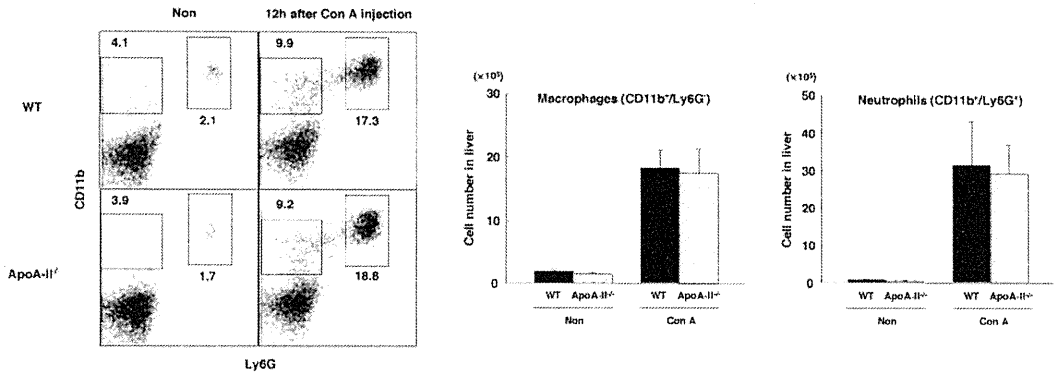
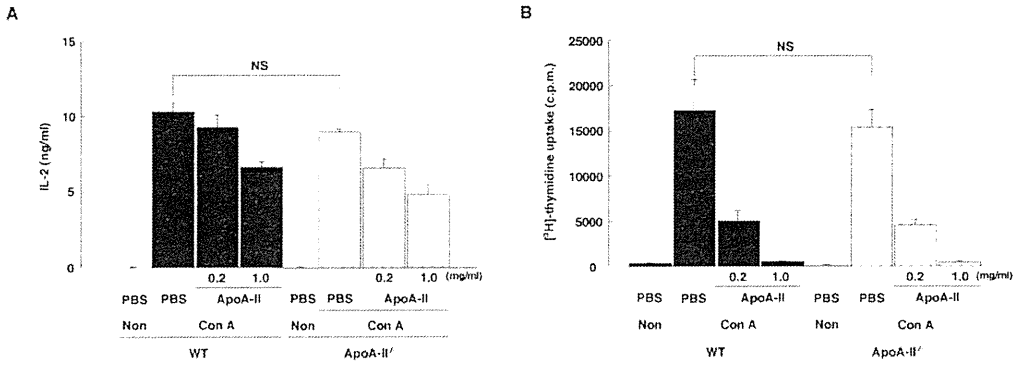
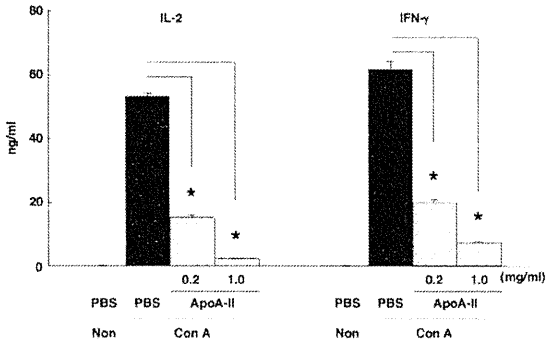


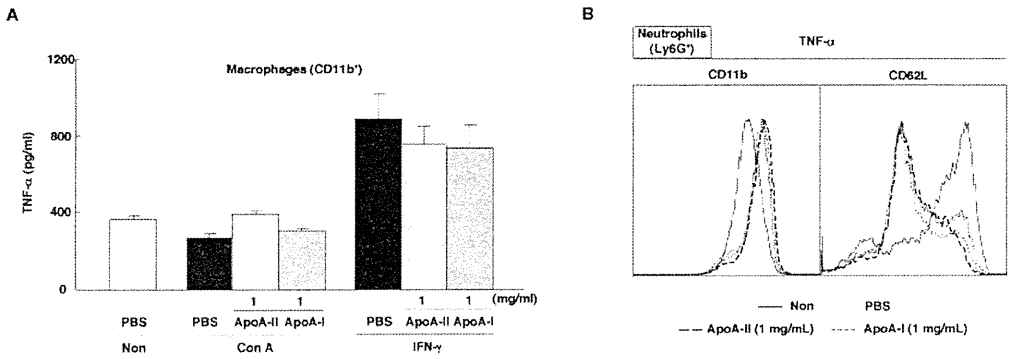
Supplemental Figure 2. Normal Con A-induced infiltration of macrophages and neutrophils into the liver in ApoA-II$^{-/-}$ mice. Con A (20 mg/kg, i.v.) was injected into ApoA-II$^{-/-}$ mice. Flow cytometric analysis was performed to assess the infiltration of macrophages and neutrophils into the liver 12 h after Con A injection. The numbers of CD11b^{+}/Ly6G^{-} cells and CD11b^{+}/Ly6G^{-} cells and were calculated using total leukocyte cell counts and flow cytometric analysis data. The results are expressed as mean ± SD (n=6).



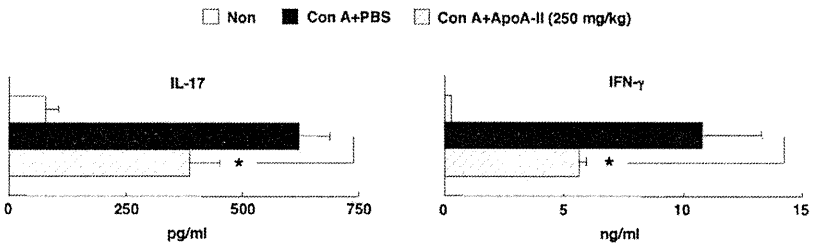
Supplemental Figure 3. The suppressive effect of ApoA-II is observed for both IL-2 production and proliferation in Con A-stimulated ApoA-II$^{-/-}$ CD4 T cells. (A), Purified splenic CD4 T cells from ApoA-II$^{-/-}$ and WT mice were stimulated with Con A (5 μg/ml) for 24 h in the presence of ApoA-II, and the amount of IL-2 in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD. **(B),** The proliferative response of splenic CD4 T cells was examined by [^{3}H]-thymidine uptake. Purified splenic CD4 T cells from ApoA-II$^{-/-}$ and WT mice were stimulated with Con A for 40 h in the presence of ApoA-II. The results are expressed as mean ± SD. Similar data were obtained from three independent experiments.



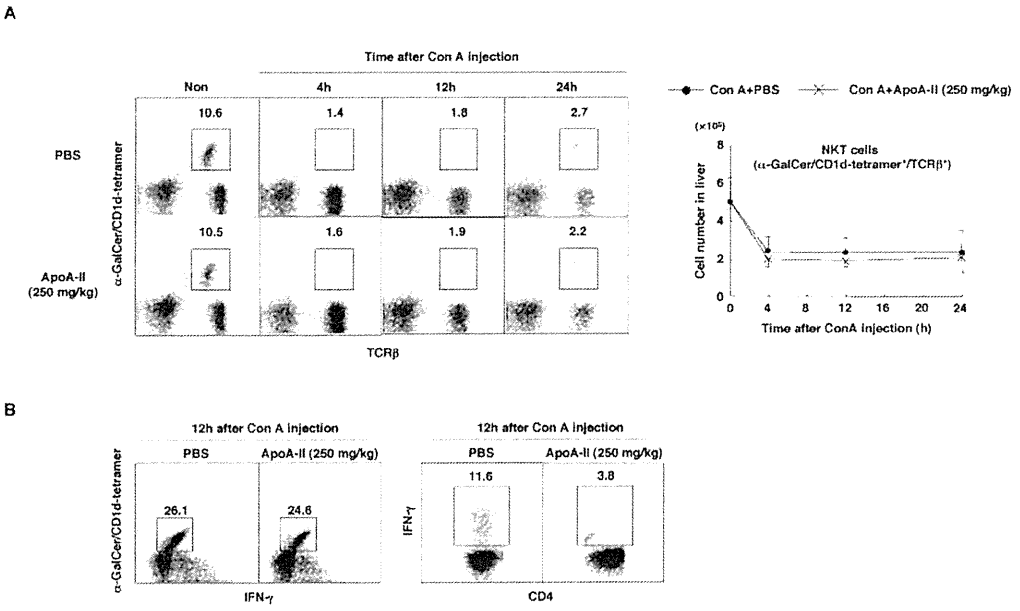
Supplemental Figure 4. Suppression of the function of mouse CD8 T cells by ApoA-II. Splenic CD8 T cells were purified from BALB/c splenocytes using PE-conjugated anti-CD8 mAb, anti-PE magnetic beads (Miltenyi Biotec), and Auto-MACS cell Sorter (Miltenyi Biotec). The purified CD8 T cells were stimulated with Con A (5 μg/ml) for 24 h in the presence of ApoA-II (0.2 or 1 mg/ml), and the amounts of IL-2 and IFN-γ in the culture supernatant were assessed by ELISA. The results are expressed as mean ± SD. *P < 0.05, compared with PBS-added CD8 T cells. Similar data were obtained from three independent experiments.



Supplemental Figure 5. ApoA-II does not suppress the function of macrophages or the activation of neutrophils. (A), To collect macrophages, 1 ml of 4% fluid thioglycollate medium (Sigma-Aldrich) was injected intraperitoneally into BALB/c mice. The peritoneal lavage cells were harvested on day 4 after the thioglycollate injection. Macrophages were purified using FITC-conjugated anti-CD11b mAb, anti-FITC magnetic beads, and Auto-MACS cell Sorting. Purified CD11b-positive cells consisted of more than 95% macrophages identified by flow cytometry and Wright-Giemsa staining, respectively (data not shown). Purified peritoneal macrophages were stimulated with Con A (5 μg/ml) or IFN-γ (2.5 ng/ml) for 24 h in the presence of ApoA-II or ApoA-I (1 mg/ml), and the amount of TNF-α in the culture supernatant was assessed by ELISA. The results are expressed as mean ± SD (n=5). **(B),** To collect neutrophils, 1 ml of 4% fluid thioglycollate medium was injected intraperitoneally into BALB/c mice. The peritoneal lavage cells were harvested 4 h after thioglycollate injection. Neutrophils were purified using PE-conjugated anti-Ly6G mAb, anti-PE magnetic beads, and Auto-MACS cell Sorting. The purified Ly6G-positive cells consisted of more than 95% neutrophils identified by flow cytometry and Wright-Giemsa staining (data not shown). Purified peritoneal neutrophils were stimulated with TNF-α (1 ng/ml) for 0.5 h in the presence of ApoA-II or ApoA-I, and the expression of CD11b and CD62L was assessed using flow cytometry.



