

Topical Application of FK506 (Tacrolimus) Ointment Inhibits Mite Antigen-Induced Dermatitis by Local Action in NC/Nga Mice

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Key Words

FK506 · Mite antigen · T helper 2 cells · Atopic dermatitis · Lymph node · NC/Nga mice

Abstract

Background: FK506 ointment (tacrolimus ointment, protopic) is a new drug therapeutically effective for patients with atopic dermatitis (AD). However, the mechanism of action of FK506 ointment on AD is not fully understood.

Methods: We examined the effect of FK506 ointment on mite antigen-induced dermatitis in NC/Nga mice. Clinical symptoms and ear thickness were recorded, and histopathological studies and in vitro analyses were performed. **Results:** Topical application of FK506 ointment (0.03–0.3%) suppressed the development of dermatitis. In the lesional skin, both interleukin (IL)-4 and interferon (IFN)- γ were detected, even though the IL-4+/IFN- γ - T helper 2 (Th2) population was predominant in the regional lymph nodes (LNs). Topical application of FK506 treatment reduced the elevated level of both IL-4 and IFN- γ in the skin, but did not decrease the expansion of the Th2 population in the LNs. **Conclusions:** Topical application of FK506 ointment suppresses dermatitis by inhibiting the activation of inflammatory cells locally, without systemic immune suppression, in this AD model.

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Introduction

Mite antigen (Ag) is a major environmental factor in atopic dermatitis (AD), with 70% of AD patients positive for skin patch tests against mite Ag [1]. Repeated environmental exposures to mite Ag are thought to maintain or exacerbate the AD symptoms, and symptoms are often improved when mites have been removed [2].

NC/Nga mice are a good model for AD, with environmental factors producing dermatitis in them. NC/Nga mice in conventional conditions, without air control, suffer from AD-like skin lesions, but these mice do not show any clinical symptoms under specific pathogen-free (SPF) conditions [3]. A recent study demonstrated that infection with fur mites could provoke dermatitis in NC/Kuj mice, a substrain of NC/Nga mice [4]. To analyze the pathogenesis of AD, we previously established a mite Ag-induced AD model with NC/Nga mice under SPF conditions [5].

Intracellular adhesion molecule-1 (ICAM-1, CD54) is expressed on Ag-presenting cells, keratinocytes and endothelial cells, and is required for infiltration and activation of leukocytes [6]. Vascular cell adhesion molecule-1 (VCAM-1, CD105) is also expressed on endothelial cells, and is required for infiltration of eosinophils. Tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and interferon (IFN)- γ induce ICAM-1 expression, and TNF- α , IL-1 β and IL-4 produce VCAM-1 expression on endothelial cells. Our mite Ag-induced AD model with NC/Nga mice shows severe infiltration of inflammatory cells, especially

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infiltration of eosinophils. Expression of the adhesion molecules may explain the severe inflammation.

FK506, a hydrophobic macrolide lactone produced by *Streptomyces tsukubaensis*, exerts profound immunosuppressive effects in vitro and in vivo [7, 8]. FK506-FKBP12 complex suppresses the activation of T cells by inhibiting calcineurin phosphatase activity. Topical application of FK506 ointment exerts good therapeutic efficacy in atopic eczema [9]. FK506 ointment penetrates through skin [10], and clinical studies have shown that FK506 reduces cytokine production and infiltration of T helper (Th) cells in the skin of AD patients [11]. Treatment with FK506 ointment in the conventional NC/Nga model also produces a decreased level of clinical signs and the reduction of Th2 cytokines in the skin [12]. However, whether FK506 ointment affects systemic immune modulation or acts only locally in the treated skin is still unclear.

The aim of this study was to analyze the mechanisms of topical FK506 in vivo. For this purpose, we analyzed the effect of FK506 ointment on cytokine production and adhesion molecule and T cell activation in the skin, then compared these results to those in the regional lymph nodes (LNs) in NC/Nga mice.

Materials and Methods

Mice

SPF NC/Nga female mice (15–20 g) at the age of 6 or 7 weeks were purchased from Charles River Japan (Yokohama, Japan). The animals were maintained in groups of 4 for at least 7 days on a 12-hour light-dark cycle (lights on from 07.00 to 19.00 h) in a controlled temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) environment. The mice were given standard laboratory food and tap water ad libitum before the experiment. All conditions and handling of the animals were approved by the Japanese Society of Laboratory Animals. In addition, all animal experimental procedures were performed according to the guidelines of the Animal Experiment Committee of Fujisawa Pharmaceutical Co. Ltd.

Reagents

FK506 ointment (0.03–0.3%) and placebo were obtained from Fujisawa Pharmaceutical (Osaka, Japan). Corticosteroid ointment, betamethasone valerate (Rinderon®-V ointment, 0.12%), was purchased from Shionogi (Osaka, Japan).

