Dendritic Cells and Pattern of Cytokines in Paracoccidioidomycosis Skin Lesions

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We demonstrated and quantified by immunohistochemistry epidermal Langerhans cells, CD34+ dermal dendrocytes (DDs), and cells expressing TNFα, interferon-γ (IFNγ), IL-5, and IL-10 in skin lesions of paracoccidioidomycosis (PCM). Sixty-one biopsies were classified in three groups according to the pattern of tissue response: Group 1, well-organized granuloma; Group 2, poorly organized granuloma; and Group 3, both kinds of granuloma. Langerhans cells had short and irregular dendrites in all groups and were decreased in number in Groups 1 and 2. CD34+ DDs did not differ in number from the control group. Group 1 was characterized by many cells expressing IFNγ. Groups 2 and 3 exhibited large numbers of cells expressing IL-5 and IL-10. The data obtained suggest that well-organized granulomas reflect a better cellular immune response, and the large number of cells expressing IL-5 and IL-10 in Group 2 indicate an ineffective response in PCM skin lesions. Both kinds of granuloma in the same cutaneous lesion probably represent an intermediate response between the anergic and hyperergic poles. Group 3 also showed higher numbers of cells expressing TNFα when compared with the control group. Some cells expressing TNFα were dendritic and localized around the granuloma similar to the factor XIIIa+ DD localization that we previously described.
Key Words: Skin—Dendritic cells—Cytokines—Paracoccidioidomycosis.

Paracoccidioidomycosis (PCM) is a human systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. It is confined to Latin America, with endemic areas in Brazil, Argentina, Colombia, and Venezuela. Primary lesions are thought to occur in the lungs. From these lesions, the fungi can disseminate via the bloodstream or lymphatics to any organ of the host, and the skin is involved in approximately 50% of cases (1). Cutaneous lesions constitute an important clinical element of the diagnosis because they represent an exteriorization of the disease.

The lesions of PCM are characterized by a granulomatous inflammatory response with formation of epithelioid granulomas with giant cells. The hyperergic pole of PCM is characterized by compact epithelioid granulomas, and the anergic pole is represented by a loose and parasite-rich granulomatous response. The skin lesions reflect a reduction in the number of Langerhans cells (LCs), resulting in an impairment in the local processing of antigens (2).

Dermal dendrocytes (DDs) constitute a group of dendritic cells with an antigen-presenting cell phenotype (3). These cells were identified by the expression of factor XIIIa (FXIIIa), a protransglutaminase involved in hemostasis, and classified as type I dendrocytes. Another dendritic cell described within the human dermis and identified by the expression of the human progenitor cell antigen CD34 was classified as type II (4–7).

Brandão et al. (8) detected more FXIIIa+ DDs in PCM oral lesions, suggesting that these cells might be related to granuloma formation. We recently described the presence of fungi inside FXIIIa+ DDs (9). These cells were hypertrophic and increased in number and were localized in the superficial and reticular dermis. The precise role of CD34+ DDs remains to be established. It seems that these cells might differentiate toward more differentiated cells such as CD1a+ LCs or FXIIIa+ DDs (10–12).

The cellular immune response mediated by CD4+ T cells producing a T helper (Th) 1 pattern of cytokines (interferon [IFN] and IL-2) represents the main resis-

tance mechanism against *P. brasiliensis*. Susceptibility is associated with the Th2 pattern of cytokine production (IL-4, IL-5, IL-6, and IL-10) (13–17). Tumor necrosis factor is a potent stimulant of leukocyte function and granuloma formation. In patients with chronic PCM, high serum levels of IL-10 and TNFα and low levels of IFNγ were related to progression of the disease (18,19).

In the current study, we investigated the correlation between skin dendritic cells (LCs and CD34+ DDs), the morphologic characteristics of the granulomatous response, and the expression of cytokines of the Th1 and Th2 patterns in the skin lesions of PCM.

MATERIALS AND METHODS

Sixty-one skin biopsies from patients with a clinical diagnosis of PCM confirmed by direct examination or fungal culture were selected from the files of the Dermatopathology Laboratory of the Department of Dermatology of the University of São Paulo Medical School according to the amount of tissue available to perform all the proposed immunohistochemical techniques. All specimens had been fixed in neutral buffered formalin and embedded in paraffin. They were classified according to tissue response (20,21) into well-organized granuloma (Group 1), poorly organized granuloma (Group 2), and both kinds of granuloma (Group 3). Ten biopsies from normal skin were used as controls.