Supplemental Figure 6. The suppressive effect of ApoA-II administration on IL-17 production by mouse liver-infiltrating CD4 T cells after Con A injection. Con A (12.5 mg/kg, i.v.) and vehicle, or Con A and ApoA-II (250 mg/kg, i.v.) were administered into BALB/c mice. 12 h after Con A injection, liver CD4 T cells were purified from mice using FITC-conjugated anti-CD4 mAb, anti-FITC magnetic beads, and Auto-MACS cell Sorter. The purified CD4 T cells were stimulated with Con A (5 μg/ml) for 24 h, and the amounts of IFN-γ and IL-17 in the culture supernatant were assessed by ELISA. The results are expressed mean ± SD (n=4). *$P < 0.05$, compared with PBS-administrated mice.



Supplemental Figure 7. ApoA-II administration did not change the activation of Vα14 NKT cell in the liver. (A), Con A (12.5 mg/kg, i.v.) and vehicle, or Con A and ApoA-II (250 mg/kg, i.v.) were administered into BALB/c mice. Flow cytometric analysis of Vα14 NKT cells in the liver 4, 12, and 24 h after Con A injection was performed to determine the expression profiles of α-GalCer/CD1d-tetramer$^+$ and TCRβ$^-$ cells. The number of α-GalCer/CD1d-tetramer$^+$/TCRβ$^+$ cells was calculated based on the total leukocyte cell counts and flow cytometric analysis data. The results are expressed as mean ± SD (n=8). **(B),** The intracellular expression of IFN-γ in α-GalCer/CD1d-tetramer$^+$ NKT cells or CD4 T cells in spleen 12h after Con A injection were analyzed using a Cytofix/Cytoperm Kit Plus (with Golgistop; BD Biosciences) according to the manufacturer's instructions.

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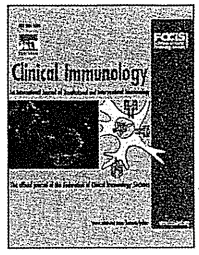

Increase of regulatory T cells and the ratio of specific IgE to total IgE are candidates for response monitoring or prognostic biomarkers in 2-year sublingual immunotherapy (SLIT) for Japanese cedar pollinosis

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Regulatory T cell;
Sublingual immunotherapy

Abstract The aims of this study were to examine the therapeutic effects of sublingual immunotherapy (SLIT) and to identify potential biomarkers that would predict the therapeutic response in a randomized, double-blind, placebo-controlled clinical trial. The trial was carried out over two pollinosis seasons in 2007 and 2008. Carry-over therapeutic effects were analyzed in 2009. SLIT significantly ameliorated the symptoms of pollinosis during the 2008 and 2009 pollen seasons. Cry j 1-specific cytokine production in a subgroup of patients with mild disease in the SLIT group was significantly attenuated. The ratio of specific IgE to total IgE before treatment correlated with the symptom-medication score in the SLIT group in 2008. Patients with increased

Abbreviations: DBPC, double-blind, placebo-controlled; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; iTreg, induced regulatory T cells; ITT analysis, intention-to-treat analysis; JAU, Japanese allergy unit; N.S., not significant; OT analysis, on-treatment analysis; PBMCs, peripheral blood mononuclear cells; RAST, radioallergosorbent test; SLIT, sublingual immunotherapy; SMS, symptom-medication score; Treg, regulatory T cells; QOL, quality-of-life.

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Cry j 1-iTreg in the SLIT group had significantly improved QOL and QOL-symptom scores. In summary, the specific IgE to total IgE ratio and upregulation of Cry j 1-iTreg are candidates for biomarker of the clinical response to SLIT.

1. Introduction

Japanese cedar (*Cryptomeria japonica*) pollinosis is a common allergy in Japan, with a prevalence estimated to be 26.5% in a nationwide survey conducted in 2008 [1].

A 2000 Japanese allergy unit (JAU) sample of standardized extract from Japanese cedar pollen is the only available allergen for subcutaneous and sublingual immunotherapy (SLIT) against pollinosis in Japan. The 2000 JAU extract contains 1.5 to 4.2 μg of the major allergen, Cry j 1 [2]. The common monthly cumulative dose for SLIT is 8000 JAU, which contains approximately 10 μg of Cry j 1. This maintenance dose is 200-fold higher than that used in traditional subcutaneous immunotherapy using 0.2 ml of a 200 JAU/ml extract, which contains approximately 50 ng of Cry j 1. Despite using a low dose of the major allergen compared with that in European trials, positive effects on pollinosis have been shown in randomized double-blind, placebo-controlled (DBPC) studies, in which SLIT significantly ameliorated the symptom score, symptom-medication score (SMS), and quality-of-life (QOL) score [3,4].

SLIT induces Cry j 1-specific IgG4 production and attenuates the seasonal increase in the number of Th2 cells specific to epitopes from Cry j 1 and Cry j 2 [3]. Involvement of antigen-specific Tr1 cells or regulatory T cells (Treg) in the therapeutic mechanism has also been suggested [5,6]. We previously found that SLIT increased the levels of Cry j 1-specific induced Treg cells (Cry j 1-iTreg; $IL10^+Foxp3^+$ cells in $CD25^+CD4^+$ leukocytes) and that the increase in Cry j 1-iTreg after the pollen season may serve as a response monitoring biomarker that correlates with a positive therapeutic effect based on the QOL-symptom score and distinguishes responders from non-responders after SLIT [6].

In this report, we examined the reproducibility of the positive therapeutic effects and safety of SLIT and upregulation of iTregs as a response monitoring biomarker, with the goal of confirming our previous results in a larger randomized DBPC study. Therefore, the safety and clinical effect of SLIT for Japanese cedar pollinosis were used as the primary endpoint, and carry-over effects, immunological changes, and biomarkers for a positive clinical effect induced by SLIT were secondary endpoints.

2. Materials and methods

2.1. Study population

The study was conducted as a randomized, DBPC, parallel-group, single center trial in subjects with Japanese cedar pollinosis. This study was performed for two pollen seasons between September 2006 and May 2008, with follow-up in the pollen season in 2009. We recruited 130 participants in September 2006. Diagnosis of Japanese cedar pollinosis was based on clinical history and the presence of IgE specific to Japanese cedar pollen of at least class 2 (CAP-RAST method, Phadia, Tokyo, Japan). Participants with a history of immunotherapy or a diagnosis of asthma, or those who were pregnant, were excluded from the study. Patients who suffered seasonal or chronic rhinitis that required medical treatment were also excluded.

A total of 103 patients were eligible for the study, and all had moderate or severe symptoms in the previous pollen season [7]. We anticipated that some participants in the SLIT group would drop out from the study due to side effects and we planned to evaluate the risk of mild or severe side effects due to the vaccination. Therefore, we randomly divided the patients into treatment (SLIT) and placebo groups with a ratio of 6:4 according to the table of random numbers prepared by the Department of Pharmacy at Chiba University Hospital (Fig. 1). The sample size was determined based on a previous study [3]. Briefly, we planned to have 50 patients in each group with anticipation of dropout. We set 1.0 as a magnitude for the difference of average SMS between that from the SLIT and placebo groups and 1.5 as a standard deviation according to the result of previous study. Therefore, when the power was set to 0.8 and the α-error to 0.05, the number of required cases was 35 in each group. A person who was not directly involved in the study was responsible for group allocation. To prevent leakage of information, the allocation table was kept by this person and a member of the ethics committee who was also not directly involved in the study, until accessed with the key after completion of the study. The protocol was approved by the Ethics Committee of Chiba University, and written informed consent was obtained from each patient prior to participation in the study.

2.2. Clinical protocols

The SLIT group included 58 patients who received standardized Japanese cedar pollen extract (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) [8], and the placebo group included 45 patients who received an inactive placebo. The protocol consisted of treatment with graded courses of the extract in 50% glycerol, followed by maintenance therapy [6]. Briefly, the extract was graded in three strengths: 20, 200, and 2000 JAU/ml. Patients received increasing doses with each vial, beginning with 0.2 ml from the 20 JAU/ml vial and increasing by 0.2 ml a day for 5 days per week. The vaccine was taken sublingually, kept in place for 2 min without a retention reagent, and then spit out. The procedure was repeated until the maximum dose (1.0 ml of 2000 JAU/ml) was reached. The maintenance dose was 1.0 ml of 2000 JAU/ml given once a week until the end of May 2008. The patients in the placebo group received inactive 50% glycerol in saline. All participants were allowed to take symptom-reducing drugs as needed.

