Clin Exp Immunol 2001; **126**:503–510

Changes in cytokine production associated with acquired immunity to *Plasmodium falciparum* malaria

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(Accepted for publication 2 August 2000)

SUMMARY

Individuals living in malaria-endemic areas eventually develop clinical immunity to *Plasmodium falciparum*. That is, they are able to limit blood parasite densities to extremely low levels and fail to show symptoms of infection. As the clinical symptoms of malaria infection are mediated in part by pro-inflammatory cytokines it is not clear whether the acquisition of clinical immunity is due simply to the development of antiparasitic mechanisms or whether the ability to regulate inflammatory cytokine production is also involved. We hypothesize that there is a correlation between risk of developing clinical malaria and the tendency to produce high levels of proinflammatory cytokines in response to malaria infection. In order to test this hypothesis, we have compared the ability of peripheral blood mononuclear cells from malaria-naive and malaria-exposed adult donors to proliferate and to secrete IFN-γ in response to *P. falciparum* schizont extract (PfSE). In order to determine how PfSE-induced IFN-γ production is regulated, we have also measured production of IL-12p40 and IL-10 from PfSE-stimulated PBMC and investigated the role of neutralizing antibody to IL-12 in modulating IFN-γ production. We find that cells from naive donors produce moderate amounts of IFN-γ in response to PfSE and that IFN-γ production is strongly IL-12 dependent. Cells from malaria-exposed donors living in an area of low malaria endemicity produce much higher levels of IFN-γ and this response is also at least partially IL-12 dependent. In complete contrast, cells from donors living in an area of very high endemicity produce minimal amounts of IFN-γ. No significant differences were detected between the groups in IL-10 production, suggesting that this cytokine does not play a major role in regulating malaria-induced IFN-γ production. The data from this study thus strongly support the hypothesis that down-regulation of inflammatory cytokine production may be a component of acquired clinical immunity to malaria but the mechanism by which this is achieved remains to be elucidated.

Keywords cytokines human protozoa T lymphocytes

INTRODUCTION

Malaria, particularly *Plasmodium falciparum*, is responsible for more deaths than any other parasitic disease and malaria-related morbidity constitutes a significant constraint on development in tropical countries. Vaccines offer a potential means of long-term malaria control but, as the clinical symptoms of malaria infection are mediated in part by pro-inflammatory cytokines [1], the design of a safe and effective vaccine requires identification of the mechanisms of antimalarial immunity and differentiation of protective from pathogenic responses [2].

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Work on murine malarias suggests that innate immune mechanisms, mediated by IFN-γ derived from natural killer (NK) cells and/or $\gamma\delta$ T cells, limit the initial phase of parasite replication [3] and that adaptive responses (mediated by $\alpha\beta$ T cells and B cells) are required for parasite elimination [4]. IFN-γ is essential for resolution of primary infection [5]. TNF-α and IFN-γ act synergistically to optimize nitric oxide (NO) production [6], which may be involved in parasite killing [7]. The difference between lethal and nonlethal malaria infections can be explained, in part, by the ability of mice to mount an early IL-12, IFN-γ or TNF-α response [8–11]. However, IFN-γ also plays a role in the acute symptoms of infection, such as fever, anorexia and weight loss, through induction of TNF-α and other endogenous pyrogens. Over-production of IFN-γ or TNF-α predisposes to severe pathology [11–13] and, in lethal infections, neutralization of

IL-12, blocking the IFN-γ pathway or down-regulation of TNF-α by administration of rTGF-β, abrogates mortality [12,14,15].

In humans, individuals living in highly endemic areas become clinically immune, i.e. they are able to control circulating parasite densities to within tolerable limits and show no significant clinical symptoms of infection. Although IFN-γ responses to some defined malaria antigens correlate with protective immunity [16,17], plasma IFN-γ concentrations are higher in symptomatic than in asymptomatic infections [18] and there is a temporal association between IFN-γ secretion and fever [19]. Similarly, plasma TNF-α and nitrogen oxide levels are associated with rapid resolution of fever and parasite clearance [20,21] but severe *P. falciparum* malaria is accompanied by high levels of circulating cytokines, including TNF-α [22–25]. Taken together, these observations suggest that there is a critical balance to be found in the inflammatory response to malaria infection and that the ability to regulate cytokine levels, within quite precise limits, may be a component of clinical immunity.

We hypothesize that there is a correlation between the risk of developing clinical malaria and the ability to produce high levels of IFN-γ in response to malaria infection, and that this can be assessed *in vitro* as the ability of PBMC to produce IFN-γ when reactivated by PfSE. In order to test this hypothesis, we have compared the ability of PBMC from malaria-naive and malaria-exposed adult donors, to proliferate and to secrete IFN-γ in response to PfSE stimulation *in vitro*. In order to determine how PfSE-induced IFN-γ production is regulated, we have also measured production of IL-12 and IL-10 from PfSE-stimulated PBMC and investigated the role of neutralizing antibody to IL-12 in modulating IFN-γ production.

SUBJECTS AND METHODS

Subjects

Malaria-naive donors ($n = 19$, aged 20–41 years, 11 male, eight female) were recruited from Edinburgh, Scotland, UK. None of the donors had knowingly been infected with malaria and the majority had never visited a malaria-endemic area. Malaria-exposed donors were recruited from two regions in Ghana with differing levels of malaria endemicity. Highly exposed donors ($n = 20$, aged 30–47 years, eight male, 12 female) were recruited from the village of Dodowa, approximately 40 miles inland from Accra, Ghana, where malaria transmission is highly endemic [26] with an entomological infection rate (EIR) of 15–20 infectious bites per year and the majority of adults show no clinical symptoms when infected with malaria. Less-exposed donors ($n = 20$, aged 21–60 years, 17 male, three female) were recruited from Accra District on the south coast of Ghana where levels of malaria transmission are much lower than in rural areas [26]; the EIR is less than one infectious bite per year and many adults still develop clinical symptoms when infected by malaria. The predominant ethnic groups in both areas are Ga and Ga-Adangbe.

Ethical approval for the study was obtained from the Ghanaian Ministry of Health.

