

Cytokine secretion profile of human keratinocytes exposed to *Malassezia* yeasts

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Abstract

The lipophilic yeast *Malassezia* is an exacerbating factor in atopic dermatitis (AD). Among organisms of the *Malassezia* species, *Malassezia globosa* and *Malassezia restricta* are particularly dominant on the skin of AD patients. However, the precise role of *Malassezia* yeasts in the pathophysiology of AD remains uncertain. Keratinocytes play a critical role in cutaneous inflammatory and immune responses by secreting cytokines. In this study, we attempted to determine the cytokine secretion profiles of human keratinocytes that were exposed to *Malassezia* yeasts. The human keratinocyte cell line PHK16-0b was cocultivated with *M. globosa* or *M. restricta* for 24 h, and the resulting cytokine secretion profile was analysed using a cytokine antibody array. The keratinocytes responded to the two *Malassezia* species with different Th2-type cytokine profiles, i.e. *M. globosa* induced IL-5, IL-10 and IL-13 secretion from the keratinocytes, whereas *M. restricta* induced IL-4 secretion. Similar results were obtained with primary normal human epidermal keratinocytes. cDNA microarray analysis confirmed that IL-5, IL-10, and IL-13 mRNAs were induced only by *M. globosa*, while IL-4 mRNA expression was induced only by *M. restricta*. These findings suggest that *M. globosa* and *M. restricta* play a synergistic role in triggering or exacerbating AD by stimulating the Th2 immune response.

Introduction

The genus *Malassezia* constitutes a family of lipophilic yeasts comprising 11 different species including *Malassezia globosa* and *Malassezia restricta* (Sugita *et al.*, 2005). These organisms are members of the normal human cutaneous microbial communities, but they are also associated with several skin diseases such as pityriasis versicolor, seborrhoeic dermatitis, *Malassezia* folliculitis and atopic dermatitis (AD) (Ashbee & Evans, 2002; Faergemann, 2002). Recently, using a nonculture-based method (nested PCR), we demonstrated that *M. globosa* and *M. restricta* were detected in c. 90% AD patients, while other *Malassezia* species were detected in < 40% AD patients, suggesting that *M. globosa* and *M. restricta* may play a particularly important role in the pathogenesis of AD (Sugita *et al.*, 2001). However, the precise mechanism by which these two species trigger or exacerbate AD remains unclear.

AD is a pruritic inflammatory skin disease that is characterized by elevated plasma levels of IgE against many types of allergens as well as the infiltration of mast cells and

eosinophils (Wuthrich, 1978; Leung *et al.*, 2004). The development and pathogenesis of AD are mainly associated with the skewing of the immune responses towards the Th2-type cytokine response, although chronic AD occasionally appears to be associated with the Th1-type cytokine response (Hamid *et al.*, 1994; Leung, 2000). Th2-type cytokines, including interleukin (IL)-4, IL-5, IL-6, IL-10 and IL-13, promote eosinophil growth, migration and activation, mast cell differentiation, and IgE production. On the other hand, Th1-type cytokines, including interferon (IFN)- γ , IL-2, IL-12 and tumour necrosis factor (TNF)- α , promote cell-mediated immunity (Chen *et al.*, 2004).

Keratinocytes play a critical role in cutaneous inflammatory and immune responses by secreting a variety of cytokines (Esche *et al.*, 2004; Albanesi *et al.*, 2005). These cells secrete several interleukins (IL-1, IL-3, IL-6, IL-7, and IL-8); colony-stimulating factors [granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF)], and growth factors [TNF- α , transforming growth factor; TGF- α , TGF- β and platelet-derived

growth factor (PDGF)] (Grone, 2002). Most of these mediators are not produced constitutively; instead, their gene expression is upregulated in response to several stimuli. Previous studies have shown that *Malassezia* yeasts can induce IL-1 α , IL-6, IL-8 and TNF- α secretion from human keratinocytes (Watanabe *et al.*, 2001).

In this study, we investigated the cytokine secretion profiles of human keratinocytes exposed to *M. globosa* and *M. restricta*.

Materials and methods

Yeast strains and culture conditions

Malassezia globosa CBS7966, *M. restricta* CBS7877, *M. furfur* CBS1878, and *M. sympodialis* CBS7222 were used in this study. These strains were cultured at 32 °C on modified Leeming and Notman agar (LNA; 20 g glucose, 50 g malt extract, 1 g polypeptone and 20 g bile salts; Oxoid, Hampshire, UK) containing 1% Tween 40, 0.2% glycerol and 50 $\mu\text{g mL}^{-1}$ chloramphenicol (Sankyo, Tokyo, Japan). Prior to use, the yeasts were harvested and suspended in Dulbecco's phosphate-buffered saline (PBS; pH 7.4) at a concentration of 1×10^8 yeast cells mL^{-1} .

Human keratinocytes and their cultivation

The human keratinocyte cell line PHK16-0b (JCRB0141) was obtained from the Japanese Collection of Research Bioresources (JCRB) cell bank and the primary normal human epidermal keratinocyte (NHEK) from PromoCell (Germany). The cells were cultured at 37 °C in 5% CO₂ in KBM-2 medium (Clonetics, Inc.) supplemented with 0.1 ng mL^{-1} EGF, 5 $\mu\text{g mL}^{-1}$ insulin, 30 $\mu\text{g mL}^{-1}$ bovine pituitary extract, 0.5 $\mu\text{g mL}^{-1}$ hydrocortisone, 50 $\mu\text{g mL}^{-1}$ gentamicin and 50 $\mu\text{g mL}^{-1}$ amphotericin.

