Suppression of RANTES in children with *Plasmodium falciparum* malaria

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Severe malarial anemia (MA) is the primary manifestation of severe malaria among children in areas of holoendemic *Plasmodium falciparum* transmission. Although overproduction of inflammatory-derived cytokines are implicated in the immunopathogenesis of severe MA, chemokines such as regulated on activation, normal T-cell expressed and secreted (RANTES, CCL5) are largely unexplored in childhood malaria. We found that RANTES is decreased during severe MA ($p<0.01$), and associated with suppression of erythropoiesis ($p<0.05$) and malaria-induced thrombocytopenia ($p<0.05$). These findings suggest that thrombocytopenia may be a source of reduced RANTES which may contribute, at least in part, to suppression of erythropoiesis in children with malarial anemia.

Key words: RANTES, malaria, anemia, erythropoiesis, reticulocyte production index, thrombocytopenia.

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Malaria is a leading cause of childhood morbidity and mortality in sub-Saharan Africa.[1] In areas of holoendemic *Plasmodium falciparum* transmission, such as western Kenya, severe malarial anemia (SMA; hemoglobin, Hb<6.0 g/dL) is the most common clinical presentation of severe malaria in children less than 3 years of age, with cerebral malaria occurring only in rare cases.[2] Markedly reduced hemoglobin concentrations in children with severe MA occur as a result of hemolysis of parasitized and non-parasitized red blood cells (RBC), increased erythrophagocytosis, and suppression of erythropoiesis.[3] Although largely unexplored in human malaria, chemokines, such as regulated on activation, normal T-cell expressed and secreted (RANTES, CCL5), are important in regulating innate and adaptive immune responses.[4] RANTES is a member of the CC- (or β-) chemokine family and is produced by a variety of cell types, including macrophages, activated natural killer (NK) cells, T cells, and platelets.[5-7] Since RANTES regulates inflammation by promoting leukocyte activation, angiogenesis, antimicrobial effects, and hematopoiesis,[4] perturbations in RANTES production may affect host immune parameters and the erythropoietic response in blood-borne infections such as malaria.

Recent investigations from our laboratory demonstrated that circulating RANTES and peripheral blood mononuclear cell RANTES mRNA levels are reduced in Gabonese children with acute malaria (defined by hyperparasitemia and mild-to-moderate forms of anemia).[8] However, the role of RANTES in mediating *severe anemia* and bone marrow responses during acute malaria has not been explored. Defining the role of RANTES in severe MA is, therefore, important since RANTES can promote migration of erythroid precursors into hematopoietic tissues[9] and prevent apoptosis of erythroid progenitors,[10] suggesting that suppression of RANTES may lead to an ineffective erythropoietic response. As such, we investigated the relationship between circulating RANTES, malarial anemia, and suppression of erythropoiesis. The erythropoietic response was determined by calculating the reticulocyte production index (RPI), a standard measure of reticulocyte production that corrects for both the degree of anemia and the early release of reticulocytes from the bone marrow in anemic patients.[11] In addition, because malarial anemia is characterized by distinct hematological changes, a number of different cellular sources that could contribute to altered RANTES production were examined. To accomplish these experimental aims, children (n=106, age<36 months) were recruited at Siaya District Hospital, western Kenya, during their first hospital contact for the treatment of malaria. All samples were collected prior to administration of antimalarial drugs and/or supportive therapy. Written informed consent was obtained from the parents and/or guardians of all participating children prior to enrollment. The study was approved by the scientific and ethical review committees of the Kenya Medical Research Institute and the University of Pittsburgh. The demographic, clinical, and hematologic characteristics of children with varying severities of malarial anemia are summarized in Table 1. Since RANTES levels have not been reported in children with malarial anemia in holoendemic *P. falciparum* regions, plasma RANTES concentra-

Table 1. Demographic, clinical, and hematological characteristics of the study participants.

