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Lymphocyte activation and subset redistribution in the peripheral blood in acute malaria illness: distinct $\gamma\delta^+$ T cell patterns in *Plasmodium falciparum* and *P. vivax* infections

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SUMMARY

Lymphocyte subset distributions and activation in the peripheral blood were studied in 39 patients with acute malaria and 16 healthy controls from Addis Ababa and Nazareth, Ethiopia. As confirmed by polymerase chain reaction (PCR), 15 patients were infected with *Plasmodium falciparum* (*Pf*), 17 with *P. vivax* (*Pv*) and seven were double-infected (Di) with both *Pf* and *Pv*. Three-colour flow cytometry was used for phenotyping. Total leucocyte and lymphocyte counts were lower in malaria patients than in controls. The T cell count was reduced in *Pf* patients, while in the *Pv* and Di patients there was a reduction in the natural killer (NK) cell count. The CD4/CD8 ratio remained unchanged. $\gamma\delta^+$ T cells were significantly elevated in *Pf* and Di patients, but not in *Pv* patients. The increase in $\gamma\delta^+$ T cells was mostly due to an increase in $V\delta1^+$ cells. Analyses of cellular activation indicated by the expressions of CD25 and HLA-DR revealed significantly higher numbers of activated $CD3^+$ cells, including $\gamma\delta^+$ T cells, in all patient groups compared with controls. Our results thus indicate that in acute malaria illness there is a complex pattern of change in lymphocyte subset distribution and activation, including $\gamma\delta^+$ T cells. These patterns in *Pf* infection seem to be distinct from those in *Pv* infection.

Keywords *Plasmodium falciparum* *Plasmodium vivax* lymphocytes flow cytometery $\gamma\delta^+$ T cell

INTRODUCTION

The protective mechanisms against malaria are complex and not fully understood. Several studies have been carried out to elucidate the functional contribution of T cells in malaria [1].

Antigen recognition by human T cells is mediated through two types of T cell receptors (TCR), defined by hetereodimeric complexes of $\alpha\beta$ or $\gamma\delta$ chains, associated with CD3 surface molecules. The vast majority of lymphocytes in the peripheral blood (> 90%) express the $\alpha\beta$ TCR, whereas about 6–8% bear the $\gamma\delta$ TCR. $\gamma\delta^+$ T cells are broadly divided into cells expressing either $V\delta1^+$ or $V\delta2^+$ chain. Cells expressing $V\delta2$ in association with $V\gamma9$ account for two-thirds of $\gamma\delta^+$ T cells in the peripheral blood [2]. $\gamma\delta^+$ T cells have been reported to produce various cytokines and to act as both MHC-restricted and non-MHC-restricted cytotoxic effector cells [2]. However, the nature of the antigens recognized as well as the process of antigen recognition by $\gamma\delta^+$ T cells are not fully understood [2].

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Recently, $\gamma\delta^+$ T lymphocytes have been reported to be elevated in peripheral blood [3–8] and spleens [9] of individuals with acute or convalescent *Plasmodium falciparum* (*Pf*) infection, and also in spleens of mice with malaria [10]. An increase in these cells has also been shown during febrile paroxysms of *P. vivax* (*Pv*) infections [8]. The expanding $\gamma\delta^+$ T cell subset was shown to be of the $V\delta2^+$ phenotype [3–5]. Proportional expansion of both $V\delta1^+$ and $V\delta2^+$ subsets has been reported in acute *Pf* infection [7]. In the situation where an increase in $\gamma\delta^+$ T cells has been described, no information is so far available on concomitant changes of the corresponding non-$\gamma\delta^+$ T cells. Moreover, the activation status of $\gamma\delta^+$ as well as non-$\gamma\delta^+$ T cells was not evaluated. Few studies on human lymphocyte subpopulation analyses have been conducted in malaria patients, with variable results [8,11–15].

The aim of this study was to characterize the lymphocyte subsets and level of activation in peripheral blood mononuclear cells (PBMC) obtained from patients with acute *Pf* and/or *Pv* malaria illnesses and healthy controls, and to relate the findings with clinical and parasitological results.

PATIENTS AND METHODS

Study population

The study subjects comprised 39 (eight females, 31 males) acutely ill adult patients attending the malaria control clinics in Addis Ababa and Nazareth, Ethiopia. Criteria for inclusion were symptoms compatible with acute malaria illness confirmed to be *Pf* or *Pv* by microscopic examination of Giemsa-stained blood films. After informed consent the clinical history and physical findings were recorded. A total of 16 healthy adult volunteers (four females, 12 males) who reported not to have had malaria or any other illness during the past 6 months were included as controls. The malaria patients received treatment according to the recommended regimens.

