

RESEARCH ARTICLE

Distinct cytokine profiles define clinical immune response to *falciparum* malaria in regions of high or low disease transmission

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ABSTRACT. The immune effector response to *Plasmodium falciparum* infection involves a finely-tuned interplay between different cell types and cytokines. However, the processes by which they mediate the development of clinical immunity, in areas of different endemicity, are poorly understood. We analyzed circulating levels of pro-inflammatory (TNF, IFN- γ , IL-12, IL-16) and anti-inflammatory (IL-4, IL-10, IL-13) cytokines in control and patient groups drawn from a *P. falciparum*-endemic and a non-endemic region of India. The endemic region control population exhibited a lower pro- to anti-inflammatory cytokine ratio, indicating a shift towards a high basal Th2 response. Levels of IL-10 contributed most towards the region-specific difference in basal cytokine response. IL-10 was also the strongest predictor of disease in the endemic region, while IL-12, along with IL-10 and IL-6, contributed most to disease outcome in the non-endemic region. A low, mean IFN- γ /IL-10 ratio was associated with disease severity in the endemic region ($p < 0.0001$). In contrast, a low mean IL-12/IL-10 ratio correlated with disease outcome in the non-endemic region ($p < 0.0001$). In the endemic region, IL-13 correlated negatively with IFN- γ in severe patients (Spearman's ρ : -0.49; p : 0.013), while in the non-endemic region, IL-13 correlated negatively with IL-6 in severe malaria patients (Spearman's ρ : -0.485; p : 0.001). In conclusion, levels of pro- and anti-inflammatory cytokines and the relative balance between the Th1 and Th2 response, illustrates how populations residing in areas of varying disease endemicity may respond to *P. falciparum*-induced immune challenge.

Keywords: cytokine, *Plasmodium falciparum*, IL-12, IFN, IL-10

The epidemiology of malaria caused by *Plasmodium falciparum* is highly complex, and largely depends on the intensity of disease transmission and level of acquired immunity. The human immune response to *P. falciparum* infection involves a complex interaction between different cell types and cytokine networks. The production of pro- and anti-inflammatory cytokines, mainly by primed CD4⁺ T-cell populations and macrophages, plays an important role in host immune-effector mechanisms. However, the balance between pro- and anti-inflammatory cytokines and their contribution to the development of disease severity and clinical immunity are poorly understood.

Blood-stage malaria parasite antigens trigger the early cytokine response and release of endogenous pyrogens including TNF, IL-1 β and IFN- γ from macrophages [1-4]. Parasite-induced, early cytokine production is necessary

for both control of parasitaemia [5] and priming of CD4⁺ T-cells (T helper cells) [6]. Pro-inflammatory cytokines, such as TNF, IL-6, IFN- γ and IL-1 β have been found to be elevated during severe malaria, and are also related to severe disease manifestations [7-11]. At low levels, TNF produced by activated macrophages and the Th1 subset of primed CD4⁺ T-cells, initiates the pyrogenic response to malaria infection and augments parasite clearance by circulating monocytes [12]. IFN- γ , the production of which by natural killer (NK) and Th1 cells, is stimulated by IL-12, triggers an effective innate immune response by promoting phagocytosis of parasitized erythrocytes by NK cells. IFN- γ also activates macrophages to release other inflammatory cytokines, including IL-1 β and IL-6, which contribute to downstream, anti-parasitic, immune responses [13, 14]. Both TNF and IFN- γ may act syner-

gistically to resolve malaria infection by assisting in effective clearance and killing of the parasite. However, elevated levels of pro-inflammatory cytokines, including both TNF and IFN- γ have been associated with severe disease manifestations [9, 15]. Excessive TNF production may lead to severe anemia, acute respiratory distress and upregulation of endothelial adhesion molecule expression resulting in cerebral, malaria-related complications [9, 16, 17]. Studies in mouse models have implicated high levels of TNF in the impairment of dendritic cell function, contributing to the immune suppression associated with malaria [18].

It is widely known that anti-inflammatory cytokines are involved in a feedback mechanism to regulate the expression of pro-inflammatory cytokines, and prevent the pathological effects that may result from their continuous secretion. IL-10, produced primarily by Th2 cells and monocytes, is an important anti-inflammatory cytokine that limits the secretion of inflammatory cytokines and acts as a regulatory switch for Th1 and macrophage cell function [19, 20]. Previous *in vitro* studies have shown that IL-10 suppresses the expression of malaria parasite-induced production of IL-1 β , IL-6 and TNF by peripheral blood mononuclear cells (PBMC) [21]. Elevated IL-10 levels have been found in severe malaria patients [8, 22, 23], suggesting its role in regulating Th1 cytokine-associated pathology.

