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Research Article

Human Monocyte/Macrophage Fungicidal Activity GM-CSF Against *Paracoccidioides brasiliensis* Depends on ROS

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Abstract

The ability of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) to activate human monocytes/macrophages for virulent *Paracoccidioides brasiliensis* killing was evaluated. Peripheral blood monocytes (MO) and monocyte-derived macrophages (MØ) were activated with different concentrations of GM-CSF. Afterwards, cells were challenged with *P. brasiliensis* strain 18 (Pb18) and the fungicidal activity was evaluated, plating and counting the Colony Forming Units (CFU) after 10 days. GM-CSF activated MO and MØ for *P. brasiliensis* killling in a concentration-dependent manner. There was an association between this fungicidal activity and the high levels of H_2O_2 release by the activated cells. Moreover, the killing effect was inhibited by Catalase (CAT), confirming the role of H_2O_2 in this process. On the other hand, L-Monomethyl-Arginine (L-NMMA) had no effect on fungicidal activity, showing that nitric oxide (NO) is not involved in killing by human cells against *P. brasiliensis*. Based on these data, the role of GM-CSF-activated human cells in the innate defense mechanisms against *P. brasiliensis* is discussed.

Keywords: Paracoccidioidomycosis; *Paracoccidioides brasiliensis*; Human monocytes; Human macrophages; GM-CSF; Fungicidal activity; H_2O_2; NO; ROS

Introduction

Paracoccidioidomycosis (PCM) is the major systemic mycosis in Latin America. Its etiological agent is the fungus *Paracoccidioides brasiliensis*, a microorganism with thermal dimorphism, behaving as yeast at body temperature [1,2]. This fungus causes a natural infection by inhalation of conidia or mycelial elements which are converted into the parasitic yeast form in lungs [3]. This disease shows multiple shapes, ranging from benign and localized to severe and disseminated ones, depending on many factors, such as the host cell immunity and strain virulence of the fungus [4].

Among the immunological mechanisms reported to this infection, innate immunity monocytes (MO)/macrophages appear to play a fundamental role, acting as the first defense line in the organism [3], depending on their state of activation [5]. Ingested conidia or yeast forms of *P. brasiliensis* readily multiply inside murine alveolar or peritoneal macrophages; however, when cells are activated by cytokines, such as IFN-γ, the multiplication is limited and conidia or yeast cells may be killed [2,3,6-8].

With regards to murine cells, some studies have shown that IFN-γ activation promotes *P. brasiliensis* killing through the L-arginine/NO pathway [9]. However, works in our laboratory have demonstrated that IFN-γ activation is not enough for the fungicidal activity of human cells against virulent *P. brasiliensis* strain (Pb18) [10]. This process is effective after cells preactivation with TNF-α or IFN-γ plus TNF-α. Moreover, these studies provided strong evidence of H_2O_2 participation as an effector mechanism, since catalase, a H_2O_2 scavenger, inhibited the intracellular killing by TNF-α or TNF-α plus IFN-γ-activated MO [11].

The role of other cytokines, besides IFN-γ and TNF-α concerning human MO/macrophage- *P. brasiliensis* interaction is still unclear. Broad evidence has indicated that GM-CSF not only promotes proliferation and differentiation of hematopoietic precursor cells, but also induces various aspects of macrophage activation [12-16], e.g., respiratory burst activity [15]. MO release Reactive Oxygen Species (ROS, such as H_2O_2, O_2- and OH), which are cytotoxic to microorganisms and tumor cells.

The effects of GM-CSF on MO/macrophage function against microbial pathogens have been studied in uncontrolled trials in humans and *in vitro* and *in vivo* experiments [12,13,16].

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Enhancement of the microbicidal activity of MO by GM-CSF was shown *in vitro* against *Candida albicans* [17], *Aspergillus fumigates* [18,19], *Histoplasma capsulatum* [20,21], *Cryptococcus neoformans* [22], *Trypanosoma cruzi* [23], *Mycobacterium avium* [24], *M. lepraemurium* [25] and *M. avium-intracellulare* Complex (MAC) [26]. Therefore, we propose that MO/macrophage activation by GM-CSF might be necessary for *P. brasiliensis* killing.

Thus, the aim of this work was to study:

A. The role of GM-CSF on human MO/macrophage fungicidal activity against a virulent strain of *P. brasiliensis* (Pb18);

B. The involvement of reactive oxygen and nitrogen intermediates in the killing of Pb18 by GM-CSF-activated MO/macrophages.

