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Effect of Japanese cedar specific immunotherapy on allergen-specific T_H2 cells in peripheral blood

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ABSTRACT

Background: The involvement of a shift from T_H2 to T_H1 responses in peripheral blood in pollen subcutaneous immunotherapy (SCIT) has been contentious, partly because of difficulties analyzing antigen-specific T_H cells.

Objectives: To use recent technical advances to establish a more direct and simple method to analyze antigen-specific T_H cells and to clarify the involvement of a T_H2/T_H1 shift in peripheral blood in pollen specific immunotherapy.

Methods: After short-term (6-hour) antigen stimulation, antigen-specific T_H cells in peripheral blood of Japanese children and young adults with Japanese cedar pollinosis undergoing SCIT were analyzed by multicolor flow cytometry for the presence of the activation marker CD154 and intracellular cytokines.

Results: Twenty-eight patients between 5 and 22 years of age were enrolled in the study; 22 had started SCIT after enrolling in the study (SCIT group), and the remaining 6 were planning to start SCIT in the next off-season (control group). The number of Japanese cedar-specific interleukin (IL) 5–, IL-4–, interferon γ –, IL-17A–, IL-10–, and tumor necrosis factor α –producing T_H cells without antigen-driven cell proliferation was determined. The seasonal increase in the number of Japanese cedar-specific IL-5– and IL-4–producing T_H cells seen in the control group was suppressed in the SCIT group ($P < .005$ and $< .001$, respectively).

Conclusion: We report a powerful method for the analysis of antigen-specific T_H cells in peripheral blood. This method will contribute to our understanding of immune mechanisms of immunotherapy and help us develop more sophisticated allergen specific immunotherapy.

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Introduction

Allergen specific immunotherapy (SIT) is the only method that can modify the course of allergic diseases.¹ Subcutaneous immunotherapy (SCIT) for seasonal allergic rhinitis reduces seasonal symptoms and the use of rescue medications.² Furthermore, SCIT prevents new allergen sensitization and progression from allergic rhinitis to bronchial asthma in children.^{3–6}

How SCIT improves allergic rhinitis is not fully understood. Several recent studies highlight the complexity of allergic immunity in pollen SCIT,^{7,8} such as a reduction in the infiltration of

effector cells into local sites, transient increases and subsequent decreases in antigen-specific IgE, induction of antigen-specific IgG4, and reductions in the T_H2/T_H1 ratio in local tissues.⁹ Interleukin (IL) 10–producing regulatory T (Treg) cells were detected in patients receiving SCIT.¹⁰ The involvement of a reduction in the T_H2/T_H1 ratio in peripheral blood remains contentious: although some early studies revealed a shift in the T_H2/T_H1 ratio in peripheral blood,^{11–13} another well-controlled study failed to confirm these findings.⁹

Analysis of antigen-specific T_H cells is technically difficult¹⁴ because of their low frequency (ie, 10^{-4} to 10^{-6} of total T_H cells), and in the past antigen-driven cell proliferation has been used to identify these rare cells. However, in vitro cell proliferation may modify the nature of the cells^{15–17} and may result in bystander cell proliferation. In addition, not all antigen-specific T_H cells may proliferate after antigen stimulation. Recent advances have enabled us to overcome these problems in identifying antigen-specific T_H cells by using techniques such as detection of the activation marker

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CD154¹⁸ and the peptide–major histocompatibility complex (MHC) class II tetramer.¹⁹ In addition, developments in multicolor flow cytometry allow for the simultaneous analysis of more than 10 fluorescence-conjugated monoclonal antibodies,²⁰ enabling the detection of a variety of cell surface markers and the cytokines produced.²¹

In the present study, we established a more direct and simple method of analyzing antigen-specific T_H cells that involves the simultaneous detection of CD154 and intracellular cytokines using multicolor flow cytometry, and determined the effects of SCIT in patients with Japanese cedar pollinosis, the most common seasonal allergic rhinitis in Japan that develops from late February to late April.²²

