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ORIGINAL RESEARCH

Differential recognition of *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris* by human innate immune cells

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María J Navarro-Arias[1]
Marco J Hernández-Chávez[1]
Laura C García-Carnero[1]
Diana G Amezcua-Hernández[1]
Nancy E Lozoya-Pérez[1]
Eine Estrada-Mata[1]
Iván Martínez-Duncker[2]
Bernardo Franco[1]
Héctor M Mora-Montes[1]

[1]Department of Biology, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, 36050, Guanajuato, Gto, México; [2]Laboratory of Human Glycobiology and Molecular Diagnostics, Centro de Investigación en Dinámica Celular, Instituto de Investigación en Ciencias Básicas y Aplicadas, Universidad Autónoma del Estado de Morelos, Cuernavaca, 62209, Morelos, México

Background: The deep-seated infections caused by the *Candida* genus are associated with a high mortality rate, and *Candida albicans* is the most frequent species associated with these diseases. The fungal wall is composed of macromolecules not synthesized by the host, and therefore is a source of ligands recognized by innate immune cells.

Methods: We performed a comparative study analyzing the cell wall composition and organization of *Candida tropicalis*, *Candida guilliermondii, Candida krusei*, and *Candida auris*, along with their ability to stimulate cytokine production and phagocytosis by human innate immune cells.

Results: We found that the wall of these species had the basic components already described in *C. albicans*, with most of the chitin and β1,3-glucan located underneath the mannan layer. However, the walls of *C. krusei* and *C. auris* were rich in chitin and the former had a lower content of mannans. *C. guilliermondii* contained changes in the mannan and the β1,3-glucan levels. These species were differentially phagocytosed by human macrophages and stimulated cytokine production in a dectin-1-dependent pathway. *C. krusei* showed the most significant changes in the tested parameters, whereas *C. auris* behaved like *C. albicans*.

Conclusion: Our results suggest that the cell wall and innate immune recognition of *C. tropicalis*, *C. guilliermondii*, *C. krusei*, and *Candida auris* is different from that reported for *C. albicans*.

Keywords: cell wall, protein glycosylation, host–fungus interplay, phagocytosis, cytokine production

Introduction

The species of the *Candida* genus are responsible for superficial and systemic candidiasis, and the latter represents a significant burden to health care systems and patients because of the high rate of morbidity and mortality associated with these infections, especially in immunocompromised patients.[1,2] Although *Candida albicans* is the most frequently isolated species from patients with diagnosed candidiasis, other *Candida* species are responsible for about 35%–65% of candidemia cases.[3,4] Among them, *Candida tropicalis, Candida parapsilosis, Candida guilliermondii, Candida glabrata,* and *Candida krusei* top the epidemiological lists of causative agents of systemic candidiasis.[5,6]

C. tropicalis is considered as an important causative agent of invasive candidiasis and colonizes 60%–80% of immunocompromised patients.[6] This species has been associated with similar or higher mortality rates than those reported for *C. albicans*.[7–9] *C. krusei* and *C. guilliermondii* are also part of the emergent group of *Candida* species related to systemic candidiasis, being the causative agents in 2%–5% of the reported cases.[6,7] A significant difference between infections associated with *C. guilliermondii*

Correspondence: Héctor M Mora-Montes
Departamento de Biología, División de Ciencias Naturales y Exactas, Campus Guanajuato, Universidad de Guanajuato, Noria Alta s/n, col Noria Alta, CP 36050, Guanajuato, Gto, México
Tel +52 473 732 0006 (ext 8193)
Fax +52 473 732 0006 (ext 8153)
Email hmora@ugto.mx

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and *C. krusei* is the mortality rate: in *C. guilliermondii* this is similar to that recorded for *C. albicans* (27%–49%),[1,10] while in *C. krusei* the mortality rate is higher (20%–67%), likely related to their poor response to standard antifungal therapies.[6,8,9,11] *Candida auris* is an emergent fungal species, firstly identified in 2009 as an etiological agent of candidiasis, and has a natural resistance to several of the antifungal drugs used to treat these infections.[12] Despite not being related to high numbers of cases of nosocomial candidiasis, it is distributed worldwide, and most of the clinical isolates have shown high minimal inhibitory concentrations for azoles, polyenes, and echinocandins.[12]

The fungal immune sensing is a key step in the establishment of a protective anti-fungal immune response, and among the first events that trigger this interaction is the recognition of the fungal cell wall.[13,14] The wall is an essential cell component that provides protection from extracellular insults, controls communication with the extracellular environment, and is the molecular scaffold that displays virulence factors in fungal pathogens.[15] As it is composed of polysaccharides which are absent in the human cells, the cell wall is the main source of pathogen-associated molecular patterns that the immune system recognizes through pattern recognition receptors (PRRs), mainly localized at the cell surface of immune cells.[13,15]

The *C. albicans* cell wall has been thoroughly studied, and nowadays, there is enough information to propose general models regarding its composition, organization, and relevance for viability, virulence, and immune sensing.[13,15–17] Despite this significant advance, little is known about the cell wall organization and composition of other species of the *Candida* genus, such as *C. guilliermondii, C. tropicalis, C. krusei,* and *C. auris*. A possible reason is that it is assumed that the *C. albicans* cell wall model should apply to all the members of this genus. In *C. albicans*, the cell wall is composed of two well-defined layers,[13,16] visible under electronic microscopy inspection: an inner layer composed of chitin, β1,3- and β1,6-glucans, and an outer layer rich in proteins modified with *N*- or *O*-linked mannans, named mannoproteins.[18] These components are not synthesized by the host cells, which in turn are recognized by a variety of PRRs that will ultimately contribute to the establishment of a protective antifungal response by the host immunity.[13] The β1,3-glucans are sensed by a heterodimer composed of toll-like receptor (TLR) 2 and TLR6 and by dectin-1, the latter being a specific receptor for this polysaccharide;[13,19] while TLR4 participates in the recognition of *O*-linked mannans[20] and mannose receptor,[20] dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin,[21] dectin-2,[22] mincle,[23] and dectin-3[24] recognize the *N*-linked mannans. The chitin–receptor interaction can block the fungal sensing,[25] or promote an anti-inflammatory state in a pathway dependent on the mannose receptor, nucleotide-binding oligomerization domain-containing protein 2, and TLR9.[26]