Materials and Methods

Reagents and Media

Recombinant human GM-CSF was purchased from R&D Systems, Minneapolis, MN, USA. RPMI 1640 Tissue Culture Medium; Histopaque (d=1.077); CAT; L-NMMA; Phorbol-Myristate-Acethate (PMA); Sulphanilamide; and Naphtyl Ethylene-Diamine-Dihydrochloride (NEED) were purchased from Sigma Chemical CO, St. Louis, MO, USA. Complete tissue culture media (CTCM) consisted of RPMI 1640 suplemmented with 10% (v/l) heat-in activated human AB serum, 20mM HEPES, 2mM of L-glutamine (Gibco Laboratories, Grand Island, NY, USA) and 40µg/ml of gentamicin.

Brain-Heart Infusion (BHI) agar medium (Difco Laboratories, Detroit, MI, USA), used for culture plating, contained gentamicin 0.5% (Neoquímica, Anápolis, GO, Brazil), 4% horse normal serum and 5% *P. brasiliensis* strain 192 culture filtrates (v/v), the latter being the source of growth-promoting factor [27]. 96-well flat-bottomed plates were purchased from Nunc, Life Tech. Inc., Maryland, MA, USA. Horseradish peroxidase (type II) was obtained from Sigma Chemical, San Diego, CA, USA. *P. brasiliensis* strain 18 (Pb 18) was maintained in the yeast-form cells at 35 °C in GPY culture medium for six days [28]. Yeast viability was determined by phase contrast microscopy and bright yeast cells were counted as viable, while dark ones were considered nonviable. Fungal suspensions containing more than 90% viable cells were used for the experiments.

Donors

MO was isolated from volunteer healthy blood donors, after informed consent from the University Hospital of the Botucatu Medical School (FMB), São Paulo State University (UNESP, Brazil). The Hospital Ethics Committee approved this study.

Isolation of peripheral blood mononuclear cells (PBMC): PBMC were isolated from heparinized venous blood by density gradient. Briefly, 10 ml of heparinized blood were mixed with an equal volume of complete tissue culture medium (CTCM). Samples were layered over 5ml of Histopaque in a 15-ml conical plastic centrifuge tube. After centrifugation at 300×g for 30' at room temperature, the interface layer of PBMC was harvested and washed twice with PBS-EDTA and once with CTCM. Cell viability as determined by 0.2% trypan blue exclusion was >95% in all experiments. The MO were stained with neutral red (0.02%) and the concentrations were adjusted to 2×10^6 MO/ml in CTCM. More than 90% of the cells were considered as MO by morphological examination, neutral red uptake, and staining for unspecific esterase [29].

MO/macrophage monolayers

100µL of MO suspension (2×10^6 MO/ml) was dispensed into 96-well flat-bottomed plates. After incubation during 2h at 37 °C in 5% CO_2, non adherent cells were removed by aspiration and each well was rinsed twice with CTCM. After adherence, MO were cultured in CTCM at 37 °C in 5% CO_2 during 18h, alone (MO) or containing GM-CSF in different concentrations (1, 10, 31.25, 62.5, 125, 250, 500 and 1000U/mL). For the MO differentiation process into macrophages, MO cultures were maintained during 7 days in CTCM, changing the culture media every other day and, subsequently activating the cells with GM-CSF (MØ), in diferente concentrations (1, 10, 31.25, 62.5, 125, 250, 500 and 1000U/mL). In other experiments, MO-derived macrophages were cultured in the presence of GMCSF 250U/mL (G-MØ), changing the culture media every other day, for 7 days. After MO-macrophage differentiation, the supernatants were removed, and cells were activated with GM-CSF in different concentrations (1, 10, 31.25, 62.5, 125, 250, 500, 1000U/mL) for 18h.

Fungicidal activity After supernatants removal of control and treated monolayers, MO/macrophages were challenged with 100 µl of 2×10^4 viable units/ml of Pb18 (ratio 50:1) in CTCM containing 10% fresh human AB serum, in absence or presence of CAT (20,000U/mL) or L-NMMA (450U/mL). After coculture during 18h (experimental cultures), cells were harvested by aspiration with sterile distilled water to lyse MO. Each culture and well washing was contained in a final volume of 2ml. The number of colony forming units (CFU) of Pb18 per culture was determined by plating 100µl of the 2-ml harvested volume, in triplicate, on BHI containing 4% normal horse serum and 5% *P. brasiliensis* strain 192 culture filtrates (v/v). A control culture only containing 100µl of yeast-form Pb18 (2×10^4 viable units/ml) was submitted to the same procedures used for the experimental cultures. Inoculated plates were incubated at 35 °C in sealed plastic bags to prevent drying. After 10 days the number of CFU per plate was counted and the percentage of fungicidal activity was determined by the formula: mean CFU of experimental culture

$$\text{Fungicidal Activity (\%)} = 1 - \frac{\text{(mean CFU of experimental culture)}}{\text{mean CFU of control culture}} \times 100$$

