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Major Article

Interaction of an opportunistic fungus *Purpureocillium lilacinum* with human macrophages and dendritic cells

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ABSTRACT

Introduction: *Purpureocillium lilacinum* is emerging as a causal agent of hyalohyphomycosis that is refractory to antifungal drugs; however, the pathogenic mechanisms underlying *P. lilacinum* infection are not understood. In this study, we investigated the interaction of *P. lilacinum* conidia with human macrophages and dendritic cells *in vitro*. **Methods:** Spores of a *P. lilacinum* clinical isolate were obtained by chill-heat shock. Mononuclear cells were isolated from eight healthy individuals. Monocytes were separated by cold aggregation and differentiated into macrophages by incubation for 7 to 10 days at 37°C or into dendritic cells by the addition of the cytokines human granulocyte-macrophage colony stimulating factor and interleukin-4. Conidial suspension was added to the human cells at 1:1, 2:1, and 5:1 (conidia:cells) ratios for 1h, 6h, and 24h, and the infection was evaluated by Giemsa staining and light microscopy. **Results:** After 1h interaction, *P. lilacinum* conidia were internalized by human cells and after 6h contact, some conidia became inflated. After 24h interaction, the conidia produced germ tubes and hyphae, leading to the disruption of macrophage and dendritic cell membranes. The infection rate analyzed after 6h incubation of *P. lilacinum* conidia with cells at 2:1 and 1:1 ratios was 76.5% and 25.5%, respectively, for macrophages and 54.3% and 19.5%, respectively, for cultured dendritic cells. **Conclusions:** *P. lilacinum* conidia are capable of infecting and destroying both macrophages and dendritic cells, clearly demonstrating the ability of this pathogenic fungus to invade human phagocytic cells.

Keywords: *Purpureocillium lilacinum*. Macrophages. Dendritic cells. Interaction *in vitro*.

INTRODUCTION

Purpureocillium lilacinum (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, comb. nov 2011, previously called *Paecilomyces lilacinus* (Thom) Samson 1974 is a filamentous, asexual hyaline fungus. The new genera *Purpureocillium* was established after a recent molecular and morphological study suggested that *P. lilacinus* was not related to the *Paecilomyces* genus[1].

Purpureocillium lilacinus is widely considered as a cosmopolitan, saprophytic fungus frequently detected in the environmental soil samples; it can cause deterioration of grains, food, and paper. The fungus can also be recovered from contaminated skin creams and lotions used clinically, and from clinical materials such as catheters and plastic implants. Currently, it is considered an important opportunistic pathogen in both immunocompromised and immunocompetent hosts[2,3]. It has been found parasitizing insects and nematodes, and hence, some researchers have described a potential use of this fungus as a biocontrol agent[4]. It can also cause infection in other animals such as cats[5].

Purpureocillium lilacinum is one of the causal agents of hyalohyphomycosis, a mycotic infection caused by a group of fungi including *Acremonium* spp, *Beauveria* spp, *Fusarium* spp, *Scopulariopsis* spp, and *Paecilomyces* spp. In this condition, the fungi are observed in the affected tissues as septate hyphae with pigmentless cell walls[6,7]. Most clinical manifestations of *P. lilacinum* hyalohyphomycosis are associated with ocular, cutaneous, or subcutaneous infections and the major risk factors are organ transplantations, corticosteroid therapy, primary immunodeficiency, diabetes mellitus, acquired immunodeficiency syndrome, intraocular lens implantation, and ophthalmic surgery[2,3,8,9]. No effective treatment has been established for this infection, and antifungal agents, including amphotericin B, flucytosine, and fluconazole, have often provided unsatisfactory results[3]. However, some of the so-called new azoles, such as posaconazole, ravuconazole, and voriconazole, have recently demonstrated good activity against *P. lilacinum in vitro*[10]. In many cases, a combination of

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antifungal agents or application of these agents together with surgical treatment was necessary to induce remission[2,10].

