Differences in therapeutic effects of topically applied corticosteroid and tacrolimus on atopic dermatitis-like symptoms in NC/Nga mice.

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This work was supported by a grant from Strategic Research Foundation Grant-aided Project for Private Universities from MEXT (Grant Number: S1311011).
The authors have no conflict of interest to declare.

Text word count: 3600 words

Number of references, tables, and figures: 38, 1, 5, respectively
Abstract

Background: Topical corticosteroid and calcineurin inhibitor have similar therapeutic benefits in atopic dermatitis (AD), but the differences in therapeutic mechanisms of action of these agents against AD symptoms are not fully understood.

Objective: This study was performed to examine the different effects of topical betamethasone valerate (BMV), clobetasol propionate (CBP), and tacrolimus (TAC) on itch-related behavior and dermatitis in NC/Nga mice with AD-like symptoms.

Methods: AD-like dermatitis was induced in the dorsal skin of NC/Nga mice by repeated topical application of Dermatophagoides farinae body (Dfb) ointment twice weekly for three weeks. Mice with dermatitis scores over 5 were divided into five groups with equal dermatitis scores and treated with BMV, CBP, TAC, or Vaseline (Vas) once daily for two consecutive days, or were not treated (NT). Scratching behavior was analyzed using a SCLABA®-Real system. Transepidermal water loss (TEWL) before and after treatment was measured using a Tewameter® TM210. Skin collected from each group was analyzed histologically.

Results: After the second treatment, dermatitis showed significantly greater improvement in the CBP and TAC-treated groups than in the Vas-treated and NT groups.
The numbers of scratching bouts were significantly lower in CBP and TAC-treated mice than in Vas-treated mice. TEWL was significantly lower in TAC-, but not in CBP-, treated mice than in Vas-treated mice. Immunohistochemical examination showed that BMV, CBP and TAC did not reduce the increased densities of epidermal protein gene product 9.5- and substance P-immunoreactive fibers. The numbers of dermal CD4-immunoreactive T cells were significantly lower in BMV and CBP-treated mice than in Vas-treated and NT mice. The numbers of dermal eosinophils were significantly lower in BMV, CBP and TAC-treated mice than in Vas-treated and NT mice, with CBP showing the strongest effect. CBP significantly reduced epidermal thickness compared with Vas and NT. There were no significant differences in the numbers of interleukin-31-immunoreactive cells and mast cells, or in expression of epidermal thymic stromal lymphopoietin among all five groups.

Conclusion: The therapeutic potency of TAC against AD-like symptoms, including pruritus, is equal to that of the corticosteroid CBP. Epidermal innervation of sensory nerves itself might not be related to the therapeutic effects of topical tacrolimus and corticosteroids in its early phase.
Key words: atopic dermatitis, corticosteroid, epidermal nerve fiber, pruritus, tacrolimus

Abbreviations: AD, atopic dermatitis; BMV, betamethasone valerate; BSA, bovine serum albumin; CBP, clobetasol propionate; DAPI, 4',6-diamidino-2-phenylindole; Dfb, Dermatophagoides farinae body; EMCSK, Eosinophil-Mast cell Stain Kit; HE, hematoxylin-eosin; IFN, interferon; IL, interleukin; NDS, normal donkey serum; NGF, nerve growth factor; NT, not treated; O.C.T., optimal cutting temperature; PBS, phosphate-buffered saline; PBS-T, PBS containing Tween 20; PFA, paraformaldehyde; PGP9.5, protein gene product 9.5; Sema3A, semaphorin3A; SD, standard deviation; SDS, sodium dodecyl sulfate; SP, substance P; TAC, tacrolimus; TB, toluidine blue; TCI, topical calcineurin inhibitor; TCS, topical corticosteroid; TB, toluidine blue; TEWL, transepidermal water loss; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; Vas, vaseline.
1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease resulting from skin barrier dysfunction and leading to eczematous and itchy lesions at the flexural folds [1]. Itch causes skin barrier damage by inducing scratching, exposing antigen-presenting cells resident in the skin to external factors, such as allergens and bacterial and viral antigens. Activated antigen-presenting cells migrate to lymph nodes and prime naive T cells to transform to Th2 cells. Elevated Th2 cytokines, along with tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), further damage skin barrier functions, leading to increased scratching [2]. Histamine is the best known pruritogen, being a main target in antipruritic therapies [3, 4], but antihistamines (i.e. histamine H1 receptor antagonists) are not usually effective in controlling pruritus of AD [5]. Therapeutic strategies are therefore needed to relieve the vicious cycle of itching and scratching in patients with AD.

