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ORIGINAL ARTICLE

Biphasic influence of *Staphylococcus aureus* on human epidermal tight junctions

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Bacterial infections (e.g., with *Staphylococcus aureus*) are serious problems in skin with a compromised barrier, such as in patients with atopic dermatitis. Previously, it was shown that tight junction (TJ) proteins are influenced by staphylococcal infection, and TJ function is impaired after infection of the keratinocyte cell line HaCaT. However, functional studies in cells or models more similar to human skin are missing. Therefore, we investigated bacterial colonization and infection with live *S. aureus* in primary human keratinocytes and reconstructed human epidermis (RHE). We show that short-term inoculation results in increased TJ barrier function—which could not be seen in HaCaT cells—hinting at an early protective effect. This is accompanied by occludin phosphorylation and sustained localization of occludin and claudin-4 at cell membranes. Long-term incubation resulted in decreased presence of claudin-1 and claudin-4 at cell membranes and decreased TJ barrier function. The *agr* regulon of *S. aureus* plays a role in the increasing but not in the decreasing effect. Proinflammatory cytokines, which are produced as a result of *S. aureus* inoculation, influence both phases. In summary, we show here that *S. aureus* can have short-term promoting effects on the TJ barrier, while in the long term it results in disturbance of TJs.

Keywords: epidermis; tight junctions; bacteria; *S. aureus*; claudin; occludin

Introduction

The skin is colonized by a broad diversity of microorganisms, which is strongly dependent on body site, age, and individual life circumstances.¹ Under healthy conditions, this skin microbiome has a protective effect that inhibits pathogen growth, educates and primes adaptive immunity, and enhances host innate immunity.² Therefore, it is also called the *microbiome barrier*.³ In addition to the microbiome barrier, the overall skin barrier also comprises two physical barriers (stratum corneum (SC) and tight junctions (TJs)), a chemical barrier (antimicrobial peptides (AMPs) and acidic surface pH), and an immunological barrier (immune cells and humoral constituents; reviewed

in Ref. 4). In diseased conditions, pathogenic microbes (e.g., *Staphylococcus aureus*) can cause serious skin infections, such as impetigo contagiosa, abscess formation, and ecthymata.^{5,6} These infections are promoted either by shifts in the microbiome or by alterations of other skin barrier components.²

TJs form a barrier in the granular cell layer of the epidermis. They play an important role in controlling the paracellular pathway of small and larger molecules,^{7–9} as well as the ion tracer lanthanum.^{10,11} For pathogenic microorganisms, they are the second physical barrier during invasion of the skin. This second barrier is of special importance when the first barrier (i.e., the SC) is

impaired (e.g., in atopic dermatitis or in wounded skin). In addition, it is known that TJs influence the SC and vice versa.^{4,12–14} While typical and functional TJ structures in the epidermis are found in the stratum granulosum, the TJ protein localization pattern is more complex. Occludin (Ocln) and claudin-4 (Cldn-4) are restricted to the stratum granulosum, whereas Cldn-1 is found in all viable layers.¹⁵ TJs and TJ proteins are altered in atopic dermatitis^{16,17} as well as other skin diseases (reviewed in Refs. 18 and 19).

Previously, we showed, via immunohistochemical staining of human *in vivo* skin diagnosed with impetigo contagiosa, a downregulation of Cldn-1 and zonula occludens 1 (ZO-1) and a partial downregulation of Ocln at areas covered with bacteria (infected areas) and an upregulation of ZO-1 and especially Ocln at neighboring areas (colonized areas).²⁰ Inoculation of porcine *ex vivo* skin with *S. aureus* resulted in a long-term decreased immunointensity of Cldn-1 and ZO-1. For Ocln, enhanced staining was observed at early time points; at later time points, enhanced staining was evident in some areas and downregulation in others. However, the impact of *S. aureus* on TJ functionality was not investigated, except for transepithelial resistance (TER) measurements in the spontaneously immortalized epidermal cell line HaCaT, which revealed an immediate decrease in TER after inoculation.²⁰ In contrast, treatment of normal human epidermal keratinocytes (NHEKs) with peptidoglycan derived from *S. aureus* resulted in an increase in TER^{21,22} and a decrease in paracellular flux.²² Additionally, treatment of reconstructed human skin resulted in reduced paracellular flux.²¹ Thus, there might be a difference on one hand between primary human keratinocytes and HaCaT cells and on the other hand between single TLR agonists and live bacteria. In general, HaCaT cells may not be the ideal test system to investigate bacterial infection, as they express pattern recognition receptors (PRRs) in a different manner than NHEKs.²³ Furthermore, they develop a lower TER than NHEKs,^{24–26} and it is still unclear whether they fully differentiate.²⁷ Therefore, we hypothesized that the influence of live *S. aureus* on primary human keratinocytes might be more complex than their effects on HaCaT cells or TLR ligand effects. Therefore, we investigated their impact on submerged cells and—to bridge the gap to *ex vivo* and *in vivo* three-dimensional

(3D) skin—in air-lifted 3D reconstructed human epidermis (RHE).

Interestingly, we observed different results in NHEKs compared with HaCaT cells. Measurement of the TER and tracer permeability assays revealed a time- and dose-dependent increase in TJ barrier function, followed by a decrease after incubation with *S. aureus*, thus revealing a biphasic influence. Increased TJ barrier function is the result of sustained localization of Cldn-4 and Ocln at the cell–cell membranes, which is accompanied by Ocln phosphorylation. Decreased TJ barrier function is characterized by an internalization of the sealing TJ proteins Cldn-1 and Cldn-4 and can be evoked by an inflammatory stimulus, but is neither caused by cell death nor by bacterial virulence factors. We also confirm the TJ protein alterations found at early and late time points after *S. aureus* inoculation in RHE. Importantly, our submerged and 3D findings reproduce what was described *in vivo* and *ex vivo*.

Materials and methods

Antibodies, primers, and bacterial strains

Detailed information about antibodies and primers used in this manuscript is listed in Tables S1 and S2 (online only). The different bacterial strains used in this study and their origin are listed in Table S3 (online only). Data shown in this manuscript are derived from *S. aureus* strain SH1000 and its mutants. However, similar results were also obtained with *S. aureus* strain 6820.

Cells

Primary human keratinocytes were isolated from juvenile foreskin. The use of these samples was approved by the ethics committee of the Aerztekkammer Hamburg (WF-61/12). Samples were used anonymously. All investigations were conducted according to the principles expressed in the Declaration of Helsinki. NHEKs were cultured in serum-free keratinocyte growth medium (KGM2, Promocell, Heidelberg, Germany) as described previously.²⁸ For TER and FD4 permeability experiments, keratinocytes were trypsinized and seeded onto Transwell membranes of 0.4 μm pore size (Corning Incorporated, Durham, NC) with a cell density of 40,000 cells/well (diameter 6.5 mm) in EpiLife medium (Life Technologies, Carlsbad, CA). When NHEKs reached confluency, they were transferred to high-calcium medium containing

1.8 mmol/L Ca^{2+} . Medium was renewed every 24 hours.

For western blot analyses, mRNA isolation, and cover slides, cells were seeded in the same density as for TER experiments in either 12- or 24-well plates (Corning Incorporated, Durham, NC).

Reconstructed human epidermis

Five hundred microliters of EpiLife medium with 1.5 mM CaCl_2 containing 3×10^5 keratinocytes in passage 3 were seeded onto Millicell cell culture inserts (0.4 μm , 12 mm diameter, Merck Millipore, Tullagreen, Ireland) placed in 6-well plates, and the basal part of the wells was filled with 2.5 mL EpiLife medium with 1.5 mM CaCl_2 . Cells were maintained at 37 °C in humidified atmosphere for 30 h and then lifted to the air–liquid interface. In parallel, medium in the basal part of the well was exchanged with 1.5 mL of EpiLife medium containing 1.5 mM CaCl_2 , 92 $\mu\text{g}/\text{mL}$ ascorbic acid (Sigma, Darmstadt, Germany) and 10 ng/mL recombinant human keratinocyte growth factor (R&D Systems, Wiesbaden-Nordenstadt, Germany). Medium was changed every second day.

Bacterial culture and inoculation

Bacteria were grown overnight at 37 °C on Columbia-Agar plates (Oxoid, Hampshire, United Kingdom). The next day, they were diluted in 5 mL of either 0.9 % NaCl solution (for RHE) or EpiLife high Ca^{2+} (for NHEKs) to an OD_{600} of 1.0 ($= 10^9$ bacteria/mL) measured with a photometer (BioRad, Hercules, CA). For further use, 1:10 dilutions were prepared in the respective medium up to the final bacterial concentration.

Bacterial inoculation of NHEKs was performed 3 days after Ca^{2+} switch when TER was substantially present ($>350 \Omega \cdot \text{cm}^2$). Bacteria were diluted in EpiLife high- Ca^{2+} medium to the indicated concentrations, and 200 μL of apical medium of the NHEKs was replaced with either sterile medium or medium containing bacteria. This was also performed in well plates (12- or 24-well plates) for the incubation of cover slides for immunofluorescence or the isolation of mRNA and protein. Bacterial concentrations were adjusted to the well sizes. Cells were then incubated for the indicated times.

For bacterial inoculation of RHE, bacteria were diluted in 0.9% NaCl solution to a final concentration of 10^7 bacteria/mL. On day 4, after lifting the models to the air–liquid interface, the models were

treated either with nothing (control), with 5 μL of sterile NaCl solution, or with 5 μL of bacterial suspension (representing 5×10^4 S. aureus/cm²). After transferring the 5 μL onto the models, these were spread with a small spatula (made from Pasteur pipettes). Models were then incubated for the indicated times.

The usage of about 5×10^4 bacteria/cm² on top of the RHE and 6×10^3 – 6×10^5 bacteria/cm² on NHEKs reflects the number of bacteria found per cm² on human skin, which fluctuates depending on the body site between 10^2 and 10^7 .²⁹

Measurement of TER in NHEKs and RHE

TER was measured at the indicated time points using a Millicell-ERS epithelial voltohmmeter (Merck, Darmstadt, Germany). TER values were corrected by subtracting the blank value (no cells). For TER measurement in RHE, models were placed in 24-well plates, and 400 μL of 37 °C PBS was added to the apical and 600 μL to the basal side of the model.

Tracer permeability assay in NHEKs

Permeability for a 4-kDa tracer (FD4, Sigma–Aldrich, Darmstadt, Germany) was measured in the same experiments in which TER measurements were performed. Two hundred microliters of FD-4 solution (1.25 mg/mL in medium) was applied to the apical side of the cells and incubated for 2 hours. Subsequently, the basal medium was collected and analyzed with a fluorescence reader (Tecan, Männedorf, Switzerland). Permeability was calculated as described before.²⁵

Tissue MTT

For investigation of cellular viability, a tissue MTT assay was performed as described previously.³⁰ As a positive control for cell death, NHEKs and RHE were treated with 1N HCl solution (NHEKs for 1 h; RHE for 2 h).

Western blot analyses

For detailed description of western blot analyses, see the Appendix (online only).

Real-time PCR analyses

For detailed description of quantitative PCR analyses, see the Appendix (online only).

Analyses of protein levels of cytokines

For description of protein level analyses, see the Appendix (online only).

Biotinylation assay

RHE was incubated with 200 μ L biotin-SH (0.5% Sulfo-NHS-LC-Biotin (Thermo Scientific, Rockford, IL) in PBS with 1 mM CaCl_2) from the membrane side for 30 minutes. RHE were subsequently fixed in Formafix solution (Grimm, Torgelow, Germany) up to 3 days at 4 °C and paraffinated.

Immunofluorescence staining and evaluation of intensity

For detailed description of immunofluorescence staining and evaluation of intensity of paraffin sections and cells grown on coverslips, see the Appendix (online only).

Evaluation of biotin-SH permeation stops

The amount of complete biotin-SH permeation stops per visual field (Axiophot II microscope (Zeiss, Göttingen, Germany), 40 $\times$) were counted. In addition, sites with a thin line of biotin-SH right after the actual stop were counted. These sites were called “half stops” (Fig. S1, online only). For calculation of the permeability in control RHE models, complete stops and half stops per visual field were counted in three models per donor with at least five visual fields per model.

Then mean number of complete stops in control models was calculated, and permeability of each individual control model determined as follows: the mean of complete stops in control models minus the complete stops in an individual control model plus the half stops in an individual control model equaled the number of permeated TJs per visual field in a distinct control model.

For treated RHE (NaCl control and *S. aureus* treatment), three models per donor and treatment with at least five visual fields per model were counted, and permeability was calculated as follows: the mean of complete stops in control models minus the complete stops in a treated model plus the half stops in a treated model equaled the amount of permeated TJs per visual field in a treated model.

Generation of *S. aureus* Δ agr and GFP strains
SH1000 Δ agr and GFP strains were constructed using phage transduction.³¹ For detailed description, see the Appendix (online only).

Determination of bacterial growth on RHE models

To investigate bacterial growth on top of the models, the amount of living bacteria was evaluated

by examining the colony-forming units (CFU). For detailed description, see the Appendix (online only).

Visualization of the bacteria

For visualization of bacteria, GFP-producing bacteria were incubated on the RHE as described above. After 24 h, the models were placed on an object slide and covered with a cover slide. Fluorescent bacteria were visualized with an Axiophot II fluorescent microscope.

