

ORIGINAL ARTICLE

Atopic Dermatitis, Urticaria and Skin Disease

Staphylococcus aureus from atopic dermatitis skin accumulates in the lysosomes of keratinocytes with induction of IL-1 α secretion via TLR9

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Abstract

Background: *Staphylococcus aureus* (*S. aureus*) is frequently detected in the skin of patients with atopic dermatitis (AD), and involved in the flare of AD. There are some evidence-specific strains of *S. aureus* affect the severity of AD. However, the mechanism of predominant colonization and the aggravation of dermatitis by certain strains of *S. aureus* in the patients with AD are still unknown.**Objective:** To reveal the characteristics of *S. aureus* from patients with AD (*S. aureus*-AD), we analyzed the interaction of *S. aureus*-AD and keratinocytes in comparison with those of *S. aureus* laboratory strains (*S. aureus*-stand.).**Methods:** We stimulated HaCaT cells, keratinocyte cell line, and human epidermal keratinocytes by heat-killed *S. aureus* strains, then evaluated immune response of keratinocytes by ELISA, immunofluorescence staining, and flow cytometry.**Results:** Upon incubation with keratinocytes, three out of four strains of heat-killed *S. aureus*-AD were strongly agglutinated inside the cytoplasm. In the cells, they are located in lysosomes and promoted the secretion of interleukin-1 α (IL-1 α). These reactions were not observed by any of four strains of *S. aureus*-stand. and *S. epidermidis* and were abolished by the treatment of *S. aureus* with proteinase K. Moreover, the IL-1 α secretion was diminished by the inhibition of Toll-like receptor 9 (TLR9).**Conclusion:** *S. aureus*-AD accumulates in lysosome of keratinocytes by means of bacterial cell wall proteins and induces IL-1 α via TLR9.

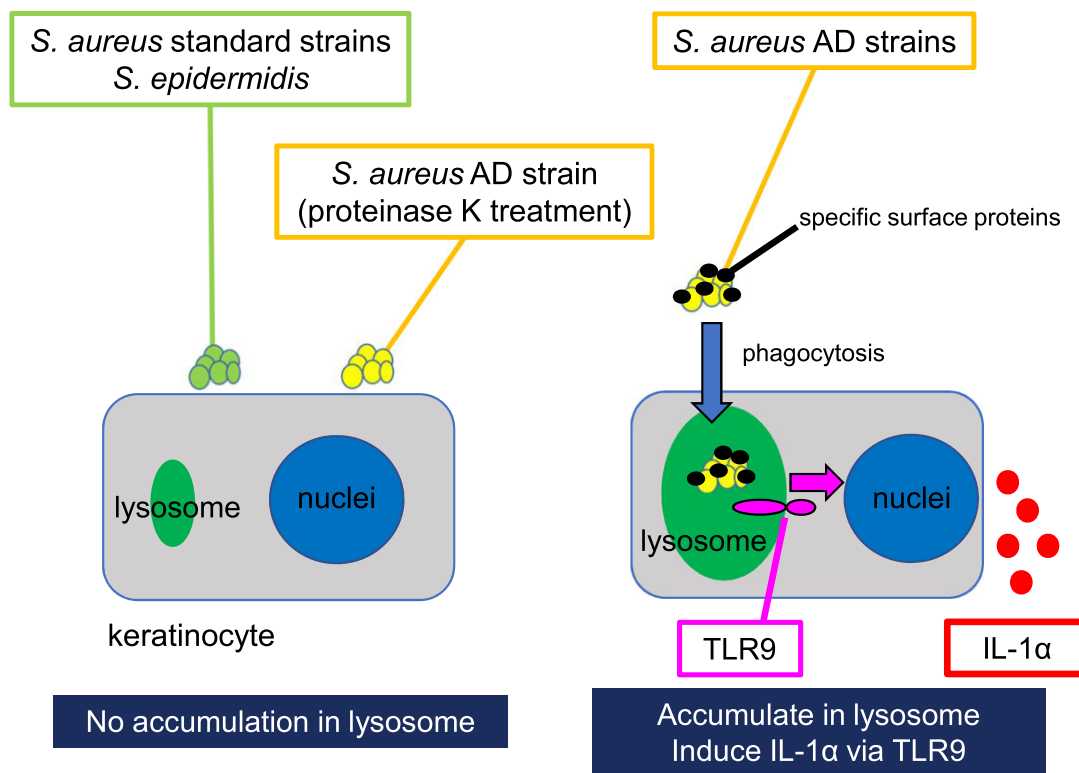
KEYWORDS

atopic dermatitis, dermatology, innate immunity, microbiome, staphylococcus aureus

1 | INTRODUCTION

Atopic dermatitis (AD) is a common chronic skin disease characterized by pruritus and recurrent eczematous lesions.¹ There is a strong correlation between *filaggrin* (*FLG*) mutations and AD, but not all carriers of *FLG* mutation develop AD,² suggesting the involvement of other causative or aggravating factors in the pathogenesis of AD. Many patients with AD frequently suffer from cutaneous infections by *Staphylococcus aureus* (*S. aureus*). Moreover, *S. aureus* is

commonly isolated from patients with AD, even from apparently non-lesion skin.³ Furthermore, *S. aureus* colonization has been shown to be associated with AD severity and observed even before the onset of AD in infants.⁴ A previous study of the skin microbiome of patients with AD revealed reduced diversity and high *Staphylococcus* proportions in flares of untreated AD,⁵ whereas *Staphylococcus epidermidis* (*S. epidermidis*) predominates on the skin surface of healthy individuals. In murine models, *S. aureus* colonization and low diversity of the microbiome drive AD-like lesions in the skin.⁶



GRAPHICAL ABSTRACT

Compared to standard *S. aureus* strains, *S. aureus* isolated from AD skin are able to induce different innate immune responses. *S. aureus* from AD skin are internalized into keratinocytes and accumulate in lysosomes with inducing IL-1α secretion via TLR9. Specific surface proteins of *S. aureus* from AD skin are responsible for their internalization.

The skin surface consists of a horny layer derived from keratinocytes, which recognize *S. aureus* by sensing receptors, such as Toll-like receptors (TLRs). They induce pro-inflammatory cytokines and antibiotic peptides to combat and eliminate *S. aureus*, by activating skin immune cells. *S. aureus*, including its exotoxins and surface proteins, can initiate the production of pro-inflammatory cytokines such as thymic stromal lymphopoietin from keratinocytes, and stimulate mast cell degranulation, resulting in the skewing of T helper 2 cells.⁷⁻⁹ Thus, *S. aureus* colonization can contribute to chronic skin inflammation in AD. However, the role of *S. aureus* strains isolated from AD skin (*S. aureus*-AD) may vary depending upon the strains. It is noteworthy that *S. aureus* strains carrying the gene for Pantone-Valentine leucocidin, but not those without this gene, cause highly lethal pneumonia.¹⁰ Moreover, several studies have shown correlations between the severity of AD and the presence of *S. aureus* that produces super-antigen on AD skin.¹¹ These findings suggest that, not only the abundance of *S. aureus* but also a certain types of *S. aureus* strains could influence AD. In fact, our previous study demonstrated that *S. aureus*-AD activate human Langerhans cells (LCs) and induce Th2-prone T-cell proliferation significantly more than strains of standard *S. aureus* (*S. aureus*-stand).¹²

In this study, we analyzed innate immune responses of keratinocytes in response to various strains of staphylococcus to verify the role of *S. aureus*-AD in the development and/or aggravation of dermatitis observed in AD.

2 | MATERIALS AND METHODS

All experiments were performed in compliance with ethical guidelines for academic research in Hiroshima University.

2.1 | Preparation of *S. aureus* and *S. epidermidis*

Eight strains of *S. aureus* TF3378, TF2962, TF2870, TF2868, NCTC8325, Mu50, FDA209P, and Newman and one strain of *S. epidermidis* ATCC12228 were used in this study. *S. aureus* TF3378, TF2962, TF2870, and TF2868 were clinically isolated from lesional skin surface of different patients with AD, and retained different sequential types as described in Table S1. *S. aureus* NCTC8325, Mu50, FDA209P, and Newman were laboratory control strains and used as control for clinical isolates. NCTC8325 was isolated from a sepsis patient.¹³ Mu50 was isolated from the purulent discharge at the sternal incision site.¹⁴ The origin of FDA209P is unknown, but used as drug-naive standard *S. aureus*.¹⁵ Newman was isolated from a case of secondarily infected tubercular osteomyelitis in man.¹⁶ All strains preserve genes for fibronectin-binding proteins (FnBPs). Newman was shown to harbor mutation in FnBPs.¹⁷

They were grown in Tryptic soy broth (TSB) (Becton Dickinson, San Diego, California) at 37°C for 24 hour with shaking and washed twice by phosphate-buffered saline (PBS). The amounts of

bacterial stimuli were adjusted by Optical Density (OD) at 660 nm to 1.0 followed by calculations of their colony-forming units (CFU). *S. aureus* strains and *S. epidermidis* were heat-killed by incubation at 57°C for 1 hour and treated with proteinase K as described previously.¹²

2.2 | Cell culture, cell stimulation, and inhibitor assay

HaCaT cells (immortalized keratinocytes) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, Carlsbad, California) with 10% regular fetal bovine serum and antibiotics (100 U/mL penicillin, 10 mg/mL streptomycin) at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂.

Clonetics™ human epidermal keratinocytes (NHEKs) were purchased from Lonza (Basel, Switzerland). NHEKs were grown in their recommended growth media (Lonza) at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂.

Cells were seeded in 24-well plates at 4×10^5 cells/well, grown to 80%-90% confluency, and treated with strains of heat-killed *S. aureus* (OD₆₆₀ = 1) and PBS for 24 hour or 74 hour. For the inhibition of lysosome acidification, HaCaT cells were pretreated with 1 $\mu\text{mol L}^{-1}$ bafilomycin A1 (Sigma, St. Louis, Missouri) for 30 minutes before *S. aureus* stimulation. For the inhibition of Toll-like receptor 9 (TLR9), HaCaT cells were pretreated with or without 1 $\mu\text{mol L}^{-1}$ Dual-iODN (Enzo, Farmingdale, New York) for 30 minute before *S. aureus* stimulation. For the inhibition of phagocytosis, cells were pretreated with 50 nmol L⁻¹ wortmannin (Cayman, Ann Arbor, Michigan) for 1 hour before stimulation. For the inhibition of fibronectin, cells were treated with human fibronectin antiserum (Cosmo Bio, Tokyo, Japan) in 500:1 dilution for 1 hour before *S. aureus* stimulation.

Cell viability was assessed by Cell Titer96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, Wisconsin) according to the manufacturer's protocol.

2.3 | Cytokine assay in culture supernatants

IL-1 α , IL-1 β , IL-6, GM-CSF, and TNF- α levels in the culture supernatants of HaCaT cells stimulated with heat-killed *S. aureus* or *S. epidermidis* were analyzed by the BD™ Cytometric Bead Array (Becton Dickinson, San Diego, California) according to the manufacturer's protocol. IL-31 and IL-33 were measured by a DuoSet® ELISA (R&D, Minneapolis, Minnesota) according to the manufacturer's protocol.

