Human Monocyte/Macrophage Fungicidal Activity of 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 (M∅) 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 M∅ for *P. brasiliensis* killing in a concentration-dependent manner. There was an association between this fungicidal activity and the high levels of H$_2$O$_2$ release by the activated cells. Moreover, the killing effect was inhibited by Catalase (CAT), confirming the role of H$_2$O$_2$ 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$_2$O$_2$; 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$_2$O$_2$ participation as an effector mechanism, since catalase, a H$_2$O$_2$ 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$_2$O$_2$, 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].
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. leprae/rium [25] and M. avium-intracellulare Complex (MAC) [26]. Therefore, we propose that MO/macroage 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/macroage 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-Acetate (PMA); Sulphanilamide; and Naphtyl Ethyene-Diamine-Dihydrochloride (NEED) were purchased from Sigma Chemical CO, St. Louis, MO, USA. Complete tissue culture media (CTCM) consisted of RPMI 1640 supplemented with 10% (v/v) heat-inactivated 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% (Neouquima, 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 300g 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 2x10^6 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/macroage monolayers

100µL of MO suspension (2x10^6 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 differente 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 2×10^6 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:

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\text{Fungicidal Activity} = \frac{\text{mean CFU of experimental culture} - \text{mean CFU of control 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,
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; 10mM of phosphate buffer, pH 7; 5.5mM of dextrose; 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 myristateacetate (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 540nm in an automatic ELISA microreader. Results were expressed in nanomols (nM) of H₂O₂/2x10⁵ 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 (NaNO₂) was used as standard. Absorbances were measured at 540nm in an ELISA microreader. Assays were carried out in quadruplicate. Results were expressed in µmols of NO₂⁻/2x10⁵ cells, comparing the optical density (OD) with a standard curve of known NO₂⁻ 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**

As shown in Table 1, H₂O₂ 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 H₂O₂ 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), H₂O₂ 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 H₂O₂ inhibition. In addition, all the cocultures treated with CAT (MO+CAT, MO+GM-CSF+CAT, MO+Pb+CAT) showed a significant inhibition in H₂O₂ levels, compared to those detected in absence of this scavenger. Concerning NO₂⁻ production, similarly to H₂O₂ 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 H₂O₂, GM-CSF (2.54±0.69µM) did not stimulate the cells to increase NO₂⁻.

**Figure 1:** Monocytes (2x10⁶/mL) were preincubated in the absence (MO) or presence of CAT (20,000U/mL) or L-NMMA (450U/mL), 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.

**Figure 2:** Monocytes (2x10⁶/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.
production, when compared to M0. Moreover, M0+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 M0+GM-CSF reinforces these results. Results shown in Figure 1 & 2 and Table 1 suggest that the fungicidal activity presented by GM-CSF-activated M0 is mediated by H$_2$O$_2$ while NO seems not to be involved.

**Table 1:** Production of H$_2$O$_2$ and NO$_2^-$ in cocultures of M0 preactivated with GM-CSF (125U/mL) for 18h, before the challenge with *P. brasiliensis* during 4h, in absence or presence of scavenger of H$_2$O$_2$ release (CAT) or inhibitor of NO production (L-NMMA). Results are expressed as M±SEM of 8 experiments.

| Monocytes treated | H$_2$O$_2$ release (nmol/2x10^6 cells) | NO$_2^-$ release (nmol/2x10^6 cells) |
|-------------------|--------------------------------------|-------------------------------------|
| MO                | 1.97±0.43                            | 2.38±0.75                           |
| MO+CAT/L-NMMA     | 0.55±0.12                            | 1.64±0.45                           |
| MO+Pd             | 0.81±0.24                            | 1.64±0.49                           |
| MO+Pd+CAT/L-NMMA  | 0.34±0.08                            | 1.91±0.31                           |
| GM-CSF            | 3.13±0.63                            | 2.54±0.69                           |
| GM-CSF+CAT/L-NMMA | 0.92±0.46                            | 1.84±0.51                           |
| GM-CSF+Pd         | 2.88±0.52                            | 1.99±0.50                           |
| GM-CSF+Pd+CAT/L-NMMA | 1.12±0.44                       | 1.63±0.39                           |

