Resolution of Eczema with Multivalent Peptides

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The integrity of the skin is an important aspect of QOL. Whether caused by genetic deficiencies or environmental insults, disruption of the surface barrier allows irritants and allergens to penetrate the skin, which initiates inflammatory responses by immune cells that often lead to life-long allergies. In this study, eczema was induced on depilated mouse skin with topical lipopolysaccharide or a mixture of Staphylococcal enterotoxin B and an extract of house dust mites, which resulted in thickening of the epidermis, epidermal disruption, and abundant neutrophils in the dermis. Within 14 days of topical treatment with 1 µM svL4, a tetravalent peptide, neutrophils were absent, and the epidermis had returned to a normal morphology. The sequence of svL4 contains glutamine residues that serve as a cross-linking substrate for transglutaminase 2, which gains access to the skin surface where the epidermis becomes disrupted. In contrast, topical application of 1 µM svH1C, a peptide mimetic of sialic acid that lacks glutamine residues, or 1 µM dexamethasone was ineffective in restoring normal epidermal morphology. The data suggest that svL4 would be a powerful treatment for resolving severe eczema.

INTRODUCTION

Terminal differentiation of keratinocytes (KCs) in the epidermis produces a nearly impermeable barrier that separates the organism from its environment. Disruption of the barrier can lead to life-threatening water loss and vulnerability to serious allergen-induced inflammatory disease. The incidence of eczema (atopic dermatitis) in children is as high as 20% in some countries, peaks at ages 2–3 years, and then declines to a few percent in adults (Nutten, 2015; Shaw et al., 2011). Genetic mutations (Bin et al., 2021; Ma et al., 2017) and environmental allergens (Sözener et al., 2020; Werfel et al., 2016) are risk factors for eczema. The prototypic genetic deficiency is loss-of-function mutations in the gene encoding the protein proFLG (Hoober and Eggink, 2022; Irvine et al., 2011; Smith et al., 2006). ProFLG is synthesized in the granular layer of the epidermis as a polyprotein that contains 10–12 repeating domains in humans (Gan et al., 1990) but 20 repeats in the mouse (Rothnagel and Steinert, 1990) and rat (Haydock and Dale, 1990). The protein is proteolytically cleaved to monomers that facilitate the aggregation of keratin intermediate microfibrils and collapse of the corneocyte cytoplasm (Steinert et al., 1981), after which the protein is further degraded to amino acids that aid in maintaining moisture in the stratum corneum (Harding and Scott, 1983; Schwartz and Friedman, 2016; Thyssen et al.,

2020). The absence of FLG leads to dry, flaky skin and a loosely packed, leaky stratum corneum (Irvine et al., 2011).

Kanemaru et al. (2019) discovered that the NC/Nga strain of mouse, which has a loss-of-function mutation in the *Clec10a* gene that encodes MGL1 (CD301a), a receptor expressed by macrophages in the dermis (Higashi et al., 2002), is particularly susceptible to dermatitis in response to allergens in house dust mites (HDMs). The major allergen in the HDM extract, Der f2 (Johannessen et al., 2005), induced the production of proinflammatory cytokines, which was mediated by toll-like receptor 4. This observation was followed by inducing inflammation with lipopolysaccharide (LPS), a well-known ligand for toll-like receptor 4, instead of the HDM extract. LPS, mediated by CD14, initiates a signaling pathway from toll-like receptor 4 that leads to the activation of NF-κB; release of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, and IL-12p40; and induction of the M1 macrophage phenotype (Ley, 2017; Liu et al., 2017; Orecchioni et al., 2019; Palma et al., 2018). A high-molecular-weight glycoprotein that is rich in terminal T (Galβ(1-3)-GalNAcα-*O*Ser/Thr) and Tn (GalNAcα-*O*Ser/Thr) antigens was purified from HDM and inhibited LPS-induced eczema in tape-stripped skin of wild-type but not of *Clec10a*$^{-/-}$ mice. Terminal galactose is a ligand for MGL1, but whether the paralog MGL2 (CD301b), which is specific for N-acetylgalactosamine, plays a role in resistance to eczema remains unclear. MGL1 and MGL2 are markers for the M2a macrophage phenotype (Knudsen and Lee, 2016; Orecchioni et al., 2019; Westcott et al., 2009), which is essential for recovery from wounds (Greenlee-Wacker, 2016; Haas et al., 2021; Shook et al., 2016, 2018) and comprises nearly 60% of all cells in healthy murine dermis (Dupasquier et al., 2006, 2004). Humans express the C-type, N-acetylgalactosamine−specific receptor CLEC10A, an ortholog of MGL2, but do not have an ortholog of MGL1.

We initiated a study with a murine model of eczema to determine whether peptide mimetics of N-acetylgalactosamine, svL4 and sv6D (Eggink et al., 2018), have activity

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Abbreviations: Ca^{2+}, calcium ion; Dex, dexamethasone; HDM, house dust mite; KC, keratinocyte; LPS, lipopolysaccharide; SEB, Staphylococcal enterotoxin B; TGM, transglutaminase

Table 1. Peptide Sequences

Sequence	Code	MW
[(VQATQSNQHTPR-GGGS)$_2$K$_2$]K-NH$_2$	svL4	6,826
[(NQHTPR-GGGS)$_2$K$_2$]K-NH$_2$	sv6D	4,369
[(VQATQS-GGGS)$_2$K$_2$]K-NH$_2$	svC1	3,893
[(NPSHPLSG-GGGS)$_2$K]$_2$K-NH$_2$	svH1C	4,592

Abbreviation: MW, molecular weight.

similar to that of the glycoprotein described by Kanemaru et al. (2019). However, we discovered that these peptides are also functional substrates of transglutaminase (TGM) 2, also called tissue TGM, and that structural disruption of the epidermis was repaired when these tetravalent peptides were applied topically as cross-linking substrates, regardless of possible interaction with MGL1 or MGL2. Peptides svC1 and sv6D, the N- and C-terminal halves of svL4, respectively, contain glutamine residues at the same position as svL4, but only sv6D, along with svL4, binds with high specificity and avidity to recombinant and native human CLEC10A (CD301) (Eggink et al., 2018; Hoober, 2020). Nevertheless, svC1 was also active in reducing the thickness of the epidermis. In contrast, peptide svH1C, a mimetic of sialic acid (Eggink et al., 2010, 2015), lacks glutamine and was not effective when applied topically. A low concentration of dexamethasone (Dex) was also not effective when applied alone, but treatment for only 5 days with a combination of the steroid and svL4 after induction of eczema with Staphylococcal enterotoxin B (SEB) and HDM extract significantly reduced the extent of disruptions and thickness of the epidermis. These data suggest that topical application of these peptides is an effective means to achieve the restoration of normal skin.

