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Thrombocytopenia May Mediate Disease Severity in *Plasmodium Falciparum* Malaria Through Reduced Transforming Growth Factor Beta-1 Regulation of Pro- and Anti-Inflammatory Cytokines

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Abstract

Background—Transforming growth factor beta-1 (TGF-β1) is an important regulator of inflammation. Platelets are a major source of TGF-β1, and are reduced in severe malaria. However, the relationships between TGF-β1 concentrations and platelet counts, pro- and anti-inflammatory cytokine and chemokine concentrations, and disease severity in malaria have not been characterized.

Methods—Platelet counts and serum concentrations of TGF-β1 and interleukin-1beta (IL–1β), IL-6, IL-10, interferon (IFN)–γ, tumor necrosis factor (TNF)- α and RANTES were measured at the time of presentation in Ugandan children with cerebral malaria (CM, n=75), uncomplicated malaria (UM, n=67) and healthy community children (CC, n=62).

Results—TGF-β1 concentrations decreased with increasing severity of disease (median concentrations (25^{th}, 75^{th} percentile) in ng/ml in CC, 41.4 (31.6, 57.4), UM, 22.7 (14.1, 36.4), CM, 11.8 (8, 21), *P* for trend<0.0001). In children with CM or UM, TGF-β1 concentrations correlated positively with platelet count (CM, *P*<0.0001, UM, *P*=0.0015). In children with CM, TGF-β1 concentration correlated negatively with IFN-γ, IL-6, and IL-10 and positively with RANTES concentrations (all *P*<0.01). TGF-β1 concentration was not associated with death or adverse neurologic or cognitive outcomes in children with CM.

Conclusions—TGF-β1 concentrations decrease with increasing *P. falciparum* disease severity. In cerebral malaria, thrombocytopenia correlates with decreased TGF-β1, and decreased TGF-β1 correlates with cytokine/chemokine changes associated with increased disease severity and death. Thrombocytopenia may mediate disease severity in malaria through reduced TGF-β1-mediated regulation of cytokines associated with severe disease.

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Keywords

cerebral malaria; platelets; TGF-beta; tumor growth factor beta

Introduction

In 2010 there were an estimated 1.24 million deaths from malaria world-wide, most of which were due to infection with *Plasmodium falciparum* [1]. The clinical presentation of malaria ranges from asymptomatic parasitemia and mild illness to cerebral malaria (CM), a condition with a mortality rate of ~15% [2]. Host immune response directly impacts the severity and outcome of disease in murine models of severe malaria [3–7], and appears to be similarly important in severe malaria in humans [8–11].

A balance between pro-inflammatory and anti-inflammatory properties appears to be necessary to combat *P. falciparum* parasitemia. Cytokines such as interferon- gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and interleukin 1-beta (IL-1β), promote inflammation and reduce parasitemia [6, 7, 12, 13]. However, an exaggerated pro-inflammatory response is associated with increased morbidity and mortality in severe malaria [10, 13, 14]. Although interleukin-10 (IL-10) is generally considered an anti-inflammatory cytokine, elevated concentrations of IL-10 have also correlated with increased mortality in several studies (14–16), highlighting the complexity of the human immune response to *P. falciparum* and its effects on disease severity.

TGF-β1 is a multi-functional protein that is important in regulating the inflammatory response. It is produced by multiple sources, including monocytes, T cells, and B cells, in an inactive precursor form with various activation pathways [15, 16]. Large quantities of TGF-β1 are stored in the alpha granules of platelets [17]. In malaria, TGF-β1 may play an important role in the regulation of the immune response and tolerance of parasitemia [18, 19]. Upon activation, TGF-β1 has concentration and environment-dependent pro- and anti-inflammatory properties. At low concentrations, TGF-β1 pro-inflammatory properties include modulating the concentrations of endothelial cell adhesion molecules, as well as recruiting monocytes, T-cells and neutrophils to sites of early infection [20]. At high concentrations, TGF-β1 has anti-inflammatory properties and assists in transition from Th-1 type to Th-2 type responses through mechanisms that include the suppression of TNF-α production from macrophages, and the inhibition of IFN-γ and TNF-α production from NK cells [21, 22]. TGF-β1 has also been shown to induce FOXp3, which is required for regulatory T cell (Treg) development and function [23]. Tregs act as important mediators of the host immune response. They are associated with increased rates of P. falciparum growth in vivo [24] and are increased in individuals with asymptomatic malaria compared to individuals who are not infected [25].

