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The effects of *Lactobacillus crispatus* on alleviation of allergic asthma

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Abstract

The effects of *Lactobacillus crispatus* on alleviation of allergic asthma

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Development of asthma is affected by the microbiota modulating immune and metabolic functions of host. Epidemiological studies have shown more frequent incidence of asthma in infants delivered by Caesarean section than in ones delivered by vaginal birth. In this study, we investigated the effect of representative vaginal lactobacilli strain, *Lactobacillus crispatus* (LC), on alleviating asthmatic responses induced by ovalbumin (OVA) and house dust mite (HDM) in mice. Daily oral administration of LC isolate from a healthy

Korean woman initiated seven days prior to day 0 (approximately 2.0 $\times 10^9$ CFU/mouse/day) and continued until the end of the experiments. In OVA-induced asthma model, mice were sensitized with OVA and alum through intraperitoneal injection on day 0 and 14, followed by OVA challenge through intranasal injection on day 18 - 20. In HDM-induced asthma model, mice were challenged with HDM on day 0, day 7-11 through intratracheal injection. In both studies, histological analysis of lung tissue revealed significantly reduced eosinophil infiltration around bronchioles in LC-administered group compared to control group. The LC-administered group displayed a significantly lower airway hyperresponsiveness level compared to HDM-WT group. Flow cytometric analysis revealed that LC treatment successfully reduced OVA/HDM-induced IL-5 and IL-13 in lungs and IL-5^+/13^+ $CD4^+$ T lymphocytes in an IL-4-independent manner. We observed a significant elevation in IL-5^+/13^+ innate lymphoid cell (ILC) population induced by OVA, but not by HDM, which was inhibited by LC treatment. We also observed a significantly reduced dendritic cells in LC-treatment group in HDM-induced asthma model. In order to confirm the underlying mechanism of anti-inflammatory action of LC, we purified and treated peptidoglycan (PGN) from LC culture to papain-stimulated murine epithelial cells *in vitro*. Both pasteurized LC pellets and LC PGN significantly reduced *il-33* expression in murine lung epithelial cells. Taken together, LC successfully alleviated

allergic asthmatic responses induced by OVA and HDM in mice and can be applied to a microbiome-based therapy for asthma.

Keywords: *Lactobacillus crispatus*, Probiotics, Allergic asthma, Ovalbumin (OVA), House-dust mite (HDM), Airway hyperresponsiveness (AHR), Innate lymphoid cells (ILC), Peptidoglycan

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I. Introduction

Asthma is a chronic inflammatory pulmonary disease characterized by various and recurrent symptoms which can display a wide extension of severity. Symptoms of asthma include wheezing, coughing, reversible airflow obstruction and bronchospasm. Worldwide, about 339 million people suffer from asthma (1), and 13.8 million disability-adjusted life years (DALYs) are estimated to be lost annually due to asthma (2). Due to its high incidence, prevalence and chronic nature, asthma is one of the major sources of global economic burden (2-4).

Allergic asthma is the most common form of asthma and often characterized by eosinophilic airway inflammation (5-7). Aeroallergens derived from house-dust mites (HDM), pollen, or dust particles trigger allergic reactions when inhaled (8). Both innate and adaptive immune responses are reported to be the main drivers of the pathogenesis of the disease; T_H2 response is traditionally believed to be responsible for pathogenesis of asthma, while there is growing evidence that innate lymphoid cells (ILC) and the related responses are also involved (9-12). The pathogenesis of allergic asthma is characterized by Interleukin (IL)-5 and IL-13 dependent lung eosinophilia, airway hyperresponsiveness (AHR), goblet cell hyperplasia, and IL-4 dependent Immunoglobulin E (IgE) elevation in serum (7, 9-11, 13-16). Popular treatments for allergic asthma include inhaled steroids such as corticosteroids (17-19), monoclonal anti-IgE antibody (20-

23) and bronchodilators (24, 25), but their efficacy is restricted to minimizing the symptoms without curing the disease (16, 19). These treatments are not 100% effective to all patients (26, 27) and possess potential side effects (28-31). Consequently, there is an increasing demand for novel treatment strategies not restricted to reducing allergic responses, but rather focus on attenuating the earlier stages of pathogenesis (16).

Probiotics, also referred to as live biotherapeutic products (LBP), are novel approaches for allergic diseases (32, 33). Human microbiome plays a crucial role in shaping and developing aspects of host immune responses (34). The bacterial colonization in human is a dynamic process which begins as soon as the time of birth; the delivery mode has been identified as a significant factor determining the initial colonization and composition of the neonatal microbiome (35, 36). There is growing evidence showing higher risk of developing asthma in children who had been delivered by Caesarean section (C-section) (37-39). Contrast to C-section, neonates delivered by a vaginal delivery contact to mothers' vaginal microbiome, which is predominantly colonized by bacteria from genus *Lactobacillus*. *Lactobacillus* species are one of the most recognized probiotics which produce lactic acid and form biofilms in the vaginal and gut microbiota. Infants delivered by C-section experience dysbiosis of the gut microbiota, which may negatively affect immune development in infants and increase their susceptibility to chronic diseases (40, 41). While the functional relationship between the human microbiota and

asthma is still poorly understood, several studies have shown that oral delivery of intestinal *Lactobacillus* species could reduce asthmatic symptoms in both animal models and human studies (42-44). In a study of atopic dermatitis, *Lactobacillus crispatus* (LC) showed the highest anti-inflammatory capacity *in vitro* among various vaginal-derived *Lactobacillus* spp. based on the prohibiting effect on production of allergy-mediating cytokines, such as IL-4 and IL-5 (45). Mice treated with LC revealed significantly decreased atopic dermatitis inflammatory scores (45). Multiple epidemiological studies suggest the sequential development of allergic diseases, that is, atopic dermatitis in infancy, and allergic rhinitis and/or asthma in later stages of life caused by allergen exposure through the epidermis (46). Considering its high anti-inflammatory capacity in atopic dermatitis studies, vagina-derived *Lactobacillus crispatus* may be a promising candidate of novel therapeutic potential for asthma.

In this study, we aimed to demonstrate the efficacy of LC on alleviating allergic asthma using ovalbumin (OVA) and house-dust mite (HDM) as allergens. The study also investigated the underlying mechanism of reduced lung inflammation due to LC administration. Daily oral dosing with LC abrogated the disease pathogenesis by decreasing infiltration of eosinophils in lungs. LC reduced both innate and adaptive immune responses. With its potential to modulate various immune responses, LC can be further

tested to be a novel therapeutic for allergic asthma.

II. Materials and Methods

1. Bacteria culture and preparation

Lactobacillus crispatus KBL693 (LC) was isolated from vagina of a healthy Korean woman. Frozen stock of LC was thawed and inoculated on Man-Rogosa-Sharpe (MRS) (5.5%; w/v, BD Difco) agar containing L-cysteine (0.05%; w/v; Sigma-aldrich) and incubated at 37℃ for 48 hours in anaerobic condition. An isolated single colony of LC was inoculated in MRS broth media and incubated at 37℃ in anaerobic condition for 16 hours. 2 times of subculture (1% v/v) of incubation subsequently followed for activation of bacterial culture. The culture was harvested by centrifugation at 4,000 rpm for 10 minutes at 4℃. Bacterial pellets were washed with 1% Phosphate Buffer Saline (PBS). Collected pellets were resuspended in PBS to 1.0×10^{10} colony forming units (CFU)/ml.

2. Animals

6 weeks old female BALB/c mice were purchased from Orient Bio Inc. (Seongnam, Republic of Korea). Upon arrival, mice were randomly allocated to individually ventilated cages that have available portable water and food ad libitum. Mice were kept in a semi specific pathogen-free facility at Seoul National University (Seoul, Republic of Korea). Mice were acclimated to the facility for 7 days; mice were 8-week-old at the initiation of the study. All experiments were in accordance with the Seoul National University Institutional Animal Care and Use Committee (IACUC) guidelines (SNU-170116-4-5). Mice were sacrificed at experimental end by isofurane (Hana Pharm Co., Ltd., Republic of Korea) overdose and cervical dislocation.

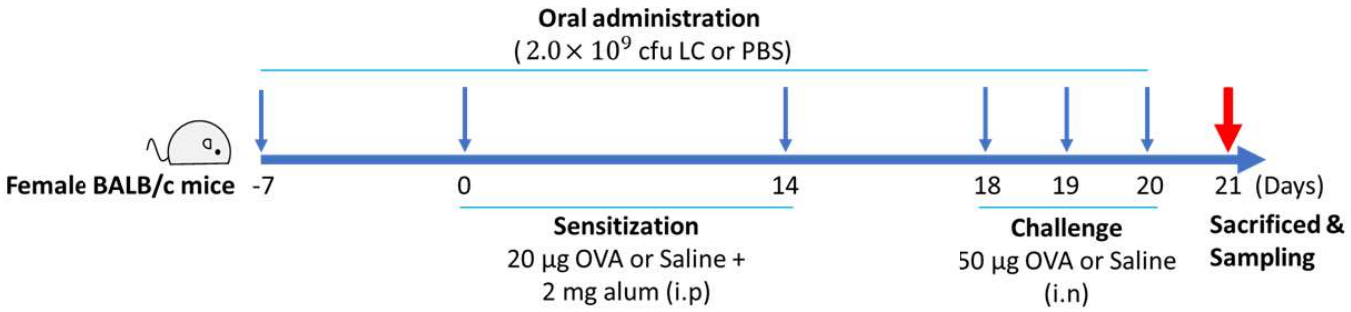
3. Ovalbumin (OVA) sensitization, challenge and treatment (Fig 1)

Mice were divided into three groups: PBS-challenged PBS treatment group (Wild-type; WT), OVA-challenged PBS treatment group (OVA-WT) and OVA-challenged LC treatment group (OVA-LC). Each mouse was received daily oral gavage of 200 μl (2.0×10^9 cfu/ml) of LC or 200 μl PBS from day -7 to day 20. General sensitization was done through intraperitoneal injection of 20 μg ovalbumin (OVA) (Sigma Aldrich) absorbed in 2 μg of Imject® Alum (Thermo Fisher Scientific) on days 0 and 14. On days 18, 19 and 20, mice were challenged intranasally with 50 μg of OVA.

4. House-dust mite (HDM) sensitization, challenge and treatment (Fig 1)

Mice were divided into three groups: PBS-challenged PBS treatment group (WT), HDM-challenged PBS treatment group (HDM-WT) and HDM-challenged LC treatment group (HDM-LC). Each mouse was received daily oral gavage of 200 μl (2.0×10^9 cfu/ml) of LC or 200 μl PBS from day -7 to day 11. General sensitization was done through intranasal injection of 10 μg of HDM (Greer) resuspended in PBS on day 0. On days 7 to 11, mice were challenged intranasally with 10 μg of HDM.

A Ovalbumin (OVA)-induced allergic asthma model



B House dust mite (HDM)-induced allergic asthma model

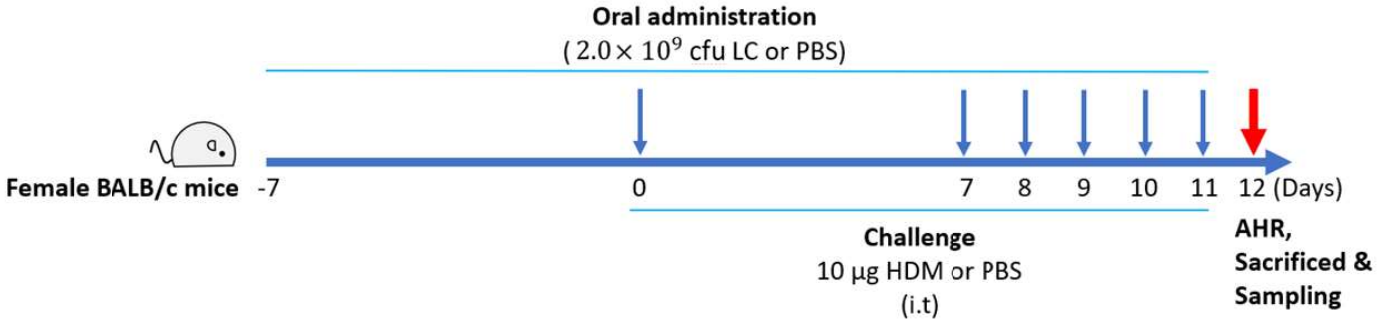


Figure 1. Experimental scheme of OVA and HDM-induced allergic asthma models.

