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# Activation and IL-1β secretion of human peripheral phagocytes infected with *Actinomadura madurae*, *Nocardia asteroides* and *Candida albicans*

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ABSTRACT

**Objective:** To evaluate the ability of *Actinomadura madurae* (*A. madurae*) and *Nocardia asteroides* (*N. asteroides*), using *Candida albicans* (*C. albicans*) as prototypic control, to elicit the activation and IL-1β secretion of blood phagocytic cells from healthy donors.

**Methods:** Microscopic evaluation of phagocytosis/activation, cell viability and spectrophotometric quantitation of endocytosis/activation, were assessed by using formazan blue test in human blood phagocytes infected with *C. albicans*, *A. madurae* or *N. asteroides* treated with either normal human serum (NHS) or with decomplemented NHS. Interlukin-1β from culture supernatants of infected polymorphonuclear was tested by ELISA kit assay.

**Results:** Microscopic assay showed that phagocytosis and activation of adherent mononuclear phagocytes were greater with *C. albicans* followed by *A. madurae* and then by *N. asteroides*. Spectrophotometric assay in polymorphonuclear phagocytes infected with NHS-treated pathogens indicated that activation was similarly higher by *C. albicans* and *A. madurae* and lower by *N. asteroides*. Kinetic assays in infected polymorphonuclear cells showed that viability was decreased by *C. albicans* and *N. asteroides* or unaffected with *A. madurae*. Levels of IL-1β at 8 h of incubation were higher with *C. albicans* followed by *A. madurae* whereas lower levels were found with *N. asteroides*.

**Conclusions:** The extent of cell-viability and activation as well IL-1β secretion may be related with the virulence of *C. albicans* and *N. asteroides* and other parameters remain to be explored for assessing the virulence of *A. madurae*.

## 1. Introduction

Actinomycetoma is a chronic granulomatous subcutaneous disease, with high prevalence in Mexico, caused by facultative intracellular filamentous bacteria such as actinomycetes, mainly *Nocardia* and *Actinomadura* spp. [1,2]. The hallmark triad of actinomycetoma includes tumefaction, fistulization of abscesses and extrusion of grains [3,4]. Tissular lesion induced by the grains is characterized by the presence of phagocytes such as neutrophils and macrophages surrounded by lymphocytes [5]. Analysis of tissue and serum samples from patients with actinomycetoma suggests that persistence may result from a predominant TH2 profile of anti-inflammatory mediators, such as IL-10, which decreases the TH1 response of IFN-γ, essential for the cellular immunity toward intracellular pathogens [6].

Experimental assays in mice infected with *Nocardia brasiliensis* (*N. brasiliensis*) suggested that IFN-γ released at the site of skin lesion by dermal cells stimulates mononuclear phagocytic cells for the secretion of pro-inflammatory cytokines, such as IL-1β. The latter collaborates for early infiltration of neutrophils and on the activation of their bactericidal mechanisms of phagocytosis

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to clear the infection [7]. The mechanisms of actinomycetoma persistence are not fully known, but *in vitro* assays in cultures of bone macrophage from mice infected with *N. brasiliensis* suggests that cell wall lipids of this pathogen enable its ability to evade phagocytosis [8]. The latter entails several key events: attachment, activation, endocytosis, endosome generation and processing, and the intracellular killing [9].

*Candida albicans* (*C. albicans*) is an opportunistic human pathogen yeast causing chronic mucocutaneous candidiasis that induce a predominant TH2 response [10]. Attachment of *C. albicans* with phagocytes leads to the expression of pro-inflammatory cytokines with a critical role in the outcome of candidiasis. *In vitro* assays in cultures of peripheral blood mononuclear cells (PBMC) from healthy donors or human oral epithelia cells infected with *C. albicans* show that IL-1β contributes to the specific T cell activation and to the recruitment of activated leukocytes and lymphocytes to the site of infection [11]. Other studies in PBMC from healthy volunteers cultured with *C. albicans* indicate that, the extent of IL-1β stimulation by *C. albicans* differs in regard other strains [12]. Analysis of *C. albicans* ability to elicit IL-1β has been proposed for detecting virulence factors as well as differences in virulence of *Candida* strains as evidenced in PBMC from healthy donors or in human oral epithelial cells [11,13]. Due to the clinical and methodological impact, this study was focused on evaluating the ability of *Actinomadura madurae* (*A. madurae*) and *Nocardia asteroides* (*N. asteroides*), using *C. albicans* as prototypic control, to elicit the activation and IL-1β secretion of blood phagocytic cells from healthy donors. This assay may contribute to the knowledge of the virulence properties of *N. asteroides*, and mainly *A. madurae* causing neglected tropical diseases.

