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Expression and function of Toll-like receptors 2 and 4 in human keratinocytes

Andor Pivarcsi[1,2], Laszlo Bodai[1], Bence Réthi[3], Anna Kenderessy-Szabó[1], Andrea Koreck[1], Márta Széll[2], Zsuzsanna Beer[4], Zsuzsanna Bata-Csörgő[1,2], Mária Magócsi[5], Éva Rajnavölgyi[6], Attila Dobozy[1,2] and **Lajos Kemény[1,2]**

[1]Department of Dermatology and Allergology, University of Szeged, 6701 Szeged, Hungary
[2]Dermatological Research Group of the Hungarian Academy of Sciences, 6701 Szeged, Hungary
[3]Department of Immunology, Eötvös Loránd University, 2131 Göd, Hungary
[4]Department of Forensic Medicine, University of Szeged, 6701 Szeged, Hungary
[5]Department of Isotope and Membrane Diagnostics, National Institute of Hematology and Immunology, 1113 Budapest, Hungary
[6]Institute of Immunology, Faculty of Medicine, University of Debrecen, 4040 Debrecen, Hungary

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Abstract

Keratinocytes have the ability to kill pathogenic fungi and bacteria by producing antimicrobial substances. Recent studies suggest that microbial components use signaling molecules of the human Toll-like receptor (TLR) family to transduce signals in various cells. Here we provide evidence that keratinocytes express both TLR2 and TLR4 at the mRNA and protein levels, and show that TLR2 and TLR4 are present in the normal human epidermis *in vivo* and that their expression is regulated by microbial components. The expression of myeloid differentiation protein gene (MyD88), which is involved in the signaling pathway of many TLR, was also demonstrated in keratinocytes. LPS + IFN-γ increased the expression of TLR2 and TLR4 50- and 5-fold respectively. Treatment of keratinocytes with *Candida albicans*, mannan, *Mycobacterium tuberculosis* or LPS with IFN-γ resulted in the activation and nuclear translocation of NF-κB. Inhibition of NF-κB blocked the *Candida*-killing activity of keratinocytes, suggesting that the antimicrobial effect of keratinocytes requires NF-κB activation. LPS + IFN-γ, *C. albicans* (4 *Candida*/KC), peptidoglycan (1 μg/ml) or *M. tuberculosis* extract significantly increased IL-8 gene expression after 3 h of treatment ($P < 0.05$). The increases over the 0-h level were 15-, 8-, 10.8- and 7-fold, respectively. The microbial compound-induced increase in IL-8 gene expression could be inhibited by anti-TLR2 and anti-TLR4 neutralizing antibodies, suggesting that TLRs are involved in the pathogen-induced expression of this pro-inflammatory cytokine. Our findings stress the importance of the role of keratinocytes as a component of innate immunity.

Introduction

The host defense against microbial infections is initiated by the innate immune system, which is rapidly activated before generation of the adaptive immune response. The innate immune system triggers a sequence of events that results in the phagocytosis and killing of pathogens, and concurrently the production of cytokines and chemokines, the activation of leukocytes, and the initiation of adaptive immunity. The recognition of invading pathogens is mediated by germline-encoded receptors that are specific for common constituents of pathogenic microorganisms. These are referred to as pattern recognition receptors (1–3). Pattern recognition receptors recognize conserved molecular structures called pathogen-associated molecular patterns that are relatively invariant within a given class of microorganisms, such as the mannans in the yeast cell wall, the mycobacterial cell wall component lipoarabinomannan, the lipopolysaccharide (LPS) of Gram-negative bacteria and the peptidoglycan (PGN) of Gram-positive bacteria. Mammalian homologues of *Drosophila* Toll, designated Toll-like receptors (TLR), which act as pattern recognition receptors were recently discovered.

Correspondence to: A. Pivarcsi, Department of Dermatology and Allergology, University of Szeged, Korányi fasor 6, 6701 Szeged, Hungary. E-mail: andor@derma.szote.u-szeged.hu

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TLRs play crucial roles in the induction of antimicrobial responses in different cells (3–6). In the past few years, 10 different human TLRs have been identified. Biochemical studies on an *in vitro* model (7) and investigations on mice deficient in TLR4 indicated that TLR4 primarily mediates cellular signaling induced by Gram-negative bacteria (4). Targeted disruption of the TLR4 gene resulted in abrogation of the responses to LPS (4). In humans, common mutations in the TLR4 gene are associated with differences in LPS responsiveness (8). While TLR4 is highly specific for LPS and it is associated with CD14, a co-receptor for LPS, TLR2 is implicated in the recognition of multiple products of Gram-positive bacteria, mycobacteria and yeast. TLR2 is required for pro-inflammatory signaling to lipoteichoic acid, PGN, lipoproteins, lipoarabinomannan and zymosan (9–16). The common downstream signaling pathway of TLR2 and TLR4 leads to the activation of NF-κB through myeloid differentiation protein (MyD88) and IL-1 receptor-associated kinase in various cell types (17–19).

The epidermis, the outermost skin layer, provides the first line of defense against the external environment. The major cell type in the epidermis is the epidermal keratinocyte (20). In addition to forming a physical barrier, keratinocytes have been demonstrated to play important regulatory roles in cutaneous inflammatory and immune responses by producing various kinds of cytokines. Keratinocyte-derived cytokines are pivotal in mobilizing leukocytes from the blood and in signaling other cutaneous cells. In addition to regulating immunologic and inflammatory responses, the epidermal keratinocytes contribute to the protective barrier of the epithelia and participate in the host defense by killing invading microorganisms. In mice, removal of the epidermis by scraping prior to inoculation with the yeast resulted in the invasion of *Candida* pseudohyphae to the dermis, indicating that suprabasal keratinocytes play an important role in the defense against cutaneous *Candida* infection (21). We earlier demonstrated that epidermal keratinocytes have direct candidacidal activity, which can be further increased by UV light (22,23), IL-1 (24), IL-8 and α-melanocyte stimulating hormone (25). IL-1, prostaglandin E_2 and platelet-activating factor have also been demonstrated to be involved in *Candida* killing by human epidermal cells (26), but the mechanism of killing remained unknown. Recently, we have shown that keratinocytes express a new type of mannose binding receptor on their surface, which has a role in the binding and subsequent killing of *C. albicans* (27). Keratinocytes have been shown to produce inducible antimicrobial products such as nitric oxide (NO) (28–30), LL-37 (31), antileukoprotease (32) and β-defensins (33,34). Keratinocyte-derived NO and antimicrobial peptides in the epidermis might be responsible for the killing of invading pathogens in the epidermis, and for the prevention of systemic invasion by microbes (33,35).

