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Original Article

Effect of Lactobacillus rhamnosus GG immunopathologic changes in chronic mouse asthma model

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KEYWORDS
Asthma;
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LGG;
RORrt;
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Abstract *Background:* Asthma is a heterogeneous inflammatory disorder of the airway. A Th2 response usually contributes to high levels of allergen-specific IgE and eosinophilic airway inflammation. Several findings have demonstrated that neutrophils, not eosinophils, are the major inflammatory cells in chronic asthma patients with steroid-resistance. Lactobacillus rhamnosus GG (LGG) exhibits anti-inflammatory properties on OVA-induced acute airway inflammation.
Objective: We hypothesized that orally administrated LGG should reduce airway remodeling in chronic experimental models.
Methods: Female Balb/c mice were sensitized with OVA. LGG was used to investigate whether oral administrations of LGG inhibited OVA-induced airway inflammation in a chronic asthma model and the different intervention times between LGG pre-treatment and post-treatment groups. BALF was analyzed with Liu's stain and ELISA assay. Lung histopathology was assayed with HE, IHC and Masson's trichrome staining. Lung tissues were assayed with PCR (T-bet, GATA3, RORrt and Foxp3). Many cytokines were detected in the serum and BALF.
Results: LGG significantly decreased the number of infiltrating inflammatory cells. We also found that the oral LGG group suppressed not only Th2 cytokine, but also IL-17, TNF-α and

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HMGB1 in the BALF levels. However, GATA3 and RORrt decreased significantly in the RNA level in the LGG groups, but the T-bet and Foxp3 increased in the RNA level.
Conclusions: LGG not only had anti-inflammatory effects on OVA-induced airway inflammation, but also improved airway remodeling and collagen expression in the chronic asthma mouse model. Moreover, LGG might be an additional or supplementary therapy for allergic airway diseases.

Introduction

Asthma is a common chronic airway disorder. Asthma is characterized by airway hyperresponsiveness, airway obstruction, airway inflammation, and airway remodeling.[1,2] The prevalence is increasing in many countries, and in 2015 it was reported that there are almost 18.4 million U.S. adults currently suffering with asthma, and 3396 adult asthma deaths.[3] Moreover, earlier studies have reported that asthma is caused by T helper type II cells (TH2 cells) and eosinophils triggered by cytokine-based airway inflammation involving cytokines such as IL-4, IL-5, IL-9 and IL-13 etc.[4,5]

In clinics, corticosteroids are widely used to treat asthma patients. However, asthma can be a distinct and overlapping phenotype syndrome with severe corticosteroid refractory asthma, such us non-eosinophilic asthma. Neutrophil mediated asthma including non-eosinophilic non-allergic asthma often does not respond to inhaled corticosteroids.[1,2,6] Therefore, there is an urgency to devise more effective compounds with fewer undesirable side effects to treat non-eosinophilic asthma.

The Food and Agriculture Organization of the United Nations define probiotics as viable micro-organisms, when administered in adequate amounts, promote or support a health benefit on the host.[7] However, that definition was modified slightly by the International Scientific Association for Probiotics and Prebiotics in 2014. Now the definition of probiotics is live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[8]

According to a bibliometric analysis, the number of publications about probiotics in pediatrics is more than 2800 articles.[9] Previous studies have shown that probiotics can modulate immune tolerance and prevent the development of disease.[9] Moreover, some studies have demonstrated that regulating the microbiota can modulate the systemic immune response of the host and reduce sensitization and allergic inflammation.[9,10] However, the prevention and treatment of specific probiotics in allergy diseases and asthma still requires furthermore study. Probiotics such as *Bifidobacterium*, *Bifidobacterium infantis* CGMCC313-2 can induce IL-10 to exert a significant inhibition of the Th2 cytokine response.[9,11] Lactobacillus strains have been widely studied and shown to have an allergy modulation effect, although inconsistent results have been reported.[12] Some studies have reported significant benefits of using probiotic supplements containing *L. rhamnosus* and have enrolled infants at high risk of allergy.[13] *Lactobacillus salivarius* PM-A0006 can significantly down-regulate the influx of eosinophils to the airway, reducethe OVA-specific IgE in BALF and up-regulate IFN-r in the serum.[14] *Lactobacillus casei* can reduce respiratory infection.[15]

Lactobacillus rhamnosus GG can lower the risk of respiratory infection and lower the number of prescribed antibiotics among children[16,17] and can also inhibit MMP9 and Th2 cytokines in animal models as shown in our previous study and others.[9,18,19]

In the present study, we hypothesized that orally administrated LGG could induce and alter the Th1/Th2 balance in the acute asthma stage and studied whether LGG could also have an effect on airway remodeling and collagen deposition in chronic stage asthma in animal models. 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 in chronic stage. Moreover, in this study we also detected IL-17, TNF-α and HMGB1 expression in the BALF and serum and measured T-bet, GATA3, RORrt and Foxp3 in RNA levels.

