

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.

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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 H₁ 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 neurons, 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 S1¹)

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-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 ($1.0 \times 10^5 \mu\text{m}^2$)

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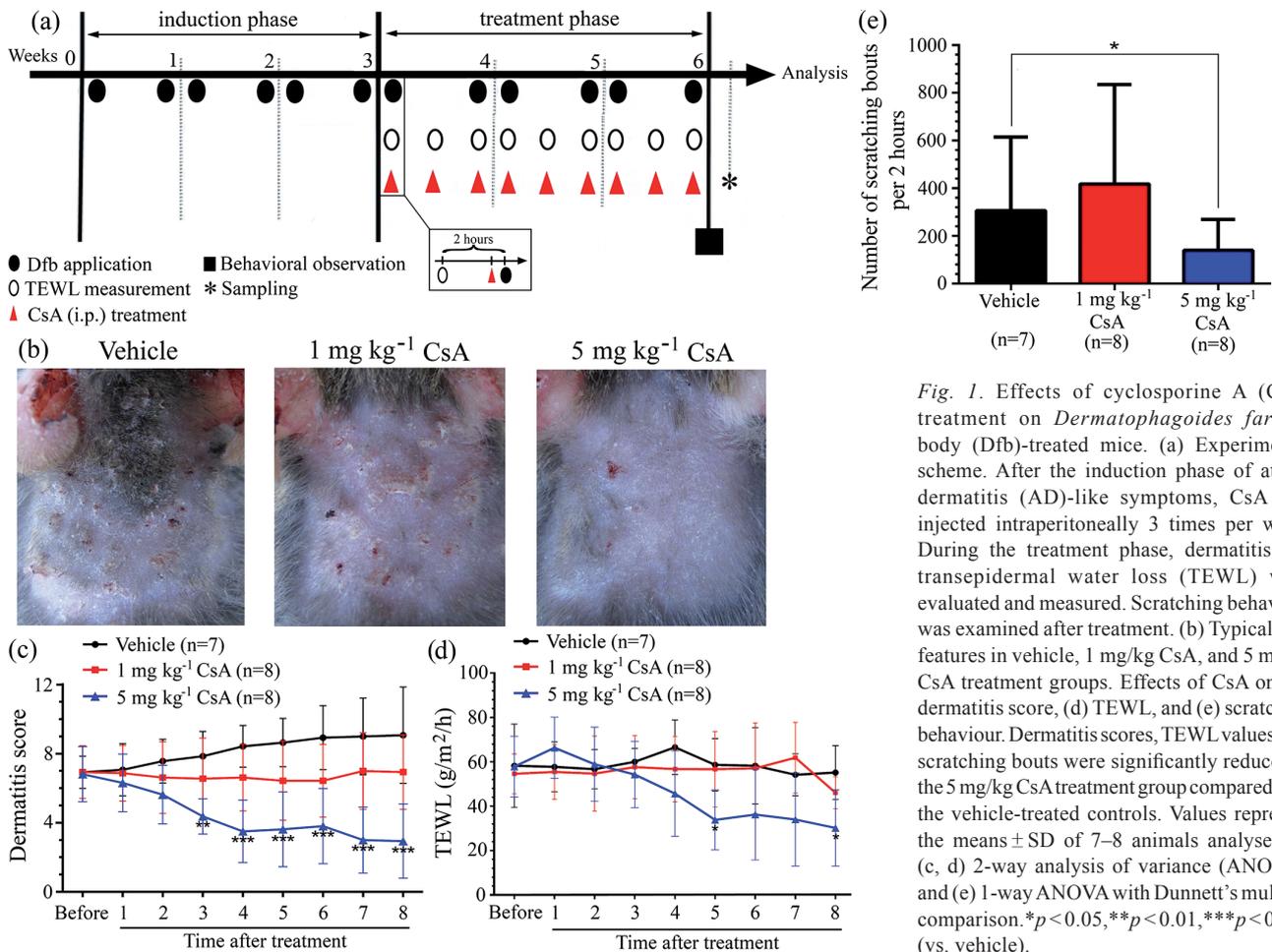


Fig. 1. Effects of cyclosporine A (CsA) treatment on *Dermatophagoides farinae* body (Dfb)-treated mice. (a) Experimental scheme. After the induction phase of atopic dermatitis (AD)-like symptoms, CsA was injected intraperitoneally 3 times per week. During the treatment phase, dermatitis and transepidermal 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 \pm 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