Statistics

Statistical analyses were performed with paired Student's *t*-test or analysis of variance (ANOVA) with Bonferroni posttest of SPSS software (IBM, Armonk, NY). The specific tests are denoted in the figure legends. Values are shown as mean $\pm$ SEM.

Results

Influence of *S. aureus* on TJs in NHEKs

***S. aureus* first increases and later decreases TJ barrier function in NHEKs.** In order to investigate the influence of *S. aureus* on TJs of NHEKs, cells were grown under high-calcium conditions on Transwell membranes. This system has been well established for the investigation of TJ barrier function in keratinocytes (e.g., Refs. 21, 22, 25, 26, and 32–35). We observed a temporary, significant dose-dependent increase in TER within the first 12 h of inoculation with *S. aureus*, which was followed by a dose-independent significant decrease (Fig. 1A). The higher concentration (6×10^5 bacteria/cm²) of *S. aureus* also resulted in an accelerated increase of TER compared with the lower concentration (6×10^3 bacteria/cm²) (Fig. 1A). Because *S. epidermidis* was shown in HaCaT cells to have a positive effect on TER, we analyzed *S. epidermidis* here as a positive control. Indeed, treatment with *S. epidermidis* also resulted in a significant dose-dependent temporary increase in TER (Fig. S2A, online only) at early time points. Of note, the increase observed after *S. aureus* inoculation was more pronounced than after *S. epidermidis* treatment. At later time points, there was also a significant decrease of TER in *S. epidermidis*-treated cells (Fig. S2A, online only).

TER comprises the resistance of the paracellular and the transcellular pathway,³⁶ but it was previously shown that, in NHEKs, TER is mainly formed by the paracellular resistance, which means by TJs.²⁵ In order to investigate whether the increase and decrease in TER after bacterial inoculation are also

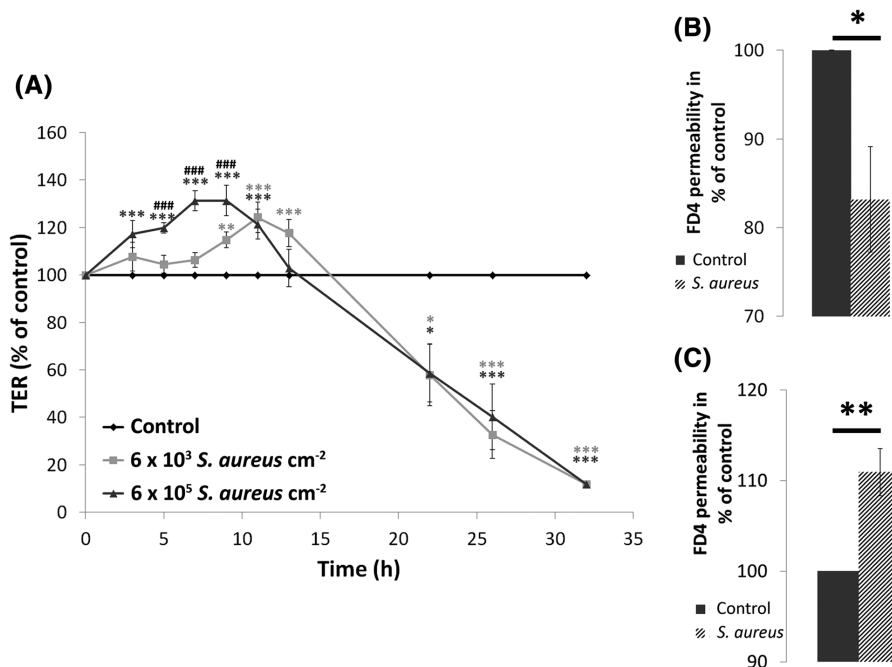


Figure 1. Influence of *Staphylococcus aureus* on TJ permeability of normal human epidermal keratinocytes (NHEKs). (A) Transepithelial resistance (TER) of NHEKs inoculated with two different concentrations of *S. aureus* normalized to the untreated control at the indicated time points. Permeability of a 4-kDa tracer at (B) 12 h and (C) 24 h of inoculation with 6×10^5 *S. aureus*/cm² normalized to control. Mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared to uninfected control, ### $P < 0.001$ when compared to the other bacterial concentration using ANOVA (A) and Student's *t*-test (B, C). $n = 5$ different donors in triplicate.

TJ-specific effects, permeability for 4-kDa FITC-dextran was investigated. This is a tracer commonly used to investigate the paracellular pathway (e.g., Refs. 37 and 38). Indeed, at early time points after *S. aureus* inoculation (12 h), when TER was increased, significantly decreased tracer permeability was found (Fig. 1B), and at late time points (24 h), when TER was decreased, a significant increase in tracer permeability was found (Fig. 1C). This suggests that TJ barrier function is altered. Similar effects on permeability were observed after *S. epidermidis* inoculation (Fig. S2B and S2C, online only).

To exclude the possibility that decreased TJ barrier function at later time points is just a result of cell death, we investigated cell viability using an MTT assay.³⁰ No decrease in cell viability was detected for *S. aureus* or for *S. epidermidis* inoculation (Fig. S3A, online only).

S. aureus inoculation results in protein relocalization but not altered TJ protein levels. To investigate putative causes for increased and decreased

TJ barrier function, we performed immunofluorescence stainings of cover slides as well as of cross-sections of paraffin-embedded cells on Transwell membranes, which had been used for the TER measurements. We observed a more pronounced localization of Occludin (Occludin) and Cldn-4 at the cell-cell membranes of NHEKs with both methods after short-term *S. aureus* inoculation (12 h; Fig. 2A and 2B). There was no clear change for Cldn-1 (Fig. 2A and 2B). Immunofluorescence stainings of NHEKs 12 h after *S. epidermidis* inoculation revealed a more pronounced localization of Cldn-4 but not of Occludin at the cell membranes (Fig. S2D, online only).

To clarify whether the increase of Occludin and Cldn-4 in *S. aureus*-treated cells within the first 12 h was due to increased mRNA or protein expression, we performed qPCR and western blot analyses. We did not observe an increase, or even a decrease, in mRNA expression of Cldn-1, Cldn-4, Occludin, or ZO-1 (Fig. S3B, online only, significant for Cldn-4 at 8 and 12 h).

On the protein level, we did not observe an alteration of Cldn-1 or Cldn-4, but the more

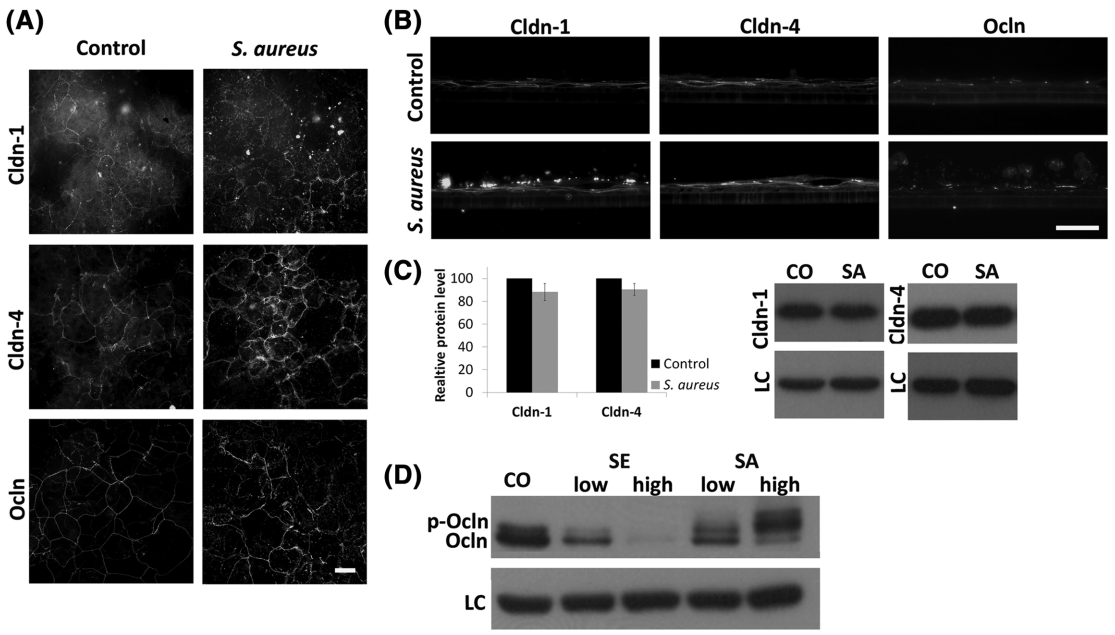


Figure 2. Influence of *S. aureus* on TJ protein localization and protein levels in NHEKs at early time points after inoculation. Immunofluorescence localization of the denoted TJ proteins in (A) cover slides (horizontal staining) and (B) vertical sections of Transwell membranes 12 h after *S. aureus* (6×10^5 bacteria/cm²) inoculation. Note a cross-reaction between Cldn-1 antibody and *S. aureus* resulting in bright fluorescent dots. (C) Semiquantitative evaluation of protein levels of Cldn-1 and Cldn-4 12 h after *S. aureus* (6×10^5 bacteria/cm²) inoculation and representative western blots for Cldn-1 and Cldn-4. (D) Western blot of Ocln 12 h after bacterial inoculation with 6×10^3 (low) or 6×10^5 (high) bacteria/cm². LC, loading control (actin); CO, control; SA, *S. aureus*; SE, *S. epidermidis*; p-Ocln, phospho-Ocln. Mean $\pm$ SEM. $n = 3$ (A, B) or 5 (C, D) different donors. Bars: 50 μ m.

phosphorylated form of Ocln was more pronounced after *S. aureus* inoculation (Fig. 2C and 2D). This can explain the increased membrane localization of Ocln, because phosphorylation of Ocln results in membrane accumulation of this protein.^{21,39,40} Interestingly, *S. epidermidis* inoculation did not result in increased levels of p-Ocln, but rather in decreased Ocln protein levels (Fig. 2D).

NHEKs inoculated for 24 h with *S. aureus* showed a decrease in Cldn-1 and Cldn-4 immunointensity at the cell–cell borders (Fig. 3A). For Ocln, a more pronounced localization at the cell–cell borders was still evident (Fig. 3A). Additionally, decreased intensity of Cldn-1 and Cldn-4 was observed for cells inoculated with *S. epidermidis* (Fig. S2E, online only).

To investigate whether decreased Cldn-1 and Cldn-4 immunolocalization after *S. aureus* inoculation was due to protein degradation, we performed western blot analyses. However, we observed only minor, nonsignificant changes at the protein level (Fig. 3B). Thus, there is an internalization of Cldn-1 and Cldn-4.

Live *S. aureus* induces expression of proinflammatory cytokines. Because it is known that NHEK treatment with AMPs (cathelicidin and h β D3)^{33,41} or short-term treatment with proinflammatory cytokines (IL-1 β and TNF- α)⁴² leads to relocalization of Ocln and Cldn-4 to the cellular membranes and thus increased TJ functionality, we investigated the induction of these molecules together with other markers of inflammation by *S. aureus* inoculation in our NHEKs. We did not see an increase of cathelicidin or h β D3 after *S. aureus* treatment. However, we observed a substantial increase in IL-1 β , TNF- α , IL-6, and IL-8 mRNAs 8 and 12 h after inoculation, while after 4 h the levels were equivalent to those of the controls (Fig. 4A). A significant increase was also observed for the cytokines on the protein level (Fig. 4B).

On the other hand, it was shown that long-term treatment of NHEKs with proinflammatory cytokines (IL-1 β and TNF- α) leads to decreased TER/TJ functionality.⁴² Therefore, we tested whether there is a prolonged upregulation of

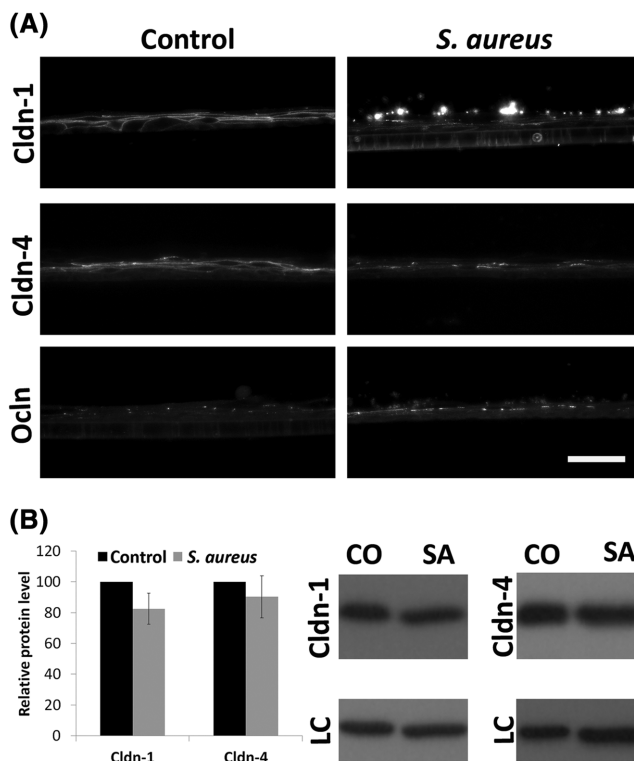


Figure 3. Influence of *S. aureus* on TJ protein localization and protein levels in NHEKs at late time points after inoculation. (A) Immunofluorescence localization of the denoted TJ proteins in vertical sections of Transwell membranes 24 h after *S. aureus* (6×10^5 bacteria/cm²) inoculation. Note a cross-reaction between Cldn-1 antibody and *S. aureus* resulting in bright fluorescent dots. (B) Semiquantitative evaluation of protein levels of Cldn-1 and Cldn-4 24 h after *S. aureus* inoculation and representative western blots. LC, loading control (actin); CO, control; SA, *S. aureus*. Mean $\pm$ SEM. $n = 3$ (A) and 6 (B) different donors. Bar: 50 μ m.

these cytokines after *S. aureus* inoculation. A strong increase of these cytokines was still observed 24 h after inoculation (Fig. 4B).