An *in vivo* study using an animal model has demonstrated that *P. lilacinum* virulence is generally low, as evidenced by the high inoculum (10^6-10^7 conidia per animal) and immunosuppression required to establish a successful infection[3]. On the other hand, we have previously shown that both immunocompetent and immunosuppressed mice that intravenously received about 10^4 *P. lilacinum* conidia developed the infection: fungal structures were observed and fungal cells were recovered from different organs[2,11]. These data indicate the need of additional studies to better comprehend the real invasive capability of *P. lilacinum*.

The antifungal immune response, although exhibiting certain species-specific variations, is generally initiated by phagocytic cells. Neutrophils, macrophages, and monocytes are important antifungal effector cells. Additional effector cells, including neutrophils and monocytes, are recruited to the sites of infection by inflammatory signals such as cytokines, chemokines, and complement components[12].

Professional antigen-presenting cells (APCs) belong to the host innate immune system and are represented mainly by macrophages and dendritic cells (DCs). These cells capture and process antigens, express lymphocyte costimulatory molecules, migrate to lymphoid organs, and secrete cytokines to initiate immune response[13]. DCs are important components of the immune system; they provide the first line of defense and are therefore essential for the onset of a strong immune response to several incoming pathogens[14,15]. DCs play an instrumental role in linking innate and adaptive responses against a variety of pathogenic fungi including *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans*[12].

This study aimed to analyze the *in vitro* interaction between *P. lilacinum* conidia and two types of human APCs, macrophages and DCs, to help elucidate the pathogenesis of infection caused by this fungus.

METHODS

A clinical *P. lilacinum* isolate from the nasal sinus, which was kindly provided by Dr. Annette W. Fothergill (Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, USA), was grown on potato-dextrose agar (Difco, Detroit, MI, USA) at room temperature for 14 days. Spores were collected by scraping the colonies, suspended in 50mM phosphate-buffered saline (PBS), pH 7.2, chilled to 4°C, and heated to 37°C. The suspension was then centrifuged at 200 × *g* for 30min, and the number of conidia in the resultant supernatant (rich in conidia but free of hyphae) was estimated by microscopy using a Neubauer hemocytometer[16]. All the conidial suspensions were freshly prepared for each experiment.

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of eight peripheral blood samples from healthy donors screened for human immunodeficiency virus (HIV) and hepatitis B virus (kindly provided by Serviço de Hemoterapia, Hospital Universitário Clementino Fraga Filho, RJ, Brazil). The cells were isolated using a Ficoll-Hypaque 1077 gradient (Sigma, St. Louis, MO, USA)[17]. Briefly, cells were washed, resuspended in Roswell Park Memorial Institute (RPMI) medium containing L-glutamine and penicillin-streptomycin (Sigma), and quantified using the Neubauer hemocytometer. Monocytes were separated from lymphocytes by cold aggregation during 30min[17]. The cells were resuspended in fresh RPMI medium containing 10% fetal bovine serum (Hyclone®; Thermo Scientific, South Logan, UT, USA) and seeded at 2×10^5 cells/well into eight-well chamber slides (Lab-Tek™ Nunc International, Rochester, NY, USA) and at 1×10^6/tube into Falcon® polystyrene tubes (Becton Dickinson Company, Franklin Lakes, NJ, USA) for cell phenotype evaluation by flow cytometry. For differentiation into macrophages, monocytes were incubated at 37°C in a humidified incubator with a 5% CO_2/95% air mixture (model MC0-19AIC-UV; Etten Leur, The Netherlands) for 7 to 10 days. For differentiation into DCs, monocytes were incubated in the presence of 100U/mL recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF; Peprotech, Rocky Hill, NJ, USA) and 1,000U/mL recombinant human interleukin-4 (rhIL-4; Peprotech) as described above[17,18]. For phenotypic evaluation, cells were washed with 200µl PBS containing 0.1% bovine serum albumin and 0.01% sodium azide and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against cluster of differentiation 14 (CD14) and peridinin-chlorophyll protein-cyanine dye (PerCP-Cy5.5)-conjugated CD209, also known as dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)[19-21], on ice for 60min. Cells were washed and analyzed using an Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI, USA) and the FlowJo™ software (Tree Star, Ashland, OR,USA).

