

## Rush Oral Immunotherapy Does Not Reduce Allergic Response in Mice with Mild Allergy to Egg White Ovomucoid

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**Summary** Oral immunotherapy (OIT) is a promising therapeutic approach for treating food allergy. Past studies have shown that OIT reduces allergic response only in severe allergy model mice. We worked to establish mild allergy model mice, and investigated whether ‘rush’ OIT for 10 d improved the allergic response and biomarkers in these mice. Balb/c mice were sensitized to ovomucoid (OM) in alum. The rush OIT was done for 10 d. Oral OM challenge was used to determine the impact of OIT on the allergic response. We measured allergic biomarkers, such as vascular permeability in the skin, plasma levels of total IgE, OM-specific IgE, IgG1 and IgG2a and cytokines in splenocyte culture supernatant. OIT for 10 d did not improve allergy symptoms and increased vascular permeability. Total IgE in the plasma of OIT-treated mice was significantly higher than in that of non-treated mice. OM-specific IgG1 and IgG2a plasma levels were not significantly different between OIT-treated and non-treated mice. Among the cytokine secretion of splenocyte from OIT-treated mice, IFN- $\gamma$  and IL-10 were significantly lower than in non-treated mice, and IL-4 and IL-5 were significantly higher. Total TGF- $\beta$  in the OIT-treated group was not detected. The IFN- $\gamma$ /IL-4 ratio of the OIT-treated group was about 1/8 that of the non-treated group. OIT for 10 d was not effective and some biomarkers showed negative responses in the mild allergy model mice. We suggest OIT should be used very carefully as this treatment carries a risk of worsening allergy symptoms for mice with mild allergy.

**Key Words** oral immunotherapy, food allergy, allergy model mice

Food allergies affect about 3% of the overall population and up to 4–6% of children (1–3). In recent times, the prevalence of reported food allergy has increased 18% among children under 18 y of age (2, 3). Dietary avoidance of specific allergens is recommended as the first-line approach in the management of food allergy (1, 2). Recently, oral immunotherapy (OIT) has been studied as a new treatment for food allergy (2, 4, 5). OIT uses small amounts of food allergen to desensitize the patient. However, OIT is not often recommended because it carries the risk of increasing allergy symptoms (2, 6).

In Japan, egg allergy has been reported as the most prevalent food hypersensitivity in the pediatric population, exceeding cow's milk allergy (7). Ovalbumin (OVA) and ovomucoid (OM) were identified as egg white allergen epitopes, constituting about 54 and 11% of egg white protein, respectively (8, 9). OM is heat resistant and has high allergenicity (10, 11). It is a well-known trypsin inhibitor (8). This suggested that OIT for OM (egg)-specific allergy patients may be a highly effective treatment and a pathway to allergen tolerance.

As research in this area has been limited, there is inadequate evidence to establish proper OIT protocols, such as allergen dosage, escalation, duration of ther-

apy and method of administration (2). OIT protocols are categorized as either ‘slow’ or ‘rush.’ In slow OIT, the allergen dose is increased at home over 1–2 y (12, 13). In rush OIT, the allergen dose is increased in about 1–2 wk under intensive management and supervision in a hospital (14–16). In some studies, rush OIT was shown to improve allergy responses (14, 15). However, Blumchen et al. reported that rush OIT was not effective in patients with peanut allergy (16).

Recently, OIT has been studied in egg white-specific allergy model mice. These studies reported decreases in allergy reaction and/or histamine release at oral challenge by the OIT treatment (17–19). However, most of the mice used in the studies were shown to exhibit either severe allergy or high IgE levels (18, 19). In general, allergic disorder is rich in diversity, including severe allergy. Therefore, it is interesting to elucidate the effect of OIT on mild allergy model mice. However, no study has evaluated whether rush OIT reduces the allergic response in mild allergy model mice.

In allergy model mice, the intensity of the allergic response can regulate sensitization conditions (20, 21). Our aim in this study was to establish mild allergy model mice, and to clarify whether rush OIT for 10 d improved the biomarkers of allergic response in these mice.

