

Letter to the Editor

Thymic stromal lymphopoietin downregulates filaggrin expression by signal transducer and activator of transcription 3 (STAT3) and extracellular signal-regulated kinase (ERK) phosphorylation in keratinocytes

To the Editor:

Atopic dermatitis (AD) is a chronic inflammatory cutaneous disease that is largely described by 2 themes of pathologic mechanisms: inflammation and barrier dysfunction.¹ Atopic skin inflammation is orchestrated by the participation of various kinds of cells and their products, cytokines and chemokines. Acute AD is particularly characterized by the T_H2 cytokines IL-4 and IL-13, which mediate immunoglobulin isotype switching to IgE synthesis. Meanwhile, atopic skin shows aberrant mechanical barrier and increased water loss through the impaired epidermis. In this regard loss-of-function mutations of the filaggrin gene (*FLG*), which encodes the epidermal barrier protein filaggrin, have been reported to be associated with early-onset, severe atopic eczema in many researches.² Although the frequency and genetic spectrum of *FLG* mutations are distinct in each population, approximately 25% to 50% of patients with AD have been reported to carry *FLG* mutations.² However, all the patients with AD have skin barrier defects. Furthermore, one study showed that the acute lesional skin of patients with AD with the *FLG* mutation exhibited lower levels of filaggrin expression compared with nonlesional skin from the same patient.³ In another report the authors noted that although there is no correlation between *FLG* mutations and psoriasis, decreased filaggrin expression has been detected in psoriatic skin, especially in acute plaques.⁴ The researchers focused on these observations and anticipated that there should be additional mechanisms to modulate filaggrin expression. As a result, to date, they found that cytokines are involved in decreased filaggrin expression in addition to genetic deficiency.^{3,4} Inversely, inflammatory cytokines produced by reattack of the sensitizing antigen also

break the skin barrier. Skin barrier dysfunction and inflammation can contribute to a vicious cycle of AD.

We were more concerned with thymic stromal lymphopoietin (TSLP) and its main source, keratinocytes, because TSLP is the initiating key molecule in immunopathogenesis of AD. TSLP is an IL-7-like cytokine that activates myeloid dendritic cells to induce an inflammatory T_H2 response.⁵ TSLP is highly expressed in acute and chronic AD lesions, mainly by keratinocytes in human skin.⁵ There is evidence to prove that TSLP is crucial in the development of AD. Mice engineered to overexpress TSLP in the skin have AD with eczematous lesions, increased circulating T_H2 cell numbers, and increased serum IgE levels.⁶ Thus keratinocytes and their product, TSLP, are in the middle of the pathogenesis of atopic inflammation from the very early stage. However, the effects of TSLP on human keratinocytes remain unknown.

The present study shows that human keratinocytes and skin express the receptor for TSLP. We investigated whether TSLP is involved in filaggrin expression in primary human keratinocytes. Furthermore, we identified the signaling molecules that conduct the inhibitory action of TSLP on filaggrin synthesis in keratinocytes.

The TSLP receptor complex consists of the IL-7 receptor (IL-7R) α chain and a TSLP-binding chain (also known as the type I cytokine receptor subunit cytokine receptor-like factor 2 [CRLF2]).⁵ To define the presence of the TSLP receptor in normal human epidermal keratinocytes (NHEKs), we investigated the expression of CRLF2 and IL-7R α using RT-PCR and immunofluorescence assay. As shown in Fig 1, A, mRNA of the 2 receptor subunits were detected by using specific primers for CRLF2 and IL-7R α . Immunofluorescence staining with mouse anti-human CRLF2 and anti-human IL-7R α antibodies also revealed the specific fluorescent signal (Fig 1, B). NHEKs were stained with 4',6-diamidino-2-phenylindole (DAPI) for nuclear localization. Our results showed the colocalization of CRLF2 and IL-7R α in NHEKs (Fig 1, B). In addition, we investigated whether CRLF2 and IL-7R α were expressed in human normal

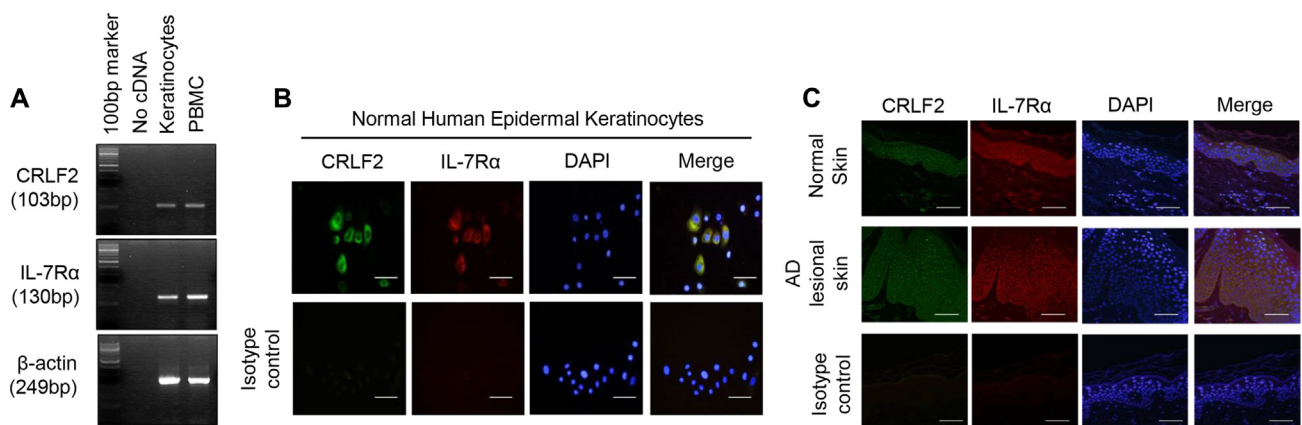


FIG 1. Keratinocytes express TSLP receptor complex. **A** and **B**, Expression of CRLF2 and IL-7R α was analyzed in NHEKs by means of RT-PCR (Fig 1, A) and immunofluorescence assay (Fig 1, B). **C**, CRLF2 and IL-7R α expression were also investigated in normal human skin and AD lesional skin by using the immunofluorescence assay. β -Actin was used as a loading control for RT-PCR. Bar = 100 μ m.

