Thrombocytopenia in early malaria is associated with GP1b shedding in absence of systemic platelet activation and consumptive coagulopathy

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coagulopathy

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Running title: GP1b shedding without platelet activation in malaria

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Summary

Thrombocytopenia develops early in malaria, but the underlying mechanisms remain incompletely understood. We studied the etiology of malaria-associated thrombocytopenia in volunteers experimentally infected with *Plasmodium falciparum* malaria, in Indonesian malaria patients and in *ex vivo* studies. In experimental human malaria, the decrease in platelet numbers was associated with a concurrent rise in young platelets (immature platelet fraction) and thrombopoietin. D-dimer concentrations were moderately elevated without a prolongation in APTT or decrease in fibrinogen. There was no increase in expression of the platelet surface markers CD62P, PAC-1 and CD63 and in plasma concentrations of the platelet factors P-selectin, CXCR4, CXCL7, RANTES and CD40L. In contrast, concentrations of soluble glycoprotein-1b (sGP1b), the external domain of the platelet receptor for von Willebrand factor (VWF), increased early. Indonesian malaria patients also had elevated concentrations of sGP1b, which correlated with VWF concentrations. Finally, incubation of platelets with parasitized erythrocytes *in vitro* failed to induce platelet aggregation or activation. We conclude that neither a compromised platelet production nor platelet activation or consumptive coagulopathy are responsible for the early thrombocytopenia in malaria. We hypothesize that the increase in sGP1b concentrations results from VWF-mediated GP1b shedding; a process that may prevent excessive adhesion of platelets and parasitized erythrocytes.

Keywords

Platelets, Thrombocytopenia, Platelet activation, Glycoprotein-1b, Malaria, VWF

Introduction

Thrombocytopenia is a common finding in patients with *Plasmodium falciparum* and *Plasmodium vivax* malaria. Apart from their central role in haemostasis, platelets contain a wide range of inflammatory, immune modulating and angiogenic factors (Smyth *et al*, 2009). Two recent studies have suggested that platelets play a distinct role in the innate host defense in the early stages of a malaria infection. McMorran and co-workers showed that purified human platelets were able to kill *P. falciparum* parasites within erythrocytes in an *in vitro* culture system, an effect that was dependent on platelet activation by adenosine 5'-diphosphate (ADP) (McMorran *et al*, 2009). Moreover, they showed that platelet deficient mice were more susceptible to death during infection with the murine malaria species *P. chabaudi*. Srivastava and co-workers showed that erythrocytes infected with the murine malaria parasite *P. berghei* interacted with and activated platelets, which resulted in secretion of CXCL4 (platelet factor 4), a platelet derived chemokine thought to be involved in the development of cerebral malaria (Srivastava *et al*, 2008).

So, platelet activation may be an important process in early malaria that contributes to the fall in platelet number and it raises concern over the use of aspirin as antipyretic in children with malaria (Greenbaum & FitzGerald, 2009; Pleass, 2009). However, to what extent platelets are activated during human malaria is uncertain. To our knowledge, there are no studies on platelet activation in early malaria, while the available data from patients with a more advanced malaria infection have been inconclusive (Lee *et al*, 1997; Supanaranond *et al*, 1992). There are also fundamental differences in the pathogenesis of human and murine malaria and caution should be taken when extrapolating findings from murine malaria models to human malaria (White *et al*, 2009).

Previously, we have reported a significant decrease in circulating platelet numbers in volunteers experimentally infected with *P. falciparum* during the earliest stages of the blood

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stage infection and we have shown that this decrease coincided with the onset of endothelial cell activation with release of the platelet binding protein von Willebrand factor (VWF) (de Mast *et al*, 2007). In the current study, we studied the occurrence of platelet activation, coagulopathy and reduced thrombopoiesis in this experimental human malaria model. Observations made in these volunteers were confirmed in patients with naturally acquired malaria in Indonesia and in *ex vivo* studies