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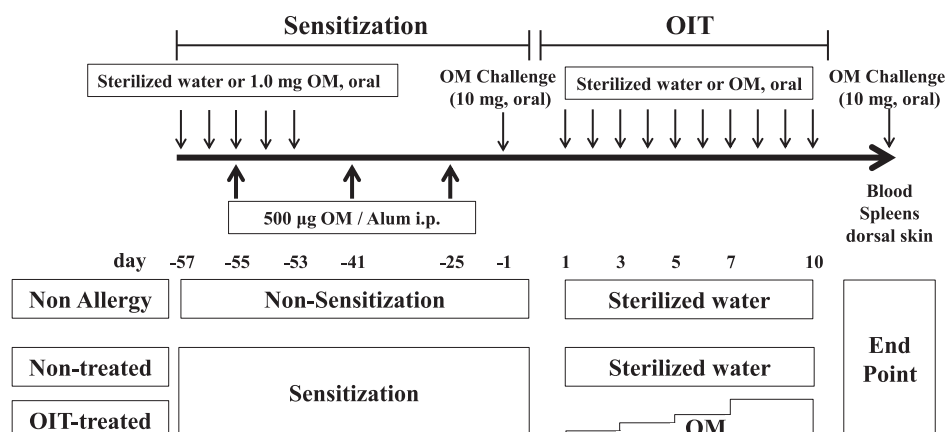


Fig. 1. Mouse experimental protocol. The protocol of sensitization included a dose of 1.0 mg OM/mouse on days -57 to -53 and sensitization by intraperitoneal (i.p.) administration of 500 µg OM/100 µL saline plus 100 µL of alum on days -55, -41, and -25. The method of rush OIT included administration of 0.5 mg OM/d during days 1-2, 1.0 mg OM/d during days 3-4, 2.5 mg OM/d during days 5-6, and 5.0 mg OM/d during days 7-10 using automatic pipettes.

## EXPERIMENTAL

**Model mice allergic to OM.** The OM used in the experiment was purified from chicken egg white using the method of Takahashi and Asao (22). Male Balb/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) at 6 wk of age and then allowed to acclimate to their new housing for 8 d before experimental protocols began. Mice were fed MF (Oriental Yeast Co., Ltd., Tokyo, Japan) which is a chicken egg-free diet. Mice were allowed access to water and food ad libitum during the course of the study.

The method we used for sensitization of the mice has been modified from that of several reports (20, 21, 23) (Fig. 1). Ten mice drank 1.0 mg OM/mouse using automatic-pipettes from days -57 to -53 and were sensitized by intraperitoneal (i.p.) administration of 500 µg OM/100 µL saline plus 100 µL of aluminum hydroxide (Alum, Wako Pure Chemical Industries, Ltd., Osaka, Japan) on days -55, -41 and -25. Five mice that received sterilized water intraperitoneally and were not sensitized, served as the non-allergy group.

Sensitized mice were divided into two groups: (1) OM-sensitized, treated with OIT (OIT-treated group) and (2) OM-sensitized, treated with a placebo (non-treated group). Mice in the OIT treatment group were treated for 10 d (Fig. 1). The OIT protocol was modified from that of Leonard et al. (17). The OIT-treated group mice were administered 0.5 mg OM/d during days 1-2, 1.0 mg OM/d during days 3-4, 2.5 mg OM/d during days 5-6 and 5.0 mg OM/d during days 7-10 using auto pipettes. Mice in the non-treated group were given sterilized water during the therapy term. Mice of the OIT-treated group were bred for 1 wk after OIT. After the final oral challenge, which was the experimental endpoint, mice were killed under isoflurane anesthesia to obtain plasma, spleen, and dorsal skin samples.

Experimental animal care and treatment conformed to Mukogawa Women's University's guidelines for the ethical treatment of laboratory animals (FSN-01-2014-

01, Hyogo, Japan). Animals were housed at 22°C with 60% humidity under a 12-h light (08:00-20:00) – dark (20:00-08:00) cycle.

**Food challenge in allergy mice.** Mice were orally challenged with 10 mg OM/50 µL on day -1 and at the final day. Mice were monitored for allergic symptoms and scored on a 0 to 5 point scale (0, no symptoms; 1, scratching around the nose and head; 2, puffiness around the eyes and mouth with reduced activity; 3, labored respiration, wheezing, cyanosis around the mouth and tail, or both; 4, no activity after prodding or tremor and convulsion; 5, death) for 30 min after oral injection.

