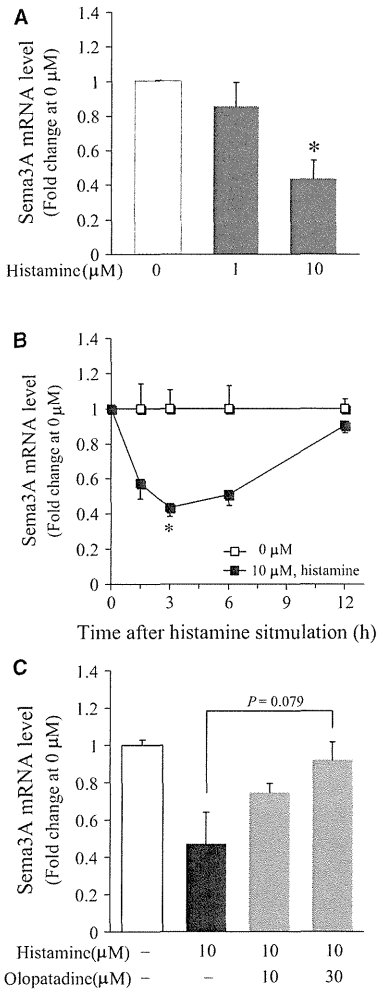

Figure 5 Effects of olopatadine on histamine-induced down-regulation of semaphorin 3A (Sema3A) expression in PAM212 cells. PAM212 cells were cultured with various concentrations of histamine for 3 h, and the level of mRNA for Sema3A was determined by real-time PCR (A). Changes in the level of Sema3A mRNA after the histamine treatment were also measured (B). The cells were cultured for 30 min in medium containing olopatadine and stimulated with histamine at 10 μM for 3 h (C). The level of mRNA is indicated as the fold difference normalized to the unstimulated control. Statistical significance; $*P < 0.05$ *vs* histamine at 0 μM.

mast cells and the late phase which is associated with infiltration by eosinophils and T cells (12). Here, we indicated that chronic allergic dermatitis was also clearly improved by a H1R antagonist plus H4R antagonist (Figs 1 and 2) with almost the same effect as prednisolone (Fig. 1). The effect of the combined treatment on the number of mast cells in skin lesion was also similar to that of prednisolone, which significantly decreased the infiltration of mast cells in skin lesion treated with PiCl repeatedly (32).

JNJ7777120 alone significantly inhibited the production of Th2 cytokines in the skin lesions (Fig. 3). We found that JNJ7777120 markedly inhibited the production of TARC and MDC by antigen-stimulated BMMC. BMMC significantly secreted histamine on stimulation with IgE and then produced TARC and MDC, which are chemokines acting through the CCR4 mainly expressed by Th2 cells. JNJ7777120 inhibited the production of TARC and MDC without inhibiting histamine release. Because BMMC have H4R but not H3R (8), the inhibitory actions of thioperamide could be attributed to H4R antagonism. The inhibition of TARC and MDC production by olopatadine, at least a part, is assumed to be due to the inhibition of degranulation. In one such example, it has been reported olopatadine inhibits IgE-mediated histamine release from human conjunctival mast cells (33). Thus, the histamine released by antigen-IgE stimulation could induce the production of TARC and MDC via H4R.

Because TARC levels in patients with AD correlate with the scoring of the AD index and eosinophil numbers (34, 35), an extracorporeal diagnostic agent, serum TARC (Shionogi & Co., Osaka, Japan), is used as a biomarker against AD in Japan. Our findings suggested that JNJ7777120 inhibited the infiltration of $CD4^+$ T cells in the skin lesions probably by inhibiting the production of TARC (Fig. 4), resulting in the alleviation of chronic dermatitis.

Prednisolone is used externally in the treatment of AD. In our model, prednisolone inhibited scratching count, dermatological score, the production of several cytokines, and the increase in serum IgE. Importantly, the combined treatment of olopatadine and JNJ7777120 could inhibit these parameters to almost similar extent. Glucocorticoids containing prednisolone frequently causes atrophy of skin and elicit side-effects by systemic administration. On the other hand, a new H4R antagonist from Palau Pharma is currently in Phase II clinical trial and has shown that the H4R antagonist is safe and very well tolerated. Therefore, our findings suggest the combined treatment of H1R and H4R antagonists might be a potent and safer therapeutic strategy to allergic diseases.

In conclusion, we clarified that histamine is involved in pruritus and the development of dermatitis in a model of chronic allergic dermatitis established in NC/Nga mice. In addition to the direct involvement of histamine in pruritus via H1R and H4R, we clarified that histamine was also associated with the regulation of Sema3A in keratinocytes and production of Th2 chemokines in BMMC, via H1R and H4R respectively. Thus, our findings indicated that combined treatment with a H1R antagonist plus H4R antagonist potentiated both anti-pruritic and anti-inflammatory effects, and had a pharmaceutical benefit equivalent to prednisolone. Our understanding of the roles of histamine in chronic dermatitis indicate that a blockade of the H4R antagonist as well as H1R antagonist might have more potential benefit for patients with AD.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

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A group of atopic dermatitis without IgE elevation or barrier impairment shows a high Th1 frequency: Possible immunological state of the intrinsic type

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SUMMARY

Background: Atopic dermatitis (AD) can be classified into the major extrinsic type with high serum IgE levels and impaired barrier, and the minor intrinsic type with normal IgE levels and unimpaired barrier.
Objective: To characterize the intrinsic type of Japanese AD patients in the T helper cell polarization in relation to the barrier condition.
Methods: Enrolled in this study were 21 AD patients with IgE < 200 kU/L (IgE-low group; 82.5 ± 59.6 kU/L) having unimpaired barrier, and 48 AD patients with IgE > 500 kU/L (IgE-high group; 8.050 ± 10.400 kU/L). We investigated filaggrin gene (*FLG*) mutations evaluated in the eight loci common to Japanese patients, circulating Th1, Th2 and Th17 cells by intracellular cytokine staining and flow cytometry, and blood levels of CCL17/TARC, IL-18, and substance P by ELISA.
Results: The incidence of *FLG* mutations was significantly lower in the IgE-low group (10.5%) than the IgE-high group (44.4%) (normal individuals, 3.7%). The percentage of IFN-γ-producing Th1, but not Th2 or Th17, was significantly higher in the IgE-low than IgE-high group. Accordingly, Th2-attracting chemokine CCL17/TARC, was significantly lower in the IgE-low than the IgE-high group. There were no differences between them in serum IL-18 levels, or the plasma substance P levels or its correlation with pruritus.
Conclusion: The IgE-low group differed from the IgE-high group in that it had much less *FLG* mutations, increased frequency of Th1 cells, and lower levels of CCL17. In the intrinsic type, non-protein antigens capable of penetrating the unimpaired barrier may induce a Th1 eczematous response.

1. Introduction

Despite a large number of clinical, laboratory and experimental studies, the pathophysiology of AD remains unfully elucidated, because AD has heterogeneous aspects. The clinical phenotype of AD can be classified into the extrinsic and intrinsic types [1,2]. Since there is still no sufficient consensus whether the intrinsic type is a distinct entity, some researchers denominate it atopiform dermatitis [3]. Nevertheless, the classification into the extrinsic and intrinsic AD has been widely used, as various kinds of clinical studies have been performed under this dichotomy in many countries, including Germany [1,4,5], Netherland [3], Hungary [6], Italy [7,8], Korea [9,10], and Japan [11].

Extrinsic and intrinsic AD are defined according to IgE-mediated sensitization, namely the presence or absence of specific IgE for environmental and food allergens [10–12]. Whereas the extrinsic patients have high levels of serum IgE, the intrinsic type shows normal IgE levels, no specific IgE, and negative skin-prick test to common aeroallergens or food allergens [13]. Since total serum IgE values are correlated with the allergen-specific IgE status [14,15], total IgE can be regarded as a clinically useful parameter to expectedly differentiate between the extrinsic and

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Table 1
Patients enrolled in this study.

Number of subjects	IgE-high group	IgE-low group	
	46 (19 men and 27 women)	21 (7 men and 14 women)	
Age (years, mean ± SD)	30.5 ± 13.2	34.8 ± 13.4	$P = 0.226$
IgE (kU/L, mean ± SD)	8.050 ± 10.400	82.5 ± 59.6	$P < 0.001$
RAST for *D. pteronyssinus* (class, mean ± SD)	5.64 ± 0.693	1.77 ± 1.73	$P < 0.001$
LDH (IU/L, mean ± SD)	269 ± 91.2	206 ± 69.0	$P = 0.0129$
Eosinophils (%, mean ± SD)	8.79 ± 6.02	5.65 ± 5.45	$P = 0.0800$
VAS (mean ± SD)	54.2 ± 27.0	50.2 ± 23.0	$P = 0.686$
SCORAD (mean ± SD)	35.4 ± 16.1	31.3 ± 11.3	$P = 0.410$
TEWL ($g/m^2/h$, mean ± SD)	14.3 ± 7.57*	8.73 ± 3.74	$P = 0.0440$
Skin surface hydration (AU, mean ± SD)	29.2 ± 7.28*	34.7 ± 5.78	$P = 0.0250$

The normal ranges or values are as follows: LDH, 119–229 IU/L; TEWL, 6.58 ± 1.78 $g/m^2/h$; skin surface hydration (capacitance), 39.2 ± 14.4 AU.
* Statistically significant, compared to normal subjects.

intrinsic types in both adults [4,11] and children [14]. There are some differences in the frequency of intrinsic AD [15] among the countries, as reported as 27% [16], 37% [14], 12% [6], and 22% [11]. The female predominance has been consistently observed in intrinsic AD by a number of studies [1,3,15,17]. Females made up 76.5% of Intrinsic AD patients in our former study [11].

The original concept of "intrinsic" type represents the non-allergic nature, however, similarly to the extrinsic type, the intrinsic type may show eosinophilia. More fundamentally, both types represent eczematous dermatitis, a manifestation of the delayed-type or late-phase reaction. Therefore, the intrinsic type AD is not a non-allergic type, but is induced *via* some immunological mechanism.

When assessed by transepidermal water loss (TEWL), skin surface hydration, and perception threshold of electric stimuli, extrinsic AD patients showed an impaired barrier function, but intrinsic AD retained the normal barrier function and sensory reactivity to external pruritic stimuli [11]. The recent identification of loss-of-function mutations in filaggrin gene (*FLG*) sheds new light on the mechanisms of AD [18,19]. These mutations represent a strong predisposing factor for AD and asthma in various countries [20–23]. Perturbation of skin barrier function as a result of reduction or complete loss of filaggrin expression leads to enhanced percutaneous transfer of allergens. *FLG* mutations are associated seemingly with the extrinsic type of AD [20] and palmar hyperlinearity, which represents a shared feature of AD and ichthyosis vulgaris. Accordingly, palmar hyperlinearity is rarely observed in the intrinsic type [3]. Therefore, it is expected that intrinsic AD patients lack a mutation in *FLG*.

