CXCL5 is increased in Candida albicans infected patients and human pulmonary epithelial cells

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Abstract: CXCL5 is critical for recruiting neutrophils and its aberrant production in lung epithelial cells has been confirmed in various mouse models of bacterial infectious diseases. The purpose of this study was to determine whether Candida albicans (C. albicans) upregulates CXCL5 expression of human lung epithelial cells. Here, we demonstrate that serum levels of CXCL5 are significantly increased and positively correlated with serum (1→3)-β-D-glucan (BG) levels in HIV patients with pulmonary candidiasis compared with patients with no evidence of Candida coinfection. Furthermore, serum CXCL5 levels were markedly decreased in patients after treatment with fluconazole for two weeks and positive correlation between serum CXCL5 and BG levels was caused by C. albicans infection. In in vitro cell culture, we further show that C. albicans significantly upregulate CXCL5 expression in both human lung epithelial cell line A549 and bronchial epithelial cell line BEAS-2B by activating PI3K, p38 MAPK, and NF-κB pathways. Together, our study provides novel insight into regulation of CXCL5 chemokine production of lung epithelial cells during C. albicans infection.

Keywords: Candida albicans, CXCL5, pulmonary epithelial cells, pulmonary candidiasis

Introduction

Pulmonary candidiasis occupies up to 86% of fungal pneumonia in HIV-infected individuals or immunosuppressed critically-ill patients [1, 2]. Among causative agents, the most common is Candida albicans (C. albicans), which was isolated from 60-70% of all samples every year [3, 4]. As the first line of defense against C. albicans infection, epithelial cells play a key role in signaling immune cells and induction of innate immune responses [5, 6]. Therefore, it is essential to evaluate the effects of C. albicans on pulmonary epithelial cells for a better understanding of C. albicans pathogenesis during pulmonary candidiasis.

The process of inflammatory cell recruitment toward the lungs in response to infection is initiated by the release of proinflammatory cytokines and a variety of chemokines like CCL2 (MCP-1), CCL5 (RANTES), CCL3/4 (MIP-1αβ), CXCL10 (IFN-inducible protein 10), and CXCL8 (IL-8) from infected epithelial cells [7]. CXCL1, -2, -3, -5, and -8 proteins are neutrophil-activating chemokines contributing to establishment of a chemotactic gradient during inflammation [8]. CXCL5/ENA-78 (epithelial neutrophil-activating peptide 78) mediated CXCR1/CXCR2-dependent neutrophil recruitment has been observed in bacterial, fungal, and viral infections [9-12]. Type I and type II alveolar epithelial cell-secreted CXCL5 plays an important role in LPS (lipopolysaccharide)-induced lung inflammation and is critical for polymorphonuclear leukocytes (PMN)-driven destructive inflammation in pulmonary tuberculosis [13-16]. In addition, CXCR1 and CXCL5 were highly induced in a Candida-infected kidney in a mouse model of systemic candidiasis and CXCR1-dependent neutrophil effector function has been shown to be a critical innate protective mechanism of fungal clearance and host survival [10, 12]. However, the effect of C. albicans on CXCL5 expression of pulmonary epithelial cells during pulmonary candidiasis remains poorly defined.

In this study, we demonstrated that serum levels of CXCL5 were significantly increased and positively correlated with serum (1→3)-β-D-
Chen et al. upregulation of CXCL5 by *Candida albicans*

glucan (BG) levels in HIV patients with pulmonary candidiasis compared with patients without pulmonary candidiasis. Furthermore, after treatment with Fluconazole for two weeks, serum CXCL5 levels were markedly decreased. We further showed that *C. albicans* upregulated CXCL5 expression in both human lung epithelial cells and bronchial epithelial cells by activating PI3K, p38 MAPK, and NF-κB pathways, while inhibitors of JNK and MEK1/2 pathways had no obvious effect on *C. albicans*-induced CXCL5 secretion. Thus, for the first time, our study describes the effect of *C. albicans* on CXCL5 chemokine production of lung epithelial cells.

**Materials and methods**

**Reagents**

JNK inhibitor (SP600125), MEK1/2 inhibitor (U0126), PI3K inhibitor (LY294002), p38 MAPK inhibitor (SB203580), NF-κB inhibitor (BAY 11-7082), and proteasome inhibitor (MG-132) were purchased from Beyotime Institute of Biotechnology (Beyotime, Shanghai, China).

