

The NLRP3 Inflammasome-Independent IL-18 Elevation Induced by *Candida Albicans* Cell Wall

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Abstract

Objective: IL-18 has a significant role in host defense and pathogenesis of many inflammatory diseases. IL-18 production initiates if a particular PRR of innate immune cell binds specific PAMP, including O-linked mannan, a *Candida albicans* cell wall component. The binding of these PAMPs by TLR4 activates the intracellular signal pathway and activates NFκB to induce the production of pro-IL-18. Pro IL-18 undergoes breakdown to IL-18 active by the most common enzyme, caspase-1. Caspase-1 is the result of NLRP3 inflammasome activation. This study aimed to prove that the *Candida albicans* cell wall can induce IL-18 production and explore the role of NLRP3 inflammasome in that process. **Methods:** The Male Wistar rats were injected with 8 mg/kg BW/day and 16 mg/kg BW C. *albicans* cell wall intraperitoneally on 5 consecutive days in 2 periods with 23 days intervals between periods. Rats were sacrificed 24 h after the end of treatment. Expressions of NLRP3 inflammasome of intraperitoneal macrophages were measured by flow cytometry, and the level of IL-18 in blood plasma was measured with the ELISA technique. **Results:** IL-18 significantly increased in groups injected with 16 mg/kg BW/day but not those with 8 mg/kg BW/day. The expression of NLRP3 inflammasome was significantly increased in the group injected with 8 mg/kg BW/days C. *albicans* cell wall but not in the group injected with 16 mg/kg BW/days. Regression analysis showed that the increasing level of IL-18 was dependent on the C *albicans* cell wall dose but independent of the NLRP3-inflammasome expression. The NLRP3-inflammasome expression did not depend on C *albicans* cell wall dose. **Conclusions:** C. *albicans*' cell wall can increase the IL-18 plasma blood level, and the production of IL-18 is independent of NLRP3-inflammasome activation. **Keywords:** IL-18, NLRP3-inflammasome, Macrophage, C. *albicans* cell wall, Wistar rat

Introduction

IL-18 was previously known as INF-inducing factor (IGIF), a potent activator that causes polarization of Th1 cell cells and induces INFγ and lymphocyte proliferation. INFγ is produced by CD4+ T cells, CD8+ T cells, and Natural Killer cells. The role of this co-stimulator for INFγ IL-18-dependent production is thought to increase the regulation of IL-18 receptors, especially b chains. The INFγ promotes caspase-1 gene expression and activation of caspase-1 from inactive precursors. IL-18 with IL-23 activates Th17 to produce IL-17 [1].

The IL-18 either cooperates with IL-12 or, with IL-15, induces the increase of IL-18Rα expression. The IL-18 alone induces the secretion of INFγ. The INFγ activates macrophages to produce inflammatory cytokines. In addition, IL-18 can directly activate chemokine secretion by macrophages and activate NK cells to induce INFγ secretion or stimulate perforin-and FasL-mediated cytotoxicity. If there is no IL-12 or IL-15, IL-18 activates Th2 to produce IL-13 and IL-4. Independently, IL-18 triggers increased cell adhesion molecules, nitric oxide synthesis, and chemokine production. IL-18 induces ICAM-1 expression in myeloid cells and VCAM-1 expression in micro endothelial cells or synovial fibroblasts *in vitro* and *in vivo* through NFκB activation. Blocking IL-18 causes reduced metastatic activity in melanoma mouse models due to decreased VCAM-1 expression. IL-18 induces CXC chemokines, macrophages or synovial fibroblasts, and angiogenic factors in rheumatoid arthritis tissue [2].

Candida albicans is an opportunistic commensal fungus found in some healthy people and healthy individuals' oropharynx, digestive and vaginal tracts, and skin. This fungus can be pathogenic and cause disease if there are predisposing factors. The clinical manifestations of *Candida* species range from localized superficial mucocutaneous disorders to life-threatening, invasive diseases involving multiple organ systems [3]. Population surveys report that the incidence of candida infection is 8 per 100,000. The incidence of candidaemia (*Candida* spp. found in circulation) in 2000 was 3.65 per 100,000 population, while in 2005, it increased significantly to 5.56 per 100,000 population [5].

