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ORIGINAL ARTICLE

Effects of Cryptococcus neoformans on activation of human alveolar basal epithelial adenocarcinoma cells (A549 cells)

Efeitos de Cryptococcus neoformans na ativação de células epiteliais basais alveolares humanas adenocarcinômicas (células A549)

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ABSTRACT

Introduction: The bronchial epithelium has defense mechanisms based on innate immunity against various airborne microorganisms, including *Cryptococcus neoformans*. Purpose: We evaluated the inflammatory effects of *C. neoformans* using human alveolar basal epithelial adenocarcinoma cells (A549 cells). **Methods:** A549 cells were exposed to *C. neoformans* (Multiplicity of Infection - MOI 1:100) and the analyses were performed 24 hours after stimulation. **Results:** *C. neoformans* increased the production of IL-6 and TGF-β1, but not IL-8 and IL-10, and activated ERK1/2 and NF-B phosphorylation in A549 cells when compared to the control group. There was a greater adhesion of *C. neoformans* when compared to internalization in A549 cells. **Conclusion:** The *C. neoformans* is able to promote changes in inflammatory parameters in human adenocarcinoma alveolar basal epithelial cells.

Keywords: A549 cells; *Cryptococcus neoformans.*

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RESUMO

Introdução: O epitélio brônquico possui mecanismos de defesa baseados na imunidade inata contra diversos microrganismos veiculados pelo ar, entre eles o *Cryptococcus neoformans*. **Objetivo:** Avaliamos os efeitos inflamatórios de *C. neoformans* usando células de adenocarcinoma epitelial basal alveolar humano (células A549). **Métodos:** Células A549 foram expostas ao *C. neoformans* (Multiplicidade de Infecção - MOI 1:100) e as análises foram realizadas 24 horas após o estímulo. **Resultados:** *C. neoformans* aumentou a produção de IL-6 e TGF-β1, mas não de IL-8 e IL-10, e ativou a fosforilação de ERK1/2 e NF-B em células A549 quando comparado ao grupo controle. Houve maior adesão de *C. neoformans* quando comparada à internalização em células A549. **Conclusão**: O *C. neoformans* é capaz de promover alterações nos parâmetros inflamatórios em células epiteliais basais alveolares de adenocarcinoma humano.

Palavras-chave: Células A549; *Cryptococcus neoformans.*

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The authors declare that there is no conflict of interest.

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Not applicable.

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Introduction

Cryptococcosis is a worldwide infectious disease with more than 70 discovered species, but only two species are considered pathogenic in humans: *Cryptococcus neoformans* and *Cryptococcus gattii*[1,2]. The vast majority of cryptococcal infections are caused by *C. neoformans* mainly in immunocompromised patients. Airway epithelial cells are the first host cells with which *Cryptococcus* spp. have close contact[3-4].

Lung infections in healthy individuals usually resolve on their own, however, in individuals with immunosuppressive conditions, such as HIV/AIDS patients, solid transplant patients, and cancer patients, *C. neoformans* may more easily escape the immune response and lungs and spread through the body, moving to its favorite place - the brain, causing cryptococcal meningitis or meningoencephalitis[5,6].

The A549 cell, a human alveolar epithelial type II cell line originating from human patient lung carcinoma tissue, is used for pharmacological, toxicological, and microbiological studies[4,7-10], including studies with *C. neoformans*[3-14]. In this study, we evaluated, in vitro, the effect of *C. neoformans* on A549 cell activation, such as the production of the cytokines IL-6, IL-8, IL-10 and TGF-β1, and the activation of phosphorylation of the transcription factors ERK1/2 and NF-kB in addition to fungal infectivity in the cell.

Methods

Cells

A549 cells (donated by Dr. David Bruce Levy from Brigham and Women's Hospital), were grown in DMEM/F-12 medium (Gibco - Life Technologies) plus 10% fetal bovine serum (FBS) (Gibco - Life Technologies) and 1% antibiotics (penicillin, streptomycin and gentamicin) (Gibco - Life Technologies) in culture flasks (TPP) with a growth area of 75 cm^2 and volume of 65 mL, kept in an incubator at 37 °C with 5% CO_2.

C. neoformans

All experiments were performed with *C. neoformans* var. grubii strain H99 (ATCC MYA-4564). *C. neoformans* was grown and maintained on Sabouraud dextrose agar (BD Biosciences). For cell stimulation, a single colony suspension in Sabouraud dextrose broth (BD Biosciences) was prepared after growth to the initial stationary phase (48h) at 37° C.

Stimulation

A549 cells (1 x 10^5 cells/mL) were incubated in 96-well plates and stimulated with *C. neoformans* (Multiplicity of Infection - MOI 1:100; 100 fungi to 1 cell)[8] for 24 h.

