

Novel Structural Features in *Candida albicans* Hyphal Glucan Provide a Basis for Differential Innate Immune Recognition of Hyphae Versus Yeast*

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Douglas W. Lowman^{‡§}, Rachel R. Greene^{‡¶}, Daniel W. Bearden^{||}, Michael D. Kruppa^{**}, Max Pottier^{‡‡}, Mario A. Monteiro^{‡‡}, Dmitriy V. Soldatov^{‡‡}, Harry E. Ensley[‡], Shih-Chin Cheng^{§§1}, Mihai G. Netea^{§§1}, and David L. Williams^{‡2}

From the [‡]Department of Surgery and ^{**}Department of Biomedical Sciences, Quillen College of Medicine, and [¶]Department of Chemistry East Tennessee State University, Johnson City, Tennessee 37614, [§]AppRidge International, LLC, Telford, Tennessee 37690, ^{||}National Institutes of Standards and Technology, Chemical Sciences Division, Hollings Marine Laboratory, Charleston, South Carolina 29412, ^{‡‡}Department of Chemistry, University of Guelph, Guelph, Ontario N1G 2W1, Canada, and ^{§§}Department of Internal Medicine and the Nijmegen Institute for Infection, Inflammation, and Immunity, Radboud University, 6525 GA Nijmegen, The Netherlands

Background: The human innate immune system can discriminate between *Candida albicans* yeast and hyphal forms.

Results: *C. albicans* hyphae possess glucan structures that are unique to the hyphae and are not found in yeast.

Conclusion: Hyphal glucan elicits robust immune responses.

Significance: These data provide a structural basis for differential immune recognition of *C. albicans* yeast versus hyphae.

The innate immune system differentially recognizes *Candida albicans* yeast and hyphae. It is not clear how the innate immune system effectively discriminates between yeast and hyphal forms of *C. albicans*. Glucans are major components of the fungal cell wall and key fungal pathogen-associated molecular patterns. *C. albicans* yeast glucan has been characterized; however, little is known about glucan structure in *C. albicans* hyphae. Using an extraction procedure that minimizes degradation of the native structure, we extracted glucans from *C. albicans* hyphal cell walls. ¹H NMR data analysis revealed that, when compared with reference (1→3,1→6) β-linked glucans and *C. albicans* yeast glucan, hyphal glucan has a unique cyclical or “closed chain” structure that is not found in yeast glucan. GC/MS analyses showed a high abundance of 3- and 6-linked glucose units when compared with yeast β-glucan. In addition to the expected (1→3), (1→6), and 3,6 linkages, we also identified a 2,3 linkage that has not been reported previously in *C. albicans*. Hyphal glucan induced robust immune responses in human peripheral blood mononuclear cells and macrophages via a Dectin-1-dependent mechanism. In contrast, *C. albicans* yeast glucan was a much less potent stimulus. We also demonstrated the capacity of *C. albicans* hyphal glucan, but not yeast glucan, to induce IL-1β processing and secretion. This finding provides important evidence for understanding the immune discrimination

between colonization and invasion at the mucosal level. When taken together, these data provide a structural basis for differential innate immune recognition of *C. albicans* yeast versus hyphae.

Candida albicans is a polymorphic fungal pathogen that can grow as a yeast, as pseudohyphae, or true hyphae (1). The ability of *C. albicans* to undergo morphogenic transformation from yeast to hyphae is a critical step in the pathogenicity of this opportunistic fungus (1). Specifically, yeast forms of *C. albicans* colonize the epithelium followed by hyphal penetration and invasion of the tissues (1). Epithelial invasion is followed by vascular dissemination, which involves hyphal penetration of blood vessels and seeding of the blood with yeast forms (1). Blood-borne *C. albicans* adhere to the vascular endothelium and form colonies followed by hyphal penetration into the tissues. Hyphae also play a role in biofilm development. Nett and Andes (2) have noted that the ability of *C. albicans* to form biofilms “has a profound impact” on the ability of the organisms to cause disease (3). Evidence also suggests that *Candida* biofilm development and maturation depends, in part, on yeast-to-hyphae transition (2). Despite the importance of *C. albicans* as a pathogen and the relevance of *C. albicans* hyphae to pathogenicity, there are very few reports on the structure and composition of *C. albicans* hyphal cell walls. Most of the published data has focused on ultrastructural analysis (electron microscopy) or immune responses to hyphae (1, 4).

We have shown that the innate immune system effectively discriminates between yeast and hyphal forms of *C. albicans* (4). We speculated that the differential recognition of *C. albicans* yeast versus hyphae is due to “differences in cell wall architecture” (4). Glucans are major structural components of the fungal cell wall (1, 5) and they are also major fungal pathogen-associated molecular patterns (6–8). The basic structure of

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² To whom correspondence should be addressed: Dept. of Surgery, East Tennessee State University, Campus Box 70575, Johnson City, TN 37614-0575. Tel.: 423-439-6363; Fax: 423-439-6259; E-mail: williamd@etsu.edu.

glucan has been extensively investigated (9–11). In general, glucans are composed of a polymer backbone containing (1→3)- β -D-linked anhydroglucose repeat units (12–14). Some, but not all, glucan polymers exhibit side chain anhydroglucose repeat units that branch from the 6-position of the backbone anhydroglucose repeat units (9–11). Most of the reports on the physicochemical/structural analysis of glucans have focused on glucans derived from yeast such as *Saccharomyces cerevisiae* and *C. albicans* (9–11). There are only a handful of reports describing the physicochemical and structural characterization of hyphal cell wall glucans (15–17).

We have determined that the method of extraction can impact the higher structure of the glucan (18). Therefore, we developed a “modified” extraction method that is much less harsh than methods previously described for the isolation of blastospore/hyphal cell wall carbohydrates. This extraction method minimizes degradation and maintains more of the native glucan structure found in the cell wall. Using this approach we compared and contrasted the structure of *C. albicans* blastospore and hyphal extracts. We discovered that glucan is a major component of the *C. albicans* hyphal cell wall and that hyphal glucan contains macromolecular structures that have never been previously identified and are unique to hyphal glucan, *i.e.* these structures are not present in *C. albicans* blastospore glucan. We also found that this unique hyphal glucan structure elicits functionally distinct cytokine induction profiles in human peripheral blood mononuclear cells and macrophages when compared with yeast glucan.

EXPERIMENTAL PROCEDURES

Ethics Statement—Human peripheral blood mononuclear cells (PBMCs)³ were obtained after written informed consent. This study has been approved by the Ethics Committee of the region Arnhem-Nijmegen under the number NL32357.091.10.

