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Decrease in circulating Th17 cells correlates with increased levels of CCL17, IgE and eosinophils in atopic dermatitis

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ABSTRACT

Background: Clinical significance of circulating $CD4^+$ T cell subsets, including T-helper (Th)1, Th2, Th17 and regulatory T (Treg) cells, in patients with atopic dermatitis (AD) remains unclear. No previous studies have simultaneously evaluated the four T cell subset profiles in AD.
Objective: The aim of the present study was to explore whether the percentage of these four subsets of $CD4^+$ T cells correlate to the severity parameters of AD patients.
Methods: Intracellular expression of interferon (IFN)-γ, interleukin (IL)-4, IL-17 and forkhead box P3 (Foxp3) in $CD4^+$ T cells was evaluated in peripheral blood mononuclear cells from normal controls and patient with AD as well as with chronic eczema using a flow cytometer. Serum CCL17 levels were measured as an objective severity parameter of AD together with percentage of eosinophils and serum IgE levels.
Results: In AD patients, the number of Th1 (IFN-γ^+) and Th17 (IL-17^+) subsets was significantly decreased, but that of Th2 (IL-4^+) and Treg (Foxp3^+) subsets was similar to that of normal controls. The T cell subset profiles of patients with chronic eczema were not different with those of normal controls. The frequency of Th17cells, particularly that of the IFN-γ^{nega}IL-17^+ subset, showed a significant negative correlation with CCL17, IgE and eosinophil levels in AD patients. This was, however, not the case in Th1, Th2 and Treg cells.
Conclusion: Decreased circulating Th17 cells might contribute to activity of AD.

1. Introduction

Atopic dermatitis (AD) is a common, chronic or chronically relapsing, severely pruritic eczematous skin disease mostly associated with hyperimmunoglobulinemia E and eosinophilia [1,2]. Specifically, AD is a diathetic and multifactorial disorder which also predisposes to bacterial and viral infections. A complex interaction between susceptibility genes encoding skin barrier molecules and markers of the inflammatory response, environmental factors, host condition, infectious agents, and specific immunologic responses are involved in the pathophysiology of AD [3]. The pivotal role of innate and adaptive immunity in the evolution and persistence of AD is currently fully appreciated. Recently, the subdivision of T cell subsets according to their cytokine-production and/or chemokine receptor expression profiles has revealed a new T-helper (Th) cell classification, namely, Th1, Th2, Th17, and regulatory T (Treg) cell subsets, that plays an important role in autoimmune, infectious and allergic disorders [3,4].

Th1 and Th2 cytokines may differentially contribute to the pathogenesis of acute and/or chronic lesions of AD. The majority of allergen-specific T cells derived from skin lesions that had been provoked by the epicutaneous application of inhalant allergens were found to produce predominantly Th2 cytokines, which was initially considered to be a specific feature reflecting immune dysregulation in AD [5]. However, the cytokine switch from Th2 in the acute phase to Th1 in the chronic phase is now generally accepted for AD and also appears to be relevant in allergic contact dermatitis [6].

Th17 cells have recently been proved to be involved in various autoimmune and inflammatory disorders as well as defense mechanisms against certain extracellular bacteria and fungi [4]. Attention has recently been drawn to a possible role of Th17 cells in allergic contact dermatitis or AD [7]. Interestingly, acute AD lesions showed more Th17 cells than chronic lesions, suggesting that interleukin (IL)-17 functions primarily in the acute Th2 phase rather than in the subsequent Th1-dominated chronic phase of AD [7–9]. Because Th17 cell differentiation is inhibited by the Th2 cytokine IL-4 [10], the question arises as to whether Th17 cells would develop in Th2 conditions. The role of Th17 cells in AD development remains very controversial considering the fact that Th17 cells are highly involved in the development and maintenance of psoriasis, which is classified into a completely different disease spectrum from AD [8].

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$CD4^+CD25^+$ T cells constitute 5–15% of peripheral $CD4^+$ T cells [11], which was referred to as natural Treg cells. However, only 1–3% of $CD4^+$ T cells express CD25 at high levels ($CD25high^+$), and only these cells have been shown to possess suppressor activity [12]. The transcription factor gene, forkhead box P3 (Foxp3), has been considered as one of the most reliable markers of $CD4^+CD25high^+$ Treg cells [13]. The frequency of circulating Treg cells in AD was controversial in previous reports [14–16].

Since the above-mentioned previous studies examined these four T cell subsets in different settings, it is difficult to compare the mutual relationship. In this study, we simultaneously measured Th1, Th2, Th17 and Treg populations in the peripheral blood mononuclear cells (PBMC) of AD patients and normal controls, and analyzed their correlation with the level of serum CCL17, an objective parameter of the disease activity of AD, as well as percentage of eosinophils and serum IgE levels.

2. Materials and methods

2.1. Subjects

Peripheral blood samples were collected from 20 AD patients (5 severe, 4 moderate and 11 mild, evaluated by physicians' global scoring). The mean age ± standard deviation of them was 30.1 ± 12.0 years old. Twenty healthy volunteers (32.3 ± 6.5 years old) were recruited as normal controls. Samples from 7 patients with chronic eczema (5 with wide-spread and 2 with localized eczema; 63.4 ± 20.6 years old) were also examined. AD was diagnosed according to the Japanese Dermatological Association criteria [17]. Routine hematological analyses of peripheral blood and serum IgE levels were also examined. The subjects received no systemic immunosuppressive drugs or corticosteroids. This study was performed after obtaining informed consent from all subjects and was approved by the Ethical Committee of Kyushu University.

2.2. Antibodies and reagents

Anti-CD3-PerCP-Cyanine5.5 (Cy5.5), anti-CD4-[Amcyan, phycoerythrin (PE) and PerCP-Cy5.5], anti-CCR6-PE, anti-CCR4-PE-Cy7, anti-CXCR3-Alexa-488, anti-CD25-Allophycocyanin (APC)-Cy7, anti-CD69-PE, and anti-IL-5-PE monoclonal antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-CD45RA-Pacific-Blue, anti-IL-4-APC, anti-IL-17-PE and anti-Foxp3-APC monoclonal antibodies and Foxp3-permeabilization kits were obtained from eBioscience (San Diego, CA, USA). Anti-interferon (IFN)-γ-FITC was procured from Beckman Coulter (Fullerton, CA, USA). Phorbol myristate acetate (PMA), ionomycin and breferdin A were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Cytofluorimetric analysis of cell surface markers and chemokine receptors

PBMC were fleshly isolated from heparinized venous blood by density gradient centrifugation on Ficoll-Paque™-Plus (GE Healthcare, Björkgatan, Uppsala, Sweden). PBMC were stained with fluorochrome-conjugated anti-CD4, anti-CD25, anti-CD45RA, anti-CXCR3, anti-CCR4, anti-CCR6, and isotype-matched control monoclonal antibodies immediately after isolation. Data were analyzed using a FACSCanto II flow cytometer and FACSDiva (BD Biosciences), and FlowJo (Tree Star, Inc., Ashland, OR, USA) software.

2.4. Analysis of intracellular Foxp3 protein

To identify the Treg population, intracellular staining for Foxp3 was performed following the manufacturer's protocol. Briefly, fleshly isolated PBMC were first incubated with monoclonal antibodies against the surface markers of CD4 and CD25 or isotype-matched controls. After extensive washing, cells were fixed and permeabilized, and then stained with anti-Foxp3 mAb. Data were analyzed using the FACSCant II flow cytometer, FACSDiva and FlowJo software.

2.5. Intracytofluorimetric analysis of cytokine production

For intracellular cytokine staining of IL-4, IFN-γ, IL-17 and IL-5, freshly isolated cells (2×10^6/mL) from PBMC were stimulated with PMA and ionomycin in RPMI-1640 with 5% fetal calf serum in the presence of brefeldin A for 5 h at 37 °C, 5% CO_2. The cells were washed, and fluorochrome-conjugated anti-CD4 mAb was added and incubated. The cells were then washed, fixed and permeabilized using a fixation & permeabilization kit (eBioscience) and stained for intracellular IL-4, IFN-γ, IL-17 and IL-5. Activated lymphocytes were confirmed with the >90% expression of the activation marker CD69 within $CD3^+$ T cells for every examination. Data were analyzed using the FACSCant II flow cytometer, FACSDiva and FlowJo software.

2.6. Quantitative analysis of serum CCL17

Concentrations of CCL17 in sera from subjects were measured using ELISA kits (R&D Systems, Minneapolis, USA) according to the manufactures' instructions. The minimum detectable level of CCL17 was 7 pg/mL.

2.7. Statistical analysis

Data are expressed as mean ± standard deviation, and statistical analyses were performed using the 2-tailed Student's *t* test or Mann–Whitney *U* test for comparison with normal controls. Because the data of total IgE levels in AD patients were not normally distributed, a logarithmic transformation value was used for analysis. Linear regression was used to correlate T-cell subset frequencies with percentage of eosinophils, serum levels of CCL17 and IgE. A P value < .05 was considered significant. Calculations were performed with Prism Graph 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Decrease in Th17 and Th1 population in AD compared with normal controls

Th1 ($IFN\text{-}\gamma^+$) and Th17 ($IL\text{-}17^+$), but not Th2 ($IL\text{-}4^+$) and Treg ($Foxp3^+$), cell populations significantly decreased in PBMC of AD patients compared to those of normal controls (Table 1). The percentages of these four T cell subsets in chronic eczema were not significantly different compared with those of normal controls

Table 1
Percentages of $CD4^+$ T cell subsets in PBMC from subjects analyzed by flow cytometry.

Population	Normal (n = 16) (%)	AD (n = 20) (%)	CE (n = 7) (%)
Th1; $IFN\text{-}\gamma^+$ cells	20.4 ± 7.2	13.1 ± 6.5**	20.9 ± 4.9
Th2; $IL\text{-}4^+$ cells	3.8 ± 1.4	3.4 ± 1.3	4.7 ± 2.0
Th17; $IL\text{-}17^+$ cells	2.0 ± 0.7	1.4 ± 0.7*	2.1 ± 0.9
Treg; $Foxp3^+$ cells	4.7 ± 1.8[a]	5.4 ± 1.5	5.0 ± 1.1

AD, atopic dermatitis; CE, chronic eczema; Treg, reguratory T; Foxp3, forkhead box P3. Data were presented as mean ± standard deviation.

* P < .05 was determined by Mann–Whitney *U* test compared with normal controls.

** P < .01 was determined by Mann–Whitney *U* test compared with normal controls.

[a] n = 20.

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Table 2
Percentages of $CD4^+CD45RA^-CD25^-$ memory T cell subsets in PBMC from subjects analyzed by flow cytometry.

