inflammation was exacerbated by downregulation of Eomes in memory Th2 cells.

DISCUSSION

We have identified functionally relevant IL-5-producing memory Th2 cells in the CD62L^{lo}CXCR3^{lo} subpopulation, which are required for the induction of Th2 cell-dependent eosinophilic airway inflammation. The possible molecular mechanisms that control IL-5 production in memory Th2 cells are depicted in the graphical abstract. Upon TCR stimulation, all four CD62L^{lo}CXCR3^{lo}, CD62L^{lo}CXCR3^{hi}, CD62L^{hi}CXCR3^{lo}, and CD62L^{hi}CXCR3^{hi} subpopulations can produce a large amount of both IL-4 and IL-13. However, only a fraction of the CD62L^{lo}CXCR3^{lo} subpopulation can produce IL-5. In this population, the expression of Eomes is limited, and high H3-K4 methylation at the *Il5*p was detected. The IL-5 nonproducing CD62L^{lo}CXCR3^{lo} subpopulation also shows high H3-K4 methylation at the *Il5*p, but this subpopulation contains two types of cells: one expresses high amounts of both Eomes and GATA3, and the other expresses high amounts of Eomes but low amounts of GATA3. The other three subpopulations do not produce IL-5 and show low H3-K4 methylation at the *Il5*p. These three populations contain roughly the same proportion of GATA3^{hi}Eomes^{lo}, GATA3^{hi}Eomes^{hi}, and GATA3^{lo}Eomes^{hi} cells as compared to the CD62L^{lo}CXCR3^{lo} population, indicating that Eomes may not play an important role in the expression of IL-5 in these populations. Moreover, GATA3 expression itself appears insufficient for the expression of IL-5 in the majority of the memory Th2 cells because very few GATA3^{hi} cells express IL-5 even in the absence of high-level Eomes expression. Therefore, other unknown factors appear to repress the expression of IL-5 or prevent the activation of IL-5 transcription in these populations, regardless of the expression of GATA3. Although the mechanisms that regulate histone modification at the *Il5* locus in memory Th2 cells remain unknown, H3-K4 methylation appears to be associated with the ability to produce IL-5.

The regulatory mechanism governing expression of Eomes in CD4$^+$ T cells has not been well established. Although previous reports suggest that IFN-γ upregulates the expression of Eomes in CD4$^+$ T cells (Suto et al., 2006), under some conditions, IL-4 appears to induce the expression of Eomes in antigen-stimulated CD8$^+$ and CD4$^+$ T cells (Takemoto et al., 2006; Weinreich et al., 2009). Based on these findings and the results of a DNA microarray analysis via IL-5$^-$ and IL-5$^+$ memory Th2 cells, we have defined several candidate molecules that may participate in control of Eomes expression in the IL-5-producing CD62L^{lo}CXCR3^{lo} subpopulation. Reduced mRNA expression of the IFN-γ receptor component *Ifngr2*, the IFN-γ downstream signaling molecule *Stat1*, and also the IL-4 receptor α chain (*Il4ra*) were detected in IL-5$^+$ memory Th2 cells as compared to IL-5$^-$ memory Th2 cells, and therefore, reduced expression of these molecules may contribute to the low expression of Eomes in IL-5$^+$ memory Th2 cells. A recent report showed that Eomes can be upregulated when GATA3 expression ceases in Th2 cells (Yagi et al., 2010). We observed a reciprocal expression profile of GATA3 and Eomes in all four subpopulations (CD62L^{lo}CXCR3^{lo}, CD62L^{lo}CXCR3^{hi}, CD62L^{hi}CXCR3^{lo}, and CD62L^{hi}CXCR3^{hi}) of memory Th2 cells. Therefore, the counterregulation of expression of GATA3 and Eomes may exist in CD4$^+$ T cells. Eomes-mediated IL-5 suppression probably occurs predominantly in GATA3 and Eomes double-expressing cells in the CD62L^{lo}CXCR3^{lo} population. However, it is also possible that other Eomes-dependent and -independent mechanisms operate in this population. Eomes may mediate indirect control of *Il5* expression by altering expression and/or function of other transcription factors or work in concert with other factors to ultimately suppress IL-5 production. The expression of Eomes was downregulated in memory Th2 cells after secondary challenge with antigen for 6 days (data not shown). In addition, the upregulation of Eomes was detected also in memory CD4$^+$ T cells induced by the immunization of antigen in vivo (data not shown).

