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(Citation)
International Journal of Food Sciences and Nutrition,72(4):478-484

(Issue Date)
2021-05-19

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90008728

Research paper

Lactobacillus plantarum 22A-3 exerts anti-allergic activity through TGF-β secretion in passive cutaneous anaphylaxis of mice

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Abbreviation

APC, antigen presenting cell;

FcεRI, Fcε Receptor I;

LAB, Lactic acid bacteria;

LP22A3, *lactobacillus plantarum* 22A-3;

TGF-β, transforming growth factor β;

PCA, passive cutaneous anaphylaxis;

Abstract

Allergy is a global issue, however, medical intervention for allergy treatment is limited. Recent studies have focused on allergy prevention with food factors. In this study, *Lactobacillus plantarum* 22A-3 (LP22A3) exerted anti-allergic effect in passive cutaneous anaphylaxis (PCA) reaction and increased transforming growth factor (TGF)-β contents in blood. Increase of TGF-β contents in blood by exogenous TGF-β injection intraperitoneally decreased Evans blue release into mice ears to the same level as LP22A3 treatment in PCA reaction. LP22A3 treatment directly to RBL-2H3 cells shows no effect on β-hexosaminidase release from RBL-2H3, but inhibited its release using the Caco-2/RBL-2H3 cells co-culture system stimulated with LP22A3 from apical side. Moreover, TGF-β treatment to RBL-2H3 inhibited β-hexosaminidase release from RBL-2H3. However, β-hexosaminidase release was cancelled by TGF-β neutralizing antibody without the influence of TGF-β mRNA expression in Caco-2 cells. These results showed that LP22A3 ameliorates allergy by TGF-β secretion through the intestine.

Keywords: Anti-allergic activity, β-hexosaminidase, *Lactobacillus plantarum* 22A-3,

passive cutaneous anaphylaxis reaction.

Introduction

Allergic disease is caused by hypersensitivity of the immune system. Hypersensitivity can be divided into four types, namely: immediate hypersensitivity (type I), cytotoxic reaction (type II), immune complex reaction (type III), and delayed hypersensitivity (type IV) (Janeway et al., 2001). Type I is initiated by the allergen, including food factors, pollens, dusts etc. When allergen gets access to the human body, it will be recognized by antigen presenting cells (APC) and presented to naïve helper T cell (Th0). Th0 is activated and differentiates into T helper 2 cell (Th2) rather than T helper 1 cell (Th1). Th2 is capable of secreting Th2 cytokines including Interlukin-4 (IL-4), IL-5 and IL-13, which can promote B cell to process immunoglobulin (Ig) class switch recombination and leading to accumulation of IgE. When the same allergen invades again, the binding of allergen to IgE activates the Fcε Receptor I (FcεRI) on mast cells and cause mast cells degranulation resulting in the release of chemical mediators like histamine (Janeway et al., 2001). Effects induced by histamine contain smooth muscle contraction, increased vascular permeability, prostaglandin generation (White, 1990), which will lead to symptoms like skin reactions (urticarial, eczema and angioedema), respiratory tract reactions (rhinitis and bronchitis), gastrointestinal tract (intestinal cramps and diarrhea) and the worst, anaphylactic shock (Janeway et al.,

68 2001).

69 Transforming growth factor (TGF)-β is a cytokine that regulates cell
70 differentiation, migration, and proliferation, and TGF-β plays a crucial role in
71 maintaining skin homeostasis (Ramirez et al., 2014). It is well known that TGF-β
72 promotes collagen production in skin (Hwang et al., 2011; Hwang et al., 2014), and
73 moreover, TGF-β is known to promote keratinocyte migration, which is essential for the
74 reconstruction of the cutaneous barrier after skin injury (Zambruno et al., 1995; Santoro
75 and Gaudino, 2005). It has reported that TGF-β acts as a negative regulator of mast cell
76 function, in part by decreasing FceRI expression (Gomez et al., 2005). However, it is
77 not well understood whether TGF-β affects allergic property.