The study was approved by the Ethical Review Committee at the Faculty of Medicine, Addis Ababa University, Ethiopia.

Standard laboratory examinations

Leucocyte and differential cell counts of fresh whole blood were determined by routine clinical laboratory procedures. Thin and thick blood films were prepared and stained with 4% Giemsa stain. The blood films were examined for malaria parasites by an experienced microscopist. The number of parasites was calculated by counting parasites against 300 leucocytes in the thick film.

Identification of parasite species by polymerase chain reaction

As a complement to microscopy, identification of the parasite species was conducted by a nested polymerase chain reaction (PCR) assay [16,17]. Briefly, portions of small sub-unit ribosomal RNA genes were specifically amplified for each parasite species (*P. falciparum, P. ovale, P. vivax and P. malariae*). The oligonucleotide primers have been described [17]. The method has been optimized in our laboratory to a sensitivity level where < 10 parasites/μl whole blood can be identified. Parasite DNA was prepared from erythrocyte pellets by phenol extraction and ethanol precipitation. The purified parasite DNA was then used as a template in the first PCR reaction with genus-specific primers, rPLU5 and rPLU6. An aliquot of the amplified product then served as template in four separate species-specific nested reactions (primer pairs rFAL 1/2, rVIV 1/2, rOVA 1/2; and rMAL 1/2). The amplified products were then analysed by agarose gel electrophoresis and visualized in UV light after ethidium bromide staining.

Preparation of peripheral blood mononuclear cells

Venous blood (16 ml) was collected from each individual. PBMC were isolated by Ficoll–Pacque (Pharmacia-Upjohn, Uppsala, Sweden) density gradient centrifugation as described elsewhere [18,19]. The isolated cells were resuspended (5–10 million cells/ml) in heat-inactivated fetal calf serum (FCS) containing 10% dimethylsulfoxide (DMSO), frozen at –80°C overnight and then transferred into liquid nitrogen. Frozen samples were later transported to Sweden for flow cytometric analysis. Frozen PBMC were thawed at 37°C and immediately washed twice in RPMI 1640. Cell viability was determined using trypan blue exclusion before staining, and also by propidium iodide (PI) exclusion at flow cytometric analysis.

Cell surface staining

The MoAbs used for phenotypic characterization of PBMC are shown in Table 1. Direct staining with FITC-, PE- and PerCp (peridin chlorophyll-A protein)-conjugated antibodies was conducted for three-colour fluorescent analysis. Antibodies were dispensed into different tubes according to the protocol (Table 1). The staining procedure has been described previously [18,19].

Flow cytometric analysis

Three-colour flow cytometric analysis was performed on a FACSort flow cytometer (Becton Dickinson, San Jose, CA). Before data acquisition, instrument parameters were checked and optimized using CaliBRITE Beads (Becton Dickinson). Optimization of colour compensation was checked each time with samples stained with MoAbs against CD3, CD56, and CD19. Data acquisition was done with Lysys II software (Becton Dickinson). For each sample, data for 10 000 lymphocytes were acquired using log amplified fluorescence and linearly amplified side and forward scatter signals. Data were analysed with the Paint -A Gate Plus software (Becton Dickinson). All samples were analysed by setting appropriate forward and side scatter gates around the lymphocyte

Table 1. MoAbs and corresponding cell populations detected in three-colour flow cytometry of peripheral blood lymphocytes†

MoAb-FITC (cell population detected)	MoAb-PE (cell population detected)
Anti-CD45 (leucocytes)	Anti-CD14 (monocytes)
IgG1 (negative isotype control)	IgG1 (negative isotype control)
Anti-TCRγ/δ1 (all $\gamma\delta^+$ cells)	Anti-HLA-DR (MHC class II, activated cells)
Anti-TCRγ/δ1 (all $\gamma\delta^+$ cells)	Anti-CD25 (β subunit of IL-2R, activated cells)
Anti-TCRγ/δ1 (all $\gamma\delta^+$ cells)	Anti-CD45RO (memory T cells)
Diversi-T δV2(a) (Vδ2/Vγ9$^+$ subset of $\gamma\delta^+$ T cell)*	Anti-TCRγ/δ1 (all $\gamma\delta^+$ cells)
Diversi-T δV1(a) (Vδ1$^+$ subset of $\gamma\delta^+$ T cells)*	Anti-TCRγ/δ1 (all $\gamma\delta^+$ cells)
Anti-CD19 (B cells)	Anti-CD56 (N-CAM$^+$, NK cells)
Anti-CD4 (MHC class II-restricted T cells)	Anti-CD8 (MHC class I-restricted T cells)
Anti-CD57 (HNK-1, larger granular lymphocytes)	Anti-CD8

MoAbs were obtained from Becton Dickinson (Mountain View, CA) unless otherwise indicated.