Eomes siRNA gene targeting experiments revealed that the expression of *Il5* is more dependent on Eomes expression as compared to *Il4* and *Il13* in both memory Th2 and MPCD4$^+$ T cells. Furthermore, enforced expression of *Eomes* suppressed the expression of IL-5 but not IL-4 and IL-13 in in vitro developing effector Th2 cells. Both GATA3^{hi} and GATA3^{lo} memory Th2 cells produced substantial amounts of IL-4, but only GATA3^{hi} cells produced IL-5. Indeed, IL-5 expression is known to be more dependent on the expression levels of GATA3 as compared to IL-4 (Inami et al., 2004). Eomes was found to interact with GATA3 in memory Th2 cells and suppress GATA3 DNA binding to the *Il5*p. These results may explain the preferential effect of Eomes on *Il5* expression as compared to *Il4* and *Il13*.

The current study indicates that the IL-5-producing CD62L^{lo}CXCR3^{lo} population of memory Th2 cells is essential for the induction of allergic eosinophilic inflammation and AHR and that Eomes plays an important role in suppression of IL-5 and the induction of eosinophilic inflammation. Better defining the IL-5-producing CD4$^+$ T cells in allergic disorders may help to identify appropriate targets for intervention and the analysis of downstream target molecules of Eomes may be of interest. The DNA microarray analysis identified several potentially functional cell surface molecules upregulated on IL-5$^+$ memory Th2 cells. Interestingly, *Il1rl1* (*ST2*) is upregulated in IL-5$^+$ memory Th2 cells. IL-1RL1 is also the receptor for IL-33, a member of the IL-1 family, and IL-33- and ST2-mediated signaling triggers the activation of NF-κB leading to the production of Th2 cytokines (Kurowska-Stolarska et al., 2008; Schmitz et al., 2005). Therefore, the IL-5-producing CD62L^{lo}CXCR3^{lo} population of memory Th2 cells identified in this study could be critical in the pathogenesis of chronic type 2 inflammation. Although preliminary, human IL-5-producing CD45RO$^+$ memory CD4$^+$ T cells in the peripheral blood showed decreased *EOMES* expression as compared to IL-5 nonproducing memory CD4$^+$ T cells, and siRNA gene targeting of *EOMES* enhanced *IL5* expression in the CD45RO$^+$ memory CD4$^+$ T cells (data not shown). Thus, further detailed studies focused on the IL-5-producing memory Th2 cells in chronic asthma models may lead to the discovery of novel therapeutic targets for the treatment of asthma.

In summary, we have identified IL-5-producing functionally relevant memory Th2 cells in the CD62L^{lo}CXCR3^{lo} subpopulation. *Il5* expression is uniquely regulated by the expression of Eomes in memory Th2 cells. Eomes plays an important role in

