



Multicolor flow-cytometric analysis of milk allergen-specific T-helper type 2 cells revealed coexpression of interleukin-4 with Foxp3

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ABSTRACT

Background: Allergen-specific T-helper type 2 (T_H2) cells play an important role in the development of allergic inflammation; however, investigations of the properties of allergen-specific T cells have been challenging in humans. Despite clear evidence that forkhead box p3 (Foxp3) is expressed in conventional effector T cells, its function has remained unknown.

Objective: To characterize allergen-specific T_H2 cells in milk allergy, with particular focus on the expression of Foxp3.

Methods: Twenty-one children with milk allergy and 11 children without milk allergy were studied. Peripheral blood mononuclear cells from subjects were stimulated with milk allergen for 6 hours and analyzed using multicolor flow cytometry to identify CD154⁺ allergen-specific T-helper cells. Simultaneously, the expression of intracellular cytokines and Foxp3 was analyzed.

Results: The milk allergy group had significantly larger numbers of milk allergen-specific interleukin (IL)-4- and IL-5–producing CD4⁺ T cells than the control group. Subjects in the milk allergy group had significantly more CD154⁺CD4⁺ IL-10–producing cells and CD154⁺Foxp3⁺CD4⁺ cells than those in the control group. In addition, the number of milk allergen-specific CD154⁺Foxp3⁺CD4⁺ cells strongly correlated with that of CD154⁺IL4⁺CD4⁺ cells. Bcl-2 expression in CD154⁺IL-4⁺Foxp3⁺ T-helper cells was significantly lower compared with that in total CD4 cells.

Conclusion: Increased numbers of IL-4–producing allergen-specific T-helper cells were found in patients with milk allergy. In addition, Foxp3 was coexpressed with IL-4 in allergen-specific T_H2 cells from patients. This coexpression was associated with lower Bcl-2 levels and could contribute to the phenotype and function of T_H2 cells.

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Introduction

Allergen-specific T-helper type 2 (T_H2) cells, which secrete T_H2 cytokines in response to activation by allergens, play an important role in the development of allergic inflammation.^{1,2} Although investigation of properties of allergen-specific T cells has been challenging in humans because of their low frequency within the T-cell repertoire, detection of the activation marker CD154 (a ligand

for CD40 and previously known as CD40L) has enabled the identification of an antigen-specific T-helper cell population.^{3–6} The CD154 molecule is primarily expressed on activated CD4⁺ T cells, and contact-dependent interactions between CD154 on T cells and CD40 on B cells or macrophages are required for T cells to function as T-helper cells.⁷ The authors established a method to analyze milk antigen-specific T_H2 cells by simultaneously detecting CD154 and intracellular cytokines using multicolor flow cytometry.

In addition to allergen-specific T-helper cells, several types of regulatory T cells (Tregs) are believed to control the development of allergy.⁸ Currently, Tregs can be classified into natural Tregs (nTregs), inducible Tregs, interleukin (IL)-10–producing Treg 1 (Tr1), CD8⁺ Tregs, and IL-17–producing Tregs.⁹ These cells share some common features, including the expression of forkhead box p3 (Foxp3; except for Tr1) and secretion of inhibitory cytokine IL-10

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and/or tumor growth factor- β . Although not all Tregs express Foxp3, Foxp3 is considered a “master” regulator for the development and suppressive functions of Foxp3⁺ Tregs.¹⁰ Foxp3 represses many genes by associating with other transcription factors, including nuclear factor of activated T cells,¹¹ the p65 subunit of nuclear factor- κ B,¹² and Runx1.¹³

Foxp3 also is transiently expressed in human conventional effector T cells (Tconv cells) after T-cell receptor activation.¹⁴ Transient Foxp3 expression in Tconv cells does not prevent cytokine production¹⁵ or confer suppressive capacity,¹⁶ although this has remained controversial.¹⁷ Thus, despite clear evidence that Foxp3 is expressed in Tconv cells, its function in Tconv cells remains unknown. Recently, McMurchy et al¹⁸ reported that Foxp3 was highly expressed in activated T_H17 cells and contributed to their phenotype by suppressing interferon- γ (IFN- γ) production and upregulating the expression of CCR4.

This study aimed to characterize allergen-specific T_H2 cells in milk allergy. While studying Foxp3 expression in milk allergen-specific T cells, the authors noticed the coexpression of Foxp3 with IL-4 in specific T-helper cells. Therefore, in this study, they investigated allergen-specific T_H2 cells in milk allergy, with particular focus on the expression of Foxp3.