(A) Experimental scheme of 21-day OVA-induced allergic asthma model. BALB/c mice were sensitized with Ovalbumin (OVA) in Imject® alum adjuvant by intraperitoneal injection on days 0 and 14. Intranasal challenge with OVA followed on days 18, 19 and 20. Animals were dosed by oral gavage with LC daily for four consecutive weeks; LC treatment began a week prior to day 0. On day 21, animals were sacrificed followed by collection of blood by cardiac puncture, and lung tissue for further analysis. During the study, there was no morbidity or mortality observed in any treatment groups.

(B) Experimental scheme of 12-day HDM-induced allergic asthma model. Oral treatment of either PBS of LC was initiated 7 days prior to day 0. BALB/c mice were sensitized and challenged with house-dust mite (HDM) by intranasal injection on days 0, 7 to 11. Daily oral treatment of LC began a week prior to day 0. animals were sacrificed followed by collection of blood by cardiac puncture, and lung tissue for further analysis. During the study, there was no morbidity or mortality observed in any treatment groups.

5. Isolation of lung, lung histology and inflammation scoring

Lungs were used for histopathological examination. 24 hours after OVA challenge, right middle lobes were removed by dissection and fixed in formalin (Sigma Aldrich). Lung tissue sections were stained with hematoxylin and eosin stain (H&E) for general morphology and identification of eosinophilia.

6. Determination of serum IgE.

Blood was collected by cardiac puncture immediately after mice were anaesthetised by isofurane (Hana Pharm Co., Ltd., Republic of Korea). The blood was coagulated for 30 minutes at room temperature and centrifuged subsequently at 13,000 g for 5 minutes. Collected serum was stored at -80°C until further analysis. LEGEND MAX™ Mouse OVA Specific IgE ELISA Kit (Biolegend®, San Diego, CA) was used to quantify the IgE levels in serum. The assay was performed according to the manufacturer's instructions.

7. RNA preparation and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated from lung tissue using easy-spin™ [DNA free] Total RNA extraction Kit (iNtRON Biotechnology). Complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Synthesized cDNA was used to perform quantitative PCR with Power SYBR® Green Master Mix (Thermo Fisher Scientific) on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Primers sequences are shown in Table 1.

8. Airway Hyperresponsiveness (AHR)

Mice were anesthetized with 150mg/kg pentobarbital sodium. Tracheas were dissected, intubated with 18-gauge catheters and mechanically ventilated at a tidal volume of 0.2ml and a frequency of 140 breaths per min. Lung resistance(R_L) was measured with invasive BUXCO FinePointe Resistance and Compliance (BUXCO Electronics) in response to aerosolized acetyl-β-methylcholine chloride methacholine (10, 20, 40 mg/ml) (Sigma-Aldrich).

Table 1. PCR Primers

Target	Sequence (from 5' to 3')	
	Forward	**Reverse**
β-actin	AGA GGG AAA TCG TGC GTG AC	CAA TAG TGA TGA CCT GGC CGT
GAPDH	AGG TCG GTG TGA ACG GAT TTG	TGT AGA CCA TGT AGT TGA GGT CA
Siglec-F	CTG GCT ACG GAC GGT TAT TC	GGA ATT GGG GTA CTG GAC TTG
T-bet	TTC CCA TTC CTG TCC TTC AC	CCA CAT CCA CAA ACA TCC TG
GATA3	GGA AAC TCC GTC AGG GCT A	AGA GAT CCG TGC AGC AGA G
FoxP3	CCT GCC TTG GTA CAT TCG TG	TGT TGT GGG TGA GTG CTT TG
RORγT	TGA GGC CAT TCA GTA TGT GG	CTT CCA TTG CTC CTG CTT TC
IL-33	TGA GAC TCC GTT CTG GCC T	CTC TTC ATG CTT GGT ACC CG
TSLP	GAG AGC AAT GAC GAT GAG GA	GAA AGC CTT GTC ACC GCT GT
TLR2	CCA AGA GGA AGC CCA AGA AAG	AGG CAT CAT AGC AAA CGT CCC
TLR4	AGC AGG TGG AAT TGT ATC GCC	CCC ATT CCA GGT AGG TGT TTC T

9. Flow cytometry

Murine lungs were chopped and incubated in RPMI1640 media with 1mg/ml type IV collagenase (Wortihngton BioChem) for at least 1hr 30min at 37°C. RBC were lysed using RBC lysis buffer (Biolegend). Lung single cells were stained with following fluorochrome-conjugated antibodies: (A) surface staining: Anti-CD45 (30-F11), Anti-CD3e (145-2C11), anti-CD11c (HL3), anti-CD11b (M1/70), anti-CD19 (ID3), anti-CD49b (DX5), anti-FcεRIα (MAR-1), anti-CD90,2 (30-H12), anti-F4/80 (BM8) and anti-Ly6G (1A8) (Biolegend). Anti-SiglecF (E50-2440) was purchased from BD Bioscience, (B) Intracellular staining: Anti-IL5 (TRFK5) and anti-IL17A (TC11-18H10.1) (Biolegend), Anti-IL13 (eBio13A) (ThermoFisher Scientific), (C) intracellular staining, Fixation/Permeabilization Solution Kit with BD GolgiPlug (BD Biosciences) was used following the manufacturer's protocol. Flow cytometry was performed using LSRFortessa™ X-20 (BD biosciences) and analyzed by FlowJo (V10.2) software.

10. Peptidoglycan extraction

Peptidoglycan (PGN) of LC was obtained using the method of Desmarais *et al*. (47) with minor modifications. LC cultured for 14 hours in 37°C was boiled in 6% Sodium Dodecyl Sulfate (SDS; Thermofisher Scientific) solution for 3 hours and left overnight. Ultrapure water was used to wash out remaining SDS from the mixture. Using ultracentrifugation at 400,000 x g for 20 minutes at room temperature, large PG polymers were pelleted and thereby separated from other cellular components. After confirming the absence of SDS residue in the sample, the sample was resuspended with 10 mM Tris-HCl (pH7.2) + 0.06% w/v NaCl, following the addition of 1 mg/ml activated Pronase E (Sigma-aldrich). After 2 hours of incubation at 60°C, 6% SDS was added to stop the Pronase E activity. The sample was washed by ultracentrifugation at 400,000 x g for 20 minutes at room temperature using ultrapure water until SDS is fully removed. On the last washing step, the sample was resuspended in 50 mM sodium phosphate buffer (pH4.9) and 1 mg/ml muramidase (Sigma-aldrich) was added. The sample was incubated overnight at 37°C. The sample was boiled to 100°C to stop the muramidase activity. After centrifugation at 16,000 x g at room temperature for 10 minutes, the supernatant was collected. The supernatant was lyophilized and collected PG was resuspended in ultrapure water to 1 mg/ml.

11. Lung epithelial cell and allergen

Murine lung epithelial cells (MLE-12 ATCC® CRL-2110™) were obtained from the American Type Culture Collection and cultured in DMEM/F-12 medium (ATCC) with following supplements: 5 μg/ml insulin, 10 μg/ml apo-transferrin, 30nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol, 2 mM L-glutamine, 10mM HEPES, and 10% fetal bovine serum. Papain (Sigma-Aldrich, St Louis, MO, USA) was reconstituted with sterile PBS to 1 mg/mL and stored at -20°C. Cells were treated with papain (0.1 - 0.25 mg/mL). Following papain stimulation, pasteurized LC pellets (1:10 ratio) or LC peptidoglycan (100 μg/ml) was treated. LC pellets were pasteurized at 70°C for 30 minutes before treatment. After 9 hours, the cells were collected and lysed. mRNA was extracted using easy-spin™ [DNA free] Total RNA extraction Kit (iNtRON Biotechnology) and complementary DNA (cDNA) was synthesized subsequently using Topscript™ RT DryMIX (enzynomics).

12. Statistical analysis

Differences between each group were compared using unpaired Student's t-test, as appropriate, or one-way analysis of variance (ANOVA) (version 8.0, GraphPad software, San Diego, CA). All values were expressed as means ± standard error of the mean (SEM). Statistical difference was accepted at $p<0.05$.

III. Results

1. LC treatment suppressed airway inflammation and eosinophilia in OVA-induced allergic asthma model.

OVA-induced eosinophilia was visually examined through histopathological studies. Histopathological studies were performed to assess the anti-inflammatory effect of LC in OVA-induced allergic asthma model. As expected, in OVA-challenged mice, eosinophil infiltration in the perivascular and peribronchial areas were observed (Fig. 2A). In LC-treated mice, a marked reduction in eosinophil infiltration in the perivascular and peribronchial areas was observed (Fig. 2A). The degree of inflammation was further analyzed using inflammation scoring; the OVA-WT mice scored significantly higher than the WT mice (Fig 2B), while the LC-treated mice showed a significantly lower inflammation score in comparison to WT mice (Fig 2B). The degree of eosinophil infiltration in peribronchial areas and bronchiolar epithelial thickness was considered for inflammation scoring (Fig 2C, 2D). In WT mice, no infiltrated eosinophils were found. We observed increased eosinophil counts in OVA-challenged mice (Fig. 2C). LC-treated mice showed a significant reduction in the number of eosinophils in comparison to WT mice (Fig 2C). Similarly, when we observed bronchiolar epithelial thickness, the OVA-WT mice had significantly thicker bronchiolar in comparison to WT mice (Fig 2D). Coinciding with the previous results, the

thickness of bronchiolar epithelial was significantly reduced comparing to OVA-WT mice (Fig 2D). The IgE level in serum was significantly increased in OVA-challenged mice (Fig 2E). Although insignificant, we observed a reducing trend in serum IgE level in LC-treated mice in comparison to OVA-WT mice (Fig 2E).