RESULTS

Tetravalent peptides

The discovery and characterization of the 12-mer tetravalent peptide svL4 were described previously (Eggink et al., 2018). The arms of tetravalent sv6D contain the C-terminal half of the svL4 sequence (Table 1). Both peptides bind specifically to human CLEC10A (Hoober, 2020) and other N-acetylgalactosamine−specific lectins (Eggink et al., 2018). svC1 contains the N-terminal half of svL4; although it does not bind to CLEC10A, it contains the same arrangement of glutamine residues one turn of an α-helix distant from each other as in svL4. svL4 and sv6D are basic peptides, with a positive charge of 12 at the acidic surface of normal skin (pH 5.0 ± 0.5) (Nguyen and Soulika, 2019; Xie et al., 2021). svH1C, a mimetic of sialic acid, binds to sialic acid−specific plant lectins (Eggink et al., 2010); and recombinant human siglecs (Eggink et al., 2015).

svL4 promoted the healing of epidermal disruptions induced by LPS

In our experiments, dorsal skin of mice was shaved, depilated with thioglycolate and sodium hydroxide (Nair), and washed with 70% ethanol. Treatment with 4% SDS as used by Kanemaru et al. (2019) caused extensive damage to the skin, which led us to treat the skin every third day with 1% SDS for
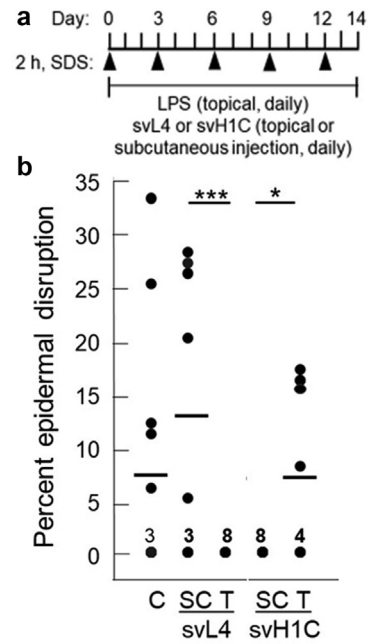

Figure 1. Extent of epidermal disruption after treatments with svL4 or svH1C. (a) Schematic of the experimental design after depilation of the skin. A 2-h treatment with 1% SDS was applied every 3 days. Eczema was induced by daily application of 200 μl of PBS containing 1 μg LPS per 1 cm^2 area of the skin. svL4 or svH1C was added to the LPS solution to a final concentration of 1 μM for topical treatment (T). The peptides were injected subcutaneously at a dose of 1 nmol/g body weight (SC). (b) Two sections, one stained with H&E and the other with anti-Ly6G, were analyzed from each of the four mice in each group after 14 days of treatment (n = 8 for each group). The cumulative area of disruption of the epidermis is expressed as a percentage of the total length of 1-cm long sections. Dots indicate the values for each section, which ranged from zero to 33%. The number of sections that did not contain a disruption of the surface barrier and received a score of 0 is indicated for each group. Horizontal bars indicate mean values for each group. C depicts LPS-treated skin, which served as the control. Statistical data were analyzed, with all values per group, by one-way ANOVA test for pairs of treatment groups indicated by the bars at the top of the figure. *$P = 0.022$ and ***$P = 0.0056$. C, control; h, hour; LPS, lipopolysaccharide; SC, subcutaneous; T, topical.

2 hours, which served as a penetration-enhancing treatment. The irritant LPS and peptides were applied daily to a 1-cm^2 area of depilated skin (Figure 1a). At the end of the 14-day treatment period, the skin was excised and sectioned for immunohistochemical analysis to determine the extent of injury. Skin treated with LPS contained a thick epidermis, and most sections revealed one or more areas of extensive epidermal damage. Our designation of epidermal disruption included breaks in the integrity of the surface barrier; disarrangement of the epidermis with loss of the basement membrane; and occasionally, nearly complete loss of the epidermal epithelium. Because we considered a disruption of a thickened epidermis an extreme state of eczema and the repair of these areas an essential process in healing, we initially quantitated the effects of 1 μM solutions of the peptides when included in the topical application with LPS or administered by subcutaneous injection of 1 nmol/g. Shown in Figure 1b is the extent of surface disruption among the sections, which typically varied from a thickened epidermis with no disruption to skin with about one third of the surface areas containing extensive damage. This variability was also
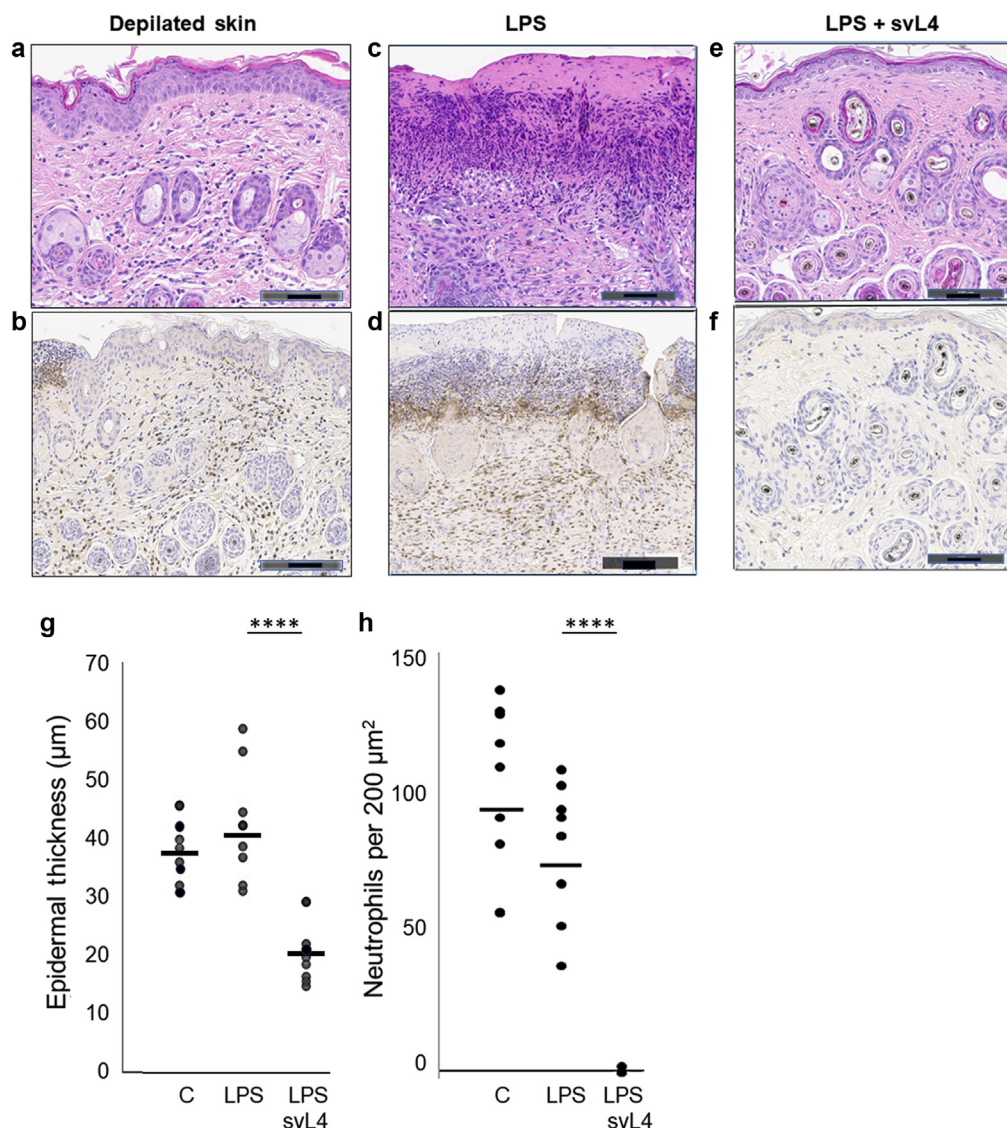
Figure 2. Changes in the morphology of skin during treatment with svL4. Images were obtained at the end of a 14-day treatment. Depilation resulted in (**a**) a thick epidermis and (**b**) frequent Ly6G+ neutrophils in the dermis, which were identified by their brown stain. (**c**) A 2-hour treatment with 1% SDS every 3 days and daily application of LPS resulted in a thick epidermis, areas where the definition of the epidermis and the basement membrane were lost and **d**) an abundance of neutrophils underlying these disruptions. Inclusion of 1 µM svL4 in the topical LPS-containing dressing resulted in (**e**) normal morphology with thin epidermis and (**f**) a dermis that was essentially free of neutrophils. Bars = 100 µm. Graphical representations of the images show (**g**) epidermal thickness under the stratum corneum measured at 10 sites along 1-cm sections of the skin stained with H/E or anti-Ly6G. Two sections from each of the four mice per group were analyzed (n = 8). Dots represent the mean of measurements for each section. (**h**) Neutrophils were counted within two defined areas of the dermis on each section stained with anti-Ly6G. The neutrophil abundance for each area is indicated by a dot. The horizontal bars indicate the mean for each group. C depicts the depilated skin treated with PBS. Statistical data were calculated for pairs of treatment groups indicated by the bars at the top of the figure. ****P = 0.000048 for **g** and 0.00001 for **h**. LPS, lipopolysaccharide.