Methods

Ethics statement

All animal experiments, care and housing requirements and all procedures were performed in accordance with the Institutional Animal Care and Use Committee at Chung Shan Medical University (No.1185).

Mice

Female BALB/c mice at 6—8 weeks of age with body weights of 20—25 g were purchased from the National Laboratory Animal Center. 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.

Probiotic- *Lactobacillus rhamnosus* GG (LGG)

Lactobacillus rhamnosus GG powder (ATCC53103, Provided by CY Biotech, Taiwan) was stored at −20 °C.

We obtained 0.18 mg LGG powder dissolved in 1 ml normal saline. The concentration of stock solution was

0.18 mg/ml stored in the 4 °C. Moreover, we were feed 100 μl/pc that the concentration of LGG was 0.018 mg.

The allergic asthma mouse model and challenges

The allergic asthma models were prepared according to our previous study.[1] The sensitization protocol (Fig. 1) was as follows: mice received an intraperitoneal injection of 50 μg of ovalbumin (OVA) complexed with alum on Days 1 and 14 and received an intranasal dose of 5% OVA 50 μl on Days 14, 17, 21, 24, 27, 60, 69, 71, 73, 74, and 75. The control group received normal saline with alum intraperitoneally on Days 1 and 14 and received normal saline intranasally on Days 14, 17, 21, 24, 27, 60, 69, 71, 73, 74, and 75. Other groups of OVA-treated mice were administered LGG. The pre-groups were treated with LGG on Days 1—14 and Days 45—60. The post-groups were treated with LGG on Days 14—28 and Days 60—75. On day 76 after the methacholine challenge, airway responses were determined in all the experimental groups, and the mice were sacrificed as well.

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. The NC group had 6 mice; (2) the positive group received 50 μg OVA plus Alum intraperitoneally and 5% OVA intranasally. The PC group had 8 mice; (3) the pre- and (4) post-LGG group received 50 μg OVA plus Alum intraperitoneally and 5% OVA intranasally, and were fed 0.018mg/pc/day of LGG. The pre-LGG and post-LGG group each had 8 mice, respectively.

In this study, we performed independent experiments more than 3 times. Therefore, we confirmed that this is a successful animal model of allergic asthma, so we used this animal model in our asthma and allergic disease study.

Bronchoalveolar lavage fluid isolation

Bronchoalveolar lavage fluid (BALF) was isolated in 1 ml of PBS. The BALF cellularity was determined using a hemocytometer. The cells were centrifuged, 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.

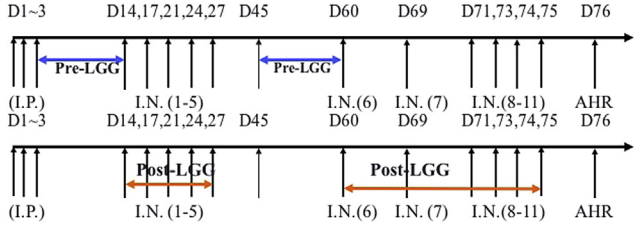

Figure 1. The allergic asthma mouse model and challenges. The allergic asthma models were prepared according to our previous study. The sensitization protocol: Pre-LGG-oral LGG on Days 0—14, and Days 45—60 or Post-LGG-oral LGG on Days 14—27, and Days 60—75.

The measurement of OVA-specific antibodies in serum

The serum levels of OVA-specific IgE and IgG2a were determined according to the pervious study protocol.[2] ELISA plate was read at 490 nm using a Bio-Rad ELISA Reader.

Analysis of airway inflammation and cytokines

Concentrations of IL-4, IL-5, IL-13, IL-17, IFN-γ and HMGB1 in the BALF were determined using Quantikine ELISA Kit (R&D system, USA) according to the manufacturer's protocol. ELISA plate was read at 450 nm using a Bio-Rad ELISA Reader.

Histological analysis

Masson's trichrome stain

To assess the pathological changes, lungs tissue were taken after the mice were sacrificed. The samples were fixed in neutrally buffered 10% formaldehyde and were embedded in paraffin. The samples were stained with Masson's trichrome to detect collagen deposition.

