

Serum Cytokine Interactions Are Implicated in the Mechanism of Action of Sublingual Immunotherapy for Japanese Cedar Pollinosis

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Objective: This study aimed to investigate whether interactions between multiple serum cytokines may be implicated in the mechanism of action (MOA) of sublingual immunotherapy (SLIT) for Japanese cedar pollinosis.

Methods: A Tokyo Metropolitan Bureau of Social Welfare and Public Health-initiated clinical study of active SLIT involving 202 patients with Japanese cedar pollinosis was jointly conducted by Tokyo Metropolitan Institute of Medical Science and Nippon Medical School between 2006 and 2008. Fifty target cytokines were quantified in serum samples collected at 6 times from baseline to the end of the study, for 300 cytokine measurements in total, using Bio-Plex Pro Human Cytokine Group I/II Panels. Therapeutic outcome was assessed based on nasal symptom scores and quality-of-life questionnaire results.

Results: Fifty-five percent of patients were free of symptoms or reported symptomatic improvements by 2 grades or greater after 2 years of SLIT treatment, while 27% showed no improvement or worsening of symptoms. Thirty-eight patients who benefited the most from treatment (responders) as well as 37 patients who benefited the least from treatment (non-responders) were identified and their serum cytokine profiles were compared. Cluster analysis of the 300 cytokine measurements identified 6 cytokine clusters that were strongly correlated with a positive response to treatment, and this correlation was consistent throughout the treatment.

Conclusion: Certain cytokine clusters are strongly correlated with a positive therapeutic outcome, suggesting they have a role in the MOA of immunotherapy. (J Nippon Med Sch 2018; 85: 250–258)

Key words: serum cytokines, sublingual immunotherapy, cluster analysis

Introduction

Immunotherapy was first applied to treat hay fever (allergic rhinitis) in humans by Noon in 1911¹. Since then, allergen immunotherapy (AIT) has remained a viable therapeutic modality for allergy and remains the only treatment that targets the allergy disease rather than the symptoms. AIT is safe and effective and can result in long-term symptom reduction^{2–9}. Despite the numerous investigations conducted to date to explore the mechanisms of action (MOA) of immunotherapy, the MOA of subcutaneous and sublingual immunotherapy remains to

be fully elucidated⁵. A deeper understanding of the MOA for these treatments may reveal biomarkers that can be used to identify patients that are good candidates for AIT and to monitor the therapeutic progress without relying solely on patient feedback⁴.

Current knowledge on the MOA of allergy immunotherapy suggests that the mechanism is complex and involves several pathways^{3–6,10–12}. There are thought to be two phases to the AIT MOA. The early phase consists of a decrease in tissue mast cells, eosinophils, basophils, and mediator release. In addition, there is an increase in

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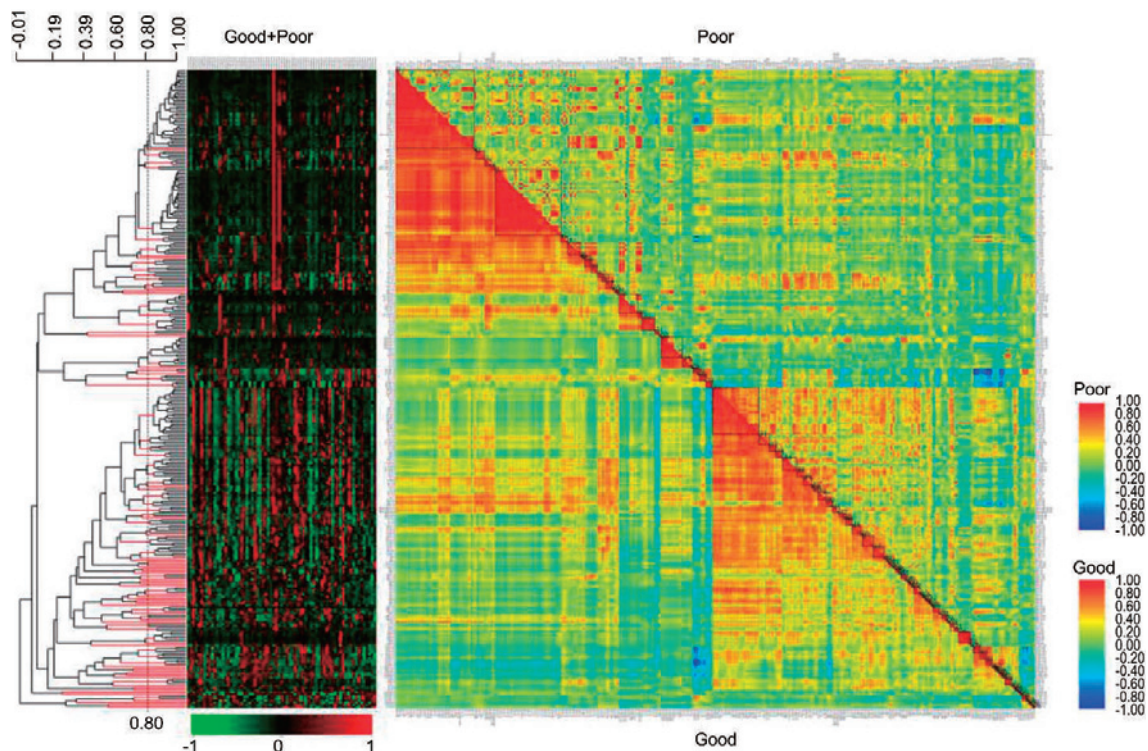