Immunohistochemistry

Four-micrometer sections were dewaxed in xylene and hydrated through a graded series of ethanol. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and preincubation with trypsin (Sigma Chemical Company, St. Louis, MO) in 0.01 M of PBS, pH 7.4, was performed to enhance antigen exposure when necessary. The following primary antibodies were applied, and sections were incubated overnight at 4°C: rabbit anti-*P. brasiliensis* (kindly supplied by Prof. Carlos Lacaz, Tropical Medicine Institute, São Paulo Medical School) diluted 1:1,000, mouse anti-CD1a (Immunotech, Marseille, France), mouse anti-CD34 (Novocastra, Newcastle, UK) diluted 1:50, rabbit anti-TNFα diluted 1:100, goat anti-IFNγ diluted 1:250, and rabbit anti-IL-5 (Genzyme, Cambridge, MA) and mouse anti-IL-10 (R&D Systems, Minneapolis, MN) used at 1:50 and 1:10 dilutions, respectively. Biotinylated antirabbit and antimouse antibody was used at a dilution of 1:1,000 for 30 minutes at 37°C (DAKO Corporation, Carpinteria, CA). For the detection of IFNγ, biotinylated antigoat antibody was used at a dilution of 1:800 (DAKO Corporation). Peroxidase-labeled streptavidin-biotin complex (SABC; DAKO Corporation) was applied for 30 minutes at 37°C at a dilution of 1:1,000. Langerhans cells and cells expressing IL-5

were better visualized by using the catalyzed signal amplification (CSA) system (DAKO Corporation) based on the peroxidase-catalyzed deposition of a biotinylated phenolic compound followed by a secondary reaction with streptavidin peroxidase (22). As a chromogen, we used 3,3 diamino-benzidine tetrahydrochloride (Sigma Chemical Company), and the slides were counterstained with Harris hematoxylin.

Specimens of PCM lesions, normal skin, Kaposi sarcoma, and reactive lymph node were used as positive controls for the immunohistochemical detection of *P. brasiliensis* antigens, LCs, CD34+ DDs, and cells expressing cytokines, respectively. The negative controls were obtained by omitting the primary antibodies for each reaction, which were replaced by PBS.

Quantitative Analysis

Cells were quantified by counting the number of immunolabeled cells in 15 randomized high-power fields for each specimen with a ×10 ocular lens with a square grid and a ×40 objective. Epidermal LCs were evaluated by determining the epidermal area fraction with positivity for CD1a (23).

Statistical Analysis

The number of positive cells was statistically analyzed by the Graph Pad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA) using a Kruskal-Wallis test with the level of significance set at 95%.

