

Expansion of FOXP3-positive CD4⁺CD25⁺ T cells associated with disease activity in atopic dermatitis

Yasunori Ito, MD, PhD*; Yuichi Adachi, MD, PhD*; Teruhiko Makino, MD, PhD†; Hiroyuki Higashiyama, MD*; Tatsuya Fuchizawa, MD, PhD*; Tadamichi Shimizu, MD, PhD†; and Toshio Miyawaki, MD, PhD*

Background: FOXP3-positive CD4⁺CD25⁺ T cells are known to have an immunoregulatory function by means of preventing T-cell reactivity to both self- and non-self-antigens. However, the role of these cells in the pathogenesis of allergic diseases is not clear.

Objective: To evaluate the quantity and quality of circulating FOXP3-positive T cells in patients with atopic dermatitis (AD).

Methods: Peripheral blood mononuclear cells were isolated from 35 AD patients (mean [SD] age, 27.1 [7.5] years) and 36 controls (mean [SD] age, 27.5 [10.0] years). Cellular FOXP3 expression was analyzed using flow cytometry. Characteristics of FOXP3-positive T cells were evaluated with respect to cytokine production capability and suppressive function.

Results: Frequencies of circulating FOXP3⁺CD25⁺ cells in the CD4⁺ T-cell population of AD patients were significantly higher than those in controls (mean [SD], 7.4% [4.6%] vs 4.5% [1.3%]; $P = .002$) and correlated with their Scoring Atopic Dermatitis (SCORAD) scores ($r = 0.74$, $P = .008$) and peripheral blood eosinophil counts ($r = 0.72$, $P < .001$). In the patients whose samples were analyzed at intervals of 1 to 2 months, frequencies of FOXP3-positive T cells were decreased as their skin lesions improved, regardless of medicines used. FOXP3-positive CD4⁺ T cells from patients, as well as those from controls, showed little capability to synthesize interferon γ and interleukin 4. No differences were found in suppression abilities of CD4⁺CD25⁺ T cells between AD patients and controls.

Conclusions: Our data suggest that dynamic fluctuation in numbers of circulating FOXP3-positive regulatory T cells might contribute to the pathogenesis of AD.

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INTRODUCTION

Immune regulation and tolerance are essential functions of the immune system to prevent and limit harmful immune responses to self- and non-self-antigens. CD4⁺CD25⁺ regulatory T (Treg) cells represent a unique lineage of immunoregulatory cells in both humans and animals and play a central role in the maintenance of immunologic self-tolerance. Most recently, FOXP3 has emerged as a master control transcription factor for the generation and function of Treg cells, as illustrated by the demonstration that naturally occurring defects in FOXP3 cause characteristic autoimmune syndrome in mice and humans.¹ Human FOXP3-expressing Treg cells, which are mostly found in the CD4⁺CD25⁺ T-cell population, are generated in the thymus and enter the circulation as naive thymus-derived Treg cells, and they convert into memory Treg cells on exposure to specific antigens.² FOXP3 expression is also induced in CD4⁺CD25[−] T cells

after activation.^{3,4} However, activation-induced FOXP3 expression is transient and the cells fail to develop suppressor function, suggesting that a sustained high level of FOXP3 expression is required for Treg phenotype and function.⁵ Another source for memory Treg cells, which are generally termed *adaptive Treg cells*, is the generation of FOXP3-expressing Treg cells from FOXP3-negative CD4⁺ T cells after T-cell receptor stimulation in the periphery.^{6,7} These cells control immune responses not only to self-antigens but also to various non-self-antigens, such as alloantigen, exogenous antigens, and tumor antigens. Although these peripherally induced adaptive Treg cells resemble naturally occurring thymus-derived Treg cells in phenotype and aspects of their function, interaction between these 2 populations has not been clarified yet.

Atopic dermatitis (AD) is a common pruritic and chronically relapsing allergic inflammatory skin disease, which is caused by impaired balance of T_H1/T_H2 immune responses to environmental factors in genetically susceptible individuals. Lack of FOXP3⁺CD4⁺CD25⁺ T cells leads to immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome,⁸ and affected patients often have AD-like skin lesions, increased IgE levels, and enhanced T_H2 responses.⁹ However, conflicting results regarding the numbers and functions of Treg cells in AD have been reported. Verhagen and

Affiliations: * Department of Pediatrics, Graduate School of Medicine, University of Toyama, Toyama, Japan; † Department of Dermatology, Graduate School of Medicine, University of Toyama, Toyama, Japan.