AD is well known as a Th2-polarized disease. However, some differences in systemic cytokine skewing have been reported between the extrinsic and intrinsic AD. Extrinsic AD shows high levels of Th2 cytokines, interleukin (IL)-4, IL-5 and IL-13, and intrinsic AD is linked with lower levels of IL-4 and IL-13 [7]. Along with the elevation of IL-5 [24], eosinophil counts [10] and eosinophil cationic protein levels [16] are increased in extrinsic AD. However, another report demonstrated that both extrinsic and intrinsic AD patients showed increased production of IL-5 and

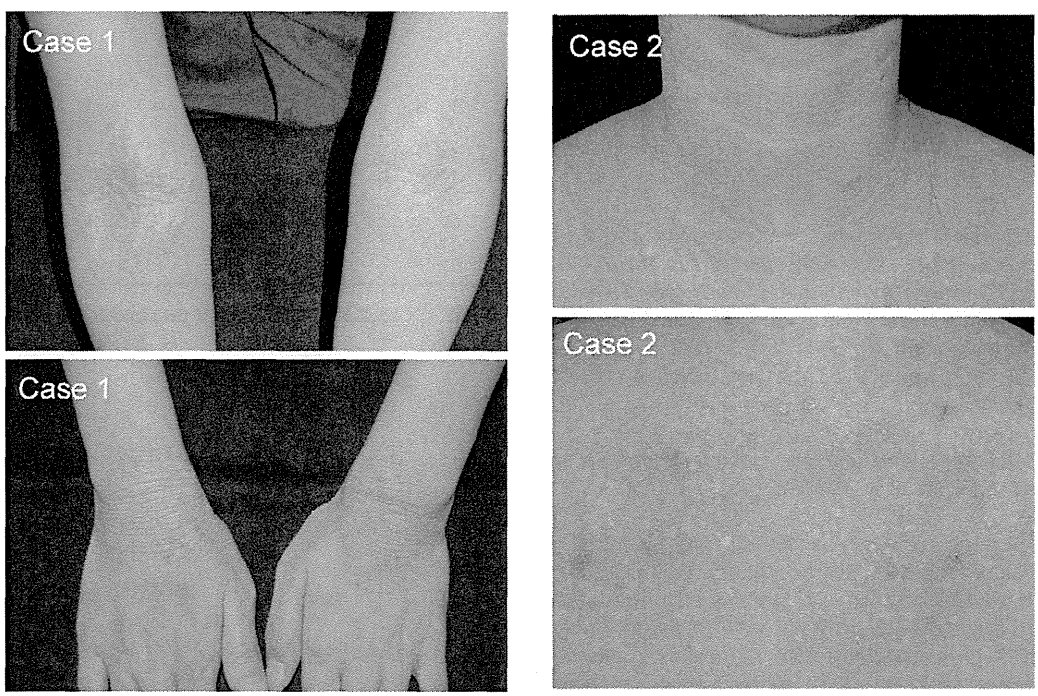

Fig. 1. Clinical photographs of the representative IgE-low AD patients. Case 1 (left): a 25-year-old female, with total serum IgE, 69 kU/L; and blood eosinophils, 10%. A lichenified eruption on the antecubital fossae (top, left) and the dorsal aspects of the wrists (bottom, left). She also had chronic eczema on the neck and trunk and was diagnosed as having AD in childhood. Case 2 (right): a 29-year-old female, with total serum IgE, 43 kU/l; and blood eosinophils, 11%. A lichenified eruption on the neck, upper chest (top, right), and back (bottom, right). The skin lesions had been diagnosed as AD since childhood.

IL-13 [25]. In addition, a more recent finding of the involvement of IL-17-producing Th (Th17) cells [26] raises an issue whether Th17 cells are more deeply associated with extrinsic or intrinsic AD.

These several different lines of evidence suggest the relationship between the skin barrier condition and helper T cell polarization in AD. We sought to investigate the immunological state of AD patients without elevation of serum IgE or impairment of barrier function. We first examined whether those patients had *FLG* mutations, and then investigated the frequencies of circulating Th1, Th2 and Th17 subsets with CCL17/TARC Th2 chemokine measurement. Results imply that the intrinsic AD is an immunological disorder fundamentally different from extrinsic AD.

2. Materials and methods

2.1. Participants and background assessments

Sixty-seven patients with AD (aged 9–59 years; 26 men and 41 women), and 10 healthy non-AD volunteers with low serum IgE levels (aged 28–38 years; 5 men and 5 women) were enrolled in this study. AD was diagnosed according to the criteria of Hanifin and Rajka classification [27]. The reported range or mean value of total serum IgE in the intrinsic type are from 22.2 to 134 kU/L, or alternatively, IgE values less than 150 or 200 kU/L have been used for an indication of intrinsic AD [15]. Our study of Japanese patients also showed that the mean value of total seurm IgE was 110.5 kU/mL (11–219 kU/L) [11]. In this study, we first selected 21 AD patients with IgE < 200 kU/L (mean ± SD, 82.5 ± 59.6 kU/L). As the control counterpart, 46 AD patients with IgE > 500 kU/L (8.050 ± 10.400 kU/L) were then selected. They were designated as the IgE-low and IgE-high groups, respectively. The disease activity was assessed by severity scoring of AD (SCORAD), and current itching was rated on a 100-mm visual analogue scale (VAS). The barrier function of stratum corneum was assessed by the skin surface hydration and TEWL [11]. The details of the patients were listed in Table 1. This study was conducted according to the Declaration of Helsinki Principles, performed after obtaining an informed consent from the patients, and approved by the Medical Ethical Committees of University of Occupational and Environmental Health and the Medical Ethical Committee of Hokkaido University Graduate School of Medicine.

2.2. FLG genotyping

FLG mutation analysis was performed in 18 patients of the IgE-high group (9 men and 9 women) and 19 patients of the IgE-low group (7 man and 12 women). All genomic DNA samples from the patients were screened for the eight previously reported Japanese-specific *FLG* mutations (R501X, 3321delA, S1695X, Q1701X, S2554X, S2889X, S3296X and K4022X) as described previously [21,28].

2.3. Intracellular cytokine staining and flow cytometric analysis of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll-Paque method. Intracellular cytokines were stained according to the protocol of Cytostain (Immunotech, Marseille, France) (26). Briefly, cells (2×10^6 cells/mL) were stained with PerCP-conjugated anti-CD8 monoclonal antibody (mAb), APC-conjugated anti-CD3 mAb (BD Biosciences), phycoerythrin-labeled anti-IL-17, IL-4, or IL-5, and FITC-labeled anti-IFN-γ mAb (all from BD Biosciences). Fluorescence profiles were analyzed by flow cytometry in FACSCanto (BD Biosciences). The percentage of $CD3^+$ $CD8^-$ cells bearing each cytokine was counted.

2.4. Naïve B cell isolation from PBMCs and quantitation of IgE in culture supernatants

Naïve B cells were separated from PBMCs of IgE-high AD patients using Naïve B Cell isolation Kit II (Miltenyi Biotec, Gladbach, Germany) and autoMACS (Miltenyi Biotec). 1×10^5 purified naïve B cells were cultured in RPMI-1640 in the presence of recombinant human IL-4 (10 μg/mL; R&D systems, Minneapolis, MN) and recombinant human CD40L (CD154) (200 μg/mL; R&D systems) for 2 weeks in the presence of 1, 5, 20 ng/mL of IFN-γ (R&D Systems, Inc). Culture supernatants were collected after 2 weeks, and IgE content was measured using Human IgE ELISA Quantitation Kit (Bethyl, Montgomery, TX).

2.5. CCL17/TARC and IL-18 measurements in sera

Serum CCL17/TARC and IL-18 levels were measured by ELISA (Special Reference Laboratories, Tokyo, Japan) in 20 AD patients with the IgE-high group (9 men and 11 women), 19 AD patients with the IgE-low group (6 men and 13 women) and 9 normal subjects (3 men and 6 women).

2.6. Substance P (SP) measurement

The concentration of plasma SP was measured by Kyowa Medex Co in 18 IgE-high AD patients (7 men and 11 women) and 12 IgE-low AD patients (4 men and 8 women).

Table 2
FLG mutations in IgE-high and IgE-low AD groups.

Case	Age	Sex	IgE(kU/L)	*FLG* mutation
IgE-high				
1	21	M	19,000	S2889X
2	19	M	2800	WT
3	24	M	675	3321delA, S2889X
4	25	F	22,000	S3296X
5	36	F	3790	WT
6	49	F	885	WT
7	29	F	53,000	WT
8	40	M	2120	3321delA, Q1701X
9	32	F	19,000	WT
10	45	M	10,000	WT
11	15	F	1600	3321delA
12	21	F	5200	WT
13	36	M	1060	WT
14	19	M	1360	S2889X
15	15	F	1090	WT
16	31	M	53,000	3321delA
17	30	F	567	WT
18	22	M	1320	WT
IgE-low				
1	28	F	43	WT
2	21	F	20	WT
3	25	F	69	WT
4	32	F	187	WT
5	59	M	81	WT
6	20	M	186	WT
7	32	F	67	K4022X
8	49	F	80	WT
9	18	F	45	WT
10	30	M	87	WT
11	37	M	6	WT
12	32	M	109	WT
13	22	F	14	WT
14	30	F	36	WT
15	46	F	44	Q1701X
16	27	F	14	WT
17	24	F	24	WT
18	43	M	148	WT
19	30	M	167	WT

WT: wild type.

2.7. Statistical analysis

Student's *t*-test (unpaired) and Fisher's test were employed to determine statistical differences. For all tests, *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Background of patients

Enrolled in this study were AD patients with he IgE-high (>500 kU/L) and IgE-low (<200 kU/L) groups with the mean values of 8.050 and 82.5 kU/L, respectively (Table 1). We first selected AD patients belonging to the IgE-low group, and their skin manifestations (Fig. 1) were indistinguishable from the IgE-high patients. IgE RAST was scored by index values 0–6 according to the manufacturer's criteria (BML, Tokyo, Japan): class 0 (≤0.34 UA/mL), class 1 (0.35–0.69 UA/mL), class 2 (0.70–3.49 UA/mL), class 3 (3.50–17.4 UA/mL), class 4 (17.5–49.9 UA/mL), class 5 (50.0–99.9 UA/mL), and class 6 (≥100 UA/mL). As a representative aeroallergen, we chose *Dermatophagoides pteronyssinus*. An index value ≥4 to *D. pteronyssinus* was obtained in 100% (18/18) of the IgE-high patients examined. Moreover, 75% of the IgE-high AD patients showed RAST score index value of 6, and none of the IgE-low patients showed this highest score. When expressed by the mean ± SD, the IgE-high group had score levels as high as class 5.64, whereas the low group had class 1.77. The RAST score for *Dermatophagoides farinae* showed virtually the same data. No significant difference was noted in age between the two groups. Women predominated in the IgE-low patients, as already noted [1,3,15,17]. SCORAD and VAS tended to be higher in the IgE-high than the IgE-low group, as reported previously in intrinsic AD [4,6,29]. Skin surface hydration was significantly lower in the IgE-high than the IgE-low AD and healthy controls, and TEWL, another assessment of the barrier function, was significantly higher in the IgE-high than the IgE-low AD and normal controls [11]. Thus, the skin barrier function was preserved in the IgE-low AD.

3.2. Significantly low incidence of FLG mutations in IgE-low group

We investigated *FLG* mutations in 18 IgE-high and 19 IgE-low AD patients with the previously reported method for Japanese AD patients [21]. Only 2 IgE-low cases (10.5%) had mutations in *FLG*, whereas 8 of 18 IgE-high patients (44.4%) possessed *FLG* mutations (Table 2). There was statistical significance between the two groups in the mutation incidence ($P = 0.0246$), suggesting that *FLG* mutations are less prevalent in the IgE-low group. The frequency of *FLG* mutations among normal controls without any history of allergy is 3.7%. In the IgE-high patients, there was no statistical difference in SCORAD or IgE level between the *FLG* mutation-bearing and *FLG* mutation-lacking patients.

3.3. Higher circulating Th1 cell frequency in the IgE-low group

The percentages of Th1, Th2 and Th17 cells in PBMCs were examined by flow cytometry after intracellular staining for IFN-γ,

