

Role of TLR2 and TLR4 in Human Neutrophil Functions Against *Paracoccidioides brasiliensis*

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Abstract

In paracoccidioidomycosis, a systemic mycosis caused by the fungus *Paracoccidioides brasiliensis* (Pb), studies have focused on the role of neutrophils that are involved in primary response to the fungus. Neutrophil functions are regulated by pro- and anti-inflammatory cytokines. The molecular mechanisms involved in this process are not fully understood, but there are strong evidences about the involvement of toll-like receptors (TLR). We aimed at evaluating TLR2 and TLR4 expression on human neutrophils activated with GM-CSF, IL-15, TNF- α or IFN- γ and challenged with a virulent strain of *P. brasiliensis* (Pb18). Moreover, we asked if these receptors have a role on fungicidal activity, H₂O₂ and IL-6, IL-8, TNF- α and IL-10 production by activated and challenged cells. All cytokines increased TLR2 and TLR4 expression. Pb18 also increased TLR2 expression inducing an additional effect to that of cytokines. On the contrary, it inhibited TLR4 expression. All cytokines increased neutrophil fungicidal activity and H₂O₂ production, but this process was not associated with TLR2 or TLR4. Neutrophils activation with GM-CSF and TNF- α resulted in a significant increase in IL-8 production, while IL-15 and IFN- γ have no effect. Pb18 alone also increased IL-8 production. None of the cytokines activated neutrophils for IL-10 release. This cytokine was only detected after Pb18 challenge. Interestingly, IL-8 and IL-10 production involved TLR2 and mainly TLR4 modulation. Our data suggest that Pb18 uses TLR4 to gain access to human neutrophils. This interaction results in IL-8 and IL-10 production that may be considered as a pathogenic mechanism in paracoccidioidomycosis.

Introduction

Paracoccidioides brasiliensis (Pb) is the aetiological agent of paracoccidioidomycosis, a systemic mycosis endemic in Latin America. The infection can be acquired by inhalation of airborne conidia that reach the lung alveoli, where they transform into yeast cells, the infective form [1]. Many people are exposed to the fungus, but only a small number develop clinical symptoms, suggesting that both innate and adaptive mechanisms are important in fungus clearance [2–5]. The host innate immune response against fungus has been well characterized, and several studies have clearly shown the role of phagocytic cells. In this context, in last years, various studies have focused on the role of neutrophils [6].

Some *in vitro* studies suggest that Pb-infected macrophages induce the onset of extravascular neutrophilia by releasing chemotactic peptides [7]. Heavy neutrophil

infiltration in the lungs of Pb-infected mice at early acute infection was correlated with the release of keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-1 α (MIP-1 α), two important neutrophil chemoattractants [8]. In consequence of these chemotactic processes, massive neutrophil infiltration is found in infected tissues from patients with paracoccidioidomycosis [9] and in the early lesions of experimentally infected animals [10, 11].

Neutrophils from infected individuals can kill Pb [12]. However, experiments using more sensitive methods showed that despite their phagocytic capacity, these neutrophils are unable to digest Pb *in vitro*, indicating that a defect of neutrophil function may represent a susceptibility factor [13]. Further, studies in mice strongly suggest that lack of fungicidal activity correlates with defect in neutrophil activation because only those neutrophils from *P. brasiliensis*-sensitized mice exhibited efficient fungicidal

activity *in vitro* [14]. In addition, neutrophil fungicidal activity is higher in resistant mice than in susceptible mice [15]. Pina *et al.* [16], in a complete study of neutrophil depletion during murine infection, have shown that these cells are essential for host defence to Pb infection and that host genetic pattern exerts an important influence on neutrophil functions.

Together, the findings reported to date clearly demonstrate that neutrophils may play an important effector and immunomodulatory role, especially in the early stages of infection, contributing to Pb host resistance. Nonetheless, some studies show that neutrophil functions, including fungus killing, require activation with cytokines and other factors. In our laboratory, IFN- γ , TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-15 have been observed to activate human neutrophils for fungicidal activity by a mechanism dependent on H₂O₂ and superoxide anion [17, 18].

The specific detection of microorganisms by innate cells is mediated by pattern recognition receptors (PRR), germ line-encoded receptors that recognize microbial structures referred to as pathogen-associated molecular pattern [19]. Toll-like receptors (TLR) are essential PRR that mediate recognition of microbial structures, such as those of fungi, as well as the subsequent inflammatory and adaptative responses [20–23].

Because neutrophils and TLR are respectively the prototypical cell and receptor of innate immune response, the role of individual TLR on neutrophil functions has been investigated [24–27], including that involved in the response of these cells to fungi [28]. Various stimuli have been shown to regulate expression of TLR in neutrophils, including pathogen structures and TLR ligands, such as lipopolysaccharide (LPS), and pro-inflammatory cytokines, such as IL-1 β , TNF- α , GM-CSF and IFN- γ [24, 26, 29–31]. In view of these observations, studies conducted to evaluate the role of TLR on neutrophil functions against Pb may contribute to a better understanding of parasite/host relationship in the mycosis. In the present study, we aimed at evaluating TLR2 and TLR4 expression on human neutrophils activated with GM-CSF, IL-15, TNF- α or IFN- γ and challenged with Pb18, a virulent strain of the fungus. Moreover, we asked if these receptors have a role on fungicidal activity, H₂O₂ and IL-6, IL-8, TNF- α and IL-10 production by activated and challenged cells.

