Candida albicans induces TLR2/MyD88/NF-κB signaling and inflammation in oral lichen planus-derived keratinocytes

Jianwei Liu¹*, Fanghui Geng²*, Hongying Sun²*, Xiaxia Wang², Hui Zhang², Qiaozhen Yang², Jie Zhang²

¹ Department of Rheumatology, Shandong University Affiliated Qilu Hospital, Jinan, Shandong, China
² Department of Stomatology, Huashan Hospital, Fudan University, Shanghai, China

* These authors equally contributed.

Abstract
Introduction: The risk of oral lichen planus (OLP), a chronic inflammatory oral mucosal disease, becoming malignant increases by 21-fold in patients with fungal infection. This study examined the impact of Candida albicans exposure on Toll-like receptor (TLR) signaling in primary keratinocyte cultures obtained from OLP patients.

Methodology: Following co-culture of primary OLP keratinocyte cultures with C. albicans for 24 hours, inflammatory cytokine concentrations were determined by ELISA. TLR2, MyD88, and NF-κBp65 mRNA and protein expression were assessed using quantitative RT-PCR and Western blot analyses, respectively. Keratinocyte apoptosis was also determined by flow cytometry.

Results: IL-10, IL-8, IL-2, and TNF-α levels were significantly higher following co-culture with C. albicans (all \( p \leq 0.034 \)). MyD88, NF-κB p65, and TLR2 mRNA (all \( p < 0.001 \)) and protein (all \( p \leq 0.004 \)) expression levels were significantly higher in OLP keratinocytes following C. albicans exposure. Finally, the apoptosis rates of OLP keratinocytes were 21.2%, 29.4%, and 25.4% for the control cells and 3.9%, 5.6%, and 4.4% for those exposed to C. albicans, suggesting that co-culture with C. albicans inhibits the apoptosis of OLP keratinocytes.

Conclusions: C. albicans activates the TLR2/MyD88/NF-κB signaling pathway in OLP keratinocytes, resulting in increased cytokine expression and decreased keratinocyte apoptosis. Two key events in the pathogenesis of OLP and its progression to malignancy, namely increased inflammation and decreased apoptosis, were induced by exposure to C. albicans. Thus, targeting this signaling pathway may represent a novel therapeutic strategy to prevent OLP malignant transformation.

Key words: Apoptosis; Candida albicans; keratinocyte; oral lichen planus; Toll-like receptor.


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Introduction
With a prevalence of 1-2% in the general population, oral lichen planus (OLP) is a chronic inflammatory oral mucosal disease that is most often observed in female middle-aged patients. Although its etiology is still unclear, involvement of CD8+ T cells, mast cell degranulation, basal keratinocyte apoptosis, atrophy of the keratin layer and matrix metalloproteinase expression has been shown [1,2]. Furthermore, OLP is characterized by chronic inflammation mediated by elevated interleukin-10 (IL-10), IL-8, IL-2, and tumor necrosis factor-α (TNF-α) levels [3,4]. Therefore, the principle mode of treatment for OLP consists of corticosteroids, immunosuppressants, and immunomodulators [1]. Because 0.4-6.5% of OLP cases will become malignant, the World Health Organization (WHO) regards OLP as a precancerous lesion [5]. The risk for malignant transformation in OLP patients with fungal infection is 21-fold higher than in patients with OLP alone [6].

Most oral infections in OLP patients are mainly caused by Candida albicans, which may be found in up to 76.7% of OLP patients [7]. Furthermore, patients with OLP have impaired immune responses to purified protein derivative and C. albicans as demonstrated by reduced interferon-γ (IFN-γ) and TNF-α secretion [8]. However, a role for C. albicans as an etiologic factor for OLP has not been confirmed [9].

Candida biofilm formation is crucial for the adhesion of C. albicans to host epithelium. Keratinocytes not only play an important role in the pathological changes that occur within an OLP lesion [10,11], but they are also crucial target cells of C. albicans and immune cells of the mucosa [12,13]. Specifically, CD8+ T cells induce keratinocyte apoptosis [14,15], resulting in degradation of the
epithelial-connective tissue interface and cleft formation [13].

