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Mizuno, Masashi
Ohto, Nobuaki
Kuwahara, Hiroshige

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Lactiplantibacillus plantarum 22A-3 isolated from pickle suppresses ovalbumin-induced food allergy in BALB/c mice and 2,4-dinitrochlorobenzene-induced atopic dermatitis in NC/Nga mice

Short title:

Inhibition of allergy by *L. plantarum* 22A-3

Authors names:

Masashi Mizuno[1,*], Nobuaki Ohto[2] and Hiroshige Kuwahara[2]

Institutes:

[1] Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

[2] Maruzen Pharmaceuticals Co. Ltd., 1089-8 Sagata, Shinnichi-cho, Fukuyama 729-3102, Japan

Corresponding author:

Masashi Mizuno

E-mail: mizuno@kobe-u.ac.jp

Postal address: Department of Agrobioscience, Graduate School of Agricultural Science, Kobe University, 1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan

Tel&Fax: +81-78-803-5835

SUMMARY

In the previous study, pickle-derived *Lactiplantibacillus plantarum* 22A-3 (LP22A3) suppressed ear edema in passive cutaneous anaphylaxis by its oral administration. Moreover, 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 the apical side. In this study, oral administration of LP22A3 decreased total IgE and ovalbumin (OVA) specific IgE contents in blood of BALB/c mice induced food allergy by OVA. Moreover, its oral administration suppressed the development of dermatitis induced by 2,4-dinitrochlorobenzene (DNCB) which was used to develop atopic dermatitis-like lesions in NC/Nga mice. This alleviation was further correlated with a reduction of elevated serum total IgE, transepidermal water loss and elevated acanthosis in the LP22A3-treated group compared with vehicle-treated positive group. In co-culture system composed of Caco-2 and RBL-2H3 cells, LP22A3 treatment on apical side before or after the sensitization with anti-dinitrophenyl (DNP) IgE antibody indicated the different effect on β–hexosaminidase release from RBL-2H3. Its treatment before the sensitization decreased β-hexosaminidase release, but not after sensitization, indicating that LP22A3 affected mast cells

sensitized with allergen through intestinal epithelial cells. These results suggest that LP22A3 may have a potential therapeutic property for Type 1 hypersensitivity and atopic dermatitis.

Key Words

Anti-allergic activity; atopic dermatitis; β-hexosaminidase; *Lactiplantibacillus plantarum* 22A-3; transforming growth factor-β

Abbreviation

AD, atopic dermatitis; APC, antigen presenting cell; CMC, carboxymethyl cellulose; FcεRI, Fcε Receptor I; LAB, Lactic acid bacteria; LP22A3, *Lactiplantibacillus plantarum* 22A-3; OVA, ovalbumin; TEWL, transepidermal water loss; TGF-β, transforming growth factor β; PCA, passive cutaneous anaphylaxis

Introduction

Allergic disorders are one of the major health problems in developed countries (1,2). Allergy is defined as an excessive immune response against normally harmless substances such as food, pollen or metals. Allergy is classified into four groups, from type I to type IV, on the basis of mediators, antigen, and effector mechanism. In particular, type I allergy patients are increasing world-wide (3). Although there are many steps in the development of type I allergy, antigen-specific immunoglobulin E (IgE) production, with subsequent fixation of IgE to FcεRI receptors on mast cells, is central to the initiation and propagation of immediate hypersensitivity reactions (4).

Atopic dermatitis (AD) occurs primarily in childhood but can persist or start in adulthood. It is a chronic inflammatory skin disease characterized by pruritic and eczematous skin lesions. Elevated serum immunoglobulin E (IgE) levels are also a characteristic feature. The incidence of AD is increasing, especially in industrialized countries, and 10–20 % of children worldwide are affected by AD (5). Although the complex interrelationships between genetic, environmental, skin barrier, pharmacological, psychological and immunological factors contribute to the pathogenesis of AD (6), the immunological basis of the disease is of considerable importance and has been extensively studied. It is well established that T helper (Th) 2

81 cytokines play important roles in the onset and development of AD (7,8).

82 Lactic acid bacteria (LAB) are the popular candidates for regulating immune
83 system. LAB is a group of Gram-positive, anaerobic bacteria including *Enterococcus*,
84 *Lactobacillus*, *Streptococcus*, and *Lactococcus*, which has been widely used in the
85 fermentation and storage of milk, meat and vegetables (9,10). Numbers of researches
86 had demonstrated that LAB can ameliorate allergic symptoms through decrease of the
87 serum levels of antigen specific IgE in animal models. LAB can influence immune
88 system by products like metabolites, cell wall component and nucleic acid (11), which
89 makes it reasonable that not only the live LAB (12,13) known as 'probiotics', but also
90 the heat-killed LAB (14,15) can have anti-allergic effect. The anti-allergic effect of
91 LAB is strain dependent with distinguished mechanisms and the most popular one is
92 through mediating Th1/Th2 cytokine expression and balance (16). It has been suggested
93 that LAB can switch the Th2 dominance to Th1 response in allergy state. This may be
94 achieved by stimulating Th1 differentiation and secretion of Th1 cytokines including
95 interferon (IFN)- γ and IL-12, which have antagonisms for IgE production promoted by
96 IL-4 (17). *Lactiplantibacillus plantarum* 22A-3 (LP22A3) is one of the plants derived
97 LAB and it was found in the rice-bran paste of eggplants pickle. Previous study
98 indicated that its oral administration prevented ear edema in passive cutaneous

anaphylaxis (PCA) reaction and increased transforming growth factor (TGF)-β contents in blood (18). Moreover, 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 the apical side.