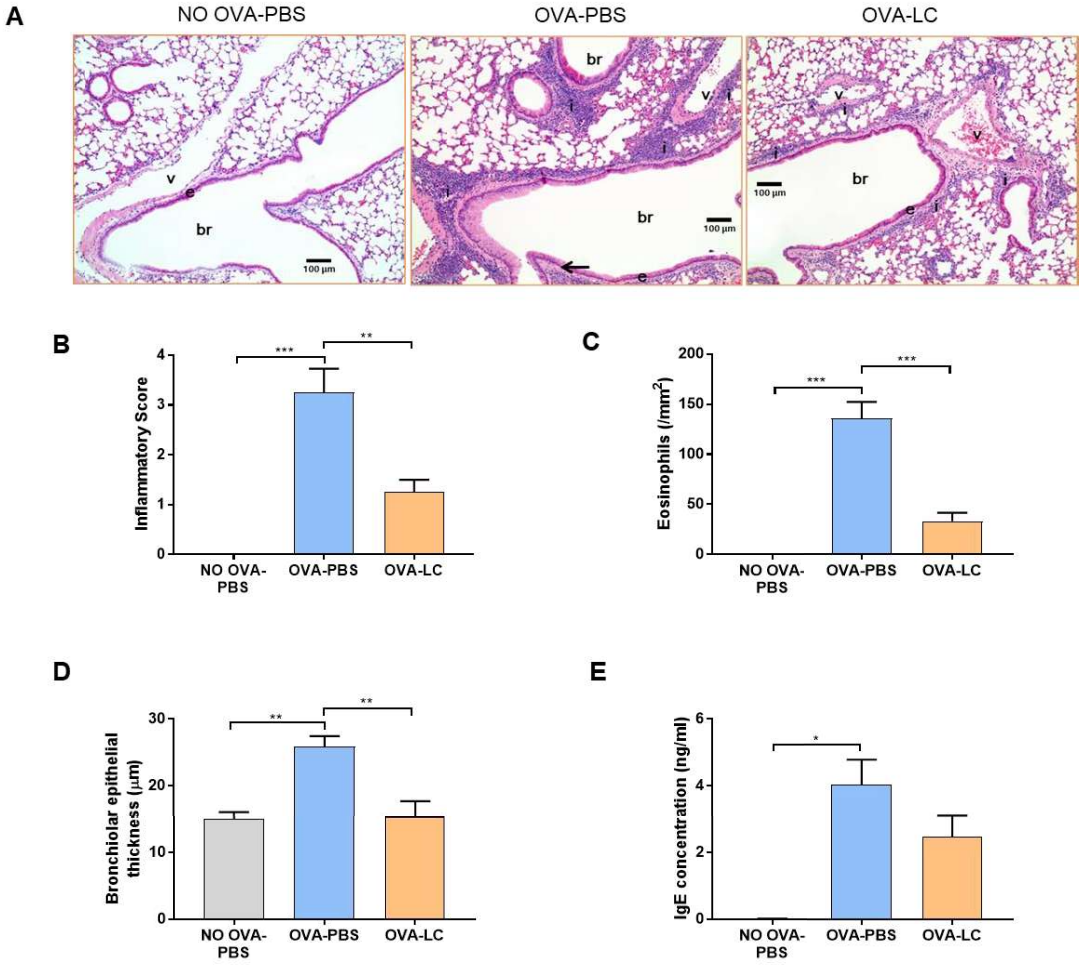

A
NO OVA-PBS
OVA-PBS
OVA-LC
br
v
e
i
100 µm
B
Inflammatory Score
0
1
2
3
4
***
**
NO OVA-PBS
OVA-PBS
OVA-LC
C
Eosinophils (/mm2)
0
50
100
150
200
***
***
NO OVA-PBS
OVA-PBS
OVA-LC
D
Bronchiolar epithelial thickness (µm)
0
10
20
30
**
**
NO OVA-PBS
OVA-PBS
OVA-LC
E
IgE concentration (ng/ml)
0
2
4
6
*
NO OVA-PBS
OVA-PBS
OVA-LC

Figure 2. Effect of LC on lung histopathology, inflammation, and IgE levels in serum of mice challenged with OVA.

Histopathological analysis of lungs of mice challenged with OVA, and treated with LC, with samples collected 24h after final challenge. (A) Representative scans of H&E stained formalin-fixed paraffin-embedded (FFPE) sections of right middle lobe of lungs. Note the inflammatory cell inflammation (i) around the vessels (v) and bronchioles (br) and thickness of smooth muscle (arrows); (B) Inflammatory score; (C) Number of eosinophils counted in 1 mm length of bronchioles in the lung from H&E stained FFPE sections; (D) Bronchiolar epithelial thickness (mm); (E) IgE levels in serum of mice. Capped bars illustrate the maximum data points within each treatment group. ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

2. LC treatment alleviated eosinophilia infiltration in lungs of OVA-challenged mice.

Using flow cytometry, we examined CD45+^{high} immune cell populations in lung which are known as key markers of pathogenesis of allergic asthmatic inflammation. The populations of eosinophils and dendritic cells increased in both OVA-challenged groups (Fig 3A), while alveolar macrophages (AM) were significantly reduced by OVA challenge (Fig 3A). We did not observe any changes in neutrophil population in three experimental groups (Fig 3A). LC treatment group showed significantly reduced eosinophils in lung compared to OVA-WT mice (Fig 3A). We performed RT-PCR analysis to measure gene expression levels of transcription factors and a surface marker which could help determine the mechanism underlying alleviated eosinophilia in lung (Fig 3B). In OVA-challenged mice, the levels of *siglec-f*, *t-bet* and *foxp3* mRNA expression were enhanced in comparison with the WT mice; no difference in the levels of *gata-3* and *rorγt* was detected (Fig. 3B). In LC treatment group, we observed a significant reduction in the expression of *siglec-f*, the prominent biomarker of eosinophils, in comparison to OVA-WT (Fig 3B).

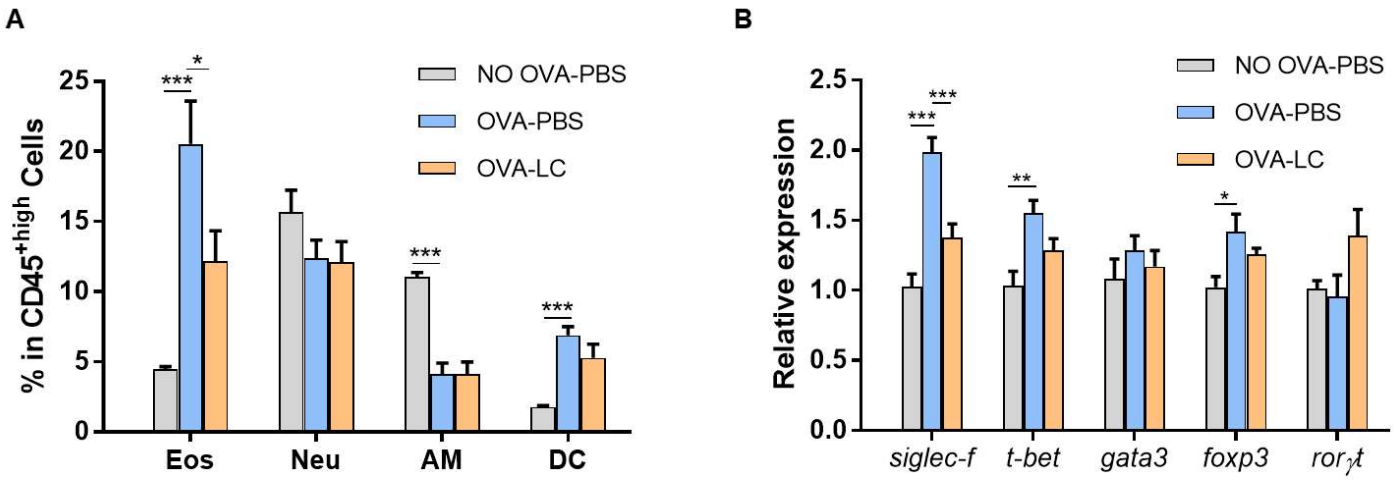

A
B
NO OVA-PBS
OVA-PBS
OVA-LC
% in CD45+high Cells
Relative expression
Eos
Neu
AM
DC
siglec-f
t-bet
gata3
foxp3
rorγt

Figure 3. Effect of LC on lung CD45+^{high} immune cell population and gene expression of surface markers and transcription factors in lungs after OVA challenge.

Lung immune cell population of mice challenged with OVA, and treated with LC, with samples collected 24h after final challenge. Flow cytometry was used to analyze CD45^{+high} immune cell population. RT-PCR was used to analyze gene expression. (A) CD45+^{high} immune cell subtype population of each treatment group shown in percentage; (B) relative gene expression levels of cell surface markers and transcription factors in lung. β-actin was used as the housekeeping gene. ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

3. LC treatment significantly reduced production of IL-5 and IL-13 in lungs of OVA-challenged mice.

We performed flow cytometry to further investigate the mechanisms underlying reduced population of lung eosinophils resulted by OVA challenge and LC treatment. The total amount of inflammatory cytokines in lung lymphocytes was examined. (Fig 4). Both PBS- and OVA-challenged mice displayed no difference in total amount of Interferon (IFN)-γ and IL-17A, proinflammatory cytokines which are produced by T_H1 CD4+ cells and T_H17 cells, respectively, in lung lymphocytes (Fig 4D, 4E). Similarly, no detectable differences in the total amount of IL-10 in lung lymphocytes among three experimental groups (Fig 4F). Flow cytometry results revealed increased total amounts of IL-5 and IL-13 in OVA-challenged mice in comparison to PBS-challenged mice (Fig 4A, 4B). IL-5 and IL-13 are type 2 cytokines which are used as biomarkers of allergic asthma. LC treated mice, however, showed significant reduction in total IL-5 and IL-13 in lung lymphocytes (Fig 4A, 4B). Notably, the OVA challenge did not change the total amount of IL-4 in lung lymphocytes despite IL-4 is one of the major T_H2 cytokines (Fig 4C).

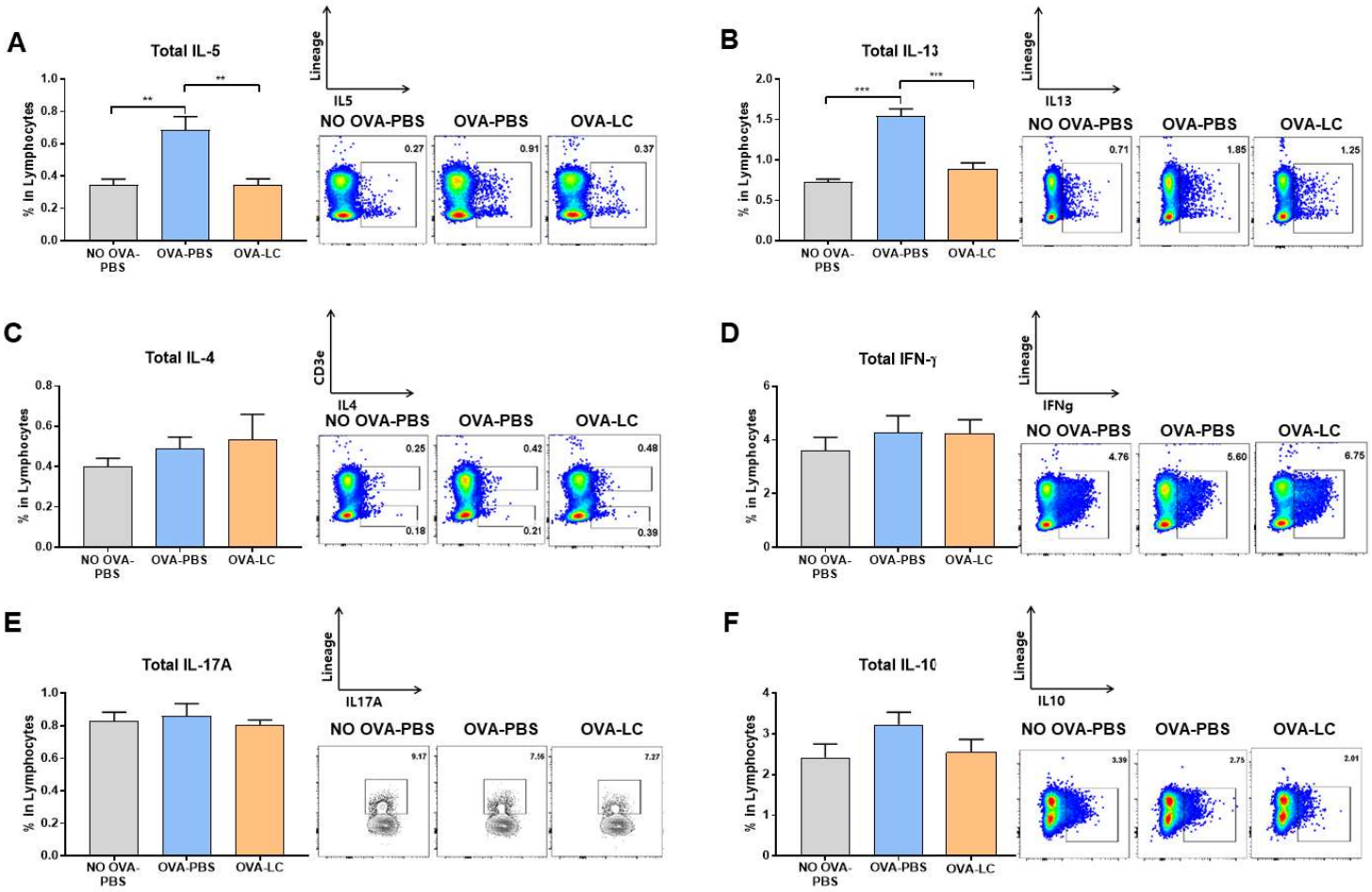

A
Total IL-5
% in Lymphocytes
NO OVA-PBS
OVA-PBS
OVA-LC
Lineage
IL5
0.27
0.91
0.37
B
Total IL-13
IL13
0.71
1.85
1.25
C
Total IL-4
CD3e
IL4
0.25
0.18
0.42
0.21
0.48
0.39
D
Total IFN-γ
IFNg
4.76
5.60
6.75
E
Total IL-17A
IL17A
9.17
7.16
7.27
F
Total IL-10
IL10
3.39
2.75
2.01

