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Perturbation and Proinflammatory Type Activation of Vδ1$^+$ γδ T Cells in African Children with *Plasmodium falciparum* Malaria

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γδ T cells have variously been implicated in the protection against, and the pathogenesis of, malaria, but few studies have examined the γδ T-cell response to malaria in African children, who suffer the large majority of malaria-associated morbidity and mortality. This is unfortunate, since available data suggest that simple extrapolation of conclusions drawn from studies of nonimmune adults ex vivo and in vitro is not always possible. Here we show that both the frequencies and the absolute numbers of γδ T cells are transiently increased following treatment of *Plasmodium falciparum* malaria in Ghanaian children and they can constitute 30 to 50% of all T cells shortly after initiation of antimalarial chemotherapy. The bulk of the γδ T cells involved in this perturbation expressed Vδ1 and had a highly activated phenotype. Analysis of the T-cell receptors (TCR) of the Vδ1$^+$ cell population at the peak of their increase showed that all expressed Vγ chains were used, and CDR3 length polymorphism indicated that the expanded Vδ1 population was highly polyclonal. A very high proportion of the Vδ1$^+$ T cells produced gamma interferon, while fewer Vδ1$^+$ cells than the average proportion of all CD3$^+$ cells produced tumor necrosis factor alpha. No interleukin 10 production was detected among TCR-γδ$^+$ cells in general or Vδ1$^+$ cells in particular. Taken together, our data point to an immunoregulatory role of the expanded Vδ1$^+$ T-cell population in this group of semi-immune *P. falciparum* malaria patients.

Human T cells express antigen receptors associated in a molecular complex to CD3. In the majority of T cells, these heterodimer antigen receptors are composed by disulfide-linked α and β chains (39), while a minority instead employ receptors composed of γ and δ chains (8). The latter population, which in healthy Caucasians normally constitutes less than 5% of peripheral T cells, can be subdivided into two largely nonoverlapping subsets (32, 37). The former of these subsets usually comprises more than two-thirds of all the γδ T cells and is characterized by disulfide-linked Vγ9 and Vδ2 chains, whereas the other, smaller subset uses a *Vδ1* gene product non-disulfide linked to products of *Vγ* genes other than *Vγ9* (12, 47, 48). In contrast, among healthy individuals in Africa, particularly children, the average frequency of TCR-γδ$^+$ cells may be as high as 10% or more and dominated by Vδ1$^+$ cells rather than Vγ9$^+$ cells (25).

Episodes of clinical *Plasmodium falciparum* malaria in adults with little or no previous malaria exposure have been reported to induce increased levels of γδ T cells, often persisting for several weeks (23, 44, 45). The Vγ9 subset of γδ T cells was found to dominate the in vivo γδ T-cell response in those studies, and several studies of nonexposed donors have shown preferential outgrowth of Vγ9$^+$ cells following malaria antigen stimulation in vitro (3, 15). In contrast, studies from areas where malaria is endemic have failed to confirm both the Vγ9$^+$ cell dominance of the TCR-γδ$^+$ response to malaria (16, 49) and persistent in vivo increases in the frequency of TCR-γδ$^+$ cells (26). Preliminary data obtained during the latter study rather suggested the presence of very transient but pronounced γδ T-cell perturbations immediately following the patient's admission to a hospital.

Based on the above observations, the present study was undertaken as a detailed examination of the γδ T-cell response to *P. falciparum* malaria in an area where malaria is endemic.

MATERIALS AND METHODS

Donors. Children (3 to 10 years old) admitted to the Department of Child Health at Korle-Bu Teaching Hospital with *P. falciparum* malaria were studied during the peak malaria season, June to August. The general inclusion criteria were asexual *P. falciparum* parasitemia (>10,000/ml), axillary temperatures that were >37.5°C, and negative sickling (HbS) test (metabisulfite method). In addition, only children with strictly defined cerebral malaria (CM) or uncomplicated malaria (UM) are included in the present report. The specific inclusion criteria for these categories have been described in detail previously (34). Children with severe malarial anemia were specifically excluded, since our previous studies have shown that transfusion affects both frequencies and absolute numbers of T cells in the peripheral blood (27). Clinically healthy and age-matched children from a nearby community (Dodowa, Ghana) were included as control donors.