RESULTS

Histopathologic Analysis

The histopathologic study revealed 22 biopsies characterized by well-organized granuloma (Group 1) (Fig. 1A), 20 characterized by poorly organized granuloma (Group 2) (see Fig. 1B), and 19 with both kinds of granuloma in the same biopsy (Group 3). The most frequent epidermal changes were hyperkeratosis, parakeratosis, acanthosis, and pseudocarcinomatous hyperplasia. Epidermal microabscesses were observed in 60% of the biopsies, some of them with fungi among the polymorphonuclear cells. Besides the granulomatous reaction, the dermis presented a perivascular inflammatory infiltrate composed predominantly of lymphocytes, plasma cells, and macrophages. Neutrophils were frequently observed, and eosinophils were present in almost 20% of the biopsies. Dermal necrosis and fibrosis were rarely seen. Vascular alterations were characterized by swelling of endothelial cells. Fungi were seen in the cytoplasm of macrophages and giant cells in all sections and were better demonstrated by anti-*P. brasiliensis* antibody.
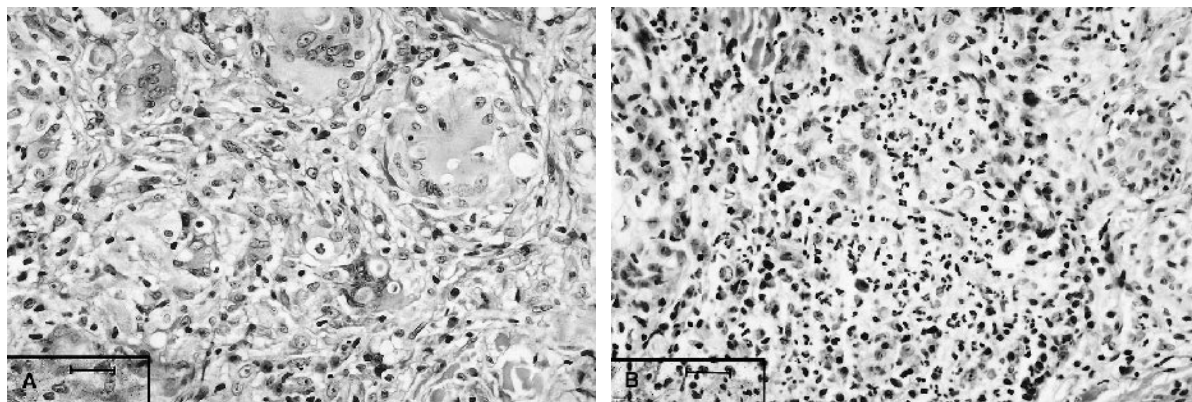
FIG. 1. Patterns of granulomatous tissue reaction in paracoccidioidomycosis. (**A**) Well-organized granuloma character-ized by compact clusters of epithelioid cells, giant cells, and mononuclear cells in a peripheral halo around the granulomas. (**B**) Poorly organized granuloma characterized by loose aggregates of epithelioid cells intermingled with mononuclear cells (hematoxylin–eosin).

Distribution of Dendritic Cells

Langerhans cells were distributed throughout the epidermis with short and irregular dendrites in Groups 1 and 2 (Fig. 2A). Dendrites were long and numerous in the control group (Fig. 2B). Quantitative analysis showed a statistically significant decrease in the number of cells immunolabeled with the anti-CD1a antibody in Groups 1 and 2 when compared with the control group ($P < 0.001$) (Fig. 3). CD34+ DDs were spindle shaped and distributed in the dermis, especially in the adventitia of sebaceous and sudoriparous glands. In the control group, these cells had the same characteristics as in PCM. Statistical analysis did not show differences between the groups in the number of CD34+ DDs.

Distribution of the Cytokines in the Skin Lesions

Cells expressing TNFα were distributed throughout the dermis in the inflammatory infiltrate. Interestingly,

some positive dendritic cells were localized around the granulomas similar to the distribution of FXIIIa+ DDs that we previously described (9). The quantitative analysis revealed a statistically significant increase in the number of cells expressing TNFα in Group 3 when compared with the control group (Fig. 4A). Cells expressing IFNγ were detected in the dermal inflammatory infiltrate of the three PCM groups. Group 1 was characterized by more of these cells when compared with Group 2 ($P < 0.001$) and the control group (Fig. 4B). Interleukin-5–stained lymphocytes were distributed in the dermal inflammatory infiltrate. A statistically significant increased number of positive cells were detected in Groups 2 and 3 when compared with the control group (Fig. 4C). Lymphocytes and some mast cells immunolabeled with the anti-IL-10 antibody were distributed in the dermis among the inflammatory infiltrate. More of these cells were present in Groups 2 and 3 when compared with the control group ($P < 0.01$) (Fig. 4D). On the whole, the
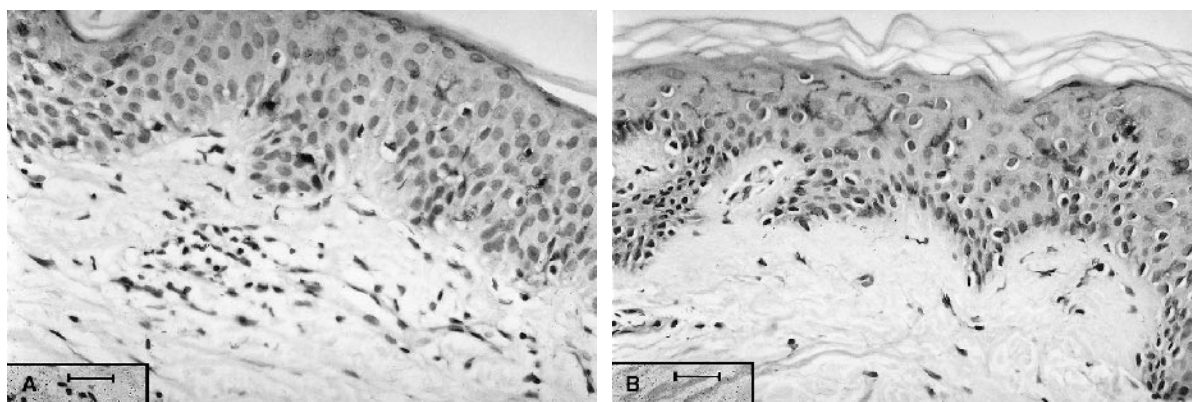