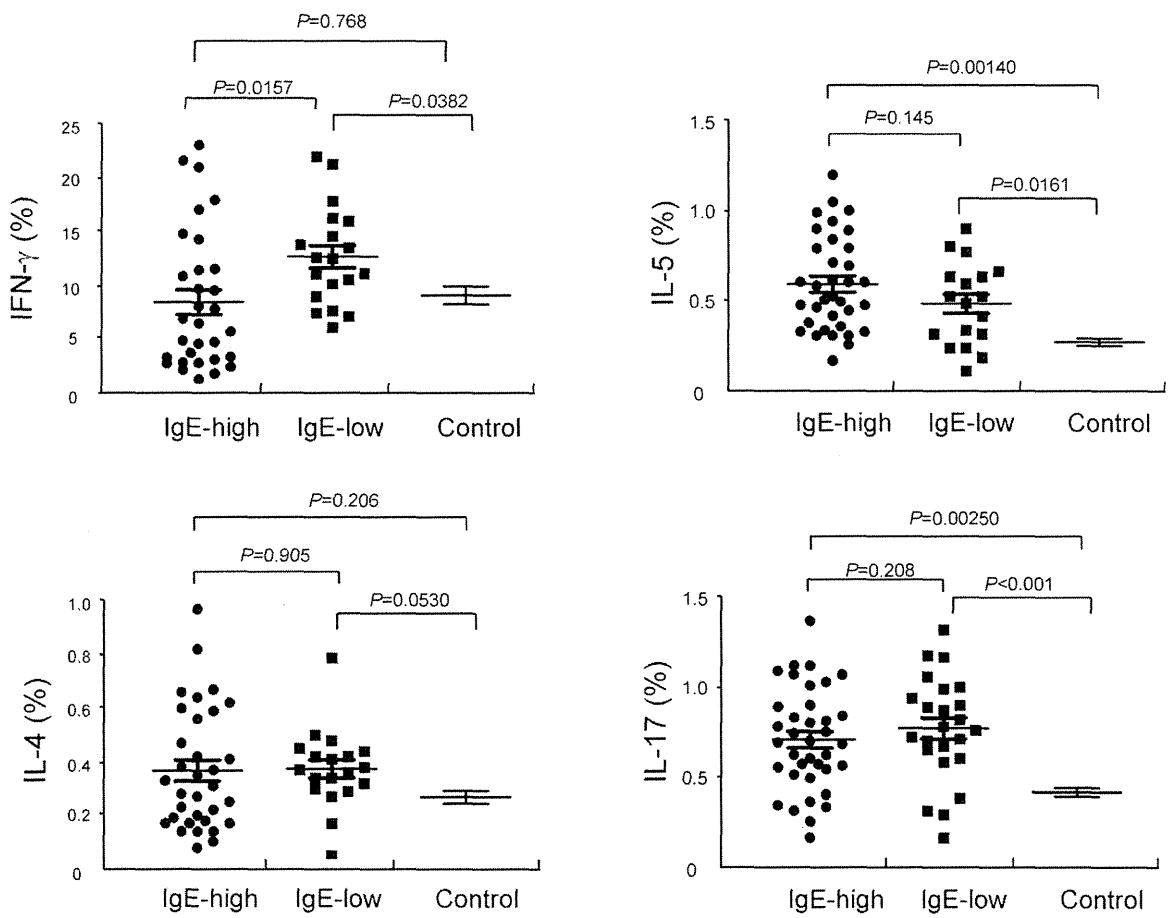

Fig. 2. Percentages of IFN-γ, IL-4, IL-5, and IL-17-positive $CD4^+$ cells. PBMCs from patients were incubated with PMA and Ca ionophore. They were then stained for CD3 and CD8, and subsequently stained intracellularly for IFN-γ, IL-4, IL-5, and IL-17. Since the stimulation with PMA and Ca ionophore downregulates the expression of CD4, $CD4^+$ cells positive for each cytokine was expressed as follows: (% $CD3^+$ cells positive for each cytokine) – (% $CD8^+$ cells positive for each cyokine). The vertical bars and error bars represent the mean ± SD.

IL-4, IL-5, and IL-17. The most intriguing finding is that the percentage of IFN-γ$^+$ T cells was significantly higher in the IgE-low than IgE-high group (Fig. 2). There was no significant difference in the IL-4, IL-5 or IL-17-positive cell frequency between the two types. These three cytokine-positive cells were increased in both groups of AD compared to the control.

3.4. Downregulation of B cell IgE production by IFN-γ

The above finding indicates that the IgE-low AD patients have high frequencies of IFN-γ$^+$ Th1 cells as well as low levels of IgE. To address the mechanism of this correlation, naïve B cells were purified from PBMCs of two patients with the IgE-high AD patients. The addition of IL-4 and CD40L (CD154) promoted class-switching and IgE production by B cells, which was inhibited by the addition of IFN-γ as low as 1 ng/mL (Fig. 3). This provides an implication that IFN-γ contributes to the normal level of IgE in the IgE-low AD.

3.5. Blood levels of CCL17/TARC, IL-18, and SP in the IgE-high and IgE-low AD groups

CCL17/TARC is a Th2 cell-attracting chemokine and its serum level is associated with the disease activity of AD [30]. IL-18 is possibly involved in the pathogenesis of AD [31], especially in the intrinsic type through the induction of IFN-γ. We therefore investigated serum CCL17/TARC and IL-18 levels in 20 IgE-high and 19 IgE-low patients. Both groups had higher levels of serum CCL17 than healthy control. Notably, its value was significantly higher in the IgE-high than the IgE-low group (Fig. 4). There was no significant difference in serum IL-18 level between the two groups of AD, as the means ± SD of IL-18 were 104.4 ± 34.97 (n = 20) in the IgE-high group and 92.40 ± 44.83 (n = 19) in the IgE-low group.

Given the original idea that external allergens are not causative in the intrinsic type, neurogenic inflammation induced by neuropeptides might be more important in this type of AD [32]. To address this issue, we measured plasma levels of SP in the two groups. The levels of SP in the IgE-high and IgE-low groups were comparable, as they were 50.6 ± 20.6 pg/mL and 59.5 ± 23.0 pg/mL, respectively. In both groups, SP levels and VAS for pruritus significantly correlated with each other (Fig. 5), indicating no dominancy of neuropeptides for the IgE-low group.

4. Discussion

The IgE-low type is a minor population of AD, but its pathophysiology is an issue of interest. In this study, we compared the IgE-low AD patients with the IgE-high patients, focusing on *FLG* mutations and the systemic Th1/Th2 polarization. The IgE-low group retained the common AD properties, *i.e.* typical clinical manifestations, eosinophilia, and increased circulating Th2 cell percentage. However, it showed a normal barrier function with a lower frequency of *FLG* mutations and an increased number of IFN-γ-producing Th1 cells. In accordance with our data, it has been reported that *FLG* mutations predispose to early-onset and extrinsic AD [20].

Regarding Th1/Th2 polarization, it has been reported that extrinsic AD shows high levels of Th2 cytokines and intrinsic AD is linked with lower levels of them [7]. The apoptosis of circulating memory/effector Th1 cells is confined to extrinsic AD patients, whereas intrinsic AD patients show no evidence for enhanced T cell apoptosis *in vivo* [33]. Another group of investigators demonstrated that both extrinsic and intrinsic AD patients had increased production of IL-5 and IL-13 [25], but when PBMCs were stimulated with anti-CD3 antibody, extrinsic AD patients had a decreased capacity to produce IFN-γ [25]. Accordingly, IFN-γ$^+$ T cell frequency was higher in the IgE-low than IgE-high group in our study. Although no significant difference was found in the percentages of IL-4$^+$, IL-5$^+$ or IL-17$^+$ T cells, Th2 cells tended to be higher in the IgE-high group. Thus, intrinsic AD may have a less Th2-skewing state and rather shows a high expression level of IFN-γ. The elevation of serum CCL17/TARC, a Th2 attractive chemokine, in the IgE-high AD further supported this notion. In the skin lesions, eosinophils infiltrate more markedly in the extrinsic than intrinsic type [9,34], suggesting the less Th2 and more Th1 preponderant state in the skin as well. The overproduction of IFN-γ may further downregulate IgE production in the IgE-low AD, as suggested by our *in vitro* study.

A non-IgE-associated, mouse AD model may be regarded as a mimicry of human intrinsic AD [35]. In this model, IL-18 contributes to the spontaneous development of skin lesions

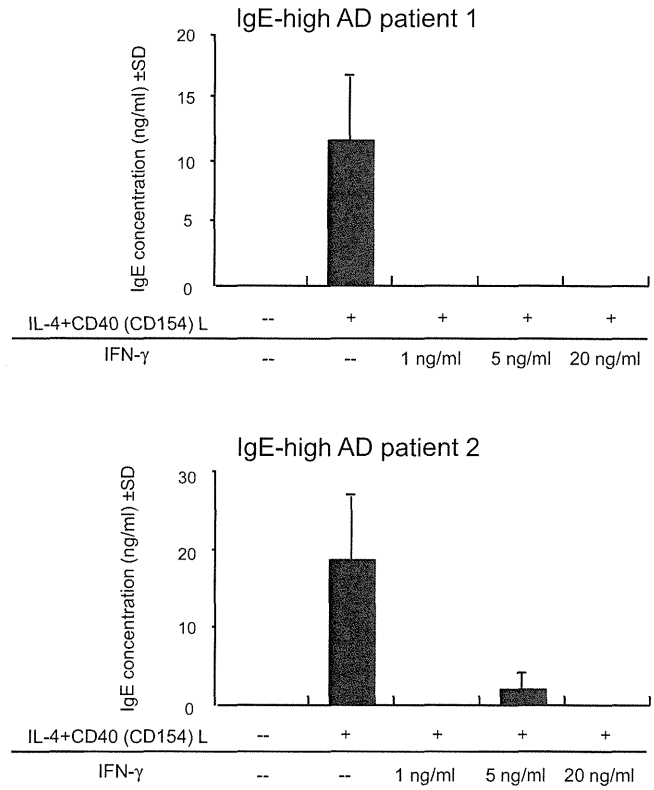

Fig. 3. IFN-γ-downregulation of IgE production by B cells from IgE-high AD patients. PBMCs from 2 patients with IgE-high AD were purified for naïve B cells. They were stimulated with IL-4 and CD40L (CD154) to produce IgE. IFN-γ was simultaneously added to the culture at a concentration of 1, 5, or 20 ng/mL.

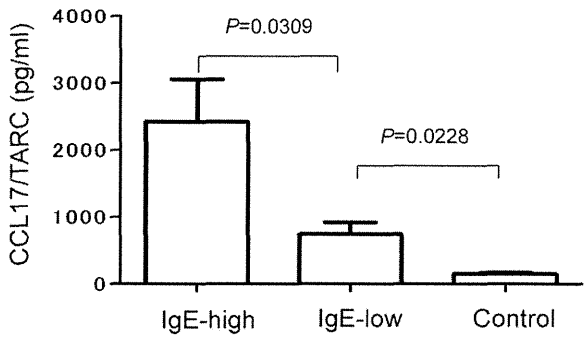

Fig. 4. Blood CCL17/TARC in IgE-high and IgE-low AD. Serum samples were obtained from IgE-high AD patients (n = 20), IgE-low AD patients (n = 19) and control subjects (n = 9). The means ± SD were 2430 ± 2820 in IgE-high AD and 851 ± 771 in IgE-low AD.

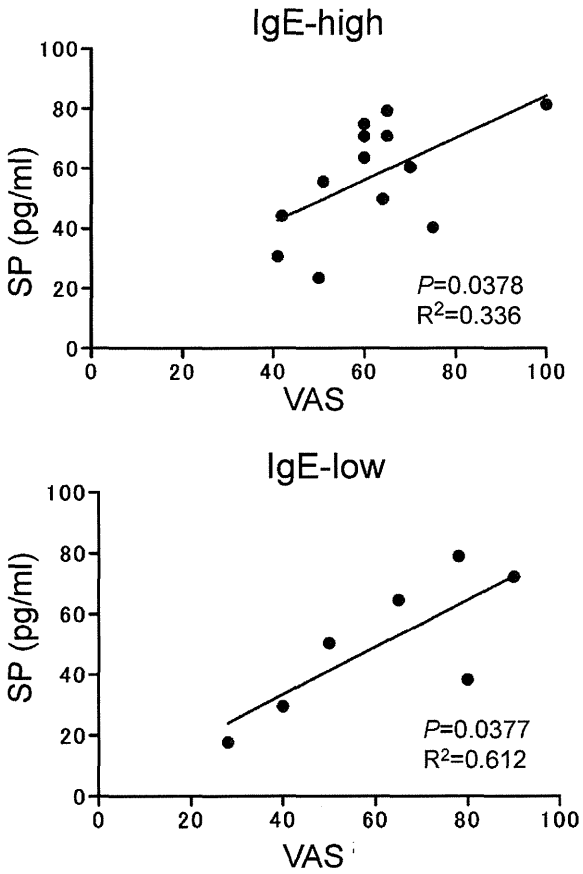

Fig. 5. Blood SP levels in IgE-high and IgE-low AD. Plasma SP levels were measured as described in Section 2, and simultaneously, VAS for pruritus was measured in the individual patients.

independently of IgE [36]. The mice can develop AD lesions with dermal infiltration of eosinophils and mast cells and showed an increase in serum levels of IL-18, but not IgE. This mouse model resembles intrinsic AD, since IFN-γ is an IL-18-promoted cytokine. However, we could not find a significant elevation of serum IL-18 in our IgE-low AD patients compared to the IgE-high patients. Thus, the IL-18 mediation remains unclear in human intrinsic AD.

In a mouse model of contact hypersensitivity, the responses to hapten are increased when a hapten is applied to the barrier-damaged skin [37]. Not only increased skin permeability but also altered immune functions of epidermal cells potentiate T-cell activation in acute barrier disruption [37]. The mRNA expression levels of Th2 chemokines and eosinophil chemoattractant are high in the epidermal cells from barrier-disrupted Th2-skewing BALB/c mice [38]. Th1 and Th2 chemokines are derived mainly from keratinocytes and Langerhans cells, respectively, and one of the crucial actions of IFN-γ is upregulation of keratinocyte production of Th1 chemokines and downregulation of Langerhans cell production of Th2 chemokines [39]. Therefore, the barrier damage likely induces infiltrates of Th2 cells and eosinophils in extrinsic AD, but their infiltrates are inhibited by IFN-γ in intrinsic AD.

