Plasmodium falciparum-specific interleukin-2 and tumor necrosis factor-α expressing-T cells are associated with resistance to reinfection and severe malaria in healthy African children

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ABSTRACT. The frequency of *P. falciparum*-specific interleukin (IL)-2-, interferon (IFN)-γ-, tumor necrosis factor (TNF)-α- and IL-10-expressing $CD3^+$ cells was studied in healthy Gabonese children segregated according to their clinical presentation at admission to a longitudinal study of severe and mild malaria. The percentage of IL-2- and TNF-α- expressing *P. falciparum*-specific $CD3^+$ cells was significantly higher in the children with prior mild malaria and less frequent reinfections compared to the children with prior severe malaria and more frequent reinfections. No differences were shown for *P. falciparum*-specific IFN-γ and IL-10 expression within $CD3^+$ cells and parasite-non-specific expression of IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, and IFN-γ within the $CD4^+$, $CD8^+$, $TCR\gamma/\delta^+$ $CD3^+$ and $CD94^+$ $CD3^-$ cell populations, indicating that immunological determinants regulating the susceptibility to malaria in age-matched children are parasite-specific. The ability of *P. falciparum*-specific T cells to mount a rapid IL-2 and TNF-α response might be of significance in preventing severe disease and reinfection.

Keywords: T cells, cytokines, *Plasmodium falciparum* malaria

INTRODUCTION

Malaria is responsible for up to two million deaths worldwide per annum [1]. In areas of Sub-Saharan Africa such as the Province of Moyen Ogooué, Gabon, where transmission of *Plasmodium falciparum* is stable and intense [2, 3], mortality is highest in infants younger than six years of age. Severe malaria in these largely non-immune Gabonese children is characterized by hyperparasitemia and severe anemia, with cerebral malaria being less frequently observed. The conditions that regulate the development of mild versus severe malaria in children are still incompletely understood, yet it is the delicate balance between protective and harmful immune responses that finally determines the outcome of malaria [4]. T cells and their cytokines have been implicated in both the pathogenesis of acute malaria attacks as well as in the development of protective immunity, which is, however, acquired only slowly even in endemic areas [5].

The pro-inflammatory cytokines IFN-γ and TNF-α, derived from both the innate and the adaptive arm of the immune system, are released early into the circulation after infection, and high levels have been associated with clinical symptoms such as fever and malaise as well as with the development of cerebral malaria and severe anemia in susceptible hosts [6-12]. However, IFN-γ and TNF-α are also components of protective immunity and it has been shown in many animal models and some studies in humans that the cellular capacity to produce these cytokines during early phases of infection is essential for the control of parasitemia [13-22]. Thus, it is the relative contribution of these pro-inflammatory cytokines, counterbalanced by the action of anti-inflammatory cytokines such as interleukin (IL)-10 or transforming growth factor (TGF)-â, that may influence the outcome of infection in terms of clinical severity or susceptibility to reinfection. Indeed, in non-immune children from the Lambaréné area, IFN-γ responses to liver-stage antigen 1 (LSA-1) and merozoite surface antigens were associated with resistance to reinfection, whereas in adolescents and adults from western Kenya, IL-10 responses to LSA-1 were predictive of resistance [23, 24]. While this switch from a protective IFN-γ -skewed response in young children to protective IL-10 production in older, malaria-exposed individuals is still to be confirmed, both studies were able to demonstrate correlates of protection using a longitudinal study design.

However, in these studies no information was provided with regard to the cellular sources of the cytokines. In addition, well defined antigens instead of whole parasite preparations were used, which allows for characterization of antigen-specific immune responses but does not reflect so closely the *in vivo* situation when host cells are confronted with a large panel of diverse antigens.

Here we studied the phenotypes and frequencies of cytokine-expressing T cells after *P. falciparum*-specific and non-specific stimulation of PBMC from healthy Gabonese children with a history of either mild or severe *P. falciparum* malaria participating in a longitudinal study. The results obtained were related to initial clinical presentation and subsequent reinfection profiles.

STUDY SUBJECTS AND METHODS

Study site and participants

The study was performed at the Albert Schweitzer Hospital in Lambaréné, Gabon, where *P. falciparum* malaria is predominantly hyperendemic, with an estimated annual entomological inoculation rate of 10-100 [2, 3]. A cohort of 15 healthy children (three males, 12 females; median age, nine years, age range, eight - 14 years) with a history of mild malaria, and of 11 healthy children (three males, eight females; median age, 9.5 years, age range, seven - 14 years), with prior severe malaria were included into the study during spring 2002. All children were recruited from a prospective longitudinal case-control study of severe and mild malaria, details of which have been published previously [23, 25]. Briefly, patients entered the study in 1995 and 1996, severe malaria on admission being defined as hyperparasitemia (> 250 000 parasites/μL) and/or severe anemia (hemoglobin < 50 g/L) and other signs of severe malaria. Criteria for mild malaria included parasitemia 1000-50 000/μL on admission, no schizontemia, hemoglobin > 80 g/L, circulating leucocytes containing malarial pigment < 50/μL, platelets > 50/nL, leucocytes < 12/nL, lactate < 3 mM, and blood glucose > 50 mg/dL. Reinfections and/or clinical malaria attacks were detected through active clinical and parasitological follow-up of individuals every two weeks following discharge from the hospital, at which times thick blood smears were routinely examined. Therefore, the period of active follow-up after admission of all children with either severe or mild malaria was at least six years. At the time of examination in 2002, all children were clinically healthy and physical examination was unremarkable. Malaria or any other severe illness within the two months prior to the study precluded participation. Blood smears were used to assure that participants were parasite-free. The rate of reinfection per year, calculated during the period of active follow-up of at least six years, was significantly higher in the group of children with prior severe malaria than in the group of children with mild malaria (median, 2.95, range, 1.19-4.79 *versus* median, 0.29, range, 0 - 4.5, $P < 0.001$) as shown by non-parametric analysis (Mann-Whitney U-test). Informed consent was obtained from the parents or guardians of participating children. Ethical clearance was given by the ethics committee of the Albert Schweitzer Hospital in Lambar éné.

