

# *Lactobacillus gasseri* attenuates allergic airway inflammation through PPAR $\gamma$ activation in dendritic cells

Miao-Hsi Hsieh<sup>1</sup> · Ren-Long Jan<sup>2,3</sup> · Lawrence Shih-Hsin Wu<sup>4</sup> · Pei-Chi Chen<sup>1,5</sup> · Hui-Fang Kao<sup>5,6</sup> · Wen-Shuo Kuo<sup>5</sup> · Jiu-Yao Wang<sup>2,5,7</sup>

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## Abstract

Lactobacilli prevent the early development of allergic diseases in children and experimental asthma in mice. However, the detailed mechanism underlying this action remains unknown. We aimed to explore the activation pathway in the host by *Lactobacillus* and identify its immunomodulation mechanism in allergic asthma. Continuous administration of  $10^7$  cfu, but not  $10^9$  cfu, of *L. gasseri* for 4 weeks prevented *Dermatophagoides pteronyssinus* (Der p)-induced airway hypersensitivity and inflammation in a mouse model of allergic asthma. DNA microarray analysis of the mesenteric and lung draining lymph nodes revealed a significant decrease in inflammatory chemokines and increase in gene expression in carbohydrate and lipid

metabolism, particularly of PPAR $\gamma$ , in  $10^7$  cfu *L. gasseri*-administered mice compared with untreated mice. Compared with WT mice, Der p-sensitized PPAR $\gamma^{L/+}$  mice showed increased airway hyperresponsiveness to methacholine, inflammatory cell infiltration, and inflammatory cytokine secretion in bronchoalveolar fluid. Moreover, the protective effects of *L. gasseri* were lost in Der p-induced airway inflammation in PPAR $\gamma^{L/+}$  mice, and *L. gasseri*-induced PPAR $\gamma$  activation in BMDCs inhibited the development of allergic airway inflammation in both PPAR $\gamma$  WT and PPAR $\gamma^{L/+}$  mice. *L. gasseri* may act via a novel PPAR $\gamma$  activation pathway in DCs to alleviate allergen-induced airway inflammation in allergic asthma.

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✉ Jiu-Yao Wang  
a122@mail.ncku.edu.tw

- <sup>1</sup> Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan
- <sup>2</sup> Department of Pediatrics, Chi-Mei Medical Center, Liou-Yin, Tainan, Taiwan
- <sup>3</sup> Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
- <sup>4</sup> Institute of Medical Sciences, Tzu Chi University, Hualien, Taiwan
- <sup>5</sup> Center for Allergy and Clinical Immunology Research (ACIR), College of Medicine, National Cheng Kung University, No.1 University Road, Tainan 70428, Taiwan
- <sup>6</sup> Department of Nursing, National Tainan Junior College of Nursing, Tainan, Taiwan
- <sup>7</sup> Graduate Institute of Integrated Medicine, School of Chinese Medicine, China Medical University, Taichung, Taiwan

## Key messages

- *L. gasseri* prevents mite allergen (Der p)-induced airway inflammation.
- Prevention of airway inflammation occurs via activation of PPAR $\gamma$  in dendritic cells.
- *L. gasseri* administration does not reverse Der p-induced airway inflammation in PPAR $\gamma^{(+/-)}$  mice.
- *L. gasseri*-induced PPAR $\gamma$  activation inhibits development of airway inflammation in WT and PPAR $\gamma^{(+/-)}$  mice.

**Keywords** Allergy asthma · Lactic acid bacillus · Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) · Dendritic cells

## Introduction

Allergic asthma is the most common chronic disease in affluent countries, and its prevalence is increasing in developing countries [1, 2]. Presently, only symptom control and anti-inflammatory agents for allergic asthma are available, but the only effective immune regulatory treatment

and prevention methods are allergen-specific immunotherapy by subcutaneous or sublingual routes [3]. The microbiome and gut microbiota have been recently reported to be related to the metabolic syndromes of diabetes mellitus, obesity, hypertension, and chronic inflammatory diseases such as asthma [4, 5]. The hygiene hypothesis [6], advocated in the last century, emphasizes that the lack of bacterial nosocomial or exogenous stimulation in early life may lead to the deviation of host immune response and development of allergic disease, as supported by recent clinical observations [7, 8] and laboratory findings [9, 10].

Probiotics, such as lactobacillus, prevent the early development of allergic diseases in children and experimental asthma in mice [11, 12]. Probiotics adhere to the intestinal mucosa and cause the local production of metabolites and short-chain fatty acids (SCFAs), which act as transducing signals for immune cell development and have an anti-inflammatory effect [13, 14]. Trompette et al. [13] reported that the microbiota and dietary fibers modulate allergic inflammation in the lungs by skewing immune cell development in the bone marrow (BM) towards a less inflammatory phenotype, and treatment with a SCFA, propionate, induced the production of BM-derived dendritic cells (BMDCs) with impaired Th2-stimulatory abilities. However, the detailed mechanism of probiotic interactions with host target molecules and the resulting immunomodulatory effects in allergic diseases remain unidentified. We previously reported that the oral intake of *Lactobacillus gasseri* for 2 months improved mucosal allergic symptoms in asthmatic children with allergic rhinitis in a double-blind, randomized controlled (DBRC) trial [15]. A study of a mouse model of allergic asthma revealed that a *L. gasseri*-attenuated allergen induced airway inflammation and suppressed Th17 cell infiltration in the lungs [12]. In this study, we used DNA microarrays to screen and compare changes in gene expression in the draining lymph nodes of the intestine and lung resulting from two dosages of *L. gasseri* that exert different protective effects in allergic asthma. We identified a group of genes involved in lipid metabolism, whose expression was associated with the administration of low-dose, but not high-dose, *L. gasseri* to mice. One of these genes, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), was significantly upregulated and is known for its anti-inflammatory roles. In this study, we used a PPAR $\gamma^{L/+}$ -deficient heterozygous mouse model to demonstrate the ability of *L. gasseri* to prevent mite (*Dermatophagoides pteronyssinus*, Der p)-induced airway inflammation through PPAR $\gamma$  activation in DCs. Our results reveal a novel mechanistic pathway by which probiotics help maintain host homeostasis and provide promising target molecules for the prevention and treatment of allergic asthma.

## Materials and methods

### Animals and reagents

Female BALB/c and C57BL/6 mice, aged 6–8 weeks, were obtained from the Laboratory Animal Center of the College of Medicine, National Cheng Kung University. The animal research in this study is according to the affidavit of approval of animal use protocol National Cheng Kung University, and the Institutional Animal Care and Use Committee (IACUC) approval No is 101096. PPAR $\gamma^{L/+}$  mice were provided by Dr. Yau-Sheng Tsai, Yale University School of Medicine [16]. The mice were generated by mutating codon 465 of mouse *Pparg* (equivalent to codon 467 of human *PPARG*) from CCC (proline) to CTG (leucine) through gene targeting in mouse embryonic stem cells, and the generated mice carried the P465L substitution. Homozygosity for the P465L mutation is lethal in utero; therefore, for the following studies, we used F1 offspring, which are termed PPAR $\gamma^{L/+}$  mice, which were derived from crosses between heterozygous *Pparg*<sup>P465L/+</sup> and C57BL/6 wild-type (WT) mice [16]. Der p extract (1 g of lyophilized whole-body extract in diethyl ether; Allergon, Engelholm, Sweden) was dissolved in pyrogen-free isotonic saline, filtered through a 0.22- $\mu$ m filter, and stored at  $-70^{\circ}\text{C}$  until use. The lipopolysaccharide concentration of the Der p preparations was  $<0.96$  ng/mL (limulus amoebocyte lysate test; E-Toxate; Sigma-Aldrich, St Louis, MO, USA).

