

Staphylococcus aureus-derived membrane vesicles exacerbate skin inflammation in atopic dermatitis

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Summary

Background Skin colonization or infection with *Staphylococcus aureus* is known to trigger aggravation of atopic dermatitis (AD). However, the exact mechanisms by which *S. aureus* can worsen AD are unknown.

Objective We investigated whether and how *S. aureus*-derived membrane vesicles (MVs) contribute to worsening of AD.

Methods Immunohistochemical and immunoelectron microscopic analyses were performed to detect staphylococcal protein A (SPA) in the epidermis of AD lesions. HaCaT cells were treated with *S. aureus* MVs and were analysed for the expression of cytokine genes. Immunopathology and cytokine gene profiles were analysed after topical application of *S. aureus* MVs to AD-like skin lesions in a mouse model.

Results The MV component SPA was detected in the keratinocytes as well as in the intercellular space of the epidermis of AD lesions colonized with *S. aureus*. Intact MVs from *S. aureus* delivered their components to keratinocytes and stimulated pro-inflammatory cytokine gene expression *in vitro*. A knock-down of Toll-like receptor 2 or nucleotide-binding oligomerization domain 2 using small interfering RNAs suppressed interleukin-8 gene expression. Topical application of intact *S. aureus* MVs to AD-like skin lesions in the mouse model induced massive infiltration of inflammatory cells and the resulting eczematous dermatitis. This inflammatory reaction was associated with a mixed Th1/Th2 immune response and enhanced expression of chemokine genes in AD-like skin lesions.

Conclusions and Clinical Relevance This study showed the importance of *S. aureus* MVs as a potent mediator for worsening of AD among many exogenous worsening factors of AD. Thus, *S. aureus* MVs may be regarded as one of the therapeutic targets for the management of AD aggravation.

Keywords atopic dermatitis, dermatology, innate immunity, membrane vesicle, *Staphylococcus aureus*

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder with a relapsing course. The pathogenesis of AD is multifactorial and involves complex interactions among susceptible genes, environmental factors, skin barrier dysfunction and aberrant immune responses

[1–3]. Although the immunopathological features of skin lesions are quite different depending on the clinical stage of AD, skin lesions of AD patients show colonization or infection of *Staphylococcus aureus* in the epidermis and exhibit an increased number of inflammatory cells in the dermis [4, 5]. Patients with AD exhibit striking susceptibility to colonization or infection with *S. aureus*. Up to 90% of patients with AD have been reported to be carriers of *S. aureus*, whereas only 5% of healthy individuals carry this

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organism on the skins [6, 7]. *Staphylococcus aureus* density in lesional and nonlesional skin is strongly associated with severity of AD [8]. These findings suggest that *S. aureus* is highly associated with the worsening of AD. Possible mechanisms by which *S. aureus* can aggravate AD include production of pro-inflammatory cytokines through the interaction of bacteria or bacterial products with keratinocytes or immune cells [9–12]. Accumulating evidence suggests that superantigens produced by *S. aureus* activate large numbers of T cells and class II antigen-expressing cells and can evoke a potent immune response [13–15], which plays a role in the aggravation of AD after a staphylococcal infection. Other bacterial molecules, including α -haemolysin [16], lipoteichoic acid [17, 18], peptidoglycan [19, 20] and staphylococcal protein A (SPA) [21, 22], are presumably involved in the induction of inflammatory reactions. Application of SPA, however, does not induce skin inflammation in a mouse model [23]. Moreover, superantigen alone does not have any significant effect on the severity of AD [24, 25]. These results led us to assume that a molecular complex derived from *S. aureus* is necessary for the worsening of AD.

Staphylococcus aureus produces membrane vesicles (MVs) during both *in vitro* culture and *in vivo* infection [26, 27], similar to outer MVs of gram-negative bacteria. *Staphylococcus aureus* MVs are bilayered spherical vesicles with the size of 20–130 nm and are considered a molecular complex that contains more than 100 proteins as well as virulence-associated molecules, including α -haemolysin, SPA and biologically active β -lactamases [26–28]. These extracellular vesicles can serve as a transport system for bacterial molecules to host cells and can induce cellular pathological changes. Lee et al. [29] have demonstrated that SPA and staphylococcal enterotoxin A (SEA) are well colocalized in the epidermis of AD lesions, suggesting that specific vehicles are responsible for the delivery of these molecules to the epidermis. Moreover, application of *S. aureus* MVs to tape-stripped skin results in AD-like skin inflammation in a mouse model [30]. These observations indicate that *S. aureus* MVs are associated with skin inflammation and pathophysiology of AD.

Many clinical and experimental studies have demonstrated that *S. aureus* triggers skin inflammation in AD lesions, but the underlying mechanism is not fully understood. Because *S. aureus* colonizing or infecting AD lesions may produce MVs, it is important to clarify whether and how *S. aureus* MVs contribute to the pathophysiology of AD. In this study, we assessed the ability of *S. aureus* MVs to induce a pro-inflammatory response *in vitro* and determined the immunopathology of AD induced by topical application of *S. aureus* MVs to AD-like skin lesions in a mouse model.

Materials and methods

Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. *Der-matophagoides farinae* extract (DFE; Greer Laboratories, Lenoir, NC, USA) was dissolved in phosphate-buffered saline (PBS) containing 0.5% Tween-20. 2, 4-Dinitro-chlorobenzene (DNCB) was dissolved in the acetone/olive oil (1 : 3) solution.

Bacterial strain and cell culture

A clinical *S. aureus* 03ST17 isolate was obtained from the skin lesions of an AD patient who had visited Kyungpook National University Hospital, Daegu, Korea. This *S. aureus* isolate produced SEA, SEI and SEN, and was resistant to oxacillin, so-called methicillin-resistant *S. aureus* (MRSA) [31]. The bacteria were cultured in Luria–Bertani broth. HaCaT cells (kindly provided by Dr. W. K. Baik, Keimyung University, Daegu, Korea) were cultured in Dulbecco's Eagle's minimum essential medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin G and 50 μ g/mL streptomycin at 37°C in a humidified 5% CO₂ atmosphere.

Patients

Skin biopsy samples were obtained from three AD patients, who had visited the Dermatology Clinics of Dongsan Hospital (Daegu, Korea). *Staphylococcus aureus* was isolated from AD lesions of two patients with eczematous dermatitis by sterile swabbing and plating in blood agar plates. The AD skin lesions were biopsied after written informed consent had been obtained. The study protocol was approved by the Institutional Review of Board of Keimyung University School of Medicine (IRB: 11-202).

Mice

Six-week-old female BALB/c mice were purchased from Bio Korea (Gyeonggi-do, Korea) and housed under specific pathogen-free conditions. Five animals were housed in each cage in a laminar air flow room, maintained at a temperature of 22 $\pm$ 2°C, with relative humidity of 55 $\pm$ 5% throughout the study. All animal experiments were approved by Institutional Animal Care and Use Committee of Kyungpook National University.