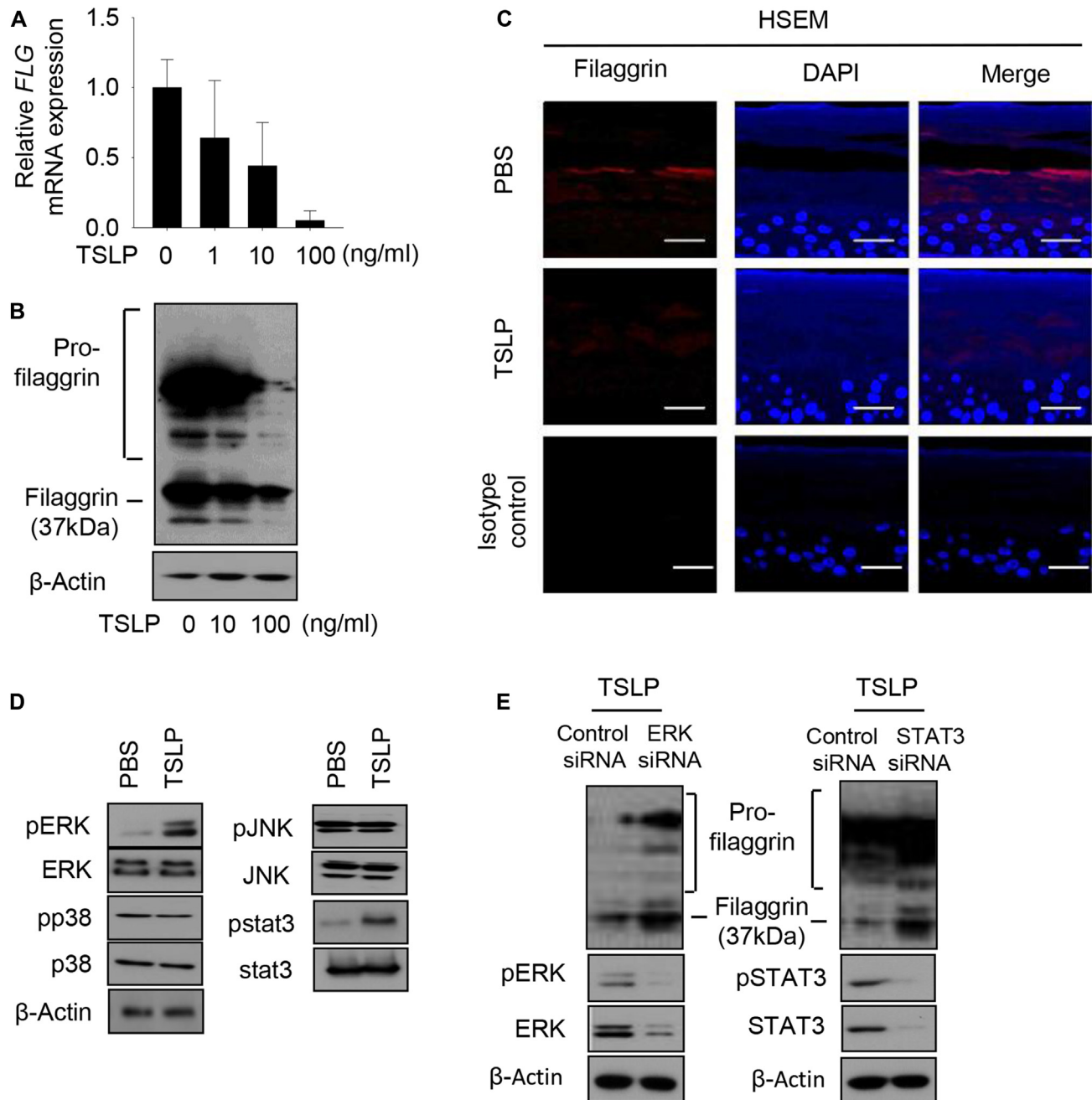


FIG 2. TSLP inhibits filaggrin expression in NHEKs through ERK and STAT3 activation. **A**, Filaggrin mRNA expression was decreased at 100 ng·mL⁻¹ TSLP. **B**, Profilaggrin and filaggrin protein expression were decreased by TSLP. **C**, TSLP treatment inhibited filaggrin expression in HSEM. **D**, TSLP stimulation increased phosphorylated (*p*) ERK and pSTAT3 expression in NHEKs. **E**, STAT3 and ERK1/2 siRNA block the TSLP-mediated downregulation of filaggrin. Columns and error bars represent means $\pm$ SEMs from 3 independent experiments. **P* < .05. Bar = 100 μ m.

skin and AD lesional skin. As shown in Fig 1, C, the 2 TSLP receptor subunits were also demonstrated in normal and AD lesional skin. A recent article also presented the expression of TSLP receptor in normal human epidermis.⁷ IL-7R α and CRLF2 mRNA expression were investigated by using real-time PCR to address the difference of the TSLP receptor expression between normal skin and AD lesional skin. Real-time PCR experiments showed that there is no difference in IL-7R α and

CRLF2 expression between normal skin and AD lesional skin (see Fig E1 in this article's Online Repository at www.jacionline.org).

To investigate the effect of TSLP on filaggrin expression, we measured filaggrin mRNA and protein levels using real-time PCR and Western blot analysis after TSLP stimulation in keratinocytes. Compared with levels in untreated cells, levels of both filaggrin and profilaggrin were downregulated by 16 hours of

exposure to TSLP, especially at a higher concentration ($100 \text{ ng} \cdot \text{mL}^{-1}$; Fig 2, A and B). Downregulation of filaggrin expression was detected from 16 hours after TSLP treatment (see Fig E2 in this article's Online Repository at www.jacionline.org).

To confirm the downregulation of filaggrin expression by TSLP in human skin, we used a human skin equivalent model (HSEM). HSEMs are similar to normal human epidermis and consist of multilayered differentiated tissue, including basal, spinous, granular, and cornified layers. Filaggrin expression was downregulated in the TSLP-treated HSEM more than in the PBS-treated HSEM (Fig 2, C). Because filaggrin is known to have single nucleotide polymorphisms (SNPs), we investigated this and confirmed that there were no abnormal SNPs in keratinocytes. The keratinocytes, which were used in this study, did not carry either the p.R501X mutation or the c.2282del4 *FLG* mutation (see Fig E3 in this article's Online Repository at www.jacionline.org). These results indicate that TSLP can reduce filaggrin expression in human skin.

Furthermore, to determine whether TSLP affects the expression of other keratinocyte differentiation-associated markers, such as involucrin, loricrin, keratin 10, and keratin 5, we measured mRNA levels of each molecule after TSLP treatment using real-time RT-PCR. The present study demonstrated that TSLP did not affect expression of involucrin, loricrin, keratin 10, and keratin 5 (see Fig E4 in this article's Online Repository at www.jacionline.org).