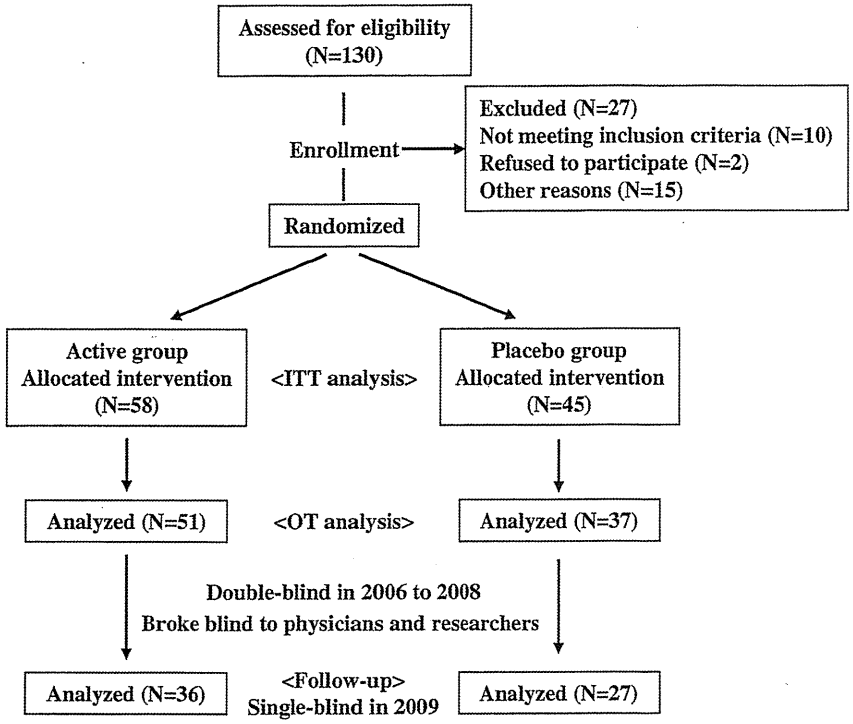

Figure 1 Flow diagram for groups and individuals in the phases of the randomized trial. Fifteen participants from the SLIT ($N=7$) and placebo ($N=8$) groups were lost to follow-up due to reasons such as moving house and transfer. The double-blind status was maintained until completion of analysis of all clinical and immunological parameters (December 2008). Follow-up analysis in 2009 was undertaken in a single-blind manner.

2.3. Clinical symptoms and safety measurements

The patients completed a pollinosis diary to record their nasal symptoms and use of symptom-reducing drugs in the 2007, 2008, and 2009 pollen seasons. The total amounts of pollen scattered from Japanese cedar and Japanese cypress (*Chamaecyparis obtusa*) in Chiba prefecture were 2777, 6596, and 5486 grains/cm^2 during the 2007, 2008, and 2009 pollen seasons, respectively, based on measurements with a Durham pollen sampler. The duration and amount of scattered Japanese cedar pollen differed greatly among these years, but the daily amount of scattered pollen typically followed a wide-based bell-shaped curve over the whole pollen season from the middle of January or early February to the middle or end of May. The duration of the peak pollen season was relatively constant in the 3 years, and therefore, we analyzed the SMS during the peak period. The peak pollen season was defined as the period from the first day that the pollen count was $\geq$20 grains/cm^2/day for 3 consecutive days until the last day that the pollen count was $\geq$20 grains/cm^2/day before a period in which the pollen count was <20 grains/cm^2/day for 7 consecutive days.

The daily SMS was calculated as described previously [3]. Briefly, daily episodes of sneezing and nose blowing were rated as 0–4: none, 0; 1–5 episodes, 1; 6–10 episodes, 2; 11–20 episodes, 3; >20 episodes, 4. Daily medication was recorded based on drug types and duration of usage using the following guidelines: antihistamines, mast cell stabilizers, and vasoconstrictors, 1; topical ocular or nasal steroids, 2. Patients with an average daily SMS in the peak pollen season of $\leq$4 were judged to have mild symptoms based on guidelines for allergic rhinitis [7].

In the middle of the 2007 and 2008 pollen seasons, the participants completed the Japanese Allergic Rhinitis QOL Standard Questionnaire No.1 (JRQLQ No.1) for assessment of QOL-symptom and total QOL scores [9]. These scores were calculated as previously described [4,6]. The total QOL-symptom score was calculated as the sum of each component score: none, 0; mild, 1; moderate, 2; severe, 3; and very severe, 4. Nasal and ocular symptoms covered by the questionnaire included runny nose, sneezing, nasal congestion, itchy nose, itchy eyes, and watery eyes. Adverse events were graded using Common Terminology Criteria for Adverse Events (CTCAE) v.3.0 [10]. Briefly, adverse events were graded as mild, grade 1; moderate, grade 2; severe, grade 3; life threatening, grade 4; death, grade 5 according to a category for allergy/immunology in the CTCAE v.3.0 scoring system.

2.4. Blood samples

Peripheral blood was obtained from each patient before treatment (September to October 2006) and before and after the pollen seasons in 2007 (December 2006 to January 2007, and May to June 2007, respectively) and 2008 (November to December 2007, and May 2008, respectively). Peripheral blood mononuclear cells (PBMCs) were isolated, frozen, and stored in liquid nitrogen [6]. However, the PBMCs isolated before treatment, and before and after the 2007 pollen season were damaged during storage and we were unable to

analyze their immunological responses. Therefore, immunological data were obtained only from PBMCs collected before and after the 2008 pollen season.

2.5. Total and antigen-specific immunoglobulin titer

The Cry j 1-specific IgE and IgG4 titers in plasma were measured by ELISA [3,11]. Total IgE and specific IgE titers for Japanese cedar, orchard grass, mugwort, and house dust mites were evaluated by the CAP-RAST method (Phadia).

2.6. Flow cytometric analysis

The levels of Cry j 1-iTreg were analyzed by flow cytometry [6]. Briefly, PBMCs were cultured with or without Cry j 1 for 3 days, followed by a culture with 10 ng/ml phorbol 12-myristate 13-acetate, 1 μM ionomycin, and 2 μM monensin for 6 h. The PBMCs were stained with PE-Cy7-anti-CD4 antibody, APC-anti-IL10 antibody (BD Biosciences, San Diego, CA, USA), PE-anti-CD25, and FITC-anti-Foxp3 (clone: PCH101) using a Foxp3 staining buffer set (eBioscience, San Diego, CA, USA).

2.7. Analysis of the number of IL4-producing cells and the concentration of cytokines

The number of IL4-producing cells stimulated with Cry j 1 was determined by enzyme-linked immunospot (ELISPOT) assay, and the concentrations of IL2, IL5, and IL13 in the culture supernatant were measured using a BD™ Cytometric bead assay (CBA) Flex system (BD Biosciences) [6]. Briefly, a 96-well sterile filter plate (Millipore, Billerica, MA, USA) was coated with monoclonal antibody to human IL4 (Mabtech AB, Nacka Strand, Sweden). The plate was pre-incubated with AIM-V medium at 37 °C for 1 h. The medium was discarded, and then PBMCs (3×10^5 cells/well) were cultured with fresh medium alone or with 10 μg/ml Cry j 1 for 17 h at 37 °C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich, St. Louis, MO, USA). The plates were then incubated with a biotinylated monoclonal antibody to human IL4 for 2 h, and then with streptavidin-conjugated alkaline phosphatase for 1 h at room temperature. After washing with PBS, the plates were incubated with BCIP/NBT^{PLUS} (Mabtech) for 5 min at 37 °C. For the CBA, isolated PBMCs were cultured at 2.5×10^6 cells/ml with or without 5 μg/ml Cry j 1 for 3 days at 37 °C in AIM-V medium containing 5% human AB serum (Sigma-Aldrich). After centrifugation at 300×*g* for 10 min, the supernatant was divided into aliquots and stored at −20 °C until the cytokine assay was performed.

2.8. Data representation

The full analysis set ($N=103$) was used for the intention-to-treat (ITT) analysis and per protocol populations ($N=88$) were used for on-treatment (OT) analysis (Fig. 1). Cry j 1-specific cytokine production is shown as the difference between cells stimulated with Cry j 1 and controls stimulated with medium only. Changes after the 2008 pollen season are shown as differences between pre- and post-pollen season values.

2.9. Statistical analysis

Two-group comparisons were performed using a Wilcoxon *t*-test or Mann–Whitney *U*-test to determine the significance of differences, or using an unpaired *t*-test as indicated. *P*-values <0.05 were considered to be significant.

3. Results

3.1. Clinical effects and adverse events

A total of 103 patients were included in the overall analysis of efficacy for the 2007 and 2008 pollen seasons. These patients were randomly divided into the SLIT ($N=58$) and placebo ($N=45$) groups at a ratio of 6:4. Diaries and QOL questionnaires for 88 patients were available at the end of the DBPC study. The overall randomized population was considered to be the ITT population. The SMS in the SLIT group did not differ significantly from that in the placebo group in ITT analysis after 2-year SLIT (*P*=N.S.; Student *t*-test, data not shown).

The final sample size included 88 subjects for OT analysis (SLIT; $N=51$, placebo; $N=37$, ratio 4:3). The demographic characteristics of the OT population before treatment are shown in Table 1. The SMS in the SLIT group did not differ significantly from that in the placebo group in the 2007 peak pollen season (February 19 to March 31, *P*=N.S.; Student *t*-test). However, the average SMS in the 2008 peak pollen season (February 29 to April 1) was significantly ameliorated in the SLIT group compared with the placebo group (4.2 vs. 5.3, $P=0.02$; Student *t*-test). The percentages of subjects with mild symptoms (SMS ≤ 4) were 55% and 28% in the SLIT and placebo groups, respectively, in the peak pollen

Table 1 Clinical data of participants at the start of the study.

Group	SLIT	Placebo	*P*-value
Number	51	37	
Sex (M/F)	17/34	8/29	N.S.[a]
Mean age	44.4	42.3	N.S.[b]
Range	16–73	19–70	
Total IgE [IU/ml]	198	258	N.S.[b]
Range	6.8–1480	8.6–2090	
Specific IgE[c]	27	29	N.S.[b]
Range	0.8–100	1.5–100	
Class [mean]	3.5	3.8	N.S.[b]
Range	2–6	2–6	
Other allergies[d] (%)			
Orchard grass	16 (31%)	11 (30%)	N.S.[e]
Mugwort	5 (10%)	3 (8%)	<0.05[f]
House dust mite	24 (47%)	13 (35%)	N.S.[e]

[a] Yates2×2 Chi-squared test.
[b] Student *t*-test.
[c] Specific IgE to Japanese cedar pollen; CAP-RAST raw value [kAU/L], mean.
[d] Number of subjects with specific IgE of at least CAP-RAST class 2.
[e] 2×2 Chi-squared test.
[f] Fisher exact probability.

season (Fig. 2A). QOL-symptom and total QOL scores were also significantly ameliorated in the SLIT group compared to those in the placebo group in the middle of the 2008 pollen season (Fig. 2B).