Subjects and methods

Healthy individuals. Twenty-eight healthy blood donors from University Hospital of the Botucatu Medical School, São Paulo State University, Brasil (age range 20–50 years) were included in the present work. The study was approved by Ethics Committee of Botucatu Medical School, and informed consent was obtained from all the blood donors.

Fungi. The high virulent strain of *P. brasiliensis* (Pb18) was used throughout this study. To ensure virulence, the isolate was used after three serial animal passages. Pb18 yeast cells were then maintained by weekly sub-cultivation in the yeast-form cells at 35 °C on 2% glucose, 1% peptone, 0.5% yeast extract and 2% agar medium (GPY medium) and used on the sixth day of culture. Yeast cells were washed and suspended in 0.15 M phosphate-buffered saline (PBS pH 7.2). To obtain individual cells, the fungal suspension was homogenized with glass beads in a Vortex homogenizer (three cycles of 10 s). Yeast viability was determined by phase contrast microscopy, and bright yeast cells were counted as viable, while dark ones were considered not viable. Fungal suspensions containing more than 95% viable cells were used in the experiments.

Isolation of human neutrophils. Heparinized venous blood samples were obtained from healthy subjects. Ten millilitres of blood was diluted in 10 ml RPMI 1640 tissue culture medium (Sigma-Aldrich, Inc., St Louis, MO, USA). The cell was layered on Percoll 85% and Histopaque – 1077 (Sigma-Aldrich). The cell fraction containing neutrophils was washed with RPMI 1640. Remaining cells were suspended in RPMI 1640 tissue culture medium supplemented with 2 mM of L-glutamine (Sigma-Aldrich), 40 μ g/ml of gentamycin and 10% heat-inactivated autologous human serum (CTCM: complete tissue culture medium). The cellular viability was assessed by trypan blue dye exclusion test, and the suspensions were adjusted for 2×10^6 cells/ml. The purity of neutrophil suspensions determined by morphological examination of May-Grunwald-Giemsa-stained slides was >98%. Then, neutrophil suspensions were dispensed into 96-well flat-bottom plates with a volume of 100 μ l/well and incubated for 18 h at 37 °C in a 5% CO₂ only with CTCM, or LPS (20 μ g/ml) or the cytokines GM-CSF (100 U/ml), IL-15 (31.2 ng/ml), TNF- α (250 U/ml) or IFN- γ (50 U/ml) (R&D Systems, Minneapolis, MN, USA) and then challenged with Pb18 at the concentration of 2×10^4 yeasts/ml of CTCM plus 10% fresh human autologous serum (1:50 fungus/neutrophils ratio) during 4 h. In the experiments for evaluating fungicidal activity, H₂O₂ and cytokines production, neutrophils were treated with anti-TLR2 (clone TL2.1) or anti-TLR4 (clone HTA125) monoclonal antibodies (Imgenex Biocarta US, San Diego, CA, USA) at 0.5 and 10 μ g/ml, respectively, for 1 h at 37 °C, before fungus challenge.

TLR2 and TLR4 expression. After Pb18 challenge, neutrophils were evaluated by TLR2 and TLR4 expression. This assay was performed by flow cytometry analysis. Neutrophils (1×10^6 neutrophils/ml) were distributed (500 μ l) into polystyrene tubes for cytometric analysis (BD Labware, San Jose, CA, USA). Cells were washed and incubated with fluorescein isothiocyanate-conjugated anti-TLR2 (Biolegend Inc., San Diego, CA,

USA), phycoerythrin-conjugated anti-TLR4 (Biolegend), according to the instructions of the manufacturer. After incubation for 15 min at room temperature, the cells were analysed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Data (an average of 10,000 events per sample) were analysed with the CELL QUEST Software (Cell Quest Software, San Jose, CA, USA).

Evaluation of fungicidal activity. After Pb18 challenge, neutrophil–fungus cocultures were harvested by aspiration with sterile distilled water to lyse neutrophils. Washing of each well resulted in a final volume of 2.0 ml, and 0.1 ml 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 (vol/vol), the latter being the source of growth-promoting factor. Inoculated plates, in triplicate of each culture, were incubated at 35 °C in sealed plastic bags to prevent drying. After 10 days, the number of colony forming units (CFU) per plate was counted. The inoculum used for the challenge was also plated according to the same conditions. The plates containing the material obtained from the neutrophil–fungus cocultures were considered as experimental plates, and those plated with the inoculum alone and counted at time zero were used as control plates. Fungicidal activity percentage was determined by the following formula:

$$\% \text{ Fungicidal Activity} = [1 - (\text{mean CFU recovered on experimental plates} / \text{mean CFU recovered on control plates})] \times 100.$$

Evaluation of H₂O₂ release. The release of H₂O₂ by neutrophils was measured by the horseradish peroxidase–phenol red oxidation method [32]. For this assay, neutrophil cultures were challenged with Pb18 suspension diluted in phenol red buffer containing 50 µg/ml of horseradish peroxidase (type II, Sigma-Aldrich) plus 10% fresh human AB serum and further incubation for 1 h in 5% CO₂ at 37 °C in humidified chamber. The reaction was stopped by addition of 10 µl of 1 N NaOH, and the absorbance at 620 nm was determined with a micro-ELISA reader (MD 5000; Dynatech Laboratories, Inc., Chantilly, VA, USA). All measurements were repeated four times, and the absorbance was converted into nanomoles of a standard curve of H₂O₂.

