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Protective role of 6-formylindolo[3,2-b]carbazole (FICZ), an endogenous ligand for arylhydrocarbon receptor, in chronic mite-induced dermatitis

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ABSTRACT

Background: Chronic eczema such as atopic dermatitis imposes significant socio-econo-psychologic burdens on the affected individuals. In addition to conventional topical treatments, phototherapy is recommended for patients with extensive lesions. Although immunosuppression is believed to explain its primary effectiveness, the underlying mechanisms of phototherapy remain unsolved. Ultraviolet irradiation generates various tryptophan photoproducts including 6-formylindolo[3,2-b]-carbazole (FICZ). FICZ is known to be a potent endogenous agonist for aryl hydrocarbon receptor (AHR); however, the biological role of FICZ in chronic eczema is unknown.
Objective: To investigate the effect of FICZ on chronic eczema such as atopic dermatitis.
Methods: We stimulated HaCaT cells and normal human epidermal keratinocytes (NHEKs) with or without FICZ and then performed quantitative reverse transcriptase polymerase chain reaction, immunofluorescence, and siRNA treatment. We used the atopic dermatitis-like NC/Nga murine model and treated the mice for 2 weeks with either Vaseline® as a control, FICZ ointment, or betamethasone 17-valerate ointment. The dermatitis score, transepidermal water loss, histology, and expression of skin barrier genes and proteins were evaluated.
Results: FICZ significantly upregulated the gene expression of filaggrin in both HaCaT cells and NHEKs in an AHR-dependent manner, but did not affect the gene expression of other barrier-related proteins. In addition, FICZ improved the atopic dermatitis-like skin inflammation, clinical scores, and transepidermal water loss in NC/Nga mice compared with those of control mice. On histology, FICZ significantly reduced the epidermal and dermal thickness as well as the number of mast cells. Topical FICZ also significantly reduced the gene expression of *Il22*.
Conclusion: These findings highlight the beneficial role of FICZ-AHR and provide a new strategic basis for developing new drugs for chronic eczema.
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1. Introduction

The skin is a sensory organ that recognizes physiologic and pathologic chemical stimuli to ensure self-defense and homeostasis. Aryl hydrocarbon receptor (AHR) is a chemical receptor that can bind and be activated by structurally diverse external and internal chemicals including dioxins as well as various phytochemicals and photo-induced chemicals with a wide range of affinities [1–4]. Upon ligand binding, the ligand-AHR complex translocates from the cytoplasm to the nucleus, binds to its specific DNA recognition site, and then upregulates the transcription of various responsive genes such as *CYP1A1*, which is a member of a multigene family of xenobiotic-metabolizing enzymes [1,2].

AHR is an evolutionarily-conserved and widely-expressed multifunctional transcription factor that profoundly modulates the functions of the epidermis and immune system [2,4,5]. A recent study by Di Meglio et al. demonstrated a significant enhancement of imiquimod-induced psoriasis-like skin inflammation in *Ahr*-null mice [6]. Moreover, their investigation stressed the importance of nonhematopoietic cell types such as epidermal cells, rather than hematopoietic cell types, in recapitulating the exaggerated skin

Abbreviations: FICZ, 6-formylindolo[3,2-b]-carbazole; AHR, aryl hydrocarbon receptor; NHEKs, normal human epidermal keratinocytes; TEWL, transepidermal water loss; FLG, filaggrin; UV, ultraviolet.

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inflammation under the *Ahr*-deleted condition [6]. In addition, the activation of AHR by the intraperitoneal injection of 6-formylindolo[3,2-b]-carbazole (FICZ, a ligand for AHR) potently inhibited the imiquimod-induced skin inflammation [6]. FICZ was originally identified as a tryptophan metabolite that forms after exposure to ultraviolet (UV) or visible light in humans [7,8]. Because of its high-affinity binding to AHR and its specific induction of *CYP1A1*, FICZ is now considered as one of the essential endogenous and physiologic ligands for AHR alongside other dietarily-supplied indoles and flavonoids [7–9]. Although sun-exposed skin is one of the major sources of FICZ, the role of FICZ in skin inflammation is not fully understood.

In the present study, we found that 1) FICZ augmented the expression of filaggrin (FLG) in cultured keratinocytes, and 2) the topical application of FICZ reduced the mite antigen-induced dermatitis and transepidermal water loss (TEWL) in NC/Nga mice.

2. Materials and methods

2.1. Reagents and antibodies

FICZ was purchased from Enzo Life Sciences (Farmingdale, NY) and was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to the concentration of 5 mM. Anti-AHR rabbit polyclonal IgG antibody (H-211) and normal mouse IgG were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-FLG (ab3137) antibody was purchased from Abcam (Cambridge, UK).

2.2. Cell culture

Human immortalized HaCaT cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum and antibiotics at 37 °C in 5% CO_2. HaCaT cells (2×10^5 cells/well) were seeded in 6-well culture plates, allowed to attach for 24 h, and subsequently treated with or without FICZ when they reached 70–90% confluence.

Normal human epidermal keratinocytes (NHEKs) obtained from Lonza (Walkersville, MD) were maintained at 37 °C in 5% CO_2. The NHEKs were cultured in serum-free keratinocyte growth medium (Lonza) containing bovine pituitary extract, recombinant epidermal growth factor, insulin, hydrocortisone, transferrin, GA-1000, and epinephrine. The culture medium was replaced every 2 days. Third-to-fifth-passage NHEKs were used in all experiments. NHEKs (1×10^5 cells/well) were seeded in 24-well culture plates, allowed to attach for 24 h, and then treated with or without FICZ when they reached 80–90% confluence.

2.3. Immunofluorescence

HaCaT cells or NHEKs (1×10^4 cells/well) were plated on an 8-well μ-slide (Ibidi, Munich, Germany) for 48 h, and treated with or without FICZ for 3 or 1 h, respectively. Then, the cells were washed with phosphate-buffered saline (PBS), fixed with acetone for 10 min, and blocked with 5% (w/v) bovine serum albumin in PBS for 30 min. The cells were then incubated with primary rabbit anti-AHR antibody (1:100) overnight at 4 °C. Specific binding was detected using a horseradish peroxidase-conjugated goat anti-rabbit antibody followed by tyramide labeling with green-fluorescent Alexa Fluor® 488 (Molecular Probes, Eugene, OR) for 1 h at room temperature in accordance with the manufacturer's instructions. The treated cells were covered with UltraCruz™ mounting medium containing 4′,6-diamidino-2-phenylindole (Santa Cruz Biotechnology). The fluorescence images were analyzed using an EVOS® FL cell imaging system (Life Technologies, Carlsbad, CA).

NHEKs ($2.5–4.0 \times 10^4$ cells/well) were cultured on an 8-well slide (Lab-Tek, Rochester, NY) with or without FICZ for 24 h. The cells were incubated with primary mouse anti-FLG antibody (1:100) in WesternBreeze® blocker diluent (Invitrogen, Carlsbad, CA) overnight at 4 °C. Slides were washed in PBS before incubation with an anti-mouse secondary antibody (Alexa Fluor® 546, Life Technologies) for 1 h at room temperature. All samples were analyzed using a D-Eclipse confocal laser scanning microscope (Nikon, Tokyo, Japan).

2.4. Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

NHEKs or HaCaT cells were treated with or without FICZ for 3 or 5 h, respectively. Total RNA was extracted using the RNeasy® Mini kit (Qiagen, Hilden, Germany). The dorsal skin of NC/Nga mice affected by mite-induced dermatitis was treated with each ointment for 2 weeks, and then total RNA was extracted using the RNeasy® fibrous tissue mini kit (Qiagen). qRT-PCR was performed with PrimeScript™ RT-reagent and SYBR® Premix Ex Taq™II (Takara Bio, Ōtsu, Japan) according to the manufacturer's instructions. qRT-PCR amplifications were performed with the following cycling conditions: 95 °C for 30 s, then 40 cycles at 95 °C for 5 s (denaturation) and 60 °C for 20 s (annealing/extension). The mRNA level of each target gene was normalized to the cycle threshold of β-actin (*ACTB*; internal control). The primer sequences are listed in Table 1.

2.5. Transfection with siRNA against AHR

Anti-*AHR* siRNA (s1200) and control siRNA (negative control #1) were purchased from Ambion (Austin, TX). NHEKs and HaCaT cells were transfected with siRNA using the Lipofectamine® RNAiMAX transfection reagent in accordance with the manufacturer's protocol. The efficiency of siRNA transfection for down-regulating *AHR* expression was $93.507 \pm 0.308\%$ as assessed by measuring *AHR* expression in NHEKs.

2.6. 3D-cultured NHEKs as human skin equivalent model

The human epidermal three-dimensionally (3D) model (EpiDem EPI-200: MatTek, Ashland, MA, USA) was incubated with or without FICZ (1 μM) for 72 h at 37 °C.

Table 1
Primers for real-time quantitative RT-PCR.

