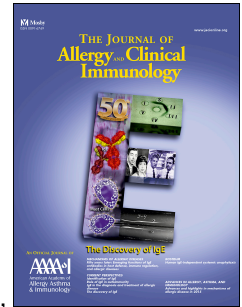


Accepted Manuscript

Blockade of TNFR1-dependent and -independent cell death is crucial for normal epidermal differentiation

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PII: S0091-6749(18)30446-9

DOI: [10.1016/j.jaci.2018.02.043](https://doi.org/10.1016/j.jaci.2018.02.043)

Reference: YMAI 13359

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 24 August 2017

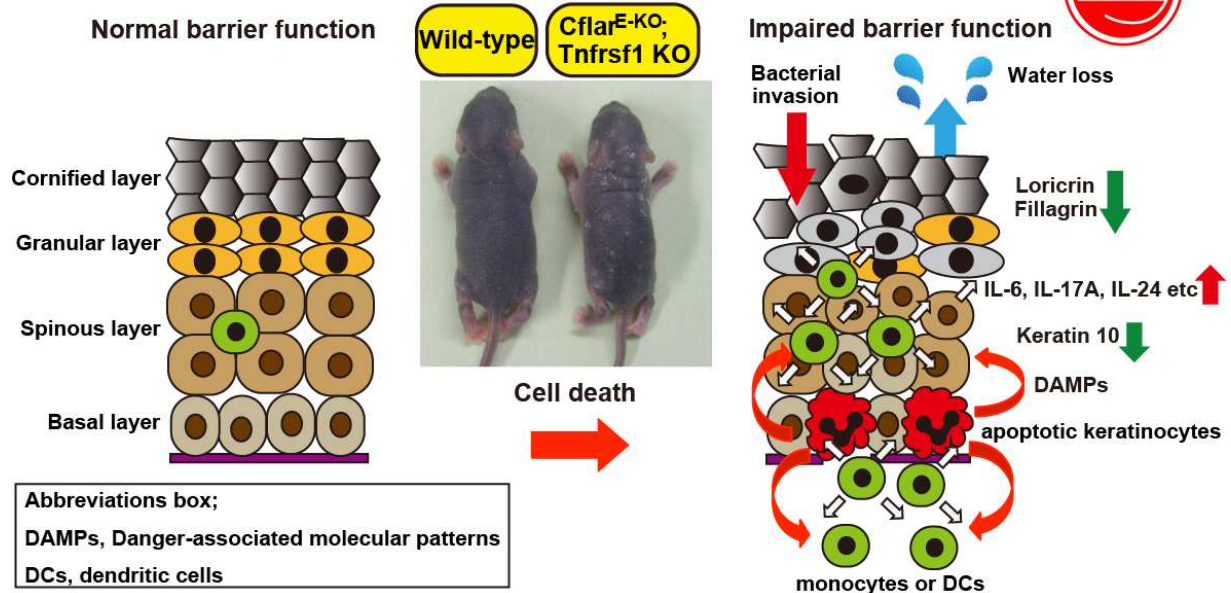
Revised Date: 13 February 2018

Accepted Date: 26 February 2018

Please cite this article as: Piao X, Miura R, Miyake S, Komazawa-Sakon S, Koike M, Shindo R, Takeda J, Hasegawa A, Abe R, Nishiyama C, Mikami T, Yagita H, Uchiyama Y, Nakano H, Blockade of TNFR1-dependent and -independent cell death is crucial for normal epidermal differentiation, *Journal of Allergy and Clinical Immunology* (2018), doi: 10.1016/j.jaci.2018.02.043.

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Enhanced cell death of keratinocytes impairs skin barrier function, resulting in the development of atopic dermatitis-like skin lesions



Blockade of TNFR1-dependent and -independent cell death is crucial for normal epidermal differentiation

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Funding: RM and RS were supported by Research Fellowships 17J00933 (to RM) and 14J12413 (to RS) from Japan Society for the Promotion of Science (JSPS), Japan, respectively. This work was supported in part by Grants-in-Aid from Scientific Research (B) 17H04069 (to HN), Challenging Exploratory Research 17K19533 (to HN), and Research Activity Start-up 26893265 (to XP) from JSPS, and Scientific Research on Innovative areas 26110003 (to HN), and Private University Research Branding project (to HN) from a MEXT (Ministry of Education, Culture, Sports, Science and Technology), Japan, research grants from NOVARTIS Foundation for the Promotion of Science (to HN), the Naito Science Foundation (to HN), the Uehara Science Foundation (to HN), and the Takeda Science Foundation (to HN).

Disclosure of potential conflict of interest: X. Piao receives grant support from JSPS. H. Nakano receives grant support from JSPS, MEXT, NOVARTIS Foundation, Naito Science Foundation, Uehara Science Foundation, and Takeda Science Foundation. The rest of the authors declare that they have no relevant conflicts of interest.

ABSTRACT

Background: Delicate balance between cell death and proliferation of keratinocytes is crucial for normal development of the skin. Previous studies have reported that cellular FLICE-inhibitory protein (cFLIP) plays a crucial role in prevention of keratinocytes from TNF α -dependent apoptosis and blocking the development of dermatitis. However, a role for cFLIP in TNF α -independent cell death remains unclear.

Objective: We investigated contribution of TNF α -dependent and -independent signals to the development of dermatitis in epidermis-specific *Cflar*-deficient (*Cflar*^{E-KO}) mice.

Methods: We examined histology, and expression of epidermal differentiation markers and inflammatory cytokines of the skin of *Cflar*^{E-KO}; *Tnfrsf1a*^{+/-} and *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice. To block TNF α -independent cell death of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice, mice were treated with neutralizing antibodies against FasL and TRAIL.

Results: *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice were born, but developed severe dermatitis and succumbed soon after birth. *Cflar*^{E-KO}; *Tnfrsf1a*^{+/-} mice exhibited embryonic lethality due to massive apoptosis of keratinocytes. While keratinocytes from *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice still died by apoptosis, neutralizing antibodies against FasL and TRAIL substantially prolonged survival of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice. Expression of inflammatory cytokines, such as *interleukin (Il)6* and *Il17a* was elevated, conversely,

expression of epidermal differentiation markers was severely downregulated in the skin of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice. Treatment of primary keratinocytes with IL-6, and to a lesser extent IL-17A suppressed expression of epidermal differentiation markers.

Conclusion: TNFR1-dependent or -independent apoptosis of keratinocytes promotes inflammatory cytokine production that subsequently blocks epidermal differentiation. Thus, blockade of both TNFR1-dependent and -independent cell death might be an alternative strategy to treat skin diseases when treatment with anti-TNF α antibody alone is not sufficient.

Key Messages: cFLIP plays a crucial role in the suppression of cell death of keratinocytes, thereby preventing a defect in epidermal differentiation triggered by inflammatory cytokines.

Capsule summary: Epidermis-specific deletion of *Cflar* gene uncovers that prevention of both TNFR1-dependent and -independent apoptosis is crucial for the maintenance of normal epidermal differentiation.

Key words: cFLIP, apoptosis, epidermal differentiation, TNF α

93 Abbreviations used

94 CC3: cleaved caspase 3

95 cFLIP: cellular FLICE-inhibitory protein

96 Flg: Filaggrin

97 Krt: Keratin

98 Lor: Loricrin

99 MLKL: Mixed lineage kinase domain-like protein

100 Nec-1: Necrostatin-1

101 RIPK3: Receptor-interacting protein kinase 3

102 TEWL: transepidermal water loss

103 TNF α : tumor necrosis factor α

104 TNFR1: TNF receptor superfamily 1

105 TRAIL: TNF-related apoptosis-inducing ligand

106

INTRODUCTION

The skin homeostasis is maintained by delicate balance between proliferation and differentiation of keratinocytes that constitutes the first line defense against microbes or various environmental stresses^{1, 2}. Cell death of keratinocytes is tightly regulated to maintain tissue homeostasis, however, the detailed molecular mechanism is not fully understood.

Psoriasis is a chronic human skin disease that is characterized by hyperproliferation of epidermal cells, parakeratosis, and massive infiltration of T cells^{3, 4}. To understand the mechanism underlying psoriasis, various murine models have been established. Accumulating studies using epidermis-specific NF- κ B activation-deficient mice reported that mice develop psoriasis-like dermatitis along with an increase in apoptosis of keratinocytes and upregulation of inflammatory cytokines⁵⁻¹¹. Importantly, the phenotypes of epidermis-specific NF- κ B activation-deficient mice are attenuated when mice are crossed with *Tnfrsf1a*^{-/-} mice or treated with neutralizing antibody against TNF α ⁵⁻¹¹. Those studies combined with others have shown that TNF α , T_H17 cells, and T_H17 signature cytokines, such as IL-17A, IL-22, and IL-23 are involved in the development of murine models of psoriasis^{12, 13}. Consistently, anti-TNF α and anti-IL-17A antibodies are used to treat psoriasis^{3, 4}. While expression of IL-6 is elevated in the skin of psoriasis patients and murine skin disease models described

above, blockade of IL-6 function rather exacerbates the severity of diseases in both human and mice^{14, 15}.

Blockade of apoptosis by deletion of *Caspase 8* or *Fadd* in the epidermis of mice culminates in the development of dermatitis^{16, 17}. Deletion of *Receptor-interacting protein kinase (Ripk)3*, an essential kinase for programmed necrosis or necroptosis, blocks the development of dermatitis in epidermis-specific *Caspase 8* or *Fadd*-deficient mice^{16, 17}. This suggests that the apoptotic signaling pathway blocks RIPK3-dependent necroptosis under normal conditions. Intriguingly, the skin phenotypes of epidermis-specific NF- κ B activation-deficient or apoptosis-deficient mice are very similar, and all mice exhibit hyperproliferation of basal cells, persistent presence of undigested nuclei in cornified cells (parakeratosis), and an impaired differentiation of epidermal cells along with an increase in cell death¹⁸. These results suggest that dying keratinocytes might release growth factors, thereby inducing proliferation and aberrant differentiation of keratinocytes. One of the candidates that links cell death and proliferation might be interleukin (IL)-24, because deletion of a receptor for IL-24 abrogates dermatitis in epidermis-specific *Ikkb*-deficient mice⁷. However, the detailed molecular signatures are not fully elucidated.

Accumulating studies including us have reported that cFLIP plays a crucial role in maintaining tissue homeostasis by preventing apoptosis and programmed

necrosis¹⁹⁻²⁴. Moreover, previous studies have shown that inducible deletion of *Cflar* gene that encodes cFLIP in the epidermis results in severe dermatitis that is blocked by neutralization of TNF α -mediated cytotoxicity^{25, 26}. Taken that cFLIP plays an essential role in protection of cell death induced by FasL and TRAIL^{27, 28}, it is unclear whether TNF α -independent signal also induces dermatitis in epidermis-specific *Cflar*-deficient mice.

In the present study, we generated epidermis-specific *Cflar*-deficient (*Cflar*^{E-KO}) mice. We found that TNFR1-dependent signal induced massive apoptosis of keratinocytes in utero, resulting in embryonic lethality of *Cflar*^{E-KO} mice. Although *Tnfrsf1a* deficiency rescued embryonic lethality of *Cflar*^{E-KO} mice, *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice still developed dermatitis and succumbed soon after birth. Notably, keratinocytes still died by apoptosis in the skin of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice. Neutralizing antibodies against FasL and TRAIL substantially prolonged survival of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice compared to control IgG-treated mice. Moreover, expression of epidermal differentiation markers, such as Loricrin, Filaggrin, and Keratin (K)10 was severely downregulated along with upregulation of inflammatory cytokines, such as *interleukin* (*IL*) 6, *Il17a*, and *Il24*. cFLIP blocks TNFR1-dependent and -independent apoptosis of keratinocytes, thereby preventing a defect in epidermal differentiation. Thus, blockade of TNFR1-dependent and -independent apoptosis is crucial for normal epidermal

164 differentiation, thereby blocking dermatitis.