2.4 | Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, blocked with 1% goat serum in PBS, and incubated with Fluorescein isothiocyanate isomer-I (FITC)-conjugated anti-*Staphylococcus aureus* antibody (ab68950 Abcam, Oxford, UK) at 1:1000 dilution and Phalloidin-Tetramethylrhodamine B isothiocyanate (Sigma) at 1:1000 dilution at 4°C for overnight. After staining, cells were washed with PBS and mounted with VECTASHIELD with DAPI (Vector, Burlingame, California). For

lysosome staining, HaCaT cells were cultured for 1 hour with Cell Navigator™ Lysosome staining Kit (AAT Bioquest®, Sunnyvale, California) according to the manufacturer's protocol before being fixed by paraformaldehyde. After fixing, cells were washed with PBS and mounted with VECTASHIELD with DAPI. To visualize the lysosomes of HaCaT cells, cells were electroporated with the LAMP1-RFP (addgene Plasmid #1818) by using Gene Pulser (Bio-Rad, Hercules, California) according to the manufacturer's protocol. Images were obtained using a confocal laser scanning microscope (FV10 Olympus, Tokyo, Japan).

2.5 | Flow cytometry

The FITC intensity of green stained lysosomes of HaCaT cells was measured by the Attune® Acoustic Focusing Cytometer (Life Technologies, Carlsbad, California) and analyzed with FlowJo V7.6.5 (Tree Star, Ashland, Oregon).

2.6 | TLRs knockdown assay by small interfering RNA (siRNA)

For transient knockdown of TLR9 and TLR2, three different siRNA duplexes (Stealth™ siRNA system; Sigma) were used. As a control, siRNAs (MISSION siRNA Universal Negative Control #1, Sigma) that do not recognize any human genes were used. The siRNAs were transfected in HaCaT cells with the Lipofectamine™ RNAiMAX Reagent (Thermo Fisher Scientific, Carlsbad, California) according to the manufacturer's protocol. The cells were used for experiments 72 hour after transfection.

2.7 | SDS-PAGE, western ligand affinity assay, and Western blotting

Cell wall-associated proteins were obtained as described previously¹² and separated by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5%-20% acrylamide gradient (Bio-Rad). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by semidry transblot system (Bio-Rad). The membrane was blocked in Blocking one™ (Nakarai tesque, Kyoto, Japan). FnBPs were detected by Western ligand affinity blotting, as described previously¹⁸ by incubation with human fibronectin (Corning, Bedford, Massachusetts) conjugated with horseradish peroxidase (HRP) using a peroxidase labeling KIT (Dojindo, Tokyo, Japan). For Western blotting, membrane was incubated with anti-fibronectin-binding protein A antibody (Antagene, Santa Clara, California) and anti-extra cellular adherence protein antibody (ab95982, Abcam). Molecular weight was estimated by protein ladder (GE Healthcare, Chalfont, UK). Detection was performed by ECL Prime Western Blotting Detection Reagent (GE Healthcare).

2.8 | Statistical analysis

Statistical analysis was performed with JMP® Pro 12.2.0 (SAS Institute Inc., Cary, North Carolina). Statistic comparison of two groups

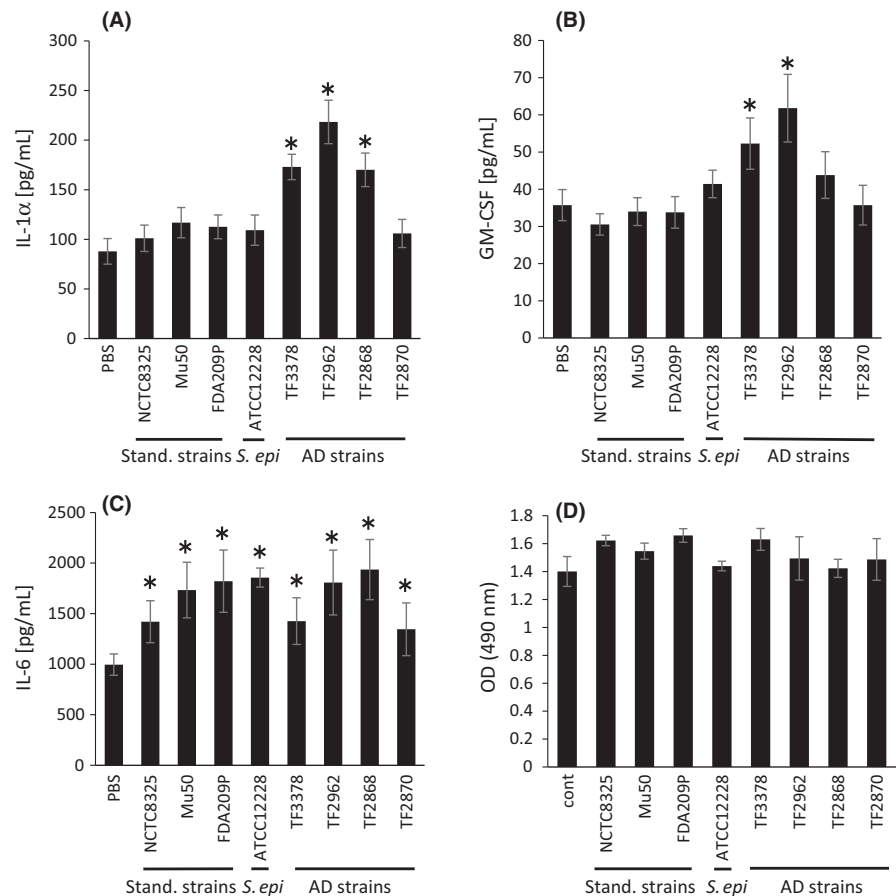


FIGURE 1 *Staphylococcus aureus* strains isolated from AD skin induce IL-1 α production. (A, B, C) HaCaT cells were stimulated with strains of *S. aureus* and *S. epidermidis* for 72 h. Cytokine production in the culture supernatants was analyzed by a Cytometric Bead Array kit. Significance was indicated compared to PBS. (* $P < 0.05$, mean $\pm$ SEM, $n = 4$). (D) HaCaT cells were stimulated with *S. aureus* for 72 h and viability was assessed by MTS assay

was performed using Mann-Whitney U test. P values less than 0.05 were considered significant.

3 | RESULTS

3.1 | Heat-killed *S. aureus* strains isolated from AD skin induce high level of IL-1 α secretion compared to standard strains of *S. aureus*

To investigate the innate immune response of keratinocytes against *S. aureus*-AD, HaCaT cells were incubated with various heat-killed staphylococci: four strains of *S. aureus*-stand. (NCTC8325, Mu50, FDA209P, and Newman), four strains of *S. aureus*-AD (TF3378, TF2962, TF2868, and TF2870), and a strain of *S. epidermidis* (ATCC12228) (Table S1).

We then quantified inflammatory and itch-inducing cytokines in the culture supernatants 72 hour after stimulation with *S. aureus* or *S. epidermidis*. Three out of four *S. aureus*-AD strains (TF3378, TF2962, and TF2868) induced a high amount of IL-1 α secretion compared to those by *S. aureus*-stand. or *S. epidermidis* (Figure 1A, S1A). Two *S. aureus*-AD strains (TF3378 and TF2962) also induced high amounts of GM-CSF (Figure 1B), but no difference was seen in the production of IL-6 (Figure 1C, S1B). The amounts of IL-1 β , TNF- α , IL-31, and IL-33 were under detection limit (data not shown). IL-1 α accumulated in a time-dependent manner (Figure S2), and low dose of *S. aureus*-AD strain (TF3378) could not induce cytokine secretion (Figure S3A).

We assessed the proliferation of HaCaT cells using MTS assay, but no difference was observed among all *S. aureus* strains and *S. epidermidis* (Figure 1D). These results revealed that heat-killed *S. aureus*-AD (TF3378, TF2962, and TF2868) induced significantly high amounts of IL-1 α secretion from HaCaT cells without cytotoxicity.

3.2 | Heat-killed *S. aureus* isolated from patients with AD was internalized into keratinocytes and accumulates in lysosome

Confocal microscopic analysis of HaCaT cells and normal human epidermal keratinocytes (NHEKs) incubated with heat-killed *S. aureus* strains and anti-*S. aureus* antibodies revealed that three strains of *S. aureus*-AD (TF3378, TF2962, and TF2868), which induced IL-1 α production, were internalized to the cytoplasm of HaCaT cells 24 hours after stimulation (Figure 2, Figure S1C, S4), and similar phenomenon was also observed in NHEKs (Figure S5). To further explore intracellular localization of *S. aureus*-AD, cells were stained using a Cell Navigator Lysosome staining Kit, which labels lysosomes green in color by the pH gradient. Microscopic analysis showed that lysosomes in HaCaT cells and NHEKs were increased and *S. aureus*-AD was colocalized with lysosome-green stained vacuole, suggesting that *S. aureus*-AD localized in lysosome (Figure 3A, S1D, S6, and S7). FACS analysis also showed a substantial amount of lysosome green population in HaCaT cells incubated with the three strains (Figure 3B, 3C S1E).

