

Effectiveness of Dp2 nasal therapy for Dp2- induced airway inflammation in mice: using oral *Ganoderma lucidum* as an immunomodulator

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Nasal immunotherapy with allergen has been reported to be effective for airway allergic disease. A group of 50 male Balb/c mice were immunized intraperitoneally with recombinant *Dermatophagoides pteronyssinus* group 2 (rDp2), then oral feeding with *Ganoderma lucidum* (known as "Ling Zhi," LZ OT) and intranasal therapy with native Dp2 (Dp2 NT) were given, the mice then received intratracheal challenge with rDp2 at 28 days and 35 days after immunization. Airway hypersensitivity to methacholine was measured 30 min (early phase) and 24 h (late phase) after the second challenge. The cytokine producing CD4 cells in PBL and interferon- γ (IFN- γ) concentrations in bronchoalveolar lavage fluid and sera were measured on 37 days after immunization. Both Dp2 NT and LZ OT downregulated total inflammatory cell infiltration in the airway. Dp2 NT reduced IL-5⁺/CD4⁺ cells and increased IFN- γ ⁺/CD4⁺ cells. When LZ OT was added to Dp2 NT, the reduction of IL-5⁺/CD4⁺ cells was diminished and the increment of IFN- γ ⁺/CD4⁺ cells was increased. LZ OT alone increased both IL-5⁺/CD4⁺ cells and IFN- γ ⁺/CD4⁺ cells. When LZ OT was added to Dp2 NT, IgG2a was further increased to a significant level. LZ OT alone significantly suppressed IgG1 and increased IgG2a production. When lung function was measured after therapy, early phase airway hypersensitivity to methacholine significantly suppressed by Dp2 NT, while late phase hypersensitivity was suppressed but not to a significant level. When LZ OT was added to Dp2 NT, the suppression of late phase airway hypersensitivity to methacholine reached a significant level. In this mouse model of Dp2-induced airway hypersensitivity, Dp2 NT downregulated airway inflammatory cell infiltration and decreased immediate airway hypersensitivity to methacholine. When LZ OT was coadministered, the airway lymphocytes and circulatory IFN- γ ⁺/CD4⁺ were both increased and late phase airway hypersensitivity was decreased. These results suggest that Dp2 NT might have a therapeutic effect on Dp2-induced airway hypersensitivity and LZ OT might also have an effect on Dp2 NT immunotherapy.

Key words: *Dermatophagoides pteronyssinus* (Dp2), *Ganoderma lucidum*, immunotherapy

Allergic disorders affect at least 20% of the population of developed countries. They include hay fever, asthma, and atopic dermatitis. These symptoms are associated with high levels of serum allergen-specific IgE and eosinophilia [1-4] and are dependent upon interleukin-4 (IL-4) and interleukin-5 (IL-5) released from allergen specific CD4 cells expressing the T helper 2 (Th2) cytokine profile [5-7]. Recent advances in the understanding of the inflammatory process in allergic diseases have led to the hypothesis that a shift in polarization of cytokine production from a Th2 to a Th1 cytokine profile by allergen-specific T cells may allow

for more specific therapy. The basis of this strategy is the supposed mutual opposition between Th1 and Th2, where Th1 cytokines down regulate Th2 function, and so would be expected to reduce IgE [8, 9]. Recent reports showed an inverse correlation between the frequency and intensity of the response to viral and bacterial infection and the development and prevalence of allergic diseases [10-12]. Immunization with the *Mycobacterium bovis*-*Bacillus Calmette Guérin* (BCG) provides protection against atopy and asthma in Japanese schoolchildren. The activation of Th1 responses may predict a lower prevalence of allergic inflammation [12].

Ganoderma lucidum, known in China as "Ling Zhi" (LZ), is a fungus herb thought to have properties that can promote longevity and enhance immune function.

