Expression of activation and cytotoxic molecules by peripheral blood lymphocytes of patients with paracoccidioidomycosis

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In a previous study, we reported an increased number of T CD8+ cells in the bronchoalveolar lavage (BAL) of patients with pulmonary paracoccidioidomycosis, suggesting a role for these cells in the local immune response. The aims of this study were to verify, by flow cytometry, the activation state, as well as the production of cytotoxic molecules by peripheral blood lymphocytes (CD8+ and CD4+). Specimens were obtained from patients with paracoccidioidomycosis (PCM), individuals with PCM-infection, i.e., healthy individuals with demonstrated strong cellular response against the fungus (PI) and controls, with studies conducted both ex-vivo and in vitro, after stimulation with Paracoccidioides brasiliensis yeast cells. The ex-vivo analysis demonstrated that PCM patients presented a lower frequency of granzyme A, B and perforin-positive cells, as compared to individuals with PCM infection (PI). P. brasiliensis stimulation led to a discrete increase in CD69+ cells and a reduction in cytotoxic granule expression in all groups. The addition of IL-15 induced an increase in the frequency of CD69+ cells only in PI individuals and controls. The effect of IL-15 on granzyme A and B expression was low, but a higher frequency of CD8+ perforin+ was detected in PI individuals than in patients with active PCM. IL-15Rα expression was lower in CD4+ T cells from patients, in relation to the PI group. Furthermore, low levels of granulysin were detected in sera from PCM patients, but a tendency for an increase in these levels was observed after antifungal therapy. Taken together, these results indicate that lymphocytes from PCM patients are poorly activated, express low levels of IL-15Rα and produce basal levels of cytotoxic granules. These findings may account for the defective cytotoxic activity in patients and, consequently, a low capacity to kill the fungus.

Keywords Paracoccidioidomycosis, CD8+ T cells, perforin, granulysin

Introduction

Paracoccidioidomycosis (PCM) is a deep mycosis that is endemic in various countries of Latin America, especially in Brazil. The etiological agent of PCM is the thermomorphogenic fungus, Paracoccidioides brasiliensis [1]. The disease presents a broad spectrum of clinical and pathological manifestations, ranging from benign and localized forms to widely disseminated disease. According to the current classification [2], the adult or chronic progressive form of PCM (AF) predominantly affects adult males, with a high frequency of pulmonary, skin, adrenal and visceral involvement. In contrast, the juvenile type (JF) equally affects young patients of both sexes and is characterized by systemic lymph node involvement, hepatosplenomegaly, and bone marrow dysfunction. Patients with AF usually exhibit low levels of specific antibodies and adequate cellular immune responses, while those with the JF typically show high levels of specific antibodies, polyclonal activation of B cells, antigenemia, and impaired cellular immune responses [3].

A protective role for CD8+ T cells was suggested in experimental PCM, since its depletion induces a more severe and/or disseminated disease in both resistant and susceptible mice [4]. Moreover, an elevated number of CD8+ T cells is detected in the lungs of patients with pulmonary PCM, in addition to a high concentration of MIP-1α in bronchoalveolar...
lavage (BAL), a chemokine known to selectively attract this cell subset [5]. Recently, Pagliari described abundant CD8+ T cells, as well as perforin and granzyme B, in cutaneous and mucosal lesions of patients with PCM, indicating an involvement of these cells in the immune response to the fungus [6].

Since cytotoxic CD8+ T cells represent a major mechanism of defense against intracellular pathogens, and since they produce IFN-γ and possess cytolytic activity [7], they may be involved in the clearance of P. brasiliensis cells. CD8+ T lymphocytes disrupt the target cell membrane through extracellular Ca2+-dependent polymerization of perforin, in addition to the uptake of granzymes. These cytotoxic granules are responsible for downstream activation of caspases and subsequent DNA fragmentation, resulting in target cell apoptosis [8,9]. Granulysin, a recently identified antimicrobial molecule produced by CD8+ and NK cells [10], has also been associated with CD8+ T cell killing of Cryptococcus neoformans and Mycobacterium tuberculosis [11–13]. Circulating granulysin levels are related to T- and NK-cell activity, and may thus reflect protection-associated cellular immune responses [14].

