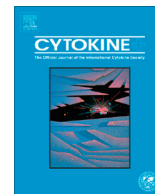




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Long-term sublingual immunotherapy for Japanese cedar pollinosis and the levels of IL-17A and complement components 3a and 5a

Masafumi Sakashita, Takechiyo Yamada*, Yoshimasa Imoto, Tomomitsu Hirota, Mayumi Tamari, Yumi Ito, Seita Kubo, Yoko Osawa, Noboru Takahashi, Shigeharu Fujieda

Department of Otorhinolaryngology, University of Fukui, Fukui 910-1193, Japan

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ABSTRACT

Allergen-specific immunotherapy is the only treatment that can alter the natural course of allergic disease. We performed long-term sublingual immunotherapy (SLIT) for patients with seasonal allergic rhinitis caused by Japanese cedar pollen (SAR-JCP), screened molecules as candidate biomarkers, and investigated serum IL-17A and complement components 3a (C3a) and C5a in order to evaluate whether these molecules show changes correlated to symptom scores. In this study, we found that the long-term SLIT reduced the serum levels of IL-17A and C3a and C5a. The levels of C3a in the patients significantly decreased from year 1 compared with those at the baseline, and their levels of IL-17A significantly decreased from year 2 compared with those at baseline. The levels of IL-17A, C3a, and C5a at year 4 of SLIT were significantly lower than not only those at baseline, but also those at year 1. A significant positive correlation was found between the symptom medication scores and the levels of IL-17A at year 4. The symptom medication scores in the group in which IL-17A levels decreased at year 4 were significantly lower than those in the group without such a decrease. The serum level of IL-17A might prove useful as a biological parameter to ascertain the effectiveness of SLIT for patients with SAR-JCP. It is necessary to produce new therapeutics for non-responders in whom serum IL-17A levels are still higher against long-term SLIT.

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1. Introduction

Seasonal allergic rhinitis (SAR) caused by Japanese cedar pollen (JCP) is the most common disease in Japan and we now consider it a national affliction [1]. Our recent study showed that over 35% of the Japanese population aged 20–50 years suffer from SAR during JCP dispersion [2]. The major allergen of SAR in Japan is Japanese cedar (*Cryptomeria japonica*) pollen. Crosslinking of antigen to IgE on the surface of mast cells and basophils induces the release of both preformed and newly generated mediators, and also manifests nasal allergic symptoms. While T_H2 dominance is a well-known SAR-related theme of study because T helper 2 (T_H2) cytokines induce IgE class switching and stimulate eosinophils

and the production of nasal mucin from the nasal glands, the roles of T_H17 cytokines are still controversial.

Both “Allergic Rhinitis and its Impact on Asthma” (ARIA) and the Japanese guidelines for allergic rhinitis recommend allergen-specific immunotherapy (AIT) for patients with severe SAR [3,4]. AIT has been used for more than 100 years as a desensitizing therapy for IgE-mediated allergic diseases and represents a potentially curative method of treatment. The mechanisms include the induction of very early desensitization of mast cells and basophils, generation of regulatory T- and regulatory B- cell responses, and regulation of IgE and IgG₄. It can prevent new sensitization/onset and provide long-term remission after the discontinuation of treatment. Sublingual immunotherapy (SLIT) has been widely used to treat patients with asthma and allergic rhinitis [5]. SLIT for SAR-JCP has also now started to be covered under the national health insurance system in Japan. The mechanisms of action of SLIT also include early desensitization effects, modulation of T cell and B cell responses, and induction of blocking antibody, as well as decreased migration of eosinophils, basophils, and mast cells to tissues and the release of their mediators [6]. Regulatory T cells have also been

Abbreviations: SLIT, sublingual immunotherapy; SAR, seasonal allergic rhinitis; JCP, Japanese cedar pollen; C3a, component 3a; T_H2 , T helper 2; ARIA, Allergic Rhinitis and its Impact on Asthma; AIT, allergen-specific immunotherapy.

* Corresponding author at: Department of Otorhinolaryngology, University of Fukui, 23-3 Matsuoka-Shimoaizuki, Eiheiji, Yoshida, Fukui 910-1193, Japan. Tel.: +81 776 61 8407; fax: +81 776 61 8118.

E-mail address: ymdtkcy@gmail.com (T. Yamada).

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identified as key regulators of immunologic processes in peripheral tolerance to allergens [7].

