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The possible effectiveness of difamilast in improving barrier dysfunction in patients with atopic dermatitis and in mouse model

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Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritus and impaired skin barrier function. Although difamilast, a topical phosphodiesterase-4 (PDE4) inhibitor, has demonstrated clinical efficacy in AD, its role in restoring barrier function remains incompletely understood. This study aimed to investigate the barrier-improving effects of topical difamilast in patients with AD and in an MC903-induced mouse model of AD-like dermatitis. We conducted a retrospective study involving seven Japanese adult patients with mild-to-moderate AD who received 1% difamilast ointment twice daily for 1 week. Transepidermal water loss (TEWL) was measured before and after treatment. Animal experiment was performed using MC903-induced AD-like dermatitis model, followed by treatment with difamilast or vehicle. Dermatitis scores, TEWL, histological changes, immunofluorescence for filaggrin and CD4, and mRNA expression of filaggrin, IL-4, IL-13 and TSLP were evaluated. Topical difamilast reduced TEWL in six out of seven AD patients. In the mouse model, difamilast markedly attenuated dermatitis severity and reduced TEWL. Histological analysis revealed suppression of epidermal thickening and inflammatory cell infiltration. Difamilast restored filaggrin expression and decreased mast cell and $CD4^+$ T-cell infiltration in lesional skin. Quantitative PCR demonstrated that difamilast normalized the MC903-induced decrease in filaggrin and increase in IL-4 mRNA expression. Our findings suggest that difamilast improves skin barrier dysfunction in both human AD and an experimental AD-like mouse model. However, large-scale controlled studies are required to validate its barrier-improving efficacy in real-world clinical settings.

KEYWORDS

retrospective study, atopic dermatitis, Japanese, difamilast, barrier dysfunction

Introduction

Atopic dermatitis (AD) is a chronic eczematous skin disorder characterized by pruritus and impaired epidermal barrier function [1]. AD has a complex pathophysiology involving genetic predisposition, barrier dysfunction, and T cell–mediated type 2 inflammation. Among these factors, epidermal barrier dysfunction plays a central role in disease development. In AD patients, filaggrin expression is suppressed as a result of genetic polymorphisms and signaling to epidermal cells by interleukin (IL)-4 and IL-13 [2]. Recent advances in understanding the pathogenesis of AD have introduced new treatments into clinical practice.

Phosphodiesterases (PDEs) are enzymes that degrade cyclic nucleotides such as cAMP and cGMP, thereby regulating intracellular signaling pathways. By controlling the levels of these second messengers, PDEs play a crucial role in various physiological processes including inflammation [3]. Difamilast, a PDE4 inhibitor, is a topical agent used to treat AD across a wide age range, from children to adults. Clinical trials have revealed the efficacy and safety of difamilast [4]. Furthermore, *in vitro* studies have demonstrated that difamilast enhances filaggrin production in keratinocytes [5]. However, the effects of difamilast on improving barrier dysfunction *in vivo* have not yet been fully elucidated. To examine this point, we analyzed 7 Japanese adult patients with AD who were treated with difamilast. We also conducted studies using MC903-induced atopic dermatitis mouse model.

Methods

Human study

Patients with mild -to-moderate AD treated with difamilast from July 2023 to July 2024 at Gunma University Hospital, Japan, were included (Table 1). All patients fulfilled the criteria for AD according to Japanese guideline [1]. Seven Japanese patients (4 males, 3 females; mean age ±standard deviation, 29.9 ± 9.5 years) were completed the 1 week treatment without interruption at our hospital. All patients received topical application of 1% difamilast twice per day. Trans epidermal water loss (TEWL) was measured on the forearm before application and after 1 week using the DERMA-LAB TEWL probe (Cortex Technology). This retrospective cohort study was approved by the Institutional Review Board of Gunma University (HS2022-169) and was conducted according to the principles of the Declaration of Helsinki.

