Journal of Inflammation Research

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ORIGINAL RESEARCH

Multiple-Cytokine Profiling: A Novel Method for Early Prediction of the Efficacy of Sublingual Immunotherapy in Allergic Rhinitis Patients

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Background: Allergic rhinitis (AR) is a common inflammatory airway disease, and allergen-specific immunotherapy (AIT) is the only disease-modifying treatment for it. However, not all AR patients respond to AIT, and early prediction of patient response is extremely important. This study aimed to example serum levels of multiple cytokines in AR and explore their association with the efficacy of AIT.

Methods: A total of 74 AR patients treated with sublingual immunotherapy (SLIT) were prospectively recruited. Serum samples were obtained before the onset of SLIT and cytokine levels detected by multiplex analysis. All patients were followed for >1 year, and associations between cytokine levels and the early efficacy of SLIT were evaluated. Significantly distinctive cytokines were further verified in another independent cohort.

Results: Sixty patients completed the visit schedule set: 35 patients were put into a responder group and 25 a nonresponder group. Multiple-cytokine profiling showed that cytokine levels differed significantly between the two groups. The responder group had higher concentrations of BAFF and CCL11 and lower levels of CCL2, CCL7, IFNγ, IL8, IL10, IL16, and IL33 than the nonresponder group ($P<0.05$). Receiver-operating characteristic curves highlighted that serum BAFF, IFNγ, IL10, and IL33 levels were strongly predictive of the efficacy of SLIT (area under the curve <0.7, $P<0.05$). Serum IL10 and IL33 were overexpressed in nonresponders in the validation cohort. Patients in the responder group exhibited significantly higher IL10 levels and lower IL33 post-SLIT than pre-SLIT ($P<0.05$), but no statistical difference was found in nonresponders ($P<0.05$).

Conclusion: Our data indicated that serum multiple-cytokine profiling was associated with response to SLIT and that IL10 and IL33 might serve as novel biomarkers for early prediction of efficacy and be involved in the therapeutic mechanisms of SLIT in AR patients.

Keywords: allergic rhinitis, sublingual immunotherapy, multiple cytokines, efficacy

Introduction

Allergic rhinitis (AR) is a common upper-airway disease that is characterized by IgE-mediated T_h2 inflammation.[1,2] Recently, epidemiological studies have revealed that AR affects 20%–30% of the population worldwide, and its prevalence continues to increase.[3,4] It has been recognized that AR has severe adverse effects on daily life, work, and study and increases the risk of asthma, allergic conjunctivitis, and chronic sinusitis.[5–7] Currently, allergen avoidance, medications, and allergen-specific immunotherapy (AIT) are the mainstays of AR treatment, and AIT is the only disease-modifying treatment that can induce allergen tolerance.[8–10] Conventional AIT can be performed subcutaneously or sublingually, and sublingual immunotherapy (SLIT) seems to be more popular because of its convenience, equivalent efficacy, and good tolerability.[11–13] Despite its widely accepted safety and effectiveness in AR patients, many users still respond poorly, and its effectiveness fluctuates widely.[9] Therefore, exploring biomarkers or methods for early identification of responders, monitor therapeutic effects in AR patients who have been assigned to SLIT is a research focus, and extremely pivotal for developing a precision medicine model and reducing the waste of medical resources.

Received: 18 November 2021
Accepted: 4 January 2022
Published: 27 January 2022

Although prior studies have identified several potential biomarkers to objectively predict the effectiveness of SLIT, including serum-specific IgE,[14] MIF,[15] metabolites,[16] and vitamin D,[17] these indicators are not clinically viable because of their poor sensitivity, specificity, and repeatability. At present, there is a lack of knowledge concerning the expression of multiple serum cytokines in AR patients and their associations with the efficacy of SLIT. We thus sought to example multiple serumcytokine levels in AR and explore their association with the effectiveness of SLIT.

Methods

Participants and Setting

A total of 74 AR patients who visited our department and underwent SLIT between May 2020 and July 2020 were prospectively included in this study. All patients met the inclusion criteria of AR diagnosis as per Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines,[18] aged 18–60 years old, skin tests positive for *Dermatophagoides farinae* and/or *D. pteronyssinus* (≥++) and/or specific IgE (>0.35 IU/mL), and symptoms typical of AR for >3 years. We excluded those with other immunologic or inflammatory disease, a history of immunotherapy, pregnant or potentially pregnant, and systemic steroid/antiallergy-medication consumption within the 4r weeks prior to enrollment. Routine examinations were performed, and demographic and clinical data were collected before the onset of SLIT. The Human Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University approved this study. All patients provided written informed consent.

