Candida albicans Phospholipomannan Is Sensed through Toll-Like Receptors

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Candida albicans is a common, harmless yeast in the human digestive tract that also causes severe systemic fungal infection in hospitalized patients. Its cell-wall surface displays a unique glycolipid called phospholipomannan (PLM). The ability of PLM to stimulate tumor necrosis factor (TNF–α) production by J774 mouse cells correlates with the activation of nuclear factor (NF–κB). We examined the involvement of Toll-like receptors (TLRs) in PLM-dependent stimulation. Compared with wild-type cells, which produced large amounts of TNF–α after incubation with PLM, the deletion of the TLR4 and TLR6 genes led to a limited alteration of the PLM-induced response. Deletion of the TLR2 gene completely abolished the cell response. Surface expression of PLM is a phylogenetic trait of C. albicans, and the recognition of PLM by TLRs, together with the unique pathogenic potential of C. albicans, suggests that this molecule may be a member of the pathogen-associated molecular pattern family.

The increasing incidence of systemic fungal infections in hospitalized patients has been a constant feature of infectious diseases over recent decades [1]. Among these, infections caused by Candida species rank fourth among nosocomial infections [1]. Candida albicans is the most frequently isolated pathogen [2] when the delicate balance between the host and this otherwise harmless commensal can turn into a parasitic relationship, resulting in the development of severe infections. C. albicans is a significant cause of morbidity and mortality in hospitals, where the manifestation and severity of infection depend on the nature and extent of the impairment to the host’s immune defense.

Clinical evidence and experimental data have indicated that both the innate and adaptive immune systems regulate the control of and resistance to Candida infections [3]. The fungus is not a mere passive participant in the infectious process. The specific abilities of C. albicans to alter its phenotype and cell shape by producing filaments, to express specific adhesins, and to secrete enzymes like secreted aspartyl proteinases and phospholipases have been well characterized [1]. Modulation of the host’s immune response by C. albicans is another important factor that influences the development of infection [3]. C. albicans or its constituents have been shown to directly modulate the response of accessory cells of innate immunity [4–8]. For example, C. albicans induces the apoptosis of neutrophils [9] and interferes with the transduction of signals necessary for the lytic activity of macrophages [10].

In a series of articles, it has been shown that C. albicans expresses a glycolipid, phospholipomannan (PLM) [11–13], that has potent activity on the innate immune response, which leads to the production of proinflammatory mediators by cells of myeloid lineage [14, 15]. An analysis of the structure of C. albicans PLM has been completed recently and showed that PLM is composed of hydroxy fatty acid amide linked to phytosphingosine, with a hydrophilic polysaccharide domain that consists...
of a linear chain of β-1,2-linked mannose residues [16]. PLM is shed by yeasts on contact with macrophages and binds to these cells, inducing tumor necrosis factor (TNF)–α secretion and protein tyrosine kinase–dependent signal transduction, similar to that induced by whole yeast cells [15].

Toll-like receptors (TLRs), a family of proteins that are homologous to Drosophila toll protein (Toll), have been shown to be critical in sensing invading surface molecules from microorganisms [17] that initiate a signaling cascade, resulting in proinflammatory cytokine production [18, 19]. Antimicrobial defenses involving macrophages are based mainly on recognition by TLR2, TLR4, and TLR6, depending on the nature of the ligands [20]. It is now agreed that TLR4 is the signal-transducing receptor for lipopolysaccharide (LPS) from gram-negative bacteria [21, 22]. TLR2 interacts with bacterial lipoproteins [23] and whole gram-positive bacteria [24], is recruited by macrophage phagosomes, and discriminates between yeasts and other pathogens [25]. Recently, TLR2 expression has been shown to be necessary for C. albicans–induced TNF–α secretion by macrophages, whereas the absence of TLR4 impaired chemokine secretion and subsequent neutrophil recruitment [26].