Methods

Patients and Study Design

Between June 2011 and January 2012, Japanese children and young adults with Japanese cedar pollinosis were invited to take part in the present study, which was conducted at Fujita Health University Hospital and The Second Teaching Hospital, Fujita Health University. A diagnosis of Japanese cedar pollinosis was made on the basis of the presence of typical seasonal allergic symptoms accompanied by Japanese cedar sensitization, as determined by the ImmunoCAP test (Phadia AB, Uppsala, Sweden). The SCIT protocol usually starts with a 2-week schedule of rush immunotherapy during the summer,²³ followed by a maintenance schedule of monthly injections of a standard 0.1 mL of 2,000 Japanese allergy units (JAU)/mL of standardized Japanese cedar extract (Torii Pharmaceutical, Tokyo, Japan). In some patients, the standard maintenance schedule of 0.1-mL injections could not be tolerated, and the injection volume was reduced to achieve tolerable doses.

The patients were divided into 2 groups (Fig 1): (1) an SCIT group comprising patients who started SCIT after enrolling in the study and (2) a control group comprising patients who were planning to start SCIT in the next off-season. The sampling points were as follows: point 1, before the start of SCIT between late June and late August; point 2, before seasonal pollen exposure between late November and early February; and point 3, after seasonal pollen exposure between late March and early June. Samples were obtained from patients in the SCIT group at all 3 points, whereas samples were obtained from the control group at points 2 and 3 only.

The present study was approved by the Research Ethics Committee of Fujita Health University. All patients or their parents provided written informed consent.

Peripheral Blood Mononuclear Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from 5- to 10-mL samples of heparinized blood using Lymphocyte Separation Medium (MP Biomedicals, Solon, Ohio) and were frozen in RPMI-1640 containing 12.5% human serum albumin (Gemini Bio-Products, Woodland, California) with 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, Missouri) according to the optimal cryopreservation protocol for intracellular cytokine staining.²⁴ To minimize experimental error, samples from the same individual were thawed on the same day. Therefore, samples were stored in liquid nitrogen for up to 12 months. Viability was evaluated using Trypan blue solution (Sigma-Aldrich), with mean viability at points 1, 2, and 3 before cryopreservation of 99.6%, 99.4%, and 99.1%, respectively, compared with 98.3%, 98.5%, and 98.5%, respectively, after thawing. Thawed PBMCs were resuspended at a concentration of 5×10^6 cells/mL in RPMI-1640 containing 10% autologous plasma and rested in an incubator overnight at 37°C under 5% carbon dioxide.

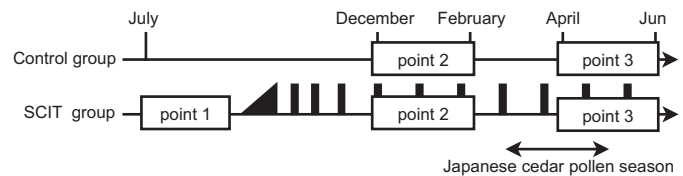


Figure 1. Study overview. Solid marks represent injections of Japanese cedar extract for subcutaneous immunotherapy (SCIT).

Antigen Stimulation and Intracellular Cytokine Staining

Antigen stimulation and intracellular cytokine staining were performed according to the intracellular cytokine staining protocol.²⁵ Briefly, 400 μ L of thawed PBMCs supplemented with anti-CD28 (1 μ g/mL; BD Biosciences, San Jose, California) were incubated with Japanese cedar antigen (600 μ g/mL) or heat-killed *Mycobacterium tuberculosis*, strain H37 Ra (100 μ g/mL; BD Biosciences) for 6 hour. After 2-hour incubation, Brefeldin A (10 μ g/mL; Sigma-Aldrich) was added to the samples. After the samples had been incubated for the full 6 hour, they were stained with ethidium monoazide bromide (EMA; Invitrogen, Carlsbad, California), fixed with IC Fixation Buffer (eBioscience, San Diego, California), permeabilized with IC Permeabilization Buffer (eBioscience), and stained with the fluorescence-conjugated antibodies. The medium control consisted of samples treated in an identical manner except that the antigen was omitted. Japanese cedar antigen was extracted from Japanese cedar pollen using ammonium bicarbonate. The endotoxin level of the final concentration of the extract used in culture, as determined by the limulus assay (endotoxin single test; Wako Pure Chemical Industries, Osaka, Japan), was 1.0 pg/mL.

