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Original Article

# *Lactobacillus paracasei*-derived extracellular vesicles alleviate neutrophilic asthma by inhibiting the JNK pathway in airway epithelium

Soyoon Sim [a, b], Hyeon Ju Park [c], Yoon-Keun Kim [c], Youngwoo Choi [d, *, 1], Hae-Sim Park [a, b, **, 1]

[a] Department of Allergy and Clinical Immunology, Ajou University School of Medicine, Suwon, South Korea
[b] Department of Biomedical Science, Graduate School of Ajou University, Suwon, South Korea
[c] MD Healthcare Inc., Seoul, South Korea
[d] Department of Biomaterials Science, College of Natural Resources and Life Science, Pusan National University, Miryang, South Korea

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BAL, bronchoalveolar lavage; Dex, dexamethasone; EA, eosinophilic asthma; EDN, eosinophil-derived neurotoxin; EVs, Extracellular vesicles; FOXP3, forkhead box P3; GC-TOF-MS, gas chromatography time-of-flight mass spectrometry; HCs, healthy controls; IBD, inflammatory bowel disease; JNK, c-Jun N-terminal kinases; *L. paracasei*, *Lactobacillus paracasei*; LpEV, *Lactobacillus paracasei*-derived extracellular vesicles; *L. rhamnosus*, *Lactobacillus rhamnosus*; LrEV, *Lactobacillus rhamnosus*-derived extracellular vesicles; MPO, myeloperoxidase; NA, neutrophilic asthma; SA, severe asthma; RORγt, retinoic-acid-receptor-related orphan nuclear receptor gamma

ABSTRACT

*Background:* *Lactobacillus paracasei* has been known to reduce airway resistance and inflammation in asthma. However, the therapeutic effect of its extracellular vesicles (EVs) in patients with asthma remains unclear.
*Methods:* To validate the clinical relevance of *L. paracasei*-derived EVs (LpEV) in asthma, the composition of gut microbial EVs was verified by metagenomics in LPS-induced C57BL/6 mice. The components of proteins and metabolites in LpEV were identified by peptide mass fingerprinting and metabolomic analysis. The serum levels of specific IgG1 or IgG4 antibodies to LpEV were compared by ELISA between patients with eosinophilic asthma (EA, n = 10) and those with neutrophilic asthma (NA, n = 10) as well as with healthy controls (HCs, n = 10). Finally, therapeutic effects of LpEV and their metabolites in asthma were validated *in vivo*/*in vitro*.
*Results:* Significantly lower proportions of EVs derived from *Lactobacillus* at the genus level were noted in mice with NA than in control mice. Moreover, the serum levels of LpEV-specific IgG4, but not IgG1, were lower in patients with NA than in those with EA or in HCs and positively correlated with $FEV_1$ (%) values. In addition, oral administration of LpEV reduced airway resistance and inflammation in mice with NA. Finally, LpEV and their 3 metabolites (dodecanoic acid, palmitoleic acid, and D-(−)-tagatose) significantly inhibited JNK phosphorylation/IL-8 production in airway epithelium *in vitro*.
*Conclusions:* These findings suggest that LpEV may have a therapeutic potential targeting NA by suppressing the JNK pathway and proinflammatory cytokine production in airway epithelium.


* Corresponding author. Department of Biomaterials Science, College of Natural Resources and Life Science, Pusan National University, 1268-50 Samrangjin-ro, Miryang, South Korea.
** Corresponding author. Department of Allergy and Clinical Immunology, Ajou University School of Medicine, 164 Worldcup-ro, Suwon, South Korea.
*E-mail addresses:* ychoi@pusan.ac.kr (Y. Choi), hspark@ajou.ac.kr (H.-S. Park).
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[1] These authors equally contributed to this work.

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## Introduction

Asthma is a chronic airway inflammation induced by various immune/cellular mechanisms, presenting heterogenous phenotypes.[1] Although asthma has been considered type 2/eosinophilic inflammation (mediated by both IgE- and non-IgE-mediated responses), neutrophilic inflammation is observed in some patients with severe asthma (SA) or steroid-resistant asthma who are classified as those with neutrophilic asthma (NA).[2,3] Neutrophilic inflammation is induced by variable factors, including viral and bacterial infections, that may work in an additive or a synergistic manner.[4–6] In addition, neutrophils induce tissue damage and mucus hypersecretion in asthmatic airway by non-immunologic mechanisms, which are not reversible by current anti-inflammatory treatment.[7] To date, there has been a lack of specific immunomodulator targeting neutrophils; new therapeutic strategies are needed to overcome steroid insensitivity and to control neutrophilic inflammation in patients with NA.

Alterations in the abundance and diversity of gut microbiota can contribute to asthma exacerbation by regulating airway inflammation and remodeling in the lungs.[6,8] Especially, the microbiology of patients with NA is characterized by increased bacterial load, but decreased microbial diversity.[9,10] Moreover, these features are consistent with other chronic lung diseases accompanied by neutrophilia, such as chronic obstructive lung disease and cystic fibrosis.[11,12] Recently, modulation of bacterial communities has been suggested as a strategy to prevent or treat asthma. Especially, probiotics, including *Lactobacillus* species, are live microorganisms that can have a potential benefit to asthmatic patients.[13] For example, *Lactobacillus rhamnosus* (*L. rhamnosus*) has contributed to asthma prevention by Treg-mediated mechanism.[14,15]

Emerging evidence has revealed that bacterial extracellular vesicles (EVs) released by all living organisms are involved in human diseases such as cancer and allergic diseases. EVs contain membrane-bound organelles carrying a cargo of various proteins and metabolites, modulating airway inflammation with close intercellular communication.[16] Moreover, they have been highlighted as a potential biomarker for chronic immunologic disease due to their high stability in environmental variation and safety in replication.[17–19] Recent studies have demonstrated that EVs derived from probiotics have therapeutic potential by suppressing mast cell activation in food allergy[20] and protecting against *Staphylococcus aureus* in atopic dermatitis.[21] Previous studies have documented that *Lactobacillus paracasei (L. paracasei*) attenuates allergic airway inflammation through rebalancing Th1/Th2 immune responses *in vivo*.[22] Moreover, EVs derived from *L paracasei* (LpEV) could attenuate inflammatory bowel disease (IBD) via modulating Th17 immune response *in vivo*,[23,24] suggesting that LpEV may be a therapeutic option for controlling neutrophilic airway inflammation. To date, there have been few reports to evaluate the therapeutic potential of LpEV in asthma. Therefore, the present study aimed to evaluate the clinical relevance of LpEV in asthma by (1) evaluating relative abundance of LpEV-specific IgG antibodies in serum of adult asthmatic patients according to asthma phenotypes, (2) identifying key molecules (proteins or metabolites) in LpEV, and (3) investigating a potential therapeutic benefit of LpEV for controlling neutrophilic airway inflammation *in vivo* or *in vitro*.

## Methods

### *Establishment of a neutrophilic asthma mouse model*

Animal studies were approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC- 2019–0024). All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by Animal and Plant Quarantine Agency, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea. To establish a LPS (Sigma–Aldrich, St Louis, MO, USA)-induced asthmatic mouse model, male 6-week-old C57BL/6 mice (Orient BIO, Seongnam, Korea) were sensitized by intraperitoneal injection of 4 mg/kg OVA (Sigma–Aldrich) with .5 mg/kg LPS, and challenged by intranasal injection of 2.5 mg/kg OVA as depicted in Supplementary Fig. 1. To measure AHR to inhaled methacholine (Sigma–Aldrich), the flexiVent System (SCIREQ, Montreal, Canada) was used.

### *Metagenomic analysis of gut microbial EV composition*

In LPS-induced asthmatic mice, EVs were isolated from feces, and 16s rDNA analysis was performed as previously described.[25,26] Briefly, fecal contents were dissolved in PBS, filtered through a .45-μm filter, and centrifuged at 150,000 *g* for 2 h at 4 °C. To perform metagenomics, microbial DNAs were amplified using primers (319F: 5′ CCTACGGGNGGCWGCAG 3′, 806R: 5′ GACTACHVGGGTATCTAATCC 3′) and the final products were sequenced by the MiSeq platform (Illumina, San Diego, CA, USA). Then, sequencing of each operational taxonomic unit was performed by BLASTN (v.2.4.0) on the reference DB (NCBI 16S Microbial). Functional gene profiles were evaluated by Phylogenetic Investigation of Communities by using Reconstruction of Unobserved States and analyzed by the Closed-reference operational taxonomic unit picking method of QIIME (v1.9).