The study was approved by the Ethical and Protocol Review Committee, University of Ghana Medical School, and by the Minister of Health, Ghana. Children were enrolled in the study only after signed, informed consent had been obtained from parents or guardians.

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Blood samples. Samples (250 to 400 μl) of EDTA-anticoagulated blood were obtained from the patients at admission (day 0) and subsequently on days 1, 2, 4, and 21 or once only (4 to 5 ml; day 2) for the analysis of cytokine production and of Vγ chain usage and CDR3 length polymorphism of Vδ1$^+$ T cells. A single 4- to 5-ml sample was obtained from the healthy control children. Thick and thin Giemsa-stained blood smears were prepared from each sample, and hematological analysis was done using an automatic hematology analyzer (Beckman Coulter, Fullerton, Calif.) before further processing. Analysis of CDR3 length polymorphism was done using peripheral blood mononuclear cells (PBMC) isolated by density centrifugation and cryopreserved in liquid nitrogen as described previously (25).

Monoclonal antibodies. Monoclonal antibodies (MAb) directed against the following determinants were used in this study: CD3 (UCHT1; DAKO, Glostrup, Denmark), CD4 (MT310; DAKO), CD8 (DK25; DAKO), CD69 (Leu-23; BD PharMingen, San Diego, Calif.), CD45RA (4KB5; DAKO), CD45R0 (UCHL1; DAKO), TcR-γδ (11F2; BD PharMingen), Vγ2,3,4 (23D12; Beckman Coulter), Vγ3,5 (56.3; a kind gift from Dieter Kabelitz, Hamburg, Germany), Vγ4 (4A11; BIOAdvance, Emerainville, France), Vγ8 (R4.5; Beckman Coulter), Vγ9 (7A5; Endogen, Woburn, Mass.), Vδ1-Jδ1 (δTCS1; Endogen), and HLA-DR (G46-6; BD PharMingen). Appropriate isotype control antibodies were always included. Most MAb were used as direct conjugates to fluorescein isothiocyanate (FITC), phycoerythrin, or Cy5. In the remaining cases, biotinylated or unconjugated primary antibody was used and labeled with FITC-streptavidin (DAKO), FITC-F(ab′)$_2$ (DAKO), or RPE-Cy5-F(ab′)$_2$ (DAKO) as second-step reagents.

Cell phenotyping. The plasma was removed following centrifugation, and the cell pellet was resuspended to its original volume in phosphate-buffered saline (PBS). Fifty-microliter aliquots were subsequently labeled (20 min; room temperature) with directly conjugated MAb or appropriate nonspecific isotype control antibodies, followed by lysis of erythrocytes (fluorescence-activated cell sorter lysing solution; BD PharMingen). Samples were then washed twice in PBS and analyzed on a FACScan flow cytometer (BD PharMingen). All samples were live gated by forward and side scatter on lymphocytes, and 5,000 to 10,000 events were collected.

Intracellular cytokine detection. One-milliliter aliquots of whole blood were incubated with monensin (1.5 μM; Sigma, St. Louis, Mo.), ionomycin (1 μM) and phorbol myristate acetate (50 μg/ml) for 90 min. Following surface staining (CD3, CD8, TCR-γδ) and erythrocyte lysis, the cells were washed twice in a freshly made saponin buffer (PBS-bovine serum albumin-NaN_3 containing 0.1% [wt/vol] saponin [Sigma]) and finally incubated with anticytokine (gamma interferon [IFN-γ] tumor necrosis factor alpha [TNF-α], or interleukin 10 [IL-10]; BD PharMingen) antibody for 30 min in the dark (4°C). Following cytokine labeling, the cells were washed twice in saponin buffer and twice in staining buffer, resuspended in the same buffer, and analyzed by flow cytometry as described above.

