

Plasma Levels of Tumour Necrosis Factor-Alpha, Interleukin-10, Interleukin-12, Macrophage Inhibition Factor and Transforming Growth Factor-Beta in Children with Severe and Uncomplicated Falciparum Malaria

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Abstract

The outcome of an infection appears to hinge on a delicate balance between appropriate and inappropriate induction of inflammatory/anti-inflammatory cytokines. However, the role of these mediators in the pathogenesis of severe malaria remains controversial. In this study, plasma levels and ratios of the pro- (TNF- α , IL-12 and MIF), and the anti-inflammatory (IL-10 and TGF- β) cytokines were determined and compared in patients with cerebral malaria (CM), severe malaria anaemia (SMA), uncomplicated malaria (UM), and healthy control (HC) children below 14 years of age.

TNF- α , IL-10 and TGF- β levels were significantly different among the four clinical groups, while IL-12 and MIF levels were similar among the clinical groups. TNF- α level were higher in SMA and UM, when compared with HC. Additionally, TNF- α levels were higher ($P=0.002$) in the combined severe malaria group (CM+SMA, 46.31 ± 44.43), compared to the combined control (UM+HC) group (25.59 ± 26.64). The HC group had lower and higher levels of anti-inflammatory cytokines, IL-10 and TGF- β respectively, when compared to each of the three patient categories. The levels of both IL-10 and TGF- β were significantly higher in UM compared to SMA. A comparison between the ratios of pro- to anti-inflammatory cytokines revealed a significantly higher TNF- α /IL-10 ratio in the HC group, compared to each of the patient categories. Malaria parasite density also correlated positively with the levels of TNF- α and IL-10, but negatively with TGF- β level. TNF- α levels also correlated positively with IL-10 and MIF, but negatively with TGF- β levels. Furthermore, a significant negative correlation was observed between IL-10 and TGF- β levels.

In conclusion, this study confirms a pathogenic role for TNF- α , with higher ratios of TNF- α to IL-10, and TGF- β associated with severe malaria anaemia in children residing in an endemic area.

Keywords: Cytokines; Severe malaria; Uncomplicated malaria; Children

Introduction

Monokines and lymphokines are crucial for induction of immune effector mechanisms against many pathogens. The outcome of an infection appears to hinge on a delicate balance between appropriate and inappropriate induction of these inflammatory/anti-inflammatory mediators [1]. In murine malaria, it has been proposed that innate immune responses mediated by natural killer (NK) cells, and/or γ/δ T cells, limit the initial phase of parasite replication [2], and that adaptive responses (mediated by T cells and B cells) are required for parasite elimination [3].

Nevertheless, excessive release of proinflammatory cytokines during a *Plasmodium falciparum* infection is reported to be the primary driving force of severe disease and eventual death [4-7]. In fact, the difference between lethal and non-lethal malaria infection can be explained, in part, by the ability of mice to stimulate an early IL-12, IFN- γ or TNF- α response [7-9]. In line with this, studies have shown that the neutralisation of IL-12 or blocking the IFN- γ pathway abrogates mortality [6,10]. Circulating levels of TNF- α have been shown to correlate with disease severity in African children [11]. However, anti-TNF monoclonal antibody is unable to reverse childhood severe malarial disease [12], suggesting that other factors may be involved in the pathogenesis of this disease. Increasing levels of the migration-

inhibition factor (MIF), a pro-inflammatory mediator, released upon macrophage ingestion of parasite-infected erythrocytes, or malaria pigment (haemozoin) correlated with severe anaemia, and thus MIF may be involved in the pathophysiology of malaria anaemia [13]. MIF is known to be a counter-regulator of gluco-corticoid action, and inhibits erythroid, multipotential and granulocyte-macrophage colony formation.

The anti-inflammatory cytokine IL-4 is not essential for limiting cytokine-induced tissue damage [14,15], but IL-10-deficient mice infected with *P. chabaudi chabaudi* as show higher mortality than their normal littermates [16], suggesting a protective role for this cytokine.

