S100A15, an Antimicrobial Protein of the Skin: Regulation by *E. coli* through Toll-Like Receptor 4

Amanda S. Büchau[1,2], Mohamed Hassan[1], Gabriela Kukova[1], Virginia Lewerenz[1], Sabine Kellermann[1], Jens U. Würthner[3,6], Ronald Wolf[4], Markus Walz[1], Richard L. Gallo[2] and Thomas Ruzicka[5]

E. coli is a Gram-negative bacterium rarely found on human skin. We investigated whether direct interaction of *E. coli* with keratinocytes might induce an innate immune response through recognition by pattern recognition receptors. The capacity of *E. coli* to activate innate immune responses and IL-8 induction was investigated. We found that *E. coli* significantly induced human S100A7 and S100A15 transcript abundance and IL-8 release in cultured primary human keratinocytes. S100A15 is a member of the S100 protein family with previously unknown function. *E. coli* induced effects could be inhibited by neutralizing Toll-like receptor 4 (TLR4) antibodies, suggesting that *E. coli*-induced IL-8 and S100A15 expression in human keratinocytes are TLR4 dependent. TLR4−/− mice lacked elevated mS100A15 expression after infection with *E. coli* in contrast to wild-type mice. *In vitro*, human S100A15 displayed antimicrobial activity against *E. coli.* Our findings suggest that *E. coli* modulates S100A15 and IL-8 expression of keratinocytes by recognition through TLR4.

Journal of Investigative Dermatology (2007) **127,** 2596–2604; doi:10.1038/sj.jid.5700946; published online 12 July 2007

INTRODUCTION

S100 proteins comprise a family of small cationic proteins and are characterized by two calcium-binding EF-hand motifs with specific calcium-dependent and calcium-independent interactions (for review see Donato, 2001; Santamaria-Kisiel *et al.*, 2006). Today, 21 proteins of the S100 family are known, of which eleven (S100A2, S100A3, S100A4, S100A6, S100A7 (psoriasin), S100A8, S100A9, S100A10, S100A11, S100A12, and S100A15) are expressed within the human epidermis or in normal human epidermal keratinocytes (NHEKs) (Wolf *et al.*, 2003; Eckert *et al.*, 2004). These proteins play a role in the pathogenesis of many epidermal diseases (Donato, 2001; Eckert *et al.*, 2004; Foell and Roth, 2004) and antimicrobial properties were found for several members of the S100 family, including S100A7, S100A8, S100A9, and S100A12 (Gottsch *et al.*, 1999; Cole *et al.*, 2001; Gläser *et al.*, 2005). The recently identified human S100A15 protein is highly homologous to S100A7 (93% identity), followed by S100A11 (34%) and S100A8 (29%) (Wolf *et al.*, 2003). Genomic analysis of S100A7 and S100A15 encoding chromosomal regions furthermore reveals that both genes must have evolved by gene duplication during primate evolution, forming an innate subfamily in man (Kulski *et al.*, 2003). Human S100A15 is mapped to the S100 gene cluster within the epidermal differentiation complex on chromosome 1q21 and has two alternatively spliced mRNA-isoforms, a short (0.5 kb), S100A15S, and a long (4.4 kb), S100A15L, isoform resulting from different non-coding regions (Wolf *et al.*, 2003), both coding for the S100A15 protein. The functional relevance of these splice variants is not known yet. Alternative splicing has been reported only for human S100A4 from the S100 family so far (Ambartsumian *et al.*, 1995).

Recently, the murine S100A15 (mS100A15) has been identified and found to parallel genomic organization, structure, gene expression, and protein-processing pattern of the human S100A7/A15 subfamily (Wolf *et al.*, 2006). The murine S100A15 was mapped to a single genomic region on chromosome 3qF1, which is the murine equivalent of the human epidermal differentiation complex. Splice variants as described for human S100A15 have not been identified for the murine mRNA of S100A15.

Little is known about the regulation and function of S100A15. We previously showed that S100A15 is expressed in healthy skin and is highly upregulated in psoriatic lesional and non-lesional skin (Wolf *et al.*, 2003, 2006), suggesting a distinct role in epidermal maturation. Many antimicrobial peptides (AMPs) and proteins, including members of the S100

[1]*Department of Dermatology, Heinrich-Heine-University, Duesseldorf, Germany;* [2]*Division of Dermatology, Department of Medicine, University of California San Diego and VA San Diego Healthcare System, San Diego, California, USA;* [3]*Department of Medical Microbiology, Heinrich-Heine-University, Duesseldorf, Germany;* [4]*Laboratory of Cancer Biology and Genetics, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA and* [5]*Department of Dermatology, Ludwig-Maximilians-University, Munich, Germany*

[6]*Current address: AstraZeneca, Global Clinical Development, Alderley Park, Macclesfield, UK.*

Correspondence: Dr Amanda S. Büchau, Division of Dermatology, University of California San Diego and VA San Diego Healthcare System, San Diego, California 92307, USA. E-mail: asbuchau@ucsd.edu

Abbreviations: AMP, antimicrobial peptide; AP-1, activator protein-1; CFU, colony-forming unit; EMSA, electromobility shift assay; LB, Luria-Bertani; LPS, lipopolysaccharide; NHEK, normal human epidermal keratinocyte; PBS, phosphate-buffered saline; RT, reverse transcription; TLR4, Toll-like receptor 4

Received 20 December 2006; revised 24 April 2007; accepted 29 April 2007; published online 12 July 2007

family (S100A7, S100A8, S100A9), β-defensins, and cathelicidin are overexpressed in psoriasis but most AMPs and proteins are also constitutively expressed in healthy skin (Frohm *et al.*, 1997; Mirmohammadsadegh *et al.*, 2000; Wolf *et al.*, 2003; de Jongh, 2005; Harder and Schröder, 2005), where they exert a broad spectrum of protective activity. However, although still many different bacterial strains survive on the skin, *E. coli* is rapidly killed (Bevins, 2005; Gläser *et al.*, 2005). *In vitro* and *in vivo E. coli* killing activity has been demonstrated for S100A7 and other AMPs (Lehrer *et al.*, 1989; Gläser *et al.*, 2005; Dorschner *et al.*, 2006). Based on these observations, we hypothesized that S100A15 may be involved in cutaneous innate immune function and might have antimicrobial function against *E. coli*. In this study, we find that *E. coli* stimulation strongly induced expression of S100A15 *in vitro* and *in vivo* and this was mediated by Toll-like receptor 4 (TLR4). In addition, we demonstrate that S100A15 is antimicrobial against *E. coli*.

