

Letter to the Editor

Induction of IL-10-producing regulatory T cells with TCR diversity by epitope-specific immunotherapy in pollinosis

To the Editor:

Specific peptide-based allergen immunotherapy is currently being used for several allergic diseases. Immunotherapy for Japanese cedar pollinosis was undertaken using sublingual application of a pool of pollen peptides containing 7 T-cell epitopes from Cry j 1 and Cry j 2, which are the major Japanese

cedar pollen allergens, and its clinical efficacy on seasonal allergic rhinitis was evaluated.¹ Interestingly, some patients undergoing immunotherapy showed reduced sensitivity to other allergens in addition to Japanese cedar pollen. Allergen-specific regulatory T cells have been suggested to play a role in this immunoprotection,²⁻⁵ but the precise mechanism is not fully understood.

IL-10-producing regulatory T cell (Tr1) is one type of acquired regulatory T cells with unclear characteristic phenotypes. In the current study, the Tr1 population percentage was compared

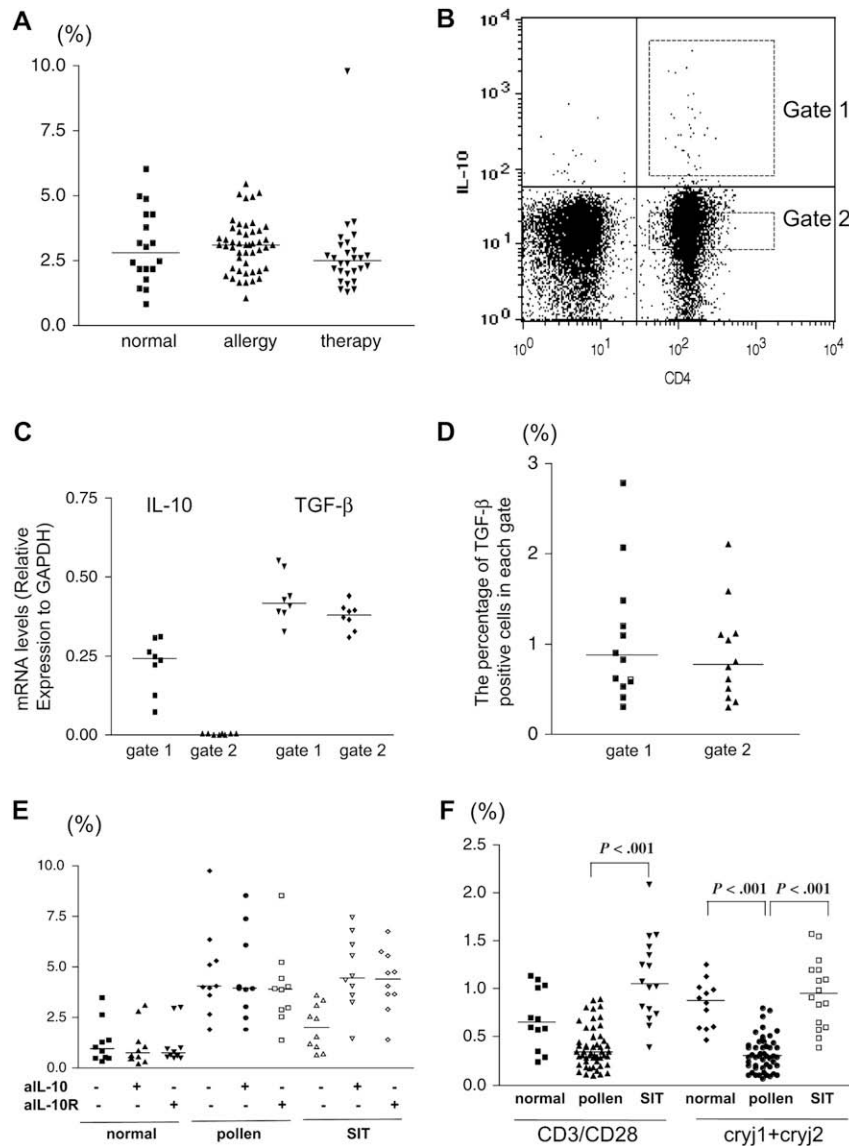


FIG 1. A, The percentage of forkhead box P3⁺CD25^{high}CD4⁺T-cells (naturally occurring regulatory T cells) was unchanged in patients with pollinosis and in those undergoing SIT compared with controls. B, Tr1 (gate 1) and non-Tr1 (gate 2) cells are gated. C, Tr1 cells show equivalent expression of TGF-β mRNA compared to non-Tr1 cells, different from that of IL-10. D, When cells were costained with anti-TGF-β antibody, the percentage of TGF-β-positive cells was similar between Tr1 (gate 1) and non-Tr1 (gate 2). E, CD4⁺ lymphocyte proliferation was significantly suppressed in PBMCs from patients who received SIT compared with patients with pollinosis. This suppressive function was abolished by the addition of anti-IL-10 neutralizing antibody or anti-IL-10 receptor antibody. F, The percentage of Tr1 cells was significantly decreased in patients with untreated pollinosis compared with normal controls and increased in patients receiving SIT.