FIG. 2. Epidermal Langerhans cells immunostained by anti-CD1a antibody. (**A**) Paracoccidioidomycosis lesion with well-organized granuloma showing epidermal Langerhans cells with short and irregular dendrites. (**B**) Langerhans cells distributed throughout the epidermis with long and numerous dendrites in the control group (streptavidin-biotin complex immunohistochemical technique).
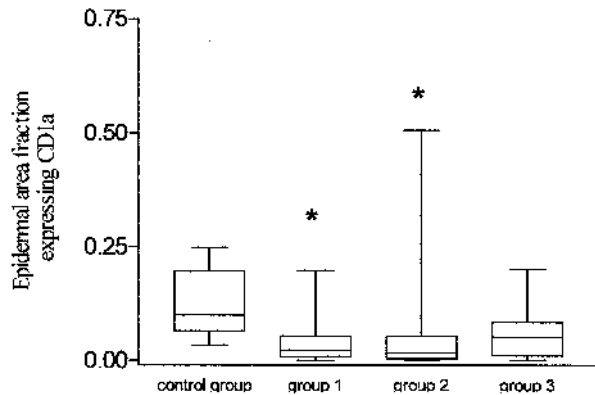
FIG. 3. Distribution of CD1a+ epidermal area fraction (Langerhans cells) in paracoccidioidomycosis lesions according to the type of granulomatous reaction and in the control group. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles, and whiskers show the highest and lowest values. *Statistically different from the control group (Kruskal-Wallis test).

morphology of cells expressing TNFα, IFNγ, IL-5, and IL-10 present in the control group was similar to that found in the PCM groups.

DISCUSSION

Cutaneous involvement in PCM is frequent. The infection begins in the lungs and reaches the skin via bloodstream dissemination of *P. brasiliensis.*

The specific cell populations that are involved in disease control in different organs have been identified so as to better understand the organ-specific immune response (24). The skin has many different immunologically active cells, and the most important are probably the dendritic cells (25).

Although it is possible to identify two well-defined poles in PCM in experimental animal models, there are a number of immune and histopathologic responses in human beings. The clinical form reflects a particular phase in a dynamic and polymorphic disease (26).

The reduction of LCs in diverse pathologic processes has been described (27–29). In our work, epidermal LCs (CD1a+) showed degenerative alterations and a reduced number in Groups 1 and 2 when compared with the control group ($P < 0.01$). This numeric reduction could be a result of factors produced by *P. brasiliensis*, or these cells may have migrated to the dermis, resulting in an immunophenotypic alteration in membrane expression of CD1a. Sandoval et al. (30) did not detect *P. brasiliensis* antigens in the cytoplasm of LCs, suggesting that they would not be capable of presenting fungal antigens efficiently. On the other hand, we could detect some yeast cells in the cytoplasm of FXIIIa+ DDs by double immunostaining (9). It was suggested that DDs might represent a possible dermal precursor of epidermal LCs (11). We

observed that Group 3 did not present numeric depletion of LCs and previously observed that FXIIIa+ DDs appeared hypertrophied and hyperplastic in this group (9). We suppose that FXIIIa+ DDs could be a reservoir for the repopulation of epidermal LCs in this PCM group.

