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Research paper

Lactobacillus rhamnosus GG induced protective effect on allergic airway inflammation is associated with gut microbiota

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ABSTRACT

Great interest has been taken in the use of beneficial bacteria for allergic diseases recently, but the underlying mechanisms through which probiotics induces immune regulation or immune tolerance are poorly understood. We aimed to explore whether *Lactobacillus rhamnosus* GG (LGG)-induced beneficial effect relates to the change of microbiota. LGG was administered orally to mouse model of ovalbumin (OVA)-induced allergic airway inflammation. Our findings manifested that LGG-treatment contributes to protect against OVA-induced allergic airway inflammation by expanding mesenteric $CD103^+$DCs and accumulating mucosal Tregs. Moreover, protective effect induced by LGG is associated with gut microbiota instead of lung flora. Collectively, our findings indicate that LGG induced protective effect is associated with gut microbiota and provide a new evidence of probiotic application in the allergic airway inflammation.

1. Introduction

Allergic diseases affect up to 30% of the population worldwide, with asthma being one of the most common chronic diseases in which affected individuals may suffer considerable morbidity [1–4]. It is estimated that asthma affects 300 million individuals worldwide, most probably as a result of changing environment and reduced exposure to microbial antigens during infancy [5,6]. The classic asthma presentation is generally regarded as a T helper 2 (Th2) airway inflammation with mucus hypersecretion and variable degrees of airflow obstruction associated with airway hyperresponsiveness (AHR) to nonspecific stimuli, all of which finally leading to bronchial remodeling [7]. Although corticosteroids are effective at managing the disease, 10% of patients do not respond to the treatment, and they are associated with severe long-term side effects [8–11]. Accordingly, there is an increasing demand for proven alternatives to pharmaceutical products from both healthcare professionals and consumers.

Alteration of microbiota contributes to the development of allergies and asthma [12,13]. Changes of microbiota could result in differences to downstream immune responses or immune development, both of which can affect immune mechanisms in distal mucosal sites such as the lung and result in asthma. However, deficiencies in the immune system can also affect the microbiota composition, which can affect distal mucosal sites and have a role in the development of atopic conditions [14]. Probiotic bacteria have been shown to modify immune responses in vitro [15–17] and in animals [18,19], and are defined as "live microorganisms which confer a health benefit on the host when administered in adequate amounts". Animal models revealed that supplementation with *Lactobacillus rhamnosus* GG (LGG) resulted in reduction in the major features of allergic airway inflammation in a murine model of experimental asthma [20]. Protective effects were also transferred to the offspring when mothers were supplemented with LGG before and during pregnancy and weaning [21]. Several human studies also demonstrated that LGG was highly effective to reduce the risk for

Abbreviations: LGG, *Lactobacillus rhamnosus* GG; OVA, ovalbumin; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; Th, T helper; DCs, dendritic cells; Tregs, regulatory T cells; 16S rRNA, 16S ribosomal RNA; PAS, periodic acid-schiff; PLN, peribronchial lymph nodes; MLN, mesenteric lymph nodes; FBS, fetal bovine serum; FCS, fetal calf serum; OTU, Operational Taxonomic Unit; MCh, Acetyl-ß-methylcholine chloride; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin/eosin; Ig, Immunoglobulin; SW, Shannon-Wiener; PCoA, principal coordinate analysis; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; MHCII, histocompatibility complex class II; ANOVA, analysis of variance; PERMANOVA, permutational MANOVA; ROC, receiver operating characteristics; AUC, area under curve

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the development of allergic asthma [22,23]. Yet despite all that, some controversial conclusions are still drew, and there have been rare reports to demonstrate whether LGG-induced protective effect on allergic airway inflammation is associated with the alteration of microbiota. Here, we aimed to explore LGG-induced beneficial effect on allergic asthma and whether it relates to the change of microbiota.

2. Material and methods

2.1. Mice

Male BALB/c mice of 6–8 weeks were obtained from the Laboratory Animal Center of the Fourth Military Medical University and housed under conventional conditions. A standard extruded pellet diet and water were provided *ad libitum*. Experimental procedures were approved by the Ethics Committee for Animal Studies of the Fourth Military Medical University (20170403) and performed in accordance with their guidelines of the Institutional Animal Care and Use Committee.

2.2. Experimental design

Allergic airway inflammation was induced as described previously [24] with minor modifications. Briefly, sensitization was made by four intraperitoneal injections of OVA (20 μg per mouse; Sigma, St. Louis, MO, USA) adsorbed with 500 μg alum at 14 day intervals (days 0, 14, 28 and 42). Beginning on the 21st day, sensitized mice received OVA challenge by 1%OVA aerosols during 30 min, three times per week (days 21–46). Challenge was carried out using an INQUA NEB plus (Omron Company limited, Dalian, Liaoning, China). The control group was given normal saline i.p. (0.2 ml per mouse) and challenged with normal saline at the same time points. Mice in the LGG-treatment group received 0.2 ml LGG drink orally (5×10^8 CFU/ml) from day 0 to day 46(Fig. 1).