Parasite preparation

P. falciparum strain S007, originally isolated from a child with severe malaria in Lambaréné, was cultured in human type 0 erythrocytes, adjusting the hematocrit to 5% and the parasitemia to 2-5% in complete parasite medium (CPM: RPMI-1640 supplemented with 25 mM HEPES, 2 mM L-glutamine, 50 μg/mL of gentamicin, 0.5% Albumax II [Gibco, Paisley, UK], with 200 μM of hypoxanthine, and 2% AB^+ serum). All cultures and media were regularly tested for *Mycoplasma* contamination by PCR amplification with genus-specific primers (GPO-1 5'-ACT CCT ACG GGA GGC AGC AGT A-3'and MGSO 5'- TGC ACC ATC TGT CAC TCT GTT AAC CTC-3') of 16S rDNA as described previously [26].

For synchronization and enrichment of *P. falciparum*-infected erythrocytes (PFE) a magnet-activated cell sorter (MACS) system was applied as described recently [27]. Parasite concentration could be increased up to 90% using CS columns and VarioMACS (Miltenyi BioTec, Bergisch-Gladbach, Germany). Yield and purity were assessed by microscopic examination of Giemsa - stained eluate and flow - through. The MACS-eluate of *P. falciparum* strain S007 was frozen at – 80 °C, thawed once and used for stimulation of the PBMC from all children.

Detection of Plasmodium falciparum-specific T cell cytokine expression by flow cytometry

PBMC were isolated from heparinized blood by ficoll-diatrizoate centrifugation and plated out in 12-well plates at 2.5×10^6 /well. Cells were cultured in Ultra Culture Medium (UCM) (Bio Whittaker, Walkersville, MD, USA) supplemented with L-glutamine (2 mM/L; Sigma, St. Louis, MO, USA), gentamicin (170 mg/L; Sigma) and 2-mercaptoethanol (3.5 μL/L; Merck, Darmstadt, Germany) for 18 hours at 37 °C in 5% CO_2, and stimulated with or without 50 μL of the MACS-eluate containing the late-stage, schizont-rich parasite preparation at a ratio of 10:1 PFE:PBMC as described recently [28, 29]. To enhance the Ag-specific response, the co-stimulatory MAb CD28 (Pharmingen, San Diego, CA, USA) was added at 10 μL to some wells (5 μg/mL final concentration) [29, 30]. Uninfected erythrocytes were processed as described above and served as controls (with and without the addition of MAb CD28). Further controls included the addition of MAb CD28 to the PBMC preparation without PFE, and medium alone without MAb CD28 or PFE. MAb CD28 in the absence of Ag had no effect on cellular cytokine responsiveness, however, it increased the frequency of PFE-induced cytokine - expressing cells up to twofold as reported previously [29].

Brefeldin A (1 μM; 10 μg/mL final concentration, Sigma) was added after six hours to block protein secretion. Cells were then harvested on ice without scraping, washed twice in phosphate-buffered saline (PBS) and fixed with 2% formaldehyde (1mL per 2×10^6 cells, Merck) for 20 min. After two additional washes in PBS, cells were then resuspended in Hank's balanced salt solution (HBSS, supplemented with 0.3% bovine serum albumine [BSA] and 0.1% sodium-azide), and stored at 4 °C in the dark until staining. Fixed cells were washed twice with PBS and made permeable with saponin (0.1%; Sigma). They were then resuspended with 50 μL of saponin-buffer-diluted

antibodies and incubated for 25 min at room temperature in the dark.

The following monoclonal antibodies were used: cytokine-specific mouse anti-human monoclonal antibody (MAb) (IFN-γ [clone: B27], fluorescein-isothiocyanate [FITC]-labeled) and rat anti-human MAb (IL-2 [MQ1-17H12], phycoerythrin [PE]-conjugated; IL-10 [JES3-9D7], PE-labeled; TNF-α [MAb11], PE-labeled). All MAb were purchased from Pharmingen. The anti-CD3-MAb was peridinin chlorophyll (PerCP), the anti-CD69-MAb was allophycocyanin (APC)-labeled (Becton Dickinson, Mountain View, CA, USA). Four-colour staining was performed and at least 10^4 cells were analyzed on a FACSCalibur (Becton Dickinson) equipped with a two laser system (488 nm and 633 nm wavelength, respectively). All cytokine combinations (IL-2/IFN-γ, IL-10/IFN-γ, TNF-α /IFN-γ) were stained in conjunction with CD3 and CD69.

T cells were defined by their side-scatter characteristics and anti-CD3 MAb staining. The specificity of cytokine staining in $CD3^+$ cells was verified by counterstaining with CD69 as a marker for activated lymphocytes. Only cells clearly positive for CD69 were classified as cytokine-producing $CD3^+$ cells. Data were analysed with CELLQuest software (Becton Dickinson), and results were expressed as the percentage of cytokine-producing cells in each $CD3^+$ $CD69^+$ cell population. Specificity of cytokine staining was confirmed by the absence of significant background in controls with isotype-matched irrelevant MAbs. Frequencies of background events within the $CD3^+$ cell population (unstimulated cells; addition of MACS-processed uninfected erythrocytes with or without anti-CD28) were < 0.03%. These values were not subtracted from those obtained after stimulation with PFE.

Detection of non-specific, mitogen-induced T cell cytokine expression by flow cytometry

PBMC were isolated from heparinized blood and stimulated in UCM with phorbol 12-myristate 13-acetate (PMA, 10 ng/mL; Sigma) and ionomycin (1.25 μM; Sigma) in the presence of brefeldin A (1 μM; Sigma) for 4 hours at 37° C in 5% CO_2 Cells were then harvested and fixed as described above. For the staining procedure, the following monoclonal antibodies (MAbs) were used: cytokine-specific mouse anti-human MAb (IFNγ [clone B27], fluorescein isothiocyanate [FITC]-conjugated); rat anti-human MAb (IL-2 [MQ1-17H12], IL-4 [MP4-25D2], IL-6 [MQ2-13A5], IL-10 [JES3-9D7], IL-13 [JES10-5A2], TNF-α [Mab11], all phycoerythrin [PE]-conjugated), the anti-CD4 MAb, anti-TCRγ/δ MAb and anti-CD94 MAb were allophycocyanin-conjugated, anti-CD3 MAb and anti-CD8 MAb were peridinin chlorophyll-conjugated; all cytokine-specific MAbs were purchased from Pharmingen (San Diego, CA, USA), the surface marker-specific MAbs from Becton Dickinson (Mountain View, CA, USA).

Samples were gated on lymphocytes according to their light scatter characteristics and the results were expressed as the percentage of cytokine-producing cells in the $CD4^+$, $CD8^+$, $TCR\gamma/\delta^+$ $CD3^+$ or $CD94^+$ $CD3^-$ cell population, respectively.