Clinically, topical corticosteroids (TCSs) are regarded as first line therapy for exacerbations of AD. The great advantage of TCSs is their rapid and high degree of effectiveness, due to their potent antiinflammatory and antiproliferative effects on inflammatory cells, keratinocytes, endothelial cells and fibroblasts. Molecularly, TCSs
block the production of inflammatory cytokines, including interleukins (IL)-2, -3, -4, and -5; IFN-γ; TNF-α; and granulocyte macrophage colony-stimulating factor [6]. TCSs also have side effects, such as skin atrophy, telangiectasia, striae, hyper- and hypopigmentation, and tachyphylaxis [7]. In extreme cases, excessive overuse of TCS may cause hypothalamic-pituitary-adrenal suppression, resulting in Cushing’s syndrome [8].

Tacrolimus (TAC) was developed as an oral drug to prevent transplant rejection. TAC binds to intracellular FK506-binding protein and suppresses calcineurin activity. The antiinflammatory effects of TAC are due to its inhibition of T-helper activity and its downregulation of the production of proinflammatory cytokines, such as IL-2, -3, and -4; IFN-γ and TNF-α [9,10]. These properties of TAC have led to the use of topical calcineurin inhibitors (TCIs) as primary antiinflammatory agents to treat AD [6]. In addition, randomized controlled clinical trials have shown that the efficacy of 0.1% TAC ointment for AD is almost equivalent to that of a strong class corticosteroid, such as betamethasone valerate (BMV) ointment [11-14]. Although both TCSs and TCIs have similar therapeutic benefits in AD, the differences in therapeutic mechanisms of action of these agents against AD symptoms are not fully understood.
This study was therefore designed to investigate the effects of topical BMV; topical CBP, a more potent corticosteroid than BMV; and TAC on itch-related scratching behavior and dermatitis in NC/Nga mice, a mouse model of AD [15]. Factors assessed included dermatitis; scratching behavior; skin barrier function; epidermal nerve fiber density; levels of expression of epidermal nerve growth factor (NGF), semaphorin 3A (Sema3A), and itch-related cytokines such as thymic stromal lymphopoietin (TSLP) and IL-31; acanthosis, and infiltration of dermal inflammatory cells.
2. Materials and methods

2.1. Animals

Male NC/Nga mice, aged 10-12 weeks (Charles River Japan, Yokohama, Japan) were kept in an animal room under conditions of controlled temperature (22–24°C), humidity (50 ± 5%) and light (lights on from 8:00–20:00). Food and tap water were provided ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at Juntendo University Graduate School of Medicine.

2.2. Reagents

Vaseline (Vas) was obtained from Maruishi (Osaka, Japan), *Dermatophagoides farinae* body (Dfb) ointment from Biostir Inc. (Kobe, Japan), 0.12% BMV ointment (Rinderon®V) from Shionogi & Co., Ltd. (Osaka, Japan), 0.05% CBP ointment (Dermovate®) from GlaxoSmithKline (MDX, UK), and 0.1% TAC ointment (Protopic®) from Astellas Pharma Inc. (Tokyo, Japan). Optimal cutting temperature (O.C.T.) compound was from Sakura Finetechanical Co., Ltd. (Tokyo, Japan), normal donkey serum (NDS) from Merck Millipore Corp. (Darmstadt, Germany), bovine serum albumin (BSA) from Sigma-Aldrich. (St. Louis, MO, USA), and Vectashield® mounting
medium from Vector Laboratories, Ltd. (Peterborough, UK). Sevoflurane was obtained from Abbott Japan (Osaka, Japan), somnopentyl from Kyoritsu Seiyaku Co. (Tokyo, Japan), Eosinophil-Mast cell Stain Kits (EMCSK) from Diagnostic BioSystems (Pleasanton, CA, USA); and 0.05% toluidine blue (TB, pH 4.1) and hematoxylin-eosin (HE) solution from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.3. Antibodies

Rabbit anti-protein gene product 9.5 (PGP9.5; 1:400 dilution) was from Enzo Life Science, Inc. (Farmingdale, NY, USA); rabbit anti-NGF (1:500 dilution), rabbit anti-TSLP (1:1000 dilution), and rat anti-substance P (SP; 1:100 dilution) were from Merck Millipore Corp.; rabbit anti-Sema3A (1:200 dilution) was from Abcam Inc. (Cambridge, MA, UK); rabbit anti-IL-31 (1:500 dilution) was from Sigma-Aldrich; and rat anti-CD4 (1:50 dilution) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were from Molecular Probes (Eugene, OR, USA).