CDR3 size polymorphism analysis. Total mRNA was prepared from 1×10^6 to 5×10^6 RNAzol-preserved PBMC according to the manufacturer's instructions (Bioprobe Systems, Montreuil, France). Single-stranded cDNA was subsequently synthesized using the superscript inverse transcriptase (Gibco-BRL, Gaithersburg, Md.). The analysis of the CDR3 size distribution of the Vδ1-Cδ rearrangements was done according to the protocol already described (7). The Vδ1- and Cδ-specific primers used, oriented 5′ to 3′, were Vδ1 (CTGTCAACT TCAAGAAAGCAGCGAAATC) and Cδ-fam (ACGGATGGTTTGGTAGAG GCTGA). To compare the Vδ1 CDR3 profiles obtained from patient and control PBMC, we used a clonality index derived from the Nei diversity index (33). This index measures the probability that two randomized Vδ1 T cells have the same CDR3 length. For each sample, the frequency of TVδ1 cells with a given CDR3 length is expressed as x_i, corresponding to the fluorescence intensity of peak number i divided by the sum of the fluorescence intensities of all peaks (n). The index of clonality (I) of the TVδ1 cells is then calculated as $I = \Sigma(x_i)^2$.

Data presentation and statistical analysis. Summary statistics are given as means and standard errors of the means (SEM). Comparison of patient groups at individual time points was done by two-factor analysis of variance (F) supplemented with the Student-Newman-Keuls test. Indices of clonality were compared by the Mann-Whitney test (T).

RESULTS

Initiation of antimalarial therapy causes a rapid, but transient, increase in frequencies and absolute numbers of γδ T cells in the peripheral circulation. In healthy Caucasians, less than 5% of circulating CD3$^+$ cells express T-cell receptor (TCR)-γδ (17, 36), but the average frequency of TCR-γδ$^+$ cells may be as high as 10% in some African populations (25). It is well established that γδ T cells from nonexposed donors respond to stimulation by malaria antigen preparations in vitro (3, 14, 15), but few studies from areas where malaria is highly endemic have examined malaria-induced changes in γδ T cells in vivo (26). In the present study, about 15% of circulating CD3$^+$ cells from *P. falciparum* malaria patients carried TCR-γδ at the time of the patient's admission to a hospital prior to initiation of chemotherapy (day 0) (Fig. 1, upper panels, and Fig. 2, left panels). This value is only slightly higher than the steady-state levels found in healthy, age-matched children in the same area (25, 26). However, within the next few days, the proportion of γδ T cells rapidly rose, peaking in most cases around day 2 (P [F] < 0.001), before returning to steady state within 1 week following admission. The increase in the frequency of TCR-γδ$^+$ cells was more pronounced with children with CM (Fig. 1, upper left panel, and Fig. 2) than with children with UM (Fig. 1, upper right panel), but this difference was not significant (P [F] = 0.15). In a few CM patients, more than 50% of the CD3$^+$ cells present in the peripheral blood on day 2 expressed TCR-γδ. Examination of absolute peripheral blood cell numbers showed that the γδ T-cell perturbation persisted longer in CM patients than in UM patients (Fig. 1, lower panels).