Staphylococcus aureus membrane vesicles

The MVs produced by *S. aureus* 03ST17 were prepared from bacterial culture supernatants as previously

described [26]. The purified MVs were checked for sterility on blood agar plates and stored at -80°C until analysis. *Staphylococcus aureus* MVs were treated with 0.1 M ethylenediaminetetraacetic acid (EDTA) for 1 h at 37°C to disrupt the MV membrane. For degradation of proteins, MVs were treated with 0.1 $\mu\text{g}/\text{mL}$ of proteinase K (Fermentas, Hanover, MD, USA) for 3 h at 50°C .

Induction of atopic dermatitis-like skin lesions and application of Staphylococcus aureus membrane vesicles

Induction of AD-like skin lesions using DNCB and a freeze-dried DFE was performed as described previously [32]. The surface of both ear lobes of each mouse was stripped five times with surgical tape (Nichiban, Tokyo, Japan). After the stripping, 20 μL of DNCB (1%) was painted on each ear and then 20 μL of DFE (10 mg/mL) was applied 4 days later. Treatment with DFE was repeated twice a week for 2 weeks. Ears were inoculated with intact *S. aureus* MVs (10 μg), disrupted MVs (10 μg) or DFE (200 μg) twice a week using a paint brush until the end of the 5 weeks of induction. Ear thickness was measured using a dial thickness gauge (Kori Seiki MFG, Tokyo, Japan).

Histological and immunohistochemical analyses

Excised mouse ears were fixed in 10% formaldehyde and embedded in paraffin. Thin 5- μm sections were stained with haematoxylin and eosin (H&E; Shandon, Pittsburgh, PA, USA) for general histopathological examination. The skin sections were stained with toluidine blue for the assessment of infiltration by mast cells. Immunohistochemical analysis was conducted using avidin–biotin immunostaining methods to detect SPA in the mouse ears and in human biopsy samples. The tissue sections were incubated with an anti-rabbit SPA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A biotin-conjugated goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) was enhanced with the avidin–biotin complex (Vector Laboratories). After the sections were washed with PBS, the reactions were visualized using 3,3'-diaminobenzidine (Vector Laboratories). The sections were counterstained with haematoxylin.

A semiquantitative reverse-transcriptase PCR (RT-PCR) and real-time quantitative PCR (RT-qPCR)

Total cellular RNA was harvested using the RNeasy Mini kit (Qiagen, Valencia, CA, USA). Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). The primers of glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

and IL-8 for a semiquantitative RT-PCR were used as previously described [33]. A semiquantitative RT-PCR was performed on an ABI 9700 machine (Applied Biosystems, Foster City, CA, USA). RT-qPCR was carried out on a Thermal Cycler Dice TP850 (Takara Bio, Shiga, Japan) with SYBR Premix Ex Taq (Takara Bio) according to the manufacturer's protocol. The PCR primers for IFN- γ , TNF- α , IL-1 β , IL-4, IL-6, IL-8, IL-13, IL-31, macrophage inflammatory protein (MIP)-1 α and monocyte chemoattractant protein (MCP)-1 were used as previously described [34–36]. The amplification specificity was evaluated using melting curve analysis. Gene expression was normalized to β -actin or GAPDH mRNA levels in each sample, and fold change was determined using the $\Delta\Delta\text{C}_t$ method.

RNA interference

HaCaT cells were seeded in 12-well plates at the density of 5×10^4 cells/well in an antibiotic-free medium. After overnight incubation, the cells were transfected with small interfering RNA (siRNA) against nontargeting control, Toll-like receptor (TLR) 2, TLR6, nucleotide-binding oligomerization domain (NOD) 1 or NOD2 at the concentration of 30 pM using the Lipofectamine RNAimax reagent (Invitrogen, Carlsbad, CA, USA). The following siRNAs obtained from Bioneer (Daejeon, Korea) were used: nontargeting control (5'-GUG UGA ACC AUG AGA AGU A-3' and 5'-UAC UUC UCA UGG UUC ACA C-3'), TLR2 (5'-GAA UUU GCC GCA ACU CAA A-3' and 5'-UUU GAG UUG CGG CAA AUU C-3'), TLR6 (5'-CUA UCA GAG UUG ACA GUU U-3' and 5'-AAA CUG UCA ACU CUG AUA G-3'), NOD1 (5'-GAA UGA UGC CAG AAA CUU A-3' and 5'-UAA GUU UCU GGC AUC AUU C-3'), and NOD2 (5'-GCA AUU GCA GAA GUU AGC U-3' and 5'-AGC UAA CUU CUG CAA UUG C-3'). After transfection for 24 h, cellular RNA was extracted for a semiquantitative RT-PCR analysis. The primer sequences, annealing temperatures and product sizes of pattern recognition receptors (PRRs) were as follows: (i) TLR2, 5'-ACA AGT TCC GTG GAA TGT TT-3' and 5'-CTT GGA ATA TGC AGC CTC CG-3', 51°C , 302 bp; (ii) TLR6, 5'-ACG ACT TGG AAA TGC CTG GT-3' and 5'-CTC CAA TTT AAC TAA CGT GG-3', 51°C , 360 bp; (iii) NOD1, 5'-GTG GGG CAA TCA AGT TGG GG-3' and 5'-TGC TTT AAC GTC TGG TTG AC-3', 51°C , 243 bp; and (iv) NOD2, 5'-TCC TCT CCT GGG AGG ACT AC-3' and 5'-ACT GGC TGA CGA AAC CCC GC-3', 57°C , 300 bp. The cells with a knock-down or down-regulation of TLR2, TLR6, NOD1 or NOD2 genes were treated with *S. aureus* MVs (15 $\mu\text{g}/\text{mL}$) for 24 h, and then, total RNA was extracted using an RNeasy Mini Kit. Expression of IL-8 gene was determined using semiquantitative RT-PCR. All siRNA experiments included negative and positive control siRNAs. The

siRNA transfection procedures and transfection reagents were not toxic to HaCaT cells, as assessed using the lactate dehydrogenase (LDH) assay.

A cytotoxicity assay

The LDH assay was performed according to the procedures described in the assay kit. Briefly, HaCaT cells were seeded in a 24-well plate at a density of 5×10^4 /well. After incubation for 24 h, the cells were treated with *S. aureus* MVs in the amounts corresponding to protein concentrations of 1, 5, 10, 20 and 50 µg/mL for 24 h. One hundred microlitres of the culture medium was collected for the LDH measurement. The absorbance was measured at 450 nm using a spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Confocal laser microscopy

The cultured HaCaT cells were seeded at a density of 5×10^4 on glass coverslips. After incubation with intact and disrupted MVs, the cells were washed with PBS and fixed in 4% paraformaldehyde. The cells were permeabilized for 10 min in PBS containing 0.25% Triton X-100 to determine intracellular localization of MV components. MVs were labelled with an anti-rabbit SPA antibody, followed by an Alexa-488-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA). The nuclei of the cells were stained with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes). The samples were examined under a laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Transmission electron microscopic (TEM) analysis

Purified MVs were applied to copper grids and stained with 2% uranyl acetate. For immunoelectron microscopy, skin biopsy samples were fixed in the solution containing 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer. The samples were washed with PBS and water, and dehydrated in a graded series of ethanol solutions. The tissue samples were embedded in the Lowicryl HM20 acrylic resin (Electron microscopy sciences, Hatfield, PA, USA). Ultrathin sections mounted on nickel grids were incubated for 1 h in 3% bovine serum albumin in PBS to block nonspecific binding. The grids were incubated with an anti-rabbit SPA antibody overnight at 4°C. A 10 nm gold particle-conjugated goat anti-rabbit IgG antibody (1 : 40; EY Laboratories, San Mateo, CA, USA) served as a secondary antibody. The sections were also stained with 2% uranyl acetate. The samples were examined under a TEM (Hitachi H-7500, Ibaraki, Japan).

