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Human TcR$\gamma\delta^+$ lymphocyte response on primary exposure to *Plasmodium falciparum*

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SUMMARY

In 29 patients experiencing their first *P. falciparum* malarial attack, blood levels of TcR$\gamma\delta^+$ lymphocytes were studied from the onset of infection to up to 6–9 months later. Blood TcR$\gamma\delta^+$ lymphocytes, revealed using the TcRδ1 monoclonal antibody (MoAb), were increased both in absolute and relative numbers. Alterations lasted for up to 3–4 months following the attack. A TiγA/BB3 reactive Vγ9 subset was preferentially amplified. *In vitro*, TcR$\gamma\delta^+$ lymphocytes from both malaria-sensitized and unprimed donors responded to *P. falciparum* schizont extract (PFSE). PFSE-stimulated polyclonal T cell lines consisted principally in TcR$\gamma\delta^+$ cells with a TiγA$^+$/BB3$^+$ phenotype. Several TcR$\gamma\delta^+$ T cell clones obtained from patients recovering from acute malarial attack were maintained in the presence of PFSE and autologous irradiated PBL. They belong to the Vγ9 subset. In long-term cultures, TcR$\gamma\delta^+$ clones progressively lost their capacity to react to PFSE antigen while they were able to proliferate and to exert cytotoxic activity in response to autologous TcR$\alpha\beta^+$, PFSE-specific T lymphocyte clones. This suggests that regulatory interactions occur between activated TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ cells generated by *P. falciparum*. Sequential variations in blood TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ lymphocyte levels after primary exposure to *P. falciparum* suggest that such regulatory interactions may occur *in vivo*.

Keywords T lymphocytes TcR$\gamma\delta$ *Plasmodium falciparum* malaria

INTRODUCTION

Interaction between *P. falciparum* and the human host's immune system results in the triggering of complex mechanisms which regulate immune responses to the parasite. T lymphocyte recognition of blood stage *P. falciparum* antigens is readily demonstrable *in vitro* when studying cells from *P. falciparum* malaria-sensitized individuals [1]. In addition, it has been shown that lymphocytes from malaria-naive previously unsensitized donors can be stimulated to proliferate and secrete lymphokines when cultured in the presence of plasmodial molecules [2–6].

Beside TcR recognition of 'cross reactive' antigenic determinants, this is consistent either with 'superantigen'-type interactions of parasitic molecules with TcR, or alternatively with mitogen-like activation via non-TcR molecules. It has also been frequently found that T lymphocytes from previously sensitized patients are hyporesponsive in the acute phase of malarial infection, especially to plasmodial antigens [7–13]. In addition to transient modifications in blood T lymphocyte counts, i.e. a decrease in CD3$^+$ and CD4$^+$ and an increase in CD8$^+$ lymphocytes, this appears, at least partially, to be a consequence of suppressor cell regulation [11–16].

In this context, the recent finding of increased TcR$\gamma\delta^+$ lymphocytes in acute malaria patients may indicate another regulatory mechanism [17,18]. Most human T cell clones specific for asexual and sexual blood stage epitopes previously described were major histocompatibility complex (MHC)-restricted, TcR$\alpha\beta^+$ cells [19–24]. Recent reports and the present work show that TcR$\gamma\delta^+$ lymphocytes are able to proliferate in response to malarial antigens [25–27].

We report here that the TcR$\gamma\delta^+$ subset is increased for months in patients experiencing their first *P. falciparum* malaria attack. Long-term culture of lymphocytes from malaria-sensitized or naive donors in the presence of *P. falciparum* schizont extract (PFSE) results in a preferential TcR$\gamma\delta^+$ lymphocyte response. In long-term cultures, TcR$\gamma\delta^+$ clones were found to be autoreactive to autologous TcR$\alpha\beta^+$ lymphocytes raised in the

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presence of PFSE. Collectively, the data suggest that TcR$\gamma\delta^+$ lymphocytes generated upon contact with *P. falciparum* regulate TcR$\alpha\beta^+$ lymphocyte-mediated immune responses to the parasite.