Fig. 1 Correlation between all pairs of cytokines quantified in responders and non-responders. A phylogenetic tree generated using clustering analysis is shown on the far left. Next to the tree, z-scores for responders and non-responders are summarized in a green-to-red heatmap. On the far right, correlation coefficients for cytokines in responders (labeled good, lower left) versus non-responders (labeled poor, upper right) are visualized as a combined matrix based on the results of the initial cluster analysis of the cytokine profiles in responders versus non-responders. This figure was previously published by Gotoh et al.¹⁵ and is reprinted here with permission from the authors.

immunoglobulin G4 (IgG4), which blocks the interaction between immunoglobulin E (IgE) and the allergen. Immunoglobulin A (IgA) is also increased in the first phase of the response to AIT^{3-6,10-12}. In the late phase, there is a shift from type 2 helper (Th2) cells to type 1 helper (Th1) cells, along with an increase in the number of regulatory T cells and an increase in some cytokines, such as interleukin-10 (IL-10), IL-35, and transforming growth factor- β (TGF- β)^{3-6,10,11}.

While IgG4 has long been implicated in the MOA of immunotherapy², it remains less well established as a biomarker for immunotherapy, given that changes in IgG4 levels are often less consistent with those in clinical symptoms in patients receiving immunotherapy⁴. Although immunotherapy has been assumed to bring about a shift in the ratio of Th1 to Th2 cells leading to the predominance of Th1 cells among T lymphocytes¹³, there is a paucity of definitive evidence to support this assumption. Furthermore, while regulatory T cell-related cytokines were recently reported to be implicated in the MOA of immunotherapy, leading to a profusion of related research¹⁴, and they have come to be assumed to play a

central role in the MOA, few studies have examined how these cytokines may account for the MOA of AIT based on a comprehensive cytokine analysis.

In this study, cytokines were comprehensively quantified to examine their involvement in the MOA of AIT, particularly, sublingual immunotherapy (SLIT). Cytokine levels from patients showing the greatest improvement in symptoms were compared with those from patients showing the least improvement. Cluster analysis was then conducted to identify cytokines that were correlated with patients responsive to AIT. A previous study showed that this approach revealed patterns in cytokine correlation present in the responders that were not present in the non-responders (Fig. 1 and Fig. 2). Figure 1 and Figure 2 were previously published by Gotoh et al.¹⁵ and are reprinted here with permission from the authors. Subtle differences were observed between the matrix containing all of the samples and those containing only the samples from the first blood collection (Fig. 1 vs. Fig. 2). These differences suggested that the patterns of cytokine correlation may change over time. In this study, correlation matrices were prepared and analyzed for time points

