Published in final edited form as:

J Allergy Clin Immunol. 2013 February ; 131(2): 421–7.e1-2. doi:10.1016/j.jaci.2012.10.030.

Filaggrin-dependent secretion of sphingomyelinase protects against staphylococcal α -toxin–induced keratinocyte death

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Abstract

Background—The skin of patients with atopic dermatitis (AD) has defects in keratinocyte differentiation, particularly in expression of the epidermal barrier protein filaggrin. AD skin lesions are often exacerbated by *Staphylococcus aureus*–mediated secretion of the virulence factor α -toxin. It is unknown whether lack of keratinocyte differentiation predisposes to enhanced lethality from staphylococcal toxins.

Objective—We investigated whether keratinocyte differentiation and filaggrin expression protect against cell death induced by staphylococcal α -toxin.

Methods—Filaggrin-deficient primary keratinocytes were generated through small interfering RNA gene knockdown. RNA expression was determined by using real-time PCR. Cell death was determined by using the lactate dehydrogenase assay. Keratinocyte cell survival in filaggrin-deficient (*fl/fl*) mouse skin biopsies was determined based on Keratin 5 staining. α -Toxin heptamer formation and acid sphingomyelinase expression were determined by means of immunoblotting.

Results—We found that filaggrin expression, occurring as the result of keratinocyte differentiation, significantly inhibits staphylococcal α -toxin–mediated pathogenicity. Furthermore, filaggrin plays a crucial role in protecting cells by mediating the secretion of sphingomyelinase, an enzyme that reduces the number of α -toxin binding sites on the keratinocyte surface. Finally, we determined that sphingomyelinase enzymatic activity directly prevents α -toxin binding and protects keratinocytes against α -toxin–induced cytotoxicity.

Conclusions—The current study introduces the novel concept that *S aureus* α -toxin preferentially targets and destroys filaggrin-deficient keratinocytes. It also provides a mechanism to explain the increased propensity for *S aureus*–mediated exacerbation of AD skin disease.

Keywords

Atopic dermatitis; skin barrier; filaggrin; acid sphingomyelinase; Staphylococcus aureus; α -toxin

Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with significant morbidity.^{1,2} AD skin lesions are frequently exacerbated by *Staphylococcus aureus*;

however, the mechanism underlying this increased host skin susceptibility to *S aureus* is poorly understood. The most prominent and destructive virulence factor produced by *S aureus* is α -toxin,³ a tissue-damaging cytolysin that is often found on the skin of patients with severe AD.⁴ α -Toxin induces cell death by forming heptameric pores that penetrate through the lipid bilayer, lysing the host cells⁵ and ultimately causing tissue destruction.⁶⁻⁹ Studies indicate that both protein- and lipid-based receptors on the host cell are involved in α -toxin binding and pore formation. One receptor is sphingomyelin,¹⁰ a constituent of the membrane lipid bilayer. The other known receptor is the protein a disintegrin and metalloprotease 10 (ADAM10).^{11,12} Sensitivity to cell death induced by α -toxin can be directly modulated by changes in the expression of either one of these receptors.¹⁰⁻¹²

Because there is a strong association between *S aureus* and atopic skin, we were interested in determining whether there is a molecular alteration in atopic skin that causes increased skin cell sensitivity to α -toxin. Recent studies on the pathophysiology of AD have highlighted a defect in the keratinocyte differentiation program.^{13,14} The deficiency in differentiation has been attributed to increased expression of the inflammatory T_H2 cytokines IL-4 and IL-13.^{14,15} In addition, genetic studies have revealed that null mutations in the epidermal skin barrier protein filaggrin¹⁶ are frequently associated with AD.¹⁶⁻²³ Filaggrin is normally expressed at late stages of the differentiation process, helping to form the epidermal barrier.¹⁶ In atopic skin keratinocyte differentiation is impaired, and levels of filaggrin are significantly reduced.¹⁴ These clinical observations prompted us to hypothesize that factors involved in the keratinocyte differentiation process, particularly filaggrin expression, might modulate keratinocyte sensitivity to *S aureus* α -toxin. Therefore the current study was carried out to assess the relative role of keratinocyte differentiation in protection of host cells against the damaging effects of staphylococcal α -toxin and to determine key factors that contribute to host defense.