MATERIAL AND METHODS

Blood donors

An acute *P. falciparum* attack was diagnosed by a trained parasitologist on blood smears obtained from 29 adult Caucasian patients (21 males and eight females, 23–49 years old). All patients lived in Europe, and experienced their first attack during or following a stay in Africa, as ascertained by the lack of previous stay in malaria-prone areas and their medical records. In 12 patients, blood was obtained on day 0, i.e. on the day of admission in the hospital. The first blood samples were taken before initiation of therapy. Subsequent studies were carried out for up to 6 months after the attack. In 17 patients, study was initiated 1–4 weeks after the beginning of therapy. All studies were conducted while patients resided continuously in the Paris area. Fifteen age-matched blood bank donors with no previous history of malaria or anti-plasmodial serum antibodies, served as control donors.

Blood lymphocyte isolation and cultures

Blood was diluted twice with RPMI 1640 medium containing 100 IU/ml of heparin (Liquemine, Roche Laboratories, France) and mononuclear cells were separated using Ficoll-metrizoate gradients (900 *g*, 20 min, 10°C). Cells were washed with heparinized medium, resuspended, and set up in short-term cultures in round-bottomed 96-well microplates at 1×10^6 cells/ml in RPMI 1640 containing penicillin and streptomycin (50 U/ml and 50 mg/ml respectively), 10% v/v heat-inactivated (56°C, 30 min) human serum from AB Rh$^+$ donors, and freshly supplemented with 2 mM glutamine. To establish cell lines, cells were incubated in 75 cm^2 tissue culture flasks at $10–15 \times 10^6$ cells per flask in the presence of PFSE alone, for 8 days (37°C, 95% humidity, and 5% CO_2). At day 9, fresh medium containing 10 IU/ml of recombinant IL-2 (rIL-2, a kind gift from Glaxo, Geneva, Switzerland) was added. As a second step, the cells were further incubated in 175 cm^2 flasks for 5–6 days until they reached a concentration close to 1×10^6/ml. They were then restimulated in the presence of irradiated autologous (ia) PBL and PFSE. T cell lines were cloned, and TcR$\alpha\beta^+$ or TcR$\gamma\delta^+$ clones were maintained in culture as previously described [24]. For proliferation measurements, cultures were pulsed with tritiated thymidine (^{3}H-TdR, 2 Ci/mmol, 1 μCi/well) and incubated for an additional 6–9 h. Cells were harvested and ^{3}H-TdR incorporation was measured in a scintillation counter.

Schizont stage antigen extract

Parasites were cultured using the original method of Trager & Jensen [28], and cultures were synchronized by treatment with sorbitol. Infected erythrocytes were isolated at the schizont stage, by centrifugation on a Percoll-sorbitol discontinuous gradient, and the schizont-enriched fractions contained more than 95% schizonts. The preparation was then pelleted, and 4 volumes of water were added to constitute a stock solution (1:1) of PFSE, which was immediately divided into 100 ml fractions and frozen in liquid nitrogen until use. Total protein concentration was, on average, 60 mg/ml, and PFSE was used in cultures at a final dilution of 1:1000. Normal erythrocyte extract treated in the same way was used as a control, as previously described [24].

Non-plasmodial antigens used in cultures

The following antigens were used: tetanus toxoid (10 μg/ml; 3000 Lf, a kind gift from Institut Merieux, Marcy l'Etoile, France), candidin (1:4000 of the stock solution; Institut Pasteur, Paris, France), tuberculin purified protein derivative (250 IU/ml; Institut Pasteur).

MoAbs to T cell surface antigens

MoAbs were directed to the following membrane molecules: CD3, CD4, CD8 (Coulter Clone, Margency, France), HLA-DR (Becton Dickinson, Pont de Claix, France), TcR$\alpha\beta$ (WT31, Becton Dickinson) which identifies a non-polymorphic determinant of the TcR$\alpha\beta$ heterodimer, and TcR$\gamma\delta$: the MoAb TcRδ1 recognizes a constant region determinant of the δ chain and therefore all $\gamma\delta^+$ T cells (T Cell Science, Cambridge, MA); the MoAb δTCS1 binds the variable Vδ1–Jδ1 or Vδ1–Jδ2 products and defines a subset of TcR$\gamma\delta^+$ cells (T Cell Science); the MoAb TigA binds a Vγ9-encoded epitope and thus delineates a subset of TcR$\gamma\delta^+$ cells (T Cell Science); the MoAb BB3 (kindly provided by Dr T. Hercend, Institut Gustave Roussy, Villejuif, France) is a Vδ2 specific MoAb. MoAbs were used either directly fluorochrome conjugated (fluorescein or phycoerythrin), or were revealed using fluorochrome-conjugated goat anti-mouse immunoglobulins (Tebu, France).

