

Effect of Culture Supernatant Derived from *Trichophyton Rubrum* Grown in the Nail Medium on the Innate Immunity-related Molecules of HaCaT

Xin-Zhu Huang, Pan-Pan Liang, Han Ma, Jin-Ling Yi, Song-Chao Yin, Zhi-Rui Chen, Mei-Rong Li, Wei Lai, Jian Chen

Department of Dermatology, the Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510630, China

Abstract

Background: *Trichophyton rubrum* is superficial fungi characteristically confined to dead keratinized tissues. These observations suggest that the soluble components released by the fungus could influence the host immune response in a cell in contact-free manner. Therefore, this research aimed to analyze whether the culture supernatant derived from *T. rubrum* grown in the nail medium could elicit the immune response of keratinocyte effectively.

Methods: The culture supernatants of two strains (T1a, T_{XHB}) were compared for the β -glucan concentrations and their capacity to impact the innate immunity of keratinocytes. The β -glucan concentrations in the supernatants were determined with the fungal G-test kit and protein concentrations with bicinchoninic acid protein quantitative method, then HaCaT was stimulated with different concentrations of culture supernatants by adopting morphological method to select a suitable dosage. Expressions of host defense genes were assessed by quantitative polymerase chain reaction after the HaCaT was stimulated with the culture supernatants. Data were analyzed with one-way analysis of variance, followed by the least significant difference test.

Results: The *T. rubrum* strains (T1a and T_{XHB}) released β -glucan of 87.530 ± 37.581 pg/ml and 15.747 ± 6.453 pg/ml, respectively into the media. The messenger RNA (mRNA) expressions of toll-like receptor-2 (TLR2), TLR4, and CARD9 were moderately up-regulated in HaCaT within 6-h applications of both supernatants. HaCaT cells were more responsive to T1a than T_{XHB}. The slight increase of dendritic cells-specific intercellular adhesion molecule 3-grabbing nonintegrin expression was faster and stronger, induced by T1a supernatant than T_{XHB}. The moderate decreases of RNase 7, the slight up-regulations of Dectin-1 and interleukin-8 at the mRNA level were detected only in response to T1a rather than T_{XHB}. After a long-time contact, all the elevated defense genes decreased after 24 h.

Conclusion: The culture supernatant of *T. rubrum* could directly and transiently activate the innate immune response of keratinocytes.

Key words: Innate Immunity; Keratinocytes; Pattern Recognition Receptors; Trichophyton

INTRODUCTION

In recent years, there has been a dramatic increase in the spread of dermatophyte. The infection affects more than 20–25% of the world's population.^[1] However, the defense mechanism of host skin against infection is not clear so far. The latest investigations suggest that keratinocytes as immune mediators play a vital role in innate immunity.^[2,3] As the first line of defense against infection, keratinocytes can directly recognize the conserved pathogen-associated molecular patterns (PAMPs) on the invading pathogens and initiate immune response by their pattern recognition receptors (PRRs).^[4] Then, keratinocytes can initiate effective immune responses through constantly secreting

some endogenous antimicrobial substances, such as NO, β -defensin, and RNase 7, to resist microorganism invasion directly,^[2,5,6] or recruit and activate neutrophils and macrophages to head to the infection site through the production of proinflammatory cytokines and chemokines. Although emerging evidence has proved a crucial role of

Address for correspondence: Dr. Jian Chen,
Department of Dermatology, the Third Affiliated Hospital of Sun Yat-Sen
University, Guangzhou, Guangdong 510630, China
E-Mail: chj75@medmail.com.cn

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

© 2015 Chinese Medical Journal | Produced by Wolters Kluwer - Medknow

Received: 02-07-2015 **Edited by:** Yuan-Yuan Ji

How to cite this article: Huang XZ, Liang PP, Ma H, Yi JL, Yin SC, Chen ZR, Li MR, Lai W, Chen J. Effect of Culture Supernatant Derived from *Trichophyton Rubrum* Grown in the Nail Medium on the Innate Immunity-related Molecules of HaCaT. Chin Med J 2015;128:3094-100.

Access this article online

Quick Response Code:



Website:
www.cmj.org

DOI:
10.4103/0366-6999.169106

keratinocyte as the participants in the innate defense against bacteria, viruses, and fungi, the precise role played by keratinocyte in fighting against dermatophyte has not been completely understood.

In vivo, colonization of dermatophyte is characteristically restricted to the horny layer of skin. This characteristic suggests that the soluble fungal substances might play an even more important role in the immunomodulation than the insoluble compounds located in the fungal cells. Analyses have indicated that the culture supernatants of dermatophytes can effectively stimulate immune response of keratinocytes.^[7,8] However, all the culture mediums used in these studies are complete mediums containing simple and absorbable nutrients. Consequently, the immunogenicity and pathogenicity of dermatophyte surviving in such a cultivation condition are distinct from those *in vivo*, where *Trichophyton rubrum* must adhere to and invade keratin to live.^[9] Therefore, these results might not be able to reflect the immune response of keratinocyte to the special substance secreted by dermatophyte during infection *in vivo*. Thus, it is necessary to investigate the immune response of keratinocyte to soluble factors released by dermatophyte grown in keratin culture medium.

