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HOLOTHURIN AND CASPOFUNGIN-INDUCED ALTERATIONS IN TTOLL-LIKE RECEPTOR 4 EXPRESSION IN THE VAGINA OF RATTUS NORVEGICUS WISTAR WITH CANDIDIASIS

Nurdiana N.[1]* Winarsih S.[2] Tri Endharti A.[3] Handayani S.[4,5]

[1] Department of Microbiology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[2] Department of Pharmacy, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[3] Department of Parasitology, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia
[4] Doctoral Program of Medical Science, Faculty of Medicine, Brawijaya University, Malang, Indonesia
[5] Department of Midwifery, Aisyiyah Surakarta University, Surakarta, Indonesia

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Abstract

Toll-like receptor 4 is a marker that indicates whether or not tissues have immunological or pro-inflammatory responses. Candida albicans has the potential to aggravate the tissue that makes up the epithelium. After candidiasis, the antifungal properties of holothurin and caspofungin have the ability to block toll-like receptor 4.

A total of 48 white rats Rattus norvegius Wistar were divided into four positive control groups (P1) and given topical C. albicans after being grown in yeast extract peptone dextrose in the vagina of white rats Rattus norvegius Wistar. P2 and P3 groups were given 3500 g holothurin and 140 g caspofugin topically in the vagina of animal models at 12-, 24-, and 48-hour intervals. Immunofluorescence was used to analyse the study results both quantitatively and qualitatively by attaching the imaging. After that, the data was processed using the SPSS statistical software version 23.

Toll-like receptor 4 expression decreased significantly in the treatment group compared to the positive control group (p0.05). This demonstrates that holothurin (P1) and caspofungin (P2) treatments reduced toll-like receptor 4 expression in C. albicans at 0.25 and 6.375 at 12 hours, 0.62 and 3. at 24 hours, and 1.68 and 4.18 at 48 hours. The mean difference in toll-like receptor 4 expression in the positive control group, on the other hand, did not differ statistically when compared to the negative (healthy) control group. This demonstrates that the treatment group's holothurin and caspofungin have the potential to reduce toll-like receptor 4 expression.

Holothurin has a potential effect compared to caspofungin on experimental animals with candidiasis experiencing significant changes in suppressing the number of toll-like receptor 4 in vaginal epithelial tissue of Rattus norvegicus Wistar.

Keywords: *TLR4, candida albicans, holothurin, caspofungin, antifungal.*

Introduction

C. albicans invades host cells via necrosis (passive and involuntary cell death caused by the uncontrolled release of inflammatory cell contents), apoptosis (an active and purposeful process of autonomic cell disassembly that avoids the beginning of inflammation), and pyroptosis (cell death caused by species infection) [*Krysan D et al., 2014*]. Necrotic dead cells release lactate dehydrogenase during

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Address for Correspondence:

Prof. Dr. Nurdiana N., M.Kes
Department of Microbiology, Faculty of Medicine Brawijaya University
Jl. Veteran, Ketawanggede, Kec. Lowokwaru, Kota Malang, Jawa Timur 65145, Indonesia
Tel.: (+62 812 3388 428)
E-mail: nurdianafarmako.fk@ub.ac.id

candida albicans injury to the mucosa. Lactate dehydrogenase is a soluble cytoplasmic enzyme found in nearly all cells that is released into the extracellular space when the plasma membrane is disrupted [*Chan F et al., 2016*]. Epithelial damage is caused when *Candida albicans* invades the body. However, necrotic cell death is different from apoptosis in that it does not cause any damage to the host [*Chan F et al., 2016*]. In general, toll-like receptor 4 (TLR4) has been long recognized to be a gram-negative lipopolysaccharide receptor. Furthermore, it binds to endogenous molecules produced as a result of tissue injury. As a result, TLR4 is a critical receptor at which infectious and non-infectious stimuli converge to cause a pro-inflammatory response [*Molteni M et al., 2016*].

Toll like receptors (TLRs) are the main recognition receptors that mediate various microorganisms. Toll like receptors are a major family of pattern recognition receptors that immune and non-immune cells become part of pathogen-associated molecular patterns (PAMPs). Some TLRs are involved in the recognition of pathogenic fungi such as *C. albicans.* The interaction between TLRs and *C. albicans* during candidiasis stimulates immune cells to produce inflammatory mediators and immunomodulators that shape the host immune response [*Choteau L et al., 2017*].

