Caspofungin Induced Cell Wall Changes of Candida Species Influences Macrophage Interactions

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Candida species are known to differ in their ability to cause infection and have been shown to display varied susceptibilities to antifungal drugs. Treatment with the echinocandin, caspofungin, leads to compensatory alterations in the fungal cell wall. This study was performed to compare the structure and composition of the cell walls of different Candida species alone and in response to caspofungin treatment, and to evaluate how changes at the fungal cell surface affects interactions with macrophages. We demonstrated that the length of the outer fibrillar layer varied between Candida species and that, in most cases, reduced fibril length correlated with increased exposure of β-1,3-glucan on the cell surface. Candida glabrata and Candida guilliermondii, which had naturally more β-1,3-glucan exposed on the cell surface, were phagocytosed significantly more efficiently by J774 macrophages. Treatment with caspofungin resulted in increased exposure of chitin and β-1,3-glucan on the surface of the majority of Candida species isolates that were tested, with the exception of C. glabrata and Candida parapsilosis isolates. This increase in exposure of the inner cell wall polysaccharides, in most cases, correlated with reduced uptake by macrophages and in turn, a decrease in production of TNFα. Here we show that differences in the exposure of cell wall carbohydrates and variations in the repertoire of covalently attached surface proteins of different Candida species contributes to their recognition by immune cells.

Keywords: chitin, β-1,3-glucan, GPI-anchored proteins, fungal cell wall, echinocandin, macrophages, immune response

INTRODUCTION

Candida species differ in their ability to cause infection. Candida albicans is the most common cause of Candida bloodstream infections (40%), followed by Candida glabrata (29%), Candida parapsilosis (11%), Candida tropicalis (4%), Candida dubliniensis (2%), and Candida lusitaniae (<1%) (Data captured from England; Health Protection Report, 2018). Candida species also have varied susceptibilities to antifungal drugs. The echinocandins act by specifically inhibiting the synthesis of β-1,3-glucan in the fungal cell wall. The inhibition of β-1,3-glucan synthesis occurs predominantly through inhibition of the catalytic Fks glucan synthase subunits (Kurtz and Douglas, 1997). Caspofungin is one of the most widely used of the echinocandins in the clinic and has fungicidal activity against the majority of Candida species. C. lusitaniae, C. parapsilosis, and Candida guilliermondii are known to have relatively reduced susceptibility compared to C. albicans and in recent years the incidence of clinical isolates of C. glabrata, which have acquired resistance...
to the echinocandins has increased (Garcia-Effron et al., 2010; Pfaller et al., 2012). Alarming echinocandin-resistant *C. glabrata* isolates (up to 38%) were also cross-resistant to fluconazole (Pfaller et al., 2012, 2013). Acquired resistance is predominantly mediated by point mutations within hotspot regions in the *FKS* genes (Park et al., 2005; Balashov et al., 2006; Garcia-Effron et al., 2010; Alexander et al., 2013; Pham et al., 2014; Marti-Carrizosa et al., 2015).

The fungal cell wall determines cell shape, maintains cell wall integrity and is recognized by the innate immune system. The cell walls of *Candida* spp. in general are composed of an inner core of chitin and β-1,3-glucan, which is covered by an outer layer of cell wall proteins, the majority of which are covalently linked to β-1,6-glucan by modified glycosylphosphatidylinositol (GPI) anchors (Gow et al., 2017). The cell wall is a dynamic structure which alters its composition in response to cell wall stress by upregulating genes involved in cell wall synthesis, in an attempt to restore the robustness of the cell wall (Walker et al., 2008). Treatment of *C. albicans* with caspofungin has been shown to lead to a compensatory increase in chitin content, in *vitro* and in *vivo* (Walker et al., 2008; Lee et al., 2012). This compensatory increase in chitin is not specific to *C. albicans* as *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, and isolates of *C. krusei* also demonstrated an elevation in chitin content in response to caspofungin treatment (Walker et al., 2013). In addition, isolates of *C. albicans*, *C. krusei*, *C. parapsilosis*, and *C. guilliermondii*, which have increased chitin content are less susceptible to caspofungin (Walker et al., 2008, 2013). *C. albicans* cells with elevated chitin contents have also been shown to be less susceptible to caspofungin in a murine model of systemic infection (Lee et al., 2012).

Putative GPI-modified cell wall proteins have been implicated in susceptibility to caspofungin as deletion of specific proteins leads to alterations in cell wall composition and subsequently to differences in susceptibility to caspofungin (Plaine et al., 2008). As a result of the cell wall remodeling that occurs in response to caspofungin treatment, chitin and β-1,3-glucan also become more exposed on the cell surface (Walker and Fink, 2006; Wheeler et al., 2008; Mora-Montes et al., 2011).

