CONCISE COMMUNICATION

Reduced Interleukin-12 and Transforming Growth Factor–β1 in Severe Childhood Malaria: Relationship of Cytokine Balance with Disease Severity

Douglas J. Perkins,[1,2] J. Brice Weinberg,[1] and Peter G. Kremsner[2,3]

[1]VA Medical Center and Duke University Medical Center, Durham, North Carolina; [2]Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon; [3]Department of Parasitology, Institute for Tropical Medicine, University of Tübingen, Tübingen, Germany

Interleukin (IL)–12 and transforming growth factor (TGF)–β1 regulate the balance between pro- and anti-inflammatory cytokines in animal models of malaria. Since the cytokine balance may be an important determinant of whether a protective or a pathogenic immune response develops, plasma cytokine ratios were examined in Gabonese children with various degrees of malarial severity. Severe disease was characterized by high-density parasitemia and severe anemia. IL-12 and TGF-β1 were significantly lower, whereas tumor necrosis factor (TNF)–α and IL-10 were significantly higher in children with severe malaria. The ratios of TGF-β1/IL-12 and IL-10/IL-12 were significantly higher in the severe, compared with the mild, malaria group. In contrast, ratios of TGF-β1/TNF-α and IL-10/TNF-α were significantly lower in the severe malaria group. These results suggest that the inflammatory cascade in severe malaria is characterized by suppression of the protective effects of TGF-β1 and IL-12, and that overproduction of TNF-α may promote deleterious effects, such as severe anemia.

Malaria is one of the most prevalent causes of morbidity and mortality of infectious origin throughout the world, with an incidence of 300–500 million clinical cases each year, and causes 1.5–2.7 million deaths [1]. Infection with *Plasmodium falciparum* in areas of hyperendemicity, such as Lambaréné, Gabon, primarily affects children <5 years old, because of their nonimmune status. Severe falciparum malaria in Gabonese children is typically characterized by high-density parasitemia and severe anemia, with cerebral malaria occurring less frequently [2].

Because of the growing problem of antimalarial drug resistance and the lack of an effective vaccine, understanding the immunologic basis of protective immunity has become increasingly important. Regulation of the host-immune response to invading pathogens depends largely on the development of acquired immunity mediated by pro- and anti-inflammatory cytokines. The protective immune response in animal models of malaria is characterized by production of pro-inflammatory cytokines, such as interleukin (IL)–12 (for review see [3]). Parasite-promoted release of IL-12 from monocytes/macrophages, B cells, and other cell types is important for initiating the inflammatory cascade [3]. IL-12–induced protection in animal models of malaria is related to the ability of IL-12 to enhance differentiation of $CD4^+$ T cells into Th1 cells for the secretion of interferon (IFN)–γ [3, 4]. Augmented release of IFN-γ stimulates monocytes to secrete tumor necrosis factor (TNF)–α, which can promote antiplasmodial properties through formation of toxic free radicals, such as nitric oxide [4].

Transforming growth factor (TGF)–β appears to be an important cytokine for maintaining the balance between protection and progression toward disease. At low levels, TGF-β has pro-inflammatory properties, whereas high levels of TGF-β are associated with anti-inflammatory effects (for review see [5]). Administration of recombinant TGF-β to *P. berghei*–infected mice significantly extends survival, perhaps by decreasing TNF-α and increasing IL-10 [5]. Conversely, treatment of malaria-infected mice with a neutralizing antibody to TGF-β increases virulence, which suggests that TGF-β protects against severe disease [5].

Although pro-inflammatory responses often are associated with protective cell-mediated immunity, and anti-inflammatory responses are associated with susceptibility to malaria, the bal-

Received 25 February 2000; revised 31 May 2000; electronically published 17 August 2000.

Presented in part: 48th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Washington, DC, December 1999 (abstract 291).

Informed consent was obtained from the parents of participating children, and the study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital and the Investigational Review Board of Duke University Medical Center.