### Probiotic preparations

*L. gasseri* A5, which exhibits probiotic characteristics, was isolated from the intestinal tract of normal, healthy neonates at ProMD Biotech Company Limited (Tainan, Taiwan) with a high-throughput detection system. Acute oral toxicity studies (FR-AC00235) conducted by the Development Center for Biotechnology (Taipei, Taiwan) in mice that were orally administered the highest dose (5000 mg/kg) of *L. gasseri* A5 for 14 days revealed no resulting side effects. To prepare a stock solution for ex vivo experiments, bacteria from frozen stocks were suspended in de Man–Rogosa–Sharpe liquid medium (Difco Laboratories, Detroit, MI, USA), plated on de Man–Rogosa–Sharpe agar, cultured anaerobically at  $37^{\circ}\text{C}$  for 24 h, inoculated into fresh de Man–Rogosa–Sharpe broth, and grown in 50-mL tubes at  $37^{\circ}\text{C}$  under anaerobic conditions for 48 h. A high-density culture system was used to produce freeze-dried powder of *L. gasseri* A5 at concentrations exceeding  $10^{11}$  colony-forming units (cfu)/g (ProMD Biotech Company Limited); the powder was stored at a temperature below  $4^{\circ}\text{C}$ . Stability tests demonstrated that cell viability could be maintained at room temperature for up to 3 months.

## Treatment protocols

Each experiment included two groups (control and allergen-challenged mice;  $n = 8$ ). The controls were sensitized; the allergen-challenged mice were subcutaneously immunized twice at 1 week intervals with a mixture containing 40  $\mu\text{g}$  of Der p in incomplete Freund's adjuvant (Difco Laboratories) and were subsequently challenged intratracheally with 50  $\mu\text{g}$  of Der p 7 days later. The treatment groups received daily oral administrations of high ( $1 \times 10^9$  cfu) or low ( $1 \times 10^7$  cfu) doses of *L. gasseri* in 700 mL of phosphate-buffered saline (PBS) by using a gavage needle, starting from 14 days before the first immunization with mite extract (day 0) until the allergen challenge (day 14). Untreated, Der p-sensitized, and challenged mice received PBS instead and served as the positive controls. The mice were subjected to measurements of airway responsiveness and bronchoalveolar lavage (BAL).

## Invasive measurement of airway hyperresponsiveness

Forty-eight hours after the last saline or Der p exposure, the mice were anesthetized with intraperitoneal injections of pentobarbital sodium (60 mg/kg). Respiratory system resistance (RrS) data were collected as an invasive measure of airway responsiveness (AHR) to increasing concentrations of aerosolized methacholine (MCh, Sigma–Aldrich) by using a computer-controlled small animal ventilator (FlexiVent System®, SCIREQ, Montreal, Quebec, Canada) [17].

## Cell infiltrates and cytokine levels in BAL fluid

BAL fluid (BALF) was collected, and total and differential cell counts in the BALF were determined as previously reported [18]. Total cells in cytocentrifuge preparations of BALF were stained with Liu's stain for differential cell counts. The concentrations of tumor necrosis factor alpha (TNF $\alpha$ ); interferon gamma (IFN $\gamma$ ); interleukin (IL)-6, -10, -13, and -17A; and thymus and activation-regulated chemokine (TARC) in BALF from the first collection were measured using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA).

## Lung histology and immunohistochemistry

Lung histology was performed as previously described [18]. In brief, lung sections were stained with periodic acid-Schiff according to the manufacturer's protocol (Sigma–Aldrich). Antibody (Ab) staining was performed with the UltraTek Alk-Phos antirabbit (permanent red) staining system (ScyTek Laboratories, Inc., Logan, UT, USA). The slides were counterstained with hematoxylin and eosin and mounted. Images were acquired using an Olympus BX50 light

microscope (Olympus, Center Valley, PA, USA) with an Optronics Magnafire digital camera (Optronics, Inc., Muskogee, OK, USA).

## Quantification of immunofluorescence staining

The amounts and expression patterns of PPAR $\gamma$  in the lung sections were analyzed after immunohistochemical staining with a primary Ab against PPAR $\gamma$  (rabbit Ab, Cayman, USA). Diaminobenzidine was used as the chromogen, and the staining was automatically performed using BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) at the Image Analysis Core Lab at the College of Medicine, National Cheng Kung University. Whole lung tissues were subsequently analyzed using a TissueFAXS Plus image cytometer (TissueGnostics GmbH, Vienna, Austria) with the TissueFAXS version 3.0.5120.119 acquisition software. Moreover, the specific PPAR $\gamma$  density in lung epithelial cells was analyzed with HistoQuest version 3.0.3.0158 software (TissueGnostics) [19]. Pulmonary PPAR $\gamma$  levels were expressed as the percentages of PPAR $\gamma$ -positive cells among the total cells in the lung sections.

## Total immunoglobulin E levels and Der p-specific IgE and IgG1 titers

Serum samples were obtained from blood collected 72 h after the final saline or Der p exposure. Der p-specific IgE, IgG1, and IgG2a titers in sera were assayed using ELISA with reagents and monoclonal Abs (mAbs) purchased from Bethyl Laboratories, Inc. (Montgomery, TX, USA). Sera were diluted two times with buffer to detect Der p-specific IgE, and sera were diluted 1000 $\times$  times with buffer to detect Der p-specific IgG1 and IgG2a.

## Flow cytometry analysis

BALF cells from the mice were incubated with 1 mg/10<sup>6</sup> cells of an Fc receptor-blocking Ab (clone 24G2; American Type Culture Collection, Manassas, VA, USA) and stained with phycoerythrin (PE)-Cy3-conjugated antimouse CD3, PE-conjugated antimouse DX5 (pan natural killer [NK] cells), PE-conjugated antimouse F4/80 Abs, allophycocyanin-conjugated antimouse c-Kit, and allophycocyanin-Cy5-conjugated antimouse CD11b. After cell surface staining, the cells were washed, fixed, and permeabilized with fix-perm solution for intracellular staining with fluorescein isothiocyanate-conjugated antimouse IL-17 Ab (clone eBio17B7, eBioscience, San Diego, CA, USA). All fluorophore-conjugated Abs and the corresponding isotypes were purchased from eBioscience. The stained cells were subjected to flow cytometry (BD LSR II).

## Culture and adoptive transfer of BMDCs

BMDCs and purified CD11c<sup>+</sup> BMDCs were generated as previously described [20]. The percentages of CD11c<sup>+</sup> BMDCs and the major histocompatibility complex (MHC) class II and CD86 expression of CD11c<sup>+</sup> BMDCs were evaluated through flow cytometry with the following mAbs: FITC anti-CD11c (clone HL3; BD Biosciences, San Jose, CA, USA), PE-Cy5 anti-MHC class II (clone M5/114.15.2), and PE-Cy5 anti-CD86 (clone GL1) (both from eBioscience). In addition,  $1 \times 10^6$  Der p-primed CD11c<sup>+</sup> BMDCs from WT and PPAR $\gamma$ <sup>L/+</sup> mice were adoptively transferred intratracheally into naïve WT and PPAR $\gamma$ <sup>L/+</sup> mice according to the protocols shown in Figs. 4a and 5a, respectively. The method of Ro treatment was that  $1 \times 10^6$  BMDCs were treated with 50 µg/ml Derp or 20 µM Ro plus 50 µg/ml Derp 24 h and then the cells were adoptively transferred intratracheally into WT or PPAR $\gamma$ <sup>L/+</sup> mice. Ten days later, the recipient mice were intranasally challenged with 20 µg/mL of Der p by instillations for 6 days. AHR was measured 24 h postchallenge, and the recipient mice were sacrificed after 72 h.

## GeneChip analysis

A GeneChip analysis was performed according to the standard Affymetrix protocol (Santa Clara, CA, USA) by using RNA extracted from the lung and mesenteric draining lymph nodes of Der p-sensitized mice treated with high or low doses of *L. gasseri*. Untreated Der p-sensitized and challenged mice served as the positive controls. Following probe preparation according to the manufacturer's instructions, fragmented cRNAs were hybridized on Affymetrix GeneChips by using quantitative controls; they were then processed and scanned according to the manufacturer's instructions. A murine Affymetrix microarray chip (no. 430A) containing 19,000 murine genes was analyzed in duplicate, and murine Affymetrix microarray chip no. U74Av2 (containing 12,000 murine genes but recognizing different probe sets) was used to further confirm the results obtained using the 430A microarray chip. The data were analyzed with GeneChip Operating Software (version 1.0; Affymetrix). In each case, low- and high-dose *L. gasseri*-administered mice were compared with the mock treatment controls. The results were calculated using GeneSpring (version 4.2.1, Silicon Genetics, Mountain View, CA, USA); they are expressed as the relative change in the treated mice compared with the controls.

