



Early Interaction of *Alternaria infectoria* Conidia with Macrophages

M. C. Almeida · D. Antunes · B. M. A. Silva · L. Rodrigues ·
M. Mota · O. Borges · C. Fernandes · T. Gonçalves

Received: 20 September 2018 / Accepted: 8 May 2019
© Springer Nature B.V. 2019

Abstract Fungi of the genus *Alternaria* are ubiquitous indoor and outdoor airborne agents, and individuals are daily exposed to their spores. Although its importance in human infections and, particularly in respiratory allergies, there are no studies of how *Alternaria* spp. spores interact with host cells. Our aim was to study the early interaction of *Alternaria infectoria* spores with macrophages, the first line of immune defense. RAW 264.7 macrophages were infected with *A. infectoria* conidia, and the internalization and viability of conidia

once inside the macrophages were quantified during the first 6 h of interaction. Live cell imaging was used to study the dynamics of this interaction. TNF- α production was quantified by relative gene expression, and the concentration of other cytokines (IL-1 α , IL-1 β , IL-6, IL-4, IL-10, IL-17, GM-CSF and INF- γ) and a chemokine, MIP-1 α , was quantified by ELISA. Conidia were rapidly internalized by macrophages, with approximately half internalized after 30 min of interaction. During the first 6 h of interaction, macrophages retained the ability to mitotically divide while containing internalized conidia. The classical macrophage-activated morphology was absent in macrophages infected with conidia, and TNF- α and other cytokines and chemokines failed to be produced. Thus, macrophages are able to efficiently phagocytose *A. infectoria* conidia, but, during the first 6 h, no effective antifungal response is triggered, therefore promoting the residence of these fungal conidia inside the macrophages.

Handling Editor: A. Vecchiarelli.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11046-019-00339-6>) contains supplementary material, which is available to authorized users.

M. C. Almeida (✉) · D. Antunes · B.
M. A. Silva · L. Rodrigues · M. Mota ·
O. Borges · C. Fernandes · T. Gonçalves
CNC - Center for Neuroscience and Cell Biology of
Coimbra, University of Coimbra, Rua Larga,
3004-504 Coimbra, Portugal
e-mail: mcalmeida@uc.pt;
mcruzalmeida90@gmail.com

L. Rodrigues · M. Mota · T. Gonçalves
FMUC - Faculty of Medicine, University of Coimbra,
Coimbra, Portugal

O. Borges
FFUC - Faculty of Pharmacy, University of Coimbra,
Coimbra, Portugal

Keywords *Alternaria infectoria* · Macrophages ·
Conidia · Infection

Introduction

The relevance of filamentous fungi in human health has been recognized in the onset of severe opportunistic respiratory infections in immunocompromised

patients [1], but millions of people suffer from respiratory pathologies related to fungal sensitization due to exposure to fungal spores or parts of mycelial hyphae [2, 3]. In fact, every day people inhale hundreds of fungal spores/particles and only a few environmental fungi are associated with fungal sensitization and respiratory allergy such as *Alternaria*, *Aspergillus*, *Penicillium* and *Cladosporium* [2]. Lately, a growing number of evidence show that fungal colonization, as a consequence of environmental exposure, increases the development of fungal sensitization and respiratory allergies [4]. Moreover, it has been shown that antifungal therapy ameliorates allergic conditions related to fungal sensitization [5]. *Alternaria* is a large and complex genus, holding several species of cell wall melanized hyphomycetes, known as dematiaceous fungi, and one of the most common airborne fungi [6]. These fungi can cause opportunistic human diseases, namely phaeohyphomycosis [7, 8], but *Alternaria* spp. is increasingly associated with fungal sensitization and asthma in both children and adults [9, 10]. Lately, we have been studying *Alternaria infectoria*, as a model organism of the genus *Alternaria*, in aspects related to its biology and to its susceptibility to antifungals, proving that the melanin content in this fungus is crucial for the susceptibility to antifungals [11–13].

Most of what is known about how filamentous fungi interact with host cells, especially with innate immune cells in the lung, was obtained in *Aspergillus fumigatus* [14, 15]. Recognition, phagocytosis and consecutive killing of conidia by phagocytic cells contribute to fungal clearance [16] and the generation of a proinflammatory immune response, such as secretion of TNF- α [17, 18], triggers local infiltration of neutrophils and their migration to the site of infection to promote further antifungal clearance. Otherwise, the germination of conidia outside the phagocytic cells disables the phagocytosis and extracellular killing mechanisms such as NETs (neutrophil extracellular traps), are activated [19]. This also proves that size matters in the response of phagocytes to filamentous fungi, whether the interacting structure is the conidia or the hyphal form [15]. The fungal cell wall composition and structure are determinant on the response triggered by the host cells but is also responsible for the survival of the fungal cells during infection [20]. Cell wall melanin plays an important role, and there are several reports describing how

Aspergillus spp. conidial cell wall melanin is crucial for its intracellular survival and dissemination of the infection [21, 22].

Despite the prevalence of *Alternaria* spp. as airborne fungi involved in respiratory allergies, there is a total lack of studies showing how its conidia interact with host cells. With that in mind, the most important goal of this work was to explore the interaction between *A. infectoria* conidia and macrophages and the early response of these cells to the conidia, even during the first stages of germination of conidia.

Materials and Methods

Strains, Media and Cell Culture Conditions

All experiments were performed with an *A. infectoria* strain, obtained from CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands (CBS 137.9). The strain was maintained in potato dextrose agar (PDA; Difco, BD, New Jersey, USA) at 30 °C with a cycle of 8 h of light with a blacklight lamp (F15 W T8BLB; Grainger, NY, USA) and 16 h of dark for at least 2 weeks.