a function of the path taken by the section through the affected area. Topical treatment with svL4 restored the integrity of the skin surface, with no epidermal disruptions (Figure 1b); however, no recovery occurred when the peptide was injected subcutaneously. In contrast, svH1C was effective when injected subcutaneously but not when applied topically. Although lesions were absent after injections of svH1C, most areas of the skin contained a thickened epidermis and abundant neutrophils in the dermis (not shown).

Immunohistochemical analyses of sections of treated skin showed that the process of depilation resulted in the thickening of the epidermis and frequent neutrophils in the dermis (Figure 2a and b). Daily treatment with LPS caused more extensive disruption of the epidermis and a high density of neutrophils in the dermis underlying these areas (Figure 2c and d). When 1 µM svL4 was included in the topical application with LPS, the epidermis at the end of the 14-day treatment was uniformly of normal thickness, and the dermis was nearly free of neutrophils (Figure 2e and f). Graphical representations of epidermal thickness and

neutrophil frequency are presented (Figure 2g and h). Measurements of neutrophil frequency were made in areas of the dermis distant from those of the disruptions of the epidermis, which contained a very high abundance, as shown in Figure 2d. Although subcutaneous injections of svL4 did not reduce epidermal thickness, an increased density of dermal collagen was noted as thicker bundles with more intense eosin staining (not shown)

svL4 reduced disrupted areas after induction with HDM and SEB

To determine whether svL4 is effective against irritants other than LPS, for example, serve as a general treatment, eczema was also induced with a combination of HDM extract and SEB, which are commonly encountered allergens (Kawakami et al., 2007). The design of the experiment is shown in Figure 3a. The allergens were applied to depilated skin for 4 days, whereafter the skin was treated with 2 µM svL4 for another 5 days alone or in combination with 1 µM Dex. With this experiment, we sought to determine the extent of repair of the epidermis during this short period of treatment and also
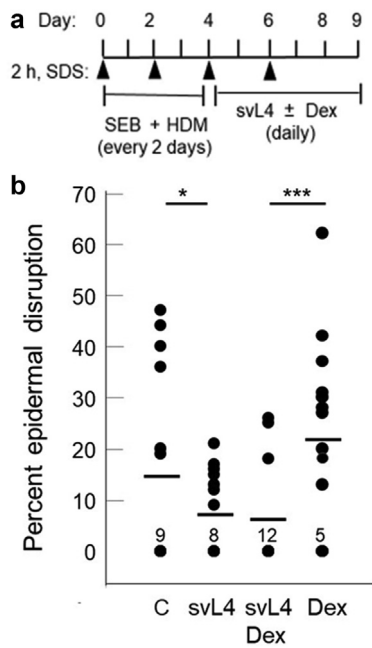
Figure 3. The extent of epidermal disruptions after topical treatment with SEB and HDM. (**a**) Schematic of the experimental design after depilation of the skin. A 2-h treatment with 1% SDS was applied every other day. SEB (100 µg) and HDM extract (10 µg) were applied in 200 µl of PBS to a 1-cm² area of skin on days 0 and 2. The allergens were removed after 4 days of induction of eczema, and then svL4 (2 µM) was applied topically alone in 200 µl of PBS or in combination with 1 µM Dex for another 5 days to the affected areas. The topical dressings were replaced every day. (**b**) The extent of disrupted areas was determined on three 1-cm long sections from each of the five mice in each group at the end of the 9-day treatment (n = 15). Dots indicate the values for sections with disruptions of the epidermis, which are expressed as a percentage of the length of the section. The number of sections that did not contain a disruption of the surface barrier and received a score of 0 is indicated for each group. C depicts the allergen-induced control group treated with PBS. Horizontal bars indicate the mean for each group. Statistical data were calculated for the paired groups indicated at the top of **b**. *$P = 0.052$ and ***$P = 0.0041$. Dex, dexamethasone; h, hour; HDM, house dust mite; SEB, Staphylococcal enterotoxin B.

whether svL4 actually reduced the severity of eczema when applied to inflamed skin or simply inhibited the action of irritants and allergens. As shown in Figure 3b, the application of svL4 dramatically reduced epidermal disruptions after the allergens had been removed. Topically applied Dex resulted in no reduction in epidermal disruption compared with that of untreated skin. However, disruptions were detected in only a few sections from animals treated with Dex plus svL4. The efficacy of treatment with svL4 alone was similar to that of the combination of svL4 and Dex, although several sections had small disruptions. The number of sections without a disruption in a group suggests a progression of healing from the Dex-treated group to the groups treated with the combination or svL4 alone (Figure 3).

