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Co-existence of specific IgE antibodies and T cells reactive to house dust mites and human transglutaminase3/tropomyosin in patients with atopic dermatitis

Background: Although specific IgE antibodies reactive to exogenous antigens are found in patients with atopic dermatitis (AD), some patients do not have such antibodies. Autoimmunity has been proposed as a possible mechanism in these patients. **Objectives:** To identify specific IgE antibodies reactive to human transglutaminase 3 (TG3) and tropomyosin (TMP) and determine whether auto-reactive T cells are induced by these proteins in patients with AD. **Materials & Methods:** Forty-two patients with AD and 27 healthy controls were included in this study. IgE antibodies against recombinant human TG3 and TMP were measured by ELISA. Cross-reactivity between allergens was determined by EdU of peripheral blood mononuclear cells (PBMCs) proliferation assays. T-cell lines were generated from PBMCs in the presence of house dust mites (HDM), TG3 and TMP. TG3/TP-specific T-cell clones were generated from T-cell lines, and were characterized by antigen specificity and cytokine pattern. **Results:** In 12 patients with anti-HDM IgE antibodies, six (50%) had anti-TG3 IgE antibody and four (33.3%) had both anti-TG3 and anti-TMP IgE antibodies. Lymphocyte proliferation was induced in 12 patients by TG3 or TMP. T-cell lines and T-cell clones from PBMCs of patients with AD who had IgE antibody reactive to HDM were fully cross-reactive with TG3 and TMP. These cell clones included both Th1 cell (producing IFN- γ) and Th2 cell (inducing IL-4) responses. TG3- and TMP-specific T-cell clones were not generated from healthy controls. **Conclusion:** Specific IgE antibody and T cell clones reactive to human TG3 and TMP were found in patients with AD, indicating that an autoimmune mechanism might contribute to AD.

Key words: atopic dermatitis, transglutaminase3, tropomyosin, TCC, auto-immunity

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Atopic dermatitis (AD) is a chronic inflammatory skin disease. The pathogenesis of AD is still not fully understood [1]. Abnormality of immune response and defects of skin barrier are considered to contribute to the formation of AD [2, 3]. A number of erogenous IgE antibodies against allergens, such as inhalant and food allergens, have been identified in patients with AD and these patients were considered to have “extrinsic AD” [4]. However, allergen-specific IgE could not be found in some patients, and these patients still have long-term chronic inflammation following strong allergen evasion [4]. In these patients, autoreactivity to human proteins has been postulated as a pathomechanism. Such autoreactivity to human proteins has been described for a repertoire of IgE-binding autoantigens that cross-react with environmental antigens [5-7]. A house dust mite (HDM) atopy patch test was shown to induce eczematous changes in non-lesional skin of AD patients [5].

More than 82 HDM-derived allergens have been identified. Der p1,2 and Der f1,2,10 are the main allergens [8-10]. HDM is the most common exogenous allergen in patients with AD. Serum IgE levels have also been found in approximately 80% patients, strongly correlating with the severity of the disease [11]. The main species of HDM includes *dermatophagoides pteronyssius*, *dermatophagoides farinae* and *blomia tropicalis* which coexist in most geographical regions. Some are found to be homologous with human proteins, such as tropomyosin (TMP) [4], and transglutaminase (TG) [12]. TG3 or epidermal TG is present in the epidermis, hair follicles, and brain. Its two domains are similar in structure to the major immunoglobulin def2 of dust mites. A 92% amino acid sequence homology was found between the two domains of TG3 and def2 [12]. TG3 knockout mice showed impaired skin barrier function [13], suggesting that TG3 is essential for skin barrier function. Tropomyosin has been shown to be a major allergen in

many invertebrates. A 73% amino acid sequence homology was found between HDM tropomyosin and human tropomyosins [14].

The cellular infiltration of eczematous reactions is dominated by CD4+ T lymphocytes. Recent studies have shown that allergen-specific T cells could be isolated from skin lesions of AD [4]. These findings suggest that an allergen-specific T-cell response is of pathophysiological relevance in sensitized patients [13]. In this study, we investigated possible cross-reactivity between HDM and human TG3 or TMP, and further examined whether T cell clones (TCC) could be induced by human TG3 and TMP.

Methods

Patients and healthy controls

A total of 27 healthy controls and 42 AD adult patients were included in this study. All patients met the Chinese criteria for AD [15]. Total serum IgE level and allergen-specific IgE antibodies to inhalant allergens were measured. Disease severity was estimated using EASI. Ethical approval for taking blood samples was obtained from the Ethics Committee of the People's Hospital of Peking University. Written consent was obtained from all patients and healthy controls.

