



Dermatophagoides farinae extract induces severe atopic dermatitis in NC/Nga mice, which is effectively suppressed by the administration of tacrolimus ointment

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease, which is accompanied by marked increases in the levels of inflammatory cells, including mast cells and eosinophils as well as T cells and macrophages. To investigate the expression pattern of chemokines in AD, a house dust mite, *Dermatophagoides farinae* extracts (DfE)-induced NC/Nga AD model was developed in mice, and this model was used to determine the expression levels of chemokines in atopic lesions using DNA microarrays and RT-PCR. When NC/Nga mice were repeatedly treated with DfE for 4 to 7 weeks on the back skin, the mRNA expression levels of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, and CCL11/eotaxin-1 were markedly induced and lesser of CCL2/MCP-1, within the inflammatory lesion of the back skin. Immunohistochemical staining revealed the expression of these chemokines in the epidermis and dermis of DfE-treated NC/Nga mice. Interestingly, repeated application of tacrolimus ointment potently inhibited DfE-induced atopic dermatitis in NC/Nga mice concomitant with the inhibition of these changes in chemokine gene and protein expression levels particularly of CCL20/LARC, CCL17/TARC, and CCL11/eotaxin-1. These data indicate that severe atopic dermatitis induced by DfE accompanies elevated chemokine levels, and it was proposed that tacrolimus ointment is beneficial for the treatment of severe AD.

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1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease accompanied by severe itching along with elevated IgE levels, and its incidence is increasing globally in infants and children [1,2]. The cause of AD is complex, while genetic predisposition and a combination of allergic and non-allergic factors appear to be important in determining disease expression. In the actual skin lesion, the symptoms are characterized by severe itching, rash, edema, hemorrhage, erosion, and desquamation. Pathological changes of atopic skin are observed as epidermal thickening and marked infiltration of inflammatory cells [3,4]. In particular, AD has been associated with the type 2 T cell (Th2) phenotype, showing the dominance of IL-4, IL-5, and IL-13 secretion [5,6]. In addition, the accumulation of large numbers of eosinophils [7], mast cells, and dendritic cells (DCs) has been observed in both the epidermis and the dermis of patients with AD [7,8].

The infiltration of inflammatory cells to the inflamed sites is known to be dependent on the local production of various member of the

chemokine family with leukocyte chemoattractant activity [9,10]. Th2 cells are attracted by CCL17/TARC and CCL22/MDC through CCR4 [11–13]. Eosinophils express CCR2, CCR3, and CXCR4 on the cell surface and migrate through these chemokine receptors. The level of CCR3 is especially increased in the skin lesions of AD patients, and its ligands, including CCL5/RANTES, CCL11/eotaxin-1, CCL13/MCP-4, CCL24/eotaxin-2, and CCL26/eotaxin-3 are considered to mediate the migration of eosinophils [14–16]. Mast cells express CCR1, CCR3, CCR4, CCR5, CXCR2, CXCR3, and CXCR4 and their chemokine ligands are thus involved in the chemotaxis of mast cells [17–19]. Furthermore, it is known that many chemokine ligands, including CCL1/I-309, CCL2/MCP-1, CCL5/RANTES, CCL13/MCP-4, CCL20/LARC, and CX₃CL1/fractalkine are candidates for the recruitment of DC precursors from the circulation to sites of atopic skin inflammation [14,15,20,21]. Thus, various members of the chemokine family with leukocyte chemoattractant activities are presumed to play important roles in the local accumulation of inflammatory cells. However, the appearance pattern of chemokines and how these chemokines are regulated by the anti-atopic drugs have not yet been clarified for the AD model mice.

NC/Nga mice were established in 1957 using a Japanese mouse strain (Nishiki-Nezumi). When kept under specific pathogen-free (SPF) conditions, these mice remain normal and healthy. However, when placed in conventional surroundings, clinical signs including

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scratching behavior appear and IgE levels are elevated from the age of 8 weeks [22,23]. Thus, NC/Nga mice are often used as a sensitive animal model for human AD. Patients with AD are highly sensitized to house dust mite allergens based on the positive results of radio-allergosorbent assay and scratch or patch tests. The major species of house dust mites in Japan is *Dermatophagoides farinae* (Df), and its body and feces are known as major environmental allergens. Previously, Matsuoka et al. reported that *Dermatophagoides farinae* extract (DfE) has the ability to induce atopic dermatitis-like skin lesion in NC/Nga mice [24].

The present study investigated whether the elevated expression levels of chemokine genes are associated with AD using DfE-treated NC/Nga mice. By performing DNA microarray analysis, it was found that the expression of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 were significantly induced in skin inflammation of NC/Nga mice. In addition, it was examined whether the administration of an immunosuppressive drug, tacrolimus ointment, is effective for these atopic model mice.

2. Material and methods

2.1. Animals

Specific pathogen free (SPF) NC/Nga mice (female) were purchased from SLC (Shizuoka, Shizuoka-city). Mice were maintained in an isolator under SPF conditions. Mice (6 weeks old) were anesthetized by muscular injection of 0.2 mg of xylazine (Bayer, Germany) and 2 mg of ketamine (Sankyo, Tokyo). The hair on their backs was shaved using an electric shaver, and followed by treatment with a skin-hair-remover (Epilat, Kanebo, Tokyo). Hair removal was performed every 2–3 weeks. All experiments with mice were performed in accordance with the regulations and with the approval of the in-house animal experiment committee of Keio University.