## Pathway analysis

A total of 682 and 765 genes of the lung and mesenteric draining lymph nodes, respectively, pre-filtered to have an average difference score of 100 and showing a mean change (both decrease and increase) in gene expression of at least

twofold between the treated and control mice were used for the network generation and pathway analysis. GenBank™ accession numbers were imported into the Ingenuity Pathway Analysis Application Tool (Ingenuity Systems, CA, USA) and mapped to the Ingenuity knowledge base, and the genes were assembled into biological networks and ranked by score. A score of 3 indicates a 1/1000 chance that a certain gene was placed in a network due to random chance. Therefore, a score of  $\geq 3$  represents 99.9% confidence of not being generated by a random chance alone. This score was used as the cutoff for identifying gene networks significantly affected by low- or high-dose *L. gasseri* administration in mice with allergic asthma. Furthermore, significantly and differentially expressed genes were grouped on the basis of molecular functions (tasks performed by the individual gene product) and biological processes (broad biological goals accomplished by ordered assemblies of molecular functions) by using the DAVID database [21].

## Real-time SYBR-green dye I polymerase chain reaction analysis

To confirm the differential expression of candidate genes identified by the GeneChip analysis among low- and high-dose *L. gasseri*-treated and control mice with allergic asthma, quantitative polymerase chain reaction (PCR) was performed using the LightCycler® FastStart DNA Master SYBR Green I kit, as recommended by the manufacturer (Roche Applied Science, Meylan, France) with 1 mL of matrix cDNA primers. The following primers were designed for PPAR $\gamma$ : ATCCAAGACAACCTGCTG (forward) and TTCCCCTCAAAAATAATAGTGC (reverse). The reactions were performed in triplicate by using three independently extracted RNA samples. Gene expression was normalized to the housekeeping genes *ACTB*, *RPLP0*, and *TBP*, which encode beta-actin, ribosomal protein P0, and TATA box-binding protein, respectively.

## Statistical analysis

Biological functions and pathways were generated and analyzed using Ingenuity Pathway Analysis® v.7.0 (IPA, Ingenuity Systems) from all the data and relevant gene sets derived from the GeneChip analysis. Mediator contents in culture supernatants and Ab production values were analyzed by one-way analysis of variance, followed by post hoc comparisons between groups. The cellular accumulation and mediator contents in BALF and serum Ab titers were analyzed using the Mann–Whitney *U* test. The results are expressed as the means and standard errors;  $p < 0.05$  was considered significant.

## Results

### Oral administration of low-dose *L. gasseri* reduced mite-induced airway inflammation and hyperresponsiveness

To demonstrate the protective effects of *L. gasseri* on allergen-induced airway inflammation, low ( $1 \times 10^7$  cfu) and high ( $1 \times 10^9$  cfu) doses of *L. gasseri* were administered orally for 4 weeks before and after Der p sensitization and challenge. Figure 1a shows that the mice that were administered low doses ( $1 \times 10^7$  cfu) of *L. gasseri* had significantly reduced airway resistance (RrS) after stimulation with the maximal dose of MCh (50 mg/mL) compared with the sensitized mice that were administered high doses of *L. gasseri* ( $1 \times 10^9$  cfu;  $p < 0.05$ ) or PBS only ( $p < 0.01$ ). A histological analysis demonstrated increases in inflammatory cell infiltration in the lung parenchyma and peribronchial areas (small arrow) as well as bronchial epithelial damages in Der p-sensitized and challenged mice (Fig. 1b). In contrast, inflammatory cell infiltration and bronchial epithelial damage were significantly reduced in the sensitized mice administered low doses of *L. gasseri* but were only slightly improved in those administered high doses (Fig. 1b). These findings were consistent with the results of inflammatory cells in BALF obtained from these mice (Fig. 1c). Significant reductions were observed in the total cell and eosinophil counts in BALF obtained from the sensitized mice treated with a low dose of *L. gasseri* compared with the sensitized mice treated with PBS ( $p < 0.05$ ). Although no significant change was observed in serum levels of anti-Der p-specific IgE Ab, the sensitized mice administered low doses of *L. gasseri* showed significantly decreased levels of anti-Der p IgG1 but increased levels of anti-Der p IgG2 $\alpha$  Abs compared with the PBS-treated mice ( $p < 0.05$ ; Fig. 1d). Low and high doses of *L. gasseri* significantly reduced TARC, TNF $\alpha$ , and IL-6 levels in BALF from the treated mice compared with the control mice (Fig. 1e). Moreover, low doses of *L. gasseri* reduced IL-17 and increased IL-10 levels in Der p-stimulated splenocytes (Fig. 1f).

### DNA microarray comparisons of draining lymph nodes of the intestine and lung

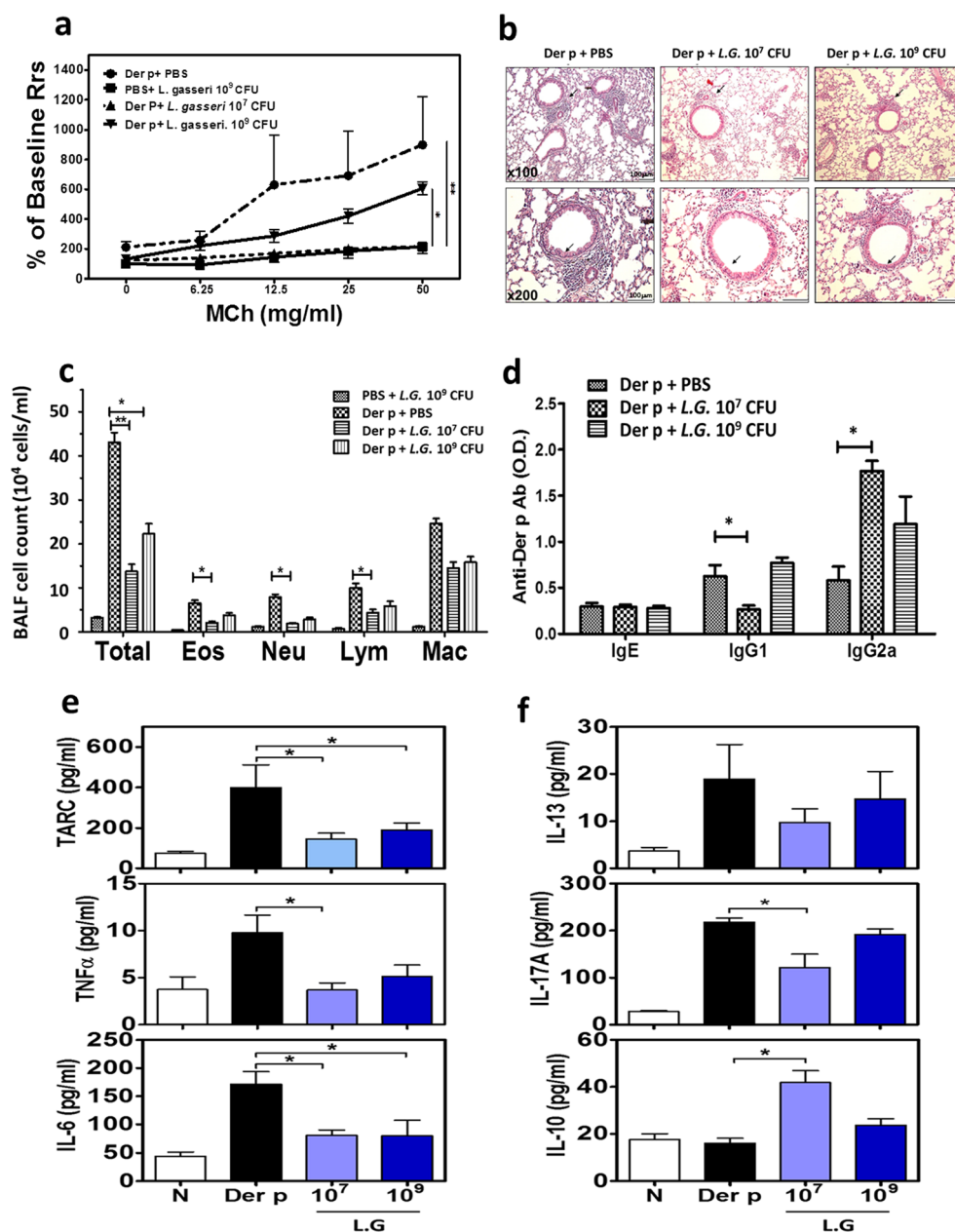
To explore the factors influencing the outcomes of allergen-induced airway inflammation due to different doses of probiotics, we used DNA microarrays to compare draining lymph nodes in the mesenteries and lungs that were obtained from Der p-sensitized mice administered high or low doses of *L. gasseri*. The DNA genetic profiling of the lung and mesenteric draining lymph nodes showed more than twofold increases and decreases in 682 and 202 genes of low-dose *L. gasseri*-treated mice and in 643 and 249 genes of high-dose *L. gasseri*-treated mice, respectively, compared with

