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Evaluation of miR-146a expression level in macrophages exposed to *Candida glabrata*

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Abstract

Background and Purpose: MicroRNAs are small non-coding RNAs with1924- nucleotides in length. Up- or down-regulation of many miRNAs has been shown by stimulation of Toll-like receptors (TLRs) in the innate immune system . Up-regulation of miR-146a has been reported by both TLR and heat-killed *Candida albicans* .In this study, we aimed to evaluate the expression of miR-146a in cultured monocyte-derived macrophages (MDMs) infected by *Candida glabrata* at 12, 24, and 48 hours.

Materials and Methods: miR-146a expression was evaluated by qRT-real time polymerase chain reaction (PCR) at three time points in *C. glabrata*-infected MDMs. The data was analyzed using repeated measures ANOVA.

Results: miR-146a expression was down-regulated in infected MDMs compared to the control group (*P<0.018*). The expression of miR-146a was at its highest level at 48 h, as compared to 12 and 24 h (*P<0.018*). The differences between the experimental group compared to the control group were statistically significant (P<0.018).

Conclusion: These results suggest that miR-146a can be involved in regulating macrophage function following TLR stimulation in *C .glabrata*-infected MDMs.

Keywords: *Candida glabrata,* Macrophage, miR-146a

Introduction

*C*andida glabrata has historically been considered as a nonpathogenic normal flora of healthy individuals. Recently, the emerging fungal pathogen, *Candida glabrata*, is the second most common cause of candidiasis after *Candida albicans*, including almost 15-25% of *Candida* infections [1-3]. The frequency of systemic infections caused by *C. glabrata* is considerably increased due to immunosuppressive diseases and antifungal resistance [4]. There are several inhibitory mechanisms for inflammatory cytokine production including the anti-inflammatory cytokines such as IL-10 [5].

Lately, it has been shown that miRNAs play the role of negative regulators in macrophages [5]. miRNAs are small, non-coding RNAs (containing about 22 nucleotides) that play an important role in the post-transcriptional regulation of gene expression. miRNAs are involved in cell cycle, differentiation, growth and development, meta-bolism, and immunity in plants, animals, and mammals. They are derived from larger stem-looped hairpin precursors that are transcribed from miRNA genes and are processed by Dicer or Dicer-like proteins [6, 7]. Some of them are expressed through the stimulation of TLRs in the innate immune system such as miR-146a_b, miR-132, and miR-155 in the THP-1 cells stimulated by lipopolysaccharide (LPS).

Increasing miR-146a expression lowers the expression of two key factors (IRAK1 and TRAF6) of the TLR signaling cascade [8]. It has been proposed by Simon et al. [5] that both *Candida albicans* and LPS are able to up-regulate miR-146, miR-155, miR-455, and miR-125a in mouse macrophages. miR-146a, as a negative regulator, can reduce TLR response to prevent further inflammation. This study aimed to evaluate miR-146a-5p expression in human macrophages infected with *Candida glabrata*, compared to non-infected macrophages.

Materials and Methods
Collection and isolation of mononuclear cells with Lymphodex

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using

Lymphodex (Inno-Train, Germany). Briefly, the fresh heparinized blood was placed into 15 ml conical centrifuge tubes. An equal volume of PBS was mixed with the heparinized blood by a sterile pipet. Then, the blood/PBS mixture was gradually layered on the Lymphodex solution by using sterile pipets. The tubes were centrifuged for 30 min at 2000 rpm (900 × g), at 20°C. The cells were counted by hemocytometer protocol and their viability was determined by trypan blue exclusion after re-suspending of mononuclear cells in complete RPMI-1640.

Monocyte-derived macrophages

Monocytes were depleted of the PBMCs suspension by adhering to plastic surfaces. In summary, PBMCs were washed and centrifuged three times in RPMI-1640 for 10 min at 1400 rpm (300 × g); then, the supernatant was removed. Monocytes were incubated in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and inactivated fetal bovine serum (Mediatech Inc, Herndon, VA) for 12 h at 37°C in 5% CO_2 humidified incubator. Non-adherent cells were removed after 12 h; tissue culture plates were slowly rinsed with complete RPMI-1640. Monocyte-derived macrophages were cultured on 6-well tissue-culture plate for 12 days (Figure 1) at 37°C in 5% CO_2 in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and inactivated fetal bovine serum.