Multiple studies have demonstrated that TGF-β1 concentrations are low in *P. falciparum* malaria, with disease severity increasing as TGF-β1 levels decrease [13, 26, 27], but the factors that lead to lower TGF-β1 concentrations in malaria are uncertain. Thrombocytopenia could contribute to the low TGF-β1 concentrations seen in severe malaria, as platelets are a major storage site for TGF-β1, and thrombocytopenia is common

in malaria and associated with disease severity and death [28]. We conducted the present study to determine the relationship of TGF-β1 concentrations to disease severity and to platelet count and parasite density in children with cerebral malaria (CM), uncomplicated malaria (UM) and community children (CC).

Subjects, Materials, and Methods

Study population and recruitment

The study was conducted at Mulago Hospital, Kampala, Uganda. Children of 4–12 years of age were recruited as part of a study assessing the complications of CM. A total of 88 children with CM, 76 children with UM, and 100 community children (CC) without evidence of acute illness were recruited. Complete details of the study cohorts were previously reported [29]. Briefly, children with CM were enrolled if they were admitted to Mulago Hospital and met the WHO criteria for CM: coma, *P. falciparum* on blood smear, and no other cause for coma. CM was treated with quinine for 7 days. Children with UM were enrolled from the Acute Care clinic or an outpatient malaria clinic at the hospital sponsored by the University of California, San Francisco (UCSF). Children were considered to have UM if they had signs and symptoms of malaria, *P. falciparum* infection on blood smear, and no evidence of malaria complications or other acute illness. Children with UM were treated in an Acute Care clinic (chloroquine plus sulfadoxine/pyrimethamine) or with combination therapy at the UCSF outpatient clinic (either amodiaquine plus sulfadoxine/pyrimethamine or amodiaquine plus artesunate). Community children were recruited from the household and extended households of children with CM or UM. Community children and children with UM were recruited to be in the same age range (4–12 years) as children with CM, and a history and physical exam was performed to ascertain that the community children were healthy at the time of enrollment.

Blood samples of 2–5 ml were obtained by venipuncture utilizing a tourniquet with the initial blood draw for routine laboratory testing prior to or as anti-malarial treatment was being given. A second sample was drawn from children with CM at 72 hours after initiation of treatment. The separated serum was pipetted into aliquots and frozen at −70 °C until testing was performed. Serum samples from 75 children with CM and 67 children with UM were available for testing, and serum samples of 62 of the 100 community children were randomly chosen for testing. Serum samples from 65 of the 75 children with CM at 72 hours after admission were available for testing. Seven of the 62 community children had asymptomatic parasitemia. All were treated and were not excluded from the study. Stool was requested from all enrolled subjects and obtained from 133 children. Stool helminth infections were assessed by microscopic examination of a stool wet mount preparation.

Written, informed consent was obtained from the parents or guardians of study participants. Ethics approval for the study was granted by the institutional review boards at Makerere University, Case Western Reserve University and the University of Minnesota.

Cytokine testing

Concentrations of total TGF-β1 (R&D Systems) and interleukin-10 (IL-10) (BD Pharmingen) were assessed by standard cytokine ELISA according to the manufacturers' instructions. Results were interpolated from standard curves generated with SoftMax software (Molecular Devices). Concentrations of interleukin-1beta (IL–1β) [3], interleukin 6 (IL-6) [8], interferon gamma (IFN–γ) [9], tumour necrosis factor alpha (TNF- α) [30], macrophage inflammatory protein alpha (MIP–1α) [31], and macrophage inhibitory protein beta (MIP-1β) [32], which have been associated with increased risk of severe disease and/or death in malaria, and of RANTES, which have been associated with a decreased risk of severe disease and death in malaria [11], were determined by a commercially available cytometric bead assay (CBA)(R&D Systems) using the Luminex system and have been previously published [11].

Neurologic and cognitive testing

A complete neurologic exam was done on children with CM at discharge, 3 months, and 6 months after hospitalization. Cognitive testing assessed attention, working memory, and tactile-based learning, as described previously [33]. Age-adjusted z-scores calculated using the scores from the healthy community children.

Statistical analysis

Data analyses were performed using STATA 10.1 (Stata Corp). Variables were assessed for normality in STATA, and those that were not normally distributed were compared using the Wilcoxon rank-sum test, and those normally distributed were compared using Student's t-test. As is typically the case, values of all cytokines/chemokines were not normally distributed. Values across groups (including TGF-β1) were compared by the Wilcoxon rank-sum test, while values comparing cytokines and T TGF-β1 were compared using Spearman's rank correlation, which does not rely on a linear relationship between the variables. P values for multiple comparisons were adjusted for with the Bonferroni correction.

