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Original Article

Topical application of activator protein-1 inhibitor T-5224 suppresses inflammation and improves skin barrier function in a murine atopic dermatitis-like dermatitis

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AD, atopic dermatitis; AP, activating protein; DNFB, 2,4-dinitrofluorobenzene; Elovl6, Elongation of long-chain fatty acids family member 6; FLG, filaggrin; JAK, janus kinase; LDH, lactate dehydrogenase; LOR, loricrin; MTT, 3,4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide; NF, nuclear factor; NHEKs, normal human epidermal keratinocytes; qRT-PCR, quantitative real-time PCR; STAT, signal transducer and activator of transcription; TYK, tyrosine kinase

ABSTRACT

Background: Selective activator protein (AP)-1 inhibitors are potentially promising therapeutic agents for atopic dermatitis (AD) because AP-1 is an important regulator of skin inflammation. However, few studies have investigated the effect of topical application of AP-1 inhibitors in treating inflammatory skin disorders.
Methods: Immunohistochemistry was conducted to detect phosphorylated AP-1/c-Jun expression of skin lesions in AD patients. In the *in vivo* study, 1 % T-5224 ointment was topically applied for 8 days to the ears of 2,4 dinitrofluorobenzene challenged AD-like dermatitis model mice. Baricitinib, a conventional therapeutic agent Janus kinase (JAK) inhibitor, was also topically applied. In the *in vitro* study, human epidermal keratinocytes were treated with T-5224 and stimulated with AD-related cytokines.
Results: AP-1/c-Jun was phosphorylated at skin lesions in AD patients. *In vivo*, topical T-5224 application inhibited ear swelling ($P < 0.001$), restored *filaggrin* (*Flg*) expression ($P < 0.01$), and generally suppressed immune-related pathways. T-5224 significantly suppressed *Il17a* and *Il17f* expression, whereas baricitinib did not. Baricitinib suppressed *Il4, Il19, Il33* and *Ifnb* expression, whereas T-5224 did not. *Il1a, Il1b, Il23a, Ifna, S100a8,* and *S100a9* expression was cooperatively downregulated following the combined use of T-5224 and baricitinib. *In vitro,* T-5224 restored the expression of *FLG* and *loricrin (LOR)* ($P < 0.05$) and suppressed *IL33* expression ($P < 0.05$) without affecting cell viability and cytotoxicity.
Conclusions: Topical T-5224 ameliorates clinical manifestations of AD-like dermatitis in mice. The effect of this inhibitor is amplified via combined use with JAK inhibitors.

Introduction

Atopic dermatitis (AD) is one of the most common inflammatory skin diseases.[1] It is pathologically characterized by dysfunctional skin barrier function and immune dysregulation resulting primarily from elevated levels of Th2 cytokines (IL-4, IL-5, IL-13, and IL-31), as well as Th17 and Th22 cytokines (IL-17 and IL-22).[2] Currently, topical agents are the mainstay of AD treatment although biologics and oral Janus kinase (JAK) inhibitors have been launched.[3] Following topical corticosteroids and tacrolimus ointment, new topical agents with different mechanisms of action, such as JAK inhibitors[4] and phosphodiesterase-4 inhibitors,[5] have been launched. The development of additional topical anti-inflammatory agents such as nuclear factor (NF)-κB inhibitor ointments[6] and non-steroidal-selective-glucocorticoid receptor agonists/modulators[7] has also been reported.

In this study, we examined the effectiveness of a selective activator protein-1 (AP-1) inhibitor, T-5224, as a topical agent in

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AD-like dermatitis. AP-1 is composed of homo- and heterodimers of Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1 and Fra2), and activating transcription factor protein families and functions as an important regulator of skin inflammation.[8] AP-1 complexes are over-expressed in lesional keratinocytes in AD, with higher levels of basal c-Jun and phorbol myristate acetate-induced c-Jun, JunB, and phosphorylated forms of c-Fos.[9]

Some studies have suggested selective AP-1 inhibition as a potential therapy for related inflammatory diseases.[10] However, little research has been conducted regarding the effect of topically applied AP-1 inhibitors in skin inflammatory diseases.

Methods

Skin specimens

This study was approved by the Ethics Committee of Okayama University (No. 2007-042) and performed in accordance with the principles of Declaration of Helsinki. Skin samples were collected with written informed consent from patients with AD or psoriasis and volunteers at Okayama University Hospital. Skin specimens from active lesions of AD patients (n = 5), psoriasis patients (n = 5) and from normal lesion of volunteers (n = 5) were used for immunohistochemical evaluation.

Animals

Female BALB/c mice (age 8–10 weeks) were purchased from Charles-River-Laboratories Japan, Inc. (Kanagawa, Japan) and housed in groups of 5 mice per cage under specific pathogen-free conditions at 23 ± 1 °C with a 12-h/12-h light/dark cycle. Water and standard laboratory diet were provided ad libitum.

Reagents

T-5224 and baricitinib were purchased from Cayman (MI, USA), whereas recombinant human IL-4, IL-13, IL-17A, and IL-22 were obtained from R & D systems (MN, USA). For *in vivo* experiments, T-5224 and baricitinib were dispersed in white petroleum/liquid paraffin (3:2)-based ointment for topical application. For *in vitro* experiments, T-5224 was dissolved in dimethyl sulfoxide.

1-fluoro-2,4-dinitrobenzene (DNFB)-induced AD-like dermatitis mouse model

A hapten-induced AD-like dermatitis mouse model was generated using female BALB/c mice as described previously.[11,12] Briefly, mouse ears were treated with 0.15 % DNFB (Combi-Blocks, CA, USA) dissolved in acetone/olive oil (3:1) once per week for 5 weeks, after which 25 μl of 0.15 % DNFB was applied to each side of both ears. 25 mg of 1 % (1.67×10^{-2} M) T-5224 ointment (about 8.3 mg/kg), 1 % baricitinib ointment or 1 % T-5224/1 % baricitinib ointment was then administered topically to each side of both ears once per day from the day of the fourth DNFB application (day 22) to the day of fifth DNFB application (day 29). Ear thickness was measured using a thickness gauge (SM-1201; Teclock, Nagano, Japan) 24 h before and after DNFB application and on days 25 and 27. Ear thickness was expressed as the increase in thickness relative to the baseline measurement. After the last measurement of ear thickness on day 30, mice were euthanized and their ears were excised for analysis of gene expression and histological evaluation. RNA was extracted from ear tissues with TRIzol reagent after homogenizing of ear sections, and the RNA was stored at −80 °C until use. All procedures performed on the animals in this study were approved by the Animal Care and Use Committee of Okayama University (No. OKU-2020402).

Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) (Thermo Fisher Scientific, MA, USA) were cultured as previously described.[13] The cells were then stimulated with IL-4, IL-13, IL-17A, IL-22 (20 ng/ml), and T-5224 (10^{-7}~10^{-6} M) in 24-well flat-bottom plates (Corning, ME, USA) for 24 h for *loricrin (LOR)* and *IL33* detection or 48 h for *filaggrin* (*FLG*) detection. Post-stimulation, RNA was extracted from the cells using TRIzol reagent (Thermo Fisher Scientific) after supernatant removal. RNA was stored at −80 °C until use.

