REVIEW

Immunology of paracoccidioidomycosis

Imunologia da paracoccidioidomicose

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Abstract: Paracoccidioidomycosis is the most prevalent systemic mycosis in Latin America, among immunecompetent patients. It's caused by the dimorphic fungus Paracoccidioiddes brasiliensis. Investigations regarding its immunopathogenesis are very important in the understanding of aspects related to natural history, as the protective immunity, and the relationship between host and parasite; also favoring the knowledge about clinical patterns and the elaboration of therapeutic strategies. The disease clinical polymorphism depends, at least, of the immune response profile according to the tissue and blood released citokynes, resulting in tissue damage.
Keywords: Allergy and immunology; Cytokines; Paracoccidioides; Paracoccidioidomycosis

Resumo: Paracoccidioidomicose é a mais prevalente micose sistêmica na América Latina, em pacientes imunocompetentes, sendo causada pelo fungo dimórfico *Paracoccidioiddes brasiliensis*. O estudo da sua imunopatogênese é importante na compreensão de aspectos relacionados à história natural, como a imunidade protetora, e à relação entre hospedeiro e parasita, favorecendo o entendimento clínico e a elaboração de estratégias terapêuticas. O polimorfismo clínico da doença depende, em última análise, do perfil de resposta imune que prevalece expresso pelo padrão de citocinas teciduais e circulantes, além da qualidade da resposta imune desencadeada, que levam ao dano tecidual.
Palavras-chave: Alergia e imunologia; Citocinas; Paracoccidioides; Paracoccidioidomicose

INTRODUCTION

Paracoccidioidomycosis (PCM) is a human systemic mycosis whose etiological agent is *Paracoccidioides brasiliensis*, a thermodimorphic fungus (Figure 1) which promotes a chronic inflammatory granulomatous disease.[1]

PCM is endemic in Latin America, with higher incidence in Brazil, and diagnosed more often in the state of São Paulo. The true incidence of the disease is not known. However, in endemic areas it is believed that the occurrence rate is that of three cases per 100,000 inhabitants.[2, 3]

PCM infection does not privilege gender or ethnicity, whereas PCM disease affects mainly adult male rural workers in constant contact with vegetation and soil during the most productive period of their lives, which implies important economic repercussions for patients and their dependents.[1, 2]

Rural workers are often among social groups of lower socioeconomic status with a lesser degree of hygiene and a higher rate of malnutrition.

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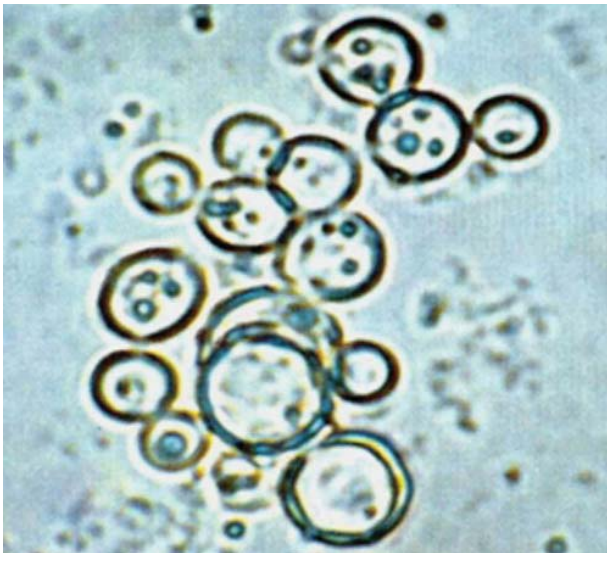

Figure 1: *Paracoccidioides brasiliensis* typical yeast form with multiple branches in the shape of a helm wheel. KOH - 400X

Furthermore, smoking and heavy alcohol consumption are commonly observed in such populations.[4, 5] These factors are considered pre-existing risk conditions and play an important role in turning infection into active disease, since they interfere with the host defense mechanisms and have implications in the quality of granuloma formation.[6] Likewise, it may be observed in individuals immunosuppressed by HIV/AIDS or iatrogenically that the fungus behaves as an opportunistic agent, with distinct clinical aspects and evolution.[7]