The cytokine profile that regulates an individual's humoral and cell-mediated immune responses, and their acquisition of clinical immunity, may be a consequence of their relative exposure to parasite infection. Populations living in malaria-endemic areas develop acquired immunity against severe malaria as a result of repeated infection and accumulation of malaria antigen-specific antibodies [24]. A recent study in the mouse-malaria model has shown regeneration of memory B cells to produce malaria-specific antibodies after re-infection with *P. chabaudi* [25]. The intensity of disease transmission, the severity of infection, levels of control of peripheral parasitaemia and development of clinical immunity that define the host immune response to malaria infection in endemic populations, may not apply to those regions where disease transmission is low. Most of the studies documenting the relative amount of cytokines produced during various manifestations of *P. falciparum* malaria infection, have been carried out on subjects from regions where malaria is hyper-endemic [7, 9, 22, 26]: there is little information on the cytokine profile and immune response to infection in patients from regions where falciparum malaria transmission is low.

In order to compare the cytokine production patterns and consequent Th1/Th2 balance during falciparum malaria in patients from areas of high or low disease transmission, we analyzed circulating levels of TNF, IFN- γ , IL-12, IL-6 (pro-inflammatory) and IL-4, IL-10 and IL-13 (anti-inflammatory) cytokines in control and patient groups from a *P. falciparum*-endemic and non-endemic region of India. Our results indicated differences between individual cytokine responses, the Th1/Th2 balance, and a cytokine-based distinction between severe and non-severe malaria cases in the two regions.

DONORS AND METHODS

Patients and controls

Approval to carry out the case-control study was obtained from the ethical committees of participating institutions. Informed consent was obtained from each volunteer/guardian prior to collection of blood from patients and controls. Patient samples were collected from a *P. falciparum*-endemic (Antagarh, Chhattisgarh and Sundargarh, Orissa), and a non-endemic (Lucknow and surrounding areas of Uttar Pradesh) region of India. Diagnosis of *P. falciparum* malaria was carried out as described elsewhere [10]. WHO guidelines [27] were followed for the categorization of severe (S) and non-severe (NS) cases [10]. Severe cases were further categorized as cerebral (CM) or non-cerebral (NCM). A total of 74 patients (63 S and 13 NS) from the non-endemic region, and 101 patients (25 S and 76 NS) from the endemic region were included in the study (table 1). Control samples (C) (90 from the non-endemic region and 102 from the endemic region) were taken from ethnically-matched, unrelated individuals (table 1) from both regions. All controls recruited were healthy individuals with no reported history of any obvious allergic manifestations. Diagnostic PCR was performed on controls from the endemic regions to determine if any were asymptomatic carriers. Patient plasma was separated immediately after collection on the first day of entry into the hospital/health centre. Plasma from control individuals was separated at the site of collection. All of the plasma samples were transported to the laboratory in liquid nitrogen and stored immediately at -70°C. Since clinical symptoms of malaria may resemble those of other infectious illness, laboratory tests were generally ordered based on clinical judgment, most commonly in our setting, an IgM ELISA test for Japanese encephalitis, and serological tests for typhoid.

Table 1
Demographic features of study participants

	Endemic region			Non-endemic region		
	Control	Non-severe	Severe	Control	Non-severe	Severe
No. of subjects	102	76	25	90	13	63
Female/Male	52/50	31/45	13/12	39/51	4/9	14/49
Mean age (years) (\pm SD)	38.9 (\pm 12.6)	28.02 (\pm 12.1)	34 (\pm 13.4)	28.7 (\pm 3.9)	29.7 (\pm 11.8)	24.3 (\pm 10.9)

Because of the low HIV prevalence in these regions (< 1%), malarial patients were not tested for HIV unless there were other clinical indications. Only malaria-positive cases, with no obvious co-infections, were included in the study.