It is already known that the inflammatory cytokines IL-4, IL-17A, and IL-22 inhibit filaggrin expression.^{3,4} In this study the inhibitory effects of TSLP, IL-4, IL-17A, and IL-22 on filaggrin expression in NHEKs were compared by using immunofluorescence assays. As shown in Fig E5 in this article's Online Repository at www.jacionline.org, TSLP and IL-4 caused a more marked decrease of filaggrin expression compared with IL-17A and IL-22 in NHEKs. These findings suggest that TSLP might be an important factor to decrease filaggrin expression.

A previous study in human airway smooth muscle cells showed that TSLP can activate signal transducer and activator of transcription (STAT) 3 and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK) 1/2, p38, and c-Jun N-terminal kinase (JNK).⁸ To investigate the involvement of STAT3 and MAPKs in TSLP-induced filaggrin downregulation in NHEKs, we conducted Western blot analyses for phosphorylated STAT3 and MAPKs. TSLP ($100 \text{ ng} \cdot \text{mL}^{-1}$) stimulation induced a significant phosphorylation of STAT3 and ERK1/2 in NHEKs; however, it did not induce noticeable phosphorylation of p38 and JNK (Fig 2, D). These results indicate that TSLP can activate the STAT3 and ERK1/2 pathways in NHEKs.

To define which signal pathway is necessary for mediating the inhibitory effect of TSLP on filaggrin expression in NHEKs, we examined the effects of signaling blockade with specific inhibitors. As shown in Fig E6 in this article's Online Repository at www.jacionline.org, blockade of STAT3 activation with the specific STAT3 inhibitor STA-21 abolished TSLP activity, which led to a substantial restoration of filaggrin expression. Similarly, suppression of ERK1/2 activation with the upstream kinase mitogen-activated protein kinase 1/2 inhibitor U0126 also markedly restored filaggrin expression. However, suppression of p38 with SB239063 or JNK with

SP600125 does not change filaggrin expression. To further establish the role of STAT3 activation, ERK1/2 activation, or both, we used a small interfering RNA (siRNA)-mediated incapacitating approach and assessed filaggrin expression. Treatment with siRNA effectively attenuated STAT3 and ERK1/2 and resulted in restoration of filaggrin expression (Fig 2, E). Recently, it was reported that caspase-14 is required for filaggrin degradation.⁹ NHEKs were treated with TSLP (0, 1, 10, or $100 \text{ ng} \cdot \text{mL}^{-1}$) for 16 hours to investigate whether caspase-14 was involved in TSLP-mediated filaggrin degradation. Expression of caspase-14 was measured by means of Western blotting with anti-caspase-14 antibody. As shown in Fig E7 in this article's Online Repository at www.jacionline.org, TSLP did not control caspase-14 expression. These data suggest that TSLP inhibits filaggrin expression through a STAT3-dependent mechanism, ERK-dependent mechanism, or both.

In conclusion, this study investigated the presence of a TSLP receptor in human epidermis and keratinocytes. Our results disclose that TSLP can downregulate filaggrin expression in human skin. The present study suggests that while skewing the immune response in a T_H2 direction, TSLP also contributes to barrier dysfunction. In addition, we demonstrated that the downregulation of filaggrin by TSLP is mediated by STAT3-dependent pathways, ERK-dependent pathways, or both. In view of these findings, TSLP and its related downstream molecules might be a target to ameliorate the disrupted skin barrier in patients with AD.

Jin Hee Kim, PhD*

Hyun Cheol Bae, PhD*

Na Young Ko, MD, PhD

See Hyun Lee, MD

Sang Hoon Jeong, PhD

Hana Lee, MSc

Woo-In Ryu, BSc

Young Chul Kye, MD, PhD

Sang Wook Son, MD, PhD

From the Department of Dermatology and Division of Brain Korea 21 Project for Biomedical Science, Korea University College of Medicine, Seoul, Republic of Korea. E-mail: skin4u@korea.ac.kr.

*These authors contributed equally to this work.

Disclosure of potential conflict of interest: S. W. Son has received research support from the National Research Foundation of Korea, which is funded by the Ministry of Science, ICT, and Future Planning (NRF-2013R1A2A2A01068137), and the Korean Health Technology R&D Project, Ministry for Health, Welfare & Family Affairs (A101334). The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Leung DY, Yassky EG. Deciphering the complexities of atopic dermatitis: shifting paradigms in treatment approaches. *J Allergy Clin Immunol* 2014;134:769-79.
2. Thyssen JP, Sanja K. Causes of epidermal filaggrin reduction and their role in the pathogenesis of atopic dermatitis. *J Allergy Clin Immunol* 2014;134:792-9.
3. Howell MD, Kim BE, Gao P, Grant AV, Boguniewicz M, Debenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. *J Allergy Clin Immunol* 2007;120:150-5.
4. Gutowska-Owsiak D, Schaupp AL, Salimi M, Selvakumar TA, McPherson T, Taylor S, et al. IL-17 downregulates filaggrin and affects keratinocyte expression of genes associated with cellular adhesion. *Exp Dermatol* 2012;21:104-10.
5. Ziegler SF. Thymic stromal lymphopoietin and allergic disease. *J Allergy Clin Immunol* 2012;130:845-52.
6. Yoo J, Omori M, Gyarmati D, Zhou B, Aye T, Brewer A, et al. Spontaneous atopic dermatitis in mice expressing an inducible thymic stromal lymphopoietin transgene specifically in the skin. *J Exp Med* 2005;202:541-9.

7. Kubo T, Kamekura R, Kumagai A, Kawata K, Yamashita K, Mitsuhashi Y, et al. Δ Np63 controls a TLR3-mediated mechanism that abundantly provides thymic stromal lymphopoietin in atopic dermatitis. *PLoS One* 2014; 29:e105498.
8. Shan L, Redhu NS, Saleh A, Halayko AJ, Chakir J, Gounni AS. Thymic stromal lymphopoietin receptor-mediated il-6 and cc/cxc chemokines expression in human airway smooth muscle cells: role of mapks (erk1/2, p38, and jnk) and stat3 pathways. *J Immunol* 2010;184:7134-43.
9. Hoste E, Kemperman P, Devos M, Denecker G, Kezic S, Yau N, et al. Caspase-14 is required for filaggrin degradation to natural moisturizing factors in the skin. *J Invest Dermatol* 2011;131:2233-41.

