

## Relationship between Plasma Interleukin-12 (IL-12) and IL-18 Levels and Severe Malarial Anemia in an Area of Holoendemicity in Western Kenya

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**In this study, we investigated whether levels of interleukin-12 (IL-12) and IL-18 in plasma are associated with severe malarial anemia outcomes in an area of holoendemicity in western Kenya. We compared plasma IL-12 and IL-18 levels in six groups of children grouped into the categories aparasitemic, asymptomatic, mild malaria, high-density uncomplicated malaria (UC), moderate malarial anemia (MMA), or severe malarial anemia (SMA). IL-12 levels were significantly reduced in children with SMA ( $P < 0.05$ ) but not in other groups compared to children in the aparasitemic control group. IL-18, a cytokine known to be critical for the induction of gamma interferon along with IL-12, was produced more frequently (70%) in children with UC ( $P = 0.06$ ) than in children in the aparasitemic control group (32%). However, in the SMA group the IL-18 response rate declined to 30%, which was similar to that in the aparasitemic control group, which showed a 32% response rate. This finding suggests that the IL-18 response may be impaired in children with SMA. In summary, the results from this study support the hypothesis that impairment of IL-12 and/or IL-18 response may contribute to the development of severe malarial anemia in areas of holoendemicity for malaria.**

Malaria remains a major global public health problem, causing 1 to 2 million deaths and 300 to 500 million clinical infections per year (26). *Plasmodium falciparum* is the most virulent human malaria parasite, causing cerebral malaria and severe malarial anemia (SMA), the two major severe disease outcomes associated with mortality (27). In areas of holoendemicity, such as western Kenya, SMA is the most common morbidity associated with *P. falciparum* malaria (1).

Cytokines are known to play an important role in the protective immunity (25) as well as in the pathogenesis of SMA (10). The development of malarial anemia appears to be complex and may involve multiple mechanisms, including dyserythropoiesis (10). Earlier studies have identified the fact that high levels of tumor necrosis factor alpha (20) and low levels of interleukin-10 (IL-10), in relation to tumor necrosis factor concentration, are associated with SMA in areas of endemicity (7, 15). The role of other cytokines in the pathogenesis of SMA remains to be investigated.

IL-12 is a major cytokine involved in the control of CD4<sup>+</sup> Th1 responses and NK cells. IL-12 has been shown to be involved in protective immunity against malaria by regulating gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha, and nitric oxide responses in experimental studies (22). In vivo administration of IL-12 was also shown to be protective against malaria in monkeys (5). IL-12 production has been shown to be defective in A/J mice, which are susceptible to *Plasmodium*

*chabaudi* infection-induced anemia, but not in resistant B6 mice (19). Mohan et al. (12) have also shown that serum obtained from *P. chabaudi*-resistant B6 mice enhanced erythropoiesis in vitro, whereas anti-IL-12 antibodies neutralized this activity. Additionally, they have shown that administration of IL-12 enhanced erythropoiesis in *P. chabaudi*-infected susceptible A/J mice (13, 14). Recent studies of African children, especially in areas with seasonal transmission, have shown that IL-12 levels are lower in children with severe disease than in children with mild malaria (8, 9, 16). Since the Gabonese study (8) combined patients with malarial anemia and those with high-density *P. falciparum* infection for the severe disease category, it was not possible to differentiate whether lower IL-12 production occurs more severely in anemic patients than in patients with high-density infection. Furthermore, it is not known whether the decline in IL-12 production occurs in children with severe disease in other areas of malarial endemicity. Therefore, it is important to determine whether the IL-12 decline is associated with the pathogenesis of SMA in different areas of endemicity.

It is becoming increasingly clear that IL-18 is an important cytokine in controlling IFN- $\gamma$  production and activation of Th1 and NK cells (3). IL-18 is structurally related to IL-1 $\beta$  and is produced by monocytes and macrophages in response to microbial products such as lipopolysaccharides (3). Experimental studies have shown that IL-18 plays a dominant role in the IL-12-mediated IFN- $\gamma$  induction in T cells (3). In mice deficient for the IL-18 gene, little or no IFN- $\gamma$  is produced despite the presence of IL-12, which suggests that IL-18 is a key player in regulation of IFN- $\gamma$  production (4, 23). Furthermore, IL-12 and IL-18 synergistically up regulate IFN- $\gamma$  production of mac-

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TABLE 1. Study participant characteristics and the summary of cytokine responses