Exposure of keratinocytes to *Malassezia* yeasts

At 18 h prior to infection, PHK16-0b or NHEK cells were seeded into 35-mm tissue culture dishes at a density of 1×10^6 cells dish^{-1} . Prior to yeast challenge, the keratinocyte monolayers that had grown in the tissue culture dishes were washed three times and incubated with antibiotic-free medium for 2 h. The keratinocyte monolayers were cocultivated with 2×10^7 yeast cells dish^{-1} [multiplicity of infection (MOI) of 20], which was shown by a preliminary experiment to be the optimal MOI for inducing cytokine secretion. Cell culture supernatants were collected after exposure for 24 h at 37 °C in 5% CO₂. In the trypan blue exclusion assay, we observed that the viabilities of the *Malassezia*-exposed keratinocytes did not differ from those of the control cells. More than 95% of the cells in all the cultures were viable (data not shown). In a separate experiment,

using the SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions, the total RNA was isolated from the cells that were exposed to *Malassezia* by cocultivation for 2 and 4 h, and was quantified spectrophotometrically.

Measurement of cytokine release using antibody arrays

Cytokine release from the keratinocytes was analysed using Human Cytokine Array VI and VII (RayBiotech Inc., Norcross, GA) according to the manufacturer's instructions. Briefly, the cytokine array membranes were blocked with $1 \times$ blocking buffer for 30 min and then incubated at 4 °C overnight with 1 mL of the samples. After incubation, the membranes were washed three times with 2 mL of $1 \times$ wash buffer I, followed by two washes with 2 mL of $1 \times$ wash buffer II at room temperature with shaking. The membranes were then incubated with 2 mL of 1:500 diluted biotin-conjugated antibodies at room temperature for 2 h and washed as described above before incubation with 1 mL of 1:40 000 diluted streptavidin-conjugated peroxidase at room temperature for 1 h. After a thorough wash, the membranes were exposed to a peroxidase substrate (detection buffers C and D) for 5 min in the dark before imaging. The membranes were exposed to an X-ray film within 30 min of exposure to the substrate. Signal intensities were quantified using the SCANALYZE software (Michael Eisen, Lawrence Berkeley National Laboratory, <http://www.microarrays.org/software.html>). Horseradish peroxidase (HRP)-conjugated antibody that was placed at six spots served as the positive substrate control, and it was also used to identify membrane orientation. For each spot, the net signal intensity was determined by subtracting the background level from the total raw signal intensity.

Enzyme-linked immunosorbent assay (ELISA)

The keratinocyte supernatants were assayed for IL-4 and IL-5 using the Human IL-4 and IL-5 ELISA kits (RayBiotech, Inc.) according to the manufacturer's instructions. The results are given in terms of pg (10^6 cells mL^{-1}).

cDNA microarray

The relative expression of cytokine mRNA was analysed using GEArray Q Series Human Inflammatory Cytokines & Receptors Gene Array (SuperArray Inc., Bethesda, MD) according to the manufacturer's instructions. In brief, 2 μg total RNA was reverse transcribed into cDNA in the presence of biotin-16-dUTP (Roche, Mannheim, Germany) using the AmpoLabeling-LPR kit (SuperArray Inc.). The obtained biotin-labelled cDNA samples were then hybridized overnight to cytokine and cytokine receptor gene-specific probes

that were spotted on the GEArray membranes. After incubation with streptavidin-alkaline phosphatase (AP) conjugate (1 : 12 500), the array image was developed using the CDP-Star chemiluminescent substrate and recorded on an X-ray film. The image was scanned using a scanner, and the obtained raw data was analysed using the SCANALYZE software and the GEARRAY Analyzer software (www.superarray.com). The signal that was obtained on the array from the expression of each gene was normalized to the signal that was derived from an internal cyclophilin A standard present on the same membrane.

Results

Cytokine profile induced in human keratinocytes by *M. globosa* or *M. restricta*

We examined the secretion of cytokines from human keratinocytes in response to *M. globosa* or *M. restricta*. PHK16-0b cells were exposed to *M. globosa* or *M. restricta* at an MOI of 20. After 24 h, the cytokine levels in the culture supernatants were detected and semiquantified on membrane arrays containing 120 different cytokine antibodies (Fig. 1a–c). Untreated human keratinocytes (control) were observed to secrete only trace or undetectable amounts of the cytokines. In this study, the differential cytokine secretion was arbitrarily defined by an intensity change of twofold or greater in secretion. Stimulation with *M. globosa* induced a pattern of cytokine secretion that was different from that induced by *M. restricta*, i.e. the exposure of PHK16-0b cells to *M. globosa* resulted in a marked secretion of IL-3, IL-5, IL-6, IL-7, IL-10 and IL-13, while exposure to *M. restricta* did not induce the secretion of these cytokines. *M. globosa* also induced the secretion of remarkable amounts of GM-CSF, IL-8, tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2, while *M. restricta* induced the secretion of these cytokines in much smaller amounts. Similarly, while *M. restricta* stimulated IL-4, monocyte inhibitory protein (MIP)-3 α , leptin, cutaneous T cell attracting chemokine (CTACK) and placental growth factor (PIGF) secretion from PHK16-0b cells, *M. globosa* did not. G-CSF was the most predominant cytokine that was detected in response to both *Malassezia* species. Consistent with the antibody array data, ELISA revealed that after exposure to *M. globosa*, keratinocytes released a significant amount of IL-5 but not IL-4 (Fig. 2a). On the other hand, a significant increase was observed in the IL-4 level but not the IL-5 level in the culture supernatant of *M. restricta*-exposed keratinocytes (Fig. 2b).