Plasma cytokine estimation

Sandwich ELISA was performed for the estimation of plasma cytokine levels in patients and controls. Plasma TNF, IL-4 and IL-13 levels were determined using capture monoclonal anti-human antibody (Pierce) and a paired, biotinylated, anti-human antibody (Pierce). Briefly, microtitre plates were coated with 100 μ L of capture monoclonal antibody and incubated overnight at room temperature. Plates were washed four times (50 mM Tris-Cl, 0.2% Tween-20) and blocked with 200 μ L of assay buffer (1X PBS with 4% BSA) for 2 h at room temperature, after which the plates were washed thoroughly four times. Fifty μ L of plasma sample or standards were added, in duplicates, in each well and plates were incubated overnight at 4°C after which plates were washed six times. Biotin-labeled detecting antibody was added and plates were incubated at room temperature for about 2 h after which plates were washed six times and then incubated for 30 min with Streptavidin linked HRP enzyme (1:20,000 dilution). Plates were washed eight times and 50 μ L TMB of substrate solution were added. The reactions were allowed to develop for about 30 min. Reactions were stopped with 7% H₂SO₄. The optical density (OD) was measured at a wavelength of 450 nm on a BioTek Microplate reader. The limit of detection was defined as two standard deviations above the mean optical density of eight replicates of the zero standard. ELISAs for IL-6, IL-10, IL-12 and IFN- γ were performed using OptEIA kits (BD Biosciences), according to the manufacturer's instructions. Standards were included in duplicates in each assay plate, and were used for the calculation of the cytokine levels in the samples. The minimum detection limit for all the assays was between 0.5-2 pg/mL.

Data analysis

Analysis was performed using GraphPad PRISM (v5.02) and an R statistical computing environment. Cytokine response values were normalized by log transformation (to the base 10), and adjusted for age prior to analysis as we had observed statistically significant differences among the three groups from both endemic and non-endemic regions (mean age \pm SD for endemic, C: 38.9 ± 12.6 ; NS: 28 ± 12.1 ; S: 34.0 ± 13.4 , $p = 0.001$; non-endemic, C: 28.7 ± 3.9 ; NS: 29.7 ± 11.8 ; S: 24.3 ± 10.9 , $p = 0.0022$). When adjusted for sex, we found no significant differences between the groups in our study population. Means of plasma cytokine values for controls and patients were compared using the Kruskal-Wallis test and within-group comparison was performed using Dunn's multiple comparison post-test. Coefficients of correlations between cytokine levels were estimated using the Spearman rank test. Multiple regression was performed using cytokine levels as the

independent variable and disease outcome (no disease, non-severe and severe malaria) as the dependent variable. Stepwise multiple regression (backward elimination) analysis was performed in order to compare disease groups and to determine which cytokine best predicted malaria severity. P-value of < 0.05 was considered to be significant for all tests.

RESULTS

Relative measurements of cytokines and the Th1/Th2 balance in the malaria-endemic and non-endemic regions

Basal levels of pro- and anti-inflammatory cytokines were compared between controls of the two regions in order to analyze the Th1/Th2 balance. When individual pro- (TNF, IFN- γ , IL-6, IL-12) to anti-inflammatory (IL-4, IL-10, IL-13) cytokine ratios were compared, the endemic region controls exhibited a lower ratio ($p < 0.0001$) (*figure 1A*) indicating a shift towards a higher basal Th2 response in the endemic region. Taking cytokine measurements as the independent variable, multiple regression analysis was performed with the controls from both regions. IL-10, IL-12 and IFN- γ contributed significantly to the region-specific difference in cytokine response, with IL-10 contributing the most (p-values; IL-10: < 0.0001, IL-12: 0.004 and IFN- γ : 0.0003). We next estimated the IL-12/IL-10 and IFN- γ /IL-10 ratios for both regions. The endemic region controls exhibited significantly lower IL-12/IL-10 and IFN- γ /IL-10 ratios when compared to non-endemic controls ($p < 0.0001$) (*figure 1B, C*), indicating the predominance of the Th2 response over Th1 in regions of high endemicity.

Comparative plasma cytokine levels in patient and control groups from endemic and non-endemic regions

Baseline plasma levels of individual cytokines were first compared between control (C) and patient (severe: S, non-severe: NS) groups from both regions. TNF levels were higher in patient groups when compared with controls, although the difference was not significant in the endemic region (*table 2*) (non-endemic; C vs S: $p < 0.001$; C vs NS: $p = 0.01$). IL-6 levels were elevated in severe patients when compared to non-severe patients or controls (endemic, S vs NS: $p < 0.01$; non-endemic, S vs NS: $p = 0.0645$) (*table 2*). Levels of IL-12 and IFN- γ were found to be significantly higher in non-severe patients when compared to severe malaria patients and control groups for both regions (*table 2*).