Financial support: National Institutes of Health (AI-07217 to D.J.P. and AR-39162 to J.B.W.); James R. Swiger Hematology Research Fund; Veterans Administration Research Service (to J.B.W.); Fortüne, Medical Faculty, University of Tübingen, Germany; and EU-INCO-DC program (to P.G.K.).

Reprints or correspondences: Dr. Douglas J. Perkins, Centers for Disease Control and Prevention, Division of Parasitic Diseases, Molecular Vaccine Section, 4770 Buford Hgwy., Mail Stop F-12, Atlanta, GA 30341 (dqp5@cdc.gov).

The Journal of Infectious Diseases 2000; 182:988–92

0022-1899/2000/18203-0050$02.00

Table 1. Clinical, parasitological, and laboratory measures of study participants.

Characteristic	Healthy controls[a]	Mild malaria	Severe malaria	*P*
No. of patients	25	19	15	
Age, years	4.6 ± 0.6	4.9 ± 0.4	4.1 ± 0.7	
Hyperparasitemia,[b]				
⩾250,000 parasites /μL	0	0	10	
Severe anemia[b]				
Hemoglobin level, ⩽6.0 g/dL	ND	0	5	
PCV, ⩽20%	ND	0	10	
Schizontemia	0	0	6	
Parasitemia/μL				
Parasites/μL	0	53,682 ± 10,988	273,546 ± 39,395	<.001
Geometric mean		41,369	275,005	
Hemoglobin level, g/dL	ND	10.3 ± 0.3	7.6 ± 0.48	<.05
PCV	ND	32.4 ± 0.8	23.3 ±1.38	<.01
Platelet count, $\times 10^6$/L	ND	208.9 ± 23.2	123.8 ± 19.9	<.01
Glucose, mg/dL	ND	87.1 ± 4.9	82.1 ± 6.1	NS
Temperature, °C	37.1 ± 0.2	38.2 ± 0.2	39.4 ± 0.2	<.01
IL-12, pg/mL[c]	54.3 ± 6.5	70.6 ± 7.8	33.8 ± 11.0	<.05
TNF-α, pg/mL[c]	82.1 ± 13.4	131.5 ± 22.5	273.7 ± 50.9	<.05
TGF-β1, ng/mL[c]	16.4 ± 1.2	9.1 ± 1.0	7.6 ± 0.5	NS
IL-10, pg/mL[c]	18.9 ± 8.4	144.0 ± 24.0	241.2 ± 28.6	<.05

NOTE. Data are mean ± SE, unless otherwise stated. ND, not determined; PCV, packed cell volume; NS, not significant; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor.

[a] Every 2 weeks, healthy controls ($n = 25$) were examined for signs and symptoms of malaria, and malaria parasites were determined by using fingerprick, Giemsa-stained, thick blood smears. Healthy controls were excluded from analysis if they had malaria or any other detectable illness within the previous 2 months.

[b] Severe cases ($n = 15$) included those children with >250,000 parasites/μL, regardless of PCV, and those children with >100,000 parasites/μL in the presence of severe anemia (hemoglobin level ⩽6.0 g/dL or PCV ⩽20%). Children in the mild malaria group ($n = 19$) all had <100,000 parasites/μL and PCV >20%. Differences between the mild and severe groups were determined by the Mann-Whitney *U* test, with statistical significance set at $P < .05$.

[c] Plasma cytokine concentrations were determined by a quantitative sandwich EIA technique by using murine monoclonal antibodies raised against human IL-12, TNF-α, TGF-β1, and IL-10 for the respective cytokines. A monoclonal antibody specific for the active heterodimer of IL-12 was used for IL-12 measurements. Polyclonal antibodies specific for human IL-12, TNF-α, TGF-β1, and IL-10 conjugated to horseradish peroxidase were used for detection of the cytokines. Differences in cytokine levels between the mild and severe groups were determined by Mann-Whitney *U* test, with statistical significance set at $P < .05$.

ance of pro- and anti-inflammatory cytokines appears to be an important determinant of whether a protective or a pathogenic response develops. Recent studies show that low levels of IL-10, in the presence of high TNF-α concentrations, are associated with severe malarial anemia [6–8]. Since IL-10 can suppress TNF-α production from monocytes [9], low levels of IL-10 may allow excessive overproduction of TNF-α, which could promote anemia. To determine how the relative expression of cytokines influences disease manifestations in malaria, we examined ratios of pro- and anti-inflammatory cytokines in Gabonese children with various degrees of malarial severity.

