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Naturally-acquired hemozoin by monocytes promotes suppression of RANTES in children with malarial anemia through an IL-10-dependent mechanism

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Abstract

Regulated upon activation, normal T-cell expressed, and secreted (RANTES, CCL-5) is an important immunoregulatory mediator that is suppressed in children with malarial anemia (MA). Although proinflammatory (e.g. TNF-α, IL-1β and IFN-γ) and anti-inflammatory (e.g., IL-4, IL-10 and IL-13) cytokines regulate RANTES production, their effect on RANTES in children with MA has not been determined. Since intraleukocytic malarial pigment, hemozoin (Hz), causes dysregulation in chemokine and cytokine production, the impact of naturally-acquired Hz (*pf*Hz) on RANTES and RANTES-regulatory cytokines (TNF-α, IFN-γ, IL-1β, IL-4, IL-10, and IL-13) was examined. Circulating RANTES levels progressively declined with increasing levels of pigment-containing monocytes (PCM) (P=0.035). Additional experiments in cultured peripheral blood mononuclear cells (PBMC) showed that monocytic-acquisition of *pf*Hz (*in vivo*) was associated with suppression of RANTES under baseline (P=0.001) and stimulated conditions (P=0.072). Although high PCM levels were associated with decreased circulating IFN-γ (P=0.003) and IL-10 (P=0.010), multivariate modeling revealed that only PCM (P=0.048, β=-0.171) and IL-10 (P<0.0001, β=-0.476) were independently associated with RANTES production. Subsequent *in vitro* experiments revealed that

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blockade of endogenous IL-10 significantly increased RANTES production (P=0.028) in PBMC from children with naturally-acquired Hz. Results here demonstrate that monocytic-acquisition of Hz suppresses RANTES production in children with MA through an IL-10-dependent mechanism.

Keywords

Malaria; Hemozoin; Monocytes; Rantes; IL-10

1. Introduction

The host innate immune response to *P. falciparum* products is mediated through the production of cytokines and chemokines from phagocytes (i.e. monocytes/macrophages and neutrophils) that condition the development and outcomes of malarial anemia (MA) [1]. Following infection by *P. falciparum*, pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ are produced during the early stages of the host immune response [2]. Although pro-inflammatory mediators elicit anti-parasitic activities, their over-production also promotes enhanced pathogenesis [3]. Elevated levels of TNF-α, interleukin (IL)-1β and IFN-γ have been associated with SMA and hyperparasitemia in children with malaria [4,5]. Conversely, antiinflammatory cytokines such as IL-10 are produced during the later stages of a malarial infection and down-regulate the potentially pathogenic inflammatory responses that are important for controlling parasitemia [6]. Although previous studies showed decreased IL-10 levels in children with SMA, a low IL-10/TNF-α ratio is associated with enhanced severity of MA [4,5,7], suggesting that the timing and magnitude of pro-inflammatory cytokine production, relative to the anti-inflammatory cytokine response, mediates the clinical outcomes of malaria.

Accumulating evidence illustrates that hemozoin (Hz)-acquisition by phagocytes suppresses cellular immunity and enhances malaria severity [8,9]. Hz is synthesized by trophozoites and early schizonts and is acquired by phagocytic and endothelial cells [9]. Our previous studies demonstrated that *P. falciparum*-derived hemozoin (*pf*Hz) and synthetic hemozoin (sHz) cause dysregulation in inflammatory mediator release from human monocytes/macrophages [10-12]. Additional investigations in our laboratory and those of others showed that RANTES suppression was associated with enhanced severity of MA, suppression of erythropoiesis, and increased mortality in children with CM [12,13]. Moreover, we also demonstrated that both *pf*Hz and sHz suppress RANTES transcripts and protein in peripheral blood mononuclear cells (PBMC) from malaria-naïve donors [12]. Thus, optimal RANTES production appears important for regulating the immune and erythropoietic responses during a malarial infection.

RANTES biosynthesis is differentially regulated by pro- and anti-inflammatory cytokines. Previous studies showed that TNF-α, IL-1β, and IFN-γ induced RANTES transcript and protein expression in human endothelial and glial cells [14,15]. Conversely, IL-10 down-regulates TNF-α- and IFN-γ-induced RANTES release by human monocytes and microglial cells [16, 17]. Consistent with these observations, IL-4, IL-10, and IL-13 inhibit RANTES expression in human endothelial cells [15], suggesting that immunoregulation of RANTES production depends on the microenvironment and/or the cellular sources in the inflammatory milieu.

To more fully characterize the role of RANTES in malaria pathogenesis, we investigated the impact of naturally-acquired Hz (*pf*Hz) on RANTES production in children with MA (Hb<11.0 g/dL) residing in a holoendemic *P. falciparum* transmission area of western Kenya. The role of intramonocytic *pf*Hz in mediating the production of the primary pro- (i.e., TNF-α, IL-1β, and IFN-γ) and anti-inflammatory (i.e., IL-4, IL-10, and IL-13) cytokines that regulate RANTES expression was also investigated. Additional studies were conducted in cultured

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PBMC from children with naturally acquired intraleukocytic *pf*Hz to determine the cytokine (s) responsible for suppression of mononuclear cell-derived RANTES suppression.

2. Materials and Methods

2.1. Study site and population

The study was conducted at the Siaya District Hospital, a rural health facility located in a malaria holoendemic region of western Kenya. The study population consisted of children (*n*=194; age 3-31 mos) with a primary diagnosis of MA defined by Hb<11.0 g/dL and presence of asexual *P. falciparum* parasitemia. In addition, all study participants were HIV-negative and abacteremic. Individuals with HbSS were also excluded from the study since this genetic variant is associated with severe anemia in African children with malaria [18]. Details of MA in this geographic region are presented in our recent report [19].

2.2. Laboratory measures

Venous blood (<3 mL) was collected into EDTA vacutainer® tubes (Becton-Dickinson, New Jersey, USA) and used for hematological (A^{c}T diff2™ Coulter, Beckman-Coulter Inc., Fullerton, USA) and laboratory investigations. Asexual stages of malaria parasites were counted/300 leukocytes in thick Giemsa-stained blood films and parasitemia was expressed per μL of blood using the patient's absolute leukocyte count. Intracellular pigment in monocytes and neutrophils was determined as previously described [4,9]. The erythropoietic response was determined by calculating the reticulocyte production index (RPI) using our previous methods [13] and the absolute reticulocyte number (ARN) was calculated using reticulocyte and erythrocyte counts.