78 *Lactobacillus plantarum* 22A-3 (LP22A3) is one of the plants derived lactic
79 acid bacteria (LAB) and it was found in the rice-bran paste of eggplants pickle. Previous
80 study indicated that its oral administration prevented DSS-induced colitis (Ohto and
81 Mizuno, 2017). Moreover, it was demonstrated that anti-inflammatory cytokine, TGF-β
82 and IL-10 productions were increased from lamina propria lymphocytes by oral
83 administration of LP22A3 to the normal mice. This report also indicated that LP22A
84 treatment in apical side of Caco-2/RAW264.7 cells co-culture system cancelled the
85 inhibition of IL-8 mRNA expression of Caco-2 cells when TGF-β neutralizing antibody

was added into basolateral side before stimulating RAW264.7 cells by lipopolysaccharide. Overexpression of TGF-β abolished the airway hyperresponsiveness and airway inflammation in murine model of allergic asthma (Hansen et al., 2014). Administration of anti-TGF-β antibody to sensitized mice before the transfer of $CD4^+CD25^+$ T regulatory cells countered the suppression effect of these cells on allergen-induced airway hyperresponsiveness (Joetham et al., 2007). Moreover, Jiménes et al., (2017) reported that the oral administration of glycomacropeptide prior to and during sensitization increases the production of TGF-β in response to allergens. Our previous study has demonstrated that oral administration of *Enterococcus faecalis* IC-1 prevents allergic symptom in passive cutaneous anaphylaxis (PCA) reaction and Caco-2/RBL-2H3 co-culture system (Yamashita et al., 2016).

The aim of this study was to investigate whether oral administration of LP22A3 can regulate mast cell activation by PCA and co-culture system, so as to understand the mechanism by TGF-β production. It was ascertained to practical application of LP22A3 for the control of allergic diseases.

Materials and Methods

Materials

104 Dulbecco's Modified Eagle's Medium (DMEM, High Glucose) with glutamine and
105 Evans blue were purchased from Wako Pure Chemical Industries (Osaka, Japan).
106 Eagle's Minimum Essential Medium (MEM) was purchased from Nissui
107 Pharmaceutical Co. Ltd., (Tokyo, Japan). Anti-dinitrophenyl (DNP) IgE, DNP-albumin,
108 and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide were purchased from Sigma (St Louis,
109 MO, USA). Trypsin, RPMI 1640 medium, MEM non-essential amino acids (NEAA)
110 ware purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS)
111 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). DNP-bovine
112 serum albumin (DNP-BSA) was purchased from Cosmo Bio (Tokyo, Japan).
113 Recombinant mouse TGF-β was purchased from BioLegend (San Diego, CA, USA).
114 Mouse anti-TGF-β antibody was purchased from R&D system (Minneapolis, MN).
115 Other chemicals and reagents were ordinary commercial and guaranteed products.
116
117 *Preparation of UV-inactivated LP22A3 and oral administration*
118 LP22A3 was cultured in Mann Rogosa Sharp (MRS) broth and incubated overnight at
119 30°C in anaerobic chamber. After incubation, bacterial cells were obtained by
120 centrifugation (4°C, 10,000 ×g, 5 min) and was resuspended in PBS and washed three
121 times in order to remove culture medium. Bacterial suspension was irradiated with an

UV germicidal lamp to inactivate before use in in vitro experiments. After UV-treatment, viable counts were below 10^2 cfu/mL indicated more than 9-log reduction of viability. Samples were stored at −80 °C until use. The inactivated LP22A3 (1 x 10^{11} cfu/g) was suspended in 0.5% carboxymethyl cellulose, and 100 μL of it was orally administered using gastric feeding tube.

Mice

Female 7-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained in filter-top cages in a specific pathogen-free condition in Kobe University Life Science Laboratory with free access to laboratory chow and water *ad libitum*. All animal experiments were approved and carried out in accordance with the Animal Experiment Ethnics Committee of Kobe University (permission number: 30-10-03-R1).

PCA reaction in mice

An IgE-dependent PCA reaction was performed in accordance with a previous study (Yamashita et al., 2016). Mice were randomly divided into four groups with 4 animals in each group and were administered with 100 μL of test sample orally for 10 days.

Anti-DNP IgE (0.1 μg) was injected subcutaneously into both ears of each mouse under anesthesia on day 10. After 24 h, the mice were injected intravenously with 0.2 mg of DNP-BSA in 100 μL PBS containing Evans blue (10 mg/mL), via tail vein. Thirty minutes after the DNP-BSA injection, mice were sacrificed and both ears were collected for measurement of the pigmented area. The ears were mixed in 250 μL of formamide at 37 °C for 24 h. After centrifugation at 10,000 rpm for 20 min at 4 °C, the supernatants were used for measurement of absorbance at 620 nm. Luteolin was used as positive control. The recombinant TGF-β (17.5 ng/ mouse) was intraperitoneally injected to increase TGF-β levels in blood every day for 10 days.