Design and methods

Experimental human P. falciparum infections

Design. Experimental human malaria infections with healthy and malaria naïve volunteers are invaluable for testing the efficacy of candidate malaria vaccines and a well established and highly reproducible model for studying pathogenic mechanisms in early malaria (Epstein *et al*, 2007;Verhage *et al*, 2005). The present study contains data from three experimental human malaria infections, which were carried out in 2005, 2007 and 2008. Details on the objectives, the characteristics of the volunteers and the clinical and parasitologic course have been described in detail before (de Mast *et al*, 2007;de Mast *et al*, 2009b;Roestenberg *et al*, 2009;McCall *et al*, 2007). The primary objective of the study in 2005 was to study immunological responses in early *P. falciparum* malaria (de Mast *et al*, 2007;McCall *et al*, 2007); the latter two were intervention studies, assessing the protective efficacy of repetitive exposure to live malaria sporozoites under chloroquine prophylaxis (Roestenberg *et al*, 2009) and of a candidate malaria vaccine (study in 2008). The current study on platelet kinetics was among the predefined exploratory objectives of all three studies. Only volunteers belonging to the control groups of these intervention studies were included to avoid a confounding effect of the intervention.

As previously described, all volunteers were infected by 5 *P. falciparum* infected mosquitoes and followed by thick blood smears and assessment of malaria symptoms three times daily until start of antimalarial treatment (Verhage *et al*, 2005). Antimalarial treatment was started upon microscopic detection of parasites in a thick blood smear and consisted of a standard, 6 dose curative regimen of artemether/lumefantrine 80/480 mg (Riamet®), given at 0, 8, 24, 36, 48 and 60 hours. The presence of *P. falciparum* was also analyzed by PCR and this revealed that submicroscopic parasitemia is usually present 3 days before thick smears become positive and treatment is given. Five volunteers who did not develop a positive malaria blood smear and thus no thrombocytopenia and who received artemether-lumefantrine at day 21, according to the study protocol, were included as controls in some experiments. The Institutional Review Board of the Radboud University Nijmegen Medical Centre and the Dutch Central Committee on Research involving Human Subjects approved the study protocols (CMO 2004/129, CMO 2006/207 and NL14715.000.06, respectively), including these supplementary platelet studies. All volunteers gave written informed consent.

Sample collection. Venous blood was collected daily between 08:00 and 10:00 AM in EDTA and in CTAD plasma tubes (Beckton Dickinson Vacutainer Systems, Europe). The latter contain the anticoagulant sodium citrate and the platelet stabilizing agents theophylline, adenosine and dipyrimadol. The CTAD tubes were kept on ice pending whole blood flow cytometric analyses or they were immediately centrifuged at 3500 rpm for 10 minutes followed by a second high speed centrifugation of the plasma to obtain platelet poor plasma for measurement of platelet circulating plasma markers. Plasma was stored at -80 °C awaiting analyses.

Laboratory procedures. The amount of blood that could be collected daily from volunteers for research purposes was limited. Therefore, platelet activation was studied by flow cytometry in 15 volunteers in the study in 2005 and by measurement of soluble platelet products in 5 volunteers in 2007. Platelet kinetics and coagulation parameters were studied in 11 volunteers in the 2007 and 2008 experimental infection.

Hemocytometry was performed on a Sysmex XE-5000 automated hematology analyzer (Sysmex Corporation, Kobe, Japan) within four hours after blood collection. Platelet production was assessed by measurement of the immature platelet fraction (IPF), a parameter for young platelets that was recently introduced on Sysmex analyzers (Abe *et al*, 2006). Quantification of the IPF was performed after addition of a polymethine dye and making use of specialized software as described in detail previously (Abe *et al*, 2006). The IPF is expressed as percentage of the total platelet number.

For measuring platelet activation by flow cytometry, whole blood in CTAD tubes was fixed with paraformaldehyde 1%, diluted with HEPES buffer and stained for 15 minutes with saturating concentrations of anti-CD41-PE, anti-CD62P-PE, PAC-1-FITC and anti-CD63-FITC. Data on 10,000 platelets in a log side-scatter versus log CD41-PE dot plot were acquired. The mean fluorescence intensity (given in arbitrary units) of each antibody was analyzed and corrected for non-specific binding of isotype control.

