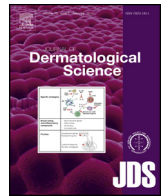




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Staphylococcus aureus inhibits terminal differentiation of normal human keratinocytes by stimulating interleukin-6 secretion

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ABSTRACT

Background: *Staphylococcus aureus* (*S. aureus*) is found on the skin of approximately 90% of patients with atopic dermatitis and approximately 20% of apparently healthy subjects. *S. aureus* induces keratinocytes and immune cells to secrete immunoregulatory factors that cause epidermal barrier dysfunction in atopic skin.

Objective: This study examined factors that cause epidermal permeability barrier dysfunction in skin colonized by *S. aureus*.

Methods: We examined the effect of *S. aureus* on keratinocyte differentiation in the stratum corneum (SC) of in vivo skin, normal human keratinocytes (NHKs) and a reconstructed human epidermis (RHE) model. The fold change in expression of the terminal differentiation markers and the level of secreted cytokines were investigated.

Results: The SC displayed decreased expression of keratin 10 (KRT 10). NHKs treated with *S. aureus* extracts increased expression of interleukin (IL)-6 and significantly reduced expression of the terminal differentiation markers KRT 1, KRT 10, loricrin (LOR), and filaggrin (FLG); however, the expression of basal layer markers (KRT 5, KRT 14) remained unchanged. Treatment of NHKs with an anti-IL-6 antibody in combination with IL-6 or the *S. aureus* extracts inhibited the decrease in KRT 10 mRNA or protein expression. After the RHEs were exposed to the *S. aureus* extracts, KRT 1 and KRT 10 protein levels decreased.

Conclusions: These findings suggest that *S. aureus* inhibits the terminal differentiation of keratinocytes by stimulating IL-6 secretion.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is the most common human skin pathogen, causing infectious skin disorders such as cellulitis, subcutaneous abscesses and ulcers [1,2]. The skin of individuals

with atopic dermatitis (AD) and psoriasis harbors significantly increased levels of *S. aureus* compared with healthy individuals [3–5]; an increased presence of *S. aureus* on the skin may be indicative of increased inflammation and epidermal barrier disruption, which are associated with the above-mentioned dermatoses. Interestingly, Shin et al. [6] revealed that the *S. aureus* count of apparently healthy human skin is significantly associated with low epidermal barrier function and increased epidermal desquamation, suggesting that the bacterium itself can induce epidermal barrier disruption. Indeed, several lines of evidence support this notion. In normal human keratinocytes (NHKs), *S. aureus* produces several virulence factors that induce inflammatory responses and tissue injury as well as various cytokines, including interleukin-6 [7]. Various extracellular proteases, such as V8 protease or V8 protease-like exfoliative toxins, cause epidermal disruption by degrading corneodesmosomes [8] and inducing the production of metalloproteases [9].

Abbreviations: *S. aureus*, *Staphylococcus aureus*; SC, stratum corneum; AD, atopic dermatitis; NHKs, normal human keratinocytes; KRT, keratin; FLG, filaggrin; LOR, loricrin.

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In contrast to the evidence demonstrating that *S. aureus* infection disrupts epidermal barrier function, little is known about the effects of *S. aureus* on keratinocyte differentiation, an important component of epidermal barrier function. The stratum corneum (SC) is composed of fully differentiated keratinocytes, intercellular lipids and natural moisturizing factors (NMFs). Because the SC maintains healthy skin function by retaining water, normal epidermal differentiation is critical to maintaining the integrity of the permeability barrier. Hyper-proliferation of undifferentiated keratinocytes interferes with epidermal barrier function; this abnormal epidermal differentiation frequently causes the dry skin associated with cutaneous diseases, such as AD [8,10] and psoriasis [10,11].

The differentiation of keratinocytes as they migrate from the basal layer to the upper layer of the epidermis is a complex process that requires the simultaneous activation and inactivation of many genes [12]. The specific phases of the differentiation process are characterized by the expression of certain marker genes [13–18]. The basal layer is characterized by expression of keratin (KRT) 5 and KRT 14, whereas KRT 1 and KRT 10 expression is predominant in the suprabasal layer. Filaggrin (FLG), type I transglutaminase (TG), involucrin (INV), and loricrin (LOR) are corneal envelope (CE) precursor proteins expressed in the suprabasal layer of the skin.

Immunohistochemical examination of skin with active AD lesions reveals evidence of epidermal hyper-proliferation [19] and expression of the cytokeratins KRT 6, KRT 16, and KRT 17; however, the suprabasal expression of KRT 10 and the basal expression of KRT 5 are reduced. In addition, expression of INV and FLG is reduced, whereas LOR expression is increased [19]. Psoriatic skin also shows reduced expression of KRT 1 and KRT 10 [11], supporting the notion that abnormal epidermal differentiation contributes to epidermal barrier dysfunction.

The present study examined epidermal differentiation in the SC of healthy human volunteers colonized by *S. aureus*. The results showed reduced expression of KRT 10 in the SC of volunteers with increased levels of *S. aureus* colonization. Moreover, the expression of KRT 1 and KRT 10 in NHKs and reconstructed human epidermis (RHE) significantly decreased after exposure to the *S. aureus* extracts. Of the cytokines secreted by *S. aureus*-treated NHKs, IL-6 appears to play an important role in suppressing keratinocyte differentiation. These findings provide important insight into the pathophysiological mechanisms underlying *S. aureus*-induced epidermal barrier dysfunction.

2. Materials and methods

2.1. Materials

One *S. aureus* strain (ATCC 6538) was purchased from ATCC, and two strains (*S. aureus*-1 and *S. aureus*-2) were isolated from human skin. IL-6, IL-8 and an anti-IL-6 antibody were purchased from R&D Systems (Minneapolis, MN). Anti-KRT 5, anti-KRT 14, anti-INV, anti-LOR, anti-caspase 14, anti-bleomycin hydrolase, anti-beta actin and anti-calpain 1 antibodies were purchased from Abcam (Cambridge, MA). Anti-KRT 1 and anti-KRT 10 antibodies were purchased from Covance Inc (Princeton, NJ). Anti-FLG was purchased from Novocastra (Newcastle upon Tyne, U.K.).