Although IL-17A had been thought to play an important role in neutrophil infiltration in tissues, IL-17A also contributes to allergic inflammation. Serum IL-17A levels were higher in the patients with SAR [8,9] and T_H17 cells responded to allergen [10]. IL-17A and T_H2 cytokines enhanced T_H2 -driven pathology, which led to more severe disease in a murine model. The severe asthma phenotype was driven by dysregulated anaphylatoxin control of the IL-23– T_H17 axis, leading complement component 3a (C3a) to enhance T_H17 responses and hyper-responsiveness [11]. C3a-induced IL-17 plays a critical role in an IgE-mediated response [12]. We previously found a significant association between single-nucleotide polymorphisms of Decay Accelerating Factor, an important regulator of C3 convertase and SAR-JCP and house dust mite-induced asthma in a Japanese population [13]. C5a can also affect the production and maintenance of IL-17-producing T cells [14]. Anaphylatoxins have various effects on cells, such as causing smooth muscle contraction, enhancing vascular permeability, and acting as chemoattractants for a wide range of leukocytes [15].

In this study, we performed SLIT for SAR-JCP patients, screened molecules as candidate biomarkers during long-term SLIT, and investigated the serum levels of IL-17A, C3a, and C5a in order to evaluate whether these molecules show changes that correlate to symptom scores during long-term SLIT.

2. Materials and methods

2.1. Subjects

For this study, twenty patients with SAR-JCP were enrolled from the general public through the Division of Otorhinolaryngology, Head & Neck Surgery, University of Fukui. These patients satisfied all of the following criteria: (a) they had Japanese cedar pollen-specific IgE; (b) they had suffered from allergic rhinitis induced by Japanese cedar pollen during February–April in Japan in the prior two years and had received medication as treatment; (c) they did not have any history of asthma; and (d) had not received prior allergen-specific immunotherapy.

Before enrolment, written informed consent was obtained from each study participant. The study was approved by the ethics committees of the Faculty of Medical Science, University of Fukui.

2.2. Study design

The patients received SLIT from a pre-seasonal period (October) through to the end of the high-pollen season (April) for four consecutive years. The induction phase lasted only 1 month in the first year, with administration of an increasing daily number of extract drops at three concentrations. One drop to 20 drops of prepared extract was held sublingually for 2 min. The antigen extract was administered sublingually daily from Week 1 to Week 4. Twenty drops of the antigen extract at 2000 Japanese Allergy Units (JAU) were administered two days per week in Week 5, once per week in Week 6 and thereafter throughout the season. The allergen for SLIT was standardized Japanese cedar pollen allergen that had been specially produced for subcutaneous immunotherapy. The concentration of 2000 JAU/ml is nearly equal to 3 μ g/ml Cry j1 (Torii Pharmaceutical Co. Ltd., Tokyo, Japan).

The patients carefully completed a diary, recording their nasal and ocular symptoms and their usage of rescue drugs. Nasal and ocular symptoms include sneezing, rhinorrhea, nasal blockage, itchy eyes, and watery eyes. Data were collected and analyzed at the Department of Otorhinolaryngology of Fukui University Hospital. The nasal and ocular symptoms were evaluated on a scale

of 0–4 in accordance with the Japanese guidelines for allergic rhinitis [3] as follows: 0 = no sensation; 1 = mild; 2 = moderate; 3 = severe; and 4 = extremely severe. Daily episodes of sneezing and nose blowing were rated on a scale of 0–4 as follows: 0 = none; 1 = 1–5 episodes; 2 = 6–10 episodes; 3 = 11–20 episodes; and 4 = more than 21 episodes. The medication scores were also recorded according to drug characteristics and the duration of usage according to the guidelines as follows: anti-histamines, mast cell stabilizers, and vasoconstrictors were listed as 1, while topical nasal, ophthalmic, or oral steroids were listed as 2 [16]. Because the five symptoms have ranges of 0–4, the daily symptom medication scores can be 20 or higher after the medication scores are summed. The peak pollen week was chosen to evaluate their symptom medication scores to represent the season. Then, seven consecutive daily symptom scores and medication scores were summed to produce symptom medication scores in the week with the highest amount of pollen. Therefore, this score could be 140 or higher after the medication scores were summed.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were obtained and centrifuged immediately to prevent degradation of complement components, and the serum was stored at -80°C within one hour of blood collection. We measured the levels of IL-17A, C3a, and C5a cytokines in the serum at the peak of pollen dispersion. Blood taken before starting SLIT had also been collected for use as a control. It is designated as “base-line” in these analyses. We conducted ELISA or a multiple assay system using kits (IL-17A: R&D Systems, Minneapolis, MN; C3a, C5a: BD Biosciences, San Jose, CA; IL-4, IL-5, IL-13, IFN- γ : Multiplex Assays, Millipore, Billerica, MA) according to the manufacturers' protocols.

