Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors

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The fungal pathogen *Candida albicans* has a multilayered cell wall composed of an outer layer of proteins glycosylated with N- or O-linked mannosyl residues and an inner skeletal layer of β-glucans and chitin. We demonstrate that cytokine production by human mononuclear cells or murine macrophages was markedly reduced when stimulated by *C. albicans* mutants defective in mannosylation. Recognition of mannosyl residues was mediated by mannose receptor binding to N-linked mannosyl residues and by TLR4 binding to O-linked mannosyl residues. Residual cytokine production was mediated by recognition of β-glucan by the dectin-1/TLR2 receptor complex. *C. albicans* mutants with a cell wall defective in mannosyl residues were less virulent in experimental disseminated candidiasis and elicited reduced cytokine production in vivo. We concluded that recognition of *C. albicans* by monocytes/macrophages is mediated by 3 recognition systems of differing importance, each of which senses specific layers of the *C. albicans* cell wall.

Introduction

Invasive *Candida albicans* infections are a serious clinical threat in patients who are immunosuppressed or undergo major surgical procedures. Mortality associated with disseminated candidiasis can be as high as 30–40%, despite the availability of new antifungal drugs (1, 2). Host defense against systemic candidiasis relies mainly on the ingestion and elimination of *C. albicans* by cells of the innate immune system, especially neutrophils, monocytes, and macrophages (3–6). After activation of these leukocyte populations by *C. albicans*, release of proinflammatory cytokines by human mononuclear cells or murine macrophages is the first step in the activation of anticanidial innate immune responses. Proinflammatory cytokines activate neutrophils and macrophages to phagocytose the fungus and to release toxic oxygen and nitrogen radicals, thus eliminating the invading pathogen (7, 8). The crucial role of proinflammatory cytokines for the host defense against *C. albicans* has been demonstrated by the increased susceptibility to candidiasis of knockout mice lacking these cytokines (9, 10). In contrast, antinflammatory cytokines such as IL-4 and IL-10 have immunosuppressive effects (11). It is currently believed that the balance between pro- and antinflammatory cytokine production is decisive in determining whether the host defense system is overwhelmed or able to eliminate the fungal pathogens (12).

Stimulation of proinflammatory cytokine production and the activation of innate immunity depend on accurate recognition of an invading pathogen. The basic strategy for recognizing pathogens by the cells of the innate immunity system consists of non-clonal recognition of conserved structures of microorganisms, called pathogen-associated molecular patterns (PAMPs), which are not present in mammalian cells. Several classes of pattern-recognition receptors (PRRs) recognize the various PAMPs, of which TLRs and C-type lectin receptors are probably the most important (13). We and others have recently identified TLR2 and TLR4 as important PRRs for *C. albicans* (14–17). The mannose receptor (MR) has also been implicated in the stimulation of cytokine production by *C. albicans* (18), and we have recently demonstrated that the C-type lectin DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) mediates the uptake of *C. albicans* by dendritic cells (19).

The outer layer of the cell wall of *C. albicans* is enriched with mannoproteins, which represent 30–40% of the cell wall dry weight (20). The inner layer is composed of chitin and β1,3- and β1,6-glucan. Earlier studies have suggested that mannosylated proteins of *C. albicans* induce cytokine production (21), and *C. albicans* mannan reportedly interacts with TLR4 (22). It has been suggested that phospholipomannan of *C. albicans* is recognized by TLR2 and induces proinflammatory cytokine production (23), whereas β-glucans were recognized by a complex of TLR2 and dendt-1 (24, 25). However, the relative contribution of these PAMPs and their respective receptors to cytokine stimulation by *C. albicans* is not known.