The human primary keratinocytes were then used as more relevant cells for determining the cytokine secretion in response to *M. globosa* or *M. restricta*. The exposure of NHEK cells to *M. globosa* induced IL-3, IL-5, IL-6, IL-10 and

IL-13 secretion; *M. restricta* did not have any remarkable effect on the secretion of these cytokines. *Malassezia restricta* induced high level of IL-4 secretion, whereas exposure to *M. globosa* did not (Table 1). These results were essentially identical to those obtained with PHK16-0b cells, although NHEK cells showed only a modest increase in secretion of IL-7, leptin and MIP-3 α in response to *Malassezia* yeasts.

Comparison of cytokine profiles of human keratinocytes in response to various *Malassezia* species

Previous work has reported that both *M. globosa* and *M. restricta* were detected in c. 90% of both AD and healthy subjects, while the other *Malassezia* species were observed in < 40% of AD (Sugita *et al.*, 2001). To assess and compare cytokine secretion changes in response to various *Malassezia* species, culture supernatants from PHK16-0b cells exposed to *M. globosa*, *M. restricta*, *M. furfur* or *M. sympodialis* were collected and analysed using human antibody array VI (Table 2). Exposure to *M. globosa* resulted in a marked increase in IL-5, IL-10 and IL-13 secretion from keratinocytes, whereas *M. restricta* induced IL-4 secretion. In contrast, no remarkable secretion of Th2-type cytokines was observed when keratinocytes were exposed to *M. furfur* or *M. sympodialis*. Only a few cytokines including BMP-6 and IL-6 were secreted from keratinocytes in response to *M. sympodialis*. This result was in line with the hypothesis that *M. globosa* and *M. restricta* play a role in the pathogenesis of AD (Sugita *et al.*, 2001).

Characteristics of cDNA microarray analysis

cDNA microarray analysis was performed to confirm the pattern of cytokine expression induced by *Malassezia* yeasts. PHK16-0b cells were exposed to *M. globosa* or *M. restricta* at an MOI of 20. The short exposure periods (2 and 4 h) were chosen to avoid secondary effects such as de novo secretion of other molecules that may alter cytokine expression. After these incubation periods, total RNA was extracted from the keratinocytes and the cytokine gene expression profile was determined by cDNA microarray analysis (Table 3). The level at which *M. globosa*-exposed cells showed the differential expression of 44 cytokine-related genes (26 genes were upregulated and 18 genes were downregulated) was threefold greater than that in nontreated cells after 2 h incubation. Similarly, *M. restricta* induced the regulation of 38 genes (27 genes were upregulated and 11 genes were downregulated) at 2 h. Almost similar results were obtained after 4 h incubation. The upregulation of several genes was observed to be specific for the *Malassezia* species, i.e. *M. globosa* was capable of inducing the gene transcription of Th2-type cytokines (IL-5, IL-10, and IL-13) in keratinocytes, while *M. restricta* specifically induced the

(a) Human Cytokine Antibody Array VI

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	POS	POS	Blank	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CKb6-1	CNTF	EGF	Eotaxin
2	NEG	NEG	NEG	NEG	Blank	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CKb6-1	CNTF	EGF	Eotaxin
3	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	IL-309	IFN- α	IGFBP-1	IGFBP-2	IGFBP-4
4	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	IL-309	IFN- α	IGFBP-1	IGFBP-2	IGFBP-4
5	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1a	IL-1b	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1a	IL-1b	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIP-1 α	MIP-3 α	NAP-2	NT-3	PARC	
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIP-1 α	MIP-3 α	NAP-2	NT-3	PARC	
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF- β 1	TGF- β 3	TNF- α	TNF- β	Blank	Blank	Blank	Blank	Blank
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF- β 1	TGF- β 3	TNF- α	TNF- β	Blank	Blank	Blank	Blank	Blank

Human Cytokine Antibody Array VII

	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	POS	POS	POS	POS	Blank	Acip30	AgRP	Angiopoietin-2	Amphiregulin	Axl	bFGF	b-NGF	BTC	CCL-28
2	NEG	NEG	NEG	NEG	Blank	Acip30	AgRP	Angiopoietin-2	Amphiregulin	Axl	bFGF	b-NGF	BTC	CCL-28
3	CTACK	Dik	EGF-R	ENA-78	Fas	FGF-4	FGF-9	G-CSF	GITR-Ligand	GITR	GRO	GRO- α	HCC-4	HGF
4	CTACK	Dik	EGF-R	ENA-78	Fas	FGF-4	FGF-9	G-CSF	GITR-Ligand	GITR	GRO	GRO- α	HCC-4	HGF
5	ICAM-1	ICAM-3	IGFBP-3	IGFBP-6	IGF-I SR	IL-1 R4/ST2	IL-1 RI	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 Ra	IL-6 R	IL-8
6	ICAM-1	ICAM-3	IGFBP-3	IGFBP-6	IGF-I SR	IL-1 R4/ST2	IL-1 RI	IL-11	IL-12 p40	IL-12 p70	IL-17	IL-2 Ra	IL-6 R	IL-8
7	LTAC	Lymphotoctin	MF	MP-1a	MP-1 b	MP-3b	MSP- α	NT-4	Osteoprotegerin	Oncostatin M	PIGF	sdp130	sTNF RI	sTNF-RII
8	LTAC	Lymphotoctin	MF	MP-1a	MP-1 b	MP-3b	MSP- α	NT-4	Osteoprotegerin	Oncostatin M	PIGF	sdp130	sTNF RI	sTNF-RII
9	TECK	TIMP-1	TIMP-2	Thrombopoietin	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	Blank	Blank	Blank	Blank	Blank
10	TECK	TIMP-1	TIMP-2	Thrombopoietin	TRAIL R3	TRAIL R4	uPAR	VEGF	VEGF-D	Blank	Blank	Blank	Blank	Blank