<http://dx.doi.org/10.1016/j.jaci.2015.04.026>

METHODS

Culture and stimulation of primary human keratinocytes

NHEKs were purchased from Millipore (Temecula, Calif). Keratinocytes, which were used in this study, did not carry the 2 widely known mutations of *FLG* p.R501X and c.2282del4 (Fig E1). Cells were cultured in a keratinocyte complete media kit (EpiGRO; Millipore, Billerica, Mass) containing 6 mmol/L L-glutamine, 0.4% EpiFactor P, 1 μ mol/L epinephrine, 0.5 ng·mL⁻¹ rhTGF- α , 100 ng·mL⁻¹ hydrocortisone hemisuccinate, 5 μ g/mL recombinant human insulin, and 5 μ g/mL Apo-Transferrin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂. Seventh- or eighth-passage keratinocytes were used. The calcium concentration was increased to 1.6 mol/L in cultures of primary cells for up to 5 days before using the cells in the experiments. Keratinocytes were stimulated with various concentrations (0, 1, 10, and 100 ng·mL⁻¹) of TSLP (Sigma, St Louis, Mo) for 16 hours to reveal the effects of TSLP on filaggrin expression. For analysis of the related signaling molecules, we investigated whether TSLP treatment (100 ng·mL⁻¹) could change the phosphorylation status of ERK, p38, JNK, and STAT3 in keratinocytes and whether 3 hours of treatment with each inhibitor against ERK (5 μ mol/L U0126, Sigma), p38 (5 μ mol/L SB239063, Sigma), JNK (1 μ mol/L SP600125, Sigma), and STAT3 (5 OM STA-21; Enzo Life Sciences, Farmingdale, NY) could restore filaggrin expression. To compare the inhibitory activities of TSLP and other cytokines on filaggrin expression, we stimulated keratinocytes with TSLP (100 ng·mL⁻¹), IL-4 (100 ng·mL⁻¹; R&D Systems, Minneapolis, Minn), IL-17A (100 ng·mL⁻¹, R&D Systems), and IL-22 (100 ng·mL⁻¹, R&D Systems) for 16 hours. The human reconstituted epidermis 3D EpiDerm models (EPI-200), which were used as HSEMs, were purchased from MatTek (Ashland, Mass). HSEMs were incubated in assay medium at 37°C and 5% CO₂ and used for immunohistochemistry analysis.

Collection of skin biopsy samples

Two-millimeter punch biopsy specimens from the normal skin of healthy volunteers (n = 5) and lesional skin of patients with AD (n = 5) were collected after obtaining written informed consent in accordance with the guidelines of the Korea University Institutional Review Board and the Declaration of Helsinki Principles. The skin samples were submerged immediately in 10% buffered formalin for immunofluorescence assay.

Preparation of siRNAs and transfection

The phosphodiester RNA oligonucleotides for ERK1, ERK2, and STAT3 molecules were synthesized by Bioneer (Daejeon, Korea). The sense strand of each siRNA duplex consisted of a 19-nt target sequence followed by a dinucleotide deoxy-thymine nucleotide (dTdT) 3' overhang. The antisense strand was composed of nucleotides complementary to the target sequence and the dTdT 3' overhang. The sense and antisense strands of siRNA were as follows: STAT3, beginning at 1108 nt, 5'-AACUUCAGACCCGUCAA CAAA-3' (sense) and 5'-TTTGTGACGGGTCTGA AGTT-3' (antisense); ERK1, beginning at 1214 nt, 5'-CCGAAGCACCAUUAAGUU CGA CAU-3' (sense) and 5'-ATGTCGAACCTGAATGGTGCTTCGG-3' (antisense); and ERK2, beginning at 569 nt, 5'-CAGAUCUUUACAAGCUCUU-3' (sense) and 5'-AAGAGCTTGTAAGATCTG-3' (antisense). P represents 5' phosphate. The RNAs were deprotected and annealed according to the manufacturer's instructions. Nonspecific control siRNA was also acquired from Bioneer (target: 5'-GCAUCAAGGUGAACUUAAdTdT-3' [sense] and 5'-UUGAAGUACCUUGAUGCdTdT-3' [antisense]). Keratinocytes on a 6-well vessel were transfected with 300 pmol of each synthesized siRNA by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions, twice at an interval of a day. The transfected cells were used for subsequent experiments 2 days later.

RT-PCR analysis

For analysis of TSLP receptor complex expression, we performed RT-PCR. Total RNA was isolated with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription reactions were conducted

with 2 μ g of total RNA. Forward and reverse specific primer sequences were used, and the size of the amplified fragment and the annealing temperature were as follows: β -actin, 5'-CCCCAGGCACCAGGGCGTGAT-3' (forward) and 5'-GGTCATCTTCTCGCGTTGGCCTTGGGGT-3' (reverse), 272 bp, 55°C; CRLF2, 5'-GAGTGGCAGTCCAAACAGGAA-3' (forward) and 5'-ACATCTCCATAGCCTTCAC C-3' (reverse), 103 bp, 62°C; and IL-17R α , 5'-TGGACGCATGTGAATTATC-3' (forward) and 5'-CATTCATCCCA GAAGCCTTT-3' (reverse), 130 bp, 57°C. PCR products were run on 2% wt/vol agarose gel electrophoresis and visualized by means of ethidium bromide staining. Quantities of all targets in test samples were normalized to the corresponding β -actin levels in the cultured keratinocytes and expressed as target gene normalized to β -actin to compare between samples and groups.

For evaluation of filaggrin, involucrin, loricrin, keratin 10, and keratin 5 expression, we performed real-time RT-PCR. Real-time RT-PCR reactions were performed according to the methods of a previous report. The reactions were carried out in a final volume of 25 μ L consisting of 12.5 to 50 ng of reverse-transcribed cDNA mixed with an optimal concentration of primers and iQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Calif) in 96-well plates on an ABI PRISM 7700 detector (Applied Biosystems, Foster, Calif). Typically, the amplification started with 2 minutes at 50°C, 10 minutes at 95°C, and then 40 cycles of the following: 15 seconds at 95°C and 1 minute at 60°C. The forward and reverse specific primer sequences were used, and the size of the amplified fragment and the annealing temperature were as follows: β -actin, 5'-CCC CAG GCA CCA GGG CGT GAT-3' (forward) and 5'-GGT CAT CTT CTC GCG GTT GGC CTTGGG GT-3' (reverse), 272 bp, 55°C; involucrin (*INV*), 5'-TCCT CCAGTCAATACCCATC-3' (forward) and 5'-AGTTGCTCATCTCTCTT GACT-3' (reverse), 363 bp, 55°C; *FLG*, 5'-TGGATCACTTGGATATAGACCA CAAC-3' (forward) and 5'-GCTTGAGCCAACCTGAATACCAT-3' (reverse), 110 bp, 55°C; loricrin (*LOR*), 5'-GGGCA CCGATGGGCTTAG-3' (forward) and 5'-GGTAGGTTAAGACATGAAGGATTTCG-3' (reverse), 70 bp, 55°C; keratin 10 (*K10*), 5'-TCGCTACTGTGTGCAGCTCT-3' (forward) and 5'-CGG AACTTCCCTCTCCTTCT-3' (reverse), 185 bp, 55°C; and keratin 5 (*K5*), 5'-TCTCGCCAGTCAAGTGTGTC-3' (forward) and 5'-ATAGCCACC CACT CCACAAG-3' (reverse), 177 bp, 55°C. Human β -actin was used as an endogenous reference. Relative quantitation values were expressed by using the 2- $\Delta\Delta$ Ct method (CFX96 real-time PCR detection system and CFX manager software, Bio-Rad Laboratories) as fold changes in the target gene normalized to the reference gene (β -actin) and related to expression of the untreated controls. The PCR efficiency in all runs was close to 100%, and all samples were performed at least in duplicate.

