Bates, Steven, Hall, Rebecca A., Lenardon, Megan D., MacCallum, Donna M., Wagener, Jeanette, Lowman, Douglas W., Kruppa, Michael D., Williams, David L., Odds, Frank C., Brown, Alistair J. P. and and others (2013) The Mnn2 Mannosyltransferase Family Modulates Mannoprotein Fibril Length, Immune Recognition and Virulence of Candida albicans. PLoS Pathogens, 9 (4). e1003276. ISSN 1553-7374.
The Mnn2 Mannosyltransferase Family Modulates Mannoprotein Fibril Length, Immune Recognition and Virulence of Candida albicans

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Abstract
The fungal cell wall is the first point of interaction between an invading fungal pathogen and the host immune system. The outer layer of the cell wall is comprised of GPI anchored proteins, which are post-translationally modified by both N- and O-linked glycans. These glycans are important pathogen associated molecular patterns (PAMPs) recognised by the innate immune system. Glycan synthesis is mediated by a series of glycosyl transferases, located in the endoplasmic reticulum and Golgi apparatus. Mnn2 is responsible for the addition of the initial α1,2-mannose residue onto the α1,6-mannose backbone, forming the N-mannan outer chain branches. In Candida albicans, the MNN2 gene family is comprised of six members (MNN2, MNN21, MNN22, MNN23, MNN24 and MNN26). Using a series of single, double, triple, quintuple and sextuple mutants, we show, for the first time, that addition of α1,2-mannose is required for stabilisation of the α1,6-mannose backbone and hence regulates mannan fibril length. Sequential deletion of members of the MNN2 gene family resulted in the synthesis of lower molecular weight, less complex and more uniform N-glycans, with the sextuple mutant displaying only un-substituted α1,6-mannose. TEM images confirmed that the sextuple mutant was completely devoid of the outer mannan fibril layer, while deletion of two MNN2 orthologues resulted in short mannan fibrils. These changes in cell wall architecture correlated with decreased proinflammatory cytokine induction from monocytes and a decrease in fungal virulence in two animal models. Therefore, α1,2-mannose of N-mannan is important for both immune recognition and virulence of C. albicans.

Introduction
Candida albicans is a polymorphic fungus that forms part of the natural human microflora. However, many adverse conditions result in predisposition to oral and vaginal infections and, under circumstances where the host immune system becomes severely compromised as a consequence of malignancy, trauma or chemotherapy, C. albicans can invade underlying epithelial cells and disseminate via the bloodstream and cause systemic disease. The associated mortality rate of systemic disease is approximately 30–40%, which is higher than that observed for many bacterial systemic infections, making C. albicans a major pathogen of the immunocompromised and a significant global health burden [1–3].

The fungal cell wall is a highly dynamic structural organelle essential for maintaining cell shape and for protection against the environment. The cell wall is also the first point of contact between the fungus and the host, and as a result the cell wall is pivotal for fungus-host interactions and immune recognition. The cell wall is comprised of an inner skeletal layer of chitin and β-glucans (β1,3- and β1,6-glucan) and an outer layer of highly glycosylated mannanproteins [4–6]. These proteins are decorated with linear O-linked mannan and highly branched N-linked mannan, which can be elaborated with additional mannan side chains attached via a phosphodiester linkage known as phosphomannan (PM). The mannan fraction of the cell wall is important for adhesion, cell wall integrity, immune recognition and comprises up to 40% of the cell wall dry weight [7–12]. All of the major cell wall carbohydrate components of fungal walls serve as pathogen associated molecular patterns (PAMPs), which are recognised by the innate immune system through pattern recognition receptors (PRRs) on the surface of immune effector cells [5,13–17]. Mannans, which are exposed on the outer surface of the cell wall, are recognised by a wide range of PRRs depending on the immune cell type. These

Citation: Hall RA, Bates S, Lenardon MD, MacCallum DM, Wagener J, et al. (2013) The Mnn2 Mannosyltransferase Family Modulates Mannoprotein Fibril Length, Immune Recognition and Virulence of Candida albicans. PLoS Pathog 9(4): e1003276. doi:10.1371/journal.ppat.1003276
Editor: Marta Feldmesser, Albert Einstein College of Medicine, United States of America
Received September 24, 2012; Accepted February 6, 2013; Published April 25, 2013
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Funding: This work was funded by the Wellcome Trust (080088, 086827, 075470 and 099215), and by a FP7-2007-2013 grant agreement (HEALTH-F2-2010-260338-ALLFUN). MDL was supported by an MRC New Investigator Award (MR/J008230/1). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Competing Interests: I have read the journal’s policy and have the following conflicts: Douglas W. Lowman is an employee of AppRidge International, LLC. The noted receipt of funding from AppRidge does not alter our adherence to all PLoS Pathogens policies on sharing data and materials.
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PLOS Pathogens | www.plospathogens.org 1 April 2013 | Volume 9 | Issue 4 | e1003276
Candida albicans is an opportunistic fungal pathogen of humans, which can cause superficial infections of the oral and genital epithelium or fatal systemic disease in immune compromised individuals. The cell wall of C. albicans consists of an inner layer of chitin and β-glucan and an outer fibrillar layer of highly glycosylated mannoproteins. These mannose-rich carbohydrates play crucial roles in fungal immune recognition. Deletion of genes encoding the Mnn2 family of α1,2-mannosyltransferases, which are involved in the biosynthesis of N-mannan outer chains, demonstrated that the addition of mannann side chains is necessary for the stabilisation and extension of the N-mannan backbone. Mutants of the MNN2 gene family had marked alterations in mannann microfibril length. Reductions in the degree of N-mannann branching resulted in decreased immune recognition by monocytes, whilst complete removal of the branched outer chains increased β-glucan exposure, and correlated with an attenuated virulence. Therefore, the Mnn2 mannosyltransferase family is critical for microfibril structure, immune recognition and virulence.

include toll-like receptor 4 (TLR4), TLR2, and the C-type lectins macrophage mannose receptor (MMR), MINCLE/CLEC4E, Dectin-2, galectin 3, and DC-SIGN/CD209 [16,18]. β-Glucan, which is in the inner sub-mannann wall layer, but is exposed at bud scars, is recognised by the lectin-like receptor, Dectin-1 [13,19]. More recently, chitin has been shown to participate in the immune recognition of C. albicans, possibly by damping the immune response during the resolution of infections [20], but the receptor(s) mediating this interaction are unknown.