In the development of infections, the host innate defense response to fungus is critical for the severity of fungal diseases. It is described that the mutation in the well-known PRRs and their intracellular signaling molecules, such as toll-like receptor-2 (TLR2), TLR4, and Dectin-1, as well as the intracellular signaling molecule of Dectin-1 CARD9, can increase the risk of fungus infection, highlighting the specific role of these host defense genes in human antifungal response.^[4,10,11] Our previous research has also found that TLR2, TLR4, and Dectin-1 of keratinocyte might participate in the recognition of the viable *T. rubrum*.^[12] The C-type lectin family of PRRs, dendritic cells-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) can recognize mannan and signal in response to dermatophyte fungi such as *Aspergillus fumigatus* conidia.^[13] The antimicrobial peptides RNase 7 is constitutive expressed in keratinocytes of the epidermis, exhibit ribonuclease activity, and antimicrobial activity.^[14] Interleukin-8 (IL-8) is an important effector molecule of the acute inflammation due to its roles in recruiting and activating neutrophils to kill microorganisms.^[15] Although these PRRs, RNase 7, and IL-8 expressions and functions in classical immune cells have been studied profoundly, very few studies have been performed to characterize PRRs on keratinocytes. So far, the ligands recognized by PRRs of keratinocytes have been found to be the same as the ones of classic immune cells.^[4,16,17] The aim of this study was to investigate the effect of *T. rubrum* culture supernatant derived from *T. rubrum* grown in the medium using keratin as the only carbon and nitrogen source on the innate immune response of keratinocytes. In this medium, *T. rubrum* have to adhere to and invade the keratin to live, which could mimic the actual condition that *T. rubrum* infect the host *in vivo*.

METHODS

Preparation of nail medium

The nail medium was prepared as previously described with some modifications.^[18] The fresh nails from healthy adults were washed with double distilled water, dried at 60°C for 24 h. Then the dried nails were milled into powder. The liquid culture medium contained the following substances (g/L, pH 7.0): Nail powder, 10.0; K₂HPO₄·3H₂O, 1.30; KH₂PO₄ 0.46; and MgSO₄·7H₂O, 0.10. The pH was adjusted to 7.0 and the medium was sterilized by autoclaving at 121°C for 15 min.

Cells and *Trichophyton rubrum* strains

The HaCaT cell line was purchased from CLS (Cell Lines Service, 300493, Eppelheim, Germany). Cell cultures, containing high glucose Dulbecco's modified Eagle's medium (Gibco, Beijing, China) supplemented with 10% fetal bovine serum, were grown at 37°C in a 5% CO₂ incubator.

Two strains of *T. rubrum* (T1a, T_{XHB}) were used in this study. The standard *T. rubrum* strain, T1a was obtained from China Medical Microbiological Culture Collection Center; T_{XHB} was isolated from a tinea corporis patient. Both strains were identified by morphological analysis, and specific DNA sequences of nuclear ribosomal internal transcribed spacer regions and the D1–D2 domain of the large-subunit ribosomal RNA genes were as previously described.^[19] *T. rubrum* was subcultured at least twice to provide enough viability. After 14 days of growths on potato dextrose agar (Oxoid, England, UK) at 28°C, cultures on agar slants were covered with sterile 0.85% saline and scraped gently to harvest conidia. The conidia suspension was filtered with Whatman filter model 1 (pore size, 11 μm) to collect microconidia. A conidial suspension (1 ml, 10⁷ microconidia/ml) was washed twice in 0.85% sterile NaCl, and then moved into the 100-ml Erlenmeyer flasks containing 30-ml nail medium and incubated on a rotary shaker at 100 r/min at 28°C for 14 days. To obtain the fungal supernatants, the *T. rubrum* and the rest nail powder were removed by centrifugation. The supernatant of T1a was then subjected to ultrafiltration with a 30,000-cutoff ultra-filtration tube (Millipore, Billerica, USA), centrifugation at 5000 ×g at 4°C for 30 min. Concentrated supernatants of both T1a and T_{XHB} were filter sterilized through 0.22-μm pore size filters (Millipore, Billerica, USA). The nail medium that had been shaken under the same condition was also filter sterilized for negative control. The filtered nail medium and fungal supernatants were freshly used or frozen at -30°C for further disposal.

Determination of β-glucan

The free β-glucan concentration in supernatant of T1a was determined through a chromogenic end-point assay, with the fungal G-test kit (Xiamen Limulus Experiment Factory, China) according to the manufacturer's instructions.

HaCaT cells stimulated with the culture supernatant of *Trichophyton rubrum*

HaCaT cells were plated in 6-well plates (Corning, New York,

USA) at a density of 2.0×10^5 cells/well. Upon reaching subconfluence, cells were exposed to the fresh medium containing 10% (v/v) nail medium or supernatants of both strains of *T. rubrum*. For time-response assay, HaCaT was treated with supernatants of both strains for 3 h, 6 h, 12 h, 24 h, and 48 h. For the dose-response assay, the final protein concentrations of T1a supernatant in cell culture wells were 10, 20, 40, 80, 100, and 120 $\mu\text{g/ml}$ diluted in phosphate-buffered saline, to stimulate HaCaT for 24 h. The protein concentrations of the supernatants were determined with bicinchoninic acid protein quantitative method (Thermo Scientific, China).