In addition to these direct antimicrobial effects, keratinocytes may be involved in the killing of pathogens by attracting professional killer cells such as neutrophil granulocytes into the epidermis, which is always a characteristic feature of acute *Candida*, staphylococcal and mycobacterial infections of the skin.

Recently, Song *et al*. have demonstrated that human keratinocytes express functional TLR4 and CD14. On contrary, Kawai *et al*. could not detect TLR4 mRNA or protein expression in cultured keratinocytes (26,37) and they supposed that the effect of LPS on keratinocytes might be due to non-LPS bacterial components contaminating commercial LPS preparations.

In this study, we provide evidence that cultured keratinocytes express TLR2, TLR4 and MyD88. The expression of TLR4 and TLR2 was increased by LPS + IFN-γ. Our results demonstrate that the activation and nuclear translocation of NF-κB occurs in keratinocytes in response to various microbes and microbial components, and that this activation is required for the killing of *C. albicans*. Moreover, we show that the expression of IL-8 is induced in the epidermal keratinocytes in response to pathogenic microorganisms and that the induction of IL-8 is dependent on TLRs. We showed that the effect of LPS on keratinocytes is dependent on TLR4, while the effect of PGN is dependent on TLR2.

Methods

Cells and reagents

Human epidermal cells were obtained from healthy individuals undergoing plastic surgery after informed consent according to Institutional Review Board protocol. After removal of the s.c. tissue and much of the reticular dermis, the tissue samples were cut into small strips and incubated in Dispase solution (grade II; Roche Diagnostics, Mannheim, Germany) overnight at 4°C. On the following day, the epidermis was peeled off the dermis. The epidermis was incubated in 0.25% trypsin solution (Sigma, St Louis, MO) at 37°C for 30 min and aspirated using a Pasteur pipette to aid cell dissociation. The number of epidermal cells and the percentage viability were determined with a hemocytometer. The viability of the cells was always >95% as determined by Trypan blue exclusion. A suspension of primary epidermal cells was prepared in keratinocyte serum-free medium (Keratinocyte-SFM; Life Technologies, Copenhagen, Denmark) supplemented with antibiotic/antimycotic solution (Sigma). Epidermal cells were seeded into 75 cm^2 tissue culture flasks (Corning, Corning, NY) at a density of 4×10^4 cells/cm^2 in Keratinocyte-SFM. Human epidermal keratinocytes were cultured in Keratinocyte-SFM in a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. In our experiments, third-passage keratinocytes were used at 70–80% confluence. At this stage, at least 99% of the cells in the culture were keratinocytes as defined by immunocytochemical staining with anti-human cytokeratin. The tissue culture dishes and pipettes used in our experiments were free of endotoxin contamination, according to the manufacturer.

HaCaT keratinocytes (kindly provided by Dr N. E. Fusenig, Heidelberg, Germany) were grown in 75 cm^2 cell culture flasks and maintained in high glucose DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies), 20 mM L-glutamine and antibiotic/antimycotic solution (Sigma) at 37°C in a humidified atmosphere containing 5% CO_2. The FBS was free of LPS, as specified by the manufacturer. The medium was changed every 2 days.

LPS (purified from *Escherichia coli*; 026:B6), IFN-γ and *Staphylococcus aureus* PGN (no. 77140) were purchased

from Sigma. *Mycobacterium tuberculosis* (clinical isolate) was kindly provided by the Department of Microbiology, University of Szeged. *C. albicans* (0656; CBS, Delft, The Netherlands) was cultured on Sabourand agar and transferred to fresh agar 24 h prior to the specified experiments. *M. tuberculosis* cell wall extract was purchased from Human (Gödöllõ, Hungary). This extract was made of the purified filtrate of *M. tuberculosis* cultured on Sauton medium. The extract contains the 19-kDa antigen of *M. tuberculosis*.

Functional-grade neutralizing anti-human TLR2 mAb TL2.1 and anti-human TLR4 HTA125 were purchased from eBioscience (San Diego, CA). Several approaches were taken to demonstrate the specificity of these antibodies to TLR2 and TLR4 (14,38). Purified mouse IgG was used as isotype control (Pharmingen, Franklin Lakes, NJ).

Quantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA concentration was determined by the A_{260} value of the sample. First-strand cDNA was synthesized from 3 μg total RNA in a 20 μl final volume by using a First Strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). After reverse transcription, real-time quantitative PCR was used to quantify the relative abundance of each genes (iCycler IQ Real Time PCR; Bio-Rad, Hercules, CA) using TaqMan probes specific for 18S, IL-8, TLR2 and TLR4. Calibration curves were constructed and linearity was achieved for all primer sets at least in 3 orders of magnitude under the conditions we used in our experiments. All PCR assays were performed in triplicate and the data were pooled. Aliquots of 2 μl of the reverse transcription reactions were used as templates for TLR2, TLR4, MyD88, IL-8 and 18S-specific PCR. The following primer sets and TaqMan probes were used for real-time PCR assays: TLR2 sense: 5′-TTTCACTGCTTTCAACTGGTA-3′, TLR2 anti-sense: 5′-TGGAGAGGCTGATGATGAC-3′, TLR2 TaqMan probe: 5′-FAM/CAAGACCCACACCATCCACAA-BHQ-1/-3′; TLR4 sense: 5′-CGATTCCATTGCTTCTTG-3′, TLR4 anti-sense: 5′-GCTCAGGTCCAGGTTCTT-3′, TLR4 TaqMan probe: 5′-FAM/CAATGCATGGAGCTGAATTTCT-BHQ-1/-3′; MyD88 sense: 5′-AAGTTATTTGTTTACAAACAGCGACCA-3′, MyD88 anti-sense: 5′-GGAAGAATGGCAAATATCGGCT-3′; IL-8 sense: 5′-CCACACTGCGCCAACA-3′, IL-8 anti-sense: 5′-GCATCTTCACTGATTCTTGGAT-3′, IL-8 TaqMan probe: 5′-FAM/CTGGGTGCAGAGGGTTGTGG-BHQ-1/-3′; 18S sense: 5′-CGGCTACCACATCCAAGGAA-3′, 18S anti-sense: 5′-GCTGGAATTACCGCGGCT-3′, 18S TaqMan probe: 5′- TexRed/ TGCTGGCACCAGACTTGCCCTC-BHQ-1/-3′. Reaction conditions were 95°C for 5 min followed by 40 cycles at 95°C for 15 s (denaturation) and 57°C for 45 s (annealing and elongation). Each primer set gave a single product. As controls, we used the reaction mixtures without cDNA. The identity of PCR products amplified by TLR2- and TLR4-specific primer pairs were confirmed by direct sequencing.