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IL-10 production also appears to be important in the induction and maintenance of immunity to *P. falciparum* in naturally exposed populations [17,18]. Importantly, down regulation of TNF- α production and consequent resistance to severe malaria, has been linked to the ability to produce the immuno-regulatory cytokine, Transforming Growth Factor (TGF)- β [19]. Low levels of TGF- β have been associated with acute [20], and severe malaria [21,22]. A relative deficiency in IL-10 [23], and lower ratios of IL-10 to TNF- α has also been recorded in patients with severe malarial anaemia [24,25]. Conversely, high ratios of TNF- α to TGF- β or IL-10 are associated with decreased risk of malaria infection [26]. Thus, the importance of TGF- β or IL-10 as immuno-regulatory molecules requires further investigation.

In this study, we determined and compared plasma levels and ratios of the pro- (TNF- α , IL-12 and MIF), and the anti-inflammatory (IL-10 and TGF- β) cytokines in patients with cerebral malaria, severe malaria anaemia, or uncomplicated malaria, and healthy control children below 14 years of age.

Materials and Methods

Study area

The study was carried out between April 2003 and December 2005 in Fako division of South West Cameroon, where malaria transmission is perennial. The study sites included hospitals (Regional Hospital Limbe and Bota District Hospital Limbe), health centres (Mount Mary Health Centre Buea, Bokova Health Centre Buea and PMI Down Beach Limbe) and primary schools (Catholic School (CS) Muea, CS Buea Station and CS Great Soppo, Government School (GS) Bolifamba, GS Bonduma, Government Practising School (GPS) Molyko I and II, GPS Muea I and II). This region experiences two seasons: the dry (November-March) and the rainy (March-October, when malaria transmission is more intense) seasons. *P. falciparum* causes more than 90% of malaria infections in this area, and *Anopheles gambiae* is the dominant vector species [27]. Malaria prevalence attains 70-85% during the peak transmission season, which drops to 30% during the low transmission season, the dry season [28].

Study population

The study participants were part of a larger study investigating the role of antibodies and gene polymorphism in the pathogenesis of severe malaria. They consisted of malaria infected and apparently healthy children 1-14 years old, reporting for medical attention at health care facilities, or attending the primary schools, respectively. Ethical clearance (2005-12-012) was obtained from the Institutional Review Board of the University of Buea, Cameroon. Authorization to conduct the school surveys was obtained from the Regional Delegate of Basic Education, South West. In addition, at each school, informed consent forms explaining in details the project objectives, protocols and benefits were sent through the children to their parents/guardians. Only children whose parents consented in writing were recruited into the study. During the school surveys, information relating to gender, age and area of residence were recorded. The axillary temperature of each pupil was obtained using a digital thermometer. Only children with a body temperature below 37.5°C were recruited as healthy controls. Febrile children with malaria parasitaemia were included in the uncomplicated malaria group.

For the malaria patients, informed consent was obtained from the parent, and/or guardian at consultation in the respective health institutions. Using a well-structured case report form, volunteers were recruited into the following groups, after due consideration of the criteria indicated below.

Case definition: Inclusion criteria for malaria patients were, fever ($\geq 37.5^\circ\text{C}$) measured within 24 hours of admission, malaria parasitaemia, and at least one other sign of malaria (vomiting, diarrhoea, malaise, etc). Study participants homozygous for the sickle cell trait were excluded.

Specific inclusion and exclusion criteria: The criteria for recruitment included the standard WHO definitions [29].

Cerebral malaria (CM) cases: Unconscious, with a Blantyre coma score of <3 and duration of coma >60 minutes, any haemoglobin value, no record of recent severe head trauma, and other cause of coma or neurological diseases.

Severe Malaria Anaemia (SMA) cases: Haemoglobin ≤ 5 g/dL, fully conscious, no incidences of severe bleeding reported, or observed convulsions.

Uncomplicated Malaria (UM) Cases: Haemoglobin value >8 g/dL, malaria parasitaemia, and fully conscious.

Controls: These consisted of apparently healthy children (HC) aged 3-14 years, attending the primary schools: afebrile, with or without malaria parasitaemia

Sample collection and processing

Venous blood (2-5 ml) was collected from the study participants into EDTA vacutainer tubes, using sterilized, disposable syringes, for parasitological, haematological and serological studies. The blood samples were centrifuged (Beckman TJ-6, USA) at $2500\times g$ for 5 minutes and plasma supernatants stored in eppendorf tubes (Brinkman Instrument Inc., Westbury, USA), at -86°C , until analysed.