Changes in TLR4 can be influenced by the balance of antigens in the cellular and environment. The work of TLR4 is known to be in the trans membrane protein. When recognizing lipopolysaccharide from antigens, TLR4 starts to activate and initiate its function. Toll like receptor-4 plays a role with lipopolysaccharide-binding protein and cluster of differentiation 14 in responding to antigens [*Ciesielska A et al., 2021*].

In addition to modulating pro-inflammatory cytokines through the production of cytokines and chemokines, TLRs are also known to function in several host immune processes such as phagocytosis, matrix metalloproteinase production and defensin production. Toll like receptors also coordinate cell activation, migration and apoptosis of macrophages and neutrophils. Toll like receptors also function to direct the development of specific adaptive immunity [*Gil M, Gozalbo D, 2006*].

In response to signals that form a complex with the leucine-rich repeat domain (LRR) and the intracellular toll receptor/interleukin-1 domain, TLR4 signaling is activated. Lipopolysaccharide stimulation triggers a sequence of interactions between the TLR4 surface complex and its auxiliary proteins [*Park B et al., 2013*].

The pattern recognition receptor (PRR) pathway activates innate and adaptive immune cells upon TLR4 activation. Toll-like receptor 4 activation by lipopolysaccharide or damage-associated molecular patterns results in the generation of pro-inflammatory cytokines via MyD88-dependent or MyD88-independent pathways [*Kuzmich N et al., 2017*]. Toll-like receptor activation is a widespread phenomenon in immune cells and other body cells, and it is the main mechanism of TLR4. Toll-like receptor location and expression are controlled in response to certain chemicals produced by infections or injured host cells. Specific intracellular signaling cascades are activated upon ligand binding to TLRs to initiate host defense [*Sun L et al., 2019*].

Toll-like receptor groups are required when dealing with infection because TLRs play an important role in innate immunity by detecting conserved pathogen-associated molecular patterns (PAMPs) in various microbes, including viruses, resulting in innate immunity activation and the orchestration of adaptive immune responses [*Iwasaki A et al., 2015*].

Toll-like receptor-4 on human epithelium can protect mucosal surfaces directly from C. albicans infection through a polymorphonuclear cell-dependent process [*Moyes D et al., 2010*]. This study aimed to determine the relationship between candidiasis and TLR4 expression in the Wistar model of Rattus norvegicus.

Material and methods

This study employed a true experimental design with a post-test-only control group design strategy. This study was conducted between January and March of 2021 with approval from the Faculty of Medicine at Universitas Brawijaya (approved number 337/EC/KEPK-S3/12/2019). Randomly selected 48 females Rattus norvegicus Wistar were acclimatized for one week prior to treatment. The entire sample is divided at random. Each group contained twelve Rattus norvegicus Wistar rats. Group 1 (K1) were negative controls, positive controls (C. albicans) (P1), C. albicans + holothurin (P2), and C. al-

bicans + caspofungin (P3), and the intervention group was subdivided three times by the treatment group (12, 24 and 48 *hours*). We removed mice who were prematurely born and died prior to receiving therapy. C. albicans was administered topically to the positive control (P1) after it was cultured in yeast extract peptone dextrose in the vagina of Rattus norvegicus Wistar rats. Holothurin 3500 *ug* and caspofugin 140 *ug* were applied topically to the vagina of animal models in Groups P2 and P3, respectively. Then, animals were chosen at random for euthanasia with 100 *mg* of intraperitoneal ketamine prior to tissue collection.

Preparation of C. albicans: C. albicans isolate (4506547065307370) was acquired from the Microbiology Laboratory, Faculty of Medicine, University of Brawijaya and inoculated overnight on Sabouraud Dextrose Agar (Sabouraud Dextrose Broth (Ph 5.6)), Crystal violet, lugol, 96 percent alcohol, aguades, and Dimethyl Sulfide. C. albicans was then propagated in yeast extract peptone dextrose medium and incubated overnight at 37°C. C. albicans was extracted, washed in sterile phosphate-buffered saline, and suspended in cell suspension (Sigma Chemical Co., St. Louis, MO, USA). Cells were prepared for further examination in 0.9 percent NaCL at a cell density of 1.0 105 CFU/mL.