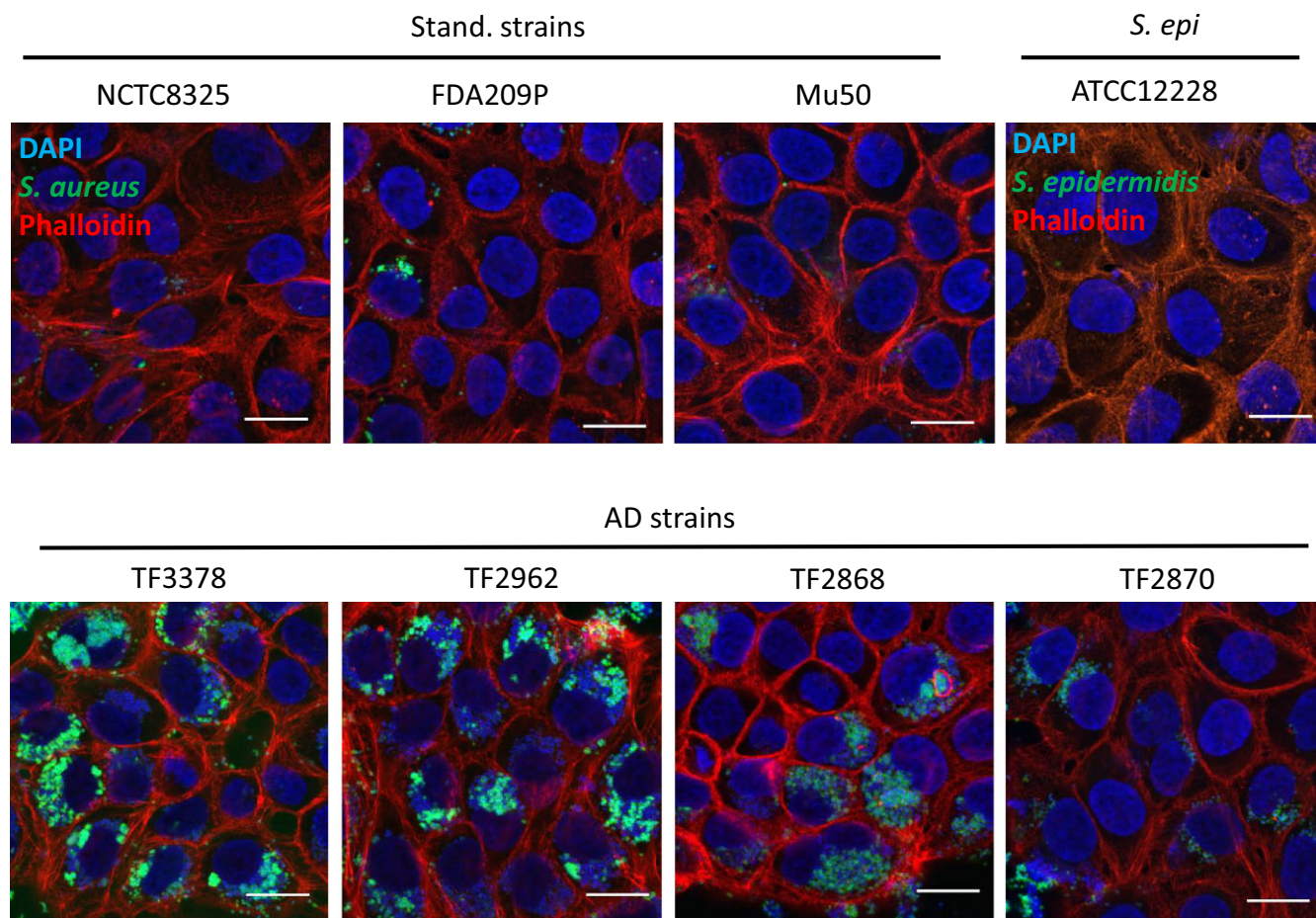


FIGURE 2 *Staphylococcus aureus* strains isolated from AD skin are internalized into keratinocytes. Immunofluorescence staining of *S. aureus* or *S. epidermidis* (Green), DAPI (Blue), and phalloidin (Red) was performed 24 h after stimulation with *S. aureus* or *S. epidermidis*. Scale bar, 20 μ m. ($\times 120$) All images were acquired by using confocal microscopy

Low dose of *S. aureus*-AD (OD = 0.1, 0.01) was less internalized into the HaCaT cells (Figure S3B,C,D). To confirm the intracellular localization of *S. aureus*-AD, we employed HaCaT cells expressing RFP-tagged lysosomal-associated membrane protein 1 (LAMP1), which is distributed on late endosomes and lysosomes.¹⁹ RFP-tagged LAMP1 was co-localized with *S. aureus*-AD (TF3378) and vacuoles stained with lysosome stain green (Figure 3D). Treatment of HaCaT cells with bafilomycin A1, a specific and potent inhibitor of vacuolar proton ATPase, abolished the intracellular localization of *S. aureus*-AD (TF3378) in low pH compartment (Figure 3E). The other *S. aureus*-AD strains, TF2962 and TF2868, were also accumulated inside the cytoplasm of HaCaT cells. (Figures 3A).

3.3 | *S. aureus*-AD induce IL-1 α production via TLR9 pathway

To investigate the mechanism of IL-1 α induction by *S. aureus*-AD, we studied the involvement of TLR9, which is expressed in endosomes and lysosomes, and recognizes CpG DNA of bacteria including *S. aureus*. The increase of IL-1 α secretion in the culture supernatant of HaCaT cells with strains of *S. aureus*-AD for 72 hour was decreased by the pretreatment with iODNs, a TLR9 antagonist²⁰ (Figure 4A). In

contrast, the release of IL-6 induced by all strains of *S. aureus* strains and *S. epidermidis* was not affected by the treatment of iODNs (Figure 4B). In line with the inhibition by iODNs, the induction of IL-1 α was canceled in TLR9 knockdown HaCaT cells (Figure 4C).

TLR2 is known to be the responsible receptor against *S. aureus*, and we tested whether TLR2 is involved with these responses. TLR2 knockdown did not affect the phenomenon induced by *S. aureus*-AD (TF3378) (Figure S8).

3.4 | Phagocytosis and internalization of *S. aureus*-AD by HaCaT cells depend on surface Protein of *S. aureus*-AD

To investigate the role of cell surface structures of heat-killed *S. aureus*-AD, we treated *S. aureus* TF3378 with proteinase K, which removes cell surface proteins, followed by washing and then exposure to HaCaT cells. *S. aureus* TF3378 treated with proteinase K significantly impaired the induction of IL-1 α (Figure 5A). On the other hand, no effect was observed on IL-6 production (Figure 5B). Immunohistochemical analysis showed that *S. aureus* TF3378 treated with proteinase K neither entered the cytoplasm nor accumulated in lysosomes (Figures 5C and 5D). Pretreatment of HaCaT cells with

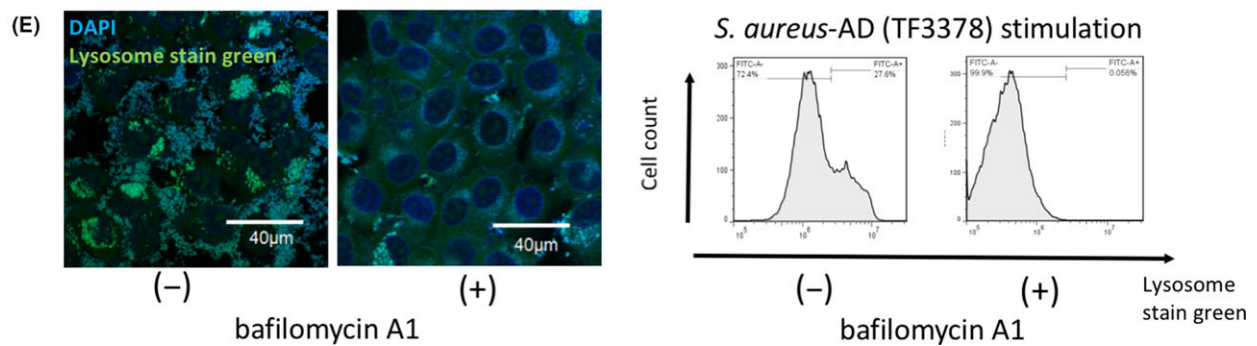
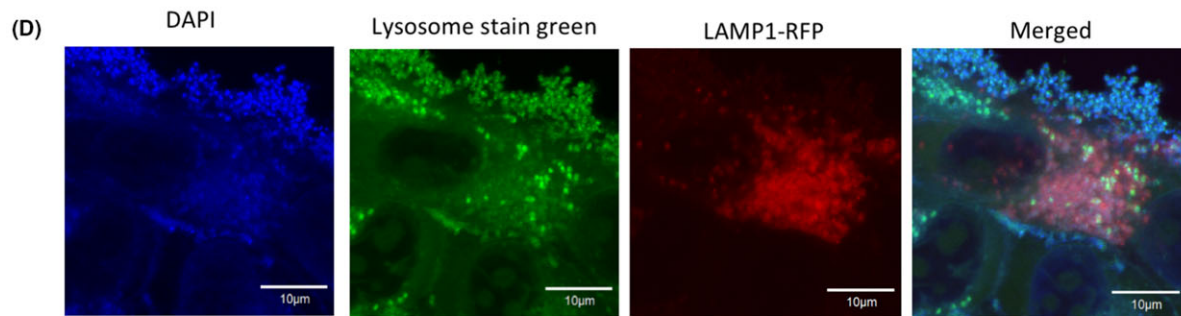
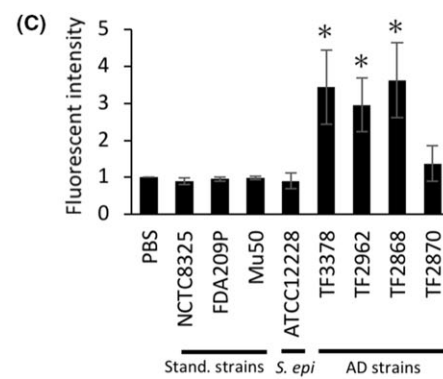
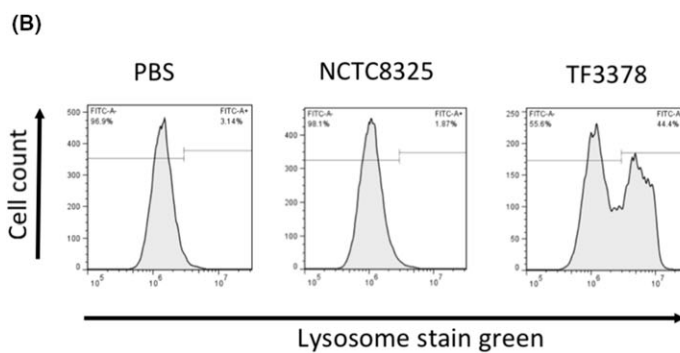
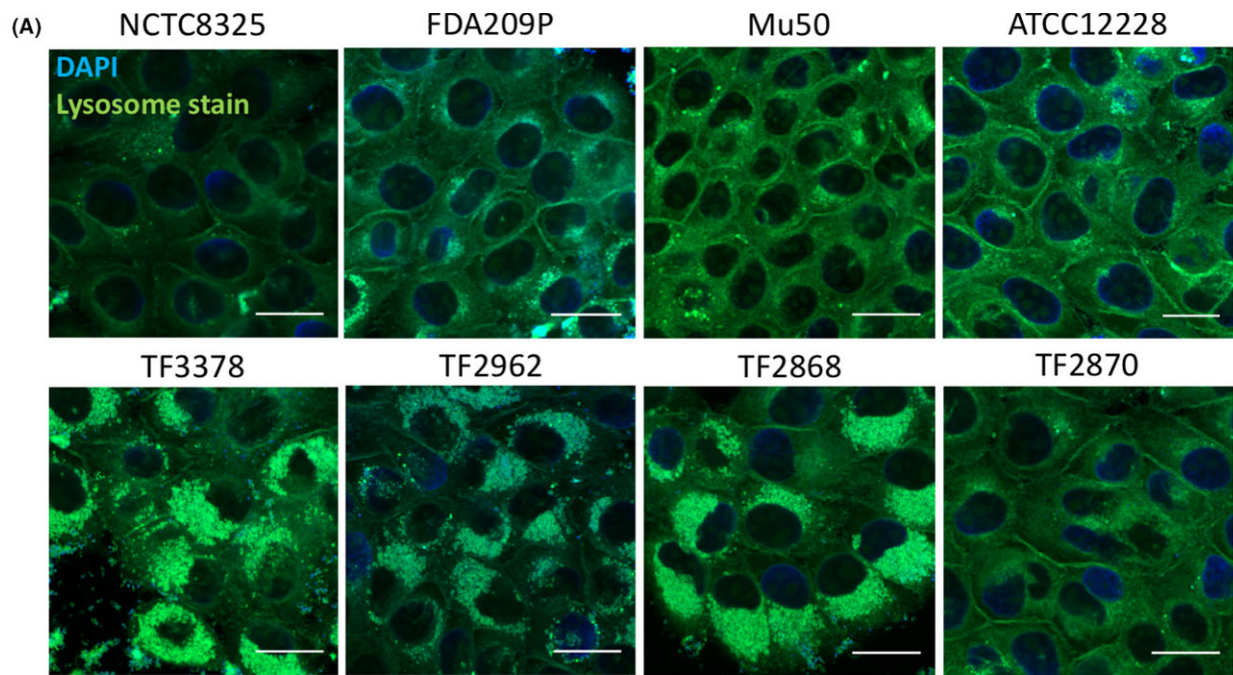