Gene (human)	Primer sequence (5′–3′)	
	Sense	Antisense
CYP1A1	TAGACACTGATCTGGCTGCAG	GGGAAGGCTCCATCAGCATC
FLG	TGAAGCCTATGACACCACTGA	TCCCCTACGCTTTCTTGTCCT
LCE1A	CCTGCAAGAGTGGCTGAGATG	GGCAGCAGATAGGTTTGTGG
LCE1B	TCTGGAGGCTGCTGCTAAAGTG	GGCCTCTGAACTCCAAGACAGAA
LCE3D	TTGATGCATGAGTTCCCAGATAC	TGACATCCTGGACATCAGACA
LCE3E	TCCAGATCCTGATGCTGAGACAA	AGCTCAGCCTGTGAAAGTCAGAA
KRT1	AGAGTGGACCAACTGAAGAGT	ATTCTCTGCATTTGTCCGCTT
KRT10	ATGTCTGTTCGATACAGCTCAAG	CTCCACCAAGGGAGCCTTTG
S100A7	AATTACCTCGCCGATGTCTTTGA	ATGGCTCTGCTTGTGGTAGTC
S100A13	CCTGAGGCTCCAGCTCACTCTA	GTCAGTGGTTCTGCTGCCATTA
S100A15	CTTCAATCCATCGCTACAGTCC	TGCCAATTGGACGGAATATTATCAG
CRNN	ATGCCTCAGTTACTGCAAAACA	TCACATCGGCAAACTCTTGCT
RPTN	ATGGGGACTGTGCCTTACTAT	TCTCGGTCTTGATCTAAGAGGTT
TCHH	CGGAGACCACATGACCCTAAG	AATCGACACGCCCATTACTGT
TCHHL1	AGACAGGTGACTAAACCAGAGAA	TCTTGAGTTGGTGAAGTTCCCA
HRNR	TCGAGACCATAACAAGAAAGTGG	GTGAGTGTCATCTCTCAGCTTTG
SPRR1B	CATTCTGTCTCCCCCAAAAA	ATGGGGGTATAAGGGAGTTG
SPRR2D	TCTGTGACAGCAAAAGATTC	ATTCAGGGAGTGAAAGATAAA
SPRR2E	ATTGGCTCACCTTGTTCCAC	TGGGAACTGACACTGCTGAG
SPRR2G	TCCACCATGCCAGGATAAAT	GCTGAAGGGAAGATGATGGA
IVL	TCCTCCAGTCAATACCCATCAG	CAGCAGTCATGTGCTTTTCCT
β-actin	ATTGCCGACAGGATGCAGA	GAGTACTTGCGCTCAGGAGGA
G6PD	CTACAGGTTCAGATGATGTC	CAGCTTCTCCTTCTCCATTG

2.7. Western blotting

Protein lysates were isolated from cells with lysis buffer (25 mM HEPES, 10 mM $Na_4P_2O_7/10H_2O$, 100 mM NaF, 5 mM EDTA, 2 mM Na_3VO_4, and 1% Triton™ X-100). The protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of protein (30 μg) were dissolved in sample buffer (Nacalai Tesque, Inc., Kyoto, Japan). The lysates were then boiled at 70 °C for 10 min and analyzed by NuPAGE® 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) at 200 V for 60 min. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), which were then probed with the anti-FLG mouse antibody (Santa Cruz Biotechnology, Dallas, TX, USA) and an anti-β-actin rabbit polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Immunological bands were identified with HRP-conjugated secondary antibodies (Cell Signaling) followed by visualization with SuperSignal® West Pico Chemiluminescence Substrate (Thermo Scientific) using a ChemiDoc touch imaging system (Bio-Rad, Hercules, CA, USA).

2.8. Mice

Female NC/Nga mice were purchased from Charles River Laboratory Japan (Yokohama, Japan). They were maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All mice were 9–10 weeks of age. The animal experiments in this study were approved by the Animal Care and Use Committee of Kyushu University School of Medicine.

2.9. Induction of dermatitis

Mice were anesthetized with sevoflurane and their hair on the upper back was shaved with a clipper and a shaver. The shaved dorsal and ear skin were treated with 150 μL of 4% sodium dodecyl sulfate (SDS) and 2 or 3 h later, 100 mg of mite antigen ointment containing *Dermatophagoides farinae* extract (Biostir Inc. Osaka, Japan) was topically applied to the barrier-disrupted skin. These procedures were repeated twice a week for 3 weeks [10]. Then, the mice were divided into three groups: Group A, ointment base (Vaseline® control) (100 mg/affected area, $n = 11$); Group B, FICZ ointment (100 mg/affected area, $n = 11$); and Group C, betamethasone 17-valerate ointment (100 mg/affected area, $n = 12$). In addition to continuing the application of 4% SDS and mite antigen twice per week, each group of mice was topically treated with the respective ointment three times per week for 2 weeks. On day 35 after the initial application of mite antigen, these mice were sacrificed.

2.10. Evaluation of skin lesions

The clinical severity of dermatitis was evaluated at weeks 3, 4, and 5. The development of 1) erythema/hemorrhage, 2) scaling/dryness, 3) edema, and 4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The sum of the individual scores was taken as the dermatitis score.

2.11. Transepidermal water loss

Transepidermal water loss (TEWL) was measured on the lesional skin using a Vapo Scan AS-VT100RS machine (Asahi Biomed, Tokyo, Japan). Measurement of TEWL was performed at room temperature (23–26 °C) and room humidity (40–55%). All data are presented as the median of three repeated recordings.

2.12. Histological observation of the skin

Portions of the dorsal skin were fixed with 10% neutral formalin, embedded in paraffin, and sectioned at 3 μm. Sections were stained with hematoxylin-eosin or toluidine blue.

2.13. Immunohistochemistry

Frozen sections (5 μm) were immersed in 3% H_2O_2 in methanol to block endogenous peroxidase activity and incubated with a polyclonal anti-mouse FLG antibody (PRB417P; Covance, Princeton, NJ) at the dilution of 1:1000 for 1 h at room temperature. The sections were incubated with a secondary antibody, N-Histofine® Simple Stain MAX-PO MULTI (Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature. Immunodetection was conducted with 3,3′-diaminobenzidine as a chromogen, followed by light counterstaining with hematoxylin.

2.14. Statistical analysis

All data are expressed as mean ± standard error. Unpaired Student's *t*-test or one-way analysis of variance was used to analyze the results. A *p*-value of less than 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. FICZ did not affect the viability of keratinocytes

Graded concentrations of FICZ (0–100 nM) did not affect the viability of HaCaT cells and NHEKs as determined by the CCK-8 assay (Supplementary Fig. S1).

3.2. FICZ induced the nuclear translocation of AHR and the upregulation of CYP1A1 expression

It is known that cytoplasmic AHR translocates to the nucleus and upregulates *CYP1A1* expression after binding to various chemicals such as dioxins, flavonoids, and tryptophan photoproducts [3,4]. To confirm the nuclear translocation of AHR and the transcriptional activation of *CYP1A1* after FICZ treatment, we treated HaCaT cells or NHEKs with DMSO (negative control) or FICZ (100 nM). As expected, in the steady state, AHR was localized mainly in the cytoplasm (Fig. 1A and C), whereas FICZ induced its nuclear translocation (Fig. 1B and D) in concert with a significant upregulation of *CYP1A1* expression (Fig. 1E and F). These results indicate the capacity of FICZ to activate AHR signaling.

3.3. FICZ upregulated FLG expression in an AHR-dependent manner

Since some AHR ligands such as coal tar and soybean tar are known to upregulate the expression of FLG and other epidermal barrier proteins [3,4,11,12], we next examined the effects of FICZ on the expression of barrier proteins. Although FICZ did not upregulate the expression of *LCE1A, LCE1B, LCE3D, LCE3E, KRT1, KRT10, S100A7, S100A13, S100A15, CRNN, RPTN, TCHH, TCHHL1, HRNR, SPRR1B, SPRR2E, SPRR2G,* and *IVL* (Supplementary Fig. S2), a significant upregulation of *FLG* and *SPRR2D* expression was observed. Since it is well known that *FLG* is important to maintain the epidermal barrier, we decided to focus on *FLG* expression. FICZ upregulated the expression of the *CYP1A1* (Fig. 2A) and *FLG* (Fig. 2B) in a dose-dependent manner in HaCaT cells. NHEKs were more sensitive than HaCaT cells in their response to FICZ and concentrations of FICZ as low as 1 nM induced a significant upregulation of *CYP1A1* (Fig. 2C) and *FLG* (Fig. 2D) expression.

