Acquisition of Hemozoin by Monocytes Down-Regulates Interleukin-12 p40 (IL-12p40) Transcripts and Circulating IL-12p70 through an IL-10-Dependent Mechanism: In Vivo and In Vitro Findings in Severe Malarial Anemia

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Severe malarial anemia (SMA) is a primary cause of morbidity and mortality in immune-naïve infants and young children residing in areas of holoendemic Plasmodium falciparum transmission. Although the immunopathogenesis of SMA is largely undefined, we have previously shown that systemic interleukin-12 (IL-12) production is suppressed during childhood blood-stage malaria. Since IL-10 and tumor necrosis factor alpha (TNF-α) are known to decrease IL-12 synthesis in a number of infectious diseases, altered transcriptional regulation of these inflammatory mediators was investigated as a potential mechanism for IL-12 down-regulation. Ingestion of naturally acquired malarial pigment (hemozoin [PfHz]) by monocytes promoted the overproduction of IL-10 and TNF-α relative to the production of IL-12, which correlated with an enhanced severity of malarial anemia. Experiments with cultured peripheral blood mononuclear cells (PBMC) and CD14+ cells from malaria-naïve donors revealed that physiological concentrations of PfHz suppressed IL-12 and augmented IL-10 and TNF-α by altering the transcriptional kinetics of IL-12p40, IL-10, and TNF-α, respectively. IL-10 neutralizing antibodies, but not TNF-α antibodies, restored PfHz-induced suppression of IL-12. Blockade of IL-10 and the addition of recombinant IL-10 to cultured PBMC from children with SMA confirmed that IL-10 was responsible for malaria-induced suppression of IL-12. Taken together, these results demonstrate that PfHz-induced up-regulation of IL-10 is responsible for the suppression of IL-12 during malaria.

Malaria is one of the leading causes of morbidity and mortality of infectious origin, resulting in approximately 0.9 to 2.8 million deaths annually (5). The majority of malaria-related mortality occurs in children under 5 years of age due to their lack of naturally acquired malarial immunity (5). In areas of holoendemic Plasmodium falciparum transmission, the primary clinical manifestation of severe malaria in young children is severe anemia, resulting in mortality rates that exceed 30% (5). Although the underlying mechanisms responsible for severe malarial anemia (SMA) are largely undefined, altered production of innate inflammatory mediators appears to play an important role in conditioning disease outcomes.

Protective immunity against malaria requires enhanced interleukin-12 (IL-12) production, which polarizes cytokine patterns towards a type I (proinflammatory) immune response (9, 44). Circulating monocytes are a primary source of bioactive IL-12p70 (10), a heterodimer of IL-12p35 and IL-12p40 subunits encoded by separate genes (18). Murine models of malaria illustrate that early and sustained induction of IL-12 provides protection against severe disease (36), at least in part, through enhanced erythropoiesis (25). Administration of recombinant IL-12 to mice infected with P. yoelli or to rhesus macaques infected with P. cynomolgi provides 100% protection against parasitemia (14, 39). Conversely, the inability of IL-12-deficient mice to resolve parasitemia is restored following treatment with recombinant IL-12 (45). Although the mechanism responsible for IL-12 suppression in human malaria is currently unknown, murine models of malaria suggest that the counter-regulatory effects of IL-10 may decrease IL-12 production (49). Experiments with cultured monocyte-derived macrophages (MDM) further illustrated that IL-12p70 is negatively regulated by IL-10 (2). Additional studies have shown that tumor necrosis factor alpha (TNF-α) release from MDM can also suppress IL-12p70 (22). Our previous investigations in an area of hyperendemic P. falciparum transmission demonstrated that Gabonese children with malarial anemia and hyperparasitemia have decreased circulating IL-12 levels and elevated levels of both IL-10 and TNF-α (20, 34). Although subsequent studies confirmed that IL-12 is suppressed during severe malaria (6, 23, 24, 28), the effect of elevated circulating IL-10 and TNF-α concentrations on IL-12 production, particularly in areas of holoendemic P. falciparum transmission,
where infants and young children develop SMA, has not been determined.

Phagocytosis of parasitic products, such as P. falciparum-derived malarial pigment (hemozoin [PHz]), alters IL-12, IL-10, and TNF-α production in cultured human peripheral blood mononuclear cells (PBMC) (3, 11, 15, 16, 26, 35, 40), suggesting that acquisition of PHz by monocytes/macrophages may be an important source of cytokine dysregulation during malaria. Hæ is an insoluble coordination polymer of heme subunits formed during hemoglobin (Hb) catabolism by Plasmodium to avoid the toxic effects of hæme (41). Circulating monocytes rapidly acquire PHz released into the bloodstream upon rupture of parasitized red blood cells (pRBC) (37) and through phagocytosis of intact pRBC (1).

