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Lactic Acid Bacteria and Natural Product Complex Ameliorates Ovalbumin-Induced Airway Hyperresponsiveness in Mice

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ABSTRACT The incidence of respiratory diseases, such as asthma, has substantially increased in recent times owing to environmental changes, such as air pollution. Induction of a chronic inflammatory response begins with production of biologically active mediators from the airway epithelium, which attracts and recruits inflammatory cells into the lung airway. In our previous study, we confirmed that *Lactobacillus casei* HY2782 and *Bifidobacterium animalis* spp. *lactis* HY8002 could improve lung inflammation in the COPD animal model. In this study, we investigated the effect of the HY2782 complex against airway hyperresponsiveness by using an ovalbumin (OVA)-induced animal model. An orally administered HY2782 complex on OVA-induced allergic asthma in a BALB/c mouse model was used. The present results showed that the HY2782 complex suppressed total immunoglobulin E in serum and bronchoalveolar lavage fluid (BALF). The cytokine production profile in BALF and serum revealed that the HY2782 complex showed reduced levels of Th2 cytokines among immune factors released due to the elevated allergic response. Levels of inflammatory mediators in BALF, *MCP-1*, *MIP-2*, and *CXCL-9* were decreased by oral administration of the HY2782 complex. Lower numbers of eosinophils and neutrophils in BALF suggested that inflammation was ameliorated by the HY2782 complex. Histological observation of lung sections also showed infiltration of fewer cells. From results, we suggested that the HY2782 complex effectively responds to improvement of the immune response and airway hypersensitivity reaction because of the anti-inflammatory effect of the *Pueraria lobata* root extract and antioxidant effect of HY2782.

KEYWORDS: • ***airway hyperresponsiveness*** • **Lactobacillus casei *HY2782*** • ***OVA*** • **Pueraria lobata**

INTRODUCTION

THE INCIDENCE OF allergic diseases is rising worldwide, with various factors such as air pollution, climate, and airborne allergens causing allergic diseases. In recent times, the abundance of air pollutants such as particulate matter, NO_2, and ozone in the atmosphere is known to cause allergic respiratory diseases, including asthma and rhinitis.[1] Among them, asthma is one of the typical, chronic respiratory diseases caused by mucus hypersecretion, airway inflammation, and airway hyperresponsiveness (AHR).[2]

The use of natural products for treatment of physiological disorders, especially in typical chronic diseases such as asthma, has been widely reported through ethnopharmacological studies.[3] Through continuous research on asthma, as many as 40% of people with allergic diseases have used natural products, such as garlic,[4] ginkgo leaves,[5] and omega-3,[6] as alternative medicines. Kudzu (*Pueraria lobata*) has been traditionally used as a medicinal food for treating cardiovascular diseases and is mainly administered in powder and oil forms. In addition, kudzu is commonly used for treating hypertension, coronary artery disease, myocardial damage, and heart failure.[7] Recently, it has been revealed that *Pueraria* root has various pharmacological properties[8] that are closely related to its various active compounds. Of these compounds, isoflavonoid compounds, including daidzin, genistin, and puerarin, exhibit many biological activities, such as antioxidant, antitumor, anti-inflammatory, and estrogenic activities.[9–13] Recent studies have reported that isoflavone compounds isolated from kudzu can effectively suppress allergic responses in an experimental asthma model.[14] These studies were based, initially, on the traditional uses of natural products, which drew the attention of pharmaceutical companies due to their easy and economical use, allowing companies to perform many studies that evaluated their therapeutic activities, toxicity, and safety. However, there are no studies on the preventive effect of Kudzu root extract against ovalbumin (OVA)-induced AHR.

Several studies have concluded that probiotics may prevent or reduce asthma in animals. *Lactobacillus rhamnosus*

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Lcr35 is beneficial in management of allergic diseases and has the potential to prevent asthma.[15] Feleszko *et al.* demonstrated that both *Bifidobacterium lactis* Bb-12 and *L. rhamnosus* GG decrease AHR.[16] Forsythe *et al.* suggested that asthma can be prevented by *Lactobacillus reuteri*, but not by *Lactobacillus salivarius*.[17] However, the beneficial effects depend on the probiotic strain and dose, and evidence was obtained mainly in murine models. However, there have been no reports on studies using a combination of probiotics and natural products. Therefore, we investigated the efficacy of a combined mixture of a dietary natural product with probiotic strains on the incidence and duration of AHR in mice. The probiotic strains used are related to strains previously suggested to have antirespiratory inflammation benefits in mice.

Therefore, the aim of this study was to explore the synergistic effect of combinations of *P. lobata* root extract and *Lactobacillus casei* HY2782 (HY2782 complex) against AHR, using an OVA-induced animal model. Furthermore, we investigated the mechanism of anti-inflammatory actions of *Lactobacillus* and *Pueraria* by measuring the expression levels of inflammation-related cytokines.

MATERIALS AND METHODS

Reagents and equipment

OVA (A5503; Sigma Aldrich, St. Louis, MO, USA), *L. casei* HY2782 (Korea Yakult Co., Ltd., Seoul, Korea), mouse interleukin (IL)-4 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (M4000B; R&D Systems, Minneapolis, MN, USA), mouse IL-5 Quantikine ELISA kit (M5000; R&D Systems), mouse IL-13 ELISA kit (BMS6015; Invitrogen, Waltham, MA, USA), mouse immunoglobulin E (IgE) ELISA kit (88-50460-88; Invitrogen), mouse leukotriene C_4 (LTC_4) ELISA kit (MBS731784; MyBioSource, San Diego CA, USA), mouse asymmetric dimethylarginine (ADMA) ELISA kit (MBS705936; MyBioSource), Easy-spin™ RNA kit (17221; iNtRON Biotechnology, Gyeonggi-do, Korea), Lysing Matrix D (6913-500; MP Biomedicals, Santa Ana, CA, USA), FastPrep-24® (MP Biomedicals), Omniscript® RT Kit (205113; Qiagen, Hilden, Germany), Gene Expression Master Mix (4639016; Applied Biosystems, Foster City, CA, USA), and QuantStudio™ 6 Flex Real-Time Instrument (Thermo Fisher Scientific, Waltham, MA, USA) were used for the experiments.

Preparation of P. lobata *root extract*

P. lobata root (PR) was purchased from Humanherb Co. Ltd. (Daegu, Korea). Dried PR was mixed by weight with water at a ratio of 1:10 and extracted under high temperature. After extraction for 6 h, the extract was filtered. The filtrate was concentrated to 13.3 Brix and made into powder using a freeze dryer. The prepared *P. lobata* root extract (PRE) was stored at −20°C until use.

Preparation of L. casei *HY2782*

The precultured HY2782 was inoculated in a sterilized medium and cultured in a fermenter. After incubation, the concentrated cells were mixed with a cryoprotectant at a ratio of 1:1. The HY2782 was made into a powder using a freeze dryer, and the number of bacteria was measured by a dilution plate method. The prepared HY2782 was stored at −20°C until use.