Cytokines and Antibodies

Monoclonal antibodies (mAbs) for mouse CD4 (PharMingen, San Diego, Calif., USA), IL-4 (PharMingen), IFN- γ (PharMingen), ICAM-1 (Seikagaku Corp., Tokyo, Japan), VCAM-1 (PharMingen), TNF- α (PharMingen) and IL-1 β (R & D, Minneapolis, Minn., USA; Genzyme, Cambridge, Mass., USA) were used for immunohistochemistry. Abs were diluted to 0.5, 25, 10, 5, 5, 10 and 1 $\mu\text{g/ml}$, respectively, and used for immunostaining. Fluorescein isothiocya-

nate (FITC)-conjugated mAbs for mouse T cell receptor (TCR) $\alpha\beta$, CD4, CD8 α , CD69, Mac-1 (CD11b), B220 (CD45R) and IFN- γ , and phycoerythrin (PE)-conjugated mAb for mouse IL-4 were purchased from PharMingen. Isotypes of both FITC-conjugated rat IgG1 and PE-conjugated rat IgG1 as negative controls were also obtained from PharMingen. All Abs were prepared for flow cytometric analysis at a concentration of 20 $\mu\text{g/ml}$.

Repeat Application of Mite Ag

Mice were immunized as described previously [5]. Briefly, NC/Nga mice were injected intradermally with saline or 5 $\mu\text{g}/10 \mu\text{l}$ of *Dermatophagoides pteronyssinus* (*Dp*) extract (Cosmo Bio LSL, Tokyo, Japan) dissolved in saline, on the ventral side of their right ear on days 0, 2, 4, 7, 9, 11, 14 and 16. 10 μl of drugs were applied onto the dorsal side of the *Dp*-treated ears 3 h before and after *Dp* treatment. The thickness of the ear injected with *Dp* was measured by an ear thickness gauge (Ozaki Mfg, Osaka, Japan) before the first injection and 24 h after each intradermal injection.

Flow Cytometric Analysis

Flow cytometric analysis was performed as described previously [5]. Briefly, freshly isolated LN cells were incubated with mAbs for TCR $\alpha\beta$, CD4, CD8 α , CD69, Mac-1 or B220 for 30 min at 4°C and analyzed by flow cytometry (FACScan, Becton Dickinson, Bedford, Mass., USA). To examine the populations of IL-4- and IFN- γ -producing cells in regional LNs, cell preparations were incubated with *Dp* (10 $\mu\text{g/ml}$) for 19 h and treated with a CytoStain™ Kit (PharMingen). First, cells were treated with GolgiStop™ (contains monensin) for 5 h. After removing the culture medium, cells were fixed and permeabilized with Cytotfix/Cytoperm™ solution (includes formaldehyde) for 20 min at 4°C . Cells were washed and resuspended with Perm/Wash™ solution (includes saponin). Then, cells were harvested and stained with FITC-conjugated anti-mouse IFN- γ and PE-conjugated anti-mouse IL-4 Ab for 30 min at 4°C . After washing twice with Perm/Wash solution, cells were suspended in staining buffer, then analyzed by flow cytometry.

Histological Analysis

Histological analysis was performed as described previously [5]. Briefly, right ears of *Dp*-injected and sham-injected mice were removed 2 days after the last injection of *Dp*. A part of the ear was fixed in 10% phosphate-buffered formalin (pH 7.2) and embedded in paraffin, and 3- μm sections were cut. Then those samples were stained with toluidine blue (pH 4.0) for detection of mast cells or congo red for eosinophils. The other part of the ear was embedded in OCT compound (Miles, Elkhart, Ind., USA) and snap frozen in dry ice ethanol for immunohistochemistry. Frozen 5- μm sections were fixed in cold acetone. After blocking of endogenous peroxidase, mAb for mouse CD4, IL-4, IFN- γ , ICAM-1, VCAM-1, TNF- α or IL-1 β was applied overnight at 4°C , and then goat anti-rat IgG conjugated with peroxidase (Jackson, West Grove, Pa., USA) was overlaid. Visualization of the enzyme reaction was performed with 3-amino-9-ethylcarbazole (Aldrich Chemical Company, Milwaukee, Wisc., USA).

Statistical Analysis

Data are expressed as mean $\pm$ SE. Statistical significance of differences was assessed by Dunnett's multiple comparison test following one-way analysis of variance and Student's t test for two-sample comparison. $p < 0.05$ was taken as the level of significance.