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Ling Zhi was reported to be an effective treatment for chronic hepatopathy, hypertension, hyperglycemia, and neoplasia. In a mouse model, LZ significantly inhibited the growth of tumor cells and reduced tumor metastasis in mice. This herb can restore DNA polymerase activity and enhance the recovery of cellular immunocompetence in gamma-irradiated-mice. Ling Zhi contains polysaccharides, nucleotides, steroid, amino acids, and polypeptides. LZ-8 is a purified protein derived from *Ganoderma lucidum* that has a mitogenic effect on human mononuclear cells. LZ-8 has been shown to have a significant effect on cellular immunity and can also increase the production of the IL-2 from T cells. It also induces cellular aggregate formation, which is correlated with increased ICAM-1 expression. Furthermore, LZ can increase the production of IFN- γ by T lymphocytes and of TNF- γ , IL-1, and IL-6 by macrophages. These cytokines may play major roles in the antitumor action of LZ. LZ-8 has an immunomodulatory effect whereby it reduces antigen induced antibody formation. Repeated LZ-8 injection into nonobese diabetic mice can completely prevent the occurrence of autoimmune diabetes mellitus [13-19]. Therefore, the activation of Th1 by LZ may have a therapeutic role in the treatment of allergic diseases.

The house dust mite, *Dermatophagoides pteronyssinus* (Dp), is the most important clinical allergen, and is the dominant species among mites collected in household environments in Taiwan [20]. A mite-induced asthma animal model has been reported which can be used for the evaluation of the effectiveness of therapy. It has long been recognized that local nasal immunotherapy (LNIT) is capable of controlling the symptoms of seasonal allergic rhinitis in a dose-dependent manner. At higher doses, LNIT can sometimes cause local adverse reactions. On the other hand, studies that used a low-dose administration schedule did not show clinical effectiveness although tolerance was found to be good [21-27]. The working mechanism of nasal immunotherapy is not clear, and the therapeutic effectiveness of coadministration of oral immunotherapy with LZ has not been determined.

The purpose of this study was to evaluate the effectiveness of immunotherapy using Dp2 NT in conjunction with LZ OT in mice with rDp2-induced allergic inflammation.

Materials and Methods

Animals

Male Balb/c mice were obtained from the National Laboratory Breeding Research Center in Taiwan and

were raised in a specific pathogen-free environment. The mice used in experiments were between 6 and 8 weeks of age. Groups of 7 mice were caged separately according to their assigned treatment. Recombinant *D. pteronyssinus* group 2 (rDp2) and native Dp2 (nDp2) were prepared as previously described [20]. Ling Zhi was purchased from Shiun Tien Tang Co. Ltd., (Taipei, Taiwan).

Induction of allergic airway inflammation

Mice were immunized by intraperitoneal (IP) injection of 1 μ g/0.1 mL rDp2 emulsified in 4 mg/0.062 mL aluminum hydroxide AL(OH)₃ (Whitehall Lab Ltd, Punchbowl, Australia) on day 0 and day 7. After immunization, mice received daily therapy with nDp2 (intranasal, 1.8 μ g/6 μ L/mouse/day) or combined therapy with nDp2 and LZ. The control groups of mice were fed with LZ 1 mg/100 μ L/day, buffer and dexamethasone (DEX) 1 μ g/100 μ L/mouse/day. On day 28 and day 35, mice were lightly anesthetized with an IP injection of 60 mg/kg of sodium pentobarbital (Sigma Chemical Co., St Louis, MO, US) and intratracheally (IT) inoculated with 1 μ g/50 μ L of rDp2. Two days after the second IT inoculation, mice were sacrificed by overdose sodium pentobarbital after pulmonary function assessment.

Sample collection and preparation

Bronchoalveolar lavage was performed by the following procedure. One separate dose of 1 mL sterile endotoxin-free saline was injected into the lungs via the trachea of each mouse. Approximately 0.85 mL of the washing solution was recovered constantly. BALF was aspirated and stored at -70°C until assay. After total leukocyte counting, cytopsin preparations of 100 μ L BALF were stained with Liu stain (Tonyar Diagnostic Inc, Taipei, Taiwan) and differential counts were performed on 200 cells. Blood samples were obtained via the orbital sinus and sera were collected and stored at -70°C until assay.

Determination of Dp2 specific immunoglobulins

Blood was obtained from retro-orbital venous plexus at the beginning and end of the experiments. Serum IgG1 and IgG2a titers of anti-Dp2 antibodies were determined using an enzyme-linked immunosorbent assay (ELISA), during which plates (Nunc Lab, Chicago, US) were coated with 100 μ L rDp2 overnight at a concentration of 0.5 μ g/mL in 4°C in a refrigerator. Detection antibodies (Horse radish peroxidase-conjugated goat anti-mouse IgG2a Ab at 1:800 and IgG1 Ab at 1:2000; Southern Biotech Assoc, Inc., Birmingham, AL, US) were added separately for each

assay and the bound enzyme substrate was detected with TMB (Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD, US). The reaction was stopped with 50 μ L 4N H₂SO₄ after 15 minutes and the optical density was measured at 405 nm using a multiscan spectrophotometer (model A-5682, SLT Lab Instruments, Salzburg, Austria). Results were expressed as ELISA units (EU). To assure reproducibility, a known serum was run with each test as a standard.