The 1st innate immune response to *C. albicans* recognizes PAMPs *C. albicans* by innate immune cells, i.e., macrophages and dendritic cells. The O-linked mannans, the most abundant component of the *C. albicans*' cell wall, are recognized by TLR4 [6]. The TLR4-LPS (lipopolysaccharide) binding is widely used to study the intracellular signaling pathway triggered by this PRR. The TLR4-LPS binding induces activation of NF- κ B, then NF- κ B upregulates transcription of the inactive precursor pro-IL-1 β , pro-IL-18, and pro-caspase-11. The DAMPs or PAMPs 2nd inflammatory signal triggers the formation of the inflammasome [7]. The inflammasome initiates activation of pro-caspase-1 to caspase-1, and this enzyme breakdowns pro IL-18 24 kDa and becomes a mature, bioactive IL-18 18 kDa and secretion from the cell [8]. If LPS is abundant or contained within vacuoles, it can enter the intracellular environment independent of TLR4. The GBPs promote vacuolar lysis, causing entry of LPS into the cell's cytosol. Pro-caspase-11 detects cytosolic LPS, initiating the formation of the inflammasome, as well as pyroptosis of the cell. The appearance of the NLRP3 inflammasome also leads to the release of cytokines by similar mechanisms to the canonical pathway [7].

Infection with *C. albicans* in BMDM (bone-marrow-derived macrophage) and macrophages J774 from mice causes pyroptosis, the result of inflammation due to the activity of IL-1 β and IL-18 [9]. When TLR 4 recognizes and binds O-link mannans of *C. albicans* cell wall, the binding can generate a similar signal, activate NLRP3 inflammation, and lead to the breakdown of procaspase-1 become active caspase-1 and caspase -1 breakdown of the pro-IL-18 to active form IL-18 [10].

This study aimed to analyze the production of IL-18 and the involvement of NLRP3 inflammasome in these processes. IL-18 enormously affects the immune response and affects an individual's health. The research is necessary to prove an increase in IL-18 and the role of NLRP3 in this process in *C. albicans* infection.

Materials and methods

The method of isolation of *C. albicans*' cell wall used in this study was a combination of physical using ultrasound homogenizer, chemical and thermal, adapted from de Groot, 2004. *C. albicans* ATCC 10231 scraped from culture were washed with cold H₂O and 10 mM Tris-HCL, pH 7.5. In the next step, the cell was resuspended in 10 mM Tris-HCL, pH 7.5, and disintegrated Ultrasound Homogenizer (Cole Parmer 4710 series). Non-covalently bound proteins and intracellular contaminants were removed by washing with 1 M NaCl and extracted with 50 mM Tris-HCL pH 7.8 containing 2 % SDS, 100 mM NA-EDTA, and 40 m β Mercapethanol, 5 min 100 °C, then washed 3 times with water and freeze-dried [11].



Figure 1 Experimental rat treatment schedule. *C. albicans* wall was injected intraperitoneally in 2 periods with intervals of 23 days. Each period consists of 5 consecutive days. On the 33rd day, the rat was sacrificed.

Wistar male rats from Independent Animal Experiment Development, Sleman, Jogjakarta, Indonesia divided into 2 treatment groups and 1 control group. The *C. albicans* cell wall administration was done in a modified technique adapted from Yoshikane, 2014 [12]. Two groups were injected intraperitoneally with 8 mg/kg BW (P1 group) and 16 mg/kg BW (P2 group) of *C. albicans* cell wall. The injection of *C. albicans* cell wall on 5 consecutive days in 2 cycles with an interval of 23 days (**Figure 1**). The control group was injected with aquadest pro injection. The rats were euthanized 24 h after the last injection with 100 mg/kg BW ketamine, i.m. The health research Ethical Clearance Commission Faculty of Dental Medicine Universitas Airlangga, Surabaya, approved the protocols on October 29th, 2017, with Certificate number 246/HRECC.FODM/X/2017.

Peripheral blood was collected from the intracardial and put in a vacutainer containing EDTA. Blood plasma was separated to measure IL-18 levels by ELISA technique. The Elisa kit for measuring IL-18 levels was from Bioassay Technology Catalogue No. E0117 Ra. The NLRP3-inflammasome and CD 68 (marker macrophage) expression on intraperitoneal macrophage were detected by flow cytometry using antibodies catalog number bs 10021RCy and bs-0649R FITC from Bioss USA.

Results and discussion

The mean level of IL-18 peripheral blood plasma is shown in **Figure 2**. The results of the ANOVA test on IL-18 levels showed significant differences between groups. The LSD test results showed significant differences between group C and P2 and no significant differences between group C and P1.

