

# Delayed expansion of V $\delta$ 2<sup>+</sup> and V $\delta$ 1<sup>+</sup> $\gamma\delta$ T cells after acute *Plasmodium falciparum* and *Plasmodium vivax* malaria

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*T lymphocytes that express T-cell receptors encoded by the  $\gamma$  and  $\delta$  T-cell receptor genes ( $\gamma\delta$  T cells), and preferentially those expressing the V $\gamma$ 9 and V $\delta$ 2 gene segments, are activated by microbial and parasitic organisms in vitro and have been implicated in the pathogenesis of the fever and rigors during acute malaria. We have found, in a cohort of nine nonimmune patients who contracted malaria during travel to endemic areas (five with *Plasmodium falciparum* and four with *P. vivax* infections) that  $\gamma\delta$  T lymphocytes expanded to comprise 17.92%  $\pm$  11% of the peripheral blood mononuclear cells (vs 3.08%  $\pm$  2.4%  $\gamma\delta$  cells in normal control subjects). Although V $\delta$ 2<sup>+</sup> cells predominated among the  $\gamma\delta$  subset,  $\gamma\delta$  lymphocytes expressing the V $\delta$ 1 gene segment also expanded significantly in some patients. Importantly, the  $\gamma\delta$  cells continued to expand for 2 months after the infection, and the mean level of  $\gamma\delta$  cells peaked during the second month after the acute clinical syndrome, when patients were free of symptoms. Thus although  $\gamma\delta$  T cells may contribute to the pathogenesis of the acute clinical syndrome, our findings suggest that  $\gamma\delta$  lymphocytes could also play a role in generating an immune response to plasmodia. (J Allergy Clin Immunol 1996;97:1387-92.)*

**Key words:** Malaria, T lymphocytes,  $\gamma\delta$  T cells, *P. falciparum*, *P. vivax*

Human malaria is a tropical parasitic disease afflicting over 400 million individuals worldwide, and it is caused by four *Plasmodium* species: *P. vivax*, *P. ovale*, *P. malariae*, and the malignant form, *P. falciparum*.<sup>1</sup> Infections with *P. falciparum* are responsible for over 1  $\times$  10<sup>6</sup> deaths annually, and the other forms of malaria are responsible for significant morbidity in developing nations.<sup>1</sup> The nature of the clinical response to *Plasmodium* infection is complex: infected individuals residing in endemic areas develop immunity during the first few years of life, and repeated infections in adulthood result in minimal clinical symptoms.<sup>2</sup> In contrast, individuals from nonendemic areas respond to infections with an acute febrile syndrome and may die during the acute episode. Both B-cell

## Abbreviations used

|        |                                    |
|--------|------------------------------------|
| mAb:   | Monoclonal antibody                |
| PB:    | Peripheral blood                   |
| PBMCs: | Peripheral blood mononuclear cells |
| TCR:   | T-cell receptor                    |

and T-cell epitopes are expressed in important malarial antigens.<sup>3</sup> Accordingly, antibodies and major histocompatibility complex-restricted CD4<sup>+</sup> and CD8<sup>+</sup>  $\alpha\beta$  T-cell receptor (TCR)-bearing lymphocytes play important roles in the immune response to malarial antigens and may influence the clinical course.<sup>3</sup>

A small subset of T lymphocytes is characterized by the expression of TCR encoded by the  $\gamma$  and  $\delta$  TCR genes ( $\gamma\delta$  TCR) and absence of expression of the CD4 or CD8 molecules.<sup>4</sup> These  $\gamma\delta$  T lymphocytes may also be involved in the immune response to *P. falciparum* infections, because in vitro experiments have shown that *P. falciparum* merozoites, schizonts, and circumsporozoites induce a polyclonal proliferation of peripheral blood (PB)  $\gamma\delta$  cells that are derived from unexposed nonimmune individuals.<sup>5-7</sup> Moreover, transient increases of

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the relative percentage of  $\gamma\delta$  cells in the PB during acute *P. falciparum* infection were also demonstrated, and some investigators have suggested a pathogenetic role for these cells in the clinical symptoms.<sup>8,9</sup> To study the possible role of  $\gamma\delta$  lymphocytes in malaria, we determined which subsets of  $\gamma\delta$  cells expand in nonimmune patients who contracted either *P. vivax* or *P. falciparum* infections during travel to endemic areas and evaluated the kinetics of the  $\gamma\delta$  cell expansion in relation to the clinical syndrome.

