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Inhibition of Human Neutrophil Apoptosis by *Paracoccidioides brasiliensis*: Role of Interleukin-8

M. J. Acorci*, L. A. Dias-Melicio*, M. A. Golim†, A. P. Bordon-Graciani*, M. T. S. Peraçoli* & Â. M. V. C. Soares*

*Department of Microbiology and Immunology, Biosciences Institute, São Paulo State University, Botucatu, SP, Brazil; and †Botucatu Blood Center, School of Medicine, São Paulo State University, Botucatu, SP, Brazil

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Correspondence to: Ângela Maria Victorian de Campos Soares, PhD, Department of Microbiology and Immunology, Biosciences Institute, São Paulo State University, Botucatu, CEP 18618-000, SP, Brazil.
E-mail: acsoares@ibb.unesp.br

Abstract

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by *Paracoccidiodes brasiliensis* that presents a wide spectrum of clinical manifestations. Because of the great number of neutrophils polymorphonuclear neutrophils (PMN) found in the *P. brasiliensis* granuloma, studies have been done to evaluate the role of these cells during the development of the infection. This fungus is found intracellularly in PMN and monocytes/macrophages, suggesting that it is capable of evading damage and surviving inside these cells. Thus, in the present study, we investigated whether *P. brasiliensis* can prolong the lifetime of PMN, and if this process would be related with IL-8 levels. PMN apoptosis and intracellular levels of IL-8 were analysed by flow cytometry and culture supernatants IL-8 levels were evaluated by enzyme-linked immunosorbent assay. We found that coincubation with *P. brasiliensis* yeast cells results in an inhibition of PMN apoptosis, which was associated with increase in IL-8 production by these cells. Cocultures treatment with monoclonal antibody anti-IL-8 reversed the inhibitory effect of *P. brasiliensis* on PMN apoptosis, besides to increase spontaneous apoptosis of these cells. These data show that, in contrast to other microbial pathogens that drive phagocytes into apoptosis to escape killing, *P. brasiliensis* can extend the lifetime of normal human PMN by inducing autocrine IL-8 production.

Introduction

Paracoccidioidomycosis is a deep mycosis which is endemic in Latin America. The clinical manifestations of the disease are those of a chronic granulomatous disease with involvement of the lung, reticulo endothelial system, mucocutaneous areas and other organs [1]. This disease is caused by *Paracoccidioides brasiliensis*, a fungus that undergoes thermal dimorphism, developing as mycelium at room temperature and in the body as yeast form [2]. The early histopathologic characteristics of PCM in humans are not fully understood, but some studies have demonstrated that *in vivo,* macrophages in contact with *P. brasiliensis* probably initiate extravascular neutrophilia by liberation of chemotactic peptides [3]. The heavy infiltration of neutrophils polymorphonuclear neutrophils (PMN) in the lung, in the early acute phase of infection with *P. brasiliensis* in mice, was correlated with the release of the chemokines KC (keratinocytes derived chemokine) and macrophage inflammatory protein-1α, known to be PMN chemoattractants [4]. Then, in a consequence of these chemotact processes these cells are found as a massive infiltration in infected tissues, of PCM patients [5], and in the early lesions of experimentally infected animals [3, 6–8]. Studies reported that PMN ingest yeast cells of *P. brasiliensis* through a typical phagocytic process [9]. However, other studies have clearly demonstrated that despite their phagocytic capacity, normal or no activated PMN and monocytes/macrophages lack antifungal activities allowing *P. brasiliensis* to survive inside them [10–14], suggesting that this fungus could have evading mechanisms against these phagocytic cells. The ability of certain intracellular microbes to survive and maintain infectivity in PMN enables these organisms to establish productive infection. These pathogens may use PMN for subsequent infection of other cells, such as macrophages [15].

Polymorphonuclear neutrophils are inherently short-lived cells, with a half-time of only ~ 6–10 h in the circulation, after which they undergo spontaneous apoptosis

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[16, 17]. The apoptotic process is well regulated and characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, membrane blebbing, and finally, decays into apoptotic bodies [16–18]. Although apoptosis is an intrinsic cell process, it is modulated by environmental signals. For example, the life span of mature PMN can be extended *in vitro* by incubation with either proinflammatory cytokines, including GM-CSF and G-CSF [19, 20], IL-8 [21], IL-1β and bacterial products such as LPS and fMLP [22, 23]. Conversely, PMN apoptosis may be induced by other mediators, such as TNF-α, IL-10, activation of the oxidative burst, or phagocytosis of bacterial species [24–27]. Several microbial pathogens have been reported to influence cellular apoptosis [28]. Some pathogens, such as *Escherichia coli* [29] and *Candida albicans* [26], were found to induce apoptosis of PMN. In contrast, inhibition of apoptosis of host cells by intracellular pathogens, such as *Chlamydia pneumoniae* [30], *Mycobacterium bovis* [31], *Mycobacterium tuberculosis* [32, 33], *Histoplasma capsulatum* [34], *Leishmania major* [35] or human cytomegalovirus (CMV) [36], can provide an intracellular niche for the pathogens by extending the life span of their host cells.