Multicolor Flow Cytometric Analyses

The 9-color panel consisted of the following: fluorescein isothiocyanate–conjugated anti-CD154 (clone TRAP1) and allophycocyanin–conjugated anti-IL-10 (clone JES3-19F1; both from BD Biosciences); phycoerythrin–conjugated anti-IL-5 (clone JES1-39D10), peridinin chlorophyll protein/Cy5.5–conjugated anti-IL-17A (clone BL168), phycoerythrin/Cy7–conjugated anti-IL-4 (clone MP4-25D2), and allophycocyanin/Cy7–conjugated anti-interferon γ (IFN- γ) (clone 4S.B3; all from BioLegend, San Diego, California); and Pacific Blue–conjugated anti-tumor necrosis factor α (TNF- α) (clone MP9-20A4), Pacific Orange–conjugated anti-CD4 (clone S3.5), and EMA (all from Invitrogen). The stained samples were acquired on a Gallios multicolor flow cytometer (Beckman Coulter, Brea, California) and were analyzed using Kaluza software (Beckman Coulter). Cell doublets were excluded by using the forward scatter integral vs forward scatter peak parameter (Fig 2A). Viable CD4⁺ lymphocytes were identified by gating on CD4 vs side scatter integral parameter (Fig 2B) and then by negative EMA gating (Fig 2C). A total of 100,000 to 290,000 (median, 210,000) viable CD4⁺ lymphocytes was acquired in each sample. The proportion of CD154⁺ cytokine-positive cells in the viable CD4⁺ lymphocyte population was determined (Fig 2D–I). Although the spectral overlap between fluorescent dyes was of concern, a fluorescence-minus-one control²⁰ showed minimal background staining with this panel (data not shown). The number of antigen-specific cytokine-producing T_H cells was determined by subtracting medium control values, with negative values taken as zero.

Statistical Analysis

Two independent groups of data were analyzed using the Mann-Whitney *U* test. Paired data from 2 or 3 groups were compared using the Wilcoxon matched-pairs signed-rank test or the