***Examination of TLR4 Expression using Immunofluorescence*:** Utilized materials included slides of vaginal tissue and labelled primary antibodies [anti-TLR4 polyclonal antibody (alexa fluor ® 647 conjugated) and rabbit anti-TLR4/CD284 polyclonal antibody (alexa fluor ® 488 conjugated)]. Observations with a 400x magnification Fluorescence microscope. Examining commences with histology preparations. The recovered tissue was then fixed with neutral buffered formalin (10% solution in water) at ambient temperature for 24 hours, dehydrated by integrating the fixed tissue into alcohol in increasing concentrations from 70% to 100%, and the internal organs were cleaned by immersion. solution of xylol for 24 hours. The tissue was infiltrated by incubating it in liquid paraffin for 12 hours and then placing it in an incubator at 55°C to 57°C. The next step is embedding the organs in solid paraffin at a temperature between 20°C and 25°C, followed by cutting them to a thickness of 4 m using a microtome. The slices were affixed to an object glass smeared with mayers albumin, labelled according to the sample code, and placed in an incubator at 37°C for one night so that they would adhere securely; they were then prepared for staining. In order to prevent the incision from detaching from the slide during immunohistochemical staining, neofren was used to adhere the incision to the slide.

Stain the slide with TLR4 antibody after it has been created. The slide is first heated for 60 minutes at 60 degrees Celsius. Then they were submerged in the following solutions in order: sterile Aquades (1 minute), Xylol (2 times 10 minutes), ethanol absolute (2 times 10 minutes), ethanol 90% (1 time 5 minutes), ethanol 80% (1 time 5 minutes), and ethanol 70% (1 time 5 minutes) (3 x 5 minutes). Citrate Buffer for Antigen Retrieval was used in this process. The slides were submerged in a pH 6.0 citrate buffer chamber before spending 20 minutes in a water bath at 95°C. After removing the slide from the water bath, give it 20 minutes to reach room temperature. Phosphate-buffered saline for three times for five minutes. The slides were washed for 1 x 5 minutes with 0.1% phosphate-buffered saline Triton-X 100. 1% bovine serum albumin was used for an incubation that lasted 30 minutes at room temperature. The serum albumin remedy was thrown out. then overnight at 4°C incubation with primary antibody that has been TLR4-labeled. Phosphate-buffered saline washed the slides three times for five minutes each. 5 minutes of 4′,6-diamidino-2-phenylindole 1:1000 incubation. Phosphate-buffered saline three times for five minutes. Cover the glass and add mounting medium. Following the completion of the preparations, observations were made using a 400x fluorescence microscope. The immunofluorescence data was entered into the ImageJ software to calculate the results.

Using an independent sample t-test on data that are univariate normally distributed, the average for numerical data is examined using the mean and standard deviation of each variable. The One-Way ANOVA test was used in statistical analysis using the SPSS 23 program.

Results

TLR4 expression varies according to treatment type and time difference

TLR4 expression in 48 Wistar rat experimental

animals treated with holothurin and capofungin, the active ingredients in mushrooms, at 12, 24, and 48 hours. (Figure 1)

Using immunofluorescence, the results of measurements of TLR4 expression after vuvlovaginal candidiasis in experimental animals within 12, 24, and 48 *hours* were analysed. At 12, 24, and 48 *hours*, TLR4 expression in the negative control group was lower than in the positive control group. In contrast, the TLR4 expression values at 12, 24, and 48 *hours* in the positive control group were significantly higher than in the negative control group, so it was concluded that the data were valid and consistent with the hypothesis.

Lower TLR4 was present in the holothurin-treated group than in the group that received a positive control at 12, 24, and 48 *hours*. Therefore, it can be said that holothurin inhibits TLR4 expression in the epithelial tissue of Rattus norvegicus Wistar vaginal candida albicans. Lower TLR4 was present in the caspofungin-treated group than in the positive control group at *hours* 12, 24, and 48. Therefore, it can be said that caspofungin inhibits the expression of TLR4 in the epithelial tissue of Rattus norvegicus Wistar vaginal candida albicans. Both holothurin and caspofungin were administered after candida albicans colonized the vaginal epithelial tissue of Rattus norvegicus Wistar, demonstrating their potential for use as antifungals.

Toll like receptor-4 data was taken from the vaginal tissue of experimental Rattus norvegicus Wistar. All inflammatory cell groups were given the same intervention with the treatment design.