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colleagues¹⁰ demonstrated the absence of FOXP3-positive T cells in skin, which suggested a dysregulated control of inflammation. In contrast, Ou and colleagues¹¹ showed the elevated number of circulating Treg cells with a normal suppressive function in patients with AD, using the expression of CD25 molecule as a marker for Treg cells. In this study, we evaluated the quantity and quality of circulating FOXP3-positive T cells in patients with AD compared with healthy individuals. Using flow cytometric analysis and an anti-FOXP3 monoclonal antibody, we explored whether frequency of FOXP3-positive cells correlated with disease activity and clinical course of AD.

METHODS

Study Participants

Peripheral blood samples were obtained from 35 AD patients (mean age, 27 years; age range, 14–41 years) and from 36 healthy volunteers (mean age, 27 years; age range, 15–49 years) with no history of allergic diseases and autoimmune diseases. Profiles of the AD patients are given in Table 1. The diagnosis of AD was based on Hanifin and Rajka's criteria.¹² No patients had received systemic glucocorticoids or immunosuppressive drugs. Disease activity was evaluated by the Scoring Atopic Dermatitis (SCORAD) index.¹³ The study was conducted according to the ethical standards of the University of Toyama, which requires informed consent from each study participant.

Flow Cytometric Analysis

Mononuclear cells (MNCs) were isolated from heparinized blood by density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St Louis, Missouri). FOXP3 expression was analyzed by flow cytometry according to the protocol described previously.^{4,8} Briefly, fresh MNCs were washed in phosphate-buffered saline, fixed in 1 mL of phos-

phate-buffered saline with 1% paraformaldehyde and 0.05% Tween 20, and kept overnight at 4°C. Cells were treated twice with 0.5 mL of DNase (100 Kunitz units/mL) according to the manufacturer's instructions (Sigma-Aldrich). Cells were incubated with a murine anti-human FOXP3 monoclonal antibody (Abcam, Cambridge, England) for 1 hour at room temperature and washed with FACS buffer (phosphate-buffered saline, 3.0% fetal calf serum, 0.5% Tween 20, and 0.05% azide). FOXP3 staining was detected using Alexa Fluor-488 goat anti-mouse IgG antibody (Invitrogen, Eugene, Oregon) and washed as described herein. Cell surface staining was then performed using PC5-conjugated anti-human CD4 (Immunotech, Marseille, France) and phycoerythrin-conjugated anti-human CD25 (Miltenyi Biotec, Bergish Gladbach, Germany) monoclonal antibodies for 20 minutes at room temperature. Cells were analyzed using a flow cytometer (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan). Intracellular synthesis of cytokines was evaluated at the single-cell level by a flow cytometric analysis with a modification of the previously described method.^{14,15} Briefly, 1×10^6 per well of fresh MNCs were suspended in RPMI 1640 (Nipro K.K., Tokyo, Japan) supplemented with 2 mL of L-glutamine, 10% fetal bovine serum (Equitch-Bio, Ingram, Texas), 2×10^{-5} M 2-mercaptoethanol, 10 U/mL of penicillin, 100 μ g/mL of streptomycin, 0.25 μ g/mL of amphotericin B, and 10 μ g/mL of gentamycin (GIBCO BRL, Grand Island, New York) and then stimulated with 20 ng/mL of phorbol myristate acetate (Sigma-Aldrich) and 1 μ g/mL of ionomycin (Sigma-Aldrich) in the presence of 10 μ g/mL of brefeldin A (Sigma-Aldrich) for 6 hours at 37°C in 5% carbon dioxide and 95% air. After washing, the stimulated cells were fixed and permeabilized, then stained with anti-FOXP3 monoclonal antibodies as mentioned herein. Finally, intracellular cytokines were stained with phycoerythrin-conjugated anti-interleukin 4 and anti-interferon γ monoclonal antibodies (Immunotech).

