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

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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; nn:nn:nn:nn)

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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. 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. Several treatments for food allergy have been proposed, including avoidance of allergenic foods, the most widely used method, and, more recently, tolerance induction through oral allergen administration.

Alternatively, treatment with probiotics has been attempted in patients with food allergy. 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. 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 Treg cells. Furthermore, a mixture of probiotics was shown to suppress experimental colitis by facilitating the generation of CD4+ regulatory T (Treg) cells in the gut. Double-stranded RNA from lactic acid bacteria triggers IFN-β production by dendritic cells (DCs), thereby inhibiting colitis.

In addition, VSL#3, a probiotic mixture consisting of 8 different gram-positive bacteria, suppresses food allergy through shifting immune responses from Th2 to Th1. 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. Bacterial EVs deliver bacterial components to host immune cells in a concentrated, protected, and targeted form. Both gram-positive and gram-negative bacteria secrete EVs, which influence the host immune system. For example, DCs exposed to EVs of *Bifidobacterium bifidum* LMG13195 strongly promote differentiation of forkhead box protein 3 (Foxp3)+ Treg cells. Additionally, EVs of *Staphylococcus aureus* cause neutrophilic pulmonary inflammation through induction of Th17 cell immune responses.

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, and *Bifidobacterium longum* KACC 91563, a subspecies of *B longum*, which is a well-known probiotic strain exhibiting positive host
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
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
TUNEL: In situ terminal deoxynucleotidyl transferase–mediated nick end-labeling

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. 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. 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 × 103 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, the supernatant was filtered with a 0.22-μm bottle top filter and then centrifuged at 100,000g 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 150,000g 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. 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 PBS, the intestinal fragments were minced and digested with 400 U/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/MHCII), T cells (MHCII+ TCRB+ CD19+), and B cells (MHCII+ TCRB+ CD19+) were sorted. For DC preparation, spleens were minced and digested with 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/MHCII), T cells (MHCII+ TCRB+ CD19+), and B cells (MHCII+ TCRB+ 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
were sorted. All sorting experiments were performed with a MoFlo Astrios (Beckman Coulter, Fullerton, Calif).

**Aptoptosis assay**

T cells, B cells, and eosinophils isolated from the small intestine and bone marrow–derived mast cells (BMMCs) were incubated with whole bacteria *B longum* KACC 91563 or EVs (2 μg/mL) of *B longum* KACC 91563 or *E faecalis* KACC 91532 for 2 hours. Apoptotic cells were stained with Annexin V and 7-aminoactinomycin D.

**In situ terminal deoxynucleotidyl transferase–mediated nick end-labeling assay**

BMMCs were incubated either with whole bacteria or EVs (2 μg/mL) from *E faecalis* KACC 91532 or *B longum* KACC 91563 for 2 hours. Mast cells were washed, and cell slides were prepared with cytopsin. Apoptotic cells were stained by using the DeadEnd Fluorometric *in situ* terminal deoxynucleotidyl transferase–mediated nick end-labeling (TUNEL) system (Promega, Madison, Wis), according to the manufacturer’s instructions. Images of cells were obtained by means of Axio Scope.A1 fluorescent microscopy (Carl Zeiss, Oberkochen, Germany) after staining with 4′-6-diamidino-2-phenylindole dihydrochloride.

**Confocal microscopy**

T cells, B cells, and eosinophils isolated from the small intestine, splenic DCs, and BMMCs were incubated with DI (Invitrogen, Carlsbad, Calif)–labeled EVs (10 μg/mL) for 2 hours at 37°C or 4°C. After washing, cell slides were prepared by using the cytospin method for confocal microscopy. Images of cells were obtained at × 400 magnification with the LSM 700 confocal microscope (Carl Zeiss) after staining with 4′-6-diamidino-2-phenylindole dihydrochloride. Cells containing DI-labeled EVs were counted in 25 different fields.