2.2. Preparation of *Dermatophagoides farinae* extract and application of mite extracts

The culture of *Dermatophagoides farinae* (Df) mites and preparation of the extract were described by Matsuoka et al. [24]. Four grams of Df with culture medium (the density of mites was 40,000–50,000 per gram) were taken from a culture flask into a plastic tube, and 40 mL of PBS was added. The tube was rotated for 30 min at room temperature, and then centrifuged at 1500 rpm for 15 min. The supernatant was used as a crude extract of Df (DfE). Culture medium was also extracted with PBS and centrifuged as a negative control (culture medium extract: MeE). After the removal of hair, DfE (200 µL/mouse) was swabbed onto the back skin three times per week using a cotton stick. The same amount of MeE or PBS was swabbed similarly onto the backs of control mice.

2.3. The administration of Tacrolimus ointment

Tacrolimus ointment /Protopic (0.1%) was a gift from Astellas Pharma Inc. (Tokyo). After DfE-application for 2 weeks, the administration of tacrolimus ointment was started on the back skin of mice three times per week. Tacrolimus ointment 100 µg was applied 30 min after DfE application.

2.4. Evaluation of scratching behavior

The scratching behaviors of mice were recorded on a digital video camera (Sony, Tokyo) for 10–30 min, immediately after the application of each extract. The scratching behavior was observed by playing back the video. The scratching of any regions of the removed-hair site by the hind paws was counted as scratching behavior. Mice rapidly scratched several times for about 1 s and a series of these movements was counted as one bout of scratching, as reported previously [25].

2.5. Measurement of total serum IgE by ELISA

Mouse serum was obtained from the axilla at sacrifice. The concentration of total IgE in mouse serum was measured using a Mouse IgE EIA kit (Yamasa, Tokyo). The ELISA was performed in accordance with the manufacturer's instructions.

2.6. Histological analysis of mast cells and eosinophils in the skin lesions

Paraformaldehyde-fixed and paraffin-embedded back skin samples from PBS-, MeE-, or DfE-treated NC/Nga mice were sectioned and stained with hematoxylin-eosin (H&E), toluidine blue, or Congo red. The number of mast cells and eosinophils in the dermis stained by toluidine blue and Congo red, respectively, in a field of 100 µm × 100 µm was counted under the microscope. The cells in 50 fields (0.5 mm² in total) of the dermis were counted.

2.7. Tissue preparation and immunostaining

After the application of PBS, MeE, or DfE for 7 weeks, the back skin was removed and cut into small pieces. Then, the tissues were embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen. Cryostat sections were cut at 6 µm thickness, air dried, and stored at –20 °C until use. After thawing and air-drying, the cryosections were fixed with acetone for 10 min. Next the sections were rehydrated in TBS and then incubated with a blocking solution (Blocking One, Nacalai Tesque, Tokyo) for 10 min. The sections were incubated with the first antibody (anti-mouse CD68 (Serotec, UK), anti-mouse CD4 (eBioscience, USA), anti-mouse CD205 (Serotec, UK), anti-CCL2/MCP-1 (Santa Cruz, USA), anti-CCL11/eotaxin-1 (R&D, USA), anti-CCL17/TARC (R&D, USA), or anti-CCL20/LARC (Santa Cruz, USA)) for 1 h at 25 °C. Thereafter, each step was followed by washing three times with PBS for 2 min. Bound antibody was detected using an alkaline phosphatase (ALP)-labeled second antibody (Jackson Immuno Research Lab., USA) for 45 min, and sections were fixed further with 1% glutaraldehyde (WAKO, Tokyo) in PBS for 30 s. After washing with distilled water, the ALP reaction was developed at 25 °C for 10–20 min in a Vector blue reaction kit solution (Vector Laboratories). After blocking, the sections were incubated further with a rabbit antibody against type IV collagen (LSL, Tokyo). To detect the antibody, the sections were incubated with porcine anti-rabbit immunoglobulins labeled with horseradish peroxidase (HRP) (DAKO, Denmark). The HRP reaction was developed at 25 °C for 5–10 min in solution of 5 mg of 3'-diaminobenzidine hydrochloride (DAB) in 40 mL PBS with 10 µL of 30% H₂O₂, and 1 mL of 0.3 M imidazole. The sections were mounted in Aquatex (Merck, USA).

2.8. DNA microarray

After the application of DfE for 3, 5, 7 weeks, skin lesions (150–250 mg) from the back were removed, frozen immediately in liquid nitrogen, and stored at –80 °C. DNA microarray was performed using a Cytokine/Chemokine array (cDNA microarray chip, Kaken Genecis, Matsudo, Chiba) as reported previously [26]. Total RNA was isolated using a QIAGEN RNeasy Midi Kit (QIAGEN, Germany) and treated with DNase I (QIAGEN, Germany). A total RNA sample (50 µg) derived from a DfE-treated mouse was labeled with Cyanine 3 (Cy3)-dUTP, control total RNA (50 µg) derived from an MeE-treated mouse was labeled with Cyanine 5 (Cy5)-dUTP by a reverse transcription reaction using reverse transcriptase, ReverScriptII (Wako, Tokyo), and an anchored oligo (dT) primer (Amersham Biosciences, Tokyo). The fluorescently labeled cDNAs were mixed and hybridized simultaneously to the cDNA microarray chip. Each RNA pair was labeled and hybridized independently in duplicate. The fluorescent images of hybridized microarrays were obtained using a ChipReader scanner (Vertek, Canada). Analysis of the microarray was conducted using MicroArray

Suite (Scanalytic, USA). The Cy3-Cy5 ratios were determined for the individual genes along with various other quality control parameters (e.g., intensity over local background). Dates were normalized by the median of the intensity of a housekeeping gene (GAPDH).

