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# Serum IL-4, IL-12 and TNF-alpha in malaria: a comparative study associating cytokine responses with severity of disease from the Coastal Districts of Odisha

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**Abstract** We investigated the role of IL-4, IL-12 and TNF-alpha in clinically well-defined groups of *Plasmodium falciparum* and *vivax* (Pf & Pv) infected patients belonging to Group I (++), Group II (+++) and Group III (++++). On the basis of hematological parameters, hyperparasitaemia, and evidence of neurological involvement, three different levels of severity were selected attributing a score from Group I (++) to Group III (++++). In each group 16 patients each of *P. falciparum* and *P. vivax* malaria were studied. As a control group for cytokine determination 30 healthy volunteers were included in the study. Serum samples were analyzed for IL-12, IL-4 and TNF-alpha using (ELISA) obtained commercially (Ray Biotech). Hb levels of Pf and Pv patients were 8 ± 1.94, 7.6 ± 1.64 g/dl and 3.6 ± 1.23 and 10.1 ± 1.21, 9.4 ± 1.43 and 7.1 ± 0.98 g/dl. Serum iron levels of Pf and Pv patients were 85.86 ± 0.86, 81.10 ± 0.70 and 70.1 ± 0.73 and 99.47 ± 0.85, 96.67 ± 1.13 and 91.7 ± 2.65 mg/dl. TNF-alpha levels of Pf and Pv patients were 155 ± 23.66, 307.5 ± 111.87 and 955 ± 261.32 and 72 ± 9.93, 140.88 ± 23.11 and 469.37 ± 416.99 pg/ml. IL-12 levels of Pf and Pv patients were 117.5 ± 8.16, 160.63 ± 20.81 and 293.13 ± 94.64 and 75.7 ± 9.25, 112.9 ± 12.05 and 200 ± 53.78 pg/ml. IL-4 levels of Pf and Pv patients were 3.7 ± 0.11, 3.2 ± 0.13 and 2.3 ± 0.63 and 5.33 ± 1.08, 4.8 ± 0.16 and 3.9 ± 0.48 pg/ml. In the control group the values of TNF-alpha, IL-12 and IL-4 were 42.9 ± 13.5, 49.8 ± 11.59 and 6.06 ± 1.32 pg/ml respectively. Cytokines and poor oxygen delivery should not be viewed as alternative theories of malarial disease pathophysiology instead poor oxygen delivery is one of the consequences of excessive release of inflammatory cytokines which is further augmented by the present work.

**Keywords** Cytokines · Malaria · TNF-alpha · IL-12 · IL-4 · Clinical severity

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## Introduction

Malaria is the world's most important parasitic infection. It remains today as it has been for centuries—a heavy burden on tropical countries like India. It affects more than 1 billion people world wide, causing more than 1–3 million deaths every year with prevalence in 103 countries (White and Breman 2001)

Malaria causes an acute systemic disease that bears many similarities, both clinically and mechanistically, to those caused by bacteria, rickettsia and viruses. Over the past few decades, a literature has emerged that argues for most of the pathology seen in all of these infectious diseases being explained by activation of the inflammatory system with the balance between the pro and anti-inflammatory cytokines being tipped towards the onset of systemic inflammation (Clark et al. 2006).

Optimal immune response to malaria infection is characterized by early intense proinflammatory cytokine-mediated effectors mechanisms that kill or clear parasite infected cells and which are then equally rapidly suppressed by anti-inflammatory effectors once parasite replication has been brought under control (Tsakonask et al. 2003).

The first characterized parasite induced cytokine was TNF-alpha induced in macrophages by erythrocytes infected by plasmodium, malarial pigment and certain glycolipids such as GPI moiety. It has been shown that GPI moiety induces NOS in macrophages and activates endothelial cells by tyrosine-kinase mediated signal transduction. IL-12 is a potent immunomodulatory cytokine which not only increases cell-mediated immune response but also affects humoral immunity by inducing isotype switching through both interferon-γ dependent and independent mechanisms.

IL-4 is produced by activated T cells of the Th-2 subtype and mast cells, it has been seen to be involved in the activation of CTL, NKcells and macrophages. Interleukin 4 and Th-2 subtype cells are important component of immune response stimulating growth of Th-2 and inhibiting Th-1 response by depressing the production of interferon-γ (Luty et al. 2000).