those from untreated Der p-sensitized mice (Fig. 2a). After eliminating replicated genes (349 and 148 genes with twofold increases and decreases, respectively) present in low- and high-dose *L. gasseri*-treated mice, the remaining genes were grouped using IPA (<http://www.ingenuity.com>) on the basis of their molecular and biological functions. The DNA microarray analysis is described in detail in the “Materials and methods” section. In a condition tree analysis of the DNA expression profiles (Fig. 2b), the increase and decrease patterns were similar in the lung draining lymph nodes but significantly different in the mesenteric lymph nodes of the low- and high-dose *L. gasseri*-treated Der p-sensitized mice (Fig. 2b). From these results, we identified a group of genes involved in lipid metabolism, whose expression was associated with the administration of low-dose, but not high-dose, *L. gasseri* to mice. One of these genes, *PPAR* $\gamma$ , had the highest upregulated gene and has known anti-inflammatory roles. Figure 2c shows significant increases in *PPAR* $\gamma$  expression in the lung draining lymph nodes in both low- and high-dose *L. gasseri*-treated mice based on the microarray analysis and real-time PCR confirmatory analyses. Contrastingly, in the mesenteric draining lymph nodes, *PPAR* $\gamma$  expression was significantly increased in low-dose *L. gasseri*-treated mice; no increase or even suppression was observed in high-dose *L. gasseri*-treated mice (Fig. 2d). To confirm *PPAR* $\gamma$  expression during Der p-induced airway inflammation, the immunohistochemical staining of the lungs using tissomicellular quantitative analysis (TissueGnostics TissueFAXS Plus<sup>TM</sup>), as described in the “Materials and methods” section (supplement Fig. S1), was used to quantify the *PPAR* $\gamma$ -stained cells in the lung sections. Figure 2e shows that *PPAR* $\gamma$ <sup>+</sup>-stained cells in bronchial epithelia of Der p-sensitized and -challenged mice were decreased compared with those in PBS-challenged mice ( $p < 0.05$ ). Contrastingly, *PPAR* $\gamma$ <sup>+</sup>-stained cells were markedly increased in Der p-sensitized and -challenged mice treated with a low dose ( $10^7$  cfu), but not in mice treated with a high dose ( $10^9$  cfu), of *L. gasseri* ( $p < 0.01$ ).

### Loss of protective effects of *L. gasseri* in Der p-sensitized and challenged *PPAR* $\gamma$ <sup>L/+</sup> mice

To confirm that the protective action of *L. gasseri* in allergen-induced asthma occurred through *PPAR* $\gamma$  activation, we administered the same dosages of *L. gasseri* or PBS to Der p-sensitized and challenged WT and *PPAR* $\gamma$ <sup>L/+</sup> mice. We found that *L. gasseri* significantly reduced AHR to MCh in PBS-treated WT mice but not in *PPAR* $\gamma$ <sup>L/+</sup> mice (Fig. 3a). The infiltration of inflammatory cells and eosinophils was only slightly reduced in the lungs (Fig. 3b) and BALF of *L. gasseri*-treated *PPAR* $\gamma$ <sup>L/+</sup> mice (Figs. 3c, d). Moreover, no significant reduction in inflammatory cytokine production (TNF $\alpha$ , TARC, and IL-17A; Fig. 4e), or increase in the anti-inflammatory cytokine IL-10 (Fig. 3f) in the BALF were

**Fig. 1** Oral administration of low-dose *L. gasseri* attenuated mite allergen-induced airway inflammation and hypersensitivity. **a** Airway resistance to MCh challenge. **b** Lung histology (H&E staining). **c** Cellular infiltration of BALF. **d** Serum concentrations of allergen-specific Abs. **e** Cytokine levels detected in BALF. **f** Cytokine production in Der p-stimulated splenocytes (48 h) from Der p-sensitized mice administered high ( $1 \times 10^9$  cfu) or low ( $1 \times 10^7$  cfu) doses of *L. gasseri*. The data are expressed as the mean  $\pm$  SEM ( $n = 8$  mice per group). \* $p < 0.05$  and \*\* $p < 0.01$ . Duplicate experiments yielded similar results



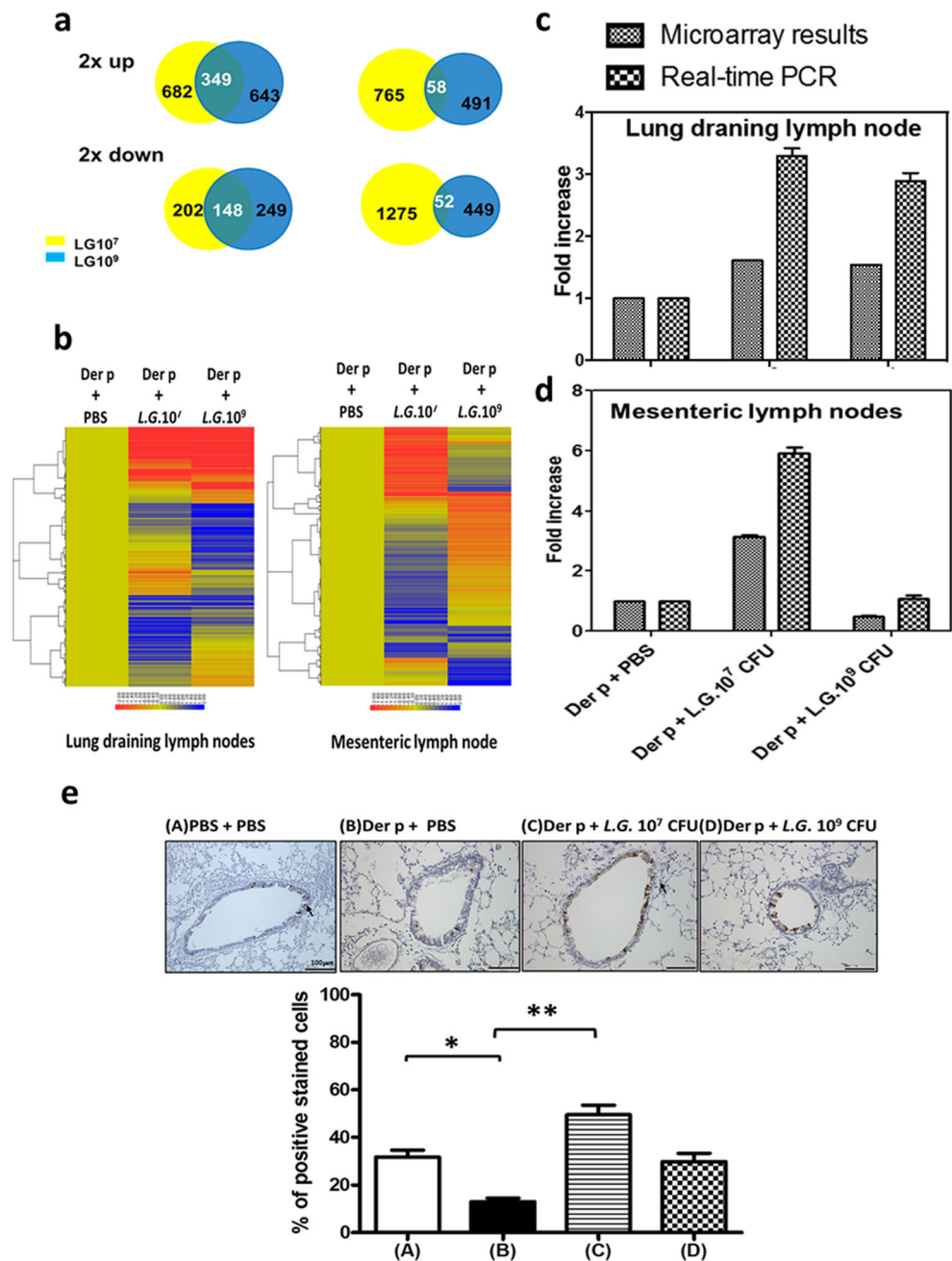
observed in *L. gasseri*-treated PPAR $\gamma^{L/+}$  mice compared with that in untreated PPAR $\gamma^{L/+}$  mice, which was in contrast to the significant increase in these indicators in *L. gasseri*-treated WT mice (Fig. 1e, f).