Methods

Participants

From February 2013 through September 2014, Japanese children with allergies were invited to participate in this study conducted at the Fujita Health University Hospital (Toyoake, Japan) and the Second Teaching Hospital, Fujita Health University (Nagoya, Japan). Milk allergy was diagnosed based on a double-blinded, placebo-controlled food challenge test given to children with suspected cow's milk allergy on the same day as flow-cytometric analysis. The control subjects, who visited the pediatric departments for allergy screening, had been consuming dairy products or drinking cow's milk on a daily basis. This study was approved by the research ethics committee of Fujita Health University and was conducted in accordance with the ethical principles of the Declaration of Helsinki. Written informed consent from parents and children at least 10 years of age and assent from children younger than 10 years were obtained.

Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Leucosep tubes (Greiner Bio-One, Frickhausen, Germany). PBMCs were suspended in AIM-V medium (5×10^6 cells/mL) and incubated overnight at 37°C in a 5% CO₂ atmosphere. Antigen stimulation and intracellular cytokine staining were performed using an intracellular cytokine staining protocol¹⁶ and a “one-step protocol for intracellular (nuclear) proteins” (eBioscience, San Diego, California). In brief, PBMCs and CD28 (1 μ g/mL; BD Biosciences, San Jose, California) were incubated with skim milk antigen (150 μ g/mL; BD Biosciences) or heat-killed *Mycobacterium tuberculosis* strain H37 Ra (100 μ g/mL; BD Biosciences) for 6 hours. After incubation for 2 hours, Brefeldin A (10 μ L/mL; Sigma-Aldrich, St Louis, Missouri) was added to the samples. After incubation for 6 hours, PBMCs were stained using ethidium bromide monoazide (Invitrogen, Carlsbad, California), fixed, and then made permeable with Fixation/Permeabilization Concentrate and Diluent (eBioscience). Then, cells were washed with IC Permeabilization buffer (eBioscience) and stained with fluorescence-conjugated antibodies. The final endotoxin concentration in the cultures was 1.3 pg/mL as determined by a limulus assay (Endotoxin Single Test; Wako Pure Chemical Industries, Osaka, Japan).

Cell staining used different combinations of labeled antibodies: fluorescein isothiocyanate anti-CD154 (clone TRAP1) from BD Biosciences; Brilliant Violet 570 anti-CD3 (clone UCHT1), Brilliant Violet 421 anti-CD4 (clone RPA-T4), peridinin chlorophyll protein (PerCP) and cyanine (Cy) 5.5 anti-IL-4 (clone MP4-25D2), allophycocyanin (APC) anti-IL-5 (clone TRFK5), phycoerythrin and Cy7 anti-IL-10 (clone JES3-9D7), and allophycocyanin and Cy7 anti-IFN- γ (clone 4S.B3) from BioLegend (San Diego, California); phycoerythrin anti-Foxp3 (clone 236A/E7) from eBioscience; and allophycocyanin anti-Bcl-2 (clone Bcl-2/100) from EXBIO (Vestec, Czech Republic). Within 3 to 4 hours after the fixation and permeabilization procedure, fluorescent signals were acquired using a Gallios Multicolor Flow Cytometer (Beckman Coulter, Brea, California), and these data were analyzed using Kaluza software (Beckman Coulter). Percentages of CD154⁺ cytokine-positive cells were determined among the viable CD4⁺ lymphocytes. Numbers of antigen-specific cytokine-producing T-helper cells were determined by subtracting the medium control values.

The gating strategy for identifying CD4⁺CD154⁺, intracytoplasmic cytokine-positive, or Foxp3⁺ cells included the following steps. Stimulated PBMCs were first gated based on forward and side scatter that identified the lymphocyte population. Then, the CD3⁺CD4⁺ T-helper cells were selected. After the doublets were removed using the forward scatter integral vs forward scatter peak parameters, the dead cells were cast away from analysis using ethidium bromide monoazide. The CD154⁺IL-4⁺ T_H2 cells, CD154⁺IL-5⁺ T_H2 cells, CD154⁺IFN- γ ⁺ T_H1 cells, CD154⁺IL-10⁺ Tr1-like cells, and CD154⁺Foxp3⁺ cells were analyzed.

Fluorescent Microscopy

For fluorescent microscopy, samples were treated in an identical manner to the flow cytometry method and were stained with Brilliant Violet 421 anti-CD4 (clone RPA-T4), fluorescein isothiocyanate anti-IL-4 (clone MP4-25D2), and Alexa Fluor 647 anti-Foxp3 (clone 150D) from BioLegend. Cells were analyzed on a Zeiss 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with a $\times 63$ oil-immersion objective lens. Images were taken from the Zen software (Carl Zeiss).