Statistical analysis

All data are presented as mean $\pm$ standard error of the mean (SEM) for two or three independent experiments. Data were analysed using R 3.3.1 (<https://www.r-project.org/>) and FSA package version 0.8.8 (<https://github.com/drog-lenc/FSA>). Expression of cytokine genes in cultured cells was analysed using a nonparametric one-way ANOVA with Dunnett's *post hoc* analysis. Expression of cytokine genes in AD-like skin lesions was analysed using a nonparametric one-way ANOVA with Tukey's *post hoc* analysis. The statistical significance of ear thickness between groups was calculated using student's *t*-test. Differences of $P < 0.05$ were considered as statistically significant.

Results

Skin barrier penetration by staphylococcal protein A and its interaction with keratinocytes in atopic dermatitis lesions colonized with Staphylococcus aureus

To verify the possible association of *S. aureus* MVs with aggravation of AD lesions in patients, we first determined whether *S. aureus*-derived MV molecules could penetrate the skin barrier in AD lesions. Two patients with AD colonized with *S. aureus* and one patient with AD without *S. aureus* were selected. Skin biopsy samples obtained from AD lesions were stained with an anti-SPA antibody. SPA was used as a marker of *S. aureus* MVs because *S. aureus* secretes a large amount of SPA via MVs [26, 31]. Immunohistochemical analysis showed that SPA not only was clearly detected in the stratum corneum, but also was present in all layers of the epidermis in AD lesions colonized with *S. aureus* (Fig. 1a). In contrast, SPA was not detected in the skin lesion of AD patient without evidence of *S. aureus* colonization or infection (Fig. 1a). Next, to determine whether SPA could interact with keratinocytes in the epidermis, we performed immunoelectron microscopic analysis of AD lesions obtained from the patient with *S. aureus* using an anti-SPA antibody. SPA was detected on the surface and in the cytoplasm of keratinocytes as well as in the intercellular space of the epidermis (Fig. 1b). Our results show that *S. aureus*-derived molecule SPA, but not bacteria itself, can penetrate the skin barrier and interact with keratinocytes in AD lesions colonized with *S. aureus*, suggesting that *S. aureus* MVs may play a role in the delivery of *S. aureus* effector molecules to the epidermis in AD lesions.

Membrane vesicles from Staphylococcus aureus isolate colonized in atopic dermatitis lesions

To determine whether *S. aureus* isolated from AD lesions produced MVs, MVs were purified from the

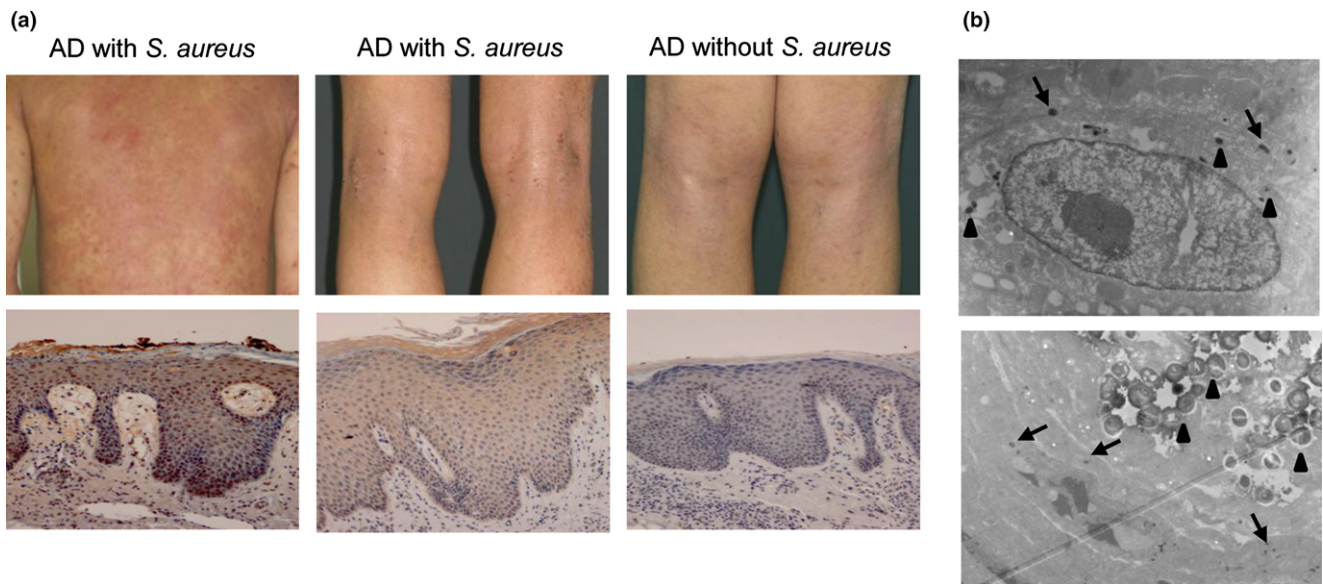


Fig. 1. Detection of staphylococcal protein A (SPA) in atopic dermatitis (AD) lesions. (a) Skin lesions of AD patients and immunohistochemical analysis of biopsy samples from AD patients using an anti-SPA antibody. Magnification of microscopic images, $\times 200$. (b) Immunoelectron microscopy of AD lesions for the analysis of SPA interacting with keratinocytes. The samples obtained from AD lesions with *Staphylococcus aureus* were stained with an anti-SPA antibody and then stained with uranyl acetate. Top, SPA was detected in the cytoplasmic membrane (arrows) and cytoplasm (arrow heads) of keratinocytes. Magnification, $\times 7000$. Bottom, SPA was detected in the intercellular space of the epidermis (arrows). The SPA-stained microorganisms were observed in the superficial layer of the skin (arrow heads). Magnification, $\times 4000$.

culture supernatant of MRSA 03ST17 isolate. We selected MRSA strain originated from the AD lesions, because there was a strong association between the presence of MRSA and increased severity of AD, and MRSA produced more superantigens than methicillin-susceptible *S. aureus* [13, 37]. TEM analysis showed that *S. aureus* produced spherical MVs (Fig. 2a). To determine whether *S. aureus* MVs could deliver bacterial molecules to host cells *in vitro*, HaCaT cells were treated with either intact MVs or EDTA-treated MVs and stained with an anti-SPA antibody, followed by an Alexa-488-conjugated secondary antibody. SPA was identified in the *S. aureus* 03ST17-derived MVs [31] and used as a marker of MVs. Green fluorescence was more clearly visible in HaCaT cells treated with intact MVs than the cells treated with disrupted MVs (Fig. 2b). Next, we determined whether *S. aureus* MVs were cytotoxic to keratinocytes. The MVs purified from *S. aureus* 03ST17 were not cytotoxic to HaCaT cells treated with up to 50 $\mu\text{g/mL}$ (Fig. 2c). These results suggest that *S. aureus* MVs play a role in the delivery of bacterial molecules to keratinocytes, but do not exhibit cytotoxicity in keratinocytes.