A questionnaire was completed for each donor, giving information on prior exposure to malaria and use of antimalarial drugs. Up to 30 ml of venous blood were collected into heparinized containers. An aliquot of plasma was removed for serology and Giemsa-stained blood films from all the African donors were examined for the presence of malaria parasites. A highly sensitive PCR method, based on the multicopy 7H8/6 gene sequence [27], was used to detect very low level *P. falciparum* infections. Details of the PCR method are given elsewhere [28].

P. falciparum *schizont antigens*

P. falciparum clone 3D7 [29] was maintained in continuous culture [30]. Cultures were routinely screened for mycoplasma using a commercial PCR kit (BioWhittaker, Wokingham, UK) and shown to be free of contamination. When parasitaemia reached approximately 6–8%, mature schizonts were separated on a 60% Percoll gradient (Pharmacia, Uppsala, Sweden), washed three times in serum-free RPMI 1640 (GIBCO, Paisley, UK), pelleted by microcentrifugation and resuspended at a concentration of 10^8 infected erythrocytes per ml. The suspension was freeze-thawed three times by immersion in liquid N_2.The entire extract (designated PfSE) was used in cell culture experiments. A portion of the extract was spun to remove the cellular debris; the supernatant was saved and used for serology (designated soluble *Pf* antigen). Both antigen preparations were aliquoted and stored at -80°C until required. Freeze thaw preparations of uninfected erythrocytes (10^8/ml) (uRBC) were used as controls.

Serology

Plasma from all donors was tested for antimalarial antibodies by ELISA. Immulon IV microtitre plates (Dynex, Billingshurst, UK) were coated with an optimal concentration of soluble *Pf* antigen (determined by titration) diluted in carbonate/bicarbonate buffer and incubated overnight at 4°C. Plates were blocked for 3 h at room temperature with PBS containing 0·5% Tween 20 (Sigma, Poole, UK) and 1% non-fat milk powder (blocking buffer). Plasma samples, diluted 1 : 1000 in blocking buffer, were added to duplicate wells, and incubated for 3 h at RT. Bound antibody was detected with rabbit antihuman IgG-horse radish peroxidase (Dako, High Wycombe, UK) and *o*-phenylenediamine/H_2O_2 as described previously [31]. Optical density was measured at 492 nm. Samples were designated positive for antimalarial antibody if the OD was greater than the mean plus 2 s.d. of the OD values of 22 control European plasmas. Antibody levels are expressed as a percentage of the value obtained for a pool of hyperimmune plasma from African adults tested on the same plate.

Cell cultures

Mononuclear cells were separated from heparinized blood by centrifugation through Lymphoprep® (Pharmacia, Uppsala, Sweden), washed in RPMI 1640 and resuspended at a concentration of 10^6 viable cells per ml in complete culture medium (RPMI 1640 with 2 mM L-glutamine, 100 U/ml penicillin, 0·1 mg/ml streptomycin, 25 mM HEPES and 0·22% (v/v) sodium bicarbonate; all Sigma) with 10% heat-inactivated, nonimmune human serum. For lymphocyte proliferation assays, cells were aliquoted (100 μl/well) into sterile, round-bottomed, 96-well microtitre plates. Antigens, at optimal concentration in 100 μl/ml complete medium, or the mitogen PHA (2 μg/ml, Sigma) were added to triplicate wells and plates incubated at 37°C in 5% CO_2 for 2, 4, 6 or 8 days. Eighteen hours before harvesting, 100 μl of supernatant was removed from each well and reserved for cytokine analysis; 1 μCi [^{3}H]-thymidine (Amersham, UK) in 100 μl fresh culture medium was added to each well. Cells were harvested onto cellulose filters and incorporation of radiolabelled thymidine assessed by scintillation counting. The geometric mean counts per minute (cpm) were determined for each antigen and the

stimulation index (SI) calculated as the ratio of PfSE-stimulated cpm to control (uRBC-stimulated) cpm. SI values $\geq 2{\cdot}5$ were considered positive [32].

In order to determine the interaction between IFN-γ production and IL-12, a neutralizing antibody to human IL-12 (R&D Systems, Abingdon, UK) or a control, isotype-matched murine IgG (R&D Systems) were added to the cultures. Optimal anti-IL-12 concentration was determined by reference to the manufacturer's information and confirmed by titration experiments (data not shown); the antibody was used at a final concentration of 5 μg/ml which was sufficient to neutralize the activity of 1 ng/ml rhIL-12. Antibodies were added at the beginning of the culture period and remained throughout. Supernatants were collected and tritiated thymidine incorporation assessed as described above.

Cytokine assays

Cell culture supernatants were tested for presence of IFN-γ, IL-12p40 and IL-10 by two-site capture ELISA. Flat-bottomed, 96-well microtitre plates (Immulon IV) were coated overnight with 100 μl per well of an optimal concentration of capture antibody diluted in PBS. Plates were washed four times with PBS plus 0·05% Tween 20, blocked with 200 μl/well blocking buffer (PBS plus 4% bovine serum albumin, Sigma) for 1 h at 37°C and washed three more times. Culture supernatants or cytokine standards (100 μl/well) were added to duplicate wells and incubated at room temperature for 3 h. After washing four times, 100 μl/well of biotinylated capture antibody (diluted in PBS) was added and incubated for 1 h at room temperature. Plates were washed six times, avidin-labelled horseradish peroxidase (1 μg/ml, Sigma) added for 30 min at room temperature and after a final eight washes, plates were developed for 15 min at room temperature with hydrogen peroxide as substrate and *o*-phenylenediamine as chromagen. The reaction was stopped with 2 M H_2SO_4 and absorbance read at 492 nm.

Antibody pairs for IL-10 and IFN-γ, and all cytokine standards, were obtained from Endogen, Woburn, MA, USA. The anti-IL-12 antibody pair was obtained from R&D systems. Coating antibodies for IL-10 and IFN-γ were used at 2 μg/ml, coating antibody for IL-12p40 was used at 4 μg/ml. Biotinylated antibody to IL-10 and IFN-γ were used at 0·05 μg/ml, antibody to IL-12p40 was used at 0·3 μg/ml.

Cytokine concentrations were calculated from standard curves using best-fit formulae. The lower limit of detection (LLD) was defined as 2 s.d. above the mean of the negative control (culture medium alone). Limited volumes of culture supernatants precluded the evaluation of additional cytokines.

