Frequency of Cytokine-Producing T Cells in Patients of Different Age Groups with *Plasmodium falciparum* Malaria

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The frequency of cytokine-producing T cells was assessed in patients of different age groups (29 infants, aged 1–5 years; 30 schoolchildren, aged 6–14 years; 26 adults, aged >15 years) with acute *Plasmodium falciparum* malaria from Gabon. By using flow cytometry for the intracellular detection of cytokines, a striking expansion was seen, in adults compared with children, of CD4⁺ and CD8⁺ T cells with the following profiles of type 1 cytokine production: interleukin (IL)-2, interferon (IFN)-γ, IL-2 /IFN-γ, and IL-2 /IFN-γ. Patients with hyperparasitemia had a significantly lower frequency of IL-2 /IFN-γ⁺ CD4⁺ cells. Type 2 cytokine expression (IL-4 /IFN-γ, IL-13 /IFN-γ) and type 0 cytokine expression (IL-4 /IFN-γ, IL-13 /IFN-γ) were also increased in adults within the CD4⁺ subset. Frequencies of IL-5⁺/IL-4⁺, IL-10⁺/IFN-γ⁺, and IL-10⁺/IFN-γ⁺ cells were similar in all groups. The increased frequency of both type 1 and type 2 cytokine–producing T cells in adults is likely to be of significance in the protection against *P. falciparum* malaria.

In areas where transmission of *Plasmodium falciparum* is both stable and intense, malaria is predominantly a disease of non-immune children. Most of the ~2 million deaths each year due to severe malaria occur in this age group [1]. During adolescence, however, natural immunity is likely to develop, first characterized by decreases in the severity of disease episodes but still high-level parasitemia. This concept of anti-disease immunity, although not uniformly accepted [2], has been attributed to the presence of antibodies to *P. falciparum* toxins released at schizogony and to differential regulation of innate immune responses in different age groups [3–5]. In adults, protective immunity acts on both the clinical and parasitologic levels, with fewer or even absent symptoms and low-grade parasitemia. The acquisition of specific antibodies to variant target antigens, such as the *P. falciparum* erythrocyte membrane protein 1, has been suggested as one of the possible mechanisms involved in protection [6]. This type of immunity, however, requires repeated exposure to the parasite to be efficient and decays rapidly once exposure to the parasite ceases.

The contribution of T cell subsets and their products to the development of natural immunity is essential both in regulating antibody formation and in inducing antibody-independent protection. The CD4⁺ T cell subset is thought to be of major importance for the induction of blood-stage immunity, while the CD8⁺ T cell subset is predominantly known for its cytolytic activity against liver stages of the parasite [7].

On the basis of their cytokine profiles, both CD4⁺ and CD8⁺ T cells have been divided into different subpopulations, namely T helper (Th) 1 and T cytotoxic (Tc) 1 (type 1) as well as Th2 and Tc2 (type 2) cells. Type 1 cells produce the cytokines interferon (IFN)-γ, interleukin (IL)-2, and lymphotoxin. Type 2 T cells are characterized by IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 production [8]. Type 1 cells producing IFN-γ are effective in activating monocytes/macrophages to kill parasites directly; the type 2 cytokine IL-4 has been shown to be crucial in the induction of *P. falciparum*–specific antibody production during blood-stage malaria [9]. In mice challenged with *Plasmodium chabaudi*, type 1 cytokines were activated during the early phase of infection to initiate parasite clearance, while type 2 cytokines appeared later on to prevent recrudescence of the primary infection [10]. Type 1 cytokines, however, contribute also to the development of potential deleterious complications, such as cerebral malaria, while type 2 cytokines, including IL-10, are believed to be beneficial in this setting by limiting overwhelming inflammation [11–14].

Whether cytokine production and the type 1/type 2 dichotomy reflect the well-known differences in clinical and parasitologic presentation between nonimmune children and semimmune adults with acute *P. falciparum* malaria is not well understood. By using flow cytometric analysis for the intra-
cellular detection of cytokines, we sought to characterize the phenotypes and the frequency of cytokine-producing T cells in a detailed cross-sectional study of P. falciparum malaria in central Africa.