The aim of this study was to investigate whether oral administration of LP22A3 can ameliorate ovalbumin-induced food allergy in BALB/c mice and 2,4-dinitrochlorobenzene-induced atopic dermatitis in Nc/Nga mice. It was ascertained to practical application of LP22A3 for the control of allergic diseases.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM, High Glucose) with glutamine, Evans blue and prednisolone were purchased from Fujifilm Wako Pure Chemical Industries (Miyazaki, Japan). Eagle's Minimum Essential Medium (MEM) was purchased from Nissui Pharmaceutical Co. Ltd., (Tokyo, Japan). Anti-dinitrophenyl (DNP) IgE monoclonal IgE, 2,4-dinitrochlorobenzene (DNCB) and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide were purchased from Sigma-Aldrich (St Louis, MO). Trypsin, RPMI 1640 medium, MEM non-essential amino acids (NEAA) ware purchased from Gibco

117 BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Thermo Fisher
118 Scientific (Waltham, MA). DNP-bovine serum albumin (DNP-BSA) was purchased
119 from Cosmo Bio (Tokyo, Japan). Mouse anti-2,4,6-trinitrophenyl (TNP) monoclonal
120 IgE was purchased from BD Biosciences (San Jose, CA). Recombinant mouse TGF-β
121 was purchased from BioLegend (San Diego, CA). Blocking One, and Chemi-Lumi One
122 L and picrylchloride (PiCl) were purchased from nacalai tesque, Inc. (Kyoto, Japan).
123 Anti-mouse IgE and anti-goat IgG-HRP conjugate antibodies were purchased from
124 Bethyl Laboratories (Montgomery, AL). Other chemicals and reagents were ordinary
125 commercial and guaranteed products.
126
127 **Preparation of UV-inactivated LP22A3 and oral administration**
128 LP22A3 was cultured in Mann Rogosa Sharp (MRS) broth and incubated overnight at
129 30°C in anaerobic chamber. After incubation, bacterial cells were obtained by
130 centrifugation (4°C, 10,000 ×g, 5 min) and was resuspended in PBS and washed three
131 times in order to remove culture medium. Bacterial suspension was irradiated with an
132 UV germicidal lamp to inactivate before use in in vitro experiments. After UV-
133 treatment, viable counts were below 10^2 cfu/mL indicated more than 9-log reduction of
134 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 (CMC), and 100 μL of it was orally administered using gastric feeding tube.

Mice

Female 7-week-old BALB/c and male 4-week-old NC/Nga 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).

Ovalbumin-induced allergic model

BALB/c mice (6 per group) were orally administrated with LP22A3(1×10^8 cfu/day) dissolved in 100 μl 0.5% CMC solution. Seven days after oral administration, mice were sensitized with 10 μg OVA + 1 mg $Al(OH)_3$ adjuvant dissolved in 300 μl phosphate-buffered saline (PBS) by intraperitoneal injection. The sensitization was continued once in 5 days for 3 more times. Two days after the last intraperitoneal sensitization, mice were challenged with OVA by intravenous injection with 3 μg OVA

153 dissolved in 100 μl PBS to trigger allergy response. One day before each OVA
154 sensitization, blood samples were collected from caudal vein. On the final day, blood
155 samples were collected by cardiac puncture.
156
157 **PCA reaction in mice**
158 An IgE-PCA reaction was performed in accordance with a previous study (19).
159 BALB/C mice (5 weeks old, female) were divided into 3 groups (3 per group). Mice
160 were intravenously administered 100 μl of anti-TNP IgE (20 μg/ml) in 0.1% BSA-PBS
161 in the tail vain and then were injected TGF-β (40 ng/mouse) in the same manner. After
162 30 min, the ear thickness was measured using an upright dial thickness gauge (Ozaki
163 MFG Co., Ltd., Tokyo, Japan). Immediately after that, 10 ml of 1.6% PiCl solution in
164 olive oil/acetone was applied on ventral side of the right ear, and the ear thickness was
165 measured again 2 h later. The ear edema was determined as the difference in the ear
166 thickness before and after the challenge. The blood samples were collected by cardiac
167 puncture to measure TGF-β contents.
168
169 **Induction of AD-like skin lesions in NC/Nga mice**
170 NC/Nga mice were randomly divided into 4 groups (4 per group): Control (normal

control vehicle), DNCB, DNCB + prednisolone, and DNCB + LP22A3. Mice were orally administered with LP22A3 (1 x 10^8 cfu/mouse/day) or prednisolone (3 mg/mouse/day) for 7 weeks. Mice were anesthetized and hairs on the back of the NC/Nga mice were completely removed and DNCB was applied to the dorsal skin to induce AD lesions. On the first day, 1% DNCB (200 μl) in the mixture of acetone and ethanol (2:3, v/v) was applied. After the first application, 0.5% DNCB (150 μl) in the mixture (acetone:olive oil = 3:1) was applied once every two days for 3 weeks. Mice were orally administrated with LP22A3(1×10^8 cfu/day) dissolved in 100 μl 0.5% CMC solution. Dorsal skin tissues obtained from the mice were subjected to histological analysis after staining with Hematoxylin and Eosin (H&E). On the final day, blood samples were collected by cardiac puncture.

