MAJOR ARTICLE

Clusters of Cytokines Determine Malaria Severity in *Plasmodium falciparum*–Infected Patients from Endemic Areas of Central India

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We investigated the role of interferon (IFN)–γ, interleukin (IL)–1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)–β in clinically well-defined groups of *Plasmodium falciparum*–infected patients manifesting mild malaria (MM), severe noncerebral malaria (SM), or cerebral malaria (CM) and in control subjects from Gondia, a malaria-endemic site in India, as well as in healthy subjects from non–malaria-endemic areas. Two-way coupled cluster analysis revealed 2 clusters of cytokines relevant to clinical subgroups of disease. The first cluster was composed of IFN-γ, IL-2, IL-5, IL-6, and IL-12, the levels of which were significantly increased during infection but were predominant in patients with MM and allowed us to distinguish them from patients with SM or CM. The second cluster was composed of TGF-β, TNF-α, IL-10, and IL-1β, the levels of which were highly correlated with each other in the different clinical groups of patients and significantly increased with disease severity, particularly in CM. Discriminant analyses allowed us to propose a minimal model. Levels of cytokines such as IL-5, IL-1β, IL-10, and IL-2 increase with infection. Levels of IL-12, IL-5, and IL-6 discriminate severe forms of malaria from MM. Finally, levels of IL-1β, IL-12, and IFN-γ are relevant for the discrimination of CM from SM: high IL-1β levels are associated with CM, and high IL-12 and IFN-γ levels are associated with SM.

Plasmodium falciparum malaria is a major public-health problem in sub-Saharan Africa and southern Asia, with ~1.7 million of the 2 billion people living in these endemic areas dying every year as a result of complications from the disease [1–3]. Although the epidemiological status of malaria in India is not precisely documented, the disease is known to be endemic and widespread in many rural areas of the country as well as in central India, where the present study was undertaken.

Disease manifestation generally differs from person to person, ranging from uncomplicated, mild malaria (MM) to severe noncerebral malaria (SM) to cerebral malaria (CM), characterized by neurological signs, repeated convulsions, and coma stage II, which can lead to death. The cause of such differential responses in the degree of severity, as well as the underlying physiopathologic process by which severe *P. falciparum* malaria progresses to cerebral complications, are not well understood. There is some evidence suggesting that the mechanical obstruction of brain microvessels by blood-stage parasites adhering to the vascular endothelium, immune responses, and genetic factors in both the mammalian host and the parasite play specific roles in disease progression [4, 5]. However, the role of parasite sequestration in the pathogenesis of CM remains controversial, because the physical presence of parasites in the brain microvessels has not been unequivocally demonstrated in all patients who die of CM. Moreover, patients with CM can recover from deep, prolonged coma associated with protracted seizures, hypoglycemia, and severe anemia, without any sequelae [6, 7]. Nonetheless, African children with severe neurological

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sequelae (e.g., spastic tetraparesis and vegetative states) usually die within a few months of discharge from the hospital [8, 9].

Proinflammatory (Th1) cytokines such as tumor necrosis factor (TNF)–α are also thought to play an important role in malaria pathogenesis, particularly in CM, by increasing the surface expression of adhesion molecules on cerebral endothelial cells that enhance parasite attachment [6, 10–12]. Excessive production of TNF-α in patients with CM seems to be a consequence of genetic variation in the host's propensity to produce this cytokine [13–16]. Furthermore, increased plasma levels of TNF-α, together with increased production of interferon (IFN)-γ, interleukin (IL)–1β, and IL-6 and reduced production of IL-4 and transforming growth factor (TGF)-β, have been reported in patients with SM [17, 18]. Conversely, IFN-γ and TNF-α are involved in effector mechanisms conferring protection against *Plasmodium* infection [19–21].

The role of anti-inflammatory cytokines in malaria severity remains controversial, because different studies have reported high concentrations of IL-10 as being associated either with SM or with protection against SM in humans [17, 22–24]. In addition, it has been shown that the balance between IL-10 and TNF-α concentrations determines the severity of anemia in infected children [25]. Along the same line of evidence, plasma levels of TGF-β, a cytokine that acts at high concentrations as an anti-inflammatory cytokine, are inversely correlated with malaria severity in murine models as well as in humans. In a recent study performed in Kenya, lower levels of TGF-β in serum and cerebrospinal fluid from children with CM were found to be associated with higher levels of TNF-α [26]. Indeed, the outcome of *P. falciparum* infection may depend on a fine balance between appropriate and inappropriate induction of these immune regulatory factors.

Although cytokines have been implicated in the pathogenesis of malaria, their specific involvement in terms of different groups/clusters correlating with MM, SM, and CM has not yet been defined. To better determine the role played by pro- and anti-inflammatory cytokine levels and their ratios in malaria severity, we analyzed the association between plasma levels of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, and TGF-β in clinically well-defined groups of *P. falciparum*–infected patients from Gondia, a district of Maharashtra State in central India, with MM, SM, or CM and compared these patients with control subjects from malaria-endemic and non–malaria-endemic areas and with subjects who have recovered from CM. We also studied the association between plasma cytokine network profiles and parameters such as age and sex of the patients, parasitemia level, and disease severity. We found that cytokines such as IL-1β, IL-12, and IFN-γ discriminated CM from SM, that high IL-1β levels were associated with CM, and that high IL-12 and IFN-γ levels were associated with noncerebral SM. Moreover, disease severity in patients with malaria was independent of parasitemia level, sex, and age.

SUBJECTS, MATERIALS, AND METHODS

Study population. The present study was performed in the Gondia District of Maharashtra State, India. Categorization of malaria cases was performed by the resident physician of the hospital on the basis of severity criteria defined by the World Health Organization. Patients with MM ($n = 33$) were outpatients and not admitted to the hospitals. Patients with SM ($n = 14$) were fully conscious and were able to respond well verbally to the doctors' questions. Patients with CM ($n = 26$) were in a comatose state. In addition, 3 groups of control subjects were defined: (1) subjects who had CM within the past 6 months and recovered ("ex-CM subjects"; $n = 8$), (2) healthy subjects from malaria-endemic areas ("endemic control subjects"; $n = 15$), and (3) healthy subjects from non–malaria-endemic areas ("nonendemic control subjects"; $n = 9$), of both sexes. The age of the patients with malaria ranged from 5 to 75 years. Blood samples from endemic control subjects were collected from patient's relatives (brothers/sisters/parents) who

Table 1. Age, sex distribution, and parasitemia in the different malaria clinical groups.