The relationship between *P. brasiliensis* and the environment has not yet been fully understood, but it is believed that the mycelium is the saprobiotic form of the fungus in nature, which would under certain conditions produce conidia, structures of asexual reproduction and propagation of the species. The spores present in soil, plants and water at room temperature are considered the infecting forms.[3, 8]

Contagion of the host occurs most often by inhalation of conidia and mycelial fragments that reach the terminal bronchioles and alveoli, where they are transformed into yeast cells, producing an infection that can spread to other tissues via lymphatic and hematogenous means. Exceptionally, there could be a skin traumatic inoculation of the fungus, which can also adhere to the skin and mucous membranes through traumatic inoculation.[9-11]

The predominance of men compared to women affected by PCM occurs due to a possible hormonal protective effect, determined by the presence of estrogen receptors in the wall of the fungus and capable of blocking the transformation of mycelia or conidia into the infecting yeast form.[12, 13] *P. brasiliensis* is devoid of mobility systems and by evolutionary means has developed antigenic characteristics to allow adhesion to and interaction with the host tissues, preventing effective defense and ensuring its survival.[11] The pathogenic mechanisms through which persistence of latent infection, intravascular invasion and the spread of the fungus throughout the organism occur have not yet been fully understood, despite numerous studies on the pathophysiology of the disease and the biology of the fungus.[11]

CLINICAL CLASSIFICATION OF PCM

PCM is classified, according to its natural course and clinical manifestations of the patient, in its acute and subacute or chronic forms (Table 1). The clinical manifestations depend on the virulence of the infecting strain of *P. brasiliensis*, the degree and type of immune response triggered, infected tissues, and intrinsic characteristics of the host.[11, 14]

There are no known clinical manifestations associated with PCM infection. The results evidenced by the finding of a positive intradermal reaction to paracoccidioidin in epidemiological surveys have shown that, in endemic areas, the infection rate can be as high as 70% of the studied population.[5] In contrast, the number of clinical cases of active disease is way below that, showing that the greater possibility is that infected individuals remain as such indefinitely, unless the host-parasite balance is changed, allowing fungal growth and progression to clinically active disease.[5, 14, 15]

The acute or subacute (juvenile type) forms of the disease can occur in young individuals of both sexes, generally developing from a primary undetec-

Chart 1: Clinical classification of paracoccidioidomycosis

1.	Paracoccidioidomycosis infection		
2.	Paracoccidioidomycosis disease		
	2.1	Acute form	
	2.1.1.		Moderate
	2.1.2.		Severe
	2.2.		Chronic form
	2.2.1.	Unifocal	Mild
			Moderate
			Severe
	2.2.2.	Multifocal	Mild
			Moderate
			Severe
3.	Paracoccidioidomycosis associated with immunosuppression		
4.	Residual form (sequela)		

Adapted source: Franco et al. 1987[16]

ted pulmonary lesion that evolves quickly with lymphatic and hematogenous spread to organs of the monocyte-macrophage system, such as spleen, liver, lymph nodes, bones and bone marrow, leading to a significant deterioration of the patient's clinical condition. High titers of specific antibodies and severe depression of cellular immunity are commonly observed.[16] The death rates associated with PCM are particularly linked to this clinical form.

The chronic form, also called "adult", is the most common in clinical practice and develops from the primary pulmonary complex or from the reactivation of a quiescent pulmonary or metastatic focus. Most cases begin in the lungs and progress slowly; they are usually observed in adult males over 30 years of age, presenting prolonged duration, typically over six months of clinical history, and often expressed by pulmonary and tegumental (cutaneous and/or mucosal) damage.[5]

Lesions may remain localized (unifocal clinical subtype) or spread to various organs and systems (multifocal), with variable degrees of severity.[16]

