Production of pro- and anti-inflammatory cytokines by monocytes from patients with paracoccidioidomycosis

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Abstract

Monocytes and macrophages can produce a large repertoire of cytokines and participate in the pathogenesis of granulomatous diseases. We investigated the production of pro- and anti-inflammatory cytokines by monocytes from patients with active paracoccidioidomycosis. Peripheral blood monocytes from 37 patients and 29 healthy controls were cultivated with or without 10 µg/ml of lipopolysaccharide (LPS) for 18 h at 37 °C, and the cytokine levels were determined in the culture supernatants by enzyme immunoassay. The results showed that the endogenous levels of tumor necrosis factor alpha (TNF-α), interleukin-1beta (IL-1β), IL-6, IL-8, IL-10 and transforming growth factor beta detected in the supernatant of patient monocytes cultivated without stimulus were significantly higher than those produced by healthy controls. These data demonstrated that monocytes from patients with active paracoccidioidomycosis produce high levels of cytokines with both inflammatory and anti-inflammatory activities. However, patient monocytes produced significantly lower TNF-α and IL-6 levels in response to LPS when compared to normal subjects, suggesting an impairment in their capacity to produce these cytokines after LPS stimulation. Concentrations of IL-1β, IL-8 and IL-10 in cultures stimulated with LPS were higher in patients than in controls. These results suggest that an imbalance in the production of pro- and anti-inflammatory cytokines might be associated with the pathogenesis of paracoccidioidomycosis.

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1. Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungus Paracoccidioides brasiliensis [1]. This mycosis is the most prevalent systemic human disease in Latin America, and available data suggest that Brazil is an important endemic area with 85% of all reported cases [2]. The disease presents as an entity with a systemic course or as a chronic localized mycosis, depending on several factors, including host immunocompetence, parasite strain and the environment [3]. PCM may be classified into different clinical forms: (i) the regressive form, with mild clinical manifestations, generally involving the lungs, (ii) the acute or subacute form, with intense involvement of the mononuclear phagocytic system, which affects young people, (iii) the chronic form, with an insidious evolution and involvement of one or more organs or systems, affecting adults older than 30 years, and (iv) the residual sequel-producing form with manifestations related to the cicatrization of old lesions. The patients with acute and chronic forms usually present generalized malaise, anorexia, and weight loss, at times so intense that it may lead to cachexia. Fever is seldom observed and may be considered as a sign of severe disease [4].

Many people are exposed to the fungus, but only a small number develop clinical symptoms, suggesting that both innate and adaptive immune mechanisms seem to be important in the clearance of the fungus [5]. Thus, cell-mediated immunity is generally a more significant factor than humoral immunity, and activated macrophages appear to provide the major line of defense.
Patients with severe disease present hypergammaglobulinemia, high levels of specific antibodies, eosinophilia, and depressed general and antigen-specific cellular immunity [3–6]. It has been suggested that in patients with PCM, high serum levels of inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) may play a role in the genesis and in the control of some aspects of the disease, including granulomatous reaction, hypergammaglobulinemia, and depression of T-cell-mediated immunity [7]. Recent work has demonstrated that peripheral blood mononuclear cells from patients with the acute or chronic form of PCM produce low levels of IL-2 and interferon-γ, but a substantial amount of IL-10. The imbalance in cytokine production may play a role in the hyporesponsiveness of PCM patients to the gp43 fungus antigen [8].

Studies on monocyte activity are still scarce in PCM. Human normal monocyte-derived macrophages treated with recombinant human IFN-γ have only a fungicidal activity against P. brasiliensis multiplication, without an evident fungicidal effect [9]. In a previous study, we demonstrated that the effects of IFN-γ and TNF-α on the fungicidal activity of human monocytes are negatively regulated by prostaglandins, which are probably secreted by the host cells in response to the pathogen and could be involved in suppressing the immune response [10].

In other mycoses, monocytes/macrophages are recognized as critically important for effective immune functions, being implicated in the innate mechanisms against fungal infections [11,12]. These cells play a pivotal role in immune regulation by producing cytokines that are both mediators of the acute inflammatory response and have inhibitory effects [13]. Several studies have reported in vitro stimulation of human monocytes/macrophages with different fungi such as Coccidioides immitis [14] and Cryptococcus neoformans [15–17] or their cell wall components, suggesting that these cells are the main source of TNF-α, IL-1, IL-6 and IL-10. These cytokines have been demonstrated to mediate the normal immune response and also to be responsible for the immunopathogenesis of systemic mycoses such as coccidioidomycosis [14].