Materials and Methods

Study participants. The study took place in the Albert Schweitzer Hospital in Lambaréné, Gabon, where P. falciparum malaria is predominantly hyperendemic [15]. Patients attending the outpatient clinic during November 1997 were enrolled if they met the following study criteria: infection with P. falciparum, no recent antimalarial treatment, and no other systemic infection. Patients' characteristics are compiled in table 1. Study participants were allocated into 3 groups according to age. Blood samples were collected immediately after P. falciparum malaria was diagnosed, followed by the administration of antimalarials. Patients with uncomplicated P. falciparum malaria were treated as outpatients with the combination of sulfadoxine-pyrimethamine, which has been shown to be curative in this area [16]. Nine patients with hyperparasitemia were hospitalized and received intravenous quinine treatment and supportive treatment as required. All patients survived.

Peripheral blood mononuclear cell (PBMC) cultures and intracellular cytokine detection by flow cytometry. Flow cytometric assessment of T cell cytokine production was done essentially according to the technique described by Jung et al. [17] and modified by Willheim et al. [18]. PBMC were isolated from heparinized blood by ficoll-diatriatrizate centrification. Cells were then cultured in medium (Ultra Culture; BioWhittaker, Walkersville, MD) supplemented with L-glutamine (2 mmol/L; Sigma, St. Louis), gentamicin (170 mg/L; Sigma), and 2-mercaptoethanol (3.5 μL/L; Merck, Darmstadt, Germany) and stimulated with phorbol 12-myristate 13-acetate (10 ng/mL; Sigma) and ionomycin (1.25 μM; Sigma) in the presence of monensin (1 μM; Sigma) for 4 h at 37°C in 5% CO2. Cells were then harvested on ice, washed twice in PBS, and fixed with 2% formaldehyde (1 mL/2 × 107 cells; Merck) for 20 min. After two additional washes in PBS, cells were resuspended in Hanks’ balanced salt solution (supplemented with 0.3% bovine serum albumin 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), resuspended with 50 μL of saponin buffer–diluted antibodies, and incubated for 25 min at room temperature in the dark. The following monoclonal antibodies (MAbs) were used: cytokine–specific mouse anti-human MAb (IFN-γ [clone: B27], fluorescein isothiocyanate [FITC]–labeled) and rat anti-human MAb (IL-2 [MQI-17H12], phycoerythrin [PE]–conjugated; IL-4 [MP4-25D2], PE–labeled; IL-4 [MP4-25D2], FITC–labeled; IL-5 [TRFK5], PE–labeled; IL-10 [JES3-9D7], PE–labeled; IL-13 [JES10-5A2], PE–labeled). All MAbs were purchased from PharMingen (San Diego). The anti-CD4 MAb and anti-CD8 MAb were allophycocyanin– and peridinin chlorophyll–labeled, respectively (Becton Dickinson, Mountain View, CA). Four-color staining was done, and at least 104 cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson) equipped with a two-laser system (488 nm and 630 nm wavelength, respectively). All intracellular cytokines were stained in conjunction with IFN-γ, except for IL-5, which was combined with IL-4. Data were analyzed (CellQuest software; Becton Dickinson), and results were expressed as the percentage of cytokine-producing cells in each CD4+ or CD8+ cell population (figure 1).

Statistical methods. Statistical analysis was done with a standard statistical package (SPSS 7.5 for Windows; SPSS, Chicago). Groups were compared by the independent-samples t test. Bonferroni’s test of multiple comparisons was applied to correct for multiple analyses. Bivariate correlations were done by computing Pearson’s correlation coefficient. P < .05 was considered significant.

Results

Study population. Three groups of patients were investigated: 29 infants (age range, 1–5 years), 30 schoolchildren (age range, 6–14 years), and 26 adults (age range, 17–74 years). Patients’ characteristics and parasitologic data are delineated in table 1. As expected, adults had significantly lower parasite densities than did both the very young and the schoolchildren (P < .001 and P < .01, respectively). Differences in parasitemia between both groups of children were not significant. Nine children were hyperparasitemic (>250,000/μL), and 3 of them were suffering from severe anemia (hemoglobin level, <50 g/L). Neither cerebral malaria, renal insufficiency, nor even multiorgan dysfunction was noted in our study collective. There was no significant influence of sex on the frequency of any of the cytokine–producing T cell subpopulations studied.