Circulating concentrations of the platelet products P-selectin, CXCL4 (platelet factor-4), CXCL7 (NAP-2), CCL5 (RANTES), CD40 ligand (CD40L) and soluble glycoprotein-1b (sGP1b; glycocalicin) were determined by using semiautomated ELISA on a TECAN RSP150 robot as previously reported in detail (Snoep *et al*, 2010). Commercially available antibody duosets Dy137, Dy795, Dy393 and Dy278 (all R&D Systems, Wiesbaden, Germany) and antibody combination BMS239 (Bender Medsystems, Vienna, Austria) were used to determine concentrations of P-selectin, CXCL4, CXCL7, CCL5 and CD40L, respectively.

Soluble glycoprotein-1b (sGP1b; glycocalicin) was determined by ELISA with an in house capture antibody (RUU-630) and a commercial detection antibody (M1852, Sanquin, Amsterdam, the Netherlands). Western blot analysis showed that this capture antibody is very specific for GP1b (data not shown). Intra- and inter-assay CV's of this assay were 4% and < 10%, respectively. Plasma thrombopoietin (TPO) concentrations were determined by a commercially available ELISA kit (R&D Systems, Wiesbaden, Germany). VWF and the VWF activation factor, which reflects the amount of VWF that is in a platelet binding conformation, was determined by ELISA as previously described (de Mast *et al*, 2007). Levels of D-dimer were measured by means of the STA Liatest D-Dimer and fibrinogen assay (Roche Diagnostics SpA, Mannheim, Germany). Activated partial thromboplastin time (APTT) was determined using a KC10A coagulometer (Amelung, Lemgo, Germany). Plasma (50 µl) was incubated for 2 minutes with 50 µl Patromtin (Behringwerke, Marburg, Germany). Coagulation was started with 50 µl of 25 µmol/l calcium chloride and the time to coagulation was measured.

Field study in Indonesia

Data obtained in the experimental human malaria studies were confirmed in patients with naturally acquired *P. falciparum* and *P. vivax* malaria, who presented to the Rumah Sakit Karitas Hospital in Weetabula, West Sumba, East Nusa Tenggara Province, Indonesia. Consecutive subjects presenting to hospital with clinical symptoms of malaria and a *P. falciparum* or *P. vivax* parasite density of at least 2500 and 500 parasites/ µL, respectively, were enrolled. A group of healthy hospital staff was enrolled as controls. Characteristics of the malaria patients and the controls have been described in detail previously (de Mast *et al*, 2009a).

Ex vivo studies

The findings from the *in vivo* studies were further confirmed by *in vitro* studies. The effect of parasitized red blood cells (pRBCs) and various Toll like receptors (TLR) agonists on platelet activation status and platelet function were examined using flow cytometry and platelet aggregometry. Citrated blood (final concentration citrate 11mM) from two healthy volunteers was used for all experiments. *P. falciparum* asexual stages of the NF54 strain were cultured in vitro as described previously (McCall *et al*, 2007). Asynchronous cultures of NF54 strain parasites were harvested at a parasitemia of ~10–20% and the mature asexual stages were purified by centrifugation on a 63% Percoll density gradient (McCall *et al*, 2007). This purification step results in preparations with 80–90% parasitemia, consisting of >95% schizonts/mature trophozoites. These preparations of pRBC were washed twice in RPMI 1640 and used fresh in stimulation assays or freeze-thawed twice to obtain parasite lysate. Mock-cultured uninfected erythrocytes (uRBC) were obtained similarly and served as control. Whole blood was also stimulated with the following TLR agonists: TLR2 with Pam3Cys (10 µg/ml; InvivoGen), TLR4 with purified *Escherichia coli* LPS (10 ng/ml), TLR9 with CpG ODN repeat (10 µg/ml; TIB MolBiol).