FIGURE 3 *Staphylococcus aureus* strains isolated from AD skin accumulate in lysosome of keratinocyte. (A) HaCaT cells were stimulated with strains of *S. aureus* or *S. epidermidis* for 24 h. Cell Navigator Lysosome staining Kit was added 1 h before fixing with paraformaldehyde (PFA). Scale bar, 20 μm . ($\times 120$) (B, C) Fluorescent Intensity (FI) of green stained lysosomes in HaCaT cells was analyzed by FACS. Representative histograms were indicated. (* $P < 0.05$, mean $\pm$ SEM, $n = 5$) (D) HaCaT cells expressing LAMP1-RFP were treated with *S. aureus* TF3378 for 24 h. Cell Navigator Lysosome staining Kit was added 1 h before fixing with PFA. Scale bar, 10 μm . ($\times 240$) (E) HaCaT cells were treated with or without bafilomycin A1 ($1 \mu\text{mol L}^{-1}$) 30 min before stimulation with *S. aureus* TF3378. Cell Navigator Lysosome staining Kit was added 1 h before fixing with PFA. Scale bar, 40 μm . ($\times 60$) FI of green stained lysosome in HaCaT cells was analyzed by FACS and representative histograms were indicated. All images were acquired by using confocal microscopy

wortmannin, a PI3K inhibitor to block phagocytosis,²¹ also abolished the internalization and lysosome accumulation of *S. aureus*-AD (Figure 5E-H).

3.5 | *S. aureus*-AD express high amount of fibronectin-binding proteins, involved in internalization

Western ligand affinity blotting and Western blotting of bacterial cell walls revealed that the levels of fibronectin-binding proteins (FnBPs) on the cell wall of *S. aureus* were higher in three strains of *S. aureus*-AD (TF3378, TF2962, and TF2868) than those of the other strains of bacteria and that *S. aureus*-AD TF3378 lost fibronectin-binding activity following proteinase K treatment (Figure 6A,B). No difference among strains was seen in extracellular adherent protein (Eap),

which is also known to be involved in the phagocytosis of *S. aureus* by keratinocytes (data not shown).

To confirm the necessity of FnBP for the *S. aureus*-AD internalization, we blocked fibronectin by using an antibody against fibronectin.²² The uptake of *S. aureus*-AD by HaCaT cells was partially inhibited by pre-treatment with a fibronectin antiserum. (Figure 6C-F)

4 | DISCUSSION

In this study, we demonstrated that *S. aureus* isolated from patients with AD (*S. aureus*-AD), but not standard *S. aureus* strains (*S. aureus*-stand.) and *S. epidermidis*, enter HaCaT cells, a human keratinocyte cell line, and NHEKs by phagocytosis. They then accumulate in the

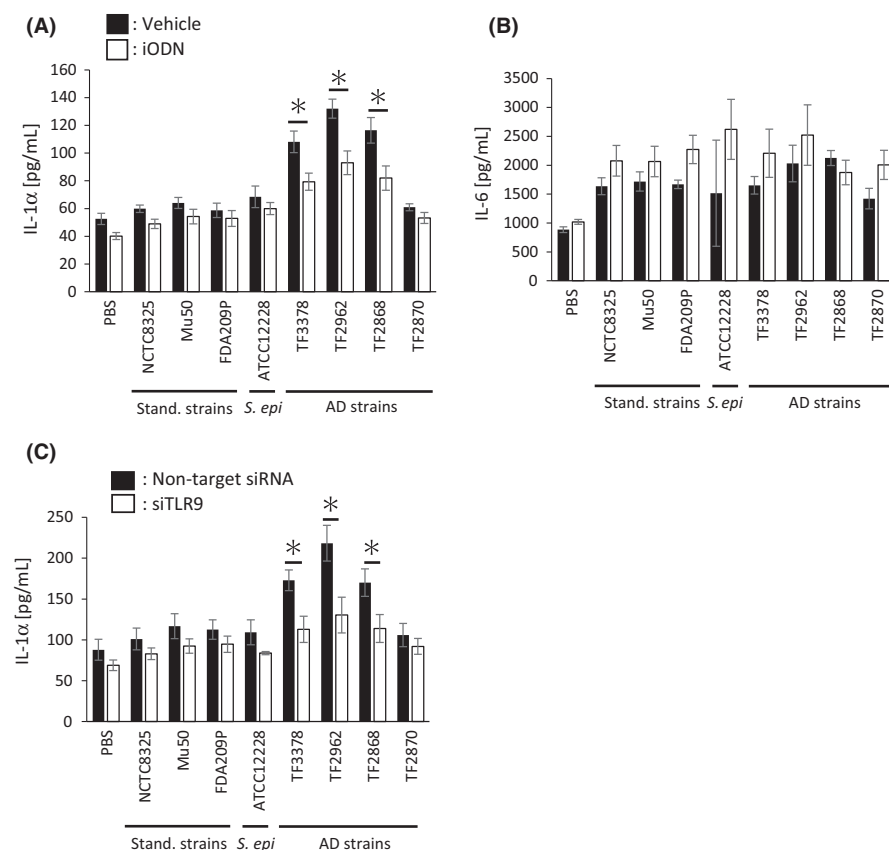


FIGURE 4 TLR9 is required for IL-1 α production by HaCaT cells in response to *S. aureus*-AD. HaCaT cells were incubated with strains of *S. aureus* and *S. epidermidis* for 72 h; amounts of IL-1 α in the culture supernatants were analyzed by a Cytometric Bead Array kit. (A, B) HaCaT cells were incubated with or without $1 \mu\text{mol L}^{-1}$ TLR9 inhibitor (iODNs) for 30 min before the stimulation with *S. aureus* and *S. epidermidis*. (* $P < 0.05$, mean $\pm$ SEM, $n = 4$) (C) HaCaT cells were lipofected with TLR9 siRNA and non-target siRNA 72 h before the stimulation with *S. aureus* strains and *S. epidermidis*. (* $P < 0.05$, mean $\pm$ SEM, $n = 4$)

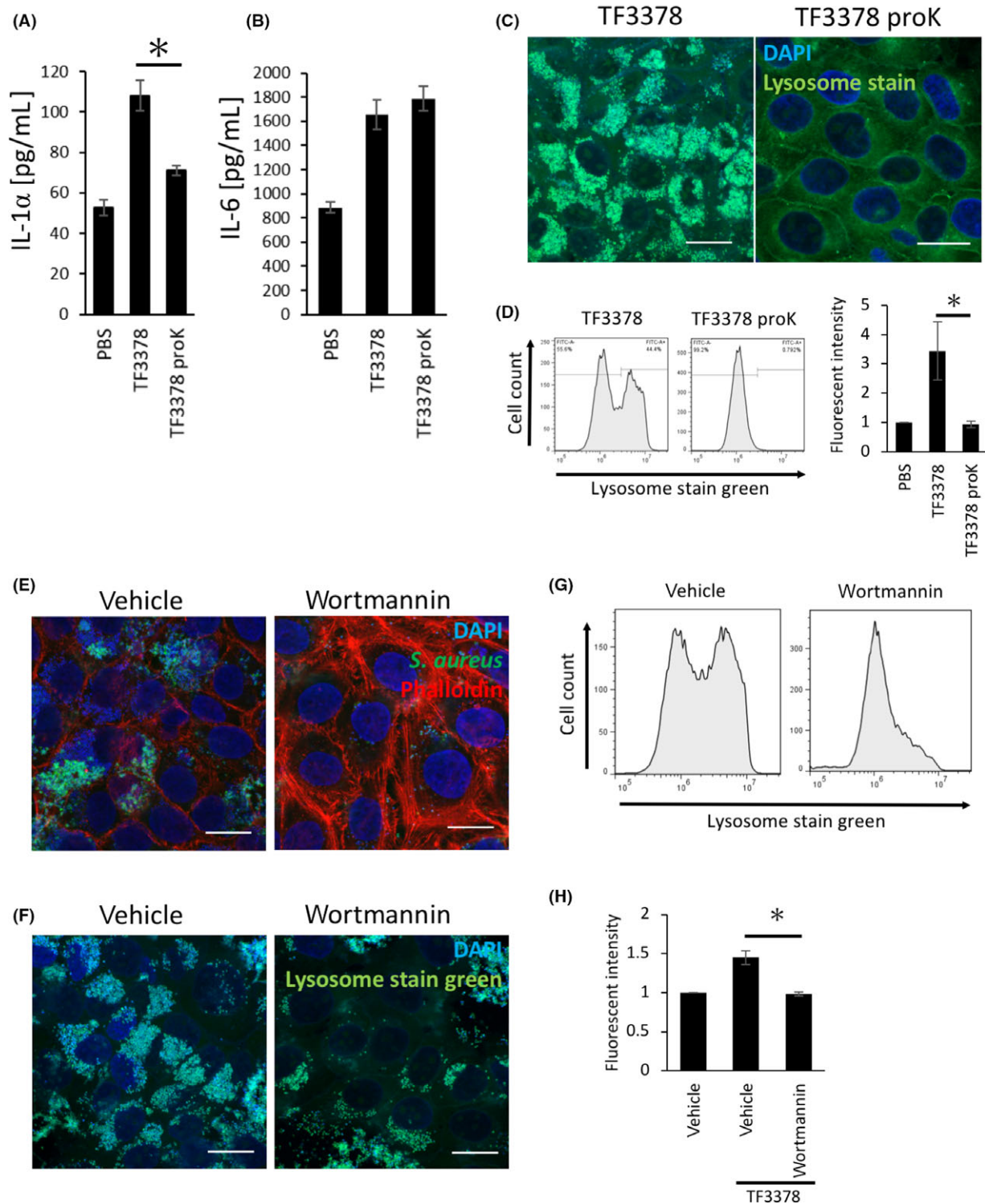


FIGURE 5 Cell wall proteins of *S. aureus*-AD retain definitive structure inducing phagocytosis in HaCaT cells. HaCaT cells were incubated with *S. aureus* TF3378 or Proteinase K-treated *S. aureus* TF3378 to remove their cell wall proteins for 24 h. (A, B) Cytokines in the supernatant were analyzed by a Cytometric Bead Array kit. (* $P < 0.05$, mean $\pm$ SEM, $n = 4$) (C) HaCaT cells were incubated for 24 h with *S. aureus* TF3378 that had been treated with or without Proteinase K. Cell Navigator Lysosome staining Kit was added 1 h before fix. Images were acquired using confocal microscopy. Scale bar, 20 μ m. (D, E) FI of green stained lysosome in HaCaT cells was analyzed by FACS and representative histograms were indicated. (* $P < 0.05$, mean $\pm$ SEM, $n = 5$) (F) HaCaT cells were treated with wortmannin (50 nmol L $^{-1}$), PI3K inhibitor, or DMSO (negative control) for 1 h before the stimulation with *S. aureus* TF3378. Immunofluorescence staining for *S. aureus* (Green), DAPI (Blue), and phalloidin (Red) 24 h after stimulation with *S. aureus* TF3378. Scale bar, 20 μ m. (F) Cell Navigator Lysosome staining Kit was added 1 h before fixing. Images were acquired using confocal microscopy. (G, H) FACS analysis of HaCaT cells after lysosome stain. Cells with green stained lysosome were analyzed. (* $P < 0.05$, mean $\pm$ SEM, $n = 4$)