To further test whether cytokines can contribute to the increase and decrease of TJ barrier function in infected keratinocytes, we combined *S. aureus* infection with IL-1 β and TNF- α treatment. Indeed, the presence of the two cytokines significantly intensified the increase in TER, and IL-1 β accelerated the effects of *S. aureus* on TER decrease (Fig. 5A).

Deletion of the accessory gene regulator in *S. aureus* diminishes the increase but not the decrease of TJ barrier function. The question arose whether the decrease in TJ barrier function might be triggered by bacterial virulence factors. These virulence factors are regulated via the accessory gene regulator (AGR) system. This regulon is activated by quorum sensing and induces the production of bacterial proteases, lipases, nucleases,

and toxins.⁴³ Therefore, we cloned a Δ *agr* *S. aureus* strain. However, we did not see differences in the decrease of TER compared with the wild-type control (Fig. 5B).

However, the increase of TER at early time points following bacterial inoculation was dependent on AGR. The Δ *agr* *S. aureus* strain revealed a significantly reduced increase in TER compared with the wild-type control strain (Fig. 5B).

Influence of live S. aureus on TJs in RHE

To investigate the effect of bacterial infection in a model that is more similar to human skin than submerged cultured keratinocytes and that also considers the influence of the SC and its interaction with TJs, we infected RHE.

Normal differentiation and functional TJs in RHE. The RHE used in this study showed structured morphology and a similar distribution of

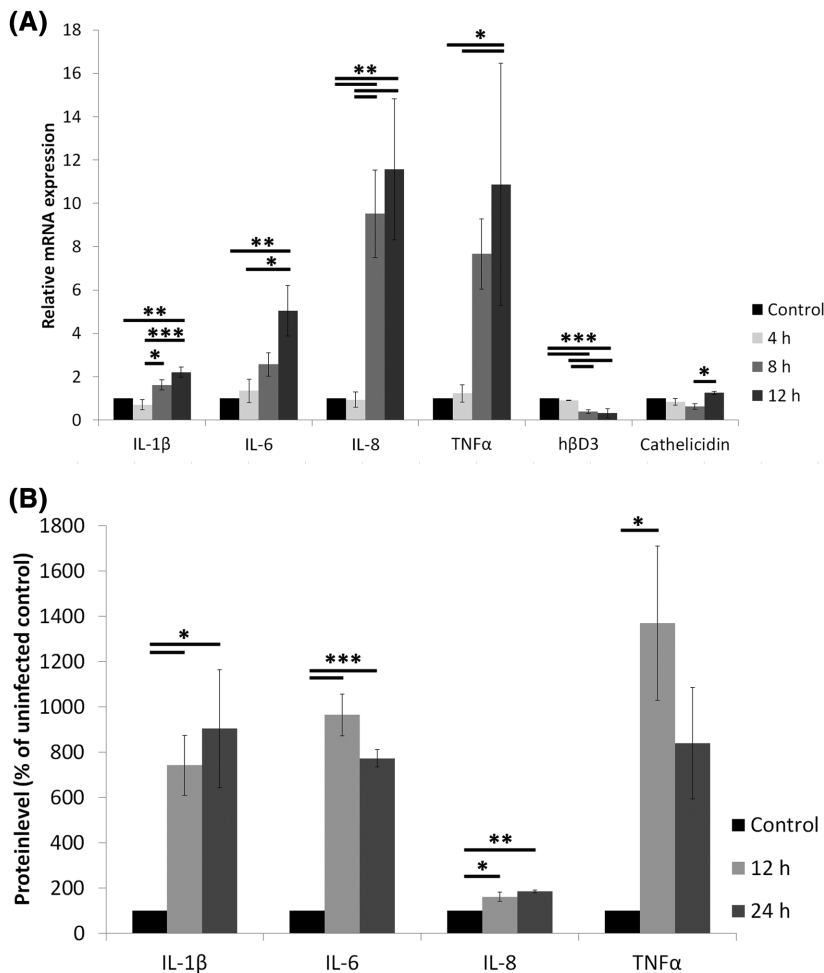


Figure 4. Influence of *S. aureus* on proinflammatory cytokines and antimicrobial peptides in NHEKs. (A) Quantitative PCR analyses of NHEKs inoculated with *S. aureus* (6×10^5 bacteria/cm²) for 4, 8, and 12 hours. (B) Analyses of secreted cytokine protein levels in apical medium of NHEKs inoculated with *S. aureus* (6×10^5 bacteria/cm²) for 12 and 24 hours. Mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ using ANOVA. $n = 3$ different donors in triplicate.

early (keratin 10), intermediate (involucrin), and late (loricrin, filaggrin) differentiation markers to human skin (Fig. S4A and B, online only). Substantial epidermal barrier function was reflected by an electrical resistance of $1785 \pm 673 \Omega \cdot \text{cm}^2$ ($n = 6$ different donors). In addition, a clear stop of biotin–SH, which was applied from the basal side of the model (biotinylation assay) at Oc1n⁺ sites, revealed functional TJs (Fig. S4C, online only).

***S. aureus* grows on the surface of RHE.** RHE was incubated for 24, 48, and 72 h with 5×10^4 bacteria/cm² *S. aureus* in physiological NaCl solution, and the CFUs of *S. aureus* were counted at

the denoted time points, displaying the amount of living bacteria. There was a significant increase of bacterial load after *S. aureus* inoculation (Fig. 6A), with an especially strong increase after 24 hours. This could also be confirmed using a fluorescent *S. aureus* strain and fluorescence microscopy (Fig. 6B).

Commensal bacteria, such as *S. epidermidis*, provide protection against the growth of pathogens (either directly or owing to the enhancement of innate immunity; reviewed in Ref. 2). In order to test whether this is also true in our RHE, the models were pretreated with *S. epidermidis* for 24 h and subsequently incubated with *S. aureus* for another 48 hours. Indeed, we observed that pretreatment

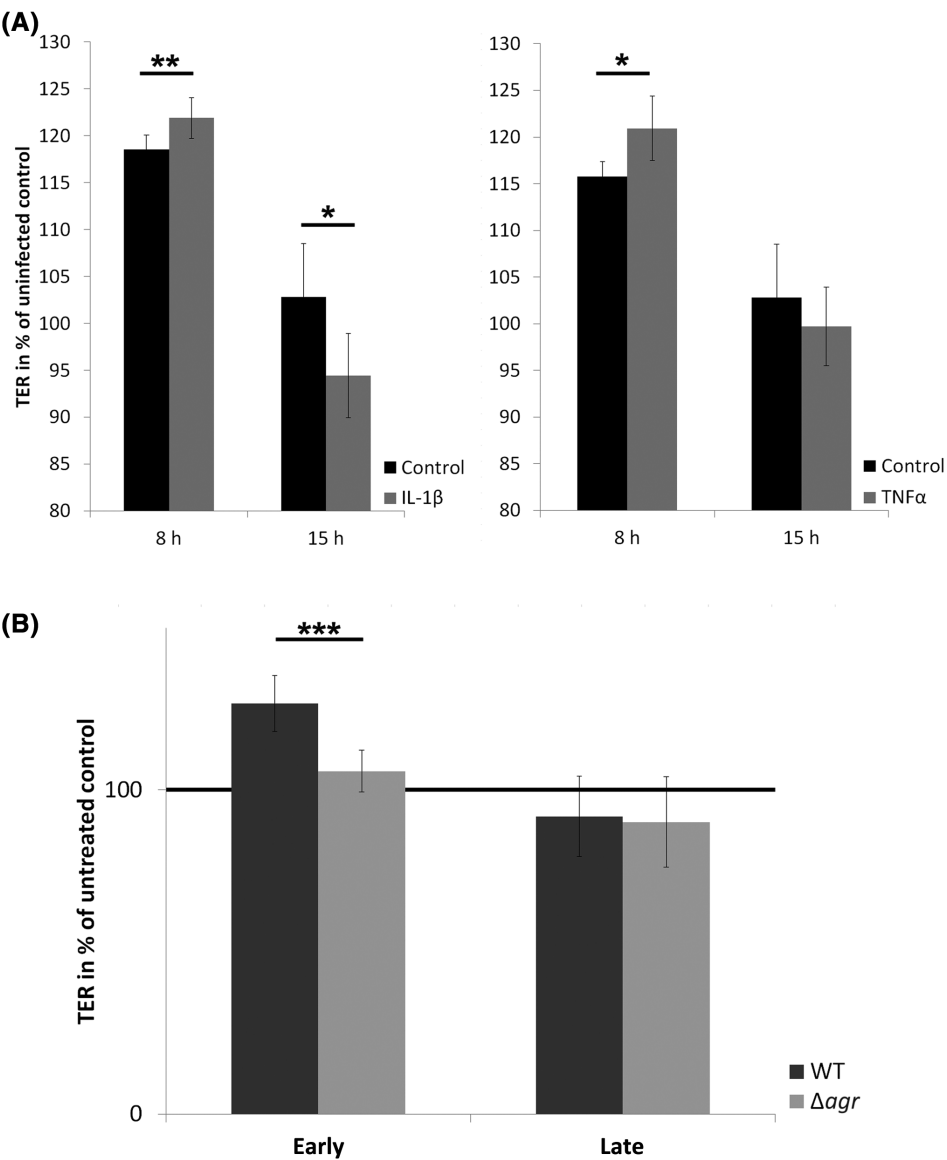


Figure 5. Influence of cytokines and *S. aureus* Δagr mutant on TER in NHEKs. (A) Influence of IL-1 β and TNF- α , respectively, on simultaneously infected NHEKs 8 and 15 h after infection/treatment. (B) Influence of *S. aureus* Δagr mutant early (8 h) and late (24 h) after inoculation. TER values are normalized to uninfected controls. Mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ using Student's *t*-test. $n = 3$ (A, TNF- α), 5 (A, IL-1 β), and 6 (B) different donors in triplicate.

with *S. epidermidis* significantly reduced growth of *S. aureus* (Fig. 6C).

Live *S. aureus* influences TJ barrier function in RHE. Evaluating the stop of biotin-SH in the stratum granulosum of RHE inoculated with *S. aureus*, we observed no changes at early time points (24 h, Fig. S5A, online only). Nevertheless, at

late time points of *S. aureus* inoculation (72 h), a significant increase in tracer permeability was found, demonstrating a decrease in TJ barrier function (Figs. 7 and 8A). The same effect was observed after 72 h of *S. epidermidis* inoculation (Fig. S6A, online only)

At early time points (24 h)—similar to NHEKs—an increase in Occludin staining at the cell–cell borders in

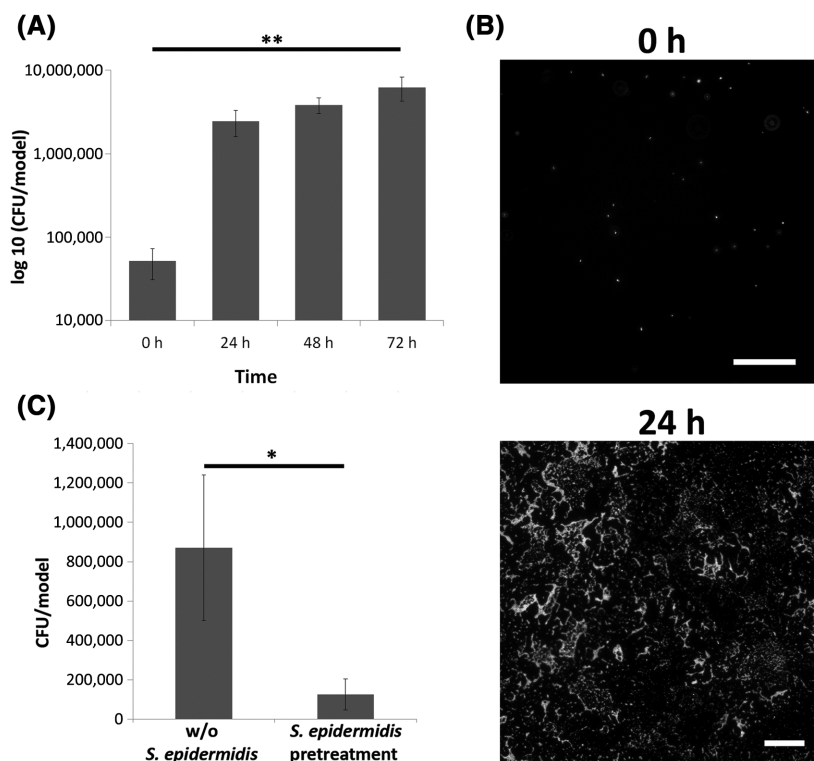


Figure 6. Growth of *S. aureus* on reconstructed human epidermis (RHE). (A) Colony-forming units (CFU) per model 24, 48, and 72 h after inoculation with 5×10^4 *S. aureus*/cm². (B) Fluorescence images of 5×10^4 *S. aureus*/cm² GFP-labeled *S. aureus* on top of the RHE immediately (0 h) and 24 h after inoculation. (C) CFU of *S. aureus* per model 48 h after inoculation with 5×10^4 *S. aureus*/cm² with and without (w/o) a preincubation of 5×10^4 *S. epidermidis*/cm² for 24 hours. Mean $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$ using ANOVA (A) and Student's *t*-test (C). $n = 5$ (A) and 3 (C) different donors in duplicate. Bar: 50 μ m.

certain areas and no change in Cldn-1 were observed after *S. aureus* inoculation (Fig. S5A, online only). For Cldn-4, no alterations were evident (Fig. S5A, online only). Western blot analyses showed—again similar to NHEKs—no changes for Cldn-1 and Cldn-4 and an increased level of p-Ocln (Fig. S5B, online only).

