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Original Article

Original Article

Differential production of interleukin-1 family cytokines (IL-1β, IL-18, IL-33 and IL-37) in patients with paracoccidioidomycosis: correlation with clinical form and antifungal therapy

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Abstract

Besides interleukin (IL)-1β and IL-18, the newly described cytokines of IL-1 family IL-33 and IL-37 can contribute to the differentiation and maintenance of different population of T cells. IL-33 acts as an allarmin and promotes a predominant Th2 inflammatory response, whereas IL-37 plays an important role as an antagonist of inflammation. In paracoccidioidomycosis (PCM), caused by the dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*, it has been shown that the acquired immune responses are associated with the diverse clinical manifestations. The severe and disseminated forms (acute form [AF] and multifocal chronic form [CF-MF]) are characterized by high Th2 cytokines and antibody production, impaired cellular immune response, and eosinophilia. In contrast, in the localized form (unifocal chronic form [CF-UF]), the cellular immune response is preserved, with high production of Th1 and Th17 cytokines, and low antibody titers. This study aimed to quantify interleukin-1 family cytokines (IL-1β, IL-18, IL-37, IL-33, and the soluble IL-33 receptor sST2) in sera of patients presenting different clinical forms of PCM before, during, and after antifungal treatment, as well as to analyze the expression of these cytokines in lesions of PCM patients. We found that AF patients presented high serum levels of IL-1β, IL-18, IL-33, sST2, and IL-37, and that these cytokines are strongly expressed

in lymph nodes lesions. Furthermore, antifungal therapy resulted in the diminution of circulating cytokines and sST2 levels in all groups of patients. These results indicate that, besides IL-1β and IL-18, IL-33, IL-37, and sST2 can be associated with the disease activity and severity.

Key words: *Paracoccidioides brasiliensis*, IL-1beta, IL-18, IL-33, IL-37.

Introduction

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungi *Paracoccidioides brasiliensis* and *P. lutzii*, endemic in Latin America with high incidence in Brazil, Colombia and Venezuela.[1,2] Typically, PCM affects immunocompetent individuals, which develop a wide range of symptoms, resulting in two main clinical forms: the acute form (AF) and the chronic form (CF).[3] The CF can be subgrouped according to severity and number of organs/systems affected in: chronic unifocal form (CF-UF), usually less severe, with isolated pulmonary or epithelial involvement (skin or mucosa); and the chronic multifocal form (CF-MF) characterized by fungal dissemination and lesions in multiple organs (mainly lungs, epithelia and lymph nodes). In contrast, the AF usually is more severe, with a rapid progression and fungal dissemination affecting principally the mononuclear-phagocytic system (lymph nodes, liver, spleen, and bone marrow), with low pulmonary involvement.[3–5]

Besides distinct clinical manifestations, several studies have shown that the clinical forms of PCM can be characterized by differences in the acquired immune responses developed after infection. The most severe forms, particularly AF but also some CF-MF patients, are characterized by impaired cellular immune responses, with a predominant Th2/suppressive profile of cytokine production (high production of IL-4, IL-5 and suppressor cytokines, such as IL-10 and transforming growth factor β [TGF-β], and low production of interferon γ [IFN-γ] and tumor necrosis factor α [TNF-α]).[6–9] These forms are associated with high production of specific antibodies (IgG4, IgE, and IgA) and an elevated number of circulating eosinophils (eosinophilia).[7, 10–12] On the other hand, mild and localized forms (CF-UF) are characterized by the production of Th1 and Th17 cytokines, such as IFN-γ, TNF-α, IL-17A, and IL-22,[6, 13] besides a reduced production of specific antibodies (mainly IgG1 and IgG2).[10, 11] Therefore, this evidence, in addition to similar results described in experimental models of the disease,[14, 15] suggest that an immune response with the presence of Th1 and Th17 cells can contribute to resistance and control of the infection, while a Th2 response can be associated with disease susceptibility.[16]

In addition to the well-established conditions necessary for TCD4$^+$ cells differentiation (IL-12p70 for Th1, IL-4 for Th2, and IL-6 plus TGF-β for Th17),[17] several studies indicate that some cytokines of the IL-1 family can also contribute to the development of the acquired immune response. In this context, IL-1beta (IL1F2) is involved in the development and maintenance of Th17 cells;[18] IL-18 (IL1F4) acts synergistically with IL-12p70 in the development of Th1[19] or in the absence of IL-12 in the differentiation of Th2 cells;[20–22] and IL-33 (IL1F11) is associated with Th2 responses.[23] Furthermore, a more recently described member of this family, IL-37 (IL1F7), can modulate the acquired immune response through its suppressive effects on Th1 and Th17 differentiation.[24]

Few studies have addressed the relation between PCM clinical forms and the production of cytokines of IL-1 family. Silva et al. (1995) demonstrated high levels of IL-1β in the circulation of PCM patients, particularly those presenting the severe forms of the disease.[25] In the experimental model of PCM, IL-18 was associated with the development of a strong inflammatory reaction, related to susceptibility and persistence of *P. brasiliensis* infection.[26] In human PCM, elevated levels of IL-18 in peripheral circulation was associated with severe forms of the disease.[27] However, to the best of our knowledge, there are no studies investigating the role of IL-33 and IL-37 in PCM.

IL-33 is constitutively produced and stored as a nuclear protein in some cells, such as epithelial and endothelial cells, presenting an important function as an allarmin (or damage-associated molecular pattern) after cell death that occurs in inflammatory conditions.[28] In addition, under some conditions, production of IL-33 can be induced in haematopoietic cells promoting Th2 inflammatory reactions,[29] activating mast cells, and inducing eosinophils production independently of IL-5.[30,31] On the contrary of other cytokines of IL-1 family, full-length IL-33 is biologically active, although enzymatic processing after release augments its biological activity.[28] IL-33 acts via a receptor called ST2 expressed in cells surfaces or found in a soluble form (sST2) that acts as a decoy receptor for this cytokine.[28] High serum or plasma levels of IL-33 and sST2 were associated with the development of several diseases, varying from

allergic to autoinflammatory, autoimmune and cardiac diseases.[32–37]

IL-37 (IL1F7) is the most recent described member of the IL-1 family of cytokines.[24] Some studies have shown that the expression of IL-37 in macrophages suppressed the production of pro-inflammatory cytokines, down-regulating the inflammatory process.[38]

In this study, we aimed to quantify serum levels of interleukin-1 family cytokines such as IL-1β, IL-18, IL-37, and IL-33 and its soluble receptor ST2 before, during, and after antifungal therapy in PCM patients with different clinical forms of the disease. Moreover, we also intended to evaluate the expression of these cytokines in oral mucosa and lymph node lesions of PCM patients.