Despite the progress in understanding the interaction of some of the fungal PAMPs with leukocyte receptors, there is no integrated view of the mechanisms by which the immune system
We hypothesized that recognition of C. albicans by monocytes/macrophages is a complex process involving multiple recognition systems and that these receptor systems recognize sequentially the various layers of the fungal cell wall. The skeleton of the C. albicans cell wall is mainly formed by chitin and β1,3- and β1,6-glucans, whereas the outer cell wall is enriched with proteins that are modified with both long-chain and highly branched N-linked mannosyl residues as well as short linear chains of O-linked mannosyl residues. Using a range of isogenic glycosylation mutants (26–29), ligand-specific blocking experiments, and PAMP receptor knockout mice, we show that recognition of C. albicans by monocytes/macrophages is a complex process involving multiple recognition systems and that these receptor systems recognize sequentially the various layers of the outer portion of the fungal cell wall.

**Results**

**Cell wall mutants of C. albicans.** We have taken a genetic approach to investigate how immune cells recognize a fungal pathogen. We have used well-defined stable isogenic mutants of C. albicans depleted in specific cell wall components to investigate cytokine responses of monocytes. The och1 mutant was defective in outer, branched N-linked glycans, and transmission electron microscopy (TEM) revealed a thicker cell wall lacking a fibrillar mannan layer (ref. 29 and compare Figure 1B with Figure 1A). The mnt1 mnt2 mutant lacks the 4 terminal O-linked α1,2-mannosyl residues, but had normal N-mannan (24) and a fibrillar outer wall surface (Figure 1C). The pmr1 mutant has defects in both N- and O-linked mannosylation, and had a thinner and less fibrillar cell wall than the control strain (Figure 1D). The mnn4 mutant lacks phosphomannan (25) and was indistinguishable by TEM from that of wild-type cells (Figure 1E). Therefore this set of mutants provided a unique molecular biological tool to dissect the immune responses of monocytes.

**Mannan stimulates cytokines in a TLR4- and MyD88-dependent manner.** Purified mannan (a mixture of both N- and O-linked oligosaccharides) from C. albicans stimulated production of TNF and IL-6 in both human mononuclear cells (MNCs) and murine peritoneal macrophages (Figure 2A). The induction of TNF in murine peritoneal macrophages was significantly decreased in mice deficient in MyD88, demonstrating that a TLR-dependent mechanism was involved in this stimulation (Figure 2B). Indeed, the diminished stimulation of macrophages harvested from TLR4-defective ScCr mice demonstrated a TLR4-dependent stimulation of TNF by C. albicans mannan, whereas TLR2+/− mice produced normal amounts of cytokines (Figure 2B). Because purified mannan is a relatively weak cytokine stimulus compared with C. albicans, we investigated whether purified mannan would inhibit C. albicans-induced cytokine production by occupying the mannan-binding sites on leukocytes. The presence of purified mannan significantly inhibited the production of TNF and IL-6 stimulated by C. albicans mannan, whereas TLR2+/− mice produced normal amounts of cytokines (Figure 2C). In control experiments, mannan had no effect on LPS-induced cytokine production (data not shown). Therefore, fungal mannan stimulated cytokine production, and pretreatment with purified mannan blocked the cytokine response induced by C. albicans yeast cells, suggesting an important role of mannan recognition receptors for the stimulation of cytokines by the fungus.