RESULTS

Various bacterial species differentially induce S100A15S, S100A15L, and S100A7 gene expression in human epidermal keratinocytes

S100A15 expression has been detected in low levels in healthy skin and at increased levels in psoriasis (Wolf *et al.*, 2003). Furthermore, S100A15 is constitutively expressed in cultured human epidermal keratinocytes and is inducible by proinflammatory cytokines and interleukin 1β (unpublished data). We therefore investigated if other inflammatory stimuli such as bacterial compounds affect expression of S100A15 splice variants. To analyze the effect of various bacteria on S100A15S, S100A15L, and S100A7 mRNA levels, cultured human keratinocytes were treated for 24 hours with or without *E. coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus* and their supernatants (Table 1). To confirm that heat-killed bacteria were biologically active, we examined *E. coli*-induced NF-κB and activator protein-1 (AP-1) activation in NHEK by electromobility shift assay (EMSA) and supershift assay (Figure 1). Here, we found that unstimulated keratinocytes contained a low but detectable, basal level of AP-1 and NF-κB binding activity, which is consistent with data of other authors (Qin *et al.*, 1999; Komine *et al.*, 2000). Exposure of keratinocytes to heat-killed *E. coli* and its supernatant led to an induction of a strong AP-1 and NF-κB binding activity within 3 and 24 hours (Figure 1a). Furthermore, examining the effect of *E. coli* and its supernatant, a more pronounced effect of *E. coli*-stimulated NHEK, was observed (Figure 1a). In addition, we ascertained the specificity of both, AP-1 and NF-κB binding, following the incubation of the nuclear extracts obtained from human keratinocytes treated with *E. coli*, with antibodies directed to c-jun in the case of AP-1 complex or to p50 and p65 subunits in the case of NF-κB complex by EMSA. The binding of the antibodies to their specific proteins c-jun or p50 or p65 shifted the specific bands of AP-1 or NF-κB to higher molecular weight (Figure 1b),

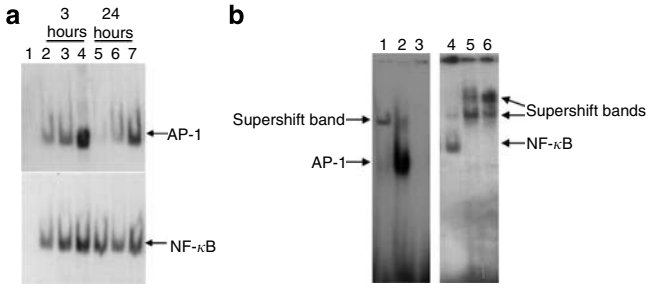

Figure 1. Induction of AP-1 and NF-κB DNA-binding activities by *E. coli* in NHEK. NHEKs were stimulated with heat-killed *E. coli* or its supernatant for 3 and 24 hours. Nuclear extracts were prepared and equal amounts of nuclear proteins (~4 μg) were analyzed for the activation of either AP-1 or NF-κB. The specificity of the DNA-binding activity was analyzed by competition with unlabeled oligonucleotides. Vehicle-treated keratinocytes contained a low but detectable basal level of AP-1 and NF-κB binding activity. (**a**) Exposure of NHEK to heat-killed *E. coli* and its supernatant led to an induction of AP-1 and NF-κB binding activity within 3 hours. (**b**) Supershift analysis and specificity of AP-1- and NF-κB-induced activities by *E. coli* in human keratinocytes. Nuclear extracts from human keratinocytes stimulated with bacterial components were incubated with antibodies to c-jun or to p50 and/or to p65. The data shown are representative of two independent experiments. (**a**) Lane 1: nuclear extracts obtained from NHEK incubated with unlabeled oligonucleotide, lanes 2 and 5: vehicle-treated NHEK, lanes 3 and 6: *E. coli* supernatant-treated NHEK, lanes 4 and 7: *E. coli*-treated NHEK; (**b**) lane 1: with anti-c-jun antibody, lane 2: without anti-c-jun antibody, lane 3: nuclear extracts obtained from NHEK incubated with unlabeled oligonucleotide, lane 4: without anti-p50 and anti-p65 antibodies, lane 5: with anti-p50 antibody, lane 6: with anti-p50 and anti-p65 antibody.

Table 1. Regulation of human S100A15S, S100A15L, and S100A7 mRNA in NHEK upon stimulation with *E. coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*

Gene bacteria	*E. coli*	*E. coli* supern.	*P. aeruginosa*	*P. aeruginosa* supern.	*S. aureus*	*S. aureus* supern.
S100A15S	2.97±0.45*	0.99±0.38	1.14±0.48	2.89±0.71*	1.67±0.43	1.94±1.03
S100A15L	10.54±1.78***	6.16±0.74**	7.35±2.34***	6.95±1.89***	3.86±0.96*	3.74±1.08*
S100A7	7.62±1.93***	3.22±1. 74	5.67±2.03**	2.34±0.56	1.54±0.84	1.24±0.63

NHEK, normal human epidermal keratinocyte; *P. aeruginosa*, *Pseudomonas aeruginosa*; RT, reverse transcription; *S. aureus*, *Staphylococcus aureus*; supern., supernatant.
Heat-killed bacteria and bacteria-derived supernatants were used to stimulate NHEK for 24 hours. Semiquantitative RT-PCR was performed. Table 1 indicates the fold increase of relative gene expression (densitometric quantification of S100A15S, S100A15L, and S100A7 mRNA each over the β-actin mRNA transcript) over constitutive expression. Each number represents the mean±SD of three independent experiments each performed in duplicates. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

demonstrating the specificity of AP-1- and NF-κB-induced activities. The levels of S100A15S, S100A15L, and S100A7 transcript abundance were analyzed by semiquantitative reverse transcription (RT)-PCR using specific primers (Table 1). Here, we found that *E. coli* and its supernatant differentially upregulated expression of S100A15S, S100A15L, and S100A7 mRNA in NHEK after 24 hours of stimulation (Table 1). *P. aeruginosa* and its supernatant induced increased mRNA levels of both isoforms of S100A15 and S100A7 (Table 1). Stimulation with *S. aureus* and its supernatant resulted in an increase in S100A15S and S100A15L mRNA, the expression of S100A7 mRNA was not significantly affected by stimulation with either *S. aureus* or its supernatant (Table 1). From all bacteria tested, *E. coli* showed the strongest induction of S100A15. In order to further evaluate *E. coli*-induced S100A15 and S100A7 gene expression, time course experiments were performed. NHEKs were stimulated with *E. coli* for 3, 24, or 96 hours (Figure 2). Here, S100A15S, S100A15L, and S100A7 were significantly upregulated after 24 hours (Figure 2a–c) and S100A15L and S100A7 were further increased after 96 hours of stimulation with *E. coli* (Figure 2b and c). *E. coli* is a Gram-negative ubiquitous bacterium that contains lipopolysaccharide (LPS) in its cell wall. To evaluate if *E. coli*-derived LPS alone is effective to regulate S100A15 and S100A7 gene expression, NHEKs were co-incubated with *E. coli*-derived LPS for 24 hours. Consistent with our data derived from heat-killed *E. coli*, also *E. coli*-derived LPS upregulated S100A15L and S100A7 gene expression, whereas lower S100A15S expression was observed (Figure 2d).

