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Trichophyton rubrum conidia modulate the expression and transport of Toll-like receptor 2 in HaCaT cell

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ABSTRACT

Trichophyton rubrum (*T. rubrum*) represents the most important agent of dermatophytosis in humans. *T. rubrum* infection causes slight inflammation, and tends to be chronic and recurrent. It is suggested that *T. rubrum* can modulate the innate immune responses of host cells, which result in the failure of host cells to recognize *T. rubrum* and initiate effective immune responses. In this study we show how *T. rubrum* conidia modulate the expression and transport of Toll-like receptor 2 in HaCaT cell. Flow cytometric analysis showed that the surface and total expression of Toll-like receptor 2 were upregulated at the very early stage when keratinocytes were exposed to *T. rubrum* conidia regardless of the dose, and the upregulation of surface TLR2 was much more significant than that of total TLR2. Moreover, TLR2 expression was suppressed after upregulation in the initial stage of *T. rubrum* exposure, and the decrease of total TLR2 was earlier than that of surface TLR2. Our results suggest that in the early stage, TLR2 of keratinocytes were upregulated and transported to the cell surface. After then, the expression of TLR2 was suppressed by *T. rubrum* conidia.

1. Introduction

Dermatophytosis refers to an infection by pathogenic dermatophytic fungi of keratinized tissues, such as the cutaneous stratum corneum, hair and nails. Most dermatophytosis are caused by *Trichophyton rubrum*. *T. rubrum* is especially well-adapted to humans sentinel immune system and causes a chronic and recurrent infection. As an extracellular pathogen, the fungal cell-wall of *T. rubrum* is the dominant target of the host innate immune system. The outermost layer of the cell-wall is composed of β-glucan, the second layer contains galactomannans and the third layer is known as chitin, giving the fungal cell-wall its rigidity [1–3]. The fungal cell-wall components are highly conserved, thus termed pathogen-associated molecular patterns (PAMPs), and play a critical role in host immune recognition against the pathogen. Why especially *T. rubrum* can cause a chronic infection is still debated. One of the significant reasons is that *T. rubrum* cell wall may have immunosuppressive effects, inhibiting inflammatory reaction and lymphoproliferation [4–7].

Besides its primary function in the formation of a barrier, keratinocytes are active players in the skin innate immune defense [8]. It expresses multiple pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs) and c-type lectin receptors (CLRs) [9–13]. TLR2 is the best-characterized Toll-like receptor, it initiates immune responses against a wide range of pathogens alone or forming heterodimers with a sort of co-receptors, such as TLR1 or TLR6 [14,15]. The antifungal function of TLR2 has been widely studied [16,17]. However, the interaction between TLR2 and *T. rubrum* is still incompletely understood, especially on keratinocytes.

In our previous study, we showed that heat-inactivated *T. rubrum* conidia reduced the surface expression of TLR2, and suppressed the secretions of interferon-inducible protein-10 (IP-10) and monocyte chemotactic protein-1 (MCP-1) of HaCaT cell after 24 h contact [18]. In this study, we focused on the impact of *T. rubrum* conidia on the expression and transport of TLR2 in HaCaT cells by analyzing the surface and total protein expression of TLR2 when HaCaTs were stimulated with heat-inactivated *T. rubrum* conidia either in a dose-manner or over the time course.

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2. Methods and materials

2.1. Cells and fungal strain

Experiments were performed using the immortalized human keratinocyte cell Line, HaCaT (purchased from China Center for Type Culture Collection). Monolayer cell cultures were grown in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA) in a humidified atmosphere containing 5% CO_2, at 37 °C.

T. rubrum strain T1a (China Medical Microbiological Culture Collection Center) was confirmed as *T. rubrum* by morphological identification and sequencing of internal transcribed spacer regions (ITS) and the D1-D2 domain of the large-subunit rRNA gene. *T. rubrum* T1a was cultured at 28 °C for 10 days on potato dextrose agar (OXOID, England) to produce conidia. Conidial suspensions were obtained from *T. rubrum* cultures by covering the fungal colonies with 5 ml sterilized phosphate buffer saline (PBS) and gently rubbing the colonies with the tip of a transfer pipette. The resulting suspensions were filtrated through a filter paper (Whatman, England) twice to remove the hyphae, and then centrifuged at 1800*g* for 10 min to harvest the conidia. The conidia were rinsed twice with sterilized PBS [19].

Inactivated *T. rubrum* conidia were prepared by heating for 60 min at 56 °C in a water bath. The viabilities of inactivated *T. rubrum* conidia were confirmed by inoculating them (approximately 1×10^6 colony-forming units (CFU)) on potato dextrose agar plates at 28 °C for 10 days. No colony was observed.

