# Age-dependency of *Plasmodium falciparum*-specific and non-specific T cell cytokine responses in individuals from a malaria-endemic area

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**ABSTRACT. In areas where *Plasmodium falciparum* malaria is highly endemic, naturally acquired immunity develops slowly with increasing age. The mechanisms that lead to this protective immunity against *P. falciparum* are under intense investigation, as they might serve as models for the development of an efficient vaccine. In this study, we aimed to investigate the potential contribution of cell-mediated immune responses to the build-up of anti-malarial immunity by comparing the phenotypes and frequencies of both *P. falciparum*-specific and non-specific, cytokine-expressing T cells in a cross-sectional study of healthy children and adults, living in a malaria-endemic area in Central Africa. An increased capacity of $CD3^+$ cells to produce interferon (IFN)-γ and tumor necrosis factor (TNF)-α, and of the $TCR\gamma\delta^+$ subset to produce TNF-α was seen in adults after stimulation of peripheral blood mononuclear cells (PBMC) with a late-stage, schizont-rich, parasite preparation. Mitogenic stimulation with PMA and ionomycin induced much higher frequencies of IFN-γ- and TNF-α- expressing $CD4^+$, $CD8^+$ as well as $TCR\gamma\delta^+$ cells in adults, while differences for interleukin (IL)-2 expression were restricted to $CD4^+$ and $CD8^+$ T cells. For IL-10, neither specific nor non-specific stimulations of PBMC were associated with significant age-dependent alterations. Impressive increases in the capacity to produce *P. falciparum*-specific and non-specific IFN-γ and TNF-α appear to be the main cellular correlates of naturally acquired immunity in Central Africa.**

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

In areas of Sub-Saharan Africa where *Plasmodium falciparum* is hyperendemic, mortality is highest in infants younger than 6 years of age [1]. During childhood and adolescence, natural immunity develops slowly, resulting in fewer or even no clinical symptoms, and only low-grade parasitemia in the adult. This kind of protective immunity however, requires repeated exposure to the parasite and wanes rapidly once this exposure ceases. The mechanisms that lead to this protective immunity against *P. falciparum* are under intense investigation, as they might serve as models for the development of an efficient vaccine [2, 3]. The accumulation of a broad repertoire of antibodies against different variant antigens such as the *P. falciparum* erythrocyte membrane protein-1 or the rifin-proteins expressed on the surface of *P. falciparum*-infected erythrocytes is thought to contribute to the build-up of immunity observed in adults living in hyperendemic areas [4-6]. In addition, age-dependent host factors such as the gradual acquisition of immunity to cross-reactive antigenic determinants might influence the immune responsiveness towards malaria, as has been suggested for adult transmigrants from malaria-free to malaria-endemic regions, who were more quickly capable of limiting parasite densities than their children [7, 8].

T cells bearing the αβ chains of antigen receptors are important components in the development of naturally acquired immunity against malaria as a consequence of their ability to provide help to B cells for antibody production and in regulating antibody-independent protection. The $CD8^+$ T cell subset has been implicated predominantly in the inhibition of liver-stage parasites, whereas $CD4^+$ T cells are important both for the induction of such $CD8^+$ T cell responses as well as the elimination of blood-stage malaria parasites [9-12].

$TCR\gamma\delta^+$ T cells have been variously implicated in host defenses against, and the pathophysiology of, malaria [13, 14]. Yet, a marked expansion early during infection with an IFN-γ-biased cytokine profile has been shown in African children [15]. Age-related differences in cytokine production of $TCR\gamma\delta^+$ T cells have, so far, not been reported from malaria-endemic areas.

The tight regulation of early IFN-γ responses has been linked to the control of parasitemia and the absence of clinical symptoms in naturally immune adults, which at the same time presupposes the balanced secretion of the predominantly counter-regulatory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β [16, 17]. Previously, we have shown age-dependent differences in the frequency of cytokine-producing T cells in both healthy individuals and *P. falciparum* malaria patients after mitogenic stimulation of PBMC [18, 19]. It appears however, of importance to correlate the development of naturally acquired immunity to the profile of *P. falciparum*-specific cellular immune responses, as those responses might be a valuable target for vaccination [2]. Therefore, the present study sought to investigate the phenotypes and frequencies of both *P. falciparum*-specific and non-specific cytokine-expressing T cells in a cross-sectional study of healthy children and adults living in a malaria-endemic area in Central Africa.

## PARTICIPANTS AND METHODS

### *Study site and participants*

The study was conducted in the Albert Schweitzer Hospital in Lambaréné, Gabon, where *P. falciparum* malaria is predominantly hyperendemic, with an estimated annual entomological inoculation rate of between 10 and 100 [20, 21]. A cohort of 14 children (5 males, 9 females; median age, 6 years, age range, 4-9 years) and 17 adults (7 males, 10 females; median age, 37 years, age range, 17-67 years) were included into the study. All participants were recruited from the Lambaréné area and were clinically healthy. Physical examination was unremarkable, leucocytes were within the normal range and C-reactive protein (CRP)-levels were not detectable in serum. Thick blood smears were used to assure that participants were parasite-free. No precise data about the frequency of HIV infection within the Lambaréné area are available, the estimated HIV rate is, however, about 5-10%. $CD4^+$ T cell counts were within the normal range in all study participants. Informed consent was obtained from the parents or guardians of participating children. Human experimentation guidelines of the authors' institutions were followed in the course of clinical research. Ethical clearance was given by the ethics committee of the Albert Schweitzer Hospital in Lambaréné.