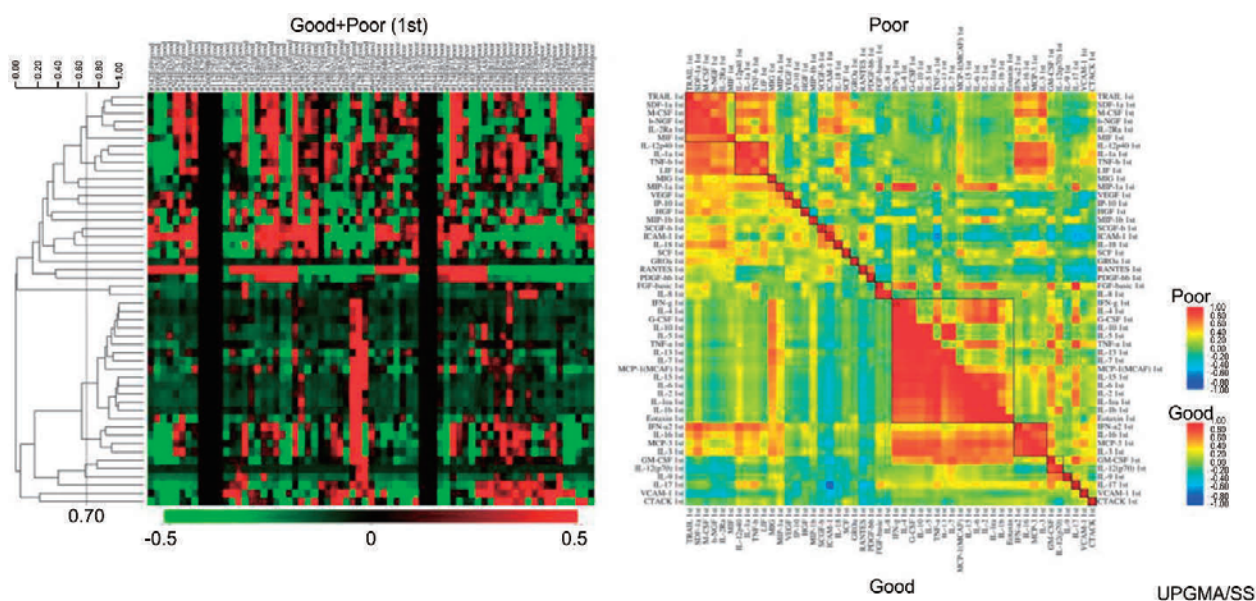


Fig. 2 Results of cluster analysis of cytokine profiles based on the first blood samples drawn from responders and non-responders. Cluster analysis was performed using cytokine data from the first blood samples alone and the results were visualized as correlation matrices. Squares within the correlation heatmap (right) indicate clusters identified when the cytokines were segmented using a dendrogram correlation coefficient of 0.7 (indicated by the dotted line), with segments thought to represent relatively large clusters being classified by Roman numerals. The axis labeled "good" represents the responder group while the axis labeled "poor" represents the non-responder group. This figure was previously published by Gotoh et al.¹⁵ and is reprinted here with permission from the authors.

throughout the course of the study.

Materials and Methods

Subjects

Patients with pollinosis who had visited the cooperating medical institutions (Tokyo Metropolitan Otsuka Hospital, Tokyo Metropolitan Komagome Hospital, Tokyo Metropolitan Hiroo Hospital, Tokyo Metropolitan Fuchu Hospital, Tokyo Metropolitan Health Medical Center Ebara Hospital, Nippon Medical School Hospital, Endo Otolaryngology/Allergy Clinic, Hirooka Clinic), met all the enrolment criteria, and did not fall under any of the exclusion criteria (see below) were enrolled in this trial.

Eligibility and Exclusion Criteria

Patients were judged eligible for the study if: 1) they were residents in or commuters to the Tokyo metropolitan area, 20 years of age or older; 2) they had had symptoms of allergic rhinitis (e.g., sneezing, rhinorrhea, nasal congestion) in the cedar pollen season during 3 consecutive years or more, including the spring of 2007; 3) they had a positive skin test to cedar pollen or a positive allergen-specific immunoglobulin E (IgE) test; 4) they understood the study objectives and voluntarily consented to participate in the 2-year study, to undergo blood tests, and to document their symptoms during the course of

the study.

Patients were excluded when: 1) they had any nasal disease that was thought likely to interfere with therapeutic outcome evaluation; 2) they could not forgo the continued use of any medication thought likely to influence therapeutic outcome evaluation; 3) they were receiving oral steroids; 4) they had a history of asthma and urticaria starting at the age of 15 years or older; 5) they had previously received AIT; 6) they were suspected of being pregnant; 7) they were a lactating woman; 8) they had hepatic, renal, or cardiac disease, respiratory infection, or any other serious disease and were judged by the investigator to be ineligible for the study; 9) they were judged by the investigator to be ineligible for the study for reasons other than those given above; 10) they were shown to have had no symptoms of pollinosis during the Japanese cedar pollen season in the spring of 2007, based on the Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ) results^{16,17}.