The mouse macrophage cell line RAW 264.7 was obtained from the European Collection of Cell Cultures (ECACC catalogue number 91062702; Salisbury, UK) and maintained in Dulbecco Minimal Essential Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% non-inactivated fetal bovine serum (FBS; Life Technologies, Paisley, UK), 10 mM HEPES, 12 mM sodium bicarbonate and 11 mg/mL sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere with 5% CO₂. In all the assays only RAW264.7 cells below fifteenth generation were used. The medium was changed every 2 days, until ~ 70% of confluence was reached.

Macrophage Infection with *A. infectoria* Conidia

Alternaria infectoria conidia were harvested in phosphate-buffered saline (PBS) by gently scrapping the surface of the culture plate, filtered through cloth filter to remove hyphal fragments and suspended in culture media to the desired concentration. RAW 264.7 cells were plated in 12 or 96-well plates with 2.5×10^5 or 1×10^5 cells/well, respectively (Corning, NY, USA)

(with or without glass 16-mm coverslips, depending on the type of measurement required) and were allowed to double for 18 h at 37 °C in a 5% CO₂ atmosphere. *Alternaria infectoria* conidia were added to the macrophages at different multiplicity of infection (MOI) depending on the assay, as follows: (1) To study the fungal conidia viability upon internalization by macrophages, a MOI of 1:2 was used in 96-well plates, and the conidia viability inside phagocytic cells was assessed by a colony-forming unit (CFU) assay. After co-culturing during 0.5, 1.5, 3 or 6 h of infection, the supernatants were discarded and, to assess the number of internalized viable conidia, the adhered macrophage cells were lysed with 0.5% Triton X-100 (BDH, Poole, England) followed by a second lysis step with sterile distilled water. The suspensions, representing the internalized viable conidia, were plated on PDA agar plates at a 1:100 dilution. Plates were incubated at 30 °C and the colonies counted after 3 days of incubation. The percentage of viable conidia inside the macrophage was calculated using the equation number of CFU/total number of conidia added to the macrophage culture. (2) To study the infection dynamics, it was used a live cell imaging strategy. For these experiments, RAW 264.7 macrophages (1 × 10⁶ cells/well) were seeded in a µ-Dish 35 mm, high glass bottom (Ibidi, München, Germany). Conidia were added to macrophages at a MOI of 1:4, immediately followed by imaging in an Observer Z1 microscope (Carl Zeiss, Jena, Germany), objective EC Plan Neofluar 40 ×/0.75 Ph2 M27 with coupled camera AxioCam HRm for 4 h with one image per min, at a 20 × magnification. The images were analyzed using the Zeiss software ZEN.

Fluorescence Microscopy

A. infectoria conidia were prepared as described previously, and RAW 264.7 macrophages were plated on 12-well plates with 16-mm glass coverslips. After 3 h of co-incubation, the plates were put on ice, the coverslips washed twice with cold PBS and then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 15 min at room temperature. Fixed cells were washed twice with cold PBS, followed by a 10-min incubation in the dark with 0.01 mg/mL wheat germ agglutinin (WGA, Invitrogen, Paisley, Scotland) Alexa Fluor® 350 conjugated in PBS. Next, cells were washed three times with PBS

and the coverslips were mounted on glass slides, using DAKO (LusoPalex, Portugal). Imaging was performed on a Carl Zeiss LSM 510 Confocal Microscope, using a 63x Plan-ApoChromat (NA 1.4) oil objective. Image analysis was achieved using Fiji software (ImageJ software).

Cytokine Production

First, the production of TNF- α , as a proinflammatory marker of macrophage activation, was studied. The relative expression of TNF-alpha gene in RAW 264.7 cells during the course of infection with *A. infectoria* conidia was performed using a real-time RT-PCR approach. After a 3-h period of infection, the 6-well plates were iced and the cells were scraped and transferred to ice-cold RNase-free Eppendorf tubes. After centrifugation at 10,000 rpm for 5 min at 4 °C, RNA was extracted using the NucleoSpin® RNA Kit protocol (Macherey–Nagel, Düren, Germany). RNA concentration was determined by the A260/280 absorbance value of the sample on NanoDrop 2000 (Thermo Fisher Scientific, Rockford, USA). Reverse transcription of total RNA into cDNA was obtained using the Transcriptor First Strand cDNA synthesis Kit (Roche, Mannheim, Germany), in a GeneAmp PCR System 2400 (PerkinElmer™, Norwalk, USA). The relative gene expression of TNF-alpha was performed by real-time quantitative RT-PCR, using the SsoFast™Eva Green® Supermix (Bio-Rad Laboratories, Inc, Hercules, USA). The primers used (final concentration of 0.5 µM) were: TNF-alpha, forward (5'-CATGATCCGCGACGTGGAAGCTG-3') and reverse (5'-AGAGGGAGGCCATTTGGGAAGCT-3') [23]; GAPDH forward (5'-GTCTTCAACCACCATG-GAGA-3') and reverse (5'-CCAAAGTTGTCATG-GATGACC-3') [24]. The process of quantitative real-time PCR was accomplished using the LightCycler® II (Roche, Portugal, Software LightCycler 2.0), with specific thermal program steps. The relative gene expression was calculated using the 2^{- $\Delta\Delta C_t$} method [25]. The concentration of TNF- α in the supernatants of co-cultures of macrophages with *A. infectoria* conidia (MOI 1:2) was quantified by ELISA. For that purpose, the supernatants were collected by centrifugation at 10,000 rpm for 2 min at 4 °C, and used to quantify the production TNF- α using the Mouse TNF-alpha ELISA Ready-SET-Go!® kit 88-7324, Bioscience, San Diego, USA). Besides TNF- α , a screen

was performed to assess for the production of other cytokines, IL-1 α , IL-1 β , IL-6, IL-4, IL-10, IL-17, GM-CSF, INF- γ and a chemokine, MIP-1 α , quantified using the same procedure of supernatant collection, but the quantification was obtained using a Bio-Plex[®] X-Plex[™] (Bio-Rad, Portugal).