In the present study, we investigated whether also *P. brasiliensis* can prolong the lifetime of normal PMN and if this process would be related with IL-8 levels. We found that coincubation with *P. brasiliensis* yeast cells results in inhibition of PMN apoptosis, which was associated with increase in IL-8 production by these cells. The effective role of IL-8 on the inhibition of PMN apoptosis was confirmed by monoclonal antibody anti-IL-8 treatment that was able to reverse the inhibitory effect of *P. brasiliensis* on PMN apoptosis.

Subjects and methods

Healthy individuals. Twenty-eight healthy blood donors from University Hospital of the Botucatu Medical School, São Paulo State University, Brazil (age range 20–50 years) were included in the present work. The study was approved by Ethics Committee of Botucatu Medical School, and informed consent was obtained from all the blood donors.

Fungi. The highly virulent *P. brasiliensis* strain 18 (Pb 18) was used throughout this study. To ensure virulence, the isolate was used after three serial animal passages. Pb 18 yeast cells were then maintained by weekly subcultivation in the yeast-form cells at 35 °C on 2% glucose, 1% peptone, 0.5% Yeast extract and 2% agar medium (GPY medium) and used on the sixth day of culture. Yeast cells were washed and suspended in 0.15 M phosphate-buffered saline (PBS pH 7.2). The fungal suspension was homogenized with glass beads in a Vortex homogenizer (3 cycles of 10 s), after left in rest for 3 min and supernatant collected in order to reduce fungal clots and facilitate counting. Yeast viability was determined by phase contrast microscopy and bright yeast cells were counted as viable while dark ones were considered not viable. Fungal suspensions containing more than 95% viable cells were used in the experiments.

Isolation of human polymorphonuclear cells. Heparinized venous blood samples were obtained from healthy subjects. 10 ml of blood were diluted in 10 ml RPMI-1640 tissue culture medium (Sigma-Aldrich, Inc., St Louis, MO, USA). The cell were layered on Percoll 85% and Histopaque–1077 (Sigma-Aldrich). The cell fraction containing PMN was washed with RPMI-1640. Remaining cells were suspended in RPMI-1640 tissue culture medium supplemented with 2 mM of L-glutamine (Sigma-Aldrich), 40 μg/ml of gentamicin and 10% heat inactivated autologous human serum (CTCM: complete tissue culture medium). The cellular viability was assessed by Trypan Blue dye exclusion test, and the suspensions were adjusted for 1×10^6 cells/ml. The purity of PMN suspensions determined by morphological examination of May-Gruwald-Giemsa stained slides was >98%. Then, these cells were incubated for 4, 12 and 18 h at 37 °C in a 5% CO_2 with CTCM, in the presence or absence of monoclonal antibody anti-IL-8 (R&D Systems, Minneapolis, MN, USA) in the concentration of 3 μg/ml. Some cultures were challenged with *P. brasiliensis* suspension at the concentrations of 2×10^4/ml (1:50 fungus/PMN ratio) during 4 h.

Apoptosis determination: Annexin V/propidium iodide assay. PMN identification control was done by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD24 or FITC-conjugated isotype-matched control IgG2a (both from BD Biosciences, San Diego, CA, USA), and selected by size (FSC) and granularity (SSC) patterns to establish the PMN gate. After this, apoptosis and necrosis were assessed by flow cytometry using FITC-conjugated Annexin V and non-vital dye propidium iodide (PI) staining (BD Biosciences) in the cocultures. Labelling of apoptotic cells with Annexin V–FITC and counterstaining with PI for necrosis were carried out according to the manufacturer's instructions. Briefly, isolated PMN were incubated for 4, 12 and 18 h at 37 °C in a 5% CO_2 with CTCM, in the presence or absence of monoclonal antibody anti-IL-8 (R&D Systems). Some cultures were challenged with *P. brasiliensis* suspension at the concentrations of 2×10^4/ml (1:50 fungus/PMN ratio). Then, the cells were stained with 5 μl FITC-Annexin V and 5 μl PI for 15 min in the dark. Fluorescence of cell surface – (Annexin V) or DNA-bound (PI) markers was analysed within 1 h of staining. Samples were acquired on a FACScalibur flow cytometry (Becton Dickinson, San Diego, CA, USA). For Annexin V/PI data as well as for CD24 PMN identification, 10,000 events were acquired and analysed using Cell Quest software (Becton Dickinson). The positive population was expressed as mean of fluorescence

intensity (MFI) for Annexin V and as percentage of apoptotic, live, necrotic and apoptotic-necrotic cells. Annexin V binds to phosphatidylserine that is expressed in the outer leaflet of the phospholipids bilayer as a consequence of apoptosis and necrosis. Cells that are late-apoptotic or early-necrotic will lose membrane integrity and stain with both FITC-Annexin V and PI. Cells that retain membrane integrity, including viable and early-apoptotic cells, will not take up PI. Therefore, the combined use of Annexin V and PI can distinguish between early-apoptotic and late-apoptotic or necrotic cells.