Of the anti-inflammatory cytokines tested, levels of IL-10 were found to be elevated in severe malaria patients when compared to controls in both regions. However, the difference between severe and non-severe patients was significant only in the endemic region (*table 2*) (endemic; S vs C: $p < 0.0001$, S vs NS: $p < 0.05$, non-endemic; S vs C: $p < 0.0001$). Levels of IL13 were elevated in both patient groups when compared to controls from the

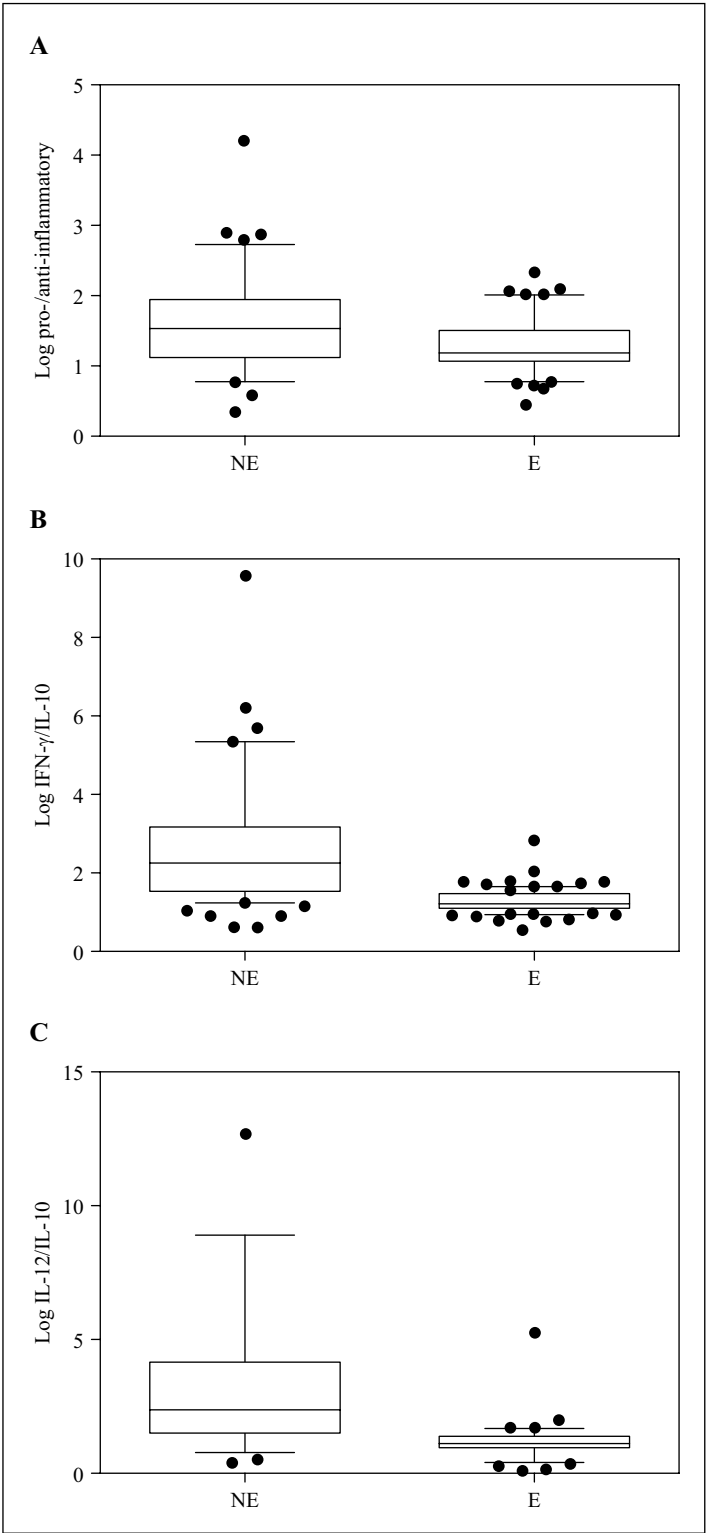


Figure 1

Comparison of cytokine ratios in control individuals from the non-endemic (NE) and endemic (E) region.

A) Ratio of pro- (TNF, IL-12, IFN-γ and IL-6) and anti-inflammatory (IL-4, IL-10 and IL-13) cytokines. Individual cytokine values were first log-transformed then followed by addition of pro- and anti-inflammatory cytokine values for each sample. The pro-/anti-inflammatory cytokine ratio was then calculated for each sample.

B) Ratio of individual log transformed IFN-γ and IL-10 levels.