Reactive oxygen intermediates (ROI) determination

ROI production was indirectly measured by assessing H_2O_2 release from MO and macrophages, according to the method previously described by PICK &KEISARI [30] and adapted by PICK & MIZEL [31]. MO or MO-derived macrophages were obtained as previously described, and cultured in duplicate in 96-well plates for 24 hours, at 37 °C in 5% CO_2 tension, with or without 100µL of recombinant human GM-CSF (rh-GM-CSF) (1, 10, 31.25, 62.5, 125,

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250, 500 and 1000U/mL). After this period, culture supernatants were used to nitric oxide (NO) determination and the adherent cells were resuspended to the original volume (0,1mL) in phenol red buffer solution containing: 140mM of NaCl; 10nM of phosphate buffer, pH 7; 5.5mM of dextrosis; 0.56mM of phenol red; 0.01mg/mL of peroxidase from radish peroxidase type II and, in the presence or absence of 1mg of phorbol myristateacethate (PMA), and were incubated at 37 °C in a dark, humid chamber. After 60 minutes, the reaction was interrupted by the addition of 0.01mL of NaOH 1N. Absorbances were measured at 620nm in an automatic ELISA microreader. Results were expressed in nanomols (nM) of $H_2O_2/2x10^5$ cells, using a standard curve.

Reactive nitrogen intermediates (RNI) determination

NO production was determined based on Griess reaction [32]. Culture supernatants were mixed in with an equal volume of Griess reagent (1% Sulphanilamide, 0.1% NEED, in 5% phosphoric acid) at room temperature for 10min [32]. Sodium nitrite ($NaNO_2$-) was used as standard. Absorbances were measured at 540nm in an ELISA microreader. Assays were carried out in quadriplicate. Results were expressed in µmols of NO_2-/$2x10^5$ cells, comparing the optical density (OD) with a standard curve of known NO_2- concentrations. Statistical Analysis: Statistical procedures were performed using Graphpad Instat software (San Diego, California - USA). Significant differences among the various groups were detected by Repeated Measures Analysis of Variance (ANOVA), followed by Tukey Kramer Multiple Correlations. Significance level was set at $p<0.05$.

Results

Role of GM-CSF on human monocyte (MO) fungicidal activity *in vitro* for high-virulent *P. brasiliensis* killing

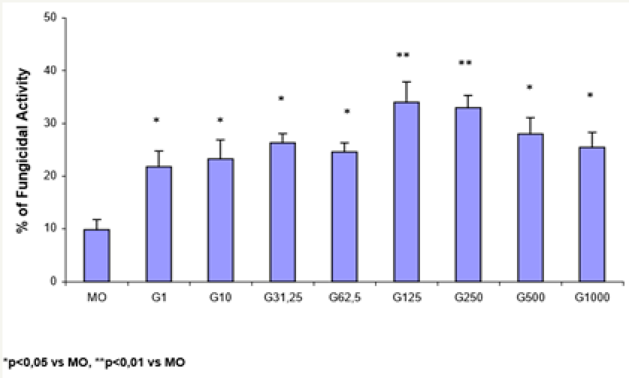

Figure 1: Monocytes ($2x10^6$/mL) were preincubated in the absence (MO) or presence of GM-CSF (G) in different concentrations (1, 10, 31.25, 62.5, 125, 250, 500 and 1000U/mL) for 18h and challenged with P. brasiliensisduring 4h for fungicidal activity *in vitro*. Results are expressed as Mean (M) ± SEM of 11 subjects.

Figure 1 shows the percentage (%) of fungicidal activity of MO newly isolated from the peripheral blood of normal donors, incubated only with CTCM (MO) or in the presence of GM-CSF (G) in different doses (1, 10, 31.25, 62.5, 125, 250, 500 and 1000U/mL). Preincubation of MO with GM-CSF during 18h induces a concentration-dependent increase in the fungicidal activity against high-virulent strain of *P. brasiliensis* (Pb18). Significant fungicidal activity was detected in every concentration of GM-CSF, and maximal activity occurred with the doses of 125 (33.9%) and 250U/mL (32.9%).