Statistical Analysis

Prism 6 (GraphPad) using a one-way analysis of variance (ANOVA) followed by Dunnett's *t* test post hoc analysis was used to compare samples with untreated macrophage cells. All results are presented as mean $\pm$ SEM with *n* = 3, unless stated otherwise. A *p* value of 0.05 or less was considered significant.

Results

A. infectoria Conidia Remain Viable and Able to Germinate Inside Macrophages

In order to study how *A. infectoria* conidia first interact with macrophages, we used two distinct approaches. First, using a CFU assay, we quantified the number of internalized *A. infectoria* conidia, and their viability. Secondly, we used a live cell imaging approach to gain insight into the dynamics of this interaction.

The live cell imaging (Supplementary Material, video S1) showed that the internalization of conidia by RAW264.7 macrophages is initiated as early as 10 min after the addition of *A. infectoria* conidia suspension to a confluent macrophage cell culture. This internalization proceeds very rapidly and, 30 min after, more than half of the conidia, 64.2%, corresponding to 1.28×10^5 conidia of the total 2×10^5 added are inside macrophages (Fig. 1a). Strikingly, during the next studied period, until 6 h of interaction, the number of conidia inside the macrophages remained unchanged, with no conidial death (Fig. 1a). Through live cell imaging, we observed that *A. infectoria* conidia were able to germinate intracellularly (Fig. 1b; Supplementary Material, video S2). The microscopic counting of the germinated conidia inside the macrophages proved inaccurate and led to a clear under-evaluation. Nevertheless, it could be assessed that, 30 min after adding the conidia to macrophages at least 1.5% of the internalized conidia were germinating (results not shown).

Macrophages internalizing *A. infectoria* Conidia Retain the Ability to Divide and Do not Change its Morphology

The live imaging approach to study the dynamics of the interaction of RAW264.7 cells with *A. infectoria* conidia also allowed the observation of events in the macrophages. During this early interaction, macrophages with internalized conidia retained the ability to divide mitotically and the phagocytosed conidia were transferred into one of the resulting daughter cells (Fig. 1c; Supplementary Material, video S3). The interaction of RAW264.7 with *A. infectoria* conidia also included cooperativity between different macrophages, increasing the efficiency of the internalization process (Fig. 1d; Supplementary Material, video S4). Another noticeable aspect was that when conidia initiated germination, with the formation of hypha, an intracellular compartment similar to a vacuole was formed near the germinating spore (Fig. 1b; Supplementary Material, video S2).

Immunofluorescence assays were used to study changes in the morphology of macrophages upon *A. infectoria* conidia, since this is one of the hallmarks of macrophage response to microorganisms. As membrane marker, wheat germ agglutinin (WGA-Alexa Fluor[®]350 conjugated) was used. The overall morphology of macrophages, either internalizing conidia or not, did not show remarkable visible changes; instead, the typical non-activated roundish morphology was kept similarly to the untreated macrophages (uninfected), although macrophages filopodia are visible (Fig. 2).

A. infectoria Conidia Do not Induce the Production of Cytokines by Macrophages

To further study the response of RAW 264.7 macrophages to *A. infectoria* conidia, we first investigated the production of TNF- α as a hallmark of inflammatory response.

The relative gene expression of TNF-alpha, the gene coding for the cytokine TNF- α , hardly changed when the macrophages were co-incubated with conidia, with 6.33 ± 6.3 -fold increase in mRNA (Fig. 3a; *n* = 2). To assess the responsiveness of the macrophage cell line, bacterial lipopolysaccharide (LPS) was used as a positive control, with a significant increase of 76-fold increase in TNF-alpha gene expression (Fig. 3a). The quantification of TNF- α release in the supernatants of