#### Activation of PPAR $\gamma$ in BMDCs inhibits Der p-induced airway inflammation

DCs are powerful antigen-presenting cells that have the unique capacity to stimulate naïve T cells and play essential roles in initiating allergic inflammation in the lungs. Following the intratracheal transfer of Der p-primed DCs from WT or PPAR $\gamma^{L/+}$  mice to naïve WT mice (protocol shown in Fig. 4a), the recipient mice showed increased AHR to MCh

(Fig. 4b) and marked inflammatory cell infiltration in the lungs (Fig. 4c). When Der p-primed BMDCs from Ro-treated WT mice, the intratracheal adoptive transfer of these BMDCs reduced AHR to MCh and inhibited inflammatory cell infiltration and cytokine production in the BALF from the recipient mice (Fig. 4d, e). Similarly, the intratracheal transfer of Der p-primed BMDCs from PPAR $\gamma^{L/+}$  mice treated with Ro (group D mice) into naïve WT mice improved Der p-induced airway inflammation, but not to a statistically significant level. Next, we administered *L. gasseri* to Der p-challenged recipient mice after the intratracheal transfer of Der p-primed BMDCs according to the protocol described in Fig. 5a. We observed significant improvements in AHR to MCh (Fig. 5b), decreased peribronchial inflammation and

**Fig. 2** DNA microarray comparisons of draining lymph nodes of the intestine and lung and quantification of immunofluorescence staining for PPAR $\gamma$ . **A** A DNA microarray comparisons of genes with twofold increased or decreased expression in the mesenteric and lung draining lymph nodes obtained from Der p-sensitized mice administered high ( $1 \times 10^7$  cfu) or low ( $1 \times 10^9$  cfu) doses of *L. gasseri*. **B** A condition tree analysis of the DNA gene expression profiles. **C** Fold increases in PPAR $\gamma$  expression in the lung and **D** mesenteric draining lymph nodes from low- and high-dose *L. gasseri*-treated mice based on microarray and real-time PCR analyses. Duplicate experiments yielded similar results. **E** The total percentages of cell positive staining for PPAR $\gamma$  in the lungs were compared among **a** Der p-sensitized and PBS-challenged mice, **b** Der p-sensitized and -challenged mice, **c** Der p-sensitized and -challenged mice treated with  $10^7$  cfu of *L. gasseri*, and **d** Der p-sensitized and -challenged mice treated with  $10^9$  cfu of *L. gasseri*. The data are expressed as the mean  $\pm$  standard error (SEM;  $n = 6$  mice per group). \* $p < 0.05$  and \*\* $p < 0.01$ . Similar results were obtained from two additional experiments



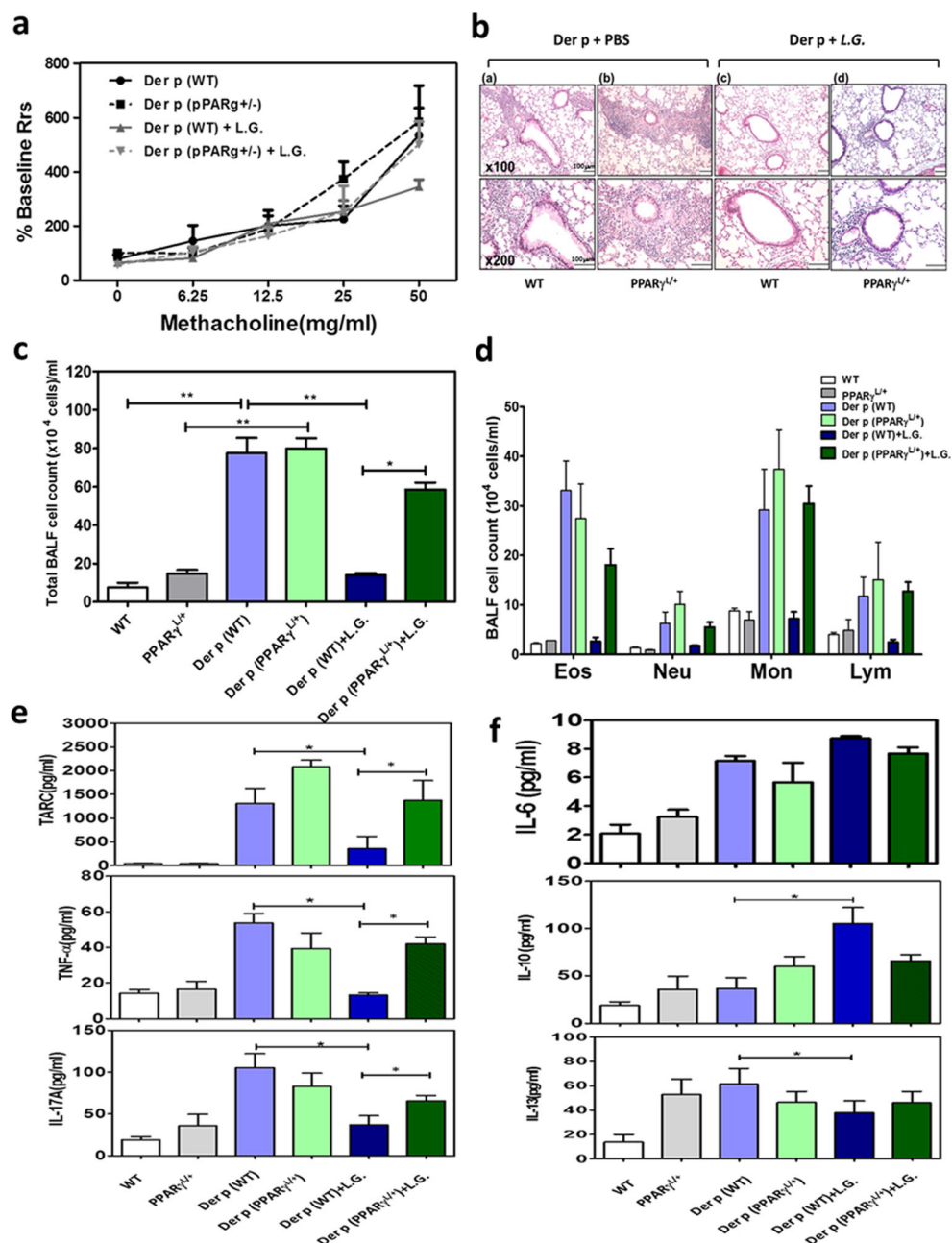
epithelial damage (Fig. 5c), reduced total cell and eosinophil infiltrations (Fig. 5d), and decreased production of TARC, TNF $\alpha$ , and IL-13 in the BALF (Fig. 5e) of WT recipient mice compared with PPAR $\gamma^{L/+}$  recipient mice. These results suggest that *L. gasseri* attenuates the DC-induced development of airway inflammation in WT mice but not in PPAR $\gamma^{L/+}$  mice.

