Interleukin-15 augments oxidative metabolism and fungicidal activity of human monocytes against *Paracoccidioides brasiliensis*

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Interleukin (IL)-15 is a pleiotropic cytokine that regulates the proliferation and survival of many cell types. IL-15 is produced by monocytes and macrophages against infectious agents and plays a pivotal role in innate and adaptive immune responses. This study analyzed the effect of IL-15 on fungicidal activity, oxidative metabolism and cytokine production by human monocytes challenged in vitro with Paracoccidioides brasiliensis *(Pb18), the agent of paracoccidioidomycosis. Peripheral blood monocytes were pre-incubated with IL-15 and then challenged with Pb18. Fungicidal activity was assessed by viable fungi recovery from cultures after plating on brain-heart infusion-agar. Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), tumour necrosis factor-alpha (TNF-α), IL-6, IL-15 and IL-10 production by monocytes were also determined. IL-15 enhanced fungicidal activity against Pb18 in a dose-dependent pattern. This effect was abrogated by addition of anti-IL-15 monoclonal antibody. A significant stimulatory effect of IL-15 on O_2^- and H_2O_2 release suggests that fungicidal activity was dependent on the activation of oxidative metabolism. Pretreatment of monocytes with IL-15 induced significantly higher levels of TNF-α, IL-10 and IL-15 production by cells challenged with the fungus. These results suggest a modulatory effect of IL-15 on pro and anti-inflammatory cytokine production, oxidative metabolism and fungicidal activity of monocytes during Pb18 infection.*

Key words: IL-15 - *Paracoccidioides brasiliensis* - monocytes - hydrogen peroxide - fungicidal activity - cytokines

Interleukin (IL)-15 is a cytokine originally discovered in 1994 as having a T cell stimulatory activity present in the culture supernatant of a simian kidney epithelial cell line that requires β and γ chains of IL-2 receptor for binding and signaling (Grabstein et al. 1994). A special feature of IL-15 is that it shares important functional attributes with IL-2, including enhanced proliferation, survival and differentiation of many distinct cell types as natural killer cells (NK), αβ and γδ T cells, B cells, macrophages and neutrophils (Carson et al. 1994, Grabstein et al. 1994, Nishimura et al. 1996, Yoshikai & Nishimura 2000, D'Agostino et al. 2004). Sharing of receptor subunits between IL-2R/IL-15R chains can explain part of the existing functional similarities. IL-15 is a cytokine of innate immunity that modulates selected adaptive immune responses (Van Belle & Grooten 2005); IL-15 mRNA is constitutively expressed in a large variety of cell types and plays a role in a broad spectrum of bio-regulatory purposes (Grabstein et al. 1994, Musso et al. 1999). Few reports describe the response of monocytes and macrophages to stimulation with IL-15 through an increase in phagocytosis and microbial clearance and increased production of IL-8, IL-12 and monocyte chemotactic protein-1, which in turn attract monocytes and neutrophils, leading to inflammatory cell accumulation (Vázquez et al. 1998, D'Agostino et al. 2004). The modulatory effect of IL-15 on the host response to infectious agents has also been reported, suggesting that this cytokine may have a potent protective role against a variety of microorganisms (Nishimura et al. 1996, Winn et al. 2003, Forcina et al. 2004). The information provided by the literature therefore indicates that IL-15 is especially produced by monocytes and macrophages in response to infectious agents and that it constitutes an important pro-inflammatory cytokine (Carson et al. 1994).

Infection with *Paracoccidioides brasiliensis* (Pb18), a dimorphic fungus, can result in paracoccidioidomycosis (PCM), which is a systemic mycosis endemic and prevalent in Latin America that infects individuals via the respiratory route. Inhaled fungal spores reach alveolar spaces, where they are ingested by resident macrophages, and depending on the subsequent immune mechanism, the disease can develop as an acute systemic or as a chronic localized mycosis (Franco 1987, Franco et al. 1989, Brummer 1994). The aetiological agent is considered an intracellular pathogen, which makes studies of its interaction with phagocytic cells essential to understand the host-parasite relationship. However, the modulation of the mononuclear phagocyte during Pb18 interactions has not been fully elucidated. Previous studies showed the lack of fungicidal activity by human monocytes against the highly virulent Pb18, even after IFN-γ activation. These results were associated with the capacity of the fungus to induce prostaglandins, as reflected in the significant fungicidal activity detected after treatment of monocytes with indomethacin, a cyclooxygenase inhibitor (Soares et al. 2001, Bordon et al. 2007). Monocytes/