Killing assay

Killing assays were performed to evaluate *Candida* killing by keratinocytes as described previously (27). Briefly, 10^6 human keratinocytes (separated as described above) and 4×10^6 *Candida* cells or *Candida* alone in DMEM (control) were incubated for 4 h at 37°C in a volume of 1 ml in the absence or presence of 0.1 or 1 mM sulfasalazine. Sulfasalazine was dissolved in the culture medium and the keratinocytes were incubated with the inhibitor for 30 min prior to the killing assay. After the incubation period, Triton X-100 was added in order to lyse the keratinocytes. *Candida* cells were then stained with methylene blue (0.1%) for 45 min at 4°C and the killed *Candida* cells were counted in a hemocytometer. To determine the accuracy of the methylene blue assay we also determined the c.f.u. (39,40) in parallel with the *C. albicans* cell counts in a separate experiment. A strong correlation was found between the two assays ($r = 0.984$, $P < 0.05$) (27). The number of *Candida* cells killed by the keratinocytes was obtained by subtracting the number of dead *Candida* cells in the controls from the number of dead *Candida* cells observed in the presence of the keratinocytes. The percentage of non-viable *Candida* cells was defined as the killing activity. To evaluate the significance of reduction of keratinocyte *Candida*-killing activity due to the presence of human anti-TLR2 or anti-TLR4, a two-tailed *t*-probe was used.

Immunocytochemistry

Cryostat sections (5 μm) of healthy human skin, fixed for 15 min in cold methanol (–10°C), were incubated overnight at 4°C in a humid chamber with the primary antibodies. Cultured human keratinocytes in the third passage were grown in slide chambers (Nalge Nunc, Roskilde, Denmark) and then fixed in cold methanol. For immunohistochemical staining, goat polyclonal antibodies raised against human TLR2 and TLR4 peptides at 1:100 dilution (clones sc-8689 and sc-8694 respectively; Santa Cruz Biotechnology, Santa Cruz, CA) were used as primary antibodies. Primary antibodies pre-incubated with the specific peptides used to generate the primary antibodies (human anti-TLR2 and anti-TLR4 + peptide) were used as controls. For neutralization, antibodies were combined with a 5-fold (by weight) excess of blocking peptide in a small volume of PBS and then the mixture was incubated for 2 h at room temperature. Immunostaining was performed using an anti-goat avidin–biotin immunoperoxidase kit (Vectastain Elite kit, Vector, Burlingame, CA). 3-Amino-9-ethylcarbazol (Sigma) was used as chromogen. The slides were counterstained with hematoxylin and mounted for light microscopy.

Immunoblotting

Total protein extracts from epidermal keratinocytes and from peripheral blood leukocytes were prepared in a lysis buffer of 1.5% SDS, 62.5 mM Tris–HCl, pH 6.8, 5 mM EDTA, 5% 2-mercaptoethanol, 1 μg/ml antipain, 1 μg/ml chymostatin and 1 μg/ml leupeptin (all from Sigma). Lysates were precleared by centrifugation and supernatants were stored at –20°C. The constituent proteins of the keratinocyte extracts were separated by SDS–PAGE on a 10% separating gel and then transferred to nitrocellulose membranes (Bio-Rad). In order to verify the equivalent loadings of proteins in the wells, the gel and the nitrocellulose was stained with Coomassie Brillant Blue and Ponceau S respectively (Sigma). Membranes were blocked by incubation in Tris-buffered saline (150 mM NaCl, 25 mM Tris pH 7.4), containing 0.05% Tween 20 (Sigma) and 3% non-fat dry milk (Fluka, Neu-Buchs, Switzerland), for 2 h at

room temperature, and subsequently incubated overnight at 4°C with 1:200 dilution of the primary antibodies (goat anti-human TLR2 and TLR4; Santa Cruz Biotechnology). For control staining, pre-immune goat serum was used. Alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma) was used as secondary antibody at 1:2500 dilution in the blocking buffer for 2 h at room temperature. Blots were developed by using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate (Sigma).

ELISA

Human IL-8 was measured at different times after stimulation in the supernatants of third-passage epidermal keratinocytes using the Quantikine human IL-8 immunoassay kit (R & D Systems, Minneapolis, MN) following the manufacturer's instructions. We used serial dilutions of recombinant human IL-8 for standard curves. The optical density of the wells was determined using a microplate reader set at 450 nm.

Isolation of nuclear extracts

Monolayers of keratinocytes or HaCaT cells were washed with ice-cold PBS supplemented with 25 mM NaF and 0.1 mM Na_3VO_4, and the cells were harvested with a cell scraper. The cell suspension was spun down and the pellet was suspended in 400 μl of freshly prepared buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 0.1 mM EGTA, pH 8.0, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain and 1 mM Na_3VO_4). The cell suspension was incubated on ice for 15 min before the addition of 5 μl of 10% NP-40. Cells were centrifuged at 800 r.p.m. for 5 min at 4°C. The nuclear pellet was suspended in 30–50 μl of buffer C (10 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, pH 8.0, 1mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain and 0.1 mM Na_3VO_4) and incubated at 4°C with shaking for 15 min. The nuclear debris was removed by centrifugation at 16,000 *g* for 10 min at 4°C. The protein concentration of each sample was determined with the Lowry assay. All nuclear extracts were stored at –70°C and multiple freeze–thawing cycles were avoided.

Electrophoretic mobility shift assay

Double-stranded 22mer oligonucleotides (30 pmol) containing the NF-κB (GGGGACTTTCCC) consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA) were 5′-end-labeled by end-filling with [γ-^{32}P]ATPs (ICN, Costa Mesa, CA) using 20 U of T4 kinase (Promega Madison, WI) at 37°C for 15 min. Labeled oligonucleotides were separated from unincorporated free [γ-^{32}P]dATP by chromatography on Sephadex G-25 columns. DNA-binding reactions were performed in a final volume of 20 μl. NF-κB-binding reactions contained 5 μg of nuclear proteins and 1 μg of poly(dI–dC) (Amersham Pharmacia Biotech, Little Chalfont, UK) in the presence of 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT and 4% glycerol. The binding reaction was carried out at room temperature for 30 min. After incubation, the binding reactions were loaded on 5% native polyacrylamide/0.5 × TBE (45 mM Tris–borate and 1 mM EDTA) gels, pre-run in 0.5 × TBE for 60 min at 100 V. The gels were then dried onto chromatography paper (Whatman 3M) and exposed to autoradiography film.