The fungal cell wall plays an important role in immune recognition as it is the first point of contact between the host and pathogen. The main innate immune cells that are involved in the recognition of invading pathogens are neutrophils, monocytes and macrophages (Neta et al., 2008). Components of the cell wall act as pathogen associated molecular patterns (PAMPs), which are recognized by pattern recognition receptors (PRRs) on host cells (Brown and Gordon, 2001; Porcaro et al., 2003; Kohatsu et al., 2006; McGreal et al., 2006; Netae et al., 2006, 2008). The two main classes of PRRs are the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs). The TLRs recognize phospholipomannan and O-linked mannan, whereas the C-type lectin receptors recognize β-1,3-glucan and mannan (Stahl et al., 1978; Wileman et al., 1986; Jouault et al., 2003; McGreal et al., 2006; Neta et al., 2006, 2008; Sato et al., 2006; Gow et al., 2007; Taylor et al., 2007). The increased exposure of chitin and β-1,3-glucan on the cell surface of *C. albicans* in response to caspofungin treatment results in altered cytokine production by immune cells, indicating that remodeling of the cell wall influences interactions with host cells (Wheeler and Fink, 2006; Wheeler et al., 2008; Mora-Montes et al., 2011; Balthc et al., 2012a,b; Fidan et al., 2014). Caspofungin treatment of *C. albicans* cells, followed by UV inactivation led to increased recognition of fungal cells by the C-type lectin, Dectin-1, which in turn increased cytokine production (Wheeler and Fink, 2006; Wheeler et al., 2008). In contrast, increased exposure of chitin on the surface of *C. albicans* has been shown to result in reduced cytokine production (Mora-Montes et al., 2011). Naturally occurring variations in the cell walls of different *C. albicans* isolates influences the dependency on dectin-1 for recognition and clearance of fungal cells (Marakalala et al., 2013). In addition the role of dectin-1 in recognition of four different *Candida* species, with differences in their cell wall carbohydrate composition, also varies (Thompson et al., 2019).

Because different *Candida* species vary in their ability to cause infection and vary in their cell wall composition, we aimed to perform a detailed comparison of the cell wall architectures of seven most prevalent *Candida* spp. and examine how their surface carbohydrates and proteomes change in response to caspofungin (CSF) treatment. In addition, the effect of caspofungin-induced cell wall changes of the different *Candida* species on the response of host immune cells was investigated.

### MATERIALS AND METHODS

#### Strains, Media, and Growth Conditions

The strains of each *Candida* species that were used in this study had been typed and their genomes sequenced, these strains are listed in Table 1. Strains were maintained on solid YPD medium (1% (w/v) yeast extract, 2% (w/v) mycological peptone, 2% (w/v) glucose, 2% (w/v) agar). RPMI-1640 medium (Gibco, Paisley, UK) was used for drug susceptibility testing and for growing cultures for HPLC and proteomic analysis.

| Species              | Strain name | Caspofungin IC50 (µg/ml) | References                      |
|----------------------|-------------|--------------------------|--------------------------------|
| *Candida albicans*   | SC5314      | 0.032                    | (Gillum et al., 1984)           |
| *Candida dubliniensis* | CD36     | 0.064                    | (Sullivan et al., 1996)        |
| *Candida glabrata*   | ATCC22001   | 0.064                    | American Type Culture Collection |
| *Candida tropicalis* | MYA-3404    | 0.125                    | American Type Culture Collection |
| *Candida lusitanae*  | ATCC42720   | 0.5                      | American Type Culture Collection |
| *Candida parapsilosis* | ATCC22019 | 1                        | American Type Culture Collection |
| *Candida guilliermondii* | ATCC8260 | 8                        | American Type Culture Collection |

*As defined by broth microdilution testing. The IC50 was defined as the concentration of caspofungin that inhibited 50% growth of each *Candida* species when cultured in RPMI-1640 for 24 h at 37°C.*
Preparation of FITC-Stained Candida spp.

Staining of Candida spp. yeast cells with fluorescein isothiocyanate (FITC) was performed as previously described (Graham et al., 2006). Briefly, Candida spp. were grown in YPD in the presence or absence of an IC$_{50}$ concentration of caspofungin (Walker et al., 2013) in YPD at 30°C for 6 h. Candida spp. cells were washed twice with PBS and stained for 10 min at room temperature in the dark with 1 mg/ml fluorescein isothiocyanate (FITC) (Sigma, Dorset, United Kingdom) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) (BDH Chemicals, VWR International, Leicestershire, United Kingdom). Cells were then washed three times in 1x PBS to remove residual FITC and were resuspended in 1x PBS. FITC-stained 6x$10^7$ Candida spp. cells that had been pre-grown with or without IC$_{50}$ caspofungin were then added to the macrophages at a multiplicity of infection (MOI) of 3 Candida spp. cells per macrophage, and incubated at 37°C for 4 h before visualization.

Phagocytosis and Cytokine Assays

Macrophage Cell Culture

J774.1 murine macrophages (European Collection of Cell Culture) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Lonza Group, Ltd., Braine-l’Alleud, Belgium), supplemented with 10% (vol/vol) fetal calf serum, 2% (wt/vol) penicillin and streptomycin antibiotics (Invitrogen, Ltd., Paisley, United Kingdom) in tissue culture flasks (Nalge Nunc, International, Hereford, United Kingdom) at 37°C and 5% (vol/vol) CO$_2$. For phagocytosis assays, 2 x $10^5$ J774.1 macrophages in 2 ml supplemented DMEM medium were seeded onto glass-based imaging dishes [Imaging dish CG 1.0; MACS Miltenyi Biotec (130-098-282), Surrey, UK] and incubated at 37°C with 5% CO$_2$ for at least 2 h to allow for macrophage adherence to the dish. Immediately prior to experiments, DMEM medium was replaced with 2 ml pre-warmed supplemented CO$_2$-independent medium (Gibco, Invitrogen, Paisley, UK) and cultures used immediately for phagocytosis assays.