Cell culture

Rat basophilic leukemia cell line, RBL-2H3 cells, were cultured in dishes in Eagle's MEM supplemented with 10% (v/v) heat-inactivated FBS (57 °C, 30 min), 100 μg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine. Cell cultures were incubated at 37 °C in a 5% CO_2 incubator. Passage numbers 14–32were used. Human intestinal epithelial cell line, Caco-2 cells, were cultured in a 75 cm^2 plastic flask in DMEM (high glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 μg/mL streptomycin, and 100 U/mL penicillin, and incubated at 37 °C in a 5% CO_2 incubator.

158 Passage numbers 48–64 were used. When either cell line reached 80% confluence, cells
159 were recovered from the culture dish or flask by trypsin digestion after washing with
160 phosphate-buffered saline (PBS). The cells were replated in a new dish or flask.
161
162 *Co-culture system of Caco-2/RBL-2H3 cells*
163 Caco-2 cells were seeded at the concentration of 0.6×10^5 cells/well onto 24-well
164 Transwell insert plates (0.33 cm^2, 0.4 μm pore size, Corning Costar Corp., Cambridge,
165 MA). Cell culture medium was changed every 3 day until the cells were fully
166 differentiated (TER value >300Ω·cm^2). RBL-2H3 cells were seeded at 2.0×10^5
167 cells/500 μL/well onto 24-well tissue culture plates in Eagle's MEM and incubated
168 overnight with 1 μg/mL at a final concentration of anti-DNP IgE. After replacing all
169 media with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl2, 1 mM $CaCl^2$,
170 40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2) the Transwell inserts on
171 which Caco-2 cells had been cultured were added into the plate wells preloaded with
172 RBL-2H3 cells. In an experiment to evaluate the anti-allergic effect of test samples, 0.2
173 mL of SB or test sample solution was applied into the apical side. After incubation for 6
174 h, the cells were challenged with 10 ng/mL final concentration of DNP albumin for 10
175 min at 37 °C. The plate was cooled in an ice bath for 10 min to stop degranulation

176 responses.

177

178 *β-Hexosaminidase activity assay*

179 RBL-2H3 cells were dispensed into 96-well plates at a concentration of 2×10^5 cells/well

180 and incubated over-night at 37℃ in 5% CO_2. Cells were sensitized with anti-DNP IgE

181 for 2 h. Cells were washed twice with Siraganian buffer before adding test sample to

182 each well and incubated for 1 h, followed by challenge with DNP-albumin, antigen for

183 1 h. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50

184 μL) was transferred into 96-well microplate and incubate with 50 μL of substrate

185 solution for 1 h. The reaction was stopped by adding stop solution. The absorbance was

186 measured with a microplate reader at 405 nm.

187

188 *Contents of TGF-β in serum*

189 TGF-β contents were measured in accordance with a previous study (Oka et al., 2020).

190 Briefly, whole blood was left undisturbed for 30 min at room temperature, and

191 subsequently centrifuged at 10,000 rpm at 4℃ for 10 min. Supernatants were collected

192 as blood serum. Serum samples were stored at -80℃ until analysis. Serum TGF-β levels

193 were measured using an ELISA (Promega, Madison, WI, USA) in accordance with the

manufacturer's recommended protocol.

RNA isolation and quantitative Real-Time PCR of TGF-β mRNA

Total RNA was extracted from Caco-2 cells using Sepasol RNA I super (Nacalai Tesque). cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Quantitative PCR assays were analyzed using Applied Biosystem 7500 Fast Real-Time PCR system with TaqMan gene expression assay. TaqMan gene expression assay were purchased from Applied Biosystems; β-actin (Mm00607939_s1) and mouse TGF-β (Mm01178820_m1).

Statistical Analysis

All the data were presented as mean ± standard deviation. Statistical significances among each group were evaluated by analysis of variance (ANOVA) and Tukey-Kramer test to determine differences between groups. Statistical significance was defined as $p < 0.05$.

Results

212

213 *Oral administration of LP22A3 suppressed anaphylaxis in PCA model mouse*

214 Oral administration of LP22A3 significantly inhibited IgE-mediated PCA reaction (Fig.
215 1). Weight and drinking amounts of LP22A3-fed mice show no significant difference
216 compared with the control group throughout experiment (data not shown). Moreover,
217 oral administration of LP22A3 for 10 days significantly increased TGF-β level in blood
218 to be approximately 2.7 fold (Fig. 2). It was assumed that anti-allergic activity of
219 LP22A3 might be associated with TGF-β secretion in blood. To confirm this hypothesis,
220 mice were injected intraperitoneally with recombinant TGF-β (17.5 ng/mouse/day)
221 every day for 10 days. It was reported in our previous study that the exogenous TGF-β
222 injection increased TGF- β contents in blood to be more than 200 pg/mL which is
223 almost equal to TGF-β levels by the oral administration of LP22A3 in this experiment
224 (Oka et al., 2020). Increase of TGF-β contents in blood by exogenous TGF-β injection
225 decreased Evans blue release into mice ears to the same level as LP22A3 treatment,
226 indicating that IgE-mediated PCA reaction was inhibited (Fig. 3).