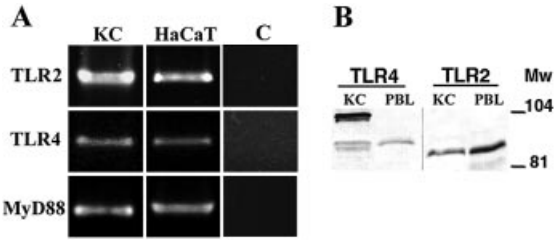

Fig. 1. TLR2, TLR4 and MyD88 are expressed in keratinocytes. (A) The expression of TLR2, TLR4 and MyD88 mRNA was examined by RT-PCR with specific primer sets in cultured keratinocytes and in the HaCaT keratinocyte cell line (C: negative control). (B) TLR2 and TLR4 proteins are present in keratinocytes. Keratinocyte total cellular extracts were analyzed by Western blotting with human anti-TLR2 and anti-TLR4 antibodies under reducing conditions. Cellular extract from peripheral blood leukocytes was used as a positive control for human TLR2 and TLR4.

Results

Human epidermal keratinocytes express TLR2, TLR4 and MyD88

Expression of TLR2 and TLR4 was examined in keratinocyte cultures and in the immortalized keratinocyte cell line, HaCaT, using RT-PCR, Western blot analyzes and immunohistochemistry. Both TLR2 and TLR4 mRNAs were expressed in keratinocytes and HaCaT keratinocytes (Fig. 1A). To ensure that the bands correspond to the expected cDNAs, the identities of the amplified fragments were verified by sequencing (data not shown).

The presence of TLR2 and TLR4 proteins in the keratinocytes was demonstrated by Western analyses (Fig. 1B). Cellular extract from peripheral blood leukocytes was used as a positive control for human TLR2 and TLR4.

The expression and localization of TLR2 and TLR4 in cultured keratinocytes and skin were examined by immunostaining (Fig. 2). Both types of TLR were found to be present on cultured human keratinocytes in the third passage (Fig. 2). The staining of skin sections revealed that TLR2 and TLR4 are present throughout the epidermis *in vivo*, but the expression of TLR2 was more pronounced in the suprabasal layers (Fig. 2). We found that both TLR2 and TLR4 are also expressed in the hair follicles (Fig. 2).

Since TLR2 and TLR4 use MyD88 to activate NF-κB, we investigated whether MyD88 mRNA is expressed in unstimulated keratinocytes and HaCaT cells. RT-PCR results demonstrated that keratinocytes and HaCaT cells expressed MyD88 mRNA (Fig. 1A).

LPS + IFN-γ induces the expression of TLR2 and TLR4 in cultured keratinocytes

To determine whether the expression of TLR2 and TLR4 could be regulated by pathogenic microorganisms, cultured keratinocytes were stimulated with LPS + IFN-γ (500 ng/ml + 10 ng/ml), *C. albicans* (4 *Candida*/KC), PGN (1 μg/ml) or

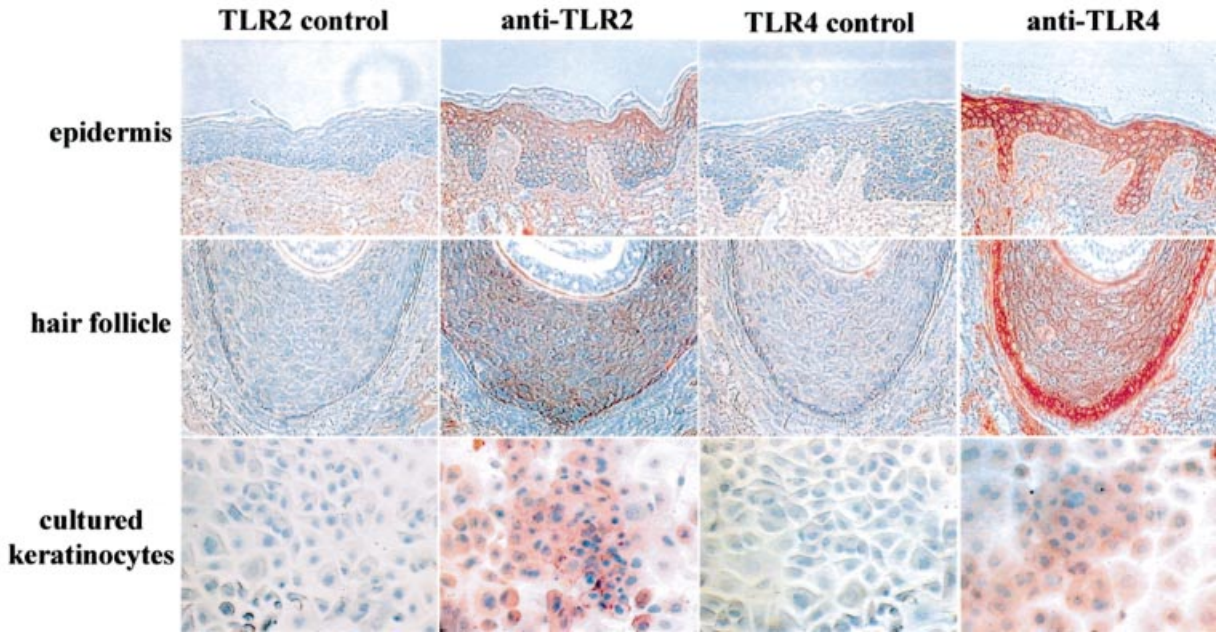

Fig. 2. Immunohistochemical analyzes of TLR2 and TLR4 in human epidermis, hair follicles and third-passage cultured keratinocytes. Slides were stained with goat polyclonal anti-human TLR2 and TLR4 antibodies targeted to TLR2- and TLR4-specific epitopes. Absorption with the specific peptide used to generate the primary antibodies was used as control. The normal human epidermis exhibited positive staining both for TLR2 and TLR4. Intense staining was observed in the hair follicles, suggesting a high level of TLR2 and TLR4 expression at these sites. Cultured keratinocytes also displayed positive staining for TLR2 and TLR4. Magnification: ×40.