Statistical methods

Differences between groups were assessed by Student's *t*-test on log-transformed data. Differences in response to different treatments within a group were assessed by paired *t*-tests comparing treated and control cells from the same donors. For antigen-specific responses, where background or uRBC values were subtracted and thus some negative values were obtained, the non-parametric Mann–Whitney *U*-test was used.

RESULTS

Donors were selected solely on the basis of their previous exposure to malaria, as determined by lifelong residence in a non-endemic country or in an area of low or high endemicity in Ghana.

Table 1. Details of blood donors

	Naive (UK)	Low-endemicity (Accra)	High-endemicity (Dodowa)
N	19	20	20
Age range (years)	20–41	21–60	30–47
Pf blood film + (%)	0 (0%)	0 (0%)	2 (10%)
Pf PCR + (%)	0 (0%)	1 (5%)	6 (30%)
Antibody + (%)	0 (0%)	13 (68%)	20 (100%)
Mean antibody* (SEM)	5 (4)	41 (6)	87 (8)
Range	4–8	13–101	24–143
Confirmed clinical malaria in the last 5 years? (%)	0 (0%)	14 (70%)	1 (5%)

*Individual antibody OD values are expressed as a percentage of the value of a high titre pool of malaria immune serum from African donors.

Accordingly, the three groups of donors were clearly distinguishable on the basis of their clinical history, malaria infection rates and serological responses to soluble malaria antigens (Table 1). The naive donors had no previous history of malaria infection and antibody levels were within the normal range defined by nonimmune sera. The majority of the low endemicity donors reported a clinical malaria infection (confirmed by microscopy and treated with antimalarial drugs) within the last 5 years. Only one of the donors had a subclinical malaria infection at the time of blood sampling. Several donors had antimalarial antibody levels below the cut-off level defined by non-immune sera and the mean antibody titre was only 41% of the hyperimmune control serum. By contrast, in the high endemicity group, only one donor reported a confirmed clinical malaria attack in the past 5 years, eight donors were subclinically infected at the time of sampling (two detected by blood film and six by PCR), all donors were seropositive and mean antibody titres were 87% of the hyperimmune control.

Lymphoproliferative responses

Proliferative responses were assessed after 3, 5 and 7 days. Cells from all donors proliferated strongly to PHA, showing that cells were viable (data not shown). In all three groups, the geometric mean SI for PfSE/uRBC increased steadily with time. Mean cpm values for the response to PfSE were consistently highest in cells from naive donors and lowest in cells from high endemicity donors but, due to the large variation within each group, these differences were not statistically significant (Fig. 1). Similarly, there was no significant difference in the mean SI between the groups ($t = 1{\cdot}62$, $P = 0{\cdot}05$). Cells from all naive and exposed donors made a significant lymphoproliferative response to PfSE (SI = 2·5) at day 7 but cells from two of the high-endemicity donors failed to proliferate (Table 2). One noticeable difference between the groups was that responses appeared slightly earlier in the low endemicity group than in the other two groups (cells from 13/19 donors gave an SI $\geq 2{\cdot}5$ at day 2 compared to 6/19 naives and 8/18 high endemicity donors).

IFN-γ production

IFN-γ in cell supernatants was measured after 2, 4 and 6 days (Fig. 2). For naive donors, IFN-γ levels in cultures stimulated

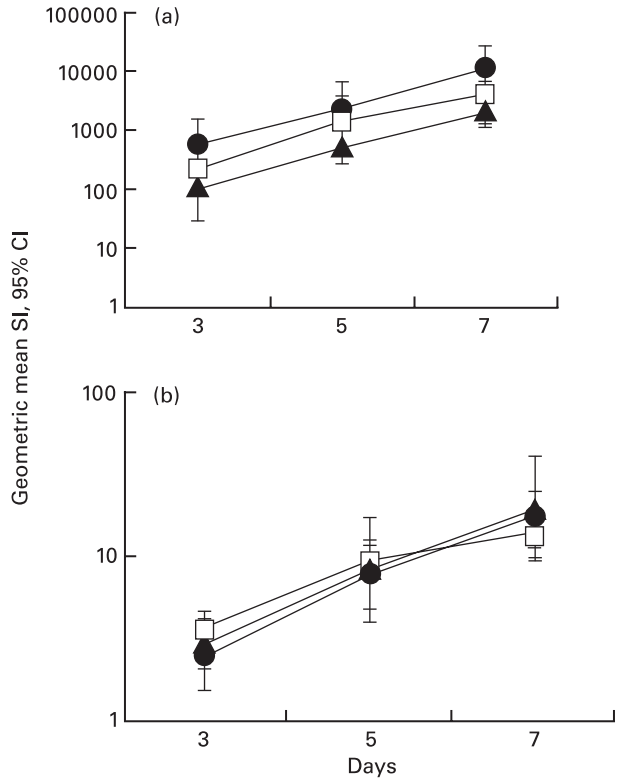

Fig. 1. Lymphoproliferative responses to PfSE by PBMC from malaria-naive (●), low-endemicity (□) and high-endemicity (▲) donors. (a) Geometric mean cpm, 95% CI; (b) geometric mean SI values, 95% CI. Data are displayed on a logarithmic scale.

with PfSE rose steadily over time. IFN-γ levels in PfSE cultures were significantly higher than in uRBC cultures on days 4 and 6 (paired $t \geq$, d.f. $= 18$, $P < 0{\cdot}002$). For low-endemicity donors, PfSE-specific IFN-γ levels rose to a mean of >900 pg/ml at 6 days, were significantly higher than in uRBC-stimulated cultures on days 2, 4 and 6 (paired $t \geq 3{\cdot}46$, d.f. ≥ 17 $P < 0{\cdot}003$ on all days) and were significantly higher than for naive donors at both 4 and 6 days (Mann–Whitney $U = 253$, d.f. $= 35$, $P < 0{\cdot}001$). By contrast, for high-endemicity donors,

Table 2. Percentage of donors whose PBMC proliferate and secrete cytokines in response to re-stimulation *in vitro* by malaria antigens

	Proliferation*	IFN$\gamma^{\dagger}$	IL-12‡
Naive			
n	19/19	14/19	2/18
%	100	74	11
Low endemicity			
n	19/19	19/20	9/20
%	100	95	45
High endemicity			
n	18/20	5/20	7/20
%	90	25	35

*Responder: SI (PfSE/uRBC) = 2·5. †Responder: PfSE-induced IFNγ at least two-fold higher than uRBC-induced IFNγ. ‡Responder: PfSE-induced IL-12 at least 20 pg/ml higher than uRBC-induced IL-12.