227

228 *Suppression of degranulation of RBL-2H3 cells by LP22A3 involves an interaction with*
229 *Caco-2*

To investigate whether LP22A3 affected mast cell directly, LP22A3 was applied to IgE-sensitized RBL-2H3 cells. As shown in Fig. 4A, LP22A3 did not show suppression of β-hexosaminidase from RBL-2H3, but luteolin did. However, LP22A3 could suppress β-hexosaminidase release from RBL-2H3 at the same levels of luteolin when LP22A3 was applied in apical side of Caco-2/RBL-2H3 co-culture system (Fig. 4B). These results suggested that LP22A3 affects RBL-2H3 cells through Caco-2 cells, indicating that the involvement of intestinal epithelial cells is an important factor in anti-allergic activity of LP22A3.

Anti-allergic activity of LP22A3 depends on TGF-β production

In PCA reaction, it was ascertained that TGF-β contents in blood was increased (Fig. 2). To confirm the involvement of TGF-β secretion through intestinal epithelial cells, RBL-2H3 cells were pretreated with an anti-TGF-β antibody for 30 min in basolateral side before adding LP22A3 into apical side of co-culture system. LP22A3 stimulation from apical side increased TGF-β mRNA expression (Fig. 5A). Moreover, pretreatments of anti-TGF-β antibody or irrelevant IgG show no effects in the expression of TGF-β mRNA which remained almost the same level as when stimulated with LP22A3. However, pretreatment with anti-TGF-β antibody in basolateral side eliminated the

248 suppression of β-hexosaminidase release although the pretreatment of irrelevant IgG did
249 not (Fig. 5B). Thus, TGF-β secretion from Caco-2 cells stimulated with LP22A3 plays
250 an inhibitory role of β-hexosaminidase release from RBL-2H3.
251
252 **Discussion**
253 Many clinical trials revealed that several *Lactobacillus* strains, including *L.*
254 *rhamnosus* GG, *L. acidophilus* L-92, and *L. casei* Shirota, were effective in prevention
255 of early atopic disease in children and of allergic symptoms in patients sensitive to
256 Japanese cedar pollen (Kalliomäki et al., 2001; Ishida et al., 2005; Tamura et al., 2007).
257 Therefore, a lot of studies mainly focused on therapeutic and preventive effects of LAB
258 in allergic diseases.
259 In this study, inhibitory effect of LP22A3 on type I allergy was evaluated by
260 PCA model as i*n vivo* and co-culture model composed of human intestinal epithelial
261 Caco-2 cells and rat basophilic leukemia RBL-2H3 cells as i*n vitro* experiments. It was
262 demonstrated that LP22A3 possessed anti-allergic activity and induced to the secretion
263 of TGF-β in blood. TGF-β is a multifunctional cytokine that plays pivotal roles in
264 diverse biological processes, including the regulation of cell growth and survival, cell
265 and tissue differentiation, development, inflammation, immunity, hematopoiesis, and

266 tissue remodeling and repair (Lee et al., 2006; Letterio and Roberts, 1998). TGF-β can
267 decreases FcεRI expression, and inhibits mast cell degranulation (Gomez et al., 2005).
268 Intraperitoneal injection of exogenous TGF-β (17.5 ng/day/ mouse) for 10 days to mice
269 demonstrated the inhibition of Evans blue exudation. This TGF-β concentration has
270 reported to increase TGF-β levels in the blood to be more than 200 pg/mL which is
271 almost equal to its concentration of mice administered with LP22A3 (Oka et al., 2020).
272 It was ascertained that the increase of TGF-β secretion in blood by LP22A3
273 administration was found to contribute the allergy suppression in PCA reaction. The
274 co-culture system composed of Caco-2 and RBL-2H3 cells has already been proven to
275 be effective in investigating intestinal-mediated anti-allergic effects (Yamashita et al.,
276 2016). LP22A3 treated in apical side indicated the suppression of β-hexosaminidase
277 release from RBL-2H3, but its direct treatment to RBL-2H3 did not. These results
278 indicated that intestinal cells are involved to exert the influence against RBL-2H3 cells
279 in β-hexosaminidase activity by LP22A3. The pretreatment of anti-TGF-β antibody and
280 irrelevant IgG indicated no influence of TGF-β mRNA in Caco-2 cells compared to
281 LA22A3 stimulation, indicating that TGF-β secretion was comparable in each group.
282 On the other hand, it was demonstrated that TGF-β neutralizing antibody could cancel
283 the inhibition of β-hexosaminidase activity by LP22A3 treatment. Collectively, these