Mouse

Animal experiments were approved by the Gunma University Animal Care and Experimentation Committee (24-062) and carried out in accordance with the approved guidelines. Eight-week-old female C57BL/6 mice were purchased from the SLC (Shizuoka, Japan). The mice were maintained in the Institute of Experimental Animal Research of Gunma University under specific pathogen-free conditions and were handled in accordance with the animal care guidelines of Gunma University.

Development of AD-like skin inflammation

AD-like skin inflammation was induced by topical application of commercially available calcipotriol (MC903; Tocris Bioscience, Bristol, UK), following previously described protocol [6]. The method for inducing AD-like skin inflammation using MC903 was adapted from our previous study [7]. Three days before initiating treatment, the dorsal fur of each mouse was shaved under anesthesia. Subsequently, 20 µL of MC903 (0.1 mM in ethanol) or an equivalent amount of ethanol (control) was applied topically once daily to a 1 cm^2 area on the back for six consecutive days. The severity of dermatitis was evaluated based on a composite scoring system encompassing erythema, edema, dryness/scaling, and

TABLE 1 Details of 7 patients with atopic dermatitis treated with difamilast.

Case	Age	Sex	Severerity of AD	Past treatment	Measurement site	Before	After
#1	31	M	Moderate	Moisturizer	Left forearm	22.8	16
#2	50	M	Mild	TCS (very strong), moisturizer	Right forearm	18.2	10.1
#3	30	M	Mild	Moisturizer	Left forearm	16.9	7.7
#4	26	F	Moderate	TCS (very strong), moisturizer	Right forearm	10.8	15.9
#5	22	M	Moderate	None	Right forearm	20.8	13.1
#6	22	F	Mild	None	Right forearm	22.6	12.3
#7	28	F	Moderate	Moisturizer	Left forearm	20.6	12.7

AD, atopic dermatitis; M, Male; F, Female; TCS, topical corticosteroids; TEWL, transepidermal water loss.

excoriation/erosion, with each parameter rated on a scale from 0 to 3, in accordance with previously described methods [7]. TEWL was measured on the back skin using the DERMA-LAB TEWL probe. To investigate the effect of difamilast on the development of AD-like skin inflammation in mice, 5 mg of difamilast or the same amount of vaseline as control were applied for 6 consecutive days.

Histological examination and immunostaining

Murine skins were removed, fixed with formalin, and embedded in paraffin. Paraffin-embedded murine skin sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and toluidine blue. Immunofluorescence staining and subsequent analysis of paraffin-embedded tissue sections were conducted as described in previous study [8]. Rabbit anti-mouse filaggrin antibody (Ab) (905804; BioLegend) and rat anti-mouse CD4 Ab (#14-9766-82; Invitrogen) was used according to manufacturer's protocol. Sections were stained with primary antibodies of interest, followed by Alexa Fluor 488–conjugated secondary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and sections were mounted using ProLong Gold Antifade Reagent (Life Technologies). Fluorescence images were captured using an FV10i-DOC confocal laser-scanning microscope (Olympus). Positive cells stained with toluidine blue and CD4 in the dermis were counted in six randomly chosen microscopic fields using ImageJ software (version 1.52a).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

To access the mRNA expression levels in the AD-like skin lesions, whole skin samples were corrected on day 6 following treatment. Total RNA was extracted using the RNeasy Mini Kits (Qiagen) and subsequently reverse-transcribed into cDNA with GoScript Reverse Transcription System, following the manufacturer's instructions. Quantitative RT-PCR was carried out using the SYBR Green system (Applied Biosystems) on ABI 7300 real-time PCR platform (Life Technologies). Primers and SYBR probes were obtained from Sigma and TAKARA BIO INC. Expression of 18S rRNA was used as an internal reference, and relative gene expression levels were calculated using the comparative Ct method.

