Extracellular vesicle–derived protein from *Bifidobacterium longum* alleviates food allergy through mast cell suppression

Jung-Hwan Kim, PhD,[a,b]* Eun-Ji Jeun, MSc,[a,b]* Chun-Pyo Hong, PhD,[a,b] Seong-Hoon Kim, MSc,[d] Min Seong Jang, DVM, PhD,[a,b] Eun-Jung Lee, MSc,[a,b] Sook Jin Moon, BSc,[a,b] Chang Ho Yun, PhD,[a] Sin-Hyeog Im, PhD,[a,b] Seok-Geun Jeong, PhD,[c] Beom-Young Park, PhD,[c] Kyong-Tai Kim, PhD,[b,d] Ju-Young Seoh, MD, PhD,[e] Yoon-Keun Kim, MD, PhD,[f] Sung-Jong Oh, PhD,[g] Jun-Sang Ham, PhD,[c]‡ Bo-Gie Yang, PhD,[a]‡ and Myoung Ho Jang, PhD[a]‡ *Pohang, Jeonju, Seoul, and Jeju, Korea*

Background: The incidence of food allergies has increased dramatically during the last decade. Recently, probiotics have been studied for the prevention and treatment of allergic disease.

Objective: We examined whether *Bifidobacterium longum* KACC 91563 and *Enterococcus faecalis* KACC 91532 have the capacity to suppress food allergies.

Methods: *B longum* KACC 91563 and *E faecalis* KACC 91532 were administered to BALB/c wild-type mice, in which food allergy was induced by using ovalbumin and alum. Food allergy symptoms and various immune responses were assessed.

Results: *B longum* KACC 91563, but not *E faecalis* KACC 91532, alleviated food allergy symptoms. Extracellular vesicles of *B longum* KACC 91563 bound specifically to mast cells and induced apoptosis without affecting T-cell immune responses. Furthermore, injection of family 5 extracellular solute-binding protein, a main component of extracellular vesicles, into mice markedly reduced the occurrence of diarrhea in a mouse food allergy model.

Conclusion: *B longum* KACC 91563 induces apoptosis of mast cells specifically and alleviates food allergy symptoms. Accordingly, *B longum* KACC 91563 and family 5 extracellular solute-binding protein exhibit potential as therapeutic approaches for food allergies. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Food allergy,* Bifidobacterium longum*, probiotics, extracellular vesicle, mast cells, family 5 extracellular solute-binding protein, ovalbumin*

Food allergy is a potentially serious disease caused by abnormal immune responses to food or food additives. Recently, hospital visits for food allergy have dramatically increased.[1] Even though it has long been known that foods, such as peanuts, milk, shellfish, wheat, and tree nuts, trigger food allergies, causative factors have not been well defined.[2] Several treatments for food allergy have been proposed, including avoidance of allergenic foods, the most widely used method,[3] and, more recently, tolerance induction through oral allergen administration.[2-4]

Alternatively, treatment with probiotics has been attempted in patients with food allergy.[5] Probiotics are live microorganisms that confer benefits to the host either directly through communication with host cells or indirectly through interaction with other gut bacteria.[6] Recent studies have suggested that probiotic bacteria affect the host immune system and suppress inflammatory and allergic responses. For example, *Bifidobacterium breve* enhances intestinal homeostasis through induction of IL-10–producing T_R1 cells.[7] Furthermore, a mixture of probiotics was shown to suppress experimental colitis by facilitating the generation of $CD4^+$ regulatory T (Treg) cells in the gut.[8] Double-stranded RNA from lactic acid bacteria triggers IFN-β production by dendritic cells (DCs), thereby inhibiting colitis.[9] In addition, VSL#3, a probiotic mixture consisting of 8 different gram-positive bacteria, suppresses food allergy through shifting immune responses from T_H2 to T_H1.[10]

Microbiota secrete extracellular vesicles (EVs), which contain DNA, protein, and cell-wall components within the nanometer-sized spherical lipid bilayer and recently have been spotlighted as key messengers.[11] Bacterial EVs deliver bacterial components to host immune cells in a concentrated, protected, and targeted form.[12] Both gram-positive and gram-negative bacteria secret EVs,[13-15] which influence the host immune system.[16] For example, DCs exposed to EVs of *Bifidobacterium bifidum* LMG13195 strongly promote differentiation of forkhead box protein 3 $(Foxp3)^+$ Treg cells.[17] Additionally, EVs of *Staphylococcus aureus* cause neutrophilic pulmonary inflammation through induction of T_H1 and T_H17 cell immune responses.[18]