outcomes indicated that LP22A3 exerted its inhibitory activity through its unique ability to promote TGF-β production from Caco-2 cells. Torii et al., (2007) reported that oral administration of *Lactobacillus acidophilus* Strain L-92 increased TGF-β production from cells Peyer's patches.

Conclusions

In conclusion, this paper demonstrates that oral administration of LP22A3 possesses anti-allergic activity in PCA reaction, and that its activity is mediated by TGF-β production through intestinal epithelial cells using co-culture system of Caco-2/RBL-2H3 cells. However, we were unable to determine the mechanism how LP22A3 induced TGF-β production in blood through intestinal epithelial cells. Clarification of the relationship between anti-allergic activity and TGF-β production by LP22A3 may provide a clue to certificate why certain LABs exert anti-allergic activity.

Disclosure statement

The authors declare that there are no conflicts of interest.

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360 Figure legends

361

362 **Fig. 1.** Anti-allergic activity of LP22A3 PCA reaction.

363 LP22A3 (1 $\times 10^8$ cfu/mouse/day) and luteolin (500 μg/mouse/day.,used as a positive
364 control) were orally administered to mice for 10 days before anti-DNP IgE sensitization.
365 After 24 h, DNP-albumin and Evans blue were injected *via* tail vein as an antigen
366 challenge. Evans blue stained ears were photographed. The ear-absorbed dye was
367 extracted with formamide, and absorbance at 620 nm was measured. Values represent
368 means ± SD (n = 4). The animal experiments were conducted with two independences.
369 Different superscript letters after values indicate statistical significance between groups
370 ($p < 0.05$).

371

372 **Fig. 2.** Upregulation of TGF-β secretion in blood by oral administration of LP22A3.

373 TGF-β level in serum prepared from mice in Fig. 1 was measured by ELISA. Values
374 represent means ± SD (n = 4). **P*<0.05 versus vehicle group.

375

376 **Fig. 3.** Influence of exogenous TGF-β on PCA reaction

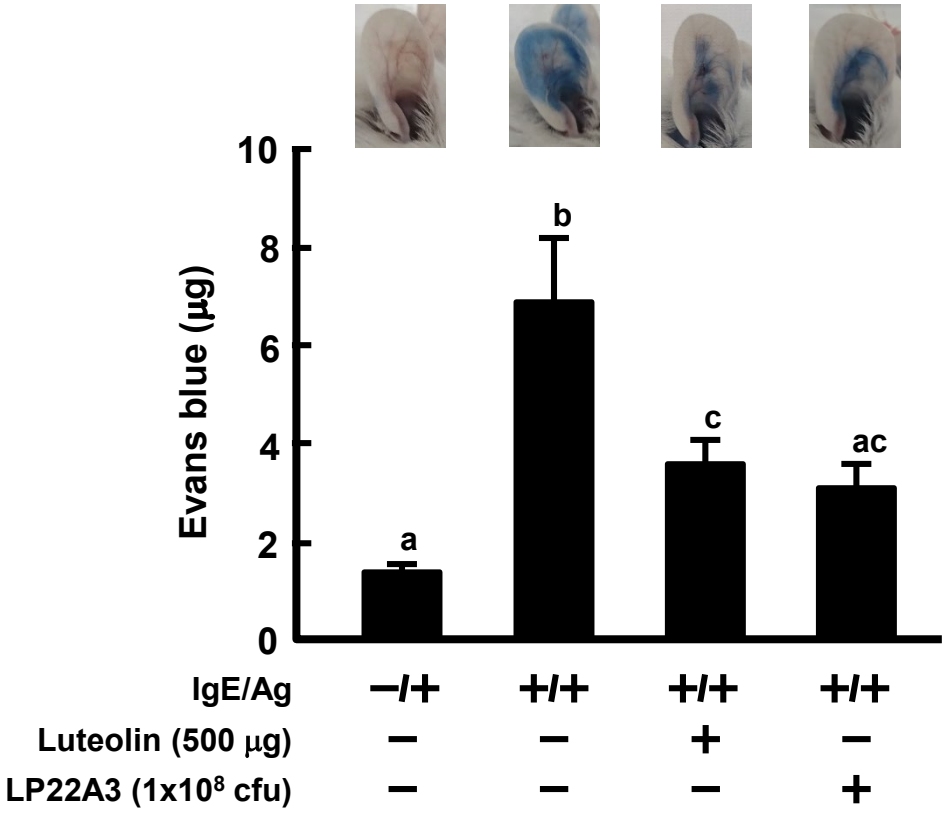
377 TGF-β (17.5 ng/mouse/day) were intraperitoneally injected to mice for 10 days. Values