* From T Cell Diagnostics (Cambridge, MA).

† In all combinations anti-CD3 peridin chlorophyll-A protein (Per-Cp) (recognizing all T cells) was used as a third colour except in CD45/14 staining. Per-Cp-conjugated IgG1 was used as negative control. Propidium iodide (PI) was added to the CD45/14 combination and cells were incubated for 5 min to determine viability.

Table 2. Leucocyte and lymphocyte counts, anti-*Plasmodium falciparum* IgG antibodies and parasite densities in patients and controls*

	Leucocytes ($10^3/\mu$l)	Lymphocytes ($10^3/\mu$l)	Anti-*Pf* IgG (μg/ml)	Parasite count ($10^3/\mu$l)
Double-infected	5·1 (3·8–6·7)**	1·5 (1·0–2·5)	94·9 (33·6–112)**†	2·3 (1·3–8·2)
P. falciparum	4·6 (4·2–6·2)**	1·4 (1·0–2·0)**	35·0 (7·7–46·5)**†	5·3 (2·9–12·7)
P. vivax	6·2 (4·5–7·1)	1·2 (0·9–2·2)**	6·30 (4·2–9·2)**	2·1 (0·4–5·3)
Controls	7·6 (4·9–9·5)	2·2 (1·6–2·5)	2·20 (1·4–4·3)	

*Median values with IQR in parentheses.
** $P < 0·05$ compared with controls; † $P < 0·05$ compared with *P. vivax*.

population using back gating on $CD45^+$ and $CD14^-$ cells. Cells stained with negative control MoAbs were used to draw the cut-off line in the histogram. Negative control cells were entirely contained within the region corresponding to first 1 to 1·1 logs of fluorescence intensity for all analysis. As a control of appropriate lymphocyte gating, the percentages of $CD3^+$, $CD56^+$ and $CD19^+$ cells within the gate were added together. For all samples analysed the sum of this gate equalled 98 ± 3%. Staining for CD3 cells was done in more than 10 tubes and staining for $\gamma\delta^+$ T cells in five tubes for each sample. The mean percentage and s.d. for $CD3^+$ cells and $\gamma\delta^+$ T cells was calculated from these observations. The average s.d. for CD3 values was 2·87 with coefficient of variation (CV) of 4·15, and the average s.d. for $\gamma\delta^+$ values was 1·12 with CV of 12·3, indicating a very low intra-assay variability. Similarly, to estimate the interassay variability, a blood sample from one single control individual was thawed and stained at each staining occasion. The s.d. for CD3 values was 3·0 with a CV of 3·96 and the s.d. for $\gamma\delta^+$ T cells was 1·80 with a CV of 13·2, indicating a low interassay variability.

Serum antibody determination by ELISA

Determination of anti-*Pf* IgG antibodies was performed as described earlier. Briefly, flat-bottomed 96-well ELISA plates (Costar, Cambridge, MA) were coated with 50 μl/well of Percoll-enriched parasite antigen (10 μg/ml), a crude *Pf* antigen extract produced as described earlier [20]. Each sample was run in duplicate. Plasmas diluted 1:1000 (100 μl) were added to the wells and incubated at 37°C for 1 h. Specifically bound antibodies were then assayed by alkaline phosphatase-conjugated rabbit anti-human IgG antibodies (Fcγ-specific; Dako A/S, Copenhagen, Denmark) with *p*-nitrophenyl phosphate as substrate. IgG levels were calculated from standard curves obtained by incubating coated plates with five known dilutions of highly purified IgG (Jackson Immuno-Research Labs, West Grove, PA) [21].

Statistical analysis

Statistical calculations were performed using the JMP program (SAS Institute Inc., Cary, NC). Distribution differences were analysed with the Wilcoxon/Kruskal–Wallis test. The Spearman rank order correlation coefficient ($r_{s)}$ was used for evaluation of correlations. Results were considered significant at $P < 0·05$. Descriptive statistics are shown in tables and figures as medians and inter-quintile ranges (IQR, 25th–75th percentile).