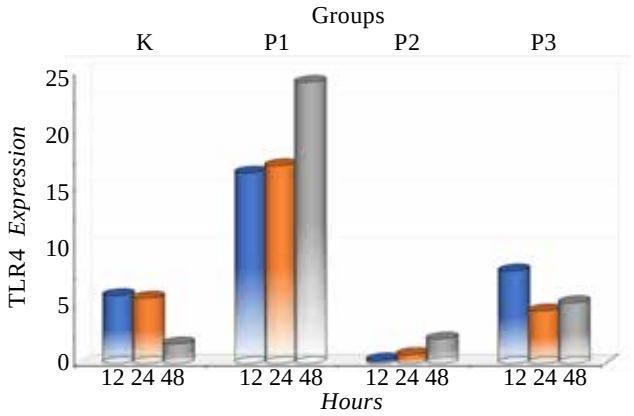

FIGURE 1 *TLR4 expression in Wistar rats.*
Notes: K: No treatment control group (healthy). P1: C. albicans positive group. P2: Holothurin therapy for fungi/C. albicans. P3: Caspofugin therapy for fungi/C. albicans. P3: Treatment of Fungi/C.albicans with Caspofugin.

Then TLR4 was analyzed using immunofluorescent. Toll like receptor-4 expression in the treatment group decreased significantly compared to the positive control group ($p<0.05$).

This demonstrates that holothurin (P1) and caspofungin (P2) treatments reduced TLR4 expression in C. albican at 0.25 and 6.375 at 12 *hours*, 0.62 and 3 at 24 *hours*, and 1.68 and 4.18 at 48 *hours*. The mean difference in TLR4 expression in the positive control group, on the other hand, did not differ statistically when compared to the negative (healthy) control group. This demonstrates that the treatment group's holothurin and caspofungin have the potential to reduce TLR4 expression. (Fig.2)

DISCUSSION

Each group's average TLR4 result percentage reveals a different interpretation. In comparison to the other groups, the positive candida control group displayed higher TLR4 expression results. One of the extracellular ligand-binding proteins, TLR4, is responsible for the release of cytokines that promote inflammation. When TLR4 levels are high, peptides such as Candidalysin or fungus poison are released, which damage the epithelium and cause the immune system to become active. Additionally, the expression of inflammatory cells in the positive control group is directly correlated with our findings. This group had higher levels of inflammatory cells than the other groups did. Therefore, it is demonstrated that after the candida invasion, there was tissue damage that led to inflammation of the rats' vaginal epithelial tissue [*Pavlidis I et al., 2020*].

Toll like receptors are known to play a role in a number of host immune processes, including phagocytosis, matrix metalloproteinase production, iron sequestration, and defensin production, in addition to modulating pro-inflammatory cytokines through the production of cytokines and chemokines. Additionally, it participates in the actin polymerization, angiogenesis, and apoptosis induction that are fundamental cellular processes. Toll like receptors also control the migration, apoptosis, and activation of neutrophils and macrophages. These receptors aid in the development of some forms of adaptive immunity [*Sokol C et al., 2015*].