### *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 25mM Hepes, 2mM 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 [22]. Nucleic acid was extracted with the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

For synchronization and enrichment of *P. falciparum*-infected erythrocytes (PFE), a magnet-activated cell sorter (MACS) system was applied as described recently [23]. 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. In addition, flow cytometry, using staining with ethidium bromide, was employed for the differentiation of uninfected from infected erythrocytes. The MACS-eluate of *P. falciparum* strain S007 was frozen at -80°C, thawed and immediately used for stimulation of the PBMC from all study participants.

### *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 x $10^6$/well. Cells were cultured in Ultra Culture Medium (UCM) (Bio Whittaker, Walkersville, Maryland, USA) supplemented with L-glutamine (2 mM/L; Sigma, St. Louis, Missouri, 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 previously [24]. The co-stimulatory MAb CD28 (Pharmingen, San Diego, CA, USA) was added at 10 μL to some wells (5 μg/mL final concentration) [25, 26]. Uninfected erythrocytes were processed as described above and served as controls. Brefeldin A (1 μM; 10 μg/mL final concentration, Sigma) was added after 6 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 (1 mL per 2x$10^6$ cells, Merck) for 20 minutes. After two additional washes in PBS, cells were then resuspended in Hank's balanced salt solution (HBSS, supplemented with 0.3% bovine serum albumin [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 minutes 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]-labelled) and rat anti-human MAb (IL-2 [MQ1-17H12], phycoerythrin [PE]-conjugated; IL-10 [JES3-9D7], PE-labelled; TNF-α [MAb11], PE-labelled). All MAb were purchased from Pharmingen. The anti-CD3-MAb was peridinin chlorophyll (PerCP), the anti-TCRγδ Mab and the anti-CD69-MAb were allophycocyanin (APC)-labelled (Becton Dickinson, Mountain View, CA, USA). Four-colour staining was performed, and at least $10^5$ cells were analyzed on a FACSCalibur (Becton

**Table 1**
Frequency *of P. falciparum*-specific $CD3^+$ cells expressing cytokine in malaria-exposed healthy children and adults[a]

| Cytokines | Children (n = 14) | Adults (n = 17) |
|---|---|---|
| IL-$2^-$/IFN-$\gamma^+$ | 0.18 ± 0.05 (0.02-0.78) | 0.18 ± 0.03 (0.04-0.51) |
| IL-$2^+$/IFN-$\gamma^-$ | 0.05 ± 0.01 (0.01-0.12) | 0.07 ± 0.01 (0.00-0.26) |
| IL-$2^+$/IFN-$\gamma^+$ | 0.04 ± 0.01 (0.01-0.11)[b] | 0.11 ± 0.02 (0.03-0.25) |
| TNF-$\alpha^+$/IFN-$\gamma^-$ | 0.23 ± 0.08 (0.04-1.25)[b] | 0.31 ± 0.04 (0.07-0.64) |
| TNF-$\alpha^+$/IFN-$\gamma^+$ | 0.19 ± 0.06 (0.03-0.85)[b] | 0.27 ± 0.04 (0.05-0.55) |
| IL-$10^+$/IFN-$\gamma^-$ | 0.03 ± 0.01 (0.01-0.08) | 0.05 ± 0.01 (0.00-0.12) |
| IL-$10^+$/IFN-$\gamma^+$ | 0.04 ± 0.01 (0.01-0.15) | 0.07 ± 0.01 (0.00-0.17) |

[a] Values indicate mean percentages of $CD3^+$ cells expressing cytokines ± SEM; respective ranges are given in parentheses.
[b] Significant different between groups, $p < 0.05$ as calculated by the Mann-Whitney U-test.

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, for the identification of *P. falciparum*-specific cytokine-expressing TCRγδ$^+$ T cells, the TNF-α/IFN-γ combination was stained in conjunction with CD3 and TCRγδ.
T cells were defined by their side-scatter characteristics and anti-CD3 MAb staining, the γδ T cell subset was additionally defined by anti-TCRγδ 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. The specificity of cytokine staining was confirmed by the absence of significant background in controls with isotype-matched, irrelevant MAbs. Data were analysed with CELLQuest software (Becton Dickinson), and results were expressed as the percentage of cytokine-producing cells in each $CD3^+$ cell (or TCRγδ$^+$CD3$^+$) population.