The C. albicans cell wall is a complex structure composed of chitin, glucans, mannanproteins, mannans, and glycolipids. Some of these molecular entities are similar in C. albicans and nonpathogenic yeasts of the same phylum. However, until now, experiments have used heat-killed yeasts, and the nature of the molecules presented to the host cells had not been determined. PLM shares most of the characteristics of the so-called pathogen-associated molecular patterns (PAMPs) [14–16, 27], which are microbial components recognized by TLRs. It was therefore tempting to speculate that PLM, like other microbial glycolipids, also uses TLRs for the activation of the immune cells. In the present study, macrophages were stimulated to produce the proinflammatory cytokine TNF–α in a TLR-dependent manner on exposure to C. albicans PLM. TLR2 was the major mediator of the proinflammatory signal induced by PLM.

MATERIALS AND METHODS

Reagents and antibodies. All reagents were obtained from Sigma-Aldrich Chimie, unless otherwise stated. Mouse monoclonal antibody (MAb) IgG specific for the p65/RelA subunit of NF-κB (clone F-6) and rabbit polyclonal IgG to TLR2 (clone H-175) were obtained from Santa Cruz Biotechnology. Horseradish peroxidase (HRPO)– and fluorescein isothiocyanate (FITC)–conjugated anti–mouse IgG and anti–rabbit IgG were obtained from Southern Biotechnology Laboratories.

Yeast culture and PLM purification. C. albicans VW32 (serotype A) was used throughout the study. Yeasts were maintained on Sabouraud’s dextrose agar at 4°C. C. albicans PLM was prepared by extensive purification partition and hydrophobic in-
teraction steps, as described elsewhere [16]. The structure of this molecule was determined by a combination of methanolysis/high-performance liquid chromatography, phosphorus/proton nuclear magnetic resonance, and matrix-associated laser desorption/ionization–time-of-flight mass-spectrometry methods. The present study used the C. albicans PLM batch recovered from those structural studies [16], after analysis by nonondenaturing methods.

J774 cell line and mouse peritoneal macrophages. The mouse macrophage-like cell line J774 (ECACC 85011428) was derived from a tumor from a female BALB/c mouse. Adherent cells were cultured at 37°C in an atmosphere that contained 5% CO₂ in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Valbiotech), 5 mmol L-glutamine, 100 µg/mL streptomycin and 50 µg/mL penicillin. Before use, cells were gently scapped off with a rubber policeman and, depending on the experiment, either plated into 8-well Labtek tissue-culture chambers (Nunc) at a concentration of 0.5 × 10⁶ cells/well for immunofluorescence assays or into 24- or 48-well tissue culture dishes at a concentration of 10⁵ cells/well in 500 µL of culture medium (for biochemical analysis or detection of TNF–α production in cell-free supernatants, respectively).

Wild-type, TLR2 knockout (KO), TLR4 KO, and TLR6 KO mice [21] were inoculated intraperitoneally with 2 mL of 4% thioglycolate (Difco Laboratories); 3 days later, peritoneal exudate cells were harvested in cold serum-free RPMI 1640 medium. Peritoneal macrophages (2 × 10⁵ cells/well) were dispensed into a 96-well plate in RPMI 1640 medium supplemented with 10% FCS. After washing, adherent cells were incubated with 50 µg/mL PLM in the presence of 30 U/mL interferon (IFN)–γ for 24 h.

Immunofluorescence analysis. After stimulation for 60 min at 37°C with PLM (10 and 50 µg/mL) or LPS (10 µg/mL, used as a control) in culture medium, J774 cells were washed with warm DMEM and fixed and permeabilized with 3.7% formaldehyde and 0.2% Triton X-100 in phosphate buffer at 20°C for 20 min. After 3 washes with phosphate buffer, 100 µL of a 1:100 dilution of either rabbit polyclonal IgG to TLR2 or anti–NF-κB p65/RelA mouse MAb IgG was added for 2 h at 20°C. After 5 washes, 100 µL of a 1:100 dilution of FITC–conjugated goat anti–rabbit or anti–mouse IgG in phosphate buffer was added for 1 h at 20°C. Slides were then washed 5 times and mounted for microscopic examination.