Measurement of IgE antibodies to HDM, TG3 and TMP

IgE antibodies to HDM were determined by ELISA, according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). IgE antibodies to TMP and TG3 were measured using an ELISA-based binding assay. Absorbency was measured at 450 nm with a molecular device reader and optical densities were converted into arbitrary ELISA units (KU)/mL.

Lymphocyte proliferation assay

Peripheral blood mononuclear cells (PBMCs) from all subjects were isolated from heparinized blood samples by means of density centrifugation on Ficoll Hypaque (Fresenius Kabi Norge AS, Oslo, Norway). The PBMCs were seeded in 96-well plates at a concentration of 5×10^5 PBMCs/100 μ L culture media/well. PBMCs were cultured routinely in RPMI 1640 medium (Gibco, Rockford, America) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Thermo scientific, Rockford) and antibiotics (100 U/mL of penicillin and 100 μ g/ml streptomycin; WELGENE) at 37 °C in a humidified 5% CO₂ incubator. PBMCs were stimulated with recombinant HDM, TG3, TMP or PHA and cultivated for five days. PBMCs were assessed by flow cytometry on a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, America) using EDU (Becton Dickinson, Franklin Lakes, NJ, America). Standard flow cytometry methods were used to determine the percentage of S-phase cells. All experiments were performed in triplicate.

Generation of T cell lines and preparation of HDM-specific T cell clones (TCC)

PBMCs from AD patients and healthy controls were seeded in 24-well flat-bottom culture plates (Costar, Cambridge, Mass) at a concentration of 2×10^5 /mL culture media/well. HDM was added to culture media at a final concentration of 4 μ g/mL. Half of the medium was changed every 48 hours. After five days, human rIL-2 was added at a final concentration of 0.2 U/mL and cultured for a further three days. T-cell blasts were enriched by means of density centrifugation and used in antigen re-stimulation tests to assess their specificity to TG3/TMP.

For generation of T-cell clones, HDM-specific TCL (2×10^5 /mL) were seeded into 24-well flat-bottom plates in the presence of 2×10^5 irradiated (5000 rad) allogeneic PBMCs. After an interval of 7–14 days, the TCLs were re-stimulated with HDM. The irradiated PBMC were added as feeder cells and rIL-2 was added to the TCL culture. All cultures were performed in triplicate.

Antigen re-stimulation test

A seven-day interval from the last round of enrichment was allowed before antigen specificity of the TCC was tested. T cells at 5×10^3 were cultured in the presence of 2×10^5 irradiated autologous PBMCs and stimulated with TG3 or TMP at a final concentration of 2.5 μ g/mL and 4 μ g/mL, respectively. After five days of culture, TCC were assessed by flow cytometry on a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, America) using a Click-iT® EdU Flow Cytometry Assay Kit (Becton Dickinson, Franklin Lakes, NJ, America). Human serum albumin (Sigma, San Francisco, America) was used as a negative control. TCC re-stimulation assays and PHA-stimulated cells (at a final concentration of 0.1 U/mL) were used as a positive control. All experiments were performed in triplicate.

Measurement of cytokines from TCC

HDM-specific TCC were washed and then incubated with irradiated autologous PBMCs in the presence of TG3 or TMP for 24 hours. Interferon- γ and IL-4 in culture supernatants were measured by ELISA.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM software and SPSS17.0 software. The specific IgE level was compared using the non-parametric Kruskal-Wallis test. The correlation analysis was performed using Pearson correlation. All experiments were performed two or three times, and representative results are shown. P values of less than 0.05 were considered significant. Data are presented as mean $\pm$ SD.

Results

IgE antibodies against HDM and TG/TP

In 12 patients with anti-HDM IgE antibodies, four (33.3%) had both anti-TG3 and TMP antibodies and six (50%)