Pioneering studies in Saccharomyces cerevisiae identified a series of mannosyltransferases, which are involved in mannann biosynthesis, and many of these enzymes are conserved in C. albicans and other pathogenic fungi. However, mannann structure differs significantly between fungal species, and this has important consequences for host-fungus interactions, and in the future development of vaccines and diagnostics. For example, S. cerevisiae O-mannan is comprised of two or more α1,2-mannann units, which are capped by several α1,3-mannann units [21–23], while the O-mannann in C. albicans is comprised solely of α1,2-mannann [11,24–25]. In addition, the N-mannann extracted from C. albicans has a higher molecular weight than that extracted from S. cerevisiae, includes β1,2-mannann in both the acid-labile and acid-stable fractions as well as higher levels of phosphomannann [26]. Therefore, studies of mannann biosynthesis in C. albicans provide important and specific insights into the host interactions and immune recognition of this fungus.

Many mannosyltransferases are fungal-specific and are encoded by gene families. This complicates investigations of mannann biosynthesis due to possible functional redundancy between family members. In addition, families of mannosyltransferases can catalyse multiple reactions [9] whose structure-function relationships cannot yet be determined from their amino acid sequences or by structural proteomics. Progress in this field therefore requires a careful, detailed, simultaneous dissection of the function of entire gene families.

As proteins transverse though the endoplasmic reticulum (ER) the α-mannann precursor (Glc3ManαGlcNAc2) is attached to specific asparagine residues of the protein. This core is subsequently processed in the ER by glycosidasers resulting in the mature α-mannann core (ManαGlcNAc2) [10]. Heavily branched outer chains are subsequently attached to the core as the protein passes through the Golgi. Initially, a single α1,6-mannose unit is attached to the core by Och1 [7]. This single mannose residue is then elaborated with α1,6-mannose residues, added by mannan polymerases I and II predominately composed of Mnn9, and form the α1,6-backbone [27]. The α1,6-backbone is further elaborated with side chains consisting of α1,2- and α1,3-mannose residues, with the α1,2-mannose extending out from the backbone. In S. cerevisiae, the initial α1,2-mannose unit is attached to the backbone by mannosyltransferase, Mnn2, which is then elaborated with additional α1,2-mannose units by Mnn5 [28]. In C. albicans, these outer chains are then capped with either β1,2-mannose or α1,3-mannose units by Bmt1, Bmt3 and Mnn1 [29–30]. Deletion of S. cerevisiae inhibits the addition of α1,2-mannose onto the mannann backbone, thus preventing the elaboration of α-N-mannann outer chains [28]. In C. albicans, complete removal of the N-mannann outer chains has significant consequences for growth, cell morphology and immune recognition [7], although it is not known which of the deleted carbohydrate epitopes in the och1Δ mutant account for the lack of immune recognition.

Here, we functionally dissect the six-member MNN2 gene family in C. albicans. To fully deduce the function(s) of each glycosyltransferase in α-N-mannann biosynthesis, we characterised a set of single, double, triple, quintuple and sextuple mutants. Deletion of even single MNN2 gene family members reduced cell wall integrity and immune recognition. These effects were enhanced when multiple family members were deleted, with the sextuple mutant showing severe defects in cell wall integrity, virulence and immune recognition. We show that mannanprotein fibril length was progressively shortened in multiple mnn2 family mutant backgrounds, that Mnn2 and Mnn26 are required for the addition of the initial α1,2-mannose residue to the α1,6-mannose backbone and that Mnn21, Mnn22, Mnn23 and Mnn24 are required for the addition of subsequent α1,2-mannose units onto the α1,6- α1,2-mannose scaffold.

The six-member C. albicans Mnn2 mannosyltransferase family

To identify orthologues of S. cerevisiae Mnn2 in C. albicans, a Blastp search [31] of ScMnn2 was performed against the C. albicans genome in the Candida Genome Database (www.candidagenome.org). Six orthologs were identified (orf19.2347, orf19.1011, orf19.3803, orf19.4874, orf19.1995, orf19.6692) and designated MNN2, MNN21, MNN22, MNN23, MNN24 and MNN26. A multiple sequence alignment for the six putative Mnn2 orthologues was generated with ClustalW2 (version 2.0.12) and from this alignment a phylogram for the C. albicans MNN2 gene family was created (Figure S1). This phylogram identified three sub-groups: group 1 comprised of MNN2, MNN22 and MNN23, group 2 comprised of MNN21 and group 3 comprised of MNN24 and MNN26. Initially each gene in the family was deleted individually and then, to test for possible functional redundancy, a series of multiple gene-disrupted mutants was constructed using the mini ura blaster protocol. This series was comprised of a double mutant for group 3 members (mnn24Δ/mnn26Δ), double mutants within groups and 3 (mnn22Δ/mnn26Δ and mnn23Δ/ mnn26Δ), a triple mutant for group 1 (mnn2Δ/mnn22Δ/mnn23Δ), a quintuple mutant for groups 1 and 3 (mnn2Δ/mnn22Δ/mnn23Δ/mnn24Δ/mnn26Δ) and the sextuple mutant (mnn2Δ/mnn22Δ/mnn23Δ/mnn24Δ/mnn26Δ/mnn21Δ). The multiple mutants were exposed to several rounds of 5-FOA treatment. Therefore, multiple colonies of each strain were tested for chromosomal loss or aneuploidy by a PCR based method [32–33]. No significant
The MNN2 gene family regulates mannan fibril length

To visualise changes in the cell wall structure, we performed two types of microscopic analyses. First, fluorescence microscopy was performed on fixed cells stained with either wheat germ agglutinin (WGA)-FITC, concanavalin A (ConA)-TR or Fc-Dectin-1 to assess whether there were any significant changes in the exposure of chitin, mannan or β-glucan on the C. albicans cell surface (Figure 3). Fluorescence microscopy showed that WGA-FITC stained the lateral cell wall of the sextuple mutant, but only stained the chitin exposed in the bud scars of the parental control strain (Figure 3A). This reflects the large size of WGA that prevents deep penetration of the wall so that only chitin that is exposed near the surface of the cell wall is accessible to this lectin. The increased amount of WGA-FITC staining of the cell wall in the sextuple mutant suggested that the chitin was no longer restricted to the inner cell wall layer, but became distributed throughout the depth of the cell wall. Previous studies have also shown that cells with increased chitin but normal mannan levels bind WGA-FITC more readily [20], confirming that the observed increase in WGA binding was not the result of increased WGA penetration due to the loss of the outer mannan layer. β-glucan exposure was visualised by FITC labelled Fc-Dectin-1 binding. Binding to wild type cells was extremely low, suggesting that the outer mannan layer shielded the β-glucan (Figure 3B). However, the mnn2Δ/ mnn2Δ double mutant, the sextuple mutants exhibited an increased patchy binding of Fc-Dectin-1 around the cell periphery (Figure 3B), suggesting that there was increased exposure of β-glucan. This increased exposure was hypothesised to result from the loss of mannan from the outer cell wall. However, ConA-TR staining of the mutants suggested that the cell wall periphery still contained a significant amount of residual mannan (Figure 3C) that was likely to be derived from the remaining N-mannan core and from O-mannans. Therefore, transmission electron microscopy (TEM) of high-pressure frozen, freeze-substituted samples was used to visualise the ultrastructure of the cell wall (Figure 4). No significant difference in the thickness of the inner cell wall layer between the parental control strain and the sextuple mannosylation mutants were observed in electron micrographs (Figure 4 A–D). However, the inner cell wall layer of the parental control strain was surrounded by an external layer of mannosylated proteins, which appeared as 74±13 nm fibrils extending out from the chitin-β-glucan layer in the electron micrographs (Figure 4A, E). These fibrils were significantly shorter (30±5 nm) in the mnn2Δ/mnn26Δ mutant (Figure 4B, E). The sextuple mutant, however, was almost completely devoid of long mannan fibrils, with only occasional, short (4±4 nm) fibrils observed on the cell wall surface (Figure 4C, E). Reintegration of both MNN2 and MNN26, was sufficient to
establish small (18±7 nm) fibrils, which covered the complete cell wall surface (Figure 4D, E).