After 4 h of co-incubation 50 µl of supernatant was removed from phagocytosis assays to allow determination of TNFα production from J774 macrophages as a result of incubation with each Candida species. TNFα concentrations were determined using enzyme-linked immunosorbent assays (R&D Systems) according to the manufacturer’s instructions.

Cell Wall Staining of Exposed Chitin and Glucan

Candida spp. yeast cells were grown in YPD in the presence or absence of an IC$_{50}$ concentration of caspofungin in YPD at 30°C for 6 h. Cells were stained with 5 µg/ml Fc:Dectin1 protein to visualize exposed β-1,3-glucan, as previously described (52). Briefly, 1 x $10^6$ cells/sample were blocked with FACS block (0.5% BSA, 5% HI-rabbit serum, 5 mM EDTA, 2 mM NaAzide in PBS) for 30 min. Cell pellets were harvested and washed 3 x with 1 ml FACS wash (0.5% BSA, 5 mM EDTA, 2 mM NaAzide in PBS) at 4°C. After washing, the cell pellet was resuspended in 100 µl of 5 µg/ml Fc:Dectin1 protein (diluted from stock in FACS block) and incubated for 1 h on ice. Cells were then washed 3 times with 1 ml FACS wash and resuspended in 200 µl FACS block plus 1/200 anti-human Fc+Alexa-488 and incubated for 45 min on ice. After incubation cells were washed three times with 1 ml FACS wash and fixed with 200 µl 1% formaldehyde. After fixing cells were also stained with 25 µg/ml Wheat Germ Agglutinin conjugated to Texas Red (WGA-TR). All samples were examined by DIC and fluorescence microscopy using a Zeiss Axiosplan 2 microscope. Images were recorded digitally using the Openlab system (Openlab v 4.04, Improvision, Coventry, UK) and a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics, Hamamatsu, Hertfordshire, UK). In all experiments the exposure time for a series of fluorescence images was fixed so the intensity of fluorescence relative to a control was used as standard. Chitin and β-1,3-glucan exposure were measured by quantifying WGA-TR and Fc:Dectin1-488 fluorescence, respectively, of individual yeast cells (Walker et al., 2008). Mean fluorescence intensities were calculated for 50 individual cells of each Candida species, for each condition.

High-Pressure Freezing (HPF)-Transmission Electron Microscopy (TEM)

Candida spp. yeast cells were grown in YPD at 30°C for 6 h and HPF was carried out as described previously (Walker et al., 2010) with the following modifications. Briefly, samples were prepared by high-pressure freezing with an EMPACT2 high-pressure freezer and rapid transport system (Leica Microsystems Ltd., Milton Keynes, United Kingdom). After freezing, cells were freeze-substituted in substitution reagent (1% [wt/vol] OsO$_4$ in acetone) with a Leica EMAFS2. Samples were then embedded in Epoxy resin and additional infiltration was provided under a vacuum at 60°C before embedding in Leica FSP specimen containers and polymerizing at 60°C for 48 h. Semithin survey sections, 0.5 µm thick, were stained with 1% toluidine blue to identify areas containing cells. Ultrathin sections (60 nm) were prepared with a Diatome diamond knife on a Leica UC6 ultramicrotome and stained with uranyl acetate and lead citrate for examination with a Philips CM10 transmission microscope (FEI UK Ltd., Cambridge, United Kingdom) and imaging with a Gatan Bioscan 792 (Gatan United Kingdom, Abingdon, United Kingdom). Image J was used to measure the thickness of the inner (chitin and glucan) and outer cell wall by averaging measurements for 30 cells, for each condition.

Antifungal Susceptibility Testing

CSF (Merck Research Laboratories, New Jersey, USA) minimum inhibitory concentrations were determined by broth micro-dilution testing using the CLSI guidelines M27-A3. Drug concentrations ranged from 0.016 to 16 µg/ml CSF. Exponentially grown cultures were diluted to 2 x $10^6$ cells/ml in 2x RPMI-1640 and 100 µl of culture was added to each well. Plates were incubated for 24 h at 37°C. After incubation, optical densities were read in a VERSAmax tunable microplate reader (Molecular Devices, California, USA) at 405 nm.

HPLC Analysis of Cell Wall Composition

Cell walls were extracted as described previously (Mora-Montes et al., 2011). Briefly, each Candida species was grown in
RPMI-1640 for 3 h at 37°C with shaking at 200 rpm. Each Candida species was then subsequently treated with a sub-MIC concentration of CSF (as determined from Table 1) for an additional 2 h, or grown without addition of drug for a further 2 h, as an untreated control. Cells were collected by centrifugation at 4,000 g for 5 min, washed once with chilled deionized water, resuspended in deionized water, and physically fractured with glass beads in a FastPrep machine (Qbiogene). The lysed cells were collected by centrifugation at 4,000 g for 5 min. The pellet containing the cell debris and walls was washed five times with 1 M NaCl, resuspended in extraction buffer (500 mM Tris-HCl buffer, pH 7.5, 2% [wt/vol] SDS, 0.3 M β-mercaptoethanol, and 1 mM EDTA), boiled at 100°C for 10 min, and freeze-dried.

To quantify glucan, mannan, and chitin the cell walls were acid hydrolyzed as previously described (Lee et al., 2012). The hydrolyzed samples were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in a carbohydrate analyzer system from Dionex (Surrey, United Kingdom) as described previously (Plaine et al., 2008). The total concentration of each cell wall component was expressed as μg per mg of dried cell wall which was determined by calibration from the standard curves of glucosamine, glucose, and mannose monomers, and converted to a percentage of the total cell wall.