**Figure 1**

Cell wall morphology in the C. albicans strains used in this study. (A–E) TEM micrographs. (A) Wild-type strain NGY152 [CAI-4 plus Clp10 vector]. (B) och1 null (strain NGY357; ref. 26) or doxycycline-regulated conditional (strain NGY361; ref. 29) mutants, which are defective in the branched outer N-linked mannosyl chains. (C) mnt1 mnt2 mutant (strain NGY337; ref. 27), which lacks 4 terminal O-linked α1,2-mannosyl residues. (D) pmr1 mutant (strain NGY355; ref. 26), which has gross defects in mannosylation, characterized by absence of phosphomannan and reduced O-linked and N-linked glycans. (E) mnn4 mutant (strain CDH15; ref. 28), which lacks phosphomannan. Scale bar: 100 nm. (F and G) Structure of the N-(F) and O-linked (G) glycans and the site of action of deleted gene products. Man, mannosyl; β-GlcNAc, β-N-acetylglucosamine.
Normal cytokine release was recovered in strains in which the deleted genes were reintegrated into their respective mutants (Figure 4, A and C). In strain TET-OCH1, the expression of OCH1 was regulated by placing the OCH1 open reading frame under the control of a promoter that was regulatable by tetracyclines. Growth in 20 μg/ml doxycycline switched off expression of OCH1 and resulted in a reduction of cytokine production to a similar level as the och1 mutant. Growth of TET-OCH1 in the absence of doxycycline resulted in expression of OCH1 and induced cytokine production similar to that of the wild-type strain CAI-4 (Figure 4B).

Similar results were obtained in experiments with live C. albicans cells. The live och1 strain induced only 29% of the TNF production induced by the control strain (0.36 ± 0.11 ng/ml versus 1.21 ± 0.23 ng/ml, P < 0.05), while the live mnt1 mnt2 strain stimulated 78% of the control TNF production (0.94 ± 0.21 ng/ml; P < 0.05). In contrast, PBMCs stimulated with the mnt4 strain released normal amounts of TNF (1.79 ± 0.64 pg/ml; P = NS). A similar stimulation pattern was found for other cytokines (data not shown). Using live fungal cells, we assessed the morphology of the fungal elements during the assay (37°C incubation in RPMI medium in the absence of plasma or serum). Whereas formation of pseudohyphae was common, no fully developed hyphae were formed. Importantly, there were no differences in hyphal formation among the various mutant strains and the control strain during the incubation.

Recognition of O-linked and N-linked mannosyl residues by TLR4 and MR. Specific anti-TLR4 and anti-MR blocking antibodies were used to investigate which PRR was involved in the recognition of mannosyl residues of the C. albicans cell wall. Blockade of either TLR4 or MR of human MNCs inhibited TNF production stimulated by the C. albicans wild-type strain NGY152. In contrast, differential blockade of cytokine release was observed in the case of the och1 and mnt1 mnt2 mutant strains. The TNF released by the PBMCs stimulated with the N-linked mannosylation-defective och1 mutant was inhibited by anti-TLR4, but not by the anti-MR antibody (Figure 5A), demonstrating that the MR recognizes N-linked mannosyl residues. The opposite was true for TNF stimulation by the O-linked mannosylation defective mnt1 mnt2 mutant, which was inhibited by anti-MR antibodies but not by anti-TLR4 antibodies, suggesting that TLR4 recognizes O-linked mannosyl residues (Figure 5A). This conclusion was confirmed in TLR4-deficient mice, which displayed lower TNF production after stimulation with CAI-4 and och1 strains but not after stimulation with the mnt1 mnt2 mutant (Figure 5B).

The role of β-glucan–dectin-1 interaction for cytokine stimulation. Interaction of β-glucans with dectin-1/TLR2 complexes has been shown to induce cytokine production, and DC-SIGN is an additional C-type
A lectin receptor that is able to recognize *C. albicans*. We hypothesized that interaction of *C. albicans* with either of these receptors may account for the residual cytokine production induced in the absence of the mannosyl residues. Accordingly, we used combinations of *C. albicans* mutant strains and receptor blockade and assessed TNF release by the *och1* mutant in TLR4–/– mice and by the *mnt1 mnt2* strain in the presence of anti-MR antibodies. In these 2 situations, the signals induced by both N-linked mannosyl/MR and O-linked mannosyl/TLR4 complexes were absent. The residual cytokine production stimulated by *C. albicans* in these 2 experimental conditions was completely blocked by laminarin, a ligand of dectin-1 that is rich in β1,3-glucan (Figure 6). In contrast, no role for DC-SIGN could be demonstrated using specific blocking antibodies (data not shown). Interestingly, when dectin-1–blocking experiments were performed with either heat-killed or live *C. albicans* microorganisms, laminarin had a much stronger inhibitory effect on cytokines.