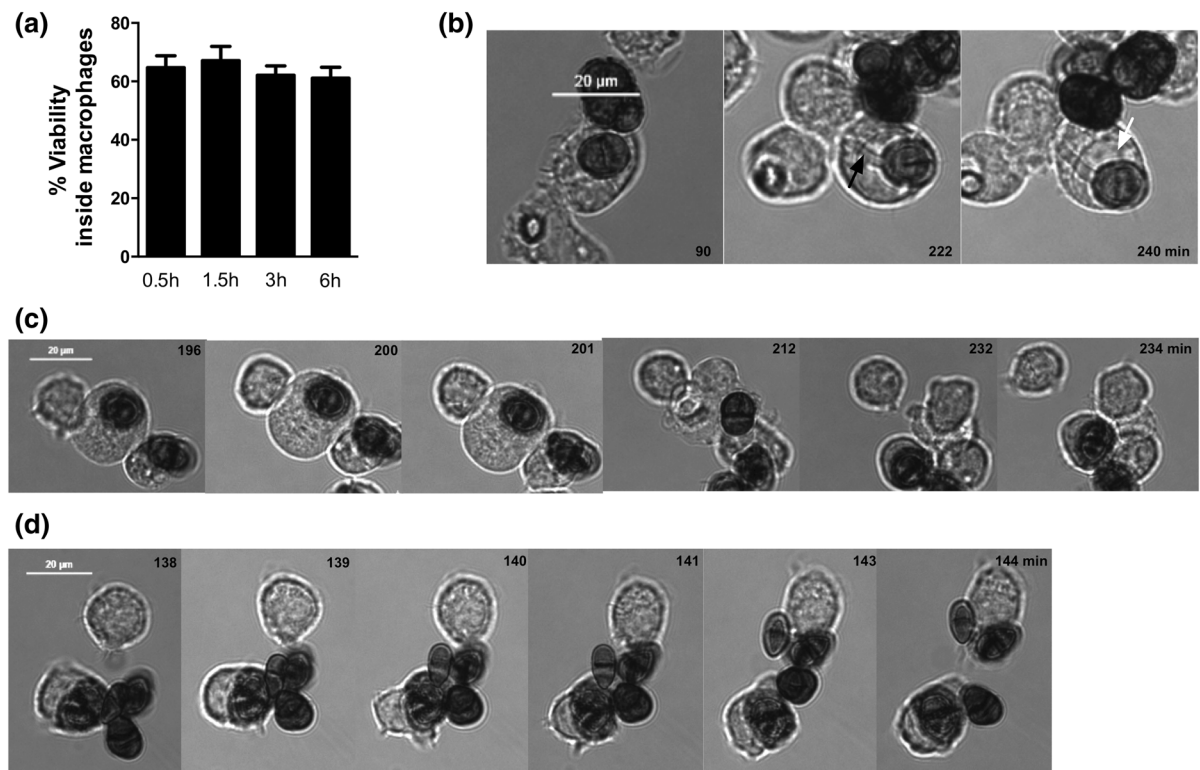


Fig. 1 *A. infectoria* conidia remain viable and able to germinate after RAW 264.7 macrophage phagocytosis. **a** Viability of *A. infectoria* conidia after RAW 264.7 macrophages infection (mean $\pm$ SEM, $n = 4$). **b** Intracellular germination of phagocytosed *A. infectoria* conidia. Black arrow shows germination, and white arrow points to vacuolar compartment. **c** Time-lapse images of RAW 264.7 macrophage cell with

internalized *A. infectoria* conidia, dividing and translocating the conidia to one of the two daughter cells. **d** Cooperativity between different macrophages. A macrophage binds 4 conidia (min 138); these conidia are shared with a second macrophage (min 139–144). Numbers in lower left corner (**b**) and upper left corner (**c** and **d**) panels express time of infection in min. Scale bar = 20 μ m

macrophages co-incubated with conidia or LPS confirmed the RT-PCR results. TNF- α production in macrophages co-incubated with conidia was below 30 pg/ml at 3 h, while in the LPS-treated macrophages (positive control) the concentration reached 600 pg/ml (Fig. 3b). Similar concentrations of TNF- α were measured at 1.5 and 6 h of co-incubation (Fig. 3b).

These results, indicating the absence of TNF- α , one of the classical inflammatory markers, lead us to screen the production of other cytokines: IL-1 α , IL-1 β , IL-6, IL-4, IL-10, IL-17, GM-CSF, INF- γ and MIP-1 α , a chemokine. This screen was carried out by multiplex immunoassay and, similarly to TNF- α , we did not find any significant changes in any of the tested cytokines and chemokine (Fig. 3c). This further suggests that *A. infectoria* conidia do not evoke neither a proinflammatory (IL-1 α , IL-1 β , IL-17, GM-CSF, INF- γ) nor an anti-inflammatory (IL-4 and

IL-10) response in RAW 264.7 macrophages at the early stages of interaction (the first 6 h of interaction).

Discussion

The present study focus was the interaction of *Alternaria*, using the model organism *A. infectoria* with macrophages, as an approach to the mode of the early interaction of the airborne *Alternaria* spp. conidia with phagocytes. We exposed RAW 264.7 macrophages to *A. infectoria* conidia and studied issues related to the infection dynamics, such as internalization, viability of phagocytosed conidia and germination upon internalization. Although we used a macrophage cell line, which raises some limitations [26, 27], only low-generation (under the 15th generation) cultures were used in this work in order to avoid

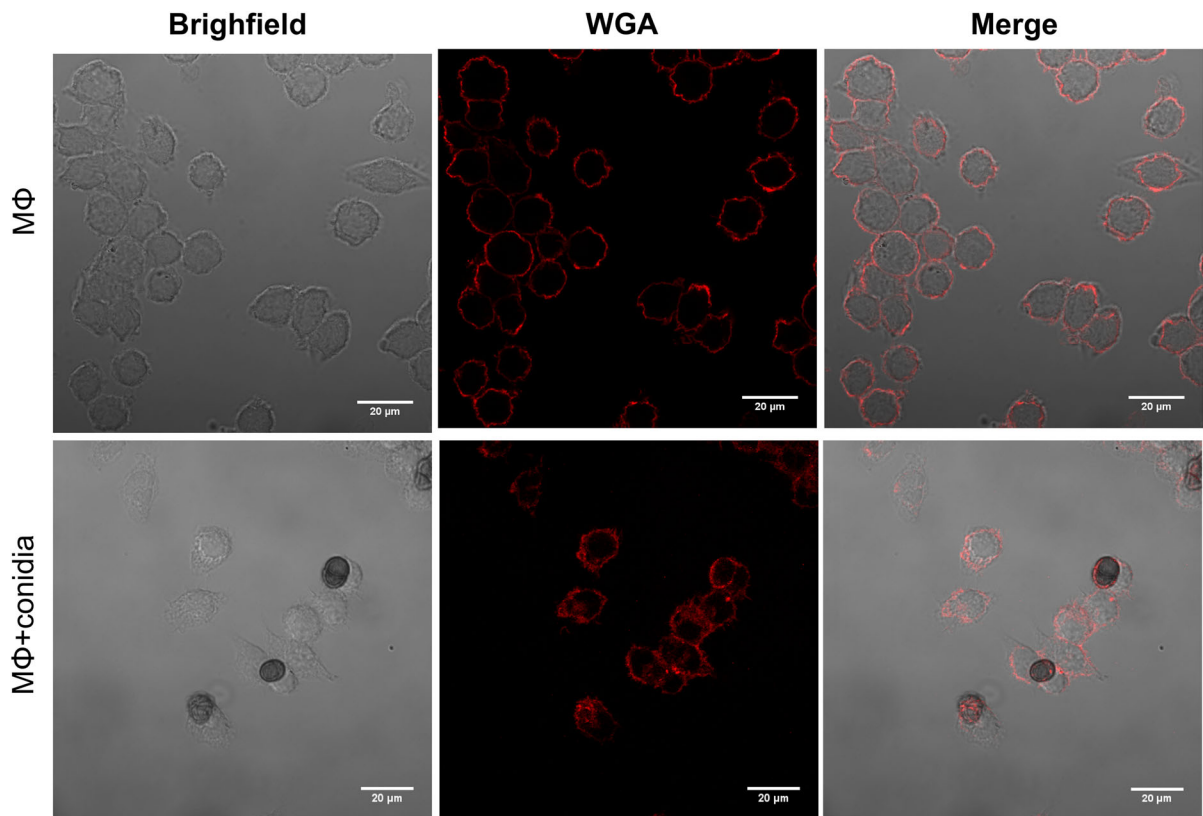


Fig. 2 *A. infectoria* conidia interaction with RAW 264.7 macrophages does not induce changes in the macrophage morphology. Wheat germ agglutinin (WGA, Invitrogen, Paisley, Scotland) Alexa Fluor® 350 conjugated-stained RAW 264.7

