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Inflammasome-mediated Inflammation by *Malassezia* in human keratinocytes: A comparative analysis with different strains

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Abstract

Malassezia species are associated with several common dermatologic conditions including pityriasis versicolor, seborrheic dermatitis, folliculitis, and atopic dermatitis and dandruff. However, its causal role remains to be established. We intended to explore the role of inflammasome activation in human keratinocytes in response to three different *Malassezia* species. We compared the different activation patterns of inflammasomes and the expression of pro-inflammatory cytokines and antimicrobial peptides by three different *Malassezia* species—*M. restricta*, *M. globosa* and *M. sympodialis*—in human keratinocytes. We found that different *Malassezia* species, especially *M. restricta* and *M. globosa* could induce nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin-domain-containing protein (NLRP3)-apoptosis-associated speck-like protein containing CARD (ASC) inflammasome activation and subsequent interleukin (IL)-1 β secretion in human keratinocytes. *Malassezia* species variably induced thymic stromal lymphopoietin, β -defensin 2, and LL-37. IL-8 mRNA and IL-22 protein significantly increased in the *M. sympodialis*-treated group, and Chemokine C-C motif ligand (CCL)17 and CCL22 mRNA were increased in response to *M. globosa*- and *M. restricta*-treated keratinocytes, respectively. Our data show that various species of *Malassezia* promote variable inflammatory responses in keratinocytes by activating NLRP3 inflammasomes, pro-inflammatory cytokines and chemokines, and antimicrobial peptides.

KEYWORDS

inflammasome, keratinocytes, *Malassezia*

1 | INTRODUCTION

Malassezia is a family of lipophilic yeasts and comprises several different species including *Malassezia restricta* (*M. restricta*), *Malassezia globosa* (*M. globosa*) and *Malassezia sympodialis* (*M. sympodialis*).^{1,2} These fungi are components of normal flora that may possibly be found on healthy human skin.^{3,4} However, these organisms are also associated with several common dermatologic diseases such as seborrheic dermatitis, folliculitis, pityriasis versicolor and atopic dermatitis (AD).^{1,5} In particular, the *Malassezia* species have been

suggested as causative organisms that aggravate head and neck dermatitis in AD.^{2,6–8} It is not well known how *Malassezia*, which usually coexist as a normal flora, exacerbates skin problems.

Malassezia is recognised by the pattern recognition receptors on the host dendritic cells or macrophages through interaction with specific fungal cell wall components.⁹ Inflammasomes are representative pattern recognition receptors and comprise Nod-like receptor (NLR) as a sensor protein, apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain (CARD) (ASC) as an adaptor protein, and caspase-1 as an effector protease protein and are

involved in the innate immune response for several pathogens.^{10–13} The NLR family includes nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin-domain-containing protein (NLRP1 and NLRP3, NLR family CARD domain-containing protein 4, and the cytosolic receptor absent in melanoma (AIM)2, which are key components of inflammasomes and recognise exogenous and endogenous danger signals.¹⁴ An influx of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) leads to assembly of inflammasomes and activation of caspase-1.^{10,11,13,15–19} Then, the caspase-1 cleaves and activates the proforms of inflammatory cytokines including pro-interleukin (IL)-1 β and pro-IL-18.^{20–22} Release of these cytokines induces an inflammatory response which subsequently leads to pathogen removal and pyroptosis.¹⁶

At first, it was thought that the activity of inflammasome existed only in immune cells, but keratinocytes were also reported to have stress-induced inflammasome activity. Recent studies showed *Dermatophagoides pteronyssinus* or ultraviolet B can induce activation of inflammasomes and result in the secretion of IL-1 β and IL-18 in human keratinocytes.^{16,23,24} NLRP3 inflammasome activation was also seen in the epidermis and dermis of patients with *Malassezia* folliculitis.²⁵

We intended to explore the role of inflammasome activation in human keratinocytes in response to three different *Malassezia* species. The expression of pro-inflammatory cytokines and chemokines, and antimicrobial peptides (AMPs) by the *Malassezia* species in human keratinocytes was also examined.