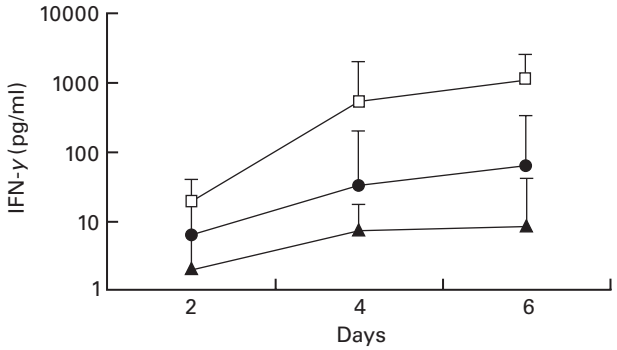

Fig. 2. IFN-γ production (pg/ml) in response to PfSE by PBMC from malaria-naive (●), low-endemicity (□) and high-endemicity (▲) donors. Geometric mean, PfSE-specific (PfSE-uRBC) IFN-γ, upper 95% CI; for clarity not all CI shown. Data are displayed on a logarithmic scale.

geometric mean IFN-γ levels increased only marginally over time. Malaria-specific IFN-γ levels were significantly lower in high-endemicity donors than in either the naive or the low-endemicity group at both day 4 and day 6 (Mann–Whitney U, $W > 230$, d.f. > 29, $P < 0{\cdot}008$ at day 4, $P < 0{\cdot}001$ at day 6).

In addition, the proportion of donors making a positive IFN-γ response to PfSE at one or more time points was statistically higher among naive and low-endemicity donors than among high-endemicity donors (Table 2, $\chi^2 = 22{\cdot}43$, $P < 0{\cdot}001$).

IL-12 production

IL-12 p40 was measured by ELISA in days 1, 2, 4, 6 and 8 supernatants of PfSE-stimulated cultures; levels peaked at day 2 but were low (< 100 pg/ml) in cells from all groups of donors. Individual donors were classified as responders if PfSE-induced IL-12 was at least 20 pg/ml greater than uRBC-induced IL-12. Using this definition, 11% of naive donors were responders but 45% of low-endemicity and 35% of high-endemicity donors responded (Table 2). The difference in the number of responders between the naive and low-endemicity groups is statistically significant ($\chi^2 = 5{\cdot}29$, d.f. $= 1$, $P < 0{\cdot}025$).

IL-10 production

To determine whether differences in anti-inflammatory cytokine production might explain either the down-regulation of the IFN-γ response in high-endemicity donors or the ability of low-endemicity individuals to avoid severe consequences of malaria despite making a strong pro-inflammatory response, we measured levels of the anti-inflammatory cytokine IL-10 in cell supernatants. Overall, levels of malaria-specific IL-10 were extremely low in all cultures on all days (< 60 pg/ml in all cultures from all donors). There were no significant differences between the three groups in mean IL-10 levels (data not shown).

Correlations between immune parameters

It is now well recognized that the absolute concentrations of particular cytokines may be less important indicators of the outcome of infection than the relative amounts of different cytokines. We have therefore looked to see whether certain immune parameters are correlated with each other and looked at the ratios of different cytokines in different donor groups. In all three donor groups there was a highly significant correlation

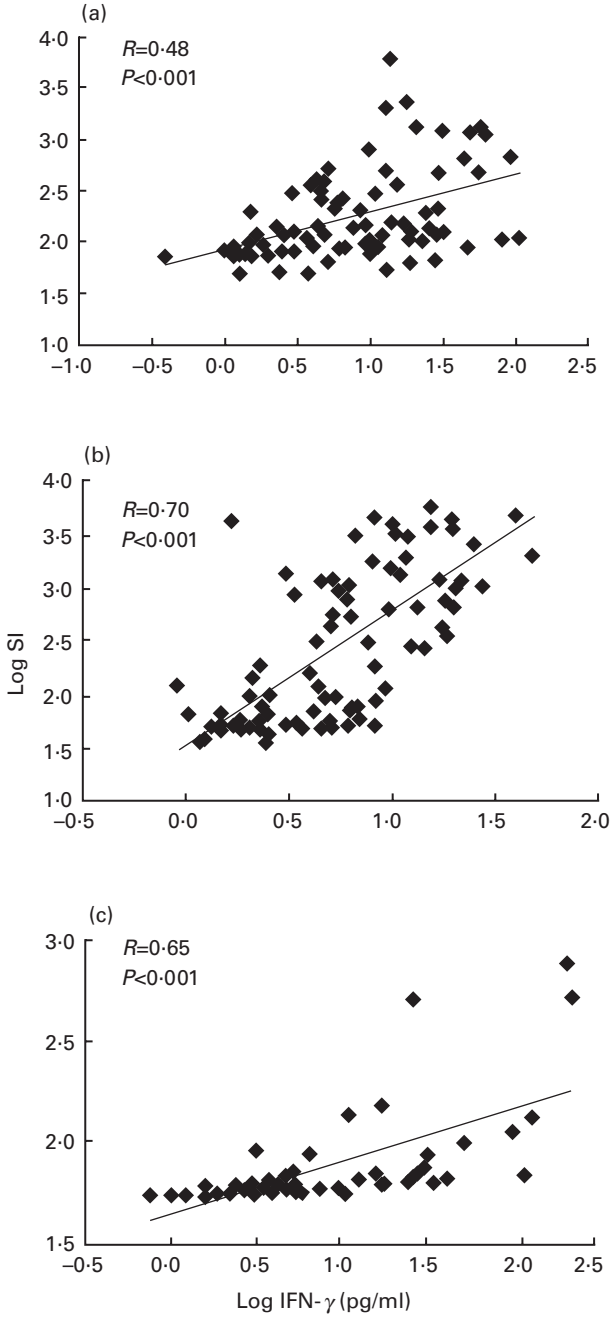

Fig. 3. Correlation between lymphoproliferative and IFNγ responses to PfSE in (a) naive, (b) low-endemicity and (c) high-endemicity donors. Data points from all time points (2, 4 and 6 days) are included. Data are displayed on logarithmic scales.

between lymphoproliferative responses and IFN-γ production (Fig. 3) suggesting that proliferating cells are the major source of IFN-γ, even though the amount of IFN-γ being produced varied from group to group. Ratios of IFN-γ to IL-12, IL-12 to IL-10 and IFN-γ to IL-10 have been calculated (Table 3). Ratios of IFN-γ to IL-12 were 6–10-fold higher in low-endemicity donors than in either of the other groups, suggesting that high levels of IFN-γ in this group do not require similarly high levels of IL-12. The very high ratio of IFN-γ to IL-10 in low-endemicity donors was due wholly to the high IFN-γ levels in this group.

