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Suppression of a Novel Hematopoietic Mediator in Children with Severe Malarial Anemia▽†

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In areas of holoendemic *Plasmodium falciparum* transmission, severe malarial anemia (SMA) is a leading cause of pediatric morbidity and mortality. Although many soluble mediators regulate erythropoiesis, it is unclear how these factors contribute to development of SMA. Investigation of novel genes dysregulated in response to malarial pigment (hemozoin [PfHz]) revealed that stem cell growth factor (SCGF; also called C-type lectin domain family member 11A [CLEC11A]), a hematopoietic growth factor important for development of erythroid and myeloid progenitors, was one of the most differentially expressed genes. Additional experiments with cultured peripheral blood mononuclear cells (PBMCs) demonstrated that PfHz decreased SCGF/CLEC11A transcriptional expression in a time-dependent manner. Circulating SCGF levels were then determined for Kenyan children ($n = 90$; aged 3 to 36 months) presenting at a rural hospital with various severities of malarial anemia. SCGF levels in circulation ($P = 0.001$) and in cultured PBMCs ($P = 0.004$) were suppressed in children with SMA. Circulating SCGF also correlated positively with hemoglobin levels ($r = 0.241$; $P = 0.022$) and the reticulocyte production index (RPI) ($r = 0.280$; $P = 0.029$). In addition, SCGF was decreased in children with reduced erythropoiesis (RPI of <2) ($P < 0.001$) and in children with elevated levels of naturally acquired monocytic PfHz ($P = 0.019$). Thus, phagocytosis of PfHz promotes a decrease in SCGF gene products, which may contribute to reduced erythropoiesis in children with SMA.

In areas of holoendemic *Plasmodium falciparum* transmission, such as some regions of sub-Saharan Africa, malaria-induced anemia is a leading cause of morbidity and mortality. In infants and young children who lack developmentally acquired malarial immunity, severe malarial anemia (SMA) (hemoglobin [Hb] level, <6.0 g/dl) is a common hematological manifestation that results in mortality rates as high as 25 to 30% (6). The underlying causes of SMA are multifactorial and include both direct and indirect destruction of parasitized and nonparasitized red blood cells (RBCs), inefficient erythropoiesis, and dyserythropoiesis (1). Results from our laboratory also showed that Kenyan children with SMA have a reduced erythropoietic response (49).

Recovery from anemia is dependent on appropriate increases in erythropoiesis. One essential factor for enhanced erythropoiesis is erythropoietin (EPO), a 30-kDa hormone produced in the kidney, which promotes growth and differentiation of early erythropoietic cells in the bone marrow (22). Although systemic EPO levels are elevated in children with malarial anemia (7, 46), other reports have suggested that these increases are insufficient for the degree of anemia (8).

In addition to EPO, additional molecules, such as growth factors and cytokines, influence the erythropoietic response. Stem cell factor, interleukin-3 (IL-3), and IL-6 are known to enhance EPO-dependent increases in erythropoiesis both in vitro and in vivo (16). In human patients with acute malaria, serum levels of stem cell factor are not significantly different from those in uninfected controls, while IL-3 and IL-6 levels are higher during a malaria infection (9, 29, 42). In addition, although macrophage migration inhibitory factor knockout mice infected with *Plasmodium chabaudi* develop less severe anemia and have improved erythroid progenitor development (32), our investigations demonstrated that migration inhibitory factor is suppressed in children with SMA (4). Reduced erythropoietic responses in children with malaria therefore do not appear to result from insufficient production of these known erythropoietic factors.

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Human stem cell growth factor (SCGF), also known as C-type lectin domain family member 11A, is an important hematopoietic growth factor with burst-promoting activity for human bone marrow erythroid progenitors (20). The cDNA for human SCGF encodes a 29-kDa polypeptide (19) expressed primarily by myeloid cells and fibroblasts (20). Human SCGF-α is a protein of 323 amino acids, while SCGF-β is a 245-amino-acid protein resulting from cleavage of the conserved carbohydrate domain (35). SCGF-α and -β exert their effects at the early stages of hematopoiesis and promote growth of erythroid and myeloid colony formation (18, 20). In addition, elevated serum concentrations of SCGF are associated with enhanced hematopoietic recovery following stem cell transplantation (21). To date, no reports have described the role of SCGF in the hematological manifestations of malaria.

One common feature of falciparum malaria is the deposition of substantial concentrations of *P. falciparum*-derived hemozoin (PfHz; malarial pigment) in bone marrow macrophages, a process that may contribute to inefficient and/or inappropriate erythropoiesis (1). Recent studies illustrated that PfHz and PfHz-conditioned media suppress the maturation of erythroid precursors (11, 12). In addition, we have recently shown that high levels of PfHz deposition in monocytes are associated with an increased risk of developing SMA (4). These studies, along with our recent investigations with cultured peripheral blood mononuclear cells (PBMCs) demonstrating that PfHz elicits dysregulation of cytokines, chemokines, and effector molecules (4, 23–26, 38, 40, 49), suggest that acquisition of PfHz by monocytes/macrophages may promote malarial anemia by altering the production of soluble mediators.