Role of CAT and L-NMMA on fungicidal activity of GM-CSF-activated MO

Role of CAT and L-NMMA on fungicidal activity of GM-CSF-activated MO To detect the effector mechanisms involved in GM-CSF-activated MO for *P. brasiliensis* killing, cocultures of MO were challenged with Pb18 and treated concomitantly with CAT (20,000U/mL), a scavenger of H_2O_2 production; or L-NMMA (450U/mL), a competitive inhibitor of nitric oxide synthase (NOS), the enzyme responsible for NO production. Results showed that CAT inhibited the fungicidal activity presented by GM-CSF-activated MO, indicating the role of H_2O_2 in this process. On the other hand, L-NMMA did not show a significant effect (Figure 2).

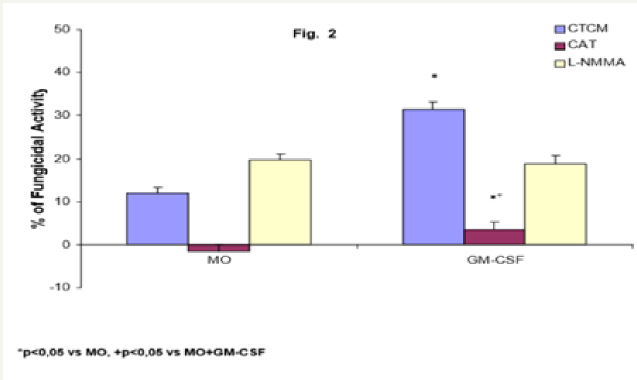

Figure 2: Monocytes ($2x10^6$/mL) (MO) activated with GM-CSF (125U/mL) during 18h were incubated in the absence or presence of CAT (20,000U/mL) or L-NMMA (450U/mL), challenged concomitantly with P. brasiliensis during 4h, and assessed for fungicidal activity *in vitro*. Results are expressed as Mean (M) ± SEM of 11 subjects.

As shown in Table 1, H_2O_2 levels were significantly diminished after non activated MO challenge with Pb18 (MO+Pb=0.81±0.24nM), when compared to non-activated MO alone (MO=1.97±0.53nM). However, when MO were pre activated with GM-CSF, a significant increase in H_2O_2 production was detected (MO+GM-CSF=3.13±0.63nM), when compared to MO or MO+Pb. Moreover, in MO pre activated with GM-CSF and challenged with Pb18 (MO+GM-CSF+Pb=2.88±0.52nM), H_2O_2 levels were like those detected in MO+GM-CSF cultures. These data show that, differently of non activated MO challenged with Pb18 (MO+Pb), the challenge of GM-CSF pre activated cells with Pb (MO+GM-CSF+Pb) did not result in H_2O_2 inhibition. In addition, all the cocultures treated with CAT (MO+CAT, MO+GM-CSF+CAT, MO+Pb+CAT) showed a significant inhibition in H_2O_2 levels, compared to those detected in absence of this scavenger. Concerning NO_2- production, similarly to H_2O_2 release, in cocultures supernatants of MO+Pb(= 1.46±0.49µM), we detected a significant inhibition in this metabolite production, compared to MO(= 2.38±0.75). However, conversely to H_2O_2, GM-CSF (2.54±0.69µM) did not stimulate the cells to increase NO_2-

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production, when compared to MO. Moreover, MO+GM-CSF+Pb did not release higher levels of NO_2-.

Together, the results showed that NO_2- levels were very low in all cocultures supernatants, suggesting no correlation with fungicidal activity. The lack of L-NMMA effect on fungicidal activity of MO+GM-CSF reinforces these results. Results shown in Figure 1 & 2 and Table 1 suggest that the fungicidal activity presented by GM-CSF-activated MO is mediated by H_2O_2, while NO seems not to be involved.

Table 1: Production of H_2O_2 and NO_2- in cocultures of MOpreactivated with GM-CSF (125U/mL) for 18h, before the challenge with *P. brasiliensis* during 4h, in absence or presence of scavenger of H_2O_2 release (CAT) or inhibitor of NO production (L-NMMA). Results are expressed as M±SEM of 8 experiments.

Monocytes treatment	H_2O_2 release (nmoles/2x10^5 cells)	NO_2^- release (µmoles/2x10^5 cells)
MO	1.97±0.43	2.38±0.75
MO+ CAT/L-NMMA*	0.55±0.12♦	1.64±0.45+♦
MO+ Pb	0.81±0.24+	1.46±0.49+
MO + Pb + CAT/L-NMMA	0.34±0.08♦	1.19±0.31+♦
MO + GM-CSF	3.13±0.63+•	2.54±0.69
MO + GM-CSF + CAT/L-NMMA	0.92±0.46♦	1.84±0.51+♦
MO + GM-CSF + Pb	2.88±0.52+•	1.39±0.52+
MO + GM-CSF + Pb + CAT/L-NMMA	1.12±0.44♦	1.16±0.39+♦

