Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading

Higher production of tumor necrosis factor alpha in hemozoin-fed human adherent monocytes is dependent on lipidic component of malarial pigment: new evidences on cytokine regulation in *Plasmodium falciparum* malaria

Prato Mauro*, Gallo Valentina, Arese Paolo

Department of Genetics, Biology and Biochemistry, University of Torino Medical School, Torino, Italy

ARTICLE INFO

Article history:
Received 3 December 2009
Received in revised form 30 December 2009
Accepted 20 January 2010
Available online 20 February 2010

Keywords:
Hemozoin
Plasmodium falciparum
Malaria
Monocyte
Phagocytosis
Tumor necrosis factor alpha (TNFalpha)
15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid (15-HETE)
Matrix metalloproteinase-9 (MMP-9)

ABSTRACT

Objective: To investigate whether the increase of tumor necrosis factor alpha is dependent on lipidic component of malarial pigment. **Methods**: Adherent human monocytes were fed for 3 hours with different meals (native hemozoin; lipid free hemozoin; and control latex particles), then tumor necrosis factor alpha was monitored in cell supernatants up to 48 hours through western blotting or specific enzyme-linked immunoadsorbent assay. In selected experiments, unfed monocytes were treated with different doses of 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid or 4-hydroxynonenal instead of phagocytosis. **Results**: Hemozoin-fed monocytes produced higher levels of tumor necrosis factor alpha than unstimulated and latex-fed cells, while lipid-free hemozoin did not reproduce these results. Additionally, hemozoin effects were mimicked dose-dependently by 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid, but not by 4-hydroxynonenal. **Conclusions**: Present data suggest an essential role for lipids in hemozoin-dependent enhanced release of tumor necrosis factor alpha from monocytes, and 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid could be one possible specific mediator.

1. Introduction

Among infective diseases, malaria is the most diffused in tropical and subtropical regions, including several areas of Asia, where each year 3 millions of new cases and 30% of global mortality occur. Etiological pathogenic agents belong to *Plasmodium(P.)* species and *P. falciparum* is responsible for the highest mortality, as a consequence of severe complications such as anemia, respiratory distress and cerebral malaria. Excessive levels of cytokines produced by infected human hosts have been related to worse clinical course leading to cerebral malaria and in several cases to death [1]. In previous works, we described an existing connection between phagocytosis of hemozoin, the malarial pigment produced by trophozoite-stage *P. falciparum* because of hemoglobin catabolism, and cytokine-related release of monocytic gelatinase granules content [2-4]. Hemozoin-dependent overproduction of tumor necrosis factor alpha and interleukin-1beta can induce higher expression and activity of matrix metalloproteinase-9, a proteolytic enzyme involved in basal lamina degradation and monocyte recruitment in brain during cerebral malaria; since matrix metalloproteinase-9 is also able to shed soluble tumor necrosis factor alpha through cleavage of its membrane-bound proform, an additional auto-alimented pathological loop which enhances levels of either cytokine or enzyme produced by human monocytes [2, 3]. Moreover, tumor necrosis factor alpha has been recently reported as a soluble mediator of hemozoin-dependent release of lysozyme, an enzyme indicated as marker of monocyte activation and stored in gelatinase granules with matrix metalloproteinase-9 [4]. Malarial pigment has a janus-like structure, either ferric or lipidic; indeed, the crystal ferriprotoporphirin IX polymer is able to generate through heme autocatalysis several peroxidation products from

*Corresponding author: Dr. Mauro Prato, PhD, Department of Genetics, Biology and Biochimistry, University of Torino Medical School, Via Santena 5 bis, 10126, Torino, Italy
Tel: +39-011-670-58-50
Fax: +39-011-670-58-45
E-mail: mauro.prato@unito.it

polyunsaturated fatty acids, including 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid and 4-hydroxynonenal[5, 6]. Recently, 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid has been showed to be involved in hemozoin-dependent increase of monocytic matrix metalloproteinase-9 and interleukin-1beta [3]. Present work investigated the involvement of lipidic component of hemozoin in tumor necrosis factor alpha enhancement, focusing on role of 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid.