Statistics

Statistical analysis was performed using Prism 5.0d (GraphPad Software, San Diego, California). The results for 2 independent groups were compared using the Mann-Whitney test. Paired data from 3 groups were compared using the Friedman test and the Dunn multiple comparisons post-tests. Possible associations between variables were assessed by Spearman rank correlation analysis (ρ). A *P* value less than .05 was considered significant.

Results

Study Participants

The present study included 21 children with milk allergy and 11 children without milk allergy. There were no significant differences in sex, age, or total IgE levels between these 2 groups (Table 1). Milk

Table 1
Patient characteristics

Characteristics	Milk allergy (n = 21)	Without milk allergy (n = 11)	<i>P</i> value
Boys/girls	13/8	7/4	>.05
Age (y), median (range)	4 (1–11)	5 (1–15)	>.05
Milk-specific IgE (UA/mL), median (range)	45.4 (3.11–735)	0.345 (0–0.75)	<.001
Total IgE (IU/mL), median (range)	501 (79.4–6110)	361 (14.9–5780)	>.05

allergen-specific IgE levels were significantly higher in the milk allergy group ($P < .001$).

Increased Milk Allergen-Specific T-Helper Cells in Patients with Milk Allergy

The milk allergy group had significantly larger numbers of milk allergen-specific IL-4–producing (median values 41 and 0 per 10^6 CD4⁺ T cell, respectively, $P < .0001$; Fig 1A) and IL-5–producing (median values 14 and 0 per 10^6 CD4⁺ T cells, respectively, $P = .0005$; Fig 1B) CD4⁺ cells than the control group. In the patients with milk allergy, there was a moderate positive correlation between numbers of milk allergen-specific IL-4–producing T_H2 cells and milk allergen-specific IgE levels ($\rho = 0.60$, $P < .005$; Fig 1F). This correlation was more apparent in subjects with higher (>40 UA/mL) milk-specific IgE levels.

CD154⁺IFN- γ ⁺ T_H1 cells were detected in 1 of the 11 subjects without milk allergy and in 8 of the 21 with milk allergy (not significant; Fig 1C, left); however, the ratio of IL-4–producing cells to IFN- γ –producing cells in those 9 subjects was apparently increased in the milk allergy group (not significant; Fig 1D). The *M tuberculosis*–specific IFN- γ –producing cells were used as positive controls because all Japanese individuals have been immunized with the Bacillus Calmette–Guérin vaccine during early childhood. There

was no significant difference in the numbers of *M tuberculosis*–specific IFN- γ –producing cells between the milk allergy and control groups (Fig 1C, right).

CD4⁺CD154⁺IL-10⁺ T Cells and Foxp3⁺ Cells in Patients with Milk Allergy

Unexpectedly, patients in the milk allergy group had significantly more milk allergen-specific IL-10–producing cells than those in the control group (median values 4 and 0 per 10^6 CD4⁺ T cells, respectively, $P = .0055$; Fig 1E).

For Foxp3⁺CD4⁺ T cells, the proportion of Foxp3⁺CD4⁺ nTregs did not differ significantly between the control and milk allergy samples (median values 7.48% and 7.91%, respectively, $P = .34$; Fig 2A) when PBMCs were not stimulated with milk allergen. However, there were significantly more milk allergen-induced CD154⁺Foxp3⁺ cells in the milk allergy group than in the control group after allergen stimulation (median values 0.0066% and 0.0002%, respectively, $P < .0001$; Fig 2B). CD154⁺Foxp3⁺ cells also were detected after allergen stimulation in approximately one half the control group, and CD154⁺IL-4⁺ and/or CD154⁺IL-5⁺ cells were detected in the same subjects. However, age, sex, other food allergy, or milk-specific IgE did not differ in these 2 cohorts within the control group (data not shown).