So far, little is known about the immune sensing of *C. tropicalis*, *C. krusei*, *C. guilliermondii,* and *C. auris*. It has been shown that neutrophils and murine phagocytic cells are capable of discriminating among *Candida* species, the former displaying reduced uptake against *C. krusei*,[27] and the latter an increased ability to kill *C. guilliermondii* and *C. krusei*.[28] In addition, *C. tropicalis* is more susceptible to damage by neutrophils than *C. albicans*.[29] Human monocytes differentially recognize *Candida* species, such as *C. albicans*, *C. parapsilosis*, *C. tropicalis,* and *C. krusei*, but not *C. glabrata* and *C. guilliermondii*, which are good stimuli for C3 and granulocyte-macrophage colony-stimulating factor.[30] On the contrary, there are studies indicating that different *Candida* species have similar abilities to interact with innate immune cells, triggering similar killing rates and phagocytosis as *C. albicans*.[31–33] Although the *C. auris*-immune effector interplay is an area that is poorly explored, it has been demonstrated that neutrophils are not capable of forming extracellular traps when interacting with *C. auris*.[34]

Here, in order to assess insights about the relevance of the cell wall in the immune sensing of *C. tropicalis, C. krusei*, *C. guilliermondii,* and *C. auris* by human peripheral blood mononuclear cells (PBMCs) and human monocyte-derived macrophages, we determined the basic composition of the cell wall of these fungal species and their ability to stimulate cytokine production and phagocytic uptake.

Materials and methods

Strains and culture media

C. albicans SC5314,[35] *C. guilliermondii* ATCC 6260,[36] *C. krusei* ATCC 6258, *C. tropicalis* ATCC MYA-3404,[37] and *C. auris* VPCI 479/P/13,[38] a genome-sequenced strain, were used in this study. Cell cultures were propagated at 30°C in Sabouraud broth (1% [w/v] mycological peptone, 4% [w/v] glucose). Unless otherwise indicated, aliquots containing 500 µL of overnight-grown cells were inoculated in 100 mL of fresh medium and further incubated at 30°C with constant shaking (200 rpm) until reaching the mid-log growth phase (typically 5–6 hours). For heat inactivation, yeast cells were incubated at 56°C for 1 hour.[39] In all cases, loss of cell viability was confirmed by incubating cells in Sabouraud medium

for 72 hours at 30°C. To remove *O*-linked mannans, cells were β-eliminated overnight with 100 mM NaOH as described.[40] Following this protocol, >96% of the cells retained their viability, as tested by cell growth before and after treatment with the alkali.[41]

Cell wall analysis

Cell homogenates were obtained in a Braun homogenizer, as previously described,[41] and walls were separated from cellular debris by centrifuging at 21,000× *g* for 10 minutes at 4°C. The cell walls were washed six times with deionized water; adsorbed intracellular components were removed with hot 2% (v/v) SDS, 0.3 M β-mercaptoethanol, and 1 M NaCl; freeze-dried; and hydrolyzed with hot 2 M trifluoroacetic acid for 3 hours, as described previously.[39] The content of N-acetylglucosamine, glucose, and mannose in the wall hydrolysates was measured by high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) in a Dionex system (Thermo Fisher Scientific, Waltham, MA, USA) using similar separation conditions to those described earlier.[41] Protein content was determined in alkali-hydrolyzed samples from the cell wall,[39] using the Bradford protein assay.

Alcian blue binding assays

Yeast cells grown at the exponential phase were pelleted, washed three times with deionized water, and the cell concentration adjusted at an OD_{600} of 0.2 in deionized water. Aliquots of 1 mL were pelleted, and cells were suspended in 1 mL of Alcian blue (30 μg·mL^{-1} in 0.02 M HCl; Sigma-Aldrich Co., St Louis, MO, USA) and assayed as described.[42]

Cell wall porosity assay

The relative cell wall porosity to polycations was estimated as described previously.[43] Cells were grown until reaching the exponential phase, washed twice with PBS, and cell concentration was adjusted at 1×10^8 cells mL^{-1}. Aliquots containing 1 mL were centrifuged, the pellet was saved and resuspended in either 10 mM Tris-HCl, pH 7.4 (buffer A), buffer A plus 30 μg·mL^{-1} poly-L-lysine (Mw 30–70 kDa, Sigma-Aldrich Co.), or buffer A plus 30 μg·mL^{-1} diethylaminoethyl (DEAE)-dextran (Mw 500 kDa, Sigma-Aldrich Co.). Cells were incubated for 30 minutes at 30°C with constant shaking (200 rpm), centrifuged, and the supernatants were saved, centrifuged again, and used to measure the absorbance at 260 nm. The relative cell wall porosity to DEAE-dextran was quantified as reported elsewhere.[43]

Analysis of the exposure of the cell wall polysaccharide at the fungal wall surface

Chitin was labeled by incubating cells with 1 mg·mL^{-1} fluorescein-5-isothiocyanate conjugated lectin from *Triticum vulgaris* (WGA-FITC; Sigma-Aldrich Co.) for 60 minutes at room temperature, as reported previously,[25] while β1,3-glucan was labeled with 5 μg·mL^{-1} IgG Fc-Dectin-1 chimera[44] for 40 minutes at room temperature. The binding of the lectin to the fungal wall was revealed by incubating cells with 1 μg·mL^{-1} donkey anti-Fc IgG-FITC (Sigma-Aldrich Co.) for 40 minutes at room temperature.[45] Samples were examined by fluorescence microscopy using a Zeiss Axioscope-40 microscope and an Axiocam MRc camera. From the pictures acquired, the fluorescence associated to 300 cells was collected using the software Adobe Photoshop™ CS6, Adobe Systems Incorporated (San Jose, CA, USA) with the formula: ([total of green pixels – background green pixels] × 100)/total pixels.[46]

Quantification of *N*- and *O*-linked mannans

Mannan trimming was performed as reported.[47] The *N*-linked mannans were removed from the cell wall by incubating yeast cells with 25 U endoglycosidase H (New England Biolabs, Ipswich, MA, USA) for 20 hours at 37°C; while removal of *O*-linked mannans was performed by β-elimination with 1N NaOH and gently shaking for 18 hours at room temperature. Mannan release and quantification was performed by HPAEC-PAD, as previously described.[48]

Ethics statement

Healthy adult volunteers were enrolled in the study and venous blood samples were withdrawn after information about the study was disclosed and written informed consent was obtained. This procedure was conducted in accordance with the Declaration of Helsinki. The use of human cells was approved by the Ethics Committee of Universidad de Guanajuato (permission code 17082011).