2.2. Detection of *S. aureus* in human skin and classification of *S. aureus* samples

Ten healthy male volunteers (mean age, 34 years; range, 24–47 years) were screened for *S. aureus* using square (3.3 cm × 3.3 cm) EasyStamp® Staph agar plates (Hanil Komed, Seoul, Korea) as described by Shin et al. [6]. Briefly, the plates were pressed onto the volunteer's right cheek and then incubated at 32 °C for 48 h.

Suspected *S. aureus* colonies (large, round, and golden-yellow) were confirmed using the Vitek identification system (BioMerieux, Marcy l'Etoile, France). The volunteers were classified into two groups: the *S. aureus*-positive group (>10 colonies detected; *n* = 3) and the *S. aureus*-negative group (0 colonies detected; *n* = 5). Two individuals who yielded ≤2 colonies were excluded from the study.

2.3. Cultivation of *S. aureus* strains

S. aureus was streaked onto tryptic soy agar (Difco Laboratories, Detroit, MI). Colonies were grown overnight in tryptic soy broth (TSB) at 32 °C in a shaking incubator (200 rpm). A sample of the overnight culture was diluted 1/100 and cultured to the mid-logarithmic phase (3 h). The final cell concentration was approximately 10⁹ colony-forming units (cfu)/mL. The *S. aureus* samples were then incubated at 75 °C for 40 min using a protocol modified from the method described by Sayama et al. [20] to prevent the bacteria from growing after they were exposed to the cells. The *S. aureus* suspension was then subjected to mild sonication and centrifugation at 13,000 × *g* for 15 min at 4 °C. The *S. aureus* extracts were obtained by resuspending the pellets in dH₂O, and the protein concentration was measured using a BSA protein assay kit (Pierce, Rockford, IL).

2.4. Biofilm formation by the *S. aureus* strains

The ability of the *S. aureus* strains to form biofilms was assessed using a protocol described by Heilmann et al. [21] with some modifications. Briefly, 100 μL of TSB was placed in each well of a flat-bottomed microtiter plate either with or without 1% glucose and 1% NaCl. A sample of the overnight bacterial culture was diluted to approximately 10⁷ cfu/mL in TSB, and 100 μL was added to each well. After incubation for 48 h at 32 °C, each well was washed twice with 100 μL of sterile water and then stained with 100 μL of crystal violet for 10 min. The stained adherent bacteria were resuspended in 100 μL of absolute ethanol, and biofilm formation was estimated by measuring the absorbance at 590 nm in a SpectraMax 190/spectrophotometer (Molecular Devices, LLC).

2.5. Tape stripping and protein extraction from SC samples

Five sequential SC samples (upper layer) were obtained from each volunteer by attaching D-squame tapes (2.3 cm × 2.3 cm; CuDerm, Dallas, TX) to the right cheek skin using a disk pressure applicator. The samples were sonicated in phosphate-buffered saline buffer and centrifuged. Soluble proteins were extracted from the SC samples using a Tris–HCl buffer (62 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol) according to the method of Redoules et al. [22].

2.6. Cultivation of NHKs

NHKs (neonatal foreskin) were purchased from Lonza (St. Louis, MO) and cultured in 60 mm dishes (0.1% precoated gelatin) with a defined keratinocyte growth medium (KGM, 0.1 mM CaCl₂) supplemented with human epidermal growth factor (hEGF), insulin, bovine pituitary extract, hydrocortisone, epinephrine, transferrin, and gentamicin/amphotericin B. The culture media of second passage keratinocytes (70–80% confluent) were treated with the *S. aureus* extracts (3 μg/mL or 6 μg/mL final protein concentration) or cytokines (10–50 ng/mL IL-6 or IL-8) for 4 days prior to differentiation marker analysis. For the neutralization experiments, keratinocytes were serum-starved in KBM medium (without supplements) for 24 h. Next, IL-6 or *S. aureus* extracts were added to the keratinocytes in the presence of 50 ng/mL anti-human IL-6 antibody. The KGM medium was changed after 2 days, and the cells were harvested 2 days later.

2.7. Real-Time RT-PCR

Total RNA was isolated from keratinocytes using Trizol (Invitrogen, Carlsbad, CA). The RNA was then quantitated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE), and 4 µg of the sample was used as a template for cDNA synthesis by reverse transcription with a SuperScript reverse transcriptase III kit (Invitrogen). Real-time PCR was performed in a solution (20 µL) containing 2× TaqMan universal PCR mixture (10 µL), 20× TaqMan expression assay mix (1 µL), cDNA (50 ng), and primers. qRT-PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). cDNA samples were analyzed for KRT 1, KRT 5, KRT 10, KRT 14, LOR, FLG, INV, Bleomycin hydrolase (BH), caspase-14 (CASP 14), and calpain 1 (CAPN 1) using the probes Hs00196158_m1, Hs00361185_m1*, Hs00166289_m1, Hs00265033_m1*, Hs01894962_s1*, Hs00856927_g1*, Hs00902520_m1, Hs00166071_m1*, Hs00201637_m1*, and Hs00559804_m1*, respectively (Applied Biosystems). Relative differences in expression were calculated using the equation from the mathematical model developed by Pfaffl et al. [23]

2.8. Antibody array

The RayBio® Human Cytokine Antibody Array 1 was purchased from RayBiotech, Inc. (Norcross, GA). Membranes (2.5 cm × 3 cm) were spotted with an array of antibodies against 23 human cytokines/chemokines (GCSF, GM-CSF, GRO, GRO-α, IL-1α, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IFN-γ, MCP-1, MCP-2, MCP-3, MIG, RANTES, TGF-β1, TNF-α and TNF-β) and placed in a tray chamber to perform the array assays according to the manufacturer's protocol. Briefly, after treatment of the NHKs with the *S. aureus* extracts, cells were centrifuged. One milliliter of each supernatant was used in the cytokine assay after protein quantification by BCA. Chemiluminescence and densitometric analyses were performed using an Image Reader LAS-3000 (Fuji Photo Film, Tokyo, Japan).

