INVESTIGATIVE REPORT

Possible Antipruritic Mechanism of Cyclosporine A in Atopic Dermatitis

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Cyclosporine A is an immunosuppressive agent that suppresses pruritus and is currently used in the treatment of patients with severe atopic dermatitis. The aim of this study was to elucidate the antipruritic mechanism of cyclosporine A using a mouse model of atopic dermatitis. Intraperitoneal injection of cyclosporine A (5 mg/kg) significantly reduced epidermal nerve density, number of scratching bouts, dermatitis scores, and transepidermal water loss, as well as decreasing the numbers of inflammatory cells in the dermis and decreasing epidermal thickness. Intraperitoneal injection of cyclosporine A dose-dependently inhibited increased itch-related receptor gene expression, such as interleukin-31 receptor A and neurokinin-1 receptor, in the dorsal root ganglion of atopic dermatitis model mice. Thus, the antipruritic efficacy of cyclosporine A may involve reduced epidermal nerve density and expression levels of itch-related receptor genes in the dorsal root ganglion, as well as improvement in acanthosis and reduction in cutaneous inflammatory cell number. ***Key words: atopic dermatitis; pruritus; cyclosporine A; epidermal nerve fibres.***

Accepted Dec 15, 2015; Epub ahead of print Dec 15, 2015

Acta Derm Venereol 2015; XX: XX–XX.

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Atopic dermatitis (AD) is a relapsing chronic inflammatory skin disease characterized by eczematous skin lesions and intense pruritus resulting in the desire to scratch frequently. Clinically, pruritus in patients with AD is often resistant to conventional treatments, such as histamine H_1 receptor antagonists. Such intractable itch is a clinical problem that reduces the quality of life in patients with AD (1–3).

Cyclosporine A (CsA) is an immunosuppressant drug widely used in the treatment of inflammatory diseases and for preventing rejection of allogeneic transplants. CsA forms a complex with the cytosolic protein cyclophilin. The CsA–cyclophilin complex inhibits calcineurin, which under normal conditions suppresses activation of T cells (4). Through the phosphorylation process, calcineurin affects nuclear factor of activated T cells (NFAT) signalling, which regulates thymic stromal lymphopoietin (TSLP) release by keratinocytes and TSLP is required for activation of sensory neurones, and thereby, itch sensation may be recognized in the brain (5). Clinically demonstrated low-dose oral CsA treatment markedly inhibits intractable pruritus in patients with severe AD (6). Therefore, low-dose CsA is currently used as a therapeutic regime in severe AD. A previous study indicated that low-dose oral CsA treatment reduced serum levels of interleukin 31 (IL-31), an itch-causing cytokine, in patients with AD, concomitant with relief of pruritus (7). However, the precise mechanism underlying the antipruritic effects of CsA is poorly understood.

The present study investigated the antipruritic mechanism of CsA in a *Dermatophagoides farinae* body (Dfb) ointment-induced AD-like mouse model (Dfb-NC/Nga). Here, we present evidence regarding the effects of CsA on epidermal nerve fibres, itch-related receptor gene expression in the dorsal root ganglion (DRG), infiltration of skin immune cells, and epidermal thickness in Dfb-NC/Nga mice after treatment with CsA.

MATERIALS AND METHODS (for complete details see Appendix S1[1])

Experimental design

Male NC/Nga mice were used in this study. The experimental schedule is shown in Fig. 1a. Induction of dermatitis was performed as described previously (8). The evaluation of clinical skin score for AD-like skin lesions was described previously (9). Scratching behaviour was observed as previously described (10). After 3 weeks of Dfb application, mice developing clinical skin scores above 5 were selected and randomly divided into 3 groups: vehicle treatment control group ($n = 7$), 1 mg/kg CsA treatment group ($n = 8$), and 5 mg/kg CsA treatment group ($n = 8$).

Tissue analyses

Skin samples were collected after the end of the 6th week in the treatment phase. The numbers of cutaneous $CD4^+$ T cells, $IL\text{-}31^+$ cells, mast cells in toluidine blue (TB)-stained sections, and eosinophils in direct fast scarlet 4BS (DFS)-stained sections were expressed as the means in 9 random fields (1.0×10^5 μm^2)