Table 3. Cytokine ratios* (± SE)

Cytokine ratio	Naive	Low-endemicity	High-endemicity
IFN-γ: IL-12	1·55 ± 0·42	9·41 ± 2·45	0·66 ± 0·21
IFN-γ: IL-10	5·81 ± 1·74	30·9 ± 7·94	2·41 ± 0·60
IL-12: IL-10	3·48 ± 0·27	5·19 ± 1·47	4·92 ± 0·44

*(pg/ml cytokine A/pg/ml cytokine B).

Effect of IL-12 neutralization on IFN-γ production

Addition of anti-IL-12 or control isotype-matched IgG, had no significant effect on lymphoproliferative responses (data not shown), but led to reduced IFN-γ production in all groups of donors. In the presence of neutralizing antibody to IL-12, IFN-γ production by cells from naive donors was reduced to background levels (Fig. 4a), indicating that IFN-γ production from naive cells is IL-12-dependent. Differences in IFN-γ production between anti-IL-12 treated and control cultures of individual donors were

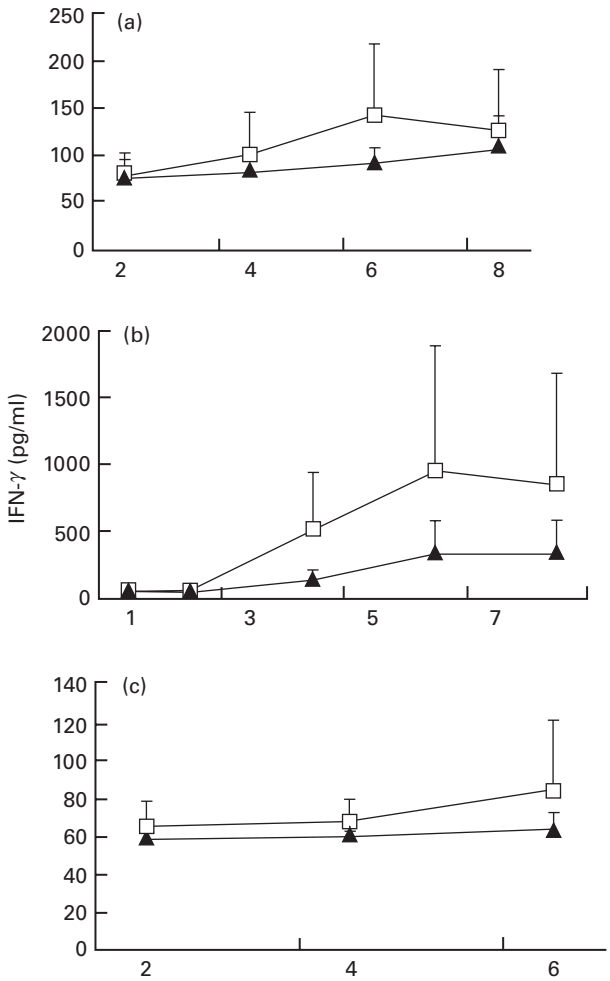

Fig. 4. Effect of neutralizing antibodies to IL-12 (5 μg/ml) on PfSE-induced IFN-γ production (pg/ml) from PBMC from (a) naive, (b) low-endemicity and (c) high-endemicity donors. (Mean, upper 95% CI). □, Cultures with control IgG (5 μg/ml); ▲, cultures with anti-IL-12 antibody (5 μg/ml).

statistically significant on days 2, 4 and 6, although the effect was most marked at day 6 (paired $t = 3{\cdot}05$, d.f. $= 13$, $P = 0{\cdot}009$). Cells from low-endemicity donors produced much higher levels of IFN-γ than cells from naive donors and in the presence of anti-IL-12, IFN-γ levels were also significantly reduced at all time points (paired $t \geq 3{\cdot}41$, d.f. $= 19$, $P \leq 0{\cdot}003$ on all days) (Fig. 4b). However, anti-IL-12 did not completely inhibit IFN-γ production and at day 6 IFN-γ levels were still significantly higher than in unstimulated cultures ($t = 5{\cdot}2$, d.f. $= 16$, $P < 0{\cdot}001$) or in PfSE-stimulated cultures of naive cells ($t = 4{\cdot}56$, d.f. $= 32$, $P < 0{\cdot}001$). Cells from high endemicity donors made very little IFN-γ; nevertheless, the addition of anti-IL-12 reduced IFN-γ levels to background values and the difference between control and anti-IL-12 cultures was significant at day 6 (paired $t = 2{\cdot}18$, d.f. $= 19$, $P = 0{\cdot}042$) (Fig. 4c).

DISCUSSION

The clinical symptoms of malaria are related to the presence of circulating pro-inflammatory cytokines such as TNF-α [1] but the risk of clinical disease also increases with increasing parasite density [33]. It is therefore not clear whether the acquisition of clinical immunity is due simply to the development of antiparasitic mechanisms, which keep parasite densities below a critical threshold or whether the ability to regulate inflammatory cytokine production is also involved. One piece of clinical evidence that points to the importance of the latter is that parasite densities which are well tolerated by immune individuals can be accompanied by severe febrile symptoms in non-immune subjects [33].

For many years it was believed that glycophospholipid components of the parasite, derived possibly from the membrane anchors of surface antigens [34], directly induced macrophages to secrete TNF-α [35,36] but the notion that this is sufficient to mediate the pathology of malaria has recently been questioned [37]. We have postulated that IFN-γ is required to induce sufficiently high levels of inflammatory mediators to cause disease and that the ability to down-regulate IFN-γ production (or its effects) may be a prerequisite for clinical immunity [1]. To date, this hypothesis is supported only by two small studies showing that levels of malaria-induced IFN-γ are lower in African adults than in European adults living in Africa [38,39].