2.9. Western blot analysis

Soluble proteins were extracted using a protocol modified from the method described by Redoules et al. [22]. Briefly, proteins were extracted from SC and keratinocytes using a Tris–HCl buffer (62 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol) and lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM ethylenediamine tetraacetic acid, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 2 µg/mL leupeptin), respectively. The total protein concentrations were measured using a BSA protein assay kit (Pierce, Rockford, IL, USA). The samples were separated by electrophoresis using 4–12% SDS-PAGE gels and transferred to a PVDF membrane. The protein levels were examined by Western blotting with anti-KRT 1 (1/2500 dilution), anti-KRT 5 (1/2500 dilution), anti-KRT 14 (1/2500 dilution), anti-INV (1/2500 dilution), anti-LOR (1/2500 dilution), anti-KRT 10 (1/2500), anti-caspase 14 (1/1000 dilution), anti-bleomycin hydrolase (1/1000 dilution), anti-calpain 1 (1/1000 dilution), β-actin (1/1000 dilution) and anti-FLG (1/200 dilution) antibodies. The density of band was quantified by densitometric analysis (AAB soft Program, Fullerton, CA).

2.10. The reconstructed human epidermis cultivation

The RHEs were purchased from MatTek (EPI-212). The SC layers of the RHEs were exposed to air for 20 days and then treated with H₂O, the *S. aureus* extracts and IL-6 (10 ng/mL) for 6 days. The SC layers of the RHEs were treated with 30 µL of each sample thrice weekly for the duration of the experiment (6 days).

2.11. Immunohistochemical staining

The RHEs were cryopreserved in frozen section compound (FSC 22®, Leica Microsystems, USA) and sectioned at 6 µm. Replicate cryosections were immunohistochemically stained using polyclonal rabbit anti-keratin 1 antibody (PRB149-P, Covance Inc., USA) or polyclonal rabbit anti-keratin 10 antibody (PRB-159P, Covance Inc., USA) respectively as the primary antibody for 40 min at room temperature. Then anti-rabbit HRP conjugated antibody (ab6802, Abcam Inc., MA, USA) was applied as the secondary antibody for 20 min. Immunoreactivity was evaluated under light microscope (Bx-41, Olympus, Japan) and photomicrographs were taken using digital camera (DP72, Olympus, Japan).

2.12. Statistical analysis

Statistical analysis was performed using a two sample *t*-test. *p* < 0.05 was considered statistically significant. All statistical analyses were performed using Minitab software, version 14 (Minitab Inc., USA).

3. Results

3.1. Reduced expression of KRT 10 in the facial SC of healthy volunteers colonized by *S. aureus*

We first examined *S. aureus* colonization in the skin samples taken from the cheeks of apparently healthy volunteers (*n* = 10, average age, 37 years) using Easy stamp® plates containing *S. aureus*-selective media. As shown in Fig. 1a, samples from five volunteers yielded large, round, golden-yellow colonies of *S. aureus*. Samples from three of these volunteers yielded more than 10 colonies and were classified as the *S. aureus*-positive group. Five volunteers yielded no colonies and were classified as the *S. aureus*-negative group. Two volunteers yielded two colonies or less and were excluded from the study. The mean number of colonies formed by the *S. aureus*-positive group was 26 ± 7.8 (Fig. 1b). We next examined the expression of the terminal differentiation markers KRT 1 and KRT 10 in the SC samples by Western blotting. Samples from the SC (upper layers) were collected from the volunteers by five rounds of tape stripping using Desquam® tape. KRT 1 expression was not detected in the SC samples from either group; however, KRT 10 expression was significantly reduced in the *S. aureus*-positive samples compared with the *S. aureus*-negative samples (Fig. 1c), suggesting that *S. aureus* induces abnormal epidermal differentiation.

3.2. *S. aureus* colonization reduces the expression of epidermal differentiation markers and increases the expression of filaggrin-processing enzymes in NHKs

To clarify whether the *S. aureus* extracts inhibit the expression of other epidermal differentiation markers in NHKs, we measured the gene and protein levels of KRT 1, KRT 10, FLG, INV, and LOR by real-time PCR and Western blotting. We also measured the mRNA and protein expression of basal epidermal keratinocyte markers (KRT 5 and KRT 14) and FLG-processing enzymes (BH, caspase-14, and calpain-1). We first isolated several *S. aureus* strains from the *S. aureus*-positive group and selected two strains, *S. aureus*-1 and *S. aureus*-2, that showed large differences in biofilm formation (significantly increased in *S. aureus*-2 compared with *S. aureus*-1). The biofilm-forming capacity of these two *S. aureus* strains, along with that of the *S. aureus*-ATCC 6538 strain, is shown in Supplementary Table 1.

RNA samples were extracted from NHKs after treatment with the *S. aureus* strains. Despite significant differences in biofilm-forming capacity, all three strains caused a decrease in the