2 | MATERIALS AND METHODS

2.1 | *Malassezia* species culture condition

Three strains of *Malassezia* species (*M. restricta*, *M. globosa* and *M. sympodialis*) were provided by Konkuk University Medical Center and grown for 3–5 days at 34°C under 5% CO₂ conditions

in modified Leeming and Notman agar composed of (per litre) 5 g glucose (Sigma-Aldrich, St. Louis, MO, USA), 10 g poly peptone (BD Bioscience, NJ, USA), 8 g bile salts (Oxoid, Hampshire, UK), 0.1 g yeast extract (Sigma-Aldrich), 0.5 g glycerol monostearate (Chemcurz, TX, USA), 12 g agar (Sigma-Aldrich), 2 g glycerol 50% (Sigma-Aldrich), 5 ml Tween 60 (Sigma-Aldrich), 5 g whole-fat cow milk and 170 μ g/ml chloramphenicol.²⁶

2.2 | HaCat cell culture and treatment

The human keratinocyte cell line (HaCat cells) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's minimal essential medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin at 37°C in a 5% CO₂ incubator. HaCat keratinocytes (1 $\times$ 10⁵ cells/well) were seeded into 6-well plates and cultured until they reached 80% confluency. Then, HaCat cells were exposed to *M. globosa* or *M. restricta* or *M. sympodialis* at a yeast cell/keratinocyte ratio of 20–30:1 for 48 h.²⁶ After cocultivation with HaCat keratinocytes, inflammasome-related molecule, pro-inflammatory cytokine and AMPs production were measured. Keratinocytes treated with polycytidylic acid (poly I:C) (Sigma-Aldrich) were used as a positive control as activator of NLRP3 inflammasome.

2.3 | Real-time PCR

Total RNA was isolated from the HaCat cells after 48 h using Trizol reagent (Invitrogen, Carlsbad, CA, US), and cDNA was synthesised using a cDNA synthesis kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The cDNA was used for real-time PCR (RT-PCR) carried out with SYBR Green (Takara, Shiga, Japan). The primer sequences are listed in Table 1. The RT-PCR experiment was repeated three times.

TABLE 1 List of primers and PCR conditions

Species	Primer name	Forward	Reverse
Human	Caspase-1	CACACAAGAAGGGAGGAGAG	TGTACCTTCACCCATGGAAC
	TGF- β 1	GCAGAAGGATCACCACAACC	TGTCTGCACTGCGGAGGTAT
	IL-1 β	GCCAATCTTCATTGCTCAAGT	ACTTCATCTGTTTAGGGCCA
	IL-6	TGTGTGAAAGCAGCAAAGAG	GCATCCATCTTTTTACGCCA
	IL-8	CTGTCTGGACCCCAAGGAAA	GAATCCATCCCCCTGTTTTCA
	IL-10	AGCCATGAGTGAGTTTGACA	CCACCTGATGTCTCAGTTT
	IL-18	AGCTGAAGATGATGAAAACCTG	ATAGAGGCCGATTTCCTTGG
	CXCL10	TGTCCACGTGTTGAGATCAT	ATGGCCTTCGATTCTGGATT
	CCL17	TTCAAAACCAGGGTGTCTCC	GGAATGGCTCCCTGAAGTA
	CCL20	ACTTTGACTGCTGTCTTGGA	TAGCATTGATGTCACAGCCT
	CCL22	AACTAATGTCCCTCCCCTCT	GGCCCATTTCTAGTGACCTT
	CCL27	CCCTACAGCAGCATTCCTAC	GGATGACCTTCCTCAGTAGC
	GAPDH	GAAGGTGAAGTCTGGAGTCAA	GCTCCTGGAAGATGGTGATG