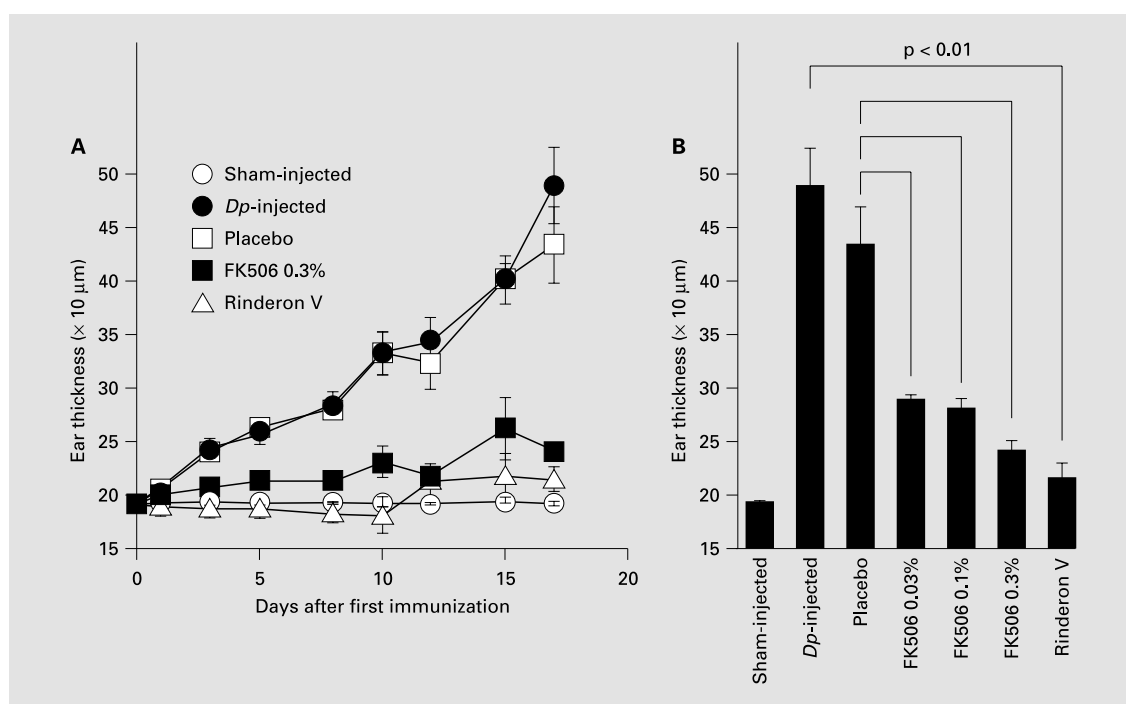


Fig. 1. FK506 ointment and steroids reduced the ear thickness of *Dp*-injected mice. NC/Nga mice were injected with *Dp* into the right ear on day 0, 2, 4, 7, 9, 11, 14 and 16. **A** Ear thickness of the right ear was evaluated by a thickness gauge 24 h after each injection. The ears of age-matched sham-injected mice were measured at the same time points as those of *Dp*-injected mice. Placebo, FK506 ointment or betamethasone valerate was applied 3 h before and after each *Dp* injection. Each data point represents the mean $\pm$ SE of 10 mice. These data are representative of 8 separate experiments. **B** Ear thickness of the right ear on day 17. Dunnett's multiple comparison was performed for statistical analysis.

Results

Effect of FK506 Ointment on Development of Dermatitis

To examine the effect of FK506 on the development of AD, we observed the clinical signs of *Dp*-injected NC/Nga mice with or without FK506 ointment treatment. *Dp* extract was injected into the ventral side of the right ears of NC/Nga mice on day 0, 2, 4, 7, 9, 11, 14 and 16, and the ear was measured 24 h after *Dp* treatment. Thickening of the ear injected with *Dp* was observed on day 5, and the thickness gradually increased until day 17 (fig. 1A). Erythema, edema, excoriation and scaling were also observed on the ear (fig. 2B). When mice were treated on the dorsal side with FK506 ointment 3 h before and after *Dp* treatment, the treated ear showed a significant decrease in thickness. On day 17, the ear thickness of *Dp*-injected mice treated with 0.03, 0.1 and 0.3% FK506 ointment was reduced by 67, 71 and 83%, respectively, compared to that of *Dp*-injected, placebo-treated mice ($487.5 \pm$

$35.6 \mu\text{m}$; fig. 1). Only slight erythema and edema, and neither scaling nor excoriation were observed on the ears treated with FK506 ointment (fig. 2C). *Dp*-injected, betamethasone ointment-treated mice did not show any clinical symptoms or ear thickening until day 17 (fig. 1, 2D).