The interaction experiments were conducted with three different ratios of conidia to human cells (5:1, 2:1, and 1:1). Briefly, the conidial suspension was added to each well of the chamber slides at the desired concentration, and incubated with APCs (macrophages or DCs) differentiated from monocytes of each donor for 1h, 6h, and 24h at 37°C in a 5% CO_2 atmosphere. Subsequently, the cells were washed gently with sterile PBS at room temperature to remove extracellular conidia, fixed with methanol for 3min, stained with Giemsa solution (Sigma) for 15min, and then examined under a light microscope (model Axiophot, Carl Zeiss Microscopy GmbH, Göttingen, Germany). Control APCs without conidia were treated and evaluated in the same manner. Quantification was performed by counting 100 fields on duplicate coverslips and the results were expressed as follows: % of infected cells = [(APC with fungus - control) ÷ control] × 100%[22]. The data were analyzed by the Student's *t*-test and the difference at $p < 0.05$ was considered statistically significant.

Ethical considerations

This study was reviewed and approved by the Research Ethics Committee of the Federal University of Rio de Janeiro, Brazil (license number 168/09).

RESULTS

Immunophenotyping of the cells was performed to monitor the differentiation of monocytes to macrophages and DCs. The differentiated DCs were characterized by the high expression of CD209 (DC-SIGN) and low expression of CD14 (**Figure 1**). In contrast, the differentiated macrophages displayed high expression of CD14 and low levels of CD209 (**Figure 1)**.

For all three tested ratios of conidia to human cells (5:1, 2:1, and 1:1), phagosome-like structures containing conidia could be observed inside the cells, indicating that conidia were phagocytized by APCs (**Figure 2)**. The control APCs presented typical morphology (**Figures 2A** and **2B**). The results obtained with the 5:1 ratio showed that conidia were phagocytized by APCs, similar to the findings for the 2:1 sample (**Figures 2C** and **2D**). However, because of the excessive number of conidia inside and outside APCs, it was not possible to quantify the internalized conidia.

For the 1:1 ratio of conidia to human cells, the infection could be followed for 1h, 6h, and 24h (**Figures 2E** to **2J**). Within 1h of interaction, *P. lilacinum* conidia were internalized by macrophages (**Figure 2E**) and DCs (**Figure 2F**); at 6h, the internalization gradually increased and some conidia became inflated (**Figures 2G** and **2H**). After 24h interaction, macrophages and DCs presented inflated conidia that formed germ tubes and hyphae (**Figures 2I** and **2J**); in many cells, they developed into septate hyphae and finally destroyed both macrophages and DCs (data not shown). This pattern of infection was observed for APCs from all the donors.

We also analyzed the percentage of infected APCs (macrophages and DCs) after 6-h interaction, because at this time point, the infection was well established and conidia were clearly observed inside the cells (**Figures 2G** and **2H)**. No significant differences between the infected macrophages and DCs containing *P. lilacinum* conidia were detected at both ratios analyzed (1:1 and 2:1, conidia:human cells) (**Figure 3**).

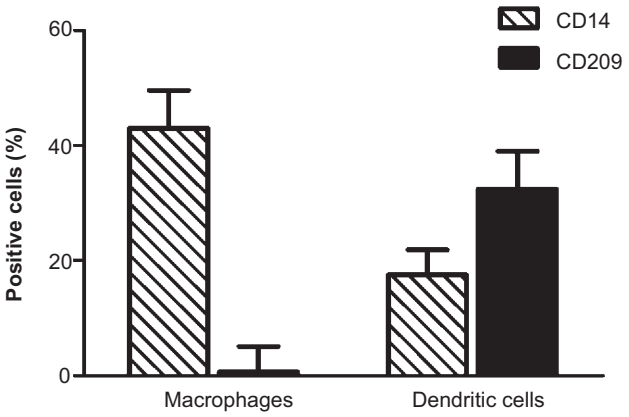

FIGURE 1 - Percentages of CD14- and CD209-expressing cells among macrophages and dendritic cells differentiated from monocytes isolated from buffy coat samples of eight healthy donors. CD: cluster of differentiation.