C. albicans Hyphae—Wild type strain SC5314 was grown at 30 °C on yeast extract/peptone/dextrose. For hyphal production, 5×10^5 cells/ml were inoculated into prewarmed medium 199, pH 7.5, and grown for 4 h for well developed hyphae at 37 °C. For hyphal growth, six 4-liter flasks containing 2.5 liters of M199 prewarmed to 37 °C were inoculated with 5×10^5 cells/ml of overnight grown *C. albicans*. Cells were grown for 4 h with gentle shaking (70 rpm). Fully developed hyphae were confirmed microscopically before harvesting each flask by filtration. Cells (15 liters) were harvested by filtration. The average yield of pooled hyphal mass was 10 g before lyophilization. Yeast were grown in 2 liters of yeast extract/peptone/dextrose in a 40-liter flask grown overnight at 30 °C and harvested by centrifugation for glucan extraction. Yeast morphology was confirmed microscopically before harvesting each flask by filtration. The average cell mass harvested was 10 g before lyophilization.

Glucan Isolation—Glucan was isolated from *C. albicans* hyphae or yeast using modifications of the methods of Lowman *et al.* (16, 19) and Mueller *et al.* (18). Briefly, hyphal or yeast cell walls were extracted with 0.1 N NaOH (1 \times for 15 min at 100 °C) followed by neutralization to pH 7.0. The neutral residue was extracted with 0.1 N H₃PO₄ (1 \times for 15 min at 100 °C). The residue was neutralized to pH 7.0. The majority of the lipids were removed from the hyphal cell wall with boiling absolute ethanol (1 \times for 15 min) as previously described (20). The glucan extractions from hyphal or yeast forms were performed in parallel using the same reagents and extraction apparatus, *i.e.* a multiposition heating mantle with stirring and temperature control. *S. cerevisiae* glucan was isolated and characterized as described by our laboratory (21). The glucans isolated using this approach are water-insoluble microparticulates (1–5 μ m). The particle distribution was similar between yeast and hyphal glucan. Protein was not detected in the glucans.

NMR Analysis—Proton and ¹³C NMR spectra were collected on a Bruker Avance II 800 NMR spectrometer using a 5-mm TCI inverse cryoprobe operating at 343 K. Approximately 25 mg of glucan was dissolved in 950 μ l of DMSO-d₆ (Sigma; “100”, p/n 156914, CAS 2206-27-1) with 40 μ l of trifluoroacetic acid-d (Cambridge Isotope Laboratories, 99.8+% deuterated, p/n DLM-46, CAS 599-00-8) to shift the exchangeable proton resonances downfield. Approximately 550 μ l of the solution was placed into a 5-mm NMR tube. Trifluoroacetic acid was added just before NMR analysis. Proton and ¹³C one-dimensional and COSY (22, 23), NOESY (24, 25) with a 150-ms mixing time, HSQC (26–28), HSQC-TOCSY (29, 30) with a 60-ms mixing time, and HMBC (31) two-dimensional NMR spectra were obtained in this study. For one-dimensional NMR experiments, chemical shift referencing used absolute referencing based on the ²H DMSO-d₆ lock signal and ¹H and ¹³C gyromagnetic ratios. For two-dimensional NMR experiments, chemical shift referencing was accomplished relative to the anomeric resonance of the (1→3)- β -linked backbone repeat unit at 4.54 ppm for ¹H and 102.49 for ¹³C relative to DMSO-d₆ residual protons as referenced above. One-dimensional and two-dimensional NMR spectra were collected and processed under conditions similar to those used previously (19). NMR spectra were processed using TOPSPIN 2.1 running on the Avance II 800 NMR and TOPSPIN 3.0.b.8 running on Windows XP Professional operating system under VMWare Fusion version 2.0.5 (VMWare, Inc., Palo Alto, CA) on a Macintosh MacBook Pro.

Monosaccharide Composition and Linkage Analysis—To analyze the sugar composition of the *C. albicans* hyphal glucan by GC-MS, the alditol acetate derivatives of the monosaccharides were analyzed (32). Polysaccharide was hydrolyzed in 4 M trifluoroacetic acid (TFA) for 4.5 h at 105 °C with frequent stirring followed by reduction with NaBD₄ in water overnight. The sample was then acetylated using acetic anhydride at 105 °C for 90 min. The alditol acetate derivatives were analyzed by GC using a Varian 3400 gas chromatograph equipped with a 30-m DB-17 capillary column (210 °C (30 min), then 240 °C at 2 °C/min) and by GC-MS in the electron-impact mode on a ThermoFinnigan PolarisQ instrument. Methylation linkage analysis was performed using the DMSO/NaOH/CH₃I procedure, and the permethylated alditol acetate derivatives were character-

³ The abbreviations used are: PBMC, peripheral blood mononuclear cell; HSQC, heteronuclear single quantum correlation; TOCSY, two-dimensional total correlation spectroscopy; HMBC, heteronuclear multiple bond coherence; SC, side chain; RU, repeat unit; RT, reducing terminus; NRT, non-reducing terminus; SRT, second glucose subunit from the reducing terminus.

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ized by GC-MS in the electron-impact mode (DB-17 column, isothermal at 190 °C for 100 min) (33).

Smith Degradation—Polysaccharide (10 mg) was dissolved in a 5-ml solution of 0.1 M sodium acetate, 0.04 M NaIO₄ at pH 4 and left at 4 °C in the dark for 3 days to oxidize the glucose units with vicinal hydroxyls (terminal and 6-linked glucose residues). The oxidized material was dialyzed against water (cut-off 1000 Da) and lyophilized. The oxidized material was reduced with NaBH₄ at room temperature for 5 days. The sample was treated with 1 M TFA at 45 °C for 1 h to cleave the acyclic acetal moieties and then dialyzed against water and freeze-dried.

Powder X-ray Diffraction Analysis—Powder diffraction measurements were conducted at room temperature on a SuperNova Agilent diffractometer equipped with a microfocus CuK_α (λ = 1.54184 Å) radiation source and Atlas CCD detector. Microsamples of glucans sized between 0.2 and 0.4 mm were mounted on glass fibers in such a way that only the glucan substance was exposed to the x-ray beam. The diffraction images were recorded in the 5–60° 2θ-range, but no features above 30° were observed. The images were processed using CrysAlisPro software v.1.171.35.8 (Agilent Technologies, Santa Clara, CA). The diffraction pattern did not show any noticeable changes for different samples of the same material or after the samples were exposed to the atmosphere, suggesting the materials investigated were structurally homogeneous and air-stable.

Human PBMC and Macrophage Stimulation—Human PBMCs were isolated by density centrifugation of buffy coats (Sanquin Biobank, Nijmegen), diluted 1:1 in pyrogen-free saline over Ficoll-Paque (GE Healthcare). Cells were washed twice in saline and resuspended in culture medium (RPMI, Invitrogen) supplemented with gentamicin 10 μg/ml, L-glutamine 10 mM, and pyruvate 10 mM. Cells were counted in a Coulter counter (Coulter Electronics), and the number was adjusted to 5 × 10⁶ cells/ml. A total of 5 × 10⁵ mononuclear cells in a 100-μl volume was added to round-bottom 96-well plates (Greiner) with RPMI and thereafter incubated at 37 °C with either 10 μg/ml β-glucan particles in suspension or culture medium with the final volume of 200 μl per well. After 24 h, supernatants were collected and stored at –20 °C until assayed. For macrophage differentiation, adherent human monocytes were cultured in complete RPMI 1640 medium (ICN Biomedicals) supplemented with 100 ng/ml human macrophage colony-stimulating factor and 10% pooled human serum for 6 days.