2.9. RT-PCR

Total RNA (2 µg) was reverse-transcribed using ReverScript I (Wako, Tokyo) in a total volume of 20 µL. Then, cDNAs were amplified with primers specific for each chemokine. Primers for PCR amplification were as follows: CCL2: 5'-CCTGCTGTTACAGTTGCC-3' /

5'-TGAGGTGGTGTGGAAAAGG-3' (Tm 57 °C); CCL11: 5'-ATG-CACCTGAAAGCCATAG-3' / 5'-AACTCGTCCCATTGTGTCC-3' (Tm 55 °C); CCL17: 5'-CAGGAAGTTGGTGAGCTGGTATA-3' / 5'-TTGTGTTGCTGTAGTGCATA-3' (Tm 55 °C); CCL20: 5'-GAAG-CAGCAAGCAACTACGA-3' / 5'-TTAGGCTGAGGAGGTTACA-3' (Tm 55 °C); CCL24: 5'-TGTTGCATCTCCCATAGA-3' / 5'-GGTGATGAA-GATGACCCCTG-3' (Tm 57 °C); GAPDH: 5'-ACTCCACTCAGGCAATTC-3' / 5'-CCTTCCACAATGCCAAAGTT-3' (Tm 57 °C).

Quantitative analysis of PCR bands was performed using the public domain NIH ImageJ program (developed at the U.S. National Institutes of Health and available from the Internet, <http://rsb.info.nih.gov/ij/>).

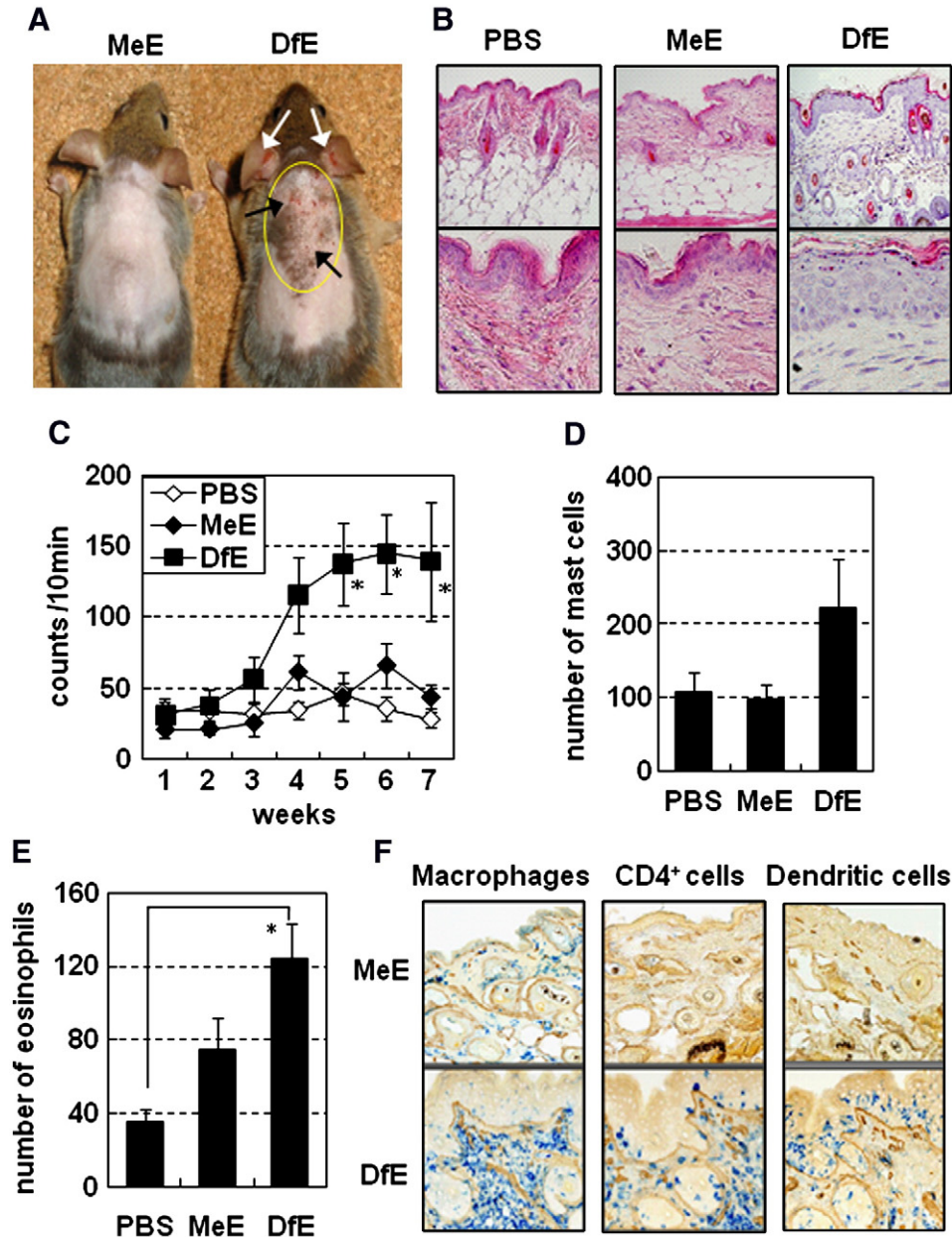


Fig. 1. Development of DfE-induced atopic dermatitis in NC/Nga mice. (A) Skin features of NC/Nga mice repeatedly treated with non-elicited (MeE) or elicited (DfE) for 7 weeks. White arrows indicate erosion and black arrows indicate desquamation. PBS-treatment did not elicit any symptoms (data not shown). (B) H&E staining of back skin treated with PBS, MeE, or DfE for 7 weeks. Upper: $\times 400$ (C) Scratching behavior of NC/Nga mice immediately after treatment with PBS, MeE, or DfE. White lozenge, PBS; black lozenge, MeE; black square, DfE. Each data point represents the mean $\pm$ S.E. of 7 mice. $*p < 0.05$ when compared with MeE (Student's *t* test). (D, E) The number of dermal mast cells (D) and eosinophils (E). Mast cells and eosinophils in the back skin of PBS, MeE, and DfE-treated NC/Nga mice were stained with toluidine blue and Congo red, respectively, and counted using a microscope at a magnification of $\times 400$. Each data point represents the mean $\pm$ S.E. of 7 mice. $*p < 0.05$ when compared with PBS or MeE (Student's *t* test). (F) Macrophages, CD4-positive cells, and dendritic cells in the back skin of PBS, MeE, and DfE-treated NC/Nga mice. Frozen sections (6 µm) were immunostained with anti-CD68 antibody, anti-CD4 antibody, or anti-CD205 antibody to detect macrophages, CD4-positive cells, and dendritic cells, respectively. Positively stained cells are shown in a blue color. Original magnification $\times 200$.