In the present study, we examined the roles of IL-12, IL-10, and TNF-α in the immunopathogenesis of SMA in infants and young children. The primary aim of this study was to determine the host-parasite interactions responsible for malaria-induced suppression of IL-12. To accomplish this aim, we determined if phagocytosis of PHz by blood mononuclear cells suppresses de novo IL-12p40 gene expression by promoting the overproduction of IL-10 and/or TNF-α. In the in vivo and in vitro results presented here for a cohort of children with malarial anemia in western Kenya illustrate that malaria-induced overproduction of IL-10, but not TNF-α, is responsible for the suppression of IL-12.

MATERIALS AND METHODS

Study participants. Children (n = 99; age, 0 to 3 years) presenting with acute P. falciparum malaria were recruited from a hospital-based longitudinal study at the Siaya District Hospital in Siaya District, western Kenya, as part of our ongoing activities examining the pathogenesis of SMA (29). The data presented here represent cross-sectional measures collected upon enrollment at the first hospital contact for the treatment of malaria. In this area of holoendemic P. falciparum transmission, residents receive up to 300 infective mosquito bites per annum (4). Children with cerebral malaria, a rare occurrence in this high transmission setting (4), were excluded from the study. In addition, since we recently demonstrated that both human immunodeficiency virus type 1 (HIV-1) exposure and PCR-confirmed presence of HIV-1 virus significantly increase the development of SMA among children (31), all children were examined for the presence of HIV-1 by proviral DNA PCR testing according to our previous methods (31). Only HIV-1-negative children were included in the study so that a malarial anemia could be examined as a single disease entity. Children presenting with malaria were given antimalarials and the appropriate supportive therapy, as required according to Kenya Ministry of Health guidelines. All blood samples were obtained prior to treatment with antimalarials and/or any other therapy, as required according to Kenya Ministry of Health guidelines. All blood samples were obtained prior to treatment with antimalarials and/or any other therapy, as required according to Kenya Ministry of Health guidelines. All blood samples were obtained prior to treatment with antimalarials and/or any other therapy, as required according to Kenya Ministry of Health guidelines. All blood samples were obtained prior to treatment with antimalarials and/or any other therapy, as required according to Kenya Ministry of Health guidelines. All blood samples were obtained prior to treatment with antimalarials and/or any other therapy, as required according to Kenya Ministry of Health guidelines.

Clinical laboratory measures. Heel/finger-prick blood (<100 μl) was obtained to determine parasitemia status with Giemsa-stained blood smears. Venipuncture blood (<3 ml) collected into EDTA-containing Vacutainer tubes was used for determinations of Hb concentration and complete blood counts with a Coulter A′ T dTz instrument (Beckman Coulter Corp., Miami, FL). Axenical malaria parasites were counted against 300 leukocytes, and parasite densities were determined by multiplying the parasite count by the absolute white blood cell count. Malarial pigment was determined by counting 100 neutrophils and 30 monocytes on thin blood smears. Although pigment-containing neutrophils (PCN) were determined, these data were not utilized in the study since the number of PCN was exceedingly low. The total number of pigment-containing monocytes (PCM)/30 monocytes was calculated, and the total pigment burden in monocytes was determined for various absolute white blood cell and differential counts. Total PCM/μl was calculated with the following formula: total PCM/μl = (number of PCM/30 monocytes) × (absolute number of monocytes/μl).

Isolation and culture of peripheral blood mononuclear cells and CD14 and CD16 cells. For in vitro experiments with healthy U.S. adults (n = 21) and children with SMA (n = 12), venous blood (40 ml and <3 ml, respectively) was drawn into EDTA-containing vials. PBMC were prepared using Ficoll-Hypaque as described earlier (45). For in vitro experiments with U.S. donors, CD14 and CD16 cells were isolated from PBMC fractions using CD14 magnetic beads (Miltenyi Biotech, Auburn, CA) per the manufacturer’s protocol. PBMC and CD14 and CD16 cells were plated at 1 × 10⁶ cells per ml in Dulbecco’s modified Eagle’s medium containing 10% pooled human sera (heat inactivated at 56°C for 30 min) from malaria-naïve donors. Cultures were stimulated with medium alone (controls) or with lipopolysaccharide (LPS [100 ng/ml]; Alexis Corp., San Diego, CA) and gamma interferon (IFN-γ [200 U/ml]; BD Pharmingen, San Diego, CA). Cultured PBMC from healthy U.S. adults were also stimulated with LPS and IFN-γ in the presence of PHz (10, 1.0, or 0.1 μg/ml) or β-hæmin (synthetic Hæ [sHæ]; 10 μg/ml). The concentrations of malarial pigment represent physiological doses of PHz calculated for children with severe (10 μg/ml) and mild (1.0 μg/ml) malaria according to our published methods (17). The effects of IL-10 and TNF-α on IL-12p70 production were determined by the addition of neutralizing antibodies for IL-10 (0.1, 0.5, and 1.0 μg/ml; R&D Systems, Minneapolis, MN) and TNF-α (1.0, 10, and 100 ng/ml; R&D Systems, Minneapolis, MN) or the exogenous addition of IL-10 (0.2, 2.0, and 20 ng/ml; Endogen, Woburn, MA) and TNF-α (0.2, 2.0, and 20 ng/ml; BD Pharmingen, Bedford, MA).