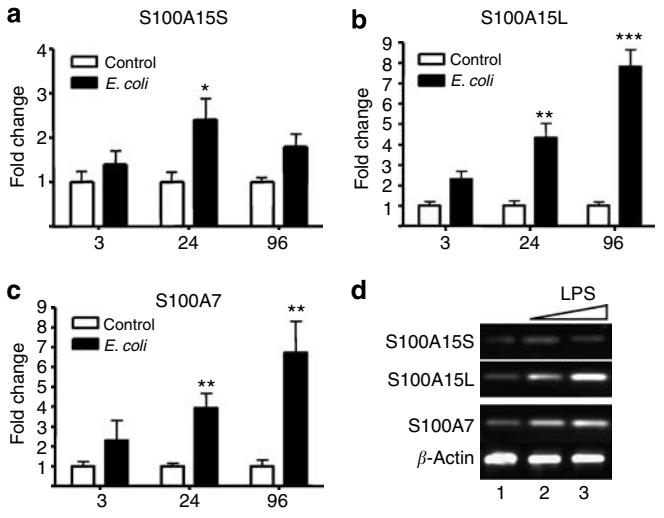

Figure 2. Regulation of human S100A15S, S100A15L, and S100A7 mRNA in NHEK by *E. coli* and LPS. (**a**–**c**) NHEKs were stimulated with *E. coli* for 3, 24, or 96 hours, respectively (**d**) or with LPS B5:055 for 24 hours. Relative transcript levels of (**a**) S100A15S, (**b**) S100A15L, and (**c**) S100A7 are shown (densitometric quantification of S100A15S, S100A15L, and S100A7 mRNA each over the β-actin mRNA transcript). Each bar represents the mean + SD of three independent experiments performed in duplicates. (**d**) In a next experiment, NHEKs were non-treated (lane 1) or were stimulated with LPS 100 ng/ml (lane 2) or 1,000 ng/ml (lane 3). Graph shows the results of one representative experiment out of three. Significance of alterations was tested using the two-way analysis of variance (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

A neutralizing TLR4 antibody suppresses *E. coli*-induced expression of S100A15L and IL-8 release in NHEK

In vitro and *in vivo* studies indicate that the pattern recognition receptor TLR4 primarily mediates cellular signaling induced by Gram-negative bacteria (Poltorak *et al.*, 1998; Takeuchi *et al.*, 1999). *E. coli* is therefore a predicted activator of TLR4 (Beutler, 2002; Takeda *et al.*, 2003). TLR4 is a member of 11 TLRs identified in man and is constitutively expressed on NHEK (Song *et al.*, 2002; Pivarcsi *et al.*, 2003; Lebre *et al.*, 2007). TLR activation initiates a signaling cascade including nuclear translocation of NFκB and proinflammatory cytokine release (Takeda *et al.*, 2003).

To investigate whether *E. coli*-mediated effects on the expression of S100A15 are mediated through TLR4, before stimulation with *E. coli*, NHEKs were treated with a TLR4 neutralizing antibody (anti-TLR4) or an isotype IgG control antibody for 30 minutes and expression of S100A15L and S100A15S and the release of IL-8 were examined (Figure 3 and data not shown). Co-incubation of NHEK with the TLR4 antibody alone or an isotype control IgG did not significantly change the expression of S100A15S or S100A15L nor the release of IL-8 (Figure 3 and data not shown). Results obtained from RT-PCR and ELISA analysis show that anti-TLR4 significantly inhibits *E. coli*-induced effects on the transcript levels of S100A15L (Figure 3b), and on release of IL-8 (Figure 3c). However, an inhibiting effect of anti-TLR4 on *E. coli*-induced upregulation of S100A15S was observed but was not statistically significant (Figure 3a).

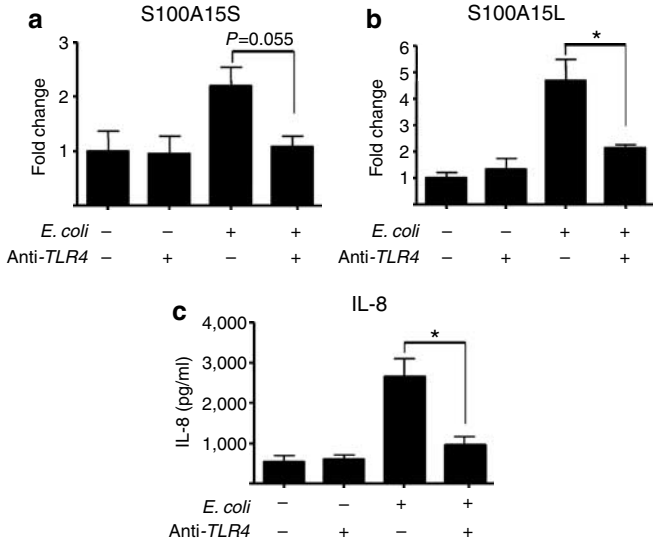

Figure 3. Inhibition of *E. coli* induced S100A15L expression and IL-8 release by TLR4 blocking antibody. NHEKs were pretreated with neutralizing antibodies against TLR4 (anti-TLR4) or control IgG (data not shown) 30 minutes before the stimulation with *E. coli* for 24 hours. Relative transcript levels of (**a**) S100A15S and (**b**) S100A15L are shown (densitometric quantification of S100A15S or S100A15L mRNA each over the β-actin mRNA transcript). Each bar represents the mean + SD of three independent experiments each performed in duplicates. (**c**) IL-8 release was measured in cell culture supernatants using ELISA. Data are means ± SD of triplicates of one representative experiment out of three (*$P<0.05$). The inhibiting effect of anti-TLR4 on *E. coli*-induced S100A15S upregulation was not statistically significant ($P=0.055$).

E. coli-induced mS100A15 expression *in vivo* is dependent on TLR4

To further elucidate the role of *E. coli*-induced mS100A15 expression in mice, *in vitro* and *in vivo* experiments were performed. To test if mS100A15, the murine ortholog of the human S100A7/A15 family, is also regulated by *E. coli,* murine keratinocytes were cultured and stimulated with heat-killed *E. coli* and expression of mS100A15 was evaluated (Figure 4a). Levels of mS100A15 mRNA were upregulated in murine keratinocytes upon stimulation with *E. coli* (Figure 4a). Based on our *in vitro* results in human keratinocytes, where inhibition of TLR4 abrogated *E. coli*-induced S100A15 expression, *in vivo* experiments were performed. C57BL/6 wild-type and TLR4−/− mice were infected with *E. coli* intracutaneously and RT-PCR and immunofluorescence staining were performed after 24 hours. In RT-PCR analyses, skin of wild-type mice but not of TLR4−/− mice, showed upregulation of mS100A15 after infection with *E. coli* (Figure 4b). These data were confirmed by immunofluorescence staining for mS100A15 (Figure 4c), using a specific antibody (Wolf *et al.*, 2006). Increased staining of mS100A15 was observed in *E. coli*-injected skin of wild-type mice but not in TLR4−/− mice. In wild-type mice, staining was localized throughout the whole epidermis after infection with *E. coli*.