Methods

Casuistic and subjects

We included sera samples and biopsies specimens from patients with proven PCM attended at the Clinical Hospital of the University of Campinas. Sera were obtained for diagnosis and/or treatment monitoring, aliquoted and stored at −20°C until use. The PCM diagnosis was confirmed by histopathological examination of biopsies or direct observation of the fungus in biological samples (skin/mucosal scrapings, sputum, or lymph nodes exudates). Sera of patients having other concomitant inflammatory or infectious diseases or neoplasm were not included. PCM patients were classified according to parameters established previously[3, 5] in: chronic form with unifocal (CF-UF - $n = 49$) or multifocal involvement (CF-MF - $n = 39$), and acute form (AF - $n = 47$) of the disease. We also included sera from 24 healthy individuals (controls), without symptoms of active inflammatory or infectious diseases and without taking anti-inflammatory drugs 30 days before blood draw. Sera from PCM patients were collected before (S1), during the antifungal treatment (S2), and after the establishment of clinical cure (S3). Time intervals between S1 and S2 were of 4.0 ± 2.2 months and between S1 and S3 were of 16.0 ± 7.5 months. From medical records, we retrieved the absolute and the relative (percentage) number of eosinophils in complete blood counts (CBC) performed in blood samples collected on the same day of the first serum (S1) used for the quantification of cytokines. Biopsies specimens were collected from 10 PCM patients for diagnosis. Five subjects with the CF (oral mucosa lesions) and five with the AF (lymph nodes) of the disease.

This study was approved by the Human Research Ethics Committee of the Faculty of Medical Sciences of University of Campinas (protocol number 140.788). The need to obtain informed consent was waived owing to the retrospective design of the study.

Immunohistochemistry

Paraffin-embedded tissues were submitted to immunohistochemistry reactions performed with monoclonal antibodies against IL-1β, IL-18 (Santa Cruz Biotechnology, Dallas, TX, USA), IL-33 (GeneTex, Inc., Irvine, CA, USA), or IL-37 (Sigma-Aldrich, St. Louis, MO, USA) with the aid of the amplification system NovoLink (Max Polymer Detection System - Novocastra Laboratories, Buffalo Grove, IL, USA). Negative control samples were processed similarly omitting primary antibodies. For quantitative analysis, high-resolution images (3136 × 2352 pixels) of three different fields (at 400 × magnification) were acquired from each biopsy specimen in a light microscope (Nikon Eclipse 50i) equipped with a CCD camera (Nikon Digital Sight DS-Ri1). The images were analyzed using GSA Image Analyser Software (GSA Bansemer and Scheel GbR, Rostock, Germany). As cytokine staining usually shows a diffuse pattern, the area of positive reaction (brown staining) was selected by color sampling. The results were expressed as the mean ± standard error of the mean (SEM) of the positive area in mm^2 (each field has a total surface area of approximately 1.6 mm^2).

ELISA for quantification of cytokines and sST2

IL-1β, IL-18, IL-33, IL-37, and sST2 levels in sera were measured by ELISA according to the manufacturer's protocol (IL-1β, IL-33, IL-37, and sST2, R&D Systems, Minneapolis, MN, USA, and IL-18 - MBL International, Woburn, MA, USA).

Statistical analysis

Statistical analysis was carried out with GraphPad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA). To compare parameters in each group we used the nonparametric Kruskal-Wallis test, followed by Dunn's post-test or the Student *t*-test. To compare parameters variation during treatment we used Friedman test with Dunn's post-test. *P* values ≤ .05 were considered statistically significant. For correlation analysis, Spearman correlation was applied; the α level was set to 0.01. Correlation was graded as weak ($r < 0.30$), moderate ($r = 0.30$ to 0.69) or strong ($r > 0.70$) according to Cohen.[39]

Results

In this study, we analyzed sera of 129 patients diagnosed with PCM, before, during, and after antifungal treatment. Analysis of medical records allowed their classification in three major groups: AF patients with lymph nodes, liver,