Enzyme-linked immunosorbent assay (ELISA)

Blood samples were stored at 4℃ overnight after placed at 22-25℃ for 30 min. Serum was collected by centrifugation of blood samples in 1.5 ml microtube at 11,000 x g at 4℃ for 10 min. Total IgE contents were measured by using Mouse IgE ELISA Set (BD Bioscience, San Jose, CA). OVA-specific IgE were measured by using DS Mouse IgE ELISA (OVA) (DS Pharma Biomedical, Osaka, Japan). Serum TGF-β levels were

189 measured by using TGFβ1 Emax ImmunoAssay System (Promega, Madison, WI). All
190 procedures were specifically following manufacturer's protocol.
191
192 **Measurement of skin barrier function**
193 To evaluate the skin barrier function, TEWL values, which increase when skin barrier
194 function is disturbed, were determined in accordance with a previous study (20). All
195 mice had TEWL values measured using a Tewameter TM300 (Courage & Khazaka,
196 Cologne, Germany) in standard conditions (23 ± 2°C and 50 ± 10% relative humidity)
197 every week.
198
199 **Histopathologic Examination**
200 Dorsal skin tissues were isolated from each mouse and fixed with 10% formalin in 50
201 mM of a phosphate buffer (pH 7.0) for 24 hours at 4°C. Skin tissues were subsequently
202 embedded in paraffin, sectioned (4 μm), and stained with H&E. Epidermal thickness
203 was measured using optical microscopy and included software (Olympus, Tokyo,
204 Japan). A minimum of three points were analyzed from each section slide for measuring
205 the epidermal thickness.
206

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. Passage numbers 48–64 were used. When either cell line reached 80% confluence, cells were recovered from the culture dish or flask by trypsin digestion after washing with phosphate-buffered saline (PBS). The cells were replated in a new dish or flask.

Co-culture system of Caco-2 and RBL-2H3 cells

Caco-2 cells were seeded at the concentration of 0.6×10^5 cells/well onto 24-well Transwell insert plates (0.33 cm^2, 0.4 μm pore size, Corning Costar Corp., Cambridge, MA). Cell culture medium was changed every 3 day until the cells were fully differentiated (TER value >300Ω·cm^2). RBL-2H3 cells were seeded at 2.0×10^5 cells/500 μL/well onto 24-well tissue culture plates in Eagle's MEM and incubated

225 overnight with 1 μg/mL at a final concentration of anti-DNP IgE. After replacing all
226 media with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl2, 1 mM $CaCl^2$,
227 40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2) the Transwell inserts on
228 which Caco-2 cells had been cultured were added into the plate wells preloaded with
229 RBL-2H3 cells. In an experiment to evaluate the anti-allergic effect of test samples, 0.2
230 mL of SB or test sample solution was applied into the apical side. After incubation for 6
231 h, the cells were challenged with 10 ng/mL final concentration of DNP albumin for 10
232 min at 37 °C. The plate was cooled in an ice bath for 10 min to stop degranulation
233 responses.
234
235 **β-Hexosaminidase activity assay**
236 RBL-2H3 cells were dispensed into 96-well plates at a concentration of 2×10^5 cells/well
237 and incubated over-night at 37℃ in 5% CO_2. Cells were sensitized with anti-DNP IgE
238 for 2 h. Cells were washed twice with Siraganian buffer before adding test sample to
239 each well and incubated for 1 h, followed by challenge with DNP-albumin, antigen for
240 1 h. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50
241 μL) was transferred into 96-well microplate and incubate with 50 μL of substrate
242 solution for 1 h. The reaction was stopped by adding stop solution. The absorbance was

243 measured with a microplate reader at 405 nm.

244

245 **Wester blotting**

246 RBL-2H3 cells (4.0×10^5 cells/ml) were plated at 1.5 ml/well in a 6-well tissue culture
247 plate in RPMI 1640 medium and cultured overnight in a 5% CO_2 incubator at 37°C. The
248 cells were sensitized with 1 μg/ml anti-DNP IgE for 2 h at 37°C. After incubation, the
249 cells were washed twice with SB and incubated with or without TGF-β for 6 h at 37°C.
250 The cells were washed twice with SB and lysed in RIPA buffer (150 mM NaCl, 0.5%
251 sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl at pH 7.4, 50 mM glycerophosphate,
252 20 mM NaF, 1 mM DTT, 10.5 mM leupeptin, aprotinin, 10 mM pepstatin A and 100 μM
253 PMSF). Proteins extracted with 50 μl RIPA buffer and quantified by the Lowry assay.
254 Total proteins (30 μg) were separated using 10% SDS-polyacrylamide gel
255 electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride
256 membranes (General Electric, Fairfield, CT). The membranes were blocked using
257 Blocking One at room temperature for 1 h. After four washes of Tris-buffered saline
258 with Tween-20 (TBST; 20 mM Tris, 150 mM NaCl, pH7.6, 0.05% Tween), the
259 membranes were incubated with the primary antibody, anti-mouse IgE or anti-β-actin
260 antibodies, at 4°C overnight. Following four washes of TBST, the membranes were

incubated with the second antibody, anti-goat or anti-mouse IgG-HRP conjugate antibodies, at 4°C for 1 h. The target signals were detected with an enhanced chemiluminescence kit, Chemi-Lumi One L, and the intensity of bands was evaluated using Image J.