PHz and sHæ preparations. Crude PHz was isolated from in vitro cultures of pD6-infected RBC as previously described (17). Cultures were isolated and spun at 2,000 rpm for 10 min, and the resulting pellets were resuspended in 40 ml of 0.01 M phosphate-buffered saline (pH 7.2) with 2 ml saponin for 10 min. Total PHz was then spun at 10,000 rpm for 15 min, and the pellets were washed with phosphate-buffered saline (four to seven times) until the resulting pellet was red and free from the white cellular components. A synthetic compound of ferriprotoporphyrin IX (sHæ), which is structurally identical to detergent-treated PHz (32, 42), was formed in a 4.5 M acetic acid solution at pH 4.5 by the method of Egan et al. (13). Hemin chloride (Sigma, St. Louis, MO) was added to a 0.1 M solution of NaOH, followed by the addition of HCl at 60°C. A solution of acetate was then added, and the mixture was incubated for 150 min at 60°C without stirring. sHæ was then spun at 14,000 rpm in a microcentrifuge, washed three times with filter-sterilized H₂O₂ and dried at 60°C under vacuum. The final PHz and sHæ pellets were weighed, resuspended at 1.0 mg/ml in filter-sterilized H₂O₂ and extensively sonicated prior to addition to cultures. Endotoxin levels in all preparations were <0.01 endotoxin unit/ml according to the Limulus amebocyte lysate test (BioWhittaker, Walkersville, MD).

Measurement of IL-12p70, IL-10, and TNF-α. Plasma and supernatant concentrations of IL-12p70, IL-10, and TNF-α were determined by quantitative sandwich enzyme-linked immunosorbent assays (ELISAs; BD Pharmingen, San Diego, CA) following the manufacturer’s specifications. The sensitivity of detection for each cytokine was ≥7.8 pg/ml.

Quantitative real-time RT-PCR. Total RNA was isolated from PBMC by the guanidinium isothiocyanate method (7). 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, Foster City, CA). cDNA (100 ng) was amplified in duplicate with specific primer/probe sets for IL-12p70, IL-12p40, IL-10, and TNF-α (assays Hs00168405_m1, Hs00233688_m1, Hs00174086_m1, and Hs00174128_m1, respectively [Applied Biosystems, Foster City, CA]). To control for nonspecific background fluorescence, no-template controls were included in triplicate. An endogenous control gene, the β-actin gene (assay 4326315E [Applied Biosystems, Foster City, CA]), was used as a reference to normalize cDNA loading between samples. Data were compared using the ΔΔCₚ method as previously described (17).

Statistical analyses. Plasma and supernatant concentrations of IL-12p70, IL-10, and TNF-α were determined in triplicate at several different dilutions. IL-12p35, IL-12p40, IL-10, and TNF-α mRNA expression was measured in duplicate. Comparisons across multiple groups were performed by Kruskal-Wallis (KW) tests, and when differences were significant, pairwise comparisons between conditions were performed by the Mann-Whitney U test. Differences
between proportions were performed using chi-square analyses. For Pearson correlation analyses, all non-normally distributed variables were transformed toward normality. This resulted in IL-10 levels being square root transformed and the following variables being logarithmically transformed: IL-12p70 and TNF-α levels and the ratios of IL-10/IL-12p70 and TNF-α/IL-12p70. The level of statistical significance for all analyses was determined using a critical alpha value of 0.05.

RESULTS

Study participants’ clinical and laboratory characteristics. The clinical and laboratory characteristics of the study participants are shown in Table 1. There was no significant difference in age (in months), gender (male versus female), or axillary temperature (°C) across the groups (Table 1). As expected based on the a priori grouping of children according to anemia status, Hb concentrations (g/dl), hematocrit values (%), and RBC numbers (10⁶/µl) differed across the groups (P < 0.001 for all tests) (Table 1). Peripheral parasitemia levels (parasites/µl) and proportions of high-density parasitemia (>10,000 parasites/µl) were not significantly different between the groups (Table 1). The percentages of children with circulating PCM were significantly different between the groups (P = 0.002) (Table 1).