Methods

Quantitative real-time RT-PCR

Total RNA was isolated by using RNeasy Mini Kits (Qiagen, Valencia, Calif), according to the manufacturer's protocol. One microgram of RNA was reverse transcribed with the Qiagen Quantiscript kit, according to the manufacturer's protocol. Real-time PCR was performed and analyzed by using the dual-labeled fluorogenic probe method with an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, Calif). Primers and probes for human actin, filaggrin, loricrin, involucrin, sphingomyelin phosphodiesterase (sphingomyelinase [SMPD]) 1, and SMPD2 were purchased from Applied Biosystems. Amplification reactions were performed in Micro-Amp optical tubes (Applied Biosystems) in a 25- μ L volume, as previously described.¹⁴ All reactions were normalized to β -actin.

Cell staining and microscopy

Immunofluorescence staining was performed on 5- μ m thin sections of formalin-fixed, paraffin-embedded skin biopsy specimens. Slides were deparaffinized with xylene/ethanol. After blocking, sections were incubated with primary Keratin 5 (K5) antibody (Covance, Princeton, NJ) overnight. Secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa) was added according to the manufacturer's protocol. Images were taken with a Leica Microscope (Leica, Wetzlar, Germany) with SlideBook software (Intelligent Imaging Innovations, Denver, Colo).

Keratinocyte cell culture

Primary human keratinocytes (Cascade Biologics, Portland, Ore) were grown in serum-free keratinocyte growth medium (EpiLife, Cascade Biologics), with 1% human keratinocyte

growth supplement (Cascade Biologics), 0.05 mmol/L CaCl₂ and 1% penicillin and streptomycin. When indicated, cells were treated with α -toxin (Sigma, St Louis, Mo) for 24 hours. For the lactate dehydrogenase assay, keratinocytes were plated in quadruplicate at 20,000 per well in a 96-well plate and allowed to adhere overnight. Cells were treated with media containing 1.0 mmol/L calcium to induce differentiation. Lactate dehydrogenase release was quantitated by using the Cyto-Tox One Kit from Promega (Madison, Wis), according to the manufacturer's instructions. Statistical significance was calculated by using the 2-tailed Student *t* test.

Small interfering RNA transfection

Third-passage keratinocytes of 50% to 60% confluence were transfected according to the manufacturer's instructions by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) with 20 μ mol/L small interfering RNA (siRNA; Dharmacon, Lafayette, Colo) in antibiotic-free media.

Assay for acid sphingomyelinase activity

The Amplex Red Sphingomyelinase Assay kit (Invitrogen) was used to determine activity in the cultured keratinocyte supernatants. The supernatants of control or filaggrin-silenced cells were harvested. The supernatants were assayed in triplicate in 96-well format according to the manufacturer's protocol. For each assay, background was determined by also performing the assay in the absence of the substrate sphingomyelin. Fluorescence was measured with a fluorescence microplate reader by using excitation at 530 nm and detection at 590 nm.

Immunoblot analysis

Acid sphingomyelinase antibody was from Abcam (Cambridge, Mass), and α -toxin antibody was from Sigma. For analysis of α -toxin heptamerization, cells growing as a monolayer were treated with α -toxin for 1 hour, washed with PBS, and harvested (without trypsin) in Triton lysis buffer and 1% Triton X-100 containing protease inhibitor (Complete; Roche, Mannheim, Germany). Cellular debris was pelleted by means of centrifugation, clarified lysates were resuspended in Laemmli buffer, and proteins were resolved on a 5% to 15% gradient gel (Bio-Rad Laboratories, Hercules, Calif). The α -toxin heptamer is stable during electrophoresis.²⁴ Proteins were transferred to a polyvinylidene difluoride membrane, blotted with a rabbit α -toxin toxin antibody (Sigma), and detected by means of enhanced chemiluminescence (Amersham, Piscataway, NJ).

