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ORIGINAL ARTICLE

Effects of immunomodulatory supplementation with *Lactobacillus rhamnosus* on airway inflammation in a mouse asthma model

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KEYWORDS
airway hyper-responsiveness;
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Lactobacillus rhamnosus GG;
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Background: Asthma is a common allergic disease. In previous studies, probiotics improved the balance of intestinal microbes, reduced inflammation, and promoted mucosal tolerance. This study investigated whether oral administrations of *Lactobacillus rhamnosus* GG (LGG) inhibited allergen (ovalbumin or OVA)-induced airway inflammation in a mouse asthma model.
Methods: The allergy/asthma animal model in this study was sensitization with OVA. After intranasal challenge with OVA, the airway inflammation and hyper-responsiveness were determined by a Buxco system, bronchoalveolar lavage fluid analysis with Liu stain, and enzyme-linked immunosorbent assay. Histopathologic changes in the lung were detected by hematoxylin and eosin staining and immunohistochemistry staining.
Results: Both pre- and post-treatment with LGG suppressed the airway hyper-responsiveness to methacholine and significantly decreased the number of infiltrating inflammatory cells and Th2 cytokines in bronchoalveolar lavage fluid and serum compared with the OVA-sensitized mice. In addition, LGG reduced OVA-specific IgE levels in serum. Oral LGG decreased matrix metalloproteinase 9 expression in lung tissue and inhibited inflammatory cell infiltration.

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Conclusion: LGG had an anti-inflammatory effect on OVA-induced airway inflammation and might be an additional or supplementary therapy for allergic airway diseases.
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Introduction

The past 3 decades have seen an increase in the prevalence of asthma, eczema, and allergic rhinitis in developed countries. A number of environmental factors including air pollution, cigarette smoking, and allergen exposure have been proposed to explain the changes in the prevalence of asthma.[1,2] In Taiwan, asthma, bronchitis, emphysema, and coronary obstructive pulmonary disease are ranked seventh among the leading causes of death.[3,4] The inhalation of allergens stimulates both bone marrow-derived cells and non-bone marrow-derived cells in the innate immune system to secrete cytokines that promote antigen expressions on CD4+ T cells, and activate the antigen presenting cells and the T cells to produce Th2 responses.[5,6] Th2 cytokines, such us interleukin (IL)-4, IL-5, IL-9, and IL-13, then induce the changes in the airways and lung parenchyma that are associated with asthma, airway eosinophilia, pulmonary lymphocytosis, mastocytosis, alternative macrophage activation, and epithelial cell proliferation with goblet cell hyperplasia.[5] Moreover, increased Th2 cytokines lead to mucus secretion, smooth muscle hyperplasia, hypertrophy, hypercontractility, subepithelial fibrosis, immunoglobulin E (IgE) secretion, increased production of con chemokines that attract T cells, eosinophils, neutrophils, mast cells, and airway hyper-responsiveness (AHR).[5] Hyper-responsiveness is defined as an increased sensitivity to agents, such as cholinergic agents and other stimuli that cause smooth muscle contractions and increased airway resistance by narrowing the airways.[7–9]

Previous studies have also shown that matrix metalloproteinases (MMPs) are members of a family of enzymes that cleave extracellular matrix proteins. Furthermore, MMPs have been implicated in many inflammatory conditions.[10] Specifically, in asthma patients MMP9 levels increase significantly.[11] MMP9 may therefore be the dominant MMP in the airways of asthmatics. This suggests that MMP9 plays an important role in monitoring treatment response and airway remodeling in asthma, but the role of MMPs in asthma and inflammation diseases remains unclear. In this study, we investigated the expression of MMP9 in bronchoalveolar lavage fluid (BALF) and serum before and after *Lactobacillus rhamnosus* GG (LGG) treatment.

An inhaled corticosteroid is the principle treatment for asthma. However, this therapy is not applicable for a considerable percentage of patients with asthma, and only about one third of patients actually benefit from the addition of leukotriene inhibitors.[12,13] Severe and corticosteroid-resistant forms of asthma actually lead to life-threatening attacks, and the mortality rate of this disease is showing an increasing trend.[14] There is thus an urgent need to devise treatments for asthma that are more effective. Due to the involvement of cytokines in chronic inflammation and airway remodeling,[13,14] they are the main targets in novel allergy and asthma therapies.

The Food and Agriculture Organization of the United Nations defines probiotics as "Live microorganisms that when administered in adequate amounts confer a health benefit on the host".[15,16] In other words, ingested probiotics could modify microbial populations to benefit the host. Previous studies have shown that probiotics can reduce allergic diseases by modifying the immune system of the host.[17] Moreover, several studies have reported that oral *Lactobacillus* strains have beneficial effects on perennial allergic rhinitis and pollen allergies,[18–22] but the mechanisms underlying the effects on the immune system are unclear.

Previous studies[23–25] have reported that administration of microbial supplements such as probiotic bacteria during pregnancy or early life might prevent allergy-related diseases.

However, unlike previous studies, our study focused on whether the effect of treatment with oral probiotics on adults is the same as prevention. Based on this idea, we used 6- to 8-week-old adult mice. The groupings of LGG-treated mice included the prevention group (pre-LGG, oral LGG on Days 1–14 during sensitized experimental process) and the treatment group (post-LGG, oral LGG on Days 14–27 during a sensitized experimental process) to analyze the effects of LGG on allergic diseases at different times. If oral probiotics can cause an improvement in adult allergy patients, it will be good news.