Group	Patients, no.	Parasitemia level, % (range)	Age, mean (range), years	Sex ratio (M/F)
NEC	9	…	34 (2.2–42.4)	8/1
EC	14	…	27 (24.9–29.2)	13/1
MM	33	1.2 (0.7–2.1)	28.4 (23.8–34)	13/20
SM	14	1.1 (0.3–3.2)	25.7 (15.85–41.8)	8/6
CM	26	2 (1–3.87)	33.3 (26–42.8)	12/14
Ex-CM	8	…	19.3 (7.35–50.9)	3/5
Total	104	…	28.9 (25.9–32.2)	…

NOTE. CM, cerebral malaria; EC, endemic control (healthy control subjects from malaria-endemic areas); ex-CM, recovered from CM; MM, mild malaria; NEC, nonendemic control (healthy control subjects from non–malaria-endemic areas); SM, noncerebral severe malaria.

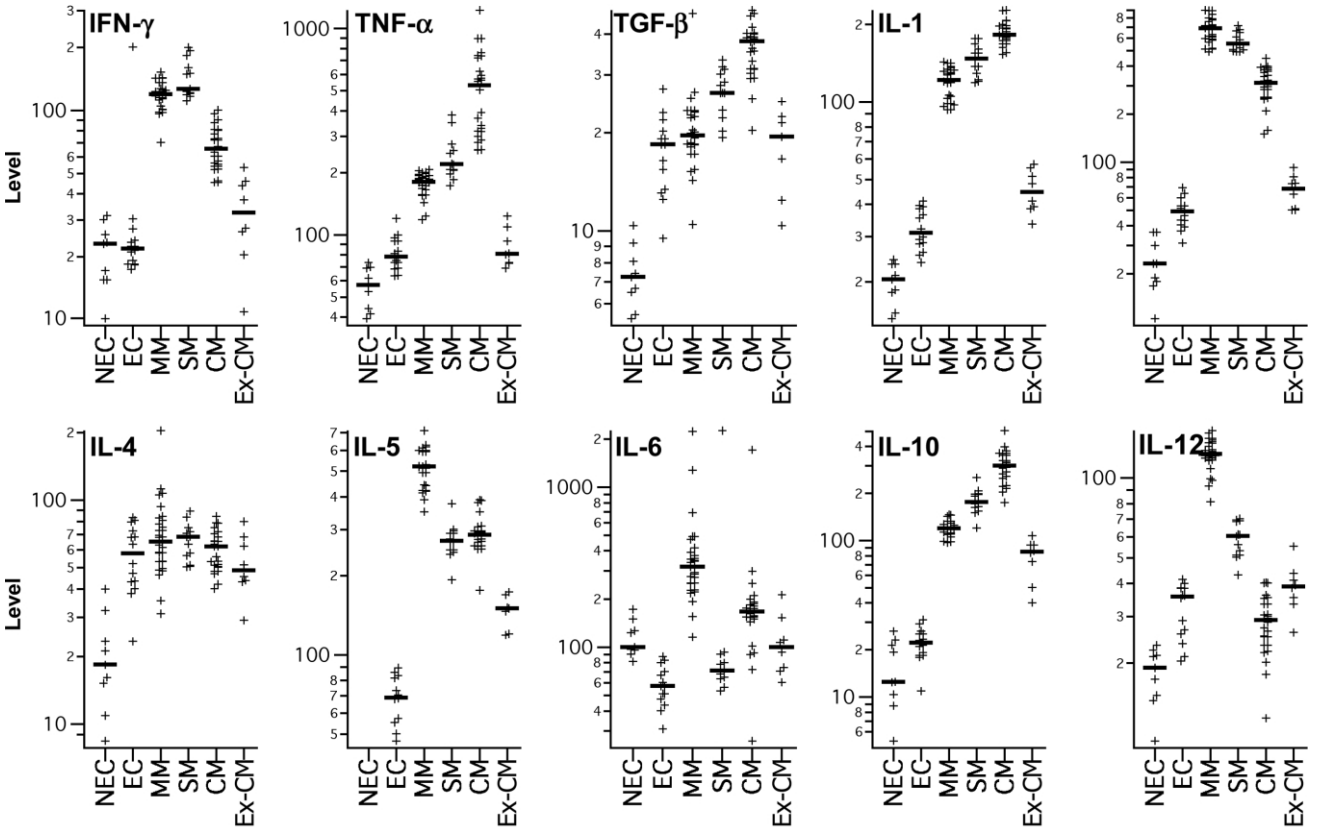

Figure 1. Differences in plasma cytokine levels in clinical malaria groups. Levels (pg/mL) of interferon (IFN)–γ, interleukin (IL)–1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)–α, and transforming growth factor (TGF)–β were determined by ELISA from 1-time blood samples collected from patients before administration of drugs. CM, cerebral malaria; EC, endemic control (healthy control subjects from malaria-endemic areas); ex-CM, recovered from CM; MM, mild malaria; NEC, nonendemic control (healthy control subjects from non–malaria-endemic areas); SM, severe noncerebral malaria. Horizontal bars indicate the respective groupwise medians.

accompanied the patient to the hospital. These persons had not had malaria for at least 2 years, nor were they clinical asymptomatic carriers; their blood smears were checked for the presence of malarial parasites. Blood samples from nonendemic control subjects (from the city of Pune) were collected from our laboratory colleagues with no history of malarial disease for $\geqslant$5 years. All consent from donors, patients, and relatives was obtained before blood collection.

Blood-sample collection. Blood samples were collected during 2001–2003 from different hospitals in Gondia: ~10–20 mL of peripheral venous blood was collected either in EDTA or in sterile vacutainers. Plasma was obtained by centrifuging the blood samples at 4500 *g* for 15 min and was stored at −80°C until further use.

Parasite assessment. A thin blood smear was prepared, air-dried, fixed in methanol, and stained with JSB1 (methylene blue) and JSB2 (eosin). The total numbers of infected and uninfected erythrocytes from ~10 fields (magnification, ×100) were counted, and parasitemia levels were calculated.

Cytokine assays. The levels of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TGF-β, TNF-α, and INF-γ) in plasma were estimated by use of Opti-EIA kits (BD-Pharmingen), used per the manufacturer's instructions. Briefly, flat-bottom 96-microwell plates (Nunc Maxisorb P/N) were coated overnight at 4°C with capture antibody diluted in 0.1 mol/L carbonate buffer, pH 9.5. After washing with PBS–0.05% Tween-20 and blocking with PBS–10% FBS for 1 h at room temperature, 100 μL of plasma from either patients or control subjects was added to each coated well and incubated for 2 h at 20°C. All assays were performed in triplicate. A standard curve was determined for each cytokine, using serial dilutions of the different human recombinant cytokines. Cytokine concentrations were calculated after incubation with peroxidase-conjugated anti–human cytokine antibodies. The peroxidase activity was revealed with tetramethyl benzidine. Unknown sample cytokine concentrations were calculated from the standard curves. Means and SDs were calculated from the values.