**Participants**

This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University, Wuhan, China. Written informed consent was obtained from all participants. Serum samples of 48 HIV-1-infected patients, with no evidence of *Candida* coinfection, and 48 HIV-1-infected patients with pulmonary candidiasis (including 29 *Candida albicans*, 9 *Candida tropicalis*, 7 *Candida glabrata*, and 3 *Candida krusei*) were collected in the Center for Disease Control and Prevention, Wuhan, China, from 2014 to 2016. All patients were previously identified as HIV-1-seropositive by commercial ELISA test and Western blotting (MP Biomedicals, Singapore), according to National Guideline for Detection of HIV/AIDS in China (edited by Chinese Center for Disease Control and Prevention), as previously described [17]. Cancer patients with HIV infection and HIV patients with tuberculosis co-infection were excluded. Baseline and clinical characteristics of HIV patients are shown in Table 1. All HIV-infected patients received antiretroviral treatment. BG levels were measured with the Glucatell kit (Glucatell; Associates of Cape Cod, Falmouth, MA, USA), according to manufacturer recommendations. The cutoff value was 80 pg/mL.

**Cell culturing**

Human lung epithelial A549 cells were maintained in complete Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS) (HyClone, CA, USA) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (100×, Beyotime, Shanghai, China). Human bronchial epithelial BEAS-2B cells were cultured in Clonetics Bronchial Epithelial Growth Medium (BEGM) (Lonza, CA, USA). Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere.

**Preparation of *C. albicans***

A lyophilized strain of *C. albicans* ATCC10231 was obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). Routine cultivation was performed on SDA culture medium and incubated at 35°C for 48 hours [2]. *C. albicans* were isolated, counted, and then washed with PBS by centrifuging at 5000× g for 10 minutes.

**Quantitative real-time PCR (qRT-PCR)**

Total RNA was isolated with TRIzol Reagent (Life Technologies, CA, USA), according to manufacturer protocol. First-strand cDNA was synthesized from total RNA using ReverTra Ace-α First-strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Relative mRNA expression of CXCL5 was performed using real-time RT-PCR with SYBR Green real-time PCR Master Mix (Toyobo, Osaka, Japan) and determined using StepOnePlus Real-Time PCR system (Applied Biosystems). Relative quantities of mRNAs were obtained using the 2^ΔΔCT method. Human CXCL5 primer sequences were as follows: 5’- GTGGTAGCCTCCCTGAAGAAC-3’, 5’-TCCTTGTTCCACCGTCCAA-3’. GAPDH was used as the endogenous control [18].

**Determination of CXCL5 levels**

CXCL5 levels in serum and culture supernatants were determined by Human CXCL5 ELISA MAX™ Deluxe set (Biolegend, CA, USA), in accordance with manufacturer instructions.

**Western blotting**

Human lung epithelial A549 cells and bronchial epithelial BEAS-2B cells were washed twice with cold PBS and lysed using RIPA buffer (Beyotime, Shanghai, China). The same amount of each sample was subjected to and electro-
Chen et al. upregulation of CXCL5 by Candida albicans

Table 1. Baseline and clinical characteristics of HIV patients

<table>
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<tr>
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<th>PC</th>
<th>Non-PC</th>
<th>χ²</th>
<th>p value</th>
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<td>48</td>
<td></td>
<td></td>
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<tr>
<td>Age (%)</td>
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<td></td>
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<td>0.6488</td>
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<td>4 (8.33)</td>
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<tr>
<td>&gt;20 and ≤60</td>
<td>41 (85.42)</td>
<td>38 (79.17)</td>
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<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>5 (10.41)</td>
<td>6 (12.50)</td>
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<td>2 (4.17 )</td>
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<tr>
<td>Widowed</td>
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<td>3 (6.25 )</td>
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<td>Profession (%)</td>
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<td>35 (72.92)</td>
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<tr>
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<td>11 (22.92)</td>
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<td>4 (8.33 )</td>
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<td>CD4⁺ T cell number per μl (%)</td>
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<tr>
<td>&gt;200 and ≤500</td>
<td>11 (22.92)</td>
<td>10 (20.83)</td>
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<td>&gt;500</td>
<td>4 (8.33 )</td>
<td>8 (16.67 )</td>
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<td>Neutrophil number (10⁹/L)</td>
<td>5.17 ± 2.08</td>
<td>3.23 ± 0.79</td>
<td>0.0193</td>
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</table>

phoresed by SDS-PAGE gel, followed by transferring to PVDF membranes (Millipore, USA). The membranes were blocked in 5% non-fat milk overnight and then incubated with CXCL5 primary antibody (1:500 dilution, Novus Biologicals, USA) [19]. HRP-conjugated IgG antibodies (Santa Cruz, USA) were used as the secondary antibody. GAPDH was used as a control (Abcam, USA). Signals were detected using ECL detection reagent (Millipore, USA), following manufacturer instructions.

