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***Staphylococcus aureus* α -toxin modulates skin host response to viral infection**

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Abstract

Background—Patients with atopic dermatitis (AD) with a history of eczema herpeticum have increased staphylococcal colonization and infections. However, whether *Staphylococcus aureus* alters the outcome of skin viral infection has not been determined.

Objective—We investigated whether *S aureus* toxins modulated host response to herpes simplex virus (HSV) 1 and vaccinia virus (VV) infections in normal human keratinocytes (NHKs) and in murine infection models.

Methods—NHKs were treated with *S aureus* toxins before incubation of viruses. BALB/c mice were inoculated with *S aureus* 2 days before VV scarification. Viral loads of HSV-1 and VV were evaluated by using real-time PCR, a viral plaque-forming assay, and immunofluorescence staining. Small interfering RNA duplexes were used to knockdown the gene expression of the cellular receptor of α -toxin, a disintegrin and metalloprotease 10 (ADAM10). ADAM10 protein and α -toxin heptamers were detected by using Western blot assays.

Results—We demonstrate that sublytic staphylococcal α -toxin increases viral loads of HSV-1 and VV in NHKs. Furthermore, we demonstrate *in vivo* that the VV load is significantly greater ($P < .05$) in murine skin inoculated with an α -toxin-producing *S aureus* strain compared with murine skin inoculated with the isogenic α -toxin-deleted strain. The viral enhancing effect of α -toxin is mediated by ADAM10 and is associated with its poreforming property. Moreover, we demonstrate that α -toxin promotes viral entry in NHKs.

Conclusion—The current study introduces the novel concept that staphylococcal α -toxin promotes viral skin infection and provides a mechanism by which *S aureus* infection might predispose the host toward disseminated viral infections.

Keywords

Atopic dermatitis; *Staphylococcus aureus*; herpes simplex virus; vaccinia virus; α -toxin; a disintegrin and metalloprotease 10

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Atopic dermatitis (AD) is a chronic inflammatory skin disease that affects up to 30% of children in certain countries and can persist into adulthood.^{1,2} A body of documented evidence suggests that skin barrier defects caused by genetic variants and acquired through immune dysregulation are associated with the pathogenesis of AD.^{3,4} Aside from being susceptible to recurrent *Staphylococcus aureus* infections, a small subset of patients with AD have life-threatening complications from disseminated viral skin infections (ie, eczema herpeticum [EH] after herpes simplex virus [HSV] infection or eczema vaccinatum [EV] after smallpox vaccination with vaccinia virus [VV]).^{5,6} The exact mechanism underlying the increased propensity for viral skin infection of patients with AD remains obscure.

S aureus is one of the most influential environmental factors on AD skin biology.⁷⁻¹² Indeed, the exacerbation of skin inflammation in patients with AD is often a result of *S aureus* infection. *S aureus* produces a myriad of virulence factors that induce inflammatory responses and tissue injury. Staphylococcal superantigens, including the staphylococcal enterotoxins (SEs) SEA, SEB, SEC, SED, SEE, and SEI and toxic shock syndrome toxin 1 (TSST-1), can cross-link T-cell receptor V β chains with MHC II molecules on antigen-presenting cells and stimulate T-cell proliferation accompanied by massive cytokine production.^{13,14} Aside from staphylococcal superantigens, the pore-forming cytolysin α -toxin has been identified as a major staphylococcal virulence factor.¹⁵ α -Toxin monomers penetrate the plasma membrane to form cylindrical heptameric pores of approximately 2 nm.^{16,17} These pores lead to cytoplasmic leaking and osmotic swelling, ultimately resulting in cell necrosis and cell death. Recently, studies with murine infection models have demonstrated that staphylococcal α -toxin is a critical virulence factor determining the severity of *S aureus* infections.¹⁸⁻²² Infections with α -toxin-producing *S aureus* strains produce significantly larger abscesses in skin and soft tissue and are associated with significantly increased mortality in pulmonary infection models. Therapeutics targeting α -toxin, such as passive and active immunization with anti- α -toxin antibody, mutated protein, or chemicals that block α -toxin binding or genetic disruption of the α -toxin cellular receptor a disintegrin and metalloprotease 10 (*ADAM10*) gene in the epithelia of the lung and skin, have shown significantly reduced tissue damage as a result of *S aureus* infection.²¹⁻²³