Histopathological Analysis of the Dermatitis

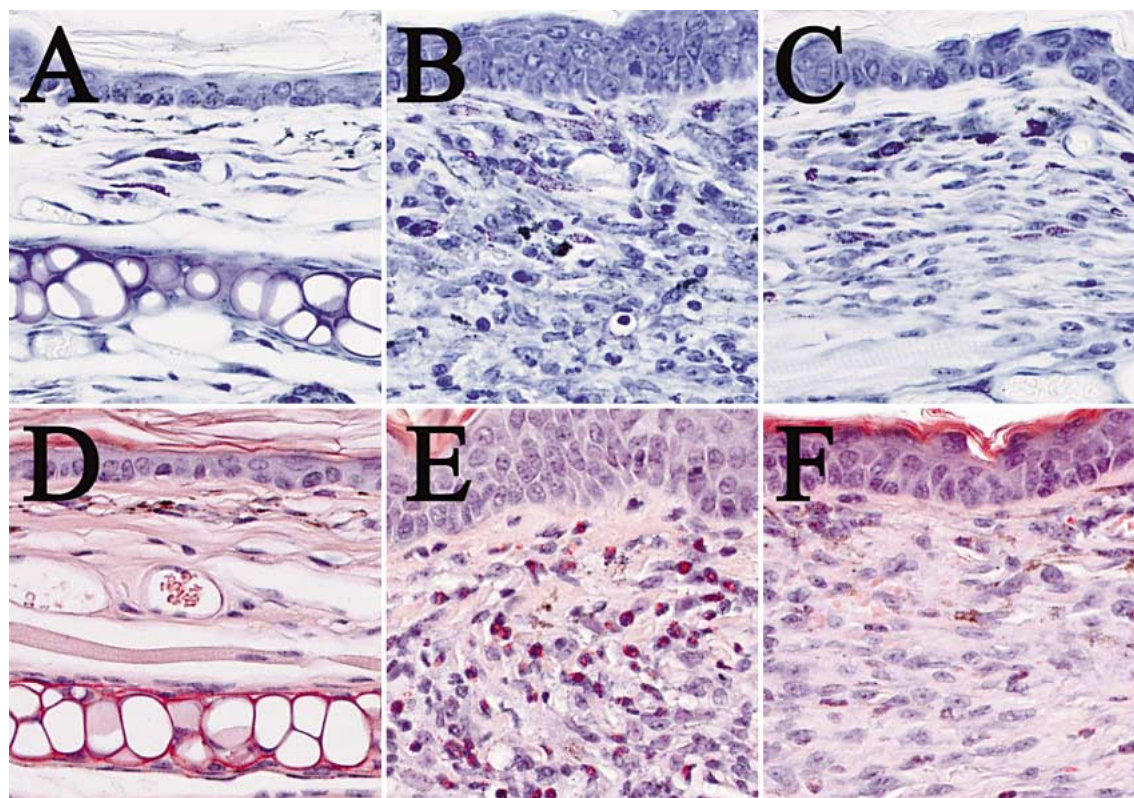
Histopathological examination of the skin lesions was performed on day 17. Congo red staining showed severe infiltration of eosinophils into the dermis of placebo-treated ears injected with *Dp*, and treatment with FK506 ointment produced a decreased eosinophil infiltration (fig. 3E, F). Most of the mast cells in the skin lesions of *Dp*-injected, placebo-treated mice were degranulated; however, degranulation of mast cells in ears treated with FK506 appeared to be relatively mild (fig. 3B, C). Treatment with steroids also demonstrated decreased eosinophil infiltration and mild degranulation of mast cells (data not shown).

Fig. 2. Topical application of FK506 ointment or steroids improved the clinical features of dermatitis in NC/Nga mice. Shown are ears of age-matched sham-injected mice (**A**), *Dp*-injected mice (**B**), *Dp*-injected, FK506 0.3% ointment-treated mice (**C**) or *Dp*-injected, betamethasone valerate-treated mice (**D**) on day 17. NC/Nga mice were injected with *Dp* on day 0, 2, 4, 7, 9, 11, 14 and 16. FK506 ointment or steroid was applied 3 h before and after each *Dp* injection. These results are representative of three separate experiments.

Fig. 3. FK506 ointment ameliorated the degranulation of mast cells and infiltration of eosinophils in *Dp*-injected mice. Sham-injected (**A**, **D**), *Dp*-injected (**B**, **E**) and *Dp*-injected, FK506 0.3% ointment-treated (**C**, **F**) ears on day 17 were analyzed with toluidine blue staining (**A–C**) or congo red staining (**D–F**). Sections were observed at a magnification of $\times 400$. These results are representative of three separate experiments.