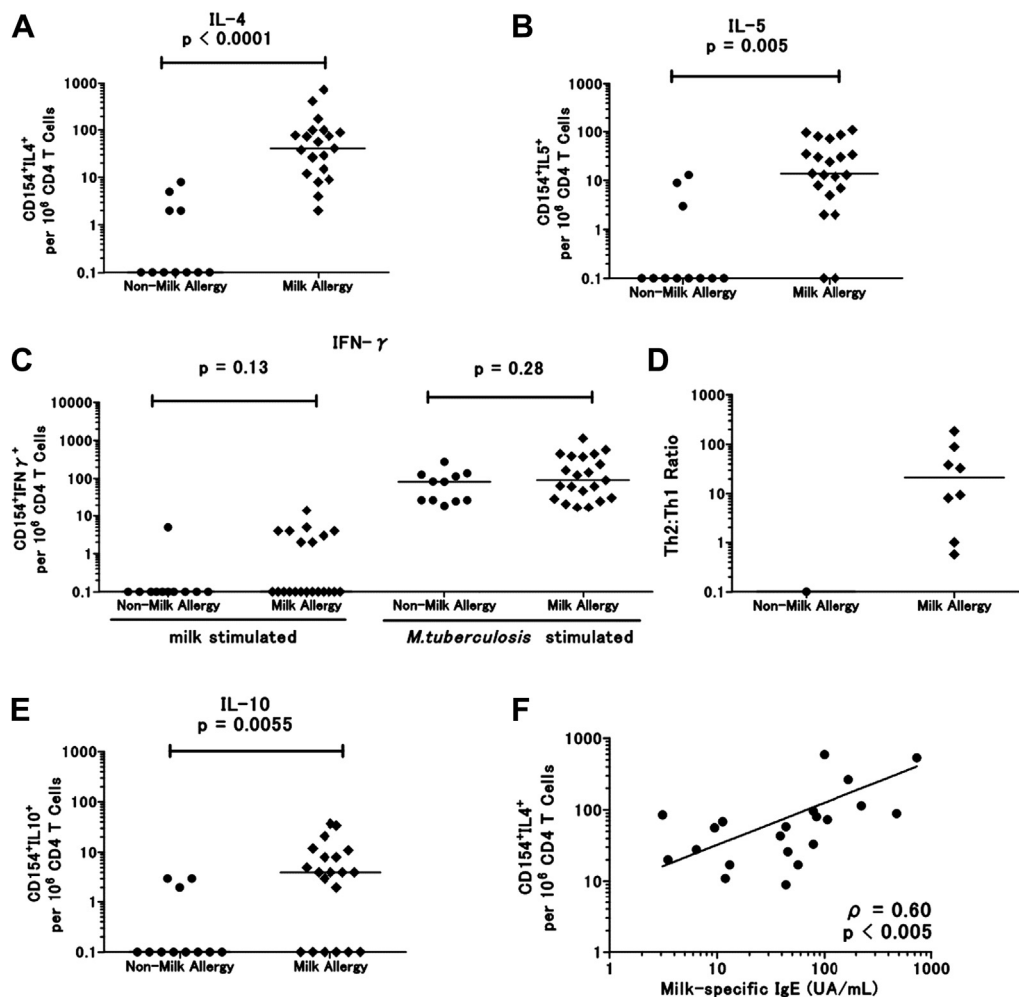


Figure 1. Numbers of milk allergen-specific cytokine-producing cells per 10^6 CD4⁺ T cells were compared between groups with and without milk allergy. The investigated cytokines were (A) interleukin (IL)-4, (B) IL-5, and (E) IL-10. (C) Numbers of milk-specific (left) and *Mycobacterium tuberculosis*–specific (right) interferon- γ (IFN- γ)–producing cells were compared. (D) In cases in which IFN- γ ⁺ cells were detected after milk allergen stimulation, the ratio of T-helper cell type 2 to type 1 was compared between groups with and without milk allergy. (F) Correlation between milk allergen-specific IL-4–producing T-helper cells and milk allergen-specific IgE levels. On the logarithmic axis (A–C, E), the value 0.1 means less than 0 per 10^6 CD4⁺ T cells.