METHODS

Reagents. Murine IL-6 (406-ML, R&D systems), IL-17A (210-17, Peprotech), IL-24 (7807-ML, R&D systems), IFN γ (315-05, Peprotech), murine and human TNF α (34-8321 and BMS301, eBioscience), z-VAD-fmk (3188-v, Peptide inc.), and Necrostatin-1 (N9037, Sigma-Aldrich) were purchased from the indicated sources. The following antibodies were used in this study and obtained from the indicated sources: anti-loricrin (PRB-145P, Covance), anti-filaggrin (PRB-417P, Covance), anti-keratin 5 (PRB-160P, Covance), anti-keratin 10 (RPB-159P, Covance), anti-cFLIP (804-127, Alexis), anti-tubulin (T5168, Sigma-Aldrich), anti-phospho-STAT1 (8826, Cell Signaling), anti-STAT1 (9172, Cell Signaling), anti-phospho-STAT3 (9145S, Cell Signaling), anti-STAT3 (sc-482, Santa Cruz), anti-phospho-I κ B α (2859, Cell Signaling), anti-cleaved caspase 3 (9664, Cell Signaling), anti-caspase 8 (804-447, Alexis), anti-CD11b (M1/70, TONBO), anti-CD3 (ab5690, Abcam), anti-F4/80 (MCA497GA, Biorad), anti-Fas (SY-001, MBL), anti-Ly-6G (1A8, TONBO), anti-RIPK1 (R41220, BD Bioscience), anti-RIPK3 (IMG-5523-2, IMGENEX), anti-Ki67 (Ab16667, Abcam), anti-IL-6 (MP5-20F3, Bio X cell), and anti-FLAG (M2, Sigma-Aldrich) antibodies, and control rat IgG (14131, Sigma-Aldrich). Anti-MLKL antibody was provided by J. Murphy and J. Silke. Anti-FasL (MFL4) and anti-TRAIL (N2B2) antibodies for in vivo administration were made in-house. Alexa 594-conjugated donkey anti-rabbit IgG

(A21207, Invitrogen), Alexa 488-conjugated donkey anti-rabbit IgG (A21206, Invitrogen), Alexa 488-conjugated goat anti-rabbit (A11070, Invitrogen), Alexa 488-conjugated donkey anti-rat IgG (A21209, Invitrogen), HRP-conjugated donkey anti-rabbit IgG (NA934, GE Healthcare), HRP-conjugated donkey anti-rabbit IgG (NA934, GE Healthcare), HRP-conjugated goat anti-rat IgG (NA935, GE Healthcare), HRP-conjugated sheep anti-mouse IgG (NA931, GE Healthcare) antibodies, biotin-conjugated goat anti-rabbit IgGs (E0432, Dako), and Streptavidin-HRP (P0397, Dako).

Cells

HaCaT cells, a human keratinocyte cell line, HeLa cells, a human cell line from cervical carcinoma, and RAW264.7 cells, a murine macrophage cell line, were maintained with DMEM containing 10% fetal calf serum.

Mice

Cflar^{F/F} mice were provided by Y-W. He and described previously²¹. *Cflar*^{F/F} mice were backcrossed with C57BL/6 mice at least 9 generations were used for experiments. *Tnfrsf1a*^{-/-} mice were provided by T-W. Mak and described previously²⁹. Keratin 5-*Cre* (*K5-Cre*) transgenic mice were generated and described previously³⁰.

To generate epidermis-specific *Cflar*-deficient under *Tnfrsf1a*-deficient background, *Cflar*^{F/F} mice were crossed with *Tnfrsf1a*^{-/-} mice, and further crossed with *K5-Cre* mice. To generate *Cflar*^{F/F}; *K5-Cre*; *Tnfrsf1a*^{+/-} mice,

Cflar^{F/+};K5-Cre;*Tnfrsf1a*^{-/-} mice were crossed with *Cflar*^{F/F}; *Tnfrsf1a*^{+/+} mice.
Cflar^{F/F};K5-Cre;*Tnfrsf1a*^{-/-} and *Cflar*^{F/F};K5-Cre;*Tnfrsf1a*^{+/-} mice were referred to as
Cflar^{E-KO}; *Tnfrsf1a*^{-/-} and *Cflar*^{E-KO}; *Tnfrsf1a*^{+/-} mice, respectively. *Cflar*^{F/F}, *Cflar*^{F/+}, or
Cflar^{F/+};K5-Cre mice under *Tnfrsf1a*^{+/-} and *Tnfrsf1a*^{-/-} genetic background were used
as control mice, and referred to as *Tnfrsf1a*^{+/-} and *Tnfrsf1a*^{-/-} mice for short,
respectively.

All experiments were performed according to the guidelines approved by the
Institutional Animal Experiment Committee of Juntendo University School of Medicine
and Toho University School of Medicine.

Measurement of TEWL

Seven-day-old mice were subjected to TEWL analysis. TEWL was measured
using a Vapometer (Delfin Technologies).

Preparation of primary keratinocytes

Preparation of keratinocytes were described elsewhere with minor
modification³¹. Briefly, the skin of mice at P0 was removed and immersed in a low
calcium medium, CnT-PR (CELLnTEC) containing Dispase I (386-02271, WAKO)
(1,000 PU/mL) at 4°C for overnight. The epidermis was peeled off from the dermis
using a forceps, and chopped into small pieces. Then, minced tissues were incubated
with Accutase (AT104, ICT) at RT for 10 min and filtrated with nylon mesh. Cells were
washed with CnT-PR several times, and cultured in the same medium.

To induce differentiation of keratinocytes, culture medium was changed to CnT-PR-D medium (CELLnTEC). Once cells became confluent, cells were further cultured in the absence or presence of TNF α , IL-6, IL-17A, or IL-24 for 96 hours.

WST assay

Primary keratinocytes (2×10^4 cells) were plated on 96-well plates and then stimulated with the indicated concentrations of TNF α , recombinant leucine zipper FasL (Alexis), or Flag-tagged TRAIL that had been crosslinked with anti-Flag antibody. Flag-tagged TRAIL was provided by T. Noguchi and previously described³². After 18 hours, cell viability was determined by WST assay (Cell Counting Kit-8, Dojindo).

HaCaT cells were transfected with control siRNA (12935112, Thermo Fisher Scientific), or siRNAs targeted against human *CFLAR* (Qiagen) by Lipofectamin 2000 (Invitrogen). Target sequences were as follows: *CFLAR* #1, 5'-AAGGAGCAGGGACAAGUUACAdTdT-3'; *CFLAR* #2, 5'-AAGCAAGGAGAAGAGUUUCUdTdT-3'. At 24 hours later, cells (1.5×10^4) were plated on 96 wells and stimulated with the indicated concentrations of anti-Fas antibody, Flag-tagged TRAIL, or human TNF α . Cell viability was determined by WST assay. Knockdown of cFLIP in HaCaT cells was verified by Western blotting with anti-cFLIP antibody.

Western blotting

The skin was homogenized with a polytron (KINEMATICA) or cells were lysed in a RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 25 mM β -glycerophosphate, 1 mM sodium

orthovanadate, 1mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml aprotinin, and 1 μ g/ml leupeptin]. After centrifugation, skin extracts or cell lysates were subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were analyzed by immunoblotting with the indicated antibodies. The membranes were developed with Super Signal West Dura Extended Duration Substrate (Thermo Scientific) and analyzed with an Amersham Imager 600 (GE Healthcare).

Histological, immunohistochemical, and immunofluorescence analyses

The skin was fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded skin sections were used for H&E staining or stained with the indicated antibodies, and then visualized by HRP-conjugated streptavidin and diaminobenzidine (for anti-CC3 and anti-Ki67 antibodies). Numbers of CC3-positive or Ki67-positive cells were counted in randomly selected three fields, and expressed as average numbers per mm² of the skin of each mouse. Epidermal thickness was evaluated by measuring the thickness of the epidermis from the basal layer to the stratum corneum as described elsewhere³³. The thickness was calculated on 10 randomly selected areas from three fields and averaged per each mouse. Pictures were obtained by Olympus BX63 and analyzed by cellSens imaging software (Olympus).

To detect phosphorylated STAT3, paraffin-embedded skin sections were stained with anti-phospho-STAT3 antibody, and their signals were enhanced by Tyramide signal amplification (TSA) Plus Fluorescence Systems according to the manufacturer's instruction (NEL741001KT, PerkinElmer, Inc.). Paraffin-embedded skin sections were stained with anti-Loricrin, anti-K10, or anti-K5 antibodies, and visualized

with Alexa 594-conjugated donkey anti-rabbit Ig antibody. Frozen skin sections were incubated with anti-CD3, anti-CD11b, anti-F4/80, or anti-Ly-6G antibodies, and visualized with Alexa 488-conjugated donkey anti-rat IgG antibody. Pictures were obtained by All in One microscope (BZ-X700, KEYENCE) or Nikon A1 confocal microscope (Nikon).

Histological analysis of human skin samples

Skin samples were collected from patients of Stevens-Jonson syndrome (n=2), atopic dermatitis with chronic lesions (n=3), and psoriasis (n=3) after obtaining an informed consent. Paraffin-embedded skin sections were stained with H&E. Frozen skin sections were stained with anti-CC3 antibody followed by Alexa 488-conjugated goat anti-rabbit antibody. The study was approved by the Ethics Committee of Niigata University Graduate School of Medical and Dental Sciences.

Transmission electron microscopy

Skin sections were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Slices of these fixed tissues were postfixed with 2% OsO₄, dehydrated in ethanol and embedded in Epok 812 (Okensohoji Co.). Ultrathin sections were cut with a ultramicrotome (ultracut N or UC6: Leica), stained with uranyl acetate and lead citrate, and were examined on a Hitachi HT7700 or JEOL JEM-1400 electron microscope.

Microarray analysis

We compared gene expression profiles of RNAs from *Cflar*^{F/F}; *Tnfrsf1a*^{+/-} and *Cflar*^{E-KO}; *Tnfrsf1a*^{+/-} mice at E18.5. The experiments were performed using two

mice per each genotype. Total RNAs were extracted from the skin of mice of the indicated genotype using Trizol according to the manufacturer's instructions (Tel-Test), then labeled with Cy3. Samples were hybridized to a Mouse Oligonucleotide Microarray (G4121B, Affymetrix) according to the manufacturer's protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Affymetrix). Data were analyzed using GeneSpring GX software (Agilent). Data were deposited in NCBI as a GEO accession number GSE99379.

qPCR

Total RNAs from the skin of indicated genotypes of mice were extracted and cDNAs were synthesized with ReverTra Ace (TOYOBO). Quantitative polymerase chain reaction (qPCR) analysis was performed with the 7500 Real-Time PCR detection system with SYBR green method of the target genes together with an endogenous control, murine *Hprt* with 7500 SDS software (Applied Biosystems). The sequences of primers used in this study are listed in Table E4. The amounts of these genes were expressed relative to those of murine *Hprt* with 7500 SDS software (Applied Biosystems).

In vivo administration of antibodies

Mice were intradermally injected with both anti-FasL and anti-TRAIL antibodies (25 µg of each antibody per mouse), anti-IL-6 antibody, or control rat IgG (50 µg per mouse) at P3, and injection was continued every other day until the pups died. To evaluate the therapeutic effects of administered antibodies, mice were sacrificed at P6 and the skin sections were examined.

324 **Statistical analysis**

325 Statistical analysis was performed by the unpaired Student's *t* test, the
326 Turkey's one-way analysis of variance (ANOVA) test, or the log-rank test, as
327 appropriate. $P < 0.05$ was considered to be statistically significant.

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RESULTS

Epidermis-specific *Cflar*-deficient mice under *Tnfrsf1a*^{-/-} background are born at the expected Mendelian ratio, but develop lethal dermatitis

While epidermis-specific *Cflar*-deficient mice die in utero²⁶, inducible deletion of *Cflar* in the epidermis results in severe dermatitis that is blocked by neutralizing antibody against TNF α ^{25, 26}. To investigate whether TNFR1-independent signal might contribute to the development of dermatitis in epidermis-specific *Cflar*-deficient (*Cflar*^{E-KO}) mice, we crossed *Cflar*^{fllox/fllox} mice with *Keratin (K)5-Cre* recombinase mice under *Tnfrsf1a*^{-/-} genetic background. *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice were born at the expected Mendelian ratios (Table E1 in this article's Online Repository at www.jacionline.org). *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice appeared to be normal at birth (Fig 1, A), but gradually developed dermatitis that was characterized by enhanced cornification on the dorsal side of the skin at postnatal day 5 to 7 (P5 to P7) (Fig 1, B). Ectopic cornification of the epidermis might impair barrier function of the skin, resulting in an increase in water loss³⁴. Indeed, transepidermal water loss (TEWL) was significantly elevated in the epidermis of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice compared to that of *Tnfrsf1a*^{-/-} mice at P7 (Fig 1, C).

Histological analysis revealed that some basal cells in the epidermis of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice were already irregularly arranged at birth (Fig 1, D). The

epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice became thickened due to enhanced cornification along with parakeratosis at P5 to P7 (Fig 1, D). While cleaved caspase (CC)3-positive cells were hardly detected in the epidermis of both genotyped mice at P0, numbers of CC3-positive cells were significantly increased in the epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to *Tnfrsf1a^{-/-}* mice at P5 to P7, suggesting that these cells died by apoptosis (Fig 1, E). Intriguingly, numbers of proliferating basal cells were also increased in *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to control mice at P5 to P7 (Fig 1, F). These results suggest that *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice developed severe dermatitis soon after birth in a TNFR1-independent manner.

Susceptibility to FasL- and TRAIL-induced cell death is increased in primary keratinocytes from *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice

To investigate the cause of apoptosis of keratinocytes in *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice, we first compared expression of cell death-regulating proteins. Expression of caspase 8 and RIPK1 in the skin was not altered between *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice and *Tnfrsf1a^{-/-}* mice at P0 or P6. Expression of RIPK3 was slightly increased in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to *Tnfrsf1a^{-/-}* mice at P5 to P7, but not P0. Moreover, Mixed lineage kinase domain-like protein (MLKL), an effector molecule of necroptosis, was undetectable in the skin of either genotyped mice at P0, whereas

MLKL was increased in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P5 to P7 (Fig 2, A).