Haematological measurements

Packed Cell Volume (PCV) and Hb measurements: Haemoglobin (Hb) levels of the study children were determined using a HemoCue haemoglobinometer (HemoCue, Anglholm, Sweden). Packed Cell Volume values were also determined using a microhaematocrit centrifuge (HHC-24, Hanshin Medical CO., Ltd, Korea) [29]. The volume of packed cells was read off a Hawksley microhaematocrit reader. White Blood Cell (WBC) and Red Blood Cell (RBC) counts were determined using a haemocytometer (improved Neubauer counting chamber) [30].

Sickle cell test: The rapid screening test [30], was used to determine the sickle cell status of study participants eligible for recruitment into the study. A drop of blood was placed on a slide and mixed with an equal volume of freshly prepared 2% w/v sodium metabisulphite. The wet preparation was placed in a petri dish with a damp piece of tissue. After 20 minutes, the slides were examined microscopically for sickled cells. Positive cases were confirmed by haemoglobin electrophoresis [30], using cellulose acetate membrane (DiaSys, Wokingham, UK).

Parasitological examination: Thick blood films were prepared for all participants, air-dried, stained with Giemsa (Sigma, St. Louis, USA), and examined for malaria parasites by light microscopy. Parasites were detected using the 10X eyepiece of a binocular Olympus microscope (Olympus Optical Co., Ltd, Japan), under oil immersion (100X objectives). Parasites were counted against a minimum of 200 white blood cells for the determination of parasite density [31]. Fifty high power fields (HPF) were scanned to confirm malaria negative slides. Urine samples were also collected from each sick case, and examined for schistosome eggs by microscopy, to eliminate anaemia cases due to schistosome infection. None of the patients were positive for schistosoma.

Enzyme-Linked Immunosorbent Assay for some plasma cytokines: Plasma levels of the cytokines Interleukin-10, IL-12, MIF, TGF- β and TNF- α were measured by ELISA, according to instructions from the manufacturer (Quantikine R&D systems for IL-10, IL-12, TGF- β and TNF- α and DuoSet ELISA Development System for MIF). Briefly, 50 μ l of assay diluent and 200 μ l of standard and samples were added per well to 96-well plates pre-coated with mouse monoclonal antibody against respective cytokine by the manufacturer. Plates were then incubated for 2 h at room temperature, 100 μ l of conjugate added per well, and plates further incubated for 2 h at room temperature. Two hundred microliters of substrate solution (hydrogen peroxide+tetramethylbenzidine) was added per well, and the plates incubated in the dark at room temperature for 20 minutes for TNF- α , and 30 minutes for IL-10. The reaction was topped with 2 N H₂SO₄ (50 μ l per well), and the optical density read at 450 nm within 30 minutes, using a Multiskan ELISA reader (Thermo Labsystems, China). All plates were washed (4 times) between incubation steps and blotted with clean paper towels. The concentrations (in pg/ml) of the cytokines in the samples were extrapolated from logarithmically transformed standard curves. In all the ELISAs, standards/samples were run in duplicates, and tested 10 non-immune Swedish & British sera were used as negative controls to check that the response was specific to malaria infection.

Statistical analysis

Analyses were performed using the software package SPSS

Statistics 17 (SPSS Inc., Chicago, IL). Malaria parasite densities were log transformed before analyses. The Pearson Chi-Square test was used to evaluate group differences in parasite rates, while differences in group means were assessed using the students't-test and analysis of variance (ANOVA). Correlation between variables was determined using Pearson correlation. Statistical significance was set at p \leq 0.05.

Results

A total of 645 age-matched children below 14 years were involved in the study. The gender, mean age and malaria parasitaemia densities of the patient categories and controls are shown in table 1. The mean age of the children was 5.30 \pm 3.22 (range: 1-13 years), with the healthy controls significantly older, when compared to each of the patient categories. The geometric mean malaria parasitaemia density of all the subjects was 4065 parasites per microliter of blood. Malaria parasite load was similar across the patient categories, although healthy controls carried lower parasitaemia, when compared to each of the patient categories (Table 1). There was no significant association between gender and study participant category (p=0.213).

