Increased hydrophobicity in *Malassezia* species correlates with increased proinflammatory cytokine expression in human keratinocytes

NARIFUMI AKAZA*,†, HIROHIKO AKAMATSU‡, SHIORI TAKEOKA†, HIROSHI MIZUTANI†, SATORU NAKATA† & KAYKO MATSUNAGA*

*Department of Dermatology, Fujita Health University School of Medicine, Toyoake, Aichi, Japan, †Research Laboratories, Nippon Menard Cosmetic Co., Ltd., Nagoya, Japan, and ‡Department of Applied Cell and Regenerative Medicine, Fujita Health University School of Medicine, Toyoake, Aichi, Japan

*Malassezia* cells stimulate cytokine production by keratinocytes, although this ability differs among *Malassezia* species for unknown reasons. The aim of this study was to clarify the factors determining the ability to induce cytokine production by human keratinocytes in response to *Malassezia* species. *M. furfur* NBRC 0656, *M. sympodialis* CBS 7222, *M. dermatis* JCM 11348, *M. globosa* CBS 7966, *M. restricta* CBS 7877, and three strains each of *M. globosa*, *M. restricta*, *M. dermatis*, *M. sympodialis*, and *M. furfur* maintained under various culture conditions were used. Normal human epidermal keratinocytes (NHEKs) (1 × 10^5 cells) and the *Malassezia* species (1 × 10^6 cells) were co-cultured, and IL-1α, IL-6, and IL-8 mRNA levels were determined. Moreover, the hydrophobicity and β-1,3-glucan expression at the surface of *Malassezia* cells were analyzed. The ability of *Malassezia* cells to trigger the mRNA expression of proinflammatory cytokines in NHEKs differed with the species and conditions and was dependent upon the hydrophobicity of *Malassezia* cells not β-1,3-glucan expression.

**Keywords** *Malassezia*, keratinocyte, mRNA, hydrophobicity, β-1,3-glucan

**Introduction**

The lipophilic yeast genus *Malassezia* is part of the cutaneous microbiota. Its taxonomy was revised in 1996 and included 14 species as of 2010 [1–7]. Nine of these species have been isolated from human skin, with *M. globosa*, *M. restricta*, *M. dermatis*, *M. sympodialis*, and *M. furfur* the most common, while *M. obtusa*, *M. slooffiae*, *M. japonica*, and *M. yamatoensis* are rarely recovered from specimens [8,9]. Members of the genus are also associated with several skin diseases such as *Malassezia* folliculitis, pityriasis versicolor, seborrheic dermatitis, dandruff, and atopic dermatitis [10,11]. *Malassezia* species are considered to act as allergens in patients with atopic dermatitis (AD) [12,13].

Indeed, AD patients have shown positive reactions to *Malassezia* in patch tests [14]. In addition, specific IgE antibodies found in AD patients were not found in healthy individuals [15,16].

The epidermis composed of keratinocytes is a physical barrier to external pathogens. Furthermore, keratinocytes act as initiators of innate immunity by producing interleukin (IL) -1α, IL-β, IL-3, IL-6, IL-8, tumor necrosis factor (TNF) -α, transforming growth factor (TGF) -α, TGF-β, and platelet-derived growth factor (PDGF) [17]. Some agents induce the production of cytokines in keratinocytes and *Malassezia* species may act as controllers of innate immunity. There are several previous reports about the proinflammatory cytokine expression triggered by *Malassezia* species in keratinocytes. Watanabe et al. reported that *M. furfur* did not induce proinflammatory cytokine production in normal human epidermal keratinocytes (NHEKs) [18]. Ishibashi et al. found that *M. globosa* induced IL-6 expression in NHEKs but *M. restricta* did not in cultures with modified LNA as the medium [19]. Walters et al.
reported that *M. furfur* serovar B, namely *M. globosa*, did not trigger IL-1α production but Baroni *et al.* noted that *M. furfur* caused IL-8 production in NHEKs [20,21]. It was also reported that exogenous innate immune defenders could control the inflammatory reaction against *Malassezia* in keratinocytes [22].