A recent study indicated that patients with AD with a history of EH had a history of *S aureus* skin infection.⁵ Although severities of viral infections have been reported to be increased when associated with *S aureus* infection, the host-pathogen interactions involving bacterial modulation of epithelial cells (eg, keratinocytes) on viral invasion have not been explored. In the current study we explored whether staphylococcal toxins were involved in modulation of viral entry and replication in human keratinocytes *in vitro* and in a murine skin infection model *in vivo*.

METHODS

S aureus toxins and viral sources

Recombinant SEB and TSST-1 were purchased from Toxin Technologies (Sarasota, Fla). α -Toxin was purchased from Sigma-Aldrich (St Louis, Mo). The H35A mutation was purified, as previously described.²⁴ The Wyeth/ACAM2000 strain of VV was obtained from the Centers for Disease Control and Prevention. HSV-1 (VR-733) was purchased from American Type Culture Collection (Manassas, Va). The firefly recombinant vaccinia virus was a generous gift from Dr Bernard Moss at the National Institutes of Health/National Institute of Allergy and Infectious Diseases.

Normal human keratinocyte culture and treatment

Normal human keratinocytes (NHKs) were maintained in serum-free EpiLife Medium containing 0.06 mmol/L CaCl_2 (Cascade Biologics, Portland, Ore). Cells were seeded in 24-well plates at 1×10^5 cells/well overnight and then treated with various toxins for 20 to 24 hours, followed by inoculation of VV and HSV-1.

S aureus strains and conditional media collection

S aureus strain MNPE (Hla-WT) was isolated from a postinfluenza pulmonary toxic shock syndrome originating from a skin source and described elsewhere previously.²⁵ The Hla-KO strain is a genetically engineered strain obtained from Hla-WT, as described in a previous report.²⁶ Hla-WT and Hla-KO *S aureus* were grown in EpiLife Media without antibiotic supplements to the same concentrations (OD of 1.0 measured at a 600-nm wavelength). The culture supernatants were then collected and filtered through 0.2- μm filters before application to NHKs.

Murine skin infection model

Six-week-old female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, Me). The animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee at National Jewish Health. After anesthetizing with isoflurane, the dorsal thoraxes of all mice were shaved, and mice were randomly divided into 2 groups. One group of mice was inoculated by means of subcutaneous injection in the upper back with 1×10^3 colony-forming units of Hla-WT *S aureus* in 50 μL of PBS; the other group of mice received 1×10^3 colony-forming units of Hla-KO *S aureus* through the same route. The injection sites were marked immediately after the injection. Mice were weighed before inoculation. Two days later, VV (1×10^7 plaque-forming units) was inoculated on the same loci of *S aureus* inoculation by means of scarification. Weight of mice and size of the skin lesions were monitored daily for 7 days. Lesion sizes were calculated by using a standard formula for area (Area = Length $\times$ Width). Mice were killed 7 days after VV inoculation, and skin biopsy specimens were collected and fixed in 10% buffered formalin.

VV immunofluorescence staining

Paraffin-embedded tissues were cut at 5 μm on frosted microscope slides. Slides were deparaffinized and rehydrated by using toluene and a series of ethanol washes. After blocking with 5% BSA in Super Block (ScyTek Laboratories, Logan, Utah) containing 10% nonimmune donkey serum (Jackson Laboratories, West Grove, Pa) for 60 minutes, skin sections were stained with a rabbit anti-A27L antibody (Abcam, Cambridge, Mass) directed against an early viral protein or control rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, Calif) at 4°C overnight. Slides were then washed with PBS/0.1% Tween 20, followed by incubation with a Cy3-conjugated donkey anti-rabbit IgG (Jackson Laboratories, Bar Harbor, Me). Finally, slides were counter-stained with fluorescence isothiocyanate-conjugated wheat germ agglutinin. The immunofluorescent signal was visualized with confocal microscopy (Leica, Wetzlar, Germany). Images were collected at $\times 40$ magnification, and levels of mean fluorescence intensity were measured with Slidebook 5.0 (Intelligent Imaging innovations, Denver, Colo).