Sphingomyelinase treatment

Undifferentiated keratinocytes were preincubated for 1 hour with 0.3 U/mL sphingomyelinase from *Bacillus cereus* (Sigma) before the addition of α -toxin.

Mice

Flaky tail (*ft/ft*) filaggrin-deficient mice²⁵ were originally from the Jackson Laboratories. Flaky tail and BALB/c control animals were killed, and 6 mmol/L punch biopsy specimens were harvested from the skin. Skin samples were cultured for 24 hours in media alone or in media containing 500 ng/mL α -toxin. After incubation, biopsy specimens were fixed, paraffin embedded, and sectioned. All mice were kept in a specific pathogen-free environment, and procedures were performed in accordance with the Animal Care and Use Committee at National Jewish Health.

Statistical analyses

All statistical analysis was conducted with GraphPad Prism software (GraphPad Software, La Jolla, Calif). Comparisons of expression levels were performed by using ANOVA techniques and Student *t* tests, as appropriate.

Results

Differentiated human epidermal keratinocytes are protected against staphylococcal α -toxin-mediated cell death

To determine whether keratinocyte differentiation affects sensitivity to staphylococcal α -toxin, primary human epidermal keratinocytes (HEKs) were differentiated *in vitro* by increasing the calcium concentration. Differentiation was monitored over an 8-day period by quantitating the induction of differentiation-specific markers. Levels of genes uniquely expressed in differentiated keratinocytes, including involucrin, loricrin, and filaggrin,¹⁶ were measured by using real-time PCR and normalized to β -actin levels (Fig 1, A). Over the course of 8 days, these markers increased from 100- to 1000-fold compared with those in undifferentiated cells.

The primary virulence factor responsible for inducing host cell death in *S aureus* infection is α -toxin.^{3,5} In Fig 1, B, we show that undifferentiated keratinocytes were highly sensitive to the toxic effects of α -toxin. In contrast, cells differentiated for 7 to 8 days were markedly resistant to the lethal effects of α -toxin. A dose response demonstrated that undifferentiated cells were sensitive to α -toxin doses as low as 1 nmol/L, whereas differentiated cells were resistant at doses that were 15-fold greater (Fig 1, C). Therefore the differentiation process protects keratinocytes against α -toxin cytotoxicity.

Filaggrin-deficient epidermal keratinocytes have increased sensitivity to α -toxin-induced cell death

To determine whether expression of differentiation-specific barrier proteins affects sensitivity to α -toxin, HEKs were transduced with siRNA directed against filaggrin, loricrin, or involucrin. Five days after siRNA transfection, 85% to 90% knockdown of these respective genes was demonstrated by using RT-PCR (see Fig E1 in this article's Online Repository at www.jacionline.org). Strikingly, filaggrin gene silencing, but not loricrin or involucrin gene silencing, was associated with a potent increase in α -toxin-mediated cell death (Fig 2, A). Although differentiated keratinocytes were resistant to α -toxin, filaggrin deficiency resulted in loss of protection. Therefore expression of filaggrin protects against α -toxin-mediated cytotoxicity.