Statistical methods

Statistical analysis was performed using a standard statistical package (SPSS 10.0 for Windows; SPSS Inc., Chicago). The Mann-Whitney U-test was applied for group differences (children with prior mild *versus* severe malaria, children with < one reinfection *versus* children with > two reinfections per year). A P value of < 0.05 was considered significant.

RESULTS

Differences in the frequency of cytokine-expressing P. falciparum*-specific CD3⁺ cells between healthy children with a history of either severe or mild malaria*

The frequency of cytokine-expressing $CD3^+$ cells was compared between 11 children with prior severe malaria and 15 children with prior mild malaria. At the time of PBMC stimulation, all patients were apparently healthy and free of parasites.

The frequency of $CD3^+$ cells exclusively expressing IL-2 and TNF-α, as well as the frequency of $CD3^+$ cells expressing IL-2 together with IFN-γ, were significantly higher in the mild malaria group when compared to the group with severe malaria *(Figures 1, 2)*. Likewise, the frequencies of overall IL-2- and TNF-α-expressing $CD3^+$ cells were significantly higher in the mild malaria than in the severe malaria group *(Table 1, Figure 1)*. As shown previously [31, 32], most IL-10-expressing cells also stained positively for IFN-γ, however, no differences were observed between groups *(Figure 1)*.

Frequency of cytokine-expressing, non-specifically stimulated PBMC in healthy children with prior severe or mild malaria

The frequencies of IL-2-, IL-4-, IL-6-, IL-10-, IL-13-, IFN-γ-, and TNF-α- expressing $CD4^+$, $CD8^+$, $TCR\gamma/\delta^+$ $CD3^+$ and $CD94^+$ $CD3^-$ cell populations after mitogenic

Table 1
Frequency of *P. falciparum*-specific cytokine expression within the $CD3^+$ cell population in healthy children with prior mild or severe malaria*

Cytokines	Prior mild malaria (n = 15)	Prior severe malaria (n = 11)
IL-2⁺ (IL-2^+/IFN-γ^+ and IL-2^+/IFN-γ^-)	0.090 ± 0.012 (0.02-0.17)[1**]	0.047 ± 0.006 (0.02-0.09)
IFN-γ⁺ (IL-2^+/IFN-γ^+ and IL-2^-/IFN-γ^+)	0.077 ± 0.015 (0.01-0.19)	0.060 ± 0.008 (0.03-0.11)
TNF-α⁺ (TNF-α^+/IFN-γ^+ and TNF-α^+/IFN-γ^-)	0.140 ± 0.022 (0.03-0.28)[2]	0.074 ± 0.014 (0.02-0.18)
IL-10⁺ (IL-10^+/IFN-γ^+ and IL-10^+/IFN-γ^-)	0.023 ± 0.006 (0.00-0.11)	0.035 ± 0.010 (0.00-0.11)

*PBMC were stimulated with MACS-separated, late stage *Plasmodium falciparum* isolates in the presence of mAb CD28.
**Values indicate mean percentages of cytokine-expressing $CD3^+$ cells ± SEM; the respective ranges are given in parentheses.
[1] significant difference between the children with prior mild or severe malaria, $P < 0.01$ as calculated by the Mann-Whitney U-test.
[2] significant difference between the children with prior mild or severe malaria, $P < 0.05$ as calculated by the Mann-Whitney U-test.

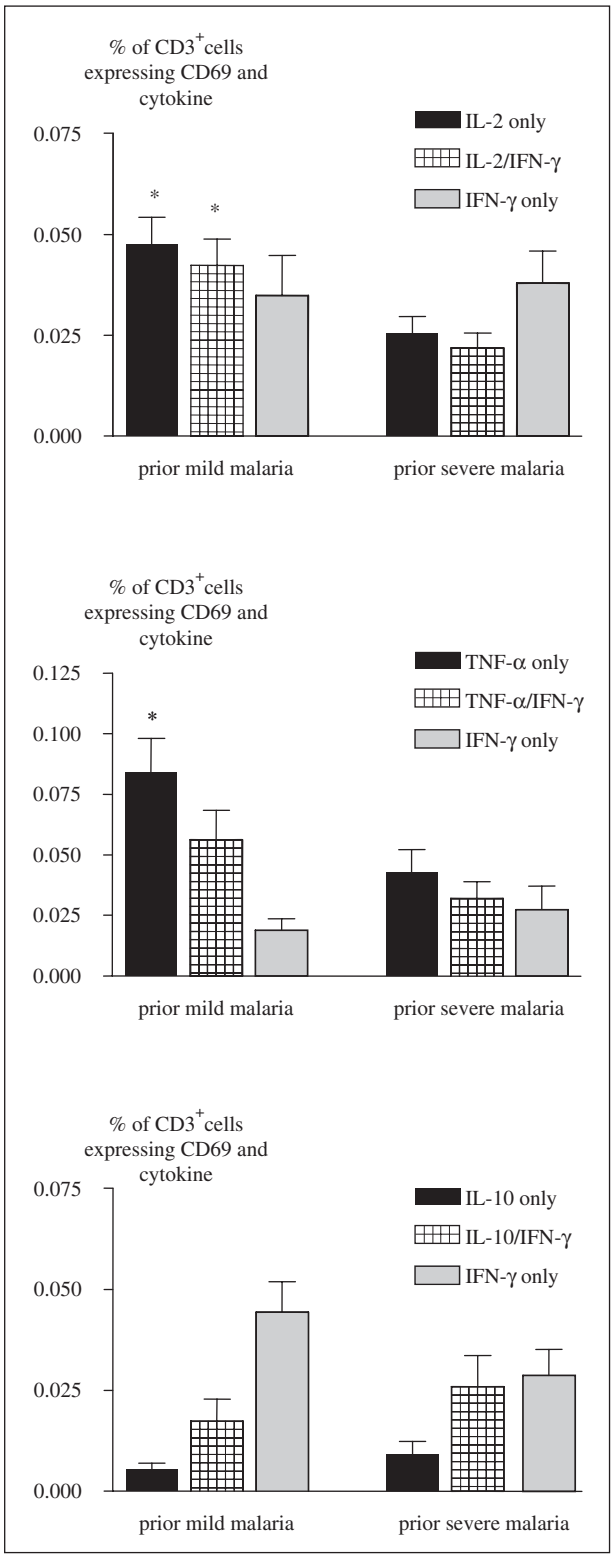

Figure 1

Frequency of cytokine-expressing, activated ($CD69^+$) $CD3^+$ cells obtained after stimulation with MACS-processed PFE in children with prior mild malaria compared to children with prior severe malaria. Each bar indicates the mean percentages ± SEM. Values that are statistically different between groups are denoted by asterisks (*$P < 0.05$, as calculated by the Mann-Whitney U-test).

stimulation with PMA and ionomycin in the presence of brefeldin A, were not significantly different between the two groups with different initial clinical presentation *(Tables 2, 3; Figure 3)*. This was apparent for both the overall production of each cytokine as well as for subsets defined by their characteristic cytokine (co-) expression patterns. Without the addition of mitogenic stimuli, no expression of cytokines was detectable in any cell population studied.