## Discussion

In the present study, we demonstrated a different attenuation effect on allergen-induced airway inflammation between low-

and high-dose *L. gasseri*-treated Der p-sensitized and -challenged mice. Continuous administrations of  $10^7$  cfu, but not  $10^9$  cfu, of *L. gasseri* for 4 weeks significantly prevented Der p-induced airway hypersensitivity and inflammatory cell infiltration in treated mice than in untreated mice. We also found both low and high doses of *L. gasseri* significantly reduced TARC, TNF $\alpha$ , and IL-6 levels in BALF from the treated mice compared with the control mice (Fig. 1e). Moreover, low doses of *L. gasseri* reduced IL-17 and increased IL-10 levels in Der p-stimulated splenocytes (Fig. 1f). IL-13 is one of the critical mediator of allergic airway disease [22]. It contributes the pathogenesis of asthma by

**Fig. 3** Loss of *L. gasseri* protective effects in Der p-sensitized and -challenged PPAR $\gamma$  mice. **a** Airway resistance to MCh challenge. **b** Lung histology (H&E staining). **c** Total cell counts in the BALF. **d** Cellular infiltration of the BALF. **e** Cytokine levels in the BALF. **f** Cytokine production in Der p-stimulated splenocytes (48 h). The data are expressed as the mean  $\pm$  SEM ( $n = 8$  mice per group). \* $p < 0.05$  and \*\* $p < 0.01$

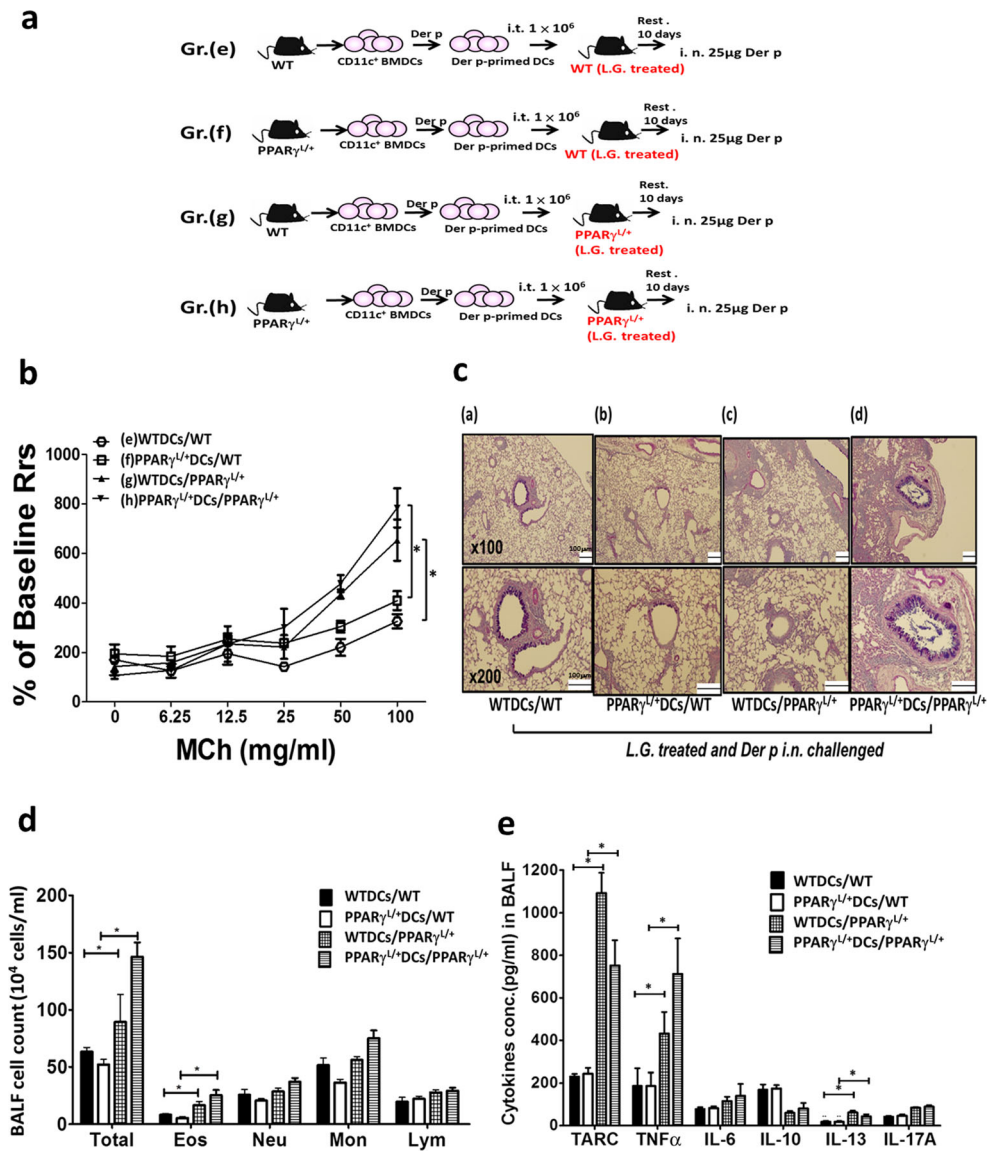


recruitment of eosinophil, IgE antibody switching, mucus hypersecretion, and airway hyperresponsiveness (AHR) and fibrosis. Our results show that administration of *L. gasseri* decreased IL-13 cytokine levels in BALF, but not to a statistical difference (Fig. 1f). In another treatment with lower dose of *L. gasseri*, the IL-13 level was significantly decreased in wild type but not in PPAR $\gamma$ <sup>L/+</sup> mice as compared to non-treated Der p sensitized wild-type mice (Fig. 3f). Recently, it has been reported that PPAR $\gamma$  can inhibit IL-13-induced collagen production in mouse airway fibroblasts [23], and IL-13 also downregulates PPAR $\gamma$  signal pathway [24]. Therefore, it was highly likely that *L. gasseri* treatment can also influence IL-13 action in allergic airway diseases by attenuated

inflammatory cell infiltration in the airways and reduced AHR as shown in this study.

The relationship between the bacterial number and probiotic effects remains unclear; therefore, the minimal dose and dose-dependent biological action required for the probiotic effects are not clear. In acute infectious diarrhea, higher doses of probiotics administered for short courses seem more effective than lower doses [25]. Contrastingly, for chronic or immunological diseases (allergic, inflammatory, and immune diseases), the effects of probiotics may depend on the duration and dose of treatment. For example, the highest concentration of bifidobacteria ( $10^7$  cfu/mL) induces peripheral blood mononuclear cells to produce less pro-inflammatory and

**Fig. 4** Activation of PPAR $\gamma$  in BMDCs inhibits Der p-induced airway inflammation. **a** Protocols for the intratracheal transfer of BMDCs from WT or PPAR $\gamma$  mice to WT mice, followed by intranasal challenge with Der p for 10 days. **b** Airway resistance to MCh challenge. **c** Lung histology (H&E staining). **d** Cellular infiltration of the BALF. **e** Cytokine levels in the BALF from mice after the intratracheal transfer of BMDCs. *L. gasseri* attenuated DC-induced airway inflammation in WT mice but not PPAR $\gamma$  mice. The data are expressed as the mean  $\pm$  SEM ( $n = 6$  mice per group). \* $p < 0.05$  and \*\* $p < 0.01$