To assess the role of Dectin-1 for cytokine induction by hyphal glucan, PBMCs isolated from either volunteers expressing the normal *Dectin1* gene or a defective premature stop-codon variant (34) were stimulated with similar amounts of the yeast or hyphal extract.

Cytokine Measurements—IL-6, TNF, and IL-1β concentrations were measured by commercial sandwich ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For intracellular IL-1β measurement, after collection of supernatant for extracellular cytokine measurement, 200 μl of fresh RPMI was added to the macrophages. Three freeze-thaw cycles were used to disrupt the cell membranes, and intracellular IL-1β was determined by ELISA.

Quantitative IL-1β Polymerase Chain Reaction—Macrophages were stimulated as described above. The supernatant was removed after 4 h, and the cells were resuspended in 200 μl of RNazolB RNA isolation solvent (Campro Scientific) and stored at –80 °C. mRNA was isolated according to the instruction of manufacturer. cDNA was synthesized from 1 μg of total RNA by use of SuperScript reverse transcriptase (Invitrogen). Relative mRNA levels were determined using the Bio-Rad i-Cycler and the SYBR Green method (Invitrogen). The following primers were used: IL-1β forward primer (5'-GCCCTAAACAGATGAAGTGCTC-3') and reverse primer (5'-GAACCAGCATCT TCCTCAG-3') and β2M forward primer (5'-ATGAGTATGCCTGCCGTGTG-3') and reverse primer (5'-CCAATGCGGCATCTTCAAAC-3') (Biologio). Values are expressed as -fold increases in mRNA levels relative to those in unstimulated cells. The expression of IL-1β was normalized to the expression of HPRT-1.

Statistics—Cytokine expression data are presented as means ± S.E. The Wilcoxon sign ranked test was used to compare differences between groups. The level of significance was set at *p* < 0.05.

RESULTS

Evidence for a Cyclic Glucan Structure in C. albicans Hyphae—Recently we reported the complete ¹H and ¹³C NMR chemical shift assignments for a linear (1→3)-β-D-glucan with long (1→6)-β-linked glucosyl side chains (SCs) isolated from *Candida glabrata* based upon extensive one- and two-dimensional NMR studies (19). The average side chain length was 4.7 (1→6)-linked repeat units with an average side chain branching frequency of 21 (1→3)-linked backbone repeat units (RUs). NMR analysis of this branched, linear (1→3,1→6)-β-D-glucan exhibited clear evidence for the presence of reducing (RT) and non-reducing (NRT) termini as well as the backbone polymer repeat unit for the glycosyl repeat unit second from RT (SRT). In addition, repeat units for the first (SC1), internal (SCInt), next to last (SC SNRT), and last (SCNRT) (1→6)-linked repeat units in SC as well as the SC branch point (Br) within the backbone chain were clearly defined. Using the chemical shift assignments derived from Lowman *et al.* (19), we were able to critically examine hyphal glucan using one-dimensional and two-dimensional NMR techniques.

The one-dimensional ¹H NMR spectrum of hyphal glucan (Fig. 1, top) clearly shows only carbohydrate resonances between 3.0 and 5.0 ppm with residual lipid resonances between 0.7 and 1.5 ppm. Examination of the anomeric ¹H spectral region of the hyphal glucan versus the linear *C. albicans* yeast glucan isolated under the same conditions (Fig. 1, bottom) clearly shows the absence of resonances assigned to the α-anomer of RT, SRT, and NRT RUs in cyclic glucan when compare with the linear glucan. Only resonances for repeat units assignable to unbranched and branched (1→3)-β-linked repeat units in the backbone were observed in the two-dimensional NMR spectra. There was no evidence for resonances assignable to SRT and NRT in the linear backbone based upon comparison of the HSQC two-dimensional NMR spectra of the linear and cyclic glucans (Fig. 2). These observations are consistent with data reported for cyclic (1→3)-β-glucans, such as the glucan

