Toll-Like Receptor Expression in Human Keratinocytes: Nuclear Factor κB Controlled Gene Activation by *Staphylococcus aureus* is Toll-Like Receptor 2 But Not Toll-Like Receptor 4 or Platelet Activating Factor Receptor Dependent

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Cultured primary human keratinocytes were screened for their expression of various members of the toll-like receptor (TLR) family. Keratinocytes were found to constitutively express TLR1, TLR2, TLR3, TLR5, and TLR9 but not TLR4, TLR6, TLR7, TLR8, or TLR10 as shown by polymerase chain reaction analysis. The expression of the crucial receptor for signaling of staphylococcal compounds TLR2 was also confirmed by immunohistochemistry, in contrast to TLR4, which showed a negative staining pattern. Next, we analyzed the activation of the proinflammatory nuclear transcription factor κB by *Staphylococcus aureus* strain 8325-4. Using nuclear extract gel shifts, RelA staining, and luciferase reporter transfection plasmids we found a clear induction of nuclear factor κB translocation by the bacteria. This translocation induced the transcription of nuclear factor κB controlled genes such as inducible nitric oxide synthetase, COX2, and interleukin-8. Transcription of these genes was followed by production of increased amounts of interleukin-8 protein and NO. Inhibition experiments using monoclonal antibodies and the specific platelet activating factor receptor inhibitor CV3988 showed that nuclear factor κB activation by *S. aureus* was TLR2 but not TLR4 or platelet activating factor receptor dependent. In line, the purified staphylococcal cell wall components lipoteichoic acid and peptidoglycan, known to signal through TLR2, also showed nuclear factor κB translocation in human keratinocytes, indicating a crucial role of the staphylococcal cell wall in the innate immune stimulation of human keratinocytes. These results help to explain the complex activation of human keratinocytes by *S. aureus* and its cell wall components in various inflammatory disorders of the skin. ***Key words: bacterial cell wall/proinflammatory activation/infection. J Invest Dermatol 121: 1389–1396, 2003***

The colonization with and infection by virulent *Staphylococcus aureus* strains is a serious complication in a variety of skin disorders especially atopic eczema. The first contact of *S. aureus* to human skin is usually initiated through adhesion structures on the bacterial surface and their interaction with human epidermal cells (Bibel *et al*, 1983; Mempel *et al*, 1998; Cho *et al*, 2001). After establishing the contact to epidermal cells several phenomena can be observed including the internalization of the bacteria into the keratinocytes (Mempel *et al*, 2002), an activation of human keratinocytes through staphylococcal components, and finally the fatal damage of the keratinocytes, most often through α- or β-hemolysins (Walev *et al*, 1993; Ezepchuk *et al*, 1996) or the induction of apoptotic cell death (Mempel *et al*, 2002).

Some of the steps leading to adhesion of the bacteria have been elucidated such as the binding of staphylococcal protein A and the fibronectin-binding proteins A and B (Bibel *et al*, 1983; Mempel *et al*, 1998; Cho *et al*, 2001). Little, however, is known of the factors leading to the inflammatory activation of keratinocytes. A proinflammatory effect has been described for hemolysin α, lipoteichoic acid (LTA), protein A, and peptidoglycan (PGN) (Ezepchuk *et al*, 1996; Uehara *et al*, 2001; Kawai *et al*, 2002), resulting in the production of β-defensin 2, tumor necrosis factor α (TNF-α), and interleukin-8 (IL-8). The mode of activation and the involved signaling pathway molecules are not clear at the moment, however.

Two major pathways have been described for the activation of epithelial cells through staphylococci and staphylococcal compounds, the first of which is the recognition of staphylococcal

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Abbreviations: LTA, lipoteichoic acid; iNOS, inducible nitric oxide synthetase; MOI, multiplicity of infections; NF-κB, nuclear factor κB; PAFR, platelet activating factor receptor; PGN, peptidoglycan; TLR, toll-like receptors.

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products through the family of toll-like receptors (TLR), which are activated by LTA and PGN (mainly through TLR2) (Schwandner *et al*, 1999; Wang *et al*, 2001). This pathway has been identified for keratinocytes (Kawai *et al*, 2002) and intestinal epithelial cells (Cario *et al*, 2002). Second, bronchial epithelial cells have been described to respond to *S. aureus* through the platelet activating factor receptor (PAFR) followed by activation of the metalloproteinase ADAM10 and the epidermal growth factor (Lemjabbar and Basbaum, 2002). For this pathway the staphylococcal LTA is the major ligand. Interestingly, both pathways lead to a downstream translocation of the nuclear factor κB (NF-κB) and the transcription of NF-κB responding genes (Wang *et al*, 2001; Lemjabbar and Basbaum, 2002). In keratinocytes, recent studies have been carried out aimed at the analysis of TLR expression and the activation of proinflammatory genes. These studies have found a constant expression of TLR2 at the surface of cells (Pivarcsi *et al*, 2001*; Kawai *et al*, 2002). The expression of TLR4 and the response to lipopolysaccharide (LPS) have been controversially reported (Kawai *et al*, 2002; Song *et al*, 2002) and the presence or absence of further members of this gene family is not clear so far.

Our work shows that primary human keratinocytes express various members of the TLR family including TLR2 and that coincubation of cultured human keratinocytes with virulent *S. aureus* leads to translocation of NF-κB followed by the enhanced transcription of NF-κB controlled genes and the production of proinflammatory mediators such as IL-8 and inducible nitric oxide synthetase (iNOS) both at the RNA and the protein level.

This proinflammatory response is mediated through TLR2 but not TLR4 as shown in blocking experiments and does not involve signaling through PAFR as evidenced with inhibition assays.

MATERIALS AND METHODS

Reagents Antibodies specific for human TLR2 and TLR4 (polyclonal goat) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and from Biocarta (Hamburg, Germany) (clone HTA125 (anti-TLR4) and clone TL2.1 (anti-TLR2)) and were used in a 1:10 and 1:50 dilution, respectively. Purified goat serum (Sigma, Deisenhofen, Germany) and purified mouse IgG (BD-Biosciences, Heidelberg, Germany) served as isotype controls. In order to evaluate the specificity of anti-TLR2 and anti-TLR4 antibodies we used HEK 293 cells transfected with a dominant expression plasmid for either hTLR2 or hTLR4 (kindly provided by C. Kirschning, Institute for Medical Microbiology and Immunology, TU Munich, Germany). As HEK 293 cells do not express any of the investigated receptors we used untransfected cells as negative control.

