Cytokine secretion profiles of human keratinocytes during *Trichophyton tonsurans* and *Arthroderma benhamiae* infections

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Dermatophytes cause intractable superficial infections in humans. *Arthroderma benhamiae*, a zoophilic dermatophyte, triggers severe inflammatory responses in humans, while *Trichophyton tonsurans*, an anthropophilic dermatophyte, triggers minimal ones. Cytokines and other factors derived from keratinocytes play important roles in inflammatory and immune responses in the skin. The authors performed an *in vitro* investigation to determine the human keratinocyte cytokine profiles during dermatophyte infection. The human keratinocyte cell line PHK16-0b was infected with *A. benhamiae* or *T. tonsurans* for 24 h, and the cytokines secreted were analysed using a human cytokine antibody array. Marked differences were observed in the cytokine profiles of the cells infected with the two dermatophytes. *A. benhamiae* infection resulted in the secretion of a broad spectrum of cytokines, including proinflammatory cytokines, chemokines, and immunomodulatory cytokines. In contrast, *T. tonsurans*-infected keratinocytes secreted only limited cytokines, including eotaxin-2, interleukin (IL)-8 and IL-16. cDNA microarray analysis confirmed that *A. benhamiae* infection upregulated genes encoding IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-13, IL-15, IL-16, IL-17 and interferon (IFN)- γ , while *T. tonsurans* infection upregulated only a few genes, such as those encoding IL-1 β and IL-16. RT-PCR demonstrated that infection by both dermatophytes enhanced IL-8 mRNA expression in keratinocytes. These results suggest that *A. benhamiae*-induced secretion of several cytokines from keratinocytes may be involved in a severe inflammatory response, and that the limited cytokine secretion from keratinocytes in response to *T. tonsurans* infection may result in a minimal inflammatory response in the skin. These cytokine profiles may aid in proving the clinical features of dermatophytosis.

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INTRODUCTION

Superficial dermatophytosis is the most common fungal infection in humans. It affects the skin, hair and nails, and is caused by keratinophilic fungi, i.e. dermatophytes (Anaisie *et al.*, 2002). The clinical presentation of dermatophytosis depends on several of the following factors: (i) the site of infection, (ii) the immunological response of the host, and (iii) the species of infecting fungus. Dermatophytes

comprise approximately 40 known species, and these are classified into three genera: *Trichophyton*, *Microsporum* and *Epidermophyton* (Richardson & Warnock, 1997). Based on their ecological characteristics, dermatophytes are divided into geophilic, zoophilic and anthropophilic species. The infections caused by the anthropophilic species tend to be chronic and intractable, and the resultant inflammation is minimal. On the other hand, the infections caused by the geophilic and zoophilic species tend to be self-healing, and the resultant inflammation is more severe (Rippon, 1988; Weitzman & Summerbell, 1995). However, the precise mechanisms by which each dermatophyte species differs in its host responses remain unclear.

Although *Trichophyton rubrum* is the most common aetiological agent of human dermatophytosis, infections

Abbreviations: CCR, CC chemokine receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM, intracellular adhesion molecule; IFN, interferon; IL, interleukin; NHEK, normal human epidermal keratinocyte; TIMP, tissue inhibitor of metalloproteinase; TNF, tumour necrosis factor.

due to two other dermatophyte species, namely *Arthroderma benhamiae* and *Trichophyton tonsurans*, have recently become prevalent and a significant health problem worldwide (Chinen & Shearer, 2005; Cruickshank *et al.*, 1991). *A. benhamiae*, a teleomorph of *Trichophyton mentagrophytes*, is a distinctly zoophilic fungus that is known to primarily infect rabbits and other lagomorphs, and guinea pigs; it also infects humans (Fumeaux *et al.*, 2004; Mochizuki *et al.*, 2002). Infected animals release infective spores in the environment, which then infect other animals or humans. Human ringworm caused by *A. benhamiae* is characterized by severe inflammatory reactions that are manifested as papules, vesicles and pustules.

T. tonsurans, an anthropophilic fungus, is the causative agent of tinea capitis and tinea corporis, which occur in combat sport players (Adams, 2002). *T. tonsurans* infection demonstrates strong infectiosity and intractability (Wagner & Sohnle, 1995). *T. tonsurans* tends to be associated more with chronic infections that are less inflammatory in nature and show slight or asymptomatic clinical manifestations (Wagner & Sohnle, 1995).

The skin responds to superficial infections by increased proliferation of the affected cells, which leads to scaling and epidermal thickening (Wagner & Sohnle, 1995). In addition, keratinocytes, which are the main constituent cells of the epidermis, play a critical role in the initiation and maintenance of the inflammatory state in the skin (Rook & Champion, 2004). Following exposure to a variety of stimuli, including fungal pathogens, keratinocytes can synthesize and release significant amounts of cytokines that are capable of modulating the inflammatory and immune responses (Grone, 2002). Human keratinocytes exposed to *T. mentagrophytes* have been shown to release interleukin (IL)-8 and tumour necrosis factor (TNF)- α (Grone, 2002; Nakamura *et al.*, 2002). However, the potential of *A. benhamiae* and *T. tonsurans* to stimulate cytokine release from keratinocytes has not been investigated in detail thus far.