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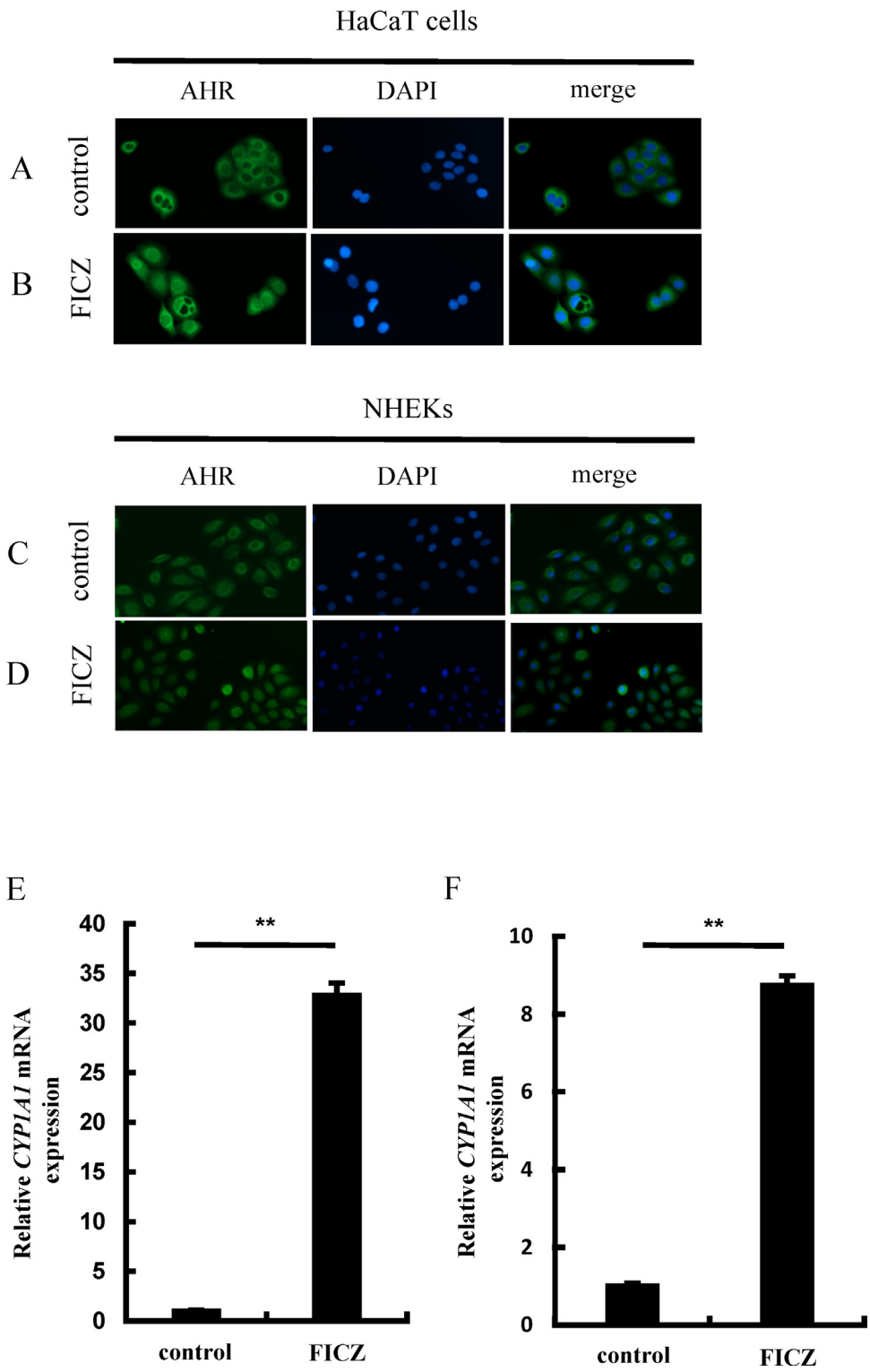

Fig. 1. 6-Formylindolo[3,2-b]-carbazole (FICZ) induced AHR nuclear translocation and the upregulation of *CYP1A1* expression. HaCaT cells (A, B) or normal human epidermal keratinocytes (NHEKs) (C, D) were stimulated with dimethyl sulfoxide (DMSO; control) or FICZ (100 nM) for 3 or 1 h, respectively, and were then fixed. Cells were stained with anti-aryl hydrocarbon receptor (AHR) antibody (green) and 4′,6-diamidino-2-phenylindole (blue). HaCaT cells (E) or NHEKs (F) were treated with DMSO (control) or 100 nM FICZ for 5 or 3 h, respectively. Then, total RNA was extracted and *CYP1A1* mRNA expression was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) normalized to the expression of β-actin (*ACTB*; internal control). Data are presented as mean ± standard error ($n = 3$ per group). $^{**}p < 0.001$ (one-way analysis of variance).

3.4. FICZ increased the abundance of filaggrin (FLG) protein

We further investigated the abundance of FLG protein. The keratinocytes or 3D cultured NHEKs were treated with DMSO (negative control) or FICZ for 24 h to 96 h, and then the abundance of FLG was examined by immunolabeling and fluorescence microscopy or western blotting. As expected, the abundance of FLG or Pro-FLG was augmented in the FICZ-treated keratinocytes

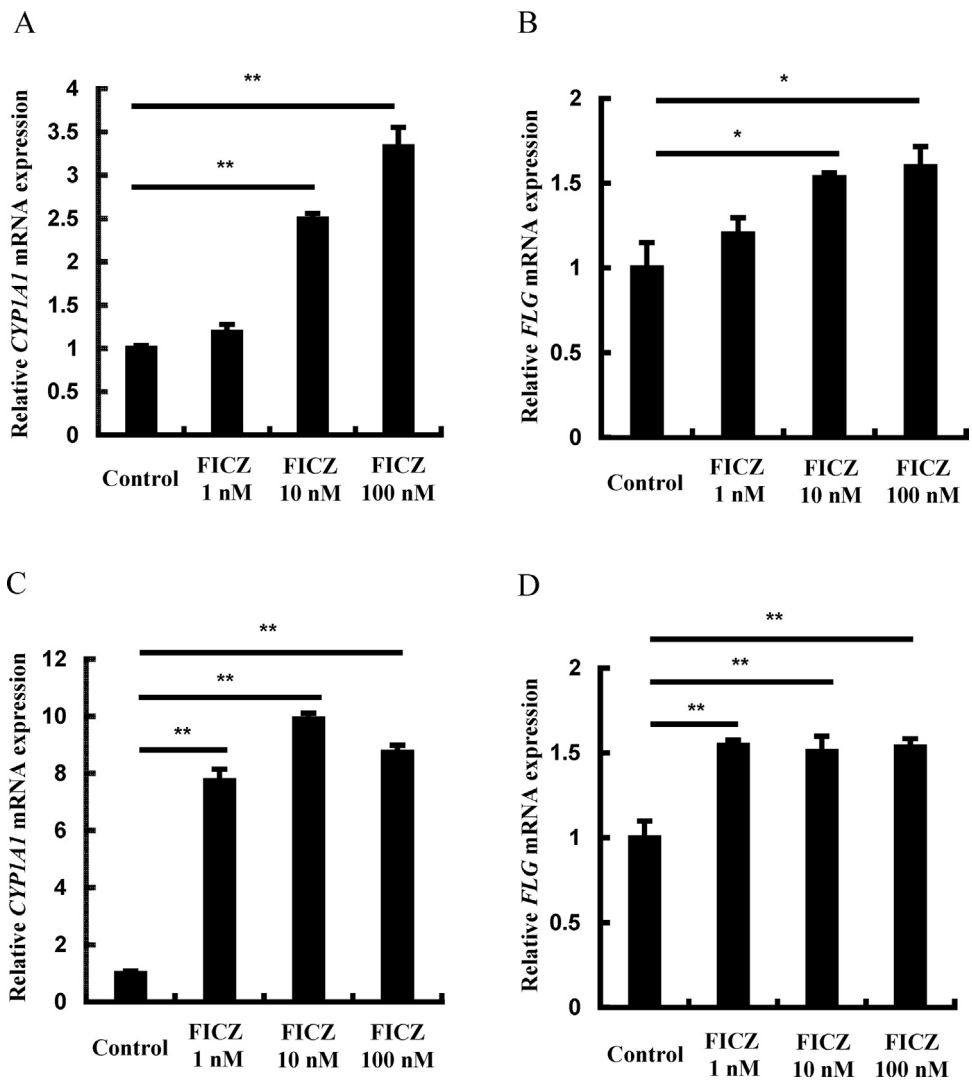

Fig. 2. FICZ upregulated *FLG* expression in HaCaT cells or NHEKs. (A, B) HaCaT cells were stimulated with DMSO (control) or FICZ (1, 10, and 100 nM) for 5 h and total RNA was extracted. (C, D) NHEKs were stimulated with DMSO (control) or FICZ (1, 10, and 100 nM) for 3 h and then total RNA was extracted. *CYP1A1* or *FLG* mRNA expression was measured by qRT-PCR normalized to the expression of β-actin (*ACTB*; internal control). Data are presented as mean ± standard error ($n = 3$ per group). $^{*}p < 0.05$ and $^{**}p < 0.001$ (one-way analysis of variance).

or 3D cultured NHEKs compared to that in their untreated counterparts (Fig. 3 and Supplementary Fig. S6).

3.5. AHR-dependency of the response to FICZ

We assessed the AHR-dependency of the induction of *FLG* expression by FICZ using AHR-knockdown keratinocytes transfected with anti-*AHR* siRNA. As expected, the enhancing effect of FICZ on *FLG* expression was completely abrogated both in HaCaT cells (Fig. 4A) and NHEKs (Fig. 4B) transfected with anti-*AHR* siRNA. These results indicate that the FICZ-induced upregulation of *FLG* is AHR-dependent.