**Physical and chemical analysis of the mnn2 mutant cell walls by GPC analysis and NMR**

To further investigate how deletion of genes encoding members of the Mnn2 mannosyltransferase family affected the structure of the mannans, X-mannans extracted from seven of the mutants (mnn2Δ, mnn24Δ, mnn26Δ, mnn2Δ/mnn26Δ, mnn24Δ/mnn26Δ, the triple, and the sextuple mutants) and the respective reconstituted control strains were analysed by gel permeation chromatography (GPC) and proton nuclear magnetic resonance (NMR). We failed to isolate sufficient purified X-mannan for analysis from all of the multiple mutants by gel permeation chromatography (GPC) due to the marked reduction in cell wall mannan content. However, we obtained sufficient mannan from all the mutants for NMR and enough mannan from mnn26Δ, mnn2Δ/mnn26Δ and mnn24Δ/mnn26Δ to perform GPC. GPC analysis for the parental control mannan confirmed that the mannan/mannoprotein had a trimodal polymer distribution (Figure 5A). The first peak had an average molecular weight (Mw) of 40×10^5 Da, but only represented 17% of the total cell wall mannan/mannoprotein. The third peak had an average Mw of 1.7×10^5 Da and represented 60% of the mannan/mannoprotein. In comparison, the mannan from the mnn26Δ and mnn24Δ/mnn26Δ mutants showed a bimodal polymer distribution, and the mannan/mannoprotein from mnn26Δ and mnn2Δ/mnn26Δ eluted primarily as a single peak resulting from a loss of the high molecular weight mannan (peaks 1 and 2, Figure 5A). In addition, the third peak of all the mnn2 mutants was shifted to the right, suggesting that the mannan/mannoprotein in the mutants had a lower Mw than the parental control strain (Figure 5A). Single peak analysis confirmed that the average Mw of the mannan/mannoprotein of the mnn26Δ, mnn24Δ/mnn26Δ and mnn2Δ/mnn26Δ mutants were 71.6, 62.3 and 93.2% of the parental control strain (CAI-4+Clp10 10.5×10^5 Da, mnn26Δ 2.9×10^5 Da, mnn24Δ/mnn26Δ 3.9×10^5 Da and mnn2Δ/mnn26Δ 0.7×10^5 Da). Therefore, the mannans produced by the mutants are less complex and more uniform than those from the wild type parental control strain.

Treatment of the mannan/mannoprotein with pronase to digest the protein attached to the mannan confirmed that all mutants had less mannan associated protein, with mnn2Δ/mnn26Δ showing the most significant decrease (Table S1).

When analysed by proton NMR, the mannans from the mutants were classified as belonging to four structural groups (Figure 5B and Table S2). The mannans in Group 1 were most structurally similar to the wild type parental control strain. This group was comprised of the reconstituted control strains of the single and double mutants, suggesting that the expression of a single copy of the respective gene partially or fully complemented the structural defects observed in the mutants (data not shown). The mnn21Δ mutant also formed part of Group 1, but showed a significant reduction in phosphorylation content compared to the parental control strain. This reduction was mainly due to the loss of mono- and di-phosphoglycans (M1-PO4 and Mβ1-2M1-PO4) and exhibited reduced resonances at 5.55, 5.47, 4.91 and 4.83 ppm, but the mutant retained some long phosphate chains (Mβ1-M1-2M1-PO4). In addition, the N-mannan of the mnn21Δ mutant also contained less α1,3-mannose residues (expressed as a reduction in the area of the resonance at 5.33 ppm), suggesting that the α1,2-mannose preceding the α1,3-mannose was missing (Figure 6 and data not shown). Group 2 consisted of the mnn24Δ and mnn26Δ/mnn2Δ mutants. The N-mannan from these mutants showed a significant reduction in α1,2-mannose side chains (represented by reduction in resonances between 5.29 and 5.13 ppm) and reduced phosphorylmannan. The larger resonance at 5.117 ppm suggested that there were regions of un-substituted α1,6-mannose residues in the backbone. There was still a strong signal at 5.068 ppm, which suggested that there were substituted α1,6-mannose units interspersed in the un-substituted regions. The area of these two resonances was roughly equivalent, indicating that the quantities of substituted and un-substituted α1,6-mannose in this mutant were similar (Figure 5B and Figure 6). The mutants in Group 3 (mnn26Δ, mnn24Δ/mnn2Δ) displayed reduced levels of α1,3-mannose in the outer chain, with the mnn24Δ/mnn26Δ mutant being almost completely devoid of α1,3-mannose. The strongest NMR resonance in these mutants was shifted from 5.067 ppm to 5.088 ppm, suggesting significant loss in α1,2-
Loss of α1,2-mannan inhibits immune recognition of *C. albicans* by PBMCs