■ *M. globosa*
■ *M. restricta*

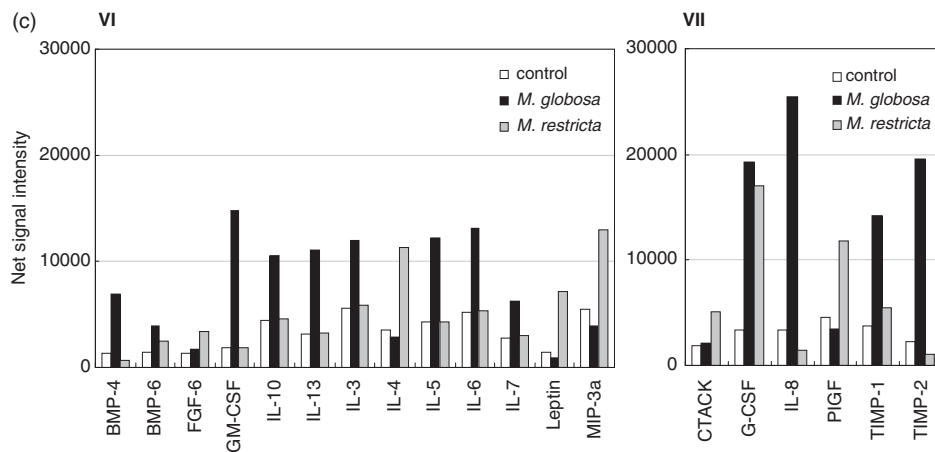
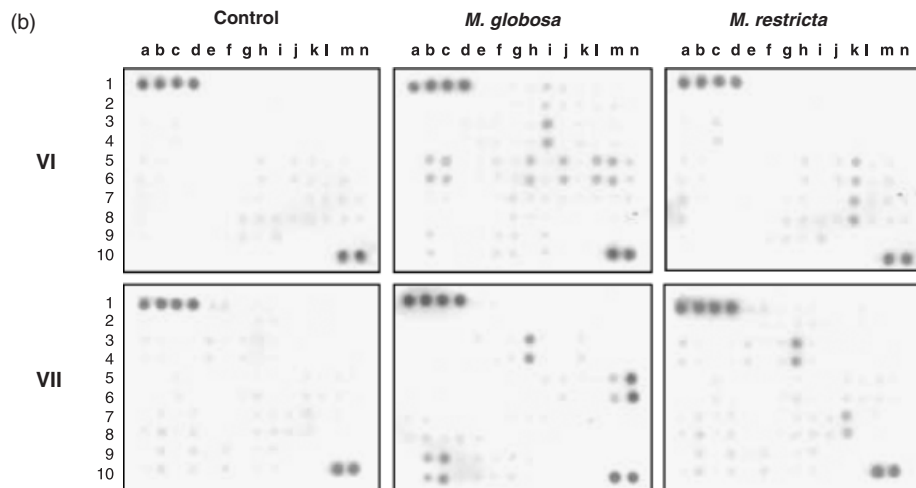


Fig. 1. Cytokine profile of human keratinocytes exposed to *Malassezia globosa* or *Malassezia restricta*. PHK16-0b cells (1×10^6 cells dish $^{-1}$) were exposed to *Malassezia globosa* or *Malassezia restricta* at a multiplicity of infection of 20 for 24 h. Untreated cell cultures were used as the control. Cell culture supernatants were subject to a cytokine antibody array. (a) Each cytokine is represented by duplicate spots in the locations shown. The cytokines exhibited a twofold or greater increase in response to *M. globosa* and *M. restricta* are represented in black and grey, respectively. (b) The cytokine array image represents the results of one of two independent experiments in which similar patterns of expression were observed. (c) The average net signal intensity for each pair of cytokine spots is shown. BMP, bone morphogenetic protein; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; MIP-3 α , macrophage inflammatory protein-3 α ; CTACK, cutaneous T cell attracting chemokine; G-CSF, granulocyte colony-stimulating factor; PIGF, placental growth factor; TIMP, tissue inhibitor of metalloproteinases.

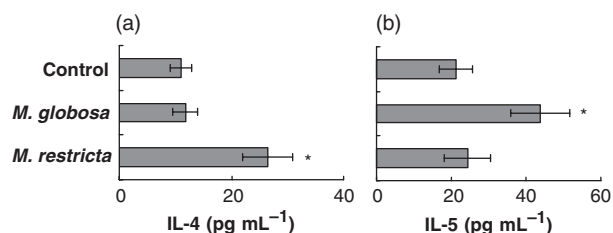


Fig. 2. IL-4 (a) and IL-5 (b) release from keratinocytes after exposure to *Malassezia globosa* and *Malassezia restricta*. Keratinocyte cultures were exposed to *M. globosa* or *M. restricta* at a multiplicity of infection of 20 for 24 h. ELISAs were performed for IL-4 and IL-5 using the culture supernatants. The values represent the means $\pm$ SE of four experiments. *, $P < 0.05$ versus control.