Population	Normal ($n=20$) (%)	AD ($n=20$) (%)	CE ($n=7$) (%)
$CXCR3^+$ cells	45.7 ± 7.7	34.7 ± 12.2**	42.1 ± 8.7
$CCR4^+$ cells	23.3 ± 6.5	22.5 ± 10.9	24.2 ± 5.6
$CCR6^+$ cells	40.3 ± 7.1	32.7 ± 12.6*	39.4 ± 5.2

AD, atopic dermatitis; CE, chronic eczema. Data were presented as mean ± standard deviation.
* $P < 0.05$ was determined by Student's t test compared with normal controls.
** $P < .01$ was determined by Student's t test compared with normal controls.

(Table 1). We also examined the percentage of IL-5-producing cells within $CD4^+$ T cells but found no significant difference between AD patients and normal controls (data not shown). We could not find out a significant correlation between the percentage of Th1 and Th17 cells in AD patients. In addition, no significant correlation was observed among the proportion of Th17, Th1, Th2 and $IL\text{-}5^+$ cells in AD patients and normal controls.

It was reported that Th1, Th2 and Th17 cells predominantly express CXCR3, CCR4 and CCR6, respectively [18,19]. We then compared the percentage of $CXCR3^+$, $CCR4^+$ and $CCR6^+$ cells in the peripheral $CD4^+CD45RA^-CD25^-$ memory T cells in PBMC from subjects. This phenotypic assay also confirmed that the percentages of $CXCR3^+$ and $CCR6^+$ cells of AD patients were significantly lower than those of normal controls, while there was no significant difference in the percentage of $CCR4^+$ cells between AD patients and normal controls (Table 2). The proportions of these three subsets in chronic eczema were similar to those in normal controls (Table 2).

Among AD subjects, we analyzed the proportion of Th1, Th2, Th17 and Treg cells in different disease severity. Th1 and Th17 cells were tended to decrease according to the severity, and a significant difference was shown in the percentage of Th17 cells between the mild and the severe group. As for the Th2 and Treg cells, there were no differences in their percentages between the groups (Fig. 1).

3.2. Negative correlation between serum CCL17 levels and Th17 population in AD

We next examined the correlation of serum CCL17 levels with the percentage of Th17, Th1, Th2 and Treg cells in AD. The serum CCL17 levels of AD patients were significantly elevated (830.4 ± 595.1 pg/mL) compared to those of normal controls (200.6 ± 99.1 pg/mL, $P < .0001$). As shown in Fig. 2, a significant negative correlation was observed between the percentage of Th17 cells and serum CCL17 levels, but that was not the case between the CCL17 levels and Th1, Th2 or Treg cell number, suggesting the decrease of Th17 cells might preferentially contribute to the disease activity of AD.

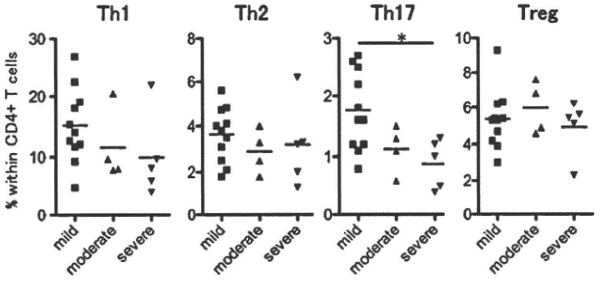

Fig. 1. Percentage of T-cell subsets within $CD4^+$ T cells in different disease severity among AD patients. Severe ($n = 5$), moderate ($n = 4$) and mild ($n = 11$) evaluated by physicians' global scoring. Th subsets (Th1: IFN-γ^+; Th2: IL-4^+: Th17: IL-17^+) were determined by intracellular cytokine production after stimulation by PMA/ionomycin and Treg cells were defined as $Foxp3^+$ cells within $CD4^+$ T cells in PBMC. * $P < .05$ by Student's t test.

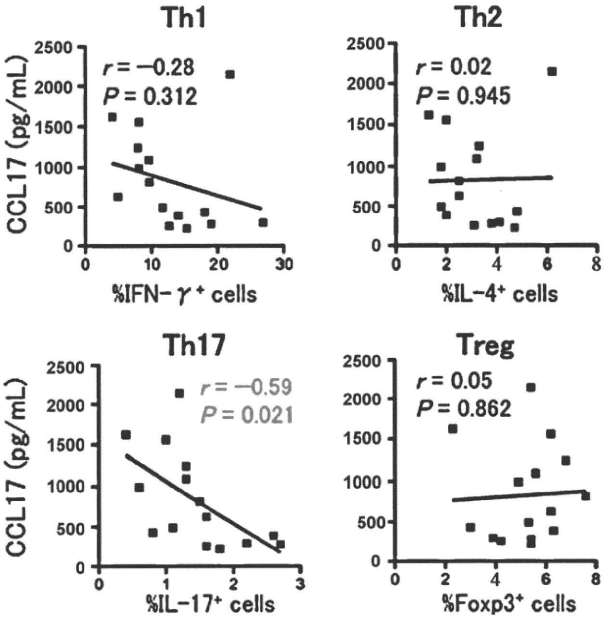

Fig. 2. Correlations of the percentages of T-cell subsets with the serum CCL17 levels in AD patients. We were not able to obtain enough samples for measurement of CCL17 levels in 5 patients ($n = 15$). Th subsets were determined by intracellular cytokine production after stimulation by PMA/ionomycin and Treg cells were defined as $Foxp3^+$ cells within $CD4^+$ T cells in PBMC. Linear regression showing correlation between the percentage of Th subsets (Th1: IFN-γ^+; Th2: IL-4^+: Th17: IL-17^+) or Treg ($Foxp3^+$) cells within $CD4^+$ T cells and the levels of serum CCL17.

3.3. Correlations of Th cell subsets with levels of serum total IgE and eosinophilia in AD

Since serum CCL17 levels have been shown to correlate with serum IgE levels and eosinophils number in AD patients [20,21], we also examined their correlation. In our AD patients, the serum CCL17 levels also significantly correlated with the serum IgE levels ($r = 0.55$, $P = .034$) and the percentage of eosinophils ($r = 0.67$, $P = .007$). As for the correlation of the frequency of T cell subsets to the serum IgE and percentage of eosinophils, we found a significant negative correlation of the percentage of Th17 subset with the percentage of eosinophils and serum IgE levels (Fig. 3). In the case of Th1, Th2 and Treg cells, however, significant correlations were not observed in both percentage of eosinophils and serum IgE levels (Fig. 3).

3.4. IFN-γ^{nega}IL-17^+ and IFN-γ^+IL-17^+ subpopulation in Th17 cells

Recent studies have demonstrated the existence of at least two subsets of Th17 cells; one is IFN-γ^{nega}IL-17^+ mono-producer and the other is IFN-γ^+IL-17^+ co-producer [18,19,22], which were readily detectable in our subjects (Fig. 4A). The former was the major subpopulation in Th17 cells in our subjects as has been previously described (Fig. 4A) [18,19,22]. Though both of the Th17 subpopulations tended to decrease in number in AD patients, the number of the IFN-γ^{nega}IL-17^+ cells was significantly decreased in AD patients compared with normal controls (Fig. 4B). The serum levels of CCL17 and IgE as well as the percentage of eosinophils again demonstrated a negative correlation to the percentage of the IFN-γ^{nega}IL-17^+ subsets in AD patients (Fig. 5). These results suggested that the IFN-γ^{nega}IL-17^+ subset might be significantly involved in the disease activity of AD than the IFN-γ^+IL-17^+ subset.

We also analyzed the percentage of IFN-γ^+IL-17^{nega} Th1 cells in our subjects. This subset was significantly decreased in AD patients

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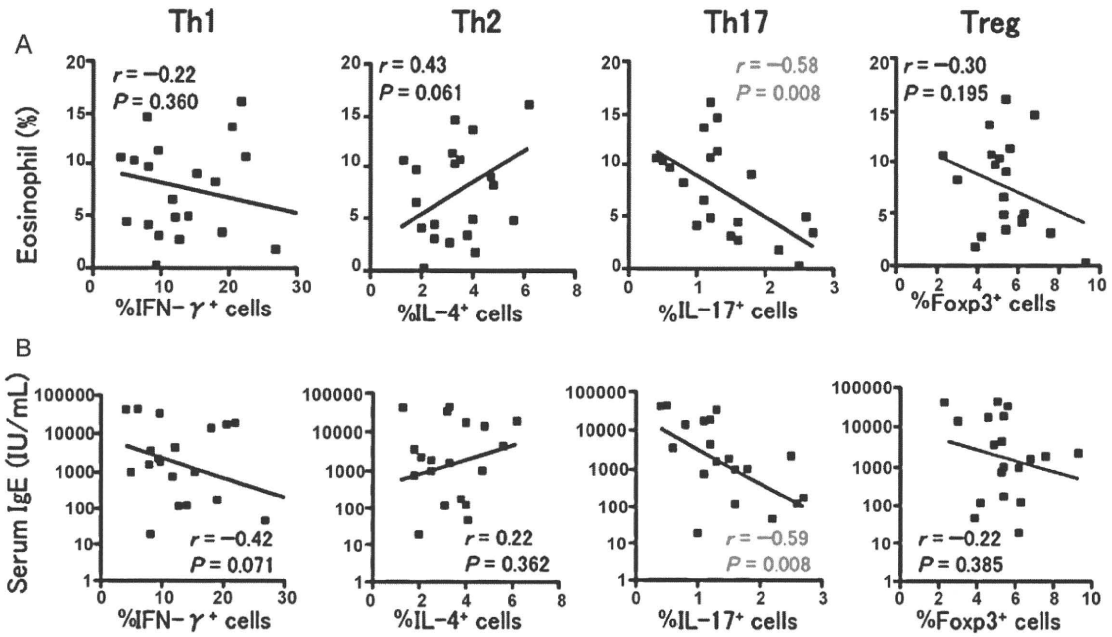

Fig. 3. Correlations of the percentages of T-cell subsets with the laboratory parameters of AD patients. Linear regression showing correlation of the percentages of Th subsets (Th1: IFN-γ^+; Th2: IL-4^+: Th17: IL-17^+) or Treg (Foxp3^+) cells within $CD4^+$ T cells in PBMC determined by flow cytometry with the percentage of eosinophils (A) and the levels of serum IgE (B).