Nuclear extract preparation and Western blot analysis of P65/RelA translocation. Nuclear protein extracts were prepared from 10⁶ J774 cells stimulated for 90 min with 10 or 50 µg/mL PLM or 10 µg/mL LPS. Cells were washed in 1.0 mL of ice-cold PBS, and the cell pellets were lysed in 400 µL of lysis buffer (10 mmol HEPES, 10 mmol KCl, 0.1 mmol EDTA, 0.1 mmol EGTA, 0.5% Nonidet P-40, 1 mmol dithiothreitol [DTT], and 0.5 mmol...
Phospholipomannan (PLM) purified from Candida albicans stimulates tumor necrosis factor (TNF-α) production by J774 cells. J774 macrophages were incubated at 37°C without (white circles) or with (black circles) 5, (black triangles) 10, or (black squares) 50 μg/mL PLM. Supernatants were collected at different times. TNF-α bioactivity was measured in a lytic assay that used the L929 cells. Results are expressed as the mean ± SD of triplicate determinations from 1 of 3 similar experiments.

Figure 1. Phospholipomannan (PLM) purified from Candida albicans stimulates tumor necrosis factor (TNF-α) production by J774 cells. J774 macrophages were incubated at 37°C without (white circles) or with (black circles) 5, (black triangles) 10, or (black squares) 50 μg/mL PLM. Supernatants were collected at different times. TNF-α bioactivity was measured in a lytic assay that used the L929 cells. Results are expressed as the mean ± SD of triplicate determinations from 1 of 3 similar experiments.

RESULTS

PLM induces TNF-α production in J774 cells. We have shown in previous studies that C. albicans PLM stimulates TNF-α production by macrophages [14, 15]. To further explore the mechanism of PLM-induced activation, we used highly purified PLM batches whose structure has been fully characterized recently [16] and murine macrophage J774 cells, which have been shown to respond to PLM [15] and express TLRs [28, 29]. The capability of PLM to induce TNF-α production by these cells was first examined in a bioassay. As shown in figure 1, significant TNF-α production was seen after incubation with 10 μg/mL PLM. The induction of cytokine production depended on the dose of PLM added to the cells; the maximum effect was obtained when cells were incubated with 50 μg/mL PLM.

PLM activates the nuclear translocation of the p65/RelA subunit of NF-κB in J774 macrophages. In macrophages, transcriptional activation and the subsequent release of TNF-α require the activation of NF-κB [30]. We therefore examined the involvement of NF-κB in the PLM-dependent activation of J774 cells. As shown in figure 2, the incubation of J774 cells with PLM or with high doses of LPS used as a positive control for induction of NF-κB activation [31] resulted in the translocation of the p65/RelA subunit of NF-κB from the cytosol to
Figure 2. *Candida albicans* phospholipomannan (PLM) induces p65/RelA translocation in J774 cells. J774 macrophages were incubated in the presence of medium alone (A), 10 μg/mL lipopolysaccharide (B), or 10 (C) or 50 (D) μg/mL PLM. After 60 min, the cells were washed 3 times with phosphate buffer. After fixation and permeabilization with 3.7% paraformaldehyde and 0.2% Triton X-100 in PBS for 15 min at 20°C, cells were incubated for 2 h at 20°C with 100 μL of a 1:100 dilution of anti–NF-κB p65/RelA mouse monoclonal antibody (MAb) IgG. Bound MAb was revealed with fluorescein isothiocyanate–conjugated goat anti–mouse IgG.