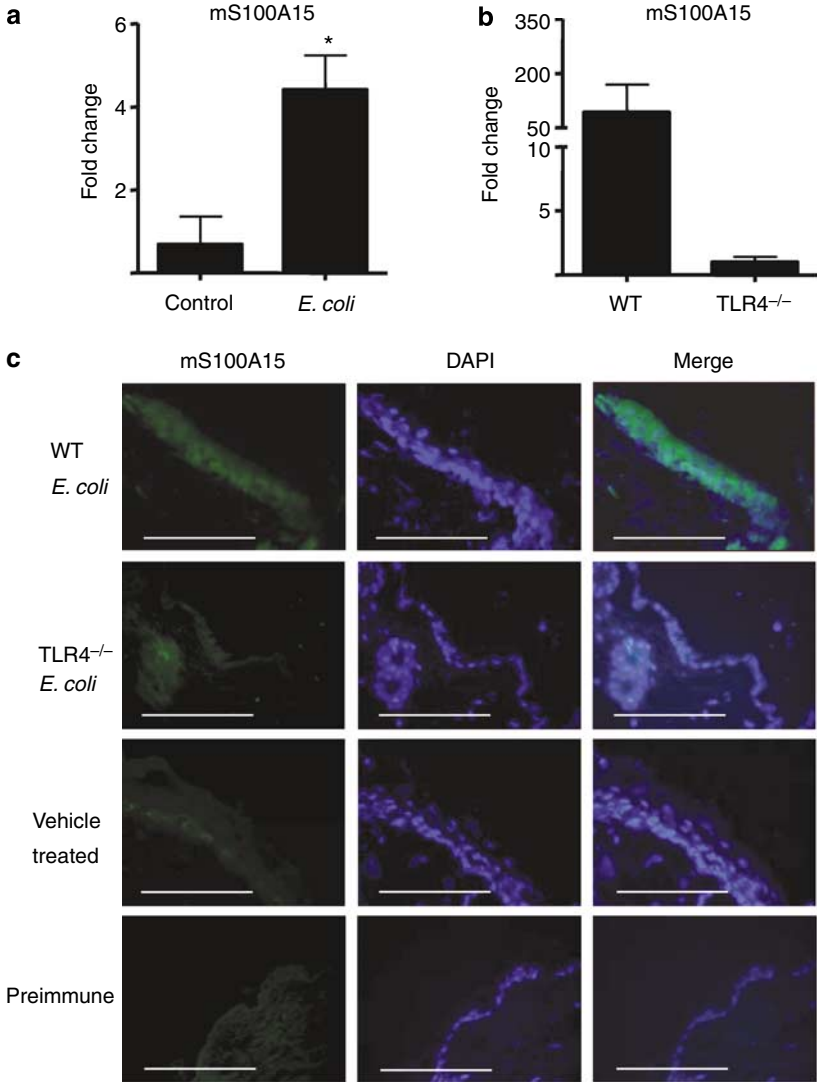

Figure 4. Expression of mS100A15 is induced by *E. coli* and mediated through TLR4. (**a**) For *in vitro* studies, murine keratinocytes were stimulated with *E. coli* for 24 hours and RT-PCR was performed. (**a**) *E. coli* stimulation induced upregulation of mS100A15 in murine keratinocytes. For *in vivo* experiments, wild-type (WT) and TLR4−/− mice were infected with *E. coli* for 24 hours and RT-PCR and immunofluorescence staining were performed. (**b**) Mouse skin of wild-type mice (WT) upregulated mS100A15 gene expression after infection with *E. coli*, whereas TLR4−/−mice did not. Mouse skin was stained for mS100A15 (green) and nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; blue). (**c**) Increased staining of mS100A15 throughout the whole epidermis was observed in *E. coli*-injected skin of wild-type mice but not in TLR4−/− mice. Fold induction of mRNA gene expression relative to vehicle-treated controls or wild-type mice was calculated using the $2^{(-\Delta\Delta C_t)}$ method, as described in Materials and Methods. *In vitro* stimulation experiments were performed in triplicates and shown are means ± SD from one representative experiment. Relative gene expression of mS100A15 in mouse skin is presented as mean ± SEM. Immunofluorescence stainings are representative for all mice tested (*$P < 0.05$). Bar = 0.1 mm.

In control mice as well as in TLR4−/− mice, a modest staining was observed in suprabasal layers and in hair follicles (Figure 4c). Thus, our data provide further evidence that mS100A15 protein expression is induced by *E. coli* and is regulated through TLR4 signaling.

S100A15 has antimicrobial activity against *E. coli*

Although human skin is capable of producing various antimicrobial proteins with a broad spectrum of activity, many bacteria survive on human skin, whereas *E. coli* is rapidly killed (Bevins, 2005; Gläser *et al.*, 2005). To investigate whether S100A15 has antimicrobial activity against *E. coli*, we performed a colony-forming unit (CFU) assay and minimal inhibitory concentration assays, using recombinant S100A15. Recombinant S100A7 and/or the AMP LL-37 were used as positive controls, because both are well-known AMPs with killing activity against *E. coli* (Gläser *et al.*, 2005; Dorschner *et al.*, 2006). Bacterial killing and growth inhibitory effects against *E. coli* were observed for S100A15 (Figure 5a and b), suggesting a role for this protein in skin defense against bacterial colonization.

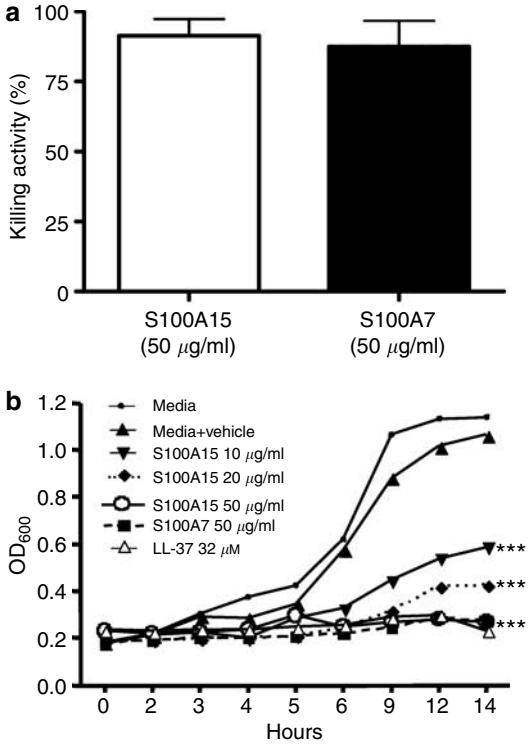

Figure 5. S100A15 has antimicrobial activity against *E. coli*. To assess antibacterial activity against *E. coli*, (**a**) an antimicrobial assay and (**b**) a minimal inhibitory concentration assay were performed. (**a**) *E. coli* was incubated for 3 hours with 50 μg/ml recombinant S100A15 or S100A7. The number of bacterial colonies was counted and percentage of killing activity was calculated as described in Materials and Methods. Data shown are means ± SD from three independent experiments. (**b**) *E. coli* was co-incubated with recombinant S100A15 (10, 20, and 50 μg/ml), S100A7 (50 μg/ml), and cathelicidin LL-37 (32 μM), and bacterial growth was monitored over time to determine antimicrobial activity. Conditions not containing recombinant proteins or containing the vehicle were used as negative controls. Bacterial growth was measured by OD_{600}. Data are means of triplicates of one representative experiment out of two (*$P<0.05$).