+= p<0,05 vs MO; • = p<0,05 vs MO+Pb, ♦= p<0,05 vs cocultures without CAT or L-NMMA. * = (CAT/L-NMMA) = CAT was used for H_2O_2 assays, and L-NMMA was used for NO_2^- assays. MO = Monocytes cultured only with CTCM; MO+Pb = MO challenged with Pb; MO+GM-CSF = MO preactivated with GM-CSF 125U/mL

Role of GM-CSF on the fungicidal activity of human MO-derived macrophages cultured in CTCM for 7 days *in vitro* (MØ)

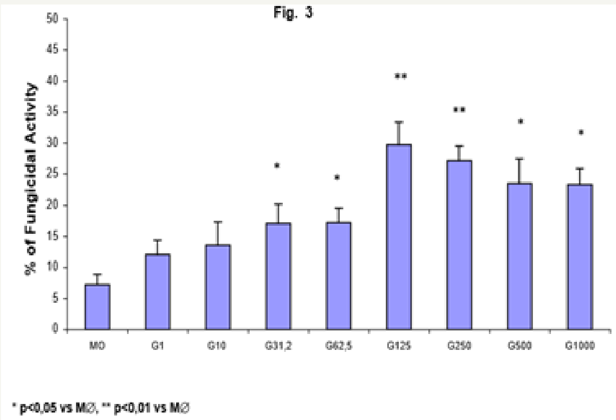

Figure 3: Monocyte-derived macrophages (2x10^6/mL) incubated with CTCM for 7 days (MØ) were activated with different GM-CSF (G) concentrations (1, 10, 31.25, 62.5, 125, 250, 500 and 1000U/mL) during 18h. Following, they were challenged with P. brasiliensisduring 4h and assessed for fungicidal activity *in vitro*. Results are expressed as Mean (M) ± SEM of 8 subjects.

Similarly, the data obtained with MO pre incubation, GM-CSF activated macrophages (MØ) displayed a dose-dependent increase in fungicidal activity, from 31.25U/mL (15.5%), to the most significant concentrations of 125 and 250U/mL (29.8% and 27.2%, respectively), when compared to control MØ (7.2%). It shall be reinforced that, compared to MO (Figure 1; 33.9 and 32.9%), the activity of activated-MØ was not statistically significant.

Role of CAT and L-NMMA on fungicidal activity of GM-CSF-activated GMØ

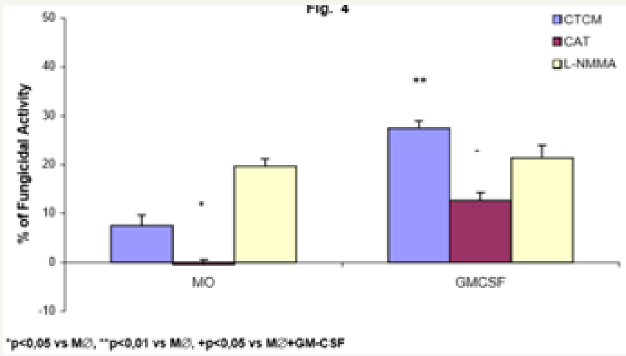

Figure 4: Monocyte-derived macrophages (2x10^6/mL) incubated with CTCM for 7 days (MØ) were activated with GM-CSF for 18h (GM-CSF); preincubated in the absence or presence of CAT (20000U/mL) or L-NMMA (450U/mL) and challenged concomitantly with P. brasiliensisduring 4h. Following, they were assessed for fungicidal activity *in vitro*. Results are expressed as Mean (M) ± SEM of 8 subjects.

To detect the effector mechanisms involved in GM-CSF-activated macrophages (MØ) for *P. brasiliensis* killing, we challenged these cocultures with Pb18 and treated them concomitantly with CAT or L-NMMA. Similarly to MO cultures (MO-Figure 2), CAT inhibited the fungicidal activity induced by GM-CSF, confirming the role of H_2O_2 in this process, as well with MØ. On the other hand, LNMMA did not change fungicidal activity of GMCSF-activated MØ, like in MO cultures (Figure 4). One may see in Table 2 that the levels of H_2O_2 were significantly diminished after non activated macrophages challenge with Pb18 (MØ+Pb=0.71±0.23nM), when compared to macrophages alone (MØ=1.24±0.53nM). However,

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when macrophages were pre activated with GM-CSF (MØ+GM-CSF=2.33±0.64nM), a significant increase in H_2O_2 production was detected, when compared to MØ or MØ+Pb.