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-2318

© 2016 The Authors. doi: 10.2340/00015555-2318
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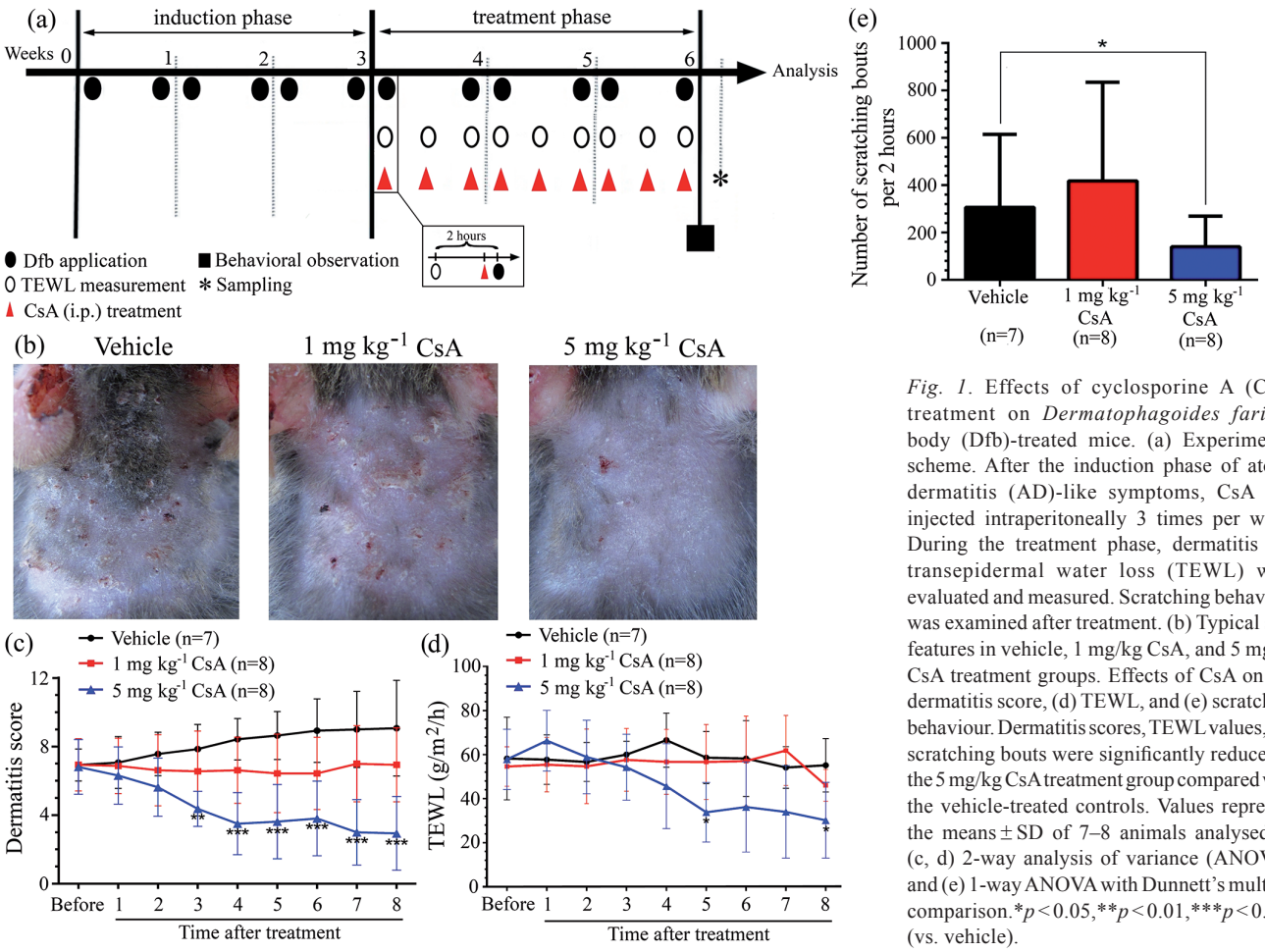


Fig. 1. Effects of cyclosporine A (CsA) treatment on *Dermatophagoides farinae* body (Dfb)-treated mice. (a) Experimental scheme. After the induction phase of atopic dermatitis (AD)-like symptoms, CsA was injected intraperitoneally 3 times per week. During the treatment phase, dermatitis and transepidermal water loss (TEWL) were evaluated and measured. Scratching behaviour was examined after treatment. (b) Typical skin features in vehicle, 1 mg/kg CsA, and 5 mg/kg CsA treatment groups. Effects of CsA on: (c) dermatitis score, (d) TEWL, and (e) scratching behaviour. Dermatitis scores, TEWL values, and scratching bouts were significantly reduced in the 5 mg/kg CsA treatment group compared with the vehicle-treated controls. Values represent the means ± SD of 7–8 animals analysed by (c, d) 2-way analysis of variance (ANOVA), and (e) 1-way ANOVA with Dunnett's multiple comparison. $*p<0.05$, $**p<0.01$, $***p<0.001$ (vs. vehicle).

per mouse. Upper to mid-cervical DRGs from each mouse under anaesthesia were collected after the end of the 6th week in the treatment phase. Culture of DRG neurones was performed in 12-well plates and neurite outgrowth assayed.

Statistical analysis

Statistical analyses were performed using the 2-tailed Student's *t*-test and 1-way or 2-way analysis of variance (ANOVA) with Bonferroni's or Dunnett's multiple comparison test using Prism 5 software (GraphPadSoftware, La Jolla, CA, USA). In all analyses, $p<0.05$ was taken to indicate statistical significance.

RESULTS

Effects of CsA on dermatitis, skin barrier, and scratching behaviour

In comparison with the vehicle control group, only the group treated with 5 mg/kg CsA showed significant improvement in dermatitis (Fig. 1b, c). TEWL values were significantly reduced in the 5 mg/kg CsA treatment group compared with vehicle-treated controls (Fig. 1d). The number of scratching bouts was significantly decreased in the 5 mg/kg CsA treatment group compared with vehicle-treated controls at the end of the treatment phase (Fig. 1e).

Effects of CsA treatment on epidermal nerve fibre density

Skin samples from each group were immunostained with anti-PGP9.5 (Fig. 2a) or anti-substance P (anti-SP) antibodies (Fig. 2b). CsA at a dose of 5 mg/kg significantly reduced the numbers of both PGP9.5-immunoreactive fibres (Fig. 2c) and SP-immunoreactive fibres (Fig. 2d) in the epidermis compared with vehicle-treated controls.

Effects of CsA on neurite outgrowth in cultured dorsal root ganglion neurones

In the presence of 10 ng/ml nerve growth factor (NGF), CsA dose-dependently inhibited NGF-induced neurite outgrowth in DRG neurones cultured for 3 days (Fig. 3a, b).CsA alone had no effect on neurite outgrowth in the absence of NGF (Fig. 3c).

