



Titan cell production in *Cryptococcus neoformans* reshapes the cell wall and capsule composition during infection

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ABSTRACT

Cryptococcus neoformans is a human fungal pathogen that often causes infections in immunocompromised individuals. Upon inhalation into the lungs *C. neoformans* differentiates into cells with altered size and morphology, including production of large titan cells. Titan cells possess thickened cell wall and dense, cross-linked capsule when compared to *in vitro* grown cells. In addition, titan cells have increased cell wall chitin that is associated with a detrimental anti-inflammatory immune response. Here we examined the cell wall and capsule composition of *in vitro*, *in vivo* typical-sized and *in vivo* titan cells using High Performance Liquid Chromatography (HPLC). The monomer composition of cell wall polysaccharides showed that *in vivo C. neoformans* cells contained more glucosamine and less glucose than *in vitro* cells, suggesting alteration in abundance of both chitin and glucans, respectively. Low levels of galactosamine were also detected in carbohydrates from both *in vivo* and *in vitro* cells. Within the *in vivo* cell population, differences in the proportions of cell wall and capsule monomers between typical and titan cells were also observed. Taken together, these results demonstrate that *C. neoformans* reshapes its cell wall and capsule composition during infection. These cell wall and capsule alterations likely help *C. neoformans* escape recognition by, and allow modulation of, the host immune system.

Introduction

Cryptococcosis is a life-threatening infection caused by *Cryptococcus neoformans* and often observed in immunocompromised individuals including patients with HIV/AIDS, cancer chemotherapy patients and organ transplant recipients (Mitchell and Perfect, 1995; Park et al., 2009; Jarvis et al., 2010). Even though the absolute number of *Cryptococcus*-related deaths has declined since previous estimates, cryptococcal meningitis still causes 15% of all HIV/AIDS-related deaths globally (Rajasingham et al., 2017). In Sub-Saharan Africa alone, the region with the greatest burden, cryptococcal meningitis kills around 140,000 people per year and global mortality is estimated at around 181,000 people (Rajasingham et al., 2017).

A critical step during cryptococcal infection involves the formation of enlarged cells called “titan cells” (Okagaki et al., 2010; Zaragoza et al., 2010). While typical *C. neoformans* cells are 5–7 μm in diameter, the titan cells can be up to 100 μm in diameter (Okagaki et al., 2010; Zaragoza et al., 2010). Titan cells are also characterized by their increased DNA content, thick cell wall, and a significantly denser

crosslinked capsule (Okagaki et al., 2010; Zaragoza et al., 2010). Changes in titan cell walls are important for survival of *C. neoformans* within the host, and increased chitin content in titan cell wall signals the host immune system to generate a detrimental immune response that promotes disease progression in mice (Wiesner et al., 2015). However, it is not known whether there are other *in vivo* cell wall changes in titan or typical cells, and how these changes might affect *C. neoformans* pathogenesis.

Mammalian cells lack a cell wall; therefore, the fungal cell wall is an ideal target for development of antifungal drugs. In fungi, the cell wall plays important roles including protecting the cell from osmotic pressure, various environmental stresses, and is also involved in interaction between the fungal cell and its environment (Bowman and Free, 2006; Erwig and Gow, 2016). *C. neoformans* also possesses a polysaccharide capsule that is attached to the cell wall and covers its cell body (Zaragoza et al., 2009). The presence or absence of capsule, as well as its size and structure depends on different environmental stimuli such as temperature, growth medium, pH, the presence of CO₂, and infected organ type (Rivera et al., 1998; Zaragoza and Casadevall 2004; O’Meara

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and Alspaugh, 2012). Capsule polysaccharides can be divided into two groups; those attached to the cell wall and exopolysaccharides that are secreted into the surrounding environment or culture media (Zaragoza et al., 2009). Exopolysaccharides are composed of 90–95% glucuronoxylomannan (GXM), 5–8% glucuronoxylomannogalactan (GXMGal), and a few mannoproteins (Juneann, 1988; Cherniak and Sundstrom, 1994, Vaishnav et al., 1998; Bose et al., 2003; McFadden et al., 2006; Heiss et al., 2009; Zaragoza et al., 2009). Most of the known information on cryptococcal capsule comes from studies that examined the polysaccharides recovered from culture media (Turner et al., 1992; Cherniak and Sundstrom, 1994, Cleare and Casadevall, 1999; McFadden et al., 2006; Heiss et al., 2009; Zaragoza et al., 2009), however one study showed that the attached capsule is different from polysaccharides secreted to the environment (Frasces et al., 2008). In addition, most, if not all, previous studies used *Cryptococcus* cells generated in *in vitro* conditions. The exact structure and composition of *Cryptococcus* capsule during infection is not well understood.

The composition and organization of individual cell wall components can vary depending on the fungal species, growth conditions and the stage of growth (Reiss et al., 1986; Abad et al., 2010; Gow et al., 2011). The cell wall of *C. neoformans* is mainly composed of α - and β -glucan, chitin and its deacetylated form chitosan (Reiss et al., 1986; Banks et al., 2005; Baker et al., 2007; Reese et al., 2007; Baker et al., 2011). In addition to these main components, the cell wall of *C. neoformans* also contains melanin, lipids and mannoproteins (Vartivarian et al., 1989; Wang et al., 1995; Siafakas et al., 2007). However, this information comes mainly from *in vitro* studies that used acapsular mutant strains or strains with mutations impacting the cell wall (Reiss et al., 1986; James et al., 1990; Reese et al., 2007). Acapsular mutants were used to avoid contamination from the capsule polysaccharides that are normally attached to the outer layer of the *C. neoformans* cell wall. This is not ideal as acapsular mutant strains are avirulent in mice (Fromtling et al., 1982; Chang and Kwon-Chung, 1994), and could possess additional defects in their cell wall structure. Thus, the complete cell wall composition of a wild-type *C. neoformans* strain is currently not known, and the potential for cell wall remodeling under different growth conditions has not been explored.

Modification of the cell wall composition/structure likely constitutes a crucial step in the initial establishment and maintenance of *C. neoformans* infection (Okagaki et al., 2010; Zaragoza et al., 2010; Okagaki and Nielsen, 2012; Zaragoza and Nielsen, 2013; Wiesner et al., 2015). Except for preliminary studies examining chitin content in titan cells, it is not known how other cell wall changes, if any, in *in vivo* *C. neoformans* cells (both titan and typical cells) affect *C. neoformans* pathogenesis and the host immune response. The aim of this study was to identify the cell wall composition of both *in vitro* grown and *in vivo* cells, both titan and typical cells, of a wild type strain of *C. neoformans*.

Materials and methods

Ethics statement

Mice were handled in accordance with guidelines defined by the University of Minnesota Animal Care and Use Committee (IACUC). All animal experiments were done in concordance with the Animal Welfare Act, United States federal law, and NIH guidelines.

Strains and media

C. neoformans KN99 α (WT) (Nielsen et al., 2003), and acapsular *C. neoformans* *cap67* and *cap59* mutant strains (Jacobson et al., 1982; Chang and Kwon-Chung, 1994) were used in this study. Strains from stored glycerol stocks (kept at -80°C) were grown at 30°C for 24–48 h on yeast-peptone-dextrose (YPD) (BD, Hercules, CA). *C. neoformans* cells from the YPD agar plate were transferred to YPD broth and grown overnight at 30°C .