Isolation of human PBMCs and cytokine stimulation

The venous blood was mixed with Histopaque-1077 (Sigma-Aldrich Co.) and density centrifugation was performed as described.[49] The PBMC–*Candida* interactions were carried out using 5×10^5 PBMCs in 100 μL of RPMI 1640 Dutch modification (added with 2 mM glutamine, 0.1 mM pyruvate, and 0.05 mg·mL^{-1} gentamycin; all reagents from

Sigma-Aldrich Co.) and 100 µL containing 1×10^5 yeast cells freshly harvested or treated. The interactions were placed in round-bottom 96-well microplates and incubated for 24 hours at 37°C with 5% (v/v) CO_2. Then, the plates were centrifuged for 10 minutes at 3,000× *g* at 4°C before the supernatants were saved and kept at –20°C until further use. The concentrations of tumor necrosis factor alpha (TNFα), IL-6, and IL-10 were quantified by ELISA using the kit ABTS ELISA Development from Peprotech, while the IL-1β levels were measured using a DuoSet ELISA Development kit (R&D systems). In all plates, mock interactions containing only PBMCs were included as controls and produced threshold levels that were subtracted to the quantifications of all the cytokines analyzed.

When indicated, human PBMCs were preincubated at 37°C for 60 minutes with 200 µg·mL^{-1} laminarin (Sigma-Aldrich Co.) before adding the fungal stimuli.

Phagocytosis assays

Human monocyte-derived macrophages were obtained by incubating the PBMCs with recombinant human granulocyte-macrophage colony-stimulating factor (Sigma-Aldrich Co.) as previously reported.[46] Yeast cells were washed twice with PBS, stained with 1 mg·mL^{-1} acridine orange (Sigma-Aldrich Co) as described,[50] washed again with PBS, and the cell concentration was adjusted at 3×10^7 yeast cells mL^{-1}. The macrophage–fungus interactions were performed in aliquots of 800 µL of DMEM medium (Sigma-Aldrich Co), in six-well plates with a macrophage-to-yeast ratio of 1:6. Plates were incubated for 2 hours at 37°C and 5% (v/v) CO_2, and then macrophages were washed twice with cold PBS and suspended in 1.25 mg·mL^{-1} trypan blue as an external fluorescence quencher, as described.[37,51] A MoFlo XDP (Beckman Coulter) fuorescence-activated cell sorting system was used to analyze samples by flow cytometry, collecting 50,000 events gated for macrophage cells. Signals were obtained using the FL1 (green fluorescence) and FL3 (red fluorescence) channels previously compensated with macrophage cells without any labeling. The phagocytosis of fungal cells was analyzed from counted events in the green channel (early stage of the phagocytic event) and red channel (cells within acidified phagolysosomes, ie, in the late stage of the phagocytic event).[37]

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. Stimulation of cytokine production and phagocytosis by human cells were carried out in duplicate with samples from six healthy donors, while the other experiments were performed at least three times in duplicate. Data represent cumulative results of all experiments performed and are shown as the mean and the SD. The Mann–Whitney *U* test was used to analyze data with a significance level set at $P<0.05$.

Results

Cell wall composition and organization of the *Candida* species under study

To quantify the basic polysaccharides and oligosaccharides of the cell wall of *C. guilliermondii*, *C. krusei*, *C. tropicalis*, and *C. auris*, the walls were extracted, depleted of cell components, acid-hydrolyzed, and analyzed by HPAEC-PAD. This methodology has been applied before in the analysis of other *Candida species*, including *C. albicans*, and allowed the quantification of chitin, glucan, and mannan, by assessment of the content of N-acetylglucosamine, glucose, and mannose, respectively.[37,39,41,42,46–48,52–54] Although it has been extensively characterized previously, here we included the analysis of the *C. albicans* cell wall for comparison purposes. The cell walls of the species analyzed showed similar levels of chitin, glucan, and mannan (Table 1), with significant differences in some cases: *C. krusei* and *C. auris* showed higher content of chitin when compared to the other species, and they were also significantly different when compared between each other (Table 1). Mannan was significantly higher in *C. guilliermondii* cell wall, but lower in *C. krusei* wall, and the former also showed lower levels of glucan (Table 1). All strains tested showed similar levels of cell wall protein content, with exception of *C. krusei* that showed low levels of cell wall protein (Table 1). To confirm the changes in the cell wall mannan content in *C. guilliermondii* and *C. krusei*, we measured the content of *O*-linked and *N*-linked mannans decorating the cell wall glycoproteins, by trimming the oligosaccharides with β-elimination and endoglycosidase H digestion, respectively.[47] Results shown in Figure 1 indicated that upon β-elimination, the *O*-linked mannan content in the five species analyzed was lower when compared to the *N*-linked mannans, and *C. guilliermondii* and *C. krusei* were the only two strains that showed changes in the mannan content: the former had increased levels of both mannans, whereas *C. krusei* showed low levels of both *N*-linked and *O*-linked mannans (Figure 1). Interestingly, in *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. auris*, *O*-linked mannans represented about 15% of the total content of cell wall mannan, but in *C. guilliermondii* these molecules were relatively more abundant than the *N*-linked mannans, representing around 33% of total mannan content.