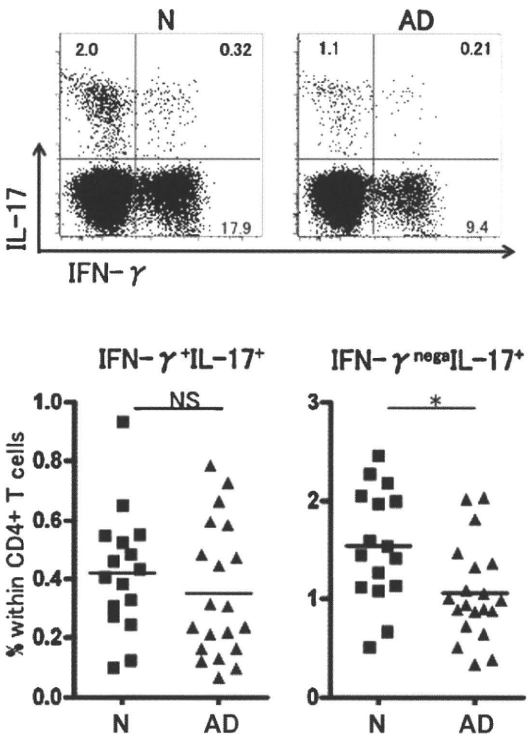

Fig. 4. Percentage of IL-17 subsets within $CD4^+$ T cells in PBMC. (A) Representative intracellular cytokine profiles (IL-17 versus IFN-γ) within $CD4^+$ T cells and determination of the frequency of the IL-17 subsets of IFN-γ^+IL-17^+ and IFN-γ^{nega}IL-17^+ cells in a normal control (N) and an AD patient. Numbers indicate percentage of cells in each quadrant. (B) Percentage of IFN-γ^+IL-17^+ and IFN-γ^{nega}IL-17^+ cells within $CD4^+$ T cells in normal controls (N, $n = 16$) and AD patients ($n = 20$). Black bars show the mean. $^*P < .05$. NS indicates "not significant" compared with normal controls as determined by Student's t test.

compared with normal controls (Fig. 6A). However, there found no significant correlation of the percentage of the IFN-γ^+IL-17^{nega} subset with the serum levels of CCL17, IgE and eosinophils (Fig. 6B).

4. Discussion

In this study, we examined whether the proportion of circulating Th1, Th2, Th17 and Treg subsets correlate with the disease parameters of AD as assessed by CCL17 levels in AD. We found that a significant decrease in Th17 and Th1 population in AD patients than those of normal controls, as detected by both in the cytokine production assays and the chemokine receptor expression. Moreover, the decrease in Th17 cells significantly correlated with serum CCL17 levels in AD.

CCL17 is a member of CC chemokines that functions as a selective chemoattractant for the recruitment and migration of $CCR4^+$ Th2 cells, and is expressed in the thymus, monocytes, dendritic cells, endothelial cells, bronchial epithelial cells and epidermal keratinocytes [20,21,23]. Many reports have demonstrated that serum CCL17 level is a very useful parameter of disease activity of AD [20,21,23,24]. In addition, the serum CCL17 levels correlate with the levels of serum IgE and eosinophilia in AD [20,21], which have been considered to be mediated by Th2-skewed immunological reaction [3,25]. We demonstrated the number of Th17 cells negatively correlated with CCL17, IgE or eosinophil levels, suggesting a mutual intimate interrelationship among these parameters in AD. Toda et al demonstrated that chronic AD lesions showed a significant increase in the number of eosinophils with a concomitant significant decrease in the number of IL-17 cells compared with acute AD lesions [9], which might also support our findings. Contrary to our data, however, it was reported that serum IL-17 levels were significantly related to clinical symptoms and peripheral eosinophil counts in allergic rhinitis [26]. The cause of this discrepancy is presently unclear, but the different phases of diseases or different methods of evaluating Th17 cells may be responsible.

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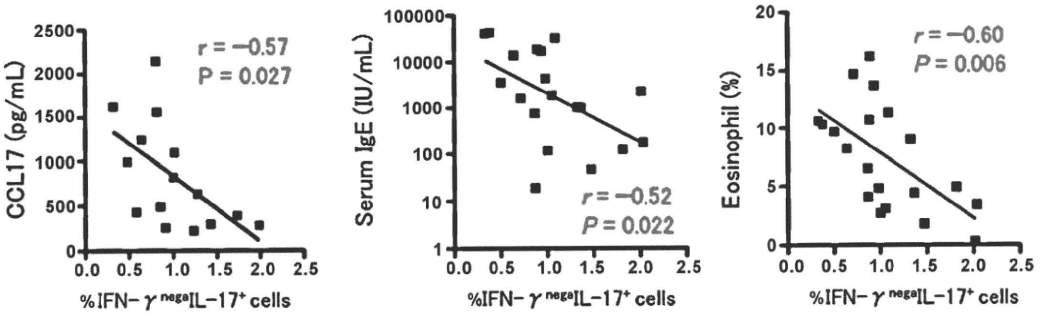

Fig. 5. Correlations of the percentage of IFN-γ^{nega}IL-17$^+$ subsets with the laboratory parameters of AD patients. Linear regression showing correlation of the percentages of IFN-γ^{nega}IL-17$^+$ cells within CD4$^+$ T cells in PBMC with the serum levels of CCL17, IgE and the percentage of eosinophils.

Psoriasis and AD have been considered opposite poles of the Th1 vs Th2 paradigm. Psoriasis has been considered a model of Th1 disease, whereas AD has been considered a polar Th2 disease in the acute phase, with a partial shift to Th1 during the chronic phase [27]. However, the classical Th1 and Th2 cell paradigm has recently been challenged with the discovery of Th17 cells. Psoriasis is the first inflammatory skin disease that had been shown to be clearly associated with Th17 cells [28], whereas the participation of Th17 cells in AD remains unclear. Interestingly, acute AD lesions showed more IL-17 cells than chronic lesions, suggesting that IL-17 functions primarily in the acute Th2 phase rather than in the subsequent Th1-dominated chronic phase of AD [7,8]. In contrast, Guttman-Yassky et al. demonstrated that IL-17 expression in AD was much lower than that in psoriasis. From the developmental point of view, Th1 and Th17 cells were closely related under the influence of IL-12 and IL-23. Presently, there is evidence that Th17 cells may be crucial in the pathogenesis of various autoimmune and inflammatory diseases, formerly categorized as Th1-mediated disorders, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and airway inflammation [29]. These notions were in accordance with our results, showing the simultaneous down-regulation of Th17 and Th1 cells in AD.

It has been reported that Th1 rather than Th2 predominates in spontaneous or older patch test lesions in AD [30]. However, recent studies have demonstrated the decrease of the levels of IFN-γ mRNA in PBMC, and of the IFN-γ producing skin-homing T cells in chronic AD [31,32]. Teramoto et al. showed a reduced ability of IFN-γ production by PBMCs was associated with an elevated serum IgE levels in AD [33]. Mauchra et al. found that decreased INF-γ production by peripheral blood in AD children was negatively correlated with the number of skin colonization of Staphylococcus aureus and SCORAD index [34]. Meanwhile, Källström et al. showed the decrease in IFN-γ$^+$ cells did not necessarily correlate with the serum levels of IgE [35]. Our results also revealed a significant decrease in Th1 cells (IFN-γ$^+$cells as well as IFN-γ$^+$IL-17^{nega} cells) in AD patients compared with normal individuals. However, the decrease in Th1 population had nothing to do with blood levels of CCL17, IgE and eosinophils in AD patients. The discrepancy between our findings and previous reports may be attributable to the differences in the methods used or investigated patient groups. The alleviative mechanisms of the Th1 axis remain unknown, but recent experiments revealed that activation-induced death of Th1 cells was accelerated in AD patients by enhanced Fas-FasL-mediated apoptosis [36]. Decrease of Th1 cells

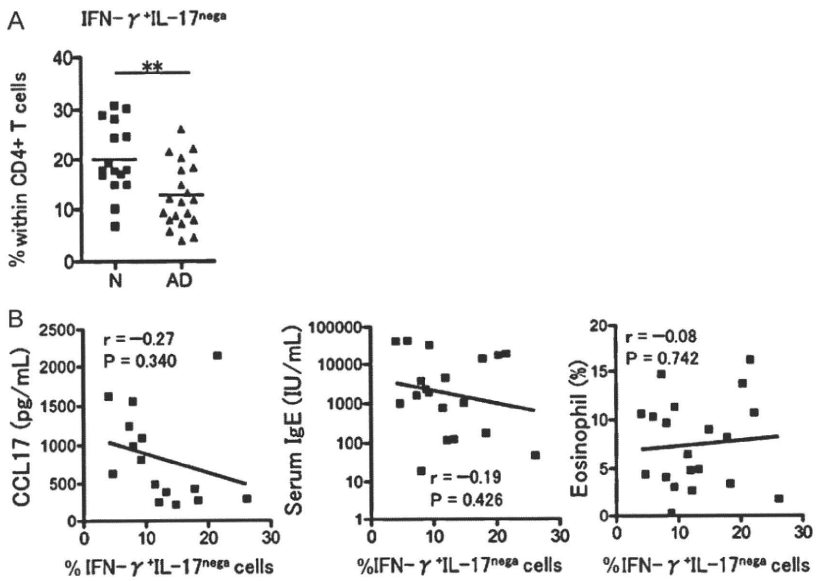

Fig. 6. (A) Percentage of IFN-γ$^+$IL-17^{nega} cells within CD4$^+$ T cells in normal controls (n = 16) and AD patients (n = 20). Black bars show the mean. $^{**}P < .01$ by Student's t test. (B) Correlations of the percentage of IFN-γ$^+$IL-17^{nega} subset with the laboratory parameters of AD patients. The percentage of IFN-γ$^+$IL-17^{nega} subset did not correlate with either the serum CCL17, IgE levels or the percentage of eosinophils.

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may contribute to make cytokine milieu toward Th2-deviated state as has been pointed out by Wong et al. [37].

In the skin, IL-17 is a master regulator of antimicrobial peptides (AMPs) in keratinocytes, playing a central role in host defense against microorganisms at the surface barrier [38]. Decreased IL-17 expression in chronic AD skin has been correlated to reduced expression of key AMPs, potentially accounting for the propensity to skin infections in this disease [28,38]. Decreased circulating Th17 cells in the present study may also contribute to the susceptibility of AD to skin infection. However, we must keep in mind that there remains a possibility that the circulating Th17 cells may decrease as a result of a tissue infiltration of these cells from circulation, because Th17 cells infiltrate to lesional skin during the acute phase of AD [7].

Several studies have demonstrated that IL-17 mono-producers and IL-17/IFN-γ co-producers are consistently detected in Th17 cells in PBMC, synovial and bronchial T cells [18,19,22]. We could also confirm these two subsets, however, the population of IFN-γ^{nega}IL-17^+ was much more abundant than that of IFN-γ^+IL-17^+ in PBMC, as previously reported [22]. Recent reports suggested a common developmental origin between Th1 and Th17 cells, because Th17 clones could be potentiated to produce IFN-γ when cultured in the presence of IL-12 [19,39]. In classical Th1 diseases, IL-17/IFN-γ co-producers were increased, suggesting that both Th1 and Th17 cells and their effector cytokines might substantially contribute to the pathogenesis [39,40]. In our study, IFN-γ^{nega}IL-17^+ subset significantly decreased in AD and it was negatively correlated with the levels of CCL17, IgE and eosinophilia. Although the accurate function of the IFN-γ^{nega}IL-17^+ and IFN-γ^+IL-17^+ subsets is still unclear, the decrease of IFN-γ^{nega}IL-17^+ subset might quantitatively and qualitatively correlate with activity of AD.