### *Bacteria culture and EV isolation*

Two probiotics, *L. paracasei* and *Lactobacillus rhamnosu* (Korean culture Center of Microorganisms, Seoul, Korea) were cultured in Ediable MRS P medium (MD Healthcare Inc., Seoul, Korea), respectively, under aerobic conditions until the optical density reached 1.5 at 600 nm. Then, their EVs were isolated as previously described.[27] To isolate EVs, the culture media were centrifuged at 10,000 *g* for 30 min. The supernatant was enriched by QuixStand (GE Healthcare, Little Chalfont, UK) and filtered through a .22-μm bottle-top filter (Sigma–Aldrich). Finally, the filtrates were centrifuged at 150,000 *g* for 2 h at 4 °C. EV morphology was observed using a JEM1011 microscope (JEOL, Akishima, Japan). EV size was measured using a Zetasizer Nano S (Malvern Instruments, Malvern, UK). EV protein pattern was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were analyzed by peptide mass fingerprinting.

### *Targeted or untargeted metabolomics using gas chromatography time-of-flight mass spectrometry*

For sample preparation, 1 mL of methanol (ThermoFisher Scientific, Pittsburgh, PA, USA) was added to 115 ul of each mouse serum or isolated LpEV. The mixtures were homogenized using an MM400 Mixer Mill (Retsch, Haan, Germany) at 30Hz for 10 min and sonicated for 5 min. The samples were centrifuged at 13,000 rpm for 10 min at 4 °C. Then, the supernatants were filtered through a .22 μm polytetrafluoroethylene syringe filter (Chromdisc, Daegu, Korea) and completely dried using a speed-vacuum concentrator (Biotron, Seoul, Korea). The dried sample were dissolved using methanol to make final concentrations for 10,000 ppm (10 mg/mL) and dried again. Then, 50 μL of pyridine-dissolved methoxyamine hydrochloride (20 mg/mL) were added to derivatized samples and incubated for 90 min at 30 °C. After that, 50 μL of N-methyl-N (trimethylsilyl)trifluoroacetamide was added and incubated for 30 min at 37 °C. The derivatized samples were analyzed by gas chromatography time-of-flight mass spectrometry (GC-TOF-MS). An

Agilent 7890A GC system (Agilent Tehnologies, Palo Alto, CA, USA) equipped with an Agilent 7693 autosampler and Pegasus HT TOF-MS (Leco Corporation, ST. Joseph, MI, USA) were utilized. Helioium was used as a carrier gas with a constant flow rate of .5 mL/min. An RTX-5MX column (Resteck Corp., St, Joseph, MI, USA) was used to separate the metabolites. 1 μL of each sample was injected in split (5:1) mode and the injector and ion source temperature were maintained at 250 °C. The column temperature was set to 75 °C for 20 min, followed by increases to 300 °C at 15 °C/min, and finally maintained for 3 min. The detector voltage was 2152.5 V and the mass was collected in the 50–600 *m*/*z* range. The experiment was performed with 3 replicate analyses for each sample. The raw data of the GC-TOF-MS analysis was converted to NetCDF and processed with the Met Align software to determine the data alignment by retention time and peak mass. The quantification was performed based on the retention time and unique mass of each metabolite. All reagent-grade chemicals and standards (dodecanoic acid, palmitoleic acid, and D-(−)-tagatose) were obtained from Sigma–Aldrich.

*LpEV and metabolite treatment in vivo*

To evaluate the effect of LpEV and their metabolites on neutrophilic inflammation, mice were orally administered with 30 or 300 μg/kg LpEV using a gastric gavage needle or intranasally treated with .5 mg/kg dodecanoic acid, palmitoleic acid, and D-(−)-tagatose (all from Sigma–Aldrich) 1 h prior to OVA challenge in comparison with 1 mg/kg dexamethasone (Dex; Sigma–Aldrich) as shown in Supplementary Figure 1. To measure immune cell numbers, BAL fluid (BALF) was collected and centrifuged at 3000 rpm for 5 min, followed by staining with hematoxylin (Dako, CA, USA) and Eosin Y (Sigma–Aldrich). Levels of CXCL1 and IL-17 in the BALF were quantified by using the ELISA kits (R&D System, Minneapolis, MN, USA). For histological analysis, lung tissues were fixed with 4% paraformaldehyde and observed using ImageJ (National Institutes of Health, Bethesda, MD, USA). Primary lung epithelial cells were isolated from mice using a commercial kit (STEMCELL technologies, Cambridge, UK) according to the manufacturer's recommendations.

*EV fluorescence imaging*

LpEV were incubated with 5 μM Cy7 mono NHS ester (GE Healthcare) for 1 h at 37 °C and then Cy7 mono NHS ester-labeled LpEV were isolated by ultracentrifugation. Then, Cy7-labeled LpEV (10 μg of total protein) was orally administered to the mice. At the indicated time point, fluorescence in the whole body and dissected organs were quantified at a wavelength of 780–800 nm using a Davinch-Invivo system (Davinch-Invivo Fluoro Chemi, Korea).

*Flow cytometric analysis*

Lung tissues were consecutively filtered through 100-μm and 40-μm nylon cell strainers (SPL Life Science, Pocheon, Korea). Then, mouse $CD4^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA) was used to purify the cells. Among them, Th17 and Treg cells were clarified by using the antibodies as follows: CD4, retinoic-acid-receptor-related orphan nuclear receptor gamma (RORγt), and forkhead box P3 (FOXP3) (all from eBioscience, San Diego, CA, USA). These cells were analyzed by FACSAria III (BD Biosciences). Graphs were produced using FlowJo software (Tree Star, Ashland, OR, USA).

*Western blot analysis*

To investigate the cell signaling pathway, the antibodies used were as follows: RORγt (ThermoFisher Scientific), FOXP3 (ThermoFisher Scientific), c-Jun N-terminal kinases (JNK; Cell Signaling Technology, Danvers, MA, USA), phospho-JNK (Cell Signaling Technology), GAPDH (Santa Cruz, Dallas, TX, USA), and actin (Santa Cruz).

*Airway epithelial cell stimulation in vitro*

A549 cells (American Type Culture Collection) were cultured in DMEM medium (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 50 μg/mL streptomycin. These cells were grown at 37 °C in humidified air with 5% $CO_2$. Then, the cells were treated with 1 μg/mL LPS (Sigma–Aldrich) with or without LpEV in 2 doses (1 and 10 ug/mL) for 24 h. To investigate the effect of metabolites, A549 cells were pretreated with dodecanoic acid, palmitoleic acid, and D-(−)-tagatose in a dose-dependent manner (5, 10, and 50 μM) for 30 min before stimulation with 1 μg/mL LPS for 24 h. The concentration of IL-8 in culture supernatants were measured using the ELISA kit (R&D System).

*Patient recruitment and clinical parameters*

The study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108). All the study subjects submitted written informed consent at the time of recruitment. In this study, 20 asthmatic patients and 10 healthy controls (HCs) were diagnosed by allergy specialists based on clinical histories including recurrent of cough, shortness of breath, chest tightness, and evidence of airway obstruction. Then, asthmatic patients were divided into eosinophilic asthma (EA) and NA according to the numbers of sputum eosinophils (≥3%) and neutrophils (≥65%). Atopy was defined as at least 1 positive result in skin prick tests using common inhaled allergens (Bencard, Bradford, UK). Serum total IgE was measured using the ImmunoCAP system (ThermoFisher Scientific). The degree of airway obstruction was evaluated using spirometry, and airway hyperresponsiveness was examined by methacholine bronchial challenge test. Levels of eosinophil-derived neurotoxin (EDN), myeloperoxidase (MPO), and IL-8 in serum were measured using the ELISA kits (EDN by SKIMS-BIO, Seoul, Korea; MPO and IL-8 by R&D Systems) according to the manufacturer's recommendations.

*Measurement of serum levels of LpEV-specific IgG antibodies by ELISA*

To evaluate levels of EV-specific IgG antibodies in serum, 100 ng/mL EVs were coated on a 96-well plate (ThermoFisher Scientific) overnight at 4 °C. After washing with PBS, the plate was blocked with 1% bovine serum albumin (Sigma–Aldrich) for 1 h and incubated with the serum samples from mice or humans for 2 h. Then, peroxidase-conjugated anti-mouse IgG antibody (Sigma–Aldrich) or anti-human IgG1/4 antibody (ThermoFisher Scientific) was added, respectively. The reaction was induced by 3,3′,5,5′-tetramethylbenzidine (BD Biosciences, San Diego, CA, USA) and stopped by using a stop solution. The intensity was evaluated by using a microplate reader (BioTek, Santa Clara, CA, USA) at 450-nm wavelength.