There were no severe adverse events that required a patient to withdraw from the study; however, some subjects reported adverse events of mild discomfort: six of grade 2 (oral pruritus: 2; gingivostomatitis: 2; asthma: 1; rash in nasal cavity: 1) in the SLIT group (6/51; 11.8%); and one of grade 1 (bitter taste) in the placebo group (1/37; 2.7%).

3.2. Immunoglobulin production

There were no significant differences in Cry j 1-specific IgE and IgG4 production between patients in the SLIT and placebo groups before treatment, or before and after the pollen seasons. The SLIT group was divided into subgroups based on the SMS in the 2008 peak pollen season: a mild subgroup with SMS ≤ 4 (classified as responders; $N=28$) and a severe subgroup with SMS >4 (non-responders; $N=23$). IgE and IgG4 production in patients in the mild subgroup were both similar to those in patients in the severe subgroup and in the placebo group at various time points (data not shown).

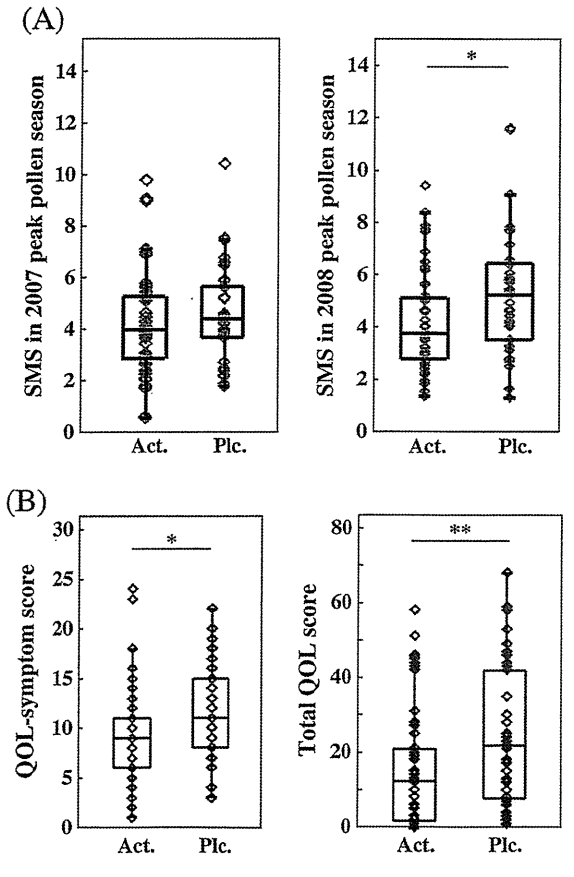

Figure 2 Clinical scores after 2-year SLIT. (A) Average daily symptom-medication scores (SMS) in the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups in the 2007 and 2008 peak pollen seasons. (B) QOL-symptom and total QOL scores from the QOL questionnaire were plotted for the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups in the middle of the 2008 pollen season. Each diamond shows a value for an individual. Two-group comparisons were performed using an unpaired Student t-test. $^{*}P<0.05$, $^{**}P<0.01$.

3.3. Cry j 1-specific cytokine production

IL2, IL5, and IL13 levels were analyzed in the culture supernatant. The number of IL4-producing cells was measured by ELISPOT because IL4 was undetectable in the supernatant. There were no significant differences between the SLIT and placebo groups in the production of each cytokine following stimulation with Cry j 1 (Fig. 3A). IL5 was significantly increased after the pollen season in all groups ($P<0.05$; Wilcoxon t-test), and the IL2 and IL13 levels and the number of IL4-producing cells were significantly increased after the pollen season in the SLIT and placebo groups and in the severe subgroup ($P<0.05$; Wilcoxon t-test). Patients in the mild subgroup (responder to SLIT) did not show significant increase of IL2 and IL13 or of IL4-producing cells after the pollen season (P=N.S.; Wilcoxon t-test). The increases in the number of IL4-producing cells and IL5 level after the pollen season in the mild subgroup were significantly less than those in the severe subgroup (non-responders) and the placebo group. The increase of IL13 in the mild subgroup was significantly less than that in the severe subgroup and showed a tendency to be attenuated compared with the placebo group (P=N.S.; Mann–Whitney U-test). The increase of IL2 in the mild subgroup was significantly less than that in the placebo group ($P<0.05$) and showed a tendency to be attenuated compared with the severe subgroup ($P=0.053$; Mann–Whitney U-test, Fig. 3B).

3.4. Prognostic biomarkers for clinical effects

The average ratio of Japanese cedar pollen-specific IgE to total IgE (sIgE/tIgE ratio) in all patients in the study was 0.193 before treatment. The SLIT group was divided into subgroups with a sIgE/tIgE ratio ≤ 0.19 (low, $N=28$) and >0.19 (high, $N=23$) before treatment. Similar subgroups were established in the placebo group. The SMS in the 2008 peak pollen season for the low subgroup was significantly improved compared to that in the high subgroup in the SLIT group ($P=0.02$; Mann–Whitney U-test); however, in the placebo group, the low and high subgroups had comparable SMSs (P=N.S.; Mann–Whitney U-test, Fig. 4A). Furthermore, the SMS was correlated with the sIgE/tIgE ratio in the SLIT group ($Rs=0.39$, $P<0.01$; Spearman correlation analysis), but not in the placebo group ($Rs=0.08$, P=N.S.; Spearman correlation analysis, Fig. 4B).

3.5. Upregulation of Cry j 1-iTreg levels as a response monitoring biomarker

A population of IL10^{+}Foxp3^{+} cells in CD25^{+}CD4^{+} leukocytes was evaluated as a potential marker for iTreg after stimulation with Cry j 1 or medium only before and after the pollen season in 2008. Neither the changes in Cry j 1-iTreg levels after stimulation with and without Cry j 1 nor the upregulation of Cry j 1-iTreg from pre- to post-pollen season differed significantly different between the groups (data not shown).

We previously reported that upregulation of Cry j 1-iTreg is a candidate biomarker that may distinguish SLIT responders from non-responders based on QOL-symptom scores [6]. Therefore, we divided the SLIT group into subgroups based on an increase (N=24) or decrease (N=27) in Cry j 1-iTreg levels from before to after the pollen season in 2008. QOL-symptom and total QOL scores in the increased iTreg subgroup significantly improved compared with those in the placebo group. In contrast, the scores in the decreased iTreg subgroup were similar to those in the placebo group (Fig. 4C).

3.6. Carry-over effects in the year after treatment

A total of 63 patients completed a pollinosis-symptom diary during the 2009 pollen season; 1 year after the 2-year SLIT treatment (Fig. 1). All participants remained blinded to their treatment with SLIT or a placebo. The SMS in the peak pollen season in 2009 (February 15 to March 6) in the SLIT group (N=36) was significantly attenuated compared to the placebo group (N=27, P=0.03). The average SMSs for the SLIT and placebo groups were 3.5 and 4.5, respectively, in the peak pollen season (Fig. 5).

4. Discussion

The primary endpoint of this randomized DBPC trial was the therapeutic effect evaluated in ITT analysis. No significant positive effect was observed between the SLIT and placebo groups after exchanging the perceived improvement of patients who dropped out with each median score from the counter group. In OT analysis, the SMS in the SLIT group was

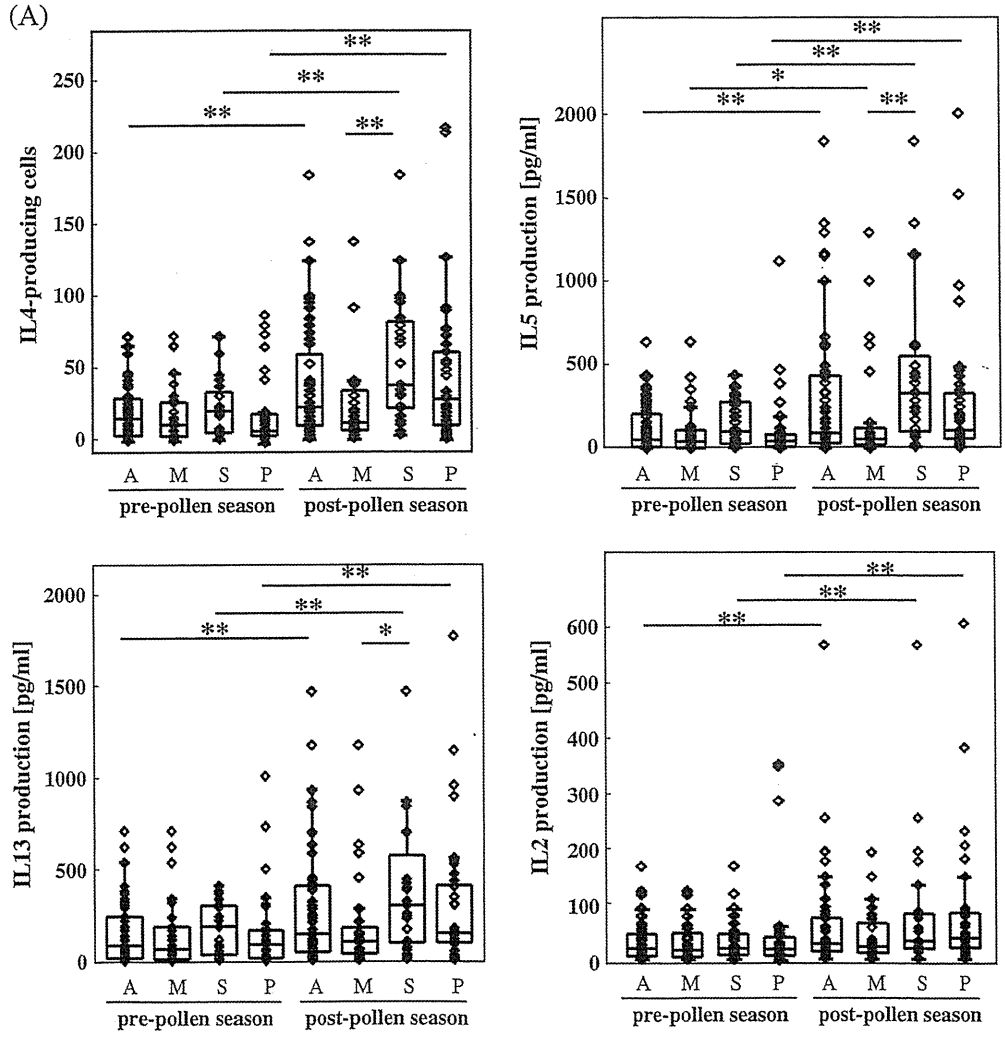

Figure 3 Cytokine production from PBMCs. (A) Number of Cry j 1-specific IL4-producing cells and Cry j 1-specific cytokine levels in the SLIT group (A; N=51), the mild subgroup of the SLIT group (M; N=28), the severe subgroup of the SLIT group (S; N=23), and the placebo group (P; N=37) at before and after the 2008 pollen season. Comparisons with a significant difference are indicated as * and **; otherwise, comparisons are not significantly different (P=N.S.). (B) Increases in the number of Cry j 1-specific IL4-producing cells and Cry j 1-specific cytokine levels occurred from before to after the 2008 pollen season in the SLIT group (Act.; N=51), the mild subgroup of the SLIT group (Mild; N=28), the severe subgroup of the SLIT group (Sev.; N=23), and the placebo group (Plc.; N=37). Each diamond shows the value for an individual. Two-group comparison was performed using a Mann–Whitney U-test. *P<0.05, **P<0.01.