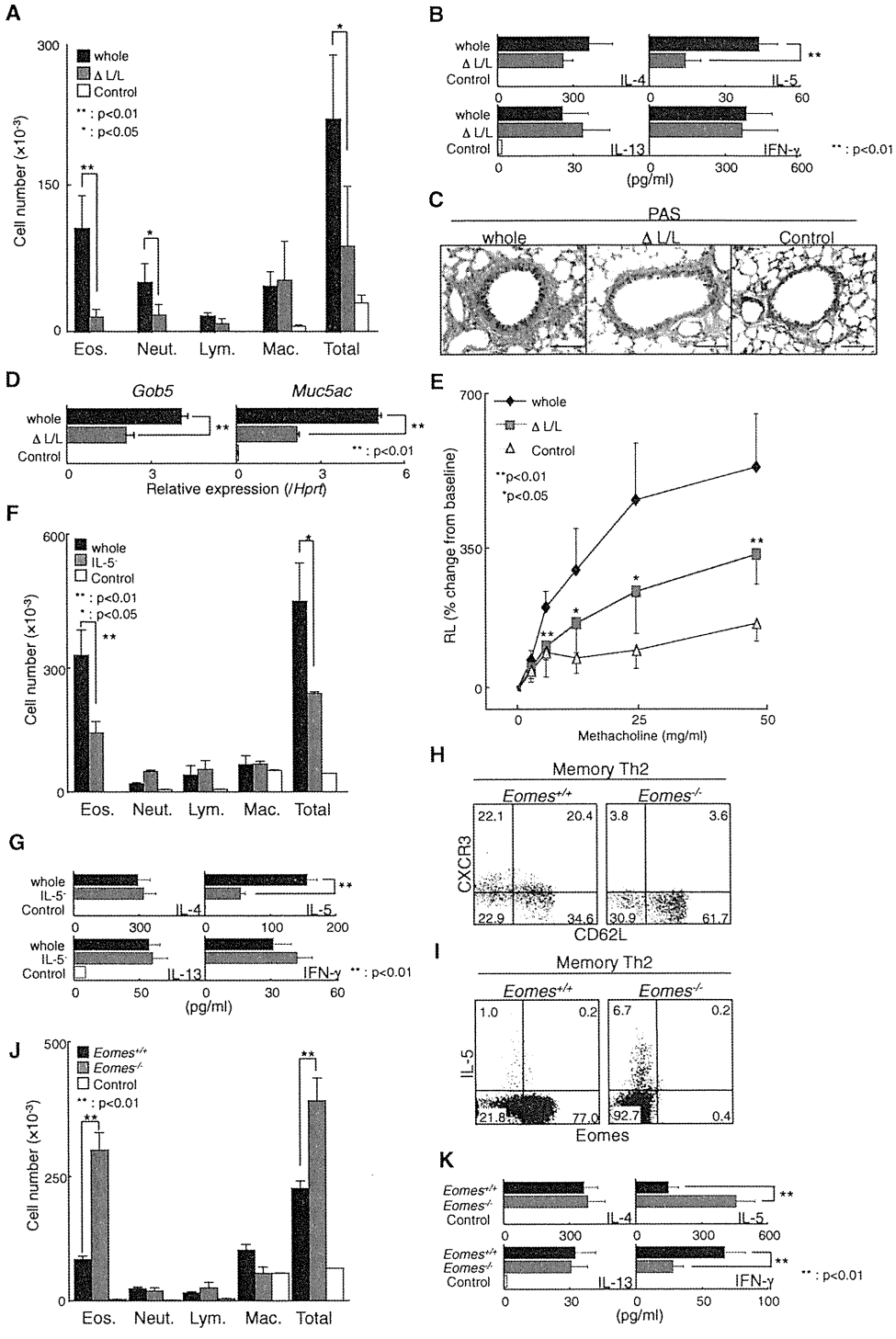

Figure 6. Memory Th2 Cell-Dependent Airway Inflammation Is Ameliorated after Depletion of the CD62L^{lo}CXCR3^{lo} Population

(A–E) OVA-specific memory Th2 cells were sorted into KJ1^{+} whole memory Th2 cells (whole) and memory Th2 cells depleted of the CD62L^{lo}CXCR3^{lo} population (ΔL/L) by FACS. These cells were intravenously transferred into BALB/c (A–D) or BALB/c *nu/nu* (E) mice. No cell transfer was performed in the control group (Control). Airway inflammation was induced with OVA challenge.

(A) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. The results were calculated with the percentages of the different cell types, the total cell number per milliliter, and the volume of the BAL fluid recovered. Samples were collected 2 days after the last OVA challenge. The mean values (five mice per group) are shown with SD.

(B) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid. Samples were collected 12 hr after the last OVA challenge. The mean values (five mice per group) are shown with SD. **p < 0.01.

the development of memory Th2 cell-dependent allergic airway inflammation demonstrating a role for Eomes in the regulation of polarized function of $CD4^+$ T cells.