**Vascular permeability.** Vascular permeability is activated by anaphylaxis. It causes the release of histamine from mast cells. We measured the vascular permeability using the method of Yamaki et al. as an index of food allergy symptom (24). Immediately following the administration of 100 µL of 4.5% fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA, Sigma-Aldrich Co., St. Louis, MO) via tail vein injection after a 10 mg OM/50 µL oral injection, 50 µL of 28 µg OM/mL in Tyrode's solution (Sigma-Aldrich Co.) and 50 µL of Tyrode's solution were administered intradermally at 4 points (test, two points; control, two points) in the shaved dorsal skin of the mice as a test substrate for vascular permeability response and as a control, respectively. The fluorescent intensity of the extract from the dorsal skin and plasma was measured automatically using a fluorescent plate reader (Infinite M200, Tecan Ltd., Männedorf, Switzerland) with 485 nm for excitation and 530 nm for emission. The vascular permeability (50 µL plasma equivalent) was calculated as follows: (higher test fluorescence value – higher control fluorescence value)/(plasma fluorescence value).

**Plasma levels of total IgE, OM-specific IgE, IgG1 and IgG2a.** We used an enzyme-linked immunosorbent assay (ELISA) to determine plasma levels of total IgE, OM-specific IgE, IgG1 and IgG2a. Total IgE level was measured by the Mouse IgE ELISA Quantitation Set

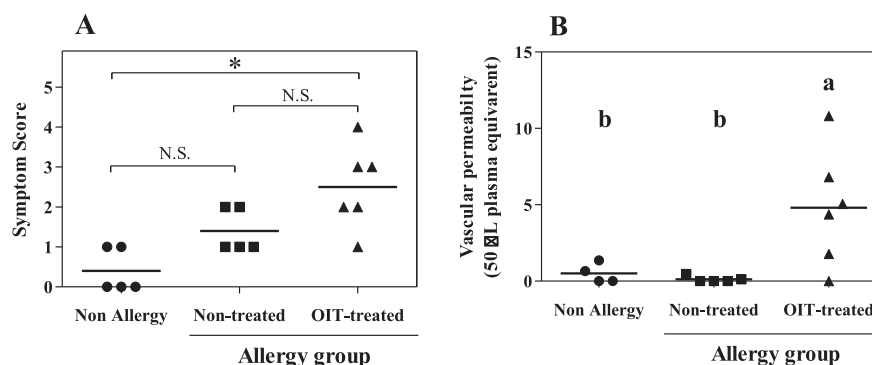


Fig. 2. Symptom score (A) and vascular permeability in dorsal skin (B) after oral challenge at endpoint. Data indicate values and mean. Significant difference of symptom score (A) was calculated via the Mann-Whitney *U* test and the Ryan method (19) and  $p < 0.05$  is indicated as \*. N.S. means not significant. Significant difference of vascular permeability (B) is indicated by different superscript letters (a, b) and was determined by one-way ANOVA and was followed by Tukey's multiple-comparison test,  $p < 0.05$ .

(Bethyl Laboratories, Inc., Montgomery, TX). The ELISA assay solutions used ELISA Starter Accessory Kit (Bethyl Laboratories, Inc.). OM-specific IgE, IgG1 and IgG2a plates were coated with the appropriate OM (10  $\mu$ g/mL) in coating buffer at 37°C for 2 h. The wells were washed 4 times with washing buffer, and then blocking buffer was added and incubated at room temperature (22–24°C) for 1 h. After washing 4 times, plasma samples diluted in dilution buffer for IgE (1 : 5 dilution), IgG1 (1 : 10,000 dilution), and IgG2a (1 : 200 dilution) were added and incubated at room temperature (22–24°C) for 16–18 h (IgE) or 2 h (IgG1 and IgG2a). After 4 washings, the detection antibody conjugated with horseradish peroxidase was added to the plate at room temperature (22–24°C) for 2 h. After 5 washings, tetramethylbenzidine (TMB) substrate solution was added to each well and incubated at room temperature (22–24°C) for 10–15 min. The reaction was stopped by 1.0 mol/L sulfuric acid and the absorbance was measured at 450 nm by a microplate reader.