Participants were selected at random from the four clinical groups for the cytokine assays and the mean plasma levels of the cytokines, compared among the groups (Figure 1). IL-10 (p<0.001) and TNF- α (p<0.001) levels were higher, while TGF- β (p<0.001) levels were lower in malaria positives, compared to malaria negatives. However, plasma

Patient category	Gender n (%)		Age (years)		Malaria parasitaemia	
	Males	Females	n	Mean \pm SD	n	GMPD ^a
CM	12 (50.0)	12 (50.0)	25	3.92 \pm 1.94	23	13617
SMA	86 (55.5)	69 (44.5)	122	2.61 \pm 2.25	138	10711
UM	136 (58.4)	97 (41.6)	189	4.55 \pm 3.39 [†]	210	6556
HC	114 (48.9)	119 (51.1)	218	7.62 \pm 1.76 [‡]	129	534 [§]
p-value	0.213			< 0.001		< 0.001

^aGMPD=Geometric mean parasite density (parasites per microliters of blood)

[†]Significantly higher than the corresponding values for UM (p<0.001), SMA (p<0.001) and CM (p<0.001)

[‡]Significantly higher than the corresponding values for SMA (p<0.001)

[§]Significantly lower than the corresponding values for UM (p<0.001), SMA (p<0.001) and CM (p<0.001)

Table 1: Basic characteristics of the study population.

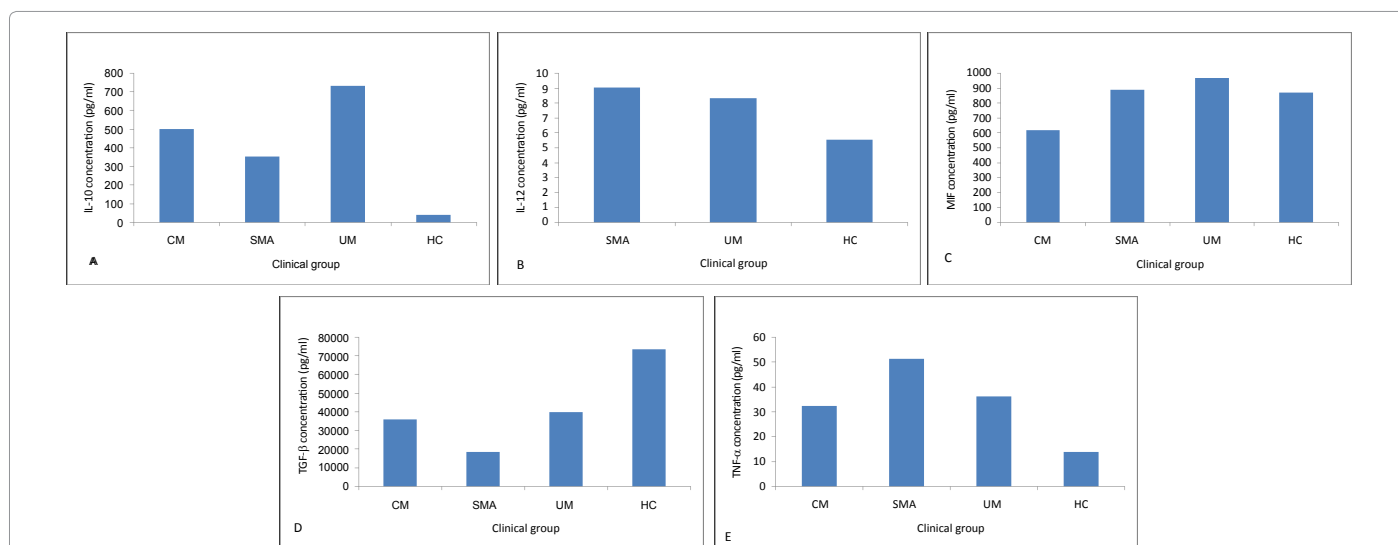


Figure 1: (A-E) Mean plasma levels (pg/ml) of IL-10, IL-12, MIF, TGF- β and TNF- α and in cerebral malaria (CM), severe malaria anaemia (SMA), uncomplicated malaria (UM), and apparently healthy control (HC) children from South Western Cameroon.

IL-12 and MIF levels were similar in malaria positives compared to malaria negatives. Our results also show that IL-10, TGF- β and TNF- α levels were significantly different ($p < 0.001$ each) among the four clinical groups, while IL-12 and MIF levels were similar among the clinical groups.

HC had significantly lower IL-10 levels than their CM ($p = 0.022$), SMA ($p = 0.048$) and UM ($p < 0.001$) counterparts, while the UM had significantly higher levels of the cytokine than the corresponding values for SMA ($p = 0.007$). Similarly, TGF- β levels were significantly higher in HC compared to CM ($p = 0.003$), SMA ($p < 0.001$) and UM ($p < 0.001$), while UM had significantly higher levels than the corresponding value for SMA ($p = 0.020$). Conversely, TNF- α levels were significantly higher in SMA ($p < 0.001$) and UM ($p = 0.040$), compared to HC (Figure 1).