Band intensities were normalized by the median of the intensity of GAPDH. Statistical comparison was done using Student *t*-test.

3. Results

3.1. DfE significantly induced skin inflammation in NC/Nga mice

To develop atopic dermatitis model mice, NC/Nga mice's shaved back skin was repeatedly swabbed with crude extract of *Dermatophagoides farinae* (DfE), medium extract (MeE), or PBS three times per week for 7 weeks. The onset of skin inflammation was observed in the DfE-treated mice from 2 to 4 weeks. Thereafter, this inflammation tended to be aggravated by the repeat application of DfE up to 7 weeks. Severe dermatitis with desquamation, erosion, and hemorrhage on the back skin and ear was observed in DfE-treated NC/Nga mice. On the other hand, no severe inflammation was observed with MeE-treated mice (Fig. 1A). Histological analysis of skin sections was performed by H&E staining and typical photographs are shown in Fig. 1B. In PBS or MeE-treated mice, no significant histological changes of the skin were observed. In contrast, significant hyperplasia of the epidermis and dermis was observed on the skin of DfE-treated NC/Nga mice during the 7 week treatment period. In addition, while no obvious scratching behavior was observed in PBS or MeE-treated mice throughout the observation periods, the scratching count of DfE-treated mice increased from 3 weeks and reached a plateau at 5 weeks (Fig. 1C). In order to investigate whether inflammatory cells had infiltrated into the skin after DfE application, tissue sections were stained with toluidine blue or Congo red to detect mast cells and eosinophils, respectively. The number of dermal mast cells and eosinophils were markedly increased in DfE-treated mice, compared with PBS or MeE-treated mice (Fig. 1D, E). Moreover, immunohistological staining revealed a high infiltration of macrophages and CD4⁺ T cells in the dermis and dendritic cells in the epidermis of DfE-treated mice (Fig. 1F). In contrast, no significant increase in the levels of these cells was observed in the skin of MeE-treated mice. Significantly increased levels of IgE production (above 1500 ng/mL) were observed in DfE-treated mice at 7 weeks, while no significant IgE production was observed in PBS- or MeE-treated mice (less than 250 ng/mL; data not shown). These data indicate that DfE application induced typical atopic-like dermatitis in NC/Nga mice from 4 weeks.

3.2. Expression of various chemokines was enhanced in DfE-treated NC/Nga mice

Next, in order to investigate which kinds of chemokines were expressed in DfE-induced NC/Nga atopic dermatitis mice model, a DNA microarray analysis for cytokine/chemokine gene expression was performed. NC/Nga mice were treated with MeE- or DfE-treated mice for 3, 5, and 7 weeks, and total RNA was prepared from the back skin of these mice. As the period of DfE application became longer, the number of expressed chemokines increased. As shown in Fig. 2, the expression of 14 chemokines was significantly induced in the skin of DfE-treated mice at 7 weeks, compared with that of MeE-treated mice. Thus, it was concluded that the progression of dermatitis is highly associated with the expression of various chemokines in skin lesions.

3.3. Expression of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 was enhanced in DfE-treated NC/Nga mice

Previous studies have suggested that CCL20/LARC and CCL17/TARC are involved in the infiltration of dendritic cells and CD4⁺ T cells, respectively [12]. In addition, CCL24/eotaxin-2 and CCL11/eotaxin-1 are presumed to be important for the infiltration of eosinophils and mast cells, while CCL2/MCP-1 is involved in the infiltration of macrophages [13]. Because mast cells, eosinophils, macrophages, CD4⁺ T cells, and dendritic cells were increased in skin lesions (Fig. 1),

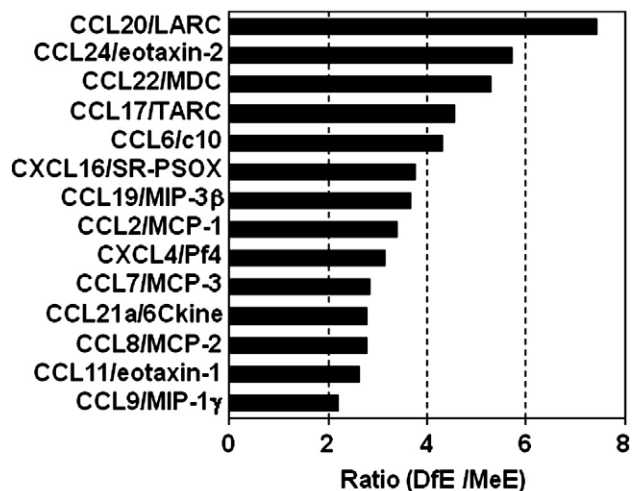


Fig. 2. Enhanced expression levels of various chemokines in DfE-induced atopic dermatitis in NC/Nga mice. The expression levels of chemokine genes in NC/Nga mice repeatedly treated with non-elicited dermatitis mouse (MeE) or elicited dermatitis (DfE) for 7 weeks were examined. The mRNA expression levels of chemokine genes were calculated by the ratio of the fluorescence intensity using a microarray, and the values were corrected using the value for GAPDH transcript as the internal control.