Table 1 Cell wall analysis of *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *and C. auris*

Cell wall abundance				Phosphomannan content (µg)[a]	Porosity (%)[b]	Protein (µg)[c]
Organism	Chitin (%)	Mannan (%)	Glucan (%)			
C. albicans	2.0±1.3	36.1±4.2	61.7±4.4	114.2±14.3	28.4±8.7[d]	146.4±14.9
C. tropicalis	2.3±1.0	36.2±3.5	62.9±5.0	139.5±19.0[d]	62.8±8.9	129.7±15.3
C. guilliermondii	2.2±1.0	47.8±4.0[d]	50.0±5.0[d]	131.2±8.9[d]	72.3±12.7	131.6±18.9
C. krusei	8.2±1.0[d]	23.9±2.0[d]	67.8±2.0	66.1±9.0[d]	57.7±10.5	76.4±14.8[d]
C. auris	4.5±1.6[d]	30.8±1.1	64.9±2.5	110.1±15.2	30.9±9.4[d]	138.7±18.2

Notes: [a]µg of alcian blue bound/OD_{600}=1. [b]Relative to DEAE-dextran. [c]µg of protein/mg of cell wall. [d]$P<0.05$, when compared with the values from the other species analyzed. Data presented as mean±SD.

Abbreviations: *C. albicans*, *Candida albicans; C. tropicalis*, *Candida tropicalis; C. guilliermondii*, *Candida guilliermondii; C. krusei*, *Candida krusei; C. auris, Candida auris*; DEAE, diethylaminoethyl.

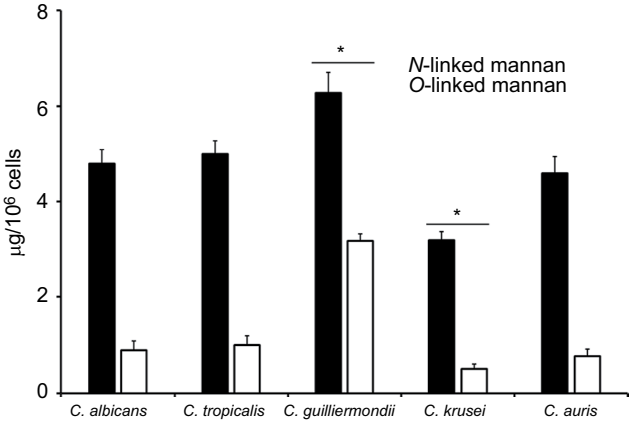

Figure 1 The content of *N*-linked and *O*-linked mannan in the cell wall of *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris*.

Notes: Yeast cells were treated either with endoglycosidase H or β-eliminated to trim *N*-linked mannans or *O*-linked mannans, respectively. The released oligosaccharides were saved and used to measure the mannose content by HPAEC-PAD. Data are mean ± SD of three independent experiments performed in duplicates. *$P<0.05$, when compared with mannans from the other species analyzed.

Abbreviation: HPAEC-PAD, high-performance anion-exchange chromatography coupled to pulsed amperometric detection.

To provide additional evidence to these observations, we next assessed the content of cell wall phosphomannan and the cell wall porosity to polycations, as these parameters have been associated with the length of cell wall mannans.[39,43,46,47,52–56] The *C. albicans* and *C. auris* walls showed similar phosphomannan levels, while *C. tropicalis* and *C. guilliermondii* showed higher content of this oligosaccharide, and the *C. krusei* walls showed lower phosphomannan abundance (Table 1). The cell wall porosity was similar for *C. tropicalis*, *C. guilliermondii*, and *C. krusei*, but this parameter was significantly lower in the walls of *C. albicans* and *C. auris* (Table 1).

To analyze the organization of the structural polysaccharide within the cell wall, the β1,3-glucan was labeled with the IgG Fc-Dectin-1 chimera,[57] and then with FITC-conjugated IgG, whereas WGA-FITC was used for chitin labeling. The five species analyzed were barely stained with both lectins (Figure 2); however, when cells were inactivated by heat, a treatment that artificially exposes chitin and β1,3-glucan on the surface of the cell wall,[58] the fluorescence associated with cells from the five species was significantly increased (Figure 2). Therefore, chitin and β1,3-glucan are localized underneath other cell wall components that impair the proper lectin–polysaccharide interaction. It is noteworthy that the labeling of β1,3-glucan in heat-killed (HK) *C. guilliermondii* cells was significantly lower compared to cells from other species under the same treatment (Figure 2), and HK *C. krusei* and *C. auris* cells were more labeled by WGA-FITC, suggesting an increased chitin content (Figure 2). These results correlated with the cell wall composition provided in Table 1. Interestingly, cells from *C. krusei* showed increased chitin labeling in the live form, suggesting that more of this polysaccharide is naturally exposed on the surface of this organism than in the other species under analysis (Figure 2). Overall, the data indicates that the cell wall of *C. tropicalis*, *C. guilliermondii*, *C. krusei*, and *C. auris* differs to which has been described in *C. albicans*.

Stimulation of cytokine production by *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei*, and *C. auris*

The differences in the cell wall described earlier are likely to affect the *Candida*–immune cell interaction when compared to that described for *C. albicans*. Thus, to get some insights about such interaction, yeast cells were coincubated with human PBMCs and the level of secreted cytokines was measured as a read-out of this interaction. *C. albicans* and *C. auris* cells barely stimulated the production of TNFα, IL-6, IL-1β, or IL-10 (Figure 3); however, *C. tropicalis*, *C. guilliermondii,* and *C. krusei* stimulated higher levels of the four cytokines analyzed (Figure 3). Among the tested strains, one that is capable of inducing a high cytokine production was *C. krusei*, and

