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Original Article

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Th17 and regulatory T cells contribute to the *in situ* immune response in skin lesions of Jorge Lobo's disease

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Abstract

Jorge Lobo's disease (JLD) is a chronic granulomatous mycosis described in various Latin American countries. The main objective of the present study was to investigate the possible role of Th17 and Foxp3+ Treg cells in the pathogenesis of Jorge Lobo's disease. Human skin biopsies were submitted to an immunohistochemistry protocol to detect Foxp3, interleukin (IL)-1beta, CD25, IL-6, IL-17, and IL-23. The epidermis presented acanthosis, hyperkeratosis, and frequent presence of fungi. The dermis presented inflammatory infiltrate comprising macrophages, lymphocytes, epithelioid and multinucleated cells, and an intense number of fungi. Foxp3+ Treg cells and IL-17+ cells were visualized in lymphocytes in the inflammatory infiltrate. IL-1, IL-2R (CD25), IL-6, and IL-23 were visualized in the dermis, intermingled with fungal cells, permeating or participating of the granuloma. Following IL-17, the most prominent cytokine was IL-6. IL-23 and cells expressing CD25 were present in fewer number. The comparative analysis between IL-17 and Foxp3 demonstrated a statistically significant increased number of IL-17+ cells. Th17 cells play a role in the immune response of JLD. IL-1beta and IL-6 added to the previously described increased number of TGF-beta would stimulate such pattern of response. Th17 cells could be present as an effort to modulate the local immune response; however, high levels of a Th17 profile could overcome the role of Treg cells. The unbalance between Treg/Th17 cells seems to corroborate with the less effective immune response against the fungus.

Key words: Jorge Lobo's Disease, immune response, Th17 cells, regulatory T cells.

Introduction

Jorge Lobo's disease (JLD) is a chronic granulomatous mycosis caused by the pathogenic fungus *Lacazia loboi*. It is usually characterized by lesions that predominate on the ears and upper and lower limbs. The disease is described in various Latin American countries, with great importance in Brazil's Amazon region and predominates in males of rural activity [1–5].

Lacazia loboi is unicellular, yeast type and is found in lesions either alone or in chains of cells connected resembling a "pearl necklace" [6].

Clinically there are differences related to the time of evolution, as well as extension of the lesions [7]. The classic clinical aspect is the keloid-like type, with isolated or confluent lesions that can form multilobular aggregates, probably related to the high expression of transforming growth factor (TGF)-beta in the lesions [8–10].

TGF-beta is an important cytokine involved in healing processes, and it also plays a role in the suppression of CD8+ T cells and differentiation of naïve T cells in Foxp3+ regulatory or Th17+ cells, depending on the stimulus of pro-inflammatory cytokines [11–15].

Studies focusing on the cell-mediated immune response in JLD demonstrate differences according to the type of lesion and clinical presentation [5,16,17]. The dermal immune response is mainly characterized by Langerhans cells, Factor XIIIa+ dermal dendrocytes, macrophages, T lymphocytes and the cytokines IL-1-beta, tumor necrosis factor (TNF)-alpha, TGF-beta, IL-10, and IFN-gamma.

The Th17 and regulatory T cells have been studied in other cutaneous mycosis that share some characteristics with JLD, for example, chromoblastomycosis and paracoccidioidomycosis and their role in both immunopathogenesis have been speculated [18,19].

We intended to contribute to the characterization of the *in situ* immune response in cutaneous lesions in Jorge Lobo's Disease, focusing on Th17 and regulatory T cells and exploring their role in the pathogenesis of this disease.

Materials and Methods

Biopsies: Forty-one skin biopsies of lower limbs from 41 patients were selected from the files of the Nucleo de Medicina Tropical, Universidade Federal do Para, Belem, PA, Brazil. The diagnosis was based on the clinical presentation, direct mycological examination, and histological analysis according to the report of each sample. Samples from patients coinfected with human immunodeficiency virus (HIV) and patients receiving any type of treatment at the time of the biopsy were excluded of the casuistic. Ten biopsies from normal skin were used as control. The mean age of patients was 52 years old, 92% male.

Immunohistochemistry: Following deparaffinization in xylene and hydration in ethanol, antigen recovery was performed in a TRIS/EDTA solution pH9.0 for 20 minutes at 95°C. The primary antibody anti-Foxp3 (EBioscience, San Diego, CA, USA) was applied at a 1:50 dilution; anti-IL-1beta (RD Systems, Minneapolis, MN, USA), anti-CD25 (Novocastra, Newcastle, UK) anti-IL-6 (Novocastra, Newcastle, UK) at a 1:20 dilution; anti-IL-17 (RD Systems, Minneapolis, MN, USA) was applied at a 1:10 dilution and anti-IL-23 (BioLegend, London, UK) at a 1:50 dilution. Following an over-night incubation at 4°C, it was applied the second antibody and a Streptavidin-Biotin peroxidase system (LSAB, Dako, Carpinteria, CA, USA). 3, 3-diaminobenzidine tetrahydroxychloride was used as chromogen and the slides were counterstained with hematoxylin. All reactions were performed with positive and negative controls. The second ones were constituted by the use of isotype controls and the omission of the primary antibody.