2.3. Airway hyperresponsiveness

AHR was assessed by measuring the total lung resistance (Rrs, cm H2O.s/mL) using the FlexiVent system (SCIREQ, Montreal, Canada) on day 47, as described previously [25]. Anaesthetized (pentobarbital sodium, 90 mg/kg) and tracheostomized (stainless steel cannula, 18 G) mice were nebulized to increasing doses1.5–100 mg/mL of acetyl-β-methylcholine chloride (MCh, Sigma). Recorded values were averaged for each dose and used to obtain dose–response curves for each group.

2.4. Bronchoalveolar lavage

Bronchoalveolar lavage was performed on day 47 after mice were sacrificed. Briefly, the airways of the mice were lavaged three times with 0.8 ml of PBS via a tracheal cannula. The total number of inflammatory cell in the bronchoalveolar lavage fluid (BALF) was determined under light microscopy, and then the BALF were centrifuged at 2500*g* for 5 min at 4 °C. The cells at the bottom were stained with Wright Giemsa stain. One hundred cells per slide were counted to classify individual leucocyte populations using the standard morphological criteria.

2.5. Enzyme-linked immunosorbent assay

BALF and serum samples were collected to assay the presence of cytokines, mouse mast cell protease 1 (mMCP-1) and immunoglobulins (Igs). The levels of BALF IL-4, IL-13, IL-10, mMCP-1, and serum OVA-specific IgE and IgG1 were measured by enzyme-linked immunosorbent assay(ELISA) Kits (ReyBiotech, Norcross, GA, USA; eBioscience, San Diego, CA, USA; Chondrex, Redmond, WA, USA) following the the instructions of the manufacturer.

2.6. OVA-specific stimulation of spleen lymphocytes

Spleen cell suspensions were obtained as previously described [26] by passing the tissue through a 40 μm-cell strainer. Erythrocytes from the spleen were eliminated with ACK lysis buffer. Spleen cells were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS), 1% L-Glutamine, and 2% Pen/Strep in round bottom 96-well plate (2×10^5 cells per animal/well) and re-stimulated with 100 mg/ml OVA for 4 days at 37 °C, 5% CO_2. As an additional readout for differences in the immune status of the animals, levels of IL-4 and IL-13 in culture supernatants were measured by ELISA(ReyBiotech).

2.7. Flow cytometry

Peribronchial lymph nodes(PLN), mesenteric lymph nodes (MLN), or trachea were processed in RPMI 1640 medium containing 2% fetal bovine serum (FBS), 400 U of type I collagenase. Cells were strained through a 70-mm cell strainer. Erythrocytes were lysed with ACK lysis buffer. Small intestine lamina propria were obtained after digestion in RPMI containing 5% FCS, 5 mM EDTA, and 2 mM dithiothreitol, as described previously [27]. Single-cell suspensions isolated from the above tissues were stained for FACS analyses. Cells were first stained for surface markers including CD4-PerCP, CD25-APC, CD11c-PerCP-Cy5.5, I-A/I-E-APC, CD103-PE, CD86-PE, CD40-PE, CCR9-FITC (BioLegend, San Diego, CA, USA). If required, cells were then fixed and permeabilized by Fixation/Permeabilization reagent(BioLegend) and stained for intracellular expression markers, Foxp3-PE. Data were acquired with BD FACSCanto (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo 10.0.7 soft (Tree Star Inc., Ashland, OR).

2.8. Histopathology

The lungs were fixed with 10% formalin for at least 24 h, after which the lungs were embedded in paraffin. Four-micrometer sections were stained with hematoxylin and eosin (H&E) for inflammation, periodic acid–schiff reagent (PAS) for goblet cell hyperplasia and Masson's trichrome for collagen fibers according to standard methods. Slides were reviewed in blinded fashion for histologic assessment using light microscopy.

2.9. Microbiome analysis

Feces and lung tissue samples from individual mice were collected

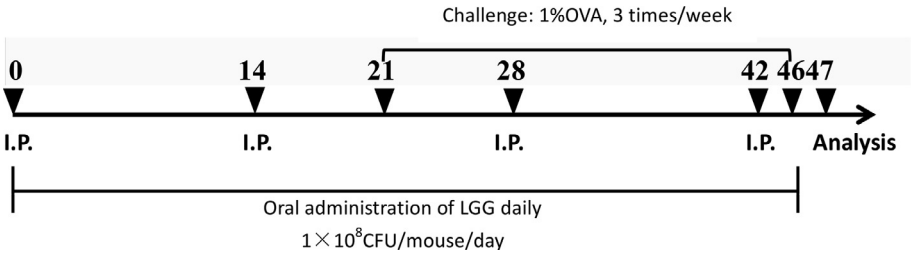

Fig. 1. Experimental procedure. Mice were sensitized with four intraperitoneal injections of ovalbumin (OVA, 20 μg per mouse) at 14-day intervals (days 0, 14, 28 and 42) and challenged with 1%OVA aerosols three times per week (days 21–46). Mice in the treatment group received bacterial suspension with three different doses or PBS orally from day 1 to day 46. Analyses were performed 24 h after the last aerosol.