Lymphocyte-mediated anti-fungal activity is dependent upon IL-15, as it is an important growth factor for T cells and NK cells [15]. IL-15 is a potent chemoattractant [16] and controls both proliferation and survival of naive and memory CD8+ T cells [17,18]. Furthermore, mice lacking IL-15, or the IL-15 receptor alpha (IL-15Rα), are deficient in memory-phenotype CD8+ T cells [17,19] and are highly susceptible to infection with M. tuberculosis [20]. Lymphocyte-mediated anti-fungal activity is dependent on IL-15, as demonstrated in C. neoformans [11,21] and Candida albicans infection [22].

In the present study, we sought to assess the activation state and production of cytotoxic granules (granzyme A, B and perforin) by the peripheral blood T cells of patients with the adult and juvenile forms of PCM, and controls. We also included a group of healthy individuals with PCM infection (PI), since it was demonstrated that these individuals exhibit a strong cellular response against the fungus and are considered resistant to the disease [23]. The comprehension of the immunoprotective mechanisms that block the development of the disease in these individuals could contribute to a better understanding of PCM physiopathology.

**Methods**

**Subjects**

We analyzed the peripheral blood cells of patients with newly-diagnosed PCM, at the University Hospital, UNICAMP, Campinas, São Paulo Brazil. The diagnosis was established by the detection of the fungus in clinical specimens and through serology (immunodiffusion test). The patients were grouped, according to clinical form, as juvenile (JF, n = 11) and adult forms (AF, n = 11). We also analyzed eight healthy individuals living in endemic areas with PCM-infection (PI), i.e., those that were presenting asymptomatic infection caused by P. brasiliensis, characterized by a positive skin test to paracoccidioidin [2] and paracoccidioidin non-reactive controls (C). This study was approved by the Ethics Committee of the State University of Campinas Medical School and informed consent was obtained from each participant.

**Delayed-type hypersensitivity (DTH) reaction**

Skin tests were performed on the left forearm by intradermal injection of 100 μl of P. brasiliensis gp43 (7.5 μg), and considered positive when an induration larger than 5 mm was observed after 24 h [24].

**P. brasiliensis yeast cells**

P. brasiliensis of the Pb18 (highly virulent) and Pb265 (low virulence) strains were maintained by weekly sub-cultivation in the semisolid culture medium at 36°C, as described elsewhere [25]. The yeast cells were washed in phosphate-buffered saline (PBS; pH 7.2) and adjusted to 2 × 10^6/ml. Viability was determined with Janus green B vital dye (Merck, Darmstadt, Germany) and was always higher than 85%.

**Culture conditions**

Peripheral blood was obtained by vein puncture from patients and healthy controls in tubes with heparin. Whole blood cultures were performed (500 μl in 1640 RPMI supplemented with 10% AB serum, 2% L-glutamine and antibiotics) and stimulated with P. brasiliensis yeast cells (Pb265 or Pb18) for 5 days at 37°C in a CO2 incubator. The cells were then stimulated with IL-15 (50 ng/ml) for 48 h. At the end of the period, the cells were harvested, washed with sodium azide-PBS (2 mM) and lysed with FACS Lysing Solution (Becton & Dickinson). Finally the cells were washed in staining buffer (PBS + BSA + azide 2 mM) and adjusted to 1 × 10^7/ml on a hemacytometer counter.