At late time points (72 h), immunohistochemical stainings revealed a significant decrease in immunointensity of the sealing TJ proteins Cldn-1 and Cldn-4 at the cell–cell membranes (Figs. 7 and 8B and 8C). This was similar to late time points in NHEKs. Of note, when intensity of the whole cells was measured, no decrease was observed (Fig. 8B and 8C). This shows that internalization of Cldn-1 and Cldn-4 was the cause of the loss of staining at the cell membranes, rather than protein degradation. This was also confirmed by western blot analyses, where no changes in Cldn-1 and Cldn-4 protein levels were observed (Fig. 8E). For *S. epider-*

midis, a decrease of Cldn-1 and Cldn-4 immunointensity at the cell membranes was observed (data not shown). For Ocln, increased immunointensity at the cell–cell borders was observed after *S. aureus* inoculation (Figs. 7 and 8D), again similar to NHEKs.

To exclude cell death as the cause of decreased TJ functionality, we performed tissue MTT staining,³⁰ which clearly showed no impaired viability of the cells (Fig. 8F). This was also supported by the extracellular localization of biotin–SH in the models, because the tracer would permeate through the membrane in the case of cell death (Fig. 7).

***S. aureus* inoculation does not influence overall barrier function in RHE.** We measured TER in the models inoculated with *S. aureus*. Of note, TER in RHE not only reflects TJ barrier function but also SC barrier function and is therefore a measure of the overall skin barrier. We did not observe differences in TER among bacterial treatment, NaCl control,

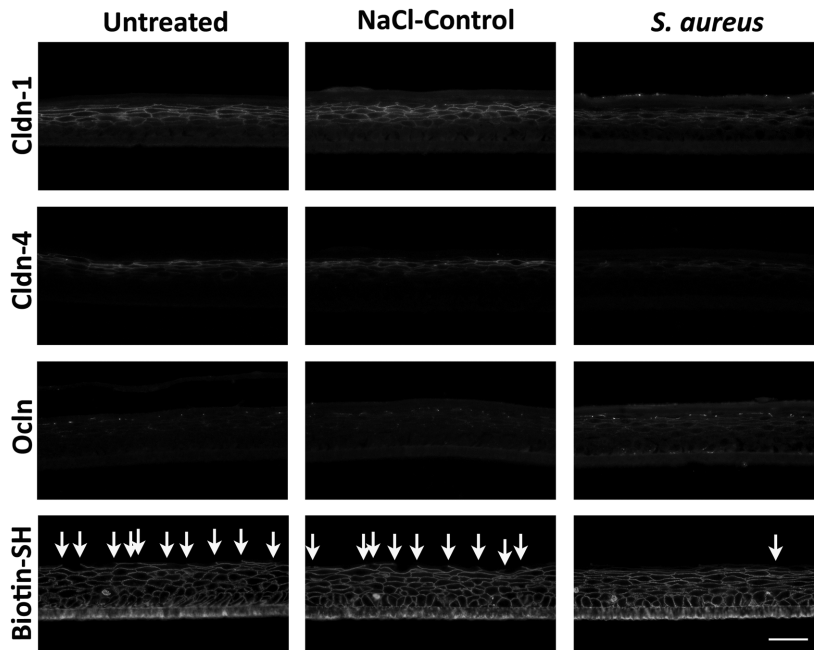


Figure 7. Immunofluorescence localization of TJ proteins and biotin-SH in vertical sections of RHE 72 h after inoculation with 5×10^4 *S. aureus*/cm². Arrows denote complete stops of biotin-SH. Bar: 50 μ m.

and untreated models, or between early and late time points (Fig. 8G), which was also the case in *S. epidermidis*-inoculated RHE (Fig. S6B, online only).

Discussion

We show here the biphasic influence of *S. aureus* inoculation on TJs in NHEKs: at early time points, *S. aureus* increases TJ barrier function, while there is a decreasing effect at later time points. The latter could also be observed in RHE. Increase and decrease are accompanied by TJ protein relocalization but not by changes in TJ protein levels.

Live S. aureus strengthens the TJ barrier at early time points

In NHEKs, we observed a TJ barrier-strengthening effect of live *S. aureus* at early time points, which can be seen in TER as well as in FITC-dextran permeability. This was also the case for *S. epidermidis*, but it was less pronounced. A TJ barrier function-enforcing effect was previously shown for lysates of *Lactobacillus* spp. and *Bifidobacterium longum* in NHEKs⁴⁴ and for live *S. epidermidis* in HaCaT cells.²⁰ In simple epithelia, a positive effect of bacteria or bacterial proteins on TJ barrier function was also observed.^{45,46} In principle, this might be a first

reaction of the epithelium to bacterial challenges with the aim of protecting the body by tightening the barrier, at least temporarily, until further protective mechanisms of innate and adaptive immunity are recruited. This is also supported by the findings that activation of several Toll-like receptors, including TLR2, results in increased TJ barrier function.^{21,22,47}

The increase in TER in NHEKs after *S. aureus* inoculation was accompanied by increased localization of Ocln and Cldn-4 at the cell-cell membranes. It was shown before that overexpression of Ocln (which was accompanied by increased intensity of the protein at the cell-cell membranes) increased TER in MDCK cells.^{48,49} In submandibular gland epithelial cells, it was shown that overexpression of Cldn-4 results in increased Cldn-4 localization at the cell membranes and in increased TER and decreased permeability for FITC-dextran.⁵⁰

Concerning the upstream signals resulting in a relocalization of Ocln to the membranes, it was shown that phosphorylation of Ocln results in this relocalization.³⁹ This fits nicely with our data, which show increased phosphorylation of Ocln in *S. aureus*-inoculated cells but not in *S. epidermidis*-inoculated cells, where this early relocalization is not seen. It also fits with data from Yuki *et al.*,²¹ who

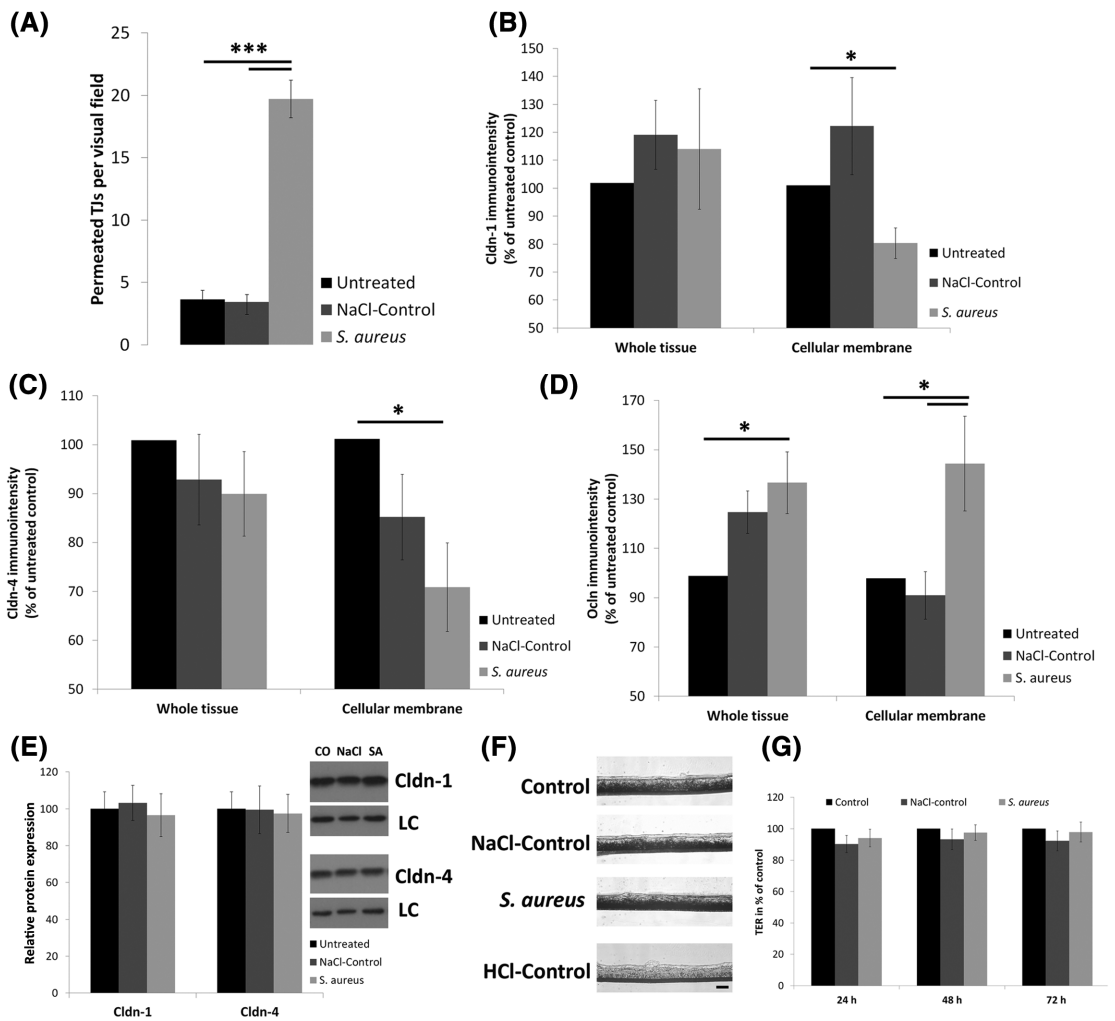


Figure 8. Influence of *S. aureus* on TJ proteins and barrier function in RHE at late time points. (A) Quantification of permeated TJs per visual field. (B, C, D) Immunofluorescence intensity of (B) Cldn-1, (C) Cldn-4, and (D) Occludin in total tissue and at the cell membranes normalized to the untreated controls. (E) Evaluation of protein levels of Cldn-1 and Cldn-4 72 h after *S. aureus* inoculation and representative western blots. (F) Tissue-MTT of RHE 72 h after inoculation with 5×10^4 *S. aureus*/cm². (G) TER of RHE 24, 48, and 72 h after treatment with 5×10^4 *S. aureus*/cm² or NaCl normalized to untreated control. Mean $\pm$ SEM. * $P < 0.05$; *** $P < 0.001$ using ANOVA. $n = 3$ (B, C, D, E, and G) and 4 (A, F) different donors in triplicate.

showed that activation of TLR2 by *S. aureus*-derived peptidoglycan results in an increase of TJ functionality, which is dependent on the kinase activity of aPKC.

An involvement of TLR2 is also supported by our experiments with a Δagr *S. aureus* strain, which showed a significantly diminished increase of TER compared with wild-type *S. aureus* at early time points. It is known that the bacterial AGR system not only positively influences secreted virulence factors when activated owing to quorum sensing but

also negatively regulates the production of staphylococcal superantigen-like proteins (Ssls).⁴³ Ssl-3 is able to specifically bind to TLR2 and inhibit its activation.⁵¹ This clearly argues for the involvement of TLR2 activation in live *S. aureus*-induced TJ barrier increase.

In addition to the above-mentioned activation of TLR2, upregulation of proinflammatory cytokines may also contribute to enhanced TJ barrier function. It has been shown that IL-1 β and TNF- α increase TER after short-term treatment in

NHEKs.⁴² This is accompanied by increased localization of Cldn-4 at the cell–cell membranes. In *ex vivo* skin, IL-1 β treatment led to a broader localization of Ocln.⁴² We found an upregulation of proinflammatory cytokines IL-1 β , IL-6, IL-8, and TNF- α upon live *S. aureus* treatment. An upregulation of gene expression of these cytokines in NHEKs was reported using *S. aureus* biofilm-secreted products,⁵² *S. aureus* biofilm-conditioned medium, or planktonic-conditioned medium.⁵³ An involvement of these cytokines in the *S. aureus*-induced increase in TER is also supported by the fact that the addition of IL-1 β and TNF- α to infected keratinocytes here produced a short-term TER-increasing effect. However, because the TER increase starts almost immediately after *S. aureus* inoculation, while significant upregulation of inflammatory cytokines can only be detected after 8 h on the mRNA level, these cytokines cannot be the initial cause for TER increase.