Statistics

P values were calculated using the Mann–Whitney U test for human data, and the unpaired Student's t-test or one-way ANOVA followed by Tukey's multiple comparisons test or Bonferroni's *post hoc* test for mouse data, using GraphPad Prism software (Version 9). Error bars represent the standard error of the mean (SEM), and the number of experiments (n) is indicated in the figure legends.

Result

First, we measured TEWL to evaluate changes in skin barrier function before and after topical application of difamilast. Result showed that topical application of difamilast significantly decreased TEWL, with six out of seven patients showing a decrease and one patient experiencing worsening (Figures 1A,B).

We next investigated the suppressive effect of topical application of difamilast of MC903-induced AD-like dermatitis mouse model. The dermatitis score was significantly decreased by difamilast at day 6 compared with those in control mice (Figures 2A,B). In addition, the elevated TEWL value in MC903-treated mice was significantly reduced by difamilast on day 6 after treatment (Figure 2C).

Next, we performed histological analyses using normal skin and MC903-treated dermatitis skin. Results showed that difamilast suppresses MC903-induced epidermal thickening and infiltration of inflammatory cells in the dermis (Figure 3A). Then, we examined the filaggrin expression in the lesional skin site by immunofluorescence staining. Results revealed that difamilast improved the impaired filaggrin expression caused by MC903 (Figure 3B). Next, we assessed the infiltration of inflammatory cells in the lesional skin. Toluidine blue staining showed that the increased number of mast cells in MC903-treated control mice significantly decreased in difamilast-treated mice (Figure 3C). Immunofluorescence revealed that the number of $CD4^+$ T-cells was also significantly decreased in difamilast-treated group compared to that in control mice in MC903 treated mice (Figure 3D).

Finally, we extracted RNA from the lesional skin and analyzed gene expression at the mRNA level using quantitative PCR. Result showed that mRNA expression of filaggrin and Interleukin (IL)-4 was elevated in MC903-treated control mice, but it was significantly reduced by difamilast (Figure 4). Increased mRNA expression of TSLP and IL-13 was observed in the MC903-treated groups compared with that in stable state; however, there was no significant difference between the control group and the difamilast-treated group in the MC903-treated model (Figure 4). These results suggest that difamilast may not protect keratinocytes from MC903-induced inflammation, and that its anti-inflammatory effect may be more closely associated with IL-4 rather than IL-13.

Discussion

In this study, we investigated the effects of barrier function improvement in human atopic dermatitis and the MC903 topical