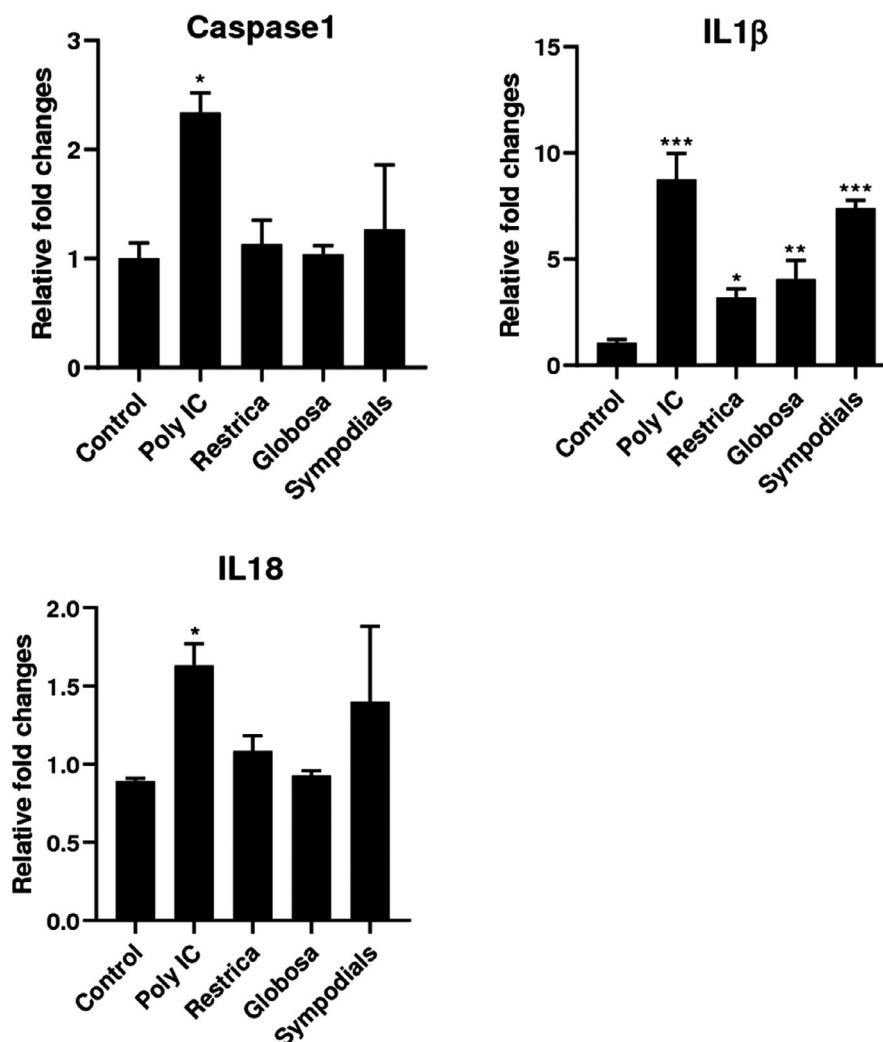


FIGURE 1 The effects of *Malassezia* on mRNA expression of *caspase-1*, *IL-1β* and *IL-18* and after exposure to *Malassezia*. (* $p < .05$ compared with the control; ** $p < .01$ compared with the control; *** $p < .001$ compared with the control)

2.4 | Western blot analysis

Protein lysates from HaCat cells were harvested and lysed using Radio-Immune Precipitation Assay (RIPA) cell lysis buffer (Pierce, Rockford, IL, USA). The concentration of protein was measured using Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. Cell lysates containing equal amounts of total protein were separated by electrophoresis on 10% SDS-polyacrylamide gels and then transferred to poly-1,1-difluoroethene membranes. The membranes were blocked with 5% bovine serum albumin in Tris Buffered Saline with Tween 20 (TBS-T) and subsequently incubated with primary antibodies against IL-4 (Invitrogen, Carlsbad, CA, US), IL-13 (Abcam, CB, UK), IL-17 (Abcam, CB, UK), IL-22 (Abcam, CB, UK), IL-1β (Cell signalling, Beverly, MA, USA), ASC (Genetex, Irvine, CO, USA), NLRP3 (Novus Biologicals, Centennial, CO, USA), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Genetex, Irvine, CO, USA), thymic stromal lymphopoietin (TSLP) (Invitrogen, Carlsbad, CA, US), β-defensin2 (Santa Cruz, CA, USA), LL-37 (Santa Cruz, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, CA, USA). The blotted membrane

was visualised with an AI600 Bio-Image analysis system (GE Healthcare Life Science, NJ, USA). The experiment was repeated three times.

2.5 | Statistical analysis

All statistical data analyses were carried out using Graphpad Prism 5 software. One-way ANOVA was used to evaluate significant differences with a p -value of $< .05$ considered to be statistically significant.

3 | RESULTS

3.1 | *IL-1β* mRNA levels are increased in *Malassezia*-stimulated human keratinocytes

A significant increase in the mRNA expression of *IL-1β* was observed in all three *Malassezia* (especially *M. restricta*) treated human keratinocytes, especially in the *M. sympodialis*-treated group (Figure 1).

Meanwhile, the changes of mRNA levels of *caspase-1* and *IL-18* were not statistically significant.