2. Materials and methods

2.1. Materials

All materials were from Sigma-Aldrich, St. Louis, MO, unless otherwise stated. Cell culture media: RPMI 1640 and macrophage-SFM media were from Invitrogen, Carlsbad, CA; Panserin 601 monocyte medium was from PAN Biotech, Aidenbach, Germany; Percoll was from Pharmacia, Uppsala, Sweden; Diff-Quik parasite stain was from Baxter Dade AG, Dudingen, Switzerland; sterile plastics were from Costar, Cambridge, UK; anti-tumor necrosis factor alpha monoclonal antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA; enzyme-linked immunoadsorbent assay kit for tumor necrosis factor alpha assay and 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid were from Cayman, Ann Arbor, MI; 4-hydroxynonenal was from Biomol, Plymouth Meeting, PA. Beta-hematin (synthetic HZ) was prepared according to the Slater *et al*[7] procedure, modified as indicated [3].

2.2. Cultivation of Plasmodium falciparum and isolation of hemozoin

P. falciparum parasites (Palo Alto strain, mycoplasma-free) were kept in culture as described [4]. Hemozoin was isolated from cultures during the first 2 days after infection of red blood cells. After centrifugation at 5,000 g on a discontinuous Percoll-mannitol density gradient, hemozoin was collected from the 0-40% interphase. Hemozoin was washed 5 times with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0) containing 10mM mannitol at 4 ℃ and once with phosphate buffered saline. Before opsonization and phagocytosis experiments, hemozoin was reincubated in RPMI 1640 for 1 h at 37 ℃.

2.3. Preparation and handling of monocytes

Human monocytes were separated by Ficoll centrifugation from freshly collected buffy coats discarded from blood donations by healthy adult donors of both sexes provided by the local blood bank (AVIS, Associazione Volontari Italiani Sangue, Torino, Italy). Separated lymphomonocytes were resuspended in RPMI 1640 medium and plated on 6-well plates. Each well received 2 mL of cell suspension containing 8×10^6cells/mL in RPMI 1640. The plates were incubated in a humidified CO_2/air-incubator at 37 ℃ for 60 min. Thereafter non-adherent cells were removed by 3 washes with RPMI 1640 and adherent cells (approximately 1×10^6 cells/well) reincubated at 37 ℃ overnight in RPMI 1640. Shortly before starting phagocytosis, wells were washed with RPMI 1640 and Macrophage-SFM medium was added (2 mL/well).

2.4. Phagocytosis by adherent monocytes of native, synthetic or delipidized hemozoin and latex particles

To each well of a 6-well plate with approximately 1×10^6 adherent monocytes, native or delipidized hemozoin (120 nmoles hemozoin heme, an amount comparable to 50μL trophozoites on heme content basis), beta-hematin (120 nmoles heme), and 50 μL amine-modified, red-fluorescent latex particles (2.5% solids, diameter 0.105 μm), were added. All phagocytic meals were opsonized with fresh autologous serum. After opsonization, they were suspended in RPMI 1640. The plates were centrifuged at low speed for 5 s to start phagocytosis and incubated in a humidified CO_2/air-incubator at 37 ℃ for 3 h. This time period maximized phagocytosis and was not sufficient to induce heme-oxygenase-mediated degradation of ingested heme[8]. As an average, each monocyte ingested hemozoin equivalent to 8-10 trophozoites in terms of ingested heme, as shown previously [9]. Thereafter noningested hemozoin, beta-hematin and latex particles were removed by 4 washes with RPMI 1640. The plates were then incubated in Panserin medium in a humidified CO_2/air-incubator at 37 ℃ for the indicated times.