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 AND DISCUSSION

Oral administration of LP22A3 alleviated allergic symptom in OVA-induced allergic model mice

To confirm whether LP22A3 can reduce IgE production in active systemic anaphylaxis (ASA), total IgE content was monitored during OVA experiment as shown in an experimental design (Fig. 1A). Total IgE content in blood of mice that administrated

279 with OVA and $Al(OH)_3$ adjuvant (OVA group) increased continually (Fig. 1B). Mice
280 orally administered LP22A3 also showed significantly the tendency of suppression in
281 total IgE production compared to OVA group from day 9. Total IgE contents on day 17
282 in the group administered LP22A3 was decreased to be approximately 50% of the
283 control group. Weight and drinking amounts of LP22A3-fed mice show no significant
284 difference compared with the control group throughout experiment (data not shown).
285 Specificity against a particular protein is an important character of IgE, meaning that a
286 certain type of IgE only triggers allergic reaction under the stimulating of the same
287 allergen (21). In order to confirm whether LP22A3 also can reduce OVA-specific IgE
288 production, blood content of OVA-specific IgE was measured. As showed in Fig. 1C,
289 LP22A3 also significantly suppressed OVA-specific IgE compared to OVA group,
290 indicating that LP22A3 possessed anti-allergic activity in OVA-induced allergic model
291 mice. Moreover, TGF-β contents in serum were increased by LP22A3 administration
292 (Fig. 1D). It has reported that oral administration of LP22A3 inhibited the ear edema in
293 PCA reaction via TGF-β production through intestinal epithelial cells (18). It was
294 shown that oral administration of *Lactobacillus sakei* LK-117 suppresses allergic
295 symptoms without affecting the amount of IgE in the blood of atopic dermatitis-induced
296 model mice (19). It was also ascertained that LK-117 upregulated the production of

297 proinflammatory cytokines such as TNF-α and IL-12. Thus, the fact that the cytokines
298 produced by different types of LAB were different is instructive when considering their
299 bioactivities. Probably, in the OVA-induced allergic system, TGF-β production was
300 expected to be a major contributor to the suppression of allergy by oral administration
301 of LP22A3.
302
303 **The effects of exogenous TGF- β on PCA reaction**
304 PCA reaction was performed to evaluate TGF-β effect *in vivo* model. TGF-β was
305 administrated intravenously at concentrations of 40 ng/mouse according to the method
306 of Masuda et al. (19). The hypersensitivity reaction was evoked by challenging PiCl on
307 the earlobe of the mice. The independent experiments were repeated twice and typical
308 result was shown in Fig. 2. The suppression of ear edema was observed with
309 statistically significant improvement by exogenous TGF-β treatment. TGF-β contents in
310 serum increased to be approximately 3.3 hold by its intravenous injection. It was
311 suggested that oral administration of LP22A3 increased the TGF-β content in the blood,
312 and that this increase may have suppressed the onset of allergy.
313
314 **Oral administration of LP22A3 alleviated atopic dermatitis in 2,4-**

dinitrochlorobenzene (DNCB)-induced atopic mice

It has reported that exogenous TGF-β suppressed AD-like skin lesion in mice (22). In order to minimize the number of mice used, independent experiments were repeated twice. The results of both experiments were almost identical. It was predicted that oral administration of LP22A3 would inhibit AD. NC/Nga mice were induced AD by DNCB application of their dorsal skins (Fig. 3A). To evaluate the serum total IgE production in DNCB-induced NC/Nga mice, a blood sample was collected by tail vain sampling on 1, 3, 5 and 7 weeks. It was observed that total IgE contents were increased in DNCB-treated NC/Nga mice when compared with that of the control group from 5 week. These increased serum IgE contents were significantly suppressed by prednisolone and LP22A3 treatment compared with DNCB-group (Fig. 3B). The TEWL values were examined as indices of dry skin. Higher TEWL is usually associated with skin barrier impairments. The TEWL values after oral administration of prednisolone and LP22A3 were increased for 3 weeks with similar to control group. However, the TEWL values for 7 weeks were significantly decreased by their treatment compared with DNCB-group. Particularly, LP22A3 treatment decreased the TEWL values lower than prednisolone-group (Fig. 3C). The hair in the dorsal skin grew a little bit on 7 day without any clinical AD symptoms in control group. As shown in Fig. 3D, DNCB application induced obvious AD symptoms such as erythema and dry skin on 3 and 7 day. The oral administration of prednisolone (positive control) and LP22A3 was prevented the development of AD symptoms on 3 day. However, the symptoms in the group treated with them seemed to return to the same level as those in the group treated with DCNB on 7 day. To further confirm the effect of LP22A3 on AD symptoms, H&E staining was applied to examine changes in epidermal thickness in the dermatitis on 7

day. The mice treated with DNCB alone had increased epidermal thickness which was restored almost to normal level by treatment with LP22A3, while prednisolone had less effective (Fig. 3E). The skin lesions of LP22A3-treated group were prevented developing AD. Thus, these data indicated that the oral administration of LP22A3 inhibited development of DNCB-induced atopic dermatitis.

Suppression of degranulation of RBL-2H3 cells by LP22A3 in co-culture composed of Caco-2 and RBL-2H3 cells

It was reported that the involvement of intestinal epithelial cells is an important factor in the anti-allergic activity of LP22A3 (18). To examine just a part of how LP22A3 can possess anti-allergic activity, co-culture system composed of Caco-2/RBL-2H3 cells was applied (23). After sensitizing RBL-2H3 cells with anti-DNP-IgE antibody and then stimulating Caco-2 cells by adding LP22A3 in the apical side of co-culture system, the addition of the antigen, DNP-BSA significantly suppressed β-hexosaminidase release of RBL-2H3 compared to the antigen-antibody group (Fig. 4A). However, when the order of treatment of LP22A3 and anti-DNP-IgE antibody was reversed, β-hexosaminidase release from RBL-2H3 did not change at all compared to that of the antigen-antibody group (Fig. 4B). This result is consistent with our previous report that LP22A3 requires interaction with intestinal epithelial cells to show anti-allergic activity (18). Furthermore, this result clearly indicated that anti-allergy of LP22A3 can only be exerted after not before mast cells are sensitized by a certain antigen-specific IgE.