Statistical analysis

Data were analyzed by Graphpad Prism V.5.00 software (GraphPad Software, CA, USA). Count data of HIV patients were analyzed using χ² test. Comparison between two groups for statistical significance was performed with unpaired or paired Student’s t-test. For more groups, one-way ANOVA followed by Neuman-Keuls post hoc test was used. P<0.05 was considered statistically significant. Spearman’s rank test was used to assess association between BG and CXCL5 levels. In addition, multivariate linear regression with enter and backward method was performed on SPSS 17.0 (SPSS Inc. Chicago, IL, USA) to adjust for other covariates (age, sex, CD4⁺ T cell number, and neutrophil number). CXCL5 was set as dependent variable.

Results

Serum CXCL5 is elevated in patients with pulmonary candidiasis

Ninety-six HIV patients were divided into two groups with or without pulmonary candidiasis (Table 1). Neutrophil number in peripheral blood was significantly higher in HIV patients with pulmonary candidiasis compared to patients with no evidence of Candida coinfection (5.17 ± 2.08 vs. 3.23 ± 0.79, P=0.0193<0.05). Then, serum levels of BG and CXCL5 were determined before antifungal therapy. Our results showed that serum BG (255.30 ± 97.93 vs. 54.09 ± 36.30, pg/ml, Figure 1A) and CXCL5 (1.77 ± 0.28 vs. 0.77 ± 0.21, ng/ml, Figure 1B) levels were significantly increased in HIV patients with pulmonary candidiasis compared to patients with no evidence of Candida coinfection. Correlation analysis further revealed that serum CXCL5 protein levels were positively correlated with BG levels in HIV patients with pulmonary candidiasis (Figure 1C, r²=0.6371, P<0.0001) but not in patients with no evidence of Candida coinfection (Figure 1D, r²=0.0131, P>0.05). In HIV patients with pulmonary candidiasis, multivariable linear regression analysis further confirmed that CXCL5 was independently positively correlated with neutrophil number (t=3.591, P=0.001) and BG levels (t=2.881, P=0.006) but no other variables such as age, sex, and CD4⁺ T cell number (Table 2). In addition, serum

levels of BG (Figure 1E) and CXCL5 (Figure 1F) were significantly decreased in 48 patients with pulmonary candidiasis after two weeks of fluconazole treatment. Positive correlation between BG and CXCL5 disappeared after fluconazole treatment (Figure 1G, P>0.05), which is the first-line therapeutic drug of localized and systemic C. albicans infection [20, 21]. Thus, positive correlation between serum CXCL5 and BG levels was caused by C. albicans infection.

C. albicans induces CXCL5 expression in lung and bronchial epithelial cells

CXCL5-secreting pulmonary epithelial cells is largely responsible for pulmonary PMN recruitment via CXCR2 [13] and, importantly, neutrophils are the major cellular mediators of innate immune response against systemic candidiasis [10]. Thus, we speculated that Candida colonized in lung tissues may have the ability to induce CXCL5 expression of lung epithelial cells. Effects of C. albicans on CXCL5 expression was evaluated in human bronchial epithelial BEAS-2B cells and human lung epithelial A549 cells. Using qRT-PCR analyses, we first demonstrated that C. albicans significantly increased CXCL5 mRNA expression at a multiplicity of infection (MOI) of 1 in both BEAS-2B cells (Figure 2A) and A549 cells (Figure 2D), in a time-dependent manner, with expression peaking after 120 minutes. We, then, further confirmed that C. albicans induced CXCL5 expression in a dose-dependent manner in both BEAS-
Chen et al. upregulation of CXCL5 by Candida albicans

C. albicans also induced upregulation of CXCL5 protein expression in BEAS-2B cells (Figure 2C) and A549 cells (Figure 2F), as quantified by ELISA. In addition, mRNA and protein expression of CXCL5 in human airway epithelial BEAS-2B cells was higher than those in human lung epithelial A549 cells, in response to C. albicans stimulation in same conditions.

C. albicans induces CXCL5 expression depending on activation of NF-κB, p38, and PI3K pathways

Next, we examined whether activation of JNK, PI3K, MEK, p38 MAPK, and/or NF-κB was required for the upregulation of CXCL5 secretion by C. albicans in both BEAS-2B cells and A549 cells. Treatment of BEAS-2B cells (Figure 3A) and A549 cells (Figure 3B) with JNK inhibitor (SP600125) and MEK1/2 inhibitor (U0126) did not significantly affect CXCL5 secretion.
Chen et al. upregulation of CXCL5 by Candida albicans


not inhibit C. albicans-mediated upregulation of CXCL5 secretion while PI3K inhibitor (LY294-002), p38 MAPK inhibitor (SB203580), NF-κB inhibitor (BAY 11-7082), and proteasome inhibitor (MG-132) significantly reduced CXCL5 secretion. Furthermore, the same results were further confirmed by Western blotting in both BEAS-2B cells (Figure 3C) and A549 cells (Figure 3D). Thus, these results suggest that C. albicans depends on activation of PI3K, P38 MAPK, and NF-κB pathways to induce CXCL5 secretion by BEAS-2B cells and A549 cells.

