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Hemozoin is a key factor in the induction of malaria-associated immunosuppression

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SUMMARY

Infection-associated immunoincompetence during malaria might result from macrophage dysfunction. In the present study, we investigated the role of macrophages as target for immunosuppression during infection, using the murine Plasmodium c. chabaudi *model. Special attention has been paid to the analysis of processing/presentation of protein antigens and presentation of peptides, using cocultures of peritoneal exudate cells (PECs) from infected mice and antigen-specific T-cell hybridomas. The results obtained indicate a defective processing of protein antigens that becomes maximal at acute parasitemias. In addition, macrophages from acutely infected mice suppress the interleukin-2 production by the antigen-activated T-cell hybridomas. This effect was independent of prostaglandin and nitric oxide production by the macrophage. The possible role of parasite components in the impaired accessory cell function of PECs was investigated and hemozoin, the end-product of the hemoglobin catabolism by intraerythrocytic malaria parasites, was found to induce similar infection-associated deficiencies* in vitro. *Moreover, hemozoin, was shown to mimic the immunosuppressive effects induced in PECs during in-vivo infections with* P. chabaudi. *In conclusion, we propose that hemozoin is a key factor in the malaria-associated immunosuppression, affecting both the antigen processing and immunomodulatory functions of macrophages.*

Keywords *malaria, immunosuppression, hemozoin*

INTRODUCTION

The occurrence of malaria-associated immunosuppression is supported by extensive experimental data. *Plasmodium* infections have been shown to induce alterations in immune reactivity and acute malaria has been associated with increased susceptibility to Salmonellosis (Bennett & Hook 1959, Mabey *et al.* 1987) and other bacterial infections (Greenwood 1974). In addition, reactivation of chronic and latent viral infections such as *Herpes zoster* (Cook 1985), *H. simplex* (Scott 1944) and Epstein–Barr virus (Whittle *et al.* 1984, 1990) were found to occur during acute *Plasmodium* infections. To date, no single mechanism can explain the suppressive effects of malaria infections on the host immune system but early studies *in vivo* have suggested that defects in macrophage accessory cell function could account for the poor immune reactivity (Loose *et al.* 1972, Warren & Weidanz 1976, Kim *et al.* 1978, Brown & Kreier 1986). Furthermore, macrophages may also act as immunosuppressor cells (Correa *et al.* 1980, Mahajan *et al.* 1986) during high levels of parasitemias, although the outcome of this immunomodulatory effect is still unknown. Experiments in which the lymphoproliferative responses of splenocytes to mitogens were evaluated have associated the immunosuppressive effects with nitric oxide (NO) and prostaglandin production (Rocket *et al*. 1994, Ahvazi *et al.* 1995).

Concerning the *Plasmodia* components responsible of the immunosuppressive effects, the most marked suppression has been described for crude schizont extracts and soluble exoantigens (Riley *et al*. 1988, Ho *et al*. 1986, Hviid *et al.* 1991). It has been also suggested that accumulation of parasite-derived hemozoin (malaria pigment) inside macrophages may inhibit their normal accessory cell functions, although the mechanism by which the suppression is mediated has not been characterized (Morakote & Justus 1988).

In the present study, we further analysed the effect of malaria infections on the accessory cell function of

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peritoneal macrophages. A convenient in-vitro antigen-presentation system, with a relevant T-cell hybridoma as a interleukin (IL)-2 producer effector cell was used to discriminate antigen processing from peptide presentation. We show that acute malaria infections affect the accessory cell function of macrophages through a defective antigen-processing and active suppression of IL-2 production by the T cell hybridoma. The inhibitory effect of the *Plasmodium* infection involves hemozoin, which readily accumulates within macrophages and which can mimic *in vitro* the effect of the malaria infection.

MATERIALS AND METHODS

Mice and parasites

Inbred female Balb/c mice aged 8–12 weeks were purchased from Harlan (Zeist, The Netherlands). The *Plasmodium c. chabaudi* AS strain was originally obtained from Dr Walliker (Edinburgh, UK). Parasites were kept as glycerol (10%) stocks at – 80°C or in liquid nitrogen. Infection of Balb/c mice with the *P. c. chabaudi* strain is characterized by a primary peak of parasitemia (28–35%) that is resolved and followed by one or two minor peaks.

Experimental mice were inoculated intravenously with 1×10^5 infected redblood cells (iRBC). Parasites were maintained by weekly passages of 10^5–10^6 iRBC (diluted in phosphate buffered saline, PBS) injected intravenously into Balb/c mice. Parasitemias were determined by examination of Giemsa-stained (10% in PBS) thin blood smears and calculated as the percentage of iRBC (% Parasitemia). For the isolation of trophozoite-infected redblood cells during day time, infected mice were kept in an inverse light schedule.