In vivo *assay*

Female BALB/c mice, aged 6 weeks, were purchased from DooYeol Biotech (Seoul, Korea) and divided into groups of 10 mice each: the phosphate-buffered saline (PBS)-sensitized and -challenged control group (PBS), OVA-sensitized and -challenged group (OVA), 0.5 mg/kg dexamethasone-treated OVA group (Dex), HY2782 (1×10^8 CFU/day mouse)-fed OVA group (HY2782), and combination of HY2782 (1×10^8 CFU/day mouse) and PRE (10 mg/kg)-fed OVA group (HY2782 complex). Tested samples were from all groups that were fed by diet. All groups, except the PBS group, were sensitized with 200 μL of 1% aluminum hydroxide [$Al(OH)_3$] containing 100 μg OVA on days 0 and 12. The PBS group was sensitized with 200 μL of PBS containing 1% $Al(OH)_3$. On days 18 and 19, the PBS and other groups were challenged with 50 μL of PBS or the same dose of OVA (50 μg/mouse). After 20 days, mice were sacrificed and the bronchoalveolar lavage fluid (BALF), blood, and lungs were extracted. The obtained organs and blood were stored at −70°C until cytokine and gene expression analyses. The experimental procedures were approved by the Ethics Review Committee of the Korea Yakult Company Limited R&D Center, Korea [AEC-2019-00066-Y(2)].

Analysis of immune cell population of BALF

The bronchial sections of sacrificed mice were cut in half vertically. A feeding needle for oral administration was inserted, and 1 mL of PBS was added and collected again. The collected BALF was centrifuged, and the supernatant was used for cytokine analysis. The pellet was resuspended in 100 μL of PBS and used for the immune cell population. Resuspended cells were analyzed using the Mindray BC-5000 Vet. The number of cells was expressed as $\times10^3$ cells/mL.

ELISA for BALF and serum cytokines

The secreted cytokines in BALF were analyzed using an ELISA kit. To separate serum, blood from mice was left at room temperature for 30 min and then centrifuged at 2000 *g* for 10 min. Subsequent steps to detect IL-4, IL-5, IL-13, IgE, ADMA, and LTC_4 were performed according to the ELISA kit instructions. The BALF was centrifuged at 3000 *g* for 10 min. Cytokine secretion from the supernatant of the BALF was analyzed.

Gene expression analysis of lung tissue

Total RNA was extracted using the easy-spin RNA kit. The lung tissue, along with lysis buffer, was added to Matrix D tubes and pulverized through the FastPrep-24 instrument. After grinding the tissue, the Easy-spin RNA kit protocol was followed. Total RNA was stored at −20°C until gene expression analysis. The extracted RNA was reverse transcribed into cDNA using an Omniscript RT Kit. cDNA was amplified with Gene Expression Master Mix and a TaqMan Probe in a QuantStudio 6 Flex Real-Time Instrument. Table 1 shows the names and catalog numbers of the genes.

Preparation of lung tissue slides and measurement of mucosal thickness

Lung tissues fixed in 10% formalin solution were used to prepare hematoxylin and eosin-stained slides by KPC (Gwangju-si, Korea). The thickness of the mucosa was measured under an Olympus CK2 microscope (Tokyo, Japan) at 200× magnification. The mucosal thickness was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are shown as the mean ± standard deviation of independent experiments. Statistical comparisons between groups were performed using Student's *t*-tests.

RESULTS

Increased cytokine and chemokine production in an OVA-induced mouse model

Induction with OVA promoted production of inflammatory factors in the respiratory tract. The secretion of IL-4, a key cytokine in allergic inflammation, was increased to 98.92 ± 13.67 pg/mL in the BALF in the OVA group, whereas its levels were at 2.56 ± 4.65 pg/mL in the PBS group. Treatment with HY2782 and PRE decreased IL-4 levels to 59.05 ± 11.33 pg/mL (HY2782) and 51.41 ± 4.17 pg/mL (HY2782 complex) (Fig. 1A). The secretion of IL-5 also showed the same pattern (Fig. 1B). Secretion of IL-13, which was increased by OVA induction, was decreased by dexamethasone treatment, but decrease in secretion was not significant ($P = .09$). However, IL-13 secretion was shown to decrease in the HY2782 ($P < .05$) and HY2782 complex groups ($P < .01$, Fig. 1C).

Total IgE secretion increased to 116.14 ± 10.91 ng/mL in the BALF of OVA-induced mice, whereas its secretion level was at 64.04 ± 15.50 ng/mL in the PBS group. IgE levels recovered to 72.54 ± 11.51 ng/mL in the Dex group The secreted IgE levels were decreased to 89.58 ± 11.82 ng/mL in the HY2782 group and to 53.07 ± 10.00 ng/mL in the HY2782 complex group. The most effective decrease in IgE secretion was observed in the HY2782 complex group (Fig. 2A) and this result was confirmed through analysis of serum levels (Fig. 2B).

Asthma-related factors, such as IL-4, increased secretion of leukotrienes in the respiratory mucosa. The secreted LTC_4 increased to 3.97 ± 0.52 ng/mL in the OVA group, while its levels were at 0.57 ± 0.11 ng/mL in the PBS group. LTC_4 levels were reduced to 2.56 ± 0.74 and 1.90 ± 0.39 ng/mL in the HY2782 group and HY2782 complex group, respectively (Fig. 3A). Serum ADMA levels decreased in the experimental group in the same manner as observed for LTC_4 (Fig. 3B).

Change in expression of antioxidant and inflammatory genes in the lungs after HY2782 and PRE complex ingestion

Reactive oxygen species (ROS) induced by OVA increases the levels of cytokines such as IL-4, IL-5, and IL-13. Therefore, ROS needs to be regulated to control airway inflammation caused by OVA exposure. Probiotics have antioxidant effects, and inhibition of ROS by probiotics may attenuate airway inflammation.

Gene expression levels of *NRF2*, a representative oxidative stress regulator, were significantly increased in the OVA group (1.51 ± 0.27) after ingestion of HY2782 (2.26 ± 0.23, Fig. 4A). Another antioxidant gene, *SOD-1*, showed the same pattern as *NRF2* (Fig. 4D), while the expression levels of *AHR* increased after OVA exposure (1.54 ± 0.39), but decreased with HY2782 ingestion (0.88 ± 0.14, Fig. 4B). The *CAT* gene showed the same

Table 1. Gene Names and Symbols Used in Gene Expression Analysis

Gene symbol	*Gene name*	*Catalog number*	*Reference sequence*
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1	NM_021283.2
SOD-1	Superoxide dismutase 1	Mm01344233_g1	NM_011434.1
CAT	Catalase	Mm00437992_m1	NM_009804.2
MCP-1	Monocyte chemoattractant protein-1	Mm00441242_m1	NM_011333.3
MIP-2α	Macrophage inflammatory protein 2-alpha	Mm00436450_m1	NM_009140.2
AHR	Aryl hydrocarbon receptor	Mm00478932_m1	NM_013464.4
NQO1	NAD(P)H quinone oxidoreductase 1	Mm01253561_m1	NM_008706.5
NRF2	Nuclear factor erythroid 2-related factor 2	Mm00477784_m1	NM_010902.3
iNOS	Inducible nitric oxide synthase	Mm00440502_m1	NM_010927.3
COX-2	Cyclooxygenase-2	Mm00478374_m1	NM_011198.3
MUC5AC	Mucin 5AC	Mm01276718_m1	NM_010844.1
CXCL9 (MIG)	Monokine induced by gamma interferon	Mm00434946_m1	NM_008599.4