Involvement of TLRs in PLM-induced cytokine production. The induction of NF-κB activation is recognized as a key for signals initiated at the macrophage membrane by TLRs. We, therefore, investigated the role of TLRs in the recognition of PLM by macrophages. Peritoneal macrophages isolated from either wild-type mice or 3 KO mice (TLR2−/−, TLR4−/−, and TLR6−/−) [21] were incubated with PLM, and the subsequent TNF-α production in cell-free supernatants was measured. The effect of PLM incubation on TNF-α secretion by macrophages from wild-type mice was first verified. Compared with unstimulated cells, significant TNF-α secretion by macrophages from wild-type mice was first verified. Compared with unstimulated cells, significant TNF-α production was observed after 24 h of incubation with 50 μg/mL PLM (figure 5). However, as had already been seen with primary macrophages [14], the presence of IFN-γ was necessary to obtain the optimal induc-
Figure 3. Nuclear extracts from phospholipomannan (PLM)–stimulated cells contained p65/RelA. J774 macrophages were incubated for 90 min in the presence of medium alone (lane 1) or in the presence of 50 (lane 2) µg/mL PLM or 10 (lane 3) µg/mL lipopolysaccharide. Sixty-microgram proteins from nuclear extracts were loaded in each lane and separated by 10% SDS-PAGE before transfer to nitrocellulose. Membranes were probed with anti–NF-κB p65/RelA mouse monoclonal antibody IgG. MW, molecular weight.

Figure 4. Candida albicans phospholipomannan (PLM) activates NF-κB in J774 cells. NF-κB activity in nuclear extracts from J774 cells was examined by gel-shift assays. A, The NF-κB probe was incubated with 1 (lanes 1 and 4), 3 (lanes 2 and 5), and 6 (lanes 3 and 6) µg of protein from nuclear extracts of cells incubated for 90 min without (lanes 1–3) or with 10 µg/mL (lanes 4–6) PLM. B, The specificity of the DNA-binding capacity of the complexes generated after incubation with PLM is shown. Before electrophoresis, a 20-fold excess of unlabeled oligonucleotides was added (lane 2) or not (lane 1) to complexes obtained with 6 µg of protein from PLM-stimulated cells. The results shown are representative of 3 independent experiments.

DISCUSSION

C. albicans has been shown to stimulate cells of the macrophage lineage to produce proinflammatory cytokines. The interaction of yeasts with macrophages is mediated by the cell wall [32], a structure that is rich in polysaccharide components. Among these, some have their counterpart host-specific receptors, such as the macrophage mannose receptor for α-mannosides [33], the β-glucan receptor for β-1,6 glucans [34], and the recently described receptor dectin-1 of β-1,3 glucans [35]. It has been shown recently that binding a special type of mannose residue present in C. albicans mannan, β-1,2–oligomannoside [36], to galectin-3 provides an alternative receptor for C. albicans [37]. β-1,2–oligomannosides are also present in C. albicans, in the glycan moiety of the glycolipid PLM [16]. Although the stimulatory activity of PLM toward macrophages has been demonstrated [14, 15], the mechanism(s) of the recognition of PLM by these cells has not been investigated.

The activation of NF-κB after stimulation by PLM was highlighted by translocation of the p65/RelA subunit from the cytosol to the nucleus of activated cells and, ultimately, binding to the DNA consensus site. Although different upstream pathways have been described in TLR-induced NF-κB activation, this central step in transduction represents one of the features of TLR-dependent signaling in macrophages [38]. We therefore proposed the possible involvement of these receptors in initiating the cell response to PLM.

Glycoconjugate-dependent cell stimulation is initiated mainly by TLR2 or TLR4, depending on the nature of the stimulus [20]. J774 cells used have been shown to express both TLRs [28, 29]. Immunofluorescence studies on our J774 cell line used as target cells for PLM have confirmed the expression of TLR2 localized at the plasma membrane and in vesicles, as has been reported elsewhere with monocyte-derived macrophages [39] (data not shown). To explore the role of these receptors in PLM-induced...
TNF-α production and to assess their discriminating role in PLM recognition, primary macrophages isolated from different TLR KO mice were used. The results obtained with these cells clearly showed that TLR2 expression was important for stimulation by PLM—the absence of this receptor led to the almost complete unresponsiveness of the cells to PLM. However, cells expressing TLR2, but not TLR4 or TLR6, also showed decreased TNF-α production after incubation with PLM but to a lesser extent.