*Dot blot analysis for LpEV-specific IgG antibodies*

To compare the presence of IgG antibodies in serum from mice or humans, 1 μg of EVs were coated on nitrocellulose membrane (GE healthcare) for 12 h. Then, each serum sample was incubated for 24 h at room temperature. After that, the membrane was

blocked with 5% skim milk in Tris buffered saline with Tween 20 for 1 h and incubated with peroxidase conjugated anti-mouse IgG antibody (Sigma–Aldrich) or anti-human IgG1/4 antibodies (ThermoFisher Scientific) overnight. The membrane was detected and visualized using the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

### *Statistical analysis*

All statistical analyses were performed using IBM SPSS software, version 26.0 (IBM Corp., Armonk, NY, USA). Differences between 2 groups were analyzed by *Student's t*-test. In addition, comparisons of data from multiple groups were made by one-way ANOVA with Bonferroni's *post hoc* test. *P* values of < .05 were considered statistically significant. Pearson's chi-square test was conducted to analyze correlations. GraphPad Prism 8.0 software (GraphPad Inc., San Diego, CA, USA) was used to create graphs.

## Results

### *Altered gut microbial EV composition in mice with NA*

Microbial EV diversity was significantly lower in mice with NA than in control mice ($P = .001$; Fig. 1A). At the phylum level, Firmicutes tended to be lower, whereas Bacteroidetes and Proteobacteria were significantly higher in mice with NA than in control mice ($P = .001$ and $P = .002$, respectively; Fig. 1B). Especially, the prevalence of Lactobacillus and Akkermansia was lower in mice with NA than in control mice, whereas that of Bacteroides was higher in mice with NA (Fig. 1C). In addition to changes in microbial EVs, airway resistance as well as CXCL1 (a chemoattractant for

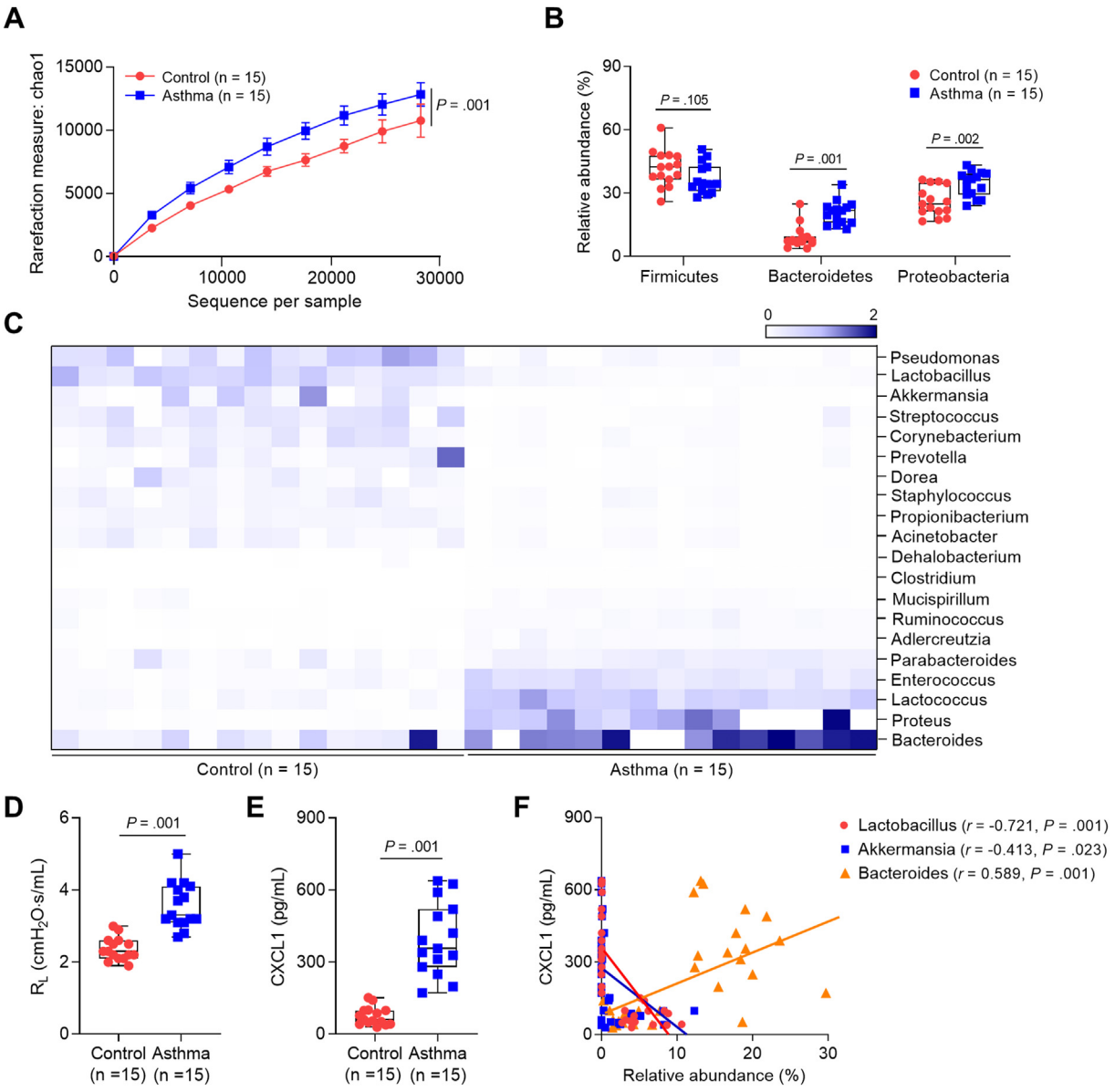


**Fig. 1.** Metagenomic analysis of gut microbial EV composition. **(A)** Chao1 index. **(B)** Relative abundance of EVs derived from microbial communities at the phylum level. **(C)** Heatmap plot of the EV origins at the genus level. **(D)** Airway hyperresponsiveness. **(E)** Concentration of CXCL1 in bronchoalveolar lavage fluid. Data are presented as box plots, n = 15. Between 2 groups, *P* values were obtained by Student's *t* test. **(F)** Correlations between concentration of CXCL1 and relative abundance of Lactobacillus, Akkermansia or Bacteroides. Data are presented as Pearson correlation coefficient *r* (*P* value). NC, normal control; NA, neutrophilic asthma; CXCL1, C-X-C motif chemokine ligand 1.

neutrophils) concentration was significantly higher in mice with NA ($P = .001$ for all; Fig. 1D, E). Moreover, the levels of CXCL1 were negatively correlated with the prevalence of Lactobacillus and Akkermansia, but positively correlated with that Bacteroides (Fig. 1F). This study further analyzed functional gene profiles and identified multiple genes involved in cellular processes and metabolism (Supplementary Fig. 2A). Among them, genes related to gondoate biosynthesis and pentose phosphate pathways were reduced, while aerobic respiration I and c-Diamide biosynthesis II were enhanced in mice with NA than in control mice ($P = .001$ for all; Supplementary Fig. 2B, C). Especially, the prevalence of Lactobacillus was significantly associated with gondoate biosynthesis and aerobic respiration I (Supplementary Fig. 2D). Furthermore, gondoate biosynthesis was negatively correlated, but aerobic respiration I was positively correlated with CXCL1 concentration (Supplementary Fig. 2E). These data suggest that low abundance of Lactobacillus in gut microbial EVs may contribute to AHR and neutrophil recruitment to the airways.