TCR CDR3 spectratyping BV2~30

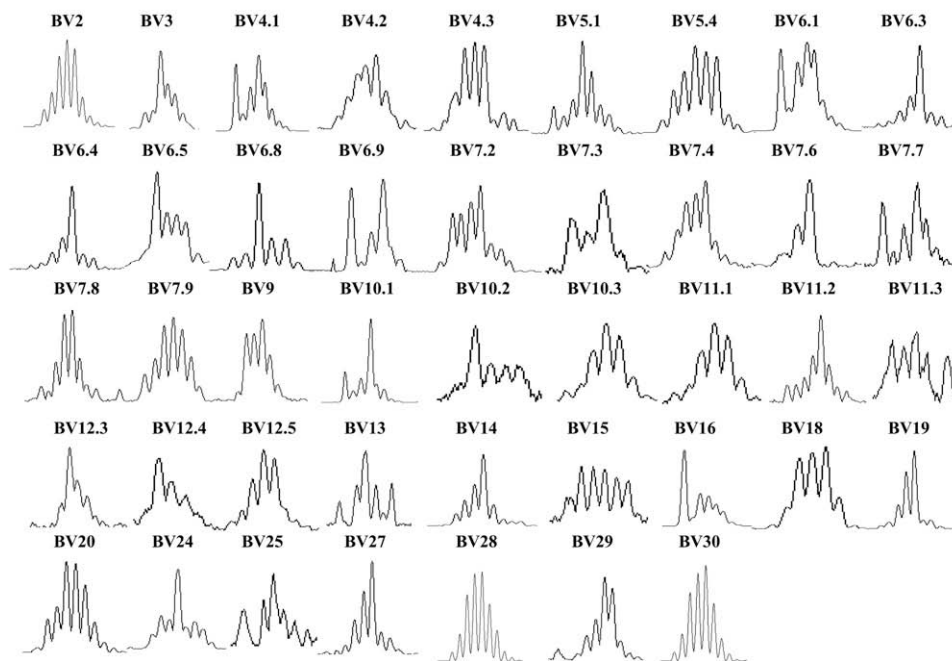


FIG 2. T-cell receptor complementarity-determining region 3 (CDR3) size spectratyping analysis of a Tr1 population from patients undergoing immunotherapy. A Gaussian distribution of CDR3 lengths was observed in many BV subfamilies, except for oligoclonal expansion in some BV subfamilies, indicating preservation of a highly diverse T-cell receptor repertoire.

among untreated patients with pollen allergy, patients undergoing immunotherapy, and healthy subjects. The results showed that the number of Tr1 cells was significantly increased in patients receiving immunotherapy compared with the other groups. In addition, the diversity of the T-cell receptor repertoire in the Tr1 population from patients undergoing immunotherapy was investigated.

Seventy-five patients from the Departments of Dermatology and Otorhinolaryngology of Mie University Hospital were enrolled (15 patients with pollen allergy receiving sublingual immunotherapy [SIT] as described, and 60 sensitive to Japanese cedar). Thirteen healthy controls were also recruited (see details in this article's [Table E1](#) in the Online Repository at www.jacionline.org). Diagnosis was based on clinical symptoms and serological results. Blood was sampled after obtaining written informed consent from all subjects, and the investigational protocol was approved by the Institutional Review Board of Mie University Hospital. Healthy volunteers had no history or subjective symptoms of atopic dermatitis or pollen allergy.

Recent studies have shown a significant decrease of forkhead box P3⁺CD25^{high}CD4⁺T cells, naturally occurring regulatory T cells in symptomatic patients with atopic dermatitis or bronchial asthma.⁶ However, the percentage of naturally occurring regulatory T cells (forkhead box P3⁺CD25^{high}CD4⁺T cells/CD4⁺T cells) remained unchanged in patients with pollinosis and in those receiving SIT compared with controls ([Fig 1, A](#)). In the current study, the IL-10-producing Tr1 cell population was characterized by flow cytometry and cell sorting. PBMCs from patients and normal donors were isolated from heparinized venous blood by density gradient centrifugation using Ficoll (for antibodies and reagents, see Methods in this article's Online Repository).