All eligible patients were fully informed about the study and gave written informed consent to participate in the study prior to their participation. All experimental procedures were approved by the Ethical Committee of Tokyo Metropolitan Institute of Medical Science (Approval No. 17-10) and the Nippon Medical School Hospital (No. 18-3). The study was registered in the University

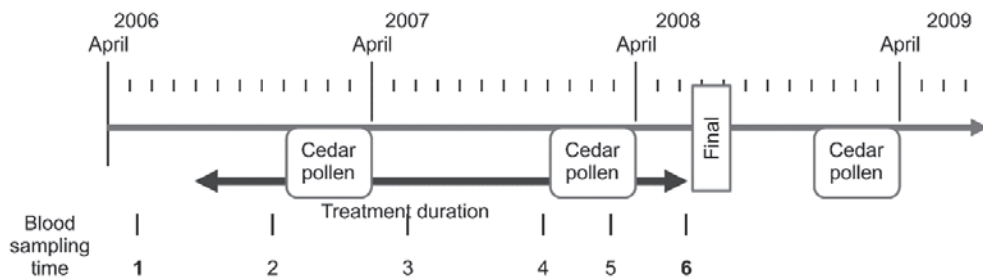


Fig. 3 Duration of sublingual immunotherapy and schedule for blood sampling. Blood samples were taken 6 times throughout the course of a 2-year study starting just before the start of the therapy in 2006 and ending with a treatment outcome assessment in 2008. The cedar pollen seasons are indicated.

Hospital Medical Information Network Clinical Trials Registry Database (UMIN000016532).

In total, 202 patients who met the eligibility criteria described above were enrolled and received SLIT in this joint clinical study conducted by Tokyo Metropolitan Institute of Medical Science and Nippon Medical School between 2006 and 2008 (Fig. 3).

Responder and Non-Responder Classification

Participating patients rated the severity of their symptoms using the JRQLQ. Nasal symptoms were recorded from February 1 to April 30 during three pollen seasons. The symptoms were investigated for allergic rhinitis using Japanese guidelines as previously described^{15,18,19}. The responses to the JRQLQ were rated according to a classification scheme developed by Okuda et al.^{15,17,20}. Briefly, the number of episodes of sneezing and nose-blowing, extent of nasal congestion and eye itchiness, symptom duration, and medication compliance were recorded in an allergy diary by the patients themselves. Quality of life scores for the patients were determined at the end of February, mid-March, and mid-April each year using the JRQLQ. SLIT efficacy was evaluated at the end of the pollen season by comparing the 5-grade severity scores (most severe, severe, moderate, mild, or asymptomatic) in the allergy diary and the JRQLQ each year with the ratings before the outset of SLIT^{17,20}.

Of the 202 patients that were initially enrolled in the study, 173 were still enrolled at 8 months and 154 patients were still enrolled at 20 months and at the therapeutic outcome evaluation (Fig. 4).

For the cytokine analysis, the pool of 154 patients was divided into quarters based on their responses in the JRQLQ. Patients with significant improvement in symptoms, complete remission, or an improvement of at least 2 symptom levels were considered responders ($n = 38$), while those patients that saw no improvements or wors-

ening of symptoms were considered non-responders ($n = 37$). The cytokine profiles of the 38 responders and 37 non-responders were examined and compared before and after immunotherapy.

Serum Sampling and Estimation of Levels of Serum Cytokines

Serum sampling was performed 6 times during the study according to the schedule shown in Figure 3. At each sampling time, 8 mL of blood was drawn from each patient and immediately centrifuged. The serum was separated, divided into 1-mL aliquots, and stored at -80°C until use.

The serum levels of cytokines were quantified using a Bio-Plex Pro Human Cytokine Group I Panel (27 target cytokines: IL-1 β , IL-1R α , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 [p70], IL-13, IL-15, IL-17, IFN- γ , TNF- α , IL-8, Eotaxin, IP-10, MCP-1 [MCAF], MIP-1 α , MIP-1 β , RANTES, Basic FGF, G-CSF, GM-CSF, PDGF-BB, and VEGF) and a Bio-Plex Pro Human Cytokine Group II Panel (23 target cytokines: IL-1 α , IL-2R α , IL-3, IL-12 [p40], IL-16, IL-18, IFN- α 2, TNF- β , TRAIL, CTACK, GRO- α , ICAM-1, MCP-3, MIF, MIG, VCAM-1, HGF, LIF, M-CSF, β -NGF, SCF, SCGF β , SDF1 α)(Bio-Rad). These 50 target cytokines were quantified in the 6 serum samples of each patient, resulting in a total of 300 cytokine measurements for each patient.

