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Increased Circulating Interleukin (IL)-23 in Children with Malarial Anemia:

In vivo and *In vitro* Relationship with Co-regulatory Cytokines IL-12 and IL-10

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Abstract

Severe malarial anemia (SMA) is a leading cause of mortality among children in sub-Saharan Africa. Although the novel cytokine, interleukin (IL)-23, promotes anemia in chronic inflammatory diseases, the role of IL-23 in SMA remains undefined. Since IL-23 and IL-12 share the IL-12p40 subunit and IL-12Rβ1 receptor, and are down-regulated by IL-10, relationships among these cytokines were explored in Kenyan children with varying severities of malarial anemia. Children with malarial anemia had increased circulating IL-23 and IL-10, and decreased IL-12 relative to healthy controls. Enhanced anemia severity and elevated parasitemia were associated with increased IL-10 relative to IL-23 and IL-12. Further exploration of the relationships among the cytokines using an *in vitro* model in which peripheral blood mononuclear cells were treated with synthetic hemozoin (sHz, malarial pigment) revealed that IL-12p35 and IL-23p19 transcripts had a sustained induction over 72 hrs, while IL-12p40 and IL-10 message peaked at 24 hrs, and rapidly declined thereafter. Taken together, results here show that IL-23 is elevated in children with malarial anemia, and that IL-10 and IL-12 appear to have important regulatory effects on IL-23 production during childhood malaria.

Keywords

Plasmodium falciparum; malarial anemia; hemozoin; cytokines; IL-23; IL-12; IL-10

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INTRODUCTION

Anemia is a common cause of childhood morbidity and mortality in sub-Saharan Africa occurring in 30-60% of children less than 5 years of age [1,2] and accounting for more than half of all hospital pediatric mortality [3,4]. One of the leading causes of pediatric anemia in sub-Saharan Africa is *Plasmodium falciparum* malaria which can culminate in life-threatening severe malarial anemia (SMA) [3,5,6]. It is estimated that SMA is responsible for 17-22% of the mortality in African children less than 5 years of age [3,7].

The etiology of SMA is multi-factorial and results from increased erythrocyte destruction and decreased red blood cell (RBC) production [8-10]. In addition, the relative production of pro- and anti-inflammatory cytokines is important for mediating the development and outcomes of malarial anemia [11-14]. Although not explored in the context of SMA, a recently discovered heterodimeric cytokine, IL-23, is important in regulating the development of anemia in autoimmune diseases [15] and chronic inflammation [16]. IL-23 is composed of p19 and p40 subunits [17] and shares common properties with IL-12, such as the p40 subunit and binding to the IL-12R β 1 receptor [18]. IL-23 and IL-12 are produced by activated myeloid antigen presenting cells and promote a type 1 immune response [17-21]. In addition, IL-23 and IL-12 are suppressed by both IL-10 [22,23] and IL-12p40 homodimers [20,24]. Although IL-23 and IL-12 share common properties, these cytokines also have distinct immunological roles [17, 25]. For example, IL-23 acts on activated memory CD4⁺ T cells, while IL-12 enhances Th1 differentiation of naïve CD4⁺T cells [17,26].

Previous investigations from our laboratory illustrate that phagocytosis of malarial pigment (hemozoin, Hz) by cultured human peripheral blood mononuclear cells (PBMC) generates a profile of cytokine production similar to that observed in children with malarial anemia [27-29]. Hz is a *Plasmodium*-derived biomineralized product of heme, a prosthetic group of hemoglobin (Hb) liberated during Hb degradation [30]. As part of the host immune response to malaria, monocytes/macrophages and neutrophils acquire Hz through ingestion of parasitized RBC (pRBC) [31], and through phagocytosis of free Hz released into the blood stream following rupture of pRBC [32]. Stimulation of cultured PBMC with Hz, therefore, offers a useful *in vitro* model for examining the immunological mechanisms that govern host-parasite interactions.