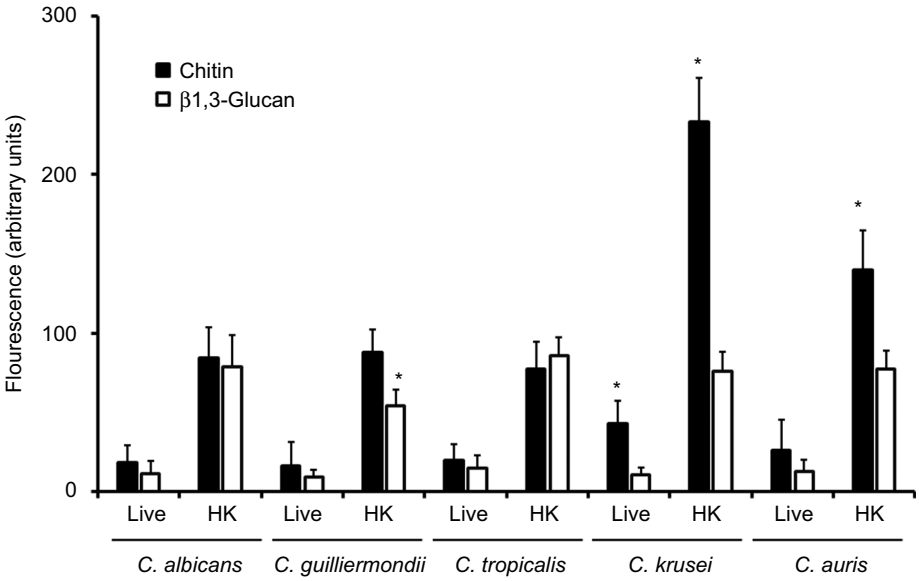

Figure 2 Fluorescent labeling of chitin and β1,3-glucan in the cell wall of *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris*.
Notes: Live or heat-killed (HK) yeast cells were incubated with either fluorescein isothiocyanate-wheat germ agglutinin conjugate (closed bars, labels chitin) or IgG Fc-Dectin-1 chimera (open bars, labels β1,3-glucan) as described in the "Materials and methods" section, inspected under fluorescence microscopy, and the fluorescence associated to 300 individual cells was recorded. *P<0.05, when compared with cells under the same treatment.

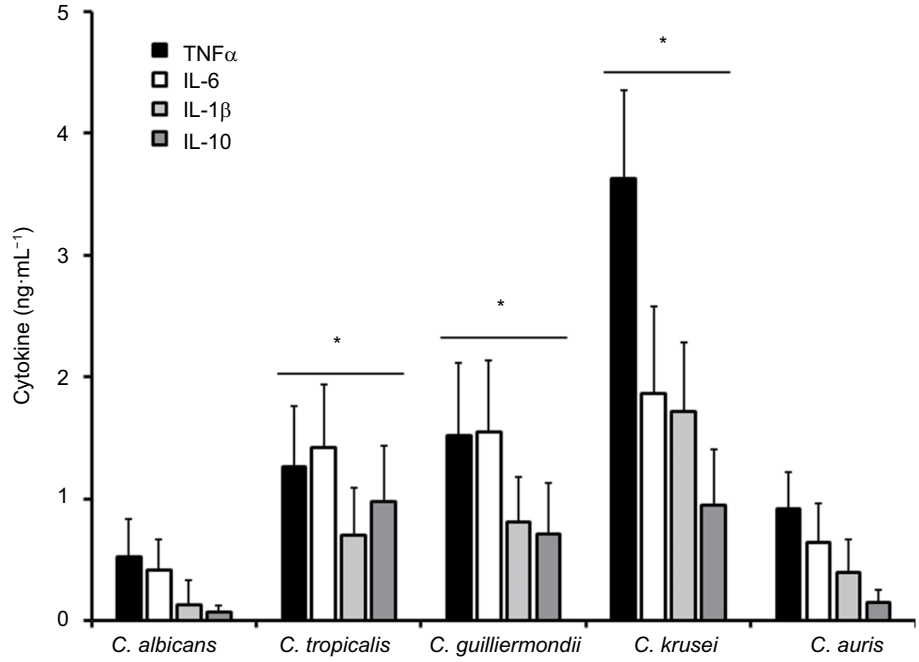

Figure 3 Stimulation of cytokine production by *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris*.
Notes: Human PBMCs were coincubated for 24 hours with live yeast cells, and then the supernatant was collected and used to quantify the cytokine levels. *P<0.05, when compared with the cytokine level stimulated by *C. albicans* cells.
Abbreviations: PBMCs, peripheral blood mononuclear cells; TNFα, tumor necrosis factor alpha.

this strain significantly stimulated the secretion of higher levels of TNFα and IL-1β (Figure 3) in comparison with the remaining strains. To assess the role of components of the inner part of the cell wall and *O*-linked mannans, we compared the cytokine profile stimulated with either live or HK cells with or without β-elimination that trimmed the *O*-linked mannans from the cell wall.[40,58] As previously reported for *C. albicans*,[41,58] HK yeast cells stimulated the production of higher levels of TNFα, IL-6, IL-1β, and IL-10 than live cells (Figure 4). The trimming of *O*-linked