Ultimately the P13K signal activates a protec-

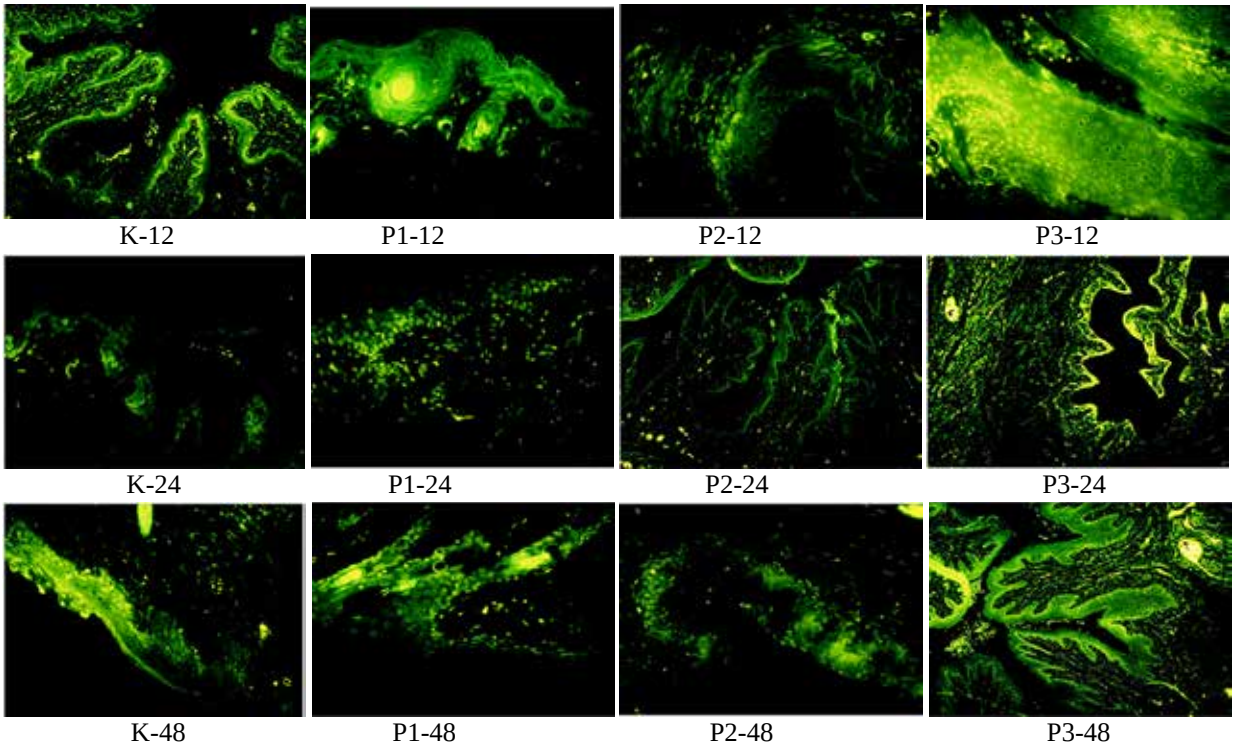

Figure 2. *Visualization of Immunofluorescent Image TLR4 expression in the mushroom treatment group and the active ingredient Holothurin and Caspofungin treatment at 12, 24 and 48 hours of experimental animals in Wistar rats. K: Control without any treatment (healthy). P1: Treatment of C. albicans. P2: Treatment of Fungi/C.albicans with Holothurin. P3: Treatment of Fungi/C. albicans with Caspofugin.*

tive/preventive mechanism against epithelial damage. Cytokines and chemokines are secreted by epithelial cells in response to C. albicans hyphal invasion, as well as the resulting damage resulting in the recruitment and activation of immune cells. IL-8 recruits GM-CSF, G-CSF, and IL-1 family activated neutrophiles [*Netea M et al., 2008*]. Through phagocytosis and the creation of the Neutrophil Extracellular Trap, neutrophils provide direct protection. They also provide indirect protection through immunological cross-talk with epithelial TLR4 [*Saffarzadeh M et al., 2012*].

Toll-like receptors are the primary recognition receptors for microorganisms. Toll-like receptors are the largest family of pattern recognition receptors, allowing immune and non-immune cells to recognize Pathogen-associated Molecular Patterns (PAMPs). Multiple TLRs are involved in the recognition of C. albicans and other fungal pathogens. During candidiasis, the interaction between TLRs and Candida albicans stimulates immune cells to produce inflammatory and immunomodulatory mediators that shape the immune response of the host [*Choteau L et al., 2017*].

Toll like receptor-4 in human epithelium can protect mucosal surfaces directly from C. albicans infection via a polymorphonuclear process that is cell-dependent [*Moyes D et al., 2010*]. TLR2 and TLR4 are expressed by various cell types of the innate immune system, including monocytes, macrophages, dendritic cells, neutrophils, CD4+ cells, and epithelial cells, and are involved in the inflammatory response induced by C. albicans.

Tumor necrosis factor, interleukin (IL)-1, and IL-10 are produced when the TLR2 signaling pathway in antigen-presenting cells is activated by ligation of C. albicans cell wall constituents like phospholipomanan. The epithelial response is primarily set up to combat candida that has gone dormant and hyphal forms. The two most important TLRs in signal cascades caused by *C. albicans* are TLR-2 and TLR-4 [*Gauglitz G et al., 2012*].