We also examined SP levels in the IgE-high and IgE-low AD patients. It has been reported that neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are increased in both extrinsic and intrinsic AD, suggesting the presence of a similar pathophysiologic background implicating a neuroimmune network [40]. However, there is a significant correlation between BDNF and SCORAD in the intrinsic but not extrinsic type [40]. Maternal NGF levels were high in both extrinsic and intrinsic AD [29]. We found that there was no difference in SP levels between the two groups, and these SP levels were correlated with the itch levels in both groups. This may imply that neurogenic inflammation is not a characteristic feature of intrinsic AD.

The normal barrier function and IFN-γ-producing potency of the IgE-low AD suggest that the patients are not sensitized with protein allergens, which are known to induce Th2 responses, but with other non-protein antigens. It is assumed that protein antigens are not allowed to enter into the skin in intrinsic AD because of the normal barrier. Instead, non-protein antigens, such as metals and haptens, can penetrate through the barrier and may induce Th1 as well as Th2 responses. In 137 atopic children, 19.3% patients were positive to metals [41], although the two types of AD were not separately studied. In 1965, Shanon reported that patients with metal allergy occasionally exhibit a skin manifestation indistinguishable from AD under the name of "pseudo-atopic dermatitis" [42]. Further investigation on this issue might clarify the pathophysiology of intrinsic AD.

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Chemokine-dependent T cell migration requires aquaporin-3–mediated hydrogen peroxide uptake

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Chemokine-dependent trafficking is indispensable for the effector function of antigen-experienced T cells during immune responses. In this study, we report that the water/glycerol channel aquaporin-3 (AQP3) is expressed on T cells and regulates their trafficking in cutaneous immune reactions. T cell migration toward chemokines is dependent on AQP3-mediated hydrogen peroxide (H_2O_2) uptake but not the canonical water/glycerol transport. AQP3-mediated H_2O_2 transport is essential for the activation of the Rho family GTPase Cdc42 and the subsequent actin dynamics. Coincidentally, AQP3-deficient mice are defective in the development of hapten-induced contact hypersensitivity, which is attributed to the impaired trafficking of antigen-primed T cells to the hapten-challenged skin. We therefore suggest that AQP3-mediated H_2O_2 uptake is required for chemokine-dependent T cell migration in sufficient immune response.

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Abbreviations used: AQP, aquaporin; CHS, contact hypersensitivity; DNFB, 2,4-dinitrofluorobenzene; DPI, diphenyleneiodonium; Nox, NADPH oxidase; Oxa, oxazolone; TNCB, 2, 4, 6-trinitro-1-chlorobenzene.

Regulated T cell migration and trafficking are of crucial importance for both steady-state T cell homeostasis and active immune responses. Although naive T cells constitutively circulate between the blood and secondary lymphoid organs in a state of immune surveillance, antigen-encountered T cells selectively migrate to extralymphoid sites to exert their secondary response to antigens (Mora and von Andrian, 2006; Pittet and Mempel, 2008). The mechanistic basis of regulated T cell trafficking involves the differential expression of adhesion molecules and chemokine receptors of naive and activated T cells (Campbell et al., 2003; Schaerli and Moser, 2005; Viola et al., 2006). The naive T cells express the LN homing receptor L-selectin (CD62L) and CCR7, enabling them to preferentially migrate to the secondary lymphoid organs (Campbell et al., 1998; Mora and von Andrian, 2006). In contrast, effector T cells express CCR4 and CCR10 instead of CD62L and CCR7, enabling them to migrate to peripheral nonlymphoid tissues, such as the gut and skin, in response to the chemokines CCL17, CCL22, and CCL27 (Campbell et al., 1999; Reiss et al., 2001). Such chemokine-dependent T cell migration requires actin-dependent changes in cell morphology and mobility, which are regulated by the Rho family GTPases, including Cdc42, Rac1, and RhoA (Burkhardt et al., 2008; Tybulewicz and Henderson, 2009).

Aquaporins (AQPs) are a family of highly conserved transmembrane channels that transport water and, in some cases, small solutes such as glycerol. Currently, 13 AQPs have been identified in mammals (AQP0–12). Numerous studies have demonstrated the fundamental importance of AQPs and have described their functions in several organs and physiological pathways, such as AQP1–3 in the urinary concentrating system, AQP1 in angiogenesis, AQP7 in obesity, and AQP4 in neuromyelitis optica and brain edema (Rojek et al., 2008; Verkman, 2009; Carbrey and Agre, 2009). More recently, some AQPs, including AQP3 and AQP8, have been found to mediate membrane

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hydrogen peroxide (H_2O_2) uptake, which is used for intracellular signaling in mammalian cells (Miller et al., 2010). Despite their importance in various biological systems, to date, AQPs have not been shown to be involved in adaptive immunity, a process in which specialized lymphocytes at different developmental stages precisely mediate protection against pathogens to maintain homeostasis. Importantly, because previous studies have shown that AQPs regulate cell migration and proliferation in some mammalian cells (Verkman, 2009), we anticipated that AQPs might play a role in the regulation of lymphocyte function.

AQP3 is abundantly expressed on the plasma membrane of kidney-collecting duct principal cells and skin epidermal keratinocytes, which facilitate water and glycerol transport (Ma et al., 2000; Hara and Verkman, 2003). Our previous studies have shown that AQP3 is necessary for keratinocyte migration and proliferation, processes which have been implicated in cutaneous wound healing and tumorigenesis (Hara-Chikuma and Verkman, 2008a,b). During the course of our study, we unexpectedly found that the AQP3 protein was expressed not only by keratinocytes but also by skin-infiltrating T cells. In this study, using genetically modified AQP3 knockout mice, we have identified a novel role of AQP3 in chemokine-dependent T cell migration, which controls cutaneous immune reactions.

RESULTS

Normal cellularity and subpopulations of T cells in AQP3-null mice

Because we noted that AQP3 protein was expressed in skin-infiltrating T cells during contact hypersensitivity (CHS) in preliminary experiments, we focused on the function of AQP3 in T cells. Quantitative real-time RT-PCR analysis showed similar AQP3 expression levels in $CD4^+$ and $CD8^+$ cells; the expression levels were lower than those found in kidney extract, which is known to exhibit high AQP3 expression levels (Fig. 1 a; Ma et al., 2000). Using a germline AQP3 knockout ($AQP3^{-/-}$) mouse as a control (Ma et al., 2000), we verified the expression of AQP3 protein in both $CD4^+$ and $CD8^+$ T cells from WT mice using flow cytometry (Fig. 1 b).

To determine the function of AQP3 in T cells in vivo, phenotypic analysis was conducted on peripheral T cells and thymocytes from WT and $AQP3^{-/-}$ mice. $CD4^+$ and $CD8^+$ lymphocyte populations in the spleen, LNs, and thymus (Fig. 1, c and d), the expression of CD3, CD25, CD44, and CD62L in the thymus and LN (not depicted), and T cell proliferation levels (Fig. 1 e) from each of these preparations were similar between WT and $AQP3^{-/-}$ mice, indicating that the germline AQP3 deficiency did not affect T cell development or homeostasis under steady-state conditions.

Impaired chemotaxis efficiency and actin polymerization in AQP3-deficient T lymphocytes

Previous studies have demonstrated that AQP regulates the migration of several cell types (e.g., epithelial and endothelial cells), although the cellular and molecular mechanisms that underlie these processes remain controversial (Saadoun et al., 2005; Hara-Chikuma and Verkman, 2006, 2008a). We found that the efficiency of T cell migration toward several ligands (CXCL12, CCL19, CCL17, and CCL27) was significantly impaired in $AQP3^{-/-}$ T cells compared with WT T cells (Fig. 2 a), whereas no difference was found in T cell migration in the absence of chemokines (not depicted). Using chemotaxis chambers, WT T cells exhibited greater directed migration in response to CXCL12 chemotactic gradients than $AQP3^{-/-}$ T cells (Fig. 2 b). In response to CXCL12, transendothelial migration of $CD4^+$ T cells through vascular endothelial cells was also attenuated by AQP3 deficiency (Fig. 2 c). Conversely, cell adhesion was similar for both WT and $AQP3^{-/-}$ T cells (not depicted). In response to chemotactic signals, T cells reorganize their actin cytoskeletons and

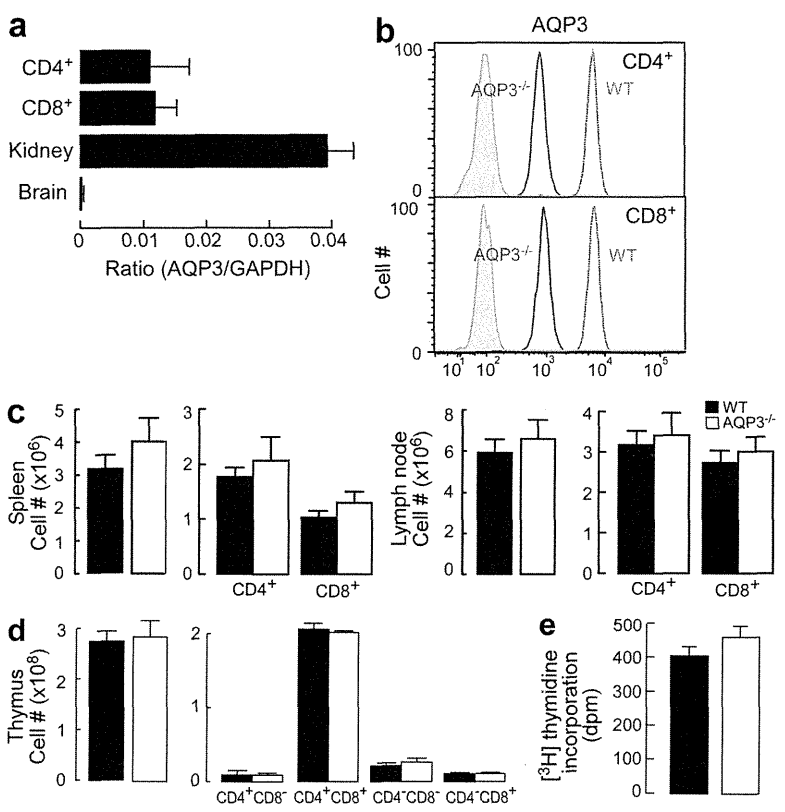

Figure 1. Normal cellularity and subpopulations of T cells in AQP3-null mice. (a) The messenger RNA expression levels of AQP3 in sorted $CD4^+$ and $CD8^+$ T cells as well as in kidney and brain tissues were assessed by real-time PCR (SE; $n = 4$). Data are expressed as the AQP3/GAPDH ratio. (b) Flow cytometric analysis of AQP3 expression in $CD4^+$ (top) or $CD8^+$ (bottom) T cells from WT and $AQP3^{-/-}$ mice. (c) Cell population analysis in the spleen (left) and LN (right). The numbers of total cells, $CD4^+$ and $CD8^+$ cells from WT and $AQP3^{-/-}$ mice (SE; $n = 4$), are shown. (d) Cell population analysis in the thymus. The numbers of total cells and indicated subsets (SE; $n = 4$) are shown. (e) [^{3}H]Thymidine incorporation in $CD4^+$ T cells from WT and $AQP3^{-/-}$ mice (SE; $n = 4$–5). Each experiment was performed three times.

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become polarized in the direction of the chemoattractant gradient, which leads to chemotaxis and T cell trafficking (Burkhardt et al., 2008; Tybulewicz and Henderson, 2009). Chemokine-stimulated WT T cells developed a polarized morphology at the leading edge, which was visualized with phalloidin staining (Fig. 2 d, left). Immunofluorescence staining showed that AQP3 was localized at the leading edge as well as in the plasma membrane in CXCL12-treated T cells, whereas control T cells tended to express AQP3 both intracellularly and on the cell surface (Fig. 2 d, left). In contrast, AQP3$^{-/-}$ T cells failed to develop distinct uropods, instead exhibiting multiple, irregularly distributed areas of patch-like F-actin staining (Fig. 2 d, left). Chemokine-induced increase in the aspect ratio of a cell shape, which is defined as the length of the major axis divided by the width of the minor axis (Yang et al., 2005), was significantly impaired in AQP3$^{-/-}$ T cells compared with WT cells (Fig. 2 d, right). Consistent with these observations, quantification of F-actin levels revealed a significant impairment in chemokine-induced actin polymerization in AQP3$^{-/-}$ T cells in comparison with WT T cells (Fig. 2 e).