## 2. Materials and methods

### 2.1. Microorganisms

This study, included yeast cultures in Sabouraud Dextrose Agar (Cat No 109-02 Difco Lab Michigan USA) of *C. albicans* ATCC 10231 strains, bacterial cultures in brain heart agar (Cat No. 13825 Merck, Naucalpan, Edo. Mex. México) of *A. madurae* ATCC 19425, and *N. asteroides* strain collected from Hospital General 'Manuel Gea González' isolated from patients. Suspensions were prepared from all strains and were adjusted at $300 \times 10^6$ CFU/mL based on the McFarland nephelometer by using RPMI 1640 (Cat No 11875 Gibco, Life Technologies Grand Island New York USA) with 7.5% $NaHCO_3$ (Cat No S5761 Sigma) used as a diluent.

For opsonization, a volume of 3 mL of $300 \times 10^6$ CFU/mL in RPIM 1640 plus 7.5% $NaHCO_3$ was centrifuged at 6 000 ×*g* 40 min at room temperature. The pellet was mixed with 2 mL of complemented or decomplemented (by heating 30 min in water bath at 56 °C) normal human serum (NHS) and incubated for 40 min at 37 °C. After that, microorganisms were centrifuged as before and suspended in the original volume of the same diluent.

### 2.2. Microscopic assay of phagocytosis and activation in adherent cells

Samples of adherent cells were prepared as following. A volume of 1 mL of defibrinated blood samples from healthy volunteers were placed onto glass slide surfaces and incubated for 40 min at 37 °C in a humid chamber with 5% $CO_2$ atmosphere. The resulting adherent cells (in most part, the mononuclear type) were washed twice with sterile saline solution. Adherent cells ($3 \times 10^3$ approximately) were treated with *A. madurae*, *N. asteroides* and *C. albicans* at $3 \times 10^4$ CFU/mL to render a proportion of one cell by 10 microorganisms tested under three different conditions: i) unopsonized or opsonized with, ii) complemented, or iii) decomplemented NHS, as described above.

For this assay, microorganisms were suspended in 1 mL of RPMI 1640/7.5% $NaHCO_3$ mixed with 0.5 mL of 0.1% *p*-nitrotetrazolium blue (NBT, Cat No. N6876 Sigma, dissolved in sterile saline). Control phagocytes were mixed with RPMI 1640/7.5% $NaHCO_3$ containing NBT. Samples were incubated for 45 min at 37 °C inside a chamber with 7% humidity and 5% $CO_2$. Finally, adherent cells on glass slides were washed twice with sterile saline solution and stained with 0.5% safranin as a contrast dye. Samples were fixed with resine and a total of 100 cells were counted under optic microscope by using 40× and 100× objective-lenses. The percentage of relative activation (dependent of NADPH oxido-reductase enzymes) was computed by dividing the number of activated cells (with intracellular blue formazan insoluble complexes) between the total numbers of phagocytic cells (un-activated (red) plus activated (blue formazan)), and multiplied by 100. Data were expressed as the mean value plus standard deviation (SD) of relative activation ($n = 3$) from one of three repeated assays.

### 2.3. Spectrophotometric assay of endocytosis and activation in polymorphonuclear cells

#### 2.3.1. Polymorphonuclear cell culture

Polymorphonuclear cells were isolated from heparinized venous blood from healthy human volunteers by using the Polymorphprep™ gradient method according to the manufacturer's instructions (Cat No. 1114683 Axis-Shield PoC AS, Oslo, Norway). Polymorphonuclear (PMN) cells recovered from the interphase were washed twice by resuspending with sterile saline solution containing heparin (143 USP/10 mL) and centrifuging at 600 ×*g* 20 min at 20 °C. Cell pellet was suspended in RPMI 1640 with 7.5% $NaHCO_3$, 143 USP/10 mL heparin and supplemented with 10% decomplemented fetal calf serum (FCS, Cat No F2442 Sigma) and incubated in culture plates of 24 wells at 37 °C with 7% of humidity and 5% $CO_2$ in a final concentration of $3 \times 10^4$ cells per well.