T-cell hybridomas and antigens

The murine 1E5.11 T-cell hybridoma (I-Ed restricted), reactive to hen egg white lysozyme (HEL) or to the HEL peptide (residues 150–120) was kindly provided by Dr A. Darji (GBF, Braunschweig, Germany). Hen egg white lysozyme (Merck) was used at a final concentration of 10 μg/ml. The HEL peptide (Dr A. Darji) was used at a final concentration of 5 μg/ml.

Antigen assays

Resident peritoneal exudate cells (PECs) were recovered from the peritoneal cavity of mice by washing with 10 ml of an ice cold 0·34 M sucrose solution. The cells were kept in complete medium supplemented with 2-mercapthoethanol (RPMI 1640 (Gibco Brl, Life Technologies, Rockville, MD, USA) + 10% FCS, 2 mM L-glutamine (Gibco), 5×10^{-5} M 2-ME (Sigma, St Louis, MO, USA), 100 U/ml of penicillin-streptomycin (Gibco). PECs were plated into 96-well plates (Falcon, Becton Dickinson, Franklin Labs, NY, USA), 100 μl per well, and the cells were allowed to adhere for 4 h. In certain experiments, nonadherent cells were removed by washing with warm RPMI. The 1E5.11 T-cell hybridoma was used at a concentration of 2×10^5 cells/ml, 100 μl per well. The antigens were added at the concentrations described above. Eighteen hours later, the supernatants were recovered and assayed for IL-2.

The porphyrins β-hematin and hemozoin were used at a final concentration of 10 μg/ml, and were added to the PECs prior to the addition of the T-cell hybridoma and the antigens.

Fixation with glutaraldehyde

The PECs were washed and resuspended to 4×10^6 cells/ml. A hundred microliters of a 0·5% glutaraldehyde (GA) solution in PBS were added to 1 ml of the cell suspension and 45 s later the cells were washed in an excess of PBS four times. The pellet was resuspended in complete medium. The fixed PECs were used as antigen presenting cells (APC) with the HEL peptide and the 1E5.11 T-cell hybridoma.

In certain experiments, PECs were preincubated with lysozyme (10 μg/ml) for 3 h, excess antigen was removed by washing, and the cells were recovered in suspension and fixed with GA as described above.

Hemozoin and β-hematin

Crude hemozoin was obtained from the blood of *P. c. chabaudi*-infected Balb/c mice. Mice with a parasitemia of 25% or more were bled when trophozoites were the predominant malaria blood form. After two washes in ice-cold PBS, the white blood cells were eliminated by passage of the blood through a column of sulphoethylcellulose (Serva, Heidelberg, Germany): sephadex G25 (Pharmacia, Uppsala, Sweden) (1 : 3 w/w in PBS). The trophozoite containing iRBC were washed twice and lysed by sonication with a soniprep 150 (1 min bursts/1 min cooling/200–300 W). Crude hemozoin, characterized by a brownish pellet, was obtained after exhaustive washes to eliminate free hemoglobin. After a final wash in 70% ethanol, the crude hemozoin was vacuum dried.

Chemically synthesized β-hematin was prepared as described (Fitch & Kanjananggulpan 1987). Briefly, 60 μmol of hematin (Sigma) were dissolved in 8 ml of 0·1 M NaOH, under N_2, and the porphyrin was precipitated by the addition of 49 mmol of acetic acid. The suspension was

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heated overnight at 70°C and the precipitate was washed four times in distilled water. Non reacting hematin was removed by extracting the precipitate twice for 3 h in 0·1 M sodium bicarbonate buffer, pH 9·1. The remaining insoluble material was recovered by centrifugation, and washed four times in distilled water. After a final wash in 70% ethanol, the insoluble material was air-dried. This method routinely converts 40–50% of the starting material into β-hematin (Slater *et al*. 1991). β-hematin and crude hemozoin (weight as a powder when dried) were resuspended in RPMI at a concentration of 1 mg/ml. To facilitate the treatment of the PECs with insoluble hemozoin or β-hematin, the compounds were then mechanically microdispersed and sonicated.

Measurements of hemozoin in PECs

PECs from noninfected and *P. chabaudi*-infected (25% parasitemia) mice were isolated as previously described and washed in PBS. The cells were lysed by three freeze-thaw cycles. Hemozoin levels in the cell lysates were measured based on the property of haem-elicited luminiscence (Schwarzer *et al*. 1994). 100 μl luminol/NaOH solution (luminol, 1 μg/ml) (Sigma), dissolved in a NaOH solution containing 0·1 M NaOH and 3 mM ethylenediaminetetraacetic acid (EDTA), and 100 μl t-buthylhydroperoxide/NaOH solution (7·26 mM t-buthylhydroperoxide (Sigma), dissolved in a solution containing 0·1 M NaOH and 3 mM EDTA), were mixed in a 96-well plate. Serial dilutions of PECs lysate (30 μl/well) were added to the plate and luminescence ouput was immediately measured at 530 nm and maximal gain, using a Cytofluor II fluorescent multiwell plate reader (PerSeptive Biosystems, Framingham, MA, USA). Results were plotted as flourescent units in function of cell concentration. As a control, synthetic β-hematine was used.