Similar to the results shown in Figure 2, skin from animals treated with HDM and SEB contained a thickened epidermis and extensive loss of structural integrity. The basement membrane between the epidermis and dermis was often obliterated, and dense populations of neutrophils were evident within damaged areas (Figure 4a). Neutrophils were essentially absent in the dermis underlying a thin epidermis

after treatment with svL4 (Figure 4b). Because CD301b⁺ macrophages are essential for healing, sections of skin were stained with antibodies against murine MGL1 (CD301a) and MGL2 (CD301b), C-type lectin receptors expressed by M2a macrophages, and dendritic cells (Higashi et al., 2002; Krzyszczyk et al., 2018; Palma et al., 2018; Zhang et al., 2011). CD301a⁺ and CD301b⁺ cells were absent in the dermis underlying areas where the integrity of the epidermis was lost (Figure 4c) but were abundant in the dermis of svL4-treated skin (Figure 4d−f). Whereas the CD301b⁺ cells were more frequent near the epidermis after treatment with svL4, these cells were more abundant in the lower dermis in the skin treated with Dex (Figure 4h). The reciprocal relationship between neutrophils and CD301b⁺ macrophages was a definitive marker of healing. The dermis contained a paucity of CD11c⁺ dendritic cells (Figure 4g), which also express CD301b.

svL4 is a substrate for TGM2
We then considered the possible mechanisms by which svL4 could provide a therapeutic action. An important aspect was the efficacy when applied topically but not when given subcutaneously. A particularly intriguing feature of the peptide is the arrangement of glutamine residues. We therefore asked whether the peptide is a substrate for TGM2. As shown in Figure 5a, TGM2 actively catalyzed a reaction in which biotinylated svL4 was linked to polylysine. A K_m of approximately 7 µM was calculated, with svL4-biotin as a substrate, which compares favorably with the K_m of 1 µM for a standard peptide, biotinyl-TVQQEL (Trigwell et al., 2004). We found that biotinylated sv6D and svC1 were poor substrates when assayed immediately after dissolving commercial lyophilized TGM2 in water containing 1 mM EDTA, pH 7.5, and 10 mM dithiothreitol (Figure 5a), but surprisingly, when the solution of TGM2 was stored over several days at 5 °C in this medium, the enzyme lost over half of the activity with svL4 but gained the ability to use sv6D and, to a lesser extent, svC1 as substrates (Figure 5b). This observation suggested that the enzyme unfolds to a less specific conformation over time. The concentration dependence also suggests higher K_m values for svC1 and sv6D with the aged enzyme than for svL4.

The tissue distribution of TGM2 was determined by immunohistochemical analysis of frozen sections after induction of eczema for 4 days with a combination of HDM extract and SEB and 5 days of treatment with svL4 (the experiment shown in Figure 3). In depilated control samples, TGM2 was detected under the epidermal/dermal boundary and around hair follicles (Figure 6a). After induction of eczema, intense staining with anti-TGM2 was often restricted to the dermis in the skin with a thickened epidermis (Figure 6b). However, where the epidermis was severely damaged, the layer of tissue containing TGM2 was exposed on the surface (Figure 6c). TGM2 was also detected among KCs in areas in which an early (Figure 6d) and later (Figure 6e) phase of re-epithelialization appeared to have occurred. The diffused appearance of the antibody stain suggests the presence of an extracellular enzyme.

We then asked whether svC1 and sv6D have activity similar to that of svL4 when applied topically to the skin. In
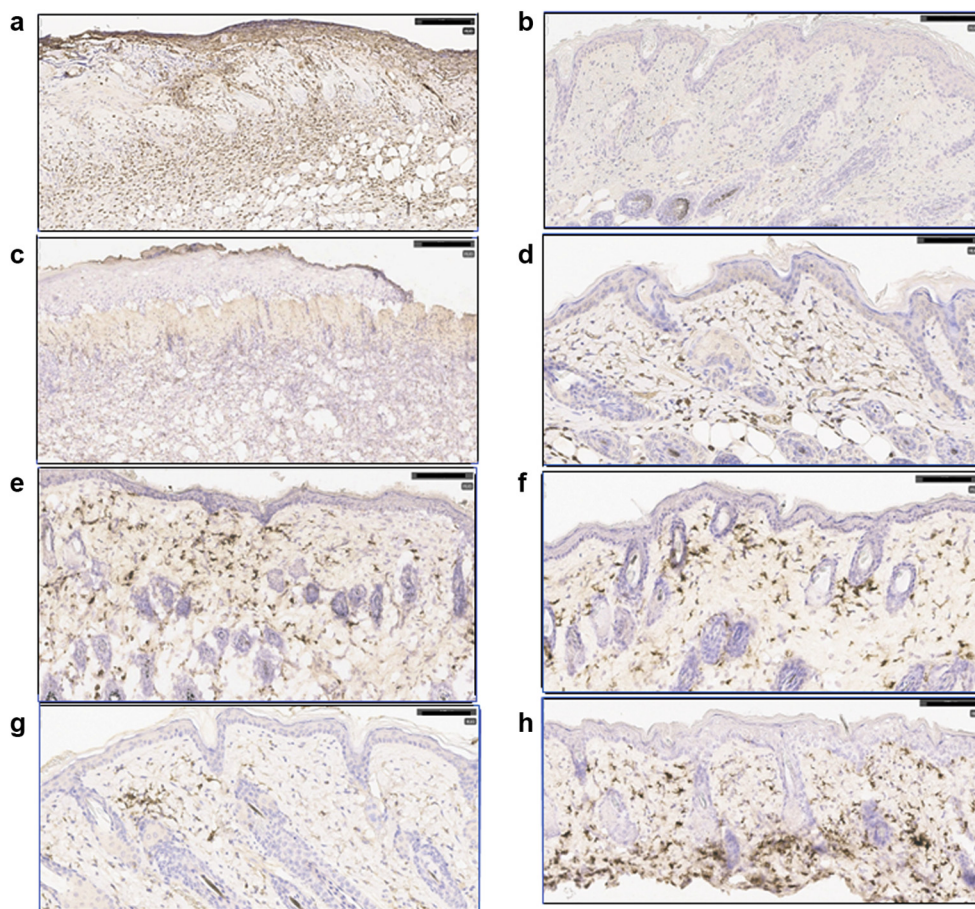
Figure 4. Induction of eczema with SEB and HDM. Shown in the Figure are sections of skin treated for 5 days with svL4 or svL4 plus dexamethasone after a 4-day induction of eczema with SEB and HDM. Sections from all mice in the experiment shown in Figure 3 were viewed to select representative images for this figure. (**a**) Section stained with anti-Ly6G from a mouse treated with PBS after induction of eczema revealed extensive epidermal damage and a dense population of neutrophils. (**b**) svL4-treated skin had a restored epidermis and a dermis essentially free of neutrophils. (**c**) Section from the same mouse as in **a** stained with ER-MP23 (anti-CD301a/b), which revealed the absence of macrophages in areas that lacked a definition of the epidermal/dermal boundary. (**d**) Section from the same mouse as in **b** treated with svL4, which had a thin epidermis and an abundance of ER-MP23–stained macrophages. (**e, f**) svL4-treated skin stained with anti-CD301b. (**g**) Section of skin from a mouse treated with dexamethasone and stained with anti-CD11c, which contained a cluster of dendritic cells. (**h**) Section from a mouse treated with dexamethasone and stained with anti-CD301b. Positive staining is indicated by the brown color. The images are representative of mice in each group (n = 5). Bar = 100 μm. HDM, house dust mite; SEB, Staphylococcal enterotoxin B.

