Paracoccidioides brasiliensis downmodulates α3 integrin levels in human lung epithelial cells in a TLR2-dependent manner

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Paracoccidioidomycosis (PCM) is the most prevalent systemic mycosis in Latin America and may be caused by the species *Paracoccidioides brasiliensis*. In the lungs, this fungus interacts with epithelial cells, activating host cell signalling pathways, resulting in the production of inflammatory mediators. This event may be initiated through the activation of Pattern-Recognition Receptors such as Toll-like Receptors (TLRs). By interacting with cell wall components, TLR2 is frequently related to fungal infections. In this work, we show that, after 24 h post-infection with *P. brasiliensis*, A549 lung epithelial cells presented higher TLR2 levels, which is important for IL-8 secretion. Besides, integrins may also participate in pathogen recognition by host cells. We verified that *P. brasiliensis* increased α3 integrin levels in A549 cells after 5 h of infection and promoted interaction between this receptor and TLR2. However, after 24 h, surprisingly, we verified a decrease of α3 integrin levels, which was dependent on direct contact between fungi and epithelial cells. Likewise, we observed that TLR2 is important to downmodulate α3 integrin levels after 24 h of infection. Thus, *P. brasiliensis* can modulate the host inflammatory response by exploiting host cell receptors and cell signalling pathways.

Paracoccidioidomycosis (PCM) is a deep human mycosis that is restricted to Latin America and affects commonly male rural workers. Although this disease has been frequently related to poverty, unfortunately, it is still not recognised as a neglected tropical disease by the World Health Organisation (WHO). Nowadays, environmental factors are changing PCM epidemiology, which is reflected in the increasing number of patients in some regions. One example was recently reported by Do Valle and co-workers (2017), who described the increase of PCM cases by sixfold after a highway construction in Rio de Janeiro (Brazil), possibly caused by deforestation and the removal of vast amounts of soil.

PCM is caused by dimorphic fungi from the genus *Paracoccidioides* that present mycelial and yeast forms. Host infection may occur by inhalation of mycelial propagules, probably contained in contaminated soil, and once in the lungs, these fungi convert into yeasts. Aiming to survive, pathogens adopt some mechanisms to exploit host cells, leading to the establishment, maintenance and dissemination of the infection.

Epithelial cell is an important cell type of host lungs that interacts with inhaled pathogens, not only primarily forming a physical barrier against microorganisms, but also acts in the innate immune response by secreting inflammatory mediators such as cytokines and chemokines, which results in the recruitment of immune cells to the site of infection.

Over the past years, our research group has focused on studying the lung epithelial cell responses to fungal infections, especially regarding the mechanisms involved in the release of inflammatory cytokines induced by yeasts of *Paracoccidioides* or *Histoplasma capsulatum*. Our data have demonstrated that different fungal species promote cytokine secretion in lung epithelial cell in distinct manners. In fact, we verified that the stimulation mechanism of cytokine release differs even among isolates of the same species.

It was also observed that *P. brasiliensis* yeasts interact with α3 and α5 integrins in A549 epithelial cells and promote the recruitment of these receptors to host cell structures known as lipid rafts. In addition, we verified that both integrins and these lipid platforms are involved in IL-8 secretion by *P. brasiliensis* infected-epithelial cells.

Besides integrins, other receptors such as Toll-like Receptors (TLRs) have been extensively studied in fungal infections. Under particular stimuli, TLRs may also collaborate with integrins to activate signalling pathways...
that culminate in cytokine secretion\textsuperscript{16,17}. Marre et al., for example, verified that the TLR2 synthetic peptide ligand PAM\textsubscript{3}CSK\textsubscript{4} induces cooperation between the heterodimers TLR2/1 and α3β1 integrin in macrophages, which results in IL-6 secretion by these cells\textsuperscript{16}.

Evidence that lung epithelial cells influence host immune responses against infections is increasing over the past decade\textsuperscript{6}. In the present work, we investigated the involvement of TLR2 in IL-8 secretion that was induced by \textit{P. brasiliensis} yeasts in lung epithelial cells. Moreover, we analysed whether TLR2 interacts with α3 integrin and modulates its expression in A549 cells during this fungal infection.