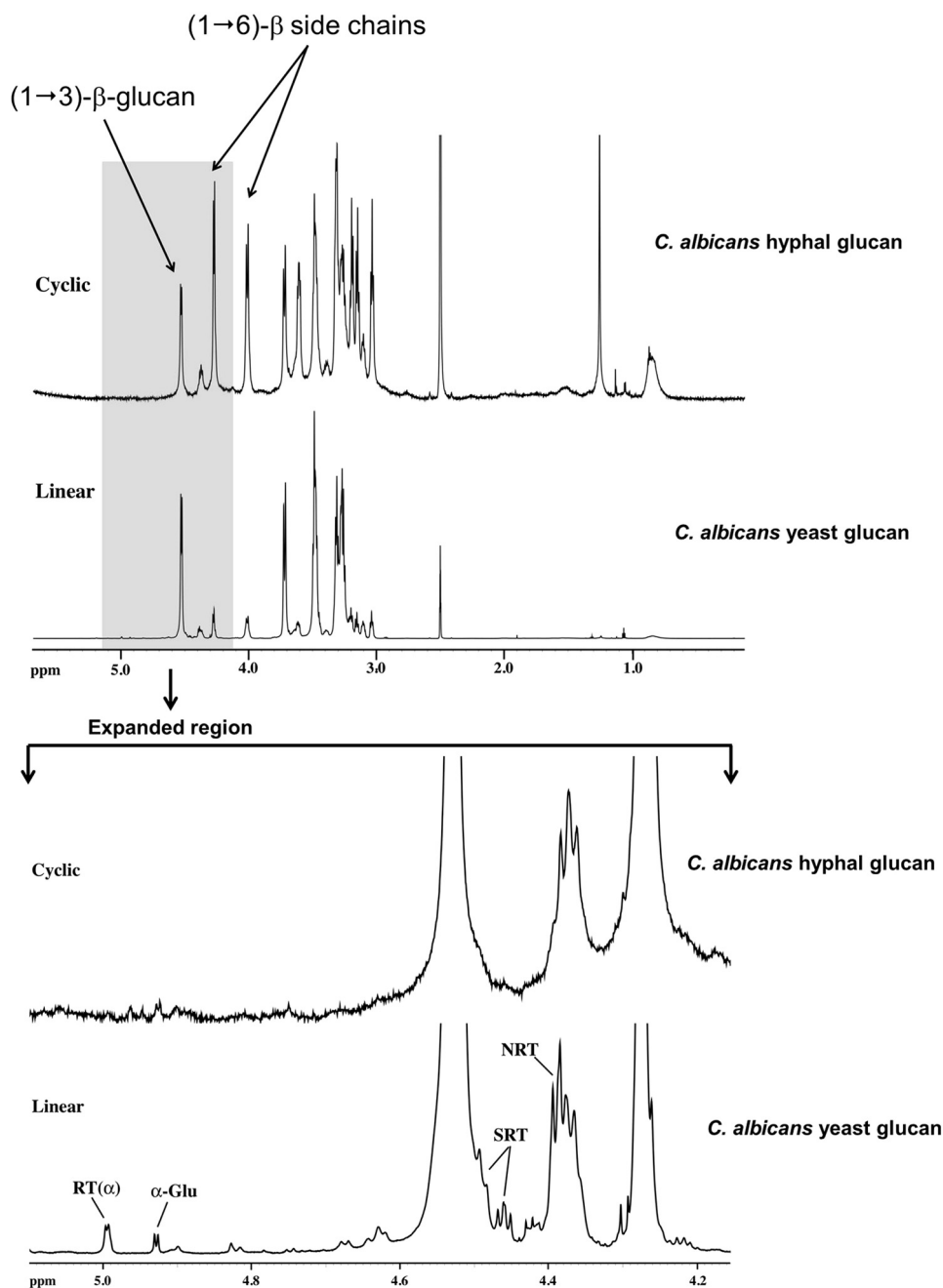


FIGURE 1. ^1H NMR analysis indicates that *C. albicans* hyphal glucans lack reducing and non-reducing termini. Specifically, there is no evidence for anomeric proton resonances (expanded region) for repeat units next to the RT, α , and β anomers of RT, NRT, or SRT side chains (arrows).

isolated from the basidiomycete *Phanerochaete chrysosporium* (35). Resonances for (1 \rightarrow 6)- β -linked side chain-related repeat units assigned to Br, SC1, SCInt, SC SNRT, and SCNRT were readily assigned by NOESY, HSQC-TOCSY, and HMBC two-dimensional NMR spectra. Resonances for the first side chain RU (1 \rightarrow 6)-linked at Br (SC1) are only observed for the anomeric ^1H and its neighboring ^1H due to resonance overlap of the remaining ^1H atoms in that RU with internal (1 \rightarrow 6)-linked side chain RUs. Protons of the internal RUs are clearly assignable. Methylene (H6 and H6') ^1H atoms of the RU (1 \rightarrow 6)-linked to SC NRT and the anomeric ^1H and its neighboring ^1H of the SC NRT are observed, whereas the remaining ^1H atoms of these two RUs are obscured due to resonance overlap. As noted above,

this detailed analysis of the two-dimensional NMR side chain spectra were compared with side chain resonances from a branched *C. glabrata* glucan (19) to confirm the ^1H and ^{13}C chemical shift assignments presented in Table 1 for the cyclic glucan.

For the cyclic glucan, the average side chain length was 13.9 (1 \rightarrow 6)-linked repeat units with an average side chain branching frequency of 12.5 (1 \rightarrow 3)-linked backbone repeat units, both calculated previously reported (19). Compared with the previously characterized linear, branched glucan (19), the cyclic, branched glucan exhibits a higher concentration of side chains that are on average three times longer and occur at twice the frequency along the linear chain, *i.e.* the branching frequency of hyphal glucan is twice that of blastopore glucan.

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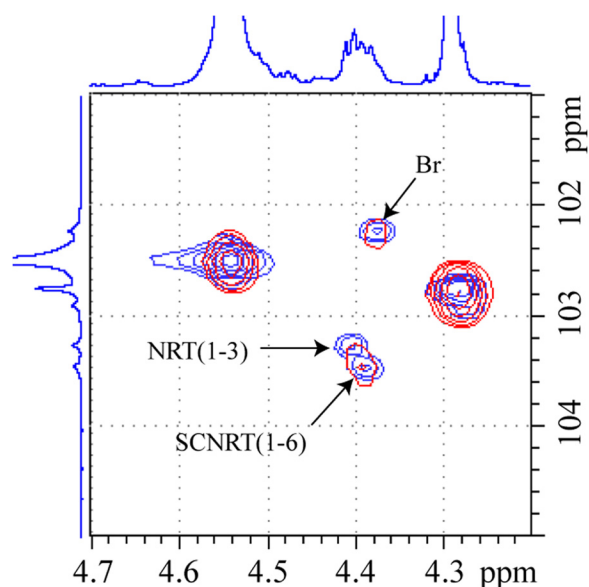


FIGURE 2. Comparison of HSQC two-dimensional NMR spectra of the branched, linear glucan (blue) and the branched, cyclic glucan (red). The one-dimensional spectra on the borders are from the linear, branched glucan. Cross-peak for resonances assigned to NRT in the linear backbone (NRT(1–3)) are labeled (blue spectrum). The absence of this resonance in the spectrum of the cyclic glucan (red spectrum) is evident. Br, branch point; SCNRT, side chain non-reducing terminus.

To confirm the reproducibility of this observation we conducted a separate experiment in which four *C. albicans* hyphal preparations were extracted simultaneously. The hyphal glucan was analyzed by NMR as described above. We identified the cyclical nature of the hyphal glucan in all four samples (data not shown), thus demonstrating that this is a consistent observation.

Monosaccharide Composition and Linkage Analysis of Hyphal Glucan—Sugar composition analysis revealed that the *C. albicans* hyphae polysaccharide isolate contains predominantly glucose (Glc) (Fig. 3A). In addition to the glucose, traces of mannose and *N*-acetyl-glucosamine (GlcNAc) were also detected. Linkage-type analysis showed two major glucose linkages, 3-substituted and 6-substituted linear units, and in lower amounts, 2,3-disubstituted and 3,6-disubstituted branch units (Fig. 3B). A very small quantity of terminal glucose was also detected.

Smith Degradation Analysis of *C. albicans* Glucan—The Smith degradation resulted in the elimination of terminal, 6-linked (linear) and 3,6-linked (branch) glucose units (Fig. 3C). The 3-linked and 2,3-linked glucose units remained intact. Also, the amount of 3-linked glucose in the Smith-degraded product increased due to the conversion of the 3,6-linked glucose branch unit to a 3-linked glucose linear residue, which indicated that the constituent attached to the 6-position of the 3,6-linked glucoses was a 6-linked glucose unit. The Smith degradation results suggest that the hyphal glucan is composed of a 3-linked backbone with 6-linked glucose chains branching from the 6 position of intermittent 3,6-linked RUs. The survival of the 2,3-linked branch glucose unit points to the fact that the components attached to the 2 and 3 position of this branch glucose are periodate-resistant. The survival of 2,3-linked glucose also confirmed that the 6-linked glucans are attached solely to the 6-position of the 3,6-linked glucose.

Powder X-ray Diffraction Analysis of *C. albicans* Hyphal Glucan—Powder x-ray diffraction analysis patterns reveal crystallinity in the hyphal and yeast glucans from *C. albicans* and glucan from *S. cerevisiae* (Fig. 4). The presence of peaks on the diffractograms indicates that there are crystal-like fragments (“crystallites”) with repeat periods of ~ 13.8 Å (from peaks at 6.4° , 12.8° , and 19.2°) and ~ 9.8 Å (peak at $\sim 9.4^\circ$). The broad peaks are consistent with small and imperfect crystallites. Although all three glucans show similarity of their crystal structure motifs, variations in the peak intensities suggest different shapes or mutual orientation of the crystallites within these glucans that may result from different length of the polymeric chains building the crystallites or different orientation of these chains with respect to each other.