For analysis of RelA (NF-κB subunit p65) translocation, the anti-p65 antibody from Rockland (Gilbertsville, PA) was used (dilution 1:200) followed by a fluorescein isothiocyanate (FITC) conjugated goat antirabbit second-step antibody (dilution 1:100) (Sigma). Griess reagent was purchased from Sigma and used as recommended by the manufacturer. LPS (*Escherichia coli*) and LTA (*S. aureus*) were obtained from Sigma and PGN from Fluka (Heidelberg, Germany). LTA and PGN were tested for LPS content using the Limulus assay from BioWhittaker, Walkersville, MD. Aliquots showed LPS contamination of approximately 0.01–0.1 μg per mg. Preliminary results using 100× higher concentration of purified LPS (10 μg per mL), however, did not show signs of keratinocyte activation in any of the assays used so that we considered LPS as not responsible for the observed biologic phenomena. The PAFR antagonist CV3988 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

Cell culture Primary cultures of human keratinocytes were obtained following standard procedures (Mempel *et al*, 2002). In brief, surgical specimens of human foreskin were cut into pieces of 0.5 cm^2 and exposed to dispase 2.4 U per mL (Roche, Mannheim, Germany) for 12 h at 4°C. The epidermis was then mechanically removed from the dermal layer, homogenized by repeated aspirations in Pasteur pipettes, and incubated in ethylenediamine tetraacetic acid (EDTA) trypsin 0.25% (Roche) for 60 min. Cells were washed three times in SFM (Gibco/Life Technologies, Eggenstein, Germany), stained for viability with 0.5% trypan blue (Sigma), and seeded into 75 cm^2 culture flasks (Becton Dickinson, Heidelberg, Germany) with keratinocyte SFM. Cells were kept at 37°C and 5% CO_2.

Bacterial strains Parent strains Newman and 8325-4 are standard *S. aureus* laboratory strains that have been described previously. All strains were kindly provided by Professor T. Foster, Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin, Ireland.

In order to maintain constant numbers of bacteria, staphylococci were treated with mitomycin C (30 μg per mL, from Sigma) for 1 h, washed extensively, and adjusted to a stock solution of OD_{570} 0.4, which corresponds to 10^8 colony-forming units per mL. 25 μL of this stock solution were used in 1 mL of SFM to stimulate the cells. This treatment has been shown to inhibit bacterial replication but not the production of virulence factors (Tokura *et al*, 1997).

Coincubation of keratinocytes and staphylococci Mitomycin C treated staphylococci were diluted at a concentration of 10^6 colony-forming units per mL and in antibiotic-free SFM and incubated for 24 h with the staphylococci. Preliminary experiments had shown that a multiplicity of infections (MOI) of 10–50 showed the best results for activation of keratinocytes avoiding cell death.

RNA preparation and RT-PCR Cells were grown in six-well dishes until approximately 90% confluency and were stimulated for the indicated conditions. Thereafter, the cells were washed and incubated with 1 mL Trizol (Gibco) for 5 min with repeated pipetting. RNA was then isolated following standard techniques including DNAse digest (Qiagen, Hilden, Germany) and dissolved in sterile H_2O. Standardized amounts of RNA (e.g., 1 μg) were then subjected to reverse transcription using the Gibco superscript kit (Gibco) and oligo dT primer. The cDNA was then amplified using Taq polymerase from Promega (Mannheim, Germany) following the recommendations of the manufacturer.

For the analysis of TLR expression the primers published by Bauer and Chuang were used with modifications (Bauer *et al*, 2001, and personal communication; Chuang and Ulevitch, 2001). Previously unpublished primers and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and iNOS were designed using the Primer Express (PE Applied Biosystems, Foster City, CA) software. COX2 primers were taken from Honda *et al* (2000). All primers were verified using LPS-stimulated peripheral blood mononuclear cells (10 μg per mL for 24 h) HEK 293 and THP-1 cells as positive control and by omitting the reverse transcription step in an otherwise identical set-up as negative control.

Of note, all TLR PCR were run to saturation (40 cycles) in order to detect very minute amounts of specific cDNA whereas reactions for COX2 (30 cycles), iNOS (34 cycles), and GAPDH (28 cycles) were established with various cycle numbers according to the expression pattern of the gene (**Table I**).

Expression of human COX2, iNOS, and GAPDH was measured using semiquantitative PCR. The following primers were used: iNOS-Fw, ATG CCA GAT GGC AGC ATC AGA; iNOS-Rv, ACC CTG CCA ACG TGG AAT TCA CTC AG; COX2-Fw, TTC AAA TGA GAT TGT GGG AAA ATT GCT; COX2-Rv, AGA TCA TCT CTG CCT GAG TAT CTT; GAPDH-Fw, CAA GTC CCT GAA GGA TGT GGA; GAPDH-Rv, GAG GAG TGG GTG TCG CTG TTT GAA GTC. All primers were obtained from MWG (Ebersberg, Germany).

Real-time PCR for IL-8 transcription As it has been reported that IL-8 production is tightly regulated after NF-κB translocation in human keratinocytes we looked in more detail at the changes in IL-8 mRNA by real-time PCR using the Taqman technique as previously described (Rad *et al*, 2002).

Taqman primers (MWG) and probes (PerkinElmer, Weiterstadt, Germany) were designed using the primer design software Primer Express. All probes were synthesized by PerkinElmer and labeled with the reporter dye 6-carboxyfluorescein at the 5′ end and the quencher dye 6-carboxytetramethylrhodamin at the 3′ end. Primers and probes were chosen to span exon junctions or to lie in different exons to prevent amplification of genomic DNA.

Five microliters of RNA were transcribed into cDNA in a total volume of 50 μL using 50 U of MultiScribe reverse transcriptase (PerkinElmer) according to the manufacturer's instructions. PCR was performed in a volume of 30 μL on the ABI PRISM 7700 sequence detection system (PerkinElmer). For each run, a master mix was prepared on ice

*Pivarcsi A, Reithi B, Szell M, *et al*: Toll-like receptors 2 and 4 are expressed on human keratinocytes and mediate the killing of pathogens. 31st Annual ESDR Meeting, Stockholm, Abstract 224, 2001.

Table I.