Production of IL-6, IL-8, IL-10 and TGF-β1

Supernatants were collected 24h after stimulation, and the concentrations of IL-6, IL-8, IL-10 and TGF-β1 were measured by enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

Expressions of phospho-NF-B and phospho-ERK1/2

The phospho-NF-B and ERK1/2 signaling pathways were evaluated by cytometry according to Oliveira et al. (2015)[12]. Briefly, 24h after stimulation with *C. neoformans*, cells were fixed with BD Cytofix Buffer (4%) for 10 min at 37 °C. After centrifugation, cells were permeabilized in ice-cold paraformaldehyde for 30 min and then stained with monoclonal anti-phospho NF-B or anti-phospho ERK1/2 (BD Biosciences Pharmingen-Phosflow, USA) or its corresponding isotype for 60 min, followed by incubation with the secondary antibody labeled with FITC or PE for another 45 min in the dark. The cells were then washed, resuspended and subjected to analysis.

The expression of phosphorylated intracellular signaling molecules in 50,000 viable cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences Pharmingen).

Phagocytosis in vitro

To determine the levels of internalization of *C. neoformans* during adhesions with A549 cells, the method of Pereira et al. (2021)[9] was followed. Briefly, yeast cells were labeled with fluorescein isothiocyanate (FITC) at 0.1 mg/mL in the dark for 30 min and after washing in PBS. A549 cells were stimulated with *C. neoformans* for 24 h at 37 °C, followed by washing with PBS (3 times using 1 mL each) to remove non-adherent fungi. Some of the fungal host cell complexes were treated for 10 min at 25 °C with trypan blue (200 µg/ml). Trypan blue is a FITC-derived fluorescence suppressing agent and, since it is not able to reach the intracellular compartment of viable cells, this dye is useful for discriminating intracellular and surface-associated *C. neoformans*, thus quenching the fluorescence of non-internalized cells. Unbound trypan blue was removed by extensive washing with PBS and the complexes were then analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed with the software "Prisma" version 5 from Graphpad (http://www.graphpad.com). The results were expressed as mean ± standard error of the mean. The evaluation of the results was performed by t tests. P values lower than 0.05 were considered statistically significant.

Results

IL-6 and TGF-β1 productions were increased in A549 cells infected with *C. neoformans*

A549 cells infected with *C. neoformans* significantly increased the concentration of IL-6 (Figure 1A) and TGF-β1 (Figure 1D) when compared to the control group. No changes in IL-8 (Figure 1B) and IL-10 (Figure 1C) production were observed between the groups.

A549 cells were stimulated with *C. neoformans* (MOI 100) for 24 h. Concentrations of IL-6 (A), IL-8 (B), IL-10 (C) and TGF-β1 (D) were evaluated by ELISA in the supernatants. Data are the mean ± SEM of three independent experiments in triplicate (n=10).

C. neoformans increased phosphorylation of ERK1/2 and NF-B in A549 cells

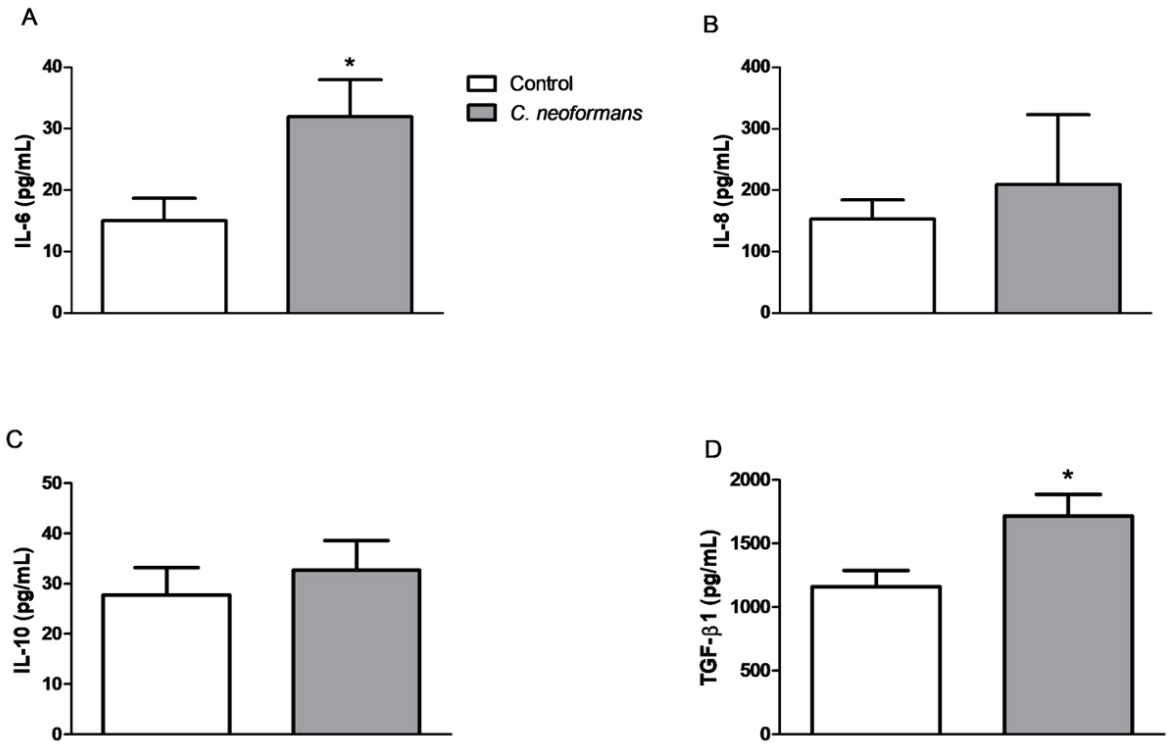
C. neoformans significantly increased the phosphorylation of ERK1/2 (Figure 2A) and NF-B (Figure 2B) in A549 cells compared with control groups.