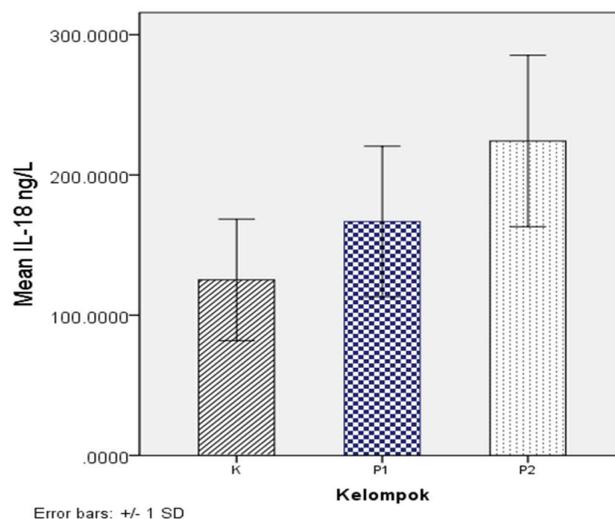


Figure 2 Mean IL-18 Level in Peripheral Blood Plasma in Rat Induced by *C. albicans* Cell Wall.

The result showed that intraperitoneal injection of *C. albicans* cell walls increased IL-18 Wistar's blood plasma significantly at 16 mg/kg BW/day but not at 8 mg/kg BW/day. The NLRP3 inflammasome on intraperitoneal macrophage increased in the P1 group but not in the P2 group. The expression of inflammatory NLRP3 did not become consistent with the amount of *C. albicans* cell wall doses given. In the 16 mg/kg BW/days dose treatment group (P2), the NLRP3 inflammation's expression was less than in the 8 mg/kg BW/days dose treatment group (P1).

This study injected the *C. albicans* cell wall into rat intraperitoneal and recognized by PRR intraperitoneal macrophages. There was a significant increase in IL-18 levels in the group injected with 16 mg/kg BW *C. albicans* cell wall ($p < 0.05$) but not in the group injected with 8 mg/kg BW *C. albicans* cell wall. The intraperitoneal *C. albicans* cell wall injection with a 16 mg/kg BW/day dose can significantly increase blood plasma IL-18 levels.

The O-link mannan in the cell wall of *C. albicans* is recognized by TLR4 and triggers the signal pathway downstream, which triggers the transcription factor NF κ B to activate the transcription of the IL-18 gene to form pro-IL-18. Mannan O-linked is one form of mannan that occupies 40 % of the composition of the cell walls of *C. albicans*. *C. albicans* strains to lack O-mannan synthesis were found to have virulence and adhesion in weaker epithelial cells and show less cytokine stimulation profiles, so neutrophil responses become less sensitive [13,14]. Pro IL-18, through both canonical and non-canonical pathways, becomes active IL-18.

The most famous effect of IL-18 is as induction of INF γ , a defense factor that is very important in defense against microbes. The role of IL-18 in the host defense against infection due to disseminated *C. albicans* has been proven through research using mice injected with *C. albicans* intravenously. The colonization of *C. albicans* in the kidneys of mice given anti-IL-18 was higher than in the mice not given anti-IL-18. This result indicates that IL-18 significantly affects *C. albicans*' systemic infection [15]. In a whole-blood model, IL-18 neutralization by IL-18 binding protein decreased *C. albicans*-induced INF- γ synthesis by 72 % [16]. On the other hand, IL-18 has many roles in the pathogenesis of many systemic diseases, i.e., inflammatory bowel disease, metabolic syndrome and diabetes type 2, atherosclerosis and

myocardial infarction, lung diseases (Asthma, Chronic Obstructive pulmonary diseases, Acute Lung injury), Sepsis, hemophagocytic syndromes, in systemic juvenile idiopathic arthritis/adult-onset Still's Disease [2]. IL-18 could potentially induce inflammatory and cytotoxic immune cell activities leading to autoimmunity. Its elevated levels have been reported in the blood of patients with some immune-related diseases, including rheumatoid arthritis, systemic lupus erythematosus, type I diabetes mellitus, atopic dermatitis, psoriasis, and inflammatory bowel disease [17].

In this study, expression of NLRP3 inflammasome was significantly higher in the groups injected with 8 mg/kg BW/day but not in the groups injected with 16 mg/kg BW/day. This result is a unique phenomenon because there has been no previous research that gives a similar outcome. The dose-independent expression may be due to the response immune is a vast complex pathway and, in a high dose, provoke a signal that inhibits the activation of NLRP3-inflammasome. We suggest investigating this phenomenon so that the pathway of the NLRP-3 activation induced by *C. albicans* becomes more clearly explained.