PBMCs were cultured in RPMI 1640 medium with L-glutamine supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, and 10% human type AB serum. These cells were stimulated with 100 ng/mL soluble anti-CD3 Ab plus 100 ng/mL anti-CD28 Ab, or 50IU Cry j 1 and Cry j 2, for 8 hours. After culture, cells were collected and incubated with anti-CD4-fluorescein isothiocyanate and phycoerythrin-conjugated IL-10 secretion antibody. Immunophenotypic analysis was performed by using a Becton Dickinson FACScan instrument and cell sorting by using a FACS Aria cell sorter (Becton Dickinson, Mansfield, Mass). The Tr1 population was gated (gate 1) as shown in [Fig 1, B](#). When PBMCs were stimulated with CD3/CD28 antibodies, these 2 sorted populations showed similar expression of TGF- β compared with non-Tr1 cells (gate 2) on the basis of quantitative PCR or intracellular staining with anti-TGF- β antibody in normal controls ([Fig 1, C and D](#)). Subsequently, cellular proliferation was evaluated. PBMCs (10^6 /mL) were first labeled with 10 μ mol/L Carboxyfluorescein Succinimidyl Ester and stimulated with Cry j 1 and Cry j 2 as discussed. After 4 days of culture, PBMCs were stained with Peridinin-chlorophyll-protein complex-conjugated anti-CD4 antibody, and the percentage of CD4⁺ lymphocyte proliferation was measured. The proliferation was significantly suppressed in patients receiving SIT compared with patients with pollen allergy ([Fig 1, E](#)). This suppressive effect was neutralized by the addition of 2 μ g/mL anti-IL-10 antibody or anti-IL-10 receptor antibody, confirming that the suppression of reactivity against cedar pollen in patients receiving SIT is IL-10-dependent (for the proliferation assay, see Methods in this article's Online Repository). The percentage of circulating Tr1 cells (IL-10⁺CD4⁺T cells/CD4⁺T cells) was significantly decreased in untreated patients with allergy compared with

normal controls, and the level was increased in patients receiving SIT compared with untreated patients with allergy (Fig 1, *F*). This elevation of Tr1 cells in SIT-treated patients was observed when PBMCs were stimulated not only with Cry j 1 and Cry j 2 but also with CD3/CD28 antibodies, and this may be 1 reason why some patients undergoing immunotherapy for Japanese cedar pollen showed reduced sensitivity to other allergens. We also performed phenotypic characterization of the identified Tr1 cells by flow cytometry; interestingly, Tr1 cells showed more skin and gut-homing tendency than the non-Tr1 population (see this article's Fig E1 on the Online Repository at www.jacionline.org).

We next performed complementarity-determining region 3 size spectratyping analysis by using established β -variable primers to investigate the diversity of the T-cell receptor repertoire in the Tr1 populations expanded or changed by immunotherapy.^{7,8} Total RNA was extracted from the sorted Tr1 population, and cDNA was prepared as previously reported.^{7,8} Fig 2 shows a representative spectratype from Tr1 cells taken from SIT-treated patients. Although the Tr1 population percentage was increased, a Gaussian distribution of complementarity-determining region 3 lengths could be observed in many BV subfamilies examined, except for oligoclonal expansion (2-4 peaks in spectratyping) in some BV subfamilies, indicating preservation of a highly diverse T-cell receptor repertoire (see this article's Fig E2 in the Online Repository at www.jacionline.org for spectratypes from other population).

Tr1 cells can be generated in the presence of locally produced IL-10 released from Tr1 cells.⁴ Tr1 cells block cellular proliferation not via cell-to-cell contact but via cytokine-mediated mechanisms (eg, IL-10). The results of the current study may explain the mechanism of suppression against multiple allergens induced by epitope-specific immunotherapy in patients with pollinosis. Specific immunotherapy blocks the allergen specific IgE and induces an allergen-specific IgG response, which is induced by IL-10 from Tr1 cells.^{2,3}

In conclusion, IL-10-producing Tr1 cells induced by specific immunotherapy play multifunctional roles in suppressing allergic reactions. We have shown here that patients undergoing specific immunotherapy have increased IL-10-producing Tr1 cell populations with high T-cell repertoire diversity, suggesting that this therapy induces tolerance to multiple allergens.

We thank Professor Peter D. Wagner, Division of Physiology, Department of Medicine, University of California, San Diego, for reviewing this article.