Results

Demographic and clinical characteristics of study participants

Children with CM were significantly more likely to younger than children with UM or CC (Table 1). Children with CM also had significantly lower hemoglobin concentrations and platelet counts than children with UM or CC, but median parasite density in children with CM and UM did not differ. Children with UM had significantly lower hemoglobin concentrations, platelet counts and higher parasitemia than CC (Table 1). Seven of the 62 CC had asymptomatic parasitemia. These asymptomatic children did not differ from the community controls in any of the variables included in Table1.

TGF-β1 concentrations according to severity of disease and clinical outcomes

Serum concentrations of TGF-β1 decreased with increasing severity of disease (median concentrations (25^{th}, 75th percentile)), ng/ml: CC, 41.4 (31.6, 57.4), UM, 22.7 (14.1, 36.4),

CM, 11.8 (8, 21), *P* for trend<0.0001, Figure 1). After adjustment for age, hemoglobin level and sex with multivariate linear regression analysis, log-transformed TGF-β1 levels remained higher in children with UM than CM (*P*=0.016, beta coefficient 0.39, 95% CI, 0.07–0.74) and higher in children with community children than UM (*P*= 0.001, beta coefficient 0.59, 95% CI, 0.35–0.833). There was no significant difference in concentrations of TGF-β1 in CC with asymptomatic parasitemia 55.7 (32.15–60.5) and CC with no detectable parasitemia (41.4 (31.6–57.4), *P*=0.5). Seventy-two hours after initiation of treatment, TGF-β1 concentrations in children with CM or UM had normalized to the concentrations seen in community children at enrollment (CM, 37.9 (27–48.1), UM 40.7 (28.6–53)).

Four children with CM died. Serum TGF-β1 concentrations did not differ significantly between the children who died (6.7, (4–11.8)) and those who survived (13.5, (8.3–21.1), *P*=0.11). TGF-β1 concentrations were not associated with any neurocognitive outcome in children with CM, UM or CC (all *P*>0.05).

Correlation of TGF-β1 concentrations with platelet count and parasite density

TGF-β1 concentrations correlated strongly with platelet count in children with CM (rho=0.61, *P*<0.0001) or UM (rho= 0.38, *P*=0.001). Platelet count correlated negatively with peripheral blood parasite density in children with CM (Spearman's rho= −0.27, *P*=0.01) and UM (rho= −0.37, *P*=0.0003), but TGF-β1 concentrations did not correlate with parasite density in either study group (CM, rho = −0.04, *P*=0.72, UM, rho= −0.19, *P* =0.13). Those who died with CM were found to have a lower median platelet count (47.5, (31–65) 10(9) cells/L) than those who survived (84.5, (55–144) 10(9) cells/L), *P*=0.039.

Correlation of TGF-β1 concentrations with concentrations of other cytokines and chemokines

In children with CM, TGF-β1 concentrations correlated negatively with IFN-γ, IL-6, and IL-10 concentrations and positively with RANTES concentrations (Table 2). In children with UM, TGF-β1 concentrations correlated negatively with IL-1β, MIP-1α and TNF-α, and positively with RANTES (Table 2 and Figure 2).

TGF-β1 concentrations according to stool helminth infection

Eleven of the 133 stool samples obtained across the 3 groups were found to have parasitic infections. Five of the 133 children (4%) had a stool helminth infection (4 *Ascaris lumbricoides*, 1*Trichuris trichiura*), including 3 of 40 children with CM, 1 of 46 children with UM and 1 of 47 CC. Six children had *Giardia lamblia* infection. In children with CM, serum TGF-β1 concentrations did not differ in those with helminth infection (median [IQR]), 13.45 (11.8–13.55) vs. those without helminth infection (11.6 (8–21.1), *P*=0.94), but the small numbers of children with CM who had helminth infection (n=3) did not allow for meaningful comparison.

Discussion

In the present study, we show that in children with cerebral malaria, thrombocytopenia correlates with decreased TGF-β1, and decreased TGF-β1 correlates with cytokine/chemokine changes associated with increased disease severity and death [8, 9, 11]. We postulate that a reduction in the platelet reservoir of TGF-β1 in severe malaria leads to impaired TGF-β1-mediated regulation of cytokine responses associated with disease severity and death. The study findings suggest a novel mechanism that might explain in part the adverse outcomes associated with thrombocytopenia in severe malaria.