Statistical analysis. Comparisons between groups were calculated by Mann-Whitney rank sum test. Coupled 2-way cluster analysis was performed using an algorithm (available at: http:

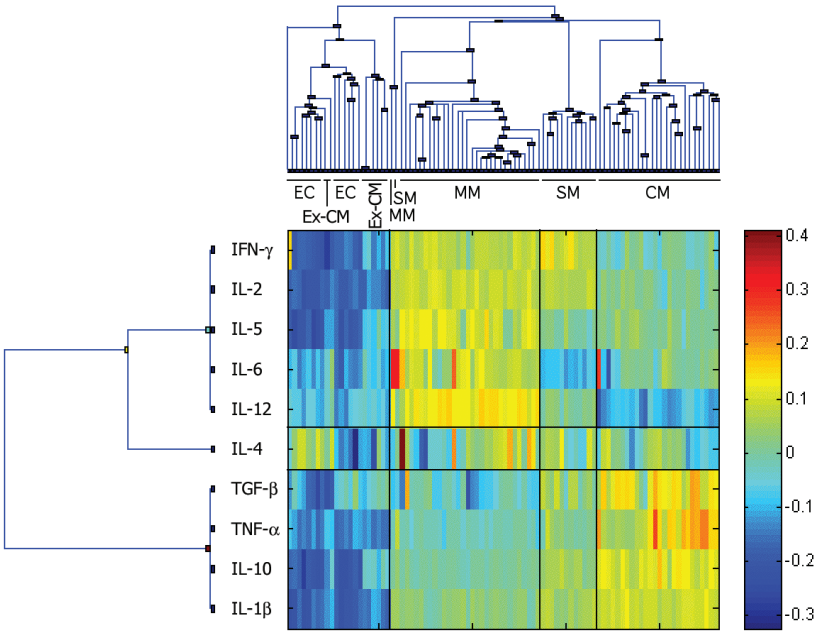

Figure 2. Two-way coupled cluster analysis. Each cell in the 2-dimensional graph indicates the measure of a single cytokine in 1 sample, with standardized levels indicated by color according to the scale on the right. Hierarchical clusters for both samples and cytokines were obtained as described (see Subjects, Materials, and Methods). Sample clustering resulting from the algorithm applied is shown at the top of the graph as a horizontal dendrogram, with an indication of the group to which each individual sample belongs. Major clusters—which discriminated the clinical groups exactly, with the exception of 1 sample—are separated throughout the graph by vertical lines. Cytokine clustering is depicted analogously in a vertical order to the left of the graph. CM, cerebral malaria; EC, endemic control (healthy control subjects from malaria-endemic areas); ex-CM, recovered from CM; IFN, interferon; IL, interleukin; MM, mild malaria; SM, severe malaria; TGF, transforming growth factor; TNF, tumor necrosis factor.

//ctwc.weizmann.ac.il), as described by Getz et al. [27] and Blatt et al. [28]. Missing data were replaced by overall means. Clustering was performed on log-transformed data with no dropout constraints until single entities were reached. Clusters were considered to be stable when they remained over 8 cycles, according to the default settings by the authors of the algorithm. Default settings were also used for the other framework conditions: T increasing from 0 to 0.25, with 0.004 increments/cycle.

Linear discriminant analysis was also performed on log-transformed data, rejecting samples with incomplete information. The significance of contributing parameters was calculated by backward elimination—that is, by partial F tests on variances explained by models with or without a respective parameter. The significance criterion was a Bonferoni-corrected P value $<.05$. For this purpose, special macros in addition to the software IgorPro (version 3.16; WaveMetrics) were used.

RESULTS

Age, sex distribution, and parasitemia in the different clinical groups. We compared the age and sex distribution of *P. falciparum*–infected patients manifesting different degrees of severity of clinical malarial disease—that is, CM, SM, and MM. The clinical severity was uniformly spread over both sexes and all age groups. Susceptibility to CM was not restricted to children and was also seen in older people (table 1). Parasitemia levels ranged from 0.1% to 7.50% in the different groups; 3 patients with CM had exceptionally high parasitemia levels (20.25%, 25.5%, and 60%). However, neither malaria disease severity nor age nor sex showed any correlation with blood parasitemia level (table 1). Thus, disease severity in Gondia *P. falciparum*–infected patients seemed to be independent of parasitemia.

Cytokine levels. Profiles of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, and TGF-β were determined by ELISA on a 1-time blood sample collected from the different groups of patients before administration of drugs. Median plasma levels of the different cytokines are shown in figure 1. The levels of IL-1β, IL-10, TNF-α, and TGF-β were observed to be most significantly increased in the CM group, compared with those in the endemic control group or in the other groups of *P. falciparum*–infected patients (for all comparisons, $P < 10^{-6}$). Interestingly, these cytokines were also present, in moderate concentrations, in plasma from the ex-CM group. The levels of IL-1β, IL-10, and TNF-α were only marginally increased in the endemic control group, compared with those in the nonendemic control group; however, levels of IL-2, IL-12, and IFN-γ were lower in the CM group than in the SM group. There was no difference in levels of IL-2 and IFN-γ between