In order to verify if cells expressing Foxp3 were CD4+ T cells, we performed a double immunostaining using the above protocol to detect Foxp3 followed by an immunohistochemistry with phosphatase and a blue chromogen to detect CD4+ T cells.

Immunolabeled cells were quantified in nine randomized fields in the dermis using a ×10 ocular lens with a square grid and a ×40 objective. The sum of the number of immunostained cells was divided by the number of fields (nine) and the result was divided by 0.0625 (area of the grid), providing the number of cells/mm^2.

Statistical analysis

The number of positive cells was statistically analyzed by using the Mann–Whitney test with the level for significance set at 95% ($P \leq .05$).

Results

The lesions were characterized by an inflammatory infiltrate, nodular and diffuse, comprising macrophages, rare asteroid corpuscles, lymphocytes, epithelioid cells, high number of multinucleated cells of Langhans and foreign

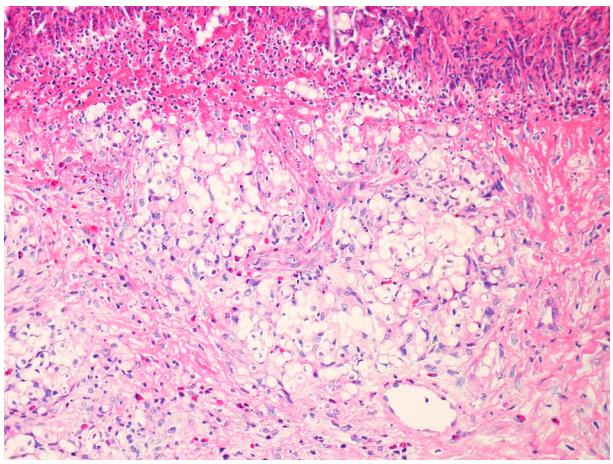

Figure 1. Jorge Lobo's disease. Histopathology aspect of lesion, characterized by inflammatory infiltrate with macrophages, lymphocytes, some eosinophils, multinucleated giant cells and high number of fungal cell and fibrosis. Note neutrophils and fungal cells infiltrating the epidermis. (Hematoxylin-Eosin, magnification ×200)

body types, with an intense number of fungal cells, in the epidermis and in granuloma or macrophages in the dermis (Figure 1).

The nuclear expression of Foxp3 was visualized in lymphocytes in all the specimens of JLD, distributed in the inflammatory infiltrate. Also, cells expressing IL-17 were detected in all specimens of lesions, in lymphocytes and neutrophils.

The control group did not present Foxp3+ cells, but a small number of cells expressing IL-17 were observed.

The double immunostaining enabled the visualization and confirmation that the predominating cells that were Foxp3+ also expressed CD4.

Cells expressing the cytokines IL-1, IL-2R (CD25), IL-6, and IL-23 were visualized in the dermis, intermingled with fungal cells, permeating or participating of the granulomatous formation. Figure 2A-G presents the immunohistochemistry expression of cells and cytokines studied.

The quantitative analysis of immunolabeled cells evidenced that, following IL-17, the most prominent cytokine was IL-6. The cytokine IL-23 and cells expressing CD25 were present in less number. Figure 2H presents the mean values for each molecule evaluated, considering nine randomized fields.

The comparative analysis between cells expressing IL-17 and Foxp3 in the group of lesions demonstrated a statistically significant increased number of IL-17+ cells when compared to Foxp3+ cells ($P = .034$). The mean values and standard deviation of each element studied both in JLD lesions and control group of normal skin are described in Table 1.

Table 1. Mean values and standard deviation of the cytokines and cells studied both in Jorge Lobo's Disease and control group of normal skin.