With regard to Treg cells, the numbers of Treg cells in AD patients were shown to be similar to or higher than those in healthy controls [14–16], likewise, we could not find a significant difference in Treg population between AD and normal controls. Although our AD patients did not receive systemic steroids or immunosuppressive drugs, we have to exclude a possible influence of standard topical steroid therapy on the interpretation of our results. In order to address this point, we measured Th1, Th2, Th17 and Treg cell subsets in the PBMC from patients with chronic eczema who were treated with long-term topical steroids. However, we found no significant difference in the population of these four subsets in the patients with chronic eczema compared with the normal controls.

In conclusion, the decrease of circulating Th17 cells may contribute to disease activity of AD as assessed by serum CCL17, IgE and eosinophil levels.

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Are lifetime prevalence of impetigo, molluscum and herpes infection really increased in children having atopic dermatitis?

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ABSTRACT

Background: Cutaneous infections such as impetigo contagiosum (IC), molluscum contagiosum (MC) and herpes virus infection (HI) appear to be associated with atopic dermatitis (AD), but there are no reports of concrete epidemiological evidence.
Objective: We evaluated the association of childhood AD with these infections by conducting a population-based cross-sectional study.
Methods: Enrolled in this study were 1117 children aged 0–6 years old attending nursery schools in Ishigaki City, Okinawa Prefecture, Japan. Physical examination was performed by dermatologists, and a questionnaire was completed on each child's history of allergic diseases including AD, asthma, allergic rhinitis and egg allergy, and that of skin infections including IC, MC and HI, as well as familial history of AD.
Results: In 913 children (AD; 132), a history of IC, MC or HI was observed in 45.1%, 19.7%, and 2.5%, respectively. Multiple logistic regression analysis revealed that the odds of having a history of IC were 1.8 times higher in AD children than in non-AD children. Meanwhile, a history of MC was significantly correlated to the male gender, but not to a personal history of AD. As for HI, we found no correlated factors in this study.
Conclusions: The lifetime prevalence of IC was indeed higher in young children with a history of AD.

1. Introduction

Atopic dermatitis (AD) is a common, chronic or chronically relapsing, severely pruritic, multifactorial eczematous skin disease, predisposing the patient to bacterial and viral infections attributable to abnormalities in both the innate and acquired immune system [1]. Epidermal barrier dysfunction, such as decreased ceramide production, decreased filaggrin production due to genetic mutation and/or atopic inflammation, defective secretion of antimicrobial peptides, and mechanical stress induced by the itch-scratch cycle, appear to be fundamentally involved in the predisposition to bacterial and viral infections in AD [1–5].

Impetigo contagiosum (IC) and molluscum contagiosum (MC) are two of the most common cutaneous infectious disorders that occur during childhood. Epidemiologic data on pediatric dermatology from Switzerland [6] and Turkey [7] suggest that IC and MC are among the ten most common diseases in children aged 3–5 years old. IC is caused by the direct inoculation of *Staphylococcus aureus* (*S. aureus*) or group A streptococci into superficial abrasions/traumas frequently associated with insect bites, eczema or burns. Lesions are highly contagious and can spread rapidly by direct contact with family and nursery school classmates [8]. The condition is reported to be more common in children with AD living in tropical climates [9], and in conditions of overcrowding and poor hygiene [10]. Meanwhile, MC is more common in children who attend swimming pools, who bathe together, and who are immunosuppressed. It is also reported that MC lesions are common and/or spread more extensively in patients with AD, and that the presence of concomitant AD is an important risk factor associated with treatment failure of MC [11]. On the other hand, the most commonly reported viral complication in subjects with AD is eczema herpeticum or Kaposi's varicelliform eruption, which

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is caused by extensive cutaneous infection with herpes virus type 1 or 2. Eczema herpeticum can be complicated by keratoconjunctivitis, viremia and sometimes multiple organ involvement with meningitis and encephalitis [12]. In contrast to IC and MC, this severe type of herpes virus infection (HI) occurs most frequently in the second and third decade of life [13]. Among the various skin infections, IC, MC and HI are empirically and clinically considered to be the three major disorders associated with AD. However, we found no previous reports on the actual association of these skin infections with childhood AD using population-based epidemiological examinations.

In 2001, we initiated an annual cross-sectional study on children aged 0–6 years old in Ishigaki City, Okinawa, Japan. We visited several nursery schools and conducted skin examinations and a questionnaire survey. This population-based study is named the Kyushu University Ishigaki Atopic Dermatitis Study (KIDS). Using the KIDS data, we have already published our findings on several important issues such as AD prevalence, serum immunoglobulin (Ig)E and thymus- and activation-regulated chemokine levels, spontaneous regression ratio and risk factors for AD among the KIDS children [14–17]. In the present study, we investigated the lifetime prevalence of IC, MC and HI among the KIDS children examined in 2006 and 2007. We then evaluated the association between a history of IC, MC and HI and a history of AD using multivariate analysis.

2. Methods

2.1. Study design

The study design has been previously reported [15]. Briefly, we contacted all nursery schools (i.e., 14 schools) in Ishigaki City, Okinawa Prefecture, Japan requesting the children's participation in the study. After obtaining written informed consent from the children's parents or guardians, we visited the 14 nursery schools during the summer and performed skin examinations on children aged 0–6 years old. In 2006 and 2007, medical skin examinations were performed on 1117 children who were enrolled in KIDS. Of these 1117 children, 913 (81.7%) returned their questionnaires, which were used for analysis in the present study. Approval for the study was obtained from the Ethics Committee of Kyushu University Hospital as well as from the directors and classroom teachers of the schools.

2.2. Questionnaire

A structured questionnaire was filled out by the children's parents or guardians, and included questions concerning data on age, gender, birth, personal history of AD, asthma, allergic rhinitis (AR) and egg allergy (EA), and familial (parents and siblings) history of AD. An additional question on the child's history of contracting infectious skin diseases (IC, MC and HI) was included in the questionnaires administered in 2006 and 2007.

2.3. Definitions

The medical skin examination of the children was performed by dermatologists from the Department of Dermatology, Kyushu University Hospital. AD was diagnosed according to the Japanese Dermatological Association criteria [18]. Children who had been diagnosed with AD as a result of the physical examination or from answers on the questionnaire were considered as AD children in this study in order to include present and cured AD. Definitions of asthma, AR and EA were assessed by questionnaire if a parent or guardian reported that a doctor had diagnosed the child as having these diseases. Furthermore, children who were reported in the questionnaire to have displayed adverse effects after eating eggs were diagnosed as having EA. The diagnosis of IC, MC and HI was made if a parent or guardian answered "yes" to the question "Has your child ever been diagnosed with IC (or MC or HI) by a doctor?"

2.4. Statistical analysis

Univariate analysis for the difference in factors between boys and girls was appropriately performed using the chi-square test for comparison between the two groups. The association between history of infectious skin diseases and gender, personal history of allergic disorders or familial history of AD was assessed using the chi-square test or Fisher's exact test. Then we examined the actual correlation between a history of skin diseases and a history of AD using multiple logistic regression analysis. Results were expressed as odds ratios with the respective 95% confidence interval (CI). To confirm the validity of the logistic regression model, we determined the post-estimation goodness-of-fit parameter for the logistic regression model using the Hosmer–Lemeshow goodness-of-fit test, and found the model to be valid ($P > 0.05$). In the multiple logistic regression analysis, the effects of age were controlled by including variables that showed significant correlation in univariate analysis as independent variables in the model. We calculated Pearson's correlation coefficients in the study variables, and found that all of the correlation coefficients ranged between −0.8 and 0.25, which implied that there was no risk of multicollinearity in the multiple logistic regression model since the risk increases when the correlation coefficients approach 1, such as $r = 0.8$ or greater (Table S1). A P value of < 0.05 was considered statistically significant. All analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Study population

Table 1 shows basic characteristics of the 913 participating children. Among these children, 759 (83.1% of our study population) who had no missing answers on the questionnaire were included in the multivariate analysis (Fig. 1). The mean (± standard deviation) age of the enrolled children was 2.9 (±1.4) years, and 55.1% were boys. The age distribution between boys and girls was not significantly different (Table 1). The proportion of children with a history of AD and AR was 14.7% and 2.8%, respectively, and showed a similar rate in both boys and girls, whereas asthma and EA were more common among boys than girls (Table 1). As for infectious skin diseases, a history of IC was the most common in all children (45.1%), and showed a similar rate in both boys and girls. However, MC and HI were more prevalent in boys than in girls (Table 1). In addition, the prevalence of any familial history of AD in boys and girls was not significantly different (Table 1).

3.2. History of IC, MC and HI in AD and non-AD children

Since a history of IC, MC and HI was assumed to show a gradual and cumulative increase with age in children, we next analyzed the age distribution of a history of IC, MC or HI. None of the children under 1 year old had a history of MC or HI (Table 2). The incidence of IC and MC gradually increased with age. Among a total of 334 children aged 4–6 years old, 177 (51.5%) and 107 (31.1%) children had a history of IC or MC, respectively. The incidence of HI was less than 5% in each age stratum (Table 2). We then evaluated the age distribution of IC, MC and HI among AD or non-AD children. A history of IC in AD children tended to overwhelm that in non-AD children in any age stratum, but a history of MC appeared to be similar in frequency between AD and non-AD subjects. The

Table 1
Characteristics of participating children.

	Number (%)			*P*-value[a]
	All (n=913)	Gender categories		
		Females (n=410)	Males (n=503)	
Age (year)				
0	12 (1.3)	5 (1.2)	7 (1.4)	
1	171 (18.7)	93 (22.7)	78 (15.5)	
2	193 (21.1)	80 (19.5)	113 (22.5)	
3	203 (22.2)	85 (20.7)	118 (23.5)	0.095
4	205 (22.5)	84 (20.5)	121 (24.1)	
5	109 (11.9)	55 (13.4)	54 (10.7)	
6	20 (2.2)	8 (2.0)	12 (2.4)	
History of allergic diseases				
AD[b]	132 (14.7)	58 (14.4)	74 (15.0)	0.803
Asthma[c]	150 (16.9)	52 (13.1)	98 (20.0)	**0.006**
AR[d]	25 (2.8)	10 (2.5)	15 (3.1)	0.632
EA[e]	37 (4.1)	10 (2.5)	27 (5.4)	**0.028**
History of infectious skin diseases				
IC	412 (45.1)	175 (42.7)	237 (47.1)	0.180
MC	180 (19.7)	60 (14.6)	120 (23.9)	<**0.001**
HI	23 (2.5)	5 (1.2)	18 (3.6)	**0.024**
Familial history of AD				
Paternal[f]	37 (4.1)	16 (4.0)	21 (4.3)	0.807
Maternal[g]	64 (7.1)	30 (7.4)	34 (6.8)	0.737
Sibling[h]	77 (9.3)	34 (9.0)	43 (9.5)	0.831

AD, atopic dermatitis; AR, allergic rhinitis; EA, egg allergy; IC, impetigo contagiosum; MC, molluscum contagiosum; HI, herpes virus infection. Significant values ($P<0.05$) are indicated in bold.