Macrophages preactivated with GM-CSF and challenged with Pb showed similar H_2O_2 levels (MØ+GM-CSF+Pb=2.17±0.6nM) to those detected in MØ+GM-CSF culture. These data show that, differently of non-activated MØ challenged with Pb18 (MØ+Pb), the challenge of activated cells (MØ+GMCSF+Pb) did not lead to H_2O_2 inhibition. In all the cocultures treated with CAT, a significant inhibition in H_2O_2 levels was obtained, compared to those detected in the absence of this scavenger. In relation to NO_2-, similarly to H_2O_2 release and also similarly to MO cultures, there was a significant inhibition in this metabolite production in cocultures supernatants of MØ+Pb (=2.46±0.53μM), compared to MØ (=3.17±0.98). However, conversely to H_2O_2, GM-CSF (3.03±0.91μM) did not stimulate the cells to increase NO2- production, compared to MØ. Moreover, MØ+GM-CSF+Pb (1.89±0.39) did not release higher levels of NO_2. Once more, as occurred in supernatants of MO cultures, the results showed that NO_2- levels were very low in all cocultures supernatants, suggesting that they were not correlated with fungicidal activity. Again, these results are associated with the lack of L-NMMA effect on fungicidal activity presented by GM-CSF-activated MØ. Together, the results presented in Table 2 and Figure S3 & 4 showed that the fungicidal activity presented by GM-CSF-activated MØ is mediated by H_2O_2, similarly to MO experiments. In experiments with MØ, once more, NO seems not to be involved.

Table 2: Production of H_2O_2 and NO_2- in cocultures of MO-derived macrophages (MØ), preactivated with GM-CSF (125U/mL) for 18h before the challenge with *P. brasiliensis* during 4h, in absence or presence of scavenger of H_2O_2 (CAT) or inhibitor of NO (L-NMMA) release. Results are expressed as M±SEM of 8 experiments.

Monocytes treatment	H_2O_2 release (nmoles/2x10^5 cells)	NO_2^- release (μmoles/2x10^5 cells)
MØ	1.34±0.53	3.17±0.98
MØ+ CAT/L-NMMA*	0.91±0.31+♦	1.94±0.47+♦
MØ+ Pb	0.71±0.23+	2.46±0.53+
MØ + Pb + CAT/L-NMMA	0.39±0.12+♦	1.42±0.41b+♦
MØ + GM-CSF	2.33±0.64+•	3.03±0.91
MØ + GM-CSF + CAT/L-NMMA	1.02±0.36♦	2.1±0.44a+♦
MØ + GM-CSF + Pb	2.17±0.6+•	1.89±0.39+
MØ + GM-CSF + Pb + CAT/L-NMMA	0.89±0.27+♦	1.22±0.27+♦

+= p<0,05 vs MØ; •= p<0,05 vs MØ+Pb, ♦= p<0,05 vs cocultures without CAT or L-NMMA. * (CAT/L-NMMA) = CAT was used in cultures for H_2O_2 dosage assays, and L-NMMA was used in cultures for NO_2^- dosage assays. MØ = Macrophages cultured only with CTCM during 7 days; MØ+Pb = MØ challenged with Pb; MO+GM-CSF = MØ preactivated with GM-CSF 125U/mL

Role of GM-CSF on the fungicidal activity of human MO-derived macrophages, after 7 days of culture in the presence of GM-CSF *in vitro* (GMØ)

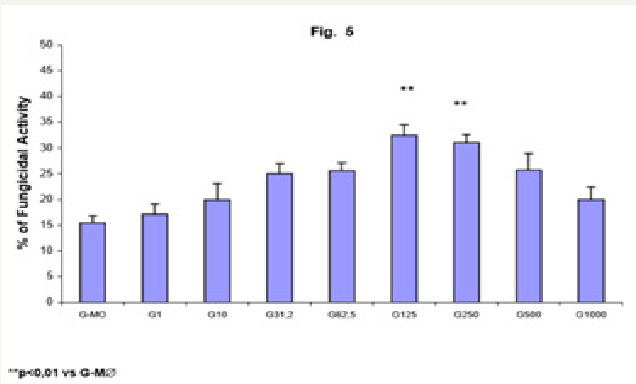

Figure 5: Monocyte-derived macrophages (2x10^6/mL) by incubation in the presence of GM-CSF for 7 days (G-MØ) were activated with GM-CSF (G) in different concentrations (1, 10, 31,25, 62,5, 125, 250, 500 and 1000U/mL) for 18h. Following, they were challenged with P. brasiliensisduring 4h and assessed for fungicidal activity *in vitro*. Results are expressed as Mean (M) ± SEM of 8 subjects.