Small interfering RNA silencing experiment

ADAM10 and Silencer Negative control 1 small interfering RNA (siRNA) duplexes were purchased from Ambion (Austin, Tex). NHKs were plated in 24-well plates at 1×10^5 cells/well the day before transfection. Cells were transfected with 5 pmol of siRNA duplexes per well by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif), according to the

manufacturer's instructions. Forty-eight hours after transfection, cells were cultured in the presence and absence of α -toxin (concentration, 20 ng/mL) for an additional 20 hours. The cells were then incubated with HSV-1 (multiplicity of infection [MOI], 0.1) and VV (MOI, 0.1) for 24 hours.

Viral plaque assays

Cells and culture supernatants were harvested together after 24 hours of viral incubation to assess the production of infectious viral particles. Cells were disrupted by 3 freeze-thaw cycles. Viral yields were determined by using a titration plaque assay, as previously described.²⁷

RNA and DNA extraction and PCR analysis

Total RNA was extracted from cells with RNeasy Mini Kits (Qiagen, Valencia, Calif), according to the manufacturer's guidelines. Genomic DNA was isolated from cells with DNeasy Mini Kits (Qiagen), according to the manufacturer's instructions. RNA was reverse transcribed into cDNA by using SuperScript III Reverse Transcriptase (Invitrogen, Grand Island, NY). Real-time PCR was conducted in an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, Calif), as previously described.²⁸ The primer sequences for transcripts of VV and HSV-1 were prepared as previously described.^{29,30} Quantities of all target genes in test samples were normalized to the corresponding glyceraldehyde-3-phosphate dehydrogenase levels.

Western blot analysis

Whole-cell extracts were prepared in RIPA buffer supplemented with 1% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated by using SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif). The blots were blocked and incubated with primary and secondary antibodies. Rabbit anti-human ADAM10 was purchased from Millipore (Billerica, Calif). Monoclonal antibodies against β -actin (Sigma Chemical, St Louis, Mo) were used as protein-loading controls.

VV luciferase assay

NHKs were seeded in 96-well plates at 5×10^3 cells/well. After incubation with various sublytic concentrations of α -toxin for 20 to 24 hours, firefly luciferase recombinant vaccinia virus was added to cells at an MOI of 10, incubated for 1 hour at 4°C, and then moved to 37°C and incubated for an additional hour. After extensive washing with PBS to remove free VV, luciferase activity was measured by using a Luciferase Assay kit purchased from Promega (Madison, Wis), according to the manufacturer's guidelines.

Statistical analysis

All statistical analyses were conducted with GraphPad Prism software, version 5.03 (GraphPad, San Diego, Calif). Comparisons of expression levels were performed by using ANOVA techniques and independent-sample *t* tests as appropriate. Differences were considered significant at a *P* value of less than .05.

RESULTS

Staphylococcal α -toxin, but not superantigens, increases viral load in keratinocytes

Previous studies have suggested that NHKs are targets of staphylococcal superantigens and α -toxin.^{2,12} Thus we investigated whether these staphylococcal toxins can modulate viral replication in keratinocytes. We first determined the cytotoxicity of these toxins. NHKs treated with up to 10 μ g/mL recombinant SEB and recombinant TSST-1 did not display cell

death (data not show). We also determined the viability of NHKs to the treatment of serial dilutions of α -toxin and found that concentrations of α -toxin of less than 25 ng/mL were sublytic to NHKs (see Fig E1 in this article's Online Repository at www.jacionline.org). NHKs were treated with recombinant SEB (10 μ g/mL), recombinant TSST-1 (10 μ g/mL), α -toxin (20 ng/mL), and media alone for 24 hours and then incubated with VV (MOI, 0.1) or HSV-1 (MOI, 0.1) for an additional 24 hours. Viral gene transcription was evaluated by using real-time PCR. As shown in Fig 1, *A* and *B*, viral gene expression of VV and HSV-1 was significantly increased in NHKs pretreated with α -toxin compared with that seen in NHKs pretreated with recombinant SEB, recombinant TSST-1, and media alone.

