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Letter to the Editor

Thrombospondin 1-mediated suppression of mast cell degranulation is involved in the efficacy of sublingual immunotherapy

Dear Editor,

After being introduced in 2014,¹ various sublingual immunotherapy (SLIT) drugs have been approved for treatment of allergic rhinitis in Japan. In addition to high efficacy rates, it is remarkable that SLIT is expected to have curative effects. However, the mechanisms by which SLIT exerts their pharmacological effects are not well-characterized. CD4⁺ T cells are candidate target cells of SLIT. Recently, Ihara *et al.* reported that a decrease in allergen-reactive ST2⁺CD45⁺CD4⁺ cells in peripheral blood was correlated with SLIT efficacy.² Upregulation of CD103⁺Foxp3⁺CD4⁺ T cells was induced by SLIT in a murine model of Japanese cedar pollinosis (JCP).³ We proposed that bitter taste receptors expressed on CD4⁺ T cells and an apoptosis pathway in CD4⁺ T cells were determinants of SLIT efficacy against JCP.^{4,5}

Contributions of another allergen recognition system, an IgE/mast cell-dependent pathway, to SLIT efficacy is controversial. Although downregulation of allergen-specific IgE and induction of IgE blocking-antibody often results from subcutaneous immunotherapy (SCIT),⁶ these effects are not always obvious in SLIT. We also observed the increment, rather than decrease, of allergen-specific serum IgE in JCP patients, regardless of decrease in disease severity, in 72% patients after SLIT for 2 years.⁷ No significant differences were observed between patients divided into high-responder (HR) and non-responder (NR) groups.

Comparison between HR and NR patients that received SLIT provided new insights into SLIT mechanisms, particularly with regard to CD4⁺ T cells.^{4,5,7} In this study, we further investigated the effects of SLIT on the IgE/mast cell-dependent pathway. Mast cells established from CD34⁺ cells in cord blood were sensitized with sera of JCP patients with high titer anti-cedar pollen IgE. Their degranulation was evoked by the crosslinkage of IgE with cedar pollen allergen. After depletion of 12 major serum proteins, including albumin and immunoglobulins, the effects of sera from HR and NR patients on allergen-induced degranulation of mast cells were examined. Degranulation was slightly suppressed by adding sera of post-SLIT NR patients, but significantly stronger suppression was induced by post-SLIT HR sera (Fig. 1A), suggesting that an inhibitory factor for mast cell degranulation was induced by SLIT, especially in sera of HR patients.

Since HR and NR patients were not distinguishable by any single serum parameter of IgE, IgG subclasses, or 50 cytokines,⁷ we investigated mast cell degranulation inhibition factors using a proteomic approach (Supplementary Methods). Sera of HR and NR patients were subjected to comparative isobaric tags for relative and absolute quantitation (iTRAQ) analysis after enriching minor proteins using ProteoMiner Protein Enrichment Kits (Bio-Rad Laboratories Inc., Hercules, CA, USA). Potential degranulation inhibition factors were present in the pre-treated and pooled samples from post-SLIT HR patients (Fig. 1B). Comparisons were made between HR

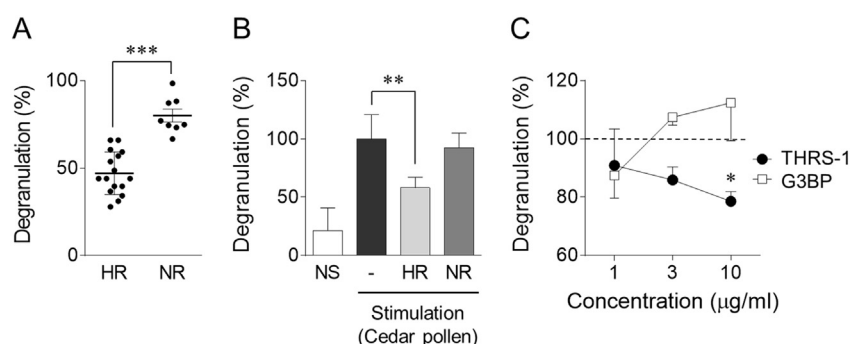


Fig. 1. Effects of sera of post-SLIT HR and NR patients on mast cell degranulation. Mast cells were immunized with high anti-cedar pollen IgE sera of JCP patients after removal of 12 abundant proteins (A). After additional enrichment of minor proteins, pooled sera were added to the cells (B). Degranulation of mast cells was induced by stimulation with 10 µg/ml cedar pollen extract. Several concentrations of THRS-1 and G3BP were added 30 min before allergen stimulation (C). Data are expressed as means $\pm$ SEM of percent of allergen-induced degranulation response in the absence of serum and inhibitory factors ($n = 4-16$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ [compared with stimulated control for (C)].