2.5. Assay of tumor necrosis factor alpha production

After termination of phagocytosis, monocytes were further incubated with Panserin 601 monocyte medium in a humidified CO_2/air-incubator at 37 ℃ for the indicated times. The level of soluble tumor necrosis factor alpha was assayed in monocyte supernatants by specific enzyme-linked immunoadsorbent assay, according the manifacturer's instructions. A standard calibration curve was generated with recombinant human tumor necrosis factor alpha. Alternatively, soluble tumor necrosis factor alpha was assayed in monocyte supernatants by western blotting. Briefly, 15 μL supernatant samples were loaded on 15 % polyacrylamide gels under denaturing and reducing conditions, with addition of Laemmli buffer, blotted on a polyvinylidene difluoride membrane, and probed with anti-tumor necrosis factor alpha mAbs at 1/1000 final dilution. Bands were visualized by enhanced chemiluminescence.

2.6. Statistical analysis

For each set of experiments, data are shown as means±SEM or one representative imagine of three independent experiments. All data were analysed by Student's *t* test (equal and unequal variances).

3. Results

3.1. Phagocytosis of hemozoin enhances time-dependently the release of soluble tumor necrosis factor alpha from human adherent monocytes

Human adherent monocytes were allowed to phagocytose hemozoin and latex particles (control meal) during 3 h. After termination of phagocytosis and elimination of noningested phagocytic meals by repeated washings, cells were incubated up to 48 h, and cell supernatants were collected at indicated times (0, 19, 32, 48 h) for following studies. Figure 1 showed

time course results from western blotting analysis of soluble tumor necrosis factor alpha release. Data were obtained from one representative experiment of three with similar results. The 17 kDa band corresponds to soluble tumor necrosis factor alpha. Cytokine band was not detectable in unstimulated and latex-fed cell supernatants, while it was observed in all hemozoin-fed monocyte supernatants, except those collected immediately after phagocytosis. Tumor necrosis factor alpha release was time-dependent, showing a progressive rate up to the observational period of 48 h. Moreover, additional measurements of tumor necrosis factor alpha production on the same samples were performed using a specific enzyme-linked immunoadsorbent assay (data not shown). Sensitivity of this assay was higher than western blotting, and led to quantify very low amounts of soluble cytokine. Results were evaluated by Student's *t*-test (equal and unequal variances). In unstimulated and latex-fed monocyte supernatants similar levels of tumor necrosis factor alpha, without significant differences, were observed (approximately from 0 ng/mL at the beginning to about 5 ng/mL at the end of the 48 h-observational period). On the other hand, hemozoin-fed monocyte produced significantly higher amounts of soluble tumor necrosis factor alphaa alpha (up to 21.6 ng/mL at the 48th hour; worst p: $P<0.05$ for hemozoin-fed versus unstimulated cells 48 h after phagocytosis) in a nonlinear but time-dependent manner, according to evidences previously reported [2].

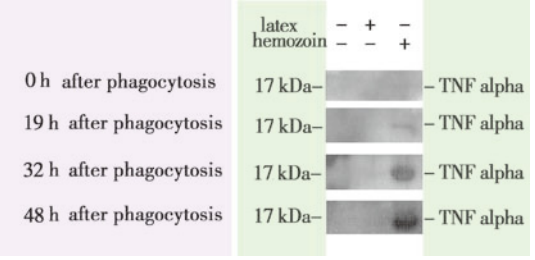

Figure 1. Time-dependent enhancement of tumor necrosis factor alpha release in hemozoin-fed human adherent monocytes.

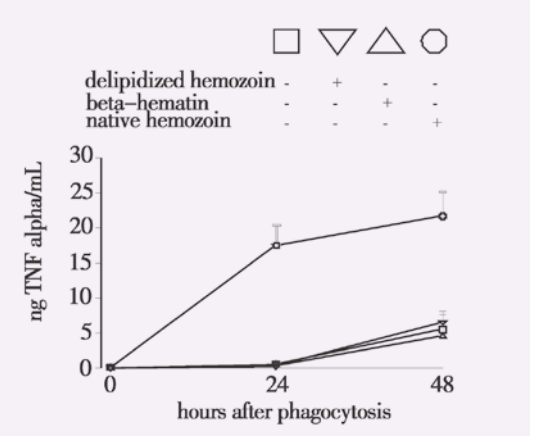

Figure 2. Role of lipidic component of malarial pigment in overproduction of tumor necrosis factor alpha in hemozoin-fed human adherent monocytes.

