Modulation of immune response in *Plasmodium falciparum* malaria: role of IL-12, IL-18 and TGF-β

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**Abstract**

The interaction between pro- and anti-inflammatory cytokines such as interleukin 12 (IL-12), interleukin 18 (IL-18) and transforming growth factor β (TGF-β) plays an important role in malaria pathogenesis and outcome, modulating the immune response in *Plasmodium falciparum* malaria. In our previous studies, we analyzed the plasmatic levels of IL-12, IL-18 and TGF-β in 105 African children with different degrees of malaria and we correlated the production of these cytokines with the severity of the disease.

The aim of the present study was to analyze with a mathematical model, taking into account all the relationships between these cytokines and the parameter variations involved in malaria pathogenesis that influence the results of each type of treatment or therapeutic protocol on patients at different stages of the disease. A mathematical correlation was demonstrated between the levels of pro-inflammatory and anti-inflammatory cytokines, and from this it was possible to build curves of reference in which each patient was positioned based on IL-12 level.

Our data, obtained in patients with mild and severe diseases, demonstrate that the levels of IL-12 represent a reliable parameter to predict the progression of the disease, which may be complemented or modulated by administration of IL-18 and/or TGF-β.

Our findings provide future implications for an immune therapy against the *P. falciparum* malaria infection, especially in the early phase of the disease showing that a more aggressive outcome may be due to the lack of a balanced immune response.

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1. Introduction

Intensive studies of the immune response against malaria parasites in humans have provided a wealth of information about the cells and cytokines implicated in the pathophysiology of either survival or fatal outcome of this infection. Complications of severe anemia and cerebral malaria are the major cause of morbidity and mortality, but recent evidence suggests that the host’s immunologic response plays a relevant role in the pathophysiology of this disease in humans [1,2].

The balance between Th1 and Th2 immune response and between pro-inflammatory and anti-inflammatory cytokines is important in determining the level of malaria parasitemia, disease outcome and rates of recovery [3,4], while the overproduction of both pro-inflammatory and anti-inflammatory cytokines can be responsible for disease severity and mortality [5,6]. In our previous studies, we demonstrated that the IL-12, IL-18 and TGF-β levels were elevated in all children with malaria, demonstrating immune activation in response to the presence of parasites [7]. However, distinguishing between the two groups according to the different levels of hemoglobin concentration, platelet count and level of parasitemia, it was possible to understand that these cytokines are linked to the reciprocal dependence, which characterizes the favorable outcome of infection.
In this study, we applied a mathematical model which analyzes the levels of these cytokines, their relationship and their correlation with the other parameters of the disease.

A provisional model based only on rough clinical analysis seems very limited, because it is not able to objectively assess those factors influencing the outcome of the disease and the entity of the immune response. On the other hand, a model based on the immunological parameters involved in the disease can also monitor the response to the treatment used. This could even verify the effects of one or more therapeutic protocols in patients at different stages of the disease.

The aim of our study was to identify the parameters required for choosing the best protocol of immune therapy for patients with acute malaria.

2. Patients and methods

The study was conducted in the city of Ouagadougou (Burkina Faso), a mesendemic area of *Plasmodium falciparum* malaria, with an intense seasonal occurrence from July to October, and was carried out in collaboration with the local Medical Center Saint Camille (CMSC). The institutional review board of CMSC obtained the ethical approval for the study. The patients were evaluated and enrolled at the Pediatric Dispensary of the same center. Informed consent for participation in this study was obtained, prior to inclusion, from parents or guardians of each individual.

2.1. Diagnosis and classification of cases

To perform this study, 105 children (55 males and 50 females) affected by acute malaria, median age 19.5 months (range 2–144), were observed during the month of October 2000 at the CMSC of Ouagadougou (Burkina Faso). Inclusion and classification of each case were based on the symptoms, physical signs and laboratory findings of malaria at the onset of the disease. On the basis of hematological parameters, hyperparasitemia and evidence of neurological involvement, two different levels of severity were classified.

(A) ‘Severe malaria’ (complicated) was established microscopically by the presence of *P. falciparum* parasite and by the clinical and physical signs according to the WHO criteria: evidence of neurological compromise (prostration, lethargy), gastrointestinal symptoms, severe anemia (Ht < 20%, Hb < 6 g/dl), hyperparasitemia corresponding to >5% parasitised red cells/100 red blood cells or >5% parasitemia, hypoglycemia (serum glucose less than 2.2 mmol/l corresponding to 40 mg/dl), acidosis with respiratory distress, oliguria, cardiovascular shock, jaundice and diffuse hemorrhages.

