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Candida tropicalis induces pro-inflammatory cytokine production, NF-κB and MAPKs pathways regulation and dectin-1 activation

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Abstract

The prevalence of *Candida* infection induced by Non-albicans *Candida* (NAC) species is increasing. However, as a common NAC species, *C. tropicalis* has received much less study in terms of host immunity than *C. albicans* has. In this study, we evaluated the pro-inflammatory cytokine responses evoked by *C. tropicalis* and determined whether dectin-1 and downstream NF-κB and mitogen-activated protein kinases (MAPKs) signaling pathways played roles in inflammation in human peripheral blood mononuclear cells (PBMCs) and THP-1 macrophage-like cells. Exposure of PBMCs and THP-1 macrophage-like cells to *C. tropicalis* led to the enhanced gene expression and secretion of TNF-α and IL-6 in a time- and dose-dependent manner. THP-1 macrophage-like cells being challenged by *C. tropicalis* resulted in the activation of the NF-κB, p38 and ERK1/2 MAPK signaling pathways. We also found that the expression of dectin-1 was increased with *C. tropicalis* treatment. These data reveal that dectin-1 may play a role in sensing the inflammation response induced by *C. tropicalis*, and that NF-κB and MAPK are involved in the downstream signaling pathways in macrophages.

**Key words:** *Candida tropicalis*; THP-1; dectin-1; pro-inflammatory cytokines; human PBMCs
Introduction

In recent years, candidemia induced by Non-albicans Candida (NAC) species have been diagnosed with increasing frequency (Ding et al. 2015; Horn et al. 2009). *C. tropicalis* is generally considered the first or second most common NAC species. *C. tropicalis* was reported to have a higher proteolytic activity than that of other NAC species (Nawaz et al. 2015), with an increasing level of fluconazole resistance and a diversity of virulence factors (Cordeiro Rde et al. 2015; Kothavade et al. 2010; Negri et al. 2012), which may be related to its pathogenicity.

The prevalence of *C. tropicalis* varies by region and patient group. Data from the China Hospital Invasive Fungal Surveillance Net (CHIF-NET) have revealed that *C. tropicalis* is the second most prevalent non-albicans Candida NAC species in China (Xiao et al. 2015). Cancer, diabetes, older patients, long periods in the intensive care unit (ICU), antibiotic administration and invasive surgery are highly related to the pathogenicity of this organism (Negri et al. 2012; Nucci et al. 2010; Viscoli et al. 1999).

Host tissues can evoke multiple responses to defense against *Candida* species, including activation of the innate immune system and cytokine release. It has been reported that the intestinal epithelial cell (IEC) line Caco-2 enhances IL-8 mRNA expression when it is co-cultured with *C. tropicalis* (Saegusa et al. 2007). Oral endothelial cells have also been found to release an enhanced level of IL-1α when they are stimulated by *C. tropicalis* (Moyes et al. 2012). Monocytes and macrophages are important effector cells in the activation and regulation of innate immunity. Whibley et al. (Whibley et al. 2015) found that the depletion of monocytes enhances the susceptibility to *C. tropicalis*. In this report, we focused on the role of macrophages in host defense against *C. tropicalis*. 
Knowledge about immunity to *Candida* infections is based primarily on studies concerning *C. albicans*. Numerous studies have reported that the dectin-1/caspase recruitment domain-containing protein 9 (CARD9) pathway plays an essential role in antifungal immunity (Plato et al. 2013), and the inflammation response elicited by *C. albicans* infection is mainly induced by pro-inflammatory mediators (Barker et al. 2005). Dectin-1 is a kind of C-type lectin receptor (CLR) that is highly expressed on the surface of phagocytes (Brown 2006; Brown et al. 2001). The cell wall component β-1, 3-glucan is considered the major pathogen associated molecular pattern (PAMP) and can be recognized by dectin-1 (Brown et al. 2002). Following dectin-1 activation, CARD9, BCL10 and MALT1 form a complex to activate downstream signaling pathways, including NF-κB activation, and increase the level of pro-inflammatory cytokines (Plato et al. 2013; Roth et al. 2013).

At present, the recognition of *C. tropicalis* by the immune system is poorly understood. Whibley et al (Whibley et al. 2015) reported that mice lacking dectin-1 or CARD9 could easily develop *C. tropicalis* infection and exhibited increased mortality, revealing the involvement of dectin-1 and CARD9 in the protection against *C. tropicalis* infection. However, no consensus has been reached about the recognition between *C. tropicalis* and host cells.