2.4. Statistical analysis

Non-parametric Wilcoxon's signed-rank test was performed to evaluate differences. Correlation analysis was performed using Spearman's correlation coefficient by rank. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. The serum levels of IL-17A during long-term SLIT

A total of 9 males and 10 females aged from 34 to 66 years old completed the study. We assessed the changes in the serum levels

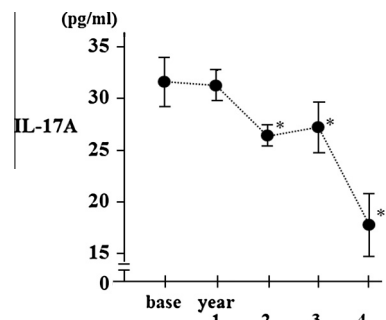


Fig. 1. The serum levels of IL-17A during long-term SLIT. The blood samples were obtained before SLIT (base) and at each high-pollen season (from year 1 to year 4) during long-term SLIT, and then the serum levels of IL-17A were measured using ELISA. The mean and SE are indicated with closed circles and lines. The levels of IL-17A were significantly lower at years 2, 3, and 4 than those at baseline (* $p < 0.05$, ** $p < 0.005$) ($n = 19$).

of IL-17A in four consecutive years. As depicted in Fig. 1, the levels of IL-17A in the patients decreased significantly by 17% at year 2 of SLIT compared with those at baseline ($p < 0.05$). The levels of IL-17A were also significantly lower at year 3 than those at baseline ($p < 0.05$). A further reduction by 44% was found at year 4 ($p < 0.005$). The levels of IL-17A at year 4 were significantly lower than those at year 1 ($p < 0.005$).

3.2. The serum levels of anaphylatoxins during long-term SLIT

Since the levels of anaphylatoxins in the serum of the patients with allergic rhinitis during SLIT remain unclear, we observed differences in the serum levels of C3a during long-term SLIT. The levels of C3a significantly decreased at year 1 compared with those at baseline ($p < 0.05$) (Fig. 2A). They showed a further decrease thereafter. Eventually, the C3a levels showed a reduction by 76% at year 4 compared with those at baseline ($p < 0.0005$). The levels of C3a at year 4 were significantly lower than those at year 1 ($p < 0.0005$).

The levels of C5a showed a significant decrease at year 4 by 84% compared with those at baseline ($p < 0.0005$) (Fig. 2B), while we could not detect any significant difference between the levels of C5a at baseline and those at year 1 to year 3. On the other hand, the levels of C5a at year 4 were significantly lower than those at year 1 ($p < 0.001$).

3.3. Serum levels of IL-17A and symptom medication scores during long-term SLIT

A biomarker should have a significant relationship to clinical symptoms. The symptom medication scores for Japanese cedar pollinosis were monitored at each high-pollen season during long-term SLIT, and we analyzed the correlation between the symptom medication scores and the serum levels of IL-17A, C3a, and C5a. The long-term SLIT significantly decreased all of the levels of IL-17A, C3a, and C5a at year 4 compared with those

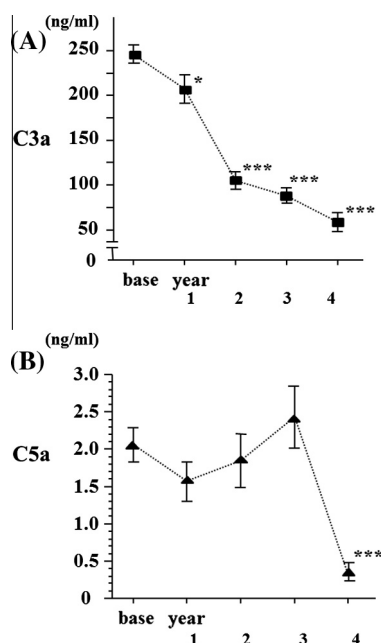


Fig. 2. The serum levels of anaphylatoxins during long-term SLIT. The serum levels of C3a (A) and C5a (B) were measured using ELISA as in Fig. 1B. The mean and SE are indicated with closed squares (A) or triangles (B), and lines. The levels of C3a were significantly lower at years 1, 2, 3, and 4 than those at baseline. The levels of C5a were significantly lower at year 4 than those at baseline (* $p < 0.05$, *** $p < 0.0005$) ($n = 19$).