The possible role of the pro-inflammatory cytokines in malaria severity was further assessed by combining the severe malaria groups (CM, SMA), and comparing with the UM or HC groups. There was however, still no difference in IL-12 and MIF levels, when the combined severe malaria group was compared with the UM or HC. When the anti-inflammatory cytokines were compared, the HC group had lower and higher levels of IL-10 and TGF- β respectively, when compared to each of the three patient categories. The levels of both IL-10 and TGF- β were significantly higher in UM compared to SMA (Figure 1).

A comparison between the ratios pro- to anti-inflammatory cytokines revealed a significantly higher TNF- α /IL-10 ratio in the HC group, compared to each the patient categories (Table 2). Further analysis however revealed a marginally significantly higher ($p = 0.055$) TNF- α /TGF- β ratio in the combined severe malaria group (0.0044 ± 0.0139), compared to the combined control (0.0006 ± 0.0009). There was no difference in the ratios of MIF or IL-12 to IL-10 and TGF- β .

Correlation analysis between the different plasma cytokine levels and malaria parasitaemia density are shown on table 3. Our results indicate that malaria parasite density correlated positively with the

Participant Category	n	TNF- α /IL-10 Mean \pm SD	n	TNF- α /TGF- β Mean \pm SD
CM	14	0.125 \pm 0.127	12	0.0018 \pm 0.0013
SMA	40	1.358 \pm 0.391	34	0.0053 \pm 0.0152
UM	35	0.109 \pm 0.174	24	0.0010 \pm 0.0011
HC	26	2.302 \pm 4.039*	22	0.0002 \pm 0.0003
Significance of Difference		$p < 0.001$		$p = 0.171$

†Significantly higher than the corresponding values for CM ($p = 0.005$), SMA ($p = 0.001$) and UM ($p < 0.001$)

For some of the participants, the sample volume was insufficient to run all the analyses.

Table 2: Mean (\pm SD) ratios of plasma TNF- α to IL-10 and TGF- β in cerebral malaria (CM), severe malaria anaemia (SMA), uncomplicated malaria (UM), and apparently healthy control (HC) children from South Western Cameroon.

levels of TNF- α and IL-10, but negatively with TGF- β level. TNF- α levels also correlated positively with IL-10 and MIF, but negatively with TGF- β levels. Furthermore, we obtained a significant negative correlation between IL-10 and TGF- β levels.

Discussion

Immune responses to malaria are likely regulated by the balance between pro- and anti-inflammatory cytokines, whose absolute levels and ratios are known to influence susceptibility to infection and clinical disease outcome [21]. However, variations in human cytokine responses and their relationship to malaria pathogenesis remain controversial [32], with heterodirectional changes in the level of cytokines during the infectious process [33]. The study sought to understand the relationship between severe malaria and cytokine level by measuring and comparing plasma levels and ratios of pro- (TNF- α , IL-12 and MIF), and the anti-inflammatory (IL-10 and TGF- β) cytokines, in well-defined patients groups (cerebral malaria, severe malaria anaemia, uncomplicated malaria) and healthy control children. We hypothesized that higher plasma levels and ratios of IL-10 and TGF- β over IL-12, TNF- α and MIF might provide protection against cerebral malaria and severe malaria anaemia, by down-regulating the severe pathologic effects associated to IL-12, TNF- α and MIF.

This study showed that children in this endemic area with severe disease (severe malaria anaemia) had significantly higher TNF- α levels than children with mild disease (uncomplicated malaria), which confirms earlier findings [25,34]. TNF- α is believed to be the principle mediator of malaria pathology [35,36], and studies suggest that it may contribute to anaemia through the suppression of bone-marrow function and increased destruction of red blood cells, characteristic to malaria. Dyserythropoietic bone-marrow changes have been described morphologically in patients with chronic malaria [37], and in an experimental model of CBA mice infected with *P. vinckei*, administration of TNF- α resulted in a similar dyserythropoiesis in bone marrow and spleen [38]. This pro-inflammatory cytokine is also thought to play a critical role in the pathogenesis of murine cerebral malaria [39]. Moreover, the neutralization of endogenously produced TNF- α with antibodies against TNF- α , and the inhibition of TNF- α production with pentoxifylline [40], prevented the development of experimental cerebral malaria. In humans, TNF- α levels are elevated in individuals with acute *P. falciparum* infections, especially persons with cerebral malaria [35,41]. There was, however, no evidence for the overproduction of TNF- α in the CM group in this study, suggesting that this cytokine may not be a critical factor in the development of cerebral malaria in children from endemic areas. Nevertheless, the plasma concentrations of cytokines correlated with parasitemia, as reported previously [36]. It is possible that the previously reported associations of high levels of TNF- α in children with cerebral malaria may in fact reflect other associated systemic complications [23].