## METHODS

### Patients

Nine consecutive patients with acute malaria were examined, eight of whom were nonimmune Israeli travelers. The ninth patient was an individual from Ethiopia who had semi-immune status. Six patients contracted malaria in Africa and three in Southeast Asia. Five patients had *P. falciparum* and four had *P. vivax* infections, diagnosed by an experienced microbiologist from PB smears. All patients were male, with a mean age of  $44 \pm 15$  years (range, 23 to 68 years). All patients responded promptly to conventional treatment, with defervescence within 4 days. The first blood samples for T-cell subset analysis in all cases were drawn during the febrile stage of the disease. In seven of the nine patients, the first blood sample for T-cell subset analysis was drawn after the initiation of treatment. Patients were followed up for up to 300 days after the acute infection. Fourteen determinations of T-cell subsets were performed in the patients with *P. falciparum* infections, and 10 in patients with *P. vivax* infections. Control subjects included nine normal healthy men with a mean age of  $39 \pm 17$  years. Five male patients with a history of malaria of at least 1 year before the examination were also studied. For the latter two groups, T-cell subsets were determined on a single occasion for each individual.

### Determination of T-cell surface antigens

Peripheral blood mononuclear cells (PBMCs) were separated from fresh heparinized blood samples by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation and stained as previously described by using a two-step procedure:  $10^5$  cells were incubated at  $4^\circ\text{C}$  in saturating concentrations of murine monoclonal antibody (mAb), washed twice, then incubated at  $4^\circ\text{C}$  with goat anti-mouse conjugated with fluorescein isothiocyanate, washed and analyzed on a Coulter fluorescence-activated cell sorter (Coulter Corp., Miami, Fla.).<sup>10</sup> The mAbs used in this study are: OKT4 (anti-CD4), OKT8 (anti-CD8), OKT3 (anti-CD3), BMA031 (anti- $\alpha\beta$  TCR), TCR $\delta$ 1 (anti- $\gamma\delta$  TCR), BB3 (anti-V $\delta$ 2) (kindly provided by Dr. Lorenzo Moretta), and TCS $\delta$ 1 (anti-V $\delta$ 1).<sup>10</sup> OKT6 (anti-CD1) was used as a nonstaining negative control for PBMCs. The results are re-

ported as the percent positive cells from the total lymphocyte population for each antigen, after subtraction of the negative control.

### Statistical analysis

Mean values of recorded T-cell subsets were compared by using Student's *t* test for paired or unpaired samples, as indicated.

## RESULTS

### Elevated $\gamma\delta$ cells in the PB after acute malaria

Fig. 1 shows the results of serial evaluations of the percentage of the  $\gamma\delta$  cells in the PBMCs of the nine patients with malaria. As can be seen, evaluations at different time points after the onset of the clinical syndrome yielded different values for this subpopulation of T cells in all but patients 5 and 7.

To analyze the changes in the  $\gamma\delta$  cell population, we calculated the mean of the peak level of  $\gamma\delta$  cells observed in each patient over the entire evaluation period. The mean of the maximal levels of the  $\gamma\delta$  cells for the nine patients was  $17.92\% \pm 11.5\%$  of the PBMCs, which is significantly greater than the percentage of  $\gamma\delta$  cells found in control subjects ( $3.08\% \pm 2.4\%$ ,  $p < 0.008$ , two-tailed Student's *t* test) (Fig. 2). Furthermore, the maximal levels significantly exceeded the normal values in both the *P. falciparum* and the *P. vivax* subgroups ( $17.02\% \pm 7\%$  and  $21\% \pm 14\%$ , respectively,  $p < 0.0001$  and  $p < 0.002$ ). In contrast, the mean of the peak levels of  $\alpha\beta$ , CD4<sup>+</sup>, and CD8<sup>+</sup> T cells did not significantly differ from the normal values ( $44\% \pm 17\%$  vs  $58\% \pm 14\%$ ,  $37\% \pm 16\%$  vs  $46\% \pm 12\%$ , and  $28\% \pm 8\%$  vs  $22\% \pm 3\%$  for the respective subsets vs the control values) (Fig. 2). These results indicate that a significant expansion of the percentage of  $\gamma\delta$  cells occurred in the patients with malaria within the period of observation after the acute infection.