2.4. Induction of dermatitis in NC/Nga mice
Dermatitis was induced in NC/Nga mice as described [16]. Briefly, the rostral part of each mouse was shaved with an electric shaver, and residual hair was depilated using a hair removal cream. Two hours later, 100 mg of Dfb ointment was applied topically to the shaved area of each mouse (antigen sensitization). Twice weekly for three weeks, growing hair was shaved with the electric shaver, followed by application of 150 µL of 4% sodium dodecyl sulfate (SDS) to the treated area (barrier disruption). Dfb ointment was applied two hours after barrier disruption (Fig. 1a and Fig. S1a). As a negative control, one group of mice was treated in the induction phase only with 4% SDS (not with Dfb) twice weekly for three weeks (SDS group).

2.5. Treatment of AD-like dermatitis

After three weeks of Dfb application, mice with dermatitis scores above 5 were selected and divided into five groups of 8–16 mice each and treated with 100 mg topical Vas, 0.12% BMV ointment, 0.05% CBP ointment, or 0.1% TAC ointment once daily for 2 to 4 days (treatment phase), or were not treated (NT).

2.6. Measurement of clinical skin score
Dermatitis scores of AD-like skin lesions were evaluated as described [17]. Briefly, the severity of dermatitis was assessed according to four symptoms: (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema and (4) excoriation/erosion. Each symptom was scored from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3). Clinical skin score was defined as the sum of the individual scores, ranging from 0 to 12.

2.7. Measurement of transepidermal water loss (TEWL)

TEWL was measured under sevoflurane anesthesia using a Tewameter® TM210 (Courage & Khazawa, Cologne, Germany), as described [18].

2.8. Evaluation of scratching behavior

Scratching behavior of each mouse after the second treatment was recorded using a SCLABA®-Real system (Noveltex Inc., Kobe, Japan), as described [15]. After an acclimation period of at least 1 hour, the behavior of each mouse was recorded for 2 hours with no experimenters present in the observation room. The number of scratching bouts was defined as the number of periodic lower limb movements lasting more than 150 milliseconds each [19]. The number of scratching bouts in the NT group was
regarded as the data of scratching behavior before the start of treatment.

2.9. Immunohistochemical and histological analyses

Following the second treatment, dorsal skin was removed from each mouse under somnopentyl anesthesia. Half of each skin sample was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hours. After washing with phosphate-buffered saline (PBS, pH 7.4), small pieces of skin were immersed in PBS containing 20% sucrose overnight at 4°C and then embedded in O.C.T. compound and frozen on dry ice.

For Sema3A staining, the other half of each skin sample was embedded in O.C.T. compound without fixation. Cryosections were made using a CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) at thicknesses of 20 μm for staining for PGP9.5 and SP, and 8 μm for staining for NGF, Sema3A, CD4, IL-31 and TSLP. Only the sections stained for Sema3A were fixed in ice-cold acetone for 10 minutes at –20°C. All sections were washed with PBS containing 0.05% Tween 20 (PBS-T), blocked by incubation in PBS containing 5% NDS, 2% BSA and 0.2% Triton X-100 (blocking solution) and incubated with primary antibodies overnight at 4°C in this order. On the next day, the sections were washed with PBS-T, incubated with secondary antibodies for one hour at
room temperature, washed again with PBS-T, and mounted in Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI). The immunoreactivities of PGP9.5, SP, NGF, Sema3A and TSLP were assessed by confocal laser scanning microscopy (DMIRE2; Leica Microsystems). The immunoreactivities of CD4 and IL-31 were assessed by BZ-9000 (Keyence, Osaka, Japan).

For histological analyses, cryosections 8 μm thick were stained with Eosinophil-Mast cell Stain Kits (EMCSK) according to the manufacturer’s protocol, toluidine blue (TB), and hematoxylin-eosin (HE), to determine eosinophil and mast cell densities and epidermal thickness. These specimens were assessed by BZ-9000 (Keyence).