represent means ± SD (n = 4). The animal experiments were conducted with two independences *P<0.05 versus degranulation group.

Fig. 4. Effect of LP22A3 treatment on degranulation of RBL-2H3 cells in monoculture and co-culture system of Caco-2/RBL-2H3 cells.

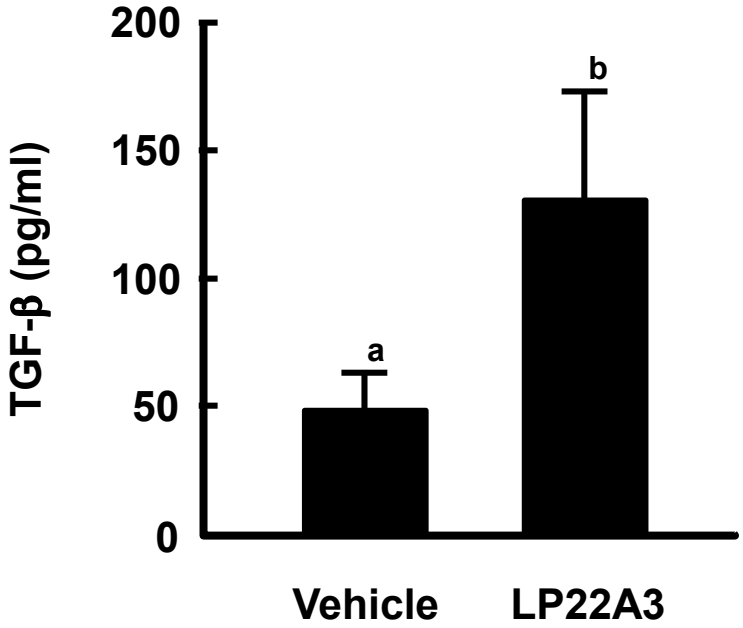
LP22A3 (1 x 10^8 cfu/mL) was applied directly to RBL-2H3 cells (A) or into apical side of Caco-2/RBL-2H3 cells (B) prior to sensitize anti DNP-IgE antibody. Degranulation of RBL-2H3 cells was evoked by DNP-albumin. Values represent means ± SD (n = 3). Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).

Fig. 5. Effect of anti-TGF-β antibody on β-hexosaminidase release from RBL-2H3 cells in co-culture system. Anti- TGF-β antibody was applied to the basolateral side of the co-culture system prior to LP22A3 treatment. (A) TGF-β mRNA expression from Caco-2 cells was measured by qPCR. (B) β-Hexosaminidase release was measured by β-hexosaminidase assay. Values represent the means±SD (n=3). Different superscript letters after values indicate statistical significance between groups ($p < 0.05$).



Up

Fujii *et al.*, Fig. 1



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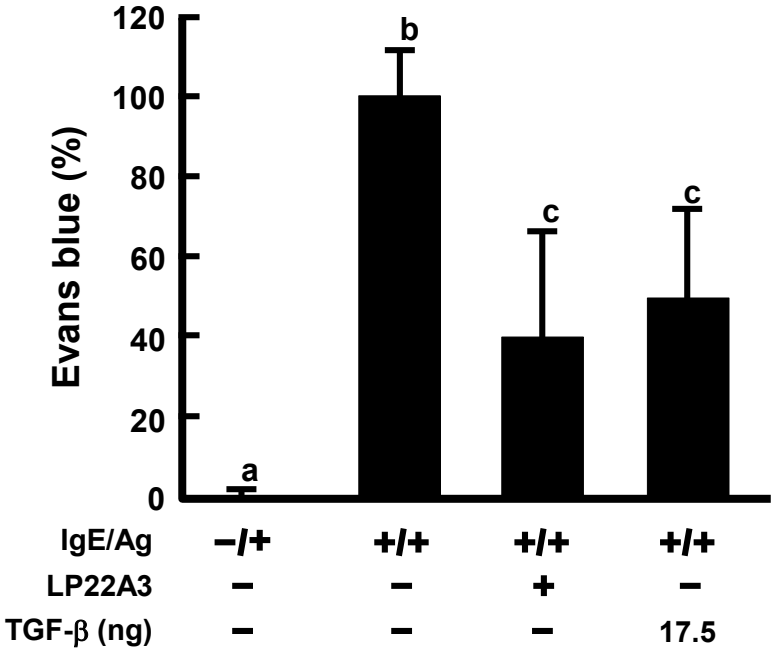