### $\gamma\delta$ Cells in relation to the clinical disease

To analyze the kinetics of the  $\gamma\delta$  cell expansion after the acute malarial infection, we calculated the mean level of  $\gamma\delta$  cells of all the samples obtained during days 1 to 10, 11 to 30, and 31 to 63 after the onset of clinical symptoms. As shown in Table I, although the mean percentage of  $\gamma\delta$  cells detected in the PBMCs during the first 10 days after the onset of the acute infection exceeded the mean found in normal individuals, the difference was not statistically significant. The mean levels of the  $\gamma\delta$  cells were, however,

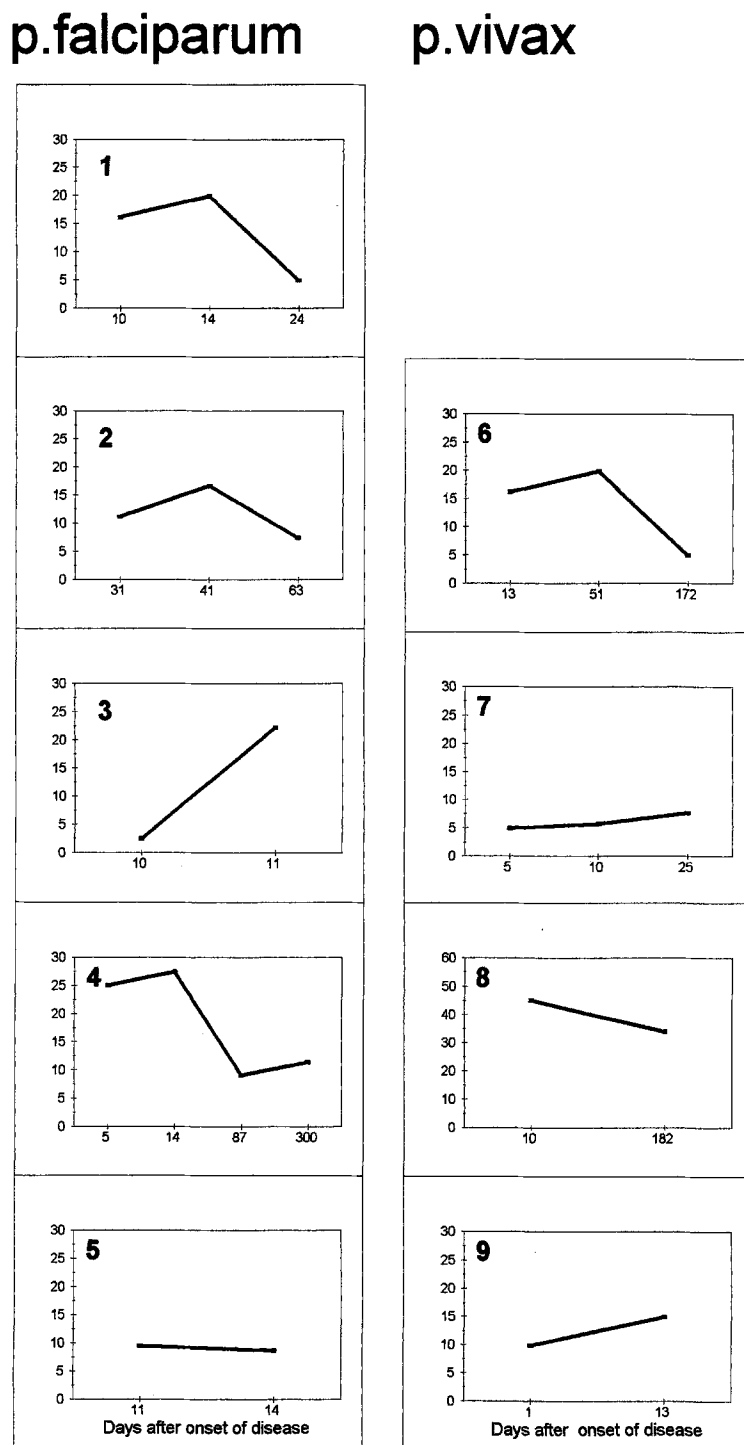


**FIG. 1.** Levels of  $\gamma\delta$  T cells in patient PBMCs as a function of time after acute infection. Percent of  $\gamma\delta$  T cells in the PBMCs of patients with *P. falciparum* (patients 1 to 5) and *P. vivax* (patients 6 to 9) infections. y axis, Percent of PBMCs; x axis, days after onset of clinical symptoms.

significantly higher than the normal values in blood samples drawn during days 11 to 30 and 31 to 63 after the infection. Thereafter, the relative number of  $\gamma\delta$  cells in the PBMC population

appeared to decline, and the mean value in five patients with a history of malaria occurring more than 1 year before the evaluation were no different from normal levels of  $\gamma\delta$  cells.