**Proteomic Analysis**

Analysis of cell wall proteins was as described previously (Dutton et al., 2014). Candida species were grown with or without CSF as described for the HPLC analysis above. Briefly, 2 mg of freeze dried cell wall was mixed with 0.5 M ammonium bicarbonate in water containing 3 mM dithiothreitol and heated at 60°C for 20 min. Iodoacetamide (30 μl of 55 mM stock solution) was then added, and the suspension was incubated in the dark, at 25°C for 10 min. Following addition of 30 μl of 20 mg/ml trypsin, the suspension was incubated at 37°C for 14 h and centrifuged at 14,000 x g for 10 min. The supernatant was freeze-dried and extracted with 10% formic acid, and peptides were purified using ZipTip mC18 pipette tips (Millipore) and dissolved in 0.1% formic acid.

Samples (3 μl) were injected into an LC-MS system which comprised an UltiMate 3000 LC instrument (Dionex Ltd., United Kingdom) fitted with a PepSwift monolithic poly(styrene-co-vinylbenzene) (PS-DVB) column (200 μm inside diameter [i.d.] by 5 cm; Dionex) coupled to an HCTultra ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) fitted with a low-flow nebulizer in the electrospray ionization (ESI) source and controlled by HyStar software (version 4.0; Bruker Daltonik). Peptides were separated at a flow rate of 2 μl/min using a linear gradient of 0–40% acetonitrile-water- formic acid (80:20:0.04) (solvent B) in water-acetonitrile-formic acid (97:3:0.05) (solvent A) over 40 min, followed by a 1 min column wash in 90% solvent B and a 12-min equilibration step in solvent A. MS/MS data (scan range, m/z 100–2,200; averages 2) were acquired in positive data-dependent AutoMS(n) mode using the esquireControl software program (version 6.2; Bruker Daltonik). Up to three precursor ions were selected from the MS scan (range, m/z 300–1,500; averages 3) in each AutoMS(n) cycle. Precursors were actively excluded after being selected twice within a 1-min window, and singly charged ions were also excluded. Peptide peaks were detected (maximum of 9,999 compounds above an intensity threshold of 50,000) and deconvoluted automatically using Data Analysis software (version 3.4; Bruker Daltonik). Mass lists in the form of Mascot Generic Format (*.mgf) files were created automatically and used as inputs to Mascot MS/MS ion searches via a local Mascot server (version 2.2; Matrix Science, London, United Kingdom) with a database built from sequence files available from Candida Genome Database (32). The Search parameters used were the following: enzyme = trypsin; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); mass values = monoisotopic; peptide mass tolerance = 1.5 Da; fragment mass tolerance = 0.5 Da; max missed cleavages = 1; instrument type = ESI-TRAP. Search results were displayed by Mascot after selection of the following parameters: standard scoring; require bold red; ion score or expect cutoff = 0.05.

**Statistical Analyses**

Results from independent replicate experiments are expressed as means ± SD. One-way ANOVA with a Dunnett’s post-hoc test was used for statistical analysis. Significance was determined as p < 0.05.

**RESULTS**

IC$_{50}$ of Candida spp to Caspofungin

The susceptibility of each of the sequenced Candida spp. strains, used in this study, to CSF was determined following the CLSI-M27-A3 guidelines. C. albicans, C. dublinensis, C. glabrata, and C. tropicalis were the most susceptible to CSF (Table 1). The IC$_{50}$ range for these susceptible species was between 0.032 and 0.064 μg/ml CSF (Table 1). C. lusitaniae and C. parapsilosis had CSF IC$_{50}$ measurements of 0.5 and 1 μg/ml, respectively, which was classified as intermediate susceptibility and C. guilliermondii was resistant to CSF (Table 1). Therefore, the order of susceptibility to CSF from most susceptible to most resistant of the isolates of the Candida spp. tested here was: C. albicans > C. dublinensis > C. glabrata > C. tropicalis > C. lusitaniae > C. parapsilosis > C. guilliermondii.

**Candida Species Have Differences in the Ultrastructure of Their Cell Wall**

Ultrastructural differences in the cell wall of each Candida species were investigated using high pressure freezing transmission electron microscopy (TEM) which conserves the fungal cell wall architecture. TEM analysis revealed that the inner core of the cell wall of C. glabrata (Figure 1A, v) and C. tropicalis (Figure 1A, ii) isolates was significantly thicker than the inner core of C. albicans isolate (Figure 1A, i; Figure 1B). In contrast to this the inner core of the C. guilliermondii cell wall (Figure 1A, vii) was significantly thinner, in comparison to C. albicans (Figure 1A, i; Figure 1B).

Overall, the largest difference in the cell wall between the Candida species was in the outer fibrillar layer (Figure 1B). The variations...
in length of the outer fibrillar layer were classified as either long (>65 nm), intermediate (30–65 nm), or short (<30 nm) (Figure 1B). *C. albicans* and *C. tropicalis* had the longest outer fibrillar layers, followed by *C. dubliniensis* and *C. lusitaniae* which were classified as having intermediate fibril length (Figure 1B). *C. glabrata*, *C. parapsilosis*, and *C. guilliermondii* isolates had short outer fibrils on the cell surface, in comparison to the *C. albicans* isolate (Figure 1B).