Immunofluorescence staining and flow fluorocytometry (FCM) analysis

For single colour direct immunofluorescence analysis, cells were incubated (15 min at 4°C) in PBS, 0·5% bovine serum albumin (BSA), washed, and reincubated (30 min, 4°C) with either the appropriate fluorescent MoAb dilution or an irrelevant control isotype. For two-colour direct immunofluorescence, cells were further incubated under the same conditions with the second fluorescent MoAb. For indirect immunofluorescence studies, unlabelled MoAb was revealed using a fluorescent second anti-mouse immunoglobulin isotype antibody [24]. After staining, all samples were washed, resuspended and fixed in 0·5% paraformaldehyde until FCM was performed. Analysis was carried out using a laser cytofluorometer (FACStar, Becton Dickinson). Dead cells and debris were excluded by conventional scatter gating or propidium iodide staining. Blood lymphocytes were identified based on the characteristic low forward angle and 90° light scatter profiles. Absolute counts of CD3$^+$ T lymphocytes and of CD3$^+$ TcR$\gamma\delta^+$ T lymphocyte subpopulations in blood samples from control donors and patients were calculated using the total lymphocyte counts performed in a haemocytometer.

Selective depletion of CD4$^+$ and CD8$^+$ cells

Depletion of CD4$^+$ or CD8$^+$ cells was performed by using magnetic beads coated with anti-CD4$^+$ or anti-CD8$^+$MoAbs (Dynal, Oslo, Norway). Briefly, washed beads were added in a test tube to isolated mononuclear cells with a bead:target cell ratio of 10:1, and incubated for 30 min at 4°C with continuous gentle rotation. After the addition of PBS with 10% decomplemented human serum, test tubes were placed for 3–5 min in a magnetic field and the supernatant depleted of CD4$^+$ or CD8$^+$ T cells was transferred into a culture flask. Two consecutive

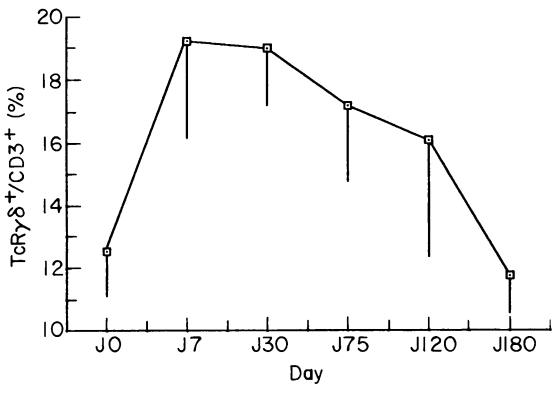

Fig. 1. Time course of blood TcRγδ⁺ T cells during the course of acute *P. falciparum* malaria. Results are given as mean TcRγδ⁺ cells/mm³. Values observed from D7 to D120 were statistically different from the D0 value ($P<0.001$). Bar indicates 1 s.d. Pooled data from a total of 29 different patients are shown. In 15 non-infected adults studied simultaneously, the mean value was 118 ± 69/mm³.

incubations with magnetic beads were completed, allowing at least 95% depletion of a particular T cell subset.

Cytotoxicity assays

Target cells were either the myeloid NK-sensitive K562 line, the NK-resistant Burkitts lymphoma Daudi, T cell leukaemia Molt-4 lines or autologous TcRαβ⁺ T cells. Cells were labelled with 100 μCi of ^{51}Cr for 45 min at 37°C, and washed three times. Cells, 1×10^4 in 100 μl, were incubated in flat-bottomed microplates with 100 μl of TcRγδ⁺ lymphocytes or autologous CD4⁺ TcRαβ⁺ T cells at 80:1, 40:1, 10:1, 5:1 effector/target ratios. After 5 h at 37°C, the plates were centrifuged and 100 μl of supernatant were removed and the activity counted in a gamma counter. Triplicates were used for each experiment, and specific lysis was calculated in percentages as the ratio: (experimental release (%) minus spontaneous release (%°)/[100−(spontaneous release (%)]×100. Spontaneous ^{51}Cr release ranged between 9 and 12% after 5 h of incubation.