Since no studies to date have reported the role of IL-23, and reciprocal interactions of IL-23 with IL-12 and IL-10 in childhood malarial anemia, absolute cytokine levels and the relative expression of these cytokines were determined in circulating peripheral blood from children with varying severities of malarial anemia. The relationship between absolute cytokine levels, cytokine ratios, Hb, and parasite burden were also examined using multivariate analyses. In addition, since the malaria parasite product(s) that regulate IL-23 are unexplored, the temporal kinetics of *de novo* IL-23p19 mRNA expression, along with IL-12p35, IL-12p40, and IL-10 transcripts were investigated in cultured PBMC stimulated with synthetic hemozoin (sHz).

MATERIALS AND METHODS

Study area

This study was conducted at Siaya District Hospital (SDH) in Siaya District, Nyanza Province, western Kenya. Siaya District is a holoendemic *P. falciparum* transmission area where residents receive up to 300 infective bites per annum [33]. SMA is the primary clinical manifestation of severe malaria in children under the age of 5 years in this region, with the peak incidence of malarial anemia occurring in children 7-24 months of age [5,34]. A detailed description of the study area and the hematological manifestations of pediatric malarial anemia (MA) in the study population can be found in our recent publication [35].

Study population

Children ($n=69$; age 0-3 years) presenting with acute *P. falciparum* malaria were recruited from an on-going hospital-based longitudinal study at the SDH, western Kenya [35]. Children with *P. falciparum* parasitemia (any density) were stratified according to Hb levels into the following categories: mild malarial anemia (MMA, $n=25$; Hb 8.0-10.9 g/dL and free from the symptoms of severe malaria such as hypoglycemia); and SMA ($n=25$; Hb<6.0 g/dL and free from the symptoms of severe malaria such as hypoglycemia). Definitions of MA were based on previous investigations that obtained >10,000 longitudinal Hb measures in children less than 48 months in western Kenya [36]. In addition, healthy, age- and gender-matched aparasitemic controls (AC, $n=19$; children with malaria-negative smears for *P. falciparum* parasitemia, Hb $\geq$ 11.0 g/dL, and free of fever or diarrhea for the last two weeks) were recruited during their visits to SDH for routine immunizations. Since our previous study indicates that HIV-1 promotes enhanced anemia in children with falciparum malaria [37], only HIV-1 negative children were included in the present study. HIV-1 status was determined by rapid serological antibody tests and HIV-1 proviral DNA PCR tests according to our described methods [37]. Also excluded from the study were children with cerebral malaria, a very rare occurrence in this high malaria transmission area [5]. Children with malaria were treated according to the Ministry of Health, Kenya (MOH) guidelines which included the use of Coartem® (artemether and lumefantrine) for uncomplicated malaria and intravenous quinine for severe malaria. Supportive care and blood transfusions were administered according to MOH guidelines. All blood samples were obtained prior to antimalarial and/or any other treatment interventions. Written informed consent was obtained from the parents or legal guardians of all children enrolled in the study. The study was approved by the Institutional Review Board of the University of Pittsburgh and the National Ethical Review Committee of the Kenya Medical Research Institute. The clinical protocol was approved by the National Institutes of Health, USA.

Hemoglobin measurements

Venous blood samples (1-3 mL) were collected into EDTA containing vacutainer tubes at the time of enrollment and a complete blood count was performed using an automated Beckman Coulter® AcT diff2™ (Beckman-Coulter Corporation, Miami, USA).

Parasitemia determination

Thick and thin peripheral blood smears were prepared from venous blood samples and stained with Giemsa reagent for malaria parasite identification and quantification by microscopy. Asexual malaria parasites were counted against 300 leukocytes, and parasite densities were determined by multiplying the parasite count by the absolute leukocyte counts from an automated hematology analyzer (Beckman Coulter® AcT diff2™, Beckman-Coulter Corporation, Miami, USA).