The causes of thrombocytopenia in uncomplicated and severe malaria remain unclear, and the question of whether thrombocytopenia is a reflection of severe disease or contributes to severe disease is also unanswered. A number of factors may be responsible for the thrombocytopenia seen in severe malaria, including host defense mechanisms against parasitemia [34], development of antibodies to platelets [35], sequestration [36], phagocytosis[37] and oxidative stress [38]. It is possible that thrombocytopenia may reflect disease severity but also subsequently contribute to increased disease severity.

In *in vitro* studies, platelets appear to be important in protection from *P. falciparum* blood stage infection, through direct anti-parasitic activity and the ability to present antigens to T cells [39, 40]. However, animal models suggest that platelets are part of disease pathogenesis. It has been proposed that timing may be important in determining whether platelets are harmful or helpful in the response to murine malaria. A recent study in a murine model of experimental cerebral malaria showed that platelets were protective early in infection, through limitation of parasite growth, but continued platelet activation contributed to inflammation at later stages of infection [41]. The present study data appear to contradict these findings, since thrombocytopenia was associated with increased disease severity. Several other studies in humans also document an association of thrombocytopenia with disease severity and mortality [28, 42], consistent with a protective and not harmful effect of platelets at later disease stages. The findings of the present study support a novel potential mechanism for platelet-associated protection from severe disease: release of TGF-β1 from platelets, modulating the inflammatory response that may lead to severe disease symptoms.

The finding of lower TGF-β1 concentrations with increasing disease severity in the present study is consistent with most [13, 26, 27] though not all [30, 43] prior studies. TGF-β1 *in vitro* inhibits development of effector T-cells and macrophage activation and has less effect once those cells have been activated [44, 45]. An initial lack of available TGF-β1 may shift the balance of inflammation towards an exaggerated inflammatory response that continues despite subsequent normalization of TGF-β1 concentrations. In the present study, TGF-β1 correlated negatively with cytokines associated with more severe disease in malaria, and this included not only the pro-inflammatory cytokines IFN-γ and IL-6 [8, 9, 13, 27], but the anti-inflammatory cytokine IL-10[8, 9]. Studies in other disease processes demonstrate TGF-β1 down-regulation of IFN-γ [46, 47]) and IL-6 [48], consistent with our findings, but contrary to our findings, most prior studies show TGF-β1 down-regulation of RANTES [49, 50] and increased levels of IL-10 in individuals with elevated TGF-β1 [51, 52]. The findings could be due to differences in timing and production of cytokines and chemokines, as cytokine

concentrations at any single time point collection could reflect concurrent or prior levels of TGF-β1, depending on the cytokine/chemokine. We did not find a correlation between parasitemia and TGF-β1, but other markers such as HRP2, which reflects parasite biomass, might be more appropriate to compare the relation of TGF-β1 concentrations to total parasite burden.

Only a small number of children (n=5) in the study had stool helminth infections and it remains unclear if they modulate the TGF-β1 response in malaria. More sensitive testing with PCR to identify stool helminth infections [53] might allow future studies to better explore the relationship between helminth infections and TGF-β1 in malaria. As with most prior human studies, a limitation of the present study is that it can show only association of factors with disease endpoints, and cannot prove causation. However, in light of the contradictory murine model and human findings regarding thrombocytopenia and morbidity in cerebral malaria, it is important to continue with human studies to assess potential mechanisms of thrombocytopenia-associated disease severity, to assess potential pathways for intervention.

In summary, the study data demonstrate that in children with cerebral malaria, thrombocytopenia correlates with decreased TGF-β1 concentrations, which in turn correlates with cytokine/chemokine changes associated with increased disease severity and death. The data suggest that thrombocytopenia may mediate disease severity in malaria in part through reduced TGF-β1-mediated regulation of cytokines associated with severe disease.