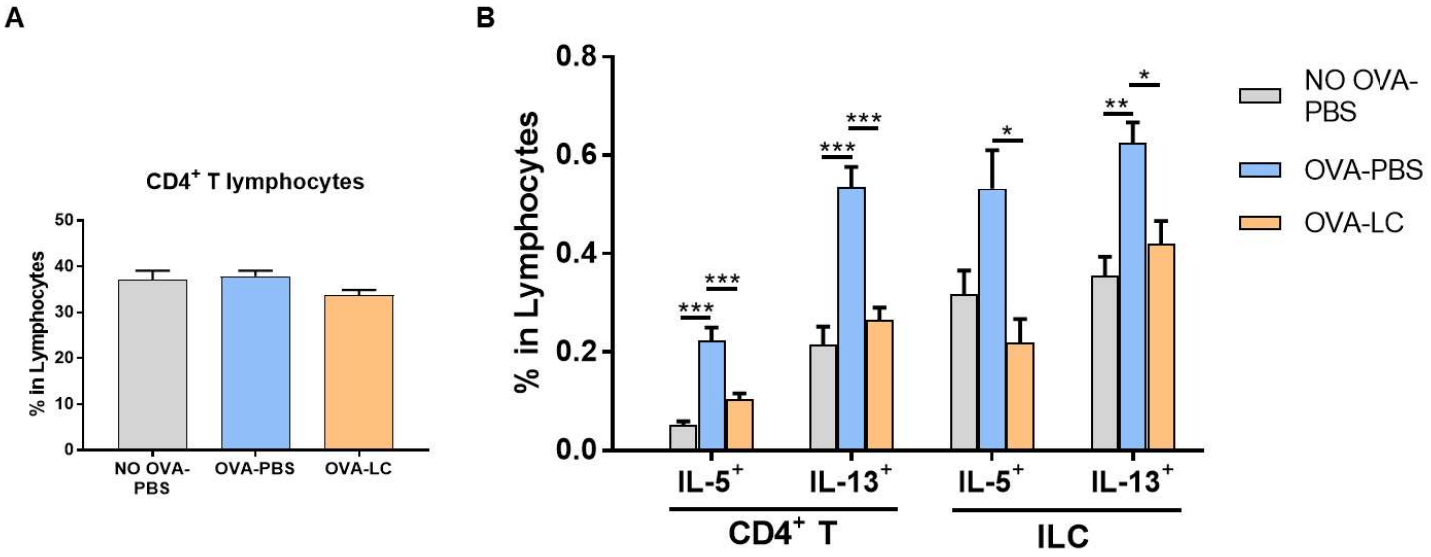
Figure 4. Impact of LC on inflammatory mediators in lungs of mice challenged with OVA.

Population of cells in lungs of mice challenged with OVA and treated with LC, with samples collected 24h after final challenge. Percentages of cytokine producing lymphocytes were determined using FACS. (A) % total IL-5 producing lymphocytes; (B) % total IL-13 producing lymphocytes; (C) % total IL-4 producing lymphocytes; (D) % total IFN-γ producing lymphocytes; (E) % total IL-17A producing lymphocytes; (F) % total IL-10 producing lymphocytes. ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

4. LC treatment significantly reduced IL-5 and IL-13 producing innate lymphoid cells in lungs of OVA-challenged mice.

We confirmed that the OVA-induced allergic asthma is mainly driven by T_H2 response, by inducing the production of IL-5 and IL-13, but not IL-4, and that LC treatment greatly reduced such induction. We then investigated which immune cell populations LC targeted. Flow cytometry analysis revealed no difference in $CD4^+$ T cell population among PBS-challenged groups and OVA-challenged groups (Fig 5A). When we investigated CD4+ subtype populations, however, we discovered that IL-5^+ and IL-13^+ $CD4^+$ lymphocytes greatly increased in OVA-challenged groups (Fig 5B). Next, we sought for different groups of cells in addition to T lymphocytes that can produce IL-5 and IL-13. Since the total amount of IL-4 remained unchanged after OVA challenge (Fig 4C), we hypothesized that T_H2 response may not be the only response that governs the immune responses when stimulated by OVA. Innate lymphoid cells (ILC) residing cells in mucosal and lymphoid tissues which produce a variety of inflammatory cytokines in response to environmental factors. We thus investigated how OVA-induced allergic asthma changed ILC populations in lungs (Fig 5B). A significant reduction in IL-5^+ ILCs in LC treatment group in comparison to OVA-WT group (Fig 5B).

LC treatment significantly reduced the population of IL-5^+ ILCs, even to a greater degree than PBS-challenged group (Fig 5B). OVA challenge significantly increased IL-13^+ ILCs, which was compromised by LC treatment (Fig 5B). Overall, the results showed that LC treatment lowers total amount of IL-5 and IL-13, and populations of IL-5 and IL-13 producing immune cells in lung in OVA-induced allergic asthma.


A
CD4+ T lymphocytes
% in Lymphocytes
50
40
30
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10
0
NO OVA-PBS
OVA-PBS
OVA-LC
B
% in Lymphocytes
0.8
0.6
0.4
0.2
0.0
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*
IL-5+
IL-13+
IL-5+
IL-13+
CD4+ T
ILC
NO OVA-PBS
OVA-PBS
OVA-LC

Figure 5. Impact of LC on population of CD4+ T lymphocytes and innate lymphoid cells (ILCs) in lungs of mice challenged with OVA.

Population of cells in lungs of mice challenged with OVA and treated with LC, with samples collected 24h after final challenge. Percentages of cytokine producing lymphocytes were determined using FACS. (A) % total CD4+ T lymphocytes; (B) IL-5+ and IL-13+ subtypes of CD4 T+ lymphocytes (left) and ILCs (right). ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

5. LC treatment alleviated eosinophilia infiltration and airway hyperresposiveness in lungs of HDM-challenged mice.

To confirm the efficacy of LC on allergic asthma and whether it works universally on other allergic asthma models, we constructed another allergic asthma model using house-dust mite (HDM) as allergen. The severity of disease and the anti-inflammatory effect of LC were measured through histopathological analysis and airway hyperresponsiveness (AHR).

Coinciding with the results of OVA-induced allergic asthma model, the whole scan image of HDM-WT mouse lungs revealed eosinophil infiltration in the perivascular and peribronchial areas (H&E staining), while there was no eosinophil infiltration observed in PBS-challenged mouse lungs (Fig 6A). In LC-treated mice, we observed a marked reduction in eosinophil infiltration in the perivascular and peribronchial areas (Fig 6A), showing much reduced inflammatory activity in lungs of LC-treated mice.

We assessed whether LC treatment has an effect on lung mechanics by measuring AHR to inhaled methacholine (Fig 6B). HDM-WT mice displayed AHR with an elevated response for the resistance of conducting airways (Rn) compared to WT mice. LC treatment mice, however, displayed significantly reduced resistance in comparison to HDM-WT mice. Taken

together, we confirmed the efficacy of LC on lowering asthmatic symptoms caused by HDM challenge.

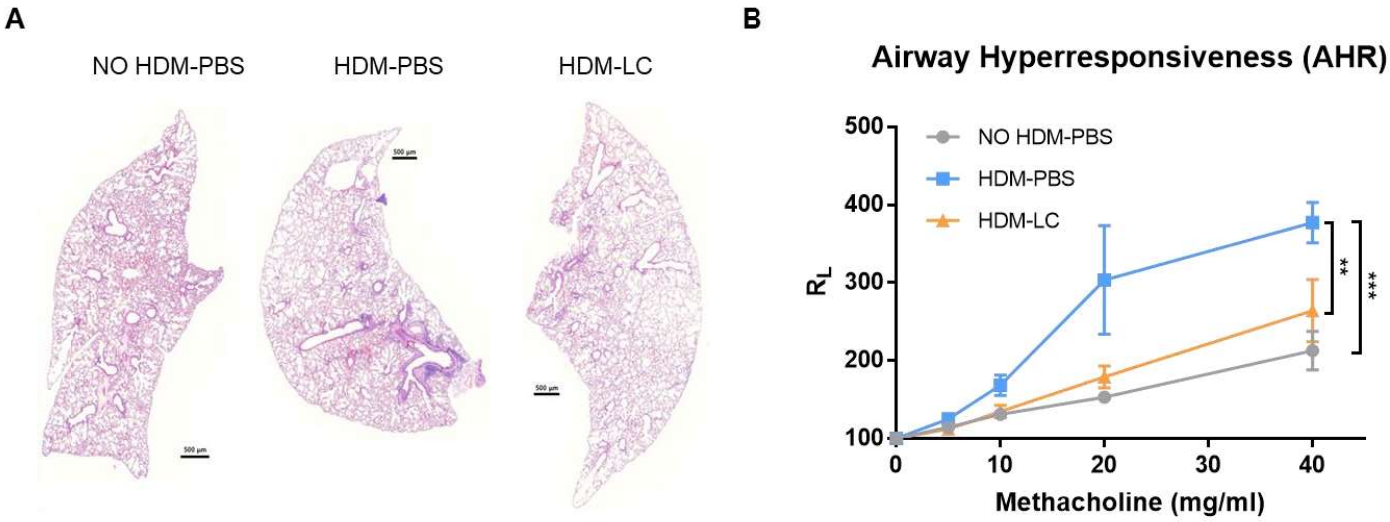

A
NO HDM-PBS
HDM-PBS
HDM-LC
B
Airway Hyperresponsiveness (AHR)
NO HDM-PBS
HDM-PBS
HDM-LC
R_L
500
400
300
200
100
0
10
20
30
40
Methacholine (mg/ml)
**
***

Figure 6. Effect of LC on lung histopathology and airway hyperresponsiveness (AHR) of mice challenged with HDM.

Lung/airway inflammation and AHR in HDM-WT and HDM-LC mice. (A) Histopathological analysis of lungs of mice challenged with HDM, and treated with LC, with samples collected 24h after final challenge. Whole scans of H&E stained formalin-fixed paraffin-embedded (FFPE) sections of right middle lobe of lungs. (B) AHR following methacholine challenge. Mice were exposed to increasing dose of aerosolized acetyl-β-methylcholine chloride methacholine (10-40 mg/ml) and lung resistance (R_L) was measured. ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

6. LC treatment significantly reduced eosinophilia and dendritic cell population in lungs of HDM-challenged mice.

Using flow cytometry, we examined CD45+^{high} immune cell populations in lung which are known as key markers of pathogenesis of allergic asthmatic inflammation. Similar to OVA model, when challenged with HDM, we observed increased populations of eosinophils and dendritic cells (DC) increased in both HDM-challenged groups (Fig 7A), and significantly reduced alveolar macrophages population in HDM-challenged groups (Fig 7A). Neutrophil population remained unchanged after HDM challenge. (Fig 7A). LC treatment group showed significantly reduced eosinophils in lung compared to HDM-WT mice (Fig 7A). Unlike to the result which we observed in Fig 3A, the population of DC was significantly lowered in HDM-LC mice in comparison to HDM-WT mice. Next, we measured the levels of inflammatory cytokines which are crucial for determining the pathogenesis of allergic asthma using flow cytometry (Fig 7B). As expected, HDM-WT mice revealed significantly elevated levels of the total IL-5 and IL-13 compared to WT mice (Fig 7B). Although insignificant, we observed a reducing trend of the total IL-5 and IL-13 in HDM-LC mice (Fig 7B).