Table 1. Cytokine profile of normal human epidermal keratinocytes (NHEK) exposed to *Malassezia globosa* or *Malassezia restricta*

	Fold increase	
	<i>M. globosa</i>	<i>M. restricta</i>
BMP-4	2.3	1.6
BMP-6	1.9	1.4
FGF-6	1.0	1.4
GM-CSF	4.4	1.3
IL-10	2.7	1.3
IL-13	2.5	1.3
IL-3	2.6	1.4
IL-4	0.9	2.0
IL-5	2.9	1.3
IL-6	2.7	1.4
IL-7	1.7	1.1
Leptin	0.8	1.6
MIP-3 α	0.7	1.9

NHEK cells (1×10^6 cells dish $^{-1}$) were exposed to *M. globosa* or *M. restricta* at a multiplicity of infection of 20 for 24 h, and the cell culture supernatants were subjected to the human cytokine antibody array VI. Each experiment was done in duplicate. Data are presented as the fold increase relative to control. The cytokines exhibited a twofold or greater increase in response to *Malassezia* yeasts are represented in bold.

upregulation of the IL-4 gene. Increased expressions of chemokine receptor genes (CCR2, CCR4, CCR5, XCR1, CX3CR1 and CXCR4) were also observed with both yeast species.

Discussion

The contribution of *Malassezia* colonization to the pathogenesis of AD has been proposed on the basis of the observation that most AD patients have a high titre of *Malassezia*-specific serum IgE antibodies (Leung, 2000), and that organisms of the *Malassezia* species, particularly *M. globosa* and *M. restricta*, are identified at high frequencies in AD patients (Sugita *et al.*, 2001). However, the precise

Table 2. Major differences of cytokine secretion in PHK16-0b keratinocytes exposed to *Malassezia* species

Cytokines	Fold increase			
	<i>M. globosa</i>	<i>M. restricta</i>	<i>M. furfur</i>	<i>M. sympodialis</i>
BMP-4	5.2	0.5	0.9	1.7
BMP-6	2.7	1.7	1.6	2.4
FGF-6	1.3	2.7	1.4	1.8
GM-CSF	8.1	1.0	1.0	0.9
IL-10	2.4	1.0	1.6	0.9
IL-13	3.6	1.0	1.2	1.0
IL-3	2.1	1.0	0.9	1.2
IL-4	0.8	3.2	1.1	1.0
IL-5	2.8	1.0	1.0	0.9
IL-6	2.5	1.0	1.0	2.4
IL-7	2.2	1.1	1.3	1.1
Leptin	0.7	5.2	1.2	0.9
MIP-3 α	0.7	2.4	1.0	0.9

PHK16-0b cells (1×10^6 cells dish $^{-1}$) were exposed to *Malassezia globosa*, *M. restricta*, *M. furfur* or *M. sympodialis* at a multiplicity of infection of 20 for 24 h. Cell culture supernatants were subject to the human cytokine antibody array VI. Each experiment was done in duplicate. Data are presented as the fold increase relative to control. The cytokines exhibited a twofold or greater increase in response to *Malassezia* yeasts are represented in bold.

mechanisms by which *Malassezia* colonization induces the immune and inflammatory cascades that lead to AD remain unclear. It is now apparent that cutaneous inflammatory and immune responses involve close interactions between immunocompetent cells and epidermal keratinocytes (Grone, 2002; Esche *et al.*, 2004; Albanesi *et al.*, 2005). By producing cytokines and chemokines (Grone, 2002) and expressing specific adhesion molecules (Banerjee *et al.*, 2004), keratinocytes play an important role in the initiation and maintenance of allergic responses in the skin. In the present study, we examined the cytokine secretion profiles of human keratinocytes exposed to *M. globosa* and *M. restricta*, and we demonstrated that the *Malassezia*-keratinocyte interaction is important to the pathogenesis of AD. Human keratinocytes can, in fact, produce Th2-type cytokines in response to *M. globosa* and *M. restricta*.

AD has been described as a Th2-type disease, at least in the initiating phase (Grewe *et al.*, 1998). Of the Th2-type cytokines, IL-4 and IL-5 are known to orchestrate the immune and inflammatory responses, including IgE synthesis, eosinophil recruitment, and the upregulation of eosinophil function, that are characteristic of the inflammatory state observed in AD (Romagnani, 1995; Koning *et al.*, 1997). Using antibody array and ELISA systems we demonstrated that *M. globosa* induced IL-5 secretion from PHK16-0b cells, while *M. restricta* induced IL-4 secretion. Similar results were obtained with NHEK cells. These findings were confirmed by cDNA microarray analysis that showed that

Table 3. Cytokine gene expression profiles of human keratinocytes exposed to *Malassezia globosa* and *Malassezia restricta*