3.6. FICZ improved mite-induced chronic dermatitis and TEWL in NC/Nga mice

Since the expression of FLG is essentially involved in human atopic dermatitis and murine experimental dermatitis models [13–16] as well as physiological epidermal barrier recovery [17], we next investigated whether the topical application of FICZ could improve mite-induced chronic dermatitis *in vivo*. The NC/Nga mice were epicutaneously treated with mite antigen and SDS twice per week for 5 weeks (Supplementary Fig. S3). This procedure induced a visible, stable, and chronic dermatitis at 3, 4, and 5 weeks after the initial application of mite antigen.

From week 3, these mice with dermatitis were topically treated with Vaseline® as a control, FICZ ointment, and a commercially available betamethasone 17-valerate ointment. Vaseline® treatment could not improve the ongoing dermatitis, while both topical FICZ and betamethasone improved the dermatitis (Fig. 5A). After 2 weeks of application (at week 5), a significant improvement was observed only in the group treated with topical FICZ (Fig. 5B). Consistent with the beneficial effect on the dermatitis score, TEWL was also improved by FICZ and betamethasone, and a significant improvement of TEWL was detected only in the group treated with topical FICZ after 2 weeks of application (Fig. 5C).

3.7. FICZ restored FLG expression and improved skin inflammation

The mite-induced dermatitis in NC/Nga mice induces a down-regulation of FLG expression alongside an increase in skin thickness and augmented mast cell infiltration [18]. The expression of FLG in normal mice was detected mainly along the thin granular layer (Fig. 6A), but it was reduced in the dermatitis-affected skin in

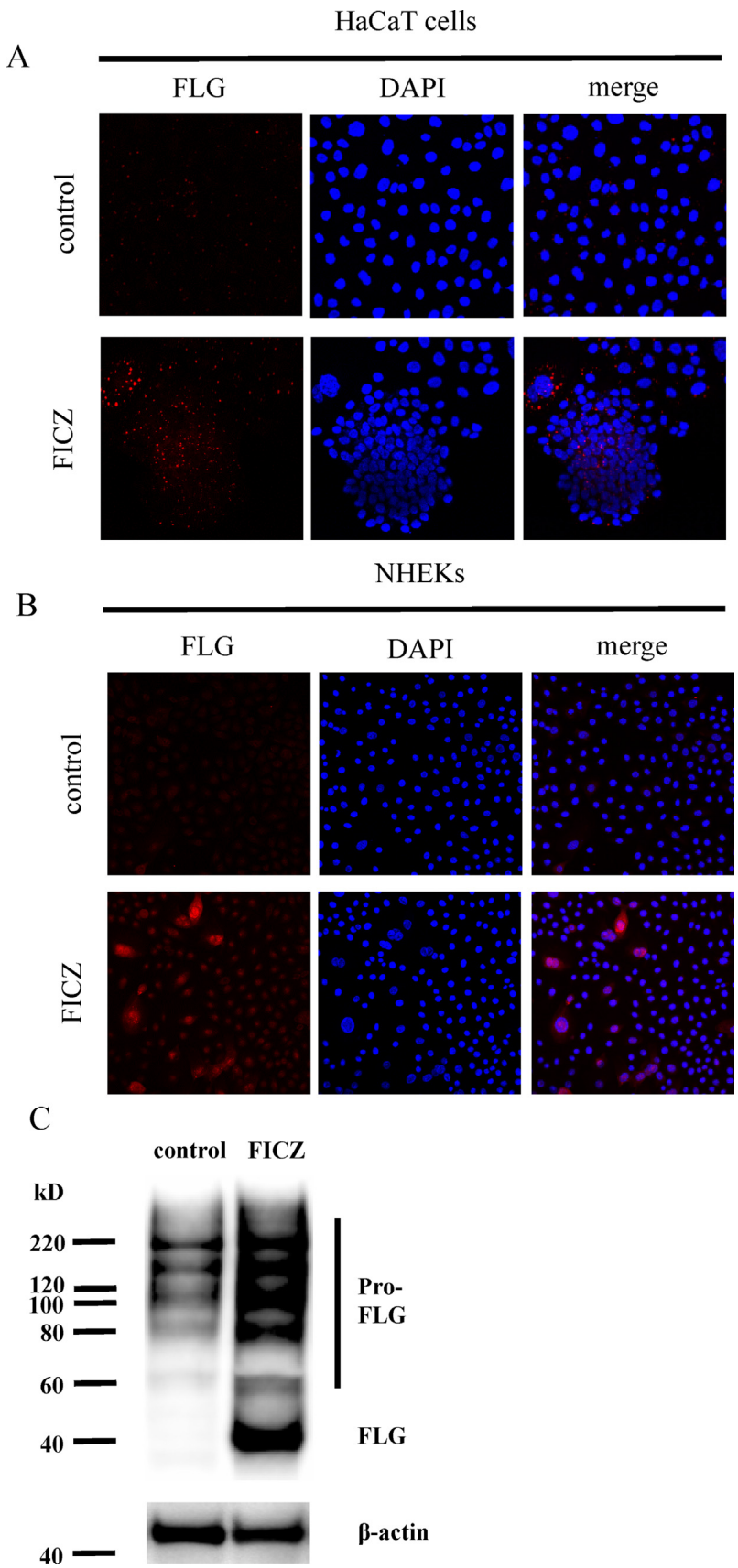

Fig. 3. FICZ increased the expression of FLG. HaCaT cells (A) or NHEKs (B) were stimulated with DMSO (control) or 100 nM FICZ for 24 h. Cells were stained with anti-FLG antibody (green) and 4′,6-diamidino-2-phenylindole (blue). 3D-cultured NHEKs (C) were treated with DMSO (control) or FICZ (1 μM) for 72 h. Total cell lysates were prepared and analyzed with an anti-FLG antibody by western blotting. Data are representative of experiments repeated three times with similar results.

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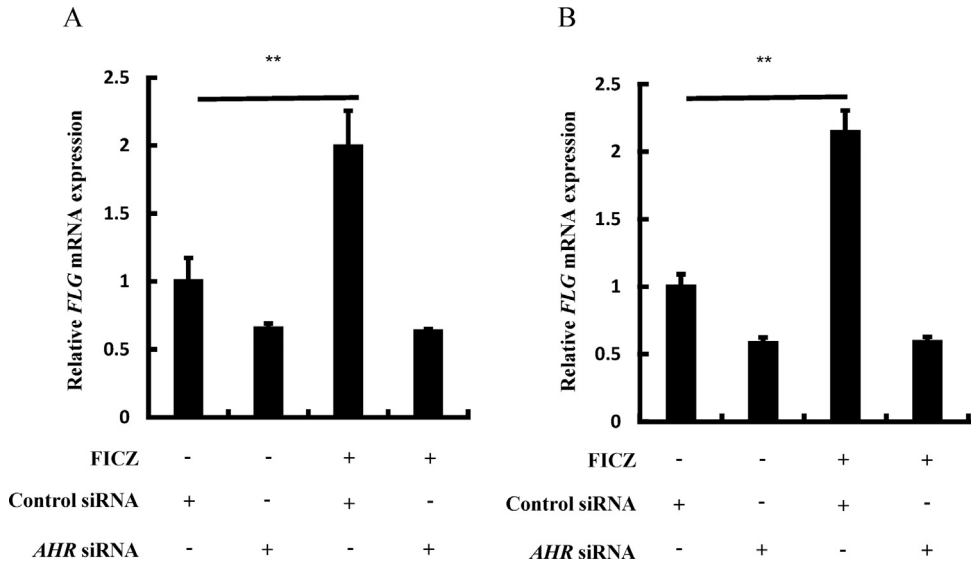

Fig. 4. FICZ-induced *FLG* expression is AHR-dependent. HaCaT cells (A) or NHEKs (B) were transfected with control siRNA or AHR siRNA for 48 h, then stimulated with 100 nM FICZ for 5 h, and total RNA was extracted. *FLG* mRNA expression was measured by qRT-PCR normalized to the expression of β-actin (*ACTB*; internal control). Data are presented as mean ± standard error ($n = 3$ per group). $^{**}p < 0.001$ (one-way analysis of variance).

the Vaseline® control group (Fig. 6B). However, topical FICZ recovered the expression of FLG in the dermatitis-affected skin (Fig. 6C). Topical betamethasone also recovered the expression of FLG but to a lesser extent (Fig. 6D). Furthermore, on histology, the thickness of skin (epidermis and dermis) was significantly reduced in the topical FICZ and betamethasone groups compared to that in the Vaseline® control group (Fig. 6E). Both topical FICZ and betamethasone also reduced the number of infiltrated mast cells and CD68$^+$ cells compared to that in the Vaseline® control group (Supplementary Fig. S4 and S5).