In this study, we have directly addressed the relationship between clinical immunity to malaria and the tendency of PBMCs to produce either pro-inflammatory or anti-inflammatory cytokines in response to stimulation with a crude preparation of malaria antigens – an antigen preparation which we believe closely represents the pool of circulating antigen released into the peripheral circulation at the time of schizont rupture. The selection of peripheral blood as the source of leucocytes is dictated by practical constraints but has been validated in many previous studies: although malaria-reactive T cells tend to disappear from the peripheral circulation during an acute infection (probably migrating to the spleen and liver) they are released back into the periphery upon resolution of the infection [40] and it is thus realistic to assume that, in healthy individuals, the peripheral T cell population is representative of the total malaria-reactive T cell pool.

Subjects were allocated to naive, low-endemicity or high-endemicity groups according to objective criteria agreed prior to commencement of the study and before any laboratory analyses were performed. Retrospective analysis of malaria infection rates and antimalarial antibody levels confirmed our allocation of individuals to appropriate groups. Selection of an appropriate naive (unexposed) control group for such studies is fraught with difficulties. Malaria is highly endemic in Ghana and even in areas of low transmission (such as urban and periurban areas) unexposed adults are impossible to find. As lack of prior exposure to malaria was our crucial criterion for the control group, we chose controls who we were certain had never been infected with malaria. However, given that there are many other differences between the control and the malaria-exposed individuals, it was important also to compare responses of Ghanaians with very different long-term exposure to malaria.

The only marked differences in cellular immune responses between the groups were in the amounts of IFNγ produced following *in vitro* stimulation with PfSE, with highly statistically significant differences being observed between all three groups. The low mean levels of IFN-γ production by cells from high-endemicity donors (who are assumed to have a high degree of clinical immunity to malaria), and the complete lack of malaria-induced IFN-γ production by 75% of these donors, strongly supports the hypothesis that clinical immunity to malaria is accompanied by down-regulation of the pro-inflammatory immune response.

The lack of detectable cytokine production in response to PfSE in immune individuals does not appear to be due to T cell anergy; PBMC from immune individuals proliferated in response to PfSE, although perhaps not quite to the same extent as cells from other donors. In fact, no major difference was seen between the groups in terms of their lymphoproliferative responses to crude malaria schizont antigens; this confirms previous findings from our own work and that of other groups [38,39]. However, it is possible that the phenotype of the proliferating cells differs between the three groups; preliminary analysis of flow cytometric data indicates that proliferating cells in naïve donors are predominantly $CD4^+$ T cells while B cells and $CD8^+$ T cells are also proliferating in cultures from malaria exposed donors (M.S.M. Rhee, unpublished data). It remains to be seen whether proliferating cells in exposed donors produce other cytokines which may down-regulate inflammatory responses.

Cells from naive donors produced moderate levels of IFN-γ, commensurate with the known risk of non-immune adults developing clinical malaria during their first infection. The IFN-γ response appeared to be strongly IL-12 dependent, suggesting that NK cells may be a source of IFN-γ in these donors. Previous studies in naive donors have shown that PfSE activates mainly $CD3^+$ $TCR\alpha\beta^+$ cells, but that $\gamma\delta^+$ T cells can also be activated under certain circumstances and that both populations can produce IFN-γ [41–44]. The $\alpha\beta$ T cells are believed to have been primed by cross-reacting antigens present in a number of commensal organisms and common pathogens [45], whereas $\gamma\delta$ T cells recognize phosphorylated nonprotein antigens [46]. Activation of NK cells during malaria infection has also been observed [47] and recent data from our own laboratory confirms that NK cells from malaria-naive donors produce IFN-γ in response to malaria antigen stimulation (Artavanis-Tsakonas and Riley, in preparation).

Interestingly, cells from low-endemicity donors produced very much higher levels of IFN-γ than naive donors; the IFN-γ response was only partly inhibited by anti-IL-12 antibodies. One explanation for this is that malaria-exposed donors possess an

additional population of IFN-γ-producing T cells which do not require IL-12. These cells may be specifically primed by malaria antigens rather than cross-reacting antigens. Despite producing very high levels of IFN-γ in response to malaria antigens, exposed donors are not expected to be at any greater risk of severe malaria than naive donors and their risk may indeed be lower. This suggests that the downstream effects of IFN-γ production – for example induction of TNF-α – may be being inhibited to avert serious pathology.

The mechanisms by which IFN-γ and its down-stream effects are regulated are not clear. Direct antagonism of IFN-γ by IL-10 seems unlikely, given that levels of IL-10 were scarcely above background levels in all groups, although IL-10 neutralization studies would be required to confirm whether this cytokine can affect IFN-γ secretion. In malaria patients plasma levels of IL-10 seem to correlate with clinical severity, being higher in severe malaria cases than in mild cases; however, levels are approximately normal in children with asymptomatic infections, suggesting that IL-10 may not be a key mediator in down-regulating the inflammatory response [48–50].

Other cytokines, such as TGF-β, may, however, play a role. We have recently shown a causal association between low TGF-β levels and severity of malaria in mice [15] and the plasma of acute *P. falciparum* patients contains lower than normal levels of circulating TGF-β [51]. n mouse malaria, TGF-β inhibits TNF-α production without any significant effect on IFN-γ levels, suggesting that TGF-β may modify the down-stream consequences of IFN-γ production [15]. Alternatively, TGF-β might down-regulate IL-12-mediated IFN-γ induction by inhibiting expression of the IL-12 receptor on T cells [52].

In summary, we have shown that there is a strong association between IFN-γ production by PBMC in response to malaria antigen stimulation and the immune status of the donor. Specifically, PBMC from heavily exposed individuals, who are able to resolve malaria infections without developing clinical disease, produce negligible levels of IFN-γ in response to stimulation by a crude malaria antigen preparation. This does not necessarily mean that IFN-γ has no role to play in protective immunity to malaria, but implies that the production of IFN-γ is strictly regulated (possibly limited to certain defined subsets of cells residing in specific organs) in order to prevent disease. By contrast, cells from non-immune individuals produce moderate levels of IFN-γ in an IL-12-dependent manner. Intriguingly, the highest levels of IFN-γ were observed in donors with low levels of prior exposure to malaria who develop clinical symptoms of infection but are at relatively low risk of severe disease, suggesting that the down-stream effects of IFN-γ may be carefully controlled in such individuals. These observations, in human cells exposed to *P. falciparum* antigens, thus strongly support the working model of antimalarial immunity developed from studies of rodent malarias [4–6,8–10]. Further studies are required to elucidate the mechanisms by which IFN-γ production is down-regulated in malaria-immune individuals and how the effects of IFN-γ are regulated in semi-immune donors.