C) Ratio of individual log transformed IL-12 and IL-10 levels. The boxes include 25th and 75th percentiles. The horizontal line in the box indicates the median, and the whiskers indicate the 10th and 90th percentiles of the ratios. Black dots represent outliers.

Table 2
Comparison of individual plasma cytokine levels (pg/mL) in patient and control groups

	Endemic region				Non-endemic region			
	Control n = 102	Non-severe n = 76	Severe n = 25	P-value	Control n = 90	Non-severe n = 13	Severe n = 63	P-value
TNF								
Median	12.02	8.76	20.37	0.4001 ^a	12.27	26.12	25.4	0.0004 ^a
Range	1.3-136.8	2.4-257.81	1.2-141.74		0.28-98.2	1.1-257.52	1.76-433.17	0.01 ^b < 0.001 ^c
IL-12								
Median	32.06	117.90	66.7	< 0.0001 ^a	26.25	137.16	61.69	< 0.0001 ^a
Range	1.2-151.31	55.4-606.03	2.85-166.22	< 0.0001 ^b < 0.01 ^c < 0.0001 ^d	1.02-132.39	43.66-320.88	17.78-150.44	< 0.0001 ^b < 0.0001 ^c < 0.01 ^d
IFN-γ								
Median	44.89	68.84	41.21	0.0203 ^a	14.89	49.11	28.92	< 0.0001 ^a
Range	4.89-248.05	3.32-980.16	8.05-765.95	< 0.05 ^b	2.63-128.58	6.84-464.37	8.56-194.89	0.0004 ^b < 0.0001 ^c
IL-6								
Median	6.76	32.47	239.75	< 0.0001 ^a	2.40	17.23	44	< 0.0001 ^a
Range	2.4-107.91	1.98-468.4	8.18-575.58	< 0.0001 ^b < 0.0001 ^c < 0.01 ^d	1.9-41.91	1.8-249.2	3.56-522.24	< 0.0001 ^b < 0.0001 ^c
IL-10								
Median	20.46	244.50	671.29	< 0.0001 ^a	3.16	183.21	178.87	< 0.0001 ^a
Range	2.28-90.84	9.08-1984.29	7.4-1691.29	< 0.0001 ^b < 0.0001 ^c < 0.05 ^d	1.7-105.32	16.53-910.92	4.14-2594.76	< 0.0001 ^b < 0.0001 ^c
IL-4								
Median	5.6	7.3	2.5	0.0005 ^a	3.2	7.45	5.1	0.0009 ^a
Range	1-43.2	1.3-68.8	1.2-30.51	< 0.01 ^c < 0.001 ^d	1.3-25.8	1.86-127.38	1.2-14.18	< 0.001 ^b < 0.05 ^c < 0.05 ^d
IL-13								
Median	7.04	15.2	13.09	< 0.0001 ^a	8.66	22.9	10.4	0.2706 ^a
Range	1.4-33.68	6.3-269.4	7.84-269.4	< 0.0001 ^b < 0.001 ^c	1.86-46.86	4.6-556.2	2.4-251.4	

^a Kruskal-Wallis test with level of significance set at $p < 0.05$.

Dunn's multiple comparison post-test with level of significance set at $p < 0.05$:

^b Control vs non-severe,

^c Control vs severe,

^d Non-severe vs severe.

endemic region, but the differences were not significant in the non-endemic region (*table 2*) (endemic; NS vs C: $p < 0.0001$, S vs C: $p < 0.001$). Consistent with a recent study on malaria patients from India [11], no difference in cytokine levels was observed when severe malaria patients were analyzed separately as CM or NCM.

Discrete patterns of cytokine profiles may define clinical immunity to falciparum malaria in regions of varying endemicity

To determine any functional association between cytokines, and their contribution to malaria-related pathology, we performed multiple regression considering cytokines as the independent variable and disease outcome as the dependent variable. In the endemic region, apart from TNF, IL-12 and IL-13, all other cytokines contributed significantly to disease outcome, with IL-10 being the strongest predictor (*table 3*). However, in the non-endemic region, IL-12, IL-10 and IL-6, were the significant predictors (*table 3*). To determine which cytokine best predicted disease severity we performed stepwise multiple regression using only non-severe and severe disease groups. IL-12 emerged as the most significant predictor of non-severe malaria outcome in both regions (endemic; $p < 0.0001$; non-endemic: 0.006).