**Candida** Species Have Species Specific GPI-Anchored Proteins Present on Their Cell Surface

To determine the differences in the cell wall proteome between the different *Candida* species, LC-MS/MS was used to identify which covalently attached, predicted GPI-modified proteins were present on the surface of each *Candida* species when they were cultured in RPMI-1640 medium (Table 2). Here we
have annotated the proteins according to their *C. albicans* orthologs based on sequence similarity according to Candida Genome Database (Skrzypek et al., 2016). A core set of predicted cell wall proteins were identified in all species (Table 2). The hyphal specific protein, Hyr1, was uniquely detected in the cell wall of *C. albicans*, consensurate with it being a *C. albicans*-specific hyphal-associated protein and *C. albicans* will undergo filamentation in RPMI-1640 medium. GPI-anchored proteins that were identified specifically in the cell wall of *C. albicans* were: Utr2 (PGUG_00573), Pga30 (PGUG_01288), C. guilliermondii Pga31 (C4_04080C_A), Ecm33 (C1_03190C_A), Rbt1 (C4_03520C_A), Pga29 (C4_04050C_A), and Ywp1/Pga24, and Pga30, a protein of unknown function was found to contain orthologs of the yeast wall protein, Ywp1/Pga24, and Pga30, a protein of unknown function (Table 2).

Caspofungin treatment has been previously shown to alter cell wall architecture and increases chitin production in *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis* (9). The effect of CSF treatment on the cell wall proteome of each Candida species was also investigated by treating each Candida spp. with their IC\textsubscript{50} concentration of caspofungin (Table 3). Certain proteins were detected only at the cell surface in response to CSF treatment in addition to the proteins listed that are found on the surface of untreated cells. The proteins expressed in response to caspofungin varied between the different Candida spp. Orthologs of the transglycosidase, Utr2, were detected on the cell surface of the majority of *Candida* species in response to CSF treatment with the exception of *C. glabrata* (Table 3). Orthologs of Pga31, a GPI-anchored protein of unknown function was detected on the surface of the species that were most sensitive to CSF, such as *C. albicans*, *C. dubliniensis*, and *C. tropicalis* in response to CSF treatment (Table 3). A protein related to Pga30, a member of the same family as Pga31 was detected in the cell wall of *C. guilliermondii* treated with CSF. *C. lusitaniae* and *C. parapsilosis*, which have intermediate susceptibility to CSF, were found to have Als-like proteins on their cell surface in response to CSF treatment (Table 3).

### Cell Wall Composition of Different Candida Species in the Presence or Absence of CSF

The carbohydrate composition of the cell walls of the different *Candida* spp., in the presence or absence of an IC\textsubscript{50} concentration of caspofungin, was determined by acid hydrolysis of isolated cell walls and analysis by high-performance liquid chromatography. *C. tropicalis* had substantially higher chitin content than *C. albicans*, whereas *C. glabrata* and *C. guilliermondii* had significantly less chitin compared to *C. albicans* (Table 4). The majority of *Candida* spp. had comparable levels of glucan in the cell wall, with the exception of *C. tropicalis*, which had significantly reduced glucon compared to *C. albicans* and *C. glabrata*, which had a higher glucon content (Table 4). The quantity of mannan in the cell wall was significantly reduced in *C. glabrata*, *C. parapsilosis*, and *C. guilliermondii* compared

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**Table 2**: Candida species-specific detection of cell wall GPI-anchored proteins by LC/MS/MS proteomics.

| Species      | Cell wall proteins specific to CSF-treated cells |
|--------------|--------------------------------------------------|
| *C. albicans*| Utr2 (C3_01730C_A) Pga31 (C4_04080C_A) Ecml33 (C1_03190C_A) Rbt1 (C4_03520C_A) Pga29 (C4_04050C_A) |
| *C. dubliniensis* | Utr2 (CD36_81610) Pga31 (CD36_43780) Pga62 (CD36_43780) Pga30 (CD36_00220) |
| *C. lusitaniae* | Utr2 (CLUG_003306) Ecml33 (C1_03190C_A) Rbt1 (CLUG_03306) Pga31 (C1_03190C_A) |
| *C. parapsilosis* | Utr2 (CPAR2_503190) Pga31 (CPAR2_00220) Pga30 (CPAR2_00220) |
| *C. tropicalis* | Utr2 (CLUG_003306) Pga31 (CLUG_00242) Pga30 (CLUG_00242) |
| *C. guilliermondii* | Utr2 (CAGL0M08492g) Pga31 (CAGL0G09449g) Pb1 (CAGL0G09449g) |