2.10. Semi-quantitative measurements

The numbers of cutaneous CD4-immunoreactive (CD4⁺) T cells, IL-31⁺ cells, and mast cells in each group were expressed as the means in six random low power fields (×200) per mouse. Eosinophils in EMCSK-stained sections were expressed as the means in six random high power fields (×400) per mouse. Epidermal thickness was measured in six random low power fields (×200) per mouse. All of these measurements
were performed using BZ-H2A software (Keyence).

To semi-quantitatively measure the fluorescence intensity of NGF, Sema3A, and TSLP in epidermis samples, six random high power fields (×400) per mouse were observed using a confocal laser-scanning microscope, with exposure and acquisition settings that avoided signal saturation. The sum of the fluorescence intensity and area of the epidermis in each field was determined using Leica Confocal Software (Leica).

To semi-quantify the numbers of epidermal PGP9.5-immunoreactive (PGP9.5⁺) and SP-immunoreactive (SP⁺) fibers, six specimens from each mouse were stained with the above-mentioned primary and secondary antibodies, and optical sections 0.9 µm thick were scanned through the z-plane by confocal microscopy. Three-dimensional images were reconstructed using Leica Confocal Software. The numbers of nerve fibers penetrating into the epidermis and the numbers of intraepidermal nerve fibers per high power field (×400) were counted as described [20, 21]. The numbers in six fields per mouse were averaged and analyzed statistically.

2.11. Statistical analyses

All results are expressed as the mean ± standard deviation (SD). Groups were
compared by unpaired t-tests or analysis of variance (ANOVA), followed by Bonferroni’s multiple comparison test. All statistical analyses were performed using Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA), with $P < 0.05$ defined as statistically significant.
3. Results

3.1. Effects of topical corticosteroid and TAC on dermatitis, scratching behavior, and TEWL

We examined whether topical application of BMV, a strong class corticosteroid, or TAC affects dermatitis and TEWL in NC/Nga mice, to which Dfb was applied repeatedly (Fig. S1a). After the second treatment, the dermatitis score was significantly lower in the TAC-treated group than in the Vas-, BMV-treated, and NT groups (Fig. S1b). After the fourth treatment, the TAC-treated group continued to show greater improvement of AD-like dermatitis (Fig. S1b). After the first treatment, TEWL was significantly lower in the TAC than in the Vas and BMV-treated groups (Fig. S1c). After the third treatment, TEWL was significantly lower in the TAC than in the Vas-treated group (Fig. S1c).

We next focused on early therapeutic effects of TAC on AD-like dermatitis, and then examined whether or not topical application of CBP, a strongest class corticosteroid, and TAC have similar therapeutic effects on AD-like dermatitis (Fig. 1a). We found that dermatitis score, TEWL, and numbers of scratching bouts were significantly higher in the NT group than in the SDS group (Fig. 1b-e). After the second
treatment, the dermatitis scores in the CBP- and TAC-treated groups were significantly lower than in the NT and Vas-treated groups (Fig. 1b and c). TEWL was significantly lower in the TAC than in the Vas group but significantly higher in the CBP than in the NT and Vas groups (Fig. 1d). The numbers of scratching bouts were significantly lower in the CBP and TAC groups than in the Vas and BMV groups (Fig. 1e).

The levels of epidermal expression of skin barrier-related genes, such as filaggrin and loricrin, were examined by quantitative RT-PCR. The level of filaggrin mRNA in the epidermis was significantly higher in the TAC than in the NT and Vas-treated groups (Fig. S2a). The level of loricrin mRNA in the epidermis was unchanged among the five groups (Fig. S2b).

3.2. Effects of topical corticosteroid and TAC on epidermal nerve densities

The distribution of epidermal nerve fibers was examined immunohistochemically in each group of mice using antibodies to PGP9.5 and SP. The numbers of PGP9.5+ and SP+ fibers penetrating into the epidermis were significantly higher in the NT than in the SDS group (Fig. 2a-f). However, treatment with BMV, CBP, and TAC did not reduce the numbers of epidermal PGP9.5+ and SP+ fibers (Fig. 2a-f).
3.3. Effects of topical corticosteroid and TAC on epidermal NGF and Sema3A expression

Epidermal expression of NGF and Sema3A was examined immunohistochemically with the respective antibodies. Epidermal expression of NGF was higher in the NT than in the SDS group, and was significantly higher in the CBP than in the Vas group (Fig. 3a and b). Epidermal expression of Sema3A was significantly lower in the NT than in the SDS group, and was significantly higher in the TAC than in the NT group (Fig. 3c and d).