IL-8 release determination. Isolated PMN (1×10^6 cells/ml) were distributed in 1.0 ml volume into 24-wells tissue culture plates and incubated for 18 h at 37 °C in a 5% CO_2 with CTCM alone or with *P. brasiliensis* suspension at the concentrations of 2×10^4/ml (1:50 fungus/PMN ratio). After incubation, supernatants were harvested and stored at −70°C, until use. IL-8 concentrations in the cultures supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Antibody-matched pairs and respective standards were purchased from R&D Systems and used according to manufacturer instructions. The sensitivity limit of the assays was 5 pg/ml.

Intracellulary IL-8 detection. Isolated PMN (1×10^6 cells/ml) were distributed in 1.0 ml volume into 24-wells tissue culture plates and incubated for 12 h at 37 °C in a 5% CO_2 with CTCM alone or *P. brasiliensis* suspension at the concentrations of 2×10^4/ml (1:50 fungus/PMN ratio). After incubation, the intracellular IL-8 was detected by flow cytometry. Briefly, cells (1×10^6 cells/ml) were centrifuged and resuspended in 100 μl of permeabilization buffer (B Fix & perm-Caltag Laboratories, Burlingame, CA, USA) and stained during 30 min with FITC-labelled IgG1 mouse monoclonal antibodies anti-human IL-8 (BD Labware, Franklin Lakes, NJ, USA). After, the cells were washed with buffer solution, centrifuged, resuspended and fixed in 500 μl of buffer solution containing 50 μl of 4.0% paraformaldehyde. The cells were analysed by flow cytometry on a FACSCalibur Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) using Cell Quest software.

Statistical analysis. Data were analysed statistically using the INSTAT software (Graph Pad, San Diego, CA, USA). The results were compared by variance analysis (ANOVA) followed by the Tukey test, with the level of significance set at $P < 0.05$.

Results

Coincubation with *P. brasiliensis* inhibits PMN apoptosis *in vitro*

It has been reported that some intracellular micro-organisms, such as *C. pneumoniae* and *H. capsulatum*, can inhibit apoptosis of infected cells [30, 34]. To examine the ability of *P. brasiliensis* to induce or inhibit PMN apoptosis, we coincubated PMN with yeast cells of the fungus during 4, 12 and 18 h. After, cells were incubated with Annexin V and PI in a binding buffer and analysed by flow cytometry. Annexin V-positive cells (apoptotic cells), PI-positive cells (necrotic cells), double-positive cells (apoptotic-necrotic cells) and double-negative cells (live cells) were determined and expressed as percentage of total cells.

In Figure 1A we showed that *P. brasiliensis* protected PMN from spontaneous apoptosis in all the periods evaluated, when compared with PMN incubated with just CTCM. The number of live cells was higher in PMN cultures challenged with *P. brasiliensis* than those incubated with CTCM alone (Fig. 1B). The percentage of necrotic cells was low in all cultures (Fig. 1C), however the percentage of apoptotic-necrotic cells was higher in both groups in the period of 4 h of culture (Fig. 1D).

Coincubation with *P. brasiliensis* induces the release of IL-8 by PMN

IL-8 production by PMN is thought to inhibit the spontaneous apoptosis of PMN [17]. Moreover, some studies demonstrated that this cytokine is involved in the apoptosis inhibition induced by some micro-organisms. Then, we evaluated the capacity of *P. brasiliensis* to induce IL-8 production by PMN *in vitro.*

Intracytoplasmic IL-8 was measured by flow cytometry after 12 h of coincubation and by ELISA in supernatants of PMN cultures after 18 h of coincubation. We did not detect intracytoplasmic production of IL-8 in the period of 12 h, probably because the IL-8, at this time, was already completely released from the cell. However, the levels of IL-8 in supernatants of PMN cultures challenged with *P. brasiliensis*, after 18 h, detected by ELISA, were significantly higher than those without challenge (Fig. 2).