Serum Multiple Cytokine–Profiling Analysis

Fasting blood (5 mL) was collected from each participant before the onset of SLIT, and processed and stored as described previously.[13] A human cytokine standard 31-Plex assay kit (Bio-Rad) was utilized to detect 31 cytokines — BAFF, CCL2, CCL3, CCL4, CCL7, CCL11, CXCL9, CXCL10, CXCL12, GM-CSF, IFNγ, IL1α, IL1β, IL2, IL2Rα, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL13, IL15, IL16, IL17, IL33, M-CSF, MIF, TNFα, and TNFβ — based on the Luminex 200 system, and data interpretation was conducted as previously described.[19] All samples were diluted at 1:3 with sample diluent, and data from the 31-Plex assays were transferred to an Excel spreadsheet for further analysis.

Immunotherapy and Efficacy

All patients were assigned to receive 3 years of standard SLIT, administered with standardized *Dermatophagoides farina* allergen drops (Wolwo Pharma Biotechnology). The entire SLIT course consists of an escalation phase and a maintainenance phase, and specific daily dosing schedules were conducted as our previous study described. During the SLIT, compliance education and follow-up were performed, medication score and total nasal symptom score (TNSS) evaluated via WeChat in the previous week and recorded weekly, and symptom and medication score (SMS) calculated as (TNSS + MS)/7, as previously described.[13] At 1-year follow-up, early efficacy of SLIT was evaluated based on change in SMS: patients who attained a 30% reduction in SMS from baseline were defined as responders and the rest nonresponders as per our previous study.[13]

Validation of Potential Cytokines

Another independent cohort consisting of 98 AR patients treated with SLIT was used to confirm significantly distinctive cytokines in the discovery cohort. At 1-year follow-up, 80 patients had completed the treatment schedule: 50 responders and 30 nonresponders. Serum samples were harvested from all patients pre-SLIT, but only 40 patients provided serum specimens at 1 year post-SLIT. Concentrations of cytokines were measured using commercial ELISA kits and changes between pre-SLIT and post-SLIT levels assessed.

Statistical Analysis

Quantitative variables are shown as means ± SD or median and interquartile range, and Student's *t*-tests or Kruskal–Wallis *H* tests were used for comparisons between two groups. Categorical variables are shown as numbers and percentages, and the $?^2$ test was used for comparison of same. Statistical analysis was performed with SPSS 23.0, and figures were constructed with GraphPad Prism 7.0. Receiver-operating characteristic (ROC) curves were constructed to evaluate cytokines with potential for predicting the efficacy of SLIT. $P<0.05$ was considered statistically significant.

Table 1 Demographic and clinical characteristics of all AR patients

	Responders (n=35)	Nonresponders (n=25)	*P*
Age (years), median (range)	28 (23–38)	29.0 (24–33)	0.423
Male, n (%)	17 (48.6)	13 (52.0)	1
BMI (kg/m^2)	22.6 (21.6–23.6)	22.7 (21.9–24.1)	0.620
Asthma, n (%)	9 (25.7)	9 (36.0)	0.409
Serum total IgE (IU/mL)	313.3 (236.9–389.5)	312.5 (228.3–359.7)	0.675
Serum-specific IgE (IU/mL)	41.3 (30.2–62.5)	49.7 (12.6–62.9)	0.923
TNSS, median (range)	9 (8–10)	9.0 (7–10)	0.170
VAS score, median (range)	7 (6–9)	7.0 (5.0–8.0)	0.319

Abbreviations: AR, allergic rhinitis; BMI, body-mass index; TNSS, total nasal symptom score; VAS, visual analogue scale.

Results

Patient Characteristics

At 1-year follow-up, 60 AR patients had completed the 1-year visit schedule and provided complete follow-up data. In sum, 35 patients were categorized as responders and 25 as nonresponders. The main demographic and clinical

Table 2 Serum cytokine concentrations in the two groups (pg/mL)