**Proteomic analysis**

EV proteins were separated by means of SDS-PAGE, and 2 major protein bands were excised. The gel pieces were treated with trypsin, according to a previously published in-gel digestion protocol.22 Samples were then injected for liquid chromatography–tandem mass spectrometry. Mass data were acquired automatically by using Analyst QS 2.0 software (Applied Biosystems, Foster City, Calif). Database searches were performed by using Proteome Discoverer (version 1.3) against the nonredundant National Center for Biotechnology Information database (NCBI). All analyses were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, Calif). For comparisons of the occurrence of diarrhea, Kaplan-Meier survival curve analysis was used, and P values were calculated by using the log-rank (Mantel-Cox) test. One-way ANOVA with the Newman-Keuls comparison posttest and Student t test were used to determine significant differences.

**RESULTS**

*B longum* KACC 91563 attenuates food allergy symptoms in a mouse model

In a previous study, *B longum* KACC 91563 and *E faecalis* KACC 91532 were isolated from fecal samples of healthy Korean neonates.19,26,27 According to TEM analysis, *B longum* KACC 91563 and *E faecalis* KACC 91532 are rod-shaped and round-shaped bacteria, respectively (see Fig E1 in this article’s Online Repository at www.jacionline.org). Because it was uncertain whether these 2 kinds of microbiota would reach the intestines of mice intact, we orally administered *E faecalis* KACC 91532 and *B longum* KACC 91563 for 2 weeks and observed bacteria in fecal samples using FISH. Both *B longum* KACC 91563 and *E faecalis* KACC 91532 were detected, and thus both *B longum* KACC 91563 and *E faecalis* KACC 91532 can reach the intestines of mice without being digested (see Fig E1, B). To assess any effects of *B longum* KACC 91563 and *E faecalis* KACC 91532 on food allergy, we adopted an allergen-induced food allergy mouse model that induces dose-dependent acute diarrhea in BALB/c mice.22 During food allergy induction, we orally treated mice with *B longum* KACC 91563 and *E faecalis* KACC 91532 daily (Fig 1, A) and found that *B longum* KACC 91563, but not *E faecalis* KACC 91532, suppressed the occurrence of diarrhea significantly (Fig 1, B). Similarly, there was a delay in the onset of diarrhea in mice treated with *B longum* KACC 91563 when mice were challenged with low-dose OVA through the intragastric route (see Fig E2 in this article’s Online Repository at www.jacionline.org). The number of bacteria seemed to be an important factor for this suppressive effect because diarrhea was not prevented at less than 5 × 10⁶ cfu of bacteria per mouse per day (data not shown). In conclusion, our data indicate that *B longum* KACC 91563 has the ability to ameliorate symptoms of food allergy.

*B longum* KACC 91563 reduces mast cell numbers in the small intestine

In this food allergy model, Th2 immune responses are induced, and there is a release of TH2 cytokines, such as IL-4, IL-5, IL-9, and Set (eBioscience), according to the manufacturer’s instructions. Stained cells were analyzed with the LSR Fortessa (BD), and data were analyzed with FlowJo software (Tree Star, Ashland, Ore).

**ELISA**

Mesenteric lymph node (MLN) cells were cultured for 72 hours at 37°C in medium containing 100 μg/mL OVA. Concentrations of IL-4, IL-5, IL-10, IL-13, IL-17A, and IFN-γ in culture supernatants were measured by using ELISA kits from eBioscience, according to the manufacturer’s instructions. Concentrations of mast cell protease 1 (MCPT-1) and OVA-specific IgE in serum were measured by using ELISA kits (MCPT-1 ELISA kit [eBioscience] and OVA-specific IgE ELISA kit [Chondrex, Redmond, Wash]), according to the manufacturer’s instructions.