Immunohistochemistry stain (IHC stain)

Samples of the lungs were taken after the mice were sacrificed. All samples were fixed in neutrally buffered 10% formaldehyde and were embedded in paraffin. The samples were stained with IHC to detect IL-17 and TNF-α in the lung tissue.

Quantitative real-time PCR analysis

The total RNA was isolated from the lung homogenates with Trizol (Invitrogen, Grand Island, NY, USA) and reverse-transcribed. Real-time PCR was done with a ABI StepOne™ System (Applied Biosystems, Foster City, Calif.).

The primers used were as follows: β-actin forward 5' -TGG AAT CCT GTG GCA TCC ATG AAAC-3′, reverse 5′-TAA AAC GCA GCT CAG TAA CAG TCCG-3'; T-bet forward 5′-GCC AGG GAA CCG CTT ATA TG-3′, reverse 5′-GAC GAT CAT CTG GGT CAC ATT GT-3'; GATA3 forward 5′TTT ACC CTC CGG CTT CAT CCT CCT 3′ reverse 5′TGC ACC TGA TAC TTG AGG CAC TCT-3'; RORrt forward 5′-TCT ACA CGG CCC TGG TTCT-3′, reverse 5′-ATG TTC CAC TCT CCT CTT CTC TTG-3'; FOXP3 forward 5′-TCA TCC GCT GGG CCA TCC TG-3′, and reverse 5′-GTG GAA ACC TCA CTT CTT GGT C-3'.

Statistical analyses

All data points represent the median ± IQR of the individual mouse groups. Analyses were performed using GraphPad Instat software (San Diego, CA), and the Mann—Whitney nonparametric test was conducted to determine the statistical significance, where appropriate. A P value < 0.05 was considered statistically significant.

Results

LGG treatment in airway hyperresponsiveness (AHR) in animal model

The mice sensitized and challenged with OVA were significantly more sensitive to methacholine exposure than the NC (Fig. 2). In this study, we detected whether LGG had a beneficial effect during the allergy challenge. According to the results, not only the pre- but also the post-groups that feed LGG had significantly lower AHR after the methacholine challenge compared with the positive mice (OVA-sensitization) (analysis at 20 mg/ml level).

LGG treatment in ovalbumin-specific antibodies in serum in animal model

A characteristic of allergic diseases was increased IgE level in serum. In this study, the serum IgE and IgG2a levels were significantly increased after the OVA sensitization/challenge. In the experimental groups that received LGG during the sensitization/challenge to OVA, the serum IgE level decreased significantly (Fig. 3). The IgG2a level in serum increased significantly compared with the positive group.

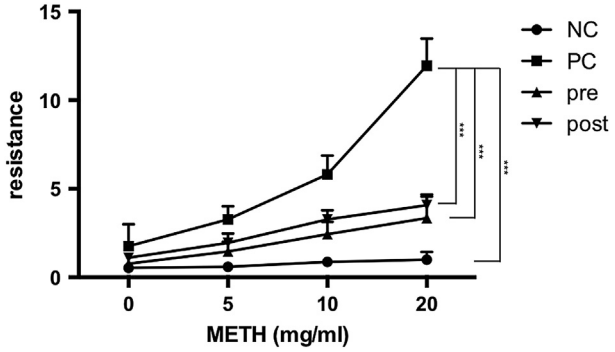

Figure 2. LGG treatment in airway hyperresponsiveness (AHR) in animal model. The female BALB/c mice received normal saline (normal control group) or were sensitized/challenged with OVA ± treatment with FIP- fve or a corticosteroid during the challenge phase. The airway hyperactivity (AHR) to methacholine was assessed with the Buxco system to record enhanced pauses (Penh). NC group was show in (●), PC group was show in (■), pre- and post-LGG group were show in (▲, ▼).The statistical analysis compared OVA-treated mice and is represented as: **$p < 0.05$; ***$p < 0.001$.

LGG treatment in infiltrating cells of the lungs in animal model

The infiltration of inflammatory cells, especially eosinophils and neutrophils in the lung was a characteristic of the asthma model. BALF was used to evaluate cell infiltration of the lung. Differential BALF cell counts included eosinophils, lymphocytes, neutrophils and monocytes. According to our results, LGG fed mice exhibited down-regulation of infiltrating cells in the lungs (Fig. 4).

