Interferon-γ Responses Are Associated with Resistance to Reinfection with *Plasmodium falciparum* in Young African Children

Adrian J. F. Luty, Bertrand Lell, Ruprecht Schmidt-Ott, Leopold G. Lehman, Doris Luckner, Bernhard Greve, Peter Matousek, Klaus Herbich, Daniela Schmid, Florence Migot-Nabias, Philippe Deloron, Ruth S. Nussenzweig, and Peter G. Kremsner

Department of Parasitology, Institute for Tropical Medicine, University of Tübingen, Tübingen, Germany; Research Unit, Albert Schweitzer Hospital, Lambaréné, and Centre International de Recherches Médicales de Franceville, Franceville, Gabon; Department of Infectious Diseases, Internal Medicine I, University of Vienna, Vienna, Austria; Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York

The contribution of T cell–mediated responses was studied with regard to resistance to reinfection in groups of Gabonese children participating in a prospective study of severe and mild malaria due to infection with *Plasmodium falciparum.* In those admitted with mild malaria, but not in those with severe malaria, production of IFN-γ by peripheral blood mononuclear cells (PBMC) in response to either liver-stage or merozoite antigen peptides was associated with significantly delayed first reinfections and with significantly lower rates of reinfection. Proliferative or tumor necrosis factor responses to the same peptides showed no such associations. Production of interferon-γ by PBMC in response to sporozoite and merozoite antigen peptides was observed in a higher proportion of those presenting with mild malaria. Differences in the Th1/Th2 cytokine balance may be linked to the ability to control parasite multiplication in these young children, helping to explain the marked differences observed in both susceptibility to infection as well as in clinical presentation.

The need to identify potential targets for a malaria vaccine has highlighted the relative dearth of knowledge concerning the interactions between the protozoan parasite and its human host. Prospective, longitudinal field-based studies are thus becoming one of the major tools used in attempts to identify naturally acquired human immune responses that correlate with some measure of protection from infection with or disease due to *Plasmodium falciparum.* In the majority of such studies carried out to date, individuals' parasitologic and clinical histories over a defined time period have been determined, and used in combination with one or more immunologic assessments. The latter have usually comprised measurements of humoral and/or cellular responses to protein antigens of either the sporozoite or the asexual blood stages. Many such studies have, in addition, included cohorts of individuals with a broad age range, to take account of the known age dependency of development of protective responses to this parasite in areas where it is highly endemic.

The success of these studies, with regard specifically to their ability to identify protective immune correlates, has varied. Attempts to show that antisporozoite responses correlate with protective effects have been equivocal in outcome [1–5]. Greater success in this context has accrued from studies that have investigated responses to asexual blood-stage antigens. Thus, for example, naturally acquired antibody responses to either neoantigens on infected red cells or to the merozoite surface antigens (MSA), MSA-1 and MSA-2, have been shown to be associated with reduced morbidity in children [6–11]. Similar studies aimed at demonstrating associations between protective effects and cellular responses to MSA-1 or MSA-2 or to other asexual-stage antigens have given conflicting results [7, 12–17]. No such studies have, to date, addressed such questions specifically with regard to the development of immunologic responses to liver-stage antigens, although both cellular and humoral responses to liver-stage antigen-1 (LSA-1), for example, can be demonstrated in exposed populations [18, 19].

Specific immunologic factors that might influence the clinical severity of malaria have also been sought through comparative studies, in some cases with a case-control, cross-sectional design. In one such study, IgM antibody profiles in those with cerebral malaria were lower than in those with noncerebral malaria [20], but in another, the lack of any difference in a range of antibody responses compared in severe and nonsevere cases suggested that the level of prior exposure, at least, was not the cause of the different clinical outcomes [21]. Numerous

Received 8 September 1998; revised 30 November 1998.

Informed consent for participation in this study was obtained, prior to inclusion, from the parent or guardian of each individual. Ethical clearance was obtained for the study from the Ethical Committee of the International Albert Schweitzer Foundation.

This study was supported in part by the *f*ortune Programme, Medical Faculty, University of Tübingen; WHO-TDR; and Malaria Programme, AUPELF-UREF.

Reprints or correspondence: Dr. Adrian J. F. Luty, University of Tübingen, Institute for Tropical Medicine, Dept. of Parasitology, Wilhelmstrasse 27, 72074 Tübingen, Germany (adrian.luty@uni-tuebingen.de).

The Journal of Infectious Diseases 1999; 179:980–8

0022-1899/99/7904-0027$02.00

cross-sectional studies have shown that higher plasma levels of several cytokines, including tumor necrosis factor (TNF), interferon-γ (IFN-γ), interleukin (IL)-1, IL-6, and IL-10, are associated with clinical and/or severe malaria [22–30]. These studies, however, have not consistently demonstrated clear correlates of protection from severe malaria. In addition, it has been noted that although such molecules may undoubtedly influence pathogenesis, they may also be seen as simply epiphenomena with no direct causal relationship to outcome [31]. The latter author also stated that in order to study specific antimalarial immunity "a logical approach is to take the actual documented experience of clinical malaria in a cohort followed over long periods of time as the index of immune status. ..." Our study took precisely this approach in an attempt to identify immunologic factors that correlate with the outcome of infection, in terms of clinical severity, or that influence susceptibility to reinfection. Thus, we carried out a matched-pair, case-control study of severe and mild malaria that included long-term active follow-up to prospectively gather information on each participants' experience of malaria over at least 1 year after inclusion in the study. Here we present data relating to cellular immunologic parameters, which were measured at admission and during the convalescent period and were compared within and between the groups in the context of reinfection profiles and clinical status.

Study Subjects and Methods

Study subjects and design. The study was carried out at the Albert Schweitzer Hospital in Lambaréné, Gabon. Transmission of *P. falciparum* in this area is perennial, with an estimated annual entomologic inoculation rate of 10–100 (Kremsner PG, et al., unpublished data). A detailed description of the participants, the inclusion criteria used, treatment given, clinical surveillance undertaken, and hematologic and biochemical methods used have been given elsewhere [32]. Briefly, 100 "cases" of severe malaria were matched for age, sex, and provenance to 100 "controls" with mild malaria. Reinfections and/or clinical malaria attacks, designated as parasitemia or parasitemia with fever (rectal temperature > 38°C), respectively, were detected through active clinical and parasitologic follow-up of individuals every 2 weeks following discharge from the hospital, at which times thick blood smears were routinely examined. Parasitemia was assessed using a calibrated thick smear technique as previously described [33]. Mothers were also urged to bring their children at any time to the hospital for any clinical event. The times to first reinfection were defined as the time from admission until the first positive thick smear. Individuals' incidence density rates of reinfection were estimated by calculating the ratio between the number of reinfections detected and the duration of follow-up observation in years.