After 24h, cells were recovered and flow cytometry showing percentages of ERK1/2 (A) and NF-B (B) were analyzed. Data are the mean ± SEM of three independent experiments in triplicate (n=4).

Adhesion and internalization of *C. neoformans* in A549 cells

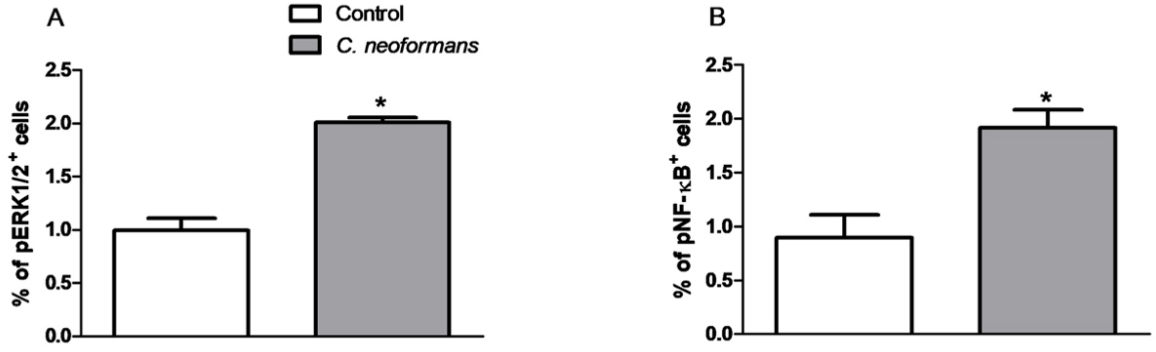
The number of cells internalized by *C. neoformans* is much smaller than the amount adhered to A549 cells.

C. neoformans was incubated with FITC (0.1 mg/mL) in the dark for 30 min A549 cells were stimulated with *C. neoformans* (MOI 100). After 24h, the cells were recovered and analyzed by cytometry to determine the amount on the surface (white bar), and intracellular (gray bar) of *C. neoformans* was then analyzed by flow cytometry. Data are the mean ± SEM of three independent experiments in triplicate (n=8).



*p<0.05 *versus* control group.

Figure 1. Modulation of cytokine production in A549 cells stimulated with *C. neoformans*.



*p<0.05 *versus* control group.

Figura 2. Phosphorylations of ERK1/2 and NF-B were increased in A549 stimulated with *C. neoformans* (MOI 100).

Figura 3. Internalization and adhesion of C. neoformans in A549 and cells.

Discussion

The role of epithelial cells in the inflammatory response to *C. neoformans* is poorly explored. *C. neoformans* is able to modulate several pulmonary responses and can exacerbate or limit the activation of certain epithelial cell mediators. Although several animal models of cryptococcal infections exist, the differences between mouse and human lung cells hinder a broader view of how *C. neoformans* interacts with the lung epithelium[14]. The A549 cell, a cell line derived from a human lung adenocarcinoma[15], has been shown to be very useful in several pharmacological, toxicological and microbiological studies[9,15] including in *C. neoformans*[3].

IL-6 plays a key role in inflammation and infection[16]. IL-6 knockout mice infected with *C. neoformans* increased blood-brain barrier breakdown, causing death faster when compared to wild-type mice[17]. Reinforcing this, in a study of human immunodeficiency virus positive patients with cryptococcal meningitis, a high concentration of IL-6 in the cerebrospinal fluid was associated with protective host responses, such as infection control and blood-brain barrier integrity[18]. *C. neoformans* increased IL-6 production in A549 cells corresponding to an innate inflammatory immune response to fungal infection. Since IL-6 plays a significant role in airway inflammation and fungal infection control, its production by innate airway immune cells could favor airway protection mechanisms and decrease susceptibility to cryptococcal development.