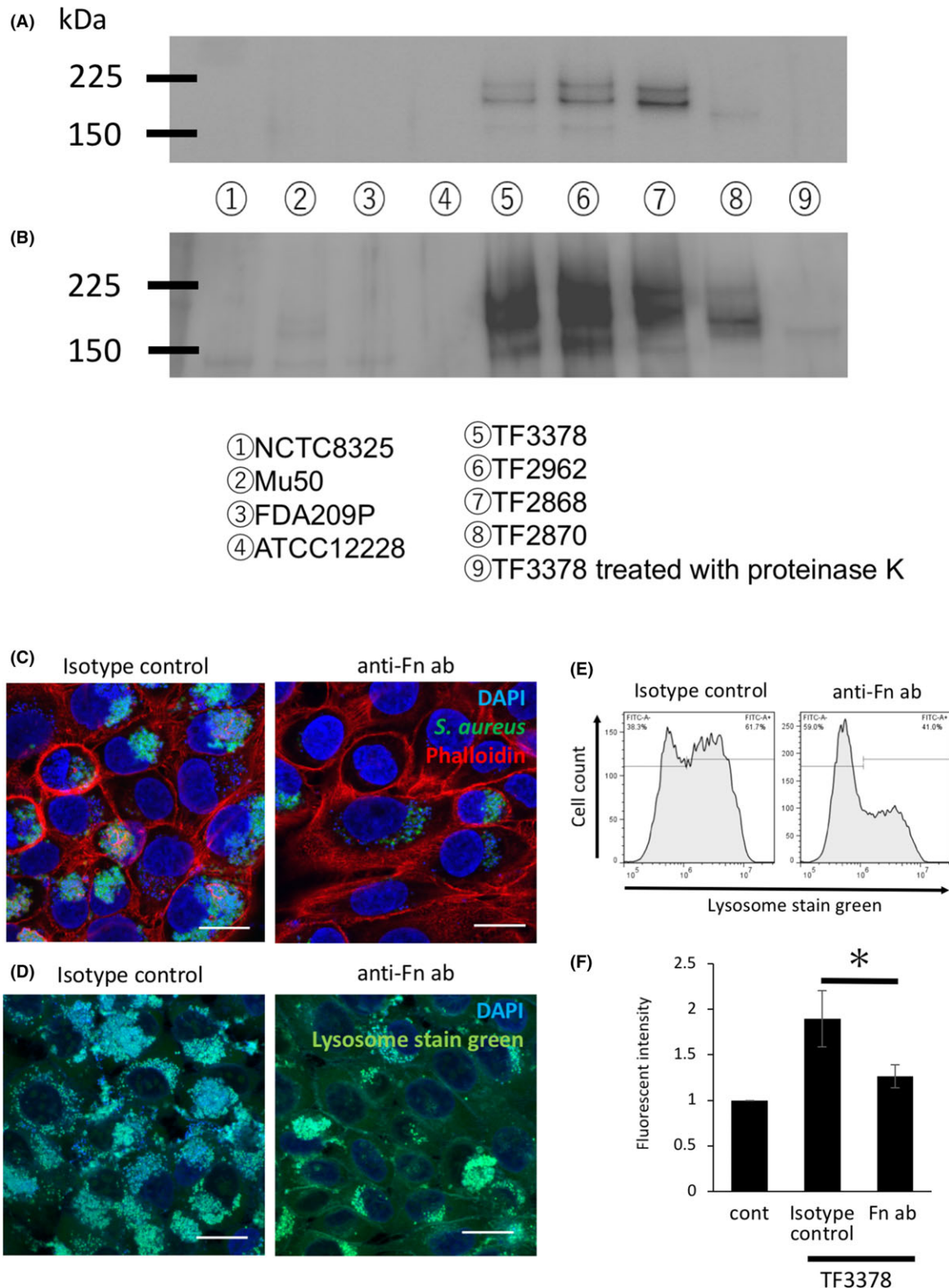


FIGURE 6 *Staphylococcus aureus*-AD express high amount of fibronectin-binding proteins. *S. aureus*-AD (TF3378) was internalized by fibronectin-binding proteins interaction. (A) Expression of fibronectin-binding proteins in *S. aureus* strains, *S. epidermidis*, and Pro K-treated *S. aureus*-AD was examined by a Western ligand affinity assay and (B) a Western blotting with anti-fibronectin-binding protein antibody. (C, D) HaCaT cells were treated with human fibronectin antiserum and rabbit IgG (for negative control) for 1 h before TF3378 stimulation. Immunofluorescence staining for *S. aureus* (Green), DAPI (Blue), and phalloidin (Red) was observed 24 h after TF3378 stimulation. Scale bar 20 μ m ($\times 120$) Cell Navigator Lysosome staining Kit was added 1 h before fixing. Images were acquired using confocal microscopy. (E, F) FACS analysis of HaCaT cells after Lysosome stain. Cells with green stained lysosome were analyzed. Significance was indicated compared to isotype control. (* $P < 0.05$, mean $\pm$ SEM, $n = 4$)

lysosome, resulting in the production of IL-1 α by stimulating intracellular TLR9.

The keratinocyte layer is the first barrier in the skin against *S. aureus* and recognizes *S. aureus* by sensing receptors such as TLRs. They produce pro-inflammatory cytokines and antibiotic peptides to eliminate *S. aureus* by activating immune cells in the skin.²³ IL- α , an IL-1 family member, activates the MyD88-dependent pathway resulting in various inflammatory responses.²⁴ Keratinocytes store IL-1 α in their cytoplasm under resting conditions and release matured IL-1 α in response to stimuli by pathogens including *S. aureus*.²⁵ The production of IL-1 α is tightly controlled in healthy subjects²⁶ but increased in the skin of patients with AD who have the *FLG* gene mutation as compared to that in the skin of healthy individuals.²⁷ Moreover, the overexpression of IL-1 α in keratinocytes results in the development of AD-like skin inflammation in mice,²⁸ suggesting a critical role of IL-1 α in the development of AD. Our data suggest that the presence of particular AD-related strains of *S. aureus* with distinct cell wall proteins is important for the development and/or aggravation of AD by inducing IL-1 α and possibly other pro-inflammatory cytokines.

Among the pattern recognizing receptors, TLR2 expressed on cell surface is known to be responsible for the host defense against *S. aureus*.²⁹ Moreover, polymorphisms in TLR2 are reported to be associated with the severity of AD³⁰ and function of TLR2 is down-regulated in AD skin.³¹ Furthermore, lipoproteins of *S. aureus* are known to induce IL-6 production in keratinocytes via the TLR1/TLR2 pathway.³² On the other hand, TLR9 is typically located within endosomes and lysosomes, and recognizes CpG DNA of bacteria.³³ Keratinocytes also express TLR9, mostly in the upper layers of the stratum corneum of human skin, and therefore may recognize CpG oligonucleotide (TLR9 ligand).³⁴ It has been reported that certain strains of *S. aureus* are internalized by macrophages and then accumulate in lysosomes.³⁵ Dendritic cells may also incorporate specific strains of *S. aureus* and induce cytokines via TLR9.³⁶ Moreover, *S. aureus* may also be internalized by various non-phagocytic human cells including keratinocytes, as well.³⁷ Furthermore, polymorphisms in the TLR9 promoter with significantly higher activity are associated with the development of AD.³⁸ Thus, it is feasible that certain strains of *S. aureus* are selectively associated with, and internalized by keratinocytes of patients with AD. The mechanism of specific internalization by *S. aureus*-AD remains a matter of discussion. The abolishment of *S. aureus* internalization and IL-1 α secretion by *S. aureus*-AD strains by the treatment with protease K and wortmannin suggests the involvement of bacterial surface proteins specific to *S. aureus*-AD, rather than exotoxins, and the PI3K pathway of keratinocytes in their internalization and IL-1 α induction.

Among the surface proteins of *S. aureus*, FnBPs and Eap are known to induce phagocytosis and internalization of *S. aureus* by human cells including keratinocytes.^{39,40} FnBPs were shown to be involved in the adhesion of *S. aureus* to AD skin,⁴¹ interacting with human $\alpha 5 \beta 1$ integrins via fibronectin.²² Furthermore, the expression level of FnBPs by *S. aureus* is known to vary among strains.⁴² In this study, we have demonstrated that the expressions of FnBPs in three

S. aureus-AD strains were larger than those in the other strain of *S. aureus*-AD and the strains of *S. aureus*-stand., which are not internalized by HaCaT cells. Moreover, the internalization of *S. aureus*-AD was partially blocked by inhibiting fibronectin. These results suggest that the expression of high amounts of FnBPs by *S. aureus*-AD is critical for internalization and induction of IL-1 α by HaCaT cells. However, the inhibition of fibronectin did not completely prevent internalization, suggesting the involvement of other proteins of *S. aureus*, as a counterpart to FnBPs that is different from fibronectin expressed on HaCaT cells, and/or specific structures of AD skin in this process.

Several trials, such as bleach baths, to improve AD by combatting *S. aureus* on AD skin have been reported.^{43,44} However, the colonization of *S. aureus* in patients with AD typically relapses a few weeks after such treatment.⁴⁵ Surface structures, which are specific to *S. aureus* strains isolated from patients with AD, may represent a new targeted approach to remove pathogenic *S. aureus*-AD with minimum disturbance of the physiological microbiome but with long-lasting efficiency.

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CONFLICT OF INTEREST

The authors have no financial conflict of interest.