Peripheral blood mononuclear cell (PBMC) cultures. Immunologic assessments were made at admission and 1 month later, during convalescence. For this purpose, 5-mL peripheral blood samples were taken by venesection into sterile collection tubes containing EDTA. PBMC were separated from whole blood by a standard density-gradient centrifugation technique using ficoll (Biochrom, Berlin, Germany). PBMC were then spin-washed with basic medium, comprising RPMI 1640 with 25 m*M* HEPES (Biochrom), and resuspended at a concentration of 1.5×10^6 cells/mL in culture medium, comprising basic medium supplemented with 10% decomplemented normal human AB^+ serum (CNTS, Paris, or Sigma, Deisenhofen, Germany) and 50 μg/mL gentamicin (GIBCO BRL, Paisley, Scotland). Cultures of PBMC were set up, in triplicate for each stimulant, in the wells of flat-bottomed 96-well plates, using 100 μL/well cell suspension mixed with 50 μl/well either culture medium alone (unstimulated cultures) or 50 μl/well the following molecules, diluted in culture medium to give the final concentrations indicated: (1) recall antigen: purified protein derivative (tuberculin; Statens Seruminstitut, Copenhagen), 10 μg/mL; (2) parasite antigen sequence-related peptides: sporozoite, $(T1B)_4$ multiple-antigen peptide, 10 μg/mL (T cell epitope, DPNANPNVDPNANPNV; B cell epitope, $[NANP]_3$); asexual erythrocytic stage, P1 (MSA-1) peptide, LNDITKEYEKLLNEI, 2 μg/mL, and P4 (MSA-2) peptide, NSTDSQKE, 1 μg/mL; liver stage (LSA-1), LSA-J peptide, ERRAKEKLQEQQRDLEQRKADTKK, 10 μg/mL, and ls6 peptide, KPIVQYDNF, 10 μg/mL.

The $(T1B)_4$ molecule, a multiple-antigen peptide based on a tetramer of conserved T and B cell epitopes of the circumsporozoite protein, has proven immunostimulatory capacity and was synthesized at New York University, as previously described [34, 35]. The P1 and P4 (MSA) and LSA peptides were custom-synthesized by the Institut Pasteur (Paris). Both merozoite antigen peptides were chosen because they represent known T cell stimulatory epitopes within conserved regions of the respective proteins [36, 37]. The LSA-1 peptides both have proven T cell stimulatory capacity and represent epitopes derived from the sequence of the T6/96 parasite clone [19]. There is no known polymorphism in the LSA-J sequence, but a single amino acid substitution ($K \rightarrow R$) has been described in the ls6 peptide sequence in a field isolate from Brazil [38].

Cell culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Pooled supernatants from triplicate wells, taken after 3 and 6 days of incubation, were stored frozen at −80°C until used for cytokine assessments (see below). Supernatants removed on day 6 were replaced with an equal volume of culture medium containing 1 μCi of tritiated thymidine (Amersham, Little Chalfont, UK), and the cultures were incubated for a further 16 h. At the end of this period, cells were collected onto glass-fiber mats using a cell harvester (PHD, Cambridge Instruments, MA) and processed for liquid scintillation counting. Proliferative responses were calculated and expressed as stimulation indices as previously described [39].

Cytokine assays. The concentrations of IL-10 and TNF were measured in supernatants of cell cultures obtained after 3 days and of IFN-γ after 6 days. For these measurements, monoclonal antibody pairs were used in a standard capture and detection sandwich ELISA, under conditions recommended by the manufacturers: IL-10 and TNF antibodies were obtained from PharMingen (Hamburg, Germany); IFN-γ antibodies were obtained from Mabtech (Nacka, Sweden). A peroxidase-conjugated avidin reagent (Extravidin; Sigma) was used in an amplification step, prior to addition of the chromogen-containing substrate mixture (TMB; Kirkegaard & Perry, Gaithersburg, MD). Reactions were stopped by adding

Table 1. Clinical and parasitologic observations of children at admission, segregated according to clinical status of malaria.

	Severe (n = 100)	Mild (n = 100)	P
Hyperparasitemia (no.)[a]	83	0	
Severe anemia			
Hemoglobin <50 g/L (no.)[a]	39	0	
Hematocrit <25% (no.)[b]	73	0	
Cerebral malaria (no.)	9	0	
Hypoglycemia (no.)	8	0	
Rectal temperature (°C)	39.8 ± 0.1	39.1 ± 0.1	<.001[c]
Heart beats/min	130 ± 2	120 ± 2	<.001[c]
Systolic blood pressure (mm Hg)	95 ± 1	103 ± 1	<.001[c]
Parasitemia/μL[d]	307,000 (217,500)	10,650 (21,750)	<.001[c]
Hematocrit (%)	21.6 ± 0.6	32.9 ± 0.4	<.001[c]
Lactate (mmol/L)	2.9 ± 0.2	2.4 ± 0.1	<.05[c]
Sickle cell trait (no.)	10	21	<.05[e]

NOTE. Values are mean ± SE unless indicated otherwise.
[a] Criteria used for case definition in this study.
[b] Criteria recently used/proposed for definition of severe anemia [40, 41].
[c] Wilcoxon signed rank test.
[d] Median (interquartile range).
[e] χ^2 test.

to each well 100 μL of 1 M H_3PO_4. Plates were read on an automated Digiscan plate reader (ASYS, Vienna) running on Mikrotek software (supplied by Dunn, Thelenberg, Germany) using a 450-nm filter with a 550-nm reference filter. The detection limit for the assays was 2 pg/mL (8 pg/mL for TNF), and values below this threshold were assigned a value of 0. For statistical analysis, after adjustment for sensitivity, cytokine concentrations in supernatants of unstimulated (control) cultures were subtracted from the values obtained for supernatants of peptide-stimulated cultures, and the resulting values were used to define responders and nonresponders.