this experiment, the skin was treated topically with 2 μM peptides along with LPS for 14 days. Sections were then stained with anti-Ly6G, and epidermal thickness was measured. As shown in Figure 7, the three peptides were essentially equally effective in reducing the epidermis to the approximate average normal thickness for murine viable epidermis, excluding the stratum corneum, of 17.5 μm (Wei et al., 2017). The mean values of the thicknesses of peptide-treated mice were 16.1 μm for svL4, 15.5 μm for svC1, and 17.1 μm for sv6D. The dermis of peptide-treated skin also lacked neutrophils (not shown).

DISCUSSION
A role for TGM2
Yokouchi et al. (2015) described a vicious cycle in which impairment of the surface barrier allows the penetration of allergens, which causes chronic inflammation, disturbed KC differentiation, and altered tight junction formation with further disruption of the barrier. Elias (2018) and Lee (2020) also argued that the inflammatory immune responses in eczema are secondary to the disruption of the surface barrier of

the skin. Elias (1983) introduced the concept of the stratum corneum as a wall composed of corneocyte bricks sealed together with a lipid mortar composed of a mixture of ceramide, cholesterol, and fatty acids, which was replicated as a topical barrier-repair therapy for eczema (Elias, 2022; Yang et al., 1995). However, integral to barrier function is the strength of the interaction between the bricks. In addition to allowing penetration of allergens, loss of the barrier function can lead to dehydration and death shortly after birth (Kasparek et al., 2017; Leyvraz et al., 2005). Restoration of the surface barrier allows the epidermal calcium ion (Ca^{2+}) gradient to form, which is critical for KC differentiation and cornification (Lee, 2020; Lee and Lee, 2018; Teshima et al., 2020).

TGM activities are essential for terminal differentiation of the epidermis and formation of the cornified layer (Lorand and Graham, 2003). Three isozymes of the Ca^{2+}-dependent TGM family—TGM1, TGM3, and TGM5—are expressed in the epidermis and function within terminally differentiating KCs (Eckert et al., 2005). Although confined to the cellular interior, these TGMs could possibly be released from KCs as a result of trauma (Gross et al.,
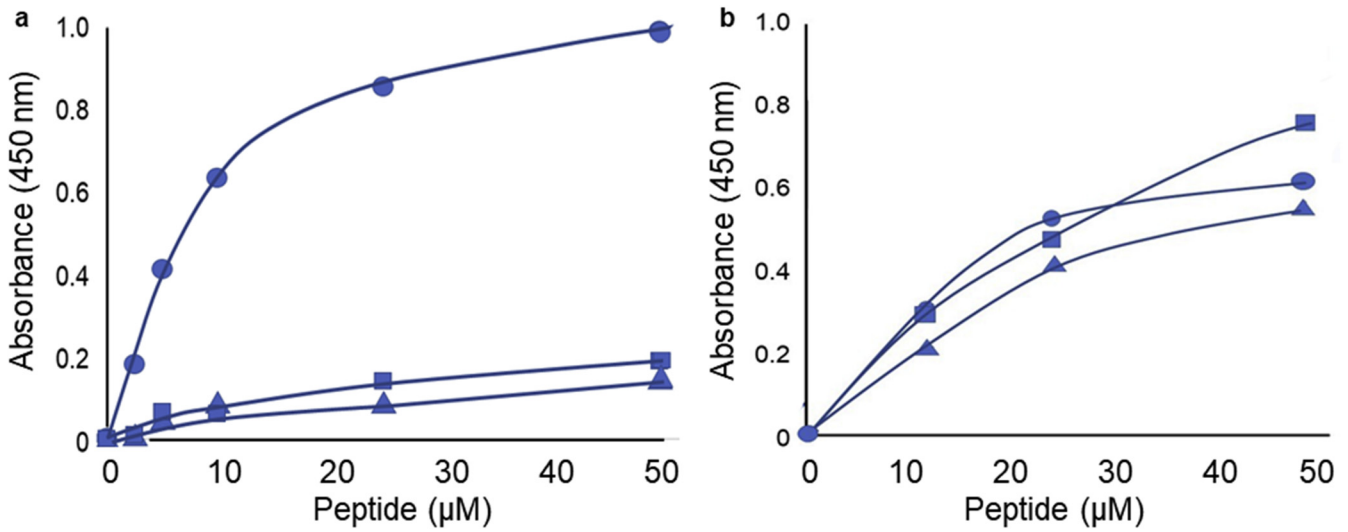
Figure 5. Assay of svL4, svC1, and sv6D as substrates for TGM2. The cross-linking reaction was performed in microtiter wells with bound polylysine as acceptor. The results shown with biotinylated svL4 (circles), svC1 (triangles), and sv6D (squares) are representative of the results of the three assays for each panel. (**a**) The assays were performed with enzyme solutions freshly prepared from a lyophilized sample from the supplier. (**b**) The assays were performed after storage of the enzyme solution for 6 days at 5 °C. TGM2, transglutaminase 2.