Keratinocyte Toll-like receptors (TLRs), which are important components of non-specific and specific immunity, play important roles in the recognition of pathogens, including C. albicans, and their metabolites as well as the response to C. albicans-induced inflammation [16]. After activation, TLR may recruit specific adapters, including MyD88, TRIF, TIRAP/MAL, TRAM and SARM, resulting in inflammatory cytokine and chemokine production, including IL-10, IL-8, IL-2, and TNF-α, and activation of long lasting inflammation in OLP [17]. Although pivotal roles for TLRs in host defense, immune regulation and tumorigenesis are well-established [18], its role in C. albicans infection is still poorly understood, and the few available findings are conflicting [19,20].

The present study was undertaken to examine the effect of C. albicans on TLR signaling and apoptosis in OLP-derived keratinocytes with the goal of elucidating the pathogenesis of OLP malignant transformation. Primary OLP keratinocyte cultures were obtained from patient samples. After co-culture with C. albicans for 24 hours, inflammatory cytokine levels and apoptosis as well as MyD88, NF-κB p65, and TLR2 expression were determined.

Methodology
Sample collection
Patients receiving a biopsy at the oral mucosa clinic of Huashan Hospital for skin lesion-free OLP with a reticulate pattern at the buccal mucosa from November 2013 to January 2014 were enrolled. The tissues from 30 patients (23 females and 7 males) with a mean age of 56.3 y (range: 37 to 88 y) were collected for evaluation of inflammatory factor secretion (n = 9), Western blot analysis (n = 9), RT-PCR analysis (n = 9), and apoptosis analysis (n = 3). Patients were excluded if they had received local or systemic therapy with corticosteroids or other immunosuppressants within 3 months prior to the study or had systemic diseases, such as diabetes and immunodeficiency. The pathological diagnosis was conducted according to the modified diagnostic criteria of the WHO [21]. This study was approved by the institutional review board of the Huashan Hospital, and all patients provided informed consent.

Establishment of primary human OLP keratinocyte cultures

Human OLP keratinocyte cultures were obtained as previously described [22]. Briefly, the tissues were washed in PBS and digested in 0.25% Dispase II (GIBCO, Carlsbad, USA) at 4°C for 11-12 h. The epithelial layer was cut into 1-4 mm² blocks, which were then digested in a mixture containing 0.05% trypsin and 0.02% EDTA (Sangon, Shanghai, China) (1:1) at 37°C. After 5 min of constant shaking, 10% calf serum was added to stop the digestion. The cells were harvested following centrifugation and resuspended in keratinocyte culture medium (GIBCO, New York, USA). Cells were seeded into 6-well plates at a density of 4×10⁵/mL and incubated at 37°C in a humidified environment with 5% CO₂. The medium was refreshed once every 2-3 days. Keratinocytes in the third passage were used for all subsequent experiments.

Culture of C. albicans

A standard strain of C. albicans (ATCC10231) was provided by the Department of Skin Fungus at the Huashan Hospital. C. albicans was maintained in Sabouraud-dextrose broth (ZhaoYuan Tuopu Biotech Co. Ltd, Shandong, China) at 37°C for 24-48 h with constant shaking. Following culture for 48 h, a C. albicans suspension was prepared in 5×10⁶ cells, and 100 μL was added to a 24-well plate containing coverslips followed by incubation at 37°C for 90 min. The fungal solution was removed, and the plate was washed with PBS thrice to remove non-adherent cells.