Immunohistochemistry and cell counting

Formalin-fixed paraffin-embedded blocks were cut into 4-μm sections and mounted on the glass slides. The sections were treated with phospho-c-Jun (Ser63) rabbit monoclonal antibody (2361S; Cell Signaling Technology, MA, USA) according to the manufacturer's recommendations. The specimens were subsequently treated using an EnVision Detection System (K5007; Dako Japan, Tokyo, Japan) according to the manufacturer's protocol for visualization with 3,3′-diaminobenzidine tetrahydrochloride chromogen. Finally, the slides were counterstained with hematoxylin. The numbers of phospho-c-Jun-positive cells in the epidermis were analyzed by counting of three randomly selected fields per slide and calculating mean number of the positive cells per mm^2. The fields were obtained with an all-in-one fluorescence microscope (BZ-X710, Keyence, Osaka, Japan) using a Plan Apochromat 40× objective (NA0.75, Keyence, Osaka, Japan). Representative images were obtained using a photo-imaging system (V-100, Olympus, Tokyo, Japan).

Quantitative real-time PCR (qRT-PCR)

Complementary DNA was synthesized from RNA, and qRT-PCR was performed as previously described.[13] GAPDH was used as an internal control. Each mRNA expression was calculated as the expression relative to GAPDH mRNA, and all data are presented as fold changes in comparison with the control. TaqMan Gene Expression Assays (Thermo Fisher Scientific) were used to analyze the expression of human *FLG* (ID:Hs06628971_s1), human *LOR* (ID:Hs01894962_s1), human *IL33* (ID:00369211_m1), murine *Flg* (ID:Mm01716522_m1), and murine *Elongation of long-chain fatty acids family member 6* (*Elovl6*) (ID:Mm00851223_s1).

NanoString nCounter analysis

The concentration and quality of RNA isolated from mouse ear tissues were assessed using a Qubit 4 fluorometer (Thermo Fisher Scientific) and TapeStation 4150 (Agilent, CA, USA). A total of 100 ng of total RNA from each sample was analyzed using the nCounter Analysis System (NanoString Technologies, WA, USA) according to the manufacturer's instructions, in combination with the Murine Immunology Panel (NanoString Technologies), which contains 561 immunology-related mouse genes, including 14 housekeeping genes. Date regarding the gene sets identified in each pathway are available online (https://nanostring.com/support-documents/ncounter-mouse-immunology-panel-gene-list/).

The expression of multiple target-genes was analyzed simultaneously using specific molecular fluorescent barcodes. All data were normalized to the 14 housekeeping genes using nSolver 4.0 software (NanoString Technologies). Clustering was performed in R software (version 3.3.2) (R-project.org), and visualized as heatmaps generated using the publicly available software Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and Java TreeView (http://jtreeview.sourceforge.net/).

Cell viability assay (3,-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide [MTT] assay)

NHEKs were seeded in 96-well plates (Corning) at 5×10^4 cells with 100 μL medium per well and incubated at 37 °C for 24 h. After incubation, the medium was changed, and the cells were treated with T-5224 (10^{-7}~10^{-6} M) or dexamethasone (10^{-7}~10^{-6} M) for 24 h. Following treatment, the cells were analyzed using a Cell Proliferation Kit I (Roche, Basel, Switzerland) according to the manufacturer's recommendations.

Cell cytotoxicity assay (lactate dehydrogenase [LDH] assay)

NHEKs were seeded in 96-well plates (Corning) at 2×10^4 cells with 200 μL of medium per well and incubated at 37 °C for 24 h. After incubation, the medium was changed, and the cells were treated with T-5224 (10^{-7}~10^{-6} M) or dexamethasone (10^{-7}~10^{-6} M) for 24 h. Following treatment, the release of LDH from cells was detected as an indicator of cell membrane damage using a CK12 Cytotoxicity LDH Assay Kit-WST (Dojindo, Kumamoto, Japan) according to the manufacturer's recommendations.

Statistical analysis

Results are expressed as the mean ± SEM. Student's t-test was used to determine significance. Mann–Whitney U-test was used to compare *Elovl6* mRNA expression in mouse tissues among each group. All analyses were performed using GraphPad Prism 4 (GraphPad Software, CA, USA). $P < 0.05$ was considered significant.

Results

Expression of phosphorylated c-Jun is increased in AD lesional skin

To assess the relevance of activated AP-1 in human AD skin, we used immunohistochemistry to evaluate the epidermal expression of phosphorylated c-Jun (Ser63), which is an indicator of AP-1 activation.[14] Abundant phosphorylated c-Jun was observed in the nuclei of epidermal keratinocytes and dermal inflammatory cells of AD lesions, and the number of the positive cells in AD lesions was significantly more than that in normal skin, suggesting that activated AP-1 is involved in the pathogenesis of AD (Fig. 1). (cf. Abundant phosphorylated c-Jun was also observed in the nuclei of epidermal keratinocytes of psoriasis lesions [Supplementary Fig. 1].)