Having determined that the members of the *MNN2* gene family are required for the addition of α1,2-mannan into the *N*-mannan outer chains, we assessed whether the loss of α1,2-mannan affected immune recognition by peripheral blood monocytes (PBMCs). Mannan is an important PAMP recognised by multiple PRRs. As a result, disruption of mannan biosynthesis is likely to have a significant impact on fungal immune recognition. To determine how the loss of α1,2-mannan affected immune recognition, heat-killed *C. albicans* cells were co-incubated with PBMCs for 24 h and the cytokine profile determined as a measure of immune cell activation. Deletion of *MNN2* resulted in a 67% reduction in TNFα secretion from PBMCs, while deletion of *MNN21*, *MNN22* or *MNN26* reduced TNFα production by 50% (Figure 7). Deletion of multiple *MNN2* gene family members also reduced TNFα production to varying degrees (Figure 7). PBMCs also secreted significantly less IL-1β when co-incubated with the various *MNN2* gene family mutants compared to the parental control strain (Figure S2A). On the other hand, secretion of IL-6 was only significantly reduced when PBMCs were co-incubated with *mnn21Δ/mnn22Δ*, the quintuple and the sextuple mutants (Figure S2B). The PBMCs elicited a greater response to the quintuple and sextuple mutants compared to the double and single mutants, suggesting that complete removal of the branched *N*-mannan, in conjunction with heat killing the cells, exposed other immune stimulatory epitopes. We therefore determined the effect of different commonly used methods of killing cells for immunological assays on the integrity of the cell wall of the parental control strain (Figure S3). Heat killed cells showed the greatest cell wall disruption, although the general architecture of the inner and outer cell wall layers was still obvious. The cell walls of thimerosal and UV-killed cells exhibited less visible perturbation of the wall structure (Figure S3). We stimulated PBMCs with a selection of mutants that had been killed by thimerosal. In agreement with the TEM images, the thimerosal killed cells elicited a lower immune response than heat killed cells (Figure S4), suggesting that a large portion of the detected immune response of heat killed cells was due to disruption of the cell wall and exposure of the underlying β-glucan. However, TNFα secretion of the mannosylation mutants was similar to that of heat-killed cells with the mannosylation mutants stimulating significantly less of a proinflammatory response than the parental control strain (Figure S4).

The *MNN2* gene family is required for virulence

To determine how the loss of α1,2-mannan seen in the mutants of the *MNN2* gene family affected the virulence of *C. albicans*, we first assessed the virulence of all the mutants using the *Galleria mellonella* infection model. Inoculation of larvae with 2.5×10⁷ cells resulted in a mean survival time of 3 days for the parental control strain. However, larvae infected with *mnn2A, mnn22A, mnn24A, mnn21Δ/mnn26Δ*, the quintuple and the sextuple mutant had mean survival times between 6–7 days, suggesting that these strains were less virulent than the parental control strain (Figure 8A).

Immunity in *G. mellonella* is a useful adjunct model for screening a large number of strains for differences in virulence, however immunity in this organism is predominantly mediated by haemocytes, and does not reflect the full immunological response of a mammalian host. Selected mutants were therefore also examined in a systemic mouse model of infection. The *mnn2A/

| Strain                  | Chitin (ppm) | Glucan (ppm) | Mannan (ppm) |
|-------------------------|--------------|--------------|--------------|
| CAI-4 Clp10             | 3.0 (±1.2)   | 73.2 (±0.7)  | 23.8 (±1.9)  |
| mnn2A                   | 4.6 (±0.02)  | 84.0 (±1.1)  | 11.5 (±1.1)  |
| mnn21Δ                  | 4.0 (±0.7)   | 79.8 (±3.2)  | 16.2 (±2.5)  |
| mnn22Δ                  | 4.9 (±4.4)   | 78.3 (±8.8)  | 16.9 (±4.5)  |
| mnn23Δ                  | 1.9 (±1.5)   | 5.4 (±3.8)   | 12.7 (±2.3)  |
| mnn24Δ                  | 4.8 (±1.6)   | 80.8 (±3.3)  | 14.4 (±1.7)  |
| mnn26Δ                  | 3.5 (±1.3)   | 84.4 (±0.6)  | 12.1 (±0.7)  |
| mnn21Δ/mnn26Δ           | 7.9 (±2.6)   | 82.6 (±1.9)  | 9.6 (±2.1)   |
| mnn23Δ/mnn26Δ           | 4.8 (±3.3)   | 85.1 (±0.9)  | 10.1 (±2.5)  |
| mnn24Δ/mnn26Δ           | 3.9 (±0.4)   | 88.8 (±3.1)  | 7.4 (±2.8)   |
| triple mutant           | 11.7 (±4.3)  | 79.0 (±5.6)  | 9.3 (±2.2)   |
| quintuple mutant        | 10.1 (±1.7)  | 83.8 (±9.0)  | 6.1 (±2.6)   |
| sextuple mutant         | 12.2 (±6.0)  | 80.8 (±4.3)  | 7.1 (±1.9)   |
| mnn21Δ+MNN2             | 3.3 (±0.9)   | 79.3 (±1.4)  | 17.3 (±2.3)  |
| mnn21Δ+MNN21            | 3.7 (±0.1)   | 82.2 (±0.8)  | 14.1 (±0.7)  |
| mnn22A+MNN22            | 4.4 (±2.0)   | 82.5 (±1.0)  | 13.1 (±1.0)  |
| mnn23Δ+MNN23            | 6.6 (±6.1)   | 76.7 (±11.0) | 16.7 (±4.9)  |
| mnn24Δ+MNN24            | 2.4 (±0.03)  | 83.4 (±0.4)  | 14.2 (±0.4)  |
| mnn26Δ+MNN26            | 3.5 (±1.0)   | 84.1 (±5.1)  | 12.4 (±4.2)  |
| mnn21Δ/mnn26Δ+MNN26     | 3.9 (±1.5)   | 81.5 (±4.3)  | 14.7 (±2.9)  |
| sextuple mutant+MNN26   | 13.4 (±3.2)  | 77.6 (±4.8)  | 9.0 (±1.7)   |

*p<0.05, **p<0.01, doi:10.1371/journal.ppat.1003276.t001
mnn26Δ mutant, its reconstituted control strain and the sextuple mutant were tested in the 3-day and 28-day murine infection model to establish the involvement of α1,2-mannan in both early infection processes and for maintenance of infection. In the 3-day infection model, virulence is expressed as an “outcome score”, which is related to weight loss and kidney fungal burdens [35]. Accordingly, the higher the outcome score the greater the kidney burden and weight loss, and therefore the greater the virulence of the strain [35]. The mnn2Δ/mnn26Δ and sextuple mutants had significantly reduced outcome scores (p = 0.004 and p = 0.002, Table 2), which correlated with reduced kidney burdens and reduced weight loss, underlining that these mutants were attenuated in virulence compared to the parental control strain. In the 28-day infection model, 80% of mice survived infection with the mnn2Δ/mnn26Δ mutant, with only 20% succumbing to infection on day 14 (Figure 8B). In contrast, all mice infected
with the sextuple mutant survived the infection (Figure 8B). The kidney burdens for mnn2Δ/mnn26Δ and the sextuple mutant at 28 days post infection were 3.9±3.0 and 4.9±1.9 log10 CFU/g, respectively, suggesting that the mutants were not completely eradicated from the mice. The mnn2Δ/mnn26Δ reconstituted control strain had a similar outcome score and kidney burden to mnn2Δ/mnn26Δ, but showed increased weight loss and only 50% survival in the 28-day infection model, indicating that ectopic expression of a single copy of MNN2 and MNN26 was insufficient to restore virulence to wild type levels (Table 2, Figure 8B).