**Mouse spleen cell cultures and cytokine secretions.** Mice spleens were removed and added to Hank's balanced salt solution (HBSS: 0.98% Hank's (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), 0.46% HEPES (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), 0.2% NaHCO<sub>3</sub>, 1.0% penicillin-streptomycin (Nacalai Tesque, Inc., Kyoto, Japan), and 10% fetal calf serum (Corning, Inc., Corning, NY)). Splenocytes were isolated in HBSS from the spleen homogenate by Lympholyte-M (Cedarlane Laboratories, Ltd., Burlington, ON). Cells were plated at a density of  $2.5 \times 10^6$ /mL in complete medium (1% RPMI-1640 (Nissui Pharmaceutical Co., Ltd.) supplemented with 1.25% NaHCO<sub>3</sub>, 1.0% penicillin-streptomycin, 10% fetal calf serum, 1% glutamine (Nacalai Tesque, Inc.) and 0.225  $\mu$ g/mL 2-mercaptoethanol) in a 24-well microplate. Cell cultures were carried out in 5.0  $\mu$ g/mL concanavalin A (ConA) or medium (negative control) and incubated at 37°C under 5% CO<sub>2</sub>. Culture supernatants were collected after 48 h. Concentrations of interleukin-4 (IL-4), IL-5, IL-10, interferon- $\gamma$  (IFN- $\gamma$ ), and total transforming growth fac-

tor- $\beta$  (TGF- $\beta$ ) were assayed by ELISA according to the manufacturer's instructions (IL-4, IFN- $\gamma$ , IL-5, IL-10 and total TGF- $\beta$ : Biolegend, San Diego, CA). The concentrations were calculated as follows: concentration stimulated by ConA – non-stimulated concentration.

**Statistical analysis.** Values are the mean  $\pm$  SE. Allergy symptom scores were analyzed using the Kruskal-Wallis test and the *p* value of multiple-comparison test was calculated via the Mann-Whitney *U* test and Ryan method (25). Statistical significance of plasma antibody levels and vascular permeability was determined by one-way ANOVA and followed by Tukey's multiple-comparison test. Cytokines analysis was followed by an unpaired *t*-test. *p*-values  $< 0.05$  were considered significant. Graph Pad Prism version 5.0 (Graph Pad Software, San Diego, CA) was used for all analyses.

## RESULTS

### Effect of OIT on mild allergy model mice

Allergy symptom scores and vascular permeability in the dorsal skin were the indices of allergic response in the oral challenge. Allergy symptom scores of the OIT-treated group were significantly higher than those of the non-allergy group and were not significantly different from those of the non-treated group (Fig. 2A). The vascular permeability of the OIT-treated group was significantly higher than that of other groups (Fig. 2B). There were no significant differences in symptom scores or vascular permeability between the non-treated group and non-allergy group (Fig. 2A and B).

### Influence of OIT on plasma levels of total IgE, OM-specific IgG1 and OM-specific IgG2a

It is well known that Type 1 and Type 2 helper T cell (T<sub>H</sub>1/T<sub>H</sub>2) imbalance causes allergic response. T<sub>H</sub>2 induces a B-cell class-switch of IgE and IgG1. Connection of a mast cell and IgE is responsible for type 1 allergies. Total IgE concentration in the non-allergy group was significantly lower level than that in the other groups (Fig. 3A). The concentration in the OIT-treated group was twice higher than that in the non-treated group (Fig. 3A). OM-specific IgE levels were not detected