Variable	Correlation coefficient and p value (n)		
	Log ₁₀ PD [‡]	MIF	TGF- β
TNF- α	0.377**; $p < 0.001$ (90)	0.276**; $p = 0.006$ (96)	-0.297**; $p = 0.004$ (92)
IL-10	0.597**; $p < 0.001$ (91)	0.046; $p = 0.654$ (98)	-0.246*; $p = 0.017$ (94)
TGF- β	-0.358**; $p = 0.001$ (87)	-0.142; $p = 0.134$ (113)	
MIF	-0.141; $p = 0.191$ (88)		

‡Log₁₀ Malaria parasite density (parasites per microliter of blood)

*Correlations is significant at the 0.05 significant

**Correlations is significant at the 0.01 significant

For some of the participants, the sample volume was insufficient to run all the analyses.

Table 3: Correlation between malaria parasite density and plasma levels (pg/ml) of IL-10, IL-12, MIF, TGF- β & TNF- α in Cameroonian children.

Successful resolution of blood stage malaria infections in mice depends first, on their ability to mount an early pro-inflammatory cytokine response, and second, on their ability to down-regulate this inflammatory response before the onset of immune pathology. T regulatory cells are important regulators if this balance is to be maintained [42]. Consistent with our findings, studies from rodent models have highlighted the significant role IL-10 plays in down-regulating severe malaria. IL-10 gene knockout mice with an intrinsic deficiency for IL-10 production were shown to succumb to severe disease and higher mortality when infected with *Plasmodium chabaudi chabaudi*, compared to their heterozygote counterparts or normal mice [16]. An enhanced Th1 response persisted throughout the course of infection in the IL-10-deficient mice, while in control mice, a Th2 response was predominant. In another study, the exogenous administration of IL-10 to susceptible CBA mice protected them from *Plasmodium berghei*-induced cerebral malaria, while an *in vivo* neutralization of IL-10 in resistant BALB/c mice induced a neurologic syndrome [43].

Collectively, these findings support the hypothesis that IL-10 is a critical factor in down-regulating the pathogenesis of severe malaria. However, we found lower IL-10 concentrations only in patients with severe anaemia (and not cerebral malaria), compared to mild disease as previously reported [24], suggesting that severe anaemia has a specific pathology. There are several mechanisms by which insufficient IL-10 production could influence the development of anaemia. IL-10 is known to have a direct synergistic effect on erythropoietin-induced erythropoiesis *in vitro* [44]. In addition, IL-10 directs murine osteogenic cells towards bone-marrow formation [45], an effect that is probably due to the down-regulation transforming growth factor production [46].

In the absence of IL-10, osteogenic cells develop into bone tissue, with diminished colony-forming units. Low IL-10 concentrations may also lead to increased production of nitric oxide [47], a molecule that seems to influence erythropoiesis *in vivo* [48], and has been implicated in the pathogenesis of malaria anaemia [49]. IL-10 is also known to suppress the production and function of a wide range of cytokines [50,51], including the mediation of negative-feedback regulation of TNF- α production, after stimulation with malaria antigens *in vitro* [52]. In line with previous reports [24,25], children with uncomplicated malaria had significantly higher IL-10-to-TNF- α ratios than children with severe malaria anemia in this study. This finding is consistent with our hypothesis that the balance between the Th1 cytokine TNF- α and the Th2 cytokine IL-10 is critical in the pathogenesis of severe disease in *P. falciparum*-infected persons resident in malaria-endemic areas. The increased ratio of TNF- α -to-IL-10 in our patients with severe anaemia may, thus, reflect defective regulation of TNF- α function.