3.2. Lipidic component of hemozoin plays a role in enhancement of tumor necrosis factor alpha production in adherent monocytes

It has been shown previously that native hemozoin produces through non-enzymatic heme catalysis potent lipoperoxidation derivatives from polyunsaturated fatty acids stably adherent to the crystalline poly-heme core [6]. To establish if lipids were involved in hemozoin-driven overproduction of tumor necrosis alpha, human adherent monocytes were allowed to phagocytose delipidized hemozoin or lipid-free synthetic hemozoin (beta-hematin) for 3 h. Native hemozoin was also used as positive control. After phagocytosis and elimination of noningested phagocytic meals, cells were incubated for 48 h, and supernatants were collected at 24th and 48th hour. Figure 2 showed the production of tumor necrosis factor alpha measured by enzyme-linked immunoadsorbent assay. Data was shown as means±SEM of three independent experiments; all data were evaluated by Student's *t*-test (equal and unequal variances). Neither delipidized hemozoin nor beta-hematin did mimick native hemozoin effect on cytokine release, suggesting an active role for lipids. Unstimulated cells (line with open square) released low levels of tumor necrosis factor alpha (approximately 5 ng/mL in the end of observational period), which were comparable to those obtained after phagocytosis of delipidized hemozoin and beta-hematin (lines with up- and down-oriented triangle, respectively) without significant differences. Otherwise, as expected, hemozoin-fed monocytes produced higher amounts of tumor necrosis factor alpha at both times (approximately 17 ng/mL at 24th hour and 22 ng/mL at 48th hour) with significant $P<0.05$ versus all other conditions at both times.

3.3. 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid mimicks hemozoin effects on tumor necrosis factor alpha release from adherent monocytes in a dose-dependent manner

Previous studies showed that15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid, a product of arachidonic acid peroxidation by hemozoin, was an active mediator of hemozoin effects in monocytes [3, 6]. As lipids seemed to be involved in hemozoin-dependent upregulation of tumor necrosis factor alpha production, 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid was added to human adherent monocytes at 0.1-10 μM (final concentration) for repeated times (every 12 h) and cells were incubated for 48 h. As positive control, monocytes previously fed for 3 h with hemozoin were incubated for the same period. Figure 3 showed tumor necrosis factor alpha production measured by enzyme-linked immunoadsorbent assay in cell supernatants at the end of the 48th hour of incubation. Data was shown as means±SEM of three independent experiments; all data were evaluated by Student's *t*-test (equal and unequal variances). As expected, hemozoin-fed monocytes (column 2) produced higher levels of cytokine than unstimulated cells (column 1), with $P<0.05$. All different doses of 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid (columns 3-5) mimicked hemozoin effects. No significant differences were calculated between values obtained from hemozoin-fed monocytes and those from cells treated with all doses of 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid. On the other hand, tumor necrosis factor alpha amounts obtained with the lowest dose of 15(S,R)-hydroxy-

6,8,11,13-eicosatetraenoic acid and with the highest one were significantly different (worst p: $P<0.05$), showing a dose-response effect. Additionally, all samples treated with 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid were significantly different when compared to unstimulated cells ($P<0.01$, $P<0.01$ and $P<0.002$ for 0.1 μM, 1 μM and 10 μM doses, respectively). The stimulatory effect of 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid appeared to be specific, as 4-hydroxynonenal, another potent polyunsaturated fatty acid derivative generated by hemozoin activity [10] was unable to mimick hemozoin effects on tumor necrosis factor alpha production when added at 0.1-10 μM (final concentration) (data was not shown).