#### 2.3.2. Spectrophotometric assay

For this assay, glass tubes (12 × 75 mm) containing PMN ($3 \times 10^4$) were mixed with microorganisms ($3 \times 10^5$ CFU/mL) opsonized with NHS suspended in RPMI plus NBT/$NaHCO_3$ (prepared as described before) to render a proportion of one cell to 10 microorganisms. Control PMN cells were incubated only with RPMI 1640 plus NBT/$NaHCO_3$ without microorganisms. Mixture samples were tested by triplicate and incubated for 45 min at 37 °C inside a chamber with 7% humidity and 5% $CO_2$.

After that, each tube was treated with 1 mL of pyridine and immersed for 15 min in a boiling water bath under reflux conditions to avoid the evaporation of pyridine and the oversaturation of formazan complexes. Absorbance of solubilized formazan complexes was evaluated spectrophotometrically at

λ = 525 nm and the concentration of formazan in μmol/mL was extrapoled from a standard curve with modifications of the original method [14].

Briefly, standards of ascorbic acid containing 2.34, 4.69, 9.38, 18.75, 37.5, 75.00 and 150 μM/mL were prepared by using 0.1% NBT as a solvent. A volume of 0.2 mL of each standard was mixed with 2 mL of 0.1 M NaOH and 2 mL of 24 mM $NaHCO_3$. Tubes were incubated for 10 min at room temperature and after the addition of 5 mL of distilled water they were centrifuged for 15 min, at room temperature and 3 000 ×*g*. After removing the supernatant, a purple insolubilized pellet from each tube was dissolved in 1 mL of pyridine and immersed for 20 min in a boiling water bath with reflux as described before. Tubes were left at room temperature to read the absorbance at λ = 525 nm using pyridine as a blank sample. Absorbance (Y axis) values compared to their corresponding ascorbic standard (X axis) were plotted for preparing the standard curve. Data of the total endocytosis of PMN were expressed as intracellular formazan in μmol/mL with microorganism or without microorganism (control). Intracellular formazan in μmol/cell was calculated as the subtraction of formazan in PMN with microorganism minus PMN without microorganism (control) divided by the total number of PMN. Finally, the percentage of actual activation was computed as a subtraction of formazan in PMN with microorganism minus PMN without microorganism (control) divided by the total PMN number and multiplied by 100.

### 2.4. Kinetic assay of cell viability in infected or uninfected PMN

Suspensions of PMN in RPMI (plus 7.5% $NaHCO_3$, 143 USP/10 mL heparin and 10% decomplemented FCS) were infected with *A. madurae*, *N. asteroides* and *C. albicans* (1 cell to 10 microorganisms). Uninfected PMN cells in the same diluent were included as controls. Culture samples tested by triplicate were taken each 60 min during 8 h of incubation at 37 °C with 5% $CO_2$ for monitoring the viability by counting dying cells in a Neubauer chamber by using a 0.4% tripan blue exclusion test. Results were expressed as the mean ($n$ = 3) plus SD of % viability from one of three repeated assays. Kinetic viability in cells was assessed in order to determine the time when the cell population is decreased to half and the IL-1β is detected in the culture supernates (see below).

### 2.5. Kinetic assays of IL-1β secretion

Purified PMN cells (35 × $10^3$ per well in 24 culture plates in RPMI plus 7.5% $NaHCO_3$, 143 USP/10 mL heparin and 10% decomplemented FCS) were infected with *A. madurae*, *N. asteroides* and *C. albicans* in a proportion of 1–10 microorganisms by pre-incubating for 15 min at 37 °C with 5% $CO_2$, while uninfected PMN in the same diluent were included as controls. Culture supernatants tested by triplicate were collected each 60 min during 8 h of incubation in order to quantify the IL-1β secretion by using an ELISA kit assay (Quantikine IL-1β Immunoassay Cat No DLB50, R&D Systems Mn, USA). Levels of IL-1β were expressed as the mean value plus standard deviation of pg/mL. Results were expressed as the mean ($n$ = 3) plus SD of pg/mL IL-1β from one of three repeated assays.

### 2.6. Statistical analysis

Data were analyzed with Student's $t$ test or one-way ANOVA by using a Sigma plot for Windows Version 11.0 software (Systat Software Incorporated, San Jose CA, USA) and $P < 0.05$ were considered to have significant differences.

### 2.7. Ethic statement and ethical approval

Samples were collected in accordance with relevance guidelines for ethical research design, confidentiality and protection human subjects. Protocol was reviewed and approved by the ethics and biosafety committees of the Universidad Autónoma Metropolitana Unidad Xochimilco. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (World Medical Association Declaration of Helsinki Ethical Principles for Medical Research, 1964).