macrophages play an important role in the immune response in certain mycoses and they are involved in the innate mechanisms against fungal infections (Smith et al. 1990, Louie et al. 1994). Several studies have reported in vitro stimulation of human monocytes/macrophages with different fungi or their cell wall components such as *Coccidioides immitis* (Dooley et al. 1994), *Cryptococcus neoformans* (Vecchiarelli et al. 1995) and *P. brasiliensis* (Anjos et al. 2002, Peraçoli et al. 2003, Kurokawa et al. 2007), suggesting that these cells are the main source of tumor necrosis factor-alpha (TNF-α), IL-1, IL-6 and IL-10 production and that they play a pivotal role in the immune response against these fungi.

The purpose of the present study was to evaluate whether human monocytes stimulated with IL-15 have the capacity to enhance fungicidal activity against Pb18 and this effect is related to the release of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which are the metabolites involved in Pb18 killing by human monocytes. In addition, the effect of IL-15 on the levels of pro and anti-inflammatory cytokines produced by monocytes such as TNF-α, IL-6, IL-15 and IL-10 was investigated.

SUBJECTS, MATERIALS AND METHODS

Subjects - Twenty-five healthy blood donors from the University Hospital of Botucatu Medical School, São Paulo State University, Brazil, who tested negative for paracoccidioidin (age range 20-50 years), were included in this study. The Research Ethical Committee of Botucatu Medical School approved the study and an informed consent was obtained from all the blood donors.

Fungus - The highly virulent Pb18 was used in this study. Pb18 yeast cells were maintained by weekly subculture in the yeast form at 35ºC on 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar medium (GPY medium, all from Gibco Laboratories, Grand Island, NY, USA) and used on the 6th day of culture. Yeast viability was determined by phase contrast microscopy and bright yeast cells were counted as viable, whereas dark cells were considered not viable (Soares et al. 2001). Fungal suspensions containing more than 95% viable cells were used in the experiments.

Monocytes culture - Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood samples by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, Inc, St. Louis, MO, USA). The PBMC suspension was stained with neutral red (0.02%), which is incorporated by monocytes and allows their identification and counting. Monocyte preparations routinely contained > 90% monocytes as determined by morphological examination and staining for nonspecific esterase (Li et al. 1973). After incubation for 2 h at 37ºC in 5% CO_2, non-adherent cells were removed from the plates and each well was rinsed twice with Roswell Park Memorial Institute (RPMI)-1640. The adherent cells, which were monocytes, were suspended to 2×10^6 or 1×10^6 monocytes/mL in a complete tissue culture medium consisting of RPMI-1640 (Sigma-Aldrich) supplemented with 2 mM of L-glutamine (Sigma-Aldrich), 40 µg/mL of gentamicin (Gibco Laboratories) and 10%

heat-inactivated autologous human serum. Monocyte suspensions were dispensed in 96-well flat-bottom plates (Nunc, Life Tech Inc, MD, USA) and treated with IL-15 at different concentrations (50, 25 and 12.5 ng/mL) (R&D Systems, Minneapolis, MN, USA) during 24 h for fungicidal activity, O_2^- and H_2O_2 assays. Addition of 1 µg/mL anti-IL-15 monoclonal antibody (MoAb) (R&D Systems) was performed in some experiments for IL-15 neutralization, according to the manufacturer's instructions. Cell suspensions containing 1×10^6 monocytes/mL were dispensed in 24-well flat-bottom plates (Nunc) for culture supernatant cytokine measurement assays.