There are differences among *Malassezia* species in the proinflammatory cytokine expression triggered in human keratinocytes [18,19]. However, the cause of the differences has not been clarified. Beta-1,3-glucan is a major fungal ligand recognized by dectin-1 [23,24]. Moreover, the hydrophobicity of yeast cells plays an important role in pathogenicity. It was reported that hydrophobic *Candida albicans* cells are more adherent than hydrophilic cells to a variety of host tissues [25]. We considered that the expression of β-1,3-glucan or lipophilic nature of *Malassezia* cells might correlate closely with the cytokine expression in human keratinocytes. The aim of this study was to clarify factors determining the ability of *Malassezia* species to induce cytokine production by human keratinocytes.

Materials and methods

**Yeast cultures**

*M. furfur* NBRC 0656, *M. sympodialis* CBS 7222, *M. dermatis* JCM 11348, *M. globosa* CBS 7966, and *M. restricta* CBS 7877 were maintained on Leeming & Notman agar medium (LNA) [26] or modified Dixon agar medium (mDA) [1] at 32 °C for 2 or 7 days. Fifteen *Malassezia* isolates, identified as described previously, were recovered from patients with *Malassezia* folliculitis (three strains each of *M. globosa*, *M. restricta, M. dermatis*, *M. sympodialis*, and *M. furfur*) and grown on LNA at 32 °C for 2 days [27]. The yeast cells were then harvested in phosphate-buffered saline (PBS; pH 7.0) for examination.

**Keratinocyte cultures**

NHEKs isolated from neonatal foreskin were obtained as cryopreserved primary cultures from Invitrogen (Carlsbad, CA, USA). They were grown in 12-well culture plates using 154S medium (Invitrogen) supplemented with 700 μl of HKGS (Invitrogen) at 37°C in an atmosphere of 5% (vol/vol) CO2 in air. The medium was replaced every 2–3 days, and NHEKs were used when they reached semi-confluence (60–80%; 1 × 10^5 cells/well).

**Co-culture of NHEKs with Malassezia species**

NHEKs (1 × 10^5 cells) and *Malassezia* species (1 × 10^6 cells) were co-cultured at 37°C in 5% CO2 in the NHEKs culture medium for 6 and 24 h. As a negative control, keratinocytes were cultured without *Malassezia* cells. Following the incubation, mRNA was isolated with Trizol reagent (Invitrogen) according to the manufacturer’s instructions.

**Quantitative RT-PCR of proinflammatory cytokines in NHEKs**

We examined the mRNA levels of the proinflammatory cytokines IL-1α, IL-6, and IL-8 in NHEKs. Total RNA concentrations were determined from A260 values. Reverse transcription and amplification were carried out using a SYBR Green Two-step qRT-PCR Kit (Invitrogen) according to the manufacturer’s instructions. qRT-PCR was used to quantify the relative abundance of each mRNA (StepOnePlus Real Time PCR system; Applied Biosystems, Foster City, CA, USA). Data were normalized to the glyceraldehyde-3-phosphate (GAPDH) mRNA level of the respective samples. Specific primers as described previously were used [28]. Prior to the experiment, we confirmed that contamination of the genome did not influence the experimental results by performing qRT-PCR with the nucleic acid solutions before the reverse transcription.

**Comparison of the hydrophobicity of Malassezia cells**

The hydrophobicity of *Malassezia* cells was assessed with a modification of the hydrophobic microsphere assay developed by Hazen and Hazen [29]. The microsphere suspension comprised 6 μl of a 10% suspension of polystyrene microspheres (0.8 μm diameter; Bangs Laboratories, Fishers, IN, USA) and 2 ml of PBS. The yeast cells (100 μl) and microsphere suspension (10 μl) were combined in glass tubes and mixed by pipetting 30 times. The attachment of microspheres was assessed under a microscope. Some 100 cells of *Malassezia* were observed, and the proportion with one or more attached spheres was recorded as percent hydrophobicity.