Circulating IL-12p70, IL-10, and TNF-α levels in children with malarial anemia. Although we have previously shown that IL-12p70 is suppressed in the presence of high peripheral blood concentrations of IL-10 and TNF-α in children with severe malaria (20, 34), the effects of IL-10 and TNF-α on suppression of IL-12p70 have not been determined previously for malaria-infected children. To examine the in vivo relationships between IL-12p70, IL-10, and TNF-α, children were stratified into groups with different severities of malarial anemia. Circulating IL-12p70 levels were significantly different across groups (KW test; P < 0.001). IL-12p70 concentrations were reduced in the MdMA and SMA groups relative to that in the UM group (P < 0.05 and P < 0.05, respectively), with the SMA group having the lowest IL-12p70 concentrations of 0.05.

Table 1. Clinical and laboratory characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value for groupa</th>
<th>P value</th>
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<tbody>
<tr>
<td>No. of participants</td>
<td>UM</td>
<td>MIMA</td>
</tr>
<tr>
<td>Age (mo)</td>
<td>13.58 (2.48)</td>
<td>13.14 (1.47)</td>
</tr>
<tr>
<td>Sex (n [%])</td>
<td>Male</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>7 (58.3)</td>
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<tr>
<td>Temp (°C)</td>
<td>37.72 (0.35)</td>
<td>37.49 (0.19)</td>
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<tr>
<td>Hemoglobin concn (g/dl)</td>
<td>11.50 (0.20)</td>
<td>9.10 (0.08)</td>
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<tr>
<td>Hematocrit (%)</td>
<td>34.13 (0.85)</td>
<td>27.73 (0.25)</td>
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<tr>
<td>No. of RBC (10⁶/µl)</td>
<td>4.62 (0.14)</td>
<td>4.16 (0.12)</td>
</tr>
<tr>
<td>Parasitemia</td>
<td>Total (parasites/µl)</td>
<td>52.130 (13,659)</td>
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<tr>
<td></td>
<td>Geometric mean (range)</td>
<td>24,080 (160–152,510)</td>
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<tr>
<td></td>
<td>High-density (n [%])</td>
<td>10 (83.3)</td>
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<tr>
<td></td>
<td>No. (%) of pigment-containing monocytes</td>
<td>0 (0.0)</td>
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a Data are means (standard errors of the means) unless otherwise noted. Children (n = 99) with P. falciparum malaria (any density) were recruited during their first hospital contact for the treatment of malaria and categorized according to Hb concentration, as defined in Materials and Methods.

b Statistical significance was determined by the Kruskal-Wallis test.
c Statistical significance was determined by chi-square analysis.

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Circulating IL-12p70, IL-10, and TNF-α levels in children with malarial anemia. Although we have previously shown that IL-12p70 is suppressed in the presence of high peripheral blood concentrations of IL-10 and TNF-α in children with severe malaria (20, 34), the effects of IL-10 and TNF-α on suppression of IL-12p70 have not been determined previously for malaria-infected children. To examine the in vivo relationships between IL-12p70, IL-10, and TNF-α, children were stratified into groups with different severities of malarial anemia. Circulating IL-12p70 levels were significantly different across groups (KW test; P < 0.001). IL-12p70 concentrations were reduced in the MdMA and SMA groups relative to that in the UM group (P < 0.05 and P < 0.05, respectively), with the SMA group having the lowest IL-12p70 concentrations (Fig. 1A). Plasma IL-10 levels did not differ between groups (KW test; P = 0.299) and were nonsignificantly reduced in the MdMA and SMA groups relative to those in the UM and MIMA groups (Fig. 1B). TNF-α progressively increased with increasing severities of malarial anemia (KW test; P = 0.011), with the SMA group having the highest TNF-α levels (P < 0.05 versus UM group) (Fig. 1C). Since our previous studies (34), as well as those of others (12, 19, 30), illustrated that the relative production of proinflammatory and anti-inflammatory cytokines in the inflammatory milieu is a better index of malaria disease severity than individual cytokine measurements, the relative expression of IL-12p70, IL-10, and TNF-α was examined. Moreover, since both IL-10 and TNF-α can suppress IL-12p70 (2, 22), the ratios examined represent biologically significant relationships. The production of IL-10 relative to that of IL-12p70 increased with the severity of malarial anemia (KW test; P = 0.027), with the SMA group having the highest IL-10/IL-12p70 ratio (P < 0.05 versus UM group) (Fig. 1D). In addition, the TNF-α/IL-12p70 ratio was progressively elevated with increasing severities of malarial anemia (KW test; P < 0.001) (Fig. 1E). The relative production of TNF-α compared to that of IL-12p70 was higher for the MdMA group (P < 0.05) and SMA groups (P < 0.01) than for the UM group (Fig. 1E). Taken together, these in vivo results support a model in which both IL-10 and TNF-α could be responsible for suppression of IL-12p70 in children with malarial anemia.