Differences in the frequency of cytokine-expressing P. falciparum*-specific CD3⁺ cells and of non-specifically stimulated PBMC between healthy children segregated according to their reinfection profile*

When participating children with < one reinfection per year ($n = 14$, all from the mild malaria group) were compared to children suffering from > two reinfections annually ($n = 9$, all from the severe malaria group), similar significant differences of cytokine expression profiles were found as shown for the groups with different clinical presentation. This was not surprising as patients with prior severe malaria had acquired many more reinfections per year than the children from the mild malaria group, during the follow-up period of six years. As shown for the groups with the history of either severe or mild malaria at admission, no significant differences with regard to the frequencies of IL-2-, IL-4-, IL-6-, IL-10-, IL-13-, IFN-γ-, and TNF-α- expressing $CD4^+$, $CD8^+$, $TCR\gamma/\delta^+$ $CD3^+$ and $CD94^+CD3^-$ cell populations after non-specific mitogenic stimulation were found when children were segregated according to their reinfection rate.

DISCUSSION

The results of this study point towards a role of IL-2- and TNF-α - expressing *P. falciparum*-specific $CD3^+$ cells in the determination of differences in clinical presentation and subsequent reinfection profiles. $CD3^+$ cells of the children who initially presented with mild malaria and who experienced less frequent reinfections during the years of follow-up were more responsive to *P. falciparum*-specific stimulation with increased expression of IL-2 and TNF-α when compared to children with initial severe malaria and more frequent reinfections. This was not only true for the overall capacity of IL-2 and TNF-α production but also for those $CD3^+$ cells exclusively expressing the respective cytokines (single IL-2 and TNF-α expression). All children were parasite-free and healthy at the time of analyses, thus results were not confounded by disease- or inflammation-associated cellular modulation, which we and others have reported to be present during acute *P. falciparum* malaria [31-34]. In addition, using non-specific mitogenic stimulation with intracellular detection of cytokines, we have shown a clear age-dependency in immune responsiveness of patients with acute malaria [32]. In the present study of age-matched healthy children, no differences in cytokine expression in the $CD4^+$, $CD8^+$, $TCR\gamma/\delta^+CD3^+$ and $CD94^+CD3^-$ cell populations were found between both groups when PBMC were non-specifically stimulated. Thus, we suppose, at least in these children and for the subsets studied, that the immunological determinants resulting in different outcomes of infection with respect to clinical severity and reinfection rates are *P. falciparum*-specific. Early type 1 cytokine production during *Plasmodium* infection has been linked to successful control of parasitemia in animal models of malaria [13-20]. This has been attributed mainly to the action of