Flow cytometry. Citrate whole blood (90µl) was incubated for 30 minutes at 37⁰ C with asynchronous pRBCs (final parasitemia ~5%), synchronized mature pRBCs (final parasitemia ~0.25%), uRBCs, lysate of synchronized pRBCs and uRBCs (2.5x10⁶ pRBCs) or phosphate buffered saline (PBS). Further platelet activation was inhibited by the fibrin polymerization inhibitor Gly-Pro-Arg-Pro peptide (GPRP; Biotrend Chemicals AG, Zurich, Switzerland). After adding saturating concentrations of antibodies, samples were incubated for 15 minutes in the dark at room temperature, followed by fixing with paraformaldehyde 10% for 10 minutes. The following antibodies were used: CD62P PECy5 (AK-4 clone; BD Pharmingen,

San Jose, CA); CD41 ECD (P2 clone), CD40 PE (MAB-89 clone) and CD36 FITC (FA-6:152 clone; all from Beckman Coulter, Immunotech, Marseille, France); TLR2 PE (CD282, TL2.1 clone), TLR4 PECy7 (CD284, HTA125 clone; eBioscience, San Diego, CA) and TLR9 FITC (CD289, 5G5 clone; Hycult Biotechnology Uden, the Netherlands). Analyses were performed using a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Miami Florida). Platelets were identified by FSC/CD41 positive staining. Isotype and fluorochrome matched controls as well as non-stained samples were used to adjust the detectors.

Aggregometry. Platelet aggregation was measured in an impedance dual channel aggregometer (Chrono-log, Model 570, Havertown, USA) and analyzed by the AggroLink software package. 750 μ l of platelet rich plasma (PRP) was pre-warmed for 5 min at 37°C in a magnetically stirred polycarbonate cuvette. After calibration, pRBCs or uRBCs were added (final hematocrit of 15%) and aggregation was monitored on two leads with ADP (22 μ M) and PBS as positive and negative control, respectively. Whole blood impedance aggregometry was chosen over optical aggregometry because the preparatory steps required for the latter technique (separation of RBCs from the PRP by extra centrifugation steps), resulted in unwanted platelet activation.

Statistical analysis

Data are presented as mean with standard deviation unless otherwise stated. Changes from baseline in serial data were analyzed with the use of a linear mixed model for repeated measures. No assumption about the shape of the time trend was made i.e. time point was included as a categorical variable. Correlation between repeated measures within subjects was assumed to only depend on the time between the repeated measures (Toeplitz correlation structure). Holm's method was used to adjust for multiple comparisons at several time points

versus baseline. Differences between groups were analyzed by Student’s T-test. All analyses were performed with SPSS version 16.0.

Results

Experimental human malaria infections

Platelet kinetics. Platelet kinetics were studied in 11 volunteers. Parasitemia induced an early and marked decrease in platelet count from a mean value of $253 \times 10^9/L$ to a mean nadir value of $113 \times 10^9/L$ (range $55 - 188 \times 10^9/L$) with a concurrent decrease in circulating leukocyte numbers (Fig 1A-B). In the early phase of the malaria infection, the IPF remained stable with a subsequent rise in the first days after the positive malaria blood smear, suggesting that the thrombopoietic capacity of the bone marrow was not suppressed (Fig 1C). Young platelets are larger and the changes in the IPF paralleled changes in the mean platelet volume (MPV; Fig 1D) and the platelet large cell ratio (P-LCR; Fig 1E). The relative thrombocytosis at week 3 was associated with a low IPF and MPV. A transient increase in plasma TPO concentrations occurred during the drop in platelet count (Fig 1F). Platelet clumps were not observed, neither by automated hemocytometry, nor by morphological examination (data not shown). Finally, in contrast to the IPF, the reticulocyte fraction decreased in the first days after the thick smear became positive and the subsequent start of antimalarial treatment, suggesting that erythrocyte and platelet production are independent processes in patients with malaria (Fig 1C).

Absence of consumptive coagulopathy. Activation of the coagulation cascade was evaluated in the same volunteers by measurement of D-dimers, fibrinogen and the APTT. These parameters were measured once at day 1 after start of antimalarial treatment, i.e. at the day platelet numbers declined markedly. Concentrations of D-dimers were above the upper

reference limit (0.5 µg/ml) in 9 out of 11 volunteers with a mean value of 2.6 µg/ml (range 0.2 – 7.5 µg/ml). The mean fibrinogen concentration was 3.3 g/l (range 2.4 – 4.4 g/l), and none of the volunteers had fibrinogen concentrations below the lower limit of the reference value (2.0 – 4.0 g/l). Compared with the corresponding baseline values, there were no relevant changes (≥ 5 seconds) in the APTT in 9 of the 11 volunteers. A prolongation in APTT of 9.3 and 19 seconds was found in the remaining 2 volunteers with the latter occurring in the volunteer with the highest D-dimers value (7.5 µg/ml).