the present study focused on the expression of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 among various genes detected by the DNA microarray. First, it was examined whether the expression levels of these chemokines were induced by DfE application by RT-PCR. Consistent with the results of the DNA microarray (Fig. 2), the expression of these chemokines was markedly and significantly induced in DfE-treated mice (Fig. 3A). In contrast, MeE-treated mice failed to induce the expression of these chemokines. Expression of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, and CCL11/eotaxin-1 was observed significantly by three independent experiments, except CCL2/MCP-1 of which increase was relatively low and not significant (Fig. 3B). In addition, to study the protein expression of these chemokines, immunostaining was performed with each specific antibody using frozen sections. High levels of CCL20/LARC, CCL2/MCP-1, and CCL11/eotaxin-1 were observed in DfE-treated dermis compared with those from MeE-treated skin. CCL17/TARC was highly expressed not only in the epidermis but also in the dermis of DfE-treated mice (Fig. 3C). These results imply that high expression levels of these chemokines, as observed in DfE-induced atopic dermatitis in NC/Nga mice, are involved in the infiltration of inflammatory cells.

3.4. Tacrolimus ointment significantly inhibited atopic dermatitis in NC/Nga mice

Tacrolimus ointment has recently been developed and approved for use in treating atopic dermatitis [27–29]. The effect of tacrolimus ointment on DfE-induced atopic dermatitis was investigated in NC/Nga mice, as was first tried by Sasakawa et al. [27]. After DfE application for 2 weeks, tacrolimus ointment was applied for 4 weeks concomitant with DfE application. No severe dermatitis, such as hyperplasia, desquamation, erosion, or hemorrhage, was observed on the back skin and ear in DfE plus tacrolimus ointment-treated mice (Fig. 4A). In addition, histological analysis revealed that tacrolimus ointment significantly inhibited DfE-induced hyperplasia of the epidermis and dermis, as shown in Fig. 4B. Furthermore, an increase in scratching behavior was not observed in DfE plus tacrolimus ointment-treated mice throughout the observation period (Fig. 4C). Interestingly, the number of dermal mast cells and eosinophils in the back skins were significantly reduced by the tacrolimus ointment treatment (Fig. 4D, E). In addition, DfE-induced infiltration of CD4⁺ T

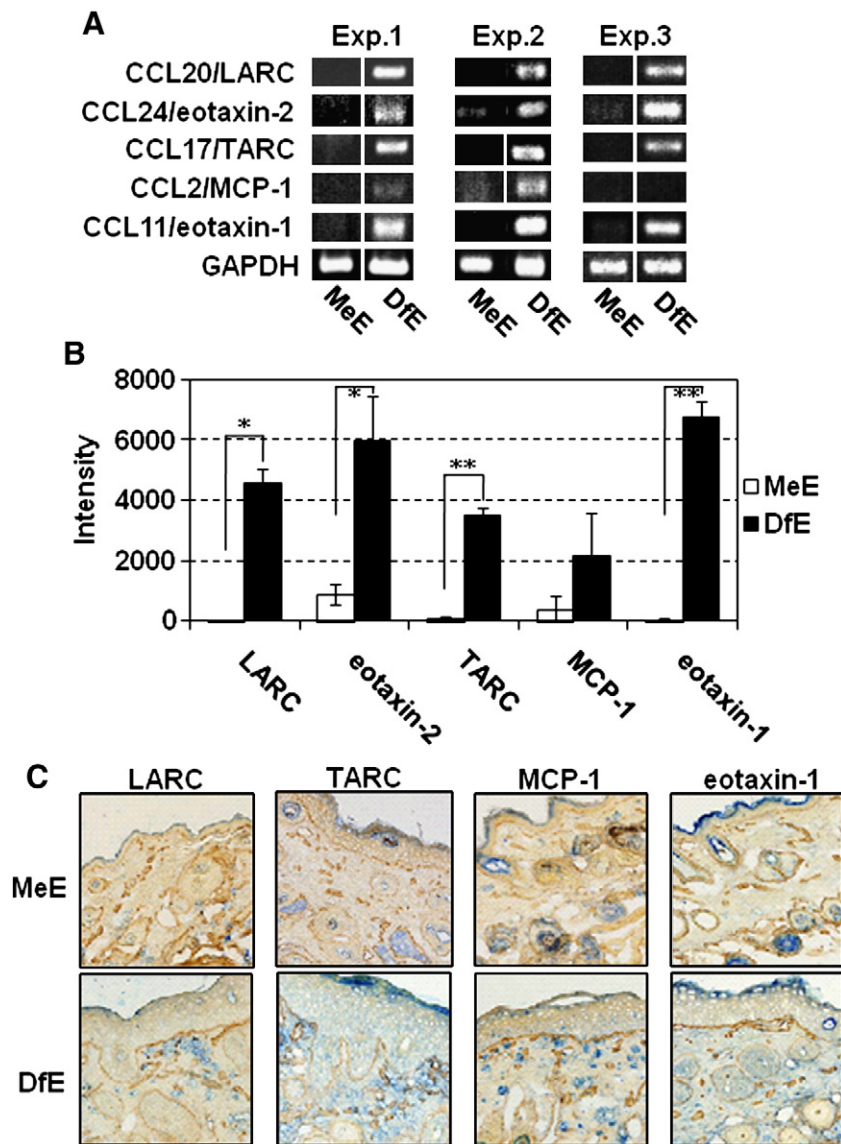


Fig. 3. Enhanced expression levels of CCL20, CCL24, CCL17, CCL2, and CCL11 in DfE-induced atopic dermatitis in NC/Nga mice. (A) The mRNA expression of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 were determined. Total RNA (2 μ g) was prepared from the back skin treated of mice treated with MeE or DfE for 7 weeks and RT-PCR (30 cycles except for GAPDH) was performed. Typical results are shown from three independent analyses. (B) The intensity of PCR bands was measured by NIH ImageJ program. Each data point represents the mean $\pm$ S.E. of 3 experiments. * $p < 0.05$ and ** $p < 0.01$ when compared with MeE (Student's t test). (C) The expression levels of CCL20/LARC, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 were determined in skin lesions. Frozen sections of the back skin treated with MeE or DfE for 7 weeks were immunostained with anti-CCL20/LARC, anti-CCL17/TARC, anti-CCL2/MCP-1, or anti-CCL11/eotaxin-1 antibodies. The blue color indicates positive staining for chemokines. Original magnification $\times 200$.