A pro-inflammatory cytokine response in keratinocytes treated with Staphylococcus aureus membrane vesicles

To determine whether *S. aureus* MVs could stimulate production of pro-inflammatory cytokines *in vitro*,

HaCaT cells were treated with *S. aureus* MVs for 6 h and the expression of pro-inflammatory cytokine genes was analysed. *Staphylococcus aureus* MVs stimulated expression of pro-inflammatory cytokine genes, including IL-1 β , IL-6, IL-8 and MIP-1 α , but expression of pro-inflammatory cytokine genes was not in a dose-dependent manner (Fig. 3a). To determine whether the delivery of bacterial molecules to host cells via MVs was prerequisite to the pro-inflammatory cytokine response, HaCaT cells were treated with either intact MVs or disrupted MVs for 6 h. Expression of IL-6 gene was significantly increased in cells treated with intact MVs or lysed MVs as compared to that of untreated control cells (Fig. 3b), but expression of IL-6 gene was higher in cells treated with intact MVs than cells treated with disrupted MVs. Nonetheless, proteinase K-treated MVs (see Fig. S1) had the same ability to induce expression of IL-6 gene as did intact MVs, although proteinase K could slightly stimulate expression of IL-6 gene in HaCaT cells (Fig. 3b). Our results suggest that cellular delivery of nonprotein molecules via MVs is essential to stimulate a pro-inflammatory response in keratinocytes *in vitro*. To determine which PRRs were responsible for induction of a pro-inflammatory response to *S. aureus* MVs, HaCaT cells were transfected with siRNA against genes encoding TLR2, TLR6, NOD1 or NOD2 for 24 h. A knock-down of corresponding genes was analysed using a semiquantitative RT-PCR (see Fig. S2). HaCaT cells with knock-down or

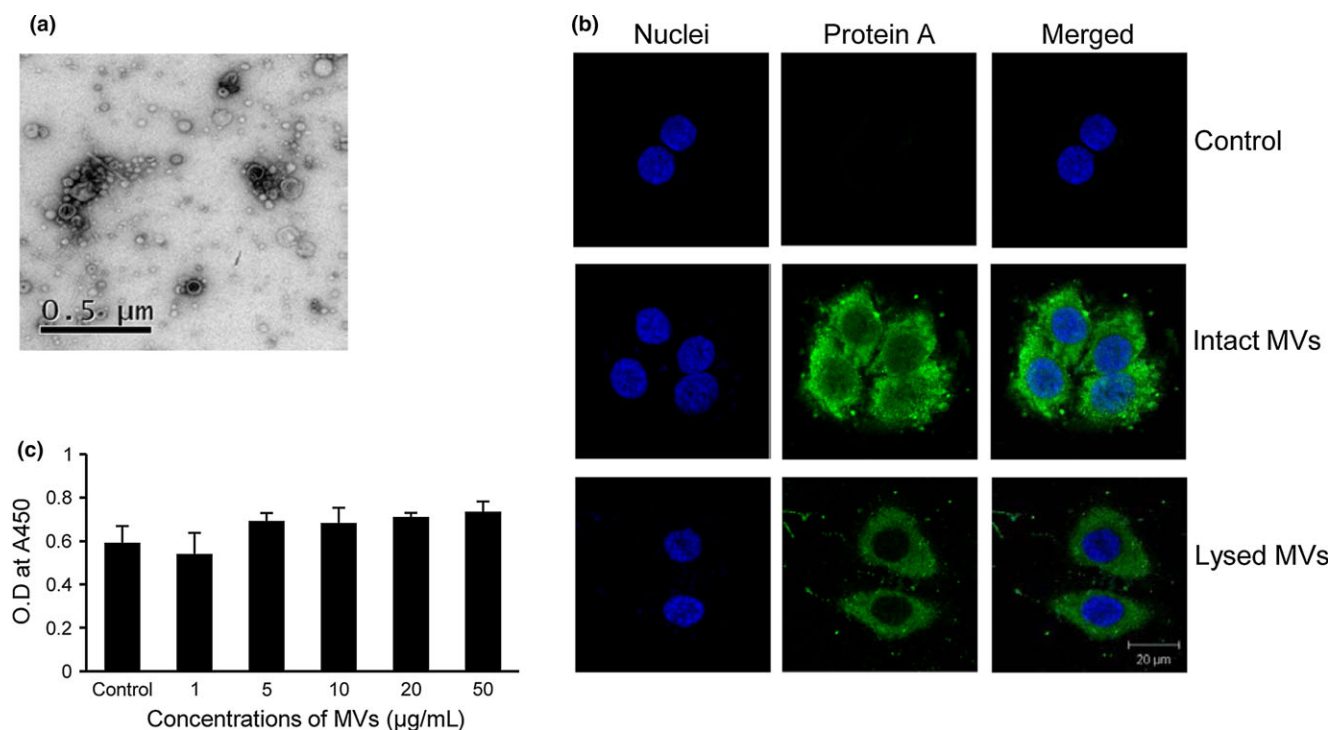


Fig. 2. Interaction of *Staphylococcus aureus* membrane vesicles (MV) with cultured keratinocytes. (a) Transmission electron microscopic analysis of *S. aureus* MVs. (b) HaCaT cells were treated with either intact or lysed MVs for 6 h. The cells were permeabilized with Triton X-100 and stained with a rabbit anti-*staphylococcal* protein A (SPA) antibody, followed by an Alexa Fluor 488-conjugated anti-rabbit IgG antibody. DAPI was used to stain the nuclei. (c) Cytotoxicity of *S. aureus* MVs. HaCaT cells were treated with various concentrations of MVs for 24 h, and then, cytotoxicity was measured using the lactate dehydrogenase assay. The data are presented as the mean $\pm$ SEM ($n = 2$) of two independent experiments.

suppression of TLR2, TLR6, NOD1 or NOD2 gene were treated with intact *S. aureus* MVs, and expression of IL-8 gene was analysed. A knock-down of TLR2 or NOD2 genes inhibited expression of IL-8 gene (Fig. 3c). Our results suggest that both TLR2 and NOD2 are responsible for the induction of a pro-inflammatory response in keratinocytes treated with *S. aureus* MVs.