In this study, we investigated the cytokine profiles of human keratinocytes after infection with *A. benhamiae* or *T. tonsurans*. The results demonstrated that keratinocytes secrete a broad spectrum of cytokines, including proinflammatory cytokines, chemokines, and immunomodulatory cytokines, in response to *A. benhamiae* infection, whereas *T. tonsurans* infection stimulates the production of only a limited number of cytokines. Such differential cytokine secretion profiles of keratinocytes in response to infection by dermatophyte species may reflect distinct inflammatory responses in the skin.

METHODS

Fungal strains. Three clinical isolates each of *T. tonsurans* (three isolates obtained from patients with tinea corporis) and *A. benhamiae* (three isolates obtained from patients with tinea capitis) were used in this study. These dermatophyte species were identified by

morphological methods, urease reaction, and molecular analysis by PCR amplification of the nuclear ribosomal internal transcribed spacer 1 (ITS1) region (Makimura, 2001). *T. tonsurans* and *A. benhamiae* were cultured at 25 °C for 2 weeks on Sabouraud dextrose agar and one-tenth Sabouraud dextrose agar, respectively. Microconidia were harvested by adding 5 ml Dulbecco's PBS, pH 7.4, to the slant culture by pipette. After extensive washing with PBS, the microconidia were counted under a microscope, and their suspension was diluted to a concentration of 1×10^8 ml⁻¹. The fungal suspension was prepared in an LPS-free fashion and was analysed by Limulus assay. No detectable LPS concentrations were found in any of the preparations.

Human keratinocytes. The human papillomavirus-16-immortalized human foreskin keratinocyte cell line PHK16-0b (JCRB0141) was obtained from the Japanese Collection of Research Bioresources (JCRB), and normal human epidermal keratinocytes (NHEKs) from PromoCell. The cells were cultured at 37 °C and 5 % CO₂ in KBM-2 medium (Clonetics) that was supplemented with 0.1 ng epidermal growth factor (EGF) ml⁻¹, 5 µg insulin ml⁻¹, 30 µg bovine pituitary extract ml⁻¹, 0.5 µg hydrocortisone ml⁻¹, 50 µg gentamicin ml⁻¹ and 50 µg amphotericin ml⁻¹.

Infection of keratinocytes with dermatophytes. The cells were seeded into 35 mm tissue culture dishes at a density of 1×10^6 cells per dish 18 h before infection. The keratinocyte monolayers grown in the tissue culture dishes were washed three times and incubated with antibiotic-free medium for 2 h prior to fungal challenge. A preliminary experiment was performed to determine the optimal microconidia:keratinocyte ratio of infection and the duration of the experiment. We chose a 1:1 ratio of infection, which allowed good viability of the infected cells for at least 24 h. Under these conditions, >95 % of the cells in all cultures were viable, as assessed by trypan blue exclusion. In the case of a 5:1 ratio of infection, dermatophytes covered the entire monolayer surface after 24 h infection, resulting in apparent cell damage. We also carried out an experiment to compare the growth rate of the two dermatophyte species in cell-free KBM-2 medium. There was no difference in growth rate between cultures of *A. benhamiae* and *T. tonsurans* in KBM-2 medium, as assessed by dry weight and spectrophotometry methods. The keratinocyte monolayers were co-cultivated with 1×10^6 microconidia per dish (m.o.i. = 1) at 37 °C and 5 % CO₂. The cell culture media were collected after 24 h exposure.

In a separate experiment, mRNA was isolated from the infected cells after co-cultivation for 3 h by using the ArrayGrade mRNA Purification kit (SuperArray) according to the manufacturer's instructions; the mRNA was then spectrophotometrically quantified.

Measurement of cytokine release by using antibody arrays.

Cytokine release from keratinocytes was analysed by using Human Inflammation Antibody Array III (Ray Biotech) according to the manufacturer's instructions. Briefly, the cytokine array membranes were blocked with $1 \times$ blocking buffer for 30 min and then incubated overnight with 1 ml sample at 4 °C. After incubation, the membranes were washed three times with 2 ml $1 \times$ Wash Buffer I (Ray Biotech) followed by two washes with 2 ml $1 \times$ Wash Buffer II (Ray Biotech) at room temperature with shaking. The membranes were then incubated with 2 ml 1:500-diluted biotin-conjugated antibodies for 2 h at room temperature and washed as described above; this was followed by incubation with 1 ml 1:40 000-diluted streptavidin-conjugated peroxidase for 1 h at room temperature. After a thorough wash, the membranes were exposed to a peroxidase substrate (detection buffers C and D; Ray Biotech) for 5 min in the dark prior to imaging. The membranes were exposed to an X-ray film within 30 min of exposure to the substrate. Signal intensities were quantified with Scanlyse software (M. Eisen, Lawrence Berkeley National Laboratory; <http://www.microarrays.org/software.html>).