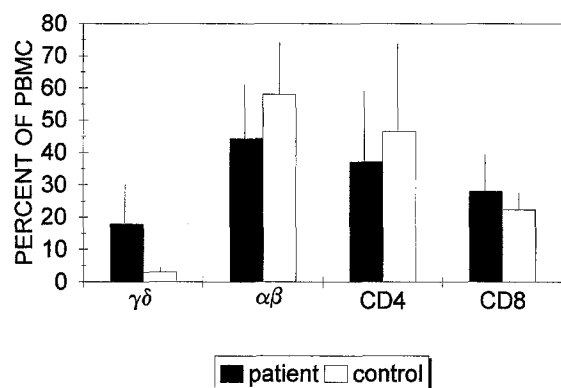


**FIG. 2.** Peak levels of T-cell subsets in malaria. Filled bars represent the mean percentage of the peak values for each of the indicated T-cell subsets in the PBMCs of the nine patients with malaria during the entire follow-up period. Open bars represent mean levels of corresponding T-cell subsets of the nine normal control subjects. Standard deviations are indicated by perpendicular lines.

### $\alpha\beta$ , CD4<sup>+</sup>, and CD8<sup>+</sup> cells in relation to the clinical disease

As shown in Table II, there were no significant changes relative to normal values in the percentages of  $\alpha\beta$ , CD4<sup>+</sup>, or CD8<sup>+</sup> cells in the PBMC population during the early 1 to 10-day period after infection. During the period in which  $\gamma\delta$  cells increased significantly (days 11 to 63 after onset of clinical symptoms),  $\alpha\beta$  T cells declined slightly but did not decrease to values significantly less than normal (Table II). However, CD4<sup>+</sup> cells did decrease to significantly low values between days 11 and 63, whereas the mean level of CD8<sup>+</sup> cells increased to values significantly above normal.

### Subsets of $\gamma\delta$ cells in acute malaria

The V $\delta$  genes expressed by the expanded  $\gamma\delta$  cells, during the 11 to 63 days after onset of the clinical syndrome, were determined by staining patient and control PBMCs with mAbs to V $\delta$ 1 and V $\delta$ 2. The results are shown in Fig. 3. The V $\delta$ 2<sup>+</sup> cells comprised 8.6%  $\pm$  5.49% of the PBMC population compared with a normal value of 2.93%  $\pm$  1.3% ( $p < 0.01$ ). V $\delta$ 1<sup>+</sup> cells comprised 3.775%  $\pm$  4.29% of the patient PBMC population and 2.76%  $\pm$  2.3% of the normal PBMC population (difference not significant). However, in one patient with *P. falciparum* infection, V $\delta$ 1<sup>+</sup> cells exceeded V $\delta$ 2<sup>+</sup> cells in three consecutive determinations (11%  $\pm$  2.8% [mean V $\delta$ 1<sup>+</sup>] vs 7.33%  $\pm$  2.4% [mean V $\delta$ 2<sup>+</sup>]  $p < 0.037$ , Student's *t* test).

**TABLE I.** Percent of  $\gamma\delta$  cells among the PBMCs during indicated time intervals after onset of clinical symptoms

| Days after clinical symptoms | Mean | SD  | <i>p</i> Value |
|------------------------------|------|-----|----------------|
| 1-10 ( <i>n</i> = 6)         | 8.7  | 7.6 | 0.07           |
| 11-30 ( <i>n</i> = 9)        | 13.6 | 6.8 | 0.0007         |
| 31-63 ( <i>n</i> = 4)        | 13.7 | 4.8 | 0.0005         |
| 87-300 ( <i>n</i> = 3)       | 8.5  | 2.7 | 0.015          |
| >360 ( <i>n</i> = 5)         | 3.4  | 3.2 | NS             |
| Control ( <i>n</i> = 9)      | 3.03 | 2.4 |                |

The number of determinations (*n*), mean, standard deviation (SD), and *p* value for comparison of the mean with the levels in the normal control subjects are indicated. The exceptionally high values of 45%  $\gamma\delta$  (day 10) and (day 182) cells for patient 8 (see Fig. 1) were omitted from the calculations, because these values exceeded the mean by more than 2 standard deviations.