Increased sGP1b concentrations in absence of systemic platelet activation. Platelet activation status was measured at four pre-specified time points by whole blood flow cytometry in 15 volunteers (Fig 2). A slightly higher PAC-1 (α IIb β 3 activation marker) surface expression was found in some volunteers, but there was no concurrent increase in expression of the α -granule membrane protein CD62P (P-selectin) or the lysosomal marker CD63.

Since activated, adherent platelets may be missed with flow cytometry, plasma concentrations of a range of platelet membrane and granule products were measured in five volunteers. No consistent increase occurred in plasma concentrations of soluble P-selectin, CXCL4, CXCL7 and RANTES during parasitemia and its treatment (Fig 3A-D). Plasma CD40L concentrations were below the assay's lowest standard value in all samples. One volunteer had a transient increase in soluble P-selectin concentration, while RANTES concentrations were transiently increased in two others. In contrast, parasitemia and its treatment resulted in a significant increase in sGP1b concentrations in all volunteers from a mean concentration of 486.6 ng/ml at baseline to 704.2 ng/ml ($p=0.04$) at day 2 after treatment initiation (Fig 3E). sGP1b is the extracellular domain of glycoprotein-1b α , the main platelet receptor for VWF. Previously, we have demonstrated a similar time course for VWF concentrations (de Mast *et al*, 2007). sGP1b concentrations depend on the circulating platelet mass and the relatively high sGP1b

concentrations at week 3 are probably due to the relative thrombocytosis at that time point.

The increase in sGP1b during parasitemia and treatment was not a direct effect of artemether-lumefantrine on platelets, because aparasitemic volunteers treated by a standard course of artemether-lumefantrine showed no increase in sGP1b concentrations (Fig 3F).

sGP1b in naturally acquired malaria

To confirm our findings of a malaria-associated increase in sGP1b, we compared plasma concentrations of this protein adjusted to the individual platelet number between patients with symptomatic *P. falciparum* or *P. vivax* malaria and healthy controls on the Indonesian island Sumba. Patients with symptomatic *P. falciparum* and *P. vivax* malaria had significantly higher sGP1b concentrations per platelet than healthy controls (Fig 4A). Total sGP1b concentrations were also significantly higher in the falciparum and vivax malaria groups than in controls with median (IQR) values of 1074 ng/ml (696 – 1511 ng/ml), 709 ng/ml (551 – 1061 ng/ml) and 512 ng/ml (420- 612 ng/ml), respectively. Recently, it was reported that shear-induced interaction of platelets with VWF results in GPIb-ectodomain shedding (Cheng *et al*, 2009). In these Indonesian malaria patients, there was indeed a significant correlation between sGP1b concentrations per platelet and VWF concentrations (Fig 4B) and VWF activation factors (Fig 4C).

Ex vivo studies

Flow cytometry. The effect of pRBCs on platelet phenotype was studied by measurement of the surface expression of various platelet receptors upon incubation of whole blood with pRBCs containing synchronous, mature asexual *P. falciparum* stages (final parasitemia of whole blood ~0.25%), pRBCs with asynchronous *P. falciparum* stages (final parasitemia ~5%) or pRBC lysate. Neither synchronized, nor asynchronous pRBCs changed CD62P

expression (Fig 1A of supporting information; only data for synchronous mature pRBCs shown). Lysate of pRBCs and uRBCs increased CD62P expression to the same degree, probably owing to the platelet activating effects of ADP and haem from erythrocytes.

Platelets express various TLRs and because *P. falciparum* contains TLR4 and TLR9 ligands, these receptors may be involved in the presumed role of platelets in the innate immune response (Aslam *et al*, 2006; Cognasse *et al*, 2005). However, there was no up- or down-regulated expression of the platelet receptors CD36, CD40 and TLR2, 4 and 9 with either intact or lysed pRBCs (Fig 1B-F of supporting information). Finally, no change in CD62P or TLR expression was observed upon incubation of whole blood with the specific TLR ligands Pam3Cys (TLR2), lipopolysaccharide (TLR4) or CPG repeat ODN (TLR9; results not shown).