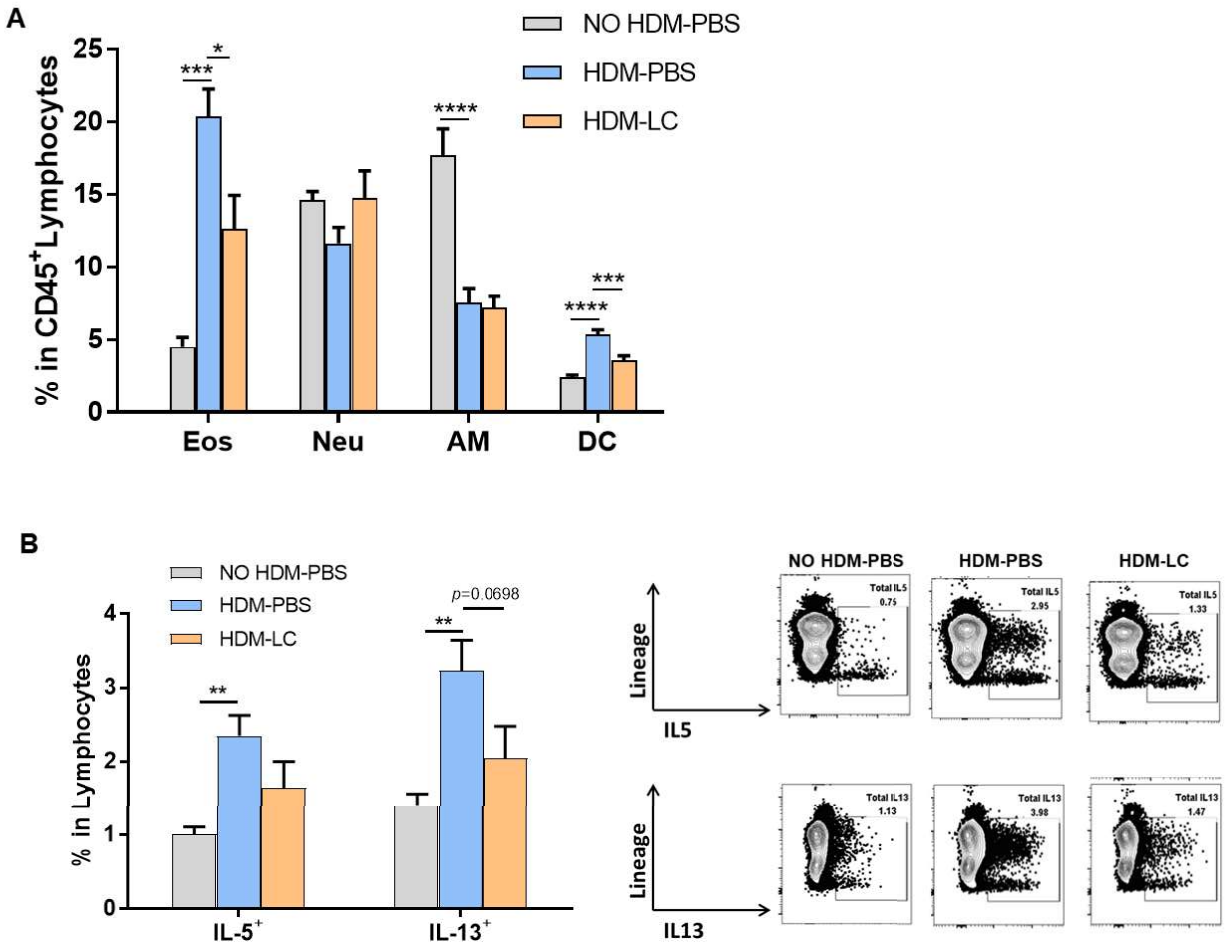

A
NO HDM-PBS
HDM-PBS
HDM-LC
% in CD45+Lymphocytes
25
20
15
10
5
0
***
*
****
****
***
Eos
Neu
AM
DC
B
NO HDM-PBS
HDM-PBS
HDM-LC
% in Lymphocytes
4
3
2
1
0
**
**
p=0.0698
IL-5+
IL-13+
NO HDM-PBS
HDM-PBS
HDM-LC
Lineage
IL5
Total IL5
0.75
Total IL5
2.95
Total IL5
1.33
Lineage
IL13
Total IL13
1.13
Total IL13
3.98
Total IL13
1.47

Figure 7. Effect of LC on CD45+^{high} immune cell population and inflammatory mediators in lungs of mice challenged with HDM.

Lung immune cell population of mice challenged with HDM, and treated with LC, with samples collected 24h after final challenge. Flow cytometry was used to analyze CD45+^{high} immune cell (A) CD45+^{high} immune cell subtype population of each treatment group shown in percentage; (B) % total IL-5 and total IL-13 producing lymphocytes. ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

7. Innate lymphoid cells were not crucial players of inducing inflammatory response in HDM-induced allergic asthma model.

After confirming anti-inflammatory effect of LC on lowering AHR and eosinophilia in lung in HDM-induced allergic asthma model, we investigated under which mechanism LC inhibits the development of asthma and which subtypes of lymphocytes are targeted by LC. In the same manner with OVA-induced allergic asthma model, we used flow cytometry to determine sub-populations of lymphocytes. Similar with the results observed in the OVA model, there was indistinguishable difference in the population of total $CD4^+$ T lymphocytes among all experimental groups (Fig 8A).The flow cytometric analysis also revealed elevated IL-5^+ and IL-13^+ subpopulations of $CD4^+$ T lymphocytes (Fig 8C). Consistent with our observation in Fig 7B, there was a reducing trend of IL-5^+ and IL-13^+ subpopulation of $CD4^+$ T lymphocytes in LC treatment group (Fig 8C).

Since we observed the similar patterns in results with the previous results of the model using OVA, we hypothesized that HDM challenge would increase the population of ILCs. Contrast to our expectation, the population of ILCs remained unaffected after HDM challenge; no effect of LC was detected (Fig 8B). Further analysis of ILC sub-populations revealed no

difference among all groups after HDM challenge; both IL-5^{+} and IL-13^{+} ILC populations remained unaffected by HDM challenge or LC treatment (Fig 8C). Taken together, we concluded that ILCs are not involved in inducing inflammatory response in HDM model and the response is rather governed exclusively by CD4^{+} T lymphocytes.

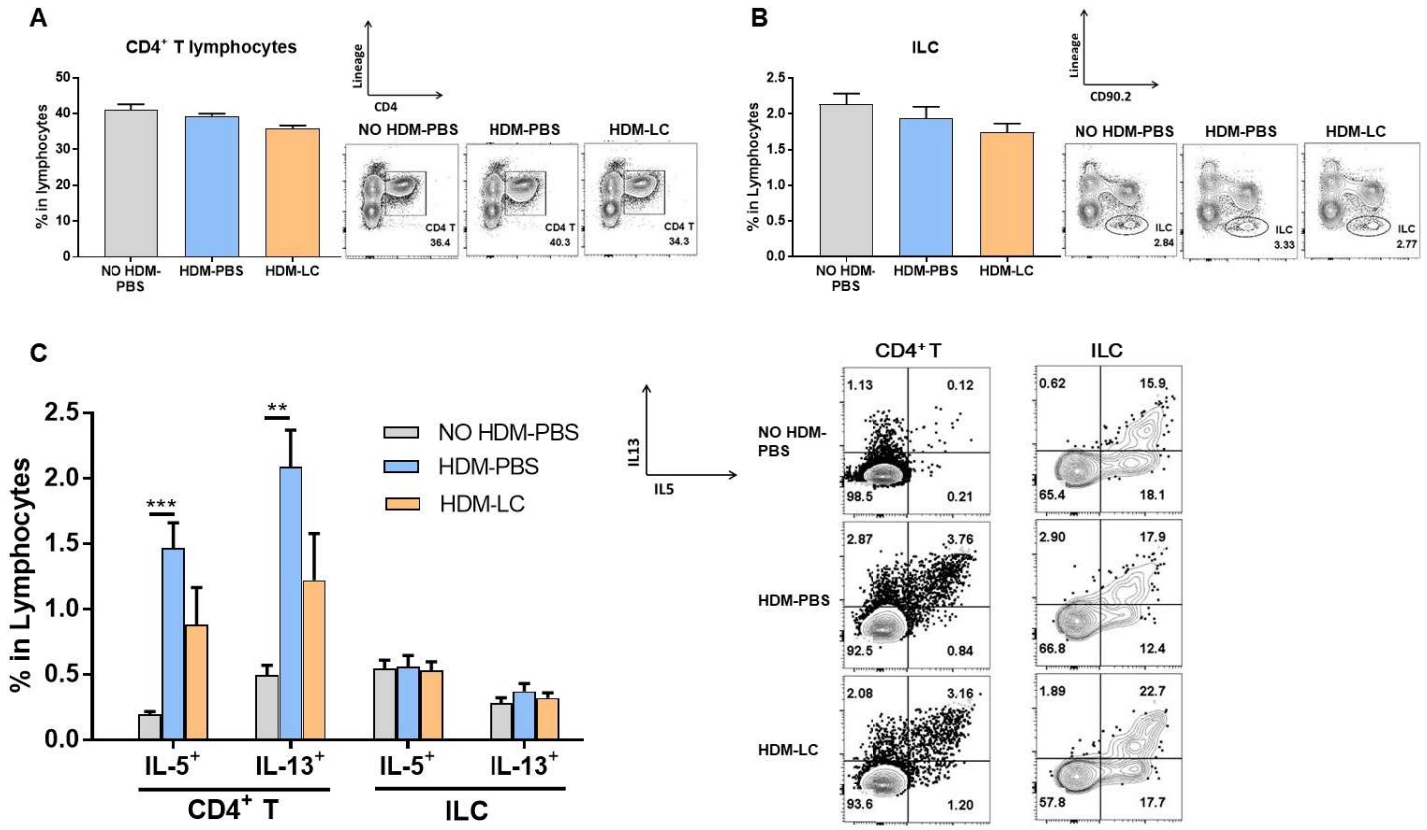

A
CD4+ T lymphocytes
% in lymphocytes
NO HDM-PBS
HDM-PBS
HDM-LC
Lineage
CD4
CD4 T 36.4
CD4 T 40.3
CD4 T 34.3
B
ILC
Lineage
CD90.2
ILC 2.84
ILC 3.33
ILC 2.77
C
% in Lymphocytes
***
**
IL-5+
IL-13+
CD4+ T
ILC
IL13
IL5
CD4+ T
ILC
1.13
0.12
98.5
0.21
0.62
15.9
65.4
18.1
2.87
3.76
92.5
0.84
2.90
17.9
66.8
12.4
2.08
3.16
93.6
1.20
1.89
22.7
57.8
17.7

Figure 8. Impact of LC on population of CD4+ T lymphocytes and innate lymphoid cells (ILCd) in lungs of mice challenged with HDM.