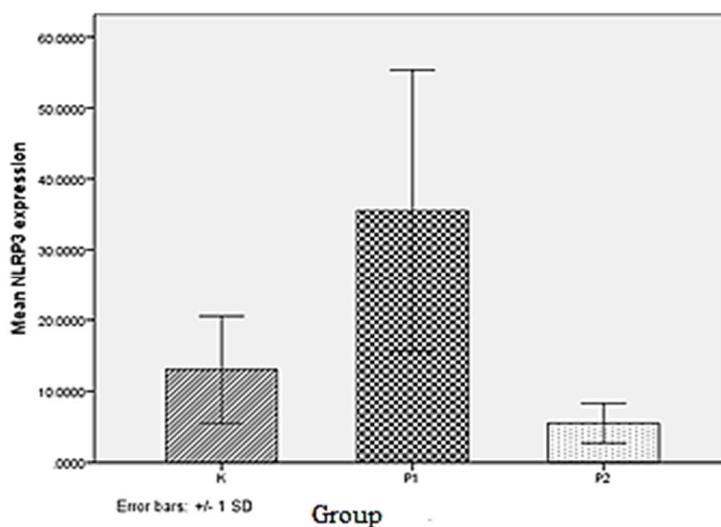


Figure 3 NLRP3-inflammasome Expression in Intraperitoneal Macrophage Rat Induced by *C. albicans* Cell Wall.

The stepwise regression test evaluated the causal relationship between inflammasome NLRP3 and IL-18 and showed no significant causality between NLRP3-inflammasome expression and IL-18 ($p > 0.05$). This result implied the mechanisms of activation of IL-18 in this research without involving NLRP3-inflammasome activation. The stepwise regression analysis showed that increasing IL-18 levels did not occur due to increasing NLRP3-inflammasome expression. IL-18 production mechanism without involving NLRP3-inflammasome has also been found in several studies. Primed murine dendritic cells (DCs) responded to p60 stimulation with reactive oxygen species (ROS) production and secretion of IL-1 β and IL-18 but not pyroptosis. Inhibitors of ROS production inhibited secretion of IL-1 β but did not impair IL-18 secretion. Furthermore, DCs from murine knockout caspase-11 (casp11) 128S6 failed in the secretion of IL-1 β in response to p60 but were fully responsive to IL-18 secretion. So, IL-18 activation can occur without involving NLRP3-inflammasome (occurs non-canonically) [18]. Studies on macrophages exposed to TLR ligands led to an increase in Fas regulation, which has made it responsive to Fas ligand-receptor involvement. Fas signaling activated caspase-8 in macrophages and dendritic cells, leading to non-canonical maturation of IL-1 β and IL-18. This activation of IL-18 is not dependent on inflammasomes but is processed in pathways that require the FADD (Fas-associated death domain) and caspase 8 maturation cells [19]. Research using NLRP3 and ASC knockout (KO) microglia exposed to *S. aureus* showed that IL-1 β production significantly decreased, but IL-18 production was unaffected. This result indicated that IL-18 production could be done without involving inflammasome activation [20]. Furthermore, caspase-1 can be activated by various canonical inflammasomes, i.e., NLRC4, NLRP-1, AIM2-like receptors, or TRIM families containing the CARD or PYD domain. IL-18 can be activated via non-inflammasome pathways. Several studies have shown that IL-18 production can occur without the assistance of inflammasomes. Activation of the IL-18 activation pathway can differ from the activation of IL-1 β [18,20].

Conclusions

C. albicans cell wall injected intraperitoneally could increase IL-18 plasma blood level in Wistar male rats through the non-NLRP3-inflammasome pathway. Mucosal *C. albicans* infection could trigger the elevation of IL-18 blood plasma levels because the *C. albicans* cell wall recognizes submucosal macrophages that aggregate at the site of infection. Because IL-18 has many pathological effects in inflammatory diseases, *C. albicans* infection in the mucous membrane should be considered in managing many inflammatory disorders.