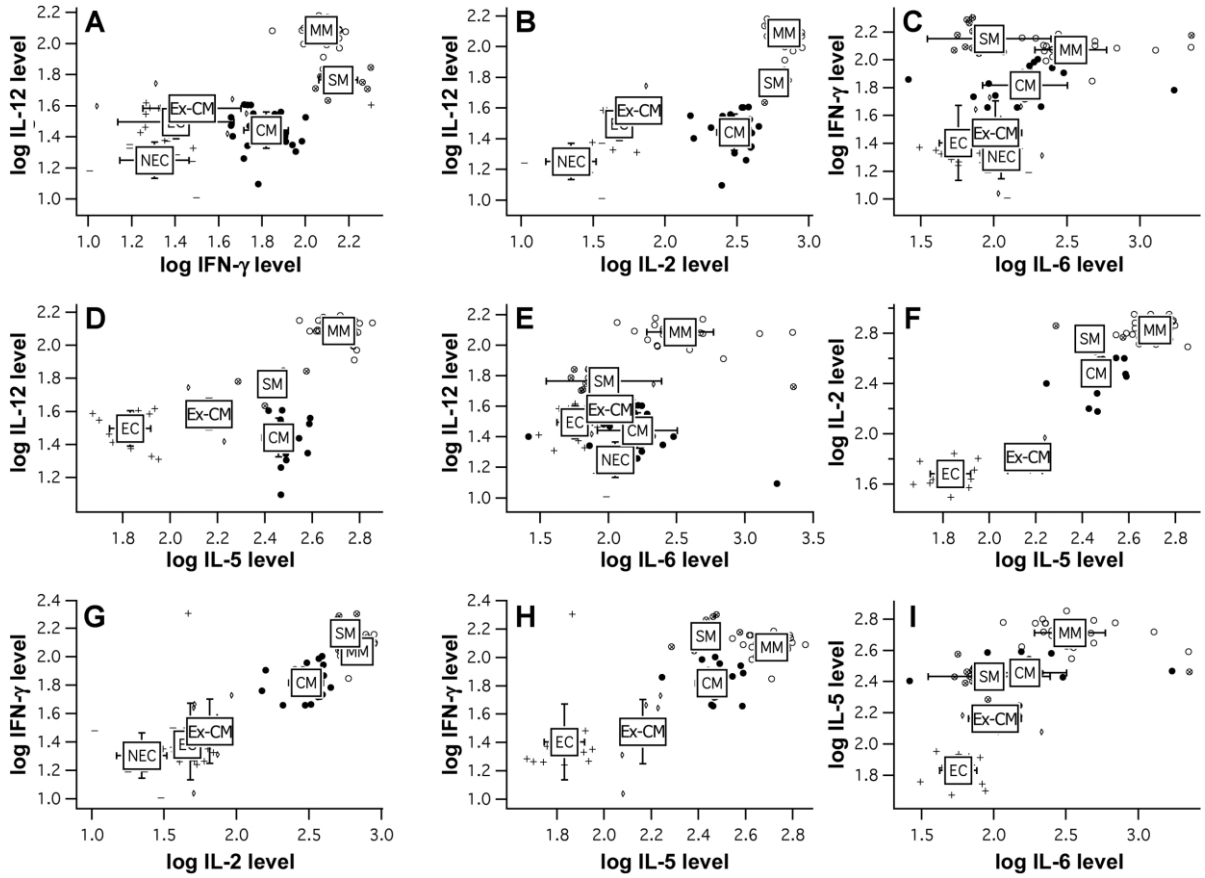

Figure 3. Relationships of cytokines represented in cluster 1. Log-transformed distributions of plasma levels (in pg/mL) of the cytokines represented in cluster 1 (interferon [IFN]–γ, interleukin [IL]–2, IL-5, IL-6, and IL-12) are depicted pairwise. Patient groups are denoted as follows: minus signs (−), nonendemic control (NEC; healthy control subjects from non–malaria-endemic areas); plus signs (+), endemic control (EC; healthy control subjects from malaria-endemic areas); white circles, mild malaria (MM); crossed circles, noncerebral severe malaria (SM); black circles, cerebral malaria (CM); diamonds, recovered from CM (ex-CM). Mean and SD for each group are denoted by error bars.

the MM group and the SM group, although IL-12 levels were significantly higher in the MM group.

Evidently, cytokine levels thus had the power to discriminate between clinical forms of malaria in the samples we studied. Therefore, to characterize the overall cytokine patterns relevant to each clinical subgroup of disease manifestation, we applied a coupled 2-way cluster analysis [27, 28]. This method allows coupled clustering of both the patients and the measured parameters without taking into account the clinical diagnosis. Since IL-5 levels were not determined for the nonendemic control subjects, only endemic control subjects and ex-CM subjects were included in addition to patients with clinical malaria. As is shown in figure 2, uninfected subjects (control and ex-CM) and patients with MM, SM, or CM segregated perfectly, with the exception of 1 patient with SM located in the cluster of patients with MM. However, this single misclassification represents 1 of 2 serum samples that had been taken from patients 1 day after commencement of antimalarial therapy.

The clustering of cytokines coupled with the classification of individuals identified 2 major clusters, which categorized all cytokines measured except IL-4 (figure 2). The first cluster included IFN-γ, IL-2, IL-5, IL-6, and IL-12, the levels of which are significantly increased during infection—predominantly in patients with MM—and decrease with malaria severity, with the exception of IL-12, the levels of which remain enhanced compared with those in the endemic control subjects and ex-CM subjects. This presents a convenient mechanism for discrimination of the MM group from the SM and CM groups. An interesting observation is that IL-6 levels were lower in the SM group than in the CM group. Levels of IFN-γ appeared to be most correlated with those of IL-2, being higher in infected patients than in control subjects but significantly lower in patients with CM than in patients with MM or SM (between which the difference was not significant) (figure 3*G*). In contrast, high IL-5 and IL-6 levels in plasma distinguished the MM group from the 2 severe-malaria groups, which exhibited low and intermediate levels of IL-6 and IL-5, respectively, whereas levels of both cytokines were low in endemic control and ex-