Cytokine measurement

IFN- γ was measured using commercially available ELISA kits (R&D Minneapolis MN, US) containing mouse monoclonal antibodies recognizing different epitopes of the cytokine molecules. The lower limit of the detectable range was 10 pg/mL. Due to their low concentration in the BALF, IL-4 and IL-5 were not measured in this part of the study.

Pulmonary function determination

Each mouse was placed inside a barometric plethysmograph (Buxco Electronics, Troy, NY, US). The plethysmograph has 2 chambers: one is the main or animal chamber (internal diameter, ID 7.5 cm and 5.5 cm height) and the other is the reference chamber (ID 7.5 cm and 3.5 cm height). A differential pressure transducer was employed to detect the pressure difference between the 2 chambers. The pressure signal was amplified, digitized via an A/D converter card, and sent to a computer for analysis using the BioSystem XA program (Buxco, Electronics, Troy, NY, US), which sampled and calculated the desired respiratory parameters. These parameters were enhanced pause (Penh), tidal volume, breathing frequency, peak inspiratory flow, peak expiratory flow, end-inspiratory pause, and end-expiratory pause.

Aerosol was generated by placing 5 mL of saline or methacholine (Sigma, 1.56 to 6.25 mg/mL) solution in the cup of an ultrasonic nebulizer (DeVilbiss, Somerset, PA, US). The aerosol was delivered via a connecting tube and a 3-way connector to the animal chamber of the plethysmograph [28]. According to information provided by the manufacturer, the median amount of the aerosol was approximately 3 μ m with a range from 1 to 5 μ m. The aerosol usually filled the chamber within 15 to 20 sec. Initially, each mouse inhaled saline aerosol for 3 min. Then the respiratory parameters were measured for 3 min. Then, inhalation of the saline aerosol was replaced by inhalation of the aerosol of methacholine solution for 3 min. In both cases, the aerosol in the chamber was cleared immediately after the exposure. Respiratory parameters

were then measured for 3 min after the inhalation of methacholine. A dose-response curve for methacholine was calculated starting from a low dose of 0 mg/dL and moving to a higher dose of 6.25 mg/dL. There was a 15 min interval between exposures. The percentage changes of Penh obtained for each dose of methacholine were represented as means $\pm$ SEM. Differences in parameters among groups were analyzed by analysis of variance.

Cell culture, immunofluorescence staining and flow cytometry analysis

Flow cytometric determination of cytokine in activated murine T helper cells was assayed according to the methods of Assenmacher *et al* [29]. Two-color staining methods were used to analyze IFN- γ and IL-5 expression in CD4 cells. Leukocytes from peripheral blood (PBL) were stimulated with phorbol myristate acetate (PMA; 50 ng/mL), ionomycin (2 μ M) and GolgiStop (Cytofix/Cytoperm Plus Cat No. 2076KK, Pharmingen, San Diego, CA, US) for 5 h and then washed twice with phosphate buffered saline (PBS). The cells were stained with CD4-FITC or IgG1-FITC at room temperature for 30 min and washed. Cells were fixed with cytofix/cytoperm at room temperature for 30 min and stained with anticytokine antibody and IgG2a-PE at room temperature for 30 min and washed. Both IgG1-FITC and IgG2a-PE, purchased from pharmingen, were used as negative control. Cells were resuspend in 0.5 mL PBS containing 0.1% w/v sodium azide. Mean fluorescence was measured by Becton Dickinson flow cytometry (Becton Dickinson, CA., US). A total of 5000 cells were analyzed in each sample.

Statistical analysis

Results were expressed as arithmetic mean $\pm$ SEM. Differences among the groups were assessed by the Mann-Whitney U test. A *p*-value less than 0.05 was considered to be statistically significant.