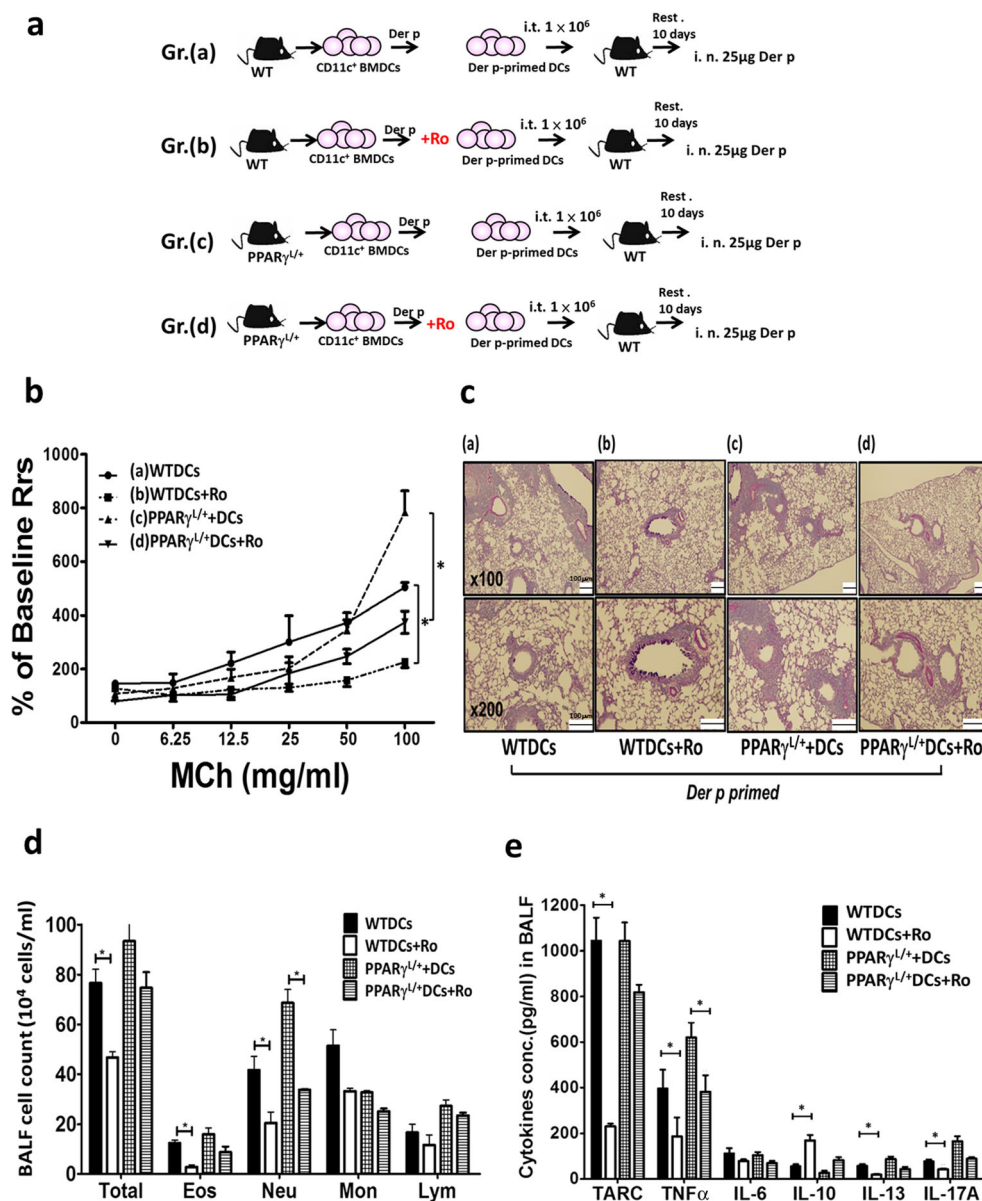


anti-inflammatory cytokines (INF $\gamma$  and IL-10) than does the lowest concentration ( $10^3$  cfu/mL) [26]. A meta-analysis reported that single probiotics at a low dose and with a short treatment duration seem more effective in improving the overall symptom response and quality of life of patients with irritable bowel diseases [27]. Notably, the dose effects of probiotics remain controversial, particularly for lactobacillus, and most reported studies were in vitro experiments. The probiotic effects on health or physiology may either be direct or indirect through modifications of the endogenous ecosystem or immune response, suggesting that a single dose-dependent action of all probiotics and effects is unlikely [28].

To explore the factors influencing the outcomes of different doses of probiotics, DNA microarray analyses of the mesenteric and lung draining lymph nodes were performed, which revealed a significant decrease in inflammatory chemokines and increase in gene expression in carbohydrate and lipid

metabolism, particularly of PPAR $\gamma$ , in the mesenteric draining lymph nodes of  $10^7$  cfu *L. gasseri*-treated mice compared with non-treated mice (Fig. 2d). By contrast, no significant difference was observed in the lung draining lymph nodes in the gene expression profile, neither in PPAR $\gamma$ , for low- or high-dose administration of *L. gasseri* (Fig. 2c). We hypothesized that probiotics in the intestinal epithelium may exert direct effects in situ on the intestinal immunogenic cells to induce tolerogenic DCs and regulatory T (T<sub>reg</sub>) cells [29], and indirectly act on the cells that migrate to the airways of the lungs for different immunological actions [30]. Hence, the biological action of probiotics on the intestine was direct and dose dependent and showed different gene expression patterns between low- and high-dose *L. gasseri* administrations. The action of probiotics on the lungs may be indirect through migratory lymphocytes and DCs that showed a similar gene expression pattern between low- and high-dose *L. gasseri* administrations (Fig. 2).

**Fig. 5** Activation of PPAR $\gamma$  in BMDCs and transfer into *L. gasseri*-treated mice. **a** Protocol of BMDCs from WT or PPAR $\gamma$  mice were intratracheally transferred to WT or PPAR $\gamma$  mice that were administered *L. gasseri* and then intranasally challenged with Der p for 10 days. **b** Airway resistance to MCh challenge. **c** Lung histology (H&E staining). **d** Cellular infiltration of the BALF. **e** Cytokine levels in BALF from mice after the intratracheal transfer of BMDCs and *L. gasseri* treatment. The data are expressed as the mean  $\pm$  SEM ( $n = 6$  mice per group). \* $p < 0.05$  and \*\* $p < 0.01$



Notably, we found a dose difference in PPAR $\gamma$  expression in *L. gasseri*-stimulated DCs isolated from Peyer's patches in the intestine and mesenteric draining lymph nodes (unpublished results). The suppressive effects of DC-expressed PPAR $\gamma$  activation on Th2 immune responses was previously shown in an ovalbumin-sensitized mouse model of asthma [31]. Furthermore, our experiments on the adoptive transfer of BMDCs from *L. gasseri*-treated WT mice but not from PPAR $\gamma^{L/L}$ -deficient heterozygous mice showed tolerogenic effects on the recipient WT mice after Der p challenge (Figs. 4 and 5). These results support our hypothesis that probiotics can activate the PPAR $\gamma$ -induced anti-inflammatory pathway mediated through DCs in the gut and migration into the lungs that alleviate allergen-induced airway inflammation.

It is established that gut microbial colonization, through interactions with intestinal epithelial cells, may have a

systemic immune regulatory effect that influences the host's metabolism, immunity, and response to infections [32]. However, how the gut microbiome affects inflammatory diseases of other remote organs, such as the skin in atopic dermatitis and airways in asthma, is unclear [33, 34]. Many experimental studies in mice clarify the communication between the gut and lungs through numerous immune cells [35–37] and bacterial metabolic products, such as SCFAs [38, 39] or polysaccharide A [40, 41]. SCFAs are well-established bacterial metabolites with effector functions in the gut mucosa; however, probiotics can also produce longer CFAs, including conjugated linoleic acid (CLA), a compound that suppresses inflammation through PPAR $\gamma$  activation [42]. For example, PPAR $\gamma$  mediates the anti-inflammatory action of *Bacteroides thetaiotaomicron*, a prevalent commensal anaerobic bacterium of the human intestine [43]. Probiotic

bacteria produce CLA locally in the gut, initiating the macrophage production of PPAR $\gamma$ , to suppress colitis [44]. The role of PPAR $\gamma$  activation in the suppression of lung inflammation has been demonstrated in experimental asthma [45–48] and under clinical conditions [49]. PPAR $\gamma$  expressed in the bronchial epithelial cells was recently found to down-regulate allergic inflammation and eosinophil activation, which may be a novel anti-inflammatory target for asthma [50, 51]. Our study not only showed that *L. gasseri*-induced PPAR $\gamma$  activation in BMDCs inhibited allergic airway inflammation in both PPAR $\gamma$  WT and -deficient heterozygous mice but also reported that this protective effect of *L. gasseri* was lost in Der p-induced airway inflammation in PPAR $\gamma^{L/+}$ -deficient heterozygous mice. These results further revealed that PPAR $\gamma$  activation is an important mediator of the anti-inflammatory and immunoregulatory actions of *Lactobacillus* in the maintenance of host homeostasis.