HISTOLOGICAL ASPECTS OF SKIN LESIONS

Contact of *P. brasiliensis* with host tissue initially triggers a congestive-exudative inflammatory reaction with a predominant afflux of neutrophils. Increasingly, these cells are replaced by macrophages, which are arranged in loose nodules, and multinucleated giant cells. With the evolution of the process epithelioid cells are found, and concomitantly a lymphoplasmacytic halo is formed. Thus, PCM granuloma consists of a nodular arrangement of epithelioid cells and multinucleated giant cells of the Langhans and foreign body types, many of them containing fungi. It is common to find a central area of suppuration and inflammatory exudate rich in lymphocytes, plasma cells and eosinophils permeating or surrounding the granulomatous reaction (Figure 2). Fibrosis of varying intensity is generally seen surrounding the granulomas or necrotic areas, which are gradually replaced by fibrous scar tissue.[6, 17]

Experimental and human studies support the hypothesis that PCM granuloma can represent a specific immune response of the host against the fungus.[18] The evolution of the granuloma is related to the host immune response to the components of the cell wall expressed by the fungus.[19] Epithelioid granulomas, obtained from skin or mucosal biopsies of PCM patients, are composed of a central cluster of HLA-DR+ cells, represented by macrophages and epithelioid cells surrounded by a peripheral mantle of T-cells, with the predominance of a CD4+ population. These findings reinforce the hypothesis that T-lymphocytes and HLA-DR+ cells are actively involved

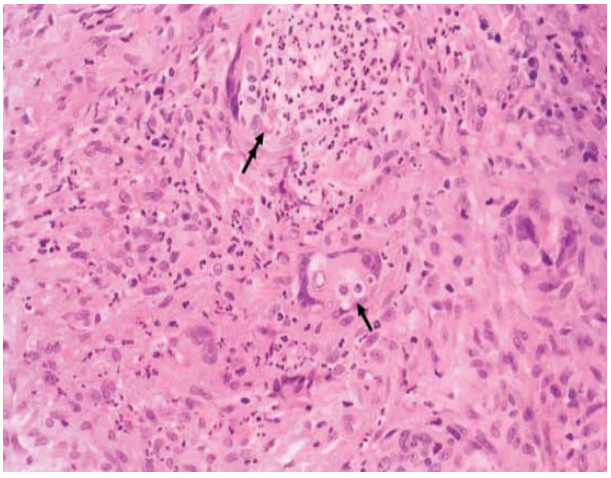

Figura 2: Skin. Suppurative and chronic inflammatory reaction, with granulomas consisting of macrophages, epithelioid cells and giant cells containing viable fungi inside (single arrow). Observe foci of suppuration in epithelioid granulomatous process (double arrow). HE - 200X

in the process of granuloma formation and host defense against *P. brasiliensis*.[20]

Therefore, macrophages and T-lymphocytes assume central importance in the morphogenesis of the inflammatory process, and the synergistic action of these cells is important in granuloma formation and modulation through the production of cytokines and other mediators at the inflammatory site.[14, 18, 21]

Morphology of the granulomatous reaction in PCM based on the pattern of immunological response can be represented by setting the two antagonistic poles. In subjects with preserved cellular immune response, compact, well-defined, sometimes confluent epithelioid granulomas can be observed, with few fungi and localized benign infection. In patients with impaired immune systems, the granulomatous inflammation is disorganized, with large numbers of fungi and loose, ill-defined granulomas, with suppurative exudation and necrotic areas. Proliferation and dissemination of the fungus occur, leading to widespread disease with bad prognosis.[5, 21, 22]

Therefore, the granulomatous reaction that occurs in human and experimental PCM represents the most specialized and efficient response of the host tissue in an attempt to block and restrain the fungus, preventing it from multiplying and spreading to tissues.