Figure 5 shows an increased fungicidal activity of human MO-derived macrophages activated with GM-CSF, after 7 days of treatment in the presence of this cytokine (G-MØ), only with 125 and 250U/mL (32.4% and 31.0%, respectively), in a similar way compared to fungicidal activity presented by GMCSF-activated-MO and MØ. One may observe that the MO differentiation process into macrophages in the presence of GM-CSF, did not change significantly the responsiveness to GM-CSF pre activation, compared to macrophages culture without this cytokine.

Role of CAT and L-NMMA on fungicidal activity of GM-CSF activated GMØ

As previous assays with MO and MØ, we attempted to detect the effector mechanisms involved in GM-CSF-activated G-MØ for *P. brasiliensis* killing, Figure 6 shows that, similarly to MO (Figure 2) and macrophage in absence of GMCSF (MØ - Figure 4) cultures, CAT inhibited the fungicidal activity presented by GMCSF-activated G-MØ, reinforcing the role of H_2O_2 as well in this process. Once again, L-NMMA could not abrogate fungicidal activity of G-MØ or GM-CSF activated G-MØ, as verified in cells treatment with CAT (Figure 6).

As seen in Table 3, H_2O_2 levels were significantly diminished after G-MØ challenge with Pb18 (G-MØ+Pb=0.82±0.24nM),

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when compared to G-MØ alone (G-MØ=1.82±0.63nM). However, when G-MØ were pre activated with GM-CSF (G-MØ+GM-CSF=3.71±0.94nM), a significant increase in H_2O_2 production was detected. In G-MØpreactivated with GM-CSF and challenged with Pb (G-MØ+GMCSF+Pb=2.83±0.73nM), the H_2O_2 levels were similar to that detected in GMØ+GM-CSF cultures. These data show that, differently of nonactivated G-MØ challenged with Pb (G-MØ+Pb), the challenge of activated cells with Pb (GMØ+GM-CSF+Pb) did not lead to H_2O_2 inhibition. In all the cocultures treated with CAT, a significant inhibition in H_2O_2 levels was observed, compared to the ones detected in absence of this scavenger.

Table 3: Production of H_2O_2 and NO_2- in cocultures of MO-derived macrophages in the presence of GM-CSF for 7 days (G-MØ), preactivated with GM-CSF (125U/mL) for 18h before the challenge with *P. brasiliensis* during 4h, in absence or presence of scavenger of H_2O_2 (CAT) or inhibitor of NO (LNMMA) release. Results are expressed as M±SEM of 8 experiments.

Monocytes treatment	**H_2O_2 release (nmoles/2x10^5 cells)**	**NO_2^- release (µmoles / 2x10^5 cells)**
G-MØ	1.82±0.63	2.93±1.05
G-MØ+ CAT/L-NMMA	0.41±0.17+♦	1.35±0.85+♦
G-MØ+ Pb	0.82±0.24+	1.23±0.71+
G-MØ + Pb + CAT/L-NMMA	0.31±0.12+♦	0.77±0.25+♦
G-MØ + GM-CSF	3.71±0.94+•	3.22±0.97
G-MØ + GM-CSF + CAT/L-NMMA	2.14±0.6♦	2.74±0.89♦
G-MØ + GM-CSF + Pb	2.83±0.73+•	0.97±0.21+
G-MØ + GM-CSF + Pb + CAT/L-NMMA	1.68±0.13♦	1.21±0.4+♦

+= p<0,05 vs MØ; •= p<0,05 vs MØ+Pb, ♦= p<0,05 vs cocultures without CAT or L-NMMA. * (CAT/L-NMMA) = CAT was used in cultures for H_2O_2 dosage, and L-NMMA was used in cultures for NO_2^- dosage. G-MØ = Macrophages cultured in CTCM+GM-CSF 250 U/mL during 7 days; G-MØ+Pb = G-MØ challenged with Pb; G-MØ+GM-CSF = G-MØ preactivated with GM-CSF 125U/mL