While the mechanism underlying an increase in expression of RIPK3 and MLKL is currently unknown, elevated RIPK3 and MLKL might increase a susceptibility to necroptosis.

We next prepared primary keratinocytes from *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P0.

We first confirmed that expression of cFLIP_L was abolished in primary keratinocytes from *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice (Fig 2, B). Although TNF α induces cell death via TNFR2 as well as TNFR1 in certain types of cells³⁵, TNF α did not induce cell death of keratinocytes from *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice (Fig 2, B). This suggests that TNFR2-dependent cell death was not responsible for TNFR1-independent cell death under these experimental conditions. Since we previously reported that TNF α -, FasL-, and TRAIL-induced liver injury is dramatically enhanced in *Cflar^{F/F};Mx1-Cre* mice after polyI:C injection²², we next treated primary keratinocytes with these death ligands. FasL- and TRAIL-induced cytotoxicity were significantly increased in primary keratinocytes from *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to those from control mice (Fig 2, B). Cell viability was significantly increased in the presence of a broad caspase inhibitor, z-VAD-fmk, and then further increased to control levels in the presence of both z-VAD-fmk and a RIPK1 inhibitor, Necrostatin (Nec)-1 (Fig 2, C). These results suggest that FasL and TRAIL might contribute to TNFR1-independent apoptosis and

necroptosis of keratinocytes from *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. We also observed that TNF α -, Fas-, and TRAIL-induced cell death was increased in human keratinocytes treated with *CFLAR* siRNAs compared to control siRNA (Fig 2, D). Consistently, numbers of CC3-positive cells were detected in Stevens-Johnson syndrome, a rare skin disease caused by drugs or viral infection³⁶ (see Fig E1 in this article's Online Repository at www.jacionline.org). However, as far as we examined, CC3-positive cells were not detected in the skins of atopic dermatitis with chronic lesions or psoriasis (Fig E1).

While expression of *Tnfsf6* that encodes FasL was not different in between the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* and *Tnfrsf1a^{-/-}* mice at P0, its expression was significantly elevated in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P5 to P7 (Fig 2, E). On the other hand, expression of *Tnfsf10* that encodes TRAIL was not different in between the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* and *Tnfrsf1a^{-/-}* mice at P0 or P5 to P7 (Fig 2, E). Thus, it is plausible that these death ligands might induce cell death of keratinocytes in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice in vivo.

Blockade of FasL and TRAIL signals ameliorates dermatitis and prolongs survival of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice

To test the possibility described above, we injected neutralizing antibodies against both FasL and TRAIL (anti-FT Abs for short, hereafter) into *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P3, and then continued injection every other day until the pups died. Treatment of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice with anti-FT Abs substantially prolonged their survival compared to control IgG-treated *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice (Fig 3, A). Thus, FasL- and TRAIL-dependent cell death contributed to, at least in part, the development of TNFR1-independent lethal dermatitis.

To further investigate the mechanism by which blockade of FasL and TRAIL signals attenuated dermatitis, we examined histology of the skin of control IgG- or anti-FT Abs-treated *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. The epidermis was thickened and CC3-positive cells were increased in control IgG-treated *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to control IgG-treated *Tnfrsf1a^{-/-}* mice. Such skin phenotypes were significantly attenuated in anti-FT Abs-treated *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice (Fig 3, B).

We next examined the infiltrated immune cells in the skin by immunohistochemistry. Few F4/80+ cells resided in the skin of both genotyped mice at P0 (Fig 3, C). However, CD11b+ cells, Ly-6G+ neutrophils, and CD3+ T cells were undetectable in the skin of either genotyped mice at P0. In sharp contrast, large numbers of CD11b+ and F4/80+ cells, but not CD3+ T cells or Ly-6G+ neutrophils infiltrated in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}*, but not control mice at P5 to P7 (Fig 3, C).

Administration of anti-FT Abs, but not control IgG attenuated infiltration of CD11b+ and F4/80+ cells in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice (Fig 3, D). Moreover, some foci containing Ly-6G+ neutrophils were detected in the skin of control IgG-treated, but not anti-FT Abs-treated *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice (Fig 3, D). Given that infiltrated CD11b+ and F4/80+ cells are considered to be bone marrow-derived inflammatory monocytes or dendritic cells³³, these cells might contribute to the exacerbation of dermatitis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. In sharp contrast to skin disease models of adult mice or human psoriasis²⁻⁴, contribution of CD3+ T cells to the development of dermatitis appears to be marginal in our skin disease model of neonatal mice.

Notably, the effect of anti-FT Abs on survival of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice was partial, and anti-FT Abs-treated *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice gradually developed severe dermatitis and eventually died at around P10 (Fig 3, A). These results suggest that other cell death pathway might also contribute to the development of TNFR1-independent lethal dermatitis. Indeed, several studies have shown that keratinocytes undergo pyroptosis^{37, 38}. Notably, expression of a component of the inflammasome, *Nlrp3*, but not *Nlrc4* or *Naip1*, was significantly elevated in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to that of *Tnfrsf1a^{-/-}* mice at P5 to P7 (Fig E2). These results suggest that inflammasome-dependent pyroptosis might also contribute to TNFR1-independent cell death.

Keratinocytes die by apoptosis in the epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice

To investigate the morphology of dying keratinocytes in more detail, we examined the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice by transmission electron microscopy. We found that a few keratinocytes in the epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice exhibited chromatin condensation at P4, but not at P1, further substantiating that keratinocytes died by apoptosis (Fig 4, A). Cohesion between keratinocytes in the epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice was impaired at P1 and persisted at P4, which was a typical feature of spongiosis (Fig 4, B). Moreover, the basal lamina of the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice was partially disrupted at P4, but appeared normal at P1 (Fig 4, C). Intriguingly, small electron dense materials accumulated in mitochondria at P1 and gradually increased in size, culminating in fully occupying the matrix of mitochondria at P4 (Fig 4, D). Taken that the electron dense materials appeared to be lipids, the metabolism of lipids in mitochondria might be impaired in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. Together, alterations in morphology of keratinocytes in the epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice already started at P1 and progressively worsened soon after birth.

Expression of epidermal differentiation markers, including loricrin, filaggrin, and K10 are diminished in the epidermis of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice

To test whether an increase in apoptosis might impair epidermal differentiation, we examined expression of epidermal differentiation markers including loricrin, K10, and K5 by immunohistochemistry. These markers were expressed in the epidermis of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice comparably to those of *Tnfrsf1a*^{-/-} mice at P0 (Fig 5, A). In sharp contrast, expression of loricrin and K10, but not K5, completely disappeared in the epidermis of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P5 to P7.

Western blotting analysis verified that expression of loricrin and K10, but not K5 in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P5 to P7, but not P0 completely disappeared (Fig 5, B). Filaggrin is produced as a proform with approximately ~400 kDa and processed into a mature form along with the development of mice³⁹. While a mature form of filaggrin was not detected in the skin of both *Tnfrsf1a*^{-/-} mice and *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P0, the processed form was detected in the skin of *Tnfrsf1a*^{-/-} mice at P5 to P7. In sharp contrast, a processed form of filaggrin was not detected in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P5 to P7 (Fig 5, B). While mRNA expression of *Loricrin* (*Lor*), *Krt10*, and *Krt5*, except for *Filaggrin* (*Flg*), was not different in the skin between these mice at P0 (Fig 5, C), expression of *Lor*, *Krt10*, and *Flg*, but not *Krt5* was severely downregulated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice

compared to *Tnfrsf1a*^{-/-} mice at P5 to P7 (Fig 5, C). Together, expression of late, but not early epidermal differentiation markers was downregulated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at both mRNA and protein levels soon after birth.

Epidermis-specific *Cflar*-deficient mice under *Tnfrsf1a*^{+/-} background results in embryonic lethality

A previous study has shown that *Cflar*^{E-KO} mice under *Tnfrsf1a*^{+/+} background die in utero at E11 to E14²⁶. However, the detailed characterization of mice has not been reported yet, prompting us to generate *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice and investigate the cause of embryonic lethality in more detail. Consistent with a previous study²⁶, we could not obtain viable *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice after birth. Timed mating revealed that *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice grew normally until E14.5 to E16.5, however, almost *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice died in utero before birth (Table E2 in this article's Online Repository at www.jacionline.org). The skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice at E18.5 appeared to be rough and pale compared to *Tnfrsf1a*^{+/-} mice (Fig 6, A). Epidermal cells appeared to be normal at E14.5 (Fig 6, B), whereas large numbers of epidermal cells died by apoptosis in *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice at E18.5 (Fig 6, C, D). Expression of *Tnf* was significantly elevated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice compared to *Tnfrsf1a*^{+/-} mice at E18.5 (Fig 6, E). While numbers of Ki67-positive

proliferating basal cells were regularly arranged in the epidermis of *Tnfrsf1a*^{+/-} mice, those cells completely disappeared in the epidermis of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice (Fig 6, F). Large numbers of Ki67-positive cells accumulated in the dermis and suprabasal layers of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice appeared to be infiltrated monocytes and macrophages. Taken that deletion of *Tnfrsf1a* completely rescued embryonic lethality of *Cflar*^{E-KO} mice (Table E1) and expression of *Tnf* was elevated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice (Fig 6, E), these results suggest that TNF α -induced apoptosis is the primary cause of embryonic lethality of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice.

Epidermal differentiation is blocked in the epidermis of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice

We found that expression of loricrin, but not K10 or K5, was diminished in the epidermis of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice compared to *Tnfrsf1a*^{+/-} mice at E18.5 by immunohistochemistry and Western blotting (Fig 7, A, B). On the other hand, consistent with a lack of processed filaggrin in the skin of *Tnfrsf1a*^{-/-} mice at P0, processed filaggrin was not detected in the skin of both *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice and *Tnfrsf1a*^{+/-} mice at E18.5. Large numbers of CD11b⁺ cells accumulated in the dermis of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice possibly in response to dead basal cells (Fig 6, D and 7, C). Among various cell death-regulating proteins, expression of MLKL was slightly increased in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice (Fig 7, D). Together, epidermal

differentiation was blocked in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice along with cell death in utero.

Gene expression profile of epidermis-specific *Cfar*-deficient mice

To investigate the mechanisms underlying blockade of epidermal differentiation in *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice, we performed genome-wide transcriptome analysis. To exclude the secondary effects of infection of the skin with commensal bacteria due to impaired barrier function on gene expression, we compared the gene expression profiles of the skin between *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice and *Tnfrsf1a*^{+/-} mice at E18.5 (Table E3 in this article's Online Repository at www.jacionline.org). We found that various cytokines and chemokines were upregulated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice compared to control mice. Among them, we confirmed that expression of *Il6*, *Il11*, *Il19*, *Il33*, and *Osm* were significantly elevated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice by qPCR (see Fig E3, A, in this article's Online Repository at www.jacionline.org). While expression of these cytokines was comparable between *Tnfrsf1a*^{-/-} and *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P0, these cytokines except for *Il33* progressively increased in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P5 to P7 (Fig E3, B, C).

IL-6, and to a lesser extent IL-17A downregulate expression of keratinocyte differentiation markers

Previous studies have shown that expression of constitutively active mutant STAT3 in the epidermis results in the development of psoriasis-like skin disease⁴⁰. Indeed, we found that STAT3 was phosphorylated in the skin of *Cflar^{E-KO};Tnfrsf1a^{+/-}* mice at E18.5 and *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P5 to P7, but not P0 by immunohistochemistry and Western blotting (see Fig E4 in this article's Online Repository at www.jacionline.org). Deletion of the receptor for IL-24 that activates STAT3 completely rescues the skin phenotypes of epidermis-specific *Ikkb*-deficient mice⁷. While TNF α , IL-6, IL-17A, and IL-22 are involved in the development of psoriasis^{12, 41}, expression of *Il22* was not elevated in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice compared to *Tnfrsf1a^{-/-}* mice at P5 to P7 (Fig E3, C). Thus, we tested whether treatment of primary keratinocytes with IL-6, IL-17A, or IL-24 blocked keratinocyte differentiation. We prepared primary keratinocytes from *Tnfrsf1a^{-/-}* and *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P0 and induced their differentiation. In contrast to previous studies⁴², at least under our experimental conditions, an increase in calcium concentrations in culture medium did not induce differentiation of primary keratinocytes (data not shown). However, primary keratinocytes underwent differentiation at 3 to 4 days after confluence based on upregulation of *Lor*, *Flg*, and

Krt10, but not *Krt5* (Fig 8, A). We found that IL-6, but not IL-24 induced phosphorylation of STAT3 in primary keratinocytes at 10 min after stimulation, whereas IL-17A only slightly induced phosphorylation of I κ B α (Fig 8, B). Under these experimental conditions, IL-6, and to a lesser extent IL-17A significantly blocked upregulation of *Lor*, *Flg*, *Krt10*, but not *Krt5* (Fig 8, C). Consistent with no effect of TNF α on cell viability of keratinocytes from *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice (Fig 2, B), TNF α did not block upregulation of differentiation markers of keratinocytes from either *Tnfrsf1a*^{-/-} or *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice (see Fig E5 in this article's Online Repository at www.jacionline.org). In contrast to dramatic amelioration of the skin phenotype of *Ikkb*-deficient mice under *Il24* receptor-deficient background⁷, IL-24 only weakly downregulated the expression of these differentiation markers. Thus, it is reasonable to speculate that primary target (s) of IL-24 are not keratinocytes, but other cell types. We also confirmed that IL-6 treatment downregulated expression of loricrin at protein levels along with phosphorylation of STAT3 at 96 hours after stimulation (Fig 8, D).