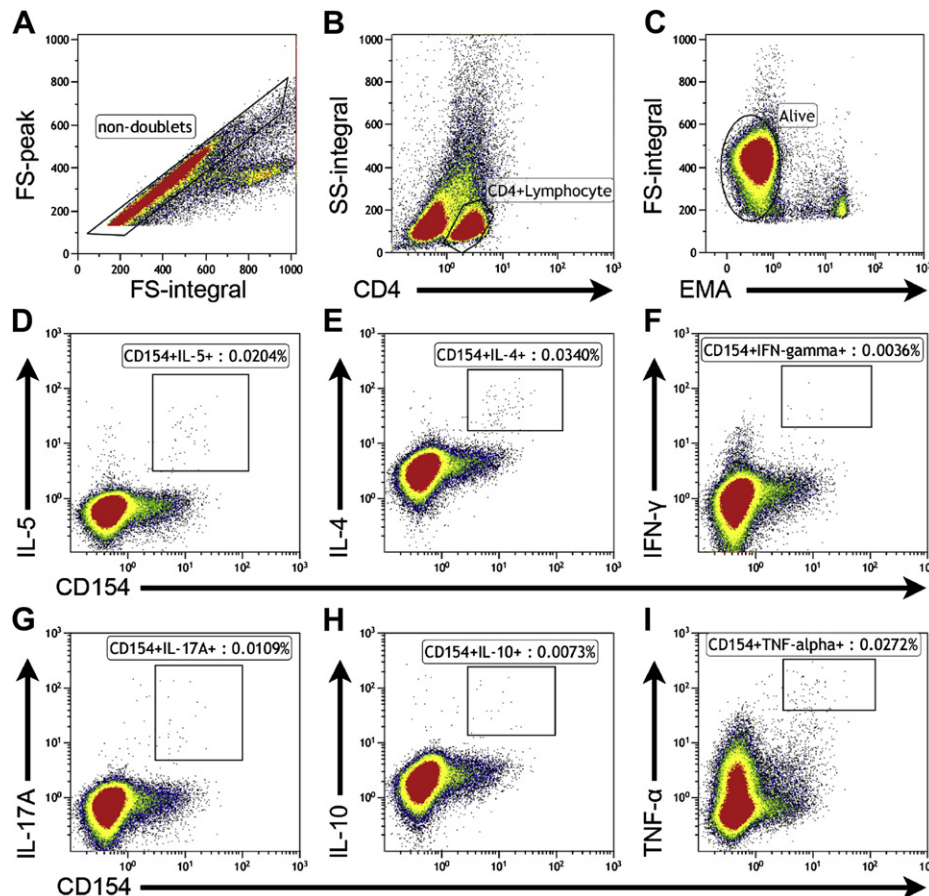


Figure 2. Detection of cytokine-producing T_H cells by multicolor flow cytometry after short-term (6-hour) stimulation with Japanese cedar antigen. A–C, Gating of viable $CD4^+$ lymphocytes. D–I, Six types of cytokine-producing T_H cells are detected simultaneously. The percentage of $CD154^+$ cytokine-positive T_H cells represents the proportion of viable $CD4^+$ lymphocytes. FS indicates forward scatter; SS, side scatter; EMA, ethidium monoazide bromide.

Friedman test with the Dunn multiple comparison posttest. Correlations were assessed by the Spearman rank correlation coefficient. $P < .05$ was considered significant. Statistical analyses were performed using Prism 5.0d (GraphPad Software, San Diego, California).

Results

Twenty-eight patients between 5 and 22 years of age were enrolled in the present study. No significant differences were found in background, age, Japanese cedar specific IgE, and total IgE between the SCIT ($n = 22$) and control ($n = 6$) groups (Table 1). In 1 patient, a conventional dose-increasing schedule was used for 2 months until the maintenance dose was reached, whereas in

another 2 patients a cluster dose-increasing schedule was used for 1 month to reach the maintenance dose.²³ They were not clinically diagnosed as having immunodeficiency.

Japanese Cedar-Specific T_H cells During the Pollen Season

The number of Japanese cedar-specific cytokine-producing T_H cells was evaluated at point 3. Patients in the control group had greater numbers of IL-5-, IL-4-, and TNF- α -producing cells than patients in the SCIT group ($P < .005$, $< .001$, and $< .05$, respectively; Fig 3A). We used *Mycobacterium tuberculosis*-specific T_H cells, which mainly produce IFN- γ and TNF- α , as a positive control because all Japanese people are immunized with the BCG vaccine during early childhood. No significant differences were found in the number of *M tuberculosis*-specific T_H cells between the SCIT and control groups at point 3 (Fig 3B).

We also investigated the correlation between the number of Japanese cedar-specific cytokine-producing T_H cells and Japanese cedar-specific IgE in the same sample obtained at point 3 in all patients, regardless of SCIT. The results indicate a weak correlation between the number of Japanese cedar-specific IL-5- and IL-4-producing cells and Japanese cedar-specific IgE ($r_s = 0.39$, $P < .05$; and $r_s = 0.41$, $P < .05$, respectively; eFig 1).

Effects of Seasonal Japanese Cedar Pollen Exposure on Japanese Cedar-Specific T_H Cells

The effect of seasonal pollen exposure on antigen-specific T_H cells was investigated in samples obtained at points 2 and 3 from the control group. The number of Japanese cedar-specific

Table 1
Summary of Patient Characteristics^a

Characteristic	SCIT group ($n = 22$)	Control group ($n = 6$)	P value
Sex (female/male)	5/17	3/3	
Age, median (range), y	10.5 (7–14)	12.5 (11–14.5)	.11
Japanese cedar-specific IgE, median (range), IU/mL	70.7 (24.5–132.8)	71.9 (39.4–222.5)	.54
Total IgE, median (range), IU/mL	452.5 (262.5–1230)	741.5 (248.3–1,174)	.82

Abbreviation: SCIT, subcutaneous immunotherapy.

^aThe SCIT group contained patients who started subcutaneous immunotherapy after enrollment in the study; the control group contained patients who did not undergo SCIT.