3.2 | Inflammasomes are activated in *Malassezia*-stimulated human keratinocytes

The increase in phospho-IL-1 β and IL-1 β protein levels was not statistically significant, but was detected in all three kinds of *Malassezia* (especially *M. restricta*) treated human keratinocytes. Whether to identify the mechanism of the increased IL-1 β level, the activation of NLRP3 inflammasome components was examined (Figure 2). The protein levels of NLRP3 and ASC showed a tendency to increase after exposure to *Malassezia*, although there was a little difference in activation patterns in NLRP3 inflammasome complex between different species of *Malassezia*-treated groups. NLRP3 increased statistically significantly in the *M. globosa*-treated group and ASC increased statistically in the *M. restricta*- and *M. globosa*-treated groups. Simultaneously, the intermediate form of caspase-1 (35kda) was detected in all three kinds of *Malassezia*-treatment groups, but the differences were not significant compared with control (Figure 2). Expression of mature caspase-1 was not detected in any groups.

On the while, NF- κ B was examined that could be associated with an increase of IL-1 β without passing through the inflammasome. The protein levels of NF- κ B did not significantly increase after exposure to the *Malassezia* species (Figure 2), suggesting that the increased in IL-1 β by *M. restricta* and *globosa* is mainly due to activation of NLRP3 inflammasomes, not of NF- κ B.

3.3 | AMPs and TSLP are increased in *Malassezia*-stimulated human keratinocytes

β -defensin 2 tended to increase on exposure to *Malassezia* although these changes were not significant. The protein levels of

TSLP inducing T helper (Th) 2 cell differentiation and AD showed a tendency to increase after exposure to *Malassezia* in the *M. restricta*-treated group and the *M. sympodialis*-treated group, but not significant. LL-37, which is a cathelicidin-related AMPs, increased on exposure to three *Malassezia* species, but these changes were not significant (Figure 3).

3.4 | Changes in Th2, Th17 and Th22 cytokines in *Malassezia*-stimulated human keratinocytes

To check changes in Th1 cell-, Th2 cell- and Th17 cell-related immune-mediated cytokines, the protein levels of IL-4, IL-13, IL-17 and IL-22 were detected after exposure to *Malassezia*. IL-4 increased slightly in the *Malassezia*-treated group, but there was no statistical significance. IL-13 did not increase in the *Malassezia*-treated group and instead decreased in the *M. restricta*-treated group compared with the control group. IL-17 also showed no significant change after exposure to *Malassezia*. On the other hand, IL-22 showed a tendency to increase in the *Malassezia*-treated group and increased statistically significantly in the *M. sympodialis*-treated group (Figure 4).

3.5 | Changes in Th1, Th17, and Th22 cytokine and chemokine mRNA after *Malassezia* exposure in human keratinocytes

The increase in *IL-6* mRNA in the *M. globosa*-treated group and *IL-8* mRNA in the *M. sympodialis*-treated group was statistically significant. For immunosuppressive cytokines, *IL-10* mRNA did not show much change in all *Malassezia*-treated groups, but *transforming growth factor (TGF- β 1)* mRNA increased significantly in *M. sympodialis*-treated group.