Production and isolation of titan and typical cells

C. neoformans titan and typical cells were produced as previously described (Okagaki et al., 2010; Wiesner et al., 2015). Briefly, overnight YPD-grown *C. neoformans* cells were collected by centrifugation, washed twice with sterile phosphate-buffered saline (PBS) and resuspended in sterile PBS at a concentration of 1×10^6 cells/ml based on hemocytometer count. Groups of 6–8-week-old female A/J mice (Jackson Laboratory, Bar Harbor, MA) were anesthetized by intraperitoneal pentobarbital injection and infected intranasally with 5×10^4 cells in 50- μl PBS. At 14 days post-infection, mice were sacrificed by CO_2 inhalation. The lungs were harvested and then homogenized in 10 ml PBS supplemented with collagenase (1 mg/ml) (Zaragoza et al., 2010). Cell homogenates were incubated for 1 h at 37°C with agitation, and washed several times with double distilled water. The *C. neoformans* cells were resuspended in sterile double distilled water.

To separate titan and normal-sized (which we refer to as “typical”) cell populations, we used a previously described method (Gerstein et al., 2015). As previously, *C. neoformans* cells (mixture of titan and typical cells) from lung homogenates were filtered using CellMicroSieves (BioDesign Inc. of New York, Carmel, NY) with a 20- μm pore size that allows typical cells to pass through the membrane. The CellMicroSieves were then rinsed with sterile PBS to remove any remaining typical cells from the filter. To recover the titan cell population, the CellMicroSieves were inverted and the membrane was washed with PBS. The resulting cell populations was concentrated by centrifugation at $12,000 \times g$ for 1 min.

γ -Radiation treatment

In vivo *C. neoformans* cells with enlarged capsule were exposed to γ -radiation to remove layers of the capsule polysaccharide as described previously with the following modifications (Zaragoza et al., 2010). Cells isolated from the lungs of infected mice were washed in PBS and resuspended in sterile double distilled water. The cells were then transferred to a 24-well flat-bottom plate and irradiated for 45 min for a total irradiation dose of 560 Gy (56,000 rad). After irradiation, cell suspensions were transferred into Eppendorf tubes, centrifuged, and the supernatant was separated from the irradiated cell pellet by centrifugation. The supernatant containing capsule polysaccharide was freeze-dried and stored at -20°C until further analysis. The irradiated cell pellet was used in cell wall extraction experiments.

Cell wall and capsule extraction

Cell wall/capsules were extracted using a modified version of previously described methods (Munro et al., 2003; Mora-Montes et al., 2007; Mora-Montes et al., 2012). Briefly, *C. neoformans* cells were either grown in YPD medium or isolated from lungs of infected mice as described above. Cells were washed with sterile double distilled water and broken with glass beads using a BIO101/Savant FastPrep FP120 machine (20 cycles of 45 s each cycle, at a speed of 4 m/s). The homogenates were centrifuged at 4000 rpm for 5 min, and the pellet (broken cells) was washed 5 times with 1 M NaCl, resuspended in buffer (500 mM Tris-HCl buffer [pH 7.5], 2% [wt/vol] SDS, 0.3 M β -mercaptoethanol, 1 mM EDTA) and boiled twice at 100°C for 10 min. Extracted cell wall pellet was collected, washed with sterile double distilled water, freeze dried and the dry weight of recovered cell walls was recorded.

Hydrolysis of cell wall/capsule polysaccharides

Purified cell wall/capsule was hydrolyzed using a modification of several previously described methods (Morrison, 1988; Mora-Montes et al., 2007; Mora-Montes et al., 2012). Six different approaches were

Table 1
Cell wall hydrolysis approaches.

	Hydrolysis method		Carbohydrates detected ($\mu\text{g}/\text{mg}$ of dry cell wall)					
	Step-1 at 37 °C	Step-2 at 100 °C	Glucosamine	Glucose	Mannose	Xylose	Galactose	Total ($\mu\text{g}/\text{ml}$)
Approach -1 (N = 6)	–	3 h	7.3 \pm 1.1	130.4 \pm 34.1	9.7 \pm 3.9	ND	ND	147.3 \pm 38.2
Approach -2 (N = 4)	20 h	–	0.6 \pm 0.5	76.7 \pm 7.9	4.2 \pm 1.5	ND	ND	81.5 \pm 9.0
Approach -3 (N = 5)	20 h	30 min	6.5 \pm 1.2	415.0 \pm 78.6	39.1 \pm 5.6	7.0 \pm 2.7	1.1 \pm 0.7	468.7 \pm 83.6
Approach -4 (N = 5)	20 h	3 h	11.3 \pm 0.5	625.9 \pm 19.8	55.5 \pm 8.6	ND	3.6 \pm 1.5	696.3 \pm 20.3
Approach -5 (N = 5)	20 h	4 h	6.5 \pm 1.6	266.8 \pm 39.7	24.8 \pm 12.4	0.4 \pm 0.2	ND	348.4 \pm 62.6
Approach -6 (N = 2)	72 h	30 min	1.3 \pm 0.5	77.0 \pm 1.2	ND	0.9 \pm 0.1	ND	79.2 \pm 0.6
Approach -7 (N = 3)	72 h	3 h	5.1 \pm 1.2	154.7 \pm 35.7	2.2 \pm 1.4	ND	ND	162.0 \pm 33.2

ND: not detected.

used to hydrolyze cell wall/capsule polysaccharide (Table 1).

Approaches 1 and 2

Trifluoroacetic acid (TFA) (0.75 ml) was added to dried cell wall/capsule polysaccharides in 1.5 ml screw-cap tubes (SARSTEDT) and incubated at 37 °C for 20 h (approach 1) or at 100 °C for 3 h (approach 2), then evaporated to dryness at 70–80 °C in a thermal block. After all acid was evaporated, double distilled water (1.0 ml) was added to each sample as a washing step, and mixed by pipetting. Samples were then evaporated to dryness by incubating open tubes at 70–80 °C. Dried hydrolyzed samples were stored at -20 °C until analyzed by High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD).

Approaches 3–7

TFA (0.75 ml) was added to dried cell wall/capsule polysaccharide and incubated at 37 °C for 20 h. Aliquots (0.375 ml) were transferred to two new tubes and myo-inositol (1.125 ml, 0.75 mg/ml) was added to each tube as a positive control to confirm hydrolysis. Preliminary studies comparing the addition of water versus myo-inositol showed no effect of the myo-inositol addition on other components within the reaction (data not shown). One aliquot was heated at 100 °C for 30 min (approach 3), and the other heated at 100 °C for 3 h (approach 4) or for 4 h (approach 5). Both samples were then evaporated to dryness. After all acid was evaporated, double distilled water (1.0 ml) was added to each sample as a washing step, and mixed by pipetting. Samples were then evaporated to dryness by incubating tubes at 70–80 °C. Dried hydrolyzed samples were stored at -20 °C until analyzed by HPAEC-PAD. Approaches 6 and 7 were similar to approaches 3 and 4 except that the dried cell wall/capsule polysaccharides were resuspended in TFA and incubated at 37 °C for 72 h, instead of 20 h, before being incubated at 100 °C for 30 min and 3 h respectively.

