

Letter to the Editor

Circulating Foxp3⁺CD4⁺ cell numbers in atopic patients and healthy control subjects

To the Editor:

We have found that the number of CD4⁺ cells expressing the transcription factor forkhead box protein P3 (Foxp3) was negatively associated with the levels of IgE, eosinophils, and IFN- γ . Also, the Foxp3⁺/CD4⁺ ratio was significantly decreased in symptomatic patients with atopic dermatitis (AD) and bronchial asthma (BA) compared with that in healthy volunteers having similar levels of IgE and eosinophils.

Simultaneous expression of surface CD4 and CD25 is a specific marker for mouse naturally occurring regulatory T cells (nTregs), which are essential for the active suppression of autoimmunity and allergic inflammation.¹ However, human nTregs are found only in CD4⁺ cells expressing high levels of CD25, and other T-cell types are contaminated in the whole CD4⁺CD25⁺ fraction.² Foxp3 is indispensable for the development of nTregs. Mice with its mutant variant had an intense multiorgan inflammatory response associated with allergic airway inflammation, striking hyperimmunoglobulinemia E, eosinophilia, and dysregulated T_H1 and T_H2 cytokine production in the absence of overt T_H2 skewing.³⁻⁵ In human subjects also, genetic defects in Foxp3 cause immune dysregulation, polyendocrinopathy, and enteropathy X-linked (IPEX) syndrome.⁶ IPEX syndrome is thought to be due to a defect in nTregs. Most patients with IPEX syndrome also have food allergy and AD-related symptoms immediately after birth.⁶ It is thus suggested that Foxp3⁺ Tregs plays an important role in regulating common allergic disorders,⁵ as well as IPEX. The number of Foxp3⁺ Tregs was recently found to be decreased in skin lesions of patients with AD and patients with psoriasis by using immunohistochemistry.⁷ The measurement of circulating Foxp3⁺ cells in human subjects also recently became possible by using anti-Foxp3 antibody.⁸ However, it remains unclear whether circulating Foxp3⁺ Tregs play a role in common allergic diseases. Here we analyzed intracellular Foxp3 protein expression in unchallenged circulating CD4⁺ T cells from allergic patients and healthy control subjects at the single-cell level to evaluate its diagnostic potential and found that the level of Foxp3⁺CD4⁺ cells correlated inversely with the levels of blood eosinophils, serum total IgE, and IFN- γ .

Samples were obtained with written informed consent after approval by the ethics review board of the hospital from 64 total donors (age, 24.0 $\pm$ 3.3 years; male/female patients, 43/21). Those donors consisted of 37 patients (age, 23.5 $\pm$ 2.7 years; male/female patients, 24/15) who were given diagnoses of AD, BA, or both based on the guidelines of the Japanese Dermatological Association and the Global Initiative for Asthma and 27 healthy control subjects (age, 24.8 $\pm$ 3.8 years; male/female patients, 19/8). All the patients were judged positive for specific IgE antibodies against at least 1 of *Dermatophagoides*

pteronyssinus, *Dermatophagoides farinae*, and Japanese cedar pollen by using the Pharmacia CAP system fluorescence enzyme-linked immunoassay (CAP-FEIA; Pharmacia, Uppsala, Sweden). Twenty-three of the 27 age-matched healthy volunteers also showed at least 1 positive reaction to those allergens by using CAP-FEIA, although they never had subjective symptoms related to allergic diseases. We were not able to recruit enough nonatopic volunteers because the majority of young adults living in the Tokyo area are sensitized to at least 1 of those allergens.⁹ The Foxp3⁺CD4⁺ ratio in PBMCs was measured by means of flow cytometry after staining with anti-Foxp3 mAb (ab20034; 236A/E7, Abcam, Cambridge, United Kingdom) and anti-CD4 mAb (BD Biosciences, San Diego, Calif). Various routine laboratory examination data, including the levels of blood eosinophils, serum IgE, and serum cytokines, such as IFN- γ , were also examined, although the levels of IL-4, IL-5, and IL-10 were often less than the detection limits.

First, we compared the circulating Foxp3⁺CD4⁺ ratios in the healthy control subjects and patients. Significant differences were detected between the 2 groups (AD: $P < .0001$, $n = 27/20$; BA: $P = .0002$, $n = 27/13$; AD plus BA: $P = .0024$, $n = 27/9$). However, comparison between the control subjects and patients also showed significant differences in total IgE and eosinophil levels (AD only: total IgE, $P < .0001$; eosinophils, $P < .0001$; BA only: total IgE, $P = 0.0076$; eosinophils, $P = .0006$; AD plus BA: total IgE, $P = .0011$; eosinophils, $P = .0008$). Not only T_H2 markers but also a T_H1 proinflammatory cytokine, IFN- γ (measured in international units per milliliter), showed significant differences between the 2 groups (data not shown). From these results, it might be possible to conclude that circulating Foxp3⁺CD4⁺ ratio is decreased in allergic patients compared with that seen in control subjects. However, the Foxp3⁺CD4⁺ ratio is also suggested to correlate directly with the levels of total IgE, eosinophils, and IFN- γ but is not directly related to the presence of allergic diseases.