The main goal of this study was to measure the level of IL-12, IL-4 and TNF-alpha in the serum of three different groups of 96 patients with falciparum and vivax malaria and to correlate the production of these cytokines with the severity of disease.

## Materials and methods

The study was conducted in S.C.B. Medical College Hospital Cuttack the largest tertiary referral government hospital in Odisha. Inclusion and classification of each case were based on symptoms, physical signs and laboratory results of malaria at the time of first presentation.

*P. falciparum* malaria was established by microscopic diagnosis of *P. falciparum* parasites in the peripheral blood and clinical signs according to the WHO criteria: evidence of neurological compromission (prostration, lethargy), gastro-intestinal symptoms, severe anaemia (Hb $< 6$ g/dl), hyper-parasitaemia corresponding to Ep $> 5 \times 10^5$ or 5 %, acidosis with respiratory distress, oliguria, cardiovascular shock, jaundice, diffuse hemorrhages.

*P. vivax* malaria was established by parasitaemia of Ep $< 5 \times 10^5$ or $< 5$ %, with fever, headache, myalgias without any finding of *P. falciparum* malaria.

On the basis of hematological parameters, hyperparasitaemia, and evidence of neurological involvement, three different levels of severity were selected attributing a score from Group I (++) to Group III (++++). In each group 16 patients each of *P. falciparum* and *P. vivax* malaria were studied.

As a control group for cytokine determination 30 healthy volunteers from Cuttack of same range of age and sex were also included in the study. All volunteers enrolled as control group were negative at the thick-smear examination for *P. falciparum* and *P. vivax*, without febrile episodes during last 6 months and without signs of anaemia (Hb $> 10$ g/dl). The above study was approved by Institutional Ethics Committee, S.C.B. Medical College Cuttack, and subjects gave informed consent to the work.

For detection of parasitaemia, a calibrated thick-smear technique was used, with standard Giemsa staining. The blood samples were collected for immunological assessment in sterile tubes containing EDTA. All the samples were centrifuged and serum was refrigerated at −40 °C and were sent to the Laboratory of Department of Biochemistry, S.C.B. Medical Cuttack for the determination of IL-12, IL-4 and TNF-alpha.

Serum samples were analyzed for IL-12, IL-4 and TNF-alpha using enzyme-linked immunosorbent assay (ELISA) obtained commercially (Ray Biotech Inc, 3607 Parkway Lane, Suite 200, Norcross GA 30092). Blood hemoglobin was measured by cyanmethemoglobin method. Ramsay's dipyridyl method was applied for determination of serum iron.

The assays were performed according to the manufacturer's protocol. Each plate included a standard curve and known positive and negative controls. Absorbance was read against a blank at 450 nm using a microtiter ELISA reader.

With these experiments, statistical significance was analyzed by using the Student's two-tailed '*t*' distribution for parametric data. The level of significance was set at a two-tailed $P < 0.05$.

## Results

In Table 1 the mean values of more important clinical, parasitological and laboratory measures are reported according to criteria related to severity of the disease.

Group I (++) Pf patients had a mean Hb level of $8 \pm 1.94$ g/dl compared to $7.6 \pm 1.64$ g/dl of Group II (+++) and $3.6 \pm 1.23$ g/dl for Group III (++++) patients. Group I (++) Pv patients had a mean of Hb level of $10.1 \pm 1.21$ g/dl compared to $9.4 \pm 1.43$ g/dl of Group II (+++) and $7.1 \pm 0.98$ g/dl for Group III (++++) patients.

Serum iron level of Pf patients belonging to Group I (++) had a mean of $85.86 \pm 0.86$ mg/dl compared to

**Table 1** Clinical, parasitological and laboratory measures in Groups I–III comprising *P. falciparum* and *P. vivax* malaria patients according to clinical severity