Yeast cell culture

Candida glabrata strain (ATCC 90030) was
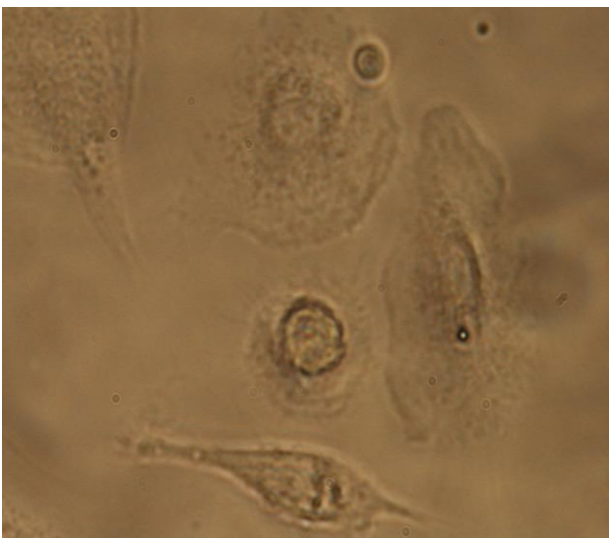
Figure 1. Monocyte-derived macrophages derived from monocytes during 12 days as the control group

cultured on Sabouraud dextrose agar (SDA) at 37°C for 24 h. Afterwards, it was sub cultured on 1% yeast extract, 2% peptone, and 2% dextrose (YPD) at 37°C in a shaking incubator at 180 rpm. Yeast peptone dextrose overnight cultures were washed twice with PBS and RPMI-1640 for 5 min at 2000 rpm (300 × g). *Candida glabrata* was counted with hemocytometer protocol.

MDMs infection with C. glabrata

Monocyte-derived macrophages were exposed to *C. glabrata* suspension. The ratio of MDMs to *C. glabrata* was 1:5. The whole solution in the 6-well tissue-culture plate was scraped and centrifuged; thereafter, the supernatant was removed. Non-infected MDMs were employed as control. Infected and non-infected MDMS were recognized by fluorescent microscope.

qRT-real time-PCR

RT-PCR was used to verify the expression of miR-146-a-5p in the MDMs. The total cellular RNA was extracted at 12, 24, and 48 h from infected and control MDMs with the miRCURY RNA Isolation Kit™ (Exiqon, Copenhagen, Denmark), and cDNA was synthesized with the Universal cDNA Synthesis Kit™ (Exiqon, Copenhagen, Denmark). Real-time PCR was performed by using SYBR® Green Master Mix Kit™ (Exiqon, Copenhagen, Denmark) and specific miR-146a-5p primers (Exiqon, Copenhagen, Denmark). The RT-PCR internal controls were synthetic RNA spike-in templates and their primers (Exiqon, Denmark). Real-time PCR was performed by ABI Step One Plus (ABI, USA). $2^{-\Delta\Delta ct}$ method was used for data analysis.

Statistical analysis

Repeated measures ANOVA was performed to evaluate differences between the groups, using SPSS version 21. P-value less than 0.05 was considered statistically significant.

Results

The macrophages were infected with *C. glabrata* (ATCC 90030), and after 3 h, 95% of the yeasts were phagocytosed. After 12, 24, and 48 h, the number of phagocytosed yeasts was increased due to replication (Figure 2).

Down-regulation

To evaluate the expression of miR-146a, MDMs were infected with *C. glabrata*, and RNA was extracted from both infected and
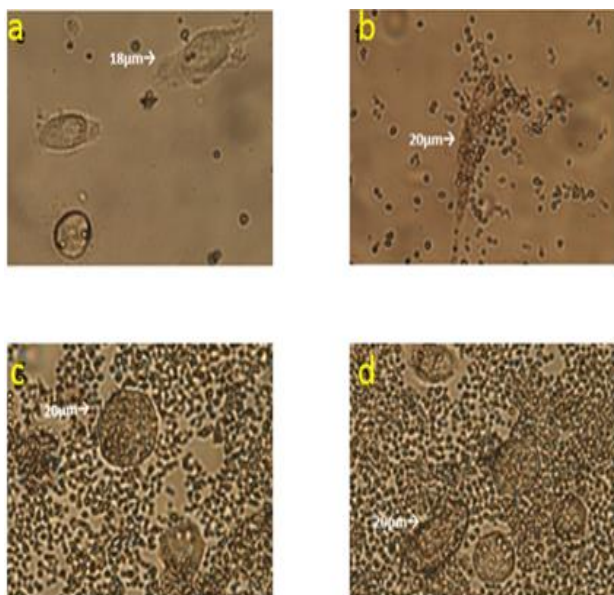
Figure 2. Control (2a), infected monocyte-derived macrophages to *Candida glabrata* after 12 (2b), 24 (2c), and 48 h (2d)