2.3. Measurement of RANTES and cytokines

Plasma samples were obtained from venous blood and stored at -70°C until chemokine and cytokine measurements were performed. RANTES and cytokine (i.e., IL-1β, IL-4, IL-10, IL-13, TNF-α, and IFN-γ) concentrations were evaluated in plasma using a 25-plex cytokine assay (Biosource International, Camarillo, USA) according to the manufacturer's instructions. Detection limits (pg/mL) for the cytokines were as follows: IL-1β (2.66); IL-4 (0.49); IL-10 (5.70); IL-13 (0.76); TNF-α (0.20); and IFN-γ (0.30). The detection limit for RANTES was 0.002 ng/mL. Production of RANTES from PBMC was determined using ELISA according to the manufacturer's protocol (Biosource International, Camarillo, USA), with a sensitivity of >0.015 ng/mL.

2.4. Isolation and culture of peripheral blood mononuclear cells

PBMC were cultured using our previously described methods [10,20]. Briefly, PBMC were cultured at 1×10^6 cells/mL in media alone or stimulated with lipopolysaccharide (LPS) (*Escherichia coli* LPS 0127B8, 100 ng/mL) (Alexis Corp., California, USA) and IFN-γ (200 U/mL) (Pharmingen, New Jersey, USA) in the presence/absence of either recombinant human IL-10 protein (rhIL-10, 2.0 ng/mL; Endogen, Rockford, USA) or IL-10 neutralizing antibodies (1.0 μg/mL; R&D Systems, Minneapolis, USA). Cultures were incubated at 37°C in 5% CO_2 atmosphere for 48 hrs, after which culture supernatants were harvested, centrifuged, and stored at -70°C until experimental measures were obtained.

2.5. Statistical analyses

Statistical analyses were conducted with SPSS, version 15.0 (SPSS Inc., Chicago, USA). Kruskal-Wallis test was used to determine across group differences, and where significant, post-hoc Mann-Whitney *U*-tests with the Bonferroni correction were performed. Chi-square analyses were used for comparing proportions between groups. Stepwise linear regression

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analyses were used to determine the impact of PCM and cytokine concentrations on circulating RANTES. Variables associated with RANTES in the bivariate analyses at (P<0.10) were entered into the model as independent variables. Before conducting inferential analyses, the following variables that deviated from univariate normality were transformed towards normality using logarithmic transformation: RANTES; TNF-α; IL-1β; IFN-γ; IL-10; parasitemia; age; gender; Hb levels; total PCM/μL; platelets; lymphocytes; monocytes; and granulocytes. P<0.05 was used to determine statistical significance.

3. Results

3.1. Presence of *pf*Hz deposition in monocytes and neutrophils

Phagocytosis of *pf*Hz enhances malaria severity and causes dysregulation in inflammatory mediator production [4,8,9,11]. In addition, we have previously shown higher baseline RANTES production in healthy, malaria-exposed aparasitemic children compared to children with malarial anemia in the same cohort [13]. Thus, Hz deposition in monocytes and neutrophils was examined in *P. falciparum*-infected children with MA (i.e., Hb<11.0 g/dL). PCM and PCN levels in the cohort were 0-86.7% [median (Q1-Q3)=1.7% (0-14.2%)] and 0-12.0% [median (Q1-Q3)=0% (0%)], respectively. Due to a substantially higher percentage of PCM vs. PCN in the cohort, we investigated the effect of naturally-acquired intramonocytic Hz on dysregulation of both RANTES and RANTES-regulatory cytokines (i.e., TNF-α, IL-1β, IFN-γ, IL-4, IL-10, and IL-13). As shown in Table 1, children were stratified according to intramonocytic *pf*Hz deposition: 0% [PCM(-), n=97]; ≤10.0% (low PCM, n=41); >10.0<26.7% (moderate PCM, n=28); and ≥26.7% PCM (high PCM, n=28). Division of the categories was based on percentile distributions of PCM in the population, and the fact that the denominator for calculating PCM levels for each individual is 30. Analysis of the three groups that contained intramonocytic Hz revealed that total PCM levels (/μL) were elevated in the high PCM group relative to both the low (P<0.0001) and moderate PCM (P<0.0001) groups.

3.2. Demographic and clinical characteristics of the study participants

The clinical and demographic features of the study participants are summarized in Table 1. Gender distribution differed across the groups (P=0.006). However, age, axillary temperature, and plasma glucose were not significantly different (P=0.059, P=0.058, and P=0.957, respectively). Although parasitemia differed across the groups (P=0.011), proportions of high-density parasitemia (HDP, ≥10,000 parasites/μL) were not significant (P=0.256). Lymphocyte counts differed across the groups (P=0.008), but monocyte and granulocyte counts were not significantly different (P=0.090 and P=0.130, respectively). Consistent with decreasing Hb levels in the presence of increasing *pf*Hz deposition, prevalence of SMA (Hb<6.0 g/dL) was most pronounced in the high PCM group (P<0.05 vs. all groups). The reticulocyte production index (RPI) non-significantly decreased with increasing monocytic-acquisition of *pf*Hz (P=0.380), such that the RPI was 28.4% and 43.8% less in the high PCM group, relative to the PCM(-) and low PCM groups, respectively. Consistent with the decreasing RPI, the absolute reticulocyte number (ARN) was reduced with increasing *pf*Hz-deposition (P=0.003). Platelet counts (P=0.292), and prevalence of thrombocytopenia (<150×10^3/μL, P=0.115) were not significantly different across the groups.

3.3. Suppression of RANTES is associated with monocytic acquisition of *pf*Hz

Our previous studies showing that *pf*Hz and sHz suppress RANTES production in PBMC from malaria-naïve individuals [12] led us to determine the impact of naturally-acquired monocytic *pf*Hz (i.e., PCM) on circulating RANTES. Circulating RANTES progressively declined with increasing PCM levels (P=0.035, across group difference), and were lowest in the high PCM group relative to the PCM(-) group (P=0.007, Fig. 1). RANTES was also inversely associated with the total PCM/μL (r=-0.258, P<0.01), suggesting that increasing monocytic deposition of

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*pf*Hz is associated with significant reductions in circulating RANTES levels in children with malarial anemia.