In RHE, we could not detect increased TJ functionality after short-term incubation with *S. aureus*, either by measuring TER or by using the biotinylation assay. However, TER values are much higher in RHE compared with NHEKs ($1785 \pm 673 \Omega \cdot \text{cm}^2$ compared with $447 \pm 47 \Omega \cdot \text{cm}^2$ ($n = 6$ different donors for both)), which indicates the contribution of the SC in RHE. An alteration of 30% in TJ-derived TER, which is seen in NHEKs and which is approximately $150 \Omega \cdot \text{cm}^2$, might not be seen in the normal standard deviation of TER in RHE and might therefore be camouflaged by the SC. This supports the value of investigating TJs in NHEKs to elucidate specific effects on TJs not covered by SC effects. For the biotinylation assay, a method commonly used to investigate TJ barrier function in skin,^{7,9,54} biotin-SH is already largely stopped at TJs at day 5 (i.e., 24 h after inoculation with *S. aureus* (8.7 ± 1.5 permeated TJs/visual field)). A decrease in tracer permeability of 15%, as can be seen for the 4-kDa FITC-dextran in NHEKs, would hardly be visible, and it is not possible to distinguish between different grades of stops except for “complete stops” and “half stops” (Fig. S1, online only). An alternative method might be the use of the dot blot system, with which Yuki *et al.*²¹ showed a decrease of biotin-SH permeability in reconstructed human skin after TLR2 activation with peptidoglycan. Unfortunately, this method did not work in our hands.

But most importantly, in our RHE, an upregulation of Ocln immunointensity at the cell–cell membranes and increased levels of p-Ocln were found after short-term inoculation with *S. aureus*. This is consistent with what was found in our NHEKs and was also observed in peptidoglycan-treated NHEKs,²¹ in *ex vivo* skin inoculated with *S. aureus* at early time points, and at sites with bacterial colonialization in impetigo contagiosa.²⁰

S. aureus impairs TJ barrier function after long-term inoculation independent of virulence factors

Investigation of the effect of long-term treatment of RHE and NHEKs with *S. aureus* revealed the same effect in both model systems (i.e., decreased TJ barrier functionality). For RHE, this decrease was detected by increased permeability of biotin-SH. In NHEKs, it was shown by decreased TER and increased permeability for 4-kDa FITC-dextran. In RHE, TER measurement did not show an influence of *S. aureus* at late time points. Again, the SC TER might camouflage the decreased TJ barrier function. This shows that a combination of both NHEKs and 3D models is ideal to reveal specific alterations of TJs but also to take into account additional possible interactions between TJs and SC.

The additional effect of the SC in RHE can also be seen by the different time points reflecting the long-term inoculation between NHEKs and RHE cells. While, in NHEKs, a decrease of TJ barrier function starts after 12–15 h and can be clearly seen at 24 h, it is only found after 72 h in RHE. This shows the better accessibility of TJs by bacteria in NHEKs compared with RHE, where bacteria meet first the SC.

The decrease of TJ barrier function is associated with decreased Cldn-1 and Cldn-4 localization at the cell membranes in both RHE and NHEKs. It was shown that knockdown of Cldn-1 and Cldn-4 in NHEKs reduces TER and increases 4-kDa FITC-dextran permeability.²⁵ Knockdown of Cldn-1 in mice increases biotin-SH permeability in the stratum granulosum.⁷ Of note, Ocln immunointensity remains increased. This clearly shows that, even though Ocln is able to strengthen the TJ barrier,^{48,49} it cannot compensate for the loss of other components.

In order to elucidate the putative causes for the decreased TJ barrier function, we first investigated

cell death. However, we could show in NHEKs and in RHE that in the course of our experiments cell death was not evident.

Another cause for decreased TJ barrier function could be destruction of TJs by bacterial virulence factors. *S. aureus* is known to secrete multiple virulence factors, such as proteases, lipases, nucleases, and toxins.⁵⁵ Loss of TJ proteins from the cellular membrane might be due to protein degradation by bacterial proteases or to changes in lipid structure due to lipases. For example, it has been shown that secreted V8 protease of *S. aureus* results in decreased Cldn-1 levels in HaCaT cells.⁵⁶ Additionally, α -hemolysin has been shown to directly decrease TER in A431 (human squamous cell carcinoma) cells.⁵⁷ Therefore, we cloned a bacterial mutant without the regulon that activates the production of secreted virulence factors by deleting the bacterial AGR system.⁴³ However, the decreased TJ functionality at later time points is independent of these bacterial virulence factors, as we did not observe a difference in decreased TER values between wild-type cells and Δagr mutants. Of note, *S. epidermidis* also shows a TJ barrier function-decreasing effect at later time points in both of our models, supporting the independence of this effect from *S. aureus*-specific virulence factors. The discrepancy with the protease/hemolysin effects mentioned above might be the use of human primary keratinocytes compared with immortalized cells. In a recent paper, it was shown that *S. aureus* induces keratinocyte-intrinsic serine protease activity (e.g., kallikreins and trypsin).⁵⁸ However, the authors concluded that TLR activation was not involved in the induction, but rather secreted virulence factors.⁵⁸ Thus, an enhanced intrinsic proteolytic activity is also unlikely to be involved in decreased TJ barrier function. This is also supported by the fact that protein levels of Cldn-1 and Cldn-4 did not decrease significantly.

Another contributing factor for decreased TJ barrier function could be inflammation. In cultured keratinocytes, IL-1 β and TNF- α show, as mentioned above, a positive effect after short-term incubation. However, long-term treatment resulted in a decrease of TER.⁴² In addition, it was shown that dermal injection of IL-1 β into healthy volunteers,⁵⁹ as well as in porcine *ex vivo* models,⁴² resulted in downregulation of Cldn-1, which is also found in our long-term *S. aureus* experiments. Additionally,

in mouse models with skin inflammation induced by allergens, decreased TJ barrier function and TJ protein membrane localization were reported.^{17,54} We show here that *S. aureus* results in an induction of IL-1 β , IL-6, IL-8, and TNF- α , which was still evident after 24 hours. In addition, we observed that the decrease in TER after *S. aureus* inoculation was accelerated when keratinocytes were simultaneously incubated with IL-1 β . This strongly hints at an influence of proinflammatory cytokines on decreased TJ barrier function. In HaCaT cells, it was shown recently that IL-1 β , used in the same concentration as in our experiment, protected the cells from *S. aureus*-secreted protease V8-mediated cell damage but not from the decrease of Cldn-1 levels.⁵⁶

Interestingly, it has been shown that Ocln, which is still present at the cell-cell membranes after long-term *S. aureus* inoculation in NHEKs and in RHE, is required for cytokine-induced regulation of TJ barriers in MDCK II cells. Overexpression of Ocln resulted in a more sensitive reaction of these cells to IFN- γ and TNF- α , while Ocln knockdown led to a less sensitive response.⁴⁹

Differences between NHEKs and HaCaT cells

NHEKs and HaCaT cells reveal different results after inoculation with *S. aureus*. While there was an immediate decrease of TER after *S. aureus* inoculation in HaCaT cells,²⁰ there was a temporary increase in NHEKs. The decrease in HaCaT cells was accompanied by a decrease of Cldn-1 and Ocln localization at the cell membranes 5 h after inoculation,²⁰ while in NHEKs these proteins are unchanged (Cldn-1) or upregulated (Ocln) within the first 12 hours. This can be explained by the different expression patterns of TLRs in these cells. Köllisch *et al.*²³ showed that both cell types express TLR2, but only NHEKs also express TLR6. Furthermore, it was shown that TLR6 interacts with TLR2 in the recognition of *S. aureus*.⁶⁰ This and the fact that it is unclear whether HaCaT cells fully differentiate²⁷ suggest that HaCaT cells may not be the ideal test system for the investigation of bacterial infection.

Bacterial infection in RHE: suitability of the model, advantages, and limitations

Investigation of the effect of *S. aureus* or its lysates/supernatants on NHEKs shows the direct effect of the organisms on this cell type and provides

important information on general mechanisms of bacterial pathogenicity.^{56,58,61} However, these studies lack details about the interplay of TJs with the SC. Therefore, we performed additional experiments with RHE that form an SC. The RHE models in our approach were used for bacterial inoculation at day 4 of air–liquid interface. This is a quite early time point for investigations of RHE models, but our models already express early (keratin 10), intermediate (involucrin), and late (loricrin and filaggrin) differentiation markers at this time point and show their correct localization. In addition, a good epidermal structuring can be observed, with palisade structure in the basal layer, functional TJs in the granular layer, and several layers of SC. The latter is also reflected by a substantial TER. However, the SC barrier is still not fully functional at this time point, as can be seen by an increasing number of SC layers and increasing TER after day 4 (data not shown). Thus, this model mimics skin with compromised SC barrier function, such as atopic dermatitis, bullous diseases, or wounded and freshly regenerated skin, but not healthy skin. We also tested bacterial inoculation at later time points (days 6, 8, or 11), and we still observed growth of bacteria, but no decrease in TJ protein localization and barrier function could be detected (data not shown). This is similar to human skin, where under healthy conditions with a completely functional SC, exfoliative toxin–negative *S. aureus* does not lead to skin infections, which confirms that the SC is of great importance for protection of the body against bacterial infections. Manifested skin infections are usually the consequence of a compromised SC barrier.⁶² Thus, when interpreting the data gained from our infected RHE, one has to keep in mind that it is a model for skin conditions with compromised SC barrier function.

The fact that the bacteria grow on top of the models shows that they are able to live and multiply on RHE. Pretreatment of RHE with *S. epidermidis* resulted in significantly decreased growth of *S. aureus*. This shows the analogy of inoculated RHE to normal skin flora. Resident microbes (like *S. epidermidis*) also help to inhibit the growth of pathogens *in vivo*.^{2,63}

Comparison of *S. aureus* and *S. epidermidis* inoculation

We observed for both *Staphylococcus* strains an increase in TER in NHEKs at early time points.

However, there seems to be a difference in mechanism as, with *S. aureus*, Ocln was phosphorylated and Ocln and Cldn-4 relocalized to the cell membranes, while, for *S. epidermidis*, Cldn-4 was also relocalized, but no phosphorylation or relocalization of Ocln was evident. Future experiments investigating the mechanism of the influence of *S. epidermidis* on TJ barrier function should address this interesting point, but this matter is beyond the scope of this manuscript, as we concentrate on *S. aureus*–related effects. At later time points, we observed a decrease in TJ barrier function in NHEKs and in RHE for both strains. It was previously shown that *S. epidermidis* is an opportunistic pathogen, which means that it can result in severe infections when it is able to penetrate into the body.⁶⁴ NHEKs, where the cells are not protected by a SC, and RHEs, where a SC is present but still weaker than in healthy skin (see above), are situations in which this pathogen can access its target cells. However, we can also show the protective side of *S. epidermidis* as a component of the skin microbiome, as it reduces the growth of *S. aureus* on the RHE surface.

Conclusions

In summary, by using NHEKs and RHE, we have shown that *S. aureus* has a biphasic influence on TJs in keratinocytes, with an enhancing effect at early time points and a decreasing effect at later time points. This suggests that strengthening of TJs is a common early answer to bacterial challenge in keratinocytes to both commensal and pathogenic bacteria. Investigation of NHEKs and RHE in parallel is beneficial, because the stepwise increase of complexity of the systems allows us to dissect distinct influences of skin barrier components.

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K.B., H.R., M.F.G., H.D., S.B.T., and J.M.B.: conception and design; K.B., S.B., S.V-y-S., B.G., E.W., P.H., G.S., and A.C.: acquisition of data; K.B., M.F.G., H.R., T.V., S.B.T., H.D., and J.M.B.: analysis and interpretation of data; K.B., M.F.G., H.R., S.B.T., S.W.S., H.D., and J.M.B.: participation in drafting the manuscript or revising its intellectual content. K.B. and J.M.B. take responsibility for the integrity of the data analyzed. This work was supported by the Deutsche Forschungsgemeinschaft (BR 1982/4-1) and Pierre Fabre Dermo-Cosmétique.

Supporting Information

Additional supporting information may be found in the online version of this article.

Appendix: Supplementary methods.

Table S1. Sources and dilutions of primary antibodies used in this study.

Table S2. Catalog numbers of target genes purchased from Applied Biosystems (Carlsbad, CA).

Table S3. The different bacterial strains used in this study and their origin.

Figure S1. Examples for “complete stops” and “half stops” of Biotin-SH in RHE.

Figure S2. Influence of *S. epidermidis* on TJ permeability of normal human epidermal keratinocytes (NHEKs).

Figure S3. Influence of *S. aureus* and *S. epidermidis* inoculation on cell death and of *S. aureus* on mRNA levels of TJ proteins in NHEKs.

Figure S4. Characterization of RHE at day 4 of air-liquid interface.

Figure S5. Influence of *S. aureus* on TJ proteins and function in RHE 24 h after inoculation.

Figure S6. Investigation of barrier function of RHE after inoculation with 5×10^4 *S. epidermidis* cm⁻².

Competing interests

The authors declare no competing interests.