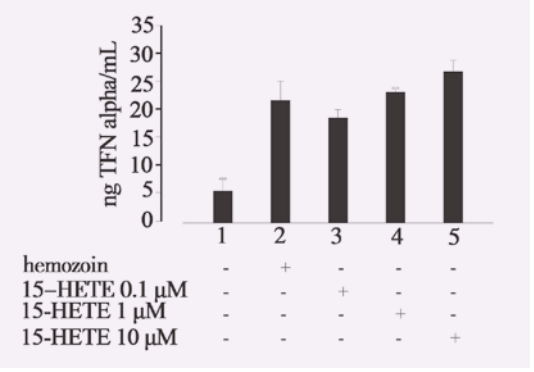

Figure 3. Dose-dependent effects of 15(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid on tumor necrosis factor alpha production in human adherent monocytes .

4. Discussion

During malaria infection, a strong proinflammatory response involving production of several cytokines, such as tumor necrosis factor alpha and interleukin-1beta occurs, leading to classical symptoms as fever and hypoglicemia [11]. These cytokines have also been related to severe complications of the disease, like cerebral malaria, a reversible encephalopathy with seizures and loss of consciousness which can lead to death [12]. According to Clark's so-called cytokine theory of cerebral malaria, which since 1981 suggested that fatal consequences of complicated malaria were triggered by excessive production of human host defensive molecules instead of toxic exogenous compounds originated from parasite [1], mean plasma tumor necrosis factor alpha levels are ten times higher in fatal cerebral malaria than in uncomplicated malaria [11].

Despite abundant evidences suggesting a role for tumor necrosis factor alpha in complicated malaria, source of excessive tumor necrosis factor alpha in malaria has been often debated, as cytokine is produced by several cell types, including leukocytes, endothelial cells, fibroblasts, smooth muscle cells [13]. A major role seems to be played by monocytes, whose functions are strongly impaired after phagocytosis of hemozoin, a crystal ferriprotoporphirin IX polymer produced by intraerythrocytic trophozoite-stage *Plasmodium parasite* after hemoglobin catabolism [5]. Indeed, among altered functions, which include impairment of antigen presentation, oxidative burst and bacterial killing [9], hemozoin-fed monocytes show an enhanced ability to produce several cytokines, including tumor necrosis factor alpha and interleukin-1beta [2-4, 14].

Present work must be plugged in this context and takes its premises from our previous study, where we described a non linear but time-dependent increase of tumor necrosis factor alpha in hemozoin-fed or hemozoin-containg trophozoites-fed human monocyte supernatants during a short term observational period (up to two hours) after phagocytosis [4]. As a first step, we expanded previous short term evidences, and levels of tumor necrosis factor alpha produced by hemozoin-fed monocyte were monitored for longer times (up to 48 h after the end of phagocytosis). As described in results section, western blotting data obtained from cell supernatants collected at indicated times (19, 32, 48 h) confirmed that time-dependent effect of hemozoin on tumor necrosis factor alpha production appears to persist also in a long-term manner. An additional quantification of cytokine accumulation was also performed, indicating that levels of soluble tumor necrosis factor alpha released by hemozoin-fed monocytes went over 20 ng/mL at the end of the 48 hours-observational period.

However, mechanism through which hemozoin promotes tumor necrosis factor alpha accumulation has been unknown for a long time. Recently, our group proposed an *in vitro* model involving matrix metalloproteinase-9, a cytokine-inducible proteolytic enzyme which is able to shed soluble tumor necrosis factor alpha after cleavage of its membrane-bound precursor [15, 16]; in this model, after phagocytosis of hemozoin or hemozoin-containing trophozoites, tumor necrosis factor alpha induces expression and activity of monocytic matrix metalloproteinase-9, which in turn promotes release of more tumor necrosis factor alpha, starting a pathological loop where both cytokine and enzyme levels are mutually upregulated [2]. Additionally, also hemozoin-dependent increase of interleukin-1beta seems to play a role in initial matrix metalloproteinase-9 activation, even though any dependence on the proteolytic enzyme was not reproduced experimentally for this cytokine, and occurrence of a cascade mechanism instead of a second additional loop is more probable [3].