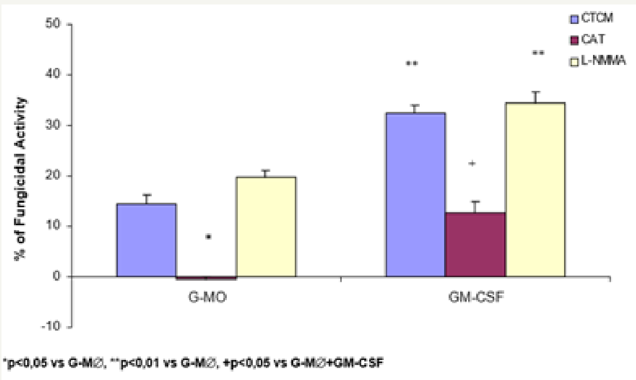

Figure 6: Monocyte-derived macrophages (2x10^6/mL) by incubation with GM-CSF 250U/mL for 7 days (G-MØ) were activated with GM-CSF 125U/mL by 18h and preincubated in the presence of scavengers, CAT (20000U/mL) or L-NMMA (450U/mL), challenged concomitantly with P. brasiliensisduring 4h, and assessed for fungicidal activity *in vitro*. The results are expressed as Mean (M) ± SEM of 8 subjects.

As to NO_2-, similarly to H_2O_2 release and similarly to MO and MØ cultures, in cocultures supernatants of MØ+Pb (= 2.46±0.53µM), we detected a significant inhibition in this metabolite production, compared to MØ (= 3.17±0.98). However, conversely to H_2O_2, MØ+GM-CSF (3.03±0.91µM) did not stimulate the cells for increased NO_2- production, compared to MØ. Moreover, MØ+GM-CSF+Pb (1.89±0.39) did not release higher levels of NO_2-, which were very low in all cocultures supernatants, suggesting that they were not correlated with fungicidal activity. Once again, these results are associated with the lack of L-NMMA effect on fungicidal activity of MO+GM-CSF cells. Therefore, it follows, from the results shown in Table 3 and Figure 5 & 6, that the fungicidal activity presented by GM-CSF-activated G-MØ is mediated by H_2O_2 similarly to MO and MØ. Again, NO seems not to be involved.

Discussion

The aim of this work was to investigate the role of GM-CSF on human mononuclear phagocytes activation and the effector mechanisms developed by these cells for *P. brasiliensis* killing. The results presented here allow us to consider the existence of an important fungicidal activity of GM-CSF-activated human MO and MO-derived macrophages against *P. brasiliensis*. Stimulation of MO/macrophage for antimicrobial activity by GM-CSF is reported by a number of studies *in vivo* and *in vitro*, evidenciating that this cytokine activates neutrophils, MO and macrophages, and enhances the ability of these cells to kill intracellular parasites, such as *Candida albicans* [17], *Aspergillus fumigates* [18,19], *Histoplasma capsulatum* [20,21], *Trypanosoma cruzi* [23], *Mycobacterium avium* [24]; *M. lepraemurium* [25], and MAC [26]. Since GM-CSF increased the fungicidal activity of human MO and macrophages against *P. brasiliensis* strain 18 (Pb18), we were interested in clarifying the mechanisms by which the cells would exert this activity.

Our results clearly demonstrated that CAT inhibited the fungicidal activity of the cells tested. Moreover, despite of the Pb18 inhibits H_2O_2 release, as shown in previous works [33], when the cells are activated by GM-CSF, a compensatory effect on this production was detected, and H_2O_2 concentrations were enough for a fungicidal effect. Thus, this work provides evidence that the mechanism by which GM-CSF activated cells kill *P. brasiliensis* is

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mediated by H_2O_2. Previous works in our laboratory [11] showed that this metabolite is also effective in *P. brasiliensis* killing for IFN-γ and TNF-α-activated human phagocytes, similarly to other microorganisms [17,20,23,34,35]. On the other hand, our results indicate that NO is not involved in Pb18 killing by human phagocytic cells, since L-NMMA did not revert the fungicidal activity presented by phagocytes and NO_2- levels in all cocultures were very low, including the ones pre activated with GM-CSF. Production of NO and subsequent formation of peroxynitrite [36] have been found to be a potent antifungal mechanism of mononuclear cells [36-38].

This mechanism has been shown to be efficient in the inhibition of replication and killing of fungi [9,39] and other microorganisms by murine mononuclear phagocytes. Previous results from our group have demonstrated that killing of Pb18 by IFN-γ and TNF-α-activated peritoneal murine macrophages is mediated by NO and H_2O_2 [40]. Although it is known that NO is abundantly synthesized by phagocytes from mice and rats, its secretion by human mononuclear phagocytes has become a controversial issue [24,37,40,41]. Mac Micking et al. [40] and Albina [42] have reported that human MO/macrophages express nitric oxide synthase type II (NOS_2) as protein synthesis, in response to various stimuli. Furthermore, while it has been found to be a potent antifungal compound of phagocytes in mice, its antifungal role has not been established in human [37-39]. Specifically, in this work, NO does not appear to be involved in the fungicidal activity of human phagocytes against Pb18 yeasts [43,44].

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