References

- Grice, E.A. & J.A. Segre. 2011. The skin microbiome. *Nat. Rev. Microbiol.* **9**: 244–253.
- Sanford, J.A. & R.L. Gallo. 2013. Functions of the skin microbiota in health and disease. *Semin. Immunol.* **25**: 370–377.
- Kuo, I.H. et al. 2013. The cutaneous innate immune response in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **131**: 266–278.
- Bäsler, K. et al. 2016. The role of tight junctions in skin barrier function and dermal absorption. *J. Control Release* **242**: 105–118.
- Murakawa, G.J. 2004. Common pathogens and differential diagnosis of skin and soft tissue infections. *Cutis* **73**: 7–10.
- Iwatsuki, K. et al. 2006. Staphylococcal cutaneous infections: invasion, evasion and aggression. *J. Dermatol. Sci.* **42**: 203–214.
- Furuse, M. et al. 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J. Cell Biol.* **156**: 1099–1111.
- Yoshida, K. et al. 2013. Functional tight junction barrier localizes in the second layer of the stratum granulosum of human epidermis. *J. Dermatol. Sci.* **71**: 89–99.
- Kirschner, N. et al. 2010. Tight junctions form a barrier in human epidermis. *Eur. J. Cell Biol.* **89**: 839–842.
- Hashimoto, K. 1971. Intercellular spaces of the human epidermis as demonstrated with lanthanum. *J. Invest. Dermatol.* **57**: 17–31.
- Kubo, A. et al. 2009. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J. Exp. Med.* **206**: 2937–2946.
- Sugawara, T. et al. 2013. Tight junction dysfunction in the stratum granulosum leads to aberrant stratum corneum barrier function in claudin-1-deficient mice. *J. Dermatol. Sci.* **70**: 12–18.
- Baek, J.H. et al. 2013. Acute modulations in stratum corneum permeability barrier function affect claudin expression and epidermal tight junction function via changes of epidermal calcium gradient. *Yonsei Med. J.* **54**: 523–528.
- Kirschner, N. et al. 2011. CD44 regulates tight-junction assembly and barrier function. *J. Invest. Dermatol.* **131**: 932–943.
- Brandner, J.M. 2009. Tight junctions and tight junction proteins in mammalian epidermis. *Eur. J. Pharm. Biopharm.* **72**: 289–294.
- De Benedetto, A. et al. 2011. Tight junction defects in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* **127**: 773–786. e1–7.
- Gruber, R. et al. 2015. Diverse regulation of claudin-1 and claudin-4 in atopic dermatitis. *Am. J. Pathol.* **185**: 2777–2789.
- Bäsler, K. & J.M. Brandner. 2016. Tight junctions in skin inflammation. *Pflugers Arch.* **469**: 3–14.
- Brandner, J.M. & J.D. Schulzke. 2015. Hereditary barrier-related diseases involving the tight junction: lessons from skin and intestine. *Cell Tissue Res.* **360**: 723–748.
- Ohnemus, U. et al. 2008. Regulation of epidermal tight-junctions (TJ) during infection with exfoliative toxin-negative *Staphylococcus* strains. *J. Invest. Dermatol.* **128**: 906–916.
- Yuki, T. et al. 2011. Activation of TLR2 enhances tight junction barrier in epidermal keratinocytes. *J. Immunol.* **187**: 3230–3237.
- Kuo, I.H. et al. 2013. Activation of epidermal Toll-like receptor 2 enhances tight junction function: implications for

- atopic dermatitis and skin barrier repair. *J. Invest. Dermatol.* **133**: 988–998.
23. Köllisch, G. *et al.* 2005. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. *Immunology* **114**: 531–541.
 24. Aono, S. & Y. Hirai. 2008. Phosphorylation of claudin-4 is required for tight junction formation in a human keratinocyte cell line. *Exp. Cell Res.* **314**: 3326–3339.
 25. Kirschner, N. *et al.* 2013. Contribution of tight junction proteins to ion, macromolecule, and water barrier in keratinocytes. *J. Invest. Dermatol.* **133**: 1161–1169.
 26. Yuki, T. *et al.* 2007. Tight junction proteins in keratinocytes: localization and contribution to barrier function. *Exp. Dermatol.* **16**: 324–330.
 27. Micallef, L. *et al.* 2009. Effects of extracellular calcium on the growth-differentiation switch in immortalized keratinocyte HaCaT cells compared with normal human keratinocytes. *Exp. Dermatol.* **18**: 143–151.
 28. Moll, I. *et al.* 1998. Characterization of epidermal wound healing in a human skin organ culture model: acceleration by transplanted keratinocytes. *J. Invest. Dermatol.* **111**: 251–258.
 29. Fredricks, D.N. 2001. Microbial ecology of human skin in health and disease. *J. Investig. Dermatol. Symp. Proc.* **6**: 167–169.
 30. Herbig, M.E. *et al.* 2015. A custom tailored model to investigate skin penetration in porcine skin and its comparison with human skin. *Eur. J. Pharm. Biopharm.* **95**(Pt A): 99–109.
 31. Winstel, V. *et al.* 2015. Transfer of plasmid DNA to clinical coagulase-negative staphylococcal pathogens by using a unique bacteriophage. *Appl. Environ. Microb.* **81**: 2481–2488.
 32. Helfrich, I. *et al.* 2007. Role of aPKC isoforms and their binding partners Par3 and Par6 in epidermal barrier formation. *J. Invest. Dermatol.* **127**: 782–791.
 33. Kiatsurayanon, C. *et al.* 2014. Host defense (antimicrobial) peptide, human beta-defensin-3, improves the function of the epithelial tight-junction barrier in human keratinocytes. *J. Invest. Dermatol.* **134**: 2163–2173.
 34. Mertens, A.E. *et al.* 2005. The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. *J. Cell Biol.* **170**: 1029–1037.
 35. Yuki, T. *et al.* 2016. Impaired tight junctions in atopic dermatitis skin and in a skin-equivalent model treated with interleukin-17. *PLoS One* **11**: e0161759.
 36. Madara, J.L. 1998. Regulation of the movement of solutes across tight junctions. *Annu. Rev. Physiol.* **60**: 143–159.
 37. Matsukawa, Y. *et al.* 1997. Size-dependent dextran transport across rat alveolar epithelial cell monolayers. *J. Pharm. Sci.* **86**: 305–309.
 38. Kawedia, J.D. *et al.* 2007. Interaction between transcellular and paracellular water transport pathways through Aquaporin 5 and the tight junction complex. *Proc. Natl. Acad. Sci. U.S.A.* **104**: 3621–3626.
 39. Wong, V. 1997. Phosphorylation of occludin correlates with occludin localization and function at the tight junction. *Am. J. Physiol.* **273**: C1859–C1867.
 40. Sakakibara, A. *et al.* 1997. Possible involvement of phosphorylation of occludin in tight junction formation. *J. Cell Biol.* **137**: 1393–1401.
 41. Akiyama, T. *et al.* 2014. The human cathelicidin LL-37 host defense peptide upregulates tight junction-related proteins and increases human epidermal keratinocyte barrier function. *J. Innate Immun.* **6**: 739–753.
 42. Kirschner, N. *et al.* 2009. Alteration of tight junction proteins is an early event in psoriasis: putative involvement of proinflammatory cytokines. *Am. J. Pathol.* **175**: 1095–1106.
 43. Painter, K.L. *et al.* 2014. What role does the quorum-sensing accessory gene regulator system play during *Staphylococcus aureus* bacteremia? *Trends Microbiol.* **22**: 676–685.
 44. Sultana, R., A.J. McBain & C.A. O'Neill. 2013. Strain-dependent augmentation of tight-junction barrier function in human primary epidermal keratinocytes by *Lactobacillus* and *Bifidobacterium* lysates. *Appl. Environ. Microbiol.* **79**: 4887–4894.
 45. Miyauchi, E. *et al.* 2012. Mechanism of protection of transepithelial barrier function by *Lactobacillus salivarius*: strain dependence and attenuation by bacteriocin production. *Am. J. Physiol. Gastrointest. Liver Physiol.* **303**: G1029–G1041.
 46. Seth, A. *et al.* 2008. Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. *Am. J. Physiol. Gastrointest. Liver Physiol.* **294**: G1060–G1069.
 47. Borkowski, A.W. *et al.* 2015. Toll-like receptor 3 activation is required for normal skin barrier repair following UV damage. *J. Invest. Dermatol.* **135**: 569–578.
 48. McCarthy, K.M. *et al.* 1996. Occludin is a functional component of the tight junction. *J. Cell Sci.* **109**: 2287–2298.
 49. Van Itallie, C.M. *et al.* 2010. Occludin is required for cytokine-induced regulation of tight junction barriers. *J. Cell Sci.* **123**: 2844–2852.
 50. Michikawa, H., J. Fujita-Yoshigaki & H. Sugiya. 2008. Enhancement of barrier function by overexpression of claudin-4 in tight junctions of submandibular gland cells. *Cell Tissue Res.* **334**: 255–264.
 51. Bardoel, B.W. *et al.* 2012. Evasion of Toll-like receptor 2 activation by staphylococcal superantigen-like protein 3. *J. Mol. Med. (Berl.)* **90**: 1109–1120.
 52. Tankersley, A. *et al.* 2014. Early effects of *Staphylococcus aureus* biofilm secreted products on inflammatory responses of human epithelial keratinocytes. *J. Inflamm. (Lond.)* **11**: 17.
 53. Secor, P.R. *et al.* 2011. *Staphylococcus aureus* biofilm and planktonic cultures differentially impact gene expression, mapk phosphorylation, and cytokine production in human keratinocytes. *BMC Microbiol.* **11**: 143.
 54. Yokouchi, M. *et al.* 2015. Epidermal tight junction barrier function is altered by skin inflammation, but not by filaggrin-deficient stratum corneum. *J. Dermatol. Sci.* **77**: 28–36.
 55. Archer, G.L. 1998. *Staphylococcus aureus*: a well-armed pathogen. *Clin. Infect. Dis.* **26**: 1179–1181.

56. Wang, B. *et al.* 2017. IL-1 β -induced protection of keratinocytes against *Staphylococcus aureus*-secreted proteases is mediated by human β -defensin 2. *J. Invest. Dermatol.* **137**: 95–105.
57. Inoshima, N., Y. Wang & J.B. Wardenburg. 2012. Genetic requirement for ADAM10 in severe *Staphylococcus aureus* skin infection. *J. Invest. Dermatol.* **132**: 1513–1516.
58. Williams, M.R. *et al.* 2017. *Staphylococcus aureus* induces increased serine protease activity in keratinocytes. *J. Invest. Dermatol.* **137**: 377–384.
59. Watson, R.E. *et al.* 2007. Altered claudin expression is a feature of chronic plaque psoriasis. *J. Pathol.* **212**: 450–458.
60. Ozinsky, A. *et al.* 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. U.S.A.* **97**: 13766–13771.
61. Wickersham, M. *et al.* 2017. Metabolic stress drives keratinocyte defenses against *Staphylococcus aureus* infection. *Cell Rep.* **18**: 2742–2751.
62. Kong, H.H. *et al.* 2012. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Res.* **22**: 850–859.
63. Lai, Y. *et al.* 2010. Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. *J. Invest. Dermatol.* **130**: 2211–2221.
64. Otto, M. 2009. *Staphylococcus epidermidis*—the “accidental” pathogen. *Nat. Rev. Microbiol.* **7**: 555–567.

Appendix

Supplementary Methods

Western Blot Analyses

RHE or NHEKs were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 5 mmol/L EDTA, 150 mmol/L NaCl, and 50 mmol/L Tris, pH 8.0) and a cocktail of protease inhibitors (Sigma, Munich, Germany). The total amount of protein was determined by using Bradfords reagent (AppliChem, Darmstadt, Germany) and 5 or 10 µg (depending on the antibody) was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. After blocking for 1 h with 5 % dry milk powder in Tris-buffered saline and Tween 20 (TBST), membranes were probed with the appropriate antibodies over night at 4 °C (see Supplementary table 1). Subsequently, membranes were washed for 3 × 5 min with TBST and incubated for 30 min with the appropriate secondary antibodies coupled with horseradish peroxidase (Jackson ImmunoResearch, West Grove, USA). After another washing for 2 × 5 minutes with TBST, immunoreactions were visualized by the ECL system (Amersham, Buckinghamshire, UK). Semi-quantification was performed by using ImageJ software version 1.41n (NHI, Bethesda, MD). β-actin was used as the loading control for correction of loading differences.

Real-Time-PCR Analyses

Total mRNA was isolated from NHEKs by using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three micrograms of total RNA were used for first-strand cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad, Munich, Germany) according to the manufacturer's specifications. 0.5 µl of the cDNA was used as a template in real-time-PCR analysis with the FAM dye-labeled TaqMan MGB probes for Cldn-1, Cldn-4, ZO-1, Ocln, IL-1β, IL-6, IL-8, TNFα and 18S RNA (Supplementary Table 2) in an iCycler iQ thermal cycler (Bio-Rad) under the conditions recommended by Applied Biosystems. All real-time-PCR analyses were performed in triplicates. Relative transcriptional levels within distinct experiments were determined by using the $2^{-\Delta\Delta C_t}$ method.¹

Analyses of protein levels of cytokines

Analyses on the protein level of cytokines was performed on sterile filtered apical supernatants of TER measurement assays with MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (Merck, Darmstadt, Germany) according to manufacturer's specifications.

Immunofluorescence stainings

Paraffin sections were deparaffinated and rehydrated. Antigen retrieval was performed by microwave oven heating (2 × 10 minutes, 600 W) in Tris-EDTA-citrate buffer (pH 7.8), followed by 0.001% trypsin for 10 minutes at 37°C. Unspecific-binding sites were blocked with a Protein Block (DAKO, Carpinteria, USA). Primary antibodies were diluted in PBS (see Supplementary table 1) and applied to the sections over night at 4 °C, followed by washing of the samples for 3 × 3 minutes in Tris-buffered saline and Tween 20 (TBST).

Cultured cells grown on coverslips were fixed in cold methanol (−20°C, 10 minutes) and acetone (15 seconds). A blocking step with 2 % normal goat serum and 0.1 % Triton X-100 for 30 min was performed at room temperature before primary antibodies were applied for 30 minutes. The samples were subsequently washed for 2 × 10 minutes in PBS.