We used the probiotic bacterium LGG because in a previous study by our team[26] the administration of LGG decreased symptoms in patients with allergic rhinitis. Moreover, some studies[27,28] have also shown that *Lactobacillus* probiotic can modulate the Th1/Th2 immune response, and it has been reported that LGG administered postnatally can reduce the risk of allergic diseases.[28]

Asthma/allergies can, however, be caused by a Th1/Th2 imbalance, and in order to advance previous research we chose LGG in this allergy/asthma study. In the current study, we hypothesized that orally-administrated LGG will induce and affect the balance of the Th1/Th2 response. To test this hypothesis, oral LGG treatment during the OVA-sensitization/challenge was evaluated for the effect of LGG in pre-established Th2 responsive mice. Moreover, in this study we also detected MMP9 expression in BALF and serum to analyze the effect of MMP9 on treatment with oral LGG.

Materials and methods

Mice

Female BALB/c mice at 5–8 weeks of age with body weights of 20–25 g were purchased from the National Laboratory

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Animal Center (Taipei, Taiwan). All animal care and housing requirements and all procedures were performed in accordance with the Chung Shan Medical University Institutional Animal Care and Use Committee (Animal Experiment Approval code: 1185), Taichung, Taiwan. Animals known to be high IgE responders were used, and the mice were maintained on an ovalbumin (OVA)-free diet and were individually housed in rack-mounted stainless steel cages with free access to food and water. The mice were divided into four experimental groups. The four groups of mice were treated as follows: (1) the normal control group received normal saline plus alum intraperitoneally and normal saline intranasally; (2) the positive group received 50 μg OVA plus alum intraperitoneally and 5% OVA intranasally; and (3) the pre- and (4) post-LGG groups received 50 μg OVA plus alum intraperitoneally and 5% OVA intranasally, and were fed 0.018 mg/pc/day of LGG.

The pre-treatment group (pre-LGG) received LGG from Day 1 to Day 14 as a method of prevention. The post-treatment group (post-LGG) received LGG from Day 14 to Day 27 as a method of treatment.

The probiotic

The LGG powder (TTY Biopharm Company Limited, Taipei, Taiwan) was stored at −20°C. Drinks were prepared using double distilled H_20 (ddH_20) only and ddH_2O plus LGG. Eighteen micrograms of LGG powder were dissolved in 10 mL ddH_2O.

The mouse model of allergic asthma

The allergic asthma model is as shown in Fig. 1A. The mice received normal saline with alum or were sensitized to OVA intraperitoneally with alum (50 μg OVA in alum) on Day 1, Day 2, Day 3 and Day 14, followed by intranasal OVA challenges (5% solution, 50 μL) or normal saline on Day 14, Day 17, Day 21, Day 24, and Day 27. The animals received either the control vehicle or LGG feeding (0.018 mg/pc/day LGG diluted in ddH_2O) from Day 1 to Day 14 (pre-LGG mice, as shown in Fig. 1B), or from Day 14 to Day 27 (post-LGG mice, as shown in Fig. 1C). The normal control group consisted of five mice; the positive control had nine mice; and the pre- and post-treatment LGG groups (pre-LGG, pre; post-LGG, post) each had seven mice.

In this study, we performed independent experiments more than three times. We therefore confirmed that this is a successful animal model of allergic asthma, so we used this animal model in our asthma and allergic disease study.

AHR and bronchoalveolar lavage fluid isolation

AHR was assessed in unrestrained mice through whole-body barometric plethysmography (Model PLY 3211; Buxco Electronic Inc., Sharon, CT, USA) to record the enhanced pause (Penh). Penh, a dimensionless parameter, was used to measure the pulmonary resistance, which was calculated by changing the chamber pressures through methacholine challenge during inspiration and expiration. After a brief acclimatization to the chamber, the mice received an initial baseline challenge of saline, followed by increasing doses of nebulized methacholine. During the exposure period, each mouse was given 0 mg/mL (saline), 5 mg/mL, 10 mg/mL, or 20 mg/mL methacholine. Mice remained in the chamber for 3 minutes. The respiratory rate was counted during the 3-minute methacholine challenge. After that, the Penh values were averaged and reported as baseline saline values in percentages.[29,30]

BALF was isolated in 1 mL of phosphate buffered saline. The BALF cellularity was determined using a hemocytometer. The cells were centrifuged (4000 × rpm, 5 min), transferred onto slides, and were fixed and stained using Liu stain. The observer counted 200—300 cells per slide using the standard morphological criteria to classify the individual leukocyte populations.[31,32]

The measurement of OVA-specific antibodies in serum

The serum levels of OVA-specific IgE and IgG2a were determined first. In short, the 96-well microtitre plates were coated with 100 mg/mL of OVA in 0.1M NaHCO3 at 4°C and