Measurement of cytokines. After Pb challenge, neutrophil culture supernatants were separated from cell debris by centrifugation at 1000 g for 15 min and stored at –70 °C. TNF-α, IL-6, IL-8 and IL-10 concentrations were measured by capture ELISA using Kit DuoSet (R&D Systems). ELISA was performed according to the manufacturer's protocol. Cytokine concentrations were determined with reference to a standard curve for serial twofold dilutions of recombinant cytokines. Absorbance values were measured at 492 nm using a micro-ELISA reader (MD 5000; Dynatech Laboratories).

Statistical analysis. Data were analysed statistically using the INSTAT software (Graph Pad, San Diego, CA, USA). The results were compared by variance analysis (ANOVA) followed by Tukey's test, with the level of significance set at $P < 0.05$.

Results

TLR2 and TLR4 expression

In the initial set of experiments, we assessed the effect of cytokines activation and/or Pb18 challenge on TLR2 and TLR4 expression by neutrophils. Before Pb18 challenge, neutrophils were pre-activated with the cytokines GM-CSF, IL-15, TNF-α or IFN-γ or LPS and evaluated by TLR2 and TLR4 expression, using flow cytometry. LPS was used as positive control for TLR2 and TLR4 expression by neutrophils. Cells treated only with CTCM expressed very low levels of TLR2 that increased after activation with cytokines or LPS. After Pb18 challenge, all cultures expressed higher TLR2 levels when compared to their respective non-challenged cultures (Fig. 1A). All cytokines and LPS increased TLR4 expression. However, after Pb18 challenge, a decrease in this expression was detected when compared to that detected in non-challenged cells (Fig. 1B). Together, the results showed that neutrophil activation with all cytokines resulted in an increase in TLR2 and TLR4 expression. However Pb18 modulation was different for TLR2 or TLR4. While this fungus increased TLR2 expression inducing an additional effect to that of cytokines, it decreased TLR4 expression.

Role of TLR2 and TLR4 on fungicidal activity

As all cytokines increased TLR2 and TLR4 expression, we performed experiments to assess the role of these receptors on antifungal activities by activated neutrophils, such as fungicidal activity, H₂O₂ release and IL-6, IL-8, TNF-α and IL-10 production. For this, before fungus challenge, neutrophils were treated with anti-TLR2 or anti-TLR4 monoclonal antibodies, for TLR2 and TLR4 blockade. Parallel experiments confirmed inhibition of TLR2 and TLR4 expression after blockade (data not presented). Figure 2 shows the results on fungicidal activity. Non-activated cells presented a very low fungicidal activity. However, this activity was significantly increased after cells activation with all cytokines. Interestingly, this response profile was not significantly altered after TLR2 or TLR4 blockade, leading us to suggest that these receptors were not involved in this activity.

Role of TLR2 and TLR4 on H₂O₂ production

Figure 3A–D show the results concerning TLR2 and TLR4 role on H₂O₂ production by neutrophil activated