(B) ‘Mild malaria’ (uncomplicated) was established microscopically by the presence of <5 parasitised red cells/100 red blood cells or <5% parasitemia, with fever, headache and myalgia without any indication of severe malaria. Patients with ‘uncomplicated malaria’ and parasitemia, were treated with chloroquine at the dosage of 10 mg/kg for 2 days, then 5 mg/kg for one more day. Patients with severe malaria or hyperparasitemia were placed under intravenous treatment with 5% glucose including quinine dichlorhydrate, according to the official therapeutic protocol of the local Programme National de Lutte contre le Paludisme (PNLP). The PNLP protocol was followed by a treatment of quinine for 7 days or chloroquine for 3 days.

As control group for cytokine determination, 40 healthy black children of the same age range (median 22 months, range 10–100) were also included in the study. All children enrolled as control group were negative at the thick-smear examination for *P. falciparum*, without febrile episodes during the previous 6 months and without signs of anemia (Hb > 10 g/dl).

2.2. Detection of parasitemia and plasma sample collection

Standard hematological parameters were determined in each patient. For the detection of parasitemia a calibrated thick-smear technique was used with standard Giemsa staining. The blood samples were collected for immunological assessment in sterile tubes containing EDTA. All samples were centrifuged and the serum was refrigerated at −40°C and were sent to the Laboratory of the Department of Biomedical Sciences, University of Catania, Italy, for the determination of IL-12, IL-18 and TGF-β.

2.3. Cytokine assays

Serum samples were analyzed for IL-12, IL-18 and TGF-β using the enzyme-linked immunosorbent assay (ELISA); the kit was obtained commercially (R&D Systems—Milan, Italy). The assays were performed according to the manufacturer’s protocol. Each plate included a standard curve and known positive and negative controls. The coefficient of variation intra-assay ranged from 1.1 to 1.4 pg/ml, from 3.4 to 6.9 pg/ml and from 2.7 to 5.9 pg/ml, respectively, for IL-12, IL-18 and TGF-β.

2.4. Statistical analysis

Within these experiments, statistical significance was analyzed by using Student’s two-tailed *t* distribution test.
for unpaired data. The level of significance was set at a two-tailed $P$ of $<0.05$. To construct the correlation curves we used a least-square fit with logarithmic functions; this was performed separately for children with severe and mild malaria. A logarithmic function was chosen in spite of the fact that it is not very flexible (it depends on only two parameters) because it guarantees the monotony of the curve and therefore the strict correlation between the data. The two curves were plotted on the same graph, together with the relative measurements. The best fit and graph were obtained by using SPSS version 10.0 for Windows (SPSS, Inc., Chicago, IL).

3. Results

Table 1 shows the mean values of more important hematological parameters according to the criteria of severity of the disease. The 77 children affected by severe malaria were the youngest and they had mean Hb levels of $3.72 \pm 1.26$ gr/dl and platelet count of $157.76 \pm 84.55$, while Hb level and platelet count were higher in the 28 children with less severe form of the disease, $8.28 \pm 1.57$ and $234.84 \pm 150.67$, respectively. The leukocyte count did not show any significant variation. A significant correlation was found between hemoglobin concentration and platelet count in all children with malaria ($P < 0.05$). IL-12 and IL-18 levels were found to be significantly lower, $21.5 \pm 10$ pg/ml and $13.2 \pm 5.53$ pg/ml, respectively, in all children with the severe form of the disease ($P < 0.001$), whereas TGF-β was higher ($28.09 \pm 22.39$ pg/ml). The levels of IL-18 and IL-12 were significantly higher ($25.7 \pm 7.6$ pg/ml and $17.1 \pm 7.8$) in children with the less severe form of the disease ($P = 0.044$); these children were older than the group with the severe form and showed a less severe anemia (Hb $8.28 \pm 1.57$ g/dl), higher platelet count ($234.84 \pm 150.67$ mm$^3$) and did not show signs of neurological involvement. In these children TGF-β was lower ($20.92 \pm 12.76$ pg/ml). The parasite density was higher (>5 parasitised red cells/100 red blood cells or >5% parasitemia) in patients with severe malaria and the IL-12 and IL-18 levels were lower. From these results it can be seen that the level of IL-18 increases up to the corresponding level of IL-12 $>20$ pg/ml (Fig. 1). The correlation between IL-12 and TGF-β and IL-18 and TGF-β showed an inverse relationship, up to the level of IL-12 and IL-18 $>20$ pg/ml (Figs. 2 and 3). When the curves of IL-12 and IL-18 of children with mild and severe malaria were plotted on the same graph, it was possible to distinguish an area which may be considered the range of favorable outcome, since all children recovered rapidly according to the official therapeutic protocol of PNLP for complicated and uncomplicated malaria.
In the control group the values of IL-18, IL-12 and TGF-β were 9.3 ± 3.5, 7.6 ± 2.5 and 6.4 ± 2.0 pg/ml, respectively.

4. Discussion

Our studies demonstrated that the IL-12, IL-18 and TGF-β levels were elevated in all children with malaria as the expression of the immune activation in response to the presence of parasites and according to the different levels of hemoglobin concentration, platelet count and level of parasitemia. A large body of evidence indicates that cytokines are determinants of malaria severity and outcome [2,8,9] and can represent potential targets for therapeutic interventions, if their effect is highlighted.