ACKNOWLEDGEMENTS

We would like to thank the people of Dodowa and the staff of the Noguchi Memorial Institute for Medical Research for their participation in these studies. We thank Kevin Tetteh for parasite diagnosis by PCR, Enid Owusu, Michael Addae, Michael Ofori, Ben Gyan and Andrew Sanderson for technical assistance. This study was funded by The Wellcome Trust. M.S.M.R was funded by a Wellcome Trust Prize PhD studentship.

REFERENCES

1 Riley EM. Is T cell priming required for initiation of pathology in malaria infections? Immunol Today 1999; **20**:228–33.
2 Omer FM, Kurtzhals JAL, Riley E. Maintaining the immunological balance in parasitic infections: a role for TGFβ? Parasitol Today 2000; **16**:18–23.
3 Fell AH, Smith NC. Immunity to asexual blood stages of *Plasmodium*: is resistance to acute malaria adaptive or innate? Parasitol Today 1998; **14**:364–9.
4 Langhorne J, Cross C, Seixas E, Li C, von der Weid T. A role for B cells in the development of T helper function in a malaria infection in mice. Proc Natl Acad Sci USA 1998; **95**:1730–4.
5 Favre N, Ryffel B, Bordmann G, Rudin W. The course of *Plasmodium chabaudi chabaudi* infections in interferon-gamma receptor deficient mice. Parasite Immunol 1997; **19**:375–83.
6 Jacobs P, Radzioch D, Stevenson MM. *In vivo* regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice. Infect Immun 1996; **64**:44–9.
7 Rockett KA, Awburn MM, Cowden WB, Clark IA. Killing of *Plasmodium falciparum in vitro* by nitric oxide derivatives. Infect Immun 1991; **59**:3280–3.
8 Stevenson MM, Tam MF, Wolf SF, Sher A. IL-12-induced protection against blood-stage *Plasmodium chabaudi AS* requires IFNγ and TNFα and occurs via a nitric oxide-dependent mechanism. J Immunol 1995; **155**:2545–56.
9 Shear HL, Srinavasan R, Nolan T, Ng C. Role of IFN-γ in lethal and nonlethal malaria in susceptible and resistant hosts. J Immunol 1989; **143**:2038–44.
10 de Souza JB, Williamson KH, Otani T, Playfair JHL. Early gamma interferon responses in lethal and nonlethal murine blood stage malaria. Infect Immun 1997; **65**:1593–8.
11 Jacobs P, Radzioch D, Stevenson MM. A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice. Infect Immun 1996; **64**:535–41.
12 Waki S, Uehara S, Kanbe K, Ono K, Suzuki M, Nariuchi H. The role of T cells in pathogenesis and protective immunity to murine malaria. Immunol 1992; **75**:646–51.
13 Kremsner PG, Neifer S, Chaves MF, Rudolph R, Bienzle U. Interferon-γ induced lethality in the late phase of *Plasmodium vinckei* malaria despite effective parasite clearance by chloroquine. Eur J Immunol 1992; **22**:2873–8.
14 Yoshimoto T, Takahama Y, Wang C-R, Yoneto T, Waki S, Nariuchi H. A pathogenic role of IL-12 in blood-stage murine malaria lethal strain *Plasmodium berghe*i NK65 infection. J Immunol 1998; **160**:5500–5.
15 Omer FM, Riley EM. TGF-beta production is inversely correlated with severity of murine malaria infection. J Exp Med 1998; **188**:39–48.
16 Riley EM, Allen SJ, Wheeler J *et al.* Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen of *Plasmodium falciparum* are associated with reduced malaria morbidity. Parasite Immunol 1992; **14**:321–38.
17 Luty AJF, Lell B, Schmidt-Ott R *et al.* Interferon-gamma responses are associated with resistance to reinfection with *Plasmodium falciparum* in young African children. J Infect Dis 1999; **179**:980–8.
18 Mshana RN, Boulandi J, Mshana NM, Mayombo J, Mendome G. Cytokines in the pathogenesis of malaria: levels of IL-1B, IL-4, IL-6, TNF-α and IFNγ in plasma of healthy individuals and malaria patients in a holoendemic area. J Clin Lab Immunol 1991; **34**:131–9.
19 Harpaz R, Edelman R, Wasserman SS, Levine MM, Davis JR, Sztein