To determine the effects of α -toxin on filaggrin-deficient epidermis, we examined skin biopsy specimens from wild-type (WT) and filaggrin-deficient (*fl/fl*) mice²⁶ treated with media alone or media containing α -toxin. Immunofluorescence shows the staining of untreated basal layer (K5⁺) cells in the epidermis of WT and filaggrin-deficient (*fl/fl*) epidermis (Fig 2, B). After 24 hours of α -toxin treatment, the reduction in the number of K5⁺ cells in the epidermis of filaggrin-deficient (*fl/fl*) mice was significantly greater than in WT animals (Fig 2, B). Furthermore, breakdown of the skin architecture was more apparent in the filaggrin-deficient mice (Fig 2, B). Quantification of results (Fig 2, C) showed that the mean number of K5⁺ cells per millimeter in WT skin decreased 19% after α -toxin treatment, whereas *fl/fl* skin showed a 63% cell loss after α -toxin treatment. Therefore filaggrin-deficient skin was more sensitive to the lethal effects of α -toxin than WT skin, demonstrating that filaggrin plays a critical role in protecting keratinocytes *in vivo* from the lethal effects of α -toxin.

Filaggrin-deficient keratinocytes have impaired secretion of acid sphingomyelinase

Our observations above demonstrate that keratinocyte differentiation results in blockade of α -toxin–induced cytotoxicity, suggesting that a receptor for α -toxin could be down regulated during the differentiation process. There are 2 known receptors for α -toxin. One receptor is the membrane lipid sphingomyelin,¹⁰ and the other receptor is the protein ADAM10.¹² Our results indicate that ADAM10 expression is not significantly modulated during keratinocyte differentiation (see Fig E2 in this article's Online Repository at www.jacionline.org). In marked contrast, cell differentiation is known to reduce the expression of sphingomyelin,^{16,27} the other α -toxin receptor.

Because reduction in sphingomyelin is primarily mediated through the enzymatic activity of sphingomyelinase,²⁸ expression of this enzyme could directly determine host cell sensitivity to α -toxin. Fig 3, A, shows that a potent, 15-fold increase in mRNA expression of acid sphingomyelinase (SMPD1) occurred upon keratinocyte differentiation. Fig 3, A, also shows that filaggrin silencing partially inhibits SMPD1 mRNA expression. In contrast, increases in neutral sphingomyelinase (SMPD2) mRNA expression were not detected (Fig 3, B). We next determined whether acid sphingomyelinase protein expression was similarly increased upon differentiation. We found that undifferentiated cells secrete very little acid sphingomyelinase, whereas differentiated cells showed dramatic increases in secretion of this enzyme (Fig 3, C, compare lanes 5 and 6). In contrast, intracellular levels of acid sphingomyelinase were relatively unchanged by differentiation (Fig 3, C, lanes 1-4). Importantly, sphingomyelinase secretion was significantly reduced in filaggrin-silenced cells (Fig 3, C, compare lanes 6 and 8). Therefore we show that the secretion of acid sphingomyelinase by differentiated cells is markedly inhibited by filaggrin deficiency. The acid sphingomyelinase activity in the supernatants was measured (Fig 3, D) to confirm a reduction in secretion in differentiated filaggrin siRNA–silenced cells. Consistent with the reduction in protein levels, acid sphingomyelinase activity was also significantly less in the supernatants from differentiated filaggrin-deficient cells. These results confirm that filaggrin silencing reduces both the amount and activity of secreted sphingomyelinase.

Sphingomyelinase protects keratinocytes against α -toxin binding and cell death

We next determined whether sphingomyelinase can protect against α -toxin–induced cell death. Undifferentiated keratinocytes were treated exogenously with sphingomyelinase, a treatment known to convert 95% of surface sphingomyelin into ceramide without adversely affecting cell viability.²⁹ Although undifferentiated keratinocytes were highly sensitive to α -toxin, cells pretreated with sphingomyelinase were resistant to α -toxin–induced host cell necrosis (Fig 4, A). Pretreatment of filaggrin-silenced cells with sphingomyelinase (Fig 4, B) also protected these cells against α -toxin–induced cell death. These results indicate that sphingomyelinase activity was sufficient to protect both control and filaggrin-deficient keratinocytes against α -toxin and suggest that sphingomyelinase causes reduced expression of an α -toxin receptor. The number of α -toxin receptors is reflected by the amount of α -toxin heptamer bound to the host cell surface.^{10,11} Therefore we analyzed α -toxin binding to control and filaggrin-deficient cells treated with sphingomyelinase (Fig 4, C). In comparison with untreated cells, control cells pretreated for an hour with sphingomyelinase bound significantly less α -toxin heptamer (Fig 4, C, compare lanes 2 and 3 with lanes 5 and 6). Parallel studies performed with filaggrin-silenced cells showed similar results. In all cases sphingomyelinase pretreatment resulted in significantly less α -toxin heptamer bound to the cells (Fig 4, C, compare lanes 8 and 9 with lanes 11 and 12). The reduced binding demonstrates that sphingomyelinase dramatically reduces the number of α -toxin receptors on the cell surface.