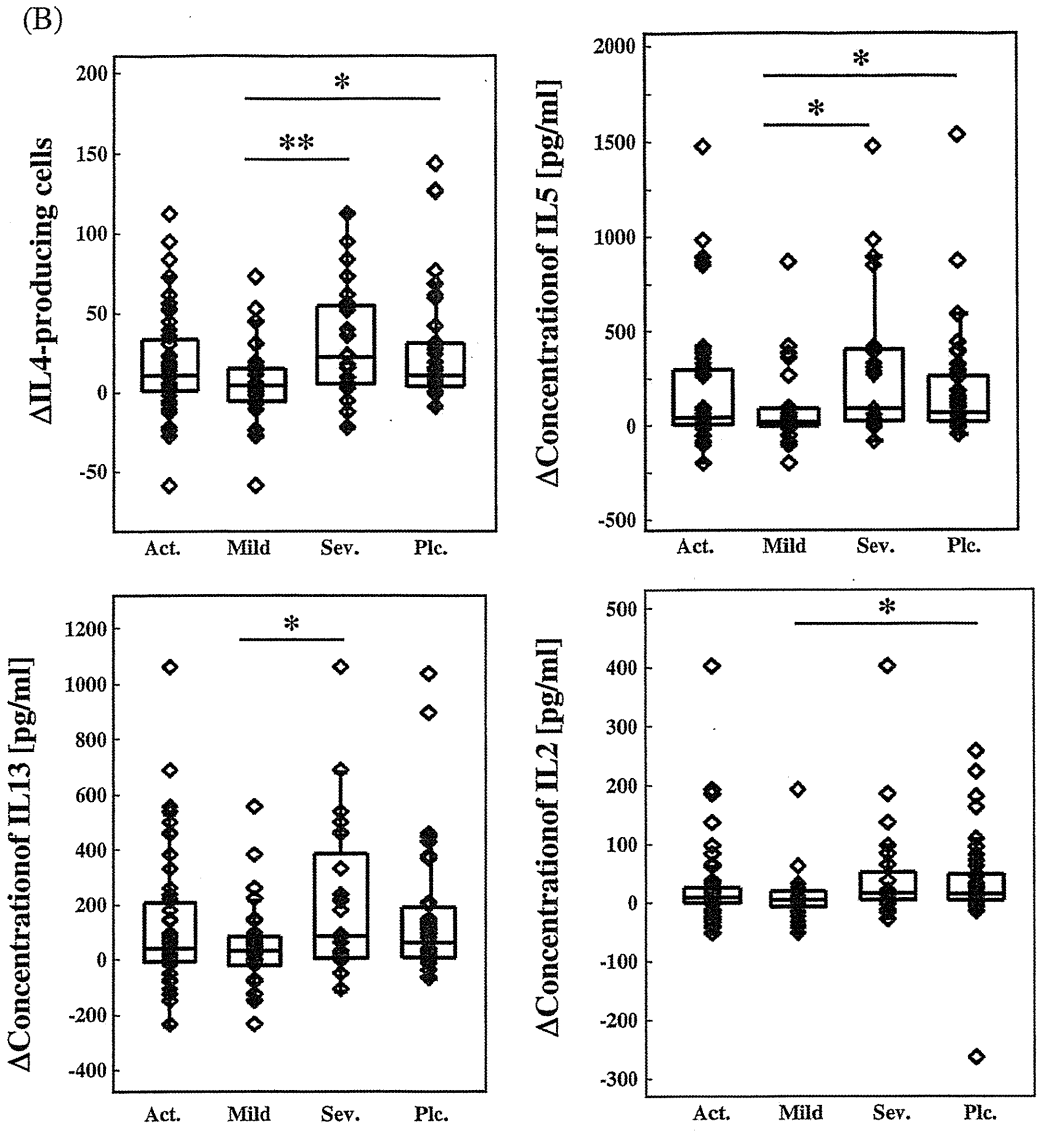

Figure 3 (*continued*).

significantly ameliorated compared to the placebo group in 2008. The percentage of mild subjects (SMS ≤ 4) in the SLIT group was 28% higher than that in the placebo group (SLIT, 55%; placebo, 27%), and the SMS was reduced by approximately 21% in the SLIT group compared with the placebo group (SLIT, 4.2; placebo, 5.3). This percentage of mild subjects differ significantly between the SLIT and placebo groups (P=0.009; 2×2 Chi-squared test). These effects following 2-year treatment were comparable to those in a trial of 1-year daily treatment using grass pollen tablets [12]. The low dose of the extract (about 1/40th of that used in Europe) may be one reason for the poor clinical outcome in the first year [13]. An extract of concentration >2000 JAU is not available for clinical use in Japan, and the clinical effects, safety, and optimum schedule for administration of an extract with a much higher allergen concentration remain unclear.

Positive clinical therapeutic effects were not obtained following 1-year treatment in our study, even in OT analysis (data not shown). In contrast, two previous reports demonstrated positive therapeutic effects after 1-year SLIT for Japanese cedar pollinosis [3,4]. However, in these studies, the annual pollen count (1154 grains/cm^2/season) [3] was less than in our study, and daily SMS was significantly attenuated on only 4 days in the pollen season [4]. The severity of SMS is affected by the amount of Japanese cedar pollen in the total and peak pollen season. Natural resolution and tolerance are not usually induced by natural exposure to Japanese cedar pollen, regardless of the amount of pollen [14].

Whether there are detectable alterations in peripheral T-cell responses after specific immunotherapy is still under debate [15–18]. The Cry j 1-specific cytokine profile from the SLIT group did not differ significantly from that in the placebo group. However, the increases in IL2, IL4, IL5, and IL13 production in the mild subgroup in the SLIT group were significantly attenuated (or showed a tendency to be attenuated) compared to the severe subgroup and the placebo

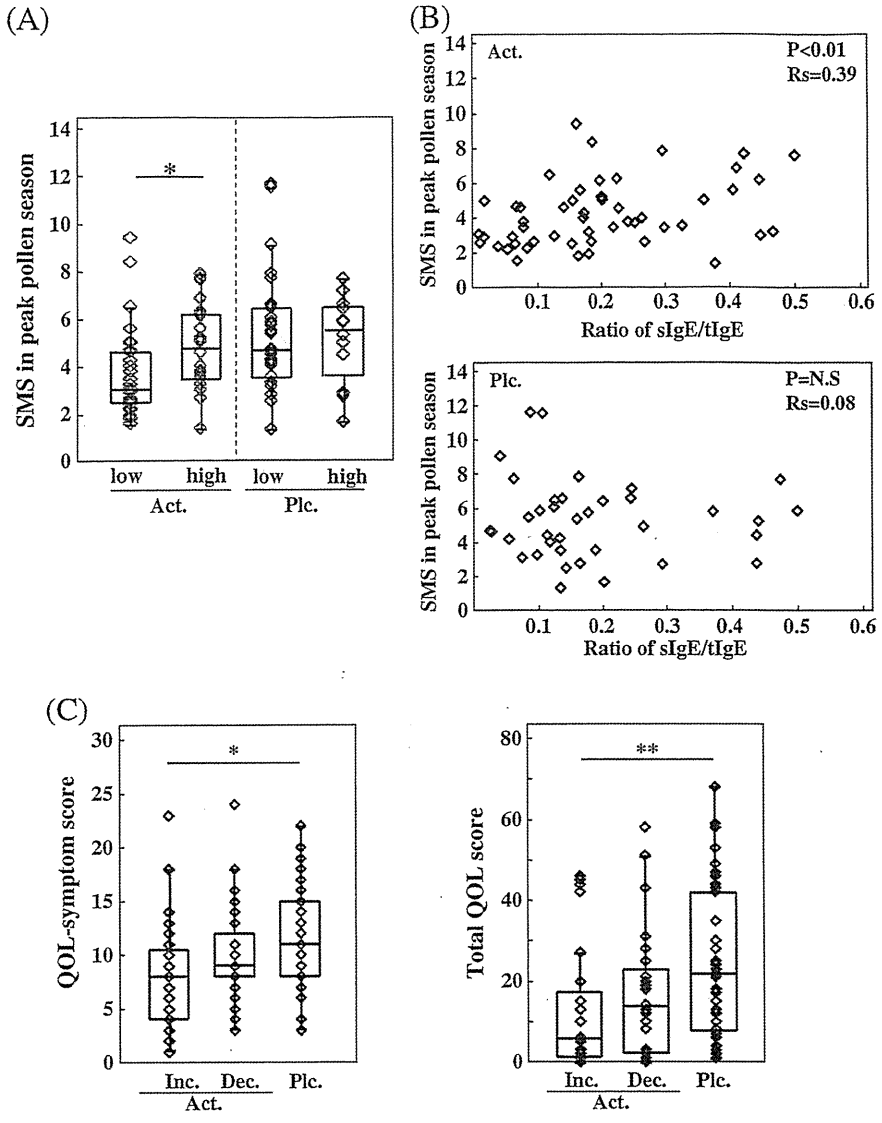

Figure 4 Biomarkers for positive therapeutic effects following SLIT. (A) SMSs in the 2008 peak pollen season for patients with low (low; $N=28$) and high (high; $N=23$) sIgE/tIgE ratios in the SLIT group (Act.), and for those with low ($N=25$) and high ($N=12$) sIgE/tIgE ratios in the placebo group (Plc.). *$P<0.05$. (B) Correlation between SMSs in the 2008 peak pollen season and sIgE/tIgE ratios before treatment in the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups. Statistical data were obtained with Spearman correlation analysis. (C) QOL-symptom and total QOL scores from the QOL questionnaire plotted for a subgroup with increased Cry j 1-iTreg in the SLIT group (Inc.; $N=24$), a subgroup with decreased Cry j 1-iTreg in the SLIT group (Dec.; $N=27$), and the placebo group (Plc.; $N=37$) in the middle of the 2008 pollen season. Each diamond shows the value for an individual. *$P<0.05$, **$P<0.01$.