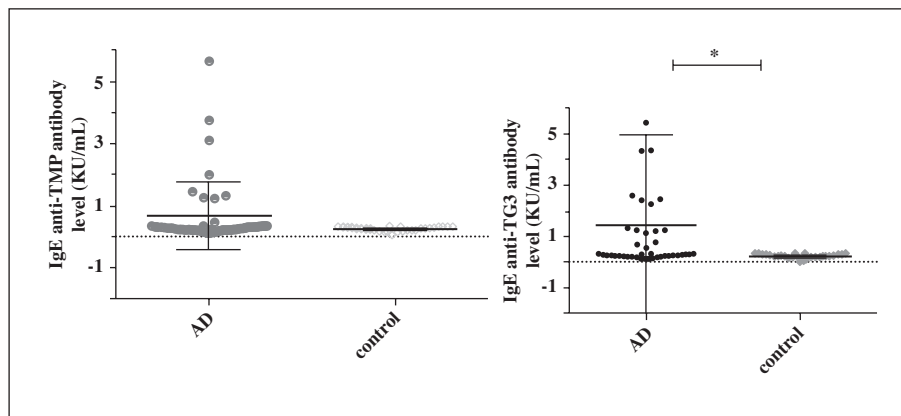


Figure 1. IgE anti-TG3 and anti-TMP antibodies in AD patients and healthy controls. The level of IgE anti-TG3 and anti-TMP antibodies is expressed as KU/mL ($p < 0.05$).

had anti-TG3 IgE antibody. In 30 anti-HDM IgE negative patients, nine (30.0%) had anti-TG3/TMP IgE antibodies, including four (13.3%) with anti-TG3 and TMP antibodies, one with (3.3%) anti-TG3 IgE antibody and four (13.3%) with anti-TMP antibody. Anti-HDM IgE-positive patients demonstrated a significantly higher incidence of anti-TG3/TMP IgE antibodies than anti-HDM IgE-negative patients ($p < 0.001$).

In 33 patients with a high level of total serum IgE, 18 (54.5%) had anti-TG3/TMP IgE antibodies including four (12.1%) with anti-TG3, five (15.1%) with anti-TG3 and nine (27.2%) with anti-TG3 and TMP antibodies. In nine patients with a normal total serum IgE level, four (44.4%) had anti-TG3/TMP IgE antibodies, including one (11.1%) with anti-TG3, one (11.1%) with anti-TMP and two (22.2%) with anti-TG3 and TMP antibodies. No statistical difference was found between the high IgE group and normal IgE group ($p > 0.05$).

TG3/TMP-specific IgE antibody and severity of AD

The TG3-specific IgE antibody level was 1.48 ± 3.53 KU/mL in AD patients, which was significantly higher than that in healthy controls (0.311 ± 0.13 KU/mL; $p < 0.05$). The TMP-specific IgE antibody level was 0.98 ± 1.33 KU/mL in AD patients, slightly higher, but not significantly higher than that in healthy controls ($p > 0.05$) (figure 1). AD patients with anti-TG3/TMP autoantibodies exhibited significantly higher total serum IgE levels than those without these autoantibodies ($p < 0.005$). The Eczema Area and Severity Index (EASI) in patients with anti-TG3/TMP autoantibodies (26.31 ± 15.5) was also significantly higher than in those without such antibodies (10.78 ± 8.2) ($p < 0.001$). There was no significant correlation between TG or TMP-specific IgE antibody level and age or eosinophils ($r = 0.24/0.08$; $-0.08/-0.06$; $0.45/0.39$; $p > 0.05$) (figure 2).

Lymphocyte proliferation on stimulation with TMP and TG3

TG3- and TMP-induced lymphocyte proliferation was found in all 12 anti-HDM-positive AD patients but in

only two of 30 anti-HDM-negative patients. In patients with anti-HDM IgE antibodies, lymphocyte proliferation was induced by TG3 in three patients (25%), by TMP in two patients (16.7%) and by both TG3 and TMP in seven patients (58.3%). TG3 and TMP failed to induce lymphocyte proliferation in healthy controls (figure 3).

Cross-reactivity between TG/TMP-specific T cell line and HDM-specific cell line

T cell lines were generated from proliferated lymphocytes upon TG/TMP stimulation. In 12 anti-HDM antibody-positive patients, T cell lines were generated in 10 (83.3%) patients upon TG3 stimulation. Moreover, T cell lines were generated in eight patients (66.7%) upon TMP stimulation. On the other hand, T cell lines were generated in only two of 30 anti-HDM antibody-negative patients. TG3 or TMP stimulation did not induce T cell lines in healthy controls (table 1).

TCC generated by stimulation with TG3/TMP

A total of 359 TCC were generated from 12 AD patients with HDM-specific IgE antibody and 53 TCCs were generated from two patients without HDM-specific IgE antibody. Antigen re-stimulation tests revealed that 341/359 (95%) TCCs were specific for TG3 and 272 /359 (75.7%) were specific for TMP. No TG3- and TMP-specific TCCs were generated from healthy controls.