In the present study, we aimed to investigate the inflammation induced by *C. tropicalis* using human PBMCs and PMA (phorbol-12-myristate-13-acetate)-induced THP-1 macrophage-like cells, including pro-inflammatory cytokine production, and tried to discover the pathways and receptors related to the inflammation induced by *C. tropicalis*. We demonstrated that the inflammation response exerted by *C. tropicalis* may be induced
by dectin-1, and that NF-κB and MAPKs are involved in the downstream signaling pathways in macrophages.

**Materials and Methods**

**C. tropicalis Strains**

Two isolates of *C. tropicalis* (C2F and C2G) were used in all experiments. They were gained from the China Medical Fungi Culture Collection Center and were cultured in SDA medium (2% glucose, 1% peptone, 1.5% agar) overnight at 28°C to obtain yeast cells. The cells were washed twice with phosphate-buffered saline (PBS) and were heat-killed for 30 minutes at 56°C. In all experiments, dead microorganisms were used to avoid the differences in growth conditions between isolates and the changes in the ratio between yeasts and THP-1 macrophage-like cells.

**Cell culture and maintenance**

The human monocyte cell line THP-1 (ATCC TIB-202, Manassas, VA, USA) was grown in RPMI-1640 growth medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin and 100 μg/mL streptomycin in a humid atmosphere of 5% CO₂ at 37°C. THP-1 cells can be differentiated into macrophage-like cells by treatment with 100 ng/mL PMA (Sigma-Aldrich, St. Louis, MO, USA) for 48 hours (Qin 2012). The cells were then replaced with medium without PMA.

**Blood Donors**

Blood samples were collected from healthy adult volunteers at Jiangsu Province Blood Center, Nanjing, China. The subjects were provided with information about the study and signed the written informed consent documents. The experiment was approved by the
Ethics Review Committee from the Institute of Dermatology, Chinese Academy of Medical Science & Peking Union Medical College and Jiangsu Province Blood Center.

**Isolation of PBMCs**

Five milliliters of blood was diluted with an equivalent volume of PBS and was layered over the lymphocyte separation solution (Axis-Shield, Dundee, UK) at the same volume. The tubes were centrifuged for 22 minutes at 2200 g. The cell interface layer was harvested carefully, and cells were washed twice in PBS and re-suspended in RPMI-1640 culture medium supplemented with 10% fetal bovine serum. Cells were counted using hemocytometer, and the concentration was then adjusted to $5 \times 10^6$ cells/mL.

**ELISA**

Specific commercial ELISA kits (Neobioscience, Shen Zhen, China) were used to measure the amount of TNF-α and IL-6 in the cell-free culture supernatants. The experimental procedure was performed according to the manufacturer’s instructions. Samples were always sufficiently diluted to ensure that values fell within the linear range of the assay. All experiments were performed in triplicate. The values were expressed as the means ± standard deviation (SD).

**Real-time RT-PCR**

After the respective treatments, cells were harvested, and total RNA was extracted with TRIZol Regent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the PrimeScript RT Master Mix (TaKaRa, Tokyo, Japan) and 1 μg of total RNA. Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-rad, Hercules, CA, USA) on a 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primer sequences are shown in Table 1. mRNA expression was calculated by fold

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changes using the $2^{-\Delta\Delta C_T}$ formula, where $\Delta C_T$ is the difference between the target gene and $\beta$-actin, and $\Delta\Delta C_T$ for the sample $=\Delta C_T$ of treated-condition $-\Delta C_T$ of the control-condition.

**Western blotting analysis**

Cells were lysed in RIPA Lysis Buffer containing protease inhibitor cocktail and the phosphatase inhibitor PhosSTOP (both from Roche Applied Science, Basel, Switzerland). The protein concentration was determined by the BCA assay (Beyotime Biotechnology, Haimen, Jiangsu, China). Equal amounts of protein from different samples were separated by 12% SDS–polyacrylamide gel electrophoresis (Beyotime Biotechnology) and were transferred onto PVDF membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin (BSA) (AMRESCO, Solon, OH, USA) and were probed with the indicated primary antibodies. Antibodies against phospho-\(\text{IkB}\alpha\), \(\text{IkB}\alpha\), phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2 MAPK, and NF-\(\kappa\B)-p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). The membranes were then incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology). The protein bands were visualized using the chemiluminescence imaging method. The band intensities were quantified using Quantity One. $\beta$-actin served as the loading control.