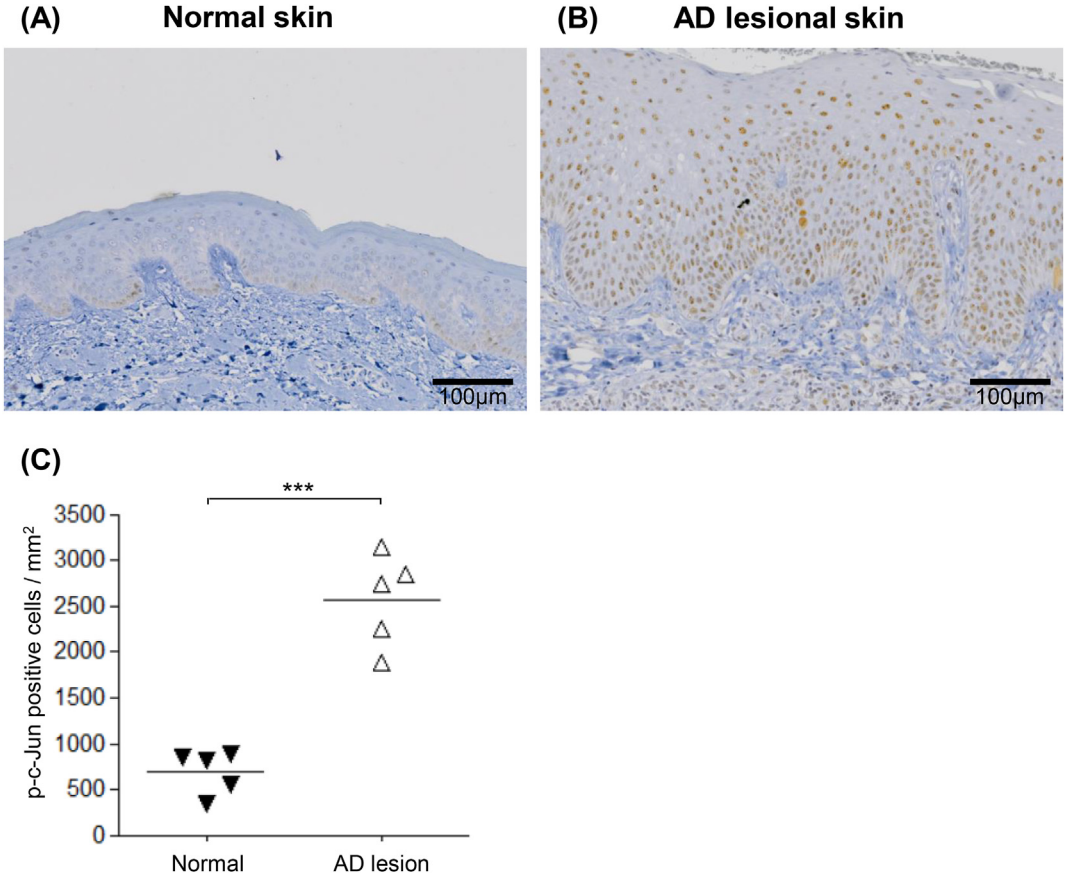

Fig. 1. Expression of phosphorylated c-Jun is increased in AD lesional skin. **(A)** Normal skin (n = 5) and **(B)** AD lesional skin sections (n = 5) were examined for phosphorylated c-Jun expression using immunohistochemistry. Results are representative of staining from the study population. Bar = 100 μm**(C)** The number of phosphorylated c-Jun-positive cells per mm^2 in normal skin and AD lesional skin. Results are expressed as mean ± SEM. ***P < 0.001. AD, atopic dermatitis; p-c-Jun, phosphorylated c-Jun.

Topical application of T-5224 and/or baricitinib ameliorates 2, 4-dinitrofluorobenzene (DNFB)-induced AD-like dermatitis in vivo

We next evaluated the effect of topical application of the AP-1 inhibitor T-5224 using AD-like dermatitis model mice. (cf. 1 % of T-5224 was effective enough to ameliorate dermatitis [Supplementary Fig. 2].) We also analyzed the effect of combination treatment with T-5224 and baricitinib. Ear thickness, which reflects the severity of inflammation, was significantly diminished at the experiment end point in mice treated with T-5224- or baricitinib-alone compared with vehicle-treated mice (Fig. 2A-C). Furthermore, combined application of T-5224 and baricitinib significantly enhanced suppression of inflammation ($P < 0.001$ vs baricitinib, $P < 0.05$ vs T-5224) (Fig. 2A-C).

We also assessed the expression of *Flg* and *Elovl6* in the ears at the end point using qRT-PCR. Expression of *Flg* and *Elovl6* was downregulated in DNFB-induced dermatitis lesions ($P < 0.001$ and $P < 0.01$, respectively). However, *Flg* expression was significantly restored by treatment with T-5224 or baricitinib ($P < 0.01$). *Elovl6* expression was also significantly restored by treatment with T-5224 ($P < 0.05$). Furthermore, expression of *Flg* and *Elovl6* was even more significantly restored by T-5224 and baricitinib ($P < 0.001$ and $P < 0.01$, respectively) (Fig. 2D, E). These data indicate that topical T-5224 is useful as an external preparation for AD and that it is even more effective when used in combination with baricitinib.

Topical application of T-5224 with baricitinib suppresses most immune response pathways in DNFB-induced AD-like dermatitis in vivo

We then investigated the differences in immune-related gene expression in the ears among the study groups using the NanoString nCounter system. The results are shown as a clustered heatmap covering all core pathways and processes of the immune response (Fig. 3). T-5224 and baricitinib generally suppressed