NS, Not significant.

**TABLE II.** Changes in  $\alpha\beta$  TCR<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells in patient PBMCs during days 1 to 10 and 11 to 63 after acute malaria

| Surface antigen                    | Mean | SD    | <i>p</i> Value |
|------------------------------------|------|-------|----------------|
| Days 1-10                          |      |       |                |
| $\alpha\beta$ TCR ( <i>n</i> = 7)  | 46.3 | 17.5  | 0.2            |
| CD4 ( <i>n</i> = 6)                | 37.3 | 15.94 | 0.2            |
| CD8 ( <i>n</i> = 6)                | 17.7 | 6.3   | 0.06           |
| Days 11-63                         |      |       |                |
| $\alpha\beta$ TCR ( <i>n</i> = 11) | 37.3 | 15.9  | 0.2            |
| CD4 ( <i>n</i> = 9)                | 30.3 | 8.5   | 0.01           |
| CD8 ( <i>n</i> = 9)                | 31.3 | 7.44  | 0.008          |

The table shows the mean, standard deviation (SD), and *p* value comparing the means with values obtained from nine normal individuals. See text and Fig. 2 for normal values of the subsets.

## DISCUSSION

This analysis of patients with acute malaria reveals a significant perturbation of the  $\gamma\delta$  T-cell subset. Strikingly, the normally small subset of  $\gamma\delta$  cells expands progressively during the first 2 months after infection, followed by successful treatment of patients with either *P. falciparum* or *P. vivax* infections. Although V $\delta$ 2<sup>+</sup> cells usually account for most of the expanding cells, V $\delta$ 1<sup>+</sup>  $\gamma\delta$  cells may also proliferate in the PB, and in some patients they constitute the majority of the cells.

The role of the  $\gamma\delta$  subset of T lymphocytes in immune responses to infectious agents is becoming

increasingly recognized, because subsets of these cells have been noted to be involved in mycobacteria, *Salmonella*, *Brucella*, *Leishmania*, and *Listeria* infections in human beings and in experimental animals.<sup>5, 11</sup> It is believed that these cells recognize superantigens or antigens of these microorganisms and are driven to proliferate.<sup>5, 12, 13</sup>

Previously, it was found that after treatment of acute *P. falciparum* infections,  $\gamma\delta$  cells became elevated in the PB for at least 3 weeks after infection.<sup>8, 9</sup> Our results, from a study of a cohort of patients infected with either *P. vivax* or *P. falciparum*, are generally concordant with this basic observation but indicate that the elevated levels of  $\gamma\delta$  cells continue to increase for up to 2 months, declining thereafter (Fig. 2 and Table I). Furthermore, we found that  $CD4^+$  cells decreased, whereas  $CD8^+$  cells increased in the late postmalaria period (Table II). Thus although patients recover clinically from *P. falciparum* or *P. vivax* infection after several days, their cellular immune system remains perturbed for several months. It is possible therefore that antigens or superantigens inducing the  $\gamma\delta$  cell expansion persist in vivo for at least several months after acute infection. Alternatively, the  $\gamma\delta$  cells may require several months to clear from the circulation. It appears likely that similar mechanisms favoring the expansion of the  $\gamma\delta$  T-cell subset occur in both *P. vivax* and *P. falciparum* infections because there was no significant difference in the degree of proliferation of  $\gamma\delta$  cells between patients with these two diseases. The decrease in the level of  $CD4^+$  cells and the slight increase in the levels of  $CD8^+$  cells concomitant with  $\gamma\delta$  cell expansion suggest that the majority of expanding  $\gamma\delta$  cells are  $CD4^-CD8^-$  or  $CD4^-CD8^+$ , phenotypes characteristic of most PB  $\gamma\delta$  T cells, or that a decline in  $CD4^+$  TCR  $\alpha\beta$  cells occurs after malarial infection, concomitant with an increase in  $CD8^+$  TCR $\alpha\beta$  lymphocytes.<sup>2</sup> Dual fluorescence studies are required to evaluate the changes in the T-cell subsets in further detail.