Western blotting

The expression of filaggrin and the phosphorylation of STAT3 and MAPKs in TSLP-treated keratinocytes were analyzed by means of Western blotting. Protein was extracted from cells with lysis buffer (50 mmol/L Tris, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% NP-40, 0.5% deoxycholic acid, 1 mmol/L sodium orthovanadate, 100 mg·mL⁻¹ phenylmethanesulfonyl fluoride, and protease inhibitors). Protein concentrations were determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill) by using BSA as the standard. Protein lysates (50 μ g) were fractionated by means of 8% SDS-PAGE. Western blot assays were carried out on polyvinylidene difluoride membranes (Millipore) using the specific antibodies for filaggrin (sc-66192, 1:5000; Santa Cruz Biotechnology, Santa Cruz, Calif), pERK1/2 (Thr202/Tyr204; Cell Signaling 4370, 1:1000), ERK1/2 (Thr202/Tyr204; Cell Signaling 9102, 1:1000), pp38 (Thr180/Tyr182; Cell Signaling 9215, 1:1000), p38 (Thr180/Tyr182; Cell Signaling 9212, 1:1000), pJNK (Thr183/Tyr185; Cell Signaling 4668, 1:1000), JNK (Cell Signaling 9252, 1:1000), pSTAT3 (Tyr705; Cell Signaling 9131, 1:1000), STAT3 (Cell Signaling 4904, 1:1000), caspase-14 (Cell Signaling 8519, 1:1000), and β -actin (1:1000; Santa Cruz sc-47778, 1:5000). The immunoreactive bands were visualized by means of an enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) reaction.

Immunofluorescence assay

Keratinocytes were fixed with acetone and then blocked with 20% goat serum. The blocked slides were incubated with mAbs for IL-7R α

(BD 557938), CRLF2 (ab56373; Abcam, Cambridge, United Kingdom), and filaggrin (Abcam ab81468). CY3-labeled goat anti-rabbit antibody (KPL, Gaithersburg, Md) was used to detect the unconjugated antibodies. Nuclei were visualized by using DAPI.

Paraffin-embedded tissue sections (4 μ m) were deparaffinized, rehydrated, and washed twice in buffer. The slides were incubated in 5% goat serum in PBS for 30 minutes and washed 4 times in buffer to reduce nonspecific background staining. The primary antibody, anti-CRLF2 antibody (1:100, anti-human CRLF2 polyclonal antibody; Abcam), or matched control mouse IgG₁ antibody was applied and incubated according to the manufacturer's recommended protocol. Then the slides were washed 4 times in buffer. Fluorescein isothiocyanate-conjugated anti-mouse IgG (1:100; BD, San Diego, Calif) antibody was applied to the slides, which were subsequently incubated for 1 hour at room temperature and washed 4 times in buffer. Phycoerythrin-conjugated IL-7R α (1:100, BD) or isotype control PE-MOPC21 (1:100, BD) antibody was applied to the slides, and the slides were incubated for 1 hour at room temperature and washed 4 times in buffer. Nuclei were counterstained with DAPI (Sigma). Samples were photographed with a fluorescence microscope (Olympus LX71 microscope; Olympus, Tokyo, Japan).

FLG mutation analysis in human keratinocytes

The analysis of *FLG* mutations was performed, as previously described, with modifications.^{E1} We used the QIAamp DNA mini kit (Qiagen, Valencia, Calif) for DNA extraction from keratinocytes. With specific primer pairs, we amplified the coding regions of the *FLG* gene harboring the 2 most frequent germline mutations: p.R501X and c.2282del4. In detail, the primers used were FIL501F-CACGGAAAGGCTGGGCTGA and FIL501R-ACCTGAGTGTCAGACCTATT for mutation p.R501X and FIL2282F-GGGAGGACTCA GACTGTTT and FIL2282R-AATAGGTCTGGACACTCAGGT for

mutation c.2282del4. PCR reactions contained 50 ng of DNA, 10 pmol of each forward and reverse primer, 4% dimethyl sulfoxide, and amfiEco PCR Premix (GenDEPOT, Barker, Tex) in a total volume of 25 μ L. For mutation p.R501X, PCR conditions were initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 15 seconds, and elongation at 72°C for 30 seconds, followed by a final elongation step at 72°C for 7 minutes. For mutation c.2282del4, PCR conditions were initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 60 seconds, followed by a final elongation step at 72°C for 7 minutes. PCR products were directly sequenced with an Automatic Sequencer ABI3730xI Applied (Applied Biosystems). Sequence analysis was performed with ABIPrism Terminator, version 3.1 (Applied Biosystems).

Statistical analysis

All experiments were conducted in triplicate and independently repeated at least 3 times. The statistical significance of the data was determined by using 1-way ANOVA with GraphPad PRISM version 4.02 for Windows (GraphPad Software, San Diego, Calif). For experiments with only 2 groups, the Student *t* test was used for comparisons of group means. Results are expressed as means $\pm$ SDs. Differences at a *P* value of less than .05 were considered statistically significant.

REFERENCE

- E1. Smith FJ, Irvine AD, Terron-Kwiatkowski A, Sandilands A, Campbell LE, Zhao Y, et al. Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. *Nat Genet* 2006;38:337-42.