| Characteristics | Group I (++) | | Group II (+++) | | Group III (++++) | | HC |
|---|---|---|---|---|---|---|---|
| | *P. falciparum* ($n = 16$) | *P. vivax* ($n = 16$) | *P. falciparum* ($n = 16$) | *P. vivax* ($n = 16$) | *P. falciparum* ($n = 16$) | *P. vivax* ($n = 16$) | ($n = 30$) |
| No of patients | 16 | 16 | 16 | 16 | 16 | 16 | 30 |
| Hyper parasitemia >250,000 parasites/µl | 05 | 0 | 09 | 01 | 14 | 05 | 0 |
| Severe anaemia Hb level <6.0 g/dl | 04 | 0 | 08 | 02 | 12 | 04 | 0 |
| Hemoglobin (g/dl) | 8.1 ± 1.94 | 10.1 ± 1.21 | 7.6 ± 1.64 | 9.4 ± 1.43 | 3.6 ± 1.23 | 7.1 ± 0.98 | 13.21 ± 0.85 |
| Serum iron (mg/dl) | 85.86 ± 0.86 | 99.47 ± 0.5 | 81.1 ± 0.70 | 96.67 ± 1.13 | 70.1 ± 0.73 | 91.7 ± 2.65 | 134.78 ± 5.18 |
| Temperature (°C) | 39.2 ± 0.2 | 38.4 ± 0.2 | 39.8 ± 0.2 | 39.0 ± 0.2 | 41 ± 0.2 | 39.2 ± 0.2 | 37.2 ± 0.2 |
| TNF-alpha (pg/ml) | 155 ± 23.66 | 72 ± 9.93 | 307.5 ± 111.87 | 140.88 ± 23.11 | 955 ± 261.32 | 469.37 ± 416.99 | 42.9 ± 13.5. |
| IL-12 (pg/ml) | 293.13 ± 94.64 | 75.7 ± 9.25 | 160.63 ± 20.81 | 112.9 ± 12.05 | 117.5 ± 8.16 | 200 ± 53.78 | 49.8 ± 11.59 |
| IL-4 (pg/ml) | 3.7 ± 0.11 | 5.33 ± 1.08 | 3.2 ± 0.13 | 4.8 ± 0.16 | 2.3 ± 0.63 | 3.9 ± 0.48 | 6.06 ± 1.32 |

*Note* Patients versus healthy controls $P < 0.05$, TNF-alpha, $P < 0.01$, IL-12, $P < 0.05$, IL-4, $P < 0.01$

81.10 ± 0.70 mg/dl of Group II (+++) and 70.1 ± 0.73 mg/dl for Group III (++++) patients. Similarly serum iron level of Pv patients belonging to Group I (++) had a mean of 99.47 ± 0.85 mg/dl compared to 96.67 ± 1.13 mg/dl of Group II (+++) and 91.7 ± 2.65 mg/dl for Group III (++++) patients.

TNF-alpha level in Pf patients belonging to Group I (++) had a mean of 155 ± 23.66 pg/ml compared to 307.5 ± 111.87 pg/ml of Group II (+++) and 955 ± 261.32 pg/ml for Group III (++++) patients. Similarly TNF-alpha level of Pv patients belonging to Group I (++) had a mean of 72 ± 9.93 pg/ml compared to 140.88 ± 23.11 pg/ml of Group II (+++) and 469.37 ± 416.99 pg/ml for Group III (++++) patients.

IL-12 level in Pf patients belonging to Group I (++) had a mean of 293.13 ± 94.64 pg/ml compared to 160.63 ± 20.81 pg/ml of Group II (+++) and 117.5 ± 8.16 pg/ml for Group III (++++) patients. In Pv patients belonging to Group I (++) IL-12 level had a mean of 75.7 ± 9.25 pg/ml compared to 112.9 ± 12.05 pg/ml and 200 ± 53.78 pg/ml for Group III (++++) patients.

IL-4 levels in Pf patients belonging to Group I (++) had a mean of 3.7 ± 0.11 pg/ml compared to 3.2 ± 0.13 pg/ml of Group II (+++) and 2.3 ± 0.63 pg/ml for Group III (++++) patients. In Pv patients belonging to Group I (++) IL-4 level had a mean of 5.33 ± 1.08 pg/ml compared to 4.8 ± 0.16 pg/ml of Group II (+++) and 3.9 ± 0.48 pg/ml for Group III (++++) patients.

In the control group the values of TNF-alpha, IL-12 and IL-4 were 42.9 ± 13.5, 49.8 ± 11.59 and 6.06 ± 1.32 pg/ml, respectively. Hb levels were 13.21 ± 0.85 g/dl and serum iron levels were 134.78 ± 5.18 mg/dl.

## Discussion

In our study TNF-alpha and IL-12 levels were elevated in all groups with malaria whereas IL-4 levels showed persistent decline, as expression of immune activation in response to the presence of parasites. However distinguishing three groups according to different levels of hemoglobin, serum iron and level of parasitaemia it is possible it is possible to understand the role of these cytokines and their relationship in the pathogenesis of malaria infection.