As shown in Fig. 3, most of the expanding  $\gamma\delta$  cells in the PB usually express the V $\delta 2$  gene, although the V $\delta 1^+$  population may also expand in some patients. The V $\delta 2^+$  gene is usually co-expressed with V $\gamma 9$ , and most malaria-responsive clones in vitro express V $\gamma 9$  and V $\delta 2$  genes.<sup>5, 13</sup> Thus the expanded V $\delta 2^+$  cells in our patients presumably include *Plasmodium*-reactive lymphocytes. Recent studies indicate that V $\gamma 9$ V $\delta 2^+$   $\gamma\delta$  T lymphocytes recognize nucleoside triphosphates and prenyl pyrophosphate derivatives, and are triggered to release lymphokines and to prolifer-

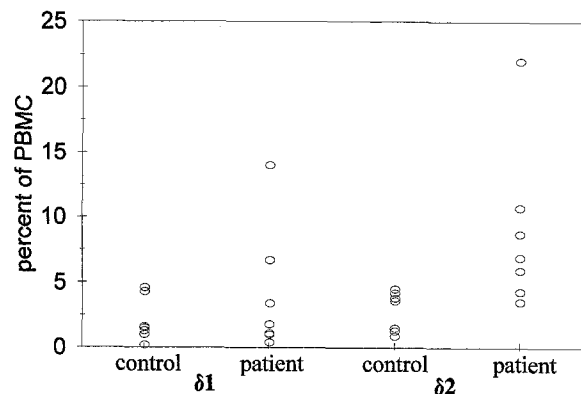


**FIG. 3.** V $\delta 1^+$  and V $\delta 2^+$  subsets of  $\gamma\delta$  cells in PBMCs of patients with malaria. PBMCs of seven patients with malaria (days 11 to 63 after acute infection) and seven normal individuals were stained with mAbs to V $\delta 1$  or V $\delta 2$ , and the percent positive cells was recorded ( $y$  axis). When two or more evaluations were available for the patients, the peak value is presented.

ate, suggesting that these compounds may be involved in the  $\gamma\delta$  cell expansion in patients with malaria.<sup>14, 15</sup> The mechanism for the expansion of the V $\delta 1^+$  population is less clear, because V $\delta 1$  is usually expressed in V $\gamma 9^-$   $\gamma\delta$  cells, although one report has shown expanded V $\gamma 9^+$ V $\delta 1^+$  cells in a patient with malaria.<sup>16</sup> Interestingly, V $\gamma 9^-$ V $\delta 1^+$  cells are increased relative to V $\delta 2^+$  lymphocytes in the PB of immune patients residing in malaria endemic regions, and moreover, the  $\gamma\delta$  cells do not expand further in the PBMCs of these patients after re-infection.<sup>17</sup> This suggests that V $\delta 1^+$  malaria-reactive cells may suppress the V $\delta 2^+$  response in these patients. In vitro studies of interactions of V $\delta 1^+$  and V $\delta 2^+$  subsets from patients with malaria may help to clarify this possibility.

A recent study showed marked elevations of  $\gamma\delta$  cells in nonimmune patients with *P. vivax* infections, which occurred primarily during the acute febrile response and correlated with clinical signs and symptoms, thus implicating  $\gamma\delta$  cells in the pathogenesis of the clinical syndrome.<sup>18</sup> In our study the samples were not specifically drawn in association with high fever, even in the early samples (obtained from days 1 to 10), which may explain the more delayed kinetics of expansion we detected. Because our results show that elevated levels of  $\gamma\delta$  cells continued to increase in the PB after subsidence of the acute infection and clearing of the clinical symptoms, our data indicate that the acute clinical symptoms cannot be attributed solely to increased levels of  $\gamma\delta$  cells in the circulation (Table I). Rather, it is likely that during the acute

infection in the presence of the antigen, triggering of  $\gamma\delta$  cells results in an early release of lymphokines, such as interferon and tumor necrosis factor, which may augment clinical symptoms, as previously suggested.<sup>5, 12</sup> The delayed expansion of the cells in the PB, however, suggests that these cells could also play a role in the development of immunity at a later phase. Recent studies support our findings.<sup>19</sup>

In summary, the bulk of the data support the idea that  $V\delta 2^+$ , and to a lesser degree,  $V\delta 1^+$   $\gamma\delta$  lymphocytes react polyclonally during acute *P. vivax* and *P. falciparum* malaria and become an increasingly major T-cell subset in the PBMCs of the patients during the first 2 months after infection. How individual subsets of these cells are involved in the clinical response and protective immunity remains to be determined.

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