CD34 antigen is present in human hematopoietic progenitor cells from bone marrow. Within the dermis, CD34 is expressed by endothelial cells, dendritic cells, and spindle-shaped cells around adnexal structures (31). Few works demonstrate the function of CD34+ DDs as well as their relation with other cutaneous dendritic cells. They do not express CD1a or S100 protein, are more frequent in the reticular and deep dermis, and only 2% express HLA-DR under normal conditions (10). It is suggested that these cells can differentiate into more specialized dendritic cells such as FXIIIa+ DDs or LCs after the stimulatory effects of TNFα and granulocyte macrophage colony-stimulating factor (GM-CSF) (10–12). We observed few CD34+ DDs in the superficial and reticular dermis, without differences between the groups. It was not possible to correlate this cell population with the other dendritic cells. Their role in skin tissue reaction to *P. brasiliensis* warrants further investigation.

Products from *P. brasiliensis* may stimulate macrophages to secrete TNFα, which is involved in granuloma development (16,21). We demonstrated the presence of dermal cells that expressed TNFα in the three groups of PCM. Some of these cells had a dendritic morphology and distribution in the periphery of granulomas similar to the distribution of FXIIIa+ DDs that we previously described (9). Group 3 showed the highest numbers of cells expressing TNFα and FXIIIa+ DDs, suggesting their participation as TNFα-producing cells following fungal stimulation.

Interferon-γ plays a pivotal role in host resistance against *P. brasiliensis* (17,32,33). Although we detected cells expressing IFNγ in the three PCM groups, Group 1 had more of these cells, correlating with a well-organized tissue response against *P. brasiliensis* with this cytokine.

Interleukin-5 is a Th2 type cytokine related to the maturation and differentiation of eosinophils (34,35). In experimental PCM, high levels of IL-5 and IL-10, eosinophilia, and low levels of IFNγ characterize the progressive disease in susceptible animals (32,36). We demonstrated more cells expressing IL-5 in Groups 2 and 3 similar to the results of cells expressing IL-10. These results suggested that poorly organized granulomas, which characterize Group 2, represent a worse response against *P. brasiliensis.*