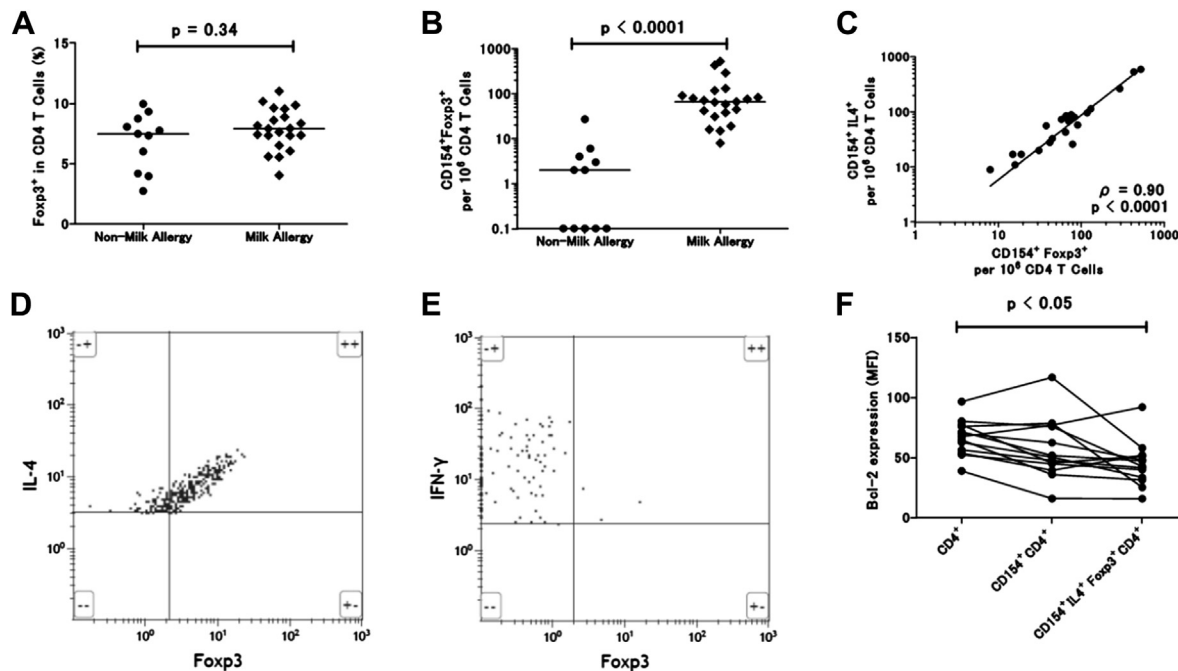


Figure 2. Fcpx3 expression in patients with milk allergy. (A) Percentages of Fcpx3⁺ T cells among unstimulated CD4⁺ T cells and (B) proportions of CD154⁺Fcpx3⁺ T-helper cells. (C) Correlation between CD154⁺IL-4⁺ and CD154⁺Fcpx3⁺ cell frequencies. (D) Representative data for Fcpx3 expression in milk-stimulated CD154⁺ cells and (E) *Mycobacterium tuberculosis*–stimulated CD154⁺ cells. (F) Mean fluorescence intensity (MFI) of Bcl-2 in stimulated CD4⁺ cells, CD154⁺CD4⁺ cells, and CD154⁺IL-4⁺ Fcpx3⁺ CD4⁺ cells. On the logarithmic axis (B), the value 0.1 means less than 0 per 10⁶ CD4⁺ T cells. IFN- γ , interferon- γ ; IL, interleukin.

The numbers of milk allergen-specific CD154⁺IL-4⁺ T-helper cells were strongly correlated with the numbers of CD154⁺Fcpx3⁺ T-helper cells ($\rho = 0.90$, $P < .0001$; Fig 2C). As shown in the representative dataset for a subject with milk allergy (Fig 2D), this strong correlation reflected the coexpression of IL-4 (detected by PerCP/Cy5.5-labeled anti-IL-4) and Fcpx3 in milk allergen-specific CD154⁺ T-helper cells. The *M. tuberculosis*–specific CD154⁺ T-helper cells from the same subject were positive for PerCP/Cy5.5-labeled anti-IFN- γ but were negative for Fcpx3 (Fig 2E). These results indicated that Fcpx3 expression in activated T-helper cells was only in T_H2 cells but not in T_H1 cells. Notably, these results also confirmed that PerCP/Cy5.5-derived fluorescence did not affect Fcpx3 positivity.

Next, expressions of IL-4 and Fcpx3 were analyzed on a sub-cellular level by confocal microscopy to confirm their coexpression. The results showed that a small proportion of CD4⁺ cells coexpressed IL-4 and Fcpx3 in their cytoplasm (Fig 3).

Bcl-2 Expression in CD154⁺IL-4⁺ Fcpx3⁺ T-Helper Cells

To investigate a possible role for Fcpx3 in milk allergen-specific CD154⁺IL-4⁺ T_H2 cells, Bcl-2 expression in CD154⁺IL-4⁺Fcpx3⁺ T-helper cells from 13 subjects with milk allergy were examined because Fcpx3 has been reported to repress the expression of prosurvival Bcl-2 molecules.¹⁹ Bcl-2 expression in CD154⁺IL-4⁺Fcpx3⁺ T-helper cells was significantly lower than that in total CD4 cells (median mean fluorescence intensity values 42.603 and 67.277, respectively, $P < .05$; Fig 2F).