Discussion

The fungal cell wall is the first point of contact between the invading pathogen and the host immune system. All the major cell wall carbohydrates have been shown to act as PAMPs that activate and modulate the host's immune response to the invading pathogen mediated via interactions with specific PRRs [5,13,17–18,36]. Previous studies using C. albicans mannosylation mutants have confirmed that both O- and N-mannan structures are important for the recognition of C. albicans by monocytes, macrophages, neutrophils and dendritic cells and established that N-linked mannan was an agonist of the MMP and DC-SIGN [7–10,37–39]. However, most PRRs bind short oligosaccharides and the precise carbohydrate epitopes that these PRRs recognise are unknown. Here, we expand on these studies by characterising the role(s) of the α1,2-mannose residues from the N-mannan in immune recognition and fungal virulence through deletion of the C. albicans MNN2 gene family.

Deletion of MNN2 gene family members resulted in increased cell aggregation - a common phenotype of glycosylation mutants [7,9–11]. Cell aggregation can result from enhanced binding of adhesions within the cell wall, or from inactivation of hydrolytic enzymes required to digest the cell wall at the bud site. High concentrations of sugars and the addition of Congo Red have been shown to compete with adhesin binding and disrupt Als5 amyloid formation decreasing cell aggregation [40–41]. However, addition of Congo Red, or high concentrations (20%) of glucose, did not disrupt the cell aggregates (data not shown), suggesting that this was not the result of Als5 amyloid formation or increased exposure of Als1 binding sites. Therefore, it is possible that these clumps of cells result from insufficient cell separation due to reduced...
hydrolytic activity of the enzymes responsible for cell wall cleavage after cell division.

The sextuple mutant has severely truncated mannoprotein fibrils, however the presence of residual mannan was confirmed by positive ConA-TR staining and HPLC analysis which showed that mannan still comprised approximately 7% of the relative cell wall dry weight. This low mannan proportion may be attributed to the O-mannan and remaining α1,6-backbone from the N-mannan. The use of high-pressure freezing and freeze substitution to prepare samples for TEM has enabled better preservation of the ultrastructure of the mannan fibril layer, which is not well preserved by traditional chemical fixation protocols. This allowed us to demonstrate that deletion of specific MNN2 gene family members generated mannan fibrils of varying length. Therefore, it is possible that each fibril may represent one N-mannan covalently attached to a cell wall protein with the length of the fibril representing the outer chain incorporating the α1,6-backbone and its side chains extending away from the cell. Assuming that one mannose unit is approximately 0.5 nm and the bond length between each mannose residue is approximately 0.15 nm, and recognising that alpha-linked complex mannan polymers are very likely to be linear molecules, then a mannan fibril of 100 nm can be estimated to have a backbone comprised of approximately 150 α1,6-mannose units. The fibrils in the mnn2D/mnn26D mutant were approximately 30 nm in length, which would correspond to approximately 46 α1,6-mannose units. The N-mannan from the reconstituted sextuple mutant contained more α1,6-mannose than the mutant and fibrils were only observed on the surface of the reconstituted strain. The NMR spectra suggest that the reconstituted strain had more side chain residues than the mutant. Therefore, it is possible that these small side chain residues are sufficient to stabilise the α1,6-backbone, allowing increased extension of the backbone by Mnn9.

Figure 4. The Mnn2 family of mannosyltransferases regulates mannan fibril length. Electron micrographs showing the ultrastructure of the cell walls of the (A) wild type (CAI-4+Clp10), (B) mnn2A/mnn26Δ, (C) sextuple mutant, (D) sextuple mutant+MNN2/MNN26 strains. The scale bar represents 500 nm. (E) Mannan fibril length was measured in 8 randomly selected cells. Each cell was measured 10 times in different locations. Data represent the means ± SD, **p<0.01.

Figure 5. Deletion of MNN2 gene family members results in a lower molecular weight and less complex N-mannan. (A) C. albicans N-mannans were extracted from cells and analysed by GPC. Solid line represents wild type (CAI-4+Clp10), dotted line represents mnn26Δ, dashed line represents mnn2A/mnn26Δ and the dashed and dotted line represents mnn24Δ/mnn26Δ. The numbers designate the peaks discussed in the text. (B) Representative proton NMR spectra for the N-mannan extracted from wild type, mnn2Δ, mnn26Δ, mnn2Δ/mnn26Δ, sextuple mutant and the reconstituted sextuple mutant.
Previous studies have proposed a model for *C. albicans* N-mannan structure [26,42–47] and additional experiments combining mild acetolysis, HPLC and 2D NMR have identified the composition and linkage of the N-mannan side chains [48]. More recently, Lowman and colleagues described a method combining 1D and 2D COSY and NOSEY NMR with published chemical shift data for specific side chains to assign resonances to non-degraded intact N-mannans from yeast [49]. This approach permitted rapid identification of structural motifs for acid liable and acid-stable fractions of N-mannan. Using this method we compared changes in the side chain motifs in our mutants and suggest structures for the different N-mannans expressed in each of the mutants (Figure 6). From our data, we hypothesise that Mnn2 is the major α1,2-mannosyltransferase responsible for the addition of the initial α1,2-mannose residue onto the α1,6-backbone, as deletion of MNN2 significantly reduced α1,2-mannose content. However, deletion of MNN2 alone did not prevent incorporation of α1,2-mannose into the side chains, suggesting a degree of functional redundancy within the family. The triple mutant displayed significant regions of un-substituted backbone compared to the mnn2Δ mutant. Therefore, it is likely that Mnn22 and Mnn23 initiate outer chain branching in the absence of Mnn2. Mnn23 has already been described as an Mnn5 orthologue because it can functionally complement the *S. cerevisiae* mnn5Δ mutant [50]. However, the mannans in *S. cerevisiae* and *C. albicans* differ significantly, so it is possible that Mnn23 has both Mnn2 and Mnn5 activity in *C. albicans*. Deletion of MNN24 and MNN26 resulted in a significant decrease of α1,3-mannan in the side chains, which was most obvious in the mnn24Δ/mnn26Δ double mutant. Therefore, we suggest a model (Figure 9) where Mnn26 is required to add the second α1,2-mannose residue onto the side chain, with the third α1,2-mannose attached by Mnn24 to generate an [α1,2-mannose]3 oligosaccharide to which α1,3-mannose residues are finally attached by members of the Mnn1 family of glycosyltransferases. In agreement with this, deletion of MNN2 and MNN26 resulted in an almost complete loss of acid liable side chains (phosphomannan), which are predominantly attached to N-mannan via the second or third α1,2-mannose in the side chain [51], confirming that the mnn2Δ and mnn26Δ mutants do not normally possess side chains longer than two α1,2-mannose.