[a] Difference between females and males using the chi-square distribution. There are missing values with respect to these variables due to incomplete answers on the questionnaires. The respective numbers for "all", "females" and "males" are as follows: [b]899, 404, 495, [c]889, 398, 491, [d]886, 396, 490, [e]905, 404, 501, [f]896, 405, 491, [g]905, 406, 499, [h]832, 377, 455.

frequency of a history of HI was very low in both AD and non-AD children (Table 2).

3.3. History of IC was significantly associated with history of AD

Next, we examined the factors associated with a history of IC, MC or HI using univariate analysis. The proportion of children with a history of AD among the children without a history of IC, MC or HI was 53/498 (10.6%), 95/721 (13.2%) and 127/877 (14.5%), respectively (Table 3). In contrast, the proportion of children with a history of AD among the children with a history of IC, MC or HI was 79/401 (19.7%), 37/178 (20.8%) and 5/22 (22.7%), respectively (Table 3). We found a higher prevalence of both a personal and familial AD history in children with IC compared with children without IC. As for MC, male gender and a history of AD and EA were more frequent among children with a history of MC than their counterparts. The factor associated with a history of HI was male gender (Table 3).

Next, to identify the actual factors correlated with a history of IC, MC or HI, we conducted a multiple logistic regression analysis including the variables that showed a significant correlation by univariate analysis shown in Table 3, adjusted for the effects of age (Table 4). The results revealed that age was a significant predictor of a history of IC and MC (Table 4). The odds of a history of IC were 1.8 times higher in AD children than in non-AD children (Table 4). Furthermore, there was a significant association between a paternal family history of AD and a history of IC (Table 4). However, AD did not show significant association with a history of MC or HI. Other allergic diseases were not associated with a history of IC, MC or HI, whereas the odds of a history of MC were 1.59 times higher in boys than in girls (Table 4).

4. Discussion

AD appears to be associated with or to aggravate skin infections, including such major types as IC, MC and HI. However, since IC and MC are common skin diseases in children, it would be necessary to perform a population-based epidemiological study to reveal any actual association. Our population-based cross-sectional study demonstrated that the odds of a history of IC were 1.8 times higher in AD children than in non-AD children. IC is a highly contagious infection that is usually transmitted by direct contact, although the exact mechanism of *S. aureus* colonization in the skin is not known. In IC, bacteria do not infect intact skin, but instead enter through sites of minor trauma. Most AD patients are colonized with *S. aureus*, which may be cultured from skin lesions and, to a lesser

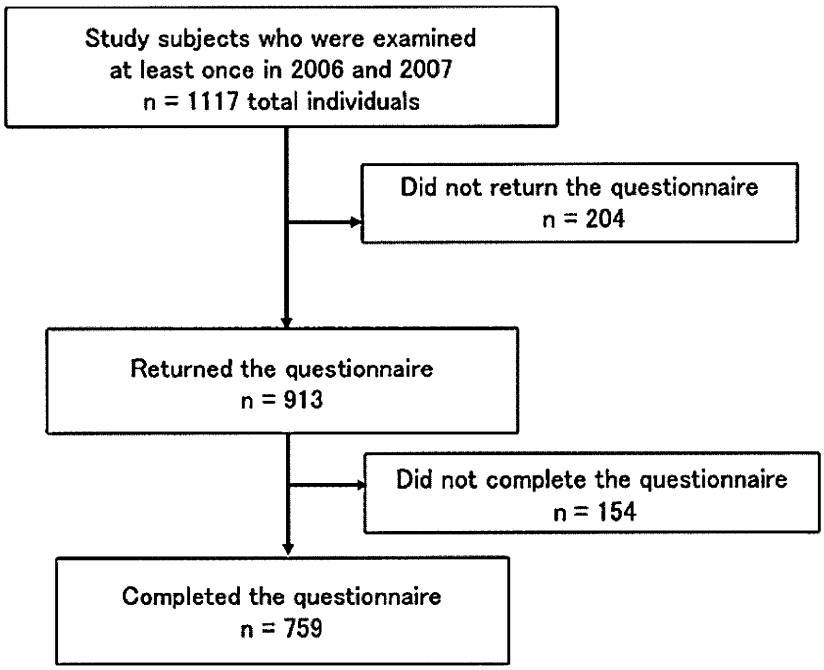

Fig. 1. Flow chart of study.

Table 2
Rate of lifetime prevalence of IC, MC and HI among AD and non-AD children.

Age (year)	All				AD categories[a]							
					Non-AD children				AD children			
	n	IC (%)	MC (%)	HI (%)	*n*	IC (%)	MC (%)	HI (%)	*n*	IC (%)	MC (%)	HI (%)
0	12	1 (8.3)	0 (0.0)	0 (0.0)	10	1 (10.0)	0 (0.0)	0 (0.0)	2	0 (0.0)	0 (0.0)	0 (0.0)
1	171	61 (35.7)	11 (6.4)	7 (4.1)	156	53 (34.0)	7 (4.5)	6 (3.9)	14	7 (50.0)	4 (28.6)	1 (7.1)
2	193	86 (44.6)	23 (11.9)	2 (1.0)	160	65 (40.7)	18 (11.3)	2 (1.3)	31	20 (64.5)	5 (16.1)	0 (0.0)
3	203	87 (42.9)	39 (19.2)	3 (1.5)	165	65 (39.4)	30 (18.2)	1 (0.6)	34	18 (52.9)	9 (26.5)	1 (2.9)
4	205	103 (50.2)	63 (30.7)	6 (2.9)	166	78 (47.0)	49 (29.5)	4 (2.4)	37	23 (62.2)	14 (37.8)	2 (5.4)
5	109	66 (60.6)	38 (34.9)	4 (3.7)	94	54 (57.5)	33 (35.1)	3 (3.2)	11	9 (81.8)	4 (36.4)	1 (9.1)
6	20	8 (40.0)	6 (30.0)	1 (5.0)	16	6 (37.5)	4 (25.0)	1 (6.3)	3	2 (66.7)	1 (33.3)	0 (0.0)

AD, atopic dermatitis; IC, impetigo contagiosum; MC, molluscum contagiosum; HI, herpes virus infection.
[a] There are missing values due to incomplete answers on the questionnaire concerning the history of AD.

Table 3
Factors associated with history of skin infections; univariate analysis.

Variables	History of IC			History of MC			History of HI		
	IC+	IC−	*P*	MC+	MC−	*P*	HI+	HI−	*P*
Male gender	237/412 (57.5)	266/501 (53.1)	0.180	120/180 (66.7)	383/733 (52.3)	**<0.001**	18/23 (78.3)	485/890 (54.5)	**0.024**
History of AD	79/401 (19.7)	53/498 (10.6)	**<0.001**	37/178 (20.8)	95/721 (13.2)	**0.010**	5/22 (22.7)	127/877 (14.5)	0.211*
History of asthma	73/401 (18.2)	77/488 (15.8)	0.337	38/175 (21.7)	112/714 (15.7)	0.056	5/21 (23.8)	145/868 (16.7)	0.272*
History of AR	13/399 (3.3)	12/487 (2.5)	0.478	5/174 (2.9)	20/712 (2.8)	0.564*	0/22 (0.0)	25/864 (2.9)	0.529*
History of EA	18/409 (4.4)	19/496 (3.8)	0.666	14/180 (7.8)	23/725 (3.2)	**0.005**	2/23 (8.7)	35/882 (4.0)	0.241*
Paternal history of AD	24/403 (6.0)	13/493 (2.6)	**0.013**	8/179 (4.5)	29/717 (4.0)	0.798	2/22 (9.1)	35/874 (4.0)	0.229*
Maternal history of AD	35/409 (8.6)	29/496 (5.8)	0.113	10/177 (5.6)	54/728 (7.4)	0.411	1/23 (4.3)	63/882 (7.1)	0.507*
Sibling history of AD	46/371 (12.4)	31/461 (6.7)	**0.005**	20/170 (11.8)	57/662 (8.6)	0.206	2/20 (10.0)	75/812 (9.2)	0.567*

Data is represented as *n*/*N* (%). AD, atopic dermatitis; AR, allergic rhinitis; EA, egg allergy; IC, impetigo contagiosum; MC, molluscum contagiosum; HI, herpes virus infection.
P values were determined by chi-square test.
* *P* values were determined by Fisher's exact test.
Significant values ($P < 0.05$) are indicated in bold.

degree, from nonlesional skin. Mechanical trauma caused by scratching due to itching is believed to result in barrier dysfunction leading to skin surface infection in AD. Furthermore, antimicrobial peptides such as human β-defensin-2 and LL-37 have an innate protective effect against skin infection, but the concentration of these peptides in AD patients is too low to effectively eradicate *S. aureus* [19]. Interleukin (IL)-4 and IL-13 produced from lesional Th2 cells and genetic predisposition appear to affect the defective synthesis of antimicrobial peptides in AD [20]. Dysfunction of the skin barrier, small traumas caused by scratching, colonization of bacteria on the skin surface and decreased levels of antimicrobial peptides in the skin might be responsible for the high lifetime prevalence of IC in AD.

We obtained the result that 20.8% of the children who had a history of MC also had a history of AD, which was similar to the results of previous hospital-based studies [21,22]. However, multiple logistic regression analysis showed no significant difference in the history of MC between AD and non-AD children. This similar association of MC between AD and non-AD individuals, of course, does not rule out the possibility that MC is exacerbated by concomitant AD, although we could not address this point in the present study protocol. MC lesions take several months to years to resolve spontaneously [11,23,24], but the presence of concomitant AD is the most significant factor associated with relapse and the risk of treatment failure of MC [11,25,26]. Children with AD and MC may visit clinics more frequently due to the easily spread or persistent tendency of the lesions, which may cause a referral bias in a hospital-based study. In addition, a history of MC was more prevalent in boys than in girls in our study, as reported previously [27,28]. The association of MC with bathing and attendance at swimming pools has been reported, and Postlethwaite et al. [28] suggested that the higher male susceptibility to MC might be due to boys going swimming more frequently than girls. We could not evaluate this possibility in this study.

Previous studies showed that the incidence of IC was 29.4 per 1000 person years (1–4 year olds) in the Netherlands [29], whereas that of MC was 18.1 per 1000 person years (1–4 year olds) in the Netherlands [29] and 1268 per 10,000 person years (0–14 year

Table 4
Factors associated with a history of IC, MC and HI: multiple logistic regression analysis.