Circulating cytokine measurements

Venous blood samples (<3.0 mL) were centrifuged and plasma was separated immediately, aliquoted, and stored at -70°C until use. Concentrations of IL-10 (Human IL-10; BD PharMingen, San Diego, CA), IL-12 [Human IL-12(p70); BD PharMingen, San Diego, CA], and IL-23 [Human IL-23p19-specific (BMS2023)]; Bender MedSystems, Vienna, Austria] were measured in plasma using sandwich enzyme-linked immunosorbent assays according to the manufacturers' instructions. Absorbances for each cytokine were measured at 450 nm and cytokine concentrations were calculated using Softmax software (Molecular Devices Corporation, Sunnyvale, CA, USA).

PBMC isolation and culture

Venous blood (70 mL) from healthy, malaria-naïve adult U.S. donors ($n=8$) was drawn into heparin-containing vials. PBMC were isolated using Ficoll-Hypaque and plated at a density of 1×10^6 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) containing 10% pooled human sera (heat-inactivated at 56°C for 30 min) according to our previous methods [29,38]. Cultures were stimulated with media alone (controls) or β -hematin (synthetic Hz, sHz; 10 μ g/mL). sHz preparations were sonicated extensively prior to addition to the cultures and were vortexed periodically during addition. Concentrations of sHz used in the present study were comparable to physiological levels of *P. falciparum*-Hz in children with severe malaria [39]. The study protocol for collection of blood samples from healthy U.S. donors was approved by the Institutional Review Board of the University of Pittsburgh.

sHz preparation

sHz was formed according to the method of Egan *et al.* [39] by dissolving hemin chloride (bovine, ICN Biomedicals, Aurora, OH) in 0.1 M NaOH, followed by the addition of HCl and acetate stock solution (12.8 M, pH 5) at 60°C. After incubation at 60°C for 150 min in the absence of stirring, the mixture was centrifuged at 10,000 rpm for 10 min, washed with filter-sterilized H₂O, and dried on a heat block at 65°C. The resulting pellet was weighed and resuspended at 1.0 mg/mL in filter-sterilized H₂O. The re-suspended sHz was sonicated thoroughly before freezing at -20°C. Endotoxin levels were determined to be less than 0.25 EU/mL (0.05 ng/mL) using the *Limulus* amoebocyte lysate test (Cambrex Bio Science, Walkersville, MD).

RNA isolation

Following the removal of culture supernatants, 1.0 mL of Solution D (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarkosyl and β -mercaptoethanol) per 2×10^6 PBMC was added to culture plates, which were stored at -20°C. Total RNA was isolated by the GITC method [40]. Briefly, thawed PBMC in Solution D were transferred to sterile baked glass tubes. Following addition of 100 μ L of 2M sodium acetate (pH 4.0), 1 mL phenol, and 200 μ L chloroform/isoamyl alcohol (49:1), glass tubes were covered in parafilm and vortexed at high speed for 10 sec. Samples were incubated on ice for 30 min, and then centrifuged at 3750 rpm for 30 min at 4°C. Three quarters (750 μ L) of the upper aqueous phase of each centrifuged sample was transferred to a sterile micro-centrifuge tube. An equal volume of cold isopropanol was added to the extracted RNA in each tube, after which the solution was mixed thoroughly and precipitated at -20°C for a minimum of 2 days. Precipitated RNA was pelleted by centrifugation at 10,000 rpm for 30 min at 4°C. Isopropanol was decanted, and tubes were centrifuged repeatedly to remove residual isopropanol with a pipette. Open tubes were placed upside-down under a fume hood for 15-30 min to allow isopropanol to evaporate without complete drying of the RNA pellet. RNA was subsequently dissolved on ice with a minimal (15 μ L) volume of DEPC-treated water. RNA samples were then tritiated, held on ice for 15 min, and heated at 65°C for 10 min to solubilize the RNA. Samples were stored at -20°C.