group (Fig. 3B). The SMS in all patients in the study correlated with the seasonal increases in IL4 ($R=0.35$, $P<0.01$), IL5 ($R=0.35$, $P<0.01$), and IL13 ($R=0.36$, $P<0.01$). The discrepancy in our current results and the results of previous studies with regard to downregulation of cytokine production from PBMCs may depend on the extent of the therapeutic effects achieved in each clinical trial.

Cry j 1-specific IgE production was not changed by treatment, even in the mild subgroup, as also found in our preliminary study [6]. We speculate that more time is required for changing antibody production following the changes of antigen-specific T cell profiles, because the alteration of T cell profiles strongly influences subsequent class switch recombination of B cells and antibody production. Another possibility is that the dose for SLIT used in this study was not high enough to alter the antibody profiles.

The sIgE/tIgE ratio has been found to be significantly higher in responders than in non-responders following 4-year immunotherapy [19]. In our trial, this ratio did not differ significantly between responders and non-responders (P=N.S.; Mann–Whitney U-test). However, subjects with a low sIgE/tIgE ratio before treatment were more likely to be responders to 2-year SLIT, and the ratio correlated with the SMS only in patients treated with SLIT (Fig. 4A, B). This suggests that SLIT was more effective in patients with a low sIgE/tIgE ratio than in those with a high sIgE/tIgE ratio. The range of total IgE levels for the participants were relatively wide (6.8–2090 IU/ml in all patients); however, the change of the total IgE for each

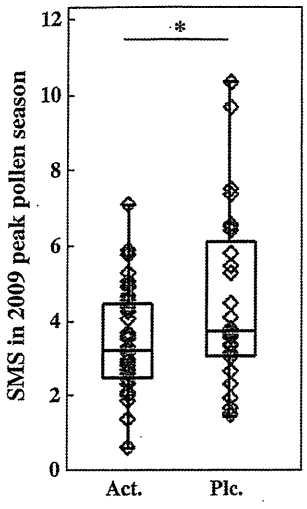

Figure 5 Carry-over effects following 2-year treatment with SLIT. SMSs in the 2009 peak pollen season were plotted for the SLIT (Act.; N=36) and placebo (Plc.; N=27) groups. Each diamond shows the value for an individual. Two-group comparisons were performed using an unpaired t-test.

individuals after 2-year treatment was not significantly different compared to before treatment (1.5±1.0 times higher, P=N.S.; paired t-test). Therefore, the wide range of total IgE levels was due to the variability on the allergic status for individuals, but not on method for measurement. The serum IgE level may affect the surface IgE level on effector cells such as mast cells and basophils, and Tregs can downregulate activation of mast cells and eosinophils [20,21]. We speculate that effector cells with a low specific IgE level are less likely to be activated by antigen crosslinking or are more susceptible to downregulation by Tregs than those with a high specific IgE level. It is also possible that the symptoms of patients with a low sIgE/tIgE ratio may be more readily attenuated by suboptimal potentiation of iTreg induced by SLIT.

We previously reported that an increased count of Cry j 1-iTregs was a candidate biomarker that could be used to distinguish between responders and non-responders to SLIT, as evaluated by the QOL-symptom score. In this report, the subgroup with increased Cry j 1-iTregs showed significant amelioration of the QOL-symptom and total QOL scores compared to the placebo group, while the subgroup with decreased Cry j 1-iTregs did not show this response (Fig. 4C). However, there was no significant difference in Cry j 1-specific cytokine production from PBMCs among patients with increased iTregs and decreased iTregs, and those in the placebo group (data not shown). Foxp3-expressing $CD25^+CD3^+$ cells and IL10-expressing $CD3^+$ cells, which are induced in the nasal mucosa after subcutaneous immunotherapy, have been linked to the clinical efficacy and suppression of seasonal inflammation [22]. Immunotherapy using an Amb a 1-immunostimulatory oligodeoxynucleotide conjugate also induced $CD4^+CD25^+$ T cells and IL10-producing cells in the nasal mucosa after the pollen season [23]. These data suggest that iTregs may downregulate effector cells at local sites of inflammation to suppress clinical symptoms. Induction of iTregs in the nasal mucosa and functional analysis of these cells may be necessary to determine the regulatory mechanisms affected by SLIT. Mucosal biopsy in the peak pollen season is useful for evaluation of local induction of iTregs and downregulation of effector cells. However, nasal biopsy in the pollen season significantly influences the daily SMS in the peak pollen season. Mucosal biopsy outside the pollen season after exposure using an artificial pollen chamber may be a powerful tool for evaluation of local regulatory mechanisms induced by SLIT [24]. Upregulation of iTregs in nasal mucosa may be difficult to determine since the evaluation may be painful for patients. However, upregulation of iTregs in peripheral blood is simple to analyze and may be a useful biomarker because an increase of peripheral Cry j 1-iTregs is correlated with QOL and QOL-symptom scores in the pollen season, as discussed here and elsewhere [6].

Cry j 1-specific IgG4 production was not induced by SLIT in this study to the same extent as that in our previous study [6]. A clinical trial showing that daily 2500 SQ-T (14 μg Phl p 5 per 4 weeks) tablets failed to induce IgG production supports our current results [13]. A change in the immunoglobulin profile may require a higher allergen dose or longer duration of exposure. However, our study suggests that detectable quantitative changes in IgG4 are not essential for the amelioration of clinical symptoms.

In summary, we suggest that the sIgE/tIgE ratio and upregulation of iTregs may be considered as prognostic and response monitoring biomarkers, respectively, for SLIT. However, further investigation of induction of iTregs at local inflammatory sites and downregulation of inflammatory cells is needed. Furthermore, validation studies with larger sample size would be required before either biomarkers should be applied widely in the clinical management of pollinosis patients. Development of a more effective vaccine and better protocols may reveal more significant differences in the Cry j 1-specific cytokine profiles and iTreg induction, and these results may increase our understanding of the roles of iTregs or Tr1 in the therapeutic mechanisms underlying the efficacy of SLIT.

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IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation

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Background: IL-22 functions as both a proinflammatory cytokine and an anti-inflammatory cytokine in various inflammations, depending on the cellular and cytokine milieu. However, the roles of IL-22 in the regulation of allergic airway inflammation are still largely unknown.

Objective: We sought to determine whether IL-22 is involved in the regulation of allergic airway inflammation.

Methods: We examined IL-22 production and its cellular source at the site of antigen-induced airway inflammation in mice. We also examined the effect of IL-22 neutralization, as well as IL-22 administration, on antigen-induced airway inflammation. We finally examined the effect of IL-22 on IL-25 production from a lung epithelial cell line (MLE-15 cells).

Results: Antigen inhalation induced IL-22 production in the airways of sensitized mice. $CD4^+$ T cells, but not other lymphocytes or innate cells, infiltrating in the airways produced IL-22, and one third of IL-22–producing $CD4^+$ T cells also produced IL-17A. The neutralization of IL-22 by anti–IL-22 antibody enhanced antigen-induced IL-13 production, eosinophil recruitment, and goblet cell hyperplasia in the airways. On the other hand, intranasal administration of recombinant IL-22 attenuated antigen-induced eosinophil recruitment into the airways. Moreover, anti–IL-22 antibody enhanced antigen-induced IL-25 production in the airways, and anti–IL-25 antibody reversed the enhancing effect of anti–IL-22 antibody on antigen-induced eosinophil recruitment into the airways. Finally, IL-22 inhibited IL-13–mediated enhancement of IL-25 expression in IL-1β– or LPS-stimulated MLE-15 cells.

Conclusion: IL-22 attenuates antigen-induced airway inflammation, possibly by inhibiting IL-25 production by lung epithelial cells. (J Allergy Clin Immunol 2011;128:1067-76.)