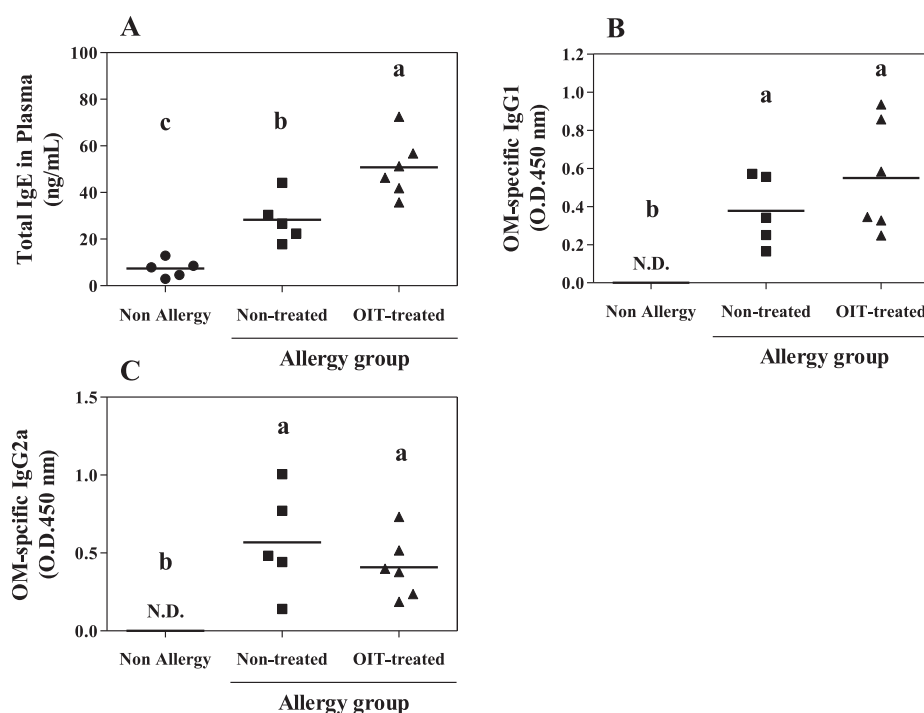


Fig. 3. Plasma concentration of total IgE (A) and levels of OM-specific IgG1 (B) and IgG2a (C). Data are presented as values and mean. Optical density (OD) for IgG1 and IgG2a in plasma diluted 1/10,000 and 1/200 respectively is expressed. Significant difference is indicated by different superscript letters (a, b, c) and was determined by one-way ANOVA followed by Tukey's multiple-comparison test,  $p < 0.05$ . N.D. means not detected.

Table 1. Cytokine concentrations of IFN- $\gamma$ , IL-4, IL-5, IL-10, and total TGF- $\beta$  following in vitro stimulation of spleen cell cultures with ConA.

|                           | Non-treated     | OIT-treated     | <i>p</i> value |
|---------------------------|-----------------|-----------------|----------------|
| IFN- $\gamma$ , ng/mL     | 4.19 $\pm$ 0.73 | 1.47 $\pm$ 0.19 | 0.0069         |
| IL-4, pg/mL               | 7.32 $\pm$ 2.71 | 43.0 $\pm$ 11.7 | 0.0330         |
| IL-5, pg/mL               | 15.5 $\pm$ 7.1  | 56.9 $\pm$ 13.3 | 0.0252         |
| IL-10, pg/mL              | 720 $\pm$ 113   | 463 $\pm$ 6.4   | 0.0023         |
| TGF- $\beta$ , pg/mL      | 57.6 $\pm$ 36.8 | N.D.            | -              |
| IFN- $\gamma$ /IL-4 ratio | 339 $\pm$ 103   | 41.5 $\pm$ 8.8  | 0.0137         |

Values were means $\pm$ SE for 4–5 mice. The values were obtained after subtraction of the concentration level from non-stimulated controls. Statistical significance was determined by the unpaired *t*-test. Differences with  $p < 0.05$  were considered statistically significant. N.D., not detected. Hyphen (-), not statistically analyzed.

in any of the groups (data not shown). OM-specific IgG1 and IgG2a levels of non-allergy were not detected (Fig. 3B and C). There were no significant differences between the non-treated group and the OIT-treated group as to OM-specific IgG1 and IgG2a levels (Fig. 3B and C).

#### *Influence of OIT on $T_h1/T_h2$ ratio in spleen culture supernatant*

Splenocyte cytokine secretions reflect the ratio of  $T_h$  cell differentiation.  $T_h1$  is related to cell-mediated immunity and releases substances such as IFN- $\gamma$ , TNF- $\alpha$ , and granulocyte-macrophage colony-stimulating fac-

tor (GM-CSF).  $T_h2$  is related to humoral immunity and releases cytokines such as IL-4 and IL-5. The concentration of IFN- $\gamma$  in the OIT-treated group was about 1/3 that in the non-treated group (Table 1). Concentrations of IL-4 and IL-5 were about 6 and 3.5 times higher than those in the non-treated group, respectively (Table 1). IL-10 is a suppressor cytokine and is involved in attaining allergen tolerance (26). The concentration of IL-10 in the OIT-treated group was significantly lower than that in the non-treated group (Table 1). TGF- $\beta$  regulates the differentiation of regulatory T cells. Total TGF- $\beta$  was detected in the non-treated group, but not in the OIT-treated group (Table 1). The IFN- $\gamma$ /IL-4 ratio reflects the  $T_h1/T_h2$  balance. The ratio of the OIT-treated group to the non-treated group was about 1 : 8 (Table 1).