## 3. Results

### 3.1. Microscopic assay of phagocytosis and activation in adherent cells

The effects of pathogens on the phagocytosis and activation were evaluated microscopically in adherent cells purified from blood of healthy donors. By comparison with unopsonized cells included as control (–), basal phagocytosis in adherent cells was significantly increased ($P < 0.05$) with *C. albicans* and *A. madurae* opsonized with whole and decomplemented NHS (DNHS), while in the case of *N. asteroides*, only with whole NHS.

Values of total phagocytosis were: for *C. albicans* [(93±7)%] (NHS) and [(77±8)%] (DNHS), for *A. madurae* [(87±8)%] (NHS) and [(63±10)%] (DNHS) and for *N. asteroides* [(54±7)%] (NHS) and [(49±6)%] (DNHS) (Table 1). Values of relative activation

**Table 1**
Microscopic evaluation of phagocytosis and activation of adherent cells.

| Opsonization | *C. albicans* | | *A. madurae* | | *N. asteroides* | |
|---|---|---|---|---|---|---|
| | Phagocytosis | Activation | Phagocytosis | Activation | Phagocytosis | Activation |
| Control | 34 ± 4 | 100 | 56 ± 10 | 52 ± 14 | 48 ± 5 | 56 ± 11 |
| NHS | 93 ± 7* | 100 | 87 ± 8* | 52 ± 8 | 54 ± 7* | 67 ± 10 |
| DNHS | 77 ± 8* | 100 | 63 ± 10* | 44 ± 20 | 49 ± 6 | 67 ± 13 |

Percentage (%) of relative phagocytosis (red plus blue intracellular formazan complexes) and activation (only blue intracellular formazan complexes) of adherent cells incubated with microbial strains opsonized with NHS or DNHS or only with diluent as control. Data ($n$ = 3) expressed as mean ± SD are representative of three repeated experiments. *$P < 0.05$ compared with the Control.

**Table 2**
Spectrophotometric analysis of endocytosis and activation in polymorphonuclear cells.

| Groups | PMN (mL) | Total formazan (µmol/mL) | Intracellular formazan (µmol/cell) | Total activation (%) |
|---|---|---|---|---|
| *C. albicans* | 2 430 000 ± 8 020 | 123.67 ± 24.58 | 0.000 026 70 ± 0.000 002 68 | 43.00 ± 1.01 |
| *A. madurae* | 2 430 000 ± 8 020 | 124.33 ± 20.65 | 0.000 018 00 ± 0.000 001 41 | 40.50 ± 4.95 |
| *N. asteroides* | 3 430 000 ± 1 530 | 309.50 ± 18.17 | 0.000 005 60 ± 0.000 001 13 | 6.00 ± 0.42 |

Total (µmol/mL) or (µmol/cel) formazan complexes in human polymorphonuclear (PMN) cells incubated with microorganism opsonized normal human serum (NHS) or only with diluent as control. Data ($n$ = 3) expressed in mean ± SD are representative of three repeated experiments.

were: with *C. albicans* of cells 100% (NHS and DNHS), with *A. madurae* [(52 ± 8)%] (NHS) and [(44±20)%] (DNHS) and with *N. asteroides* [(67±10)%] (NHS) and [(67±13)%] (DNHS) whereas with (DNHS) (Table 1).