**Role of CAT and L-NMMA on fungicidal activity of GM-CSF-activated M∅**

Figure 4: Monocyte-derived macrophages (2x10$^6$/mL) incubated with CTCM for 7 days (M∅) were activated with GM-CSF for 18h (GM-CSF); preincubated in the absence or presence of CAT (2000U/mL) or L-NMMA (450U/mL) and challenged concomitantly with *P. brasiliensis* during 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 (M∅) for *P. brasiliensis* killing, we challenged these cocultures with Pb18 and treated them concomitantly with CAT or L-NMMA. Similarly to M0 cultures (MO-Figure 2), CAT inhibited the fungicidal activity induced by GM-CSF, confirming the role of H$_2$O$_2$ in this process, as well with M∅. On the other hand, LNMMA did not change fungicidal activity of GM-CSF-activated M∅, like in M0 cultures (Figure 4). One may see in Table 2 that the levels of H$_2$O$_2$ were significantly diminished after non activated macrophages challenge with Pb18 (M∅+Pb=0.71±0.23nM), when compared to macrophages alone (M∅=1.24±0.53nM). However,
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 (GM∅)

Table 2: Production of $\text{H}_2\text{O}_2$ and NO$\text{2}^-$ in cocultures of MO-derived macrophages (M∅), preactivated with GM-CSF (125U/mL) for 18h before the challenge with *P. brasiliensis* during 4h, in absence or presence of scavenger of $\text{H}_2\text{O}_2$ (CAT) or inhibitor of NO (L-NMMA) release. Results are expressed as M±SEM of 8 experiments.

| Monocytes treatment | $\text{H}_2\text{O}_2$ release (nmol/2x10^5 cells) | NO$\text{2}^-$ release (µmol/2x10^5 cells) |
|---------------------|-----------------------------------------------|----------------------------------------|
| M∅                  | 1.34±0.53                                   | 3.17±0.98                              |
| M∅+CAT/L-NMMA      | 0.91±0.31*                                   | 1.49±0.47*                             |
| M∅+Pb               | 0.71±0.23                                   | 2.46±0.53*                             |
| M∅+GM-CSF          | 3.33±0.64*                                   | 0.30±0.91                              |
| M∅+GM-CSF+Pb       | 0.39±0.12                                   | 1.42±0.41*                             |
| M∅+GM-CSF+CAT/L-NMMA| 0.89±0.27*                                  | 1.22±0.27*                             |

Figure 5: Monocyte-derived macrophages (2x10^6/mL) by incubation in the presence of GM-CSF for 7 days (G-M∅) 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. brasiliensis* during 4h and assessed for fungicidal activity in vitro. Results are expressed as Mean (M) ± SEM of 8 subjects.

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

As previous assays with M∅ and M∅, we attempted to detect the effector mechanisms involved in GM-CSF-activated G-M∅ for *P. brasiliensis* killing, Figure 6 shows that, similarly to M∅ (Figure 2) and macrophage in absence of GMCSF (M∅ - Figure 4) cultures, CAT inhibited the fungicidal activity presented by GMCSF-activated G-M∅, reinforcing the role of $\text{H}_2\text{O}_2$ as well in this process. Once again, L-NMMA could not abrogate fungicidal activity of G-M∅ or GM-CSF activated G-M∅, as verified in cells treatment with CAT (Figure 6).