Statistical methods. Individuals with <1 year of follow-up were excluded from analyses except, where comparisons of time to first reinfection were made, for those who were reinfected but lost from the study before the completion of 1 year of follow-up. Kaplan-Meier analyses were used to determine differences between the groups in the observed delay to first reinfection, using clinical status or dichotomized immunologic parameters as the grouping variable and the log rank test to determine the significance of differences between the groups. The association between immunologic parameters and reinfection profiles was also assessed by segregation into responders and nonresponders, followed by comparisons of reinfection data using the nonparametric Mann-Whitney U test. For the purposes of these analyses, we were interested specifically in the ability of an individual's PBMC to respond to a given stimulus, regardless of the time point. Thus, in all figures and tables, data from the acute- and convalescent-phase samples were combined so that individuals whose PBMC produced IFN-γ on either occasion, designated IFN-positive, were defined as responders, regardless of the presence or absence of an IL-10 response to the same molecule. Those producing IL-10 alone, or neither IL-10 nor IFN-γ, were designated IFN-negative. Contingency tables, using either Fisher's exact test or the McNemar test, were used to compare proportions between the groups.

Results

Clinical and parasitologic observations at admission and during follow-up. The matched patient groups comprised 61 female and 39 male patients, with a mean age of 44 ± 2 months. Selected clinical and parasitologic data at admission are shown in table 1. The frequencies of clinical signs and symptoms reflect the rigorous inclusion criteria and disease severity of the 2 study groups. Thus, rectal temperature and heart beat frequency were significantly higher and systolic blood pressure significantly lower in those with severe malaria. As expected, there was a significantly higher proportion of those with mild malaria who had the sickle-cell trait. During the follow-up period, no asymptomatic parasitemia was observed in any child (i.e., parasitemia was always associated with malaria symptoms). Thus, in all cases, reinfections referred to below are synonymous with clinical attacks.

Reinfection profiles according to clinical presentation. The reinfection profiles in the 2 groups, segregated according to clinical presentation at admission, are shown in table 2. Kaplan-Meier analysis showed that first reinfections occurred significantly earlier (P = .007) in those who presented with severe malaria. Pairwise comparison confirmed this finding, showing that a significantly higher number of first reinfections occurred during the first year of follow-up in those who presented with severe malaria, compared with those who had mild malaria (60/82 vs. 41/79; McNemar, P = .003). As shown in table 2, nonparametric analysis showed also that the group presenting with severe malaria had a significantly higher incidence density rate than did their counterparts with mild malaria (1.4 vs. 0.5, P < .001).

Reinfection profiles and PBMC proliferative responses. Comparison of proliferative responses in acute and convalescent phases combined showed that there was no difference in the proportions of responders in the groups with mild or with severe malaria for tuberculin (74/86 vs. 64/85), LSA-1 (62/78 vs. 55/70), the P1 peptide (27/81 vs. 27/79), or the P4 peptide (35/81 vs. 37/80). In Kaplan-Meier analyses and/or comparisons of groups segregated according to the presence or absence of proliferative responses, no significant differences were detected in either the times to reinfection or the rates of reinfection within either group (mild or severe malaria) (data not shown).

Time to first reinfection and PBMC cytokine responses. The medians and ranges of IFN-γ and IL-10 responses in PBMC to parasite antigen stimulation are shown in table 3. The amounts of either cytokine produced in response to the different peptides did not differ significantly between the groups segre-

Table 2. Times to first reinfection and annual reinfection rates in children segregated according to their clinical presentation with malaria at admission.

	Time to first reinfection	Rate of reinfection
Mild	43 (59) (n = 79) P = .028[a]	0.51 (1.59) (n = 71) P < .001[a]
Severe	29 (47) (n = 82)	1.39 (1.83) (n = 75)

NOTE. Values are medians (interquartile ranges) of times to reinfection in weeks and of incidence density rates of reinfection per year.
[a] Differences between groups were assessed by nonparametric (Mann-Whitney) analysis.

Table 3. Concentrations of cytokines produced by parasite antigen–stimulated PBMC from children with mild or severe malaria.

	IFN-γ		IL-10	
	Mild	Severe	Mild	Severe
$(T1B)_4$	56 (2–2030)	39 (2–833)	134 (2–2329)	72 (1–1761)
LSA-J	9 (1–155)	8 (1–53)	23 (1–296)	45 (1–800)
ls6	10 (3–126)	15 (2–252)	27 (1–482)	27 (1–152)
P1	19 (1–235)	18 (2–332)	22 (1–1056)	15 (1–261)
P4	17 (3–287)	10 (1–104)	19 (1–260)	14 (1–360)

NOTE. Values are medians (ranges) in pg/mL of cytokine production of peripheral blood mononuclear cells (PBMC) obtained in either acute or convalescent phase (data from responders only). IFN, interferon; IL, interleukin.

gated according to clinical presentation. In those with mild malaria, Kaplan-Meier analysis showed a highly significant association between delayed reinfection and the presence or absence of an IFN-γ response to the two LSA-1 peptides, with acute- and convalescent-phase data combined as the grouping variable (figure 1A; $P < .001$). Similar analyses of responses to the P1 and P4 merozoite (MSA1 and MSA2) peptides, in the same group, showed nonsignificant trends toward longer delays to reinfection in those with IFN-γ responses (figures 2A, 3A). In those who presented with severe malaria, such analyses showed that the delay to first reinfection was similar, regardless of the presence or absence of IFN-γ responses to either the LSA or MSA peptides (figures 1B, 2B, 3B).

Nonparametric analyses using dichotomization of IFN-γ responses of PBMC are shown in table 4. In the cohort with mild malaria, those whose PBMC produced IFN-γ in response to either of the LSA-1 peptides had a significantly longer delay to first reinfection. In the same group, there were nonsignificant trends toward longer delays to reinfection in those with IFN-γ responses to the MSA-1 and MSA-2 peptides. The presence or absence of cytokine responses to the circumsporozoite protein–derived $(T1B)_4$ molecule was not associated with any significant difference in the delay to first reinfection in this group. As seen in table 4, no such associations were found for those with severe malaria. Times to reinfection were not influenced in either group by the presence or absence of TNF responses to any of the stimuli used (data not shown). Controlling for the presence of the sickle cell trait (HbAS) did not alter the associations described above. The possible influence of blood group (ABO) or of the recently described point mutation in the inducible nitric oxide synthase (NOS2) promoter region, designated the $NOS2^{Lambaréné}$ [42], was assessed by contingency table analyses. There was an equal distribution of these genetic parameters in all the subgroups defined by dichotomization of cytokine responses (data not shown).