**Flow cytometry**

Ex-vivo and cultured cells were collected and labeled with monoclonal antibodies against surface molecules (CD69-FITC, CD4-PE, CD8-CyChrome, anti-IL-15Rα and isotype controls) and were fixed and permeabilized. Cells were then labeled with monoclonal antibodies against cytoplasmic granules and cytokines (granzyme A-FITC, granzyme B-FITC, perforin-PE). All labeled antibodies were from BD – Biosciences (San José, CA, USA), except biotinylated...
anti-IL-15Ra (R&D Systems, Minneapolis, MN, USA). Cell fluorescence was detected with a flow cytometer (FACScalibur, Becton & Dickson) and analyzed with De Novo Software – FCS Express (Los Angeles, CA, USA). In all cases at least 10,000 gated events were acquired. Results were expressed as the percentage of positive cells.

Sera

The detection of granulysin was determined in a group of individuals that included those used for cell analysis, as described above. Serum samples were obtained at baseline (T0) and after 1–2 years of antifungal therapy (T1). Sera from patients with the JF (n = 18) and AF (n = 16) of PCM were analyzed, as well as sera from PI individuals (n = 8) and controls (n = 9).

Granulysin detection by ELISA

Ninety six-well plates were coated with anti-granulysin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), (1:5,000 dilution in Tris Buffer Saline, TBS), without protein addition (pH 7.2), overnight at 25°C. The plates were washed twice with TBS (pH 7.2). The remaining binding sites were blocked with TBS-5% nonfat dry milk for 2 h at 37°C. After three washes with TBS (pH 7.2), serum samples were added in duplicate, and the plates were incubated for 1.5 h at 37°C. The wells were washed four times with TBS (pH 7.2) and the anti-granulysin antibody (rabbit anti-human, a gift from C. Clayberger, Stanford University) was added, diluted at 1:5,000 in TBS-BSA-0.1%, pH7.2. After 1.5 h of incubation at 37°C, the plates were washed again, and anti-rabbit biotinylated antibody (1/1000 dilution, in TBS-BSA-0.1% pH7.2) was added. After 1.5 h incubation at 37°C and four washes with TBS, the streptavidin-peroxidase conjugate bronchoalveolar lavage was added (1:1,000 dilution in TBS-BSA-0.1%, pH7.2), the plates were then incubated for 1.5 h at 37°C and washed four times with TBS-0.05% Tween 20, pH 7.2. The substrate solution (TMB-H2O2, in sodium-acetate buffer, pH 5.5) was added, and the reaction was stopped by the addition of H2SO4. The optical densities (ODs) were read in an ELISA reader (Bio Rad) at 450 nm. Results were expressed as granulysin units (an arbitrary value calculated as the absorbance of the test sample minus the absorbance of low concentration serum pool divided by the absorbance of the high concentration serum pool). The low and high concentration pools were prepared using at least four different sera from patients with PCM (low) or normal controls (high) and used in all plates.

Statistical analysis

Patients (AF and JF), PI individuals and the control group were compared before and after IL-15 treatment using the paired t test. In order to compare the granulysin production for the groups, ANOVA followed by the student t-test was used. Significance was defined as P≤0.05.

Results

(a) CD69 expression

The ex-vivo expression of CD69, an activation marker, on peripheral blood lymphocytes of all individuals was very low and no significant differences were detected among the groups. Numbers of CD8+CD69+ cells, however, tended to be lower in JF patients. Peripheral blood cells cultured for 5 days in the absence of P. brasiliensis yeast cells also exhibited low expression of CD69 with the lowest levels observed in CD8+ cells from controls and patients with the JF of PCM and for CD4+ cells in patients with JF and AF of the disease (Fig. 1).

IL-15 is required to elicit an efficient activation and development of CD8+ T cell-mediated response. In cells from controls and PI individuals, cultured in the absence or in the presence of P. brasiliensis yeast cells (strain Pb18 or Pb265), the addition of IL-15 promoted a significant enhancement in CD69+ expression in CD8+, as well as in CD4+ T cells, under all conditions. However, significant differences were detected mainly in PI and control individuals (Fig. 1). In patients with the adult form of PCM, IL-15 induced a lower increase in CD69 expression, as compared with the controls and the PI group. The analyses of cells from JF and AF patients showed a less pronounced effect of IL-15 on CD69 expression, with a significant increase only in non-stimulated CD8+ cells from JF patients (Fig. 1A) and non-stimulated CD4+ cells from AF patients (Fig. 1B). Moreover, CD8+ and CD4+ cells from AF patients presented an increase in the number CD69 molecules per cell (as demonstrated by Mean Fluorescence Intensity; MFI), which were expressed after IL-15 addition in the presence of Pb265 (data not shown).