3.8. FICZ regulated the expression of cytokines including Il22 and Ifng

Finally, we addressed the cytokine milieu in the dermatitis-affected skin treated with Vaseline®, FICZ, and betamethasone by measuring the mRNA expression levels of *Il1A, Il1B, Il4, Il6, Il13, Il17A, Il22, Ifng*, and *Tnfa*. Topical FICZ significantly reduced the expression of *Il22* and *Ifng*, but did not affect the expression of the other cytokine genes (Fig. 7). Topical betamethasone inhibited the expression of *Ifng* only (Fig. 7).

4. Discussion

In this study, we demonstrated that FICZ upregulates the expression of FLG in keratinocytes in an AHR-dependent fashion and that the topical application of FICZ potently inhibits the ongoing mite-induced chronic dermatitis in the NC/Nga murine model. In this model, topical FICZ improved the TEWL of the treated dermatitis lesions and maintained a normal expression of FLG.

AHR is a xenobiotic chemical sensor that is abundantly expressed in barrier-forming epithelial tissues such as skin, trachea, and intestine [3,19–23]. Various xenobiotic and natural compounds such as dioxins, dietary polyphenols including flavonoids, and microbiota-derived factors can activate AHR and upregulate *CYP1A1* expression leading to the induction of oxidant and/or antioxidant signaling pathways in a ligand-dependent manner [3,5,24]. AHR is also an important regulator of the development and function of both innate and adaptive immune cells and is indispensable for the maturation of intestinal immunity [5,21,22,25,26].

In addition, AHR is an essential transcription factor for human epidermal differentiation complex genes including *FLG* [4,27–29]. Some medicinal ligands for AHR such as coal tar and soybean tar actively upregulate *FLG* expression [11,12]. The barrier function is significantly disrupted in *Ahr*-null mice, indicating that AHR plays a pivotal role in skin barrier integrity [23]. Since the tryptophan photoproduct FICZ is a high-affinity endogenous ligand for AHR [7,8,30], we examined the effect of FICZ on the expression of *FLG* in keratinocytes. The present study confirmed that *FLG* expression is augmented by FICZ and that the induction of *FLG* expression by FICZ does not occur in AHR-knockdown keratinocytes.

Previous studies have shown that the systemic injection of FICZ inhibits dextran sulfate sodium-induced colitis [31], ovalbumin-induced allergic asthma [32], and topical imiquimod-induced psoriasis [6] in mice. In parallel with these reports, topical FICZ significantly inhibited mite-induced chronic dermatitis in the present study. The mite-induced dermatitis in NC/Nga mice has been reported to be associated with an enhanced skin thickness, an accumulation of dermal mast cells, an increased TEWL, and a decreased expression of FLG [18]. In addition, a high number of CD68$^+$ macrophages has been detected in the dermis of atopic dermatitis skin lesions [33]. All these features were significantly normalized by the topical application of FICZ in the present study. Although FLG is one of many barrier proteins [29], it is likely to be involved in the functional integrity of the epidermal barrier [14]. A loss-of-function mutation of *FLG* causes dry skin and is critically involved in the pathogenesis of atopic dermatitis and experimental animal disease phenotypes [13,15,34]. Moreover, the topical application of apigenin, a phytochemical AHR activator, improves epidermal barrier homeostasis by upregulating FLG expression in normal murine skin [17]. These studies suggest that the AHR-mediated FLG upregulation by FICZ may be an integral part of the therapeutic modality of FICZ as a potential treatment for dermatitis.

Our analysis of gene expression in the treated dermatitis-affected skin revealed that FICZ significantly downregulated the expression of *Il22* and *Ifng*. Betamethasone treatment significantly inhibited the expression of *Ifng*, but not that of *Il22*. Considering the weak and insignificant clinical effect of betamethasone, the inhibitory action of FICZ on *Il22* expression may be meaningful in our experimental model. Recent studies have stressed the pathogenetic role of IL-22 in human atopic dermatitis and mite-induced murine dermatitis [35–38]. IL-22 markedly

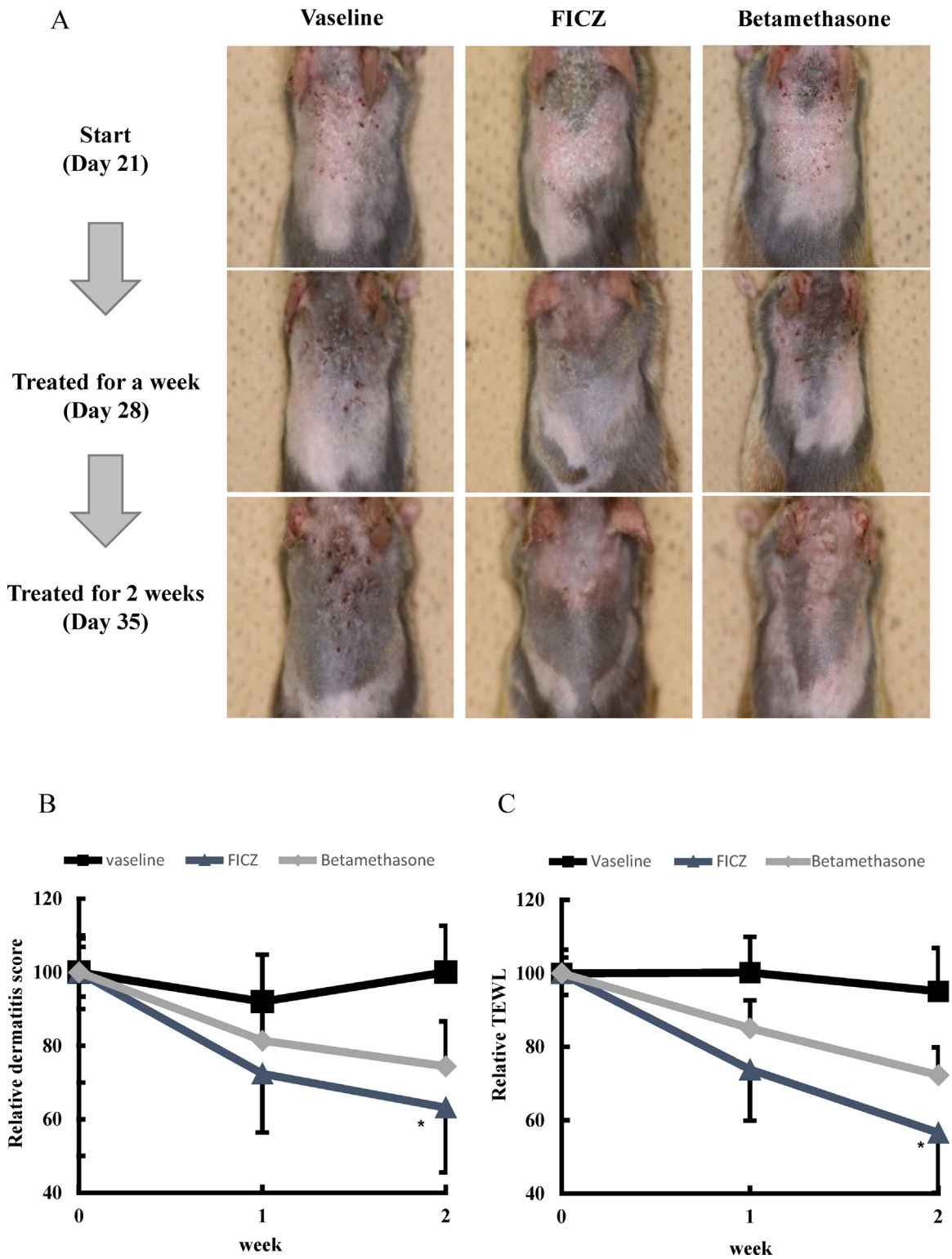

Fig. 5. Effect of topical FICZ ointment on the mite-induced dermatitis of NC/Nga mice (A) Macroscopic features of skin lesions on day 21 (week 0), day 28 (week 1), and day 35 (week 2). Vaseline® control (left panel), FICZ ointment at a dose of 10^{-3} mM (center panel), and betamethasone 17-valerate ointment at a dose of 2.5 mM (right panel). (B) Dermatitis scores were assessed on day 21 (week 0), day 28 (week 1), and day 35 (week 2). (C) Transepidermal water loss (TEWL) was measured on the lesional skin by a Vapo Scan instrument on day 21 (week 0), day 28 (week 1), and day 35 (week 2). Data were presented as the average of three repeated recordings. All data are presented as mean ± standard error (n = 11–12 per group). $^{*}p < 0.05$ (one-way analysis of variance) compared to the Vaseline® control group..

downregulates FLG expression [39,40]. Moreover, therapeutic success in atopic dermatitis is associated with the downregulation of IL-22, the enhancement of FLG expression, and the improvement of TEWL [37]. Therefore, the downregulation of *Il22* may potentially be related to the effectiveness of FICZ treatment. In accordance with this notion, the FICZ-induced downregulation of *Il22* was evident in the imiquimod-induced psoriasis model [6]. However, it is enigmatic that FICZ augments IL-22 production by T cells and innate lymphoid cells *in vitro* [41,42].