Monoclonal antibody anti-IL-8 treatment reverts the inhibitory effect of *P. brasiliensis* upon PMN apoptosis

In view of the above data and the reported capacity of IL-8 to inhibit PMN apoptosis [17], we hypothesized that the generation and autocrine action of IL-8 may contribute to the inhibitory effect of *P. brasiliensis* on PMN apoptosis. Using a specific neutralizing antibody, monoclonal antibody anti-IL-8 (3 μg/ml), we showed that the effect of *P. brasiliensis* on the inhibition of PMN apoptosis was reversed in all periods of incubation (Fig. 3). Besides, the anti-IL-8 treatment increased the PMN spontaneous apoptosis. Figure 3 shows the MFI of PMN labelled with Annexin V that were treated with anti-IL-8 and challenged with *P. brasiliensis*. Figure 3D and E

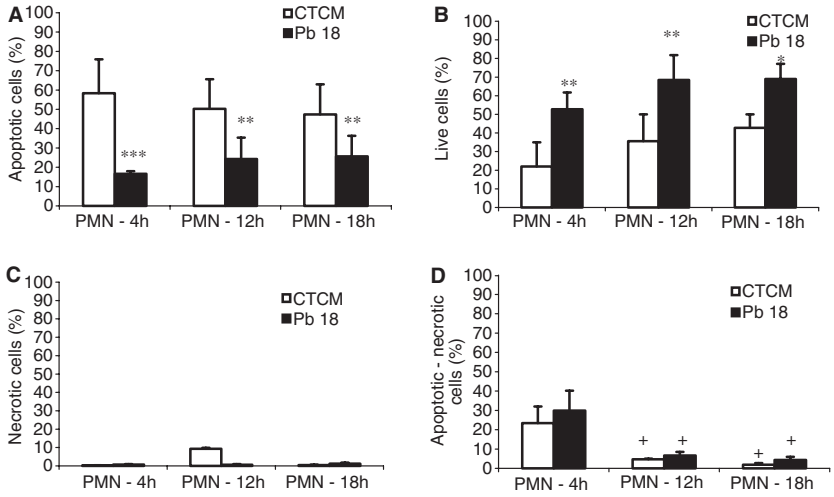

Figure 1 *Paracoccidioides brasiliensis* inhibits PMN apoptosis. Human PMN were incubated during 4, 12 and 18 h with CTCM or with *P. brasiliensis*. After these periods, cells were incubated with Annexin V and PI. PMN cocultures were labelled with FITC-conjugated mouse anti-human CD24 and selected by size (FSC) and granularity (SSC) patterns. The percentage of apoptotic (A), live (B), necrotic (C) and apoptotic-necrotic cells (C) was determined by flow cytometric analysis. Results are presented as means ± SEM of twenty blood donors. Statistically significant differences between groups are indicated. *$P < 0.05$ versus its control; **$P < 0.01$ versus its control; ***$P < 0.001$ versus its control. $^{+}P < 0.05$ versus PMN – 4 h.

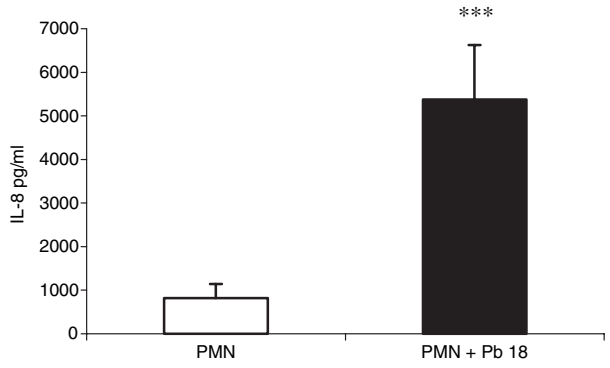

Figure 2 *Paracoccidioides brasiliensis* induces IL-8 release by PMN. PMN were incubated for 18 h with or without *P. brasiliensis*. The IL-8 content of the supernatants was measured using ELISA. The figure shows the mean ± SEM obtained from twenty blood donors. Statistically significant differences between groups are indicated. ***$P < 0.001$.

represents a typical flow cytometry profile of PMN stained with Annexin V, showing the effect of *P. brasiliensis* on the inhibition of PMN apoptosis (Fig. 3D) and the reversal of this effect when anti-IL-8-neutralizing antibodies were applied (Fig. 3E).

Discussion

It has been demonstrated that *P. brasiliensis* is able to be phagocytosed by human PMN [9]. Dias *et al.* [9] observed that PMN from healthy subjects showed a high capability to ingest *P. brasiliensis* yeast forms. Even after an interaction time of 150 min, the PMN showed a normal appearance. Adhesion and ingestion occurred at a high speed during the first 30 min of interaction. However, normal PMN lack antifungal activities against *P. brasiliensis* allowing its survival inside them, and just activation with cytokines such as IFN-γ, TNF-α and GM-CSF results in PMN fungicidal activity against this fungus [10, 13, 14, 37]. Thus, investigations about the mechanisms that allow or not *P. brasiliensis* to survive in PMN will contribute for understanding PCM pathogenesis.