Human PBMCs Show Differential Cytokine Expression in Response to *C. albicans* Yeast and Hyphal Glucan— β -Glucan is a major cell wall component of *C. albicans*, and extensive work has been carried out to examine the functional effect of β -glucan in terms of its receptor and downstream signaling (36–40). However, most of the β -glucan studied in the past was extracted from yeasts, and few studies have been performed with hyphal glucans. Based on the fundamental structural differences between *C. albicans* yeast and hyphal glucan, we hypothesized that differential recognition of the two different glucans by the host might lead to different downstream responses. To critically evaluate this hypothesis, cytokine profiles induced by yeast and hyphal glucan in human PBMCs were assessed (Fig. 5A). In agreement with previous results (4), *C. albicans* yeast glucan is not a potent stimulus for human PBMCs by itself and induces only small amounts of TNF- α , IL-6, and IL- β . In contrast, hyphal glucan is able to induce strong proinflammatory cytokine production (Fig. 5A). Interestingly, yeast glucan induces higher IFN- γ than hyphal glucan, thus emphasizing the differential response of PBMCs to hyphal versus yeast glucan. The hyphal glucan-induced cytokine production is fully dependent on Dectin-1 signaling. Specifically, human PBMCs from Dectin-1-deficient patients failed to produce cytokines upon hyphal glucan stimulation (Fig. 5B).

Human Monocyte-derived Macrophages Show Differential Cytokine Expression in Response to *C. albicans* Yeast and Hyphal Glucan—We next examined whether the differential cytokine induction observed in PBMCs holds true when human monocyte-derived macrophages were used as the target cells. Similar patterns were observed with almost no induction of cytokine by yeast glucan and strong induction by hyphal glucan (Fig. 5C).

***C. albicans* Hyphal Glucan Is a Potent Stimulus for Human Macrophage IL-1 β Responses**—We have demonstrated previously that yeast-to-hyphae transition triggers inflammasome activation in macrophages and induces IL-1 β processing and secretion (4). The differential induction of IL-1 β by yeast and hyphal β -glucan could, therefore, be an alternative recognition mechanism at the level of the mucosae. We assessed IL-1 β mRNA expression in human macrophages. Yeast glucan is able to induce IL-1 β mRNA expression, although the mRNA level is lower than with the hyphal glucan-treated group (Fig. 5D). Intracellular IL-1 β measurement (including the inactive proIL-1 β) clearly demonstrated that although there is intracellular

TABLE 1

Proton (¹H) and ¹³C NMR chemical shift assignments for *C. albicans* hyphal glucan

SC SNRT indicates side chain second glucose residue from the non-reducing terminus, and SC NRT indicates side chain non-reducing terminus.

	Chemical shift					
	(1→3)-β-Linked backbone chain	Br	SC1	SC internal (1→6)-β-linked side chain	SC SNRT (1→6)	SC NRT (1→6)
Proton assignment				ppm		
H1	4.542	4.374	4.255	4.282	^a	4.364
H2	3.321	3.258	3.038	3.045	^a	3.107
H3	3.502	3.408	^a	3.202	^a	^a
H4	3.265	3.327	^a	3.163	^a	^a
H5	3.322	3.225	^a	3.328	^a	^a
H6	3.729	4.012	^a	4.021	4.012	^a
H6'	3.483	3.642	^a	3.619	3.640	^a
Carbon assignment						
C1	102.49	102.16	102.91	102.73	^a	103.28
C2	72.93	71.75	^a	72.89	^a	73.31
C3	85.72	86.70	^a	76.01	^a	^a
C4	67.95	67.71	^a	69.57	69.71	69.70
C5	74.96	74.50	^a	75.01	^a	^a
C6	60.41	67.67	^a	68.03	^a	60.60

^a Resonance was not observed due to resonance overlap.

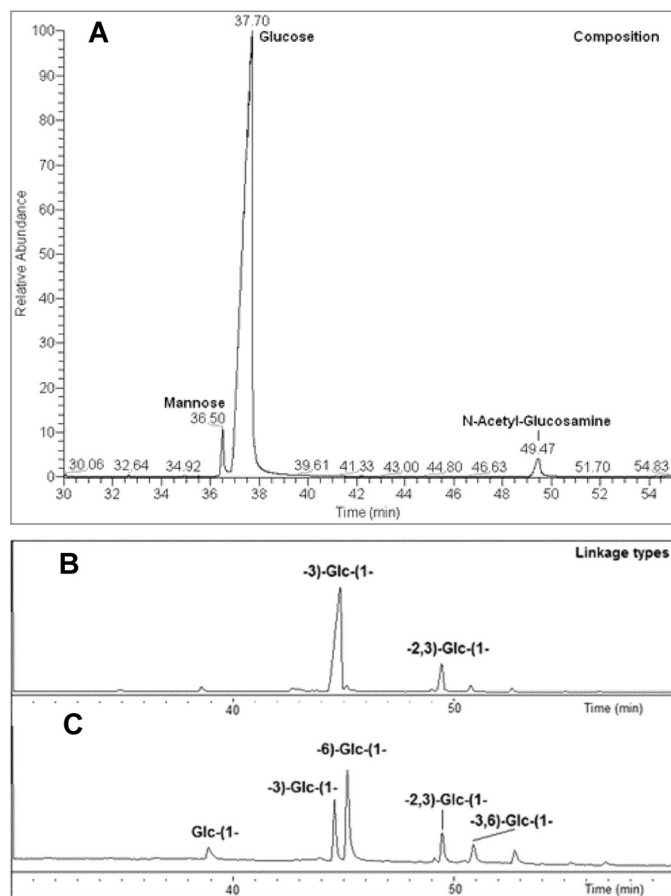


FIGURE 3. Monosaccharide composition and linkage analysis of *C. albicans* hyphal glucan. A, GC profile showing glucose as the dominant monosaccharide unit in the *C. albicans* hyphal glucan with traces of mannose and *N*-acetyl-glucosamine. B, Smith degradation of *C. albicans* hyphal glucan confirms the presence of the 2,3 linkage. C, GC profile of the linkage analysis of the Smith degradation product shows the elimination of 6-linked, 3,6-linked, and terminal glucose compared with the parent polysaccharide (bottom). These data confirm the backbone of the polysaccharide is composed of the 3-linked glucose and that the 6-linked chains branch off the 6-position of the 3,6-linked units.