Quantitative real time RT-PCR

Total RNA (1 μ g) was reverse-transcribed into cDNA using a PTC-100 Peltier Thermal Cycler (MJ Research, Inc., Waltham, MA). Cytokine gene expression was analyzed by quantitative real time PCR on an Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). cDNA was amplified in duplicate with the following primer/probe sets (Applied Biosystems, Foster City, CA): IL-12p35 (Hs00168405m1), IL-12p40 (Hs00233688m1), IL-10 (Hs00174086m1), and IL-23p19 (Hs00372324m1). The endogenous control gene β -actin [Accession Number NM001101 (Applied Biosystems, Foster City, CA)] was used as a reference gene to normalize the expression levels of target genes by correcting

differences in the amount of cDNA loaded between samples. Controls without cDNA were included in duplicate for each gene to control for nonspecific background fluorescence. Data were analyzed using the comparative C_T method ($\Delta\Delta C_T$) [28]. The change in C_T (ΔC_T) for each experimental sample was derived by subtracting the endogenous control gene (β -actin) C_T from the experimental gene C_T . The ΔC_T for each experimental sample was then subtracted from the ΔC_T of the control (medium alone) sample. Fold change was expressed as $2^{-\Delta\Delta C_T}$ relative to control conditions.

Statistical analyses

Comparison of the continuous variables between groups was performed using Kruskal-Wallis test. Pair-wise comparisons of cytokine levels and cytokine ratios between groups were performed using Mann-Whitney U test. Differences in the proportional measurements were determined using Pearson's chi-square test. Relationships between plasma cytokine levels, cytokine ratios, Hb levels, and parasitemia were performed using multivariate linear regression models. Parasitemia, plasma cytokine levels, and cytokine ratios were transformed toward normality prior to correlation analyses. Cytokine transcriptional expression was measured in duplicate and presented as mean $\pm$ standard error of the mean.

RESULTS

Characteristics of the study participants

The demographic and clinical characteristics of the study participants are listed in Table 1. Children in the different clinical categories were comparable in age and gender ($P=0.514$ and $P=0.990$, respectively). However, there were significant differences in axillary temperature, Hb concentrations, and red blood cell (RBC) counts ($P<0.001$ for all comparisons) across the groups. Post-hoc testing revealed that relative to the AC group, the M/MA and SMA groups had higher axillary temperatures ($P<0.001$ and $P=0.002$, respectively), and decreased Hb levels ($P<0.001$ for both groups) and RBC counts ($P<0.001$ for both groups). Mean peripheral parasitemia was not significantly different between the M/MA and SMA groups ($P=0.421$), although children with SMA had a higher geometric mean parasitemia than children with M/MA.

Absolute cytokine levels and cytokine ratios in the clinical groups

To investigate the role of IL-23 in the pathogenesis of malarial anemia and to explore the relationship between IL-23, IL-12, and IL-10, plasma cytokine levels and cytokine ratios were determined in children with varying severities of malarial anemia. As shown in Figure 1A, IL-23 levels were elevated in children with M/MA ($P<0.05$) and SMA groups ($P=0.095$), relative to AC. IL-12 levels progressively declined with increasing disease severity ($P<0.05$, M/MA vs. AC and $P<0.01$, SMA vs. AC, Figure 1B), while IL-10 concentrations were significantly higher in children with malarial anemia ($P<0.001$ for both the M/MA and SMA vs. AC, Figure 1C). The absolute IL-23, IL-12 and IL-10 plasma cytokine levels did not differ significantly between the M/MA and the SMA groups ($P>0.05$).