We next examined the Foxp3⁺CD4⁺ ratios in comparison with the levels of blood eosinophils, serum IgE, and IFN- γ within the control and patient groups. The Foxp3⁺CD4⁺ ratios often tended to correlate inversely with the levels of those markers, although the correlations were not statistically significant (healthy control subjects [$n = 27$]: serum total IgE, $P = .272$ and $r = 0.2193$; eosinophils, $P = .119$ and $r = 0.3071$; serum IFN- γ , $P = .733$ and $r = 0.0686$; patients [$n = 37$]: total IgE, $P = .832$ and $r = 0.0361$; eosinophils, $P = .502$ and $r = 0.1140$; serum IFN- γ , $P = .707$ and $r = 0.0640$). We then combined our data for the 2 groups. Analysis showed that the Foxp3⁺CD4⁺ ratios correlated inversely at statistically significant levels with the serum total IgE level ($P = .037$, $r = 0.2611$), eosinophil ratio ($P = .027$, $r = 0.2762$), and serum IFN- γ level ($P = .017$, $r = 0.2973$)

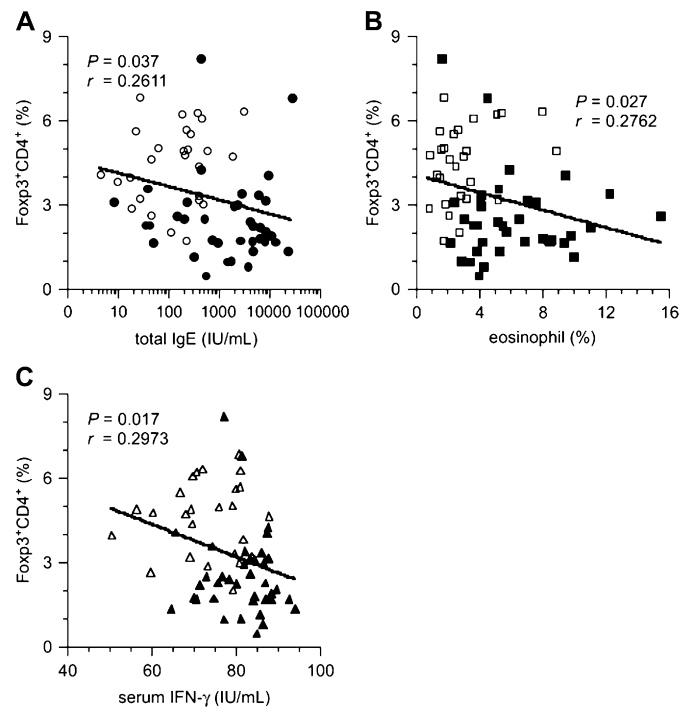


FIG 1. An inverse correlation of Foxp3⁺CD4⁺ cell ratios with T_H1/T_H2 markers *in vivo*. Analysis of Foxp3⁺CD4⁺ cell ratios versus total IgE levels, eosinophils (T_H2), and serum IFN- γ levels (T_H1) in the total population, including patients. Simple linear regression revealed significant differences: total IgE (A), eosinophils (B), serum IFN- γ (C) (n = 64). Open symbols represent the healthy control subjects, and solid symbols represent the allergic patients.