RESULTS

Patients and controls

Out of the 39 patients, 19 were microscopically diagnosed as *Pf* and 20 as *Pv* infections. In nested PCR analysis, seven of the patients were found to be double-infected (Di) with both *Pf* and *Pv* parasites. Three of those Di had been microscopically diagnosed as only *Pf*- and four as only *Pv*-infected. One patient who was diagnosed as *Pf* by microscopy, with a very low parasite load of 130/μl, was found to be positive only for *Pv* by PCR. Thus, patients were regrouped based on the PCR results into *Pf*-infected ($n = 15$), *Pv*-infected ($n = 17$) and Di ($n = 7$). All controls remained negative

Table 3. Percentages (%) and absolute counts (*n*) of major lymphocyte subsets and T cell subsets in the peripheral blood of patients and controls*

		$CD3^+$ (T cells)	$CD19^+$ (B cells)	$CD3^-CD56^+$ (NK cells)	$CD3^+CD4^+$	$CD3^+CD8^+$	$CD3^+CD56^+$	$CD3^+$ $CD4^-CD8^-$
Double-infected	%	89 (82–91)	7 (6–8)	6 (4–11)**	35 (23–51)	51 (37–64)	7 (5–11)	13 (10–14)
	n	1372 (693–2255)	107 (62–179)	108 (61–153)**	479 (315–783)	541 (298–1141)	82 (33–248)	178 (61–335)
P. falciparum	%	78 (68–86)	5 (3–9)	16 (8–24)†	40 (26–49)	47 (42–56)	10 (6–14)	11 (7–17)
	n	1261 (681–1681)**	69 (39–158)	210 (134–294)	361 (190–681)	630 (293–709)**	122 (48–170)	158 (55–247)
P. vivax	%	82 (78–87)	7 (4–11)	8 (6–14)	42 (33–47)	50 (43–63)	8 (5–14)	8 (5–11)
	n	1014 (795–1813)	63 (40–160)	132 (47–255)**	428 (240–599)	525 (355–1142)	91 (48–176)	102 (46–148)
Controls	%	74 (64–84)	7 (3–9)	16 (8–26)	37 (25–49)	54 (43–60)	12 (7–18)	8 (5–11)
	n	1728 (1446–1914)	115 (79–230)	344 (167–488)	465 (382–943)	914 (540–1033)	172 (85–265)	126 (78–197)

* Median values with IQR in parentheses. Percentages of T cells, B cells and NK cells calculated from total $CD45^+CD14^-$ cells.
** $P < 0·05$ compared with controls; † $P < 0·05$ compared with *P. vivax*.

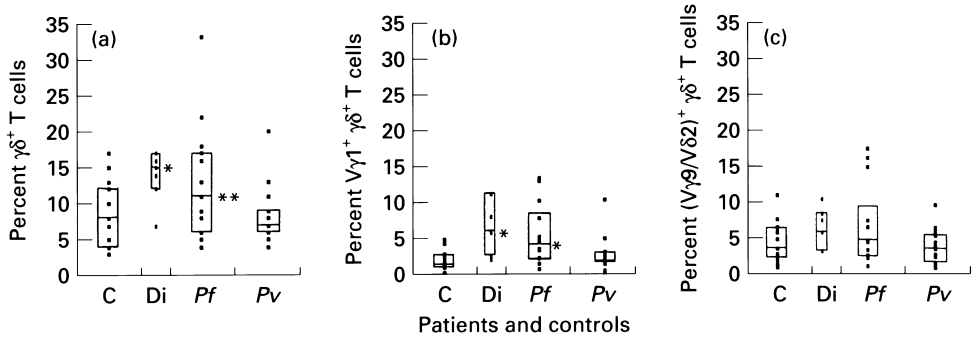

Fig. 1. Percentage of $\gamma\delta^+$ T cells (a) and $V\delta1^+$ (b) and $V\gamma9/V\delta2^+$ (c) cells of $CD3^+$ cells in the peripheral blood of *Plasmodium falciparum* (*Pf*), *P. vivax* (*Pv*), double-infected (Di) patients and controls (C). Median values and interquintile range are shown by the box plots. Note that a single point in scatter plot may represent more than one observation. $*P < 0{\cdot}05$; $**P = 0{\cdot}05$ compared with controls.

by PCR. The median age of the study population was 27 years (22–32 years, IQR) and did not differ between groups. All patients presented with classical symptoms of acute malaria.

Antibody responses

Significantly elevated anti-*Pf* IgG antibodies were detected in the plasma obtained from Di and *Pf* patients (Table 2). Two of the controls and 11 of the *Pv*-infected patients had low but detectable anti-*Pf* antibodies. Anti-*Pf* concentrations were not correlated to any of the clinical findings, parasitaemia or phenotypic characteristics of lymphocytes.

Leucocyte counts

Total leucocyte counts in malaria patients were generally lower than in controls (Table 2), although only significantly so for *Pf* patients. Similarly, absolute lymphocyte counts were significantly lower in *Pf* and *Pv* patients than in controls (Table 2).