Key words: Allergic inflammation, asthma, IL-22, eosinophils, IL-25

Asthma is chronic airway inflammation characterized by eosinophil infiltration, mucus hypersecretion, and airway hyperresponsiveness (AHR) to a variety of stimuli.[1-3] These characteristics are mainly mediated by antigen-specific T_H2 cells and their cytokines, including IL-4, IL-5, and IL-13.[1-3] In addition, a number of studies have revealed that the airways of patients with severe asthma exhibit neutrophil infiltration accompanied by IL-17A production.[4-6] Moreover, we and others have shown that T_H17 cells induce neutrophilic airway inflammation in part through the production of IL-17A.[7,8] More recently, IL-22, one of the T_H17 cell–derived cytokines,[9] has been detected in the airways in a murine model of asthma.[10,11]

IL-22 is a member of the IL-10 cytokine family with multiple functions in various inflammatory diseases.[12,13] The fact that IL-22 markedly increases the expression of antimicrobially acting proteins in various epithelia suggests a role for this cytokine in innate immune defense.[12,13] Although previous studies have demonstrated that IL-22 is mainly produced by T_H1 and T_H17 cells,[9,12,13] recent studies have shown that skin-homing $CCR10^+$ T cells also produce IL-22 without IL-17A production and that these IL-22–producing $CD4^+$ T cells (T_H22 cells) show a stable and distinct phenotype from T_H1, T_H2, and T_H17 cells.[14-16] In addition, it has been demonstrated that a population of natural killer (NK) cells, $CD11c^+$ myeloid cells, and lymphoid tissue inducer (LTi)–like cells produce IL-22.[9,17-19]

IL-22 mediates its effects through a heterodimeric transmembrane receptor complex composed of IL-22 receptor 1 (IL-22R1) and IL-10 receptor 2 (IL-10R2) and subsequent JAK–signal transducer and activator of transcription (STAT) signaling pathways, including Jak1, Tyk2, and STAT3.[12,13] IL-10R2 has been shown to function as a receptor component not only of IL-22 but also of IL-10, IL-26, IL-28, and IL-29 and to be ubiquitously expressed in a variety of cells.[12,13,20] On the other hand, it has been shown that IL-22R1 is a receptor component of IL-22, IL-20, and IL-24, and its expression is restricted to nonimmune cells, such as epithelial cells in the intestine and lung and keratinocytes in the skin.[12,13,20,21] Indeed, it has been reported that neither resting nor activated immune cells, including T cells, B cells, NK cells, macrophages, and dendritic cells, express IL-22R1.[20] These findings suggest that IL-22 acts on nonimmune cells in the skin, intestine, and lung.

Importantly, recent studies have shown that IL-22 exhibits both proinflammatory and anti-inflammatory properties.[13,22] The

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Abbreviations used
AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
BMDC: Bone marrow–derived dendritic cell
IL-10R2: IL-10 receptor 2
IL-22R1: IL-22 receptor 1
LTi: Lymphoid tissue inducer
NK: Natural killer
OVA: Ovalbumin
PAS: Periodic acid–Schiff
qPCR: Quantitative real-time PCR
SOCS3: Suppressor of cytokine signaling 3
STAT: Signal transducer and activator of transcription
TARC: Thymus and activation-regulated chemokine
TSLP: Thymic stromal lymphopoietin

proinflammatory properties of IL-22 have been supported by the finding that IL-22–deficient mice exhibited decreased acanthosis and reduced neutrophil infiltration in the inflamed skin after repeated treatments with IL-23.[9] In addition, it has been shown that IL-22–producing T cells are involved in the pathogenesis of inflammatory skin diseases through an IL-22– and TNF-α–dependent manner.[23] On the other hand, IL-22 production by NKp46$^+$ NK cells has been shown to be involved in mucosal defense mechanisms.[24] The beneficial properties of IL-22 are further underscored by the findings that IL-22 is involved in protection against bacterial pneumonia,[21] acute liver injury,[25] and murine models of inflammatory bowel disease.[26,27] These findings suggest that the functions of IL-22 are influenced by the cellular and cytokine milieu.

Recently, it has been shown that IL-22 is detected at the site of allergic airway inflammation.[10,11] In addition, Zhao et al[28] have reported that serum levels of IL-22 are higher in patients with severe asthma than those seen in patients with mild asthma and healthy control subjects. Moreover, it has been reported that IL-22 inhibits inflammatory responses in a murine model of asthma by modulating the function of dendritic cells.[10] Furthermore, Besnard et al[29] have recently shown that IL-22 is required for the sensitization phase of allergic inflammation but exerts inhibitory functions in the effector phase. However, the mechanisms by which IL-22 regulates allergic airway inflammation remain largely unknown.

In this study we sought to determine whether IL-22 regulates allergic airway inflammation in a murine model of asthma and, if so, to determine the mechanism by which this occurs. We found that IL-22 was produced by CD4$^+$ T cells infiltrating the airways on antigen challenge, that the neutralization of IL-22 by anti–IL-22 antibody in the effector phase enhanced antigen-induced eosinophil recruitment in the airways, and that intranasal administration of recombinant IL-22 inhibited antigen-induced eosinophil recruitment in the airways. We also found that anti–IL-22 antibody enhanced antigen-induced IL-25 production in the airways, which is known to enhance T_H2-type immune responses in the airways,[30-32] and indeed, coinjection of anti–IL-25 antibody reversed the enhancing effect of anti–IL-22 antibody on antigen-induced eosinophil recruitment into the airways. Finally, we found that IL-22 inhibited IL-13–mediated enhancement of IL-25 expression in an IL-1β– or LPS-stimulated lung epithelial cell line (MLE-15 cells). Our results suggest that IL-22 attenuates antigen-induced airway inflammation in part by inhibiting the expression of IL-25 in lung epithelial cells.

METHODS

Mice

BALB/c mice (Charles River Laboratories, Atsugi, Japan) were housed in microisolator cages under pathogen-free conditions. The Chiba University Animal Care and Use Committee approved the animal procedures used in this study.

Reagents

Polyclonal anti–IL-22 antibody and anti–IL-25 (IL-17E) antibody were obtained from R&D Systems (Minneapolis, Minn) and BioLegend (San Diego, Calif), respectively. The anti–IL-22 mAb (clone MH22B2) was described previously.[33] Recombinant cytokines were purchased from PeproTech (Rocky Hill, NJ). A murine lung epithelial cell line (MLE-15 cell) was a kind gift from Dr Jeffrey Whitsett (University of Cincinnati).[34] See the Methods section in this article's Online Repository at www.jacionline.org for further details.

Antigen-induced allergic inflammation in the airways

BALB/c mice (aged 6-8 weeks) were immunized intraperitoneally with ovalbumin (OVA) and challenged once with inhaled OVA, as described previously (see the Methods section in this article's Online Repository for further details).[35] For the analysis of goblet cell hyperplasia and AHR, OVA-sensitized mice were challenged with inhaled OVA 3 times at a 48-hour interval.[7] Where indicated, mice were injected intraperitoneally with anti–IL-22 antibody (20 μg per mouse), anti–IL-25 antibody (20 μg per mouse), or control antibody (BD Biosciences, San Diego, Calif) at 24 hours before the inhaled OVA challenge. In other experiments recombinant IL-22 (0.1 μg per mouse) or saline (as a control) was administered intranasally twice at 48 and 2 hours before the inhaled OVA challenge, respectively. The numbers of eosinophils, neutrophils, lymphocytes, and CD4$^+$ T cells recovered in bronchoalveolar lavage fluid (BALF) were evaluated at 48 hours after OVA inhalation, as described previously.[7]

Cytokine assay

The amounts of IL-5, IL-13, IL-22, IL-25, IL-33, IFN-γ, and thymic stromal lymphopoietin (TSLP) in BALF were determined by mean of ELISA, according to the manufactures' instructions (see the Methods section in this article's Online Repository for further details).

Measurement of airway responsiveness

Airway responsiveness to aerosolized acetylcholine was assessed by using a computer-controlled small animal ventilator system (flexiVent; SCIREQ, Inc, Montreal, Quebec, Canada), as described elsewhere.[36]

Cytokine production and chemokine receptors of CD4$^+$ T cells

CD4$^+$ T cells were isolated from BALF cells, inguinal lymph node cells, or lung homogenates by means of magnetic cell sorting.[7] For intracellular cytokine analysis, CD4$^+$ T cells were stimulated with phorbol 12-myristate 13-acetate (20 ng/mL; Calbiochem, San Diego, Calif) plus ionomycin (1 μg/mL, Calbiochem) at 37°C for 4 hours in the presence of brefeldin A (10 μmol/L, BD Bioscience). Cytokine profiles (IFN-γ, IL-4, IL-17A, and IL-22) and the expression of chemokine receptors (CCR3, CCR5, CCR6, and CCR10) of CD4$^+$ T cells were evaluated by means of flow cytometry (see the Methods section in this article's Online Repository for further details).

Histologic and immunohistologic analysis

The number of goblet cells was counted on periodic acid–Schiff (PAS)–stained lung sections, as described elsewhere.[31] Immunostaining of cryosections was performed as described previously (see the Methods section in this article's Online Repository for further details).[37]

Culture of MLE-15 cells

A murine lung epithelial cell line (MLE-15 cells) was grown in HITES medium, as described previously.[34] MLE-15 cells were stimulated with IL-1β (20 ng/mL) or LPS (100 ng/mL), IL-13 (20 ng/mL), or both in the presence or absence of IL-22 (20 ng/mL) in HITES medium for 6 hours.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qPCR) was performed with a standard protocol on an ABI PRISM 7300 instrument (Applied Biosystems, Foster City, Calif; see the Methods section in this article's Online Repository for further details).

Western blotting

Western blotting was performed as described previously[38] by using anti-STAT3 antibody, anti–phospho-STAT3 antibody, and anti–phospho-STAT6 antibody (Cell Signaling Technology, Boston, Mass).

Preparation of bone marrow–derived dendritic cells

Bone marrow–derived dendritic cells (BMDCs) were prepared as described previously (see the Methods section in this article's Online Repository for further details).[39]

Data analysis

Data are summarized as means ± SDs. The statistical analysis of the results was performed by using the unpaired *t* test. *P* values of less than .05 were considered significant.

RESULTS

Lung-infiltrating $CD4^+$ T cells produce IL-22 in a murine model of asthma

To determine whether IL-22 is involved in the regulation of antigen-induced airway inflammation, we first examined the expression of IL-22 at the site of allergic airway inflammation. Consistent with recent reports,[10,29] levels of IL-22 were increased in the BALF of OVA-sensitized mice at 48 hours after OVA inhalation (n = 5 mice in each group, $P < .05$; Fig 1, *A*). Even in the absence of OVA inhalation (saline inhalation), IL-22 was detected at low levels in BALF (Fig 1, *A*). The induction of IL-22 expression in the lungs of OVA-sensitized mice by OVA inhalation was confirmed at mRNA levels by means of qPCR analysis (data not shown).