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

Flow cytometric assessment of intracellular cytokine expression was performed essentially according to the technique described previously [19, 27]. 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 (IFN-γ [clone B27], fluorescein isothiocyanate [FITC]-conjugated); rat anti-human MAb (IL-2 [MQ1-17H12], IL-4 [MP4-25D2], IL-10 [JES3-9D7], IL-13 [JES10-5A2], TNF-α [Mab11], all phycoerythrin [PE]-conjugated), the anti-CD4 MAb and the anti-TCRγδ MAb were allophycocyanin-conjugated, the 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). All cytokine combinations were stained in conjunction with CD4 and CD8, as well as CD3 and TCRγδ. Data were analysed with CELLQuest software (Becton Dickinson). 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 $CD4^+$, $CD8^+$ or TCRγδ$^+$CD3$^+$cell population, respectively.

### *Statistical methods*

Statistical analysis was performed using a standard statistical package (SPSS 11.5 for Windows; SPSS Inc., Chicago, USA). The Mann-Whitney U-test was applied for group differences (children *versus* adults). Bivariate correlations were done by computing a Spearman's correlation coefficient. A p value of $< 0.05$ was considered significant.

## RESULTS

### *Frequency of P. falciparum-specific CD3$^+$ cells expressing cytokines: differences between healthy children and adults*

Adults showed an overall increased frequency of activated ($CD69^+$) $CD3^+$ cells expressing cytokines after stimulation with MACS-separated, late stage parasites of *P. falciparum* when compared with children. This was significant for the type 1 cytokine IFN-γ and the pro-inflammatory cytokine TNF-α, but not for IL-2 and IL-10 (*table 1* and *figures 1, 2*). Frequencies of background events within the $CD3^+$ cell population (unstimulated cells; addition of MACS-processed, uninfected erythrocytes) were always < 0.04%.

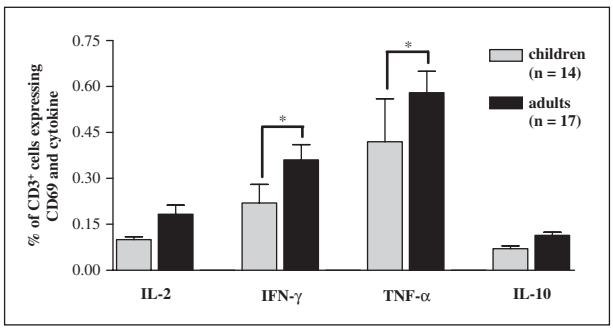


**Figure 1**
Frequency of cytokine-expressing, activated ($CD69^+$) $CD3^+$ cells obtained after stimulation with a MACS-processed, late stage parasite preparation of *P. falciparum* in healthy children compared to healthy adults from a malaria-hyperendemic area in Central Africa. Each bar indicates 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).