M. tuberculosis extract (1 μg/ml) for 0, 3, 6, 12 or 24 h (n = 3; Fig. 3). The levels of TLR2 and TLR4 mRNAs were analyzed by quantitative real-time RT-PCR using Taqman probes specific for TLR2 and TLR4. Treatment of cultured keratinocytes with LPS + IFN-γ increased the expression level of the TLR4 gene. At 6 h after the treatment with LPS + IFN-γ, the increase in TLR4 expression was 3-fold (Fig. 3). At 24 h after LPS + IFN-γ treatment the TLR4 mRNA level was 5-fold higher than at 0 h (P = 0.006). Stimulation with *C. albicans*, PGN or *M. tuberculosis* extract did not increase the expression of TLR4 significantly.

LPS + IFN-γ caused an 8-fold increase in the level of TLR2 mRNA 6 h after stimulation (P = 0.003). At 12 h after the treatment, the increase in TLR2 expression was 26-fold (P = 0.003) and it reached a >60-fold value at 24 h (P = 0.0002). Expression of TLR2 gene was not changed significantly by treatments with *C. albicans*, PGN or *M. tuberculosis* extract (Fig. 3). LPS alone also induced the expression of TLR2 and TLR4 in keratinocytes, but the induction was somewhat lower as compared to that of LPS + IFN-γ (data not shown).

Microbial compounds and C. albicans induce nuclear translocation of NF-κB in cultured keratinocytes and in HaCaT cells

Binding of microbial components directly to TLR or to receptors associated with TLR is known to activate NF-κB in different cell types. Therefore we were interested in whether bacteria and fungi could also induce nuclear translocation of NF-κB in keratinocytes. Human epidermal keratinocytes and HaCaT keratinocytes at 70–80% confluence were stimulated with *C. albicans* (4:1 *Candida*/KC), heat-killed *M. tuberculosis*

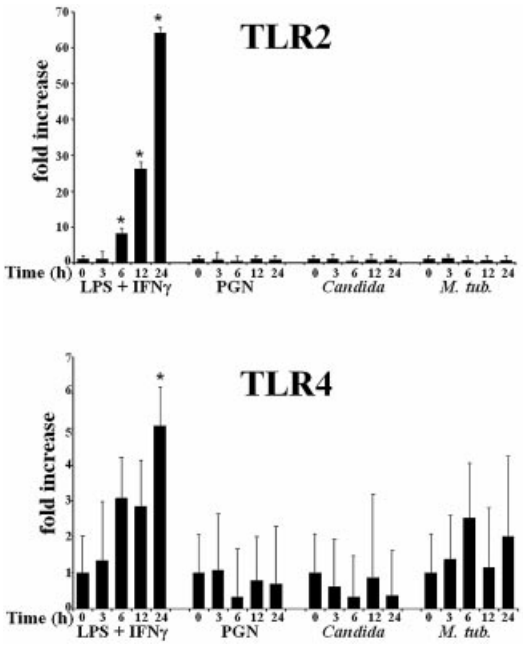

Fig. 3. LPS + IFN-γ regulate the expression of TLR4 and TLR2. Expression of TLR2 and TLR4 genes was analyzed by quantitative real-time RT–PCR using TaqMan probes. Total RNA from third-passage keratinocytes treated with LPS + IFN-γ, heat-killed *C. albicans*, PGN or *Mycobacterium* extract for specified times was reverse transcribed and subjected to real-time RT–PCR. The levels of TLR4 and TLR2 mRNAs were normalized to the 18S rRNA level, and are presented as fold increases over the 0-h values. The chart shows the mean ± SE of three independent experiments (*P < 0.05, Student's t-test).

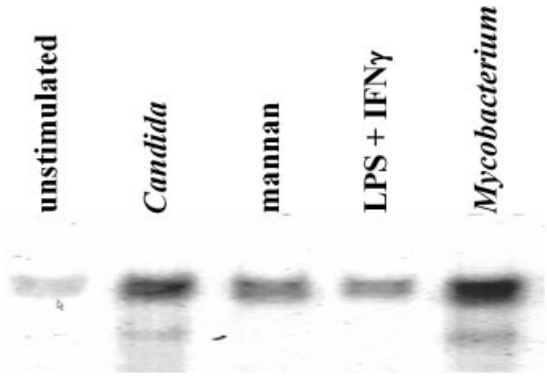

Fig. 4. Microbial compounds and *C. albicans* activate NF-κB in keratinocytes. Activation of NF-κB was examined by electromobility shift assay in cultured keratinocytes at the third passage in response to various microbial compounds. Keratinocytes were stimulated with LPS + IFN-γ, *C. albicans*, mannan and *M. tuberculosis* for 2 h. Nuclear extracts were prepared from unstimulated and stimulated cells, and NF-κB DNA-binding activity was measured by electrophoretic mobility shift assay with a radiolabeled probe containing the consensus NF-κB-binding site. Results of one typical experiment out of three.

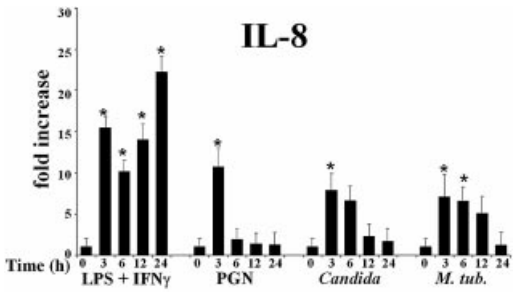

Fig. 5. Microbial compounds and *C. albicans* induce the expression of IL-8 in keratinocytes. We determined the effects of LPS + IFN-γ, *C. albicans*, *Staphylococcus aureus* PGN and *Mycobacterium* extract on IL-8 mRNA expression in keratinocytes. Total RNAs were extracted and reverse transcribed at 0, 3, 6, 12 and 24 h after the specified treatments. The levels of IL-8 and the internal control (18S rRNA) were analyzed by quantitative real-time RT-PCR with TaqMan probes. IL-8 expression values are expressed as fold increases over the 0-h values. The chart shows the mean ± SE of three independent experiments (*$P < 0.05$, Student's *t*-test).

(8:1 bacteria/KC) or LPS together with IFN-γ (500 ng/ml + 10 ng/ml) for 2 h. We found that the unstimulated keratinocytes contained a low, but detectable, basal level of NF-κB activity (Fig. 4), which is consistent with the data of other authors (41). Exposure of keratinocytes to *C. albicans* or heat-killed *M. tuberculosis* led to a strong induction of NF-κB both in normal keratinocytes (Fig. 4) and in HaCaT cells (data not shown). Interestingly, the treatment of keratinocytes with LPS + IFN-γ or mannan led to a relatively weak induction of NF-κB.