In addition, since the relative expression of cytokines in the inflammatory milieu is important for mediating malarial anemia pathogenesis [11,13,14,41], and IL-12p70 down-regulates IL-23 production [26], the IL-12 to IL-23 ratio was examined in the different clinical groups. Although there was a decrease in the relative expression of IL-12 to IL-23 in the M/MA and SMA groups relative to AC, the across group comparison was not significant ($P=0.215$, Figure 1D). Additional relationships were explored between the cytokines since IL-10 suppresses both IL-23 and IL-12 [22,23,26]. These analyses revealed that there was a significant difference across the groups for IL-10 production relative to both IL-23 ($P<0.001$, Figure 1E) and IL-12 ($P<0.001$, Figure 1F). The IL-10/IL-23 and IL-10/IL-12 ratios showed a similar pattern

characterized by an increased IL-10/IL-23 and IL-10/IL-12 ratio in children with M/MA ($P<0.001$ for both ratios for M/MA vs. AC) and SMA ($P<0.001$ for both ratios for SMA vs. AC). However, IL-10 production relative to both IL-23 and IL-12 was not significantly different between the M/MA and SMA groups ($P>0.05$ for both groups). These results suggest that malarial anemia is characterized by elevated levels of IL-10 relative to both IL-23 and IL-12.

Relationship between absolute cytokine levels, cytokine ratios, and parasitemia

To determine the influence of peripheral parasitemia on the patterns of cytokine expression, children were stratified according to parasite density. For these analyses, children were divided into three groups that most appropriately represented the range of parasite burdens among the study participants: aparasitemic, healthy controls (AC, $n=19$), 1-50,000 parasites/ μL ($n=24$), and $>50,000$ parasites/ μL ($n=26$). As shown in Figure 2A, IL-23 levels progressively increased with elevated peripheral parasitemia ($P=0.063$ for 1-50,000 parasites/ μL vs. AC and $P<0.05$ for $>50,000$ parasites/ μL vs. AC). Circulating levels of IL-12 decreased with increasing parasite burden ($P=0.069$ for 1-50,000 parasites/ μL vs. AC and $P<0.01$ for $>50,000$ parasites/ μL vs. AC, Figure 2B), while plasma IL-10 concentrations progressively increased with elevated peripheral parasitemia ($P<0.001$ for both 1-50,000 parasites/ μL vs. AC and $>50,000$ parasites/ μL vs. AC, Figure 2C).

Additional analyses were performed to determine the relationship between the relative cytokine production and parasitemia. The expression of IL-12 to IL-23 progressively declined with increasing parasite burden ($P=0.330$ for 1-50,000 parasites/ μL vs. AC and $P<0.050$ for $>50,000$ parasites/ μL vs. AC, Figure 2D). The relative expression of IL-10 to both IL-23 and IL-12 showed a similar pattern in which the IL-10/IL-23 and IL-10/IL-12 ratios increased in the presence of enhanced parasitemia ($P<0.001$ for 1-50,000 parasites/ μL vs. AC for both ratios and $P<0.001$ for $>50,000$ parasites/ μL vs. AC for both ratios, Figures 2E and F, respectively).

To further explore the relationship between cytokine patterns and parasitemia, multivariate linear regression analyses were conducted with parasitemia as the dependent variable and age, gender, cytokine levels, and cytokine ratios as independent predictors. For these analyses, a separate model was constructed for absolute cytokine levels and cytokine ratios to maintain a stable regression equation in the sample population ($n=69$). The multivariate model identified IL-12 (standard partial regression coefficient, β -weight=-0.181, $P=0.035$) and IL-10 (β -weight=0.724, $P<0.001$) as significant predictors of parasitemia. In addition, the IL-12/IL-23 (β -weight=-0.616, $P=0.004$) and IL-10/IL-23 (β -weight=0.876, $P<0.001$) emerged as predictors of parasitemia. However, in a model containing the significant predictor variables (i.e., IL-10, IL-12, IL-12/IL-23 ratio, and IL-10/IL-23 ratio), only IL-12 (β -weight=-0.881, $P=0.036$) and IL-10 (β -weight=0.880, $P<0.001$) remained as significant predictors of parasitemia. Taken together, these results suggest that there is a counter-regulatory mechanism between peripheral parasite density and IL-10 and IL-12 in children with malarial anemia. It is possible that the IL-12 and IL-10 levels also regulate peripheral parasite density, and conversely that peripheral parasite density in turn mediates the production of these cytokines.