cells co-incubated (during 3 h) with *A. infectoria* conidia showed a non-activated morphology as in untreated RAW 264.7 macrophages. Scale bar = 20 μ m

the underresponsiveness previously described by others [28]. Live imaging was used, since it constitutes a valuable tool to study the dynamics of host–pathogen interaction, namely in the elucidation of the *C. albicans*–host cells interaction [29], *Candida krusei* interaction with macrophages [30] or *A. fumigatus* conidia and hyphae interaction with phagocytic cells [31]. Since *Alternaria* spp. are usually associated with respiratory fungal allergy and fungal sensitization, previous studies were limited to the response of host cells to fungal extracts of these fungi [32, 33]. However, comprehensibly, people are mostly exposed to spores, not to fungal homogenates that include not only the fungal surface-cell wall structures, but also membranes and cytoplasmic content. So, our purpose was to elucidate how live *Alternaria* spp. large fungal conidia (7–10 $\times$ 23–34 μ m) [34] interacted with macrophages. We describe that the internalization of these large fungal elements is early initiated, after

10 min of co-culture; most of these conidia survive the macrophage, at least during the early period of interaction of 6 h, and retain the ability to germinate inside the phagocyte. Rarely, it was observed that the growing hyphae of germinating conidia push the macrophage membrane outward. A possible interpretation of this is that it is an event preceding the piercing of the macrophage plasma membrane and the resulting cell lysis, as described in the first 12 h of infection for *A. fumigatus* but not in *A. terreus* [35]. As described by these authors [35], we also believe that once the *A. infectoria* germ tube/nascent hyphae pierce the macrophage, a response is triggered because, assays in which we prolonged the period of infection until 18 h, showed that at this time period of infection most of the spores were germinating, in the form of hyphae; this was concomitant with a massive loss of viability of the macrophages internalizing conidia, and macrophages surrounding the hyphae and remaining non-

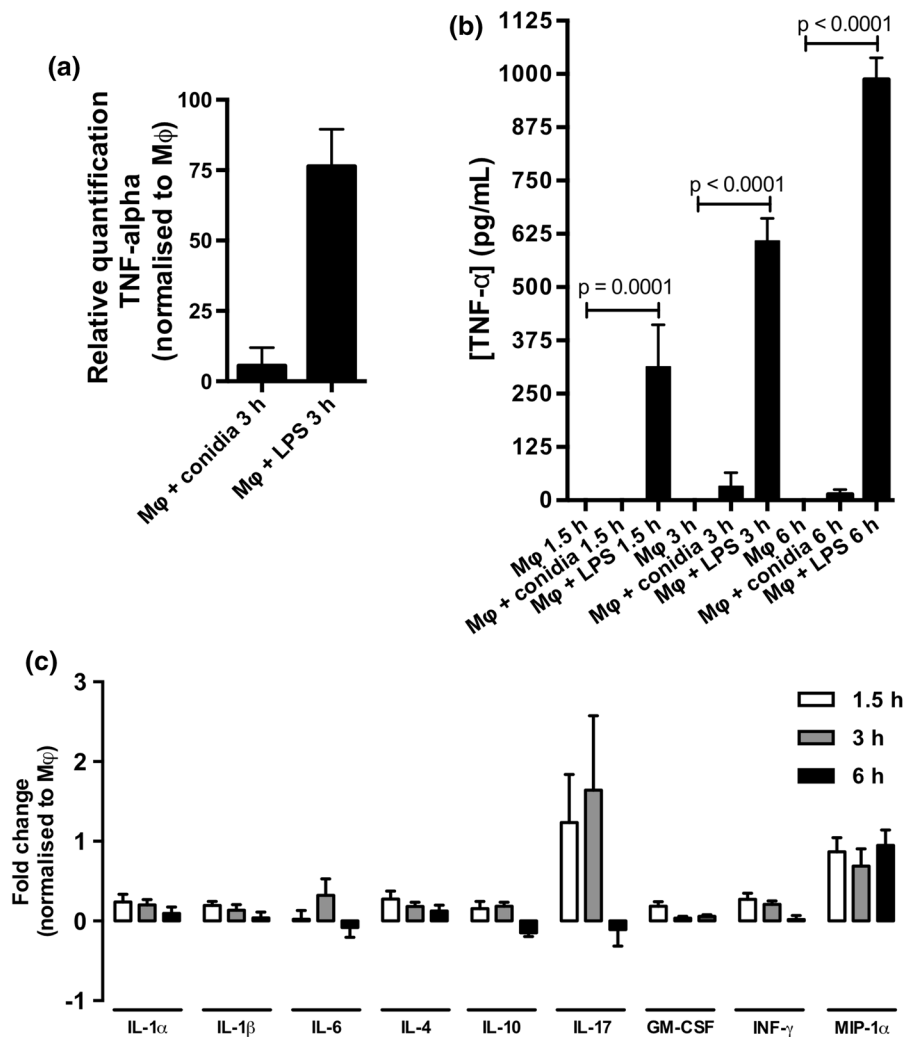


Fig. 3 *A. infectoria* conidia interaction with RAW 264.7 macrophages does not induce cytokine production. **a** Relative gene expression of TNF- α in RAW 264.7 macrophages co-incubated with *A. infectoria* conidia or LPS (100 ng/ml) for 3 h, normalized to untreated RAW 264.7 macrophages. GAPDH gene was used as reference gene. Data were analyzed by relative quantification using the $2^{-\Delta\Delta C_t}$ method (mean $\pm$ SEM, $n = 2$). **b** *In vitro* TNF- α production by macrophages co-incubated with