cells, but not much of macrophages and dendritic cells into the skin was clearly inhibited by tacrolimus ointment (Fig. 4F). Thus, these results suggest that tacrolimus ointment is highly effective for the treatment of atopic dermatitis in NC/Nga mice induced by DfE, particularly for the suppression of scratching behavior and infiltration of mast cells and eosinophils.

3.5. Tacrolimus ointment significantly inhibited DfE-induced expression of CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 in NC/Nga mice

It was further examined whether the tacrolimus ointment affected the increased expression of chemokine genes in the skin of DfE-treated mice. Tacrolimus ointment markedly inhibited DfE-induced expression of CCL20/LARC, CCL17/TARC, CCL11/eotaxin-1 at the mRNA level, but much less mRNA level of CCL2/MCP-1, CCL24/eotaxin-2, and (Fig. 5A, B). Furthermore, it was also observed that

the expression of CCL20/LARC, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 proteins in the dermis of DfE-treated mice were clearly suppressed by tacrolimus ointment (Fig. 5B). Taken together, the present study demonstrated that tacrolimus ointment suppressed atopic dermatitis by inhibiting the chemokine expression that is required for the infiltration of inflammatory cells.

4. Discussion

This study investigated the role of chemokines in atopic dermatitis (AD) using NC/Nga mice treated with DfE as a model of severe AD. When NC/Nga mice are kept in conventional conditions, they develop spontaneous dermatitis at the age of 8 weeks, as reported previously elsewhere [22,30]. To elicit skin inflammation more clearly in a shorter time-frame than in conventional housing conditions, extract of Df was used that includes highly active house-dust-mite antigens. Although this mite does not bite humans, most of the population of