To understand better the relative measurements of cytokine production and Th1/Th2 balance, we compared the ratios of pro-/anti-inflammatory cytokines that significantly contributed to disease outcome in the regression

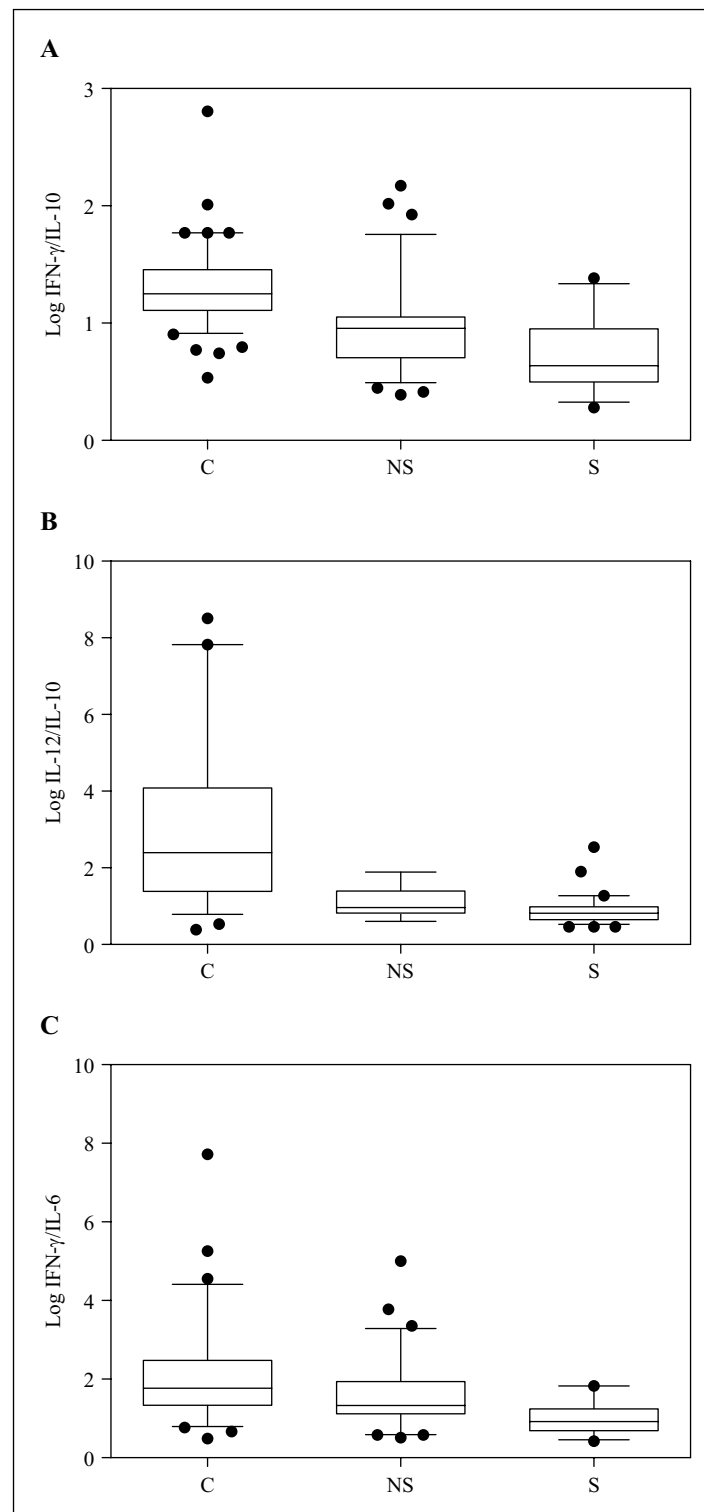
analysis. Since IL-10 is known to be an important regulator of the Th1 response and an inhibitor of inflammatory cytokine production, we determined individual IL-6/IL-10 and IFN- γ /IL-10 ratios for each control and patient group in the endemic region, and IL-6/IL-10 and

Table 3

Multiple regression analysis to determine relative contribution of cytokines to disease outcome. Plasma cytokine values and disease outcome were taken as independent and dependent variables, respectively

	Regression coefficient	Standard error	P-value
<i>Endemic region participants</i>			
TNF	0.020	0.0473	0.658
IL-12	0.028	0.0475	0.548
IFN- γ	0.255	0.0619	0.001
IL-6	0.251	0.0454	< 0.0001
IL-4	- 0.234	0.0827	0.005
IL-10	0.578	0.0545	< 0.0001
IL-13	- 0.202	0.0769	0.416
<i>Non-endemic region participants</i>			
TNF	0.055	0.0625	0.373
IL-12	0.174	0.0626	0.006
IFN- γ	-0.068	0.1095	0.532
IL-6	0.249	0.0745	0.001
IL-4	-0.126	0.1041	0.225
IL-10	0.630	0.0599	< 0.0001
IL-13	0.021	0.0731	0.764

Significant p-values are indicated in bold.