A. infectoria conidia or LPS (100 ng/ml). RAW 264.7 macrophage production of cytokines after 1.5, 3 and 6 h of exposure to conidia or LPS (100 ng/ml) (mean $\pm$ SEM, $n = 3$). **c** Multiplex for IL-1 α , IL-1 β , IL-6, IL-4, IL-10, IL-17, GM-CSF, INF- γ and MIP-1 α in RAW 264.7 macrophage cells after 1.5, 3 and 6 h of co-incubation with *A. infectoria* conidia or LPS (100 ng/ml), normalized to untreated RAW 264.7 macrophage cells (mean $\pm$ SEM, $n = 3$)

germinated spores (Supplementary Material, S5). Nevertheless, after 6 h of infection most of the macrophages kept the non-activated morphology. The typical polarized morphology and elongated forms of activated macrophages were absent (or rare) during the 6-h interaction of RAW 264.7 macrophages with *A. infectoria* spores. Moreover, macrophages infected with *A. infectoria* large spores retained the ability to divide and pass the internalized spore to one of the daughter cells. This was also observed during

the early period of *C. krusei*-macrophage interaction, with macrophage division followed by fusion [30]. We also observe conidial transfer between macrophages, an event common to other fungal pathogens [30]. The observation of an intracellular compartment similar to a vacuole formed near the germinating spore can be interpreted as a phenomenon recently described as “renitence vacuoles” [36], because the compartments observed by us reside adjacent to the neighboring phagosome containing the fungal conidia, but do not

surround it, as the structures characterized by those authors. Wong and collaborators described that the renitence vacuoles fuse with the lysosomes whenever the phagosome is damaged preventing the expected resulting injury to the cell. Although this is an issue that deserves further investigation, we can speculate that the germination of the spore inside the phagosome could destroy the phagosomal membrane and the arising vacuole formation would prevent cell damage due to plasma membrane piercing.

In order to further characterize the macrophage response to *A. infectoria* conidia, we first studied the production of TNF- α and then screened for the production of other mediators of the immune system, cytokines and chemokines that render different macrophages phenotypes [37] and that are involved in the recruitment of leukocytes to the site of infection [17]. TNF- α is an important cytokine in the immunological response to fungi, and patients under anti-TNF- α therapy are more prone to fungal infections [38, 39]. Under the working conditions adopted in our study, during the first 6 h of interaction, we concluded that there was no production of TNF- α (TNF-alpha gene expression or TNF- α release), or of other cytokines or chemokines that could indicate which stimulatory pathways were activated when macrophages are exposed to *A. infectoria* spores, even when the spores were initiating their germination inside the macrophages. Moreover, some cytokines related to allergic responses, such as IL-4, also failed to be produced. When compared with *A. fumigatus*, these results indicate that *A. infectoria* conidia have a lower macrophage stimulatory effect. As recently reviewed [14], *A. fumigatus* conidia trigger the production of inflammatory chemokines and cytokines, such as TNF- α , IL-12, IFN- γ , IL-18, IL-6, IL-1 β , MIP-1, MIP-1 α , MIP-2, G-CSF, and GM-CSF by alveolar macrophages. Nonetheless, *A. fumigatus* conidia are also described as immunologically inert due to a surface rodlet layer of hydrophobic RodA protein and the pigment DHN-melanin [40]. In fact, it is known that fungi use the cell wall outer layer to prevent immune recognition of the underlying components by immune cells, by modulating their fungal cell wall composition and 3D rearrangement [20, 41, 42]. External layers such as the rodlet layer lose its integrity when *A. fumigatus* conidia swell, leading to the exposure of the underlying components allowing their immune recognition by macrophages [40]. The

results obtained in this study indicate that *A. infectoria* conidia have a low immunological potential, at least during the first 6 h of interaction and germination. This lack of proinflammatory response induced by *A. infectoria* conidia may be due to the masking of immunogenic components of the conidial and hyphal cell wall by immunologically inert molecules. We have been conducting studies to endeavor the nature and the architecture of both the conidial and the hyphal cell wall surface of *A. infectoria* [12, 13]. *A. infectoria* cell wall is enriched in DHN-melanin [13], but the complete cell wall surface composition remains elusive, being plausible a camouflage layer playing an important role in the early stages of interaction with the host cells, namely macrophages.

In summary, here we provide the first interaction study of macrophages and *Alternaria* sp. (*A. infectoria*) conidia. Our results suggest that *A. infectoria* do not trigger a proinflammatory response in RAW 264.7 macrophages. It can also be concluded that albeit the current advances in the *Aspergillus* interaction with host cells, there are still unknown mechanisms by which other filamentous fungi, such as *Alternaria* spores, are proficient in persisting inside host cells, namely prolonging the opportunity to induce fungal sensitization.

Acknowledgements This study was partly supported by FEDER funds through the Operational Programme Competitiveness Factors-COMPETE and national funds by FCT-Foundation for Science and Technology under the strategic project UID/NEU/04539/2019 and by HealthyAging 2020:CENTRO-01-0145-FEDER-000012. This work was also financed through Program Santander-Totta/FMUC (FMUC-BST-2017). C. Fernandes and L. Rodrigues were recipients of fellowships from FCT-Fundação para a Ciência e Tecnologia, respectively, SFRH/BPD/63733/2009 and SFRH/BD/74181/2010. We acknowledge Luísa Cortes and Margarida Caldeira for helpful technical microscopy support.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

References

1. Pegorie M, Denning DW, Welfare W. Estimating the burden of invasive and serious fungal disease in the United Kingdom. *J Infect*. 2017;74(1):60–71.
2. Denning DW, Pashley C, Hartl D, Wardlaw A, Godet C, Del Giacco S, et al. Fungal allergy in asthma—state of the art and research needs. *Clin Transl Allergy*. 2014;4:1–23.