Toll like receptors have the ability to identify different pathogens and trigger defense mechanisms, such as the release of proinflammatory cytokines in response to infection. TLR4 binds to mannans from microorganisms (*C. albicans* on epithelial cells) when *C. albicans* comes into contact with the surface of the epithelial cell and causes the production of cytokines by the epithe-

lial cells. When Candida blastoconidia transform into hyphal form, TLR4-mediated proinflammatory signals are lost [*van der Graaf C et al., 2005*].

Stimulation of host responses by *C. albicans* at the cell membrane is mediated by toll-like receptors (TLRs) and C-type lectin receptors. TLR4 induces primarily pro-inflammatory signals in monocytic cell types (monocytes, macrophages and dendritic cells via the MyD88-Mal-mediated NF-κB and mitogen-activated protein kinase pathways. TLR4 stimulates the production of pro-inflammatory cytokines on contact with mannas [*Gauglitz G et al., 2012*] and *C. albicans* hyphae produce adhesin, which is crucial to the pathogenesis of the infection with *C. albicans*. Aglutinin-like sequences and hyphae wall protein 1 (Hwp1p) are two of the main adhesins on the cell wall of *Candida albicans* [*Cleary I et al., 2011*].

In response to *C. albicans* infection, epithelial cells secrete IL-1 and activate an innate type 17 cellular response, resulting in the release of IL-17. Neutrophils interact with C. albicans via pattern recognition receptors (toll-like receptors, C-type lectin receptors, and NOD-like receptors), phagocytose and destroy yeast and short hyphae, and then release inflammatory cytokines. Signals from cytokines stimulate the release of Tumor necrosis factor - from neutrophils, which regulates TLR4 expression in epithelial cells so that it can provide additional defense. In a process known as NETosis (a program for formation of neutrophil extracellular traps), hyphae that are too large to be phagocytosed stimulate the production of neutrophil extracellular traps. C. albicans that has been phagocytosed by macrophages can avoid damage by inducing pyroptosis and hyphal growth; this enables C. albicans to avoid macrophages [*Richardson J et al., 2019*].

In addition to its ability to invade epithelial cells, Candida albicans can suppress epithelial TLR4 expression, which in turn makes epithelial cells more susceptible to infection by Candida albicans [*Cheng S et al., 2012*]. The anti-inflammatory properties of some TLR activations contribute to homeostasis maintenance in the presence of commensalism [*Gaffen S et al., 2009*]. TLR4 represents one of the pattern recognition receptors because of its role as the main receptor for bacterial lipopolysaccharide [*Gauglitz G et al., 2012*]. Epithelial cells express many pattern recognition receptors such as toll like receptors, including TLR2, TLR4, and dectin-1 which can change expression after Candida attack. These pattern recognition receptors recognize yeast cells and hyphae through pathogen associated molecular patterns (PAMPs), namely mannans and β-glucans. However, this PAMP-PRR interaction does not appear to activate the c-Fos/MKP1 signaling pathway or immune-modulating cytokine secretion [*Richardson J et al., 2019*].

For direct recognition of *C. albicans* PAMPs, cells of the innate immune system are equipped with cytoplasmic, membrane-bound pattern recognition receptors [*Zheng N et al., 2015*]. Indirect recognition is also possible, in which soluble components, such as complement and antibodies, bind to C. albicans and are subsequently detected by opsonizing receptors. Pattern recognition receptors can be subdivided into several families, including type C lectin receptors, toll-like receptors, NOD-like receptors and RIG I-like receptors [*Iwasaki A et al., 2004*], and its role in C. albicans recognition.

Ligand binding leads to fungal uptake and production of anti-inflammatory IL-10 and modulation of TLR signaling, via a Raf-1-dependent pathway [*Gringhuis S et al., 2007*]. Toll signaling pathways in host defense against fungal infection, ten human homologues called toll-like receptors have been extensively characterized and studied. TLRs can be broadly divided into two groups, which are mainly expressed on cellular surfaces involved in lipid and protein recognition (TLRs 1, 2, 4, 5, 6 and 10) [*Netea M et al., 2008*], and which are mainly expressed in the intracellular compartment involved in recognition. nucleic acids (TLR3, 7, 8, 9). TLRs recognize PAMPs by an extracellular domain containing leucine-rich repeats, followed by a transmembrane region and finally an intracellular toll receptor/interleukin-1 homology domain involved in signal transduction [*Gringhuis S et al., 2007*]. Most TLRs are expressed by various cells of the immune system including macrophages, monocytes, neutrophils, dendritic cells, T cells and epithelial cells [*Iwasaki A et al., 2004*].