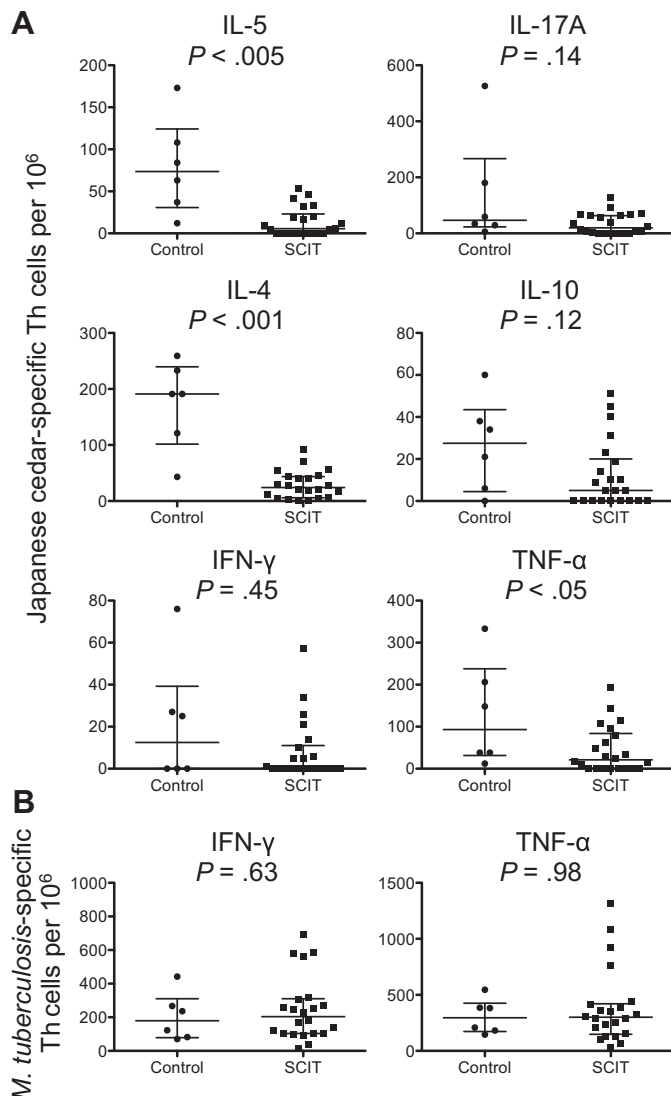


Figure 3. Antigen-specific cytokine-producing T_H cells in the control and subcutaneous immunotherapy (SCIT) groups during the Japanese cedar pollen season. The number of Japanese cedar–(A) and *Mycobacterium tuberculosis*–specific (B) cytokine-producing T_H cells.

IL-5– and IL-4–producing T_H cells increased significantly in all patients in the control group from point 2 to point 3 ($P < .05$ for all; Fig 4A). In the SCIT group, the number of antigen-specific T_H cells was followed from point 1 to point 3. A significant decrease was noted in the number of Japanese cedar–specific IL-5–producing T_H cells at point 2 ($P < .01$, posttest), and there was no increase in the number of either IL-5– or IL-4–producing T_H cells at point 3, despite seasonal pollen exposure (Fig 4A). Conversely, neither seasonal Japanese cedar pollen exposure nor Japanese cedar–specific SCIT had any effect on the number of *M. tuberculosis*–specific IFN- γ – and TNF- α –producing T_H cells (Fig 4B).

Effects of SCIT on Seasonal Increases in Japanese Cedar–Specific T_H Cells

To determine the effects of SCIT more directly, we assessed changes in antigen-specific T_H cells from point 2 to point 3. There was an increase in the number of Japanese cedar–specific IL-5–, IL-4–, and IL-17A–producing T_H cells in the control group from point 2 to point 3, which was suppressed in the SCIT group ($P < .005$, $< .001$, and $< .05$, respectively; eFig 2A). However, no significant differences

were found in the number of Japanese cedar–specific T_H cells between the 2 groups at point 2 (data not shown). In addition, there were no significant differences in the number of *M. tuberculosis*–specific IFN- γ – and TNF- α –producing T_H cells between the SCIT and control groups (eFig 2B).

Discussion

In the present study, the number of Japanese cedar–specific IL-5–, IL-4–, and TNF- α –producing T_H cells was lower in the SCIT group than in the control group during the Japanese cedar pollen season. Furthermore, seasonal Japanese cedar pollen exposure increased the number of Japanese cedar–specific IL-5– and IL-4–producing T_H cells in patients who did not undergo SCIT. However, in patients who did undergo SCIT, the seasonal increase in the number of Japanese cedar–specific IL-5–, IL-4–, and IL-17A–producing T_H cells after pollen exposure was suppressed. These findings indicate that Japanese cedar–specific SCIT shifted the Japanese cedar–specific T_H cell subtype from T_H2 to T_H1 by reducing levels of Japanese cedar–specific T_H2 clones in peripheral blood.