DISCUSSION

Epithelial surfaces like the skin depend on two major mechanisms of innate immune responses for protection against microbes: (1) the existence of receptors to recognize pathogens (Medzhitov and Janeway, 2000; Zhang and Ghosh, 2000) and (2) employment of mechanisms that kill pathogens, such as AMPs and proteins (Braff *et al.*, 2005). This is supported by observations in humans and animals where deficient AMP expression is associated with increased risk for infection (Morrison *et al.*, 1999; Nizet *et al.*, 2001; Ong *et al.*, 2002; Pütsep *et al.*, 2002). Similarly, mice deficient in pattern recognition by lacking TLRs have been shown to be more susceptible to infections than wild-type mice (Branger *et al.*, 2004). However, little is known about the mechanisms that control AMP expression and function in the skin upon infection with *E. coli*.

In this study, we demonstrate the differential regulation of human S100A15 and S100A7 as well as the murine S100A15 in response to stimulation with microbes and bacterial components. In primary human keratinocytes, *E. coli* induced the strongest upregulation of S100A15L from all bacteria tested. Furthermore, we demonstrate that murine and human S100A15 orthologs are similarly regulated in keratinocytes, supporting our previous results (Wolf *et al.*, 2006). *In vivo* studies, using TLR4−/− and wild-type mice, revealed that TLR4 signaling is important for mS100A15 regulation. These data are in accordance with *in vitro* results obtained from human keratinocytes. Induction of S100A15S and S100A15L transcript abundance, both coding for the human protein S100A15, was suppressed by neutralizing TLR4 antibodies. In our experiments, this blocking effect was more pronounced for the long splice variant S100A15L compared to the short splice variant S100A15S. Alternative splicing is a versatile mechanism to create diversity and flexibility in the regulation of gene expression. It has been already described that the composition and length of splice variants influence mRNA transcription, processing efficiency, and half-life (Ayoubi and Van de Ven, 1996). Hence, it is possible that one or more of these mechanisms are responsible for the different levels of the splice variants of human S100A15. Currently, antibodies recognizing specifically human S100A15 are under development and will be of great interest for future studies.

Our studies strongly suggest involvement of the TLR4 pattern recognition receptor in S100A15 regulation in skin. TLR4 was the first human homologue of Toll to be described (Rock *et al.*, 1998) and was subsequently characterized as a receptor for LPS signaling (Tapping *et al.*, 2000; Takeda *et al.*, 2003). Controversial results have been reported regarding expression and role of LPS reactivity mediated by TLR4 in primary human keratinocytes. Some authors found TLR4 to be expressed by primary and HaCaT keratinocytes (Song *et al.*, 2002; Pivarcsi *et al.*, 2003; Lebre *et al.*, 2007), whereas other authors showed no TLR4 expression and no LPS reactivity in cultured keratinocytes (Kawai *et al.*, 2002; Köllisch *et al.*, 2005). Our own results are consistent with this

controversy, as semiquantitative PCR analysis of TLR4 expression in NHEK from mammary surgery showed positive expression, whereas NHEKs from commercial sources were negative for TLR4 (unpublished data). These controversial results may be due to different culture conditions, differentiation status of the cells, and origin of keratinocytes, an issue previously described (Pivarcsi *et al.*, 2004). In this study, we demonstrated that in NHEK neutralizing antibodies against human TLR4 significantly inhibited *E. coli*-induced IL-8 secretion and expression of S100A15L but not that of S100A15S. We hereby demonstrate that TLR4 has a functional role in human keratinocytes, confirming the results described by several authors (Song *et al.*, 2002; Pivarcsi *et al.*, 2003; Lebre *et al.*, 2007). In a mouse model using wild-type and TLR4−/− mice, we demonstrated that only wild-type mice upregulate mS100A15 expression upon *E. coli* infection of the skin indicating that TLR4 is important for initiation of an innate immune response in keratinocytes. However, we cannot exclude that pattern recognition receptors other than TLR4 contribute to the *E. coli*-induced effects on S100A15 expression. The dependence upon TLR4 for keratinocyte responsiveness for *E. coli*, as demonstrated in this work, may be direct or indirect. *In vivo* recognition of pathogens by TLRs leads to the release of proinflammatory cytokines and chemokines (Takeda *et al.*, 2003; Akira and Takeda, 2004; Beutler, 2004), which are responsible for the recruitment of inflammatory cells to sites of infection and the initiation of an enhanced immune response in keratinocytes. It is thereby possible that upregulation of mS100A15 expression *in vivo* is caused by both or either, a direct effect on keratinocytes or an indirect effect mediated by proinflammatory cytokines produced after *E. coli* infection in the skin. The inability of *E. coli* to colonize healthy human skin may be due to the efficiency of the innate immune system that employs at least two mechanisms: first, keratinocytes rapidly recognize pathogens and mount an antimicrobial response; second, the same mechanism of pathogen recognition by TLRs is used to induce an inflammatory immune response. Production of proinflammatory IL-8 in NHEK following stimulation with *E. coli* in *in vitro* conditions was observed here and induction *in vivo* remains to be studied. However, the hypothesis of this ''dual'' mechanism of innate immune function may contribute to certain skin diseases. Many AMPs have been shown to be overexpressed in skin states going along with a strong inflammatory component, such as psoriasis and wound healing (Wolf *et al.*, 2003; Lee and Eckert, 2007; Schauber *et al.*, 2007). Thus, recognition of pathogens in diseased skin may result in enhanced antimicrobial and inflammatory immune responses and may thereby result in exacerbation of inflammation.

In order to evaluate if upregulated S100A15 expression through bacterial components reflects protection against *E. coli*, we performed antimicrobial assays identifying S100A15 as a new antimicrobial protein. Antimicrobial properties have already been described for several members of the S100 family, including S100A7, S100A8, S100A9, and S100A12 (Gottsch *et al.*, 1999; Gläser *et al.*, 2005). Notably, antimicrobial activity of these calcium-binding proteins is not dependent on calcium concentrations, but has been shown for few S100 proteins to be associated with zinc ion concentrations (Sohnle *et al.*, 2000; Gläser *et al.*, 2005). Also, it is possible that in addition to S100A15, several antimicrobial proteins synergize with each other, as previously suggested for defensins and cathelicidins (Nagaoka *et al.*, 2000).

In summary, our results complement previous observations that *E. coli* has a strong and underappreciated effect on cutaneous innate immunity through regulation of S100 proteins. In recent years, much has been learned about how microbes and bacteria-derived molecules activate the innate immune system. AMPs and proteins play a key role in the innate immune response and confer resistance to bacterial infection of the skin. Understanding the regulation and function of S100 proteins as effector molecules of the innate antimicrobial response might lead to new treatments of infectious skin disease.