To further explore potential pathogenic mechanisms of SMA, gene expression profiling was performed with pooled fractions of PBMCs stimulated with PfHz. Based on these results, additional in vitro investigations determined the impact of leukocytic acquisition of PfHz or β-hematin (synthetic hemozoin [sHz]) on the temporal kinetics of SCGF transcription. Circulating levels of SCGF were also examined in children with various degrees of *P. falciparum*-induced malarial anemia. Furthermore, the relationships between SCGF and Hb concentrations, reticulocyte production, and pigment-containing monocytes (PCM) were determined.

MATERIALS AND METHODS

Study area and participants. Children ($n = 90$; aged 3 to 36 months) were recruited at the pediatric ward in Siaya District Hospital, western Kenya, as part of an ongoing hospital-based longitudinal study examining the pathogenesis of SMA. This area is holoendemic for *P. falciparum* transmission, with the primary clinical manifestations of severe malaria in this study population being severe anemia and high-density parasitemia (HDP) (5, 33, 39). A complete description of the study area and malarial anemia in this population can be found in our previous report (39).

Prior to enrollment and after the parent or guardian of the child consented to the child's participation in the study, a questionnaire was used to collect relevant demographic and clinical information, including prior exposure to malaria. Based on this questionnaire and existing medical records, children visiting the hospital for their first malaria episode were recruited. Healthy children (HC) (Hb level of ≥11.0 g/dl and no parasitemia) were also recruited during their routine immunizations. Parasitemic children were stratified into the following three groups, based on Hb concentration: (i) uncomplicated malaria (UM) (Hb level of ≥11.0 g/dl), (ii) malarial anemia (MA) (11.0 g/dl > Hb level ≥ 6.0 g/dl), and (iii) SMA (Hb level of <6.0 g/dl). Based on a previous large-scale, longitudinal study examining the distribution of >14,000 Hb measurements in an age-matched and geographically matched reference population in western Kenya, SMA in children from this geographic location is best defined as a Hb level of <6.0 g/dl with parasitemia at any density (33). Children with human immunodeficiency virus type 1 (HIV-1) and cerebral malaria were excluded from the current study. Cerebral malaria was defined according to the World Health Organization definition (50), i.e., presence of an unarousable coma persisting for at least 30 min, inability to localize a painful stimulus, presence of peripheral asexual *P. falciparum* parasitemia, and no other identified causes of encephalopathy (e.g., bacterial or viral meningitis or toxic or metabolic encephalopathy). Pre- and posttest HIV counseling was provided, and informed consent was obtained from the parents and/or guardians of the participating children prior to enrollment.

Healthy, malaria-naïve adult donors ($n = 11$) were recruited from the University of Pittsburgh. This study was approved by the ethics committees of the University of Pittsburgh and University of New Mexico institutional review boards and the Kenya Medical Research Institute Ethical Review Board.

Laboratory procedures. Peripheral blood samples (<3.0 ml) were obtained prior to any treatment interventions, and appropriate supportive therapy was provided as required. Children were treated according to the guidelines of the Ministry of Health, Kenya. Peripheral blood smears were stained with 3% Giemsa stain, and trophozoites were counted against 300 leukocytes. Parasite density was estimated as follows: parasite density/μl = white blood cell (WBC) count/μl × number of trophozoites/300 leukocytes. Complete hematological parameters were determined with a Beckman Coulter AcT diff2 counter (Beckman Coulter Corporation). Reticulocyte counts were determined with new methylene blue staining of thin blood films. The reticulocyte production index (RPI) was calculated as described previously (49). The absolute reticulocyte number (ARN) was calculated as follows: ARN = [% reticulocytes × (RBC × 1,000)]/100. HIV-1 testing was performed according to our previously described methods (41).

Isolation and culture of PBMCs. PBMCs were purified from venous blood (<3.0 ml from Kenyan children [$n = 55$] and 40 ml from malaria-naïve U.S. adults [$n = 11$]) by use of Ficoll-Hypaque as described previously (48). Cells were plated at 1×10^6 cells per ml in Dulbecco's modified Eagle's medium containing 10% pooled human serum (heat inactivated at 56°C for 30 min). Cultures from Kenyan children ($n = 55$) were stimulated with medium alone, while cultures from healthy, malaria-naïve U.S. donors ($n = 6$) were cultured with medium alone in the absence and presence of PfHz (10 μg/ml) or sHz (10 μg/ml). For the membrane array studies (described below), PBMCs from healthy, malaria-naïve U.S. donors ($n = 5$) were stimulated with lipopolysaccharide (100 ng/ml; Alexis Corp.) and gamma interferon (200 U/ml; BD Pharmingen) in the absence or presence of PfHz (10 μg/ml). The concentration of PfHz used in the present studies is in the physiological range for children with severe malaria (10 μg/ml Hz), based on our previous results (25).

PfHz and sHz preparation. Crude PfHz was isolated from in vitro cultures of *P. falciparum*-infected RBCs as previously described (25). sHz was formed in a 4.5 M acidic acetate solution at pH 4.5 by the method of Egan (15). The final pellets of PfHz and sHz were dried, weighed, and resuspended in filter-sterilized H_2O at a final concentration of 1.0 mg/ml, followed by extensive sonication to disperse the preparations. Endotoxin levels in the PfHz and sHz preparations were <0.01 EU/ml by the *Limulus* amebocyte lysate test (BioWhittaker). In addition, cultures were routinely checked per the manufacturer's instructions (R&D Systems) to ensure that no mycoplasma contamination was present.