Although a shift from the T_H2 to T_H1 subtype in peripheral blood was not thought to play an important role in pollen SCIT, recent studies using more sensitive methods have demonstrated its involvement. For example, based on the detection of CD154 and intracellular cytokines, Campbell et al²⁶ found that ragweed SIT with Amb a 1 immunostimulatory oligodeoxynucleotide conjugate affected antigen-specific T_H2 cytokine-producing T_H cells in peripheral blood. In another study, using peptide-MHC class II tetramer with multicolor flow cytometry, Wambre et al²⁷ reported that alder pollen allergy patients who had undergone SCIT had fewer allergen-specific T_H2 cytokine-producing T_H cells in peripheral blood than patients who had not undergone SCIT. On the other hand, Bonvalet et al failed to show the involvement of a T_H2/T_H1 shift in peripheral blood at an early stage of sublingual immunotherapy by using peptide-MHC class II tetramer.²⁸ This consequence may be attributed to the difference of the antigen-dosing routes.

With recent technical advances, analysis of antigen-specific T_H cells has shifted from bulk culture analysis to the analysis of single cells.¹⁴ Although the peptide-MHC class II tetramer¹⁹ can be used to detect antigen-specific T_H cells correctly, there are some limitations to its use. Indeed, this method requires the examination of MHC class II and predominant epitopes in each individual. Furthermore, Wambre et al²⁷ recently reported the need for cell proliferation before the analysis of intracellular cytokines of MHC class II tetramer–positive T cells because the number of antigen-specific T_H cells that can be detected using the peptide-MHC class II tetramer method is estimated to be 10-fold lower than that detected using the activation marker CD154.^{29,30}

A major limitation of the present study is the question of whether we were able to detect all antigen-specific T_H cells. Although IL-10–producing Treg cells are thought to play an important role in the establishment of tolerance in pollen SCIT,^{10,31–33} we did not find any evidence of their involvement. Although the first report regarding the usefulness of CD154¹⁸ confirmed its sensitivity in an experimental model, a small population of cells, such as IL-10–producing Treg cells, may not be detected correctly. The time course of CD154 expression on IL-10–producing Treg cells also may differ from that of effector T_H cells. Indeed, Litjens et al³⁴ reported that CD154 expression of FOXP⁺ natural Treg cells was detectable 6 hours after stimulation, reaching maximal levels after 24 hours. Another limitation is its relatively small sample size. A larger verification study will be needed to show the effect on a T_H2/T_H1 shift and may shed light on the role of other T_H cell subsets in allergen SIT.

