SLIT Improves Cedar Pollinosis by Restoring IL-10 Production from Tr1 and Monocytes—IL-10 Productivity Is Critical for Becoming Allergic—

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ABSTRACT
Background: Allergen-specific immunotherapy (SIT) is currently used for several allergic disorders and IL-10-producing regulatory T cells (Tr1) induced by SIT suppress allergic reactions. We investigated the relation between IL-10 production and acquiring allergy.

Methods: A prospective study was undertaken to evaluate the effect of SIT on IL-10 production in T cells and other cell fractions in children with pollinosis. In addition, blood samples were collected from non-allergic healthy controls and patients with pollinosis to compare the levels of IL-10 production. PBMC were cultured with pollen peptides or control allergens, and the IL-10 production from monocyte and CD4 T cell was analyzed.

Results: Monocytes and CD4 T cells from SIT group of patients produced high levels of IL-10, suggesting that the induction of IL-10 is essential for inducing T cell tolerance. IL-10 production from monocytes and T cells was significantly increased in non-allergic controls compared to patients with pollinosis. This high IL-10 production was observed even when PBMC were stimulated with antigens other than pollen peptides.

Conclusions: IL-10 is critical for induction of specific T cell tolerance, and increased production of IL-10 by monocytes and T cells during inflammatory responses or after SIT may influence effector cells in allergy. Present data implicates that the low productivity of IL-10 by monocytes and T cells is closely related with sensitivity to multiple allergens, and resistance to allergic diseases. Augmentation of constitutive IL-10 production from immune system is a potential therapeutic approach for allergic disorders.

KEY WORDS
allergen-specific immunotherapy, interleukin-10, monocytes, regulatory T cells, tolerance

ABBREVIATIONS
SIT, allergen-specific immunotherapy; Tr1, IL-10 producing regulatory T cell; SLIT, sublingual allergen-specific immunotherapy; PBMC, peripheral blood mononuclear cells; nTregs, naturally occurring regulatory T cells; CFSE, 5 (and 6)-carboxyfluorescein diacetate, succinimidyl ester; SMS, symptom medication score; TNSS, total nasal symptom score.
INTRODUCTION

IL-10 is a regulatory cytokine secreted during inflammatory responses where they inhibit T cell proliferation. IL-10 plays a pivotal role in the acquisition and maintenance of specific T cell tolerance.1 IL-10 is produced by a variety of cells including monocytes/macrophages, T cells (Th1, Th2, Th17),2,3 B cells,4 and mast cells.5,6 Peptide-based allergen-specific immunotherapy (SIT) is currently being used for the treatment of several allergic diseases. IL-10-producing regulatory T cells (Tr1) induced by SIT suppress allergic reactions.7,9 Immunotherapy for Japanese cedar pollinosis is performed by sublingual application of a pool of pollen peptides containing Cry j1 and Cry j2, which are the major Japanese cedar pollen allergens, and its clinical efficacy on seasonal allergic rhinitis has been previously appraised.10-12 Sublingual allergen-specific immunotherapy (SLIT) is very suitable to children with allergy because it is safe (eg, no risk of anaphylaxis) and its administration is not painful. Allergen-specific regulatory T cells play important roles in the mechanism of immunoprotection.7,13-15 We have recently demonstrated that one of the mechanism of the beneficial effect of SLIT depends on its ability to increase the number of circulating Tr1 (IL-10+CD4+ T cell).9 The increased Tr1 population in SLIT-received patients functionally suppresses T cell proliferation when stimulated by pollen peptides compared to that from pollen allergy patients, and this suppressive effect was neutralized by the addition of anti IL-10 antibody or anti-IL-10 receptor antibody, confirming that the suppression of reactivity against cedar pollen in SLIT-received patients is IL-10-dependent; however, whether its effect is time-dependent remains still unclear. In the present study, we performed a prospective SLIT in children with pollinosis, to compare the percentage of Tr1 cells and other cell fractions between pre-treatment, during-treatment, and post-treatment. In addition, to evaluate whether low IL-10 production is associated with development of allergic disease, the level of IL-10 production was compared between patients with allergy to Japanese cedar pollen and non-allergic healthy controls. We discussed the possible mechanism of becoming sensitive to multiple allergens.