## DISCUSSION

OIT has been studied recently in the treatment of food allergy, and in humans and mice to establish a mechanism for oral tolerance and a protocol of treatment. In the present study, we performed the following experiments to establish mild allergy model mice and to evaluate the effects of rush OIT in these mice.

First, we identified the conditions required to effectively generate a model mouse with a mild allergy to OM. In this experiment, the symptom scores of all the mice in the non-treated group were  $>1$  and the total IgE of the non-treated group was 4 times higher than that of non-allergy group (Fig. 3A,  $p < 0.05$ ). The total IgE level in allergy mice was lower than those in previous studies (20, 27). The above results were consistent with

what we had predicted, since oral administration before sensitization suppressed the increase in total IgE (28, 29). In this experiment, alum was used as the adjuvant for sensitization. In the manufacturer's instructions for alum, the amount of allergen should be 5.0 mg/mouse. In some reports, the low amount of allergen used in the sensitization reduced specific IgE levels and allergy responses (20, 21). Accordingly, we decided to use 0.5 mg/mouse, which was 1/10 of the recommended amount of alum. Most allergy model mice are female since female mice are known to develop allergies more easily than male mice (30). Therefore, we decided to use male mice in the experiment. We confirmed allergy reactions in all the allergy mice and the symptom scores were 1 or 2. Leonard et al. reported that the symptom score of the allergy model mice was 4, which showed no activity after prodding or tremor and convulsion, after oral challenge (17). Rupa and Mine reported the mean of symptom score in allergy model mice was 3, which showed wheezing, labored respiration, and cyanosis (19). From previous reports and our results, it appears that we succeeded in regulating the allergic response in mice and established mild allergy model mice.

The target amount of allergen used in rush OIT is not defined. The maximum dose of OM in this OIT protocol was 0.151 g OM/kg of body weight (5.0 mg OM/mouse, mouse body weight 33 g). This amount is approximately the same as that used in human OIT (15). Thus, the result of the OIT protocol shows that the present OIT protocol may be applicable OIT for humans allergic to eggs.

Recently, there have been many studies regarding human OIT (4–6, 12–16, 31) and allergy model mice (17–19, 29, 30, 32, 33). Nurmatov et al. reported that OIT can promote allergen desensitization, but the risk of local adverse reactions increased with OIT in a meta-analysis (4). Failure of OIT, in which allergen desensitization was not achieved, has also been reported (16, 31). In this experiment, the allergic response and some allergy biomarkers in the OIT-treated mice were found to be worse than those of the non-treated mice. Allergy model mice treated with OIT for 1 wk exhibited gastrointestinal symptoms (i.e. weight loss and soft stools) but these symptoms improved after 1 wk (33, 34). Therefore, we suggest that oral intake of allergen in the early stages of OIT carries the risk of exacerbating the allergy condition.

It has been reported that rush OIT improved allergic responses in human patients (14, 15). Itoh et al. used rush OIT for severe allergy as a therapeutic treatment (15). Leonard et al. demonstrated that rush OIT reduced allergy response in severe allergy model mice (17). As described above, we examined the effect of rush OIT in mild allergy model mice, but the improvement of the allergic responses was not observed. The present finding suggests that rush OIT may be effective in severe allergy mice, but that the OIT is not valid in mild allergy model mice.

IL-10 is secreted by a wide variety of cells and has many functions (26). TGF- $\beta$  promotes the induction

of IL-10-secreting CD4<sup>+</sup> regulatory T cells by modulating Foxp3 expression (18, 26). In this experiment, significant positive correlation was observed between the IFN- $\gamma$ /IL-4 ratio and IL-10 concentration in the culture supernatant of the spleen ( $p < 0.001$ ,  $r = 0.9022$ ). Total TGF- $\beta$  was not detected from the spleen secretion in the OIT-treated group. The underlying mechanism is unknown, but it appears that IL-10 modulated by TGF- $\beta$  might play a role in the maintenance of T<sub>H</sub>1/T<sub>H</sub>2 balance.

In conclusion, rush OIT of 10 d is not effective in mild allergy model mice. Some biomarkers showed negative responses to allergen desensitization. We suggest that OIT should only be used very carefully as this treatment carries the risk of worsening the allergy symptoms for mild allergy mice.

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