Table 1. Primers used for real-time quantitative PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5´-3´)</th>
<th>Product length (bp)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GGAAACTGTGGCCTGATGGGCCG&lt;br&gt;GGGACAGGGAGAAG</td>
<td>140</td>
<td>66.68</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>65.82</td>
</tr>
<tr>
<td>TLR2</td>
<td>CTCCATCCCACGTGCGTGCC&lt;br&gt;GAAACGTGCGCCAGGACC</td>
<td>140</td>
<td>65.99</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>65.81</td>
</tr>
<tr>
<td>MYD88</td>
<td>GGAGATCCCGCAACTTGGAGAC&lt;br&gt;GCCCAGCTTGTAAGCAGCTC</td>
<td>115</td>
<td>62.5</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>63.9</td>
</tr>
<tr>
<td>P65</td>
<td>CTGGGGGCTTTGGCTGGCCAAC&lt;br&gt;CAGGGAATCCATCATCAGCATGGGC</td>
<td>145</td>
<td>66.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65.06</td>
</tr>
</tbody>
</table>
Keratinocyte exposure to C. albicans

Keratinocytes in logarithmic phase were randomly divided into either a control group or a co-culture group. Keratinocytes in the control group were cultured alone at a density of 1×10⁶/well. Those in the co-culture group were seeded into 6-well plates at a density of 1×10⁶/well and then co-cultured with C. albicans (5×10⁵/well) for 24 hours after which they were processed for real-time quantitative PCR, Western blot analysis, or an apoptosis assay.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-10, IL-8, IL-2, and TNF-α were determined using corresponding ELISA kits following the manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA). Briefly, 50 μL of blank buffer, standards, and samples were added to a 96-well plate followed by 50 μL of biotin-conjugated antibody. After 2 h at room temperature, the wells were washed, and 100 μL of Streptavidin-HRP was added to each well for 1 h at room temperature. After washing, 100 μL of TMB Substrate Solution was added to each well and incubated in the dark at room temperature. After 10 min, 100 μL of stop solution was added to each well. The absorbance at 450nm was measured. Standard curves of the mean OD values versus the standard concentration along with the corresponding linear prediction were calculated independently for IL-10, IL-8, IL-2, and TNF-α.

Real-time quantitative PCR analysis

Total RNA was extracted with Trizol reagent (Thermo Fisher, Waltham, USA) and reverse transcribed into cDNA using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio Inc., Kusatsu, Shiga, Japan). Fluorescence quantitative PCR was conducted with an iCycler iQ thermal cycler (Bio-Rad, Hercules, USA) with a 50-μL mixture from the PrimeScript RT reagent Kit (TaKaRa Bio Inc., Kusatsu, Shiga, Japan) containing 1.0 μL of cDNA and the primers, which were designed by Shanghai Yusen Biotech Co., Ltd (China; Table 1). The conditions were as follows: pre-denaturation at 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30-34 s. The 2−ΔΔCt method was used to calculate the mRNA expression of each target gene as follows: ΔCt = Ct target gene−Ct GAPDH, ΔΔCt = ΔCt hypoxia−ΔCt control.

Western blot assay

Cells were harvested, and total protein was extracted with RIPA (50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150mM NaCl; 1mM EDTA; 1 μg/mL of Aprotinin, leupeptin, and pepstain; 1mM Na₃VO₄; 1mM NaF; 1mM PMSF) on ice. After centrifugation, the supernatant was collected. Following determination of the protein concentration using the BCA method (Beyotime Biotechnology, Shanghai, China), 30 μg of total protein was subjected to 8-10% SDS-PAGE. After the proteins were transferred onto a PVDF membrane, the membrane was next incubated with a goat anti-rabbit IgG secondary antibody (1:5000) (Santa Cruz, USA). The membrane was then blocked in 5% non-fat milk for 1 h at room temperature and incubated with the following primary antibodies overnight at 4°C: anti-TLR2 antibody (1:1000), anti-MyD88 antibody (1:1000), anti-NF-κB p65 antibody (1:1000) (all purchased from Santa Cruz Biotechnology, Santa Cruz, USA). The membrane was then incubated with a goat anti-rabbit IgG secondary antibody (1:5000; Santa Cruz Biotechnology) at room temperature for 1 h, and visualization was done with the Enhanced Chemiluminescence kit (Pierce, ThermoFisher Scientific, Milwaukee, USA). Protein bands were scanned, and their optical density was analyzed with Quantity One software (Bio-Rad).