Microbial compounds and C. albicans induce the production of IL-8 in keratinocytes

It is known that NF-κB is an important regulator of IL-8 (42). We have shown that NF-κB can be induced in keratinocytes by various microbial substances. Therefore, we set out to investigate the effects of microbial components and heat-killed *C. albicans* on the expression of IL-8 in cultured human keratinocytes. Keratinocytes were treated with LPS + IFN-γ (500 ng/ml+10 ng/ml), *C. albicans* (4 *Candida*/KC), PGN (1 μg/ml) and *M. tuberculosis* extract for 0, 3, 6, 12 or 24 h, and the level of IL-8 mRNA was analyzed by quantitative real-time RT-PCR using TaqMan probes ($n = 3$; Fig. 5). LPS + IFN-γ, *C. albicans* (4 *Candida*/KC), PGN (1 μg/ml) or *M. tuberculosis* extract significantly increased ($P < 0.05$) IL-8 gene expression after 3 h treatment (Fig. 3). The increases over the 0-h level were 15-, 8-, 10.8- and 7-fold, respectively (Fig. 3). The maximal increases in IL-8 expression were observed at 3 h, with the exception of LPS + IFN-γ, which induced the highest expression 24 h after the treatment. At 24 h, the expression of IL-8 decreased near to the 0-h level in all samples, with the exception of LPS + IFN-γ (Fig. 3).

Next, we aimed to investigate the effect of LPS + IFN-γ, *C. albicans*, PGN or *M. tuberculosis* extract on the IL-8 production of keratinocytes at the protein level. Samples were taken at the same time points as for the IL-8 RT-PCR experiment and the amount of secreted IL-8 protein in the cell culture supernatants was measured by ELISA ($n = 3$). We found that LPS + IFN-γ and *C. albicans* induced a marked increase in keratinocyte IL-8 production (Fig. 6). The amount of IL-8 started to rise 3 h after the treatment and reached its maximum 24 h after the induction, with the exception of heat-killed *C. albicans*. PGN and *M. tuberculosis* cell wall extract also induced the production of IL-8 in keratinocytes (Fig. 6). LPS alone also induced the expression of IL-8 in keratinocytes, but the induction was somewhat lower as compared to that of LPS + IFN-γ (data not shown).

Microbial compound-induced up-regulation of IL-8 expression is dependent on TLR in keratinocytes

We tested the functional roles of keratinocyte TLR2 and TLR4 in response to microbial compounds and heat-killed *C. albicans*. The addition of LPS (500 ng/ml), PGN (1 μg/ml), *Mycobacterium* extract (1 μg/ml) or heat-killed *C. albicans* (4 *Candida*/KC) resulted in the up-regulation of IL-8 expression in keratinocytes 3 h after the treatment ($n = 3$) (Fig. 6). However, when cultured keratinocytes were preincubated with the anti-TLR2 mAb TL2.1 (10 μg/ml) for 1 h before the addition of PGN or *Mycobacterium* extract, the up-regulation of IL-8 expression was abrogated (Fig. 7). The presence of anti-TLR2 antibodies could not inhibit the cellular responses to *C. albicans*. Anti-TLR4 mAb HTA125 (10 μg/ml) inhibited LPS-induced up-regulation of IL-8 expression in keratinocytes after exposure to LPS, suggesting that TLR4 is required for cell responses to LPS (Fig. 7). Anti-TLR4 antibody also inhibited the *Mycobacterium*-induced up-regulation of IL-8 in keratinocytes.

C. albicans killing of keratinocytes is inhibited by blocking NF-κB with sulfasalazine

We have shown previously that keratinocytes are able to kill the human pathogen yeast *C. albicans* (22). In order to investigate whether NF-κB may be involved in the killing of *C. albicans* by keratinocytes, killing assays were performed in the presence or absence of 0.1 or 1 mM sulfasalazine, an inhibitor

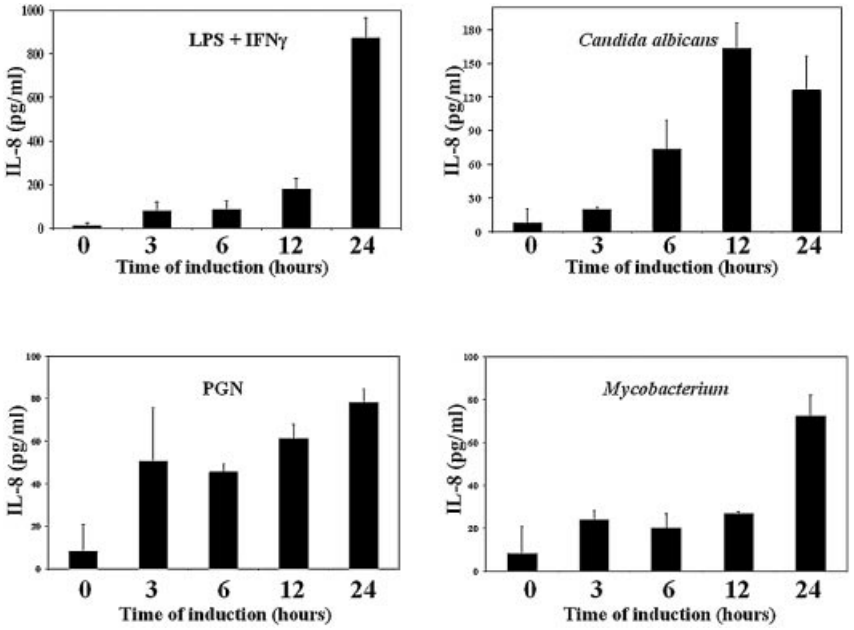

Fig. 6. Microbial compounds and *C. albicans* induce the production of IL-8 in keratinocytes. Third-passage keratinocytes were treated with LPS + IFN-γ, *C. albicans*, *S. aureus* PGN or *M. tuberculosis* extract. The amount of IL-8 in the supernatant medium was measured by Quantikine human IL-8 ELISA. The chart shows the mean ± SE of three independent experiments.