To further extend our findings in vivo, we injected newborn *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice with anti-IL-6 antibody from P3 and continued injection every other day until the pups died. Treatment of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice with anti-IL-6 antibody slightly prolonged their survival compared to those mice treated with control IgG (Fig 8, E). Phosphorylation of STAT1, but not I κ B α was also induced in the

575 skin of *Cflar*^{E-KO};*Tnfrsf1a*+/- mice at E18.5 and *Cflar*^{E-KO};*Tnfrsf1a*-/- mice at P5 to P7,
576 but not P0 (see Fig E6 in this article's Online Repository at www.jacionline.org), Thus,
577 combined blockade of the signals triggered by IL-6 and other cytokines, such as IFN γ
578 might be required for prolonging survival of *Cflar*^{E-KO};*Tnfrsf1a*-/- mice.

579

DISCUSSION

In the present study, we have shown that *Cflar*^{E-KO} mice died in utero, and *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice were born at the expected Mendelian ratio, but still developed severe dermatitis and succumbed soon after birth. Treatment of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice with neutralizing antibodies against FasL and TRAIL substantially prolonged their survival compared to control IgG-treated mice. Expression of inflammatory cytokines, such as IL-6, IL-17A, and IL-24 was elevated in the skin of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice at P5 to P7, conversely, expression of epidermal differentiation markers including Loricrin, Filaggrin, and K10 was downregulated. Treatment of primary keratinocytes with IL-6, and to a lesser extent IL-17A, resulted in a decrease in expression of these epidermal differentiation markers. Together, cFLIP blocks TNFR1-dependent and -independent apoptosis of keratinocytes, thereby preventing blockade of epidermal differentiation. Thus, suppression of both TNFR1-dependent and -independent cell death might be an alternative strategy to treat human skin diseases when treatment with anti-TNF α antibody alone is not sufficient.

Deletion of *Tnfrsf1a* gene rescued embryonic lethality of *Cflar*^{E-KO} mice. Consistent with the results of *Cflar*^{F/F}; *Mx1-Cre* mice²², suppression of both TRAIL and FasL signals by their neutralizing antibodies substantially prolonged survival of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice. While large numbers of CD11b⁺ and F4/80⁺ cells infiltrated

in the skin of *Cflar^{E-KO};Tnfrsf1a*^{-/-} mice, blockade of cell death signals triggered by FasL and TRAIL substantially attenuated infiltration of these cells along with a decrease in numbers of apoptotic keratinocytes. Infiltrated CD11b⁺ and F4/80⁺ cells appear to be bone marrow-derived inflammatory monocytes and Langerhans cells, and play a dominant role in the development of psoriasis³³. Hence, apoptotic keratinocytes might release chemokines that recruit inflammatory monocytes, which further exacerbated dermatitis.

Taken that the effect of neutralizing antibodies against FasL and TRAIL on survival of *Cflar^{E-KO};Tnfrsf1a*^{-/-} mice was partial, other signaling pathway(s) leading to cell death might also be involved in premature lethality and the development of lethal dermatitis. Although TNF α mediates cytotoxicity in certain cells through TNFR2³⁵, TNF α did not decrease cell viability or block upregulation of differentiation markers of keratinocytes from *Cflar^{E-KO};Tnfrsf1a*^{-/-} mice. Thus, death ligand (s) other than TNF α , FasL, or TRAIL might also contribute to the development of skin diseases.

Accumulating studies have shown that keratinocytes undergo pyroptosis in response to bacterial infection^{37, 38}. Indeed, expression of a component of the inflammasome, such as *Nlrp3*, and *Il1b* were significantly elevated in the skin of *Cflar^{E-KO};Tnfrsf1a*^{-/-} mice. Moreover, caspase 8 other than caspase 1 or caspase 11 might also be involved in activation of inflammasome under certain conditions⁴³. Taken that caspase 8 activation

is easily induced in under cFLIP-deficient conditions, pyroptotic cell death of keratinocytes might also contribute to lethal dermatitis. Further study will be required to address this issue.

Although we stained human skin samples of dermatitis with anti-CC3 antibody, we could not detect apoptotic cells in the skin of chronic lesions of atopic dermatitis or psoriasis. One might surmise that vicious cycle between inflammation and impaired skin barrier function might be caused by a yet unknown mechanism other than cell death of keratinocytes. In contrast, a previous study reported that T-cell dependent apoptosis is detected in acute lesions of eczematous dermatitis⁴⁴. Taken that apoptotic cells are rapidly cleared by phagocytes in vivo⁴⁵, we cannot formally exclude the possibility that small numbers of apoptotic keratinocytes per se might play a role, at least in part, in the development of atopic dermatitis and psoriasis. Further study will be required to address this issue.

The phenotype of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice is reminiscent of those of epidermis-specific NF-κB activation-deficient mice, in which enhanced cell death appears to block epidermal differentiation⁵⁻¹¹. Taken that deficiency of *Tnfrsf1a* significantly prolongs survival of epidermis-specific NF-κB activation-deficient mice⁵⁻¹¹, the phenotype of *Cflar* deficiency in the epidermis was very severe compared to epidermis-specific NF-κB activation-deficient mice. We found that expression of

inflammatory cytokines was increased, and conversely, expression of *Lor*, *Flg*, and *Krt10* was severely downregulated in the epidermis of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at P5 to P7. However, expression of inflammatory cytokines and epidermal differentiation markers was not different between *Tnfrsf1a^{-/-}* and *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice at birth. Together, these results suggest that commensal bacterial infection triggers FasL- or TRAIL-induced cell death and/or pyroptosis that subsequently induce inflammatory cytokine production. Produced inflammatory cytokines might impair normal skin barrier function, resulting in further exacerbation of commensal bacterial infection. Treatment of primary keratinocytes from either genotyped mouse with IL-6 dramatically downregulated expression of epidermal differentiation markers, such as *Lor* and *Flg*. However, administration of neutralizing antibody against IL-6 only weakly prolonged survival of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. This suggests that cytokines other than IL-6, such as IL-17A, IL-24, IL-11, IFN γ , or OSM might also contribute to the skin phenotype of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. Alternatively, taken that inflammatory cytokine production and subsequent blockade of epidermal differentiation are secondary events of cell death, suppression of cell death, but not prevention of cytokine-dependent blockade of epidermal differentiation, might be essential for preventing lethal dermatitis.

It would be intrigued to investigate the mechanism by which IL-6 downregulated expression of *Lor*, *Flg*, and *Krt10*. Epidermis-specific *Stat3*-deficient mice do not show a defect in skin development, but an impaired wound healing response³⁰. Conversely, expression of constitutively active mutant STAT3 in the skin results in the development of psoriasis-like dermatitis of mice⁴⁰. Moreover, a STAT3 inhibitor ameliorates skin xenografts from human psoriasis patients, and attenuates blockade of epidermal differentiation^{40, 46}. Together, these results suggest that STAT3-dependent signals promote proliferation of keratinocytes, but downregulate expression of *Lor*, *Flg* and *Krt10*. Although the mechanisms underlying STAT3-dependent downregulation of these genes remain unclear, expression of these genes might be regulated by microRNA (miRNA). Indeed, STAT3 induces expression of miR-21 that plays a causal role in the development of psoriasis through reduction of epidermal TIMP-3 (tissue inhibitor of matrix metalloproteinase 3) expression⁴⁷. Thus, it would be intrigued to test whether miRNA(s) induced by STAT3 promotes degradation of mRNAs of *Lor*, *Flg*, and *Krt10*. Alternatively, STAT3 suppresses expression of *Lor*, *Flg*, and *Krt10* through epigenetic regulation including histone modification or promoter methylation. Taken that STAT3 inhibitor (s) might affect broad biological effects, understanding of the mechanisms underlying STAT3-dependent suppression of

673 epidermal differentiation may be crucial to develop a novel strategy to treat human skin

674 diseases such as psoriasis.

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Acknowledgments

We thank Y.-W. He for *Cflar*^{F/F} mice, T.-W. Mak for *Tnfrsf1a*^{-/-} mice, T. Noguchi for recombinant Flag-TRAIL, and J. Murphy and J. Silke for anti-MLKL antibody, respectively. We thank M. Nishio and A. Suzuki for kindly transferring *K5-Cre* transgenic mice to our lab. We thank M. Kikkawa and Y. Tanno for technical assistance. We also thank S. Yamazaki, K. Kabashima, A. Ishiko, Y. Matsunaga for helpful suggestion.

Author contributions. XP, RM, SM, SK-S, RS, AH, MK, Acquisition of data, Analysis and interpretation of data; RA, CN, TM, YU, Analysis and interpretation of data; JT, HY, Contributed reagents; HN, Conception and design, Analysis and interpretation of data, Drafting or revising the article.

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FIGURE LEGENDS

FIG 1. *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice are born at the expected Mendelian ratio, but develop lethal dermatitis soon after birth. **A, B**, Macroscopic analysis of mice at P0 (**A**) and P5 (**B**) (n=20 mice per each genotype). White arrowheads indicate areas showing desquamation. **C**, A significant increase in TEWL of 7-day-old *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice.. Results are mean $\pm$ SEM (n=6 mice per each genotype). **D**, Hematoxylin & Eosin (HE)-stained skin sections (n=10 mice per each genotype) at P0 (left) and P5 to P7 (right). Red and white arrowheads indicate irregularly arranged basal cells and pyknotic nuclei, respectively. Scale bars, 100 μ m. **E, F**, Cleaved-caspase (CC)3-positive (**E**) or Ki67-positive (**F**) cells in the skin of mice at P0 and P5 to P7 (brown) (n=4 mice per each genotype). Red arrowheads indicate CC3-positive cells. Nuclei were stained with Hematoxylin. Numbers of CC3-positive or Ki-67-positive cells were counted and are expressed as numbers per mm² in the skin of mice. Results are mean $\pm$ SEM (n=4 mice per each genotype). ***p*<0.01, ****P*<0.001, ns, not significant. Scale bars, 100 μ m.

FIG 2. An increase in susceptibility to FasL- and TRAIL-induced apoptosis of *Cflar*-deficient keratinocytes. **A**, Extracts from the skin of mice at P0 and P5 to P7 were

analyzed by immunoblotting. Each number indicates individual mouse. N=4 mice per each genotype. **B**, Lysates of primary keratinocytes from mice were analyzed by immunoblotting. Primary keratinocytes were stimulated and cell viability was determined by WST assay. Results are mean $\pm$ SD of triplicate samples. **C**, Primary keratinocytes were stimulated as in **B** in the absence or presence of z-VAD-fmk (50 μ M), Nec-1 (20 μ M), or z-VAD-fmk + Nec-1. Cell viability was determined by WST assay. Results are mean $\pm$ SD of triplicate samples. **D**, HaCaT cells treated with the indicated siRNAs were stimulated with the indicated concentrations of TNF α , anti-Fas Ab, or TRAIL. Cell viability was determined by WST assay. Results are mean $\pm$ SD of triplicate samples. **E**, mRNA expression of the indicated genes of the skin was determined by qPCR. Results are mean $\pm$ SE (n=4 to 10 mice per each genotype). Results are representative of two independent experiments (A to D). * P <0.05, ** P <0.01, *** P <0.001, ns, not significant.

FIG 3. Administration of both anti-FasL and anti-TRAIL antibodies (anti-FT Abs) attenuates dermatitis and prolongs survival of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice. **A**, Survival of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice treated with control IgG (dotted line, n=11) or anti-FT Abs (thick line, n=7). Statistical significance was determined by the log-rank test. **B**, H&E-stained skin sections (upper panels) and CC3-positive cells in the skin (lower

panels) of mice at P6. Mice were treated with control IgG or anti-FT Abs (n=4 mice per each group). Red arrowheads indicate CC3-positive cells (brown). Nuclei were stained with Hematoxylin. Numbers of CC3-positive cells were counted and epidermal thickness was measured in the skin of mice. Results are mean $\pm$ SEM (n=4 mice per each group). Scale bars, 100 μ m. **C**, Skin sections of mice at P0 and P5 to P7 were stained with the indicated antibodies (green) (n=4 mice per each genotype). Nuclei were stained with DAPI. Scale bars, 100 μ m. Asterisks indicate non-specific staining. **D**, Skin sections of mice treated with control IgG or anti-FT Abs at P6 were stained as in **C** (n=4 mice per each genotype). White arrowheads indicate small foci of Ly-6G⁺ neutrophils. ** P <0.01, *** P <0.001, ns, not significant. Scale bars, 100 μ m.