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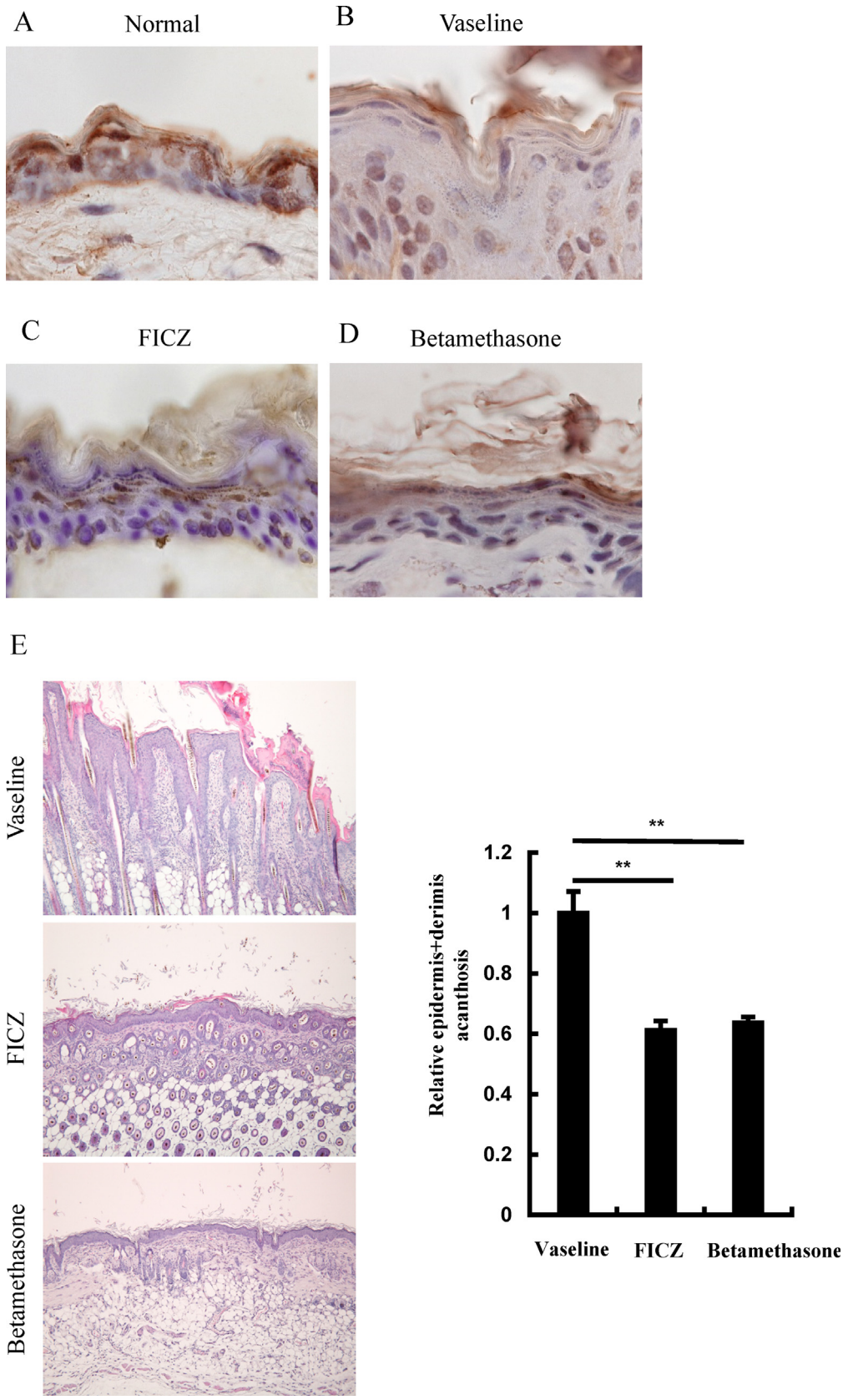

Fig. 6. FICZ ointment improved atopic dermatitis-like inflammation in NC/Nga mice (A–D) Immunohistochemical staining of filaggrin in the normal skin (A) and the lesional skin treated with each ointment for 2 weeks. Vaseline® as a control (B), FICZ (C), and betamethasone 17-valerate (D). (E) Histological changes of the dorsal skin treated with each ointment for 2 weeks. Skin sections were stained with hematoxylin-eosin. Vaseline® control (upper left panel), FICZ (upper center panel), and betamethasone 17-valerate (upper right panel). Measurements of the epidermal plus dermal thickness of the representative area were presented as the average of five repeated recordings. All data are presented as mean ± standard error ($n = 11–12$ per group). $^{**}p < 0.001$ (one-way analysis of variance) compared to the Vaseline® control group.

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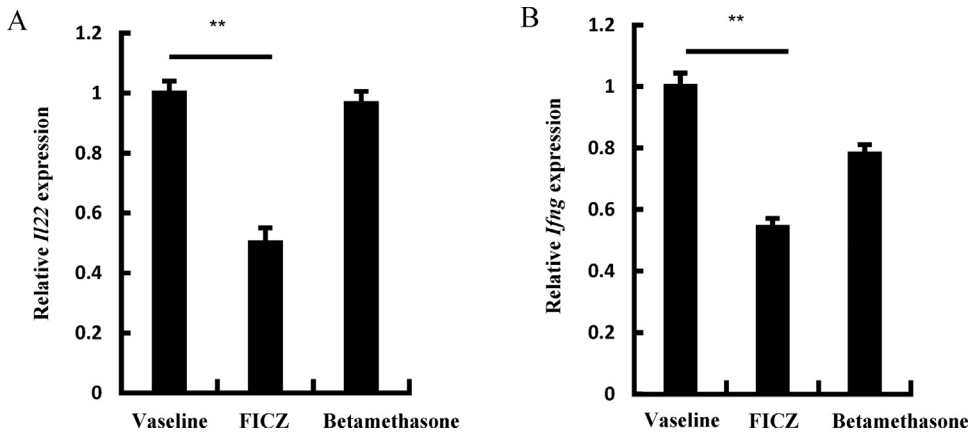

Fig. 7. Effect of FICZ on cytokine production in mite-induced dermatitis. Total RNA of the lesional dorsal skin was extracted. *Il22* (A) or *Ifng* (B) mRNA expression was measured by qRT-PCR normalized to *G6pd* expression (internal control). Data are presented as mean ± standard error (n = 11–12 per group). $^{**}p < 0.001$ (one-way analysis of variance) compared to the Vaseline® control group.

Phototherapy is useful for inflammatory skin diseases [43–45]. Suberythematous doses of UV radiation cause immunosuppression [46]. It is known that AHR is involved in this UV-mediated immunosuppression [47]. Moreover, suberythematous doses of UV radiation can upregulate FLG expression and reinforce the function of the epidermal barrier [48]. Since FICZ is the main tryptophan photoproduct generated by UV radiation and is a high-affinity endogenous ligand for AHR [7], the immunosuppressive and barrier-protective effects of UV may be mediated, at least in part, by FICZ.

In conclusion, topical FICZ inhibits the ongoing mite-induced chronic dermatitis in NC/Nga mice in concert with upregulating FLG expression via AHR, improving TEWL, and reducing *Il22* gene expression in the treated site. The beneficial effects of FICZ may recapitulate the salubrious properties of UV radiation. The development of AHR-targeting drugs may be promising for the treatment of inflammatory skin diseases.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jdermsci.2018.02.014.

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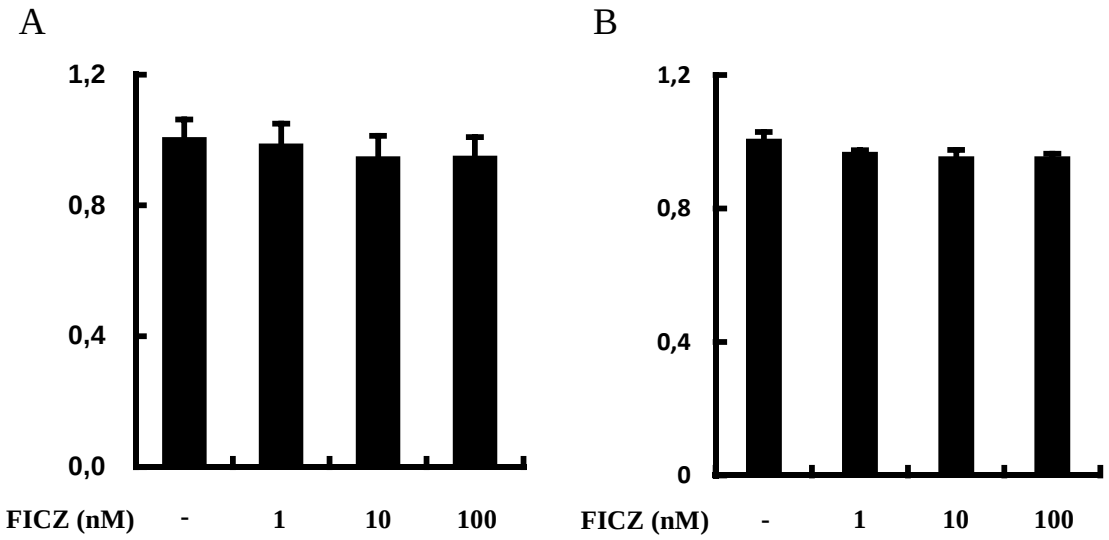
Supplementary Figure S-1