Characteristic	*Healthy controls*	*mild MA*	*moderate MA*	*severe MA*	*p*
No. of subjects	24	28	27	27	
Gender (male/female)	11/13	16/12	9/18	13/14	0.357
Age (months)	10.87 (1.63)	13.57 (1.45)	12.37 (1.14)	9.30 (0.85)	0.089
Axillary temperature (°C)	36.98 (0.18)	37.47 (0.20)	37.90 (0.22)*	37.59 (0.19)†	<0.05
Glucose (mM/L)	4.89 (0.17)	4.65 (0.18)	4.50 (0.22)	4.60 (0.23)	0.679
Parasite density (/μL)	0	34,183 (7,909)	37,771 (8,169)	38,874 (7,685)	0.578
Geometric mean (/μL)	0	13,927	21,402	20,655	
Hemoglobin (g/dL)	11.77 (0.14)	9.12 (0.09)§	7.18 (0.08)§	4.88 (0.11)§	<0.0001
RBC ($\times 10^6$/μL)	4.87 (0.08)	4.22 (0.13)§	3.45 (0.10)§	2.31 (0.09)§	<0.0001
WBC ($\times 10^3$/μL)	11.67 (0.99)	10.82 (0.75)	11.44 (0.75)	13.22 (1.14)	0.402
Lymphocytes ($\times 10^3$/μL)	7.18 (0.75)	5.13 (0.36)	5.36 (0.41)	7.07 (0.67)	0.082
Monocytes ($\times 10^3$/μL)	0.80 (0.07)	0.94 (0.10)	0.99 (0.10)	1.45 (0.17)*	<0.01
Granulocytes ($\times 10^3$/μL)	3.70 (0.37)	4.76 (0.65)	5.09 (0.39)*	4.71 (0.48)	<0.05
Platelets ($\times 10^3$/μL)	380.33 (32.15)	213.21 (30.58)§	219.48 (27.75)§	161.41 (11.50)§	<0.0001

Data are means (SEM) unless otherwise indicated. Multiple group comparisons were performed using the Kruskal-Wallis test and χ^2 test for proportions. Paired comparisons were performed by the Mann-Whitney U test. Study participants were categorized according to anemia status using the standard definition of anemia for children less than 5 years old in developing nations (Hb<11.0 g/dL).[17] Children with malarial anemia were stratified into anemia categories using results from a previous large-scale study examining the distribution of Hb concentrations in children less than 4 years of age in western Kenya.[18] The anemia in the study participants with P. falciparum parasitemia (any density) was classified as follows: severe, Hb<6.0 g/dL; moderate (6.0≤Hb<8.0 g/dL); and mild (8.0≤Hb<11.0 g/dL). Healthy controls (HC) were recruited at hospital during routine childhood vaccinations and defined by having a Hb≥11.0 g/dL, and being aparasitemic, and free of fever and/or diarrhea for the past 14 days. Giemsa-stained thin and thick venous blood smears were used to determine number and species of asexual Plasmodium parasites/300 WBC, with parasite density/1L calculated using the WBC count for each individual. Complete blood counts were determined in venipuncture blood (<3.0 mL) using a Coulter® AcT diff2™ (Beckman Coulter Corp.).
**Moderate and severe MA and SMA vs. HC, p<0.01; †severe MA vs. HC, p<0.05; §severe. moderate and mild MA vs. HC, p<0.0001. RBC: red blood cells; WBC: white blood cells.*

tions were compared across the clinical groups. As shown in Figure 1A, RANTES decreased with increasing malarial anemia severity (p<0.01), with the severe MA group having lower circulating RANTES than children with moderate MA (p<0.01), mild MA (p<0.01), or healthy controls (p<0.001).