Carbohydrate quantification by HPAEC-PAD

The hydrolyzed cell wall/capsule samples were analyzed by HPAEC-PAD as previously described with minor modifications (Munro et al., 2003; Mora-Montes et al., 2007; Mora-Montes et al., 2012). For monosaccharide analysis of the *Cryptococcus* cell wall component, 10 μl aliquots of acid-hydrolyzed samples were injected in a Dionex HPAEC system equipped with a CarboPac SA-10 analytical column (4 \times 250 mm) and a CarboPac SA-10 guard column (4 \times 50 mm). The CarboPac SA-10 analytical column was used because the most commonly used CarboPac PA-10 column was unable to separate xylose and mannose (Supplemental Table 1 and Supplemental Fig. 1). Samples were eluted with 12 mM NaOH at a flow rate of 1.2 ml/min for 10 min. The column was washed with 100 mM NaOH for 5–10 min and equilibrated with 12 mM NaOH for 10 min prior to the next run. Monosaccharides were detected by their oxidation at a gold electrode surface of the PAD. Seven monomers were used as standards: D-fucose, D-glucose, D-glucosamine, D-mannose, D-galactose, D-galactosamine, and D-

xylose. The monomer standards were dissolved in double distilled water and run under the same conditions. Myo-inositol was used as a hydrolysis control for Approaches 3–7.

Results

Detection of *C. neoformans* carbohydrates depends on the method used

To analyze the total *C. neoformans* cell surface carbohydrate content, we used a wild type strain, KN99 α , that is virulent in the mouse model of infection (Nielsen et al., 2003). Using previously described methods (Munro et al., 2003; Mora-Montes et al., 2007; Mora-Montes et al., 2012), we measured the total carbohydrate content of *C. neoformans* cells from *in vitro* cultures and from lungs of infected mice (*in vivo* cells). The major characteristic differentiating *in vivo* and *in vitro*-grown cells is the presence of a large capsule surrounding the *C. neoformans* cell body *in vivo* (Fig. 1A). *C. neoformans* capsule is predominantly composed of GXM and GXMGal polysaccharides (Frases et al., 2008; Zaragoza et al., 2009). Preliminary analysis of carbohydrates of both *in vitro* and *in vivo* cells identified different proportions of glucosamine, glucose and mannose in the two cell populations (Fig. 1B). However, monosaccharides such as galactose and xylose that would be expected to come from *in vivo* capsule were not detected (Fig. 1B). Different hydrolysis methods that varied the temperature and time of hydrolysis were tested to optimize detection of all expected monosaccharides (Table 1). These experiments showed that hydrolysis for 20 h at 37 °C followed by a 30-min incubation at 100 °C (Approach 3) recovered all carbohydrates expected to be present in the cell wall and capsule of *C. neoformans* (Table 1). In addition, a CarboPac SA 10 column was needed to differentiate galactose, galactosamine, glucose, glucosamine, mannose, and xylose simultaneously (Supplemental Table 1 and Supplemental Figs. 1 and 2).

We next measured the carbohydrate content of the wild type strain (KN99 α) and the previously used acapsular mutants *cap67* and *cap59* grown in the same *in vitro* conditions. The results show that, when grown *in vitro*, *C. neoformans* KN99 α , *cap67* and *cap59* strains had similar glucosamine (monomer component of cell wall chitin and chitosan), and glucose (monomer component of α - or β -glucans) (Fig. 2A and B). The mannose content was significantly higher in the wild type strain compared to the acapsular mutant strains (Fig. 2C). Low levels of galactose and xylose (components of GXM and GXMGal) were detected in the wild type strain and, as expected, xylose was absent in the acapsular mutant strains (Fig. 2D and E). Surprisingly, low levels of galactose, a component of the capsular GXMGal were detected in the acapsular mutants (Fig. 2E). Very low levels (less than 0.1% of total carbohydrate content) of galactosamine were also detected in both the wild type and acapsular mutants, with acapsular mutants having more galactosamine than the wild type strain (data not shown). These results show that KN99 α , *cap67* and *cap59* strains have overall similar cell wall carbohydrate composition when grown *in vitro*. As the wild type strain is known to form a small capsule *in vitro* (Fig. 1A), the presence of

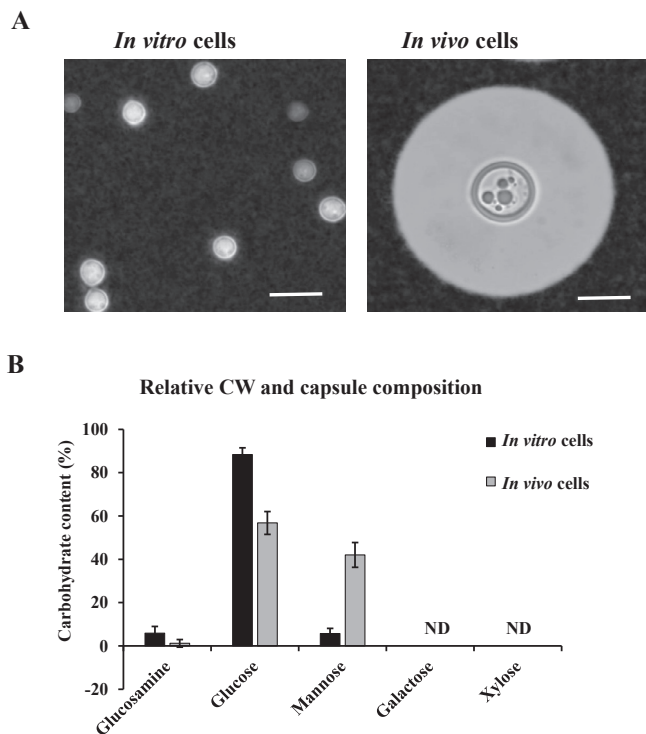


Fig. 1. Total carbohydrate content of *in vitro* and *in vivo* *C. neoformans* cells. A, *In vitro* grown and *in vivo* *C. neoformans* cells stained with India ink. B, Carbohydrate content of *in vitro* and *in vivo* *C. neoformans* cells. C, *neoformans* KN99 α strain was grown *in vitro* overnight at 30 °C in YPD broth, or isolated from the lungs of infected mice at 14 days post-infection. Cell walls were extracted, freeze-dried and hydrolyzed with TFA for 3 h at 100 °C. Glucosamine, glucose, mannose, galactose and xylose were quantified using high-performance anion-exchange chromatography with pulse amperometric detection (HPAEC-PAD). Data represent means of results from 2 independent biological replicates (N = 6 for *in vitro* samples, and N = 8 for *in vivo* samples). ND: not detected, YPD: yeast-peptone-dextrose, CW: cell wall. Scale bar = 10 μ m.

xylose in the wild type, but not in the acapsular mutant strains indicates that xylose is mainly a capsular component. The presence of galactose in the acapsular cell wall extracts could be because the GXMGal was secreted into the environment or was still loosely attached to the cell wall in these mutants, as previously suggested (Turner et al., 1984; Cherniak, 1988).