SCGF determination. Plasma and supernatant concentrations of SCGF were determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates were coated overnight at room temperature with an affinity-purified polyclonal antibody (goat anti-human) specific for SCGF-β (PeproTech Inc.), followed by blocking for 1 h and subsequent washing. Samples and standards (recombinant human SCGF-β; PeproTech Inc.) were incubated at room temperature for 2 h, washed, and incubated with a biotin-conjugated SCGF-β-specific detection antibody (PeproTech Inc.) for 2 h. Plates were washed and incubated with a horseradish peroxidase-streptavidin conjugate (ExtraAvidin; Sigma) and TMB substrate solution (Pharmingen) for 30 min while being protected from light. Absorbance was determined at 405 nm. The detection limit for SCGF was 0.2 ng/ml.

cDNA membrane array. Total RNA was isolated from cultured PBMCs (stimulated with PfHz for 48 h) by the guanidinium isothiocyanate method (13) and pooled to a total concentration of 2.0 mg/ml (0.4 mg/ml/individual). Gene expression profiles were determined using a human cDNA cytokine expression array (R&D Systems) according to the manufacturer's instructions. Briefly, total RNA was reverse transcribed in the presence of ^{33}P-labeled dCTP. Radiolabeled cDNA was purified on a Sephadex-G25 column and hybridized to a membrane overnight. Following hybridization, membranes were stringently washed and exposed to a phosphor screen for 24 h (Molecular Dynamics). Gene expression

was analyzed, and densitometry measurements were obtained for each gene. Densitometry readings for gene expression were determined using commercially available software (ArrayVision 7.0; Imaging Research Inc.). Gene expression was normalized to the expression of a panel of housekeeping genes (β_2-microglobulin, β-actin, cyclophilin A, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase, and L19 genes). The threshold of detection was set at 1,000 absorbance units, based on background densitometry readings. Genes containing expression levels below the threshold were not included in the data analyses.

Quantitative real-time RT-PCR. Total RNA was isolated from PBMCs by the guanidinium isothiocyanate method (13). Total RNA (1 μg) was reverse transcribed into cDNA, and cytokine gene expression was analyzed by quantitative real-time reverse transcription-PCR (RT-PCR) on an ABI Prism 7700 sequence detection system (Applied Biosystems). cDNA (100 ng) was amplified in duplicate with specific primer-probe sets for SCGF (GenBank accession number NM_002975) (Applied Biosystems). To control for nonspecific background fluorescence, no-template controls were included in triplicate. An endogenous control gene, the β-actin gene (GenBank accession number NM_001101) (Applied Biosystems), was used as a reference gene to normalize cDNA loading between samples. Data were compared using the $^{-\Delta\Delta}C_T$ method as previously described (25).

Statistical analyses. Plasma and supernatant concentrations of SCGF (ng/ml) were measured in triplicate at 1:5 and 1:10 dilutions. SCGF mRNA expression was measured in duplicate. Across-group comparisons were evaluated using the Kruskal-Wallis test, while pairwise comparisons between conditions, including pairwise tests of dose-dependent effects, were performed by the Mann-Whitney U test. Spearman's correlations were used to assess the association between circulating SCGF and hematological indices. *P* values of ≤0.05 were considered statistically significant.

RESULTS

Identification of novel gene expression profiles. To identify novel genes that are dysregulated in the context of malaria, a human cDNA cytokine expression array that recognizes 864 inflammatory-related genes was utilized. PBMCs from healthy, malaria-naïve U.S. donors ($n = 5$) were cultured in medium alone or with a physiologically relevant concentration of PfHz (10 μg/ml) (25). PBMCs were collected at 48 h, and mRNAs from the five individuals were isolated and pooled. The cDNA expression array revealed that SCGF was one of the most highly dysregulated genes. As shown in Fig. 1A, gene expression profiles of SCGF were >2.5-fold lower following stimulation with PfHz. The additional gene expression profiles can be found in the supplemental material.

Effect of PfHz acquisition on SCGF gene expression. To further examine the effect of PfHz acquisition by mononuclear cells on SCGF production, SCGF transcripts were quantified in cultured PBMCs stimulated with a physiologically relevant concentration of PfHz or sHz (10 μg/ml) (25), and SCGF transcripts were temporally determined over 48 h by real-time RT-PCR. Both PfHz and sHz significantly suppressed SCGF transcripts at 2, 8, 24, and 48 h ($P < 0.05$) (Fig. 1B), confirming the results obtained in the gene expression profiling experiments. Taken together, the results presented here show that PfHz is responsible, at least in part, for suppression of PBMC SCGF gene expression.