Fig. 1. Representative cytokine profile of PHK16-0b keratinocytes infected with dermatophyte species. PHK16-0b cells (1×10^6 cells per dish) were infected for 24 h with *A. benhamiae* AB1 or *T. tonsurans* TT1 at an m.o.i. of 1. Levels of cytokines released from keratinocytes were analysed by using the Human Inflammation Antibody Array. (a) The cytokine array image was obtained by a 15 min exposure of membrane to X-ray film. Each cytokine is represented by duplicate spots in the following locations: A1–2 and B1–2, positive control; C1–2 and D1–2, negative control; E1–2, eotaxin; F1–2, eotaxin-2; G1–2, G-CSF; H1–2, GM-CSF; I1–2, ICAM-1; J1–2, IFN- γ ; K1–2, I-309; L1–2, IL-1 α ; A3–4, IL-1 β ; B3–4, IL-2; C3–4, IL-3; D3–4, IL-4; E3–4, IL-6; F3–4, IL-6sR; G3–4, IL-7; H3–4, IL-8; I3–4, IL-10; J3–4, IL-11; K3–4, IL-12p40; L3–4, IL-12p70; A5–6, IL-13; B5–6, IL-15; C5–6, IL-16; D5–6, IL-17; E5–6, IP-10; F5–6, MCP-1; G5–6, MCP-2; H5–6, M-CSF; I5–6, MIG; J5–6, MIP-1 α ; K5–6, MIP-1 β ; L5–6, MIP-1 γ ; A7–8, RANTES; B7–8, TGF- β ; C7–8, TNF- α ; D7–8, TNF- β ; E7–8, sTNFR1; F7–8, sTNFR2; G7–8, PDGF-BB; H7–8, TIMP-2; I7–8 and J7–8, blank; K7–8, negative control; L7–8, positive control. (b) The average net signal intensity of each pair of cytokine spots was determined on the basis of the greyscale levels using Scanlyse software.

Table 1. Cytokine secretion profiles in PHK16-0b keratinocyte cultures infected with dermatophyte species

PHK16-0b cells (1×10^6 cells per dish) were infected for 24 h with three isolates each of *A. benhamiae* (isolates AB1, AB2 and AB3) and *T. tonsurans* (isolates TT1, TT2 and TT3) at an m.o.i. of 1. Levels of cytokines released from keratinocytes were analysed by using the Human Inflammation Antibody Array. The antibody array analysis was performed once on each of the isolates. Each experiment was done in duplicate. Data are presented as the fold increase relative to the control. Data pooled from three experiments are presented as the mean $\pm$ SEM.

Cytokine	Fold increase relative to control											
	<i>A. benhamiae</i>						<i>T. tonsurans</i>					
	AB1	AB2	AB3	Mean $\pm$ SEM	TT1	TT2	TT3	Mean $\pm$ SEM	TT1	TT2	TT3	Mean $\pm$ SEM
Eotaxin	3.2	2.6	1.9	2.6 $\pm$ 0.4*	1.1	0.6	1.4	1.0 $\pm$ 0.2				
Eotaxin-2	4.1	3.2	2.6	3.3 $\pm$ 0.5*	2.2	1.3	2.8	2.1 $\pm$ 0.4*				
G-CSF	3.1	2.6	2.8	2.8 $\pm$ 0.2*	0.9	1.7	0.8	1.1 $\pm$ 0.3				
GM-CSF	3.6	2.3	3.0	3.0 $\pm$ 0.4*	1.6	1.7	0.6	1.3 $\pm$ 0.4				
ICAM-1	3.7	1.9	2.0	2.6 $\pm$ 0.6*	1.7	1.5	0.8	1.3 $\pm$ 0.3				
IFN- γ	1.9	2.4	1.9	2.1 $\pm$ 0.1*	0.6	1.1	1.9	1.2 $\pm$ 0.4				
I-309	0.9	1.1	1.4	1.1 $\pm$ 0.1	0.4	1.0	0.9	0.8 $\pm$ 0.2				
IL-1 α	0.8	1.0	1.4	1.1 $\pm$ 0.2	0.6	1.0	0.9	0.8 $\pm$ 0.1				
IL-1 β	2.0	2.7	3.2	2.6 $\pm$ 0.4*	0.5	1.1	1.0	0.8 $\pm$ 0.2				
IL-2	1.7	1.3	2.1	1.7 $\pm$ 0.2*	1.0	0.6	1.7	1.1 $\pm$ 0.3				
IL-3	2.1	1.3	2.0	1.8 $\pm$ 0.3*	0.7	0.7	1.7	1.0 $\pm$ 0.3				
IL-4	2.8	2.7	2.4	2.6 $\pm$ 0.1*	0.7	1.6	1.3	1.2 $\pm$ 0.3				
IL-6	4.6	2.3	2.2	3.0 $\pm$ 0.8*	1.4	0.7	1.3	1.1 $\pm$ 0.2				
IL-6sR	2.4	2.3	2.6	2.4 $\pm$ 0.1*	0.3	1.6	1.9	1.3 $\pm$ 0.5				
IL-7	3.9	2.5	3.7	3.4 $\pm$ 0.4*	1.1	1.7	0.8	1.2 $\pm$ 0.3				
IL-8	3.4	3.0	3.8	3.4 $\pm$ 0.2*	2.7	1.6	2.1	2.1 $\pm$ 0.3*				
IL-10	1.2	0.8	1.3	1.1 $\pm$ 0.1	1.2	0.9	1.0	1.0 $\pm$ 0.1				
IL-11	1.2	1.1	0.8	1.0 $\pm$ 0.1	0.2	1.0	0.6	0.6 $\pm$ 0.2				
IL-12p40	0.6	1.1	1.1	0.9 $\pm$ 0.2	0.2	1.0	0.8	0.7 $\pm$ 0.3				
IL-12p70	0.4	1.3	0.9	0.9 $\pm$ 0.3	0.8	0.7	1.0	0.9 $\pm$ 0.1				
IL-13	1.7	1.3	1.3	1.4 $\pm$ 0.1*	0.8	0.5	1.3	0.9 $\pm$ 0.2				
IL-15	2.0	1.9	2.4	2.1 $\pm$ 0.2*	1.1	1.0	1.3	1.1 $\pm$ 0.1				
IL-16	4.7	2.6	3.3	3.5 $\pm$ 0.6*	5.5	2.5	1.4	3.2 $\pm$ 1.2*				
IL-17	2.4	3.0	2.2	2.5 $\pm$ 0.3*	1.3	1.0	1.2	1.2 $\pm$ 0.1				
IP-10	2.3	1.5	2.4	2.1 $\pm$ 0.3*	1.0	1.3	1.6	1.3 $\pm$ 0.2				
MCP-1	2.0	2.8	1.5	2.1 $\pm$ 0.4*	0.6	1.9	1.1	1.2 $\pm$ 0.4				
MCP-2	0.9	1.3	1.0	1.1 $\pm$ 0.1	0.4	1.0	1.2	0.9 $\pm$ 0.2				
M-CSF	0.9	1.4	1.3	1.2 $\pm$ 0.2	0.8	0.9	1.3	1.0 $\pm$ 0.2				
MIG	0.8	1.3	1.2	1.1 $\pm$ 0.1	0.4	1.0	1.0	0.8 $\pm$ 0.2				
MIP-1 α	0.7	1.3	1.2	1.1 $\pm$ 0.2	0.5	1.1	1.0	0.9 $\pm$ 0.2				
MIP-1 β	0.7	1.2	1.1	1.0 $\pm$ 0.1	1.5	0.5	0.9	1.0 $\pm$ 0.3				
MIP-1 γ	0.5	0.9	1.3	0.9 $\pm$ 0.2	0.3	0.9	1.3	0.9 $\pm$ 0.3				
RANTES	1.6	1.1	1.0	1.2 $\pm$ 0.2	0.7	0.7	1.5	1.0 $\pm$ 0.3				
TGF- β 1	1.4	1.2	0.8	1.1 $\pm$ 0.2	0.5	0.6	1.4	0.9 $\pm$ 0.3				
TNF- α	1.5	1.2	0.7	1.1 $\pm$ 0.2	1.2	0.7	0.5	0.8 $\pm$ 0.2				
TNF- β	1.2	1.2	1.0	1.2 $\pm$ 0.1	1.0	1.1	0.6	0.9 $\pm$ 0.2				
sTNFRI	1.1	1.3	1.3	1.2 $\pm$ 0.1	0.4	1.5	0.8	0.9 $\pm$ 0.3				
sTNFRII	0.6	1.3	1.3	1.1 $\pm$ 0.2	0.3	0.6	1.5	0.8 $\pm$ 0.4				
PDGF-BB	0.4	1.0	0.7	0.7 $\pm$ 0.2	0.3	0.5	1.0	0.6 $\pm$ 0.2				
TIMP-2	1.0	1.2	1.0	1.1 $\pm$ 0.1	0.3	0.4	0.7	0.4 $\pm$ 0.1*				