Differences in *C. neoformans* cell wall composition *in vitro* and *in vivo*

C. neoformans titan cells from *in vivo* mouse models possess a thicker cell wall compared to *in vitro* grown cells (Zaragoza et al., 2010). Furthermore, we previously showed that titan cells have increased cell wall chitin compared to *in vivo* typical cells (Wiesner et al., 2015). However, it was not known whether other cell wall components also change in *in vivo* *C. neoformans* cells. We used hydrolysis approach-3 to measure the cell wall composition of the wild type strain KN99 α grown *in vitro*, and isolated from the lungs of infected mice. *In vivo* cells contained significantly higher glucosamine and less glucose than *in vitro* grown cells (Fig. 3A and B). *In vivo* cryptococcal cells also contained increased mannose and xylose compared to *in vitro* grown cells (Fig. 3C and D). Galactose content was slightly higher in *in vitro* cells than in *in vivo* cells (Fig. 3E). To determine whether these differences were due to the capsule on the *in vivo* cells, we also analyzed the cell wall composition of irradiated *in vivo* cells in which most of the capsule was removed (Fig. 4). Irradiated *in vivo* cells contained similar mannose and xylose as the *in vitro* cells (Fig. 3C and D), showing the increase in these components in the non-irradiated *in vivo* cells was due to additional capsule formation. Galactose was not detected in irradiated cells suggesting that galactose is a capsular component that was removed by the irradiation (Fig. 3E).

These data show that *in vivo* and *in vitro* cells have different cell wall and capsule composition (Fig. 3A–E). *In vivo* cells have increased glucosamine and decreased glucose indicating higher chitin and lower glucan content of the *in vivo* cell wall compared to the *in vitro* cell wall. In addition, the *in vivo* cells have a thickened capsule with increased levels of mannose, xylose and galactose when compared to *in vitro* cells.

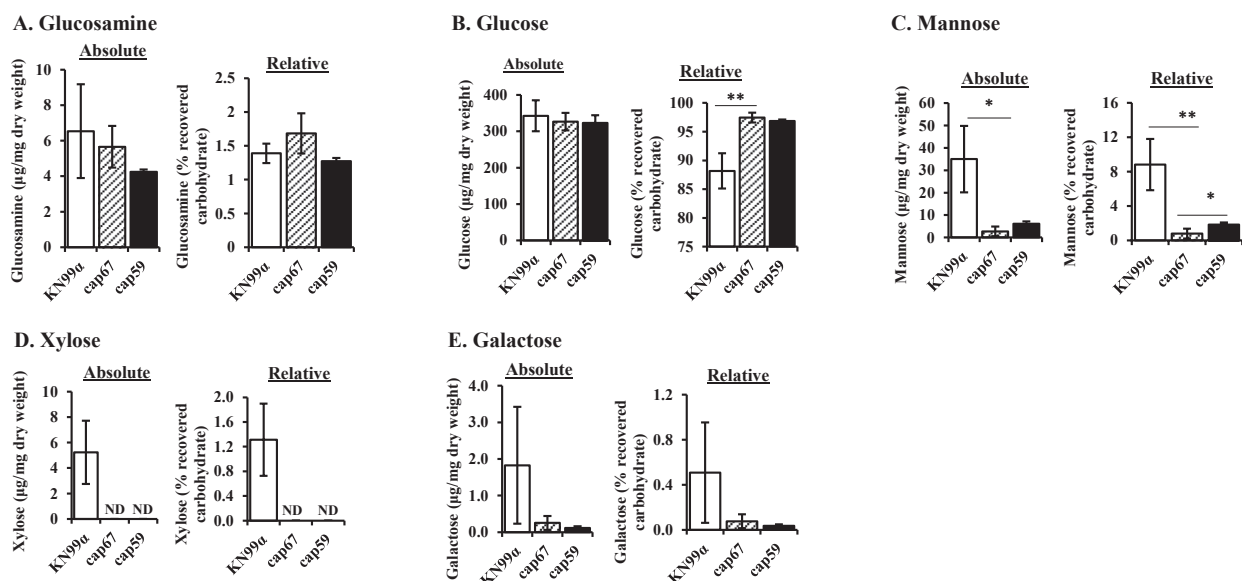


Fig. 2. Cell wall composition of *in vitro* grown *C. neoformans* KN99 α , *cap67* and *cap59* strains. *C. neoformans* KN99 α , *cap67* and *cap59* strains were grown *in vitro* overnight at 30 °C in YPD broth. Cell walls were extracted, freeze-dried and hydrolyzed with TFA. Absolute monosaccharide amounts are expressed as micrograms per 1 mg of dry weight. Relative carbohydrate content expressed as percentages of the recovered total carbohydrate materials. Glucosamine (A), glucose (B), mannose (C), xylose (D), and galactose (E) were quantified using HPAEC-PAD. Data represent mean of results from at least 3 replicates (N = 5 for KN99 α , N = 3 for *cap67* and *cap59*). T-test was used to compare mean of the wild type (WT) strain to those of acapsular mutants. * P < 0.05, ** P < 0.01. YPD: yeast-peptone-dextrose, ND: not detected.