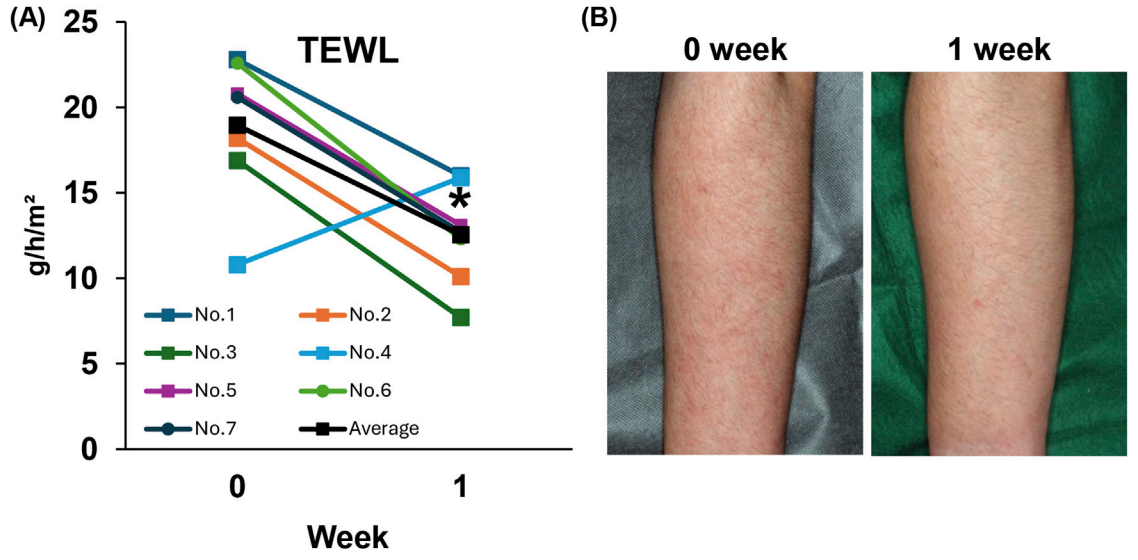

FIGURE 1
(A) Trans Epidermal Water Loss (TEWL) in patients with atopic dermatitis. n = 7. **(B)** Representative images of skin manifestation at day 7 in mice treated with MC903 and treated with vaseline or 1% difamilast. Statistical analysis was performed by Wilcoxon matched-pairs signed rank test.

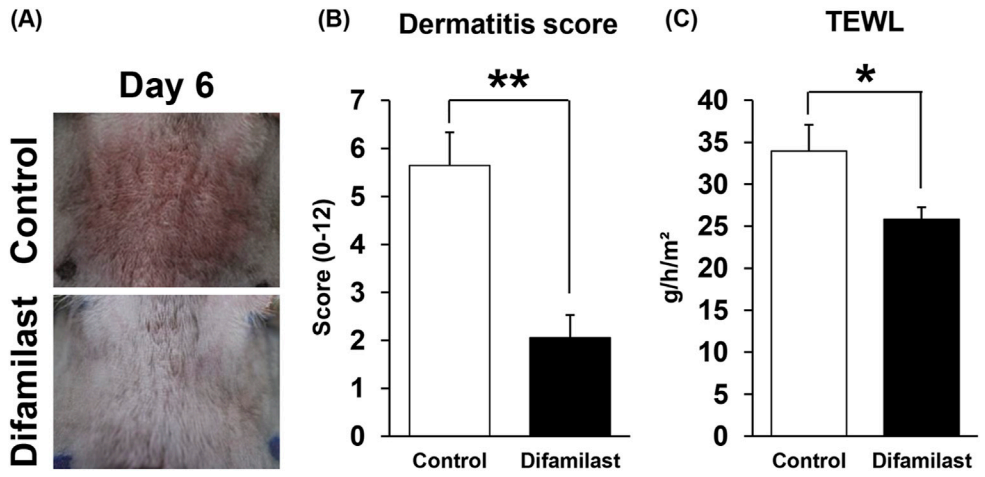

FIGURE 2
(A) Representative images of dermatitis skin in each groups. **(B)** Cumulative dermatitis score and **(C)** TEWL at day 6 in each group. n = 9 for each. Statistical analysis was performed by Mann Whitney test. Values represent mean ± standard error of the mean. *$p < 0.05$, **$p < 0.01$.

application-induced model. In double-blind vehicle-controlled study, topical application of difamilast ointment 1% showed significantly higher value of improvement of investigator global assessment score than with the vehicle at week 4 (38.46% vs. 12.64%, respectively, P < 0.0001). Moreover, significant improvements of least-square (LS) mean percent change in overall EASI score from the baseline was observed at week 1. These rapid improvements support our result of improvement of TEWL at week 1. PDE4 is involved in various cells, including epidermal cells, fibroblasts, and inflammatory cells, difamilast is thought to exert multiple effects [9]. Recent report has identified a novel mechanism by which difamilast regulates the expression of filaggrin and loricrin in human keratinocytes via cAMP-responsive element binding protein (CREB) and keratinocyte proline-rich protein (KPRP) axis [5]. The disruption of the skin barrier by harmful substances