LGG affects inflammation cytokines in BALF in animal model

The Th1 cytokines (IL-2, IL-12 and IFN-γ), Th2 cytokines (IL-4, IL-5, IL-13) and TGF-β were detected in the BALF and IL-17, TNF-α and HMGB1 were also detected in the BALF. In our acute allergy mouse model, the Th1 cytokines were increase and the Th2 cytokines were decreased.[2] Similarly, fed LGG decreased Th2 cytokines and TGF-β in our chronic allergy animal model (Fig. 5). The Th1 cytokines were also up regulated in chronic animal model (Table 1).

Moreover, IL-17, TNF-α, and HMGB1 also decreased in LGG fed groups (Fig. 5c, d).

LGG affects IL-17 and TNF-α with IHC stain in animal model

The effect of the LGG treatment in the OVA-sensitized/challenged mice on overall IL-17 and TNF-α was evaluated through histological staining with *IHC* (Fig. 6). The positive group (Fig. 6B and F) had high IL-17 and TNF-α expression. After being fed LGG, there was significantly less IL-17 and TNF-α expression in not only the pre-treated groups but also the post-treated groups (Fig. 6C,D,G and H).

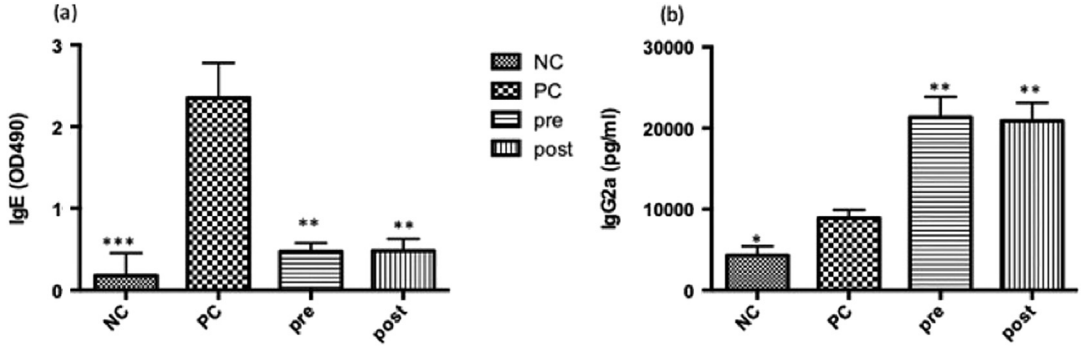

Figure 3. LGG treatment in ovalbumin-specific antibodies in serum in animal model. Serum in OVA-specific-IgE and OVA-specific-IgG2a concentrations was obtained from all groups Fig. 3(a) The OVA-specific-IgE in each group. Fig. 3(b) The OVA-specific-IgG2a expression in serum in each group. The statistical analysis compared PC with NC and treated with LGG, it is represented as: *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

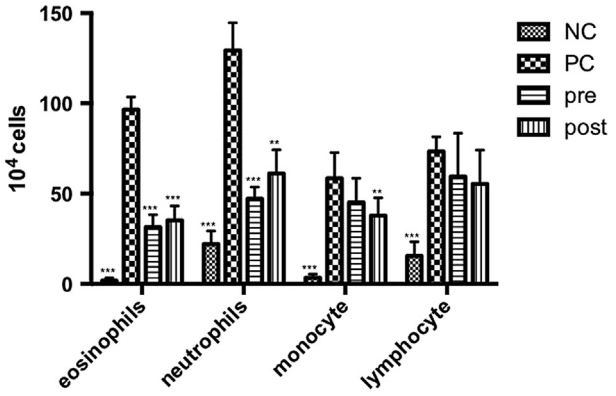

Figure 4. LGG treatment in infiltrating cells of the lungs in animal model. All cell counts were obtained from all groups. The total cells and inflammatory cells were counted ($x10^4$) from the BALF in millimeters by morphometric evaluations of cytospin preparations. The statistical analysis compared PC and mice treated with LGG, it is represented as: *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

LGG affects collagen deposition in lung tissue in animal model

To evaluated the effects of the LGG treatment on the OVA-sensitized/challenged mice with collagen deposition in the lung were used histological staining with Masson's trichrome stain (Fig. 7). The positive group which sensitized/challenged with OVA in the allergic asthma models (Fig. 7B) had more collagen deposition. In the pre-treated and post-treated LGG groups (Fig. 7C and D) collagen deposition decreased significantly.

To quantify the histological and immune stained images, we randomly selected 10 fields for each sample, and used Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA) for morphometric analysis (7G).