**Figure 6. Predicted structures of the *C. albicans* N-mannan in the respective mutants as identified by NMR.** The N-mannan structure is based on the work by Shibata and colleagues [26,43,45,48]. doi:10.1371/journal.ppat.1003276.g006
residues. Deletion of MNN21 did not significantly alter the NMR spectra, suggesting little change in side chain composition. It is possible that Mnn21 contributes to N-mannan biosynthesis through capping some of the α1,3-mannose units with α1,2-mannose residues (Figure 9).

Single mutants of the MNN2 gene family elicited a lower immune response when co-incubated with PBMCs, suggesting that these mutants lacked important PAMPs required for immune recognition. On the other hand, heat-killing the sextuple mutant resulted in a increase in the production of pro-inflammatory cytokines compared to thimerosal killed cells, suggesting that more β-glucan was exposed upon heat treatment due to the reduced integrity of the cell wall. Staining exponentially growing cells with soluble Fc-Dectin-1 confirmed that there was more β-glucan exposed on the surface of the sextuple mutant than the parental control strain. However, PBMCs elicited a significantly reduced pro-inflammatory response when the sextuple mutant was killed by thimerosal, which maintains cell wall integrity, suggesting that in these assays conditions mannan and not β-glucan is the major PAMP recognised by the PBMCs. These results are in agreement with previously published data for the och1Δ mutant which has significantly increased amounts of β-glucan and reduced amounts of mannan in the cell wall, but still elicits a reduced pro-inflammatory cytokine profile upon co-incubation with PBMCs [7,16]. The immunological signature of these mutants, therefore, confirm that N-mannan is both an immunostimulatory PAMP (in the case of PBMCs) and a shield that masks the highly proinflammatory β1,3-glucan layer (in the case for macrophages) [8,52]. To determine the importance of the α1,2-mannan in fungal virulence, all mutants were tested for their ability to infect G. mellonella. This infection model has been shown to be a valuable tool for screening virulence defects in C. albicans strains, with results correlating well with those obtained from murine systemic infection models [53]. Deletion of all MNN2 gene family members increased the average survival of the moth larvae by 50%, suggesting that α1,2-mannose is important for virulence. The 28-day murine systemic infection model confirmed that the sextuple mutant was attenuated in virulence with 100% of mice surviving infection with the sextuple mutant. Mice infected with mnn2Δ/ mnn26Δ and the sextuple mutant often displayed at least one swollen kidney. Kidney enlargement and unilateral kidney involvement in systemic candidiasis has previously been observed when low inocula of SC5314 are used [54]. After intravenous inoculation of yeast cells into the blood circulation, the majority of C. albicans are removed rapidly from the bloodstream with only low levels of C. albicans found in the blood 2 h post infection [54]. C. albicans cells which reach the kidney are likely to be phagocytosed and cleared by patrolling macrophages, with only the few cells that manage to evade macrophage killing left to establish infection. Increased exposure of β-glucan in the sextuple mutant may enhance macrophage recognition and phagocytosis resulting in rapid clearing and limitation of infection.

Complementation of the mutants with a single copy of the respective gene did not fully restore wild type phenotypes in some of the assays. This is likely to be due to the ectopic expression of the wild type gene at a non-native locus. In addition, our reintegration constructs used 1 kb of DNA upstream from the start codon and 300 bp of terminator sequence. Such constructs may lack some of the regulatory sequences that are required for the normal expression profile of the MNN2 gene family members. It was noted however that the majority of the cytokine profiles were elevated in the reconstituted strains compared to the mutant. Therefore, expression levels in these strains were sufficient to partially restore mannan biosynthesis to levels where it was sufficient to drive PAMP mediated immune responses but was not sufficient to fully restore virulence.

In conclusion, the addition of α1,2-mannose onto the α1,6-backbone is mediated by the MNN2 family of α1,2 mannosyltransferases. We present a model (Figure 9) where Mnn2 is required for the addition of the majority of the α1,2-mannose residues onto the backbone of the N-mannan which is then elaborated with additional α1,2-mannose units by Mnn26 and Mnn24. Deletion of all six MNN2 gene family members resulted in the termination of α1,6-mannan elongation, presumably due to reduced stabilisation of the backbone as a result of the loss of mannan side chains, with cells appearing devoid of mannan fibrils. The loss of mannan fibrils from the cell wall surface resulted in increased exposure of the underlying β-glucan layer, and
consequential virulence attenuation perhaps due to the mutant being cleared from the kidney by patrolling macrophages as a result of increased Dectin-1 dependent phagocytosis.

This work contributes to the identification of specific carbohydrate epitopes, which are recognised by the PRRs and is relevant in the context of the development of novel immunotherapies and diagnostics. Furthermore, because most fungal mannosyltransferases are not found in mammalian cells, characterisation of the enzymes involved in N-mannan biosynthesis may represent novel antifungal drug targets.

**Table 2. Deletion of MNN2 gene family members attenuates virulence in the 3-day murine infection model.**

| Strain                        | Kidney burden (log_{10} CFU/g) | % weight change | Outcome score |
|-------------------------------|---------------------------------|-----------------|---------------|
|                               | Mean                            | Stdev           | Mean          | Stdev           | Mean   | Stdev           |
| CAI-4+Clp10                   | 5.0                             | 0.3             | -8.6          | 3.9             | 9.3    | 2.1             |
| mnn2Δ/mnn26Δ                  | 4.2                             | 0.7             | -1.6          | 2.7             | 5.0    | 1.1             |
| mnn2Δ/mnn26Δ+MNN2/MNN26       | 3.3                             | 0.7             | -0.8          | 1.4             | 3.7    | 1.0             |
| Sextuple mutant               | 4.3                             | 0.4             | -2.5          | 2.0             | 5.5    | 1.2             |

Figure 8. Mnn2 members are required for virulence of C. albicans. (A) Mutants were screened for virulence defects in the G. mellonella infection model. Larvae were infected with 2.5 x 10^5 C. albicans cells and incubated at 37°C. Data represent the mean larvae survival times ± SD from 3 independent experiments, *p<0.05, **p<0.01. (B) wild type (CAI-4+Clp10), mnn2Δ/mnn26Δ, mnn2Δ/mnn26Δ+MNN2/MNN26 and the sextuple mutant were tested for their ability to cause infection in the 28 day mouse model of systemic infection.

doi:10.1371/journal.ppat.1003276.g008
Materials and Methods

Ethics statement

All animal experimentation conformed to UK Home Office legislation requirements and was approved by the UK Home Office and the Ethical Review Committee of the University of Aberdeen. All animals had free access to food and water throughout the experiments.