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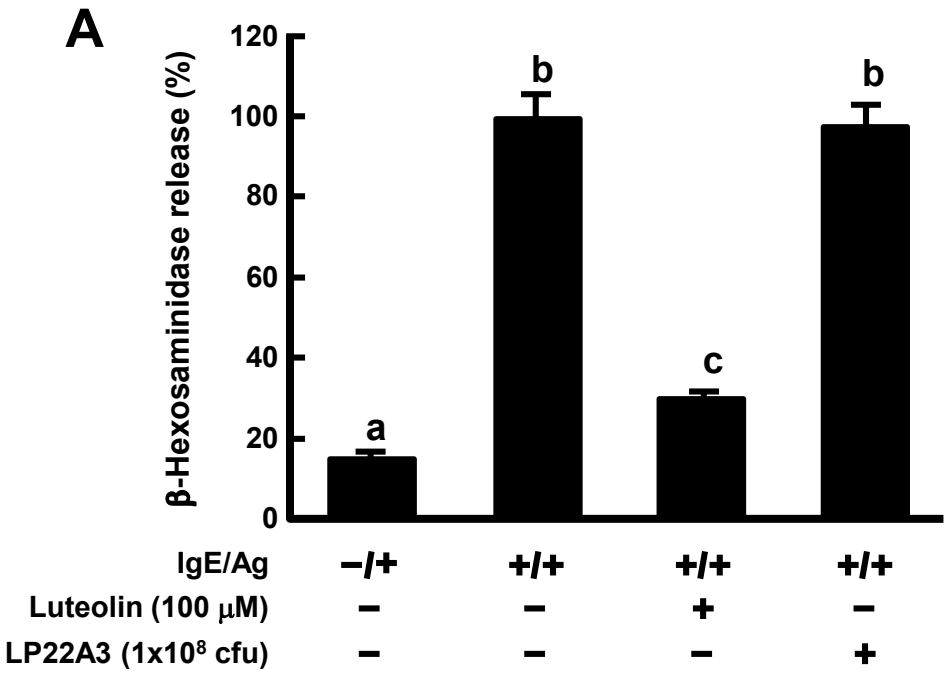
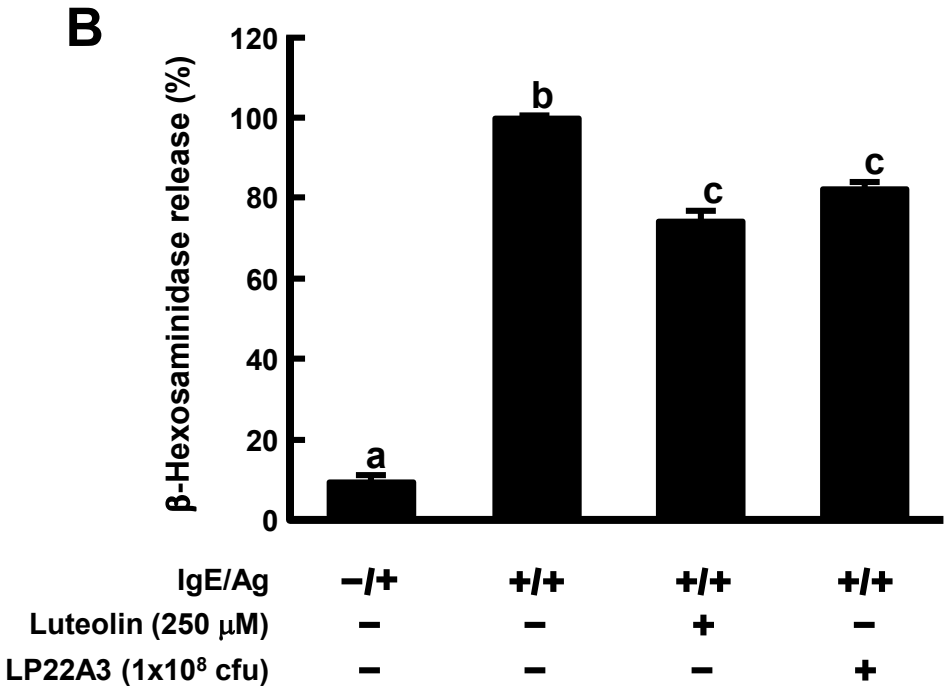