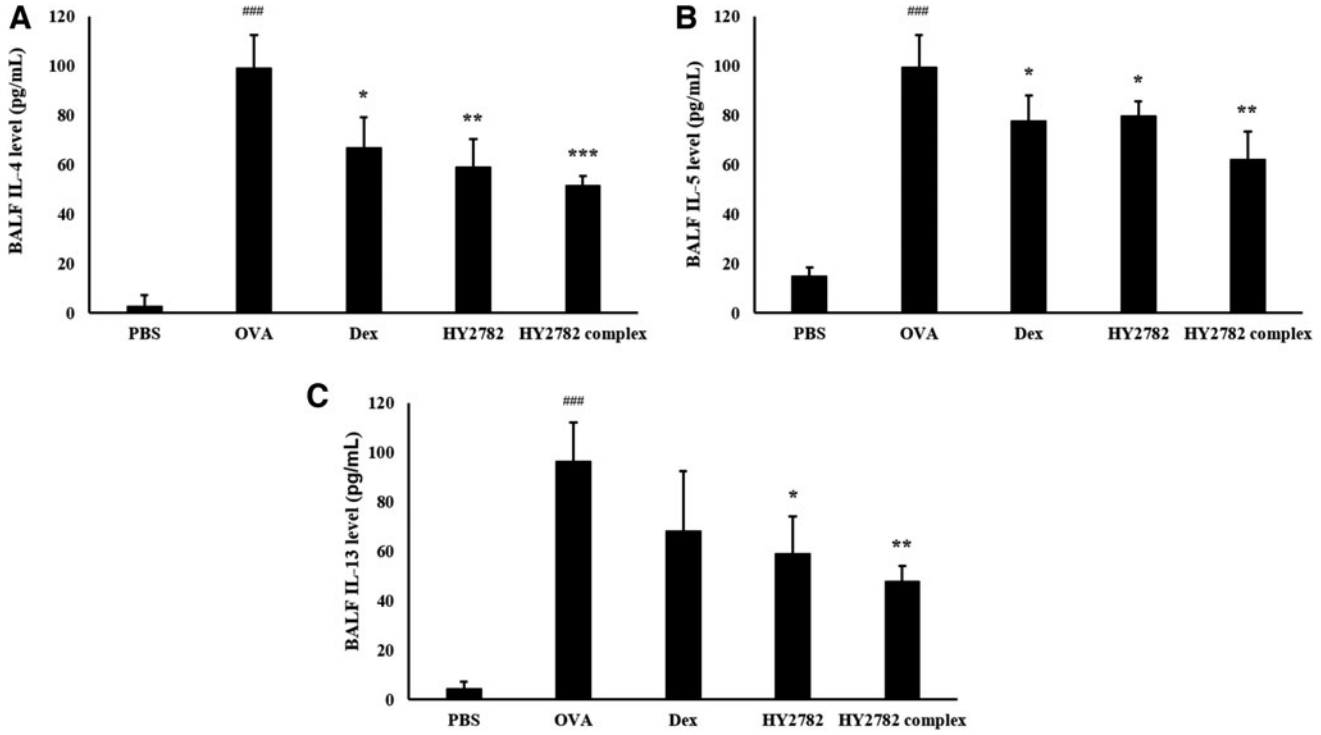

FIG. 1. Cytokine levels in BALF. BALF was collected and analyzed to determine IL-4, IL-5, and IL-13 secretion. **(A)** Increased secretion of IL-4 by OVA exposure. **(B)** IL-5 secretion in BALF. **(C)** IL-13. Data are shown as the mean ± SD. Significant differences are indicated as $^{###}P < .001$ when compared with the PBS group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group. BALF, bronchoalveolar lavage fluid; IL, interleukin; OVA, ovalbumin; PBS, phosphate-buffered saline; PRE, *Pueraria lobata* root extract; SD, standard deviation.

results as *AHR* (Fig. 4C). The expression of *NQO1* increased in the OVA group (1.54 ± 0.30, $P < .05$), but did not change in the other groups ($P > .05$, Fig. 4E).

Gene expression of *MCP-1*, a key factor in regulation of immune cell migration and infiltration, increased in the OVA group (2.86 ± 0.42). However, its expression significantly decreased in the experimental groups (HY2782: 1.80 ± 0.49, HY2782 complex: 1.17 ± 0.18, Fig. 5A). Gene expression levels of *MIP-2*, which plays an important role in the chemotactic effect of leukocytes and hematopoietic cells, were also increased in the OVA group (2.45 ± 0.32, Fig. 5B). In addition, the gene expression levels of *CXCL9* (*MIG*), an inflammatory chemokine that regulates immune cell migration, differentiation, and activation, showed the same expression pattern as that shown by *MCP-1* and *MIP-2* (Fig. 5C).

Expression of inducible nitric oxide synthase (*iNOS*) and cyclooxygenase-2 (*COX-2*) associated with mucosal inflammation increased in the OVA group, but decreased in

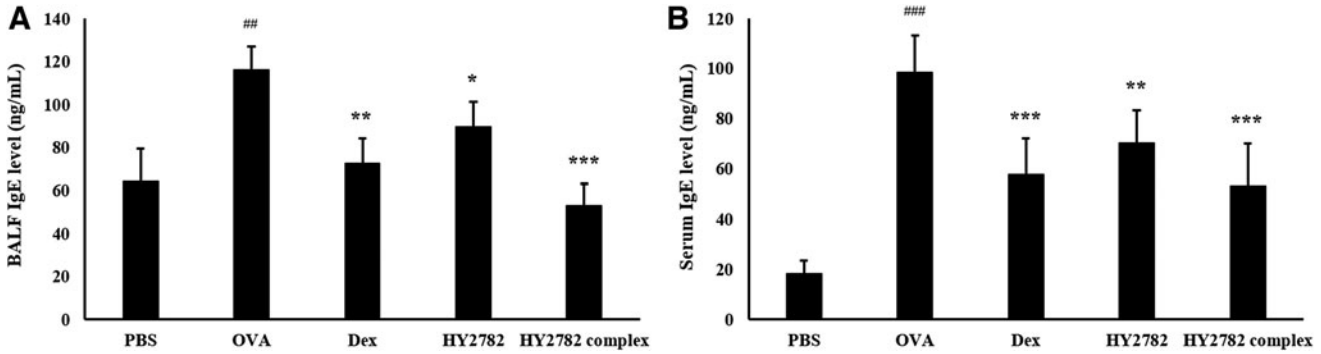

FIG. 2. IgE secretion in BALF and serum. **(A)** IgE secretion in BALF. **(B)** Serum. Data are shown as the mean ± SD. Significant differences are indicated as $^{##}P < .01$ and $^{###}P < .001$ when compared with the PBS group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group. IgE, immunoglobulin E.