Discussion

Damaging effects caused by the pathogen *S aureus* are driven by secretion of α -toxin. Although the cell death induced by α -toxin has long been described, very little is known about the host defense mechanisms that protect against α -toxin-mediated pathogenicity. We report here that filaggrin protects epidermal keratinocytes against the lethal effects of α -toxin. Furthermore, we show that undifferentiated human keratinocytes become resistant to cell death upon differentiation and expression of filaggrin. Finally, our studies with *fl/fl* mice confirm that α -toxin is particularly lethal to epidermal cells deficient in filaggrin expression. Our results therefore demonstrate a novel function for filaggrin in protection against staphylococcal α -toxin-induced cell death.

We propose that cell differentiation induces changes at the molecular level that protect the cell against α -toxin. As keratinocytes differentiate, expression of filaggrin, a protein critical for epidermal skin barrier function,²⁰ is induced. Filaggrin plays a key role in excluding foreign compounds and protecting the inner basal cells and underlying tissue against damage. However, filaggrin-deficient or atopic skin has flaws in the barrier that allow proteins to penetrate into the epidermis.^{25,26,30-32}

In addition to filaggrin, we find that the keratinocyte differentiation process also leads to a 15-fold increase in expression of acid sphingomyelinase mRNA. In addition to these increases in gene expression, levels of secreted protein are also significantly increased during the process of keratinocyte differentiation. Our data demonstrate that in comparison with undifferentiated cells, acid sphingomyelinase is efficiently secreted only in differentiated keratinocytes. Importantly, we also demonstrate that filaggrin plays a critical role in this process because differentiated filaggrin-silenced cells are unable to efficiently secrete sphingomyelinase.

Recent studies have indicated that filaggrin is required for efficient secretion of lamellar bodies.^{30,32} These organelles contain lipid-modifying enzymes³³ that on secretion, alter the membrane surface and promote barrier function. Filaggrin-deficient cells are demonstrably less efficient in secretion of lamellar bodies.^{30,32} Therefore the reduced secretion of lamellar bodies likely accounts for the reduced secretion of sphingomyelinase that we observe in filaggrin-deficient cells.

Previous studies have demonstrated that sphingomyelinase is the predominant enzyme that cleaves cell-surface sphingomyelin into ceramide and phosphocholine.²⁸ Sphingomyelinase-mediated cleavage of sphingomyelin reduces the number of α -toxin binding sites on the cell surface. Our data directly demonstrate that sphingomyelinase enzymatic activity prevents α -toxin binding and protects keratinocytes against α -toxin-induced cytotoxicity. Filaggrin plays a crucial role in this process by mediating the secretion of sphingomyelinase, an enzyme that reduces α -toxin binding. By mediating secretion of sphingomyelinase, filaggrin provides a crucial function in protection of the host skin against the destructive consequences of α -toxin and *S aureus*.