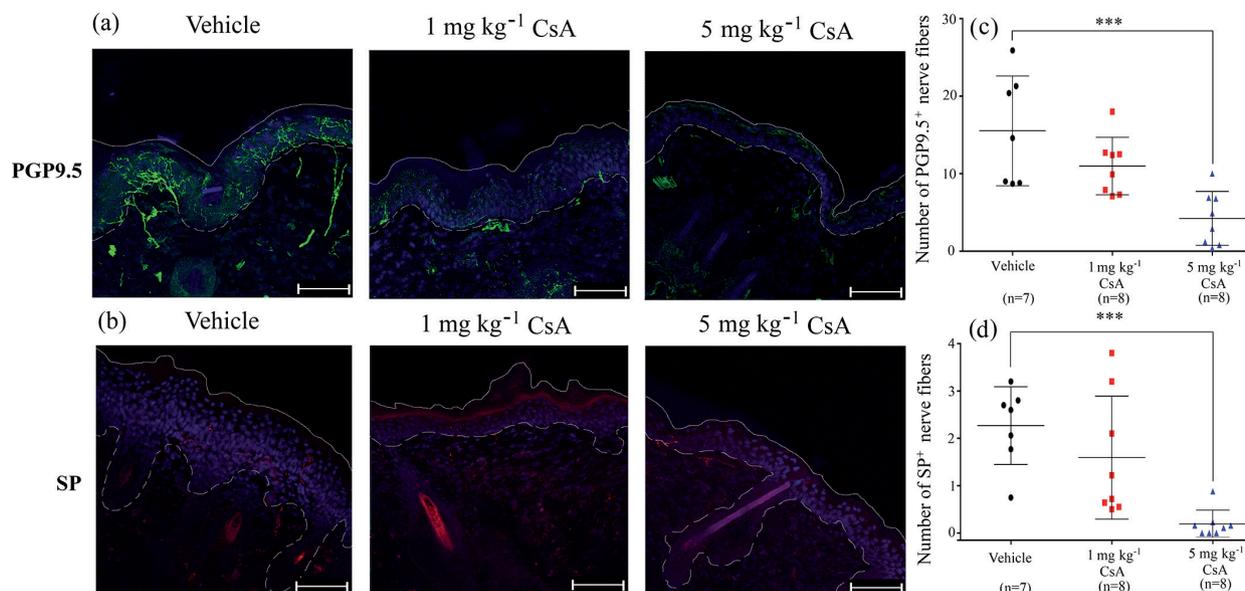


Fig. 2. Effects of cyclosporine A (CsA) treatment on epidermal nerve densities in lesional skin of *Dermatophagoides farinae* body (Dfb)-treated mice. The figure shows immunolabelling with anti-PGP9.5 (green in panel a) and substance P (SP, red in panel b) antibodies of intraepidermal nerve fibres on 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 μ m. 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 \pm 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 μ m.

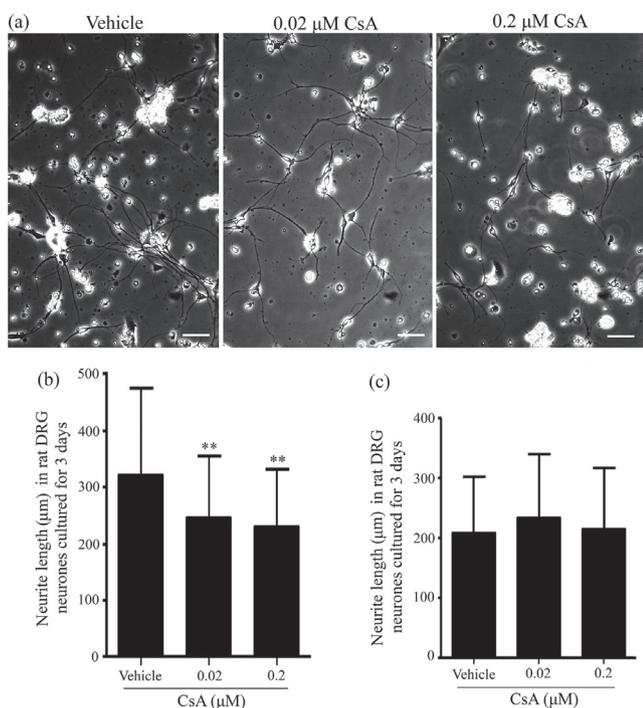


Fig. 3. Inhibition of nerve growth factor (NGF)-induced neurite out-growth in cultured dorsal root ganglion (DRG) neurons by cyclosporine A (CsA). (a) The length of the longest process was measured in DRG neurons incubated with vehicle, 0.02 μ M CsA, and 0.2 μ M CsA in the presence of NGF (10 ng/ml) for 3 days. (b) The neurite length in cultured DRG neurons 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 \pm 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 μ m.

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 S1¹). 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).