**Results**

**TLR2 expression levels in lung epithelial cells infected with \textit{P. brasiliensis} yeasts, and involvement of this receptor in IL-8 secretion.** After incubation of A549 epithelial cells with \textit{P. brasiliensis} yeasts (isolate Pb 18), we evaluated by Western blot whether this fungus modulates the expression of TLR2. Figure 1\textsuperscript{a} shows that, after 5 h of fungal infection, TLR2 levels of epithelial cells were very similar to uninfected cells. However, after 24 h of A549-\textit{P. brasiliensis} interaction, we verified higher TLR2 levels (3.55-fold) than A549 cell basal levels (Fig. 1\textsuperscript{b}).

Next, we analysed whether secretion of IL-8 by A549 cells, promoted by \textit{P. brasiliensis}, is TLR2-dependent. For this, epithelial cells were transfected with negative control (NC)- or with TLR2-directed small interfering RNA (siRNA), and then, infected with \textit{P. brasiliensis} yeasts. After 24 h, IL-8 concentrations were measured by ELISA. As expected, this fungus induced an increase of this chemokine levels by 5.2-fold when compared to uninfected cells (Fig. 2\textsuperscript{b}, samples NC). When TLR2 was silenced, we verified a reduction of 70% of IL-8 secretion when compared to \textit{P. brasiliensis}-infected NC cells (Fig. 2\textsuperscript{b}), indicating that this fungus promotes release of this cytokine in a TLR2-dependent manner.

To confirm that A549 cells are able to secrete IL-8 through TLR2 cell signalling pathway, we incubated A549 cells with the ligand PAM\textsubscript{3}CSK\textsubscript{4}, which specifically interacts with TLR2/1 heterodimer\textsuperscript{18,19}. First, we observed higher TLR2 levels than control cells only after 24 h incubation of epithelial cells with PAM\textsubscript{3}CSK\textsubscript{4} (Fig. 1\textsuperscript{c,d}). Then, we analysed IL-8 secretion by A549 cells after incubation with this peptide. As expected, PAM\textsubscript{3}CSK\textsubscript{4} promoted an increase of IL-8 levels by 7.3-fold when compared to levels of NC cells without this ligand. Moreover, in PAM\textsubscript{3}CSK\textsubscript{4}-stimulated A549 cells, TLR2-directed siRNA reduced IL-8 secretion by 81.3% when compared to PAM\textsubscript{3}CSK\textsubscript{4}-NC cell cultures (Fig. 2\textsuperscript{d}), corroborating that A549 epithelial cells may secrete chemokine IL-8 in a TLR2-dependent manner.

By Western blot, efficiency of TLR2 silencing was analysed, and we verified that, when compared to transfected NC cells, the levels of this receptor were decreased by 75% or 59.1% in TLR2-siRNA transfected A549 cells incubated with \textit{P. brasiliensis} yeasts or PAM\textsubscript{3}CSK\textsubscript{4}, respectively (Fig. 2\textsuperscript{a,c}).

**Paracoccidioides brasiliensis** increases interaction between α3 integrin and TLR2 in A549 cells. Since \textit{P. brasiliensis} yeasts induce IL-8 secretion in A549 cells in α3 integrin\textsuperscript{9} and TLR2-dependent manners (Fig. 2\textsuperscript{b}), we analysed whether these two receptors interact with each other during fungal infection. For this, after \textit{P. brasiliensis}-A549 cell incubation, α3 integrin was immunoprecipitated and TLR2 was detected by Western blot. Figure 3\textsuperscript{a} shows an increase of the co-immunoprecipitated α3 integrin-TLR2 complexes, obtained
from A549 epithelial cells incubated for 5 h with \( P. \) brasiliensis yeasts, when compared to uninfected cells. On the other hand, after 24 h \( P. \) brasiliensis-A549 cell infection, we did not observe α3 integrin-TLR2 complexes (Fig. 3b). This last result was unexpected for us, since 24 h-infected A549 cells presented notable TLR2 protein levels (Fig. 1b).