A large body of evidences indicates that cytokines are determinants of malaria severity and outcome (Day et al. 1999; Gogos et al. 2000; Malaguarnera and Musumeci 2002; Riley 1999) and can represent potential targets for therapeutic interventions, if their effect will be highlighted.

Complications of severe anaemia and cerebral malaria are thought to be major cause of morbidity and mortality but recent evidence suggests that the host's immunological response could also contribute to the pathophysiology of the disease (Malaguarnera et al. 2002). The pathogenic manifestations during a malaria crisis are due proinflammatory cytokines released by T cells and macrophages in response to malaria parasites and their products including glycosylphosphatidylinositol (GPI) moieties malaria pigment and plasmodium-derived nitric oxide synthase (NOS)-inducing factor.

Several studies suggest that the balance between proinflammatory cytokines such as TNF-alpha and anti-inflammatory cytokines such as IL-4 determines the degree of malaria parasitaemia, level of anaemia and clinical severity (Winkler et al. 1998). Other evidences suggest that

malaria outcome depends on cytokine overproduction and not on the balance between them, since high levels of anti-inflammatory as well as pro-inflammatory cytokines may be associated with disease severity and mortality (Day et al. 1999). In human malaria altered immune reactivity appears late in the acute phase of the disease and can last a long time after the clearance of parasites from the circulation. An explanation for the poor acquisition of malaria immunity in naturally exposed populations is that the parasite actively modulates the immune system of the host, preventing the development of specific immune responses (Plebanski and Hill 2000). The inflammatory response that is needed to remove parasites leads to considerable tissue damage and activation of phagocytes to kill intracellular or extracellular parasites requires the production of inflammatory cytokines, which can cause systemic effects such as severe anaemia and cerebral malaria (Gogos et al. 2000; McGuire et al. 1994). The outcome of infection depends on a delicate balance between appropriate and inappropriate induction of these mediators.

Our results, indicating the levels of TNF-alpha, IL-12 and IL-4 in three groups of patients comprising falciparum and vivax malaria according to clinical severity reveals a prognostic significance in malaria infection. TNF-alpha levels in falciparum and vivax malaria patients pertaining to all three groups (I–III) showed significant elevation [Pf: Group I = 155 ± 23.66 pg/ml, Group II = 307.5 ± 111.87 pg/ml and Group III = 955 ± 261.32 pg/ml. Pv: Group I = 72 ± 9.93 pg/ml, Group II = 140.88 ± 23.11 and Group III = 469.37 ± 416.99 pg/ml] compared to healthy controls [42.9 ± 13.5 pg/ml]. Further TNF-alpha levels were higher in Pf patients compared to Pv patients in all three groups. The intensity of elevation in TNF-alpha values was more steeper in falciparum malaria patients compared to vivax malaria patients in all three groups, studied signifying that TNF-alpha may be an important component in the pathogenesis of severe falciparum malaria and in particular in the cerebral syndrome and hypoglycemia which can complicate this disease (Grau et al. 1989). TNF-alpha induces migration inhibitory factor (MIF) and TNF-alpha, IL-1beta and LT generate the inducible form of nitric oxide synthase i NOS adding to the inflammatory cascade. Cytokines such as TNF-alpha increased in Pf and Pv malaria can induce a late onset, but long-acting wave of a cytokine termed the high mobility group box1 (HMGB1) protein which prolongs and amplifies inflammation (Andersson et al. 2000). It has now been clearly established that TNF-alpha increases the antimicrobial activity of neutrophils and since neutrophils have been shown to phagocytose and inhibit growth of *P. falciparum*, it indicated the ability of TNF-alpha to modulate the antimalarial activity of human neutrophils (Kumaratilake et al. 1990). The capacity of TNF-alpha to alter the Fc complement and other adherence receptors is likely to be important in its effects on neutrophil damage to malarial parasites. TNF-alpha has a role in the regulation of macrophage interleukin 12 production and it has been shown that TNF-alpha is an important cofactor for interleukin 12 induced production of interferon $\gamma$ by NK cells which is a macrophage-activating factor involved in the innate immune response to malaria.