Horseradish peroxidase (HRP)-conjugated antibody served as the positive substrate control at six spots and was also used to identify the membrane orientation. For each spot, the net signal intensity level was determined by subtracting the background signal intensity levels from the total raw signal intensity levels.

ELISA. The level of IL-1 β , IL-6, IL-7 and IL-8 in the culture supernatants was measured using ELISA kits (Biosource International) according to the manufacturer's instructions. The detection limits for IL-1 β , IL-6, IL-7 and IL-8 were 1, 2, 9 and 5 pg ml⁻¹, respectively. All results were expressed as pg ml⁻¹.

cDNA microarray. The relative mRNA expression of the cytokines was analysed using the GEArray Q series Human Inflammatory Cytokines and Receptors Gene Array (SuperArray) according to the manufacturer's instructions. In brief, 1 μ g mRNA was reverse-transcribed into cDNA by using the AmpoLabelling-LPR kit (Superarray) in the presence of biotin-16-dUTP (Roche). The 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 conjugate (1:12 500), the array image was developed with CDPStar chemiluminescent substrate (Superarray) and recorded on X-ray film. The image was scanned into raw data by a scanner and analysed by Scanalyze software and GEArray Analyser software (Superarray). The signal from the expression of each gene on the array was normalized to the signal derived from an internal cyclophilin A standard on the same membrane.

RT-PCR. The expression of mRNA for IL-8 was determined by RT-PCR by using a One-step RT-PCR kit (Qiagen) according to the manufacturer's protocol. The primers for IL-8 were as follows: sense, 5'-ATG ACT TCC AAG CTG GCC GTG GCT-3'; antisense, 5'-TCT CAG CCC TCT TCA AAA ACT TCT-3'. The primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: sense, 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'; antisense, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' (Zhu et al., 2004). The thermocycling programme of the one-step RT-PCR consisted of reverse transcription at 50 °C for 30 min, an initial denaturation at 94 °C for 3 min, 35 cycles (denaturation at 94 °C for 40 s, annealing at 58 °C for 40 s, and elongation at 72 °C for 60 s), and a final 6 min elongation at 72 °C. The PCR products were visualized by ethidium bromide after electrophoresis on a 2% agarose gel.

RESULTS AND DISCUSSION

The cytokine levels in the culture supernatants of dermatophyte-infected cells were determined using membrane arrays containing 40 different anti-cytokine antibodies. Fig. 1 provides an overview of the cytokine secretion patterns in PHK16-0b cells infected with *A. benhamiae* isolate AB1 or *T. tonsurans* isolate TT1. Uninfected cells secreted only trace or undetectable amounts of cytokines. Table 1 shows the collected data of cytokine secretion profiles of PHK16-0b keratinocytes obtained with three isolates of each dermatophyte species. The keratinocytes responded to the two dermatophyte infections with different cytokine secretion profiles. *A. benhamiae* infection of keratinocytes resulted in a significant secretion of cytokines, including proinflammatory cytokines (IL-1 β , IL-6, IL-6sR

*Significant difference ($P < 0.05$) versus the control as determined by a Mann-Whitney U test.