2.2. Stimulation of kerationcyte lineage with heat-inactivated T. rubrum

HaCaT cells were seeded at a density of 0.3×10^6 cells per well in a 6-well plate. Monolayer cell cultures reached approximately 70%–80% confluence, and then the cultures were stimulated with heat-inactivated *T. rubrum* conidia (at the ratios of 1.5, 3 and 6, namely 0.45×10^6, 0.9×10^6 and 1.8×10^6, respectively) for 90 min. On the other hand, HaCaT cells were stimulated with heat-inactivated *T. rubrum* conidia at a ratio of 3 for 90 min, 6 h and 24 h. And unstimulated HaCaT cells at the same time point served as controls.

2.3. Flow cytometry

HaCaT cells were analyzed by flow cytometry for the surface and total expression of TLR2. The cells were stained with anti-human TLR2 APC (T2.1, eBioscience, USA). Isotype control IgG antibody was used to assess background staining. The cells were washed and fixed with paraformaldehyde (2% in PBS), and then analyzed by flow cytometry. In the experiments performed to determine total protein expression, after the surface staining , the cells were treated with Tween-20 (0.5% in PBS) solution for 10 min to increase the permeability of the cells, incubated with the same antibodies for 30 min, and washed with PBS.

The experiment was performed according to the manufacturer's instructions. Data were acquired on a FACSCalibur (BD Biosciences, USA) and analyzed using FlowJo software (USA).

2.4. Statistical analysis

The unpaired Wilcoxon's rank-sum test or Student's *t*-test (SPSS 16.0, IBM Acquires SPSS Inc. USA) was used to compare two different groups. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Expression of surface and total TLR2 affected by the T. rubrum conidia in a dose-manner

The previous experiments have verified that the expressions of TLRs at the transcriptional level were unaffected by stimulation with heat-inactivated *T. rubrum* conidia, but the expression of TLR2, IP-10 and MCP-1 at the protein level were down-regulated after stimulation with heat-inactivated *T. rubrum* conidia for 24 h. In the first set of experiments, we intended to demonstrate the dose effect of heat-inactivated *T. rubrum* conidia on the expression of TLR2. The surface and total expressions of TLR2 were measured by flow cytometry in HaCaT cells stimulated with heat-inactivated *T. rubrum* conidia for 90 min in the indicated ratios. The surface and total expression of TLR2 at 90 min after treatment represented an early up-regulation. Both surface and total expression of TLR2 were significantly enhanced and peaked at the ratio of 3 ($76.67 \pm 8.67\%$ and $87.73 \pm 2.40\%$, respectively. Fig. 1A). TLR2 surface and total expressions increased 3.81 and 2.06 times at the ratio of 3, respectively, when presented as the fold increases over those of the unstimulated cells (Fig. 1B).

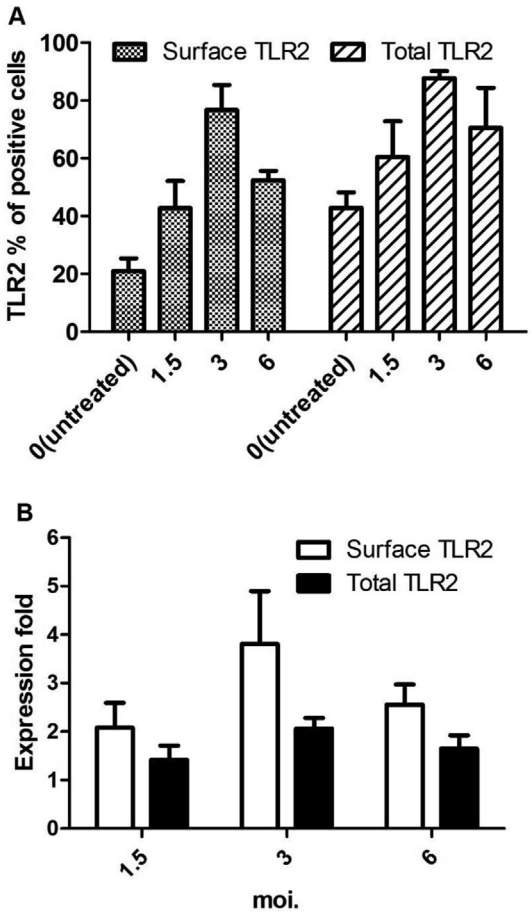

Fig. 1. Expression of TLR2 in HaCaT cells stimulated with heat-inactivated *T. rubrum* conidia in an initial stage (90 min). HaCaT cells were treated with heat-inactivated *T. rubrum* conidia in the indicated concentrations (X-axis: multiplicity of infection, moi). HaCaT cells were collected 90 min after challenge and the expression of TLR2 was analyzed by flow cytometry. A: The figure shows the percentage of TLR2-positive HaCaT cells obtained from three independent experiments. B: TLR2 surface and total expressions were presented as the fold increases over those of the unstimulated cells.