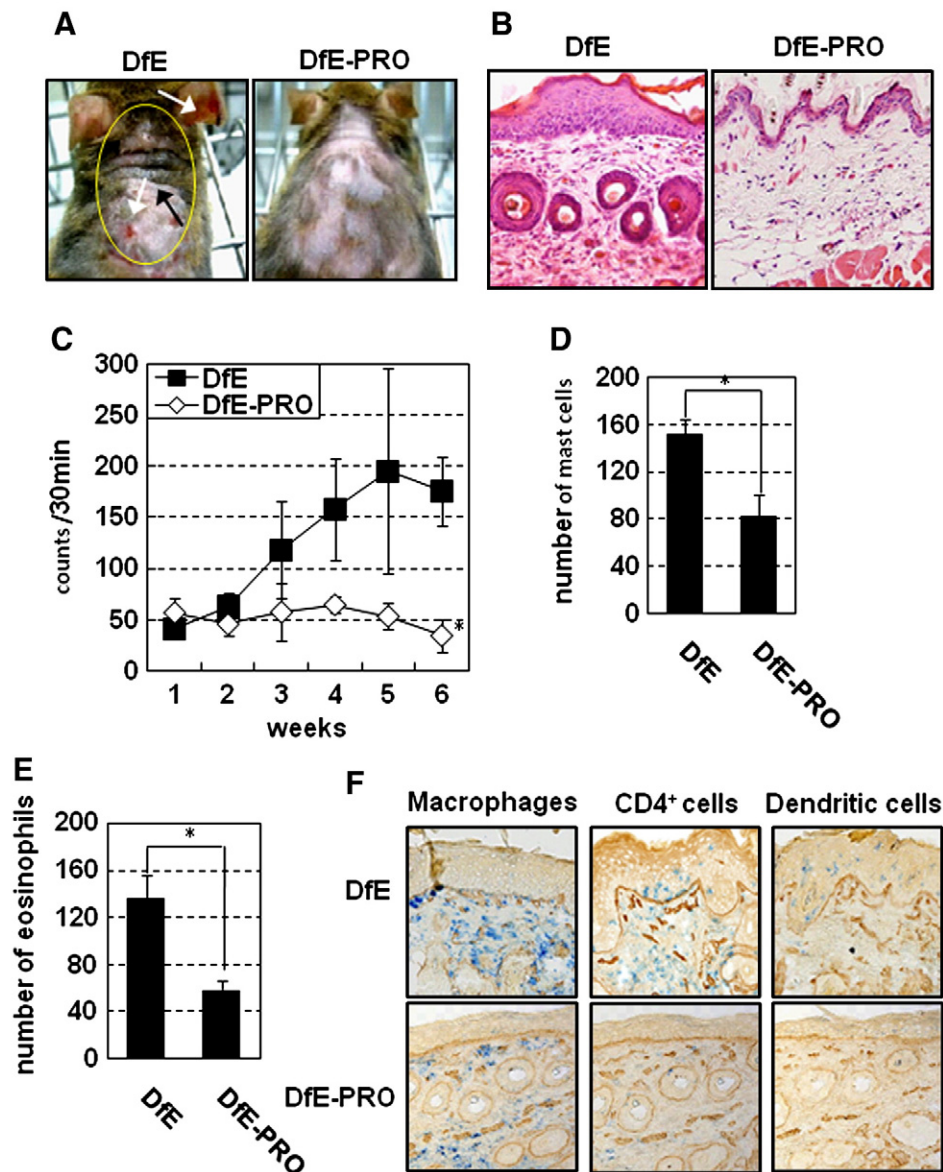


Fig. 4. Inhibition of DfE-induced atopic dermatitis in NC/Nga mice by tacrolimus ointment. (A) The effect of tacrolimus ointment /Protopic (PRO) on the features DfE-treated skin was assessed. Thirty minutes after DfE application tacrolimus ointment was applied for 2 weeks, tacrolimus ointment (PRO) was applied repeatedly after DfE application for 4 weeks. White arrows indicate erosion and black arrows indicate desquamation. (B) H&E staining of the back skin treated with DfE-treated or DfE plus tacrolimus ointment (PRO)-treated NC/Nga mice. Original magnification $\times 200$. (C) Effect of tacrolimus ointment (PRO) on scratching behavior of DfE-treated NC/Nga mice. Each data point represents the mean $\pm$ S.E. of 4 mice. $*p < 0.05$ when compared with MeE (Student's *t* test). (D, E) The effect of tacrolimus ointment (PRO) on the number of dermal mast cells (D) and eosinophils (E). Mast cells and eosinophils in the back skin of DfE-treated and DfE plus tacrolimus ointment (PRO)-treated NC/Nga mice were examined. Mast cells and eosinophils were stained with toluidine blue and Congo red, respectively, and counted using a microscope at a magnification of $\times 400$. Each data point represents the mean $\pm$ S.E. of 4 mice. $*p < 0.05$ when compared with DfE (Student's *t* test). (F) Macrophages, CD4-positive cells and dendritic cells were counted in the back skin of DfE-treated and DfE plus tacrolimus ointment (PRO)-treated NC/Nga mice. Frozen sections were immunostained with anti-CD68 antibody, anti-CD4 antibody, or anti-CD205 antibody to detect macrophages, CD4-positive cells, and dendritic cells, respectively. Positively stained cells exhibit a blue color. Original magnification $\times 200$.

atopic dermatitis patients has high titers of specific IgE to *Dermatophagoides* mites. Therefore, DfE-induced AD-like NC/Nga mice are thought to be an excellent mouse model to understand AD in humans. Previously, Matsuoka et al. performed swabbing with DfE under conventional conditions [24] to develop AD-like NC/Nga, but the present study was performed under SPF conditions to avoid the risk of spontaneous dermatitis and exposure to unexpected environmental factors. In the present system, the phenomena of AD, such as IgE hyperproduction, hyperplasia, desquamation, erosion, and hemorrhage, began to appear in NC/Nga mice at 4 weeks. Histologically, 7 weeks after DfE treatment, hyperplasia of epidermis and dermis was observed in the skin lesions (Fig. 1B). These changes were considered

to be associated with the infiltration of inflammatory cells into the dermis. Actually, massive infiltration of mast cells, eosinophils, macrophages, CD4⁺ T cells, and dendritic cells was observed in DfE-induced dermatitis (Fig. 1D, E, F). Allergens invading the epidermis are known to be engulfed by Langerhans cells [31]. Thereafter, epidermal Langerhans cells work as antigen presenting cells that induce sensitization to the antigen. The activated Th2-type cells release cytokines including IL-4 and IL-13. Then, the Th2 cytokines induce the proliferation and activation of mast cells and eosinophils in skin inflammation [6,22].

While chemokines are necessary for infiltration of the inflammatory cells into the skin lesion, the expression of chemokines in DfE-