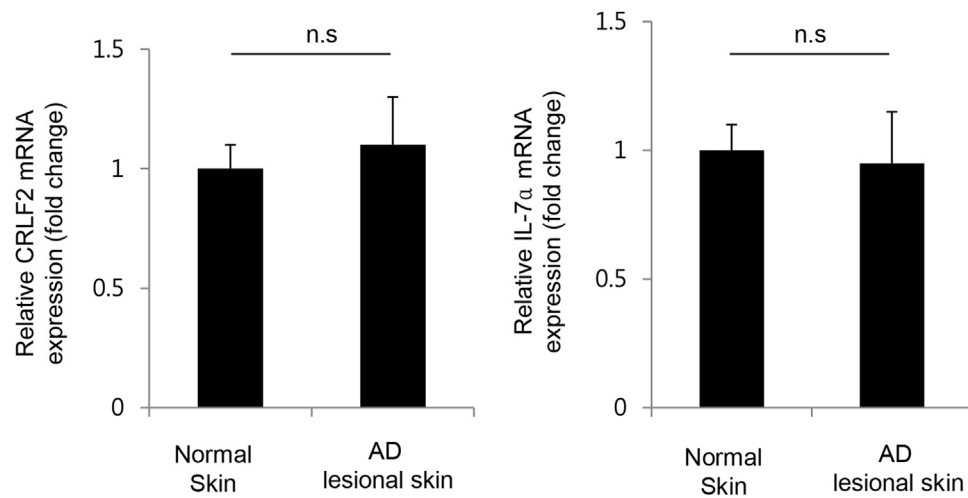


FIG E1. CRLF2 and IL-7 α mRNA expression in normal skin and AD lesional skin. Total mRNA was extracted from normal human skin and AD lesional skin. Expression of CRLF2 and IL-7 α was measured by using real-time PCR. *n.s.*, Not significant ($P > .05$).

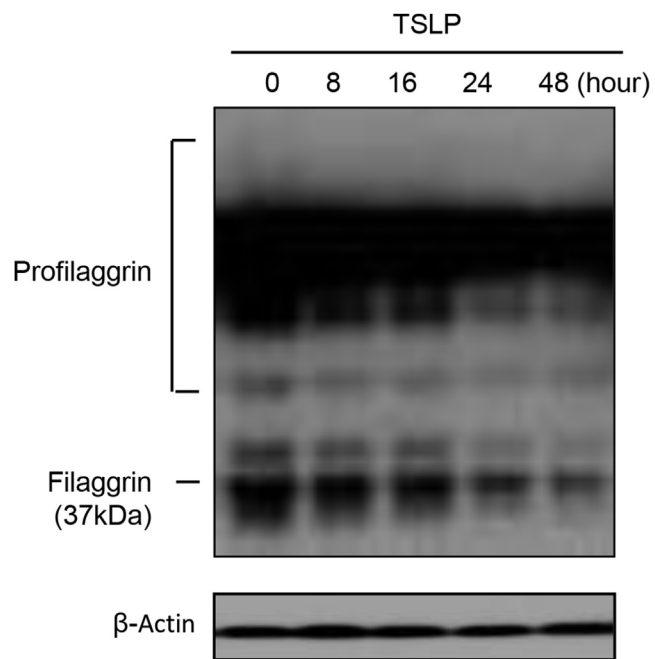


FIG E2. Time course of filaggrin expression after TSLP treatment in NHEKs. NHEKs were treated with $100 \text{ ng} \cdot \text{mL}^{-1}$ TSLP for each time point. The expression of filaggrin was measured by means of Western blotting with anti-filaggrin antibodies.

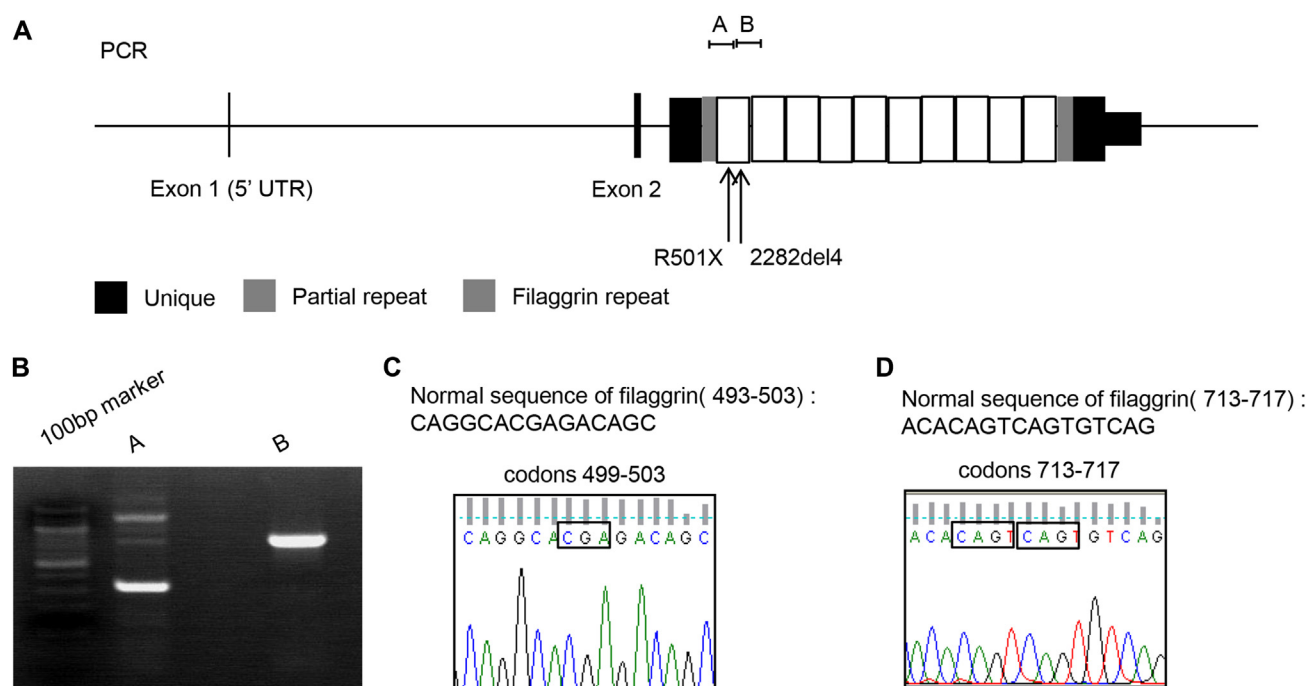


FIG E3. *FLG* mutation confirmation. **A**, Schematic diagram of *FLG*. **B**, PCR product from genomic DNA covering the exon 3 A and B region. **C**, Normal sequence from filaggrin repeat 1 in exon 3 corresponding to codons 499 to 503 (upper portion). In the lower portion of Fig E3, C, the same region of *FLG* as seen in the upper region is shown, with normal sequence of filaggrin (499-503) in NHEKs, which were used in this study. **D**, Normal sequence from filaggrin repeat 1 in exon 3, corresponding to codons 713 to 717 (upper portion). In the lower portion of Fig E3, D, the same region of *FLG* as seen in the upper region is shown, with the 2282del4 filaggrin sequence in NHEKs, which were used in this study.