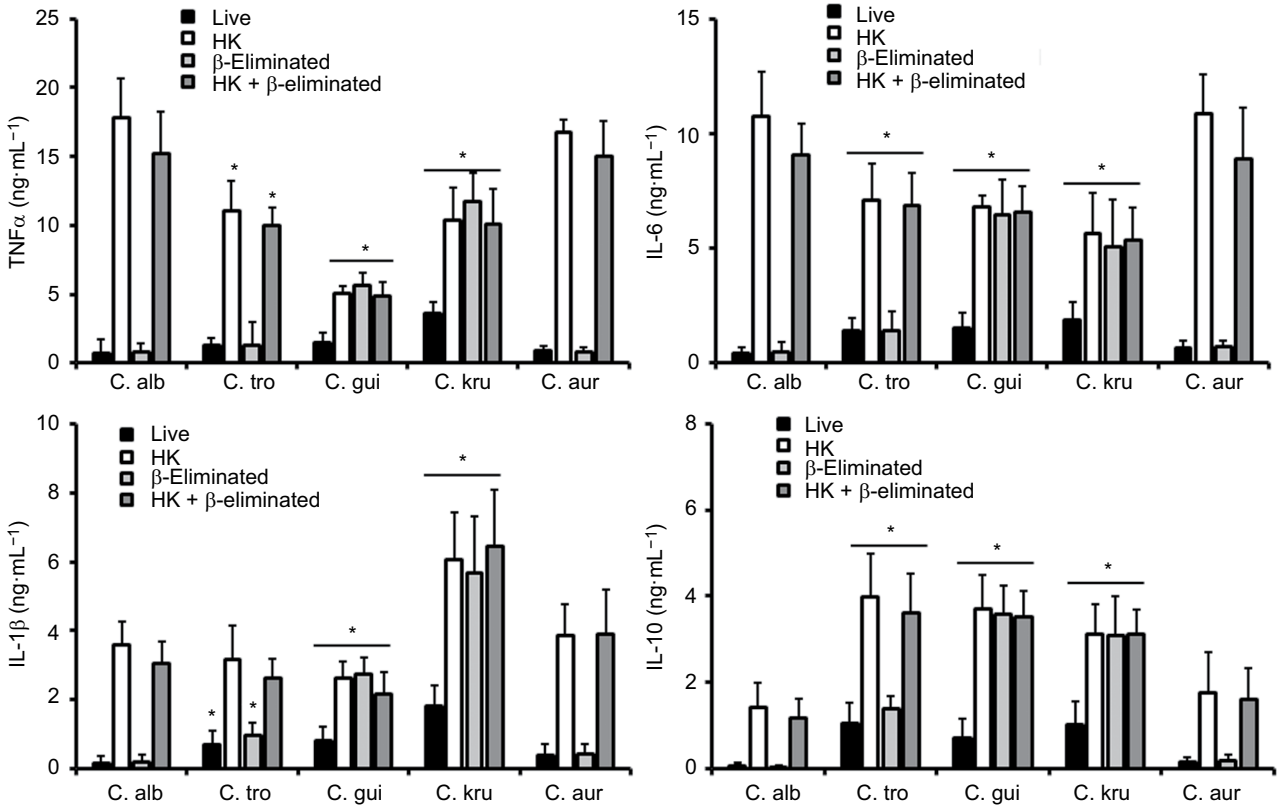

Figure 4 Stimulation of cytokine production by heat-killed and β-eliminated cells from *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris*.

Notes: Yeast cells were heat-killed (HK), β-eliminated, or subjected to both treatments before being coincubated with human PBMCs for 24 hours. The supernatants of interactions were collected and used to quantify the cytokine levels. * $P<0.05$, when compared with the cytokine level stimulated by *C. albicans* cells under the same treatment.

Abbreviations: PBMCs, peripheral blood mononuclear cells; TNFα, tumor necrosis factor alpha; C. alb, *Candida albicans*; C. tro, *Candida tropicalis*; C. gui, *Candida guilliermondii*; C. kru, *Candida krusei*; C. aur, *Candida auris*.

mannan did not affect significantly the cytokine profile stimulated either by live or HK *C. albicans* cells (Figure 4). The *C. auris* cells showed similar ability to *C. albicans* to stimulate cytokine production, as the profiles of the four cytokines tested were similar for both species when live, HK, β-eliminated or HK + β-eliminated cells were used for the interaction with the human PBMCs (Figure 4). For *C. tropicalis*, *C. guilliermondii,* and *C. krusei*, the HK cells stimulated higher cytokine levels than live cells (Figure 4), but some differences were observed when their cytokine profiles were compared with those stimulated by *C. albicans*. These three species stimulated lower production of TNFα and IL-6 than *C. albicans*, including when cells were HK and β-eliminated (Figure 4). Interestingly, upon β-elimination, live cells stimulated high levels of these two cytokines, comparable with those obtained with HK cells (Figure 4). In the case of the production of IL-1β, the HK *C. tropicalis* cells stimulated similar levels of this cytokine to those produced upon interaction of the human cells with *C. albicans*, but live and β-eliminated cells stimulated higher levels of this cytokine when *C. tropicalis* cells were used during the coincubation period (Figure 4). For *C. guilliermondii*, HK and HK + β-eliminated cells stimulated less IL-1β production than *C. albicans*, but higher levels of this cytokine were observed when β-eliminated cells were used in the interactions (Figure 4). The HK, β-eliminated, and HK + β-eliminated cells from *C. krusei* stimulated similar IL-1β levels that were significantly higher than those produced by *C. albicans* cells (Figure 4). A similar observation could be drawn for the stimulation of IL-10 production by cells of *C. guilliermondii* and *C. krusei*, whereas only HK and HK + β-eliminated *C. tropicalis* cells stimulated high production of this anti-inflammatory effector (Figure 4).

To explore whether the significant increment in the ability to stimulate cytokine production by HK cells was dependent on the recognition of β1,3-glucan by dectin-1, the human PBMCs were preincubated with laminarin, an antagonist of dectin-1,[41,46,47] and then used in the interaction with the yeast cells. Results given in Table 2 indicated that preincubation of the human cells with laminarin did not affect

Table 2 Effect of laminarin on the ability of *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei, and C. auris* to stimulate cytokine production