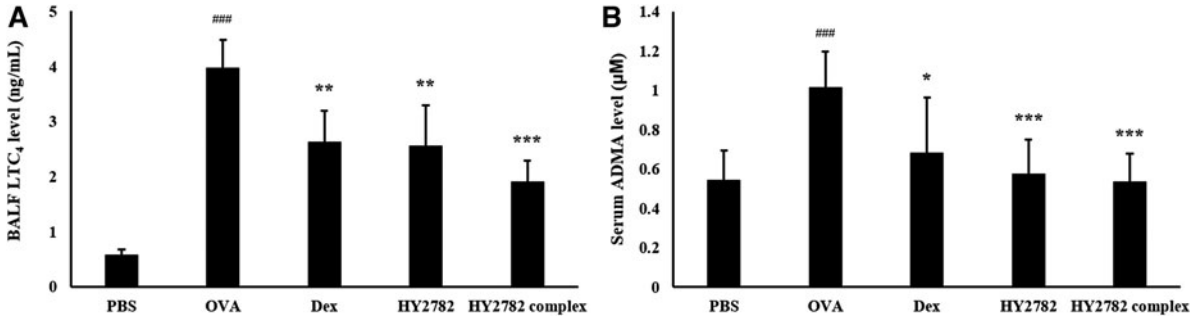

FIG. 3. Chemotactic factors in serum and BALF **(A)** LTC_4 in BALF. **(B)** Serum ADMA level. Increased ADMA by OVA decreased in the experimental groups. Data are shown as the mean ± SD. Significant differences are indicated as $^{###}P < .001$ when compared with the PBS group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group. ADMA, asymmetric dimethylarginine; LTC_4, leukotriene C_4.

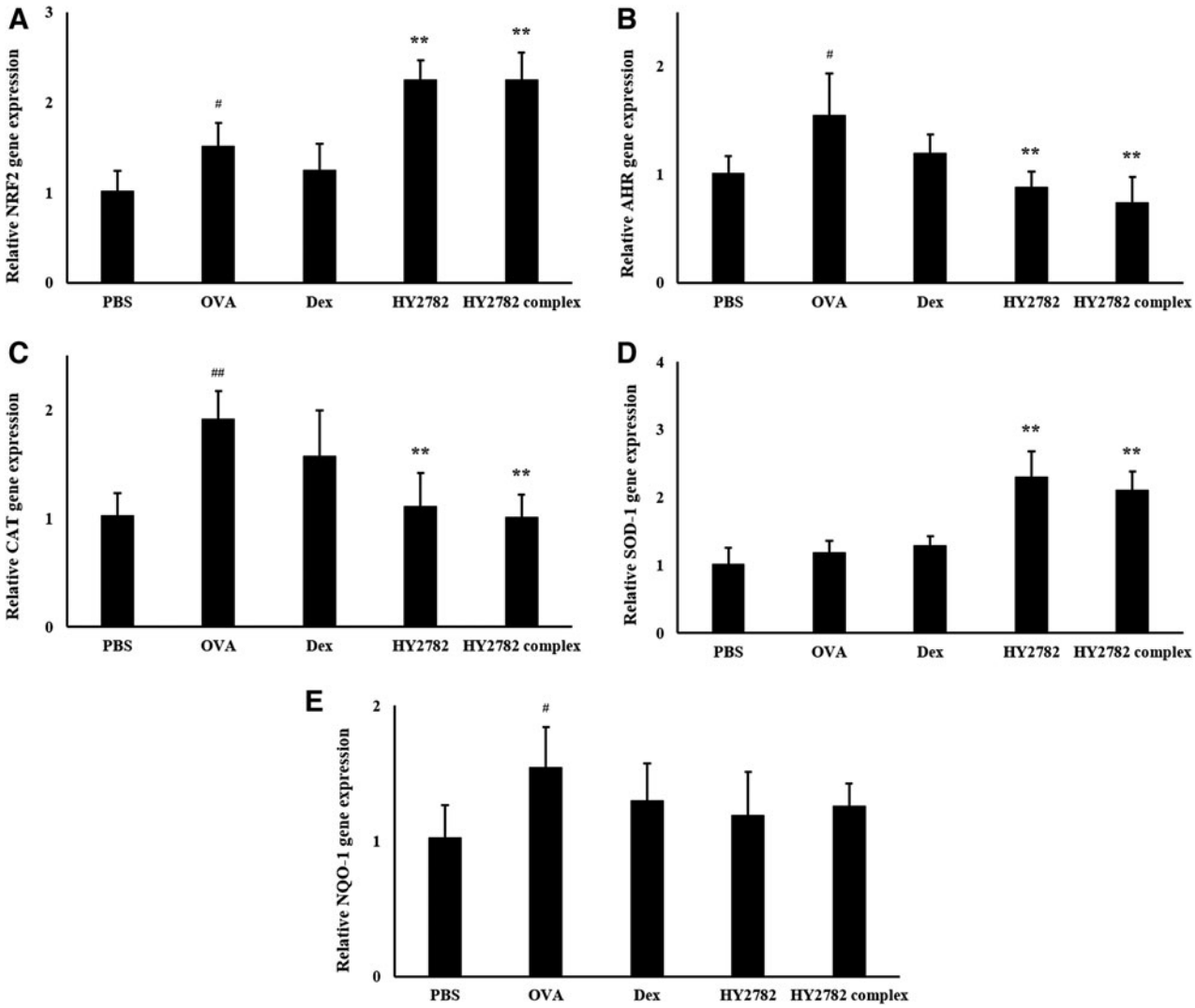

FIG. 4. Antioxidant gene expression levels in the lung. **(A)** *NRF2* increased in the OVA group, but increased further in the experimental groups. **(B)** *AHR* increased in the OVA group, but decreased in the experimental groups. **(C)** Changes of antioxidant gene *CAT* expression level. **(D)** *SOD-1* expression level. **(E)** *NQO-1* expression level. Data are shown as the mean ± SD. Significant differences are indicated as $^{#}P < .05$ and $^{##}P < .01$ when compared with the PBS group and $^{**}P < .01$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group. *AHR*, aryl hydrocarbon receptor; *NRF2*, nuclear factor erythroid 2-related factor 2.