Induction of eczematous dermatitis by topical application of *Staphylococcus aureus* membrane vesicles to atopic dermatitis-like skin lesions in a mouse model

To determine whether *S. aureus* MVs triggered aggravation of AD *in vivo*, an AD mouse model was set up by treatment with DNCB and subsequent topical application of a freeze-dried DFE for 2 weeks. Thereafter, the mice were treated with either intact or disrupted MVs of *S. aureus* topically for 3 weeks. The schematic experimental procedures are shown in Fig. 4a. Mice treated with DFE alone for 5 weeks showed AD-like dermatitis (Fig. 4b). Topical application of intact *S. aureus* MVs to AD-like skin lesions induced severe eczematous dermatitis, including swelling, redness, bullae and eschar formation. In contrast, the disrupted MVs did not induce eczematous dermatitis. Thickening of skin lesions was most prominent in mice treated with

DFE plus intact MVs (Fig. 4c). Histological analysis showed that intact MVs induced massive infiltration of inflammatory cells (Fig. 5a) and mast cells (Figs 5b and S3) into the dermis. In contrast, the disrupted MVs caused minor skin alterations, such as thickening of epidermis and appearance of a small number of inflammatory cells in the dermis. Tissue penetration of SPA was the most prominent feature in mice treated with DFE plus intact MVs, although SPA was nonspecifically stained in the dermis and blood vessels of mice treated with DFE alone (Fig. 5c). These results suggest that topical application of *S. aureus* MVs induces eczematous dermatitis in AD-like skin lesions *in vivo*.

Inflammatory cytokine profiles after topical application of *Staphylococcus aureus* membrane vesicles to atopic dermatitis-like skin lesions

To determine the types and levels of inflammatory responses in AD-like skin lesions treated with *S. aureus* MVs, RT-qPCR was performed to measure gene expression of Th1/Th2 cytokines (IFN- γ for Th1; IL-4, IL-13 and IL-31 for Th2), pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6) and chemokines (IL-8, MIP-1 α and MCP-1). All the cytokine genes tested were up-regulated in the three groups of mice treated with DFE

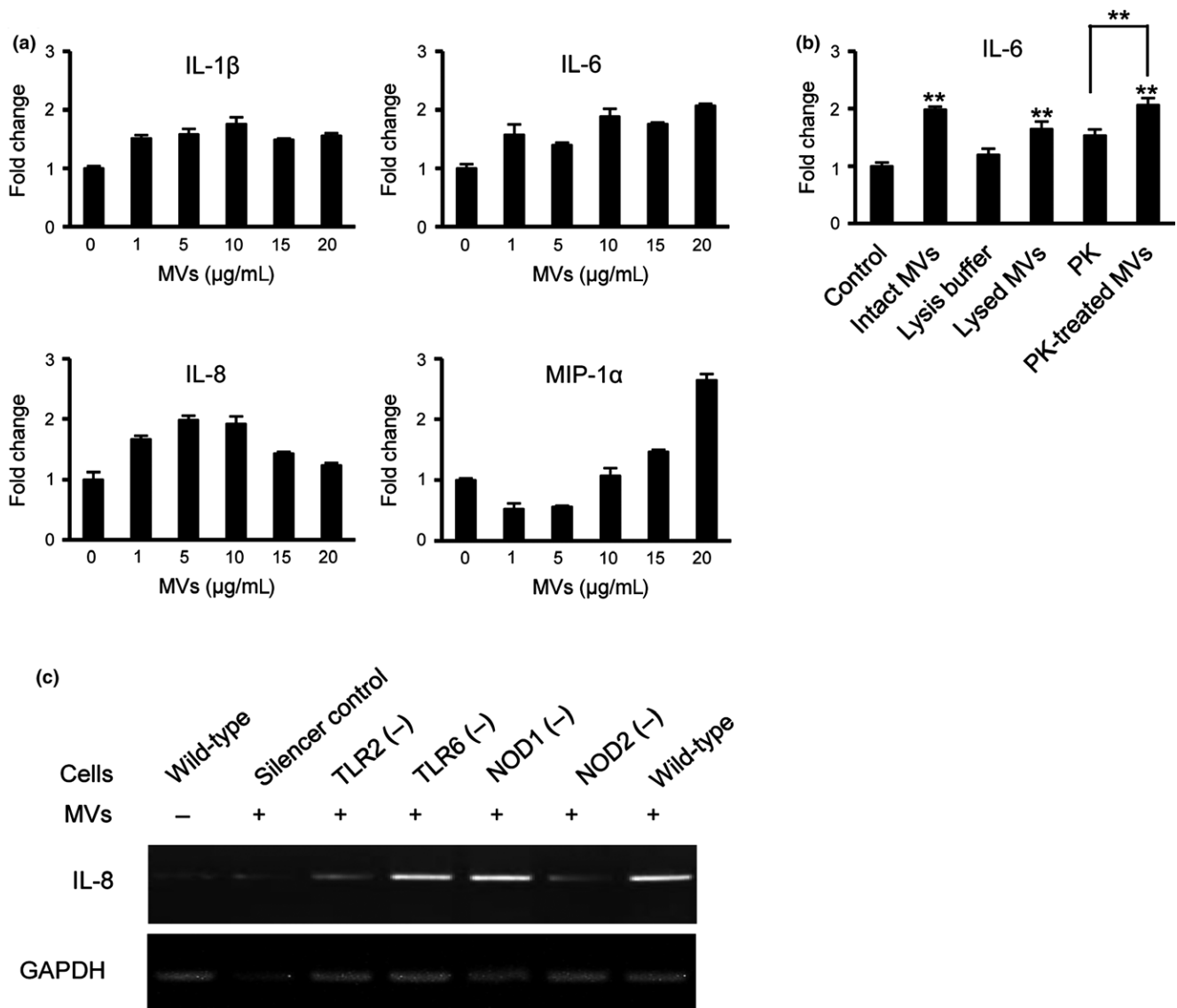


Fig. 3. Expression of pro-inflammatory cytokine genes in HaCaT cells treated with *Staphylococcus aureus* membrane vesicles (MV). (a) HaCaT cells were treated with *S. aureus* MVs for 6 h. Gene expression was assessed by RT-qPCR. The data are presented as the mean $\pm$ SEM ($n = 2$) of two independent experiments. (b) HaCaT cells were treated with 15 μ g/mL of intact MVs, lysed MVs and proteinase K (PK)-treated MVs for 6 h, and expression of IL-6 gene was analysed using a RT-qPCR. As a control, the cells were treated with the same concentration of ethylenediaminetetraacetic acid (EDTA; lysis buffer) and PK for protein degradation in MVs (PK). The data are presented as the mean $\pm$ SEM ($n = 2$) of two independent experiments. ** $P < 0.01$ compared with the control. ** $P < 0.05$ between the PK-treated cells and cells treated with PK-treated MVs. (c) HaCaT cells were transfected with a small interfering RNA against silence control, TLR2, TLR6, NOD1 or NOD2 gene. After a knock-down of each PRR gene, the cells were treated with *S. aureus* MVs (15 μ g/mL) for 24 h. Expression of IL-8 gene was assessed by a semiquantitative RT-PCR.