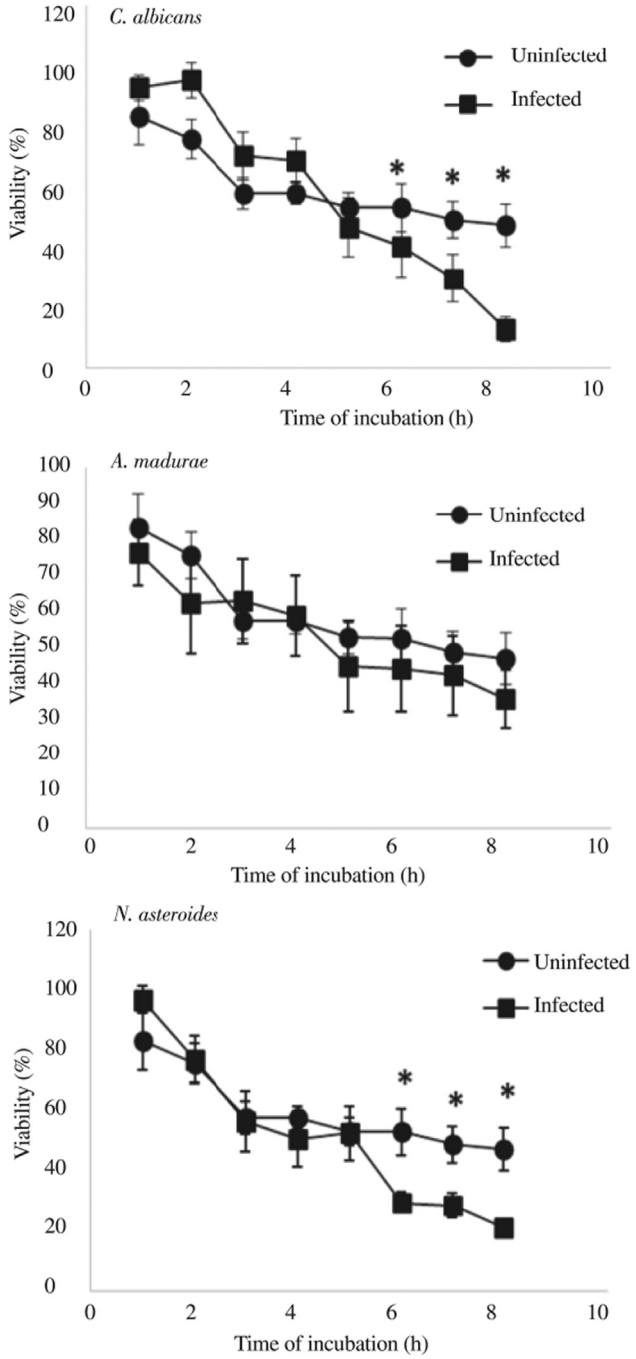


**Figure 1.** Kinetic of viability monitored during 8 h of PMN cells infected with *C. albicans*, *A. madurae* or *N. asteroides* or uninfected included as controls.
*$P < 0.05$ compared with the uninfected cells.

### 3.2. Photometric assay of endocytosis and activation in polymorphonuclear cells

The effect of microorganisms on endocytosis (total µmol/mL red plus blue formazan complexes) and activation (µmol/cell blue formazan complexes) in PMN cells evaluated by photometric assays are summarized in Table 2. Percentage of activation of PMN cells was significantly lower ($P < 0.05$) by the infection with *N. asteroides* (6.0% ± 0.42%) in comparison to *C. albicans* (43.0% ± 1.01%) and *A. madurae* (40.5% ± 4.95%).

### 3.3. Kinetic assay of cell viability in infected or uninfected PMN

The kinetic of cell viability of PMN cells infected with pathogens was evaluated in order to assess their viability during the infection. From the first 5 h of incubation, viability of PMN cells from the infected and uninfected followed similar kinetic patterns (Figure 1). From the 6 to 8 h of incubation and by comparison with uninfected conditions, viability of PMN infected with *C. albicans* and *N. asteroides* was significantly decreased ($P < 0.05$) whereas no differences were found in cells infected with *A. madurae*.

### 3.4. Kinetic assays of IL-1β secretion

The kinetic pattern of IL-1β secretion by PMN cells showed that by comparison with uninfected control cells, secretion of IL-1β was higher in PMN infected: at 1 h by *C. albicans* and *A. madurae*; at 2 h by all strains; at 4 and 5 h only by *A. madurae*; at 6 h by *A. madurae* and *N. asteroides*; at 7 h by *C. albicans* and *N. asteroides*; and at 8 h by all strains. Comparisons within strains indicated that the levels of IL-1β were significantly higher ($P < 0.05$) in cells infected by *C. albicans* (1, 2, 7 and 8 h), *A. madurae* (4 and 5 h) and *N. asteroides* (6 h). No other differences were found (Figure 2).

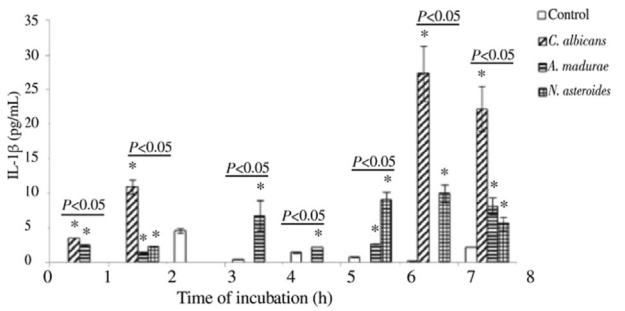


**Figure 2.** Kinetic of IL-1β secretion in PMN cells infected with *C. albicans*, *A. madurae* or *N. asteroides* or uninfected control cells.
*$P < 0.05$ compared with the control uninfected cells or among the strains $P < 0.05$ value on bar.