METHODS

PATIENTS AND SLIT PROTOCOL

This prospective study comprised 46 children (age, 14.1 ± 7.7 years; male/female, 35/11) sensitive to Japanese cedar referred to the Department of Otorhinolaryngology of Mie University Hospital. Diagnosis was based on clinical symptoms and serological results. Blood was drawn after obtaining written informed consent from all subjects and the investigational protocol was approved by the Institutional Review Board (IRB) of Mie University Hospital. The protocol details of SLIT are described previously.12 In brief, the allergen administration was every day from beginning to the forth week. On the first week, 2 JAU of allergen was administered from 1 drop to 10 drops, on the second week, 20 JAU of allergen was administered from 1 drop to 10 drops, on the third week, 200 JAU of allergen was administered from 1 drop to 10 drops, and then on forth week 2000 JAU of allergen was administered from 1 drop to 20 drops, as the final dose. On the fifth week twice a week, after the sixth week, 2000 JAU/ml was administered to sublingual 20 drops as the final highest dose by once a week. Blood samples were collected pre- (November to December), during- (at the fifth week, January), and after-completing SIT (February).

In other experiments, fresh blood was also collected from 21 adult non-allergic healthy volunteers (age, 31.1 ± 5.7 years; male/female, 14/7) and 25 adult patients (age, 33.1 ± 7.1 years; male/female, 20/5) with allergy to Japanese cedar pollen. This protocol was also approved by IRB.

PBMC CULTURE

Peripheral blood mononuclear cells (PBMC) were isolated from 20 mL of heparinized venous blood by density gradient centrifugation using Ficoll (Sigma, St. Louis, MO, USA). PBMC were cultured in RPMI 1640 medium (Nikken Bio Medical Laboratory, Kyoto, Japan) containing L-glutamine supplemented with 100 U/mL penicillin, 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 10% Human AB serum (Gemini Bio-Products, West Sacramento, CA, USA). The cells were plated on 24-well tissue culture plates at a density of 2 × 10⁶ cells/1 mL/well and were incubated with 50 IU of Cry j1 and Cry j2 (Torii, Tokyo, Japan) for 8 h at 37°C in an atmosphere of 5% CO₂. In a different experiment, PBMC were stimulated with 1 μg/mL of tetanus toxoid (Kaketsukun, Kumamoto, Japan) or 250 ng/mL of PPD derived from Mycobacterium Bovis (Japan BCG, Tokyo, Japan) as control antigens. Endotoxin level was approved to be less than 0.1 ng/µg (1 EU/µg) of the protein in Cry j1 and Cry j2, tetanus toxoid, and PPD.

STAINING OF NATURALLY OCCURRING REGULATORY T CELLS (nTregs)

For identification of nTreg (FoxP3+CD25highCD4+ T cells), freshly isolated PBMC were directly stained with monoclonal antibodies to CD4-PE/Cy7 and CD25-PE in cell surface staining buffer containing 0.1 M PBS and 2% FCS (Biowest, Nuaille, France). Then, intracellular staining with Foxp3-PE/Cy5 antibody was performed according to the manufacturer’s instruction (eBioscience, San Diego, CA, USA). The population of nTreg was analyzed using a Becton Dickinson FACScan instrument (Becton Dickinson, Mansfield, MA, USA).
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Fig. 1  a, Naturally occurring regulatory T cells (nTregs). The percentage of FoxP3+CD25^{high}CD4^{+} T cells/nTreg was unchanged during SLIT in patients with pollinosis.  

b, Proliferation assay. The proliferation was significantly suppressed with the addition of Tr1, and this suppressive effect was neutralized by the addition of 2 μg/mL of anti IL-10 antibody, confirming that categorized Tr1 has the suppressive function.  
c, The percentage of IL-10-producing T cells, B cells, and monocytes in children undergoing SLIT. The percentage of circulating IL-10 producing monocytes was significantly increased during and after SLIT compared to pre-treatment values. The percentage of circulating Tr1 cells was significantly increased after the completion of SLIT. The number of IL-10 producing CD8^{+} T cells and B cells remained unchanged during SLIT.

### Key Points
- Naturally occurring regulatory T cells (nTregs) were unchanged during SLIT in patients with pollinosis.
- Proliferation assay: Tr1 significantly suppressed proliferation, which was neutralized by anti IL-10 antibody.
- IL-10 production increased in monocytes during and after SLIT.
- Circulating Tr1 cells increased after SLIT completion.
- CD8^{+} T cells and B cells remained unchanged during SLIT.
PROLIFERATION ASSAY
In order to confirm that IL-10 producing T cells defined Tr1 is truly functional or not, the proliferation assay was performed. 2 x 10^6/mL of PBMC were cultured with 50 IU of Cry j1 and Cry j2 as mentioned above. On the day 2, PBMC were collected and washed, and then Tr1 was purified with using CD4 T cell isolation kit II followed by IL-10 secretion assay cell enrichment and detection kit (Miltenyi Biotec, Auburn, CA, USA). CD4+IL-10+T cells were categorized as non-Tr1. Simultaneously fresh PBMC were collected from the same donor on the day 2, and then CD4 T cells were purified in the same way. Collected CD4 T cells were labeled with 10 μM of 5 (and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Eugene, OR, USA) and then 2 x 10^4/200 μL of CFSE-stained CD4 T cells were stimulated by 10 IU of Cry j1 and Cry j2 in the presence of 2 x 10^4 of non-Tr1 cells, with or without 2 x 10^4 of Tr1 and 2 μg/mL of anti-IL-10 antibody in 96-well culture plate. After 4 days culture, cells were collected and cell proliferation was analyzed by Becton Dickinson FACScan instrument. The percentage of proliferative cells was calculated (n = 6 per each population).