Human TLR	Primer
1	5′-TTT GAA AAT TGT GGG CAC CTT ACT G-3′
	5′-AAG CAA CAT TGA GTT CTT GCA AAG C-3′
2	5′-TGT GAA CCT CCA GGC TCT G-3′
	5′-GTC CAT ATT TCC CAC TCT CAG G-3′
3	5′-AGC CGC CAA CTT CAC AAG-3′
	5′-AGC TCT TGG AGA TTT TCC AGC-3′
4	5′-ACA GAA GCT GGT GGC TGT G-3′
	5′-TCT TTA AAT GCA CCT GGT TGG-3′
5	5′-CAT GAC CAT CCT CAC AGT CAC AAA G-3′
	5′-GGG CAT AAC TGA AGG CTT CAA GG-3′
6	5′-CAT GAC GAA GGA TAT GCC TTC TTT G-3′
	5′-TAT TGA CCT CAT CTT CTG GCA GCT C-3′
7	5′-TTG GCT TCT GCT CAA ATG C-3′
	5′-CTA AAG GTT GGA ATT CAC TGC C-3′
8	5′-GTC GAC TAC AGG AAG TTC CCC-3′
	5′-GGG TAA CTG GTT GTC TTC AAG C-3′
9	5′-GTG CCC CAC TTC TCC ATG-3′
	5′-GGC ACA GTC ATG ATG TTG TTG-3′
10	5′-CTT GAC CAC AAT TCA TTT GAC TAC TC-3′
	5′-CAT CTT CCT TCT CTA GAT TGG GGA TC-3′

containing 15 μL of Universal Master Mix (PE Applied Biosystems), primers (1 μmol per L), fluorogenic probes (0.32 μmol per L), and H_2O. To each well of a 96-well plate, 25 μL of master mix and 5 μL of cDNA samples were added. All PCR were performed in duplicate. Thermal cycling was initiated with an incubation step at 50°C for 2 min, followed by a first denaturation step at 95°C for 10 min, and continued with 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s.

The following primers and probes were used: forward, GCCAACAC-AGAAATTATTGTAAAGCTT; reverse, AATTCTCAGCCCTCTTCAA-AAACTT; labeled probe, AGAGCTCTGTCTGGACCCCAAGGAAAAC.

NF-κB translocation We used three different techniques to screen for NF-κB translocation. First, nuclear extract gel shifts were performed as previously published (Brand *et al*, 1997; Mackman, 2000). In brief, nuclear extracts from 5×10^6 cells were prepared and analyzed as described. Protein concentrations were determined by the Bradford method (Bio-Rad, Munich, Germany). The prototypic immunoglobulin κ-chain oligonucleotide was used as a probe (5′-CAGAGGGACTTTCCGAGA-3′) and labeled by annealing of complementary primers followed by primer extension with the Klenow fragment of DNA polymerase I (Boehringer Mannheim, Germany) in the presence of [α-^{32}P]dCTP (>3000 Ci per mmol; DuPont, Brussels, Belgium) and deoxynucleoside triphosphates (Boehringer Mannheim). Nuclear extracts (5 μg protein) were incubated with radiolabeled DNA probes (≈10 ng; 10^5 cpm) for 30 min at room temperature in 20 μL of binding buffer (20 mmol per L Tris–HCl, pH 7.9; 50 mmol per L KCl; 1 mmol per L dithiothreitol; 0.5 mmol per L EDTA; 5% glycerol; 1 mg per mL bovine serum albumin; 0.2% Nonidet P-40; 50 ng of poly(dI-dC) per μL). Samples were run in 0.25 × TBE buffer (10 × 890 mmol per L Tris; 890 mmol per L boric acid; 20 mmol per L EDTA, pH 8.0) on nondenaturing 4% or 6% polyacrylamide gels at 125 V. Nuclear extracts from LPS-stimulated THP-1 cells were used as positive controls. As an additional control, samples were incubated with an excess (10×, 100×) of nonlabeled κB oligonucleotide, which completely abolished binding of the radiolabeled oligonucleotide to the nuclear proteins. To control the nuclear protein content, the nuclear extracts were incubated with a blunt end double-stranded Sp-1 oligonucleotide that was labeled with [α-^{32}P]ATP (>5000 Ci per mmol, DuPont) and T4 polynucleotide kinase (Boehringer Mannheim). Gels were dried and analyzed by autoradiography.

Second, we used the immunostaining technique for p65 translocation as described by Song *et al* (2002), and third, in order to quantitate the induction of NF-κB translocation, we established the transient transfection of primary keratinocytes with an NF-κB–luciferase reporter plasmid from Clontech (Palo Alto, CA). The plasmid was grown using the Topo cloning kit from Invitrogen (Gibco, Eggenfelden, Germany) and purified with the Quiagen Maxi Prep kit. Various amounts of the plasmid were transfected into the keratinocytes using DMRIE-C reagent from Invitrogen following the instructions of the manufacturer. To control for transfection efficacy, a plasmid expressing green fluorescent protein constitutively (kindly provided by Dr Markus Braun-Falco, Department of Dermatology and Allergy, TU Munich) was used in parallel experiments. Usually, this control yielded between 20% and 30% of transfected cells after 24 h. Thereafter, the cells were incubated with the various stimuli for 24 h before the cells were harvested and prepared for