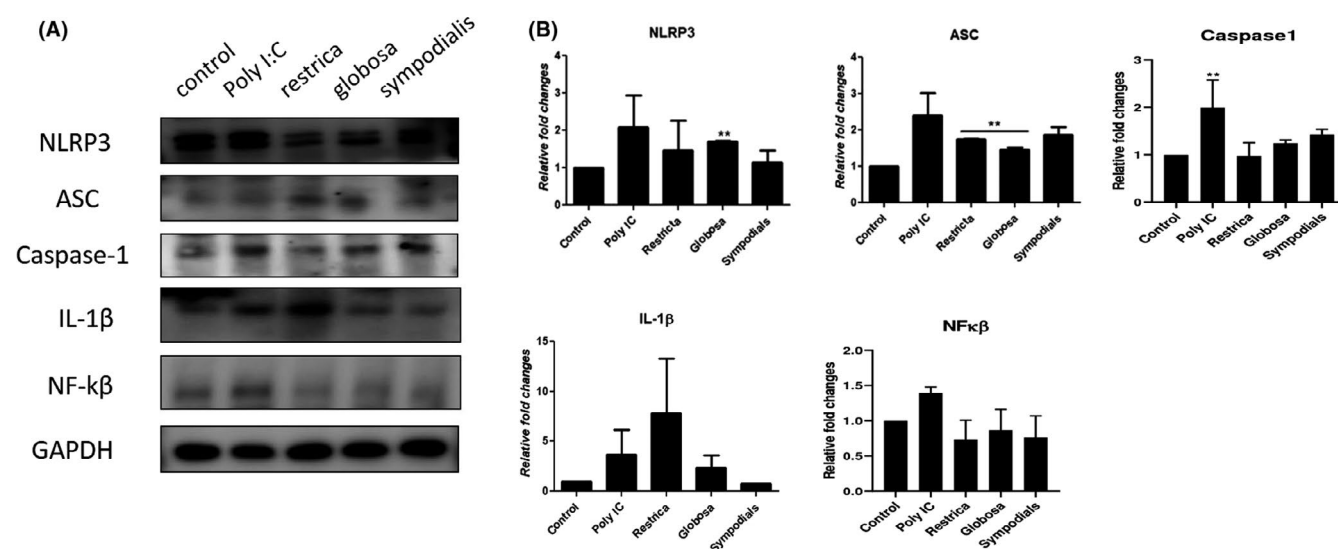


FIGURE 2 Changes in inflammasome-related molecules after exposure to *Malassezia*. (**p < .01 compared with the control)

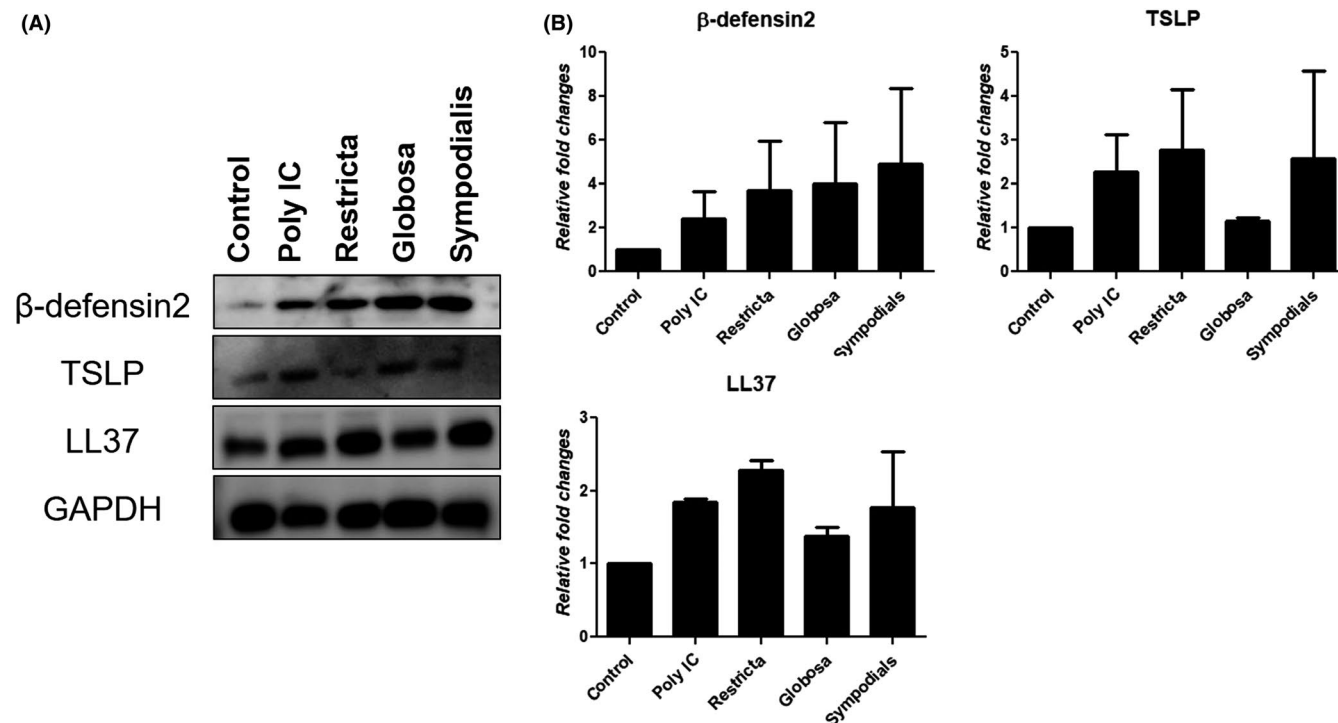


FIGURE 3 The effects of *Malassezia* on AMPs and TSLP were detected by (A) Western blot analysis, and the relative fold change graphs are shown in (B)