STATING OF IL-10 IN T CELLS, B CELLS, AND MONOCYTES
After 8 h culture with antigens, PBMC were collected and incubated with PE-conjugated IL-10 secretion assay kit according to the manufacturer’s instructions (Miltenyi Biotec). Cells were also co-stained with anti-CD4-FITC and anti-CD8-PerCp antibody (Becton Dickinson, Franklin Lakes, NJ, USA) and anti-CD14-FITC (eBioscience, San Diego, CA, USA) and anti-CD19 PECy5 antibody, or anti-CD144-FITC (eBioscience, San Diego, CA, USA) and anti-CD8-PerCp antibody (Becton Dickinson, Franklin Lakes, NJ, USA). The percentage of circulating IL-10 producing monocytes and IL-10 producing CD4+ T cells, CD8+ T cells, B cells, and monocytes were analyzed by flow cytometry.

EFFICACY OF SLIT AND THE PERCENTAGE OF TR1 OR IL-10 PRODUCING MONOCYTES
In order to investigate the correlation between the efficacy of SLIT and the percentage of Tr1 and IL-10 producing monocytes, symptom medication score (SMS) and total nasal symptom score (TNSS) were employed in children’s SLIT cases. Nasal and ocular symptoms and medication(s) prescribed were classified from score 0 to 4 and score 0 to 3, respectively. The sum of these scores was used as the symptom-medication score (SMS). On the other hand, nasal symptoms of sneezing, nasal discharge, and nasal congestion were recorded each day. Symptoms were categorized on a 5-point scale (0: none, 1: mild, 2: moderate, 3: severe, 4: extremely severe) according to Practical Guideline for the Management of Allergic Rhinitis in Japan.11 The average of TNSS for the three nasal symptom items in the cedar pollen season of February and March 2008 was calculated.

STATISTICAL ANALYSIS
Statistical analysis was performed using the Kruskal-Wallis nonparametric ANOVA and Dunn’s multiple comparison test for post hoc analysis. A p value of less than 0.05 was considered as statistically significant. The relation between SMS or TNSS and the percentage of Tr1 or IL-10 producing monocytes was analyzed by relative correlation test.

RESULTS
NATURALLY OCCURRING REGULATORY T CELLS (nTregs)
The percentage of nTreg (FoxP3+CD25highCD4+ T cells/CD4+ T cells) remained unchanged during SLIT in patients with pollinosis (Fig. 1a).

PROLIFERATION ASSAY
The percentage of CD4+lymphocytes proliferation was measured and found that the proliferation was significantly suppressed when Tr1 was supplemented in the culture, and this suppressive effect was neutralized by the addition of 2 μg/mL of anti-IL-10 antibody, confirming that IL-10 producing CD4 T cells categorized as Tr1 has the inhibitory function (Fig. 1b).

IL-10 PRODUCTION IN T CELL, B CELL, AND MONOCYTES DURING SLIT
The percentage of circulating IL-10 producing monocyte (IL-10+CD14+ monocytes/CD14+ monocytes) was significantly increased during and after SLIT compared to pre-treatment values. The percentage of circulating Tr1 (IL-10+CD4+ T cells/CD4+ T cells) cells was significantly increased after SLIT. However, the number of IL-10 producing CD8+ T cells and B cells remained unchanged during the course of SLIT (Fig. 1c).

CORRELATION BETWEEN THE EFFICACY OF SLIT AND THE PERCENTAGE OF TR1 OR IL-10 PRODUCING MONOCYTES
The correlation between SMS or TNSS and the percentage of Tr1 or IL-10 producing monocytes did not reach the significance (Fig. 2).

IL-10 PRODUCTION ANTIGEN STIMULATION IN SUBJECTS WITH AND WITHOUT ALLERGY
The percentage of both IL-10+CD14+monocyte/CD14+ monocytes and IL-10+CD4+ T cell/CD4+ T cells was significantly increased in PBMC from normal controls compared to patients with pollinosis when they were stimulated with Cry j1 and Cry j2. These significant elevations in the IL-10 producing cell population were also observed when PBMC were stimulated with tetanus toxoid or PPD (Fig. 3).