Since RANTES regulates hematopoiesis,[9,10] and was reduced in children with malarial anemia, the relationships between RANTES, anemia status (Hb concentrations), and suppression of erythropoiesis (RPI) were examined. Prior to conducting inferential analyses, variables were examined for departures from univariate normality, and all non-normal variables were transformed toward normality. There was a significant association between RANTES and Hb (r=0.319, p<0.01). The mean (SEM) RPI for anemic children (Hb<11.0 g/dL) was 1.77 (0.29) in the mild MA group, 1.88 (0.23) in the moderate MA group, and 1.49 (0.18) in the severe A group. Furthermore, there was a significant relationship between circulating RANTES and the RPI (r=0.231, p<0.05). Since a RPI<2.0 is indicative of suppression of erythropoiesis, while a RPI ≥3.0 reflects appropriate erythropoietic responses during anemia,[11] children were stratified into these two categories. The percent of children with RPI<2.0 was 66% in the mild MA group, 60% in the moderate MA group, and 70% in the severe MA group, while those with a RPI ≥3.0 in MlMA, M*d*MA, and SMA groups was 7%, 15%, and 11%, respectively. Moreover, the levels of RANTES were significantly lower in children with suppression of erythropoiesis (RPI<2.0) than in those with an appropriate erythropoietic response (RPI ≥3.0, p<0.05, Figure 1B), demonstrating that decreased circulating RANTES is associated with suppression of erythropoiesis. To investigate the potential cellular source(s) of altered RANTES concentrations in children with malarial anemia, relationships between RANTES and numbers of lymphocytes, monocytes, granulocytes, and platelets were determined. There were no significant relationships between RANTES levels and lymphocytes, monocytes, or granulocytes (*data not presented*). However, decreasing RANTES levels paralleled the decline in platelet numbers with increasing severity of anemia (Figure 2A); there was a significant correlation between RANTES levels and platelet counts (r=0.504, p<0.001). Since previous investigations showed that childhood malaria is characterized by thrombocytopenia (platelets<150×10^3/μL),[12] children were stratified according to thrombocytopenia status. Consistent with studies demonstrating that platelets are a primary source of RANTES,[6,13] RANTES levels were significantly lower in children with platelet counts <150×10^3/μL (p<0.05, Figure 2B). Thus, malaria-induced thrombocytopenia may contribute, at least in part, to suppression of RANTES in children with malarial anemia.

Comprehensive examination of the relationship between RANTES and clinical and hematologic indices in children with malarial anemia (n=82) was performed by hierarchical multiple regression analysis with RANTES levels as the dependent variable, age as a covariate, and the following independent (or predictor) variables: temperature, parasitemia, Hb, RBC, RPI, lymphocytes, monocytes, granulocytes, and platelets. Age was entered first into the model followed by the block of predictor variables. The full regression model was statistically significant (F (10, 65)=4.27, p<0.001) with the entire set of variables (age plus predictors) accounting for 39.6% of the variance in RANTES production (R=0.629, R2=0.396). In addition, age (β=0.256, p<0.03) and two of the predictor variables, RBC