Patient characteristics. To determine if SCGF played a role in mediating hematological outcomes in children with falciparum malaria, a series of experiments were then performed at our study site in western Kenya. The demographic, parasitological, and hematological characteristics of the Kenyan study participants are listed in Table 1. Although ages were not statistically different across the groups ($P = 0.091$), children with SMA were relatively younger. Gender distribution was

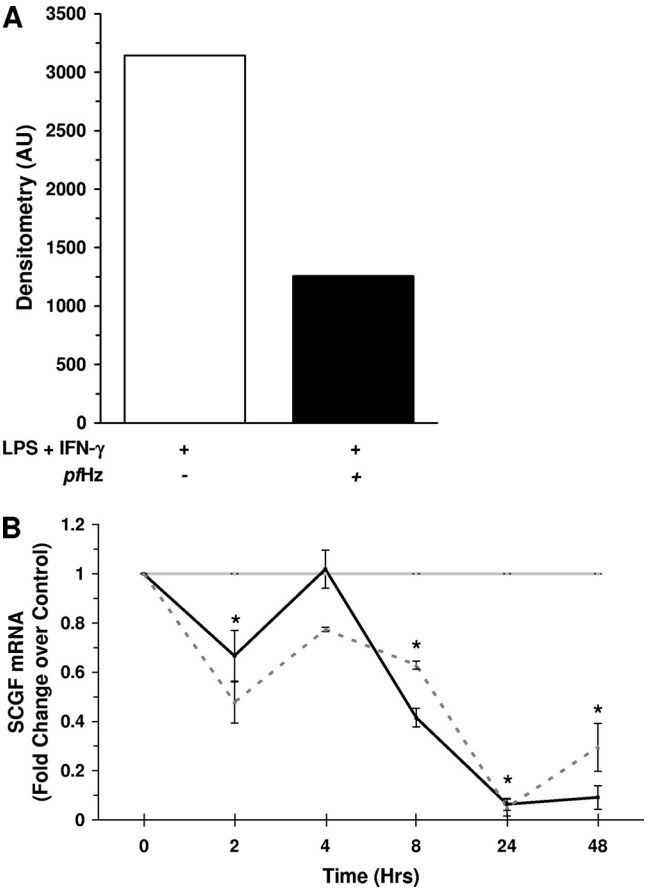

FIG. 1. Effect of PfHz on SCGF gene expression. (A) Gene expression profiles of SCGF in PBMCs (1×10^6 cells/ml) from healthy, malaria-naïve U.S. donors ($n = 5$) stimulated with PfHz (10 μg/ml). Gene expression profiles were determined for pooled samples from five individuals by use of a cDNA membrane array. Densitometry readings (arbitrary units [AU]) were obtained, and data were normalized to a panel of housekeeping genes. Values are presented as means. IFN-γ, gamma interferon. (B) Temporal effect of PfHz and sHz on SCGF transcriptional expression in cultured PBMCs from healthy, malaria-naïve U.S. donors ($n = 6$). SCGF mRNA was quantified by real-time RT-PCR. PBMCs (1×10^6 cells/ml) were cultured in medium alone (solid gray line; controls) and following stimulation with PfHz (10 μg/ml; solid black line) or sHz (10 μg/ml; dashed gray line) for 48 h. Values are presented as means ± standard errors of the means. Statistical significance was determined by Student's *t* test. *, $P < 0.05$ compared to unstimulated conditions.

not significantly different across the groups ($P = 0.836$). In addition, parasitemia and the proportion of children with HDP were not significantly different across the parasitemic groups ($P = 0.103$ and $P = 0.076$, respectively). As expected based on the clinical groupings, RBC numbers and Hb levels were significantly different across the groups ($P < 0.0001$ for both variables). In addition, ARN differed nonsignificantly across the groups ($P = 0.063$). Evaluation of leukocyte counts revealed that WBC numbers were not significantly different between the groups ($P = 0.126$). However, the numbers of circulating lymphocytes, monocytes, and granulocytes differed significantly across the groups ($P < 0.001$, $P < 0.001$, and $P < 0.0001$, respectively).

TABLE 1. Demographic, parasitological, and hematological characteristics of study population[a]

Parameter	Value for group				*P* value
	HC (n = 18)	UM (n = 11)	MA (n = 45)	SMA (n = 16)	
Age (mo)	12.00 (11.00)	12.00 (8.0)	11.00 (10.5)	7.00 (4.0)	0.091[b]
Gender					
Male (n [%])	7 (38.9)	5 (45.5)	23 (51.1)	7 (43.8)	0.836[c]
Female (n [%])	11 (61.1)	6 (54.5)	22 (48.9)	9 (56.2)	
Parasitemia (parasites/μl)	0	49,662 (82,823)	15,165 (30,104)	22,014 (25,017)	0.103[b]
HDP (n [%])	0	9 (81.8)	27 (60.0)	14 (87.5)	0.076[c]
Erythrocyte indices					
Hb concn (g/dl)	11.65 (0.9)	11.10 (0.9)	7.80 (1.8)	4.95 (0.8)	<0.0001[b]
RBCs (10^6/μl)	4.83 (0.3)	4.54 (0.9)	3.74 (0.9)	2.16 (0.7)	<0.0001[b]
ARN (10^9/liter)	134.37 (52.3)	110.11 (173.5)	108.69 (123.8)	58.52 (81.5)	0.063[b]
Leukocyte counts					
WBCs (10^3/μl)	10.20 (5.3)	12.20 (6.6)	10.30 (4.2)	13.00 (8.5)	0.126[b]
LY (10^3/μl)	6.30 (5.3)	4.30 (1.8)	4.90 (2.2)	6.95 (4.0)	<0.001[b]
MO (10^3/μl)	0.80 (0.5)	0.60 (0.3)	0.80 (0.6)	1.30 (1.3)	<0.001[b]
GR (10^3/μl)	3.05 (1.3)	6.10 (5.6)	4.80 (2.3)	4.60 (3.6)	<0.0001[b]