Association between absolute cytokine levels, cytokine ratios, and anemia

One of the primary goals of immunological research in children residing in holoendemic *P. falciparum* transmission areas is to identify the biomarkers associated with SMA. As such, we performed similar multivariate linear regression analyses as those outlined above in which Hb was the dependent variable and age, gender, parasitemia, cytokine levels, and cytokine ratios were the independent predictors. These analyses revealed that none of the cytokines, i.e., IL-10 (β -weight=0.175, $P=0.243$), IL-12 (β -weight=0.022, $P=0.827$), or IL-23 (β -weight=0.106, $P=0.288$), or cytokine ratios i.e., IL-12/IL-23 (β -weight=0.122, $P=0.701$), IL-10/IL-23 (β -

weight=-0.105, $P=0.741$), or IL-10/IL-12 (β -weight=0.186, $P=0.633$) were significantly associated with Hb levels.

Effect of sHz on PBMC cytokine gene expression

We have previously shown that parasite-derived products, such as *p*fHz, cause dysregulation of *de novo* cytokine transcription in cultured PBMC [27-29,38]. IL-23 and IL-12 share the IL-12p40 subunit [17] and are both antagonized by IL-10 [22,23] and secreted forms of IL-12p40 [20,24]. Aste-Amezaga et al. [22] further reported that IL-10 suppression of IL-12p40 and IL-12p35 occurred at the transcriptional level, with both the *IL-12 p40* and *IL-12 p35* genes having different requirements for *de novo* protein synthesis. As such, we examined the effect of sHz on the transcriptional kinetics of IL-23p19, IL-12p35, IL-12p40, and IL-10. PBMC from healthy malaria-naïve adult U.S. donors, ($n=8$), were stimulated with medium alone (control) or sHz. Cells were harvested at 4, 8, 24, 48, and 72 hrs for determination of transcript levels using real time RT-PCR. Treatment of PBMC with sHz caused a gradual increase in IL-23p19 and IL-12p35 transcripts that were 3.3 and 3.5-fold higher than control conditions at the end of the 72-hr time course experiment (Figures 3A and B). In addition, sHz treatment induced expression of IL-12p40 transcripts that peaked at 24 hrs (~350-fold vs. control) and rapidly declined to near baseline levels thereafter (Figure 3C). Examination of IL-10 transcripts revealed that IL-10 expression peaked at 24 hrs (3.7-fold vs. controls), but was down-regulated at 72 hrs relative to control conditions (Figure 3D). These results suggest that acquisition of hemozoin by blood mononuclear cells may be, at least in part, responsible for altered production of IL-23, IL-12, and IL-10 in children with malaria. In addition, these results show that sHz causes only marginal increases in IL-23p19, IL-12p35, and IL-10 transcripts, while having a substantial effect on IL-12p40 gene expression.

DISCUSSION

Recent studies suggest that IL-23 may be important for promoting anemia in chronic inflammatory diseases [15,42]. Consistent with these investigations, results presented here illustrate that children with malarial anemia have increased levels of IL-23 in circulation relative to healthy, age-matched controls. Although IL-23 and IL-12 share the p40 subunit [17], levels of IL-23 were elevated in children with malarial anemia, while plasma IL-12 concentrations progressively declined with increasing anemia severity. The decrease in IL-12 with increasing disease severity parallels previous findings in African children residing in areas with intense malaria transmission [12-14,29,43-45]. In addition, examination of the IL-12 to IL-23 ratio revealed that SMA is characterized by low levels of IL-12 relative to IL-23. This divergent pattern of IL-23 and IL-12 production in children with malarial anemia may offer important insight into the pathogenesis of SMA since both IL-23 and IL-12 share a common p40 subunit [17] and, as such, may be expected to yield similar directional patterns during active disease.