The present study was undertaken in order to determine the extent of monocyte activation in patients with active PCM by investigating the spontaneous and lipopolysaccharide (LPS)-stimulated production of pro- and anti-inflammatory cytokines in cultures of monocytes isolated from peripheral blood.

2. Material and methods

2.1. Patients and controls

We studied 37 patients with a diagnosis of active PCM at the Infectious Diseases Service, University Hospital of the Botucatu Medical School, São Paulo State University, Brazil. The diagnosis of the mycosis was confirmed by direct microscopic observation of the causative agent in clinical specimens and/or by the demonstration of typical fungi in biopsy material. Thirty patients were males and seven were females (age range 12–60 years). Twenty-nine healthy blood donors matched for sex and age to the patients were included as control. The study was approved by the Ethics Committee of the Botucatu Medical School, and informed consent was obtained from all patients and controls.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated by density gradient centrifugation at 400 × g for 30 min on Histopaque (density (d) = 1.077) (Sigma Chemical Co., St. Louis, MO, USA). Briefly, 5 ml of heparinized blood was mixed with an equal volume of RPMI-1640 tissue culture medium (Gibco Laboratories, Grand Island, NY) containing 2 mM L-glutamine, 10% heat-inactivated human AB serum (Sigma Chemical Co., St. Louis, MO, USA), 20 mM HEPES and 40 µg/ml gentamicin (complete medium). Samples were layered over 6 ml of Histopaque in a conical plastic centrifuge tube. After centrifugation at 400 × g for 30 min at room temperature, the interface layer of cells was harvested twice with phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA) and once with complete medium. Cell viability, as determined by 0.2% trypan blue was >95% in all experiments. The monocytes were counted using 0.02% neutral red and the mononuclear cells were suspended to a concentration of 1 × 10⁶ monocytes/ml in complete medium. Monocyte preparations routinely contained >90% monocytes as determined by morphologic examination and staining for nonspecific esterase [18].

2.3. Monocyte monolayers

The monocyte suspension (1 × 10⁶ ml⁻¹) was dispensed at 1 ml/well in Linbro Titertek (Flow Laboratories, Inc., Mclean, VA) 24-well flat-bottomed plates. After incubation for 2 h at 37 °C in 5% CO₂, non-adherent cells were removed by aspiration and each well was rinsed twice with complete medium. After adherence, the monocytes were cultured in the presence or absence of 10 g/ml Escherichia coli O₅B₅ LPS (Sigma Chemical Co., St Louis, MO). The supernatants were harvested after 18 h culture and kept at –70 °C until cytokine determination.

2.4. Enzyme-linked immunosorbent assay for cytokines

Cytokine concentrations were determined in culture supernatants by enzyme-linked immunosorbent assay (ELISA). Antibody-matched pairs and respective standards were purchased from Genzyme (IL-1β, IL-6, IL-8 and TNF-α) (Cambridge, MA, USA) and from R&D Systems (IL-10, transforming growth factor (TGF)-β1) (Minneapolis, MN, USA) and used according to the manufacturer’s instructions. The sensitivity limit of the assays was 10 pg/ml for TNF-α, IL-10 and TGF-β1 and 10 ng/ml for IL-1β, IL-6 and IL-8.
2.5. Statistical analysis

Differences in the cytokine responses between patients and controls were analyzed by the non-parametric Mann-Whitney U test. Differences in cytokine production between monocytes cultured in the absence or presence of LPS were analyzed by the paired non-parametric Wilcoxon sign rank test. The level of significance was set at $P < 0.05$.

3. Results

3.1. Spontaneous in vitro production of cytokines by monocytes from patients with PCM

To assess the function of monocytes from patients with PCM in the context of the existing chronic monocyte activation, we evaluated the constitutive production of cytokines (IL-1β, IL-6, IL-8, IL-10, TNF-α and TGF-β1) by isolated monocytes cultured in the absence of any exogenous stimuli for 18 h [Fig. 1]. The levels of all cytokines studied and spontaneously produced by monocytes of PCM patients were significantly higher than those produced by monocytes from healthy individuals. The results suggest an in vivo activation state of patient monocytes.