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Disclosure of potential conflict of interest: A. Yuta receives grants for scientific research from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare. K. Okubo is a medical advisor for Kyowa Hakkou Kirin, Zeria Shinyaku, and Meiji Nyugyo and receives grant support from the Ministry of Health, Welfare, and Labor. The rest of the authors have declared that they have no conflict of interest.

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doi:10.1016/j.jaci.2009.06.020

METHODS

Antibodies and reagents

Ficoll was purchased from Sigma (St Louis, Mo), and the IL-10 secretion assay kit was from Miltenyi Biotec (Auburn, Calif). Cell surface staining buffer contains 0.1 mol/L PBS and 2% FCS (Biowest, Nuaille, France). mAbs to CD4-fluorescein isothiocyanate, CD25-phycoerythrin, and forkhead box P3-phycoerythrin-Cy5 were purchased from eBioscience (San Diego, Calif). Antibody against TGF- β -Peridinin-chlorophyll-protein complex was purchased from R&D (Minneapolis, Minn). The cells were stimulated with 100 ng/mL soluble anti-CD3 plus 100 ng/mL soluble anti-CD28 (BD Biosciences, San Jose, Calif), or 50 IU Cry j 1 and Cry j 2 (Torii, Tokyo, Japan). Cells were cultured in a final volume of 200 μ L RPMI 1640 medium with L-glutamine supplemented with 5 mmol/L HEPES, 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen, Carlsbad, Calif), and 10% FCS.

Real-time quantitative PCR

RNA was isolated by using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Total RNA 1 μ g (A260/A280 = 1.7-2.0) was reverse-transcribed with oligo-dT primers and the SuperScript III First-Strand Synthesis System (Invitrogen) in a final volume of 20 μ L. Quantitative real-time PCR was performed with a TaqMan by using ABI gene expression assays (Applied Biosystems, Foster City, Calif) according to the manufacturer's instructions. GAPDH was used as a control for cDNA input.

Proliferation assay

PBMCs (10^6 /mL) were initially labeled with 10 μ mol/L CFSE and then stimulated with 50 IU Cry j 1 and Cry j 2 with or without 2 μ g/mL anti-IL-10 neutralizing antibody or anti-IL-10 receptor antibody (R&D) for 4 days. PBMCs were collected and stained with CD4-PerCP antibody (BioLegend, San Diego, Calif). Cell proliferation was analyzed by a CellQuest flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentage of proliferative cells was

calculated by dividing CD4⁺ proliferative lymphocytes by the total number of CD4⁺ lymphocytes ($n = 10$ per each population).

Statistical analysis

Statistical analysis was performed by using the Kruskal-Wallis nonparametric ANOVA test with *post hoc* analysis with the Dunn multiple comparison test. A *P* value less than .05 was considered statistically significant.

Complementarity-determining region 3 spectratyping analysis

T-cell receptor BV segments were amplified with 1 of 43 BV subfamily-specific primers and constant-variable primer recognizing both CB1 and CB2 regions. The sequences of BV primers and fluorescent CB primer have been previously described.^{E1,E2} PCR products were applied to a 5% polyacrylamide sequencing gel, and the size distribution of each fluorescent PCR product was determined by electrophoresis on an automated 377 DNA sequencer (Applied Biosystems). In this technique, an amplified TCR BV subfamily migrates as a series of bands, each one corresponding to a different complementarity-determining region 3 length separated from one another by 3 nucleotides. Data were analyzed by using the GeneScan software (Applied Biosystems), which assigns a size and peak area to the different PCR products.