TGF-β suppresses directly β-hexosaminidase release from RBL-2H3

It was ascertained that the oral administration of LP22A3 promotes TGF-β production

363 in blood (Fig. 1D). Therefore, the direct association of TGF-β in LP22A3 anti-allergic
364 activity was examined using RBL-2H3 monolayer culture. As shown in Fig. 5, the
365 direct TGF-β treatment after sensitizing anti-DNP-IgE to RBL-2H3 caused the
366 suppression of β-hexosaminidase release triggered by DNP-BSA with dose dependent
367 manner. It is known that TGF-β inhibits the expression of Fcε-RI, a high affinity
368 receptor for IgE expressed on the surface of mast cells (24). Therefore, to confirm
369 whether TGF-β affects directly FcεRI expression on the surface of RBL-2H3 cells, the
370 amount of IgE bound to RBL-2H3 cells after sensitization was measured by Western
371 blotting. When RBL-2H3 cells were sensitized with anti DNP-IgE antibody and then
372 were directly treated with TGF-β, the amount of IgE bound to RBL-2H3 was found to
373 be constant regardless of the amount of TGF-β added (Fig. 6). Although this result was
374 an indirect proof, it was assumed that the amounts of FcεRI in RBL-2H3 was not
375 changed by TGF-β treatment. This result in the present study was inconsistent with the
376 results reported by Gomez et al (24). Presumably, in their case, TGF-β treatment time
377 was 3 days, while in our case it was only 6 h, which was predicted to have caused no
378 change in FcεRI expression levels. Taken together, it was predicted that TGF-β affects
379 the downstream signaling after cross-linking by the exposure of the same allergen to
380 mast cells sensitized with the allergen-specific IgE.

381 Although it is certain that more research needs to be done on how oral
382 administration of LP22A3 promotes TGF-β production and suppresses allergy, it is very
383 important to note that oral administration of LP22A3 suppresses both type I allergy and
384 atopic dermatitis. In this study, we used LP22A3 inactivated by UV irradiation as in our
385 previous report (18), but we are currently investigating whether the same effect can be
386 seen with live LP22A3 in another experiment. Moreover, based on the literature which

LAB stimulate the immune system through interaction with Toll-like receptors (25), we hypothesize that the cell wall fraction of LP22A3 is the active fraction, and we are currently conducting research. If the inactivated LP22A3 will be found to be more bioactive in anti-allergic activity, it is expected to be more effective in developing products using LP22A3, considering the stability of the products. By clarifying the mechanism of action of LP22A3 in the future, it may be able to be used as a food ingredient to suppress allergy and atopic dermatitis, which have been becoming social problems.

ACKNOWGEDGMENTS

The authors declare no conflict of interest.

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475

476 Figure legends

477

478 **Fig. 1.** Anti-allergic activity of LP22A3 in OVA-induced allergic mice.

479 LP22A3 (1×10^8 cfu/mouse/day) were orally administered to BALB/c mice for 17 days.

480 mice were sensitized with 10 μg OVA + 1 mg $Al(OH)_3$ adjuvant dissolved in 300 μL
481 phosphate-buffered saline (PBS) by intraperitoneal injection. The sensitizations were
482 continued once every 5 days for 2 more times. Five days after the last intraperitoneal
483 sensitization, mice were challenged with OVA by intravenous injection with 3 μg OVA
484 dissolved in 100 μL PBS to trigger an allergy response. (A) Experimental design. Total
485 IgE (B), OVA specific IgE (C), and TGF-β (D) contents were measured by ELISA. Values
486 represent means ± SD (n = 6). Items with different letter were significantly different (p
487 < 0.05).
488
489 **Fig. 2.** Effect of exogenous TGF-β on PCA reaction.
490 Values represent means ± SD (n = 3). Items with different letter were significantly
491 different ($p < 0.05$).
492
493 **Fig. 3.** Effect of LP22A3 administration on DNCB-induced atopic mice
494 Mice were orally administered with LP22A3 (1 x 10^8 cfu/mouse/day) or prednisolone (3
495 mg/mouse/day) for 7 weeks. DNCB was applied to the dorsal skin to induce AD lesions.
496 DNCB (1%, 200 μl) in the mixture of acetone and ethanol (2:3, v/v) was applied. After
497 the first application, 0.5% DNCB (150 μl) in the mixture (acetone:Olive oil = 3:1) was

498 applied once every two days for 3 weeks. Mice were orally administrated with
499 LP22A3(1×10^8 cfu/day) dissolved in 100 μl 0.5% CMC solution. (A) Experimental
500 design. (B) Total IgE contents were measured by ELISA. (C) The TEWL values were
501 measured using a Tewameter TM300. (D) Changes in atopic dermatitis symptoms at the
502 dorsal skin lesion on week 0, 3 and 7. (E) Change in skin epidermal thickness elicited by
503 DNCB. Values represent means ± SD (n = 4). Items with different letter were
504 significantly different ($p < 0.05$).
505
506 **Fig. 4.** Effect of different timing of LP22A3 treatment on degranulation of RBL-2H3 cells
507 in co-culture system of Caco-2/RBL-2H3 cells.
508 LP22A3 (1 x 10^8 cfu/mL) was applied into apical side of Caco-2/RBL-2H3 cells after (A)
509 and before (B) the sensitization of anti DNP-IgE antibody. Values represent means ±
510 SD (n = 3). **P<0.01, *P<0.05 versus anti DNP-IgE/DNP-BSA group.
511
512 **Fig. 5.** Effect of exogenous TGF-β on β-hexosaminidase release from RBL-2H3 cells.
513 **P<0.01, *P<0.05 versus anti DNP-IgE/DNP-BSA group.
514
515 **Fig. 6.** Effect of exogenous TGF-β on IgE contents binding RBL-2H3 cells.