In a previous study, we isolated probiotics from fecal samples of healthy Korean neonates. Among them, we selected *Enterococcus faecalis* KACC 91532, a major strain,[19] and *Bifidobacterium longum* KACC 91563, a subspecies of *B longum*, which is a well-known probiotic strain exhibiting positive host

From [a]the Academy of Immunology and Microbiology, Institute for Basic Science, Pohang; [b]the Division of Integrative Biosciences and Biotechnology and [d]the Division of Molecular and Life Science, Department of Life Sciences, Pohang University of Science and Technology; [c]the National Institute of Animal Science, Rural Development Administration, Jeonju; the Departments of [e]Microbiology and [f]Medicine, Ewha Womans University Graduate School of Medicine, Seoul; and [g]the Faculty of Biotechnology, College of Applied Life Science, Jeju National University.

*These authors contributed equally to this work.

‡These authors contributed equally to this work.

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Corresponding author: Myoung Ho Jang, PhD or Bo-Gie Yang, PhD, Academy of Immunology and Microbiology, Institute for Basic Science, 77 Cheogam-ro, Nam-gu, Pohang, Gyeongbuk 790-784, Republic of Korea. E-mail: jangmh@ibs.re.kr or: yangbg@ibs.re.kr. Or: Jun Sang Ham, PhD, National Institute of Animal Science, RDA, 1500 Kongjwipatjwi-ro, Iseo-myeon, Wanju-gun, Jeollabuk-do, Korea. E-mail: hamjs@korea.kr.

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Abbreviations used
ASBP: ABC transporter, substrate-binding protein
BMMC: Bone marrow–derived mast cell
cfu: Colony-forming unit
DC: Dendritic cell
DC-SIGN: Dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin
ESBP: Family 5 extracellular solute-binding protein
EV: Extracellular vesicle
FISH: Fluorescence *in situ* hybridization
Foxp3: Forkhead box protein 3
GST: Glutathione-S-transferase
LP: Lamina propria
MCPT-1: Mast cell protease 1
MHCII: MHC class II
MLN: Mesenteric lymph node
OVA: Ovalbumin
TCR: T-cell receptor
TEM: Transmission electron microscopy
Treg: Regulatory T
TUNEL: *In situ* terminal deoxynucleotidyl transferase–mediated nick end-labeling

effects.[20] The effects of these bacterial strains on food allergy have not been investigated previously. Here we show that oral administration of *B longum* KACC 91563 markedly decreases the severity of food allergy in a mouse model. *In vitro*, *B longum* KACC 91563–derived EVs containing family 5 extracellular solute-binding protein (ESBP) interact with mast cells and specifically induce cell death. ESBP is one of the extracellular solute-binding receptors of bacteria that function in chemoreception, transmembrane transport, and initiation of sensory transduction pathways and that recognize peptides.[21] However, any immunomodulatory function of ESBP has not been reported. In this study, we confirmed that ESBP decreases the severity of food allergy by reducing the number of mast cells in the small intestinal lamina propria (LP). Accordingly, we suggest a new mechanism of food allergy regulation by probiotics through the reduction of mast cells with no effect on T-cell responses.

METHODS

Mice

Six- to 8-week-old BALB/c wild-type mice were provided by the Pohang University of Science and Technology mouse facility. All experiments were performed under specific pathogen-free conditions. We followed experimental protocols approved by the Animal Care and Use Committee of the Pohang University of Science and Technology.

Food allergy model

Food allergy was induced in mice, as described previously.[22] In brief, we intraperitoneally injected ovalbumin (OVA; 50 μg, grade V; Sigma-Aldrich, St Louis, Mo) with aluminum potassium sulfate adjuvant (1 mg; A-7210, Sigma-Aldrich) to mice once and then again 2 weeks later. Two weeks after the injections, OVA (10 or 50 mg) was administered orally every other day. Mice were deprived of food for approximately 3 to 4 hours before oral challenge with OVA. The degree of food allergy was evaluated based on diarrhea occurrence, which was assessed by visually monitoring mice for up to 1 hour after oral challenge. Multiple observers blinded to the experimental protocol scored the occurrence of diarrhea.