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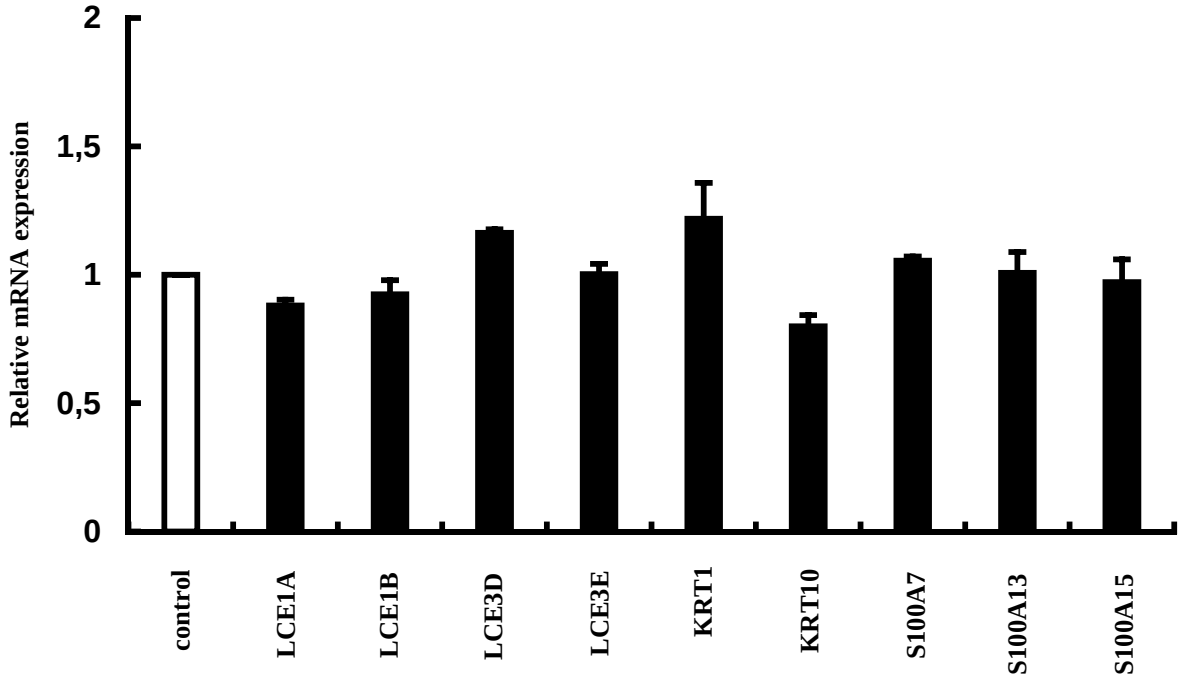

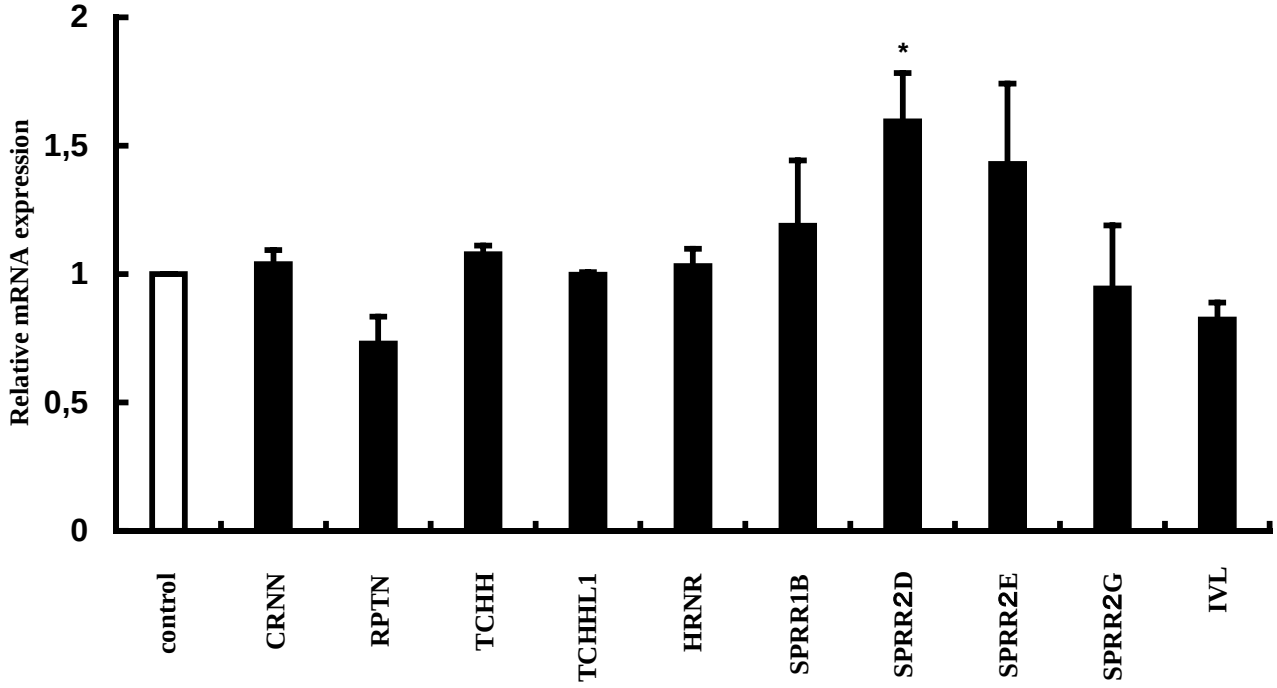

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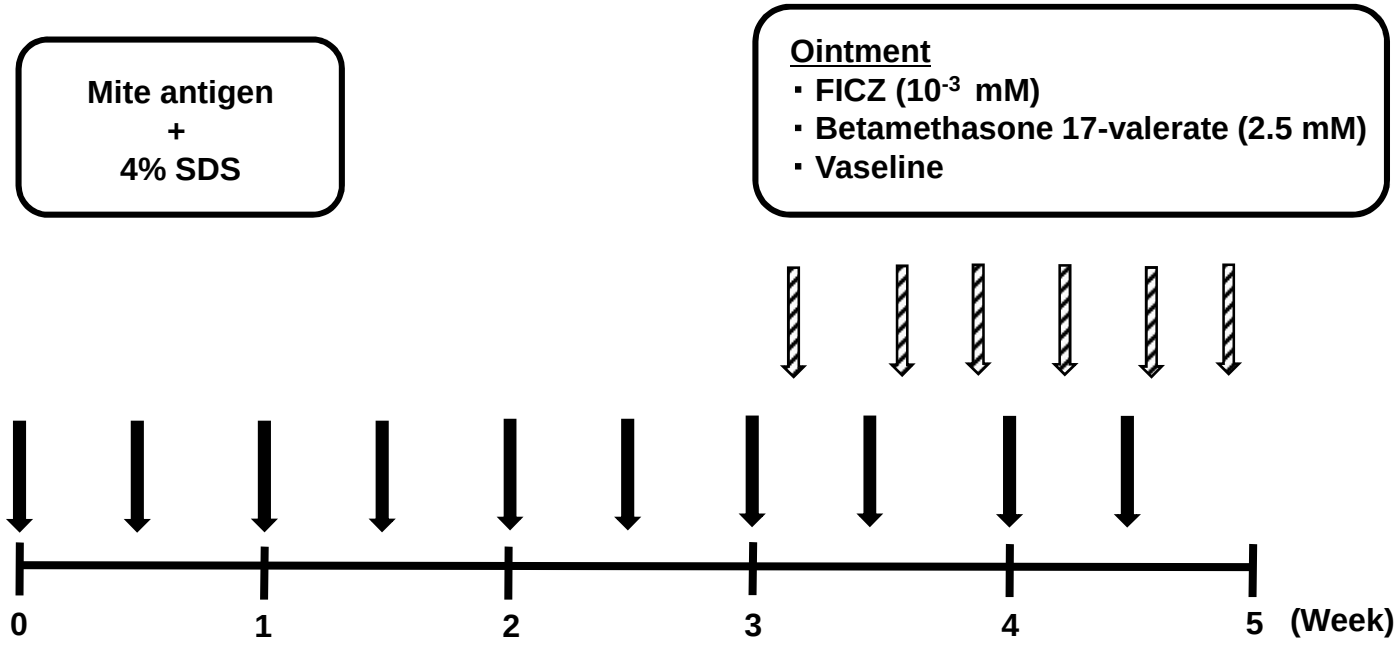