Detection of apoptosis

Apoptotic cells were detected using an Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, USA), according to the manufacturer’s instructions. The proportion of apoptotic cells was determined using a FACS Canto flow cytometer (BD, San Jose, USA).
Figure 2. Co-culture with *C. albicans* increases MyD88, NF-κB p65, and TLR2 mRNA expression by OLP keratinocytes. MyD88, NF-κB p65, and TLR2 mRNA expression was determined by quantitative RT-PCR analysis following culture alone (control) or with *C. albicans* (experimental) for 24 hours. Data represent the mean and standard deviation of three independent experiments.

Figure 3. Co-culture with *C. albicans* increases MyD88, NF-κB p65, and TLR2 protein expression by OLP keratinocytes. MyD88, NF-κB p65, and TLR2 protein expression was determined by Western blot analysis following culture alone (control) or with *C. albicans* (experimental) for 24 hours. (A) Representative data. (B) Data represent the mean and standard deviation of three independent experiments.
Statistical analysis

Data were summarized as mean ± standard deviations (SD) of the control and experimental groups. Differences between the groups were compared using a two-sample t-test. Statistical assessments were two-tailed, and \( p < 0.05 \) was considered significant. All statistical analyses were carried out using IBM SPSS statistical software version 19.0 for Windows (IBM Corp., New York, USA).

Results

Exposure to \( C. \text{ albicans} \) increases inflammatory cytokine secretion by OLP keratinocytes

The impact of \( C. \text{ albicans} \) exposure on the secretion of inflammatory cytokines by keratinocytes was determined by ELISA. As shown in Figure 1, IL-10, IL-8, IL-2, and TNF-\( \alpha \) levels were significantly higher following co-culture with \( C. \text{ albicans} \) (all \( p \leq 0.034 \)).

Co-culture with \( C. \text{ albicans} \) increases MyD88, NF-\( \kappa B \) p65, and TLR2 expression by OLP keratinocytes

To examine the effect of co-culture with \( C. \text{ albicans} \), MyD88, NF-\( \kappa B \) p65, and TLR2 mRNA and protein expression levels were determined using quantitative real-time PCR and Western blot analyses. As shown in Figure 2, MyD88, NF-\( \kappa B \) p65, and TLR2 mRNA expression levels were significantly higher with \( C. \text{ albicans} \) exposure (\( p < 0.001 \)). Moreover, MyD88, NF-\( \kappa B \) p65, and TLR2 protein levels were also higher in cells co-cultured with \( C. \text{ albicans} \) (\( p \leq 0.004 \); Figure 3).

\( C. \text{ albicans} \) decreases OLP keratinocyte apoptosis

We next examined the impact of \( C. \text{ albicans} \) on OLP keratinocyte apoptosis. Representative flow cytometry data are shown in Figure 4A. As shown in Figure 4B, the apoptosis rates were 21.2%, 29.4%, and 25.4% for the control group and 3.9%, 5.6%, and 4.4% for the experimental group, suggesting that co-culture with \( C. \text{ albicans} \) inhibited the apoptosis of OLP keratinocytes.

Discussion

The role of TLRs in the host defense against \( C. \text{ albicans} \) infections in OLP patients is still poorly understood with some studies showing an increased prevalence of \( \text{Candida} \) species [23] while others reporting no such association [24,25]. Therefore, the present study was undertaken to examine the effect of \( C. \text{ albicans} \) on TLR signaling in OLP-derived keratinocytes. Co-culture with \( C. \text{ albicans} \) not only induced inflammatory cytokine secretion, but also dramatically increased MyD88, NF-\( \kappa B \) p65, and TLR2 expression by OLP keratinocytes. Furthermore, decreased apoptosis by OLP keratinocytes was noted with \( C. \text{ albicans} \) exposure. Taken together, these results suggest that \( C. \text{ albicans} \) induces TLR signaling in OLP keratinocytes, which may lead to malignant transformation via increased inflammation and decreased keratinocyte apoptosis.