Figure 2. Effect of TLR2 silencing on IL-8 secretion by A549 cells incubated with \( P. \) brasiliensis yeasts or PAM\(_3\)CSK\(_4\). A549 cells were transfected with siRNA directed to TLR2 (siRNA TLR2) or negative control siRNA (NC), and then incubated with \( P. \) brasiliensis yeasts (MOI = 2.5) (Pb 24 h +) or with 5 μg/mL of the TLR2-specific ligand PAM\(_3\)CSK\(_4\). After 24 h, epithelial cells were lysed and aliquots were submitted to SDS–PAGE and Western blot, using anti-TLR2 antibodies (a,c). β-actin was used as loading protein control. Relative TLR2 levels were determined by densitometric analysis of the bands (a,c). (b,d) Cell supernatants were collected, and IL-8 levels of siRNA-transfected A549 cells, during incubation with \( P. \) brasiliensis yeasts or with PAM\(_3\)CSK\(_4\), were analysed by ELISA. Values represent the mean of triplicate experiments ± standard deviation. *, \( p < 0.01 \) when compared to A549 cells transfected with NC in the absence of \( P. \) brasiliensis or PAM\(_3\)CSK\(_4\). **, \( p < 0.01 \) when compared to A549 cells transfected with NC and incubated with \( P. \) brasiliensis yeasts or PAM\(_3\)CSK\(_4\). Uncropped images are presented in Supplementary Fig. S2.

Figure 3. Interaction of α3 integrin with TLR2 in A549 cells infected with \( P. \) brasiliensis yeasts. A549 cells were incubated (Pb) or not (C) with \( P. \) brasiliensis yeasts (MOI = 2.5). After 5 h (a) or 24 h (b), cells were lysed and samples were incubated with anti-α3 integrin (α3 int) antibodies (IP). After 16 h, agarose beads conjugated with A/G protein were added. Then, sample buffer was added, and the resultant supernatant was submitted to SDS–PAGE. TLR2 was analysed by Western blot (WB). Similar results were obtained from three independent experiments. Uncropped images are presented in Supplementary Fig. S3.
**Paracoccidioides brasiliensis** yeasts modulate α3 integrin levels in epithelial cells. Next, we investigated α3 integrin protein levels in infected-A549 cells after 5 h and 24 h. Corroborating previous data\(^9\), *P. brasiliensis* yeasts induced an increase (5.4-fold) of α3 integrin levels in A549 cells after 5 h incubation (Fig. 4a). However, after 24 h, this integrin levels decreased by 98.3% in *P. brasiliensis*-infected A549 cells (Fig. 4b). Longer exposure time of this PVDF membrane enabled to detect a band, recognised by an anti-α3 integrin antibody. This band (Fig. 4c, arrow) presented a lower molecular weight than α3 integrin (150 kDa), suggesting a degradation of this receptor that was induced by *P. brasiliensis* yeasts in A549 epithelial cells.

In addition, we verified whether the TLR2-specific ligand PAM3CSK4 could modulate α3 integrin levels in A549 cells. As shown in Fig. 4d, after 5 h of incubation, PAM3CSK4 increased α3 integrin levels by two fold when compared to basal levels, and by 5.2-fold when the peptide was incubated for 24 h with epithelial cells (Fig. 4e), indicating that PAM3CSK4 can upmodulate α3 integrin expression.

As we found that *P. brasiliensis* yeasts downmodulate α3 integrin levels in A549 epithelial cells (Fig. 4b, 4c), we evaluated whether TLR2 may participate in this process. Therefore, after silencing this PRR by siRNA, A549 cells were incubated with *P. brasiliensis* for 24 h, and then, we analysed α3 integrin levels. Under these conditions, we observed that α3 integrin levels were 3.4-fold higher in TLR2-silenced cells than in *P. brasiliensis*-infected-NC cells (Fig. 5a), indicating that downmodulation of this integrin occurs in a TLR2-dependent manner.

As shown in Fig. 5b, when compared to PAM3CSK4 incubated-NC cells, we observed that TLR2-siRNA promoted a reduction of α3 integrin levels up to 80%. In this manner, unlike *P. brasiliensis*, PAM3CSK4 upmodulates α3 integrin levels and TLR2 is also important for this process.

*As* *P. brasiliensis* secretes compounds that may promote different effects in the host\(^11,12\), we verified if the decrease of α3 integrin levels in A549 cells after 24 h could be a result of this fungal secretion. For this, A549 cells were cultured in 6-well plates, and *P. brasiliensis* yeasts were placed in the upper compartment of Transwell platforms or in direct contact with the epithelial cells. As expected, *P. brasiliensis* incubated in direct contact with A549 cells promoted reduction of α3 integrin levels by 97.3% after 24 h (Fig. 6, Pb). However, when fungi were arranged in the Transwell upper compartment and, consequently, without contact with A549 cells, we observed a 2.8-fold increase in α3 integrin levels (Fig. 6, PbUP). Thus, the direct contact between A549 cells and *P. brasiliensis* secreted compounds may promote different effects in the host.
is mandatory for a yeast-induced reduction of α3 integrin levels in epithelial cells. Moreover, in a different manner, secreted factors by this fungus promoted an increase of α3 integrin levels in A549 cells.