Aggregometry. The effect of direct interaction of platelets with pRBCs on platelet aggregation was studied with impedance aggregometry. Incubation of pRBCs with non-synchronized asexual stages (hematocrit 15%; parasitemia 15%) with PRP (ratio 1:1) did not induce any detectable platelet aggregation (Fig 2A of supporting information). In addition, there was no difference in the degree of platelet aggregation to 22 μ M ADP between PRP incubated with pRBCs or uRBCs (Fig 2B of supporting information).

Discussion

Recent evidence from murine malaria and *in vitro* studies has suggested that platelets and platelet activation play a role in the pathogenesis of early malaria. Studies in humans are however sparse. From our current study, in which the decrease in platelet numbers was associated with an early increase in the IPF and TPO concentrations, we conclude that thrombopoiesis is not suppressed in early malaria, unlike the erythropoiesis. Increased peripheral platelet clearance thus seems responsible for the malaria-induced thrombocytopenia, a finding supported by an earlier scintigraphic platelet study showing a strongly reduced platelet life span in malaria patients (Skudowitz *et al*, 1973). Second, we demonstrate that systemic platelet activation and consumptive coagulopathy are not responsible for this enhanced clearance.

Previously, in the same human malaria model, we have shown that endothelial cell activation with release of active VWF, i.e. VWF in a platelet binding conformation, is an early event in malaria and that VWF concentrations correlated negatively with platelet numbers (de Mast *et al*, 2007). Multiple other recent *in vivo* and *in vitro* studies have also implicated VWF release as a pathogenic mechanism in malaria (Bridges *et al*, 2009; de Mast *et al*, 2009a; Larkin *et al*, 2009; Lowenberg *et al*, 2010). Given this presumed role for VWF, the increase in plasma concentrations of sGP1b is an interesting observation. sGP1b, also known as glyocalicin, is the external domain of the GP1b receptor, the main platelet receptor for VWF and the leukocyte integrin Mac-1 (Wang *et al*, 2005). The external domain of the GP1b receptor can be shed from the platelet surface by cleavage by the metalloproteinase TNF- α -converting enzyme (TACE/ADAM17) and this leads to a clear attenuation of normal platelet function (Bergmeier *et al*, 2004). Recently, Cheng and co-workers reported data from *in vitro* studies in which they showed that interaction of platelets with VWF under flow condition induces GPIb-ectodomain shedding, a process that could be inhibited by inhibitors of metalloproteases

(Cheng *et al*, 2009). The similar kinetics of sGP1b and VWF plasma concentrations in experimental malaria and their positive correlation in Indonesian malaria patients support these recent findings by Cheng *et al*. This novel mechanism may not only explain the elevated sGP1b concentrations in absence of other signs of platelet activation in our volunteers, but may also prove important for the negative regulation of both excessive platelet adhesion and VWF-mediated cytoadherence of infected red blood cells - a mechanism that has recently been reported (Bridges *et al*, 2009). Future studies will need to establish the functional consequences of GP1b shedding in malaria.

There is a paucity of data on the occurrence of platelet activation in human malaria. To our knowledge, no previous studies have used flow cytometry to examine platelet activation in malaria patients. Our findings suggest that platelet activation does not contribute to the thrombocytopenia in early malaria. We cannot exclude that some degree of platelet activation occurs in severe malaria, a condition associated with organ dysfunction and high mortality. No significant difference in absolute CXCL4 (PF4) and CXCL7 (β -TG) levels between patients with severe malaria and healthy controls was found in a well performed study in Thailand, in which special attention was paid to prevent ex vivo platelet activation (Supanaranond *et al*, 1992). The occurrence of some low grade in vivo platelet activation was suggested, however, by the fact that malaria patients had a lower circulating platelet mass and a slightly higher CXCL4/CXCL7 ratio. Thus, while our study found no platelet activation in early malaria, further studies are required to clarify the role of platelet activation in more advanced stages of malaria. In contrast to studies in humans, marked platelet activation has been described in several malaria animal models (e.g. von Zur Muhlen *et al*, 2009). There are however important differences in the pathology of (cerebral) murine malaria compared with severe human malaria. Murine malaria is primarily an immunopathological process with marked inflammation and little or no sequestration of pRBCs, while endothelial activation

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with pRBC sequestration is a central feature of human malaria (White *et al*, 2009). These fundamental differences in pathology can explain the discrepancy in results of platelet activation studies between human and murine malaria.