**Comparison of β-1,3-glucan expression on Malassezia cells**

Test yeasts were dispersed in PBS containing bovine serum albumin (2% BSA-PBS), and incubated for blocking at 4°C for 1 h. Yeast solutions (30 μl) and 10 μg/ml of monoclonal anti-β-1,3-glucan antibody (mouse IgG; Biosupplies, Victoria, Australia) (90 μl) were mixed, and incubated at 4°C for 30 min. As a negative control, monoclonal Mouse IgG (R&D systems) was used. After two washes, 100 μl of Alexa Fluor 488-labeled anti-mouse IgG antibody (1/500, Invitrogen) was added, and the cells were incubated at 4°C for 30 min. After being washed two more times, the yeast cells were fixed with 10% formalin-PBS. The stained cells were analyzed by fluorescence-activated cell sorting (FACS) with a Cell Lab Quanta SC MPL.
system (Beckman Coulter, Brea, CA, USA) and an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Statistics

All experiments were carried out in triplicate, and results are expressed as the mean ± SD. Comparisons between tests were made using an Analysis of variance (ANOVA) and the Dunnett method.

Results

Effects of Malassezia species on mRNA levels of pro-inflammatory cytokines in NHEKs

IL-1α, IL-6 and IL-8 mRNA levels in NHEKs co-cultured for 6 h with \( M. \) furfur \( \text{CBS} \) 7222, \( M. \) sympodialis \( \text{CBS} \) 7222, \( M. \) dermatis \( \text{JCM} \) 11348, \( M. \) globosa \( \text{CBS} \) 7966, and \( M. \) restricta \( \text{CBS} \) 7877 maintained under various culture conditions is shown in Fig. 2A. There were statistically significant differences among the culture conditions for \( M. \) furfur \( \text{CBS} \) 0656, \( M. \) sympodialis \( \text{CBS} \) 7222, and \( M. \) dermatis \( \text{JCM} \) 11348.

The hydrophobicity of the hydrophilic microsphere assay developed by Hazen and Hazen [25] of \( M. \) furfur \( \text{CBS} \) 0656, \( M. \) sympodialis \( \text{CBS} \) 7222, \( M. \) dermatis \( \text{JCM} \) 11348, and \( M. \) restricta maintained for 2 days with LNA is shown in Fig. 2B. There was a statistically significant difference among Malassezia species.

Comparison of \( \beta \)-1,3-glucan expression on Malassezia cells

Beta-1,3-glucan expression on the surface of \( M. \) furfur \( \text{CBS} \) 0656, \( M. \) sympodialis \( \text{CBS} \) 7222, \( M. \) dermatis \( \text{JCM} \) 11348, \( M. \) globosa \( \text{CBS} \) 7966, and \( M. \) restricta \( \text{CBS} \) 7877 cells maintained for 2 days with LNA is shown in Fig. 3A. There was no difference among the cells cultured for 2 and 7 days with LNA and mDA for \( M. \) furfur \( \text{CBS} \) 0656, \( M. \) sympodialis \( \text{CBS} \) 7222, \( M. \) dermatis \( \text{JCM} \) 11348, \( M. \) globosa \( \text{CBS} \) 7966, and \( M. \) restricta \( \text{CBS} \) 7877 (data not shown).

The results of a FACS analysis of \( \beta \)-1,3-glucan expression on each of three strains of clinically isolated \( M. \) furfur, \( M. \) sympodialis, \( M. \) dermatis, \( M. \) globosa, and \( M. \) restricta maintained for 2 days with LNA were summarized in Fig. 3B. There was a statistically significant difference among Malassezia species.

Discussion

Watanabe et al. previously concluded that there are differences among Malassezia yeasts in their ability to evoke cytokine production by human keratinocytes. Our results support this finding at the mRNA level, but in addition we propose that the culture conditions of Malassezia strains affect the ability to induce cytokine production. We consider that the innate in vivo immunity response of human keratinocytes might also differ with the species and conditions of cutaneous Malassezia.