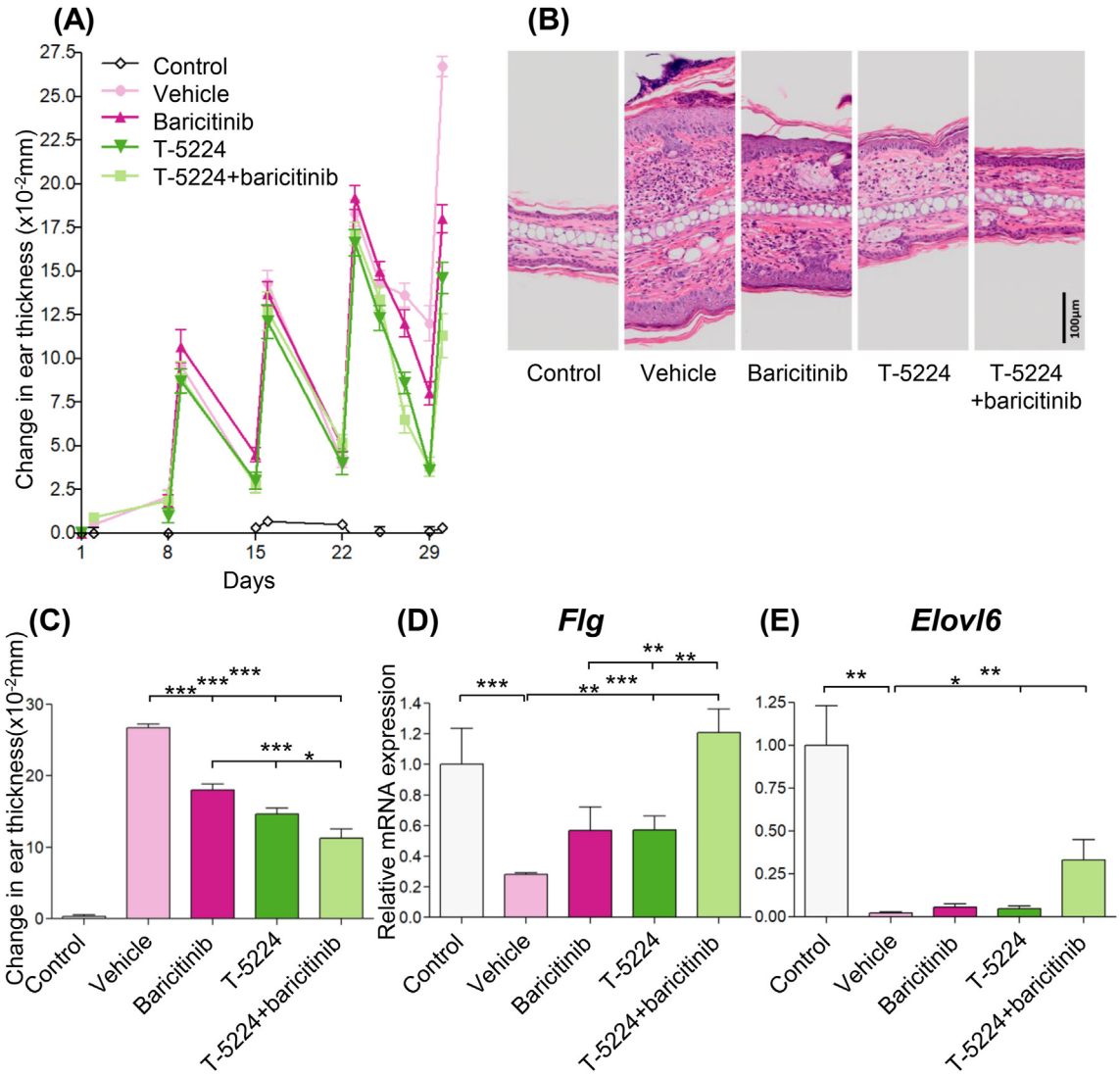

Fig. 2. Topical application of T-5224 and baricitinib ameliorates DNFB-induced AD-like dermatitis. Mice received topical application of 0.15 % DNFB in acetone/olive oil or vehicle (control) on the ear once a week for 5 weeks. Vehicle or T-5224 and/or baricitinib was administered topically once a day for 8 days from day 22 to day 29 (n = 5 in each group). **(A)** Ear thickness change from day 1 to day 30. Data are expressed as the increase in ear thickness from baseline. **(B)** Histological analysis at 24 h after the fifth DNFB application. Bar = 100 μm **(C)** Ear thickness difference between day 1 and day 30. **(D, E)** Relative mRNA expression of *Flg* and *Elovl6* to *GAPDH* in ear tissues 24 h after the last DNFB application was measured by qRT-PCR. Results are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. DNFB, 1-fluoro-2,4-dinitrobenzene; Flg, filaggrin; Elovl6, Elongation of long-chain fatty acids family member 6; qRT-PCR, quantitative real-time PCR.

immune response-related gene expression compared with vehicle treatment. The combination of T-5224 and baricitinib suppressed immune response-related gene expression more effectively than either single-treatment. The combination treatment markedly suppressed the mRNA expression of almost all immune-related pathways compared with vehicle treatment. However, the opposite result was observed with regard to genes of pathways related to major histocompatibility complex class II antigen presentation and transforming growth factor-β signaling.

We further analyzed the ear expressions of genes of specific pathways related to the pathogenesis of AD, including cytokine signaling, innate immune system, and adaptive immune system (Fig. 3B-D and Supplementary Fig. 3A–C). In all pathways examined, the expression of numerous genes was strongly suppressed by combined treatment with T-5224 and baricitinib. In contrast, the expression of some genes in these pathways was suppressed even under treatment with T-5224 or baricitinib alone.

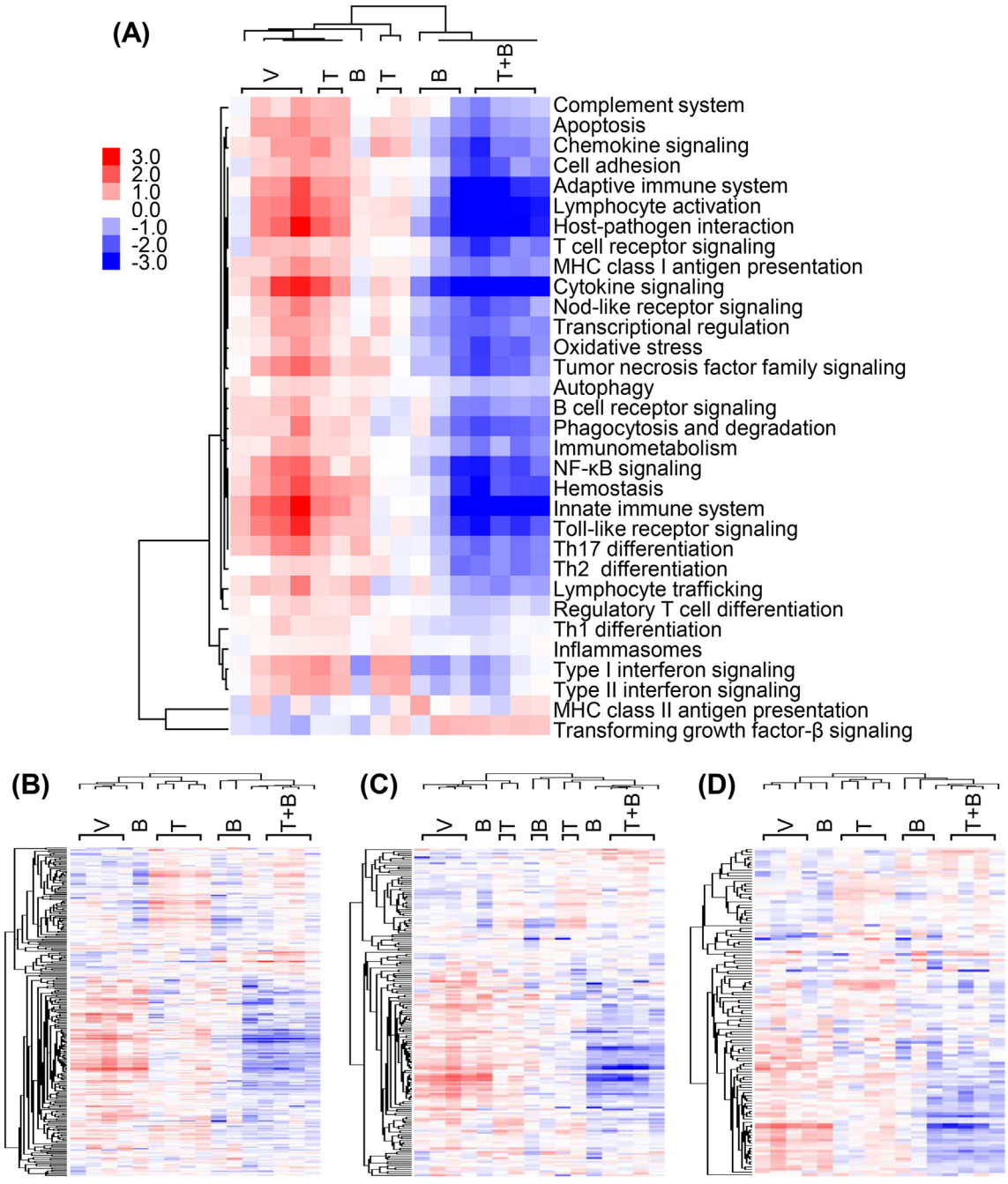

Fig. 3. Effects of topical T-5224 and baricitinib on immunological pathways in DNFB-induced AD-like dermatitis. **(A)** mRNA expression in the ears of mice shown as a heatmap covering core immune response pathways and processes. Heatmap illustrating the changes in mRNA expression of genes related to **(B)** cytokine signaling, **(C)** the innate immune system and **(D)** the adaptive immune system. Information about gene profiles of each pathway is based on the manufacturer. Expression levels are presented on a log2 scale. Direction of change in expression is shown by the color scale. V, Vehicle; B, Baricitinib; T, T-5224; MHC, *major histocompatibility complex;* DNFB, 1-fluoro-2,4-dinitrobenzene.