Subjects and Methods

Subjects. Children ($n = 59$, 2–7 years old) were recruited at the Albert Schweitzer Hospital in Lambaréné, Gabon, an area hyperendemic for *P. falciparum* transmission. Healthy donors ($n = 25$; 12 men, 13 women) were recruited from an ongoing longitudinal study examining the occurrence of malaria in Lambaréné and the surrounding area. Patients who sought treatment for falciparum malaria were categorized into 2 groups: mild ($n = 19$; 9 men, 10 women) and severe ($n = 15$; 8 men, 7 women) malaria, which were defined by modified World Health organization guidelines ([10] see table 1). There were no cases of cerebral malaria in the present study. Children were given antimalarials and appropriate supportive therapy, as required. All cytokine measurements were performed on samples isolated before therapeutic interventions.

Eligible children were enrolled in the study, and venous blood samples (2–3 mL) from each were collected into sterile EDTA-containing vacutainer tubes. Plasma was separated immediately and frozen in liquid nitrogen, to avoid denaturation of cytokines.

Cytokine measurements. Plasma cytokine concentrations for human IL-12, TNF-α, TGF-β1, and IL-10 were determined by quantitative sandwich EIA technique with commercially available reagents (R&D Systems, Minneapolis). A monoclonal antibody specific for the active heterodimer of IL-12 was used for IL-12 measurements. Since activation of platelets can cause release of TGF-β1 from platelet granules, plasma was collected into EDTA as an anticoagulant, to prevent platelet activation; platelet-poor samples were generated by centrifugation of plasma at 10,000 *g*

Table 2. Pro- and anti-inflammatory cytokine ratios in children with mild and severe malaria.

Cytokine ratios	Mild malaria	Severe malaria	*P*
TGF-β1/IL-12			
Mean ± SE	0.38 ± 0.26	4.20 ± 1.07	<.01
Median (range)	0.12 (0.00–4.40)	4.02 (0.05–10.14)	
IL-10/IL-12			
Mean ± SE	4.20 ± 1.07	110.31 ± 33.01	<.01
Median (range)	2.10 (0.10–82.50)	104.27 (1.18–374.77)	
TGF-β1/TNF-α			
Mean ± SE	0.36 ± 0.21	0.04 ± 0.01	<.01
Median (range)	0.12 (0.02–0.39)	0.03 (0.00–0.09)	
IL-10/TNF-α			
Mean ± SE	1.78 ± 0.33	0.98 ± 0.09	<.05
Median (range)	1.51 (0.63–5.75)	1.08 (0.48–1.62)	

NOTE. Differences between the mild ($n = 19$) and severe malaria ($n = 15$) groups were determined by the Mann-Whitney *U* test, with statistical significance set at $P < .05$. Although there were 5 children in the severe malaria group who had high-density parasitemia with hemoglobin and hematocrit levels slightly above the cut-off criteria for severe anemia, removal of these children from the analysis did not change the results. TGF, transforming growth factor; IL, interleukin; TNF, tumor necrosis factor.

for 15 min. Plasma samples for TGF-β1 determinations were activated by incubation with 2.5 N acetic acid/10 N urea for 10 min, followed by neutralization with 2.7 N NaOH/1 *M* HEPES. Polyclonal antibodies specific for human IL-12, TNF-α, TGF-β1, and IL-10 conjugated to horseradish peroxidase were used for detection of the cytokines. Sensitivity of detection for the cytokine assays was as follows: IL-12, <5.0 pg/mL; TNF-α, <4.4 pg/mL; TGF-β1, <7.0 ng/mL; and IL-10, <3.0 pg/mL.