CUTANEOUS LESIONS

The clinical significance of skin lesions depends on their number, pattern and location. Multiple lesions of the papular-acneiform type suggest hematogenous dissemination of *P. brasiliensis* and reflect the

severity of the disease. Basically, the frequency, number and characteristics of the skin lesions are consequences of host-parasite interactions, including the pathogenesis of the lesions, the site involved and the duration of the disease.[5]

The characterization of the morphological patterns of cutaneous lesions in PCM is not easily established due to the clinical polymorphism of the disease, the dynamics of lesion progression and also the dermatological criterion employed. However, ulcerated lesions are considered the most prevalent and may represent a typical semiological pattern, i.e., the appearance of clear sores, not secondarily infected and with the presence of stippled hemorrhage mimicking the so-called moriform stomatitis (Figure 3), thus supporting clinical diagnosis.[5] Skin lesions are more frequent in the cephalic segment, and present around the nose and mouth.[1, 5, 23]

Disseminated forms are associated with a less efficient immune response, worse prognosis and more frequent recurrence, as is the case in both the acute and subacute forms, as well as in chronic severe forms of the disease (Figure 4).

IMMUNOPATHOGENESIS OF PCM

The establishment of the disease, its spread and severity depend on factors inherent to the fungus itself, as its virulence, antigenic composition, environmental conditions and especially those factors related to the host's ability to develop an effective immune response. With regard to the latter aspect, we can consider that *P. brasiliensis* synthesizes metabolic antigens which interact with the host immune system, causing an immunological response that is both highly complex and multifactorial.[14] Clinical and experimental studies have suggested an interaction between specific and nonspecific defense mechanisms in determining resistance to *P. brasiliensis*.[11, 24]

P. brasiliensis presents a complex antigenic structure, with epitopes that are related to pathogenicity. The main antigenic component of *P. brasiliensis* is a surface glycoprotein of fungal wall with 43 kDa (gp43), an immunodominant antigen associated with virulence factor and/or escape by which the fungus evades the host defense mechanisms and lodges itself in the tissues.[25] The glycoprotein gp43 has proteolytic effects on collagen, elastin and casein. This digestion of structural proteins seems to play an important role in the implantation of the fungus in tissues.[26]

Several innate immune mechanisms such as the activation of complementary system proteins, microbicidal activity of natural killer cells (NK) and phagocytes act significantly in the fight against pathogenic fungi. Innate response cells, such as natural killer cells (NK), neutrophils, monocytes and macrophages play a central role in the resistance to *P. brasiliensis*. The participation of these cells in inflammatory reaction and antifungal activity is induced by the fungus and by cytokines produced by the cells during their interaction with phagocytes.[27-29] The role of NK cells has been studied with regard to the peripheral blood of patients with PCM and the experimental hamster model. These cells have their cytotoxic activity decreased during PCM disease, suggesting immune disorder associated with depression of cellular immunity both in patients and in the experimental model.[30]

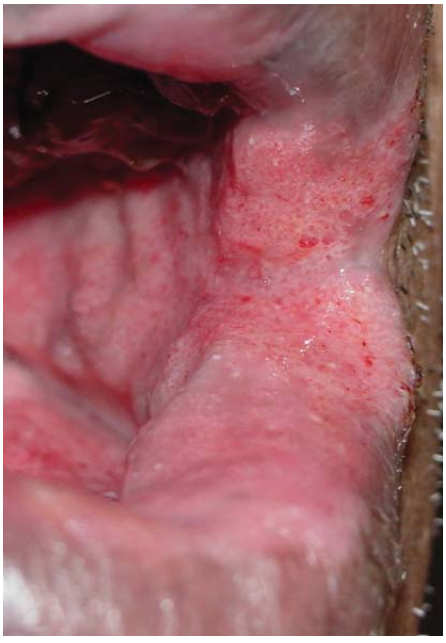

Figura 3: Ulcers on the lip and gingival mucosa presenting a clear bed with stippled hemorrhage (moriform stomatitis)