DISCUSSION

In the present study, we performed *in vitro* analysis of the interaction between *P. lilacinum* conidia and human professional APCs derived from human monocytes, and demonstrated that fungal conidia were capable of infecting and destroying both macrophages and DCs.

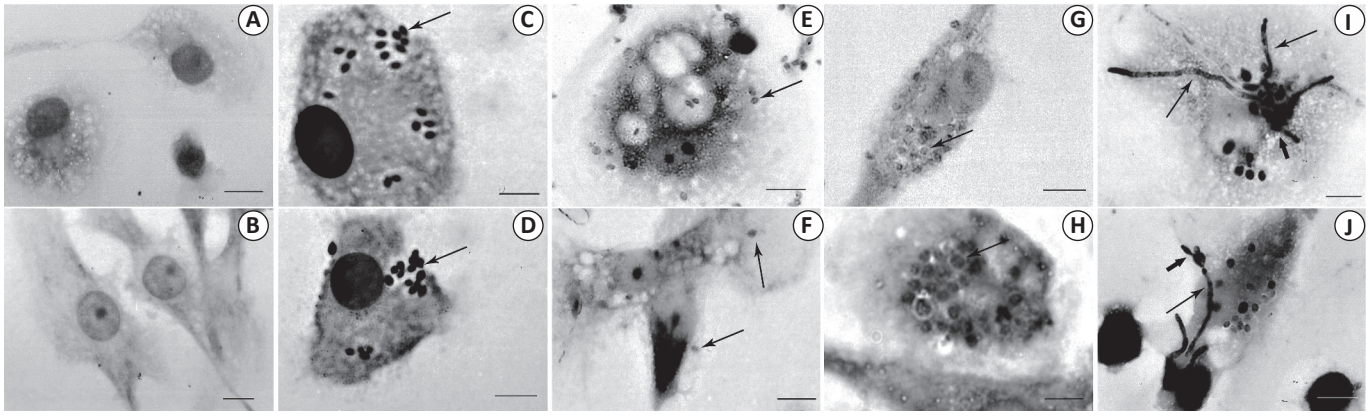

FIGURE 2 - Macrophages (A, C, E, G, I) and dendritic cells (B, D, F, H, J) derived from monocytes isolated from human mononuclear cells were infected in vitro with conidia of a *Purpureocillium lilacinum* human isolate, stained using Giemsa staining, and analyzed by light microscopy at ×1,000 magnification. Macrophages (A) and dendritic cells (DCs; B) incubated without the fungi were used as negative controls and did not show any changes throughout the experiment. The conidia were phagocytized by macrophages and DCs, as evidenced by the presence of phagosome-like structures containing conidia (→) after 1h of interaction at the 2:1 (C and D) and 1:1 (E and F) ratios of conidia to human cells. At 6h of contact using the 1:1 ratio, the internalization gradually increased and some conidia became inflated (→) inside macrophages (G) and DCs (H) and at 24h of interaction, the conidia with germ tubes (➔) and hyphae (→) were observed in macrophages (I) and DCs (J). Scale bar, 10µm.