In this study, the detection of apoptosis assessed by Annexin V binding, as well as the death-associated DNA fragmentation by non-vital dye PI, allowed to demonstrate for the first time that coincubation of PMN with *P. brasiliensis* led to an inhibition of their spontaneous apoptosis *in vitro*, showing that this fungus is able to prolong PMN lifetime. The decreased percentage of cells labelled with Annexin V and PI indicated that apoptosis and necrosis of PMN is downregulated by the pathogen. Micro-organisms may trigger apoptosis of phagocytic cells to avoid their destruction [26, 29]. However, other intracellular microbes may block or delay apoptosis of these cells to keep an environmental for their survival and replication [30, 31, 34–36]. *Histoplasma capsulatum* induces an antiapoptotic state on human and murine PMN that may represent an escape mechanism for the fungus by delaying cell death and allowing the fungus to survive inside leucocytes [34]. It was described that *C. pneumoniae* [30], *L. major* [35], *M. bovis* [31], human CMV [36] significantly decreased the ratio of apoptotic PMN.

Although apoptosis is an intrinsic cell process, it can also be modulated by cytokines such as IL-8, which was shown to delay the spontaneous apoptosis of PMN in a dose-dependent fashion [17, 21, 38, 39]. It was

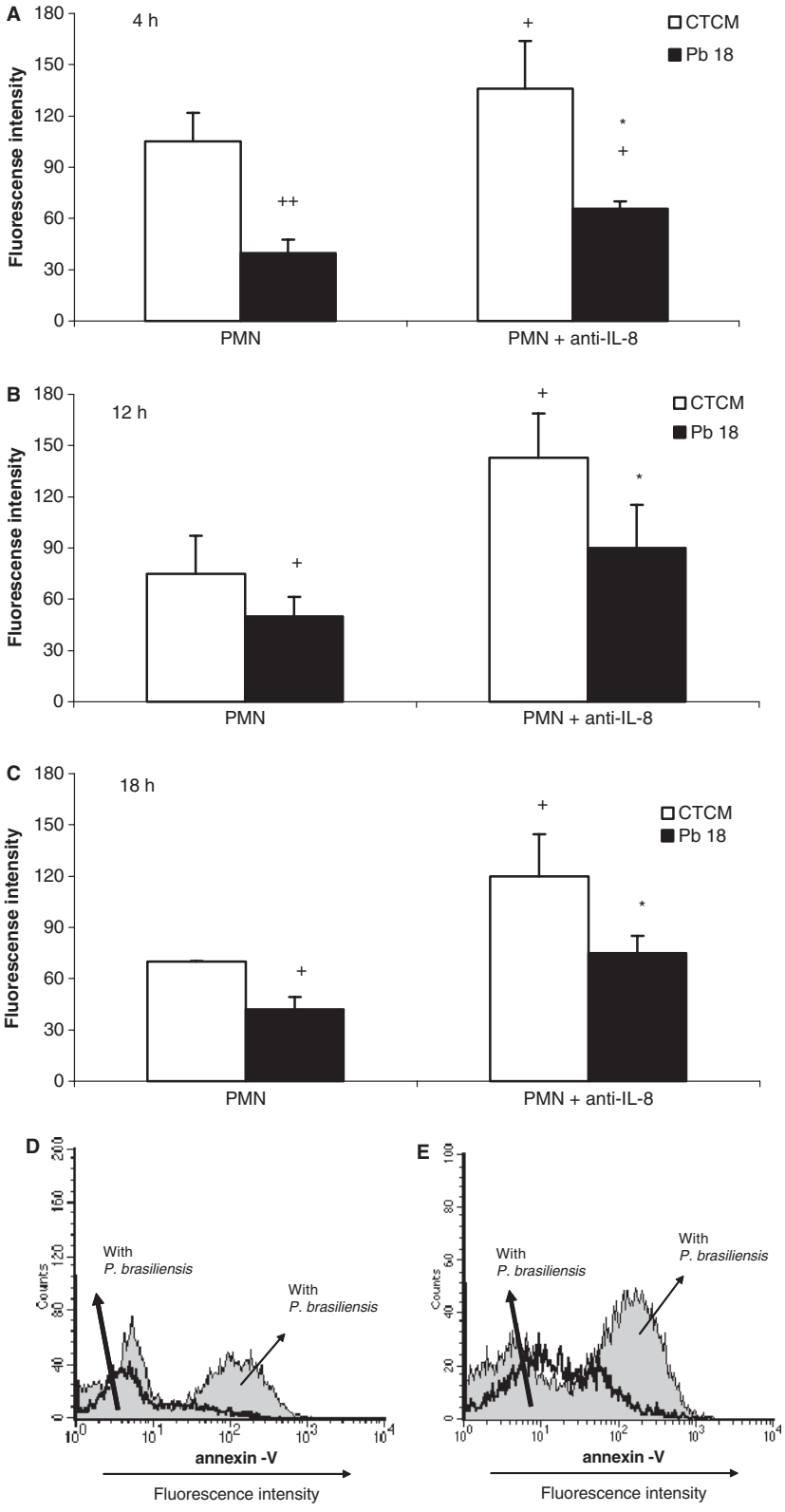

Figure 3 Antibody anti-IL-8 reverts the antiapoptotic effect of *Paracoccidioides brasiliensis* upon human PMN. Human PMN were incubated during 4 (A, D and E), 12 (B) and 18 h (C) with CTCM or with *P. brasiliensis*, in the absence (D) or presence (E) of monoclonal antibody anti-IL-8 (3 μg/ml). After these periods, cells were incubated with Annexin V and PI. PMN cocultures were labelled with FITC-conjugated mouse anti-human CD24 and selected by size (FSC) and granularity (SSC) patterns. Results are presented as mean fluorescence intensity (MFI) ± SEM of Annexin V staining from eight blood donors. In Figures D and E, the X-axis shows the green MFI (FL-1) of cells stained with Annexin V – FITC, and the numbers indicate the ratio of Annexin V-positive cells. Statistically significant differences between groups are indicated. $^{+}P < 0.05$ versus PMN (CTCM). $^{++}P < 0.01$ versus PMN (CTCM). $*P < 0.05$ versus PMN (Pb 18).

demonstrated that IL-8 inhibited PMN apoptosis by suppressing the proapoptotic function of Fas-FasL interactions [38]. More recently, it was showed that a cross-talk between NF-Kappa B and PI3-Kinase pathways in TNF-alpha-stimulated PMN, resulting from NF-Kappa B/ERK1/2-dependent IL-8 production which acts in an autocrine manner to drive PI3-Kinase-dependent survival [39].