Hydrophobicity plays an important role in the pathogenicity of micro-organisms. Hydrophobic Candida albicans cells are more adherent than hydrophilic cells to a variety of host tissues [25]. A number of assays of hydrophobicity have been reported [29–31]. FACS methods [30,31] are not suitable for the examination of Malassezia, because some strains form clumps and do not disperse as single cells. We considered the hydrophobic microsphere assay developed by Hazen and Hazen [29] suitable for this study. However, it could not distinguish among Malassezia strains because the adhesion rate of spheres to Malassezia cells was too
Fig. 1  Proinflammatory cytokine mRNA expression in NHEKs co-cultured with Malassezia spp. IL-1α, IL-6, and IL-8 mRNA levels in NHEKs co-cultured with Malassezia species were determined by qRT-PCR. The levels were normalized to GAPDH, and are presented as fold increases over the control (untreated cells) values. Data are the mean ± SD. Comparisons between tests were made using the Dunnett method. Asterisks indicate statistically significant differences (* P < 0.05, ** P < 0.001) versus the control. (A) Proinflammatory cytokine mRNA levels in NHEKs co-cultured for 6 h with M. furfur NBRC 0656, M. sympodialis CBS 7222, M. dermatis JCM 11348, M. globosa CBS 7966, and M. restricta CBS 7877 maintained for 2 or 7 days using LNA or mDA. (B) Proinflammatory cytokine mRNA levels in NHEKs co-cultured for 6 and 24 h with each of three strains of clinically isolated M. furfur, M. sympodialis, M. dermatis, M. globosa, and M. restricta maintained for 2 days using LNA.

high. Therefore, we evaluated hydrophobicity using a revised method, in which the number of spheres was reduced. The results clarified that the hydrophobicity of Malassezia cells differs according to the species and culture conditions. Furthermore, the differences in hydrophobicity related to proinflammatory cytokine mRNA expression in NHEKs exposed to Malassezia. These results indicate that hydrophobicity may contribute to the
proinflammatory cytokine expression induced by *Malassezia* in keratinocytes. Moreover, the hydrophobicity of each *Malassezia* species differed according to the culture conditions including the medium. We consider elucidation of the relation between hydrophobicity and culture conditions as an interesting future theme to study for controlling the proinflammatory cytokine expression in keratinocytes against *Malassezia* species.

Beta-1,3-glucan is a major fungal ligand recognized by dectin-1, a glycoprotein C-type lectin receptor [23,24]. Dectin-1 is expressed in not only phagocytes and neutrophils but also keratinocytes [32,33]. It was also reported that β-1,3-glucan from *Saccharomyces cerevisiae* induced IL-1α, IL-6, and IL-8 expression in NHEKs [34]. In this study, we compared β-1,3-glucan expression on *Malassezia* cells but found that the expression did not differ with the culture conditions, and did not relate to proinflammatory cytokine mRNA expression in NHEKs exposed to *Malassezia* cells. In the proinflammatory cytokine expression in keratinocytes against *Malassezia* cells, β-1,3-glucan of *Malassezia* may not be a significant factor. There is also a report that some yeasts such as *Candida albicans* did not require the dectin-1, β-1,3-glucan receptor for host defense [35]. We consider the most important factor contributing to the proinflammatory cytokine expression in keratinocytes to be the hydrophobicity of *Malassezia* cells, namely the ability to adhere to keratinocytes, not β-1,3-glucan expression.