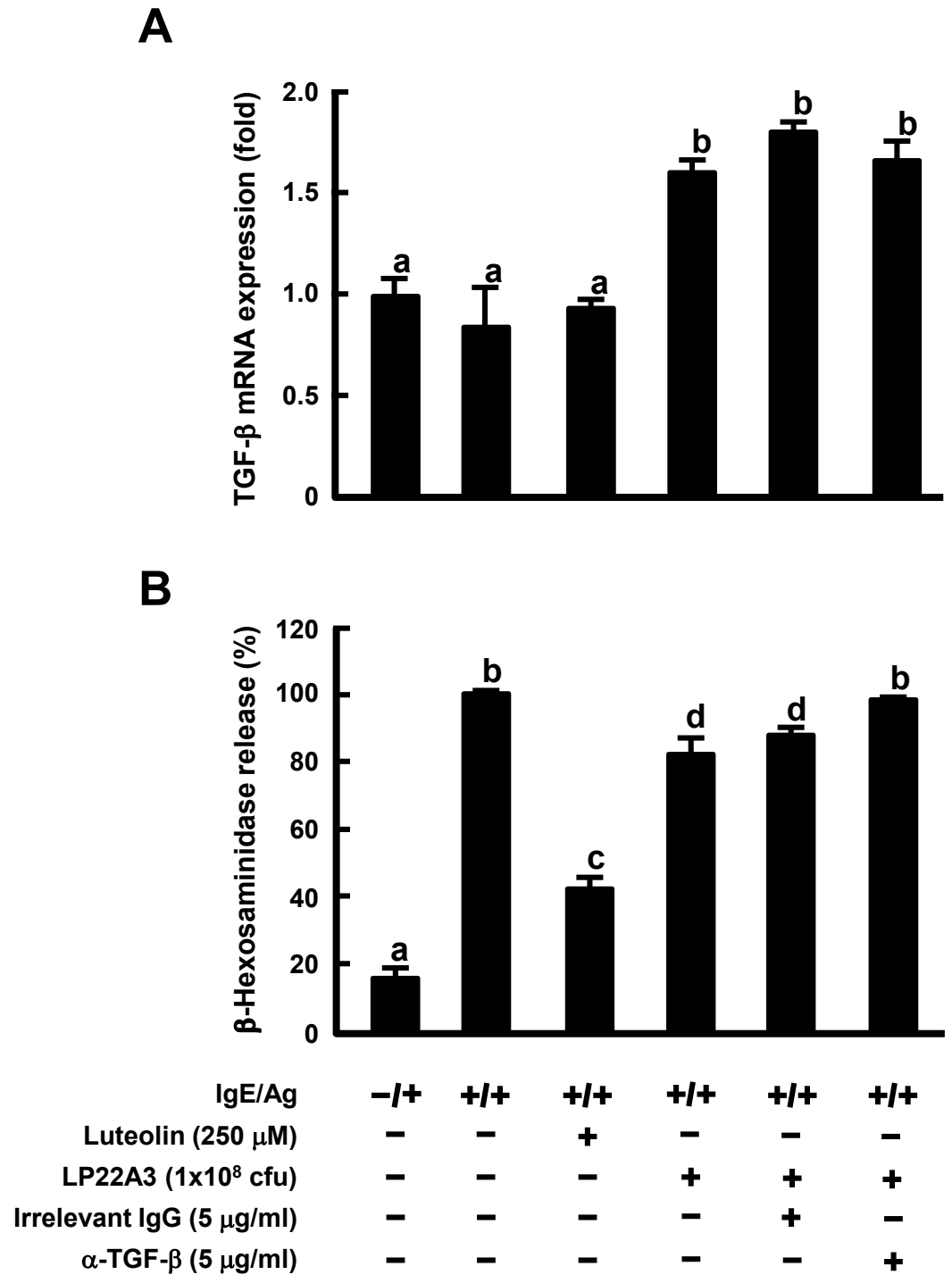




Up

Fujii *et al.*, Fig. 4



Up

Fujii *et al.*, Fig. 5