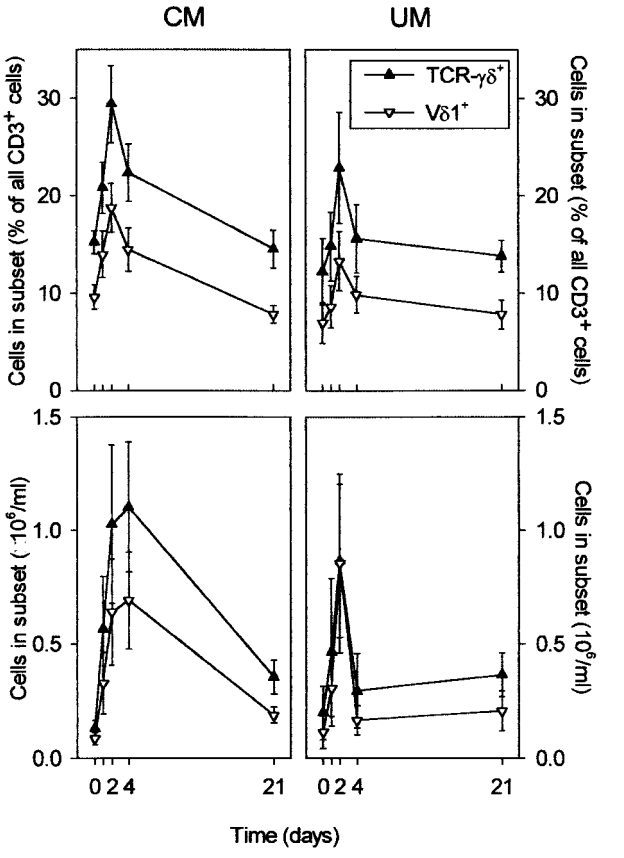

FIG. 1. Proportions (upper panels) and absolute numbers (lower panels) of CD3$^+$ T cells expressing TcR-γδ (▲) or Vδ1 (▽) at various time points after initiation of chemotherapy in 14 patients with CM (left panels) and in 7 patients with UM (right panels). Data are given as means and SEM.

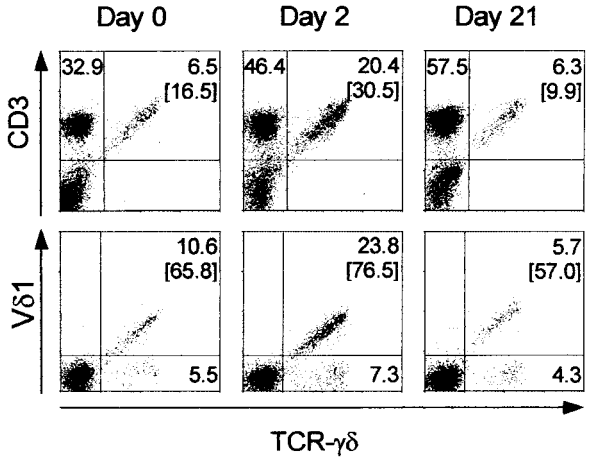

FIG. 2. Proportions of lymphocytes (FSC/SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] gated) expressing CD3 and TcR-γδ (upper panels) and of $CD3^+$ cells expressing Vδ1 and TCR-γδ (lower panels) in a CM patient. Samples shown were obtained on the day of admission (left panels) and 2 (middle panels) and 21 days (right panels) after initiation of chemotherapy. Numbers indicate percentages of cells in quadrants.

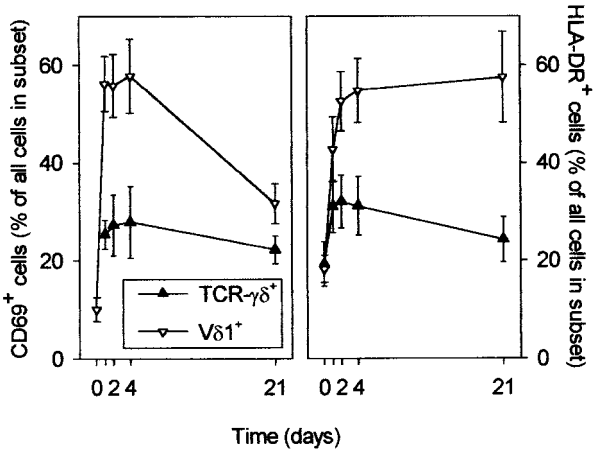

FIG. 3. Proportions of TCR-$\gamma\delta^+$ (▲) and $V\delta1^+$ (▽) T cells expressing the early activation marker CD69 (left panel) and the late activation marker HLA-DR (right panel) at various time points after initiation of chemotherapy in eight *P. falciparum* malaria patients. Data are given as means and SEM.