FIG 4. Transmission electron microscopy of the skin of *Tnfrsf1a*^{-/-} and *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P1 and P4. **A**, Morphology of keratinocytes of mice at P4. Red arrowheads indicate apoptotic cells. Scale bars, 5 μ m. **B**, Cohesion between keratinocytes of mice at P1 and P4. Red arrowheads indicate loosened structures. Scale bars, 5 μ m. **C**, Disruption of the basal lamina of the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice at P4. Red arrowheads and arrows indicate intact and disrupted basal lamina, respectively. Scale bars, 500 nm. **D**, Accumulation of electron dense materials in mitochondria of keratinocytes of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice. Red arrowheads indicate

lipid droplets. Scale bars, 500 nm. All results are representative of two to three mice per each genotype.

FIG 5. Epidermal differentiation is blocked in the skin of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice. **A**, Skin sections of mice at P0 and P5 to P7 were stained with the indicated antibodies (red) (n=4 mice per each genotype). Nuclei were stained with DAPI. Scale bars, 100 µm. **B**, Extracts from the skin of mice at P0 and P5 to P7 were analyzed by immunoblotting. Each number indicates individual mouse. Results are representative of two independent experiments (n=4 mice per each genotype). **C**, mRNA expression of the indicated genes of the skin of mice was determined by qPCR. Results are mean ± SE (n=4 mice per each genotype). **P*<0.05, ***P*<0.01, ****P*<0.001, ns, not significant.

FIG 6. Epidermis-specific *Cflar*-deficient mice under *Tnfrsf1a*^{+/-} genetic background exhibit embryonic lethality. **A**, Macroscopic analysis of mice of the indicated genotype at E18.5 (n=4 mice per each genotype). **B**, **C**, H&E-stained skin sections (n=4 mice per each genotype) at E14. 5 (**B**) and E18.5 (**C**). Scale bars, 100 µm. **D**, Skin sections were stained with anti-CC3 antibody (brown) (n=4 mice per each genotype). Nuclei were stained with Hematoxylin. Scale bars, 100 µm. **E**, mRNA expression of the indicated genes of the skin of mice at E18.5 was determined by qPCR. Results are mean ± SE

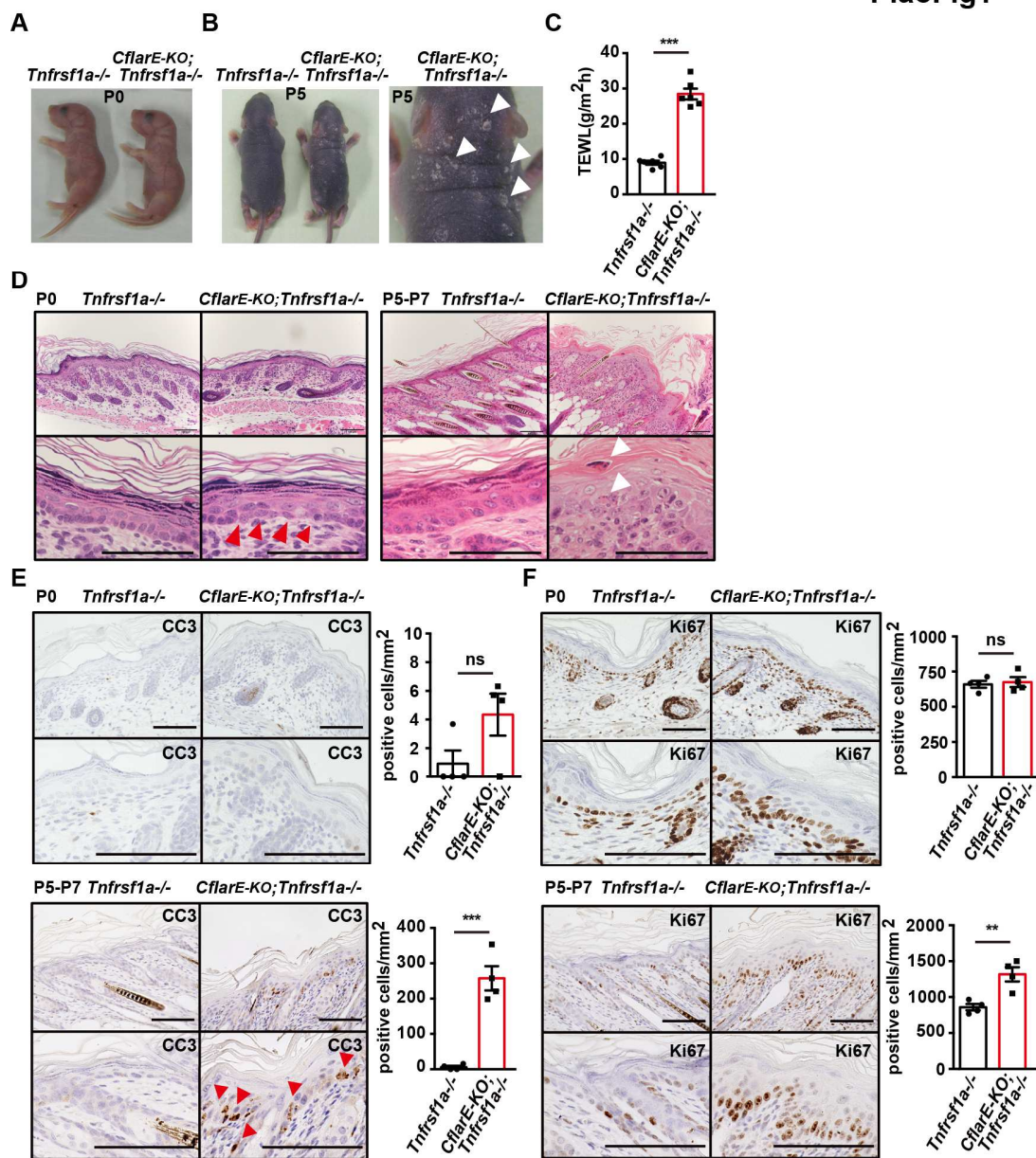
(n=4 to 6 mice per each genotype). * $P < 0.05$. ND, not detected. **F**, Skin sections at E18.5 were stained with anti-Ki67 (brown) (n=4 mice per each genotype). Red arrowheads and arrows indicate Ki67-positive (proliferating) basal cells and disappearance of Ki67-positive basal cells, respectively. Nuclei were stained with Hematoxylin. Scale bars, 100 μ m.

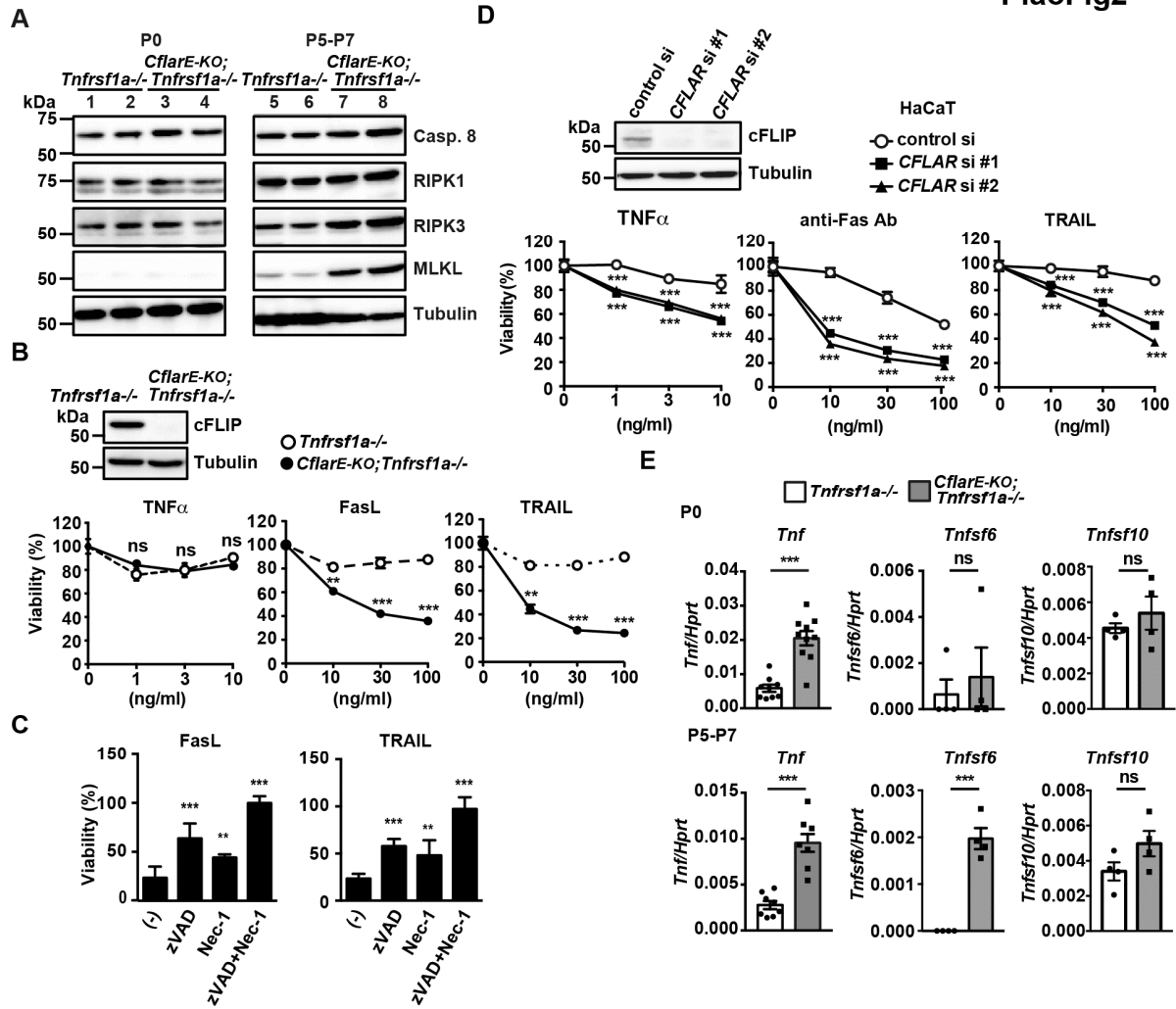
FIG 7. Epidermal differentiation is blocked in the skin of *Cflar^{E-KO};Tnfrsf1a^{+/-}* mice. **A**, **C**, Skin sections of mice at E18.5 were stained with the indicated antibodies (red or green) (n=4 mice per each genotype). Nuclei were stained with DAPI. Scale bars, 100 μ m. Asterisks indicate non-specific staining. **B**, **D**, Extracts from the skin of mice at E18.5 were analyzed by immunoblotting. Each number indicates individual mouse. Results are representative of two independent experiments (n=4 mice per each genotype).

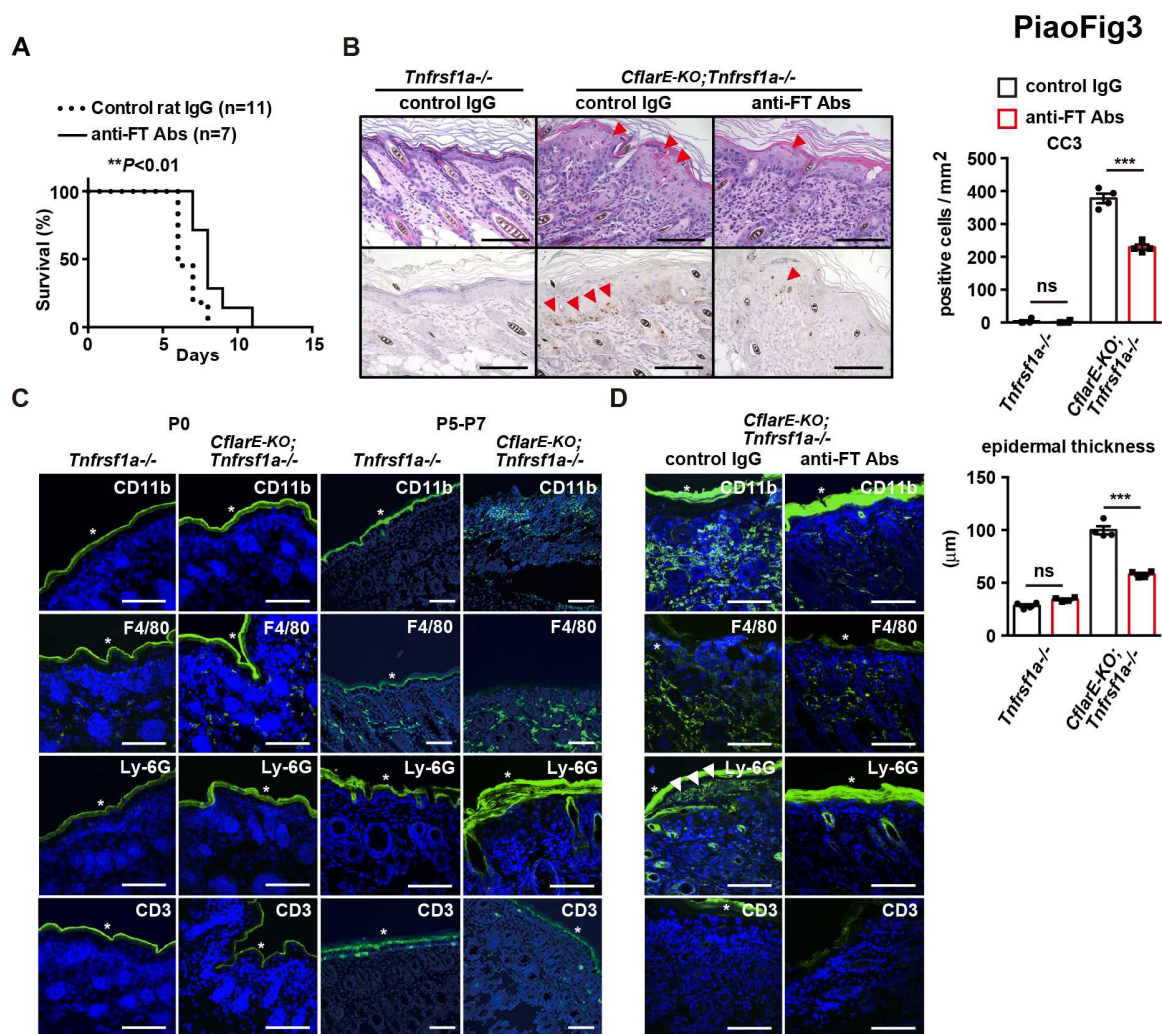
FIG 8. IL-6 downregulates expression of epidermal differentiation markers. **A**, In vitro differentiation of primary keratinocytes. Primary keratinocytes from mice at P0 were cultured for 96 hours after cells became confluent. Expression of the indicated genes at 0 and 96 hours after differentiation was quantified by qPCR. Results are mean $\pm$ SD of triplicate samples. **B**, IL-6 induces STAT3 phosphorylation. Primary keratinocytes were