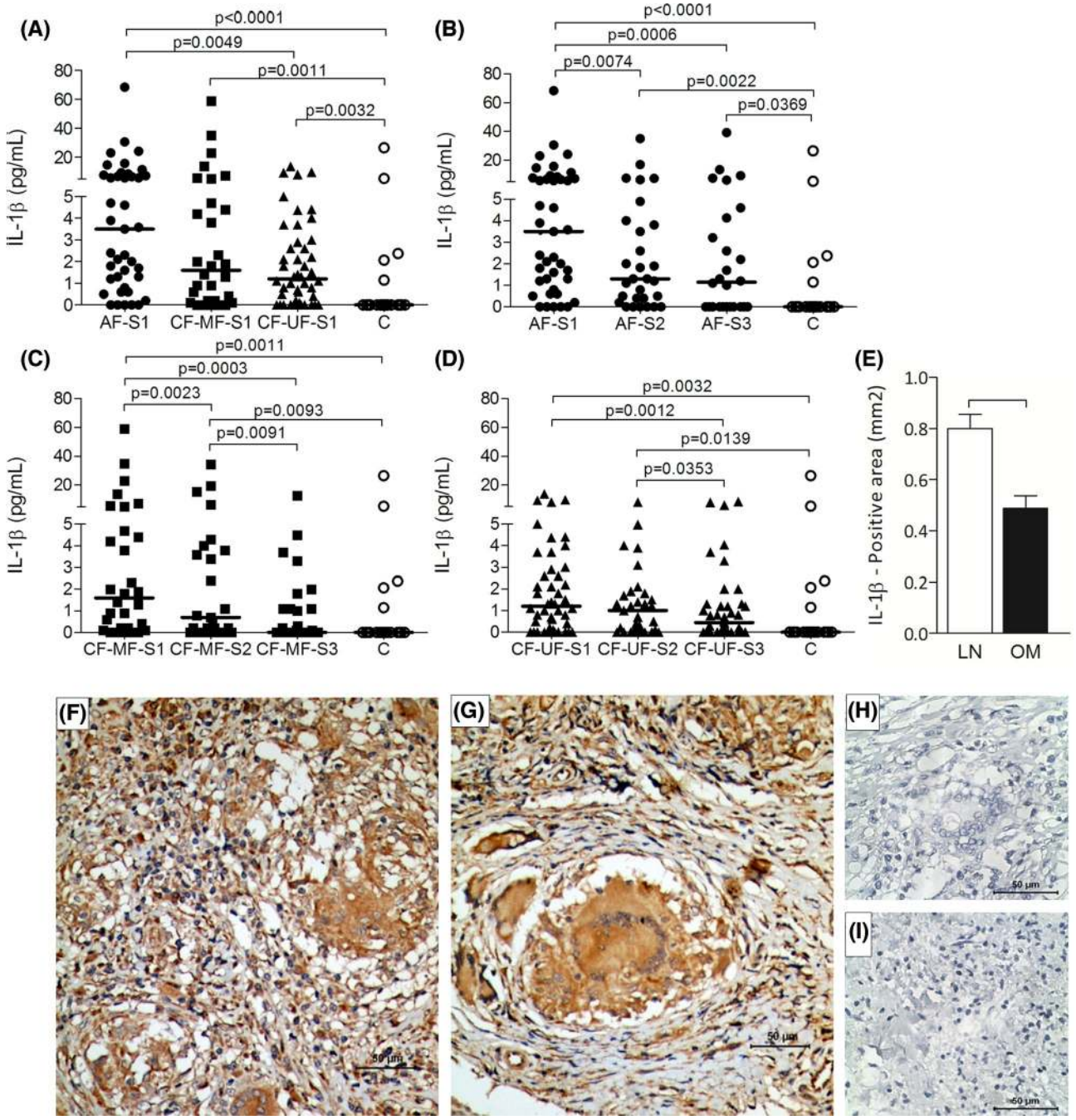

Figure 1. Evaluation of IL-1β in sera samples by ELISA and in lesions by immunohistochemistry. (A) Quantification by ELISA of IL-1β in sera samples of PCM patients with the Acute Form (AF), Chronic Form Multifocal (CF-MF) or Chronic Form Unifocal (CF-UF) collected before antifungal treatment (S1) and healthy controls (C). Horizontal bars represent the median. Statistical analysis: Kruskal-Wallis, *P* values are depicted above brackets. (B–D) Comparison among IL-1β levels in sera of AF (B), CF-MF (C), and CF-UF (D) patients collected before (S1), during (S2), or after (S3) antifungal treatment, and in sera of healthy individuals (controls). Horizontal bars represent the median. Statistical analysis: Comparison among S1, S2, and S3 - Friedman with Dunn's post-test. Comparison between sera S1, S2, or S3 with sera of controls -Kruskal-Wallis. *P* values are depicted above brackets. (E) Quantitative analysis of the immunohistochemistry reactions for IL-1β in lymph node (LN) and oral mucosa (OM) lesions. Results expressed as mean±SEM of positive areas for IL-1β. Statistical analysis - Unpaired *t* test. Bracket represents $P < .05$. (F-I) Representative results of the immunohistochemistry analysis for IL-1β in lymph node (F) and oral mucosa (G) lesions of PCM patients. Scale bars, 50 μm. (H–I) Negative controls (H, lymph node, I, oral mucosa). Scale bars, 50 μm.

Table 1. Epidemiological characteristics and number of blood eosinophils of PCM patients and controls.

	AF ($n = 47$)	CF-MF ($n = 39$)	CF-UF ($n = 49$)	Control ($n = 24$)
Median age (range) (years)	19* (5–48)	46 (32–75)	52 (28–79)	28 (18–72)
Male/Female (ratio)	26/21* (1.2/1)	36/3 (12/1)	46/3 (15.3/1)	14/10* (1.4/1)
Blood Eosinophils (x$10^3/\mu$L) Median (range)	1.1* (0.0–12.9)	0.5 (0.0–1.9)	0.3 (0.1–2.3)	ND
Blood eosinophils (%) Median (range)	8.0* (0.0–61.0)	5.7 (0.0–23.8)	4.2 (0.9–32.6)	ND

AF, acute form; CF-MF, chronic form multifocal; CF-UF, chronic form unifocal; ND, not determined.
*$P < .05$ in relation to CF-MF and CF-UF groups.

spleen, and bone marrow involvement, and CF patients divided into two groups: multifocal (CF-MF) with lung, mucosal and/or skin lesions; and unifocal (CF-UF) with isolated pulmonary lesions or skin/mucosal involvement (data not shown). Both groups of CF patients were predominantly males (ratio male/female:12-15.3/1) and older than AF patients (Table 1). The analysis of the absolute and the relative number of circulating eosinophils showed that the majority of AF patients had values above the normal range (Supplementary Fig. 1). Furthermore, relative and absolute number of eosinophils were statistically higher in blood from AF group compared to CF-MF and CF-UF patients (Table 1 and Supplementary Fig. 1).

Our data showed that, independently of the clinical form, levels of IL-1β were higher in sera from PCM patients than in sera the from control group (Fig. 1A). Moreover, sera from AF patients group before the treatment (S1) have higher amounts of IL-1β (median: 3.5 pg/ml; range: 0.0–68.5 pg/ml) than sera from CF-UF patients (median: 1.2 pg/ml; range: 0.0–13.7 pg/ml) and controls (median: 0.0 pg/ml; range: 0.0–26.3 pg/ml) (Fig. 1A). In relation to antifungal therapy, all groups showed a diminution of IL-1β (Figs. 1B–1D and Supplementary Table 1), during (S2), and after treatment (S3). However, though the levels of this cytokine after treatment (S3) in sera of CF-MF (median: 0.0 pg/ml; range: 0.0–12.7 pg/ml) and the CF-UF (median: 0.0 pg/ml; range: 0.0–25.6 pg/ml) were similar to controls (median: 0.0 pg/ml; range: 0.0–26.3 pg/ml), IL-β in sera from AF patients remained higher than those observed in control group (median: 1.20 pg/ml; range: 0.0–26.6 pg/ml) (Fig. 1B and Supplementary Table 1). Immunohistochemistry analysis showed expression of IL-1β in both lymph nodes and oral mucosa lesions of PCM patients, mainly in giant cells (Figs. 1F and 1G, respectively). Moreover, the area stained for IL-1β was greater in lymph nodes compared to oral mucosa (Fig. 1E).