LGG affects T-bet, GATA3, RORrt and Foxp3 *in lung tissue in animal model*

The effects of the LGG treatment on the OVA-sensitized/challenged mice with T-bet, GATA3, RORrt and Foxp3 in the lung were evaluated through PCR (Fig. 8). The mice sensitized/challenged with OVA in the allergic chronic asthma models had more GATA3 and RORrt expression. In the treated LGG groups the T-bet and Foxp3 increased significantly and down regulated GATA3 and RORrt.

Discussion

In this study, OVA-induced airway inflammation in a mouse model in chronic stage was not only used to explain the role of OVA in mediating airway inflammation, inflammatory cell infiltration, inflammatory cytokine expression and airway remodeling, but also to explain the effect of the probiotic LGG in airway inflammation in chronic stage.

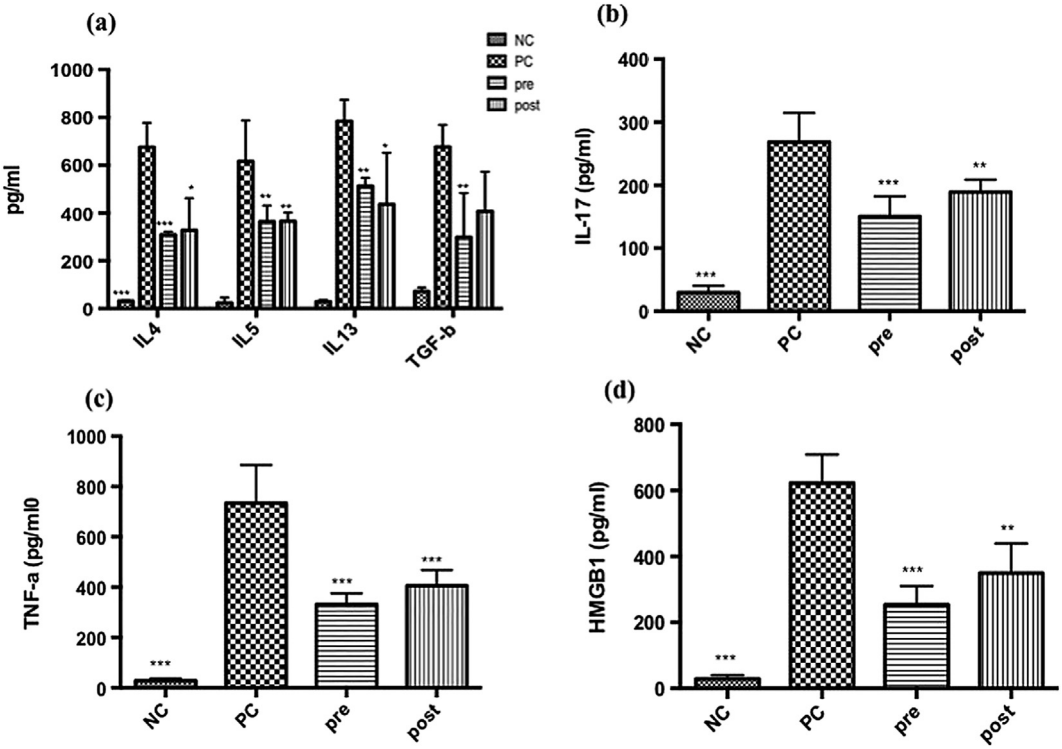

Figure 5. LGG affects inflammation cytokines in BALF in animal model. Th2 cytokines, TGF-β, IL-17, TNF-α and HMGB1concentrations in the BALF were obtained from all groups. Fig. 5 (a) the Th2 cytokines and TGF-β detected in the BALF. Fig. 5 (b), (c) and (d) the IL-17, TNF-α and HMGB1concentrations in the BALF. The statistical analysis compared OVA-treated mice (PC group) and mice treated with LGG, it is represented as: *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$.

Table 1 The effects of the LGG treatment on Th1 cytokines expression in the BALF. Data points represent the median ± IQR of the individual mouse groups.

Group/cytokine	NC (n = 6)	PC (n = 8)	pre-LGG (n = 8)	post-LGG (n = 8)
IL-2 (pg/ml)	22.00 ± 1.24*	68.53 ± 9.53	242.50 ± 37.25***	302.45 ± 45.7***
IL-12 (pg/ml)	47.74 ± 17.66*	104.80 ± 24.33	337.54 ± 32.74***	277.75 ± 31.45**
IFN-r (pg/ml)	54.19 ± 21.64	79.65 ± 7.89	327.29 ± 53.55***	368.82 ± 77.69***

BALF was obtained from the PC group, pre or post-LGG groups. The statistical analyses compared PC group Represented as: *p value $\leq$ 0.05, **p value $\leq$ 0.01, ***p value $\leq$ 0.001.