non-infected MDMs. The results demonstrated down-regulation of miR-146a in infected MDMs compared to the control group (Figure 3). The level of miR-146a expression increased at 12 h after infection of MDMs with *C. glabrata,* and then slowly reduced at 24 h. The expression of miR-146a was at its highest level within 48 h, as compared to the 12 and 24 h time points (Figure 3). The differences between the experimental and the control groups were statistically significant (*P<0.018*).
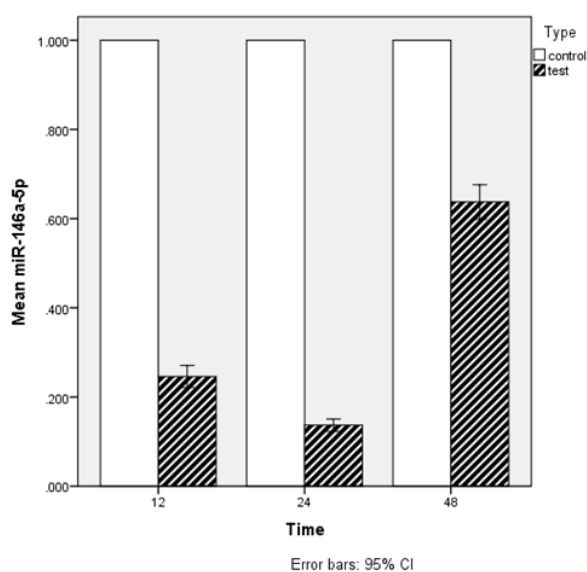
Figure 3. miR-146a expression after 12 (25%), 24 (10%) and 48 h (65%); the $2^{-\Delta\Delta ct}$ method was applied for data analysis and the control group was considered as the reference for 12, 24, and 48 h (*P<0.018*)

Discussion

In the innate immunity as the first line of host defense, the primary recognition of pathogens is facilitated via dedicated pattern recognition receptors (PRRs), which are at the surface of phagocytic cells such as monocytes and macrophages. Pattern recognition receptors recognize microbe-specific pathogen-associated molecular patterns (PAMPs) and trigger the activation of some intracellular signaling cascades such as the MAPK and NF-*kB* pathways.

Subsequently, the production of cytokines including pro-inflammatory cytokines causes inflammation at the site of infection [9-11]. NF-*kB* is the main transcription factor in the inflammatory signaling pathways and plays an essential role in the regulation of inflammatory cytokines production.

The inflammatory response is essential to combat pathogens, but its inappropriate response can lead to severe problems [12-15]. The miRNAs can control many cellular decision-making networks by targeting the key molecules [16, 17].

miRNAs are also involved in the regulation of the NF-*kB* pathways (e.g., miR146a, miR155, miR-125b, miR-9, and miR-29) [15]. Many of them such as miR-146, miR-155, miR-181b, miR-21, and miR-301a in the NF-*kB* system are considered negative regulators [18, 19]. Previous studies have shown that the expression of miR-155, miR-146a and miR-146b are induced in response to viral stimuli through TLRs [20, 21]. Monk et al. [5] exhibited that miR-155, miR-146a, miR-146b, miR-125a, and miR-455 can be up-regulated by both TLR and heat-killed *C. albicans.* miR-146a is known as a target key element of NF-*kB* pathways just as IRAK1 and TRAF6. Generally, the regulatory function of miR-146a leads to the regulation of pro-inflammatory cytokines production. Contrary to the present study, up-regulation of miR-146a in infected macrophages with *C. albicans* has been reported by Monk et al. [5], that is, various species may have different effects on the expression of special microRNA such as miR-146a [22].

Conclusion

In the present study, the expression of miR-146a was down-regulated in infected macrophages with *Candida glabrata*. We can conclude that down-regulation of miR-146a can reduce pro-inflammatory cytokines production, which can help *C. glabrata* survive in the macrophages.

Acknowledgments

Author's contribution

B.A. and M S. performed tests, M.S. was a consultant and edited the manuscript, and R.M. supervised the research stages and revised the manuscript.

Conflicts of interest

None declared.

Financial disclosure

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