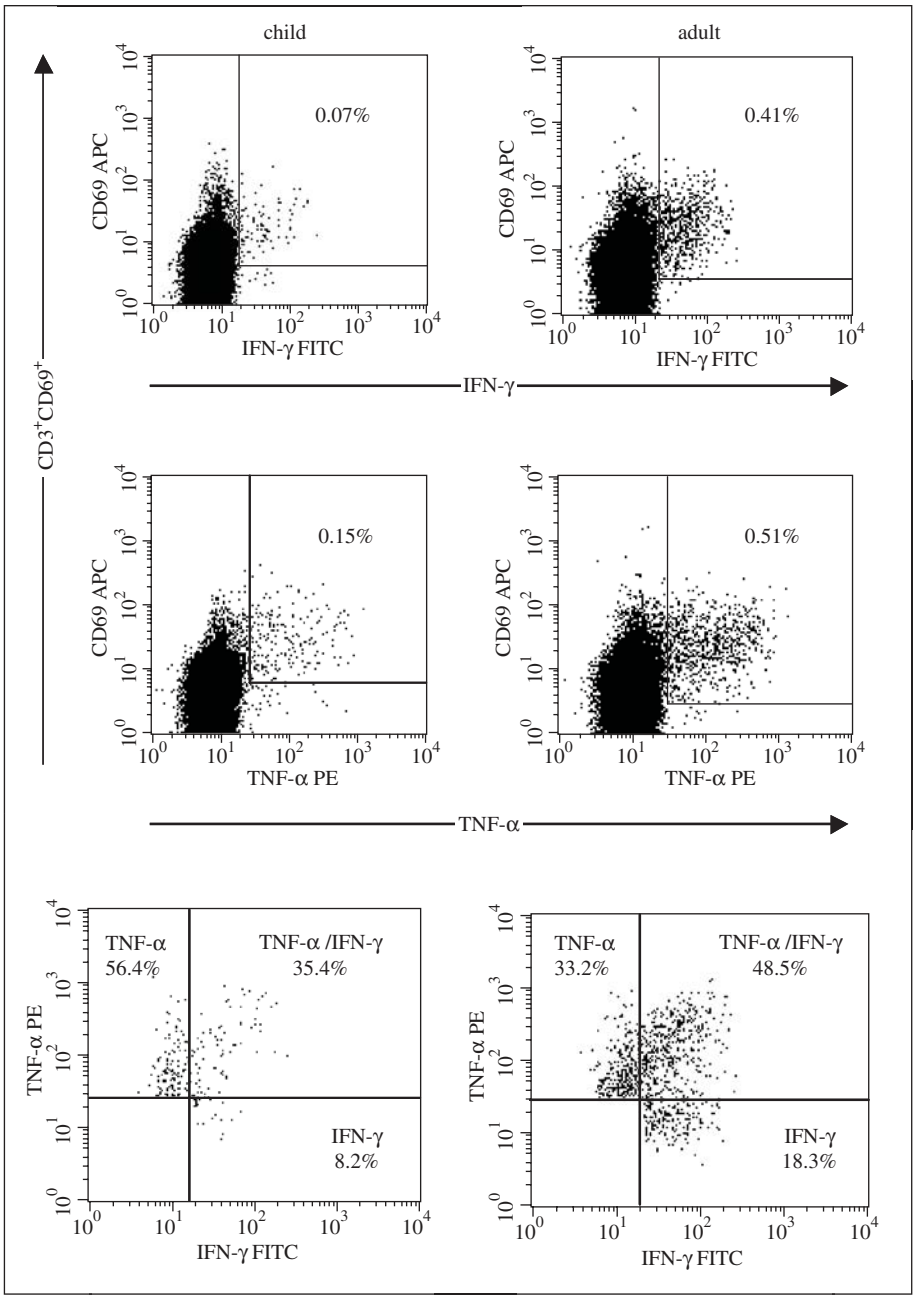


**Figure 2**

Two-parameter dot plots indicating the frequency of IFN-γ-and TNF-α-expressing *P. falciparum*-specific, activated ($CD69^+$) $CD3^+$ T cells of one representative child and one adult from a malaria hyperendemic area in Central Africa.

Note the marked age-related differences in the expression of IFN-γ (first horizontal column) and TNF-α (second horizontal column). The third horizontal column displays the cytokine distribution pattern of IFN-γ together with 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*.

### *Frequency of P. falciparum-specific TCRγδ⁺CD3⁺ cells expressing TNF-α and IFN-γ: differences between healthy children and adults*

The frequency of $TCR\gamma\delta^+CD3^+$ cells expressing TNF-α was significantly increased in adults when compared with children (mean, 0.21%; range, 0-0.75% *versus* mean, 0.09%; range, 0-0.48%; $p < 0.05$). However, only 12 adults (71%) and 5 children (36%) displayed frequencies of > 0.1% TNF-α-expressing $TCR\gamma\delta^+CD3^+$ cells in response to PFE stimulation. In addition, a frequency of > 0.1% IFN-γ-expressing $TCR\gamma\delta^+CD3^+$ cells responsive to specific stimulation was seen in only 5 adults and 4 children (29%, respectively). Mean values for the frequencies of IFN-γ-expressing cells were 0.08% for both adults and children. In the presence of medium alone or when stimulated with uninfected erythrocytes, frequencies of cytokine-responsive $TCR\gamma\delta^+CD3^+$ cells were always below 0.02%.

### *Frequency of P. falciparum-non-specific CD4$^+$ and CD8$^+$ cells expressing cytokines: differences between healthy children and adults*

The frequencies of cytokine-expressing CD4$^+$ and CD8$^+$ cells after mitogenic stimulation with PMA and ionomycin in the presence of brefeldin A, were significantly different between adults and children with regard to the type 1 cytokines IFN-γ and IL-2, as well as for the pro-inflammatory cytokine TNF-α (*table 2* and *figures 3, 4*). IL-4-expressing CD8$^+$ cells were more frequently observed in adults, most of them however, were positive for IFN-γ *(table 2)*.

### *Frequency of P. falciparum-non-specific TCRγδ$^+$CD3$^+$ cells expressing cytokines and the relationship of T cell-cytokine responses after P. falciparum-specific and non-specific stimulation*

As shown for the CD4$^+$ and CD8$^+$ T cellular subsets, a significant, age-related increase in the frequency of TCRγδ$^+$CD3$^+$ cells expressing cytokine after short-term stimulation with PMA and ionomycin in the presence of brefeldin A was noted (*table 3* and *figure 3*). Again, the differences were obvious for IFN-γ and TNF-α, but were also significant for the classical, type 2 cytokines IL-4 and IL-13. As seen for CD8$^+$ cells, most type 2 cytokine-expressing cells also stained positively for IFN-γ (IFN-γ/IL-4 or IFN-γ/IL-13 co-producers). Low percentages of TCRγδ$^+$CD3$^+$ cells expressed IL-10 (*table 3* and *figure 3*). Within both groups of study participants, the frequency of cytokine-expressing CD4$^+$ CD8$^+$ and TCRγδ$^+$CD3$^+$ T cells, after non-specific, mitogenic stimulation, did not correlate with the frequency of specifically activated, cytokine-expressing CD3$^+$ and TCRγδ$^+$CD3$^+$ T cells.