By the way, as nature of malarial pigment is either ferric (heme core of haemoglobin-derived ferriprotoporphirin IX polymer) or lipidic (monohydroxy-derivatives of polyunsaturated fatty acids generated from polyunsaturated fatty acids peroxidation carried out by heme autocatalysis) [5, 6], it appears intriguing to discern what component of hemozoin is responsible for the enhancement of cytokines and enzyme. An involvement of lipids has been recently described for hemozoin upregulation of interleukin-1beta and matrix metalloproteinase-9 [3]. Present work integrates these evidences expanding the study on tumor necrosis factor alpha. To ascertain role of lipidic fraction of hemozoin on tumor necrosis tumor necrosis factor alpha modulation, human monocytes were allowed to phagocytose lipid-free hemozoin, either native or synthetic, and tumor necrosis factor alpha amounts were dosed in cell supernatants 48 h after phagocytosis. In both cases, cytokine amounts were similar to control levels, and hemozoin effects were not reproduced, suggesting that only heme moiety is not sufficient for enhancing release of tumor necrosis factor alpha, while lipidic component is essential.

Complex pattern of oxygenated lipids produced through heme-catalysis, which is favoured by high concentrations of hemozoin and the acidic pH in the digestive vacuole, includes six hydroxy-eicosatetraenoic acid isomers, two hydroxyoctadecadienoic acid isomers, and several terminal hydroxyaldehydes [6]. Some of these molecules have

been related to toxic effects of hemozoin on monocytes: for example, 12- and 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acids inhibited oxidative burst [6], while 4-hydroxynonenal blocked nicotinamide adenine dinucleotide phosphate oxidase through downregulation of protein kinase C[10]. Additionally, 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid has been indicated as possible mediator of interleukin-1beta and matrix metalloproteinase-9 hemozoin-dependent enhancement[3]. Native hemozoin contains 0.24 mmol 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid/ mol heme, while hemozoin-fed monocytes contain about 10 μM 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid, assuming a mean phagocytosis of 10 trophozoites for each monocyte [6].

Here, 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid supplemented to monocytes at different doses (0.1-10 μM) enhanced tumor necrosis factor alpha release in a dose-dependent manner, indicating that 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid could play a role in hemozoin-triggered overproduction of the cytokine. On the other side, similar doses of 4-hydroxynonenal did not mimick hemozoin and 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid effects, suggesting that role of 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid might be specific. Mechanisms through which 15-(S,R)-hydroxy-6,8,11,13-eicosatetraenoic acid promotes cytokine and matrix metalloproteinase production are not well understood. However, genes of tumor necrosis factor alpha, interleukin-1beta and matrix metalloproteinase-9 are usually under control of nuclear factor-kappa B transcription system [10]. Since evidences of nuclear factor-kappa B activation in hemozoin-fed murine macrophages have been described [17], an involvement of such a pathway should be considered.

Taken together, present data could help to better understand etiology of excessive tumor necrosis factor alpha production, which triggers a complex cascade of events leading to cerebral malaria. Moreover, a major knowledge of mechanisms through which hemozoin alters monocyte functions is certainly required in a realistic perspective for specific design of new antimalarial drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This work was supported in the context of the Italian Malaria Network by grants from Compagnia di San Paolo-IMI and from Regione Piemonte, Ricerca Sanitaria Finalizzata 2008bis to PA. Thanks are due to Associazione Volontari Italiani Sangue (AVIS, Torino, Italy) for providing freshly discarded buffy coats, and to Elena Valente and Dr. Oscar Akide-Ndunge for helping with cultures.