Cytokines	Responders (n=35)	Nonresponders (n=25)	*P*
BAFF	6,404.1 (5,614.4–7,958.5)	5,039.5 (4,224.4–6,040.3)	0.001
CCL2	28.0 (19.8–44.2)	44.9 (27.2–120.2)	0.005
CCL3	6.5 (3.7–6.5)	7.4 (5.1–13.2)	0.066
CCL4	145.2 (130.2–168.5)	155.5 (135.0–196.6)	0.215
CCL7	0.3 (0.2–0.4)	0.8 (0.3–5.7)	0.018
CCL11	46.3 (31.7–65.5)	32.8 (23.9–49.9)	0.025
CXCL9	144.8 (110.7–232.9)	159.5 (128.2–239.5)	0.653
CXCL10	284.5 (231.3–472.3)	381.2 (282.9–431.3)	0.910
CXCL12	772.0 (644.3–966.7)	834.6 (742.8–1076.0)	0.148
GM-CSF	0.7 (0.4–1.5)	1.0 (0.4–1.8)	0.132
IFNγ	3.7 (3.0–4.7)	6.1 (3.7–7.4)	0.006
IL1α	8.7 (5.0–14.0)	10.1 (6.5–15.2)	0.636
IL1β	2.0 (1.4–2.6)	2.0 (1.6–2.8)	0.900
IL2	0.6 (0.4–0.6)	0.6 (0.4–0.8)	0.130
IL2Rα	69.3 (50.1–95.8)	61.5 (52.4–89.2)	0.722
IL4	2.0 (1.7–2.3)	1.7 (1.4–2.3)	0.222
IL5	2.3 (1.0–2.6)	2.4 (1.2–3.1)	0.449
IL6	0.3 (0.3–0.7)	0.6 (0.3–1.0)	0.130
IL7	3.8 (2.6–6.2)	3.8 (2.6–6.2)	0.881
IL8	43.0 (19.5–118.2)	79.8 (54.4–131.7)	0.041
IL9	238.4 (220.2–249.8)	241.8 (227.7–250.8)	0.266
IL10	1.0 (0.4–1.2)	2.0 (1.5–3.6)	0.004
IL13	1.7 (1.0–2.9)	1.5 (0.9–2.2)	0.121
IL15	32.6 (25.4–45.4)	32.7 (20.5–52.8)	0.385
IL16	79.8 (51.2–110.4)	113.4 (66.6–184.3)	0.020
IL17	5.5 (4.8–6.7)	5.9 (4.8–7.0)	0.537
IL33	67.5 (34.0–103.8)	142.5 (54.7–178.1)	0.001
M-CSF	21.8 (17.6–30.1)	23.1 (20.3–37.7)	0.216
MIF	1,131.0 (681.3–1,565.4)	1,420.0 (785.6–1,822.0)	0.335
TNFα	16.5 (14.1–21.2)	19.4 (14.1–24.9)	0.412
TNFβ	226.0 (208.2–240.0)	231.6 (210.6–238.9)	0.644

Abbreviations: BAFF, B cell activating factor; CCL, CC motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; GM-CSF, granulocyte monocytecolony stimulating factor; IFN, interferon; IL, interleukin; M-CSF, macrophage colony stimulating factor; MIF, macrophage migration inhibitory factor; TNF, tumor necrosis factor.