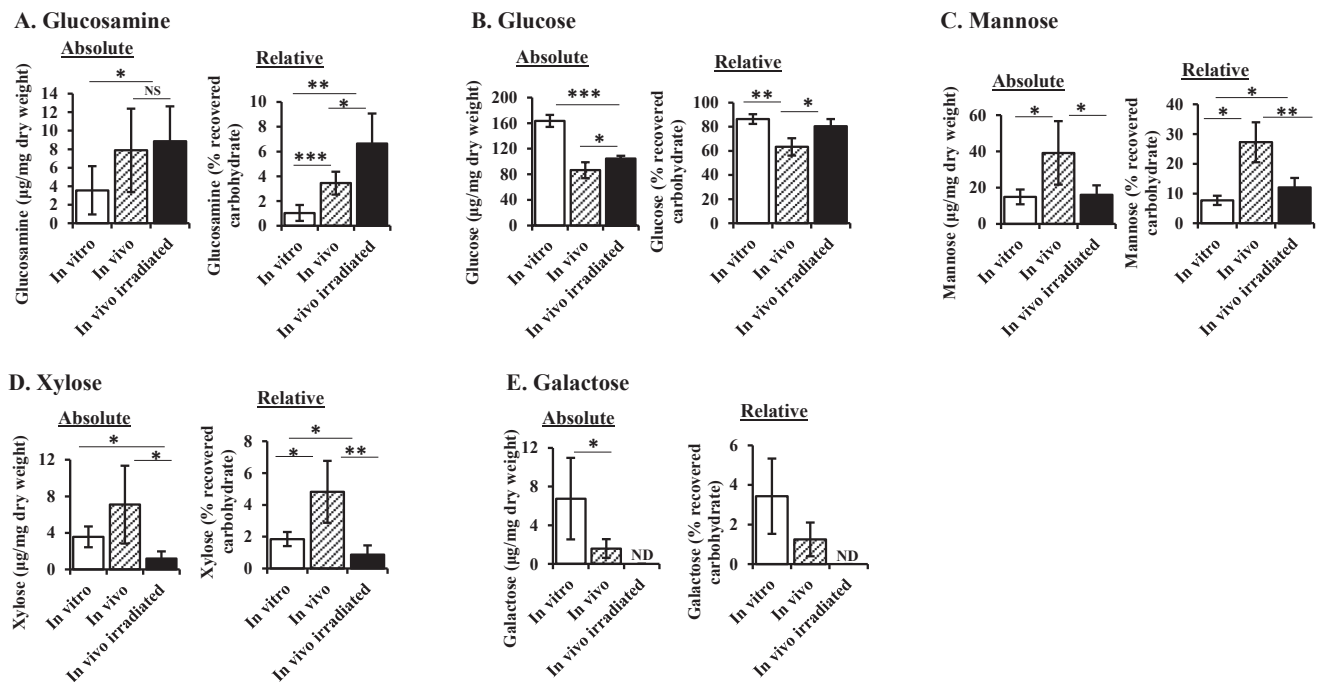


Fig. 3. Differences between cell wall and capsule carbohydrate composition of *in vivo* and *in vitro* *C. neoformans* cells. *C. neoformans* KN99α strain was either grown *in vitro* overnight at 30 °C in YPD broth, or isolated from lungs of infected mice. A portion of the *in vivo* *C. neoformans* cell sample was treated with γ -radiation for 45 min to remove the capsule polysaccharides. Cell walls were extracted from irradiated and non-irradiated *C. neoformans* cells, freeze-dried and hydrolyzed with TFA. Absolute monosaccharide amounts are expressed as micrograms per 1 mg of dry weight. Relative carbohydrate content is expressed as percentage of the recovered total cell wall material. Glucosamine (A), glucose (B), mannose (C), xylose (D), and galactose (E) were quantified using HPAEC-PAD. Data represent mean of at least 4 replicates per group. T-test or Mann-Whitney test were used to compare mean carbohydrate composition of *in vitro*-grown, *in vivo* and *in vivo* irradiated *C. neoformans* cells. *P < 0.05, **P < 0.01, ***P < 0.001. YPD: yeast-peptone-dextrose, TFA: trifluoroacetic acid.

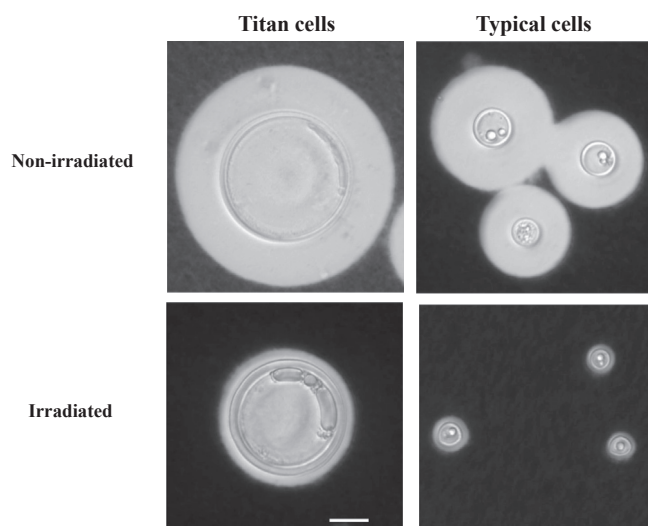


Fig. 4. Irradiation removes *C. neoformans* capsule. *C. neoformans* KN99α cells were isolated from the lungs of infected mice 14 days after infection. A portion of the cell sample was treated with γ -radiation (560 Gy) for 45 min to remove capsule polysaccharides. Both irradiated and non-irradiated cells were stained with India ink, and imaged using differential interference contrast (DIC). Scale bar = 10 μ m.

Titan and typical *C. neoformans* cells have different cell wall composition

In vivo *C. neoformans* cells vary in size, from cells less than 3 μ m in cell body diameter to cells more than 100 μ m in diameter (Feldmesser et al., 2001; Okagaki et al., 2010; Zaragoza et al., 2010; Alanio et al., 2015). The *in vivo* *C. neoformans* cells were divided into two groups; typical cells (less than 10 μ m of cell body diameter), and titan cells (greater than 10 μ m in cell body diameter). The titan cell population has increased DNA content, a dense cross-linked capsule and thick cell wall (Okagaki et al., 2010; Zaragoza et al., 2010). We examined the cell wall

composition of both typical and titan cells after γ -irradiation to remove most of the capsule. Glucosamine was significantly increased in titan cell walls compared to typical cells (Fig. 5A), confirming previous studies showing increased levels of chitin in titan cells (Wiesner et al., 2015). While the absolute glucose content was higher in titan cells (Fig. 5B), the relative cell wall glucose content as percentage of the total recovered cell wall material decreased in titan cells (78.5%) compared to typical cell walls (93.8%) (Fig. 5B). The cell wall of titan cells also contained significantly higher mannose and xylose (Fig. 5C and D). Galactose was not detected in these γ -irradiated cell wall samples (Fig. 5E), suggesting no GXMGal capsule component remained associated with the cell wall after γ -irradiation. The detected mannose and xylose could be structural components of the actual *C. neoformans* cell wall, cell wall mannoproteins, or the small portion of the capsule still attached to the irradiated *C. neoformans* cells (Fig. 4), also described previously (Zaragoza et al., 2010).

Capsule composition differences between titan and typical cells

Treatment of *C. neoformans* cells with γ radiation removes most of the capsule (Fig. 4). We analyzed the composition of this dissociated capsule from *in vivo* typical and titan cells. Glucosamine was not detected in the dissociated capsule of either titan or typical cells (Fig. 6A), suggesting the capsule preparation was not contaminated with cell wall debris. Analysis of the dissociated capsule revealed that titan cell capsule contained significantly higher relative mannose content than that of typical cells (Fig. 6C). Conversely, the relative amount of glucose and galactose decreased in the capsule of titan cells compared to typical cells, although this difference was not significant when absolute values were compared (Fig. 6B and E). Absolute xylose content was increased in the titan cell capsule (Fig. 6D), however the relative xylose content (percentage) was not significantly different from typical cells (15.1% vs 15.7% respectively) (Fig. 6D). These results show that, in addition to the cell wall composition, capsule composition differs between titan

