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Journal of Dermatological Science

journal homepage: www.elsevier.com/jds



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

Rieko Kabashima-Kubo^a, Motonobu Nakamura^a, Jun-ichi Sakabe^{a,b}, Kazunari Sugita^a, Ryosuke Hino^a, Tomoko Mori^a, Miwa Kobayashi^a, Toshinori Bito^{a,c}, Kenji Kabashima^{a,d}, Koetsu Ogasawara^e, Yukiko Nomura^f, Toshifumi Nomura^f, Masashi Akiyama^{f,g}, Hiroshi Shimizu^f, Yoshiki Tokura^{a,b,*}

^a Department of Dermatology, University of Occupational and Environmental Health, Kitakyushu, Japan

^b Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan

^c Department of Dermatology, Kobe University Graduate School of Medicine, Kobe, Japan

^d Department of Dermatology, Faculty of Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan

^e Department of Immunobiology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan

^f Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

^g Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan

ARTICLE INFO

Article history:

Received 29 November 2011

Received in revised form 15 March 2012

Accepted 10 April 2012

Keywords:

Atopic dermatitis

Extrinsic

Intrinsic

IgE

Filaggrin

Th1

Th2

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.

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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

* Corresponding author at: Department of Dermatology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. Tel.: +81 53 435 2303; fax: +81 53 435 2368.

E-mail address: tokura@hama-med.ac.jp (Y. Tokura).

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 $\pm$ SD)	30.5 $\pm$ 13.2	34.8 $\pm$ 13.4	$P = 0.226$
IgE (kU/L, mean $\pm$ SD)	8.050 $\pm$ 10.400	82.5 $\pm$ 59.6	$P < 0.001$
RAST for <i>D. pteronyssinus</i> (class, mean $\pm$ SD)	5.64 $\pm$ 0.693	1.77 $\pm$ 1.73	$P < 0.001$
LDH (IU/L, mean $\pm$ SD)	269 $\pm$ 91.2	206 $\pm$ 69.0	$P = 0.0129$
Eosinophils (% , mean $\pm$ SD)	8.79 $\pm$ 6.02	5.65 $\pm$ 5.45	$P = 0.0800$
VAS (mean $\pm$ SD)	54.2 $\pm$ 27.0	50.2 $\pm$ 23.0	$P = 0.686$
SCORAD (mean $\pm$ SD)	35.4 $\pm$ 16.1	31.3 $\pm$ 11.3	$P = 0.410$
TEWL (g/m ² /h, mean $\pm$ SD)	14.3 $\pm$ 7.57*	8.73 $\pm$ 3.74	$P = 0.0440$
Skin surface hydration (AU, mean $\pm$ SD)	29.2 $\pm$ 7.28*	34.7 $\pm$ 5.78	$P = 0.0250$

The normal ranges or values are as follows: LDH, 119–229 IU/L; TEWL, 6.58 $\pm$ 1.78 g/m²/h; skin surface hydration (capacitance), 39.2 $\pm$ 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 serum 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 $\pm$ 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 men 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 μ g/mL; R&D systems, Minneapolis, MN) and recombinant human CD40L (CD154) (200 μ g/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)	<i>FLG</i> 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 the 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 $\pm$ 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 ,