Topical application of T-5224 and/or baricitinib markedly reduce immune-related gene expression in DNFB-induced AD-like dermatitis in vivo cooperatively or independently

Finally, we focused on the expression of representative genes that exhibited characteristic and significant changes. T-5224, but not baricitinib, significantly suppressed the expression of *Il17a* and *l17f* which induce AP-1 activation (Fig. 4A, B). By contrast, baricitinib, but not T-5224, significantly suppressed the expression of *Il4, Il19, Il33,* and *Ifnb1* (Fig. 4C-F). *Il4, Il19,* and *Ifnb* which activate JAK-signal transducer and activator of transcription (STAT) signaling. In addition, the expression of *Il1a, Il1b, Il23a, Ifna1,*

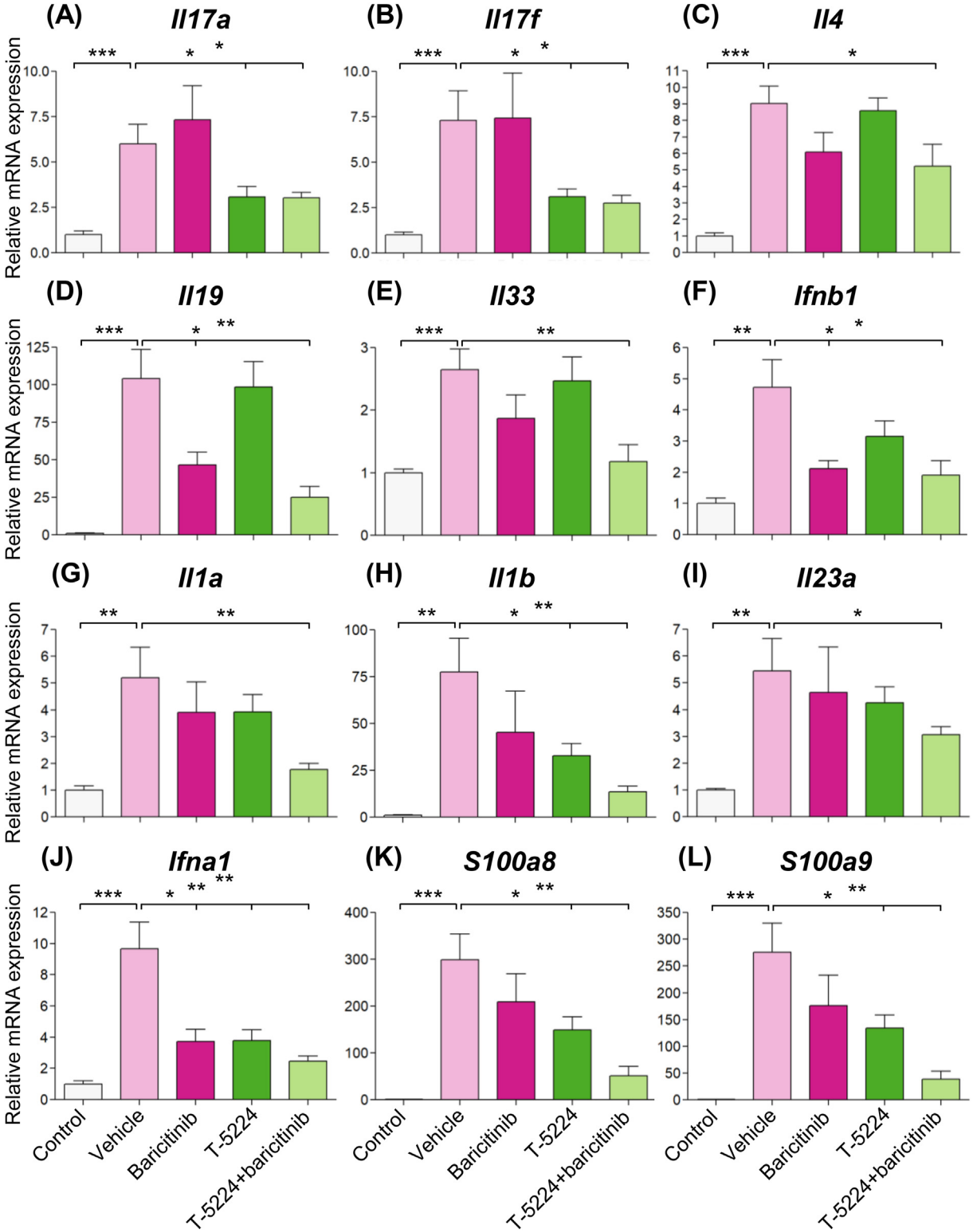

Fig. 4. Topical application of T-5224 and baricitinib to the ears of DNFB-induced AD-like dermatitis markedly reduced the expressions of immune-related genes. **(A–L)** Relative mRNA expression of genes normalized according to that of 14 housekeeping genes in ear tissues 24 h after the fifth DNFB application. Results are expressed as mean ± SEM (n = 4 for baricitinib group and n = 5 for all other groups). *P < 0.05, **P < 0.01, ***P < 0.001. DNFB, 1-fluoro-2,4-dinitrobenzene.

S100a8, and *S100a9* expression was significantly and cooperatively downregulated by combined treatment with T-5224 and baricitinib (Fig. 4G-L).

T-5224 restore barrier-related genes expression and suppress IL-33 expression in epidermal keratinocytes stimulated with AD-related cytokines

We also investigated the effect of T-5224, in epidermal keratinocytes, which would be most affected by topical agents, stimulated with AD-related cytokines *in vitro*. NHEKs were treated with T-5224 and simultaneously stimulated with IL-4, IL-13, IL-17A, and IL-22 (4 mix), representative cytokines involved in the pathogenesis of AD.[15] As shown in Figure 5, the expression of *FLG* and *LOR* was significantly restored by T-5224 treatment in a dose-dependent manner (Fig. 5A, B). On the other hand, dexamethasone further suppressed the expression of *FLG* and *LOR* in the cells (Supplementary Fig. 4A, B). In contrast, *IL33* expression was significantly suppressed by these inhibitors in a dose-dependent manner (Fig. 5C). We then performed MTT and LDH assays to evaluate the cell proliferation and cytotoxicity under these conditions, respectively. A 24-h exposure to T-5224 did not affect cell proliferation or produce signs of cytotoxicity (Fig. 5D, E). Dexamethasone also did not significantly change the proliferation or cytotoxicity (Supplementary Fig. 4C, D).