	Mean ± SD – JLD	Mean ± SD – Normal skin
Foxp3	61.36 ± 42.67	0.00
IL-1 beta	15.93 ± 49.73	0.87 ± 1.35
CD25	13.44 ± 17.67	0.88 ± 0.96
IL-6	57.73 ± 50.91	7.46 ± 15.72
IL-17	114.5 ± 93.40	36.44 ± 36.50
IL-23	13.63 ± 9.95	37.92 ± 53.07

Discussion

The mean age that characterized the present casuistic was 52 years old with a predominance of males (92%). The biopsies were obtained from patients of the Amazon region in Brazil, in which rubber gatherers, gold prospectors, and rural workers are frequent activities. This is in accordance with previous works that related such activities to the predominance of the disease [20]. Similarly, in a previous study of 249 patients, the mean age was 53 years old. However, in "Caiabi" Brazilian Indians, the disease is very frequent among women and predominates in young people up to 20 years old, suggesting an earlier infection when compared to other population [21].

The histopathologic findings were constituted by an intense histiocytic infiltrate of the dermis, with a prominent number of parasites, similar to other fungal infections such as chromoblastomycosis and paracoccidioidomycosis. The epidermis presented areas of acanthosis, hyperkeratosis, spongiosis, and frequent presence of neutrophil and fungus, revealing their capacity of transepidermal elimination.

In the last few years, many studies about the characterization of the *in situ* immune response in cutaneous lesions of patients with Jorge Lobo's disease have been performed. The classification of lesions is frequently done according to the distribution: local or isolated and disseminated forms [20].

It is speculated that the genesis of symptoms of JLD is related to the host defective cellular immune response [17,18]. The lesions begin as papules and can result in plaques or nodules. Also, they can present a verrucous, gummatous, ulcerated, or infiltrative aspect [22].

It was demonstrated that the cell-mediated immune response and the pattern of cytokines is different according to the clinical presentation of lesions and can vary from a Th1 profile, intermediate and Th2 pattern [17].

Some elements of the local immune response were suggested as modulating this infection, such as the Langerhans cells, which could help the fungus to escape the immune system [23]. The Th2 pattern of cytokines, mainly IL-4 and