MATERIALS AND METHODS

Reagents

The S100A15 and S100A7 cDNA sequences, containing a 6× histidine tag sequence, were ligated into PQE30-vector (Qiagen, Hilden, Germany). *E. coli* M15 were transformed with each resulting expression vectors, and recombinant S100A15 and S100A7 proteins were produced by *E. coli* M15, according to the manufacturer's protocol (Qiagen). *E. coli* M15 itself was not killed by production of S100A15 and S100A7 probably due to strain-specific antimicrobial activity, as it has been already observed for S100A7 (Gläser *et al.*, 2005). Recombinant proteins were purified by Ni-NTA-agarose. The amounts of the purified proteins were quantified by NuPAGE (10% Bis-Tris gel) and Western Blot detection. Each recombinant protein was kept in a solution of 1:1 phosphate-buffered saline (PBS)/glycerine and stored at −20°C until use. LPS (O55:B5) derived from *E. coli* (Sigma-Aldrich, Steinheim, Germany) was used at concentrations of 100 and 1,000 ng/ml that have been shown to induce a significant cell response (Shimazu *et al.*, 1999; Song *et al.*, 2002; Lebre *et al.*, 2007). Functional-grade neutralizing anti-human TLR4 antibody (anti-TLR4), clone HTA 125, was purchased from Serotec (Düsseldorf, Germany) and used at a concentration of 10 μg/ml (Shimazu *et al.*, 1999) and several approaches were taken to demonstrate the specificity of this antibody (Shimazu *et al.*, 1999). As isotype control for the TLR4 antibody, purified mouse IgG was used (Pharmingen, Heidelberg, Germany).

Keratinocyte culture and stimulation of keratinocytes

NHEKs were obtained from skin samples of healthy individuals undergoing breast reduction plastic surgery after informed consent and the use of NHEKs was approved by the ethical committee for human research at the University of Duesseldorf. The study was conducted according to the Declaration of Helsinki Principles. Cells were seeded in 175 cm^2 flasks at a density of 4×10^4 cells/cm^2 maintained in a serum-free 1:1 mixture of keratinocyte-SFM (Gibco, Eggenstein, Germany) and MCDB 153 (Biochrom, Berlin, Germany) containing supplements (epidermal growth factor, bovine pituitary gland extract, glutamine, and antibiotic/antimycotic mixture, all purchased from Gibco). Keratinocytes were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Keratinocytes were

cultured for three passages and were kept in supplement-free medium for 24 hours before stimulation experiments. Normal murine keratinocytes were a kind gift by Dr Jamora (University of California, San Diego, CA). All bacterial strains were kindly provided by the Department of Medical Microbiology, Heinrich Heine University Duesseldorf, Germany and the Division of Dermatology, University of California San Diego and VA San Diego Healthcare System, San Diego, CA. We used standard laboratory strains for *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 15442), and *S. aureus* (ATCC 25923). For *in vitro* stimulation experiments, bacterial cultures were allowed to grow in Luria-Bertani (LB) medium at 37°C to a optical density $(OD)_{600}$ of 0.8. Bacteria were harvested and washed twice in PBS, diluted to working solutions and heat-inactivated at 68°C for 20 min. For some experiments, bacteria were further centrifuged at $3,000 \times g$ for 20 minutes to obtain a supernatant and a pellet. Heat-killed bacterial compounds were used to stimulate NHEK corresponding to approximately 3.5×10^4 CFU/ml for *E. coli*, 2.5×10^4 CFU/ml for *P. aeruginosa*, and 1.8×10^4 CFU/ml for *S. aureus* per well.

RNA preparation, semiquantitative and real-time PCR

Total RNA was extracted from human keratinocytes by using the RNAeasy Minikit (Qiagen) or from mouse skin samples and murine keratinocytes that were homogenized in TRIZOL reagent (Invitrogen, Karlsruhe, Germany). For mouse skin samples, a dismembrator was used. For human keratinocytes, 1 μg of total RNA was reversibly transcribed by Superscript II (Invitrogen), according to the manufacturer's protocol. For amplification of human S100A15S, S100A15L, S100A7, and β-actin primer pairs were used as described previously (Wolf *et al.*, 2003; Gläser *et al.*, 2005) and were commercially synthesized by MWG-Biotech (MWG Biotech, Ebersberg, Germany). PCRs were carried out in a total volume of 20 μl, containing 1 μl of the RT sample, 2 μl 10 × buffer (Qiagen), 0.2 mM dNTPs, 2 U *Taq* polymerase (Qiagen), and 0.2 μM of specific primer pairs for the human S100A15 long isoform (7.1 sense 5′-ACGTCA CTCCTGTCTCTCTTTGCT-3′, 6.2 antisense 5′-TCATGAATCAACCC ATTTCCTGGG-3′), the S100A15 short isoform (NCBP5F sense 5′-CA AGTTCCTTCTGCTCCATCTTAG-3′, NCBP4R antisense 5′-AGCCTT CAGGAAATAAAGACAATC-3′), S100A7 (sense 5′-AGACGTGATG CAAGATTGAC-3′, antisense 5′-TGTCCTTTTTCTCAAAGACGTC-3′, β-actin (F(A) sense 5′-AGAGATGGCCACGGCTGCTT-3′, R(A) antisense 5′-ATTTGCGGTGGACGATGGAG-3′). PCRs were performed in duplicate and each PCR was performed at two cycle numbers within the log-linear phase. The PCR conditions were 31 and 33 cycles for S100A15L, 37 and 39 cycles for S100A15S and S100A7, and 20 and 22 cycles for β-actin at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute (+time increment of 2 seconds/cycle) followed by an additional extension step at 72°C for 5 minutes. A negative control was set up for each reaction. Amplicons were subjected to electrophoresis in a 1.5% agarose gel containing 0.002% ethidium bromide. The density of amplicons was measured by UV transillumination of the stained bands, using a digital camera analyzing system (Multianalyst, Bio-Rad Laboratories, Hercules, CA). The amount of each S100A15 isoform was normalized by β-actin expression. For murine keratinocytes and mouse skin samples, an iScript cDNA synthesis kit was used (Bio-Rad). For amplification of mS100A15, real-time RT-PCR was performed. A predeveloped Taqman assay probe was used for mS100A15 (ABI, Foster City, CA). 18S (forward primer: CGGAGGTTCGAAGACGAT CA, reverse primer: CATCGTTTATGGTCGGAACTACG) expression was evaluated using a SYBR Green protocol, according to the manufacturer (ABI). Analyses were performed in triplicate in an ABI Prism 7000 Sequence Detection System. Fold induction relative to the vehicle-treated control was calculated using the $2^{(-\Delta\Delta Ct)}$ method, where $\Delta\Delta C_t$ is $\Delta C_{t(\text{stimulant})} - \Delta C_{t(\text{control})}$, ΔC_t is $C_{t(\text{target gene})} - C_{t(\text{housekeeping gene})}$, and CT is the cycle at which an arbitrary detection threshold is crossed. Data are presented as means + SD for human and murine keratinocytes, for mouse skin as means + SEM. For statistical analysis, a Student's *t*-test was performed unless otherwise indicated. We considered a $P < 0.05$ to be statistically significant.