stimulated with IL-6 (20 ng/ml), IL-24 (20 ng/ml), or IL-17A (20 ng/ml) for 10 min, and phosphorylation of STAT3 and I κ B α were analyzed by immunoblotting. **C**, IL-6, and to a lesser extent, IL-17A downregulate mRNA expression of epidermal differentiation markers. Primary keratinocytes were cultured as in **B** for 96 hours and expression of the indicated genes was analyzed as in **A**. Results are mean $\pm$ SD of triplicate samples. **D**, IL-6 downregulates expression of loricrin. Primary keratinocytes were treated as in **C**, and cell lysates were analyzed by immunoblotting. **E**, Survival of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice treated with control IgG (dotted line, n=7) or anti-IL-6 antibody (thick line, n=11). Statistical significance was determined by the log-rank test. * p <0.05, ** p <0.01, *** p <0.001, ns, not significant. All results are representative of two to four independent experiments.

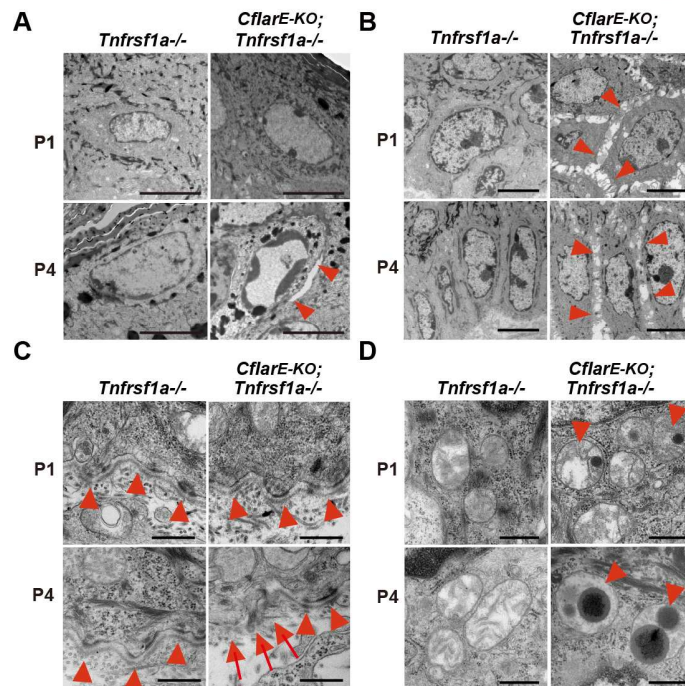
PiaoFig1

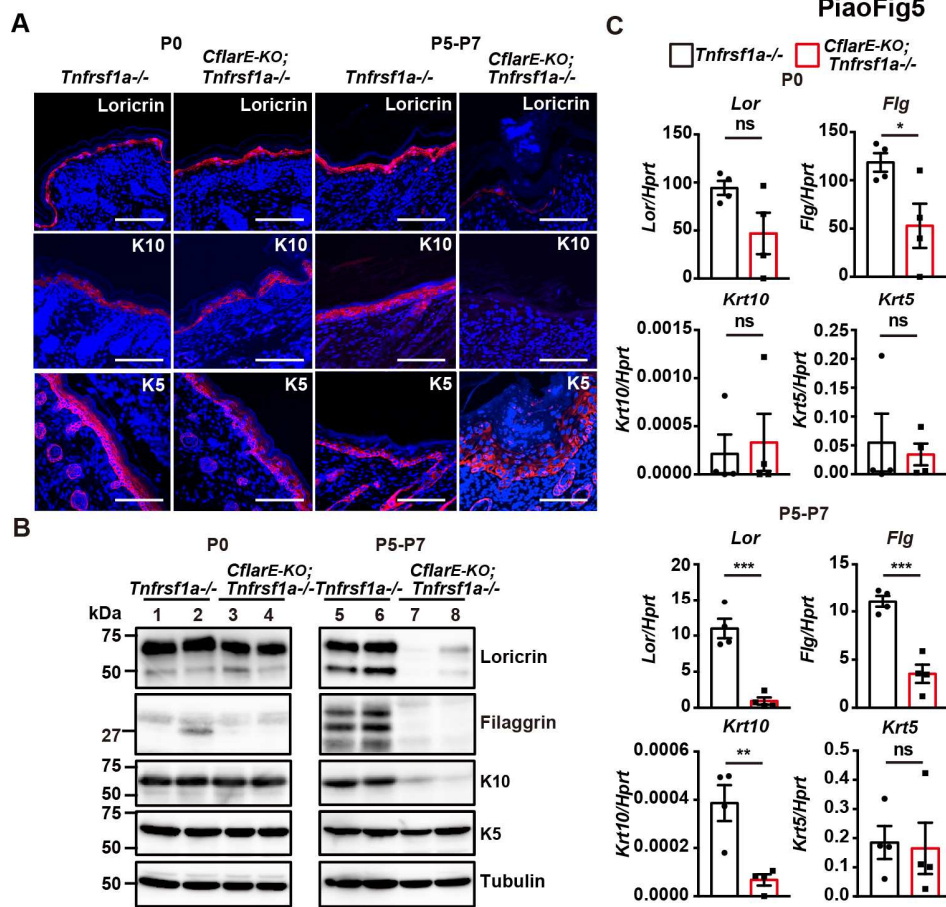




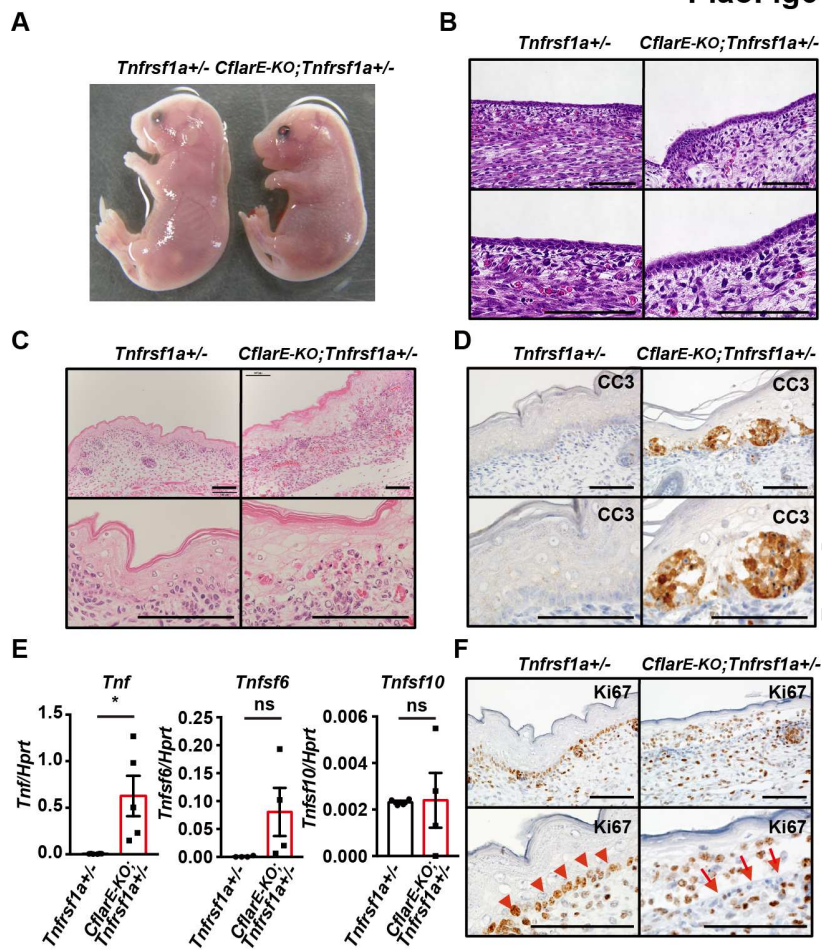


PiaoFig4

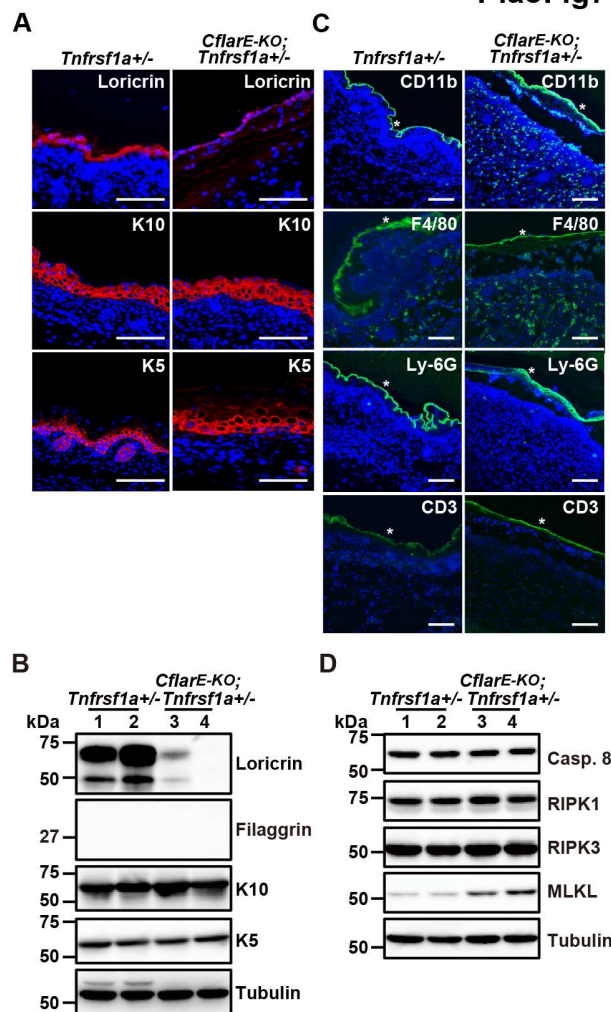




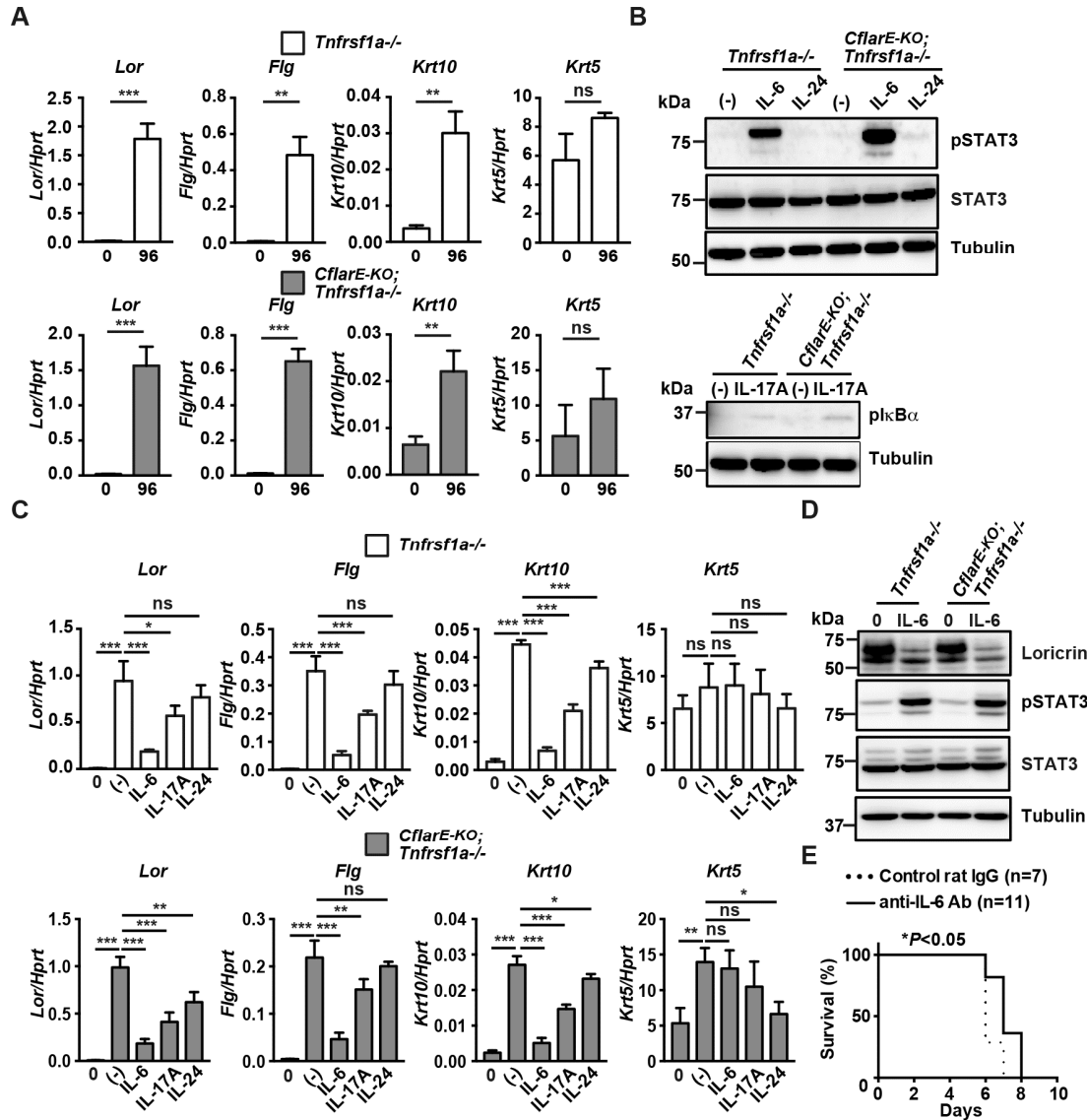
PiaoFig6



PiaoFig7



PiaoFig8



1 **Table E1.** Genotyping of *Cflar*^{E-KO} mice under *Tnfrsf1a*^{-/-} background.

Genotypes	P0		P5-P10		3 weeks~		Total	
(<i>Tnfrsf1a</i> ^{-/-} background)	No.	%	No.	%	No.	%	No.	%
<i>Cflar</i> ^{F/F}	18	23.4	15	22.3	20	32.8	53	25.8
<i>Cflar</i> ^{F/+}	18	23.4	22	32.8	17	27.9	57	27.8
<i>Cflar</i> ^{F/F} ;K5-Cre	26	33.8	19	28.3	0	0.0	45	21.9
<i>Cflar</i> ^{F/+} ;K5-Cre	15	19.5	11	16.4	24	39.3	50	24.3
Total	77	100.0	67	100.0	61	100.0	205	100.0

2 *Cflar*^{F/+};K5-Cre;*Tnfrsf1a*^{-/-} mice were crossed with *Cflar*^{F/F}; *Tnfrsf1a*^{-/-} mice.

3 Genotyping of progeny at the indicated days after birth was determined by PCR.

4

5 **Table E2.** Genotyping of *Cflar*^{E-KO} mice under *Tnfrsf1a*^{+/-} background.

Genotypes	E14.5		E16.5		E18.5		P0		3 weeks~	
(<i>Tnfrsf1a</i> ^{+/-} background)	No.	%	No.	%	No.	%	No.	%	No.	%
<i>Cflar</i> ^{F/F}	2	22.2	1	10	14	20.6	4	26.7	23	27.1
<i>Cflar</i> ^{F/+}	1	11.1	4	40	14	20.6	5	33.3	28	32.9
<i>Cflar</i> ^{F/F} ;K5-Cre	3	33.3	3	30	21	30.9	1*	6.7	0	0.0
<i>Cflar</i> ^{F/+} ;K5-Cre	3	33.3	2	20	19	27.9	5	33.3	34	40.0
Total	9	100	10	100	68	100	15	100	85	100.0

6 *Cflar*^{F/+};K5-Cre;*Tnfrsf1a*^{-/-} mice were crossed with *Cflar*^{F/F}; *Tnfrsf1a*^{+/+} mice.
 7 Genotyping of progeny at the indicated days after coitus (E) or after birth (P) were
 8 determined by PCR. Asterisk indicates that the pup died at birth.

Table E3. Upregulated genes in the skin of *Cflip*^{E-KO}; *Tnfrsf1a*+/- mice compared to those of *Tnfrsf1a*+/- mice.

Gene symbol	Accession No.	Exp. 1	Exp. 2
<i>S100a8</i>	M83218	66.14200849	74.13382509
<i>Spp1</i>	BC002113	61.4381428	89.14106377
<i>Cxcr2</i>	BC051677	60.27835644	75.3503302
<i>Arg1</i>	BC013341	59.76183231	74.43532764
<i>Slfn4</i>	BC044865	48.26969767	25.95804559
<i>S100a9</i>	M83219	42.4581905	48.20617855
<i>Tslp</i>	AF232937	31.76357331	19.21233139
<i>Il24</i>	AF235006	30.81146725	12.86341842
<i>Itgam</i>	AK040921	21.32559047	24.25829939