The acquired γδ T-cell response is dominated by Vδ1 cells, with negligible involvement of other γδ T-cell subsets. While $V\gamma9^+$ is by far the dominant γδ T-cell subset in healthy Caucasians, this is not the case in healthy individuals living in areas where malaria is endemic (16, 25). Furthermore, increased proportions of both the $V\gamma9^+$ subset and the largely complementary $V\delta1^+$ subset have been reported following *P. falciparum* malaria in patients from areas where malaria is endemic (23, 49). Only the former of these subsets responds to malaria antigen stimulation in vitro (3, 15). As shown in Fig. 1 and 2, $V\delta1^+$ cells dominated among TCR-$\gamma\delta^+$ cells at all time points, whether expressed as fractions of $CD3^+$ cells or as absolute numbers of cells. Furthermore, essentially all the disturbances in the proportions and numbers of TcR-$\gamma\delta^+$ cells that were observed in the patients within the first week after admission were due to perturbations within the Vδ1 subset (Fig. 1 and 2) (P [F] < 0.001 [between days]; P [F] $= 0.10$ [between patient categories]).

The γδ T cells present in the peripheral circulation initially have an activated phenotype. We have previously hypothesized that the rapid therapy-induced correction of the lymphopenia in malaria patients reflects the emergence of T cells into the peripheral circulation from sites of disease-induced sequestration and proliferation (11, 27–29). Since T-cell adhesion is a consequence of previous activation, and since our data suggest that γδ T cells are released very rapidly following treatment, we proceeded to examine the level of expression of activation markers on the γδ T cells at various time points. A large proportion of TCR-$\gamma\delta^+$ cells, and in particular the $V\delta1^+$ subset of these cells, obtained at various time points after initiation of therapy expressed the early (CD69) and late (HLA-DR) markers of activation (Fig. 3). There was no marked difference in the level of γδ T-cell activation between CM and UM patients, and in Fig. 3 the data from all donors are shown together. The expression of CD69 and HLA-DR on TCR-$\gamma\delta^+$ cells and on T cells in general was similar (not shown), with the exception of $V\delta1^+$ cells which showed a much higher level of expression of both markers (Fig. 3).

$V\delta1^+$ T cells use all expressed Vγ chains. The very high frequencies and absolute numbers of $V\delta1^+$ cells around day 2 after initiation of therapy prompted us to examine the TCR usage of these cells in more detail. As seen in Fig. 4, $V\delta1^+$ cells expressing all *V*γ gene products could be identified in all children examined this way at day 2, without marked differences between UM and CM patients. The most common phenotype among the $V\delta1^+$ cells was cells labeled by MAb 23D12, which reacts with cells expressing Vγ2, Vγ3, or Vγ4, but all expressed Vγ chains were identified among the $V\delta1^+$ cells, as observed previously (25). The data did not suggest expansion of $V\delta1^+$

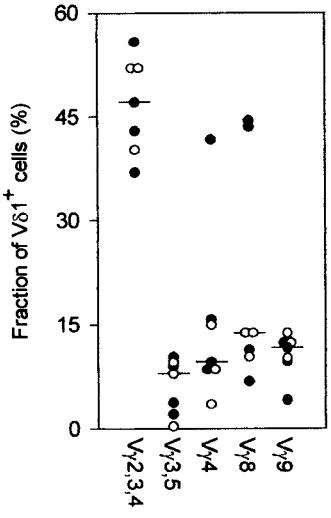

FIG. 4. Expression of Vγ gene products by $V\delta1^+$ T cells obtained at day 2 following initiation of chemotherapy from four patients with cerebral (●) and three patients with uncomplicated (○) *P. falciparum* malaria. Individual data points and medians are given.