These findings in human volunteers were supported by the finding of *in vitro* studies, in which incubation of whole blood with pRBCs failed to induce direct platelet activation or platelet sensitization to ADP. In contrast to our results, enhanced platelet aggregation and increased surface expression of CD62P and PAC-1 after incubation of platelets with pRBCs were found in previous studies (Inyang *et al*, 1987;Srivastava *et al*, 2008). Differences in the used protocols may be responsible for these conflicting results. *In vivo* conditions were simulated as much as possible in our current study by using impedance aggregometry to measure aggregation of platelets after addition of RBCs, instead of PRP in optical aggregometry. In addition, parasite densities in the range of those normally found in clinical malaria infections were used, which limits the possible confounding effects of excessive release of free hemoglobin and ADP, both potent platelet activators, from pRBCs during the incubation time.

In recent years, platelets were shown to express various TLRs, although their precise functional importance remains elusive (Aslam *et al*, 2006;Cognasse *et al*, 2005). *P. falciparum* contains known TLR2/4/9 ligands (Parroche *et al*, 2007;Gowda, 2007). Incubation of whole blood with pRBCs did not change the surface expression of TLR2/4/9, while incubation of whole blood with specific TLR2/4/9 agonists did not upregulate CD62P expression. Two earlier studies also found no induced platelet CD62P expression after stimulation with TLR2 and TLR4 agonists (Cognasse *et al*, 2008;Ward *et al*, 2005). Therefore, to date, it remains uncertain whether TLRs on platelets play a role in malaria pathogenesis.

The contribution of activation of the coagulation cascade to the malaria-induced thrombocytopenia has also been disputed in the past (Francischetti, 2008). In our study, coagulation activation did occur, as indicated by the elevated D-dimer concentrations in 9 of 11 volunteers. However, a consumptive DIC did not develop, given the absence of a clear prolongation in the APTT in all except one volunteer and the high-normal fibrinogen concentrations. Earlier field studies had already shown that frank DIC is only rarely encountered (Vreeken & Cremer-Goote, 1978). So, coagulation activation does occur in early malaria, as it occurs in almost every infectious disease, but its role in the marked thrombocytopenia in malaria seems only minor. The same holds true for splenic pooling of platelets; a scintigraphy platelet study in malaria patients was consistent with diffuse platelet sequestration, instead of splenic or hepatic pooling (Karanikas *et al*, 2004), while asplenic persons also develop pronounced thrombocytopenia when infected with malaria (Demar *et al*, 2004). Finally, immune-mediated mechanisms have also been implicated in the pathogenesis of malaria-associated thrombocytopenia. Antiplatelet IgG antibody concentrations were reported to correlate with platelet number (Kelton *et al*, 1983), although this finding could not be confirmed in another study (Looareesuwan *et al*, 1992). Antiphospholipid antibodies also occur in malaria patients. While antiplatelet antibodies may play a role in later stages of a malaria infection, it seems unlikely that they are responsible for the early decrease in platelet number when parasite densities are still extremely low. Previously, we found no induction of anticardiolipin antibodies in our experimental human malaria model (Damoiseaux *et al*, 2005).

In conclusion, there is growing evidence that platelets play a distinct role in the pathogenesis of malaria during the early phases of the blood infection. Thrombocytopenia occurs in absence of platelet activation or consumptive coagulopathy. We hypothesize that the increase

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in sGP1b results from VWF-mediated GP1b shedding; a process that may prevent excessive adhesion of platelets and parasitized erythrocytes.