Results

Effect of immunotherapy on Dp2-induced airway inflammation

After immunotherapy, the body weights of both experimental and control groups of mice were similar to the naive group of mice. Dp2 NT alone downregulated the total inflammatory cell infiltration in the airway. Not only eosinophils but also neutrophils were decreased after therapy. However, lymphocytes were increased after Dp2 NT. When LZ OT was added

Table 1. Effect of immunotherapy on the total cells and cell subpopulations in BALF

	Total cells	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Naïve	12.3 ± 0.3	11.3 ± 0.3	1.0 ± 0	0	0
Buffer	102.5 ± 3.4	16.5 ± 2.1	16.0 ± 2.7	38.8 ± 4.2	31.0 ± 2.6
Dp2 NT	63.2 ± 3.4 ^a	14.8 ± 1.8	19.0 ± 2.5 ^b	20.2 ± 1.4 ^a	9.4 ± 2.4 ^a
Dp2 NT + LZ OT	73.6 ± 5.7 ^a	22.2 ± 2.9 ^b	23.6 ± 3.1 ^b	21.0 ± 1.6 ^a	8.2 ± 1.3 ^a
LZ OT	44.0 ± 1.6 ^a	11.0 ± 1.0	11.3 ± 1.3 ^a	13.5 ± 0.6 ^a	8.0 ± 0.7 ^a
DEX	51.5 ± 4.0 ^a	15.0 ± 1.2	16.3 ± 2.1	15.5 ± 0.6 ^a	4.8 ± 0.8 ^a

Abbreviations: BALF = bronchial alveolar lavage fluid; NT = nasal therapy; LZ = Ling Zhi; OT = oral therapy; DEX = dexamethasone; Dp2 = *Dermatophoides pteronyssinus* group 2

Note: Data are mean ± SEM of total cell number obtained from four animals (×10⁴)

^a*p* < 0.05 when compared with buffer.

^b*p* < 0.05 when increased numbers compared with buffer.

to Dp2 NT, further increase of lymphocytes in the BALF was noted, LZ OT alone also down-regulated the total inflammatory cell infiltration. However, all subpopulations of leukocytes were decreased after LZ OT (Table 1).

Effect of immunotherapy on cytokine producing CD4 cells in PBL

Dp2 alone reduced the IL-5⁺/CD4⁺ cells and increased the IFN-γ⁺/CD4⁺ cells in PBL cultured with PMA and ionomycin for 5 h. When LZ OT was added to Dp2 NT, the reduction of IL-5⁺/CD4⁺ cells was diminished, but further increase of IFN-γ⁺/CD4⁺ cells occurred. LZ OT alone increased both IL-5⁺/CD4⁺ cells and IFN-γ⁺/CD4⁺ cells (Table 2). When the ratios of these 2 types of cells were compared, the lowest ratio occurred in mice that received Dp2 NT.

Effect of immunotherapy on immunoglobulin production

Dp2 NT alone suppressed IgG1 and increased IgG2a production in sera. However, these alterations did not reach significant levels. When LZ OT was added to Dp2 NT, the IgG2a was further increased reaching a significant level. LZ OT alone also suppressed IgG1 and increased IgG2a production to a significant level (Fig. 1). The IgE level was too low to be detected in this experiment.

Effects of immunotherapy on IFN-γ levels in sera and BALF

Dp2 NT alone was able to enhance IFN-γ release in sera and BALF. When LZ OT was added to Dp2 NT, the enhancement of IFN-γ was diminished. LZ OT alone was also able to enhance IFN-γ release in sera and BALF. However, this enhancement was less than that of Dp2 NT (Fig. 2).

Effect of immunotherapy on pulmonary function

Airway hypersensitivity to methacholine was evaluated 30 min (early phase) and 24 h (late phase) after Dp2 IT challenge. Although both early and late phase airway hypersensitivity were suppressed by Dp2 NT, only early phase suppression reached a significant level. When LZ OT was added to Dp2 NT, the late phase airway hypersensitivity to methacholine was further suppressed to a significant level. LZ OT alone had no significant effect on the airway hypersensitivity to methacholine (Fig. 3).

Discussion

In this study, we assessed the effectiveness of immunotherapy with Dp2 NT and Dp2 NT in conjunction with LZ OT in treating rDp2-induced airway hypersensitivity and inflammation in mice. We found that Dp2 NT can downregulate Dp2 induced early phase airway

Table 2. The percentage of cytokine-producing CD4 cells in PBL

	Naïve	Buffer	Dp2 NT	Dp2 NT + LZ OT	LZ OT	DEX
CD4	52.78	44.22	65.12	43.56	51.16	47.24
IL-5/CD4	0.46	12.96	5.92	10.98	14.72	7.18
IFN-γ/CD4	0.44	5.86	13.02	17.48	14.9	12.16
IL-5/IFN-γ	1.04	2.21	0.45	0.63	0.99	0.59

Abbreviations: PBL = peripheral blood leukocytes; IL = interleukin; IFN = interferon; NT = nasal therapy; Dp2 = *Dermatophoides pteronyssinus* group 2; LZ = Ling Zhi; OT = oral therapy; DEX = dexamethasone

Note: These results are for pooled sera of 6 mice, all data represent the percentage of positive cells in PBL.