Relationship between cytokine dysregulation and disease severity. To further investigate the roles of IL-12p70, IL-10, and TNF-α in the immunopathogenesis of SMA, the relationships between circulating cytokine concentrations (and ratios) and the two primary end points of disease severity in areas with holoendemic transmission (i.e., the Hb concentration and parasitemia level) were examined by correlation analyses. Plasma levels of IL-12p70 (r = 0.313; P < 0.01) and TNF-α (r = −0.345; P < 0.01), but not IL-10 (r = 0.174; P = 0.09), were significantly correlated with the Hb concentration (Table 2). The relative expression of both IL-10 and TNF-α compared to that of IL-12p70 was also significantly associated with the Hb level (r = −0.298 and P < 0.01 for IL-10; and r = −0.435 and P = 0.001 for TNF-α) (Table 2). Examination of absolute
FIG. 1. Peripheral blood cytokine concentrations in children with malarial anemia. Plasma was obtained from children \( (n = 99) \) with various severities of acute malaria. Children were categorized according to their anemia status, as follows: UM \((n = 12)\), MlMA \((n = 28)\), MdMA \((n = 30)\), and SMA \((n = 29)\). IL-12p70 (A), IL-10 (B), and TNF-\(\alpha\) (C) levels were determined by ELISA. The relative production of IL-10/IL-12p70
cytokine levels and cytokine ratios revealed that only IL-10 was significantly correlated with parasitemia (r = 0.259; P < 0.01) (Table 2). These results demonstrate that the IL-10/IL-12p70 and TNF-α/IL-12p70 ratios are significantly associated with malaria anemia, independent of the parasite burden.

**Role of naturally acquired PfHz in cytokine dysregulation.**

Recent studies of phytohemagglutinin-stimulated PBMC showed that phagocytosis of parasitic products, such as PfHz, is associated with inhibition of proliferative responses and decreased IL-12 production (11). To determine if suppression of IL-12 production associated with inhibition of proliferative responses and decreased IL-12p70, IL-10, and TNF-α were not detected in CD14+ cultures under any of the conditions examined (data not shown). Stimulation of PBMC or CD14+ cells with LPS and IFN-γ elevated IL-12p70 production (P < 0.01 and and P < 0.01, respectively) (Fig. 3A and B). The addition of a high dose (10 μg/ml) of PfHz or sHz decreased LPS- and IFN-γ-promoted IL-12p70 production (P < 0.05 and P < 0.05, respectively) (Fig. 2A and B). IL-12p70 was not detectable in culture supernatants from PfHz- or sHz-treated PBMC in the absence of LPS and IFN-γ stimulation (data not shown). LPS and IFN-γ stimulation increased IL-10 and TNF-α production in PBMC and CD14+ cell cultures (P < 0.01 for all tests) (Fig. 3C to F). A high dose of PfHz or sHz significantly augmented IL-10 and TNF-α production (P < 0.05 for all tests) (Fig. 3C to F). The results presented here illustrate that physiological concentrations of PfHz and sHz decrease PBMC-derived IL-12p70 and increase IL-10 and TNF-α levels and that monocytes are the primary source of cytokine production.

**Temporal kinetics of PfHz-induced dysregulation of de novo cytokine transcripts.**

Regulation of cytokine production during an inflammatory event occurs primarily at the level of de novo transcription. As such, the effect of PfHz on the temporal kinetics of IL-12p35, IL-12p40, IL-10, and TNF-α gene expression was examined in PBMC by real-time RT-PCR. Cells were stimulated with medium alone (controls), with LPS and IFN-γ, or with LPS and IFN-γ in the presence of PfHz (10 μg/ml). Although stimulation of PBMC with LPS and IFN-γ significantly augmented IL-12p35 mRNA expression at all time points examined (P < 0.05) (Fig. 4A), the addition of PfHz did not significantly alter IL-12p35 transcript levels relative to those observed under LPS- and IFN-γ-stimulated conditions (Fig. 4A). IL-12p40 transcripts were elevated at 8 h (P < 0.01) and peaked at 24 h (P < 0.01) in cells stimulated with LPS and IFN-γ (Fig. 4B). Treatment of PBMC with PfHz significantly down-regulated IL-12p40 mRNA levels at both 8 (P < 0.01) and 24 h (P < 0.01) (Fig. 4B). LPS and IFN-γ increased IL-10 transcripts at 8, 24, and 48 h (P < 0.01 for all time points) (Fig.