IL-2-specific enzyme-linked immunosorbent assay

96-well plates (Nunc, Roskilde, Denmark) were coated with the rat antimouse IL-2 monoclonal antibody (18161D, Pharmingen, San Diego, CA, USA) (0·05 μg/ml in 0·1 M sodium hydrogen carbonate, pH 9·6), overnight at 4°C. After two washes in PBS + 0·05% Tween 20, the plates were blocked with PBS + 10% FCS (Gibco) for 2 h at 37°C. Subsequently, the wells were incubated with 100 μl of supernatants from the antigen presentation tests (triplicates), or with 100 μl of a recombinant murine IL-2 standard (19211T, Pharmingen, diluted in PBS + 10% FCS) for 1 h at 37°C. Following four washes, 100 μl of the biotinilated rat antimouse IL-2 monoclonal antibody (18172D, Pharmingen) (0·05 μg/ml in PBS + 10% FCS) were applied into each well, and allowed to react for 1 h at 37°C. A hundred microliters of streptavidin-peroxidase conjugate (Jackson Immuno Research, West Grove, PA, USA) (0·025 μg/ml in PBS + 10% FCS) were added for 30 min. Once the plates were washed several times, the bound complexes were detected by reaction with tetramethyl-benzidine (TMB, Sigma) and H_2O_2. The absorbencies were read at 450 nm/690 nm and the IL-2 concentration in the samples was calculated as pg/ml using the recombinant murine IL-2 standard. Statistical analysis of the experimental data was performed using Student's *t*-test.

Uptake and catabolism of protein antigen

Lysozyme was radiolabelled with ^{125}I (to a specific activity of 1·1106–4·7106 cpm/μg) as specified by the manufacturer (Pierce Chemicals, Rockford, IL, USA). PECs (2106 per ml) were plated on 35 mM tissue culture plates (Falcon) and incubated with 6 μg of radiolabeled lysozyme for 4 h at 37°C.

Non-adherent PECs and nonincorporated lysozyme were removed by washing the culture plates with warm RPMI several times until no radioactivity could be detected in the washing medium. The adherent PECs were then solubilized in Triton X-100 (0·5% in PBS) and the radioactivity from the total cell extracts was measured. To study protein catabolism, cells incubated with the radioactive lysozyme for 4 h were lysed with Triton X-100 and the 10% TCA-soluble and insoluble radioactivity present in cells and in culture media were measured. In control experiments, PECs were incubated with ^{125}I-lysozyme in the presence of 0·2 mM sodium azide to block endocytosis. Statistical analysis of the experimental data was performed using ANOVA.

RESULTS

Peritoneal antigen-presenting cells from *P. chabaudi*-infected exhibit a defective accessory cell function that correlates with the level of parasitemia

PECs harvested from *Plasmodium chabaudi*-infected mice (early, moderate and acute infections) were tested for their capacity to process/present the lysozyme protein or to present a lysozyme-derived peptide (HELpep residues 105–120) to a relevant T-cell hybridoma (1E5.11). The results shown in Table 1 indicate that during *P. chabaudi* infections, PECs become gradually defective in their ability to stimulate, via antigen processing/presentation, IL-2 production by a relevant T-cell hybridoma. Indeed, at moderate parasitemias, a reduced IL-2 production is recorded when assessing processing/presentation of lysozyme and an increase in parasitemia concomitantly causes a further impairment in IL-2 production by the T-cell hybridoma. When testing presentation of the HEL-peptide, a significant

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Table 1 Peritoneal antigen-presenting cells from *P. chabaudi*-infected mice are suppressed in their capacity to induce IL-2 production by relevant T-cell hybridomas. The defective accessory cell function correlates with the level of parasitemia

		IL-2 production (pg/ml)					
		Stimulation with HEL			Stimulation with HEL-peptide		
% Parasitemia	PECs[a] × 10^4/well	Control	Infected mice	% Reduction in IL-2 production[b]	Control	Infected mice	% Reduction in IL-2 production[b]
2	2·5	689 ± 42	623 ± 65	10	849 ± 85	790 ± 63	7
	1·25	471 ± 36	385 ± 53	18	646 ± 53	655 ± 42	0
15	2·5	780 ± 53	650 ± 39	17	910 ± 76	820 ± 56	10
	1·25	630 ± 33	415 ± 45	34	580 ± 42	520 ± 73	10
36	2·5	507 ± 45	92 ± 23	82	612 ± 56	165 ± 45	73
	1·25	457 ± 32	114 ± 40	75	457 ± 65	246 ± 42	46

[a]Peritoneal exudate cells (PECs) were harvested from non-infected or *P. chabaudi* AS-infected mice at different days of infection. The PECs were co-cultured with the 1E5.11 T-cell hybridoma in the presence of hen egg white lysozyme (HEL, 10 μg/ml) or a derived peptide (5 μg/ml). Eighteen hours later, supernatants from the culture cells were recovered, and their content in IL-2 was estimated by enzyme-linked immunosorbent assay. Background IL-2 values, in the absence of antigen were zero. Values are expressed as means of triplicate measurements ± SD.
[b]% Reduction = (IL-2 concentration (infected mice)]/[IL-2 concentration (control mice)]) × 100.

defective IL-2 production also is observed at high parasitemias.