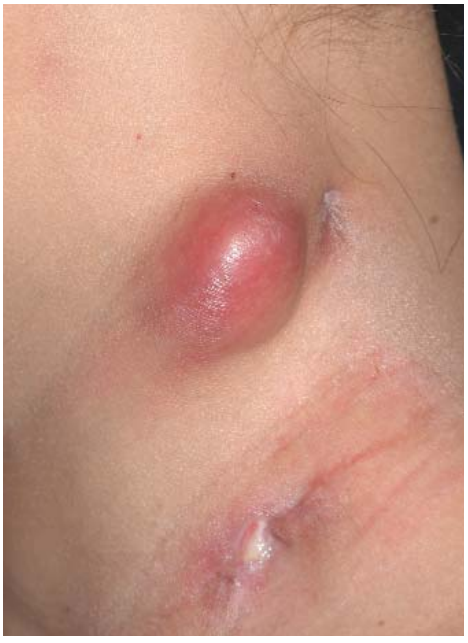

Figura 4: Patient with acute PCM, with preauricular and cervical lymph node with abscess and fistula

The interaction between the parasite surface molecules and the homologous receptors present in the cellular membrane of phagocytic cells, including neutrophils, modulates phagocytosis and the activation of those cells. Among these receptors Toll-like receptors (TLRs) and lecithin C-like receptors (CLR) stand out, which are transmembrane proteins that interact with the molecular structures of pathogens by activating phagocytic cells. TLRs are capable of recognizing pathogen-associated molecular portions (PAMPs) and inducing signals that result in gene expression of innate immune response, and in the production of cytokines with inflammatory and anti-inflammatory properties that regulate the adaptive immune response.[31]

Although TLRs promote an immune response against infectious agents, experimental models suggest that yeast fungi penetrate the host's macrophages through TLR2 and TLR4 receptors. The interaction between TLR and *P. brasiliensis* is considered an escape mechanism developed by the fungus for survival inside phagocytic cells.[32]

Involvement of TLR2, TLR4 and dectin-1 in the recognition and internalization of *P. brasilienses* with consequent activation of neutrophils was shown. The less virulent strain of the fungus was preferably recognized by TLR2 and dectin-1, with balanced production of TNF-α and IL-10. However, the more virulent strain induced production of only TNF-α. The authors suggest that the less virulent strain would trigger a more controlled, less damaging response to the host through induction of IL-10 production. [33]

Human polymorphonuclear cells (PMN) are present in large amounts in infected tissues and they play an important role in fungicidal activity against *P. brasiliensis* through mechanisms dependent on oxygen metabolites such as H_2O_2 and superoxide anion, when these cells are stimulated with IFN-γ, TNF-α, GM-CSF and IL-15. [34, 35] PMN are essential in the early stages of infection, conferring resistance to the host and contributing to the development of an effective immune response against *P. brasiliensis.* PMN also produce large amounts of prostaglandin E2 and leukotrienes, perpetuating edema and inflammation but minimizing cell damage caused by monocytes.

However, when challenged with *P. brasiliensis in vitro,* PMN produce high levels of IL-8, triggering an anti-apoptotic process of neutrophils and favoring the proliferation and survival of the fungus within the phagocytic cell. [36] Similarly, the production of nitric oxide (NO) by monocytes stimulated by *P. brasiliensis* seems to exert a negative modulatory effect on the formation of granulomas and a positive effect on fungal spread, leading to the dissemination of PCM. [37]

Experimental studies in patients with PCM indicate that resistance to the fungus is dependent on activities of *T Helper* cells and macrophages/monocytes, mediated by IFN-γ and TNF-α. [27] The synergistic effect between these two cytokines is essential for host resistance and effective fungicidal activity against *P. brasiliensis.* [27, 28] During the course of the infection, CD4 Th1 lymphocytes synthesize cytokines such as IFN-γ, TNF-a and IL-12 that protect the host by preventing the spread of the fungus. [14, 18, 27, 38]

TNF-α acts on macrophage function in human and experimental PCM modulating and amplifying the immune response, promoting a granulomatous reaction and fungicidal activity mediated by macrophages. [27, 39] The absence of this cytokine results in the impairment of defense mechanisms associated with the inability to develop granulomatous reactions, which are effective to contain fungal multiplication. [40]