We further used a viral plaque-forming assay to investigate whether α -toxin enhanced viable replicating virions. As shown in Fig 1, *C*, viral plaques were significantly increased after pretreatment of NHKs with 20 ng/mL α -toxin. These results demonstrated that a sublytic concentration of α -toxin significantly enhanced viral load in NHKs.

Sublytic α -toxin is the major staphylococcal virulence factor enhancing viral load in keratinocytes

Because *S aureus* produces multiple toxins beyond superantigens or α -toxin, we carried out additional experiments with the wild-type *S aureus* strain MNPE that expresses α -toxin (Hla-WT)²⁵ and its genetically engineered mutant strain that had a deletion of its α -toxin gene (Hla-KO).²⁶ Hla-WT and Hla-KO produce multiple identical exoproteins, including superantigens and proteases, except that the Hla-KO strain does not produce α -toxin.²⁶ In these experiments Hla-WT and Hla-KO *S aureus* were grown in NHK culture media overnight, and culture supernatants were then collected after the bacteria were grown to identical concentrations. The protein of α -toxin was detected only in supernatants collected from the Hla-WT strain but not in supernatants collected from the Hla-KO strain; however, TSST-1 protein was detected in the supernatants collected from both strains (Fig 2, *A*). The *S aureus* culture supernatants had no effect on the viability of NHKs (data not shown). As shown in Fig 2, *B*, viral loads of both VV and HSV-1 were significantly greater in NHKs treated with supernatants from Hla-WT *S aureus* than NHKs treated with supernatants from Hla-KO *S aureus* or sham-treated cells.

α -Toxin-producing *S aureus* enhances VV replication in murine skin

To investigate further whether our observation in NHKs was biologically important *in vivo*, we used a BALB/c murine infection model. Mice were administered subcutaneously with either 1×10^3 colony-forming units of Hla-WT *S aureus* or Hla-KO *S aureus* in the shaved backs. There was no abscess formation at the injection sites after 2 days. At this time point, we inoculated VV by means of scarification at the site of *S aureus* infection. Seven days after VV inoculation, mice were killed, and biopsy specimens of the skin lesions were taken and analyzed for VV by using immunofluorescent staining. Although there was no significant difference in body weight loss between mice that received Hla-WT *S aureus* versus mice that received Hla-KO *S aureus* (data not shown), skin lesions were significantly larger in mice receiving Hla-WT *S aureus* and VV compared with mice receiving Hla-KO *S aureus* plus VV (Fig 3, *A* and *B*). Importantly, VV immunostaining intensity was significantly greater in skin lesions from mice treated with Hla-WT *S aureus* than from mice treated with Hla-KO *S aureus* (Fig 3, *C* and *D*).

Pore formation by α -toxin is essential for its virus-enhancing effect

ADAM10 was recently identified as the cellular receptor for α -toxin in epithelial cells.^{15,31} The binding of α -toxin to ADAM10 is the initial step before penetration of the α -toxin monomer into the cell membrane and is followed by the formation of α -toxin heptameric pores. To investigate further whether the viral enhancement effect of α -toxin is ADAM10

mediated, we silenced *ADAM10* gene expression in NHKs by using a siRNA technique. As shown in Fig 4, A, ADAM10 protein was successfully knocked down in NHKs treated with *ADAM10* siRNA compared with NHKs treated with control siRNA. α -Toxin treatment did not change *ADAM10* gene expression in NHKs. In addition, silencing *ADAM10* expression diminished α -toxin heptamer formation on NHKs (Fig 4, A). Both HSV-1 and VV viral enhancement by α -toxin were blocked in ADAM10-deficient NHKs (Fig 4, B and C). These results indicated that α -toxin-mediated viral enhancement is a receptor-dependent event and requires its binding to the NHK membrane.

To further delineate whether α -toxin's pore formation is essential for its viral enhancement effect, we used the H35A mutated α -toxin to treat NHKs. Previous studies reported that mutated α -toxin 35 histidine to alanine or leucine leads to loss of function accompanied by a lack of pore formation.^{24,31} We found that although the H35A α -toxin could still form heptamers on NHKs at higher concentrations (Fig 5, A), it had no virus-enhancing effect for either VV or HSV-1 (Fig 5, B and C), even at concentrations 5 times greater than wild-type α -toxin.