Child group	No. of subjects	Median age (range) (yr)	Temp <sup>a</sup> (°C) (n) <sup>i</sup>	Parasitemia <sup>a</sup> (10 <sup>3</sup> parasites/μl)	Hemoglobin concn <sup>a</sup> (g/dl)	IL-12 concn <sup>a</sup> (pg/ml)	No. of IL-18 responders/total no. of subjects (%)
Aparasitemic <sup>b</sup>	19	9 (3–12)	36.7 ± 0.4 (19)	0	11 ± 1.8	254 ± 334	6/19 (32)
Asymptomatic <sup>c</sup>	20	8 (5–11)	36.5 ± 0.3 (19)	1.9 ± 4.5	10.5 ± 3.3	82 ± 84	3/20 (15)
Mild malaria <sup>d</sup>	17	6 (2–11)	38.6 ± 0.9 (17)	3.2 ± 4.2	10.9 ± 2.9	197 ± 252	9/17 (53)
UC <sup>e</sup>	10	4 (3–11)	38.3 ± 1.3 (10)	40 ± 28.4	11.8 ± 1.6	88 ± 139	7/10 (70)
MMA <sup>f</sup>	13	3 (2.5–7)	38.3 ± 1 (12)	32.3 ± 49.1	7 ± 0.9	98 ± 142	6/13 (46)
SMA <sup>g</sup>	10	3 (2–6)	37.6 ± 0.9 (10)	27.9 ± 28.6	4 ± 0.6	36 ± 54	3/10 (30)

<sup>a</sup> Data are arithmetic means ± standard deviations.

<sup>b</sup> Aparasitemic children were those who had neither detectable levels of *P. falciparum* parasitemia nor fever.

<sup>c</sup> Asymptomatic children had *P. falciparum* parasitemia but no febrile symptoms.

<sup>d</sup> Mild malaria was defined as a febrile illness (temperature > 37.5°C) in the presence of asexual parasitemia (<8,000 parasites/μl) without severe anemia.

<sup>e</sup> UC malaria was defined as presence of high-density *P. falciparum* parasitemia (≥8,000 parasites/μl) with fever (axillary temperature >37.5°C) but no severe anemia (Hb > 8 g/dl).

<sup>f</sup> The MMA group included children with any densities of *P. falciparum* infection, fever, and hemoglobin levels of <8 but >5 g/dl.

<sup>g</sup> The SMA group included children with fever and any levels of *P. falciparum* parasitemia with anemia (Hb level of ≤5 g/dl).

<sup>h</sup> The arithmetic means ± standard deviations are given. The median values were significantly different between the aparasitemic control group and the SMA group ( $P < 0.05$ ).

<sup>i</sup> n, total number of subjects in each group.

rophages, T cells, and NK cells including the enhancement of NK- and T-cell-mediated cytotoxic activities (6, 11, 18). In a recent preliminary study, IL-18 was found to be elevated in adults with uncomplicated *P. falciparum* malaria (24). However, the role of IL-18 in the pathogenesis of malarial anemia is not yet known. In this study, we investigated whether plasma cytokine levels of IL-12 and IL-18 are altered in Kenyan children with a spectrum of *P. falciparum* malaria infections.

#### MATERIALS AND METHODS

**Study area.** This study was conducted in the population living in and around Kisumu Town of western Kenya. *P. falciparum* accounts for approximately 97% of the malarial infections in this area. Malaria is transmitted perennially in this area, with two peak transmissions occurring during and after rainy seasons in March to May and October to November. This is one of the areas with the highest malaria transmission rates in the world, with an estimated 100 to 300 infective mosquito bites per year (2).

**Study population.** More than 90% of the residents in the area of Kisumu are of the Luo tribe. Children aged 2 to 12 years admitted to the Kisumu District hospital were enrolled for the study after informed written consent was obtained. The Institutional Review Boards of the Kenya Medical Research Institute (KEMRI) and Centers for Disease Control and Prevention approved the study. Participants were divided into the following six categories: healthy aparasitemic control, asymptomatic control, mild malaria, high-density uncomplicated malaria (UC), moderate malarial anemia (MMA), and SMA. Aparasitemic and asymptomatic control children were recruited from rural communities in the study area, while children in the rest of the groups were enrolled in the Kisumu District hospital. Aparasitemic children were those who did not have any detectable levels of *P. falciparum* parasitemia or fever. Asymptomatic children had *P. falciparum* parasitemia but had no febrile symptoms. The mild malaria group included children with an asexual *P. falciparum* parasitemia of <8,000 parasites/μl with fever (temperature > 37.5°C) but without any other symptoms of severe malaria. The UC group included children with an asexual *P. falciparum* parasitemia of ≥8,000 parasites/μl with fever (temperature > 37.5°C) but without any other symptoms of severe malaria (hemoglobin [Hb] level > 8 g/dl). The MMA group included children with any densities of *P. falciparum* infection, fever, and Hb levels <8 g/dl but >5 g/dl. The SMA group included children with fever and any levels of *P. falciparum* parasitemia with anemia (Hb level ≤ 5 g/dl). Children who were severely immunosuppressed, malnourished, or had other concurrent infections that may cause fever, as well as children admitted for malaria but already on medication for a period of more than 4 h, were excluded from the study. In the aparasitemic, asymptomatic, MMA, and SMA groups the male and female children were approximately in equal proportions. In the mild malaria group and the UC group about 60% of the children were males and 40% of the children were females. Patient characteristics are described in Table 1.