TLR is a pattern recognition receptor of congenital immunity, recognizing and defending against a variety of exogenous microorganisms. TLRs may also bind to molecular patterns related to tissue injury and cell apoptosis [26]. TLR2 can bind to different exogenous ligands, including the chitin of \( C. \text{ albicans} \). After TLR2 activation, two downstream signaling pathways may be activated: MyD88-dependent and MyD88-independent (TRIF-dependent) pathways. The MyD88-dependent pathway plays an important role in \( C. \text{ albicans} \)-induced OLP [27]. MyD88 may bind to IL-1 Receptor-Associated Kinase (IRAK) via its death domain at N terminal, leading to the autophosphorylation of IRAK. IRAK binds to the downstream adapter, TNF Receptor-
Associated Factor 6 (TRAF-6), to activate Inhibitor of NF-κB Kinases (IKKs), resulting in the phosphorylation and degradation of IκB and subsequent translocation of NF-κB into the nucleus [28]. NF-κB activation induces gene expression, including pro-inflammatory TNF-α, COX2 and iNOS, which play important roles in the occurrence and development of OLP. In the present study, the expression of TLR2, MyD88 and NF-κB was significantly increased in OLP keratinocytes co-cultured with C. albicans, suggesting that C. albicans infection plays an important role in the OLP-induced inflammation. The increase in TLR2 expression observed with C. albicans exposure in the present study is consistent with data presented by Adami et al. [29] in which increased TLR1 expression was noted in OLP. It is also consistent with Li et al. [30] in which phospholipomannan, a glycolipid expressed on the cell wall of C. albicans, upregulated TLR2 and activated NFκB signaling.

Previous studies have shown that keratinocyte β-defensin-2, 3 and LL-37 were induced by phospholipomannan [31]. Similarly, phospholipomannan induced the expression and secretion of IL-6 and IL-8 by keratinocytes [30]. In the present study, IL-10, IL-8, IL-2, and TNF-α levels were significantly higher following co-culture with C. albicans. The increased inflammatory response with C. albicans co-culture is consistent with a previous study that noted increased IL-8 levels in OLP tissue as compared controls [29]. However, it is in contrast with another study in which peripheral blood mononuclear cells (PBMCs) isolated from OLP patients showed impaired immune responses with exposure to purified protein derivative and C. albicans [8]. It is possible that these inconsistencies may be due to differences in the cell types analyzed (PBMCs vs. keratinocytes).

The present study found that exposure to C. albicans reduced keratinocyte apoptosis. Because altered apoptotic signaling is a hallmark of the malignant transformation of many cell types, this may represent an important event in OLP malignant transformation. Indeed, NF-κB-induced chronic inflammation (i.e., increased TNF-α, IL-1α, IL-6, and IL-8) and inhibition of apoptosis have been shown in OLP patients to favor metastatic phenotypes [32]. However, it is not clear if TLR signaling is mediating the anti-apoptotic effects of C. albicans, especially given that NF-κB may be protective against keratinocyte apoptosis [33]. Thus, further studies are necessary to examine if inhibition of this signaling pathway impacts the effect of C. albicans on OLP keratinocyte apoptosis.

Conclusions

In conclusion, C. albicans activates the MyD88-dependent pathway in OLP keratinocytes, resulting in increased cytokine expression and decreased keratinocyte apoptosis, which are both key steps in the pathogenesis of OLP and its progression to malignancy. These data may be helpful for the identification of potential targets for the therapy of OLP and provide theoretical basis for further studies on TLR and its downstream targets.

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References


Corresponding author
Hongying Sun
Department of Stomatology, Huashan Hospital, Fudan University, No.12, Middle Wulumuqi Road, Shanghai 200040, China.
Phone: +86 (21) 5288-7810
Fax: +86 (21) 6248-9191
Email: huashan110926@163.com

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