2003). However, unlike other members of the family, TGM2 is ubiquitously expressed and is secreted into the extracellular space (Chou et al., 2011; Griffin et al., 2002; Johnson et al., 1998; Zemskov et al., 2011). The activity of TGM2 is dependent on Ca^{2+}, with an activation constant, K_A, of 6.9 mM (Folk et al., 1967). A concentration this high is achieved at the peak of the Ca^{2+} gradient in the granular layer of the epidermis (Elias et al., 2002; Lee and Lee, 2018; Mauro et al., 1998), but the gradient is dissipated by disruption of the barrier. However, sufficient Ca^{2+} is apparently retained for some level of activity of TGMs.

Figure 6. Immunohistochemical localization of TGM2. Eczema was induced on depilated mouse skin with SEB/HDM for 4 days as described in the legend of Figure 3. The induction period was followed by treatment with (**a**) PBS or (**b–e**) 2 μM svL4 in PBS for another 5 days. Accumulation of TGM2 is indicated by the brown stain on frozen sections that were incubated with anti-TGM2. Bar = 180 μm for **a–c** and 100 μm for in **d** and **e**. HDM, house dust mite; SEB, Staphylococcal enterotoxin B; TGM2, transglutaminase 2.
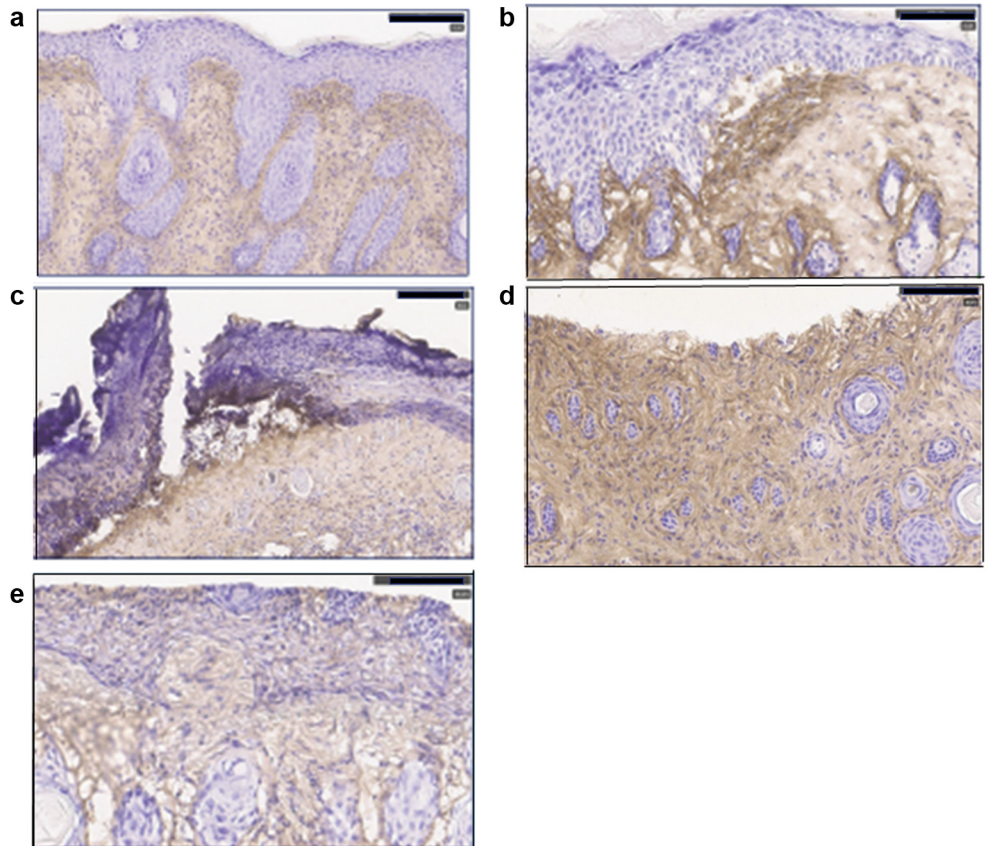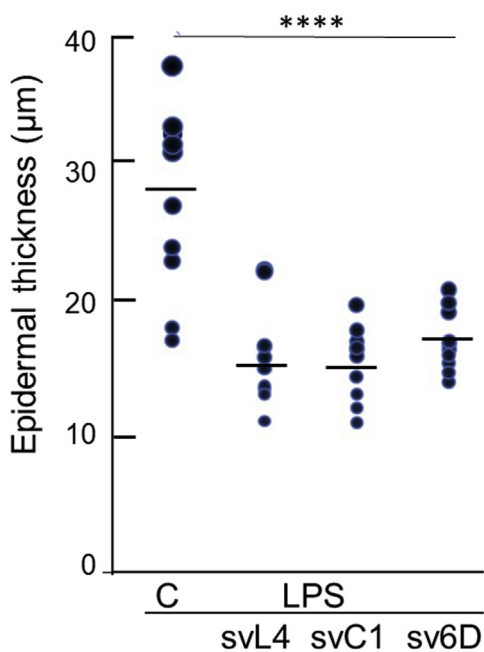
Figure 7. Reduction in epidermal thickness by topical treatment with peptides. The experimental design was similar to the schematic shown in Figure 1. Peptides (2 μM) were added to LPS treatment for 14 days. Measurements of thickness under the stratum corneum were made at 20 sites along 1-cm long sections stained with anti-Ly6G. Two sections were analyzed from each of the five mice in each group (n = 10). Dots represent the mean values for each section. C depicts the depilated LPS-treated control. Statistical data were calculated for peptide-treated epidermis compared with the LPS control. ****P = 0.00018 for svL4, 0.000056 for svC1, and 0.0002 for sv6D. LPS, lipopolysaccharide.

Substrate specificity of TGM

As KCs terminally differentiate into corneocytes, an early step in the formation of the cornified envelope is an attachment of involucrin to the inner surface of the cell membrane in a reaction catalyzed by the essential enzyme, TGM1 (Candi et al., 2005; Eckert et al., 2005; Kalinin et al., 2001; Lee, 2020). Highlighting the specificity of TGM1, involucrin contains 39 repeats of a 10 amino acid sequence, with each containing three glutamine residues (Eckert and Green, 1986), but only one glutamine residue within this 585-amino acid protein—in the sequence ELPEQ**Q**VGQP (reactive Q is in bold)—is reactive in vitro unless the protein is proteolytically degraded (Simon and Green, 1988). The activity of TGM1 is required for the formation of cross-links between structural proteins and loricrin during the formation of the intracellular cornified envelope (Candi et al., 2005; Eckert et al., 2005; Ishitsuka and Roop, 2020; Kalinin et al., 2001; Lee, 2020).