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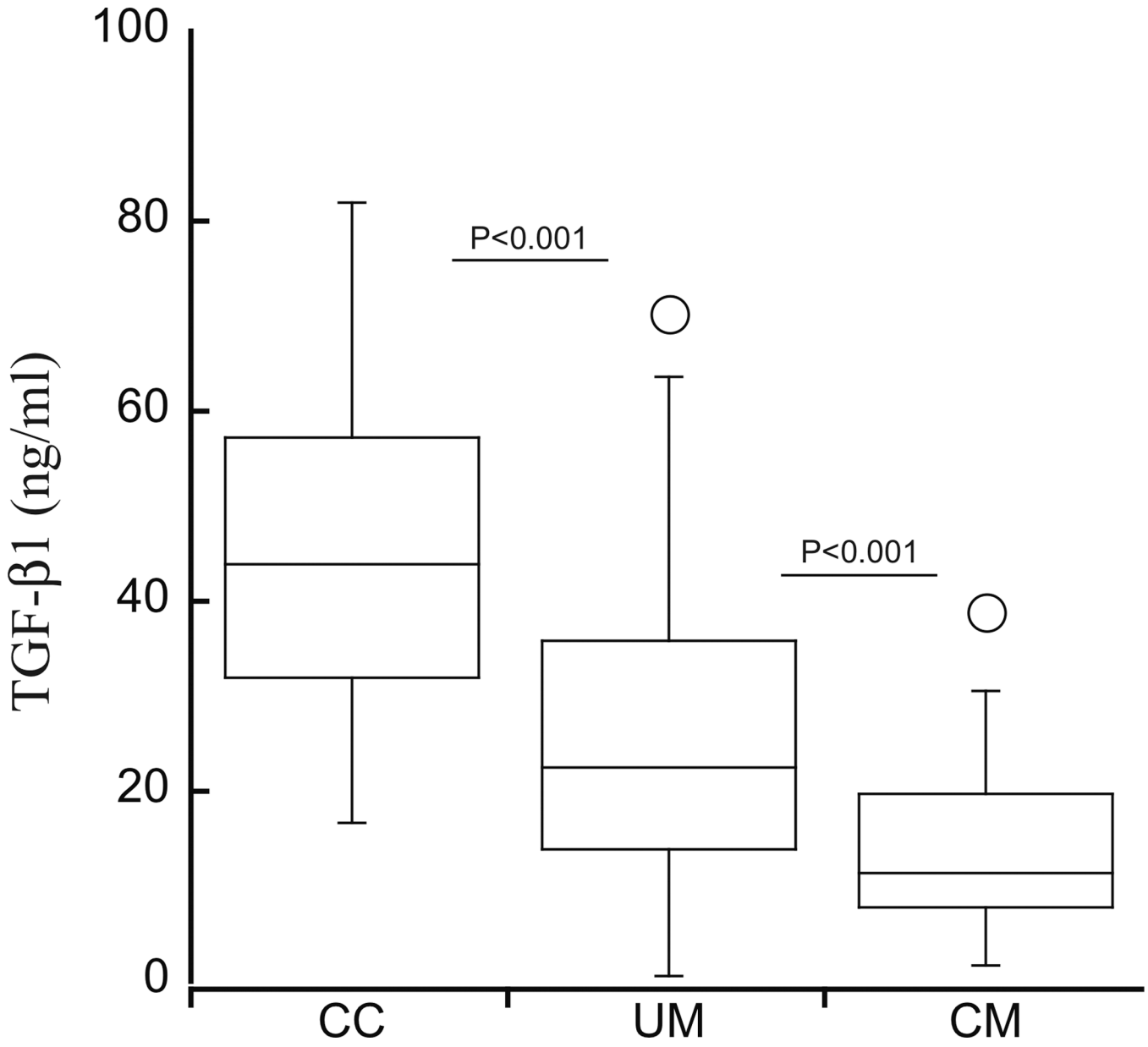

Figure 1.

TGF-β1 concentrations decrease with increasing disease severity. CC, community children; UM, uncomplicated malaria; CM, cerebral malaria. In this box and whisker plot, the box represents the 25th and 75th percentiles, the central line the median, and the whiskers the 5th and 95th percentiles.