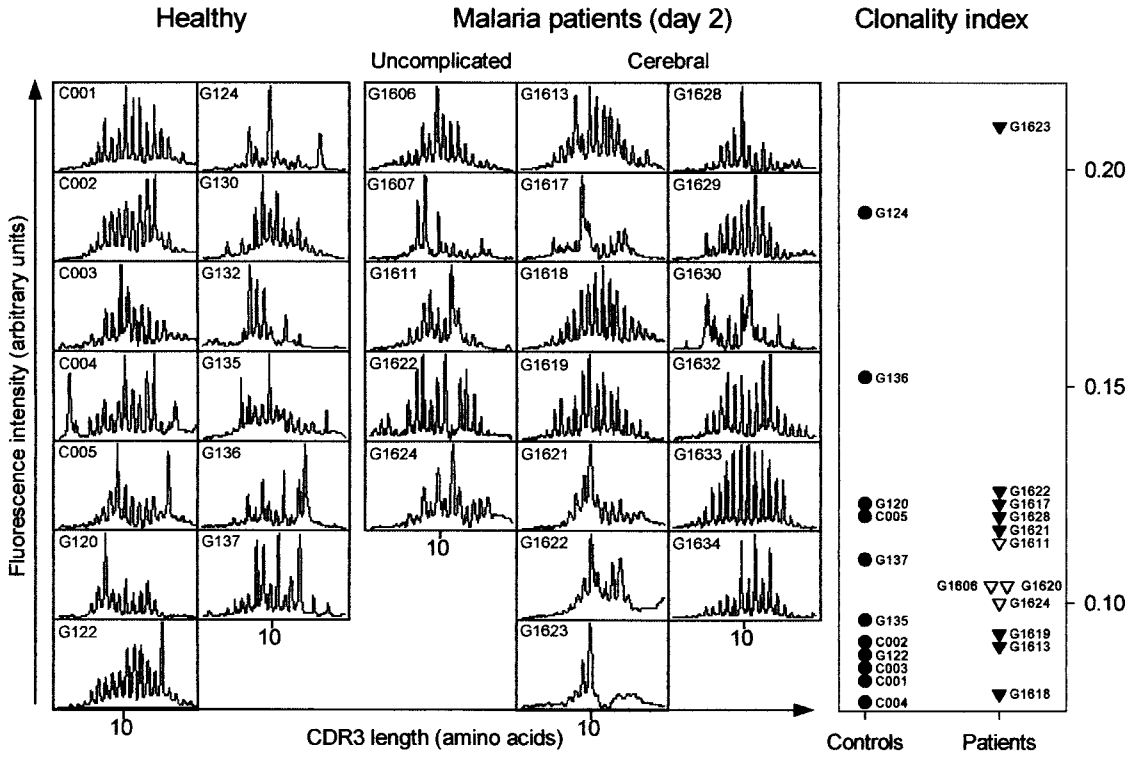

FIG. 5. Vδ1 CDR3 characterization of T cells obtained from 13 healthy children and from 18 *P. falciparum* malaria patients (5 with UM and 13 with CM) on day 2 following initiation of chemotherapy. Individual CDR3 length polymorphism (Immunoscope profiles; left panel) and Vδ1 clonality indices (right panel) from healthy children (●) (two data points missing) and patients with UM (▼) (one data point missing) and CM (▽) (five data points missing) are shown.

cells bearing particular Vγ chains. Cocktails of all the Vγ antibodies labeled only about 85% of the Vδ1$^+$ cells, in contrast to experiments done in parallel on healthy children, where essentially all Vδ1$^+$ cells were labeled by the antibody cocktail. The reason for this difference is unclear and is under investigation.

The high proportion of Vδ1$^+$ cells is not caused by conventional antigen-driven proliferation. In the absence of evidence of unusual dominance of particular Vγ-Vδ combinations, we proceeded to investigate whether the high proportion of Vδ1$^+$ cells was driven by antigen recognized by Vδ1 alone. This was done by size spectratyping of the CDR3 antigen recognition domain of Vδ1 (43). This analysis was completed for day-2 samples from 5 UM and 13 CM patients, in addition to samples from 13 healthy control children, and revealed no obvious differences between patients and control donors or between patient groups (Fig. 5). Supplementary clonality index analysis, taking into consideration both the degree of CDR3 length polymorphism and the percentage of Vδ1$^+$ cells, did not show significant differences (P [T] = 0.29) between patients and controls or among patient groups (Fig. 5).