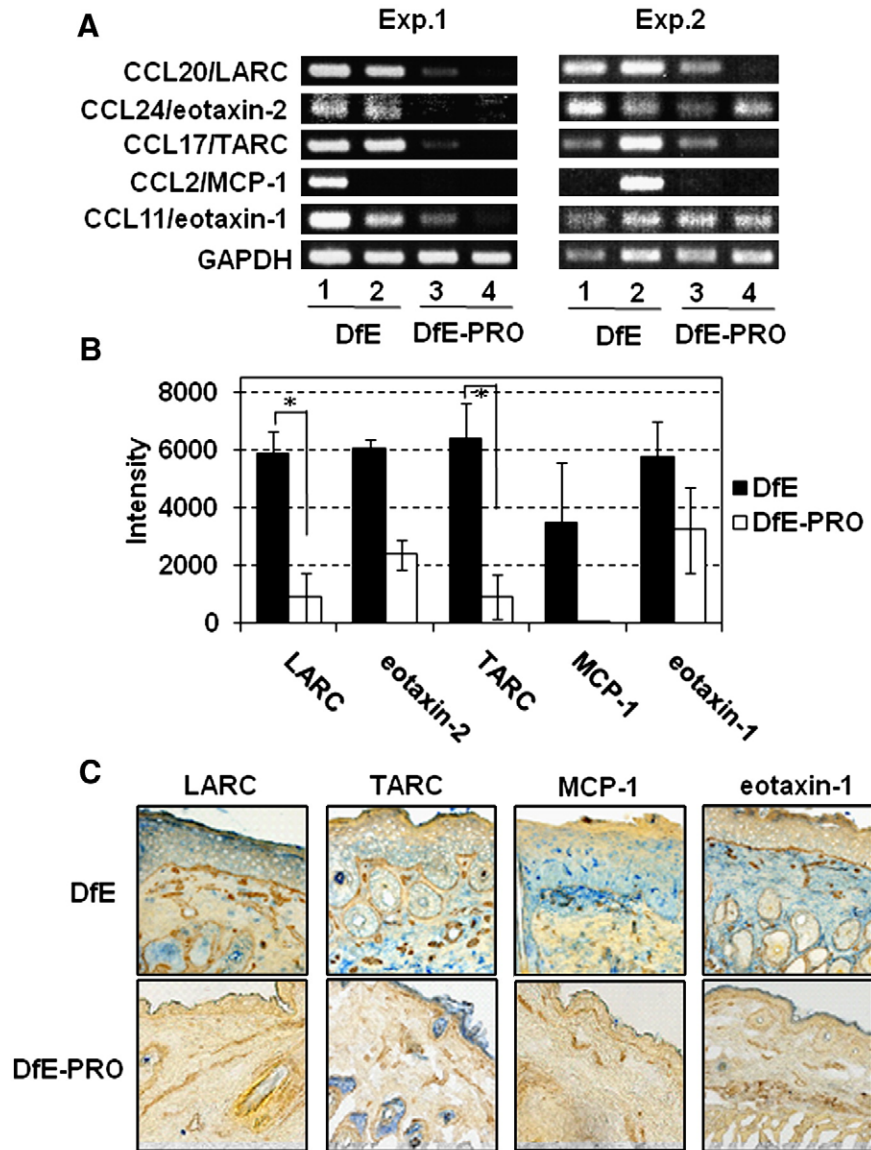


Fig. 5. Inhibition of DfE-induced expression levels of CCL20, CCL24, CCL17, CCL2, and CCL11 in the back skin of NC/Nga mice treated with tacrolimus ointment. (A) The effect of tacrolimus ointment/Protopic (PRO) on DfE-induced mRNA expression levels of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 was assessed. After DfE application for 2 weeks, tacrolimus ointment (PRO) was applied repeatedly 30 min after DfE application, for 4 weeks. Total RNA was prepared from the back skin of DfE-treated and DfE plus tacrolimus ointment (PRO)-treated NC/Nga mice, and RT-PCR was performed. Typical RT-PCR results are shown from three independent analyses. (B) The intensity of PCR bands was measured by NIH ImageJ program. Each data point represents the mean $\pm$ S.E. of 4 mice. * $p < 0.05$ when compared with DfE (Student's *t* test). (C) The effects of tacrolimus ointment (PRO) on the expression levels of CCL20/LARC, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 in skin lesions are shown. Frozen sections of the back skin treated with DfE and DfE plus tacrolimus ointment (PRO) were immunostained with anti-CCL20/LARC antibody, anti-CCL17/TARC antibody, anti-CCL2/MCP-1 antibody, or anti-CCL11/eotaxin-1 antibody. The blue color indicates positive staining for chemokines. Original magnification $\times 200$.

induced dermatitis was investigated, since no such experiments have been tested so far [28]. We found that various chemokines were highly expressed in the skin lesions of DfE-treated mice by DNA microarray assays (Fig. 2). In particular, the present study focused on five kinds of chemokines, CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1. Previously, it has been reported that CCL2/MCP-1 is involved in the recruitment of monocytes, memory T lymphocytes, and natural killer cells [32–34]. While CCL11/eotaxin-1 and CCL24/eotaxin-2 have been shown to be potent eosinophils chemoattractants [35–37], they all utilize a common receptor, CCR3, which is highly expressed on eosinophils and also on mast cells, basophils, and a subpopulation of Th2 lymphocytes [17,38,39]. In AD-like NC/Nga mice, significant infiltration of macrophages, mast cells, and eosinophils into the skin lesions was observed (Fig. 1D, E, F), and high expression levels

of CCL2/MCP-1, CCL11/eotaxin-1, and CCL24/eotaxin-2 were also detected (Figs. 2 and 3). Namely, these data imply that the above chemokines potentially induce tissue inflammation through the infiltration of macrophages, mast cells, and eosinophils. CCL17/TARC is a ligand of CCR4, which is predominantly expressed on Th2 lymphocytes, basophils, and natural killer cells [40–42]. Interestingly, a previous report showed that the expression of CCL17/TARC in keratinocytes was increased in the skin lesions of AD-like mouse model [22,43]. Furthermore, Liddiard et al. showed that activated monocytes and macrophages produce CCL17/TARC at local inflammation sites [44]. CCL20/LARC is the only chemokine known to interact with CCR6 [45]. By the production of CCL20/LARC, immature dendritic cells, CD4⁺ T lymphocytes, and B lymphocytes migrate to the site of inflammation to encounter the invading pathogens [46]. It has been reported that

CCL20/LARC expression by airway epithelium cells is inducible by TNF- α , IL-1 α , and Th2 cytokines [47], and the level of CCL20/LARC is significantly higher in bronchoalveolar lavage fluid from patients with cystic fibrosis [47]. Although their detailed induction mechanism is still unknown, it was demonstrated here that high expression levels of chemokines, including CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1, were observed in DfE-induced AD in NC/Nga mice, implying their important role in the accumulation of inflammatory responses.

Tacrolimus ointment/Protopic is a topical calcineurin inhibitor that was developed specially for the treatment of atopic dermatitis [28,29]. Tacrolimus ointment/Protopic has been found to exhibit potent inhibitory effects on spontaneously developed dermatitis [48]. Therefore, the present study aimed to examine the effect of Tacrolimus ointment/Protopic on DfE-induced AD in NC/Nga mice, particularly emphasizing to investigate the effect on the chemokine gene expression and production. In conclusion, we revealed that histological changes such as epidermal thickening and infiltration of inflammatory cells were significantly inhibited by the application of Tacrolimus ointment/Protopic, which was mainly due to the inhibition of chemokine production, that is, the expression levels of CCL20/LARC, CCL24/eotaxin-2, CCL17/TARC, CCL2/MCP-1, and CCL11/eotaxin-1 in the skin lesion were effectively reduced by Tacrolimus ointment/Protopic in the AD model mice. We proposed that the results of the present study will be useful for the elucidation of the pathogenic mechanism and therapeutic targets in AD.

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