α -Toxin promotes viral entry into keratinocytes

To determine the mechanism or mechanisms by which α -toxin enhances viral load in NHKs, we tested whether α -toxin treatment would lead to increased gene expression of viral entry receptors or reduced gene expression of antimicrobial peptides or other antiviral genes. We evaluated gene expression levels of the antimicrobial peptides LL37 and HBD3³²; the antiviral genes 2'5'-oligoadenylate synthetase 1 (*OAS1*), *OAS2*, *OAS3*, and double-stranded RNA-dependent protein kinase; and the HSV-1 binding receptor nectin-1 in NHKs treated with or without sublytic concentrations of α -toxin.³³⁻³⁵ Importantly, expression of these genes was not changed in NHKs treated with α -toxin (see Fig E2 in this article's Online Repository at www.jacionline.org).

Next we investigated whether α -toxin treatment increased the entry of viruses into host cells. We used a recombinant VV strain that contains the firefly luciferase gene regulated by a synthetic early promoter to determine VV entry.³⁶ The luciferase activity can be measured after 1 hour of VV entry. As shown in Fig 6, A, VV luciferase activity was significantly increased in NHKs treated with 20 ng/mL α -toxin.

Because we did not have a recombinant HSV-1 virus to determine entry, we performed a kinetic assay of HSV-1 replication in NHKs in the presence and absence of acyclovir, an HSV-1 polymerase inhibitor that blocks HSV replication. As shown in Fig 6, B, in the absence of acyclovir, HSV-1 DNA copies significantly increased with increasing time of viral incubation. Cells treated with α -toxin had higher numbers of HSV-1 DNA copies compared with those seen in control cells. In the presence of acyclovir, HSV-1 DNA copies did not increase with longer periods of viral incubation because viral DNA replication was inhibited; however, cells treated with α -toxin still had greater numbers of HSV-1 DNA copies compared with those seen in control cells without α -toxin treatment. These data indicate that original viral entry is greater in cells treated with α -toxin and demonstrated that α -toxin treatment promoted viral entry.

DISCUSSION

As the threat of a terrorist attack with smallpox as a bioweapon became a serious public health concern, the National Institute of Allergy and Infectious Diseases funded the multicenter Atopic Dermatitis and Vaccinia Network to investigate mechanisms resulting in EV and to identify the subgroup of patients with AD at high risk of disseminated viral infection. Publications through the Atopic Dermatitis and Vaccinia Network have suggested

that EH is a complex disease determined by aberrant immunologic responses and defects in the skin barrier. EH was found to be associated with genetic variants in skin terminal differentiation markers, including filaggrin and claudin-1, genes of the interferon-signaling pathway, including *IFNAR1*, *IFNGR1*, *IRF2*, and T_H2-promoting cytokine thymic stromal lymphopoietin.³⁷⁻⁴¹ These genetic traits can lead to easy penetration of allergens and an enhanced T_H2 response. In addition, reduction in the expression of transcription factor specificity protein 1, leading to decreased expression of the antiviral genes *OAS2* and double-stranded RNA-dependent protein kinase in skin biopsy specimens from patients with EH, might also contribute to the increased propensity for disseminated viral infections.²⁷ Beck et al⁵ recently showed that *S aureus* might be an important environmental factor associated with EH. This observation prompted us to investigate whether *S aureus* colonization and infections contribute to the pathogenesis of EH and EV.