<table>
<thead>
<tr>
<th>Cytokine parameter</th>
<th>Correlation with disease outcome</th>
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<tr>
<td></td>
<td>Hemoglobin concn</td>
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<tr>
<td></td>
<td>r value</td>
</tr>
<tr>
<td>IL-12p70 level</td>
<td>0.313</td>
</tr>
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<td>IL-10 level</td>
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</tr>
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<td>TNF-α level</td>
<td>-0.345</td>
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<td>IL-10/IL-12p70 ratio</td>
<td>-0.298</td>
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<td>TNF-α/IL-12p70 ratio</td>
<td>-0.435</td>
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* Relationships between absolute cytokine levels (and ratios) and anemia (Hb concentration) or parasitemia (parasites/μl) were determined for children with *P. falciparum* malaria (n = 99), using Pearson correlation analyses.

(D) and TNF-α/IL-12p70 (E) was determined. Each box represents the interquartile range, the line through the box is the median, whiskers show 10th and 90th percentiles, and closed circles are outliers. Statistical significance was determined by the Kruskal-Wallis test. For tests that were found to be significant, pairwise comparisons were conducted using Mann-Whitney U tests.
FIG. 2. Effects of naturally acquired PfHz on circulating cytokine concentrations. Children with acute *P. falciparum* malaria (*n* = 99) were categorized according to the absence or presence of PCM, as follows: PCM−, *n* = 68; PCM+, *n* = 31. Plasma concentrations of IL-12p70 (A), IL-10 (B), and TNF-α (C) and the relative production of IL-10/IL-12p70 (D) and TNF-α/IL-12p70 (E) were determined by ELISA. Each box represents the interquartile range, the line through the box is the median, whiskers show 10th and 90th percentiles, and closed circles are outliers. Statistical significance was determined by the Mann-Whitney U test.
The addition of PfHz to LPS- and IFN-γ-stimulated PBMC significantly up-regulated peak IL-10 transcript levels at 24 h (P < 0.01) (Fig. 4C). TNF-α transcripts had the earliest induction, with LPS and IFN-γ stimulation significantly increasing gene expression at 2, 4, and 8 h (P < 0.01) (Fig. 4D). In the presence of PfHz, the TNF-α message was significantly up-regulated at each of these time points (P < 0.01) (Fig. 4D). These results illustrate that PfHz down-regulates IL-12 transcripts and up-regulates IL-10 and TNF-α mRNAs.

Role of IL-10 and TNF-α in mediating PfHz-induced IL-12p70 suppression. Since exogenous IL-10 and TNF-α suppress IL-12 production in MDM (2, 22), we postulated that PfHz-induced increases in IL-10 and/or TNF-α may suppress IL-12p70 production. To test this hypothesis, neutralizing antibodies for IL-10 (0.1, 0.5, and 1.0 μg/ml) and TNF-α (1.0, 10, and 100 ng/ml) or exogenous IL-10 (0.2, 2.0, and 20 ng/ml) and TNF-α (0.2, 2.0, and 20 ng/ml) were added to stimulated PBMC in the presence of a high dose (10 μg/ml) of PfHz. IL-12p70 production was measured in culture supernatants at 48 h. Stimulation of PBMC with LPS and IFN-γ increased IL-12p70 production (P < 0.05), which was significantly decreased by PfHz (P < 0.05) (Fig. 5A to D). The addition of intermediate (0.5 μg/ml) and high (1.0 μg/ml) doses of IL-10 neutralizing antibody significantly increased PfHz-induced IL-12p70 production (P < 0.05) (Fig. 5C). IL-10 levels were dose-dependently decreased in the presence of increasing concentrations of IL-10 neutralizing antibody (data not shown). Exogenous addition of IL-10 dose-dependently suppressed IL-12p70 production in PfHz-treated PBMC (Fig. 5B). No concentration of TNF-α neutralizing antibody restored PfHz-induced suppression of IL-12p70 (Fig. 5C). TNF-α levels were dose-dependently decreased by increasing doses of TNF-α neutralizing antibody (data not shown). In addition, exogenous TNF-α failed to significantly reduce IL-12p70 production at all of the concentrations examined (Fig. 5D).

Based on these results, experiments were performed with cultured PBMC from children with SMA (n = 12) to determine if IL-10 was responsible for the suppression of IL-12p70 during natural malaria infection. Cultures were stimulated with medium alone (controls), with LPS (100 ng/ml) and IFN-γ (200 U/ml), or with LPS and IFN-γ in the presence of IL-10 neutralizing antibody (0.5 μg/ml) or exogenous IL-10 (2.0 ng/ml). Consistent with experiments with cells from malaria-naïve donors, LPS and IFN-γ significantly increased IL-12p70 production (P < 0.05) (Fig. 5). The addition of IL-10 neutralizing antibody caused a significant increase in IL-12p70 production (P < 0.05), while exogenous IL-10 decreased IL-12p70 production in cultured PBMC from children with SMA (P < 0.05) (Fig. 5E). These experiments definitively demonstrate that IL-10 is responsible for the suppression of IL-12p70 production in PfHz-treated PBMC and in cultured PBMC from children with SMA.