EXPERIMENTAL PROCEDURES

Mice

The animals used in this study were backcrossed to BALB/c or C57BL/6 mice 10 times. Anti-OVA-specific TCR-αβ (DO11.10) transgenic (Tg) mice were provided by D. Loh (Washington University School of Medicine, St. Louis) (Murphy et al., 1990). *Eomes*$^{fl/fl}$ mice were kindly provided by S. Reiner (Pennsylvania University) (Intlekofer et al., 2008). Ly5.1 mice were purchased from Sankyo Laboratory. CD4-Cre mice were purchased from Taconic Farms (Germantown, NY). All mice were used at 6–8 weeks old and were maintained under SPF conditions. BALB/c and BALB/c *nu/nu* mice were purchased from Clea Inc. (Tokyo). Animal care was conducted in accordance with the guidelines of Chiba University.

The Generation of Effector and Memory Th1 and Th2 Cells

Effector and memory Th1 and Th2 cells were generated as previously described (Inami et al., 2004; Yamashita et al., 2006). The detailed protocols are described in the Supplemental Experimental Procedures.

Flow Cytometry and Sorting

Memory Th2 cells were stained with anti-CD62L-APC and anti-CXCR3-PE, and four subpopulations ($CD62L^{lo}CXCR3^{lo}$, $CD62L^{lo}CXCR3^{hi}$, $CD62L^{hi}CXCR3^{lo}$, and $CD62L^{hi}CXCR3^{hi}$) were purified by FACS. Memory Th2 cells were stimulated with immobilized anti-TCRβ for 6 hr, and $IL\text{-}5^+$ and $IL\text{-}5^-$ cells were purified with an IL-5 secretion assay kit (130-091-175, Miltenyi Biotec.) and FACS. The other reagents used in flow cytometry are listed in the Supplemental Experimental Procedures.

Quantitative Real-Time PCR and ELISA for the Measurement of Cytokine Expression

Quantitative RT-PCR and ELISA were performed as described previously (Yamashita et al., 2006).

Chromatin Immunoprecipitation Assay

ChIP assays were performed as described previously (Yamashita et al., 2002). The antibodies and primer pairs used in the ChIP assays are listed in the Supplemental Experimental Procedures.

siRNA Gene Targeting Analysis

siRNA was introduced into memory Th2 or $MPCD4^+$ T cells by electroporation with a mouse T cell Nucleofector Kit and Nucleofector I (Amaxa). Memory Th2 cells and $MPCD4^+$ T cells were transfected with 675 pmole of control random siRNA or siRNA for *Eomes* and *Tbx21* (Applied Biosystems) and cultured for 24 hr.

Immunoprecipitation, Immunoblotting, and Pull-Down Assay

The detailed protocol is described in the Supplemental Experimental Procedures.

Assessment of Memory Th2 Cell Function In Vivo

OVA-specific memory Th2 cells were first generated in vivo (Nakayama and Yamashita, 2009). AHR was assessed on day 4 as described previously (Yamashita et al., 2008). The mRNA expression of *Gob5* and *Muc5ac* in the lung was assessed on day 5 (Yamashita et al., 2006). BAL fluid for the analysis of cytokine production by ELISA was collected 12 hr after the last inhalation and that for the assessment of inflammatory cell infiltration was collected on day 5. Lung histology was assessed on day 5.

Statistical Analysis

Student's t test was used for all comparisons, data represented as mean ± SD.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE33516.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2011.08.017.