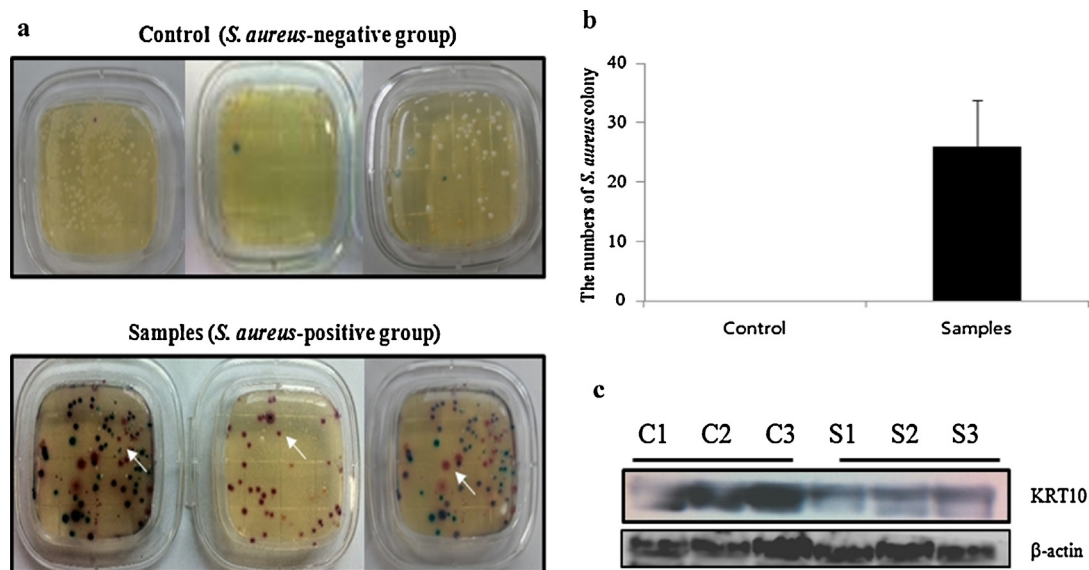


Fig. 1. KRT 10 expression in the *S. aureus*-positive group compared with the *S. aureus*-negative group (samples taken from apparently healthy human skin). (a) Samples were taken from the right cheek of each volunteer (with apparently healthy skin), and *S. aureus* colonization was detected using Easystamp[®]. (b) The number of *S. aureus* colonies in the control and *S. aureus*-positive samples. (c) KRT 10 expression was reduced in the SC of *S. aureus*-positive skin samples (S1, S2 and S3) compared with the *S. aureus*-negative skin samples (C1, C2 and C3).

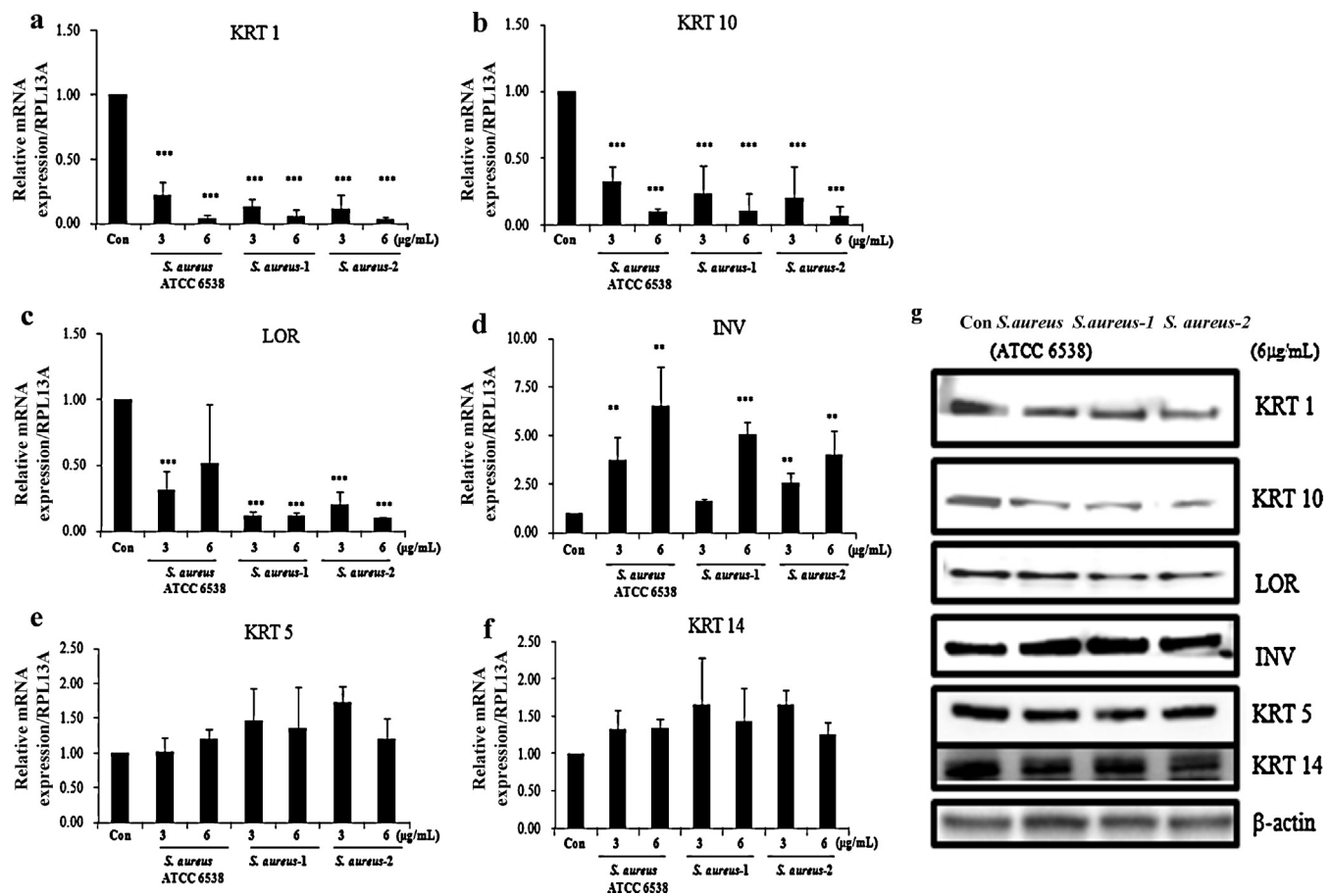


Fig. 2. The effects of the *S. aureus* extracts on the mRNA and protein expression of terminal differentiation markers in NHKs. NHKs were treated with the *S. aureus* extracts (3 μg/mL or 6 μg/mL) for 4 days. Expression of mRNA for the following genes was assessed by qRT-PCR: (a) KRT 1, (b) KRT 10, (c) LOR, (d) INV, (e) KRT 5 and (f) KRT 14. (a)–(d) and (e)–(f) represent terminal differentiation marker genes and basal epidermal keratinocyte markers, respectively. (g) The expression levels of the KRT 1, KRT 10, INV, LOR, KRT 5 and KRT 14 protein were measured by Western blotting ($n = 2$). The mRNA levels were normalized to the ribosomal protein RPL13A. Data represent the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with the control group).