**Figure 3**
The role of N- and O-linked mannosyl residues for cytokine stimulation by *C. albicans*. MNCs were stimulated for various time points with the various *C. albicans* strains: the wild-type parent NGY152 strain; the och1 mutant (strain NGY357; ref. 29), defective in N-linked mannan; the mnt1 mnt2 mutant (strain NGY337; ref. 27), defective in O-linked mannan; and the mnn4 mutant (strain CDH15; ref. 28), defective in phosphomannan. (A and C) *C. albicans* concentration-dependent stimulation curves for TNF (A) and IFN-γ (C) after stimulation for 24 hours. (B and D) Time-dependent stimulation curves for the 2 cytokines when MNCs were stimulated with the various *C. albicans* strains. Results (mean ± SD) are pooled triplicate data from 2 separate experiments with a total of 8 volunteers per group. *P < 0.05; **P < 0.01 versus wild-type.

**Figure 4**
Reintegration of the defective genes restores cytokine production. (A) MNCs were stimulated with the parent NGY152 strain, the N-linked mannosyl-defective *C. albicans* strain (*och1*; strain NGY357; ref. 29), and the complemented reintegrant *och1/och1/OCH1* strain (strain NGY358). (B) Stimulation was also performed with the conditional doxycycline-dependent mutant (*pTET/och1*; strain NGY361; ref. 29) in both the absence and the presence of doxycycline (doxy). (C) Comparison of the *mnt1 mnt2* mutant (strain NGY337; ref. 27), defective in O-linked mannosyl residues, with the *mnt1 mnt2 + MNT1* reintegrant strain (strain NGY335; ref. 27). After 24 hours’ stimulation at 37°C, supernatants were collected, and cytokines were determined by RIA or ELISA. Results (mean ± SD) are pooled triplicate data from 2 separate experiments with a total of 8 volunteers per group. *P < 0.05; **P < 0.01 versus wild-type.
induced by heat-killed *C. albicans* yeasts compared with live fungi (data not shown). This finding strongly sustains the recent findings of Gantner et al., who reported that the β-glucans of the cell wall in live cells are shielded from recognition by the mannann layers but become exposed in heat-killed *C. albicans* (31).

The role of mannosyl residues in the virulence of *C. albicans*. Published experiments from our groups have established the role of mannosyl residues in the virulence of *C. albicans*. We have shown that the och1, pmr1, and mnt1 mnt2 strains are attenuated in virulence in a mouse model (26, 27), but that the mnn4 mutant is unaffected in virulence (28). We extended this in the present study by characterizing the course of disseminated candidiasis and cytokine production in mice infected with the och1 *C. albicans* strain. Compared with the wild-type strain, the och1 strain induced significantly less mortality in a model of disseminated candidiasis in mice (Figure 7A) and had a reduced fungal load in the organs of the mice (Figure 7B). However, viable cells were still present in kidneys with little sign of disease. Virulence was fully recovered in the control strain that contained a reintegrated copy of OCH1 (Figure 7). These virulence parameters were paralleled by significantly decreased cytokine levels in the kidneys of the mice infected with the och1 *C. albicans* mutant (Figure 7C).