<i>Il33</i>	BC003847	20.59917348	21.64005708
<i>Il1r2</i>	CT010182	18.91996271	26.82022032
<i>Cd300lf</i>	BC057864	17.59826509	20.94306771
<i>Slfn1</i>	BC052869	15.95261041	11.48381967
<i>Clec4d</i>	AF061272	13.05133887	21.48618938
<i>Clec7a</i>	AF262985	11.06607425	13.41639814
<i>Fosl1 Ccdc85b</i>	BC052917	10.93171043	10.70251786
<i>Il1b</i>	BC011437	10.73776864	6.302967925
<i>Il1f8</i>	BC119517	10.54868405	4.527963097
<i>Il4ra</i>	M27960	10.09583161	11.41066825
<i>Tlr8</i>	AY035890	9.992811557	15.53200813
<i>Il1a</i>	BC003727	9.867398013	4.613543454
<i>Il1f9</i>	AY071843	9.44486917	7.328971006
<i>Cd300ld</i>	BC019814	9.408179963	10.3854507
<i>Areg</i>	BC009138	8.974178269	7.583628453
<i>Pyhin1</i>	BC096384	8.502306981	9.238052141
<i>Clec4e</i>	AB024717	8.17510559	11.14258818
<i>Tnfrsf23</i>	AY165625	7.875668011	8.040625055
<i>Duox1</i>	AK037033	7.45831387	3.855580549
<i>Il1f6</i>	BC117102	6.392627046	2.969194453

<i>Osm</i>	BC099866	5.542262318	5.35542011
<i>Il1rn</i>	BC042532	5.161512349	4.273803866
<i>Slfn2</i>	AF099973	4.940298023	6.348417069
<i>Naip1</i>	AF007769	4.699676105	1.491106513
<i>Naip2</i>	AF135490	4.663798565	5.965593931
<i>Il17ra</i>	U31993	4.536390206	4.786845777
<i>Tnfrsf1b</i>	M59378	4.487830205	5.946235992
<i>Il6</i>	J03783	4.165534392	3.132248369
<i>Naip6 Naip7</i>	AF135494	4.018502699	4.850162806

14 RNAs were extracted from the skin of indicated mice at E18.5 and analyzed using
 15 oligonucleotide arrays. Fold changed were calculated using expression levels of each
 16 gene in the skin of *Cflar^{E-KO};Tnfrsf1a^{+/-}* mice compared to those of *Tnfrsf1a^{+/-}* mice
 17 and top 38 genes are shown. Experiments were performed using two mice per each
 18 genotype.
 19

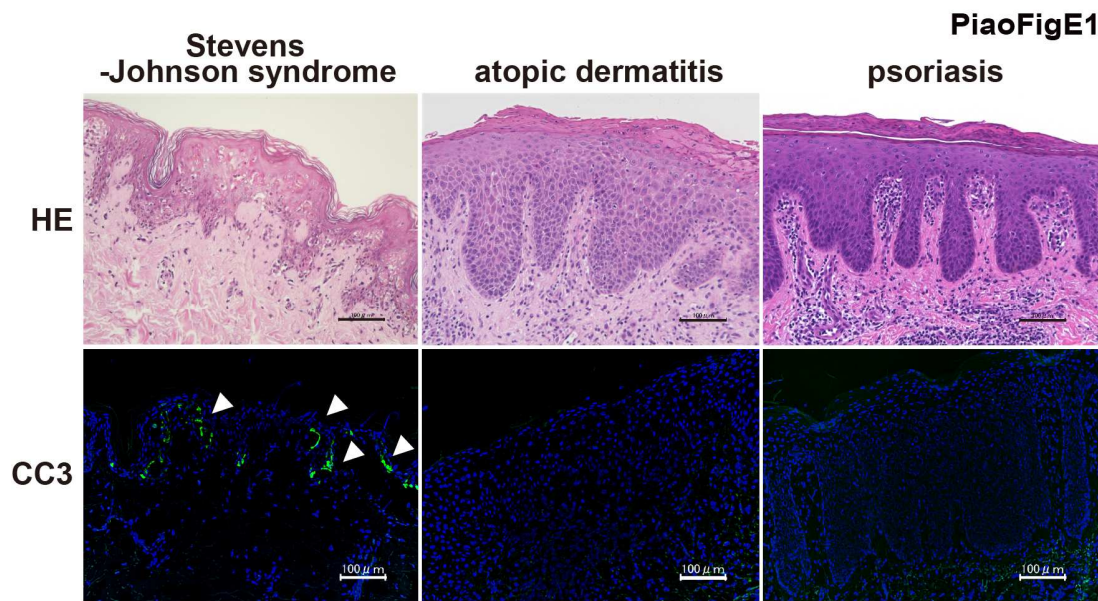
20 **Table E4.** List of primers for qPCR used in the study.

21	<i>Filaggrin (Flg):</i>	5'- ATGTCCGCTCTCCTGGAAAG-3'
22		5'- TGGATTCTTCAAGACTGCCTGTA-3'
23	<i>Il1b:</i>	5'- GCAACTGTTCTGAACTCAACT-3'
24		5'- ATCTTTTGGGGTCCGTCAACT-3'
25	<i>Il6:</i>	5'- GTATGAACAACGATGATGCACTTG-3'
26		5'- ATGGTACTCCAGAAGACCAGAGGA-3'
27	<i>Il11:</i>	5'- CTGCACAGATGAGAGACAAATTCC-3'
28		5'- GAAGCTGCAAAGATCCCAATG-3'
29	<i>Il17a:</i>	5'-CTGGAGGATAACACTGTGAGAGT-3'
30		5'-TGCTGAATGGCGACGGAGTTC-3'
31	<i>Il18:</i>	5'- GACTCTTGCGTCAACTTCAAGG-3'
32		5'- CAGGCTGTCTTTTGTCAACGA-3'
33	<i>Il19:</i>	5'- GCCAACTCTTTCCTCTGCGT-3'
34		5'- GGTGGCTTCCTGACTGCAGT-3'
35	<i>Il22:</i>	5'-TCC GAG GAG TCA GTG CTA AA-3'
36		5'-AGA ACG TCT TCC AGG GTG AA
37	<i>Il24:</i>	5'- TTTGGATGCTCCGACTGACC-3'
38		5'- TACGGTTTCCAGGGAAGGTG-3'
39	<i>Il33:</i>	5'- ATGGGAAGAAGCTGATGGTG-3'
40		5'- CCGAGGACTTTTTGTGAAGG-3'
41	<i>Keratin 5(Krt5):</i>	5'- TCTGCCATCACCCCATCTGT-3'
42		5'- CCTCCGCCAGAACTGTAGGA-3'
43	<i>Keratin 10(Krt10):</i>	5'- CGTACTGTTTCAGGGTCTGGAG-3'
44		5'- GCTTCCAGCGATTGTTTCA-3'
45	<i>Loricrin:</i>	5'- CCGATGGGCTTAACTTTCTC-3'
46		5'- AACAGGATACACCTTGAGCG-3'
47	<i>Naip1:</i>	5'- TGCCCAGTATATCCAAGGCTAT-3'
48		5'- AGACGCTGTCGTTGCAGTAAG-3'
49	<i>Nlrc4:</i>	5'- GCGGAGGTGGGAGATATG-3'
50		5'- CGTAGAAGGTTTGGAACAGC-3'
51	<i>Nlrp3:</i>	5'- AGAGCCTACAGTTGGGTGAAATG-3'
52		5'- CCACGCCTACCAGGAAATCTC-3'