Impaired CHS with decreased T cell trafficking to challenged skin in AQP3-null mice

To determine the role of AQP3-mediated T cell trafficking in ongoing immune reactions, we used an experimental CHS model; specifically, we examined the T cell–mediated cutaneous immune/inflammatory reaction to haptens (Grabbe and Schwarz, 1998; Martin, 2004). WT and AQP3$^{-/-}$ mice were sensitized by applying haptens to the abdomen and were challenged on the ear 5 d later. Ear swelling (evidence of CHS) in response to the challenge with 2,4-dinitrofluorobenzene (DNFB), 2, 4, 6-trinitro-1-chlorobenzene (TNCB), and oxazolone (Oxa) was significantly decreased in AQP3$^{-/-}$ mice compared with WT mice (Fig. 3 a). Histological examination revealed pronounced spongiosis and extensive infiltration of lymphocytes in the dermis of WT mice, whereas such inflammation was mild in AQP3$^{-/-}$ mice (Fig. 3 a, right). A single application of DNFB or the nonspecific irritant croton oil induced identical ear swelling, indicating that non–T cell–mediated inflammatory responses were unaffected by AQP3 expression (not depicted).

AQP3 is expressed not only in T cells but also in epidermal keratinocytes, where it regulates cell proliferation (Hara-Chikuma and Verkman, 2008a,b). To exclude the possibility that the impaired CHS response in AQP3$^{-/-}$ mice was a result of compromised keratinocyte function, lethally irradiated C57BL/6 WT mice were

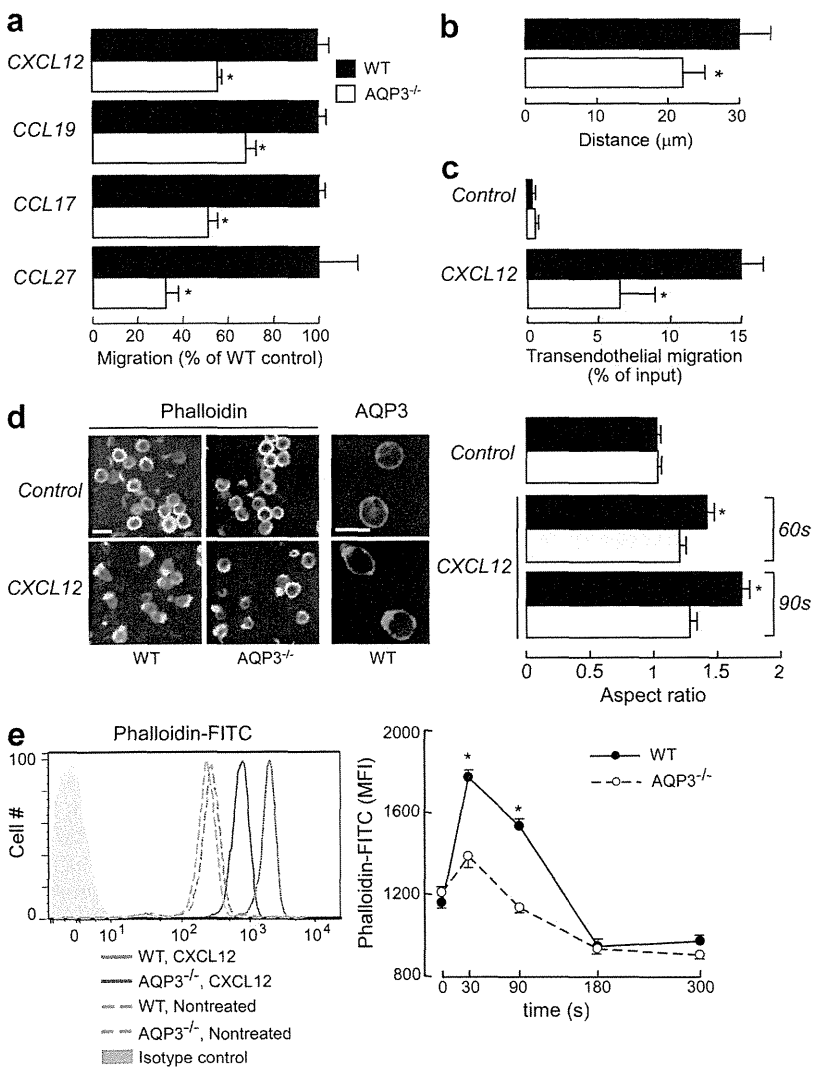

Figure 2. Impaired chemotaxis efficiency and F-actin polymerization levels of AQP3-deficient T lymphocytes. (a) Chemotaxis assay. The migration efficiency of CD4$^+$ T cells from WT and AQP3$^{-/-}$ mice toward the ligands CXCL12 (100 ng/ml), CCL19 (100 ng/ml), CCL17 (80 ng/ml), or CCL27 (80 ng/ml) was examined using a transwell chamber with 5-μm pores. Data are expressed as the percentage of WT control migration levels (SE; $n = 5$; *, $P < 0.01$). (b) Three-dimensional chemotaxis assay in response to CXCL12 gradient for 60 min. Accumulated distances for each cell (SE; $n = 50$; *, $P < 0.01$) are shown. (c) Transendothelial migration of CD4$^+$ T cells through mouse vascular endothelial cells (F-2 cells) in the presence of 100 ng/ml CXCL12 (SE; $n = 5$; *, $P < 0.01$). (d) CD4$^+$ WT and AQP3$^{-/-}$ T cells were stimulated with 500 ng/ml CXCL12 and stained with phalloidin-FITC (90 s) or with anti-AQP3 (3 min; cy3). (left) Representative immunofluorescence microscopy. Bars, 10 μm. (right) T cells were stimulated for 60 or 90 s with CXCL12. The aspect ratio of cell shape was quantified by measuring the length of the major axis divided by the width of the minor axis (SE; $n = 50$; *, $P < 0.01$). (e) T cells from WT and AQP3$^{-/-}$ mice were stimulated with 500 ng/ml CXCL12 and stained with phalloidin-FITC and CD4-Pacific blue. (left) Flow cytometry analysis of phalloidin-FITC gated on CD4$^+$ cells. One of four representative experiments is shown. (right) The mean fluorescence intensity (MFI) of phalloidin-FITC in the CD4$^+$ cells was analyzed (SE; $n = 5$; *, $P < 0.01$, WT vs. AQP3$^{-/-}$ cells). Each experiment was performed three times.

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reconstituted with BM cells from either WT or AQP3$^{-/-}$ mice. Ear swelling was impaired in the chimeric mice reconstituted with AQP3-deficient BM cells compared with the response in mice reconstituted with WT BM cells (Fig. 3 b). These data indicate that the CHS response requires AQP3 expression on hematopoietic cells but not on keratinocytes.

We further examined the sensitization and elicitation phases of the CHS model. First, T cells from the draining LNs of WT and AQP3$^{-/-}$ mice were isolated 5 d after DNFB sensitization, and their responses to 2,4-dinitrobenzene sulfonic acid, a water-soluble compound with the same antigenicity as DNFB, were compared in vitro. We found comparable proliferative responses and IFN-γ production levels between WT and AQP3$^{-/-}$ mice (not depicted), suggesting that T cell priming occurred at equal levels in both groups of mice during the sensitization period.

Next, we investigated the elicitation phase of CHS using intravenous adoptive transfer. As shown in Fig. 3 c, the transfer of primed AQP3$^{-/-}$ T cells resulted in reduced ear swelling irrespective of AQP3 expression in the recipients compared with that resulting from the transfer of WT donor cells, suggesting that the cell-intrinsic defects in AQP3$^{-/-}$ T cells resulted in a defective elicitation of the immune response. To determine the involvement of AQP3 in T cell trafficking/homing during a secondary challenge in vivo, we adoptively transferred sensitized WT or AQP3$^{-/-}$ T cells that were labeled with a cell-tracking dye (CMFDA). Comparable numbers of CD4$^+$ CMFDA$^+$ T cells from WT and AQP3$^{-/-}$ mice were found in the LNs, whereas fewer skin-infiltrating T cells from AQP3$^{-/-}$ mice were observed than from WT mice (Fig. 3 d). The chemokine expression levels in the DNFB-challenged skin (not depicted), the chemokine receptor expression levels on the T cells (not depicted), and naive T cell homing into LNs (not depicted) were similar between WT and AQP3$^{-/-}$ mice.

Finally, we performed subcutaneous adoptive transfer experiments. When we injected sensitized LN cells directly into the ears of recipient WT or AQP3$^{-/-}$ mice, the AQP3$^{-/-}$ T cells overcame the defect and induced the same strong CHS that was observed with WT T cells (Fig. 3 e). Collectively, our findings provide evidence that AQP3 expression is important in the trafficking of T cells to antigen-challenged skin sites during CHS responses.

Impaired chemokine-induced Cdc42 activation in AQP3-deficient T lymphocytes

Our data suggest that AQP3 plays a fundamentally critical role in actin polymerization and subsequent T cell chemotaxis. The pathways that regulate actin dynamics are well characterized and involve Rho family small

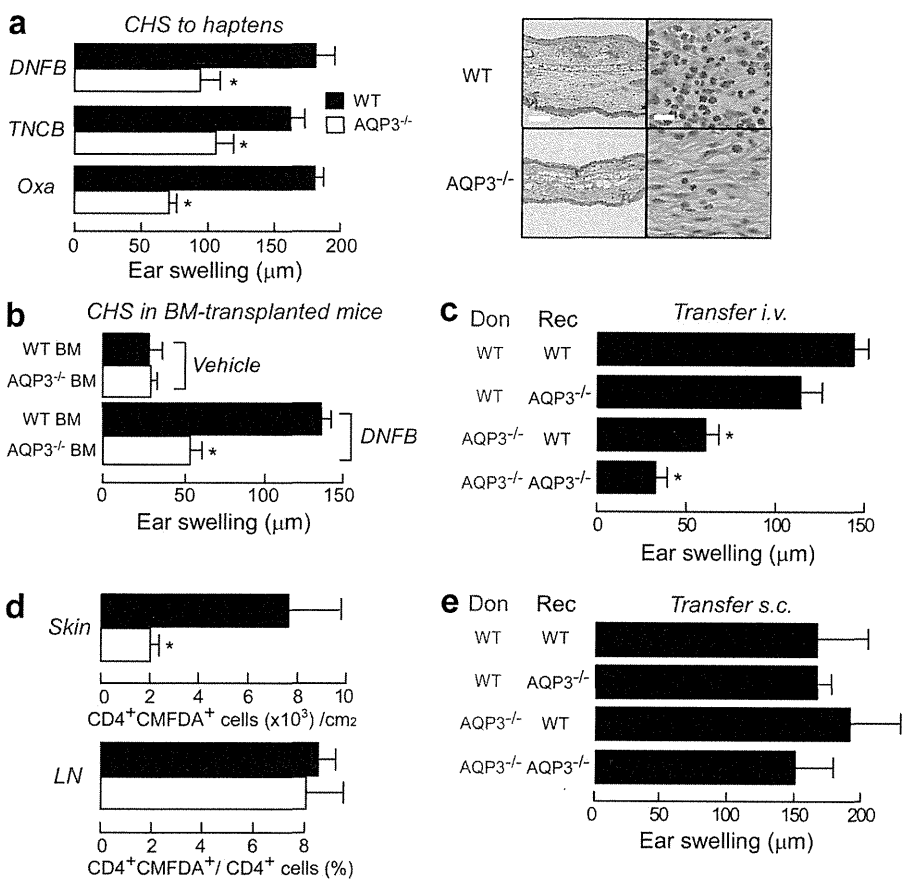

Figure 3. Impaired CHS with decreased T cell trafficking to a challenged skin site in AQP3-null mice. (a, left) Mice were sensitized with DNFB, TNCB, or Oxa and challenged 5 d later on the ear. The ear thickness was measured in micrometers 24 h after the challenge (SE; $n = 5$; *, $P < 0.01$). (right) Hematoxylin and eosin staining of the ears of sensitized WT and AQP3$^{-/-}$ mice at 24 h after challenge with DNFB. Bars: (left) 100 μm; (right) 20 μm. (b) CHS test using BM cell-transferred mice. C57BL/6 mice received transplants of BM cells from WT and AQP3$^{-/-}$ mice. The CHS test was performed with DNFB 2 mo later (SE; $n = 4$; *, $P < 0.01$). (c) Adoptive transfer experiments by intravenous injection. LN cells from sensitized donor WT and AQP3$^{-/-}$ mice (Don) were injected intravenously (3×10^7 cells/head) to recipient mice (Rec). Ear swelling at 24 h after challenge (SE; $n = 3$–5; *, $P < 0.01$) is shown. (d) Adoptive transfer experiments by intravenous injection. LN cells from sensitized WT and AQP3$^{-/-}$ donors were stained with CMFDA and injected into recipient WT mice, and these mice were challenged with 0.3% DNFB. The ear skin, which was painted with DNFB, and LNs were excised 24 h after challenge. CD4$^+$ and CMDFA$^+$ cells were analyzed by flow cytometric analysis (SE; $n = 4$; *, $P < 0.01$). (e) Adoptive transfer experiments by subcutaneous injection (2×10^5 cells) into the ears. Ear swelling at 24 h after challenge is shown (SE; $n = 4$). Experiments in a, c, and e were performed in two other independent experiments and in b and d in one other experiment with similar results.