## DISCUSSION

This study clearly illustrates the significance of ageing in the development of *P. falciparum*-specific and non-specific cellular immune responses in individuals from a malaria-hyperendemic area. The increased capacity of various T cell subsets to produce cytokines in adults was especially seen after mitogenic stimulation, but also when PBMC were incubated with a late-stage, schizont-rich parasite preparation. According to their intrinsic capabilities, CD3$^+$, CD4$^+$ and CD8$^+$ T cells as well as the TCRγδ$^+$ subset contributed to the impressive, age-dependent differences with an emphasis on the type 1 cytokine IFN-γ and on pro-inflammatory TNF-α. Although, a trend towards higher expression of IL-2 in adult T cells was also noted after specific stimulation, differences between children and adults were highly significant only in CD4$^+$ and CD8$^+$ cells (not in TCRγδ$^+$ T cells) after non-specific stimulation.

The role of IFN-γ and TNF-α as determinants in age-associated cellular responses is especially notable, as the capacity to produce the counteracting cytokine IL-10 appears to be independent of age. These findings accord well with another study from the Lambaréné area using the *P. falciparum,* liver-stage antigen (LSA)-1-derived T cell epitope stimulation for the assessment of cellular immune responses in healthy children and adults: an age-related increase in the proportion of individuals capable of producing IFN-γ, while the proportion of children and adults producing IL-10 remained similar, was noted [28]. Once more, in children from a malaria holoendemic area in Kenya, IFN-γ responses to LSA-1 and the blood-stage antigen merozoite-surface protein (MSP)-1, required increased age and/or repeated exposure, and IL-10 responses were again independent of age [29]. Such an age-dependent decline in the influence of IL-10 was seen, even in *P. berghei*-infected rats, when higher levels of IFN-γ-dependent IgG2c antibodies and lower IL-10 serum levels were associated with resistance of adult animals to a primary infection, whereas young rats with high IL-10 levels succumbed to disease [30].

Thus, there are several indications that naturally immune residents from hyper-or holo-endemic areas generally possess an increased capacity to mount an effective, antiparasitic, probably type 1- and TNF-α-driven, host response when compared to more disease-susceptible children, while the contribution of IL-10 is maintained at a similar level throughout life and probably more closely associated with clinical disease. Indeed, high initial levels of IL-10

**Table 2**
Frequency of CD4$^+$ and CD8$^+$ T cells expressing cytokine in malaria-exposed healthy children and adults from the Lambaréné area after non-specific stimulation with PMA and ionomycin in the presence of brefeldin A[a]

| Cytokines | % of CD4$^+$ | | % of CD8$^+$ | |
|---|---|---|---|---|
| | Children (n = 14) | Adults (n = 17) | Children (n = 14) | Adults (n = 17) |
| IL-2$^-$/IFN-γ$^+$ | 5.4 ± 0.5 (2.4-9.2) | 5.8 ± 0.4 (2.8-9.8) | 25.4 ± 2.5 (11.9-42.0) | 34.9 ± 3.5 (8.7-63.9) |
| IL-2$^+$/IFN-γ$^+$ | 5.8 ± 0.4 (2.8-9.8)[b] | 19.4 ± 1.5 (10.9-37.6) | 5.3 ± 0.6 (3.1-10.3)[b] | 12.2 ± 1.2 (4.7-20.4) |
| IL-2$^+$/IFN-γ$^-$ | 29.7 ± 1.6 (21.2-43.1)[b] | 44.1 ± 1.6 (32.8-57.9) | 3.3 ± 0.4 (1.1-6.7)[d] | 7.0 ± 1.3 (1.4-24.1) |
| IL-4$^+$/IFN-γ$^-$ | 4.5 ± 0.5 (1.6-9.0) | 5.2 ± 0.4 (2.0-8.4) | < 1 | < 1 |
| IL-4$^+$/IFN-γ$^+$ | 2.1 ± 0.2 (0.9-3.4) | 3.3 ± 0.4 (0.4-6.6) | < 1[c] | 1.7 ± 0.3 (0-4.4) |
| IL-10$^+$/IFN-γ$^-$ | < 1 | < 1 | < 1 | < 1 |
| IL-10$^+$/IFN-γ$^+$ | 1.1 ± 0.2 (0.1-3.5) | 1.1 ± 0.3 (0.3-6.2) | < 1 | < 1 |
| IL-13$^+$/IFN-γ$^-$ | 4.1 ± 0.5 (1.7-8.0) | 4.3 ± 0.4 (1.6-7.2) | < 1 | < 1 |
| IL-13$^+$/IFN-γ$^+$ | < 1 | < 1 | < 1 | < 1 |
| TNF-α$^+$/IFN-γ$^-$ | 26.2 ± 1.8 (16.6-36.4)[c] | 32.7 ± 149 (19.5-41.9) | 1.8 ± 0.3 (0.3-5.4) | 2.5 ± 0.3 (0.5-5.4) |
| TNF-α$^+$/IFN-γ$^+$ | 12.0 ± 1.2 (4.3-23.7)[b] | 21.7 ± 1.9 (10.5-45.8) | 21.1 ± 3.0 (6.8-47.5) | 30.4 ± 3.1 (9.9-56.3) |

[a] Values indicate mean percentages of cytokine expressing CD4$^+$ and CD8$^+$ ± SEM and ranges (in parentheses).
[b] significant different between children and adults, $p < 0.001$ as calculated by the Mann-Whitney U-test.
[c] significant different between children and adults, $p < 0.05$ as calculated by the Mann-Whitney U-test.
[d] significant different between children and adults, $p < 0.01$ as calculated by the Mann-Whitney U-test.