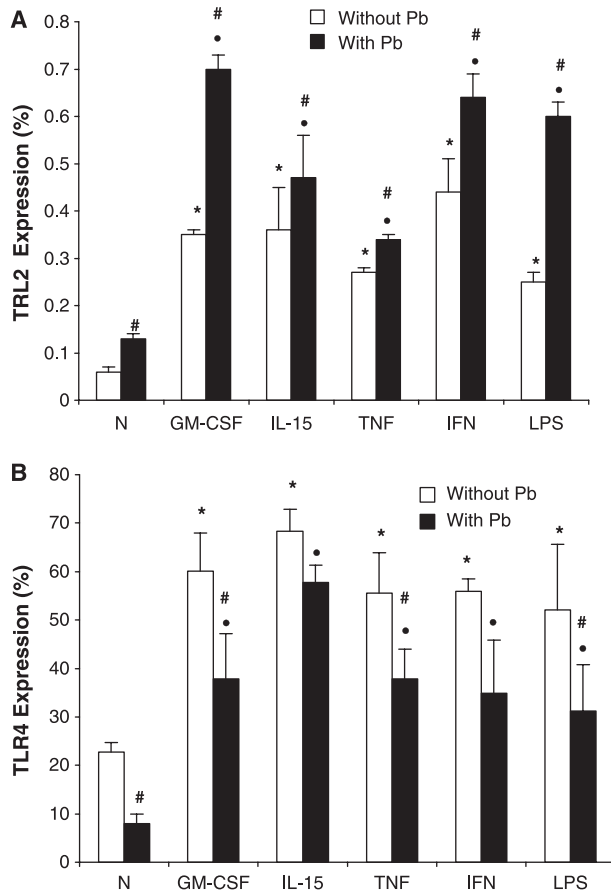


Figure 1 TLR2 (A) and TLR4 (B) expression by human neutrophils. Cells were non-activated (N) or activated with the cytokines GM-CSF (100 U/ml), IL-15 (31.2 ng/ml), TNF- α (250 U/ml) or IFN- γ (50 U/ml) or LPS (20 μ /ml) for 18 h, challenged or not with Pb18 for 4 h (2×10^4 yeasts/ml: 1:50 fungus/PMN ratio) and evaluated by TLR2 and TLR4 expression by flow cytometry. The figure shows the mean \pm SEM obtained from 25 blood donors. Statistically significant differences between groups are indicated. * $P < 0.05$ versus N; • $P < 0.05$ versus N + Pb; # $P < 0.05$ versus N without Pb.

with GM-CSF, IL-15, TNF- α or IFN- γ , respectively. A similar response profile was detected for all assays, because H₂O₂ levels were significantly increased after activation with the four cytokines, but differences among them not being detected. Moreover, there was a tendency towards Pb18 to increase metabolite release and to induce an additional effect to that of cytokines (data not statistically significant). However, as detected for fungicidal activity, H₂O₂ release was not significantly altered with TLR2 or TLR4 blockade showing the non-involvement of these receptors on this neutrophil activity.

Participation of TLR2 and TLR4 on IL-6, IL-8, TNF- α and IL-10 production

We also studied the possible role of TLR2 and TLR4 on IL-6, IL-8, TNF- α and IL-10 production by human

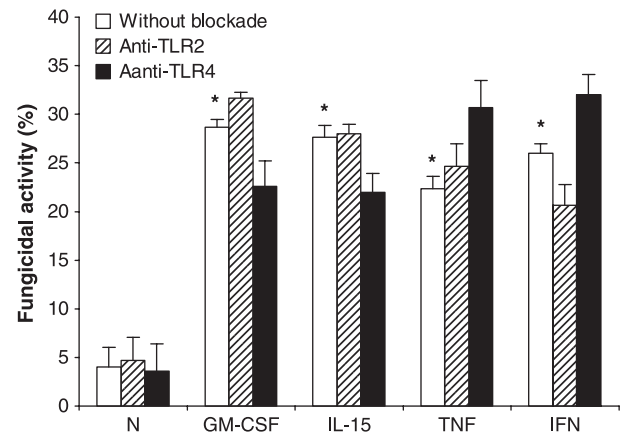


Figure 2 Role of TLR2 and TLR4 on fungicidal activity. Cells were non-activated (N) or activated with the cytokines GM-CSF (100 U/ml), IL-15 (31.2 ng/ml), TNF- α (250 U/ml) or IFN- γ (50 U/ml), in the absence or presence of anti-TLR2 or anti-TLR4 monoclonal antibodies, challenged or not with Pb18 for 4 h (2×10^4 yeasts/ml:1:50 fungus/PMN ratio) and evaluated for fungicidal activity. The figure shows the mean \pm SEM obtained from 25 blood donors. Statistically significant differences between groups are indicated. * $P < 0.05$ versus N without anti-TLR2 or anti-TLR4.

neutrophils activated with the different cytokines and Pb18 challenged. IL-6 and IL-8 were not detected in neutrophil supernatants. Then, Figs. 4A–E and 5A–E show the results regarding IL-8 and IL-10 production, respectively. Neutrophil activation with GM-CSF and TNF- α resulted in a significant increase in IL-8 production, while IL-15 and IFN- γ have no effect. Pb18 alone also increased IL-8 production. Moreover, it was detected a tendency towards the fungus exhibit an additional effect in relation to this cytokine production in GM-CSF-treated cultures. None of the cytokines activated neutrophils for IL-10 release. This cytokine was only detected after Pb18 challenge. Interestingly, in most cultures, IL-8 and IL-10 production induced by cytokines and/or Pb was diminished after TLR2 and mainly TLR4 blockade. These results suggest that IL-8 and IL-10 production by neutrophils in response to *P. brasiliensis* is dependent on TLR2 and mainly on TLR4.

Discussion

Neutrophils are essential components of the innate immune response against fungi, because they are the first immune cells to arrive at sites of infection, where they initiate antimicrobial and pro-inflammatory functions. A variety of receptors are involved in innate immune responses to fungal infections, including the mannose receptor, complement receptor 3 (CR3), TLR and β -glucan receptor (β GR), and dectin-1 [6, 33, 34]. Then, neutrophils activated by some of these receptors may limit infection via fungus phagocytosis and by releasing