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Figure legends

Fig 1. Time course of (A) platelet and (B) leukocyte counts, (C) immature platelet fraction and reticulocyte fraction, (D and E) platelet indices and (F) plasma thrombopoietin concentrations in 11 volunteers during an experimental human *P. falciparum* malaria infection. Data presented are mean $\pm$ SE at the following time points: parasite inoculation (baseline), the period between 3 days (d T-3) before thick smear positivity and subsequent start of antimalarial treatment (d T) until 3 days thereafter (d T+3), day 7 after start of antimalarial treatment (d T+7) and at week 3 and 4 post-parasite inoculation. The median time to thick smear positivity and subsequent start of antimalarial treatment was 10.2 days post-parasite inoculation. * $p < 0.05$, ** $p < 0.01$ compared with corresponding baseline value, determined by linear mixed model for repeated measures.

Fig 2. Time course of the mean fluorescence intensity (X-mean) of the platelet activation markers CD62P (P-selectin), PAC-1 (α IIb β 3 activation marker) and CD63 (lysosomal marker) in 15 volunteers during an experimental human *P. falciparum* malaria infection. Antimalarial treatment was started after a median time of 9.7 days (range, 7.3–11.3 days) after parasite inoculation. Expression was determined by whole blood flow cytometry at the following time points with the corresponding relative (percentage of baseline value) mean platelet count between parentheses: start of antimalarial therapy (d T) or the day thereafter (d T+1; 76%); day 2 or 3 after start of antimalarial treatment (d T+2 or d T+3; 62%); week 3 post-parasite inoculation (140%) and week 6 post-parasite inoculation (97%). This latter time point corresponds with the normal, healthy situation after treatment of malaria. For all three markers, no significant differences between the week 6 value and the other time points were found by paired *t* test with Bonferroni correction. Only 9 blood samples were available for analysis at time point d T+2 or T+3.

Fig 3. Time course of plasma concentrations of (A-E) platelet α -granule and membrane factors in 5 volunteers during an experimental human *P. falciparum* malaria infection and (F) soluble glycoprotein-1b concentrations in 5 aparasitemic volunteers at the start of a standard course of artemether-lumefantrin and two days thereafter. Plasma concentrations shown in panel A-E were determined at the following time points: parasite inoculation (baseline), the period between 2 days (d T-2) before start of antimalarial treatment (d T) until 3 days thereafter (d T+3) and at week 3 post-parasite inoculation. The median time of start of antimalarial treatment was at day 10.2 post-parasite inoculation.

Fig 4. (A) Scatter dot plot with line at mean of plasma concentrations of soluble glycoprotein-1b (sGP1b) per platelet in patients with symptomatic *P. falciparum* malaria (n=26), *P. vivax* malaria (n=11) and healthy Sumbanese controls (n=10). Mean platelets counts (interquartile range) in the first 3 groups were $122 \times 10^9/L$ (70 – $154 \times 10^9/L$), $117 \times 10^9/L$ (77 – $220 \times 10^9/L$) and $237 \times 10^9/L$ (172 – $258 \times 10^9/L$). Group comparisons were done using students *t* test. One outlier in the falciparum group was excluded (sGP1b concentration of 84.7 ng/ml). (B-C) Correlation (Pearson *r*) of sGP1b concentrations per platelet with von Willebrand factor antigen and von Willebrand activation factors.

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Expression (shown as the mean fluorescence intensity) of the platelet receptors CD62P (P-selectin), CD36, CD40 and Toll like receptors-2/4/9 upon incubation of whole citrate blood with synchronized, mature parasitized red blood cells (parasitemia ~0.25%, black line) and uninfected red blood cells (grey line) and their lysate. Isotype fluorochrome matched platelets were used as a negative control (dotted line).
158x160mm (96 x 96 DPI)

Impedance aggregometry using platelet rich plasma (PRP) incubated with asynchronous, asexual parasitized red blood cells (pRBCs; parasitemia 15%; red line) or uninfected red blood cells (uRBCs; blue line). (A) Incubation of PRP did not result in platelet aggregation. (B) No difference in the degree of platelet aggregation was observed after incubation of PRP with pRBC or uRBC and subsequent stimulation with 22 μ M ADP. Changes in impedance were 9 and 8 Ω for the pRBC and uRBC group, respectively.

118x54mm (150 x 150 DPI)