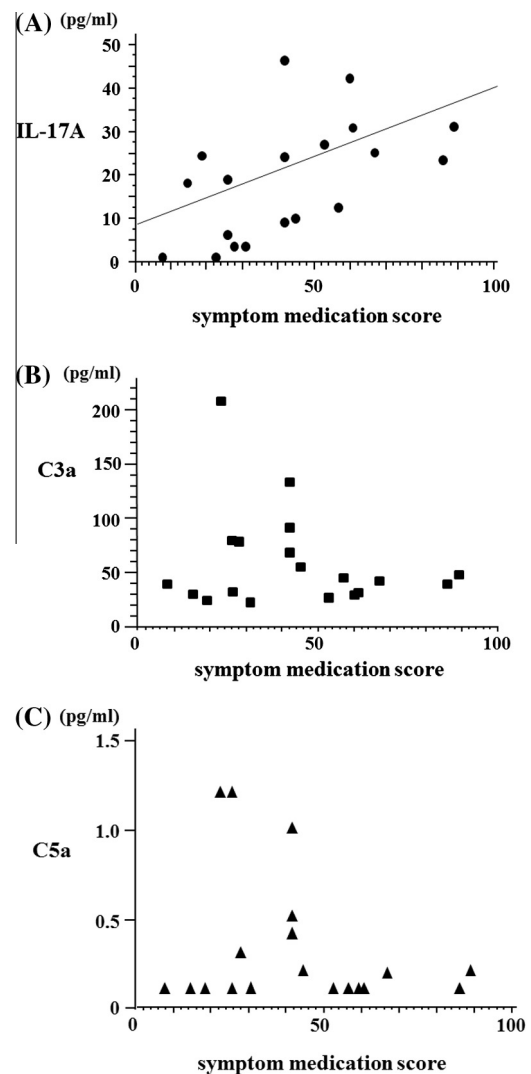


Fig. 3. Correlation between symptom medication scores and serum levels of IL-17A. The symptom medication scores were monitored at each high-pollen season during long-term SLIT, while the serum levels of IL-17A, C3a, and C5a were observed as in Figs. 1 and 2. The correlations between the symptom medication scores and the levels of IL-17A (A) ($\gamma = 0.57$, $p < 0.01$), C3a (B) ($\gamma = -0.05$, $p = 0.83$), and C5a (C) ($\gamma = -0.10$, $p = 0.67$) ($n = 19$) were examined using Spearman's correlation coefficient by rank.

both at baseline and at year 1. As shown in Fig. 3A, a significant positive correlation was found between the symptom medication scores and the levels of IL-17A at year 4 ($\gamma = 0.57$, $p < 0.01$). We could not detect any correlation between the symptom medication scores and the levels of C3a, nor those of C5a at year 4 (Fig. 3B and C). In this study, there was no significant correlation between the levels of IL-17 and the levels of C3a and C5a, from baseline to year 4.

In Fig. 4, we have divided the enrolled patients into two groups according to the serum levels of IL-17A at the high-pollen season in year 4. The serum levels of IL-17A in all patients were more than 20 pg/ml at baseline and at year 1. In group L ($n = 10$), the serum levels of IL-17A decreased to less than 20 pg/ml at year 4. In group H ($n = 9$), they were still more than 20 pg/ml at year 4. Fig. 4A shows that the symptom medication scores were significantly lower in group L than those in group H at year 4 ($p < 0.05$). There were no significant differences in the symptom medication scores at year 1 during the peak of the pollen season among the groups. The symptom medication scores were affected by the cumulative