	TNFα (%)[a]	IL-6 (%)[a]	IL-1β (%)[a]	IL-10 (%)[a]
C. albicans				
Live	100	100	100	100
Live + laminarin[b]	99.1±0.1	99.2±0.1	99.3±0.2	95.8±0.6
HK	100	100	100	100
HK + laminarin[b]	15.7±4.4[c]	22.8±5.4[c]	26.8±6.4[c]	23.7±7.8[c]
β-eliminated	100	100	100	100
β-eliminated + laminarin[b]	98.4±1.1	97.8±0.9	96.8±2.8	98.7±0.8
HK + β-eliminated	100	100	100	100
HK + β-eliminated + laminarin[b]	17.2±10.4[c]	21.8±6.0[c]	22.1±3.2[c]	29.3±9.4[c]
C. tropicalis				
Live	100	100	100	100
Live + laminarin[b]	98.1±0.3	97.2±0.1	98.8±0.3	97.4±0.2
HK	100	100	100	100
HK + laminarin[b]	26.7±6.1[c]	28.4±8.1[c]	21.8±9.1[c]	29.4±10.1[c]
β-eliminated	100	100	100	100
β-eliminated + laminarin[b]	94.2±0.9	95.0±2.4	98.1±0.7	94.6±2.1
HK + β-eliminated	100	100	100	100
HK + β-eliminated+ laminarin[b]	27.9±7.1[c]	29.4±4.1[c]	30.4±10.5[c]	31.7±8.5[c]
C. guilliermondii				
Live	100	100	100	100
Live + laminarin[b]	99.8±0.1	93.0±4.4	98.4±0.2	90.8±4.4
HK	100	100	100	100
HK + laminarin[b]	34.8±9.4[c]	17.5±9.4[c]	19.5±4.5[c]	20.6±6.1[c]
β-eliminated	100	100	100	100
β-eliminated + laminarin[b]	35.6±7.4[c]	27.3±5.6[c]	18.5±3.7[c]	22.4±8.8[c]
HK + β-eliminated	100	100	100	100
HK + β-eliminated + laminarin[b]	27.2±5.4[c]	25.4±8.4[c]	20.2±6.1[c]	21.9±3.7[c]
C. krusei				
Live	100	100	100	100
Live + laminarin[b]	93.3±2.4	90.4±9.4	9.7±1.8	93.1±5.2
HK	100	100	100	100
HK + laminarin[b]	23.2±6.1[c]	25.8±5.1[c]	19.5±4.5[c]	26.1±4.8[c]
β-eliminated	100	100	100	100
β-eliminated + laminarin[b]	21.8±5.5[c]	16.4±2.4[c]	27.4±7.6[c]	23.1±6.0[c]
HK + β-eliminated	100	100	100	100
HK + β-eliminated + laminarin[b]	22.2±11.4[c]	22.0±5.7[c]	30.4±10.5[c]	32.5±8.3[c]
C. auris				
Live	100	100	100	100
Live + laminarin[b]	97.0±0.5	97.4±0.4	96.9±0.6	99.1±0.1
HK	100	100	100	100
HK + laminarin[b]	20.4±8.4[c]	27.0±8.1[c]	24.1±9.4[c]	28.8±7.9[c]
β-eliminated	100	100	100	100
β-eliminated + laminarin[b]	92.8±6.5	95.1±1.7	92.4±8.0	97.2±3.7
HK + β-eliminated	100	100	100	100
HK + β-eliminated + laminarin[b]	21.4±7.5[c]	28.0±9.4[c]	27.2±6.6[c]	21.0±5.6[c]

Notes: [a]The 100% value corresponds to the cytokine concentration produced when laminarin was not included in the stimulation assays. [b]Human PBMCs were preincubated for 60 minutes at 37°C with 200 μg·mL^{-1} laminarin before adding the fungal stimuli. [c]$P<0.05$, when compared with the assay with no laminarin included. Data presented as mean±SD.

Abbreviations: PBMCs, peripheral blood mononuclear cells; TNFα, tumor necrosis factor alpha; HK, heat-killed; *C. albicans*, *Candida albicans; C. tropicalis*, *Candida tropicalis; C. guilliermondii*, *Candida guilliermondii; C. krusei*, *Candida krusei; C. auris, Candida auris.*

the ability of live *C. albicans*, *C. tropicalis*, and *C. auris* to stimulate cytokine production, but a significant reduction in the cytokine levels were observed when HK cells were used for the interactions. Similar data were obtained when cells were subjected to β-elimination (Table 2). For the case of live and HK cells from *C. guilliermondii* and *C. krusei*, similar results were observed, but the cytokine production stimulated by both live β-eliminated and HK + β-eliminated cells was significantly affected by preincubation of human PBMCs with laminarin (Table 2).

Human monocyte-derived macrophages differentially uptake *Candida* spp.

Next, to assess whether other cellular players of the innate immunity differentially interact with the analyzed *Candida* species and thus affecting the recognition and phagocytosis, we evaluated the ability of human monocyte-derived macrophages to uptake the yeast cells, using a flow cytometry-based protocol that has been used previously to characterize the phagocytic process of fungal cells.[37,51,59] Yeast cells were labeled with acridine orange, which emits a green fluorescence that turns reddish once they are in an acidified microenvironment of the mature phagolysosomes.[37,59] Results given in Figure 5 shows that most of the fungal species were uptaken and internalized into acid phagolysosomes, but the number of fungal cells undergoing this process was significantly higher for *C. tropicalis*, *C. guilliermondii*, and *C. krusei* than for *C. albicans* and *C. auris*, which showed a similar behavior when interacting with these human cells. Similar results were observed for green cells associated to macrophages, ie, recently phagocyted, with low cell numbers associated to *C. albicans* and *C. auris* and relatively high cell numbers associated to *C. tropicalis*, *C. guilliermondii*, and *C. krusei* (Figure 5). Collectively, these data indicate a differential ability of human monocyte-derived macrophages to uptake *Candida* spp. cells.

Discussion

C. albicans is a model organism popular to study fundamental aspects of a human fungal pathogen, and a vast amount of information about its biology is currently available. Because of their taxonomical classification, it is usual to find literature assuming that most of the members of the *Candida* genus should have biological traits like those described in *C. albicans*, with minimal differences in the phenotype or the molecular determinants related to virulence.[41] Nonetheless, the genomic evidence clearly suggests that these organisms have significant differences in the fitness and metabolic processes,[60] which are likely to affect the interaction with the host's components, such as the immune system. The comparative analysis performed here underscored the fact that even though the species under study had a similar composition of the cell wall, they have differences that are likely to affect the interaction with components of the innate immunity such as PPRs. As a proof of the concept, we have the case of *C. guilliermondii* cells, which had lower levels of β1,3-glucan and as a consequence, they induced the lowest levels of cytokines when this polysaccharide was exposed on the cell surface. Since the protein content in the *C. guilliermondii* cell wall was similar to the one obtained from the other species under analysis, we hypothesized that the increment in both *N*- and *O*-linked mannans in the cell wall was related to the different variety of cell wall proteins that are likely to contain more sites of these posttranslational modifications. Supporting this notion, a different cell secretome has been predicted for *C. guilliermondii*, which may affect the kind of proteins associated with the wall.[61] The lower *C. krusei* cell wall protein content fits with the low mannan levels, which is likely to be the reason behind this observation. In agreement, the phosphomannan content in this species was also low. The high porosity of the cell wall has been associated with mannans containing short lateral chains;[41,46,47] therefore, it is likely this will be the case of mannans on the surface of *C. tropicalis*, *C. guilliermondii*, and *C. krusei*.