[a] Data are median values (interquartile ranges) unless otherwise noted. Study participants were categorized as described in Materials and Methods. For parasitemia levels and proportions of children with HDP (≥10,000 parasites/μl), statistical analyses were determined only for the parasitemic groups. LY, lymphocytes; MO, monocytes; GR, granulocytes.

[b] Statistical significance was determined by Kruskal-Wallis test.

[c] Statistical significance was determined by chi-square analysis.

SCGF production in children with malaria. To determine if SCGF production was associated with malaria disease severity, circulating SCGF levels were examined in the clinical groups. As shown in Fig. 2A, SCGF levels differed across the groups with various degrees of anemia (P = 0.013) (Fig. 2A). Further analyses revealed that SCGF was reduced in children with SMA relative to that in the HC and UM groups (P = 0.001 and P = 0.034, respectively), with a borderline difference with the MA group (P = 0.057) (Fig. 2A).

Additional experiments were performed to examine the potential source of decreased SCGF in the circulation. Since our previous studies have shown that PBMCs are an important source of circulating cytokines, chemokines, and effector molecules in children with malaria (4, 23–26, 38, 40, 49), PBMCs were isolated from children in the HC (n = 6), MA (n = 37), and SMA (n = 12) groups and cultured for 48 h. SCGF production in cultured PBMCs differed across the clinical groups (P = 0.016) (Fig. 2B). PBMCs from children with MA and SMA had decreased SCGF production relative to those from the HC group (P = 0.067 and P = 0.004, respectively) (Fig. 2B). Although the difference was not statistically significant (P = 0.102), SCGF levels were also lower in the SMA group than in the MA group (Fig. 2B). The findings presented here support the notion that PBMCs are an important source of circulating SCGF in children with malaria.

Association between SCGF, anemia, and reticulocyte production. To further examine the association between SCGF and malarial anemia severity, the relationship between circulating SCGF and Hb concentrations was determined for the entire population (n = 90). SCGF was positively associated with the Hb concentration (r = 0.241; P = 0.022) (Fig. 3A). Since previous in vitro studies showed that SCGF augments erythroid formation (20), the relationship between circulating SCGF and the RPI was determined for children with anemia (Hb level, <11.0 g/dl; n = 61). The RPI for anemic individuals (Hb level, <11.0 g/dl) is an effective measure of the erythropoietic response: an RPI of ≥3.0 is associated with an appropriate erythropoietic response, while an RPI of <2 indicates suppression of erythropoiesis (27). Circulating SCGF was positively correlated with the RPI (r = 0.280; P = 0.029) (Fig. 3B). To determine if reduced circulating SCGF concentrations were associated with suppression of erythropoiesis (RPI of <2), parasitemic children with anemia (n = 61) were stratified into two categories, those with an RPI of <2 and those with an RPI of ≥3.0. Children with an RPI of <2 had reduced plasma SCGF (P < 0.0001) (Fig. 3C). These results illustrate that reduced plasma concentrations of SCGF are associated with decreased Hb concentrations and a reduced erythropoietic response in children with malarial anemia.

Effect of in vivo acquisition of PfHz on circulating SCGF. Our previous studies (4, 23–26, 38, 40, 49) as well as those of others (17) showed that PfHz is an important parasitic product that alters inflammatory mediator gene expression profiles in children with malaria and in cultured PBMCs. Since SCGF production progressively declined with increasing malaria severity and PBMCs were an important source of SCGF production, we postulated that acquisition of PfHz by monocytes may be responsible, at least in part, for decreased SCGF production in children with malaria. Stratification of children according to the percentage of PCM (i.e., none [0%], low [<10%], and high [≥10%]) revealed that SCGF production progressively declined with increasing monocytic PfHz deposition (P = 0.031) (Fig. 4). Further analyses revealed that children in the no-PCM group had higher circulating SCGF concentrations than did children in the low- and high-PCM groups (P = 0.036 and P = 0.019, respectively) (Fig. 4), demonstrating that reduced SCGF production in children with malaria is associated with monocytic ingestion of PfHz.