3.4. Effects of topical corticosteroid and TAC on infiltrating inflammatory cells, acanthosis, and epidermal TSLP expression

The numbers of dermal CD4$^+$ T cells, IL-31$^+$ cells, mast cells, and eosinophils, and epidermal thicknesses were higher in the NT group than in the SDS group, but there were no significant inter-group differences in the fluorescence intensity of TSLP (Fig. 4a-f).

The number of dermal CD4$^+$ T cells was significantly lower in the BMV and CBP
groups than in the TAC, NT and Vas groups (Fig. 4a). The numbers of dermal IL-31+ cells and mast cells did not differ significantly among the five groups (Fig. 4b and c). The numbers of eosinophils in the dermis were significantly lower in the BMV, CBP, and TAC groups than in the NT and Vas groups (Fig. 4d). Epidermal thickness was significantly lower in the CBP group than in the NT and Vas groups (Fig. 4e). The level of TSLP expression in the epidermis was similar among the five groups (Fig. 4f).
4. Discussion

The present study showed that topical corticosteroids, such as BMV and CBP, and TAC have different therapeutic effects on AD-like symptoms in Dfb-induced NC/Nga mice (Table 1). During the early treatment phase, TAC, but not BMV, reduced dermatitis scores, scratching bouts, and TEWL. In contrast, CBP reduced dermatitis scores and scratching bouts. The efficacy of CBP in treating dermatitis and scratching behavior was similar to that of TAC, suggesting that the therapeutic potency of TAC against the symptoms of AD is equal to the strongest corticosteroids such as CBP.

Although TAC and CBP reduced scratching behavior after the second treatment (Fig. 1e), neither reduced epidermal nerve densities, including SP+ fibers (Fig. 2). We also showed that TAC treatment significantly upregulated Sema3A compared with NT (Fig. 3d), and that CBP-treatment significantly increased NGF expression compared to VAS-treatment (Fig. 3b). Yamaura et al demonstrated, using repeated hapten induced dermatitis model, that the levels of NGF mRNA in the ears of the mice were increased by topical application of dexamethasone [22]. Therefore, these findings suggest that the imbalance of NGF and Sema3A expressions in the epidermis is not normalized after the treatment in TAC- and CBP-treated groups. This might be explained by the fact that
epidermal innervation was not improved. We also analyzed itch-related cytokines, such as IL-31 [23, 24] and TSLP [25, 26]. TAC and CBP did not affect the numbers of dermal IL-31$^+$ cells or epidermal TSLP levels (Fig. 4). Calcineurin inhibitors such as TAC have been shown to attenuate neuronal functions, such as synaptic vesicle recycling and calcium currents [27, 28]. Moreover, TAC treatment was shown to deplete SP, which is involved in neurogenic inflammation and/or enhancement of itch [29], as well as to attenuate itch sensation through desensitization of TRPV1 [27]. Kido et al demonstrated by hapten-induced dermatitis mouse model that scratching behavior did not necessarily correlate with epidermal nerve fiber sprouting or inflammatory cell infiltration [30]. Therefore, topical TAC may exert immediate antipruritic effects, by inhibiting sensory nerve functions such as SP release and calcium influx, rather than by reduction of epidermal nerve fibers.

BMV and CBP also reduced the infiltration of inflammatory cells such as CD4$^+$ T cells and eosinophils (Fig. 4a, d). CBP was more effective in improving acanthosis than were BMV and TAC. Epidermal keratinocytes can produce some pruritogens such as leukotriene B4 [31, 32]. Thus, these findings suggest that the antiscratching activity of CBP is related to the improvement of acanthosis. In contrast, BMV failed to reduce the
number of scratching bouts, regardless of a decrease in infiltration of inflammatory cells, such as CD4+ T cells and eosinophils.

We found that TAC treatment improved dermatitis, concomitant with a decrease in the number of dermal eosinophils, although its inhibitory effect on inflammatory cells was less than that of topical corticosteroids. BMV reduced the infiltration of inflammatory cells, but did not improve dermatitis in Dfb-NC/Nga mice. Removing the claws of NC/Nga mice, thus preventing scratching, successfully inhibited the induction and aggravation of dermatitis [33]. Recently, epicutaneous allergic sensitization was promoted by interaction between allergen protease activity and mechanical skin barrier damage [34]. In addition, scratching induces the release from sensory nerve terminals of SP [35], which is involved in the development and aggravation of skin inflammation. Thus, these findings suggest that topical TAC improves dermatitis by suppressing scratch-evoked mechanical stimuli rather than by inhibiting the infiltration of inflammatory cells. This mechanism of action may also be applicable to corticosteroids such as CBP.