**DISCUSSION**

SMA is the primary clinical manifestation of severe malaria in infants and young children in areas of holoendemic *P. falciparum* transmission (5). SMA is responsible for the greatest burden of malaria-related morbidity and mortality, as evidenced by the 17.2% mortality rate for children <5 years of age residing in regions of holoendemic malaria (27). However, the mechanisms that govern the pathogenesis of severe anemia in children with falciparum malaria are largely undefined, particularly for regions of sub-Saharan Africa where HIV-1 is now endemic. Since our recent findings demonstrated that HIV-1 dramatically enhances the development of severe anemia during acute malaria (31), the current investigations were performed with HIV-1-negative children to define the immunological response to malaria in children with SMA as a single disease entity.

The successful resolution of malaria requires a rapid and efficient type 1 immune response for the control of parasitemia, followed by a type 2 immune response that prevents enhanced pathogenesis through attenuation of proinflammatory cytokine production (for a review, see reference 43). This notion is supported by previous studies illustrating that low levels of anti-inflammatory (i.e., IL-10) relative to proinflammatory (i.e., TNF-α) cytokine production are associated with an enhanced severity of malarial anemia (19, 30, 34). We have previously shown that low levels of IL-10 relative to those of IL-12 are also associated with an enhanced severity of disease in children with hyperparasitemia and mild to moderate forms of malarial anemia (34). These findings appear important since IL-12 polarizes the inflammatory cascade towards a type 1 immune response, at least in part, through the generation of IFN-γ, which is important for the control of parasitemia (9, 44). Decreased production of IL-12 in murine models of malaria is also associated with severe disease and increased mortality (36) as well as with ineffective erythrocytosis (25). Previous studies of Gabonese children demonstrated that the acquisition of PfHz by neutrophils was the most significant factor associated with the suppression of IL-12 (20). Unlike the results of previous investigations in Gabon (20), there was a relatively small number of children in the current cohort with PCN (<4%) (data not presented), suggesting that PCM rather than PCN are the likely source of IL-12 suppression in children with SMA in this region. Since monocytes are a primary cellular source of circulating IL-12p70 (10), we hypothesized that ingestion of PfHz by monocytes is one of the primary mechanisms responsible for suppression of IL-12p70. The results presented here demonstrate that PfHz naturally acquired by monocytes is associated with increased production of IL-10 and TNF-α relative to that of IL-12p70. These in vivo results
support a model in which PfHz-induced increases in both IL-10 and TNF-α could be responsible for suppression of IL-12.

Previous studies with cultured PBMC demonstrated that phagocytosis of Hz causes dysregulation in IL-12, IL-10, and TNF-α production (3, 11, 15, 16, 26, 35, 40), suggesting that the acquisition of Hz by blood mononuclear cells may be responsible for the altered levels of cytokine production observed in children with natural infections. To extend these findings and to determine the importance of cellular interactions between monocytes and lymphocytes, in vitro experiments were performed with cultured PBMC and CD14⁺ and CD14⁻ cells from malaria-naive donors. These experiments showed that the acquisition of PfHz and sHz by monocytes (CD14⁺ cells) suppresses IL-12p70 production and increases IL-10 and TNF-α production, while cultured CD14⁻ cells (primarily CD4⁺ and CD8⁺ T cells and B cells) failed to generate detectable amounts of IL-12p70, IL-10, and TNF-α. Thus, although CD3⁺ T cells are important in the early phases of the immune response to pRBC (38), the results presented here illustrate that cytokine dysregulation generated by mononuclear cell ingestion of PfHz occurs independently of direct T-cell interactions.

Additional investigations examining the temporal kinetics of transcript formation in PfHz-treated cells revealed that the ingestion of physiologically relevant concentrations of PfHz down-regulated de novo IL-12p40 transcript formation and up-regulated IL-10 and TNF-α gene expression. The suppression of IL-12 in our in vitro model is in contrast to previous results showing that detergent-purified PfHz increases IL-12 production in activated dendritic cells (8). However, it is important that mature dendritic cells comprise ~0.7% of blood mononuclear cells in children with severe malaria (46) and would therefore not be likely to contribute substantially to

**FIG. 3.** In vitro effects of PfHz on PBMC and CD14⁺ cell cytokine production. PBMC (1 × 10⁶ cells/ml) (A, C, and E) or CD14⁺ cells (1 × 10⁶ cells/ml) (B, D, and F) from healthy, malaria-naive donors (n = 15 and n = 3, respectively) were stimulated with medium alone or with LPS (100 ng/ml) and IFN-γ (200 U/ml) in the absence or presence of various concentrations of PfHz (10, 1.0, and 0.1 μg/ml) or sHz (10 μg/ml). Supernatants were removed at 48 h for IL-12p70 (A and B), IL-10 (C and D), and TNF-α (E and F) determinations. Values are means ± standard errors of the means (SEM). Statistical significance was determined by the Mann-Whitney U test. *, P < 0.05 compared to unstimulated conditions; **, P < 0.05 compared to LPS and IFN-γ stimulation.