Rate of reinfection and PBMC cytokine responses. Preliminary analyses, using a logistic regression model, suggested that there was an independent association of mild malaria with IFN-γ responses. This was assessed here through comparisons using McNemar paired analysis with cumulated data from the acute and convalescent stages; these showed a significantly higher proportion of responders in the mild compared with the severe group, when considering IFN-γ responses to $(T1B)_4$ or to the MSA-1/MSA-2 peptides ($P = .006$ and $P = .027$, respectively). The observed rates of reinfection in relation to dichotomized cytokine responses, in groups segregated according to their clinical status at admission, are shown in table 5. In the group who presented with mild malaria, individuals whose PBMC produced IFN-γ to either of the LSA-1 peptides had a significantly lower rate of reinfection compared with those whose cells produced no IFN-γ, and there was a similar but less marked as-

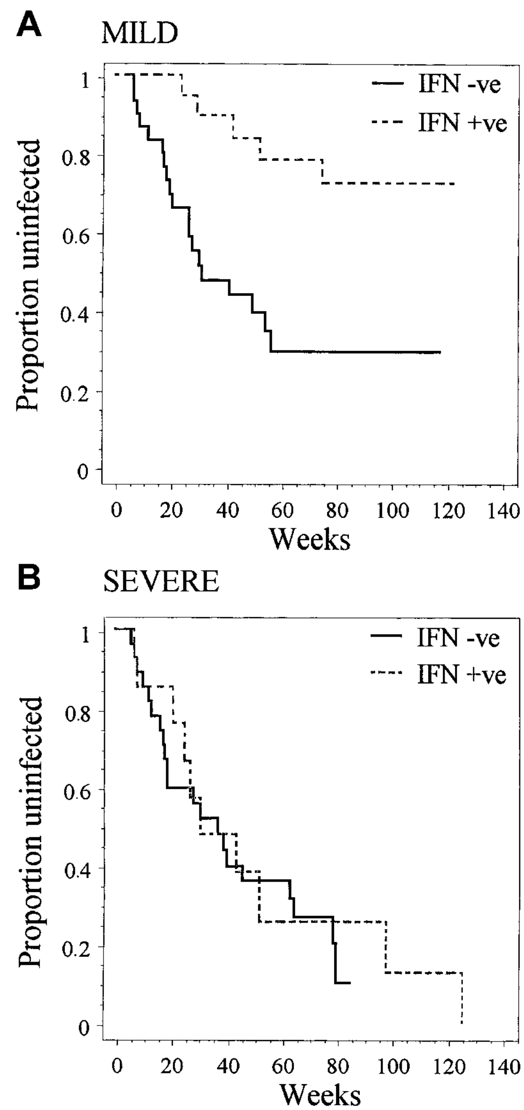

Figure 1. Kaplan-Meier analyses of times to first reinfection according to presence (positive, +ve) or absence (negative, -ve) of IFN-γ responses to liver-stage antigen-1 peptides (LSA-J & ls6), using acute- and convalescent-phase data combined. *A,* Children who presented initially with mild malaria; log rank, $P < .001$ for difference between groups. *B,* Children who presented initially with severe malaria; log rank, not significant.

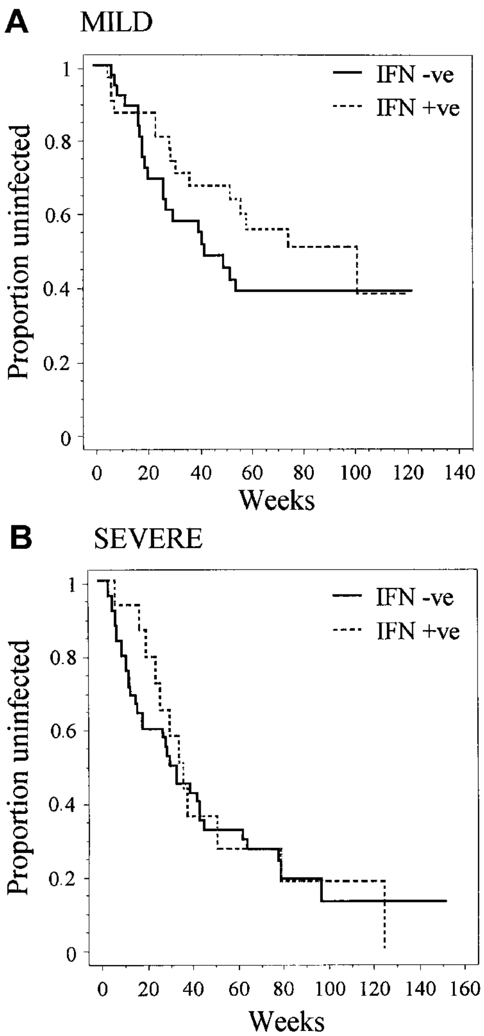

Figure 2. Kaplan-Meier analyses of times to first reinfection according to presence (positive, +ve) or absence (negative, -ve) of IFN-γ responses to merozoite surface antigen-1 peptide P1, using acute- and convalescent-phase data combined. *A,* Children who presented initially with mild malaria. *B,* Children who presented initially with severe malaria. Log rank, not significant for either group.

sociation for those with IFN-γ responses to the MSA peptides. In those who presented with severe malaria, rates of reinfection were lower in individuals with IFN-γ responses to the LSA-J and P1 peptides, but in neither case did the differences approach statistical significance. In both groups, those with IFN-γ responses to the $(T1B)_4$ molecule had lower rates of reinfection, but here again the differences did not approach statistical significance. No associations were found in either group between the rates of reinfection and TNF responses to the different stimuli (data not shown). Contingency table analyses revealed no differences in the distributions of genetic parameters (HbAS, ABO blood group, $NOS2^{Lambaréné}$) in comparisons between subgroups defined by cytokine responses (data not shown).

Discussion

This is the first study, to our knowledge, to have simultaneously combined a matched-pair case-control study of severe malaria with a prospective evaluation of reinfection profiles and their association with immunologic responses in the same individuals. Its value, we feel, essentially lies in three fundamental aspects of its design. First, the very strict inclusion criteria we used allowed a direct comparison of matched pairs of children who differed initially in their clinical status with regard to *P. falciparum* infection [32]. Second, the posttreatment, prospective, long-term follow-up we undertook self-evidently demon-