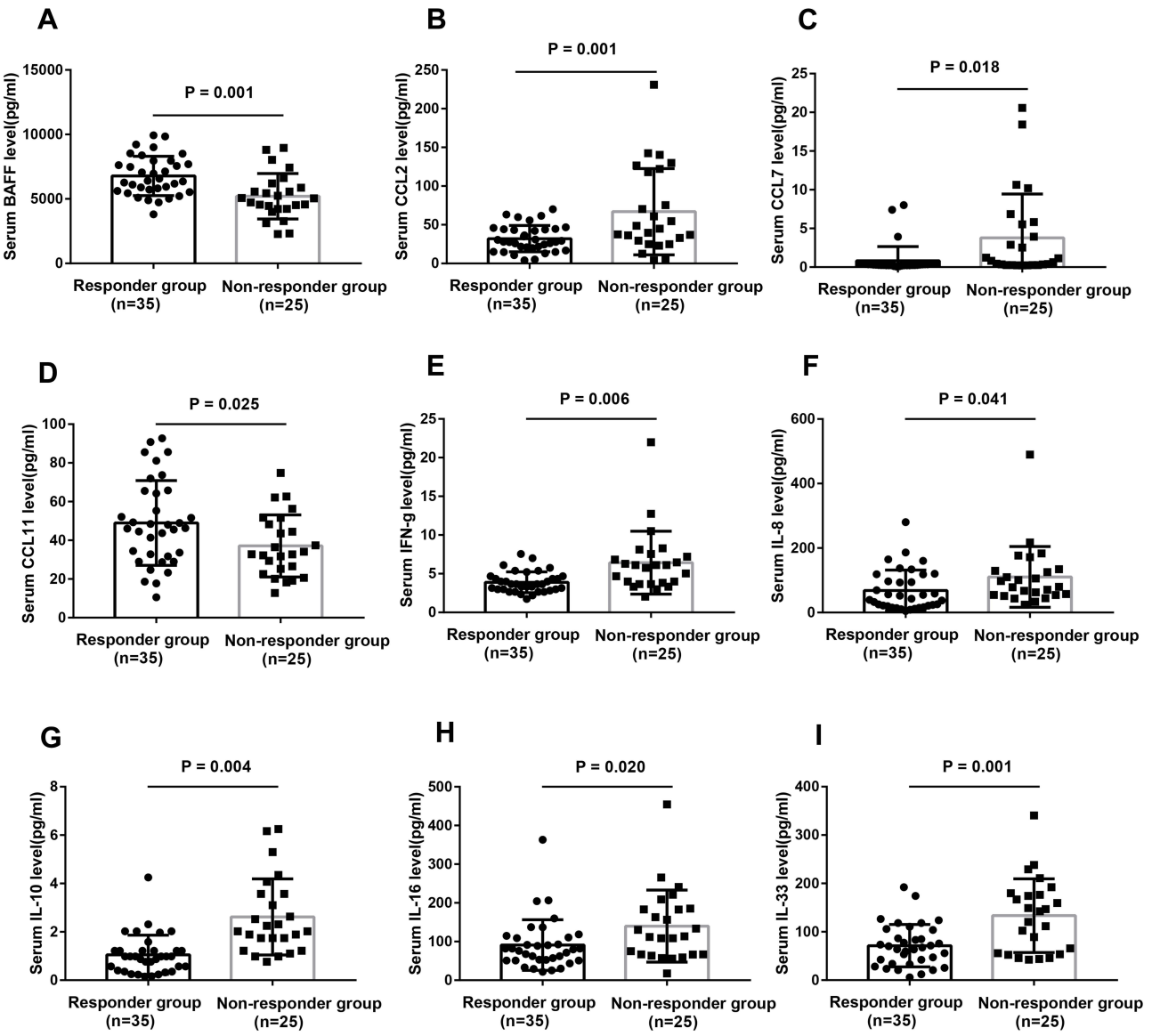

Figure 1 Serum levels of 9 differential cytokines between responder group and nonresponder group (**A**–**I**).
Abbreviations: BAFF, B cell activating factor; CCL, CC motif chemokine ligand; IFN, interferon; IL, interleukin.

characteristics of all patients in the prospective cohort are shown in Table 1. No significant differences in age, sex, BMI, asthma, serum total and specific IgE levels, TNSS, or VAS scores were observed.

Cytokine Profiling Differs Between Responders and Nonresponders

Levels of 31 cytokines in responders and nonresponders are displayed in Table 2. As shown in Figure 1, nine cytokines were significantly different between the two groups, with responders having higher concentrations of BAFF and CCL11 and lower levels of CCL2, CCL7, IFNγ, IL8, IL10, IL16, and IL33 than nonresponders ($P<0.05$). ROC curves highlighted that serum BAFF, IFNγ, IL10, and IL33 exhibited strong ability for predicting the efficacy of SLIT (area under the curve <0.7, $P<0.05$; Figure 2), and detailed parameters are given in Table 3.

Validation of Potential Cytokines

In order to validate the predictive abilities of BAFF, IFNγ, IL10, and IL33, another independent cohort was studied. Table 4 presents the demographic and clinical characteristics of all participants. As shown in Figure 3, responders