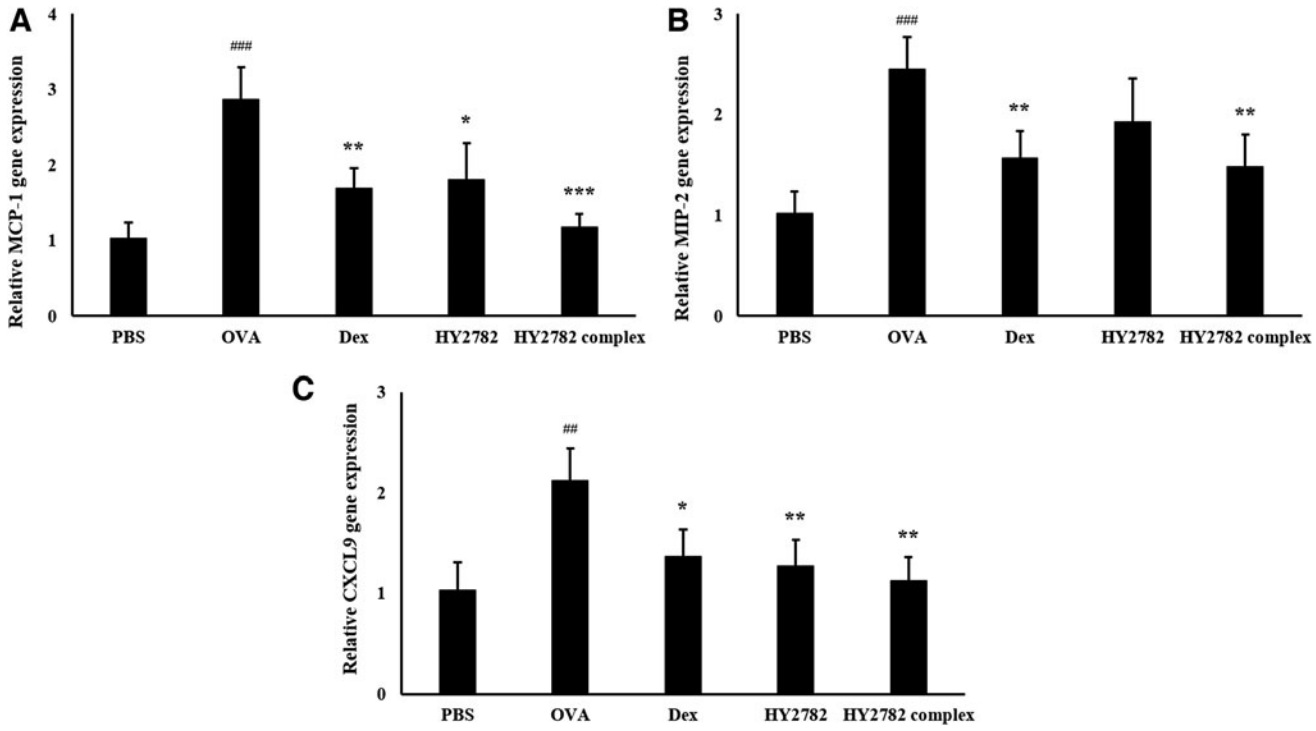

FIG. 5. Changes in the chemotactic gene expression by HY2782 and PRE complex. **(A)** *MCP-1* decreased in the HY2782+PRE group. **(B)** *MIP-2* expression level. **(C)** *CXCL9* increased in the OVA group, but decreased in the experimental groups. Data are shown as the mean ± SD. Significant differences are indicated as $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ when compared with the PBS group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group.

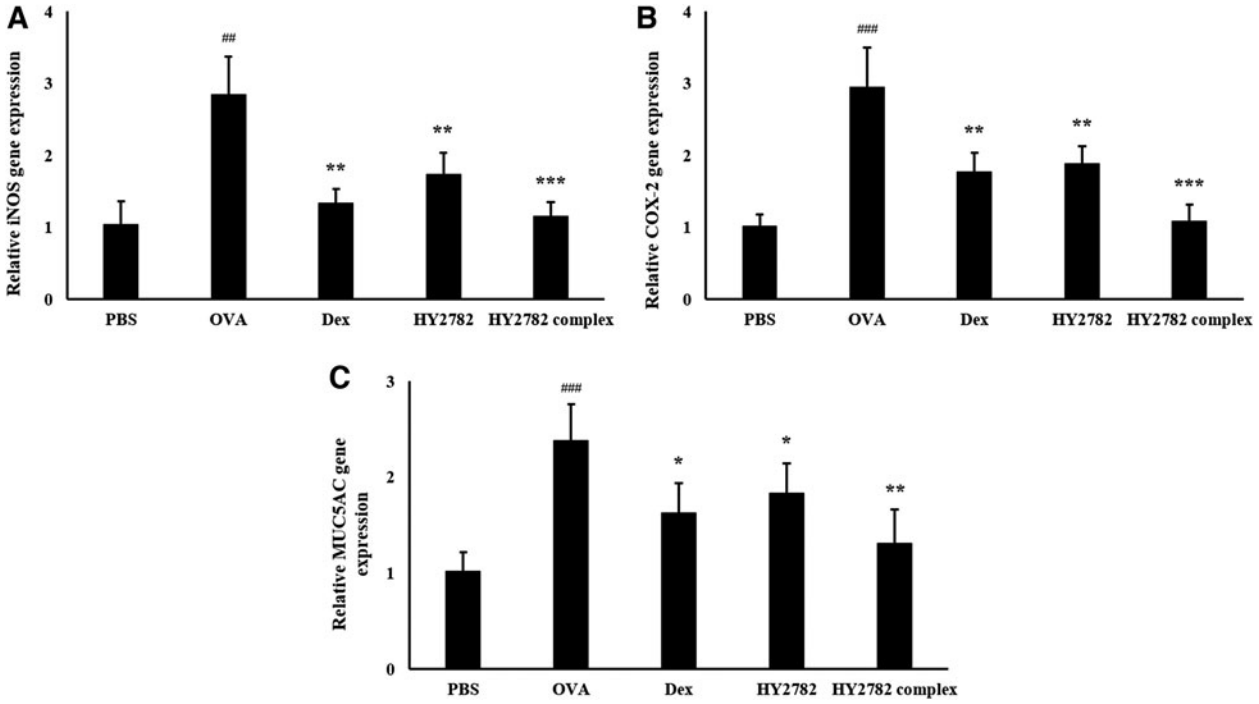

FIG. 6. Mucosal inflammation-related gene expression levels in the lung. **(A)** *iNOS* was increased in the OVA group. **(B)** *COX-2* increased by OVA decreased in the experimental groups. **(C)** Reduction of *MUC5AC* by HY2782 treatment. Data are shown as the mean ± SD. Significant differences are indicated as $^{\#\#}P < .01$ and $^{\#\#\#}P < .001$ when compared with the PBS group and $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group. *COX-2*, cyclooxygenase-2; *iNOS*, inducible nitric oxide synthase.

the other groups (Fig. 6A, B). Thus, the reduced secretion of LTC_4 in BALF was due to decreased expression of *iNOS* and *COX-2*. The expression of the mucosal gene, *MUC5AC*, significantly increased in the OVA group (2.38 ± 0.38, $P < .001$). HY2782 intake decreased the expression levels of *MUC5AC*, but not significantly (1.82 ± 0.31, $P = .069$). However, PRE ingestion significantly reduced the expression of *MUC5AC* (1.31 ± 0.36, $P < .01$, Fig. 6C).

Taken together, ROS and airway inflammation increased by OVA were controlled by HY2782 and PRE treatment. Therefore, HY2782 is a key factor in regulation of ROS induced by OVA, and the complex with PRE could be excellent for inflammation control.

Changes in mouse immune cell composition by OVA exposure

To confirm the anti-inflammatory effects of HY2782 and PRE complex on lungs, mice were exposed to OVA, and immune cells in the BALF were counted. Neutrophils, lymphocytes, macrophages, and eosinophils in the BALF significantly increased in the OVA group compared with the PBS group. The number of neutrophils in the BALF was $105.50 \pm 22.18 \times 10^3$ cells/mL in the OVA group, and this number significantly decreased to $68.75 \pm 15.82 \times 10^3$ cells/mL after HY2782 intake ($P < .05$). The number of immune cells decreased to $64.33 \pm 15.00 \times 10^3$ cells/mL in the HY2782 complex group ($P < .01$). Ingestion of PRE and HY2782 complex was shown to reduce immune cell recruitment in the respiratory tract (Fig. 7).

HY2782 and PRE complex attenuate lung inflammation in the OVA mouse model

We confirmed that more inflammation occurred in the lung tissues of mice exposed to OVA. In contrast, feeding of probiotics and PRE complex to mice relieved lung tissue inflammation. In the PBS group, the lung mucosa was $18.57 \pm 3.65\ \mu$m thick, whereas in the OVA group, the thickness increased to $44.06 \pm 3.62\ \mu$m ($P < .001$). Intake of samples decreased mucosal thickness to $22.24 \pm 4.09\ \mu$m in the HY2782 group ($P < .001$) and $23.35 \pm 2.42\ \mu$m in the HY2782 complex group ($P < .001$, Fig. 8).