Fungicidal activity assay - Following IL-15 and anti-IL-15 IgG1 MoAb treatments, monocyte cultures were washed with RPMI medium and challenged for 4 h in 5% CO_2 at 37ºC with 100 µL of a Pb18 suspension containing 4×10^4 fungal cells/mL (in a ratio of 50 monocytes per 1 fungal cell) prepared in complete medium plus 10% fresh human AB serum, as the source of complement for yeast opsonization. Co-cultures of monocytes and fungus were harvested by aspiration with sterile distilled cold water to lyse the monocytes. Each well resulted in a final volume of 2.0 mL and 100 µL of this preparation was plated on supplemented brain-heart infusion-agar medium (Difco Laboratories, Detroit, MI, USA) plates containing 0.5% of gentamicin, 4% horse normal serum and 5% *P. brasiliensis* strain 192 culture filtrate (v/v), which constituted the source of growth-promoting factor (Singer-Vermes et al. 1992). Experiments using neutralizing anti-IL-15 MoAb were performed to analyze the role of IL-15 on fungicidal activity. The inoculum used for the challenge was plated under the conditions described above. The plates containing the material obtained from the monocyte-fungus co-cultures were considered the experimental plates and those plated with the fungus inoculum alone and counted at time zero, were used as controls. The inoculated plates were incubated in triplicates for each culture at 37ºC in sealed plastic bags to prevent drying. After 10 days, the number of colony forming units (CFU) per plate was counted. Fungicidal activity percentage was determined by the following formula: % fungicidal activity = [1 - (mean CFU recovered on experimental plates/mean CFU recovered on control plates)] x 100.

Respiratory burst assay - O_2^- production was measured with an assay for superoxide dismutase-inhibitable cytochrome c reduction and H_2O_2 release was measured by the horseradish peroxidase-phenol red oxidation method described by Russo et al. (1989). Briefly, after monocyte activation for 24 h in 5% CO_2 at 37ºC with IL-15 at different concentrations (50, 25 and 12.5 ng/mL), the monocyte cultures were washed with RPMI medium and challenged with 100 µL of a Pb18 suspension prepared with 4×10^4 yeast/mL plus 10% fresh human AB serum diluted in cytochrome c partially acetylated from horse heart (Sigma-Aldrich) for O_2^- assay. For H_2O_2 assay, 100 µL of Pb18 suspension were prepared with 4×10^4 yeasts/mL in phenol red buffer containing 50 µg/mL of horseradish peroxidase type II (Sigma-Aldrich). The O_2^- and H_2O_2 reactions were incubated for 60 min in 5% CO_2 at 37ºC and the H_2O_2 assay was stopped by addition

of 10 μL of 1N NaOH. The absorbance at 550 nm and 620 nm for the O_2^- and H_2O_2 assays, respectively, were determined spectrophotometrically for each sample supernatant with a micro-enzyme-linked immunosorbent assay (ELISA) reader (MD 5000; Dynatech Laboratories Inc, Chantilly, VA, USA). The O_2^- was calculated by using the extinction coefficient for reduced cytochrome c and transformed into nanomoles $O_2^-/2$ x 10^5 cells. An H_2O_2 standard curve was produced with serial dilutions from 0.5-8 nanomoles to convert absorbance into nanomoles $H_2O_2/2$ x 10^5 cells.

Cytokine measurement - The monocytes were cultured in the presence of 10 μg/mL *Escherichia coli* O55B5 lypopolisaccharide (LPS) (Sigma-Aldrich), which was used as the positive control culture and 50 ng/mL IL-15 (R&D Systems) during 24 h. Then, monocyte cultures were washed with RPMI medium and challenged with Pb18 for 18 h. Cytokine concentrations were determined in monocyte culture supernatants by ELISA, using Quantikine ELISA kits (R&D Systems) for TNF-α, IL-6, IL-15 and IL-10 according to the manufacturer's instructions.

Statistical analysis - The results were compared by analysis of variance (ANOVA) followed by Tukey's test using INSTAT 3.05 software (GraphPad San Diego, CA, USA). The level of significance was set at $p < 0.05$.

RESULTS

IL-15 increases fungicidal activity - The fungicidal activity of human monocytes against Pb18 was significantly enhanced when the cells were treated with IL-15 for 24 h. Monocytes activated with IL-15 concentrations of 50, 25 and 12.5 ng/mL killed 47.3, 36.3 and 23.2% of growing Pb18 ($p < 0.05$), respectively, showing a dose-dependent pattern. Experiments using a neutralizing antibody against IL-15 showed that the fungicidal activity was inhibited by treatment with the anti-IL-15 IgG1 MoAb (Fig. 1).