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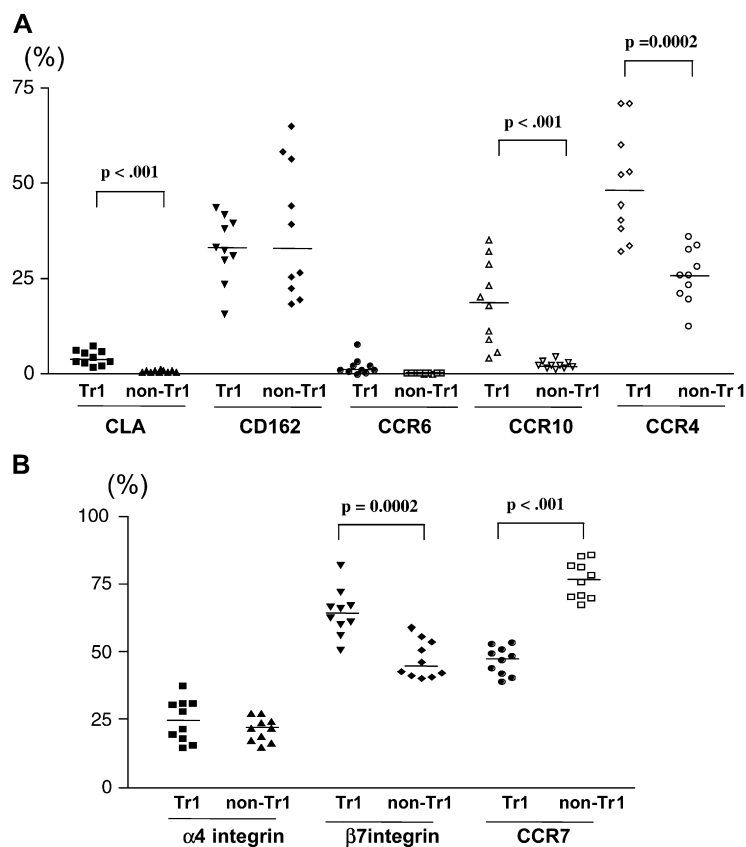
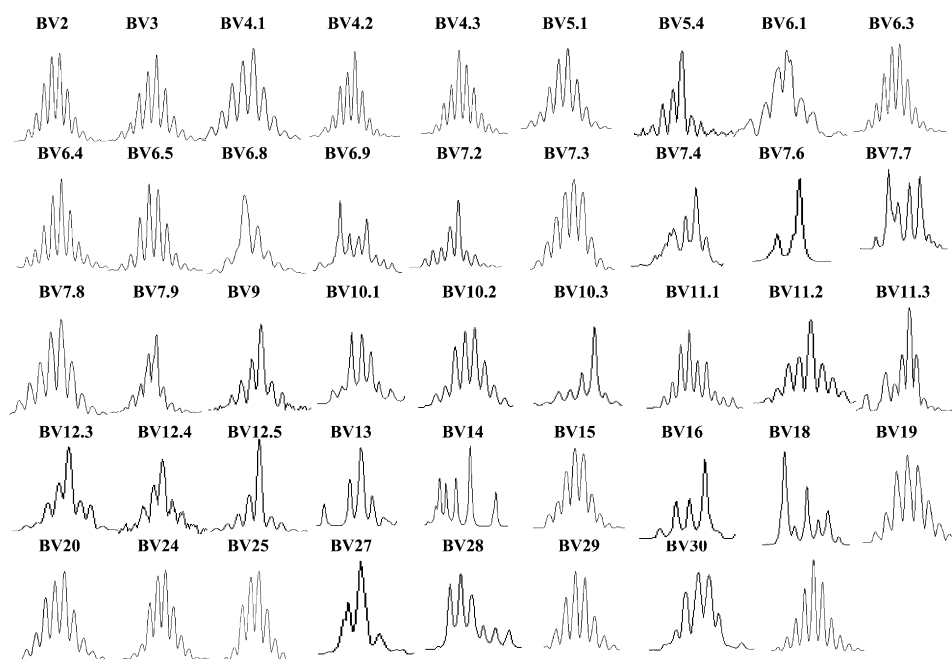


FIG E1. Phenotypic characterization of identified Tr1 cells. $CD4^{+}$ T cells were purified from normal PBMCs and cultured with CD3/CD28 antibodies for 8 hours as described in the text. We analyzed the expression levels of skin-homing receptors and chemokine receptors (cutaneous lymphocyte-associated antigen (CLA), a sialyl Lewis^x-related epitope expressed on P-selectin glycoprotein ligand 1, CCR6, CCR10, and CCR4), as well as gut ($\alpha 4$ and $\beta 7$ integrins) and lymph node (CCR7)-homing receptors on $CD4^{+}$ T cells. Levels of the skin-homing markers CLA, CCR10, and CCR4 were elevated in the Tr1 population compared with the non-Tr1 population. The expression level of $\beta 7$ integrin was also increased in the Tr1 population, but the levels of the lymph node marker CCR7 were decreased ($n = 10$, Mann-Whitney test).