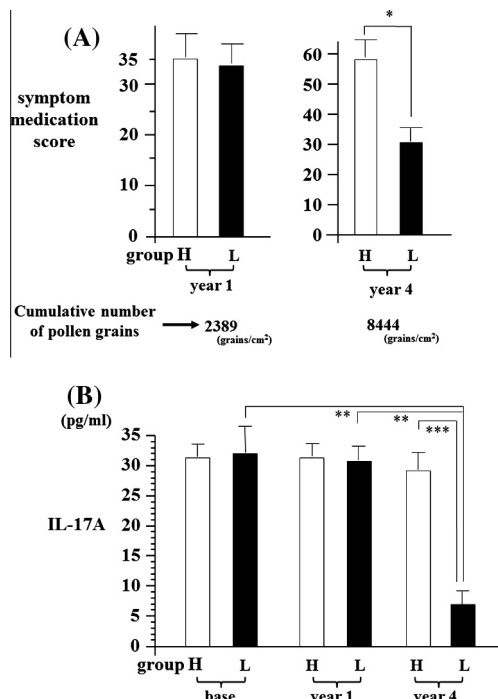


Fig. 4. Comparison of symptom medication scores among high- and low-IL-17A-level groups. The patients were divided into two groups according to the serum levels of IL-17A at the high-pollen season in year 4, from the findings in Fig. 3. In group H, the serum levels of IL-17A were ≥ 20 pg/ml. In group L, the levels were < 20 pg/ml. The symptom medication scores were significantly lower in group L than in group H at year 4 (A) (* $p < 0.05$). The cumulative number of pollen grains (grains/cm²) in each year is shown with the arrow. The mean and SE of IL-17A levels at baseline, year 1, and year 4 are respectively indicated with open (group H) ($n = 9$) and closed (group L) ($n = 10$) bars and lines (B) (** $p < 0.01$, *** $p < 0.005$).

number of pollen grains. Although there were no significant differences in IL-17A levels among groups both at baseline and at year 1, the IL-17A levels at year 4 were significantly lower than those at baseline or year 1 only in group L (Fig. 4B). In addition, there were no significant differences in the levels of C3a or C5a among groups at baseline, year 1, and year 4 (data not shown).

3.4. The serum levels of IL-4, IL-5, IL-13 and IFN- γ during long-term SLIT

We also examined the T_H2 and T_H1 cytokines during long-term SLIT. As shown in Table 1, the levels of IL-4 showed a significant decrease at year 4 compared with those at year 1 ($p < 0.005$). On the other hand, the levels of IL-5, IL-13, and IFN- γ at year 4 did not change significantly compared with those at year 1.

4. Discussion

In this study, we found that long-term SLIT reduced the serum levels of IL-17A, C3a, and C5a. A significant positive correlation was

Table 1
The serum levels of IL-4, IL-5, IL-13 and IFN- γ during long-term SLIT.

Cytokine	Serum levels at year 1 (pg/ml)	Serum levels at year 4 (pg/ml)
IL-4	170.9 $\pm$ 25.6	110.6 $\pm$ 13.2*
IL-5	16.8 $\pm$ 5.4	17.2 $\pm$ 5.4
IL-13	28.4 $\pm$ 10.8	24.5 $\pm$ 9.6
IFN- γ	45.0 $\pm$ 13.2	47.4 $\pm$ 12.3

The samples were obtained at year 1 and year 4 of long-term SLIT, and the serum levels of IL-4, IL-5, IL-13, and IFN- γ were measured using multiple assay system. The mean and SE are indicated. The levels of IL-4 were significantly lower at years 4 than those at year 1 (* $p < 0.005$) ($n = 19$).

found between the symptom medication scores and the levels of IL-17A during long-term SLIT. The scores in the group in which the IL-17A levels decreased were significantly lower than those in the group in which these levels did not decrease. IL-17A might be involved in pathological features of SAR-JCP or mechanisms of SLIT efficacy, suggesting that it could be one of the suitable biological parameters for SLIT efficacy.

The potential roles of IL-17A in the pathogenesis of allergic disorders have been described in several reports. Allergic patients exclusively sensitized to birch presented significantly higher median levels of IL-17 than healthy control subjects [9]. The serum IL-17A concentration was significantly related to the visual analog scale score of nasal symptoms [8]. In our study, we found that long-term SLIT significantly reduced the levels of IL-17A and those levels were significantly related to the symptom medication scores.

Complement activation of nasal mucosa has been reported in rapid allergic reaction to pollen [17]. Ragweed extract has been shown to produce complement activation in the sera of allergic patients and healthy controls [18]. Proteases from *Dermatophagoides* species (particularly Der p3 and Der f3) have the ability to cleave C3 and C5 into their active fragments *in vitro* and to promote the generation of C3a/C5a during allergen sensitization [19]. Proteases from Japanese cedar pollen have been identified, and they might activate a complementary reaction [20]. Anaphylatoxins are effective inflammatory mediators targeting a broad spectrum of immune and nonimmune cells. C3a and C5a are important chemoattractants for cell populations, such as eosinophils and mast cells, involved in the development of allergic responses. Eventually, C3a and C5a promote smooth muscle cell contraction and mucus production and enhance vascular permeability [15]. Complement activation has effects on allergic conditions through the complement activation pathways, including the classical pathway, alternative pathway, and direct protease activity of allergens. Here, in this study, long-term SLIT significantly reduced the levels of C3a and C5a in the patients.