Statistical analysis. Plasma cytokine concentrations were determined in triplicate and expressed as mean ± SE of the mean. Comparisons between groups were made using the Mann-Whitney *U* test, with statistical significance set at $P < .05$. Regression analysis was used to determine the correlation between variables.

Results

Patient characteristics. Children were recruited from the Albert Schweitzer Hospital and the surrounding community and were classified into 3 groups: healthy donors ($n = 25$; mean age, 4.6 ± 0.6 years), mild malaria ($n = 19$; mean age, 4.9 ± 0.4 years), and severe malaria ($n = 15$; mean age, 4.1 ± 0.7 years). Patient characteristics are summarized in table 1.

IL-12 and TNF-α. Plasma IL-12 was nonsignificantly increased in children with mild malaria, compared with healthy donors ($P = .21$; table 1). However, IL-12 was significantly lower in children with severe malaria ($P < .05$ vs. controls and vs. mild malaria; table 1). Circulating IL-12 concentrations were detected in all 25 healthy donors, in 18 (95%) of 19 children with mild malaria, and in only 6 (40%) of 15 children with severe malaria. IL-12 levels were inversely correlated with peripheral parasitemia ($P < .01$, $r = -0.52$).

Compared with levels in healthy donors, TNF-α levels were significantly elevated in children with mild ($P < .05$) and severe malaria ($P < .01$; table 1). Children with severe disease also had significantly higher concentrations of TNF-α than did children with mild disease ($P < .05$; table 1). TNF-α levels were correlated with peripheral parasitemia ($P < .01$, $r = 0.43$). The within-patient levels of IL-12 and TNF-α were not associated ($P > .05$).

TGF-β1 and IL-10. TGF-β1 was reduced in the mild ($P < .01$) and severe malaria groups ($P < .01$; table 1). TGF-β1 levels were not significantly different between the control group and the mild and severe malaria groups ($P > .05$; table 1). TGF-β1 levels were inversely correlated with peripheral parasitemia ($P < .01$, $r = -0.44$) and TNF-α ($P < .05$, $r = -0.34$) but not associated with IL-12 ($P > .05$).

IL-10 was significantly increased in the mild and severe malaria groups ($P < .01$ for both groups vs. controls), with the highest IL-10 concentrations in the severe malaria group ($P < .05$ vs. mild malaria; table 1). IL-10 levels were positively associated with peripheral parasitemia ($P < .01$, $r = 0.68$) and TNF-α ($P < .01$, $r = 0.58$) and inversely correlated with TGF-β1 ($P < .01$, $r = -0.60$). Circulating IL-10 was not associated with IL-12 ($P > .05$).

Ratios of pro- and anti-inflammatory cytokines. Because anti-inflammatory cytokines can decrease the pro-inflammatory response, we examined TGF-β1 relative to IL-12 and found that the TGF-β1/IL-12 ratio was significantly higher in children with severe malaria ($P < .01$; table 2). In addition, since IL-10 can suppress IL-12 [11], we examined the IL-10/IL-12 ratios in mild and severe disease. The IL-10/IL-12 ratio was also significantly higher in children with severe disease ($P < .01$; table 2).

The ratios of TGF-β1/TNF-α and IL-10/TNF-α also were determined, because suppression of TNF-α by anti-inflammatory cytokines may be important for controlling overproduction of TNF-α. The ratios of TGF-β1/TNF-α and IL-10/TNF-α were significantly lower in the severe malaria group ($P < .01$ and $P < .05$, respectively; table 2).