Non-Tr1 from SIT-patient 1 TCR CDR3 spectratyping BV2~30



Tr1 from SIT-patient 2 TCR CDR3 spectratyping BV2~30

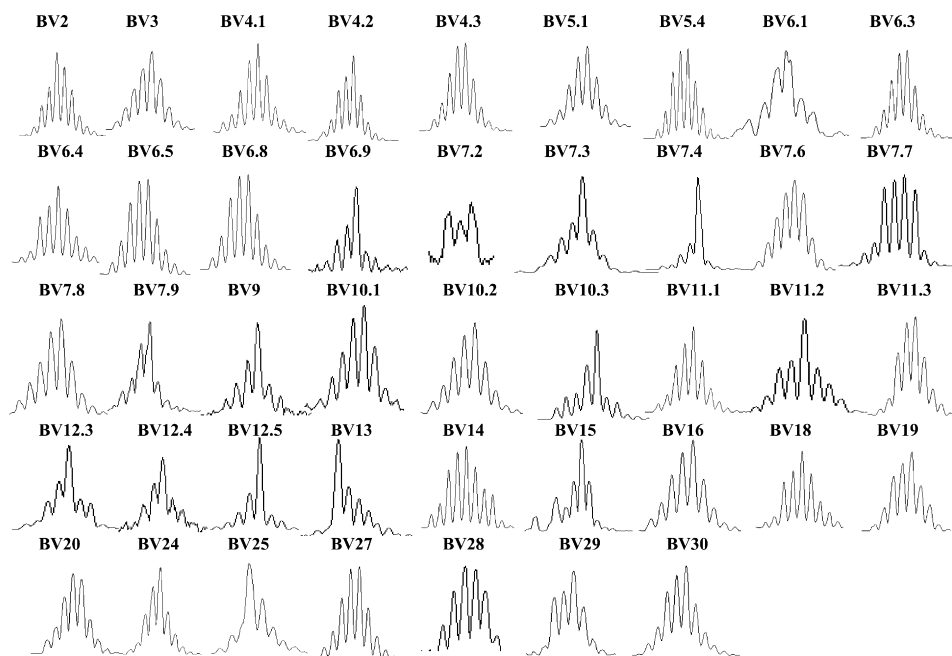
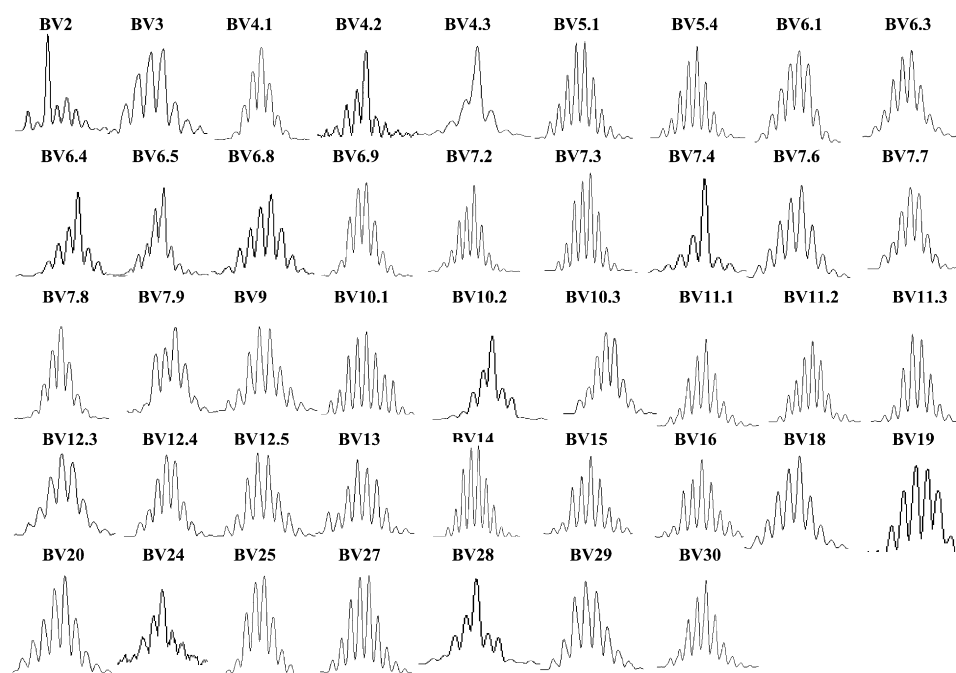


FIG E2. T-cell receptor complementarity-determining region 3 (CDR3) size spectratyping analysis of Tr1 or non Tr1-population from SIT-patient, pollen allergy-patient, or normal control.