Cytokine profile in TG3/TMP-induced TCC

HDM-specific TCC were stimulated with TG3/TMP for 36 hours, and the cytokines in supernatants were measured. The results showed that 50% TCC stimulated by TG3 produced IFN- γ at >500 pg/mL and 28.6% produced IL-4 at >50 pg/mL. In addition, 14.3% TCC stimulated by TG3 produced both IFN- γ and IL-4, 42.8% TCC stimulated by TMP produced IFN- γ , and 28.5% produced IL-4. Finally, 14.3% TCC stimulated by TMP produced both IFN- γ and IL-4 (table 2)

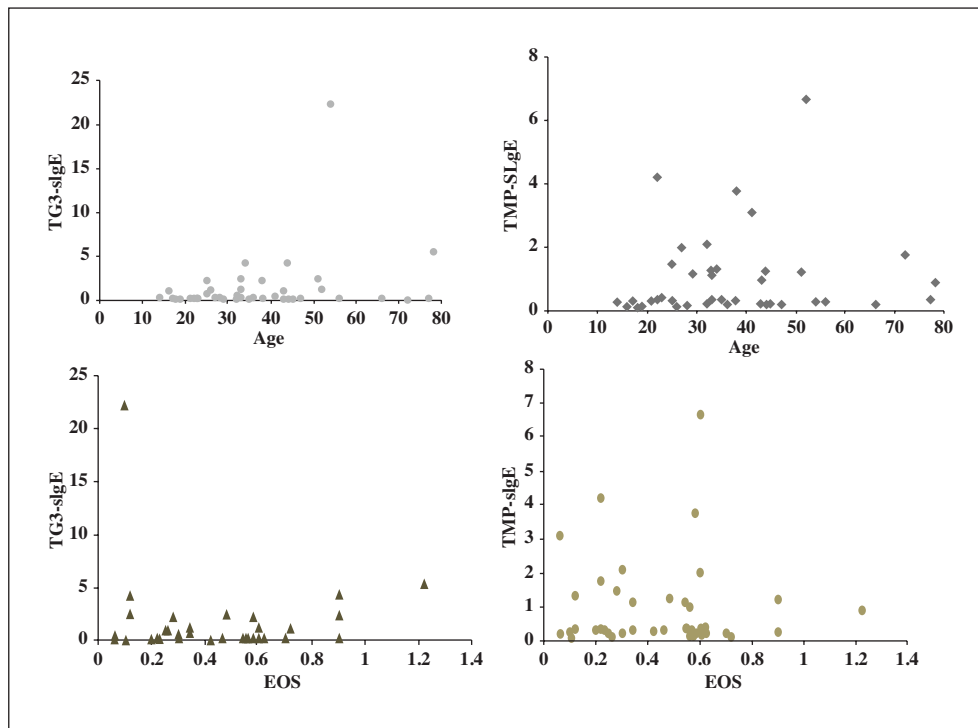


Figure 2. No significant correlation between TG or TMP-specific IgE antibody level and age or eosinophils.