Moreover, the difference in β-1,3-glucan expression on cells in response to *Malassezia* species was inversely proportional to the proinflammatory cytokine mRNA levels in NHEKs. This result may indicate that ligands other than β-1,3-glucan on the cell surface participate in the proinflammatory cytokine expression induced in response to *Malassezia* in keratinocytes. The absence of immunolabelling may suggest that β-1,3-glucan is hidden by another compound. It was reported that the *Malassezia* cell wall contains polysaccharides, β-1,6-linked linear galactofuranosyl polymers with a small amount of mannan, on the outside of the β-1,3-glucan and chitin layer [31]. Conversely, intelectin was identified as a human galactose-binding lectin from heart, small intestine, colon, and thymus [36]. Moreover, mincle, a C-type lectin, on activated macrophages that specifically react with *Malassezia* cells has also been reported [37]. Mincle recognizes the specific geometry of α-mannosyl residues in the genus *Malassezia*. The galactofuranosyl and α-mannosyl residues expressed on *Malassezia* cells may be important to the response of keratinocytes to *Malassezia* cells, if intelectin and mincle are expressed in human keratinocytes.

In the present study, *M. sympodialis*, *M. dermatis*, and *M. restricta* did not induce proinflammatory cytokine expression. However, this does not mean that these species do not have proinflammatory cytokine expression-inducing capabilities. We set the ratio of NHEKs to *Malassezia* cells at 1:10, but proinflammatory cytokine expression in NHEKs was found for all *Malassezia* species when this ratio was changed to 1:30 (data not shown). In the study of proinflammatory cytokine expression in *vitro*, the ratio of keratinocytes to *Malassezia* cells in co-cultures is an important factor to consider.

*Malassezia* species induce some inflammation in skin diseases such as *Malassezia* folliculitis and pityriasis versicolor [10,11]. It is considered that this inflammatory response may also be the result of the ability of *Malassezia* lipase to hydrolyze triglycerides into free fatty acids other than the innate immunity-inducing ability of *Malassezia* species. *Malassezia* lipase is associated with insoluble fractions, and its optimal pH was 5.0 [38]. We reported that the lipase activities of each cutaneous *Malassezia* species were higher than that of *Propionibacterium acnes*, and
Cytokine expression induced by Malassezia in keratinocytes

Fig. 2  Hydrophobicity of Malassezia spp. The hydrophobicity of various Malassezia species was determined with a modified hydrophobic microsphere assay. Data are the mean ± SD of percent hydrophobicity values. Comparisons between tests were made using ANOVA. (A) Hydrophobicity of Malassezia furfur NBRC 0656, M. sympodialis CBS 7222, M. dermatis JCM 11348, M. globosa CBS 7966, and M. restricta CBS 7877 maintained for 2 or 7 days using LNA or mDA. There were statistically significant differences among the culture conditions for M. furfur NBRC 0656, M. sympodialis CBS 7222, and M. dermatis JCM 11348 (**P < 0.001). (B) Hydrophobicity of each of three strains of clinically isolated Malassezia furfur, M. sympodialis, M. dermatis, M. globosa, and M. restricta maintained for 2 days using LNA. There was a statistically significant difference among Malassezia species (P < 0.001).

differed according to the species of Malassezia [39]. For example, the lipase activity of M. globosa was the highest among five cutaneous Malassezia species which is different from the result of this study regarding proinflammatory cytokine expression in human keratinocytes. Moreover, temperature, the fatty acids included in sebum, and sweat had differing influences on the growth of Malassezia species [39]. Malassezia is a common cutaneous microorganism. We consider that each species of cutaneous Malassezia induces skin inflammation in different ways, such as via lipase activity or the innate immune response, when the environment on human skin is changed.

This study clarified that the mRNA expression of proinflammatory cytokines in NHEKs in response to Malassezia cells differs with the species and environmental conditions. The greatest factor contributing to the proinflammatory cytokine expression in human keratinocytes may be the hydrophobicity of Malassezia cells, namely the ability to adhere to keratinocytes, not β-1,3-glucan expression. However, the possibility that
β-1,3-glucan is an important ligand for the proinflammatory cytokine expression in keratinocytes can not be entirely excluded based on results obtained from one experimental model. Moreover, the hydrophobicity of Malassezia cells was analyzed by a microsphere assay in this study, although a comparative analysis of cellular
zeta potential was also considered. More studies are necessary to elucidate the mechanism of proinflammatory cytokine expression in response to *Malassezia* species in keratinocytes.

**Declaration of interest:** The authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**References**


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