Non-Tr1 from SIT-patient 2 TCR CDR3 spectratyping BV2~30



Tr1 from pollen allergy-patient TCR CDR3 spectratyping BV2~30

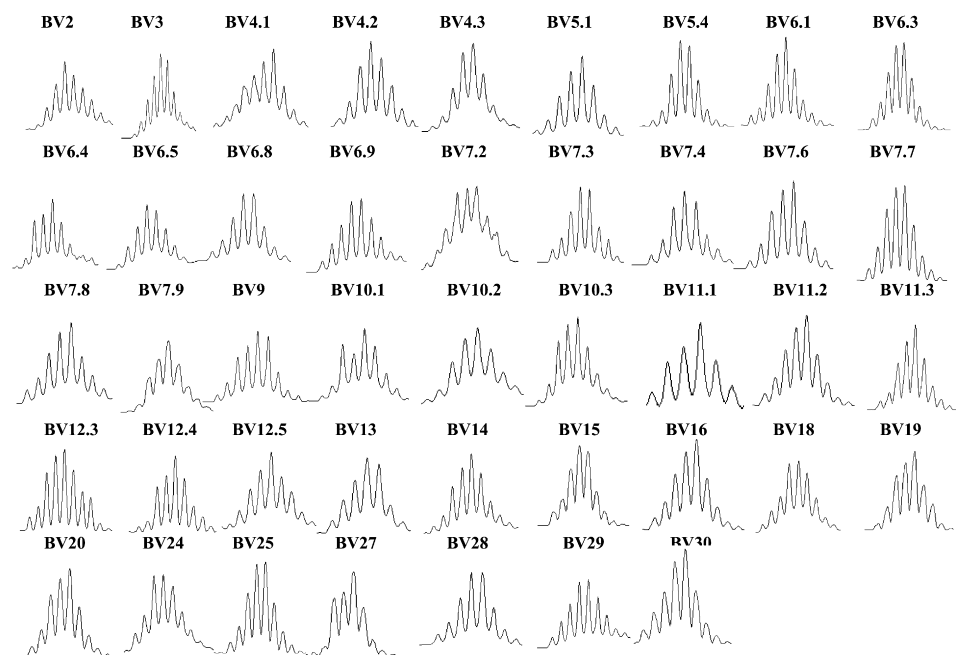
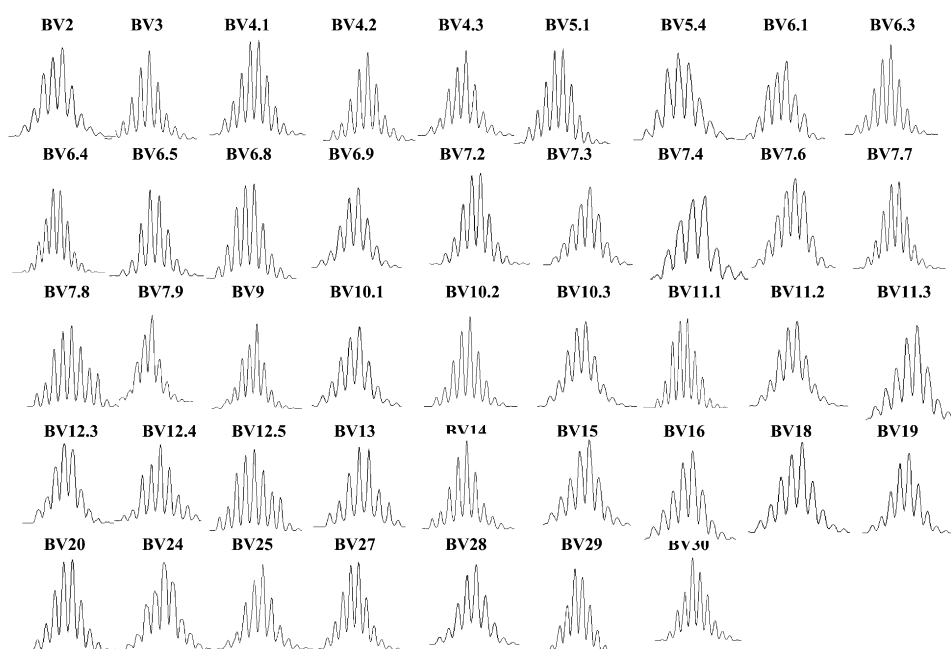