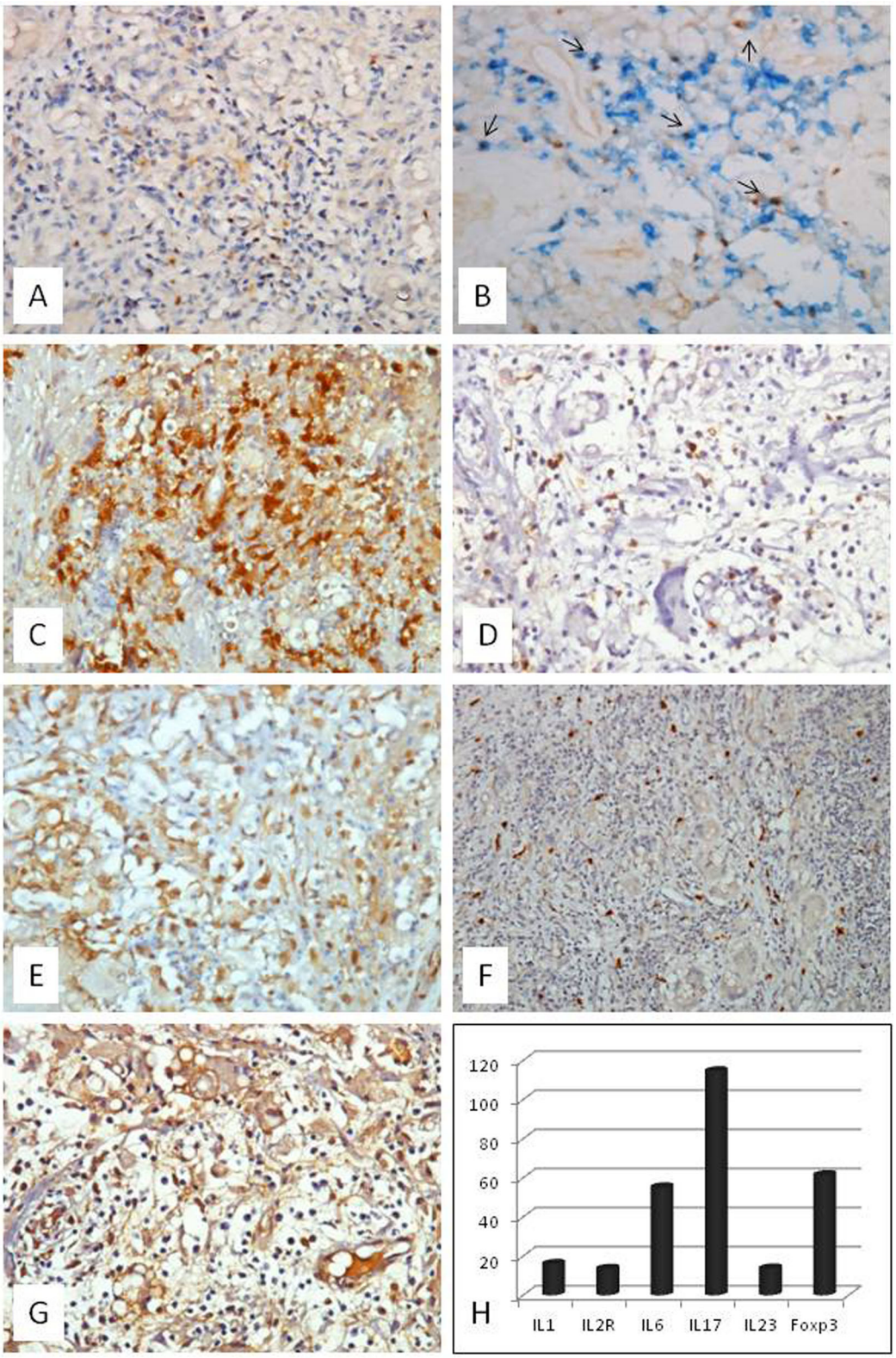

Figure 2. Jorge Lobo's disease. Immunohistochemistry characterization of inflammatory infiltrate. **A)** Foxp3+ regulatory T cells intermingled with fungal cells. **B)** Double stain evidencing the colocalization (arrows) of CD4+ T cells (in blue) and Foxp3 nuclear expression (in brown). **C)** High number of cells expressing IL-1beta and granulomatous area with intense expression of this cytokine. **D)** Expression of IL-2R (CD25). **E)** Expression of IL-6. **F)** Cells expressing IL-17 in superficial and reticular dermis. **G)** Expression of IL-23 in mononuclear and giant cells. (Streptavidin-Biotin peroxidase, ×400). **H)** Quantification of cells expressing cytokines and Foxp3 in the dermis considering nine randomized fields. Values expressed as number of cells/mm^2.

IL-6, and also the presence of melanin in the fungus wall could also collaborate to a noneffective response, keeping the infection. It is noteworthy that fungal viability indices range from 20% to 50% [4].

In this study, we intended to explore the participation of both Treg and Th17 cells in JLD and could observe the high number of cells expressing IL-6, a proinflammatory cytokine, expressive number of IL-17 over Foxp3+ cells and the presence of IL-23. Previously, it was described that high numbers of cells expressed TGF-beta in skin lesions of JLD [8]. In this context of cytokines, IL-10 is also present and acts with TGF-beta by inhibiting the effective cellular immune response [10].

The Th17 cells are characterized by the production of IL-17 and are involved in processes in which the immune profiles of Th1 and Th2 cells are not effective in the host defense against intracellular pathogens. They have been considered in many pathologic processes such as fungal infections [18,19,24,25]. This group of cells plays an inflammatory role and seems to be associated with the chronic inflammatory response and fungal persistence in candidiasis and aspergillosis, with defective fungal clearance.

The induction of a Th17 response is mediated by IL-23, which promotes the expression of IL-17 in T cells [26]. IL-23, on the other hand, shares the p40 subunit to IL-12, a Th1-related cytokine [27]. Also, TGF-beta, IL-1beta and IL-6 constitute an important group of cytokines that induces the activation of Th17 cells [28,29]. The high concentration of TGF-beta is also crucial for the development of regulatory T cells, in the presence of low expression of proinflammatory cytokines.

Our results allow us to conclude that Th17 cells participate and seem to play a role in the immune response of Jorge Lobo disease in human skin lesions. The presence of the proinflammatory cytokines IL-1beta and IL-6 added to the previously described increased number of cells expressing TGF-beta would stimulate such pattern of response. Some works have also demonstrated that TGF-beta can play a role in the genesis of the intense collagen tissue observed in injuries [8].

The Th17 pattern of cytokines could be presented as an effort to modulate the local immune response; however, it is also important to consider that the effects of Th17 immunity can also contribute to pathology with detrimental effects [30], which is still a controversial field, as we recently verified in Chromoblastomycosis, another important human fungal infection that shares some characteristics with JLD [21].

Similar to that, in JLD we could also observe the presence of Foxp3+ T cells, but it seems to have an imbalance with respect to the Th17 response. The Foxp3+ T regulatory cells are important inducer of an appropriate environment for the development and chronicity of the lesion, since such cells control negatively the immune response.

The characterization of a local immune response with high expression of IL-17 could be at least in part, an attempt to help the immune system against this fungal infection. However, high levels of a Th17 profile could overcome the role of Treg cells. The unbalance between Treg/Th17 cells seems to corroborate with the least effective immune response against the pathogenic fungus *Lacazia loboi*.

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Declaration of interest

None of the authors have any potential conflict of interest or financial support in the subject matter.

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