alone, DFE plus intact MVs and DFE plus disrupted MVs, compared to control mice treated with PBS (Fig. 6a–c). There was no significant difference in expression of Th1/Th2 (Fig. 6a) and pro-inflammatory cytokine genes (Fig. 6b) between the mice treated with DFE alone and DFE plus intact MVs. However, expression levels of chemokine genes were significantly different between the mice treated with DFE alone and mice treated with DFE plus intact MVs (Fig. 6c). Expression of Th2 cytokine genes, including IL-13 and IL-31,

was significantly different between the mice treated with intact MVs and disrupted MVs (Fig. 6a). Moreover, expression of chemokine genes was also significantly different between the mice treated with DFE plus intact MVs and mice treated with DFE plus disrupted MVs (Fig. 6c). Our results suggest that intact *S. aureus* MVs, but not disrupted MVs, are an effective inducer of chemokines in AD-like skin lesions, which is related to infiltration of inflammatory cells and inflammatory responses.

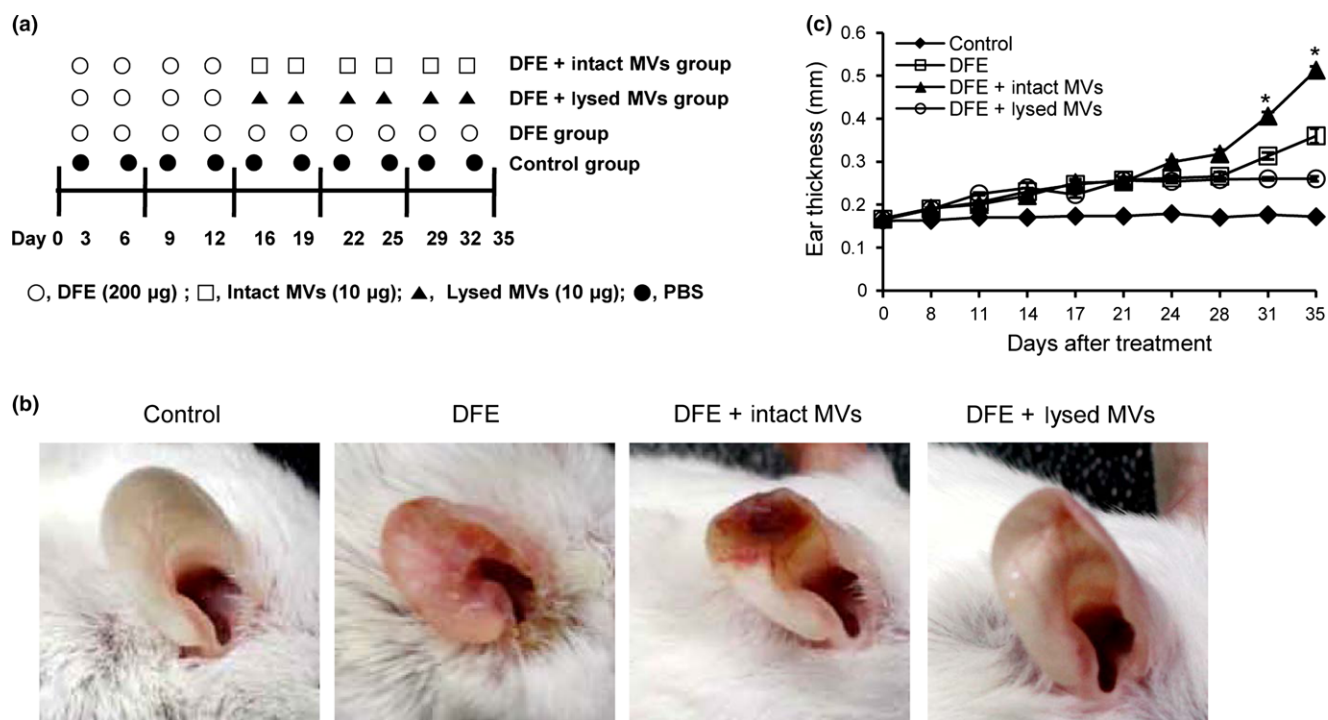


Fig. 4. Worsening of atopic dermatitis (AD)-like skin lesions by *Staphylococcus aureus* membrane vesicles (MVs) in a mouse model. (a) The experimental schedule for the application of mite extracts (DFE) and *S. aureus* MVs. (b) Photographs of mouse ears treated with DFE alone or DFE plus *S. aureus* MVs. (c) Effects of *S. aureus* MVs on ear thickness. The data are presented as mean $\pm$ SE of triplicate measurements of five mice. * $P < 0.05$ compared with the mice treated with DFE. DFE, *Dermatophagoides farinae* extract.

Discussion

Staphylococcus aureus is a pivotal exacerbating factor in AD. Many attempts have been made to characterize the role of *S. aureus* in worsening of AD. Several *S. aureus*-derived molecules, especially superantigens, have been implicated in skin inflammation *in vitro* and *in vivo* [14, 15, 29]. However, there is no evidence demonstrating specific delivery of *S. aureus* molecules to effector cells and the association of those molecules with an inflammatory response. The present study demonstrates that MVs play a role in the delivery of *S. aureus*-derived effector molecules to host cells and that nonprotein molecules packaged in the MVs contribute to pro-inflammatory responses in keratinocytes *in vitro*. Furthermore, topical application of *S. aureus* MVs specifically induces expression of chemokine genes in AD-like skin lesions *in vivo* animal model.

Previous studies demonstrated the possible association of *S. aureus* MVs with the pathophysiology of AD [29, 30]. In particular, *S. aureus* MVs induce AD-like skin inflammation in a mouse model [30], suggesting that *S. aureus* MVs are inducing factor of AD, but not a worsening factor. *Staphylococcus aureus* MV components, SPA and SEA, are colocalized in the epidermis and are detected with increased immunoreactivity in

the upper part of the epidermis of AD lesions [29]. In the present study, we also found that *S. aureus* MV component SPA is present in all layers of the epidermis in AD lesions colonized with *S. aureus*. SPA seems to interact with keratinocytes through the cytoplasmic membrane and cytoplasm. The spherical intact *S. aureus* MVs deliver SPA to keratinocytes, whereas the disrupted MVs cannot deliver SPA to keratinocytes effectively *in vitro*, according to our present results. Furthermore, intact *S. aureus* MVs deliver more SPA to deep tissues than do disrupted MVs, in our *in vivo* animal model. Although the mechanical disruption of the skin can facilitate the penetration of bacteria and bacterial products into the epidermis, our results suggest that *S. aureus* MVs participate in the delivery of bacterial effector molecules to host cells. *Staphylococcus aureus* MVs are cytotoxic to human laryngeal epithelial HEp-2 cells [26] and human cervical carcinoma HeLa cells [28]. In this study, however, MVs purified from a clinical *S. aureus* isolate from AD lesions are not cytotoxic to keratinocytes. *Staphylococcus aureus* 03ST17 MVs were not cytotoxic to HEp-2 cells treated with up to 15 µg/mL [31]. Instead, these extracellular vesicles elicit a pro-inflammatory response in keratinocytes. The apparent discrepancy in cytotoxicity of *S. aureus* MVs may be due to differences in *S. aureus* strains or in cell lines used in different studies.