Suppression Assay

In vitro suppression assays were performed as previously reported.¹¹ Briefly, CD4⁺ T cells purified by means of negative selection with immunomagnetic beads (by depletion of CD8⁺, CD11b⁺, CD16⁺, CD19⁺, CD36⁺, and CD56⁺ cells) were separated into CD4⁺CD25⁺ and CD4⁺CD25⁻ fractions by positive selection with immunomagnetic beads with a CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec). The purity of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells was always more than 90%. All steps were performed according to the manufacturer's instructions. To analyze the suppressive capacity of CD4⁺CD25⁺ T cells, CD4⁺CD25⁻ T cells (6×10^4) were stimulated with soluble 1 μ g/mL of anti-CD3 (Nihirei, Tokyo, Japan) in the presence of irradiated (30 Gy) autologous MNCs as antigen-presenting cells (6×10^4 per well) for 72 hours. Variable numbers of CD4⁺CD25⁺ T cells were added, giving final CD25⁺ cell to CD25⁻ cell ratios of 1:1, 0.5:1, 0.2:1, 0.1:1, or 0:1. Proliferation was determined using a bromodeoxyuridine proliferation enzyme-linked im-

Table 1. Characteristics of Study Participants^a

Characteristic	Patients (n = 35)
Age, mean (SD), y	27.1 (7.5)
M/F	22/13
Allergic disease	
Bronchial asthma	8 (22.8)
Rhinitis	5 (14.3)
Conjunctivitis	1 (2.8)
Treatment	
Oral	
Corticosteroids	0 (0)
Antihistamines	22 (62.8)
Topical	
Corticosteroids	25 (71.4)
Calcineurin inhibitors	6 (17.1)
PUVA therapy	2 (5.7)

Abbreviation: PUVA, psoralen-UV-A.

^a Data are given as number (percentage) except where indicated otherwise. Of the 36 control subjects, 24 were men and 12 were women (mean [SD] age, 27.5 [10.0] years).

munosorbent assay kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Briefly, bromodeoxyuridine was added into the incubated cells 2 hours before the termination of the culture. After transferring into 96-well flat bottom plates, the cells were treated with FixDenat solution for 30 minutes and were subsequently incubated with anti-bromodeoxyuridine-peroxidase antibodies for 90 minutes at room temperature. Substrate solution was added, and the absorbance of samples was measured with an enzyme-linked immunosorbent assay reader at 450 nm (reference wavelength, 690 nm). Suppression was expressed as percentage proliferation compared with CD4⁺CD25⁻ stimulated T cells.

Statistical Analysis

Comparison between unpaired groups was performed using the Mann-Whitney *U* test. Linear regression analysis was performed to determine whether a relationship existed among frequencies of FOXP3-positive CD4⁺CD25⁺ T cells, serum IgE levels, peripheral eosinophil counts, and SCORAD scores. Differences were considered statistically significant at *P* < .05.

RESULTS

FOXP3-Positive CD4⁺CD25⁺ T Cells in the Peripheral Blood of Patients With AD

Flow cytometric analysis of peripheral blood mononuclear cells revealed that FOXP3 expression was detectable mainly within the CD4⁺CD25⁺ T-cell population and only rarely in CD4⁺CD25⁻ T cells (Fig 1-A). It was marked that percent-

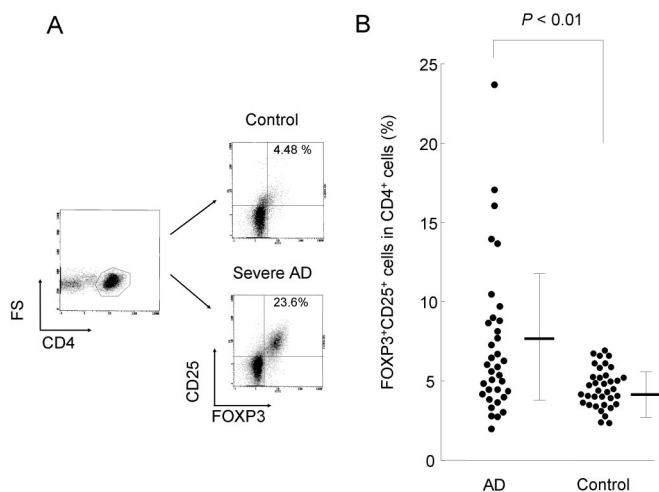


Figure 1. Flow cytometric analysis of FOXP3 expression in circulating CD4⁺ T cells. A, Representative data of a healthy individual and a patient with severe atopic dermatitis (AD). The numbers in the upper right quadrant indicate percentages of FOXP3-positive CD25⁺ T cells in a CD4⁺ T-cell population. B, Percentages of FOXP3-positive CD25⁺ T cells in a CD4⁺ T-cell population of patients with AD (*n* = 35) and control subjects (*n* = 36). Thick horizontal bars indicate the mean for each group, and error bars indicate the SD.