Discussion

In the present study, we have shown that 3 components of the cell wall of a pathogenic fungus, N-linked mannans, O-linked mannans, and β-glucans, are involved in the recognition by monocytes/macrophages and for the subsequent induction of pro- and anti-inflammatory cytokine release. To investigate the role of cell wall mannosyl groups for the recognition of *C. albicans*, we used isogenic mutant strains of *C. albicans* with specific defects in the mannosylation of cell wall proteins. A gross defect in protein mannosylation was investigated using *C. albicans* Pmr1p, which encodes a Mn³⁺/Ca²⁺ transporter necessary for the activity of several Golgi-bound mannosyl transferases and shows defects in both N-linked and O-linked mannosylation (29). The role of phosphomannan was investigated in the mnn4 mutant (28), while the role of O-linked mannosylation was assessed using a mnt1 mnt2 mutant strain lacking 2 partially redundant α1,2-mannosyl transferases that are required to add the second and third mannose residues to a linear oligomannoside (27). Finally, the effects of N-linked mannosylation were investigated in the och1 *C. albicans* strain, which lacks an α1,6-mannosyl transferase that is required for initiation of the synthesis of the branched outer mannan chains (29).

We demonstrated that N-linked and O-linked mannosyl groups of glycoproteins of the outer surface of the cell wall were responsible for most of the cytokine-stimulating activity by the yeast

Figure 5

Differential recognition of O- and N-linked mannosyl residues by TLR4 and MR. (A) Human MNCs were stimulated with the various *C. albicans* strains — the parent NGY152 strain; the och1 mutant (strain NGY357; ref. 29), defective in N-linked mannosylation; and the mnt1 mnt2 mutant (strain NGY337; ref. 27), defective in O-linked mannosylation — in the presence of monoclonal antibodies against TLR4 or MR or a isotype-matched control antibody. After 24 hours' stimulation at 37°C, supernatants were collected, and TNF concentration was measured by RIA. Results are pooled triplicate data from 2 separate experiments with a total of 8 volunteers per group. *P < 0.05; **P < 0.01; ***P < 0.001 versus stimulation in the presence of control antibodies (A) or versus TLR4⁻/⁻ mice (B).

Figure 6

The role of β-glucan/dectin-1 interaction for cytokine stimulation by *C. albicans*. The role of β-glucan/dectin-1 interaction for *C. albicans*-induced TNF was investigated using 2 approaches with combinations of mutant *C. albicans* strains and receptor blockade: (A) stimulation with the och1 NGY357 strain (29) in TLR4⁻/⁻ mice and (B) stimulation with mnt1 mnt2 NGY337 strain (27) in the presence of anti-MR antibodies in human MNCs. In both situations, the signals induced by N-linked mannosyl/MR and O-linked mannosyl/TLR4 complexes were deficient. The residual cytokine production stimulated by *C. albicans* in these 2 experimental conditions was completely blocked by laminarin, a ligand of dectin-1. Results (mean ± SD) are pooled data from 2 separate experiments with a total of 10 mice per group (A) or 8 human volunteers (B). *P < 0.05; **P < 0.01; ***P < 0.001 versus wild-type.
Mannosyl residues are the most PAMPs recognized by these receptors when yeasts, which previously that differential activation of PRRs such as TLR2 and other receptors. We found that purified mannan isolated from C. albicans stimulated cytokine production in a manner dependent partly on MyD88, an intracellular TLR adaptor molecule (37), and on TLR4. This extends previous studies that also suggested TLR4-dependent cytokine production by mannan (22). Second, we demonstrated that mannan-binding sites on the surface of leukocytes were important for recognition of the yeast cell surface (5). Third, mutants lacking N- or O-linked mannosyl residues were markedly affected in their ability to induce cytokines in monocytes.

We demonstrated that a global defect in the mannosylation of cell wall mannoproteins due to in the absence of the pmr1 Golgi transporter resulted in strongly reduced cytokine induction. Perhaps surprisingly, considering the reported immunostimulatory effects attributed to mannosylphosphate (38), a mannosylphosphate-deficient mnn4 mutant induced normal cytokine production. This was in agreement with the previous observation that this mutant is recognized normally by macrophages (28). In contrast, approximately 70% less cytokine production was stimulated by och1 mutants lacking branched N-linked mannan, and 30% less cytokine production was seen using a C. albicans mutant with truncated O-linked mannan. A similar difference in cytokine production as found between heat-killed C. albicans strains was seen with the live microorganisms. This is an important observation, because heat-killed C. albicans was present exclusively as yeasts, whereas large numbers of pseudohyphae were present in the preparations using live C. albicans. Therefore, mannans are the most important structure for the induction of cytokines by both live and heat-killed C. albicans, in accord with the localization of these epitopes at the exterior face of the fungal cell wall and with the modifications of the superficial cell wall structure visible by electron microscopy (Figure 1).