Statistical Analysis

Quantile normalization was conducted on the results for the pre- and post-treatment sample groups. Cytokine levels were then normalized with a z-score using the equation $z = [v - \mu] / \sigma$, where v is the raw value, μ is the mean and σ is the standard deviation in the population.

The Pearson's product-moment correlation coefficient was used to calculate correlation coefficients (r) between any two cytokines measured. Cluster analyses were conducted using these correlation coefficients, and a phylo-

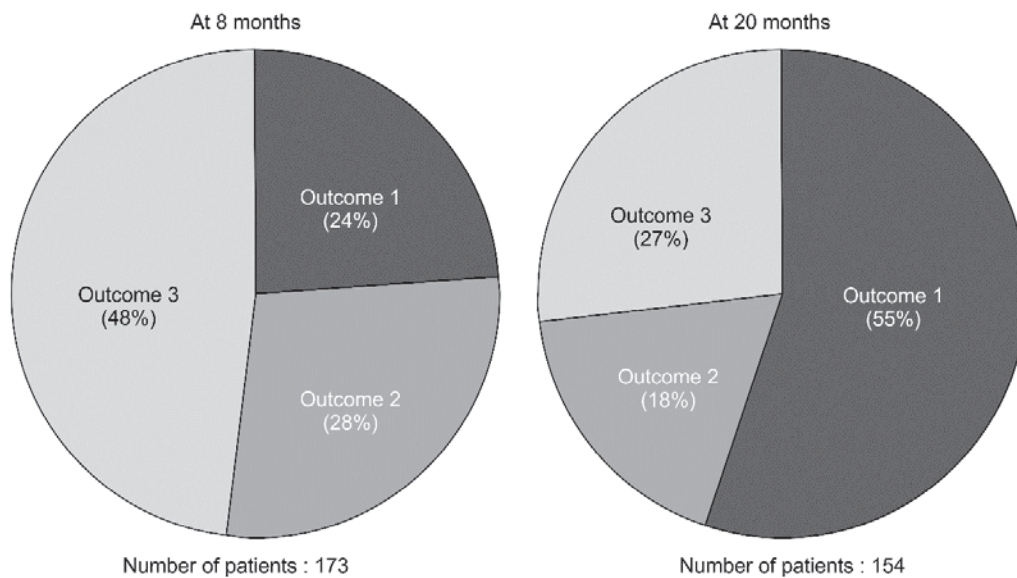


Fig. 4 Comparison of therapeutic outcomes during treatment. The therapeutic outcomes of the patients were assessed using the Japanese Rhinoconjunctivitis Quality of Life Questionnaire (JRQLQ). Three outcomes were observed: Outcome 1 included patients that were asymptomatic or had 2-grade or greater symptomatic improvement, outcome 2 included patients that had 1-grade symptomatic improvements, and outcome 3 included patients with no improvement or worsening of symptoms.

genetic tree was constructed using the unweighted pair-group method with an arithmetic mean¹⁵.

Results

Sublingual Immunotherapy Reduced Allergy Symptoms for Most Participants

In total, 202 patients entered the SLIT study; however, only 154 patients completed the full 2-year study. Of the 202 originally enrolled patients, 173 were available for therapeutic evaluation at 8 months and 154 were available for evaluation at 20 months (Fig. 4).

At 20 months of treatment, about two-thirds of the patients benefited from treatment, with 55% becoming asymptomatic or showing symptomatic improvements of 2 grades or greater on the JRQLQ, 18% with 1-grade symptomatic improvements, and 27% with no improvement or worsening of symptoms. Of those showing symptomatic improvements 2 grades or greater, 38 patients who benefited the most from treatment were determined as responders, while of those not benefiting from treatment, 37 patients who benefited the least were determined as non-responders. The annual mean symptom scores for responders versus non-responders are shown in Figure 5.