Administration of probiotics

B longum KACC 91563 and *E faecalis* KACC 91532 were provided by the National Institute of Animal Science, Rural Development Administration. They were isolated from feces of healthy Korean infants and anaerobically cultured at 37°C. Although *E faecalis* KACC 91532 was cultured in MRS broth (BD Biosciences, San Jose, Calif), *B longum* KACC 91563 was cultured in MRS broth containing 0.05% cysteine. These cultured bacteria were freeze-dried with protectant based on skim milk and administered daily by means of intragastric gavage (5×10^9 colony-forming units [cfu] per mouse). In some experiments freeze-dried bacteria mixed with powdered mouse food were provided at the same cfu as bacteria in the oral gavage experiment, with fresh food administered every 3 days.

Fluorescence *in situ* hybridization

Bifidobacterium species and *E faecalis* in fecal samples were detected by using fluorescence *in situ* hybridization (FISH) kits (10MEH001 and 10MEH015; Ribo Technologies, Groningen, The Netherlands), according to the manufacturer's instructions.

Quantification of mast cells in the small intestine

After the fifth OVA intragastric challenge (50 mg) in a food allergy model, mice were sacrificed, and jejunal portions of the small intestines were prepared for paraffin sections. Jejunal tissue paraffin sections were stained with naphthol AS-D chloroacetate esterase (91C, Sigma-Aldrich), according to the manufacturer's instructions. Mast cells were counted in at least 3 different sections per mouse (magnification ×400).

Isolation of EVs

After culture of *B longum* KACC 91563 or *E faecalis* KACC 91532, culture medium was centrifuged at 8000 rpm for 20 minutes. As described previously,[23] the supernatant was filtered with a 0.22-μm bottle top filter and then centrifuged at 100,000*g* for 2 hours by using 0.8 and 2.5 mol/L sucrose solutions. After sucrose gradient centrifugation, the interlayer of the 0.8 and 2.5 mol/L sucrose was collected. The collected interlayer was diluted in PBS, and sucrose gradient centrifugation was performed one more time. Then the collected interlayer was centrifuged at 150,000*g* for 2 hours. Protein concentrations were measured with a BCA assay Kit (Thermo Scientific, Waltham, Mass). Size distribution of EVs was determined by using the dynamic light scattering assay, and imaging of EVs was conducted with transmission electron microscopy (TEM).

Cell preparation

LP cells of the small intestine were prepared, as described previously.[24] In brief, after fat tissues and Peyer patches were removed from the small intestine, the intestine was opened longitudinally, washed in PBS, and cut into approximately 1- to 2-cm lengths. For removal of epithelial cells, fragments of the small intestine were treated with PBS containing 10 mmol/L EDTA for 20 minutes at 37°C with continuous stirring. After washing with PBS, the intestinal fragments were minced and digested with 400 U/mL collagenase D (Roche, Mannheim, Germany) and 100 μg/mL DNase I (Roche) at 37°C for 45 minutes. LP cells were enriched through density gradient centrifugation in approximately 40% to 75% (vol/vol) Percoll (GE Healthcare Life Science, Fairfield, Conn). Then LP cells were stained with antibodies against CD11b, CD11c, CD19, MHC class II (MHCII), and T-cell receptor (TCR) β, and eosinophils ($CD11b^+CD11c^{int}MHCII^-$), T cells ($MHCII^-TCR\beta^+CD19^-$), and B cells ($MHCII^-TCR\beta^-CD19^+$) were sorted. For DC preparation, spleens were minced and digested with collagenase D (400 U/mL) and DNase I (100 μg/mL) at 37°C for 45 minutes. After enrichment by means of centrifugation through 17.5% Accudenz solution (Accurate Chemical & Scientific Corporation, Westbury, NY), DCs ($MHCII^+CD11c^+$) were sorted. For mast cell preparation, bone marrow cells were cultured in the presence of mIL-3 (10 ng/mL; R&D Systems, Minneapolis, Minn) and mSCF (50 ng/mL, R&D Systems) for more than