It was interesting to observe that Group 3, the one with both kinds of granuloma formation, did not differ from Group 1 in the number of IFNγ-expressing cells. We could demonstrate that the number of FXIIIa+ DDs was increased in Group 3 of skin lesions (9) and so was the number of TNFα-expressing cells, some of them with
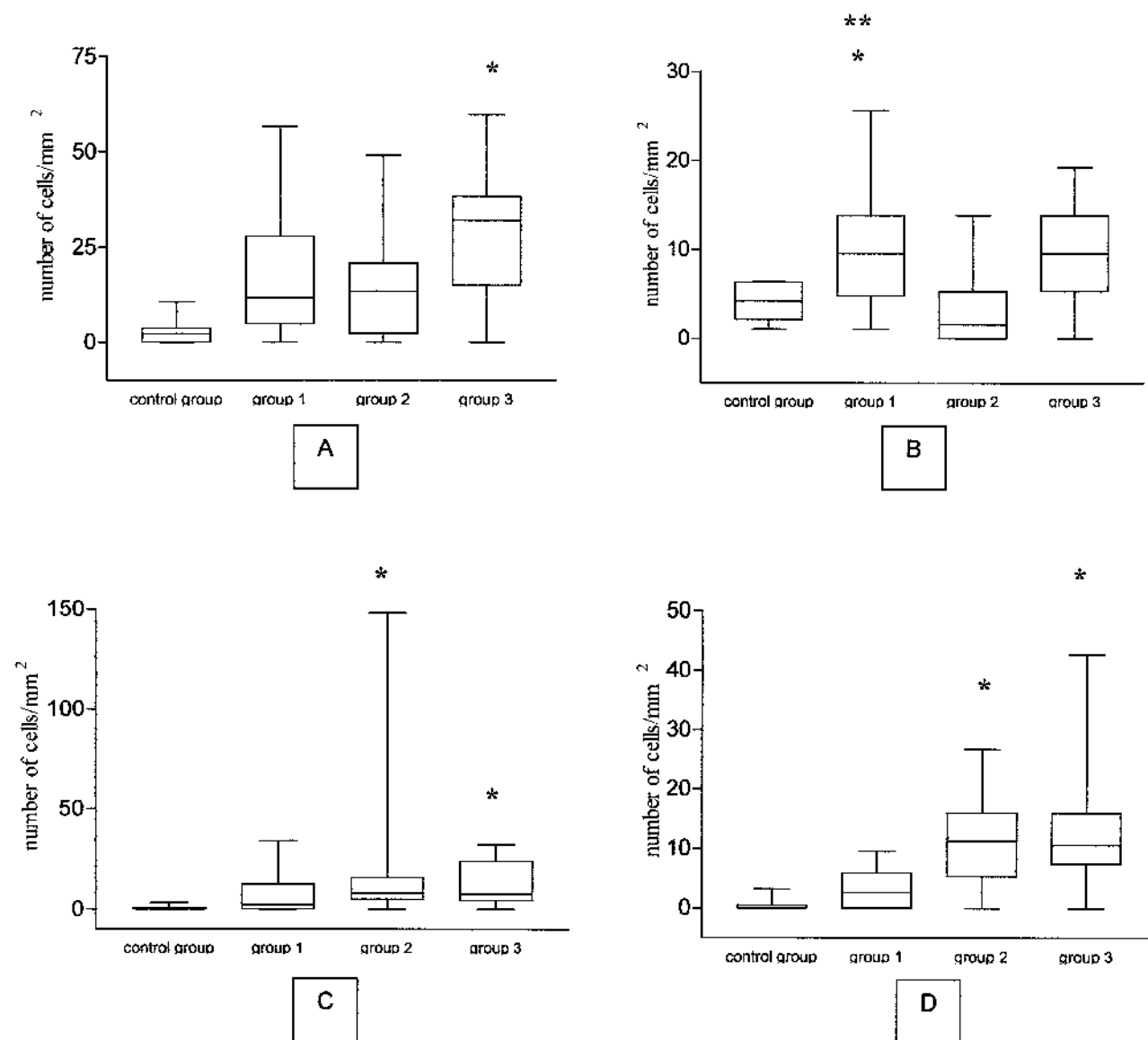
FIG 4. Distribution of the number of cytokine-expressing cells per square millimeter in paracoccidioidomycosis skin lesions according to the type of granulomatous reaction and control group. (**A**) Tumor necrosis factor-α–expressing cells. (**B**) Interferon-γ–expressing cells. (**C**) Interleukin-5–expressing cells. (**D**) Interleukin-10–expressing cells. Horizontal lines represent the median values, boxes represent the 25th to 75th percentiles, and whiskers show the highest and lowest values. *Statistically different from the control group (Kruskal-Wallis test). **Group 1 greater than Group 2 (Kruskal-Wallis test).

dendritic morphology. FXIIIa+ DDs have been related to Th1 type cytokine cutaneous processes (37). We suppose that these cells expressing TNFα in PCM skin lesions might be activated to improve the immune response against *P. brasiliensis*.

There are many factors that can regulate Th1/Th2 cell expression such as genetic background, the type of antigen-presenting cell interacting with T cells, and the cytokine microenvironment at the time of activation. Calich et al. (36) related the difference in the immune response to *P. brasiliensis* antigens to the interaction between the antigens and different populations of anti-

gen-presenting cells. Almeida and Lopes (38) studied dendritic cells in resistant and susceptible mice following inoculation with GP43 and demonstrated that the preferential activation of Th1 and Th2 responses, respectively, by those cells seems to be determined by the costimulatory microenvironment of cytokines in the early phases of infection. The presence of IFNγ would stimulate these cells to produce IL-12, inducing a Th1 response. On the other hand, IL-10, prostaglandin E, or IL-4 stimulates a Th2 response (39).

The presence of different populations of dendritic cells in the skin and their antigen-presenting capacity consti-

tute important factors in the immune response against *P. brasiliensis*. Understanding the correlation between these cells and cytokines could contribute to possible treatment targeting. The immunologic mechanisms that confer resistance to PCM are dependent on various factors, and dendritic cells seem to play a role.

Our results suggest the participation of LCs and FXIIIa+ DDs in the pathogenesis of the PCM cutaneous tissue response. These cells could act as antigen-presenting cells and/or fungal target cells. They would be related to the production and induction of the cytokines that modulate the immune response against the parasite.

The cutaneous tissue response in PCM correlates with the mechanisms that occur in other organs but with characteristic mechanisms that are related to its own immune system.   □

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