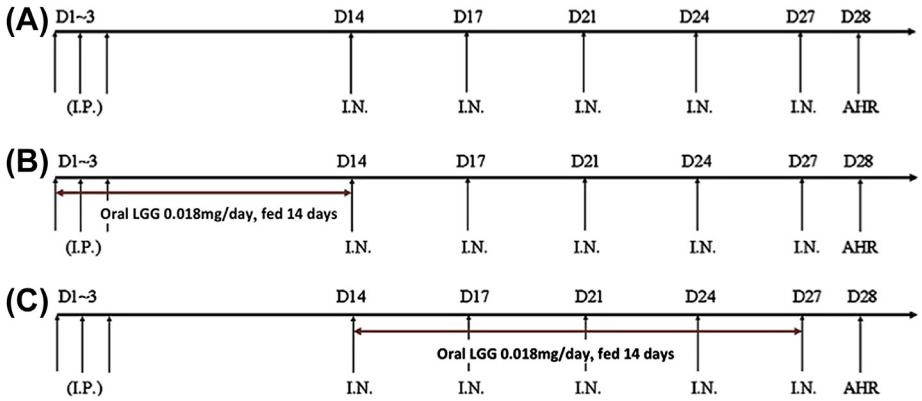

Figure 1. The protocols used for the mouse models of allergic asthma. (A) The protocols used for the mouse models of allergic asthma. (B) The protocols used for the mouse models of allergic asthma and pre-treatment with *Lactobacillus rhamnosus* GG (LGG). (C) The protocols used for the mouse models of allergic asthma and post-treatment with LGG. In (B) and (C), mice were fed 0.018 mg/pc/day of LGG. Ovalbumin (OVA)-sensitized mice were given 50 μg/pc I.P. (intraperitoneal) and 5% OVA solution I.N. (intranasal).

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left overnight. The plates were washed with Tween 20 solution (0.05%) and then blocked with bovine serum albumin (3%) for 1 hour at 37°C. After washing, 50 μL of serially diluted sera in 3% bovine serum albumin was added and incubated at 4°C and the plates were left unattended overnight. After washing, horseradish peroxidase conjugated with anti-mouse isotype-specific antibody (BD Pharmingen, San Jose, CA, USA) and optimal dilutions were added. The plates were incubated at 37°C for 2 hours and were then washed. To determine the IgE/IgG2a, p-nitrophenyl phosphate substrate (Sigma Chemical Co., St Louis, MO, USA) was added and the absorbance at 490 nm was measured. The detection antibody was alkaline phosphatase-labeled.

The measurement of cytokines

Serum and BALF samples were collected from each group of mice after the mice were sacrificed. The samples were assayed for the presence of cytokines using the R&D system (Boston, MA, USA) following the manufacturer's protocol. After that, interferon-gamma (IFN-γ, limit of detection: 9.5 pg/mL), IL-4 (limit of detection: 8.0 pg/mL), IL-5 (limit of detection: 16.1 pg/mL), IL-10 (limit of detection: 15.9 pg/mL), IL-12p70 (limit of detection: 8.3 pg/mL), IL-13 (limit of detection: 41 pg/mL), and transforming growth factor beta (TGF-β, limit of detection: 31.2 pg/mL) levels were assayed according to the manufacturer's protocol (Quantikine ELISA Kit, R&D Systems, Boston, MA, USA), and the enzyme-linked immunosorbent assay (ELISA) plate was read at 450 nm using a Bio-Rad ELISA Reader (Bio-Rad, Hercules, CA, USA). The data were then analyzed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). The unknowns were compared using a standard curve containing at least five to seven dilution points, which were the relevant recombinant cytokines on each assay plate.

The measurement of MMP9

The serum and BALF samples were collected from each group of mice after the mice were sacrificed. The R&D Systems Quantikine ELISA Kit was used to analyze the samples. However, MMP9 levels (limit of detection: 31.3 pg/mL) were assayed according to the manufacturer's protocol, and the ELISA plate was read at 450 nm using a Bio-Rad ELISA Reader. The data were then analyzed using SoftMax Pro software (Molecular Devices). The unknowns were compared using a standard curve containing at least five to seven dilution points, which were the relevant recombinant cytokines on each assay plate.

Histological analysis

To assess the pathological changes, samples of the lungs were taken after the mice were sacrificed. The samples were fixed in neutrally buffered 10% formaldehyde and were embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin to detect inflammatory cell infiltration in the lung tissue. The figure for airway inflammation was detected by hematoxylin and eosin staining with a magnification ratio of 1:200.

Statistical analysis

All data points represent the mean ± standard error of the mean of the individual mouse groups. Analysis was performed using GraphPad Instat software (San Diego, CA, USA), and the Mann-Whitney nonparametric test was conducted to determine the statistical significance, where appropriate. A p-value of < 0.05 was considered significant.

Results

The effect of LGG on the AHR

The Buxco system was applied to detect lung function. We also wanted to analyze whether administrated LGG would suppress AHR in sensitized mice. The OVA-sensitized and challenged mice were significantly more sensitive to methacholine exposure than the normal control group (Penh value: 10.37 ± 4.2 vs. 0.27 ± 0.13, respectively; $p < 0.007$). In this study, we investigated whether LGG had a beneficial effect on inflammation. The mice that received LGG during Days 1—14 as a means of prevention (pre-LGG, Penh value: 4.02 ± 2.9), had significantly lower AHR to methacholine compared with the OVA mice tested at the 20 mg/mL level ($p < 0.0012$) and comparable responses in the normal control animals were observed at the same dose. Similar results were observed in terms of significant inhibition of AHR when LGG was administered during Days 14—27 as a means of treatment (post-LGG; Penh value: 4.32 ± 3.3; Fig. 2).