EMSA and supershift assay

EMSAs have been performed as described previously (Hassan *et al.*, 2004, 2005). Double-stranded oligonucleotides containing the AP-1 or NF-κB consensus sequence (Santa Cruz Biotechnology, Santa Cruz, CA) were 5′-end-labeled with [γ-^{32}P] ATPs (Hartmann Analytica, Hilden, Germany). DNA-binding reactions were performed, using 4 μg nuclear extract and 0.2 ng probe in a total volume of 30 μl for 30 minutes at room temperature in binding buffer (10 mM Tris, pH 7.5; 50 mm NaCl; 1 mM EDTA; 1 mM $MgCl_2$; 0.5 mM dithiothreitol; and 4% glycerol). The specificity of binding was analyzed by competition with an unlabeled oligonucleotide. After incubation, the binding reactions were loaded on a 6% native polyacrylamide/0.5 × TBE (45 mM Tris-borate and 1 mM EDTA) gel, and run in 0.5 × Tris-borate/EDTA buffer at room temperature. The dried gel was visualized by exposure to high-performance autoradiography film.

Supershift assays were performed by incubation of nuclear extracts (obtained from human keratinocytes following stimulation with *E. coli*) with either anti-c-jun antibody (Sc-1694) or with anti-p50 (Sc-7178) and/or p65 (Sc-109) antibodies (all from Santa Cruz Biotechnology), to prove specificities of AP-1 and NF-κB DNA-binding activities, respectively. The antibodies were preincubated for 30 minutes at 37°C with the nuclear extracts and then allowed to incubate with 0.2 ng of the labeled oligonucleotide of AP-1 or NF-κB for 30 minutes at room temperature. Samples were subjected to EMSA as described above for AP-1 and NF-κB gel shift assay.

IL-8 ELISA

Human IL-8 was assayed in supernatant-stimulated NHEK using the Quantikine human IL-8 immunoassay kit (R&D Systems, Wiesbaden, Germany), according to the manufacturer's instructions. The standard curves were performed using serial dilutions of recombinant human IL-8. The optical density of the wells was determined using a microplate reader set at 450 nm.

Antimicrobial assay

E. coli (ATCC 25922) was grown until log-phase in LB medium, centrifuged $350 \times g$ for 5 minutes, and diluted in PBS (Gibco, calcium and magnesium free, pH 7.4) to an end concentration of 1.5×10^6 CFU/ml. A 50 μg/ml portion of recombinant S100A15 or S100A7 protein or their vehicle (control) was added to the bacterial dilutions and incubated for 3 hours. A 10 μl portion of bacterial solution with or without recombinant proteins was plated in serial dilutions on agar plates overnight at 37°C for enumeration of CFU.

The killing activity of S100A15 and S100A7 was calculated as the percentage of bacterial death compared to vehicle-incubated controls expressed as [1−(survival after recombinant protein incubation)/(survival after vehicle incubation) × 100].

Minimal inhibitory concentration assay

Minimal inhibitory concentration assays were performed as described previously (Dorschner *et al.*, 2006) with slight modifications. Growing bacteria in media containing $NaCOH_3$ and $NaPOH_4$ increases the sensitivity of bacteria to AMPs (Dorschner *et al.*, 2006). Bacteria were seeded in a 96-well plate and recombinant proteins of S100A15, S100A7, and synthetic LL-37 were added in concentrations as indicated. As a negative control, bacteria were grown in media only or in media containing the protein vehicle (PBS/glycerine) for recombinant S100A15 and S100A7. OD_{600} was measured at indicated time points.

Mice and *E. coli* infection model

All mice experiments were approved by the Veteran Affair San Diego subcommittee on animal studies. Female C57BL/6 wild-type mice and TLR4−/− mice on C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME) and kept under specified pathogen-free conditions. Three mice of 10 weeks were used per group. Mouse hair was removed 48 hours before the experiments were started. For infection, *E. coli* was grown in LB at 37°C overnight and then subcultured until log phase. Bacteria were harvested, washed twice in PBS, and were kept in 1:1 dilution with cytodex beads. Bacteria (0.9×10^7) were injected per mouse in a volume of 100 μl intracutaneously in the left flank, injection of the vehicle followed in the right flank. Twenty-four hours after infection, mice were killed and skin punch biopsies (0.8 mm diameter) were obtained from bacteria- and vehicle-injected sites. Half of the skin sample was used for immunofluorescence; the other half was used for RT-PCR analyses.

Fluorescence immunostaining

Immunofluorescence was performed on serial 10 μM frozen sections of mouse skin, fixed in acetone as described previously with slight modifications (Wolf *et al.*, 2006). Briefly, sections were blocked in 5% goat serum, and incubated overnight with mS100A15 rabbit-derived polyclonal antibody (5 μg/ml, antibody 3961) or preimmune serum. After washing in PBS for three times 15 minutes, slides were reprobed with an FITC-labeled goat anti-rabbit antibody (Sigma-Aldrich, St Louis, MO). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR). Slides were mounted in ProLong Anti-Fade reagent (Molecular Probes) and evaluated with an Olympus BX41 microscope (Olympus, Melville, NY) at ×100 original magnification.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Professor Dr med. Bernhard Homey from the Department of Dermatology, Heinrich-Heine-University, Düsseldorf, Germany for support and comments. We also thank all members from our laboratories for their helpful and critical suggestions. This work was supported by a grant from Deutsche Forschungsgemeinschaft (DFG) for R.W. (Emmy-Noether Program, Wo 843/2-1) and by a grant from Deutsche Forschungsgemeinschaft (DFG) for A.S.B. (BU 2212/1-1).

REFERENCES

Ambartsumian N, Tarabykina S, Grigorian M, Tulchinsky E, Hulgaard E, Georgiev G *et al.* (1995) Characterization of two splice variants of metastasis-associated human mts 1 gene. *Gene* 159:125–30

Akira S, Takeda K (2004) Toll-like receptor signalling. *Nat Rev Immunol* 4:499–511

Ayoubi TA, Van de Ven WJ (1996) Regulation of gene expression by alternative promoters. *FASEB J* 10:453–60

Beutler B (2002) TLR-4 as the mammalian endotoxin sensor. *Curr Top Microbiol Immunol* 270:109–20

Beutler B (2004) Innate immunity: an overview. *Mol Immunol* 40:845–59

Bevins CL (2005) An important clue: fingerprints point to psoriasin in defense against *E. coli*. *Nat Immunol* 6:12–3

Braff MH, Di Nardo A, Gallo RL (2005) Keratinocytes store the antimicrobial peptide cathelicidin in lamellar bodies. *J Invest Dermatol* 124:394–400

Branger J, Leemans JC, Florquin S, Wejer S, Speelman P, Van der Poll T (2004) Toll-like receptor 4 plays a protective role in pulmonary tuberculosis in mice. *Int Immunol* 16:509–16

Cole AM, Kim YH, Tahk S, Hong T, Weis P, Waring AJ, Ganz T (2001) Calcitermin, a novel antimicrobial peptide isolated from human airway secretions. *FEBS Lett* 504:5–10

de Jongh J, Zeeuwen PLJM, Kucharekova M, Pfundt R, van der Valk PG, Blokx W *et al.* (2005) High expression levels of keratinocyte antimicrobial proteins in psoriasis compared with atopic dermatitis. *J Invest Dermatol* 125:1163–73

Donato R (2001) S100: a multigenic family of calcium-modulated proteins of the EF-hand type with intracellular and extracellular functional roles. *Int J Biochem Cell Biol* 33:637–68