RNA isolation and complementary DNA synthesis

Total RNA was extracted with TriPure Isolation Reagents (Roche, Mannheim, Germany) according to the manufacturer's protocol. Purity and concentration of the RNA were determined by measuring 260/280 absorbance ratios using a spectrophotometer (DU 730 Beckman Coulter, Fullerton, USA). Complementary DNA (cDNA) synthesis was achieved using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with oligo-dT primers according to the manufacturer's protocol.

Relative quantification real-time polymerase chain reaction analysis

Quantitative polymerase chain reaction (qPCR) was performed on a 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, USA) in a 20- μl reaction volume using FastStart Universal SYBR Green Master (ROX) (Roche, Mannheim, Germany). The PCR setup was 10 min at 95°C for activation of FastStart Taq DNA Polymerase, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. Melting curve analysis was performed to confirm the amplification of a single product in each reaction. Relative real-time PCR data were analyzed using the $2^{-\Delta\Delta C_t}$ method^[20] to calculate the relative level of each messenger RNA (mRNA) in each sample and expressed as a ratio relative to endogenous GAPDH housekeeper genes. The primer sequences (in 5'-3' direction) used are shown in Table 1.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). The statistical significance was performed by one-way analysis of variance (ANOVA), followed by the least significant difference test for comparison of mRNA expression using SPSS 19.0 software (SPSS, Chicago, USA). A $P < 0.05$ was considered statistically significant.

RESULTS

Beta-glucan concentrations detected in nail medium culture supernatants of *Trichophyton rubrum*

The *T. rubrum* strains (T1a and T_{XHB}) released β -glucan of 87.530 ± 37.581 $\mu\text{g/ml}$ and 15.747 ± 6.453 $\mu\text{g/ml}$, respectively to the media.

HaCaT cells treated with supernatant of *Trichophyton rubrum* for 24 h exhibited morphogenetic transition in a dose-dependent manner

Usually, HaCaT is polygonal-shaped, adherent cells

Table 1: Primers used in quantitative PCR

Gene	Nucleotide sequence
Dectin-1	5'-ACACTTCGACTCTCAAAGCA-3' 5'-TACCCAGGACCACAGCTATC-3'
CARD9	5'-CCCTCACGCATCACACCTTAC-3' 5'-GCACACCCACTTCCGTTTG-3'
TLR4	5'-GTGATGTTTGATGGACCTCTGA-3' 5'-AAGGAGCATTGCCAACAG-3'
TLR2	5'-ACTTCTCCCATTTCCGCTTT-3' 5'-GGACTTTATCGCAGCTCTCA-3'
RNase 7	5'-GGAGTCACAGCACGAAGACCA-3' 5'-CATGGCTGAGTTGCATGCTTGA-3'
DC-SIGN (CD209)	5'-AATGGCTGGAACGACGACAAA-3' 5'-CAGGAGCTGCGGACTTTT-3'
IL-8	5'-GCCAACACAGAAATTATTGTAAAGCTT-3' 5'-AATTCTCAGCCTCTTCAAAACTT-3'
GAPDH	5'-AAGGCTGGGGCTCATTGTC-3' 5'-GCTGATGATCTTGAGGCTGTTG-3'

PCR: Polymerase chain reaction; TLR: Toll-like receptor; DC-SIGN: Dendritic cells-specific intercellular adhesion molecule 3-grabbing nonintegrin; IL-8: Interleukin-8.

growing as a confluent monolayer (0 $\mu\text{g/ml}$) [Figure 1a]. Our results showed that treatment with concentrations lower than 20 $\mu\text{g/ml}$ of *T. rubrum* supernatant for 24 h, no change or only moderate morphological changes could be discerned, meaning that cells were viable [Figure 1b-d]. At higher concentrations (40 $\mu\text{g/ml}$), however, T1a supernatant significantly increase the refractivity of the cell nucleus [Figure 1e]. When the concentrations rose to 80–120 $\mu\text{g/ml}$, the highly refractive nucleus was much more obvious. Meanwhile, the increase of the refractivity of the edge of cells was accompanied with the widened cell-to-cell contact, which implies the cells began to detach from the bottom. Also, more cells with visible polynuclear, atypical vacuoles, or granules in cell plasma appeared as more cells turned round [Figure 1f-h].

HaCaT cells treated with *Trichophyton rubrum* supernatant for immunity analysis were normal in morphology

As shown in Figure 2, HaCaT cells with normal morphology were seen in the negative control group. After cells were treated with supernatants from the two strains of *T. rubrum* (T1a, T_{XHB}) for 3 h, 6 h, 12 h, 24 h, and 48 h, the morphology showed no significant change when compared with nail-treated controls. Repetition of this experiment revealed similar changes in cellular morphology, indicating that the observed effects of supernatants were reproducible.