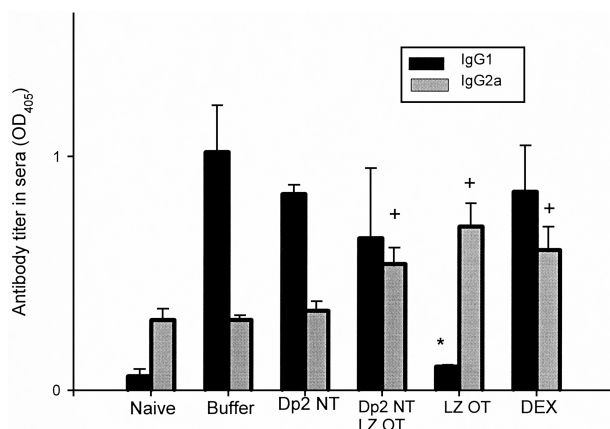


Fig 1. Effects of immunotherapy on Dp2 specific IgG1 and IgG2a production in sera.

* $p < 0.05$ compared with buffer IgG1.

* $p < 0.05$ compared with buffer IgG2a.

hypersensitivity. When Dp2 NT was co-administered with LZ OT, the rDp2 induced late phase but not early phase airway hypersensitivity was diminished. Although LZ OT alone reduced IgG1 production and increased IgG2a and IFN- γ production, it had only a trivial effect on airway hypersensitivity. Dp2 NT alone also increased IFN- γ production in BALF and sera, it had no effect on late phase airway hypersensitivity.

When mice received oral immunotherapy with LZ alone, there was a significant reduction of IgG1 and elevation of IgG2a production in the sera. The IFN- γ producing CD4 cells in the PBL and IFN- γ concentrations in BALF or sera were also increased. The total leukocyte, eosinophil and neutrophil counts in the BALF were also diminished. These results indicated that oral LZ therapy had anti-inflammatory and immunomodulatory effects in this animal model. The

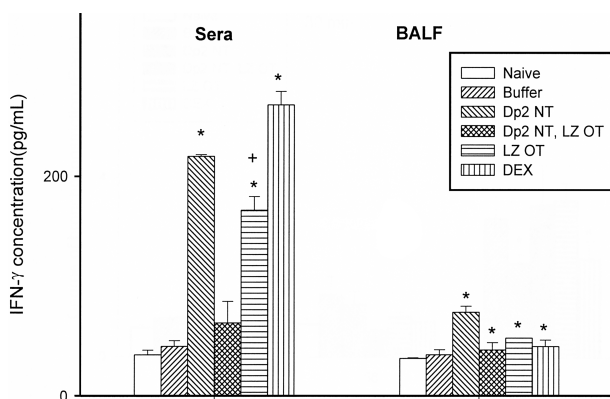


Fig 2. Effects of immunotherapy on IFN- γ concentration in sera and BALF.

* $p < 0.05$ compared with buffer.

* $p < 0.05$ compared with Dp2 NT.