IL-1β in yeast glucan-treated macrophages, no extracellular IL-1β was detectable. In contrast, IL-1β was present both intracellularly and extracellularly when macrophages were stimu-

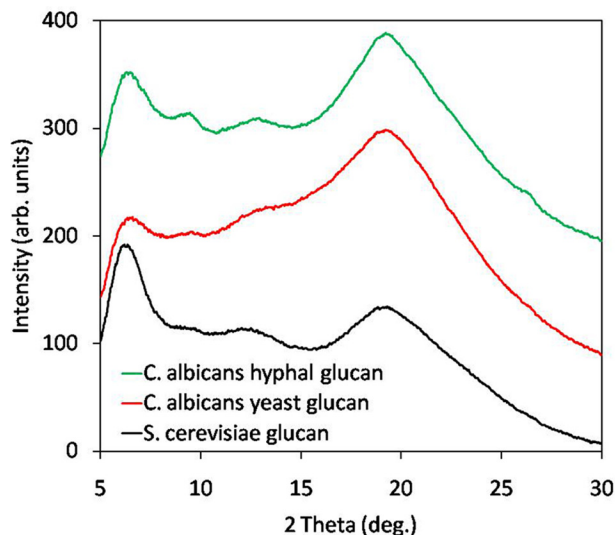


FIGURE 4. Powder x-ray diffractograms of the hyphal and yeast glucans from *C. albicans* with glucan from *S. cerevisiae* used as a reference. Although all three glucans show similarity of their crystal structure motifs, the variations in the peak intensities suggest different shape or mutual orientation of the crystal-like fragments (crystallites) in these glucans. This in turn may result from different length of the polymeric chains building the crystallites or their different orientation with respect to each other.

lated with *C. albicans* hyphal glucans. In fact, intracellular IL-1β was significantly ($p < 0.05$) greater in the hyphal glucan-treated cells. This demonstrates that although both yeast and hyphal glucans can induce the first steps of transcription and translation of proIL-1β, only the hyphal glucan can induce processing and release of bioactive IL-1β by macrophages.

DISCUSSION

Several new and important observations have emerged from this study. First, using a new, milder extraction approach, we have isolated and purified the glucan from *C. albicans* hyphae. Our data are consistent with a glucan that is composed of a cyclic (1→3)-β-linked polymer backbone with long (1→6)-β-linked side chains stemming from the 6-position of 3,6 branches that occur at twice the frequency observed for linear blastospore glucan (Fig. 6). Second, glucan appears to be a

C. albicans Hyphal Glucan Is Structurally Unique

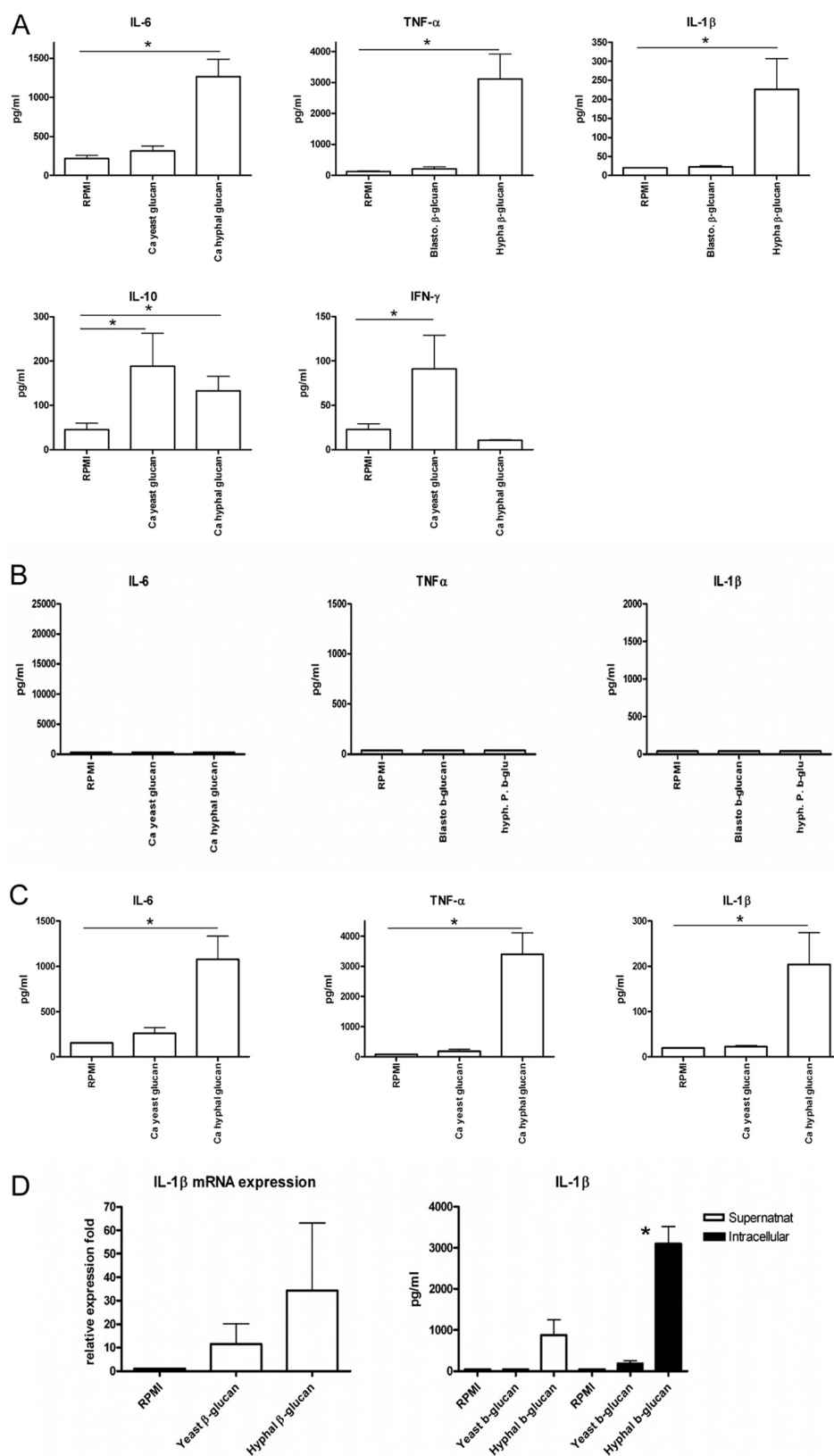


FIGURE 5. Differential cytokine responses to *C. albicans* (Ca) hyphal versus yeast glucan is mediated through a Dectin-1-dependent mechanism. *A*, hyphal glucan stimulates cytokine expression from normal human PBMCs, whereas yeast glucan does not. *B*, PBMCs from Dectin-1-deficient patients do not respond to *C. albicans* hyphal or yeast glucan, indicating that the hyphal glucan response is Dectin-1-dependent. *C*, *C. albicans* hyphal glucan stimulates cytokine expression in human monocyte-derived macrophages. Monocyte-derived macrophages were incubated with hyphal or yeast glucan. Media alone served as control. Supernatant was collected for TNF α , IL-6, and IL-1 β ELISA after 24 h stimulation. * $p < 0.05$ versus RPMI control. *D*, *C. albicans* hyphal glucan is a more potent stimulus for IL-1 β production in human monocyte-derived macrophages than yeast glucan. IL-1 β mRNA expression was measured by RT-PCR after 4 h of stimulation. Extracellular and intracellular IL-1 β production was measured by ELISA after 24 h of stimulation. Data are presented as the mean \pm S.E. Results are from at least three sets of experiments, with a minimum of six volunteers were pooled and analyzed using GraphPad Prism software. * $p < 0.05$.