After induction of AD-like symptoms in NC/Nga mice, filaggrin expression was higher in the NT than in the SDS group (Fig. S2a). Similarly, a recent study showed that
filaggrin expression was not decreased in NC/Nga mice with AD-like symptoms [36]. In contrast, the expression of *filaggrin* mRNA was significantly higher in the TAC group than in the NT and Vas groups (Fig. S2a). Thus, TAC may improve skin barrier function through upregulating the expression of *filaggrin* mRNA.

CBP treatment increased TEWL (Fig. 1d). S.E. Lee et al reported that CBP reduced the levels of tight junction proteins, such as claudin-1 and -4 and occludin, and their mRNAs. In contrast, TAC did not affect the levels of expression of claudin-1 and -4 mRNA and protein, although TAC downregulated occludin mRNA and protein to a lesser extent than did CBP [37]. This might constitute one of the mechanisms by which topical CBP increases TEWL.

In conclusion, the results of the present study suggest that the therapeutic potency of TAC against AD-like symptoms including pruritus is as high as that of strong corticosteroids such as CBP. Epidermal innervation of sensory nerves itself might not be related to the therapeutic effects of topical tacrolimus and corticosteroids in its early phase.
Acknowledgements

This work was supported by a grant from Strategic Research Foundation Grant-aided Project for Private Universities from MEXT (Grant Number: S1311011).
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Figure Legends

Fig. 1. Effects of topical BMV, CBP and TAC ointment on dermatitis, TEWL and scratching behavior in Dfb-treated NC/Nga mice.

(a) Experimental schedule for topical application of BMV, CBP and TAC. After the induction phase of AD-like symptoms, Vas, BMV, CBP or TAC was topically applied once daily for two consecutive days. During the treatment phase, dermatitis score and TEWL were evaluated just before each treatment and skin sampling. (b) Skin photographs taken at the third evaluation after the second treatment. (c) Effects of treatment on dermatitis score. Values are shown as means ± SD (n = 8 per group) and compared by two-way ANOVA with Bonferroni’s multiple comparison test. (d) Effects of treatment on TEWL. Values are shown as means ± SD (n = 8 per group) and compared by unpaired t-test or two-way ANOVA with Bonferroni’s multiple comparison test. (e) Number of scratching bouts after the second treatment. Values are shown as means ± SD (n = 8-14 per group) and compared by one-way ANOVA with Bonferroni’s multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001 (vs NT); †P < 0.05; ‡P < 0.01 (vs Vas); #P < 0.05 (vs SDS); *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: SDS, sodium dodecyl sulfate; NT, not treated; Vas, vaseline;
BMV, betamethasone valerate; CBP, clobetasol propionate; TAC, tacrolimus.

**Fig. 2.** Effects of topical BMV, CBP and TAC ointment on epidermal nerve densities in Dfb-treated NC/Nga mice.

(a) Immunolabeling of skin samples obtained after the second treatment with anti-PGP9.5 antibody (green). White and broken lines indicate the skin surface and the basement membrane, respectively. (b, c) Numbers of PGP9.5\(^+\) fibers penetrating into the epidermis (b) and within the epidermis (c); fiber densities were not lower in the BMV-, CBP-, and TAC-treated samples than in the control samples. (d) Immunolabeling of skin samples after the second treatment with anti-SP antibody (red). White and broken lines indicate the skin surface and basement membrane, respectively. (e, f) Numbers of SP\(^+\) fibers penetrating into the epidermis (e) and within the epidermis (f); fiber densities did not differ significantly among the treated and control groups. The nuclei in each panel were counterstained with DAPI (blue). Scale bars, 100 \(\mu\)m. All data are shown as means ± SD (\(n = 8\) per group) and compared by one-way ANOVA with Bonferroni’s multiple comparison tests. \(^#P < 0.05\) (vs SDS). Abbreviations: SDS, sodium dodecyl sulfate; NT, not treated; Vas, vaseline; BMV, betamethasone valerate, CBP, clobetasol propionate;
TAC, tacrolimus.