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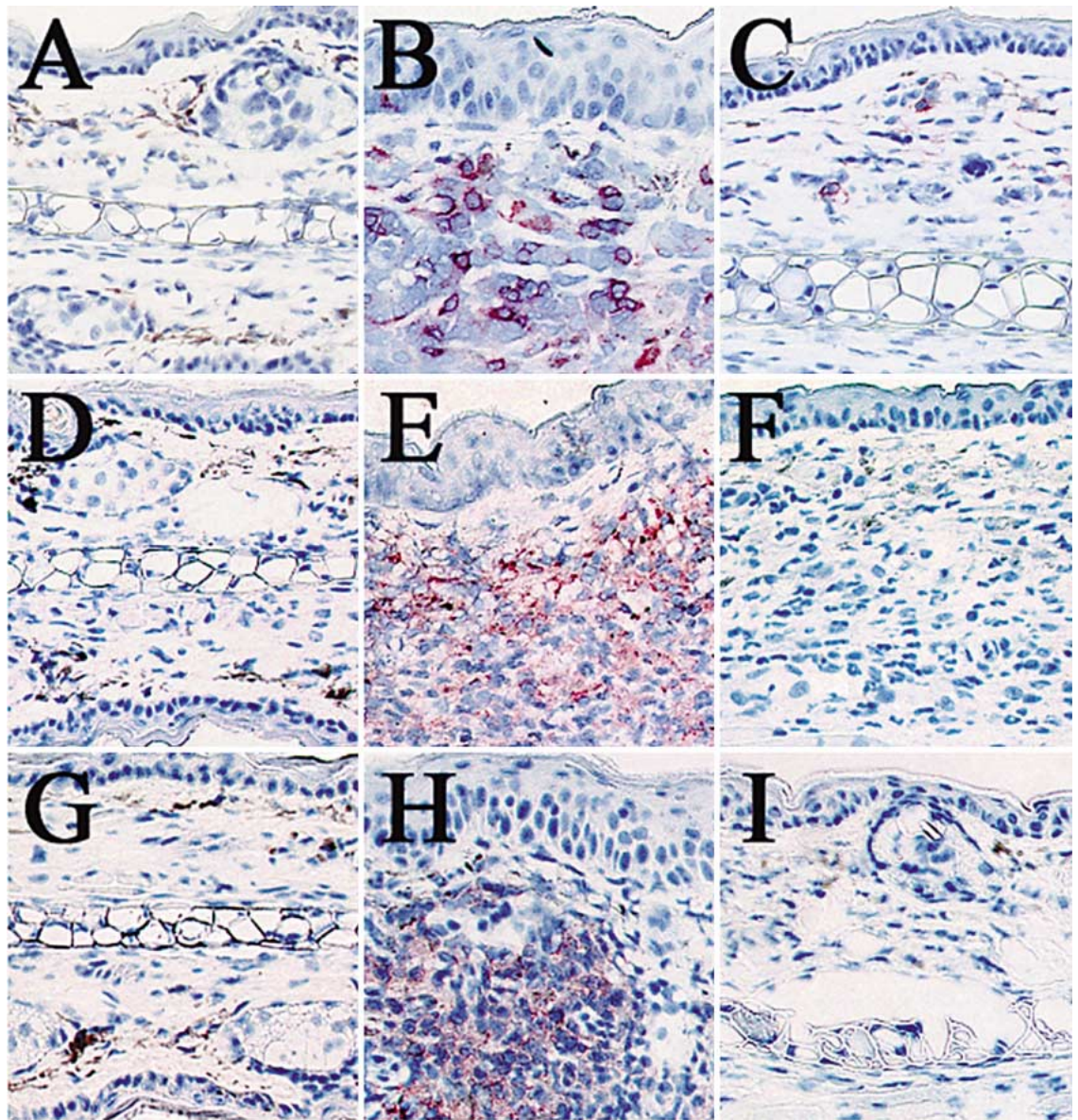


Fig. 4. FK506 ointment suppressed infiltration of Th cells and cytokine production. Sham-injected (**A, D, G**), *Dp*-injected (**B, E, H**) and *Dp*-injected, FK506 0.3% ointment-treated (**C, F, I**) ears on day 17 were stained with anti-CD4 mAb (**A–C**), anti-IL-4 mAb (**D–F**) or anti-IFN- γ mAb (**G–I**). Sections were observed at a magnification of $\times 200$. These results are representative of three separate experiments.

Next, we assessed the lesional skin by immunohistochemical analysis. There was an increased number of CD4+ cells in skin lesions of *Dp*-injected, placebo-treated mice. FK506 ointment reduced the infiltration of CD4+ cells (fig. 4B, C). IL-4 and IFN- γ were also strongly stained in the dermis of *Dp*-injected, placebo-treated mice, but not in that of sham-injected mice. FK506 treatment reduced the staining of both IL-4 and IFN- γ

(fig. 4F, I). Steroid treatment also reduced the infiltration of CD4+ cells and staining of both IL-4 and IFN- γ (data not shown).

We investigated the expression of ICAM-1 and VCAM-1 in *Dp*-injected, placebo-treated skin, and compared it to that in sham-injected skin. Both ICAM-1 and VCAM-1 were expressed in the dermis of sham-injected mice, with expression of both increased in *Dp*-injected,