Although IL-10 can suppress both IL-23 and IL-12 [22,23], IL-23 can also promote IL-10 production by activated lymphocytes [46]. Results presented here showing that the relative expression of IL-10 to IL-23 progressively increased with increasing anemia severity may suggest that IL-10 could have a suppressive effect on IL-23 production. Our previous investigations illustrate that phagocytosis of malarial pigment by monocytes causes enhanced production of IL-10 that, in turn, suppresses IL-12 [29]. These results have important implications in the pathogenesis of malarial anemia since IL-12 is required for promotion of erythropoiesis through its ability to enhance erythroid burst (BFU-E) and colony forming units (CFU-E) [47,48]. Results presented here showing high levels of IL-10 relative to IL-12 in children with SMA support previous investigations in children with malarial anemia [12,14, 29]. Furthermore, findings presented here extend these results by showing that SMA is also

characterized by high levels of IL-10 relative to IL-23. However, additional experiments in malaria-infected children that directly examine the effect of IL-10 on IL-23 production are required to confirm the association reported here.

To further explore the relationship between IL-23 and its counter-regulatory cytokines, IL-12 and IL-10, in the context of malaria, children were stratified according to parasite density. These analyses revealed an identical pattern of both absolute cytokine levels and cytokine ratios as those observed when the children were stratified according to anemia severity. However, across group differences in cytokine production and cytokine ratios were more pronounced in children stratified according to parasite density, suggesting that parasitemia, rather than anemia, may be more closely associated with IL-23, IL-12, and IL-10 production. To more quantitatively elucidate the potentially important relationship between parasitemia, absolute cytokine levels, and cytokine ratios, multivariate linear regression analyses were performed. These analyses demonstrated that IL-10, IL-12, IL-12/IL-23 ratio, and IL-10/IL-23 ratio were independent predictors of parasitemia. However, when all of the significant predictor variables were placed in a multivariate model, only IL-10 and IL-12 emerged as significant predictors of parasitemia. The strong positive association between IL-10 and parasitemia and the strong negative association between IL-12 and parasitemia suggest that the overall magnitude of parasitemia may be an important determinant for promoting increased IL-10 and decreased IL-12 production, respectively.

Using a similar modeling approach, we determined the relationship between Hb concentrations, absolute cytokine levels, and cytokine ratios, while controlling for parasitemia. Although IL-23 and IL-10 were increased in children with malarial anemia, while IL-12 levels were suppressed, the multivariate model demonstrated that none of the absolute cytokine levels, or their respective ratios was significant predictors of Hb concentrations after controlling for parasitemia. These results suggest that the pattern of IL-23, IL-12, and IL-10 production observed in children with malarial anemia in the current study were significantly influenced by the strong relationship between parasite density and production of these cytokines. It is also important to point out that in this population of children, we have consistently observed that parasitemia is not strongly associated with malarial anemia severity [29,35,38]. This could explain, at least in part, our observation that the absolute levels of these cytokines were comparable between the M/MA and SMA groups. However, there is a need for additional studies to elucidate the precise role of malaria parasites and/or malaria parasite products in conditioning anemia in young children in holoendemic settings.

A number of studies from our laboratory [27-29,38], as well as others [49-51], have shown that Hz is an important parasitic product that causes dysregulation of *de novo* cytokine production in human PBMC. As such, Hz was examined as a potential parasitic product that could alter the transcriptional expression of IL-23, IL-12, and IL-10. These results illustrated that IL-23p19 and IL-12p35 have similar transcriptional profiles, characterized by low levels of expression that gradually increased throughout the 72 hr time-course experiment. In contrast, IL-12p40 transcripts were increased over ~350-fold at 24 hrs prior to rapidly declining to near baseline levels by 48 hrs. Since IL-23 and IL-12p70 share a common p40 subunit [17], these results suggest that IL-12p40 gene products may be the rate-limiting factor for formation of both IL-23 and IL-12p70. Results showing that IL-10 transcriptional expression also peaked at 24 hrs is consistent with the fact that IL-10 is capable of suppressing IL-12p40 [22]. Thus, IL-10 may exert its suppressive effects on IL-23 and IL-12 by down-regulating IL-12p40 gene products. Additional studies in samples collected prospectively in malarial-infected children at different time points throughout the course of infection are required to more definitively examine the inter-relationships among IL-23, IL-12, and IL-10 *in vivo*. Since IL-23 and IL-12 have distinct roles in the induction of immunity to intracellular pathogens [21,52-54], as well as different downstream signaling pathways [55], it is important that these pathways be further