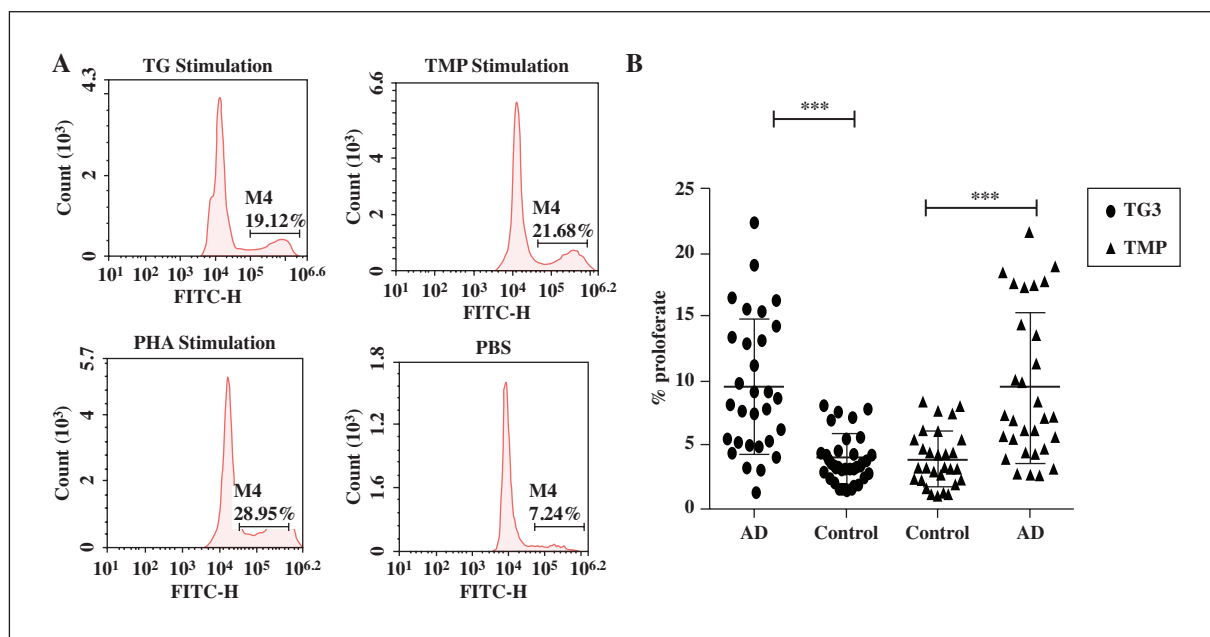


Figure 3. A) PBMC proliferation after five days exposure to TG3 or TMP in a patient with AD. B) Proliferation of lymphocytes with atopic dermatitis was measured by flow cytometry. The percentage of proliferating lymphocytes stimulated by TG/TP in AD patients was higher than that in healthy controls ($p < 0.001$).

Discussion

Immunoglobulin E (IgE) is a biomarker of AD. About 80% patients with AD have an increased IgE level. About two thirds of patients have specific IgE antibodies against exogenous antigens. A total of 52.9% of infants have raised

total IgE, and 55.5% are sensitized to at least one allergen. There is a significant difference in total IgE values and sensitization rates to foods and aeroallergens among infants from different countries. The highest prevalence rates of allergen-sensitized infants were found in Australia (83%), the UK (79%) and Italy (76%) [16]. However, allergen-specific IgE could not be found in some patients, and

Table 1. PBMC proliferation upon TG3 and TMP stimulation in patients with anti-HDM IgE antibody (expressed as percentage of proliferating cells).

	TG3 stimulated	TMP stimulated
Patient 1	19.12%	21.68%
Patient 2	16.5%	14.42%
Patient 3	14.32%	13.54%
Patient 4	16.30%	17.84%
Patient 5	15.43%	18.91%
Patient 6	11.21%	18.54%
Patient 7	9.85%	17.75%
Patient 8	13.16%	17.43%
Patient 9	22.35%	17.55%
Patient 10	13.42%	9.88%
Patient 11	15.64%	10.15%
Patient 12	12.89%	11.35%

Table 2. Cytokine profile of TG3-specific and TMP-specific TCCs.

	TG3(+) clones		TMP(+) clones	
	IFN- γ (pg/mL)	IL-4 (pg/mL)	IFN- γ (pg/mL)	IL-4 (pg/mL)
1	203.8	61.2 $\uparrow$	299.6	78.1 $\uparrow$
2	2094.9 $\uparrow$	102.8 $\uparrow$	1186.3 $\uparrow$	141.2 $\uparrow$
3	15	77.9 $\uparrow$	53.28	79.1 $\uparrow$
4	1145.7 $\uparrow$	4.7	1778.2 $\uparrow$	19.1
5	26.9	79.3 $\uparrow$	2.89	121.3 $\uparrow$
6	506.7 $\uparrow$	13.8	557.3 $\uparrow$	44.7
7	550.3 $\uparrow$	12.9	625.0 $\uparrow$	5.6
8	765.1 $\uparrow$	22.4	630.9 $\uparrow$	12.1
9	2705.1 $\uparrow$	17.2	2672.2 $\uparrow$	3.8
10	3019.5 $\uparrow$	10.1	2983.6 $\uparrow$	23.3
11	7.2	210.1 $\uparrow$	19.53	342.5 $\uparrow$
12	616.7 $\uparrow$	43.4	84.6	21.3
13	376.6	28.9	301.2	6.7
14	712.2 $\uparrow$	163.2 $\uparrow$	1253.8 $\uparrow$	3203.3 $\uparrow$