Lymphocyte subsets

A statistically significant decrease in absolute number of T ($CD3^+$) cells was observed in *Pf* patients compared with controls, with a similar trend in Di and *Pv* patients. The percentage of T cells was, however, not significantly lower. The absolute counts of natural killer (NK) ($CD3^-CD56^+$) cells were significantly lower in Di and *Pv* patients than in controls (Table 3). B ($CD19^+$) cell percentages and counts were similar in patients and controls.

T cell subsets

The percentages of $CD4^+$ and $CD8^+$ cells out of $CD3^+$ cells were not different in patients and controls (Table 3). Neither was there any difference in the CD4/CD8 T cell ratio (data not shown). However, absolute numbers of both $CD4^+$ and $CD8^+$ cells were generally lower in the patient groups. In *Pf* patients the $CD8^+CD3^+$ count was significantly lower than in controls (Table 3). $CD8^+CD57^+$ cells accounted for 43% (35–53%, IQR) of total $CD8^+$ cells, and no difference was seen between patients and controls. Similarly, no significant difference was seen in the percentage of $CD3^+CD56^+$ cells (Table 3).

$\gamma\delta^+$ T cells

There was a great individual variation in the percentage of $\gamma\delta^+$ T cells within all groups, with the highest value of 33% for *Pf* patients and 20% for *Pv* patients (Fig. 1a). A statistically significant increase in $\gamma\delta^+$ T cells was seen in Di and *Pf* patients. Despite lower leucocyte and absolute lymphocyte counts the absolute counts of $\gamma\delta^+$ T cells were higher in *Pf* and Di patients than in *Pv* patients and controls (Table 4). The percentage of double-negative $CD4^-CD8^-CD3^+$ cells was directly proportional and positively correlated ($r_s = 0{\cdot}76$, $P = 0{\cdot}0001$) to the percentage of $\gamma\delta^+$ T cells. The percentages of $\gamma\delta^+$ T cells were negatively correlated to the $CD4^+/CD8^+$ T cell ratio in *Pf* and Di patients ($r_s = -0{\cdot}68$, $P = 0{\cdot}0006$).

Table 4. Percentages (%) and absolute counts (*n*) of $\gamma\delta^+$ T cells in $CD3^+$ cells and $\gamma\delta^+$ T cell subsets in the peripheral blood of patients and controls*

		$CD3^+$ $\gamma\delta^+$	$V\gamma9V\delta2^+/\gamma\delta^+$	$V\delta1^+/\gamma\delta^+$	Ratio $V\delta1^+ : V\gamma9V\delta2^+$
Double-infected	%	15 (12–17)**†	49 (22–57)	38 (32–60)**	0·78 (0·57–2·32)**
	n	233 (75–316)†	45 (31–186)	77 (28–153)**	
P. falciparum	%	11 (6–17)††	42 (29–60)	29 (25–71)**	0·59 (0·37–2·2)
	n	158 (34–265)	73 (18–115)	50 (17–99)	
P. vivax	%	7 (6–9)	46 (24–59)	31 (19–42)	0·59 (0·32–1·43)
	n	73 (47–147)	32 (24–61)	29 (14–50)	
Control	%	8 (4–12)	47 (39–80)	23 (16–27)	0·48 (0·22–0·66)
	n	110 (68–175)	52 (40–89)	22 (18–36)	

* Median values with IQR in parentheses.

** $P < 0{\cdot}05$ compared with controls; † $P < 0{\cdot}05$ compared with *P. vivax*; †† $P = 0{\cdot}050$ compared with controls.