MB. Serum cytokine profiles in experimental human malaria. J Clin Invest 1992; **90**:515–23.
20 Kremsner PG, Winkler X, Brandts C *et al.* Prediction of accelerated cure in *Plasmodium falciparum* malaria by the elevated capacity of tumour necrosis factor production. Am J Trop Med Hyg 1995; **53**:532–8.
21 Kremsner PG, Winkler X, Wilding E, Prada J, Bienzle U, Graninger W, Nussler A. High plasma levels of nitrogen oxides are associated with severe disease and correlate with rapid parasitological and clinical cure in *Plasmodium falciparum* malaria. Trans Roy Soc Trop Med Hyg 1996; **90**:44–7.
22 Grau GE, Taylor TE, Molyneux ME *et al.* Tumor necrosis factor and disease severity in children with falciparum malaria. N Engl J Med 1989; **320**:1586–91.
23 Kwiatkowski D, Hill AVS, Sambou I *et al.* TNF concentration in fatal, non-fatal cerebral and uncomplicated *Plasmodium falciparum* malaria. Lancet 1990; **336**:1201–4.
24 Wenisch C, Parschalk B, Narzt E, Looareesuwan S, Graninger W. Elevated serum levels of IL-10 and IFNγ in patients with acute *Plasmodium falciparum* malaria. Clin Immunol Immunopathol 1995; **74**:115–7.
25 Friedland JS, Ho M, Remick DC, Bunnag D, White NJ, Griffin GE. Interleukin-8 and *Plasmodium falciparum* malaria in Thailand. Trans Roy Soc Trop Med Hyg 1993; **87**:54–5.
26 Afari EA, Appawu MA, Dunyo S, Baffoe-Wilmot AB, Nkrumah FK. Malaria infection, morbidity and transmission in two ecological zones in Southern Ghana. Afr J Health Sci 1995; **2**:312–6.
27 Limpaiboon T, Shirley MW, Kemp DJ, Saul A. 7H8/6, a multicopy DNA probe for distingushing isolates of *Plasmodium falciparum*. Mol Biochem Parasitol 1991; **47**:197–206.
28 Wagner G, McGuinness D, Koram K, Bennett S, Nkrumah FK, Riley E. High incidence of asymptomatic malaria infections in a birth cohort of children under 1 year of age in Ghana, detected by multicopy gene polymerase chain reaction. Am J Trop Med Hyg 1998; **59**:115–23.
29 Walliker D, Quakyi IA, Wellems TE *et al.* Genetic analysis of the human malaria parasite *Plasmodium falciparum*. Science 1987; **236**:1661–6.
30 Lambros J, Vanderberg JP. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 1979; **65**:418–20.
31 Riley EM, Wagner GE, Ofori *et al.* Lack of association between maternal antibody and protection of African infants from malaria infection. Inf Immun 2000; **68**:5856–63.
32 Bennett S, Riley EM. The statistical analysis of data from immuno-epidemiological studies. J Immunolog Meth 1992; **146**:229–39.
33 Schellenberg JA, Smith T, Alonso PL, Hayes R. What is clinical malaria? Finding case definitions for field research in highly endemic areas. Parasitol Today 1994; **10**:439–42.
34 Schofield L, Hackett F. Signal transduction in host cells by a glycophosphatidylinositol toxin of malaria parasites. J Exp Med 1993; **177**:145–53.
35 Taverne J, Bate CAW, Kwiatkowski D, Jakobsen PH, Playfair JHL. Two soluble antigens of *Plasmodium falciparum* induce TNF release from macrophages. Infect Immun 1990; **58**:2923–8.
36 Kwiatkowski D, Cannon JG, Manogue KR, Cerami A, Dinarello CA, Greenwood BM. Tumour necrosis factor production in falciparum malaria and its association with schizont rupture. Clin Exp Immunol 1989; **77**:361–6.
37 Scragg I, Hensmann M, Bate C, Kwiatkowski D. Early cytokine induction by *Plasmodium falciparum* is not a classical endotoxin-like process. Eur J Immunol 1999; **29**:2636–44.
38 Riley EM, Jepsen S, Andersson G, Otoo LN, Greenwood BM. Cell-mediated immune responses to *Plasmodium falciparum* antigens in adult Gambians. Clin Exp Immunol 1988; **71**:377–82.
39 Chizzolini C, Grau G, Geinoz A, Schrijvers D. T lymphocyte interferon-gamma production induced by *Plasmodium falciparum* antigen is high in recently infected non-immune and low in immune subjects. Clin Exp Immunol 1990; **79**:95–9.
40 Hviid L, Theander TG, Abdulhadi NH, Abu-Zeid YA, Bayoumi RAL, Jensen JB. Transient depletion of T cells with high LFA-1 expression from the peripheral circulation during acute *Plasmodium falciparum* malaria. Eur J Immunol 1991; **21**:1249–53.
41 Dick S, Waterfall M, Currie J, Maddy A, Riley E. Naive human $\alpha\beta$ T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of IFNγ. Immunol 1996; **88**:412–20.
42 Waterfall M, Black A, Riley E. $\gamma\delta+$ T cells preferentially respond to live rather than killed malaria parasites. Infect Immun 1998; **66**:2393–8.
43 Currier J, Sattabongkot J, Rosenberg R, Good MF. Natural T cells responsive to malaria: evidence implicating immunological cross reactivity in the maintenance of TCR$\alpha\beta+$ malaria specific responses from non-exposed donors. Int Immunol 1992; **4**:985–94.
44 Goodier M, Lundqvist C, Hammarstrom M-L, Troye-Blomberg M, Langhorne J. Cytokine profiles for human Vγ9+ T cells stimulated by *Plasmodium falciparum*. Parasite Immunol 1995; **17**:413–23.
45 Currier J, Beck H-P, Currie B, Good MF. Antigens released at schizont burst stimulate *Plasmodium falciparum*-specific CD4+ T cells from non-exposed donors: potential for cross-reactive memory T cells to cause disease. Int Immunol 1995; **7**:821–33.
46 Behr C, Poupot R, Peyrat M-A, Poquet Y, Constant P, Dubois P, Bonneville M, Fournie J-J. *Plasmodium falciparum* stimuli for human $\gamma\delta$ T cells are related to the phophorylated antigens of mycobacteria. Infect Immun 1996; **64**:2892–6.
47 Orago A, Facer C. Cytotoxicity of human natural killer (NK) cell subsets for *Plasmodium falciparum* erythrocytic schizonts: stimulation by cytokines and inhibition by neomycin. Clin Exp Immunol 1991; **86**:22–9.
48 Baptista JL, Vanham G, Wery M, Van Marck E. Cytokine levels during mild and cerebral falciparum malaria in children living in a mesoendemic area. Trop Med Int Health 1997; **2**:673–9.
49 Ho M, Sexton MM, Tongtawe P, Looareesuwan S, Suntharsamai P, Webster HK. Interleukin-10 inhibits tumor necrosis factor production but not antigen-specific lymphoproliferation in acute *Plasmodium falciparum* malaria. J Infect Dis 1995; **172**:838–44.
50 Peyron F, Burdin N, Ringwald P, Vuillez JP, Rousset F, Banchereau J. High levels of circulating IL-10 in human malaria. Clin Exp Immunol 1994; **95**:300–3.
51 Wenisch C, Parschalk B, Burgmann H, Looareesuwan S, Graninger W. Decreased serum levels of TGFβ in patients with acute *Plasmodium falciparum* malaria. J Clin Immunol 1995; **15**:69–73.
52 Gorham JD, Guler ML, Fenoglio D, Gubler U, Murphy KM. Low dose TGF-beta attenuates IL-12 responsiveness in murine Th cells. J Immunol 1998; **161**:1664–70.