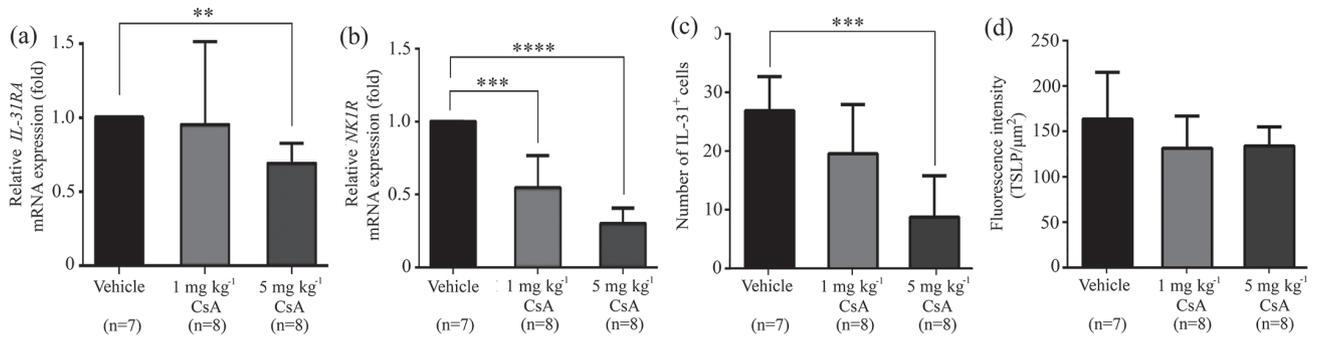


Fig. 4. Effects of cyclosporine A (CsA) treatment on itch-related receptor expression in dorsal root ganglion (DRG) of *Dermatophagoides farinae* body (Dfb)-treated mice. Expression levels of (a) interleukin 31 receptor A (*IL-31RA*) and (b) *NK1R* genes in DRG were examined in vehicle, 1 mg/kg CsA, 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 \pm standard deviation (SD) of 3 independent experiments, and were compared by 1-way analysis of variance (ANOVA) with Bonferroni's comparison. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (vs. vehicle).

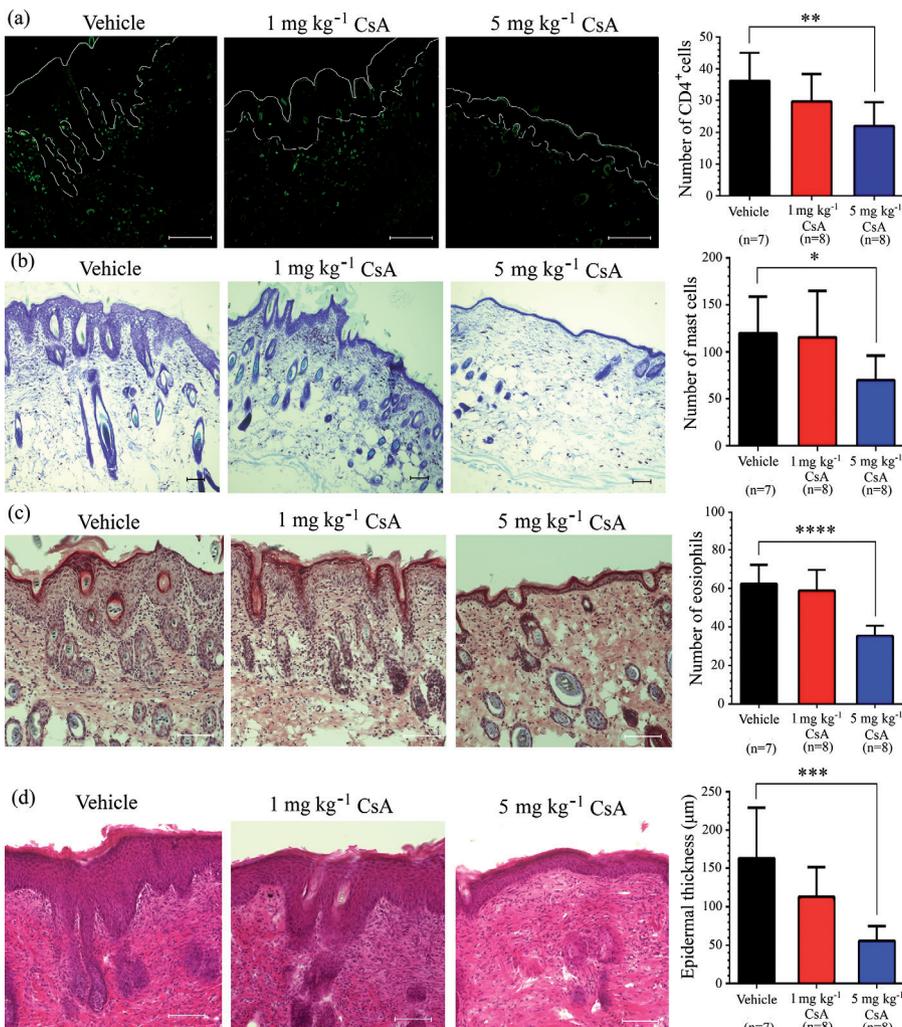


Fig. 5. Effects of cyclosporine A (CsA) treatment on infiltrating immune cells and acanthosis. Numbers of (a) CD4⁺ T cells (green), (b) mast cells, and (c) eosinophils were significantly reduced in skins of 5 mg/kg CsA-treated group. (d) Measurement of epidermal thickness after haematoxylin-eosin staining 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 \pm 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 S1¹). 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. S1¹). 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.

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The authors declare no conflicts of interest.

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