Effects of CsA treatment on itch-related receptor gene expression in dorsal root ganglion, number of IL-31$^+$ cells and epidermal thymic stromal lymphoprotein levels of Dfb-NC/Nga mice

Expression of itch-related receptor genes was examined in DRG of Dfb-NC/Nga mice by quantitative reverse

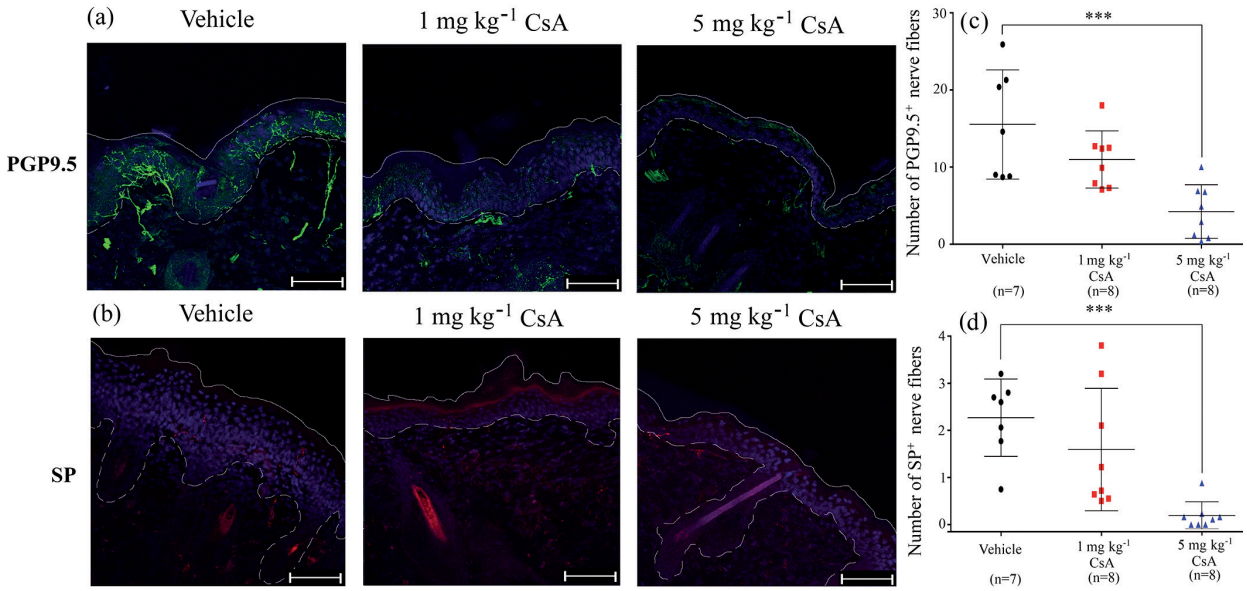


Fig. 2. Effects of cyclosporine A (CsA) treatment on epidermal nerve densities in lesional skin of *Dermatophagoides farinae* body (Dfb)-treated mice. The figure shows immunolabelling with anti-PGP9.5 (*green in panel a*) and substance P (SP, *red in panel b*) antibodies of intraepidermal nerve fibres on lesional skin after CsA treatment. *White and broken lines* indicate the skin surface and the border between the epidermis and dermis (basement membrane), respectively. *Scale bars* = 75 µm. Treatment with 5 mg/kg CsA significantly reduced the numbers of (c) PGP9.5- and (d) SP-immunoreactive fibres in the epidermis of Dfb-treated NC/Nga mice compared with vehicle-treated controls. Values represent the means±standard deviation (SD) of 7–8 animals and were compared by 1-way analysis of variance (ANOVA) with Bonferroni's comparison. ***$p < 0.001$ (vs. vehicle). *Scale bar* = 75 µm.

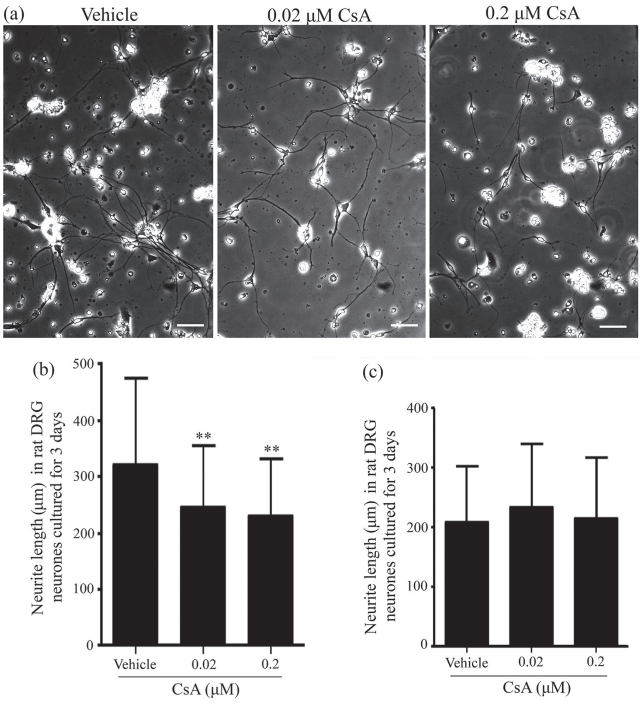


Fig. 3. Inhibition of nerve growth factor (NGF)-induced neurite out-growth in cultured dorsal root ganglion (DRG) neurones by cyclosporine A (CsA). (a) The length of the longest process was measured in DRG neurones incubated with vehicle, 0.02 µM CsA, and 0.2 µM CsA in the presence of NGF (10 ng/ml) for 3 days. (b) The neurite length in cultured DRG neurones was significantly reduced by CsA in the presence of NGF, whereas (c) there were no differences among the 3 groups in the absence of NGF. Values are expressed as the means ± standard deviation (SD) of 3 independent experiments, and were compared by 1-way analysis of variance (ANOVA) with Dunnett's multiple comparison. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$(vs. vehicle). *Scale bar* = 100 µm.

transcription PCR (qRT-PCR), and we found increased expression levels of some itch-related receptor genes, i.e. *MrgprA3, IL-31RA, PAR2, TRPA1, TGR5* and *NK1R*, in the DRG of Dfb-NC/Nga mice compared with controls (Table SI[1]). We next examined the effects of CsA treatment on increased itch-related receptor gene expression in the DRGs. The expression level of *IL-31RA* in the DRG was decreased in the 5 mg/kg CsA treatment group compared with vehicle-treated controls (Fig. 4a). In addition, CsA dose-dependently decreased the expression level of *NK1R* in the DRG of Dfb-NC/Nga mice compared with vehicle-treated controls (Fig. 4b). The number of IL-31$^+$ cells was significantly decreased in the skin of the 5 mg/kg CsA treatment group compared with vehicle-treated controls (Fig. 4c). There was no statistically significant difference in the fluorescence intensity of TSLP in the epidermis among the 3 groups (Fig. 4d).