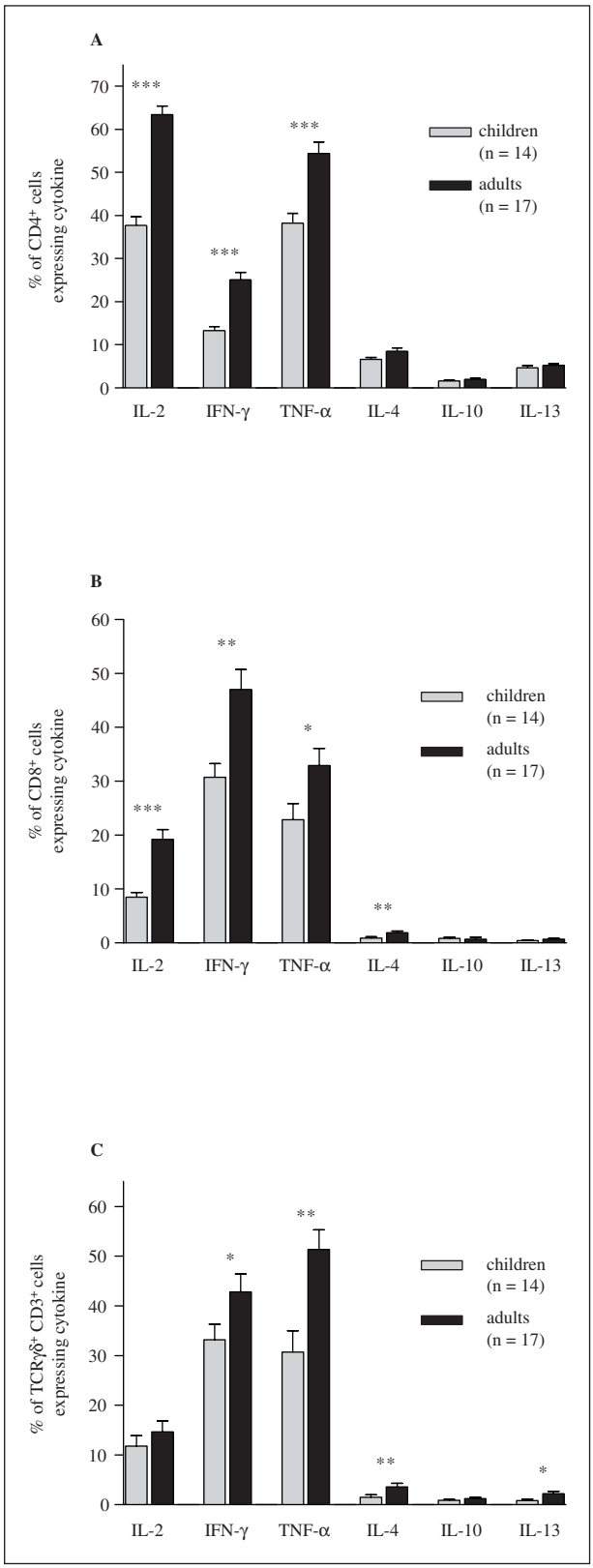


**Figure 3**

Frequency of (**A**) $CD4^+$, (**B**) $CD8^+$, and (**C**) $TCR\gamma\delta^+CD3^+$ T cells expressing cytokine, following non-specific stimulation with PMA and ionomycin in the presence of brefeldin A, in healthy children compared to healthy adults from a malaria-hyperendemic area in Central Africa.

Each bar indicates the mean percentages ± SEM. Values that are statistically different between groups are denoted by asterisks (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, as calculated by the Mann-Whitney U-test).

were strongly associated with less effective clearance of *P. falciparum* parasites in African children [31], which was attributed to its down-regulating activity on antigen-presenting cells or the induction of suppressive, regulatory T cells.

The importance of early type 1 cytokine production during *Plasmodium* infection has been linked to successful control of parasitemia in many animal models of malaria [30, 32-37], and "appropriate" IFN-γ as well as TNF-α production appears to be protective and necessary for limiting parasitemia in human malaria too [38-42]. The increased capacity of IFN-γ and TNF-α production in naturally immune individuals might be crucial in the rapid mounting of antiparasitic effector mechanisms such as the synthesis of nitric oxide and reactive oxygen intermediates, thus allowing a more efficient control of parasitemia and subsequently the prevention of clinical disease.