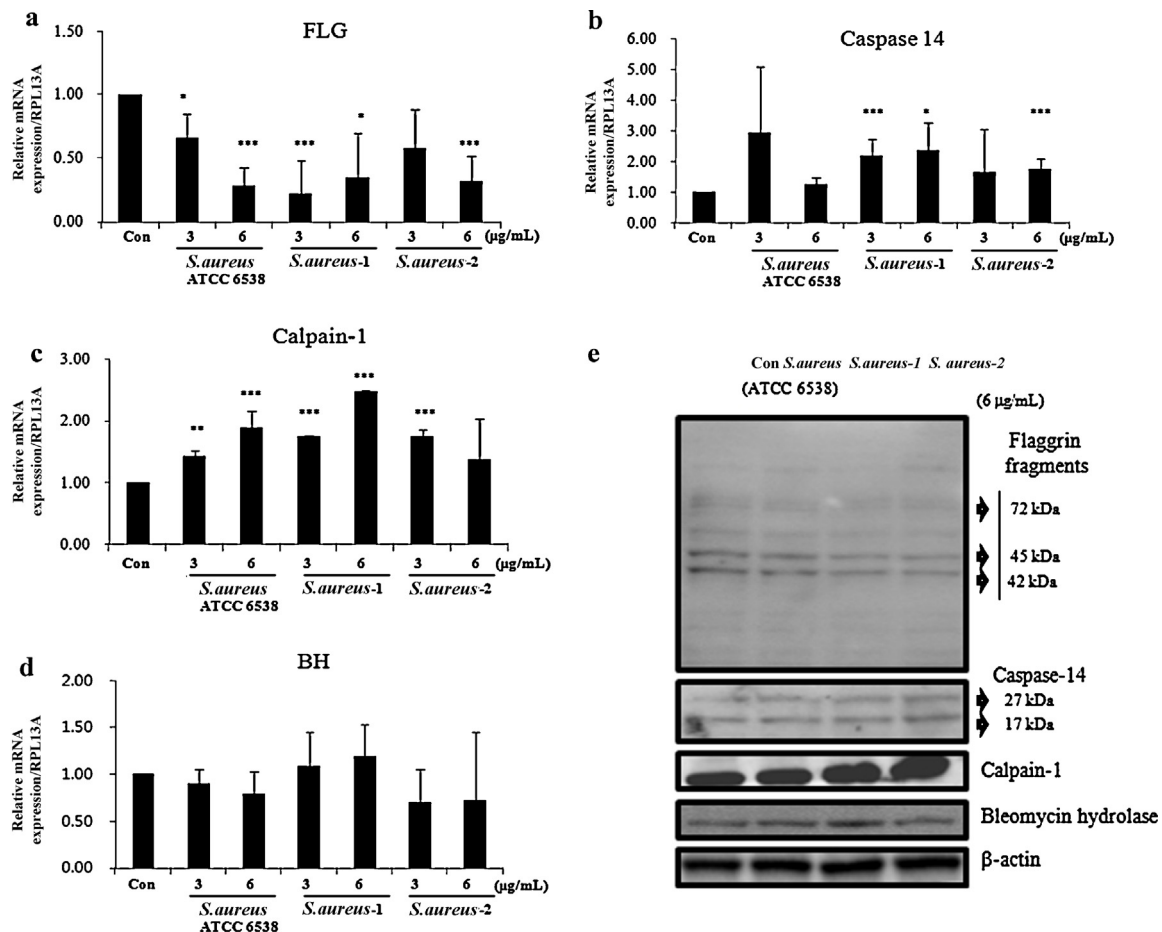


Fig. 3. The effects of the *S. aureus* extracts on the mRNA and protein expression of filaggrin and filaggrin-processing enzymes in NHKs. NHKs were treated with the *S. aureus* extracts (3 μ g/mL or 6 μ g/mL) for 4 days. (a) The expression of FLG mRNA decreased after exposure to the *S. aureus* extract. The expression of the filaggrin-processing enzymes caspase-14 (b) and calpain-1 (c) slightly increased. Bleomycin hydrolase (d) mRNA levels were not significantly altered after *S. aureus* extract treatment. (e) The expression levels of the filaggrin, caspase-14, calpain-1 and bleomycin hydrolase protein were measured by Western blotting ($n = 2$). The mRNA expression levels were normalized to RPL13A. Data represent the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared with the control group).

expression of KRT 1, KRT 10, LOR and FLG mRNA (by 70–90%) compared with the non-treated control (Figs. 2a–c and 3a). By contrast, INV mRNA expression increased significantly (Fig. 2d). Expression of the basal epidermal keratinocyte markers KRT 5 and KRT 14 was unchanged (Fig. 2e and f). The levels of KRT 1, KRT 10, LOR, INV, KRT 5 and KRT 14 protein expression were similar to those of mRNA expression (Fig. 2g). Expression of the FLG-degrading enzymes caspase-14 and calpain-1 mRNA increased slightly upon exposure to the *S. aureus* extracts (Fig. 3b and c), but BH expression was unaffected (Fig. 3d). The expression of 45 and 48 kDa filaggrin fragments were slightly decreased by *S. aureus* extract treatment although we could not detect profilaggrin in our experimental condition (Fig. 3e). Expression levels of caspase-14, calpain-1 and BH proteins were similar to those of mRNA expression upon exposure to *S. aureus* extracts (Fig. 3e). These results suggest that *S. aureus* affects terminal epidermal differentiation in the upper layer of the SC.