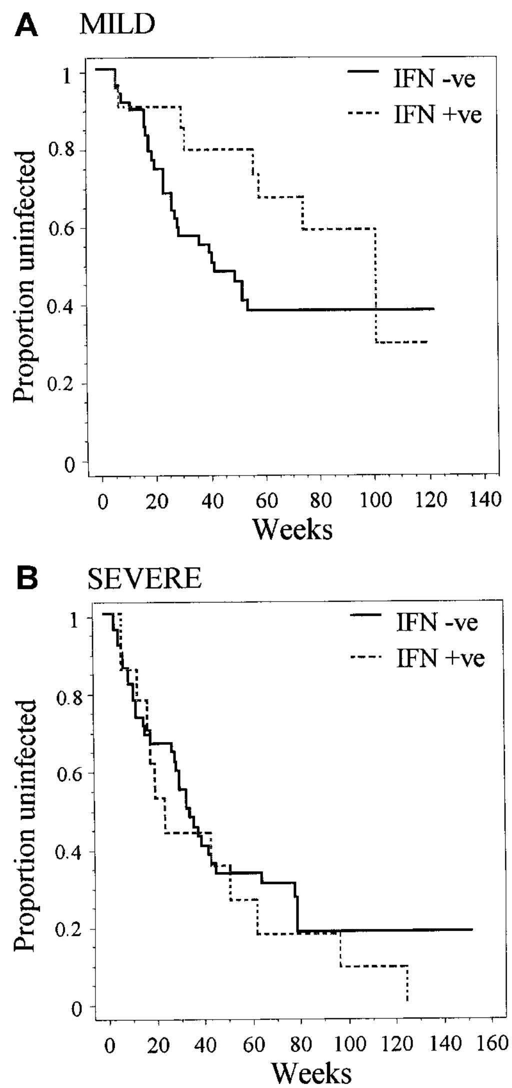

Figure 3. Kaplan-Meier analyses of times to first reinfection according to presence (positive, +ve) or absence (negative, -ve) of IFN-γ responses to merozoite surface antigen-2 peptide P4, using acute- and convalescent-phase data combined. *A,* Children who presented initially with mild malaria. *B,* Children who presented initially with severe malaria. Log rank, not significant for either group.

Table 4. Relationship between times to first reinfections and presence or absence of parasite antigen–stimulated IFN-γ responses of peripheral blood mononuclear cells from children presenting with either mild or severe malaria.

	Mild			Severe		
	IFN-γ–negative[a]	IFN-γ–positive[b]	P[c]	IFN-γ–negative[a]	IFN-γ–positive[b]	P[c]
$(T1B)_4$	28 (48)	53 (61)	NS	29 (31)	34 (58)	NS
n	6	56		23	33	
LSA-J	42 (51)	92 (41)	.007	28 (30)	31 (77)	NS
n	18	15		21	10	
ls6	31 (39)	95 (34)	.001	34 (53)	42 (92)	NS
n	31	14		24	8	
P1	43 (65)	59 (63)	NS	30 (53)	35 (35)	NS
n	32	29		42	13	
P4	42 (68)	64 (43)	.098	33 (55)	23 (42)	NS
n	43	17		42	12	

NOTE. Values are medians (interquartile ranges) of times to first reinfection in weeks. Response status is based on combination of acute- and convalescent-phase responses. NS, not significant.
[a] No interferon (IFN)-γ response.
[b] IFN-γ response with or without interleukin-10.
[c] Differences between groups were assessed using Mann-Whitney *U* test.

strated its utility through the results generated, which allowed us to identify clear differences in reinfection profiles, both within and between the clinically defined groups. Third, one of the major aims of this study was to assess immunologic responses as a function of clinical presentation following *P. falciparum* infection, to try to define factors that influence the differing outcomes. We concluded, as have others [43], that it is important to measure not only acute- but also convalescent-phase responses, as the latter, especially, could provide potentially the most informative data. Numerous studies have shown that acute infection with *P. falciparum* results in modulation of cellular responsiveness [15, 44–46], although the ability to produce cytokines may be maintained, as we have seen here and as others have also noted [28, 45]. Following effective antimalarial treatment, peripheral lymphocyte counts do normalize; the speed at which this happens is a function of disease severity, suggesting that sequestration of T cells explains the reduced reactivity in at least a proportion of individuals [43, 47]. For these reasons, therefore, by including observations in both acute and convalescent phases, we are confident that we have maximized our chances of detecting the individuals' ability to mount cellular immune responses to the stimulants used.

It should be stressed that the conclusions we draw from the findings presented here are based on an assumption that the reinfection profiles observed cannot be explained by fundamental differences in the participants' exposure to infection. We have made this assumption for three reasons. First, the inclusion criteria strictly stipulated residency within a defined geographic area around the hospital study base, restricting to a certain extent the possibility of variations in interindividual transmission patterns. Second, as part of the study, we conducted a simultaneous socioeconomic assessment of participants and their families. Analysis of these data failed to demonstrate any differences between the groups according to

Table 5. Relationship between annual rates of reinfection and presence or absence of parasite antigen–stimulated IFN-γ responses of PBMC from children presenting with either mild or severe malaria.

	Mild			Severe		
	IFN-γ–negative[a]	IFN-γ–positive[b]	P[c]	IFN-γ–negative[a]	IFN-γ–positive[b]	P[c]
$(T1B)_4$	1.41 (2.42)	0.46 (1.40)	NS	1.47 (1.22)	1.08 (1.64)	NS
n	5	52		20	31	
LSA-J	1.29 (1.76)	0.00 (0.49)	.021	2.02 (1.36)	1.24 (1.03)	NS
n	18	15		18	10	
ls6	0.77 (1.58)	0.00 (0.43)	.009	1.29 (1.28)	1.41 (2.00)	NS
n	29	14		22	8	
P1	0.76 (1.57)	0.00 (0.53)	.041	1.24 (1.49)	1.01 (1.61)	NS
n	31	25		36	13	
P4	0.57 (1.56)	0.00 (0.63)	.064	1.02 (1.65)	1.39 (1.52)	NS
n	40	16		37	11	

NOTE. Values are medians (interquartile ranges) of incidence density rates of infection/year. Response status is based on combination of acute- and convalescent-phase responses. NS, not significant.
[a] No interferon (IFN)-γ response.
[b] IFN-γ response with or without interleukin-10.
[c] Differences between groups were assessed using Mann-Whitney *U* test.

socioeconomic status that could explain the different outcomes of infection with respect to clinical severity and times to first reinfection [48]. Finally, in detailed analyses of humoral responses to a range of parasite-specific molecules, we found no evidence to suggest a higher degree of prior exposure in those with severe malaria (Luty AJF, unpublished data). We nevertheless accept that differences in individuals' exposure resulting from microenvironmental variation can not be entirely excluded as confounding influences, although we consider it unlikely that such factors could adequately explain the associations between indices of reinfection and immunologic responses we detected.