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References

- [1] D Boraschi and CA Dinarello. IL-18 in autoimmunity: Review. *Eur. Cytokine Netw.* 2006; **17**, 224-52.
- [2] G Kaplanski. Interleukin-18: Biological properties and role in disease pathogenesis. *Immunol. Rev.* 2018; **281**, 138-53.
- [3] J Talapko, M Juzbašić, T Matijević, E Pustijanac, S Bekić, I Kotris and I Škrlec. *Candida albicans*-the virulence factors and clinical manifestations of infection. *J. Fungi* 2021; **7**, 79.
- [4] N Yapar. Epidemiology and risk factors for invasive candidiasis. *Therapeut. Clin. Risk Manag.* 2014; **10**, 95-105.
- [5] C Bourgeois and K Kuchler. Fungal pathogens-a sweet and sour treat for toll-like receptors. *Front. Cell. Infect. Microbiol.* 2012; **2**, 142.
- [6] L Mukaremera, KK Lee, HM Mora-Montes and NAR Gow. *Candida albicans* yeast, pseudohyphal, and hyphal morphogenesis differentially affect immune recognition. *Front. Immunol.* 2017; **8**, 629.
- [7] E Seki, H Tsutsui, H Nakano, NM Tsuji, K Hoshino, O Adachi, K Adachi, S Futatsugi, K Kuida, O Takeuchi, H Okamura, J Fujimoto, S Akira and K Nakanishi. Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1 β . *J. Immunol.* 2001; **166**, 2651-7.
- [8] M Wellington, K Koselny, FS Sutterwala and DJ Krysan. *Candida albicans* trigger NLRP3-mediated pyroptosis in macrophages. *Eukaryot. Cell* 2014; **13**, 329-40.
- [9] CE Diamond, HJ Khameneh, D Brough and A Mortellaro. Novel perspectives on non-canonical inflammasome activation. *ImmunoTargets Ther.* 2015; **4**, 131-41.
- [10] PWJD Groot, ADD Boer, J Cunningham, HL Dekker, LD Jong, KJ Hellingwerf, CD Koster and FM Klis. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot. Cell* 2004; **3**, 955-65.
- [11] Y Yoshikane, M Koga, K Imanaka-Yoshida, T Cho, Y Yamamoto, T Yoshida, J Hashimoto, S Hirose and K Yoshimura. JNK is critical for the development of *Candida albicans*-induced vascular lesions in a mouse model of Kawasaki disease. *Cardiovasc. Pathol.* 2015; **24**, 33-40.
- [12] CC Sheth, R Hall, L Lewis, AJP Brown, FC Odds, LP Erwig and NAR Gow. Glycosylation status of the *C. albicans* cell wall affects the efficiency of neutrophil phagocytosis and killing but not cytokine signaling. *Med. Mycol.* 2011; **49**, 513-24.
- [13] NAR Gow, FLVD Veerdonk, AJP Brown and MG Netea. *Candida albicans* morphogenesis and host defence: Discriminating invasion from colonization. *Nat. Rev. Microbiol.* 2012; **10**, 112-22.
- [14] RJL Stuyt, MG Netea, I Verschuere, G Fantuzzi, CA Dinarello, JWMVD Meer and BJ Kullberg. Role of interleukin-18 in host defense against disseminated *Candida albicans* infection. *Infect. Immun.* 2002; **70**, 3284-6.
- [15] MG Netea, RJL Stuyt, SH Kim, JWMVD Meer, BJ Kullberg and CA Dinarello. The role of endogenous interleukin (IL)-18, IL-12, IL-1 β , and tumor necrosis factor- α in the production of interferon- γ induced by *Candida albicans* in human whole-blood cultures. *J. Infect. Dis.* 2002; **185**, 963-70.
- [16] SA Ihim, SD Abubakar, Z Zian, T Sasaki, M Saffarioun, S Maleknia and G Azizi. Interleukin-18 cytokine in immunity, inflammation, and autoimmunity: Biological role in induction, regulation, and treatment. *Front. Immunol.* 2022; **13**, 919973.

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- [17] RL Schmidt and LL Lenz. Distinct licensing of IL-18 and IL-1 β secretion in response to NLRP3 inflammasome activation. *PLoS One* 2012; **7**, e45186.
- [18] L Bossaller, C Ping-I, C Schmidt-Lauber, S Ganesan, WJ Kaiser, VAK Rathinam, ES Mocarski, D Subramanian, DR Green, N Silverman, KA Fitzgerald, A Marshak-Rothstein and E Latz. FAS mediates non-canonical IL-1beta and IL-18 maturation via caspase-8 in a Rip3-independent manner. *J. Immunol.* 2012; **189**, 5508-12.
- [19] R Hanamsagar, V Torres and T Kielian. Inflammasome activation and IL-1 β /IL-18 processing are influenced by distinct pathways in microglia. *J. Neurochem.* 2011; **119**, 736-48.