Discussion

The immune response to malaria likely is regulated by the balance of pro- and anti-inflammatory cytokines that culminate in either immunoprotection or pathogenesis. Results presented here show that IL-12 and TGF-β1 were significantly lower, whereas TNF-α and IL-10 were significantly higher, in children with severe malaria. In addition, high ratios of TGF-β1/IL-12 and IL-10/IL-12 were associated with severe malaria, whereas low ratios of IL-10/TNF-α and TGF-β1/TNF-α were associated with severe disease. Thus, we postulate that the anti-inflammatory response may suppress IL-12 but be insufficient for preventing excessive TNF-α production, which could promote anemia in children with acute malaria.

Elimination of intracellular pathogens appears to require cell-mediated immunity initiated by pro-inflammatory molecules, such as IL-12 [3]. Induction of immunity in susceptible A/J mice after administration of recombinant IL-12 strongly sup-

ports the necessity of a pro-inflammatory response for controlling malaria [4]. In addition, administration of recombinant IL-12 before inoculation of rhesus monkeys with *P. cynomolgi* provides 100% protection through an IFN-γ–dependent (and perhaps nitric oxide–dependent) antiplasmodial mechanism [12]. The association between low IL-12 and severe malaria reported here suggests that IL-12 may also be important for promoting immunoprotection in human malaria.

Our previous studies show that plasma IL-12 levels are equivalent during the convalescent phase of disease in Gabonese children who had either mild or severe malaria [2]. The cause of low circulating IL-12 in children with severe malaria, as reported here, may be related to reduction of IL-12 by an inhibitory factor(s). Since previous studies show that TGF-β1 can reduce the pro-inflammatory response [13], we reasoned that TGF-β1 may suppress IL-12. The significantly higher ratio of TGF-β1/IL-12 in the severe malaria group supports this hypothesis. However, it is also possible that a common feature of malaria, such as macrophage ingestion of malarial pigment, may suppress both cytokines.

The high levels of circulating immune complexes documented in severe falciparum malaria [14], and the finding that immune complexes are potent inhibitors of IL-12 by human monocytes via TNF-α–induced increases of IL-10 [11], may also explain our results. The significant positive correlation between TNF-α and IL-10 and the high IL-10/IL-12 ratio in children with severe disease supports this notion. Thus, immune complex–promoted increases in TNF-α and IL-10 may explain the decreased IL-12 reported here.

Low levels of TGF-β1 in Gabonese children with malaria are comparable to results obtained in Southeast Asian adults with acute falciparum malaria [15], which suggests that reduced TGF-β1 may be a common feature of malaria. Previous experiments in rodent malaria show that TGF-β is inversely correlated with severity of infection and that TGF-β is important for regulating cytokine expression; administration of recombinant TGF-β to *P. berghei*–infected mice significantly decreases plasma levels of TNF-α [5]. Although it is difficult to extrapolate a sequence of events from a single measurement, the low TGF-β1/TNF-α ratio shown here in children with severe disease parallels the findings in murine malaria, which suggests that lack of a TGF-β response in children with severe malaria may contribute to their overproduction of TNF-α.

Although IL-10 and TNF-α increased with disease severity, a low IL-10/TNF-α ratio was associated with severe disease. An earlier study illustrates that a low IL-10/TNF-α ratio is associated with severe anemia among children living in a holoendemic region of malaria transmission [7]. The association between a low IL-10/TNF-α ratio and severe malaria in Gabonese children residing in a hyperendemic region of malaria transmission, as presented here, confirms those findings and extends previous observations by illustrating that a low TGF-β1/TNF-α ratio is also associated with severe malaria. Thus, while IL-10 and TGF-β1 were inversely correlated and appear to suggest opposite trends for severe disease, their expression relative to TNF-α reveals that low ratios for both cytokines are associated with severe malaria. These findings underscore the importance of considering the relative balance of cytokines when trying to characterize the immunopathogenesis of malaria.