Population of cells in lungs of mice challenged with HDM and treated with LC, with samples collected 24h after final challenge. Percentages of cytokine producing lymphocytes were determined using FACS. Percentages and representative flow cytometry gating strategies of (A) total CD4+ T lymphocytes; (B) total ILCs; (C) IL-5+ and IL-13+ subtypes of CD4 T+ lymphocytes (left) and ILCs (right). ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

8. Cell wall component of LC downregulated the alarmin cytokine that directly affects recruitment of ILCs *in vitro*

Significant reduction in the population of IL-5^+ and IL-13^+ ILCs observed in Fig 5B led us to further investigation of the anti-inflammatory effect of LC on cellular level. ILCs, members of innate immune system, are known to be stimulated by alarmin cytokines such as IL-33 and TSLP produced by epithelial cells in the pathogenesis of asthma. We hypothesized that orally administered cellular components of LC bypass the liver, enter the bloodstream, and ultimately reach to the lungs where they may have impact on lung epithelial cells to secrete alarmin cytokines. We set up an *in vitro* experiment using MLE-12 lung epithelial cells, using papain, another allergen which can induce asthma, as stimulant. We treated MLE-12 cells with pasteurized LC and analyzed the gene expression using RT-PCR.

As expected, papain greatly induced IL-33 mRNA expression in MLE-12 cells (Fig 9A). Notably, treatment of pasteurized LC significantly reduced IL-33 mRNA expression (Fig 9A). In contrast, TSLP, another cytokine produced by lung epithelial tissue, was not upregulated by papain stimulation (Fig 9A), showing that the LC specifically targets the production of IL-33. We also analyzed the gene expression of patter-recognition

receptors (PRRs) to study which receptors of innate immune system are stimulated by papain and LC. Addition of papain did not induce the expression of *tlr2* nor *tlr4*. We observed significantly upregulated expression of *tlr2* in pasteurized LC treatment group (Fig 9A). There was no difference in the gene expression of *tlr4* in all experimental groups (Fig 9A). TLR2 is a receptor in the innate immunity which recognizes the peptidoglycan structure of the bacterial cell wall, while TLR4 recognizes lipopolysaccharide (LPS) structure of the Gram-negative bacterial cell wall.

Given these results, we hypothesized that hypothesized that peptidoglycan (PGN) from the cell wall structure of LC will downregulate the gene expression of alarmin cytokines. MLE-12 cells were stimulated with papain before treated with PBS or LC PGN. Treatment of LC PGN greatly increased the expression of *tlr2*, but not *tlr4*, as expected, confirming the MLE-12 cells recognized the structure of LC PGN (Fig 9B). Papain did not have an affect on upregulation of *tlr2* and *tlr4* (Fig 9B). Congruent with the results in Fig 9A, treatment of LC PGN significantly reduced the expression of *il-33*, but not *tslp*.

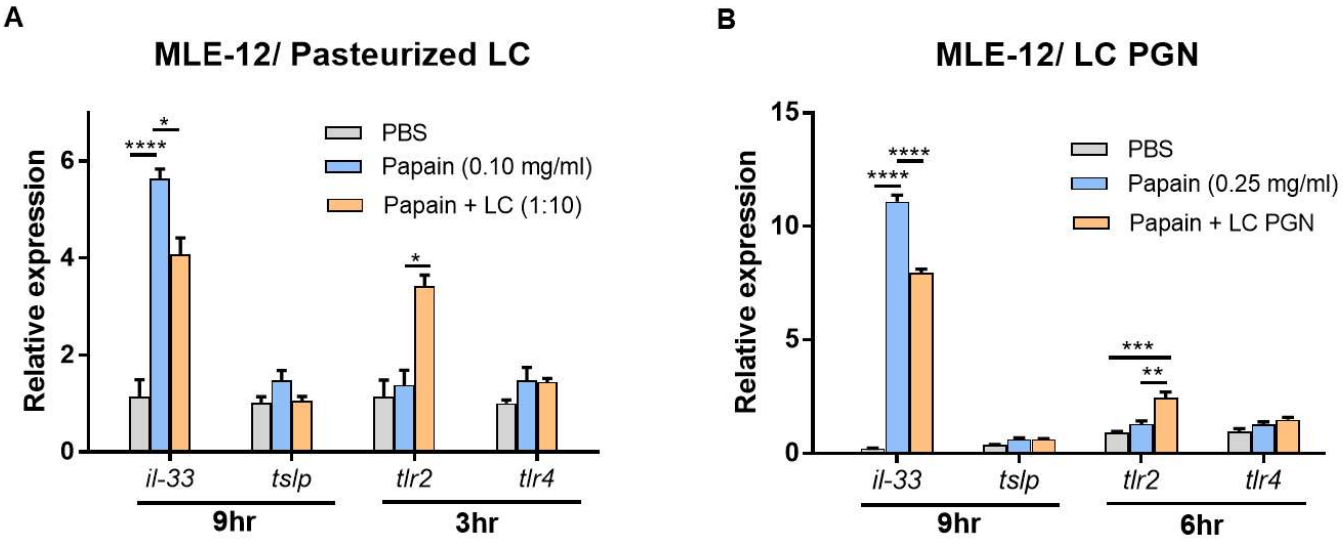

A
MLE-12/ Pasteurized LC
Relative expression
PBS
Papain (0.10 mg/ml)
Papain + LC (1:10)
il-33
tslp
tlr2
tlr4
9hr
3hr
B
MLE-12/ LC PGN
Relative expression
PBS
Papain (0.25 mg/ml)
Papain + LC PGN
il-33
tslp
tlr2
tlr4
9hr
6hr

Figure 9. Anti-inflammatory effect of pasteurized LC and peptidoglycan of LC on gene expression in lung epithelial cells *in vitro*.

RT-PCR analysis of MLE-12 cells. GAPDH was used as the housekeeping gene. MLE-12 cells were grown to confluence prior to papain stimulation. (A) MLE-12 cells were stimulated with PBS or papain (0.25 mg/ml), followed by either PBS or pasteurized LC (1:10) treatment. Cells were harvested after 3 hours and 9 hours. (B) MLE-12 cells were stimulated with PBS or papain (0.10 mg/ml), followed by either PBS or LC peptidoglycan. Cells were harvested after 6 hours and 9 hours. ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ between treatment groups.

IV. Discussion

In this study, we demonstrated the effect of LC on alleviating the allergic asthmatic symptoms and inhibiting the disease pathogenesis. Furthermore, we aimed to discover the cellular component of LC which may reduce development of ILCs, one of the key players of innate immunity and the pathogenesis of allergic asthma. Two known allergens to trigger allergic reactions were used in animal studies: OVA and HDM. Both OVA and HDM are well characterized, widely used allergen to induce acute allergic asthma in mice. OVA, the chicken egg white derivative, induces a robust allergic reaction in mice which leads to pulmonary inflammation (48). In this study, BALB/c mice were sensitized to OVA in Imject® Alum intraperitoneally and challenged with OVA intranasally to generate key features of clinical asthma. Since HDM is often preferably chosen over OVA in many asthma studies for its clinical relevance (49), we performed an additional experiment using HDM as allergen to prove the efficacy of LC on relieving allergic asthmatic symptoms in a more clinically relevant model. BALB/c mice were sensitized and challenged with HDM intratracheally. In both models, oral delivery of LC reduced prevented disease development, represented by reduced airway inflammation and lung eosinophil infiltration. Through our results, we also confirmed the differences between OVA and HDM in earlier stages of disease pathogenesis, and how the oral treatment of LC had the alleviating effect accordingly.

OVA induction increased pulmonary inflammatory infiltrates, despite the reduction in alveolar macrophage. We confirmed a significant increase in lung eosinophilia and bronchiolar epithelial thickness induced by OVA challenge, which were used to determine the inflammatory scores of each group. A striking effect of LC on the significant reduction lung eosinophilia and bronchiolar epithelial thickness was confirmed through lung histopathology and flow cytometric analysis. This suggests that the disease was induced in a strongly eosinophil-dependent manner, and LC acts on prevention of disease development by inhibiting recruitment of eosinophils in lung. This was supported by the significant reduction in mRNA expression levels of *siglec-F*, a surface marker of eosinophil which is used to regulate levels of eosinophils (13).

The level of IgE in serum is often measured to determine the severity of disease. Our observation in serum IgE level revealed modest reduction in LC treatment group, however statistically insignificant. During OVA stimulation, IgE is mainly produced by plasma B cells which are activated as a result of T_H2-type response (50). Our result therefore suggests that there may be an alternative response other than T_H2-type response on which LC acted to lower OVA-induced eosinophil infiltration in the lungs of LC treatment group.

Multiple mechanisms have been proposed to provide insight into our

understanding in development of allergic asthma and resulting phenotypes, which, eventually, suggest potential therapeutic targets. They are not restricted to adaptive immunity represented by $CD4^+$ T helper cells; innate immunity also plays a pivotal role. Traditionally, T_H2 asthma hypothesis has been used to explain the disease development of asthma. It is based on dysregulation of the T_H1/T_H2 balance (51). It states that IgE and eosinophils are regulated by T_H2 cytokines and play the major part in disease pathogenesis. T_H2 cells activated by allergen produces T_H2 cytokines, mainly IL-4, IL-5 and IL-13, which leads to eosinophilic lung inflammation, goblet cell hyperplasia, and mucous production. The recruitment of eosinophils in lung and the degree of eosinophilia are closely related to AHR. Recently, however, there is growing evidence that innate immunity also plays an important role in development of allergic asthma. ILC2 have been reported to significantly contribute to the IL-5 and IL-13 production in murine models of allergic asthma (52), thus activating and recruiting eosinophils. ILCs may act the innate counterparts of T_H1, T_H2, and T_H17 cells, yet they lack antigen receptors (53). While allergens presented by DCs induce differentiation of $CD4^+$ naïve T-cells into T_H2 cells, cytokines secreted by lung epithelial cells, such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP), directly activate ILCs (53). When ovalbumin in alum is used to induce asthma in mice, both T cells and ILCs seem to contribute equally to the production of IL-5 and IL-13. Thus, immune response pathways of either T_H2 cells or ILCs can

be therapeutic targets of preventing or treating asthma.

Our RT-PCR and flow cytometric analysis suggest that OVA induced a strong induction of T_H2-type response especially, but not exclusively. We observed a significant reduction of the total levels of IL-5 and IL-13 in lung, but not IL-4. We also observed a significant decrease in IL-5^+ and IL-13^+ $CD4^+$ lymphocytes, but not in the total amount of $CD4^+$ lymphocytes. These cytokines are the main T_H2 cytokines; IL-4, especially, is crucial in naïve T cell differentiation to T_H2 cells. No marked changes in total levels of IL-4, therefore, implies that an alternate pathway other than T_H2 pathway largely contributed to disease development, and the effect of LC was not limited to T_H2 response. Furthermore, the gene expression of *gata3*, the signature transcription factor of T_H2 lymphocytes, was not affected by OVA nor LC, emphasizing the roles of non-T_H2 pathway in the disease development. OVA failed to induce T_H1 response; there was no observable difference in levels of IFN-γ among WT and OVA groups, despite the upregulation of *t-bet*, the transcription factor of IFN-γ. Moreover, OVA did not induce the IL-17A and its transcription factor *rorγt* expression, suggesting T_H17 response was not involved in the disease pathogenesis in OVA model. Although we did observe an increase in the gene expression of foxp3, the transcription factor of T_{reg} cells, we concluded that T_{reg} response was not induced by OVA challenge, supported by the evidence displaying no change in total amount of IL-10 after OVA challenge. The results, therefore, have led us to hypothesize that the

innate immune response, ILCs especially, is involved in the earlier stage of disease pathogenesis of the OVA model, and LC can reduce ILC development as it does to T_H2 development.

As expected, we confirmed that OVA challenge induced elevation of IL-5^+ and IL-13^+ ILCs, and the effect of LC on reducing ILC population in lung. The effect of LC was reflected on a significant reduction in the population of IL-5^+ and IL-13^+ lung ILCs. There is growing evidence that ILCs can act in the upstream of the immune pathway involving adaptive T_H2 immune response (53). ILC2-deficient mice lacked ability to induce T_H2 immune response in response to papain allergen (54). While large amounts of IL-13 and IL-5 were produced by ILC2 under papain stimulation, change in the level of IL-4 was negligible; the mechanism of papain-induced T_H2 responses was independent of IL-4. IL-13 produced by ILCs acted as a main promoter of T_H2 cell differentiation in the draining lymph nodes (54). Coincidingly, our results suggest the role of LC on production of IL-5 and IL-13 by ILCs under OVA stimulation, which then interfere with induction of T_H2 responses and eosinophils, ultimately reducing the disease severity.