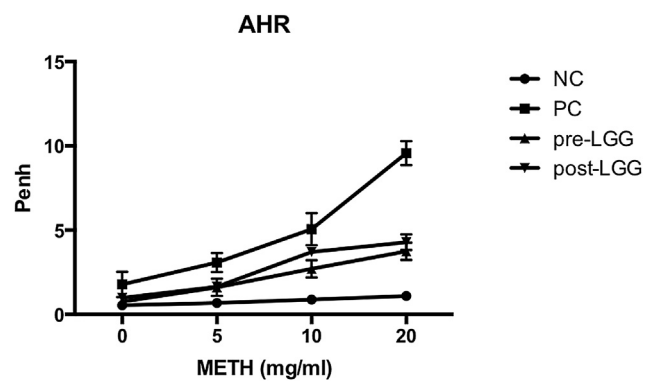

Figure 2. The effects of *Lactobacillus rhamnosus* GG (LGG) on the pre- and post-treatment mouse models of allergic asthma. The female bronchoalveolar lavage fluid (BALB)/c mice were given normal saline (normal control group) or were sensitized/challenged with ovalbumin (OVA) ± treatment with LGG. The airway hyper-responsiveness to methacholine was assessed in a Buxco system to record the enhanced pause (Penh). The Penh value in the positive control (PC, ■) group was higher than the other groups. However, the Penh value in the oral pre- and post-LGG groups (▲, ×) was significantly decreased. The normal control (NC, ◆) group was lower than all of the groups. NC, $n = 5$ for the NC group; PC, $n = 9$ for OVA only treatment group; pre-LGG, $n = 7$ for pre-treatment LGG; and post-LGG, $n = 7$ for post-treatment LGG. The statistical analysis compared OVA-treated mice and is represented as: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Airway hyperresponsiveness (AHR).

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The effect of LGG treatment on the reversal of allergen-induced serum OVA-specific antibodies in serum

Herein, we detected whether oral LGG would decrease serum levels of IgE, and increase the Th1 response by measuring IgG2a. ELISA was used for data analysis. The serum levels of OVA-specific IgE and IgG2a were significantly elevated after the OVA sensitization/challenge compared with the normal control group. In the group that received oral LGG treatment (pre- and post-treatment) during the OVA sensitization/challenge, the serum IgE was significantly decreased ($p < 0.05$, Fig. 3A). Moreover, the serum IgG2a was also significantly increased ($p < 0.005$, Fig. 3B).

The effect of LGG on the infiltrating cells of the lungs

Previous studies have reported that cell infiltration of the lung is a hallmark of asthma. In order to analyze the infiltration of cells into the lung, we used cytospin and Liu stain to evaluate BALF. Differential cell counts (Fig. 4) revealed that total cells, eosinophils, lymphocytes and monocytes were significantly increased in the OVA-sensitized mice compared to the control group. However, the infiltrating cells of the lung were significantly decreased in the two groups treated with LGG ($p < 0.05$).

The effect of LGG treatment on cytokines in serum and BALF

Th1 and Th2 system imbalance is characteristic of asthma/allergic diseases. In this study, we tested the Th1 and Th2 cytokines after LGG treatment to determine whether oral LGG would decrease Th2 cytokine secretion and increase Th1 cytokine.

Th2 cytokines in the OVA-sensitized/challenged mice were increased compared with the normal control group. IFN-γ was significantly decreased in the OVA-sensitized group. Besides this, not only Th1 cytokines but also regulatory T (Treg) cytokines including IL-12, IFN-γ and TGF-β were significantly increased in the LGG pre- and post-treatment groups (Fig. 5). Moreover, the LGG treatment administered during the challenge phase significantly decreased the Th2 cytokines, including IL-4, IL-5, and IL-13. However, IL-10 was significantly decreased in the serum and BALF after LGG treatment (Fig. 6).

The effect of LGG on lung inflammation

Next, we assessed the inflammation of lung tissue. One of the hallmarks of asthma is lung inflammation, so we analyzed by histology whether LGG would improve lung inflammation.

The effect of LGG treatment on the OVA-sensitized/challenged mice on overall lung inflammation was evaluated through histological hematoxylin and eosin staining (Fig. 7). The normal control group (Fig. 7A) and the mice in the OVA-sensitized/challenged (Fig. 7B) group (positive control) with allergic asthma had severe inflammation; in the pre- and post-treatment LGG models (Fig. 7 C and D) of allergic asthma after LGG treatment, there was significantly less inflammation. A morphometric examination of the airway sections revealed that the OVA exposure (Fig. 7B) significantly increased the epithelial cell thickness. In both groups (Fig. 7C and D) treated with LGG, there was a decrease in epithelial cell thickness compared to the OVA mice.

The effect of LGG on MMP9 expression

The MMP9 expression was highly associated with inflammation, asthma, and airway remodeling. We therefore detected the MMP9 levels in serum and BALF to analyze the MMP9 concentration after LGG treatment.