**Fig. 3.** Effects of topical BMV, CBP and TAC on epidermal expression of NGF and Sema3A in Dfb-treated NC/Nga mice.

(a) Immunolabeling of skin samples obtained after the second treatment with anti-NGF antibody (green). (b) Fluorescence intensity of NGF in the epidermis was significantly higher in the CBP than in the VAS-treated group. (c) Immunolabeling of skin samples obtained after the second treatment with anti-Sema3A antibody (green). (d) Fluorescence intensity of Sema3A in the epidermis was significantly higher in the TAC than in the NT group. White and broken lines in each panel indicate the skin surface and basement membrane, respectively. Scale bars, 100 μm. Data are shown as means ± SD (n = 8 per group) and compared by one-way ANOVA with Bonferroni’s multiple comparison tests. *P < 0.05 (vs SDS), *P < 0.05. Abbreviations: SDS, sodium dodecyl sulfate; NT, not treated; Vas, vaseline; BMV, betamethasone valerate; CBP, clobetasol propionate; TAC, tacrolimus.

**Fig. 4.** Effects of topical BMV, CBP and TAC on infiltrating immune cells, acanthosis,
and epidermal TSLP expression in the lesional skin of Dfb-treated NC/Nga mice after the second treatment.

(a) Immunolabeling of skin samples with anti-CD4 antibody. The number of dermal CD4\(^+\) T cells was significantly lower in the BMV- and CBP-treated groups than in the other three groups. (b) Immunolabeling of skin samples with anti-IL-31 antibody. There were no significant differences in the numbers of dermal IL-31\(^+\) cells among the five groups. (c) Staining of mast cells in the dermis with toluidine blue. There were no significant differences in the numbers of dermal mast cells among the five groups. (d) Staining of eosinophils infiltrating the dermis with EMCSK. The number of dermal eosinophils was significantly lower in the BMV, CBP and TAC group than in the NT and Vas groups. (e) Epidermal thickness measured in skin specimens stained with hematoxylin-eosin. Epidermal thickness was significantly reduced by CBP treatment. (f) Immunolabeling of skin samples with anti-TSLP antibody. The fluorescence intensity of TSLP did not differ significantly among the five groups. Values are shown as means ± SD (\(n = 8\) per group). Statistical analyses were performed by one-way ANOVA with Bonferroni’s multiple comparison tests. \(^{#}P < 0.05\) (vs SDS), \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.001\). Abbreviations: SDS, sodium dodecyl sulfate; NT, not treated; Vas,
vaseline; BMV, betamethasone valerate; CBP, clobetasol propionate; TAC, tacrolimus.
Figure 1. Noguchi et al.

(a) Induction phase

- Dfb application
- Treatment (Yaseline, Betamethasone valerate, Clobetasol propionate, Tacrolimus)
- Evaluation (Dermatitis score, TEWL)
- Sampling
- SCLABA (Scratching behavior)

2 times per week, total 3 weeks

(b) SDS  NT  Vas  BMV  CBP  TAC

(c) Dermatitis score vs time after treatment (day)

- SDS
- NT
- Vas
- BMV
- CBP
- TAC

(0.05 vs NT)

(0.05 vs Vas)

(d) TEWL (g/cm².h)

Before treatment

SDS  NT

(e) Improvement ratio of TEWL vs time after treatment (day)

VT  Vas  BMV  CBP  TAC

(0.05 vs NT)

(0.05 vs Vas)

(f) Scratching bouts per 2 hrs after 2nd time treatment

- SDS
- NT
- Vas
- BMV
- CBP
- TAC

(0.05 vs SDS)
Figure 2. Noguchi et al.

(a) PGP9.5

(b) Number of PGP9.5+ fibers penetrating into epidermis

(c) Number of PGP9.5+ fibers in epidermis

(d) SP

(e) Number of SP+ fibers penetrating into epidermis

(f) Number of SP+ fibers in epidermis
Figure 4. Noguchi et al.