In summary, we illustrate that the relative balance of pro- and anti-inflammatory cytokines appears to be an important indicator of malarial severity in children with high-density parasitemia and severe anemia. On the basis of results presented here, we postulate that low levels of IL-12 and TGF-β1 contribute to increased disease severity in malaria. In addition, our results suggest that the anti-inflammatory response may decrease IL-12 but be insufficient for controlling overproduction of TNF-α. 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. It is 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. We are currently conducting longitudinal studies to assess this possibility.

Acknowledgments

We thank the following staff members of the Albert Schweitzer Hospital in Lambaréné, Gabon, for their cooperation and technical assistance: Anita van den Biggelaar, Judith Jans, Hanna Knoop, Doris Luckner, Barbara Moritz, Anselme Ndzengue, Marcel Nkeyi, Daniela Schmid, and Milena Sovric. We also thank James B. Hittner, Department of Psychology, College of Charleston, South Carolina, for his statistical expertise and critical evaluation of the manuscript.

References

1. World Health Organization. World malaria situation in 1994. Wkly Epidemiol Rec **1997**;72:269–76.
2. Perkins D, Kremsner P, Schmid D, Misukonis M, Kelly M, Weinberg JB. Blood mononuclear cell nitric oxide production and plasma cytokine levels in healthy Gabonese children with prior mild or severe malaria. Infect Immun **1999**;67:4977–81.
3. Crutcher JM, Stevenson MM, Sedegah M, Hoffman SL. Interleukin-12 and malaria. Res Immunol **1995**;146:552–9.
4. Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12–induced protection against blood-stage *Plasmodium chabaudi* AS requires IFN-γ and TNF-α and occurs via a nitric oxide–dependent mechanism. J Immunol **1995**;155:2545–56.
5. Omer F, Kurtzhals J, Riley E. Maintaining the immunologic balance in parasitic infections: a role for TGF-β. Parasitol Today **2000**;16:18–23.
6. Mordmuller BG, Metzger WG, Juillard P, et al. Tumor necrosis factor in *Plasmodium falciparum* malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance. Eur Cytokine Netw **1997**;8:29–35.
7. Othoro C, Lal A, Nahlen B, Koech D, Orago A, Udhayakumar V. A low interleukin-10 tumor necrosis factor–α ratio is associated with malaria anemia in children residing in a holoendemic region in western Kenya. J Infect Dis **1999**;179:279–82.

8. Kurtzhals J, Abadayeri V, Goka B, et al. Low plasma concentrations of interleukin 10 in severe malarial anemia compared with cerebral and uncomplicated malaria. Lancet **1998**; 351:1768–72.
9. Ho M, Sexton M, Tongtawe P, Looareesuwan S, Suntharasamai P, Webster K. Interleukin-10 inhibits tumor necrosis factor production but not antigen-specific proliferation in acute *Plasmodium falciparum* malaria. J Infect Dis **1995**; 172:838–44.
10. World Health Organization. Severe and complicated malaria. Trans R Soc Trop Med Hyg **1990**; 84(Suppl 2):1–65.
11. Berger S, Chandra R, Ballo H, Hilenbrand R, Stutte H. Immune complexes are potent inhibitors of interleukin-12 secretion by human monocytes. Eur J Immunol **1997**; 27:2994–3000.
12. Hoffman S, Crutcher J, Puri S, et al. Sterile protection of monkeys against malaria after administration of interleukin-12. Nat Med **1997**; 3:80–3.
13. Bogdan C, Nathan C. Modulation of macrophage function by transforming growth factor–β, interleukin-4, and interleukin-10. Ann NY Acad Sci **1993**; 685:713–39.
14. Perlmann P, Perlmann H, Flyg BW, et al. Immunoglobulin E, a pathogenic factor in *Plasmodium falciparum* malaria. Infect Immun **1997**; 65:116–21.
15. Wenisch C, Parschalk B, Burgman H, Looareesuwan S, Graninger W. Decreased serum levels of TGF-β in patients with acute *Plasmodium falciparum* malaria. J Clin Immunol **1995**; 15:69–73.