Discussion

In this study, we first showed that AP-1 is activated in epidermal keratinocytes of AD lesions. We next revealed that T-5224 significantly ameliorates the symptom of DNFB-induced AD-like dermatitis in model mice in which phosphorylated c-Jun is upregulated.[16] T-5224 also suppresses the expression of a variety of proinflammatory cytokines, chemokines, and antimicrobial peptides in the AD lesions. We then demonstrated that T-5224 restore the expression of barrier-related genes and suppress *IL33* expression in epidermal keratinocytes, which would be most affected by topical agents, stimulated with AD-related cytokines. T-5224 selectively inhibits c-Fos, and specifically inhibits the DNA-binding activity of c-Fos/c-Jun.[17] As various inflammatory cytokines, such as those of the IL-1 family (IL-1α/β, IL-18, IL-33, IL-36α/β/γ),[18] IL-17 family (IL-17A-F),[19] and TNF family[20] activate AP-1 to induce cytokine, chemokine and antimicrobial peptide expression, AP-1 inhibition is thought to suppress proinflammatory reactions by inhibiting these cytokines signaling pathways. Several reports have shown that T-5224 is a promising candidate for the treatment of inflammatory disorders. Systemic T-5224 treatment ameliorated rheumatoid arthritis in a mouse model[21] and attenuated IgE-related allergic responses in anaphylaxis models and mast cell-mediated AD in mice.[22]

In general, topical administration seems to be safer rather than systemic therapies in minimizing adverse events, when dealing

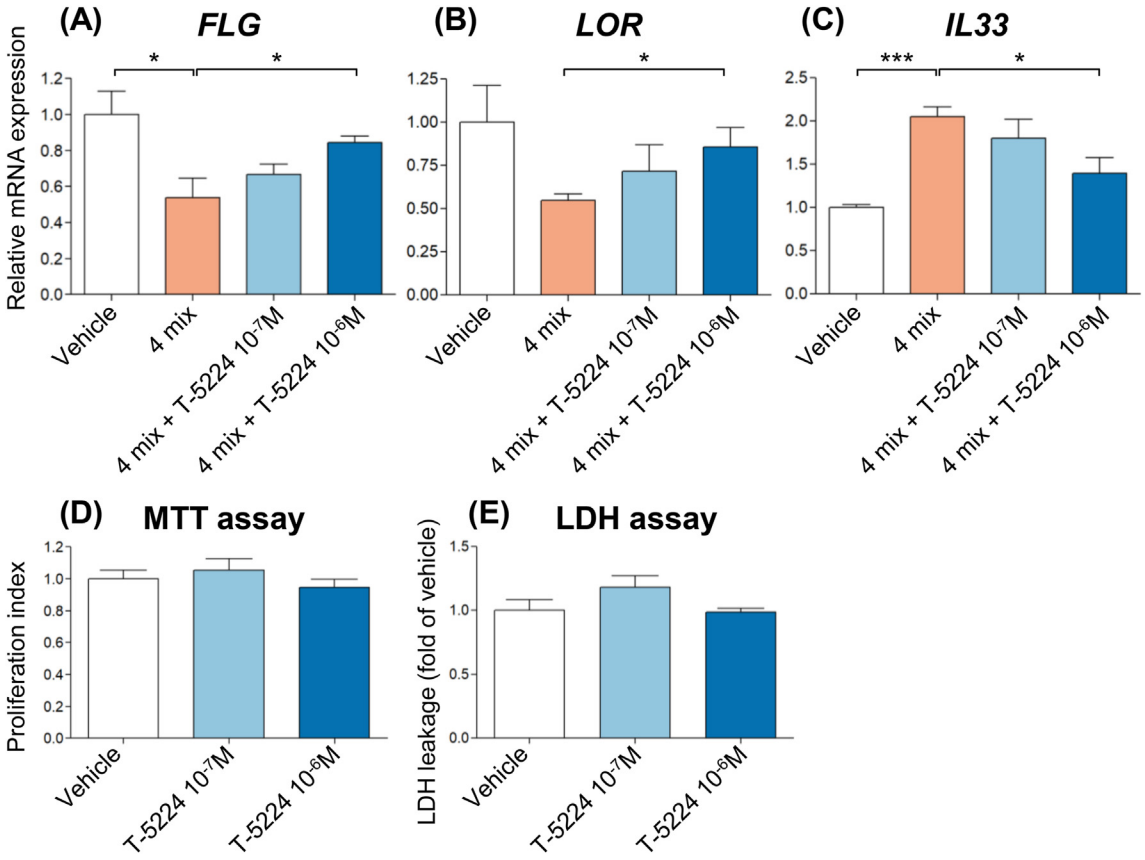

Fig. 5. T-5224 restore *FLG* and *LOR* expression and suppress *IL33* expression in epidermal keratinocytes stimulated with IL-4, IL-13, IL-17A, and IL-22. **(A–C)** NHEKs were treated with T-5224 and simultaneously stimulated with vehicle or IL-4, IL-13, IL-17A, and IL-22 (4 mix). The relative mRNA expression of *FLG*, *LOR* and *IL33* to *GAPDH* was measured using qRT-PCR. **(D)** Proliferation of NHEKs was examined using the MTT assay. Cell proliferation index was defined as the absorbance ratio of T-5224 treated cells relative to vehicle treatment. (**E**) Cytotoxicity against NHEKs was examined using the LDH assay. LDH leakage was expressed as the ratio relative to that of vehicle-treated cells. Data represent at least three independent experiments, each performed in triplicate. Results are presented as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. NHEKs, normal human epidermal keratinocytes; FLG, filaggrin; LOR, loricrin; qRT-PCR, quantitative real-time PCR; MTT, 3,-4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide; LDH, lactate dehydrogenase.

with skin inflammation. In addition, epidermal keratinocytes are an important therapeutic target in the treatment of AD because they contribute to pathogenesis by secreting several cytokines such as IL-1β, TSLP, IL-25, and IL-33, which activate and recruit inflammatory cells such as T cells, ILCs, mast cells, eosinophils and dendritic cells.[23] Therefore, the use of AP-1 inhibitors for topical application is quite reasonable. However, we have not evaluated potential adverse events of T-5224, such as the development of infections and malignancies which could be caused by the long-term use. Further evaluation is needed for its safety, as AP-1 regulates a wide range of cellular processes, including proliferation, differentiation, apoptosis and tumor progression.[10]

IL-4 and IL-13 bind IL-4Rα/IL-13Rα1, activate JAK1, JAK2, and tyrosine kinase (TYK)2, and induce the phosphorylation of STAT6 and STAT3.[15] Several JAK inhibitors that suppress the JAK/STAT pathway are clinically available as therapeutic options for treating AD via both topical and systemic application.[24] Also, in our AD-like dermatitis model experiments, topical application of baricitinib suppressed the expression of *Il4,* although the AP-1 inhibitor T-5224 showed little effect.

IL-17A activates the *c-fos* and *c-jun* promoters, resulting in increased AP-1 activity.[25] IL-17A/F and IL-17F are also known to exhibit the similar proinflammatory functions.[26] In our AD-like dermatitis model experiments, topical T-5224 suppressed the expression of *Il17*a and *Il17f,* although baricitinib showed little effect.

IL-23 is a heterodimeric cytokine consisting of p19 and p40.[27] This cytokine induces the phosphorylation of JAK2 and STAT3, leading to the proliferation and differentiation of Th17 cells and group 3 ILCs, which are main cell sources of IL-17.[28] In our AD-like dermatitis model experiments, topical T-5224 and baricitinib cooperatively suppressed the expression of *Il23a*.

IL-19, a pro-inflammatory cytokine known to stimulate JAK1, TYK2, and STAT3,[29] is significantly upregulated in AD and positively correlated with the disease severity.[30] Both IL-17A and IL-4 increase IL-19 mRNA expression in human epidermal keratinocytes.[31] Similar to *Il4*, *Il19* expression was suppressed by baricitinib, but not T-5224, in our experiments.