A substantial proportion of the early, innate cytokine responses during malaria has been ascribed to $TCR\gamma\delta^+$ T cells. Here, this particular subset participated in the generally observed, age-dependent increased capacity for IFN-γ and TNF-α production, but also showed increased frequencies of IL-4 and IL-13 expression in adults after mitogenic stimulation. However, most type 2 cytokine-expressing $TCR\gamma\delta^+$ T cells also co-produced IFN-γ, so these cells can not be referred to as classical type 2 cells. The overall, low specific responsiveness of $TCR\gamma\delta^+$ T cells in this study might be due to the use of freeze-thawed rather than live parasites in the field experiments, which has been shown to make an important difference in the cellular response to parasite-preparations [24, 43]. Nevertheless, TNF-α expression was again more frequently observed in $TCR\gamma\delta^+$ T cells of adults when compared with children. $CD4^+$ T cell-derived IL-2 has been shown to play a role in the activation of human $TCR\gamma\delta^+$ T cells when stimulated with freeze-thawed schizont extracts [44], which would offer one possible explanation for the somewhat more frequent cytokine responses to parasitic stimulation in the adult group. However, whether $TCR\gamma\delta^+$ T cells play a role in cell-mediated immunity as has been postulated in rodent models, or rather exert immune-regulatory activities during human *P. falciparum* malaria has to be further elucidated.

Although a wealth of data confirm the importance of T cell-derived cytokines in the pathophysiology and host defenses against *Plasmodium* spp., the significance of cell-mediated immunity to the overall development of naturally acquired immunity in individuals from endemic areas is currently unknown, as the latter appears to develop largely due to the acquisition of a broad repertoire of antibodies to variant surface antigens [4-6]. Therefore, our data may be best seen as complementary to those many studies trying to link humoral immune responses to the degree of naturally acquired immunity, although more recently protection from natural *P. falciparum* infection and disease was also correlated with a strong $CD4^+$ T cell response directed to a conserved epitope in the circumsporozoite protein [45]. We are well aware that the increased frequency of *P. falciparum*-specific cytokine responses in adults may just reflect the more frequent contact and increased cellular reactivity to antigens expressed by both malaria parasites and commensal organisms. It has been speculated, however, that even such periodic exposure to cross-reacting antigens can maintain some degree of immunity [46]. The

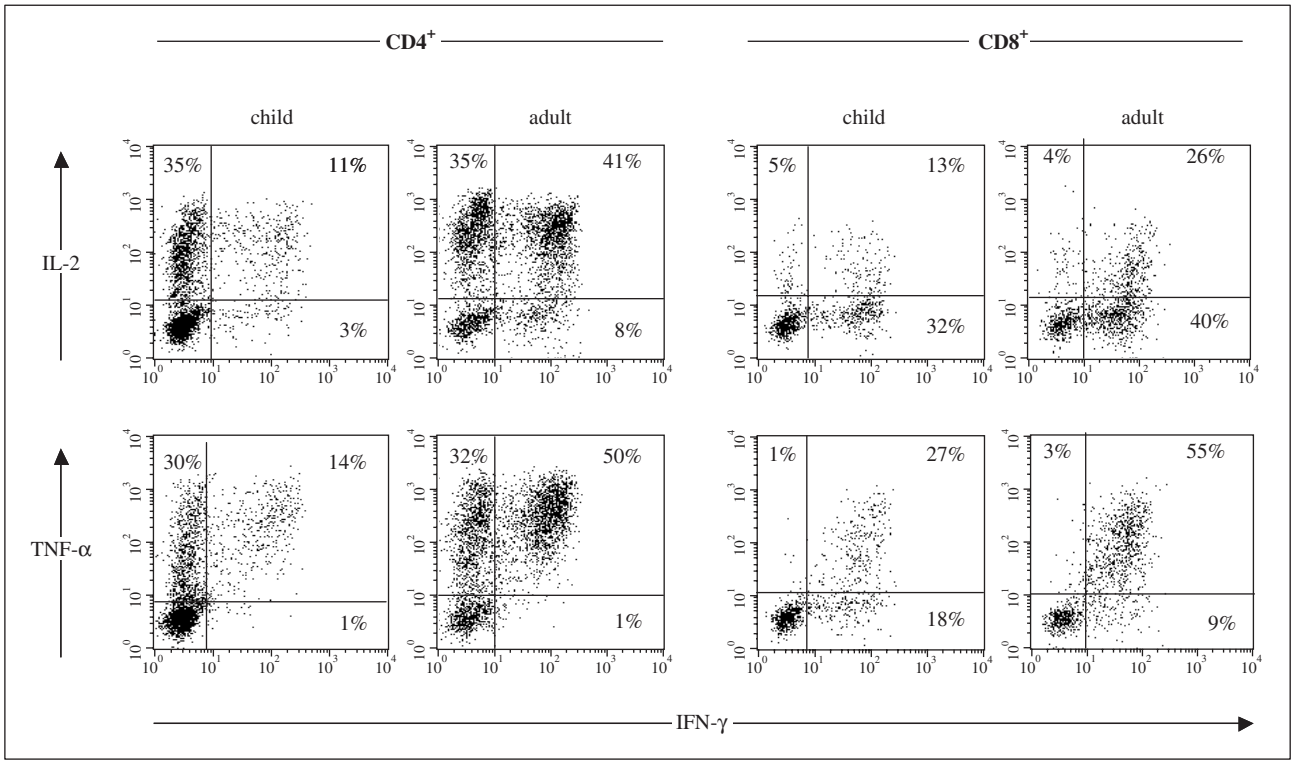


**Figure 4**

Representative, two-parameter dot plots indicating the profile of IL-2-, IFN-γ- and TNF-α- expressing $CD4^+$ and $CD8^+$ T cells from one representative child and one adult following non-specific stimulation with PMA and ionomycin in the presence of brefeldin A.
Similar characterizations were performed for the cytokines IL-4, 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 *table 2* and *figure 3*.