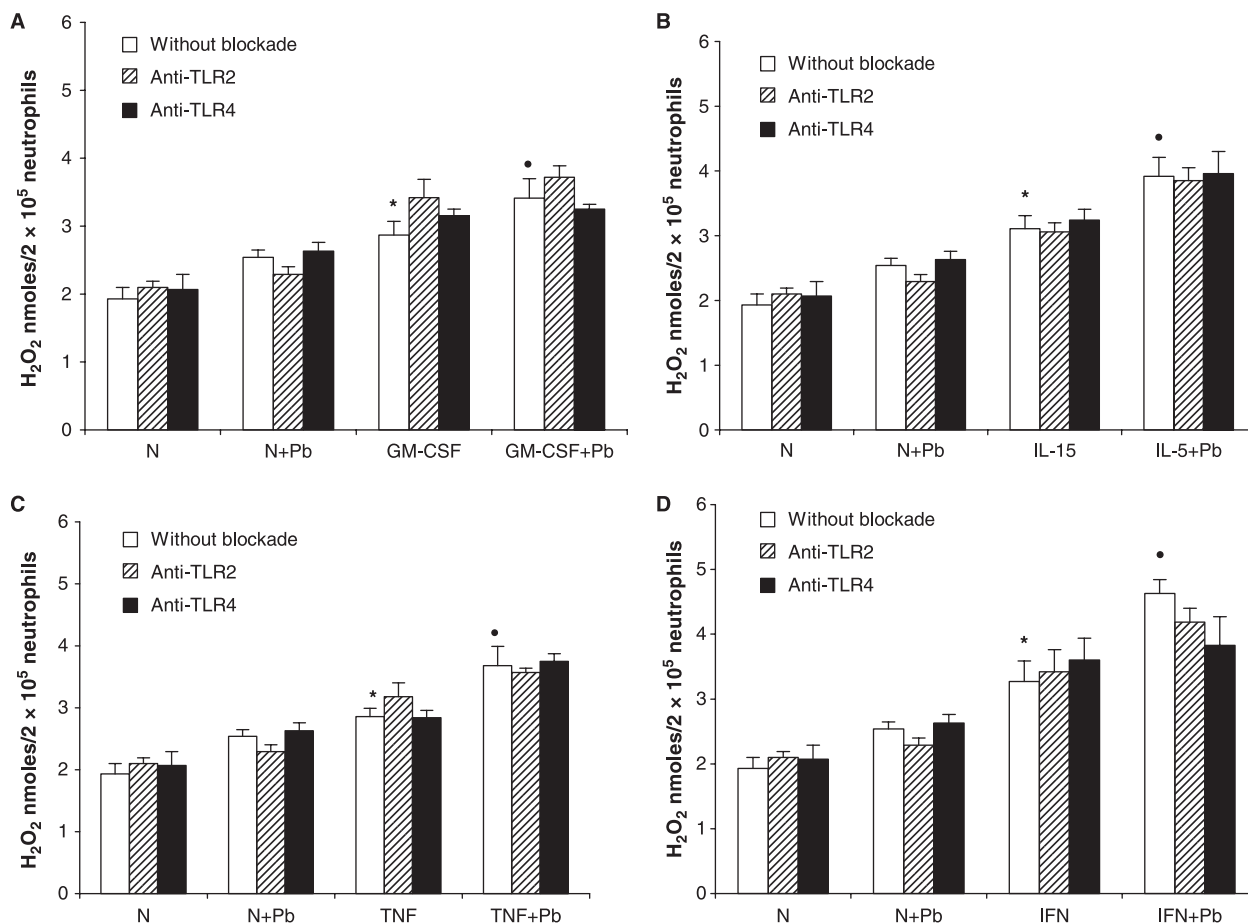


Figure 3 Role of TLR2 and TLR4 on H_2O_2 production. Cells were non-activated (N) or activated with the cytokines GM-CSF (100 U/ml) (A), IL-15 (31.2 ng/ml) (B), TNF- α (250 U/ml) (C) or IFN- γ (50 U/ml) (D) for 18 h, in the absence or presence of anti-TLR2 or anti-TLR4 monoclonal antibodies, challenged or not with Pb18 for 4 h (2×10^4 yeasts/ml:1:50 fungus/PMN ratio) and evaluated for H_2O_2 production. The figure shows the mean \pm SEM obtained from 25 blood donors. Statistically significant differences between groups are indicated. * $P < 0.01$ versus N; • $P < 0.001$ versus N.

antimicrobial peptides, reactive oxygen intermediates and pro-inflammatory cytokines. Through chemokines production, they may recruit and activate other immune cells, and finally they have an important role on modulating adaptive immune response [28, 35]. In this context, we aimed at evaluating TLR2 and TLR4 expression on human neutrophils activated with the cytokines GM-CSF, IL-15, TNF- α or IFN- γ and challenged with a virulent strain of Pb. Moreover, we asked if these receptors have a role on fungicidal activity, H_2O_2 and IL-6, IL-8, TNF- α and IL-10 production by activated and challenged cells. We detected that cells expressed both TLR2 and TLR4 receptors and that this expression is significantly increased after GM-CSF, IL-15, TNF- α and IFN- γ activation. These results are in agreement with others showing that human neutrophils express almost all known TLR, including TLR2 and TLR4 [26], and that cytokines such as IL-1, and TNF- α [29], GM-CSF [24, 26, 31] and IFN- γ [31] increased this expression.