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GTPases such as Cdc42, Rac1, and RhoA. To gain insight into the molecular mechanism that regulates AQP3-mediated actin polymerization, we examined the activation of each GTPase upon CXCL12 stimulation. Quantification of the activated forms of each GTPase using a colorimetry-based assay revealed a complete impairment in Cdc42 activation in response to CXCL12 in AQP3$^{-/-}$ T cells (Fig. 4 a). Compared with WT cells, AQP3$^{-/-}$ cells exhibited minimal activation of Rac1, although this difference was not statistically significant (Fig. 4 b). CXCL12-induced Rac1 activation was lower in AQP3$^{-/-}$ than in WT T cells ($P < 0.07$). We considered that Rac1 activation was also attenuated by AQP3 deficiency. No difference was found between AQP3$^{-/-}$ and WT cells for CXCL12-induced RhoA activation (Fig. 4 c).

Cdc42 activates WASP (Wiskott-Aldrich syndrome protein), which, through the Arp2/3 protein complex, leads to actin polymerization (Burkhardt et al., 2008; Tybulewicz and Henderson, 2009). AQP3$^{-/-}$ T cells also exhibited a defect in CXCL12-induced phosphorylation of WASP and Arp2 (Fig. 4 d). Moreover, Tec family tyrosine kinase Itk has been suggested to be one of the upstream signaling molecules of CXCL12-induced Cdc42 and Rac1 activation pathways (Fischer et al., 2004; Takesono et al., 2004). Fig. 4 e showed the impairment of CXCL12-induced Itk phosphorylation in AQP3$^{-/-}$ T cells.

We next sought to examine the implications of AQP3-mediated Cdc42 activation in terms of actin polymerization using dominant-active (V12-Cdc42) mutants of Cdc42. Given the technical difficulty of transfecting primary mouse T cells, we established an experimental protocol in which endogenous AQP3 was efficiently knocked down by siRNA and subsequently transfected with exogenous Cdc42-cDNA in human T cells. AQP3 knockdown in human T cells reproduced the impaired chemokine-induced actin polymerization (Fig. 4 f) and chemotaxis (not depicted) observed in AQP3$^{-/-}$ mouse T cells. In this setting, impaired actin polymerization was restored by the expression of V12-Cdc42 (Fig. 4 g). These findings suggest that AQP3 is required for chemokine-induced actin polymerization and polarization of T cells through the activation of pathways involving Cdc42.

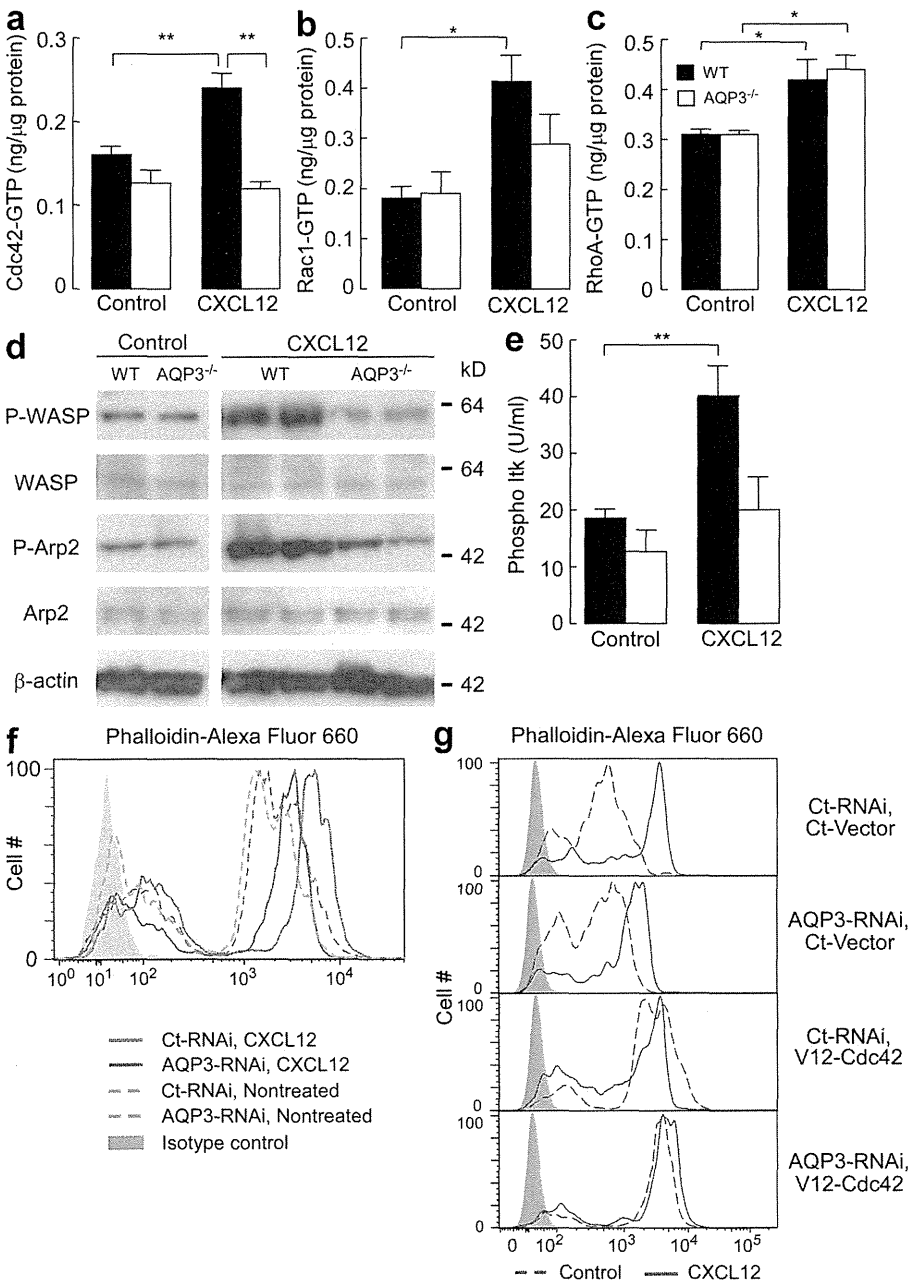

Figure 4. Impaired chemokine-induced Cdc42 activation in AQP3-deficient T cells. (a–c) Quantifications of Cdc42, Rac1, and RhoA activation. T cells were incubated with 250 ng/ml CXCL12 for 1 min, and GTP-bound active form of Cdc42 (a), Rac1 (b), and RhoA (c) were assessed using the G-LISA activation assay kit (SE; $n = 4$–5; *, $P < 0.05$; **, $P < 0.01$). (d) Immunoblot analyses to detect the phosphorylation of WASP and Arp2. T cells were incubated with 250 ng/ml CXCL12 for 3 min and were analyzed using antibodies against phospho-WASP (P-WASP), WASP, phospho-Arp2, Arp2, and β-actin. The blots shown are representative of three separate sets of experiments. (e) Phosphorylated Itk in response to CXCL12 (250 ng/ml, 1 min) was quantified with BD cytometric bead array (SE; $n = 4$–5; **, $P < 0.01$). (f and g) F-actin polymerization in response to 500 ng/ml CXCL12 in AQP3 knockdown (f) and/or V12-Cdc42–positive human primary T cells (g). One representative experiment of three experiments is shown. Experiments in a–c and e were performed in two other independent experiments.

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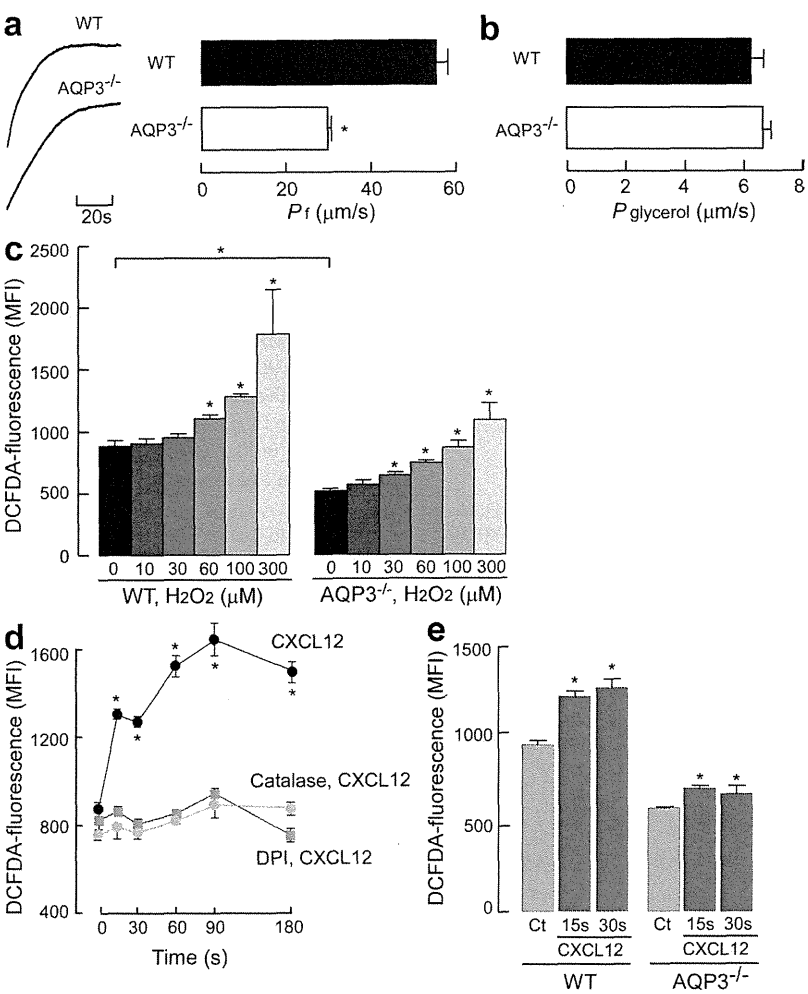
Water/H_2O_2 permeability is dependent on AQP3 expression in T cells

To further investigate the mechanism underlying AQP3-mediated T cell chemotaxis, we examined whether the membrane transport of water, glycerol, and H_2O_2, which may affect cellular activities, occurred in T cells in an AQP3-dependent manner. The osmotic water permeability of $CD4^+$ T cells was measured using the kinetics of scattered light intensity in response to osmotic challenge (Fig. 5 a, left), as previously described (Yang et al., 2001). Water transport in response to a 150-mM inwardly directed mannitol gradient was significantly higher in WT than in $AQP3^{-/-}$ T cells (Fig. 5 a, right). In contrast, no difference was found in the glycerol transport levels between the WT and $AQP3^{-/-}$ T cells (Fig. 5 b). In T cells, glycerol permeability might be facilitated primarily by diffusion through the membrane or other AQPs because the permeability levels were much higher than those observed in other cells such as erythrocytes (Yang et al., 2001). We measured intracellular H_2O_2 levels using CM-H2DCFDA fluorescence and identified reduced cellular H_2O_2 levels in $AQP3^{-/-}$ $CD4^+$ T cells (Fig. 5 c). Exogenous H_2O_2 supplementation for 15 s increased cellular H_2O_2 levels to a significantly greater extent in WT than in $AQP3^{-/-}$ $CD4^+$ T cells, suggesting the involvement of AQP3 in H_2O_2 transport in T cells (Fig. 5 c).

We next investigated the effects of a chemokine on cellular H_2O_2 concentration and its production in T cells. As shown in Fig. 5 d, CXCL12 stimulation markedly increased intracellular H_2O_2 levels in WT $CD4^+$ T cells within 15 s. Previous studies have reported that T cells generate H_2O_2 via NADPH oxidase (Nox), which was considered to regulate TCR signaling or adaptive immune responses (Jackson et al., 2004; Purushothaman and Sarin, 2009). Pretreatment with diphenyleneiodonium (DPI), a general Nox inhibitor, or incubation with catalase, which removes any extracellular H_2O_2, significantly suppressed CXCL12-induced intracellular H_2O_2 levels in WT cells (Fig. 5 d). This suggested that CXCL12 stimulation activates Nox for the extracellular production of H_2O_2. $AQP3^{-/-}$ $CD4^+$ T cells also showed a significant increase in cellular H_2O_2 levels at 15–30 s after CXCL12 stimulation, although this increase was minimal as compared with that in WT cells (Fig. 5 e). Collectively, our results suggest that CXCL12 stimulation increases the intracellular H_2O_2 level, which was dependent on AQP3 in T cells.