Statistical analysis

Variance analysis was performed using 'parametric' (Student's *t*-test) or 'non-parametric' (Mann–Whitney) test when appropriate. The Spearman rank order correlation coefficient R_s was used for evaluation of parameter association. For all tests, P values less than 0·05 were considered significant.

RESULTS

In vivo *increase of TcRγδ⁺ blood lymphocytes in patients with acute* P. falciparum *malaria*

On day 0, total and CD3⁺ lymphocyte counts averaged 1435/mm³ (range 315–2808) and 949/mm³ (range 250–1580) respectively. On day 7, mean values for blood total and CD3⁺ lymphocytes returned to usual values for healthy adults (1000–3500 lymphocytes/mm³ and 740–2900 CD3⁺ lymphocytes/mm³ respectively). From day 0, the percentage of TcRγδ⁺ lymphocytes (reactive with the TcRδ1 antibody) increased and peaked on day 7. It then remained at a plateau for several weeks and gradually decreased over 3 to 4 months following the attack (Fig. 1). Within TcRγδ⁺ T cells, less than 9% also expressed CD8 antigen while none of them were CD4⁺ (data not shown). The TiγA reactive population was preferentially amplified since δTCS1⁺ (Vγ1) and TiγA⁺ (Vγ9) lymphocytes accounted for 26·5–31% and 57–73·5% of TcRγδ⁺ respectively in three representative patients (Table 1). Reactivities with γTCS1 and TiγA were mutually exclusive. More than 97% of cells reacting with TiγA were also labelled with BB3. Interestingly, all TcRγδ⁺ lymphocytes with detectable membrane HLA-DR molecules were TiγA⁺ and δTCS1 negative. From sequential measurements in the weeks following the *P. falciparum* attack, a negative correlation was found between the numbers of CD4⁺ (TcRαβ⁺) lymphocytes (expressed as CD4⁺/CD3 ratios) and of TcRγδ⁺ lymphocytes ($R_s=-0.65$, $P<0.05$). No correlation was found between CD8⁺ and TcRγδ⁺ T lymphocytes ($R_s=0.091$, $P=0.803$).

In vitro *amplification of TcRγδ⁺ cells in short term lymphocyte cultures stimulated with PFSE*

In cultures of PBL from control subjects, as well as malaria patients, addition of PFSE resulted in an amplification of the TcRγδ⁺ cell subset (Table 2 and Fig. 2). Percentages of TcRγδ⁺ lymphocytes were comparatively high in cultures established with lymphocytes obtained from patients undergoing an acute malarial attack, and to a more variable extent, in cultures of PBL from individuals studied 1 to 6 months after exposure to *P. falciparum*. Most blastic cells reacted with the TiγA or BB3 MoAbs, and $82\cdot7\pm5\cdot3\%$ of TcRγδ⁺ lymphocytes were HLA-DR positive. On average, the number of TcRγδ⁺ cells in culture on day 18 exceeded the initial figure 10-fold.

In control cultures of PBL from the same donors performed in the presence of non-parasitized erythrocyte extract or the soluble antigens tetanus toxoid, candidin or tuberculin-purified protein derivative, percentages of TcRγδ⁺ cells observed in cultures at days 9, 18 and 27 averaged 5%, 2%, 1%, and 8·5% respectively (data not shown).

Characterization of TcRγδ⁺ T cell polyclonal lines and clones

Long-term T cell lines restimulated with PFSE in the presence of ia PBL usually exhibited a regular increase in the percentage of TcRγδ⁺ lymphocytes (Table 2). From day 30 of culture, repeated restimulation by ia PBL and PFSE was not consistently successful in maintaining the enrichment in TcRγδ⁺ cells, especially in cultures from control donors. For this reason, cloning was optimally performed before day 30. In established cloned lines tested regularly, more than 80% of cells were labelled with the TcRδ1 MoAb. The majority of clones reacted with TiγA and BB3 MoAb, whereas less than 2% reacted with the δTCS1 MoAb.