**Immunofluorescence assay for detecting the nuclear translocation of NF-\(\kappa\B\)**

For NF-\(\kappa\B\) detection, THP-1 macrophage-like cells were cultured on glass dishes. After the respective treatments, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (w/v), and blocked with 3% BSA. These cells were incubated with monoclonal antibodies against NF-\(\kappa\B\)-p65 over night at 4°C followed by 2 hours of
incubation with anti-rabbit IgG conjugated to Cy3 antibody (excitation wavelength 550nm; emission wavelength 570nm) (Beyotime Biotechnology) on a rocker. The cells were washed three times with PBS for 5 minutes, and the nuclei were stained with DAPI (excitation wavelength 359 nm; emission wavelength 461 nm) for 15 minutes. Subsequently, the cells were visualized by confocal microscopy.

**Flow cytometry**

THP-1 macrophage-like cells were collected and labeled with phycoerythrin (PE)-conjugated anti-dectin-1 monoclonal antibody (eBioscience, San Diego, CA, USA) for 30 minutes at 4°C. After washing, the cells were resuspended in PBS and analyzed by flow cytometry (BD FACSVerse™) using the Flow Jo software (TreeStar, Ashland, OR, USA).

**Statistical analysis**

The independent experiments were performed at different times. Similar results were obtained from at least three independent experiments for the statistical analysis. The data are expressed as the means ± SD and were analyzed by univariate ANOVA or Student’s t-test using the GraphPad Prism 5 Software (GraphPad Software, San Diego, CA). *P* values < 0.05 were considered statistically significant.

**Results**

**Effect of *C. tropicalis* on gene expression and secretion of the pro-inflammatory mediators TNF-α and IL-6 in PBMCs**

We found that co-culturing PBMCs with two *C. tropicalis* isolates, C2F and C2G (10:1, fungi to cell), enhanced the expression of mRNAs encoding TNF-α and IL-6 (Fig. 1), suggesting that PBMCs could exert antifungal immune responses. The dectin-1 ligand
Curdlan (100 μg/ml) was selected as the positive control, and untreated cells served as the negative control.

**Effect of *C. tropicalis* on the gene expression and secretion of pro-inflammatory mediators TNF-α and IL-6 in THP-1 macrophage-like cells**

To determine whether the enhancement of pro-inflammatory mediators also occurred in macrophages, we used THP-1 macrophage-like cells to co-culture with *C. tropicalis* for different durations and assayed the mRNA expression of TNF-α and IL-6 in THP-1 macrophage-like cells exposed to *C. tropicalis* (1:1 and 10:1, fungi to cell). The up-regulated mRNA expression of TNF-α and IL-6 in THP-1 macrophage-like cells began 3 hours after stimulation, and peaked at 6 hours, and fungi induced the cytokine levels in a dose-dependent manner (Table 2).

After 24 hours of incubation, the culture supernatants of THP-1 macrophage-like cells exposed to *C. tropicalis* (10:1, fungi to cell) were harvested. The concentration of TNF-α and IL-6 was up-regulated by *C. tropicalis* (Fig 2), and THP-1 macrophage-like cells could exert an almost similar or stronger effect than PBMCs. We then applied THP-1 macrophage-like cells as the main target cells of our study.

**Activation of the NF-κB, p38 MAPK and ERK1/2 MAPK signaling pathways by *C. tropicalis* in THP-1 macrophage-like cells**

To determine the activation of NF-κB, we measured the level of phosphorylation and degradation of IκB-α in THP-1 macrophage-like cells. Our results showed that significant IκB-α phosphorylation and degradation was detected at 30 minutes and reached a peak 1 hour post stimulation (Fig. 3a).

To further elucidate the molecular mechanism by which *C. tropicalis* modulates gene
expression in THP-1 macrophage-like cells, the effect of yeasts on NF-κB was investigated. The results presented in Fig. 4 clearly indicated that C. tropicalis and Curdlan could induce the translocation of NF-κB in THP-1 macrophage-like cells. The first panel of confocal images of Fig. 4 showed that NF-κB-p65 was mostly located in the cytoplasm of un-induced cells, and it was obviously transferred to the nuclei in the C. tropicalis isolates C2F- and C2G-stimulated cells. These results demonstrated that activation of NF-κB was involved in the C. tropicalis-elicited inflammatory response.

The positive control Curdlan showed a similar response (Fig. 4, 2nd panel).

We also examined the effects of C. tropicalis on the activation of p38 MAPK and ERK1/2 MAPK. Our results found that p38 MAPK and ERK1/2 MAPK phosphorylation was induced after 30 minutes in THP-1 macrophage-like cells (Fig. 3a).