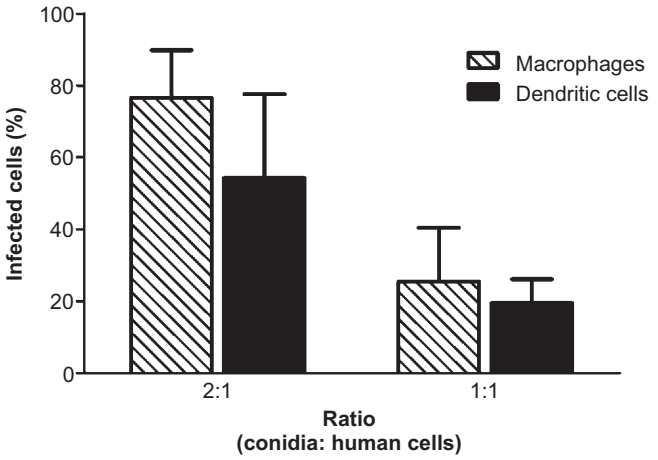

FIGURE 3 - Phagocytosis of *Purpureocillium lilacinum* conidia by human macrophages and dendritic cells (DCs). The results are presented as the mean ± standard deviation of the percentage of infected cells after 6-h interaction between conidia and human cells at the ratios of 2:1 and 1:1. No significant differences were detected between the percentages of infected macrophages and DCs.

After a 1h interaction period, we found conidia inside human APCs as described previously with *Penicillium marneffei, Fusarium solani, F. oxysporum*, and *Verticillium nigrescens*[23,24]. A recent study showed that phagocytosis and cell death of *Aspergillus fumigatus, A. terreus*, and *A. flavus* occurred within macrophages and DCs after 30min of interaction[25].

Here, we observed that, once internalized, the conidia swelled over time and started producing germ tubes in an attempt to generate mycelia. The ability to produce mycelia and sporulate in the infected tissue is a peculiar feature of *P. lilacinum*[26]. According to Latgé, conidial swelling is a prerequisite step for the development of hyphae[27]. This researcher showed that inhaled conidia of *A. fumigatus* could reach the alveoli and that, at this stage of infection, *Aspergillus* germinated and showed the growth of small germ tubes, followed by the generation of hyphal fragments[28]. Furthermore, conidia can germinate in monocytes, suggesting an essential role of phagocytic cells such as neutrophils, in containing conidia that resist intracellular killing[29-31].

Previous studies have also demonstrated the ability of DCs to ingest latent *A. fumigatus* that develop swollen conidia and hyphae[32,33]. Macrophages and DCs have been described as efficient phagocytic cells with regard to *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Candida albicans*, and *A. fumigatus*[34] and, until this study, also to *P. lilacinum*.

After 24h of interaction with human cells, the conidia of *P. lilacinum* destroyed macrophages and DCs, demonstrating that the fungus was able to remain viable and active inside both types of APCs, evading the innate cell defense responses by an as-yet unknown mechanism. A similar phenomenon has been observed for *Fusarium solani*, one of the species phenotypically related to *P. lilacinum*[1], after phagocytosis by human macrophages (unpublished observations).

Purpureocillium lilacinum is generally described as a fungus of low virulence, but our group has demonstrated its capacity to infect murine macrophages and to produce mycelium within 24h of *in vitro* interaction[16]. The present observations with human cells indicate the rapid germination of *P. lilacinum* conidia and complete destruction of all macrophages and DCs, in sharp contrast to the data obtained for *Aspergillus* spp., including *A. fumigatus*, which were efficiently killed by human monocyte-derived macrophages within 120min[29]. This difference is interesting considering that *A. fumigatus* is likely more invasive than *P. lilacinum*, causing infection that often leads to fatal invasive aspergillosis in humans[27].

Previous studies on hyalohyphomycosis caused by *P. lilacinum* have focused on clinical manifestations, treatment, prognosis, and drug-susceptibility testing[2], while little is known about the fungus interaction with the host. To the best of our knowledge, this is the first study to address the fate of *P. lilacinum* after phagocytosis by human APCs. We are currently investigating the immune evasion mechanisms of *P. lilacinum*, in particular the factors that support its survival within the host cells and are responsible for the pathogenicity of infection. The elucidation of these mechanisms will certainly help in the development of new antifungal treatment strategies.

In conclusion, *P. lilacinum* conidia were capable of infecting and destroying both macrophages and DCs, clearly demonstrating the ability of this fungus to invade human phagocytic cells.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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