Increased expression of IL-22 is also a characteristic finding in AD.[32] IL-22 binds to its receptor IL-22R, which is abundantly expressed on keratinocytes and some fibroblasts,[32] inducing the phosphorylation of JAK1 and TYK2 and activating STAT3.[4] IL-22 also synergistically induces the transcription of AP-1 family members in cooperation with TNF-α.[33] IL-22 directly upregulates the expression of epithelial-derived Th2 cytokines, such as TSLP and IL-33, in primary keratinocytes.[32] IL-33 is upregulated in the lesional skin of AD.[34] This cytokine induces the expression of proinflammatory cytokines and chemokines via NF-κB and AP-1.[35] IL-33 activates ILC2 to produce Th2 cytokines, especially IL-5 and IL-13.[36] IL-33 also activates mast cells and eosinophils and reduces the expression of FLG in epidermal keratinocytes.[34] In our AD-like dermatitis model experiments, *Il33* expression was also suppressed by baricitinib, but not T-5224, in our AD-like dermatitis model experiment.

The most significant expression change observed in this study was that of the antimicrobial proteins S100A8 and S100A9. S100A8 and S100A9 promote cytokine production and decrease the expression of FLG and LOR via the NF-κB, and MAPK pathways leading to AP-1.[37] IL-17 and IL-22 induce keratinocytes to produce S100A8 and S100A9, which are significantly associated with AD disease activity.[38] In our AD-like dermatitis model experiments, topical T-5224 and baricitinib synergistically suppressed the expression of *S100a8* and *S100a9*.

Taken together, our data indicate that various cytokines that activate AP-1 and/or the JAK/STAT pathway are involved in the pathogenesis of AD. Some additive or synergistic effects between AP-1 and JAK inhibitors were observed in our analyses. Our gene analysis data showed that inhibition of AP-1 and/or JAK in chronic dermatitis effectively suppresses a wide range of immune-related pathways. The pathogenesis of AD appears to involve orchestrated activity of both the innate and adaptive immune responses, as previously proposed.[39] As JAK inhibitors do not suppress AP-1 directly, and vice versa, the combined use of T-5224 and baricitinib seems reasonable to inhibit both the innate and adaptive immune systems.

Allergic inflammation is known to downregulate skin barrier function in AD.[40] IL-4 and IL-13, IL-17A and IL-22 all play roles in skin barrier dysfunction by inhibiting the expression of barrier-related molecules such as FLG, LOR and involucrin.[15] AP-1 is known to suppress the expression of these epidermal barrier proteins.[41] A previous report showed that pre-treatment with the AP-1 inhibitor tanshinone IIA, increases FLG expression in particulate matters(SRM1649b)-stimulated human keratinocytes in which FLG expression was downregulated by activation of AP-1.[42] We confirmed that *FLG* and *LOR* expression was suppressed by stimulation with IL-4, IL-13, IL-17A, and IL-22 in NHEKs and restored by T-5224 treatment. In addition, topical application of T-5224 and baricitinib cooperatively increased *Flg* expression in our AD-like dermatitis model experiments. These results suggest that topical application of AP-1 inhibitors improves the skin barrier function and suppresses allergic skin inflammation and that combination use with a JAK inhibitor further enhances these effects.

In conclusion, we have shown that topical application of selective AP-1 inhibitors ameliorates AD-like dermatitis by reserving the dysregulation of innate and adaptive immune responses. In addition, combination use with a JAK inhibitor synergistically suppresses the symptoms of AD-like dermatitis. Our data suggest that targeting AP-1 selectively via topical treatment is a new and promising strategy for treating AD.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2023.12.006.

Conflict of interest

SM has received research support from Mitsubishi Tanabe Pharma, Sanofi and Maruho, and honoraria for lectures from Eli Lilly Japan, AbbVie, Pfizer Japan, Torii Pharmaceutical, Sanofi and Maruho. DE has received research support from Nippon Shinyaku and Chugai Pharmaceutical, and honoraria for lectures from Eisai, Kyowa Kirin, Chugai Pharmaceutical, SymBio Pharmaceuticals and Bristol Myers Squibb. ST has received honoraria for lectures from Chugai Pharmaceutical and Illumina. The rest of the authors have no conflict of interest.

Authors' contributions

SM designed the study, MS, HU, KT, KI, KH, YM and KS contributed to data collection. MS, DE and ST analyzed and visualized data; MS and SM wrote the manuscript; All authors read and approved the final manuscript.

References

1. Fishbein AB, Silverberg JI, Wilson EJ, Ong PY. Update on atopic dermatitis: diagnosis, severity assessment, and treatment selection. *J Allergy Clin Immunol Pract* 2020;**8**:91–101.
2. Hassan Z, Luvsannyam E, Patel D, Nukala S, Puvvada SR, Hamid P. Review of prominent cytokines as superior therapeutic targets for moderate-to-severe atopic dermatitis. *Cureus* 2020;**12**:e9901.