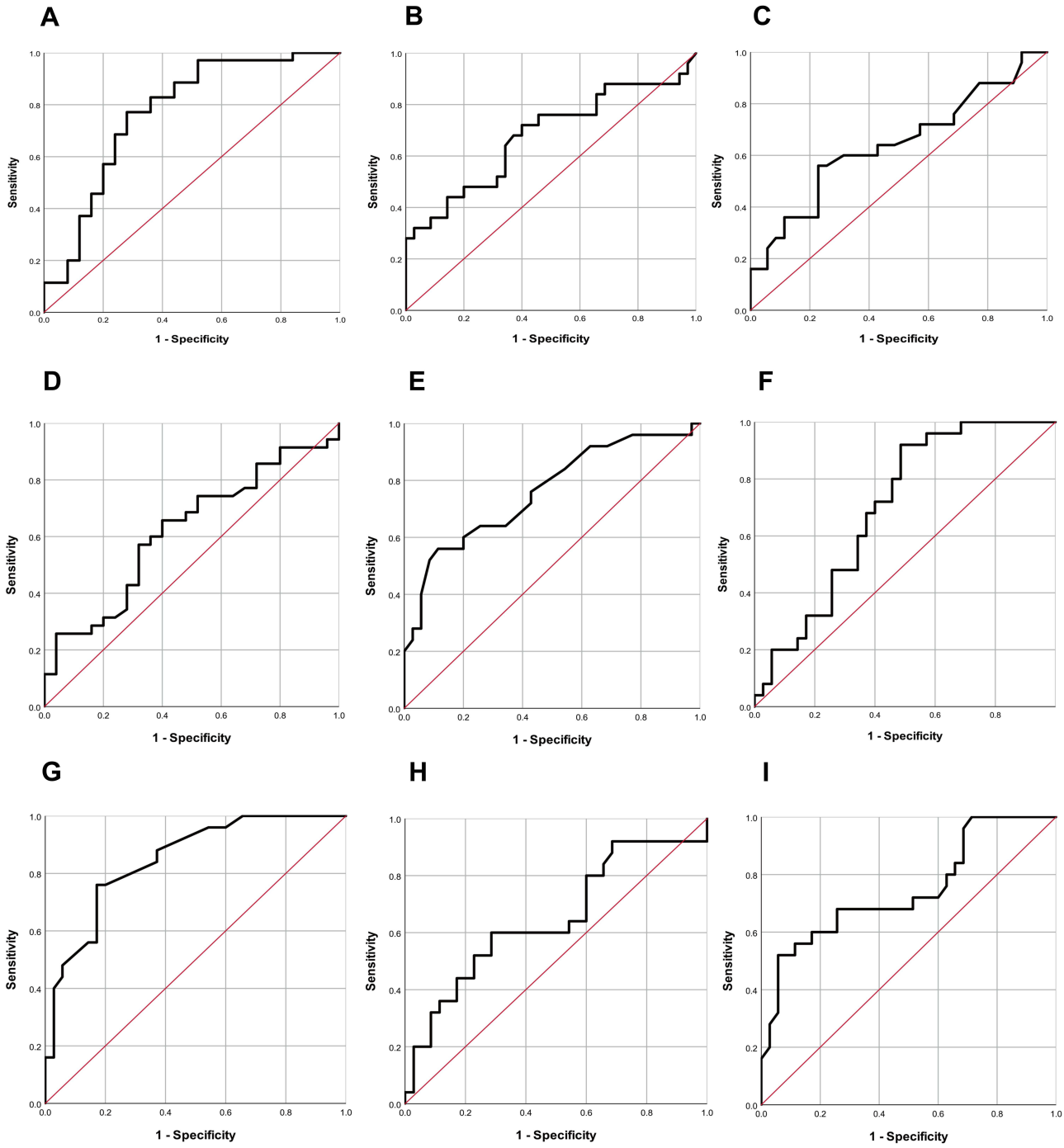

Figure 2 ROC curves of nine cytokines in predicting the efficacy of SLIT. (**A**) BAFF; (**B**) CCL2; (**C**) CCL7; (**D**) CCL11; (**E**) IFNγ; (**F**) IL8; (**G**) IL10; (**H**) IL16; (**I**) IL33.
Abbreviations: ROC, receiver-operating characteristic; SLIT, sublingual immunotherapy.

exhibited higher concentrations of IL10 and IL33 than nonresponders ($P<0.05$), but serum BAFF and IFNγ levels were not significantly different between the two groups. Moreover, respondes exhibited significantly higher IL10 levels and lower IL33 post-SLIT than pre-SLIT ($P<0.05$), but no significant difference was found in nonresponders. ROC curves revealed that serum IL10 and IL33 presented reliable accuracy for predicting the efficacy of SLIT in the validation cohort (Figure 4 and Table 5).

Table 3 ROC results for ytokines in terms of SLIT efficacy (pg/mL)

	AUC (95% CI)	*P*	Cutoff	Sensitivity	Specificity
BAFF	0.770 (0.642–0.899)	<0.001	5591.1	0.771	0.680
CCL2	0.678 (0.533–0.822)	0.020	35.6	0.680	0.629
CCL7	0.642 (0.495–0.788)	0.075	0.6	0.560	0.771
CCL11	0.619 (0.476–0.763)	0.073	44.5	0.571	0.680
IFNγ	0.760 (0.634–0.886)	0.001	5.6	0.560	0.886
IL8	0.699 (0.569–0.830)	0.009	43.1	0.920	0.514
IL10	0.847 (0.751–0.944)	<0.001	1.7	0.760	0.829
IL16	0.647 (0.502–0.793)	0.053	100.7	0.600	0.714
IL33	0.745 (0.615–0.875)	0.001	134.5	0.520	0.943

Abbreviations: ROC, receiver-operating characteristic; SLIT, sublingual immunotherapy; AUC, area under the curve.