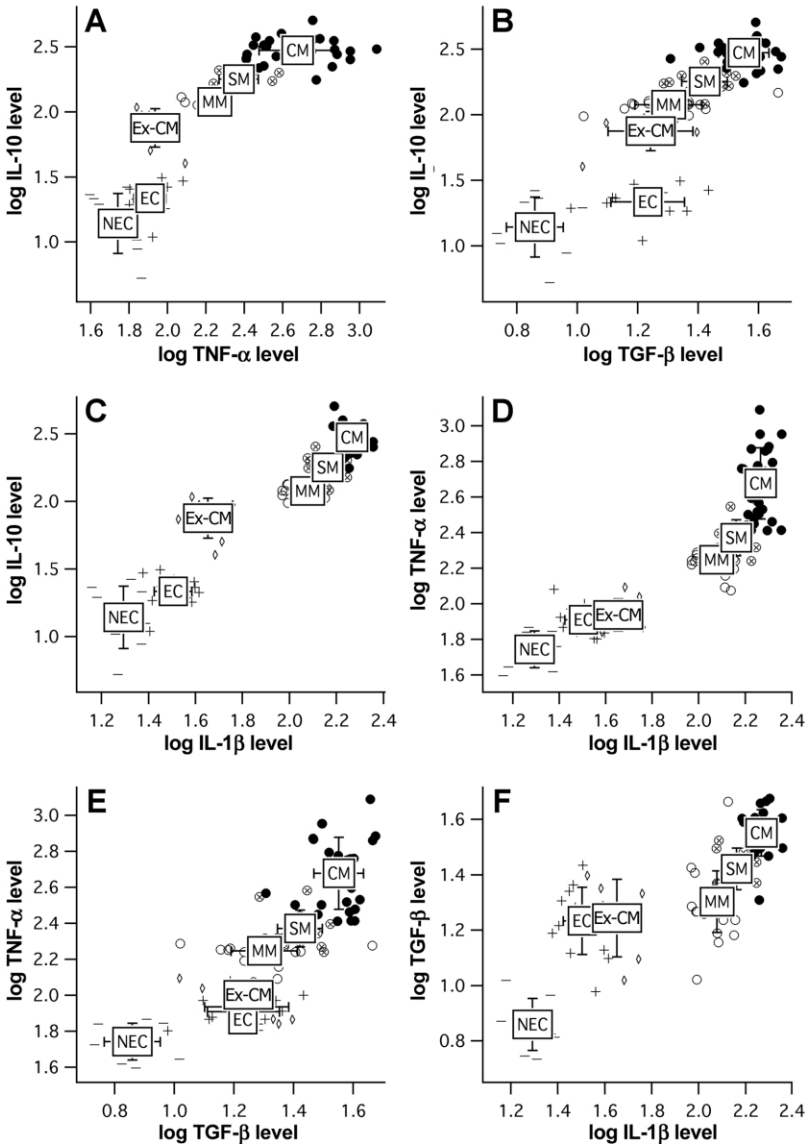

Figure 4. Relationships of cytokines represented in cluster 2. Log-transformed distributions of plasma levels (in pg/mL) of the cytokines represented in cluster 2 (transforming growth factor [TGF]–β, tumor necrosis factor [TNF]–α, interleukin [IL]-10, and IL-1β) are depicted pairwise. Mean and SD for each group are denoted by error bars. For symbols and abbreviations, see figure 3.

CM subjects (figure 3*I*). Most remarkably, IL-12 completely discriminated all 3 clinical forms, in that its levels were inversely proportional to disease severity—that is, they continuously decreased with increasingly severe malaria manifestation. This trend paralleled one seen with another cytokine in this group—namely, IL-2—but IL-12 was clearly more characteristic of the clinical form and was less influenced by the level of infection per se (figure 3*B*).

The second cluster consisted of TGF-β, TNF-α, IL-10, and IL-1β. Levels of all of these cytokines were highly correlated with each other when all the different clinical groups of patients were considered, and they significantly increased with disease severity. It is worthy of note that, in the ex-CM group, the levels of these cytokines (except IL-10) decreased to levels comparable to those in the endemic control groups. Levels of TGF-β in the MM group did not differ from those in the endemic control groups, whereas they were lower in the nonendemic control group (figure 4). The general results can be best represented by the distribution of IL-12 versus IL-10 in the different groups studied: a continuous increase in levels of cluster-2 cytokines with disease severity is associated with reductions in levels of cluster-1 cytokines that are individually fine-tuned (figure 5).

Discriminant analysis of cytokine profiles in patients with malaria. Cytokines were represented in the same cluster, because of correlations among them (figure 2). Such correlation

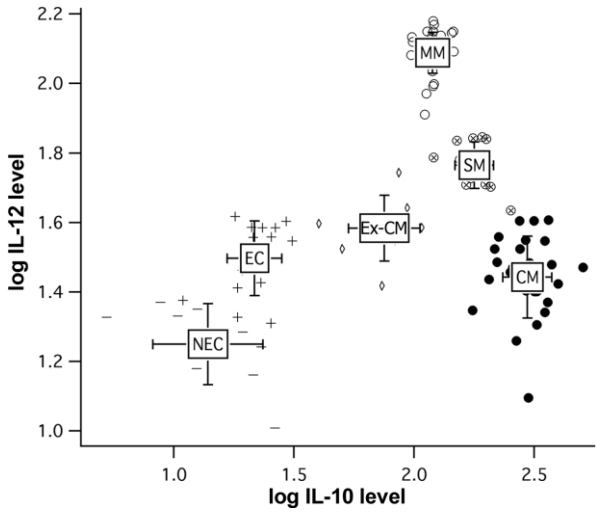

Figure 5. Relationship between distributions of interleukin (IL)–12 and IL-10. Shown is the 2-dimensional distribution of IL-12 and IL-10, as the most characteristic representatives for cluster-1 and cluster-2 cytokines, respectively, in pg/mL. Mean and SD for each group are denoted by error bars. For group symbols, see figure 3. A continuous increase in levels of cluster-2 cytokines (transforming growth factor [TGF]–β, tumor necrosis factor [TNF]–α, IL-10, and IL-1β) with disease severity is associated with a reduction in levels of cluster-1 cytokines (interferon [IFN]–γ, IL-2, IL-5, IL-6, and IL-12).

implies that differences found for a single cytokine in relation to clinical forms may indicate either (1) a functional association between the cytokine and a specific pathology or (2) that it follows the same general trend as any another functionally associated factor. Discriminant analysis is a method that is, in principle, capable of estimating these 2 possibilities within a given panel of parameters. Thus, to identify the most likely genuinely associated cytokines among the panel reported above, rather than those that are secondarily associated with a particular manifestation of malaria, we performed a series of discriminant analyses. First, we performed a linear discriminant analysis, including the same groups as the above cluster analysis, with the exception of the ex-CM group.

The resulting 2-factor representation is depicted in figure 6*A*, which shows complete separation of 4 groups and shows that 2 resulting factors account for infection (factor 1, horizontal) and clinical forms (factor 2, vertical). To resolve the different possible effects separately, we performed more-specific analyses, as indicated in the figure by dashed lines, for infection (discrimination A), severity (discrimination B), and CM (discrimination C). Cytokines relevant to each type of discrimination are annotated. When discriminating infection, we found that levels of IL-5, IL-1β, IL-10, and IL-2 increased with infection, thereby contributing significantly (in the order indicated, with IL-5 having the highest impact). When discriminating SM from MM, we noted that IL-12, IL-5, and IL-6 were relevant, in that levels of all 3 were relatively increased in the MM group. Finally, IL-1β, IL-12, and IFN-γ seemed relevant for the discrimination of CM from SM. High IL-1β levels were associated with CM, and high levels of IL-12 and IFN-γ were associated with SM. These results are summarized in a minimal model (figure 6*B*).