Taken together, the ROS induced by OVA increased levels of asthma-related factors such as IL-4, IL-5, COX-2, LTC_4, and ADMA and induced lung inflammation along with recruitment of immune cells. Ingestion of HY2782 increased the levels of antioxidant factors such as *NRF2* for regulation of ROS *in vivo*. Increased *NRF2* appeared to reduce lung inflammation by increasing the levels of antioxidant-related factors such as *CAT* and *SOD*, inhibiting intracellular ROS production, and decreasing chemokine and cytokine secretion, thereby inhibiting immune cell infiltration.

DISCUSSION

Asthma is a complex and heterogeneous disease of the airways that affects millions of people worldwide. Respiratory allergens induce production of proinflammatory cytokines from airway epithelial cells.[18,19] Airway epithelial cells activate Th2 cytokine production in mast cells.[20] The functional roles of B cells in allergic airway inflammation have been controversial, whereas their ability to produce the IgE antibody is well established. When the B cell encounters an allergen, it produces IgE and binds with mast cells to activate degranulation[21,22]; subsequently, various types of autacoids (such as histamine and leukotriene) are secreted to mediate allergens and inflammatory reactions.[23,24] In kudzu root extract, flavonoids such as genistein, daidzin, daidzein, and puerarin are known as representative functional ingredients.[25] In immune hypersensitivity reactions such as allergies, these flavonoids are known to inhibit the release of histamine by suppressing mast cell/basophil activation and degranulation of eosinophils.[26] Utilizing a mouse model of allergic asthma, a

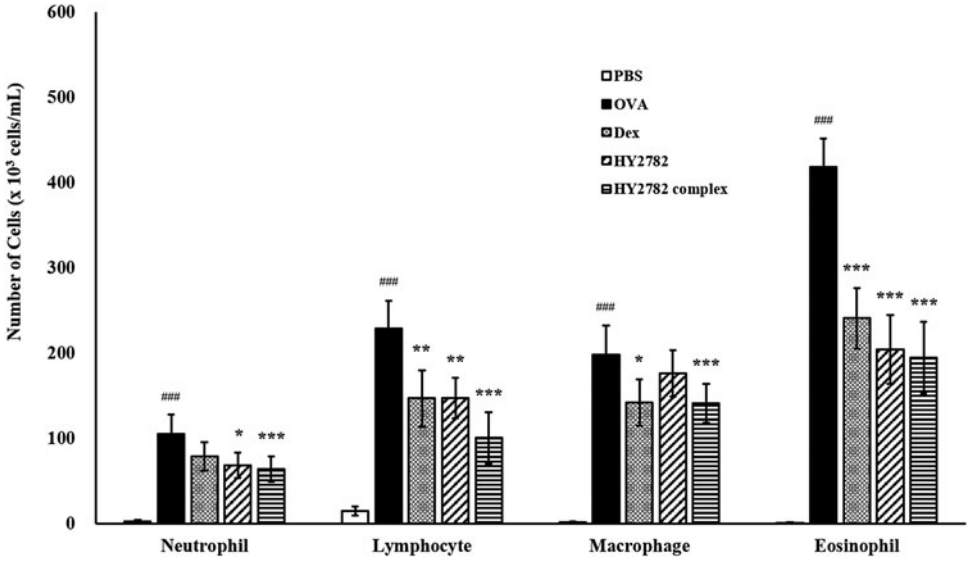

FIG. 7. Immune cell composition in the BALF. The immune cell count was increased by OVA exposure and was decreased in experimental groups. Data are shown as the mean ± SD. Significant differences are indicated as $^{###}P < .001$ when compared with the PBS group and $*P < .05$, $**P < .01$, and $***P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group.

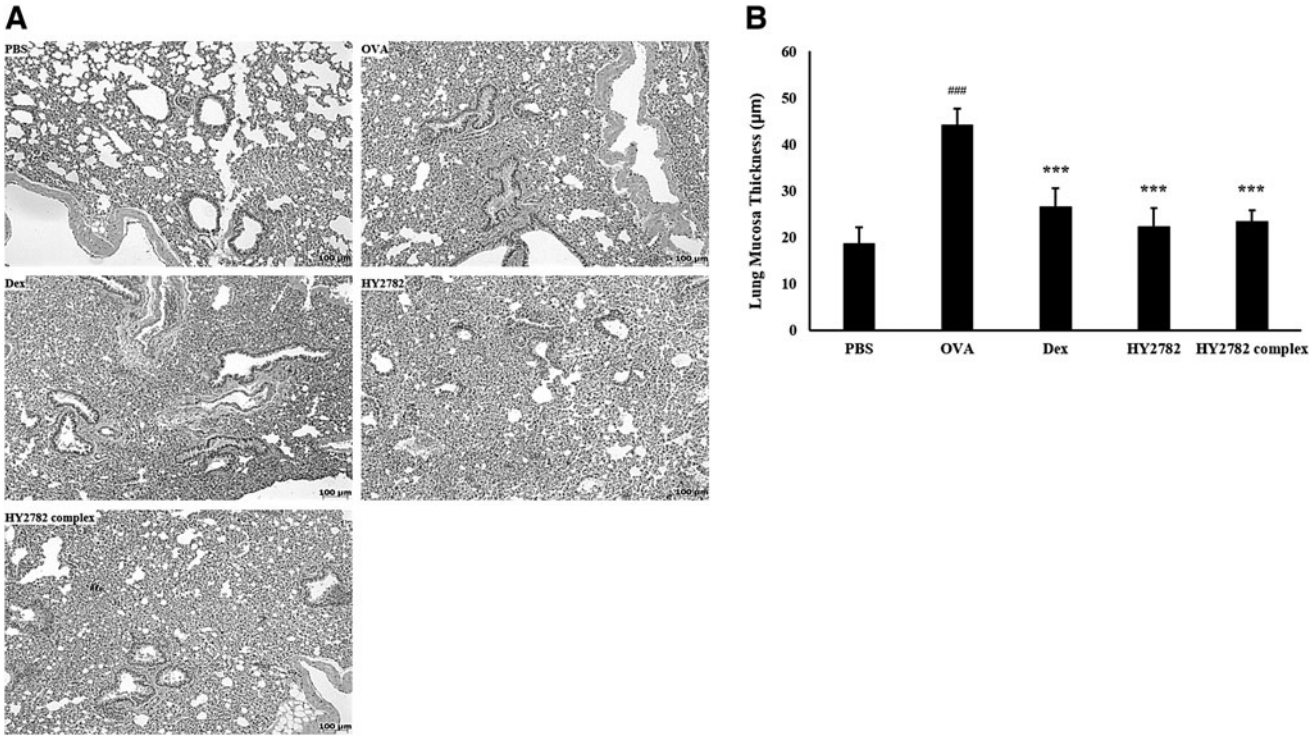

FIG. 8. Effect of HY2782 and HY2782+PRE on histology of lung tissue in the OVA mouse model. Female BALB/c mice were challenged with OVA intranasal administration. **(A)** Lung was stained with H&E (200×magnification). **(B)** Mucosal thickness was measured using ImageJ software. Data are shown as the mean ± SD. Significant differences are indicated as $^{###}P < .001$ when compared with the PBS group and $^{***}P < .001$ when compared with the OVA group. PBS, normal mice; OVA, OVA-exposed mice; Dex, dexamethasone-treated OVA group; HY2782, *Lactobacillus* HY2782-fed OVA group; HY2782 complex, HY2782 and PRE-fed OVA group.

recent study indicated that puerarin treatment attenuated OVA-induced airway inflammation by regulation of eotaxin-3, which suggested its potential application in allergic inflammation, although the detailed mechanism was unknown.[12] In this study, HY2782 probiotics have not been shown to effectively lower IgE produced in excess by OVA exposure in BALF and blood. On the other hand, the HY2782 complex exhibited a more effective IgE reduction effect than dexamethasone, the positive control (Fig. 2). This suggested that the HY2782 complex will more effectively modulate immune hypersensitivity reactions than will administration of just probiotics.