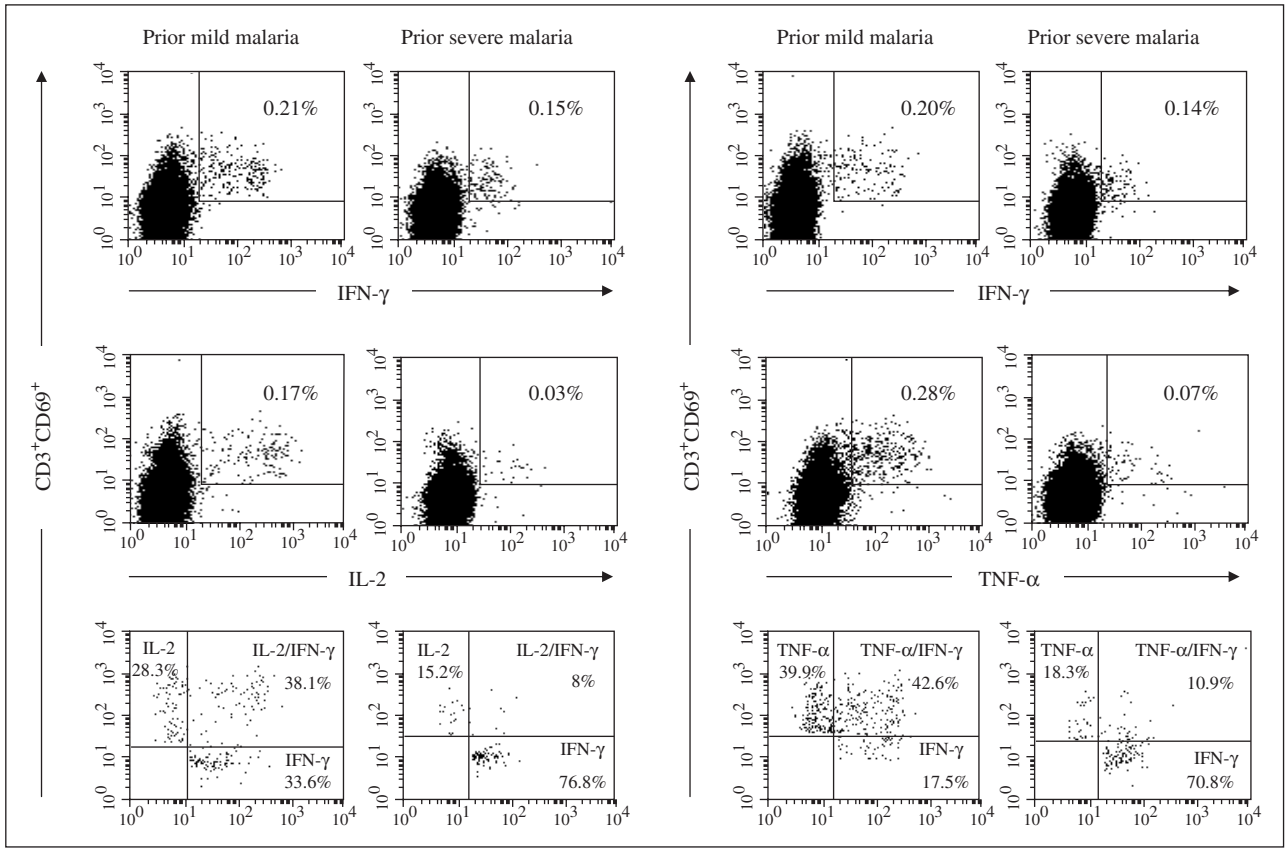

Figure 2

Two-parameter dot plots indicating the frequency of IL-2-, IFN-γ- and TNF-α- expressing *P. falciparum*-specific activated ($CD69^+$) $CD3^+$ T cells of one representative child with prior mild and one child with prior severe malaria. Note the marked differences in the expression of IL-2 and TNF-α between the two children (second horizontal column). The third horizontal column displays the cytokine distribution pattern of IFN-γ together with IL-2 or TNF-α. PBMC were stimulated with a MACS-enriched *P. falciparum* late stage parasite preparation and the co-stimulatory MAb anti-CD28. Numbers indicate the percentage of $CD3^+$ cells positive for the respective cytokine (first and second horizontal column) or $CD3^+$ cells co-expressing two cytokines (third horizontal column). Corresponding data and statistics are depicted in *Table 1*, as well as in *Figure 1*.

Table 2
Frequency of mitogen-induced cytokine expression within the $CD4^+$ and $CD8^+$ T cell subsets in healthy children with prior mild (n = 15) or severe malaria (n = 11)[1]

Cytokines	% of $CD4^+$		% of $CD8^+$	
	Prior mild malaria	Prior severe malaria	Prior mild malaria	Prior severe malaria
IFN-γ only	3.5 ± 0.3 (1.6-5.9)*	3.2 ± 0.3 (1.9-5.2)	17.0 ± 2.1 (8.9-34.3)	17.5 ± 2.6 (4.7-35.0)
IL-2/IFN-γ	9.9 ± 0.8 (4.5-15.8)	9.2 ± 1.0 (5.9-17.4)	8.3 ± 0.8 (3.7-14.7)	8.9 ± 1.5 (3.0-18.8)
IL-2 only	38.9 ± 1.1 (31.1-44.5)	40.3 ± 1.5 (30.8-47.2)	6.9 ± 0.5 (4.1-11.6)	6.4 ± 0.6 (4.2-9.4)
IL-4 only	4.3 ± 0.4 (2.0-8.0)	4.7 ± 0.5 (2.0-7.0)	< 1	< 1
IL-4/IFN-γ	1.6 ± 0.2 (0.5-2.7)	2.0 ± 0.2 (1.0-3.4)	< 1	< 1
IL-6 only	< 1	< 1	< 1	< 1
IL-6/IFN-γ	< 1	< 1	< 1	< 1
IL-10 only	< 1	< 1	< 1	< 1
IL-10/IFN-γ	< 1	< 1	< 1	< 1
IL-13 only	3.8 ± 0.3 (2.0-6.3)	4.3 ± 0.4 (2.3-6.8)	< 1	< 1
IL-13/IFN-γ	< 1	< 1	< 1	< 1
TNF-α only	26.5 ± 1.6 (16.4-41.2)	28.5 ± 1.9 (15.9-36.5)	1.2 ± 0.1 (0.5-2.5)	1.3 ± 0.2 (0.2-2.6)
TNF-α/IFN-γ	11.9 ± 0.8 (8.4-18.2)	10.9 ± 1.1 (6.4-20.1)	16.1 ± 2.1 (6.1-36.3)	17.8 ± 2.9 (4.7-38.9)

*Values indicate mean percentages of cytokine-expressing $CD4^+$ and $CD8^+$ ± SEM; the respective ranges are given in parentheses. PBMC were stimulated with PMA and ionomycin in the presence of brefeldin A.

[1]Differences between groups reached no significance as calculated by the Mann-Whitney U-test.

Table 3
Frequency of mitogen-induced cytokine expression within the CD3$^+$TCRγδ$^+$ and CD3$^-$ CD94$^+$ cell populations in healthy children with prior mild (n = 15) or severe malaria (n = 11)[1]

Cytokines	% of CD3$^+$TCRγd$^+$		% of CD3$^-$CD94$^+$ (NK cells)	
	Prior mild malaria	Prior severe malaria	Prior mild malaria	Prior severe malaria
IFN-γ only	34.7 ± 3.0 (18.6-615)	25.6 ± 4.7 (7.5-590)	47.2 ± 3.7 (13.8-681)	43.2 ± 4.9 (14.9-74.2)
IL-2/IFN-γ	6.7 ± 0.6 (2.5-2.2)	4.9 ± 0.6 (1.3-8.7)	< 1	1.2 ± 0.3 (0.4-4.5)
IL-2 only	7.9 ± 1.1 (2.4-9.0)	9.9 ± 1.3 (4.0-7.2)	< 1	< 1
IL-4 only	< 1	< 1	< 1	< 1
IL-4/IFN-γ	1.3 ± 0.2 (0.3-2.9)	< 1	< 1	< 1
IL-6 only	< 1	< 1	< 1	< 1
IL-6/IFN-γ	< 1	< 1	< 1	< 1
IL-10 only	< 1	< 1	< 1	< 1
IL-10/IFN-γ	< 1	< 1	< 1	< 1
IL-13 only	< 1	< 1	< 1	< 1
IL-13/IFN-γ	< 1	< 1	< 1	< 1
TNF-α only	4.8 ± 0.7 (1.4-10)	4.3 ± 1.1 (1.0-14.9)	8.0 ± 1.0 (3.1-15.0)	8.3 ± 1.6 (1-20.0)
TNF-α/IFN-γ	31.8 ± 3.8 (12.8-69.7)	23.9 ± 4.5 (6.8-48.4)	20.9 ± 2.5 (5.3-39.3)	19.6 ± 3.3 (12.2-45.3)

*Values indicate mean percentages of cytokine-expressing CD3$^+$TCRγδ$^+$ and CD3$^-$CD94$^+$ (NK cells) ± SEM; the respective ranges are given in parentheses. PBMC were stimulated with PMA and ionomycin in the presence of brefeldin A.
[1] Differences between groups reached no significance as calculated by the Mann-Whitney U-test.