DISCUSSION

The balance between pro- and anti-inflammatory cytokines plays a pivotal role in the regulation of immune responses and pathogenesis in *P. falciparum* malaria, although, to date, their role in disease pathogenesis and relationship to host protection have remained unclear. In the present study, we directly addressed the relationship between the clinical severity of malaria and the complex pro- and anti-inflammatory cytokine network in subjects from the Gondia District of Maharashtra State, India. As reported above, malaria disease severity in patients was found to be independent of parasitemia, sex, and age.

Multivariate cluster and discriminant analyses suggest a 2-component model of clinical forms of malaria-related cytokine responses. As a first component, a response with protective properties against severe clinical forms is clearly indicated in our analysis by cytokine cluster 1, which is best represented by IL-12. IL-12 has been shown to be down-regulated in SM but not in mild *P. falciparum* malaria [29]. A study performed in Gabonese children revealed low IL-12 levels in SM, suggesting that the inflammatory cascade in SM is characterized by suppression of protective effects of IL-12 [30]. Additionally, an increase in IL-12 levels during the acute phase of uncomplicated *P. falciparum* malaria is thought to reflect an early and effective immune response regulated by proinflammatory Th1 cytokines [31]. Cluster 1 also includes IFN-γ and IL-2 and appears to be a Th1-type cytokine network. IFN-γ is a critical mediator of immunity to malaria and is generally associated with protective mechanisms [32, 33]. This association between proinflammatory cytokines and MM is in agreement with previous studies indicating that an increase in IL-2 levels during the acute phase of uncomplicated *P. falciparum* malaria could affect an early and effective response regulating Th1 cytokines [31]. Increased levels of IFN-γ were detected in serum from patients with uncomplicated malaria but were also detected in those with SM and CM in a study in Bangkok [34]. In our study, this network also involved the induction of IL-5 and IL-6, levels of which were found to be inversely correlated with disease severity as well. This is in contrast to previous studies showing levels of IL-6 to be increased in SM [35]. It would be interesting to explore further the association between malaria severity and IL-5, which has so far been studied to a very limited extent in this context.

Pathogenic Th1-type responses have been suggested to be down-regulated by factors such as IL-10 [36], but protective Th1-type immunity can be also down-regulated and suppressed

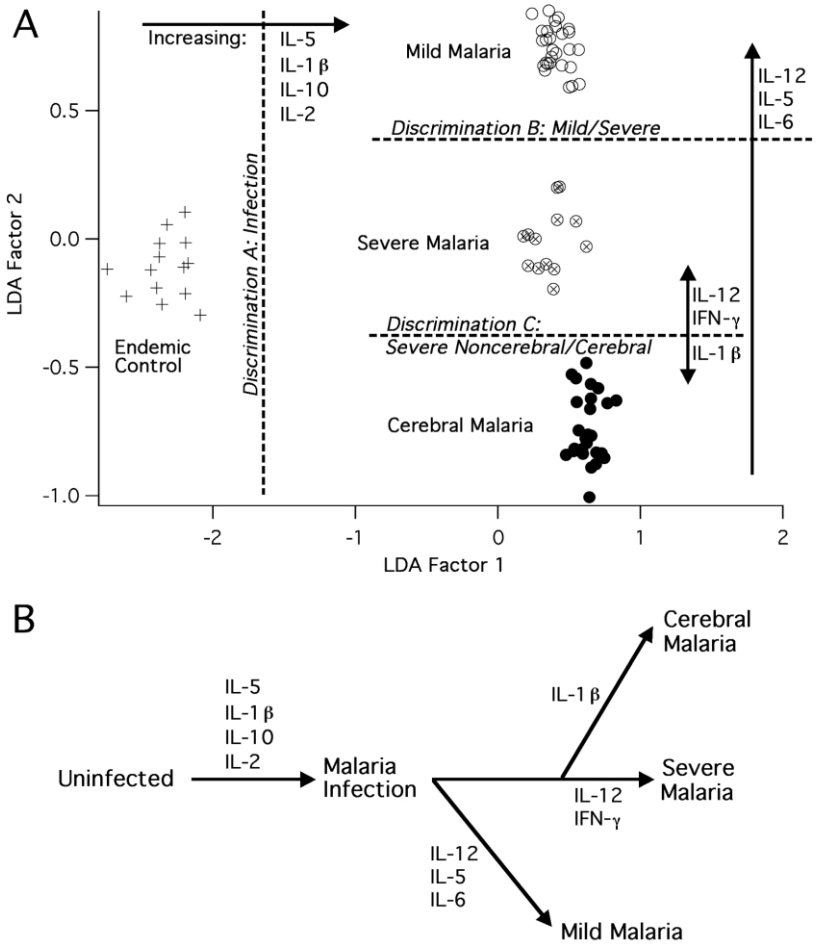

Figure 6. Linear discriminant analysis (LDA) of the cytokine profiles in *Plasmodium falciparum*–infected patients and healthy control subjects from malaria-endemic areas (endemic control [EC]). *A,* Factors 1 and 2 resulting from LDA, discriminating the EC, mild malaria (MM), severe noncerebral malaria (SM), and cerebral malaria (CM) groups according to their cytokine profiles. The completely separated 4 groups were further studied by more-specific discriminant analyses, indicated in the figure by dashed lines, for infection (between EC and all other groups; discrimination A), severity (between MM and severe forms; discrimination B), and CM (against SM; discrimination C). Annotations indicate the cytokines that turned out to be relevant for each type of discrimination, in their respective order of significance calculated by backward elimination. *B,* Minimal model summarizing the cytokine effects that were found to be significant for each type of discrimination.

in severe forms of malaria by a second type of response triggered by plasmodial infection [37, 38]. Our results are in agreement with the interpretation that such a "regulatory" response, as it was recently referred to [38], is represented by cytokine cluster 2. We found the highest levels of TNF-α, IL-1β, TGF-β, and IL-10 in the CM group, followed by SM group and then by the MM group. These results are in line with earlier findings showing increased levels of TNF-α, IL-10, and IL-1β in plasma from patients with SM [37–40]. Immunohistochemical staining of brain sections from patients with CM indicates tissue-localized production of TNF-α, IL-1β, and IL-10 (besides IFN-γ) in association with malaria pathology [41]. The most characteristic cytokine of this cluster is IL-10, which is known to be a pleiotropic immunomodulatory cytokine regulating not only Th1- but also Th2-type reactions in many instances [42–45]. However, the role of IL-10 in malaria is still controversial, because high levels have been shown to be associated with both severe disease and protection against *P. falciparum* infections [17, 30, 37, 40, 46]. IL-10 also shows the best evidence of a direct relationship with parasitemia, as has been demonstrated in children with high-density parasitemia [25] that reflects functionally less effective parasite clearance [47]. Our results fall in line with those of earlier studies, in which plasma levels of IL-10 were high in patients with CM [17]. However, a study performed in Bangkok indicated that serum levels of IL-10 and IFN-γ were markedly increased in patients with acute malaria [41]. The high levels of IL-10 associated with significantly low percentages of lymphocytes producing IL-4 during malaria suggest an immunoregulatory role rather than a Th2 activity [48]. In line with this hypothesis, IL-10 has been shown to inhibit the production of