Discussion

In an infection process, inside the host, a pathogen may interact with a particular cell, engaging several receptors and highjacking host cell signalling pathways in order to survive. One family of receptors extensively studied in infection models is the Toll-Like Receptor (TLR) family. In fungal infections, by promoting the secretion of cytokines and chemokines, these receptors are important for the host immune response, recognising several ligands in these pathogens, such as O-linked mannans, phospholipomannans, and unmethylated DNA with CpG motif 20,21. In the present study, we demonstrated that *P. brasiliensis* modulates TLR2 expression in A549 epithelial cells and, by silencing this receptor, we also found that TLR2 participates in IL-8 secretion by these cells. In fact, some groups described the participation of TLR2 in the immune response against *P. brasiliensis* 22–24.
example, the work developed by Balderramas and co-workers (2014) showed that *P. brasiliensis* binds to TLR2 to induce the production of IL-12 by neutrophils. Another group, Loures and co-workers (2009), showed that TLR2 is an important receptor for the innate immune system in a *P. brasiliensis* infection, and also this receptor may have a productive effect in pulmonary fungal infections.

In the literature, several groups have shown that TLRs and integrins pathways communicate with each other in different cell processes. For example, demonstrated that the TLR2 ligand PAM3CSK4 induces cell migration of the rheumatoid arthritis synovial fibroblast cells (RASF), and also increases β1 integrin levels in these cells. Moreover, these authors observed that RASF cell migration, induced by PAM3CSK4, was inhibited by β1 integrin neutralizing antibodies, demonstrating that activation of TLR2 induces RASF migration that occurs in a β1 integrin dependent manner.

In the present work, we verified in A549 lung epithelial cells that the TLR2 ligand PAM3CSK4 increased α3 integrin levels, which was inhibited by silencing TLR2. Both data indicate that the heterodimer TLR2/1 upmodulates this integrin levels in A549 epithelial cells. However, when these epithelial cells were infected with *P. brasiliensis* yeasts for 24 h, we observed higher levels of TLR2 and also that α3 integrin was almost undetectable by Western blot. The latter result was not expected since, in a previous study, we verified an augmentation of the expression of α3 and α5 integrins in A549 epithelial cells infected with this fungus for 5 h. Regarding IL-8 secretion, although we observed the decrease of α3 integrin levels after 24 h of incubation with *P. brasiliensis* yeasts, the upmodulation of α3 integrin levels in the first 5 h of A549 cell infection seems to be important to promote this cytokine secretion, since after α3 integrin silencing, we observed a reduction of IL-8 levels.

In summary, this work shows that during early infection (5 h), *P. brasiliensis* yeasts induce a raise in α3 integrin levels in A549 epithelial cells, while TLR2 levels are similar to those of uninfected cells. Moreover, interaction of α3 integrin with TLR2 occurs during this early period. However, in longer periods of infection (24 h), *P. brasiliensis* downmodulates α3 integrin levels in direct contact- and TLR2-dependent manners. Together, these results indicate that *P. brasiliensis* yeasts differently modulate the activity of host cell receptors and signalling pathways during the course of the infection. Interestingly, Kim and co-workers (2009) described that α3β1 integrin is involved in the formation of myofibroblasts and lung fibrosis. These authors showed that mice lacking α3 integrin-pulmonary epithelial cells, upon bleomycin stimulus, presented lower accumulation of myofibroblasts and did not progress to lung fibrosis. Moreover, lack of lung fibrosis is seen in acute cases of PCM, a severe form of this mycosis. In this manner, the reduction of α3 integrin in lung epithelial cells promoted by *P. brasiliensis* might contribute to the pathogenesis of PCM acute form. Therefore, a better understanding of the cellular mechanisms that promote fungal infection will lead to the development of new therapies in a scenario where there are no vaccines against fungal infections, antifungal drugs are limited, and some fungi are already developing resistance to these drugs.

**Methods**

**Epithelial cell culture.** A549 epithelial cell line (human lung adenocarcinoma) was cultured in Dulbecco’s Modified Eagle's Medium—DMEM (Sigma-Aldrich/Merck, MO, USA) with 10% fetal bovine serum – FBS (Vitrocell, SP, Brazil), 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich/Merck, MO, USA) (complete DMEM) at 37 °C, in 5% CO2 atmosphere. For *P. brasiliensis*-A549 interaction assays, cells were grown in 100 mm2 dishes, 6-well or 24-well plates.