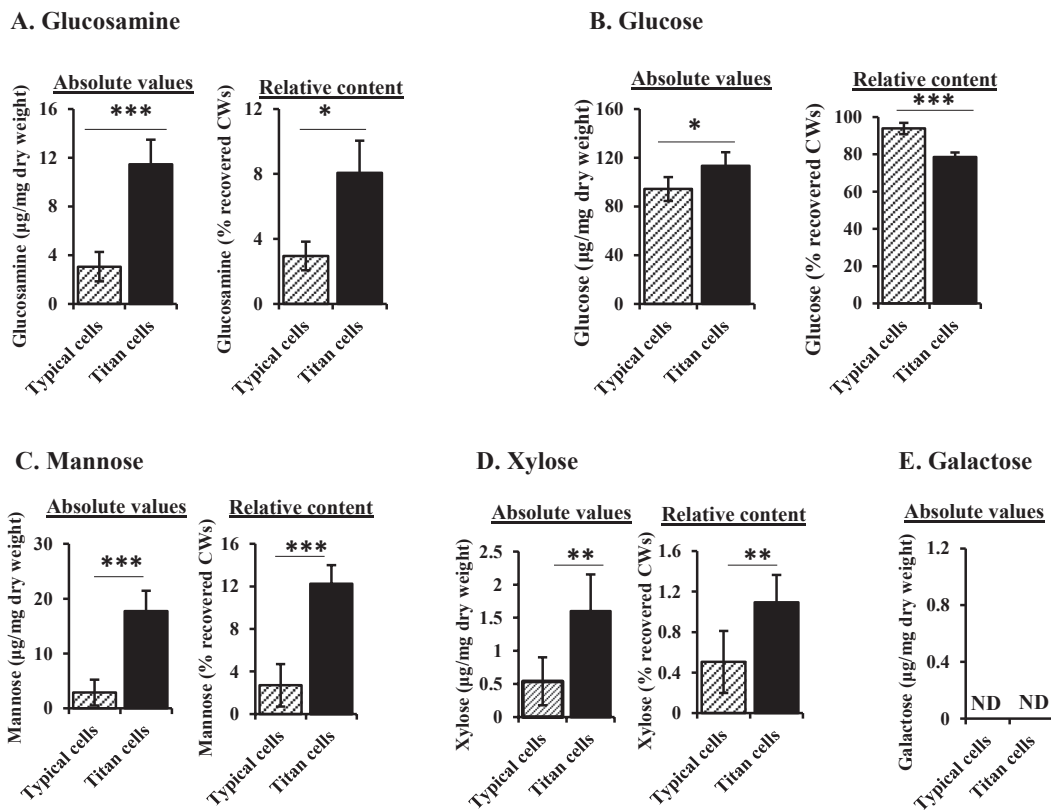


Fig. 5. Cell wall composition of *C. neoformans* typical and titan cells. *C. neoformans* KN99 α cells were isolated from lungs of infected mice. Titan cells were separated from typical cells by filtration and treated with γ -radiation for 45 min to remove capsule polysaccharides. Cell walls were extracted from irradiated *C. neoformans* cells, freeze-dried and hydrolyzed with TFA. Absolute monosaccharide amounts are expressed as micrograms per 1 mg of dry weight. Relative carbohydrate content is expressed as percentage of the total recovered cell wall material. Glucosamine (A), glucose (B), mannose (C), xylose (D), and galactose (E) were quantified using HPAEC-PAD. Data represents the mean of 4 replicates per group. T-test was used to compare mean carbohydrate composition of typical and titan cell walls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. TFA: trifluoroacetic acid, ND: not detected.

and typical cells, with titan cells containing higher mannose and xylose than typical cells.

Discussion

Morphology changes play an important role in the initiation and maintenance of *C. neoformans* infections. During infection, *C. neoformans* cells of varying cell body size and capsule size are observed (Feldmesser et al., 2001; Okagaki et al., 2010; Zaragoza et al., 2010; Alanio et al., 2015). These structural and morphological characteristics allow *C. neoformans* to evade and modulate the host immune system (Almeida et al., 2001; Okagaki and Nielsen 2012; Wiesner et al., 2015). We previously showed that increases in chitin, a *C. neoformans* cell wall component, during titan cell formation induces a detrimental Th2 immune response in mice (Wiesner et al., 2015). However, it was not known if there are other cell wall changes in *in vivo C. neoformans*. Here we examined the cell wall composition of both *in vitro*-grown and *in vivo C. neoformans* cells to identify changes that occur when *C. neoformans* transitions from *in vitro* to *in vivo* growth conditions. Cell walls of *C. neoformans* cells from infected mice contained significantly higher glucosamine, and lower glucose than the cell wall of *in vitro*-grown cells. In addition to differences between *in vitro* and *in vivo* cell walls, we found that *in vivo* titan cells have a different cell wall composition than that of *in vivo* typical cells. Titan cell walls contained higher glucosamine, mannose and xylose than typical cells.

The HPLC analysis used here identified changes in the total carbohydrate content between different *C. neoformans* cell types. However, this technique cannot determine the origin of the observed changes for specific polysaccharides with similar monomer carbohydrate components. For example, we observed that the cell wall glucosamine content

increased in *in vivo C. neoformans* cells, but HPLC cannot determine whether these changes in glucosamine come from changes in chitin, chitosan, chito-oligomers, or glucosamine-derived polysaccharides. We also observed changes in total cell surface glucose and mannose content. Similarly, our method cannot specify if these carbohydrate changes reflect changes in α - or β -glucans, or changes in cell wall mannans, mannoproteins, or capsule components (GXM, GXMGal) trapped in the cell wall, respectively. In spite of these limitations, this study identified substantial cell surface modifications that occur during *C. neoformans* infection and likely dramatically impact the way *C. neoformans* is perceived by the host immune system.

The increase in total glucosamine in *in vivo* cell wall, especially in titan cells, supports our previous observation using different methods (Wiesner et al., 2015) that *C. neoformans* has increased chitin (polymer of *N*-acetylglucosamine) *in vivo*. This increase in chitin correlates with a detrimental Th2 immune response in mice (Wiesner et al., 2015). In addition, the glucosamine-derived molecules chitin and chitosan are known to play key roles in cell wall integrity, capsule structure/attachment to the cell wall, and in the virulence of *C. neoformans* (Baker et al., 2007; Rodrigues et al., 2008; Fonseca et al., 2009; Baker et al., 2011; Wiesner et al., 2015). Thus, modification of *in vivo C. neoformans* not only changes cell wall composition but also interactions with the host immune system and the resulting immune response.

Increase in fungal cell wall chitin can also affect how the fungus reacts to antifungal drugs. In another fungal pathogen, *Candida albicans*, increased chitin is associated with increased resistance to echinocandins, a class of antifungal drugs targeting cell wall β ,1–3 glucans (Walker et al., 2010). The observed increase in *in vivo* cell wall glucosamine, and decreased glucose mirror cell wall changes observed when *C. albicans* is treated with Caspofungin, an antifungal that inhibits cell