Correlation between the Serum Cytokines Quantified

Of all cytokines measured, only IL-12 showed a significant difference between the responders and non-responders (Fig. 6). Correlation matrices were generated

for cytokines in responders and non-responders to examine how cytokines were correlated in both groups. The matrices were folded along the diagonal and the resulting triangles for non-responders (upper) and responders (lower) were joined together to visualize differences in patterns of correlation between all pairs of cytokines in responders (good) versus non-responders (poor) (Fig. 1). From this combined matrix, it became clear that while the cytokines correlated well in responders as indicated by the abundant red color in the lower triangle—even though their levels varied over time—this was not the case in non-responders. Thus, correlation analysis of the serum cytokines suggested different patterns of correlation for cytokines in responders versus those in non-responders.

Changes in Correlation Coefficients for Serum Cytokines Over Time

The results of a similar cluster analysis using data obtained from the first blood samples drawn from responders and non-responders are shown in Figure 2. Pearson correlation coefficients of the cytokine data in the responder and non-responder groups were calculated and were visualized in a heatmap showing the mutual relationships. Cytokine groups were extracted by hierarchical clustering using a dendrogram correlation coefficient of 0.7. In doing so, the analogous relationship (correlation) of cytokines was defined, and cytokine clusters in com-

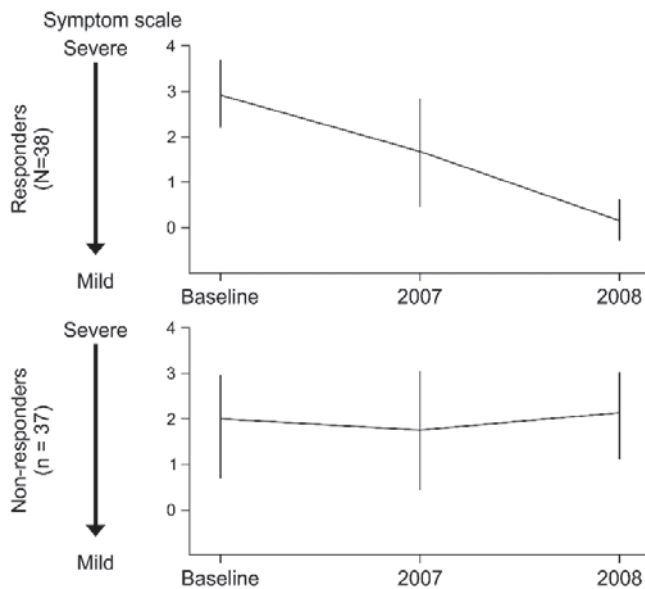


Fig. 5 Determination of responders and non-responders. Symptom severity for the responders (upper graph) and non-responders (lower graph).

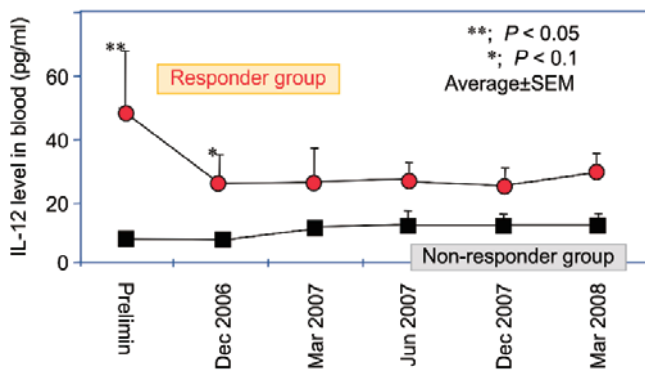


Fig. 6 Correlation between IL-12 levels in blood following allergy immunotherapy evaluation. IL-12 levels were significantly elevated in the responder group at the preliminary ($P < 0.05$) and second ($P < 0.01$) evaluations. IL-12 was the only cytokine to show a significant difference between the responder and non-responder groups. The cytokine levels of the responders are shown as circles and those of the non-responders as squares.

mon between both groups were extracted using the average of the correlation coefficient in the responder and that in the non-responder group. Cytokines that were assumed to form cytokine networks are listed in **Table 1**.