Fig. 2. ELISA detection of cytokines secreted by PHK16-0b keratinocytes after infection with dermatophyte species. PHK16-0b cells (1×10^6 cells per dish) were infected with three isolates each of *A. benhamiae* and *T. tonsurans* at an m.o.i. of 1. The keratinocyte culture supernatants were collected after 24 h. Levels of IL-1β (a), IL-6 (b), IL-7 (c) and IL-8 (d) were measured by ELISA. The values represent the mean $\pm$ SEM from three independent experiments. Each experiment was done in duplicate. Statistical significance was determined using the two-tailed Student's *t* test. * $P < 0.05$.

and IL-17), chemokines (IL-8, MCP-1, eotaxin and eotaxin-2), immunomodulatory cytokines (IL-7, IL-15, IL-16 and IP-10), and colony-stimulating factors [granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF)]. In contrast, human keratinocytes infected with *T. tonsurans* secreted only a limited number of cytokines (eotaxin-2, IL-8 and IL-16). Consistent with the antibody array data, ELISA revealed that *A. benhamiae* stimulated significantly the secretion of IL-1β, IL-6 and IL-7 from keratinocytes in a species-specific manner, while none of the *T. tonsurans* strains showed an increased secretion of these cytokines (Fig. 2). Both *A. benhamiae* and *T. tonsurans* showed an enhanced IL-8 secretion, regardless of the strain. When the cytokine response of keratinocytes was compared between two growth phases, exponential phase (3–5 days' culture) and stationary phase (2 weeks' culture), of each dermatophyte species, no difference was seen in IL-6 and IL-8 release from keratinocytes in both species (data not shown), indicating that the metabolic state of dermatophytes does not influence the cytokine response. NHEK cells were also used as more relevant cells for determining the cytokine secretion in response to *A. benhamiae* or *T. tonsurans*. The cytokine profiles of NHEK cells were essentially identical to those obtained with PHK16-0b cells (Table 2). cDNA microarray

analysis was performed to confirm the pattern of cytokine expression that was induced by the two dermatophytes (Table 3). *A. benhamiae* infection induced greater than twofold upregulation of 48 cytokine-related genes compared

Fig. 3. IL-8 mRNA expression in cultured PHK16-0b keratinocytes during a dermatophyte infection. PHK16-0b cells (1×10^6 cells per dish) were infected for 3 h with *A. benhamiae* or *T. tonsurans* at an m.o.i. of 1. The mRNA was extracted and IL-8 mRNA expression was analysed by RT-PCR. GAPDH was used as the internal mRNA control.

Table 2. Major differences of cytokine secretion in NHEK cells infected with dermatophyte species

NHEK cells (1×10^6 cells per dish) were infected for 24 h with two isolates each of *A. benhamiae* (isolates AB1 and AB2) and *T. tonsurans* (isolates TT1 and TT2) at an m.o.i. of 1. Levels of cytokines released from keratinocytes were analysed by using the Human Inflammation Antibody Array. Data are presented as the average fold increase relative to the control obtained from two experiments.

Cytokine	Average fold increase relative to control	
	<i>A. benhamiae</i>	<i>T. tonsurans</i>
Eotaxin	2.8	1.0
Eotaxin-2	3.7	2.2
G-CSF	2.3	1.2
GM-CSF	3.1	1.0
ICAM-1	2.2	1.2
IFN- γ	2.3	1.0
IL-1 β	2.8	1.2
IL-2	1.5	1.1
IL-3	1.8	1.0
IL-4	2.3	1.1
IL-6	3.2	1.2
IL-6sR	2.2	1.2
IL-7	3.5	1.1
IL-8	3.1	2.4
IL-13	1.5	1.0
IL-15	1.9	1.0
IL-16	3.2	2.4
IL-17	2.3	1.1
IP-10	2.0	0.9
MCP-1	2.2	1.1
TIMP-2	1.0	0.5

to the non-infected cells, while *T. tonsurans* infection induced the differential regulation of 12 genes (four genes were upregulated and eight genes were down-regulated). Notable characteristics of these patterns included features that appeared to reflect the inflammatory response, immune response, tissue remodelling and wound healing in *A. benhamiae* infection, and features that reflected the insufficient inflammatory response to *T. tonsurans* infection.

Inflammatory cytokine response to *A. benhamiae* infection

Proinflammatory cytokines and chemokines play a central role in defining the nature of the inflammatory infiltrate in the skin (Baumann & Gauldie, 1994). We observed that *A. benhamiae*-infected keratinocytes secreted a variety of proinflammatory cytokines (IL-1 β , IL-6, IL-6sR and IL-17) and chemokines (IL-8, MCP-1, eotaxin and eotaxin-2) (Fig. 1, Table 1). cDNA microarray analysis and RT-PCR confirmed the enhanced expression of IL-1 β , IL-6, IL-17 and IL-8 genes in *A. benhamiae*-infected keratinocytes (Fig. 3,