Discussion

The present study explored multiple-cytokine profiling in the sera of AR patients treated with SLIT and evaluated associations with SLIT effectiveness. We firstly observed that cytokine levels were significantly different between responders and nonresponders, and that serum BAFF, IFNγ, IL10, and IL33 might be associated with the efficacy of SLIT. Further validation results revealed that serum IL10 and IL33 were elevated in nonresponders in the validation cohort, and patients in the responder group exhibited significantly higher IL10 and lower IL33 post-SLIT than pre-SLIT. Taken together, our observations indicate that serum IL10 and IL33 might serve as potential biomarkers for objectively predicting the efficacy of SLIT and contribute to its therapeutic mechanisms.

IL10 is an anti-inflammatory cytokine that acts as a pivotal immunoregulatory molecule and is involved in inflammatory and allergic diseases.[20,21] Previous studies have demonstrated that IL10 levels are dysregulated in nasal lavage fluid, nasal mucosa, and peripheral blood of AR patients and animal models and associated with the occurrence and development of AR.[22–24] Boonpiyathad et al[25] reported that IL10 activated innate lymphoid cells and the number of innate $IL10^+$ lymphoid cells was associated with the efficacy of AIT in house dust-mite AR patients. A recent study found that serum IL10 levels were enhanced in AR patients who were treated with SLIT and vitamin D supplementation, which implied that IL10 expression might be involved in the development of immunotolerance.[26] In the present study, serum IL10 levels were strongly linked with the effectiveness of SLIT and IL10 concentrations significantly enhanced in responders after 1 year's SLIT, but did not change in nonresponders between pre-SLIT and post-SLIT, in line with most prior conclusions.[25,26] Development of immunotolerance is a critical process in immunotherapy. The induction of T_{reg} and B_{reg} cells have been proven to be modified during AIT and correlated with its clinical efficacy.[27–29] An elevated number of T_{regs} and B_{regs} promotes the secretion of IL10 to dampen the T_h2 phenotype and reinforce the T_h1 phenotype.[30–32] Moreover, accumulating evidence has shown that IL10-producing T_{reg}, B_{reg}, and group 2 innate lymphoid (ILC2) cell counts correlate inversely with symptom severity after AIT.[33,34] These observations suggest that serum levels might serve as an indicator of the success of AIT; however, the underlying mechanisms remain unclear.

Table 4 Demographic and clinical characteristics of subjects in the validation cohort

	Responders (n=50)	Nonresponders (n=30)	*P*
Age, years	28 (24–33)	30 (25–37)	0.307
Male, n (%)	23 (46)	19 (63.3)	0.927
BMI (kg/m^2)	22.1 (21.2–23.2)	22.0 (21.1–23.0)	0.618
Asthma, n (%)	14 (28)	12 (40)	0.327
Serum total IgE (IU/mL)	291.7 (218.8–372.5)	279.4 (219.5–372.1)	0.782
Serum-specific IgE (IU/mL)	33.6 (15.8–60.9)	36.0 (27.8–54.6)	0.423
TNSS, median (range)	9 (6–10)	6 (5–8)	0.213
VAS score, median (range)	7 (5–8)	5 (4–7)	0.975

Abbreviations: BMI, body-mass index; TNSS, total nasal symptom score; VAS, visual analogue scale.