The effect of the LGG treatment in the OVA-sensitized/challenged mice on the MMP9 expression in serum and BALF was detected using ELISA (Table 1). The mice that were OVA-sensitized/challenged in the allergic asthma models had stronger expression of MMP9 compared with the normal

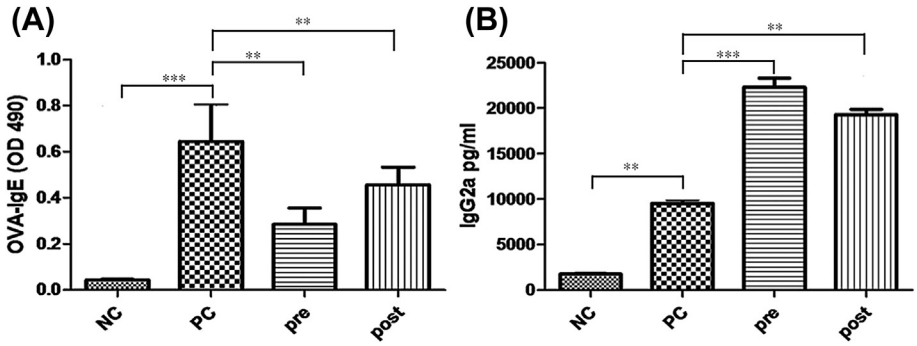

Figure 3. The effects of *Lactobacillus rhamnosus* GG (LGG) treatment on the specific anti-OVA immunoglobulin (Ig)E and IgG2a serum levels in the ovalbumin (OVA)-treated mice. Serum OVA-specific IgE and IgG2a concentrations were obtained from the normal control group and the OVA sensitized/challenged mice (positive control) or presence of treatment with LGG. (A) There was significant expression of OVA-specific IgE in the positive control group, and groups with pre- and post-treatment LGG had decreased expression. (B) OVA-specific IgG2a expression in serum; the groups treated with LGG increased significantly. The statistical analysis compared OVA-treated mice and is represented as: $*p < 0.05$; $**p < 0.01$, and $***p < 0.001$.

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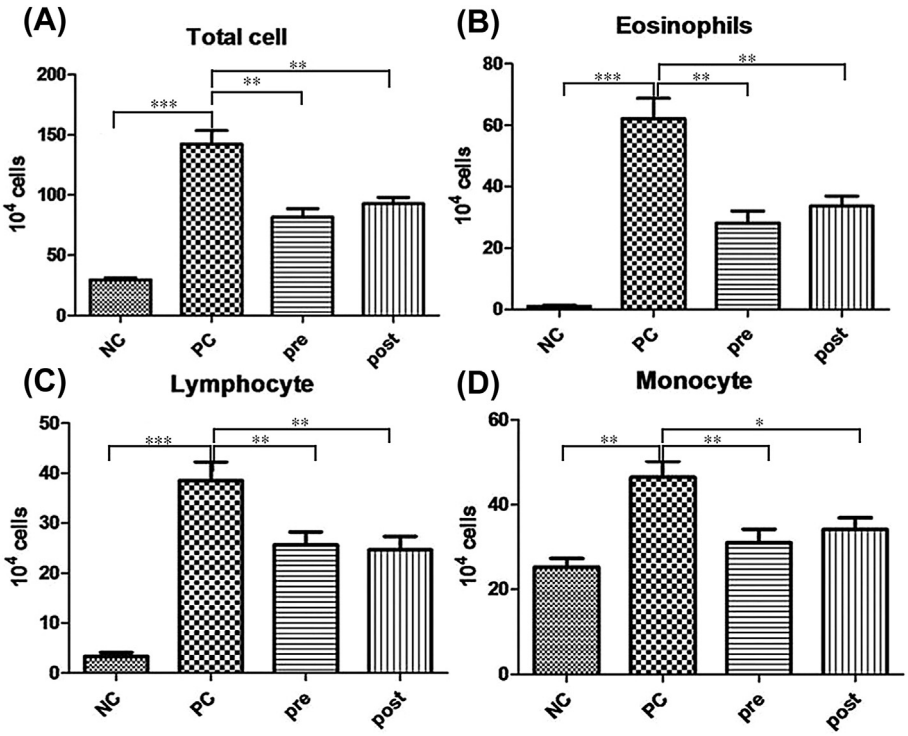

Figure 4. The effects of the *Lactobacillus rhamnosus* GG (LGG) treatment on the inflammatory cell infiltrations in bronchoalveolar lavage fluid (BALF) of ovalbumin (OVA)-treated mice. All cell counts were obtained from the normal control group and OVA sensitized/challenged mice (positive control, PC) or presence of treatment with LGG. The total cells and inflammatory cells were counted ($\times 10^4$) in BALF in millimeters by the morphometric evaluations of cytospin preparations. (A) Total cell count in BALF. A large number of cells was expressed in BALF of the PC group; however, the total cells in the BALF of LGG treated groups decreased. (B) The eosinophil is the hallmark of asthma. In the PC group the eosinophils significantly increased compared to LGG treated groups. (C and D) The monocyte and lymphocyte cell counts. The statistical analysis compared OVA-treated mice and is represented as: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