IL-15 increases the oxidative metabolism of human monocytes - To determine whether the Pb18 killing by IL-15-activated monocytes was dependent on oxidative metabolism, the levels of O_2^- and H_2O_2 were evaluated. Notably, treatment of monocytes with IL-15 led to higher levels of O_2^- release when compared with monocytes cultured without stimulus. Human monocytes treated with IL-15 at concentrations of 50, 25 and 12.5 ng/mL for 24 h showed a significant increase in O_2^- production for all doses of IL-15 employed when compared to untreated control cultures (Fig. 2). Higher levels of O_2^- production were also observed in cultures of monocytes challenged with Pb18 in comparison with control cultures. When these infected cells were pretreated with IL-15, a significant increase in O_2^- was observed with 50 ng/mL of IL-15 in comparison with the Pb18 culture alone.

Monocytes cultured with RPMI medium alone released basal levels of H_2O_2, whereas incubation with IL-15, especially at the concentration of 50 ng/mL, induced significantly higher levels of H_2O_2. Fig. 3 shows that the Pb18 challenge had an inhibitory effect on H_2O_2 release from monocytes, with levels significantly lower than those observed under other culture conditions. However, activation of these cells with different concentrations of IL-15 for 24 h followed by challenge with the fungus resulted in higher levels of H_2O_2 released by monocytes in comparison to cells without stimulus and with Pb18. The results therefore indicate that the inhibitory effect of Pb18 on H_2O_2 release was reversed by IL-15, especially at a concentration of 50 ng/mL.

IL-15 modulates cytokine production by monocytes - Supernatants recovered from cells treated with IL-15 (50 ng/mL) for 24 h and challenged with Pb18 for 18 h were evaluated for TNF-α, IL-6, IL-15 and IL-10 production. LPS was employed as the positive control in all
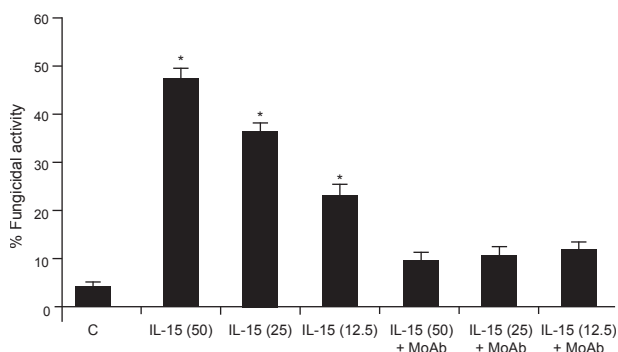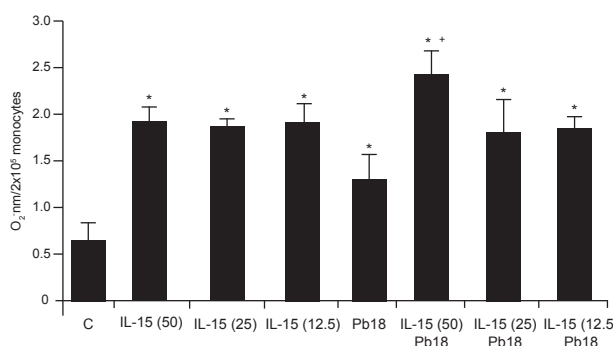

Fig. 1: interleukin (IL)-15 increased fungicidal activity of human monocytes against *Paracoccidioides brasiliensis* (Pb18). Monocytes were treated with IL-15 or IL-15 plus anti-IL-15 monoclonal antibody (MoAb IgG1) during 24 h and then challenged with Pb18 in a ratio of 50 monocytes per one fungus for 4 h. The results are expressed as mean ± standard error of mean from triplicate cultures of monocytes obtained from 26 subjects. Asterisk means $p < 0.05$ vs. control (C) and IL-15 + MoAb (50, 25 and 12.5 ng/mL).