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(C) Two days after the last OVA challenge, the lungs were fixed and stained with periodic-acid-Schiff (PAS). A representative staining pattern is shown. Scale bars represent 100 μm.
(D) Quantitative RT-PCR analysis of *Gob5* and *Muc5ac* from the lung tissue 2 days after the last OVA challenge. $^{**}p < 0.01$.
(E) One day after the last OVA inhalation, changes in lung resistance (RL) were assessed. The mean values (five mice per group) are shown with standard deviations.
The experiments were performed twice with similar results (A, B, D, and E).
(F) The number of infiltrated leukocytes in the BAL fluid from whole or $IL\text{-}5^-$ memory Th2 cell transferred group are shown as in (A).
(G) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid from each experimental group is shown in (F).
(H and I) *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cells were generated by transferring OT-II Tg-CD4-Cre-$CD45.1^+$ or OT-II Tg-CD4-Cre-*Eomes*$^{fl/fl}$-$CD45.1^+$ effector Th2 cells into *TCRbd*$^{-/-}$ mice.
(H) *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cells were stained with CD62L and CXCR3 mAbs.
(I) *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cells were stimulated in vitro with immobilized anti-TCRβ for 6 hr. Intracellular staining profiles of Eomes and IL-5 are shown with the percentage of cells in each area.
(J) The absolute cell numbers of leukocytes recovered in the BAL fluid of *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cell transferred groups are shown as in (A).
(K) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid of the experiments shown in (J).
The experiments were performed twice with similar results (F–K).

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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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KEYWORDS
Allergic rhinitis;
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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.

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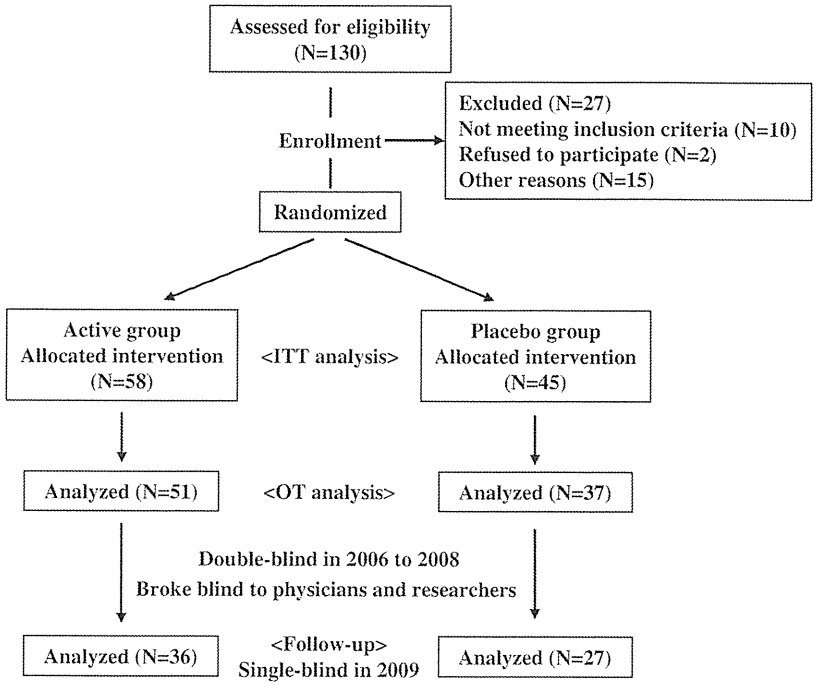

Figure 1 Flow diagram for groups and individuals in the phases of the randomized trial. Fifteen participants from the SLIT ($N=7$) and placebo ($N=8$) groups were lost to follow-up due to reasons such as moving house and transfer. The double-blind status was maintained until completion of analysis of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

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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.

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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).