We also found that Pb18 increased TLR2 expression inducing an additional effect to that of cytokines. In contrast, Pb challenge resulted in a decrease in TLR4 expression in non-stimulated neutrophils and cells treated with GM-CSF, TNF- α and LPS but not IL-15 and IFN- γ . A possible explanation for this result is that Pb can use TLR4 to bind and enter inside neutrophils with consequent diminution in TLR4 levels on cells surface. This idea is supported by recent studies showing that TLR4 and TLR2 are involved in Pb recognition by phagocytic cells [36].

As cytokines and/or Pb modulated TLR4 and TLR2 expression, we had interest in assessing whether these receptors are involved in neutrophil antifungal functions induced by these cytokines. We confirmed our previous studies showing that GM-CSF, IL-15, TNF- α and IFN- γ activate human neutrophils inducing these cells to release higher H_2O_2 levels and fungicidal activity against Pb [17, 18, 37]. However, both H_2O_2 release and fungicidal

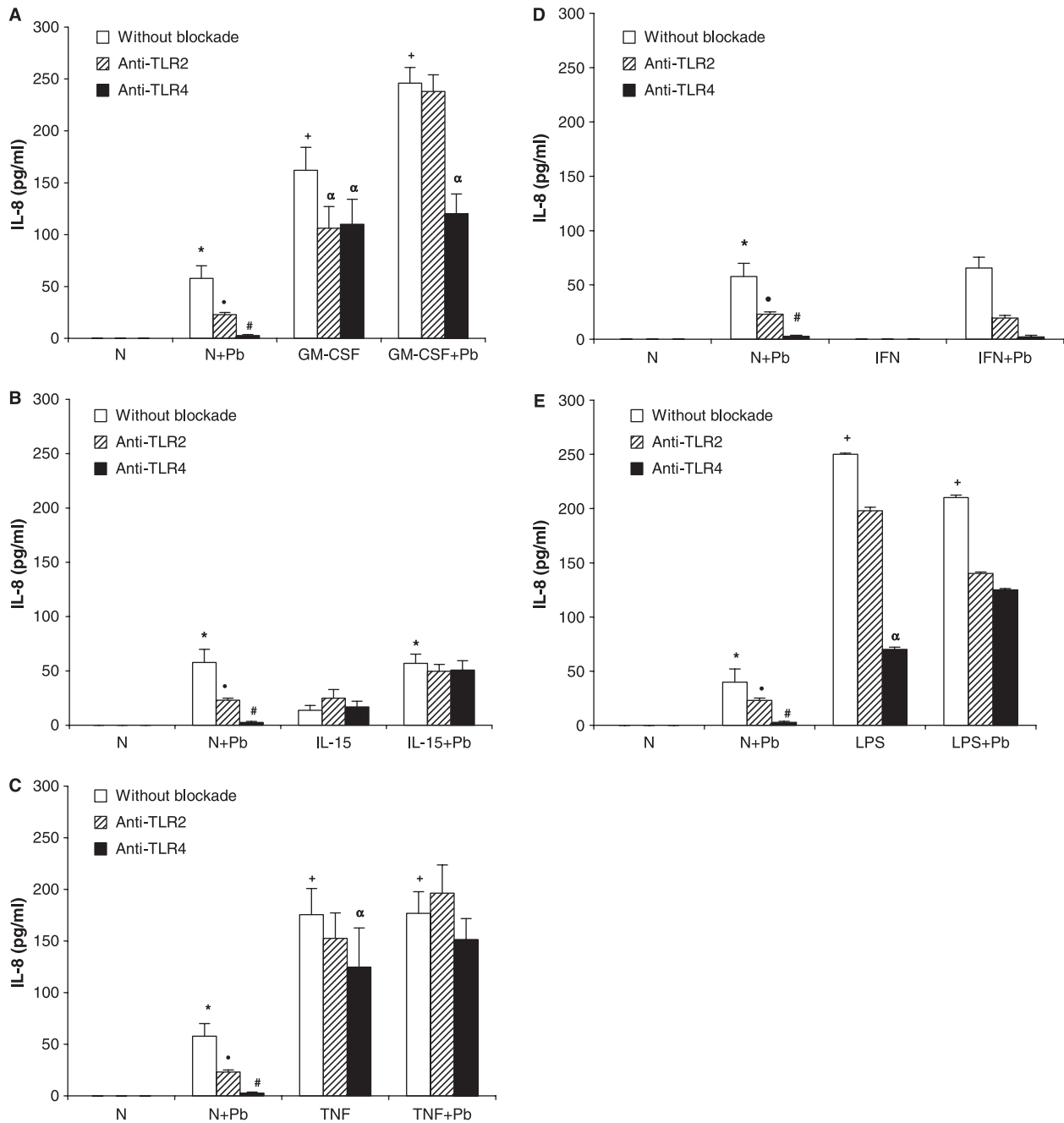


Figure 4 Participation of TLR2 and TLR4 on IL-8 production. Cells were non-activated (N) or activated with the cytokines GM-CSF (100 U/ml) (A), IL-15 (31.2 ng/ml) (B), TNF- α (250 U/ml) (C) or IFN- γ (50 U/ml) (D) or LPS (20 μ g/ml) for 18 h, in the absence or presence of anti-TLR2 or anti-TLR4 monoclonal antibodies, challenged or not with Pb18 for 4 h (2×10^4 yeasts/ml:1:50 fungus/PMN ratio) and evaluated for IL-8 production by ELISA. The figure shows the mean \pm SEM obtained from 25 blood donors. Statistically significant differences between groups are indicated. * $P < 0.05$ versus N; + $P < 0.01$ versus N; • $P < 0.05$ without blockade; # $P < 0.01$ without blockade; $\alpha P < 0.001$ without blockade.