3.4. Reduced RANTES biosynthesis in cultured PBMC is associated with *in vivo* phagocytosis of *pf*Hz

To further examine the effect of *pf*Hz on RANTES production, PBMC from children with MA were cultured in either media alone or concomitant stimulation with LPS+IFN-γ. Children were stratified into: PCM(-) (0%, n=36); low PCM (≤10%, n=7); moderate (Mod) PCM (>10<26.7%, n=6); and high PCM (≥26.7%, n=3). RANTES production under baseline (P=0.001) and stimulated (P=0.072) conditions decreased with increasing PCM level (Fig. 2). Baseline RANTES production was reduced in the low (P=0.005), moderate (P=0.004), and high PCM (P=0.021) groups relative to the PCM(-) group (Fig. 2). These *in vitro* results support the *in vivo* findings showing that reduced RANTES in circulation is associated with monocytic acquisition of *pf*Hz.

3.5. Monocytic acquisition of *pf*Hz alters pro-inflammatory cytokine profiles

Since we have previously shown that phagocytosis of *pf*Hz by monocytes is associated with dysregulation in soluble inflammatory mediators in children with MA [10,11], the impact of PCM on circulating pro-inflammatory cytokines (i.e., TNF-α, IL-1β, and IFN-γ) known to up-regulate RANTES expression [14,15] was investigated. TNF-α increased with elevated PCM levels (P=0.029, Fig. 3A), while IL-1β (P=0.05, Fig. 3B) and IFN-γ (P=0.003, Fig. 3C) decreased with increasing percentages of PCM. In addition, IFN-γ was lower in the high PCM group relative to the PCM(-) group (P=0.001, Fig. 3C). Thus, acquisition of *pf*Hz by monocytes is associated with significant changes in circulating levels of TNF-α, IL-1β, and IFN-γ.

3.6. Monocytic acquisition of *pf*Hz alters anti-inflammatory cytokine profiles

To further define the inflammatory milieu associated with altered RANTES production, circulating antiinflammatory cytokines (i.e., IL-4, IL-10, and IL-13) known to down-regulate RANTES expression [16,17] were examined. IL-10 differed across the groups (P=0.010) and was significantly reduced in children with high PCM levels relative to the low PCM group (P=0.008, Fig. 4A). Neither IL-4 (P=0.568, Fig. 4B) nor IL-13 (P=0.741, Fig. 4C) differed significantly across the groups. These results illustrate that monocytic acquisition of *pf*Hz may alter circulating IL-10 production, a cytokine with potent down-regulatory effects on RANTES [16,17].

3.7. Modeling of pro- and anti-inflammatory cytokines reveal that IL-10 is associated with circulating RANTES

Since phagocytosis of *pf*Hz by monocytes caused dysregulation in a number of cytokines known to regulate RANTES production, the association between these cytokines and RANTES was further explored. To establish the predictor(s) of circulating RANTES in children with MA, stepwise linear regression analyses were used to examine the impact of pro- (i.e., TNF-α, IL-1β, and IFN-γ) and anti-inflammatory (i.e., IL-10) cytokines, acquisition of monocytic *pf*Hz (i.e., PCM), and clinical variables associated with circulating RANTES. Only variables with differences at the P<0.10 level in the bivariate analyses were included in the model. Circulating RANTES levels were entered in the model as the dependent variable, followed by the block of predictor variables: age; gender; parasitemia; Hb levels; TNF-α; IL-1β; IFN-γ; IL-10; total PCM/μL; platelets; monocytes; lymphocytes; and granulocytes. The overall model was significant (R=0.507, R^2=0.257, P=0.048) with PCM (standardized regression coefficient, β=-0.171, P=0.048) and IL-10 (β=-0.476, P<0.0001) independently associated with circulating RANTES. These analyses suggest that suppression of circulating RANTES occurs, at least in part, through acquisition of PCM and altered IL-10 production.

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3.8. Suppression of RANTES biosynthesis is due to endogenous production of IL-10

Based on the modeling results suggesting that IL-10 is closely associated with circulating RANTES, *in vitro* experiments examined the effect of IL-10 on RANTES production in PBMC isolated from children without [PCM(-), *n*=6] and with [PCM(+), *n*=6] monocytic *pf*Hz accumulation. PBMC were cultured in media alone or concomitant stimulation with LPS (100 ng/mL) and IFN-γ (200 U/mL) with either rhIL-10 (2.0 ng/mL) or IL-10 neutralizing antibodies (1.0 μg/mL), for 48 hrs. Culture conditions were based on our previous studies demonstrating that monocytic phagocytosis of *pf*Hz results in maximal inflammatory mediator dysregulation at 48 hrs [10,20]. As shown in Fig. 5, concomitant stimulation with LPS+IFN-γ increased RANTES production in both the PCM(-) and PCM(+) groups (P=0.027 and P=0.041, respectively). RANTES production in stimulated PBMC from the PCM(-) group was not significantly altered by blockade of endogenous IL-10 production (P=0.752, Fig. 5). However, RANTES production in stimulated cells from the PCM(+) group were significantly elevated (P=0.043) in the presence of IL-10 neutralizing antibodies (Fig. 5). Addition of exogenous rhIL-10 did not significantly decrease RANTES production in stimulated cells from the PCM (+) group (P=0.141). In contrast, rhIL-10 caused significant reductions (P=0.028) in RANTES biosynthesis in stimulated cells from the PCM(-) group (Fig. 5). Taken together, these results suggest that suppression of RANTES production in children with intramonocytic acquisition of *pf*Hz is due, at least in part, to endogenous IL-10 production.

4. Discussion

Our recent reports were the first to demonstrate that RANTES is suppressed in children with falciparum malaria [12,13]. Subsequent investigations revealed that reduced circulating RANTES in children with cerebral malaria is associated with enhanced mortality [21]. Results presented here extend previous studies by illustrating that naturally-acquired *pf*Hz by monocytes is, at least in part, an important mechanism through which RANTES levels are reduced in children with MA.