Effects on the expression of defense gene of HaCaT keratinocytes stimulated with *Trichophyton rubrum* culture supernatant

To investigate whether the defense gene expression in HaCaT keratinocytes was regulated by *T. rubrum* culture supernatant, Dectin-1, TLR2, TLR4, RNase 7, IL-8, CARD9, and DC-SIGN mRNA were analyzed by real-time PCR [Figure 3]. Based on the results obtained [Figure 2], the medium contained

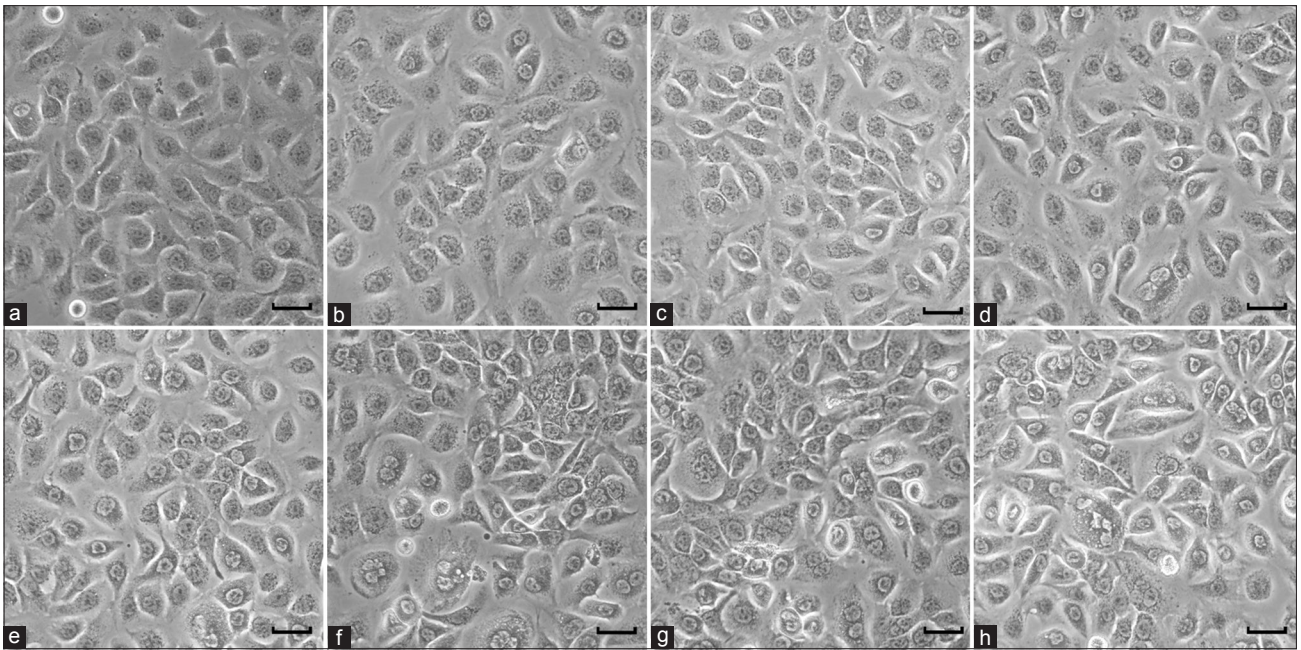


Figure 1: The effect of different concentrations of T1a supernatant on HaCaT morphology. The final concentrations of *Trichophyton rubrum* proteins in medium were 0 µg/ml (a), 5 µg/ml (b), 10 µg/ml (c), 20 µg/ml (d), 40 µg/ml (e), 80 µg/ml (f), 100 µg/ml (g), and 120 µg/ml (h). Nail medium treated cells were used as negative control (a) (bar, 20 µm).