The parasite-derived factor hemozoin causes a deficient accessory cell function in PECs

The observation that acute malaria infections induced a defective accessory cell function in peritoneal antigen-presenting cells, led us to investigate whether in-vitro treatment of naive PECs with malaria parasite products could induce a similar cellular dysfunction. Peritoneal macrophages from noninfected mice incubated with crude hemozoin, readily ingested and accumulated the malaria pigment (observed under the light microscope as a dark-brown product retained within the cytoplasm of the phagocyte). The engulfed hemozoin persisted within the cells for at least 72 h, but did not kill the cells (the viability was assessed by trypan blue staining), nor alter their adherence properties. Similar features were observed when PECs were incubated with β-hematin, the synthetic form of hemozoin (results not shown).

PECs loaded with crude hemozoin were severely affected in their ability to activate the 1E5.11 T-cell hybridoma via protein antigen processing/presentation or peptide

Table 2 Hemozoin is the parasite-derived factor involved in the deficient accessory cell function. In-vitro treatment with crude or synthetic hemozoin induces a defective activation of the T-cell hybridomas by peritoneal antigen-presenting cells

	IL-2 production (pg/ml)		% Reduction in IL-2 production	
PECs × 10^4/well	HEL	HELpep	HEL	HELpep
Control				
2·5	725 ± 54	716 ± 43		
1·25	470 ± 28	632 ± 32		
Hemozoin-treated[a]				
2·5	220 ± 9·5	221 ± 11	70	69
1·25	190 ± 18	247 ± 19	59	61
β-hematin treated[b]				
2·5	80 ± 6	64 ± 4	89	91
1·25	55 ± 8	10 ± 5	88	98

[a,b]PECs were plated in 96-well plates, and crude hemozoin (trophozoite lysate) or synthetic β-hematin were added at a final concentration of 10 μg/ml, prior to the addition of the T-cell hybridoma lysozyme (HEL) or the HELpeptide. Values are expressed as means of triplicate measurements ± SD.

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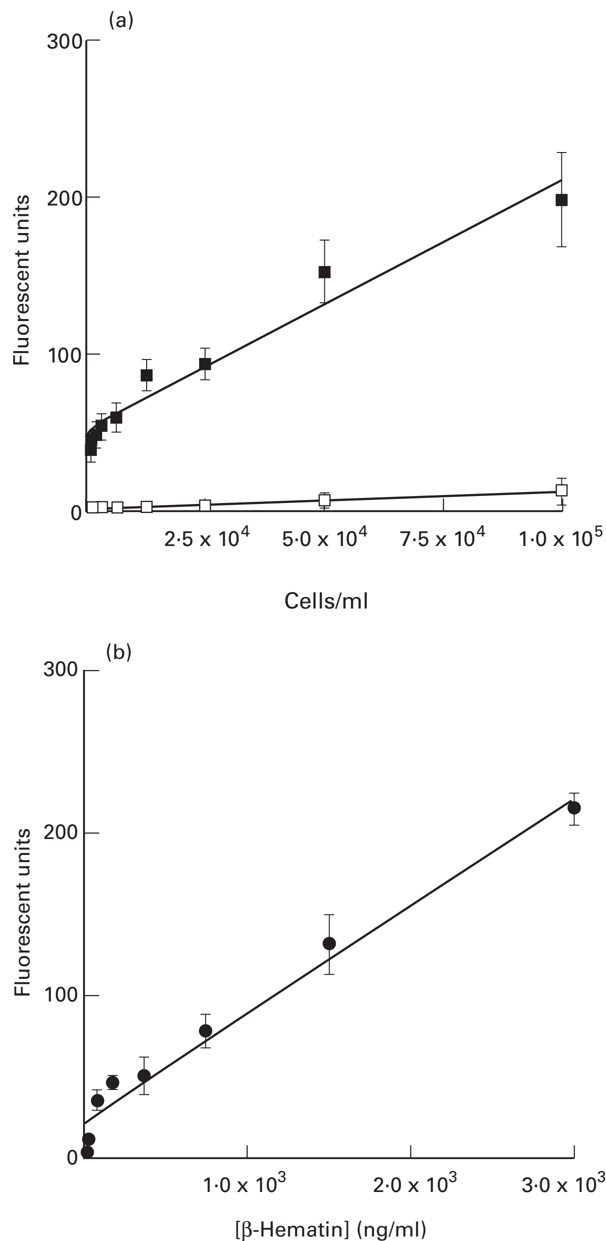