**Blood sample collection.** The presence of *P. falciparum* parasitemia was determined using a Giemsa-stained thick blood smear of blood obtained by finger

prick. The number of asexual-stage parasites per 300 white blood cells was counted, and the density of parasitemia per microliter of blood was estimated by the following formula: (parasite count/300) × 8,000 (where 8,000 is the mean number of white blood cells/μl of blood). Hb levels were determined using the HemoCue spectrophotometer (Hemocue AB, Angelholm, Sweden). Five milliliters of venous blood was collected from the enrolled children using sterile heparinized Vacutainers (Becton Dickinson, Rutherford, N.J.). The plasma was immediately separated by centrifuging at 209 × g for 10 min, aliquoted, and frozen rapidly in a liquid nitrogen container.

**Cytokine enzyme-linked immunosorbent assay.** A double sandwich enzyme-linked immunosorbent assay was used to measure plasma cytokine levels as previously described (14). IL-12 was detected using the OptEIA kit from BD Pharmingen (San Diego, Calif.). IL-18 was detected using a capture mouse monoclonal anti-human IL-18 antibody (clone 52713.11) and a biotinylated goat anti-human IL-18 antibody from R&D Systems Inc. (Minneapolis, Minn.). Cytokine concentrations were determined using a standard curve obtained from the known concentration of cytokine standards included in each assay plate.

**Statistical analysis.** Each sample is considered to be independent from the others. Data were analyzed using SAS (Cary, N.C.) software (version 8.2). *P* values of <0.05 were considered statistically significant. The IL-12 data were analyzed using nonparametric methods. Overall association of IL-12 levels between the groups was assessed using the Kruskal-Wallis one-way analysis of variance by ranks, comparing the sample medians of IL-12. Between groups, comparisons of median values were assessed, adjusting for multiple comparisons using proc multtest in the SAS package. The association between age and IL-12 concentration was assessed using the Spearman rank correlation coefficient.

Since only 38% of the subjects had an IL-18 response, we used a logistic regression predicting the probability an IL-18 response versus the probability of no response rather than the difference in the IL-18 concentrations. The overall association between groups (for IL-18 responder rate) was assessed, and differences between the specific groups were compared using the SimIntervals macro to adjust for multiple testing. The association between age and the probability of IL-18 response was assessed, but there was no significant difference.

#### RESULTS

Plasma cytokine levels were compared among six different clinical groups—aparasitemic children; asymptomatic children; and children with mild malaria, UC, MMA, and SMA—as described in Table 1.

**IL-12 levels.** The mean IL-12 concentrations in different groups are given in Table 1, and the actual cytokine concentrations in each individual tested are given in Fig. 1A. Ninety-two percent of the participants had detectable levels of IL-12. The highest level of plasma IL-12 was found in the aparasitemic group, and this cytokine level declined progressively as