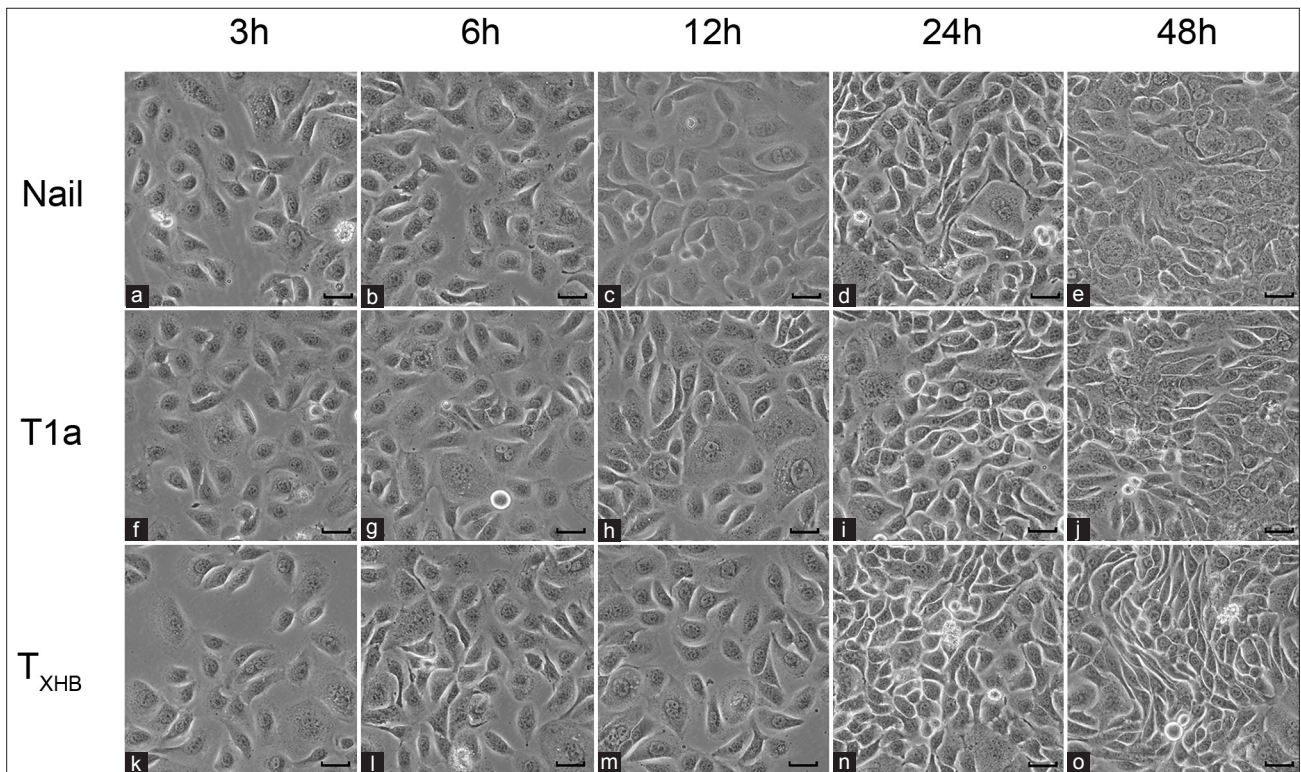


Figure 2: Photomicrograph of HaCaT cells after exposure to supernatants of *Trichophyton rubrum* for 48 h. The final concentration of *Trichophyton rubrum* proteins in the media was 20 µg/ml. HaCaT cells were incubated with nail medium (a-e), supernatants from two strains of *Trichophyton rubrum*, T1a (f-j) and T_{XHB} (k-o). Nail medium was used as negative control. After 3 h (a, f, and k), 6 h (b, g, and l), 12 h (c, h, and m), 24 h (d, i, and n), and 48 h (e, j, and o) continuous exposure, changes in cellular morphology were assessed using light microscopy (bar, 20 µm).

20 µg/ml *T. rubrum* proteins was chosen for the treatment group, and the nail medium was used for negative control. As shown in Figure 3a-c, short periods of 6 h incubation with

T1a or T_{XHB} supernatant significantly enhanced the mRNA expression of TLR2, TLR4, and CARD9 in HaCaT cells. The respective ANOVA *F*-values were 12.080 (TLR2 6 h,

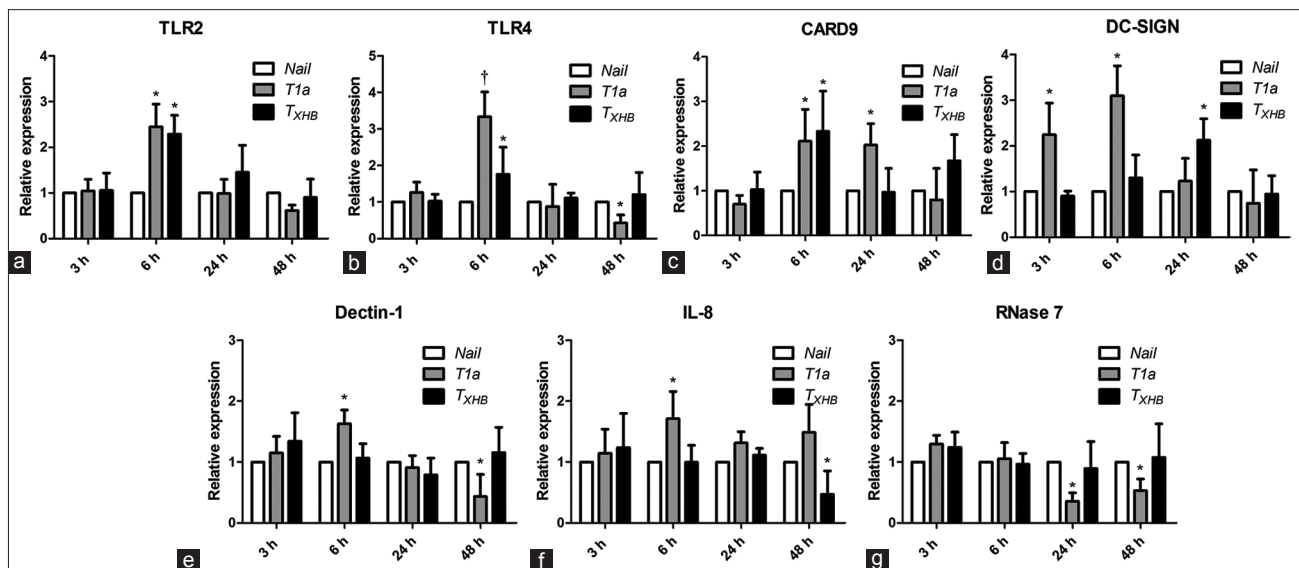


Figure 3: The relative expression of TLR2 (a), TLR4 (b), CARD9 (c), DC-SIGN (d), Dectin-1 (e), RNase 7 (f) and interleukin-8 (g) messenger RNA in HaCaT cells cultured with the supernatants of nail medium (nail), T1a or T_{XHB} for 3, 6, 24, and 48 h. The expression of each gene in HaCaT treated with nail medium was regarded as the standard control and normalized for the housekeeping gene GAPDH (RQ = 1). Mean values \pm standard deviation of four different experiments are performed. (* $P < 0.05$, † $P < 0.001$, least significant difference).

