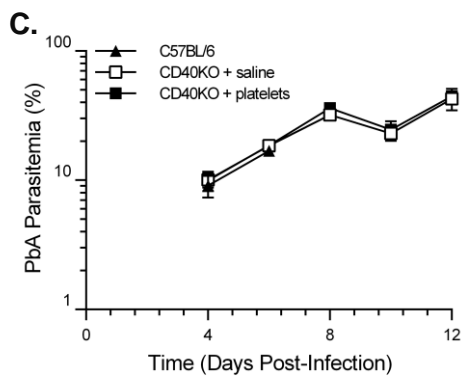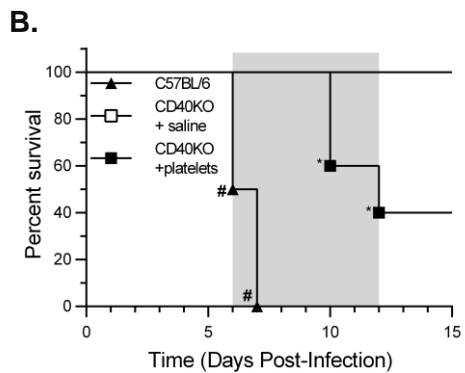
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Figure 1

Figure 2

Figure 3
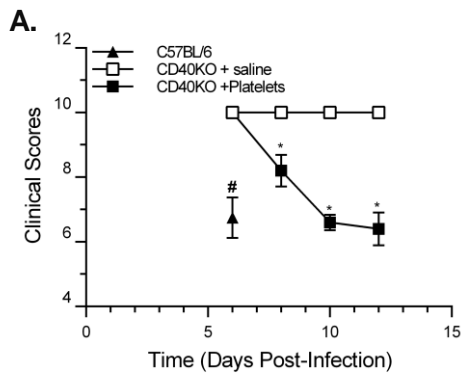
Figure 4

Figure 5

A.

Clinical Scores vs Time (Days Post-Infection)

Legend:
- C57BL/6
- CD40KO + saline
- CD40KO +Platelets

B.

Percent survival vs Time (Days Post-Infection)

Legend:
- C57BL/6
- CD40KO + saline
- CD40KO +platelets

C.

PbA Parasitemia (%) vs Time (Days Post-Infection)

Legend:
- C57BL/6
- CD40KO + saline
- CD40KO + platelets

Figure 6

Platelets activate a pathogenic response to blood-stage *Plasmodium* infection but not a protective immune response.

Irene Gramaglia, Joyce Velez, Valery Combes, Georges E.R. Grau, Melanie Wree and Henri C. van der Heyde

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