Th2 cells are thought to be the central immune cells that regulate allergic airway inflammation in asthma.[27,28] Th2 cells secrete IL-4, IL-5, and IL-13 and promote humoral immune response and increase cytokine secretion during allergic inflammatory reactions.[29–31] Th2 cytokines play an important role in amplifying the allergic inflammation in asthma by activating eosinophil and inflammatory cell infiltration and mucus overproduction.[32] In our study, HY2782 complex treatment downregulated IL-4, IL-5, and IL-13 levels in the BALF (Fig. 1) and also downregulated *MUC5AC* mRNA expression (Fig. 3). These results suggest that the HY2782 complex effectively decreases inflammation induced by OVA and inhibits mucus hypersecretion and also improves allergic responses in BALF by reducing Th2 cytokine levels.

Chemokines also play an important role in inducing inflammatory cells into the lesion site. *MCP-1* is a CC chemokine that selectively induces immune cells, including monocytes, lymphocytes, and basophils. In asthma, a disturbed hemostatic balance is one of the important factors for perpetuation of allergic inflammation.[13,14] In our study, HY2782 complex treatment downregulated expression of *MCP-1*, *MIP-2*, and *CXCL9* mRNA in serum (Fig. 5). This result suggests that the HY2782 complex helps to suppress the hypersensitivity reaction by alleviating the inflammatory response.

In respiratory hypersensitivity reactions, leukotriene plays a role in relaxing the smooth muscle of the nasal mucosa and promotes the movement of eosinophils in blood and bronchial lavage of asthmatic patients.[33] Therefore, leukotriene modulators have been used as asthma treatments in many studies. ADMA is an endogenous NOS inhibitor that competes with L-arginine for binding to NOS. It has been suggested that ADMA contributes to inflammation, collagen deposition, nitrosative stress, and lung function in murine models.[34] In this study, the HY2782 complex showed the effect of reducing the respiratory inflammatory response by increasing the expression of *iNOS* and *COX-2* genes in the acute inflammatory response induced by OVA and thus reduced the concentration of ADMA in serum. In addition, it showed an effect of reducing the expression of *COX-2*, which is involved in the inflammatory response. In

addition, it was confirmed that the expression of *MUC5AC* and LTC_4 was also decreased in BALF. These results are assumed to indicate that the HY2782 complex can effectively respond to immune hypersensitivity reactions in the bronchus and trachea.

Probiotics are emerging as a safe and natural strategy for allergy prevention and treatment. However, clinical, probiotic intervention studies have so far yielded conflicting results. Administration of probiotics in mice has been explored for preventing or reducing the development of the hallmarks of allergic diseases in animal studies. To gain insight into the probiotic- and allergy-associated mechanisms responsible for the observed effects, researchers analyzed several parameters, such as allergen-specific antibody production[35]; Th1, Th2, and Treg cytokine levels in the airways and lymph nodes[35–37]; effector and regulatory T cell population counts[35,37]; and airway function.[38–41]

It is known that most people with respiratory hyperresponsiveness such as asthma are very susceptible to oxidative stress caused by ROS production compared with healthy individuals.[42] AhR is a ligand-activated transcription factor, and after ligation of dioxins to AhR, the receptor translocates from the cytosol to the nucleus. AhR ligands are also concentrated in bronchial epithelial cells, suggesting that the respiratory system is sensitive to AhR ligands.[2]

In this study, the HY2782 complex-treated mice showed a decrease in the expression of *AhR*, one of the main indicators for confirming a respiratory hypersensitivity reaction, which is judged as a reduction effect by HY2782 as well as an antioxidant effect.

In our previous study, we confirmed that *L. casei* HY2782 improves lung inflammation in the COPD animal model.[43] Consequently, it was hypothesized that HY2782 might exhibit anti-AHR effects *in vivo*. In the current study, we demonstrated that the HY2782 complex treatment improves *AHR*, using an acute asthma-induced animal model. Although the mouse model of asthma does not completely reproduce human diseases, the known allergic pulmonary inflammation and immunological information of AHR can be applied to humans, so the OVA-induced asthma mouse model is used. This study also confirmed that the HY2782 complex relieves airway hypersensitivity and helps improve airway inflammation through strong antioxidant activity.

In conclusion, through this study, we confirm that the HY2782 complex effectively improves the immune response and airway hypersensitivity reaction by the anti-inflammatory effect of the *P. lobata* root extract and antioxidant effect of HY2782. In this study, we provide *in vivo* data for development of effective and new therapeutic agents for AHR treatment. However, further studies are required to elucidate its detailed mechanism of action.