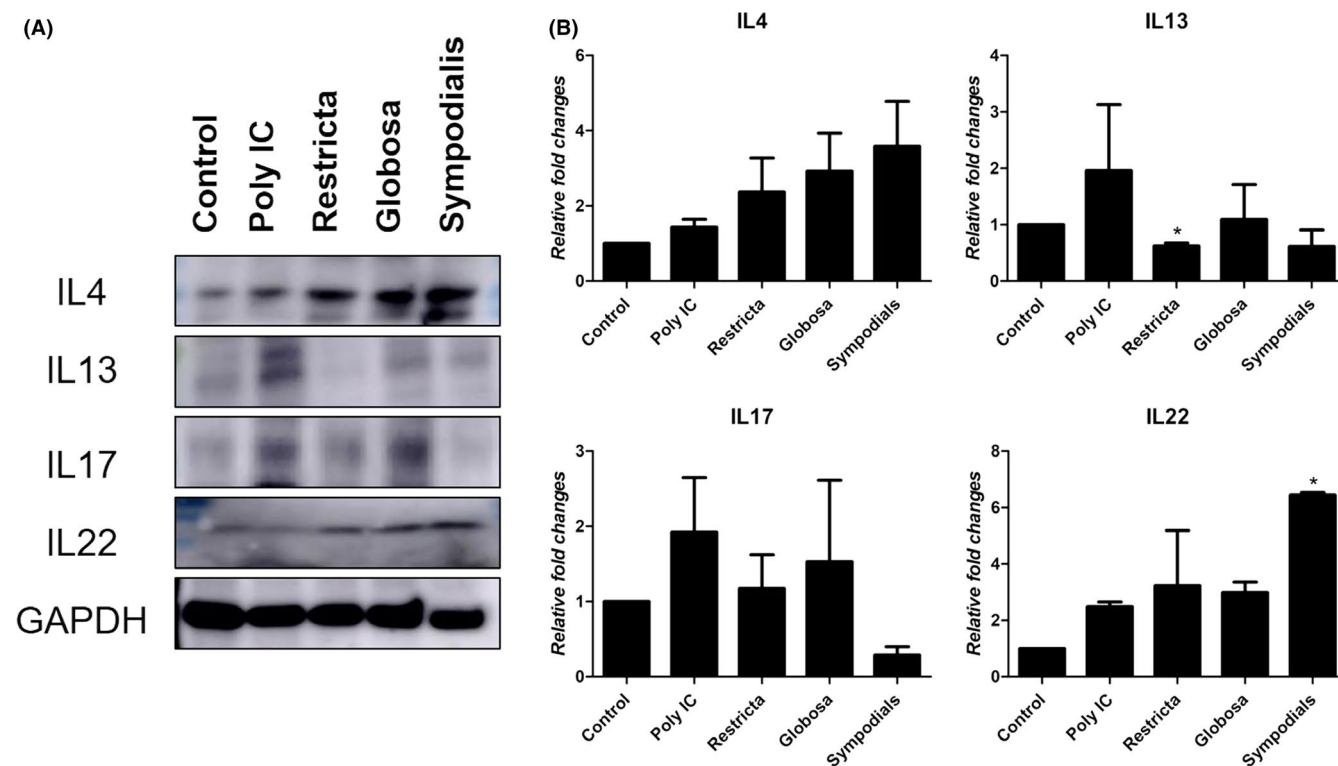


FIGURE 4 Changes in T helper (Th) cell-related cytokine levels after exposure to *Malassezia* were detected by (A) Western blot analysis, and the relative fold change graphs are shown in (B). (* $p < .05$ compared with the control.)