Description	Gene name	Fold regulation in			
		<i>M. globosa</i>		<i>M. restricta</i>	
		2 h	4 h	2 h	4 h
Homo sapiens Burkitt lymphoma receptor 1, GTP-binding protein (BLR1)	CXCR5 (BLR1)	2.9	1.8	4.5	4.2
Chemokine (C-C motif) receptor 1	CCR1	2.8	1.5	5.3	3.9
Chemokine (C-C motif) receptor 2	CCR2	14.1	10.3	11.6	9.3
Chemokine (C-C motif) receptor 3	CCR3	2.8	3.3	4.5	5.9
Chemokine (C-C motif) receptor 4	CCR4	14.0	15.9	18.6	23.4
Chemokine (C-C motif) receptor 5	CCR5	10.2	13.0	8.4	11.9
Chemokine (C-C motif) receptor 6	CCR6	1.9	2.5	2.1	3.3
Chemokine (C-C motif) receptor 7	CCR7	-2.7	-1.9	-1.0	-1.5
Chemokine (C-C motif) receptor 8	CCR8	1.1	1.3	4.1	3.1
Chemokine (C-C motif) receptor 9	CCR9	1.2	1.6	1.2	1.8
Homo sapiens chemokine (C motif) XC receptor 1 (CXCR1)	XCR1	6.4	6.7	8.0	9.2
Chemokine (C-X3-C) receptor 1	CX3CR1	3.4	3.1	6.3	6.1
Chemokine (C-X-C motif), receptor 4 (fusin)	CXCR4	4.7	5.5	4.9	6.5
Interferon, γ	IFN- γ	6.8	8.1	4.2	5.7
Interleukin 10	IL-10	3.4	3.0	-4.9	-2.4
interleukin 10 receptor, α	IL-10Ra	1.3	2.1	1.3	2.3
Interleukin 10 receptor, β	IL-10Rb	7.1	4.4	6.0	4.4
Interleukin 11	IL-11	-1.9	-1.9	-1.3	-1.1
Interleukin 11 receptor, α	IL-11Ra	-1.4	-1.3	-1.3	-1.2
Interleukin 12A, p35	IL-12A	7.1	4.4	18.3	12.1
Interleukin 12B, p40	IL-12B	4.9	2.1	12.9	6.6
Interleukin 12 receptor, β 1	IL-12Rb1	1.4	1.1	4.1	2.5
Interleukin 12 receptor, β 2	IL-12Rb2	-1.1	-1.0	-1.8	-1.4
Interleukin 13	IL-13	3.4	3.9	-1.7	-1.1
Interleukin 13 receptor, α 1	IL13RA1	-3.5	-1.5	1.1	1.4
Interleukin 13 receptor, α 2	IL-13Ra2	-11.1	-3.5	3.2	4.1
Interleukin 15	IL-15	-5.4	-3.4	-2.0	-2.1
Interleukin 15 receptor, α	IL-15Ra	20.6	32.9	11.2	20.8
Interleukin 16 (lymphocyte chemoattractant factor)	IL-16	6.6	22.5	22.9	25.7
Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	IL-17	1.0	1.0	1.8	2.2
Homo sapiens IL-17 receptor mRNA	IL-17 R	-1.3	-1.1	-1.8	-1.1
Interleukin 18 (interferon- γ -inducing factor)	IL-18	-2.8	1.2	1.6	1.8
Interleukin 18 receptor 1	IL18R1	-5.7	-3.7	-2.5	-2.4
Interleukin 1, α	IL-1a	-23.4	-5.2	1.9	2.9
Interleukin 1, β	IL-1b	-3.6	-1.1	-2.1	-1.3
Interleukin-1 receptor type I	IL-1R1	-1.5	-1.8	1.9	2.1
Interleukin-1 receptor type II	IL-1R2	-1.0	-1.0	2.8	2.6
Interleukin 2	IL-2	-11.1	-7.5	-1.6	-1.5
Interleukin 20	IL20	-1.2	-1.2	5.3	10.0
Homo sapiens interleukin 21 (IL21)	IL21	1.1	1.8	-1.2	-1.5
Likely ortholog of mouse interleukin 25	IL25	-16.0	-9.2	-4.3	-2.1
Interleukin 2 receptor, α	IL-2 Ra	-51.4	-27.7	-22.7	-20.2
Interleukin 2 receptor, β	IL-2 Rb	8.8	18.2	1.7	2.9
Interleukin 2 receptor, γ (severe combined immunodeficiency)	IL-2 Rr	-5.6	-1.3	-1.8	-2.6
Interleukin 4	IL-4	1.2	1.9	3.7	6.8
Interleukin 5 (colony-stimulating factor, eosinophil)	IL-5	3.5	4.5	2.1	2.2
Interleukin 5 receptor, α	IL-5 Ra	3.2	6.7	14.0	33.2
Interleukin 6 (interferon, β 2)	IL-6	5.0	3.9	2.6	2.7
Interleukin 6 receptor	IL-6 Ra	-1.9	-1.7	-4.4	-3.9
Interleukin 6 signal transducer (gp130, oncostatin M receptor)	gp130	-8.7	-8.0	-1.0	-1.0
Interleukin 9	IL-9/p40	-1.6	1.1	3.0	5.1
Interleukin 9 receptor	IL-9 Ra	-3.6	-4.1	1.0	-1.9

continued

Table 3. Continued.