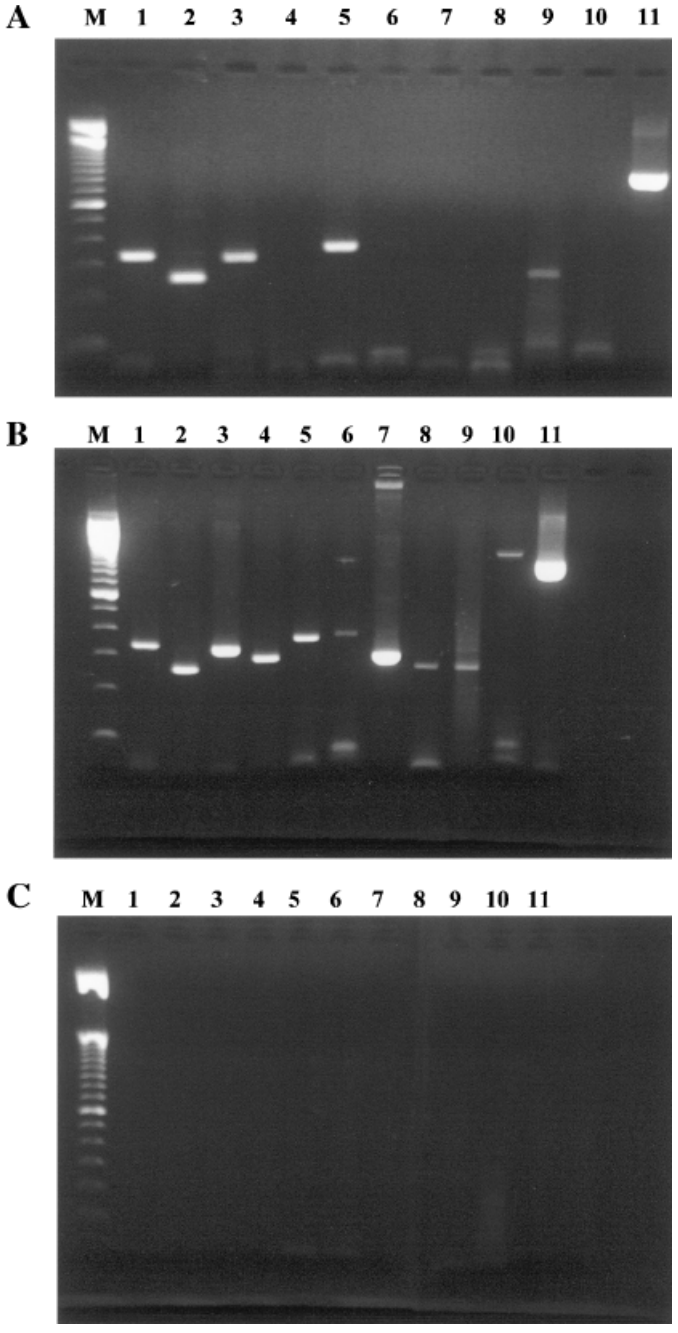

Figure 1. Transcription of TLR1–TLR10 was investigated in primary human keratinocytes by RT-PCR. Repeatedly a high expression of TLR1, TLR2, TLR3, and TLR5 was found together with a low transcription signal of TLR9. Interestingly, no transcription signal was found for TLR4. Experiments were repeated in 10 different donors with similar results (M, size marker; 1–10, TLR1–TLR10; 11, GAPDH) (*A*). As positive control for primer specificity, combined RNA of LPS (10 μg per mL) stimulated peripheral blood mononuclear cells, HEK293 and THP-1 cells was used (*B*). The negative control was performed with an identical reaction mix omitting the reverse transcription step (*C*). All PCR were run to saturation (40 cycles).

both luciferase quantification (using the luciferase reaction buffer from Invitrogen and a luminometer from Berthold Tech, Bad Harzburg, Germany) and total protein determination using the Bradford assay from Bio-Rad.

IL-8 ELISA and Griess reaction IL-8 protein was quantified using the ELISA from R & D (Wiesbaden, Germany) according to the manufacturer's specification. To this end, cells were grown in 12-well plates and incubated with the respective stimulus for 24 h. Then the cells were washed and the medium was aspirated. Aliquots were used to determine the amounts of IL-8 and the formation of NO using Griess reagent (Sigma) as recommended by the manufacturer. In brief, the accumulation of NO_2^-, a stable end product of NO formation, was measured as an indicator for NO production. 100 μL of cell-free culture supernatant were incubated for 15 min with 100 μL Griess reagent at room temperature and the absorbance at 540 nm was measured in a microplate reader.

RESULTS

TLR RNA expression in primary human keratinocytes RNA from unstimulated human keratinocytes was reverse transcribed using oligo dT primers. A panel of hTLR specific primers targeting TLR1–TLR10 specific cDNA was used. This analysis revealed expression of TLR1, TLR2, TLR3, TLR5, and TLR9 but not TLR4, TLR6, TLR7, TLR8, and TLR10 (**Fig 1*A***). The lack of TLR4 transcription under nonstimulatory conditions was surprising but has been previously described by others (Kawai *et al*, 2002). Repeatedly, TLR3 showed the strongest transcription signal as evaluated in primary keratinocytes of various donors. In order to control for the sensitivity and the specificity of our primers we included HEK293, THP-1 and LPS-stimulated peripheral blood mononuclear cells (**Fig 1*B***) as positive and the corresponding RNA without addition of the reverse transcriptase enzyme (**Fig 1*C***) as negative control.

Immunohistochemical staining of cultured human keratinocytes Primary human keratinocytes were grown on glass slides until confluency. Cells were stained with anti-TLR2 and anti-TLR4. We obtained a clear positive staining for anti-TLR2 (**Fig 2*A***). In contrast, the staining for anti-TLR4 (**Fig 2*D***) showed virtually no difference compared to the isotype control (purified goat serum) (**Fig 2*G***). The specific staining pattern of the antibodies used was verified by using TLR2- and TLR4-transfected HEK 293 cells (**Fig 2*B, E***) as well as untransfected HEK 293 cells (**Fig 2*C, F***). These results paralleled our RT-PCR findings.

NF-κB translocation after exposure to *S. aureus* Keratinocytes were incubated with 10^6 staphylococci per mL in SFM. Thereafter, cells were harvested and nuclear extracts were prepared. Gel shift analysis of the separated nuclear extracts showed a clear band for NF-κB when the keratinocytes were exposed to *S. aureus* strain 8325-4 or 50 ng per mL of IL-1β and TNF-α (used as positive control) but not when the cells were incubated with medium alone (used as negative control) (**Fig 3**). Next we screened for translocation of the NF-κB subunit p65 (RelA) by immunohistochemistry. As shown in **Fig 4** the