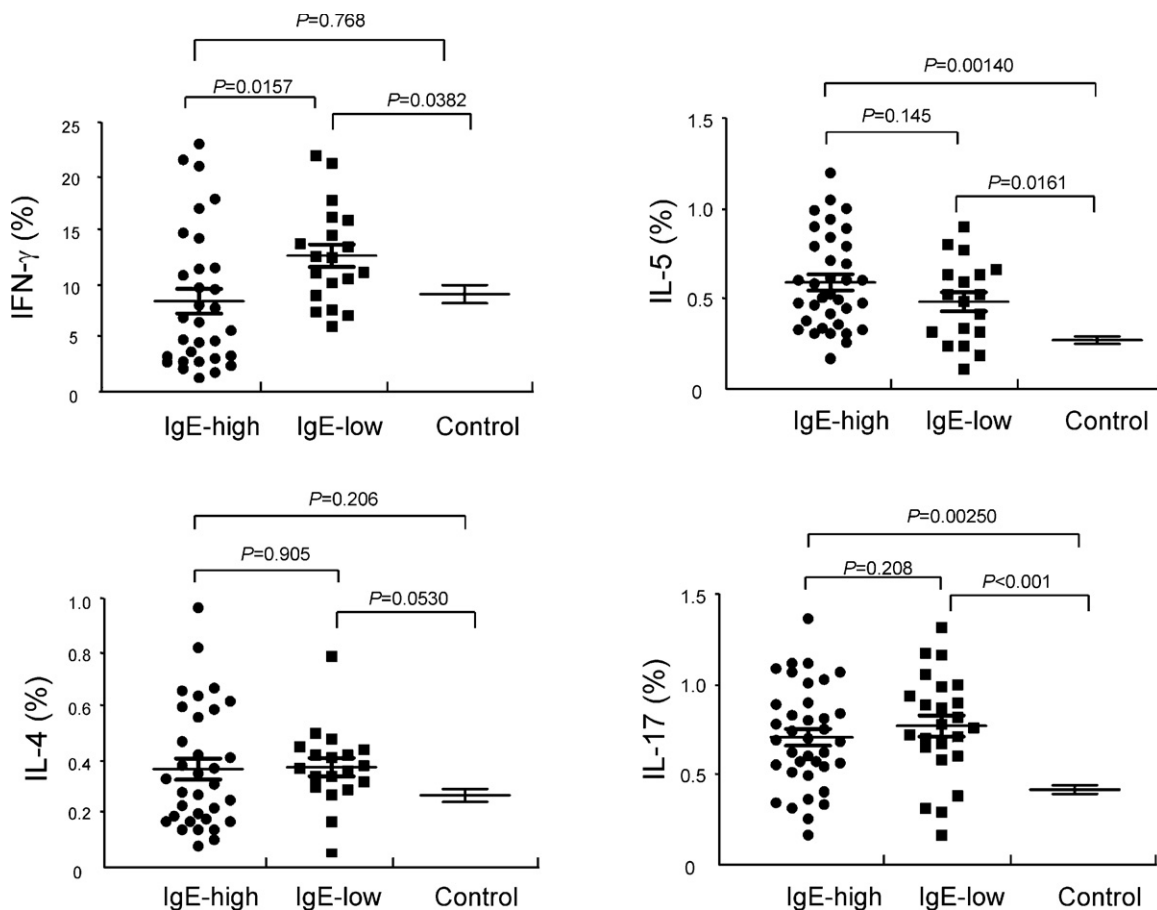


Fig. 2. Percentages of IFN- γ , IL-4, IL-5, and IL-17-positive CD4⁺ cells. PBMCs from patients were incubated with PMA and Ca ionophore. They were then stained for CD3 and CD8, and subsequently stained intracellularly for IFN- γ , IL-4, IL-5, and IL-17. Since the stimulation with PMA and Ca ionophore 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 cytokine). The vertical bars and error bars represent the mean $\pm$ 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 $\pm$ 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 dominance 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