In addition to demonstrating the role of protein mannosylation for C. albicans-induced cytokine production, we also identified the PRRs involved in the specific recognition of N-linked and O-linked mannosyl residues. Using a combination of mannosyl-defective C. albicans strains, anti-receptor blocking antibodies, and knockout mice, we demonstrated that MR recognized the highly branched N-linked mannosyl chains whereas TLR4 bound and recognized the linear O-linked mannosyl phosphate-deficient mutants lacking branched N-linked mannan. A similar difference in cytokine production was found between heat-killed C. albicans strains was seen with the live microorganisms. This is an important observation, because heat-killed C. albicans was present exclusively as yeasts, whereas large numbers of pseudohyphae were present in the preparations using live C. albicans. Therefore, mannans are the most important structure for the induction of cytokines by both live and heat-killed C. albicans, in accord with the localization of these epitopes at the exterior face of the fungal cell wall and with the modifications of the superficial cell wall structure visible by electron microscopy (Figure 1).

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recognize specific structures of the fungal cell wall, and we are currently investigating how this recognition is translated into differentiated immune responses.

Using combinations of mutant C. albicans strains and receptor blockade (testing the och1 strain in TLR4+/− mice and the mnt1 mnt2 strain in the presence of anti-MR antibodies), we also demonstrated that the residual cytokine production stimulated by C. albicans in the absence of signals mediated by mannosyl residues was mediated by the β-glucan receptor dectin-1. Dectin-1 has been reported to be the major receptor for β-glucan (42), and recent studies have demonstrated that it forms a receptor complex with TLR2, amplifying its effects (24, 25). Moreover, TLR2-independent activity of dectin-1 has also been recently reported (43). In addition, the recognition of yeasts, but not hyphae, by dectin-1 has been proposed to represent an escape mechanism of the fungus (31).

The differences in pattern recognition and cytokine production among different C. albicans strains were reflected by significant differences in the virulence in in vivo models of disseminated candidiasis. In earlier studies we have demonstrated reduced virulence of the pmr1, och1, and mnt1 mnt2 strains (26, 27). In the present study we confirmed a significant reduction in the virulence of the och1 strain and showed that this strain induced fewer cytokines in vivo (Figure 7). However, the experimental model of candidiasis is more complex than the in vitro experiments. Whereas stimulation of monocytes/macrophages in vitro specifically investigates the role of mannosyl residues for the recognition of C. albicans, the outcome of the experimental infection is influenced by multiple mechanisms, including pattern recognition and cytokine production as well as other factors such as adherence to host endothelial cells and growth rate of the various mutants. The och1 strain not only was defective in cytokine induction, as shown in the present study, but also displays hypersensitivity to agents that perturb the cell wall (29), which may be reflected in a lower resistance to candidal infection mechanisms. Thus a combination of these factors most likely contributes to the lower virulence of the och1 C. albicans mutant.

In conclusion, we show that C. albicans induced cytokine stimulation in mammalian MNCs via 3 pathways, each recognizing 1 of the multilayered structures of the fungal cell wall: N-linked mannosyl polymers are recognized by MR, O-linked chains by TLR4, and β-glucans by dectin-1/TLR2. Phosphomannan is apparently not involved in pro- or antiinflammatory cytokine induction. The specific activity of the cytokine induction response to these cell wall components reflects their abundance and accessibility in the C. albicans yeast cell wall. This study is the first to our knowledge that describes in totality the recognition pathways of a fungal pathogen and can serve as model for future studies of the innate recognition of other microorganisms.