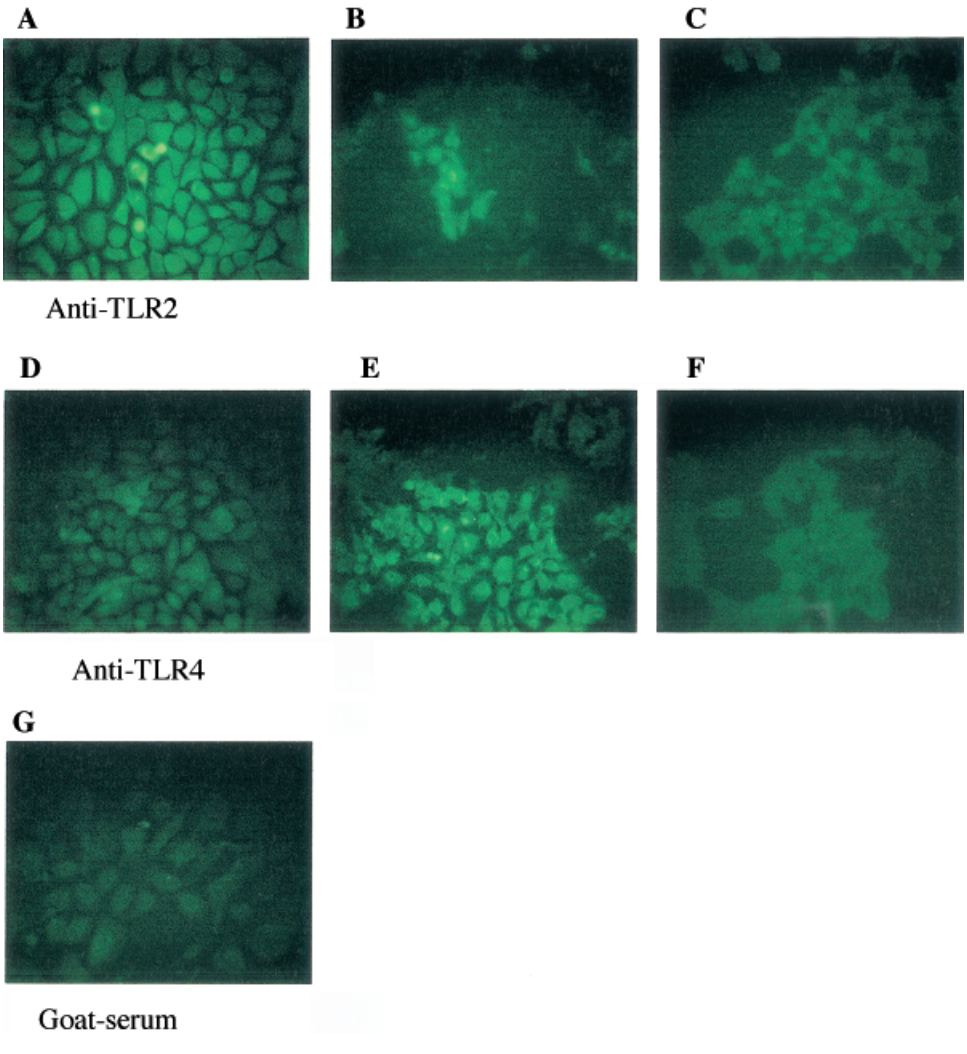

Figure 2. Staining for TLR2 and TLR4 on primary human keratinocytes. Cells were grown on glass slides to confluency. Cells were stained with a polyclonal primary goat anti-TLR antibody (dilution 1:10) and an FITC-labeled rabbit antigoat secondary antibody (dilution 1:50). Repeatedly, the keratinocytes were found to express TLR2 (*a*) but no specific staining was detected for TLR4 (*D*). Purified goat serum was used as isotype control (*G*) and HEK 293 cells transfected with hTLR2 (*B*) and hTLR4 (*E*) and untransfected HEK 293 cells (*C*, *F*) were used as control for antibody specificity and sensitivity.

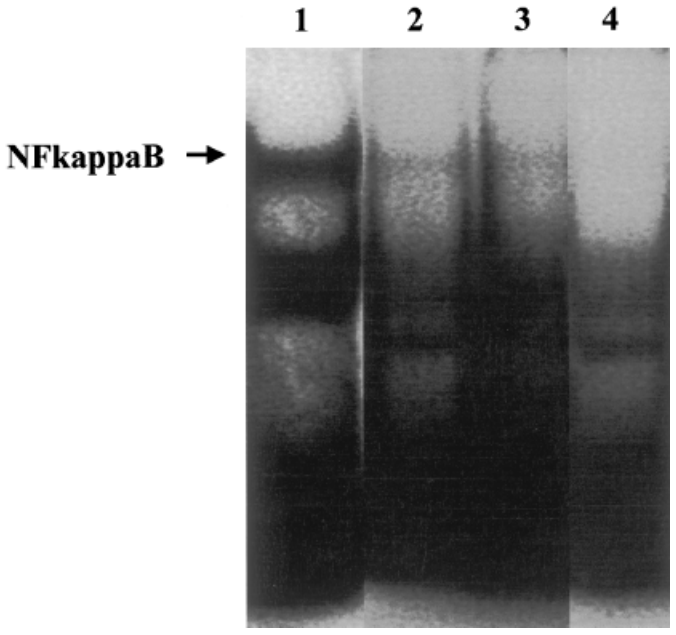

Figure 3. Nuclear extract gel shift for NF-κB. Primary human keratinocytes were grown to confluency in sterile Petri dishes (10^6 cells) and incubated with TNF-α (50 ng per mL)/IL-1β (50 ng per mL) (*lane 2*), *S. aureus* strain 8325-4 (*lane 3*), and medium (*lane 4*) for 2 h before they were harvested by scraping them off the dish with a sterile cell scraper. Preparation of nuclear extracts and gel shifts were carried out as described in *Materials and Methods*. LPS-stimulated THP-1 cells served as positive control (*lane 1*). This experiment showed translocation of NF-κB for cytokine and *S. aureus* stimulated keratinocytes but not for medium-stimulated control cells.

incubation with *S. aureus* resulted in a significant translocation of RelA from the cytoplasm into the nucleus of the keratinocytes. Again, IL-1β/TNF-α was used as positive control whereas medium alone did not induce a significant recruitment of the p65 subunit to the nucleus.

In order to quantitate the NF-κB activation following incubation with *S. aureus*, we developed an NF-κB–luciferase reporter assay using transient transfections with the luciferase reporter plasmid. This assay showed a significant increase in the activation of NF-κB by *S. aureus* strain 8325-4 and to a lesser extent by strain Newman (**Fig 5**).

NF-κB translocation can be blocked by anti-TLR2 but not anti-TLR4 or a specific inhibitor of the PAFR pathway We then used the NF-κB reporter assay to identify pathogen recognition molecules involved in the activation of human keratinocytes by *S. aureus*. To this end, keratinocytes were incubated with either anti-TLR2 or anti-TLR4 or the well-described inhibitor of PAFR signaling CV3988. As shown in **Fig 6**, preincubation with anti-TLR2 but not with anti-TLR4 or the PAFR antagonist CV3988 completely inhibited NF-κB activation in the keratinocytes. This inhibition was *S. aureus* specific as it did not impair the translocation of NF-κB following exposure to IL-1β/TNF-α (data not shown). This experiment showed that NF-κB translocation of human keratinocytes is mediated through TLR2 and not TLR4 or PAFR.