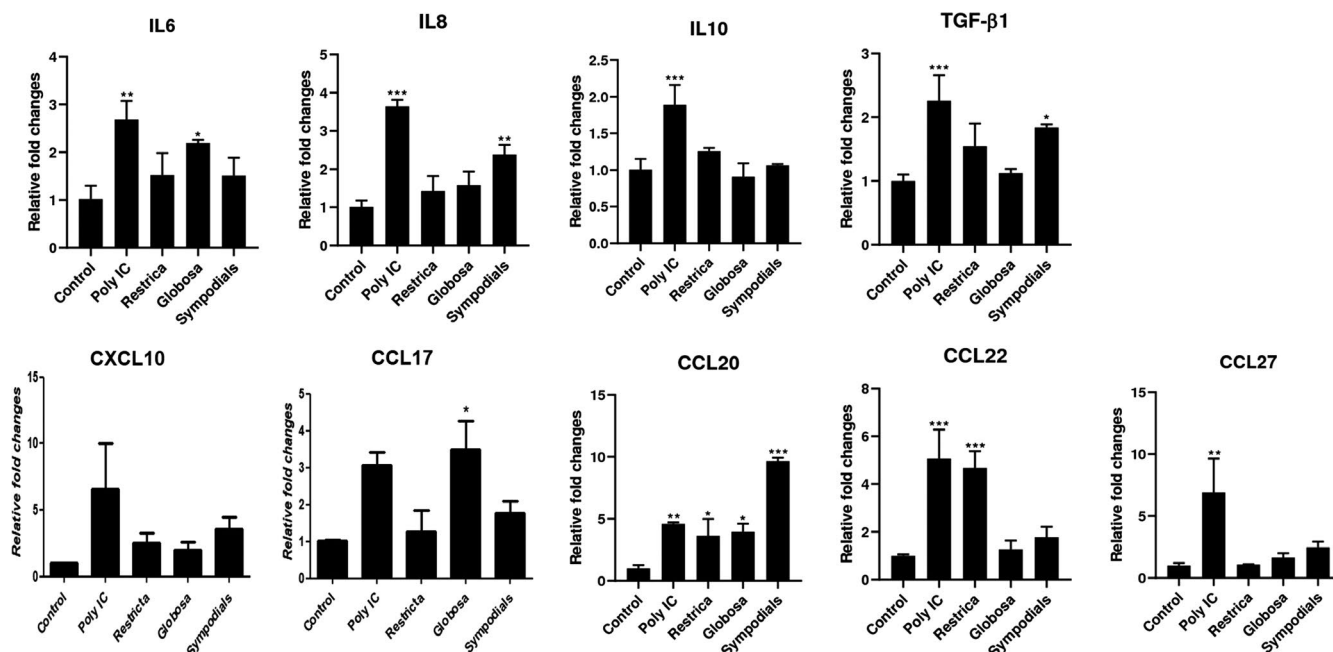


FIGURE 5 The mRNA expression changes of Th1, Th17, Th22 and immunoregulatory cytokine and chemokine levels after exposure to *Malassezia*. (* $p < .05$ compared with the control; ** $p < .01$ compared with the control; *** $p < .001$ compared with the control)

Chemokine C-C motif ligand (CCL 17) showed a significant increase in mRNA expression after exposure to *M. globosa*. CCL 20 mRNA was increased by all *Malassezia*-stimulated groups and CCL22 mRNA was increased by *M. restricta*. These findings suggest that *Malassezia* may be involved in the recruitment of Th22 cells and induction of chronic inflammation (Figure 5). Meanwhile, the mRNA level of chemokine C-X-C motif ligand (CXCL)10, and CCL 27 did not increase significantly in the *Malassezia*-stimulated groups.

4 | DISCUSSION

We investigated the interaction between different *Malassezia* species and human keratinocytes through NLRP3 inflammasome, AMPs and cytokines. Overall, different *Malassezia* species variably stimulated NLRP3-ASC-caspase1- IL-1 β axis. *M. globosa* and *M. restricta*, known as the most virulent species significantly upregulated NLRP3 and ASC expression and tended to increase in caspase1 and IL-1 β in HaCat cells. Meanwhile, the activation of NLRP3 inflammasome component was not significantly increased by *M. sympodialis*. This may result from the differences between species in the structures of the *Malassezia* cell wall component.^{27,28} IL-1 β secretion can be induced not only by canonical NLRP3 inflammasome activation pathway, but also by NF- κ B pathway independent of inflammasome activation.²⁵ Since the levels of NF- κ B were not significantly changed in response to *Malassezia* species, *Malassezia*-induced IL-1 β production in this study appears to result from the activation of canonical NLRP3 inflammasome pathway.

We treated *Malassezia* species in co-culture methods instead of using *Malassezia* extract or antigens in order to create an

environment similar to the physiological conditions of *Malassezia* in vivo skin. Interestingly, we demonstrated that yeast-type *Malassezia*, known for its low pathogenicity, variably activates NLRP3 inflammasomes in vitro skin keratinocytes. The responsiveness of inflammasome to invasive hyphae is assumed to be stronger than that by colonising yeast.