3. Bush RK. Fungal sensitivity: new insights and clinical approaches. *J Allergy Clin Immunol Pract.* 2016;4(3):433–4.
4. Goldman DL, Huffnagle GB. Potential contribution of fungal infection and colonization to the development of allergy. *Med Mycol.* 2009;47(5):445–56.
5. Denning DW, O'Driscoll BR, Powell G, Chew F, Atherton GT, Vyas A, et al. Randomized controlled trial of oral antifungal treatment for severe asthma with fungal sensitization. *Am J Respir Crit Care Med.* 2009;179(1):11–8.
6. Kasprzyk I, Rodinkova V, Šaulienė I, Ritenberga O, Grinn-Gofron A, Nowak M, et al. Air pollution by allergenic spores of the genus *Alternaria* in the air of central and eastern Europe. *Environ Sci Pollut Res Int.* 2015;22(12):9260–74.
7. Pastor FJ, Guarro J. *Alternaria* infections: laboratory diagnosis and relevant clinical features. *Clin Microbiol Infect.* 2008;14(8):734–46.
8. Hipolito E, Faria E, Alves AF, de Hoog GS, Anjos J, Gonçalves T, et al. *Alternaria infectoria* brain abscess in a child with chronic granulomatous disease. *Eur J Clin Microbiol Infect Dis.* 2008;28(4):377–80.
9. Vicencio AG, Santiago MT, Tsirilakis K, Stone A, Worgall S, Foley EA, et al. Fungal sensitization in childhood persistent asthma is associated with disease severity. *Pediatr Pulmonol.* 2014;49(1):8–14.
10. Stern DA, Morgan WJ, Halonen M, Wright AL, Martinez FD. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. *Lancet.* 2008;372(9643):1058–64.
11. Anjos J, Fernandes C, Silva BMA, Quintas C, Abrunheiro A, Gow NAR, et al. $\beta(1,3)$ -glucan synthase complex from *Alternaria infectoria*, a rare dematiaceous human pathogen. *Med Mycol.* 2012;50(7):716–25.
12. Fernandes C, Anjos J, Walker LA, Silva BMA, Cortes L, Mota M, et al. Modulation of *Alternaria infectoria* cell wall chitin and glucan synthesis by cell wall synthase inhibitors. *Antimicrob Agents Chemother.* 2014;58(5):2894–904.
13. Fernandes C, Prados-Rosales R, Silva BM, Nakouzi-Naranjo A, Zuzarte M, Chatterjee S, et al. Activation of melanin synthesis in *Alternaria infectoria* by antifungal drugs. *Antimicrob Agents Chemother.* 2016;60(3):1646–55.
14. Heinekamp T, Schmidt H, Lapp K, Pätz V, Shopova I, Köster-Eiserfunke N, et al. Interference of *Aspergillus fumigatus* with the immune response. *Semin Immunopathol.* 2015;37(2):141–52.
15. Erwig LP, Gow NAR. Interactions of fungal pathogens with phagocytes. *Nat Rev Micro.* 2016;14(3):163–76.
16. Brakhage AA, Bruns S, Thywissen A, Zipfel PF, Behnken J. Interaction of phagocytes with filamentous fungi. *Curr Opin Microbiol.* 2010;13(4):409–15.
17. Phadke AP, Mehrad B. Cytokines in host defense against *Aspergillus*: recent advances. *Med Mycol.* 2005;43:S173–6.
18. Hasenberg M, Behnken J, Krappmann S, Brakhage A, Gunzer M. Phagocyte responses towards *Aspergillus fumigatus*. *Int J Med Microbiol.* 2011;301(5):436–44.
19. Bruns S, Kniemeyer O, Hasenberg M, Aimaniananda V, Nietzsche S, Thywissen A, et al. Production of extracellular traps against *Aspergillus fumigatus in vitro* and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathog.* 2010;6(4):e1000873.
20. Latgé JP. Tasting the fungal cell wall. *Cell Microbiol.* 2010;12(7):863–72.
21. Volling K, Thywissen A, Brakhage Axel A, Saluz Hans P. Phagocytosis of melanized *Aspergillus* conidia by macrophages exerts cytoprotective effects by sustained PI3K/Akt signalling. *Cell Microbiol.* 2011;13(8):1130–48.
22. Akoumianaki T, Kymizi I, Valsecchi I, Gresnigt Mark S, Samonis G, Drakos E, et al. *Aspergillus* cell wall melanin blocks LC3-associated phagocytosis to promote pathogenicity. *Cell Host Microbe.* 2016;19(1):79–90.
23. Ghosh S, Howe N, Volk K, Tati S, Nickerson KW, Petro TM. *Candida albicans* cell wall components and farnesol stimulate the expression of both inflammatory and regulatory cytokines in the murine RAW264.7 macrophage cell line. *FEMS Immunol Med Microbiol.* 2010;60(1):63–73.
24. Jaïdane H, Caloone D, Lobert PE, Sane F, Dardenne O, Naquet P, et al. Persistent infection of thymic epithelial cells with coxsackievirus B4 results in decreased expression of type 2 insulin-like growth factor. *J Virol.* 2012;86(20):11151–62.
25. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc.* 2008;3(6):1101–8.
26. Merly L, Smith SL. Murine RAW 264.7 cell line as an immune target: are we missing something? *Immunopharmacol Immunotoxicol.* 2017;39:55–8.
27. Berghaus LJ, Moore JN, Hurley DJ, Vandenplas ML, Fortes BP, Wolfert MA, Boons GJ. Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. *Comp Immunol Microbiol Infect Dis.* 2010;33:443–54.
28. Taciak B, Białasek M, Braniewska A, Sas Z, Sawicka P, Kiraga Ł, Rygiel T, Król M. Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. *PLoS ONE.* 2018;13:e0198943.
29. Lewis LE, Bain JM, Lowes C, Gillespie C, Rudkin FM, Gow NAR, et al. Stage specific assessment of *Candida albicans* phagocytosis by macrophages identifies cell wall composition and morphogenesis as key determinants. *PLoS Pathog.* 2012;8(3):e1002578.
30. García-Rodas R, González-Camacho F, Rodríguez-Tudela JL, Cuenca-Estrella M, Zaragoza O. The interaction between *Candida krusei* and murine macrophages results in multiple outcomes, including intracellular survival and escape from killing. *Infect Immun.* 2011;79(6):2136–44.
31. Knox BP, Huttenlocher A, Keller NP. Real-time visualization of immune cell clearance of *Aspergillus fumigatus* spores and hyphae. *Fungal Genet Biol.* 2017;105:52–4.
32. Murai H, Qi H, Choudhury B, Wild J, Dharajiyi N, Vaidya S, et al. *Alternaria*-induced release of IL-18 from damaged airway epithelial cells: an NF- κ B dependent mechanism of Th2 differentiation? *PLoS ONE.* 2012;7(2):e30280.
33. Kouzaki H, Iijima K, Kobayashi T, O'Grady SM, Kita H. The danger signal, extracellular ATP, is a sensor for airborne allergen and triggers IL-33 release and innate Th2-type responses. *J Immunol.* 2011;186(7):4375–87.
34. Larone DH. Medically important fungi—a guide to identification. 3rd ed. Washington: ASM Press; 1995.
35. Slesiona S, Gressler M, Mhlan M, Zaehle C, Schaller M, Barz D, et al. Persistence versus escape: *Aspergillus terreus*