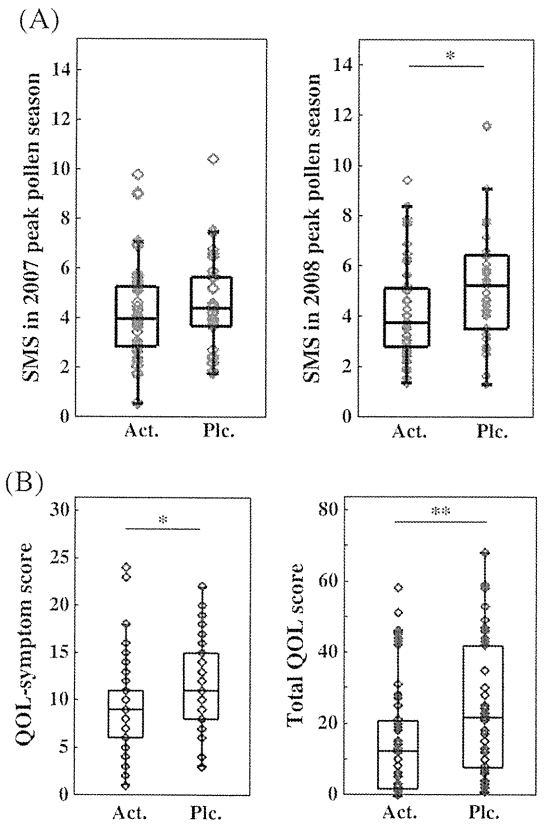

Figure 2 Clinical scores after 2-year SLIT. (A) Average daily symptom-medication scores (SMS) in the SLIT (Act.; $N=51$) and placebo (Plc.; $N=37$) groups in the 2007 and 2008 peak pollen seasons. (B) QOL-symptom and total QOL scores from the QOL questionnaire were plotted for the SLIT (Act.; $N=51$) 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).

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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

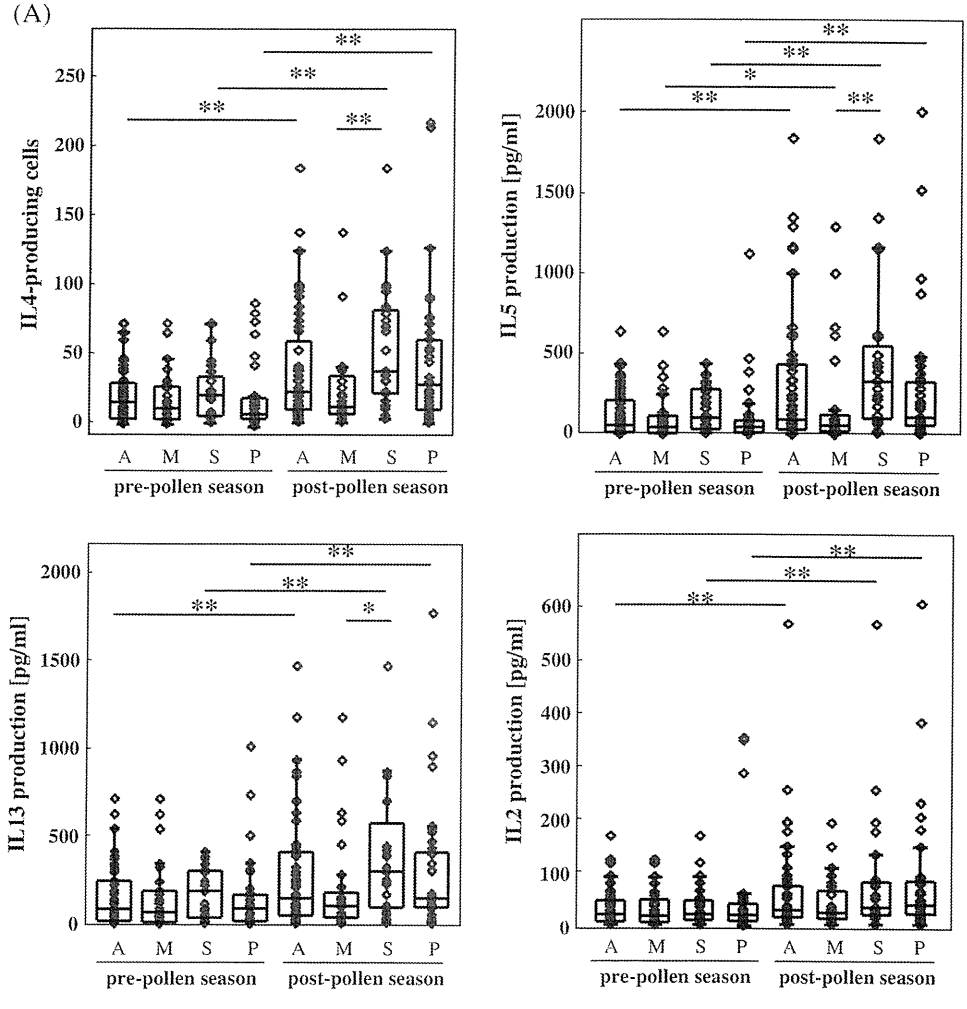

Figure 3 Cytokine production from PBMCs. (A) Number of Cry j 1-specific IL4-producing cells and Cry j 1-specific cytokine levels in the SLIT group (A; $N=51$), the mild subgroup of the SLIT group (M; $N=28$), the severe subgroup of the SLIT group (S; $N=23$), and the placebo group (P; $N=37$) at before 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. *$P<0.05$, **$P<0.01$.