A similar analysis was conducted for cytokine data from the blood samples taken at sampling times 2 to 6. **Figure 7** demonstrates that the cytokines were consistently correlated throughout the study. Of note, Th1/Th2 cytokines, such as IFN- γ , IL-4, IL-5, and IL-13, which were shown to be well correlated in responders but less

Table 1 Cytokine clusters assumed to form cytokine networks

Cluster	Cytokines assumed to form a cytokine network
I	TRAIL, SDF-1 α , M-CSF, β -NGF, IL-2R α , MIF
II	IL-12 (p40), IL-1 α , TNF- β , LIF
III	FGF-basic, IL-8
IV	IFN- γ , IL-4, G-CSF, IL-10, IL-5, TNF- α , IL-13, IL-7, MCP-1, IL-15, IL-6, IL-2, IL-1R α , IL-1 β , Eotaxin
V	IFN- α 2, IL-16, MCP-3, IL-3
VI	GM-CSF, IL-12(p70)

well correlated in non-responders, were all located in cluster IV (**Table 1**).

Discussion

Multiple studies have examined changes in single cytokines in an attempt to provide clues to the MOA of allergen immunotherapy. However, very few have examined correlations among multiple cytokines based on their comprehensive quantification. However, given that multiple cytokines are assumed to be functionally correlated, it appears worthwhile to examine changes in cytokine levels of a multitude of cytokines simultaneously, as we did in this study.

Multivariate analyses are commonly employed to identify or clarify relationships among three or more variables related to the study subject. Of these, hierarchical clustering is of interest because it is highly convenient, allowing cases to be classified on the basis of any available factors that characterize them. Indeed, hierarchical clustering has come to be commonly employed in a wide range of areas, allowing uniform classification of cases that would previously be classified on an empirical basis alone²¹⁻²³.

In this study, cytokine profiles for responders versus non-responders were analyzed using hierarchical clustering. Cluster analysis of Pearson correlation coefficients for cytokines from the blood samples taken at the first sampling time demonstrated low correlation among cytokines for non-responders versus high correlation for responders, suggesting that there is a population of strongly correlated cytokines in responders that is not observed in non-responders. Moreover, the patterns of cytokine correlation were consistent in all subsequent blood samples.

Participants in this study were residents or commuters in the Tokyo metropolitan area between 2006 and 2008. The allergic rhinitis symptoms observed by patients enrolled in this study would be affected by the cedar pollen