We next examined IL-18 levels in the same groups of patients. We found higher levels of IL-18 in the disseminated forms of PCM (AF - median: 113.0 pg/ml; range: 6.3–1357.0 pg/ml and CF-MF - median: 84.1 pg/ml; range: 2.4–502.4 pg/ml) than in sera from CF-UF (median: 41.7 pg/ml; range: 8.1–230.9 pg/ml) and healthy controls (median: 52.0 pg/ml; range: 17.9–150.6 pg/ml) (Fig. 2A). There were no differences between the levels of IL-18 in sera from CF-UF patients and sera from healthy controls (Fig. 2A). Moreover, antifungal treatment resulted in decreased levels of IL-18 in all groups, including the CF-UF (Figs. 2B–2D and Supplementary Table 1). Biopsies analysis showed IL-18 staining in giant cells (lymph nodes; Fig. 2F) and macrophages surrounding the granulomas (oral mucosa; Fig. 2G). Quantitative analysis showed a higher IL-18 expression in lymph nodes compared with oral mucosa (Fig. 2E).

We also evaluated IL-33 levels in PCM patient's sera and lesions. Our results showed elevated levels of this cytokine only in sera from AF patients (median: 554.5 pg/ml; range: 6.4–3308.0 pg/ml), when compared to the other groups (CF-MF - median: 117.4 pg/ml, range: 0.0–6747.0 pg/ml; CF-UF - median: 56.7 pg/ml, range: 0.0–1256.0 pg/ml; Control - median: 45.9 pg/ml, range: 34.7–324.1 pg/ml) (Fig. 3A). As for the aforementioned cytokines, antifungal treatment resulted in significant decrease of IL-33 (Fig. 3B and Supplementary Table 1). In *P. brasiliensis* infected tissues, IL-33 was mostly found in giant cells in lymph nodes (Fig. 3F) and in both, giant cells and in isolated cells in oral mucosa (Fig. 3G). Moreover, labeled areas for IL-33 were larger in lymph nodes when compared to oral mucosa (Fig. 3E). We also found a moderate correlation between IL-33 levels and the relative/absolute number of eosinophils (Table 2).

Differently of IL-33, the evaluation of IL-33 soluble receptor (sST2) showed increased levels in patients of all groups (AF - median: 532.2 pg/ml, range: 63.3—4168.0 pg/ml; CF-MF - median: 198.1 pg/ml, range: 69.1–3365.0 pg/ml; CF-UF - median: 186.0 pg/ml, range: 25.1–608.1 pg/ml) in comparison to controls (median: 92.7 pg/ml; range: 0.0–592.0 pg/ml). As for IL-1β, IL-18, and IL-33, the highest production of sST2 was found in AF patients (Fig. 4A). Decreased levels of sST2 were observed in all groups after the antifungal treatment (S3), reaching levels observed in the control group (Figs. 4B–4D and Supplementary Table 1). Additionally, we found a moderate positive