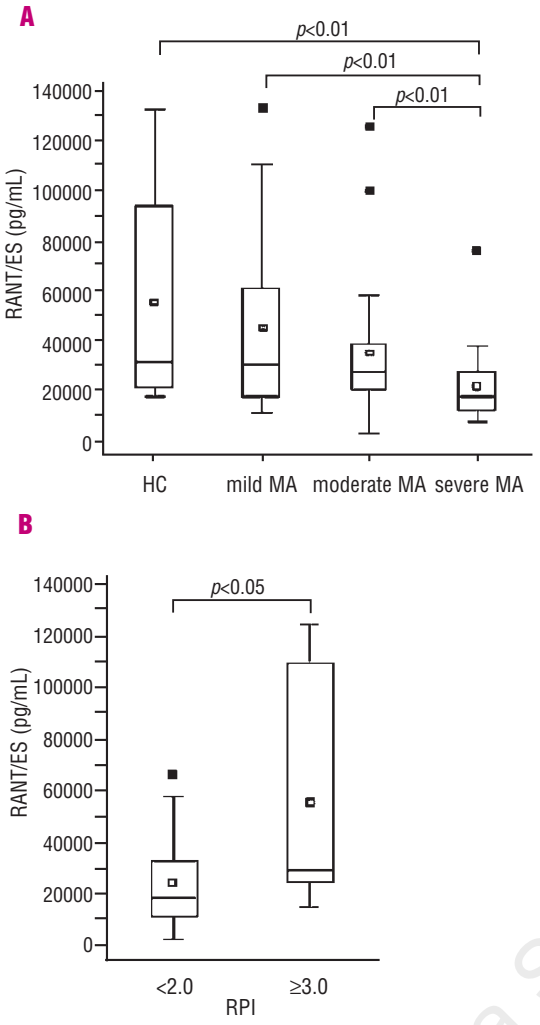

Figure 1. Association between RANTES, malarial anemia, and suppression of erythropoiesis. (A) Venipuncture blood (<3.0 mL) was collected and plasma was immediately obtained by spinning at 10,000 rpm. Plasma RANTES (pg/mL) was determined by ELISA for healthy controls (HC) (n=24), and patients with mild MA (n=28), moderate MA (n=27), and severe MA (n=27) according to the manufacturer's specifications (Human RANTES Cytoset™ kit, detection limit ≥15pg/mL; Biosource International). 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, solid circles represent outliers, and the open circles represent the mean. Statistical significance was determined by the Mann-Whitney U test. **denotes significant differences between groups ($p<0.01$, Kruskal Wallis test). (B) Relationship between RANTES concentrations (pg/mL) and reticulocyte production index (RPI) in children with malarial anemia; RPI<2.0 (n=39) indicates insufficient erythropoiesis, while RPI≥3.0 denotes an appropriate erythropoietic response (n=10).[11] Statistical significance was determined by the Mann-Whitney U test. RPI was calculated as follows: RPI=reticulocyte index (RI)/maturation factor (MF), where RI = [reticulocyte count (%) x hematocrit (Hct)/0.36]; and MF=b+(m)(x), where b=1, m=0.05, and x=(avg. normal population Hct - patient's Hct).(19) Although the standard Hct used to calculate the RPI in adults in western populations is 45%, the standard Hct used in our calculations was 36% since this value was appropriately age- and geographically-matched by calculating the average Hct value in a cohort of non-anemic (Hb>11.0 g/dL), aparasitemic children in western Kenya (n=107).

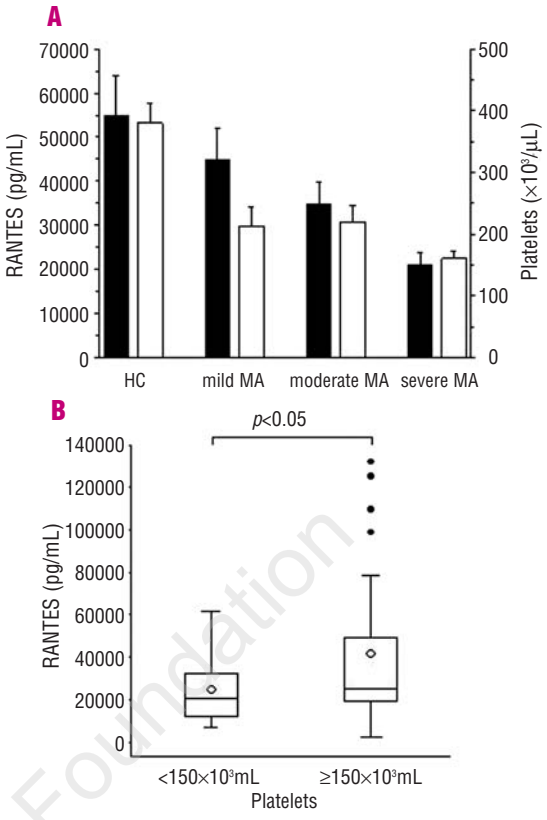

Figure 2. Relationship between RANTES and thrombocytopenia. (A) Association between RANTES levels (pg/mL) and platelet count ($\times 10^3/\mu L$) was determined for HC (n=24), and patients with mild MA (n=28), moderate MA (n=27), and severe MA (n=27). Data are presented as mean (SEM), closed bars indicate RANTES levels and the open bars represent platelets. (B) Relationship between RANTES concentrations and thrombocytopenia in children with malarial anemia. Thrombocytopenia was defined as platelets $<150\times10^3/\mu L$ (n=35), while non-thrombocytopenic children were classified as those with a platelet count $\geq150\times10^3/\mu L$ (n=47). Data are presented as box plots with statistical significance determined by the Mann-Whitney U test.