Staphylococcal LTA and PGN both activate human keratinocytes As various components of staphylococci have been described to act in an immunostimulatory way, we included two commercially available purified cell wall components in our NF-κB activation assay. As shown in **Fig 7**, staphylococcal LTA and PGN both induced NF-κB translocation.

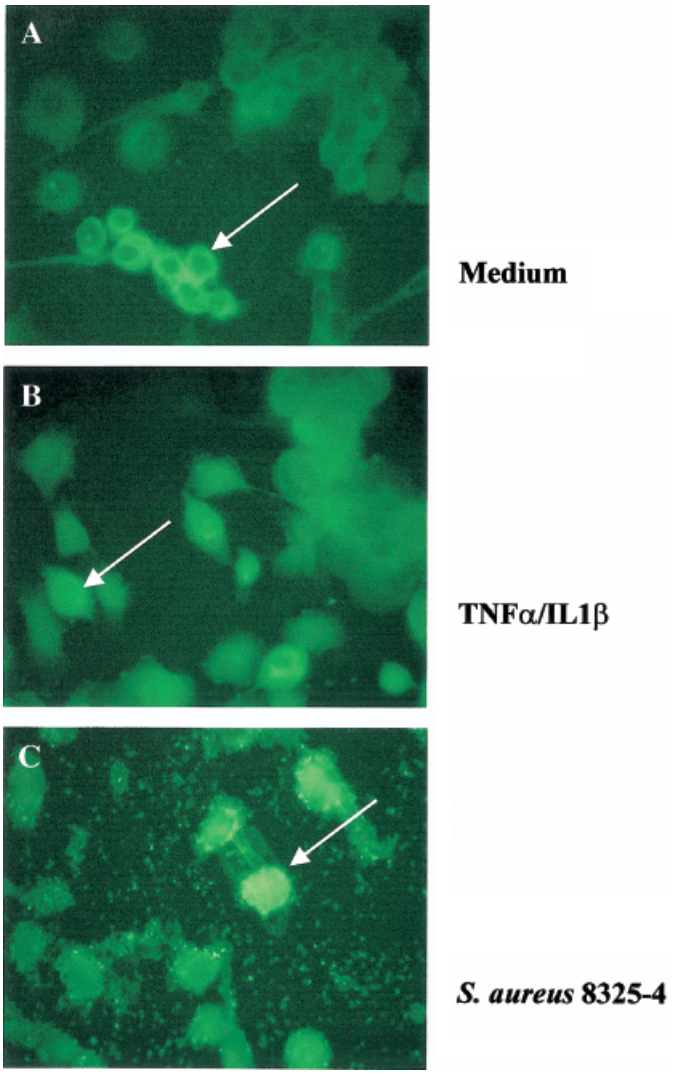

Figure 4. Staining for RelA in primary human keratinocytes. Cells were incubated with medium (negative control, *A*), with TNF-α (50 ng per mL)/IL-1β (50 ng per mL) (positive control, *B*), and with *S. aureus* strain 8325-4 (*C*) for 2 h before the cells were stained with a primary rabbit anti-p65 antibody followed by an FITC-labeled goat antirabbit antibody. A positive reaction was found after stimulation with cytokines and *S. aureus* as seen by the changes in staining patterns (unstimulated cells, cytoplasmatic staining pattern; stimulated cells, nuclear staining pattern as denoted by *arrows*). Note the staining of *S. aureus* bacteria by their capacity to fix the antibodies through protein A on their surface (*C*).

Exposure of keratinocytes to *S. aureus* and its cell wall components results in increased transcription of NF-κB controlled genes Our experiments so far established the translocation of NF-κB by *S. aureus* in primary human keratinocytes through a TLR2-dependent mechanism. We next looked for the transcription of NF-κB controlled genes by semiquantitative RT-PCR (compared to GAPDH transcription). This experiment revealed for iNOS and COX2 an increase in specific RNA transcription after stimulation. This increase was moderate for COX2 but more pronounced for iNOS. A possible explanation for this difference might be the high level of basal transcription for COX2 already present in unstimulated keratinocytes (**Fig 8*A***).

In order to analyze transcription of IL-8, the major mediator of neutrophil attraction, in more detail, we used the quantitative real-time Taqman PCR. This experiment also showed a clear

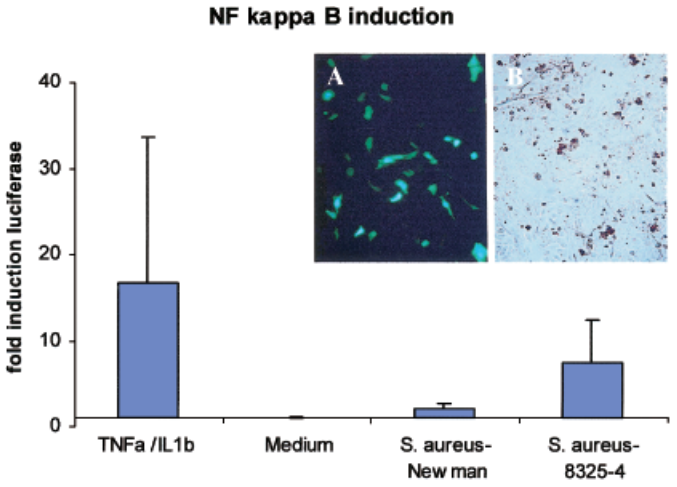

Figure 5. NF-κB translocation was quantified by introducing a luciferase reporter plasmid into the keratinocytes. Transfection efficacy was controlled by parallel transfection of the green-fluorescent-protein-containing control plasmid. Transfection rates were found between 20% and 30% of the cells (as shown in the inserted panel as *A* and *B*). Cells were then incubated with *S. aureus* strains 8324-5 and Newman (mitomycin C inactivated) at an MOI of approximately 50 for 24 h before luciferase units were measured. This experiment showed translocation of NF-κB after incubation with *S. aureus* strain 8325-4 and to a lesser extent with strain Newman. TNF-α/IL-1β served as positive control and incubation medium as negative control. Experiments were carried out in triplicate and were repeated at least three times with similar results.