Filaggrin deficiency is the primary feature of AD, a skin disease characterized by loss of barrier function. In addition to reduced levels of filaggrin, AD skin also has reduced levels of important hydrophobic lipids, including ceramides.^{34,35} Sphingomyelinase is the primary enzyme responsible for producing ceramide,²⁸ and levels of this enzyme are reportedly decreased in atopic skin.³⁶ These results are consistent with our data demonstrating that efficient secretion of sphingomyelinase hinges on filaggrin expression. Reduced secretion of sphingomyelinase and reduced production of ceramide in filaggrin-deficient cells might contribute to the increased antigen penetration also observed in these cells.^{25,26,30-32}

AD skin lesions are frequently colonized with α -toxin-producing *S aureus*.⁴ Our current study demonstrates that filaggrin deficiency also enhances the cytotoxic effects of α -toxin, further compromising the epidermal barrier and exacerbating the disease state. Although very rare in normal skin, the majority of atopic patients have appreciable levels of *S aureus* growing on their skin surface^{37,38} that produces α -toxin.⁴ Multiple lines of evidence suggest that α -toxin is the primary agent released by *S aureus* that triggers the keratinocytic lesions associated with AD.^{4,38} Confirming evidence is shown here in a mouse model system because generation of dermal lesions is induced by α -toxin. Although α -toxin-producing strains cause severe dermonecrotic skin lesions, there is little or no dermonecrosis in mice infected with *S aureus* strains deficient in α -toxin.^{8,39}

α -toxin penetration through the skin requires a host microenvironment with deficient barrier function. The skin from patients with acute AD expresses increased levels of inflammatory T_H2 cytokines,¹⁴ which not only inhibit filaggrin expression but also cause inflammation. The increased inflammation contributes to breakdown of tight junction-based adhesive and scaffolding proteins that comprise a second barrier in the epidermis.^{13,40}

The current report is the first description of naturally occurring events that determine vulnerability of host skin cells to *S aureus* α -toxin-induced cell death. Indeed, it is apparent that filaggrin expression, occurring as the result of keratinocyte differentiation, significantly inhibits staphylococcal α -toxin-mediated pathogenicity. Furthermore, we have determined that filaggrin plays a crucial role in mediating the secretion of sphingomyelinase, an enzyme that reduces the number of α -toxin binding sites on the cell surface. Finally, we have demonstrated directly that sphingomyelinase enzymatic activity prevents α -toxin binding and protects keratinocytes from α -toxin-induced cytotoxicity. By mediating secretion of sphingomyelinase, filaggrin provides a crucial function in protection of the host skin against the destructive force of α -toxin and *S aureus* infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Shih-Yun Lyman for her assistance in the preparation of this manuscript. We also thank Cliff Hall and Joanne Streib for excellent technical support.

Supported by National Institutes of Health (NIH) grant R01 AR41256 and the Atopic Dermatitis Research Network (NIH/National Institute of Allergy and Infectious Diseases [NIAID] contract NIH/NIAID HHSN272201000020C).

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Abbreviations used

AD	Atopic dermatitis
ADAM10	A disintegrin and metalloprotease 10
HEK	Human epidermal keratinocyte
K5	Keratin 5

siRNA	Small interfering RNA
SMPD	Sphingomyelin phosphodiesterase (sphingomyelinase)
WT	Wild-type

Key messages

- *Staphylococcus aureus* α -toxin preferentially targets and destroys filaggrin-deficient or undifferentiated keratinocytes.
- Filaggrin plays a crucial role in protecting cells by mediating the secretion of sphingomyelinase, an enzyme that reduces the number of α -toxin binding sites on the keratinocyte surface.
- The epidermis of filaggrin-deficient (*fl/fl*) mice shows increased cell damage in response to α -toxin.
- The study provides a mechanism to explain the increased propensity for *S. aureus*-mediated exacerbation of AD skin disease.

Fig 1.