Frequency of the CD4+ and CD8+ T cell subsets within groups. Figure 2 shows the percentage of cells within the lymphocyte scatter gate positively stained for either CD4 or CD8 in each age group. Striking was the increased CD4+ subset in both adults and schoolchildren compared with infants.

Differential frequency of type 1 cytokine–producing cells within groups. The frequency of T cells producing IFN-γ or IL-2 or co-producing both cytokines was investigated (figures 1, 3A, 3B, 4A, 4B). With respect to the CD4+ subset, a substantial increased capacity of type 1 cytokine production was observed in circulating cells from schoolchildren compared with infants and in adults compared with both groups of children (figures 1, 3A, 3B). Most strikingly, the percentage of overall IFN-γ–producing CD4+ cells was ~2-fold higher in adults than in infants (20% vs. 10%; P < .001), with 67% of these cells co-producing IL-2 (14% vs. 6%; P < .001) (figures 1, 3A). Within the CD8+ subset, no significant differences were seen between
Figure 1. Representative two-parameter dot plots displaying differences in frequency of cytokine-producing CD4⁺ cells between study groups. Each vertical row depicts results of cytometric analyses from 1 representative patient. Interferon (IFN)-γ (x axis) was stained in combination with interleukin (IL)-2, IL-4, IL-10, and IL-13 (all y axis). IL-4 (x axis) was also combined with IL-5 (y axis). Quadrant statistics were set on basis of corresponding negative controls. Box plots of figure 3 show corresponding statistics. FITC, fluorescein isothiocyanate.
Infants and schoolchildren, but differences were significant between adults and both groups of children (figures 4A, 4B).

**Differential frequency of type 2 cytokine-producing cells within groups.** The frequency of IL-4, IL-5, IL-10, and IL-13-producing T cells was assessed (figures 1, 3C–3F). In addition, the capacity of CD4+ and CD8+ cells to produce both IL-4 and IL-5 was studied (figures 1, 3E). Significant differences were seen between adults and both groups of children and were restricted to the CD4+ subset. In adults, IL-4+/IL-5− and IL-13+/IFN-γ− CD4+ cells were markedly expanded, and differences were also shown between adults and schoolchildren for the exclusively IL-5-producing subpopulation (figures 1, 3C, 3E). The frequency of IL-10–expressing cells was similar in all groups, as was the percentage of IL-4+/IL-5− cells within the CD4+ subset (figures 1, 3E, 3F). The frequencies of IL-4+/IFN-γ−, IL-13+/IFN-γ−, IL-4+/IL-5−, and IL-10+/IFN-γ− CD8+ cells were <1% in all 3 groups.

**Differential frequency of type 0 lymphocytes within groups.** The type 0 subset of lymphocytes, which is characterized by its ability to produce a combination of type 1 and type 2 cytokines, was investigated by determining the coexpression of type 2 cytokines together with IFN-γ (figures 1, 3D, 3F). As shown for type 2 cytokines, type 0 cytokine expression of CD4+ cells was equal in both groups of children but was increased in adults, except for the IL-10+/IFN-γ− subpopulation (figures 1, 3D, 3F). Small differences, albeit statistically significant, were found for CD8+ cells producing cytokines in a type 0 manner between infants and both schoolchildren and adults (IL-10+/IFN-γ− and IL-13+/IFN-γ−, 2% vs. 1%; P < .05).

**Frequency of cytokine-producing T cells in severe versus uncomplicated malaria in children.** By comparing children with hyperparasitemia (n = 9) with the other children, significant differences were shown for the CD4+ subset, as cells exclusively expressing IFN-γ were lower in patients with hyperparasitemia (5% vs. 3%; P < .05). Overall, IL-10–producing CD4+ cells were equally decreased in hyperparasiticemic patients (4% vs. 3%; P < .05). Higher percentages, however, of IL-5–producing CD8+ cells were found in the hyperparasitemic group, mainly because of an expansion of the exclusively IL-5–producing subpopulation (4% vs. 2%; P < .05).