**Table 3**: Caspofungin treatment specific proteins in different Candida species in RPMI-1640 medium.

| Species      | Cell wall proteins specific to CSF-treated cells |
|--------------|--------------------------------------------------|
| *C. albicans*| Utr2 (C3_01730C_A) Pga31 (C4_04080C_A) Ecml33 (C1_03190C_A) Rbt1 (C4_03520C_A) Pga29 (C4_04050C_A) |
| *C. dubliniensis* | Utr2 (CD36_81610) Pga31 (CD36_43780) Pga62 (CD36_43780) Pga30 (CD36_00220) |
| *C. lusitaniae* | Utr2 (CLUG_003306) Ecml33 (C1_03190C_A) Rbt1 (CLUG_03306) Pga31 (C1_03190C_A) |
| *C. parapsilosis* | Utr2 (CPAR2_503190) Pga31 (CPAR2_00220) Pga30 (CPAR2_00220) |
| *C. tropicalis* | Utr2 (CLUG_003306) Pga31 (CLUG_00242) Pga30 (CLUG_00242) |
| *C. guilliermondii* | Utr2 (CAGL0M08492g) Pga31 (CAGL0G09449g) Pb1 (CAGL0G09449g) |
to *C. albicans* (Table 4) in agreement with the TEM analysis which suggested these species have shorter mannan fibrils on their outer surfaces. In most cases treatment with CSF resulted in an increase in chitin content, with the exception of *C. glabrata* and *C. parapsilosis* (Table 4). As expected all *Candida* spp. isolates tested had a reduction in β-1,3-glucan in response to CSF treatment. Exposure to an IC<sub>50</sub> concentration of CSF led to a significant increase in mannan content in all *Candida* spp., with the exception of *C. tropicalis* and *C. dubliniensis* isolates (Table 4).

### Relationship Between Phagocytosis and Exposure of Chitin and Glucan

To determine the effect of exposure of chitin and glucan on phagocytosis, the uptake of isolates of each *Candida* species by J774 macrophages was determined. The yeasts were cultured with and without sub-MIC CSF treatment and phagocytosis correlated to the exposure of chitin and β-1,3-glucan on the cell surface (Figure 2, Supplementary Table 1). The majority of *Candida* species had low levels of chitin and β-1,3-glucan exposed naturally on the cell surface, under the growth conditions used (Figure 2A). The exception was *C. glabrata* and *C. guilliermondii* which had significantly more β-1,3-glucan exposed on the cell surface, compared to *C. albicans*, which correlated with these *Candida* species having the highest phagocytosis by J774 macrophages (Figure 2A). To determine the effect of altered exposure of β-1,3-glucan and chitin, the phagocytosis of CSF-treated cells was also measured (Figure 2B). In all cases the combined increase in exposure of chitin and β-1,3-glucan, in CSF-treated cells, resulted in a significant decrease in phagocytosis by macrophages (Figure 2B). Treatment of *C. glabrata* and *C. parapsilosis* with CSF had little effect on the exposure of the outer cell wall components and consequently there was no significant change in their phagocytosis by macrophages (Figure 2B). Pearson Correlation analysis demonstrated that there was a positive correlation between glucan exposure and chitin exposure and caspofungin treatment \( r_{(12)} = 0.703, p = 0.005 \); \( r_{(12)} = 0.699, p = 0.005 \) respectively but a negative correlation between % phagocytosis and caspofungin treatment \( r_{(12)} = -0.763, p = 0.002 \).
To determine the cytokine production stimulated by the different Candida species, TNF-α production was measured when untreated live cells of each Candida species were exposed to J774 macrophages for 4 h (Figure 3). Live cells of C. albicans and C. dubliniensis stimulated the highest levels of TNF-α production (Figure 3). The other Candida species all stimulated very low levels of TNF-α (Figure 3), which remained unchanged even after 24 h (data not shown). Treatment with an IC_{50} concentration of CSF, specific to each Candida species, led to a significant decrease in TNF-α production in C. albicans and C. dubliniensis, compared to untreated cells (Figure 3). Treatment with CSF had no effect on TNF-α production in the other Candida species isolates, with the exception of the C. tropicalis isolate which had a significant increase in TNF-α production compared to untreated cells (Figure 3). C. tropicalis also had the highest level of surface-exposed β-(1,3)-glucan when treated with CSF.

### DISCUSSION

This study aimed to directly compare the structure and composition of the cell walls of different Candida species, examine how the cell wall was altered in response to caspofungin treatment and assess how this impacted on fungal: murine macrophage interactions. We demonstrated that the length of the outer fibrillar layer varied between the species isolates tested and that, in most cases, reduced fibril length correlated with increased exposure of β-(1,3)-glucan on the cell surface. C. glabrata and C. guilliermondii, which had naturally more β-(1,3)-glucan exposed on the cell surface, were phagocytosed more efficiently by J774 macrophages. This is in agreement with a previous study that showed that in a mixed population of C. albicans and C. glabrata, C. glabrata is preferentially phagocytosed over C. albicans (Kepler-Ross et al., 2010). Similar to our observations, Estrada-Mata et al. (2016) also demonstrated that C. parapsilosis had less mannan content but more β-(1,3)-glucan exposed on the surface compared to C. albicans which correlated with an increase in cytokine production by human PBMCs (Estrada-Mata et al., 2016). Dectin-1 has been shown to play an important role in recognition of C. parapsilosis by human PBMCs (Toth et al., 2013). In our study C. parapsilosis induced little cytokine production from J774 macrophages at 4 h, but differences in the source of immune cells and the time that Candida cells were exposed to immune cells before performing the ELISAs may account for these differences.

There were also notable differences in the cell wall proteins that reside on the surface of the different Candida species, for example, the GPI-anchored proteins, Epα3, Epα6, and Awp4, were identified specifically on the cell wall of C. glabrata under the growth conditions tested. These are known to be adhesion proteins in C. glabrata which are associated with biofilm formation (de Groot et al., 2008; Kraneveld et al., 2011). The presence of cell wall proteins on the cell surface of C. glabrata, C. parapsilosis, and C. tropicalis has been shown to be dependent on the growth conditions tested (Karkowska-Kuleta et al., 2019).