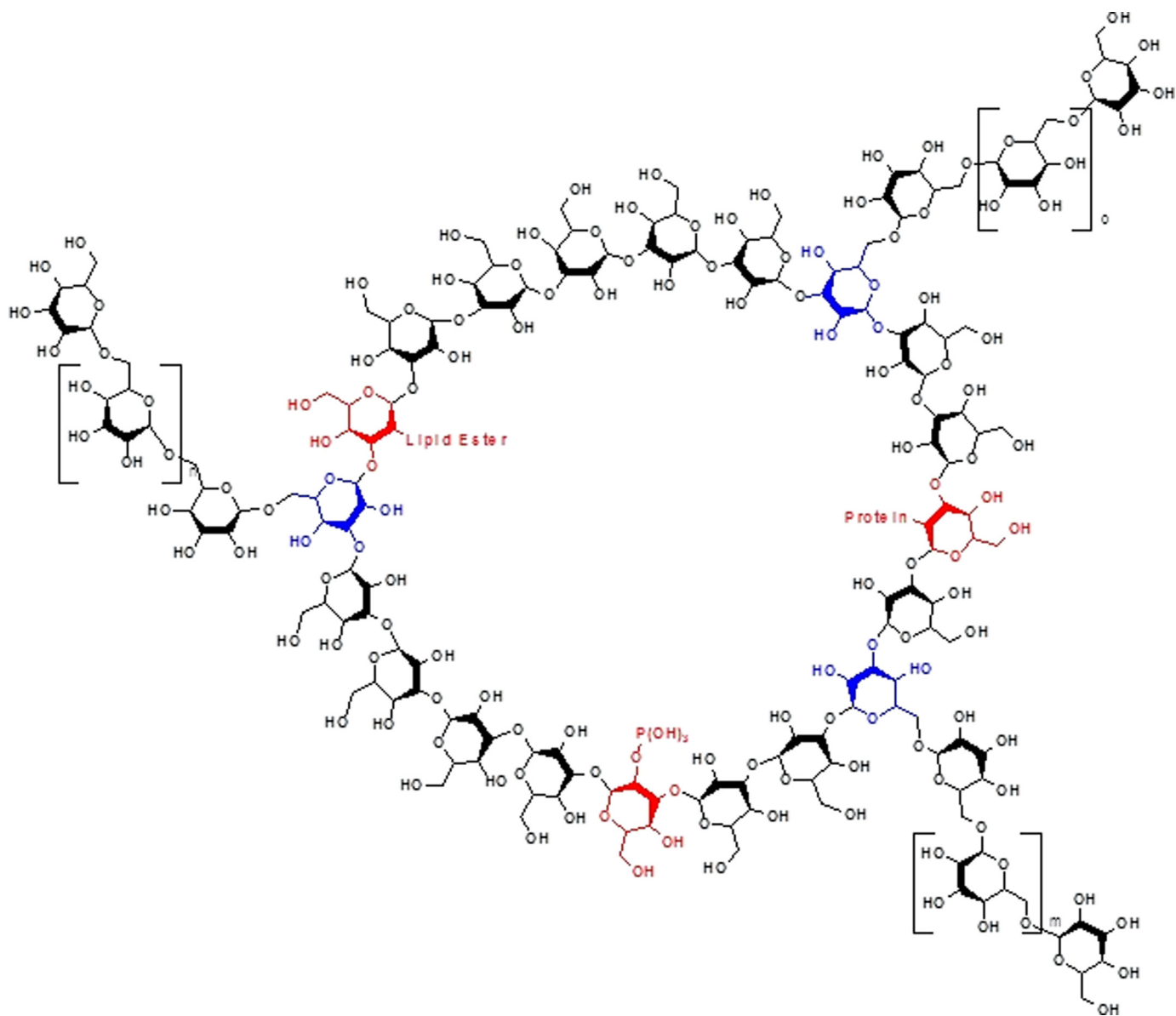


FIGURE 6. Conceptual model of *C. albicans* hyphal glucan showing the cyclical (1→3)-β-linked polymer backbone with long (1→6)-β-linked side chains. In addition to the expected (1→3), (1→6), and 3,6 linkages (blue), we identified a 2,3 linkage (red) in hyphal glucan that has not been reported previously in *C. albicans* or any other fungi. Specifically, the 2,3 structure is a glucose subunit in the (1→3)-β-linked polymer backbone that also has a linkage point at the 2-position (hence, the 2,3 designation) rather than the 6-position, *i.e.* the (1→6)-β-linked side chains. The model does not represent the molar mass, *i.e.* molecular weight, of the hyphal glucan; rather the model is intended to be a conceptual representation of the cyclical or closed chain nature of the hyphal glucan.

major structural carbohydrate in the cell wall of *C. albicans* hyphae. Third, *C. albicans* hyphal glucan does not exhibit the reducing termini that are routinely observed in (1→3)-β-glucans isolated from *C. albicans* blastospores. Consequently, *C. albicans* hyphal glucan is structurally distinct from *C. albicans* blastospore glucan. NMR data indicate that the hyphal glucan structure is consistent with a cyclical (1→3)-β-glucan. To the best of our knowledge, this is the first report of a cyclical glucan in *C. albicans*. Fourth, in addition to the (1→3), (1→6), and 3,6 linkages, we identified a 2,3 linkage in hyphal glucan that has not been reported previously in *C. albicans*. Finally, we compared and contrasted immune responsiveness to yeast and hyphal glucans. We found that hyphal glucan induces stronger proinflammatory cytokine production in human PBMCs and

macrophages than does *C. albicans* yeast glucan. In addition, hyphal glucan can induce processing and release of bioactive IL-1β by human macrophages, but yeast glucan does not. This indicates that *C. albicans* hyphal glucan dictates structure-specific immune responses distinct from those elicited by blastospore glucan. We conclude that hyphal glucan is a major pathogen-associated molecular pattern in hyphae.

At the present time we do not fully understand the structural significance of the cyclical hyphal glucan. However, we know that hyphae are produced rapidly, and our data clearly show that glucan is a major structural carbohydrate in *C. albicans* hyphal cell wall. We speculate that hyphal cell wall glucan is structurally different from blastospore cell wall glucan due to the different kinetics of its induction during hyphal growth,

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with hyphal cell wall integrity being almost entirely dependent on the glucan.

It is well established that the *C. albicans* yeast cell walls contain substantial amounts of mannan (41). Using single-molecule atomic force probe microscopy Beaussart *et al.* (42) have recently reported that mannans are more abundant on *C. albicans* hyphae versus yeast. As part of this investigation we attempted to isolate mannan from *C. albicans* hyphae using mannan specific extractions methods previously reported by our group (43–45). We were not able to isolate hyphal mannans, except in trace quantities that were too small to be analyzed. The difference between our results and those of Beaussart *et al.* (42) may reflect differences in methodology. It is also possible that hyphal mannan has a lower molecular weight than yeast mannan. This may make it more difficult to isolate using standard mannan extraction methods. Whatever the case, our results indicate that mannans are present in low abundance on the hyphal cell wall.