**Influence of age on the frequency of cytokine-producing T cell subpopulations within groups.** Within the adult group, older age was associated with a higher frequency of exclusively IL-5−, IL-10−, and IFN-γ− and IL-10/IFN-γ− CD4+ cells (P < .05, except for IL-10 [P < .01]) as well as of total IL-10 expression in CD4+ cells (P < .05). IL-2+/IFN-γ− cells were equally increased within the CD8+ subset as an age-related modification (P < .05). In the infant group, an age-dependent increase in IL-4+/IFN-γ− and IL-13+/IFN-γ− cells and in IL-4+/IL-5− cells was observed (P < .01 for both IL-4+/IFN-γ− and IL-13+/IFN-γ− and P < .05 for IL-4+/IL-5−). In schoolchildren, the percentage of CD4+ cells was positively correlated with aging (P < .05). The percentage of CD8+ cells showed no age-related modifications in the 2 groups of children.

**Correlation of cytokine-producing T cell subpopulations within groups.** The most consistent finding was the uniformly positive correlation of all type 2 cytokines within both T cell subsets; the same, however, was shown also for the type 1 cytokines IL-2 and IFN-γ (data not shown). Patients who exhibited increased frequencies of type 1 cytokine–producing cells had generally also higher type 2 cytokine expression. Also, type 0 cytokine production capacity was correlated with both type 1 and type 2 cytokine expression.

**Discussion.** The specific immune response to the pathogenic asexual blood stages of *P. falciparum* is largely dependent on T cells, especially those of the CD4+ subset. The aim of the present study was to investigate the type I/type 2 dichotomy of T cells in patients of different age groups with acute *P. falciparum* malaria for the purpose of examining immunoregulatory mechanisms that ultimately result in different levels of antimalarial protection. By studying cytokine-producing T cell subpopulations, the most impressive finding was the striking increased frequency of type 1 cytokine–expressing cells in adults compared with children. Adults displayed a 2-fold higher frequency of IFN-γ–expressing CD4+ cells, most of which were also IL-2 producers, than did infants. These differences were not restricted to CD4+ T cells but were also evident for the CD8+ subset. The gradual expansion of type 1 cytokines and especially IFN-γ–producing cells with age is further suggested by the finding that the IFN-γ production capacity of both CD8+ and CD4+ cells was positively correlated with aging within the adult group. It is tempting to hypothesize from our data that the development of protective immunity against *P. falciparum* is at least in part dependent on an effective type 1 response. In that, early high IFN-γ production as part of a type 1–driven immune response has been associated with a more favorable
Figure 3. Median and quartile distributions of frequency of cytokine-producing CD4⁺ cells within 3 study groups. Line through box shows median, with other quartiles at either end. Values that varied by >1.5 times length of box are not depicted. Significant differences between groups: IL-2⁺/IFN-γ⁺, adults vs. both schoolchildren and infants, $P < .001$; IL-2⁺/IFN-γ⁺, adults vs. both groups of children, $P < .001$ (A). IL-2⁺/IFN-γ⁺, adults vs. schoolchildren, $P = .001$; adults vs. infants, $P < .001$ (B). IL-4⁺/IFN-γ⁺, adults vs. both children's groups, $P < .05$; IL-13⁺/IFN-γ⁺, adults vs. schoolchildren, $P < .05$; adults vs. infants, $P < .001$ (C). IL-4⁺/IFN-γ⁺, adults vs. schoolchildren, $P = .001$; adults vs. infants, $P < .001$; IL-13⁺/IFN-γ⁺, adults vs. schoolchildren, $P < .01$; adults vs. infants, $P < .05$ (D). IL-5⁺/IL-4⁺, adults vs. schoolchildren, $P < .05$ (E). Differences of IL-10⁺/IFN-γ⁻ and IL-10⁺/IFN-γ⁺-expressing CD4⁺ cells were not significant between the age groups (F).
outcome in most animal models of malaria [19–24], and the addition of this particular cytokine greatly enhanced the effect of antimalarial chemotherapy in murine *Plasmodium vinckei* infection [25]. This has been attributed to its monocyte/macrophage-activating capacities, with rapid killing of the malarial blood-stage parasites by reactive oxygen and nitrogen intermediates [26, 27]. The role of IFN-γ as a key antiparasitic molecule is further underlined by the fact that children with hyperparasitemic malaria displayed an even lower frequency of IFN-γ-producing CD4+ cells than did children with the uncomplicated form of the disease.