**Figure 2**

Comparison of cytokine ratios.

A) Ratio of individual log transformed IFN- γ and IL-10 values in endemic region subjects.

B) Ratio of log transformed IL-12 and IL-10 values in non-endemic region subjects.

C) Ratio of log transformed IFN- γ and IL-6 values in endemic region subjects. The boxes include the 25th and 75th percentiles. The horizontal line in the box indicates the median and the whiskers indicate the 10th and 90th percentiles of the ratios.

Black dots represent outliers. C: control, NS: non-severe malaria, S: severe malaria.

IL-12/IL-10 ratios in the non-endemic region and then compared their means. In the endemic region, the mean IFN- γ /IL-10 ratio was lower in patients when compared to control groups ($p < 0.0001$ for all comparisons) (figure 2A), while no significant difference was observed

between severe and non-severe patients. In the non-endemic region, the mean IL12/IL10 ratio was significantly lower in patients when compared to controls ($p < 0.0001$) (figure 2B). For both regions, IL-6/IL-10 comparisons were non-significant. However, IL-6 and

IL-10 showed positive correlations in severe patients from the non-endemic region (Spearman's ρ : 0.4; p : < 0.0001). Since TNF and IFN- γ induce secretion of IL-6, we compared the relative production of IL-6 to that of TNF and IFN- γ . Significantly higher ratios of IFN- γ /IL-6 in non-severe patients from the endemic region (p : 0.0001) were observed (*figure 2C*). Interestingly, in the endemic region, IL-13 correlated negatively with IFN- γ in severe patients (Spearman's ρ : -0.49; p : 0.013), while in the non-endemic region, IL-13 correlated negatively with IL-6 in severe malaria patients (Spearman's ρ : -0.485; p : 0.001).

DISCUSSION

The incidence of malaria in India is high and falciparum malaria alone accounts for more than 80% of all cases of malaria in specific areas, particularly regions in western, central, eastern and north-eastern India [28, 29]. There are limited reports of patterns of cytokine profiles and immune responses in Indian populations, and those available refer to endemic regions [11, 22, 28, 29]. Mechanisms involved in the development of protective immunity against malaria remain unclear, but criteria that define clinical immunity in populations from malaria-endemic regions may not apply to regions of low disease intensity and transmission. In areas where *P. falciparum* malaria is endemic, people gradually develop mechanisms to control severe inflammatory responses related to the parasite as a consequence of repeated infection. When basal level ratios of pro- to anti-inflammatory cytokines were compared between controls from the two regions in this study, the endemic region population exhibited lower ratios and higher levels of anti-inflammatory cytokines. The half-life of most of the cytokines is relatively short and they are rapidly cleared from the circulation by the liver or kidneys. The level of circulating cytokines depends on many factors including disease severity and the level of immune challenge. It is believed that populations living in malaria-endemic regions, control peripheral parasitaemia in such a way that they do not show clinical symptoms, which includes production of pro-inflammatory cytokines [24, 30]. Higher basal levels of anti-inflammatory cytokines (lower Th1/Th2 ratio) in controls from the malaria-endemic region indicate their relevance in the development of acquired immunity. Among the anti-inflammatory cytokines tested, IL-10 contributed the most to the region-specific difference in basal cytokine levels, indicating its role in controlling the stimulation of endogenous pyrogens and the downstream immune effector cascade that are triggered due to the presence of peripheral malaria antigens in the otherwise asymptomatic endemic-region individuals.

Significantly elevated levels of TNF in non-endemic patients validate its role in both the resolution of infection and induction of the pyrogenic response. Although TNF levels do not vary significantly between patients and control groups in the endemic region, its positive correlation with IFN- γ in severe patients may be associated with *P. falciparum*-related pathology. It was interesting to