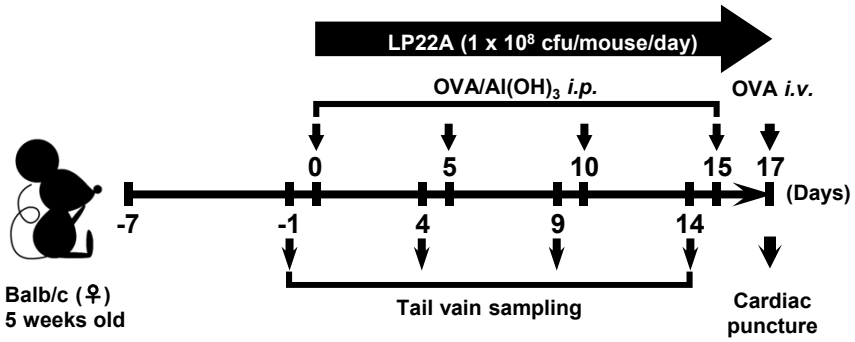
516 RBL-2H3 cells were sensitized with anti DNP-IgE (1 μg/mL) for 2 h, and then TGF-β

517 (50, 100 pg/ml) was added and incubated for another 6 h. The cells were collected and

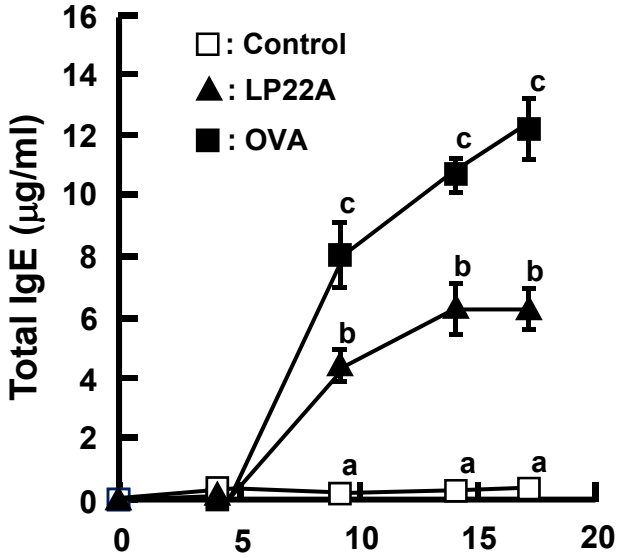
518 the amount of IgE bound to RBL-2H3 cells was measured by Western blotting. Values

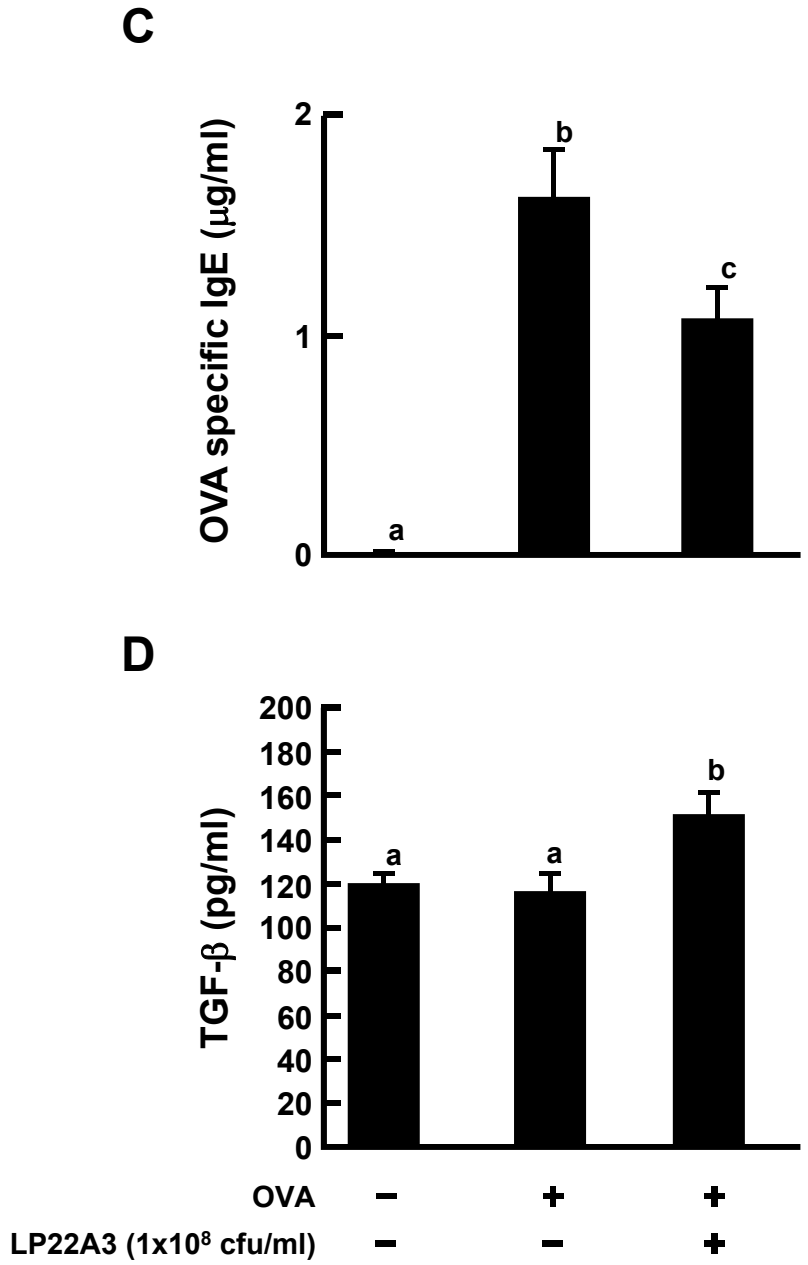
519 represent means ± SD (n = 3).