Variable	OR (95% CI)		
	History of IC	History of MC	History of HI
Age	**1.23 (1.10–1.37)**[c]	**1.53 (1.33–1.76)**[c]	1.11 (0.78–1.58)
Male gender	–	**1.59 (1.09–2.32)**[a]	2.71 (0.88–8.39)
History of AD	**1.80 (1.16–2.81)**[b]	1.64 (1.00–2.68)	–
History of EA	–	2.08 (0.92–4.68)	–
Paternal history of AD	**2.44 (1.07–5.56)**[a]	–	–
Sibling history of AD	1.53 (0.90–2.60)	–	–
Hosmer–Lemeshow test	$\chi^2 = 7.96$, $P = 0.24$	$\chi^2 = 10.12$, $P = 0.26$	$\chi^2 = 13.26$, $P = 0.10$

This analysis covers 759 children who had no missing answers on their questionnaires. AD, atopic dermatitis; AR, allergic rhinitis; EA, egg allergy; IC, impetigo contagiosum; MC, molluscum contagiosum; HI, herpes virus infection; OR, odds ratio; CI, confidence interval.
[a] $P < 0.05$ (significant values are indicated in bold).
[b] $P < 0.01$ (significant values are indicated in bold).
[c] $P < 0.001$ (significant values are indicated in bold).

olds) in the UK [30]. In Fiji, the prevalence of IC was 25.6% (5–15 year olds) [9], and in Japan, that of MC was 5.6% (4–5 year olds) [27]. However, it is difficult to compare the results considering the difference in study area and participant age. Our children were likely to have a high frequency of lifetime episodes of IC (45.1%) and MC (19.7%). One reason for this is that the other studies showed the prevalence or incidence, but not the lifetime prevalence. In accordance with this notion, Steer et al. demonstrated that the cumulative incidence of IC among school children 5–15 years old in Fiji was 47.5% [9]. In general population of the Netherlands, the cumulative incidence of MC in children under 15 years old was 17% [31]. Another possible factor is the climate of Ishigaki City which is located in a subtropical area with high temperature and humidity, since IC and MC are more common in tropical climates [9,32].

As for HI, we found no significant difference in the history of HI between AD and non-AD children. As described previously, HI is not common in children 0–6 years old [29], as was also the case in the present study. It might be difficult to confirm the actual association between HI and AD due to the low prevalence of HI in young children. It should be emphasized that large-scale epidemiological studies on HI and AD using adult subjects indicated that HI is actually exacerbated by concomitant AD, and vice versa. In adult AD patients, it is well known that the most clearly delineated risk factor for the severe form of HI (eczema herpeticum) is disruption of the epidermal barrier [33]. Previous reports have shown that a high serum IgE level and early onset of AD are both risk factors for eczema herpeticum [34] Peng et al. [13] also found that skin lesion area and total IgE levels were significantly higher in AD patients than in those not suffering from eczema herpeticum. Moreover, a recent report by Beck et al. [12] demonstrated that, compared to AD patients without eczema herpeticum, AD subjects with eczema herpeticum had more severe skin scores, more body surface area affected, and higher biomarkers such as thymus- and activation-regulated chemokine levels.

We also investigated whether a history of IC, MC and HI was correlated with a history of other allergic diseases including asthma, AR and EA, which are commonly associated with AD [35–37]. However, we found no significant correlation of these infections with either asthma, AR or EA, suggesting that a local skin condition was a major factor for the preponderant association of IC with AD. The fact that this study is based on a general population is a strong point, but it should be emphasized that there are possible limitations in interpreting the results. Information on the personal history of asthma, AR, EA, IC, MC and HI was based on the questionnaire results, and AD was determined by the physical examination and questionnaire results in order to include present and cured AD, because the resolution/remission rate of AD is around 80% in children aged 0–6 years old [15]. There might be a recall bias in the parents' reports of prior allergic diseases and skin infections. Questionnaire-based prevalence studies could overestimate the data. Another limitation is that the present study was a cross-sectional investigation and hence the results must be interpreted with caution regarding the time sequence, because this study does not assess "present" IC and AD. In conclusion, we consider this to be the first demonstration that the lifetime prevalence of IC, but not that of MC or HI, did indeed increase in AD children compared with their non-AD counterparts. Our findings may support the presence of defective cutaneous innate immunity in AD.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2010.09.003.

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Scratching behavior does not necessarily correlate with epidermal nerve fiber sprouting or inflammatory cell infiltration

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ABSTRACT

Background: Increased sprouting of epidermal nerve fibers of lesional skin are thought to be associated with persistent pruritus in chronic inflammatory dermatitis such as atopic dermatitis as supported by a murine study using tacrolimus (or FK506: FK) which was shown to inhibit both epidermal sprouting of nerves and scratching behavior or by immunohistochemical observations of lesional skin in the patients with atopic dermatitis or prurigo, etc.
Objectives: To examine a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 (MEK1/2) inhibitor (CX-659S: CX) for a possible anti-pruritic property in vivo since some MEK1/2 inhibitors have been reported to inhibit neurite growth *in vitro*.
Methods: CX, FK and corticosteroids (betamethasone valerate: BV) were topically applied on inflamed skin in a mouse model of chronic dermatitis using repetitive hapten painting to examine anti-pruritic property and anti-inflammatory effects. Scratching behaviors were assessed using MicroAct automatic measuring system, and epidermal sprouting of nerves and skin inflammation was assessed histologically.
Results: FK significantly decrease scratching behavior, but CX and BV failed to do so despite of their ability to significantly inhibit epidermal nerve fiber sprouting and skin inflammation, respectively. In addition, CX + BV mixture synergistically inhibited epidermal nerve fiber sprouting and skin inflammation even more potently than FK without decreasing scratching behavior.
Conclusions: These findings suggest that the scratching behavior does not necessarily correlate with epidermal nerve fiber sprouting or inflammatory cell infiltration.

1. Introduction

Controlling pruritus and scratching is important for the treatment of skin diseases with severe and persistent pruritus such as atopic dermatitis. Scratching causes excoriation of the skin, and the excoriation exacerbates skin inflammation generating further pruritus which induces scratching almost inevitably, setting up so-called "itch-scratching vicious cycle" [1]. Histamine, the best known itch-mediator, has been a main target in anti-pruritic therapies [2,3], but it has been known widely that antihistamines is not always effective in controlling pruritus [4]. There have been accumulating evidences that factors other than histamine are involved in the pathophysiology of pruritus, such as neuropeptides, proteases, opioids, growth factors, cytokines or abnormal structure of peripheral afferent nerves in the lesional skin [3,5]. Increased intraepidermal nerve fibers, so-called epidermal nerve fiber sprouting, have been a current topic for pathogenesis of persistent pruritus in various pruritic skin diseases including atopic dermatitis, psoriasis or the skin lesions of an atopy model mouse, NC/Nga [6–8].

Tacrolimus (FK506: FK) is a new curative of atopic dermatitis and is known to exert an anti-pruritic property [9]. Ability to inhibit epidermal nerve fiber sprouting and depletion of substance P by FK is currently considered a potential mechanism for the exertion of anti-pruritic property [10,11]. While topical glucocorticoid, such as betamethasone valerate (BV), have a strong anti-inflammatory effect and is used as a fundamental treatment for the various inflammatory skin diseases including atopic dermatitis, but its anti-pruritus effect has not been constantly proved in the animal models [10,12]. Nerve growth factor (NGF) derived from stimulated keratinocytes appears a potential cause for such epidermal nerve fiber sprouting [13], and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2

Abbreviations: PC, picryl chloride; CX, CX659S; FK, FK506 (tacrolimus); BV, betamethasone valerate; SP, substance P; CGRP, calcitonin gene-related peptide; NGF, nerve growth factor; PGP9.5, protein gene product 9.5; MEK1/2, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2.

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(MEK1/2) pathway is a downstream of NGF receptor signaling. MEK1/2 pathway is closely associated with neural proliferation and differentiation [14]. Indeed, some MEK1/2 inhibitors are able to inhibit neurite growth *in vitro* [15]. CX-659S (CX), a MEK1/2 inhibitor, is a currently developed anti-inflammatory agent [16–18], and we hypothesized that the MEK1/2 inhibitor might have anti-pruritic property similar to that seen by FK.

In the present study, we examined the inhibitory effects of FK, BV and CX on the scratching behavior, epidermal nerve fiber sprouting and inflammatory cell infiltration in murine chronic dermatitis model induced by repetitive hapten application.

2. Materials and methods

2.1. Mice

C57BL/6NCrj mice (8–9-week-old female) were obtained from Charles River (Tokyo, Japan) and were maintained in specific pathogen-free condition. The protocol was approved by the Committee of Ethics on Animal Experiments in the Graduate School of Medical Sciences, Kyushu University.

2.2. Reagents

Picryl chloride: PC (Sigma, St. Louis, MO, USA) was dissolved in acetone at the concentration of 0.5%. A MEK 1/2 inhibitor; CX (Japan Energy Co., Tokyo, Japan; Kowa Co. Ltd., Tokyo, Japan), FK (Astellas Pharma Inc., Osaka, Japan) and betamethasone valerate: BV (Sigma, St. Louis, MO, USA) were dissolved in acetone at the concentrations of 2.0% for CX- and FK-solution, or of 0.2% for BV solution, based on the degree of anti-inflammatory effects examined in our previous [16] and preliminary experiment.

2.3. Induction of skin inflammation

Ears and shaved back skin of each mouse were painted every other day (days 0, 2, 4, 6, 8, 10, 12) with 0.5% PC solution of 20 μl and 50 μl, respectively and were also painted everyday with CX, FK, BV or the vehicle (acetone) of 20 μl and of 100 μl, respectively, 30 min before the application of PC, until day 12. Negative control group was painted with vehicle alone. Thickness of the painted ears was measured with a dial thickness gauge (G-1A, Ozaki MFG Co. Ltd., Tokyo, Japan) before any paintings on day 0 and at 24 h after the last painting. The painted ears and back skin samples were harvested immediately afterwards for histological analyses which were performed in a blinded manner.

2.4. Histological analysis for skin inflammation

Skin samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin, sectioned at 3 μm-thick, and stained with hematoxylin-eosin or with toluidine-blue for mast cells. Some skin samples were sectioned at 6 μm-thick and stained with anti-IL-4- or with anti-IFNγ antibodies (Biolegend, SA, USA) with peroxidase-diaminobenzidine method. Sections were counterstained with hematoxylin. To assess the number of dermal infiltrating cells, mast cells, IL-4 positive cells and IFNγ positive cells, five high power fields (HPFs) per skin section from each mouse were randomly selected and mean cell numbers per field were counted.