Effects of CsA on infiltration of inflammatory cells and acanthosis

CD4$^+$ T cells, mast cells, eosinophil infiltration, and epidermal thickness of the skin were examined histologically in 3 groups, i.e. vehicle, 1 mg/kg CsA, and 5 mg/kg CsA. The numbers of CD4$^+$ T cells, mast cells, and eosinophils were significantly lower in the lesional skin of the 5 mg/kg CsA treatment group compared with the vehicle-treated controls (Fig. 5a–c). In addition, CsA treatment at a dose of 5 mg/kg significantly improved acanthosis compared with control mice (Fig. 5d).

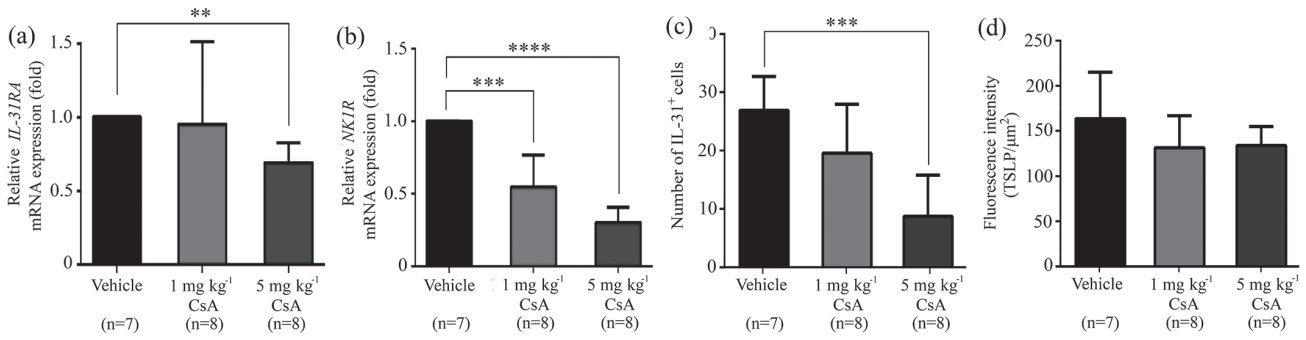


Fig. 4. Effects of cyclosporine A (CsA) treatment on itch-related receptor expression in dorsal root ganglion (DRG) of *Dermatophagoides farinae* body (Dfb)-treated mice. Expression levels of (a) interleukin 31 receptor A (*IL-31RA*) and (b) *NK1R* genes in DRG were examined in vehicle, 1 mg/kg CsA, and 5 mg/kg CsA treatment groups. (a) Among the 3 groups, *IL-31RA* expression was significantly decreased in the DRG of the 5 mg/kg CsA treatment group compared with vehicle-treated controls. (b) *NK1R* expression was dose-dependently inhibited by CsA treatment. (c) The number of IL-31$^+$ cells was significantly decreased in the skin of the 5 mg/kg CsA treatment group compared with vehicle-treated controls. (d) There was no statistically significant difference in the intensity of TSLP in the epidermis among the 3 groups. Values are expressed as the means± standard deviation (SD) of 3 independent experiments, and were compared by 1-way analysis of variance (ANOVA) with Bonferroni's comparison. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.001$ (vs. vehicle).