Monovalent peptides were identified as specific substrates for several TGM isozymes (Fukui et al., 2013; Sugimura et al., 2008; Tanabe et al., 2019). TGM2 is also remarkably specific for the glutamine residues that provide the substrate for the first step in the cross-linking reaction. The sequences glutamine-X (any amino acid)-proline (QXP), QQ, and QXXQ are preferred targets for TGM2, whereas QP and QXXP are poor substrates (Fleckenstein et al., 2002; Vader et al., 2002). The sequence in sv6D (QXXP, see Table 1) is unfavorable, whereas svC1 contains a favorable sequence

(QXXQ), but both were poor substrates in vitro with freshly prepared enzyme solutions. However, both contain an active Q in position 2 of the peptide (Hitomi et al., 2009; Pastor et al., 1999). The short arms of sv6D and svC1 may be hindered from entering the active site by the remainder of the tetravalent peptides. Alternatively, the low activity of sv6D and svC1 with freshly prepared enzyme may result from K_m values for these peptides much higher than that of 7 μM for svL4 when assayed under the same conditions. For example, a frequently used substrate to study TGM2, carbobenzoxy-L-glytaminylglycine, has a K_m of 22 mM (Folk and Cole, 1965). The kinetics of the reaction suggest that glutamines that appear unreactive may simply react more slowly (Christensen et al., 2014).

The acceptor amine group for the second step in the cross-linking reaction is less restrictive, although N-terminal amino groups of peptides are not active acceptors. Primary alkyl amines such as putrescine, spermidine, or the side chain of lysine are active second substrates (Schrode and Folk, 1978). The reaction catalyzed by TGM2, shown in Figure 5, demonstrated that the enzyme can use glutamine from the arms of svL4, svC1, and sv6D to link with the ε-amine groups of lysine residues to form ε(γ-glutamyl)lysine isopeptide bonds. This reaction with the peptides would form cross-links without the restrictive specificity for endogenous glutamine.

Possible functions of other TGM

Expression of TGM2 is induced in inflamed and wounded tissues (Haroon et al., 1999; Griffin et al., 2002), by Dex (Johnson et al., 1998), and in KCs under inflammatory conditions (Bowness and Tarr, 1997; Eckert et al., 2005) and is reported to facilitate wound healing (Eckert et al., 2005; Griffin et al., 2002; Gross et al., 2003; Haroon et al., 1999; Stephens et al., 2004; Verderio et al., 2004). Knockout of *TGM2* normally has no significant physiological consequence, which suggests that other members of the TGM family compensate for the absence of TGM2 (De Laurenzi and Melino, 2001; Nanda et al., 2001; Odii and Coussons, 2014), although closure of wounds was significantly impaired in *Tgm2*-knockout mice (Iismaa et al., 2009; Mearns et al., 2002; Sarang et al., 2009). Furthermore, processes required for wound healing and extracellular matrix remodeling are dramatically reduced when fibroblasts and macrophages are rendered TGM2 deficient by transfection with antisense RNA (Stephens et al., 2004; Telei and Griffin, 2006). TGM2 normally occurs in an inactive, closed conformation but is rapidly converted to the open, active form by injury (Griffin et al., 2002; Pinkas et al., 2007).

Although we have emphasized a possible role for TGM2 in recovery from eczema, TGM3 may also be important. TGM2 and TGM3 are expressed in all layers of the epidermis in humans (Chermnykh et al., 2020; Su et al., 2017), but TGM2 is expressed predominantly in the dermis of mice (Figure 6). As with TGM2, knockout mutation of *Tgm3* causes no significant physiological effect in mice, but in contrast to *Tgm2*, this mutation has no obvious effect on wound healing (John et al., 2012). TGM3 is expressed at high levels in KCs in inflamed skin. Liedén et al. (2012) and Su et al. (2020) showed a dramatic increase in the expression of TGM in the epidermis of patients with atopic dermatitis. TGM3 has

also been detected extracellularly and is present in the cornified layer (John et al., 2012). The lack of apparent involvement of TGM3 in the formation of the epidermal barrier may result from a paucity of suitable glutamine-containing substrates.

The absence of TGM1 in mice leads to death within a few hours after birth (Candi et al., 2005; Matsuki et al., 1998) and causes congenital ichthyosis, a severe skin disease, in humans. Remarkably, therapy by topical replacement of TGM1 corrected skin architecture and dramatically reduced transepidermal water loss (Aufenvenne et al., 2013).

A possible target for cross-linking

A possible target to which TGMs may form cross-links with svL4 is lysine residues in corneodesmosin, a major component along with desmoglein 1 and desmocollin 1 of corneodesmosomes (Caubet et al., 2004; Igawa et al., 2013), which occur at the tips of villus-like structures on the surface of corneocytes (Riethmüller et al., 2015). Corneodesmosin is synthesized in the lower granular layer of the epidermis and is secreted in vesicles from KCs as a glycoprotein (Guerrin et al., 1998; Simon et al., 1997). Loops in the N-terminal domain are exposed on the cell surface and are binding sites for colonizing *Staphylococcus aureus* in atopic dermatitis lesions (Towell et al., 2021). Several lysine residues lie within the sequence near these loops and conceivably would provide the amino groups for cross-linking. Whereas corneodesmosomes occur at the periphery of flattened wild-type corneocytes, they cover the entire surface of loosely packed homozygous FLG-deficient ($FLG^{-/-}$) mutant cells. Moreover, these structures are approximately five-fold more abundant on $FLG^{-/-}$ cells than on wild-type cells (Riethmüller et al., 2015). The villus projections extend up to several hundreds of nanometers into the extracellular space between cells and conveivably would be readily available to engage in cross-linking.

To our knowledge, this is a previously unreported use of tetravalent peptides as a treatment for eczema. The efficacy of topical application of svL4 (Figure 1) supports a role for the physical presence of the peptide on the surface of the skin. The most likely role of the peptide is to provide a glutamine-containing cross-linking substrate for TGMs. Further analysis of the details of the mechanism of the svL4/TGM2 effect in the reconstruction of the surface barrier, and questions that this study has raised regarding the involvement of the immune system require additional research.

In summary, our data suggest that the ability of svL4 to serve as a treatment for eczema is provided by the tetravalent properties of the peptide, which allow cross-linking of multiple proteins and cells catalyzed by extracellular TGMs. TGM2 was exposed on the skin surface in areas where the epidermis was disrupted or disarranged and was expressed in epidermal tissue during re-epithelialization. Without exogenous glutamine residues provided by the peptides, repair of the epidermis did not occur within the 2-week period of treatment. Foremost among the observations during treatment with svL4 was the repair of areas of structural disruption of the epidermis, reduction of the thickness of the epidermis, and a loss of neutrophils in the dermis. Efficacy of topical svL4 supports a role for the peptide on the skin surface. Thus,

we propose that the primary role of the peptides in resolving eczema is related to their ability to serve as a substrate for TGMs to achieve cross-linking of structural proteins or cells to reconstruct a functional stratum corneum. Toxicity and antigenicity have not been detected in our studies with the peptides in animals (Eggink et al., 2018).