The heterogeneous aspect of the lesions seen in the skin of patients with PCM is probably associated to immunopathological events that occur during contact *of* P. brasiliensis with host tissue. The agent-host interaction stimulates the production of cytokines such as TNF-α by phagocytic cells, leading to tissue damage through the exacerbated production of intermediate metabolites of oxygen, collagenase and activation of adhesion molecules and fibroblast proliferation. Thus, in PCM, similarly to what occurs in other granulomatous diseases such as tuberculosis [41], leprosy [42] andschistosomiasis, [43] TNF-α seems to be involved in the protection against infectious agents through the induction of microbicidal and fungicidal activity of mononuclear phagocytes and tissue macrophages, activation of fibroblasts in the initiation and maintenance of the granulomatous response, expression of adhesion molecules, as well as in tissue immunopathogenesis. [18, 27, 39]

To corroborate the importance of the mediating role of TNF-α in the development of an effective immune response against intracellular pathogens, we may cite the immunobiological treatment of inflammatory diseases with anti-TNF-α immunoglobulin, which increases the risk of developing severe forms of infectious diseases . [44]

In granulomatous lesions of skin and mucous membranes of patients with PCM, the expression of TNF-α is diffusely distributed in the dermis and is expressed in the mononuclear cells of the inflammatory infiltrate around the granuloma and in keratinocytes, indicating the involvement of this cytokine in the genesis and maintenance of granulomas. [18] Large numbers of dendritic cells expressing TNF-α are arranged around granulomas, suggesting early involvement of this cytokine after contact *of* P. brasiliensis with the host. [45] The expression of anti-inflammatory cytokines such as IL-10 and TGF -β in the lymph nodes

of patients with the acute form of the disease is associated with the mechanism by which the fungus evades host immune response, contributing to the disseminated form of PCM. [46] Detection of immunostaining for IL-10 and IL-5 in loose granuloma of patients indicates an ineffective immune response in curbing the fungal infection. [45, 47] In the mucocutaneous forms of PCM, the presence of TGF-β in areas of fibrosis after treatment with trimethoprim-sulfamethoxazole suggests that this cytokine is involved in the healing process through stimulation of fibroblast proliferation. It also exerts regulatory and reparative functions of lesions, modulating the inflammatory response, reducing tissue destruction and contributing to repair and lower intensity of tissue sequela. [18]

The successful implantation of *P. brasiliensis* in host tissue depends on characteristics of the fungus such as virulence of the infecting strain, inoculant volume and, especially, the cytokines produced during confrontation between the fungus and phagocytic cells. Therefore, if the fungus precociously induces cytokine synthesis with suppressive or anti-inflammatory activity, such as TGF-β and IL-10, the result could be the suppression of the macrophage response, allowing the installation and reproduction of the fungus in tissues and its dissemination to various organs and systems. [14]

There is evidence to indicate the capacity of internalization of the fungus by keratinocytes, epithelial cells of other tissues and by the endothelium, without triggering an effective phagocytic response, which may indicate another mechanism by which the agent evades phagocytosis by leukocytes and thus enters the bloodstream and spreads to other tissues. [11]

Immunoregulation in PCM is associated with patterns of immune response regulated by T *helper* cells (Th1, Th2) and $CD4^+$ CD25 regulatory T $cells^+$ (Treg). Healthy individuals who come in contact with *P. brasiliensis* can resolve the infection at the site of the inoculant by an efficient innate immune response and the development of Th1 response pattern, with formation of dense granulomas. [14]

Most clinical forms of the disease occur due to the inability to develop an effective Th1 response and, therefore, unsuitable for the formation of dense granulomas. In these cases, there is the possibility of diversion to other patterns of immune responses, such as Th2, which turns out to be inefficient to contain the spread of infection. [45]

Patients with active disease have depression of cellular immune response characterized by decreased synthesis of Th1 cytokines such as IL-2, IFN-γ and IL-12 and increased levels of IL-4, IL-5 and IL- 10, corresponding to Th2 response, which does not protect the host. [48]

The humoral immune response against the fungus is not effective. It is characterized by the production of high titers of antibodies of the IgG4, IgA and IgE classes, associated with the prevalence of cytokines that suppress granuloma such as IL-4, IL-5 and TGF-β, in addition to marked eosinophilia. It is observed in patients with more severe forms of PCM, which reinforces the non-protective role of Th2 response. [49, 50]