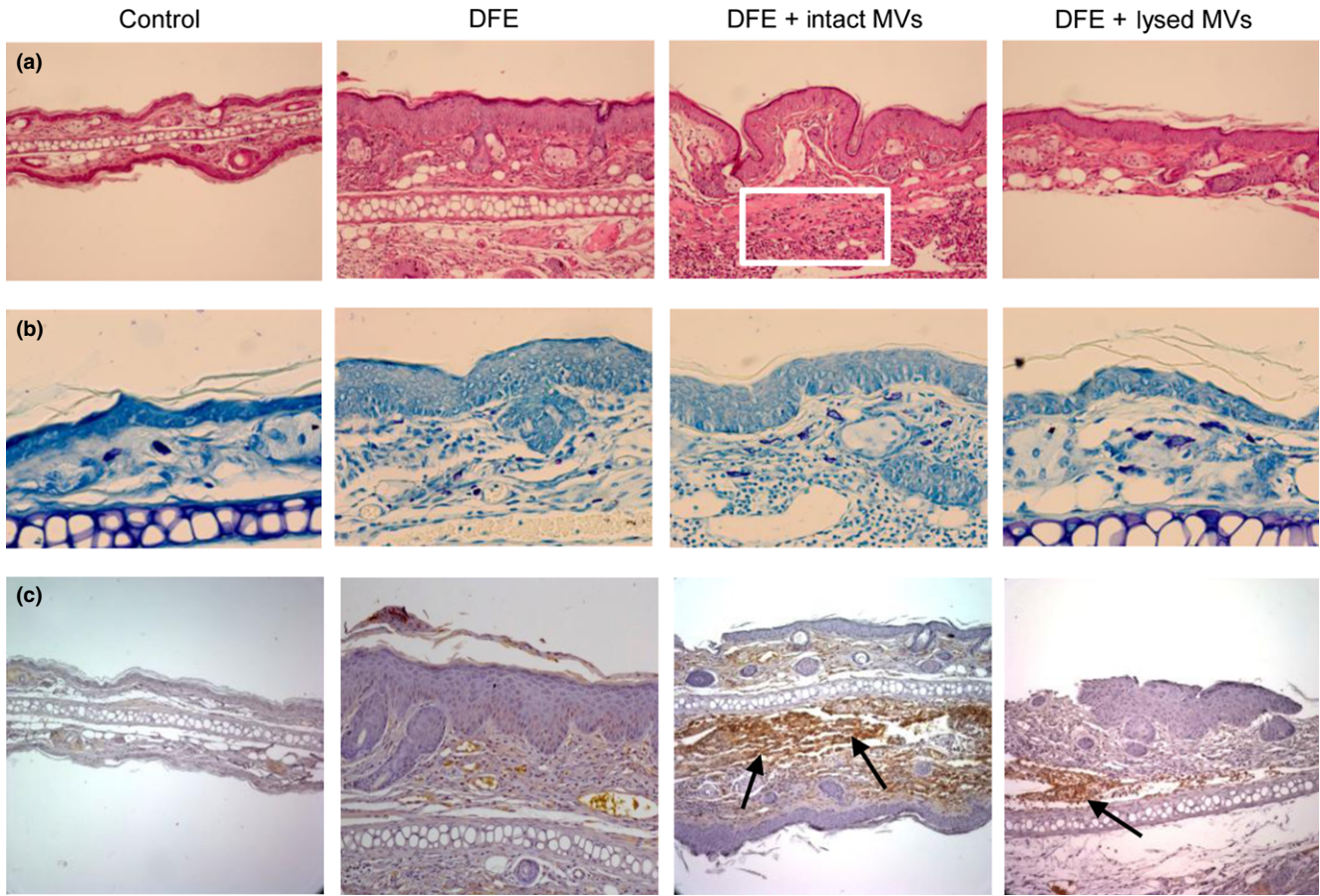


Fig. 5. Histological analysis of ear sections in mice treated with mite extracts (DFE) alone or DFE plus *Staphylococcus aureus* membrane vesicles (MVVs). The samples were stained with haematoxylin and eosin (a; magnification, $\times 200$), toluidine blue (b; magnification, $\times 200$) or an anti-staphylococcal protein A (SPA) antibody (c; magnification, $\times 200$). White box indicates the massive infiltration of polymorphonuclear cells. Arrows indicate the penetration of SPA by *S. aureus* MVVs. DFE, *Dermatophagoides farinae* extract.

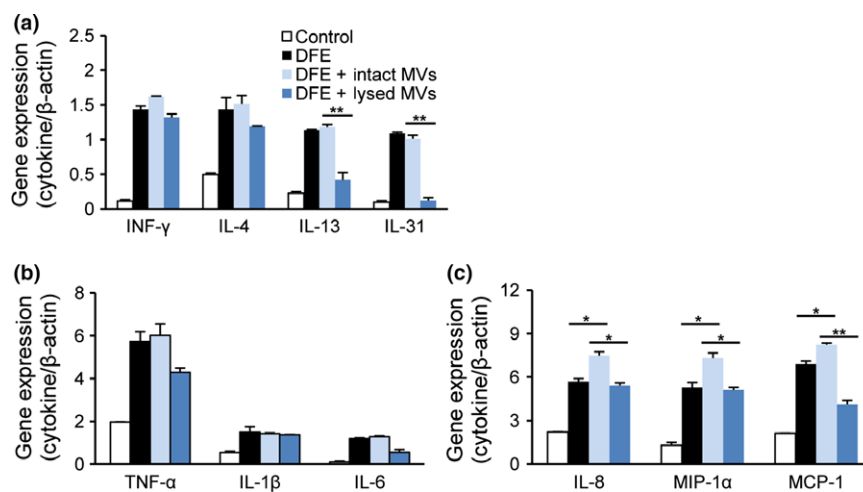


Fig. 6. Expression of cytokine genes in atopic dermatitis (AD)-like skin lesions treated with *Staphylococcus aureus* membrane vesicles (MVVs). The ears of mice treated either with mite extracts (DFE) alone or with DFE plus *S. aureus* MVVs were excised, and total RNA was isolated. RT-qPCR was performed. Expression of Th1/Th2 cytokine genes (a), pro-inflammatory cytokine genes (b) and chemokine genes (c) was measured. The data are presented as mean $\pm$ SEM of triplicate measurements of five mice. Significant differences are denoted by asterisks (* $P < 0.05$, ** $P < 0.01$). DFE, *Dermatophagoides farinae* extract.