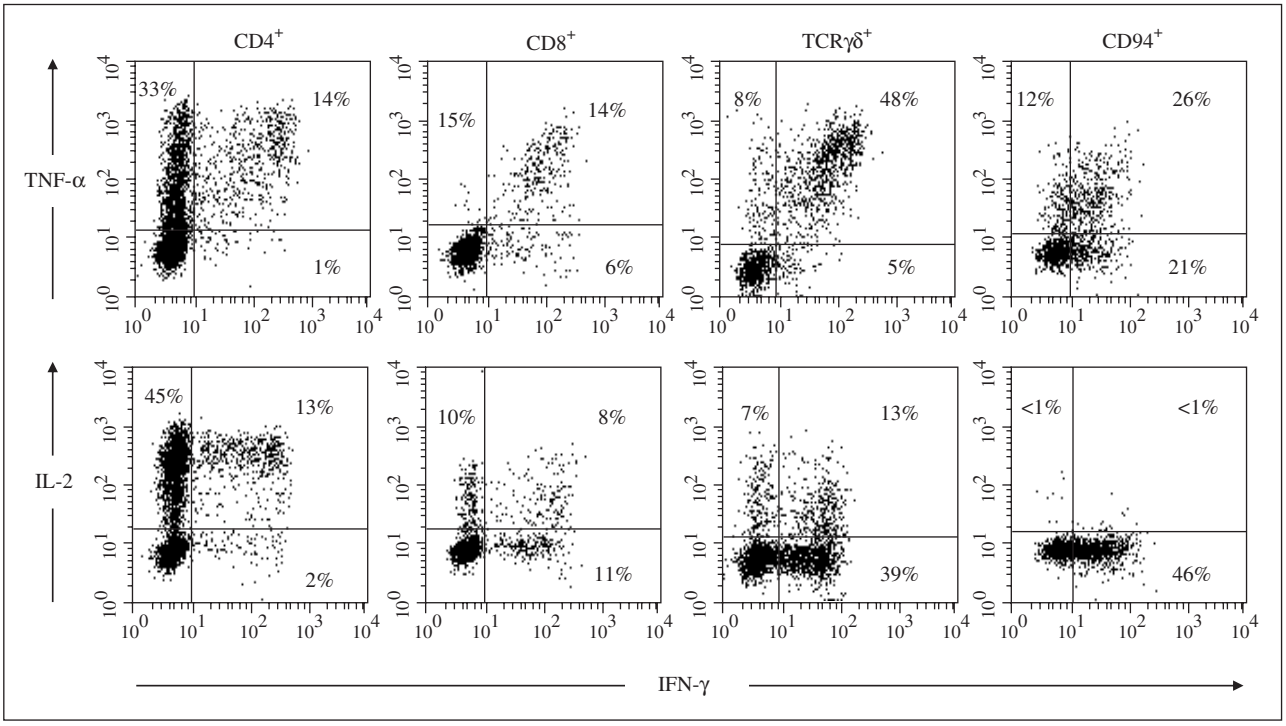

Figure 3
Representative t wo-parameter dot plots indicating the profile of IL-2-, IFN-γ- and TNF-α- expressing CD4$^+$, CD8$^+$, TCRγ/δ$^+$CD3$^+$ and CD94$^+$CD3$^-$ cell populations from one child following non-specific stimulation with PMA and ionomycin in the presence of brefeldin A. Similar characterizations were performed for the cytokines IL-4, IL-6, IL-10, and IL-13. The numbers in each quadrant represent the percentage of gated cytokine-expressing cells with the right upper quadrant including cells co-expressing two cytokines. Corresponding data and statistics are depicted in *Tables 2* and *3*.

IFN-γ and TNF-α. Indeed, IFN-γ was associated with resistance to reinfection in a cohort of children (including those children studied here), when PBMC were stimulated with defined liver-stage and blood-stage antigens [23]. After several years of intense follow-up, only a selection of these children were still available for the present examination, nevertheless much care has been taken to assure comparability of groups with regard to age, socioeconomic status and parasitological factors such as the estimated annual entomological inoculation rate [2, 35]. As we were unable to find differences in the expression of IFN-γ between groups, we suggest that in older children, other cytokines such as IL-2 and TNF-α might play important roles in the protection against reinfection as well. Some evidence for an age-dependent switch of the type of cytokines involved in antimalarial host defenses comes from a

study of adult Kenyans, where IL-10 and not IFN-γ responses to liver-stage antigen-1 were associated with protection against reinfection [24].

IL-2, a product predominantly of $CD4^+$ cells, has originally been described as T cell growth factor [36]. The effects of IL-2 on its cell targets include mitogenesis in type 1 cells, stimulation of cytotoxicity in cytotoxic T lymphocytes and NK cells, as well as generation of lymphokine-activated killer cells. Its role during human malaria has not been very well exploited, as IL-2 is barely detectable in plasma, and most studies focused on the contribution of other cytokines to the pathogenesis of malaria infection. Earlier studies showed a parasite-specific and non-specific defect in IL-2 production during acute *P. falciparum* malaria [37-39], which was thought to provide the basis of malaria-associated cellular immune hyporesponsiveness. By using non-specific stimulation of PBMC from Gabonese patients with uncomplicated malaria, we have found preserved capacity for IL-2 production by $CD4^+$ cells, which, however, was downregulated during clearance of parasitemia [31]. In addition, while IL-2 is termed a classical type 1 cytokine together with IFN-γ, we have shown that both cytokines are reciprocally regulated during the course of an acute *P. falciparum* infection. A role for $CD4^+$ T cell-derived IL-2 has been shown for the activation of human $TCR\gamma/\delta^+$ cells when stimulated with freeze-thawed schizont extracts [40-42]. In addition, treatment of *P. yoelii*-infected mice with IL-2 preferentially accumulated $TCR\gamma/\delta^+$ cells in their brains and rendered them susceptible to cerebral malaria [43]. Therefore, a link between adaptive and innate responses mediated by IL-2 can be suggested. However, while the role of early IL-2 expression in response to *P. falciparum* infection remains rather speculative until now, more precise data are available with regard to early TNF-α production.