$CD4^+$ T cells in BALF were isolated from OVA-sensitized mice at 48 hours after OVA inhalation, stimulated with phorbol 12-myristate 13-acetate plus ionomycin, and analyzed by means of intracellular cytokine staining to identify the cellular source of IL-22 expressed at the site of allergic airway inflammation. As shown in Fig 1, *B*, 1.6% ± 0.5% of $CD4^+CD3^+$ T cells in BALF were positive for intracellular IL-22 staining, whereas only 0.2% ± 0.3% of $CD4^+$ T cells from inguinal lymph nodes in the same mice were positive for intracellular IL-22 staining (means ± SDs, n = 4, $P < .05$; Fig 1, *B*). Among IL-22–producing $CD4^+$ T cells in BALF, 33.2% ± 8.5% of cells were also positive for IL-17A staining, whereas few IL-22–producing $CD4^+$ T cells were positive for IFN-γ or IL-4 staining (Fig 1, *C*). Further analysis revealed that 21.6% ± 6.3% of IL-22–producing $CD4^+$ T cells expressed CCR6, a representative chemokine receptor expressed on T_H17 cells and LTi cells (Fig 1, *D*). In contrast, IL-22–producing $CD4^+$ T cells did not express CCR10 (Fig 1, *D*), which has been shown to be expressed on skin-homing IL-22–producing T cells in human subjects.[14,16] Moreover, IL-22–producing $CD4^+$ T cells lacked the expression of CCR5 and CCR3 and representative chemokine receptors expressed on T_H1 cells and T_H2 cells, respectively.

Previous studies have shown that not only $CD4^+$ T cells but also alveolar macrophages from patients with inflammatory lung disease and a population of NK cells in secondary lymphoid tissues produce IL-22.[40,41] To confirm IL-22 production by $CD4^+$ T cells, we performed immunostaining on BALF cells recovered from OVA-sensitized, OVA-inhaled mice. Although the frequency of cells showing a positive signal for anti–IL-22 staining was less than 1% of cells in BALF, approximately 80% of signals with anti–IL-22 staining were colocalized with $CD4^+$ cells (Fig 1, *E*). In addition, IL-22 mRNA was detected in $CD4^+$ cells but not in $CD8^+$ cells, $B220^+$ cells, $DX5^+$ cells, or $CD11c^+$ cells recovered from the lung of OVA-sensitized, OVA-inhaled mice (see Fig E1 in this article's Online Repository at www.jacionline.org). Moreover, IL-22 mRNA was not detected in $CD4^+CD3\epsilon^-$ cells in the lung (see Fig E1), suggesting that LTi-like cells are not responsible for IL-22 production in this asthma model. These results suggest that $CD4^+$ T cells, including T_H17 cells, are a major source for IL-22 in patients with allergic airway inflammation.

Anti–IL-22 antibody enhances antigen-induced eosinophil recruitment into the airways and AHR

To address the role of IL-22 in antigen-induced airway inflammation, we next examined the effects of IL-22 neutralization on the development of antigen-induced airway inflammation. Anti–IL-22 polyclonal antibody (20 μg per mouse), which neutralizes the activity of IL-22,[42] or control antibody was injected intraperitoneally into OVA-sensitized mice, and 24 hours later, the mice were challenged with OVA inhalation. As shown in Fig 2, *A*, anti–IL-22 antibody significantly enhanced antigen-induced eosinophil and $CD4^+$ T-cell recruitment into the airways (n = 10 mice in each group, $P < .05$). Similarly, anti–IL-22 mAb (clone MH22B2), which exhibits IL-22–neutralizing capacity equivalent to polyclonal anti–IL-22 antibody, enhanced antigen-induced eosinophil and $CD4^+$ T-cell recruitment into the airways (data not shown). In addition, anti–IL-22 antibody significantly enhanced the levels of IL-13 in BALF (n = 10 mice in each group, $P < .05$; Fig 2, *B*). On the other hand, IL-5 levels in BALF in anti–IL-22 antibody-treated mice were similar to those seen in control antibody-treated mice (Fig 2, *B*). The expression of IL-23 p19 and IL-12/IL-23 p40 mRNA in the lungs in anti–IL-22 antibody–treated mice was also similar to that seen in control antibody–treated mice (data not shown).

Histologic analysis revealed that increased eosinophil counts in BALF in anti–IL-22 antibody–treated mice were associated with enhanced peribronchial and perivascular inflammatory cell infiltration, which consists of lymphocytes, eosinophils, and some neutrophils, compared with that seen in control mice (Fig 3, *A*). PAS staining of lung sections revealed that goblet cell hyperplasia was also enhanced in anti–IL-22 antibody–treated mice (n = 5, $P < .05$; Fig 3, *A*). To determine whether IL-22 is involved in antigen-induced AHR, we examined the effects of anti–IL-22 antibody on airway responsiveness to acetylcholine in OVA-sensitized mice after OVA inhalation. Consistent with the enhanced eosinophil recruitment and IL-13 production in anti–IL-22 antibody–treated mice (Fig 2), airway responsiveness to acetylcholine was significantly enhanced in anti–IL-22 antibody–treated mice (n = 10, $P < .05$; Fig 3, *B*). These results

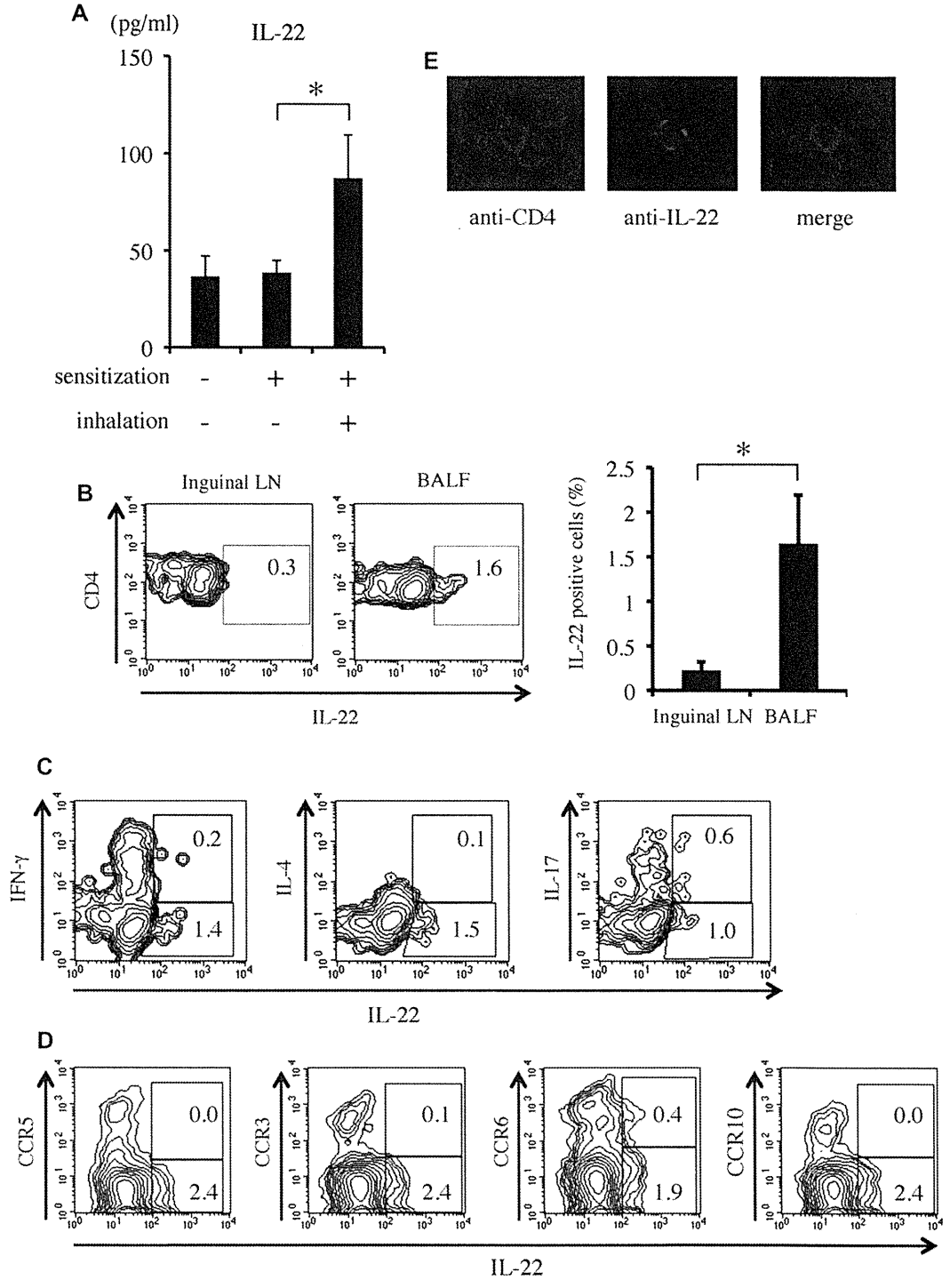

FIG 1. Lung-infiltrating $CD4^+$ T cells produce IL-22 in allergic airway inflammation. OVA-sensitized BALB/c mice were challenged with inhaled OVA or saline (control). IL-22 levels and $CD4^+$ T-cell numbers in BALF were then analyzed at 48 hours after OVA inhalation. **A**, IL-22 levels are increased in the lungs of sensitized mice on antigen inhalation. The amounts of IL-22 in BALF were determined by means of ELISA. Data are presented as means ± SDs for 5 mice in each group. $*P < .05$. **B**, IL-22–producing $CD4^+$ T-cell numbers are increased in allergic airway inflammation. $CD4^+$ T cells in BALF or inguinal lymph nodes (control) were collected by means of magnetic cell sorting and analyzed for the expression of CD4 and intracellular IL-22. Representative CD4 versus IL-22 staining of cells from BALF and inguinal lymph nodes *(LN)* and means ± SDs of the percentage of $IL\text{-}22^+$ cells among $CD4^+$ T cells are shown (n = 4 each). $*P < .05$. **C**, $CD4^+$ T cells in BALF were analyzed for intracellular IL-22 together with IFN-γ, IL-4, and IL-17. **D**, $CD4^+$ T cells in BALF were analyzed for intracellular IL-22 together with CCR5, CCR3, CCR6, and CCR10. **E**, Cells in BALF were stained with anti-CD4 antibody and anti–IL-22 antibody. Representative photomicrographs from 4 mice are shown.