3. Eichenfield LF, Tom WL, Berger TG, Krol A, Paller AS, Schwarzenberger K, et al. Guidelines of care for the management of atopic dermatitis. *J Am Acad Dermatol* 2014;**71**:116–32.
4. Nakashima C, Yanagihara S, Otsuka A. Innovation in the treatment of atopic dermatitis: emerging topical and oral Janus kinase inhibitors. *Allergol Int* 2022;**71**:40–6.
5. Yang H, Wang J, Zhang X, Zhang Y, Qin ZL, Wang H, et al. Application of topical phosphodiesterase 4 inhibitors in mild to moderate atopic dermatitis: a systematic review and meta-analysis. *JAMA Dermatol* 2019;**155**:585–93.
6. Tanaka A, Muto S, Jung K, Itai A, Matsuda H. Topical application with a new NF-kappaB inhibitor improves atopic dermatitis in NC/NgaTnd mice. *J Invest Dermatol* 2007;**127**:855–63.
7. Eirefelt S, Stahlhut M, Svitacheva N, Carnerup MA, Da Rosa JMC, Ewald DA, et al. Characterization of a novel non-steroidal glucocorticoid receptor agonist optimized for topical treatment. *Sci Rep* 2022;**12**:1501.
8. Zenz R, Eferl R, Scheinecker C, Redlich K, Smolen J, Schonthaler HB, et al. Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Res Ther* 2008;**10**:201.
9. Pastore S, Giustizieri ML, Mascia F, Giannetti A, Kaushansky K, Girolomoni G. Dysregulated activation of activator protein 1 in keratinocytes of atopic dermatitis patients with enhanced expression of granulocyte/macrophage-colony stimulating factor. *J Invest Dermatol* 2000;**115**:1134–43.
10. Ye N, Ding Y, Wild C, Shen Q, Zhou J. Small molecule inhibitors targeting activator protein 1 (AP-1). *J Med Chem* 2014;**57**:6930–48.
11. Nagai H, Hiyama H, Matsuo A, Ueda Y, Inagaki N, Kawada K. FK-506 and cyclosporin A potentiate the IgE antibody production by contact sensitization with hapten in mice. *J Pharmacol Exp Ther* 1997;**283**:321–7.
12. Guo JZ, Wang WH, Li LF, Yang SM, Wang J. The role of metallothionein in a dinitrofluorobenzene-induced atopic dermatitis-like murine model. *Sci Rep* 2018;**8**:11129.
13. Kajita AI, Morizane S, Takiguchi T, Yamamoto T, Yamada M, Iwatsuki K. Interferon-gamma enhances TLR3 expression and anti-viral activity in keratinocytes. *J Invest Dermatol* 2015;**135**:2005–11.
14. Cho Y-Y, Tang F, Yao K, Lu C, Zhu F, Zheng D, et al. Cyclin-dependent kinase-3–mediated c-jun phosphorylation at Ser63 and Ser73 enhances cell transformation. *Cancer Res* 2009;**69**:272–81.
15. Furue M. Regulation of filaggrin, loricrin, and involucrin by IL-4, IL-13, IL-17a, IL-22, AHR, and NRF2: pathogenic implications in atopic dermatitis. *Int J Mol Sci* 2020;**21**:5382.
16. Wang Z, Yi T, Long M, Ding F, Ouyang L, Chen Z. Involvement of the negative feedback of IL-33 signaling in the anti-inflammatory effect of electroacupuncture on allergic contact dermatitis via targeting MicroRNA-155 in mast cells. *Inflammation* 2018;**41**:859–69.
17. Aikawa Y, Morimoto K, Yamamoto T, Chaki H, Hashiramoto A, Narita H, et al. Treatment of arthritis with a selective inhibitor of c-Fos/activator protein-1. *Nat Biotechnol* 2008;**26**:817–23.
18. Mantovani A, Dinarello CA, Molgora M, Garlanda C. Interleukin-1 and related cytokines in the regulation of inflammation and immunity. *Immunity* 2019;**50**:778–95.
19. Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. *Immunity* 2011;**34**:149–62.
20. Kyriakis JM. Activation of the AP-1 transcription factor by inflammatory cytokines of the TNF family. *Gene Expr* 1999;**7**:217–31.
21. Makino H, Seki S, Yahara Y, Shiozawa S, Aikawa Y, Motomura H, et al. A selective inhibition of c-Fos/activator protein-1 as a potential therapeutic target for intervertebral disc degeneration and associated pain. *Sci Rep* 2017;**7**:16983.
22. Wang HN, Ji K, Zhang LN, Xie CC, Li WY, Zhao ZF, et al. Inhibition of c-Fos expression attenuates IgE-mediated mast cell activation and allergic inflammation by counteracting an inhibitory AP1/Egr1/IL-4 axis. *J Transl Med* 2021;**19**:261.
23. Chieosilapatham P, Kiatsurayanon C, Umehara Y, Trujillo-Paez JV, Peng G, Yue H, et al. Keratinocytes: innate immune cells in atopic dermatitis. *Clin Exp Immunol* 2021;**204**:296–309.
24. Traidl S, Freimooser S, Werfel T. Janus kinase inhibitors for the therapy of atopic dermatitis. *Allergol Select* 2021;**5**:293–304.
25. Kim G, Khanal P, Lim SC, Yun HJ, Ahn SG, Ki SH, et al. Interleukin-17 induces AP-1 activity and cellular transformation via upregulation of tumor progression locus 2 activity. *Carcinogenesis* 2013;**34**:341–50.
26. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 family of cytokines in health and disease. *Immunity* 2019;**50**:892–906.
27. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000;**13**:715–25.
28. Rosine N, Miceli-Richard C. Innate cells: the alternative source of IL-17 in axial and peripheral spondyloarthritis? *Front Immunol* 2020;**11**:553742.
29. Bharadwaj U, Kasembeli MM, Robinson P, Tweardy DJ. Targeting Janus kinases and signal transducer and activator of transcription 3 to treat inflammation, fibrosis, and cancer: rationale, progress, and caution. *Pharmacol Rev* 2020;**72**:486–526.
30. Konrad RJ, Higgs RE, Rodgers GH, Ming W, Qian YW, Bivi N, et al. Assessment and clinical relevance of serum IL-19 levels in psoriasis and atopic dermatitis using a sensitive and specific novel immunoassay. *Sci Rep* 2019;**9**:5211.
31. Oka T, Sugaya M, Takahashi N, Nakajima R, Otobe S, Kabasawa M, et al. Increased interleukin-19 expression in cutaneous T-cell lymphoma and atopic dermatitis. *Acta Derm Venereol* 2017;**97**:1172–7.
32. Lou H, Lu J, Choi EB, Oh MH, Jeong M, Barmettler S, et al. Expression of IL-22 in the skin causes Th2-biased immunity, epidermal barrier dysfunction, and pruritus via stimulating epithelial Th2 cytokines and the GRP pathway. *J Immunol* 2017;**198**:2543–55.
33. Eyerich S, Wagener J, Wenzel V, Scarponi C, Pennino D, Albanesi C, et al. IL-22 and TNF-alpha represent a key cytokine combination for epidermal integrity during infection with Candida albicans. *Eur J Immunol* 2011;**41**:1894–901.
34. Imai Y. Interleukin-33 in atopic dermatitis. *J Dermatol Sci* 2019;**96**:2–7.
35. Griesenauer B, Paczesny S. The ST2/IL-33 Axis in immune cells during inflammatory diseases. *Front Immunol* 2017;**8**:475.
36. Klonowska J, Glen J, Nowicki RJ, Trzeciak M. New cytokines in the pathogenesis of atopic dermatitis-new therapeutic targets. *Int J Mol Sci* 2018;**19**:3086.
37. Kim MJ, Im MA, Lee JS, Mun JY, Kim DH, Gu A, et al. Effect of S100A8 and S100A9 on expressions of cytokine and skin barrier protein in human keratinocytes. *Mol Med Rep* 2019;**20**:2476–83.
38. Guttman-Yassky E, Bissonnette R, Ungar B, Suarez-Farinas M, Ardeleanu M, Esaki H, et al. Dupilumab progressively improves systemic and cutaneous abnormalities in patients with atopic dermatitis. *J Allergy Clin Immunol* 2019;**143**:155–72.
39. Honda T, Kabashima K. Reconciling innate and acquired immunity in atopic dermatitis. *J Allergy Clin Immunol* 2020;**145**:1136–7.
40. Kim BE, Leung DYM. Significance of skin barrier dysfunction in atopic dermatitis. *Allergy Asthma Immunol Res* 2018;**10**:207–15.
41. Kypriotou M, Huber M, Hohl D. The human epidermal differentiation complex: cornified envelope precursors, S100 proteins and the 'fused genes' family. *Exp Dermatol* 2012;**21**:643–9.
42. Lee CW, Lin ZC, Hu SC, Chiang YC, Hsu LF, Lin YC, et al. Urban particulate matter down-regulates filaggrin via COX2 expression/PGE2 production leading to skin barrier dysfunction. *Sci Rep* 2016;**6**:27995.