*Characterization of LpEV composition and measurement of LpEV-specific IgG4 antibodies in NA*

LpEV are spherical lipid bi-layered membranes with an average diameter of 43.82 ± 12.90 nm (Fig. 2A, B). Moreover, LpEV were

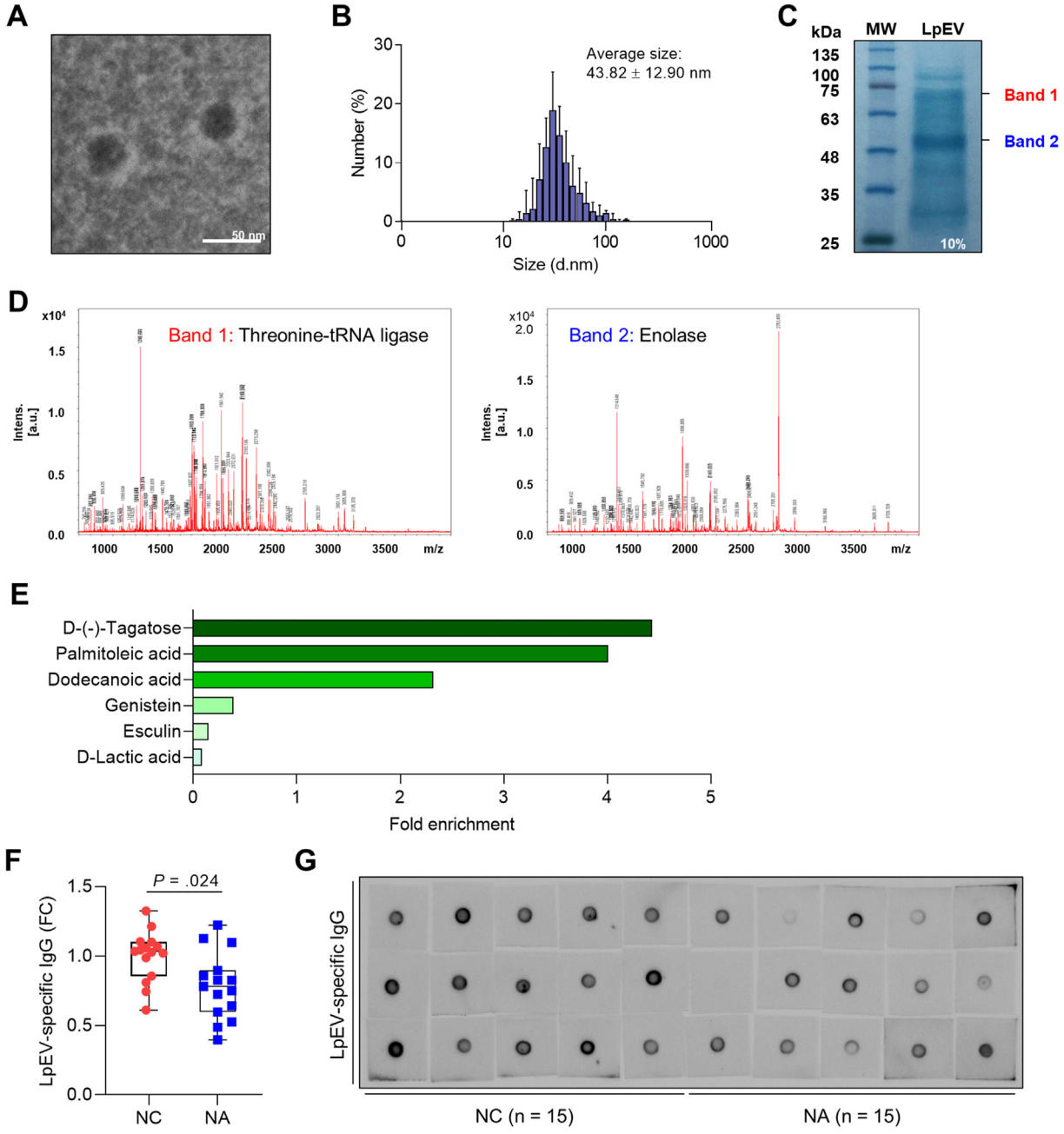


**Fig. 2.** Identification of LpEVs and evaluation of LpEV-specific IgG in mice. **(A)** EV morphology observed using transmission electron microscopy. Scare bar, 60 nm. **(B)** EV size measured using a dynamic light-scattering analyzer. Data are represented as means ± SD, n = 5. **(C)** EV protein patterns analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis. **(D)** Peptide mass fingerprinting for investigating specific proteins in the EVs. **(E)** Untargeted metabolomic analysis for finding metabolites in the EVs. **(F)** Relative abundance of LpEV-specific IgG in the serum of mice. Data are presented as box plots, n = 15. *P* value was obtained by Student's *t* test. **(G)** Dot blot analysis of LpEV-specific IgG. LpEV, *Lactobacillus paracasei*-derived extracellular vesicles; MW, molecular weight.

composed of multiple proteins including threonine-tRNA ligase and enolase (Fig. 2C, D). By metabolomic analysis, metabolites, such as D-(−)-tagatose, palmitoleic acid, and dodecanoic acid, were found to be enriched in the EVs (Fig. 2E). To determine whether *Lactobacillus* species are involved in the development of NA, the prevalence of IgG specific for EVs derived from *L. paracasei* or *L. rhamnosus* (LrEV) was evaluated in the serum of mice. As a result, mice with NA showed lower LpEV-IgG, but not LrEV-specific IgG, compared to control mice (Fig. 2F, G and Supplementary Fig. 3A, B). In humans, asthmatic subjects were classified into patients with EA and those with NA (Table 1). Between the 2 groups, there were no differences in sex, age, or lung function; however, higher blood/sputum eosinophils and serum EDN levels in patients with EA and higher sputum neutrophils and serum MPO levels in patients with NA were noted ($P < .05$ for all). Furthermore, significantly lower serum levels of LpEV-specific IgG4 were noted in asthmatics than in HCs as well as in patients with NA than in those with EA ($P = .001$ for all), while no differences were noted in LpEV-specific IgG1 levels between the 2 phenotypes (Fig. 3A, B). Moreover, the levels of LpEV-specific IgG4 were positively correlated with $FEV_1$ (%) values ($r = .432$, $P = .016$; Fig. 3C). In addition, the serum levels of IL-8 (a neutrophil-activating cytokine) were significantly higher in patients with NA than in those with EA and HCs ($P = .030$ and $P = .016$, respectively; Fig. 3D) and negatively correlated with those of LpEV-specific IgG4 (Fig. 3E). These indicate that the serum levels of LpEV-specific IgG4 may represent low abundance of LpEV in patients with NA, which is associated with lower $FEV_1$ values (%) and higher IL-8 levels.

### *Effects of LpEV on airway resistance and inflammation in mice with NA*

To evaluate effects of LpEV on neutrophilic inflammation *in vivo*, mice were orally treated with LpEV. As a result, these EVs were delivered to the lungs and even brain within 24 h (Fig. 4A). In mice with NA, AHR as well as neutrophil counts and CXCL1 and IL-17 production in the BALF were significantly increased. Moreover, enhanced immune cell infiltration and epithelial thickness in the lung tissues were noted However, LpEV treatment significantly attenuated all these findings in a dose dependent manner with more suppressive effects compared to Dex treatment (Supplementary Fig. 4A–E, 5A and Fig. 4B–E). In addition, LpEV reduced the expression and proportion of $CD4^+ROR\gamma t^+$ Th17 cells and $FOXP3^+$ Treg cells in the lungs as confirmed by Western blot and flow cytometric analysis (Fig. 4F and Supplementary Fig. 5B). These data suggest LpEV as a novel candidate immunomodulator for targeting NA.

### *Inhibition of the JNK pathway by LpEV metabolites in airway epithelium*

To investigate how LpEV regulates neutrophilic inflammation in asthmatic airways, human airway epithelial cells (AECs) were treated with LPS. As a result, enhanced JNK phosphorylation and IL-8 production were noted, which were significantly suppressed by LpEV treatments (in a dose-dependent manner) as well as Dex treatment (Supplementary Fig. 6A, B). As dodecanoic acid, palmitoleic acid, and D-(−)-tagatose were enriched in LpEV, we compared the concentration of these metabolites in serum between control and LPS-induced asthmatic mice by targeted metabolomic analysis using GC-TOF-MS. Finally, the levels of dodecanoic acid were significantly higher while those of the levels of palmitoleic acid and D-(−)-tagatose were lower in asthmatic mice than control mice (Fig. 5A–C). Especially, D-(−)-tagatose could be a potential biomarker discriminating the asthmatic group from control group (AUC = .963, $P = .002$; Fig. 5D). Then, the study further verified anti-inflammatory functions of LpEV metabolites *in vitro* and *in vivo*. In AECs stimulated with LPS, 3 metabolites significantly inhibit the JNK pathway and IL-8 secretion in AECs (Supplementary Fig. 6C–H). Moreover, when mice with NA were intranasally administered with LpEV metabolites, all metabolites markedly reduced neutrophil counts as well as CXCL1 and IL-17 concentrations in BALF (Fig. 5E–G). Furthermore, these metabolites significantly inhibited JNK phosphorylation in the lung tissues, especially in primary AECs isolated from mice (Fig. 5H, I). Finally, lung histological pictures revealed significantly decreased immune cell infiltration, epithelial thickness, and mucus production in the tissues by LpEV metabolite treatment (Fig. 5J). Here, we provide a possible mechanism of action by which LpEV attenuates airway inflammation in NA (Fig. 6).