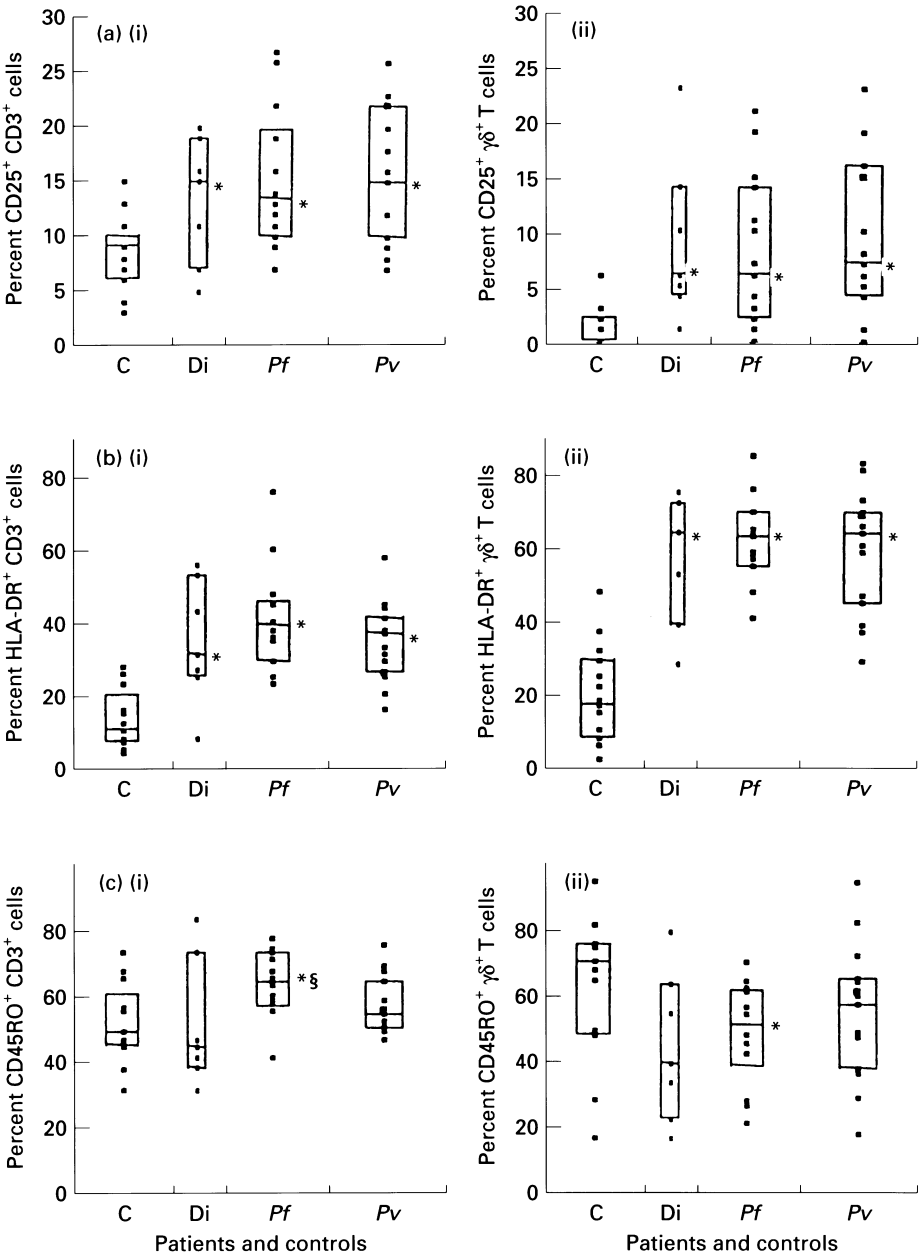

Fig. 2. Percentage of $CD25^+$ (i), HLA-DR^+ (ii) and $CD45RO^+$ (iii) of $CD3^+$ (a) and $\gamma\delta^+$ T cells (b) in the peripheral blood of *Plasmodium falciparum* (*Pf*), *P. vivax* (*Pv*), double-infected (Di) patients and controls (c). Median values and interquintile range are shown by box plots. Note that a single point in scatter plot may represent more than one observation. *$P < 0{\cdot}05$ compared with controls; §$P < 0{\cdot}05$ compared with *P. vivax*.

Subset distribution of $\gamma\delta^+$ T cells

The percentage of $V\delta1^+$ $\gamma\delta^+$ cells out of $CD3^+$ cells and the percentage $V\delta1^+$ $\gamma\delta^+$ cells out of total $\gamma\delta^+$ T cells were significantly higher in *Pf* and Di patients (Fig. 1b, Table 4). Absolute counts of $V\delta1^+$ cells and the median $V\delta1^+$:$V\gamma9/V\delta2^+$ ratio were also higher in patients than in controls (Table 4). This difference was significant in Di patients.

CD25 (IL-2 receptor) expression

The absolute number and percentage of $CD3^+$ cells and also $\gamma\delta^+$ T cells expressing CD25 were significantly higher in all patient groups than in controls (Fig. 2a(i), (ii)). The percentage of $CD25^+$ T cells was positively correlated to the percentage of $CD4^+$ T cells ($r_s = 0{\cdot}46$, $P = 0{\cdot}03$) in *Pf* patients.

HLA-DR expression

The percentage and absolute number of $CD3^+$ cells and also $\gamma\delta^+$ T cells expressing HLA-DR were significantly higher in all patient groups than in controls (Fig. 2b(i), (ii)). A significant positive correlation was also seen between the percentage of HLA-DR-expressing $CD3^+$ cells and the percentage of $CD8^+CD3^+$ cells ($r_s = 0{\cdot}66$, $P = 0{\cdot}0009$).