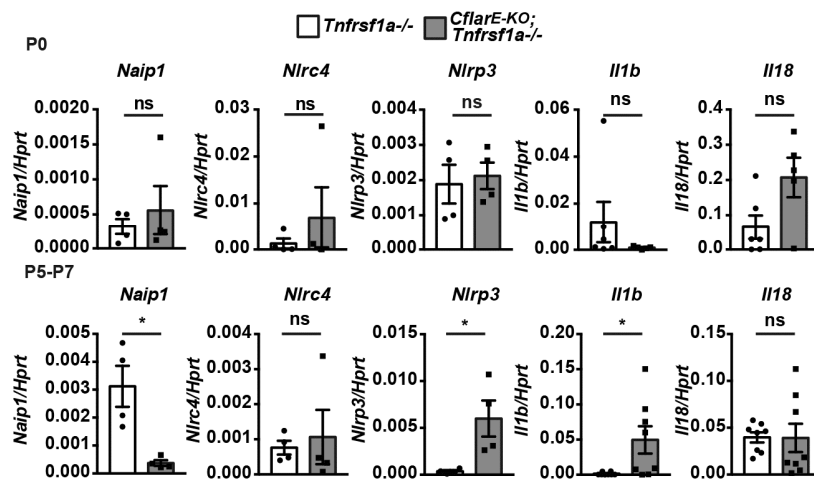
53 *Osm*: 5'- CGGCACAATATCCTCGGCATAAGG-3'
54 5'- GAAGCGATGGTATCCCCAGAGAAAG-3'
55 *Reg3b*: 5'- CTCCTGCCTGATGCTCTTAT-3'
56 5'- TTGTTACTC-CATTCCCATCC-3'
57 *Reg3g*: 5'- ACGAATCCTTCCTCTTCCTCAG-3'
58 5'- GTCTTCACATTTGGGATCTTG-C-3'
59 *Tnf*: 5'- GAAAAGCAAGCAGCCAACCA-3'
60 5'- CGGATCATGCTTTCTGTGCTC-3'
61 *Tnfsf6 (FasI)*: 5'- TCCGTGAGTTCACCAACCAAA -3'
62 5'- GGGGGTTCCTGTAAATGGG -3'
63 *Tnfsf10 (Trail)*: 5'- ATGGTGATTGTCATAGTGCTCC -3'
64 5'- GCAAGCAGGGTCTGTTCAAGA -3'
65 *Tslp*: 5'- AGGCTACCCTGAAACTGAG-3'
66 5'- GGAGATTGCATGAAGGAATACC-3'

67

68

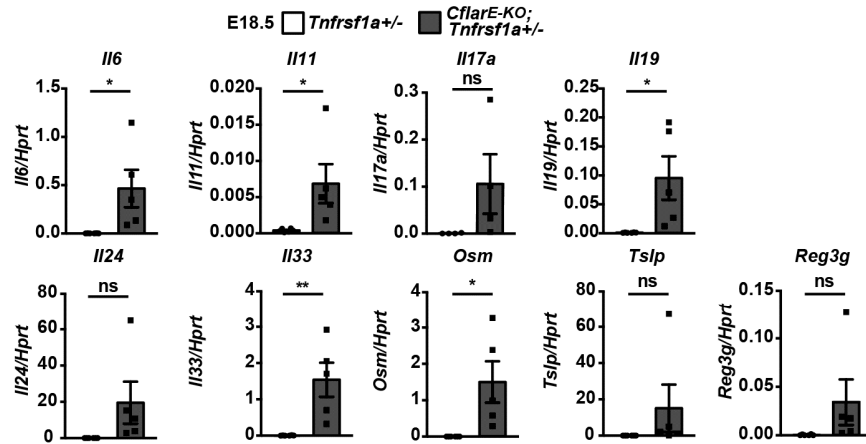


PiaoFigE2

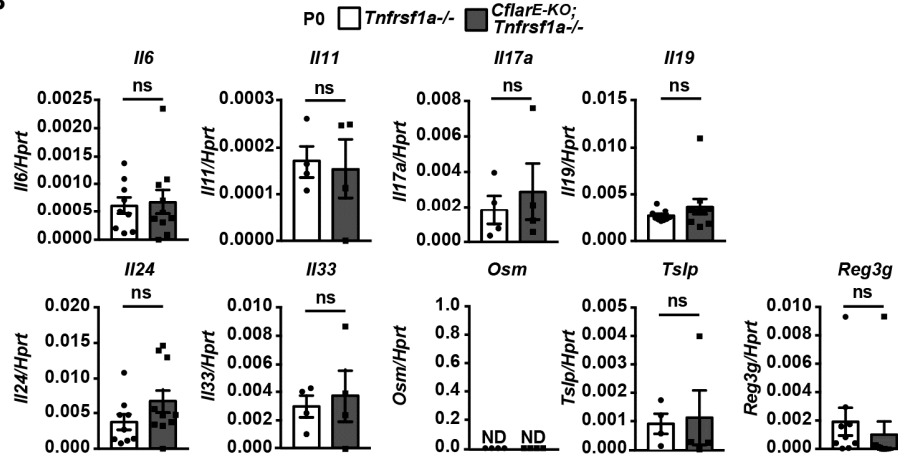


PiaoFigE3

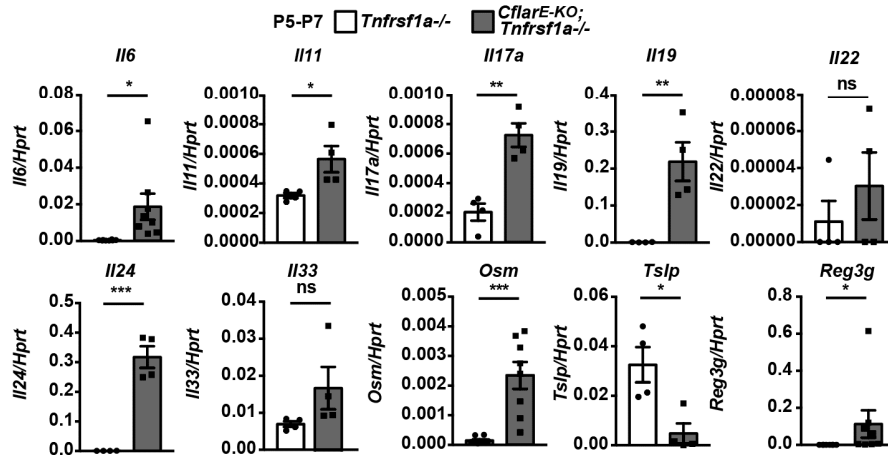
A



B

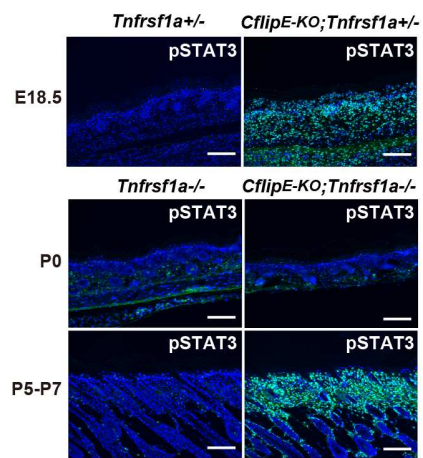


C

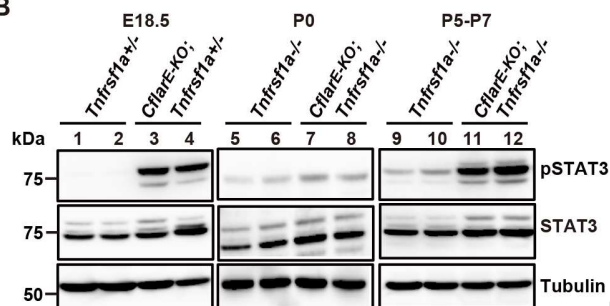


PiaoFigE4

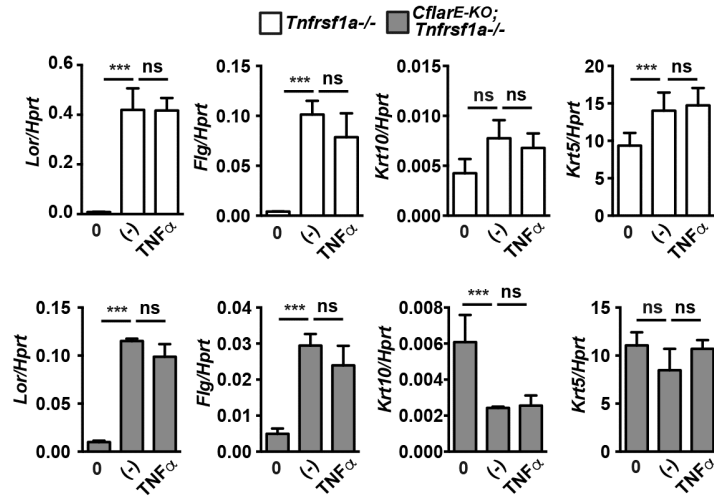
A



B

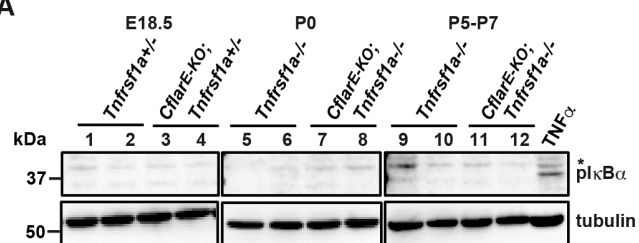


PiaoFigE5

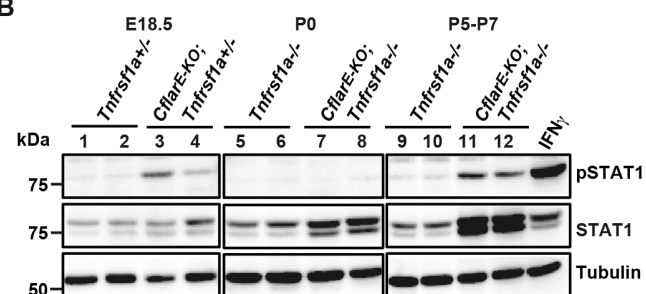


PiaoFigE6

A



B



Online Repository Materials

Blockade of TNFR1-dependent and -independent cell death is crucial for normal epidermal differentiation

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Online Repository Figure Legends

FIG E1. Apoptotic cells are detected in the skins of Stevens-Johnson syndrome, but not atopic dermatitis, or psoriasis. Human skin sections of the indicated diseases were stained with H&E (A) or anti-CC3 antibody (B). Results are representative of the skin sections of the indicated diseases (n=2 for Stevens-Johnson syndrome, n=3 for atopic dermatitis with chronic lesions, n=3 for psoriasis). Scale bars, 100 μ m.

FIG E2. Expression of *Nlrp3* and *Il1b* is elevated in the skin of *Cflar*^{E-KO}; *Tnfrsf1a*^{-/-} mice at P5 to P7. mRNA expression of the indicated genes in the skin of mice at P0 or

P5 to P7 was determined by qPCR. Results are mean $\pm$ SE (n=4 to 8 mice per each genotype). * P <0.05, ns, not significant.

FIG E3. Expression of cytokines and tissue repair genes are upregulated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{+/-} mice and *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice. **A-C**, mRNA expression of the indicated genes in the skin of mice at E18.5, P0, and P5 to P7 was determined by qPCR. Results are mean $\pm$ SEM (n=4 to 10 mice per each genotype). * p <0.05, ** p <0.01, *** p <0.001, ns, not significant. ND, not detected.

FIG E4. Phosphorylation of STAT3 is elevated in the skin of *Cflar*^{E-KO};*Tnfrsf1a*^{-/-} mice. **A**, Skin sections of mice at E18.5, P0, and P5 to P7 were stained with anti-phospho-STAT3 antibody (green) (n=6 mice per each genotype). Nuclei were stained with DAPI. Scale bars, 100 μ m. **B**, Extracts from the skin of mice as described in **A** were analyzed by immunoblotting. Results are representative of two independent experiments (n=4 mice per each genotype).

FIG E5. TNF α does not downregulate mRNA expression of epidermal differentiation markers of primary keratinocytes under *Tnfrsf1a*-deficient genetic background. Primary keratinocytes were cultured in the absence (-) or presence of TNF α (10 ng/ml) for 96 hours after cells became confluent. Keratinocytes were harvested before (0) and after differentiation. Expression of the indicated genes was quantified by qPCR. Results are mean $\pm$ SD of triplicate samples. Results are representative of two independent experiments. *** p <0.001, ns, not significant.

FIG E6. Phosphorylation of STAT1, but not I κ B α is elevated in the skin of *Cflar^{E-KO};Tnfrsf1a^{-/-}* mice. Extracts from the skin of mice at E18.5, P0, and P5 to P7 were analyzed by immunoblotting with phospho-I κ B α (**A**) or phospho-STAT1 antibodies (**B**). Lysates of HeLa cells treated with TNF α (10 ng/ml) for 15 min and RAW264.7 cells treated with IFN γ (10 ng/ml) for 15 min were used for positive control samples of pI κ B α and pSTAT1, respectively. Results are representative of two independent experiments (n=4 mice per each genotype). Asterisk indicates non-specific bands.