Discussion

Neutrophils are the most abundant subpopulation of leukocytes and are essential for maintaining innate immune surveillance. They also represent a major contributor to tissue damage during inflammation caused by invading pathogens [14]. Recently, a growing number of studies have been focused on examining the role of CXCL5-driven neutrophil recruitment in infectious diseases. CXCL5 appears to be the dominant effector of neutrophil influx to the lungs. During mycobacterium tuberculosis infection, CXCR2 and CXCL5 are strongly upregulated in the lungs [22] and pulmonary epithelial cell-secreted CXCL5 drives destructive neutrophilic inflammation in tuberculosis [13]. Furthermore, CXCL5 exerts a predominant role in mediating neutrophil influx to lungs during inflammation after LPS inhalation and anti-CXCL5 antibodies attenuates LPS-induced neutrophil accumulation in acute lung injury (ALI) [23]. In contrast, high serum levels of CXCL5 also have the potential to increase plasma concentrations of CXCL1/CXCL2, two other receptors for CXCR2, and impair formation of chemokine gradients for neutrophil influx to the lungs by binding to
Chen et al. upregulation of CXCL5 by *Candida albicans*

Erythrocyte duffy antigen receptors for chemokines (DARC) [15]. Thus, upregulated secretion of CXCL5 by alveolar type II epithelial (AE II) cells increased lung bacterial burden and mortality in a severe *Escherichia coli* (*E. coli*) pneumonia mouse model [15, 16].

In a mouse model of systemic candidiasis, CXCL5 and CXCR1 were highly induced in *C. albicans*-infected kidneys and CXCR1-dependent neutrophil effector function was shown to be a critical innate protective mechanism of fungal clearance and host survival [10]. However, the mechanisms of how CXCL5 mediates CXCR1-dependent neutrophil effector function remains poorly clarified. In this study, we compared serum levels of CXCL5 in HIV patients with or without pulmonary candidiasis and found that CXCL5 was significantly increased and positively correlated with serum BG levels in HIV patients with pulmonary candidiasis. More importantly, serum CXCL5 levels were markedly decreased after two weeks of Fluconazole treatment, a first-line management option for treatment and prophylaxis of localized and systemic *C. albicans* infection [20, 21]. Thus, *C. albicans* infection induced upregulation of CXCL5 in HIV patients. However, the role of elevated CXCL5 in neutrophils accumulation in patients with pulmonary candidiasis still need to be further clarified in future studies.

Activation of AEII cells via TLR4-dependent p38, STAT3, and c-Jun signaling is important for producing CXCL5 in lungs exposed to LPS [16] or in murine lungs during *E. coli* pneumonia [24]. In addition, non-typeable Haemophilus influenzae (NTHi) upregulates CXCL5 expression by activating NF-κB and p38 MAPK pathways in middle ear epithelial cells [11]. In cellular studies, we have further shown that *C. albicans* infection significantly induced CXCL5 expression in both human lung epithelial cells and bronchial epithelial cells and could be inhibited by presence of PI3K, p38 MAPK, and NF-κB inhibitors while the presence of JNK, MEK1/2 showed no significant effects. Thus, activation of PI3K, p38 MAPK, and NF-κB pathways are involved in *C. albicans*-induced CXCL5 expression in lung epithelial cells. TLR2, TLR4, and Dectin-1 are the most important receptors of epithelial cells or immune cells involved in interaction with *C. albicans* [25-27]. Furthermore, these cell surface receptors are also important upstream molecules of PI3K, p38 MAPK, and NF-κB pathways. Therefore, the detailed molecular mechanisms involved with *C. albicans*-induced CXCL5 expression in lung epithelial cells still need to be clarified in future studies.

In conclusion, for the first time, our study demonstrates that serum CXCL5 is elevated in HIV patients with pulmonary candidiasis. Furthermore, we also confirm that *C. albicans* upregulate CXCL5 expression in both human lung epithelial cells and bronchial epithelial cells by activating PI3K, p38 MAPK, and NF-κB pathways. Hence, the results of our study provide valuable information for a better understanding of the pathogenesis of pulmonary candidiasis.

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**Disclosure of conflict of interest**

None.

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