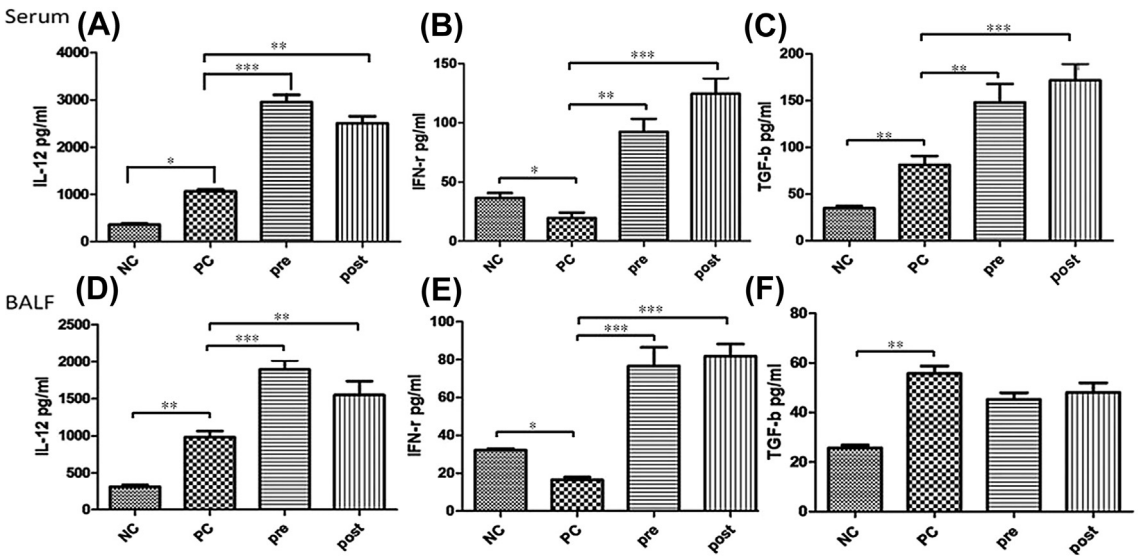

Figure 5. The effects of the *Lactobacillus rhamnosus* GG (LGG) treatment on Treg and Th1 cytokine levels [transforming growth factor (TGF)-β, interferon (IFN)-γ, and interleukin (IL)-12] in the ovalbumin (OVA)-treated mice in serum and bronchoalveolar lavage fluid (BALF). Serum and BALF in Th1 and Treg cytokine concentrations were obtained from the normal control group and OVA sensitized/challenged mice (positive control, PC) or in the presence of treatment with LGG. (A—C) the Th1 (IL-12, IFN- γ) and Treg (TGF-β) cytokines detected in serum and (D—F) the Th1 and Treg cytokines detected in BALF. In LGG-treated groups the Th1 cytokines in the serum and BALF increased significantly compared with the PC group. In the oral LGG groups, however, the Treg cytokines in serum and BALF decreased compared with PC group. The statistical analysis compared OVA-treated mice and is represented as: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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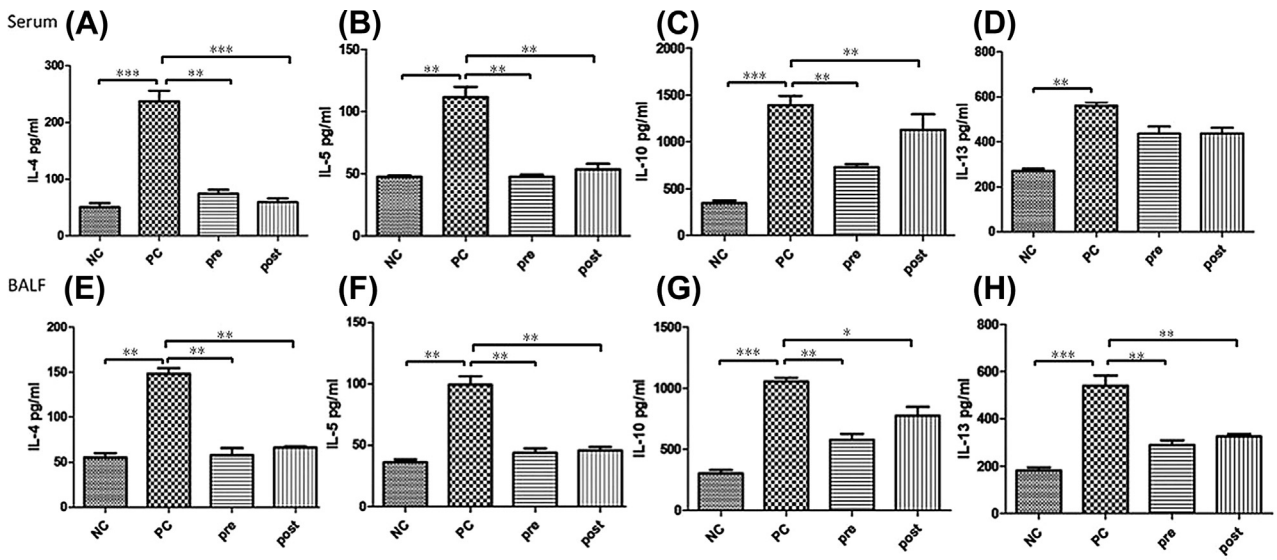

Figure 6. The effects of the *Lactobacillus rhamnosus* GG (LGG) treatment on Th2 cytokine levels [interleukin (IL)-4, IL-5, IL-10, and IL-13] in the ovalbumin (OVA)-treated mice in serum and bronchoalveolar lavage fluid (BALF). Serum and BALF in Th2 cytokine concentrations were obtained from the normal control group and OVA sensitized/challenged mice (positive control) or in the presence of treatment with LGG. (A—D) The Th2 (IL-4, IL-5, and IL-13) cytokines detected in serum and (E—H) in BALF. In LGG-treated groups the Th2 cytokines in serum and BALF were significantly decreased compared with positive control group. The statistical analysis compared OVA-treated mice and is represented as: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

control group. After the LGG treatment, in both of the groups treated with LGG in the allergic asthma models, there was significantly less MMP9 expression. LGG treatment therefore led to a significant decrease in MMP9 expression in serum and BALF.