DISCUSSION

Our efforts aimed at identifying novel patterns of gene expression by use of a human cDNA cytokine expression array revealed that SCGF was one of the most significantly dysregu-

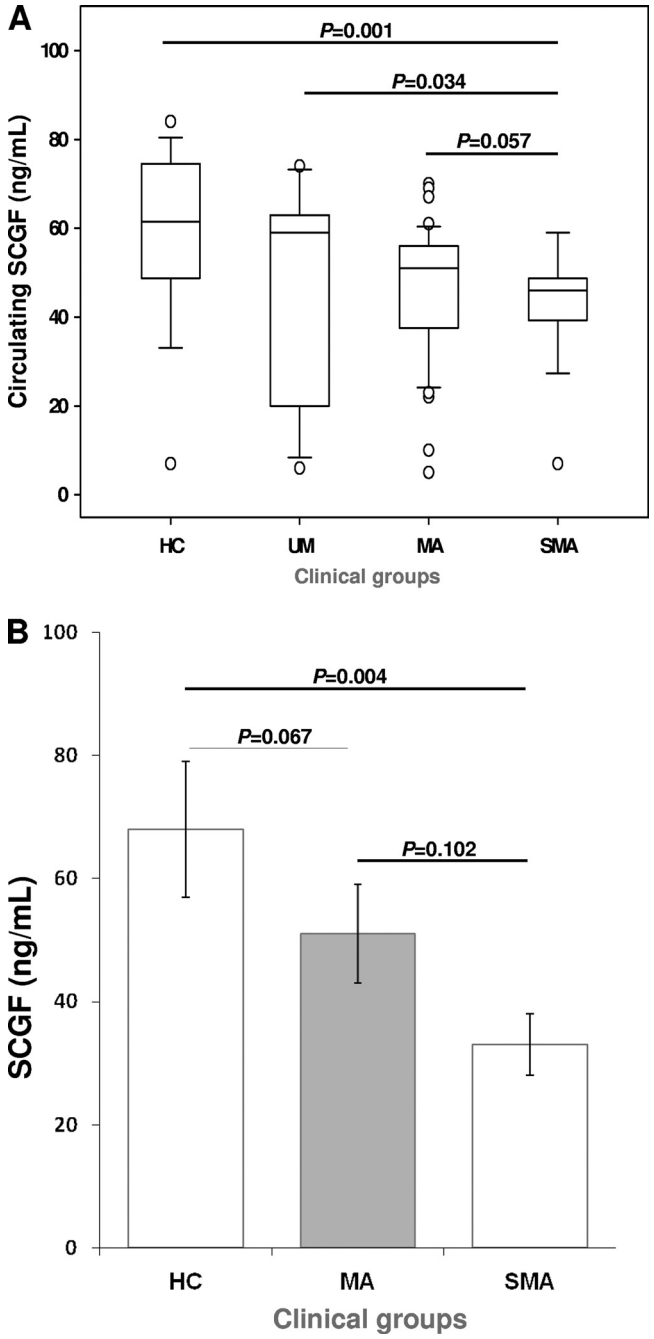

FIG. 2. SCGF production in children with malaria. Plasma was obtained from healthy, aparasitemic children and children with acute malaria, and SCGF concentrations were determined by ELISA. (A) Children were categorized according to Hb concentrations, as defined in Materials and Methods. Clinical groups were HC ($n = 18$), UM ($n = 11$), MA ($n = 45$), and SMA ($n = 16$). Each box represents the interquartile range, the line through the box is the median, whiskers show the 10th and 90th percentiles, and circles are outliers. Statistical significance was determined by the Kruskal-Wallis test, followed by post hoc comparisons using the Mann-Whitney U test. (B) PBMCs were isolated from peripheral blood (<3.0 ml) of children with various degrees of malarial anemia and cultured (1×10^6 cells/ml) under baseline conditions. Children were categorized according to Hb concentrations, as defined in Materials and Methods. Clinical groups were HC ($n = 6$), MA ($n = 37$), and SMA ($n = 12$). Supernatants were obtained at 48 h for SCGF determination by ELISA. Data are presented as means ± standard errors of the means. Statistical significance was determined by Student's t test.

lated genes in cultured PBMCs from healthy U.S. donors stimulated with PfHz. Based on these results, SCGF transcript expression was examined temporally in PBMCs from healthy, malaria-naïve U.S. blood donors by real time RT-PCR. Both PfHz and sHz suppressed SCGF gene expression over 48 h in culture, confirming the findings of the gene expression profiling experiments. These results are consistent with our previous studies (4, 23–26, 38, 40, 49), as well as those of others (17), demonstrating that ingestion of PfHz by monocytes results in dysregulation of host-derived cytokines, chemokines, and effector molecules.

Since profiles of inflammatory mediators generated in cultured PBMCs stimulated with PfHz closely resemble patterns of inflammatory mediators known to promote enhanced anemia in children with malaria (4, 23–26, 38, 40, 49), we hypothesized that SCGF may be altered in children with malaria and that perturbations in SCGF may be associated with distinct hematological outcomes. The findings presented here for a cohort of children of less than 3 years of age from an area of holoendemic *P. falciparum* transmission in western Kenya where the primary clinical outcome of severe malaria is SMA demonstrate that circulating SCGF concentrations progressively decline with increasing severity of anemia and are lowest in children with SMA. Consistent with this finding, plasma SCGF levels were positively correlated with Hb levels. In addition, cultures of PBMCs from children with various degrees of malarial anemia showed that SCGF production decreased with increasing severity of anemia, further suggesting that PBMCs may be an important source of circulating SCGF in children with malaria. These findings, in the context of previous investigations showing that SCGF promotes the growth of erythroid and myeloid colonies (20), suggest that reduced SCGF production may contribute to the anemia observed in the cohort.