Treatment with CSF is known to result in a compensatory increase in cell wall chitin levels and result in increased exposure of chitin and β-(1,3)-glucan on the surface of C. albicans cells (Wheeler and Fink, 2006; Wheeler et al., 2008; Mora-Montes et al., 2011). All of the Candida species isolates tested, with the exception of C. glabrata and C. parapsilosis isolates, demonstrated a compensatory increase in cell wall chitin content in response to CSF treatment. Likewise, treatment with CSF also resulted in an increase in exposure of chitin and β-(1,3)-glucan on the cell surface for the majority of species tested. Notable exceptions were C. glabrata and C. parapsilosis with no changes in glucan exposure in response to caspofungin treatment. This increase in exposure of the inner cell wall polysaccharides did not increase the number of macrophages that had engulfed the fungal cells in fact caspofungin treatment resulted in a reduction in % phagocytosis.

### Table 5

| Candida species | Glucan exposure (mean fluorescence intensity) | Chitin exposure (mean fluorescence intensity) |
|-----------------|---------------------------------------------|---------------------------------------------|
|                 | No treatment | Caspofungin treatment | No treatment | Caspofungin treatment |
| C. albicans     | 179 ± 20     | 450 ± 34*            | 167 ± 17     | 488 ± 51*            |
| C. dubliniensis | 211 ± 14     | 374 ± 52*            | 160 ± 26     | 404 ± 40*            |
| C. glabrata     | 273 ± 21*    | 257 ± 39             | 89 ± 18*     | 79 ± 20              |
| C. tropicalis   | 185 ± 41     | 607 ± 66*            | 178 ± 22     | 518 ± 54*            |
| C. krusei      | 171 ± 29     | 468 ± 49*            | 159 ± 31     | 438 ± 35*            |
| C. parapsilosis | 201 ± 13     | 189 ± 30             | 172 ± 28     | 202 ± 45             |
| C. guilliermondii | 263 ± 36* | 537 ± 72*            | 160 ± 29     | 445 ± 49*            |

*The average relative β-(1,3)-glucan and chitin contents of individual cells from different Candida species were determined by measuring the intensity of Fc:Dectin1–Alexa488 and WGA-Texas Red fluorescence. Measurements were made on untreated control cultures and after growth with caspofungin at the specific IC_{50} for each species (as determined in Table 1). Statistical differences are shown for comparison to untreated cells of C. albicans (*P = 0.05) or untreated cells of the same species (†P = 0.05), and data are means with standard deviations (n = 50).
FIGURE 2 | Correlation between exposure of chitin and glucan on the cell surface of Candida species and phagocytosis by J774 macrophages. The values for exposure of chitin and β-1,3-glucan were taken from Table 5. Phagocytosis of Candida spp. by macrophages was determined by fluorescence microscopy. All cells were stained with 10 µg/ml FITC prior to exposure to macrophages. FITC-stained cells were exposed to J774 macrophages for 1 h, after which non-phagocytosed cells were stained with 25 µg/ml CFW. The number of macrophages which had phagocytosed Candida cells were then counted (n = 300). (A) Untreated Candida spp. cells. (B) CSF treated Candida cells. Black shapes = chitin (WGA-TR), gray shapes = glucan (Fc:dectin1-alexa488).

The majority of live Candida spp. cells stimulated low levels of TNFα production. In comparison the C. albicans and C. dublinensis isolates stimulated elevated levels of TNFα but these levels were significantly reduced when macrophages were exposed to the same isolates after caspofungin treatment. A notable exception to this trend was CSF-treated C. tropicalis isolate which stimulated significantly elevated TNFα production compared to untreated cells attaining a level comparable to C. albicans and C. dublinensis. C. tropicalis had the highest level of Fc-dectin1-Alex488 staining with and without caspofungin treatment. These findings suggested that the immune stimulatory properties of the different Candida species is not solely dependent upon β-1,3-glucan exposure and recognition by Dectin-1 and other factors may come into play (Wagener et al., 2014). Recently, treatment with either fragments of chitin or β-1,3-glucan from Aspergillus fumigatus were shown to stimulate production of TNFα. In addition, combined treatment with both chitin and β-1,3-glucan resulted in a synergistic increase in TNFα production (Dubey et al., 2014). This is in contrast with the findings here where other changes at the cell surface including increased chitin exposure appeared to override any stimulation of the immune response elicited by glucan exposure. The differences in stimulation of the immune response may be due to variations in size of the cell wall polysaccharides and the way the polymer is presented to immune cells. Small particles of chitin are known to illicit a greater cytokine response compared to larger
Candida species stimulate different immune responses. Live cells of each Candida species, which had been grown with or without an IC_{50} concentration of caspofungin for 6 h, were exposed to J774 macrophages for 4 h. After 4 h of co-incubation, supernatants were collected and cytokine production was measured. Significant differences (#p < 0.05) compared to C. albicans. Significant differences (*p < 0.05) compared to untreated cells of the same species. Error bars are SD (n = 3).