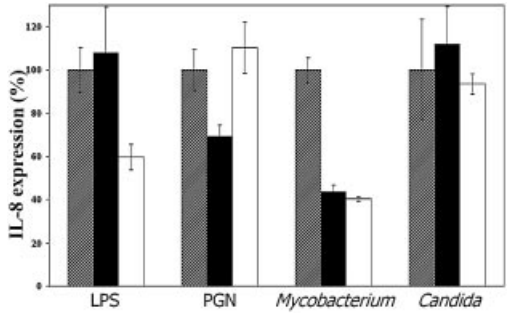

Fig. 7. Blocking of TLR2 and TLR4 inhibits the microbial compound-induced expression of IL-8 in keratinocytes. Cultured keratinocytes were grown on six-well plates and pretreated with anti-TLR2 mAb (filled bars) (10 μg/ml), anti-TLR4 mAb (empty bars) (10 μg/ml) or isotype-matched control mouse IgG antibodies (stripped bars) (10 μg/ml) for 1 h, and then stimulated with LPS, PGN, *M. tuberculosis* extract or *C. albicans* for 3 h. The relative expression of IL-8 was measured by quantitative real-time RT-PCR. PGN-induced IL-8 expression was blocked by anti-TLR2 antibody; LPS-induced IL-8 expression was blocked by anti-TLR4 antibody. Both anti-TLR2 and anti-TLR4 antibodies blocked the effect of *Mycobacterium* extract; none of these antibodies could block the effect of heat-killed *C. albicans*. The IL-8 expression values are expressed relative to the values measured in the mIgG-treated controls. 18S rRNA expression was used as internal control. The chart shows the mean ± SE of three independent experiments.

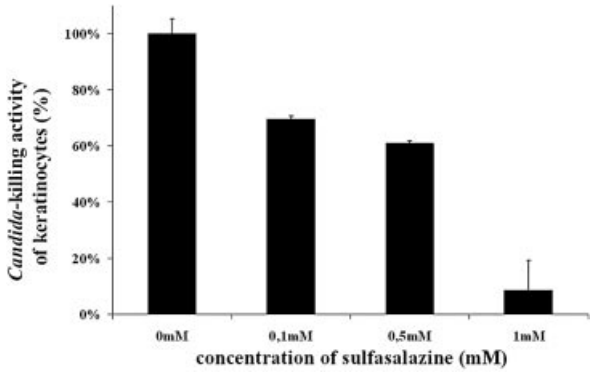

Fig. 8. Inhibition of NF-κB decreases the *Candida*-killing activity of keratinocytes. The presence of sulfasalazine, a specific NF-κB inhibitor, inhibited the killing of *C. albicans* by keratinocytes dose dependently. *Candida*-killing activity is expressed as the percentage of killed yeast cells. The extent of *Candida*-killing activity shows a strong negative correlation (r = –0.96; P < 0.01) with the concentration of sulfasalazine administered (n = 12).

of NF-κB. Incubation of keratinocytes with 0.1 mM sulfasalazine prior to the assay resulted in a 27% decrease in the *C. albicans*-killing activity of keratinocytes compared to the control (Fig. 8). Nearly complete inhibition of the killing activity was observed at 1 mM sulfasalazine (81%). The extent of *Candida*-killing activity shows a strong negative correlation (r = –0.96; P < 0.01) with the concentration of sulfasalazine administered.

Human anti-TLR2 and anti-TLR4 antibodies inhibit the Candida*-killing ability of keratinocytes*

In order to investigate whether TLR2 or TLR4 is involved in the killing of *Candida* by keratinocytes, we performed *Candida*-killing assays in the presence or absence of functional-grade human anti-TLR2 (TL2.1, 10 or 20 μg/ml) or anti-TLR4 (HTA125, 10 or 20 μg/ml) antibodies. Mouse IgG antibodies were used as isotypic controls. We found that both human anti-TLR2 and anti-TLR4 antibodies significantly inhibited the *Candida*-killing activity of keratinocytes (n = 3, P = 0.007 and 0.0005 respectively) (Fig. 9).

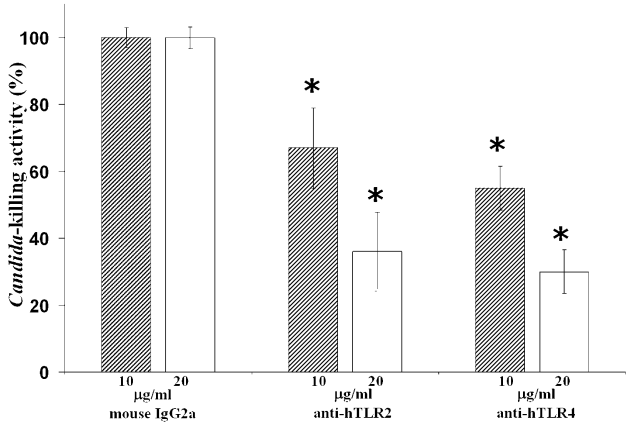

Fig. 9. Blockade of human TLR2 and TLR4 inhibits the *Candida*-killing activity of keratinocytes. Functional-grade human anti-TLR2 or anti-TLR4 antibodies inhibited the killing of *C. albicans* by keratinocytes dose dependently compared to isotype control antibodies. *Candida*-killing activity is expressed as the percentage of killed yeast cells. The chart shows the mean ± SE of three independent experiments (*$P < 0.05$, Student's *t*-test).

Discussion

The epidermis is an important site of microbe–host interactions and the keratinocytes participate in the host defense. Epidermal keratinocytes do not merely form a passive physical barrier, but additionally initiate and regulate the cutaneous inflammation and immune responses by producing cytokines. In addition to their regulatory role, keratinocytes are able to kill invading microorganisms by producing antimicrobial products, such as NO, LL-37 and β-defensins (31,33,35). However, little is known about the receptors responsible for the recognition of pathogens by keratinocytes. We recently demonstrated that a new mannose-binding receptor is expressed by keratinocytes, which is involved in the recognition and subsequent killing of *C. albicans* (27).

The expression of TLR4 on keratinocytes was shown recently by Song *et al*. (37). However, Kawai *et al*. could not detect TLR4 expression in cultured keratinocytes. In our study we demonstrated that TLR4 is expressed in cultured keratinocytes both at the mRNA and protein levels (36). Kawai *et al*. suggested that the effects of LPS on keratinocytes might be mediated by TLR2-dependent recognition of contamination in commercial LPS preparations. Here we show that LPS-induced IL-8 expression in keratinocytes can be inhibited by functional-grade anti-human TLR4 antibodies, suggesting that the effect of LPS on keratinocytes is mediated by TLR4 and not by TLR2. In the present study, we provide experimental evidence that epidermal keratinocytes express TLR2 and the PGN-induced expression of IL-8 is TLR2 dependent.