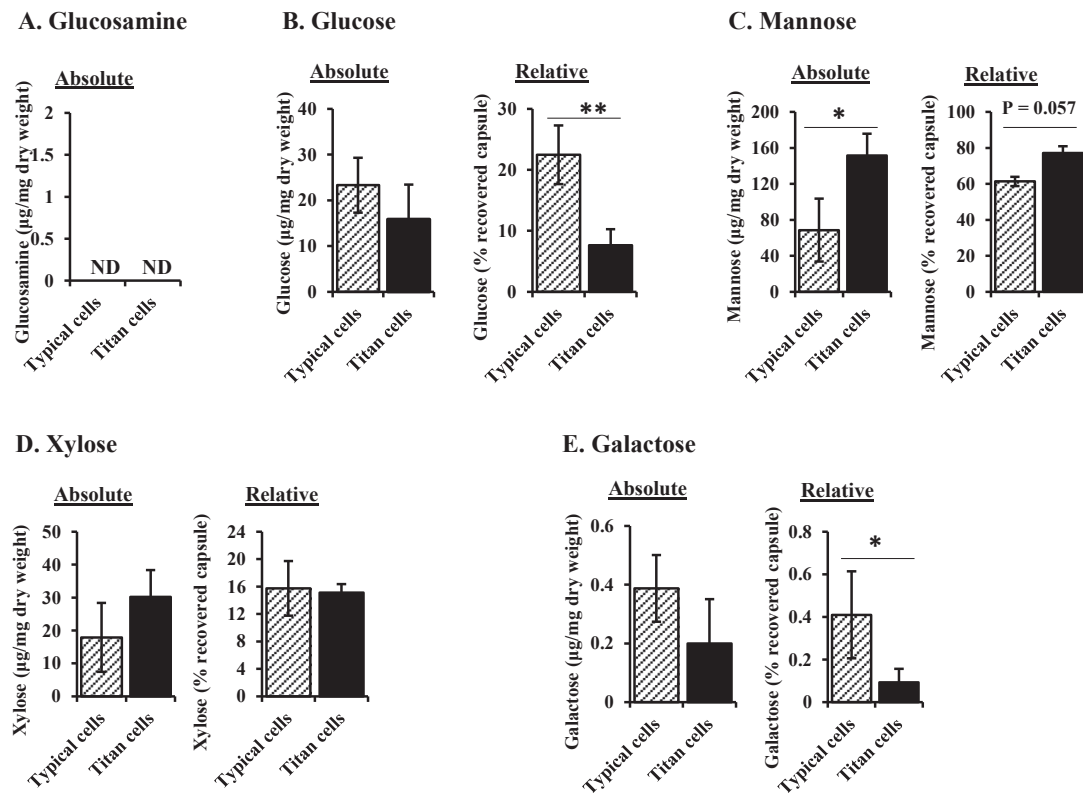


Fig. 6. Typical and titan capsule composition. *C. neoformans* KN99 α cells were isolated from lungs of infected mice. Titan cells were separated from typical cells by filtration and treated with γ -radiation for 45 min to remove the capsule polysaccharides. The dissociated capsule was collected in the supernatant, freeze-dried and hydrolyzed with TFA. Absolute monosaccharide amounts were expressed as micrograms per 1 mg of dry weight. Relative carbohydrate content is expressed as percentage of the total recovered capsule material. Glucosamine (A), glucose (B), mannose (C), xylose (D), and galactose (E) were quantified using HPAEC-PAD. Data represent mean of at least 3 replicates in each group. (N = 4 for typical cells and N = 3 for titan cells). T-test or Mann-Whitney test were used to compare mean carbohydrate composition of the capsule of typical and titan cells. *P < 0.05, **P < 0.01. TFA: trifluoroacetic acid, ND: note detected.

wall β ,1–3 glucan synthesis (Walker et al., 2008), and likely explains why echinocandin drugs show promising anti-cryptococcal activity *in vitro* (Bartizal et al., 1997; Franzot and Casadevall, 1997), but are ineffective *in vivo* (Abruzzo et al., 1997).

Glucans are polysaccharides of glucose monomers, and the cell wall of *C. neoformans* is composed of both α - and β -glucans (Bacon et al., 1968; James et al., 1990; Reese et al., 2007). In our experiments, we showed that *in vivo* cell wall contains less glucose than *in vitro* cell wall, and titan cell wall contained less glucose than typical cell wall. Cell wall α -glucans are important for capsule attachment to the *C. neoformans* cell body (Reese and Doering, 2003). Disrupting α -1,3-glucan production in *C. neoformans* produces cells that shed capsule material into the environment but lack the surface capsule normally attached to the cell body (Reese and Doering, 2003; Reese et al., 2007). Titan cells have a thick and highly cross-linked capsule attached to their cell wall (Fig. 7 and (Zaragoza et al., 2010)). Thus, the reduction we see in total glucose content of the titan cell wall is unlikely due to a lack of α -glucans as capsule attachment appears to be unaffected. *C. neoformans* cell wall also contains two types of β -glucans; β -1,3-glucans and β -1,6-glucans (James et al., 1990; Feldmesser et al., 2000). The proportion of each β -glucan type can change depending on the cryptococcal strain, growth condition, and exposure to caspofungin, an antifungal drug that targets β -1,3-glucans (Feldmesser et al., 2000). The specific function of β -1,6-glucan in the *C. neoformans* cell wall is not well understood, but disruption of β -1,6-glucan production results in capsules that are enlarged, more diffuse, and more permeable than wild type cells (Gilbert et al., 2010). *In vivo* titan cells have large cross-linked capsules with reduced permeability (Zaragoza et al., 2010), suggesting that β -1,6-glucans are either unaffected or increased in our *in vivo* samples. Fungal β -glucans are recognized by the Dectin-1 receptor of host immune cells (Brown

et al., 2003; Gow et al., 2007). Fungal pathogens such as *C. albicans* are known to evade Dectin-1 recognition by switching to hyphal forms that do not expose β -glucans at the cell surface (Gantner et al., 2005). Similarly, our data show *C. neoformans* cell wall contains lower glucan levels, which likely explains why Dectin-1 receptor has no function in the immune response to *C. neoformans* (Nakamura et al., 2007). The combination of low glucan in *in vivo* cell walls and a thick capsule likely reduce the surface exposure of β -glucans on *in vivo* *C. neoformans* cells and prevent direct contact between β -glucans and the Dectin-1 receptor. Direct contact between Dectin-1 receptor and the microbial surface, not soluble ligands, is required for the activation of the receptor and subsequent signaling (Goodridge et al., 2011; Lin et al., 2016).

Large increases in mannose were detected in the titan cell wall, and could be components of cell wall mannans, mannoproteins, or capsule components trapped in the cell wall during extraction. Mannoproteins are located in the inner layer of the *C. neoformans* cell wall (Vartivarian et al., 1989). These mannoproteins were suggested to be non-structural cell wall components that eventually migrate to the cell surface, and are secreted in the environment (Vartivarian et al., 1989). Mannoproteins are also found in the supernatant and cell wall extracts from *in vitro* *C. neoformans* cultures, and small amounts of mannose were found among monosaccharides extracted from *C. neoformans* cells (Murphy et al., 1988; James et al., 1990; Levitz et al., 2001; Park et al., 2012). Mannose detected in titan cell walls accounted for approximately 12% of the total cell wall. This amount seems high for residual capsule GXM and GXMGal transiting the cell wall for subsequent secretion. An alternate hypothesis is that titan cell walls contain a significant amount of mannoproteins or structural mannans. The presence of significant mannoprotein fractions in the cell walls could also explain the presence