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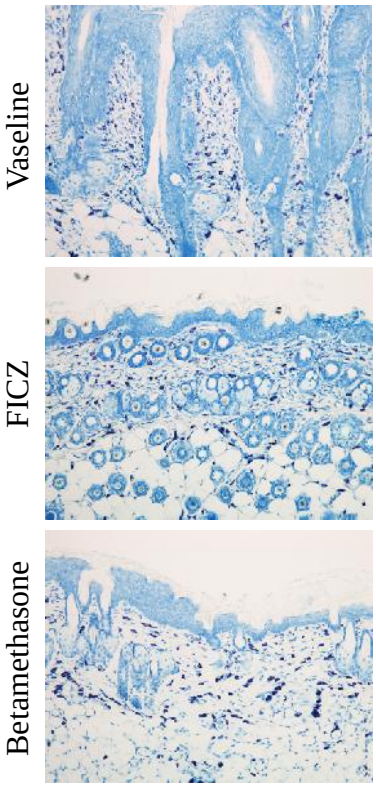
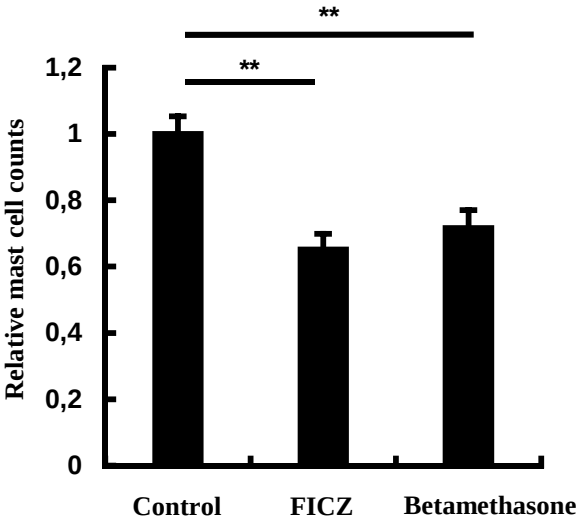
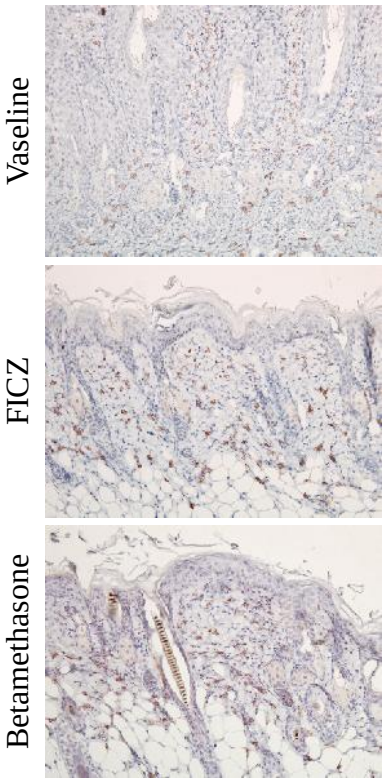
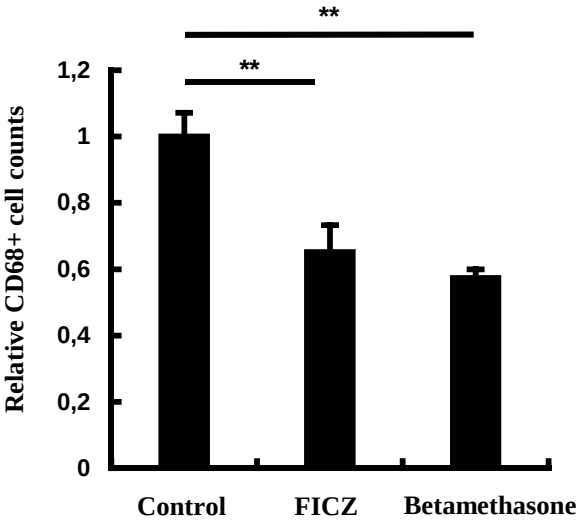
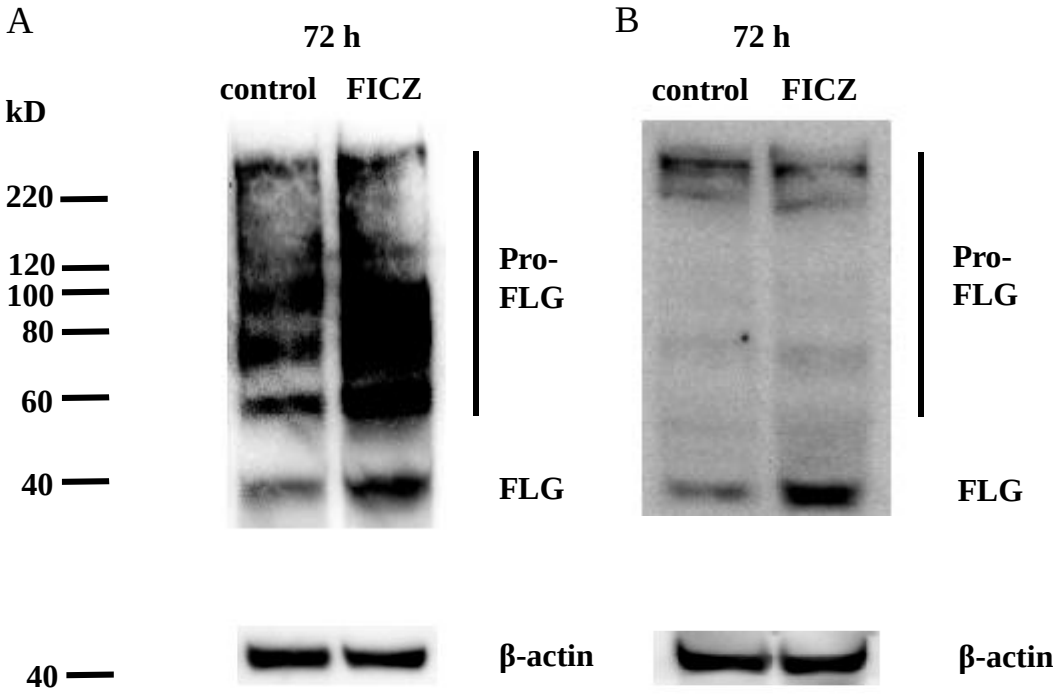
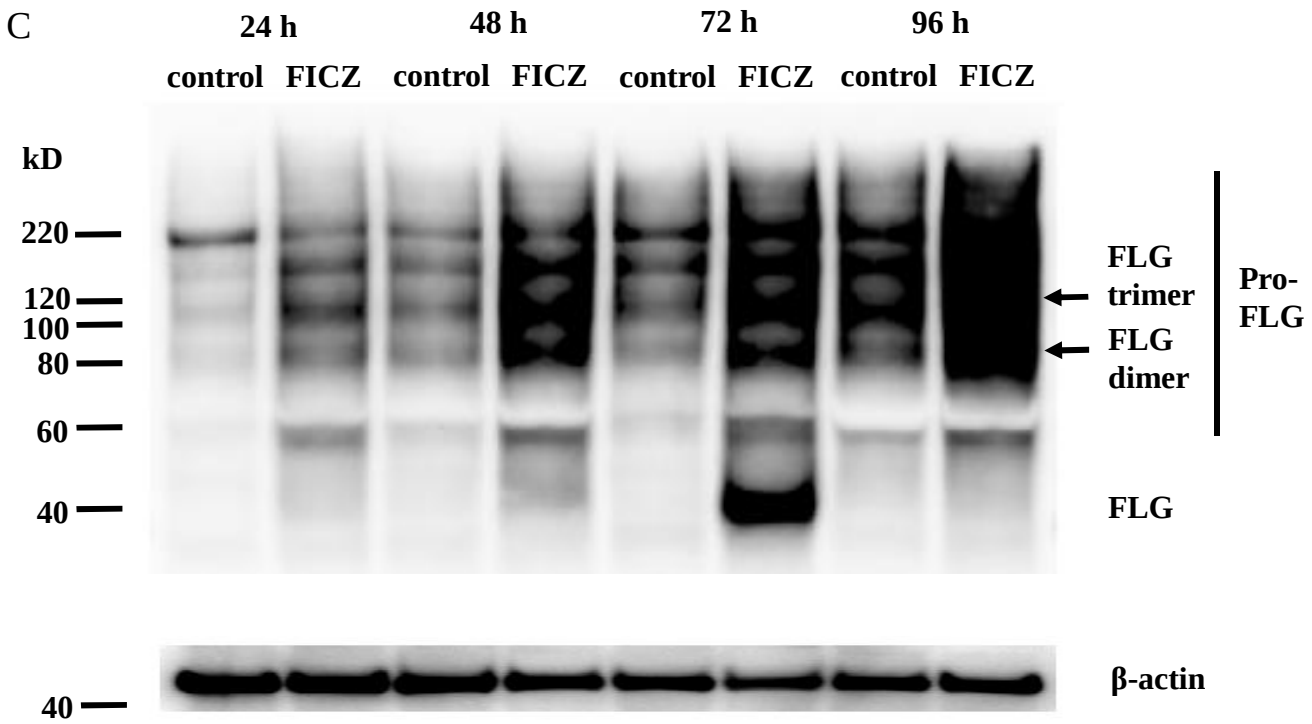




Supplementary Figure S-5





Supplementary Figure S-6