AUTHOR CONTRIBUTIONS

MM, KI, and MH designed the experiments of keratinocytes and wrote the manuscript. MM and AM performed the experiments of keratinocytes. MM and YY analyzed the results. YN, JH, and MS designed and prepared the experiments of *S. aureus* and *S. epidermidis*. All authors contributed to interpretation of the research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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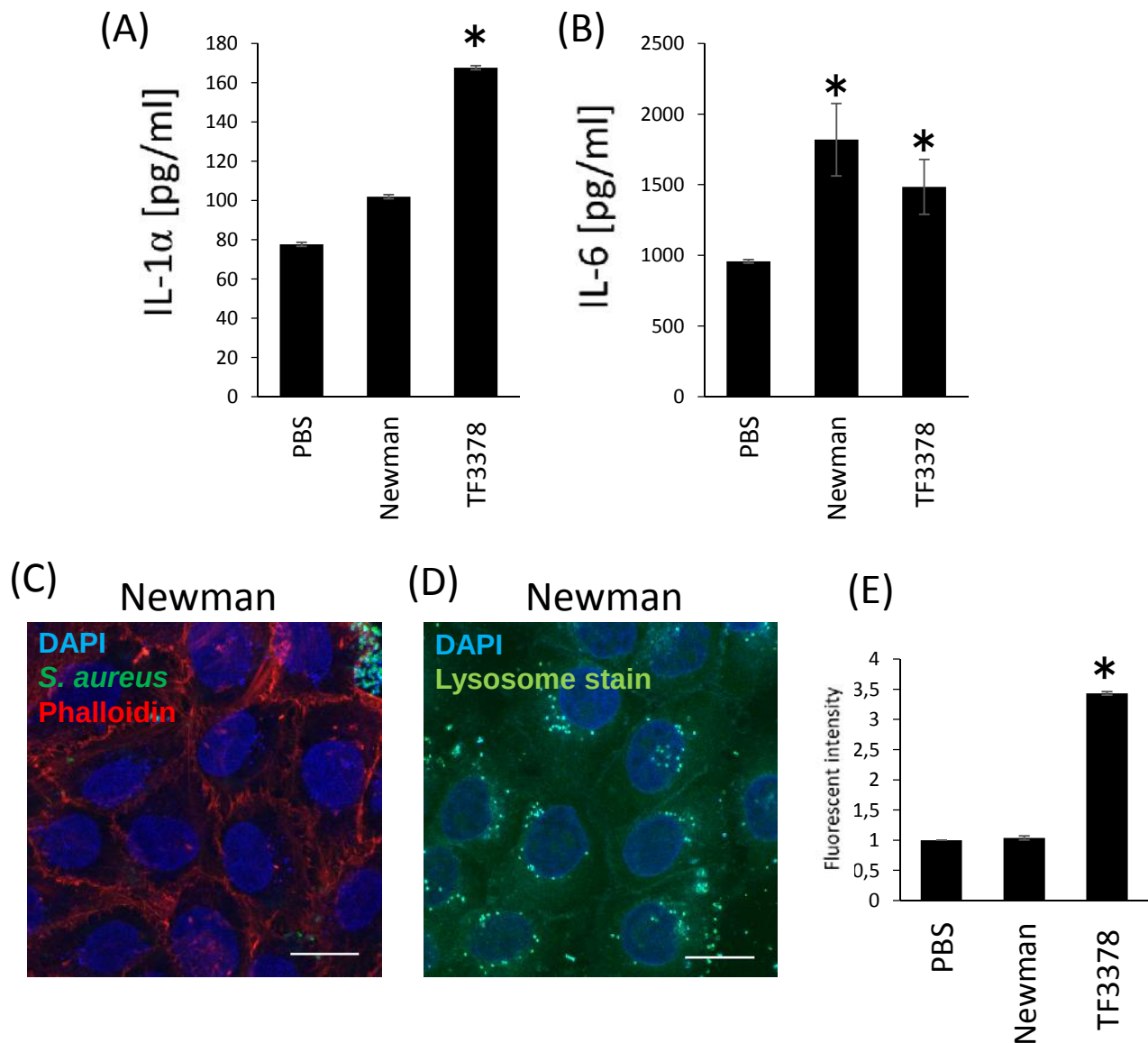


Figure S1: *S. aureus* Newman does not induce IL-1α secretion from HaCaT cells. *S. aureus* Newman is less internalized into HaCaT cells compared to *S. aureus*-AD (TF3378).

(A) (B) HaCaT cells were stimulated with *S. aureus* Newman and *S. aureus*-AD (TF3378) for 72 h, followed by the analysis of cytokine production in culture supernatants. Significance is calculated by Dunn's test. (* $p < 0.05$, mean $\pm$ SEM, $n = 3$) (C) Immunofluorescence staining of *S. aureus* (Green), DAPI (Blue) and phalloidin (Red) was performed 24 h after stimulation with *S. aureus*. Scale bar, 20 μm. ($\times 120$) All images were acquired by using confocal microscopy. (D) HaCaT cells were stimulated with strains of *S. aureus* for 24 h. Cell Navigator™ Lysosome staining Kit was added 1 h before fixing with paraformaldehyde. Scale bar, 20 μm. (E) Fluorescent Intensity of green stained lysosomes in HaCaT cells was analyzed by FACS. (* $p < 0.05$, mean $\pm$ SEM, $n = 4$)

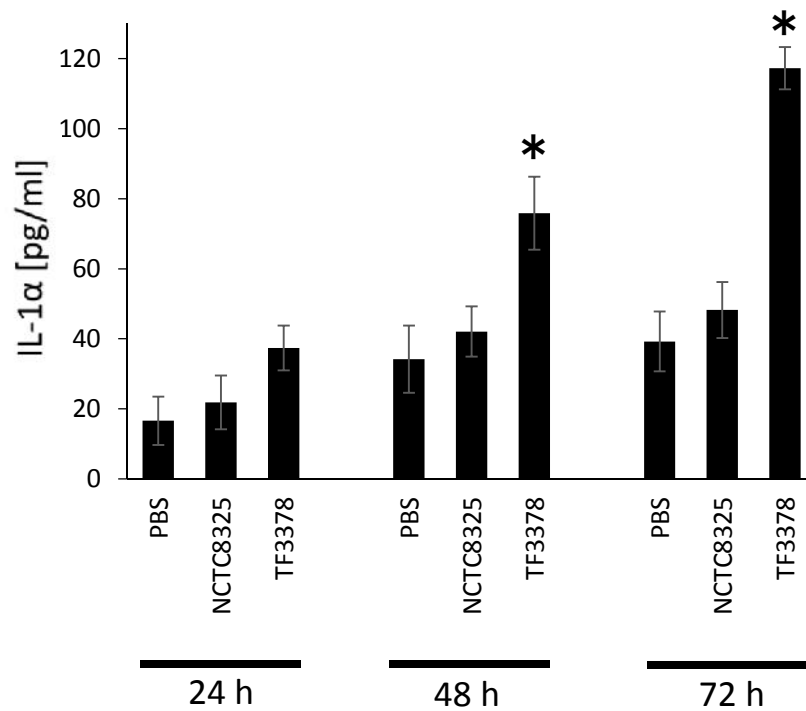


Figure S2: *S. aureus*-AD (TF3378) induces IL-1 α from HaCaT cells in a time dependent manner. HaCaT cells were stimulated with *S. aureus*-stand. and *S. aureus*-AD for 24, 48 and 72 h, followed by the analysis of cytokine production in culture supernatants. Significance was indicated compared to PBS. (* $p < 0.05$, mean $\pm$ SEM, n = 4)

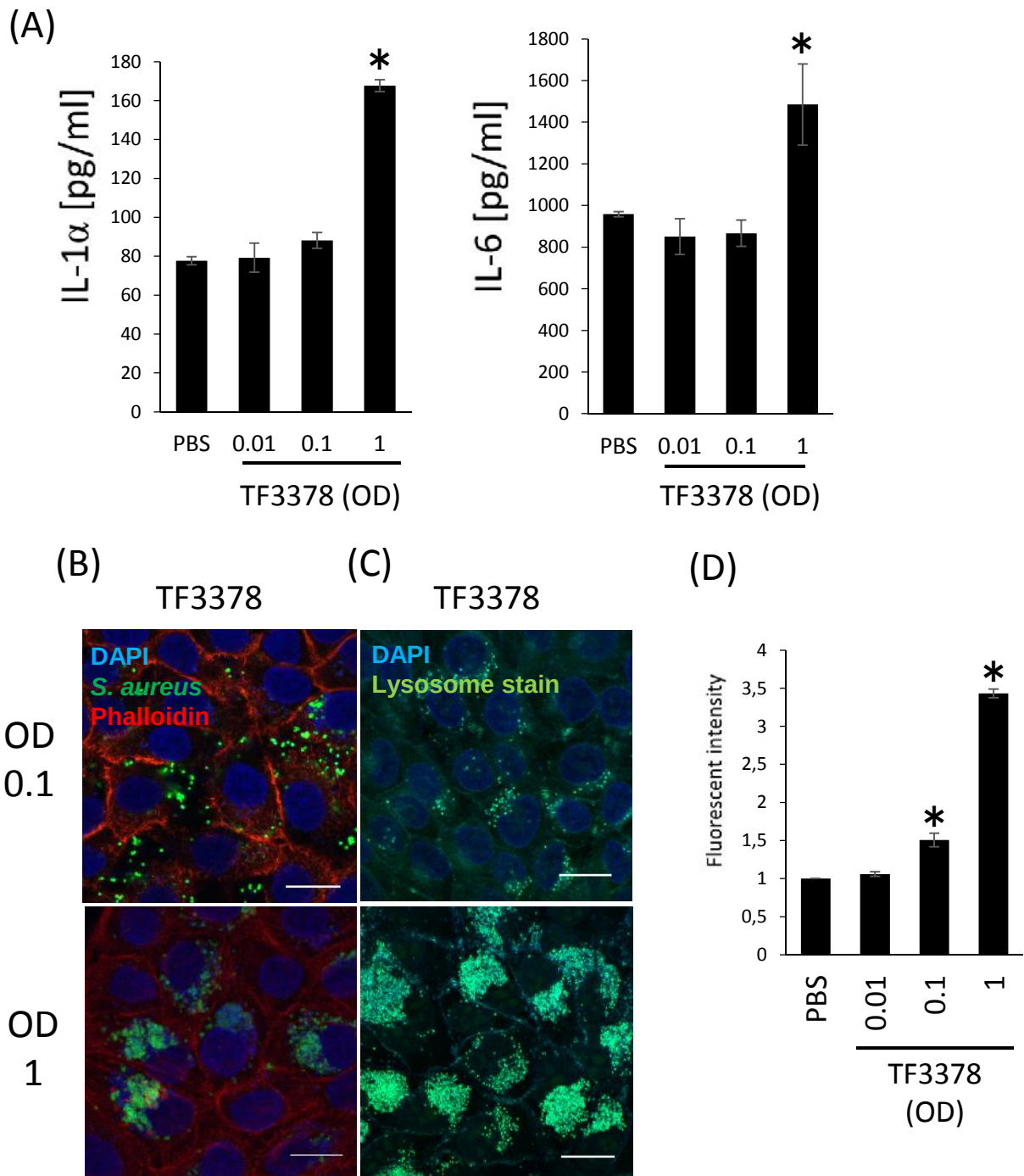


Figure S3: Low dose of *S. aureus*-AD does not induce cytokine secretion from HaCaT cells and internalization was depend on *S. aureus* concentration.