$df=2.9$), 24.618 (TLR4 6 h, $df=2.9$), and 7.710 (CARD9 6 h, $df=2.9$) and all F with $P < 0.05$. Curiously, HaCaT cells were more responsive to T1a than T_{XHB} [Figure 3c-g]. The expression of CARD9 by T1a was upregulated at 6 h and 24 h, longer than 6 h by T_{XHB} [Figure 3c] (ANOVA CARD9 6 h, $F = 7.710$, $df = 2.9$, $P < 0.05$; CARD9 24 h, $F = 8.436$, $df = 2.9$, $P < 0.05$). The mRNA expression of DC-SIGN was slightly up-regulated after application of T1a supernatant for 3 and 6 h, the increases were 2–4 times higher than in the control groups, while the slightly increase happened only at 24 h with T_{XHB} supernatant [Figure 3d] (ANOVA DC-SIGN 3 h, $F = 363.865$, $df = 2.9$, $P < 0.05$; DC-SIGN 6 h, $F = 26.450$, $df = 2.9$, $P < 0.05$). The mRNA expression of Dectin-1 was slightly up-regulated after application of T1a supernatant for 6 h ($P < 0.05$), and then downregulated after 48 h ($P < 0.05$), while no significant change was seen with T_{XHB} supernatant [Figure 3e] (ANOVA Dectin-1 6 h, $F = 15.040$, $df = 2.9$, $P < 0.05$; Dectin-1 48 h, $F = 6.506$, $df = 2.9$, $P < 0.05$). IL-8 was minimally upregulated applied with T1a supernatant for 6 h ($P < 0.05$), and moderately declined at 48 h with T_{XHB} supernatant [Figure 3f] (ANOVA IL-8 6 h, $F = 8.154$, $df = 2.9$, $P < 0.05$; IL-8 48 h, $F = 8.678$, $df = 2.9$, $P < 0.05$). Moderate decrease was detected at 24 h and 48 h for RNase 7 at mRNA level respectively in response to T1a except T_{XHB} [Figure 3g] (ANOVA RNase 7 24 h, $F = 7.790$, $df = 2.9$, $P < 0.05$; RNase 7 48 h, $F = 5.500$, $df = 2.9$, $P < 0.05$).

DISCUSSION

In this study, whether the culture supernatant derived from *T. rubrum* grown in the nail medium could enhance the immune response of keratinocyte was examined. It is reported that high concentration of the extracellular lipase

from culture supernatant of *Candida albicans* can damage cell viability, which will also influence the innate immune response.^[21,22] Therefore, an analysis is performed in the first part to select a suitable dosage of *T. rubrum* culture supernatants so as to stimulate HaCaT under different concentrations of *T. rubrum* culture supernatant with morphological method. The supernatant of T1a seems to be able to exert cytotoxic effects on HaCaT depending on the applied dose, as the concentrations were higher than 40 $\mu\text{g/ml}$. Then morphological examination of HaCaT exposed to supernatants of 20 $\mu\text{g/ml}$ from two strains of *T. rubrum* within 48 h revealed that cell viability was not likely to be affected. Since the immunity analysis requires functional, metabolically active cells without obvious injury, the HaCaT was stimulated by culture supernatants of *T. rubrum*, in which the final concentration of *T. rubrum* proteins was 20 $\mu\text{g/ml}$.

The culture supernatants of a standard strain T1a and a clinical strain T_{XHB} were compared for β -glucan concentrations and their capacity to impact the innate immunity of stimulated keratinocytes. Both of their supernatants could induce transient upregulation of TLR2 and TLR4 mRNA in keratinocytes. It is reported that many cell types, including corneal epithelial cells, can upregulate mRNA expression of TLR2 and TLR4 by antigens from the supernatant of *A. fumigatus*.^[23] The above results support the hypothesis that TLR2 and TLR4 could play an important role for epithelial cells *in vitro* responses to the fungal culture supernatants. As an adaptor of Dectin-1,^[10] CARD9 was elevated at mRNA levels after incubation for 6 h and 24 h with both supernatants. However, only the corresponding enhancement in Dectin-1 at 6 h with supernatant from T1a could be detected, without any enhancement at any given point of time with supernatant from T_{XHB} . This is probably because

that CARD9 can be activated by other receptors unknown yet, and the different immunomodulation of keratinocyte corresponding to the two strains could be the result of the intraspecies diversity of PAMPs in the supernatants.