After verifying the alleviating effect of LC on OVA-induced allergic asthma, we proceeded to verify its effect on HDM-induced allergic asthma. Similar to OVA, HDM is known to induce a strong T_H2 response. Therefore, we expected orally administered LC would inhibit the disease development

by inhibiting T_H2 response. As expected, LC reduced HDM-induced lung inflammation and eosinophilia, as supported by histopathological and flow cytometric analysis. AHR, closely related to eosinophilia in lung, was greatly reduced in LC treatment mice, strongly insisting LC's effect on reducing asthmatic symptoms induced by HDM. The population of IL-5^+ and IL-13^+ lymphocytes significantly increased by HDM challenge, while there was a notable reduction of them with a statistical significance. The results suggest that LC affects on recruitment and proliferation of IL-5^+ and IL-13^+ lymphocytes, as we discovered in the results of the OVA model. Unlike in OVA model, however, LC significantly reduced the population of DC in lung. Upon foreign antigen exposure, DC presents the antigen and send signals to naïve T lymphocytes which then differentiate into functional, mature T lymphocytes (55). Due to its ability to determine the type of T_H response elicited on inhalation of aeroallergen, it affects the development and continuation of allergic asthmatic inflammation (55). A significant reduction of DC in LC treatment mice, therefore, may imply a great amount of adaptive immune response involving T_H response was reduced. That is, HDM-elicited immune responses were largely predisposed to T_H response.

How the pathogenesis of HDM-induced asthma was mainly governed by adaptive immunity was verified by the flow cytometric results of $CD4^+$ T lymphocyte and ILC populations. As OVA did, challenge with HDM did not cause a change in population of $CD4^+$ T lymphocytes; only the subpopulations, IL-5^+ and IL-13^+, remarkably increased. Although statistically insignificant, LC treatment induced a modest reduction in IL-5^+ and IL-13^+ $CD4^+$ lymphocytes. Meanwhile, the population of ILCs and subpopulations of ILCs remained unchanged after HDM challenge, suggesting that there was a miniscule influence of innate immunity on initiating the cascade of immune response; production of proinflammatory cytokines, IL-5 and IL-13, and disease development was done by T_H2 lymphocytes. These results coincide with our earlier observation of significantly reduced DC population in lungs of LC treatment mice. It is assumed that lung DCs are originated from two sources: the circulating pre-conventional DC (pre-cDC) progenitor expressing hematopoietic cytokine receptor Flt3, and monocyte-derived DC (moDC) (56, 57). Under inflammatory conditions, monocytes largely contribute to the lung DC pool (58); proinflammatory chemokines like CCL2 and CCL7 are produced by lung epithelial barrier cells. These chemokines upregulate the release of $CCR2^{hi}$ monocytes from the bone marrow, which then migrate to the airway mucosa and differentiate into moDC (59-61). During HDM challenge, moDC direct allergic inflammation by presenting allergen in the lung and produce

proinflammatory cytokines (58). Taken together, we concluded that HDM-induced allergic asthma is developed by T_H2 response which initiate from DC uptake of inhaled allergen. We also concluded that LC may inhibit differentiation of DC precursors like monocytes, and recruitment to mucosal surfaces. As a result, DC activities which are crucial for earlier stages of allergic reaction, such as antigen presentation and migration to lymph nodes where naïve and mature T_H lymphocytes reside may be inhibited, hence preventing the progress of the allergic cascade.

Lastly, we aimed to discover the cellular component of LC which can directly affect the innate immunity, more specifically, ILC development. Orally administered LC is broken down in the digestive system into cellular components, which will travel through blood stream. We hypothesized that the cellular component may directly affect the lung epithelial cells, and as a result, the levels of alarmin cytokines secretion by lung epithelial cells under allergen stimulation will be altered. Substantially decreased gene expression of *il-33,* in MLE-12 cells after treatment of pasteurized LC suggests that the strain's anti-inflammatory effect which we observed *in vivo* studies may be due to the cellular components, not its metabolic activities. The elevated expression level of *tlr2* by pasteurized LC led us to investigate the anti-inflammatory effect of PGN purified from LC. LC, with all the other lactic acid bacteria, is categorized as Gram-positive bacteria. The cell wall of Gram-positive bacteria consists of a think peptidoglycan (PGN) sacculus. The

composition and cross-linking of peptidic chains and their residues can vary among species; the structural variations of PGN are often associated with increasing resistance of bacteria against certain antibiotics (62). A previous study has reported the association between the protective capacity of *Lactobacillus salivarius* Ls33 in inflammatory bowel disease (IBD) and the purified PGN of the strain (63).

Consistent with our hypothesis, LC PGN treatment significantly reduced *il-33* expression in MLE-12 cells under papain stimulation, suggesting the role of LC PGN in IL-33 production and its potential role in earlier stages of allergic asthma development. IL-33 is an important cytokine for regulation of both innate and adaptive immunity. It promotes T_H2 differentiation of naïve $CD4^+$T lymphocytes in the IL-4 independent manner, enhancing IL-5 and IL-13 production (64). It also regulates development and the function of ILC2; IL-13 production in chronic rhinosinusitis with nasal polyps is largely dependent on epithelial cell-derived IL-33 and IL-33-responsive ILCs (65). Our results suggest that LC PGN may directly act on lung epithelial cells and down-regulate their IL-33 production, leading to downregulation of T_H response and ILC response, and overall disease development.

In summary, this study successfully confirmed the alleviating effects of LC on development of allergic asthma induced by OVA and HDM and

provided novel therapeutic targets for allergic asthma. The anti-inflammatory effects of LC were verified by diminished allergic asthma phenotype. The alleviating effects of LC acted on inhibiting both innate and adaptive immunity, which may be due to the ability of PGN purified from LC to downregulate the expression of *il-33*. We suggest the future studies to use LC PGN on alleviating asthmatic symptoms *in vivo* in order to verify its effects on alleviating asthmatic symptoms.

V. References

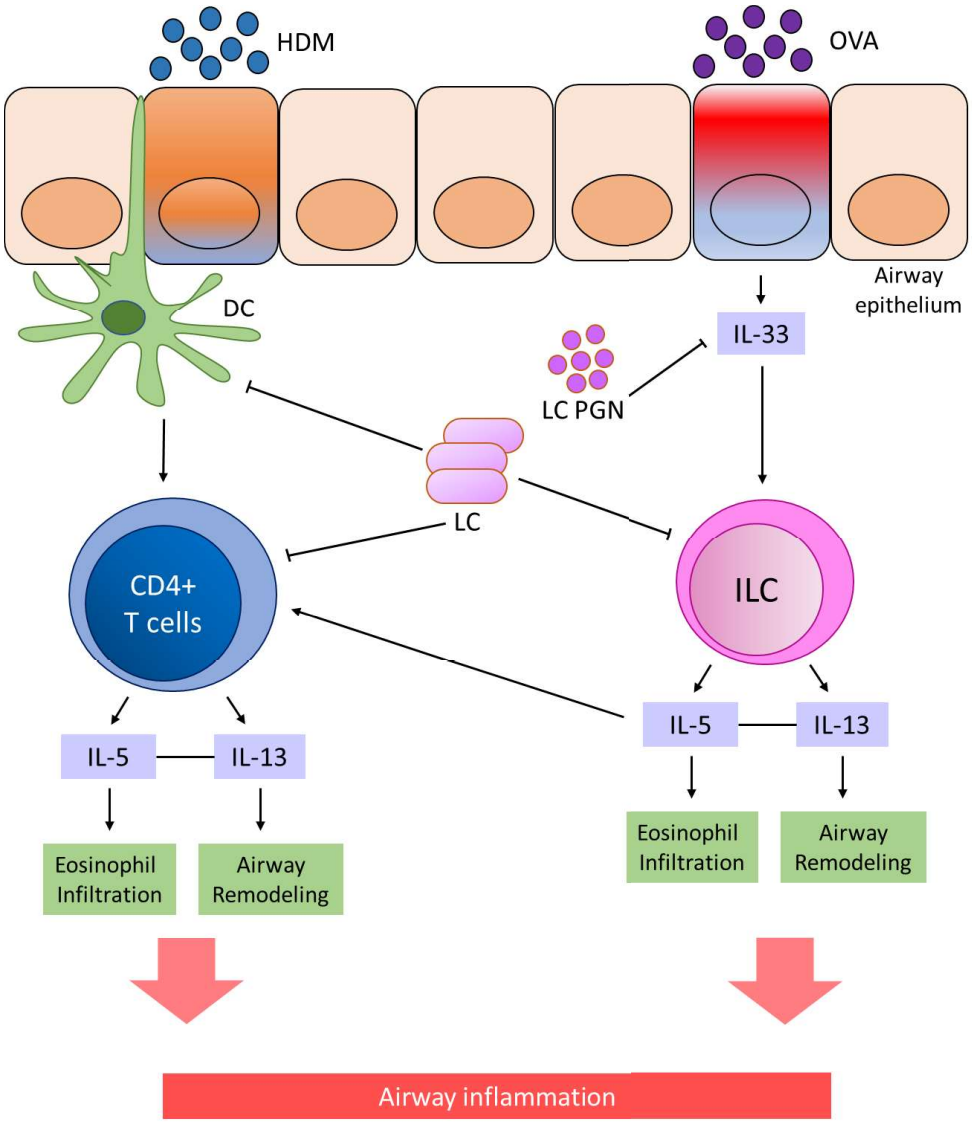
1. Masoli M, Fabian D, Holt S, Beasley R, & Program GIfA (2004) The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 59(5):469-478.
2. Bahadori K*, et al.* (2009) Economic burden of asthma: a systematic review. *BMC pulmonary medicine* 9(1):24.
3. Nunes C, Pereira AM, & Morais-Almeida M (2017) Asthma costs and social impact. *Asthma research and practice* 3(1):1.
4. Braman SS (2006) The global burden of asthma. *Chest* 130(1):4S-12S.
5. Asthma GIf (2018) Global strategy for asthma management and prevention. Updated 2018.
6. Bousquet J*, et al.* (1990) Eosinophilic inflammation in asthma. *New England Journal of Medicine* 323(15):1033-1039.
7. Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, & Young IG (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *Journal of Experimental Medicine* 183(1):195-201.
8. Kay AB (2001) Allergy and allergic diseases. *New England Journal of Medicine* 344(1):30-37.
9. Bosnjak B, Stelzmueller B, Erb KJ, & Epstein MM (2011) Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir Res* 12:114.
10. Barnes PJ (2001) Th2 cytokines and asthma: an introduction. *Respir Res* 2:64-65.
11. Wenzel SE (2012) Asthma phenotypes: the evolution from clinical to molecular approaches. *Nature medicine* 18(5):716.
12. Holgate ST (2012) Innate and adaptive immune responses in asthma. *Nature medicine* 18(5):673.
13. Rosenberg HF, Phipps S, & Foster PS (2007) Eosinophil trafficking in allergy and asthma. *Journal of Allergy and Clinical Immunology* 119(6):1303-1310.
14. Walter DM*, et al.* (2001) Critical role for IL-13 in the development of allergen-induced airway hyperreactivity. *The Journal of Immunology* 167(8):4668-4675.
15. Webb DC*, et al.* (2000) Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *The Journal of Immunology* 165(1):108-113.
16. Olin JT & Wechsler ME (2014) Asthma: pathogenesis and novel drugs for treatment. *Bmj* 349:g5517.
17. Kerrebijn KF, van Essen-Zandvliet E, & Neijens HJ (1987) Effect of long-term treatment with inhaled corticosteroids and beta-agonists on the bronchial responsiveness in children with asthma. *Journal of Allergy and Clinical Immunology* 79(4):653-659.