![Spontaneous production of cytokines by monocytes from patients with PCM](image1.png)

**Fig. 1.** Spontaneous production of cytokines by monocytes from patients with PCM ($n = 37$) and healthy controls ($n = 29$). Isolated monocytes ($1 \times 10^6$ ml$^{-1}$) from patients (filled bars) and from healthy controls (open bars) were cultured without a stimulus for 18 h. The concentrations of TNF-α, IL-1β, IL-6, IL-8, IL-10 and TGF-β1 were measured by specific ELISA. The results are expressed as median values. * Statistically significant ($P < 0.05$) when compared with healthy controls using the Mann-Whitney $U$ test.

Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Patients</th>
<th>Healthy controls</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>774.9 (379.3–1148.0)</td>
<td>1028.9 (487.1–1264.7)</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>IL-1β (ng/ml)</td>
<td>8.3 (1.9–13.8)</td>
<td>5.9 (2.1–7.7)</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>IL-6 (ng/ml)</td>
<td>25.4 (12.0–48.2)</td>
<td>33.4 (17.6–53.6)</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>IL-8 (ng/ml)</td>
<td>245.9 (77.5–399.9)</td>
<td>88.4 (33.6–143.3)</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>1735.7 (118.1–5869.7)</td>
<td>873.7 (216.8–2499.1)</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>TGF-β1 (pg/ml)</td>
<td>1010.5 (224.2–2298.7)</td>
<td>1173.1 (744.3–1866.5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Isolated monocytes ($1 \times 10^6$ ml$^{-1}$) were cultured in the presence of LPS (10 µg/ml) for 18 h. The results were expressed as medians and range (in parentheses).

* Difference between patients and healthy controls by the Mann-Whitney $U$ test.

3.2. Cytokine production by LPS-stimulated monocytes from patients with PCM

To determine the changes in the capacity of monocytes to produce cytokines, we studied the in vitro cytokine production by LPS-stimulated monocytes isolated from patients and healthy controls [Table 1]. Both patient and control monocytes respond to in vitro LPS stimulation, producing significantly higher levels of pro- and anti-inflammatory cytokines detected in the supernatant cultures than did non-stimulated monocytes.

The production capacity of IL-1β, IL-8 and IL-10 by monocytes after LPS stimulation was significantly higher in patients than in healthy controls. On the other hand, monocytes from patients produced lower levels of TNF-α and IL-6 than controls. Among the cytokines studied no significant differences between patients and controls were observed when the levels of TGF-β1 produced by LPS-stimulated monocytes were determined. These observations indicate that monocytes from patients with PCM respond to LPS stimulation, but the balance of cytokines may be altered.
4. Discussion

In this study we evaluated cytokine production by peripheral blood monocytes from PCM patients. The levels of IL-1β, IL-6, IL-8, IL-10, TNF-α and TGF-β1 detected in the supernatant of patient monocytes cultured without a stimulus were significantly higher than those produced by control individuals. The results provide evidence that monocytes from patients are in a state of in vivo activation.

Elevated levels of TNF-α, IL-1 and IL-6 in serum of PCM patients have been previously reported [7,19]. The augmented production of these cytokines in serum and the spontaneous production by monocytes detected in culture supernatants could be associated with peripheral blood cell stimulation by the fungal load, during the course of the mycosis. Antigenemia in PCM has been previously described [20,21] to be associated with the pathogenesis of PCM. Previous studies have shown that macrophages from hamsters [22] or mice [23] produced high levels of TNF-α after in vitro stimulation with P. brasiliensis or its cell wall components.

The systemic overproduction of TNF-α, IL-1β and IL-6 cytokines may be responsible for the non-protective effects and constitutional symptoms of fever, anorexia and weight loss commonly associated with coccidioidomycosis [14,24] and tuberculosis [25]. In PCM several of these symptoms are present, mainly in the severe forms of the disease, and might be attributed to inflammatory effects of the cytokines produced by activated monocytes. High levels of TNF-α were observed during the later stages of infection in Syrian hamsters with experimental PCM, associated with loss of weight and vitality and resulting in animal death. High levels of this cytokine were probably deleterious and contributed to the pathogenesis of the mycosis [22].

Besides the high levels of endogenous TNF-α produced by monocytes from PCM patients, we demonstrated that these cells also synthesized elevated concentrations of IL-1β, IL-6, IL-8, IL-10 and TGF-β1 in contrast to those of healthy control individuals. To our knowledge, there are no reports in the literature showing spontaneous cytokine production by monocytes detected in culture supernatants of non-PCM patients. The elevated production of IL-1β, IL-6, IL-8, IL-10, TNF-α and TGF-β1 detected in the supernatant of patient monocytes cultured without a stimulus were significantly higher than those produced by control individuals. The results provide evidence that monocytes from patients are in a state of in vivo activation.