(β=0.456, $p<0.04$) and platelets (β=0.321, $p<0.01$) were significantly associated with RANTES levels. Squared semi-partial correlations revealed that the unique amount of variance in RANTES levels accounted for by age, RBC, and platelets was 6.6%, 4.2%, and 7.3%, respectively, suggesting that age, RBC numbers, and platelet counts are significant predictors of circulating RANTES concentrations.

Taken together, the results presented here demonstrate that circulating RANTES levels progressively decline in children with increasingly severe malarial anemia, and that suppression of RANTES is significantly associated with suppression of erythropoiesis and thrombocytopenia. The association between suppression of circulating RANTES and increasing severity of malarial anemia, along with the significant positive correlation between RANTES and Hb, and the RPI suggest that decreased production of RANTES may play a role in the development of malarial anemia by promoting suppression of erythropoiesis. This rationale is supported by our findings demonstrating that RANTES levels were significantly lower in children with erythropoietic suppression (RPI<2.0) than in those with an appropriate erythropoietic response (RPI ≥3.0). Although myelosuppression could account for reduced erythropoiesis,

results here showing that malaria was characterized by monocytosis and granulocytosis suggest that this mechanism does not explain the decreased RPI in our study participants. While not examined in the current study, additional mechanisms such as reduced RANTES from CD34+ progenitor cells may promote decreased erythropoiesis.[10] Results presented here in children with symptomatic malaria differ from those of previous studies in Kenyan children with asymptomatic malaria showing that the erythropoietic response is appropriate for the degree of anemia.[14] Since a decreased RPI could result from both ineffective erythropoiesis and suppression of erythropoiesis, additional studies are required to determine the mechanism(s) through which decreased RANTES may affect the erythropoietic response. Moreover, determination of the relationship between RANTES and soluble transferrin receptor (an indirect index of total erythropoiesis) may provide important information about the role of RANTES in mediating erythropoiesis in childhood malaria. Additional results demonstrating that circulating RANTES levels were significantly associated with platelet counts, and that thrombocytopenia was associated with lower RANTES levels, suggest that malaria-induced thrombocytopenia may contribute to decreased RANTES during acute malaria. Previous studies showing that children with severe MA have increased megakaryocytes in the presence of thrombocytopenia,[3] along with the present results demonstrating a significant positive relationship between RANTES and platelets, illustrate that decreased RANTES does not promote inadequate production of platelets, but rather decreased platelets may contribute to reduced RANTES. The hierarchical regression analysis examining the relationship between different hematologic and clinical indices and RANTES further illustrated that platelets were one of the strongest predictors of circulating RANTES concentrations. The findings presented here parallel those of previous investigations showing that thrombocytopenia is associated with decreased circulating RANTES in adults with sepsis.[15] However, our results differ from those of studies showing that RANTES transcripts and protein are elevated in brain tissue from children with cerebral malaria.[16] It remains to be determined why RANTES levels in circulation apparently differ from those found in brain tissue during childhood malaria. Additional investigations aimed at defining the role of RANTES in regulating malaria pathogenesis may offer important insight into the molecular mechanisms that govern the development and clinical outcomes of malaria.

TW: student who conducted the study, analyzed the data, and drafted the manuscript; JBH: conducted the statistical analyses; CO: involved in conducting the assays; ROO: involved in conducting the assays; ASSO: assisted in design of experiments and analysis of data; JMO: on-site supervisor who assisted in analyzing and interpreting data, and writing of the manuscript; JMV: assisted in study design and analysis; CCK: post-doctoral associate who assisted in analyzing and interpreting data, and writing of the manuscript; DJP: PI of the study, involved in experimental design, interpretation of data, and drafting of the manuscriptWe sincerely thank all the parents, guardians, and children who participated in this study.

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