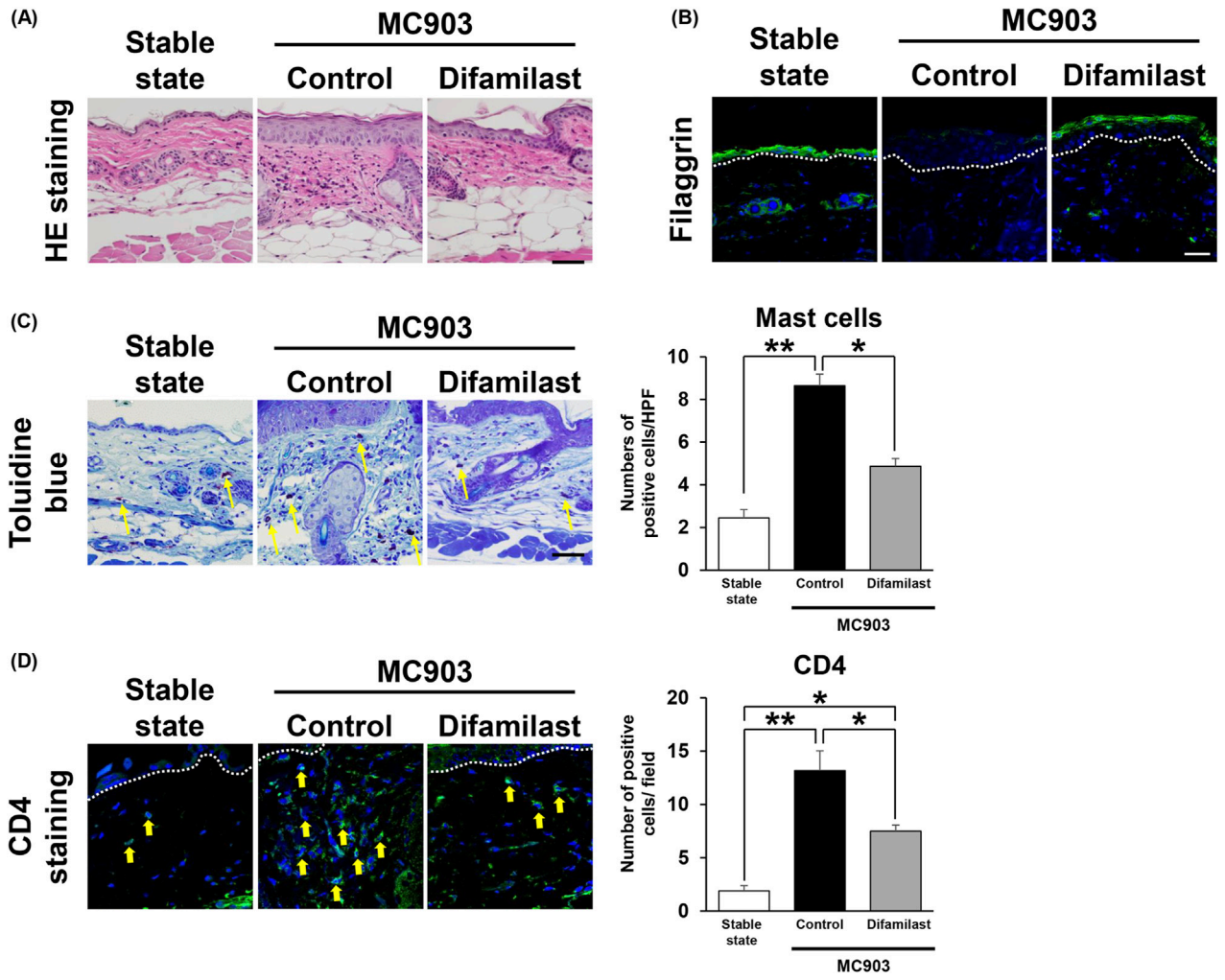

FIGURE 3
(A) Representative images of H&E staining of skin sections in stable state and at day 6 in mice. Scale bar = 50 μm. **(B)** Representative immunofluorescence staining image of filaggrin of skin in stable state and at day 6. Scale bar = 50 μm. **(C)** Representative images of toluidine blue staining of skin in stable state and at day 6. n = 3 for stable state and n = 5 for control and difamilast. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's *post hoc* test. Arrows indicate positive cells (yellow). **(D)** Representative immunofluorescence staining image of CD4 of skin in stable state and at day 6. Scale bar = 25 μm. n = 3 for stable state and n = 4 for control and difamilast group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparisons test. Arrows indicate positive cells (yellow).