In all three groups (I–III) IL-12 levels both in falciparum and vivax malaria patient showed substantial increase [Pf: Group I = 293.13 ± 94.64 pg/ml, Group II = 160.63 ± 20.81 pg/ml and Group III = 117.5 ± 8.16 pg/ml. Pv: Group I = 75.7 ± 9.25 pg/ml, Group II = 112.9 ± 12.05 pg/ml and Group III = 200 ± 53.78 pg/ml] compared to healthy controls [49.8 ± 11.59 pg/ml]. IL-12 is a proinflammatory cytokine that has been shown to be involved in protective immunity and in the cellular immunity response to the blood-stage of the infection (Kumaratilake et al. 1990; Crutcher et al. 1995). Although IL-12 was shown to play a significant role in the adaptive immune response in *P. falciparum* malaria and to correlate with the severity of the disease (Winkler et al. 1998) paradoxically in our study Group III (++++) Pf patients with high parasite burden were reported to have lower levels of IL-12 (117.5 ± 8.16 pg/ml) compared to Group II (+++) Pf patients (160.63 ± 20.81 pg/ml) and Group I (++) Pf patients (293.13 ± 94.64 pg/ml). It has been demonstrated that early events in the cell-mediated immune response required for defense against malaria initiate with the release of IL-12 from monocytes/macrophages, B cells and other cell types (Tsakonask et al. 2003) and consequently, the level of IL-12 reveals a prognostic significance in the malaria infection. Moreover there is evidence that children with *P. falciparum* hyperparasitemia have lower levels of CD 4 + T cell secreting IFN-$\gamma$ than children with uncomplicated malaria. It is possible that reduced IL-12 levels in patients with hyperparasitemia and severe malaria are associated with reduced T cell mediated IFN-$\gamma$ activity. Our results establish a critical role for IL-12 in the adaptive immune response to malaria, inducing development, proliferation and activity of Th1 cells. The outcome of the disease such as susceptibility to severe anaemia and other aspects of malarial pathophysiology could depend on the response of host macrophages to parasite products and consequently impaired IL-12 production.

IL-4 levels in all three groups (I, II and III) both in falciparum and vivax malaria patients showed significant reduction [Pf: Group I = 3.7 ± 0.11 pg/ml, Group II = 3.2 ± 0.13 pg/ml and Group III = 2.3 ± 0.63 pg/ml. Pv: Group I = 5.33 ± 1.08 pg/ml, Group II = 4.8 ± 0.16 pg/ml and Group III = 3.9 ± 0.48 pg/ml] compared to healthy controls (6.06 ± 1.32 pg/ml). IL-4 was the first recognized for its effect on B cell growth. It increases or induces their expression of Class II MHC molecules (Malaguarnera et al. 2002) and of the low affinity receptor for IgE (Fc$\varepsilon$RII) (Lyke et al. 2004) as well as increasing the number of IL-4 receptors found on the

surface of the cell. It appears likely that these responses on resting B cells play an important role in their function. IL-4 functions as a switch factor for IgE and IgG1. Mouse B cells treated with LPS and IL-4 produce substantial amount of IgE and IgG1 whereas treatment with LPS only gives rise to virtually no IgE and to modest amounts of IgG1. This effect of IL-4 in directing B cells to produce IgE and IgG1 can also be observed when the B cell costimulant is an activated T cell rather than LPS. IL-4 is a T cell growth factor, in addition, IL-4 appears to enhance the proliferation of precursors of cytotoxic T cells (CTL) and their differentiation into active CTL. The growing understanding of the biologic and pathophysiologic roles of IL-4 indicate that these molecules are key to the regulation of protective immune responses. The wide range of functions of IL-4 mandate that their in vivo regulation must be complex. Understanding these functions in detail will provide many opportunities for intervention in pathophysiologic processes for malaria.

In conclusion, the essential mechanism of death in malaria is agreed by many researchers: a functional tissue hypoxia that forces an unsustainable dependence on anaerobic metabolism. An unresolved key question is whether tissue hypoxia arises (a) because of insufficient oxygen reaches the mitochondria through either vascular occlusion from sequestered parasitized red cells acting alone or in combination with anaemia or (b) because excessive release of inflammatory cytokines induced by malarial toxins render mitochondria unable to use oxygen to generate energy from oxidative phosphorylation. Current basic literature suggests that inflammatory are very much dominant partner, having amongst their powers the capacity to shut down bone marrow, make red cells prematurely poorly deformable and channel sequestration towards certain sites (Clark et al. 2006). Thus cytokines and poor oxygen delivery should not be viewed as alternative theories of malarial diseases pathophysiology instead poor oxygen delivery is one of the consequences of excessive release of inflammatory cytokines which is further augmented by the present work.

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