Membrane vesicles purified from *S. aureus* ATCC 14458 induce production of pro-inflammatory mediators in dermal fibroblasts [30]. On the other hand, keratinocytes, the main cell population in the epidermis, are the main producers of cytokines and chemokines in the skin, which are necessary for attracting other immune cells to participate in the immune responses against pathogens [38]. Our present results show that intact *S. aureus* MVs stimulate expression of pro-inflammatory cytokine genes in keratinocytes *in vitro*. Intact MVs deliver SPA to keratinocytes, whereas a relatively small amount of SPA is detectable in keratinocytes when the cells are treated with the disrupted MVs. Differences in the delivery of MV components to keratinocytes may result in small difference of expression of IL-6 gene between intact and disrupted MVs (Fig. 3b). This finding suggests that cellular delivery and subsequent recognition of MV components by specific PRRs perform an important function in pro-inflammatory responses.

Our present results show that protein-degraded MVs can elicit a pro-inflammatory response, suggesting that protein components in the *S. aureus* MVs do not exert a pro-inflammatory response in keratinocytes. A knock-down of TLR2 or NOD2 gene suppressed expression of IL-8 gene in keratinocytes treated with *S. aureus* MVs. TLR2 forms homodimers and heterodimers with TLR1 and TLR6 for recognition of bacterial ligands on the cell surface. TLR1/TLR2 heterodimers recognize triacylated lipoproteins and lipopeptides, whereas TLR2/TLR6 heterodimers recognize diacylated lipoproteins such as lipoteichoic acid [38, 39]. Peptidoglycan is a major constituent of the cell wall of gram-positive bacteria, and PRRs such as TLR2, NOD1 and NOD2 can sense peptidoglycan [39, 40]. NOD molecules are intracellular PRRs involved in the intracellular recognition of pathogen-associated molecular patterns. NOD1 detects a unique diaminopimelate-containing *N*-acetylglucosamine (GlcNAc)-*N*-acetylmuramic acid (MurNAc) tripeptide muropeptide found mostly in the gram-negative bacterial peptidoglycan [41]. NOD2 detects the GlcNAc-MurNAc dipeptide that corresponds to the minimal motif found in both gram-positive and gram-negative bacterial peptidoglycan [42]. Accordingly, our results indicate that nonprotein molecules, possibly peptidoglycan, in *S. aureus* MVs are responsible for the pro-inflammatory responses mediated by the TLR2 and NOD2 signalling pathways.

We also found here that topical application of intact *S. aureus* MVs to AD-like skin lesions induces eczematous dermatitis characterized by a mixed Th1/Th2 immune response. IgE-mediated extrinsic AD that accounts for 70–80% of AD cases gravitates towards Th2 responses together with accumulation of Th2 chemokines in the AD lesions [43, 44]. In the present

study, both AD animal groups treated with DFE alone and DFE plus intact MVs showed a mixed Th1/Th2 response in AD-like skin lesions. Kim et al. [32] demonstrated that treatment with DFE alone for 4 weeks stimulated expression of genes associated with Th1 cytokine (IFN- γ), Th2 cytokines (IL-4, IL-13 and IL-31) and pro-inflammatory cytokine (TNF- α) in a mouse model, suggesting that DFE alone induces a mixed Th1/Th2 inflammatory response. In our study, treatment with DFE for 2 weeks and intact MVs for 3 weeks also stimulated expression of Th1/Th2 cytokine and pro-inflammatory cytokine genes. Infiltration of inflammatory cells was clearly observed in AD-like skin lesions treated with DFE plus intact MVs. This inflammatory response may be associated with enhanced expression of chemokine genes, including IL-8, MIP-1 α and MCP-1. However, the disrupted MVs did not induce eczematous skin lesions, thickening of the ear and histological changes observed in mice treated with intact MVs. Treatment with DFE plus disrupted MVs significantly decreased the expression of genes of Th2 cytokines (IL-13 and IL-31) and chemokines in AD-like skin lesions, compared to treatment with DFE plus intact MVs. However, there was no significant difference in the expression of IL-4 gene between intact MVs and disrupted MVs. Gene expression of Th1 cytokines, pro-inflammatory cytokines and chemokines was not significantly different between the mice treated with DFE alone and DFE plus disrupted MVs. These results suggest that the disruptive *S. aureus* MVs induce a pro-inflammatory response in AD-like skin lesions observed in mice treated with DFE alone, but are not possibly associated with worsening of AD. Altogether, our findings indicate that intact MVs produced by *S. aureus* colonizing or infecting the superficial skin can penetrate skin barriers and interact with effector cells; this interaction induces production of pro-inflammatory cytokines and chemokines, which may result in recruitment of inflammatory cells and local inflammation in the AD lesions. However, it should be determined the potential of *S. aureus* MVs to stimulate inflammatory response, because *S. aureus* MVs induced relatively low expression of inflammatory cytokine genes *in vitro* and *in vivo*.

In conclusion, our results will advance the understanding of the pathophysiology of AD and may lead to identification of one of the therapeutic targets for the management of AD. Further clinical and experimental research will be needed to elucidate the relationship between *S. aureus* MVs and clinical severity of AD.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Protein profiles of *Staphylococcus aureus* MVs.

Figure S2. A knockdown of genes encoding PRRs using a siRNA.

Figure S3. Infiltration of master cells induced by *Staphylococcus aureus* MVs.

(kDa)

M

1

2

250

75

50

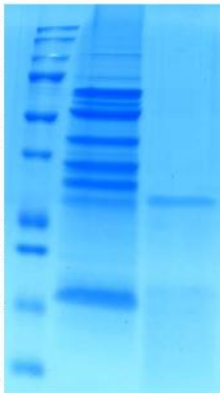
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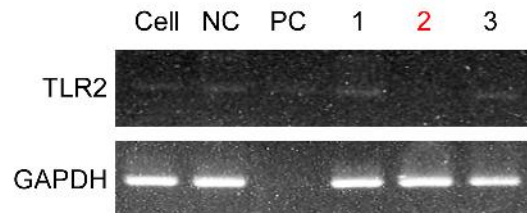
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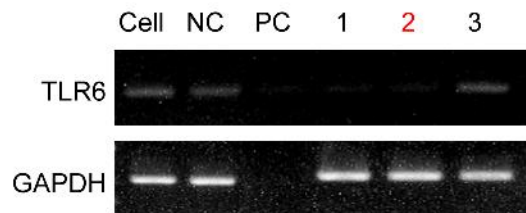
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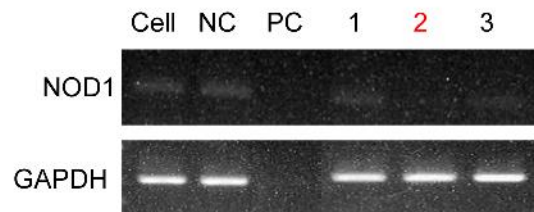
A. A knockdown of TLR2 gene



B. A knockdown of TLR6 gene



C. A knockdown of NOD1 gene



B. A knockdown of NOD2 gene