In the current study we first tested whether staphylococcal superantigens and sublytic α -toxin alter the viral loads of HSV-1 and VV. We found that sublytic α -toxin, but not superantigens, significantly increases viral loads in keratinocytes. Using cell culture supernatants collected from an α -toxin-producing *S aureus* strain and an isogenic α -toxin-deleted strain, we further demonstrated that sublytic α -toxin is the major virulence factor enhancing the viral load of HSV-1 and VV in NHKs. Importantly, these *in vitro* observations were confirmed *in vivo* in a murine skin infection model. We inoculated a small number of live *S aureus* bacteria into BALB/c murine skin to mimic the condition of skin colonization and mild infection, which are often seen in the skin of patients with AD^{2,12}; we then inoculated VV by means of scarification at the site of *S aureus* infection. We found that VV replication was significantly increased in murine skin with prior inoculation of α -toxin-producing *S aureus* strain compared with that seen in murine skin with prior inoculation of the isogenic α -toxin knockout *S aureus* strain. These results highlight a mechanism through which staphylococcal α -toxin might increase the risk of patients with AD to have life-threatening disseminated viral skin infections and is relevant to conditions such as EH, which is often associated with *S aureus* colonization or infection.⁵ In addition, we postulate that α -toxin promotes influenza virus infection in lung epithelial cells in which fulminate influenza-induced pneumonias are accompanied by *S aureus* infection.^{25,42-44} Our studies are likely to open many new avenues of research as investigators evaluate the influence of numerous bacteria and their virulence factors on multiple viral infections.

Recently, ADAM10 has been identified as a functional receptor on epithelial cells for α -toxin.^{15,23,31} Consistent with this recent report, we found that silencing the gene expression of *ADAM10* blocked α -toxin-induced viral enhancement in NHKs. The requirement of a functional cellular receptor indicates that α -toxin-mediated viral enhancement acts on host cell receptors rather than through direct effects on viruses. In addition, this result demonstrates that the virus-enhancing effect is specifically mediated by α -toxin through its receptor, ADAM10, thus excluding the possibility that other contaminating staphylococcal proteins are causing this effect.

Previous studies have found that the³⁵ histidine of α -toxin is critical for its cytotoxicity. The H35A and H35L mutated α -toxins have no cytolytic function. Liang et al²⁴ demonstrated that the H35A mutated α -toxin could internalize but could not form pores in the cell plasma membrane. Interestingly, preincubation of the H35A mutated α -toxin with cells could block wild-type α -toxin-induced cell death, suggesting that the H35A mutated α -toxin can either saturate the cellular binding sites for α -toxin or interact with wild-type α -toxin to interfere with its function. We found that the H35A mutated α -toxin had no virus-enhancing effect despite the fact that it still formed heptamers on the cell membrane. In line with the previous finding that the H35A mutated α -toxin could not form pores, our data

suggest that pore formation of α -toxin on cell membrane is important for its virus-enhancing effect.

In the current study we demonstrated that entry of both VV and HSV-1 was significantly increased in sublytic α -toxin-treated NHKs, suggesting that there might be a common mechanism resulting in enhanced entry of the 2 viruses that is most likely the result of altered host cell membrane structure. Aside from the proteinaceous receptor ADAM10, the cholesterol/sphingomyelin-rich domain of cell plasma membrane is also indispensable for α -toxin binding and function.⁴⁵ Interestingly, previous studies demonstrated that the entry of both VV and HSV-1 requires the participation of cholesterol/sphingomyelin domains.^{46,47} We postulate that the incorporation of α -toxin in the keratinocyte membrane might change the cell plasma membrane structure and enhance formation of cholesterol/sphingomyelin domains with pores, thus leading to increased opportunity for viral entry. In addition, the protease activity of ADAM10 can be activated even by sublytic concentrations of α -toxin, leading to cleavage of E-cadherin, an important component for epithelial adherens junctions.²³ Loss of cell-surface expression of E-cadherin would cause decreased keratinocyte cohesion and subsequently lead to exposure of receptors for viral binding.⁴⁸

In summary, the current study demonstrates that sublytic α -toxin enhances viral load in NHKs by promoting viral entry into host epithelial cells. This action is mediated by its receptor, ADAM10. These data advance our understanding of the virulence of α -toxin in *S. aureus* infections and its possible role in the promotion of disseminated viral infections. Identification of the molecular targets by which staphylococcal toxins promote viral replication will provide new therapeutic strategies to interfere with the microbial subversion of host defense.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AD	Atopic dermatitis
ADAM10	A disintegrin and metalloprotease 10
EH	Eczema herpeticum
EV	Eczema vaccinatum
HSV-1	Herpes simplex virus 1
MOI	Multiplicity of infection
NHK	Normal human keratinocyte
OAS	2'5'-Oligoadenylate synthetase
SE	Staphylococcal enterotoxin
siRNA	Small interfering RNA
TSST-1	Toxic shock syndrome toxin 1
VV	Vaccinia virus

Key messages

- *S aureus* α -toxin, but not superantigens, increases viral load in NHKs.
- VV load is increased in murine skin inoculated with α -toxin-producing *S aureus*.
- The pore-forming property of α -toxin is essential for its virus-enhancing effect.
- Sublytic *S aureus* α -toxin promotes viral entry into keratinocytes.