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Table 1
HR-specific inducible proteins.

Protein name	Fold difference	
	HR-post/NR-post	HR-post/HR-pre
Anti-thrombin III	5.2	4.1
Thrombospondin 1 (THRS-1)	5.1	1.7
Galectin-3-binding protein (G3BP)	2.5	3.3
Serum amyloid P	2.4	1.6

Proteins specifically induced in the sera of HR patients following SLIT were isolated by iTRAQ proteomics analysis. Representative proteins in HR patients after SLIT (HR-post), which were expressed at levels >1.5-fold higher than those in both NR patients after SLIT (NR-post) and HR patients before SLIT (HR-pre) are listed.

and NR patients after SLIT and between HR patients before and after SLIT, resulting in identification of four candidate proteins (Table 1). Although anti-thrombin III, thrombospondin 1 (THRS-1), galectin-3-binding protein (G3BP), and serum amyloid P were not identified in our previous study in which differentially expressed genes and proteins between HR and NR patients were analyzed,^{4,5,7} these proteins potentially correlated with the difference in sensitivity of patients may become biomarkers indicating the efficacy of SLIT. The expression levels of these proteins in individual patients deserve further investigation.

Previous studies have shown that THRS-1 and G3BP are implicated in allergic diseases or immune functions.^{8–10} Based on these studies, we used recombinant THRS-1 and G3BP to evaluate degranulation of mast cells. THRS-1 exerted a weak, but dose-dependent suppression of allergen-induced mast cell degranulation, whereas G3BP did not suppress degranulation (Fig. 1C). The suppressive effect of THRS-1 but not G3BP on degranulation was also seen by using rat mast cell line, RBL-2H3 (Supplementary Fig. 1). Inhibitory effects of THRS-1 on mast cell activity had not been reported previously, and the corresponding mechanisms are unknown. However, the effect of THRS-1 on other immune cells has been demonstrated. THRS-1 behaved as an autocrine negative regulator of dendritic cells.⁸ For CD4⁺ T cells, THRS-1 promoted the generation of regulatory T cells from CD4⁺ T cells.⁹ Interaction with CD47 expressed on target cells is responsible for these THRS-1 functions.^{8,9} Whether the suppressive effect of THRS-1 on mast cell degranulation is mediated through CD47 is unknown.

Our study clearly demonstrates that induction of factors that inhibit mast cell degranulation, including THRS-1, is a mechanism underlying SLIT efficacy. In conjunction with our previous studies showing the involvement of taste receptors and apoptosis pathways in CD4⁺ T cells and with future studies for the effects of anti-thrombin III and serum amyloid P, this study showed that SLIT displays its strong pharmacological effect via multiple cell types and pathways.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2019.03.007>.

Conflict of interest

The authors have no conflict of interest to declare.

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Supplementary Methods

Clinical study of sublingual immunotherapy (SLIT) on Japanese cedar pollinosis (JCP)

Study design, patient recruitment, administration of cedar pollen extract, and evaluation of clinical efficacy were recently described.¹ Briefly, 202 adult patients with JCP lived in Tokyo, Japan, were over 20 years old, and attended Nippon Medical School Hospital, Tokyo Metropolitan Hospitals, and clinics in Tokyo. All patients suffered from symptoms of allergic rhinitis, such as sneezing, rhinorrhea, and nasal congestion during the February to April pollen season for at least three consecutive years. They were skin-test positive and IgE-positive for cedar pollen allergen. The study was registered in the University Hospital Medical Information Network Clinical Trials Registry Database (UMIN000016532) and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All experimental procedures were approved by the ethical committee of Nippon Medical School Hospital, Tokyo Metropolitan Institute of Medical Science, and other clinics participated. All patients provided informed written consent prior to participation.

All subjects were administered a cedar pollen allergen.¹ Briefly, patients held the allergen with bits of bread (about $1.5 \times 1.5 \times 1.5$ cm) under the tongue for 2 min and then, spat it out. Starting at one drop of 2 Japanese allergy units (JAU)/ml, the amount of allergen administered to patients was gradually increased and reached up to 20 drops of 2,000 JAU/ml as a maintenance dose at five weeks. Administration of the allergen started from July 2006 and continued for two years.