Our results point to a defining role for IFN-γ responses in determining reinfection profiles and possibly also clinical outcome in this cohort of nonimmune children. Within the group who presented with mild malaria, those who had IFN-γ responses to the LSA or MSA peptides both were slower to get reinfected and had fewer reinfections than those producing no IFN-γ to these molecules. Since the determination of reinfections in our study relied on detection of parasites in thick blood smears, we cannot distinguish between effector mechanisms operating on preerythrocytic stages or those that may act on the asexual erythrocytic stages in the immediate post–hepatocyte-rupture phase. We would, nevertheless, interpret our observations as representing the result of the cumulated effects of IFN-γ responses on both liver and asexual blood stages. The associations with reinfection indices, judged by the high levels of statistical significance, were very strong for IFN-γ responses to LSA-1 and to the combination of MSA-1/MSA-2. LSA-1 is thought to be produced uniquely during the liver stage [49], which would rule out any confounding influence of cross-reactive responses between this and the asexual blood-stage antigens. MSA-1, however, is produced during hepatocytic development, and cross-reactive, potentially protective responses thus cannot be entirely ruled out in this case. Furthermore, our results, with respect to a possible association between LSA-1–induced IFN-γ responses and protection from reinfection, are consistent with those reported in a recent study of responses of adult Papua New Guineans to an N-terminal peptide of LSA-1 [50].

One of the principal distinguishing features we found between those with mild and severe malaria, at the level of cytokine activity, was in the ability to produce IFN-γ in response to different parasite-antigen stimuli. Thus, a significantly higher proportion of children with mild malaria produced IFN-γ, in particular to the circumsporozoite protein-derived $(T1B)_4$ molecule, with an associated trend, albeit statistically nonsignificant, toward protection from reinfection. The protective effects of naturally acquired human antisporozoite cell-mediated responses have been demonstrated in one study with a treatment/reinfection design [2]. It is, however, difficult to envisage how sporozoites, which disappear very rapidly from the blood, could themselves be affected by cell-mediated responses. This has led to the widely held view that the targets of such responses are infected hepatocytes expressing sporozoite antigen-derived peptides on their surface. We would thus speculate that the sporozoite antigen-induced responses we have observed represent a surrogate marker for activity directed at developing liver stages. Individuals with a predominantly IFN-γ–led response to sporozoite antigens, as may be the case here in those with mild malaria, may thus be better able to control infections at the liver stage.

Evidence for the antiparasitic effects of IFN-γ, in the context of preerythrocytic stages, has come from in vitro experiments demonstrating an inhibitory effect on the development of hepatic-stage parasites [51]. Subsequent studies have implicated nitric oxide as the effector molecule targeting parasites within hepatocytes in this system [52, 53]. Other studies have, in addition, suggested that IFN-γ may mediate protection against preerythrocytic stages in vivo [54, 55]. There are numerous potential cellular sources of the IFN-γ we detected here in response to LSA-1. Obvious candidates, which have putative protective effects, are HLA class I–restricted $CD8^+$ cytotoxic T lymphocytes, such as those identified by Hill et al. [18]. The HLA-B53 cytotoxic T lymphocytes characterized in the latter study recognize, among other molecules, the ls6 nonapeptide we used for in vitro PBMC stimulations in the current study. As we and others have seen, stimulation of PBMC with LSA-1–derived peptides can induce both proliferation and IFN-γ production, which may be dissociated from each other but which nevertheless involve a substantial proportion of $CD8^+$ cells [19, 50]. Since LSA-1 accumulates in the parasitophorous vacuole space during the parasite's intrahepatic development and is released as a flocculent matrix surrounding merozoites when the hepatocytes rupture [19], it seems reasonable to assume that this material will also be processed to induce $CD4^+$ T cell responses. A study of chronically exposed Kenyans detected an association between IL-10 responses to LSA-1 polypeptides and resistance to reinfection (J. Kurtis and P. Duffy, personal communication). This would suggest, in the context of our results with nonimmune children, that there may be a modulation of or switch away from inflammatory (Th1)-type responses to given epitopes as immunity to *P. falciparum* evolves. Other studies have shown that chronically exposed individuals do indeed down-regulate IFN-γ responses, which could be taken as evidence for the existence of such a modulatory process [25, 56]. It will clearly be of great interest to know if the associations between protection from reinfection and anti–LSA-1 cytokine responses we and others have described are seen consistently in other areas with different levels of endemicity and varying transmission profiles.

In this study we also observed that a significantly greater proportion of those with mild malaria were able to produce IFN-γ in response to peptides derived from the merozoite antigens. IFN-γ is not known to have a direct effect on asexual erythrocytic forms, but an indirect effect could be envisaged through its ability to activate macrophages and neutrophils.

There is substantial evidence for distinct roles of the latter two cell types in recognition and removal of either merozoites or parasitized erythrocytes [57, 58, reviewed in 59]. In addition, the idea of a protective effect of IFN-γ during the early phase of blood-stage malaria infection is strongly supported by laboratory experiments with murine models. The consensus, although somewhat dependent on the combination of mouse and parasite strains used, is that effective control of parasitemia in mice given primary blood-stage infections requires an early IFN-γ–driven Th1-type response [reviewed in 60].

To summarize, the study described here demonstrated marked differences in the susceptibility to reinfection in 2 matched groups of nonimmune African children who differed in their initial clinical presentation. We believe that parasite density is perhaps the most important parameter associated with the development of severe malaria and that, for this reason, determining which factors are involved in the control of parasite multiplication is essential for an understanding of the disease process. On the basis of the marked differences in the reinfection profiles we observed between those who presented initially with severe compared with mild malaria, we feel our findings provide compelling evidence to support the idea that the principal factor distinguishing them apart as groups is therefore the ability, or lack of such, to control parasitemia. We have shown, on one hand, that in some children IFN-γ responses to both preerythrocytic and asexual blood stage parasite antigens are strongly associated with protection from reinfection. On the other hand, as a group, the children who presented initially with mild malaria and who have significantly lower reinfection rates are better able to mount such IFN-γ responses than those admitted with severe malaria. We therefore feel that these findings demonstrate a pivotal role for IFN-γ in controlling parasitemia in some of these nonimmune children, and that this goes some way toward explaining the differences in clinical outcome.

Acknowledgments

We thank the children and their families for their participation in this study, Anselme Ndzengué and Marcel Nkeyi for their excellent technical assistance, and Swissair for free transport of study material.