In vitro *interactions between autologous, PFSE-stimulated TcRαβ⁺ and TcRγδ⁺ clones*

In cultures studied sequentially, the proportion of CD4⁺ lymphocytes decreases progressively (Table 2). To further explore interactions between CD4⁺ and TcRγδ⁺ cells in culture, depletion of CD4⁺, TcRαβ⁺ lymphocytes was performed using anti-CD4 coated magnetic beads. Early depletion (1st to 3rd day of culture) resulted in a 1·8- to 3·6-fold decrease in the percentage of TcRγδ⁺ cells observed on day 7 (mean decrease $66\cdot5+14\%$). CD4⁺ depletion after culture day 4 did not significantly affect the ratio of TcRγδ⁺ T cells observed on day

Table 1. Evolution of blood TcR$\gamma\delta^+$ lymphocytes and lymphocyte subsets in three patients in the course of acute *P. falciparum* malaria (mean absolute count/mm^3 ± 1 s.d.)

	TcR$\gamma\delta^+$ cells	δTCS1 reactive cells	TiγA reactive cells	TiγA$^+$/δTCS1$^+$
D0	113±85	16±11	96±20	6
D7	339±143	59±22	293±89	5
D14	375±57	86±17	284±46	3·3
D30	309±31	55±7	257±102	4·6
D180	121±21	29±16	99±15	3·4
Control adults	93±60	24±15	74±20	3·1

Table 2. *In vitro* amplification of TcR$\gamma\delta^+$ lymphocytes in lymphocyte cultures in the presence of PFSE

Culture time (day)	CD4$^+$	CD8$^+$	TcR$\gamma\delta^+$
Control donors (n=4)			
DO	66·7±3·1	26·4±2·4	6·4±0·3
D7	59·6±5·3	23·8±5·0	16·7±0·3
D12	49·1±8·2	29·1±8·9	21·8±2·1
D17	28·7±9·7	27·3±8·6	43·3±5·3
Recovering patients (n=5)			
D0	67·2±2·6	26·3±2·9	6·2±1·3
D7	61·4±3·5	25·6±4·1	13·9±2·9
D16	49·1±8·2	29·1±8·9	21·8±2·1
D24	50·3±9·5	16·5±9·1	33·3±2·4
D32	42·5±13·1	13·4±11·9	44·1±3·7
D45	13·8±5·9	31·9±8·2	54·3±3·3
D60	18·8±7·8	15·7±9·7	65·7±4·9

Results expressed in percentages (mean values ± 1 s.d.) in per cent of the CD3$^+$ lymphocyte counts.

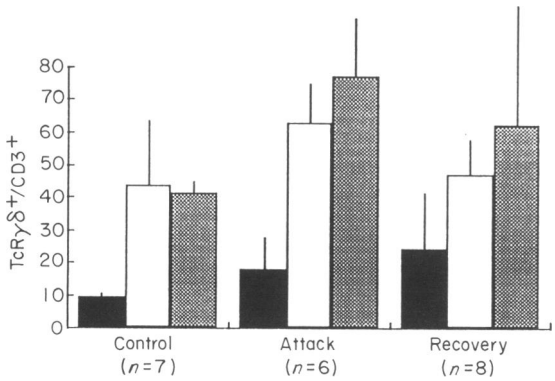

Fig. 2. Increase of TcR$\gamma\delta^+$ T lymphocytes in PBL cultures from naive control donors, patients studied on day 0 of an acute *P. falciparum* attack, and adults who experienced an attack 1–6 months before the study. Days in culture: (■) = 9, (□) = 18, (▨) = 27. n = number of donors. Bar indicates 1 s.d.

10. Whatever the culture day, CD8$^+$ cell depletion did not alter the number of TcR$\gamma\delta^+$ cells (data not shown). Interactions between TcR$\alpha\beta^+$ and $\gamma\delta^+$ lymphocytes were then explored at the clonal level. TcR$\alpha\beta^+$ clones consistently responded to PFSE in association with presenting ia PBL. In contrast, the ability of TcR$\gamma\delta^+$ clones to proliferate in the presence of PFSE and ia PBL, decreased progressively in the weeks following the cloning procedure. Out of six PFSE-unreactive TcR$\gamma\delta^+$ clones tested, four were capable of proliferation in response to autologous, irradiated, PFSE specific TcR$\alpha\beta^+$ clones. Table 3 shows results obtained from experimental and control cultures performed simultaneously using a typical, antigen-unreactive TcR$\gamma\delta^+$ clone. In contrast, when two TcR$\gamma\delta^+$ clones were tested under