Strains, media and growth conditions

All strains used in this study are listed in Table S3. Strains were maintained as glycerol stocks at −80°C and when required plated onto YPD agar (1% yeast extract, 2% glucose, 2% mycological peptone and 2% agar) and grown at 30°C for 48 h. For the majority of assays, cells were inoculated in YPD, grown to stationary phase at 30°C, 200 rpm and then diluted 1:100 in fresh YPD and grown to mid exponential phase. For morphology assays, stationary phase YPD cultures were diluted and plated onto Spider medium (1% nutrient broth, 1% mannitol, 0.2% K2PO4, 2.5% agar, pH 7.2) and incubated at 30°C, or Dulbecco’s modified Eagle medium (DMEM; 1.34% DMEM, 2% glucose, 3.57% HEPES, pH 7) supplemented with 5% foetal calf serum and DMEM in the presence of a 5% CO2 atmosphere and incubated at 37°C.

Construction of mutant strains

All mutants were generated in the CAI-4 background using the mini ura blaster method as previously described [55]. Primers were designed to have 70 bp of homology with the gene of interest and where possible the full open reading frame was deleted from start (ATG) to stop (TAA) (Table S4). To restore the URA3 status of the mutants, StuI digested CIp10 was integrated at the RPS1 locus of all mutants. Reconstituted strains were generated by integration of the full-length open reading frame together with 1 kb of the promoter and 500 bp of terminator sequence into the SacI and NotI sites of the CIp10 plasmid. All strains were verified by Southern analysis and qRT-PCR.

Alcian Blue binding assay

Alcian Blue assays were performed as described previously [34]. Briefly, an aliquot of 1 x 10^6 stationary phase cells was washed with PBS and resuspended in 30 µg/ml Alcian Blue and incubated at room temperature for 10 min. Samples were centrifuged and 200 µl aliquots of the supernatant used to determine the concentration of unbound dye, with reference to a standard curve. The amount of Alcian Blue bound to the cell wall of C. albicans cells was then calculated to give an estimate of the amount of cell wall phosphomannan. Data were analysed by a one-way ANOVA, followed by post-hoc analysis including Tukey test with a 95% confidence level.

Cell wall integrity assays

Strains were grown for 16 h in YPD media, 30°C, 200 rpm and diluted to an OD600 of 0.5 and 3 µl of 1:10 serial dilutions spotted onto YPD agar and incubated at 30, 37 and 42°C for 48 h, or grown on YPD supplemented with either 100 µg/ml CFW, 0.03% SDS, 100 µg/ml Congo Red and 1 M NaCl and incubated at 30°C for 48 h.

Activation of the cell wall integrity pathway

Activation of the cell wall integrity pathway MAP kinase Mkc1 was assessed using an phospho-p44/42 MAPK (Thr202/Tyr204) antibody (Cell Signalling Technology). Strains were grown for 16 h in YPD at 30°C, 200 rpm and diluted 1:100 in fresh YPD medium and grown for 4 h at 30°C. As a control for Mkc1 activation, CAI-4+CIp10 was exposed to 0.032 µg/ml caspofungin for 10 min. Cells were harvested by centrifugation (3500 rpm, 5 min) and resuspended in 250 µl RE buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100), supplemented with Roche complete proteinase cocktail. Samples were lysed with a bead beater (6 x 6.0 for 30 s) with 4 min on ice between each cycle. Lysates were cleared by centrifugation (13,000 rpm, 5 min) and protein concentration estimated by Bradford assay with a BSA standard curve. Proteins (15 µg) were separated by SDS-PAGE on 4–12% NuPAGE Bis-Tris gels and stained with Coomassie Blue. Proteins were transferred to a PVDF membrane overnight and probed with the appropriate antibody.
Samples were washed 3 times in Buffer B and then incubated on ice for 45 min with 100 ng of anti-human Fc antibody conjugated antibody was added and incubated at 4°C overnight. For detection an anti-rabbit IgG-HRP conjugated antibody was used at a 1:2000 dilution in PBS-T for 2 h at room temperature. Membranes were stripped, blocked in 10% Minitab version 15.

Data were analysed by a one-way ANOVA followed by post-hoc analysis including Tukey test with a 95% confidence level using Minitab version 15.

Fluorescence microscopy

To stain the cell wall for chitin and mannan, exponentially growing cells were washed and suspended in water and pH adjusted to 6.5–7.0 after which the samples were washed and resuspended in 8:1 methanol:acetic acid solution until the precipitate was white to colourless. The precipitate was dried, re-suspended in 20 mM HCl. The precipitated carbohydrate and copper were removed by washing in 8:1 methanol:acetic acid solution with 1:100 in fresh YPD and grown to mid-exponential phase at 37°C. Cells were washed 4 times with sterile water and lysed in a bead beater (15 cycles of 6000 rpm, 40 s with 3 min on ice between cycles). Cell pellets were washed 5 times with 1 M NaCl and pellets boiled in protein extraction buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 1 mM EDTA and 0.3 M 2-mercaptoethanol) for 10 min to remove contaminating cytoplasmic proteins. Cell wall pellets were washed with water, snap-frozen in liquid nitrogen and freeze-dried. The cell wall (2-3 mg) was hydrolysed with trifluoroacetic acid for 3 h at 100°C. Hydrolysed samples were washed and resuspended in water to a concentration of 10 mg/ml and 1:10 dilutions analysed by HPLC as described previously [56] and the relative proportions of sugars in the cell wall calculated. Data were analysed by a one-way ANOVA followed by post-hoc analysis including Tukey test with a 95% confidence level using Minitab version 15.

Mannan purification

Mannan was extracted from cells as described previously [57–58]. Briefly, cells were delipidated in acetone and disrupted by bead beating in deionised water, followed by autolysing for 3 h. The cell extract was centrifuged and the supernatant split in two. To one sample, 100 mg of pronase (heated to 70°C for 30 min to remove glycosidic activity) was added and incubated at 37°C for 18 h. The other sample was untreated. The supernatant was then exposed to Fehling precipitation [57] and then dissolved in 3 M HCl. The precipitated carbohydrate and copper were removed by washing in 8:1 methanol:acetic acid solution until the precipitate was white to colourless. The precipitate was dried, re-suspended in water and pH adjusted to 6.5–7.0 after which the samples were lyophilised and stored at –20°C.