A


LP22A (1 x 10^8 cfu/mouse/day)
OVA/Al(OH)3 i.p.
OVA i.v.
0
5
10
15
17
(Days)
-7
-1
4
9
14
Balb/c (♀)
5 weeks old
Tail vain sampling
Cardiac puncture

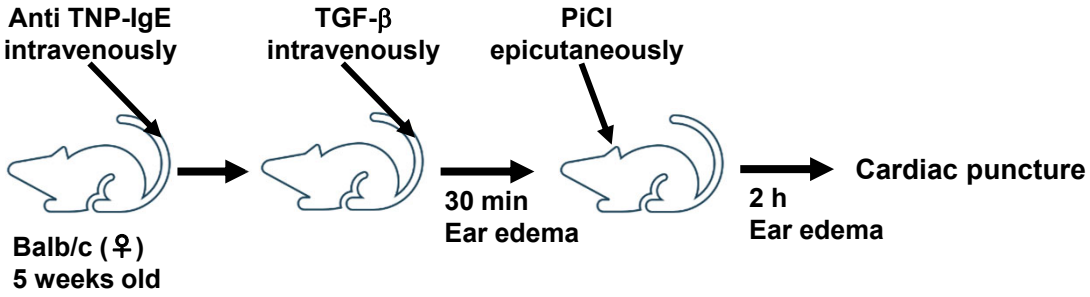
B

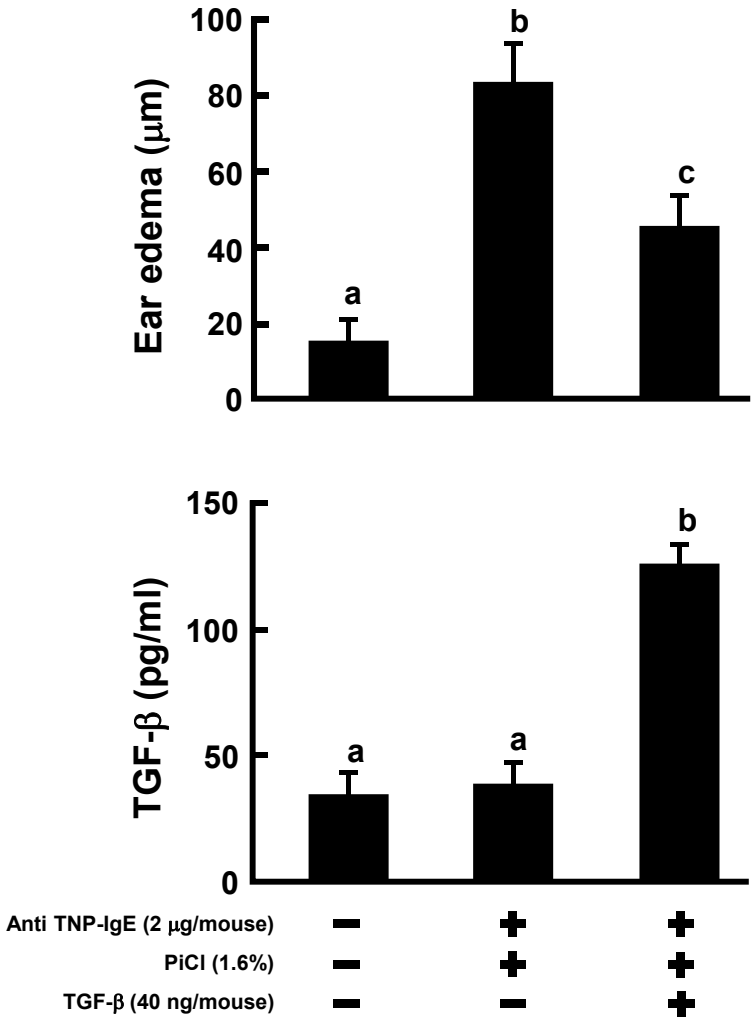

□: Control
▲: LP22A
■: OVA
Total IgE (μg/ml)
16
14
12
10
8
6
4
2
0
0
5
10
15
20
a
a
a
b
b
b
c
c
c