On the other hand, C3a and C5a regulated the development of maladaptive T_H2 and T_H17 immune responses during allergen sensitization and the effector phase. C3a drove IL-23 production and promoted the development of T_H17 cells [11]. Treatments with a C3a receptor antagonist or anti-C3a mAb during the challenges inhibited the increase in IL-17-positive CD4-positive cells [12]. C5a is considered to be an anaphylatoxin that is critical for the maintenance of inhalation tolerance at the mucosal surface through regulation of the activation status of immunizing and tolerogenic pulmonary DC subsets. Once an allergic environment has been established, the main effect of C5a appears to be pro-allergic [21]. C3a receptor and C5a receptor signaling is required for IL-23 production [22]. In addition, T_H17 development in experimental asthma is regulated not only by C5a receptor but by the second C5a receptor, C5L2 [23]. In our study, the levels of C3a in the patients significantly decreased from year 1 compared with those at baseline, and the levels of C3a and C5a at year 4 of SLIT were significantly lower than those at baseline and at year 1. However, we could not detect any correlation between the symptom medication scores and the levels of C3a and C5a during long-term SLIT.

T_H1/T_H2 balance has been regarded as important for homeostatic maintenance of the immune system. Furthermore, a non-T_H1/T_H2 cell-based mechanism related to T_H17 cells and Treg cells was found to explain many complicated pathological situations that cannot be understood simply using the T_H1 cell and T_H2 cell paradigm [24]. These mechanisms to induce an allergic response might fit the induction of upper airway allergic responses such as seasonal allergic rhinitis. In a challenge to the traditional discrete categorization of T_H subtypes, populations of CD4-positive T cells

that express both IL-17A/F and type 2 cytokines have been identified in allergic airway disease [25,26]. IL-4/IL-17 dual-positive T_H cells were demonstrated by dual-cytokine expression and the combined expression of transcription factors and cell-surface markers with T_H2 (GATA3, signal transducer and activator of transcription 6, and chemoattractant receptor-homologous molecule expressed on T_H2 cells) and T_H17 (retinoic acid-related orphan receptor γ t, signal transducer and activator of transcription 3, and CCR6) cells [27]. In addition, the increased frequency of dual-positive T_H2/T_H17 cells characterized a population of patients with severe airway disease. We have revealed that the levels of IL-17A at year 4 of SLIT were significantly lower than both those at baseline and those at year 1, and the levels of IL-4 showed a significant decrease at year 4 compared with those at year 1. In contrast, there was no significant correlation between the levels of IL-4 and IL-17 during long-term SLIT here in this study. Further experiments are needed concerning dual-positive T_H2/T_H17 cells using other methods including use of a cell sorting system for long-term SLIT.

In addition to dual-positive T_H cells, other cell types, including IL-17 single-positive T_H17 cells and innate immune cells, also produce IL-17 [28]. Future studies to characterize IL-4/IL-17 dual-positive cells more deeply, analyzing the production of additional type 2 cytokines (IL-5 and IL-13) and the expression of IFN- γ , IL-22, and surface IL-23 receptor, have implicated them in the pathologic effects of T_H17 cells [29]. In this study, there was no significant difference in the levels of IL-5, IL-13, and IFN- γ at year 4 of SLIT compared with those at year 1.

In a previous prospective open controlled study of SLIT, patients with respiratory allergy mono-sensitized to mites were followed up for 15 years, and it was suggested that a 4-year duration of SLIT is the optimal choice because it induced a long-lasting clinical improvement similar to that seen with a 5-year course and greater than that of a 3-year vaccination [30]. Here, we performed long-term SLIT, screened molecules as candidates, and found that the levels of IL-17A, C3a, and C5a significantly decreased and a significant positive correlation was found between the symptom medication scores and the levels of IL-17A. The serum level of IL-17A might prove useful as a biomarker to ascertain the effectiveness of SLIT for patients with SAR-JCP. However, further studies are needed to evaluate the difference between responders and non-responders in SLIT. In addition, collaboration among scientists and pharmaceutical companies is necessary to produce new therapeutics for non-responders in whom serum IL-17A levels are still higher at year 4 for SAR-JCP.

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