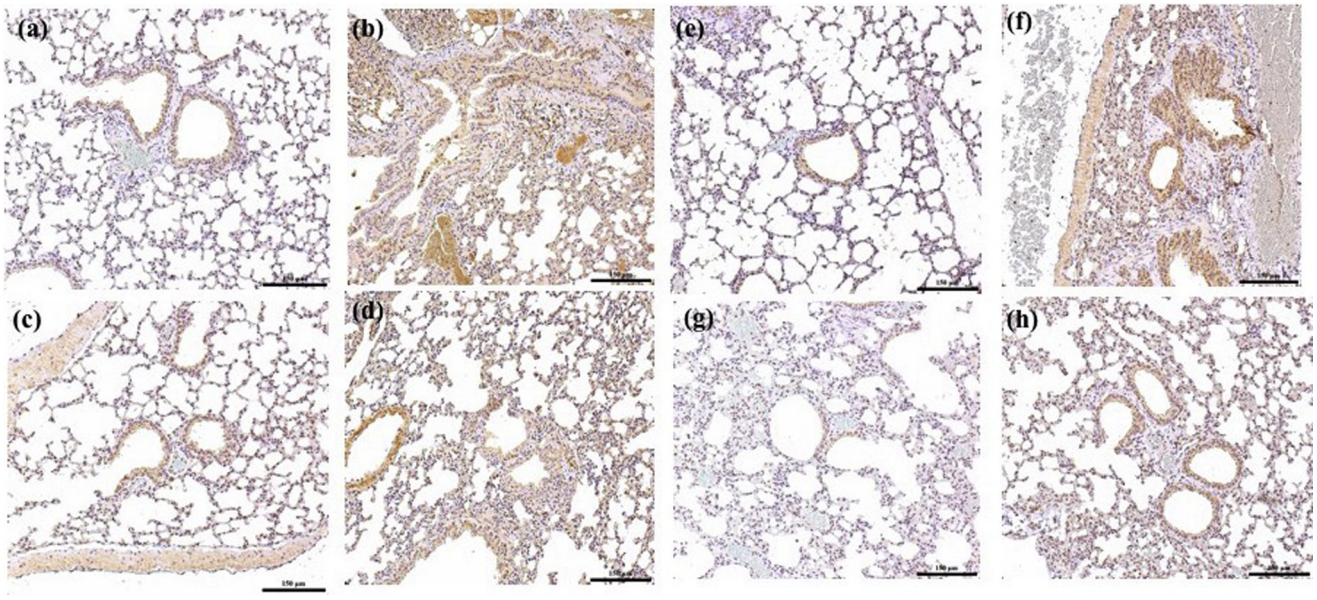

Figure 6. LGG affects IL-17 and TNF-α expression in animal model. IL-17 and TNF-α were evaluated through histological staining with *IHC* (Fig. 6). The mice in the OVA-sensitized/challenged (Fig. 6b; 6f) group had severe IL17 and TNF-α expression compared with NC (Fig. 6 a, c). After being fed LGG, there was significantly less IL17 and TNF-α expression in not only the pre-groups (Fig. 5c and d) but also in the post-groups (Fig. 6g and h). The analysis was used Image pro plus 5.

Acute animal models have shown that oral administration of LGG during allergen sensitization induces a Th1-predominant allergen-specific immune response, such as increased IFN-r, down regulated Th2 cytokines and decreased MMP9 expression in lung tissue in mice with allergic asthma.[18] In a recent study, we developed a mouse model of airway inflammation and remodeling as a representation of patients with chronic asthma: eosinophil and neutrophil infiltration in the lung, release of inflammatory cytokines, goblet cell hyperplasia in the airway and increased collagen deposition in the lung. We demonstrated that oral LGG in an animal model of allergic chronic stage could alleviate airway remodeling and airway inflammation. According to the results, oral LGG could suppress the level of IgE in the serum and also reduce the Th2 cytokines (IL-4, IL-5 and IL-13) and IL-17, TNF-α and HMGB1. In RNA levels, LGG could also decrease GATA3 and RORrt expression. Moreover, oral LGG could reduce inflammatory cell infiltration in the lung, especially eosinophils and neutrophils in pre-treated and post-treated LGG groups in chronic stage.