- and *Aspergillus fumigatus* employ different strategies during interactions with macrophages. PLoS ONE. 2012;7(2):e31223.
36. Wong AO, Marthi M, Mendel ZI, Gregorka B, Swanson MS, Swanson JA. Renitence vacuoles facilitate protection against phagolysosomal damage in activated macrophages. Mol Biol Cell. 2018;29(5):657–68.
 37. McWhorter FY, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. Proc Natl Acad Sci USA. 2013;110(43):17253–8.
 38. Filler SG, Yeaman MR, Sheppard DC. Tumor necrosis factor inhibition and invasive fungal infections. Clin Infect Dis. 2005;41(3):S208–12.
 39. Ali T, Kaitha S, Mahmood S, Ftesi A, Stone J, Bronze MS. Clinical use of anti-TNF therapy and increased risk of infections. Drug Health Patient Saf. 2013;5:79–99.
 40. Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K, Elluru SR, et al. Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature. 2009;460(7259):1117–21.
 41. Romani L. Immunity to fungal infections. Nat Rev Immunol. 2011;11(4):275–88.
 42. Ballou ER, Avelar GM, Childers DS, Mackie J, Bain JM, Wagener J, et al. Lactate signalling regulates fungal β -glucan masking and immune evasion. Nat Microbiol. 2016;2:16238.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

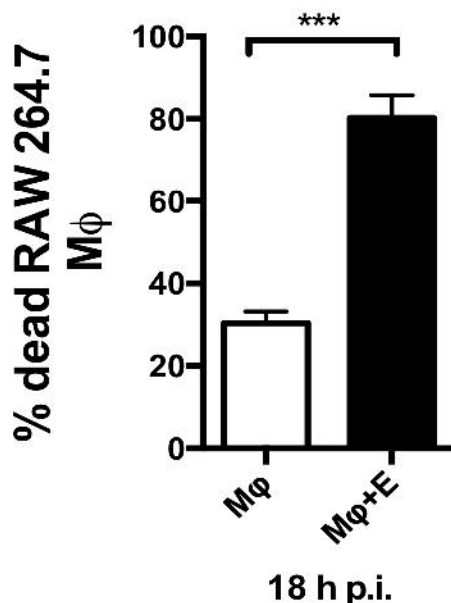
Supplementary Material S5

Interaction of *Alternaria infectoria* conidia with RAW 264.7 macrophages (18 h)

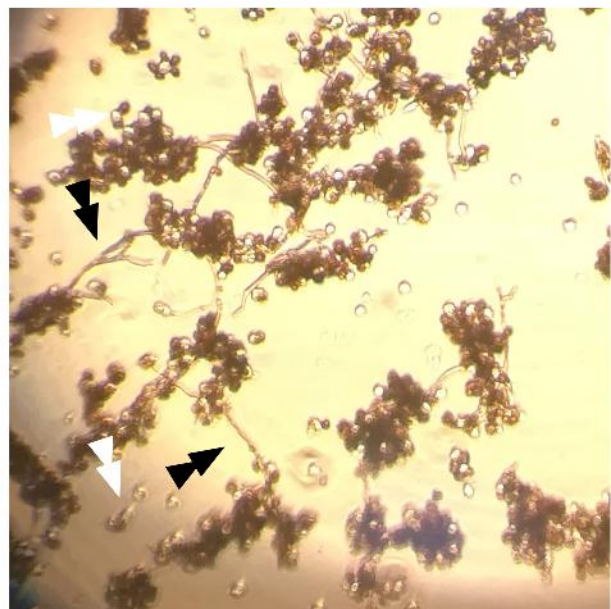
Methodology

In this set of experiments, the methodology of infection was the same as described under viability assays (CFU's) but prolonged up to 18 h. After 18 h, trypan blue was used to assess macrophage viability by counting positive blue cells (dead).

A



B



Legend. RAW 264.7 macrophages viability is decreased when infected during 18 h with *A. infectoria* conidia. A) Trypan blue positive macrophages were quantified after 18 h of infection. B) Microscopic image showing filamentation of *A. infectoria*. Black arrows show hyphae and white arrows show macrophages. Results are shown as mean $\pm$ SD, $n = 3$, **** $p < 0,0001$. Statistical analysis unpaired t-test.