Causes of anemia in malaria-infected children are multifactorial and likely differ according to the degree of malarial immunity, based largely on *P. falciparum* endemicity (31, 39, 45). However, reduced erythropoiesis in acute malaria has been documented for more than 60 years (47). Previous studies of Thai adults with malaria showed that low reticulocyte counts were accompanied by a suppression of erythropoiesis (10). Additional evidence for suppression of erythropoiesis comes from investigations conducted with bone marrow aspirates from Gambian children demonstrating that individuals with acute malaria do not have a significantly larger number of total erythroblasts than uninfected controls, a requirement for meeting the physiological demand of anemia (1, 2). Since our previous studies of children with malarial anemia from this region showed that children with SMA have a reduced erythropoietic response (as evidenced by an RPI of <2), the relationship between circulating SCGF and the RPI was investigated. These analyses revealed a significant positive correlation between SCGF and the RPI. In addition, stratification of malaria-infected children according to their RPI scores (i.e., RPIs of <2 versus RPIs of ≥3) showed that individuals with an RPI of <2 had lower peripheral SCGF concentrations. The positive association between SCGF and RPI, as well as reduced SCGF production in children with RPIs of <2, illustrates that decreased SCGF production may contribute to suppression of the erythropoietic response in children with malaria.

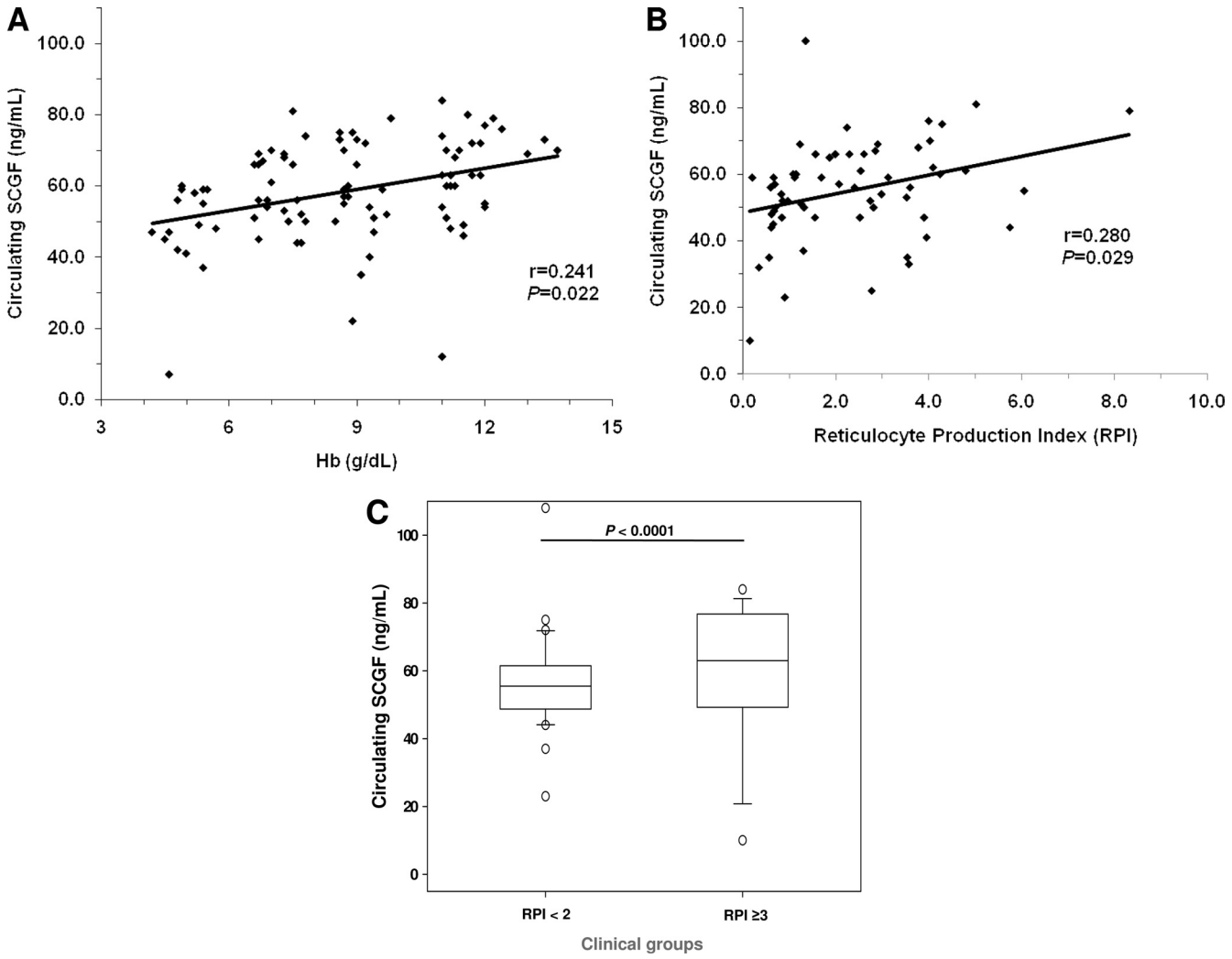

FIG. 3. Association of circulating SCGF with anemia outcomes. Plasma was obtained from healthy, aparasitemic children and children with acute malaria, and SCGF concentrations were determined by ELISA. (A) Correlation between SCGF concentrations and Hb levels for all clinical groups ($n = 90$). (B) Association between SCGF and RPI for anemic children ($n = 61$). Relationships between SCGF, Hb, and RPI were determined by Spearman's correlation coefficient. (C) Circulating SCGF levels in anemic children ($n = 48$) were stratified according to RPI (RPIs of <2 versus RPIs of ≥ 3). Each box represents the interquartile range, the line through the box is the median, whiskers show 10th and 90th percentiles, and circles are outliers. Statistical significance was determined by the Mann-Whitney U test.

Our previous investigations in western Kenya demonstrated that elevated levels of PCM were associated with SMA and that PCM levels were a better predictor of Hb concentrations than was peripheral parasite density (4). These studies support previous reports from areas with lower degrees of malaria endemicity than western Kenya showing that acquisition of PfHz by monocytes is a better index of malaria disease severity than parasitemia is (3, 