In general, the increase in CD69 expression promoted by IL-15 was higher in non-stimulated cells than those following stimulation by P. brasiliensis yeast cells. Moreover, the inhibition promoted by the fungus was more evident in CD8+ than in CD4+ cells.

(b) Granzyme A expression

The constitutive expression of granzyme A was high in all groups, especially in CD8+ cells. No differences were detected among cultured cells in the presence or in the absence of P. brasiliensis yeast cells. Addition of IL-15 to the cells promoted a slight increase in granzyme A expression. The only significant differences induced by IL-15 were in non-stimulated CD8+ cells of the
Fig. 1 Percentage of CD8+ T cells (A), and CD4+ (B) expressing CD69 ex-vivo or after 5 days in culture, when not stimulated (no fungus) or stimulated with *Paracoccidioides brasiliensis* (Pb18 or Pb265) yeast cells in the presence (+) or absence (−) of IL-15 (50 ng/ml) in controls (C), individuals with PCM-Infection (PI), and patients with the adult (AF) or juvenile form of PCM (JF). Data are expressed as means ± SEM, t-test *P < 0.05, **P < 0.005.

control group and in Pb18-stimulated CD8+ cells from PI individuals (Fig. 2A). In relation to the CD4+ subset, a significant difference was induced by IL-15 in Pb18-stimulated cells from patients with the adult form of PCM (Fig. 2B).

(c) **Granzyme B expression**

*Ex-vivo* expression of granzyme B+ was high in CD8+ cells, as compared to CD4+ cells, in all groups. In contrast to granzyme A, the effect of IL-15 on granzyme B expression was more obvious. The frequency of CD8+ granzyme B+ T cells in the control and PI groups was enhanced after 5 days of their incubation in the absence or in the presence of Pb265 yeast cells (Fig. 3A). Interestingly, CD4+ T cells of AF patients presented a significant increase in granzyme B expression, even in the presence of the highly virulent strain of *P. brasiliensis* (Pb18) (Fig. 3B).

(d) **Perforin expression**

The *ex-vivo* expression of perforin was higher in CD8+ T cells from PI individuals and JF patients than in the other groups, but there was a great variability among subjects in each group. After IL-15 stimulation the frequency of CD8+ perforin+ cells increased only in PI individuals in the presence of both Pb18 and Pb265 yeast cells. In patients with the severe form of PCM (JF), IL-15 induced a significant increase in perforin expression only in CD4+ cells in the presence of the highly virulent strain of *P. brasiliensis* (Fig. 4B).

(e) **Ex-vivo expression of IL-15Rα**

Considering the low response of lymphocytes of patients with PCM to IL-15, we next examined the *ex-vivo* expression of IL-15Rα. The analysis of total lymphocytes and CD8+ T cell did not show any significant difference among the groups. However, in relation to the CD4+ T cell
population, patients with the adult and juvenile forms of PCM showed a significantly lower expression of IL-15Rα than PI individuals (Fig. 5).

(f) Serum granulysin levels
Granulysin is an antimicrobial molecule that is able to directly kill microorganisms. Patients with PCM had significantly lower serum granulysin levels as compared to controls (C and PI) but no significant differences were observed between the juvenile and adult forms of the disease (Fig. 6A). We also examined serum granulysin levels in some patients with the juvenile and adult forms of PCM before and after antifungal treatment. As a result we observed that for almost 70% of patients, granulysin concentrations tended to increase, although in different degrees, by the end of treatment (around 1.5 years) (Fig. 6B).