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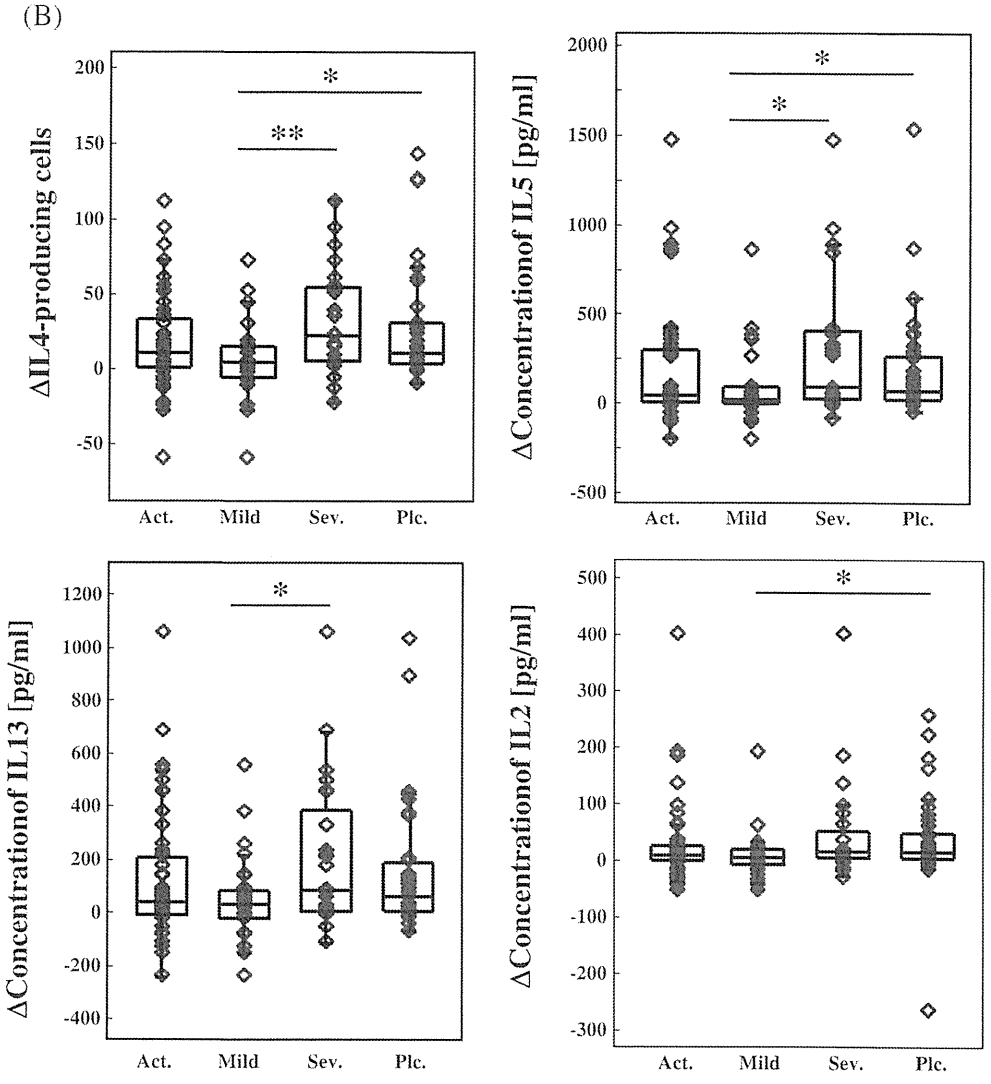

Figure 3 (*continued*).