investigated to delineate the role of IL-23 and IL-12 in malarial anemia pathogenesis. As such, we are currently investigating the role of the IL-23 and IL-12 pathways in prospectively collected samples from children naturally exposed to *P. falciparum*.

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Figure 1. Absolute cytokine levels and cytokine ratios in the clinical groups

Data are presented as box plots with whiskers and outliers. The box represents the interquartile range, while the whiskers represent the 10th and 90th percentiles, including the outliers (closed circles). The horizontal line in the box indicates the median value. (A) IL-23 levels, (B) IL-12 levels, (C) IL-10 levels, (D) IL-12/IL-23 ratio, (E) IL-10/IL-23 ratio, and (F) IL-10/IL-12 ratio in relation to anemia severity. The group comparisons were done using Kruskal-Wallis test while pair-wise comparisons were done using Mann-Whitney U test.

Figure 2. Plasma cytokine levels/ratios versus parasitemia

Data are presented as box plots with whiskers and outliers. The box represents the interquartile range while the whiskers represent the 10th and 90th percentiles including the outliers (closed circles). The horizontal line across the box indicates the median value. (A) IL-23 levels, (B) IL-12 levels, (C) IL-10 levels, (D) IL-12/IL-23 ratio, (E) IL-10/IL-23 ratio, and (F) IL-10/IL-12 ratio in relation to parasitemia levels. Differences between groups were tested using Mann-Whitney U test.

Figure 3. Effect of sHz on PBMC cytokine gene expression

PBMC (1×10^6 cells/mL) from healthy, malaria-naïve adult U.S. donors ($n=8$) were stimulated with medium alone (control; grey line) or with sHz (10 μ g/mL; black line). Cells were collected at 4, 8, 24, 48, and 72 hrs for determination of IL-23p19 (A), IL-12p35 (B), IL-12p40 (C), and IL-10 (D) transcript levels by real time RT-PCR (determined in duplicate for each time point). Values represent mean $\pm$ SEM.

Table 1
Demographic and clinical characteristics of the study participants

	Aparasitemic Healthy Controls (AC)	Mild Malarial Anemia (M/MA)	Severe Malarial Anemia (SMA)	<i>P value</i>
Number of individuals	19	25	25	
Age (<i>mos</i>)	6 (4.0 - 11.0)	8 (6.0 - 12.5)	8 (6.0 - 11.0)	0.514 ^a
Gender (<i>female</i> , %)	57.9	56.0	56.0	0.990 ^b
Axillary temperature (°C)	36.50 (36.1 - 36.9)	37.50 (36.8 - 38.5)	37.30 (36.5 - 38.3)	<0.001 ^a
Hemoglobin (g/dL)	11.6 (11.4 - 12.0)	9.0 (8.4 - 9.9)	4.2 (3.6 - 4.2)	<0.001 ^a
Red blood cells ($\times 10^{12}/L$)	5.19 (4.82 - 5.52)	4.13 (3.67 - 4.57)	1.75 (1.43 - 1.75)	<0.001 ^a
Parasitemia (μL)	0	38593 (6040 - 130162)	66130 (12747 - 140622)	0.421 ^c
Geometric mean parasitemia	0	25883	37490	

Data are presented as median (interquartile range) unless stated otherwise.

^a Differences in the age, temperature, Hb levels, and red blood cell counts were compared using Kruskal-Wallis test.

^b Differences in the proportion of gender were compared using Pearson's χ^2 test.

^c Parasitemia between the two malaria groups was compared using Mann-Whitney U test.