Concomitant with the expansion of type 1 cytokine-producing T cells, which is in line with previously obtained results in the murine system [10, 19, 28], an increased frequency of IL-4- and IL-13-producing CD4+ cells was observed in the adult group compared with both infants and schoolchildren. Similar increased frequencies in adults were found for CD4+ cells producing cytokines in a type 0 manner (IL-4+/IFN-γ-, IL-13+/IFN-γ-). While the possible functions of the latter T cell subpopulations in malaria are unknown, it has been shown that type 2 cytokine-producing cells prevent recrudescences in mice challenged with *P. chabaudi chabaudi* by enhancement and acceleration of specific IgG1 production [10]. IL-4-deficient mice, challenged with the same *Plasmodium* species, however, were able to clear a primary infection, and in vitro, IL-4 even facilitated *P. falciparum* parasite survival by suppressing macrophage activity [29, 30]. These apparent divergences concerning the function of IL-4 in blood-stage disease led to the hypothesis that another type 2 cytokine, namely IL-13, might replace to some extent IL-4 in providing B cell help in switching to specific IgG1 synthesis [30]. This would suggest a distinct role of IL-13 in malarial infection, and indeed we found this particular cytokine significantly expressed with a very close correlation to the expression of IL-4.

No differences between groups were found for the frequency of IL-10-producing CD4+ and CD8+ T cells, except for the subgroup of hyperparasitemic patients with decreased expression of the respective cytokine. The role of IL-10 in human malaria appears not well-defined, yet IL-10 has been shown to be protective by limiting tumor necrosis factor-α and type 1 cytokine-induced pathology in murine models of cerebral malaria [14]. About half of the IL-10-producing CD4+ and CD8+ cells in our study coexpressed IFN-γ, which might reflect an indirect feedback mechanism for inhibition of IFN-γ expression, since IL-10 suppresses antigen-presenting cell function by down-regulating class II major histocompatibility complex antigens and costimulatory signaling through CD80, as well as by blocking IL-12 production [31–33]. The complexity of these interactions is underlined by the fact that IL-12 itself has been found to induce this particular subpopulation of IL-10+/IFN-γ- T cells [34].

IL-5 was substantially produced by both T cell subsets. Within the CD4+ subset, however, the majority of IL-5 producers also expressed IL-4. Surprisingly, we found an expansion of IL-5-producing CD8+ cells in the hyperparasitic patients, the clinical significance of which remains unknown. It is clear from our data, however, that IL-5 expression is not downregulated in *P. falciparum* malaria, as has been suggested by a study revealing eosinopenia during the acute phase of the disease [35]. Indeed, healthy subjects from our study area displayed

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**Figure 4.** Median and quartile distributions of frequency of type 1 cytokine-producing CD8+ cells within 3 study groups. Line through box shows median, with other quartiles at either end. Values that varied by >1.5 times length of box are not depicted. Significant differences between groups: IL-2+/IFN-γ-, adults vs. schoolchildren, *P* < .01; adults vs. infants, *P* < .05 (A). IL-2+/IFN-γ-, adults vs. both children’s groups, *P* < .01; IL-2+/IFN-γ-, adults vs. schoolchildren, *P* < .01; adults vs. infants, *P* < .01 (B).
a significantly lower frequency of IL-5–producing T cells than did malaria patients (unpublished data).

A mechanism responsible for the overall lower frequency of cytokine-producing T cells in the bloodstream of children could be their enhanced sequestration in the periphery, as suggested in some studies [36–38]. This transient disease-induced depletion of T cells has been associated with high expression of the adhesion molecule LFA (leukocyte function–associated molecule)-1 on these cells [36]. Some support for these observations comes from our data, as the frequency of CD4+ cells within the gated cell population was significantly lower in very young children, who are more susceptible to severe disease manifestations. Whether this particular subset, as suggested in these studies, reemerges into the peripheral circulation following drug cure and potentially exhibits another cytokine profile is currently under investigation.

T cells and their products represent the major immunoregulatory host defense strategy against blood-stage malaria. The striking differences in the capacity of cytokine production observed within our study population during acute \( P. falciparum \) infection are therefore likely to be of clinical significance. Taken from our data, this is especially true for IFN-\( \gamma \), although it appears obvious that both type 1 and type 2 cytokines are engaged in an effective immune response against the blood-stage forms.

References

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