**Statistical analysis**

All analyses were performed with GraphPad Prism 5 software (GraphPad Software, San Diego, Calif). For comparisons of the occurrence of diarrhea, Kaplan-Meier survival curve analysis was used, and P values were calculated by using the log-rank (Mantel-Cox) test. One-way ANOVA with the Newman-Keuls comparison posttest and Student t test were used to determine significant differences.
and IL-13. Examine whether a reduction in Th2 immune responses is involved in suppression of diarrhea, we measured levels of OVA-induced Th1, Th2, and Th17 cytokines secreted from MLN cells. Th2 cytokines, such as IL-4, IL-5, IL-9, and IL-13, were strongly induced, whereas in contrast, the Th1 cytokine IFN-\(\gamma\) and the Th17 cytokine IL-17 were not detected in the food allergy group. Interestingly, even though \(B\) longum KACC 91563 alleviated the diarrhea, Th2 cytokine levels were not decreased (Fig E3, A). We hypothesized that mast cell–specific apoptosis induced by \(B\) longum KACC 91563 and its EVs specifically induce apoptosis of BMMCs.

\(B\) longum enhances intestinal defense and protects the host from the infection through production of short-chain fatty acids, such as acetate. Therefore we hypothesized that the prevention of food allergy was caused by acetate produced by \(B\) longum KACC 91563; however, diarrhea was not attenuated by administration of acetate during the entire period or the effector phase of the food allergy model (see Fig E5 in this article’s Online Repository at www.jacionline.org).

In a recent study, \(Bacteroides\) fragilis–derived EVs were shown to contain polysaccharide A, which is known to induce Treg cells, suggesting that EVs perform similar functions to the cells from which they originate through the transport of crucial effector molecules. We hypothesized that EVs of \(B\) longum KACC 91563 and \(E\) faecalis KACC 91532 had a measureable effect on the production of OVA-specific IgE (Fig 2, C). Collectively, these data indicate that \(B\) longum KACC 91563 suppresses allergic diarrhea through selective reduction of mast cell numbers in the small intestine.

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91563–derived EVs specifically. Thus, we incubated BMMCs, splenic DCs, eosinophils, T cells, and B cells with DiI-labeled EVs of *B. longum* KACC 91563 and *E. faecalis* KACC 91532. Specifically, BMMCs showed high uptake ability compared with other cell populations and efficiently took up EVs of *E. faecalis* KACC 91532, as well as *B. longum* KACC 91563 (Fig 4), indicating that mast cells are capable of internalizing EVs of bacteria, irrespective of their origin. These results also implied that a certain component in EVs of *B. longum* KACC 91563 is likely to be responsible for mast cell apoptosis. In fact, although DCs are well-known phagocytes, BMMCs took up the labeled EVs more readily than did DCs (Fig 4). To exclude the possibility of phagocytosis, we incubated EVs with each type of cell under the phagocytosis-inhibiting conditions at 4°C. Although the uptake ability of DCs was significantly impaired at 4°C, uptake by mast cells was not markedly affected by temperature (Fig 4). These results suggest that mast cells specifically take up EVs through an undefined receptor on their surface.

**ESBP, a major protein present in *B. longum* KACC 91563–derived EVs, attenuates food allergy symptoms**

To address which component in EVs of *B. longum* KACC 91563 participates in mast cell apoptosis, we separated proteins of EVs...
using SDS-PAGE. EVs of *B. longum* KACC 91563 were found to be mainly comprised of 2 proteins (Fig 5, A) that were identified as ESBP and ASBP through proteomic analysis (Fig 5, A). To perform functional studies, GST-tagged recombinant proteins of ESBP and ASBP were expressed in *E. coli* and purified by using glutathione Sepharose beads (see Fig E6, A, in this article’s Online Repository at www.jacionline.org). The purified proteins were intraperitoneally injected twice before the first and fifth OVA oral challenges to examine whether ESBP and ASBP suppress food allergy (Fig 5, B). ESBP, but not ASBP, dramatically reduced the occurrence of diarrhea in a dose-dependent manner (Fig 5, C, and see Fig E6, B and C). In