Fig. 2: interleukin (IL)-15 augments superoxide anion (O_2^-) production by monocytes challenged with *Paracoccidioides brasiliensis* (Pb18). Cells were assayed after monocytes treatment with IL-15 (50, 25 and 12.5 ng/mL) during 24 h and challenge with Pb18 in a ratio of 50 monocytes per one fungus. The results are expressed as mean ± standard error of mean from triplicate cultures of monocytes obtained from 25 subjects. Asterisk means $p < 0.05$ vs. control (C). +: $p < 0.05$ vs. Pb18.
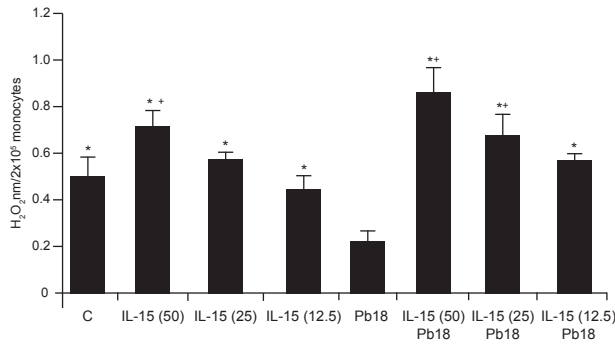
Fig. 3: interleukin (IL)-15 stimulate hydrogen peroxide (H_2O_2) release by monocytes challenged with *Paracoccidioides brasiliensis* (Pb18). Cells were assayed after monocytes incubation with IL-15 (50, 25 and 12.5 ng/mL) during 24 h and challenged with Pb18 in a ratio of 50 monocytes per one fungus. The results are expressed as mean ± standard error of mean from triplicate cultures of monocytes obtained from 25 subjects. Asterisk means $p < 0.05$ vs. control (C). +: $p < 0.05$ vs. Pb18.

cultures. As shown in Fig. 4A, TNF-α production by human monocytes was significantly increased after IL-15 activation. High levels of TNF-α were observed after the fungus challenge, showing an enhanced effect of IL-15 on cell stimulation with Pb18. The results show that IL-15 is able to increase TNF-α production by uninfected cultures after 24 h of treatment and by cells challenged in vitro with Pb18.

The production of IL-6 by human monocytes was significantly higher after activation with IL-15 than in control cultures. A significant increase in IL-6 release was observed when monocytes were challenged with Pb18 or were pre-activated with IL-15 and challenged with the fungus in comparison with cultures without stimulus. However, treatment with IL-15 did not affect the production of this cytokine when monocytes were challenged with Pb18 (Fig. 4B).

Monocyte cultures treated with IL-15 for 24 h reflected the autocrine effect of IL-15, as significantly higher levels of IL-15 were observed in these cell cultures without stimulus (Fig. 4C). Pb18 induced higher levels of IL-15 in human monocytes and when these cells were treated with IL-15, the cytokine production was significantly increased.

The levels of IL-10 detected after treatment with IL-15 are shown in Fig. 4D. Pb18 induced higher levels of IL-10 when compared with non-stimulated cells. However, IL-10 production in monocytes activated by IL-15 and challenged with Pb18 was significantly higher than in cultures challenged only with the fungus.

DISCUSSION

Phagocytic cells play a critical role in host defenses, enhancing their function mainly after cell stimulation with recombinant inflammatory cytokines. Because IL-15 has low cytoxicity in vivo (Munger et al. 1995), the ability of this cytokine to modulate human monocyte function against Pb18 was examined to understand the
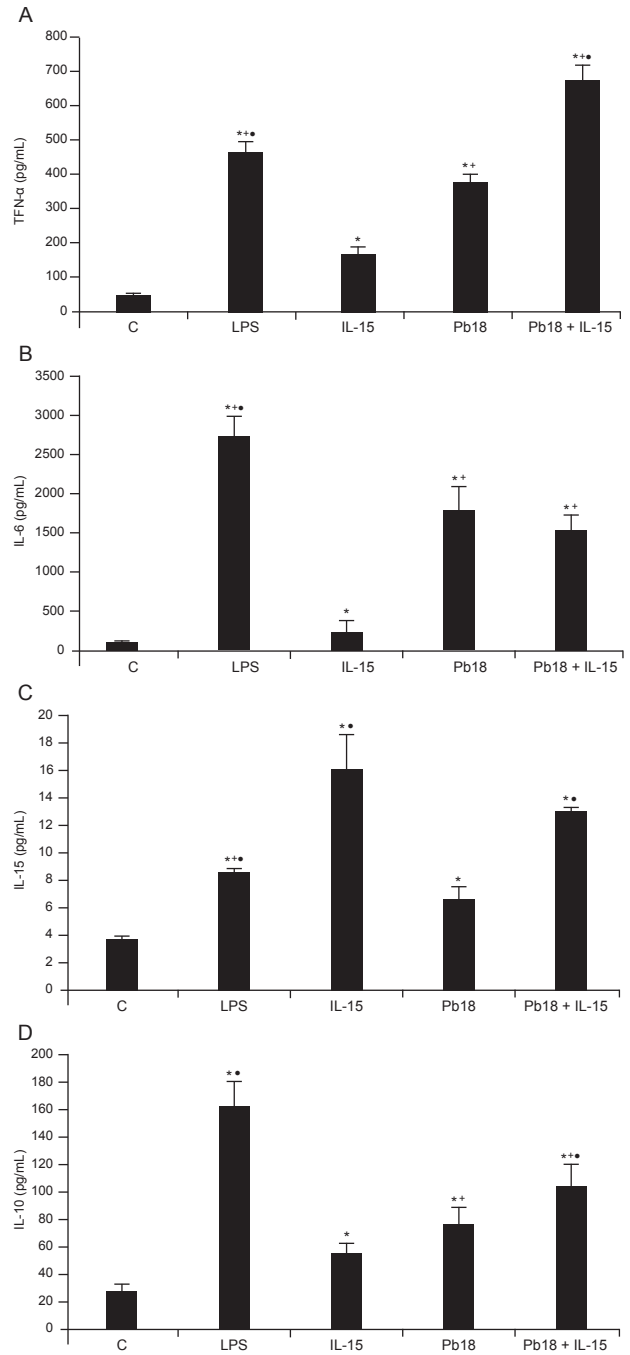