In this study, we used the probiotic strain *L. gasseri*, which has been well-characterized for its genomic and phenotypic effects on human health [52]. *L. gasseri* has been reported to elicit immunological changes in both allergen-sensitized animal models and atopic patients [53, 54]. In a DBRC clinical trial, children with allergic diseases who were administered probiotic yogurt containing *L. gasseri* and *L. coryniformis* demonstrated, in addition to decreased IgE and increased IgA in sera, an increase in both CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells and NK cells [55]. These results are compatible with our previous study of *L. gasseri*, which when administered alone alleviated allergic symptoms in asthmatic children with allergic rhinitis [15].

In summary, we demonstrated different attenuation effects on allergen-induced airway inflammation in low- and high-dose *L. gasseri*-treated Der p-sensitized mice. We exploited this advantage to explore the factors influencing the outcomes of different doses of probiotics by conducting DNA microarray comparisons and found that PPAR $\gamma$  expression in the mesenteric draining lymph nodes was the major differential factor between the two doses of *L. gasseri* administered to the mice. The increase in PPAR $\gamma$  expression in the lungs of mice after *L. gasseri* administration validated the novel finding that the anti-allergic effect of *L. gasseri* is mediated through the activation of PPAR $\gamma$  signal transduction (Fig. 2e). Furthermore, the anti-inflammatory effect was significantly reduced in PPAR $\gamma^{L/+}$ -deficient heterozygous mice (Fig. 3) under Der p sensitization and challenge. Moreover, we report that *L. gasseri*-treated BMDCs can have tolerogenic effects on the recipient WT mice after Der p challenge (Figs. 4 and 5). Based on these results, our study revealed a novel mechanistic pathway through PPAR $\gamma$  activation, by which probiotics help maintain host homeostasis and provide promising target molecules for the treatment and prevention of allergic asthma.

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**Compliance with ethical standards** The animal research in this study is according to the affidavit of approval of animal use protocol National Cheng Kung University, and the Institutional Animal Care and Use Committee (IACUC) approval No is 101096.

**Competing interests** The authors declare that they have no competing interests.

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## Electronic supplementary material (ESM)

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Title: ***Lactobacillus gasseri* attenuates allergic airway inflammation through PPAR $\gamma$  activation in dendritic cells**

Miao-Hsi Hsieh,<sup>1,\*</sup> Ren-Long Jan,<sup>2,3,\*</sup> Lawrence Shih-Hsin Wu,<sup>4</sup> Pei-Chi Chen,<sup>1,5</sup>  
Hui-Fang Kao,<sup>5,6</sup> Wen-Shuo Kuo,<sup>5,6</sup> and Jiu-Yao Wang.<sup>5,7,8,#</sup>

<sup>1</sup> Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan;

<sup>2</sup> Department of Pediatrics, Chi-Mei Medical Center, Liou-Yin, Tainan;

<sup>3</sup> Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan;

<sup>4</sup> Institute of Medical Sciences, Tzu Chi University, Hualien, Taiwan;

<sup>5</sup> Center for Allergy and Clinical Immunology Research (ACIR), College of Medicine, National Cheng Kung University, Tainan;

<sup>6</sup> Department of Nursing, National Tainan Junior College of Nursing, Tainan

<sup>7</sup> Division of Allergy and immunology, Department of Pediatrics, National Cheng Kung University Hospital

<sup>8</sup> Graduate Institute of Integrated Medicine, School of Chinese Medicine, China Medical University, Taichung, Taiwan

\*These authors equally contributed to the work

# Corresponding authors:

Jiu-Yao Wang

a122@mail.ncku.edu.tw

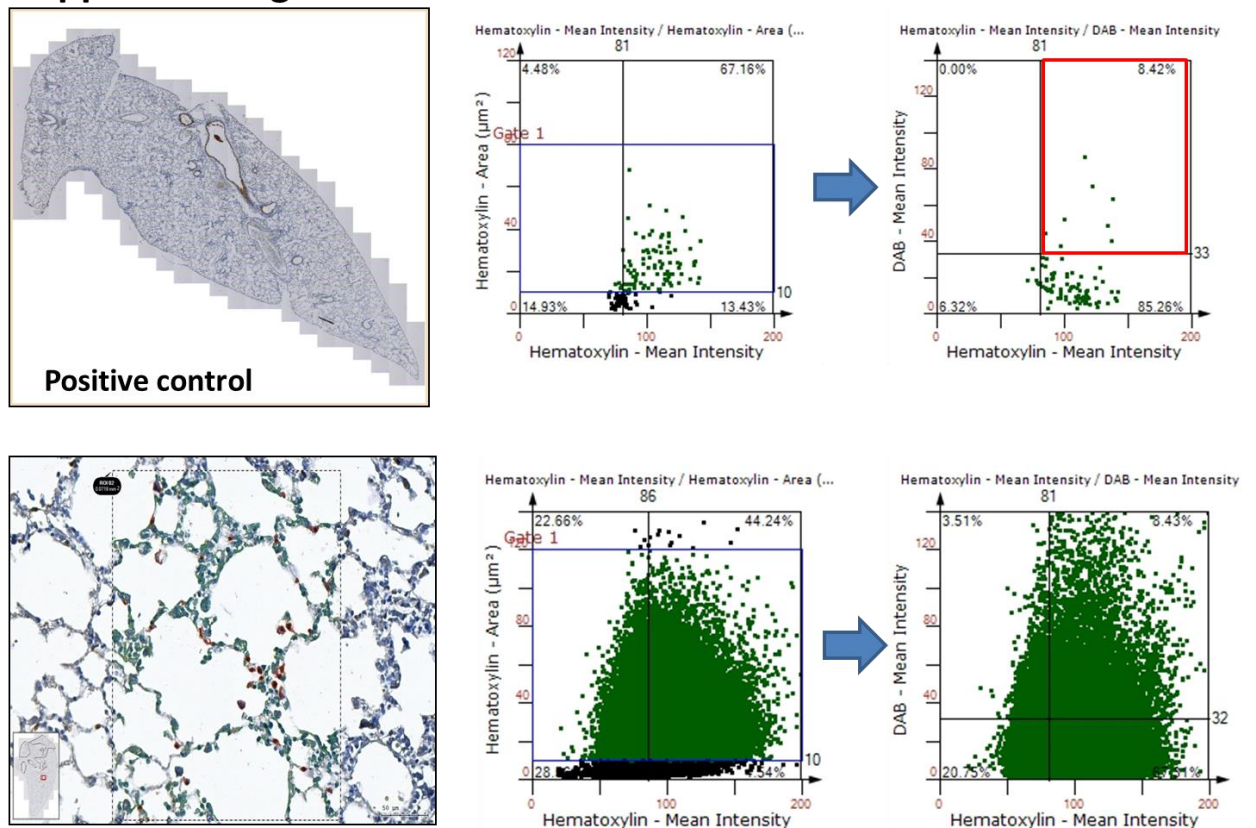
Tel. (+886) 2353535

Fax. (+886) 2753083

## Supplementary materials

### Supplement figure

**Supplement Fig. S1**



## SUPPLEMENTAL FIGURE LEGENDS

**Supplemental Figure S1** *PPAR* $\gamma$  expression detected through immunofluorescence staining and quantified using an objective, accurate, and reproducible tissomic cellular quantitative analysis (TissueGnostics TissueFAXS Plus™).