Table 3). IL-1 is a pivotal proinflammatory cytokine that induces the expression of vascular endothelial cell adhesion molecules (Sauder, 1989). IL-8 has been described as a potent chemoattractant for neutrophils (Kemeny *et al.*, 1994). The CC chemokines, such as MCP-1 and eotaxins, probably contribute to the chemotaxis of the CC chemokine receptor 3 (CCR3)-expressing eosinophils, macrophages and Th2 lymphocytes into the skin (Chinen & Shearer, 2005; Gu *et al.*, 2000; Horuk & Ng, 2000). It has been demonstrated that IL-17 stimulates the production of IL-6, IL-8, GM-CSF and prostaglandin E2 (PGE2) in epithelial, endothelial and fibroblastic cells, and induces intracellular adhesion molecule (ICAM)-1 (CD54) expression on fibroblasts (Fossiez *et al.*, 1996; Jovanovic *et al.*, 1998; Schwarzenberger *et al.*, 1998). Taken together, it is probable that these keratinocyte-derived cytokines contribute to the inflammatory cell infiltration in the skin during *A. benhamiae* infection.

Immunomodulatory cytokine response to *A. benhamiae* infection

Cell-mediated immune responses play a critical role in the eradication of dermatophyte infection, while humoral immune responses are non-protective. Our study demonstrated that *A. benhamiae*-infected keratinocytes secreted T-cell trophic cytokines, such as IL-7, IL-15, IL-16 and IP-10 (Fig. 1, Table 1). cDNA microarray analysis confirmed the upregulation of IL-15, IL-16 and IP-10 genes in *A. benhamiae*-infected keratinocytes (Table 3). Keratinocyte-derived IL-7 is considered to play a significant role in T-cell trafficking (Moller *et al.*, 1996; Takashima *et al.*, 1995). IL-16 is a chemoattractant for CD4⁺ T cells (Cruikshank *et al.*, 1991). Human IP-10, which is a CXC chemokine, differs from most chemokines in its apparent specificity for activated T lymphocytes (Loetscher *et al.*, 1996). Additionally, IP-10 selectively enhances antigen-stimulated IFN- γ synthesis by human T cells, but not that of IL-4 (Deng *et al.*, 1994). Keratinocytes have been reported to be the main source of IL-15, which induces IFN- γ production by T cells and natural killer (NK) cells (Ohteki *et al.*, 2001). We also observed that *A. benhamiae* infection induced the secretion of both Th1 (IL-2) and Th2 (IL-4 and IL-13) cytokines from keratinocytes. These findings suggest that the T-cell trophic cytokines derived from keratinocytes may participate in the predominant induction of a cell-mediated immune response against *A. benhamiae* infection. Although cDNA microarray analysis showed an enhanced expression of IL-12 mRNA, no significant secretion of this protein was seen in *A. benhamiae*-infected keratinocytes. This may be due to post-transcriptional mechanisms (Smith *et al.*, 2003).

A. benhamiae-induced secretion of cytokines involved in tissue remodelling

Certain keratinocyte-derived cytokines, including IL-6 and IL-6sR, may play a role in tissue remodelling and wound healing (Kishimoto *et al.*, 1992). We found that *A. benhamiae*-infected keratinocytes secreted IL-6 and IL-

Table 3. Cytokine gene expression profiles in PHK16-0b keratinocytes infected with dermatophyte species

Keratinocyte monolayers (1×10^6 cells per dish) were infected for 3 h with *T. tonsurans* or *A. benhamiae* at an m.o.i. of 1. Differential expressions of transcripts were determined by calculating the fold change between the signal intensity values from the infected cells and the corresponding control cells. The upregulated genes are represented in bold and the downregulated ones in italic.

Category	Symbol	Description	Fold change	
			<i>A. benhamiae</i>	<i>T. tonsurans</i>
Chemokine receptors	BLR1	<i>Homo sapiens</i> Burkitt's lymphoma receptor 1, GTP-binding protein (BLR1)	1.0	0.6
	CCR1	Chemokine (C-C motif) receptor 1	1.0	1.0
	CCR2	Chemokine (C-C motif) receptor 2	1.8	1.7
	CCR3	Chemokine (C-C motif) receptor 3	3.2	1.1
	CCR4	Chemokine (C-C motif) receptor 4	6.4	0.8
	CCR5	Chemokine (C-C motif) receptor 5	4.2	1.1
	CCR6	Chemokine (C-C motif) receptor 6	2.7	0.3
	CCR7	Chemokine (C-C motif) receptor 7	1.9	0.4
	CCR8	Chemokine (C-C motif) receptor 8	2.9	1.9
	CCR9	Chemokine (C-C motif) receptor 9	1.3	1.4
	CCXCR1	<i>Homo sapiens</i> chemokine (C motif) XC receptor 1 (CCXCR1)	2.0	1.4
	CX3CR1	Chemokine (C-X3-C) receptor 1	3.8	1.0
	CXCR4	Chemokine (C-X-C motif), receptor 4 (fusin)	3.3	0.5
	IFN- γ	Interferon, gamma	2.0	1.1
Interleukins and receptors	IL-10	Interleukin 10	2.5	0.1
	IL-10R α	Interleukin 10 receptor, alpha	2.0	0.6
	IL-10R β	Interleukin 10 receptor, beta	1.7	1.4
	IL-11	Interleukin 11	2.4	1.7
	IL-11R α	Interleukin 11 receptor, alpha	3.5	1.0
	IL-12A	Interleukin 12A, p35	4.1	0.7
	IL-12B	Interleukin 12B, p40	2.8	0.8
	IL-12R β 1	Interleukin 12 receptor, beta 1	2.5	0.6
	IL-12R β 2	Interleukin 12 receptor, beta 2	2.2	0.6
	IL-13	Interleukin 13	0.6	0.1
	IL-1 β	Interleukin 1, beta	2.0	2.3
	IL-16	Interleukin 16 (lymphocyte chemoattractant factor)	2.3	4.0
	IL-15	Interleukin 15	2.6	1.5
	IL-15R α	Interleukin 15 receptor, alpha	3.3	1.0
	IL-13R α 1	Interleukin 13 receptor, alpha 1	3.1	2.0
	IL-17	Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	2.9	0.5
	IL-17R	<i>Homo sapiens</i> IL-17 receptor mRNA	1.1	1.1
	IL-18	Interleukin 18 (interferon-gamma-inducing factor)	1.5	0.8
	IL-18R1	Interleukin 18 receptor 1	2.5	1.4