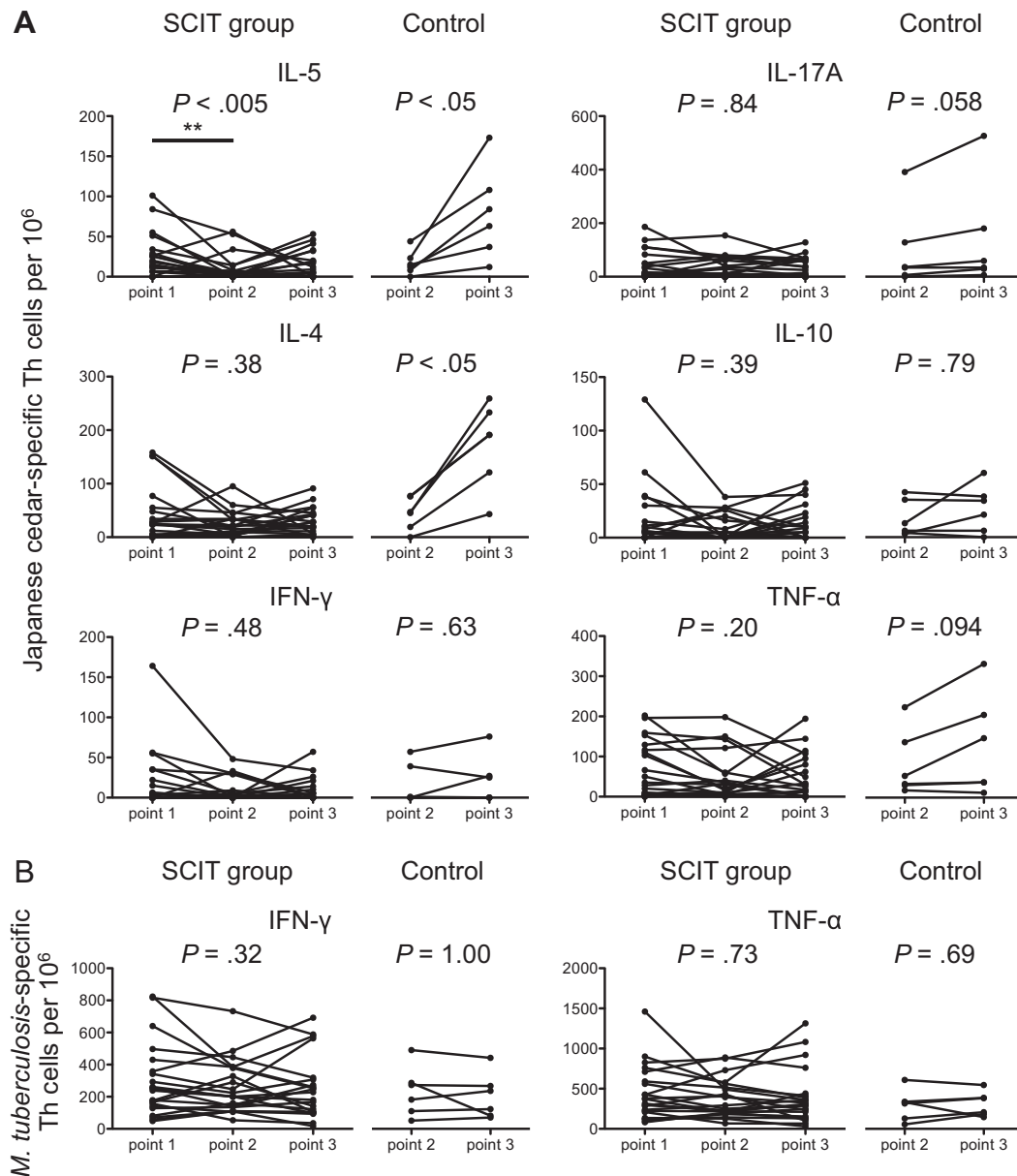


Figure 4. Effects of Japanese cedar-specific subcutaneous immunotherapy (SCIT) and seasonal Japanese cedar pollen exposure on antigen-specific cytokine-producing T_H cells in the SCIT and control groups. The number of Japanese cedar-(A) and *Mycobacterium tuberculosis*-specific (B) cytokine-producing T_H cells. ** $P < .01$.

Despite these limitations, there are 2 benefits of the present study. First, this study is the first to reveal the involvement of a T_H2/T_H1 shift in peripheral blood following pollen SCIT in children and young adults. Because the modification of the natural course of allergy by pollen SCIT has been reported in trials performed in children,^{3–6} we need to determine the mechanism of action of pollen SCIT in children, which may differ from that in adults. Second, the method used in the present study is the simplest reported to date for the analysis of the major T_H cell subtypes after pollen SCIT and does not require cell proliferation of antigen-specific T_H cells. This method will enable us to investigate immunologic mechanisms in large SIT trials without the restrictions associated with MHC class II determination in individuals, irrespective of the allergen responsible.

In conclusion, the present study describes a powerful method for the analysis of allergen-specific T_H cells in peripheral blood. Use of this method to monitor allergen SIT will provide new

insights into the central mechanisms of complex allergic immunity and help us develop more sophisticated allergen SIT.