CD45RO expression

The percentage of $CD3^+$ cells expressing CD45RO was significantly higher in *Pf* patients than in *Pv* patients and in controls (Fig. 2c(i)). The percentage of $\gamma\delta^+$ T cells expressing CD45RO was lower in all patient groups than in controls (Fig. 2c(ii)). This difference was statistically significant only for *Pf* patients. The percentage of $CD45RO^+$ $\gamma\delta^+$ T cells in *Pf* and Di patients was negatively correlated to the percentage of $V\delta1^+$ cells out of $\gamma\delta^+$ T cells ($r_s = -0{\cdot}46$, $P = 0{\cdot}026$). A significant positive correlation was seen between the percentage of $CD45RO^+$ $\gamma\delta^+$ T cells and the percentage of $V\gamma9/V\delta2^+$ cells out of $\gamma\delta^+$ T cells ($r_s = 0{\cdot}76$, $P = 0{\cdot}0001$)

DISCUSSION

Our analysis of lymphocyte subset distribution in relation to infecting malaria species contributes new information, in particular on the $\gamma\delta^+$ T cell subset and the activation of $\gamma\delta^+$ and non-$\gamma\delta$ T cells. *Pf* infection was associated with an increase in $\gamma\delta^+$ T cells, especially $V\delta1^+$ T cells, despite a decreased T lymphocyte level. In contrast, *Pv* infection was associated with an unchanged number of $\gamma\delta^+$ T cells and distribution of T cell subset and a decreased NK cell level. In Di patients a mixed pattern was found, with a decreased NK cell level as in *Pv* infection and an increase in $\gamma\delta^+$ T cells with increased $V\delta1^+$:$V\gamma9V\delta2^+$ ratio, similar to that seen in the *Pf*-infected group. A significant fraction of T lymphocytes including $\gamma\delta^+$ T cells was activated *in vivo*, as indicated by the increased expression of CD25 and HLA-DR in all patient groups.

Our results also show the importance of PCR-based parasite species diagnosis when analysing immune responses to a different malaria infection. The high sensitivity and specificity of PCR assay have been described [16,17].

Our finding of significantly increased levels of $\gamma\delta^+$ T cells in the peripheral blood of Ethiopian patients with Di and *Pf* corroborates previously reported data from other geographical regions [3–7]. The increased number of $\gamma\delta^+$ T cells in spite of lower absolute lymphocyte counts suggests either a proliferative response or a selective recruitment of $\gamma\delta^+$ T cells to the circulation. *In vitro* studies have shown that crude *Pf* antigens induce proliferation of $\gamma\delta^+$ T cells [7,22,23], supporting the former. However, redistribution of $\gamma\delta^+$ T cells between peripheral blood and tissues has also been reported [24]. In agreement with others, we found no increase in the level of $\gamma\delta^+$ T cells in *Pv*-infected patients [8]. The significantly elevated number of $\gamma\delta^+$ T cells in the Di patients compared with those with *Pv* infection suggests that *Pf* infection specifically stimulates the increase in blood $\gamma\delta^+$ T cell number.

The percentage of $\gamma\delta^+$ T cells was negatively correlated to the percentage of $CD4^+$ cells, as has previously been reported [5,25]. The positive correlation between the percentage of $CD4^-CD8^-$ T cells and $\gamma\delta^+$ T cells suggests that most $\gamma\delta^+$ T cells were negative for CD4 and CD8.

We found a higher increase of $V\delta1^+$ than $V\gamma9/V\delta2^+$ subset of $\gamma\delta^+$ T cells in Di and in *Pf*-infected patients, resulting in an increased $V\delta1^+$:$V\gamma9/V\delta2^+$ ratio. Previously, a predominance of $V\delta1^+$ T cells has been reported in malaria-immune asymptomatic African adults [26]. However, in our controls and in the *Pv*-infected individuals the $V\gamma9/V\delta2^+$ subset was predominant. Thus, the shift to $V\delta1^+$ in *Pf* and Di patients is probably due to an increase, either due to expansion or redistribution, of $V\delta1^+$ T cells in acute *Pf* infection. Our finding is different from those in previous reports [3–5,7], suggesting that the $\gamma\delta^+$ T cell response is not uniform but may depend on host- and/or parasite-related factors.