(A) HaCaT cells were stimulated with various dose (OD=1, 0.1, 0.01) of *S. aureus*-AD (TF3378) for 72 h, followed by the analysis of cytokine production in culture supernatants. Significance was indicated compared to PBS. (* $p < 0.05$, mean $\pm$ SEM, $n = 3$) (B) Immunofluorescence staining of *S. aureus* (Green), DAPI (Blue) and phalloidin (Red) was performed 24 h after stimulation with *S. aureus*-AD (TF3378) (OD= 0.1). Scale bar, 20 μ m. All images were acquired by using confocal microscopy. © HaCaT cells were stimulated with strains of *S. aureus*-AD (TF3378) (OD= 0.1) for 24 h. Cell Navigator™ Lysosome staining Kit was added 1 h before fixing with paraformaldehyde. Scale bar, 20 μ m. (D) Fluorescent Intensity of green stained lysosomes in HaCaT cells was analyzed by FACS. (* $p < 0.05$, mean $\pm$ SEM, $n = 4$)

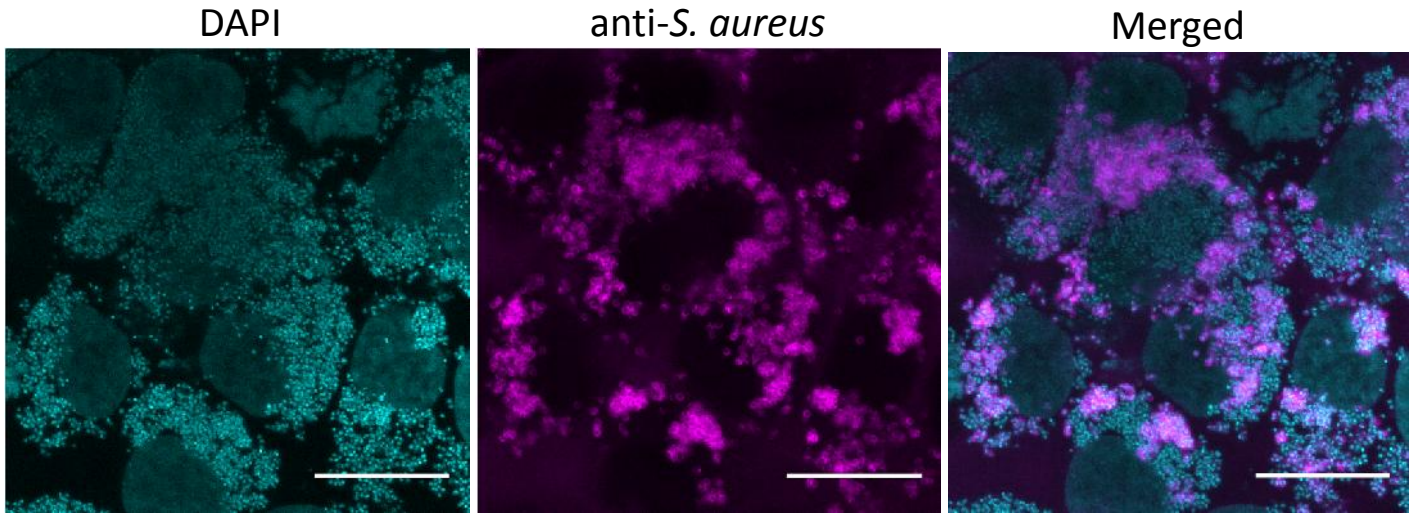
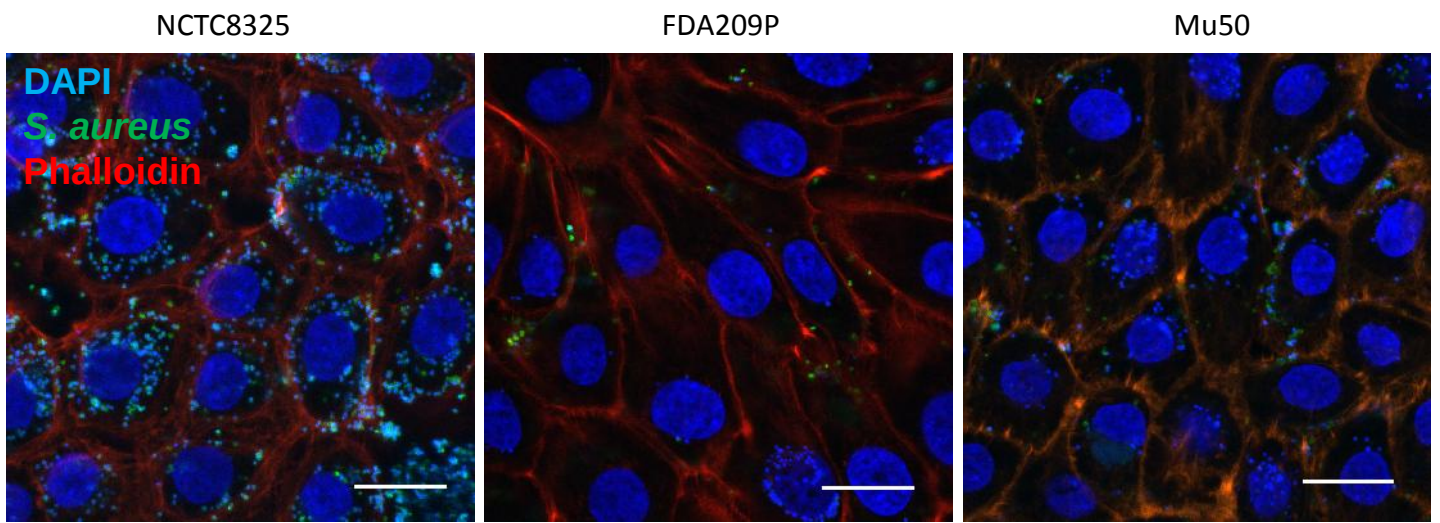


Figure S4: Colocalization of *S. aureus*-AD with cytoplasm of HaCaT cells.

HaCaT cells treated with *S. aureus*-AD (TF3378) were stained by DAPI and antibody against *S. aureus*. Images of DAPI and anti-*S. aureus* antibody are shown respectively (left and middle panel) and together as merged images (right panel). Scale bar, 20 μm . ($\times 240$) All images were acquired by using confocal microscopy.

Stand. strains



AD strains

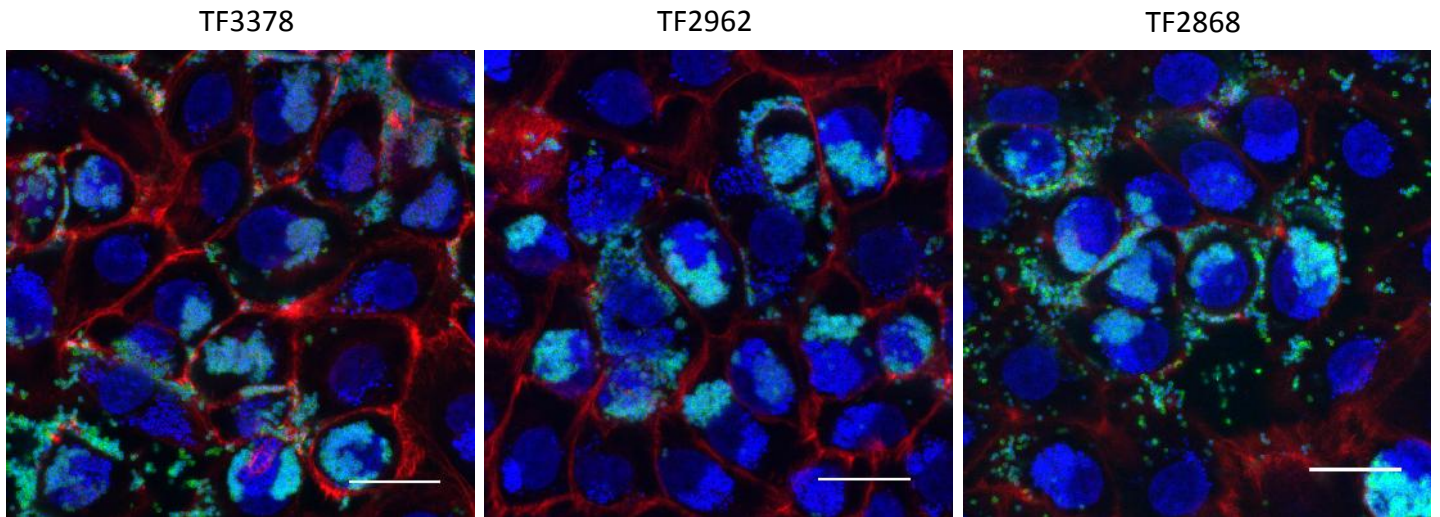


Figure S5: *S. aureus* strains isolated from AD skin are internalized into keratinocytes.

Human epidermal keratinocytes were stimulated with strains of *S. aureus* for 24 h.

Immunofluorescence staining of *S. aureus* (Green), DAPI (Blue) and phalloidin (Red) was performed 24 h after stimulation with *S. aureus*. Scale bar, 20 μm ($\times 120$). All images were acquired by using confocal microscopy.

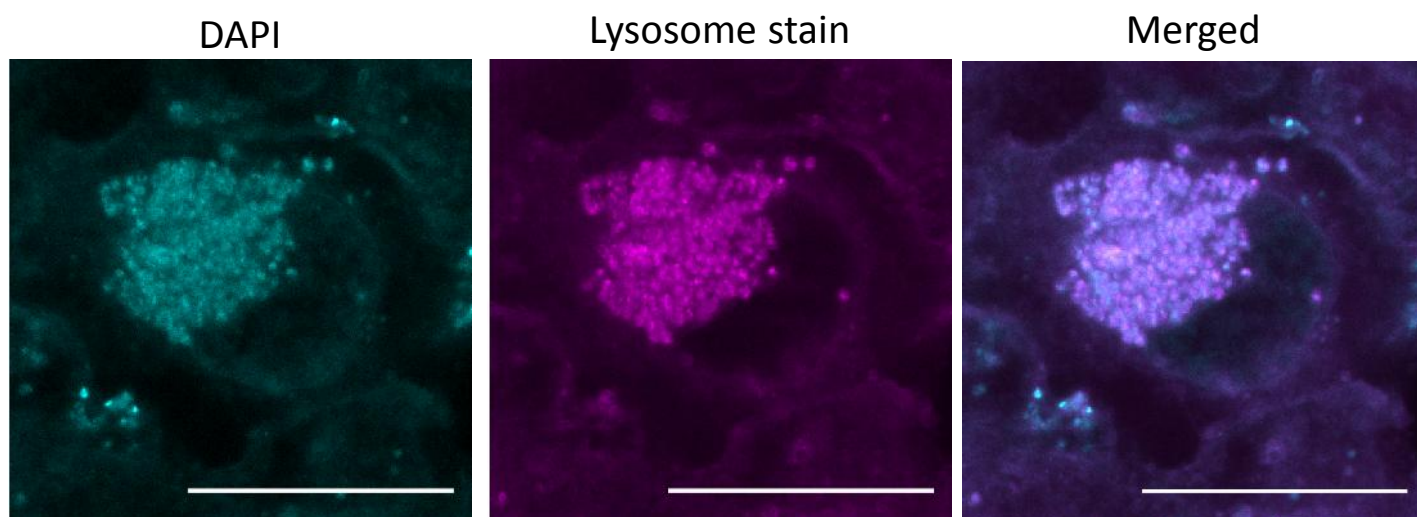


Figure S6: Colocalization of *S. aureus*-AD with lysosomes of HaCaT cells.