Stratification of children according to *pf*Hz accumulation in their monocytes revealed that circulating RANTES levels progressively declined with increasing PCM. In addition, presence of intramonocytic *pf*Hz was associated with significantly elevated rates of SMA. These results are consistent with our previous investigation illustrating that *pf*Hz deposition in monocytes is a significant predictor of SMA [11]. Findings presented here also support our previous study showing that circulating RANTES levels decrease with increasing severity of MA [13], and that circulating RANTES is suppressed in children with acute falciparum malaria relative to healthy, malaria-exposed controls [12]. Additional results presented here demonstrate that naturally-acquired *pf*Hz by monocytes is associated with reduced RANTES expression in cultured PBMC from children with MA under both baseline conditions and stimulated conditions. This finding parallels our previous *in vitro* study in malaria-naïve individuals showing that phagocytosis of hemozoin (*pf*Hz and synthetic Hz) by PBMC suppresses RANTES transcription and protein expression [12]. Thus, results presented here, and in our previous investigation, suggest that hemozoin deposition in monocytes may be an important mechanism for both reduced RANTES biosynthesis and enhanced severity of malarial anemia, at least in part, through suppression and/or reduced erythropoiesis.

The immunopathology of malaria infection involves interactions between a number of cell types important for both innate and adaptive immunity including monocytes, dendritic, γδ T and NK cells, and conventional and regulatory T cell activities [22-27]. Although a number of these cell populations, and their respective interactions, were not directly examined here due to limited blood volumes available from small, anemic children, our previous investigation showed that cytokine dysregulation in peripheral blood following acquisition of *pf*Hz by leukocytes occurs primarily through monocytes (i.e., CD14+ cell populations) [28]. In addition,

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we have previously demonstrated that phagocytosis of *pf*Hz by cultured PBMC and CD14+ cells causes dysregulation in cytokine (IL-10, IL-12, and TNF-α) and chemokine (MIF, MIP-1α, MIP-1β, and RANTES) production [10,12,28], suggesting that altered cytokine and chemokine profiles during a malaria infection are largely due to acquisition of hemozoin by monocytes.

To explore the potential mechanism(s) through which *pf*Hz suppresses RANTES, we examined a panel of pro- (i.e., TNF-α, IL-1β, and IFN-γ) and anti-inflammatory (i.e., IL-4, IL-10, and IL-13) cytokines known to regulate RANTES [14-17]. These studies demonstrated that children with elevated concentrations of PCM had reduced circulating IL-10 levels. The pattern of IL-10 production in children with MA in the current study is consistent with our previous investigation showing that PCM(+) children had decreased IL-10 levels [28]. These results also parallel our previous study in women with placental malaria showing that high levels of *pf*Hz in intervillous blood mononuclear cells (IVBMC) was associated with decreased IL-10 production [29]. Thus, high levels of intramonocytic *pf*Hz accumulation may render mononuclear cells anergic and/or refractory to IL-10 production. It is important to note that the relative expression of IL-10 in the environment will impact on RANTES production. Thus, even in the presence of low IL-10 levels, these reduced levels may be sufficient to suppress RANTES production. Data presented here in children with malarial anemia also parallel previous investigations in placental malaria illustrating a dose-dependent suppression of IFN-γ production in the presence of increasing intraleukocytic *pf*Hz-deposition [30]. Taken together, these studies and results presented here illustrate that there are both common and differing effects of naturally-acquired *pf*Hz on inflammatory cytokine pathways in various mononuclear cells.

After determining that TNF-α, IL-1β, IFN-γ, and IL-10 differed in children with varying amounts of intramonocytic *pf*Hz, a model was developed to determine the relationship between cytokine dysregulation and circulating RANTES. Stepwise linear regression analyses revealed that IL-10 and PCM were the only significant predictors of circulating RANTES levels. The negative *β*-weight for IL-10 in the analyses suggests that IL-10 may be responsible, at least in part, for decreased production of RANTES in children with MA. These results are consistent with the fact that IL-10 suppresses RANTES production from human monocytes [17].

Based on results obtained in the cytokine modeling, the effect of IL-10 on RANTES production was directly examined by culturing PBMC from children with MA stratified into two categories: PCM(-) and PCM(+). Children in the PCM(+) were not further stratified due to limited sample size. The *in vitro* experiments demonstrated that blockade of endogenous IL-10 in stimulated cells from PCM(+) children caused a significant increase in RANTES production, providing further evidence that IL-10 is an important mechanism for decreasing RANTES biosynthesis in pigmented mononuclear cells. In contrast, stimulated cells from children in the PCM(-) group produced very high levels of RANTES that were unaffected by IL-10 neutralizing antibodies, suggesting that LPS and IFN-γ stimulation induce maximal levels of RANTES in the absence of intramonocytic pigment that cannot be further augmented through blockade of IL-10. Additional experiments revealed that addition of exogenous IL-10 decreased stimulated RANTES production in both the PCM(-) and PCM(+) groups, although to a greater degree in the PCM(-) group. This finding appears to be due to the fact that intramonocytic *pf*Hz has a strong suppressive effect on RANTES production that cannot be further downregulated by exogenous IL-10 since RANTES levels are already substantially reduced. Taken together, the *in vitro* results suggest that suppression of RANTES following natural-acquisition of *pf*Hz by monocytes is mediated, at least in part, by IL-10.

The precise underlying molecular mechanism(s) by which IL-10 suppresses RANTES remains to be defined. IL-10 could potentially suppress RANTES production through inhibition of

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transcriptional factors and/or indirectly through suppression of RANTES-regulatory cytokines [16,17]. This hypothesis is supported by our previous results and those of others showing that Hz-induced up-regulation of IL-10 suppresses cytokine production and cellular responses of monocytes and PBMC [28]. Although not examined in the current studies, we have previously shown that TGF-β, an anti-inflammatory cytokine that decreases TNF-α-induced RANTES production in a manner similar to IL-10 [16], is suppressed in children with severe malaria [31], suggesting that TGF-β may not be a primary source of reduced RANTES in the cohort.

Previous studies demonstrated that altered production of erythropoietic mediators and acquisition of hemozoin by phagocytic cells are associated with enhanced pathogenesis of MA [8], suggesting that perturbations in hematopoietic cytokine production plays an important role in MA pathogenesis. We recently showed that reduced RANTES production in children with MA was associated with suppression of erythropoiesis [13]. These investigations further revealed that decreased RANTES was due, at least in part, to reduced numbers of platelets, an important source of circulating RANTES production [32]. Results presented here extend the previous findings by demonstrating that naturally-acquired intramonocytic *pf*Hz also suppresses RANTES biosynthesis through an IL-10-dependent mechanism, illustrating that there are, at least, several different mechanisms for decreased RANTES in children with MA: thrombocytopenia and monocytic phagocytosis of *pf*Hz. It is important to note that additional parasite-derived products, such as glycosylphosphatidylinositol and malarial antigens were not examined in the current study. Since these parasitic products can cause dysregulation in the inflammatory cascade [33], it is possible that these molecules may also contribute to altered RANTES production in children with MA. Subsequent studies investigating the impact of additional parasite-derived products and the molecular mechanism(s) through which phagocytosis of hemozoin generates altered cytokine and RANTES production may provide additional insights into the pathogenesis of MA.