Fig. 4: effect of interleukin (IL)-15 on tumor necrosis factor-alpha (TNF-α) (A), IL-10 (B) IL-6 (C) and IL-15 (D) production. Monocytes were cultured with IL-15 (50 ng/mL) during 24 h and challenged with *Paracoccidioides brasiliensis* (Pb18) in a ratio of 50 monocytes per one fungus during 18 h. The results are expressed as mean ± standard error of mean and derived from triplicates cultures of monocytes obtained from 25 subjects. Asterisk means $p < 0.05$ vs. control (C). LPS: lypopolisaccharide; +: $p < 0.05$ vs. IL-15; •: $p < 0.05$ vs. Pb18.

host defense mechanisms against the fungus. The present study therefore focused on investigating the effect of IL-15 on the induction of Pb18 killing by human monocytes, which is closely associated with the innate immune response.

The present results indicate that in vitro treatment with IL-15 significantly increases monocyte fungicidal activity against Pb18, mainly at a concentration of 50 ng/mL, showing that IL-15 potentiates antimicrobial activity in these cells. In addition, the results demonstrated that monocyte treatment with a MoAb anti-IL-15 abrogated this fungicidal activity. Prior studies showed that IL-15 and IL-2 significantly contributed to lymphocyte proliferation and lymphocyte-mediated anticryptococcal activity in both, encapsulated and acapsular *C. neoformans* (Mody et al. 1998). Interestingly, IL-15 restored lymphocyte proliferation and anticryptococcal activity that had been abrogated by blocking IL-2. The mechanism underlying this anticryptococcal activity was described by Ma et al. (2002), who reported that the antifungal activity triggered by IL-15 over T CD8 cells correlated with the up-regulation of granulysin, located in the acidic granules. Furthermore, antibodies against IL-15 were able to neutralize this effect. IL-15 may also directly induce Pb18 killing by human neutrophils, a different cellular population also involved in Pb18 resistance, by an oxidative pathway (Tavian et al. 2008).

The mechanisms involved in Pb18 killing by human monocytes activated with IL-15 were investigated in the present study. IL-15 enhanced the generation of O_2^- by monocytes challenged or not with Pb18 in all the concentrations employed. Vázquez et al. (1998) showed that IL-15 stimulates oxidative metabolism, increasing candidacidal activity and O_2^- production in monocytes, which is in agreement with the present data. Our results suggest a correlation between O_2^- production and monocyte fungicidal activity against Pb18, as high levels of O_2^- were observed in IL-15 activated co-cultures and these cells presented high fungicidal activity in all IL-15 concentrations, showing that this metabolite is involved in human monocyte fungicidal activity.