Figure 1 Presence of hemozoin in lysate of malaria infected mice. (a) Cell lysates of PECs isolated from *P. chabaudi*-infected mice (■) and non infected control mice (□) were analysed for their hemozoin content, by haem-enhanced fluorescence. Results of triplicate measurements are expressed as fluorescent units (mean ± SD), and (b) compared to the fluorescent signal obtained with synthetic β-hematin (●).

presentation (Table 2). A more pronounced effect was attained when using synthetic β-hematin. The reduction in IL-2 production by the T-cell hybridoma was not due to toxic effects since neither crude hemozoin nor the synthetic β-hematin interfered with the proliferation of the T-cell hybridoma (results not shown).

Hemozoin-treated PECs can be conveniently used as an in-vitro model for the analysis of in-vivo effects of malaria infections on the antigen-presenting capacity of PECs. The hemozoin content of PECs from both noninfected and infected mice was measured by a quantitative haem-enhanced luminescence method (Schwarzer *et al.* 1994). The results presented in Figure 1 show that indeed PECs from infected mice contain high levels of hemozoin, while PECs from control mice do not.

The defective accessory cell function of PECs from *P. chabaudi*-infected mice is not due to an impaired uptake and catabolism of protein antigens

The defective activation of the T-cell hybridoma by PECs from *P. chabaudi*-infected mice when using protein antigen could reflect the incapacity of such PECs to take up and process antigens. To test this possibility, normal PECs and PECs from *P. chabaudi*-infected mice (32–38% parasitemia) were incubated with radiolabeled HEL and the uptake and catabolism of the protein was followed. Peritoneal exudate cells from *P. chabaudi* acutely infected mice incorporated radiolabeled lysozyme as efficiently as normal PECs, and were similarly capable to degrade the protein. No significant differences were observed between the efficiency of control PECs and PECs from infected mice or hemozoin-treated PECs, at the level of protein uptake and degradation protein ($P > 0{\cdot}05$) (Table 3).

The defective accessory cell function of PECs is not due to an impaired antigen presentation capacity

Activation of a T-cell hybridoma by peritoneal antigen-presenting cells requires a proper processing of protein antigens, and a presentation of the antigenic peptides in the context of MHC class II molecules. The suboptimal IL-2 production by the 1E5.11 T-cell hybridoma was not due to an incapacity of the PECs from *P. chabaudi*-infected mice to present appropriately the antigenic peptide on their surface. Indeed, fixation by GA treatment of PECs from *P. chabaudi*-infected mice, prior to addition of the HEL peptide yielded a similar IL-2 production as control PECs (Figure 2a,b). Similarly, fixation of hemozoin and β-hematin treated PECs rendered these cells capable of inducing IL-2 levels by the T-cell hybridoma comparable to control PECs (Figure 2a,b).

By treating the PECs with GA after incubation with lysozyme, processing of the antigen is allowed to occur and eventually presentation of derived antigenic peptides on the surface of the antigen-presenting cells. Following this

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Table 3 Uptake and catabolism of protein antigen is not affected during acute *P. chabaudi* infection

PECs	Total associated radioactivity[d]	Non-degraded[e]	% Total radioactivity	Degraded[f]	% Total radioactivity
Control[a]	6588 ± 384	3879 ± 384	58·8 ± 7.4	2346 ± 565	35·6 ± 6.4
Infected[b]	6549 ± 119	4613 ± 739	70·4 ± 10	1706 ± 424	26 ± 6
Hemozoin[c]	6791 ± 144	4447 ± 544	65·4 ± 9.4	2058 ± 484	30·3 ± 7.5

[a,b]Peritoneal exudate cells were harvested from non-infected mice and mice with acute parasitemia (32–38%). [c]PECs were treated with crude hemozoin (10 μg/ml) overnight. [d]After 2 h of incubation with $Na^{125}I$-labeled lysozyme, the PECs were washed extensively and the radioactivity associated with the cells was measured. [e,f]The incorporated radioactivity was measured after lysis with triton X-100 and treatment with 10% tricloroacetic acid (TCA). The radioactivity associated with the TCA soluble (degraded lysozyme) and insoluble (non-degraded lysozyme) fractions was measured separately. Values are expressed as means of triplicate measurements ± SD.

treatment, PECs from acutely infected mice were still impaired in their capacity to activate optimally the 1E5.11 T-cell hybridoma (Figure 2c,d). This result indicates that the malaria infection affects the late stage processing of proteins by PECs. Similar results were obtained with hemozoin and β-hematin treated PECs pulsed with lysozyme (Figure 2c,d).

The defective accessory cell function of PECs from infected mice does not involve prostaglandins or nitric oxide

GA fixation of PECs from infected mice and pulsing with the HEL peptide led to normal IL-2 production by the T-cell hybridoma. Therefore it was further investigated whether when viable, the cells produced factors capable of inducing the inhibition.