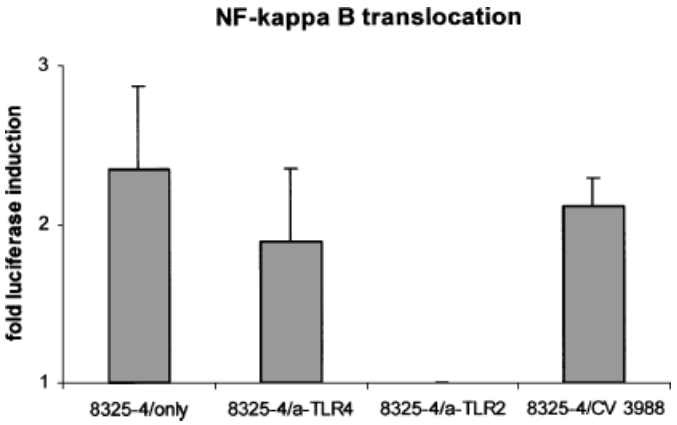

Figure 6. Inhibition of NF-κB translocation. Keratinocytes were preincubated for 2 h with the respective anti-TLR2- or anti-TLR4-specific antibody or CV3988 before identical concentrations (MOI ≈ 50) of *S. aureus* strain 8324-5 (mitomycin C inactivated) were added to all culture conditions for a further 24 h. Then, the cells were harvested for determination of luciferase activity. Translocation of NF-κB by *S. aureus* 8325-4 was blocked by preincubation with 10 μg per mL of anti-TLR2 (MAB clone TL2.1) but not with anti-TLR4 (MAB clone HTA125) or with the specific PAFR inhibitor CV3988 (10 μg per mL), indicating an activation of primary human keratinocytes by *S. aureus* through the pattern recognition molecule TLR2.

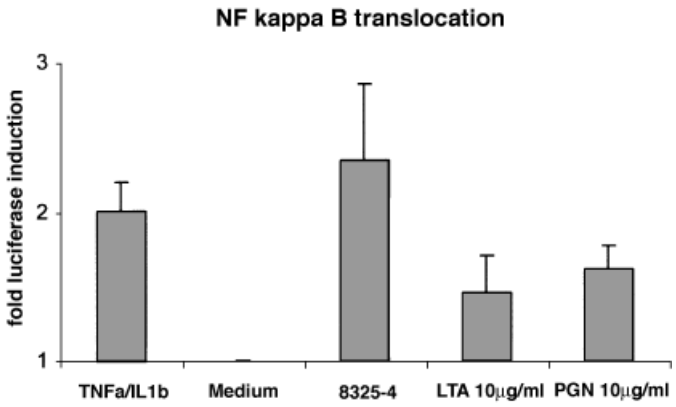

Figure 7. Translocation of NF-κB by *S. aureus* in primary human keratinocytes can be mimicked by the known TLR2 ligands PGN and LTA. Cells were incubated for 24 h with the staphylococcal cell wall components (both in a concentration of 10 μg per mL) or strain 8324-5 (MOI ≈ 50 and mitomycin C inactivated) before luciferase activity was measured. TNF-α/IL-1β and medium served as positive and negative control, respectively.

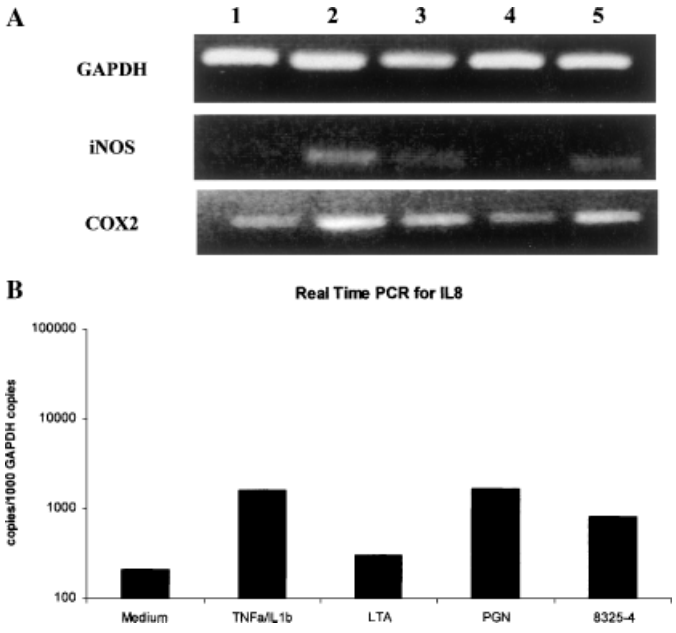

Figure 8. Increased transcription of NF-κB controlled genes. (*A*) *S. aureus* 8325-4 (*lane 5*) and its cell wall components LTA (*lane 3*) and PGN (*lane 4*) induce transcription of the NF-κB controlled genes iNOS and COX2. Semiquantitative PCR showed increased transcription of these genes compared to GAPDH. (*B*) IL-8 transcription was analyzed in more detail by real-time Taqman PCR. Again, we found induction of this gene after stimulation with *S. aureus* and its components. The figure shows one representative experiment out of three. Medium (*A*, *lane 1*) and TNF-α (50 ng per mL)/IL-1β (50 ng per mL) (*A*, *lane 2*) served as negative and positive control, respectively.

induction of IL-8 by *S. aureus* and its cell wall components (**Fig 8*B***).

The NF-κB-dependent transcription of IL-8 and iNOS is followed by protein translation In order to search for an enhanced production of the gene products for the iNOS and IL-8 gene we harvested cell culture supernatants after 24 h of stimulation and subjected them to quantification of IL-8 by ELISA and iNOS production by using the Griess reaction (**Fig 9*A, B***). These experiments showed that *S. aureus* strain 8325-4 as well as the cell wall components LTA and PGN induced IL-8 and iNOS in primary human keratinocytes.