3.3. *S. aureus* extracts induce the expression of various proinflammatory cytokines and chemokines in NHKs

It has been previously reported that various cytokines, including IL-6 and IL-8, are present in the culture supernatants of NHKs after stimulation with heat-killed *Staphylococcus* [7]. To confirm these results in our experimental conditions, we used cytokine arrays to test the culture medium from *S. aureus* extract-

treated NHKs. Increased levels of IL-5, IL-6 and IL-10 were secreted by *S. aureus* extract-treated NHKs (Supplementary Fig. 1a). In particular, the expression of IL-6 was 6-fold higher than in the control. The expression of the chemokines GCSF, GM-CSF, IL-8, MCP-2, -3 and RANTES was also increased compared with non-treated NHKs (Supplementary Fig. 1a). The expression of IL-6 and IL-8 mRNA increased 3–6-fold in *S. aureus* extract-treated NHKs compared with the control (Supplementary Fig. 1b and c).

3.4. IL-6 mediates the *S. aureus*-induced suppression of KRT 1, KRT 10, LOR and FLG expression

IL-6 levels have been reported to increase during wound repair, playing an important role in the repair process by downregulating keratinocyte differentiation rather than proliferation. IL-6 treatment decreases the expression of KRT 1 and KRT 10 in NHKs [24]. Therefore, we speculated that the *S. aureus*-induced suppression of keratinocyte differentiation might be partially mediated by the *S. aureus*-induced increase of cytokines and chemokines. Among the cytokines and chemokines upregulated by NHK treatment with the *S. aureus* extracts, the levels of IL-6 and IL-8, respectively, experienced the most significant increase (Supplementary Fig. 1a). Therefore, we examined whether IL-6 or IL-8 modulates the expression of genes encoding the terminal differentiation markers. NHKs were treated with IL-6 or IL-8, and the levels of KRT 1 and KRT 10 mRNA were examined. No differences in mRNA levels were

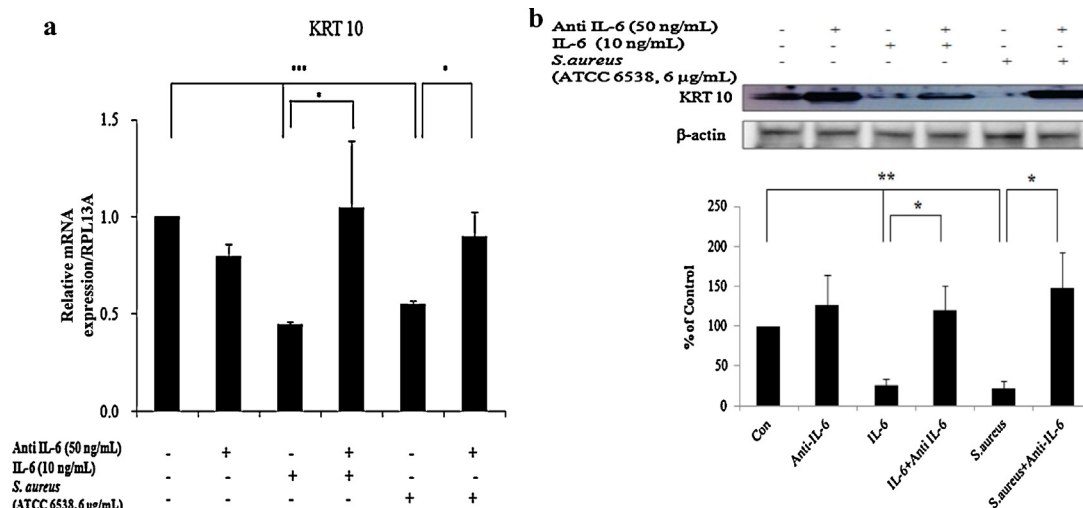


Fig. 4. The effects of the IL-6 antibody on the expression of KRT 10 mRNA and protein in NHKs exposed to IL-6 or *S. aureus*. NHKs were treated with 50 ng/mL anti-IL-6 antibody in combination with either 10 ng/mL IL-6 or 6 µg/mL *S. aureus* extracts for 4 days in KGM medium. (a) Total RNA was extracted, and KRT 10 expression was analyzed by qRT-PCR. mRNA levels were normalized to RPL13A ($n = 3$). (b) Total protein was extracted with lysis buffer, and western blotting was performed with a mouse anti-KRT 10 antibody (1/2500). Data are expressed as ratios of KRT 10 to β -actin, and as percentage relative to control group ($n = 3$). Data represent the mean $\pm$ SD of three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ (compared with the control group).

detected at 24 h (data not shown). However, IL-6 significantly inhibited the expression of KRT 1, KRT 10, LOR and FLG mRNA at 48 h (Supplementary Fig. 2a and b), whereas IL-8 treatment had no effect (Supplementary Fig. 2a and b). To confirm that IL-6 is involved in the *S. aureus* extract-induced suppression of KRT 1 and KRT 10 expression, NHKs were treated with 50 ng/mL anti-IL-6 antibody in combination with 10 ng/mL IL-6 or 6 µg/mL *S. aureus* extract for 4 days. Treatment with the anti-IL-6 antibody prevented the *S. aureus* extract-mediated suppression of KRT 10 mRNA expression (Fig. 4a) but not KRT 1 mRNA expression (data not shown). Western blot analysis also revealed that treatment with the anti-IL-6 antibody rescued the expression of KRT 10 in cells exposed to the *S. aureus* extracts (Fig. 4b).

3.5. *S. aureus* extracts decrease the expression of KRT 1 and KRT 10 proteins in the RHEs

We also examined the effects of the *S. aureus* extracts and IL-6 treatments on the RHEs. The expression of KRT 1 and KRT 10 in the RHEs was detected by immunohistochemistry using the anti-KRT 1 or anti-KRT 10 antibodies. Treatment with the *S. aureus* extracts (Fig. 5c) and IL-6 (Fig. 5d) decreased the expression of KRT 1 and KRT 10 in the RHEs compared with control groups (non-treated or dH₂O) (Fig. 5a and b). We performed image analysis for presenting the differential expressions of KRT 1 and KRT 10 (Fig. 5e and f).