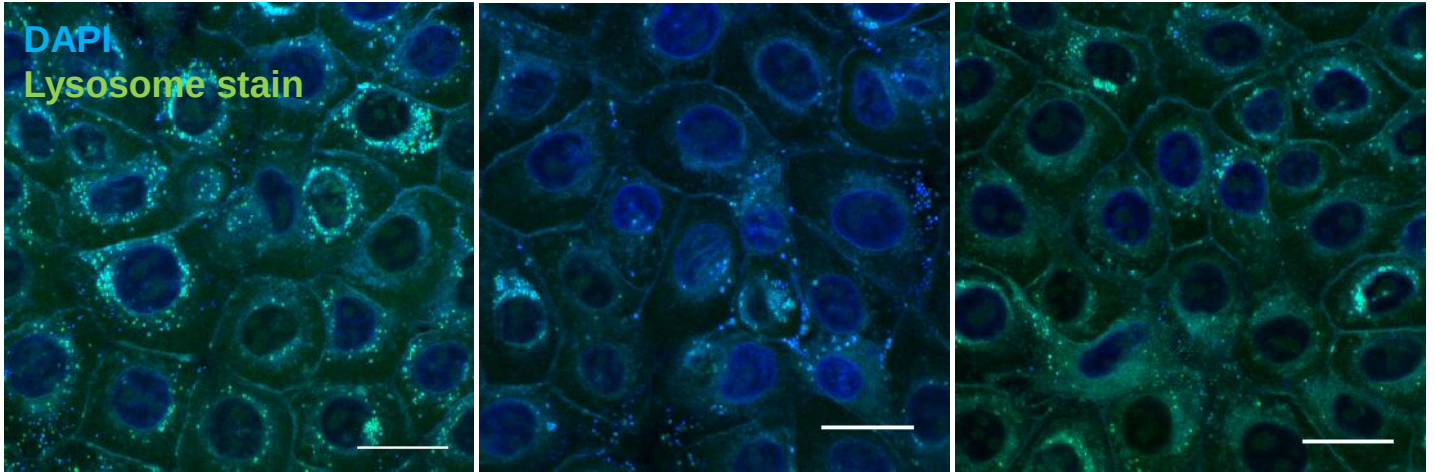
HaCaT cells treated with *S. aureus*-AD (TF3378) were stained by a Cell Navigator Lysosome staining Kit and DAPI. Images of DAPI and lysosome-stain green are shown respectively (left and middle panel) and together as merged images (right panel). Scale bar, 20 μm ($\times 240$) All images were acquired by using confocal microscopy.

Stand. strains

NCTC8325

FDA209P

Mu50



AD strains

TF3378

TF2962

TF2868

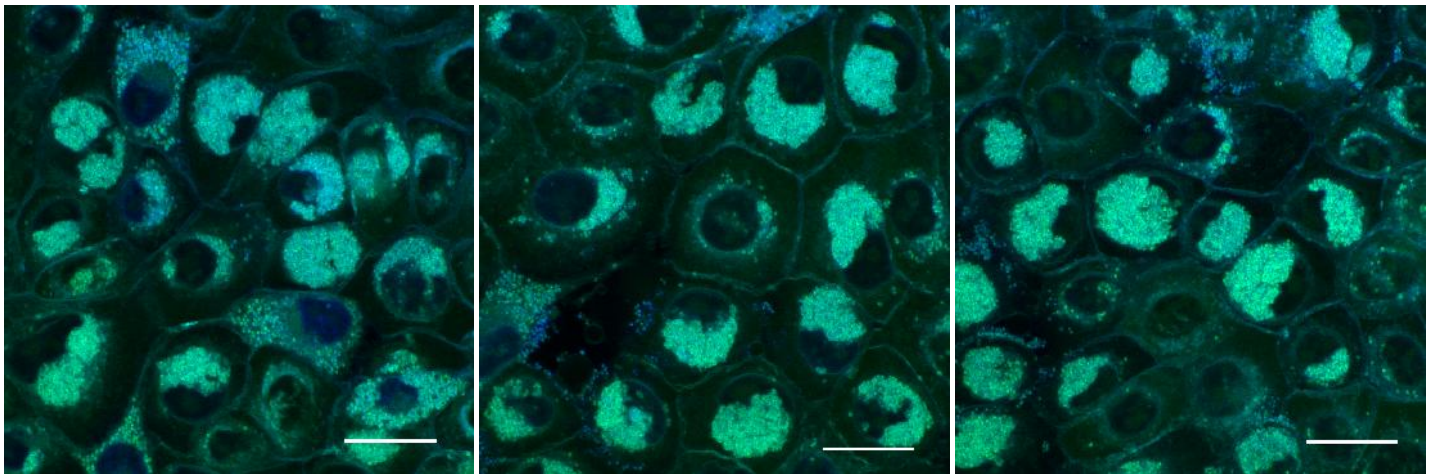


Figure S7: *S. aureus* strains isolated from AD skin accumulate in lysosome of NHEK.

Human epidermal keratinocytes were stimulated with strains of *S. aureus* for 24 h. Cell Navigator Lysosome staining Kit was added 1 h before fixing with paraformaldehyde (PFA). Scale bar, 20 μm . ($\times 120$) All images were acquired by using confocal microscopy.

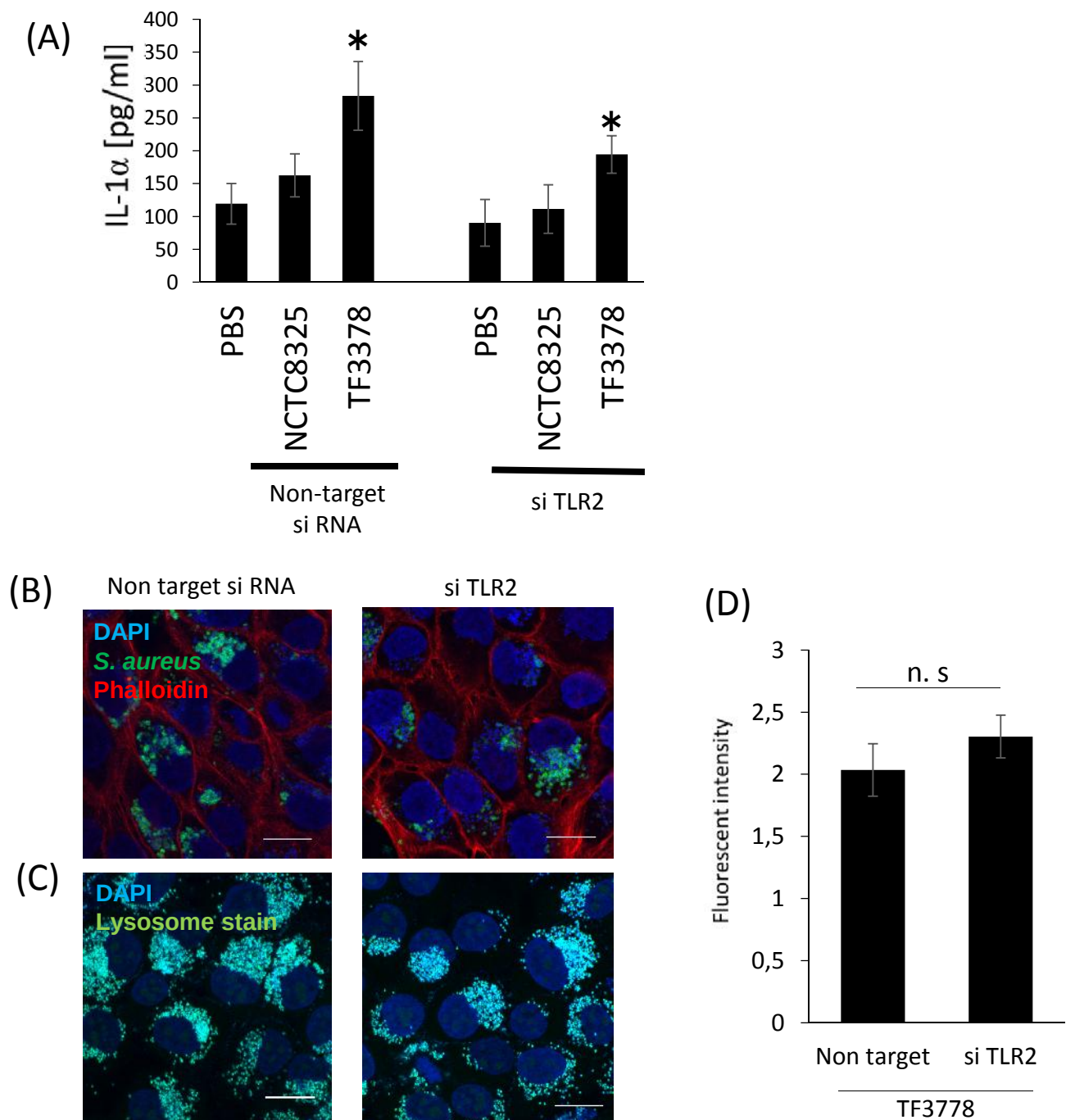


Figure S8: TLR2 knockdown does not affect IL-1 α secretion or internalization of *S. aureus*-AD.

HaCaT cells were lipofected with TLR2 siRNA and non-target siRNA 72 h before the stimulation with *S. aureus* strains. (A) HaCaT cells were incubated with strains of *S. aureus* for 72 h. Amounts of IL-1 α in the culture supernatants were analyzed by a Cytometric Bead Array kit. (* p <0.05, mean $\pm$ SEM, n = 3) (B) Immunofluorescence staining of *S. aureus* (Green), DAPI (Blue) and phalloidin (Red) was performed 24 h after stimulation with *S. aureus*-AD (TF3378). Scale bar, 20 μ m. All images were acquired by using confocal microscopy. (C) HaCaT cells were stimulated with *S. aureus*-AD (TF3378) for 24 h. Cell Navigator™ Lysosome staining Kit was added 1 h before fixing. Scale bar, 20 μ m. (D) Fluorescent Intensity of green stained lysosomes in HaCaT cells was analyzed by FACS. (mean $\pm$ SEM, n = 4)

Table S1: *S. aureus* strains and *S. epidermidis* used in this study

Strain	Character	Sequence Type
NCTC8325	Laboratory strain MSSA*	ST8
FDA209P	FDA standard strain MSSA*	ST464
Newman	Laboratory strain MSSA*	ST8
Mu50	Laboratory strain MRSA**, VISA***	ST5
ATCC12228	<i>S. epidermidis</i> standard strain	
TF3378	MSSA* isolate from AD skin	ST59
TF2962	MSSA* isolate from AD skin	ST59
TF2868	MSSA* isolate from AD skin	ST121
TF2870	MSSA* isolate from AD skin	ST20

* Methicillin-susceptible *S. aureus*

** Methicillin-resistant *S. aureus*

*** Vancomycin-intermediated *S. aureus*