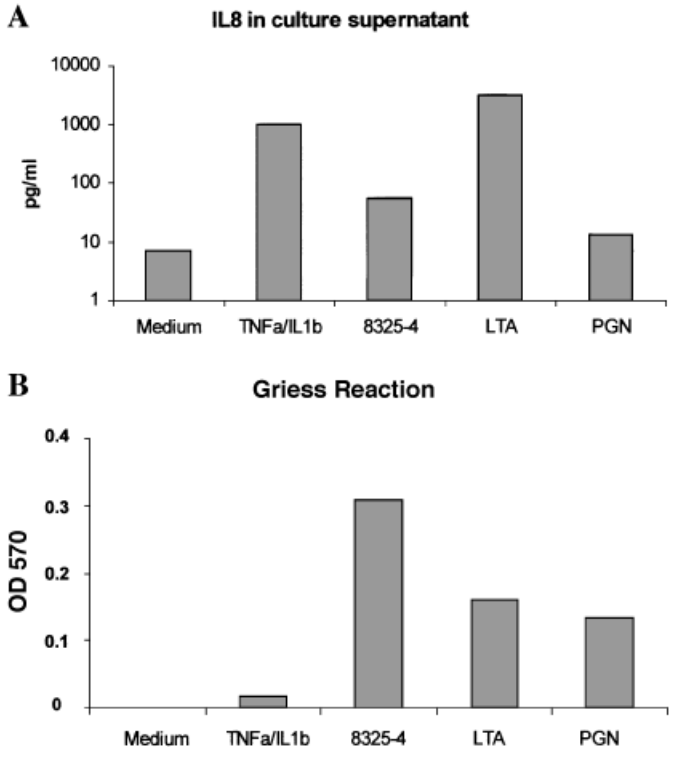

Figure 9. To investigate staphylococci-induced protein production IL-8 and NO (which reflects iNOS induction) concentrations in culture supernatants of stimulated keratinocyte cultures were evaluated. As shown in *panel A*, IL-8 was induced by *S. aureus* and its cell wall components. NO production as measured by the Griess reaction also paralleled the RT-PCR findings of increased proinflammatory gene activation (*B*). Of note, after challenge with TNF-α and IL-1β only a slight increase in NO production was observed. Experiments were carried out in duplicate and were repeated three times.

DISCUSSION

We have investigated the molecular mechanisms leading to the proinflammatory response of human keratinocytes after exposure to *S. aureus*. These experiments clearly showed that the NF-κB-dependent activation of keratinocytes requires TLR2 but not TLR4 or PAFR signaling. Following the contact of the bacteria to this receptor we observed a translocation of the crucial proinflammatory transcription factor NF-κB and a transcription as well as a translation of NF-κB downstream genes. Interestingly, we found transcription of several TLR genes in the cultured keratinocytes with TLR1, TLR2, TLR3, TLR5, and TLR9 being constitutively transcribed but not TLR4, TLR6, TLR7, TLR8, and TLR10. This study is to our knowledge the first systematic analysis of functional TLR expression in cultured human keratinocytes.

The expression of TLR2 on human keratinocytes has been previously described and the activation of keratinocytes by known TLR2 ligands has been reported (Pivarcsi *et al*, 2001; Uehara *et al*, 2001; Kawai *et al*, 2002). We extended these results by unambiguously identifying TLR2 as the crucial recognition pattern receptor for staphylococci in human keratinocytes. The signaling through TLR2 is followed by the translocation of the proinflammatory transcription factor NF-κB and the induction of a proinflammatory response at the mRNA and protein level. This signaling is TLR2 dependent as it can be blocked by an anti-TLR2 specific antibody (but not by anti-TLR4) and cannot be impaired by the specific inhibitor of the PAFR pathway although human keratinocytes have been described to express PAFR (Shimada *et al*, 1998).

Our finding that primary human keratinocytes do not constitutively express TLR4 mRNA and protein is in accordance with a recent report in which similar results have been published (Kawai *et al*, 2002) but contrasts with previous publications describing expression of TLR4 as well as signaling of bacterial LPS through this receptor (Pivarcsi *et al*, 2001; Uehara *et al*, 2001; Song *et al*, 2002). One possible explanation might be the inducibility of TLR4 (Kawai *et al*, 2002), which could account for the different findings.

Given this immediate and reproducible response of cultured keratinocytes to staphylococci and staphylococcal products mediated through TLR2 and given the presence of TLR2 ligands even in coagulase-negative staphylococci (Hajjar *et al*, 2001), which are present on human skin in high numbers even when it is not inflamed, as well as the estimated 30% of healthy *S. aureus* carriers, the question remains why the normal human skin does not constantly react to the colonizing bacteria. A possible answer can be found in the expression pattern of TLR2 in normal human skin (Kawai *et al*, 2002). The expression of this molecule has been found to be strongest in the basal layer(s) of the epidermis whereas only marginal expression was seen in suprabasal layers. Thus, the bacterial products would have to enter through the barrier of the stratum corneum and through the stratum granulosum to gain access to TLR2-expressing keratinocytes. This usually happens only in skin diseases with disturbed barrier functions such as atopic eczema where the staphylococci may contribute to the TH1 inflammatory pattern seen in chronic lesions (which are very often colonized with high numbers of staphylococci) (Thepen *et al*, 1996; Herz *et al*, 1998).

Many of the ligands for the TLR molecules have been recently identified and their signaling through the various TLR has been defined in several cell types (for review see Janeway and Medzhitov, 2002). For example, TLR9 has been described to recognize unmethylated CpG DNA (Hemmi *et al*, 2000), a pathogen-associated signal that is widely suppressed in mammalian DNA. Interestingly, recent work has shown a TH1-like activation of keratinocytes after exposure to such CpG-containing DNA (Mirmohammadsadegh *et al*, 2002). It is thus conceivable that the expression of TLR9 on the keratinocytes enables the cells to react to pathogen-derived DNA molecules.

TLR5 has been described as a receptor for bacterial flagellin (Hayashi *et al*, 2001), a microbial component that also plays an important role in the colonization of several bacteria to body surfaces such as the skin.

TLR3 has been identified as a receptor for double-stranded RNA (Alexopoulou *et al*, 2001). The role of its expression in keratinocytes is not clear at the moment but as for other TLR the recognition of further pattern-associated molecules cannot be excluded.

TLR1 on the other hand has been described to compete with TLR6 for the formation of dimers with TLR2 (Hajjar *et al*, 2001). For this TLR molecule, an inhibitory effect for a TLR2/TLR6 heterodimer mediated signaling and to a lesser extent for the TLR2 homodimer signaling has been described (Hajjar *et al*, 2001). As our experiments only found expression of TLR2 but not TLR6 in primary human keratinocytes further experiments are under way to investigate a possible inhibitory signal on TLR2 activation.

As the human skin represents the major barrier to a potentially hostile environment, the development of innate defense mechanisms such as a distinct set of pattern recognition receptors is of vital interest for the host defense. Obviously, the expression of functional receptors for pathogen-associated molecular patterns is required not only on professional epidermal immune cells (e.g., Langerhans cells) but also on epidermal keratinocytes to guarantee normal skin homeostasis.

Future projects are set to elucidate the various interactions of the different TLR in keratinocytes and the mechanisms that orchestrate and regulate the immune response through this family of molecules in human skin.

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