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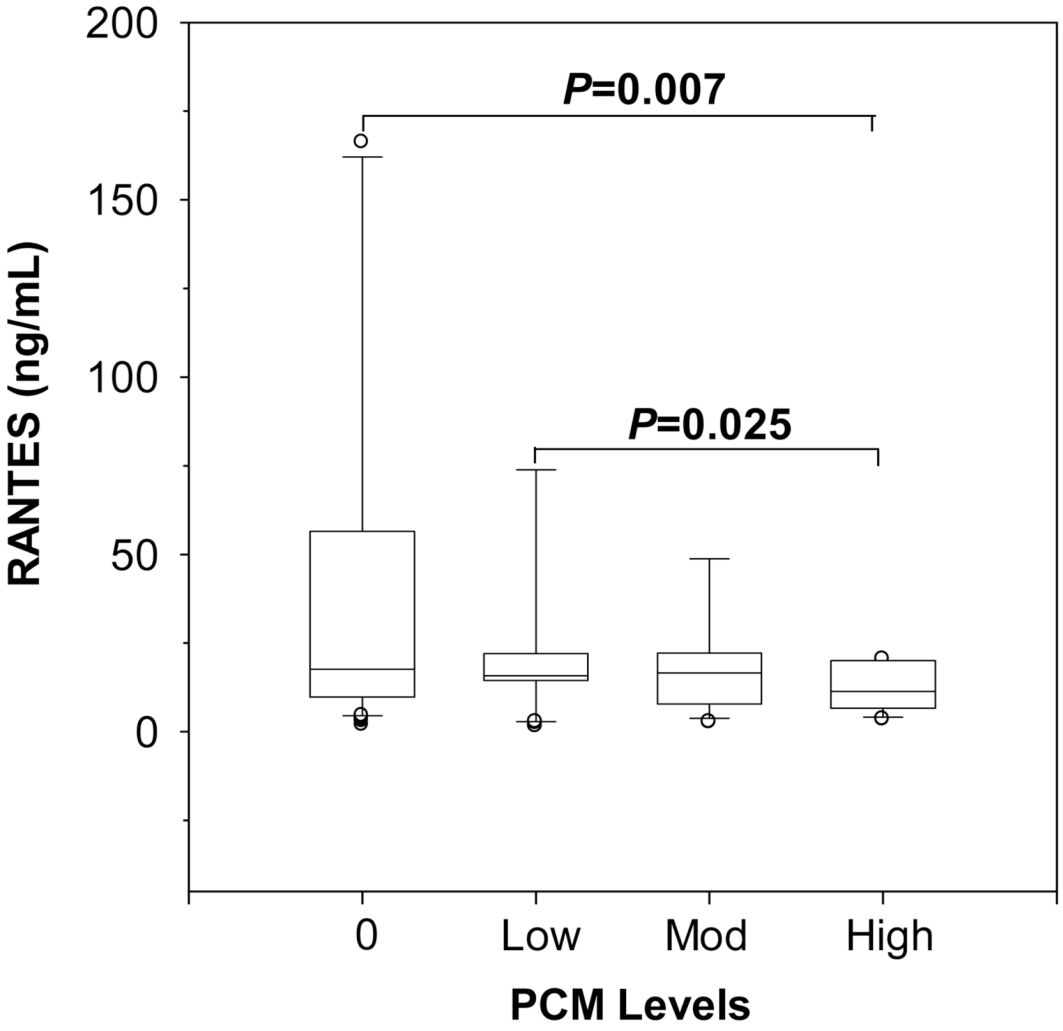

Figure 1. Circulating RANTES stratified according to intramonocytic *pf*Hz

Children with malarial anemia (Hb<11.0 g/dL) were stratified based on PCM levels: PCM(-) (0%, *n*=97); low PCM (≤10%, *n*=41); moderate (Mod) PCM (>10<26.7%, *n*=28); and high PCM (≥26.7%, *n*=28). Data are presented as box plots, where the box represents the interquartile range, the line through the box represents the median, whiskers indicate the 10^{th} and 90^{th} percentiles, and the open circles represent outliers. RANTES levels (ng/mL) decreased with PCM level (*P*=0.035, Kruskal-Wallis test), and were significantly lower in children with high PCM relative to the PCM(-) group (*P*=0.007, post-hoc Mann-Whitney *U*-test with the Bonferroni correction).