**Effect of C. tropicalis on dectin-1 expression**

Because dectin-1 is one of the most important receptors involved in the recognition of C. albicans (Netea et al. 2006), we tried to study whether it plays a role in the immune sensing of C. tropicalis. Flow cytometry analysis was used to assess dectin-1 expression on the surface of THP-1 macrophage-like cells after treatment with C. tropicalis for different durations. The expression of dectin-1 was up-regulated after stimulation by C. tropicalis isolates C2F and C2G, and reached a peak at 6 to 12 hours (Fig. 5). Therefore, our results indicated that this receptor may play an important role in the immune recognition of C. tropicalis.

**Discussion**

NAC species are inducing increasing numbers of infections, and there is an urgent need to explore immunity against these emerging species. Studies concerning C. albicans were
set as the skeleton diagram to further explore and understand the immunity to other
*Candida* species. There is a similarity between *C. tropicalis* and *C. albicans* because both
species can escape from the immune response by forming blastoconidia, hyphae and
pseudohyphae, and it has been found they can both induce a specific CD4<sup>lo</sup>CD8<sup>hi</sup> T
lymphocyte response upon infection (Misme-Aucouturier et al. 2016). Studies have
shown that neutrophils and monocytes served as the first cells to sense and kill *C.
tropicalis* (Lindemann et al. 1991; Whibley et al. 2015). Comparatively, little attention
was paid to the interaction between macrophages and *C. tropicalis*. In this article, we
sought to explore the inflammation induced by *C. tropicalis* in human macrophages.

In this study, our data showed that *C. tropicalis* treatment in human PBMCs and THP-1
macrophage-like cells reveals an enhanced level of the production of two
pro-inflammatory cytokines (TNF-α and IL-6). Whibley *et al* (Whibley et al. 2015) have
found that *C. tropicalis* could induce the enhanced production of TNF-α, which plays a
role in limiting fungal disease during disseminated *C. tropicalis* infection. Other
researchers have examined the effect of *C. tropicalis* on the expression of other
pro-inflammatory cytokines, such as IL-8, IL-1α and IL-6 (Foster *et al*. 2015; Moyes *et
al*. 2012; Saegusa *et al*. 2007). In this regard, these cytokines may be involved in the
process of protection from *C. tropicalis* infection.

NF-κB is an important transcriptional regulator and controls the expression of various
pro-inflammatory mediators (DiDonato *et al*. 2012). IκB acts as an inhibitory protein to
prevent NF-κB migration into the nucleus, and the activation of IκB-α leads to the release
of NF-κB p65/p50 dimers into the nucleus (Hinz *et al*. 2012). Studies have reported that
MAPK subfamilies ERK1/2 and p38 participate in multiple functional responses and
induce the expression of various genes that regulate the inflammatory response (Arthur et al. 2013). A striking finding of our research was the activation of p38 MAPK, ERK1/2 MAPK and IκB-α following the recognition of *C. tropicalis*. Wang et al (Wang et al. 2016) found that the hyphae and yeast forms of *C. tropicalis* could induce NF-κB activation and cytokine (IL-6, TNF-α and IL-10) production, and demonstrated that dectin-3 was required for CARD9/Bcl10 complex formation and NF-κB activation. Our research partly supported their conclusion, and we considered that p38 MAPK and ERK1/2 MAPK may participate in the immunologic defense against *C. tropicalis*.

Because our experiment used the heat-killing yeast form of *C. tropicalis* for research, we wanted to demonstrate whether the exposure of β-glucan could induce the recognition of dectin-1. Our study showed that dectin-1 is indeed an important receptor for the immune sensing of this species because the stimulation experiments resulted in the significantly increased expression of dectin-1. After dectin-1 recognize β-1,3-glucan, the tyrosine residue within the cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) is phosphorylated and results in the recruitment of syk, leading to the activation of the downstream signaling pathway. Thus, our study indicated that the activation of dectin-1 and downstream signaling pathways after challenge by *C. tropicalis* might occur in THP-1 macrophage-like cells.

CARD9 is an important adaptor protein that participates in the downstream signaling pathway of several ITAM-associated CLRs, such as dectin-1, dectin-2, dectin-3 and mincle (Gross et al. 2006; Jia et al. 2014; Robinson et al. 2009; Wang et al. 2016). Whibley et al (Whibley et al. 2015) have reported that mice lacking CARD9 showed 100% mortality in disseminated *C. tropicalis* infection, and mice lacking dectin-1 showed
50% mortality, but dectin-2 did not influence the susceptibility. Thus, dectin-1 plays a major role in the recognition of both *C. albicans* and *C. tropicalis*. Additionally, dectin-3 deficiency was found to reduce the phagocytic and fungicidal abilities in macrophages (Wang et al. 2016). Thus, there may be several CLRs involved in *C. tropicalis* induced immunity, and these need further investigation.