IL-8 is a chemotactic mediator, mainly for neutrophils, which acts in host defense against fungi, bacteria and viruses[19,20,21]. In the work by Guillot et al. (2008)[22], it was observed that viable acapsular *C. neoformans* are potent activators of human bronchial epithelial cells (BEAS-2B), inducing a significant secretion of IL-8, which corresponds to an acute inflammatory response in the cell. However, in our experiment, no significant change in IL-8 production was observed between control and infected cells.

IL-10 is a cytokine with anti-inflammatory and immunosuppressive effects, aiding in the reduction of inflammation. Increased production of IL-10 in peripheral blood samples from patients with cryptococcosis is usually related to disease spread and early mortality[22,23]. IL-10 is part of Th2 immunity related to disease dissemination in the host. *C. neoformans* did not increase IL-10 production in A549 cells. This result may suggest that *C. neoformans* does not modulate the anti-inflammatory mechanism of IL-10 in A549 cells in favor of susceptibility to cryptococcal infection.

TGF-β1 is a multifunctional cytokine that plays a central role in the pathogenesis of several chronic diseases in regulating inflammatory responses[24,25]. *C. neoformans* increased TGF-β1 production in A549 cells. Increased TGF-β1 may contribute to the persistence of cryptococcal infection during chronic phases of infection, as seen in Shao et al. (2005)[26].

NF-B acts in inflammation and host response to various infections[26,27]. Pereira et al. (2021)[9] observed that NF-B activity in bronchial epithelial cells (BEAS-2B cells) might be unnecessary for the development of airway inflammation in response to *C. neoformans*. This work is in agreement with McDermot et al. (2018)[28], who demonstrated in the mouse model of cryptococcosis that NF-B signaling in lung epithelial cells is completely dispensable for airway inflammation in response to *C. neoformans*. In our study, *C. neoformans* increased phospho-NF-B activation in A549 when compared to controls. These results are at odds with previous studies and may be related to differences in models (in vivo and in vitro) or cells (BEAS-2B and A549 cells) used. However, these results are associated with an increase in IL-6, which may be produced by NF-B[28,29].

The other important signaling pathway for inflammation is extracellular signal-regulated kinase 1/2 (ERK), which participates in important regulatory processes such as cell adhesion, cell cycle progression, and cell migration[30]. Studies have shown that the *C. neoformans* GXM capsule has the ability to decrease ERK1/2 activation in CD45+ wild-type mice[4,10]. Pereira et al. (2021)[9] observed that ERK1/2 activity in bronchial epithelial cells (BEAS-2B cells) may be unnecessary for the development of airway inflammation in response to *C. neoformans*. We observed increased phosphorylation of ERK1/2 by *C. neoformans* in A549 cells. The differences between our results and others may be related to the cell types used in the in vitro and in vivo experiments and models, as well as to the use of fungal polysaccharide instead of live fungal cells. The increase in ERK1/2 could be associated with the inflammatory process in favor of controlling *C. neoformans* infection in the airways.

The ability of cryptococcal cells to adhere to the lung surface of the host is important for the establishment of the disease[3,4]. Several papers have been published attempting to investigate the mechanisms of adhesion and internalization of the fungus to *C. neoformans* in respiratory tract epithelial cells[4,31-34]. We observed both internalization and adhesion of *C. neoformans* in A549 cells which could favor primary airway lesions[35,36].

Conclusion

The lung epithelium is a component of the innate immune response and demonstrates a multifunctional role beyond that of a protective physical barrier between the external environment and tissues through the release of an array of mediators, such as cytokines and chemokines, which can orchestrate and influence innate and adaptive immune responses in the modulation of airway inflammation. Hence, our results demonstrated that *C. neoformans* is able to active and interact with human adenocarcinoma alveolar basal epithelial cells modulating the airways' innate immune response in its favor to promote the development of cryptococcal infections.

Authors' contributions

The authors' contributions are structured according to the taxonomy (CRediT) described below:

Conceptualization, Investigation, Methodology, Visualization & Writing - review and editing: KCCF. Conceived and designed the analysis; collected the data, carried out the analysis, wrote the article: Carried out the analysis and writing: WMS. Carried out the analysis and writing: ABMP. Carried out the analysis and writing: ALEA-S. Writing and acquisition of resources and funding: PRS. Supervision, writing and acquisition of resources and funding: MLSV. Project management, supervision, writing and acquisition of resources and funding: APR. All the authors discussed, read and approved the final version of the chapter.

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