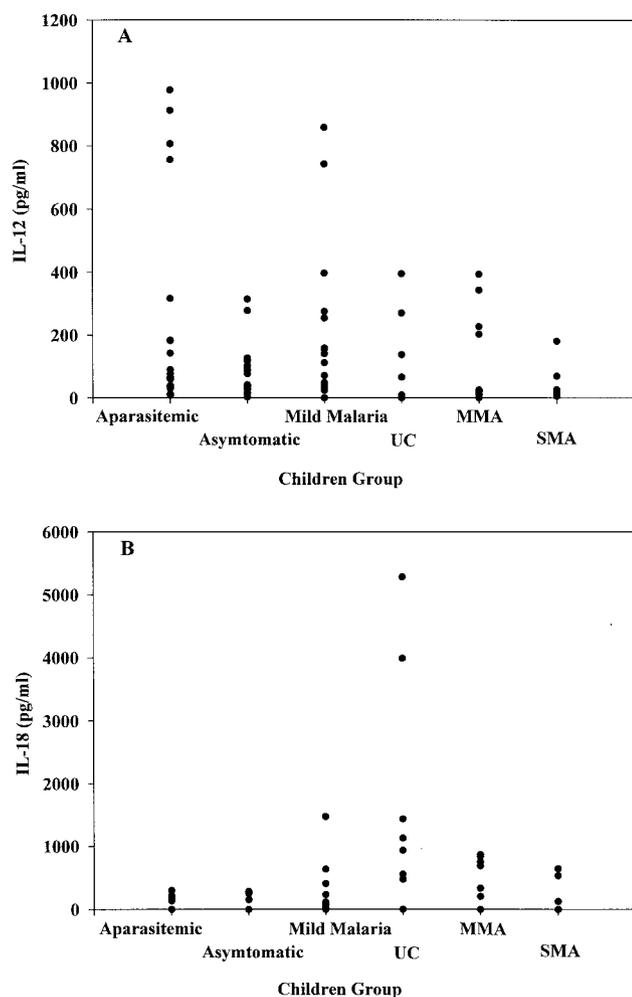


FIG. 1. (A) IL-12 levels in plasma of children with malaria. The values represent the actual plasma IL-12 concentration in each individual tested. The mean IL-12 levels are reported in Table 1. The median and interquartile range (in parentheses) for the aparasitemic, asymptomatic, mild malaria, UC, MMA, and SMA groups were 76 (239), 59 (83), 111 (221), 7 (137), 20 (201), and 15 (16) pg/ml, respectively. The difference between the aparasitemic and SMA group was significantly different ( $P < 0.05$ ). There were no significant differences between other groups. (B) IL-18 levels in plasma of children with malaria. The values are actual plasma IL-18 concentrations in each individual tested. Since most of the children in the aparasitemic and asymptomatic control groups did not have detectable IL-18, only the difference in the response rate was determined, and this is reported in Table 1.

the severity of the disease increased (Fig. 1A). The median IL-12 concentration was significantly lower in children with SMA compared to children in the aparasitemic control group ( $P < 0.05$ ). Age was not significantly associated with IL-12 concentration. The rank correlation coefficient (0.16) was not significantly different from zero ( $P = 0.13$ ).

**IL-18 levels.** Unlike IL-12 levels, levels of IL-18 in plasma were below the level of detection in a majority of control children (aparasitemic and asymptomatic groups). Among all the subjects tested, only 38% of the children had detectable levels of plasma IL-18 (Table 1). The plasma concentrations of IL-18 in each individual tested are given in Fig. 1B. It is evident

from this figure that levels of IL-18 in plasma showed an increased trend following symptomatic malaria infection (mild malaria group) and peaked in the UC group. Interestingly, in the MMA and SMA groups a progressive decline in the IL-18 responses was noticed. Since many children had IL-18 levels below the detection limit, we compared the probability of the presence of IL-18 responses instead of mean IL-18 concentrations (Table 1). The UC group had the highest responder rate, 70%, and a marginal difference was noted when compared to the asymptomatic control group ( $P = 0.06$ ). Among symptomatic malaria-infected children, the SMA group showed the lowest responder rate, 30%, which was similar to the 32% responder rate in the aparasitemic control group (Table 1). Age was not statistically significant in predicting the probability of an IL-18 response ( $P = 0.46$ ).

**IFN- $\gamma$  levels.** In all the groups, the mean IFN- $\gamma$  levels were very low ( $<15$  pg/ml) as reported in our previous study (15) and there was no significant difference in the levels of IFN- $\gamma$  among the groups (data not shown). Due to the poor IFN- $\gamma$  response, it was not possible to determine if plasma IFN- $\gamma$  levels are directly related to plasma IL-12 and IL-18 levels.

## DISCUSSION

Our study showed that levels of IL-12 and IL-18 in plasma were lower in children with SMA than in the aparasitemic and UC groups, respectively. The finding that IL-12 was significantly lower in children with SMA is in agreement with rodent studies (13, 19) and previous human studies involving Gabonese children (8, 16). Since the Gabonese study combined patients with SMA and those with high-density *P. falciparum* infection (irrespective of anemia status) for the severe disease category, it was not possible to differentiate whether lower IL-12 production occurs more severely in anemic patients than in patients with high-density infection but without signs of anemia. Since our study included different clinical groups, it was possible to determine how the IL-12 response alters in relation to clinical status of malaria. Our study illustrated that the lowest level of IL-12 was in children with SMA although children with MMA and UC also had lower levels of IL-12. Thus, our findings closely agree with the results from a recent study conducted in Burkina Faso, where the levels of IL-12 have been shown to be significantly reduced in children with severe disease compared to those with mild malaria (9). Overall, the results from our investigation were consistent with the hypothesis that defective IL-12 production may contribute to the development of malaria-associated anemia in humans as it has been demonstrated in experimental model systems (12–14, 19).