ages of FOXP3-positive CD25⁺ cells within the circulating CD4⁺ T cells in patients with AD were significantly higher than those in control subjects (mean [SD], 7.4% [4.6%] vs 4.5% [1.3%]; *P* = .002) (Fig 1B). Because our clinical observation suggested that an increase of FOXP3-positive CD4⁺CD25⁺ T cells might be related to the severity of AD, we evaluated the relationship between the percentage of FOXP3-positive cells and disease activity. Frequencies of FOXP3-positive CD4⁺CD25⁺ T cells significantly correlated with SCORAD scores (*r* = .74, *P* = .008) and peripheral blood eosinophil counts (*r* = .72, *P* < .001) but not with serum IgE levels (*r* = .22, *P* = .37) (Fig 2). We next examined whether numbers of circulating FOXP3-positive CD4⁺CD25⁺ T cells in AD patients were changing during their clinical course (Table 2). Of 35 patients, 6 had been chosen because of their poor adherence to treatment. After thorough education, adherence improved except for one, and these samples were reanalyzed 1 to 2 months later. Regardless of medicines used for the treatment, frequencies of FOXP3-positive CD4⁺CD25⁺ T cells clearly decreased in 5 patients whose skin lesions were improved as their SCORAD scores decreased but increased in 1 patient who had refused treatment and experienced deterioration of the skin symptoms (patient 1).

Phenotypes of FOXP3-Positive CD4⁺CD25⁺ T Cells in Patients With AD

We next sought to determine whether expanded FOXP3-positive CD4⁺CD25⁺ T cells of AD patients elicited the characteristics compatible with Treg cells. It has been shown that FOXP3-positive Treg cells lack an ability to synthesize T_H1 and T_H2 cytokines by themselves¹⁶; thus, we evaluated cytokine production patterns of circulating T cells on stimulation with phorbol myristate acetate and ionomycin. Extent of FOXP3 expression was not affected by these stimuli. Although patients with AD had a higher ratio of interleukin 4- and interferon γ -producing CD4⁺ T-cell numbers, reflecting their T_H2-skewed conditions, FOXP3-positive CD4⁺ T cells from patients, as well as those from control subjects, showed little capability to synthesize both cytokines (Fig 3A

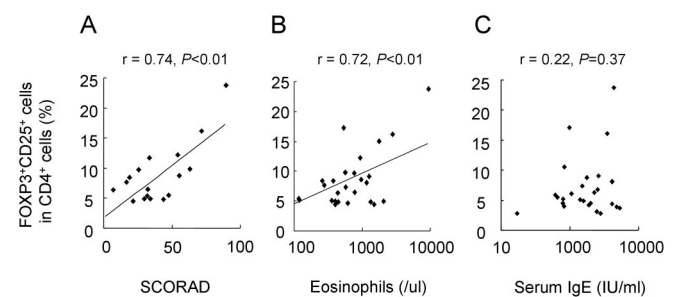


Figure 2. Correlations between the frequencies of FOXP3-positive CD25⁺ T cells in a CD4⁺ T-cell population of patients with atopic dermatitis and their Scoring Atopic Dermatitis (SCORAD) scores (A; *n* = 17), peripheral blood eosinophil counts (B; *n* = 29), and serum IgE levels (C; *n* = 27).

Table 2. Profiles of 6 Patients Whose Samples Were Analyzed Twice

Patient No./ sex/age, y	SCORAD score		FOXP3 ⁺ CD25 ⁺ cells, %		Treatment			Interval, mo
	First	Second	First	Second	TCS	TCI	PUVA	
1/M/31	53.5	79.0	10.8	12.9	—	—	—	2
2/M/24	17.0	12.5	8.7	6.7	+	+	—	1
3/M/34	64.5	54.4	8.5	7.2	—	+	—	1
4/F/14	66.6	37.1	7.2	6.9	+	—	—	1
5/M/33	82.8	41.2	14.9	6.9	+	—	—	1
6/F/29	89.0	21.2	23.6	7.3	+	—	—	2

Abbreviations: SCORAD, Scoring Atopic Dermatitis; TCS, topical corticosteroids; TCI, topical calcineurin inhibitor; PUVA, psoralen-UV-A. Symbols: Minus sign, negative; plus sign, positive.