4. Discussion

The present study examined the expression of terminal differentiation markers in the SC of *S. aureus*-colonized healthy human skin and *S. aureus* extract-treated NHKs to address the role of this bacterium in the destruction of the epidermal permeability barrier observed not only in skin dermatoses but also in apparently healthy human skin. We found that *S. aureus* extracts suppressed terminal differentiation of NHKs by increasing IL-6 secretion.

Previous studies of skin barrier dysfunction induced by *S. aureus* focused on SC desquamation mediated by proteases such as *S. aureus* V8 protease and metalloproteinases [8,9]. Here, we showed that *S. aureus* may induce skin barrier dysfunction directly by inhibiting terminal epidermal differentiation. Expression of KRT 10 in the SC of *S. aureus*-colonized skin significantly decreased

compared with *S. aureus* non-colonized skin (Fig. 1c). NHK exposure to the extracts of two clinically isolated *S. aureus* strains (*S. aureus*-1 and *S. aureus*-2) and one standard strain (*S. aureus*-ATCC 6538) resulted a significant reduction in KRT 1, KRT 10, and LOR mRNA expression (Fig. 2a, b, and d), indicating abnormal keratinization. Extracts from all three *S. aureus* strains were not cytotoxic to NHKs at a concentration of 6 µg/mL but did show toxicity at 12 µg/mL (Supplementary Fig. 3a–c). We also examined the NHK proliferation after *S. aureus* extract treatment and found that *S. aureus* was not significantly affecting the proliferation of NHKs (Supplementary Fig. 3d). We also confirmed that the *S. aureus* strains existed in the inner layers of the SC as well as on the upper layers of the facial skin SC (Supplementary Fig. 4).

Keratinization is an important process that maintains the skin barrier function of the SC, which contains intercellular lipids [25] and NMFs that prevent water evaporation and maintain skin hydration [26,27]. Abnormalities in keratinization induced by hyper-proliferation or abnormal differentiation are often observed in individuals with dry skin [28,29] or cutaneous skin diseases, such as AD [8,10,30] and psoriasis [10,11]. Therefore, abnormal keratinization by NHKs upon exposure to *S. aureus* can affect the expression of FLG (a critical precursor of NMFs) and the FLG-processing enzymes. Indeed, FLG expression was significantly reduced in *S. aureus* extract-treated NHKs (Fig. 3a). However, the mRNA expression of FLG-processing enzymes, such as caspase-14 and calpain-1, was slightly increased upon exposure to the *S. aureus* extracts (Fig. 3b and c), whereas BH mRNA expression did not change (Fig. 3d). The increases in caspase-14 and calpain-1 gene expression may compensate for the reduced expression of NMFs resulting from decreased FLG expression. Taken together, these results suggest that *S. aureus* may affect terminal epidermal differentiation. Moreover, *S. aureus* is potentially involved in the increased trans-epidermal water loss (TEWL), decreased hydration, rough skin, and increased desquamation observed in apparently healthy human skin [6].

Biofilm formation by *S. aureus*-2 was significantly increased compared with *S. aureus*-1 or *S. aureus*-ATCC 6538 (Supplementary Table 1), and the expression of V8 protease mRNA, as determined by microarray analysis, was increased by >30-fold in *S. aureus*-2 relative to *S. aureus*-1 (unpublished data). This result suggests certain biological differences between the individual *S. aureus*