Previous studies have shown that LGG could induce a Th1- predominant response, balance the Th1/Th2 immune response and inhibit allergen-induced IgE and Th2 cytokines during allergen sensitization[20,21] and newer studies also report that LGG could modulate immunity and reduce the chance of inflammation.[22–24] These studies support that effect of LGG in allergic diseases.

Previous studies report that TNF could increase in serum and tissue levels with inflammatory and infectious conditions but is not detected in healthy individuals. There are a wide range of cells that can produce TNF such as neutrophils, T and B lymphocytes, mast cells, natural killer … etc.[25,26] However, some studies have reported that TNF may play a role in airway inflammation, remodeling, neutrophil infiltration and bronchial hyperresponsiveness.[26–28] Moreover, a previous study also reported that an important marker of acute and chronic inflammation was TNF-α.[25] In our study, oral LGG decreased TNF- α significantly and reduced neutrophil, eosinophil infiltration in the airway in pre-treated and post-treated LGG groups. These results confirmed that LGG has the capability for anti-inflammation in allergy inflammation. However, a recent study reported that the LGG inhibition of TNF-α product was by means of the p38 MAPK pathway.[29] Furthermore study is necessary to determine whether LGG works through this path to improve asthma.

Moreover, regulatory T cells and cytokine TGF-β have been a focal point of considerable investigation as both a

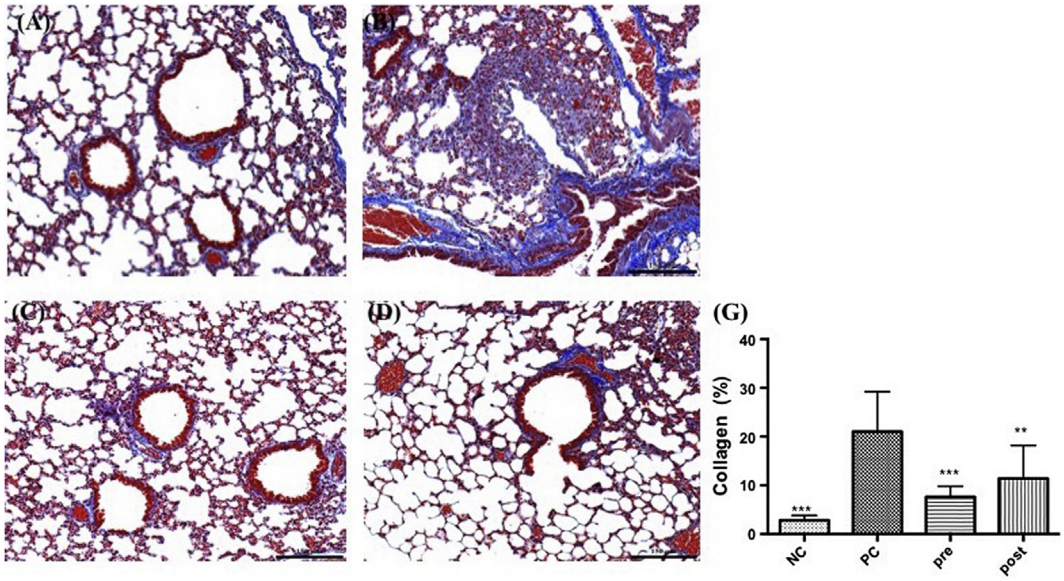

Figure 7. LGG affects collagen deposition in lung tissue in animal model. Lung tissues for detection of airway remodeling and collagen deposition were obtained on day 76. Fig. 7(A) the NC group 7(B) the OVA sensitized/challenged mice (PC), 7(C) and 7(D) the groups pre- or post-treated with LGG which were stained with **Masson's trichrome**. Fig. 7(G) collagen deposition analyzed statistically. The analysis was used Image pro plus 5 and Prism 6. The statistical analysis compared OVA-treated mice (PC group) and mice treated with LGG, it is represented as: *p $<$ 0.05; **p $<$ 0.001; ***p $<$ 0.0001.