Differentiated HEKs are resistant to staphylococcal α -toxin-mediated cell death. **A**, Primary HEKs were differentiated for the indicated times (0-8 days), and levels of filaggrin (*FLG*; circles), loricrin (*LOR*; squares), and involucrin (*IVL*; triangles) were measured by using quantitative RT-PCR. Fold induction was determined relative to time 0 (undifferentiated cells). **B**, HEKs were differentiated for the indicated times (0-8 days) and then treated for an additional 24 hours with media alone or media containing 8 nmol/L α -toxin. Cell death was measured based on lactate dehydrogenase (*LDH*) release. **C**, α -toxin dose-response curve in undifferentiated (diamonds) and calcium-differentiated HEKs (squares). Statistical analysis was performed with the Student *t* test. Error bars delineate the SEM of 4 replicates. Significant decreases in α -toxin-induced cell death were observed after 5, 7, and 8 days of calcium-induced differentiation. ***P* < .0001 versus undifferentiated cells.

Fig 2.

Filaggrin deficiency increases α -toxin-induced cell death. Primary HEKs were transfected with control, filaggrin (*FLG*), involucrin (*IVL*), or loricrin (*LOR*) siRNA and then differentiated for the indicated times. α -toxin (1.5 nmol/L) or medium was added for 24 hours, and cell death was determined based on lactate dehydrogenase (*LDH*) release. Error bars delineate the SEM of 4 replicates. The increase in cell death in *FLG* siRNA-treated cells versus control cells was statistically significant. $**P < .0001$. **B**, Biopsy specimens from WT or *ft/ft* (filaggrin-deficient) mice were treated for 24 hours with media alone or media containing 15 nmol/L α -toxin. Biopsy specimens were visualized by means of immunofluorescence microscopy. Nuclei (blue, DAPI, 4'-6-diamidino-2-phenylindole), epidermal architecture (green, wheat germ agglutinin), and keratinocytes (red, K5) are indicated. **C**, Quantitation of the number of K5⁺ keratinocytes for both WT and *ft/ft* skin in the presence and absence of α -toxin. In WT skin a 19% reduction in K5⁺ cell numbers occurred after α -toxin treatment (mean with media: 133.2 ± 7.11 cells/mm [open triangles]; mean with α -toxin: 108.0 ± 12.73 cells/mm). In contrast, *ft/ft* skin showed a 63% reduction in K5⁺ cell numbers after α -toxin treatment (mean with media: 173.6 ± 11.93 cells/mm; mean with α -toxin: 63.56 ± 12.51 cells/mm). Error values represent the SEM. The increased cell loss in filaggrin-deficient epidermis was statistically significant. $*P = .032$.

Fig 3.

Filaggrin-silenced keratinocytes have reduced secretion of acid sphingomyelinase. HEKs were transfected with control or filaggrin (*FLG*) siRNA and then left undifferentiated or differentiated with calcium for 5 days. **A** and **B**, Levels of acid sphingomyelinase (SMPD1; Fig 3, A) and neutral sphingomyelinase (SMPD2; Fig 3, B) were measured by using quantitative RT-PCR. Fold induction was determined relative to undifferentiated cells and normalized to actin. **C**, Cell lysates and supernatants were collected from undifferentiated (*U*) and differentiated (*D*) cells. Acid sphingomyelinase (SMPD1) protein levels were measured by means of immunoblotting. **D**, Cell-culture supernatants were analyzed for acid sphingomyelinase activity by using the Amplex Red Sphingomyelinase Assay kit and quantitated by means of fluorescence.

Fig 4. Sphingomyelinase protects keratinocytes against the lethal effects of α -toxin. **A** and **B**, HEKs transfected with control (Fig 4, A) or filaggrin (*FLG*) siRNA (Fig 4, B) were either left untreated or treated with 0.3 Units/mL sphingomyelinase for 1 hour. Untreated (diamonds) and sphingomyelinase-treated (squares) keratinocytes were then treated for an additional 24 hours with media containing the indicated concentrations of α -toxin. Cell death was measured based on lactate dehydrogenase (LDH) release. **C**, Control or FLG siRNA-transfected HEKs were left untreated or treated with sphingomyelinase for 1 hour. Cells were then incubated with α -toxin for 1 additional hour. α -toxin heptamer binding was determined by immunoblotting the cell lysates.