MATERIALS AND METHODS
Peptides
Synthesis and purification of endotoxin-negative svL4, svC1, and sv6D (Eggink et al., 2018) and svH1C (Eggink et al., 2015) were described previously.

Animals
The animal studies were conducted at Biomodels LLC (Waltham, MA), an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility in Watertown, MA. Approval for this work was obtained from Biomodels LLC Institutional Animal Care and Use Committee (protocol number 17-0613-4). Female C57BL/6J mice aged 10 weeks were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed at Biomodels LLC.

Induction of disease

LPS. On day minus-1, all mice were anesthetized with inhaled isoflurane, and the hair was removed from the dorsal skin using electric clippers followed by depilation with thioglycolate and sodium hydroxide (Nair). The skin was then washed with 70% ethanol followed by PBS to ensure no depilation reagent remained. On day 0, mice were again anesthetized with inhaled isoflurane, and a 1-cm^2 square was drawn on the center of the back skin to direct the placement of gauze. A 1% SDS solution was applied to a 1-cm^2 sterile piece of gauze secured to the hair-free back skin with a bio-occlusive dressing (Tegaderm). Two hours after SDS treatment, mice were again anesthetized with inhaled isoflurane, and the Tegaderm and gauze were removed. The skin was blotted dry with sterile gauze. For treatment groups, 0.1 ml of a 10 μg/ml solution of LPS (*E. coli* 0111:B4, catalog number L2630, MilliporeSigma, Burlington, MA) and 0.1 ml of PBS or 2 μM peptide in PBS were applied to a 1-cm^2 sterile piece of gauze secured to the hair-free back skin with a bio-occlusive dressing (Tegaderm). Every 3 days, the SDS application was repeated 2 hours before topical gauze application of the treatment solution, which was replaced every 24 hours with fresh LPS and peptide. On day 14, all mice were killed by carbon dioxide inhalation, and skin was collected for histological analysis.

HDM and SEB. A similar experiment was conducted with 0.1 ml of 1 mg/ml SEB (100 μg, catalog number BT202, Toxin Technology, Sarasota, FL) and 0.1 ml of 0.1 mg/ml HDM extract (10 μg, catalog number B81, Greer Laboratories, Lenoir, NC), in which eczema was induced by a 2-hour treatment with 0.2 ml of 1% SDS followed by replacement of the allergen mixture in 0.2 ml for 2 days. The SDS/allergen treatment was then repeated for a second 2-day period. The skin was then washed with PBS. The 2-hour SDS treatment was continued every other day, with 2 μM svL4 in PBS alone or mixed with 1 μM Dex sodium phosphate (catalog number 2480645, Henry Schein, Melville, NY) in 0.2 ml of PBS applied daily for another 5 days.

Histological analyses
At the end of the treatment period, mice were killed by carbon dioxide inhalation, and a 1-cm^2 portion of the skin was excised. One half was fixed in formalin for histopathological analysis, whereas the

Table 2. Antibodies used for Immunohistochemistry

Ab Target	Cell Type	Clone	Catalog Number	Vendor
Ly6G	Neutrophil	1A8	551459	BD Biosciences
CD301a	Macrophage	ER-MP23[1]	NB100-64874	Novus Biologicals
CD301b	Macrophage	11A10-B7	50-3011-82	Thermo Fisher Scientific
CD11c	Dendritic cell	D1V9Y	97585S	Cell Signaling Technology
TGM2		D11A6	3557S	Cell Signaling Technology

Abbreviation: Ab, antibody.

[1]ER-MP23 reacts with a common epitope in CD301a and CD301b (Denda-Nagai et al., 2010).

other half was flash frozen. Histological analysis by H&E staining, measurement of epidermal thickness, and immunostaining were performed by Inotiv Boulder (Boulder, CO) with the Leica Bond RX/RXm platform using standard chromogenic methods or by Dallas Tissue Research (Farmers Branch, TX). Neutrophils were stained on fixed sections with monoclonal anti-Ly6G, whereas frozen samples were embedded in optimal cutting temperature compound, and sections were stained with monoclonal anti-CD301b or anti-TGM2 (See Table 2 for the antibodies used in this study). Image capture and analysis were performed with Aperio's ImageScope program or with EasyScan Pro 6 digital slide scanner.

Analysis of tissue sections

The cumulative length of epidermal disruptions on 1-cm long sections of fixed tissues was measured at a magnification of ×20 and related to the total length of the section. Epidermal thickness of each sample was measured at 10 or 20 sites along 1-cm long, H&E- or anti-Ly6G-stained sections without histological artifacts, perpendicular to the long axis of the sections, encompassing the basal layer through all cell layers, excluding the stratum corneum, and averaged to obtain the mean thickness for each section. The number of neutrophils in square areas of the dermis, 200 μm on a side, was counted with a magnification of ×320 on images of sections stained with anti-Ly6G.

TGM assay

The assay was performed in polylysine-coated microtiter wells. The reaction mixture contained, in 50 μl, 100 mM Tris hydrogen chloride, pH 7.5, 10 mM calcium chloride, 5 mM dithiothreitol, 1 mM EDTA, 150 mM sodium chloride, and 0.05% Tween-20. Lyophilized TGM2 from guinea pig liver (1.5 units/mg, catalog number T5398, Sigma-Aldrich, St. Loius, MO) was dissolved in 1 mM EDTA, pH 7.5, containing 10 mM dithiothreitol. Freshly prepared TGM2 was assayed with 10 ng protein/well, whereas 40 ng protein/well was used with enzyme stored at 5 ºC for 6 days. After a 2-minute activation period, biotinylated peptides were added to provide a series of concentrations from 0.5 to 50 μM. After 30 minutes of incubation, biotin bound in the wells was detected with streptavidin conjugated with horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine. The reaction was stopped with 1 N sulfuric acid and read immediately at 450 nm.

Statistical analyses

Quantitation of disrupted areas, epidermal thickness, and neutrophil abundance was analyzed by the one-way ANOVA test. Significance was set at $P \leq 0.05$ for all tests.

Data availability statement

No large datasets were generated or analyzed during this study. Minimal datasets necessary to interpret and/or replicate data in this paper are available on request to the corresponding author.

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Disclaimer

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