Patients with chronic disease of moderate severity present an intermediate immune response between Th1 and Th2 patterns. Individuals with PCM living in endemic areas who do not develop the disease show a Th1 response pattern, suppressing fungal replication and maintaining a balance between host and parasite. In addition, patients with acute/subacute disease develop Th2 response. [51]

The importance of Th1 response in PCM was demonstrated *in vivo* by development of disseminated disease in a patient with congenital deficiency of the b1 subunit of the IL-12/IL-23 receptor. This deficiency results in additional loss of synthesis of IFN-γ and susceptibility to intracellular infections by mycobacteria and salmonella. [52]

$CD4^+$ $CD25^+$ Regulatory T cells (Treg) are important in controlling the immune response. The absence of these cells is associated with exacerbation of the inflammatory response and, consequently, the development of autoimmune diseases. On the other hand, excessive activation may be associated with susceptibility to pathogens. [53]

Patients with chronic PCM show high levels of cell phenotype typical of Treg cells ($CD4^+$ CD25 $^+$), both in peripheral blood and lesions, suggesting that these cells may control the local and systemic immune response in chronic PCM. The expression of CTLA-4 receptors (Cytotoxic T-lymphocyte-associated antigen-4 immunoglobin) in Treg cells acts as a negative regulator of T cell activation in patients with PCM. [53, 54]

Within the context of susceptibility of PCM, class II human leukocyte antigens (DRB1 * 11) are associated with the chronic unifocal mild form of the disease, suggesting that this allele may confer resistance against the spread *of* P. brasiliensis. [55]

Cytokines such as IL-1b, IL-6, IL-8, IL-10, IL-12 and TNF-α in PCM have been reported by several authors and suggest that monocytes/macrophages are important sources of these peptides capable of promoting a systemic inflammatory response. [56] The systemic production of TNF-α may be responsible for symptoms such as fever, anorexia, weight loss and tissue damage, commonly associated with moderate and severe forms of PCM. [39, 57] Elevated levels of IL-18 and sTNF -RII in patients with the acute form are associated with severity, [58] and sTNF-R1, sTNF-R2 and the chemokine CXCL9 in the chronic form indicate active

disease. [59] In a recent study, Siqueira et al 2009 showed that addition of IL-6 to monocyte culture allowed the proliferation *of* P. brasiliensis when these cells were challenged with a virulent strain of the fungus. The mechanisms by which monocytes allow the proliferation of the fungus in the presence of IL-6 are not known; however, the elucidation of this process might contribute to the understanding of the role of this cytokine in the pathogenesis of PCM. [60]

Although the immune response is essential for host defense, immunopathological aspects are related to the exacerbation of the immune response resulting in tissue damage. Proinflammatory cytokines such as IL-1, IL-8 and TNF-α may be involved in metabolic disorders such as fever, elevated C-reactive protein, asthenia and weight loss seen in patients with more severe disease. [56] In contrast, the anti-inflammatory cytokines IL- 10 and TGF-b probably have the function of controlling and modulating the inflammatory response in patients with active disease. [56]

A summary of the effects of cytokines resulting from the interaction between phagocytes and *P. brasiliensis* is found in Figure 5.

As stated, the interaction of the components of the fungus wall with receptors of the host immune system induces production of cytokines, which act by stimulating cellular defense mechanisms against the fungus, causing its elimination and regulating the intensity of the granulomatous response, thus preventing tissue injury . On the other hand, the involvement of cytokines during confrontation between the fungus and phagocytic cells may promote growth of the fungus in host tissues, leading to disease progression. [60] However, a balance between pro-and anti-inflammatory signals is crucial for a successful interaction between host and fungus.