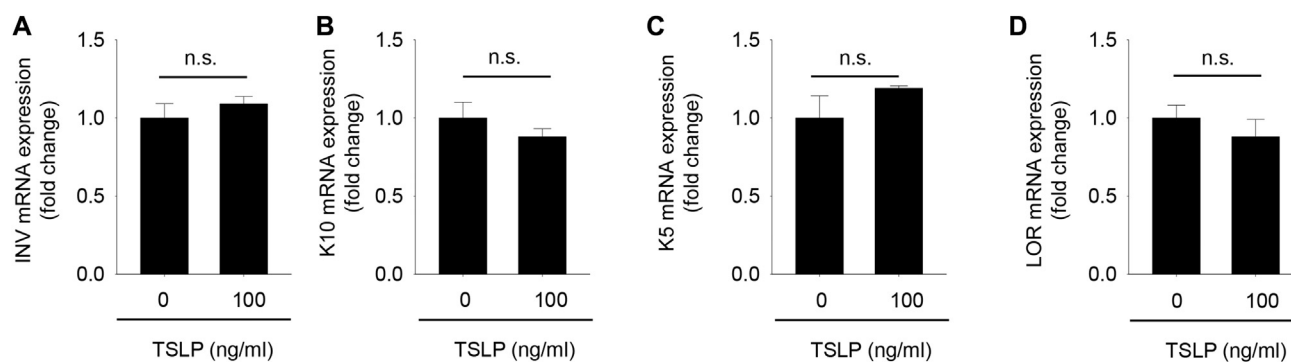


FIG E4. Effects of TSLP on skin barrier proteins. NHEKs were treated with TSLP ($100 \text{ ng} \cdot \text{mL}^{-1}$) for 16 hours. Gene expression of involucrin (*IVL*; **A**), keratin 10 (*K10*; **B**), keratin 5 (*K5*; **C**), and loricrin (*LOR*; **D**) was examined by means of real-time RT-PCR. *n.s.*, Not significant ($P > .05$).

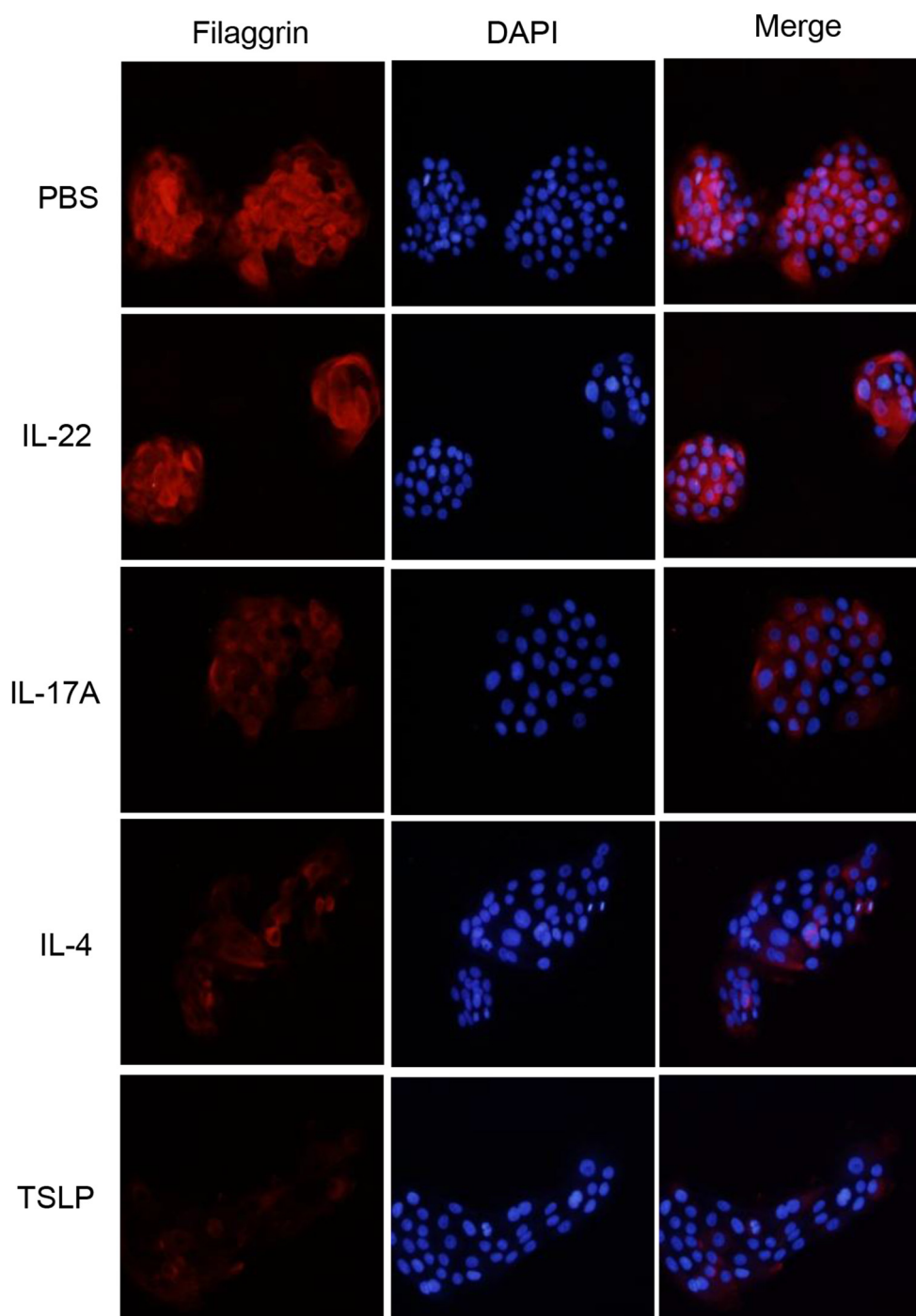


FIG E5. Comparison of inhibitory activity on filaggrin expression between TSLP and other inflammatory cytokines. Filaggrin expression levels were examined by using immunofluorescence. Filaggrin expression was decreased in all keratinocytes treated with TSLP ($100 \text{ ng} \cdot \text{mL}^{-1}$), IL-4 ($100 \text{ ng} \cdot \text{mL}^{-1}$), IL-17A ($100 \text{ ng} \cdot \text{mL}^{-1}$), and IL-22 ($100 \text{ ng} \cdot \text{mL}^{-1}$). TSLP and IL-4 showed a stronger inhibitory activity on filaggrin expression compared with IL-17A and IL-22.