Dorschner RA, Lopez-Garcia B, Peschel A, Kraus D, Morikawa K, Nizet V *et al.* (2006) The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. *FASEB J* 20:35–42

Eckert R, Broome A, Ruse M, Robinson N, Ryan D, Lee K (2004) S100 proteins in the epidermis. *J Invest Dermatol* 123:23–33

Foell D, Roth J (2004) Proinflammatory S100 proteins in arthritis and autoimmune disease. *Arthritis Rheum* 50:3762–71

Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H *et al.* (1997) The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* 272:15258–63

Gläser R, Harder J, Lange H, Bartels J, Christophers E, Schröder JM (2005) Antimicrobial psoriasin (S100A7) protects human skin from *Escherichia coli* infection. *Nat Immunol* 6:57–64

Gottsch JD, Eisinger SW, Liu SH, Scott AL (1999) Calgranulin C has filariacidal and filariastatic activity. *Infect Immun* 67:6631–6

Harder J, Schröder JM (2005) Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *J Leukoc Biol* 77:476–86

Hassan M, Ghozlan H, Abdel-Kader O (2004) Activation of RB/E2F signaling pathway is required for the modulation of hepatitis C virus core protein-induced cell growth in liver and non-liver cells. *Cell Signal* 16: 1375–85

Hassan M, Ghozlan H, Abdel-Kader O (2005) Activation of c-Jun NH_2-terminal kinase (JNK) signaling pathway is essential for the stimulation of Hepatitis C Virus (HCV) non-structural protein 3 (NS3)-mediated cell growth. *Virology* 333:324–36

Kawai K, Shimura H, Minagawa M, Ito A, Tomiyama K, Ito M (2002) Expression of functional Toll-like receptor 2 on human epidermal keratinocytes. *J Dermatol Sci* 30:185–94

Köllisch G, Kalali BN, Voelecker V, Wallich R, Behrendt H, Ring J *et al.* (2005) Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* 114:531–41

Komine M, Rao LS, Kaneko T, Tomic-Canic M, Tamaki K, Freedberg IM *et al.* (2000) Inflammatory versus proliferative processes in epidermis. Tumor necrosis factor alpha induces K6b keratin synthesis through a transcrip-

tional complex containing NFkappa B and C/EBPbeta. *J Biol Chem* 275:32077–88

Kulski JK, Lim CP, Dunn DS, Bellgrad M (2003) Genomic and phylogenetic analysis of the S100A7 (Psoriasin) gene duplications within the region of the S100 gene cluster on human chromosome 1q21. *J Mol Evol* 56:397–406

Lebre MC, van der Aar AM, van Baarsen L, van Capel TM, Schuitenmaker JH, Kapsenberg ML *et al.* (2007) Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol* 127:331–41

Lee KC, Eckert RL (2007) S100A7 (Psoriasin) – mechanism of antibacterial action in wounds. *J Invest Dermatol* 127:945–57

Lehrer RI, Barton A, Daher KA, Harwig SS, Ganz T, Selsted ME (1989) Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. *J Clin Invest* 84:553–61

Medzhitov R, Janeway CA (2000) Innate immunity. *N Engl J Med* 343:338

Mirmohammadsadegh A, Tschakarjan E, Ljoljic A, Bohner K, Michel G, Ruzicka T *et al.* (2000) Calgranulin C is overexpressed in lesional psoriasis. *J Invest Dermatol* 114:1207–8

Morrison GM, Davidsom DJ, Dorin JR (1999) A novel mouse beta defensin, Defb2, which is upregulated in the airways by lipopolysaccharide. *FEBS Lett* 442:112–6

Nagaoka I, Hirota S, Yomogida S, Ohweda A, Hirata M (2000) Synergistic actions of antimicrobial neurtophil defensins and cathelicidins. *Inflamm Res* 49:73–9

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudsill J, Dorschner RA *et al.* (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414:454–7

Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T *et al.* (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 347:1151–60

Pivarcsi A, Koreck A, Bodai L, Szell M, Szeg C, Belso N *et al.* (2004) Differentiation-regulated expression of Toll-like receptors 2 and 4 in HaCaT keratinocytes. *Arch Dermatol Res* 296:120–4

Pivarcsi A, Bodai L, Rethi B, Kenderssy-Szabo A, Koreck A, Szell M *et al.* (2003) Expression and function of Toll-like receptors 2 and 4 in human keratinocytes. *Int Immun* 15:721–30

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X *et al.* (1998) Defective LPS signalling in C3H/Hej and C57BL/10ScCR mice: mutations in Tlr4 gene. *Science* 282:2085–8

Pütsep K, Carlsson G, Boman HG, Andersson M (2002) Deficiency of antimicrobial peptides in patients with morbus Kostmann: an observation study. *Lancet* 360:1144–9

Qin JZ, Chaturverdi V, Denning MF, Choubey D, Diaz MO, Nickoloff BJ (1999) Role of NF-kappaB in the apoptotic-resistant phenotype of keratinocytes. *J Biol Chem* 274:37957

Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF (1998) A family of human receptors related to *Drosophila* Toll. *Proc Natl Acad Sci USA* 95:588–93

Santamaria-Kisiel L, Rintala-Dempsey AC, Dhaw GS (2006) Calcium-dependent and -independent interactions of the S100 protein family. *Biochem J* 396:201–14

Schauber J, Dorschner RA, Coda AB, Büchau AS, Liu PT, Kiken D *et al.* (2007) Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J Clin Invest* 117: 803–811

Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K *et al.* (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med* 189:1777–82

Sohnle PG, Hunter MJ, Hahn B, Chazin WJ (2000) Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor-related proteins 8 and 14). *J Infect Dis* 182:1272–5

Song PI, Park YM, Abraham T, Harten B, Zivony A, Nepardize N *et al.* (2002) Human keratinocytes express functional CD14 and Toll-like receptor 4. *J Invest Dermatol* 119:424–32

Takeda K, Kaisho T, Akira S (2003) Toll-like receptors. *Annu Rev Immunol* 21:335–76

Takeuchi O, Hoshino K, Kawaei T, Sanjo H, Takada H, Ogawa T *et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity* 11: 443–451

Tapping RI, Akashi S, Miyake K, Godowski PJ, Tobias PS (2000) Toll-like receptor 4, but not Toll-like receptor 2, is signalling receptor for *Escherichia* and *Salmonella* lipopolysaccharides. *J Immunol* 165:5780–7

Wolf R, Mirmohammadsadegh A, Walz M, Lysa B, Tartler U, Remus R *et al.* (2003) Molecular cloning and characterization of alternatively splice mRNA isoforms from psoriatic skin encoding a new member of the S100 family. *FASEB J* 17:1969–71

Wolf R, Voscopopulos CJ, FitzGerald PC, Goldsmith P, Cataisson C, Gunsior M *et al.* (2006) The mouse S100A15 ortholog parallels genomic structure, gene expression, and protein-processing pattern of the human S100A7/A15 subfamily during epidermal maturation. *J Invest Dermatol* 126:1600–8

Zhang G, Ghosh S (2000) Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. *J Endotoxin Res* 6:453–7