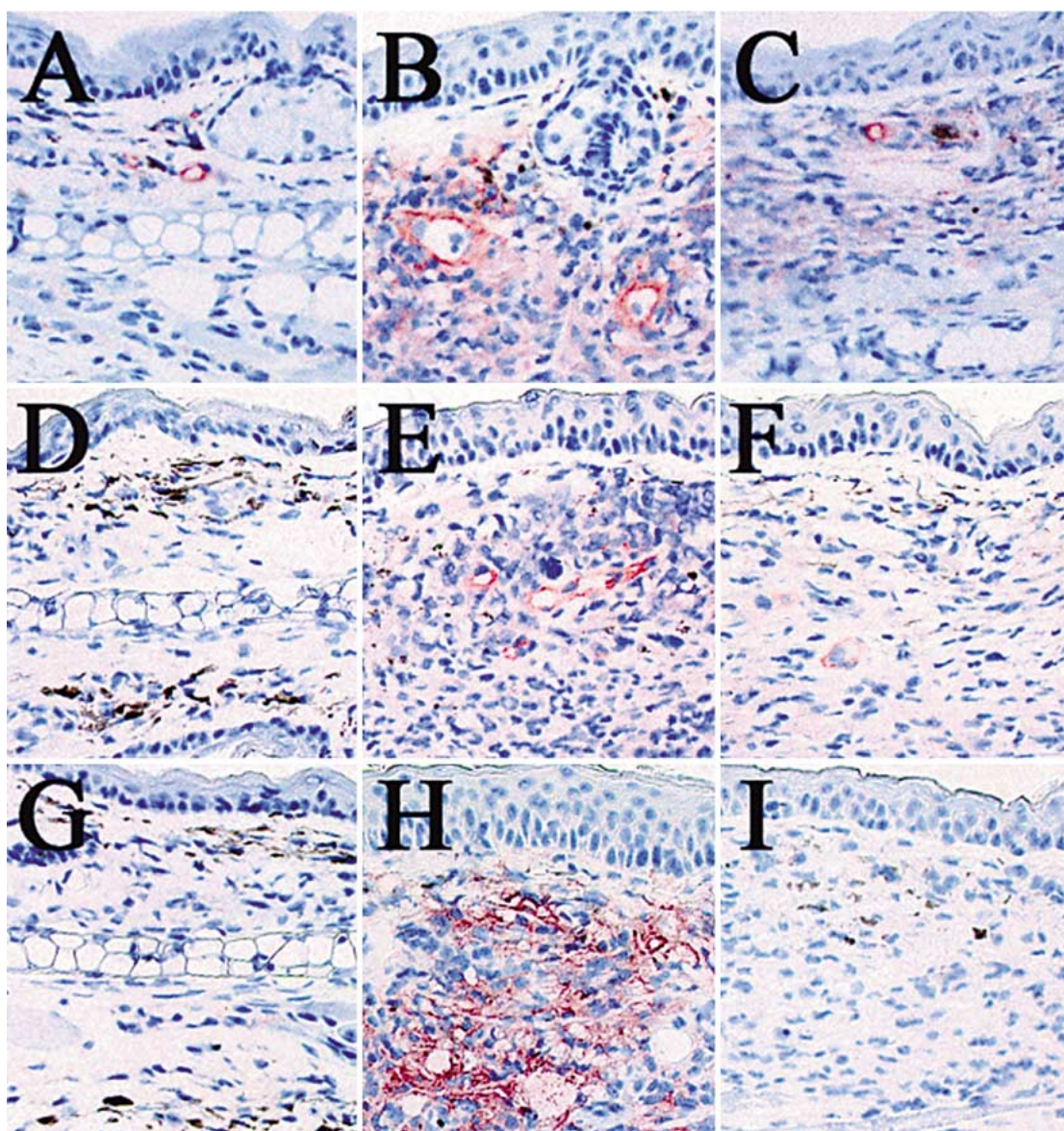


Fig. 5. FK506 ointment inhibited the expression of adhesion molecules and TNF- α . Sham-injected (**A, D, G**), *Dp*-injected (**B, E, H**) and *Dp*-injected, FK506 0.3% ointment-treated (**C, F, I**) ears on day 17 were stained with anti-ICAM-1 mAb (**A–C**), anti-VCAM-1 mAb (**D–F**) and anti-TNF- α mAb (**G–I**). Sections were observed at a magnification of $\times 200$. These results are representative of three separate experiments.

placebo-treated mice (fig. 5B, E). Treatment with FK506 ointment reduced the expression of ICAM-1 and VCAM-1 (fig. 5C, F). TNF- α was detected in the dermis of *Dp*-injected, placebo-treated mice, but not in the dermis of sham-injected mice (fig. 5G, H). FK506 ointment reduced the expression of TNF- α in the dermis (fig. 5I).

Effect of FK506 on Reactivity of LN Cells

To determine the relationship between dermatitis and regional LNs, we investigated the cell number and cell population of regional LNs in *Dp*-injected, placebo-treated mice, and compared them to those in *Dp*-injected, FK506-treated mice. The LN cell number for *Dp*-injected, placebo-treated mice was markedly increased compared to sham-injected mice (fig. 6). The LN cell number for

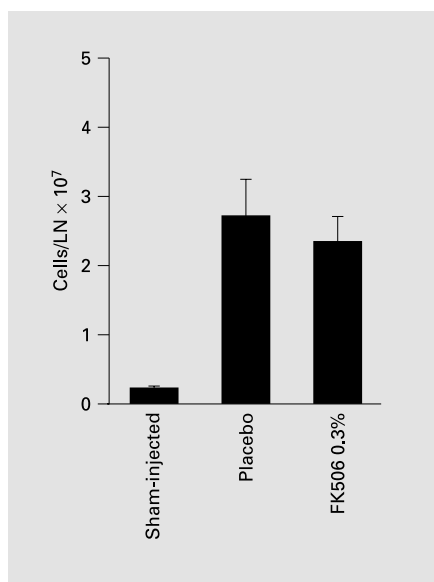


Fig. 6. FK506 ointment did not change the cell number in regional LNs. LNs of sham-injected mice, *Dp*-injected, placebo-treated (placebo) mice and *Dp*-injected, FK506 0.3% ointment-treated (FK506 0.3%) mice were collected and homogenized to make a single cell suspension. Freshly isolated LN cells were counted with 0.25% trypan blue solution. These data are representative of four separate experiments.