AQP3-mediated H_2O_2 transport is required for CXCL12-induced Cdc42 activation and chemotaxis

Recent evidence has suggested a role of cellular H_2O_2 as a signaling intermediate in the regulation of a variety of biological processes, including growth, differentiation, and migration

Figure 5. Water and H_2O_2 permeability depended on AQP3 expression in T cells. (a) The osmotic water permeability of $CD4^+$ T cells isolated from WT and $AQP3^{-/-}$ mice was measured based on the time course of scattered light intensity in response to a 150-mM inwardly directed mannitol gradient generated by stopped flow at 22°C. (left) Representative time course data showing responses to rapid changes in perfusate osmolality between 300 and 450 mOsm. (right) Averaged osmotic water permeability coefficients (P_f; SE; $n = 5$; *, $P < 0.01$). (b) Glycerol permeability was measured in response to a 150-mM inwardly glycerol gradient by stopped flow at 30°C (SE; $n = 4$). (c) H_2O_2 uptake into T cells. $CD4^+$ T cells were incubated with 10–300 μM H_2O_2 for 15 s, and cellular H_2O_2 levels were detected using CM-H2DCFDA reagent by flow cytometric analysis. The mean fluorescence intensity (MFI) of CM-H2DCFDA fluorescence (SE; $n = 4$; *, $P < 0.01$, H_2O_2 added vs. control cells) is shown. (d) WT $CD4^+$ cells were incubated with 5 μM DPI or 2,000 U/ml catalase for 30 min and followed with 500 ng/ml CXCL12 for 15–180 s at 37°C. $CD4^+$ cellular H_2O_2 levels were detected using CM-H2DCFDA reagent by flow cytometry analysis (SE; $n = 5$; *, $P < 0.01$, CXCL12 treated vs. control cells). (e) Cellular H_2O_2 levels in WT and $AQP3^{-/-}$ $CD4^+$ cells after CXCL12 stimulation (500 ng/ml, 15 or 30 s; SE; $n = 5$; *, $P < 0.01$ vs. control cells). Each experiment was performed three times.

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(Stone and Yang, 2006; Veal et al., 2007; Poole and Nelson, 2008; Paulsen and Carroll, 2010). We first sought to determine whether intracellular H_2O_2 is involved in CXCL12-induced downstream cellular signaling. Treatment with catalase or DPI suppressed CXCL12-induced Cdc42 activation and Itk phosphorylation (Fig. 6, a and b). CXCL12-mediated actin polymerization was also prevented by treatment with catalase or DPI (Fig. 6 c). Furthermore, treatment of WT T cells with catalase or DPI attenuated the chemotactic efficacy in response to CXCL12 (Fig. 6 d).

Next, we determined whether the exogenous addition of H_2O_2 could modulate Itk and Cdc42 activation. Fig. 6 (e and f) shows that an exogenously added high dose of H_2O_2 (100 µM) induced Itk phosphorylation and Cdc42 activation in WT T cells, whereas no activation was found in AQP3$^{-/-}$ T cells (not depicted). These results confirmed the involvement of intracellular H_2O_2 in CXCL12-induced cell signaling for chemotaxis in T cells.

Having observed the robust uptake of extracellular H_2O_2 through AQP3 in T cells, we next examined the significance of AQP3-mediated H_2O_2 uptake in CXCL12-induced cell signaling and T cell chemotaxis. As shown in Fig. 5 c, when AQP3$^{-/-}$ T cells were exogenously supplemented with 100 µM H_2O_2 for 15 s, the intracellular concentration of H_2O_2 was equivalent to that observed in control WT T cells in which H_2O_2 might be transported through other AQPs or may diffuse across the plasma membrane. CXCL12 stimulation together with exogenous addition of 100 µM H_2O_2 for 15 s remarkably increased the level of DCFDA in AQP3$^{-/-}$ T cells. This was equal to that observed in WT T cells with 100-µM-H_2O_2 supplementation or CXCL12 stimulation (Fig. 7 a). In this context, as shown in Fig. 7 (b and c), AQP3$^{-/-}$ T cells exhibited significant improvements in CXCL12-induced Itk phosphorylation and Cdc42 activation. Furthermore, impaired F-actin polymerization in response to CXCL12 observed in AQP3$^{-/-}$ T cells was recovered by adding exogenous 100 µM H_2O_2 and CXCL12 stimulation (Fig. 7 d). Finally, we found that pretreating AQP3$^{-/-}$ T cells with 100 µM H_2O_2 improved decreased chemotaxis in response to CXCL12 resulting from AQP3 deficiency (Fig. 7 e). These results provide evidence that AQP3-mediated H_2O_2 transport plays an important role in T cell chemotaxis.

DISCUSSION

Our current data reveal a previously unrecognized role of AQP3 in T cell–mediated immunity. First, AQP3 deficiency impaired the water/H_2O_2 permeability and migration of T cells, which was accompanied by defective F-actin dynamics and Cdc42 activation in response to chemokines. Second, AQP3$^{-/-}$ mice showed a severe resistance to CHS development. A direct injection of primed AQP3$^{-/-}$ T cells to the site of the secondary challenge completely reconstituted CHS, suggesting that this defect was based solely on the trafficking of T cells to regional sites. Third, the attenuation of the intracellular H_2O_2 level suppressed chemokine-induced Cdc42 activation, F-actin dynamics, and cell migration. Last, exogenous supplementation with H_2O_2, which restored the reduced cellular H_2O_2 levels in AQP3$^{-/-}$ T cells to the levels observed in WT T cells (presumably by passive diffusion), rescued all the defects in AQP3-deficient T cells, including chemokine-induced Cdc42 activation, actin dynamics, and chemotaxis. Collectively, these findings suggest that AQP3-mediated H_2O_2 transport is required for Cdc42 activation and chemokine-dependent T cell migration, which regulates T cell trafficking during CHS.

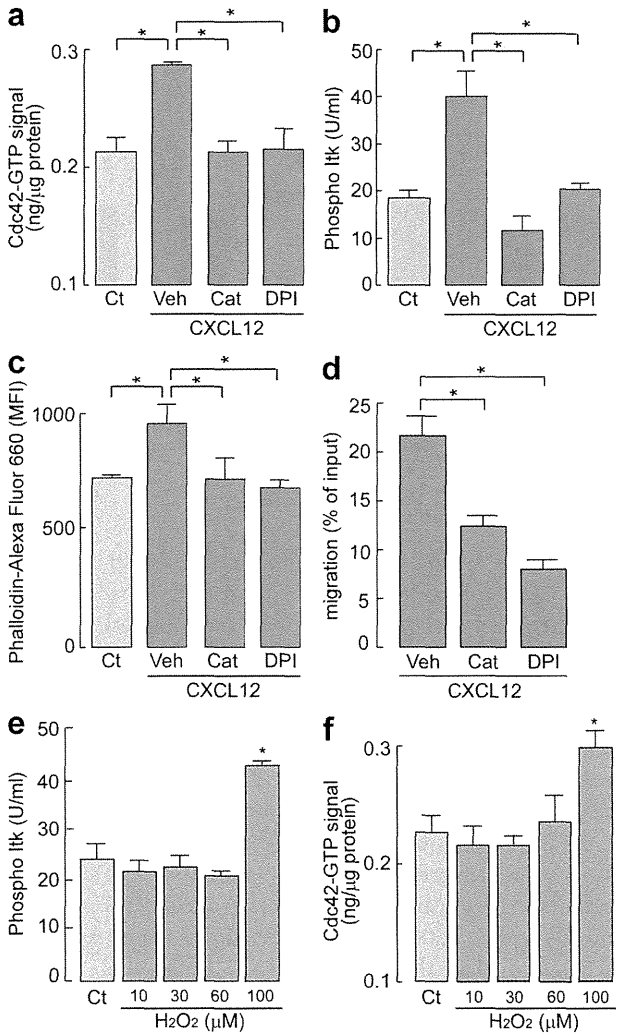

Figure 6. Intracellular H_2O_2 affects CXCL12-induced cell signaling and chemotaxis. (a–c) CD4$^+$ T cells were incubated with 2,000 U/ml catalase (Cat), 5 µM DPI, or vehicle (Veh) for 30 min at 37°C and stimulated with 250 or 500 ng/ml CXCL12. (a) Quantification of Cdc42 active form (GTP bound) with G-LISA activation assay kit (SE; n = 4–5; *, $P < 0.01$). (b) Cytometric bead array–based quantification of phosphorylated Itk (SE; n = 4–5; *, $P < 0.01$). (c) Mean fluorescence intensity (MFI) of phalloidin-Alexa Fluor 660 in the CD4$^+$ cells (SE; n = 5; *, $P < 0.01$). (d) Chemotaxis assay toward 100 ng/ml CXCL12 for 1 h (SE; n = 5; *, $P < 0.01$). (e and f) CD4$^+$ T cells were incubated with 10–100 µM H_2O_2 for 1 min at 37°C. (e) Quantification of Cdc42 active form (GTP bound; SE; n = 4; *, $P < 0.01$). (f) The amount of phosphorylated Itk (SE; n = 4; *, $P < 0.01$). Experiments in a–d were performed in two other independent experiments and in e and f in one other experiment with similar results.

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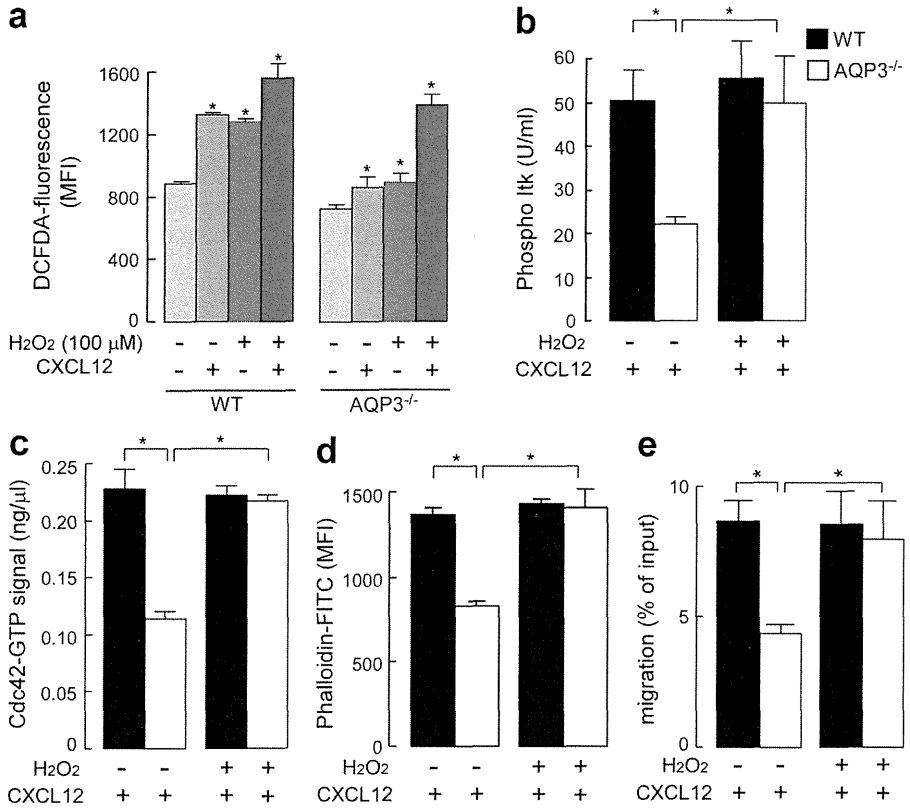

Figure 7. H_2O_2 supplementation restored the impaired cell signaling and chemotaxis in AQP3-deficient T cells. (a–d) $CD4^+$ T cells from WT and $AQP3^{-/-}$ mice were stimulated by 500 ng/ml CXCL12 together with/without exogenous addition of 100 μM H_2O_2. (a) Intracellular H_2O_2 levels for 15 s (SE; $n = 4$; *, $P < 0.01$, treated vs. control cells). (b) The amount of phosphorylated Itk (SE; $n = 4$; *, $P < 0.01$). (c) Quantification of Cdc42 active form (GTP bound; SE; $n = 4$; *, $P < 0.01$). (d) Mean fluorescence intensity (MFI) of phalloidin-FITC in the $CD4^+$ cells was analyzed (SE; $n = 4$; *, $P < 0.01$). (e) T cells were incubated with 100 μM H_2O_2 for 15 s, washed, and assayed for chemotaxis for 30 min toward 200 ng/ml CXCL12 (SE; $n = 5$; *, $P < 0.01$). Experiments were performed in one other experiment with similar results.