Significantly higher percentages of T cells, including $\gamma\delta^+$ T cells, positive for CD25 and HLA-DR, were seen in all malaria-infected patients. An increase in $HLA\text{-}DR^+$ T cells in acute malaria infection has been reported [5,7,27]. In *Pv* patients $\gamma\delta^+$ T cells were activated but not increased in cell number. Similar findings have been seen after stimulation with Staphylococci antigen *in vitro* [28]. The positive correlation between the percentage of $CD25^+CD3^+$ cells and $CD4^+$ cells and between the percentage of $HLA\text{-}DR^+$ cells and $CD8^+$ T cells indicates that in malaria CD25 expression is more prominent on $CD4^+$ T cells and HLA-DR is preferentially expressed on $CD8^+$ cells. Similar findings have previously been described in shigellosis, and may thus be a general feature of acute infections [19]. Activated $HLA\text{-}DR^+$ $CD8^+$ cells have been described to contain cytotoxic effector cells [29,30], but their functional contribution in malaria remains to be determined. The percentage of CD25-expressing $\gamma\delta^+$ T cells was five to six times higher in patients than in controls, while the level of CD25-expressing non-$\gamma\delta^+$ T cells was increased less than two-fold. Similarly, the percentage of HLA-DR-expressing cells was higher among $\gamma\delta^+$ T cells than non-$\gamma\delta^+$ T cells. This suggests a more prominent activation of $\gamma\delta^+$ T cells than non-$\gamma\delta^+$ T cells, or that activated non-$\gamma\delta^+$ T cells are more prone to leave the circulation, as suggested previously [31]. High levels of soluble IL-2R in plasma during acute malaria infection have been reported [31–33], but no increase in IL-2R-expressing cells has previously been described.

It has been described that the majority of $V\delta2^+$ cells express $CD45RO^+$, whereas only a minority of $V\delta1^+$ cells are $CD45RO^+$ [34,35]. Thus, the presence of a dominant $V\delta1^+$ subset of $\gamma\delta^+$ T cells in these patients might explain the lower percentage of $CD45RO^+$ $\gamma\delta^+$ T cells in these groups. Moreover, the low expression of CD45RO might suggest redistribution rather than actual increase. However, activation of $V\delta1^+$ cells has been reported in spite of a lack of CD45RO expression, implying a different mode of activation in $\gamma\delta^+$ T cells [36], and a shift to CD45RO expression has been shown to be a later event in cellular activation [37]. Based on this evidence, the possibility of expansion of these cells can not be excluded.

The functional significance of $\gamma\delta^+$ T cells during malaria infection is not yet understood. Preferential expansion and activation of this subset of cells were also shown after *in vitro* stimulation of PBMC from non-malaria-exposed donors with crude extract of *Pf* cultures [22,23,38,39]. Studies in mice have suggested a protective role of $\gamma\delta^+$ T cells in malaria infection [40–42]. In humans, *in vitro* activated $\gamma\delta^+$ T cells (both $V\delta2^+$ and $V\delta1^+$) can inhibit re-invasion of merozoites into new erythrocytes *in vitro* [43] (unpublished observation). Taking all data into account, this might suggest a protective function of $\gamma\delta^+$ T cells. Considering the non-MHC-restricted killing capability of $\gamma\delta^+$ T cells, their possible role in recognition of parasite antigens expressed on infected erythrocytes and on free merozoites is expected. This may be of particular importance, since these cells may not carry appropriate MHC molecules. In addition, the high level of activation of these cells suggests significant participation in production of the cytokine milieu. *In vitro* studies have shown that $\gamma\delta^+$ T cells activated with malaria antigens produce an array of cytokines [39] implicated in immunity and/or pathogenesis of malaria. Thus, further studies are needed to elucidate the functional significance of $\gamma\delta^+$ T cells in human malaria.

Our finding of a significantly lower absolute count of NK cells in Di and *Pv* patients than in *Pf* patients may suggest that this change is associated with *Pv* infection, and may indicate that NK cells play a different role in *Pf* and *Pv* infections. Previously decreased levels of $CD3^{-}CD56^{+}$ cells have been reported in *Pf* infection [44]. Down-regulation of NK cells by parasite factor(s) could potentially be a strategy of the parasite to avoid host immune protection.

The level of $CD4^{+}$ cells was lower and that of $CD8^{+}$ cells was higher in our study population, irrespective of malaria infection, than most reports of European and North American healthy subjects [45,46]. Our findings are more similar to those of Bangladeshi healthy subjects [19]. Differences in the spectra and levels of exposure to microorganisms may explain this disparity.

No correlation was found between cellular phenotypic pattern and clinical findings, or parasite count. However, our findings of distinct and significantly different cellular response patterns in *Pf*, *Pv* and double infections thus suggest that different malaria species use different strategies in the interaction with the host immune system, and that this is reflected in the relative activation of the different arms of the host non-MHC-restricted cytotoxic effector mechanisms, including $\gamma\delta^{+}$ T cells and NK cells. This difference may be important for the understanding of the different pathological and clinical profiles in the two infections.

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