Table 3. cont.

Category	Symbol	Description	Fold change	
			<i>A. benhamiae</i>	<i>T. tonsurans</i>
Chemokines	IL-1 α	Interleukin 1, alpha	1·1	1·6
	IL-13R α 2	Interleukin 13 receptor, alpha 2	9·1	3·4
	IL-1R1	Interleukin-1 receptor type I	1·7	1·3
	IL-1R2	Interleukin-1 receptor type II	2·7	1·7
	IL-2	Interleukin 2	2·2	0·5
	IL-20	Interleukin 20	2·2	1·1
	IL-21	<i>Homo sapiens</i> interleukin 21 (IL21)	1·5	1·3
	IL-25	Likely orthologue of mouse interleukin 25	0·6	1·3
	IL-2R α	Interleukin 2 receptor, alpha	1·8	1·2
	IL-2R β	Interleukin 2 receptor, beta	1·6	1·5
	IL-2R γ	Interleukin 2 receptor, gamma (severe combined immunodeficiency)	1·8	1·5
	IL-4	Interleukin 4	2·3	1·4
	IL-5	Interleukin 5 (colony-stimulating factor, eosinophil)	1·7	1·2
	IL-5R α	Interleukin 5 receptor, alpha	1·5	1·0
	IL-6	Interleukin 6 (interferon, beta 2)	2·3	0·9
	IL-6R	Interleukin 6 receptor	2·2	1·5
	IL-6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	1·1	0·9
	IL-9	Interleukin 9	0·8	1·1
	IL-9R	Interleukin 9 receptor	1·1	0·9
	LEP	Leptin (murine obesity homologue)	1·9	1·0
	LT α	Lymphotoxin-alpha (TNF superfamily, member 1)	2·6	0·8
	LT β	Lymphotoxin-beta	1·9	1·0
	LT β R	<i>Homo sapiens</i> lymphotoxin beta receptor (TNFR superfamily, member 3) (LTBR)	1·5	1·0
	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	1·2	1·1
	SCYA1	Small inducible cytokine A1 (I-309, homologous to mouse Tca-3)	1·5	1·1
	SCYA11	Small inducible cytokine subfamily A (Cys-Cys), member 11 (eotaxin)	1·2	1·1
	SCYA13	Small inducible cytokine subfamily A (Cys-Cys), member 13	1·5	1·1
	SCYA14	Small inducible cytokine subfamily A (Cys-Cys), member 14	3·3	0·6
	SCYA15	Small inducible cytokine subfamily A (Cys-Cys), member 15	2·1	0·7
	SCYA16	Small inducible cytokine subfamily A (Cys-Cys), member 16	2·7	1·0
	SCYA17	Small inducible cytokine subfamily A (Cys-Cys), member 17	3·0	0·8
	SCYA18	Small inducible cytokine subfamily A (Cys-Cys), member 18, pulmonary and activation-regulated	1·3	1·0
	SCYA19	Small inducible cytokine subfamily A (Cys-Cys), member 19	2·3	1·2
	SCYA2	Small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je)	1·5	1·0
	SCYA20	Small inducible cytokine subfamily A (Cys-Cys), member 20	1·0	1·1
	SCYA21	Small inducible cytokine subfamily A (Cys-Cys), member 21	2·0	0·8
	SCYA22	Small inducible cytokine subfamily A (Cys-Cys), member 22	1·9	0·7
	SCYA23	Small inducible cytokine subfamily A (Cys-Cys), member 23	1·7	1·3

Table 3. cont.