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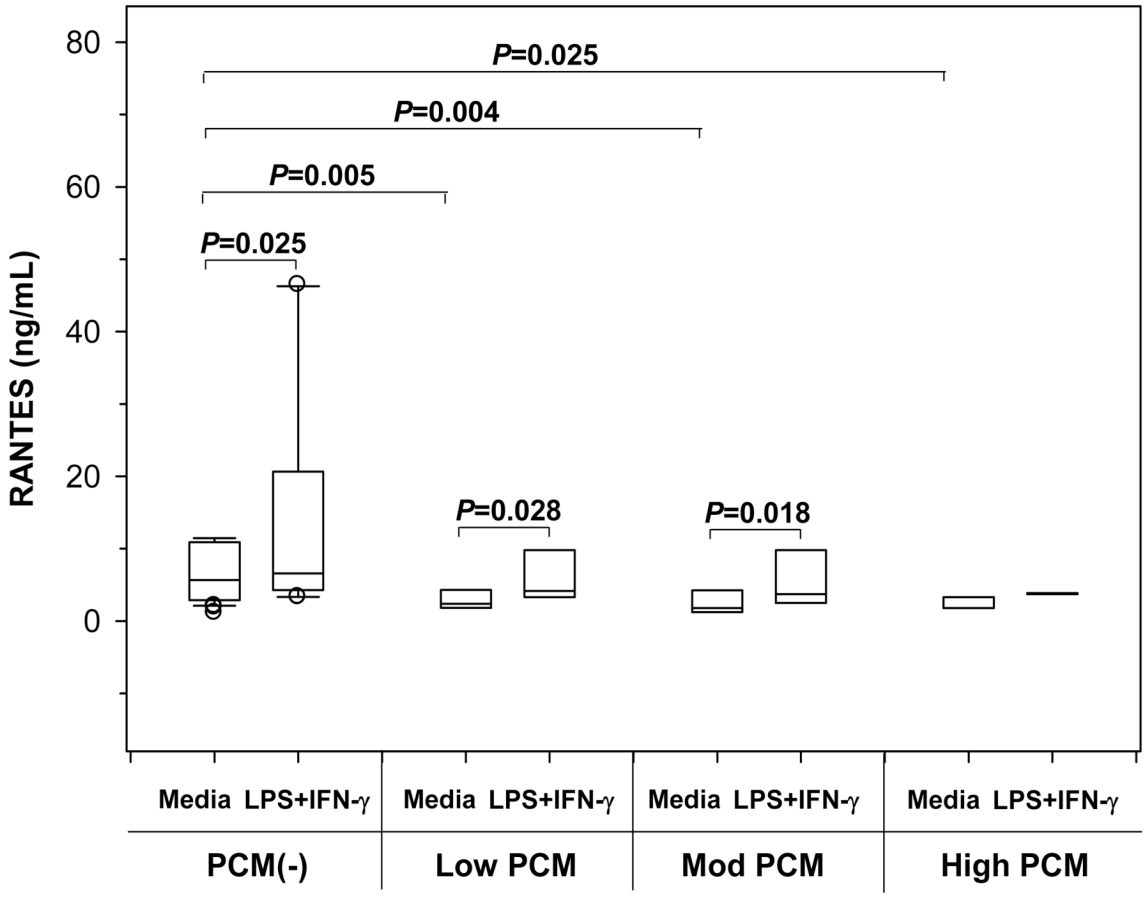

Figure 2. Effect of naturally-acquired *pf*Hz on PBMC RANTES production

RANTES synthesis (ng/mL) by PBMC from children with malarial anemia (Hb<11.0 g/dL) was stratified according to *pf*Hz-containing monocytes: [PCM(-), (0%, *n*=36)]; low PCM (≤10%, *n*=7); moderate (Mod) PCM (>10<26.7%, *n*=6); and high PCM (≥26.7%, *n*=3)]. PBMC were cultured at 1×10^6 cells/mL in media alone or stimulated with a combination of lipopolysaccharide (LPS, 100 ng/mL) and interferon (IFN)-γ (200 U/mL). Data are presented as box plots, where the box represents the interquartile range, the line through the box represents the median, whiskers indicate the 10^{th} and 90^{th} percentiles, and the open circles represent outliers. RANTES production under baseline (*P*=0.001, Kruskal-Wallis test) and stimulated (*P*=0.072, Kruskal-Wallis test) conditions decreased with increasing PCM level. Post-hoc Mann-Whitney *U*-test showed that baseline RANTES production was lower in the low (*P*=0.005), moderate (*P*=0.004), and high PCM (*P*=0.025) groups relative to the PCM(-) group. RANTES biosynthesis was higher in stimulated conditions in the PCM(-) (*P*=0.025, Wilcoxon rank sum test), low (*P*=0.028, Wilcoxon rank sum test), and moderate PCM (*P*=0.018, Wilcoxon rank sum test) groups.

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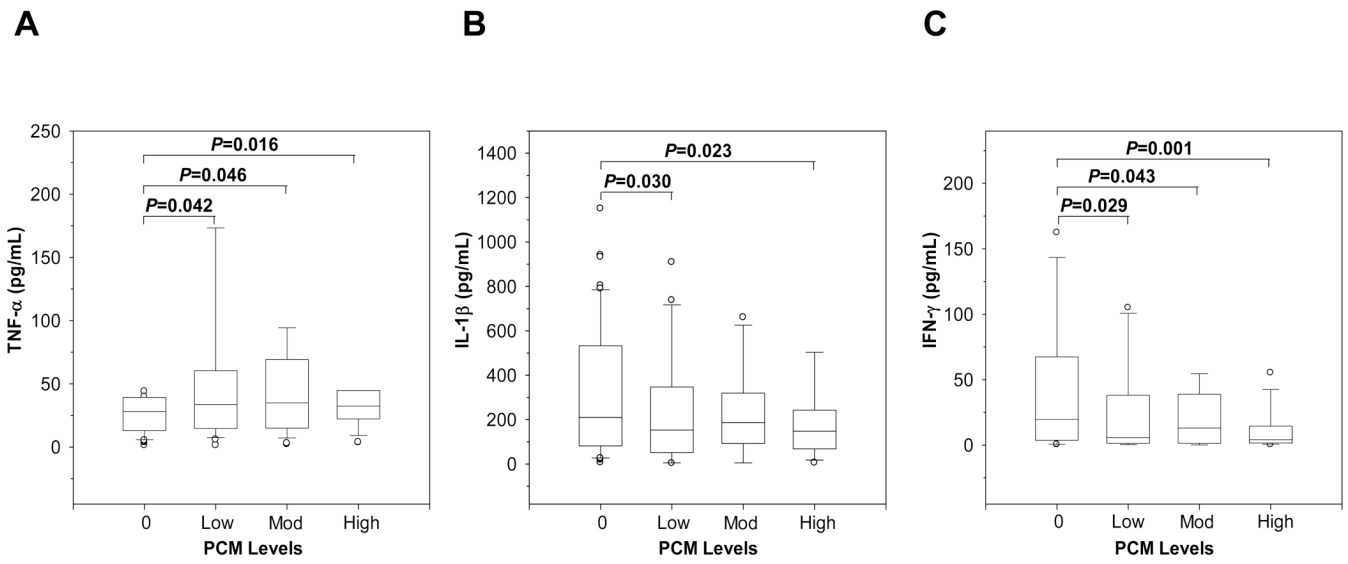

Figure 3. Circulating pro-inflammatory cytokine profiles in children with naturally-acquired *pf*Hz

Children with malarial anemia (Hb<11.0 g/dL) were stratified based on PCM level as follows: PCM(-) (0%, *n*=97); low PCM (≤10%, *n*=41); moderate (Mod) PCM (>10<26.7%, *n*=28); and high PCM (≥26.7%, *n*=28). Data are presented as box plots, where the box represents the interquartile range, the line through the box represents the median, whiskers indicate the 10th and 90th percentiles, and the open circles represent outliers. **(A)** Circulating TNF-α levels (pg/mL) in children with varying PCM levels differed across groups (*P*=0.029, Kruskal-Wallis test). **(B)** Circulating IL-1β concentrations (pg/mL) differed in children with increasing PCM levels (*P*=0.05, Kruskal-Wallis test). **(C)** Plasma IFN-γ levels (pg/mL) in children with different PCM levels. IFN-γ levels decreased gradually with increasing levels of PCM (*P*=0.003, Kruskal-Wallis test) and were significantly lower in the high PCM group compared to the PCM(-) group (*P*=0.001, post-hoc Mann-Whitney *U*-test with the Bonferroni correction).