The release of TNF-α in response to parasites has been shown to be dependent upon the presence of $CD3^+$ cells and monocytes [44]. $TCR\gamma/\delta^+CD3^+$ cells have been identified as the major lymphocyte subset involved in t his early TNF-α production [28]. Our previously obtained data and those shown here however, ascribe considerable capacity for TNF-α production to other T cell subsets [29]. "Appropriate" and early TNF-α production appears to be protective and necessary to limit parasitemia [21, 22], while high levels in plasma have been linked to cerebral malaria and in the presence of low IL-10 levels, with severe anemia in African children [6, 7, 10, 11, 22, 45]. An increased capacity of TNF-α production might explain the greater capacity of PBMC from children with prior mild malaria to express nitric oxide (NO) synthase and to produce NO [46]. This may allow a more efficient reduction of parasitemia, thus preventing progression to severe disease in this group of children. Anti-inflammatory activities, here represented by both specifically and non-specifically induced IL-10 were not different between groups, neither were the non-specifically induced classical type 2 cytokines IL-4 and IL-13. Most IL-10-expressing $CD3^+$ cells also co-expressed IFN-γ, suggesting an immune-regulatory role in response to stimulation with *P. falciparum* [47]. Intracellular IL-6 was not detected in either T cells or within the NK cell subset.

In all, we have detected considerable frequencies of cytokine-expressing, activated T cells in healthy children upon specific stimulation with late-stage *P. falciparum* parasite preparations. Mean frequencies of responding $CD3^+$ T cells were 1/5000 after staining for IL-10, and 1/700 cells expressing TNF-α, which is comparable to the precursor frequencies previously obtained *in vitro* after six days of incubation with *Plasmodium*-infected erythrocytes [48]. Marked differences were shown for the frequency of IL-2- and TNF-α- producing *P. falciparum*-specific activated T cells between children with prior mild malaria and less frequent reinfections on the one hand and children with initial severe malaria and more frequent reinfections on the other, pointing towards a protective role of both cytokines in host defense against malaria. Studies are underway that examine specific cellular cytokine responses both during acute malaria as well as in individuals with different degrees of immunity to *P. falciparum*.

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REFERENCES

1. Snow RW, Craig M, Deichmann U, Marsh K. 1999. Estimating mortality, morbidity and disability due to malaria among Africa′s non-pregnant population. *Bull World Health Org.* 77: 624.
2. Sylla EH, Kun JF, Kremsner PG. 2000. Mosquito distribution and entomological inoculation rates in three malaria-endemic areas in Gabon. *Trans R Soc Trop Med Hyg.* 94: 652.
3. Wildling E, Winkler S, Kremsner PG, Brandts C, Jenne L, Wernsdorfer WH. 1995. Malaria epidemiology in the province of Moyen Ogooué, Gabon *Trop Med Parasitol.* 46: 77.
4. Miller LH, Baruch DI, Marsh K, Doumbo OK. 2002. The pathogenic basis of malaria. *Nature.* 415: 673.
5. Troye-Blomberg M, Berzins K, Perlmann P. 1994. T-cell control of immunity to the asexual stages of the malaria parasite. *Crit Rev Immunol.* 14: 131.
6. Grau GE, Taylo r TE, Molyneux ME, Wirima JJ, Vassalli P, Hommel M, Lambert PH. 1989. Tumor necrosis factor and disease severity in children with falciparum malaria. *N Engl J Med.* 320: 1586.
7. Kwiatkowski D, Hill AV, Sambou I, Twumasi P, Castracane J, Manogue KR, Cerami A, Brewster DR, Greenwood BM. 1990. TNF concentrations in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet.* 336: 1201.
8. Kwiatkowski D, Molyneux ME, Stephens S, Curtis N, Klein N, Pointaire P, Smit M, Allan R, Brewster DR, Grau GE. 1993. Anti-TNF therapy inhibits fever in cerebral malaria. *Q J Med.* 86: 91.
9. Day NPJ, Tinh Hien T, Schollaardt T, Loc PP, Chuong LV, Chau TT, Mai NT, Phu NH, Sinh DX, White NJ, Ho M. 1999. The prognostic and pathophysiologic role of pro- and anti-inflammatory cytokines in severe malaria. *J Infect Dis.* 180: 1288.
10. Kurtzhals JAL, Adabayeri V, Goka BQ, Akanmori BD, Oliver-Commey JO, Nkrumah FK, Behr C, Hviid L. 1998. Low plasma concentrations of interleukin 10 in severe malarial anaemia compared with cerebral and uncomplicated malaria. *Lancet.* 351: 1768.
11. Othoro C, Lal AA, Nahlen B, Koech D, Orago AS, Udhayakumar V. 1999. A low interleukin-10 tumor necrosis factor-α ratio is associated with malaria anemia in children residing in a holoendemic malaria region in western Kenya. *J Infect Dis.* 179: 279.
12. Harpaz R, Edelman R, Wasserman SS, Levine MM, Davies JR, Sztein MB. 1992. Serum cytokine profiles in experimental human malaria. *J Clin Invest.* 90: 515.