## Discussion

This is the first study to demonstrate the therapeutic potential of LpEV in NA. Here, we found the lower proportion of *Lactobacillus* species in gut microbial EVs of mice with NA and decreased levels of LpEV-specific IgG4 in patients with NA. Moreover, LpEV treatment attenuated airway inflammation by reducing neutrophil infiltration

**Table 1**
Demographic data of the study subjects according to asthma phenotypes.

| Variables | HCs (n = 10) | Asthmatics (n = 20) | *P* value | EA (n = 10) | NA (n = 10) | *P* value |
|---|---|---|---|---|---|---|
| Age (y) | 43.0 ± 11.1 | 46.3 ± 17.2 | .539 | 43.3 ± 15.6 | 49.2 ± 19.1 | .459 |
| Female sex (%) | 30.0 | 45.5 | .429 | 50.0 | 40.0 | .653 |
| Atopy (%) | 40.0 | 75.0 | .061 | 70.0 | 80.0 | .606 |
| IgE (IU/mL) | 97.6 ± 108.2 | 428.1 ± 396.2 | .016 | 458.0 ± 438.0 | 398.3 ± 371.0 | .746 |
| TEC (cells/μL) | 89.2 ± 52.3 | 566.2 ± 432.1 | .002 | 794.0 ± 445.3 | 338.4 ± 283.6 | .015 |
| Sputum Eos (%) | ND | 32.7 ± 38.6 | ND | 53.2 ± 39.0 | .4 ± .5 | .001 |
| Sputum Neu (%) | ND | 42.1 ± 32.2 | ND | 29.2 ± 21.9 | 81.1 ± 11.3 | .001 |
| Lung function | | | | | | |
| Baseline $FEV_1$ (%) | 104.3 ± 5.4 | 92.2 ± 12.2 | .006 | 94.5 ± 12.6 | 90.0 ± 12.0 | .425 |
| $PC_{20}$ (mg/mL) | ND | 8.9 ± 6.1 | ND | 6.9 ± 6.3 | 11.0 ± 5.4 | .136 |
| Granule protein | | | | | | |
| EDN (ng/mL) | 74.9 ± 32.9 | 146.5 ± 118.2 | .021 | 182.1 ± 147.4 | 89.1 ± 43.5 | .034 |
| MPO (ng/mL) | 138.5 ± 75.4 | 304.1 ± 244.6 | .047 | 167.8 ± 201.9 | 340.1 ± 248.4 | .018 |

Values are given as n (%) for categorical variables and as mean ± SD for continuous variables.
*P* values were applied by Pearson chi-square test for categorical variables and Student's t test for continuous variables.
HC, healthy control subjects; EA, eosinophilic asthma; NA, neutrophilic asthma; IgE, immunoglobulin E; TEC, total eosinophil count; Eos, eosinophil; Neu, neutrophil; $FEV_1$, forced expiratory volume in 1 s; methacholine $PC_{20}$, the provocative concentration of methacholine required to cause a 20% fall in $FEV_1$; EDN, eosinophil-derived neurotoxin; MPO, myeloperoxidase; ND, no data.

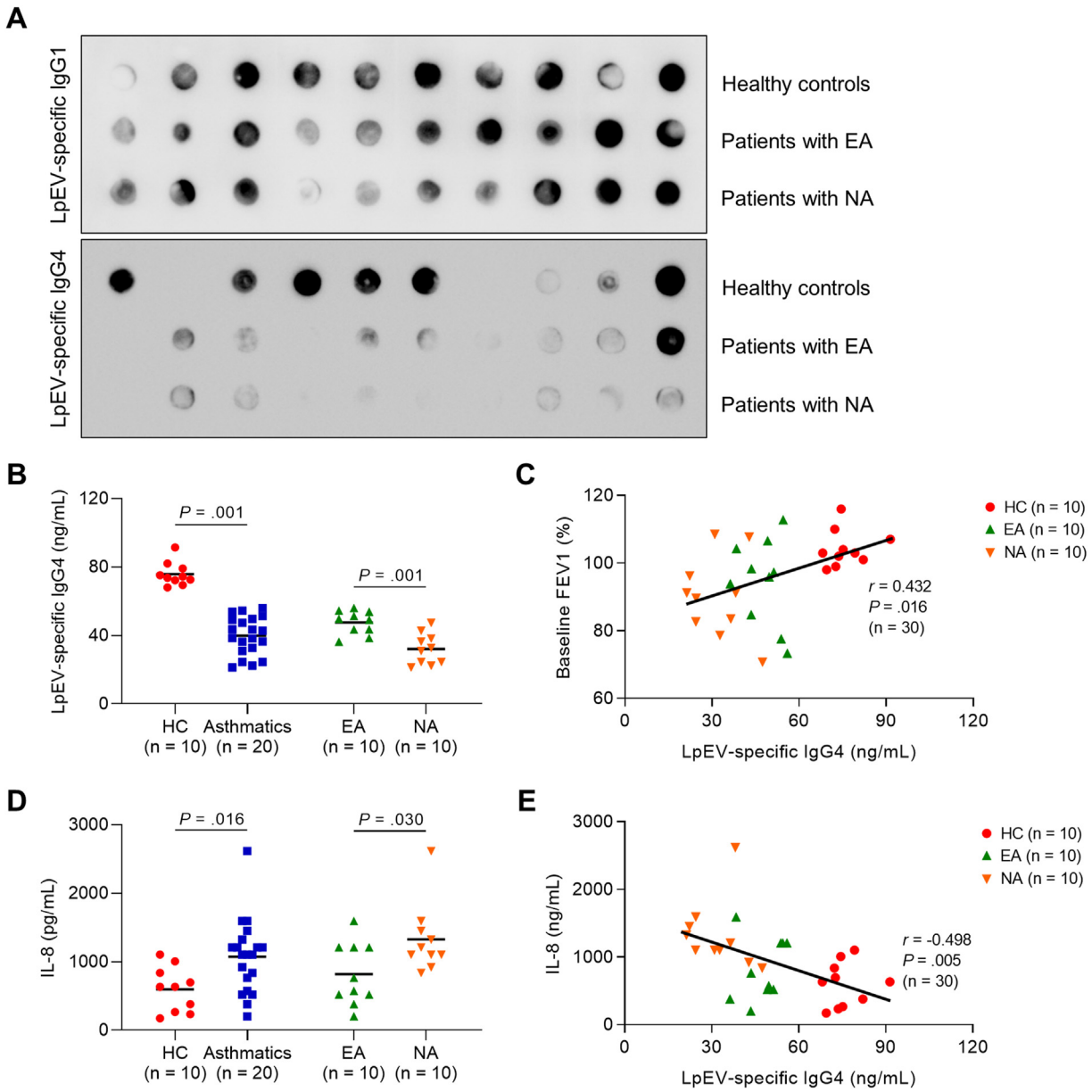


**Fig. 3.** Low levels of LpEV-specific IgG4 but high levels of IL-8 in patients with neutrophilic asthma. **(A)** Dot blot analysis of LpEV-specific IgG1 (upper panel) and IgG4 (lower panel). **(B)** The levels of LpEV-specific IgG4 in serum of the study subjects. Data are presented as means. *P* value was obtained by Student's *t* test. **(C)** A correlation between the levels of LpEV-specific IgG4 and baseline $FEV_1$ (%). Data are presented as Pearson correlation coefficient *r* (*P* value). **(D)** The levels of IL-8 in serum of the study subjects. Data are presented as means. *P* value was obtained by Student's *t* test. **(E)** A correlation between the levels LpEV-specific IgG4 and those of IL-8. Data are presented as Pearson correlation coefficient *r* (*P* value). EA, eosinophilic asthma; HC, healthy controls; NA, neutrophilic asthma.

and pro-inflammatory cytokine production in the lungs. Especially, LpEV-derived metabolites suppressed the JNK pathway in AECs *in vivo* and *in vitro*. These findings indicate that LpEV can be a new candidate immunomodulator in patients with NA.