FIG 3. Apoptosis of BMMCs induced by *B. longum* KACC 91563 and its EVs. A, TEM image of EVs derived from *B. longum* KACC 91563 and *E. faecalis* KACC 91532. B, Size of EVs. Error bars indicate means ± SDs from 5 measurements. C, Apoptosis assay. Annexin V+ apoptotic cells were measured by means of fluorescence-activated cell sorting (n = 3). Values represent means ± SEMs. Statistical analysis was performed by using 1-way ANOVA with the Newman-Keuls posttest. **P < .01 and ***P < .001. Data are representative of 2 to 3 independent experiments. D, BMMCs were incubated with whole bacteria or EVs of *B. longum* KACC 91563 or *E. faecalis* KACC 91532 for 2 hours. Apoptotic cells were stained with the DeadEnd Fluorometric TUNEL system. Representative data are shown (magnification ×400). Nuclei of cells are blue, and TUNEL is green. The number of apoptotic cells was counted in 7 different fields (magnification ×100, n = 3 per group). Values represent means ± SEMs. Statistical analysis was performed by using 1-way ANOVA with Newman-Keuls posttest. *P < .05 and **P < .01. B. l, *B. longum* KACC 91563; E. f, *E. faecalis* KACC 91532.
addition, like *B. longum* KACC 91563, the recombinant ESBP protein reduced the number of mast cells in the small intestine without affecting proportions of Foxp3+ Treg cells and eosinophils in the small intestine (Fig 5, D and E, and see Fig 3 in this article's Online Repository at www.jacionline.org). MCPT-1 levels in serum were also decreased by recombinant ESBP protein compared with recombinant GST and ASBP protein (Fig 5, F). These data show that ESBP specifically reduces
the number of mast cells and alleviates diarrhea in this food allergy model.

**DISCUSSION**

Recently, one study suggested that reconstitution of mice with human microbiota attenuates diarrhea through induction of Treg cells. This result indicates that human microbiota are compatible with mouse microbiota, the mouse model could be a powerful method for investigation of allergic disease and the microbiota. Vulnerability to allergic disease is controlled by species and degree of exposure of colonized microbiota. One way to control the composition and effects of the microbiota is to take in probiotics that assist the host in attenuating allergic disease by modulating the intestinal immune response. In this report, we suggest that administration of a single strain of human probiotic bacteria, *B. longum* KACC 91563, suppresses allergen-induced food allergy in mice.

It is critical to understand the mechanisms of action of these bacteria within the host to increase the application and clinical utility of probiotics to improve human health and prevent disease. The Th2 immune response plays a critical role in the development...
The reduction of mast cells results in suppression of food allergy. The EVs penetrate through intestinal epithelial cells and specifically induce apoptosis of mast cells in the lamina propria. This suggests that BMMCs internalize EVs through a specific receptor-mediated pathway; however, the underlying molecular mechanisms remain unclear.

Previously, many publications have suggested that probiotic-derived proteins affect the immune system in the gastrointestinal tract. For example, S layer protein A expressed on the surface of the Lactobacillus acidophilus strain NCFM binds to the DC-SIGN receptor and induces production of IL-10. Mutated L acidophilus NFCM lacking the S layer protein A induced proinflammatory cytokines in human DCs. Furthermore, Lactobacillus salivarius UCC118 produces a bacteriocin in vivo that can dramatically protect mice against infection with the invasive pathogen Listeria monocytogenes. In the present study, we characterized 2 proteins, ESBP and ASBP, from B longum KACC 91563–derived EVs. Of these, only ESBP had the ability to attenuate the food allergy symptoms in the mouse model. Details regarding the binding receptor for ESBP and any downstream signaling molecules affected by its binding remain to be determined. Thus, further analysis of the characteristics of ESBP, as well as the detailed molecular mechanisms underlying its anti–food allergy functions, will be required in future studies.