Discussion

In this study, the authors established for the first time a method to analyze milk antigen-specific T_H2 cells by simultaneously detecting CD154 and intracellular cytokines using multicolor flow cytometry. Using this method, the authors confirmed that the milk allergy group had larger numbers of milk allergen-specific IL-4- and IL-5–producing CD4⁺ cells than the control group, as reported

previously for various food allergens, including milk.^{20–24} There also was a fairly definite positive correlation between the numbers of milk allergen-specific IL-4–producing T_H2 cells and milk allergen-specific IgE levels. This correlation could confirm the accuracy of the present analysis because IL-4 is an essential cytokine for the immunoglobulin class switch to IgE.²⁵ Although nearly one half the patients with milk allergy had IFN- γ –producing cells, the ratio of IL-4–producing cells to IFN- γ –producing cells increased in the milk allergy group, suggesting that the T_H2/T_H1 ratio was more important than the absolute numbers of T_H1 cells, as reported previously.²²

Unexpectedly, patients in the milk allergy group had significantly more milk allergen-specific (CD154⁺) IL-10–producing cells than those in the control group. Because antigen-specific IL-10–producing Tr1 cells participate in peripheral tolerance,⁹ true Tr1 cells might not be detected as CD154⁺IL-10⁺. Another possibility was that Tr1 cells play an important role in developing induced tolerance achieved by immunotherapy rather than the natural tolerance of subjects without milk allergy. Changes in IL-10–producing CD154⁺ cells after oral immunotherapy are currently being investigated in the authors' laboratory. Although nearly two thirds of patients with milk allergy and one third of the control group had CD154⁺IL-10⁺ cells, the biological role and pathologic significance of these cells remain to be clarified. One possibility is that IL-10 produced in CD154⁺ cells in patients with milk allergy might help downregulate IFN- γ production.

Subsequently, the authors investigated Fcpx3⁺CD4⁺ T cells. The proportion of Fcpx3⁺CD4⁺ T cells in the unstimulated CD4⁺ T cells was not significantly different between the control and milk allergy samples, suggesting that the nTreg cell number was not decreased in patients with milk allergy. This result was contrary to the findings of Stelmazczyk-Emmel et al²⁶ who reported that the frequency of Tregs in patients with symptoms of atopic dermatitis and/or food allergy was significantly lower, particularly in children with multiorgan involvement. Milk allergy alone might not affect the frequency of nTreg cells. In addition, the control group visited

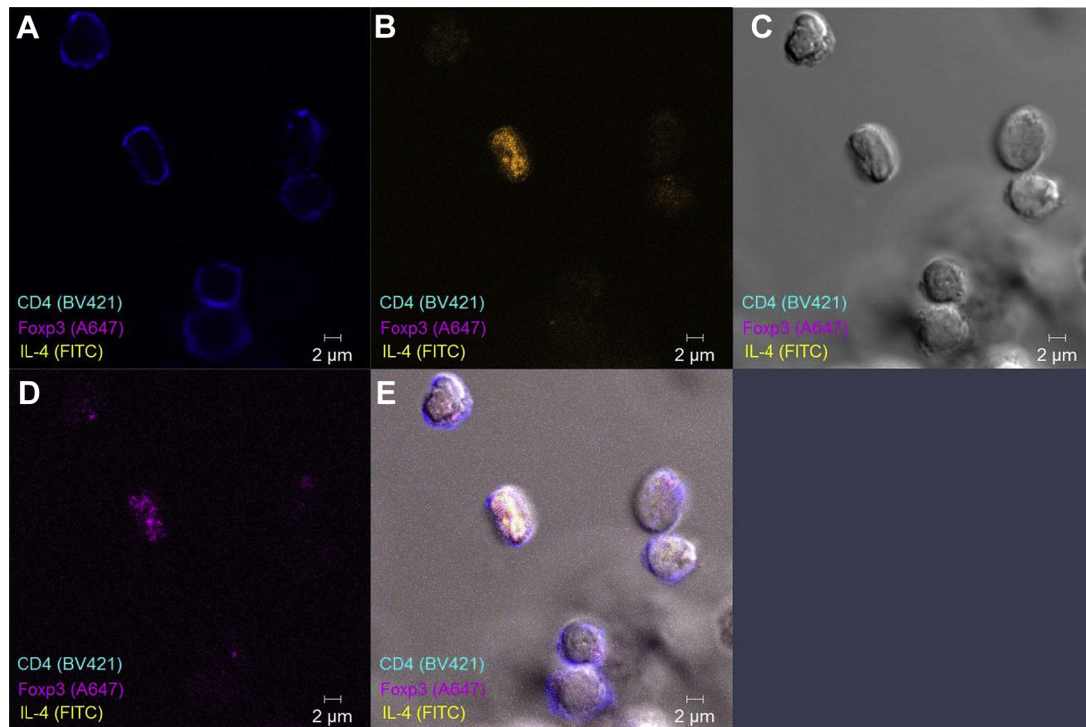


Figure 3. Confocal microscopic analysis of allergen-stimulated peripheral blood mononuclear cells. Representative data from a patient with milk allergy are shown. (A) CD4 was stained with Brilliant Violet 421 conjugated anti-CD4 (clone RPA-T4). (B) Foxp3 was stained with Alexa Fluor 647 conjugated anti-Foxp3 (clone 150D). (C) Nomarski phase-contrast image. (D) Interleukin-4 (IL-4) was stained with fluorescein isothiocyanate (FITC) conjugated anti-IL-4 (clone MP4-25D2). (E) Merged images.

the authors' pediatric departments for allergy screening. Therefore, most had some kind of allergic disease except milk allergy.