The increase in immunosuppressive cytokines such as TGF- β mRNA after exposure to *Malassezia* species may be a mechanism by which *Malassezia* generally do not induce an inflammatory response to the skin.²⁸ Recent data demonstrated that synthetic cationic AMPs inhibit the NLRP3 inflammasomes and subsequent inflammatory responses in the lung and liver.²⁹ AMPs in the skin may play a role in protecting the skin from inflammatory reactions induced by *Malassezia*-induced inflammasome activation and maintaining skin homeostasis under healthy state. The inhibitory effects of AMPs on NLRP3 inflammasomes may not be sufficient for AD lesional skin with low AMPs and high Th2 cytokine environments. Previous studies have revealed that human keratinocytes released β -defensin 2 by different *Malassezia* species in a toll-like receptor 2-dependent manner,^{30–32} but the increase of β -defensin 2 and LL-37 in this study was not significant. The interaction between AMPs and NLRP3 should be further studied.

It is not much known how NLRP3 inflammasomes affect adaptive immune systems. Secreted IL-1 β , caspase-1, and IL-18 via canonical NLRP3 inflammasome pathway directly and indirectly influence immune cells such as T cells, eosinophils, mast cells, and macrophages and could worsen local inflammation.^{33,34} Activation of inflammasomes by fungal infection can affect the response of Th1 or Th17 cells by recruiting neutrophils and can act a host protective role.³⁵ Emerging evidences support that inflammasomes also promote Th2 responses³³ and exacerbate the inflammation of asthma.³⁶

M. globosa and *M. restricta* are the most commonly isolated *Malassezia* species from trunk and scalp among Chinese and Korean patients with seborrheic dermatitis.³⁷ *M. globosa* and *M. restricta* has been found to be involved in the pathogenesis of dandruff and seborrheic dermatitis by secreting IL-8 in other studies.^{37,38} *M. restricta* extract is known to induce CXCL10 and the signal transducer and activator of transcription 1 expression, thus enhancing Th1-type inflammation.³⁹ *M. globosa* and *M. restricta* have also been found to exacerbate AD by inducing a Th2 cytokines and TSLP in some studies.^{1,40} In this study, the CCL17 and CCL22 mRNA, which are Th2 chemokines, were significantly increased in *M. globosa*- and *M. restricta*-treated keratinocytes, respectively. However, we did not observe a significant increase in Th2 cytokines, TSLP or CXCL10 in response to any *Malassezia* species in this study. This discrepancy between studies may be related to the different *Malassezia* strains or to other characteristics such as yeast adaptation and growth conditions.³⁰

M. sympodialis is known as the main causative organism in head and neck dermatitis in AD. Human keratinocytes exposed to *M. sympodialis* release IL-1 β , IL-6, IL-8 and tumour necrosis factor- α ,⁴¹ however, whether the source of IL-1 β is through activation of NLRP3 inflammasome in skin keratinocytes has not been studied. The level of IL-1 β mRNA was the greatest in *M. sympodialis* compared with other species in this study, but protein levels of IL-1 β and activation of NLRP3 inflammasome and was not prominent. Post-transcriptional regulation of IL-1 β seems to be finely controlled by more complex factors. We found that IL-8 mRNA and IL-22 protein were significantly increased after *M. sympodialis* exposure. These Th1 and Th2 cytokines are known to cause chronic inflammation in various skin diseases.

5 | CONCLUSION

This is the first study that revealed *Malassezia* yeasts stimulate NLRP3 inflammasome activation in human keratinocytes. Differences in fungal cell wall components and metabolites between species may cause differences in immune response. NLRP3 inflammasome could be a possible target for the treatment of *Malassezia*-related skin diseases. Further studies are needed to identify on how *Malassezia*-induced NLRP3 inflammasome activation affects innate and adaptive inflammatory responses.

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CONFLICT OF INTEREST

In relation to this article, I declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

Hye Ree Park: Formal analysis (equal); Writing-original draft (equal). **Jee Hye Oh:** Investigation (lead); Methodology (supporting); Visualization (lead). **Yu Jin Lee:** Formal analysis (equal); Investigation (equal). **Song Hee Park:** Data curation (equal); Investigation (equal). **Yang Won Lee:** Conceptualization (supporting); Methodology (lead); Resources (lead). **Seongju Lee:** Conceptualization (supporting); Project administration (equal); Supervision (supporting). **Hoon Kang:** Resources (equal); Software (equal); Supervision (equal). **Jung Eun Kim:** Conceptualization (lead); Formal analysis (equal); Funding acquisition (lead); Project administration (lead); Writing-original draft (equal); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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