Subsequently to both, paraffin sections and cover slips, Alexa 488- and Alexa 594- or Cy3-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, USA) were applied for 30 minutes at room temperature, followed by washing in PBS 1 x 5 min and a subsequent incubation with 4,6-diamidino-2-phenylindole (DAPI; 1:5000 in PBS) for 1 min. In case of biotinylated samples, streptavidin labeled with Texas red (Rockland, Limerick, USA) (1:700 in PBS) was applied together with the secondary antibody onto the sections in order to visualize Biotin-SH. The slides or coverslips were washed again with PBS once for 5 min followed by aqua dest. twice for 3 min and mounted with Fluoromount (Southern Biotechnology Associates, Inc. Birmingham, AL). Isotype-matched antibodies were used as negative controls.

Evaluation of intensity

An Axiophot II microscope (Zeiss, Göttingen, Germany) and the software Openlab 2.0.9 (Improvision, Coventry, UK) were used for the evaluation of stainings. All images of stainings from a series of experiments were acquired and processed with the same settings, and representative areas were photographed. For quantification, ImageJ software version 1.41n (NHI, Bethesda, MD) was used. For measurement of "whole tissue" intensity, 3 regions of

interest (ROIs) were defined per raw image (Cldn-1: from suprabasal to granular cell layer; width 79.7 μm ; for Cldn-4 and Oc1n: ROIs of 1060 μm^2 located in the granular cell layer) and at least 3 images were evaluated per RHE. For evaluating intensities of membrane staining 3 ROIs (90 μm^2) within cells (excluding the plasma membranes) were defined and the mean intensity of these ROIs were subtracted from the intensity of the larger ROIs measuring the whole tissue. For general background subtraction two ROIs (1060 μm^2) were defined at areas without tissue. Intensity measurements were then normalized to the mean of untreated models.

*Generation of a *S. aureus* Δagr and GFP strains*

SH1000 Δagr and SH1000 GFP strains were constructed using phage transduction. Overnight cultures of *S. aureus* RN6390 Δagr or RN4220xpCM29 (GFP producing strain) at an OD600 of 1.0 were infected with phage 11 in STA-softagar (Nutrient broth 20 g/l, NaCl 5 g/l, CaCl_2 0.4 g/l, Bactoagar 7 g/l) and incubated over night at 30 °C. In order to resuspend the plaque containing softagar, 5 ml of NB^{2+} -medium (Nutrient broth 20 g/l, CaCl_2 0,4 g/l) were added and the resulted suspension sonificated on ice (10 sec at 35 % amplitude; Branson sonifier, G Heinemann, Schwäbisch Gmund, Germany). Subsequently the softagar was pelleted by centrifugation (30 min; 5000 g). The supernatants which contained the plasmid-bearing phage particle lysate were filtrated with a 0.2 μm filter (Thermo Fischer, Rockford, USA). *S. aureus* SH1000 at OD600 8.0 in NB^{2+} -medium was mixed 1:1 with the phage lysate and incubated for 30 min at 37°C allowing phage particle adsorption to bacterial host cells. After adding sodium citrate to a final concentration of 20 mM, bacteria were washed 3 times in brain heart infusion broth (BHI) and incubated for 1 h at 37 °C in BHI-broth containing 10 $\mu\text{g}/\text{ml}$ tetracycline (for Δagr) or 10 $\mu\text{g}/\text{ml}$ chloramphenicol (for xPCM29 (GFP)). Afterwards, the bacteria-phage suspension was plated onto selective BHI-medium (Oxoid, Hampshire, United Kingdom) containing tetracycline or chloramphenicol (10 $\mu\text{g}/\text{ml}$) and incubated for 24 h at 37°C. Plates were screened for resistant colonies, which were transferred to BHI selective medium for further investigation. Bacterial Δagr mutants were confirmed by using PCR.

Determination of bacterial growth on RHE models

To investigate bacterial growth on top of the models, the amount of living bacteria was evaluated by examining the colony forming units (cfu). Therefore models were cut out of the insert and placed together with the membrane in 2 ml reaction tubes filled with 500 μl 0.9 %

NaCl solution and a metallic ball on ice. Using the TissueLyser (Quiagen, Hilden, Germany) for 2 min at 20 times per second models were shredded at 4 °C. Afterwards the suspension was placed for 1 min in an ultrasonic bath (Bandelin, Hagen, Germany) in order to separate the bacteria. Subsequently serial 1:10 dilutions of the bacterial suspension in NaCl-solution were performed on ice and plated on BHI-Agar plates. After incubation at 37 °C for 24, 48 and 72 h the cfu/model were counted.

Supplementary Tables:

Table S1: Sources and dilutions of primary antibodies used in this study.

Antibody	Species/Clone	Company	Dilution WB	Dilution IHC
Cldn-1	Rabbit/Jay.8	Invitrogen (Darmstadt, Germany)	1:1500	1:900
Cldn-4	Mouse/3E2C1	Invitrogen (Darmstadt, Germany)	1:200	1:500
Ocln	Goat/N-19	Santa Cruz (Dallas, USA)		1:500
Ocln	Mouse	Invitrogen (Darmstadt, Germany)	1:100	
CK10	Mouse	Progen (Aachen, Germany)		1:200
Involucrin	Mouse/Sy5	Leica (Nussloch, Germany)		1:200
Loricrin	Rabbit/AF 62	BioLegend (Dedham, USA)		1:500
Filaggrin	Rabbit/NBP1-21310	Novus (Wiesbaden, Germany)		1:2000
β -Actin	Mouse/AC-15	Sigma-Aldrich (Darmstadt, Germany)	1:30 000	

Table S2: Catalog numbers of target genes purchased from Applied Biosystems (Carlsbad, CA).

Target Gene	Catalog number
<i>CLDN-1</i>	Hs00221623_m1
<i>CLDN-4</i>	Hs00533616_s1
<i>OCLN</i>	Hs00170162_m1
<i>ZO-1</i>	Hs01551861 TJP1
<i>TNFα</i>	Hs001113624_g1
<i>IL-1β</i>	Hs01555410_m1
<i>IL-6</i>	Hs00985639_m1
<i>IL-8</i>	Hs00174103_m1
<i>18S rRNA</i>	Hs99999901_s1

Table S3: The different bacterial strains used in this study and their origin.

Species	Strain	Description	Origin
<i>Staphylococcus epidermidis</i>	PIA97		Rohde et al., 2007 ²

<i>Staphylococcus aureus</i>	SH1000		Horsburgh et al., 2002 ³
<i>Staphylococcus aureus</i>	SH1000	xpCM29	This study
<i>Staphylococcus aureus</i>	SH1000	$\Delta agr::tetM$	This study
<i>Staphylococcus aureus</i>	6820	MRSA	Clinical isolate
<i>Staphylococcus aureus</i>	RN6390	$\Delta agr::tetM$	Novick et al., 1993 ⁴
<i>Staphylococcus aureus</i>	RN4220	xpCM29	Malone et al., 2009 ⁵

Supplementary References

1. Livak, K. J. & T. D. Schmittgen. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*. 25: 402-408.
2. Rohde, H., *et al.* 2007. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials*. 28: 1711-1720.
3. Horsburgh, M. J., *et al.* 2002. σB Modulates Virulence Determinant Expression and Stress Resistance: Characterization of a Functional *rsbU* Strain Derived from *Staphylococcus aureus* 8325-4. *Journal of Bacteriology*. 184: 5457-5467.
4. Novick, R. P., *et al.* 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *The EMBO Journal*. 12: 3967-3975.
5. Malone, C. L., *et al.* 2009. Fluorescent Reporters for *Staphylococcus aureus*. *Journal of microbiological methods*. 77: 251-260.

Supplementary figures

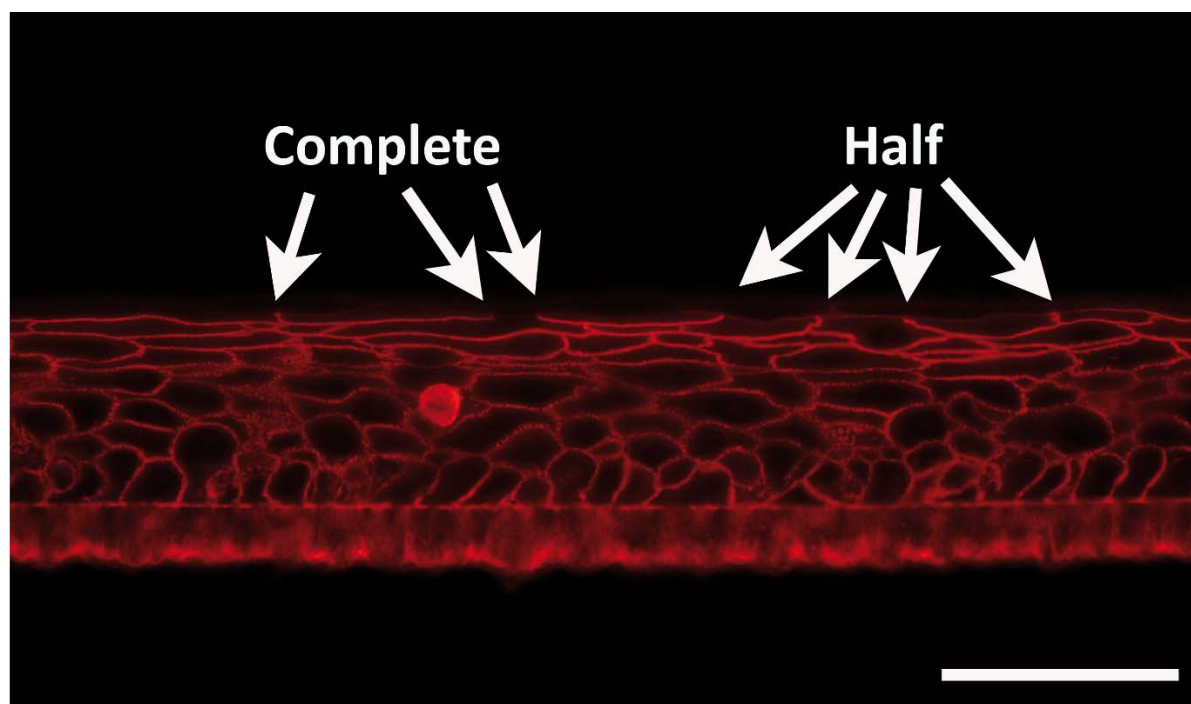


Figure S1: Examples for “complete stops” and “half stops” of Biotin-SH in RHE. Bar: 50 μm .

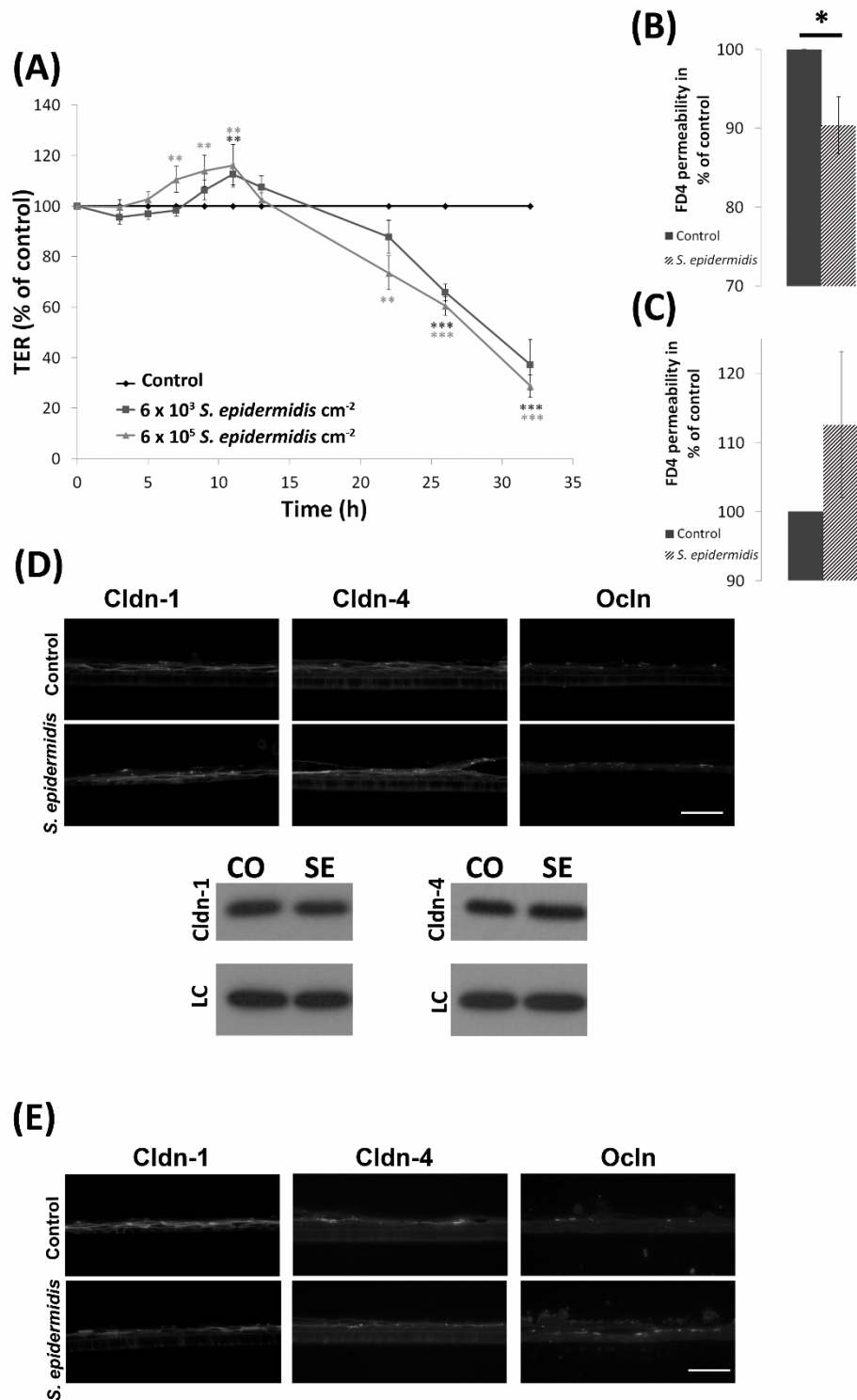


Figure S2: Influence of *S. epidermidis* on TJ permeability of normal human epidermal keratinocytes (NHEKs). (A) Transepithelial electrical resistance (TER) of NHEKs inoculated with 2 different concentrations of *S. epidermidis* normalized to the untreated control at indicated time points. (B, C) permeability of a 4 kDa tracer at (B) 12 h and (C) 24 h of inoculation with 6×10^5 *S. epidermidis* cm⁻² normalized to control. (D, E) Immunohistochemical stainings and Western Blots of denoted TJ proteins in vertical sections of transwell membranes (D) 12 and

(E) 24 h after *S. epidermidis* (6×10^5 bacteria cm^{-2}) inoculation. Mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using ANOVA (A) and Student's t-test (B, C). $n = 5$ different donors in triplicates. Bar: 50 μm .