The results obtained so far suggest a potential role of immunomodulatory substances, such as the use of effective vaccine that can modulate the immune system in order to amplify the effective response against *P. brasiliensis.* Molecules, associated with heat shock proteins (HSP), are the most attractive option, since they are associated with different phenomena of innate and adaptive immunity. [61] Members of this family of proteins have been tested in the prophylaxis and/or immunotherapy of diseases such as tumors, autoimmune diseases and mycoses. [62, 63]

The potential of DNAhsp65 vaccine has been tested in a murine model of PCM as an immunomodulator factor. Immunization with DNAhsp65 induced Th1 response associated with reduction of pulmonary fungal load. These results indicate that DNAhsp65 is a promising and attractive candidate in prevention and even therapy, as an adjunct to the treatment of PCM. [64] Another molecule, called HSP60, when used in the model of pulmonary PCM, induced a protective immune response against infection, suggesting that this immunodominant antigen is also considered a candidate for vaccine development against *P. brasiliensis.* [65]

The treatment of systemic mycoses is done with systemic antifungal agents and, in cases of immuno-

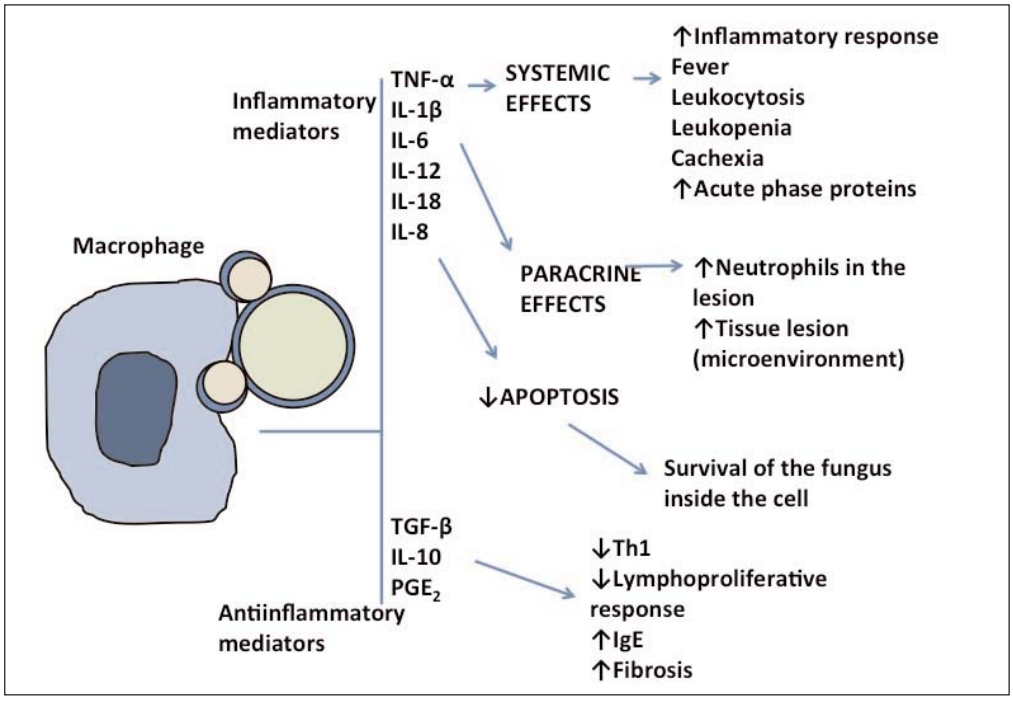

Figura 5: Microenvironment of cytokines resulting from the interaction between P. brasiliensis and mononuclear phagocyte

suppressed patients, the yeast forms intensely proliferate due to deficiency of innate and adaptive immune response. Under these conditions, treatment is very little efficient and long periods of treatment are necessary with the risk of relapses. A therapeutic vaccine with fungal antigens may lead to an efficient cellular immune response, preventing a possible relapse of the disease. DNA and peptide vaccines appear to be quite promising, since they are obtained in large quantities and with a high degree of purification for human immunization. [66]

The study of PCM immunology has provided subsidies for understanding the natural course of the disease and its clinical manifestations. It has also helped in the development of therapeutic approaches and of protective measures such as vaccines. While all these pathophysiological aspects are not clarified, PCM remains a disease with a high morbidity rate, thus causing a significant impact on society. ❑

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