In an attempt to evaluate the role of autocrine IL-8 on the inhibition of apoptosis induced by *P. brasiliensis* infection, in our assay system, we first analysed the concentrations of this cytokine on cocultures supernatants. High IL-8 levels were detected suggesting that the antiapoptotic effect of *P. brasiliensis* can be mediated by the autocrine production of IL-8 by PMN, as it has been shown for other pathogens [30, 31, 35, 36]. The role of IL-8 on this process was confirmed by neutralization of this cytokine with anti-IL-8 antibodies that restored PMN apoptosis. Moreover, the monoclonal antibody treatment increased the spontaneous apoptosis of PMN.

Previous studies on experimental model of this infection have demonstrated increased apoptosis induced by *P. brasiliensis* in resistant mice [40]. Verícimo *et al.* [40] suggested that the high levels of apoptotic cytokines, such as IL-1β and IFN-γ, produced by peritoneal cells of resistant mice, could induce the apoptosis process in T-cell populations. In our system, we used a purified population of PMN that produced high levels of IL-8, an important antiapoptotic cytokine. Thus, the reason for the different results may be related to the differences in the cellular populations analysed that released different patterns of cytokine in response to *P. brasiliensis in vivo*.

The idea that inhibition of phagocytic cells apoptosis by pathogens seems to be a strategy to facilitate intracellular survival is well established in the literature. Although this could be a beneficial characteristic for the fungus, permitting plenty of time for multiplication and dissemination, we believe that it is also favourable to the host, as it permits PMN and monocytes/macrophages to synthesize mediators and become activated, and then kill the fungus. Macrophages have receptors for apoptotic cells, and after phagocytosis of these cells, they become deactivated by the production of anti-inflammatory cytokines [41], and could not efficiently participate in the immune response. Then, apoptosis could be seen as a negative modulator of the host immune response against infectious agent, by controlling inflammation delaying the start of an efficient immune response.

Dias *et al.* [42] showed that PMN from patients with treated PCM degenerate during the phagocytosis process demonstrating the existence of a functional PMN deficiency against *P. brasiliensis* in susceptible individuals. Our group showed that monocytes from PCM patients produced high levels of IL-8 when compared with healthy individuals [43], and monocytes from healthy donors challenged with *P. brasiliensis* produced high levels of this cytokine [44]. These results indicate that PCM patients could have a factor that blocks IL-8 action on apoptosis inhibition, seen that despite of higher IL-8 production, their PMN degenerate in presence of the fungus. Following this idea, PMN apoptosis could be related to susceptibility.