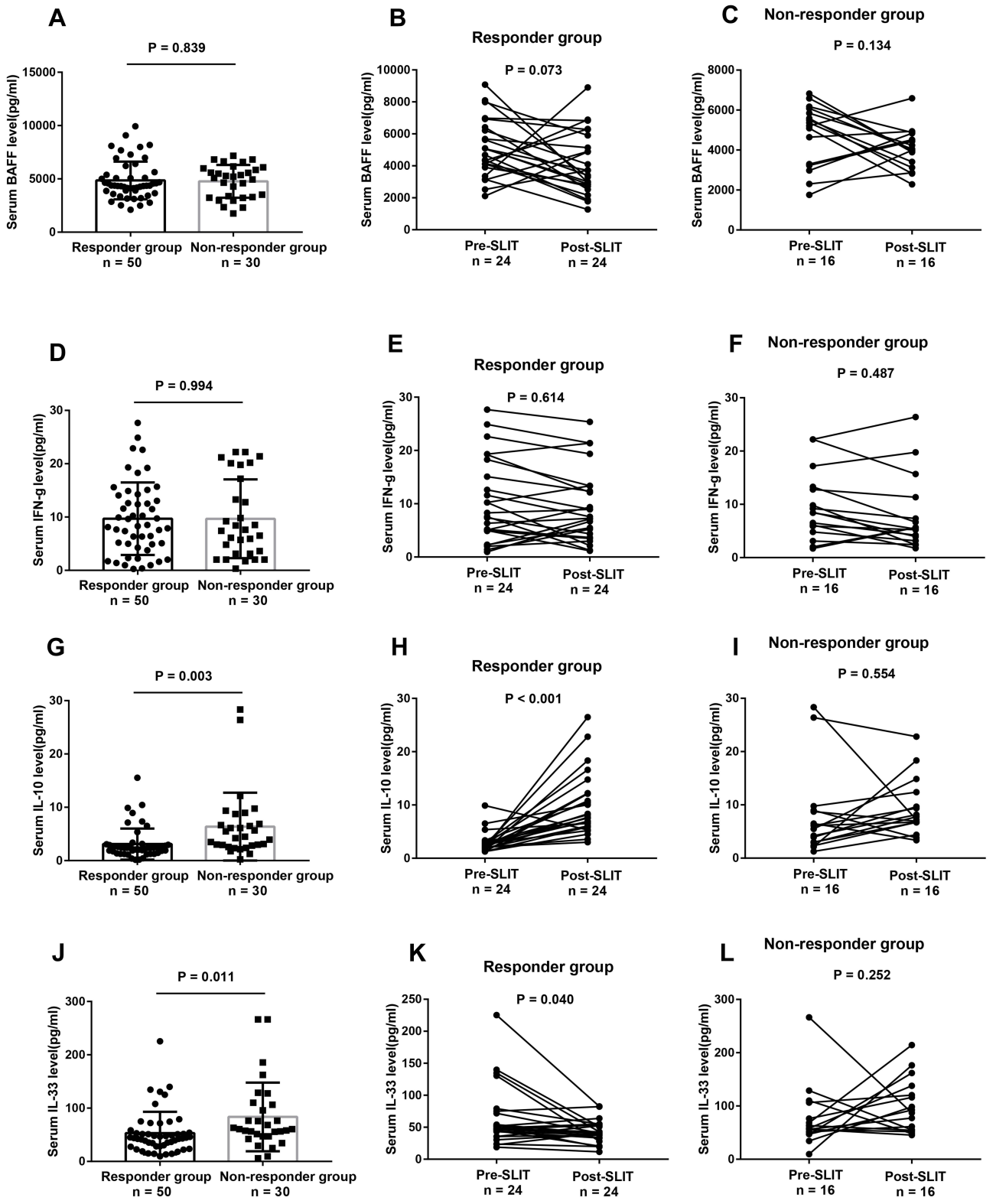

Figure 3 Serum levels of four potential cytokines in validation cohort. (**A–C**) BAFF; (**D–F**) IFNγ; (**H** and **I**) IL10; (**J–L**) IL33.
Abbreviation: SLIT, sublingual immunotherapy.

Another important finding in the present study was that serum IL33 levels were associated with the effectiveness of SLIT and that concentrations were reduced when patients responded to SLIT. IL33 is known to be an epithelial alarmin cytokine that is released upon external irritants, contributes to activation of ILC2 and T_h2 cells, and involved in mucosa