Conditioned media of cultures from PECs from infected mice, as well as from normal PECs treated with hemozoin inhibited the IL-2 production by 1E5.11 T-cell hybridoma (Table 4). No inhibition was observed when using conditioned media from control PECs (data not shown).

Prostaglandins could be responsible for the deficient T-cell hybridoma via their inhibitory effect on IL-2 production by T-cells (Rappaport & Dodge 1982). The presence in hemozoin of small amounts of free heme (Fitch & Kanjananggulpan 1987) and the observation that free heme activates the prostaglandin endoperoxide synthetase (Ueno *et al*. 1982) led us to investigate the role of prostaglandins on the observed immunosuppression. In general, treatment of PECs from malaria-infected mice with indomethacin (IM) during the antigen presentation assay did not restore significantly the IL-2 production by the T-cell hybridoma ($P > 0{\cdot}05$) (Table 5). Only at the lowest PEC concentration, a partial restoration of IL-2 production could be obtained by addition of IM ($P = 0{\cdot}02$). Defective IL-2 production induced by hemozoin-fed PECs or β-hematin fed PECs was also insensitive to indomethacin. Again, only at the lowest PEC concentration, and only in the case of hemozoin, a partial restoration of IL-2 production could by obtained by IM addition ($P < 0{\cdot}01$). Thus, these results indicate that prostaglandins are not the main cause of the impaired malaria-associated accessory cell function.

Both PECs from *P. chabaudi*-infected mice or PECs treated with hemozoin produced nitrite when cultured for 24 h. Nitric oxide has been shown to suppress IL-2 production by T-cells (Taylor-Robinson 1997) and to inhibit the

Table 4 Peritoneal macrophages from *P. chabaudi*-infected mice or from PECs treated with hemozoin produce soluble factors that inhibit IL-2 production

	Infection IL-2 production (pg/ml)[a]		Hemozoin (lysate) IL-2 production (pg/ml)[b]	
PECs × 10^4/well	Control	+50% CM	Control	+50% CM
2·5	650 ± 54	430 ± 40	625 ± 44	343 ± 20
1·25	382 ± 35	262 ± 21	350 ± 28	217 ± 19

[a,b]IL-2 production (pg/ml) by the 1E5/11 T-cell hybridoma when naive PECs are used to process/present HEL (10 μg/ml) in the presence of conditioned media (50% V/V) from 24-h cultures of PECs from infected mice (32–40% parasitemia) or naive PECs treated with hemozoin (10 μg/ml), respectively. Values are expressed as means of triplicate measurements ± SD.

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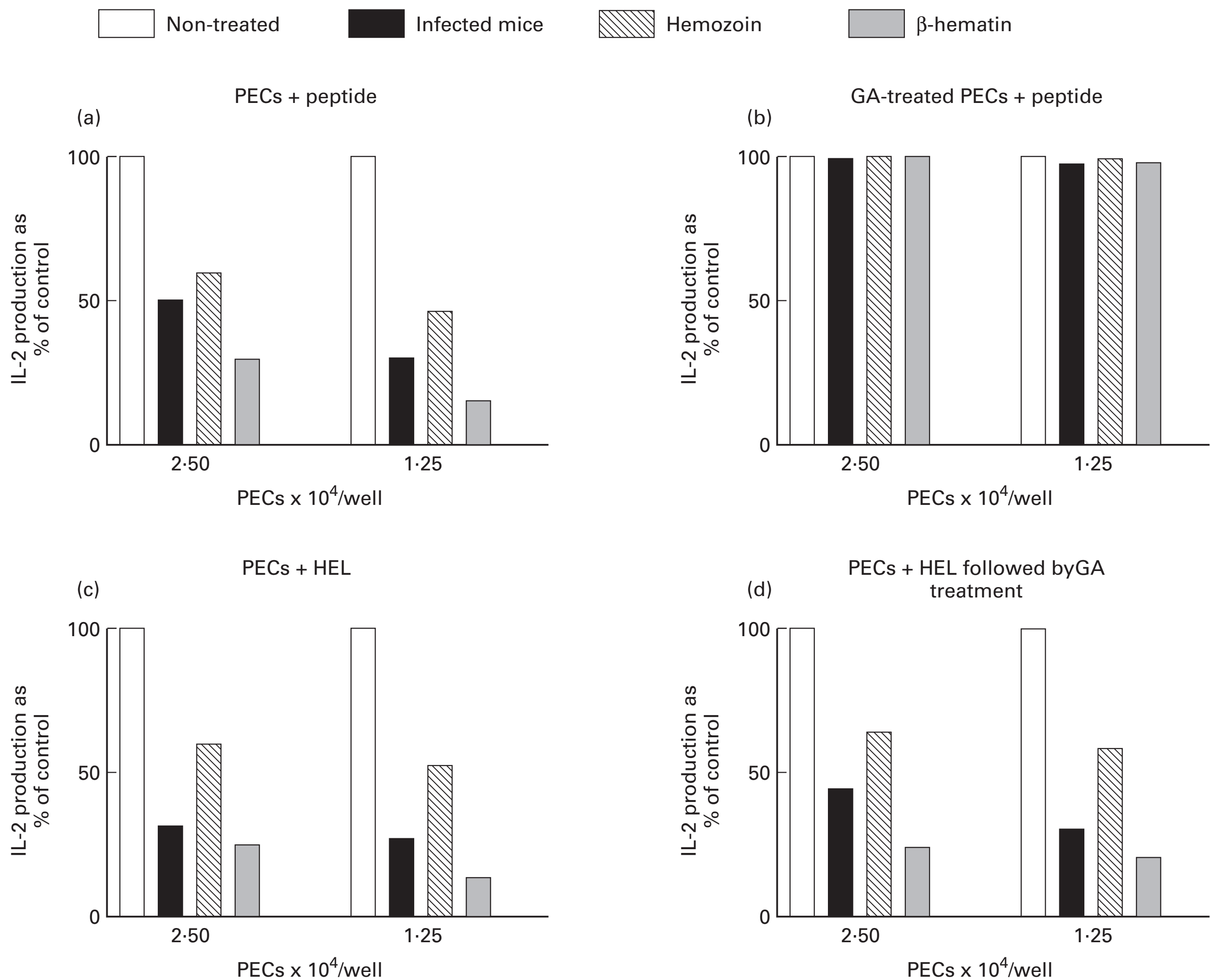