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2013.02.015>

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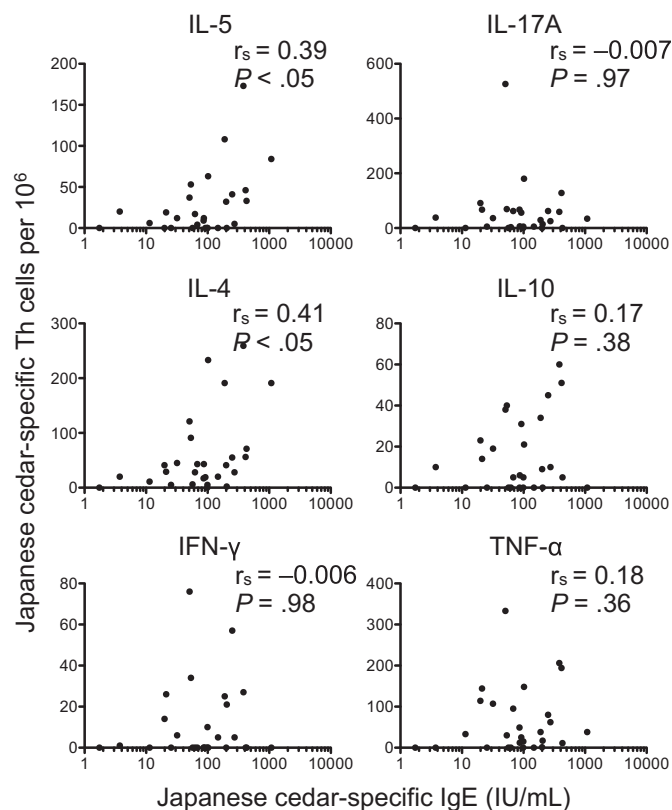


Figure 1. Correlation between Japanese cedar-specific cytokine-producing T_H cells and Japanese cedar-specific IgE during the Japanese cedar pollen season.

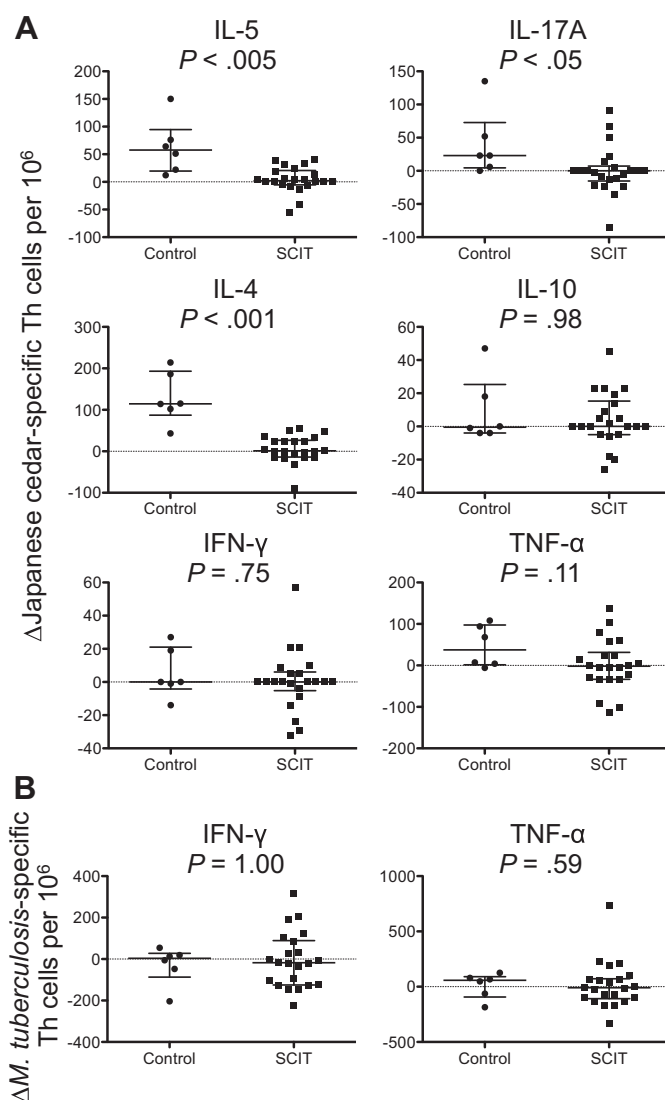


Figure 2. Changes in the number of antigen-specific cytokine-producing T_H cells around the time of seasonal Japanese cedar pollen exposure. Changes in the number of Japanese cedar–(A) and *Mycobacterium tuberculosis*–specific (B) cytokine-producing T_H cells in the control and subcutaneous immunotherapy (SCIT) groups.