Studies of murine malaria [19] indicate that the principal immunoregulatory mechanism that allows parasite clearance to occur without overwhelming pathology is specific induction of TGF- β , during the transition from the innate to the adaptive phase of the immune response. Severe malaria pathology occurs either when TGF- β is not induced, or when TGF- β is induced too early in the course of the infection, which results in down-regulation of the nascent inflammatory response, and allows uncontrolled parasite growth. The low levels of TGF- β in malaria positives compared to malaria negatives found in this study is in agreement with previous reports in Gabonese children with malaria [21], and Southeast Asian adults with acute falciparum malaria [20]. This suggests that reduced TGF- β may be a common feature of malaria. Our results also showed that children with severe malaria

anaemia had significantly lower TGF- β levels than their mild disease counterparts. Previous experiments in rodent malaria show that TGF- β is inversely correlated with severity of infection, and is important in regulating cytokine expression; administration of recombinant TGF- β to *P. berghei*-infected mice significantly decreases plasma levels of TNF- α [1]. Although it is difficult to extrapolate a sequence of events from a single measurement, the low TGF- β /TNF- α ratio shown here in children with severe disease parallels the findings in murine malaria and Gabonese children [21], which suggests that lack of a TGF- β response in children with severe malaria may contribute to their overproduction of TNF- α . The timing of such TGF- β production, which derives from regulatory T cells, seems to be crucial for the successful resolution of murine malaria infections [42].

The analysis of baseline cytokine levels showed strong positive associations between the levels of TNF- α , IL-10 and TGF- β , and malaria parasitaemia density. The tight correlation between TNF- α and TGF- β levels suggests an efficient and accurate counter-regulatory mechanism in most patients, matching the anti-inflammatory cytokine response to the degree of activation of the pro-inflammatory cytokine cascade. The strong correlations we observed between the plasma levels of both TNF- α and IL-10 and parasitemia, confirm earlier findings [24,34,36,53]. The positive correlation between TNF- α and IL-10 has been explained by the high levels of circulating immune complexes documented in severe falciparum malaria, that inhibit IL-12 by human monocytes via TNF- α -induced increases of IL-10 [11]. TGF- β concentrations were however, inversely correlated with malaria parasite density, as reported previously in children from Gabon [21] and Tanzania [22]. TGF- β may thus play a crucial role in establishing the pro-inflammatory/anti-inflammatory cytokine balance that is required for the successful resolution of malaria infections [42].

Since MIF, a regulator of innate immunity [54], is now established as a putative host factor inhibitor of erythropoiesis: the suppression of Erythropoietin-dependent erythroid (BFU-E) and multipotential (CFUGEMM) progenitor cells *in vitro* [13], we hypothesized that MIF produced by macrophages in response to malarial infection could be a factor involved in severe anaemia. However, MIF can also exert its protective activities through the induction of other pro-inflammatory cytokines, such as TNF- α and IL-1 and reactive oxygen intermediates [55]. This is supported by the observed positive correlation between MIF and TNF- α in this study (Table 3). Contrary to previous reports [13], MIF levels were similar in the three clinical disease categories used in the present study, with a trend of increasing concentration with decreasing disease severity. Further studies are needed to clarify the role of MIF in severe malaria pathogenesis. Although lower levels of IL-12, a potent inducer of pro-inflammatory responses [56], have been reported previously in children with severe disease (SMA), compared to mild disease [21,33], IL-12 levels were similar in the patient groups in this study. It is possible that the small sample size used in this study was too small to draw any meaningful conclusions.

In conclusion, this study confirms a pathogenic role for TNF- α , with higher ratios of TNF- α to IL-10 and TGF- β associated with severe malaria anaemia in children residing in an endemic area. These suggest that imbalances in the IL-10 and TGF- β cytokine regulatory network may contribute to the pathogenesis of severe malaria anaemia. The observation of an antagonistic relationship between IL-10 and TGF- β confirm previous reports that this cytokine may perform both pro- and anti-inflammatory roles. Although it is possible that plasma cytokine levels examined here may not represent the local tissue milieu, we believe that measurement of plasma cytokines as indicators of disease severity

for human blood-stage malaria is very useful. Understanding how the balance of these cytokines is regulated during a malarial infection may lead to novel approaches to immunotherapy and immunoprophylaxis. It is also possible that ratios of pro- and anti-inflammatory plasma cytokines will be good predictors of immunity for use in development and evaluation of malaria vaccines.

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