Nasal symptoms of individual patients from February 1 to April 30 across four pollen seasons from 2006 to 2009 were investigated using the Japanese guideline for allergic rhinitis.^{2, 3} Briefly, the number of sneezes, nose blowing, extent of nasal congestion, and eye itchiness were self-recorded daily by patients using an allergy diary according to the classification means developed by Okuda et al.⁴ QOL of the patients were determined three times: at the end of February, middle of March, and middle of

April, in each year using the Japan Rhinoconjunctivitis Quality of Life Questionnaire No.1 (JRQLQ No.1). At the end of each pollen season, the clinical efficacy of SLIT was evaluated based on the descriptions in the allergy diary and JRQLQ No.1 as described previously.⁵ Consistent with the results of previous investigations,^{6, 7} approximately 30% of patients did not show any improvements in their symptoms, even after undergoing two years of SLIT treatment. The first 33 HR patients whose conditions were extremely improved, and the last 34 NR patients whose conditions were either unchanged or exacerbated, were selected.¹

Cultured human mast cells

Human mast cells were obtained from CD34⁺ cells as previously described.⁸ Briefly, CD34⁺ cells purified from cord blood using anti-CD34⁺ microbeads with a magnetic cell sorting system were cultured in methylcellulose medium supplemented with 2 µg/ml stem cell factor (SCF, PeproTech, NJ, USA), 500 ng/ml IL-6 (PeproTech), and 10 ng/ml IL-3 (PeproTech). After four weeks, IMDM containing 1% insulin-transferrin-selenium, 50 µM 2-mercaptoethanol, 100 U/ml penicillin + streptomycin, 2% FCS, 100 ng/ml SCF, and 50 ng/ml IL-6 was added onto the resulting cells. After two weeks, cells were recovered and cultured in IMDM supplemented with 2% FCS, SCF, and IL-6 for 12 weeks. Half of the medium was replaced with fresh medium every seven days. The resulting live cells were used as mature mast cells.

Mast cell degranulation

Mast cells (5×10^5 /ml) were sensitized by overnight incubation with 1/100 volume of JCP patient sera with anti-cedar pollen IgE levels of 29.9-36.9 U/ml. Cells were washed three times with Tyrode's salt solution containing 0.03% human albumin and suspended in the same buffer (6.25×10^5 /ml). Eighty microliters of cell suspension (5×10^4) was treated with 1 µl of sera from post-SLIT HR

or NR patients after removal of top 12 abundant proteins by Top 12 Abundant Protein Depletion Spin Columns (ThermoFisher Scientific, Waltham, MA, USA). In some experiments, pooled sera from five HR or NR patients were treated with ProteoMiner Protein Enrichment Kits (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. Following incubation at 37°C for 30 min, cells were stimulated with 10 µg/ml cedar pollen extract (Cosmo bio CO., LTD. Tokyo, Japan) or left unstimulated for 2 h. Degranulation was evaluated by measurement of β-hexosaminidase released from mast cells as described previously.⁹ Mast cell degranulation experiments were also performed in the presence of several concentrations of recombinant thrombospondin 1 (THRS-1, R&D Systems, Minneapolis, MN, USA) and galectin-3-binding protein (G3BP, R&D Systems). Since normal human sera contain microgram-submicrogram/ml order these proteins,^{10, 11} 1-10 µg/ml of them were used in this experiment. Effects of THRS-1 and G3BP on the degranulation was also examined by using rat mast cell line, RBL-2H3, upon sensitization with 1 µg/ml anti-DNP IgE (Cosmo bio CO., LTD.) and stimulation with 0.5 ng/ml DNP-BSA (Cosmo bio CO., LTD.).

Proteomics analysis

Comparative proteomics analysis of sera of HR and NR patients was performed as described previously.¹² Briefly, 20 µg of protein from each of the three sample pools (HR-pre, HR-post, and NR-post) were treated with ProteoMiner Protein Enrichment Kits, then reduced and cysteine-blocked using Tris (2-carboxyethyl) phosphine and S-methyl methanethiosulfonate, respectively. Proteins were digested with trypsin and resulting peptides were labeled using iTRAQ Reagent Multiple Kit (AB Sciex, Framingham, MA, USA) as follows: iTRAQ 114 tag – HR-post, iTRAQ 115 tag – NR-post, and iTRAQ 116 tag – HR-pre. The respective labeled peptides were mixed, then purified by cation-exchange solid phase extraction to remove interfering substances prior to mass spectrometry analysis. Eluted samples were desalted using a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), dried by vacuum

centrifugation, and analyzed on a TripleTOF 5600 system with an Eksigent cHiPLC-nanoflex system (AB Sciex). Protein identification and relative quantitation were processed by Paragon algorithm in ProteinPilotTM software (version 4.5, AB Sciex). Among the proteins identified with greater than 95% confidence, proteins in which the value of HR-post/NR-post and HR-post/HR-pre exhibited a significant ($p < 0.05$) >1.5-fold change were selected as candidate biomarkers.

Statistical analysis

Results are presented as the means or means $\pm$ SEM. Statistical analyses were performed using Student's *t*-test or one-way ANOVA and Dunnett's or Dunn's multiple comparison test. A *p* value <0.05 was considered statistically significant.

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