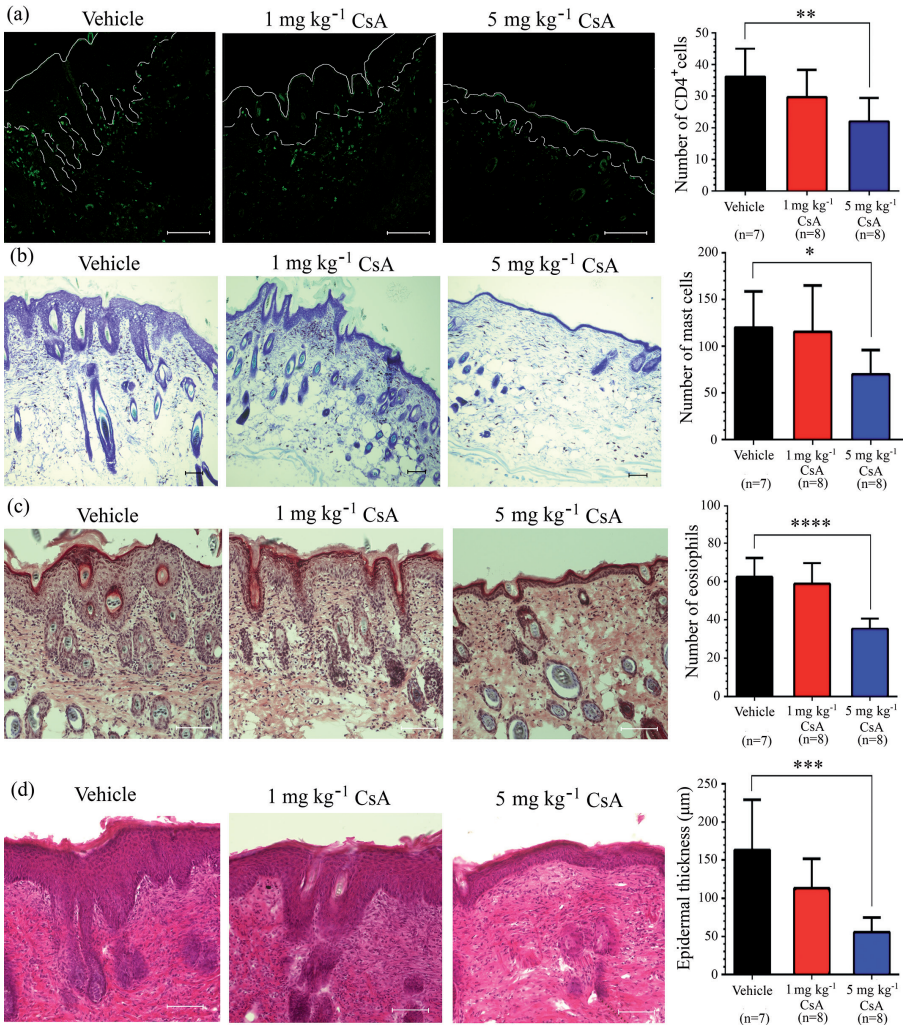


Fig. 5. Effects of cyclosporine A (CsA) treatment on infiltrating immune cells and acanthosis. Numbers of (a) CD4$^+$ T cells (*green*), (b) mast cells, and (c) eosinophils were significantly reduced in skins of 5 mg/kg CsA-treated group. (d) Measurement of epidermal thickness after haematoxylin-eosin staining showed reduction of epidermal thickness in the 5 mg/kg CsA treatment group compared with vehicle-treated controls. In *panel a*, *white* and *broken lines* indicate the skin surface and the border between the epidermis and dermis, respectively. Values represent the means± standard deviation (SD) of 7–8 animals and were compared by 1-way analysis of variance (ANOVA) with Bonferroni's comparison. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ (vs. vehicle). *Scale bars* = (a) 75 μm, (b–d) 100 μm.

DISCUSSION

The results of this study showed that 5 mg/kg CsA reduced scratching behaviour and improved skin barrier function and dermatitis in Dfb-NC/Nga mice (Fig. 1). These findings were consistent with those of previous clinical studies demonstrating the efficacy of low-dose CsA in the treatment of severe AD patients with pruritus (6), unlike immunosuppression, which requires high-dose administration (11). Patients with AD frequently scratch at the sites of dermatitis (12). Removing the claws of NC/Nga mice thus prevents scratching and inhibits the induction and progression of dermatitis (13). These findings suggest that reduction or prevention of scratching is important in improvement of skin barrier function (14). Therefore, the decreased number of scratching bouts observed after CsA treatment, and its ability to interrupt the itch–scratch–itch cycle, suggests that this treatment method would be clinically useful.

Peripheral nerve fibres are responsible for itch sensation in humans and animals (15). Histologically, epidermal PGP9.5- and SP-immunoreactive nerve fibre densities are high in the

lesional skin of individuals with AD compared with controls (2, 16, 17). SP is also involved in neurogenic inflammation and/or enhancement of itch sensation (2, 18). Our immunohistochemical findings showed that both PGP9.5- and SP-immunoreactive fibres were abundant in the epidermis of non-treated Dfb-NC/Nga mice, whereas the numbers were dose-dependently reduced in the epidermis of CsA-treated Dfb-NC/Nga mice (Fig. 2). Several studies have reported that increased levels of NGF and decreased levels of semaphorin 3A (Sema3A) induce neurite growth to penetrate into the epidermis and exacerbate itch. Meanwhile, anti-NGF, Sema3A replacement and several existing treatments, such as ultraviolet-based therapies, can ameliorate pruritus in patients with AD and atopic NC/Nga mice via the reduction in epidermal nerve fibre density (19–24). Our *in vitro* experiments showed that the NGF-induced neurite outgrowth in cultured DRG neurones was inhibited by CsA (Fig. 3). Thus, although we could not completely exclude its indirect effects, CsA may directly inhibit the elongation of nerve growth in the epidermis, thereby at least partly improving itch sensitization in the periphery in AD. This was supported by a previous report that calcineurin/NFAT signalling is involved in neurotrophin-dependent outgrowth of embryonic axons (25).

Recent studies identified a series of itch-related ligands and receptors as well as peripheral neurones and spinal afferents specialized in transmitting this sensation and distinguishing it from pain (26–28). In this study, we found that expression levels of some itch-related receptors were increased at the mRNA level in the DRG of Dfb-NC/Nga mice compared with controls (Table SI[1]). Among them, increased levels of *IL-31RA* and *NK1R* expression were inhibited dose-dependently in the CsA-treated group, especially at a dose of 5 mg/kg (Fig. 4). Intradermal injection of both IL-31 and SP, ligands for these receptors, evoked itching in humans and animals (29–31). A recent study indicated that increased expression of *IL-31RA* in DRG induces scratching behaviour in mice (32). In addition, NK1R-immunoreactive fibres have also been shown to increase in the lesional skin of patients with AD and atopic NC/Nga mice (33). Taken together, our data suggested that CsA partly attenuates pruritus in AD via inhibitory effects on gene expression of both IL-31RA and NK1R in the DRG, in addition to reduced production of itch-related ligands, such as IL-31 and TSLP caused by calcineurin blockade (5, 7, 34). In fact, although TSLP levels did not reduce by CsA treatment, IL-31$^+$ cells in the dermis were lower in the 5 mg/kg CsA-treated group than in the others (Fig. 4).

CD4$^+$ T cells, mast cells, and eosinophils have been shown to produce itch-related ligands and modulators in humans and animals, and these cells were found to induce allergic inflammation in AD (35–37). Increased numbers of these cells have been observed in both patients with AD and Dfb-NC/Nga mice (17, 23). Our study showed that CsA treatment induced significant reductions in the numbers of CD4$^+$ T cells, eosinophils and mast cells in the lesional skin of Dfb-NC/Nga mice (Fig. 5). Previous studies have shown that Th2 cytokines, including IL-4 and IL-13, were also inhibited by CsA treatment (38, 39). Some studies indicated that CsA also inhibits cell proliferation, migration, and invasion (40–42). These results suggest that CsA affects not only sensory nerve fibres in the epidermis, but also immune cells and other cells, such as keratinocytes. We also found that CsA dose-dependently normalized acanthosis, possibly by homeostasis and integrity regulation of epidermal keratinocytes (40, 41, 43). In fact, 5 mg/kg CsA was effective for promoting the integrity of the skin barrier in this study (Fig. 1d). In addition, abnormal expressions of keratin-14 and keratin-10 in the epidermis of Dfb-NC/Nga mice were normalized in the CsA-treated group, especially 5 mg/kg CsA (Fig. S1[1]). These findings were also supported by a previous report that non-immunosuppressive CsA is useful in the treatment of hyperproliferative epidermal diseases (40).