Chitin fragments (Da Silva et al., 2009). Likewise, differences in polysaccharide structure may influence the immune response. The chitin microfibrils of A. fumigatus and C. albicans may have a different architecture, which could influence the immune response (Gow and Gooday, 1983; Lenardon et al., 2007). Recently, hyphal β-1,3-glucan of C. albicans was found to have a unique cyclical structure that is absent in β-1,3-glucan from the yeast form (Lowman et al., 2014). Compared to β-1,3-glucan from yeast cells, hyphal glucan was shown to induce an immune response in macrophages and human peripheral blood mononuclear cells through a Dectin-1-dependent mechanism. In addition CSF treatment has also been shown to not only effect β-1,3-glucan exposure but also its nano-structure with the density of dectin-1 binding sites increasing leading to increased phagocytosis by dendritic cells (Lin et al., 2016). To dissect further whether glucan and/or chitin plays the main role in caspofungin-mediated dampening of the immune response, it would be interesting to evaluate the effect of blocking receptors of both polysaccharides on interaction with macrophages. Another consideration is that the change in GPI-modified proteins on the cell surface, in response to CSF treatment, may also influence the interaction with immune cells. For example, the transglycosidase, Utr2, was detected on the surface of the majority of Candida species in response to CSF treatment. Utr2 is involved in crosslinking chitin and β-1,3-glucan and forms part of a compensatory mechanism activated in response to cell weakening induced by echinocandin treatment (Pardini et al., 2006). A C. albicans utr2Δ mutant was found to have the same susceptibility to micafungin as the wild type (Alberti-Segui et al., 2004). Deletion of UTR2 in C. albicans resulted in attenuated virulence in a mouse model of systemic candidiasis. Despite this attenuation in virulence the fungal burdens from the kidneys of mice infected with the utr2Δ mutant were comparable to that of mice infected with wild-type C. albicans. This suggests that in the absence of UTR2, cells do not elicit the same inflammatory response as wild-type cells and enables the utr2Δ mutant to be tolerated by the host, leading to survival of the infected mice (Pardini et al., 2006). Therefore, the increased abundance of Utr2 on the cell surface in response to caspofungin treatment may also contribute to altered interactions with host cells. Candida guilliermondii and C. parapsilosis are known to be intrinsically less sensitive to the echinocandins. Both species had thin walls and their cell wall proteomes differed from CSF-susceptible species. Pga24/Ywp1 orthologs were detected on their cell surfaces when grown on RPMI-1640 medium. In C. albicans Pga24 is associated with the yeast cell wall (Granger et al., 2005) and here was not detected when C. albicans was grown on RPMI-1640 medium. Mutation of PGA24 did not alter echinocandin susceptibility of C. albicans (Plaine et al., 2008). The role of this surface protein in echinocandin susceptibility of C. guilliermondii and C. parapsilosis remains to be determined.

The ability of live cells of each isolate of the different Candida species to stimulate cytokine production varied significantly (Figure 3). C. albicans and C. dubliniensis isolates, tested here, elicited the highest production of TNFα. In contrast to this the other isolates representing the different Candida species
stimulated negligible levels of TNFα when exposed to J774 macrophages for 4 h. Previous work has demonstrated that C. parapsilosis, C. tropicalis, C. lusitaniae, and C. guilliermondii in addition to C. albicans and C. dubliniensis, stimulated production of TNFα from murine peritoneal macrophages and human PBMCs after 24 h (Aybay and Imir, 1996; Navarro-Arias et al., 2019). The most likely reason for the difference observed at the 4 h time point is that C. albicans and C. dubliniensis are the only two species which form true hyphae and were capable of germinating within macrophages, and consequently escaping from macrophages, faster than any of the other Candida species. For example, C. glabrata yeast cells have been shown to replicate within macrophages for up to 3 days before causing lysis of the macrophage (Seider et al., 2011). Therefore, the low levels of cytokine production that have been observed as a result of exposure of macrophages to live C. glabrata is thought to be due to C. glabrata evading host defenses by hiding within immune cells (Seider et al., 2011, 2014; Brunke et al., 2014). However, it is possible for C. glabrata to filament under certain conditions. Prolonged exposure of C. glabrata to macrophages led to filamentous growth which was a result of a point mutation in CHS2 (Lewis et al., 2013). Treatment of C. albicans and C. dubliniensis with CSF resulted in a decrease in production of TNFα and reduced phagocytosis by macrophages. Exposure of C. albicans to caspofungin has previously been shown to reduce cytokine production in both live and heat killed C. albicans cells, as a result of increased exposure of chitin on the cell surface (Mora-Montes et al., 2011).

Here we examined caspofungin induced changes at the cell surface of representative isolates of different Candida species. We detected drug-induced changes in the repertoire of covalently attached surface proteins and alterations in the exposure of cell wall carbohydrates of contributing to their recognition by J774 macrophages. The cell surface is highly variable both between and within species and so these investigations should be expanded to a wider range of isolates of the different species to determine the extent of cell surface variability and how it impacts on host interactions and drug susceptibility.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

LW and CM contributed to acquisition, analysis, and interpretation of data for the work. All authors contributed to the design of the work and approved the submitted and final version.

FUNDING

We acknowledge funding from the British Society for Antimicrobial Chemotherapy, the Medical Research Council (G0400284) and Wellcome Trust Strategic Award for Medical Mycology and Fungal Immunology 097377.

ACKNOWLEDGMENTS

We thank Gillian Milne from the University of Aberdeen Microscopy and Histology facility for help with EM, Dr. David Stead (Aberdeen Proteomics) for proteomics analysis and Prof. Gordon Brown for Fc:Dectin1. We thank Dr. Judith Bain and Prof. Lars Erwig for advice on the macrophage assays.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.00164/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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