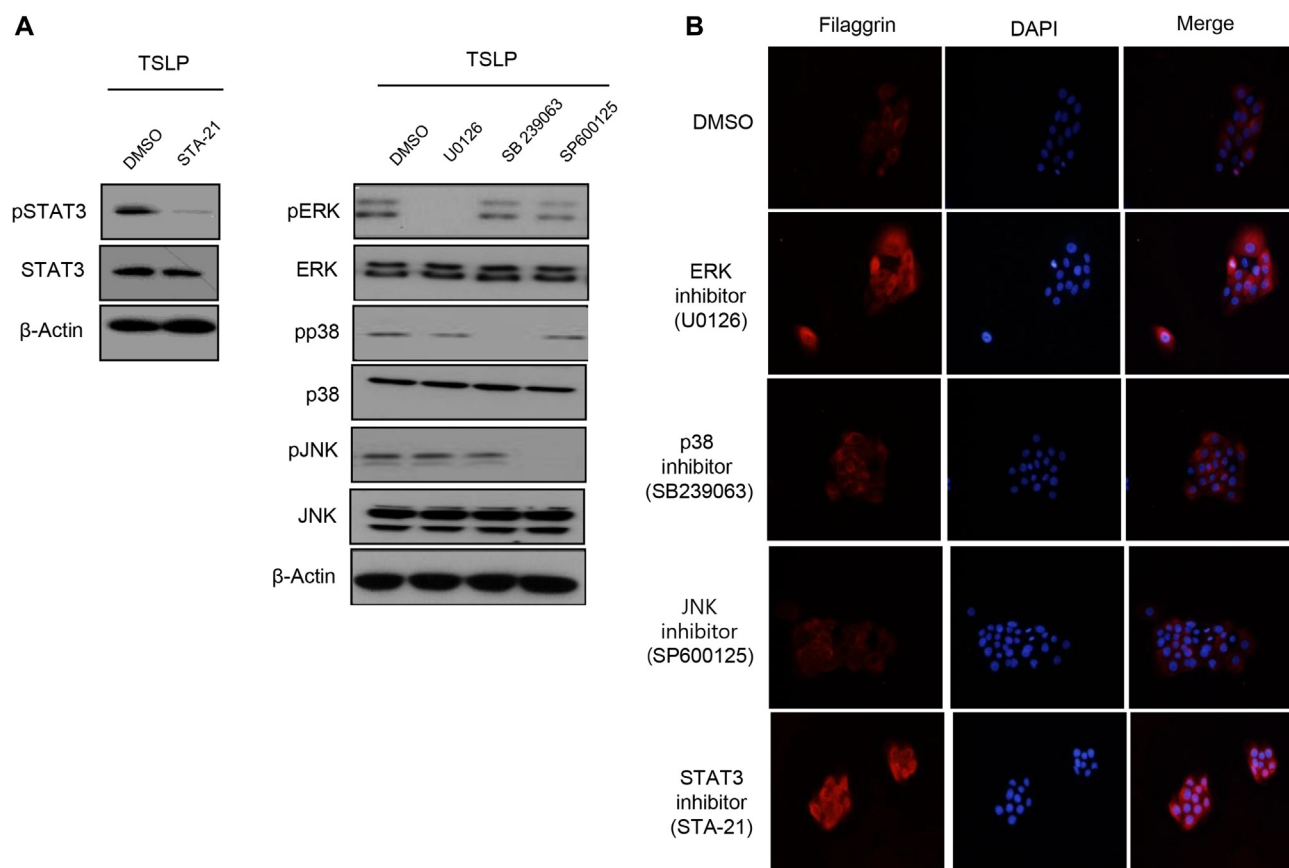


FIG E6. STAT3 and ERK1/2 inhibitors or siRNA block the TSLP-mediated downregulation of filaggrin. **A**, Western blot analysis characterized the expression of total ERK, phosphorylated (*p*) ERK (Thr202/Tyr204), total p38 MAPK, pp38 MAPK (Thr180/Tyr182), total JNK, pJNK (Thr183/Tyr185), total STAT3, and pSTAT3 (Tyr705). NHEKs were preincubated with specific inhibitors against ERK (U0126, 5 $\mu\text{mol/L}$), p38 (SB239063, 5 $\mu\text{mol/L}$), JNK (SP600125, 1 $\mu\text{mol/L}$), and STAT3 (STA-21, 5 $\mu\text{mol/L}$) for 3 hours before stimulation with TSLP (100 $\text{ng}\cdot\text{mL}^{-1}$). **B**, Expression of filaggrin was visualized by using immunofluorescence staining. β -Actin was used as a loading control. When ERK and STAT3 signaling was blocked, filaggrin expression was restored. Data are representative of 3 independent experiments. *DMSO*, Dimethyl sulfoxide.

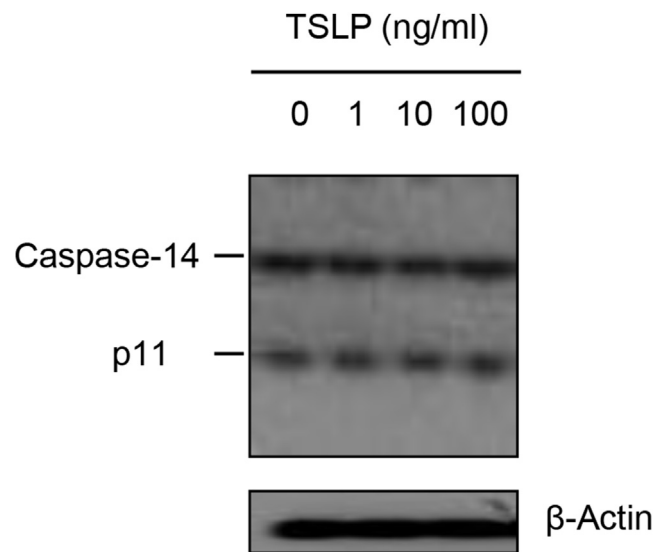


FIG E7. TSLP treatment does not control caspase-14 expression in NHEKs. NHEKs were treated with TSLP (0, 1, 10, and 100 ng·mL⁻¹) for 16 hours. Expression of caspase-14 was measured by means of Western blotting with anti-caspase-14 antibodies.