Our results, in children with different degrees of the disease, suggest that a fine mechanism of regulation...
modulates the production of IL-12, IL-18 and TGF-β. Moreover, the study of the IL-12 plasmatic level represents a suitable tool to monitor the immune response to *P. falciparum*. In fact, our recent results establish a critical role for IL-12 in the adaptive immune response to malaria [7] and confirm the association between levels of IL-12 and macrophage activation with the production of IFN-γ [10], directly related to the effects of hemozoin, the most important metabolite of the parasite derived from hemoglobin digestion, by these cells [11]. The correlation between cytokines confirms that, in a very early phase of *P. falciparum* infection, when the production of IL-12 is uncontrolled, the IL-18 production is not synchronous to the variation of IL-12 (Fig. 1). This fine mechanism of control downregulates the immune response, influencing the overproduction of pro-inflammatory and anti-inflammatory cytokines, which may be associated with disease severity and mortality. Also the production of TGF-β becomes necessary in the modulation of the immune response since it inhibits IFN-γ production and upregulates IL-10 [12]. Studies in murine models showed that low levels of TGF-β production were associated with lethal outcome, whereas sustained production of TGF-β was associated with resolving infections [13]. Moreover, levels of TGF-β inversely correlate with rates of parasite replication; neutralization of TGF-β leads to more rapid parasite growth, while the treatment with optimal doses of r-TGF-β slows the rate of parasite replication [14]. Therefore, this demonstrates that TGF-β has two distinct roles in malaria infection depending on the time of infection. Early in the infection TGF-β promotes Th-1 mediated mechanisms that control parasite growth. Later TGF-β downregulates the Th-1 like response to limit inflammation associated pathologies [15] (Figs. 2 and 3).

Our results show that TGF-β downregulates IL-12 more than IL-18, and thereby modulates the immune response to *P. falciparum* preventing the susceptibility to the side effects of the disease, such as severe anemia and cerebral malaria. The synchronous IL-12 and IL-18 production, and the early production of TGF-β may play important roles in the favorable outcome of malaria, reducing the systemic damage induced by an uncontrolled immunoresponse. Recently, the use of IL-12 has been proven to be effective in experimental malaria [16], but the dose of IL-12 appears to be critical, given the potential toxic effects of this cytokine [17,18]. Our results showing a synchronous IL-12 and IL-18 production during the disease suggest that the administration of IL-12 alone is not sufficient. Thus, in the treatment of severe *P. falciparum* malaria it is necessary to associate the administration of IL-18 to IL-12, reproducing the physiological regulation of the immune response. However, the two regression curves (Fig. 4) of children with severe and mild malaria are well separated with regard to the large concentration of IL-12 (>20 pg/ml). In fact, as is shown, the IL-18 concentration is higher in patients with severe malaria than in patients with mild malaria. This result could be used for its prognostic significance: the higher level of IL-18 may be a parameter that correlates with severity of the disease. A similar procedure was applied correlating IL-12 and TGF-β (Fig. 5). We observed that these two parameters correlate negatively (for IL-12 < 20 pg/ml). In this case lower

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**Fig. 3.** Correlation between IL-18 and TGF-β in 77 children with severe and in 28 children with mild *P. falciparum* malaria.

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y = -0.6406x + 40.44 \\
R^2 = 0.2462
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values of TGF-β were found in children with severe malaria and consequently also IL-12/TGF-β can represent a prognostic parameter. The prognostic significance of both the measurements (IL-12, IL-18 or IL-12, TGF-β) is greater in the area where the two curves diverge. This experimental method could be useful for a new immunological approach in the treatment of acute malaria, which could permit the control of the immune response, increasing TGF-β and lowering IL-18, in a disease where the entity of this response is determinant in the fatal evolution [19]. In addition, the hyperparasitemia in the first phase of the disease can also be controlled by the previous administration of TGF-β, which induces Th1 cells to produce IFN-γ. In the last phases of disease the use of TGF-β to downregulate Th1 cells decreases the proinflammatory response. The correlation between TGF-β and IL-12 in severe and mild malaria delimits the area where the TGF-β level must be corrected (Fig. 5). Taking into account the variation of these three cytokines in severe and mild malaria, our mathematical model became interesting since it suggests that the combined treatment of cytokines with chloroquine or quinine could be more effective in patients at the first infection. In conclusion, the monitoring of the immune response becomes important starting from the patient’s initial clinical status when there is a high parasitemia and an unbalanced cytokine production. The realization of an efficient mathematical model requires an exhaustive knowledge of the specific effect of the pro- and anti-inflammatory cytokines since the omission of
some of the parameters involved in the immune response would lead to the creation of an inadequate model. Further experimental procedures are necessary to construct more accurately fitting curves in order to determine the prognostic values of these parameters.

References