activity were not altered after TLR2 or TLR4 blockade showing the non-involvement of these receptors on these neutrophil activities. In agreement with our results, some studies have demonstrated a non-association between TLR2, TLR4 and fungal killing mechanisms. TLR4 was shown to be involved in protection in disseminated candidiasis. However, an association between this receptor

and the mechanisms involved in *Candida albicans* killing, such as nitric oxide and superoxide anion, was not detected [38]. It was also shown that Pb yeasts are recognized by TLR2 and TLR4 resulting in increased phagocytic ability, NO secretion and fungal infection of macrophages. However, this effect did not result in fungal growth control [36].

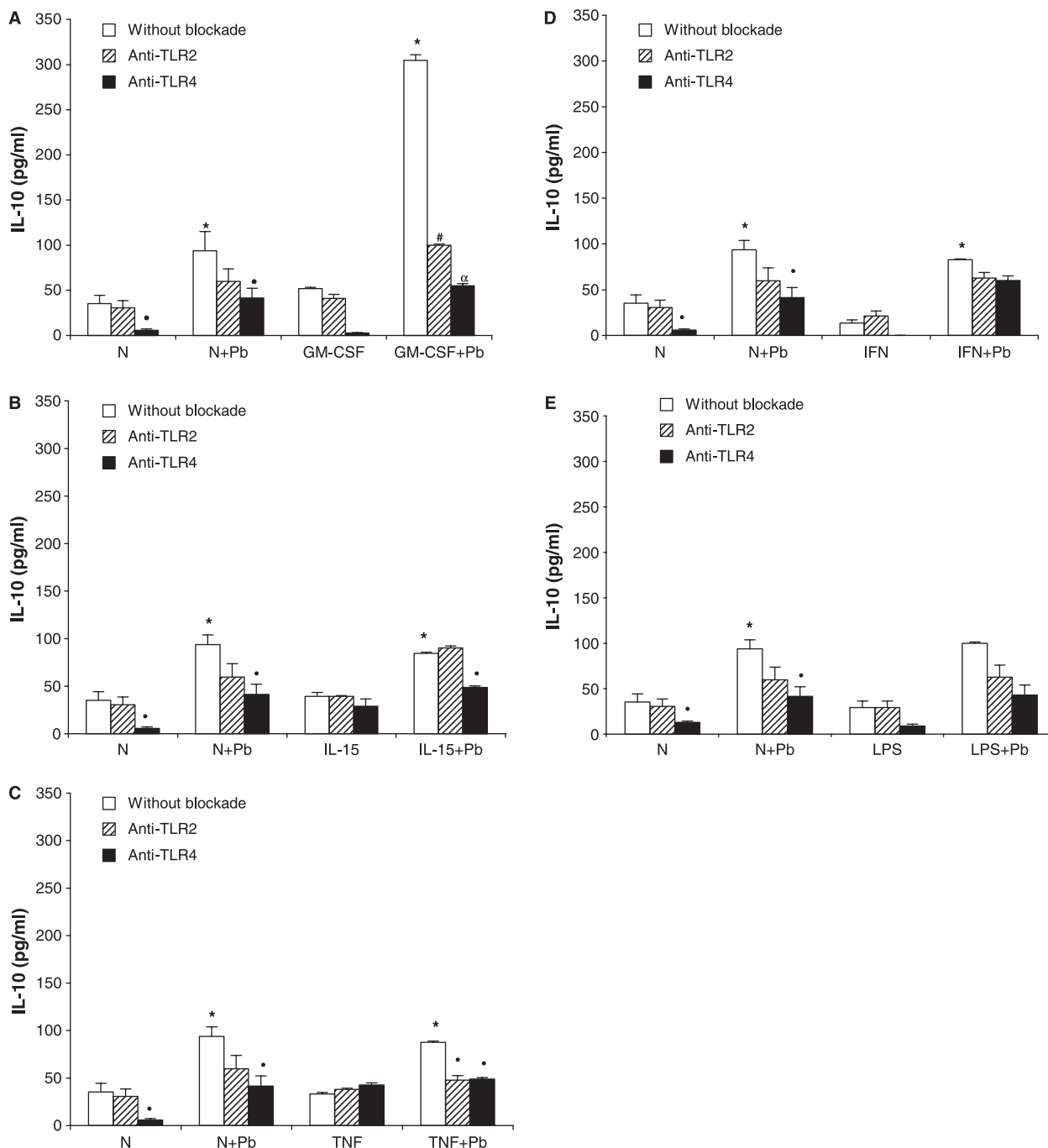


Figure 5 Participation of TLR2 and TLR4 on IL-10 production. Cells were non-activated (N) or activated with the cytokines GM-CSF (100 U/ml) (A), IL-15 (31.2 ng/ml) (B), TNF- α (250 U/ml) (C) or IFN- γ (50 U/ml) (D) or LPS (20 μ g/ml) for 18 h, in the absence or presence of anti-TLR2 or anti-TLR4 monoclonal antibodies, challenged or not with Pb18 for 4 h (2×10^4 yeasts/ml:1:50 fungus/PMN ratio) and evaluated for IL-10 production by ELISA. The figure shows the mean \pm SEM obtained from 25 blood donors. Statistically significant differences between groups are indicated. * $P < 0.05$ versus N; + $P < 0.01$ versus N; • $P < 0.05$ without blockade; # $P < 0.01$ without blockade; $\alpha P < 0.001$ without blockade.