13. Langhorne J, Gillard S, Simon B, Slade S, Eichmann K. 1989. Frequencies of $CD4^+$ T cells reactive with *Plasmodium chabaudi chabaudi*: distinct response kinetics for cells with Th1 and Th2 characteristics during infection. *Int Immunol.* 1: 416.
14. Stevenson MM, Tam MF. 1993. Differential induction of helper T cell subsets during blood-stage *Plasmodium chabaudi* AS infection in resistant and susceptible mice. *Clin Exp Immunol.* 92: 77.
15. Stevenson MM, Tam MF, Wolf SF, Sher A. 1995. 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.* 155: 2545.
16. Perlman H, Kumar S, Vinetz JM, Kullberg M, Miller LH, Perlmann P. 1995. Cellular mechanisms in the immune response to malaria in *Plasmodium vinckei* infected mice. *Infect Immun.* 63: 3987.
17. Jacobs P, Radzioch D, Stevenson MM. 1996. In vivo regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. *Infect Immun.* 64: 44.
18. Jacobs P, Radzioch D, Stevenson MM. 1996. A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. *Infect Immun.* 64: 535.
19. Mohan K, Moulin P, Stevenson MM. 1997. Natural killer cell cytokine production, not cytotoxicity, contributes to resistance against blood-stage *Plasmodium chabaudi* AS infection. *J Immunol.* 159: 4990.
20. Kremsner PG, Neifer S, Schermuck S, Ferreira Chaves M, Sliwa K, Bienzle U. 1991. Interferon-γ enhances the effect of antimalarial chemotherapy in murine *Plasmodium vinckei* malaria. *J Infect Dis.* 163: 1161.
21. Kremsner PG, Winkler S, Brandts C, Wildling E, Jenne L, Graninger W, Prada J, Bienzle U, Juillard P, Grau GE. 1995. Prediction of accelerated cure in *Plasmodium falciparum* malaria by the elevated capacity of tumor necrosis factor production. *Am J Trop Med Hyg.* 53: 532.
22. Mordmüller BG, Metzger WG, Juillard P, Brinkman BM, Verweij CL, Grau GE, Kremsner PG. 1997. 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.* 8: 29.
23. Luty AJ, Lell B, Schmidt-Ott R, Lehman LG, Luckner D, Greve B, Matousek P, Herbich K, Schmid D, Migot-Nabias F, Deloron P, Nussenzweig RS, Kremsner PG. 1999. Interferon-γ responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. *J Infect Dis.* 179: 980.
24. Kurtis JD, Lanar DE, Opollo M, Duffy PE. 1999. Interleukin-10 responses to liver-stage antigen 1 predict human resistance to *Plasmodium falciparum*. *Infect Immun.* 67: 3424.
25. Kun JF, Schmidt-Ott RJ, Lehman LG, Lell B, Luckner D, Greve B, Matousek P, Kremsner PG. 1998. Merozoite surface antigen 1 and 2 genotypes and rosetting of *Plasmodium falciparum* in severe and mild malaria in Lambaréné, Gabon. *Trans R Soc Trop Med Hyg.* 92: 110.
26. Van Kuppeveld FJ, van der Logt JT, Angulo AF, van Zoest MJ, Quint WG, Niesters HG, Galama JM, Melchers WJ. 1992. Genus- and species-specific identification of mycoplasmas by 16S rRNA amplification. *Appl Environ Microbiol.* 58: 2606.
27. Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. 1999. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. *Cytometry.* 35: 329.
28. Hensmann M, Kwiatkowski D. 2001. Cellular basis of early cytokine response to *Plasmodium falciparum Infect Immun.* 69: 2364.
29. Ramharter M, Willheim M, Winkler H, Wahl K, Lagler H, Graninger W, Winkler S. 2003. Cytokine profile of *Plasmodium falciparum*-specific T cells in non-immune malaria patients. *Parasite Immunol.* 25: 211.
30. Suni MA, Picker LJ, Maino VC. 1998. Detection of antigen-specific cytokine expression in whole blood by flow cytometry. *J Immunol Methods.* 212: 89.
31. Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W, Kremsner PG. 1998. Reciprocal regulation of Th1- and Th2-cytokine-producing T cells during clearance of parasitemia in *Plasmodium falciparum* malaria. *Infect Immun.* 66: 6040.
32. Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W, Kremsner PG. 1999. Frequency of cytokine-producing T cells in patients of different age groups with *Plasmodium falciparum* malaria. *J Infect Dis.* 179: 209.
33. Hviid L, Kurtzhals JA, Goka BQ, Oliver-Commey JO, Nkrumah FK, Theander TG. 1997. Rapid reemergence of T cells into peripheral circulation following treatment of severe and uncomplicated *Plasmodium falciparum* malaria. *Infect Immun.* 65: 4090.
34. Kemp K, Akanmori BD, Adabayeri V, Goka BQ, Kurtzhals JA, Behr C, Hviid L. 2002. Cytokine production and apoptosis among T cells from patients under treatment for *Plasmodium falciparum* malaria. *Clin Exp Immunol.* 127: 151.
35. Luckner D, Lell B, Greve B, Lehman LG, Schmidt-Ott RJ, Matousek P, Herbich K, Schmid D, Mba R, Kremsner PG. 1998. No influence of socioeconomic factors on severe malarial anemia, hyperparasitemia or reinfection. *Trans R Soc Trop Med Hyg.* 92: 478.
36. Smith KA. 1988. Interleukin-2: inception, impact, and implications. *Science.* 240: 1169.
37. Troye-Blomberg M, Andersson G, Stoczkowska M, Shabo R, Romero P, Patarroyo ME, Wigzell H, Perlmann P. 1985. Production of IL-2 and interferon-gamma by T cells from malaria patients in response to *Plasmodium falciparum* or erythrocyte antigens in vitro. *J Immunol.* 135: 3498.
38. Ho M, Webster HK, Green B, Looareesuwan S, Kongchareon S, White NJ. 1988. Defective production of and response to IL-2 in acute human falciparum malaria. *J Immunol.* 141: 2755.
39. Kremsner PG, Zotter GM, Feldmeier H, Graninger W, Rocha RM, Jansen-Rosseck R, Bienzle U. 1990. Immune response in patients during and after *Plasmodium falciparum* infection. *J Infect Dis.* 161: 1025.
40. Jones SM, Goodier MR, Langhorne J. 1996. The response of γδ T cells to *Plasmodium falciparum* is dependent on activated $CD4^+$ T cells and the recognition of MHC class I molecules. *Immunology.* 89: 405.
41. Elloso MM, Wallace M, Manning DD, Weidanz WP. 1998. The effects of interleukin-15 on human γδ T cell responses to *Plasmodium falciparum* in vitro. *Immunol Lett.* 64: 125.
42. Waterfall M, Black A, Riley E. 1998. $\gamma\delta^+$ T cells preferentially respond to live rather than killed malaria parasites. *Infect Immun.* 66: 2393.
43. Haque A, Echchannaoui H, Seguin R, Schwartzman J, Kasper LH, Haque S. 2001. Cerebral malaria in mice: interleukin-2 treatment induces accumulation of γδ T cells in the brain and alters resistant mice to susceptible-like phenotype. *Am J Pathol.* 158: 163.
44. Scragg IG, Hensmann M, Bate CA, Kwiatkowski D. 1999. Early cytokine induction by *Plasmodium falciparum* is not a classical endotoxin-like process. *Eur J Immunol.* 29: 2636.
45. May J, Lell B, Luty AJ, Meyer CG, Kremsner PG. 2000. Plasma interleukin-10: Tumor necrosis factor (TNF)-α ratio is associated with TNF promoter variants and predicts malarial complications. *J Infect Dis.* 182: 1570.
46. Perkins DJ, Kremsner PG, Schmid D, Misukonis MA, Kelly MA, Weinberg JB. 1999. Blood mononuclear cell nitric oxide production and plasma cytokine levels in healthy Gabonese children with prior mild or severe malaria. *Infect Immun.* 67: 4977.
47. Trinchieri G. 2001. Regulatory role of T cells producing both interferon γ and interleukin 10 in persistent infection. *J Exp Med.* 194: F53.
48. Zevering Y, Amante F, Smillie A, Currier J, Smith G, Houghten RA, Good MF. 1992. High frequency of malaria-specific T cells in non-exposed humans. *Eur J Immunol.* 22: 689.