2.5. Analysis of epidermal nerve fiber extension

Paraformaldehyde-fixed skin specimens were washed in gradient PBS containing 10%, 15% and 20% sucrose at 4 °C overnight, embedded in OCT compound (Sakura Fineteck Japan, Tokyo, Japan) and stored at −80 °C. The frozen specimens were sectioned at 40 μm-thick and stained with anti-protein gene product 9.5 (PGP 9.5) polyclonal- (UltraClone Limited, Wellow Isle of Wight, UK), anti-calcitonin gene-related peptide (CGRP) polyclonal- (Chemicon International, Inc., Temecula, CA, USA) or anti-substance P (SP) monoclonal (Chemicon International, Inc.) antibodies, visualized by Alexa 488 (Invitrogen, Tokyo, Japan). The degree of epidermal nerve fiber sprouting of PGP 9.5-, SP- and CGRP-positive fibers were observed and evaluated using μRadiance confocal laser scanning system (Bio-RAD, Tokyo, Japan), Laser sharp 2000 software ver. 3.4. For quantification of epidermal nerve fiber extension, five random photographs were taken per specimen and the length of the immunoreactive epidermal nerve fiber was measured using Image J software ver.1.37 (National Institutes of Health, Bethesda, Maryland, USA) and calculated per millimeter horizontal width of epidermis.

2.6. Evaluation of scratching behavior

Scratching behavior of mice was measured for 2 h immediately after the painting on day 12 using Microact® system (Neuroscience Inc., Tokyo, Japan), as previously reported [19]. Briefly, a small teflon-coated columnar magnet (Ø1 mm × 3 mm) was implanted subcutaneously into the back of both hind legs of the mouse under ether anesthesia at least 5 days before the first application of PC solution. The mouse was placed into a small observation chamber with a round coil. The electric current induced by the movement of the magnet-implanted hind legs was recorded and automatically evaluated by Microact® software ver. 1.0.

2.7. Statistical analysis

Results were shown as mean ± standard deviation (SD). Statistical analysis of the data was performed using Bonferoni multiple comparison test using MS Excel 2003. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Inhibiting epidermal nerve fiber sprouting does not necessarily result in controlling scratching behavior

Repetitive hapten painting for 12 days could induce a Th2-shifted chronic dermatitis as evidenced by Th2-polarized dermal infiltrating cells (Fig. 1A). Topical application of FK significantly inhibited scratching behavior at day 12 (Fig. 1B), while both CX and BV showed no decrease of scratching behavior. Similar results were seen at day 6 (data not shown). Both CX and FK, but not BV, significantly inhibited epidermal nerve fiber sprouting of PGP 9.5 positive nerves (Fig. 1C and D). The insignificant inhibition by BV was the case in SP- or CGRP-positive sensory nerve sprouting (Fig. 1E and F). There was no significantly positive correlation between scratching behavior and epidermal nerve fiber sprouting of PGP9.5-, SP- or CGRP-positive nerves at day 12 as assessed by Pearson's correlation coefficient analysis (data not shown).

FK and BV significantly inhibited PC-induced ear swelling (data not shown), the number of total dermal infiltrating cells and the number of mast cells (Fig. 2A–C), while CX could significantly inhibit only the number of total dermal infiltrating cells (Fig. 2B).

3.2. Mixture of corticosteroids and MEK1/2 inhibitors significantly suppresses both inflammation and nerve fiber sprouting, but fails to reduce scratching behavior

Since FK, which inhibited scratching behavior, showed both strong anti-nerve fiber sprouting property and anti-inflammatory

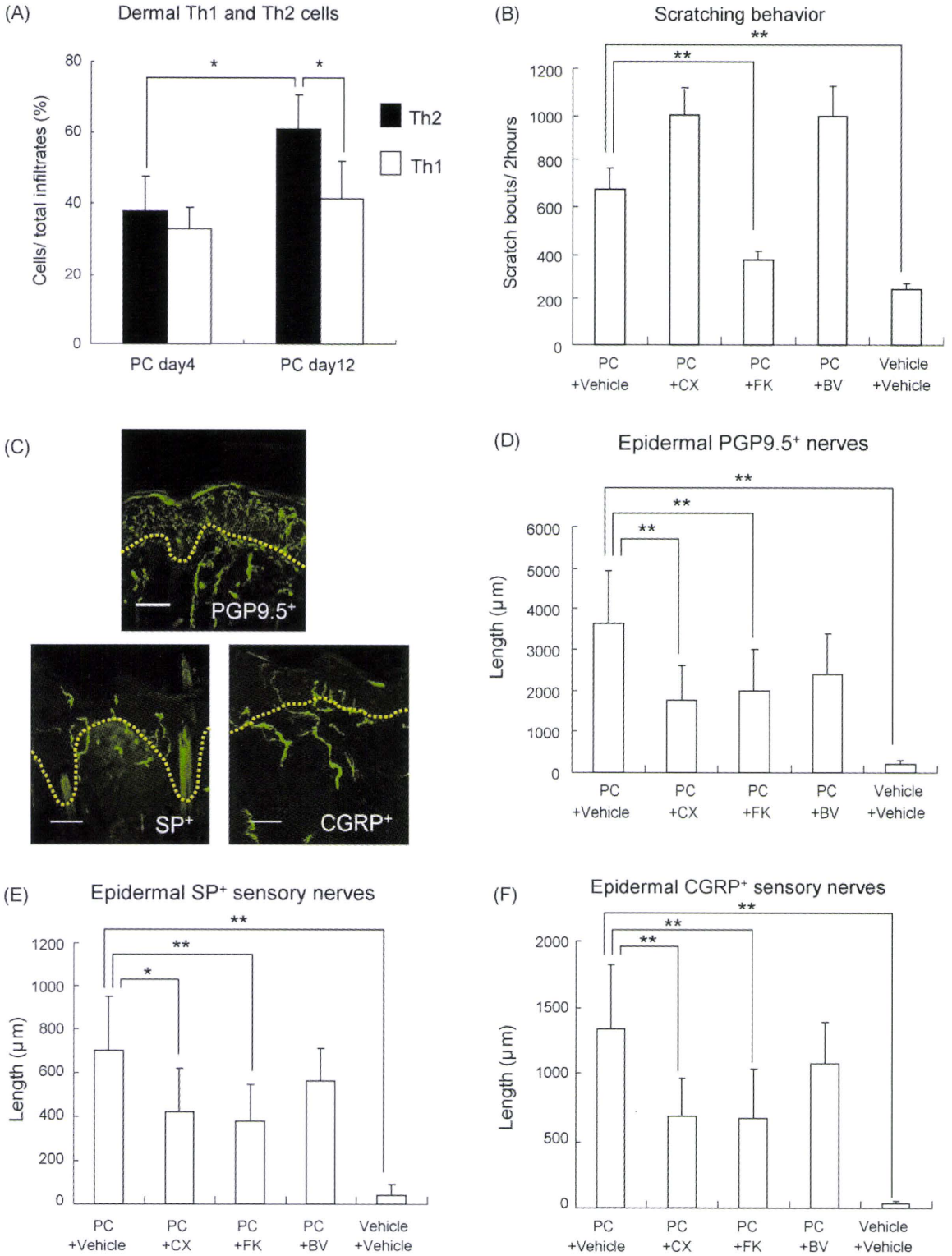

Fig. 1. Only FK significantly inhibits scratching behavior. (A) Repetitive hapten painting induced a Th2-shifted chronic dermatitis by day 12. (B) FK, but not CX or BV, significantly inhibited scratching behavior at day 12. (C) Representative pictures of PGP9.5-, substance P- and CGRP-positive nerves in PC painted back skin of mice. (D–F) Both FK and CX significantly inhibited epidermal nerve fiber sprouting of PGP9.5-, substance P- and CGRP-positive nerves. Values are expressed as mean ± SD of 8–9 animals. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with PC. Note that some description of statistical analysis, such as those between negative control group and others, were omitted for clarify in B, D–F.

property, we next hypothesized that the use of a CX and BV mixture (hereafter referred as "CX + BV mixture") might exert anti-pruritic effects, expecting that the combination would have both strong anti-nerve fiber sprouting effects and anti-inflammatory effects as well as FK. However, CX + BV mixture showed no decrease of scratching behavior (Fig. 3A), while FK significantly inhibited scratching behavior. CX + BV mixture, as well as FK, significantly inhibited epidermal nerve fiber sprouting of PGP 9.5

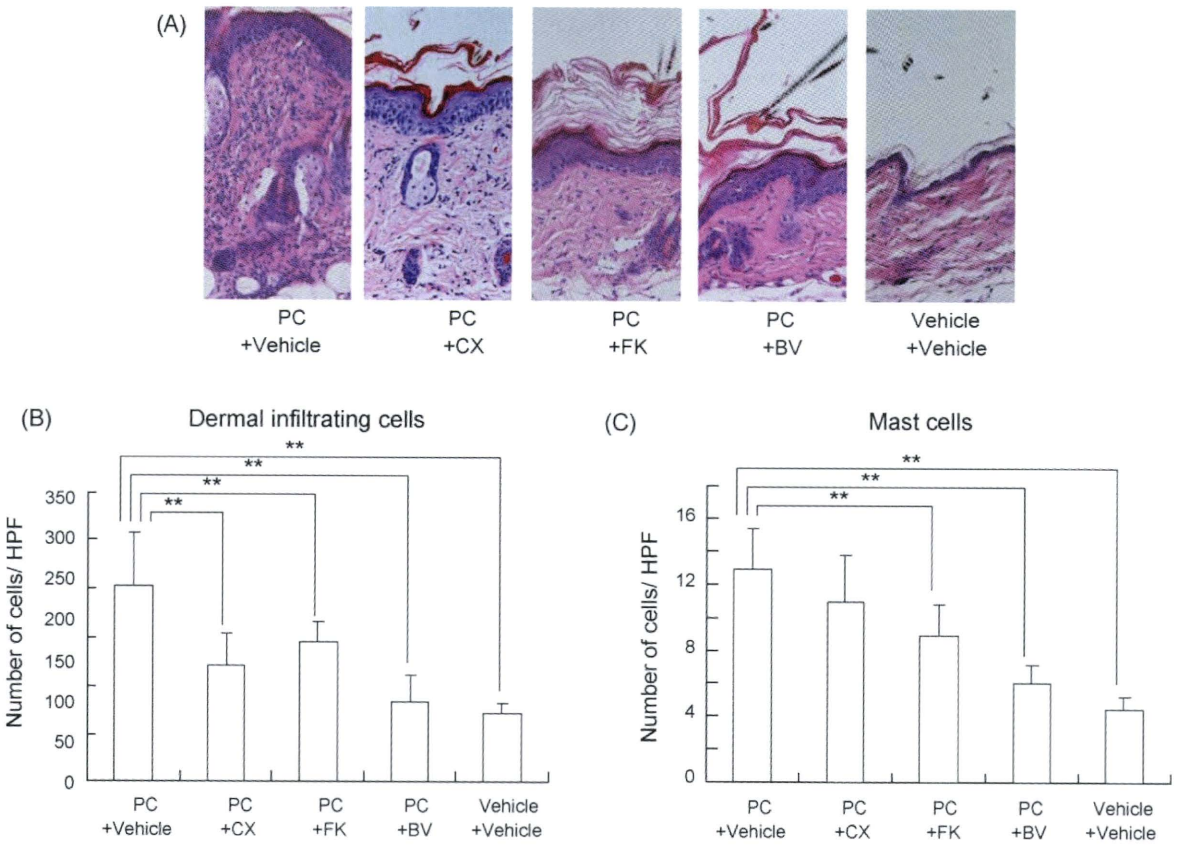

Fig. 2. Both FK and BV show strong anti-inflammatory effects. (A) The difference in ear thickness and degree of skin infiltration in differently treated mice. (B and C) FK and BV significantly inhibited dermal infiltrating cells and mast cells. Values are expressed as mean ± SD of 8–9 animals. $^{**}p < 0.01$, when compared with PC.