Dp-injected, FK506-treated mice was comparable to that of *Dp*-injected, placebo-treated mice. The cell number in the spleen was not different amongst the groups (data not shown).

Next, we analyzed freshly isolated LN by flow cytometry and compared the cell populations (table 1). The CD69+ population in *Dp*-injected, placebo-treated mice was enhanced compared to that in sham-injected mice. An analogous enhancement was observed in *Dp*-injected, FK506-treated mice. The absolute number of TCRαβ+ cells from both *Dp*-injected, placebo-treated mice and *Dp*-injected, FK506-treated mice was increased due to the increase in LN cells (fig. 6, table 1).

Topical application of FK506 ointment did not diminish either the cell expansion or cell activation in LNs. Next, we analyzed the Th1 to Th2 ratio in LNs and compared *Dp*-injected, FK506-treated mice with *Dp*-injected, placebo-treated mice (table 2). Regional LN cells were incubated with *Dp* for 19 h, and intracellular IL-4 and IFN-γ were evaluated by flow cytometric analysis. The *Dp*-specific IL-4+/IFN-γ- Th2 population in *Dp*-injected, placebo-treated mice ($6.78 \pm 0.93\%$) was greater than that in sham-injected mice ($0.66 \pm 0.2\%$). The *Dp*-spe-

Table 1. Cell population in LNs

Cell type	Untreated	Placebo	FK506 0.3%
TCRαβ, %	64.24 ± 2.27	39.56 ± 4.30	37.26 ± 3.50
CD4, %	44.02 ± 3.14	27.55 ± 1.73	26.22 ± 2.68
CD8α, %	12.31 ± 6.12	18.43 ± 0.03	13.96 ± 0.41
CD69, %	7.86 ± 1.58	23.08 ± 1.87	25.54 ± 3.41
Mac-1, %	5.95 ± 2.58	1.06 ± 0.53	1.28 ± 0.59
B220, %	32.57 ± 4.48	59.59 ± 5.27	60.53 ± 6.32

LNs were freshly obtained on day 17. LNs of one group were collected as one population of LN cells. The cells were stained with FITC-conjugated mAb and cell preparations were analyzed by a flow cytometer. The percentage of positive cells was calculated from the control. Data are presented as mean ± SE of three individual experiments.

Table 2. The Th1/Th2 population in LNs

Cell type	Untreated	Placebo	FK506 0.3%
IL-4-/IFN-γ-, %	99.22 ± 0.19	93.12 ± 0.91	93.14 ± 1.79
IL-4+/IFN-γ-, %	0.66 ± 0.20*	6.78 ± 0.93	6.71 ± 1.75
IL-4-/IFN-γ+, %	0.02 ± 0.02	0.01 ± 0.01	0.03 ± 0.02
IL-4+/IFN-γ+, %	0.09 ± 0.05	0.09 ± 0.02	0.11 ± 0.04

LNs were isolated from mice on day 17. LNs of one group were collected as one population of LN cells. LN cells were stimulated with *Dp* (10 μg/ml) for 19 h. Then, cells were harvested and stained with FITC-conjugated anti-mouse IFN-γ mAb and PE-conjugated anti-mouse IL-4 mAb, and analyzed by FACScan. Data are presented as mean ± SE of three individual experiments. *p < 0.01 compared with the placebo group.

cific Th2 population in *Dp*-injected, FK506-treated mice ($6.71 \pm 1.75\%$) was equivalent to that in *Dp*-injected, placebo-treated mice. The *Dp*-specific IL-4-/IFN-γ+ Th1 populations in LN cells of *Dp*-injected, placebo-treated mice and *Dp*-injected, FK506-treated mice were not different compared to those in sham-injected mice (table 2).

Effect of FK506 on the Untreated Ear

Though FK506 ointment did not modulate cell expansion, cell population or the Th1 to Th2 ratio in drainage LNs, it was effective on clinical signs and histopathological changes of the skin in this model. We hypothesized that FK506 ointment acts locally and does not exhibit a systemic effect by diffusion into the blood. To determine the local action of FK506 ointment, we examined skin

lesions in *Dp*-injected mice treated with FK506 on the contralateral ear. The thickness of the *Dp*-injected ear of mice treated with FK506 on the contralateral ear was similar to that in *Dp*-injected, placebo-treated mice (fig. 7). Clinical signs and histopathology of *Dp*-injected ears of mice treated with FK506 on the contralateral ear developed similarly to those in *Dp*-injected, placebo-treated mice.

Discussion

In this study, we demonstrated that FK506 ointment as well as steroid treatment were effective in our newly developed mite Ag-induced dermatitis model in NC/Nga mice [5]. FK506 ointment reduced erythema, edema, scaling and excoriation. The effect of FK506 ointment could be exerted by topical mechanisms without any systemic immune suppression.