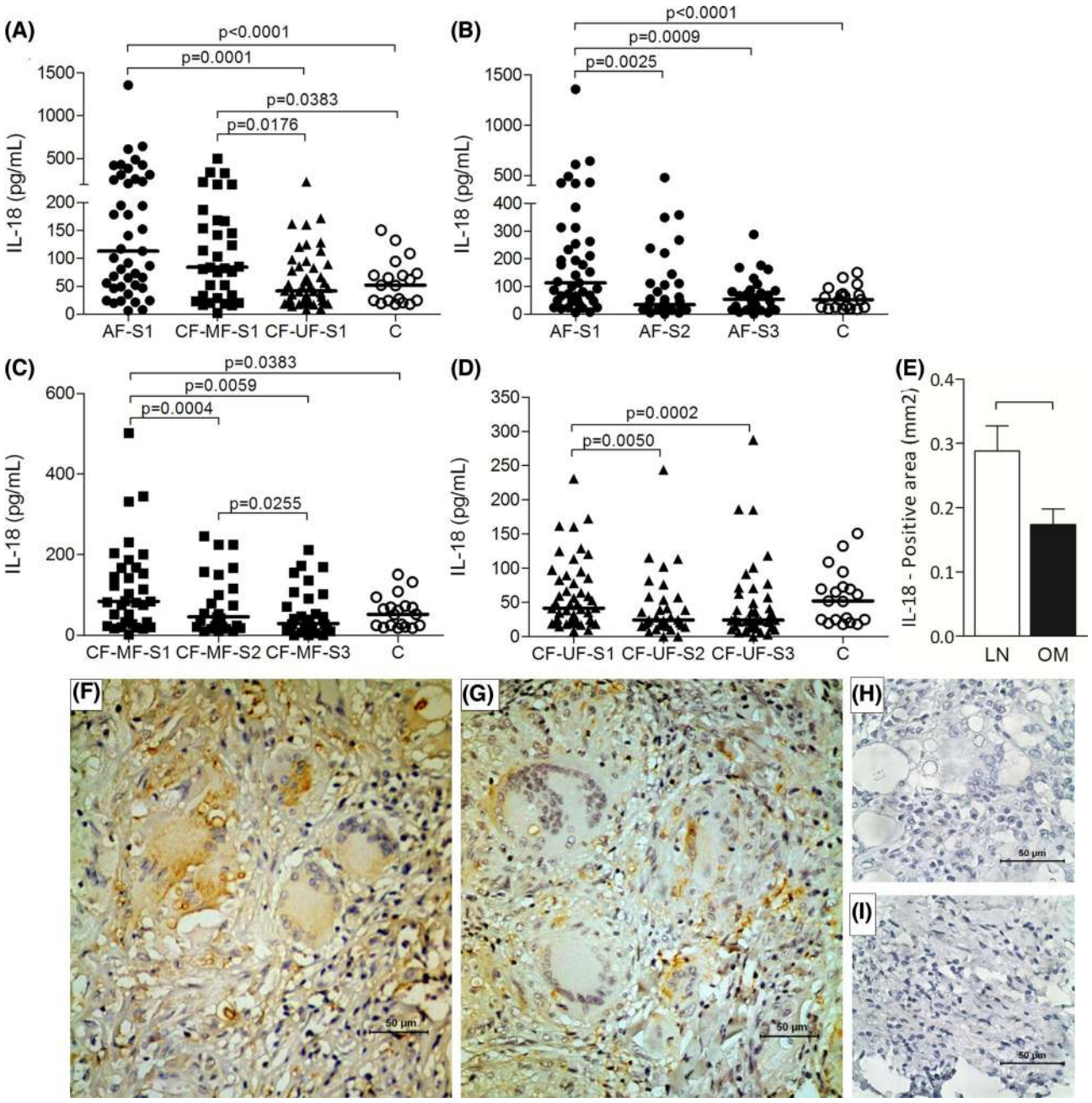

Figure 2. Evaluation of IL-18 in sera samples by ELISA and in lesions by immunohistochemistry. (A) Quantification by ELISA of IL-18 in sera samples of PCM patients with the Acute Form (AF), Chronic Form Multifocal (CF-MF), or Chronic Form Unifocal (CF-UF), collected before antifungal treatment (S1) and healthy controls (C). Horizontal bars represent the median. Statistical analysis: Kruskal-Wallis, *P* values are depicted above brackets. (B–D) Comparison among IL-18 levels in sera of AF (B), CF-MF (C), and CF-UF (D) patients collected before (S1), during (S2) or after (S3) antifungal treatment, and in sera of healthy individuals (controls). Horizontal bars represent the median. Statistical analysis: Comparison among S1, S2, and S3 - Friedman with Dunn's post-test. Comparison between sera S1, S2, or S3 with sera from controls -Kruskal-Wallis. *P* values are depicted above brackets. (E) Quantitative analysis of the immunohistochemistry reactions for IL-18 in lymph node (LN) and oral mucosa (OM) lesions. Results expressed as mean ± SEM of positive areas for IL-18. Statistical analysis - Unpaired *t* test. Brackets represents $P < .05$. (F–I) Representative results of the immunohistochemistry analysis for IL-18 in lymph node (F) and oral mucosa (G) lesions of PCM patients. Scale bars, 50 μm. (H–I) Negative controls (H, lymph node, I, oral mucosa). Scale bars, 50 μm.

correlation between IL-33 and sST2 levels in sera from PCM patients (Table 2).

AF group also has increased levels of IL-37 (median: 1109.0 pg/ml, range: 23.5–3374.0 pg/m) in sera before the antifungal treatment in relation to the other PCM patient groups (CF-MF - median: 175.0 pg/ml, range: 4.6–3617.0 pg/ml; CF-UF - median: 91.7 pg/ml, range: 0.1–4200.0 pg/ml) and controls (median: 122.4 pg/ml, range:

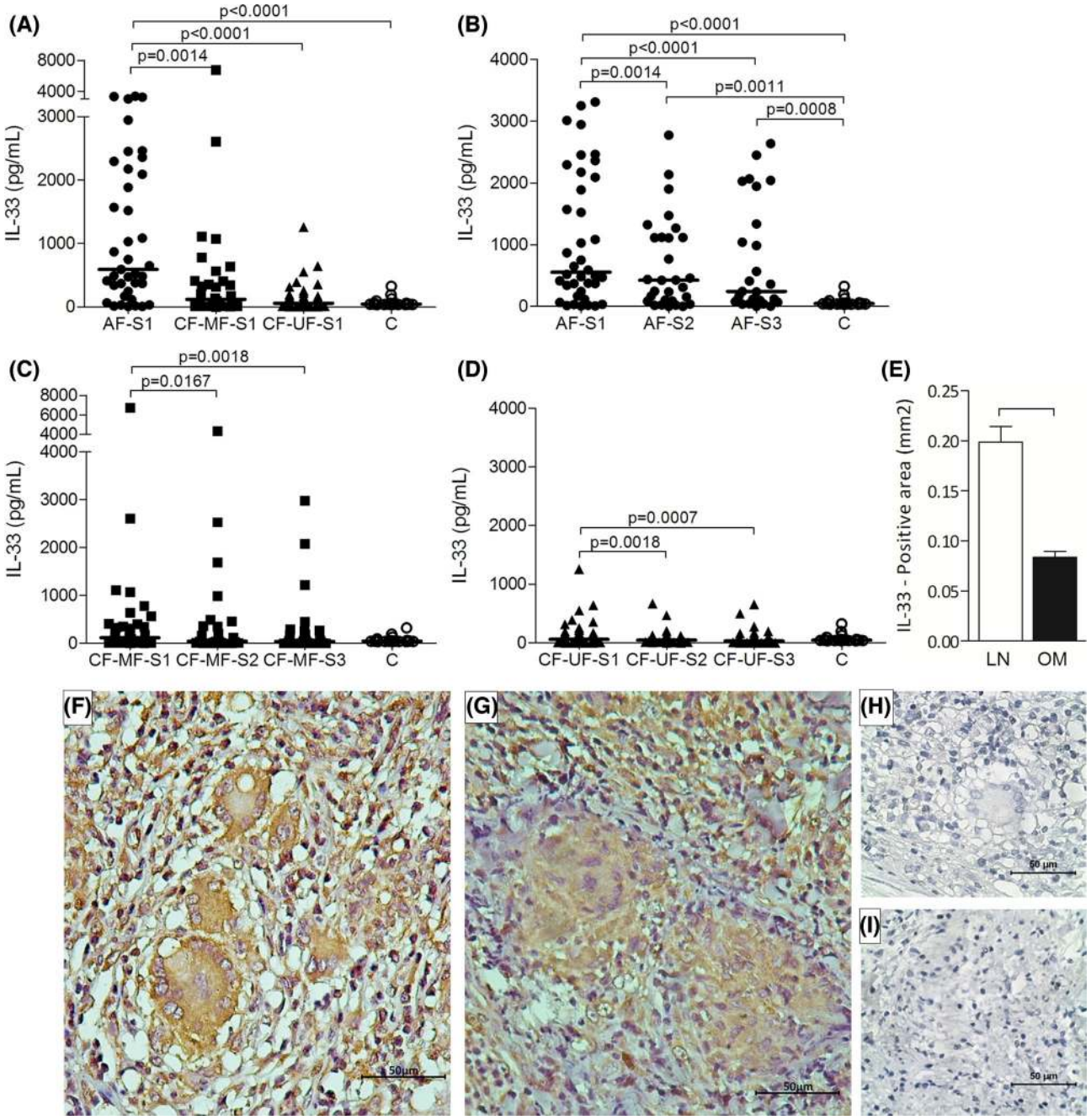

Figure 3. Evaluation of IL-33 in sera samples by ELISA and in lesions by immunohistochemistry. (A) Quantification by ELISA of IL-33 in sera samples of PCM patients with the Acute Form (AF), Chronic Form Multifocal (CF-MF) or Chronic Form Unifocal (CF-UF), collected before antifungal treatment (S1) and healthy controls (C). Horizontal bars represent the median. Statistical analysis: Kruskal-Wallis, *P* values are depicted above brackets. (B–D) Comparison among IL-33 levels in sera of AF (B), CF-MF (C), and CF-UF (D) patients collected before (S1), during (S2) or after (S3) antifungal treatment, and in sera of healthy individuals (controls). Horizontal bars represent the median. Statistical analysis: Comparison among S1, S2, and S3 - Friedman with Dunn's post-test. Comparison between sera S1, S2, or S3 with sera of controls -Kruskal-Wallis. *P* values are depicted above brackets. (E) Quantitative analysis of the immunohistochemistry reactions for IL-33 in lymph node (LN) and oral mucosa (OM) lesions. Results expressed as mean ± SEM of positive areas for IL-33. Statistical analysis - Unpaired *t* test. Brackets represents $P < .05$. (F–I) Representative results of the immunohistochemistry analysis for IL-33 in lymph node (E) and oral mucosa (F) lesions of PCM patients. Scale bars, 50 μm. (H–I) Negative controls (H, lymph node, I, oral mucosa). Scale bars, 50 μm.

40.9–1384.0 pg/ml) (Fig. 5A). Comparison among sera collected before and after antifungal treatment showed diminution of IL-37 levels in all groups of patients (Figs. 5B–5D and Supplementary Table 1), but in AF patients the IL-37 levels were maintained elevated even after the establishment of clinical cure (S3-median: 419.1 pg/ml, range: 0.0–3052.0 pg/ml) (Fig. 5B and Supplementary Table 1). In addition, we observed a moderate positive correlation

Table 2. Correlation between serum levels of cytokines, sST2 and number of eosinophils in PCM patients before antifungal treatment (S1).

	IL-18 *r (p)*	IL-33 *r (p)*	IL-37 *r (p)*	sST2 *r (p)*	Eosinophil (cells/μl blood) *r (p)*	Eosinophil (%) *r (p)*
IL-1	0.2783 (0.0024)	0.2791 (0.0034)	0.3125 (0.0005)	0.2642 (0.0220)	0.1607 (0.1325)	0.1129 (0.2922)
IL-18	...	0.2725 (0.0034)	0.2103 (0.0260)	0.2703 (0.0190)	0.2463 (0.0167)	0.2270 (0.0278)
IL-33	...	...	0.5372 (<0.0001)	0.6013 (<0.0001)	0.5661 (<0.0001)	0.6674 (<0.0001)
IL-37	...	...	...	0.0394 (0.7441)	0.2929 (0.0053)	0.3927 (0.0001)
sST2	...	...	...	...	0.1966 (0.0543)	0.2858 (0.0344)

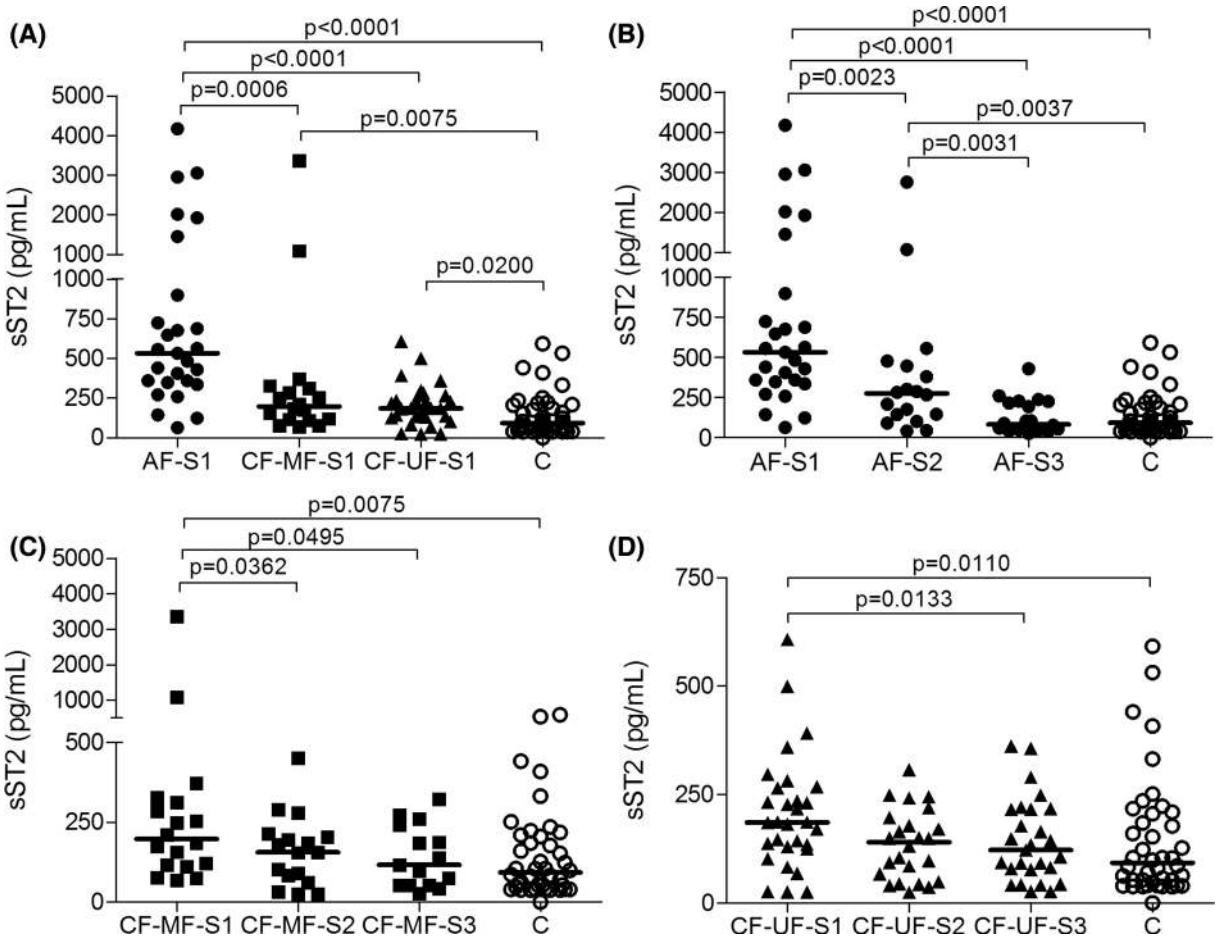

Figure 4. Evaluation of sST2 in sera samples by ELISA. (A) Quantification by ELISA of sST2 in sera samples of PCM patients with the acute form (AF), chronic form multifocal (CF-MF) or chronic form unifocal (CF-UF), collected before antifungal treatment (S1) and healthy controls (C). Horizontal bars represent the median. Statistical analysis: Kruskal-Wallis, *P* values are depicted above brackets. (B–D) Comparison among sST2 levels in sera of AF (B), CF-MF (C), and CF-UF (D) patients collected before (S1), during (S2), or after (S3) antifungal treatment, and in sera of healthy individuals (controls). Horizontal bars represent the median. Statistical analysis: Comparison among S1, S2, and S3 - Friedman with Dunn's post-test. Comparison between sera S1, S2, or S3 with sera of controls -Kruskal-Wallis. *P* values are depicted above brackets.