Our results showing non-TLR2 or non-TLR4 requirement for neutrophil killing mechanisms lead us to ask about the role of other receptors. Some studies have demonstrated the importance of mannose receptors [39, 40] and CR3 [40, 41] in Pb phagocytosis. However, in our

study, we can discard mannose receptors involvement, because this receptor is not expressed by human neutrophils. In contrast, studies have shown CR3 and dectin-1 expression by these cells [42, 43]. Moreover, dectin-1 is involved in *C. albicans* killing by human

neutrophils [35]. Studies are being conducted in our laboratory to test the role of both CR3 and dectin-1 on fungal killing by human neutrophils.

We aimed at studying TLR2 and TLR4 requirement for IL-6, TNF- α , IL-8 and IL-10 production. However, in our assays, neutrophils failed to release IL-6 and TNF- α . Studies on the literature are controversial in relation to release of some cytokines by human neutrophils [44]. However, we are suggesting that lack of TNF- α and IL-6 detection in our assays may be related to the period of culture for supernatant analysis (at least 18 h). It is possible that this period was very late for TNF- α and IL-6 detection. Neutrophil activation with GM-CSF and TNF- α resulted in a significant increase in IL-8 production, while IL-15 and IFN- γ have no effect. Pb18 also increased IL-8 production. Moreover, there was a tendency towards Pb 18 exhibiting an additive effect in GM-CSF-treated cultures. None of the cytokines activated neutrophils for IL-10 release. This cytokine was only detected after Pb18 challenge. Interestingly, in most assays, cytokines production was inhibited after receptors blockade. However, in relation to this effect, we must consider the most evident role of TLR4 in relation to TLR2. Some studies have shown TLR2 and TLR4 requirement for cytokines production by phagocytic cells in response to several stimuli, including fungi. TLR4 is important for murine protection to *C. albicans*, by chemokines production such as KC and MIP-2, important for neutrophils influx [37]. Yet, in candidiasis, TLR2 is involved in TNF- α , MIP-2 [45], IL-12, IFN- γ [46] and IL-10 production [47]. In relation to paracoccidioidomycosis, our data showing preferential involvement of TLR4 in cytokines production are not in agreement with some studies showing that IL-10 production by dendritic cells or monocytes/neutrophils in response to Pb involves a preferential fungus recognition by TLR2 and dectin-1 instead of TLR4 [48, 49]. The possible explanation for these differences might be related for differences in experimental protocols such as evaluation periods and the blockade or not of receptors.

In paracoccidioidomycosis, as in other infections, IL-10 production in response to Pb has been considered as an escape mechanism from host defence. High levels of this cytokine are detected in serum and culture supernatants of patients [50–52], and patients' monocytes spontaneously release high levels of this cytokine *in vitro* [53]. In experimental model of the mycosis, higher levels of IL-10 were released by susceptible mice when compared to those of resistant mice [54]. In our laboratory, this cytokine has been demonstrated to inhibit Pb killing by IFN- γ -activated and TNF- α -activated human monocytes and neutrophils [36, 55]. However, we cannot discard the possible beneficial role of IL-10, controlling excessive inflammatory response induced by pro-inflammatory cytokines. In a recent study, less virulent strain of Pb was

shown to be recognized by TLR2 and dectin-1 with consequent balanced production of TNF- α and IL-10. On the other hand, more virulent strain induced only TNF- α production. Thus, less virulent strain, by IL-10 production, induced a more controlled response, beneficial for the host [49].

Regarding IL-8, studies in our laboratory have demonstrated that this cytokine is involved in an anti-apoptotic effect of Pb on neutrophils, resulting in a delay on cells death, a process that could allow the fungus to survive intracellularly [56]. In addition, some studies showed that delayed neutrophil apoptosis induced by IL-8 involves signalling by TLR4 [57].

In summary, our data suggest that Pb18 uses TLR4 to gain access to human neutrophils. However, this process does not result in an increase in killing mechanisms by these cells. On the other hand, it is involved in IL-8 and IL-10 production by human neutrophils in response to activator cytokines and/or Pb. Considering that IL-10 and IL-8 are preferentially involved in escape mechanisms of Pb from neutrophil functions, our study points to the idea that Pb interaction with TLR4 on human neutrophils could be considered as a pathogenicity mechanism of this fungus, which would use host receptors of innate immunity to infect cells and to guarantee its own multiplication.

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Conflict of interest

None.

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