The yeast form of Pb18 has been shown to be susceptible to the effects of H_2O_2 at μM concentrations in the H_2O_2-horseradish peroxidase-halide system (McEwen et al. 1984). The assessment of H_2O_2 production revealed that IL-15 directly increased H_2O_2 production. In the present study when cells were pre-activated with IL-15 and challenged with the fungus, H_2O_2 production was lower in all groups, excepted in cells treated with IL-15 at 50 ng/mL. The fungus decreased H_2O_2 levels in cultures of non treated monocytes, or cells activated with IL-15 at 25 and 12.5 ng/mL. However, besides directly increasing H_2O_2 production by monocytes, IL-15 at the highest dose (50 ng/mL) restored the H_2O_2 production that was inhibited by Pb18. The inhibition of H_2O_2 production caused by the fungus in monocyte cultures can be explained by its ability to produce cytosolic and peroxisomal catalase isoenzymes, showing that high-dose treatments with H_2O_2 lead to an early increase in total catalase enzymatic activities, which is indicative of post-transcriptional regulation (Campos et al. 2005, Felipe et al. 2005, Dantas et al. 2008).

Another potential mechanism to explain the IL-15-induced enhancement of fungicidal activity could be its ability to stimulate the release of other proinflammatory cytokines that maintain monocyte activation. In the pres-ent study, monocytes challenged with Pb18 released both pro and anti-inflammatory cytokines and cell activation with IL-15 induced high levels of TNF-α, the main cytokine involved in the effective killing of Pb18 in vitro (Calvi et al. 2003, Carmo et al. 2006). In experimental PCM models, persistent TNF-α production has been associated with resistance to Pb18 infection, which is also essential for the control of fungal dissemination (Bocca et al. 1998, Souto et al. 2000, Nascimento et al. 2002).

High IL-6 levels were observed only when cells were challenged with the fungus; monocyte activation with IL-15 and challenge with Pb18 did not stimulate IL-6 production. This finding is significant because monocytes from PCM patients produce high levels of IL-6 during the active stages of the disease (Peraçoli et al. 2003) and IL-6 deactivates macrophages with the consequent reduction of microbicidal activity (Blanchard et al. 1991, Bermudez et al. 1992). Furthermore, in a previous study we demonstrated that IL-6 increases Pb18 growth in human monocyte cultures, suggesting that this cytokine may contribute to the pathogenesis of Pb18 infection by promoting fungal growth during interaction with phagocytes. The effect of IL-6 on suppressing fungicidal activity was correlated with the downregulation of TNF-α production by monocytes (Siqueira et al. 2009). Thus, the results of the present study suggest that IL-15 might compensate for the IL-6 induced suppression of monocyte activation caused by Pb18 by increasing TNF-α production.

Treatment of monocytes with IL-15 (50 ng/mL) led to an increase of its release even after the challenge with Pb18. This is an important finding because a minimal endogenous IL-15 production by human monocytes is required for optimal production of IFN-γ by NK cells, which are likely to have a significant role during the innate immune response to certain infections (Carson et al. 1994). The present observation that IL-15 may synergize with other inflammatory cytokines to activate the effector cells for protection against microbial infections is in agreement with findings reported by other authors (Yoshikai & Nishimura 2000).

Higher levels of IL-10 were detected in monocyte cultures challenged with Pb18 and a more significant increase was detected in cultures of IL-15-activated monocytes. Considering the suppressor effects of IL-10, its production in response to various microorganisms, including fungi, has been regarded as an important host evasion mechanism (Redpath et al. 2001). The same mechanism could explain the activity of Pb18, as high concentrations of this cytokine were detected in patients' serum (Fornari et al. 2001) and in peripheral blood cell culture supernatants (Benard et al. 2001, Oliveira et al. 2002). Moreover, monocytes from patients (Peraçoli et al. 2003) and monocytes from normal individuals challenged with the fungus (Kurokawa et al. 2007) spontaneously release high concentrations of this cytokine in vitro. In experimental models of PCM infection, susceptibility was associated with high levels of IL-10 (Calich & Kashino 1998). Thus, IL-10 has important regulatory roles in immunological and inflammatory responses by inhibiting the production of pro-inflammatory cytokines by monocytes (de Waal

Malefyt et al. 1991). The production of IL-10 observed in the present study may play a role in the control of the Pb18-induced inflammatory immune response and IL-15 could be involved in potentiating this effect.

Our findings suggest that IL-15 may induce Pb18 killing by human monocytes through the activation of oxidative metabolism dependent on TNF-α production. Further studies are required to test monocyte functions and cytokine activation in patients with PCM to enable a better understanding of host-parasite interactions during mycosis.

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