11, 30, 34, 37). Phagocytosis of PfHz by leukocytes appears to be an important mechanism for suppression of the erythropoietic cascade. For example, PfHz causes the release of 15(*S*)-hydroxyeicosatetraenoic acid and 4-hydroxy-nonenal from monocytes, which can lead to impairment of erythroid growth (43, 44). PfHz may also impact erythropoiesis through dysregulation of cytokines (reviewed in reference 28). In vitro studies in our laboratory showing that PfHz promotes decreased IL-12 and increased tumor necrosis factor alpha (TNF-α) production from cultured PBMCs parallel in vivo findings in which elevated PCM levels are associated with suppressed circulating IL-12 and enhanced TNF-α concentrations in children with malarial anemia (26). Since IL-12 promotes enhanced erythroid growth (36), while TNF-α can directly suppress all stages of erythropoiesis (14), PfHz-induced perturbations in cytokine production appear to be an important causal factor for promoting malarial anemia. The findings presented here showing that SCGF was negatively correlated with the amount of PfHz phagocytosed by monocytes (i.e., PCM) and that stimulation of PBMCs with PfHz decreased SCGF transcript expression support this hypothesis and suggest that PfHz may impair the erythropoietic response through an additional soluble mechanism, i.e., suppression of SCGF production.

Taken together, the results presented here demonstrate that naturally acquired PfHz decreases mononuclear cell SCGF production and that reduced SCGF may contribute to the

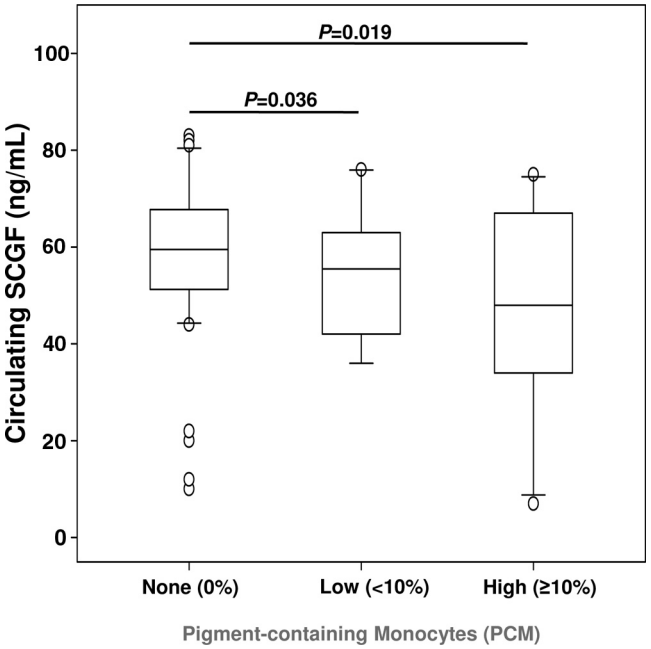

FIG. 4. Circulating SCGF levels in children with various levels of intramonocytic PfHz. Circulating concentrations of SCGF were determined for children with various degrees of malarial anemia and stratified according to the percentage of PCM (none [0%], $n = 52$; low [≤10%], $n = 13$; and high [>10%], $n = 7$). Each box represents the interquartile range, the line through the box is the median, whiskers show 10th and 90th percentiles, and circles are outliers. Statistical significance was determined by the Kruskal-Wallis test, followed by post hoc comparisons using the Mann-Whitney U test.

development of SMA. In this study, SCGF levels were decreased in children with SMA, with the levels and correlations showing variability, pointing to potential complex interactions between immune mediator levels, immune responses, and the pathogenesis of SMA. Future studies directly examining the effects of SCGF on bone marrow progenitor cells from children with malarial anemia may provide additional insight into the direct role of SCGF on the erythropoietic cascade. At present, it remains to be determined if SCGF is also reduced during other clinical manifestations of severe malaria, such as cerebral malaria.

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