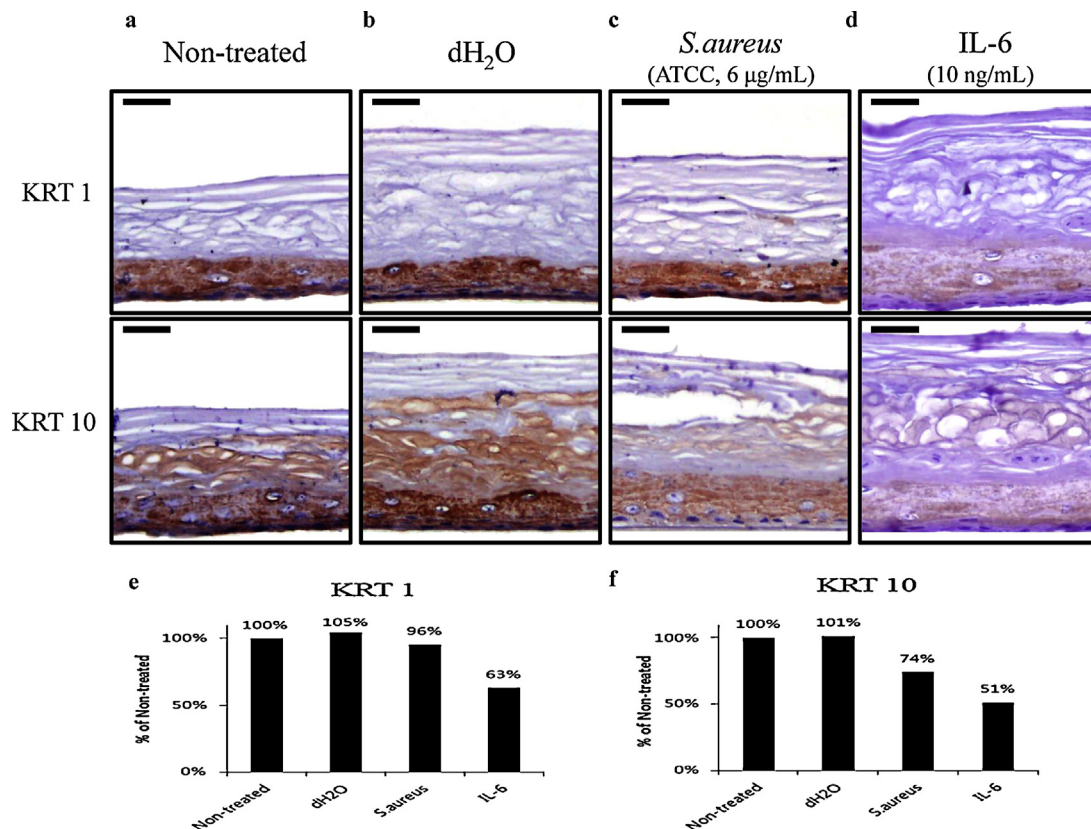


Fig. 5. The effects of the *S. aureus* extracts and IL-6 on the expression of KRT 1 and KRT 10 in the RHEs. The SC layers of the RHEs were exposed to air for 20 days to ensure complete differentiation. The RHEs were then treated with dH₂O, the *S. aureus* extracts and 10 ng/mL IL-6 for an additional 6 days. Cryotome-embedded sections were immunohistochemically stained. (a) Non-, (b) dH₂O-, (c) *S. aureus* extract- and (d) IL-6-treated samples. (e) Image analysis results of KRT 1 staining. (f) Image analysis results of KRT 10 staining. Immunohistochemical staining of KRT 1 and KRT 10 using anti-KRT 1 and anti-KRT 10 antibodies, respectively (scale bar = 20 μ m).

strains. However, we did not observed any relationships between these differences and the expression patterns of the genes encoding terminal differentiation markers in NHKs. This result suggests that the inhibition of terminal differentiation is related to factors other than V8 protease expression by *S. aureus* or its biofilm formation capacity.

Secretion of proinflammatory cytokines and chemokines by NHKs exposed to the *S. aureus* extracts was increased (Supplementary Fig. 1a) in a manner similar to the increase of IL-6 and IL-8 secretion previously reported by Sasaki et al. [7]. In that study, IL-6 and IL-8 stimulated the epidermal infiltration of neutrophils in cutaneous infection with staphylococci, but the epidermal differentiation of skin was not assessed. Moreover, keratinocytes express Toll-like receptor 2 (TLR 2), which forms heterodimers with TLR 1 or TLR 6. These heterodimers then recognize triacyl and diacyl lipopeptides expressed by pathogens [31]. In addition to TLRs, keratinocytes also express nucleotide oligomerization domain 2 (NOD2), which recognizes peptidoglycans within bacterial cell walls. *S. aureus*-induced activation of these TLRs and NOD2 induces an immune response mediated by the myeloid differentiation primary response gene 88 (MyD88). This response results in the production of proinflammatory cytokines (IL-1 α , IL-1 β , and IL-17), chemokines (CXCL1, CXCL2 and IL-8), and antimicrobial peptides (β -defensin 2 and 3, cathelicidin, and RNase 7) [32]. The current study confirms that treatment with the *S. aureus* extracts increases the expression of TLR 2 and MyD88 mRNAs in NHKs (Supplementary Fig. 5). To address whether *S. aureus* extract induce IL-6 secretion through TLR pathway, we tested the protein level of total I κ B- α after *S. aureus* treatment to NHKs or MyD 88 siRNA transfected NHKs. The protein level of total

I κ B- α was decreased after *S. aureus* treatment (Supplementary Fig. 6a). However, in MyD88 siRNA transfected NHKs, the protein level of total I κ B- α was not decreased by *S. aureus* treatment (Supplementary Fig. 6b). These results suggest that *S. aureus*-induced secretion of IL-6 in KC may be mediated via TLR-MyD88 pathway.

We also measured the phosphorylation level of STAT3 after *S. aureus* extract treatment and observed that the phosphorylation of STAT3 was increased by *S. aureus* extract treatment (Supplementary Fig. 7). This result suggests that *S. aureus*/IL-6-mediated inhibition of NHK differentiation is probably, at least in part, mediated via STAT3 activation.

The expression of IL-6 and IL-8 is substantially increased in AD skin [33], and the observed increases in IL-6, IL-8, GM-CSF and RANTES expression are similar to those reported by Vu et al. [31]. In particular, IL-6 and IL-8 mRNAs were the most highly expressed cytokine and chemokine, respectively, among those significantly increased in *S. aureus*-treated NHKs (Supplementary Fig. 1a). Therefore, we examined whether IL-6 and IL-8 modulated epidermal differentiation in NHKs and found that IL-6 was partially responsible for suppressing epidermal differentiation. Treatment with an anti-IL-6 antibody prevented the *S. aureus* extract-mediated suppression of KRT 10 (Fig. 4) but not KRT 1 (data not shown), implying that factors other than IL-6 could influence the downregulation of KRT 1 in *S. aureus* extract-treated NHKs. The other *S. aureus*-induced cytokines and chemokines in NHKs are potential candidates.

The effects of *S. aureus* and IL-6 on the expression of epidermal differentiation markers examined in the current study are different from those reported by Grossman et al. [34]. The study by

Grossman et al. [34] focused on the high level of IL-6 expression in psoriatic skin and found that IL-6 stimulated the proliferation of cultured human keratinocytes. Although they showed that IL-6 induces hyper-proliferation of keratinocytes, the present study revealed that the expression of epidermal differentiation markers in NHKs was significantly inhibited by *S. aureus* extracts or IL-6. The differences between the two studies may be attributed to differences in the cell culture conditions. IL-6 induces cell proliferation in growth-arrested cells in the absence of supplements and has no significant effect on cells cultured in complete medium. The cells in the present study were cultured in complete medium. We also observed that IL-6 but not *S. aureus* extract treatment increased the epidermal thickness of the RHEs, although both treatments successfully inhibited the expression of KRT 1 and KRT 10 in the RHEs (Fig. 5d).

In summary, *S. aureus* extracts induced abnormal epidermal differentiation by inhibiting the expression of terminal differentiation markers in NHKs, and IL-6 plays an important role in this process. Taken together, these results suggest that *S. aureus* causes skin barrier disruption, in part, by inducing expression of IL-6 in keratinocytes.

Funding statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jdermsci.2013.12.004>.

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