induction of immunity appears unlikely as it has been known for many years that malaria-specific T cells can be detected, even in non-exposed and obviously non-immune individuals [26, 47].

As lifetime cumulative exposure to *P. falciparum* evolves co-linearly with ageing in malaria hyperendemic areas, it is impossible to disentangle the effects of ageing from the consequences of exposure. Age-related changes in antimalarial immune responses, independent of prior exposure, have been reported in migrant populations from Indonesia and from newly established endemic areas in Africa [8, 48]. There, adults acquired clinical immunity after a rather brief period of heavy exposure, whereas children remained susceptible to disease manifestations, suggesting constitutional and obviously malaria-relevant differences between the immune system of children and adults. In line with this, pubertal development associated with increased dehydroepiandrosterone sulphate levels were found necessary for maximal expression of antimalarial resistance in both females and males from Kenya [49, 50]. Unfortunately, our data are not suitable for resolving the fundamental question, as to whether ageing itself, cumulative exposure with

**Table 3**
Frequency of $TCR\gamma\delta^+$ $CD3^+$ T cells expressing cytokine after non-specific stimulation with PMA and ionomycin in the presence of brefeldin A[a]

| Cytokines | % of $TCR\gamma\delta^+$ $CD3^+$ | |
|---|---|---|
| | Children (n = 14) | Adults (n = 17) |
| $IL\text{-}2^-/IFN\text{-}\gamma^+$ | 27.2 ± 3.4 (10.9-47.3)[b] | 35.7 ± 3.8 (7.9-76.8) |
| $IL\text{-}2^+/IFN\text{-}\gamma^+$ | 3.7 ± 0.4 (0.6-7.5) | 6.5 ± 0.9 (0.8-14.6) |
| $IL\text{-}2^+/IFN\text{-}\gamma^-$ | 8.1 ± 1.8 (1.8-24.1) | 8.1 ± 1.5 (0-27.9) |
| $IL\text{-}4^+/IFN\text{-}\gamma^-$ | < 1 | 1.4 ± 0.3 (0.1-5.8) |
| $IL\text{-}4^+/IFN\text{-}\gamma^+$ | < 1[c] | 2.2 ± 0.4 (0-7.2) |
| $IL\text{-}10^+/IFN\text{-}\gamma^-$ | < 1 | < 1 |
| $IL\text{-}10^+/IFN\text{-}\gamma^+$ | < 1 | < 1 |
| $IL\text{-}13^+/IFN\text{-}\gamma^-$ | < 1 | 1.4 ± 0.4 (0-5.7) |
| $IL\text{-}13^+/IFN\text{-}\gamma^+$ | < 1 | < 1 |
| $TNF\text{-}\alpha^+/IFN\text{-}\gamma^-$ | 7.5 ± 2.0 (0.6-25.9)[c] | 15.4 ± 2.1 (2.9-38.5) |
| $TNF\text{-}\alpha^+/IFN\text{-}\gamma^+$ | 23.2 ± 2.8 (6.9-47.9)[b] | 35.9 ± 3.2 (7.7-62.6) |

[a] values indicate mean percentages of cytokine expressing $TCR\gamma\delta^+$ $CD3^+$ T cells ± SEM and ranges (in parentheses).
[b] significant different between children and adults, $p < 0.05$ as calculated by the Mann-Whitney U test.
[c] significant different between children and adults, $p < 0.01$ as calculated by the Mann-Whitney U-test.

acquisition of antibodies/memory T cells, or both, are implicated in the development of naturally acquired immunity to *P. falciparum*. Likewise, it remains speculative, whether our *in vitro* findings represent reliable correlates of cell-mediated, protective immunity.

Nevertheless, we propose that immune interventions including vaccines should consider the profound, age-related differences in the capacity of T cell-cytokine production in areas where *P. falciparum* malaria is hyperendemic. Our findings would argue for a vaccine that efficiently induces "adult-like", strong T cell-derived IFN-γ and TNF-α (IL-2?) responses in order to be protective in the recipient. Even if this response might not be sufficiently efficient to prevent infection and the development of blood-stages, it might reduce parasite densities and thus complications of malaria.

***Acknowledgements***. *The study was, in part supported by a grant from the Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Stadt Wien (project 1931), from fortüne program, Medical Faculty, University of Tübingen, Germany and from EU-INCODEV.*

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