Description	Gene name	Fold regulation in			
		<i>M. globosa</i>		<i>M. restricta</i>	
		2 h	4 h	2 h	4 h
Leptin (murine obesity homolog)	Leptin	-1.4	1.6	3.1	2.1
Lymphotoxin- α (TNF superfamily, member 1)	TNF-b/Lta	19.5	21.6	16.7	21.2
Lymphotoxin- β	LT-b	4.4	8.8	2.6	6.0
Homo sapiens lymphotoxin β receptor (TNFR superfamily, member 3(LTBR)	LTbR	-27.6	-19.0	-6.0	-4.0
Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	MIF	-2.4	-1.5	-2.1	-1.6
Small inducible cytokine A1 (I-309, homologous to mouse Tca-3)	I-309	-3.8	-2.0	-2.2	-1.1
Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin)	Eotaxin	-1.4	1.1	-1.7	-1.0
Small inducible cytokine subfamily A (Cys-Cys), member 13	MCP-4	2.2	2.9	1.2	1.7
Small inducible cytokine subfamily A (Cys-Cys), member 14	HCC-1	-4.8	-2.8	-5.9	-2.8
Small inducible cytokine subfamily A (Cys-Cys), member 15	MIP-1 delta	-3.1	-1.5	-4.3	-2.0
Small inducible cytokine subfamily A (Cys-Cys), member 16	HCC-4	-2.0	-1.5	-2.9	-2.0
Small inducible cytokine subfamily A (Cys-Cys), member 17	TARC (SCYA17)	-1.1	-1.1	-2.3	-2.0
Small inducible cytokine subfamily A (Cys-Cys), member 18, pulmonary and activation-regulated	PARC	1.0	2.4	1.0	2.7
Small inducible cytokine subfamily A (Cys-Cys), member 19	SCYA19	2.7	4.1	1.2	1.8
Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	MCP-1(SCYA2)	2.9	1.0	-2.5	1.0
Small inducible cytokine subfamily A (Cys-Cys), member 20	MIP-3a	1.8	2.4	3.1	4.2
Small inducible cytokine subfamily A (Cys-Cys), member 21	MIP-2 (SCYA21)	1.0	1.6	-1.1	1.5
Small inducible cytokine subfamily A (Cys-Cys), member 22	MDC	-1.2	-1.1	-1.4	-1.2
Small inducible cytokine subfamily A (Cys-Cys), member 23	MPIF-1	-1.5	-1.2	-3.0	-1.6
Small inducible cytokine subfamily A (Cys-Cys), member 24	MPIF-2	1.9	2.5	1.6	4.4
Human chemokine (TECK)	TECK	1.7	4.0	1.2	2.4
Small inducible cytokine A3 (homologous to mouse Mip-1a)	MIP-1a	1.1	1.9	-1.8	-1.0
Small inducible cytokine A4 (homologous to mouse Mip-1b)	MIP-1b	6.8	12.0	4.0	7.8
Small inducible cytokine A5 (RANTES)	SCYA5 (RANTES)	4.5	8.9	2.4	2.4
Homo sapiens mRNA for monocyte chemotactic protein-3 (MCP-3)	MCP-3	-1.7	-1.9	1.7	1.6
Small inducible cytokine subfamily A (Cys-Cys), member 8 (monocyte chemotactic protein 2)	MCP-2	2.2	2.3	2.9	3.7
γ -interferon inducible early response gene (small inducible cytokine subfamily B (Cys-X-Cys)	P10 (IP 10)	-2.7	-2.0	-7.5	-3.0
Small inducible cytokine subfamily B (Cys-X-Cys), member 11	I-TAC (IP9) (SCYB11)	-1.7	-1.2	-1.5	-1.2
Small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant)	SCYB13	-2.9	-1.3	1.7	2.3
Small inducible cytokine subfamily B (Cys-X-Cys), member 5 (epithelial-derived neutrophil-activating	ENA-78	3.3	5.6	-1.0	-2.1
Human chemokine α 3 (CKA-3) mRNA	GCP-2	-2.3	-1.2	-2.8	-1.3
Small inducible cytokine subfamily C, member 1 (lymphotactin)	Lymphotactin	2.6	3.9	2.1	3.4
Small inducible cytokine subfamily C, member 2	SCYC2	3.1	4.7	2.6	4.3
Small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (fractalkine, neurotactin)	Fractalkine	1.4	1.9	1.4	1.8
Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)	SCYE1	-1.4	-1.2	1.5	1.6
Stromal cell-derived factor 1	SDF1	1.7	1.5	1.2	1.0
Homo sapiens mRNA for SDF2	SDF2	4.1	6.5	4.1	7.1
Transforming growth factor, α	TGF-a	-2.5	-2.5	-2.1	-2.2
Transforming growth factor, β 1	TGFb1	-17.1	-10.0	-13.5	-7.3
Transforming growth factor, β 2	TGF b2	1.3	2.3	1.3	1.9
Transforming growth factor, β 3	TGF b3	4.6	15.8	5.0	17.3
Tumor necrosis factor (TNF superfamily, member 2)	TNFa	-1.0	1.1	1.3	1.3
Tumor necrosis factor receptor superfamily, member 1A	TNFR1	-6.2	-3.7	-7.1	-4.5
Human tumor necrosis factor receptor 2	TNFR2 (TNFSF1B)	-1.3	-1.5	-1.6	-1.3

PHK16-0b monolayers (1×10^6 cells dish $^{-1}$) were exposed to *M. globosa* and *M. restricta* at a multiplicity of infection of 20 for 2 and 4 h. The differential expression of the cytokine transcripts was determined by comparing the calculated fold change in the signal intensity values of the exposed cells and the corresponding control cells. The upregulated genes are represented in black, and the downregulated ones are represented in grey.

■, up-regulated; ■, down-regulated.