DISCUSSION
Here, we showed that monocytes and CD4+ T cells from patients with good response to SLIT produce
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Fig. 2 Correlation between the efficacy of SLIT and the percentage of Tr1 and IL-10 producing monocytes. In order to analyze the efficacy of SLIT, SMS and TNSS were employed for children’s SLIT cases. The correlation between SMS or TNSS and the percentage of Tr1 or IL-10 producing monocytes did not reach the significance.

high levels of IL-10; suggesting that production of IL-10 is essential for inducing T cell tolerance. In addition, we also discovered that IL-10 production by monocytes and T cells is significantly decreased in patients with Japanese cedar pollen allergy compared to non-allergic healthy controls. This low IL-10 production was observed even when PBMC were stimulated with antigens other than cedar pollen peptides. These observations suggest that low productivity of IL-10 may be critical for becoming sensitive to multiple allergens and for contracting allergic diseases.

In the first experiment, the percentage of circulating Tr1 (IL-10+CD4+ T cell/CD4+ T cell) cells was evaluated and found that it was significantly increased in patients after SLIT. The categorized Tr1 was proved to have functionally suppressive ability; which was neutralized with the addition of anti-IL-10 antibody. This increase in the Tr1 population may naturally be the result of clonally expanded antigen specific T cells. However, Tr1 cells can be generated in the presence of IL-10 locally released from Tr1 cells, and it is known that the mechanism of cell proliferation inhibition by Tr1 cells is cytokine-mediated but not via cell-to-cell contact. We have previously shown that Tr1 cells with high production of IL-10 induced by SLIT conserve high T cell repertoire diversity without showing monoclonal expansion. This observation suggests that, SLIT causes tolerance to multiple allergens by inducing Tr1 cells with high production of IL-10. Specific immunotherapy blocks allergen-specific IgE and induces an allergen-specific IgG response, which is stimulated by IL-10.
The percentage of IL-10-producing monocytes and T cells in healthy control and patients with pollinosis. PBMC from 21 healthy volunteers (N) and 25 patients with Japanese cedar pollen allergy (A) were cultured with Cry j1 and Cry j2 (Cry), tetanus toxoid (TT), or PPD. The percentages of IL-10 producing monocytes and CD4 T cells were significantly increased in PBMC from normal healthy individuals compared to those from patients with pollinosis, even when PBMC were stimulated with Cry j1 and Cry j2, tetanus toxoid, or PPD.

Fig. 3 The percentage of IL-10-producing monocytes and T cells in healthy control and patients with pollinosis. PBMC from 21 healthy volunteers (N) and 25 patients with Japanese cedar pollen allergy (A) were cultured with Cry j1 and Cry j2 (Cry), tetanus toxoid (TT), or PPD. The percentages of IL-10 producing monocytes and CD4 T cells were significantly increased in PBMC from normal healthy individuals compared to those from patients with pollinosis, even when PBMC were stimulated with Cry j1 and Cry j2, tetanus toxoid, or PPD.

from Tr1 cells. Another surprising finding is the induction of monocyte population with high IL-10 production ability by SLIT. The mechanism of this effect is not clear but function of monocytes is probably controllable by immunotherapy. Monocytes recognize antigens via Toll like receptors, digest antigen with intrinsic enzyme, and some monocyte population such as CD14+CD16+ monocyte presents antigens combined with its expressing MHC class II like antigen presenting cells. During this process, IL-10 production may be increased. The relative percentage of IL-10 positive fraction is higher in monocytes than in the Tr1 population, suggesting monocytes as potential therapeutic target of allergic disorders. Considering that the percentage of Tr1 and IL-10 producing monocytes population is correlated to clinical symptom or not can be answered with using SMS and TNSS scores. As shown in Figure 2, the significance was not observed among SLIT-receiving patients unfortunately, suggesting other factors influence the severity of clinical symptoms, and we need further investigation.

We then investigated the grade of IL-10 production in non-allergic healthy controls. As shown in Figure 3, the percentages of IL-10 producing monocyte and CD4 T cells were significantly elevated in Cry j1- and Cry j2-stimulated PBMC from normal healthy controls compared to those from patients with pollinosis. In addition, abundant IL-10 production was recognized even when PBMC were stimulated with control antigens such as tetanus toxoid or PPD. In addition to allergen specific suppressive effect, IL-10 may have broad potential in the immune modulations.

This study demonstrated that IL-10 is critical for induction of specific T cell tolerance, and that the increased production of IL-10 by monocytes and T cells may affect to effector cells during inflammatory responses to allergen or after therapy with SIT. These observations suggest that low production of IL-10 by monocytes and T cells may play a critical role in the initiation of allergic responses.

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