As seen in Table 3, $\text{H}_2\text{O}_2$ levels were significantly diminished after G-M∅ challenge with Pb18 (G-M∅+Pb=0.82±0.24nM), supernatants of M∅+Pb (=2.46±0.53µM), compared to M∅ (=3.17±0.98). However, conversely to $\text{H}_2\text{O}_2$, GM-CSF (3.03±0.91µM) did not stimulate the cells to increase NO2- production, compared to M∅. Moreover, M∅+GM-CSF+Pb (1.89±0.39) did not release higher levels of NO2-. Once more, as occurred in supernatants of M∅ cultures, the results showed that NO2- 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 M∅. Together, the results presented in Table 2 and Figure S3 & 4 showed that the fungicidal activity presented by GM-CSF-activated M∅ is mediated by $\text{H}_2\text{O}_2$, similarly to MO experiments. In experiments with M∅, once more, NO seems not to be involved.

Figure 6 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-M∅), only with 125 and 250U/mL (32.4% and 31.0%, respectively), in a similar way compared to fungicidal activity presented by GMCSF-activated-M∅ and M∅. 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 macrophage culture without this cytokine.
when compared to G-M∅ alone (G-M∅=1.82±0.63nM). However, when G-M∅ were pre activated with GM-CSF (G-M∅+GM-CSF=3.71±0.94nM), a significant increase in H$_2$O$_2$ production was detected. In G-M∅ preactivated with GM-CSF and challenged with Pb (G-M∅+GM-CSF+Pb=2.83±0.73nM), the H$_2$O$_2$ levels were similar to that detected in G-M∅+GM-CSF cultures. These data show that, differently of nonactivated G-M∅ challenged with Pb (G-M∅+Pb), the challenge of activated cells with Pb (GM∅+GM-CSF+Pb) did not lead to H$_2$O$_2$ inhibition. In all the cocultures treated with CAT, a significant inhibition in H$_2$O$_2$ levels was observed, compared to the ones detected in absence of this scavenger.

**Table 3**: Production of H$_2$O$_2$ and NO$_2^-$ in cocultures of MO-derived macrophages in the presence of GM-CSF for 7 days (G-M∅), preactivated with GM-CSF (125U/mL) for 18h before the challenge with *P. brasiliensis* during 4h, in absence or presence of scavenger of H$_2$O$_2$ (CAT) or inhibitor of NO (LNMMMA) release. Results are expressed as M±SEM of 8 experiments.

**Figure 6**: Monocyte-derived macrophages (2x10$^6$/mL) by incubation with GM-CSF 250U/mL for 7 days (G-M∅) were activated with GM-CSF 125U/mL by 18h and preincubated in the presence of scavengers, CAT (20000U/mL) or LNMMMA (450U/mL), challenged concomitantly with *P. brasiliensis* during 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$_2$O$_2$ release and similarly to M0 and M∅ cultures, in cocultures supernatants of M∅+Pb (= 2.46±0.53µM), we detected a significant inhibition in this metabolite production, compared to M∅ (= 3.17±0.98). However, conversely to H$_2$O$_2$, M0+GM-CSF (3.03±0.91µM) did not stimulate the cells for increased NO$_2^-$ production, compared to M∅. Moreover, M∅+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 LNMMMA 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-M∅ is mediated by H$_2$O$_2$ similarly to MO and M∅. 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*, evidencing 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$_2$O$_2$ 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$_2$O$_2$ 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...
mediated by $\text{H}_2\text{O}_2$. 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-NMA 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 macrophages is mediated by NO and $\text{H}_2\text{O}_2$ [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]. Macrophages from mice and rats, its secretion by human mononuclear phagocytes is mediated by NO and group have demonstrated that killing of Pb18 by IFN-γ and TNF-α-activated human macrophages is mediated by NO and group have demonstrated that killing of Pb18 by IFN-γ and TNF-α-activated human macrophages is mediated by NO and group have demonstrated that killing of Pb18 by IFN-γ and TNF-α-activated human macrophages is mediated by NO and group have demonstrated that killing of Pb18 by IFN-γ and TNF-α-activated human macrophages is mediated by NO and references 43,44].

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