Several articles have demonstrated the involvement of TLR2 in the recognition of glycolipids from a large number of phylogenetically unrelated pathogens, such as lipoarabinomannan from *Mycobacteria* species [40], lipoteichoic acid from *Staphylococcus* species, glycolipids from *Treponema* species [41], and glycosylinositolphospholipid from *Trypanosoma* species [42]. However, the complex formation of TLR2 with other molecules involved in pattern recognition—such as CD14, MD2, TLR1, TLR4, and TLR6—has been demonstrated [43]. These different possible molecular associations appear to be the foundation of PAMP recognition and of their discrimination by macrophages. For example, the interaction of TLR2 with TLR1 is necessary for the recognition of the lipid configuration of the mycobacterial lipoprotein [44]. The combined expression of TLR2 and TLR4 allows the recognition of microbial components with different structures such as glycolipids or proteins [43]. In the case of *Pseudomonas aeruginosa* mannuronic acid polymers, TLR4 nonetheless had a predominant role, given that the alteration of TLR4 expression completely suppressed cytokine production, whereas release from TLR2 KO macrophages was only half of that seen with wild-type cells [45]. For yeast recognition, both TLR2 [25, 26] and TLR4 [26] have been shown to be involved. However, although TNF-α production could be inhibited by anti-TLR2 antibodies, no such alteration was observed in cells presenting defective expression of TLR4. Conversely, the chemokine response was shown to depend on TLR4 expression but did not involve TLR2 [26].

A differential cell response has already been shown for *C. albicans* but was more related to which *C. albicans*-derived molecules were involved. In particular, chemokine secretion is induced mainly by β-glucans, either β-1,6 [34] or β-1,3 [46] glucan. These components, which are present in the deepest layers of the *C. albicans* cell wall, are only accessible for interaction with macrophages when heat-killed yeasts are used, a procedure that removes surface components [47]. Glucans components have only limited activity on TNF-α production [46]. Zymosan, another yeast product that stimulates macrophages, is recognized by a TLR2-TLR6 heterodimer [48].

The complete involvement of TLR2 and the partial involvement of TLR4 and TLR6, with a balance between the respective importance of each receptor, was seen here for a single *C. albicans*-derived molecule. This molecule consists of PLM, which is a phylogenetically unique glycolipid composed of phytoceramide and long linear chains of mannose residues with unusual β-1,2 linkage types [16]. This composition confers to the molecule a highly specific structure [49]. β-1,2-oligomannosides present in PLM have been shown to interact with galectin-3 [37], whereas the ubiquitous α-mannosides expressed on other *C. albicans* molecules as well as by numerous microorganisms, among which nonpathogenic yeasts are prominent. Current innovative research concerns the pleiotropic activities of galectin-3 and its ability to modulate mammalian responses through its interaction with self-molecules. Considering the structure of PLM and the role of the lectin as a receptor for its glycan moiety, the hypothesis that galectin-3 could act as a coreceptor for TLR2 is currently being explored. The mecha-
nisms involved could be similar to those involved in LPS sensing and signaling, which require both the presence of a TLR (TLR4) and another secreted molecule, MD2 [50].

In conclusion, PLM, which is present at the cell-wall surface [12] and is shed from C. albicans in contact with host cells [15], directly initiates proinflammatory cytokine production through an interaction with TLR. The demonstration that TLR2 is the main receptor for C. albicans PLM may elucidate, at least in part, the triggering mechanism for this recently demonstrated receptor in C. albicans–induced proinflammatory cytokine production [26]. Together with its glycolipidic nature, these findings make it a new member of the PAMPs family. Because this molecule, which is specific to the most common commensal opportunistic yeast pathogen, has such a status, it is likely to have a key role in the early engagement of cells of innate immunity during the saprophytic-parasitic transition.

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References