results in the increased expression of alarmins, including TSLP, IL-25, IL-33, and type 2 chemokines. These alarmins activate ILC2s and Th2 cells, initiating type 2 inflammatory responses [10]. Previous study revealed that elevated TSLP expression was observed from Day 2 after topical application of MC903 [6]. In our study, TSLP expression and dermatitis scores were assessed only on Day 6, which may be insufficient for a comprehensive evaluation. To fully clarify the inhibitory effect of difamilast on TSLP production from MC903-induced epidermal cells, it is necessary to evaluate multiple time points.

Results showed that difamilast treatment decreased the number of infiltrating $CD4^+$ T-cells. IL-4 is well known to induce barrier dysfunction in atopic dermatitis, and previous studies have shown that PDE4 inhibitors reduce IL-4 production in PBMCs derived from patients with atopic dermatitis *in vitro* [11]. IL-13 also is known to play a key role in inducing barrier dysfunction in AD. IL-13 is produced by both $CD4^+$ T-cells and ILC2 in lesional skin site. The lack of reduction in IL-13 expression following difamilast application may be due to the absence of an inhibitory effect of difamilast on IL-13 production or a limited impact on ILC2 activity. However, further evaluation is necessary to clarify this point. Mast cell activation and migration are primarily regulated by stem cell factor (SCF), while chemokines such as CCL2, CXCL8, and CXCL12 have also been shown to play contributory roles [12, 13]. Apremilast, an oral phosphodiesterase 4 (PDE4) inhibitor, has been shown to restore CCL2 expression to normal levels in a