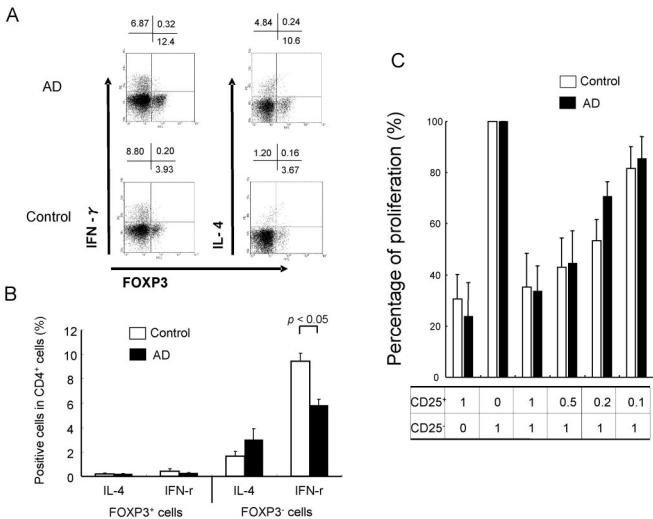


Figure 3. Phenotypes of FOXP3-positive CD4⁺ T cells in patients with atopic dermatitis (AD). Flow cytometric analysis of intracellular interferon γ and interleukin 4 production of CD4⁺ T cells. A, Representative data of 3 patients with AD and 3 control subjects. The numbers in each quadrant indicate percentages of the respective subpopulation in gated CD4⁺ T cells. B, Combined data with SEM within each cell population. C, Suppressive capabilities using CD4⁺CD25⁺ T cells as a surrogate for FOXP3-expressing T cells. Variable numbers of CD4⁺CD25⁺ T cells were added, giving the indicated final CD25⁺ to CD25⁻ cell ratios. Suppression was expressed as percentage proliferation compared with CD4⁺CD25⁻ stimulated T cells. Results (mean $\pm$ SEM) are shown for patients with AD (n = 3) and control subjects (n = 3).

and B). It also has been known that FOXP3-positive Treg cells have an ability to suppress the activation and proliferation of other T cells in a contact-dependent manner.¹⁷ In vitro suppression assay was performed using CD4⁺CD25⁺ T cells as a surrogate for FOXP3-expressing Treg cells. Proliferation of CD4⁺CD25⁻ T cells in response to soluble anti-CD3 monoclonal antibody in the presence of accessory cells was suppressed as the numbers of cocultured CD4⁺CD25⁺ T cells increased. No differences were found in the suppression abilities of CD4⁺CD25⁺ T cells between AD patients and control subjects (Fig 3C).