18. Barnes PJ, Pedersen S, & Busse WW (1998) Efficacy and safety of inhaled corticosteroids: new developments. *American journal of respiratory and critical care medicine* 157(3):S1-S53.
19. Guilbert TW*, et al.* (2006) Long-term inhaled corticosteroids in preschool children at high risk for asthma. *New England Journal of Medicine* 354(19):1985-1997.
20. Busse W*, et al.* (2001) Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *Journal of allergy and clinical immunology* 108(2):184-190.
21. Milgrom H*, et al.* (1999) Treatment of allergic asthma with monoclonal anti-IgE antibody. *New England Journal of Medicine* 341(26):1966-1973.
22. Djukanovic R*, et al.* (2004) Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma. *American journal of respiratory and critical care medicine* 170(6):583-593.
23. Jutel M*, et al.* (2015) International consensus on allergy immunotherapy. *Journal of Allergy and Clinical Immunology* 136(3):556-568.
24. Cazzola M & Matera MG (2008) Novel long-acting bronchodilators for COPD and asthma. *British journal of pharmacology* 155(3):291-299.
25. Cazzola M, Page CP, Calzetta L, & Matera MG (2012) Pharmacology and therapeutics of bronchodilators. *Pharmacological reviews* 64(3):450-504.
26. Martinez FD (2011) New insights into the natural history of asthma: primary prevention on the horizon. *Journal of Allergy and Clinical Immunology* 128(5):939-945.
27. Warner SM & Knight DA (2008) Airway modeling and remodeling in the pathogenesis of asthma. *Current opinion in allergy and clinical immunology* 8(1):44-48.
28. Lipworth BJ (1999) Systemic adverse effects of inhaled corticosteroid therapy: a systematic review and meta-analysis. *Archives of Internal Medicine* 159(9):941-955.
29. Kelly HW*, et al.* (2012) Effect of inhaled glucocorticoids in childhood on adult height. *New England Journal of Medicine* 367(10):904-912.
30. Partridge MR, van der Molen T, Myrseth S-E, & Busse WW (2006) Attitudes and actions of asthma patients on regular maintenance therapy: the INSPIRE study. *BMC pulmonary medicine* 6(1):13.
31. Rabe K, Vermeire P, Soriano J, & Maier W (2000) Clinical management of asthma in 1999: the Asthma Insights and Reality in Europe (AIRE) study. *European Respiratory Journal* 16(5):802-807.
32. Toh ZQ, Anzela A, Tang ML, & Licciardi PV (2012) Probiotic therapy as a novel approach for allergic disease. *Frontiers in pharmacology* 3:171.
33. Majamaa H & Isolauri E (1997) Probiotics: a novel approach in the management of food allergy. *Journal of Allergy and Clinical Immunology* 99(2):179-185.
34. Lee YK & Mazmanian SK (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science* 330(6012):1768-1773.
35. Dominguez-Bello MG*, et al.* (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in

newborns. *Proceedings of the National Academy of Sciences* 107(26):11971-11975.
36. Mueller NT, Bakacs E, Combellick J, Grigoryan Z, & Dominguez-Bello MG (2015) The infant microbiome development: mom matters. *Trends in molecular medicine* 21(2):109-117.
37. Thavagnanam S, Fleming J, Bromley A, Shields MD, & Cardwell C (2008) A meta-analysis of the association between Caesarean section and childhood asthma. *Clinical & Experimental Allergy* 38(4):629-633.
38. Renz-Polster H*, et al.* (2005) Caesarean section delivery and the risk of allergic disorders in childhood. *Clinical & Experimental Allergy* 35(11):1466-1472.
39. Arrieta M-C*, et al.* (2015) Early infancy microbial and metabolic alterations affect risk of childhood asthma. *Science translational medicine* 7(307):307ra152-307ra152.
40. Kelly D, King T, & Aminov R (2007) Importance of microbial colonization of the gut in early life to the development of immunity. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 622(1-2):58-69.
41. Martin R*, et al.* (2010) Early life: gut microbiota and immune development in infancy. *Beneficial microbes* 1(4):367-382.
42. Yu J*, et al.* (2010) The effects of Lactobacillus rhamnosus on the prevention of asthma in a murine model. *Allergy, asthma & immunology research* 2(3):199-205.
43. Jang S-O*, et al.* (2012) Asthma prevention by Lactobacillus rhamnosus in a mouse model is associated with CD4+ CD25+ Foxp3+ T cells. *Allergy, asthma & immunology research* 4(3):150-156.
44. Jan R-L*, et al.* (2012) Lactobacillus gasseri suppresses Th17 pro-inflammatory response and attenuates allergen-induced airway inflammation in a mouse model of allergic asthma. *British Journal of Nutrition* 108(1):130-139.
45. 고우리 (2018) Inhibitory effects of Lactobacillus crispatus isolated from vaginal microbiota on atopic dermatitis. (서울대학교 대학원).
46. Spergel JM (2010) From atopic dermatitis to asthma: the atopic march. *Annals of allergy, asthma & immunology* 105(2):99-106.
47. Desmarais SM, Cava F, de Pedro MA, & Huang KC (2014) Isolation and preparation of bacterial cell walls for compositional analysis by ultra performance liquid chromatography. *JoVE (Journal of Visualized Experiments)* (83):e51183.
48. Nials AT & Uddin S (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. *Disease models & mechanisms* 1(4-5):213-220.
49. Johnson JR*, et al.* (2004) Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *American journal of respiratory and critical care medicine* 169(3):378-385.
50. Gould HJ & Sutton BJ (2008) IgE in allergy and asthma today. *Nature Reviews Immunology* 8(3):205.

51. Kidd P (2003) Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Alternative medicine review* 8(3):223-246.
52. Wolterink RGK*, et al.* (2012) Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *European journal of immunology* 42(5):1106-1116.
53. Eberl G, Colonna M, Di Santo JP, & McKenzie AN (2015) Innate lymphoid cells: A new paradigm in immunology. *Science* 348(6237):aaa6566.
54. Halim TY*, et al.* (2014) Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40(3):425-435.
55. Gill MA (2012) The role of dendritic cells in asthma. *Journal of Allergy and Clinical Immunology* 129(4):889-901.
56. Ginhoux F*, et al.* (2009) The origin and development of nonlymphoid tissue CD103+ DCs. *Journal of Experimental Medicine* 206(13):3115-3130.
57. Jakubzick C*, et al.* (2008) Blood monocyte subsets differentially give rise to CD103+ and CD103− pulmonary dendritic cell populations. *The Journal of Immunology* 180(5):3019-3027.
58. Plantinga M*, et al.* (2013) Conventional and monocyte-derived CD11b+ dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38(2):322-335.
59. Serbina NV & Pamer EG (2006) Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nature immunology* 7(3):311.
60. Hammad H*, et al.* (2009) House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nature medicine* 15(4):410.
61. León B, López-Bravo M, & Ardavín C (2007) Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* 26(4):519-531.
62. Chapot-Chartier M-P & Kulakauskas S (2014) Cell wall structure and function in lactic acid bacteria. *Microbial cell factories*, (BioMed Central), p S9.
63. Fernandez EM*, et al.* (2011) Anti-inflammatory capacity of selected lactobacilli in experimental colitis is driven by NOD2-mediated recognition of a specific peptidoglycan-derived muropeptide. *Gut* 60(8):1050-1059.
64. Kurowska-Stolarska M*, et al.* (2008) IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. *The Journal of Immunology* 181(7):4780-4790.
65. Shaw JL*, et al.* (2013) IL-33–responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. *American journal of respiratory and critical care medicine* 188(4):432-439.

VI. Graphical Abstract



VII. Appendix

List of Abbreviations

Airway hyperresponsiveness (AHR)

CC chemokine ligand (CCL)

Cluster of differentiation (CD)

Colony forming unit (CFU)

Complementary DNA (cDNA)

Dendritic cell (DC)

Deoxyribonucleic acid (DNA)

enzyme-linked immunosorbent assay (ELISA)

Fluorescence-activated cell sorting (FACS)

Formalin-fixed paraffin-embedded (FFBE)

Haematoxylin and eosin (H&E)

House-dust mite (HDM)

Immunoglobulin E (IgE)

Innate lymphoid cell (ILC)

Interleukin (IL)

Lactobacillus crispatus (LC)

Live biotherapeutic product (LBP)

Lung resistance (R_L)

Man-Rogosa-Sharpe (MRS)

Ovalbumin (OVA)

Peptidoglycan (PGN)

Phosphate buffer saline (PBS)

Polymerase chain reaction (PCR)

Real-time polymerase chain reaction (RT-PCR)

Ribonucleic acid (RNA)

Sodium dodecyl sulfate (SDS)

T helper cells (T_H cells)

Toll-like receptor (TLR)

Wild type (WT)

국문초록

락토바실러스 크리스파투스의
알러지성 천식 증상 완화 효과

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지도교수 고 광 표

천식의 발달은 숙주의 면역 및 대사 기능을 조절하는 미생물군총의 영향을 받는다. 제왕절개 분만으로 태어난 아기가 자연분만으로 태어난 아기에 비해 높은 천식 발병률을 보인다는 역학 조사 보고가 잇따르고 있다. 이 연구에서는 여성 질내 균총의 대표적인 락토바실러스 균주인 락토바실러스 크리스파투스 (LC)의 오브알부민 (OVA)과 집먼지 진드기 (HDM)로 유도한 알러지성 천식의 완화 효과를 쥐를 대상으로 한 *in vivo* 모델에서 확인하였다. 건강한 한국 여성의 질에서 분리한 LC를 유도 실험 7일 전부터 실험 종료일까지 매일 경구 투여 (약 2.0×10^9 CFU/쥐/일) 하여 쥐의 장 내에 균주의 정착을 유도하였다. OVA 실험 에서는 0일과 14일에 쥐에게 OVA와 알럼을 복강 투여하여 감작을 진행하고, 18일부터 20일까지 비강주사로 OVA를 주입하여 질병을 유도하였다. HDM 실험 에서는 0일과 7일부터 11일까지 기관내 주사를 통해 HDM을 주입하여 질병을 유도하였다. 조직학적 분석 결과 두 실험 모두에서 LC를 투여한 그룹에서 호산구의 폐 침투율이 유의하게 감소한 것을 확인하였다. HDM 실험 에서는 폐의 기능성을 측정하기 위해 기도과민성을 측정하였는데, LC를 투여한 그룹이 양성 유도군에 비해 유의하게 감소하였다. 유동세포분석법을 통하여 OVA와 HDM에 의해 증가된 IL-5, IL-

13 및 IL-5^{+}/13^{+} CD4^{+} T림프구가 LC를 투여했을 때 IL-4와 독립적인 방식으로 감소시키는 것을 확인하였다. 또한, OVA에 의해 증가된 IL-5^{+}/13^{+} 선천성 림프세포를 LC가 유의하게 감소시키는 것을 확인하였다. HDM 모델에서는 LC가 수지상 세포를 유의하게 감소시키는 것을 확인하였다. 다음으로 LC의 항염증성 작용에 직접적인 관련이 있는 LC의 세포물질을 파악하고 관련 기전을 연구하고자 하였다. LC의 세포벽의 펩티도글리칸을 분리하여 파파인으로 자극시킨 폐 상피세포 (MLE-12)에 처리하였다. 살균된 LC와 LC의 펩티도글리칸을 처리했을 때 *il-33*의 유전체 발현량이 유의하게 감소하는 것을 확인하였다. 따라서 본 연구는 LC가 OVA와 HDM으로 유도한 알러지성 천식을 효과적으로 완화시킬 수 있음을 확인하였고 마이크로비옴을 기반으로 한 천식 치료에 본 연구의 결과를 이용할 수 있음을 시사한다.

주요 단어: *락토바실러스 크리스파투스*, 프로바이오틱스, 알러지성 천식, 오브알부민, 집먼지진드기, 기도과민성, 선천성 림프세포, 펩티도글리칸

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