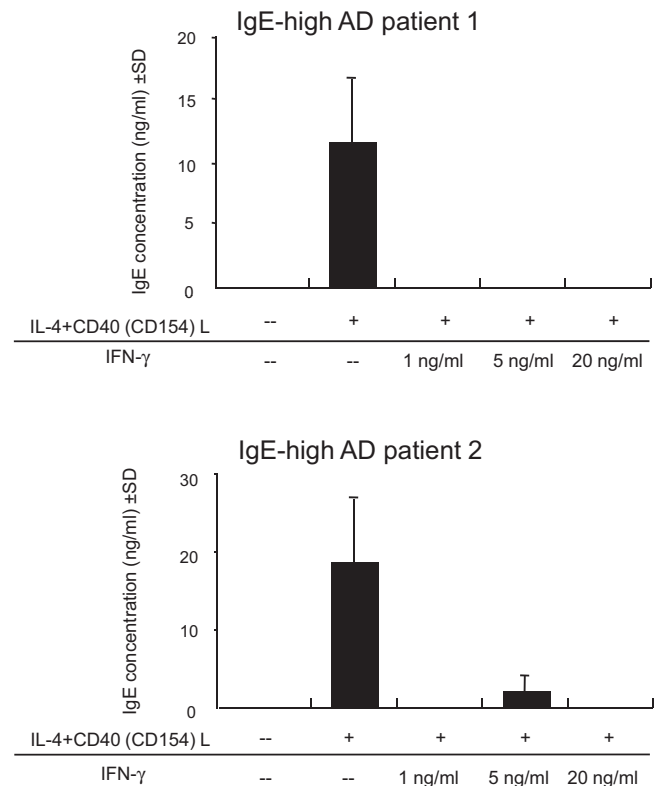


Fig. 3. IFN- γ -downregulation of IgE production by B cells from IgE-high AD patients. PBMCs from 2 patients with IgE-high AD were purified for naïve B cells. They were stimulated with IL-4 and CD40L (CD154) to produce IgE. IFN- γ was simultaneously added to the culture at a concentration of 1, 5, or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

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

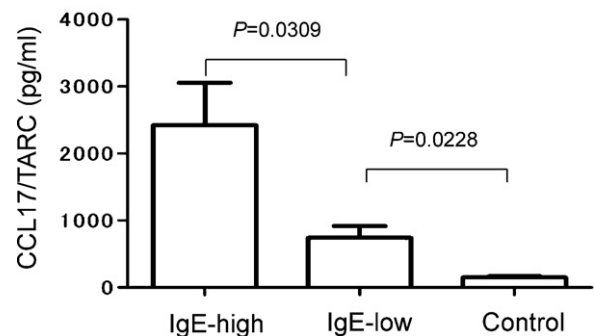


Fig. 4. Blood CCL17/TARC in IgE-high and IgE-low AD. Serum samples were obtained from IgE-high AD patients ($n = 20$), IgE-low AD patients ($n = 19$) and control subjects ($n = 9$). The means $\pm$ SD were 2430 ± 2820 in IgE-high AD and 851 ± 771 in IgE-low AD.

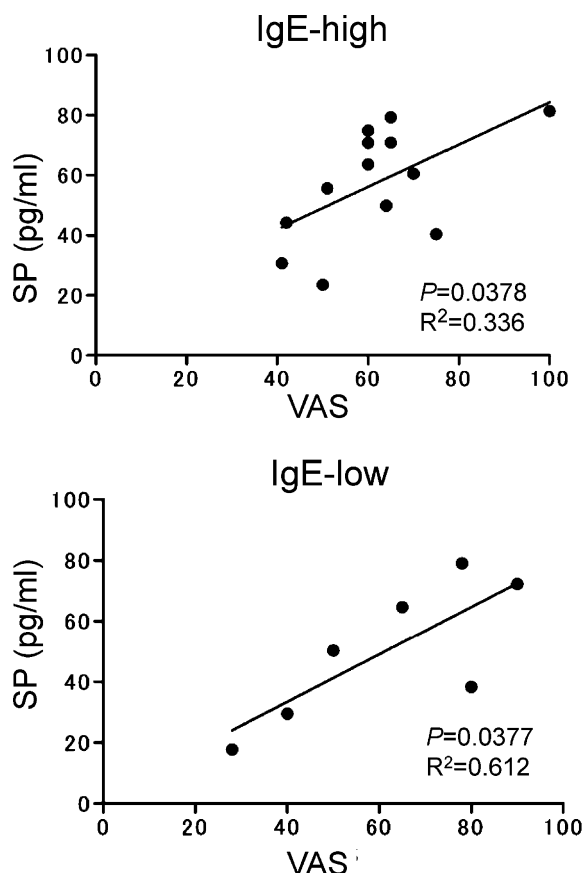


Fig. 5. Blood SP levels in IgE-high and IgE-low AD. Plasma SP levels were measured as described in Section 2, and simultaneously, VAS for pruritus was measured in the individual patients.

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.

Funding sources

None.

Acknowledgments

This work is supported by Grants-in Aid for Science Research from the Ministry of Health, Labour, and Welfare of Japan; and the Ministry of Education, Science, Sports, and Culture of Japan. We thank Ms. Rie Murase, Ms. Yukako Miyazaki, and Ms. Tamae Oishi for their technical assistance.

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