Methods

Animals. TLR4-deficient C57BL/ScCr mice were from a local colony at Radboud University Nijmegen, and control TLR4-competent C57BL/10J mice and C57BL/6J mice were obtained from The Jackson Laboratory. MyD88−/− mice and TLR2−/− mice on a C57BL/6 background were kindly provided by S. Akira (Tokyo University, Tokyo, Japan). All mice weighed 20–25 g and were 6–8 weeks old. The mice were fed sterilized laboratory chow (Hope Farms) and water ad libitum. The experiments were approved by the ethics committee on animal experiments of Radboud University Nijmegen.

C. albicans strains and growth conditions. Homozygous null mutants in glycosylation genes were constructed in the C. albicans CAI-4 serotype A background by targeted gene disruption (44). Control strains were used in which the wild-type genes, under the control of their own promoters, and the URA3 selectable marker were reintroduced at the neutral RPS1 locus via the Clp10 integrative plasmid (45). Parental strain CAI-4 was also transformed with empty Clp10, generating strain NGY152, making all strains isogenic with regard to Ura status. In addition, a conditional och1 mutant was also used in which 1 remaining functional allele was regulated by the tetracycline/doxycycline–repressable TET promoter (strain NGY361; ref. 29). Mutants had defects in outer chain N-mannosylation (och1; strain NGY357, ref. 29), O-mannosylation (mnt1 mnt2; strain NGY337, ref. 27), and phosphomannan biosynthesis (mnt4; strain CDH15; ref. 28) or were downregulated in glycosylation due to low levels of Man1− in the Golgi (pmr1; strain NGY355; ref. 26). C. albicans was grown with continuous shaking at 200 rpm at 30°C in Sabouraud broth (1% mycological peptone/4% glucose) overnight, transferred to fresh medium, and incubated for 4 hours. All mutant strains showed similar morphology (yeasts and hyphae) to the control C. albicans strain, either when incubated in growth medium or together with PBMCs, as described previously in detail (26–29). The cells were harvested by centrifugation, and the pellets were washed twice in 20 ml sterile PBS and resuspended to a density of 1 × 10^6 cells/ml before heat-killing at 56°C for 1 hour. The TET-OCH1 strain was pregrown overnight in the presence of 20 μg/ml doxycycline. In separate experiments, live C. albicans yeast cells were washed and resuspended in RPMI 1640 at a concentration of 1 × 10^6 CFU/ml and used for the stimulation of cytokine production. The mnt1 mnt2, och1, and pmr1 mutant strains tended to aggregate more easily in culture medium, and we cannot completely exclude a certain degree of aggregation even after disrupting them by vigorous vortexing. This may have resulted in some underestimation of the yeast number in the suspension containing these mutant strains. In contrast, the mnt4 mutant did not aggregate.

Freeze substitution TEM. Midexponential phase yeast cells were grown in yeast extract, peptone, and dextrose medium and harvested by centrifugation, and the pellets were resuspended in 1% agarose and transferred to flat specimen carriers. The samples were frozen in liquid nitrogen at high pressure using a Leica EM PACT high-pressure freezer (Leica Microsystems). Freeze substitution of the frozen cells was carried out in an automatic temperature-controlled freeze substitution system (AFS; Leica Microsystems) in dried acetone containing 1% OsO4 at −90°C for 48 hours. The samples were gradually warmed to −30°C and then processed in a Lynx tissue processor to finish in acetone/resin at a ratio of 1:2. The samples were transferred to a Lynx tissue processor to embed in TAA8812 epoxy resin (TAAB Laboratory Equipment Ltd.). Ultra-thin sections (60 nm) were cut with a Leica ultracut E, and the sections were stained with uranyl acetate and lead citrate. Samples were imaged in a Philips CM10 transmission microscope (FEI UK Ltd.), and the images were recorded with a Gatan BioScan 792 (Gatan).