The discovery of the 2,3 linkage may provide important insights into hyphal cell wall organization, inter-connections, and structural integrity. It is currently thought that the (1→6)-glucan side chains serve as attachment points for cell wall proteins through GPI remnants (46). However, the (1→6) side chains terminate in non-reducing termini that are not reactive. This raises the question of how proteins and other macromolecules are attached to non-reactive side chain termini. In contrast, the 2,3 structure may be ideally suited for linkage of the hyphal glucan with other macromolecules, such as cell wall proteins or lipids or perhaps as bridges between the 3-linked cyclic glucans forming a macromolecular structure (Fig. 6). Additional studies will be required to unambiguously determine the identification of components attached to the two position. The linkage analysis data indicate that the 2,3 linkage is present in hyphal glucan at levels that should be adequate for linking to other macromolecules, *i.e.* carbohydrates, proteins, or lipids, within the hyphal cell wall.

Only a few types of β -glucans showing crystallinity have been reported in the literature (47–49). Only (1→2)- β glucans have been reported to form cyclic molecules, but no diffraction data have been reported for these glucans (50–53). In the present study, the powder x-ray diffraction analysis data (Fig. 4) make it possible to compare the studied glucans with other crystalline or semi-crystalline glucans reported previously (48, 49, 54–58). The linear (1→4)- β glucan, cellulose, exhibits several crystalline forms with the polymeric chains combined in H-bonded sheets (54, 55). These forms are monoclinic with the longest repeat distance of ~ 10 Å (54, 55). Two linear natural product (1→3)- β glucans, paramylon (*Euglena* species) and curdlan (*Alcaligenes* bacteria), also exhibit a number of crystalline forms, but the polymeric molecules form helices (48, 56, 57). Paramylon forms a structure where the longest repeat period is 13.6 Å (48, 56, 57). The most recent report confirms a hexagonal structure with $a = 15.547$ Å and $c = 18.587$ Å for never dried and $a = 14.543$ Å and $c = 5.853$ Å for dried paramylon (57). Curdlan was isolated in three forms (47), two being identical to the never dried and dried forms of paramylon (48, 59). The third form is reported to be hexagonal with $a = 17.01$ Å and $c = 22.70$ Å (47, 58). A recent study reported two forms of

curdlan with repeat periods of 15.4 and 13.6 Å (49). To the best of our knowledge, no diffraction data were reported for this linear (1→6)- β glucan. Pelosi *et al.* (60) have reported powder x-ray diffraction analysis images of (1→3,1→6)- β glucan from *Saprolegnia monoica* and *Rubus fruticosus*, with the longest repeat distances of ~ 13.5 and ~ 15 Å, respectively. Although naturally occurring cyclic glucans have been isolated and studied, no evidence of their crystallinity was reported.

From these comparisons, the crystallites in the glucans we studied were formed by the helices of either (1→3) or (1→6) chains but not by cyclic fragments. If the 13.82 ± 0.14 Å repeat period is the a parameter of a hexagonal unit cell, then the distance between the helices (and hence the diameter of the helix) is ~ 16 Å. The crystallites in hyphal, and yeast glucans seem to have a similar crystal structure, but they differ in size and mutual orientation. This is consistent with a different orientation of side chains attached to either cyclic or linear backbone.

Evidence indicates that the innate immune system effectively discriminates between yeast and hyphal forms of *C. albicans* (4). However, the mechanistic basis for the ability of the immune system to differentiate between *C. albicans* yeast and hyphal forms is the subject of debate. Several immune mechanisms and pattern recognition receptors have been proposed to mediate this discriminatory activity, including C-type lectin receptors Dectin-1 (4), TLR2 (61), or Dectin-2 (62–64), and/or MAPK (63). Recent data have also documented differential inflammasome activation by *C. albicans* yeast and hyphae (1, 4, 46, 65). We have previously demonstrated that the inflammasome is differentially activated by *C. albicans* yeast and hyphae in macrophages by comparing wild type and yeast-locked *C. albicans* strains (4). In the present study we provide a structural explanation for this differential recognition of hyphae versus yeast. By demonstrating the capacity of hyphal glucan to induce IL-1 β processing and secretion, we provide an important piece of evidence for understanding the immune discrimination between *C. albicans* colonization and invasion at the mucosal level (1).

It is well established that the innate immune system recognizes and responds to fungal glucans based on structural features including, but not limited to, the (1→3)- β -linked backbone and/or the presence, frequency, and length of (1→6)- β -linked side chains (40, 66–68). The present data clearly demonstrate that hyphal glucan has at least three unique structural features, *i.e.* cyclical structure, 2,3 linkages, and (1→6)- β -linked side chains that are longer and of greater frequency, any or all of which may contribute to the immune recognition of hyphae. We found that hyphal glucan, but not yeast glucan, induces IL-1 β secretion by macrophages, implying that the unique structure of this glucan might serve as a hyphal specific pathogen-associated molecular pattern to activate inflammasomes in macrophages. Moreover, hyphal glucan induces higher levels of proinflammatory cytokines in PBMCs. This indicates that the recognition of hyphal glucan represents a danger signal for the innate immune system to respond in a more robust way. Dectin-1 recognition is involved in this process; however, the stronger cell stimulation by hyphal glucan suggests that a different receptor complex containing Dectin-1 is engaged when cells

are stimulated by hyphae. Presumably, this not only happens at the site of the mucosal barrier where tissue macrophages are located but also in the blood where monocytes are present. Dectin-1 is known to interact with a variety of other pattern recognition receptors (69–71). We speculate that differential engagement of pattern recognition receptors by hyphal *versus* yeast glucan may explain, in part, the difference in immunological responses that we observed. It also possible that the other pattern recognition receptors may recognize hyphal proteins and/or lipids present at very low levels in the hyphal glucan.

In summary, our data indicate that *C. albicans* hyphal glucan contains structures that have not been previously identified and appear to be unique to hyphal glucan, *i.e.* these structures are not present in *C. albicans* blastospore glucan. In fact, these structures have not been previously identified in any *Candida* species or other pathogenic fungus for that matter. We have shown that the innate immune system effectively discriminates between yeast and hyphal forms of *C. albicans* (4). We speculated that the differential recognition of *C. albicans* yeast *versus* hyphae was due to differences in cell wall architecture (4). The present data provide compelling evidence that the structural differences in hyphal glucan induces greater proinflammatory cytokine production in PBMCs and inflammasome activation in macrophages. We conclude that hyphal glucan is a major pathogen-associated molecular pattern in *C. albicans* hyphae. To the best of our knowledge, these are entirely new and novel findings which advance our understanding of hyphal cell wall structure and composition. These data also shed light on the biology and immune recognition of hyphae, and they provide a structural basis for the differential recognition of *C. albicans* yeast *versus* hyphae.

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