References

1. Marsh K, Hayes RH, Carson DC, et al. Anti-sporozoite antibodies and immunity to malaria in a rural Gambian population. Trans R Soc Trop Med Hyg **1988**;82:532–7.
2. Hoffman SL, Oster CN, Mason C, et al. Human lymphocyte proliferative response to a sporozoite T cell epitope correlates with resistance to falciparum malaria. J Immunol **1989**;142:1299–303.
3. Hollingdale MR, Hogh B, Petersen E, Wirtz RA, Bjorkmann A. Age-dependent occurrence of protective anti–*Plasmodium falciparum* sporozoite antibodies in a holoendemic area of Liberia. Trans R Soc Trop Med Hyg **1989**;83:322–4.
4. Snow RW, Shenton FC, Lindsay SW, et al. Sporozoite antibodies and malaria in children in a rural area of The Gambia. Ann Trop Med Parasitol **1989**;83:559–68.
5. Riley EM, Allen SJ, Bennett S, et al. Recognition of dominant T cell–stimulating epitopes from the circumsporozoite protein of *Plasmodium falciparum* and relationship to malaria morbidity in Gambian children. Trans R Soc Trop Med Hyg **1990**;84:648–57.
6. Marsh K, Otoo L, Hayes RH, Carson DC, Greenwood BM. Antibodies to blood stage antigens of *Plasmodium falciparum* in rural Gambians and their relation to protection against infection. Trans R Soc Trop Med Hyg **1989**;83:293–303.
7. Riley EM, Allen SJ, Wheeler JG, et al. Naturally acquired cellular and humoral immune responses to the major merozoite surface antigen (PfMSP1) of *Plasmodium falciparum* are associated with reduced malaria morbidity. Parasite Immunol **1992**;14:321–37.
8. Tolle R, Früh K, Doumbo O, et al. A prospective study of the association between the human humoral response to *Plasmodium falciparum* blood stage antigen gp190 and control of malarial infections. Infect Immun **1993**;61:40–7.
9. Al-Yaman F, Genton B, Anders RF, et al. Relationship between humoral response to *Plasmodium falciparum* merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. Am J Trop Med Hyg **1994**;51:593–602.
10. Al-Yaman F, Genton B, Kramer KJ, et al. Assessment of the role of naturally acquired antibody levels to *Plasmodium falciparum* merozoite surface protein-1 in protecting Papua New Guinean children from malaria morbidity. Am J Trop Med Hyg **1996**;54:443–8.
11. Egan AF, Morris J, Barnish G, et al. Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. J Infect Dis **1996**;173:765–9.
12. Chougnet C, Lepers JP, Astagneau P, Rason MD, Savel J, Deloron P. Lymphoproliferative responses to synthetic peptides from merozoite ring-infected erythrocyte surface antigen and circumsporozoite protein: a longitudinal study during a falciparum malaria episode. Am J Trop Med Hyg **1991**;45:560–6.
13. Riley EM, Allen SJ, Troye-Blomberg M, et al. Association between immune recognition of the malaria vaccine candidate antigen Pf155/RESA and resistance to clinical disease: a prospective study in a malaria endemic region of West Africa. Trans R Soc Trop Med Hyg **1991**;85:436–43.
14. Riley EM, Jakobsen PH, Allen SJ, et al. Immune response to soluble exoantigens of *Plasmodium falciparum* may contribute to both pathogenesis and protection in clinical malaria: evidence from a longitudinal, prospective study of semi-immune African children. Eur J Immunol **1991**;21:1019–25.
15. Mshana RN, Boulandi J, Mayombo J, Mendome G. In vitro lymphoproliferative responses to malaria antigens: a prospective study of residents of a holoendemic area with perennial malaria transmission. Parasite Immunol **1993**;15:35–45.
16. Al-Yaman F, Genton B, Taraika J, Anders R, Alpers MP. Cellular immunity to merozoite surface protein 2 (FC27 and 3D7) in Papua New Guinean children. Temporal variation and relation to clinical and parasitological status. Parasite Immunol **1997**;19:207–14.
17. Al-Yaman F, Genton B, Taraika J, Anders R, Alpers MP. Association between cellular response (IL-4) to RESA/Pf155 and protection from clinical malaria among Papua New Guinean children living in a malaria endemic area. Parasite Immunol **1997**;19:249–54.
18. Hill AVS, Elvin J, Willis AC, et al. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature **1992**;360:434–9.
19. Fidock DA, Gras MH, Lepers JP, et al. *Plasmodium falciparum* liver stage antigen-1 is well conserved and contains potent B and T cell determinants. J Immunol **1994**;153:190–204.
20. Brasseur P, Ballet JJ, Druilhe P. Impairment of *Plasmodium falciparum*–specific antibody response in severe malaria. J Clin Microbiol **1990**;28:265–8.