between serum levels of IL-33 and IL-37 (Table 2). In lesions, IL-37 was detected in giant cells in lymph nodes (Fig. 5F) and with a diffuse pattern in oral mucosa biopsies (Fig. 5G). Comparison of labeled areas showed higher expression in lymph node than in oral-mucosa biopsies (Fig. 5E).

Discussion

Clinical and epidemiological characteristics of PCM patients included in this study were similar to those previously described by us[40] and others.[41,42] The high proportion of males in both CF groups of patients was previously

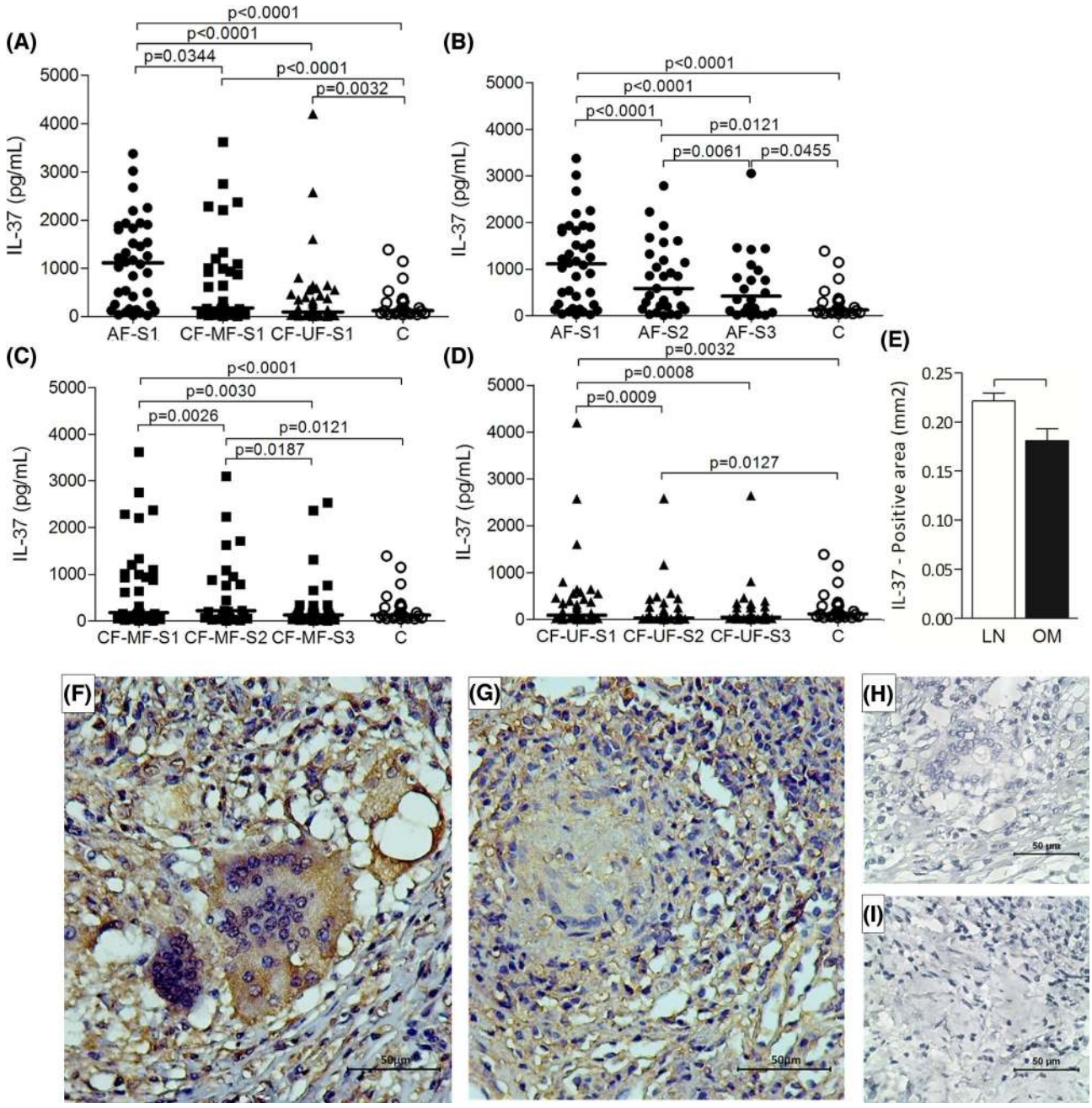

Figure 5. Evaluation of IL-37 in sera samples by ELISA and in lesions by immunohistochemistry. (A) Quantification by ELISA of IL-37 in sera samples of PCM patients with the Acute Form (AF), Chronic Form Multifocal (CF-MF) or Chronic Form Unifocal (CF-UF), collected before antifungal treatment (S1) and healthy controls (C). Horizontal bars represent the median. Statistical analysis: Kruskal-Wallis, *P* values are depicted above brackets. (B–D) Comparison among IL-37 levels in sera of AF (B), CF-MF (C), and CF-UF (D) patients collected before (S1), during (S2), or after (S3) antifungal treatment, and in sera of healthy individuals (controls). Horizontal bars represent the median. Statistical analysis: Comparison among S1, S2, and S3 - Friedman with Dunn's post-test. Comparison between sera S1, S2, or S3 with sera of controls -Kruskal-Wallis. *P* values are depicted above brackets. (E) Quantitative analysis of the immunohistochemistry reactions for IL-37 in lymph node (LN) and oral mucosa (OM) lesions. Results expressed as mean±SEM of positive areas for IL-37. Statistical analysis - Unpaired *t* test. Brackets represents $P < .05$. (F–I) Representative results of the immunohistochemistry analysis for IL-37 in lymph node (F) and oral mucosa (G) lesions of PCM patients. Scale bars, 50 μm. (H–I) Negative controls (H, lymph node; I, oral mucosa). Scale bars, 50 μm.