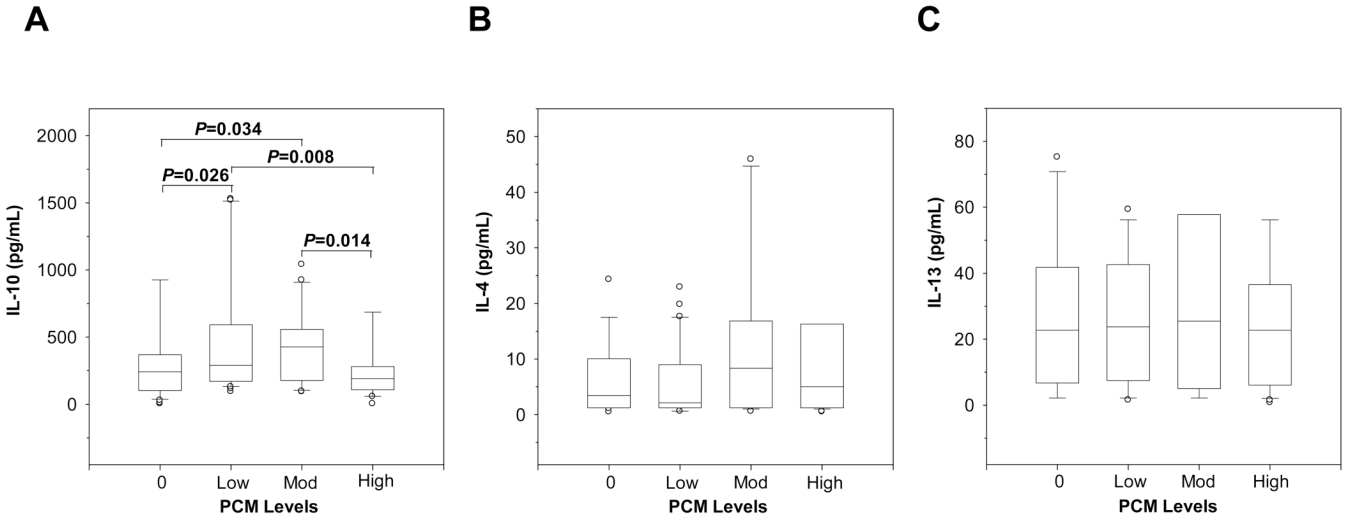

Figure 4. Circulating anti-inflammatory cytokine profiles in children with naturally-acquired *pf*Hz

Children with malarial anemia (Hb<11.0 g/dL) were stratified based on PCM level as follows: PCM(-) (0%, *n*=97); low PCM (≤10%, *n*=41); moderate PCM (>10<26.7%, *n*=28); and high PCM (≥26.7%, *n*=28). Data are presented as box plots, where the box represents the interquartile range, the line through the box represents the median, whiskers indicate the 10th and 90th percentiles, and the open circles represent outliers. **(A)** Plasma IL-10 levels (pg/mL) differed in children with varying PCM levels (*P*=0.010, Kruskal-Wallis test). IL-10 levels decreased in the high PCM group relative to the low (*P*=0.008, post-hoc Mann-Whitney *U*-test with the Bonferroni correction). **(B)** Plasma IL-4 (pg/mL) concentrations were not significantly different across the groups (*P*=0.568, Kruskal-Wallis test). **(C)** IL-13 levels (pg/mL) were also not significantly different across the groups (*P*=0.741, Kruskal-Wallis test).

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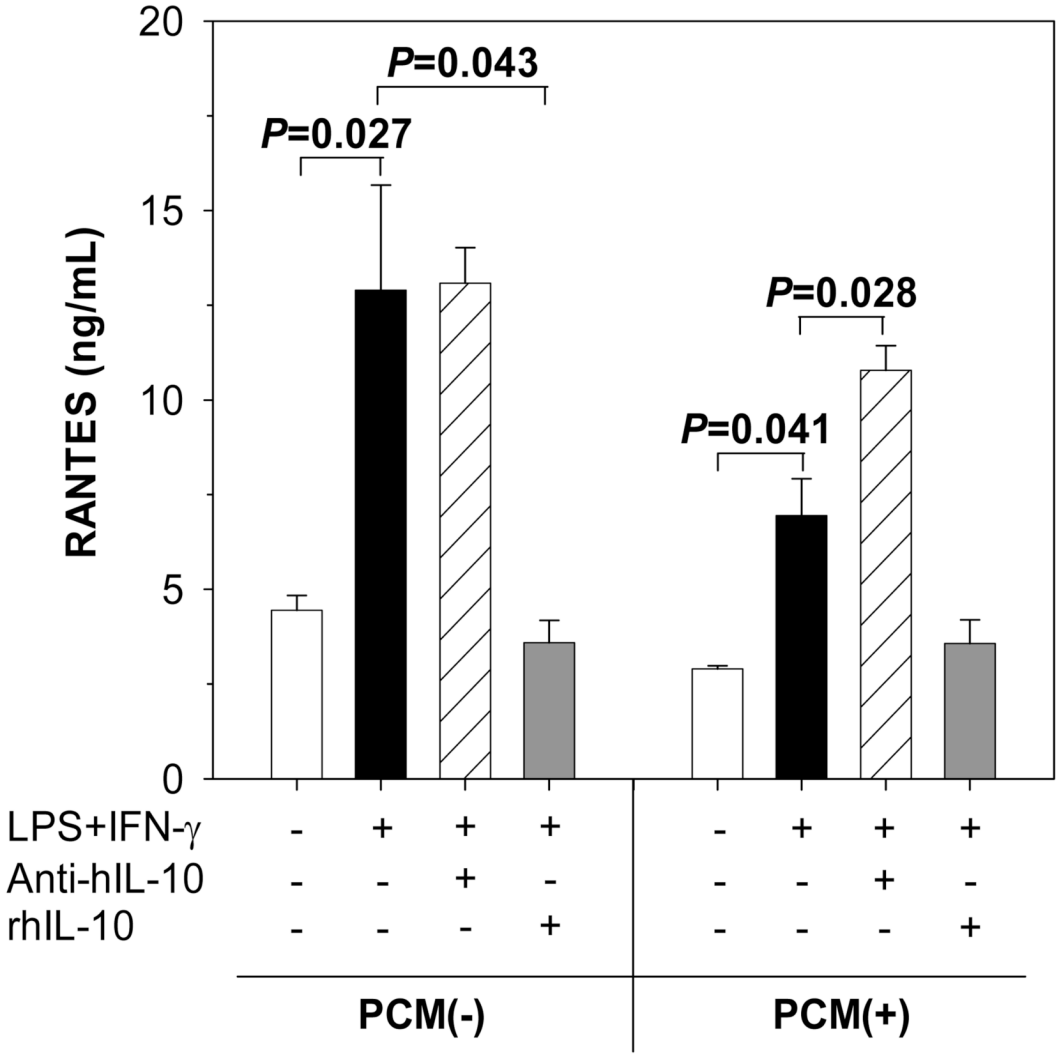