Discussion
In diseases caused by intracellular microorganisms, CD8+ T cells are known to be essential in infection control. It has been shown in experimental PCM, that fungal loads are mainly controlled by CD8+ T cells, whereas antibody production and DTH reactions are regulated by CD4+ T cells [26].

The aims of this study were to verify the activation state, both ex-vivo and in vitro, as well as the production of cytotoxic molecules, by peripheral blood lymphocytes (CD8+ and CD4+) from patients with PCM, PI individuals and controls, after stimulation with P. brasiliensis yeast cells.

The ex-vivo data showed a tendency towards a higher constitutive expression of the activation marker CD69, granzyme A, B and perforin in PI individuals, than in PCM patients (IF and AF). This condition could enable CD4+ and CD8+ cells from PI individuals to mount a more effective cytotoxic response against the fungus, as compared to patients. We have recently shown that CD8+ and NK cells...
from patients with active PCM present a lower fungicidal and cytotoxic response to *P. brasiliensis* than PI individuals [27].

The ability of CD8\(^+\) T cells to respond to IL-15 is critical to the development of protective immunity, considering that this cytokine is essential for activation and expansion of effector and memory CD8\(^+\) T cells [28]. The addition of IL-15 to the cell cultures induced a significant increase in the activation marker, CD69, only in CD8\(^+\) and CD4\(^+\) T cells from controls and PI individuals. This occurred in the absence of stimulation, as well as in the presence of both strains of *P. brasiliensis*. On the other hand, IL-15 induced an increase in the frequency of CD69\(^+\) cells in patients, but a statistically significant increment was observed only in the absence of stimulation. In general, the influence of IL-15 was greater in non-stimulated cultures than in the presence of fungal cells. These data are in accordance with those of Liu *et al.* who showed that IL-15 is not only a crucial growth factor, but is also an antigen-independent activator of effector functions in CD8\(^+\) memory T cells [29].

Major fungal inhibition of cell activation in response to IL-15 was observed in the CD8\(^+\) T cells subset in the patients with the juvenile form of the disease. The mean fluorescence intensity (MFI) of CD69 was also increased by IL-15 but only in cultures with the low virulent strain of *P. brasiliensis*. These results confirm the highly virulent potential of the Pb18 strain (data not shown). PI individuals presented higher CD8\(^+\) CD69\(^+\) T cells number after IL-15 addition, even in the presence of the *P. brasiliensis* highly virulent strain. In experimental tuberculosis, it has been demonstrated that IL-15 is necessary to regulate the expansion and the effector mechanisms of CD8\(^+\) T cells [30,31]. IL-15 is a potent inducer of both pro-inflammatory and antifungal activities of polymorphonuclear cells, activating several antimicrobial functions involved in the cellular response against *C. albicans* [32]. Neutrophils from healthy donors pretreated with IL-15 show enhanced fungicidal activity against *P. brasiliensis* with elevated production of superoxide and H\(_2\)O\(_2\) [33].

IL-15 treatment induced a modest increase in granzyme A and B expression, although an increased on MFI expression

Fig. 3 Percentage of CD8\(^+\) (A), and CD4\(^+\) (B) T cells expressing granzyme B *ex-vivo*, or after 5 days in culture, when not stimulated (no fungus) or stimulated with *Paracoccidioides brasiliensis* (Pb18 or Pb265) yeast cells in the presence (+) or absence (−) of IL-15 (50 ng/ml) in controls (C), individuals with PCM-Infection (PI), and patients with the adult (AF) or juvenile form of PCM (JF). Data are expressed as mean ± SEM, t-test *P < 0.05, **P < 0.005.
Fig. 4 Percentage of CD8⁺ (A), and CD4⁺ (B) T cells expressing perforin ex-vivo, or after 5 days in culture, when not stimulated (no fungus) or stimulated with *P. brasiliensis* (Pb18 or Pb265) yeast cells in the presence (+) or absence (−) of IL-15 (50 ng/ml) in controls (C), individuals with PCM-Infection (PI), and patients with the adult (AF) or juvenile form of PCM (JF). Data are expressed as mean ± SEM, t-test *P < 0.05, **P < 0.005.

was verified in CD8⁺ T cells from PI individuals, even in presence of highly virulent yeasts (data not shown). Patients with the adult form of the disease showed a better capacity to respond to the Pb18 strain than those with the juvenile form of PCM. The CD4⁺ T cells of these individuals increased their granzyme A and B expression after IL-15 addition, confirming different responses between the two clinical forms of PCM. In experimental PCM, the CD4⁺ T cells display a protective role in the resistant and intermediate mouse strains, whereas in susceptible mice they are deleted or anergic [34].