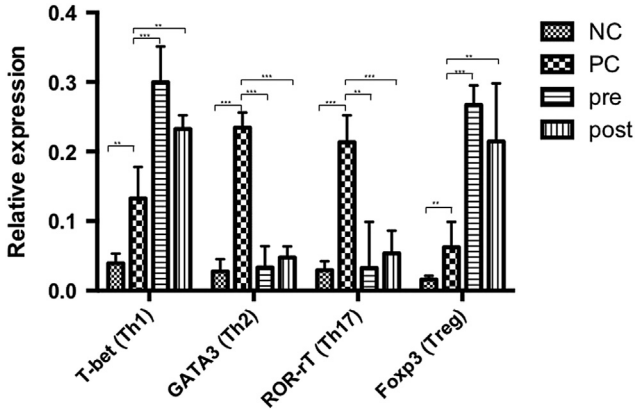

Figure 8. LGG affects T-bet, GATA3, RORrt and Foxp3 in lung tissue in animal model. T-bet, GATA3, ROR-rt and Foxp3 RNA level in lung tissues. The RNA levels were detected in lung tissue, OVA sensitized/challenged mice (PC) or the mice treated with LGG. The statistical analysis compared OVA-treated mice (PC group) and mice treated with LGG, it is represented as: *p $<$ 0.05; **p $<$ 0.001; ***p $<$ 0.0001.

mediator and effector molecule in the Th2 driven immune cascade, and TGF-β is believed to play an important role in most of the cellular biological processes leading to airway remodeling.[30] In our earlier study[18] in which oral LGG was used during acute allergic stage, TGF-β was up-regulated in the pre-LGG group. In this study, oral LGG administered during chronic allergic stage decreased TGF-β expression in the BALF and RNA levels in the pre-LGG group. However, oral LGG in the post group also down-regulated TGF-β in serum and RNA levels but was not statistically significant in the BALF (Fig. 4).

Earlier studies have shown that different strains of bacteria have the ability to induce a discrete production of cytokines by myeloid DCs, which in turn would differently polarize the subsequent adaptive immune response toward specific T helper cell subsets (Th1, Th2, Th17) or even T regulatory cells. These observations, especially the ability to polarize a T cell response, support probiotics as interesting candidates in the treatment of immune-mediated disease.[31,32] Moreover, a previous study explains that LGG could balance Th17 and Treg cells or inhibit the IL-17 response and it was suggested LGG be used in place of T cell polarization toward Th17 [32, 33]. Moreover, some studies have also demonstrated significantly high levels of Th17 cells in patients with chronic severe asthma,[33] and Th2 cells can produce IL-17 in response to inflammatory stimuli and IL-17 producing Th2 cells induce severe allergic phenotypes in chronic mouse models.[33–35]

A previous study has shown that Foxp3 was expressed in lung epithelial cells and Foxp3 protected against cell infiltration, inflammatory cytokine release and goblet cell metaplasia in allergen-induced asthma models.[36] In this study, LGG groups had significantly increased Foxp3 expression in RNA levels from the lung tissue which may be one of the reasons LGG improves airway inflammation.

However, LGG not only suppressed Th17 (IL-17, RORrt), but also affected Th1 (T-bet), Th2 (GATA3) and Foxp3 (Treg), and LGG also significantly up-regulated T-bet and Foxp3 expression in RNA levels from the lung tissue. These results were similar with previous studies in which LGG regulated Treg and Th1/Th2 balance.[37–40] Because a result, LGG could be used as an anti-inflammation therapy.

Nevertheless Treg cells could exhibit Foxp3 and secrete TGF-β to regulate related immune responses. However,

Halwani R et al.[30] have shown that almost all structural immune cells, and inflammatory cells recruited to the airways are able to express and secrete TGF-β in the lungs, leading a large increase in TGF-β when asthma is worsening, and TGF-β is also involved a number processes in collagen synthesis, deposition and remodeling of the new extracellular matrix.[41] Moreover, some studies had shown IL-6 suppresses Foxp3 expression and iTreg cell generation to release the suppression of active immunity by TGF-β cells and induces Th17 cell differentiation, resulting in neutrophilic asthma. That is combination of IL-6 and TGF-β could suppress FoxP3 expression and enhances IL-17 production.[42] Therefore, in our studies, we demonstrated that whether LGG could also play beneficial in airway. In asthma modes of acute stage, LGG could increase highest Th1 cytokines and TGF-β, and decrease Th2 cytokines. However, compared with chronic stage LGG were decrease Th2 cytokines and increase high Th1 cytokines and Treg Foxp3 expression, but the TGF-β was small rise.

Moreover, the probiotic LGG had the ability to decrease collagen deposition in the lung tissue in our study. A previous study also reported that LGG decreases collagen deposition in chronic liver disease.[43]

In conclusion, we developed a chronic asthma mouse model of OVA-sensitizations and discovered that LGG might play an important role in reversing the chronic stage of airway inflammation and airway remodeling. LGG could be an alternative agent to prevent and treat allergic diseases.

Conflicts of interest

The study had no conflicts of interest.

Acknowledgements

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