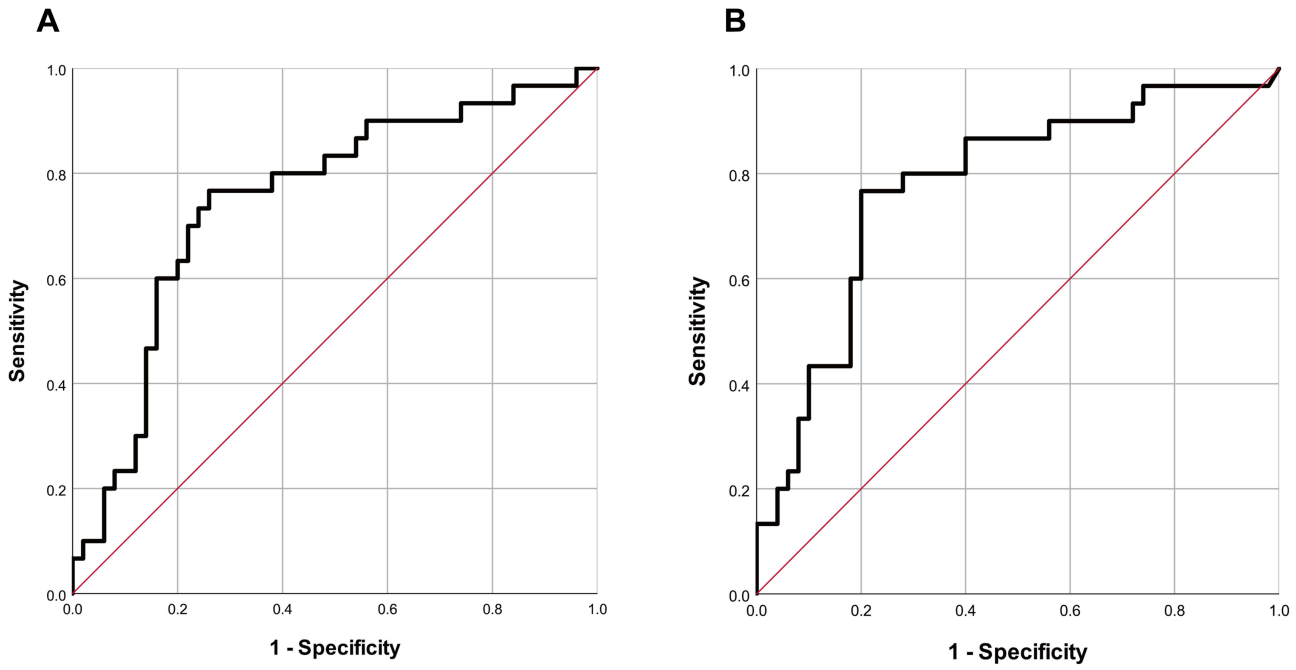

Figure 4 ROC curves of serum IL10 (**A**) and IL33 (**B**) in predicting the efficacy of SLIT in the validation cohort.
Abbreviations: ROC, receiver-operating characteristic; SLIT, sublingual immunotherapy.

eosinophilia and immunoresponse.[35] Studies have reported a crucial role of IL33 in the inflammatory response and tissue remodeling in asthma and AR.[36,37] A recent publication found that serum IL33 and its receptor ST2 were lower in AR patients than healthy controls and that SLIT enhanced IL33 expression, but did not change ST2 levels.[38] However, Wang et al[39] proved that IL33 levels were decreased during SLIT in the nasal lavage of AR children and positively correlated with increased IL10 expression. In this study, we found that serum IL33 levels were closely associated with the efficacy of SLIT in AR patients and reduced after 1 year's treatment, in accordance with Wang et al's conclusion. Accordingly, IL33 has been found to be an essential promoter in T_h2-based inflammation.[40] High concentrations of IL33 can activate its ST2 receptor and facilitate the production of T_h2 cytokines and IgE, then aggravate eosinophil recruitment and airway hyperreactivity, resulting in refractory inflammation in AR and a failure of SLIT. As such, we can speculate that IL33 might play a decisive role in the underlying therapeutic mechanism of SLIT and serum IL33 is a reliable biomarker for predicting the efficacy of SLIT in AR patients.

Although we observed that responders exhibited higher serum BAFF and lower IFNγ levels than nonresponders in the discovery cohort, these tendencies were not found in the validation cohort. We speculate that these two cytokines might serve a limited role in the therapeutic effects of SLIT in AR patients. More studies are needed to clarify their functions in AR.

There are several limitations to this study. First, the sample is relatively small. All patients were from a single medical center and of the same ethnicity, which may have increased the risk of selection bias and limit generalization. Second, follow-up was relatively brief, and we evaluated only the early efficacy of SLIT. Associations between multiple-cytokine levels and more prolonged efficacy of SLIT are not clear.

Table 5 ROC analysis results for IL10 and IL33 in terms of SCIT efficacy (pg/mL)

	AUC (95% CI)	*P*	Cutoff	Sensitivity	Specificity
IL10	0.750 (0.636–0.864)	<0.001	2.8	0.767	0.740
IL33	0.778 (0.669–0.886)	<0.001	55.4	0.767	0.800

Abbreviations: ROC, receiver-operating characteristic; SLIT, sublingual immunotherapy; AUC, area under the curve.

In conclusion, we firstly confirmed that serum multiple-cytokine profiling was associated with the response to SLIT and that serum IL10 and IL33 might serve as novel biomarkers for early prediction of efficacy. We also found that serum IL10 and IL33 levels changed during SLIT in AR patients, which suggests that they were involved in the therapeutic mechanisms of SLIT. Further multicenter studies with a large samples and longer follow-up are needed to confirm our conclusions.

Data Sharing

The data used to support the observations of this study are available from the corresponding author upon request.

Ethics

This study was conducted in accordance with the recommendations of the Declaration of Helsinki. The Human Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University approved the study, and all participants provided informed consent.

Funding

This work was supported by the National Natural Science Foundation of China (62076198) and the General Projects of Shaanxi Provincial Key Research and Development Program (2020GXLH-Y005).

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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