In contrast, there were significantly more milk allergen-induced CD154⁺Foxp3⁺ cells in the milk allergy group than in the control group. These results were contrary to the idea that CD154⁺Foxp3⁺ cells are a type of regulatory cells. In addition, Noyan et al.²⁷ recently showed that CD154 was not expressed on naive and activated Treg cells. Rather, the numbers of milk allergen-specific CD154⁺Foxp3⁺ T-helper cells were strongly correlated with the numbers of CD154⁺IL-4⁺ T-helper cells. This strong correlation seemed to reflect the coexpression of IL-4 and Foxp3 in milk allergen-specific CD154⁺ T-helper cells. In contrast to milk allergen-specific CD154⁺ T_H2 cells, *M. tuberculosis*-specific CD154⁺ T_H1 cells were negative for Foxp3, indicating that Foxp3 expression in activated T-helper cells was present only in T_H2 cells but not in T_H1 cells. Because activated human T cells are known to transiently express Foxp3,¹⁴ these results could reflect the activation of allergen-stimulated CD154⁺ cells. Using confocal microscopic examination, coexpression of IL-4 and Foxp3 was confirmed in the cytoplasm of allergen-stimulated CD4⁺ cells. These results are concordant with those of Magg et al.²⁸ who reported that human effector T cells expressed Foxp3 mainly in the cytoplasm at activation, in sharp contrast to nTreg cells, where Foxp3 was predominantly expressed in the nucleus.

Despite clear evidence that Foxp3 was expressed in Tconv cells, its function remains unknown. One possibility is that Foxp3 functions as a proapoptotic protein, as suggested by Tai et al.¹⁹ They reported that Foxp3 repressed the expression of prosurvival Bcl-2 molecules and induced cell death in newly arising murine Treg cells in the thymus if the γ c-dependent survival signals were deficient. The present finding that Bcl-2 expression in the CD154⁺IL-4⁺Foxp3⁺ T-helper cells was decreased compared with that in the total number of CD4 cells could extend this idea; Foxp3 might be involved in the apoptosis of terminally differentiated allergen-specific human T_H2 cells by decreased Bcl-2 levels when repeatedly activated T cells are further stimulated in vitro.²⁹ An

alternative possible role of Foxp3 expression in activated T_H2 cells could be the maintenance of T_H2 lineages through the suppression of IFN- γ production because Foxp3 expression in human T_H17 cells has been reported to suppress IFN- γ production.¹⁸ However, the authors did not find a correlation between the expression of Foxp3 and T-bet,³⁰ the T_H1 master regulator (data not shown). Further investigations will be required to elucidate the actual role of Foxp3 in T_H2 cells.

In conclusion, increased numbers of IL-4-producing allergen-specific T-helper cells were found in patients with milk allergy. In addition, Foxp3 was coexpressed with IL-4 in allergen-specific T_H2 cells from these patients. This coexpression was associated with lower Bcl-2 levels and could contribute to the phenotype and function of T_H2 cells.

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References

- Woodfolk JA. T-cell responses to allergens. *J Allergy Clin Immunol*. 2007;119:280–294.
- Wambre E, James EA, Kwok WW. Characterization of CD4⁺ T cell subsets in allergy. *Curr Opin Immunol*. 2012;24:700–706.
- Frentsch M, Arbach O, Kirchhoff D, et al. Direct access to CD4⁺T cells specific for defined antigens according to CD154 expression. *Nat Med*. 2005;11:1118–1124.
- Campbell JD, Buchmann P, Kesting S, Cunningham CR, Coffman RL, Hessel EM. Allergen-specific T cell responses to immunotherapy monitored by CD154 and intracellular cytokine expression. *Clin Exp Allergy*. 2010;40:1025–1035.
- Smith KA, Gray NJ, Cheek E, et al. Characterisation of CD154⁺ T cells following ex vivo birch allergen stimulation defines a close relationship between T cell subsets in healthy volunteers. *BMC Immunol*. 2013;14:14.
- Nomura T, Tsuge I, Inuo C, et al. Effect of Japanese cedar specific immunotherapy on allergen-specific T(H)2 cells in peripheral blood. *Ann Allergy Asthma Immunol*. 2013;110:380–385.