Vδ1$^+$ cells have a cytokine profile different from that of CD3$^+$ cells in general. As a final element in our characterization of the Vδ1$^+$ cells involved in the perturbation of this subset following malaria, we evaluated subset-specific production of three major T-cell cytokines, IFN-γ, TNF-α, and IL-10. Figure 6 shows that the proportions of cytokine-producing CD3$^+$ cells, TCR-γδ$^+$ cells, and Vδ1$^+$ cells were significantly different (P [F] < 0.001). Thus, intracellular IFN-γ could be detected in almost twice as many TCR-γδ$^+$ cells and Vδ1$^+$ cells as among CD3$^+$ cells in general. Conversely, TCR-γδ$^+$ cells and Vδ1$^+$ cells produced substantially less TNF-α than CD3$^+$ cells in general (P [F] < 0.001). We did not detect any IL-10 production by TCR-γδ$^+$ cells (not shown).

DISCUSSION

γδ T cells have been implicated in the immune response to malaria (reviewed in reference 35), but few studies have examined individuals exposed to malaria parasites in areas where the disease is endemic. This is problematic, since the limited evidence available from such studies indicates that a simple extrapolation from studies of nonexposed individuals may not always be possible (16, 26, 49). Thus, the sustained and Vγ9$^+$-cell-dominated responses seen in nonexposed individuals caused by cells activated by phosphorylated nonpeptide antigens present in malaria parasites (4) were not seen in the above-cited studies in Africa.

Here we present evidence that children from areas where malaria is endemic show a very pronounced, but transient, γδ T-cell response during treatment of *P. falciparum* malaria. This perturbation peaked around day 2 after initiation of antimalarial chemotherapy and thus occurred considerably earlier than the TCR-αβ lymphopenia-to-lymphocytosis-to-homeostasis sequence reported from the same area (27). It is likely that the perturbation is induced by a therapy-mediated

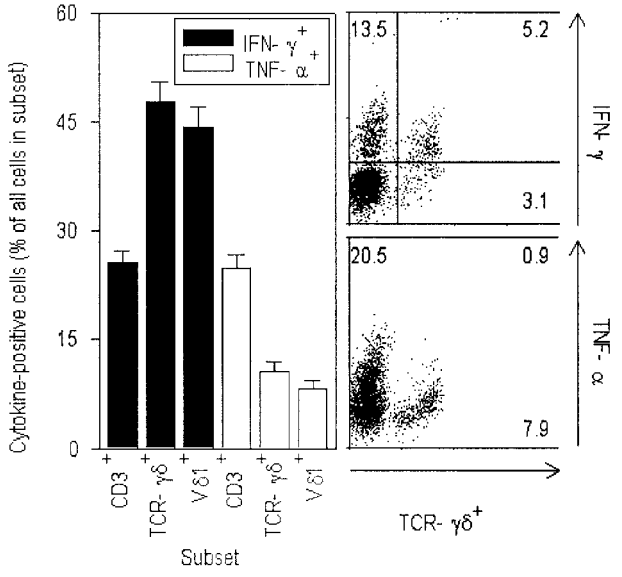

FIG. 6. Cytokine expression by T-cell subsets following 90 min of stimulation in vitro. Left panel, proportion of subsets producing IFN-γ (filled bars) and TNF-α (open bars). Right panels, production of IFN-γ (upper panel) and TNF-α (lower panel) by $CD3^+$ cells from a UM patient (G1611). Numbers indicate percentages of all $CD3^+$ cells in quadrants.

alleviation of tissue inflammation and that the transiently increased frequency and absolute numbers represent cells emerging from sites of malaria-induced sequestration and expansion (reviewed in reference 24).

Subset analysis at the time of peak frequency and the number of TCR-$\gamma\delta^+$ cells revealed that the peak was totally dominated by changes in cells using Vδ1 in their TCR, while only very minor perturbations were seen among Vδ1-negative γδ T cells. In contrast to the healthy children that we have previously studied in the same area (25), the $V\delta1^+$ cells obtained at day 2 of treatment from the malaria patients studied here showed marked evidence of activation. The expanded $V\delta1^+$ population at this time was not characterized by particular Vγ-chain usage and did not show significant dominance of certain CDR3 lengths. In all these respects, our patients resemble those from other studies of infectious diseases, such as HIV infection and autoimmune disorders (6, 10, 21, 38, 41).