M. globosa induced the transcription of the IL-5 gene in keratinocytes, while *M. restricta* induced the upregulation of the IL-4 gene. Our observation that *M. globosa* induced both the gene transcription and protein secretion of other Th2-type cytokines, such as IL-10 and IL-13, provides additional evidence for a possible relationship between *Malassezia* colonization and the Th2-type immune response occurring in AD. IL-10 promotes the development of a Th2 response to antigens and is essential for eosinophil infiltration into the skin in allergic dermatitis (Laouini *et al.*, 2003). IL-10 is expressed by keratinocytes as well as by hemopoietic cells including dendritic cells (DCs), macrophages, mast cells and lymphocytes (Enk & Katz, 1992; Moore *et al.*, 1993). The biological effects of IL-13 on B cells, macrophages and monocytes are very similar to those of IL-4, probably because the IL-4 and IL-13 receptors share a common chain (Schnyder *et al.*, 1996). In B cells, IL-13 promotes proliferation, differentiation and Ig heavy chain class switching to IgE and IgG4 (Minty *et al.*, 1993; Zurawski & deVries, 1994). In addition, IL-13 enhances CD23/Fc ϵ RII expression on resting B cells (Defrance *et al.*, 1994). It was also noteworthy that *M. globosa* induced the secretion of IL-6 from keratinocytes because the role of IL-6 has been demonstrated in IL-4-dependent IgE synthesis (Vercell *et al.*, 1989). Therefore, it is likely that *M. globosa* and *M. restricta* play a synergistic role in triggering or exacerbating AD by inducing the secretion of differential Th2-type cytokines from human keratinocytes. As expected, using ELISA we found that mixed exposure to *M. globosa* and *M. restricta* induced simultaneous secretion of IL-4 and IL-5 at extents similar to those observed in the single exposures (data not shown). Our study also revealed that other *Malassezia* species such as *M. furfur* and *M. sympodialis* failed to induce secretion of Th2-type cytokines from keratinocytes. These results are in good agreement with the previous finding that both *M. globosa* and *M. restricta* are identified in c. 90% of the AD patients, while *M. furfur* and *M. sympodialis* are detected less frequently, suggesting that *M. globosa* and *M. restricta* play a role in the pathogenesis of AD.

Another important connection between *Malassezia* colonization and AD is the increased secretion of colony-stimulating factors and chemokines from keratinocytes. We observed that *M. globosa* induced GM-CSF secretion from keratinocytes. GM-CSF is essential for the differentiation and maturation of Langerhans cells (LCs) and DCs (Witmer-Pack *et al.*, 1987; Caux *et al.*, 1992). It is known that in AD, GM-CSF is overproduced by keratinocytes (Girolomoni, 1997). The current hypothesis is that the lesional abundance of GM-CSF primarily contributes to the maintenance of the chronic inflammatory process in AD by enhancing the antigen-presenting capacity of LCs and DCs (Witmer-Pack *et al.*, 1987). In addition, the mitogenic effect of GM-CSF on keratinocytes (Braunstein *et al.*, 1994) would

result in epidermal hypertrophy, which is a characteristic of chronic inflammation. We also observed that *M. restricta* induced the secretion of CTACK. CTACK is another Th2-specific chemokine that belongs to the CC chemokine family and selectively attracts cutaneous lymphocyte antigen-positive memory T cells to the inflammatory sites (Morales *et al.*, 1999). A recent finding has revealed that CTACK is upregulated in AD patients (Kakinuma *et al.*, 2003). Taken together, these findings suggest that the secretion of GM-CSF and CTACK from keratinocytes in response to *M. globosa* and *M. restricta* colonization may also contribute to the pathogenesis of AD. IL-8, a CXC chemokine, is known to be involved in the inflammatory process by stimulating the migration of neutrophils and lymphocytes (Larsen *et al.*, 1995; Keller *et al.*, 2005). Our present finding that *M. globosa* can induce the secretion of IL-8 from keratinocytes further extends the proinflammatory role of *Malassezia* colonization in AD pathogenesis.

We observed that human keratinocytes could produce TIMP-1 and TIMP-2 in response to *M. globosa*. TIMPs block the activities of matrix metalloproteinases (MMPs) that degrade the various components of the connective tissue matrix (Woessner, 1991). The appropriate regulation of MMPs and their TIMPs during the processes of tissue repair is important for tissue remodelling (Edwards *et al.*, 1996). Elevated levels of serum TIMPs have been documented in AD patients (Katoh *et al.*, 2002). The increased production of TIMPs could contribute to the formation of chronic eruptions such as lichenified plaques in AD.

The molecular mechanisms underlying the upregulation of cytokine production are presently unclear. It has been reported that Toll-like receptor 2 (TLR2) mediates intracellular signalling in human keratinocytes in response to *M. furfur*, leading to the expression of human β -defensin-2 (hBD2) and IL-8 mRNA (Baroni *et al.*, 2006). Since our study revealed the differential cytokine production from keratinocytes in response to *M. globosa* and *M. restricta*, it appears likely that several different microbial components and the corresponding keratinocyte receptors may be involved in triggering cytokine secretion. Further studies will be attempted to identify the keratinocyte receptors and signalling pathways involved.

In conclusion, the results of this study demonstrate that *M. globosa* and *M. restricta* induce the secretion of distinct Th2-type cytokines from human keratinocytes; *M. globosa* induces IL-5, IL-10 and IL-13 secretion, while *M. restricta* induces IL-4 secretion. We hypothesize that *M. globosa* and *M. restricta* play a synergistic role in triggering or exacerbating AD by stimulating the Th2 immune response. Elucidation of the role of *Malassezia*-induced cytokine secretion from keratinocytes will shed light on the pathogenesis of AD and provide clues for the development of new therapeutic strategies.

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