Thus, our results presented here about inhibition of normal PMN apoptosis could indicate a strategy that keeps the immune system activated representing a resistance mechanism of the host against this fungus. Taking the data altogether, we can suggest that the antiapoptotic activity of *P. brasiliensis* is a way found by the host to combat the fungus, but, we can not discard that, at the same time, this process could allow the fungus to survive intracellularly. Investigations about the molecular processes involved in delayed apoptosis induced by *P. brasiliensis*, such as caspases activity and BCL2 expression, are been conducted in our laboratory.

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Conflict of interest

None.

References

1 Franco M, Mendes RP, Moscardi-Bacchi M, Rezkallah-Iwasso MT, Montenegro MR. Paracoccidioidomycosis. *Bailliere's Clin Trop Med Commun Dis* 1989;4:185–220.
2 Carbonell LM. Ultrastructure of dimorphic transformation in *Paracoccidioides brasiliensis*. *J Bacteriol* 1969;100:1076–82.
3 Calich VLG, Singer-Vermes LM, Siqueira AM. Susceptibility and resistence of inbred mice to *Paracoccidioides brasiliensis*. *Br J Exp Pathol* 1985;66:585–94.
4 Souto JT, Aliberti JC, Campanelli AP *et al.* Chemokine production and leukocyte recruitment to the lungs of *Paracoccidioidomicose brasiliensis*-infected mice is modulated by interferon-gamma. *Am J Pathol* 2003;163:583–90.
5 Franco M, Montenegro MRG. Anatomia Patológica. In: Del Negro G, Lacaz CS, Fiorillo AM, eds. *Paracocoidioidomicose: Blastomicose Sul-Americana*. São Paulo: Sarvier-EDUSP, 1982:97–117.
6 Iabuki K, Montenegro MR. Experimental paracoccidioidomycosis in the Syrian hamster: morphology, ultrastructure and correlation of the lesions with presence of specific antigens and serum levels of antibodies. *Mycopathologia* 1979;67:131–41.
7 Peraçoli MTS, Mota NGS, Montenegro MR. Experimental paracoccidioidomycosis in the Syrian hamster: morphology and correlation of lesions with human and cell-mediated immunity. *Mycopathologia* 1982;79:7–17.
8 Kerr IB, Araripe PCO, Lenzi HL. Paracoccidioidomycosis in experimentally-infected rats. *Rev Inst Med trop São Paulo* 1988;30: 336–50.
9 Dias MF, Figueira AL, de Souza W. A morphological and cytochemical study of the interaction between *Paracoccidiodes brasiliensis* and neutrophils. *Microsc Microanal* 2004;10:215–23.
10 Goihman-Yahr M, Essenfeld E, Albornoz MC *et al.* Defects on *in vitro* digestive ability of polymorphonuclear neutrophils in paracoccidioidomycosis. *Infect Immun* 1980;28:557–66.
11 Brummer E, Hanson LH, Restrepo A, Stevens DA. Intracellular multiplication of *Paracoccidioides brasiliensis* in macrophages: killing