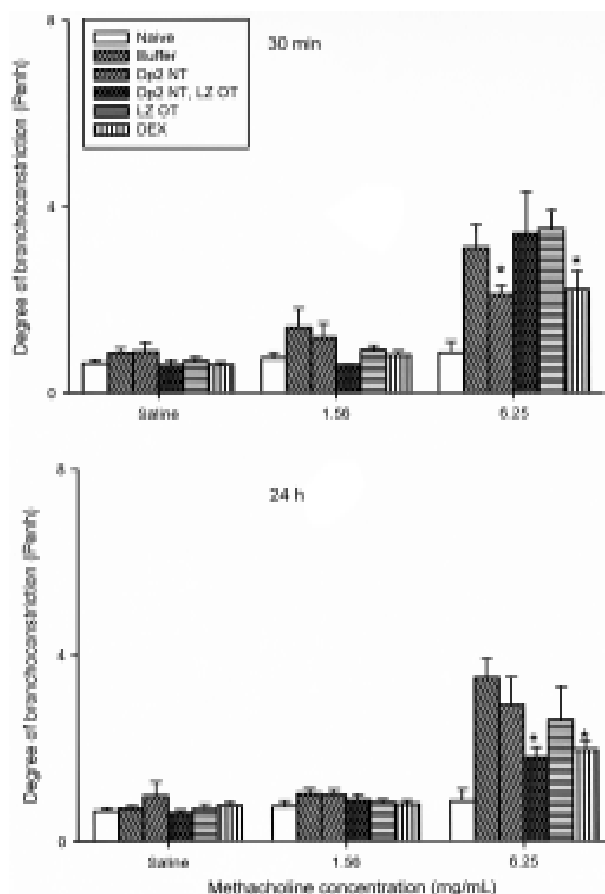


Fig 3. Effects of immunotherapy on the pulmonary function. Airway hypersensitivity to methacholine was measured 30 min and 24 hrs after rDp2 IT challenge. Penh was measured after each dose of methacholine challenge.

* $p < 0.05$ compared with buffer.

ratio of IL-5/IFN- γ producing CD4 cells also indicated that LZ might be able to down-regulate rDp2 induced allergic airway inflammation through IFN- γ production. This effect is consistent with previous studies which demonstrated LZ to have immunomodulatory effects and to be useful in cancer prevention [13-19]. Despite the inhibition of IgG1 production and inflammatory cell infiltration in the airway after oral LZ therapy in this study, no significant improvement was found in the lung function or airway hyperreactivity. This discrepancy may be due either to the anti-inflammatory effect or the presence of other inflammatory components in the airway. It has been reported that airway hypersensitivity can be determined by three independent pathways of airway hyperreactivity in mice [30]. The first pathway is dependent on IL-4 and mast cells, the second is IL-5 and eosinophils and the third is mediated by IL-13. Whether these different cytokines can be down-regulated by LZ OT remains to be clarified.

Nickelsen JA *et al* reported that specific nasal immunotherapy has prophylactic effect against the development of bronchial symptoms in patients with rhinitis or rhinoconjunctivitis. In their study, the dose was 100 times higher than that of conventional subcutaneous immunotherapy used for a treatment period of similar length [23]. In this study, we had similar findings that Dp2 NT decreased airway inflammation. Allergic asthma is characterized by airway hyperresponsiveness to specific stimuli, chronic eosinophilic inflammation, elevated serum IgE levels and excessive mucus production. The inflammatory component of this disease is characterized by increased numbers of activated T lymphocytes, mast cells, and eosinophils within the airway lumen and bronchial mucosa [31, 32]. In this study, when Dp2 NT alone, or LZ OT and Dp2 NT were administered, the airway hypersensitivity to methacholine was decreased. Lymphocytes were increased in BALF and PBL, which indicated that these therapies might be mediated by lymphocytes. It has been previously reported that coimmunization with a mixture of antigens and the immunostimulatory DNA sequences ISS elicits a Th1 response [33-34], and this treatment has also been shown to be effective in animal models considered relevant to the human allergic disease state [35-39]. Amb A1, a major ragweed allergen which has been conjugated to ISS has recently been reported to reflect an enhanced Th1-biased immunogenicity and a reduced antigenicity [40]. In this study, the concentration of IFN- γ was elevated not only in sera and BALF, but also in IFN- γ producing CD4 cells, which indicated that LZ OT might be relevant to the Th1 cell activity. Whether this working mechanism is similar to that of intradermal injection will remain unknown until injectable LZ is available for study. Results obtained using the mouse model of Dp2-induced airway hypersensitivity in this study suggest that the nasal delivery of an allergen in conjunction with oral delivery of a Th1 response-inducing adjuvant may amplify the therapeutic effect of allergen immunotherapy.

This study found slight inflammation was still present in groups of mice treated with DEX, which might have been due to insufficient concentration to suppress the rDp2-induced airway inflammation.

In conclusion, in this Dp2 induced airway hypersensitivity mouse model, Dp2 NT down-regulated airway inflammatory cell infiltration and decreased immediate airway hypersensitivity to methacholine. When LZ OT was coadministered, the airway lymphocytes and circulatory IFN- γ^+ /CD4 $^+$ were both increased and late phase airway hypersensitivity was

decreased. These results indicate that Dp2 NT might have a therapeutic effect on Dp2-induced airway hypersensitivity and that LZ OT might also have an effect on Dp2 NT immunotherapy.

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