Further studies are required to understand the mechanisms involved in the down regulation of IL-12 responses in children with severe manifestations of malaria. Luty et al. (8) have suggested that malaria pigments may contribute to the suppression of IL-12 production. Using a rodent model, Xu et al. (28) have shown that IL-12 p40 gene expression is profoundly inhibited by *Plasmodium berghei* infection. They have further shown that IL-10 may play a role in this inhibition and that it may be regulated by transcriptional regulation of the IL-12 gene (28).

Although the exact mechanism by which lower IL-12 pro-

duction may contribute to anemia development is not known, several possibilities can be considered. Since IL-12 is involved in directly activating protective immunity against both liver stage and blood stage parasites, a severe defect in the IL-12 response could directly affect protective immune pathways and contribute to anemia development. Another potential mechanism may involve a direct role for IL-12 in the erythropoiesis. This view is supported by experimental studies that have shown involvement of IL-12 in the regulation of erythropoiesis (12). Serum obtained from *P. chabaudi*-resistant B6 mice enhanced erythropoiesis in vitro, whereas this activity was neutralized by anti-IL-12 antibodies (12). In addition, therapy with low doses of combined IL-12 and chloroquine was shown to cure parasitemia as well as significantly up regulate erythropoiesis in *P. chabaudi*-infected mice (14). Therefore, further studies are warranted to determine the mechanisms associated with down regulation of IL-12 and its significance in the manifestation of SMA.

We also have shown that IL-18 levels were lower or absent in healthy children but elevated following symptomatic *P. falciparum* infection as reported in a previous study (24). In contrast to IL-12, which was reduced especially in children with severe malaria outcomes, IL-18 was up regulated in children with UC malaria but progressively declined in children with MMA and SMA. One potential explanation for this observation is that IL-18 may be induced in response to active malaria infection, and those who mount a strong IL-18 response may be able to compensate for the poor IL-12 response and clear parasitemia through IFN- $\gamma$ -dependent activation of T cells or NK cells. It is evident from previous studies that IL-18 is a critical factor for the activation of naive human T cells and NK cells (3). Mice deficient in IL-18 cannot mount a strong IFN- $\gamma$  response despite the presence of normal levels of IL-12 (23). Furthermore, IL-18 can compensate for IL-12 in knockout mice, and both of these cytokines can synergistically activate both T and NK cells (3, 6, 11, 18). More importantly, in a rodent study, treatment with IL-18 has been shown to increase IFN- $\gamma$  levels in mice infected with *Plasmodium yoelii* 265 or *P. berghei* ANKA. This also led to a delay in the onset of parasitemia and conferred protection (21). IL-18 knockout mice were more susceptible to *P. berghei* ANKA than were wild-type mice of the same genetic background. (21). Altogether, the results from our present study and the experimental findings in the rodent study (21) are consistent with the hypothesis that down regulation of the IL-18 response may lead to severe malaria disease outcome such as SMA.

Since the levels of IFN- $\gamma$  in plasma were generally poor in these children, it was not possible to determine whether lack of an IL-12 or IL-18 response also contributed to the defective IFN- $\gamma$  production. This observation is consistent with our previous report, which showed a general lack of serum IFN- $\gamma$  response in children residing in the area (15). A recent study has also shown that peripheral blood mononuclear cells from individuals residing in an area of high malarial endemicity produced very low levels of IFN- $\gamma$  compared to individuals from an area of low endemicity when stimulated with malarial antigen in vitro (17). It is possible that levels of IFN- $\gamma$  in plasma may not be strong predictors of systemic IL-12 and IL-18 responses, and in vitro cellular studies may be needed to

test whether poor IL-12 and IL-18 responses in malaria anemic patients lead to defective IFN- $\gamma$  production.

In summary, our findings are consistent with the hypothesis that impairment of IL-12 and/or IL-18 may significantly contribute to the development of anemia in malaria patients. Further studies are needed in other areas of endemicity to validate this hypothesis and to find out the mechanism by which impairment of IL-12 and IL-18 responses occur in vivo.

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