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REFERENCES

1. Zhao YX, Zhang HR, Yang XN, *et al.*: Fine particulate matter-induced exacerbation of allergic asthma via activation of T-cell immunoglobulin and mucin domain 1. *Chinese Med J Peking* 2018;131:2461–2473.
2. Alexandrov K, Rojas M, Satarug S: The critical DNA damage by benzo(a)pyrene in lung tissues of smokers and approaches to preventing its formation. *Toxicol Lett* 2010;198:63–68.
3. Duan W, Chan JH, Wong CH, Leung BP, Wong WS: Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model. *J Immunol* 2004; 172:7053–7059.
4. Shin IS, Hong J, Jeon CM, *et al.*: Diallyl-disulfide, an organosulfur compound of garlic, attenuates airway inflammation via activation of the Nrf-2/HO-1 pathway and NF-kappaB suppression. *Food Chem Toxicol* 2013;62:506–513.
5. Babayigit A, Olmez D, Karaman O, *et al.*: Effects of *Ginkgo biloba* on airway histology in a mouse model of chronic asthma. *Allergy Asthma Proc* 2009;30:186–191.
6. Farjadian S, Moghtaderi M, Kalani M, Gholami T, Hosseini Teshnizi S: Effects of omega-3 fatty acids on serum levels of T-helper cytokines in children with asthma. *Cytokine* 2016;85: 61–66.
7. Tian F, Xu LH, Zhao W, Tian LJ, Ji XL: The optimal therapeutic timing and mechanism of puerarin treatment of spinal cord ischemia-reperfusion injury in rats. *J Ethnopharmacol* 2011;134: 892–896.
8. Jin SE, Son YK, Min BS, Jung HA, Choi JS: Anti-inflammatory and antioxidant activities of constituents isolated from *Pueraria lobata* roots. *Arch Pharm Res* 2012;35:823–837.
9. Liu XJ, Zhao J, Gu XY: The effects of genistein and puerarin on the activation of nuclear factor-kappaB and the production of tumor necrosis factor-alpha in asthma patients. *Pharmazie* 2010; 65:127–131.
10. Zhang J, Guo W, Tian B, *et al.*: Puerarin attenuates cognitive dysfunction and oxidative stress in vascular dementia rats induced by chronic ischemia. *Int J Clin Exp Pathol* 2015;8:4695–4704.
11. Choo MK, Park EK, Yoon HK, Kim DH: Antithrombotic and antiallergic activities of daidzein, a metabolite of puerarin and daidzin produced by human intestinal microflora. *Biol Pharm Bull* 2002;25:1328–1332.
12. Tong J, Hu XJ, Cai WQ, Dai X, Wang L: Puerarin alleviates delayed-type hypersensitivity via cytokine inhibition by modulating Th1/Th2 balance. *Exp Ther Med* 2018;15:4441–4447.
13. Miadoková E: Isoflavonoids—An overview of their biological activities and potential health benefits. *Interdiscip Toxicol* 2009; 2:211–218.
14. Dong F, Wang C, Duan J, *et al.*: Puerarin attenuates ovalbumin-induced lung inflammation and hemostatic unbalance in rat asthma model. *Evid Based Complement Alternat Med* 2014;2014: 726740.
15. Yu J, Jang SO, Kim BJ, *et al.*: The effects of *Lactobacillus rhamnosus* on the prevention of asthma in a murine model. *Allergy Asthma Immunol Res* 2010;2:199–205.
16. Feleszko W, Jaworska J, Rha RD, *et al.*: Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clin Exp Allergy* 2007;37: 498–505.

17. Forsythe P, Inman MD, Bienenstock J: Oral treatment with live *Lactobacillus reuteri* inhibits the allergic airway response in mice. *Am J Respir Crit Care* 2007;175:561–569.
18. Drake LY, Iijima K, Hara K, *et al.*: B cells play key roles in Th2-type airway immune responses in mice exposed to natural airborne allergens. *PLoS One* 2015;10:e0121660.
19. Caraballo L, Valenta R, Puerta L, *et al.*: The allergenic activity and clinical impact of individual IgE-antibody binding molecules from indoor allergen sources. *World Allergy Organ J* 2020;13: 100118.
20. Nagarkar DR, Poposki JA, Comeau MR, *et al.*: Airway epithelial cells activate TH2 cytokine production in mast cells through IL-1 and thymic stromal lymphopoietin. *J Allergy Clin Immunol* 2012; 130:225–232.
21. da Silva EZ, Jamur MC, Oliver C: Mast cell function: A new vision of an old cell. *J Histochem Cytochem* 2014;62:698–738.
22. Albert-Bayo M, Paracuellos I, González-Castro AM, *et al.*: Intestinal mucosal mast cells: Key modulators of barrier function and homeostasis. *Cells* 2019;8:135.
23. Branco A, Yoshikawa FSY: Role of histamine in modulating the immune response and inflammation. *Mediators Inflamm* 2018; 2018:9524075.
24. Jozefowski S, Biedroń R, Bobek M, Marcinkiewicz J: Leukotrienes modulate cytokine release from dendritic cells. *Immunology* 2005;116:418–428.
25. Park HS, Kim SR, Kim JO, Lee YC: The roles of phytochemicals in bronchial asthma. *Molecules* 2010;15:6810–6834.
26. Wang J, Zhang T, Ma C, Wang S: Puerarin attenuates airway inflammation by regulation of eotaxin-3. *Immunol Lett* 2015;163: 173–178.
27. Kaufman PB, Duke JA, Brielmann H, Boik J, Hoyt JE: A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: Implications for human nutrition and health. *J Altern Complement Med* 1997;3:7–12.
28. Kirakosyan A, Kaufman P, Warber S, Bolling S, Chang S, Duke J: Quantification of major isoflavonoids and L-canavananine in several organs of Kudzu vine (*Pueraria montana*) and in starch samples derived from kudzu roots. *Plant Sci* 2003;164: 883–888.
29. Steinke JW, Borish L: Th2 cytokines and asthma. Interleukin-4: Its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res* 2001;2:66–70.
30. Zhu J: T helper 2 (Th2) cell differentiation, type 2 innate lymphoid cell (ILC2) development and regulation of interleukin-4 (IL-4) and IL-13 production. *Cytokine* 2015;75:14–24.
31. Herrick CA, MacLeod H, Glusac E, Tigelaar RE, Bottomly K: Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J Clin Invest* 2000; 105:765–775.
32. Schuijs MJ, Willart MA, Hammad H, Lambrecht BN: Cytokine targets in airway inflammation. *Curr Opin Pharmacol* 2013;13: 351–361.
33. Wang HB, Akuthota P, Kanaoka Y, Weller PF: Airway eosinophil migration into lymph nodes in mice depends on leukotriene C(4). *Allergy* 2017;72:927–936.
34. Scott JA, North ML, Rafii M, *et al.*: Asymmetric dimethylarginine is increased in asthma. *Am J Respir Crit Care Med* 2011; 184:779–785.
35. Pellaton C, Nutten S, Thierry AC, *et al.*: Intragastric and intranasal administration of *Lactobacillus paracasei* NCC2461 modulates allergic airway inflammation in mice. *Int J Inflam* 2012;2012:686739.
36. Jan RL, Yeh KC, Hsieh MH, *et al.*: *Lactobacillus gasseri* suppresses Th17 pro-inflammatory response and attenuates allergen-induced airway inflammation in a mouse model of allergic asthma. *Br J Nutr* 2012;108:130–139.
37. Karimi K, Inman MD, Bienenstock J, Forsythe P: *Lactobacillus reuteri*-induced regulatory T cells protect against an allergic airway response in mice. *Am J Respir Crit Care Med* 2009;179: 186–193.
38. Mendes E, Acetturi BG, Thomas AM, *et al.*: Prophylactic supplementation of *Bifidobacterium longum* 5(1A) protects mice from ovariectomy-induced exacerbated allergic airway inflammation and airway hyperresponsiveness. *Front Microbiol* 2017;8:1732.
39. Nunes CF, Nogueira JS, Vianna PHO, *et al.*: Probiotic treatment during neonatal age provides optimal protection against experimental asthma through the modulation of microbiota and T cells. *Int Immunol* 2018;30:155–169.
40. Wu CT, Chen PJ, Lee YT, Ko JL, Lue KH: Effects of immunomodulatory supplementation with *Lactobacillus rhamnosus* on airway inflammation in a mouse asthma model. *J Microbiol Immunol Infect* 2016;49:625–635.
41. Spacova I, Ceuppens JL: Probiotics against airway allergy: Host factors to consider. *Dis Model Mech* 2018;11:dmm034314.
42. Misso NL, Thompson PJ: Oxidative stress and antioxidant deficiencies in asthma: Potential modification by diet. *Redox Report* 2005;10:247–255.
43. Nam W, Kim H, Bae C, *et al.*: *Lactobacillus* HY2782 and *Bifidobacterium* HY8002 decrease airway hyperresponsiveness induced by chronic PM2.5 inhalation in mice. *J Med Food* 2020; 23:575–583.