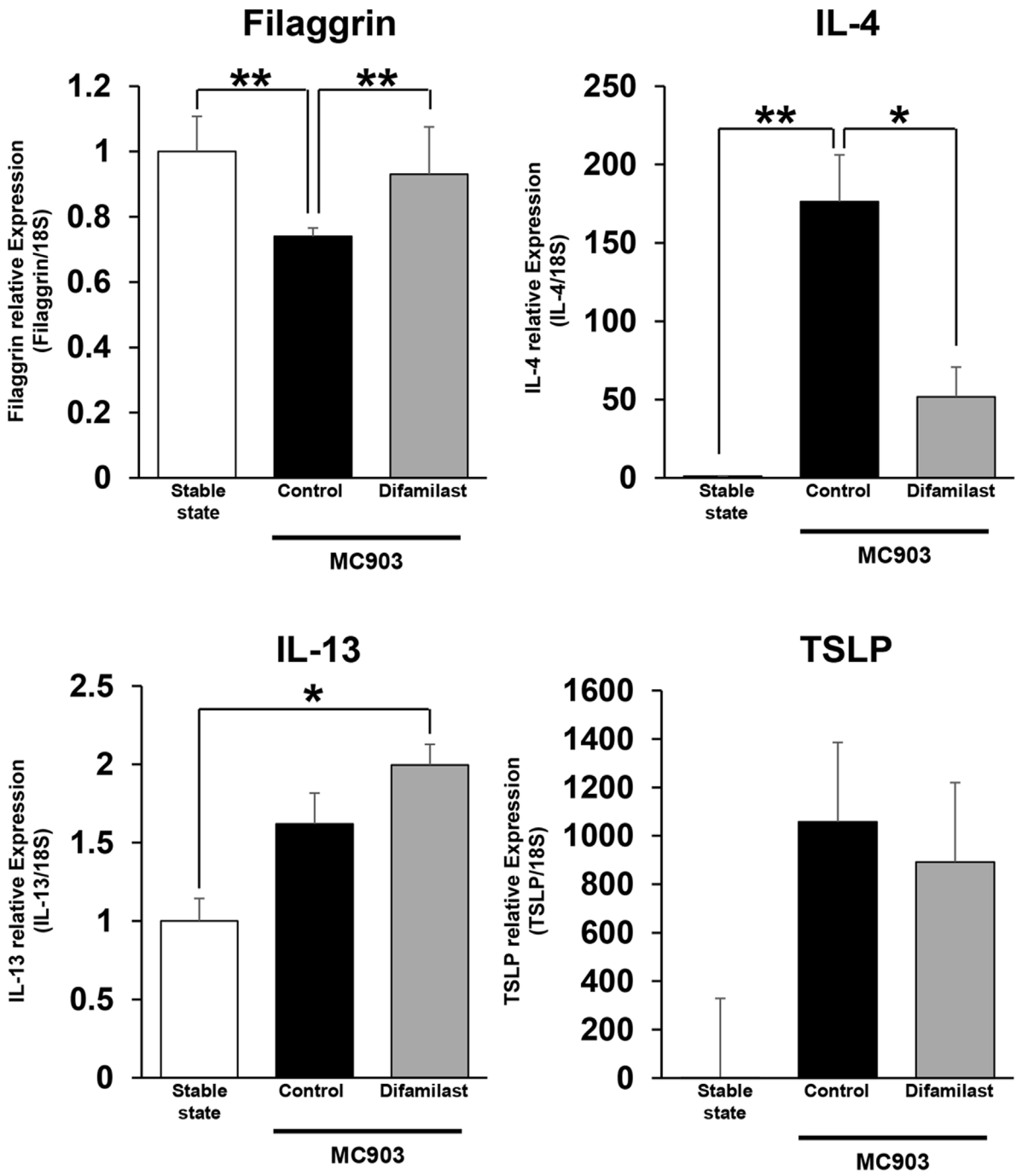

FIGURE 4
mRNA levels of filaggrin, IL-4, IL-13 and TSLP in the stable state and lesional skin on day 6. n = 3-5 for filaggrin, IL-4, TSLP, and IL-13 in each group. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons test.

DNCB-induced murine model of atopic dermatitis [14]. Cilomilast, a phosphodiesterase-4 (PDE4) selective inhibitor, has suppressive effects on RV16-induced ICAM-1 and CXCL8 expression [15]. These results indicate that PDE4 inhibitors might suppressed the infiltration of mast cells via suppressing those cytokines and chemokines. These combined effects suggest that difamilast may improve barrier dysfunction *in vivo* in both humans and mice.

There are several limitations in this study. It was very small numbers of samples, short durations, non-blinded, and non-controlled designs in human experiments. With regard to animal

studies, we have not examined models other than the MC903-induced model. It should also be taken into account that there are substantial differences in transdermal drug absorption rates between humans and mice. Therefore, further research is needed to elucidate the mechanisms by which difamilast regulates barrier dysfunction in AD *in vivo*.

In conclusion, our results demonstrate the potential of difamilast to improve barrier dysfunction in AD. However, further investigation is required for long-term evaluation and control study for human AD.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://figshare.com/articles/figure/Data_for_manuscript_/30112927.

Ethics statement

The studies involving humans were approved by Institutional Review Board of Gunma University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Gunma University Animal Care and Experimentation Committee. The study was conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

AUc and S-IM conducted the design. AUc, BT, and KK carried out the studies and analysis of the data. AU wrote the manuscript with support from MI, AUe, MS, and KR. S-IM confirmed the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

S-IM serves as Editor-in-Chief of the Journal of Cutaneous Immunology and Allergy.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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