FIG E2. (Continued)

non-Tr1 from pollen allergy-patient TCR CDR3 spectratyping BV2~30



Tr1 from normal control TCR CDR3 spectratyping BV2~30

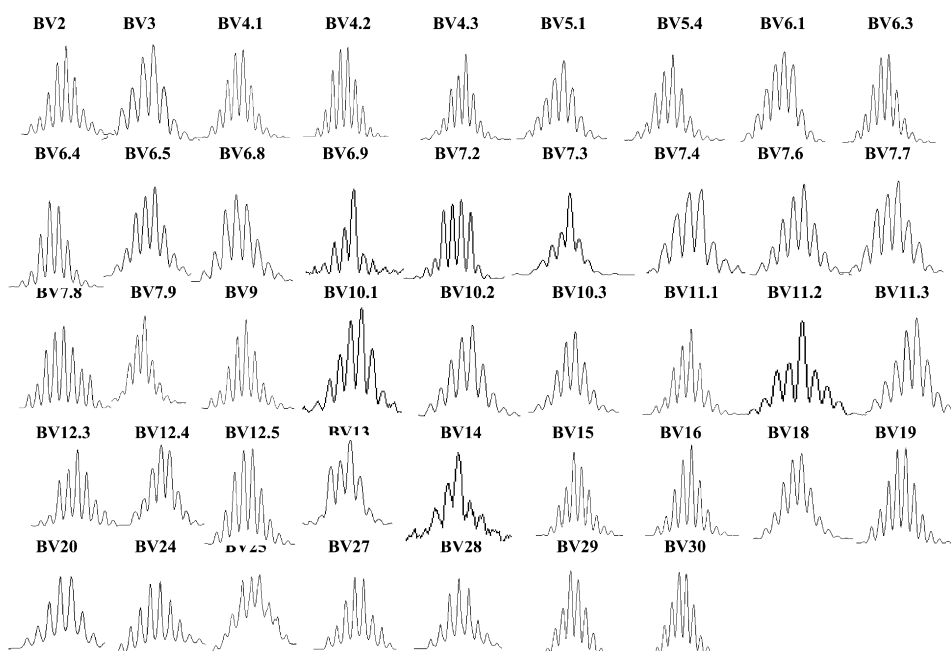


FIG E2. (Continued)

non-Tr1 from normal control TCR CDR3 spectratyping BV2~30

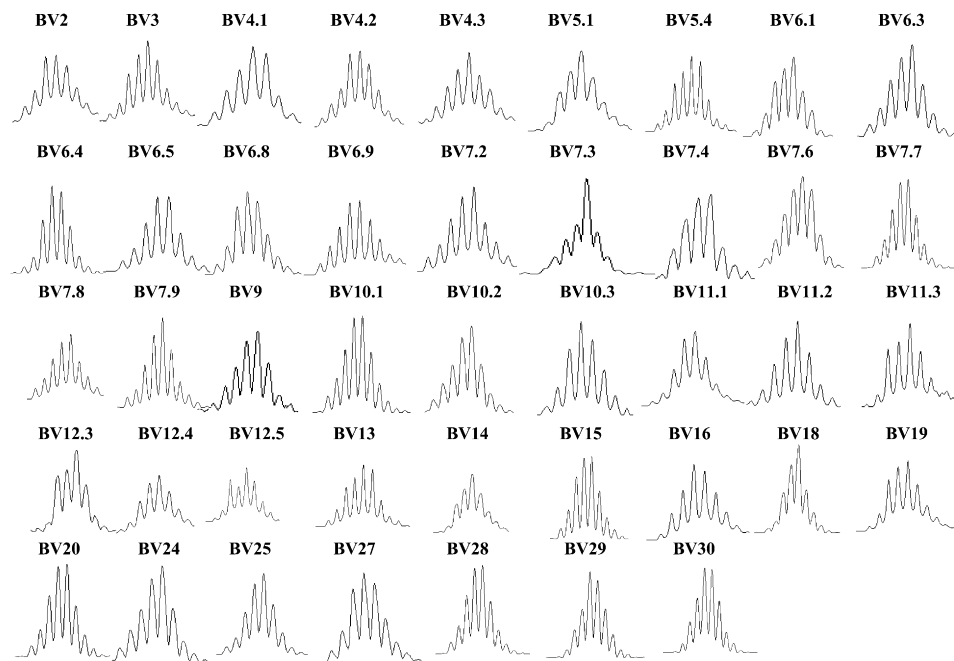


FIG E2. (Continued)

TABLE E1. Patients' information

	Normal	Allergy	SIT
N	13	60	15
Age (y)	38.4 ± 6.1	26.8 ± 5.7	39.1 ± 14.5
Male and female	7 and 6	43 and 17	5 and 10
TNSS	0	4.34 ± 1.61	2.21 ± 1.85

Nasal symptoms of sneezing, nasal discharge, and nasal congestion were recorded every day. Symptoms were categorized on a 5-point scale (0, none; 1, mild; 2, moderate; 3, severe; 4, extremely severe) according to practical guidelines for the management of allergic rhinitis in Japan. The average of total nasal symptom score (TNSS) for the 3 nasal symptoms in the cedar pollen season of February and March 2008 was calculated.^{E3}