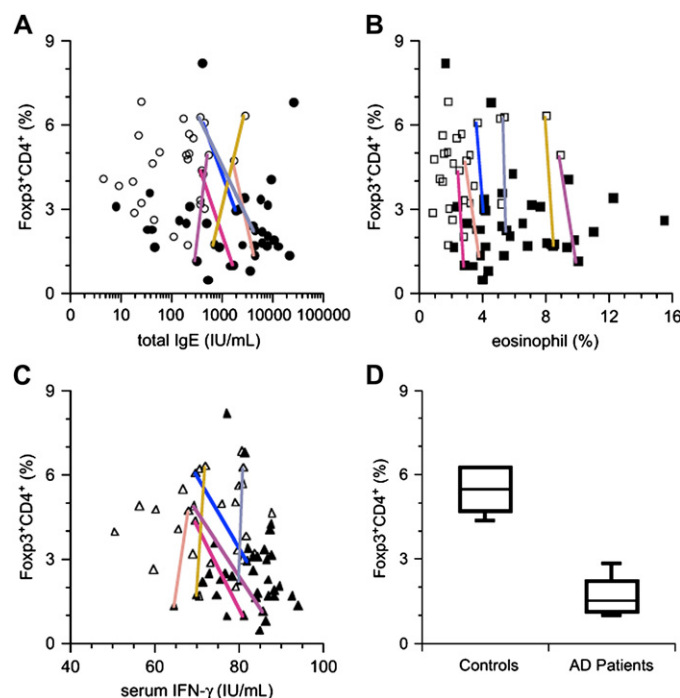


FIG 2. A case-control study using matched-pair analysis of patients with AD only. We were able to identify 6 pairs who had similar levels of both total IgE (A) and eosinophils (B) within $\pm 20\%$. No difference was detected in serum IFN- γ levels (C). Each color line indicates one selected pair, and the same color is used for that pair in each of the 3 graphs. D, Significantly lower Foxp3⁺CD4⁺ cell ratios were seen in patients than in control subjects (Mann-Whitney U test, $P = .037$).

in the total population ($n = 64$, Fig 1). Although this inverse correlation is considered to be a novel finding, it remain unclear whether circulating $\text{Foxp3}^+\text{CD4}^+$ cells can be a diagnostic marker for allergic diseases.

To examine whether the $\text{Foxp3}^+\text{CD4}^+$ ratios are affected by the presence of allergic diseases independently of the levels of IgE and eosinophils, we attempted to perform a case-control study using matched-pair analysis to eliminate the influence of the $\text{Foxp3}^+\text{CD4}^+$ level correlations with the total IgE level and eosinophil ratios. In the subgroups of patients with AD only or AD plus BA and the control subjects, 6 case-control pairs were found showing similar levels in both of 2 parameters (total IgE and eosinophil levels) within levels of $\pm 20\%$ log scale for total IgE; (Fig 2, A and B; see also Fig E1, A and B, in this article's Online Repository at www.jacionline.org). These selected matched pairs showed no significant difference in the serum IFN- γ level or the levels of IgE and eosinophils (Fig 2, C; see also Fig E1, C, in this article's Online Repository at www.jacionline.org). As for BA, because few patients had isolated BA and their number was not sufficient for matched-pair selection by 2 factors, only 1 parameter (total IgE level), which matched within $\pm 3\%$ (log scale), was chosen for 4 pairs (see Fig E2, A, in this article's Online Repository at www.jacionline.org). The selected matched pairs also showed no significant difference in the eosinophil ratio or serum IFN- γ level or the level of IgE (see Fig E2, B and C in this article's Online Repository). However, significant differences in the $\text{Foxp3}^+\text{CD4}^+$ ratios were detected between the patients and control subjects in the matched pairs (AD only: $P = .0039$, $n = 6$; BA only: $P = .0202$, $n = 4$; AD plus BA: $P = .0104$, $n = 6$; Fig 2, D; see also Figs E1 and E2, D, in this article's Online Repository at www.jacionline.org). Regarding treatment of the diseases, none of the subjects were treated with oral corticosteroids, which upregulate Foxp3 expression.¹⁰ All the patients selected for the matched-pair analysis were treated with a topical glucocorticoid (6/6 in patients with AD, 4/4 in patients with BA, and 6/6 in patients with AD plus BA; dexamethasone ointment was used for patients with AD, and fluticasone propionate inhaler was used for patients with BA), an H_1 -histamine receptor antagonist (4/6 in patients with AD and 6/6 in patients with AD plus BA), or a β_2 -adrenergic bronchodilator (2/4 in patients with BA and 4/6 in patients with AD plus BA). Nevertheless, topical glucocorticoid treatment is unlikely to have affected the present results because the patients showed significantly lower levels of $\text{Foxp3}^+\text{CD4}^+$. Although there is a possibility that other concomitant drugs might have had an effect, $\text{Foxp3}^+\text{CD4}^+$ cells might influence the present illness of AD, BA, or both independently of the levels of total IgE and eosinophils.

In conclusion, we were able to demonstrate that atopic patients with active disease had a lower $\text{Foxp3}^+\text{CD4}^+$ ratio than asymptomatic control subjects having similar levels of serum IFN- γ , total IgE, and eosinophils. These results suggest that circulating $\text{Foxp3}^+\text{CD4}^+$ cells regulate unknown factors affecting the present illness, which are unrelated to these $\text{T}_\text{H}1/\text{T}_\text{H}2$ markers. Measurement of $\text{Foxp3}^+\text{CD4}^+$ cells has the potential to aid in evaluating the presence of active inflammation, which cannot be evaluated by known $\text{T}_\text{H}1$ - and $\text{T}_\text{H}2$ -related markers in patients with allergic diseases.