21. Erunkulu OA, Hill AVS, Kwiatkowski DP, et al. Severe malaria in Gambian children is not due to lack of previous exposure to malaria. Clin Exp Immunol **1992**; 89:296–300.
22. Grau GE, Taylor TE, Molyneux MR, et al. Tumor necrosis factor and disease severity in children with falciparum malaria. N Engl J Med **1989**; 320: 1586–91.
23. Kern P, Hemmer CJ, Van Damme J, Gruss HJ, Dietrich M. Elevated tumor necrosis factor-α and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. Am J Med **1989**; 87:139–43.
24. Kwiatkowski D, Hill AVS, Sambou I, et al. TNF concentration in cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. Lancet **1990**; 336:1201–4.
25. Mshana RN, Boulandi J, Mshana NM, Mayombo J, Mendome G. Cytokines in the pathogenesis of malaria: levels of IL-Iβ, IL-4, IL-6, TNF-α and IFN-γ in plasma of healthy individuals and malaria patients in a holoendemic area. J Clin Lab Immunol **1991**; 34:131–9.
26. Ringwald P, Peyron F, Vuillez JP, Touze JE, Le Bras J, Deloron P. Levels of cytokines in plasma during *Plasmodium falciparum* malaria attacks. J Clin Microbiol **1991**; 29:2076–8.
27. 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.
28. Ho M, Sexton MM, Tongtawe P, Looareesuwan S, Suntharasamai 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.
29. Kremsner PG, Winkler S, Brandts C, et al. Prediction of accelerated cure in *Plasmodium falciparum* malaria by the elevated capacity of tumor necrosis factor production. Am J Trop Med Hyg **1995**; 53:532–8.
30. Mordmuller BG, Metzger WG, Juillard P, et al. Tumor necrosis factor in *Plasmodium falciparum* malaria: high plasma level is associated with fever, but high production capacity is associated with rapid fever clearance. Eur Cytokine Netw **1997**; 8:29–35.
31. Marsh K. Malaria—a neglected disease? Parasitology **1992**; 104:S53–69.
32. Kun JFJ, Schmidt-Ott RJ, Lehman LG, et al. Merozoite surface antigen 1 and 2 genotypes and rosetting of *Plasmodium falciparum* in severe and mild malaria in Lambarene, Gabon. Trans R Soc Trop Med Hyg **1998**; 92:110–4.
33. Kremsner PG, Zotter GM, Feldmeier H, Graninger W, Rocha RM, Wiedermann G. A comparative trial of three regimens for treating uncomplicated falciparum malaria in Acre, Brazil. J Infect Dis **1988**; 158:1368–71.
34. Munesinghe DY, Clavijo P, Calvo Calle M, Nussenzweig RS, Nardin EH. Immunogenicity of multiple antigen peptides (MAP) containing T and B cell epitopes of the repeat region of the *P. falciparum* circumsporozoite protein. Eur J Immunol **1991**; 21:3015–20.
35. de Oliveira GA, Clavijo P, Nussenzweig RS, Nardin EH. Immunogenicity of an alum-adsorbed synthetic multiple-antigen peptide based on B- and T- cell epitopes of the *Plasmodium falciparum* CS protein: possible vaccine application. Vaccine **1994**; 12:1012–7.
36. Rzepczyk CM, Csurhes PA, Saul AJ, et al. Comparative study of the T cell response to two allelic forms of a malarial vaccine candidate protein. J Immunol **1992**; 148:1197–204.
37. Kabilan L, Sharma VP, Kaur P, Ghosh SK, Yadav RS, Chauhan VS. Cellular and humoral immune responses to well-defined blood stage antigens (major merozoite surface antigen) of *Plasmodium falciparum* in adults from an Indian zone where malaria is endemic. Infect Immun **1994**; 62:685–91.
38. Yang C, Shi YP, Udhayakumar V, et al. Sequence variations in the nonrepetitive regions of the liver stage–specific antigen-1 of *Plasmodium falciparum* from field isolates. Mol Biochem Parasitol **1995**; 71:291–4.
39. Luty AJF, Mayombo J, Lekoulou F, Mshana R. Immunologic responses to soluble exoantigens of *Plasmodium falciparum* in Gabonese children exposed to continuous intense infection. Am J Trop Med Hyg **1994**; 51: 720–9.
40. Menendez C, Kahigwa E, Hirt R, et al. Randomised placebo-controlled trial of iron supplementation and malaria chemoprophylaxis for prevention of severe anaemia and malaria in Tanzanian infants. Lancet **1997**; 350: 844–50.
41. Stoltzfus RJ. Rethinking anaemia surveillance. Lancet **1997**; 349:1764–6.
42. Kun JFJ, Mordmuller B, Lell B, Lehman LG, Luckner D, Kremsner PG. Polymorphism in promoter region of inducible nitric oxide synthase gene and protection against malaria. Lancet **1998**; 351:265–6.
43. Hviid L, Kurtzahls JAL, Goka BQ, Oliver-Commey JO, Nkrumah FK, Theander TG. Rapid reemergence of T cells into peripheral circulation following treatment of severe and uncomplicated *Plasmodium falciparum* malaria. Infect Immun **1997**; 65:4090–3.
44. Brasseur P, Agrapart M, Ballet JJ, Druilhe P, Warrell MJ, Tharavanij S. Impaired cell-mediated immunity in *Plasmodium falciparum*–infected patients with high-parasitemia and cerebral malaria. Clin Immunol Immunopathol **1983**; 27:38–50.
45. Riley EM, Andersson G, Otoo LN, Jepsen S, Greenwood BM. Cellular immune responses to *Plasmodium falciparum* antigens during and after an acute attack of falciparum malaria. Clin Exp Immunol **1988**; 73:17–22.
46. Kremsner PG, Zotter GM, Feldmeier H, et al. Immune response in patients during and after *Plasmodium falciparum* infection. J Infect Dis **1990**; 161: 1025–8.
47. Chougnet C, Tallet S, Ringwald P, Deloron P. Kinetics of lymphocyte subsets from peripheral blood during a *Plasmodium falciparum* attack. Clin Exp Immunol **1992**; 90:405–8.
48. Luckner D, Lell B, Greve B, et al. No influence of socio-economic factors on severe malarial anaemia, hyper-parasitaemia and reinfection. Trans R Soc Trop Med Hyg **1998**; 92:478–81.
49. Guerin-Marchand C, Druilhe P, Galey B, et al. A liver-stage–specific antigen of *Plasmodium falciparum* characterized by gene cloning. Nature **1987**; 329:164–7.
50. Connelly M, King CL, Bucci K, et al. T-cell immunity to peptide epitopes of liver-stage antigen-1 in an area of Papua New Guinea in which malaria is holoendemic. Infect Immun **1997**; 65:5082–7.
51. Schofield L, Villaquiran J, Ferreira A, Schellekens H, Nussenzweig R, Nussenzweig V. γ-interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. Nature **1987**; 330:664–8.
52. Seguin MC, Klotz FW, Schneider I, et al. Induction of nitric oxide synthase protects against malaria in mice exposed to irradiated *Plasmodium berghei* infected mosquitoes: involvement of interferon-gamma and $CD8^+$ T cells. J Exp Med **1994**; 180:353–8.
53. Klotz FW, Scheller LF, Seguin MC, et al. Co-localization of inducible nitric oxide synthase and *Plasmodium berghei* in hepatocytes from rats immunized with irradiated sporozoites. J Immunol **1995**; 154:3391–5.
54. Ferreira A, Schofield L, Enea V, et al. Inhibition of development of exoerythrocytic forms of malaria parasites by γ-interferon. Science **1986**; 232: 881–4.
55. Deloron P, Chougnet C, Lepers JP, Tallet S, Coulange P. Protective value of elevated levels of γ interferon in serum against exoerythrocytic stages of *Plasmodium falciparum.* J Clin Microbiol **1991**; 29:1757–60.
56. Chizzolini C, Grau GE, Geinoz A, Schrijvers D. T lymphocyte interferon-γ 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.
57. Bouharoun-Tayoun H, Oevray C, Lunel F, Druilhe P. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. J Exp Med **1995**; 182:409–18.
58. Druilhe P, Perignon JL. A hypothesis about the chronicity of malaria infection. Parasitol Today **1997**; 13:353–7.
59. Kumaratilake LM, Ferrante A. T-cell cytokines in malaria: their role in the regulation of neutrophil- and macrophage-mediated killing of *Plasmodium falciparum* asexual blood forms. Res Immunol **1994**; 145:423–9.
60. Taylor-Robinson AW. Regulation of immunity to malaria. Valuable lessons learned from murine models. Parasitol Today **1995**; 11:334–42.