TNF-α, IL-1β, IL-6, and IFN-γ [49], the latter 2 of which are cytokines found in cluster 1, associated with MM in our groups of patients. Interestingly, the "regulatory" response in our samples did not appear to follow a strict Th1/Th2 cytokine network scheme but was instead characterized by parallel increases in levels of IL-1β, TNF-α, and TGF-β. A burst of TGF-β, the presence of $CD4^+CD25^+FOXP3^+$ regulatory T cells, and a decrease in proinflammatory cytokine production have recently been found to be associated with higher rates of parasite growth in *P. falciparum*–infected volunteers [50].

Discriminant analysis showed that TNF-α and TGF-β did not significantly contribute to any group discrimination, a finding that is compatible with the possibility that these cytokines follow a pattern that is, rather, determined by other factors (as is the case with IL-10) without necessarily influencing the pathogenic process directly. The critical role of TNF-α in human CM has indeed been brought into question by the failure of TNF-α–neutralizing reagents to decrease the incidence of clinical CM [51, 52]. In contrast, increased IL-1β levels contributed significantly to the discrimination of CM in our population, supporting a direct pathogenic relevance. Significantly higher IL-1β levels have been observed in serum samples from patients with SM [53]. IL-1β mRNA has been detected in postmortem brain tissue samples from patients with CM, whereas it was absent from normal brain tissue [54].

Finally, our results suggest that the inflammatory cascade in SM is characterized by suppression of the protective effects of type 1 cytokines, such as IL-12, and that overproduction of TNF-α and IL-1β may promote deleterious effects. These molecules have an effective role in the pathogenesis of malaria, and their levels can be useful as diagnostic markers for malaria and for monitoring the severity of the disease [55]. Although each cytokine has its unique function, most cytokines exhibit a wide range of biological effects that act simultaneously on a particular cell type. This creates a complex cytokine network, which makes a precise correlation with malaria severity difficult. In conclusion, mechanisms linking cytokines with biochemical aspects of cell activation, regulation, and adhesion-molecule expression in the microvasculature seem to emerge as potential modulators of malaria severity. On the basis of these observations, measurement of panels of cytokines could be helpful for diagnosis.

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References

1. Snow RW, Craig M, Deichmann U, Marsh K. Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. Bull World Health Org **1999**; 77:624–40.
2. WHO Expert Committee on Malaria. 20th report. Geneva: WHO, **2000**.
3. Breman JG, Egan A, Keusch GT. The intolerable burden of malaria: a new look at the numbers. Am J Trop Med Hyg **2001**; 64:iv–vii.
4. Mazier D, Nitcheu J, Idrissa-Boubou M. Cerebral malaria and immunogentics. Parasite Immunol **2000**; 22:613–23.
5. Miller LH, Baruch DI, Marsh K, Doumbo OK. The pathogenic basis of malaria. Nature **2002**; 415:673–9.
6. Grau GE, De Kossodo S. Cerebral malaria: mediators, mechanical obstructions or more. Parasitol Today **1994**; 10:408–9.
7. Clark IA, Rockett KA. The cytokine theory of human cerebral malaria. Parasitol Today **1994**; 10:410–2.
8. Bondi FS. The incidence and outcome of neurological abnormalities in childhood cerebral malaria: a long term follow up of 62 surviviors. Trans R Soc Trop Med Hyg **1992**; 86:17–19.
9. Brewster DR, Kwiatkowski D, White NJ. Neurological sequelae of cerebral malaria in children. Lancet **1990**; 336:1039–43.
10. Beremdt AR, Simmons DL, Tansey J, Newbold CL, Marsh K. Intracellular adhesion molecule-1 is an endothelial cell receptor for *Plasmodium falciparum*. Nature **1989**; 341:57–9.
11. Hommel M. Cytoadherence of malaria-infected erythrocytes. Blood Cells **1990**; 16:605–19.
12. Miller KL, Silverman PH, Kullgren B, Mahlmann LT. Tumor necrosis factor alpha and the anemia associated with murine malaria. Infect Immun **1989**; 57:1542–6.
13. McGuire W, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature **1994**; 371:508–10.
14. McGuire W, Knight JC, Hill AV, Allsopp CE, Greenwood BM, Kwiatkowski D. Severe malarial anemia and cerebral malaria are associated with different tumor necrosis factor promoter alleles. J Infect Dis **1999**; 179:287–90.
15. Knight JC, Udalova I, Hill AV, et al. A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria. Nat Genet **1999**; 22:145–50.
16. Ubalee R, Suzuki F, Kikuchi M, et al. Strong association of a tumor necrosis factor-alpha promoter allele with cerebral malaria in Myanmar. Tissue Antigens **2001**; 58:407–10.
17. Peyron F, Burdin N, Ringwald P, Vuillez JP, Rousset F, Banchereau J. High levels of circulating IL-10 in human malaria. Clin Exp Immunol **1994**; 95:300–3.
18. De Kossodo, Grau GE. Role of cytokines and adhesion molecules in malaria immunopathology. Stem Cells **1993**; 11:41–8.
19. Taylor-Robinson AW, Phillips RS, Severn A, Moncada S, Liew FY. The role of Th1 and Th2 cells in a rodent malaria infection. Science **1993**; 260:1931–4.
20. Sedegah M, Finkelman F, Hoffman SL. Interleukin-12 induction of interferon gamma dependent protection against malaria. Proc Natl Acad Sci USA **1994**; 91:10700–2.
21. Winkler S, Willhem M, Baier K, Schmid D, Aichelburg A, Kremsner PG. Frequency of cytokine-producing T cells in patients of different age groups with *Plasmodium falciparum*. J Infect Dis **1999**; 179:209–16.
22. Ho M, Schollaardt T, Snape S, Looreesuwan S, White NJ. Endogenous interleukin-IL-10 modulates proinflammatory response in *Plasmodium falciparum* malaria. J Infect Dis **1998**; 178:520–5.
23. Kurtis JD, Lanar DE, Opollo M, Duffy PE. Interleukin-10 responses to liver-stage antigen 1 predict human resistance to *Plasmodium falciparum*. Infect Immun **1999**; 67:3424–9.
24. Nussenblatt V, Mukasa G, Metzger A, Ndeezi G, Garrett E, Semba DR. Anemia and interleukin-10, tumor necrosis factor alpha, and erythropoitein levels among children with acute, uncomplicated *Plasmodium falciparum* malaria. Clin Diagn Lab Immunol **2001**; 8:1164–70.
25. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, Udhayakumar V. A low interleukin-10, tumor necrosis factor-alpha ratio is associated with anemia in children residing in a holoendemic malaria region in western Kenya. J Infect Dis **1999**; 180:1753–5.
26. Esamai F, Ernerudh J, Janols H. et al. Cerebral malaria in children:

serum and cerebrospinal fluid TNF-alpha and TGF-beta levels and their relationship to clinical outcome. J Trop Pediatr **2003**; 49:216–23.
27. Getz G, Levine E, Domany E. Coupled two-way clustering analysis of gene micro array data. Proc Natl Acad Sci USA **2000**; 97:12079–84.
28. Blatt M, Wiseman S, Domany E. Superparamagnetic clustering of data. Phys Rev Lett **1996**; 76:3251–4.
29. Luty AJ, Perkins DJ, Lell B, et al. Low interleukin-12 activity in severe *Plasmodium falciparum* malaria. Infect Immun **2000**; 68:3909–15.
30. Perkins DJ, Weinberg JB, Kremsner PG. Reduced interleukin-12 and transforming growth factor–β1 in severe childhood malaria: relationship of cytokine balance with disease severity. J Infect Dis **2000**; 182: 988–92.
31. Torre D, Speranza F, Giola M, Matteelli A, Tambini R, Biondi G. Role of Th1 and Th2 cytokines in immune response to uncomplicated *Plasmodium falciparum* malaria. Clin Diagn Lab Immunol **2002**; 9:348–51.
32. Ferreira A, Schofield L, Enea V, et al. Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. Science **1986**; 232:881–4.
33. Herrera MA, Rosero F, Herrera S, et al. Protection against malaria in *Aotus* monkeys immunized with antigens fused to a universal T-cell epitope: correlation of serum gamma interferon levels with protection. Infect Immun **1992**; 60:154–8.
34. Nagamine Y, Hayano Kashiwamura S, Okamura H, et al. Involvement of interleukin-18 in severe *Plasmodium falciparum* malaria. Trans R Soc Trop Med Hyg **2003**; 97:236–41.
35. Lyke KE, Burges RB, Cissoko Y, et al. HLA-A2 supertype-restricted cell-mediated immunity by peripheral blood mononuclear cells derived from Malian children with severe or uncomplicated *Plasmodium falciparum* malaria and healthy controls. Infect Immun **2005**; 73: 5799–808.
36. Hunt NH, Grau GE. Cytokines: accelerators and brakes in the pathogenesis of cerebral malaria. Trends Immunol **2003**; 24:491–9.
37. Winkler S, Willheim M, Baier K, et al. Reciprocal regulation of Th1- and Th2-cytokine-producing T cells during clearance of parasitemia in *Plasmodium falciparum* malaria. Infect Immun **1998**; 66:6040–4.
38. Good MF. Identification of early cellular immune factors regulating growth of malaria parasites in humans. Immunity **2005**; 23:241–2.
39. Baptista JL, Vanham G, Wery M, Van Marck E. Cytokine levels during mild and cerebral falciparum malaria in children living in a mesoendemic area. Trop Med Int Health **1997**; 2:673–9.
40. Wenisch C, Parschalk B, Narzt E, Looareesuwan S, Graninger W. Elevated serum levels of IL-10 and IFN-γ in patients with acute *Plasmodium falciparum* malaria. Clin Immunol Immunopathol **1995**; 74: 115–7.
41. Maneerat Y, Pongponratn E, Viriyavejakul P, Punpoowong B, Looareesuwan S, Udomsangpetch R. Cytokines associated with pathology in the brain tissue of fatal malaria. Southeast Asian J Trop Med Public Health **1999**; 30:643–9.
42. de Waal Malefyt R, Yssel H, Roncarolo MG, Spits H, Vries JE. Interleukin-10. Curr Opin Immunol **1992**; 4:314–20.
43. Fiorentino DF, Bound MW, Mosmann TR. Two types of mouse T helper cells. IV. Th2 clones secrets a factor that inhibits cytokine production by Th1 clones. J Exp Med **1989**; 170:2081–95.
44. Fiorentino DF, Zlotnik A, Vieeira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol **1991**; 146:3444–51.
45. Villegas EN, Wille U, Craig L, et al. Blockade of costimulation prevents infection-induced immunopathology in interleukin-10 deficient mice. Infect Immun **2000**; 68:2837–44.
46. Ho M, Sexton M, Tongtawe P, Looareesuwan S, Suntharasamai P, Webster HK. Interleukin-10 inhibits tumor necrosis factor production but not antigen-specific lymphoproliferation in acute plasmodium malaria. J Infect Dis **1995**; 172:838–44.
47. Hugosson E, Montgomery SM, Premiji Z, Troye-Blomberg M, Bjorkman A. Higher IL-10 levels are associated with less effective clearance of *Plasmodium falciparum* parasites. Parasite Immunol **2004**; 26:111–7.
48. Jason J, Archibald LK, Nwanyanwu OC, et al. Cytokines and malaria parasitemia. Clin Immunol **2001**; 100:208–18.
49. Moore KW, O'Garra A, de Waal Malefyt AR, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol **1993**; 11:165–90.
50. Walther M, Tongren JE, Andrews L. Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. Immunity **2005**; 23:287–96.
51. Kwiatkowski D, Molyneux ME, Stephens S, et al. Anti-TNF therapy inhibits fever in cerebral malaria. Q J Med **1993**; 86:217–8.
52. van Hensbroek MB, Palmer A, Onyiorah F. The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria. J Infect Dis **1996**; 174:1091–7.
53. Vogetseder A, Ospelt C, Reindl M, Schober M, Schmutzhard E. Time course of coagulation parameters, cytokines and adhesion molecules in *Plasmodium falciparum* malaria. Trop Med Int Health **2004**; 9: 767–73.
54. Brown H, Turner G, Rogerson S, et al. Cytokine expression in the brain in human cerebral malaria. J Infect Dis **1999**; 180:1742–6.
55. el-Nashar TM, el-Kholy HM, el-Shiety AG, Al-Zahaby AA. Correlation of plasma levels of tumor necrosis factor, interleukin-6 and nitric oxide with the severity of human malaria. J Egypt Soc Parasitol **2002**; 32: 525–35.