Table 3. Proliferative response of the TCR$\gamma\delta^+$ TK19 clone to TCRab, PFSE specific T cell clones

Cells in culture	^{3}H-TdR incorporation
TK19 alone	410
TK19+PFSE	450
TK19+ia PBL	230
TK19+PFSE+ia PBL	760
TK3+ia PBL	1200
TK3+ia PBL+PFSE	42900
iTK3+ia PBL+PFSE	1510
TK6+ia PBL	1400
TK6+ia PBL+PFSE	9230
iTK6+ia PBL+PFSE	1850
TK7+ia PBL	1300
TK7+ia PBL+PFSE	11150
iTK7+ia PBL+PFSE	550
TK19+iTK3*	23870
TK19+iTK6*	5800
TK19+iTK7*	13320
TK19+ia PBL+PFSE+iTK19	240

Results are given in mean ct/min per culture well (rounded up to the nearest 10 value). In all experiments, 1 s.d. averaged 11·8% of the mean.

20000 cloned cells and 10^5 irradiated (ia PBL or ia TK clone) cells were added per well. TK19 is a TCR$\gamma\delta^+$ (CD4$^-$, CD8$^-$, TCR$\alpha\beta^-$) T cell clone. TK3, TK6, TK7 are TCR$\alpha\beta^+$ (CD4$^+$, CD8$^-$), PFSE-specific T cell clones.

* TK3, TK6 and TK7 were obtained from 3-day cultures in the presence of ia PBL and PFSE, and irradiated.

Table 4. Cytotoxic activity of two PFSE-specific TcRγδ⁺ clones to CD4⁺, αβ⁺ autologous clones

	CD4⁺, TcRαβ⁺ target cells											
	TK7 clone				TK6 clone				TK1 clone			
	80:1	40:1	10:1	5:1*	80:1	40:1	10:1	5:1*	80:1	40:1	10:1	5:1*
Effector (TcRγδ⁺, CD4⁻, CD8⁻) cells												
TK19 clone	25†	30	5	0	10	27	49	0	18	20	5	0
TK25 clone	32	35	7	0	9	20	34	5	13	17	0	0

* Effector/target ratio.

† See expression of results in Material and Methods.

the same conditions in the presence of three TcRαβ⁺ clones with an irrelevant specificity (one specific for tetanus toxoid, two reactive to allogeneic stimulus), no proliferative response was observed (data not shown).

The reactivity of TcRγδ⁺ lymphocytes to autologous TcRαβ⁺ T cells was also evaluated in a cell-mediated cytotoxicity assay. The clones TK19 and 25 exerted a significant cytotoxic effect against autologous TcRαβ⁺ cell clones (Table 4). In addition, the TK19, TK25 and two additional TcRγδ⁺ clones tested had a low cytotoxic activity against lymphoblastoid cell lines. At a 50:1 effector/target cell ratio, average lysis ratios were 24%, 10% and 40% for the K562, the Daudi and the Molt-4 cell lines respectively.

DISCUSSION

The majority of T lymphocytes express the TcRαβ heterodimer, whereas the TcRγδ is found on a minor subset of T lymphocytes with defined tissue localization [29,30]. Increased TcRγδ⁺ cells have been observed in the periphery and occasionally in the spleen of *P. falciparum*-infected patients, as well as in the spleen of *P. chabaudi*-infected mice, suggesting that TcRγδ cells are involved in the immune response to malarial parasites [17,18,31,32].

In this report, our previous observation of long-lasting increases in blood levels of TcRγδ-bearing lymphocytes in patients during and following an acute *P. falciparum* attack was confirmed. In man, *in vivo* local or restricted accumulation of Tγδ⁺ T lymphocytes was previously observed in other infections, including leprosy and leishmaniasis [33–35]. Increased numbers of TcRγδ⁺ cells have been shown in rheumatoid arthritis, sarcoidosis and in the gut epithelium of patients with coeliac disease [36–38]. In malaria patients, this TcRγδ⁺ T cell expansion appears to be more systemic.