these patients still have long-term chronic inflammation following strong allergen evasion [4]. IgE-mediated auto-reactivity against human auto-antigens has been postulated as a possible mechanism involved in the persistence and exacerbation of AD. It has been reported that auto-antigens could either cross-react with exogenous antigens specific to IgE, or directly activate T cells [6, 7]. Several studies have shown that not only exogenous allergens but also ‘auto-allergens’ play a role in the pathogenesis of AD [4, 6, 17]. Auto-reactivity has been shown to be a consequence of cross-reactivity and molecular mimicry [18]. It has been hypothesized that tissue damage results in release of cellular proteins such as TG3 and TMP, which might be exposed to the immune system followed by activation of auto-reactive T cells [19, 20]. Amino acid sequence homology of 92% was found between the two domains of

human TG3 and HDM protein def2. Amino acid sequence homology of 73% was found between HDM tropomyosin and human tropomyosins [14]. IgE auto-antibodies have been found in patients with AD, correlating with elevated total serum IgE and severity of disease [6]. In this study, we demonstrated that in patients with anti-HDM IgE antibody, 83.3% had anti-TG3 IgE antibody and 33.3% had anti-TMP IgE antibody, suggesting that an autoimmune or autoallergy mechanism might be induced by molecular mimicry. In this study, a high level of IgE-mediated cross-reactivity between HDM and human protein TG3/TMP was found. It is likely that environmental HDM induced primary sensitization. Skin damage allowed auto-antigen release such as TG3/TMP, which might contribute to maintenance and potentiation of skin inflammation. IgE cross-reactivity between human and fungal allergens has been well characterized on a molecular basis in patients with AD [21]. Human manganese superoxide dismutase (MnSOD) has been shown to elicit positive skin reactions and specific T-cell proliferation in patients sensitized to the *Aspergillus fumigatus* MnSOD and to cross-reactive MnSODs of different origin [22]. Hari *et al.* [17] provided evidence for cross-reactive T cells between *M sympodialis* thioredoxin (Mala s 13) and its human counterpart (human thioredoxin [hTrx]). Here, we report cross-reactivity of IgE antibody and TCC generation between HDM and its human homologue protein TG3 and TMP. It was demonstrated that TG3/TMP induced significant lymphocyte proliferation in PBMC from AD patients. HDM-specific T lymphocytes showed high cross-reactivity with TG3 and TMP. Interestingly, we also found that two patients had TG3 auto-reactivity who otherwise had neither increased total serum IgE level nor anti-HDM IgE antibody. These patients were referred to as “intrinsic AD” or “non-AD”. Our study suggests that autoimmune reactions can occur in patients with or without antibodies against common inhalants or food allergens. We failed to generate comparable numbers of TG3/TMP-specific TCC from peripheral blood of healthy controls, suggesting that this might be AD-specific. Our study also suggests that not only the anti-TG3/TMP IgE antibodies, but also TG3/TMP-specific TCCs might play a role in AD. The majority of the TCCs generated in our study were of the Th1 phenotype, while some were Th2 phenotype. Analysis of the cytokine pattern of the supernatants from TCC confirmed that the TG3/TMP-related auto-allergic immune response involved both Th2 and Th1 cells. A predominance of Th2 cells and a decreased ability of peripheral CD4+ T cells to produce IFN- γ have been postulated in patients with AD [23]. It was proposed that Th2 cells are predominant in the early phase of AD and that Th1 cells play a role in maintenance of AD [24]. Analysis of the cytokine pattern of the supernatants from TCC confirmed that the TG3/TMP-reactive TCCs were heterogeneous. IFN- γ could induce keratinocyte apoptosis [25, 26] and skin inflammation. The acute phase of Th2-predominant reactions has been observed to transit to a chronic phase of Th1-predominant reactions with related cytokines (IFN- γ and IL-12) in abundance. Such a transit could result from the development of auto-reactive T cells, potentially through epitope spreading, secondary to inflammation. In summary, our study provides new evidence for IgE- and T-cell-mediated cross-reactivity between human TG3/TMP and HDM exogenous antigen. Human epidermal

protein TG3 and TMP might act as auto-allergens to induce both IgE auto-antibody and auto-reactive T cells, and play important roles in initiation and maintenance of AD. Tissue damage induced in the context of allergic inflammation through exogenous agents (*e.g.* exogenous allergens, pruritus, scratching, infections) may result in the release of intracellular proteins, such as TG3/TMP and other auto-antigens. These auto-allergens might then be exposed and presented to the immune system, thereby perpetuating and aggravating the chronicity of the disease.

Our study has several limitations. The sample size was small. Also, the screening for IgE antibodies and T cells clones for human TG3 and TMP was undertaken solely based on *in vitro* studies. Further study may help to elucidate the mechanism of AD.

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