The synthesis of the anti-inflammatory cytokines IL-10 and TGF-β1 detected in culture supernatants of non-PCM patients was also elevated in PCM patients. The elevated amount of IL-10 spontaneously produced by monocytes from patients with PCM confirmed recent studies. Benard et al. [8] demonstrated that peripheral blood mononuclear cells from patients with both the acute and chronic forms of PCM produced high levels of IL-10, but low concentrations of IL-2 and IFN-γ. IL-10 plays a role as an anti-inflammatory and suppressor agent [28] capable of downregulating a broad spectrum of pro-inflammatory cytokines and cytokine production by Th1 cells [29,30]. High levels of IL-10 in serum of patients with chronic hepatitis are associated with a hepatic inflammatory response [31]. In PCM, high levels of endogenous IL-10 and the elevated capacity of cytokine production by patient monocytes probably represent an attempt by the organism to control the inflammatory reaction against P. brasiliensis.

The high production of TGF-β1 in patients suggests that monocytes were activated in vivo and partially responsible for the immunosuppression detected in active PCM. Spontaneous production of this cytokine by monocytes was observed in the active form of tuberculosis [32] and attributed to in vivo monocyte activation by circulating mycobacterial products [32,33]. This cytokine has an inhibitory effect on macrophage, eosinophil, neutrophil, T and B lymphocyte populations [34] and mainly on T-cell proliferation induced by IL-2 [35]. Based on the paracrine nature of cytokine production, the elevated synthesis of TGF-β1 and IL-10 by monocytes from patients with PCM might be involved in the impairment of IFN-γ production [8,36] of lymphocyte proliferation, and of the production of IL-2 and its receptor previously described in this mycosis [5].

Our results also demonstrated a significant increase in cytokine production after in vitro monocyte stimulation with LPS in both patients and controls, showing the capacity of monocyte activation after exogenous stimulation with the endotoxin. LPS is a wall constituent of Gram-negative bacteria and represents a naturally occurring endotoxin which mainly stimulates monocytes in the blood [37]. However, the TNF-α and IL-6 concentrations produced by monocytes from patients after the LPS stimulus were significantly lower than those produced by healthy subjects. These results provide evidence that these cells may be functionally altered and demonstrate an impairment in the capacity of monocytes to produce these cytokines after LPS stimulation. The low reactivity of monocytes to LPS was also observed in patients with septic shock and was correlated with the severity of sepsis [38,39].

Our results of in vivo monocyte activation in patients with PCM represented by elevated endogenous production of pro- and anti-inflammatory cytokines and the lower reactivity to LPS evidenced by low levels of TNF-α and IL-6 synthesis are in accordance with results in the literature concerning sepsis and septic shock. Monocytes from patients with sepsis presented tolerance to LPS stimulation when previously treated with low levels of LPS and after being...
challenged with high doses of the endotoxin. According to Randow et al.\textsuperscript{[39]}, this tolerance may be induced by cell production of IL-10 and TGF-β1 both in vivo and in vitro and can be prevented by the addition of anti-IL-10 or anti-TGF-β1 antibodies to the monocyte cultures. Thus, the hyporesponsiveness or tolerance to in vitro LPS stimulation is considered a consequence of IL-10, TGF-β1 and PGE2 production by monocytes.\textsuperscript{[39,40]}

Mediators such as IL-10, PGE2 and TGF-β1 are liberated during the course of several infections, including sepsis caused by Gram-negative and -positive microorganisms, and are responsible for the impairment of monocyte activation, including a lower expression of HLA-DR molecules and a reduced production of pro-inflammatory cytokines by these cells.\textsuperscript{[39,41]}

Taken together, the results of the present study demonstrate that monocytes from patients with active PCM are an important source of pro-inflammatory cytokines such as IL-1, IL-8 and TNF-α, as well as others with regulatory activity such as IL-6, IL-10 and TGF-β1. These cytokines may be involved in metabolic disturbances such as fever, C-reactive protein, weight loss and alterations in both innate and adaptive immune responses observed in PCM. The activation state of monocytes detected during the active disease might be responsible for the systemic immunosuppression observed previously in PCM.\textsuperscript{[5,42]} Thus, anti-inflammatory cytokines IL-10 and TGF-β1 produced by monocytes probably play a role in the control of monocyte activation state and modulate the systemic inflammatory response observed in PCM. The chronic activation of monocytes and the complex imbalance of the cytokines they produce may contribute to the impaired immune competence observed in PCM.

References