Although the ligands of PRRs had been extensively studied in classical immune cell, such as macrophages and dendritic cell subsets, it is still presently unknown how keratinocyte could recognize the threatened *T. rubrum* without direct contact with living fungus. The outermost layer at the surface of *T. rubrum*, β -glucan is known to elevate Dectin-1 mRNA expression of keratinocytes.^[17,24] Studies on *C. albicans* had presented the native phospholipomannan from the surface of *C. albicans* cell wall can augment the keratinocyte mRNA expression of TLR2 and trigger inflammatory responses of human keratinocytes in the TLR2 signaling-pathway dependent manner.^[25] Chitin could induce the expression of the TLR4 on keratinocytes at mRNA and protein level.^[26] The mannan receptor DC-SIGN was found to recognize several pathogenic fungi, including *C. albicans* and *A. fumigatus* conidia.^[13] All these ligands (β -glucan, phospholipomannan, chitin, mannan, and galactomannan) are located in the cell wall or cell surface of *Trichophyton*.^[27,28] All the results above indicate that there are certain soluble factors, PAMPs derived from the fungal wall and other stimulating components released by *T. rubrum*.

In this work, we found that the mRNA expressions of DC-SIGN enhanced by the supernatants of both T1a and T_{XHB}, but the time and extent of enhancement were different. This could be because the ligands for DC-SIGN in the supernatant of T1a were different with those in the supernatant of T_{XHB}, which might in turn reflect the various capacities to trigger the innate immune response of keratinocytes.

Besides, IL-8 expression in keratinocytes was slightly and transiently increased only in exposure to the supernatant of T1a but decreased by supernatant of T_{XHB}. However, some other findings found that the culture supernatant of *T. rubrum* can strongly enhance IL-8 secretion of keratinocytes.^[29] As found from previous reports, β -glucan, a ligand for TLR2 and Dectin-1, can promote the production of IL-8 in keratinocyte effectively at an optimal concentration of 10 μ g/ml. Lower or higher amounts of β -glucan (1 or 100 μ g/ml) could not enhance the IL-8 significantly.^[17] In this study, the final content of β -glucan in the HaCaT cultivation medium was <20 pg/ml, far below the optimal level to elevate IL-8. Furthermore, the difference in the composition of the culture supernatant between *T. rubrum* that grows in the complete medium and those in the mineral medium with keratin might also attribute to the variety among study results.

Also, according to the clinical features of dermatophytosis, *T. rubrum* may only cause mild or asymptomatic symptoms in human infections.^[30] These results show the mRNA expression of IL-8 and Dectin-1 was only slightly up-regulated by the standard strain, even the elevated defense genes (Dectin-1, TLR2, TLR4, IL-8, CARD9, and DC-SIGN) were all decreased over 24 h. The mechanisms of the weak

immune response and the subsequent decline of host defense genes expression in response to *T. rubrum* supernatants beyond 24 h are unclear. It is possible that *T. rubrum* might suppress the immune response of keratinocyte with some inhibitory factor. Although whether the fungal wall mannan can also be detected in the culture supernatant of *T. rubrum* as in that of *C. albicans* has not been reported yet, mannan from *T. rubrum* can inhibit IL-2 production and proliferation of keratinocytes.^[31,32] The production of inhibitory factors by *T. rubrum* may induce a limited inflammatory response in the skin, which prolongs the survival of *T. rubrum* in the long term. Thus, further studies are required to analyze the culture supernatant components of *T. rubrum* that account for the limited immune response of keratinocytes.

Financial support and sponsorship

This work was supported by a grant from the Foundation for Young Scientists of Hospital Support Plan (No. A1286).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Vena GA, Chieco P, Posa F, Garofalo A, Bosco A, Cassano N. Epidemiology of dermatophytoses: Retrospective analysis from 2005 to 2010 and comparison with previous data from 1975. *New Microbiol* 2012;35:207-13.
2. Pivarsci A, Kemény L, Dobozy A. Innate immune functions of the keratinocytes. A review. *Acta Microbiol Immunol Hung* 2004;51:303-10.
3. Sanford JA, Gallo RL. Functions of the skin microbiota in health and disease. *Semin Immunol* 2013;25:370-7.
4. de Koning HD, Simon A, Zeeuwen PL, Schalkwijk J. Pattern recognition receptors in infectious skin diseases. *Microbes Infect* 2012;14:881-93.
5. Firat YH, Simanski M, Rademacher F, Schröder L, Brasch J, Harder J. Infection of keratinocytes with *Trichophyton rubrum* induces epidermal growth factor-dependent RNase 7 and human beta-defensin-3 expression. *PLoS One* 2014;9:e93941.
6. Szolnoky G, Bata-Csörgő Z, Kenderessy AS, Kiss M, Pivarsci A, Novák Z, et al. A mannose-binding receptor is expressed on human keratinocytes and mediates killing of *Candida albicans*. *J Invest Dermatol* 2001;117:205-13.
7. Kano R, Hasegawa A, Watanabe S, Sato H, Nakamura Y. *Candida albicans* induced interleukin 8 production by human keratinocytes. *J Dermatol Sci* 2003;31:233-5.
8. Wollina U, Künkel W, Bulling L, Fünfstück C, Knöll B, Vennewald I, et al. *Candida albicans*-induced inflammatory response in human keratinocytes. *Mycoses* 2004;47:193-9.
9. Martinez-Rossi NM, Persinoti GF, Peres NT, Rossi A. Role of pH in the pathogenesis of dermatophytoses. *Mycoses* 2012;55:381-7.
10. Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spruiel AB, Venselaar H, et al. Human dectin-1 deficiency and mucocutaneous fungal infections. *N Engl J Med* 2009;361:1760-7.
11. Lantermier F, Pathan S, Vincent QB, Liu L, Cypowyj S, Prando C, et al. Deep dermatophytosis and inherited CARD9 deficiency. *N Engl J Med* 2013;369:1704-14.
12. Li Y, Chen J, Wan MJ, Lai W, Zheng Y, Li MR, et al. The immune response of human keratinocytes to *Trichophyton rubrum* conidia is partially mediated by toll-like receptor-2, 4, dectin-1 and cytokines (in Chinese). *J South Med Univ* 2011;31:678-81.
13. Serrano-Gómez D, Leal JA, Corbi AL. DC-SIGN mediates the binding of *Aspergillus fumigatus* and keratinophytic fungi by human dendritic cells. *Immunobiology* 2005;210:175-83.
14. Zanger P, Holzer J, Schleucher R, Steffen H, Schitteck B, Gabrysch S. Constitutive expression of the antimicrobial peptide RNase 7 is