FIG 1. Staphylococcal α-toxin, but not superantigens, enhances viral load in NHKs. NHKs were pretreated with indicated *S aureus* superantigens and α-toxin, followed by VV (MOI, 0.1) and HSV-1 (MOI, 0.1) inoculations. **A** and **B**, The viral mRNA expression levels of HSV-1 (Fig 1, **A**) and VV (Fig 1, **B**) were evaluated by using real-time PCR. **C**, Viral plaque formation was determined by using the viral plaque assay. The *left panel* shows crystal violet staining of viral plaques; the *right panel* shows quantitative results of viral plaques. All data are presented as mean ± SEM values. Data from 1 representative experiment of 3 independent experiments performed are shown. ** $P < .01$ and *** $P < .001$.

FIG 2. Sublytic α -toxin is the major staphylococcal virulent factor enhancing viral load in keratinocytes. **A**, Western blot showing α -toxin and TSST-1 protein expression in Hla-WT and Hla-KO *S. aureus* culture supernatants. **B**, Viral gene expression in NHKs pretreated with supernatants collected from sham, Hla-WT, and Hla-KO *S. aureus* cultures. Data are presented as mean $\pm$ SEM values. Data from 1 representative experiment of 3 independent experiments performed are shown. ** $P < .01$ and *** $P < .001$.

FIG 3.

VV load is greater in murine skin inoculated with Hla-WT *S aureus* strain than with Hla-KO *S aureus* strain. **A**, Representative picture of murine skin lesions at day 7 of VV inoculation. **B**, Quantitative results of the sizes of murine skin lesions at day 7 of VV inoculation. Data are presented as mean $\pm$ SEM values. **C**, Representative images of VV immunofluorescent staining of murine skin lesion biopsy specimens taken at day 7 after VV inoculation (magnification $\times 40$). **D**, VV immunofluorescent intensity. Data are expressed as mean $\pm$ SEM values. *MFI*, Mean fluorescence intensity.

FIG 4.

Silencing *ADAM10* expression blocks sublytic α -toxin's effect on viral enhancement. **A**, Western blot showing ADAM10 protein level and α -toxin in indicated conditions. β -actin was used as a loading control. **B**, HSV-1 mRNA expression in indicated conditions. **C**, VV mRNA expression in indicated conditions. Data are presented as mean $\pm$ SEM values. Data from 1 representative experiment of 3 independent experiments performed are shown.

FIG 5.

H35A mutated α -toxin has no viral enhancement effect. NHKs were treated with indicated concentrations of wild-type (*WT*) α -toxin and H35A mutated α -toxin for 20 hours followed by 24 hours of viral incubation. Cells were then harvested for Western blot assay and real-time PCR. **A**, Western blot showing α -toxin in indicated conditions. β -actin was used as a loading control. **B** and **C**, HSV-1 (Fig 5, *B*) and VV (Fig 5, *C*) mRNA expression in indicated conditions. Data are presented as mean $\pm$ SEM values. Data from 1 representative experiment of 3 independent experiments performed are shown.

FIG 6.

α -Toxin promotes viral entry into keratinocytes. **A**, VV luciferase activity was measured in α -toxin-pretreated NHKs after incubation with VV luciferase at an MOI of 10 for 1 hour. **B**, NHKs were pretreated with or without α -toxin (20 ng/mL) for 20 hours, and cells were then subjected to 100 μ mol/L acyclovir treatment for 2 hours. Finally, HSV-1 (MOI, 0.05) was added into the cells for additional 5- and 10-hour incubations. Intracellular HSV-1 DNA copies were evaluated by using real-time PCR. Data are presented as mean $\pm$ SEM values. Data from 1 representative experiment of 3 independent experiments performed are shown.