associated with the female hormone 17-beta-stradiol influence in the morphological transition of *P. brasiliensis* mycelia/conidia to yeast.[43]

In relation to the cytokines, our results showed that the levels of IL-1β in circulation were higher in the AF group. IL-1β was initially described as an important inflammatory mediator in response to infectious process. This cytokine acts on endothelial cells increasing the expression of adhesion molecules and chemokines production.[44] Furthermore, together with TNF-α and IL-6, IL-1β acts systemically inducing the production of acute phase proteins.[44] In PCM, previous *in vitro* studies showed that human monocytes stimulated with *P. brasiliensis* yeast cells produce large amounts of IL-1β and that peripheral blood mononuclear cells (PBMCs) stimulated with IL-1β increased their fungicidal activity.[45,46] In addition, Silva et al. (1995) showed that PCM patients with active disease have high levels of this cytokine in circulation, mainly those with the severe and disseminate forms of the disease.[25] Our results corroborate these findings since we found that patients with the AF form of the disease have higher amounts of IL-1β in peripheral blood when compared with CF-UF and controls. Recent studies have shown a role for IL-1β in inducing Th17 cells differentiation and maintenance.[47] In a previous study we found that Th17 cells are predominantly found in patients presenting the CF of PCM.[6] These apparently contradictory results may indicate that in PCM high circulating levels of IL-1β are associated with the magnitude of the inflammatory process and to the activity of disease, rather than to the differentiation of T helper cells.

Levels of IL-18 also were higher in sera of AF patients, and these results are in agreement with a previous study of our group.[27] IL-18 plays an important role in the differentiation of Th1 cells, IFN-γ production, and activation of natural killer (NK) cells, in synergy with IL-12.[22] However, in absence of IL-12 this cytokine can induce the differentiation of Th2 cells.[21, 22] Interestingly, in a previous study our group showed that after the stimulus with *P. brasiliensis* derived antigen (PbAg), PBMCs from PCM patients with the CF of the disease produced higher quantities of IL-12p40/p70 than cells from AF patients.[13] In experimental leishmaniasis, IL-18 was associated with the increased number of Th2 cells[20] and in tuberculosis, high levels of this cytokine were associated with severe forms of the disease.[48] Furthermore, experimental studies have shown that the presence of IL-18 is related to the susceptibility to *P. brasiliensis* and persistence of the infection by means of promoting a vigorous inflammation that interferes with the acquired immune response.[26]

To the best of our knowledge, this is the first study that examined the IL-33/ST2 axis in PCM. Our results showed that elevated levels of both IL-33 and sST2 were observed almost exclusively in AF patients. In the experimental model of pulmonary infection with *Cryptococcus neoformans*, deficiency of the IL-33 receptor (ST2) resulted in a decreased production of IL-4 by eosinophils and an increased differentiation of Th1 and Th17 cells.[49] Additionally, Flaczyk et al. (2013) showed that the pulmonary infection by *C. neoformans* led to IL-33 production, associated with accumulation of innate lymphoid cells type-2, alternative activation of macrophages (M2 macrophages) and Th2 differentiation.[50] Also, mice deficient for ST2 have increased survival rates, lower fungal burden and less disseminated disease, suggesting a detrimental role for IL-33/ST2.[50] IL-33 also present a role modulating antibody production and class switch, increasing the production of IgE by B cells.[51]

Intriguingly, higher levels of IL-33 and sST2 were described in autoimmune diseases associated with Th1 and Th17 responses, such as psoriasis and rheumatoid arthritis,[52–54] but also in Th2 mediated pathologies such as allergic asthma[35,55,56] and ulcerative colitis.[57] As mentioned, under inflammatory conditions IL-33 acts as an allarmin when released after cell death, leading to the activation and migration of T cells to inflammatory sites.[28] Our results can indicate that in PCM the intense inflammatory response observed in AF patients can promote the release of IL-33 by cells, that in addition to the *de novo* production of IL-33 can induce the differentiation of Th2 cells and provide support to IgE production by B cells, characteristics previously described for this group of patients.[10] Eosinophilia is also a common finding in patients with the AF of PCM.[12,58] In this study, we found a moderate positive correlation between IL-33 levels and the relative/absolute number of eosinophils. These findings suggest that IL-33 could be related to eosinophils production and recruitment in PCM patients, mainly in the AF of the disease.

Interestingly, levels of sST2 were also found higher in the circulation of AF patients, presenting a moderate positive correlation with the levels of IL-33. As mentioned, sST2 acts as a decoy receptor, blocking IL-33 functions. Increased seric levels of sST2 were observed in cardiovascular,[59] autoimmune,[52] and bacterial infections,[60] suggesting its potential use as a disease severity/activity marker.[52,59,60] Our results indicate that sST2 can also be used in PCM as a marker of disease severity and activity.

IL-37 was recently described and its role in the immune response remain unclear. Our data showed that this cytokine is produced mainly by AF patients. Apparently, this cytokine plays an important role in the suppression and regulation of the inflammatory response.[24,61] The expression of IL-37 on macrophages suppresses the production of other pro-inflammatory cytokines, without altering the

production of cytokines with anti-inflammatory activity.[38] Furthermore, silencing of IL-37 leads to an increased production of pro-inflammatory cytokines.[38] Previous studies showed that AF patients present a predominant Th2 acquired immune response, characterized by an impaired cellular immune response, which is associated with the susceptibility to infection.[8,10,13,62] This response seems to interfere with macrophages effectors functions, diminishing the production of reactive oxygen species necessary for the effective fungicidal activity. [63]

In summary, our results showed that cytokines of the IL-1 family are differentially produced by PCM patients. In general, patients with the acute form of the disease, which is more severe and disseminated, presented high circulating levels of IL-1β, IL-18, IL-33, and IL-37, and of the soluble IL-33 receptor (sST2). High levels of IL-37, IL-33, and IL-18 in the absence of IL-12, in AF PCM patients, may contribute to the development of the Th2 response characteristic of this group and, consequently, may be associated with the susceptibility to the infection. On the other hand, elevated IL-1β and sST2 seems to be related to the increased systemic inflammatory response. Although further studies will need to be carried out to determine the effect of these cytokines (particularly IL-33 and IL-37) in the effector functions of innate and acquired immune responses in PCM, these results suggest their utility as markers of disease activity and severity.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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