- [7] Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev*. 2009;229:152–172.
- [8] Akdis M, Verhagen J, Taylor A, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med*. 2004;199:1567–1575.
- [9] Zhang H, Kong H, Zeng X, Guo L, Sun X, He S. Subsets of regulatory T cells and their roles in allergy. *J Transl Med*. 2014;12:125.
- [10] Feuerer M, Hill JA, Mathis D, Benoist C. Foxp3⁺ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol*. 2009;10:689–695.
- [11] Wu Y, Borde M, Heissmeyer V, et al. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*. 2006;126:375–387.
- [12] Bettelli E, Dastrange M, Oukka M. Foxp3 interacts with nuclear factor of activated T cells and NF- κ B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci U S A*. 2005;102:5138–5143.
- [13] Ono M, Yaguchi H, Ohkura N, et al. Foxp3 controls regulatory T cell function by interacting with AML1/Runx1. *Nature*. 2007;446:685–689.
- [14] Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4⁺ T cells. *Eur J Immunol*. 2007;37:129–138.
- [15] Allan SE, Crome SQ, Crellin NK, et al. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol*. 2007;19:345–354.
- [16] Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4⁺FOXP3⁺ T cells by T-cell receptor stimulation is transforming growth factor- β dependent but does not confer a regulatory phenotype. *Blood*. 2007;110:2983–2990.
- [17] Walker MR, Kasprrowicz DJ, Gersuk VH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁺ T cells. *J Clin Invest*. 2003;112:1437–1443.
- [18] McMurchy AN, Gillies J, Gizzi MC, et al. A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood*. 2013;121:1265–1275.
- [19] Tai X, Erman B, Alag A, et al. Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals. *Immunity*. 2013;38:1116–1128.
- [20] Schade RP, Van Ieperen-Van Dijk AG, Van Reijssen FC, et al. Differences in antigen-specific T-cell responses between infants with atopic dermatitis with and without cow's milk allergy: relevance of TH2 cytokines. *J Allergy Clin Immunol*. 2000;106:1155–1162.
- [21] Tiemessen MM, Van Ieperen-Van Dijk AG, Bruijnzeel-Koomen CA, et al. Cow's milk-specific T-cell reactivity of children with and without persistent cow's milk allergy: key role for IL-10. *J Allergy Clin Immunol*. 2004;113:932–939.
- [22] Tsuge I, Kondo Y, Tokuda R, et al. Allergen-specific helper T cell response in patients with cow's milk allergy: Simultaneous analysis of proliferation and cytokine production by carboxyfluorescein succinimidyl ester dilution assay. *Clin Exp Allergy*. 2006;36:1538–1545.
- [23] Sicherer SH, Wood RA, Stablein D, et al. Immunologic features of infants with milk or egg allergy enrolled in an observational study (Consortium of Food Allergy Research) of food allergy. *J Allergy Clin Immunol*. 2010;125:1077–1083.
- [24] Michaud B, Aroulandom J, Baiz N, et al. Casein-specific IL-4- and IL-13-secreting T cells: a tool to implement diagnosis of cow's milk allergy. *Allergy*. 2014;69:1473–1480.
- [25] Kühn R, Rajewsky K, Müller W. Generation and analysis of interleukin-4 deficient mice. *Science*. 1991;254:707–710.
- [26] Stelmaszczyk-Emmel A, Zawadzka-Krajewska A, Szypowska A, Kulus M, Demkow U. Frequency and activation of CD4⁺CD25⁺ FoxP3⁺ regulatory T cells in peripheral blood from children with atopic allergy. *Int Arch Allergy Immunol*. 2013;162:16–24.
- [27] Noyan F, Lee YS, Zimmermann K, et al. Isolation of human antigen-specific regulatory T cells with high suppressive function. *Eur J Immunol*. 2014;44:2592–2602.
- [28] Magg T, Mannert J, Ellwart JW, Schmid I, Albert MH. Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells. *Eur J Immunol*. 2012;42:1627–1638.
- [29] Hayashi N, Liu D, Min B, Ben-Sasson SZ, Paul WE. Antigen challenge leads to in vivo activation and elimination of highly polarized TH1 memory T cells. *Proc Natl Acad Sci U S A*. 2002;99:6187–6191.
- [30] O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4⁺ T cells. *Science*. 2010;327:1098–1102.