*Paracoccidioides brasiliensis* yeast culture and preparation for interaction assays with epithelial cells. *P. brasiliensis* yeasts, Pb18 isolate, were kindly provided by Dr. Wagner Luiz Batista, from Universidade Federal de São Paulo (Diadema, SP, Brazil). Fungi were cultured in PGY medium (5 g/L neopeptone, 5 g/L yeast extract, 15 g/L glucose—Becton Dickinson, NJ, USA) containing 1.4 g/L asparagine and 0.1 g/L thiamine (Sigma-Aldrich/Merck, MO, USA) for 5–7 days in incubator shaker at 37 °C, 120 rpm, as previously described by Ywazaki and co-workers (2011). Fungal virulence was maintained by reisolating *P. brasiliensis* from infected B10.A mice. For epithelial cell-fungus interaction assays, single fungal mother and daughter yeasts from *P. brasiliensis* were obtained as previously described by Barros and co-workers (2016). The resultant supernatant contained only single fungal cells, which were washed three times with DMEM.

**Interaction between epithelial cells and *P. brasiliensis* yeasts.** A549 epithelial cells were seeded in tissue culture plates. After 48 h, cells were washed three times with PBS-free DMEM, and incubated in this medium overnight, at 37 °C, 5% CO2 (starving). A549 cells were then incubated for 5 h or 24 h with: (1) 5 μg/mL of TLR2/1 ligand PAM3CSK4 (InvivoGen, CA, USA); or (2) *P. brasiliensis* yeasts with a multiplicity of infection (MOI) of 2.5 fungi for 1 epithelial cell. Controls without stimuli were also performed. Then, epithelial cells (or supernatant culture) were analysed by ELISA, co-immunoprecipitation or Western blot.

**Enzyme-linked immunosorbent assay (ELISA).** IL-8 secretion of A549 cell cultures was analysed by sandwich ELISA. After 24 h of incubation with PAM3CSK4 or *P. brasiliensis* yeasts, A549 cell culture supernatants were collected, centrifuged to remove fungi or cell debris, and then analysed using Duo Set Kit (R&D Systems/Bio-Techne, MN, USA), according to manufacturer’s instructions.

**Western blot.** After *P. brasiliensis*-A549 cell interaction for 5 or 24 h, cells were washed with PBS, collected using a cell scraper, and incubated with RIPA lysis buffer (25 mM Tris–HCl pH 7.4 containing 150 mM NaCl, 0.1% sodium dodecyl sulphate—SDS—, 1% Triton X-100 and 0.5% sodium deoxycholate) containing protease inhibitors as previously described by Barros and co-workers (2016). After 30 min at 4 °C, samples were centrifuged, and the resultant supernatant containing proteins was quantified using QuantiPro BCA Assay kit.
PAM3CSK4 or (2) tion, cells were washed three times with DMEM and incubated for 24 h with: (1) 5 µg/mL of TLR2/1 ligand, which has a sequence that does not target any gene, was used as a negative control. After 24 h of transfection, 5 nM. Silencer Select Negative Control siRNA (4390843, Invitrogen/Thermo Fisher Scientific, CA, EUA), designed siRNA for TLR2 (s-169, Invitrogen/Thermo Fisher Scientific, CA, EUA) at the final concentration for 5 h. Then, cells were transfected using a mix of Lipofectamine RNAiMAX Reagent and Silencer Select Pre-

Statistical analyses. Statistical analyses were performed using ANOVA or Student’s t test through Graph-Pad Prism Software (CA, USA). Values were considered statistically significant at p ≤ 0.01.

Received: 10 December 2019; Accepted: 29 October 2020
Published online: 10 November 2020

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Acknowledgements
This work was supported by São Paulo Research Foundation (FAPESP Grants 2015/25652-6, 2016/06285-5, and 2019/26693-9), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq Grant 306163/2015-2), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). This study was approved by the Research Ethics Committee of Universidade Federal de São Paulo (1185200217).

Author contributions
B.C.S.C.B and E.S. designed the study, B.C.S.C.B and B.R.A. performed the experiments. B.C.S.C.B and E.S. analysed the data, wrote the manuscript and prepared the figures. All authors reviewed, edited and critically discussed the results showed in this paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-76557-6.

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