Figure 2 Interleukin-2 production by the 1E5.11 T-cell hybridoma when using (a,c) nonfixed or (b,d) GA-fixed PECs as antigen-presenting cells. The GA treatment was effectuated prior to the addition of (b) HEL-pep or (d) following incubation (3–4 h) with HEL.

Table 5 Indomethacin does not correct significantly the malaria-associated defective accessory cell function

	Infection		Hemozoin (lysate)		β-hematin	
PECs × 10^4/well	−IM	+IM	−IM	+IM	−IM	+IM
5	71 ± 5	68 ± 8	84 ± 6	73 ± 5	90 ± 8	89 ± 4
2·5	61 ± 10	41 ± 8	92 ± 7	61 ± 21	89 ± 5	79 ± 7
1·25	76 ± 9	47 ± 10[1]	90 ± 5	54 ± 11[2]	88 ± 6	78 ± 8

Percentages of reduction in IL-2 production by the 1E5.11 T-cell hybridoma when processing/presentation of HEL is effectuated by PECs from *P. chabaudi* infected mice (35% parasitemia), and PECs treated with crude hemozoin or β-hematin (10 μg/ml). Indomethacin (IM) was added at a final concentration of 1 μmg/ml. Values are expressed as mean of triplicate measurements ± SD. All *P*-Values > 0.05, except when indicated.
[a]$P = 0.02$; [b]$P < 0.01$.

Table 6 Inhibition of nitric oxide production does not restore the IL-2 production by the T-cell hybridoma

PECs × 10^4/well	Infection		Hemozoin (lysate)	
	−AMG	+AMG	−AMG	+AMG
5	78 ± 7	87 ± 4	59 ± 6	60 ± 5
2·5	75 ± 6	79 ± 8	59 ± 4	55 ± 3
1·25	80 ± 5	80 ± 7	69 ± 4	64 ± 5

Percentages of reduction in IL-2 production by the 1E5.11 T-cell hybridoma when processing/presentation of HEL is effectuated by PECs from *P. chabaudi* infected mice (35% parasitemia), and PECs treated with crude hemozoin (10 μg/ml). Aminoguanidine (AMG) was added at a final concentration of 1 mM. Values are expressed as mean of triplicate measurements ± SD.

binding of NF-κβ factors to DNA (Matthews *et al*. 1996). However, addition of aminoguanidine (a structural analogue of L-arginine that inhibits the NO synthase), although strongly decreasing NO production by the PECs, did not alter the deficient T-cell activation (Table 6).

DISCUSSION

Numerous experimental observations suggest that during malaria infections the accessory cell function of macrophages becomes defective, and that consequently, *Plasmodium* infections may impair the optimal activation and proliferation of T cells. Here, we show that during malaria infections, peritoneal exudate cells (PECs) become defective in their capacity to stimulate an antigen specific T cell hybridoma.

The first deficiency in the accessory cell function of PECs seems to occur at the level of antigen processing. Indeed impaired processing/presentation of the HEL protein was already obvious with the PECs from moderate-infected mice while impaired presentation of the HEL peptide occurred mainly during the acute phase of the infection that corresponds to the peak of parasitemia. The impaired presentation of the HEL peptide does not reflect a deficient presentation capacity since GA treatment restored completely the ability of HEL peptide pulsed PECs from acutely infected mice to stimulate the T cell hybridoma. Hence *Plasmodium* infections do not alter the intrinsic antigenic presenting capacity of PECs but rather modulate such cells to actively suppress IL-2 production.