Discussion

In this study, the allergen-induced airway inflammatory mouse model was used to explain the role of OVA in mediating pulmonary inflammation, promoting airway

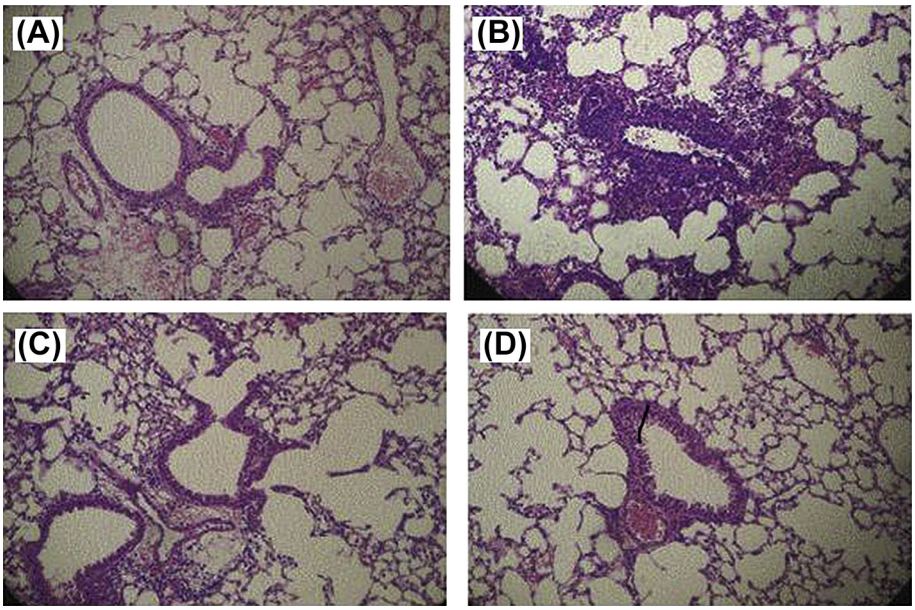

Figure 7. The effects of the *Lactobacillus rhamnosus* GG (LGG) treatment on allergen-induced airway inflammation. Lung tissues were obtained on Day 28 from (A) the normal control group and (B) the ovalbumin (OVA) sensitized/challenged mice in the absence of, (C) pre-treatment with LGG, or (D) post LGG treatment, which were stained with hematoxylin and eosin and with a magnification ratio of 1:200. The positive control group shows severe inflammation in the airway. However, the oral LGG groups show significantly decreased airway inflammation compared with the positive control group.

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Table 1 The effects of the *Lactobacillus rhamnosus* GG (LGG) treatment on MMP9 expression in serum and bronchoalveolar lavage fluid (BALF)[a]

Samples	Group			
	Normal control ($n = 5$)	Positive control ($n = 9$)	Pre-LGG ($n = 7$)	Post-LGG ($n = 5$)
Serum (ng/mL)	0.0798 ± 1.4**	4.37 ± 3.31	2.34 ± 1.79**	2.97 ± 2.2**
BALF (ng/mL)	0.0973 ± 2.01**	5.792 ± 6.32	2.91 ± 3.32**	3.06 ± 1.42*

[a] Serum and BALF were obtained on Day 28 from the normal control group, the ovalbumin (OVA)-sensitization group and pre- or post-treat LGG groups, which were detected with enzyme-linked immunosorbent assay. The positive control group had shown more MMP9 expression in serum and BALF. However, groups receiving oral LGG had significantly decreased MMP9 compared with the positive control group. The statistical analyses are compared with OVA-treated mice and represented as: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

remodeling, and promoting airway hyper-reactivity to methacholine. The BALB/c mice were found to be susceptible to the IgE-mediated allergic responses.[33] In addition to the responses of the OVA sensitized/challenged mice following an increase in the serum IgE level, the eosinophils displayed hyperplasia in the mouse asthma model. Previous studies have shown that the oral administration of LGG during allergen sensitization induces a Th1-predominant allergen-specific immune response in food-allergic mice and balances the Th1/Th2 immune response.[17] Previous studies have also reported that in some atopic children oral probiotics increase Th1 cytokines and inhibit allergen-induced IgE and Th2 cytokines.[34,35] Similarly, our results showed the Th1/Th2 immune response in the allergen animal model. Moreover, our study also showed that oral LGG decreased AHR and inflammation cells in the pre- and post-treatment LGG groups. These results support the theory that LGG may be an effective anti-allergy therapy.

Lammers et al[18] reported that several probiotic therapies decrease IL-1β, IL-8, and IFN-γ mucosal gene expression, whereas the proinflammatory cytokines TNF-α, IL-6, IL-12, and regulatory cytokines IL-10 and TGF-β are not altered. In recent studies,[36–39] IFN-γ, IL-12, and TGF-β increased significantly after treatment with LGG. These different results may be caused by different probiotic treatments or different allergic diseases but the mechanisms of probiotic therapy should become clearer in the future. In other studies with an allergy mouse model, however, oral administration of probiotics has been shown to suppress OVA-specific IgE production, and inhibit subsequent allergic sensitization that is caused by inducing Treg-associated TGF-β production.[40] After oral probiotics, the levels of IFN-γ and IL-10 become significantly higher than those in non-treated mice.[41] Th2 cytokine-IL-4 is known to directly promote the features of asthma, such as eosinophil infiltration, goblet cell metaplasia, AHR, IgE production in serum, mastocytosis, airway remodeling, and Th2 induction/maintenance.