It is now well established that TLR4 mediates signaling events upon LPS stimulation (4). TLR2, in contrast, has been mainly implicated as the receptor for yeast, Gram-positive bacterial and mycobacterial cell wall components (5,6,9–11). In order to test the function of TLR2 and TLR4 in keratinocytes, blocking experiments were performed with human anti-TLR2 or anti-TLR4 mAb, and the expression of the IL-8 gene was examined after stimulation with microbial compounds (LPS, PGN and *Mycobacterium* extract) and heat-killed *C. albicans*. The anti-TLR2 mAb inhibited IL-8 expression in keratinocytes after exposure to PGN or *Mycobacterium* extract. These data support the hypothesis that TLR2 could play an important role in *in vivo* responses to Gram-positive and mycobacterial bacterial structures. Anti-TLR2 antibody did not block LPS-induced IL-8 expression in keratinocytes, suggesting that the expression of TLR2 (unlike TLR4) is not required for cell responses to LPS. Anti-TLR2 antibodies could not inhibit the cellular responses to *C. albicans* despite the fact that TLR2 is involved in the recognition of yeast cell wall component, mannan. Other receptors than TLRs are known to be expressed in keratinocytes that might also participate in the recognition of this pathogen. One such receptor could be the keratinocyte mannose-binding receptor, as we could show it earlier (27). Binding of the yeast to only one of these receptors may be sufficient for the induction of IL-8 expression and this could explain why anti-TLR2 antibodies alone could not inhibit the up-regulation of IL-8 in keratinocytes after exposure to *C. albicans*.

The anti-TLR4 antibodies inhibited LPS-induced up-regulation of IL-8 expression after exposure to LPS, suggesting that TLR4 is required for cell responses to LPS in keratinocytes. Anti-TLR4 also inhibited the *Mycobacterium*-induced up-regulation of IL-8 in keratinocytes.

With the aid of TLRs, keratinocytes could be the first sensors of various pathogenic microorganisms in the epidermis. CD14, which is known to associate with TLR4, could also be involved in the mediation of LPS effects in keratinocytes. We earlier demonstrated that keratinocytes express CD14 under specific circumstances (43). After the recognition of pathogens, keratinocytes could alarm the immune system by producing pro-inflammatory cytokines and by attracting leukocytes to the site of infection. On the other hand, keratinocytes themselves are able to kill pathogens. Various antimicrobial products and chemokines are expressed by keratinocytes in an inducible manner (29,31,34). The signaling pathway of the TLR family leads to the activation of NF-κB through the adaptor proteins MyD88 and IRAK (3). MyD88 is required for the intracellular signaling of TLR2 and TLR4 (5,6,36,44). We have shown here for the first time that MyD88 mRNA is also expressed in keratinocytes.

Exposure to bacterial compounds such as LPS or lipoarabinomannan purified from *Mycobacterium* or to pro-inflammatory cytokines such as IFN-γ increased the expression of TLR4 in human monocytes and polymorphonuclear leukocytes, but failed to influence the expression of TLR2 in monocytes (45). Interestingly, the gene expression of TLR2 was increased significantly after LPS treatment, while TLR4 was expressed constitutively and remained constant after various stimulations in mouse macrophages (46). We showed that the expression of TLR4 in keratinocytes was increased after stimulation with LPS + IFN-γ, while *C. albicans*, PGN or *M. tuberculosis* extract failed to increase its expression. LPS + IFN-γ also increased the expression of TLR2.

We have also demonstrated that incubation of cultured keratinocytes with LPS + IFN-γ, heat-killed *C. albicans* or *M. tuberculosis* extract resulted in the expression and secretion

of the potent chemokine, IL-8. As IL-8 is an important chemotactic factor for neutrophils and, to a lesser extent, for lymphocytes, we presume that the induction of IL-8 in keratinocytes by pathogens could lead to the recruitment of inflammatory cells to the site of infection (47). Indeed, during acute *Candida*, staphylococcal and even in rare primary *M. tuberculosis* infections, neutrophils are detected in the suprabasal layers of the epidermis (48,49). The most frequent infections of the skin are caused by *S. aureus*, which may cause symptoms in the epidermis (impetigo) or in the hair follicles (folliculitis). The typical histological feature of both impetigo and folliculitis is the presence of numerous neutrophils (48). In addition to attracting leukocytes to the skin, IL-8 has been shown to increase the *Candida* killing activity of keratinocytes (27).

Interestingly, when *Candida*-killing assay was performed in the presence of functional-grade human anti-TLR2 or anti-TLR4 antibodies, the *Candida*-killing activity of keratinocytes decreased. Since these antibodies could not inhibit the *Candida*-induced up-regulation of IL-8 gene expression, we hypothesize that the *Candida*-induced up-regulation of IL-8 expression and the killing of *Candida* are mediated by distinct pathways.

Since only a small fraction of skin pathogens belong to the Gram-negative family of bacteria, we speculate that the function of TLR4 might be less important than that of TLR2 in the epidermis *in vivo*.

NF-κB regulates the expression of a wide variety of genes that play important roles in innate immune responses. Such NF-κB-regulated genes are those encoding cytokines (IL-1, IL-2, IL-6, IL-8 and tumor necrosis factor-α), inducible enzymes (iNOS), antimicrobial peptides (50) and adhesion molecules. Since the expression of both iNOS and antimicrobial peptides is regulated by NF-κB, the pathogen-induced activation of this transcription factor could be an important step toward the killing of pathogens by keratinocytes. We have demonstrated that sulfasalazine, an inhibitor of NF-κB, was able to inhibit the *Candida*-killing activity of keratinocytes. The inhibitory effect of sulfasalazine on the killing of *C. albicans* was observed in the same concentration range as that for the inhibitory effect of sulfasalazine on the NF-κB-binding activity seen by electromobility shift assay (51).

In summary, here we demonstrated that human homologues of *Drosophila* Toll, TLR2 and TLR4, are expressed in epidermal keratinocytes and are involved in pathogen-induced cellular responses. Keratinocyte TLR2 and TLR4 may have important functions in the protection of the skin against pathogenic fungi, and Gram-positive and Gram-negative bacteria. The recognition of pathogens by TLR in keratinocytes leads to the production of the pro-inflammatory cytokine IL-8, which might be responsible for the recruitment of inflammatory cells to the sites of infections. In the epidermis, the killing of pathogens may be performed by the epidermal keratinocytes themselves and by the infiltrating neutrophils attracted to the site of infection by keratinocyte-derived chemokines, such as IL-8. In both cases, the presence and action of keratinocytes is indispensable for the protection of the body. Our findings stress the importance of the keratinocyte as a component of the innate immune response.

Acknowledgements

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Abbreviations

LPS	lipopolysaccharide
MyD88	myeloid differentiation protein
PGN	peptidoglycan
TLR	Toll-like receptor

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