When PECs were incubated with lysozyme (3–4 h) prior to fixation, the impaired IL-2 production induced by the infection still persisted, indicating a blockage at the level of antigen uptake/processing. However, protein antigen uptake and catabolism by PECs from *P. chabaudi*-infected mice was not affected. Taken together, these results suggest that (1) a factor secreted by nonfixed PECs from malaria infected mice plays a crucial role in the observed immunosuppression at acute parasitemias, and (2) that in addition late steps of antigen processing subsequent to endocytosis and catabolism seem also to be affected during both moderate and acute *Plasmodium* infection stages. The infection could hamper, for example, the formation of the adequate antigenic peptides, a process generated at multiple stages within the endocytic and biosynthetic pathways. It has been demonstrated for instance that processing of HEL is distinct in early and late endocytic compartments, generating peptides with different clonal specificities (Griffin *et al*. 1997). In addition, loading of the adequate peptides to form peptide:MHC-II complexes could also be affected during the infection.

Conditioned media obtained from cultures of PECs from acutely infected mice inhibited the IL-2 production by the antigen-activated T-cell hybridoma. In respect to macrophage-derived inhibitory factors, reduced IL-2 responses could be explained by the inhibitory activity of NO on the DNA binding activity of the NF-κβ transcription factor family (Matthews *et al*. 1996). However, further investigation into the nature of the soluble secreted factor(s) involved in the malaria induced immunosuppression excluded a role for NO since the observed inhibition in IL-2 production was refractory to aminoguanidine treatment. In addition indomethacin did not abrogate completely the defective IL-2 production. These two results contrast with two earlier reports describing a correlation between the immunosuppressive role of macrophages during malaria infections and the production of prostaglandin and NO (Rockett *et al*. 1994, Ahvazi *et al*. 1995). Thus, the nature of the soluble factors secreted by PECs from malaria-infected mice and responsible for the reduction of IL-2 secretion in the used antigen presentation assays remains to be identified.

In order to unravel part of the biochemical mechanism involved in malaria induced immunosuppression, attention was paid to the parasite components responsible for the

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observed effects. Since hemozoin is one of the most important parasite catabolites and was suggested to be capable to promote a defective antigen-processing presentation (Morakote & Justus 1988), we further investigated its effect in our antigen-presentation system. Similar to PECs from malaria-infected mice that were shown to contain high levels of hemozoin, hemozoin (or β-hematin)-fed PECs were affected in their capacity to induce optimal IL-2 production by the T-cell hybridoma when the HEL protein was used as antigen. Also, GA fixation of hemozoin-treated PECs prior to addition of the HELpeptide, completely abrogated the reduction in IL-2 production, suggesting an active inhibition of IL-2 production by the pigment-loaded macrophages. This inhibitory effect was never reported before and was found to be independent of prostaglandin or NO production by the hemozoin-fed macrophages. Interestingly, NO production was not affected by treatment of naive PECs with hemozoin, this in contrast to observations described by Prada *et al*. (1996). These authors did not specify the quantity of malaria pigment used in their experiments to load the macrophages, and only describe having added very high amounts of hemozoin. It is probable that at very high concentrations, hemozoin can affect more dramatically antimicrobial macrophages functions such as phagocytosis and production of reactive nitrogen intermediates. It seems that the mechanisms involved in the malaria-induced immunosuppression can be mimicked by loading naïve PECs with crude hemozoin or with synthetic β-hematin prior to antigen processing/presentation. Although hemozoin was considered for several years as an inert parasite catabolic product, it was shown to impair the phagocytic capacity of human monocytes, as well as the generation of phorbol-ester induced oxidative burst. Furthermore, both the activity of protein kinase C and NADPH-oxidase are drastically reduced in hemozoin-loaded monocytes, while viability, ATP levels and protein synthesis remained unaltered (Turrini *et al.* 1993).

The precise mechanism accounting for the suppressive effects of hemozoin remains to be elucidated. In the present study, we show that protein uptake and catabolism are unaltered in hemozoin-treated macrophages, and are therefore not the cause of the observed immunosuppression. Recent data demonstrate that hemozoin-loaded monocytes exhibit markedly increased levels of lipoperoxides and of 4-hydroxynonenal (HNE), a very toxic aldehyde originating from lipoperoxidation of unsaturated fatty acids (Schwarzer *et al*. 1996). HNE was shown to down-regulate PKC activity, and to reduce NADPH oxidase activity and oxidative burst in hemozoin-loaded monocytes. Considering that PKC exerts a vast number of cellular functions (Nishizuka 1992), it will be of interest to assess the effect of HNE in macrophage functions such as antigen-processing/presentation, and their immunomodulatory properties.

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