Neutrophils, the most abundant circulating leukocytes in human blood, are involved in the first line of defense.[28,29] Neutrophilic inflammation in the asthmatic airways is related to persistent asthmatic symptoms and steroid resistance.[30] In particular, the level of IL-8, a chemoattractant and activator of neutrophils, has positive correlations with the number of airway neutrophils and the frequency of asthma exacerbations in asthmatic patients.[31] For IL-8 production in AECs, the activation of the mitogen-activated protein kinase families including ERK and JNK is required.[32] In this study, LpEV inhibited JNK phosphorylation induced by LPS in both human and murine AECs. Previously, selective JNK inhibitors have been suggested to modulate leukocyte infiltration and inflammatory cytokine production in pulmonary diseases.[33] Taken together, our data indicate that LpEV could be a novel therapeutic strategy to regulate neutrophil recruitment/infiltration in the asthmatic airways by suppressing the JNK pathway in AECs.

Much evidence has emphasized the significance of bacteria as a regulator of the host immune system.[34] Especially, probiotics known as a safety strain may prevent allergic diseases, such as food allergy or atopic dermatitis.[35,20,21] Thus, most studies to evaluate functional effect of *L. paracasei* in asthma have been focused on improving allergic airway inflammation. For example, intragastric administration of *L. paracasei* significantly reduced allergic

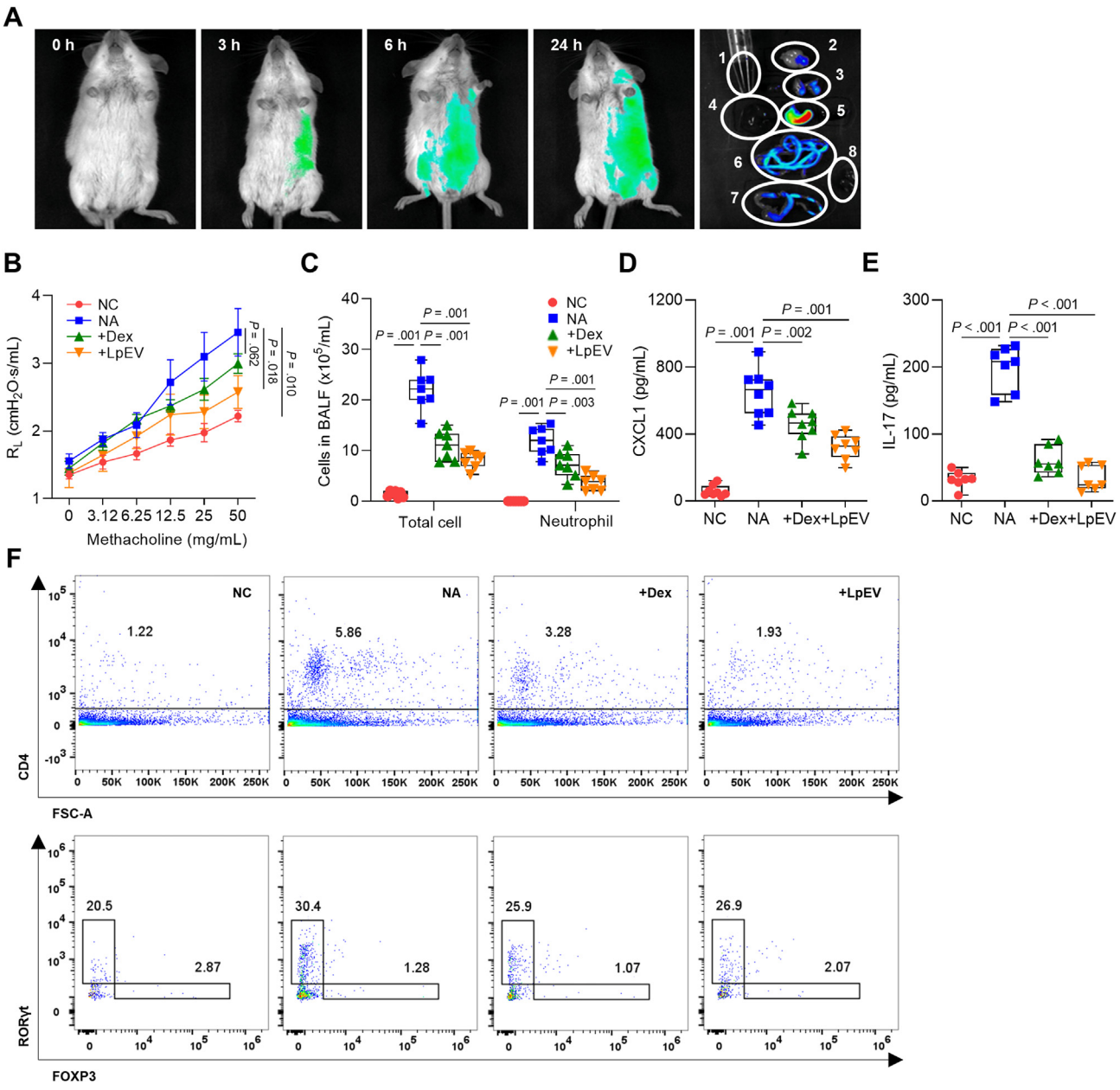


**Fig. 4.** Effects of LpEV on airway inflammation in mice with neutrophilic asthma. **(A)** Distribution and remaining time of LpEV in mice after oral administration. 1: blood; 2: brain; 3: lung; 4: liver; 5: stomach; 6: small intestine; 7: large intestine; 8: kidney. **(B)** Airway hyperresponsiveness. **(C)** Differential cell count. Concentration of **(D)** CXCL1 and **(E)** IL-17 in BALF. Data are presented as box plots, n = 8. *P* values were obtained by one-way ANOVA with Bonferroni's *post hoc* test. **(F)** Flow cytometric analysis of RORγt$^+$ Th17 and FOXP3$^+$ Treg cells in lung CD4$^+$ T cells. BALF, bronchoalveolar lavage fluid; Dex, dexamethasone; FOXP3, forkhead box P3; RORγt, retinoic-acid-receptor-related orphan nuclear receptor gamma.

inflammatory markers, such as eosinophil recruitment to the lungs and IL-5 production in the BALF.[36] Although there have been a few studies regarding immunomodulating effects of *L. paracasei* or its EVs on NA, several studies have shown that *L. paracasei* could reduce not only eosinophil counts and IL-4/5/13 levels, but also neutrophil counts and IL-17 levels in the BALF in Th2/Th17 mixed asthmatic mice model,[22,37] suggesting a possible role of *L. paracasei* to suppress neutrophilic inflammation in the airway. Recent studies have reported that Th17 cells contribute to neutrophil recruitment to the airway by releasing IL-17.[38] Especially, the levels of IL-17 were increased in serum and sputum of patients with NA, which drives persistent asthma symptoms and steroid resistance.[39] In this study, oral gavage of LpEV reduced the levels of IL-17 in the BALF as well as the proportion of Th17 cells in the lungs from mice with NA. These data indicate that LpEV may have a therapeutic potential for improving Th17-mediated neutrophilic airway inflammation in asthma.

IgG1 is the most abundant subclass in human sera and known as a mediator for antibody responses against viral pathogen. Although IgG4 is the least abundant subclass, this antibody could be a biomarker for representing the degree of exposure to external factors as it is often formed by long-term exposure to non-infectious organisms.[40] Recently, the serum levels of IgG4 specific to EVs derived from *Micrococcus luteus* were found to be decreased in patients with NA. Moreover, they have been suggested as a novel molecule to inhibit neutrophilic airway inflammation by regulating