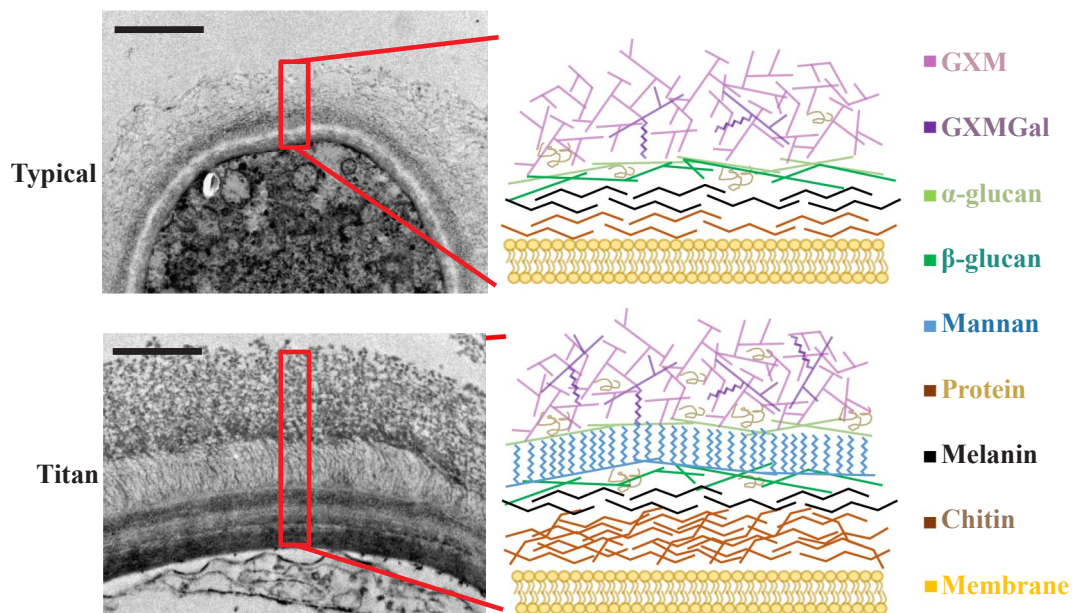


Fig. 7. Schematic representation of *C. neoformans* typical and titan cell surface. Electron microscope (EM) images and schematic diagram of titan and typical cell wall and capsule. The cell wall of *C. neoformans* is surrounded by a thick polysaccharide capsule composed of GXM and GXMGal. The cell wall is composed of polysaccharides arranged into inner and outer layers. The inner wall contains chitin, chitosan, β -glucans, melanin and cell wall proteins. The outer layer is composed of α -glucans that mediate the attachment of the capsule to the cell wall. Based on our findings, titan cells contain more chitin in their inner wall, and the outer wall of titan cell walls also have a layer of mannan fibrils in addition to α -glucans. Attached to α -glucans is the capsule that is more dense and highly cross-linked in titan cells than in typical cells. Scale bar = 1 μ m.

of xylose as previous studies showed that *C. neoformans* mannoproteins might contain xylose and xylose-phosphate structures (Reilly et al., 2011; Park et al., 2012). Some mannoproteins are recognized as primary cryptococcal antigens (Murphy et al., 1988; Levitz et al., 2001; Mansour et al., 2002), while other mannoproteins such as MP4 and App1 dampen the host immune response against *C. neoformans* (Coenjaerts et al., 2001; Luberto et al., 2003). During infection, titan cells confer protection from phagocytosis to the entire cryptococcal population (Crabtree et al., 2012; Okagaki and Nielsen, 2012). Whether this protection is associated with increased production of specific mannoproteins and their impact on immune responses poses an intriguing idea. Finally, and perhaps most intriguing is the possibility that titan cells contain increased structural mannans. Studies in both *Pneumocystis* and *C. albicans* have revealed structural mannan layers that generate distinct vertical fibril structures when imaged by electron microscopy (EM) (Gow et al., 2011; Erwig and Gow, 2016; Ma et al., 2016). EM images of titan and typical cell walls (Fig. 7) show similar vertical structures on the exterior surface of the titan cell wall (adjacent to the capsule), and these structures are absent in typical cells.

Capsule dissociated from titan cells contained more mannose, but less galactose and glucose, than capsule from typical cells. All these carbohydrate monomers were previously found to be components of the *Cryptococcus* capsule (Cherniak and Sundstrom, 1994; Vaishnav et al., 1998; Frases et al., 2008). Glucose represented up to 20% of capsule dissociated from typical cells. This glucose may represent glucans released from the cell wall by γ -radiation treatment (Frases et al., 2008). Alternatively, the glucose may be a structural component of the capsule essential for the attachment of the capsule to the cell wall. Our observation that glucose levels were higher than xylose, a known capsule component of GXM and GXMGal, and the absence of glucosamine in the detached capsule argue for the latter hypothesis. Differences in capsule polysaccharides between *in vivo* typical and titan cells may represent different epitopes between these two types of cells, and likely impact how these two types of cells are perceived by and/or interact with the host immune system.

In addition to the carbohydrate content of the known cell wall and capsule polysaccharides, we observed smaller amounts of galactosamine in both *in vitro* and *in vivo* cell extracts. To our knowledge

galactosamine has not been previously detected in *C. neoformans* cell wall or capsule, although one study identified a *UGT1* (UDP-galactose transporter) gene responsible for the transport of both UDP-galactose and UDP-galactosamine *in vitro* (Li et al., 2017). This finding together with our observation suggest the presence of both galactose and galactosamine in *C. neoformans* polysaccharides. The significance of this observation in relation to possible differences in host immune response remain to be explored. Studies in *Aspergillus fumigatus* found that galactosaminogalactan (GAG), a polysaccharide of galactose and *N*-acetyl-galactosamine, impacts the immune response (Fontaine et al., 2011; Gravelat et al., 2013; Lee et al., 2015). This observation supports the need to study galactosamine in *C. neoformans* and its potential role in pathogenesis.

Our data, both EM images and HPLC analysis, and previous studies support a model in which *C. neoformans* typical and titan cells possess a different cell surface (Fig. 7). Upon initial pulmonary infection, a subset of *C. neoformans* cells differentiate into enlarged titan cells with a thick cell wall and a highly crosslinked capsule (Feldmesser et al., 2001; Okagaki et al., 2010; Zaragoza et al., 2010). The cell wall of *C. neoformans* is composed of (a) an inner cell wall layer that contains chitin/chitosan, melanin and β -glucan, and (b) an outer layer that contains α -glucans that mediate the attachment the capsule to the cell wall (James et al., 1990; Nosanchuk et al., 2000; Rosas et al., 2000; Reese and Doering, 2003; Banks et al., 2005; Baker et al., 2007; Reese et al., 2007; Erwig and Gow, 2016). Titan cell walls contain increased chitin and melanin and less β -glucan than typical cells (Fig. 5 and (Zaragoza et al., 2010; Wiesner et al., 2015)). EM images suggest that titan cells, but not typical cells, also possess structural mannan in their outer cell wall in addition to α -glucans (Figs. 5 and 7). Lastly, our results suggest that titan cells have increased capsule GXM, but similar GXMGal than typical cell capsule (Figs. 6 and 7). In addition to polysaccharides the *C. neoformans* cell surface also contains proteins and lipids (Vartivarian et al., 1989; Levitz et al., 2001; Djordjevic et al., 2005), however these were not analyzed in the present study. The proposed model of *in vivo* *C. neoformans* cell wall and capsule presented in Fig. 7 was deduced from our observations, together with observations from earlier studies that measured total carbohydrate content. The exact organization and localization of individual cell wall and capsule components needs to be

verified by further studies.

The *in vivo* cell surface analysis performed here was from *C. neoformans* cells isolated from the lungs of infected mice. Previous studies specifically examining capsule structure have shown differences based on anatomical location with differences in capsule structure of cells grown *in vitro* and those isolated from the lungs, spleen, kidney, liver, heart and brain of infected mice (Rivera et al., 1998; Charlier et al., 2005). Further research is necessary to investigate whether similar cell wall changes occur across different organs. Importantly, we have shown dramatic differences in the composition of the cell wall *in vivo* versus *in vitro*, and between different *in vivo* cell types that likely impact interaction with the host. Better understanding of how these differences alter the host-pathogen interaction and the immune response will highlight key changes necessary for *C. neoformans* pathogenesis.

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Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tcs.2017.12.001>.

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