Moreover, skin inflammatory cells, together with sensory nerve fibres, are partly responsible for pruritus in AD, as SP secreted from sensory nerve fibres stimulates the production of various chemical mediators from mast cells (2, 44). Eosinophils may also have direct interaction with nerve fibres, and eosinophils themselves directly release itch-related mediators, such as NGF, cytokines, and proteases (45). Thus, CsA may at least partly contribute to amelioration of pruritus in AD via suppression of inflammatory cells, such as CD4$^+$ T cells, mast cells, and eosinophils, interacting with sensory nerves fibres in the atopic skin.

In conclusion, the results of the present study indicate a possible antipruritic mechanism of CsA treatment for AD. In addition, CsA may exert a pleiotropic antipruritic effect through inhibition of many events related to evocation and/or enhancement of itch.

ACKNOWLEDGEMENTS

This work was partly supported by a grant of Strategic Research Foundation Grant-aided Project for Private Universities from MEXT (Grant Number S1311011), and by an Inohana foundation from Chiba University.

The authors declare no conflicts of interest.

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Appendix S1

MATERIALS AND METHODS

Animals

Male NC/Nga mice (10–12 weeks old; Oriental Yeast, Tokyo, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine under a 12-h light: 12-h dark cycle at a regulated temperature of 22–24°C with food and tap water provided *ad libitum*. Care and handling of all animals conformed to the National Institutes of Health (NIH) guidelines for animal research, and all animal procedures were approved by the Institutional Animal Care and Use Committee of Juntendo University Graduate School of Medicine.

Reagents

The following reagents were used in this study: Dfb ointment (Biostir Inc., Osaka, Japan), bovine serum albumin (BSA), CsA, nerve growth factor (NGF), poly-D-lysine, laminin, 5-fluro-2-deoxyuridine, uridine, penicillin, and streptomycin (Sigma, St Louis, MO, USA), optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd, Tokyo, Japan), normal donkey serum (NDS; Chemicon, Temecula, CA, USA), sevoflurane (Abbott Japan, Osaka, Japan), somnopentyl (Kyoritsu Seiyaku Corporation, Tokyo, Japan), Vectashield® mounting medium (Vector Laboratories Ltd, Peterborough, UK), Dulbecco's modified Eagle's medium (DMEM)/F12 and N-2 supplement (Invitrogen, Carlsbad, CA, USA).

Antibodies

The following antibodies were used in this study: rabbit anti-protein gene product 9.5 (PGP9.5) (1:400 dilution; Enzo Life Science, Inc., Farmingdale, NY, USA), rabbit anti-TSLP (1: 1000 dilution, Millipore), rabbit anti-IL-31 (1: 500; Sigma-Aldrich), guinea pig anti-keratin-14 (1:100 dilution, Progen Biotechnik), guinea pig anti-keratin-10 (1:100 dilution, Progen Biotechnik), rat anti-substance P (SP) (1:100 dilution; Chemicon), and rat anti-CD4 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR, USA).

Experimental design

AD-like symptoms were induced by repeated application of Dfb twice per week for 3 consecutive weeks (induction phase), followed by repeated application of Dfb twice per week for the following 3 consecutive weeks for maintenance of AD-like symptoms. CsA was injected intraperitoneally 3 times per week for 3 consecutive weeks (treatment phase) from weeks 3 to 6. During the treatment course, dermatitis score and transepidermal water loss (TEWL) were evaluated before each application of Dfb ointment and measured 3 times per week. Scratching behaviour was evaluated after CsA treatment.

Induction of dermatitis

In the first induction, mice were anaesthetized with sevoflurane and the dorsal skin of the nape of the neck was shaved with an electric shaver. The residual hair was depilated by applying hair removal cream, which was then followed by application of ointment containing 100 mg Dfb to the shaved area and both surfaces of each ear. In the second induction, residual growing hair in the shaved area was shaved again with an electric shaver, followed by barrier disruption by treatment with 4% sodium dodecyl sulphate (SDS) solution 2 h before application of Dfb ointment. The above procedure was repeated twice per week for 3 consecutive weeks.

Measurement of TEWL

TEWL was measured by pressing the Tewameter® TM210 (Courage-Khazaka, Cologne, Germany) against the rostral part of the back skin for 30 s. TEWL was measured 3 times per week, starting before CsA treatment and ending after 3 weeks of CsA treatment.

Observation of scratching behaviour

Mice in each group were placed individually in acrylic cages consisting of 4 cells (13×9×35 cm). A camcorder (HDR-SR11; Sony, Tokyo, Japan) was positioned above the cages to record the behaviour of the mice. After an acclimation period of at least 1 h, the behaviour of mice was recorded on video for 2 h with no experimenters present in the observation room. Scratching behaviour was assessed by monitoring and counting the replays of each video. Each incidence of scratching behaviour was defined as the raising to lowering of a leg and changes in number of scratching behaviours after treatment were recorded.

Treatment with CsA

Individual doses of CsA were prepared by dissolving in a minimal amount of 95% ethanol and diluted as a fine suspension by adjusting to the final volume with 30% glycerin in distilled water. In the treatment phase, all of the mice in each group were injected with 1 mg/kg CsA or 5 mg/kg CsA (0.25 ml, intraperitoneal) 3 times weekly for 3 consecutive weeks, immediately followed by application of 100 mg of Dfb ointment twice a week for 3 consecutive weeks. TEWL measurement and dermatitis score were evaluated after each CsA treatment, starting from the 3rd week and ending on the 6th week. Scratching behaviour was observed in the 3rd week (before CsA treatment) and the sixth week (end of CsA treatment).