Figure 5. Effect of IL-10 on PBMC RANTES production

Effect of IL-10 on RANTES production in PBMC with PCM [PCM(+), range 6.7–23.3% (*n*=6)] and without PCM [PCM(-), 0%, *n*=6]. PBMC were cultured at 1×10^6 cells/mL in media alone or lipopolysaccharide (LPS, 100 ng/mL) and interferon (IFN)-γ (200 U/mL) in the presence of either recombinant human IL-10 protein (rhIL-10, 2.0 ng/mL) or IL-10 neutralizing antibodies (Anti-hIL-10, 1.0 μg/mL) for 48hrs. Data are shown as mean (SEM). RANTES biosynthesis in LPS- and IFN-γ-stimulated cells was higher in the PCM(-) and PCM(+) groups (*P*=0.027 and *P*=0.041, respectively, Wilcoxon rank sum test) relative to baseline conditions. Compared to cells stimulated with LPS and IFN-γ, exogenous IL-10 decreased RANTES production only in the PCM(-) group (*P*=0.043, Wilcoxon rank sum test), while IL-10 neutralizing antibody increased RANTES production in the PCM(+) group (*P*=0.028, Wilcoxon rank sum test).

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Table 1

Clinical and demographic characteristics of the study participants

Characteristic	PCM (%)				*P*
	PCM(-) (0%)	**Low PCM (≤10%)**	**Moderate PCM (>10<26.7%)**	**High PCM (≥26.7%)**	
No. of subjects	97	41	28	28	
Total PCM/μL	0	113 (44-264)	1258 (749-1950)[a]	6082 (3450-11747)[a,b,c]	**<0.0001**
Gender, male/female (%)	63/37	44/56	61/39	29/71	**0.006**
Age, months	10.0 (7.0-16.0)	10.0 (7.0-16.0)	8.0 (5.0-13.8)	8.0 (5.3-10.8)	0.059
Axillary temperature, °C	38.3 (36.8-38.8)	37.4 (36.8-38.4)	37.8 (36.7-38.2)	37.0 (36.3-38.0)	0.058
Glucose, mM	4.9 (4.4-5.5)	4.8 (4.6-5.5)	5.0 (4.4-5.9)	4.9 (4.2-5.7)	0.957
Parasites/μL	17,893 (7,216-46,967)	25,935 (9,660-56,839)	47,222 (14,850-101,745)	46,481 (10,774-122,583)	**0.011**
Geometric mean/µL	15,991	23,680	31,193	35,206	
HDP, n (%)	64 (66.0)	31 (75.6)	23 (82.1)	22 (78.6)	0.256
Lymphocytes ($\times10^3/\mu$L)	6.8 (4.0-7.4)	4.5 (3.6-7.5)	6.7 (5.1-8.1)	7.4 (5.5-9.1)[a,c]	**0.008**
Monocytes ($\times10^3/\mu$L)	1.0 (0.7-1.6)	0.9 (0.5-1.5)	1.2 (0.9-2.0)	1.2 (0.9-2.1)	0.090
Granulocytes ($\times10^3/\mu$L)	4.3 (3.2-5.4)	4.2 (3.1-5.6)	6.0 (2.8-9.2)	4.7 (3.3-7.8)	0.130
Hemoglobin, g/dL	7.50 (5.50-8.80)	5.50 (4.80-7.30)[c]	5.70 (5.03-8.0)	4.75 (4.03-5.60)[a,b,c]	**<0.0001**
SMA (Hb<6.0 g/dL), n(%)	27 (27.8)	29 (70.7)	17 (60.7)	26 (92.6)	**<0.0001**
RPI	1.02 (0.49-1.76)	1.30 (0.48-2.11)	0.84 (0.33-1.54)	0.73 (0.39-1.93)	0.380
ARN ($\times10^9$/L)	59.7 (31.0-102.8)	59.1 (25.7-102.8)	39.4 (16.3-84.2)	28.5 (17.7-87.1)[a]	**0.003**
Platelets ($\times10^3/\mu$L)	159 (108-231)	137 (92-203)	154 (104-194)	128 (109-216)	0.292
Thrombocytopenia, n (%)	41 (42.3)	24 (58.5)	13 (46.4)	18 (64.3)	0.115

Data are presented as median (Q1-Q3) unless otherwise indicated. Data analysis was performed by χ^2-test and, Kruskal-Wallis tests for across group differences, and post-hoc Mann-Whitney *U*-tests with Bonferroni correction for paired comparisons. PCM, pigment-containing monocytes; HDP, high-density parasitemia (≥10,000 parasites/μL); SMA, severe malarial anemia (Hb<6.0 g/dL); RPI, Reticulocyte production index; ARN, reticulocyte production number; Thrombocytopenia, platelets <150×$10^3/\mu$L;

[a] $P<0.00833$ vs. low PCM;

[b] $P<0.00833$ vs. moderate PCM;

[c] $P<0.00833$ vs. PCM(-).