The transient increase in circulating $V\delta1^+$ T cells in the absence of evidence of conventional antigen-driven expansion of $V\delta1^+$ T cells during *P. falciparum* malaria raises the question of its cause. In vitro stimulation of T cells from unexposed donors by crude *P. falciparum* antigen causes expansion of $V\gamma9^+$ $V\delta2^+$ cells but not of $V\delta1^+$ cells (3, 15). In line with this, we did not find a significant relation between in vivo frequencies of $V\delta1^+$ cells and asymptomatic parasitemia (25).

The $V\gamma9^+$ $V\delta2^+$ T-cell response to stimulation by *P. falciparum* preparations is caused by recognition of nonpeptide phosphorylated antigens found in numerous bacteria and protozoa, including *P. falciparum* (4, 9). While the antigens that are recognized by these cells are thus well characterized, little is known about the antigens recognized by $V\delta1^+$ cells (reviewed in reference 40). It has been proposed that these cells respond to stress- or infection-induced molecules on a variety of cell types, including epithelial cells and B cells. Specifically, $V\delta1^+$ cells have been reported to recognize the weakly polymorphic major histocompatibility complex class I-like molecules MICA and MICB (18, 19). However, the same authors have recently shown that it is NKG2D rather than TCR-γδ that is the MICA and MICB receptor (2). Other recent work has demonstrated recognition of CD1c molecules expressed on dendritic cells and B cells by $V\delta1^+$ clones expressing various Vγ chains (46).

Finally, it should be noted that although the conditions that have been described as being characterized by locally or systemically increased levels of $V\delta1^+$ T cells are seemingly very heterogeneous, they all share evidence of polyclonal B-cell activation. This is not least the case in malaria (1, 13), and it is thus of interest that activated B cells are an antigenic target of $V\delta1^+$ T cells (20, 30, 42). Although T-cell activation is a general feature of *P. falciparum* malaria (11, 29), we found particularly high levels of activation among $V\delta1^+$ T cells. The lack of evidence of particular antigens driving the expansion of $V\delta1^+$ cells, and their preponderance in situations where B-cell activation is a major feature, make it tempting to speculate that they are in fact regulatory cells responding to self antigens expressed by activated B cells. An autoregulatory role for γδ T cells has been proposed in several recent reviews (5, 22). The antigens recognized by such putative B-cell-regulatory $V\delta1^+$ T cells remain unknown, but the observation that certain cytotoxic T cells expressing TCR-γδ recognize idiotype is intriguing in this context (31, 50). A B-cell-cytotoxic role for the $V\delta1^+$ T cells in our malaria patients is consistent with the observed ex vivo cytokine production profile of these cells, which resembles that of $CD8^+$ cells, and is furthermore in line with our preliminary in vitro experiments (unpublished data). We are currently characterizing a panel of $V\delta1^+$ γδ T-cell clones from an individual from our study area to pursue this issue further.

In conclusion, we have shown that patients with previous parasite exposure and who are undergoing treatment for *P. falciparum* malaria transiently show markedly increased levels of highly activated $V\delta1^+$ T cells having a proinflammatory cytokine profile. These cells are not restricted by particular Vγ chains and do not appear to expand in response to a specific antigen recognized by a limited set of $V\delta1^+$ clones. Taken together, the data point to an immunoregulatory role of these cells. This is the first detailed longitudinal study of the γδ T-cell response to *P. falciparum* malaria in children from Africa, i.e., those who suffer most of the morbidity and essentially all the mortality from this disease. Our findings are at variance with most previous data from ex vivo and in vitro studies of nonimmune adults and thus emphasize the extreme caution necessary when extrapolating from model laboratory investigations to the situation in the main target population of this major health problem.

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