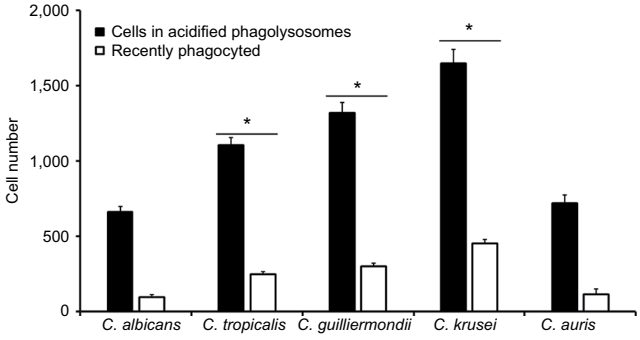

Figure 5 Phagocytosis of *Candida albicans*, *Candida tropicalis*, *Candida guilliermondii*, *Candida krusei*, and *Candida auris* by human monocyte-derived macrophages.

Notes: Acridine orange-labeled yeast cells were incubated with the human cells at an MOI ratio of 1:6 for 2.5 hours at 37°C under a CO_2 atmosphere. Then, macrophages were gated by FACS system and 50,000 cells were counted/sample. Results represent macrophages interacting with at least one green fluorescent cell (recently phagocyted), and those associated with red fluorescence that were classified as macrophages with yeast cells within acidified phagolysosomes. The data represent the mean ± SD of three independent biological replicates performed in duplicate. * $P<0.05$, when compared with *C. albicans* cells.

Abbreviations: MOI, multiplicity of infection; FACS, fluorescence-activated cell sorter.

Results presented here clearly demonstrate that in an in vitro setting, the human PMBCs are differentially stimulated to produce cytokines by the analyzed *Candida* species. It was interesting to observe that *C. albicans* and *C. auris* had similar abilities to interact with these immune cells and with monocyte-derived macrophages, even though they are species relatively distant in the phylogeny.[62] Thus far, only the interaction of *C. auris* with neutrophils has been studied, and differences in the ability to evade the neutrophil attack have been documented.[34] Nonetheless, our results reported here suggest that if escaped from interaction with these immune cells, the next line of defense, the mononuclear cells and macrophages are likely to control this pathogen. Further experiments are required to confirm this hypothesis.

Live cells of *C. tropicalis*, *C. guilliermondii*, and *C. krusei* stimulated higher cytokine levels than *C. albicans* cells, which might be related to the increased wall porosity. In addition, these three species showed different wall phosphomannan levels that might also contribute to this observation. It was interesting to note that upon removal of *O*-linked mannans from live cells from these three species, the ability to stimulate cytokine production changed, which in some cases was comparable to the quantified value when HK cells were used for immune stimulation. These data suggest that *O*-linked mannans play a negative role during recognition of these cell walls by the human mononuclear cells, and once they are removed, relevant ligands for cytokine production are accessible for immune receptors. Here, we demonstrated that the pathway behind this observation requires dectin-1 engagement with β1,3-glucan. Similar observations have been reported for *Candida parapsilosis sensu lato* and *sensu stricto*,[41,46] and for the interaction of *C. albicans* with macrophages.[63]

The therapeutic strategies to control candidiasis are extremely limited when compared to the wide repertoire of drugs to treat bacterial infections, and this has negatively impacted the sensitivity of fungal cells to the currently available drugs. Moreover, some *Candida* species are naturally resistant to some antifungal drugs or have the ability to rapidly develop resistance, such as *Candida glabrata* and *C. kruseii*.[64,65] Therefore, it is imperative to expand the portfolio of antifungal drugs to treat candidiasis. An alternative approach to control these infections has been explored using immunomodulators such as β1,3-glucan. Administration of this cell wall polysaccharide primes PBMCs and monocytes, which acquire prolonged enhanced functional state mediated by epigenetic mechanisms.[66] Although these could lead to a promising strategy to help in the control of this disease, our data here demonstrated that this approach might not be beneficial for the treatment of infections caused by *C. guilliermondii*, as this species does not induce a strong cytokine production via engagement of dectin-1 with β1,3-glucan.

Since the *O*-linked mannans of the cell wall of the species studied here showed a masking effect on the β1,3-glucan exposure at the cell surface and therefore precluded a proper activation of the cytokine production via dectin-1, it is tempting to speculate that inhibitors of the *O*-linked mannosylation pathway may have a positive effect on controlling infections caused by these fungal species.

The evaluation of the phagocytic process by human monocyte-derived macrophages pointed out again that *C. tropicalis*, *C. guilliermondii*, and *C. krusei* are species with a different ability to interact with these immune cells when compared to *C. albicans*. We have recently demonstrated that *C. tropicalis* is more phagocytosed than *C. albicans* by a phosphomannan-dependent mechanism,[37] and results reported here confirmed this previous observation. Since *C. guilliermondii* also showed higher phosphomannan content than *C. albicans* cells, it is tempting to offer a similar explanation for this observation. Although we do not currently have a proper explanation for data generated with *C. krusei*, a similar observation for this species and *C. guilliermondii* has been reported when interacting with murine macrophages.[28]

Conclusion

In conclusion, our study shows that the current knowledge about *C. albicans* cell wall and its interaction with innate immune cells cannot be extrapolated to *C. tropicalis*, *C. guilliermondii*, *C. krusei*, and *C. auris*. These species have differences in the cell wall composition and with exception of *C. auris*, displayed different abilities to stimulate cytokine production by human PBMCs and to be phagocytosed by human monocyte-derived macrophages.

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Disclosure

The authors report no conflicts of interest in this work.

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