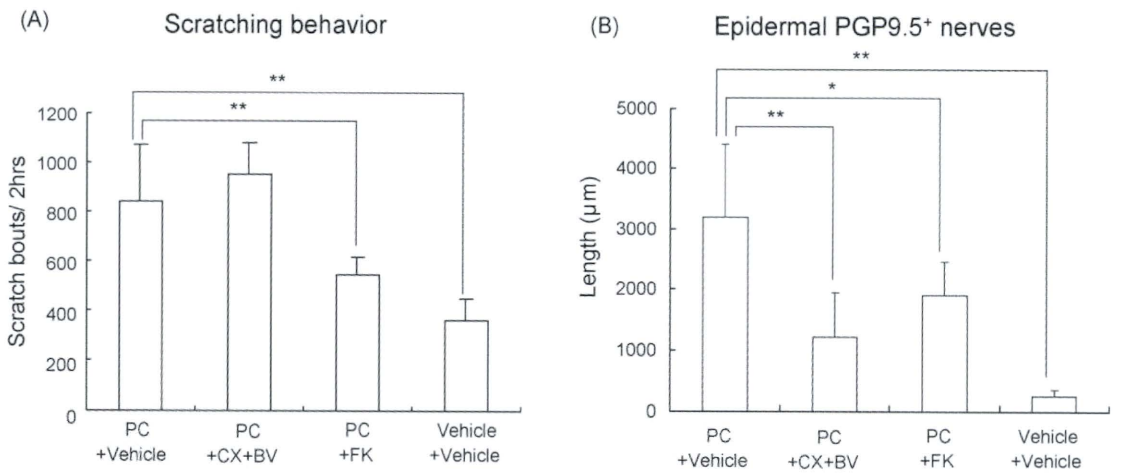

Fig. 3. CX + BV mixture does not inhibit scratching behavior. (A) FK, but not CX + BV mixture, significantly inhibited scratching behavior at day 12. (B) The anti-nerve fiber sprouting property by the CX + BV mixture was equal, or possibly even slightly greater, to that seen by FK. Values are expressed as mean ± SD of 9–10 animals. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with PC.

positive nerves (Fig. 3B) as well as that of SP- or CGRP-positive nerves (data not shown). The mixture also excessively inhibited the number of total dermal infiltrating cells and mast cells, even to the significantly greater extent as compared to those seen by FK (Fig. 4A–C).

4. Discussion

The present study demonstrated a clear dissociation among scratching behavior, epidermal nerve fiber sprouting and skin inflammation in the chronic dermatitis model, suggesting that careful interpretation may be needed. FK significantly inhibited scratching behavior, epidermal nerve fiber sprouting and inflammatory cell infiltration, but BV inhibited neither scratching nor epidermal nerve fiber sprouting despite of its strong inhibitory effects on skin inflammation. CX failed to inhibit scratching behavior despite of the significant inhibition of epidermal nerve fiber sprouting and skin inflammation. We considered that the ineffectiveness of CX might be due to the lack of inhibitory action on mast cell infiltration, because mast cells are thought to contribute to induction and modulation of pruritus. However, CX + BV mixture, which strongly inhibited the infiltration of mast

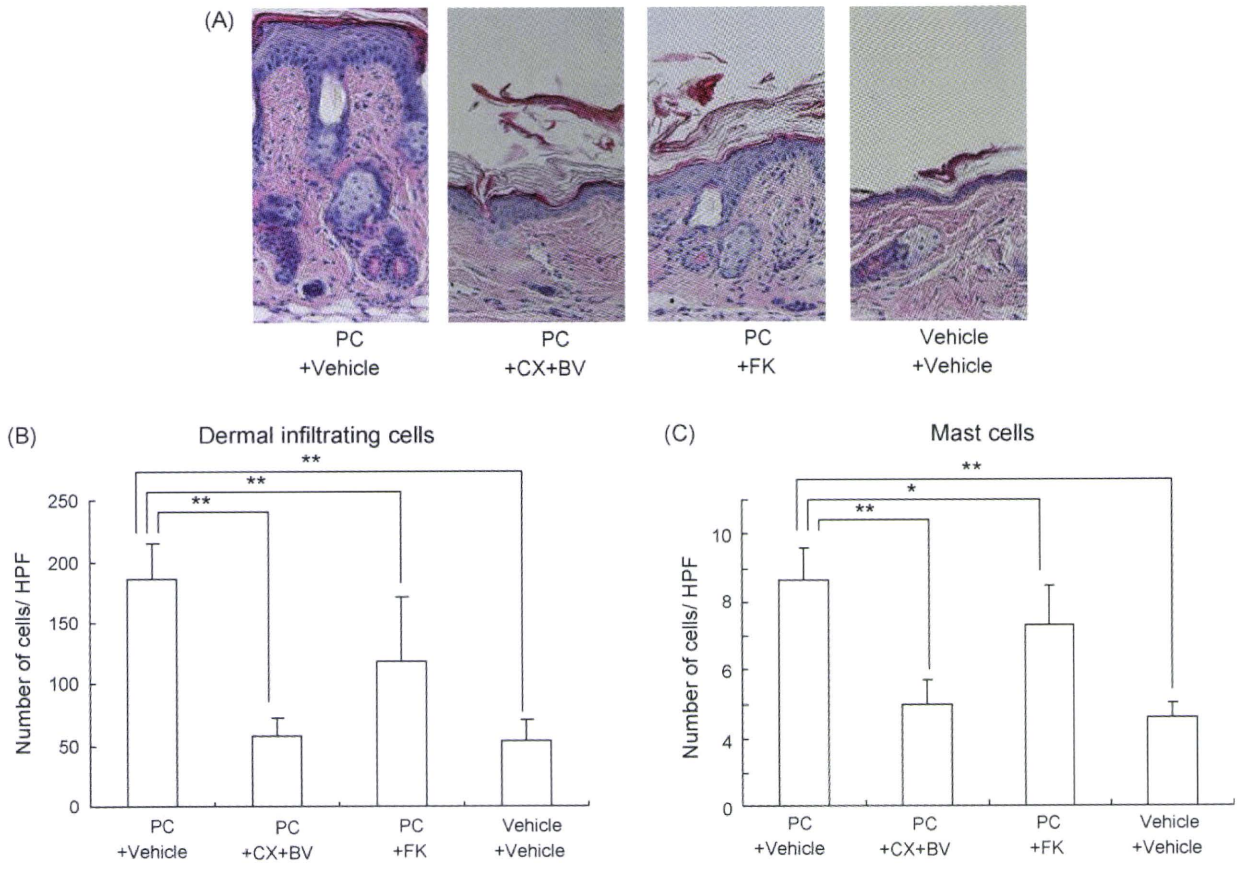

Fig. 4. CX + BV mixture shows stronger anti-inflammatory effects than FK. (A–C) CX + BV mixture significantly suppress dermal infiltrating cells and mast cells greater than FK. Values are expressed as mean ± SD of 9–10 animals. $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with PC.

cells and other inflammatory cells as well as epidermal nerve fiber sprouting, could not inhibit scratching behavior.

Several possibilities are conceivable to explain our results. First, it should be noted that anti-pruritic effects of corticosteroids seem to depend on the mice strain and/or condition for pruritus induction [10,12]. These evidences might indicate that the mediator which most contribute to induction of pruritus might change depending on the condition of the dermatitis. Thus it is possible that pruritus might be linked more closely to neuronal sensitization that not necessarily is combined with structural changes rather than to inflammatory cell infiltration in the mouse model used in the present study. In addition, FK might have a mechanism, which BV and CX miss, other than inhibiting epidermal nerve fiber sprouting for the exertion of anti-pruritic property. In fact, FK was reported to induce depletion of substance P in the skin of a murine allergic dermatitis model [11]. The inhibition of calcineurin which is a target of FK could be related to its anti-pruritic ability, because calcineurin is present at central and peripheral nervous system as well as immune cells and may be involved in affecting functions of sensory nerves [20]. FK was also reported to transiently activate capsaisin-sensitive DRG neurons and cutaneous C-fibers partially via transient receptor potential vanilloid receptor 1 (TRPV1) [21], which is another evidence indicating that FK could affect neurons functionally. The activation of C-fibers by tacrolimus, which is observed as "burning sensation" in humans, could suppress itch sensation.

Increased epidermal nerve fiber sprouting was reported in various pruritic skin diseases such as atopic dermatitis and psoriasis [6,7]. Elevated serum neurotrophins such as NGF is so far commonly observed in both human [13] and mice [8] under the condition with allergic dermatitis. Furthermore, blocking NGF system in NC/Nga mice resulted in a significant inhibition of both scratching behavior and epidermal nerve fiber sprouting [22,23]. Needless to say, sensation of pruritus is transmitted by nerve fibers and is recognized by central nervous system, and nerve fibers play pivotal roles in mechanism of pruritus. However, a recent report showed that the degree of pruritus in psoriasis was not correlated with the number of epidermal sensory nerve fibers [24]. NGF could modulate neural functions such as neural sensitization by enhancing sodium channels and TRPV1 [25] other than just elongating nerve fibers. It is interesting because NGF-blocking treatment might partially exert its anti-pruritic effects by down-regulating TRPV1 which could modulate histamine-induced pruritus [26] as well as burning sensation. Anyway, these and our results suggest that other mechanisms such as the functional modulation of nerve fibers rather than the mere inhibition of the nerve fiber sprouting might lead reducing pruritus/scratching.

In conclusion, only FK which inhibits epidermal nerve fiber sprouting could inhibit pruritus/scratching behavior in this model. However, the anti-pruritic effects by FK did not seem to occur simply through either the anti-epidermal nerve fiber sprouting or the anti-inflammatory property since CX or CX +BV could not suppress scratching behavior. Scratching behavior does not necessarily correlate with epidermal nerve fiber sprouting or inflammatory cell infiltration, emphasizing that the anti-pruritic effects might be exerted through affecting neurons functionally rather than structurally.

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