Because B longum KACC 91563 was isolated from neonates and targets mast cells, a major effector cell population important for the allergy response (also known as a T cell–independent immune response), use of this probiotic for treatment of food allergy is likely safe. Also, bacterial EVs are extremely small and transfer bacterial components inside their lipid bilayers to target cells in a concentrated and protected form. Thus, we propose a possible mechanism of action in which EVs of B longum KACC 91563 penetrate through intestinal epithelial cells and induce apoptosis of mast cell selectively (Fig 6). Additionally, ESBP from B longum KACC 91563 induced a reduction in mast cell counts and alleviated food allergy symptoms in the effector phase of the food allergy model (Fig 5). However, administration of B longum KACC 91563 only during the effector phase did not alleviate food allergy symptoms (see Fig E8 in this article’s Online Repository at www.jacionline.org), and B longum KACC 91563 seemed not to be efficient in the treatment of food allergy. Therefore, recombinant ESBP protein might be used as a drug for the treatment of food allergy, although additional investigations in food allergy models sensitized through physiologic routes, such as oral and cutaneous priming with adjuvant, are required.

Despite numerous attempts at clinical approaches to cure allergic diseases and a growing commercial market, there are few viable treatment options. Our research suggests that proteins derived from EVs might be a novel treatment option for allergic diseases targeting mast cells.

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FIG 6. Proposed mechanism of attenuation of food allergy by B longum KACC 91563. B longum KACC 91563 in the intestinal lumen secretes small-sized EVs that contain bacterial components. The EVs penetrate through intestinal epithelial cells and specifically induce apoptosis of mast cells in the LP. The reduction of mast cells results in suppression of food allergy.

of allergic disorders through the release of cytokines, such as IL-4, IL-13, and IL-9. These cytokines induce mastocytosis and IgE class-switching in the intestine. Even though depletion of Th2 cytokines, such as IL-4 and IL-13, attenuates food allergy, we could not detect the suppression of IL-4, IL-5, or IL-9 in the B longum KACC 91563–treated group. Furthermore, a recent study has reported that maintenance of oral tolerance by Foxp3 Treg cells is important for the prevention of food allergy. Treg cells are induced by the probiotics Lactobacillus reuteri and Lactobacillus casei, which bind to dendritic cell–specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) on DCs. In addition to DCs and Treg cells, mast cells play a major role during allergic disease; however, thus far, clinical application of probiotics has focused on the modulation of T-cell polarization through DCs and not on mast cells. Here we suggest that administration of B longum KACC 91563 before food allergy induction alleviates symptoms of food allergy by reducing the number of mast cells present in the LP of the small intestine. In contrast, no evidence of an effect of this probiotic on Th2 or Treg cells was found in these studies. If daily intake of probiotics led to suppression of steady-state T-cell activity, this could be detrimental because a successful immune response in the intestines is the first line of defense to many pathogens. However, B longum KACC 91563 is able to maintain the immune response while suppressing food allergies by affecting only mast cells, the major effector cells of this disease.

EVs are enriched for molecules, such as proteins, DNA, and lipids, inside their lipid bilayer. Compared with the soluble forms of these molecules, EVs more stably preserve and effectively transport these molecules. Although the component-sorting system of EVs remains to be elucidated, released EVs showed similar effects as whole bacteria. This suggests that EVs might be a supporter or even a substitute for the probiotic effects of the bacteria. Indeed, B fragilis–derived EVs alleviated colitis similarly to B fragilis and were directly internalized by DCs in vitro.
Clinical implications: ESBP isolated from the EVs of *B longum* KACC 91563 is a potential therapeutic molecule that suppresses food allergies by targeting mast cells.