Immunohistochemical and histological analyses

Skin from each mouse was removed under anaesthesia with somnopentyl. After removing the skin, samples were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 4 h at 4°C. After washing with phosphate-buffered saline (PBS, pH 7.4), small pieces of skin were immersed successively in PBS containing 20% sucrose overnight at 4°C. The samples were then embedded in OCT compound and frozen on dry ice. Cryosections 8 μm, and 20 μm thick were cut using a CM1850 cryostat (Leica, Wetzlar, Germany) and mounted on silane-coated glass slides. After blocking in PBS with 5% NDS, 2%BSA, and 0.2% Triton X-100, the cryosections on the glass slides were incubated with primary antibodies overnight at 4°C. The next day, the primary antibodies were washed with PBS containing 0.05% Tween 20 (PBS-T), and incubated with secondary antibodies for 1 h at room temperature. The sections were washed again with PBS-T and mounted in Vectashield® mounting medium. Then, the immunoreactivity was assessed by confocal laser scanning microscopy (DMIRE2; Leica). In histological analyses, cryosections 8μm thick were stained with direct fast scarlet 4BS (DFS), toluidine blue (TB), and haematoxylin-eosin (HE).

Semi-quantitative measurements

Epidermal thickness was measured in 9 random fields (1.0×10^5 μm^2) per mouse. All of these measurements were

performed using BZ-H2A software (Keyence, Osaka, Japan). To perform semi-quantitative measurements of the fluorescence intensity of TSLP in epidermis, 9 random fields (1.6×10^5 μm^2) per mouse were observed with a confocal laser-scanning microscope, with exposure and acquisition settings such that no signal saturation occurred. The sum of the fluorescence intensity of the epidermis and the area of the epidermis in each field was measured using Leica Confocal Software. Fluorescence intensity per unit area was determined by dividing the total fluorescence intensity by the total area. To semi-quantify the number of epidermal nerve fibres, 9 specimens from each mouse were stained with the above-mentioned primary antibodies, and optical sections 0.9 μm thick were scanned through the z-plane of the stained specimens by confocal microscopy. The images were reconstructed in 3 dimensions using Leica Confocal Software (Leica). For measurement of the number of epidermal nerve fibres, we analysed at least 15 confocal images per group per experiment. The number of epidermal nerve fibres in each 1.6×10^5 μm^2 section of the epidermis was quantified. The numbers of inflammatory cells and epidermal nerve fibres were counted manually. All values represent the means ± SD.

Quantitative RT-PCR (qRT-PCR) analysis

Total RNA from the DRGs was isolated with an RNeasy Fibrous Tissue Minikit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. Gene expression analyses for itch-related receptors in DRGs were performed by qRT-PCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The protocols for qRT-PCR analysis were performed according to the manufacturer's instructions. The primers used in this study were synthesized by Perfect Real-Time support system (TaKaRa, Kyoto, Japan) and are listed in (STable I). The levels of gene expression were calculated relative to expression of ribosomal protein S18 (RPS18). The qRT-PCR analyses in this study were performed at least 3 times.

STable I. *Sequences of primer pairs used for qRT-PCR analysis*

Genes	Sequence (5'–3')
H1R	(forward) ACACATCAGAGGCATGGACAGAC
	(reverse) TCTTCGGAGGCAGAAGAGGTG
H4R	(forward) TTTGTGGTGGACAGAAACCTTAGAC
	(reverse) AACATGCAGATTCCACTTCCAA
Mrgpra3	(forward) AAGCCAGCAACCTACATCCAG
	(reverse) TTCCAGGGATGGTTTCGTTC
IL-31RA	(forward) CCAGAAGCTGCCATGTCGAA
	(reverse) CTCGGTGTCCCAACACTGGATA
Osmr	(forward) GATTCGCATCACAGCCAACAA
	(reverse) CCAGATACGGGCTCCCAAGA
PAR2	(forward) GCTGGGAGGTATCACCCTTCTG
	(reverse) GAGGATGGACGCAGAGAACTCA
TSLPR	(forward) CCAGAAGCTGCCATGTCGAA
	(reverse) CTCGGTGTCCCAACACTGGATA
Trpv1	(forward) CAAGGATGACTTCCGGTGGTG
	(reverse) CCTGGGTCCTCGTTGATGATG
TrpA1	(forward) TGGGCAGCTTATTGCCTTCAC
	(reverse) GGACCTCTGATCCACTTTGCGTA
TGR5	(forward) TGCCCAAAGGTGTCTACGAGTG
	(reverse) GCTGCATTGGCTACTGGTGTG
NK1R	(forward) CAGTCAGCCCTGGGAACCTA
	(reverse) GGTCCACCTCATCTTCGCTCATA

Culture of DRG neurones

Postnatal rat-derived DRG neurones (5×10^3 cells: Cambrex, Walkersville, MD) were placed into each well for neurite growth assay. The DRG neurones were maintained in serum-free medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10 ng/ml NGF, 87.5 ng/ml 5-fluro-2-deoxyuridine, 37.5 ng/ml uridine, 50 U/ml penicillin, and 50 mg/ml streptomycin supplemented with 5% N-2 at 37°C in 5% CO_2.

Assay of neurite outgrowth in cultured DRG neurones

DRG neurones were cultured in basal medium with vehicle (dimethyl sulphoxide), 0.02 μM CsA, and 0.2 μM CsA in the presence of 10 ng/ml NGF. After 3 days, the cells were fixed with 4% PFA in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature, and then examined under phase-contrast microscopy (DMIL; Leica). For semi-quantitative determination of neurite outgrowth, 9 random photographs were taken per well and the length of the longest process of DRG neurones was measured with BZ-H2A software (Keyence). In each experiment, at least 100 cells from triplicate wells per group were analysed. The data from 3 independent experiments were used for statistical analysis.