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

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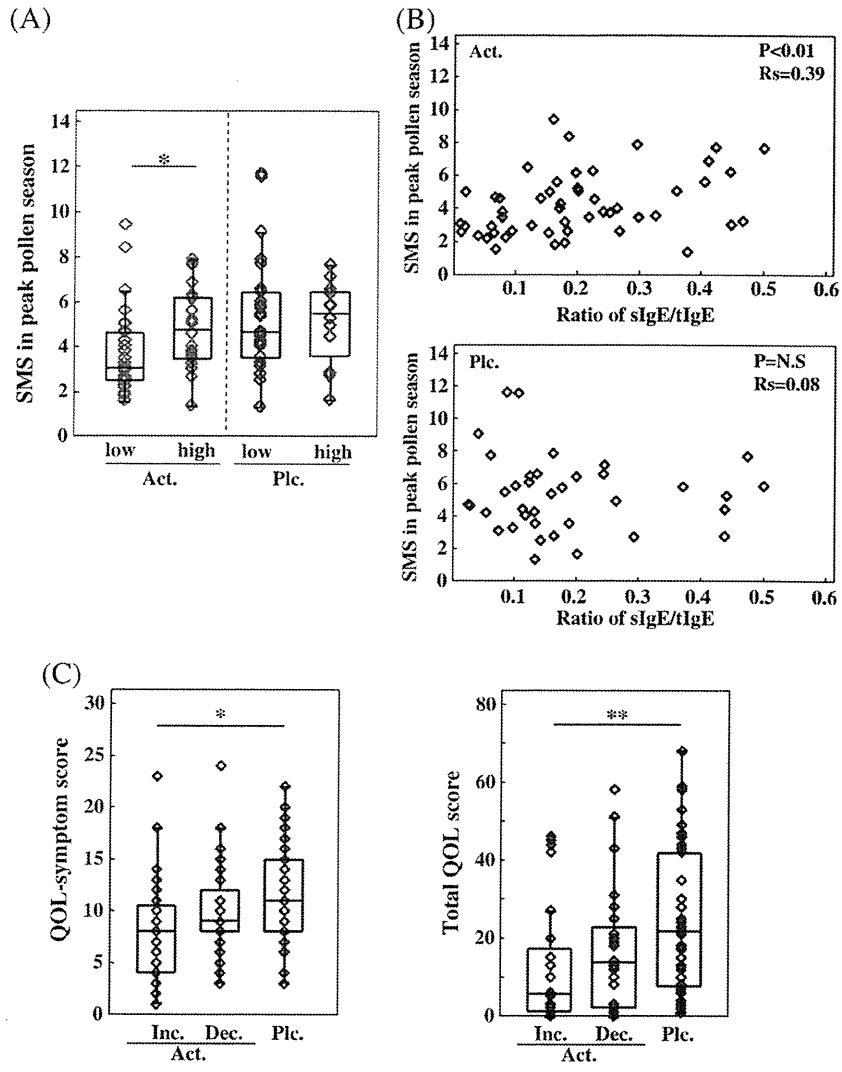

Figure 4 Biomarkers for positive therapeutic effects following SLIT. (A) SMSs in the 2008 peak pollen season for patients with low (low; $N=28$) and high (high; $N=23$) sIgE/tIgE ratios in the SLIT group (Act.), and for those with low ($N=25$) and high ($N=12$) sIgE/tIgE ratios in the placebo group (Plc.). *$P<0.05$. (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

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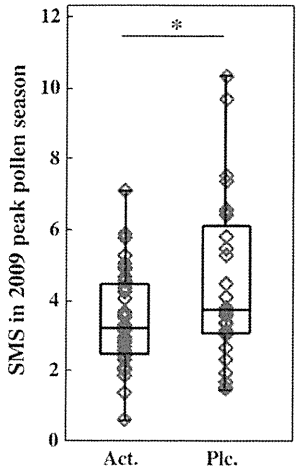

Figure 5 Carry-over effects following 2-year treatment with SLIT. SMSs in the 2009 peak pollen season were plotted for the SLIT (Act.; $N=36$) and placebo (Plc.; $N=27$) groups. Each diamond shows the value for an individual. Two-group comparisons were performed using an unpaired t-test.

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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