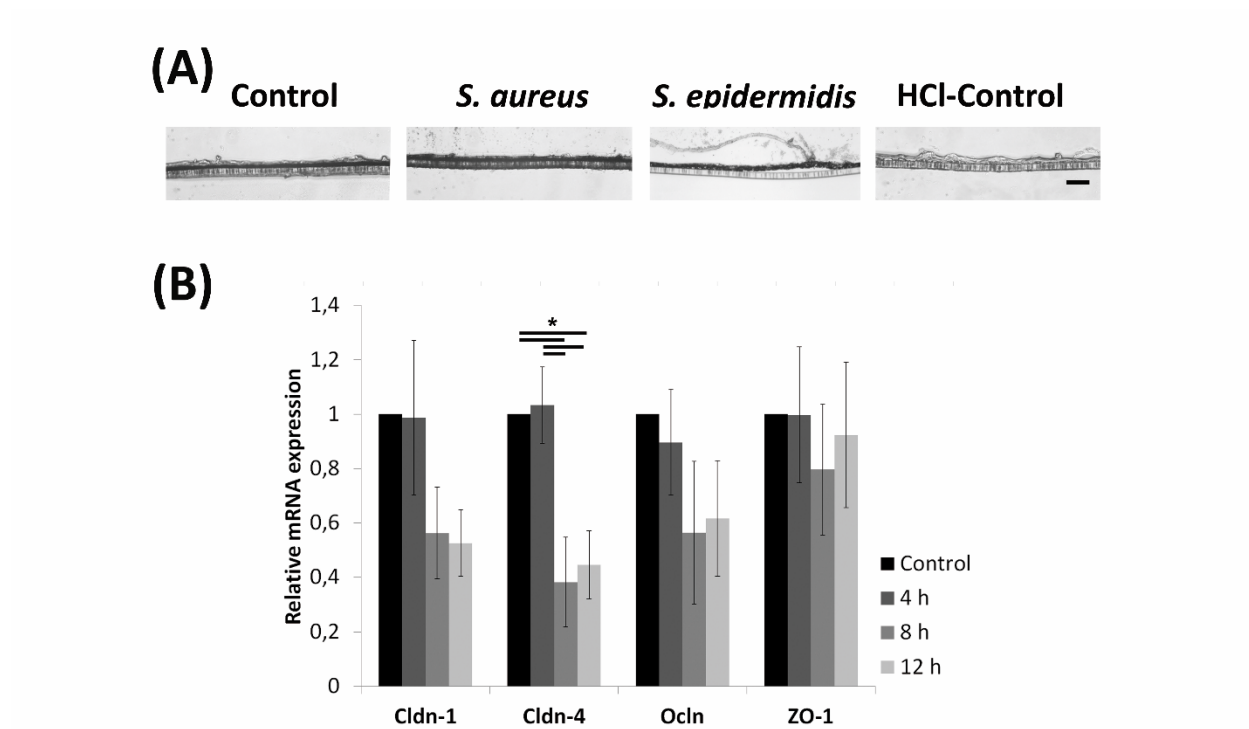


Figure S3: Influence of *S. aureus* and *S. epidermidis* inoculation on cell death and of *S. aureus* on mRNA levels of TJ proteins in NHEKs. (A) MTT-assay of NHEKs grown on transwell filters 24 h after *S. aureus* or *S. epidermidis* inoculation (6×10^5 bacteria cm^{-2}). HCl was used as a control resulting in cell death. (B) qPCR analyses of the mRNA levels of Cldn-1, Cldn-4, Ocln and ZO-1 after inoculation with *S. aureus* at the denoted time points normalized to the control. * $p < 0.05$ using ANOVA. $n = 3$ different donors. Bar: 50 μm .

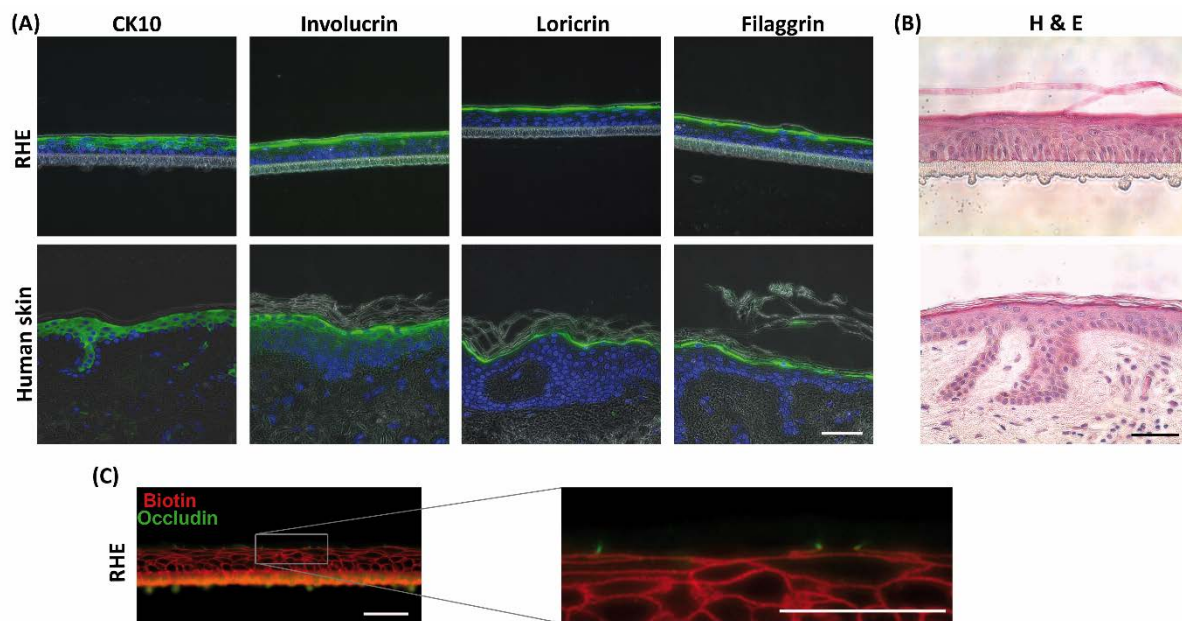


Figure S4: Characterization of RHE at day 4 of air-liquid interface. (A) Immunohistological localization of the denoted proteins (green) in the RHE (upper row) and in human skin (lower row). Blue: DAPI staining of nuclei; Overlay of epifluorescence pictures and phase contrast pictures. (B) H+E staining of the RHE and human skin. (C) Biotinylation assay of RHE. Note the stop of the tracer (red) in the granular cell layer at Ocln-positive sites (green). Bar: 50 μ m.

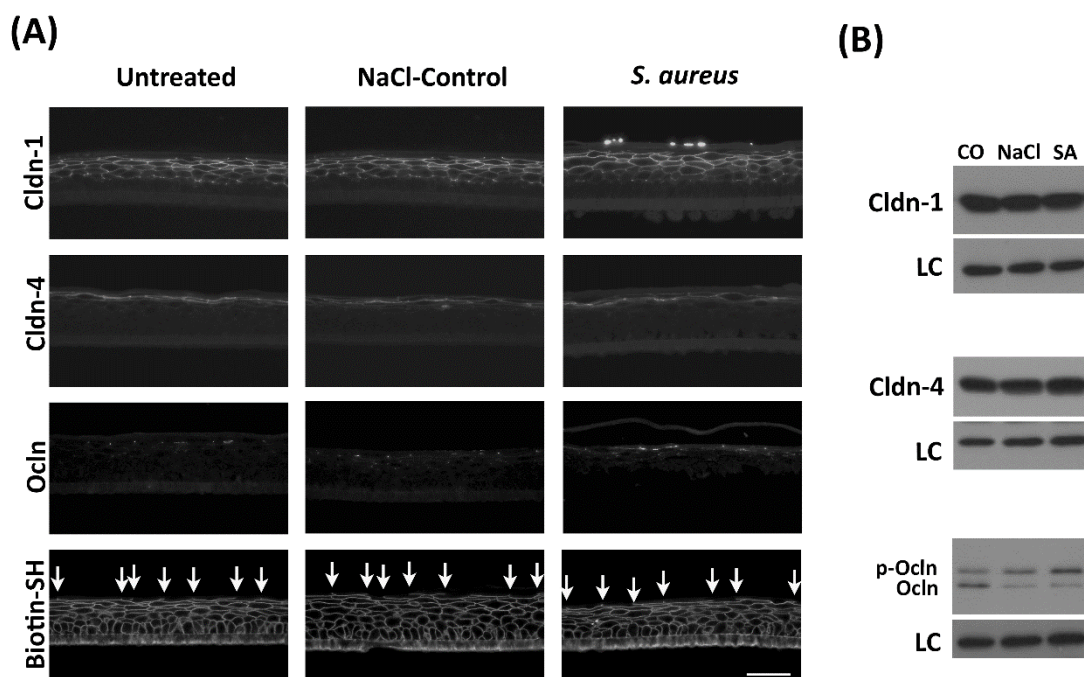


Figure S5: Influence of *S. aureus* on TJ proteins and function in RHE 24 h after inoculation. (A) Immunofluorescence localization of the denoted TJ proteins as well as Biotin-SH in vertical sections of RHE 24 h after inoculation with 5×10^4 *S. aureus* cm^{-2} . Arrows denote the stop of Biotin-SH in the granular cell layer. Note a cross-reaction between Cldn-1 antibody and *S. aureus* resulting in bright fluorescent dots. (B) Representative Western blots of Cldn-1, Cldn-4 and Occl. LC: loading control (actin), CO: untreated RHE, NaCl: NaCl treated RHE, SA: *S. aureus* treated RHE. n = 3 different donors. Bar: 50 μm .

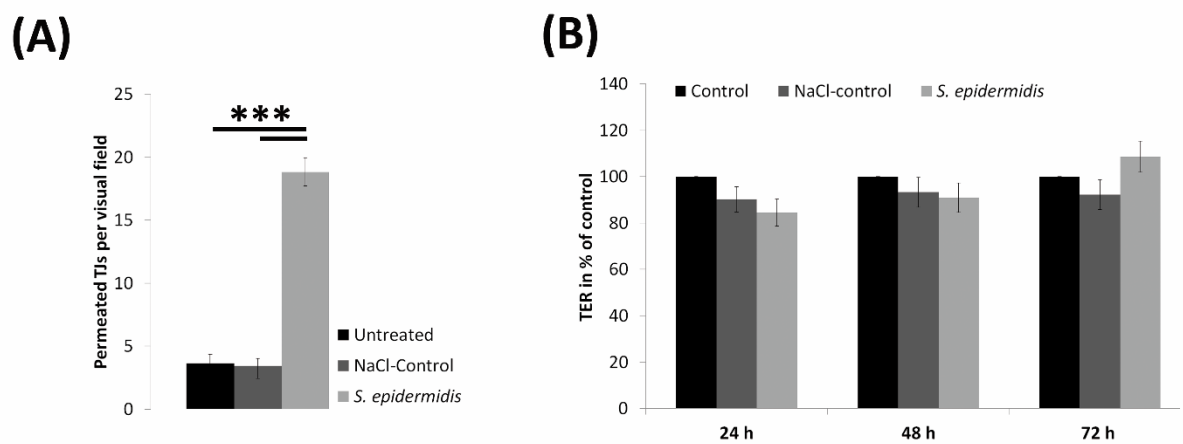


Figure S6: Investigation of barrier function of RHE after inoculation with 5×10^4 *S. epidermidis* cm^{-2} . (A) Quantification of permeated TJs per visual field after 72 h of inoculation with *S. epidermidis*. (B) TER measurement of RHE inoculated with *S. epidermidis* for 24, 48 and 72 h. Mean $\pm$ SEM. *** $p < 0.001$ using ANOVA. n = 4 different donors in triplicates.