and restriction of multiplication by activated macrophages. *Infect Immun* 1989;57:2289–94.
12 Moscardi-Bacchi M, Brummer E, Stevens DA. Support of *Paracoccidioides brasiliensis* multiplication by human monocytes or macrophages: inhibition by activated phagocytes. *J Med Microbiol* 1994;40:159–64.
13 Kurita N, Biswas SK, Oarada M *et al.* Fungistatic and fungicidal activities of murine polymorphonuclear leucocytes against yeast cells of *Paracoccidioides brasiliensis*. *Med Mycol* 1999;37:19–24.
14 Kurita N, Oarada M, Ito E, Miyaji M. Antifungal activity of human polymorphonuclear leukocytes against yeast cells of *Paracoccidioides brasiliensis*. *Med Mycol* 1999;37:261–7.
15 Laskay T, van Zandbergen G, Solbach W. Neutrophil granulocytes: Trojan horses for *Leishmania major* and other intracellular microbes? *Trends Microbiol* 2003;11:210–4.
16 Savill JS, Wyllie AH, Henson JE *et al.* Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 1989;83:865–75.
17 Payne CM, Glasser L, Tischler ME *et al.* Programmed cell death of the normal human neutrophil: an *in vitro* model of senescence. *Microsc Res Tech* 1994;28:327–44.
18 Squier MK, Sehnert AJ, Cohen JJ. Apoptosis in leukocytes. *J Leukoc Biol* 1995;57:2–10.
19 Cox G, Gauldie J, Jordana M. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils *in vitro*. *Am J Respir Cell Mol Biol* 1992;7:507–13.
20 Kobayashi SD, Voyich JM, Whitney AR, DeLeo FR. Spontaneous neutrophil apoptosis and regulation of cell survival by granulocyte macrophage-colony stimulating factor. *J Leukoc Biol* 2005;78:1408–18.
21 Kettritz R, Gaido ML, Haller H *et al.* Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. *Kidney Int* 1998;53:84–91.
22 Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 1992;80:2012–20.
23 Lee A, Whyte MK, Haslett C. Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* 1993;54:283–8.
24 Cox G. IL-10 enhances resolution of pulmonary inflammation *in vivo* by promoting apoptosis of neutrophils. *Am J Physiol* 1996;271:L566–71.
25 Lundqvist-Gustafsson H, Bengtsson T. Activation of the granule pool of the NADPH oxidase accelerates apoptosis in human neutrophils. *J Leukoc Biol* 1999;65:196–204.
26 Rotstein D, Parodo J, Taneja R, Marshall JC. Phagocytosis of *Candida albicans* induces apoptosis of human neutrophils. *Shock* 2000;14:278–83.
27 Salamone G, Giordano M, Trevani AS *et al.* Promotion of neutrophil apoptosis by TNF-alpha. *J Immunol* 2001;166:3476–83.
28 Monack D, Falkow S. Apoptosis as a common bacterial virulence strategy. *Int J Med Microbiol* 2000;290:7–13.
29 Watson RW, Redmond HP, Wang JH, Condron C, Bouchier-Hayes D. Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. *J Immunol* 1996;156:3986–92.
30 van Zandbergen G, Gieffers J, Kothe H *et al. Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J Immunol* 2004;172:1768–76.
31 Suttmann H, Lehan N, Bohle A, Brandau S. Stimulation of neutrophil granulocytes with *Mycobacterium bovis* bacillus Calmette-Guerin induces changes in phenotype and gene expression and inhibits spontaneous apoptosis. *Infect Immun* 2003;71:4647–56.
32 Rojas M, Barrera LF, Puzo G, Garcia LF. Differential induction of apoptosis by virulent *Mycobacterium tuberculosis* in resistant and susceptible murine macrophages: role of nitric oxide and mycobacterial products. *J Immunol* 1997;159:1352–61.
33 Keane J, Remold HG, Kornfeld H. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. *J Immunol* 2000;164:2016–20.
34 Medeiros AI, Bonato VL, Malheiro A *et al. Histoplasma capsulatum* inhibits apoptosis and Mac-1 expression in leucocytes. *Scand J Immunol* 2002;56:392–8.
35 Aga E, Katschinski DM, van Zandbergen G *et al.* Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J Immunol* 2002;169:898–905.
36 Saez-Lopez C, Ngambe-Tourere E, Rosenzwajg M *et al.* Immediate-early antigen expression and modulation of apoptosis after *in vitro* infection of polymorphonuclear leukocytes by human cytomegalovirus. *Microbes Infect* 2005;7:1139–49.
37 Rodrigues DR, Dias-Melicio LA, Calvi SA, Peraçoli MT, Soares AM. *Paracoccidioides brasiliensis* killing by IFN-gamma, TNF-alpha and GM-CSF activated human neutrophils: role for oxygen metabolites. *Med Mycol* 2007;45:27–33.
38 Leuenroth S, Lee C, Grutkoski P, Keeping H, Simms HH. Interleukin-8-induced suppression of polymorphonuclear leukocyte apoptosis is mediated by suppressing CD95 (Fas/Apo-1) Fas-1 interactions. *Surgery* 1998;124:409–17.
39 Cowburn AS, Deighton J, Walmsley SR, Chilvers ER. The survival effect of TNF-alpha in human neutrophils is mediated via NF-kappa B-dependent IL-8 release. *Eur J Immunol* 2004;34:1733–43.
40 Verícimo MA, França MK, Arnholdt ACV, Kipnis TL. Increased apoptosis during the early phase of experimental paracoccidioidomycosis as a phenotypic marker of resistance. *Microbes Infect* 2006;8:2811–20.
41 McDonald PP, Fadok VA, Bratton D, Henson PM. Transcriptional and translational regulation of inflammatory mediator production by endogenous TGF-beta in macrophages that have ingested apoptotic cells. *J Immunol* 1999;11:6164–72.
42 Dias MF, Mesquita J, Filgueira AL, De Souza W. Human neutrophils susceptibility to *Paracoccidioides brasiliensis*: an ultrastructural and cytochemical assay. *Med Mycol* 2008;46:241–9.
43 Peraçoli MT, Kurokawa CS, Calvi SA *et al.* Production of pro- and anti-inflammatory cytokines by monocytes from patients with paracoccidioidomycosis. *Microbes Infect* 2003;5:413–8.
44 Kurokawa CS, Araujo JP Jr, Soares AM, Sugizaki MF, Peraçoli MT. Pro- and anti-inflammatory cytokines produced by human monocytes challenged *in vitro* with *Paracoccidioides brasiliensis*. *Microbiol Immunol* 2007;51:421–8.