Herein, our results showed that the OVA-sensitized mice expressed higher levels of IL-4 in serum and BALF than the naïve mice, but the IL-4 levels decreased after treatment with LGG. Furthermore, previous studies have shown that IL-10 and TGF-β production are associated with T cell tolerance and Tregs, which are induced by mucosal exposure to antigens.[42] However, IL-10 was initially described as a Th2 type cytokine, but further evidence suggests that IL-10 is not specific to Th2 cells or Treg cells but instead is a much more broadly expressed cytokine. IL-10 is expressed by many cells including Th1, Th2, and Th17 cell subsets, Treg cells, CD8+ T cells, and B cells.[43] Other studies have shown that IL-10 cannot only downregulate production of IL-4 and IL-5 but can also stimulate B cell differentiation and immunoglobulin secretion.[44,45]

Makela et al[46] reported that OVA-sensitized and challenged IL-10−/− mice develop a pulmonary response but fail to exhibit AHR. Reconstitution of these deficient mice with the IL-10 gene fully restores development of AHR to a level comparable to control mice. Some studies, however, have shown that in atopic children oral LGG results in elevated IL-10 concentrations, indicating that specific probiotics may have anti-inflammatory effects *in vivo* and may also enhance regulatory or tolerance-inducing mechanisms.[34,47]

Probiotics can increase Th1 cytokines and inhibit IgE and Th2 cytokines. Conversely, in allergens produced by house dust mites, oral probiotics reduce IL-10 responsiveness.[48] Our study showed that after treatment with LGG, IL-10 levels in serum and BALF were significantly decreased compared to OVA-sensitized mice. Previous study with LGG, such as that by Szajewska et al,[49] have demonstrated that LGG is successful in decreasing the duration of acute diarrhea in children aged 1–36 months, and LGG is more efficient in reducing diarrhea caused by rotavirus.

Previous studies have shown that MMP9 may play a role in airway inflammation and remodeling in asthma; moreover MMP9 concentrations increase in severe, persistent asthma and following allergen challenge.[50] Ohno et al[51] stated that MMP9 is highly expressed in bronchial biopsies or BALF from asthma patients, and MMP9 activity increases after allergen challenge.[52] In order to determine whether MMP9 activity increases after an allergen challenge such as OVA and to carry out further study of chronic asthma, we examined MMP9 in this study. The MMP9 levels not only increased in serum but also in BALF after OVA challenge; these results are similar to those of previous studies.[50–52] More importantly, in our study orally-administrated LGG decreased MMP9 levels in BALF and serum.

In a probiotic study of a clinical trial with infants at high risk for allergies, mothers received a probiotic mixture (*Bifidobacterium* spp. and *Propionibacteria* spp.) or placebo during the past few months of pregnancy, and their infants received the same mixture from birth until 6 months

of age. According to the study, no preventive effect of asthma was observed until up to 5 years of age in those who received the probiotic.[53] Rosenfeldt et al[54] in another allergic study, demonstrated that oral intake of *L. rhamnosus* and *Lactobacillus reuteri* improves clinical symptoms in patients with atopic dermatitis. In addition to these results, a large cohort study of LGG administration for 4 weeks revealed decreased SCORing Atopic Dermatitis results in pediatric patients with atopic dermatitis.[55] Many animal studies have used various strains of probiotics in animal models of asthma. Oral administration of probiotics attenuates the symptoms of allergic asthma in a mouse model and induces immune regulation by a CD4(+)CD25(+) Foxp3(+)Treg cellmediated mechanism.[56,57] Similar to our results, Yu et al[58] reported that oral *Lactobacillus casei rhamnosus* Lcr35 attenuates airway inflammation and AHR in a mouse model of allergic airway inflammation.

In a previous study of the probiotic LGG, oral LGG administration particularly suppressed allergen-induced proliferative responses.[59] Moreover, previous clinical studies have suggested that probiotic microorganisms suppress Th2-type effector mechanisms.[60] Our study had similar results. Another study, however, showed that with recurrent wheezing and an atopic family history, oral LGG has no clinical effect on atopic dermatitis or asthma-related events.[61] It may be helpful to investigate molecular pathways after probiotic treatment in order to understand the different study results. Another study reported that there are insufficient current data to support the use of probiotics for the treatment of established allergic diseases.[61] We have therefore designed definitive intervention studies of LGG combined therapy in allergic diseases for treatment and prevention. The results demonstrated that oral LGG may inhibit and prevent airway inflammation, Th2 cytokine production, MMP9, etc. However, the mechanism of oral LGG in inflammation remains unclear, so further studies are needed.

Asthma is a complicated disease that requires further investigation. This study found that oral LGG can inhibit OVA-induced airway inflammation. Nevertheless, some studies have reported that oral probiotics have little or no clinical effect on allergic diseases. Our results suggested that oral probiotics might be an additional or supplementary therapy to other clinical allergy therapies.

Conflicts of interest

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