(a) Number of CD4+ T cells

(b) Number of IL-31+ cells

(c) Number of mast cells

(d) Number of eosinophils

(e) Epidermal thickness (µm)

(f) Fluorescence intensity (TSLP/µm²)
**Table 1** Therapeutic effects of BMV, CBP and TAC on AD-like dermatitis

<table>
<thead>
<tr>
<th></th>
<th>BMV (vs Vas)</th>
<th>CBP (vs Vas)</th>
<th>TAC (vs Vas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dermatitis score</td>
<td>ns</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Scratching bouts</td>
<td>ns</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>TEWL</td>
<td>ns</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>PGP9.5 nerve fibers</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>SP nerve fibers</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>NGF expression</td>
<td>ns</td>
<td>↑</td>
<td>ns</td>
</tr>
<tr>
<td>Sema3A expression</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CD4 T cells</td>
<td>↓</td>
<td>↓</td>
<td>ns</td>
</tr>
<tr>
<td>IL-31 cells</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Mast cells</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Epidermal thickness</td>
<td>ns</td>
<td>↓</td>
<td>ns</td>
</tr>
<tr>
<td>TSLP expression</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

AD: atopic dermatitis; BMV: betamethasone valerate; CBP: clobetasol propionate; IL: interleukin; NGF: nerve growth factor; ns: no significance; NT: nontreated; PGP9.5: protein gene product 9.5; Sema3A: semaphorin3A; SP: substance P; TAC: tacrolimus; TEWL: transepidermal water loss; TSLP: thymic stromal lymphopoietin; Vas: vaseline; ↓: lower; ↑: higher.
Supplementary data

Supplemental materials and methods

Quantitative RT-PCR (qRT-PCR) analysis

Epidermal sheets from each mouse were collected after the second treatment, as described [1]. Total RNA was isolated from the epidermal sheets using RNeasy Fibrous Tissue Mini kits (Qiagen KK, Tokyo, Japan), according to the manufacturer’s instructions. The protocols for qRT-PCR analysis have been described [1]. The primers used in this study are listed in Table S1. The levels of gene expression were calculated relative to expression of ribosomal protein S18 (RPS18).

Supplemental figure

Fig. S1. Effects of topical BMV and TAC ointment on dermatitis and TEWL in Dfb-treated NC/Nga mice.

(a) Experimental schedule for topical application of BMV and TAC. After the induction phase of AD-like symptoms, Vas, BMV or TAC was topically applied once daily for four consecutive days. During the treatment phase, dermatitis score and TEWL were evaluated just before each treatment and the next day of the last treatment. (b) Effects of treatment on dermatitis score. Values are shown as means ± SD (n = 8 per group) and
compared by two-way ANOVA with Bonferroni’s multiple comparison test. (c) Effects of treatment on TEWL. Values are shown as means ± SD (n = 8 per group) and compared by two-way ANOVA with Bonferroni’s multiple comparison test. *P < 0.05, **P < 0.01 (vs NT); +P < 0.05, ++P < 0.01, +++P < 0.001 (vs Vas), #P < 0.05, ##P < 0.01 (vs BMV). Abbreviations: NT, not treated; Vas, vaseline; BMV, betamethasone valerate; TAC, tacrolimus.

**Fig. S2.** Effects of topical BMV, CBP and TAC on epidermal expression of (a) filaggrin and (b) loricrin mRNAs in Dfb-treated NC/Nga mice.

(a) The expression of filaggrin mRNA was significantly higher in TAC-treated than in Vas-treated and NT mice. (b) The expression of loricrin mRNA in the epidermis was unchanged among the five groups. All data are shown as means ± SD (n = 8 per group) and compared by one-way ANOVA with Bonferroni’s multiple comparison tests. *P < 0.05, **P < 0.01. SDS: sodium dodecyl sulfate; NT: not treated; Vas: vaseline; BMV: betamethasone valerate; CBP: clobetasol propionate; TAC: tacrolimus.

**Supplemental References**

Supplemental figure 1. Noguchi et al.

(a) Induction phase and Treatment phase

2 times per week, total 3 weeks

(b) Dermatitis score over time after treatment

(c) TEWL (g/cm²/h) over time after treatment
<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mFilaggrin</em></td>
<td>(forward) AGACTGGGGAGGCAAGCTACA</td>
</tr>
<tr>
<td></td>
<td>(reverse) CCTGCCTCCTTCAGAGTCAC</td>
</tr>
<tr>
<td><em>mLoricrin</em></td>
<td>(forward) TCATCTTCCCTGGTGCTTCA</td>
</tr>
<tr>
<td></td>
<td>(reverse) GAGGTCTTTTCCACAAACCCACA</td>
</tr>
<tr>
<td><em>mRPS18</em></td>
<td>(forward) CCAAGAAGGGAAGACGACTG</td>
</tr>
<tr>
<td></td>
<td>(reverse) CGGATGAACTGACTGAGCAA</td>
</tr>
</tbody>
</table>