CD8⁺ T cells of PI individuals also showed an increase in perforin expression in response to IL-15, as compared to patient and control cells. The ability to produce high levels of perforin in response to IL-15 may suggest an increased capacity to kill *P. brasiliensis* and indicates that perforin is an important effector molecule in this system. The expression of perforin by CD4⁺ T cells was also higher in PI individuals after IL-15 stimulation but, due to a great variability among the subjects, the differences among the groups were not statistically significant.

CD4⁺ T cells exert a regulator and an effector function during the immune response, mediated by the production
of cytokines [35]. However, the ability of CD4+ T cells to become cytotoxic against alloantigens, microbe-infected cells and cancer has been reported over the past two decades in both mouse and human studies [36,37]. Previous studies in mice demonstrate that CD4+ cytotoxic T lymphocytes (CTL) cause target cell lysis primarily via Fas/FasL interactions [36]. However, other studies on human CD4+ T cells have demonstrated that CD4+ CTL also employ perforin and a granzyme-based granule exocytosis pathway [38,39].

The deficient response of PCM patients to IL-15 stimulation led us to investigate the expression of IL-15Rα on their lymphocytes. Constitutive IL-15Rα expression was low and similar on total lymphocytes, as well as on the CD8+ T cell subset. However, a higher frequency of CD4+IL-15Rα+ cells was observed in PI individuals compared to the JF and AF PCM patients. In addition to its role in adaptive immunity, IL-15 is able to stimulate the oxidative burst of neutrophils against P. brasiliensis [33], Aspergillus [40], Fusarium spp. and Saedosporium spp. [41]. Therefore, the low expression of IL-15Rα by lymphocytes could have important impact on innate, as well as cytotoxic responses, as observed in PCM patients. It has been shown that coordinate expression of IL-15 and IL-15Rα in the same cell increases the stability and secretion of both molecules as a complex. In the absence of co-expressed IL-15Rα, a large portion of the IL-15 produced is rapidly degraded immediately after synthesis, impairing the expansion of NK and T cells in the lungs, liver and spleen of mice [42]. The murine infection by P. brasiliensis results in heavy fungal loads in these organs. The lungs are also frequently involved in human PCM, thus, lower IL-15 Rα expression could impair sustained IL-15 activity and consequently the expansion and activation of cytotoxic effector cells in these sites [43].

The relationship between granulysin production and host defense against microbial pathogens has been reported in leprosy. Granulysin-expressing T cells were detected in cutaneous leprosy lesions at a six-fold greater frequency in patients with the localized tuberculoid form, as compared with the disseminated lepromatous form of the disease [44]. Plasma granulysin levels correlate with curative host response in tuberculosis, and are reported to increase after completion of tuberculosis therapy [45]. In this study, the granulysin concentration in PCM serum showed reduced levels, as compared with the PI individuals and controls. In addition, a slight but not significant increase in granulysin levels were observed during antifungal treatment, in most of the patients analyzed, indicating the association of high granulysin levels in serum with response to antifungal therapy and improvement of the cytotoxic response.

Taken together, these results indicate that lymphocytes of PCM patients present a lower activation status, reduced expression of the IL-15 receptor and low cytotoxic granule protein expression (granzyme A, B, perforin and granulysin), as compared with the PI group. These findings, in association with other mechanisms that compromise cellular immunity, could result in a deficient cytotoxic activity, and a consequently reduced capacity to kill the fungus.

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