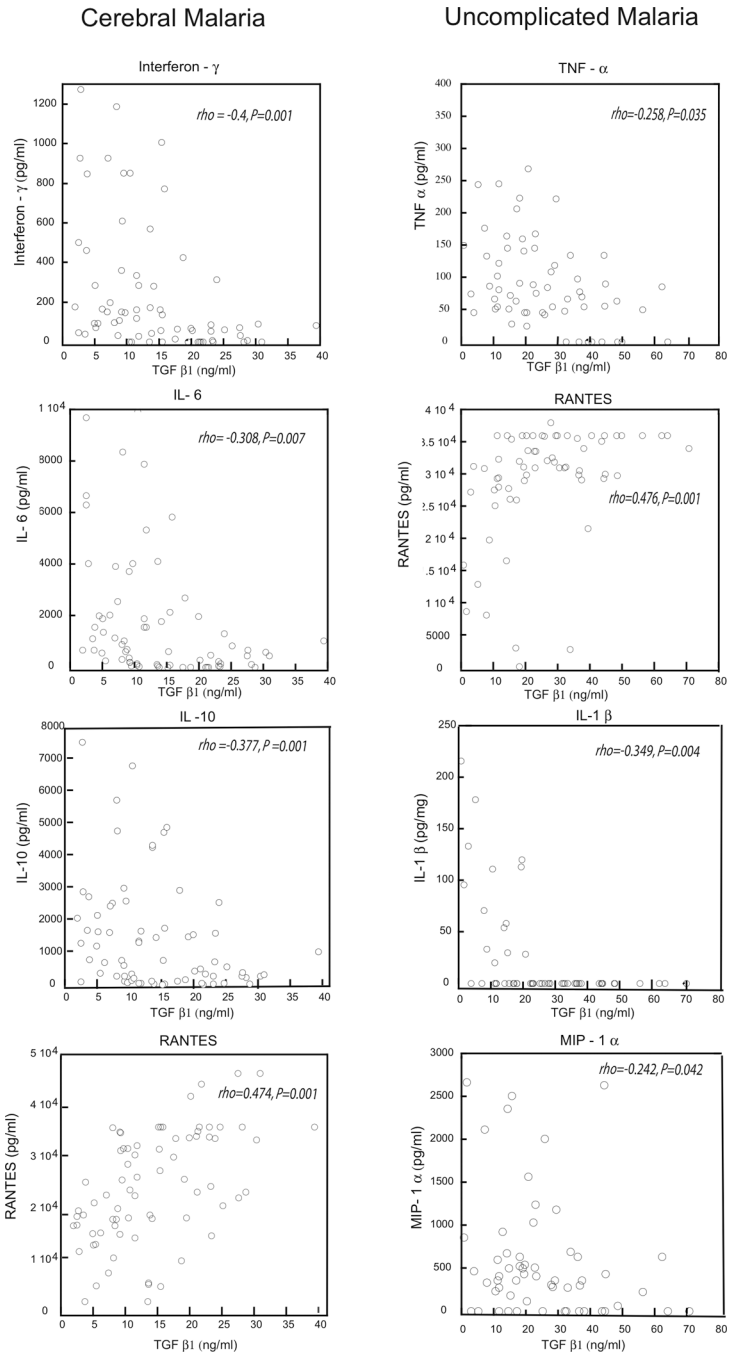

Figure 2.

TGF-β1concentrations and pro- and anti-inflammatory cytokines with in UM, uncomplicated malaria; CM, cerebral malaria. IFN-γ, (Interferon- gamma), TNF-α (tumor necrosis factor-alpha), IL–1β (interleukin-1beta), IL-6 (interleukin 6), MIP–1α (macrophage inflammatory protein alpha), refer to table 2 for spearman correlations.

Table 1

Demographic and clinical characteristics of study participants.

	CC (n = 62)	UM (n = 67)	CM (n = 75)
Sex, % male	46.7	38.8	57.3
Age, years[a]	7.8 (2.1)	7.6 (2.6)	6.4 (2.4)[c,d]
Hemoglobin mg/dL[a]	11.9 (1.5)	11.2 (1.8)[b]	8.4 (2)[c,d]
Platelet count[a] 10(9)cells/L	345.9 (103.5)	144 (85)[b]	81 (75.6)[c,d]
Parasite density, median (IQR)	0 (0)	33,920 (111,625)[b]	29,760 (136,060)[d]

[a] Mean, SD,

[b] P<0.05 for UM vs. CC,

[c] P<0.05 for CM vs. UM,

[d] P<0.05 for CM vs. CC

Table 2

Correlation with TGF-β1 concentrations with concentrations of other cytokines and chemokines in children with cerebral malaria (CM) or uncomplicated malaria (UM).

	CM Group N, Spearman's rho, p value	UM Group N, Spearman's rho, p value
IL-6	75, −0.3082, 0.007	67, 0.0214, 0.864
IL-10	73, −0.3765, 0.001	67, −0.0397, 0.740
IL-1β	75, 0.1376, 0.239	67, −0.3485, 0.004
IFN–γ	75, −0.4004, 0.004	67, −0.1454, 0.240
MIP-1α	75, −0.1950, 0.094	67, −0.2416, 0.042
MIP-1β	75, −0.1397, 0.232	67, −0.2075, 0.092
RANTES	75, 0.4754, 0.001	67, 0.4676, 0.001
TNF-α	75, 0.1342, 0.251	67, −0.2581, 0.035