REFERENCES


A. Morphologic features of B. longum KACC 91563 and E. faecalis KACC 91532 were analyzed by using TEM.

B. B. longum KACC91563 and E. faecalis KACC 91532 were administered for 2 weeks, and FISH analysis was performed on fecal samples.
FIG E2. Effects of *B. longum* KACC 91563 and *E. faecalis* KACC 91532 on the low-dose food allergy model. For food allergy induction, 10 mg of OVA was orally administered 7 times (OVA vs OVA + *B. longum*: *P* < .05, n = approximately 6-8 mice per group).
FIG E3. Alleviation of food allergy by *B. longum* KACC 91563 was not associated with T cell–mediated immune responses. **A**, After the fifth intragastric challenge of OVA, MLN cells were cultured with OVA for 72 hours, and concentrations of IL-4, IL-5, IL-9, IL-10, IL-13, IL-17, and IFN-γ in the culture supernatants were measured. **B**, Cells were isolated from spleens and MLNs and stained for CD4 and Foxp3. Proportions of Foxp3+ Treg cells were assessed by using fluorescence-activated cell sorting. Values represent means ± SEMs (n = 4 per group). Data are representative of 2 independent experiments. *B. l.*, *B. longum* KACC 91563; *E. f.*, *E. faecalis* KACC 91532.
FIG E4. Effect of *B. longum* KACC 91563 on mast cells in the intestine under homeostatic conditions. *B. longum* KACC 91563 was administered into mice without induction of food allergy. A, Intestinal mast cell population was assessed by using fluorescence-activated cell sorting (n = 5 mice per group). B, Paraffin-embedded sections of the jejunum were stained for mast cells, and the number of mast cells was counted in 3 different fields (n = approximately 4-5 mice per group; original magnification ×400). ND, Normal diet. Values represent means ± SEMs.
FIG E5. Effect of acetate, a metabolite of *B. longum*, on food allergy. A and C, Experimental schedule for food allergy induction and acetate administration in mice. B, Acetate (150 mmol/L) was administered in drink to mice every day from day 19 of the food allergy model and occurrence of allergic diarrhea was analyzed to examine the effect of acetate on food allergy symptoms (n = 5 mice per group). D, Acetate (150 mmol/L) was administered in drink to mice every day from day 0 of the food allergy model and occurrence of allergic diarrhea was analyzed to examine the effect of acetate on food allergy symptoms (n = approximately 5-10 mice per group). i.g., Intragastric; i.p., intraperitoneal.
FIG E6. ESBP attenuates food allergy in a dose-dependent manner. A, Purification of GST-tagged recombinant ESBP and ASBP. B and C, Injection of ESBP and ASBP into the mouse that was part of the food allergy model at various concentrations (0.5, 5, and 50 μg) and measurement of diarrhea occurrence (OVA + GST 50 μg vs OVA + ESBP 50 μg: **P < .01, n = 6 mice per group).
FIG E7. Effects of ESBP on mast cells, eosinophils, and Foxp3+ Treg cells in the small intestine. Mast cells, eosinophils, and Foxp3+ Treg cells in the LP were analyzed by using flow cytometry. A, Mast cells were gated as IgE+c-kit+ cells. B, Eosinophils were gated as Siglec-F+CCR3+ cells. C, Foxp3+ Treg cells were gated as CD4+Foxp3+ cells. Values represent means ± SEMs. Statistical analysis was performed by using 1-way ANOVA with the Newman-Keuls posttest. **P < .01. Data are representative of 2 independent experiments.
FIG E8. Administration of *B. longum* KACC 91563 during the effector phase of the food allergy model has no suppressive effect. 

**A**, Experimental schedule for food allergy induction and *B. longum* KACC 91563 administration in mice. 

**B**, Diarrhea occurrence (n ~ approximately 4-9 mice per group). 

**C**, Proportion of mast cells in the small intestine was determined by using fluorescence-activated cell sorting analysis (n ~ approximately 4-7 mice per group). 

**D**, MCPT-1 levels in serum. 

**E**, Paraffin-embedded sections of the small intestine were stained for mast cells. Numbers of mast cells were counted in 3 different fields (n ~ approximately 3-4 mice per group; original magnification ×400). Values represent means ± SEMs. 

*i.g.*, Intragastric; *i.p.*, intraperitoneal.