Abnormalities in the development of lymphocyte lineages and regulatory interactions are presumably also responsible for alterations of the TcRγδ⁺ subset since increased blood TcRγδ⁺ levels have been reported in primary immunodeficiency states and in allogeneic bone marrow recipients [30,39,40].

In vitro, expansion of TcRγδ⁺ lymphocytes from malaria patients in the presence of PFSE mimics that observed in blood. TcRγδ⁺ T cell heterogeneity was delineated using MoAbs TiγA and BB3 which detect the product of rearranged Vγ9 segment and δTcR1 (corresponding to a Vδ1 segment). Both *in vivo* and *in vitro*, the majority of TcRγδ⁺ lymphocytes generated on contact with *P. falciparum* expressed Vγ9-encoded epitopes and HLA-DR, but not usually CD8.

In agreement with previous observations of the stimulation of TcRαβ⁺ cells from control, naive donors by *P. falciparum*, we and others have found that TcRγδ⁺ lymphocytes from naive donors recognized *P. falciparum in vitro* [25–27]. Similarly, with the *in vivo* situation, we found a preferential amplification of the Vγ9⁺ subset. This may be consistent with responses to mitogen-type (or 'superantigen') molecules, as the Vγ9 subset is predominantly represented among peripheral blood TcRγδ⁺ lymphocytes [41–43]. Against this hypothesis are reports that the majority of proliferating lymphocytes triggered by *P. falciparum* belong to the CD45RO⁺ subset, mainly involved in the 'memory' compartment of the immune system [44,45]. In addition, it has been shown that not all Vγ9⁺ lymphocytes are stimulated by *P. falciparum*, suggesting an antigen-specific response [46,47].

The parasite component(s) which stimulates Vγ9⁺ T cells is still a matter of speculation. Like others, we found that *P. falciparum* culture supernatants generate TcRγδ lymphocyte expansion, which suggests a soluble component (data not shown, and [25–27]). From previous studies, with other micro-organisms, a distinctive antigenic TcRγδ repertoire is suggested by the observation that some of these cells recognize defined microbial antigens such as mycobacterial moieties and mycobacterial heat shock proteins [48]. Vγ9 TcRγδ⁺ T cells, however are also stimulated by staphylococcal enterotoxin A, mycobacteria and tularaemia antigens [37,48,49].

From our data, the long term TcRγδ expansion observed *in vivo* cannot be attributed to the given parasite component(s). That regulatory interactions occur between autologous TcRαβ⁺ by TcRγδ⁺ lymphocytes is supported by several observations both *in vivo* and *in vitro*. First, blood levels of CD4⁺, TcRαβ⁺ and TcRγδ⁺ lymphocytes were negatively associated during the course and recovery of plasmodial infection. Another suggestive piece of *in vivo* evidence was the month-duration of increased TcRγδ⁺ cells in spite of the disappearance of malarial antigens in recovering patients. *In vitro*, we found that some TcRγδ⁺ clones were stimulated by autologous TcRαβ⁺ lymphocytes, not only to proliferate but also to exhibit cytotoxic activity against autologous TcRαβ⁺ lymphocytes activated with the same parasite extracts. To test for non-MHC-restricted cytotoxicity, lymphoblastoid target cells (both NK sensitive and non-sensitive) were studied, and although some lymphoblastoid cell lysis was detected, the higher lysis of autologous TcRαβ⁺ clones

suggests the involvement of MHC encoded molecules in the cytotoxicity of TcR$\gamma\delta^+$ clones against TcR$\alpha\beta^+$ cells. This is consistent with previous observations suggesting that anti-MHC class II antibodies inhibit TcR$\gamma\delta$ response to *P. falciparum in vitro*, and that TcR$\gamma\delta^+$ T cells regulate the functions and development of TcR$\alpha\beta^+$ T cells [49–51].

As TcR$\gamma\delta$ T cells secrete cytokines, another regulatory mechanism may involve these mediators [52–54]. Preliminary experiments have shown that the PFSE-reactive TcR$\gamma\delta^+$ clones secrete high concentrations of IFN-γ (data not shown). Interferon-γ and TNF-mediated consequences of TcRyδ^+ lymphocyte activation during and following a malaria attack may include temporary depression of TcR$\alpha\beta^+$ lymphocyte responses in the acute period, enhancement of HLA class II expression, initial lymphocytopenia, and TcR$\gamma\delta^+$ cell accumulation.

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