- associated with *Staphylococcus aureus* infection of the skin. *J Infect Dis* 2009;200:1907-15.
15. Harada A, Sekido N, Akahoshi T, Wada T, Mukaida N, Matsushima K. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol* 1994;56:559-64.
 16. Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitemaker JH, Kapsenberg ML, *et al.* Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol* 2007;127:331-41.
 17. Kobayashi M, Yoshiki R, Sakabe J, Kabashima K, Nakamura M, Tokura Y. Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes. *Br J Dermatol* 2009;160:297-304.
 18. Chen J, Yi J, Liu L, Yin S, Chen R, Li M, *et al.* Substrate adaptation of *Trichophyton rubrum* secreted endoproteases. *Microb Pathog* 2010;48:57-61.
 19. Huang XQ, Yi JL, Yin SC, Chen RZ, Li MR, Gong ZJ, *et al.* Exposure to heat-inactivated *Trichophyton rubrum* resulting in a limited immune response of human keratinocytes. *Chin Med J* 2013;126:215-9.
 20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods* 2001;25:402-8.
 21. Paraje MG, Correa SG, Albesa I, Sotomayor CE. Lipase of *Candida albicans* induces activation of NADPH oxidase and L-arginine pathways on resting and activated macrophages. *Biochem Biophys Res Commun* 2009;390:263-8.
 22. Paraje MG, Correa SG, Renna MS, Theumer M, Sotomayor CE. *Candida albicans*-secreted lipase induces injury and steatosis in immune and parenchymal cells. *Can J Microbiol* 2008;54:647-59.
 23. Zhao J, Wu XY. *Aspergillus fumigatus* antigens activate immortalized human corneal epithelial cells via toll-like receptors 2 and 4. *Curr Eye Res* 2008;33:447-54.
 24. Smijs TG, Pavel S. The susceptibility of dermatophytes to photodynamic treatment with special focus on *Trichophyton rubrum*. *Photochem Photobiol* 2011;87:2-13.
 25. Li M, Chen Q, Shen Y, Liu W. *Candida albicans* phospholipomannan triggers inflammatory responses of human keratinocytes through Toll-like receptor 2. *Exp Dermatol* 2009;18:603-10.
 26. Koller B, Müller-Wiefel AS, Rupec R, Korting HC, Ruzicka T. Chitin modulates innate immune responses of keratinocytes. *PLoS One* 2011;6:e16594.
 27. Willment JA, Brown GD. C-type lectin receptors in antifungal immunity. *Trends Microbiol* 2008;16:27-32.
 28. Wu-Yuan CD, Hashimoto T. Architecture and chemistry of microconidial walls of *Trichophyton mentagrophytes*. *J Bacteriol* 1977;129:1584-92.
 29. Li HC, Wang BX, Sun QN. Effect of the metabolic substances produced by common dermatophytes on the immune activity of human keratinocytes *in vitro* (in Chinese). *Chin J Dermatovenereology* 2006;8:001.
 30. Mignon B, Tabart J, Baldo A, Mathy A, Losson B, Vermout S. Immunization and dermatophytes. *Curr Opin Infect Dis* 2008;21:134-40.
 31. Watanabe T, Takano M, Murakami M, Tanaka H, Matsuhisa A, Nakao N, *et al.* Characterization of a haemolytic factor from *Candida albicans*. *Microbiology* 1999;145(Pt 3):689-94.
 32. Blake JS, Dahl MV, Herron MJ, Nelson RD. An immunoinhibitory cell wall glycoprotein (mannan) from *Trichophyton rubrum*. *J Invest Dermatol* 1991;96:657-61.