Toll-like receptor-2 recognizes both C. albicans yeast and hyphal forms via phospholipomannan glycolipids and induces the induction of pro-inflammatory cytokines [*Jouault T et al., 2001; Netea M et al., 2008; Gil M et al., 2009*]. However, conflicting results have been reported regarding the role of

TLR2 in susceptibility to disseminated candidiasis in infection models. In one study, TLR2-deficient rats were more resistant to intravenous *C. albicans* infection, and this was associated with increased chemotaxis and increased candidacidal capacity due to decreased regulatory T-cell response [*Sutmuller R et al., 2006; Netea M et al., 2008*].

In studies by other groups, mice lacking TLR2 have been shown to be more susceptible to *C. albicans* infection [*Villamón E, 2004; Gil M et al., 2009*], and the differences may be explained by differences in *C. albicans* strains [*Gil M et al., 2009*].

Toll-like receptor-2 can form heterodimers with TLR1 and TLR6 for pathogen recognition. Meanwhile, in vitro data shows the role of TLR6 in the recognition of *C. albicans* [*Jouault T et al., 2001; Netea M et al., 2008*], mice lacking either TLR1 or TLR6 show normal susceptibility to disseminated *C. albicans* infection [*Netea M et al., 2008*].

In addition, in humans, single-nucleotide polymorphisms in TLR1, but not in TLR6, lead to reduced cytokine production by peripheral blood mononuclear cells in response to C. albicans and are associated with increased susceptibility to candidemia [*Plantinga T et al., 2012*]. A polymorphism in TLR2 is not associated with candidemia [*Plantinga T et al., 2012*], but was found to be associated with increased susceptibility to recurrent vulvovaginal candidiasis, due to reduced production of interferon and IL-17 in response to C. albicans [*Rosentul D et al., 2014*].

The role of TLR4 in C. albicans recognition is also a matter of debate. In vitro studies by one group demonstrated that TLR4 recognizes Candida O-linked mannans leading to the production of pro-inflammatory cytokines [*Netea M et al., 2008*], whereas others observed subtle differences of TLR4-deficient macrophages in response to C. albicans [*Gil M et al., 2009*].

Similarly, in an in vivo model for disseminated candidiasis, TLR4-deficient mice were observed to be more susceptible to infection, due to impaired chemokine expression and neutrophil recruitment by a single group [*Netea M et al., 2008*], whereas no difference in survival was observed by the others [*Gil M et al., 2009*]. The reason for choosing the difference between these groups could be the variable dependence on TLR4 for the introduction of C. albicans strains [*Netea M et al., 2008*].

Furthermore, a single nucleotide polymorphism in TLR4 has been associated with increased susceptibility to Candida bloodstream infections [*van der Graaf C et al., 2005*], however, larger studies were unable to confirm this association [*Plantinga T et al., 2012*]. Nucleic acid recognition TLRs 3, 7 and 9 are also involved in C. albicans recognition. In vitro stimulation of peripheral blood mononuclear cells carrying a single nucleotide polymorphism in TLR3 showed reduced interferon response to C. albicans [*Nahum A et al., 2011*], and was found to be associated with susceptibility to skin candidiasis [*Nahum A et al., 2011*].

Toll-like receptor-7 recognizes C. albicans RNA, resulting in a decreased IL-12 response, and receptor-deficient mice are more susceptible to C. albicans infection in a model of disseminated infection [*Biondo C et al. 2014*]. In vitro, by inhibition or genetic manipulation of the receptor, TLR9 was shown to be involved in C. albicans recognition. In the same study, using TLR9-deficient mice, the receptor was found to be overexpressed in an in vivo disseminated candidiasis model [*van de Veerdonk F et al., 2008*], whereas another study found that receptor-deficient mice had a higher mortality rate [*Biondo C et al. 2014*].

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Armen A. Muradyan

Address for correspondence:

Yerevan State Medical University
2 Koryun Street, Yerevan 0025,
Republic of Armenia

Phones:

(+37410) 582532 YSMU
(+37493 588697 Editor-in-Chief
Fax: (+37410) 582532
E-mail:namj.ysmu@gmail.com, ysmiu@mail.ru
URL:http//www.ysmu.am

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Acharyan St. 44 Bulding,
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