**FIG. 4.** Temporal kinetics of PfHz-induced IL-12p35, IL-12p40, IL-10, and TNF-α transcripts. PBMC (1 × 10⁶ cells/ml) were stimulated with medium alone (dashed black lines), with LPS (100 ng/ml) and IFN-γ (200 U/ml) (dashed gray lines), or with LPS and IFN-γ in the presence of PfHz (10 μg/ml) (solid black lines). Cells were collected at 2, 4, 8, 24, and 48 h for IL-12p55 (A), IL-12p40 (B), IL-10 (C), and TNF-α (D) mRNA determinations by real-time RT-PCR. Values are means ± SEM (n = 3). Statistical significance was determined by the Mann-Whitney U test. *, P < 0.01 compared to unstimulated conditions; **, P < 0.01 compared to LPS and IFN-γ stimulation.
circulating IL-12p70 concentrations during the erythrocytic stage of malaria.

Since the in vivo and in vitro findings supported a model in which both IL-10 and TNF-α could be responsible for IL-12 suppression, we examined the effects of neutralizing antibodies and the addition of exogenous IL-10 and TNF-α on IL-12 production. These investigations demonstrated that neutralization of endogenous IL-10 restored IL-12p70 production, while the exogenous addition of IL-10 dose-dependently decreased IL-12p70 synthesis in PHFz-treated PBMC from malaria-naïve donors. In contrast, the blockade of TNF-α and exogenous addition of TNF-α had no effect on IL-12p70 production, suggesting that PHFz-induced overproduction of TNF-α is not responsible for the suppression of IL-12p70. These findings were then extended to children with SMA to confirm if IL-10

FIG. 5. Role of PHFz-induced IL-10 and TNF-α overproduction in IL-12p70 suppression. PBMC (1 × 10^6 cells/ml) were stimulated with medium alone, with LPS (100 ng/ml) and IFN-γ (200 U/ml), or with LPS and IFN-γ in the presence of PHFz (10 μg/ml). IL-10 neutralizing antibody (0.1, 0.5, or 1.0 μg/ml) (A), exogenous IL-10 (0.2, 2.0, or 20 ng/ml) (B), TNF-α neutralizing antibody (1.0, 10, or 100 ng/ml) (C), or exogenous TNF-α (0.2, 2.0, or 20 ng/ml) (D) was added to stimulated PBMC in the presence of PHFz (solid black lines), and supernatant concentrations of IL-12p70 were measured at 48 h. Values are means ± SEM (n = 3). Statistical significance was determined by the Mann-Whitney U test. *P < 0.05 compared to unstimulated conditions; **, P < 0.05 compared to LPS and IFN-γ stimulation; ***P < 0.05 compared to PHFz-treated conditions. (E) PBMC (1 × 10^6 cells/ml) were obtained from children with SMA (n = 12) and cultured in the presence of medium alone (controls), LPS (100 ng/ml) and IFN-γ (200 U/ml), or LPS and IFN-γ in the presence of IL-10 neutralizing antibody (IL-10 Ab; 0.5 μg/ml) or exogenous IL-10 (exo. IL-10; 2.0 ng/ml). IL-12p70 production was determined in supernatants at 48 h. Statistical significance was determined by the Mann-Whitney U test. *P < 0.05 compared to unstimulated (control) conditions; **P < 0.05.
was responsible for the suppression of IL-12. Experiments with cultured PBMC from children with SMA demonstrated that the blockade of IL-10 restores IL-12p70 production and that the exogenous addition of IL-10 suppresses LPS- and IFN-γ-induced IL-12p70 formation. These results are consistent with findings for cultured splenic macrophages from malaria-infected mice illustrating that IL-10 is responsible for decreased IL-12 production (49). In addition to IL-12, other inflammatory mediators, such as prostaglandin E2 (PGE2), can down-regulate IL-12 production (47). Our previous studies, however, showed that PGE2 is suppressed in children with severe malaria (33, 34) and that PfHz down-regulates cylooxygenase-2-derived PGE2 production (16), suggesting that PGE2 is not responsible for suppression of IL-12p70 in children with SMA.

We show here for the first time that PfHz-induced overproduction of IL-10, but not TNF-α, is responsible for suppression of IL-12p70 through decreased expression of IL-12p40 transcripts. Although current research is focused on designing/implementing effective vaccines for the prevention and treatment of malaria, the present study suggests that suppression of IL-12 by PfHz is an important factor that must be considered in the design and testing of malaria vaccine constructs.

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