Fungal carbohydrate polymers. The mannan from C. albicans was isolated as previously described by Kogan et al. (46). Briefly, 100 g wet wt of yeast biomass was suspended in 400 ml 2% (wt/vol) KOH and heated for 1 hour at 100°C. Insoluble residues were separated by centrifugation, and mannann was precipitated from supernatant with Fehling’s reagent. The sedimented mannann-copper complex was dissolved in a minimum volume of 3 M HCl and added dropwise to methanol/acetic acid at a ratio of 8:1 (vol/vol). The procedure of dissolution and precipitation was repeated twice. Finally, the sediment was separated, dissolved in distilled water, and dialysed for 24 hours. Mannan contained no nitrogen as determined by elementary analysis. Laminarin was purchased from Sigma-Aldrich. The mannann and laminarin were chemically characterized to confirm their molecular weight and chemical structure (47, 48) and were assayed to confirm the absence of endotoxin.

Antibodies. The monoclonal anti-TLR4 HTA125 antibody was a kind gift of K. Miyake (Saga Medical School, Saga, Japan). The monoclonal mouse anti-human MR antibody and the isotype-matched IgG antibody used as a control in all experiments were purchased from Sigma-Aldrich.
Stimulation of cytokine production in human MNCs. Isolation of MNCs was performed as described previously (49). Venous blood was collected from cubital veins of 8 healthy volunteers. All volunteers gave informed consent prior to participating in the study. Samples of 5 x 10^5 MNCs in a 100-μL volume were added to round-bottomed 96-well plates (Greiner Bio-One) and incubated for 24 hours with 100 μL of the various strains of live or heat-killed (30 minutes at 56°C) C. albicans at a concentration 1 x 10^5 yeast cells/ml unless otherwise indicated. In receptor-blocking studies, MNCs were preincubated for 1 hour at 37°C with the various monoclonal antibodies (anti-TLR4, anti-MR, or control IgG; 10 μg/ml) before stimulation with C. albicans. After 24 hours’ incubation at 37°C, the MNC/C. albicans cell suspensions were centrifuged, and the supernatants were collected and stored at -70°C until assayed. Human TNF-α concentrations were determined by specific RIAs as described previously (50). IL-6, IL-10, and IFN-γ concentrations were measured by commercial ELISA kits (Sanquin).

Cytokine production by murine peritoneal macrophages. Resident peritoneal macrophages from the various mouse strains were harvested by injecting 4 ml sterile PBS containing 0.38% sodium citrate (8). After centrifugation and washing, the cells were resuspended in RPMI 1640. Cells were cultured in 96-well plates and stimulated with 100 μL of the various C. albicans strains for 24 hours at 37°C (50). Murine IL-1α, IL-1β, and TNF-α were determined by specific RIAs (detection limit, 20 pg/ml) (51).

C. albicans infection model. We used a C. albicans infection model as described previously (15). Briefly, 1 x 10^5 (for survival experiments) or 1 x 10^6 CFUs (for fungal burden) of C. albicans strains (wild-type, och1, or the reintegrant strain) were injected i.v. into mice on day 0. Survival was assessed daily. On days 3, 7, and 14, kidneys and livers of subgroups of mice were aseptically removed, weighed, and homogenized in sterile saline in a tissue grinder. The number of viable C. albicans cells was determined by plating serial dilutions on Sabouraud dextrose agar plates. The colonies were counted after 24 hours at 37°C, and results were expressed as CFU/g tissue. In addition, tissue homogenates were centrifuged, and cytokines were measured in the supernatant by ELISA (see above).

Statistics. The human experiments were performed using triplicate samples in 2 experiments with a total of 8 volunteers. The mouse experiments were performed twice in 10 mice per group. The differences between groups were analyzed by Mann-Whitney U test. The level of significance between groups was set at P < 0.05. Data are given as means ± SD.

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