Taken together, our study contributes to the better understanding of the inflammation response against the emerging pathogen *C. tropicalis*. It was verified that *C. tropicalis* induce significantly enhanced IL-6 and TNF-α production in vitro in THP-1 macrophage-like cells and human PBMCs. Moreover, we found that *C. tropicalis* induces immune responses by activation of dectin-1 as well as the NF-κB, p38 MAPK and ERK1/2 MAPK signaling pathways in THP-1 macrophage-like cells. Although the underlying molecular mechanisms remain to be clarified, these findings may partly explain the inflammatory response of *C. tropicalis* infection. By studying the precise native responses against this type of NAC infection in macrophages, our findings may enable the exploration and development of novel antifungal strategies.

**Acknowledgements**

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

References


Figure legends

**Fig. 1** Effect of *C. tropicalis* on the gene expression and secretion of pro-inflammatory mediators TNF-α and IL-6 in PBMCs. Real-time PCR was used to determine the TNF-α and IL-6 mRNA expression levels in PBMCs challenged with *C. tropicalis* isolates C2F and C2G (10:1, fungi to cell) and Curdlan (100 µg/mL) for 6 hours (a). PBMCs were exposed to *C. tropicalis* (10:1, fungi to cell) and Curdlan for 24 hours, and the concentrations of the secreted of TNF-α and IL-6 proteins in the cell-free culture supernatants were determined (b, c). (**) *P*<0.01, (***) *P*<0.001.

**Fig. 2** Effect of *C. tropicalis* on the secretion of pro-inflammatory mediators TNF-α and IL-6 in THP-1 macrophage-like cells. THP-1 macrophage-like cells were exposed to *C. tropicalis* isolates C2F and C2G (10:1, fungi to cell) and Curdlan for 24 hours. The protein concentrations of TNF-α and IL-6 in the cell free culture supernatants were determined. (***) *P*<0.001.

**Fig. 3** *C. tropicalis* triggers the activation of NF-κB, p38 MAPK and ERK MAPK in THP-1 macrophage-like cells. Cellular extracts from THP-1 macrophage-like cells stimulated with *C. tropicalis* isolates C2F and C2G (10:1, fungi to cell) for 30 minutes and 1 hour were prepared and analyzed for the phosphorylation and degradation of IκB-α, p38 MAPK and ERK1/2 MAPK by western blotting (a). The positive control Curdlan can trigger similar responses (b).

**Fig. 4** Confocal microscopic images demonstrate the effect of *C. tropicalis* on the translocation of NF-κB in THP-1 macrophage-like cells. NF-κB-p65 translocation was analyzed by staining with NF-κB-p65 (red), and nuclei were colored with DAPI (blue). Images of un-treated cells are shown in panel 1. THP-1 macrophage-like cells treated
with Curdlan for 1 hour are shown in the 2nd panel. Cells challenged by *C. tropicalis*
isolates C2F and C2G are shown in the 3rd and 4th panels, respectively. The merged
images show that *C. tropicalis* can induce the translocation of NF-κB in THP-1
macrophage-like cells. Scale bar=10 µm.

**Fig. 5** *C. tropicalis* induces increased expression of dectin-1 on the surface of THP-1
macrophage-like cells. Flow cytometry was used to assess dectin-1 expression on the
surface of THP-1 macrophage-like cells after treatment with *C. tropicalis* isolates C2F
and C2G for 24 hours. The expression of dectin-1 was up-regulated after stimulation by
C2F and C2G, and reached a peak at 6 to 12 hours. (*P<0.05, **P<0.01, ***P<0.001).
Table 1. Sequence information for real-time RT-PCR primers

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Table 2. Effects of *Candida tropicalis* on mRNA encoding pro-inflammatory cytokines in THP-1 macrophage-like cells

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<td>292.2***</td>
<td>292.2***</td>
<td>292.2***</td>
<td>292.2***</td>
</tr>
</tbody>
</table>

Real-time RT-PCR was used to determine the TNF-α, IL-6 mRNA expression in THP-1 macrophage-like cells challenged with *C. tropicalis* (1:1 and 10:1, fungi to cell) and Curdlan (100 µg/mL) for 1, 3, 6 hours. C2F and C2G were two isolates of *C. tropicalis* used in all experiments. The mRNA level was calculated using $2^{-\Delta\Delta C_{T}}$ formula. (*$P<0.05$, **$P<0.01$, ***$P<0.001$).
206x148mm (300 x 300 DPI)