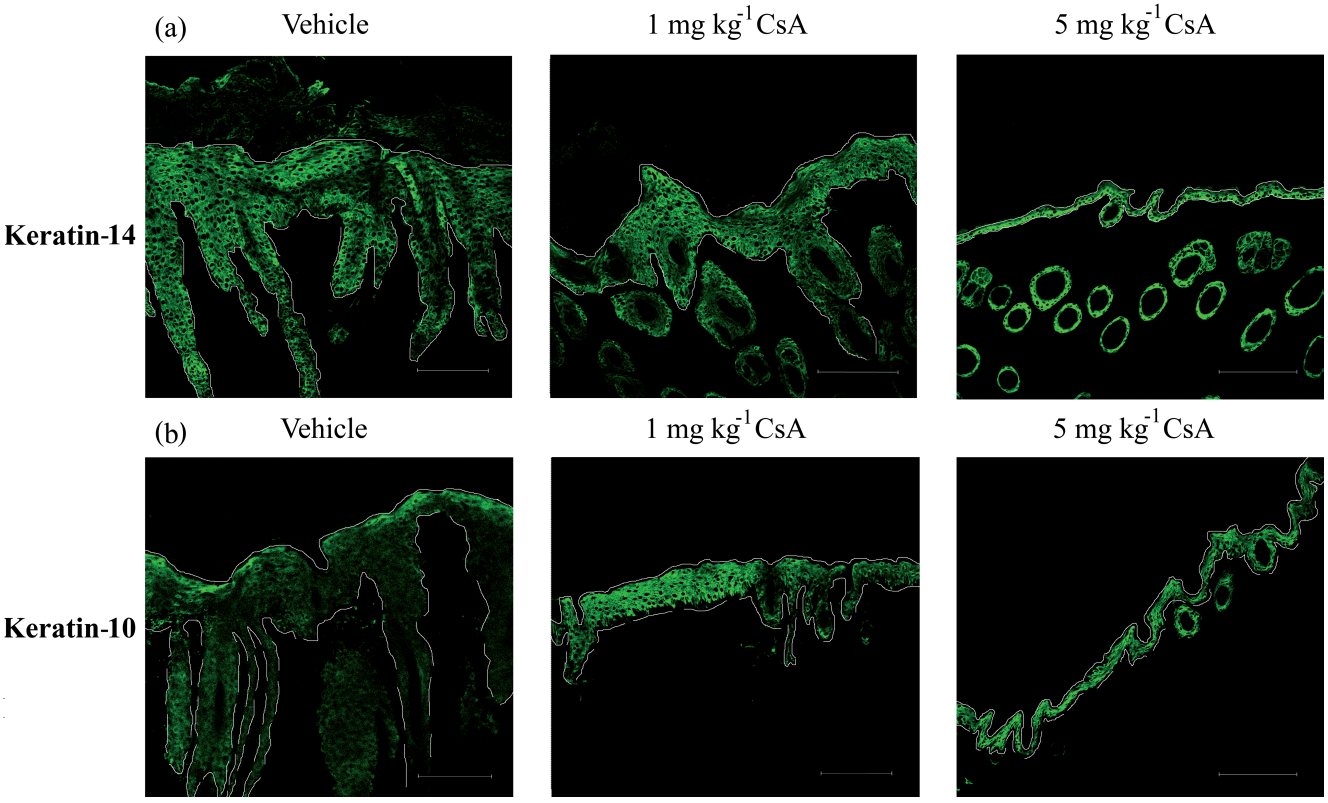


Fig. S1. Effect of cyclosporine A (CsA) treatment on keratin-14 and keratin-10 expression in lesional skin. The figure shows immunolabelling with (a) keratin-14 and (b) keratin-10 expression in the lesional skin of the 3 groups after CsA treatment course. Abnormal expressions of keratin-14 and keratin-10 in the epidermis of Dfb-NC/Nga mice were normalized in the CsA-treated group, especially 5 mg/kg CsA. *White* and *broken lines* indicate the skin surface and the border between the epidermis and dermis (basement membrane), respectively. *Scale bars*=150 μm.

Table SI. *Comparison of gene expression between control and atopic dorsal root ganglions (DRGs). Expression levels of itch-related receptor genes including Mrgpra3, IL-31RA, PAR_2, TrpA1, TGR5 and NK1R in atopic DRGs were significantly higher than those in control DRGs. Expression level of H_4Rin atopic DRGs was significantly lower than that in control DRGs*

Genes	Comparison	Mean ± SD	*p*-value (*t*-test)
H1R	Control	1	0.543
	AD	1.27 ± 0.52	
H4R	Control	1	0.0002*
	AD	0.25 ± 0.09	
Mrgpra3	Control	1	0.0003*
	AD	2.33 ± 1.09	
IL-31RA	Control	1	0.0004*
	AD	2.85 ± 1.06	
Osmr	Control	1	0.6983
	AD	0.95 ± 0.24	
PAR2	Control	1	0.004*
	AD	1.46 ± 0.95	
TSLPR	Control	1	0.641
	AD	0.75 ± 0.48	
Trpv1	Control	1	0.125
	AD	1.49 ± 1.31	
TrpA1	Control	1	0.0220*
	AD	1.37 ± 0.68	
TGR5	Control	1	0.0057*
	AD	1.41 ± 0.92	
NK1R	Control	1	0.0302*
	AD	2.5 ± 0.84	

*$p < 0.05$.

Control: DRGs from SDS-treated mice; AD: DRGs from Dfb-treated mice. AD: atopic dermatitis; SDS: sodium dodecyl sulphate; Dfb: *Dermatophagoides farinae* body; DRG: dorsal root ganglion; SD: standard deviation; AD: atopic dermatitis.