DISCUSSION

There is accumulating evidence that Treg cells participate in allergic diseases.¹⁸ In this study, we demonstrated that there was an increase in the numbers of FOXP3-positive CD4⁺CD25⁺ T cells in the peripheral blood of patients with AD using FOXP3 monoclonal antibody. Although Ou and colleagues¹¹ showed similar results, the reported frequencies of Treg cells from patients with allergic diseases are inconsistent. Children with asthma had decreased numbers of CD4⁺CD25⁺ T cells compared with healthy individuals without allergy.¹⁹ In adults, the rate of FOXP3⁺ cells in CD4⁺ cells was lower in symptomatic patients with AD and asthma compared with that in healthy volunteers who had similar levels of IgE and eosinophils.²⁰ In contrast, other studies showed that numbers of circulating CD4⁺CD25⁺ T cells in patients with allergic rhinitis²¹ and AD²² were comparable to those in nonatopic individuals. The differences in these observed frequencies might be related to the disease status. Pediatric patients with more severe asthma had more CD4⁺CD25⁺ T cells in their peripheral blood than did patients with less severe disease, and there was a positive correlation between numbers of CD4⁺CD25⁺ T cells and serum IgE levels.¹⁹ These findings are consistent with ours in that the frequencies of FOXP3-positive CD4⁺CD25⁺ T cells in patients with AD were correlated with their SCORAD scores and peripheral eosinophil counts, which reflect disease severity. Also, frequencies of FOXP3-positive CD4⁺CD25⁺ T cells in AD patients of this study normalized after short periods as their skin lesions were improved. These rapid changes in numbers of FOXP3-positive CD4⁺CD25⁺ T cells might be explained by rapid peripheral turnover of Treg cells, which was reported by Vukmanovic-Stejic and colleagues.²³ They explored the origin of circulating FOXP3-expressing Treg cells and demonstrated that these cells are continuously recruited from the memory CD4⁺ T-cell pool and are susceptible to apoptosis. It also has been reported that FOXP3 is transiently induced by stimulation of anti-CD3 and CD28 in FOXP3-negative CD4⁺CD25⁻ T cells, but this transient expression is not able to lead to the suppression of cytokine production in vitro.^{24,25} However, expanded FOXP3-positive CD4⁺CD25⁺ T cells in AD patients of this study were hyporesponsive for proliferation and cytokine production, and

their suppressive capacity was equal to healthy volunteers, suggesting that these cells could be functional as Treg cells.

Treatments for AD, such as glucocorticoid steroids and immune suppressants, might affect dynamics of Treg cell expansion. It had been reported that adult asthmatic patients receiving inhaled or systemic glucocorticoid had higher FOXP3 messenger RNA expression in circulating CD4⁺ T cells compared with that seen in untreated asthmatic patients or healthy volunteers²⁶ and that 4 weeks of treatment with inhaled glucocorticoids increased percentages of CD4⁺CD25⁺ T cells and FOXP3 messenger RNA expression levels in peripheral blood and bronchoalveolar lavage fluid.²⁷ In contrast, Caproni and colleagues²⁸ performed immunohistochemical evaluation of eczematous skin lesions before and after topical treatment with calcineurin inhibitor or corticosteroid and demonstrated that both treatments did not modify the number of FOXP3-positive cells in the skin. In this study, increased numbers of FOXP3-positive cells in AD patients clearly decreased as their skin lesions improved even when they were treated with topical glucocorticoids or calcineurin inhibitor. These results suggest that FOXP3-positive Treg cells could be expanded in response to systemic and local allergic inflammation and might play a role in the negative feedback loop of T-cell activation.

It is not clear whether our results obtained using peripheral blood T cells directly reflect the topical inflammatory response in the skin. Verhagen and colleagues reported the absence of FOXP3-positive T cells in AD skin specimens.¹⁰ However, Treg cells express a wide variety of trafficking receptors, including CLA, CCR4, and CCR6, which are well known as skin homing receptors.^{29,30} Recent literature shows that FOXP3-positive cells are present in the skin of inflammatory dermatoses, including AD and psoriasis.^{28,31–33} Interestingly, Chen and colleagues³⁴ demonstrated that frequencies of Treg cells in the peripheral blood and skin concordantly changed with the clinical features of psoriasis. Consistent with our findings, Reefer and colleagues³⁵ recently reported that CD25^{high} CD4⁺ T cells in the peripheral blood of AD patients were increased compared with control subjects and that the frequency of CD25^{high} CD4⁺ T cells decreased after allergen avoidance in parallel with improvement of skin condition. Furthermore, they demonstrated that among these increased cells CCR6-negative cells had capability to promote T_H2 responses, speculating that activated CD25^{high} T cells in the skin might lose expression of CCR6 and leave the skin to join the circulation. Analysis of T cells at the skin level in the context of skin homing receptors, including CCR6, may provide insights into the role of FOXP3-positive T-cell subtypes in immune homeostasis at this site. Collectively, dynamic migration of FOXP3-positive Treg cells may contribute to the pathogenesis of AD. Further studies are required to improve our understanding of the role of Treg cells in allergic inflammation.

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Requests for reprints should be addressed to:
 Yuichi Adachi, MD, PhD
 Department of Pediatrics
 Graduate School of Medicine
 University of Toyama
 2630 Sugitani
 Toyama 930-0194, Japan
 E-mail: yadachi@med.u-toyama.ac.jp