Kanami Orihara, MSc^{a,c}
Masami Narita, MD, PhD^b
Takashi Tobe, PhD^c
Akira Akasawa, MD, PhD^b
Yukihiro Ohya, MD, PhD^b
Kenji Matsumoto, MD, PhD^a
Hirohisa Saito, MD, PhD^a

From ^athe Department of Allergy and Immunology, National Research Institute for Child Health and Development, ^bthe National Center for Child Health and Development, and ^cthe School of Pharmaceutical Sciences, Showa University, Tokyo, Japan. E-mail: hsaito@nch.go.jp.

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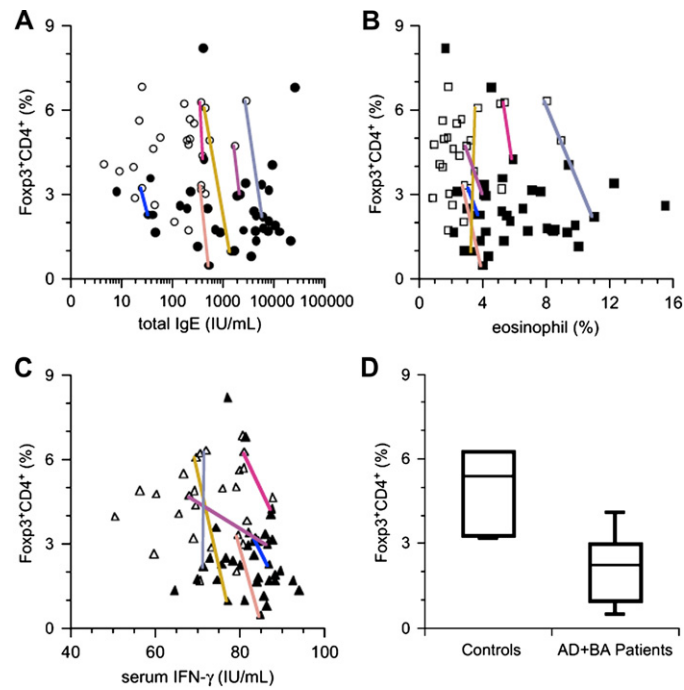


FIG E1. A case-control study using matched-pair analysis of patients with AD plus BA. We were able to identify 6 pairs who had similar levels of both total IgE (**A**) and eosinophils (**B**). No difference was detected in serum IFN- γ levels (**C**). Each color line indicates one selected pair, and the same color is used for that pair in each of the 3 graphs. **D**, Significantly lower FcγR3+CD4+ cell ratios were seen in patients than in control subjects (Mann-Whitney U test, $P = .017$).

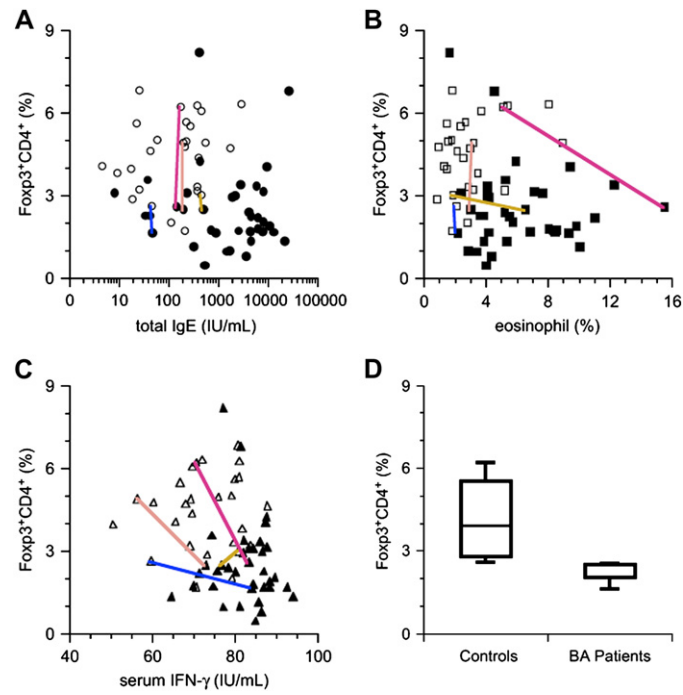


FIG E2. A case-control study using matched-pair analysis of patients with BA only. We were able to identify 4 pairs who had similar levels of total IgE within $\pm 3\%$ (log scale) (**A**). No difference was detected in eosinophils (**B**) or serum IFN- γ levels (**C**). Each color line indicates one selected pair, and the same color is used for that pair in each of the 3 graphs. **D**, Significantly lower Fcγ3⁺CD4⁺ cell ratios were seen in patients than in control subjects (Mann-Whitney *U* test, *P* = .027).