Gel permeation chromatography (GPC) analysis

Duplicate mannans were diluted to 3 mg/ml in mobile phase and incubated at room temperature. The mannans were then incubated in acetone for 2 h and the membrane block was washed with 5% BSA in PBS-T for 1 h. Mkk1 phosphorylation was detected with a 1:2000 dilution of phospho-P44/P42 (Erk1/2) (Thr-202/Tyr-204) rabbit monoclonal antibody (New England Biolabs, Hitchin, Hertfordshire, UK) in PBS-T with 5% BSA. Membranes were incubated overnight at 4°C. For detection an anti-rabbit IgG-HRP conjugated antibody was used in a 1:2000 dilution in PBS-T, 5% BSA at room temperature. To detect Act1 levels, membranes were stripped, blocked in 10% Minitab version 15.

Isotope Laboratories, 99.8% deuterated). Proton 1D and COSY spectra were acquired on a Bruker Avance III 700 MHz at 25°C and assigned using the Bruker TopSpin software. Two-dimensional (2D) spectra were used to assign all cross-peaks and determine the chemical shift of all resonances.
2D NMR spectra were collected on a Bruker Avance III 600 NMR spectrometer using a CH cryoprobe operating at 333 K (60°C) in 5-mm NMR tubes. Spectral data were collected and processed as reported previously [49].

Stimulation of peripheral blood mononuclear cells

PBMCs were isolated from the blood of healthy volunteers using Ficoll plaque as described previously [59]. C. albicans strains were grown in Sabouraud broth (4% glucose, 1% mycological peptone) for 16 h, 30°C, washed in PBS and resuspended to a final cell density of 5×10^6 cells/ml in RPMI 1640 supplemented with 5% foetal calf serum and incubated at 25°C, 200 rpm for 3 h. Cells were washed in PBS and resuspended at the desired cell concentration. Yeast cells were heat killed at 65°C for 2 h and 1×10^7 cells added to 5×10^5 PBMCs in a final volume of 200 μl (MOI = 0.4). Cells were incubated at 37°C, 5% CO2 for 24 h. Samples were centrifuged (1600 rpm, 8 min) and the supernatant removed to a fresh plate. Extracellular cytokines were measured by enzyme-linked immunosorbant assay commercial kits (R&D) according to the manufacturer's recommendations. Data were analysed by two-way ANOVA, followed by post-hoc analysis including Tukey test with a 95% confidence level. Minitab version 15 was used for the statistical analyses.

Galleria mellonella infection model

G. mellonella infections were performed as described previously [60]. Briefly, fungal strains were grown to stationary phase in YPD at 30°C, 200 rpm, washed with PBS and resuspended to a final concentration of 2.5×10^7 cells/ml. Groups of 20 G. mellonella larvae per strain were inoculated with 10 μl of cell suspension (2.5×10^5 cells) by injection in the last pro-leg. Larvae were incubated at 37°C for 8 days and infection progression monitored every 12 h. Three independent biological replicates were performed and data pooled and analysed by the Kaplan-Meier Log-rank statistic and Dunnet's T-tests with a 95% confidence level using SPSS statistics version 19.

Murine infection model

For infection experiments, C. albicans strains were grown in NGY medium (0.1% Neopeptone, 0.4% glucose, 0.1% yeast extract) in a rotating wheel at 30°C for 16 h. Fungal cells were harvested, washed twice in saline and then sonicated to disrupt any cell clumps. Cell suspensions were adjusted to the desired concentration by addition of saline.

Female BALB/c mice (6–8 weeks old; Harlan, UK) were randomly assigned into groups of 12 animals, with each group intravenously infected with 2×10^4 CFU/g mouse body weight. Inoculum levels were confirmed by viable cell counts. Six mice (mice 7–12) from each group were culled on day 3, with weight change and kidney fungal burdens determined at this time to allow the infection outcome scores to be calculated [35]. The remaining six animals from each group (mice 1–6) were monitored over 28 days to determine gross virulence of the fungal strains. Mice were weighed and checked at least once daily, with any mice showing signs of severe illness and distress, or losing more than 20% of their initial body weight, being culled immediately and the kidney fungal burdens determined. Day 3 kidney fungal burdens, body weight change and infection outcome scores were compared by Mann-Whitney U test and the 28 day survival data compared by Kaplan-Meier Log-rank statistic using IBM SPSS statistics version 19.

Supporting Information

**Figure S1** Phylogram of the C. albicans MN2 gene family. A multiple sequence alignment for the six putative MNN2 orthologues was generated using ClustalW2 (version 2.0.12) and the phylogram was created in ClustalW2. (TIF)

**Figure S2** Deletion of MNN2 gene family members reduces monocyte immune recognition. Purified PBMCs were co-incubated with C. albicans strains at an MOI of 0.4 for 24 h. Cytokines was measured by ELISA. A) IL-1β, B) IL-6. Data represent the means ± SEM from 8 independent experiments, *p<0.05, **p<0.01. (TIF)

**Figure S3** Heat killing C. albicans significantly alters the cell wall structure exposing underlying PAMPs. Electron micrographs showing the ultrastructure of the cell walls of CAI-4+Clp10 after different killing methods (A) live, (B) heat killed, (C) UV killed and (D) thimerosal killed. Scale bar represents 0.5 μm. (TIF)

**Figure S4** Thimerosal treatment significantly lowers the pro-inflammatory response. Purified PBMCs were co-incubated with thimerosal killed C. albicans strains at an MOI of 0.4 for 24 h. TNFα was measured by ELISA. Data represent the means ± SD from 4 independent experiments *p<0.01. (TIF)

**Table S1** Mannan/mannoprotein distribution in the mannosylation mutants. (DOCX)

**Table S2** Summary of N-mannan side chain composition. (DOCX)

**Table S3** Strains used in this study. (DOCX)

**Table S4** Primers used in this study. (DOCX)

Acknowledgments

We thank Gordon Brown for a gift of purified Fc Dectin-1 and Kanya Preechasuth and Keunsook K. Lee for help with HPLC.

Author Contributions

Conceived and designed the experiments: RAH NARG DMM DLW DWL AJPB FCO. Performed the experiments: RAH SB MDL DMM NARG DWL. Analyzed the data: RAH DMM NARG DWL. Contributed reagents/materials/analysis tools: MDK DWL NARG AJPB FCO. Wrote the paper: RAH MDL DMM DWL NARG.

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