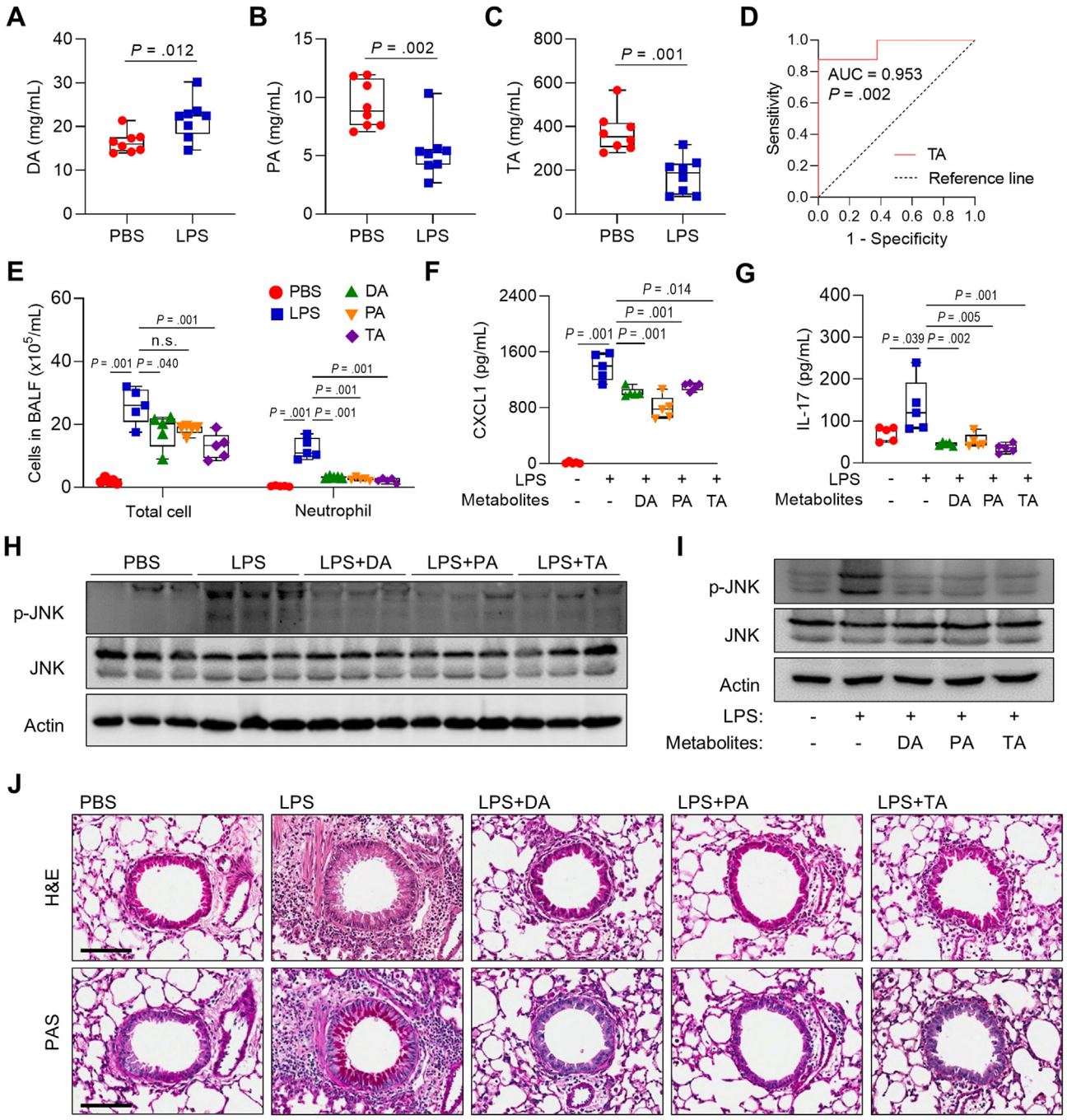


**Fig. 5.** Effects of 3 major metabolites of LpEV on airway inflammation in mice with neutrophilic asthma. The serum levels of **(A)** Dodecanoic acid, **(B)** palmitoleic acid, and **(C)** D-(−)-tagatose by targeted metabolomic analysis. Data are presented as box plots, n = 8. *P* values were obtained by Student's *t* test. **(D)** Receiver operating characteristic curve of serum levels of D-(−)-tagatose. **(E)** Differential cell count. Concentrations of **(F)** CXCL1 and **(G)** IL-17 in BALF. Data are presented as box plots, n = 5. *P* values were obtained by one-way ANOVA with Bonferroni's *post hoc* test. Phosphorylation of JNK **(H)** in the lung tissues and **(I)** epithelial cells isolated from the lungs of mice. **(J)** Lung tissues stained with H&E and PAS. Scale bar, 100 μm. AUC, area under the ROC Curve; DA, dodecanoic acid; H&E, hematoxylin and eosin; JNK, c-Jun N-terminal kinase; PA, palmitoleic acid; PAS, periodic acid Schiff; TA, D-(−)-Tagatose.

miRNA expression in AECs.[41] Similarly, this study showed that the serum levels of LpEV-specific IgG4 were significantly lower in patients with NA than in those with EA, while no differences were noted in LpEV-specific IgG1 between the groups. In this aspect, our findings suggest that low exposure to LpEV may contribute to high serum levels of IL-8 in patients with NA. Finally, we evaluated the effect of LpEV on AECs to control NA focused on neutrophil recruitment to the airway by its chemoattractant, IL-8/CXCL1, because this phenotype presents severe clinical symptoms and treatment resistance, but biologics targeting severe NA are not available.

The present study clarified various proteins and metabolites abundantly found in LpEV. Among them, dodecanoic acid is known to enhance Th1/Th17 cell differentiation.[42] Moreover, it was decreased in the serum of OVA-induced asthmatic mice, suggesting its potential as a biomarker in allergic asthma.[43] In contrast, palmitoleic acid could reduce IFN-γ, IL-6, and IL-17 production in human $CD4^+$ T cells and suppressed NF-κB phosphorylation in

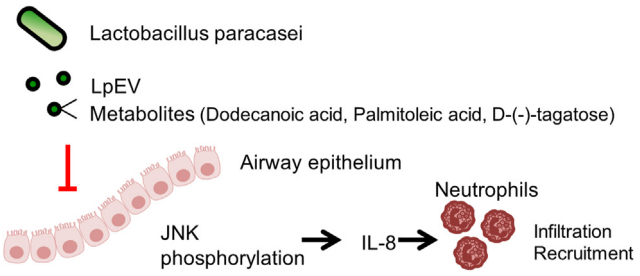


**Fig. 6.** Schematic of LpEV inhibiting airway inflammation in neutrophilic asthma. Suppression of neutrophilic inflammation in the airway via JNK pathway by LpEV.

murine lungs.[44,45] Although the function of D-(−)-tagatose in inflammation is not determined, it acts as a prebiotic to modulate gut microflora by increasing beneficial bacteria, but decreasing pathogenic bacterial load.[46] In the study, neutrophilic asthmatic mice showed increased levels of dodecanoic acid, but decreased levels of palmitoleic acid and D-(−)-tagatose in serum. However, unlike previous studies focusing on T cells, our study emphasized the anti-inflammatory effects of metabolites on AECs. Furthermore, both *in vitro* and *in vivo* studies demonstrated that these 3 metabolites could attenuate neutrophilic recruitment by reducing IL-8/CXCL1 production via the JNK pathway, indicating that the suppressive effects of LpEV on neutrophilic airway inflammation may be derived from these 3 metabolites.

In conclusion, LpEV metabolites could attenuate AEC activation, which suppresses neutrophil recruitment and pro-inflammatory cytokine production in patients with NA. These findings might provide an insight into new therapeutic strategies using microbial EVs for asthma treatment.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2023.10.008.

*Conflict of interest*

The authors have no conflict of interest to declare.

*Authors' contributions*

SS designed the experiments, analyzed the data, and wrote the paper. HJP isolated microbial EVs. YKK helped design the experiments. YC and HSP provided overall supervision for the entire study.