Category	Symbol	Description	Fold change	
			<i>A. benhamiae</i>	<i>T. tonsurans</i>
Growth factors	SCYA24	Small inducible cytokine subfamily A (Cys-Cys), member 24	2·4	0·8
	SCYA25	Human chemokine (TECK)	2·4	0·8
	SCYA3	Small inducible cytokine A3 (homologous to mouse Mip-1a)	1·5	1·2
	SCYA4	Small inducible cytokine A4 (homologous to mouse Mip-1b)	1·3	0·9
	SCYA5	Small inducible cytokine A5 (RANTES)	1·6	0·8
	SCYA7	<i>Homo sapiens</i> mRNA for monocyte chemotactic protein-3 (MCP-3)	1·9	0·7
	SCYA8	Small inducible cytokine subfamily A (Cys-Cys), member 8 (monocyte chemotactic protein 2)	2·2	0·6
	SCYB10	Gamma-interferon inducible early response gene (small inducible cytokine subfamily B (Cys-X-Cys)	2·4	0·6
	SCYB11	Small inducible cytokine subfamily B (Cys-X-Cys), member 11	2·5	1·1
	SCYB13	Small inducible cytokine B subfamily (Cys-X-Cys motif), member 13 (B-cell chemoattractant)	1·2	1·1
	SCYB5	Small inducible cytokine subfamily B (Cys-X-Cys), member 5 (epithelial-derived neutrophil-activating)	1·4	0·5
	SCYB6	Human chemokine alpha 3 (CKA-3) mRNA	2·6	0·5
	SCYC1	Small inducible cytokine subfamily C, member 1 (lymphotactin)	2·9	0·7
	SCYC2	Small inducible cytokine subfamily C, member 2	1·6	0·9
	SCYD1	Small inducible cytokine subfamily D (Cys-X3-Cys), member 1 (fractalkine, neurotactin)	1·5	1·4
	SCYE1	Small inducible cytokine subfamily E, member 1 (endothelial monocyte activating)	1·5	1·0
	SDF1	Stromal cell-derived factor 1	3·1	1·0
	SDF2	<i>Homo sapiens</i> mRNA for SDF2	1·3	0·8
	TGF α	Transforming growth factor, alpha	1·6	0·9
	TGF β 1	Transforming growth factor, beta 1	1·1	1·1
	TGF β 2	Transforming growth factor, beta 2	2·3	0·7
	TGF β 3	Transforming growth factor, beta 3	1·9	1·2
	TNF	Tumour necrosis factor (TNF superfamily, member 2)	1·6	0·9
	TNFRSF1A	Tumour necrosis factor receptor superfamily, member 1A	1·6	1·1
	TNFRSF1B	Human tumour necrosis factor receptor 2	2·4	0·7

6sR. IL-6 binds to its soluble-form receptor IL-6sR, and the complex associates with two gp130 molecules on the target cells, thus initiating intracellular signalling. The gp130 receptor is expressed ubiquitously, whereas IL-6sR expression is restricted (Kishimoto *et al.*, 1995). It has been demonstrated that IL-6 stimulates keratinocyte proliferation in diseases associated with epidermal hyperplasia and wound healing (Gottlieb, 1988; Krueger *et al.*, 1991). We also found that *A. benhamiae*-infected keratinocytes secreted G-CSF and GM-CSF, both of which can stimulate endothelial cell proliferation and migration and may, therefore, play a role in angiogenesis (Bussolino *et al.*, 1991). These factors also stimulate growth and migration of fibroblast precursor cells (Dedhar *et al.*, 1988) and keratinocytes (Olaniran *et al.*, 1995). Thus, it is likely that these keratinocyte-derived cytokines may participate in tissue remodelling and wound healing during *A. benhamiae* infection.

Insufficient inflammatory cytokine response to *T. tonsurans* infection

T. tonsurans is known to trigger a minimal inflammatory reaction (Wagner & Sohnle, 1995). *T. tonsurans* generally causes an endothrinx pattern of hair invasion and can result in low-grade-inflammatory or non-inflammatory infections (Elewski, 2001; Wagner & Sohnle, 1995). If untreated, non-inflammatory *T. tonsurans* may persist for years, and the host becomes a normal carrier (Babel & Baughman, 1989; Ghannoum *et al.*, 2003). We found that *T. tonsurans* stimulates the secretion of limited cytokines only, including eotaxin-2, IL-8 and IL-16 (Fig. 1, Table 1). It was also found that IL-1 β mRNA accumulation was not associated with the release of significant IL-1 β protein by *T. tonsurans*-infected keratinocytes (Tables 1 and 3), suggesting that post-transcriptional events play an important role in IL-1 β protein production by these cells (Elias *et al.*, 1989). In addition, a significant decrease in tissue inhibitor of metalloproteinase (TIMP)-2 secretion was seen in *T. tonsurans*-infected keratinocytes. TIMP-2 plays a role in tissue remodelling (Saarialho-Kere *et al.*, 1992). This insufficient cytokine production by keratinocytes may be responsible for the minimal inflammatory response in the skin. Infection by *T. rubrum*, which is also an anthropophilic dermatophyte, elicits a lower inflammatory response and is less likely to elicit an intense delayed-type hypersensitivity response (Dahl & Grando, 1994; Schwinn *et al.*, 1995; Wagner & Sohnle, 1995). It has also been reported that the cell wall mannan of *T. rubrum* can inhibit cell-mediated immunity and proliferation of keratinocytes; it also enhances the ability of the organism to overcome the natural host defences (Ikuta *et al.*, 1997). Thus, further studies are required to examine the influence of the cell surface components of *T. tonsurans* on the cytokine secretion profiles of keratinocytes.

Conclusions

Our results indicate that keratinocytes are capable of secreting a broad spectrum of cytokines, including proinflammatory

cytokines, chemokines and immunomodulatory cytokines, in response to *A. benhamiae* infection, while *T. tonsurans* infection induces the production of only a few cytokines. The differential cytokine secretion profiles of keratinocytes in response to a dermatophyte infection may reflect the distinct inflammatory responses in the skin. The molecular mechanisms underlying the upregulation of cytokine production are at present unclear. Keratinocytes recognize pathogens through different pattern recognition receptors, such as Toll-like receptors (TLRs) and the mannose receptor, leading to the production of cytokines and chemokines (Pivarcsi *et al.*, 2005). Further studies will be undertaken to identify the keratinocyte receptors and the fungal components involved in the cytokine secretion. Elucidation of the role of keratinocyte-derived cytokines will provide clues to understanding the host responses during a dermatophyte infection and shed light on the pathogenesis of these fungi.

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