note that cytokine levels, when compared individually between controls and patient groups, did not exhibit any region-specific patterns. However, when analyzed relatively, we found that the cytokine response was region-specific indicating that there exists a critical balance between cytokine production and the immune response that may depend on the level of disease transmission. IFN- γ , IL-10, IL-6, and IL-4 were predictors of disease outcome in the endemic region. IFN- γ production has been shown to be instrumental in controlling parasitaemia and activating macrophages to produce endogenous pyrogens that mediate the inflammatory cascade [14]. In endemic populations, primary infection with the malaria parasite induces IFN- γ production primarily by innate immune cells (NK cells, macrophages), and, at the same time, T-cells are primed [31]. Upon reinfection, excessive IFN- γ production is induced predominantly by malaria-specific memory T-cells, along with NK cells and macrophages [3, 32], and is potent for parasite killing and resolution of infection. Elevated levels of IFN- γ relative to IL-6 in non-severe endemic patients, suggest that IFN- γ production is optimized for evasion of acute phase symptoms. Memory T-cell-derived IFN- γ can also contribute to adaptive immunity in asymptomatic individuals since we observed significantly higher levels of IFN- γ in control endemic populations compared to controls from the non-endemic region. Early IFN- γ production by NK cells is predominantly dependent on IL-12 secretion by activated macrophages and dendritic cells (DC). IL-12 has been shown to initiate a number of protective immune responses to malaria including cell-mediated cytotoxicity (*e.g.* oxidative burst, nitric oxide release and enhancement of phagocytosis), up-regulation of IFN- γ production by NK and T-cells, and differentiation of CD⁺ T-cells towards a Th1 response [33, 34]. Low levels of plasma IL-12 have been correlated with severe forms of falciparum malaria in a number of studies [4, 35], possibly due to down-regulation of the Th1 response. We also found elevated levels of IL-12 in non-severe patients from both regions, indicating the protective role of IL-12 against severe malaria. A positive correlation between plasma IL-12 and IFN- γ (Spearman's ρ : 0.46) was also seen in non-severe patients from the non-endemic region, although the correlation was not significant (probably due to the small sample size). IL-12 is regulated in a negative feedback mechanism by IL-10 since uncontrolled IL-12 production leads to excessive production of pro-inflammatory cytokines resulting in detrimental downstream effects [36]. High IL-10 levels have been associated with severe falciparum malaria [8, 22]. The low levels of *P. falciparum*-induced IL-12 relative to IL-10 (low IL-12 /IL-10 ratio) in severe patients from non-endemic regions suggest that IL-12 might be a very important mediator of the Th1 response, with low levels resulting in down-regulation of pro-inflammatory cytokines and their anti-parasitic effects. Since early production of IFN- γ is also an attribute of acquired immunity in endemic populations, the low levels of IFN- γ , relative to IL-10 (low IFN- γ /IL-10 ratio) in severe endemic region patients, indicate its indirect regulation by IL-10, possibly due to deactivation of macrophage cell function by IL-10 leading to low IL-12 production.

We observed an interesting correlation between plasma IL-13 and other cytokines in both regions. IL-13 is a Th2, anti-inflammatory cytokine and not much is known about its role in falciparum malaria-induced immune responses. Since IL-13 is closely related to IL-4 (~ 30% homology), and is also involved in IgE and IgG4 switching in human B cells [37], the negative correlation between IL-13 and IFN- γ in severe patients from the endemic region may indicate its counter-regulatory role and its possible involvement in development of clinical immunity. However, in the non-endemic region elevated levels of *P. falciparum*-induced IL-6, relative to IL-13, may be associated with febrile illness.

The observed heterogeneity in the levels of individual cytokines between patients and control groups could have been confounded by some factors. All blood samples were collected at the time of admission and since cytokine production varies with circadian rhythm and the time course of the illness, the level of any particular cytokine observed may not have been an exact reflection of disease status. Thus, additional studies may be required to explain the marked variations in individual cytokine measurements within groups. Although the levels of various pro- and anti-inflammatory cytokines are a consequence of the individual's immunity and intensity of exposure, they may also be attributable to his/her genetic makeup. The region-specific variations in the cytokine profile observed in our dataset may also be explained by genetic variants of the genes that encode these cytokines. Since malaria is known to be one of the strongest forces of selection [38], it will be interesting to understand the observed immune response to malaria infection from the perspective of the genetic background of populations from the two regions. Previously, we have shown the correlation of TNF promoter variants with malaria susceptibility and elevated TNF production [10]. Preliminary results (unpublished) from our genetic study on the same endemic and non-endemic populations have revealed significant, region-specific differences in allele frequencies of some genetic variants of several cytokine-encoding genes.

Taken together, our results are clearly indicative of the wide variation in relative pro- and anti-inflammatory cytokine responses to falciparum malaria infection in regions of varying disease endemicity, and suggest that it is the overall 'balance' between the two classes of cytokines that reflects how differently-exposed populations may respond to *P. falciparum*-induced immune challenge.

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