Histopathological study showed that FK506 ointment reduced the infiltration of CD4⁺ T cells and secretion of IL-4 and IFN- γ in the dermis of NC/Nga mice with mite Ag-induced dermatitis. CD4, IL-4 and IFN- γ were detected in the lesional skin of this model. However, our previous study showed that there was only a small population of IFN- γ + cells in LNs [5]. In AD patients, IFN- γ has also been detected in severe eczema [13], but decreased production of IFN- γ from peripheral blood mononuclear cells has been observed [14]. IFN- γ in the skin closely correlates to delayed-type hypersensitivity, which exacerbates AD symptoms. However, systemic treatment with IFN- γ decreases the dermatitis of NC/Nga mice [15]. Thus, IFN- γ may have different roles in the local skin and the systemic immune system. It is possible that the suppression of local IFN- γ together with IL-4 by FK506 treatment may contribute to improvement of dermatitis. FK506 ointment also decreased CD4⁺ T cells in the local skin, suggesting that FK506 ointment inhibits both the infiltration and activation of CD4⁺ T cells in this model.

FK506 ointment reduced the infiltration of eosinophils and expression of ICAM-1 and VCAM-1, important adhesion molecules for eosinophil infiltration, in the dermis. Expression of ICAM-1 and VCAM-1 increased in the lesional skin of this model, and also occurs in the injured skin of AD patients. Although TNF- α and IL-1 β are potent inducers of ICAM-1 and VCAM-1 in endothelial cells in vitro, we could detect TNF- α in this model (fig. 5), but not IL-1 β (data not shown). This suggests that TNF- α rather than IL-1 β might be important for the expression of ICAM-1 and VCAM-1 in this model. Since FK506

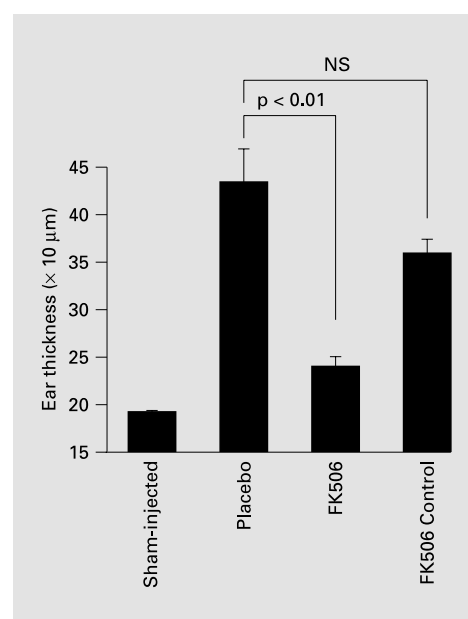


Fig. 7. Application of FK506 ointment to the contralateral ear did not affect the thickness of the Ag-treated ear. NC/Nga mice were treated and evaluated on day 17 as described in the legend to figure 1. Sham-injected mice, *Dp*-injected, placebo-treated (placebo) mice, *Dp*-injected, FK506 0.3% ointment-treated (FK506) mice and *Dp*-injected mice with FK506 0.3% ointment on the contralateral ear (FK506 Cont.) were investigated. Drugs were applied 3 h before and after each *Dp* injection. Each data point represents the mean $\pm$ SE of 10 mice. These data are representative of three separate experiments. NS = Not significant.

ointment suppressed TNF- α production and the infiltration of CD4⁺ T cells (potent producers of TNF- α) in the dermis, the suppressive effect on the expression of adhesion molecules could be attributed to inhibition of cytokine production and/or T cell migration.

Application of FK506 ointment to the contralateral ear did not suppress the increase in ear thickness of the Ag-treated ear, suggesting that FK506 ointment exerted a localized effect on the area of application under our experimental conditions. It is unlikely that the effect of FK506 ointment applied to the ear was due to oral uptake of the ointment by licking, since oral administration of FK506 was only effective at more than 10 mg/kg (data not shown), about 7 times more than the content of FK506 in the applied ointment. More importantly, treatment with FK506 ointment did not affect regional LN cells. Both CD69⁺ and IL-4⁺/IFN- γ ⁺ Th2 populations in LNs increased after stimulation with *Dp*, and FK506 did not reduce this increase. These results indicate that topical application with FK506 ointment acts only locally under

our experimental conditions. It has been reported that topical FK506 decreases the expression of FcεRI and CD36 on CD1a+ epidermal dendritic cells in AD patients [16], indicating that topical FK506 treatment lowers the level of Ag uptake by dendritic cells. This suggests that topical FK506 treatment may modulate the immune response in LNs in clinical settings, since dendritic cells taking up Ag migrate into LNs and control the immune response. We did not detect any significant effect of FK506 ointment on LN cells. Since we injected rather a large amount of Ag into the skin to establish the model, this may mask the effect of FK506 on dendritic cells.

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