References

[1]Clark IA, Rockett KA. The cytokine theory of human cerebral malaria. *Parasitol Today* 1994; **10**: 410 – 2.

[2]Prato M, Giribaldi G, Polimeni M, Gallo V, Arese P. Phagocytosis of hemozoin enhances matrix metalloproteinase-9 activity and TNF-alpha production in human monocytes: role of matrix metalloproteinases in the pathogenesis of falciparum malaria. *J Immunol* 2005; **175**: 6436 – 42.

[3]Prato M, Gallo V, Giribaldi G, Arese P. Phagocytosis of haemozoin (malarial pigment) enhances metalloproteinase-9 activity in human adherent monocytes: role of IL-1beta and 15-HETE. *Malar J* 2008; **7**:157.

[4]Prato M, Giribaldi G, Arese P. Hemozoin triggers tumor necrosis factor alpha-mediated release of lysozyme by human adherent monocytes: new evidences on leukocyte degranulation in *P. falciparum* malaria. *Asian Pac J Trop Med* 2009; **2**: 35-40.

[5]Arese P, Schwarzer E. Malarial pigment (haemozoin): a very active 'inert' substance.*Ann Trop Med Parasitol* 1997; **91**: 501-6.

[6]Schwarzer E, Kuhn H, Valente E, Arese P. Malaria-parasitized erythrocytes and hemozoin nonenzymatically generate large amounts of hydroxy fatty acids that inhibit monocyte functions. *Blood* 2003; **101**: 722-8.

[7]Slater AF, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, Cerami A, et al. An iron-carboxylate bond links the heme units of malaria pigment. *Proc Natl Acad Sci USA* 1991; **88**: 325-9.

[8]Schwarzer E, De Matteis F, Giribaldi G, Ulliers D, Valente E, Arese P. Hemozoin stability and dormant induction of heme oxygenase in hemozoin-fed human monocytes. *Mol Biochem Parasitol* 1999;**100**: 61-72.

[9]Schwarzer E, Turrini F, Ulliers D, Giribaldi G, Ginsburg H, Arese P. Impairment of macrophage functions after ingestion of *Plasmodium falciparum*-infected erythrocytes or isolated malarial pigment. *J Exp Med* 1992; **176**: 1033-41.

[10]Schwarzer E, Muller O, Arese P, Siems WG, Grune T. Increased levels of 4-hydroxynonenal in human monocytes fed with malarial pigment hemozoin. A possible clue for hemozoin toxicity. *FEBS Lett* 1996; **388**: 119-22.

[11]Kwiatkowski D. Tumor necrosis factor, fever and fatality in *falciparum* malaria. *Immunol Lett* 1990; **25**: 213-6.

[12]Gimenez FS, Barraud de Lagerie S, Fernandez C, Pino P, Mazier D. Tumor necrosis factor-alpha in the pathogenesis of cerebral malaria. *Cell Mol Life Sci* 2003; **60**: 1623-35.

[13]McDermott MF. TNF and TNFR biology in health and disease. *Cell Mol Biol* 2001; **47**: 619-35.

[14]Pichyangkul S, Saengkrai P, Webster HK. *Plasmodium falciparum* pigment induces monocytes to release high levels of tumor necrosis factor-alpha and interleukin-1beta. *Am J Trop Med Hyg* 1994; **51**: 430-5.

[15]Bond M, Fabunmi RP, Baker AH, Newby AC. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kB. *FEBS Lett* 1998; **435**: 29-34.

[16]Gearing AJ, Beckett P, Christodoulou M., Churchill M, Clements JM, Davidson AH, et al. Matrix metalloproteinases and processing of pro-TNFalpha. *J Leukocyte Biol* 1995; **57**: 774-7.

[17]Jaramillo M, Gowda DC, Radzioch D, Olivier M. Hemozoin increases IFN-gamma-inducible macrophage nitric oxide generation through extracellular signal-regulated kinase- and NF-kappa B-dependent pathways. *J Immunol* 2003; **171**: 4243-53.