## 4. Discussion

Due to the critical role of phagocytosis in the outcome of infections caused by *A. madurae* [3], C. *albicans* [15] and *N. asteroides* [16], the ability of these pathogens to modulate the function of phagocytic cells from healthy donors was explored. According to the findings, phagocytosis of adherent cells (mostly of mononuclear type) was higher when *C. albicans*, *A. madurae* and *N. asteroides* were opsonized with NHS (containing antibodies and complement), although lesser extent was seen with DNHS (depleted of complement). Previous assays to assess the effect of opsonization of pathogens showed that phagocytosis and the killing of *C. albicans* by human mononuclear cells appear to be optimal when the yeast is opsonized with NHS [17]. Opsonization with antibodies triggers the killing activity of macrophages only with *N. asteroides* in log-phase cells but not in stationary-phase cells [16]. Phagocytosis of mononuclear cells of opsonized *A. madurae* is unknown, although in some actinomycetes opsonization with complement fragments and specific antibodies is correlated with the reduction in viability of *Actinomyces viscosus* by PMN cells [18]. Under host conditions, the substantive impact of opsonization to *N. asteroides* and *C. albicans* for enabling the phagocytosis by mononuclear cells is not fully clear [15,16] or unknown for *A. madurae*. Thus, under our experimental conditions, *in vitro* assays suggest that opsonization with antibodies and complement may contribute to phagocytosis of the pathogens by mononuclear cells.

In this study *C. albicans*, *A. madurae* and *N. asteroides* elicited differential patterns of activation of adherent and PMN cells. These effects may result, in part, from the expression of surface molecules of attachment with phagocyte receptors able to elicit signal pathways leading to engulfment and activation [9]. The presumable role of surface molecules on the interaction with phagocytic cells in *C. albicans* may include mannoproteins and/or β-glucans [19,20]. Surface components of interaction with phagocytes in *N. asteroides* and *A. madurae* are not yet described, but may include cell wall lipids reported from *N. brasiliensis* [8] or type-2 fimbriae lectin described in actinomycetes like *Actinomyces* [21].

Kinetic assays showed that viability was decreased in PMN cells infected with *C. albicans* and *N. asteroides*. Decreased PMN cell viability may result from components associated with the virulence including hydrolytic enzymes from *C. albicans* [19,20] or mycolic compounds described in *N. brasiliensis* [8,22]. During the kinetic assay, cell viability was unaffected by the infection with *A. madurae*, however, it contains cell wall components with potential deleterious effects on cell viability [4].

Interaction of surface components of pathogens with phagocytes via pattern recognition receptors elicits signal pathways leading to pro-inflammatory cytokines [9]. In this regard, elicitation of pro-inflammatory cytokines like IL-1β has been related with mannoproteins and β-glucans from *C. albicans* in human phagocytic cells [13,23] and mycolyl glycolipids as described in *Nymphaea rubra* in mouse peritoneal macrophages [24].

Accordingly, kinetic assays of IL-1β secretion were evaluated in PMN cells infected with pathogens. An overall analysis showed that decreased viability occurred along with increased IL-1β levels at (7–8) h of infection with *C. albicans* or at (6–8) h with *N. asteroides*. These findings might result from variations in the extent of the accumulative virulence effects from each pathogen that accounted for the increased IL-1β levels and decreased cell viability. Analysis of the ability to elicit IL-1β is useful for detecting virulences factors as well as differences in the virulence of *Candida* strains as described in human mononuclear cells and oral human epithelia [11,13]. In this assay conducted in PMN cells, findings suggest then that higher levels of IL-1β and lower viability may be associated with the virulence of C. *albicans* and *N. asteroides*. Levels of IL-1β and PMN cell viability were in general terms unaffected by the infection with *A. madurae* what reflect reduced virulence of this pathogen in regard C. *albicans* and *N. asteroides*.

Pathogen viability after the infection of phagocytes was not evaluated. However, in spite of this limitation, findings of this assay may contribute to designing methodological strategies with clinical impact to evaluate the virulence based on the ability of pathogens associated with subcutaneous or mucocutaneous chronic infections to elicit the response of phagocytic cells and the concomitant generation of pro-inflammatory cytokines. This assay may also contribute to the knowledge about the virulence properties of *N. asteroides* and *A. madurae* causing neglected tropical diseases.

## Conflict of interest statement

We declare that we have no conflict of interest.

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