In earlier studies (Schröder and Eaton, 2008), H_2O_2 was appreciated only as a marker for oxidative stress, and it was shown to be associated with various pathological conditions such as angiogenesis, cancer, and aging. Recent evidence revealed that H_2O_2 is not only important for such pathologies, but also plays an essential role in cellular physiology as a signaling molecule (Rhee, 2006; Stone and Yang, 2006; Veal et al., 2007; Schröder and Eaton, 2008; Yoo et al., 2011). In the past, extracellular H_2O_2 was thought to passively diffuse across the plasma membrane. Recently, however, yeast and plants were reported to actively transport H_2O_2 through membrane AQPs such as TIP1 (Bienert et al., 2007; Ludewig and Dynowski, 2009). Moreover, mammalian AQP3 was shown to mediate the uptake of H_2O_2 (Miller et al., 2010). In this study, we found that AQP3 transports H_2O_2 and regulates the intracellular H_2O_2 level in T cells, which is essential for chemokine-induced cell signaling, at least in terms of the activation of Cdc42. The precise mechanism by which AQP3-mediated H_2O_2 is involved in CXCL12-induced Cdc42 activation remains unknown. Although the protein tyrosine phosphatase family has been recognized as one of the potential targets of H_2O_2 in cell signaling, other molecular targets of H_2O_2 or H_2O_2-based signal transduction are still largely unknown (Rhee, 2006; Poole and Nelson, 2008; Paulsen and Carroll, 2010). The membrane activation of the Tec family kinase Itk in T cells is believed to require CXCL12-induced activation of Cdc42 and Rac1 (Fischer et al., 2004; Takesono et al., 2004). Indeed, we found not only the defect of Cdc42 activation and the attenuation of Rac1 activation, but also a loss of Itk phosphorylation in response to CXCL12 in $AQP3^{-/-}$ T cells. In addition, our data suggests that CXCL12 stimulation induces a substantial amount of H_2O_2 production, probably because of the activation of Nox, which is transported inward by AQP3 in T cells. AQP3 immunostaining suggested that upon CXCL12 stimulation, AQP3 may be translocated to the leading edge of a cell membrane where it could facilitate H_2O_2 transport. Although further studies are required to elucidate the mechanism underlying the involvement of AQP3-mediated H_2O_2 transport in cell signaling pathways, we hypothesize that AQP3-facilitated intracellular H_2O_2 is involved in chemokine-induced Itk and Cdc42/Rac1 activation and subsequently regulates the downstream protein WASP and the Arp2/3 protein complex. Together, these play a key role in the formation of filopodia and lamellipodia during T cell trafficking.

We propose that AQP3, in addition to its known biological importance, should be considered a target for T cell–mediated diseases. For example, inflammatory skin diseases, such as atopic dermatitis and psoriasis, are characterized by the infiltration of T cells into both the dermis and the epidermis of affected skin, whereas different subsets of T cells are associated with each disease (Guttman-Yassky et al., 2011). In our study, we found abundant T cell infiltrates with AQP3 expression in the skin of the atopic dermatitis and psoriatic lesions (unpublished data), suggesting that these pathologies also involve AQP3-mediated T cell migration to the skin. Future studies may provide novel therapeutic strategy for controlling unwanted immune reactions in the skin, including atopic dermatitis and psoriasis, and other autoimmune diseases.

MATERIALS AND METHODS

Mice. $AQP3^{-/-}$ mice (C57BL/6 genetic background) were generated by targeted gene disruption (Ma et al., 2000). All comparisons were between

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littermates. All animal experiments were approved by the Committee on Animal Research of Kyoto University.

Flow cytometry analysis. Single cell suspensions were stained with monoclonal antibodies against CD3, CD4, CD8, CD25, CD44, or CD62L (eBioscience). To assess their cellular F-actin content, we fixed lymphocytes in 4% formalin in PBS, permeabilized with 0.1% Triton X-100, and stained with Alexa Fluor 488– or Alexa Fluor 660–conjugated phalloidin (Invitrogen). The samples were analyzed using the flow cytometry FACSCanto II system (BD).

Immunohistochemistry and immunofluorescence microscopy. Paraffin-embedded sections were stained with hematoxylin and eosin. For cell polarization, cells were cultured on poly-lysine–coated coverslips and stimulated with CXCL12. Cells were fixed with 4% formalin in PBS, permeabilized with 0.1% saponin, and stained with Alexa Fluor 488–phalloidin or anti-AQP3 (Millipore).

Immunoblotting, GTPase assays, and Itk assay. To analyze chemokine-induced protein phosphorylation, cells were lysed with ice-cold lysis buffer (Cell Signaling Technology). The supernatant (14,000 *g*, 10 min, 4°C) was used for immunoblotting with antibodies against phospho-Arp2, phospho-WASP (ECM Biosciences), Arp2 (Millipore), WASP (Santa Cruz Biotechnology, Inc.), and β-actin (Sigma-Aldrich). The activation of Rho family small GTPases was detected using G-LISA Rho, Rac1, or Cdc42 activation Biochem kit (Cytoskeleton) according to the manufacturer's instructions. The amount of phospho Itk was assayed with Itk BD cytometric bead array (BD) according to the manufacturer's instructions.

Water, glycerol, and H_2O_2 permeability assay. Water and glycerol permeability were measured using an SX20 stopped-flow spectrometer (Applied Photophysics). The sorted T cells in RPMI medium were subjected to a 150-mM inwardly directed Mannitol or glycerol gradient. The kinetics of the decreasing cell volumes was measured from the time course of 90° scattered light intensity at 450 nm wavelength. Osmotic water/glycerol permeability coefficients (P_f) were calculated as described previously (Yang et al., 2001). Cellular H_2O_2 was assayed using the CM-H2DCFDA reagent (Invitrogen) according to the manufacturer's instructions.

Chemotaxis and transendothelial migration assays. T cells from the LNs of WT and AQP3$^{-/-}$ mice (10^6 cells) were deposited on the upper chamber containing a polycarbonate transwell membrane filter (5-μm pore size; Corning). The lower chamber contained 100 ng/ml CXCL12, 100 ng/ml CCL19, 80 ng/ml CCL17, or 80 ng/ml CCL27 in RPMI complete medium. The recovered cells were analyzed with flow cytometry analysis.

Three-dimensional chemotaxis assay was performed using the μ-Slide chemotaxis chamber (Ibidi) according to the manufacturer's instructions. WT or AQP3$^{-/-}$ T cells in collagen gel (3×10^6 cell/ml) were applied to the chamber. Cell movement in response to CXCL12 gradient was tracked for 60 min and analyzed using National Institutes of Health ImageJ.

For transendothelial migration, mouse vascular endothelial cell line (F-2; Toda et al., 1990) was seeded onto a 5-μm transwell filter and grown to confluence. T cells (10^6 cells) were deposited on the upper chamber, and RPMI complete medium containing 100 ng/ml CXCL12 was added to the lower chamber. After 8 h, the transmigrated cells were counted by flow cytometry analysis.

RNAi and plasmid DNAs. To knock down the AQP3 gene, human T cells were incubated with Accell AQP3 siRNA or Accell Non-Targeting siRNA in Accell siRNA delivery medium (Thermo Fisher Scientific) for 48–72 h. AQP3 expression was confirmed by flow cytometry analysis and quantitative PCR. The cDNA from plasmid for dominant-active mutants of Cdc42 (V12-Cdc42; a gift from S. Narumiya, Kyoto University, Sakyo-ku, Kyoto, Japan) was transfected with TransIT (Takara Bio Inc.).

CHS. Mice were sensitized with 50 μl of 0.5% DNFB, 5% TNCB, or 5% Oxa solution on the abdomen. 5 d later, 20 μl of each hapten was applied to the left ear, and the vehicle (acetone/olive oil, 4:1) was applied to the right ear. Ear swelling was measured with a spring-loaded micrometer (Mitutoyo) 24 h after challenge.

Adoptive transfer experiments. Cell suspensions obtained from the LNs of DNFB-sensitized mice were injected subcutaneously (2×10^5 per 20 μl PBS) or intravenously (3×10^7 cells/head) into the ears of naive WT and AQP3$^{-/-}$ mice. The ears were immediately challenged by applying 20 μl of 0.3% DNFB or vehicle to both sides of the ear. Ear thickness was measured after 24 h. To track the transferred cells, the isolated cells derived from DNFB-sensitized mice were stained with CMFDA for 20 min, washed, and injected intravenously (4×10^7 cells/head). The ear and regional LN were excised at 24 h after DNFB application, and the cells were isolated with collagenase and trypsin treatment. CD4$^+$ and CMFDA$^+$ cells were analyzed by flow cytometry analysis.

BM transplantation. BM cells from WT and AQP3$^{-/-}$ mice were subjected to hypotonic red blood cell lysis. C57BL/6 recipients (8–10 wk old) were γ irradiated with two doses of 600 rad (3 h apart). After irradiation, the mice received 10^6 BM cells intravenously. This protocol constantly gave >90% reconstitution of the recipient by donor hematopoietic cells, as evaluated by a separate transplantation experiment using BM from C57BL/6-CD45.1 congenic mice. The CHS test was performed 2 mo later.

Statistical analysis. Statistical analysis was performed using the two-tailed Student's *t* test or analysis of variance.

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Allergic contact dermatitis caused by a skin-lightening agent, 5,5′-dipropylbiphenyl-2,2′-diol

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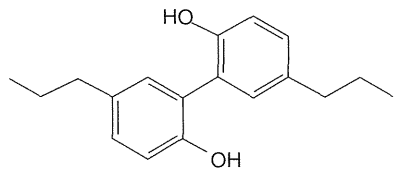
Key words: allergic contact dermatitis; cosmetics; 5,5′-dipropylbiphenyl-2,2′-diol; skin-lightening agent.

Many skin-lightening agents are currently available, including kojic acid, arbutin, hydroquinone, and 5,5′-dipropylbiphenyl-2,2′-diol (Fig. 1); the last of these is a relatively new skin-lightening agent that has been used in Japanese cosmetics since 2006. 5,5′-Dipropylbiphenyl-2,2′-diol is a biphenyl derivative, and downregulates melanin synthesis by inhibiting tyrosinase maturation, leading to accelerated tyrosinase degradation (1). Here, we report a case of allergic contact dermatitis caused by 5,5′-dipropylbiphenyl-2,2′-diol.



Molecular formula: $C_{18}H_{22}O_2$

Molecular weight: 270.372

CAS number: 20601-858-8

Fig. 1. Chemical formula of 5,5′-dipropylbiphenyl-2,2′-diol.

Case Report

A 45-year-old female presented with a 2-month history of an itchy erythematous rash on her cheek and neck. She had a negative history for dermatitis. Previously, she had been instructed to apply 0.3% prednisolone valerate acetate ointment twice daily by another dermatologist, but as cosmetic dermatitis was not suspected at the time, she had continued to use her cosmetics, with gradual worsening of the symptoms. We treated her with a regimen of oral prednisolone 20 mg daily for 4 days, olopatadine hydrochloride 10 mg daily for 1 week, and 0.1% hydrocortisone butyrate ointment, and advised her to stop using her cosmetics; her symptoms subsequently improved. Patch tests were performed with her personal cosmetics and 17 cosmetic allergens, using Finn Chambers® on Scanpor® tape. Readings were performed on D2 and D4 according to the International Contact Dermatitis Research Group guidelines. The patient showed positive reactions to a cream (+ on D2 and D4) and beauty essence (+ on D2 and D4), both of which were started 2 months prior to appearance of the rash. In the second patch test, with cosmetic ingredients provided by the cosmetic supplier, she reacted only to 5,5′-dipropylbiphenyl-2,2′-diol (0.5% pet., + on D2 and D4), which was present in both the cream and the beauty essence as a skin-lightening agent. The skin did not lighten over the area of the patch test with this chemical. Other cosmetic ingredients and cosmetic allergens gave negative reactions. Patch tests with 5,5′-dipropylbiphenyl-2,2′-diol 0.5% pet. in 4 control subjects gave negative results.

Discussion

Cosmetics with skin-lightening agents are popular in Japan. Cosmetics containing skin-lightening agents such as arbutin (2), hydroquinone and kojic acid have been reported to cause allergic contact dermatitis. 5,5′-Dipropylbiphenyl-2,2′-diol, an inhibitor of melanin synthesis synthesized by an oxidative coupling method (3), is a relatively new skin-lightening agent that has been marketed in Japan since 2006. Initial studies on this agent did not find adverse skin reactions. A double-blind test

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