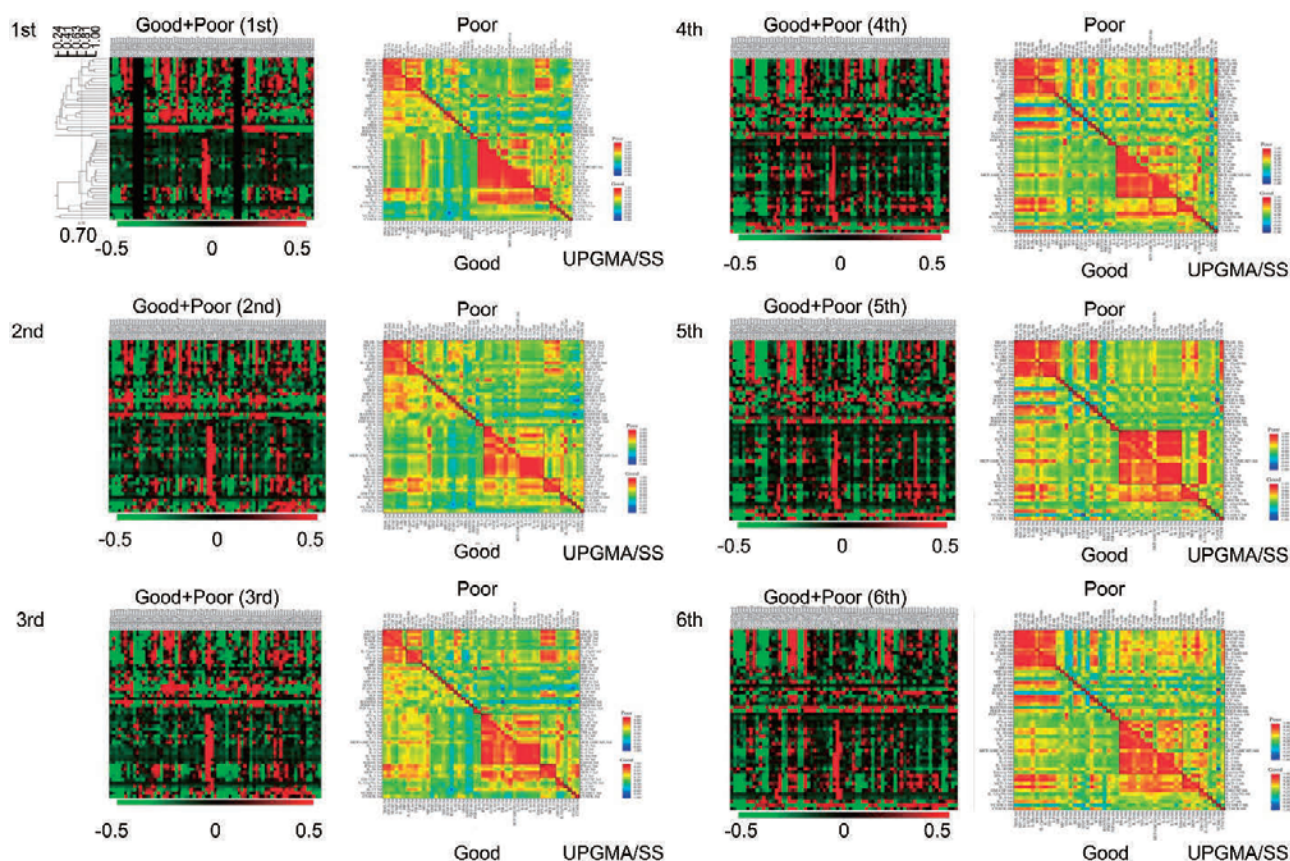


Fig. 7 Cluster analysis of cytokine profiles from all blood samples. Matrices were generated for correlation coefficients for cytokines from blood samples taken at sampling times 1 to 6, with the cytokines arranged in the same order on the longitudinal and horizontal axes as those in the matrix generated for cytokine data from the first blood samples in Fig. 6. Patterns of cytokine correlations are shown to be consistent from the first through all subsequent blood samples. The axis labeled “good” represents the responder group while the axis labeled “poor” represents the non-responder group.

dispersal during the study period. Studies of cedar pollen dispersal show that 2006 had a moderate level of pollen in the air with more than 1,000 grains/cm²/year but less than 5,000 grains/cm²/year. The following years had at least the same pollen count or higher with 2007 measuring at around 5,000 grains/cm²/year and 2008 measuring more than 5,000 grains/cm²/year but less than 10,000 grains/cm²/year. These figures come from a study based in the Sagami-hara Hospital in the Kanagawa prefecture which is considered part of the greater Tokyo area²⁴. Another study performed within Tokyo reported similar levels for 2006 and 2007 but did not contain information about 2008²⁵. Based on the pollen counts, it is unlikely that a reduction in pollen year-to-year could be responsible for the decreased symptoms reported by the responder group in this study.

Correction of the Th1/Th2 imbalance and subsequent inhibition of allergic inflammation is thought to account for the core MOA of immunotherapy. One cytokine that is involved in regulation of Th1 is IL-10, which was identified

within cluster IV of our mathematical analysis (**Table 1**). In our mathematical analysis of cytokines, IFN- γ , IL-10, IL-4, IL-5, and IL-13, which were shown to be highly correlated in responders but poorly correlated in non-responders, were located in a single cluster, suggesting that these cytokines cooperate in cytokine networks contributing to treatment success in responders, while they fail to do so in non-responders (**Table 1**). Several of the cytokines identified in this analysis are also involved in the regulation of IL-10 including IL-4²⁶, which was identified in cluster IV and IL-12 which was the only cytokine identified with a statistically significant difference between responders and non-responders. Th1, IL-10, and IL-4 have all been studied for their involvement in the MOA of AIT; however, for the most part these studies have focused on only one or a few parts of our proposed network of cytokines^{14,26,27}. The cytokine clusters I to VI shown in **Table 1** are suggested to be strongly correlated with the MOA of AIT for Japanese cedar pollinosis. These cytokines need to be further examined in biologi-

cal functional analyses to provide evidence for their causal relationships.

Conclusions

Currently, AIT is the only established radical treatment modality for allergic diseases. However, the MOA involved remains poorly elucidated. In this study, based on a comprehensive assessment of cytokine profiles in relation to the MOA of SLIT for Japanese cedar pollinosis, it is suggested that, contrary to current thinking, certain clusters of cytokines may be implicated in the pathogenesis and control of allergy.

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Conflict of Interest: The authors declare no conflict of interest to declare.

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