## References

1. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;**18**:716–25.
2. Ray A, Kolls JK. Neutrophilic inflammation in asthma and association with disease severity. *Trends Immunol* 2017;**38**:942–54.
3. Lee Y, Quoc QL, Park HS. Biomarkers for severe asthma: lessons from longitudinal cohort studies. *Allergy Asthma Immunol Res* 2021;**13**:375–89.
4. Mikhail I, Grayson MH. Asthma and viral infections: an intricate relationship. *Ann Allergy Asthma Immunol* 2019;**123**:352–8.
5. Drescher B, Bai F. Neutrophil in viral infections, friend or foe? *Virus Res* 2013;**171**:1–7.
6. Edwards MR, Bartlett NW, Hussell T, Openshaw P, Johnston SL. The microbiology of asthma. *Nat Rev Microbiol* 2012;**10**:459–71.
7. Crisford H, Sapey E, Rogers GB, Taylor S, Nagakumar P, Lokwani R, et al. Neutrophils in asthma: the good, the bad and the bacteria. *Thorax* 2021;**76**:835–44.
8. Park HK, Choi Y, Lee DH, Kim S, Lee JM, Choi SW, et al. Altered gut microbiota by azithromycin attenuates airway inflammation in allergic asthma. *J Allergy Clin Immunol* 2020;**145**:1466–9. e8.
9. Yang X, Li H, Ma Q, Zhang Q, Wang C. Neutrophilic asthma is associated with increased airway bacterial burden and disordered community composition. *Biomed Res Int* 2018;**2018**:9230234.
10. Taylor SL, Leong LEX, Choo JM, Wesselingh S, Yang IA, Upham JW, et al. Inflammatory phenotypes in patients with severe asthma are associated with distinct airway microbiology. *J Allergy Clin Immunol* 2018;**141**:94–103. e15.
11. Einarsson GG, Comer DM, McIlreavey L, Parkhill J, Ennis M, Tunney MM, et al. Community dynamics and the lower airway microbiota in stable chronic obstructive pulmonary disease, smokers and healthy non-smokers. *Thorax* 2016;**71**:795–803.
12. Zemanick ET, Wagner BD, Robertson CE, Ahrens RC, Chmiel JF, Clancy JP, et al. Airway microbiota across age and disease spectrum in cystic fibrosis. *Eur Respir J* 2017;**50**:1700832.
13. Chung KF. Airway microbial dysbiosis in asthmatic patients: a target for prevention and treatment? *J Allergy Clin Immunol* 2017;**139**:1071–81.
14. Yu J, Jang SO, Kim BJ, Song YH, Kwon JW, Kang MJ, et al. The effects of Lactobacillus rhamnosus on the prevention of asthma in a murine model. *Allergy Asthma Immunol Res* 2010;**2**:199–205.
15. Jang SO, Kim HJ, Kim YJ, Kang MJ, Kwon JW, Seo JH, et al. Asthma prevention by Lactobacillus rhamnosus in a mouse model is associated with CD4(+)CD25(+) Foxp3(+) T cells. *Allergy Asthma Immunol Res* 2012;**4**:150–6.
16. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science* 2020;**367**:eaau6977.
17. Chronopoulos A, Kalluri R. Emerging role of bacterial extracellular vesicles in cancer. *Oncogene* 2020;**39**:6951–60.
18. Choi Y, Park HS, Jee YK. Urine microbial extracellular vesicles can Be potential and novel biomarkers for allergic diseases. *Allergy Asthma Immunol Res* 2021;**13**:5–7.
19. Choi Y, Park HS, Kim YK. Bacterial extracellular vesicles: a candidate molecule for the diagnosis and treatment of allergic diseases. *Allergy Asthma Immunol Res* 2023;**15**:279–89.
20. Kim JH, Jeun EJ, Hong CP, Kim SH, Jang MS, Lee EJ, et al. Extracellular vesicle-derived protein from Bifidobacterium longum alleviates food allergy through mast cell suppression. *J Allergy Clin Immunol* 2016;**137**:507–16. e8.
21. Kim MH, Choi SJ, Choi HI, Choi JP, Park HK, Kim EK, et al. Lactobacillus plantarum-derived extracellular vesicles protect atopic dermatitis induced by Staphylococcus aureus-derived extracellular vesicles. *Allergy Asthma Immunol Res* 2018;**10**:516–32.
22. Wang X, Hui Y, Zhao L, Hao Y, Guo H, Ren F. Oral administration of Lactobacillus paracasei L9 attenuates PM2.5-induced enhancement of airway hyperresponsiveness and allergic airway response in murine model of asthma. *PLoS One* 2017;**12**:e0171721.
23. Choi JH, Moon CM, Shin TS, Kim EK, McDowell A, Jo MK, et al. Lactobacillus paracasei-derived extracellular vesicles attenuate the intestinal inflammatory response by augmenting the endoplasmic reticulum stress pathway. *Exp Mol Med* 2020;**52**:423–37.
24. Fournier BM, Parkos CA. The role of neutrophils during intestinal inflammation. *Mucosal Immunol* 2012;**5**:354–66.
25. Chelakkot C, Choi Y, Kim DK, Park HT, Ghim J, Kwon Y, et al. Akkermansia muciniphila-derived extracellular vesicles influence gut permeability through the regulation of tight junctions. *Exp Mol Med* 2018;**50**:e450.
26. Lee DH, Park HK, Lee HR, Sohn H, Sim S, Park HJ, et al. Immunoregulatory effects of Lactococcus lactis-derived extracellular vesicles in allergic asthma. *Clin Transl Allergy* 2022;**12**:e12138.
27. Choi Y, Kwon Y, Kim DK, Jeon J, Jang SC, Wang T, et al. Gut microbe-derived extracellular vesicles induce insulin resistance, thereby impairing glucose metabolism in skeletal muscle. *Sci Rep* 2015;**5**:15878.
28. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* 2011;**11**:519–31.
29. Ham J, Kim J, Ko YG, Kim HY. The dynamic contribution of neutrophils in the chronic respiratory diseases. *Allergy Asthma Immunol Res* 2022;**14**:361–78.
30. Manni ML, Trudeau JB, Scheller EV, Mandalapu S, Elloso MM, Kolls JK, et al. The complex relationship between inflammation and lung function in severe asthma. *Mucosal Immunol* 2014;**7**:1186–98.
31. Ordonez CL, Shaughnessy TE, Matthay MA, Fahy JV. Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: clinical and biologic significance. *Am J Respir Crit Care Med* 2000;**161**:1185–90.
32. Li J, Kartha S, Iasvovskaia S, Tan A, Bhat RK, Manaligod JM, et al. Regulation of human airway epithelial cell IL-8 expression by MAP kinases. *Am J Physiol Lung Cel Mol Physiol* 2002;**283**:L690–9.
33. Bennett BL. c-Jun N-terminal kinase-dependent mechanisms in respiratory disease. *Eur Respir J* 2006;**28**:651–61.
34. Belkaid Y, Hand TW. Role of the microbiota in immunity and inflammation. *Cell* 2014;**157**:121–41.
35. Sharma G, Im SH. Probiotics as a potential immunomodulating pharmabiotics in allergic diseases: current status and future prospects. *Allergy Asthma Immunol Res* 2018;**10**:575–90.
36. Pellaton C, Nutten S, Thierry AC, Boudousquie C, Barbier N, Blanchard C, et al. Intragastric and intranasal administration of Lactobacillus paracasei NCC2461

modulates allergic airway inflammation in mice. *Int J Inflam* 2012;**2012**:686739.
37. Lin CH, Tseng CY, Chao MW. Administration of Lactobacillus paracasei HB89 mitigates PM2.5-induced enhancement of inflammation and allergic airway response in murine asthma model. *PLoS One* 2020;**15**:e0243062.
38. Laan M, Cui ZH, Hoshino H, Lotvall J, Sjostrand M, Gruenert DC, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *J Immunol* 1999;**162**:2347–52.
39. Wei Q, Liao J, Jiang M, Liu J, Liang X, Nong G. Relationship between Th17-mediated immunity and airway inflammation in childhood neutrophilic asthma. *Allergy Asthma Clin Immunol* 2021;**17**:4.
40. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 2014;**5**:520.
41. Sim S, Lee DH, Kim KS, Park HJ, Kim YK, Choi Y, et al. Micrococcus luteus-derived extracellular vesicles attenuate neutrophilic asthma by regulating miRNAs in airway epithelial cells. *Exp Mol Med* 2023;**55**:196–204.
42. Hammer A, Schliep A, Jorg S, Haghikia A, Gold R, Kleinewietfeld M, et al. Impact of combined sodium chloride and saturated long-chain fatty acid challenge on the differentiation of T helper cells in neuroinflammation. *J Neuroinflammation* 2017;**14**:184.
43. Yu M, Cui FX, Jia HM, Zhou C, Yang Y, Zhang HW, et al. Aberrant purine metabolism in allergic asthma revealed by plasma metabolomics. *J Pharm Biomed Anal* 2016;**120**:181–9.
44. Passos ME, Alves HH, Momesso CM, Faria FG, Murata G, Cury-Boaventura MF, et al. Differential effects of palmitoleic acid on human lymphocyte proliferation and function. *Lipids Health Dis* 2016;**15**:217.
45. Souza CO, Teixeira AA, Biondo LA, Silveira LS, Calder PC, Rosa Neto JC. Palmitoleic acid reduces the inflammation in LPS-stimulated macrophages by inhibition of NFkappaB, independently of PPARs. *Clin Exp Pharmacol Physiol* 2017;**44**:566–75.
46. Roy S, Chikkerur J, Roy SC, Dhali A, Kolte AP, Sridhar M, et al. Tagatose as a potential nutraceutical: production, properties, biological roles, and applications. *J Food Sci* 2018;**83**:2699–709.