Up

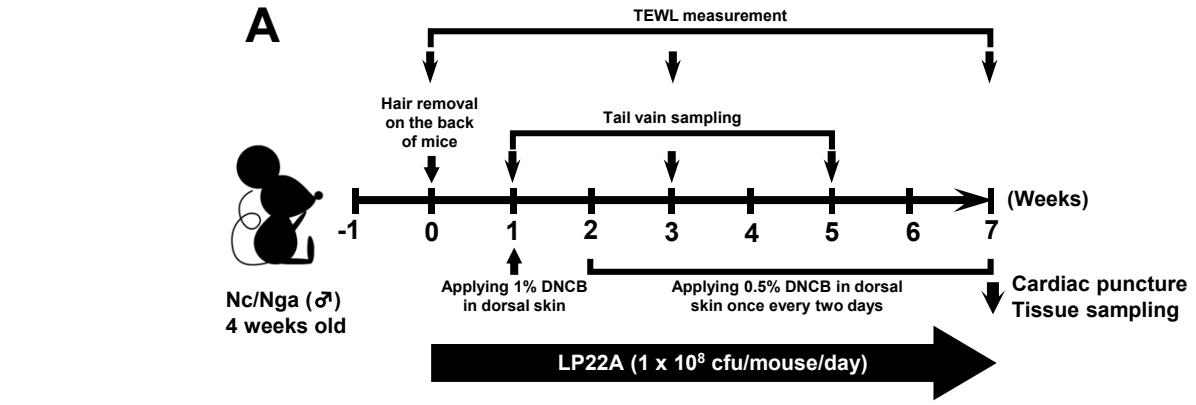
Mizuno *et al.*, Fig. 1

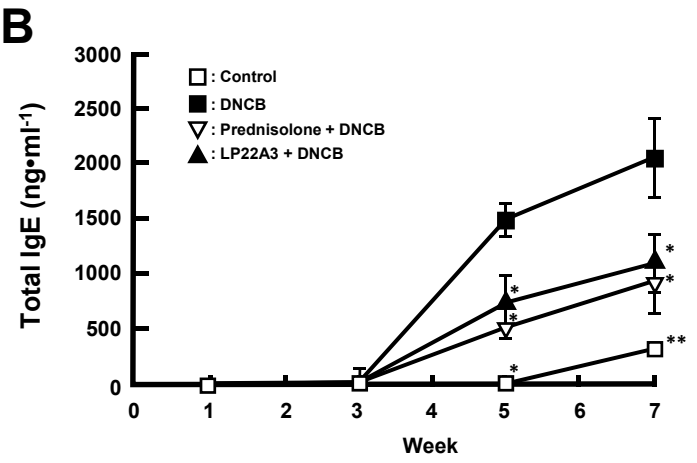


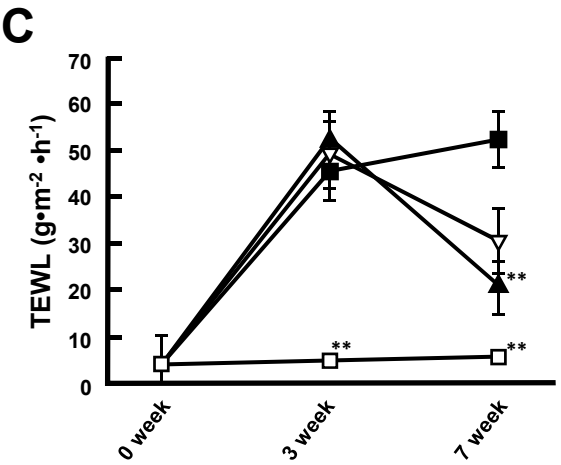


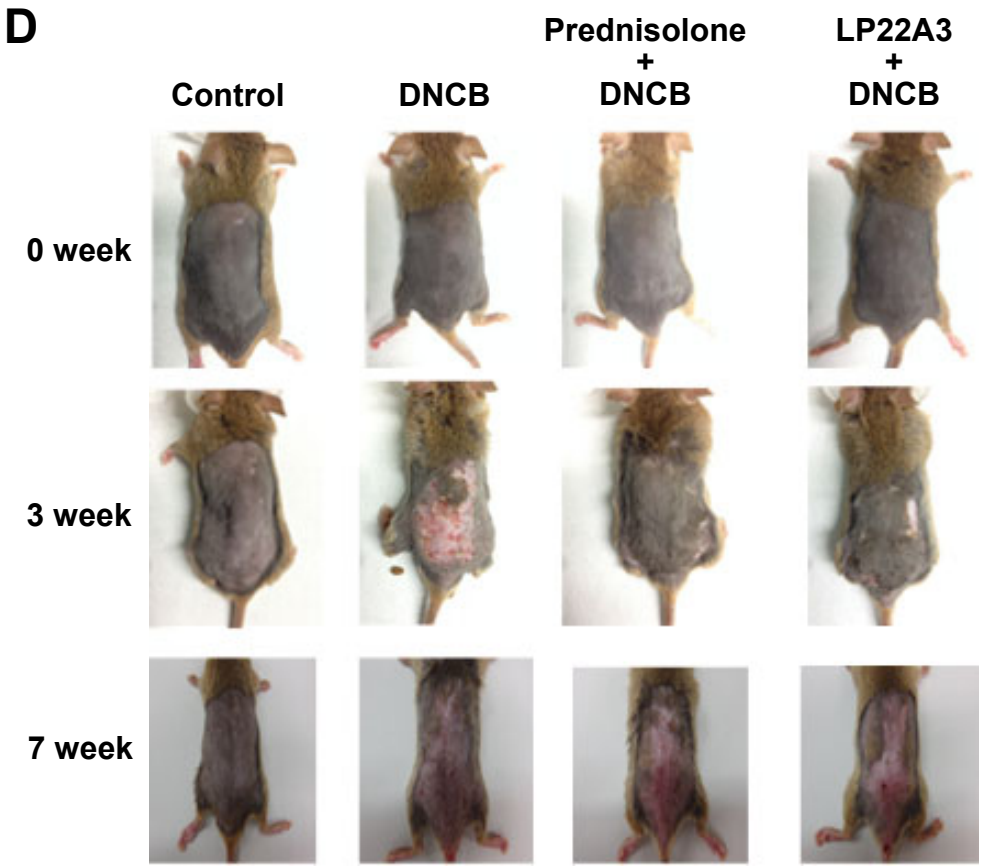
Up

Mizuno *et al.*, Fig. 2

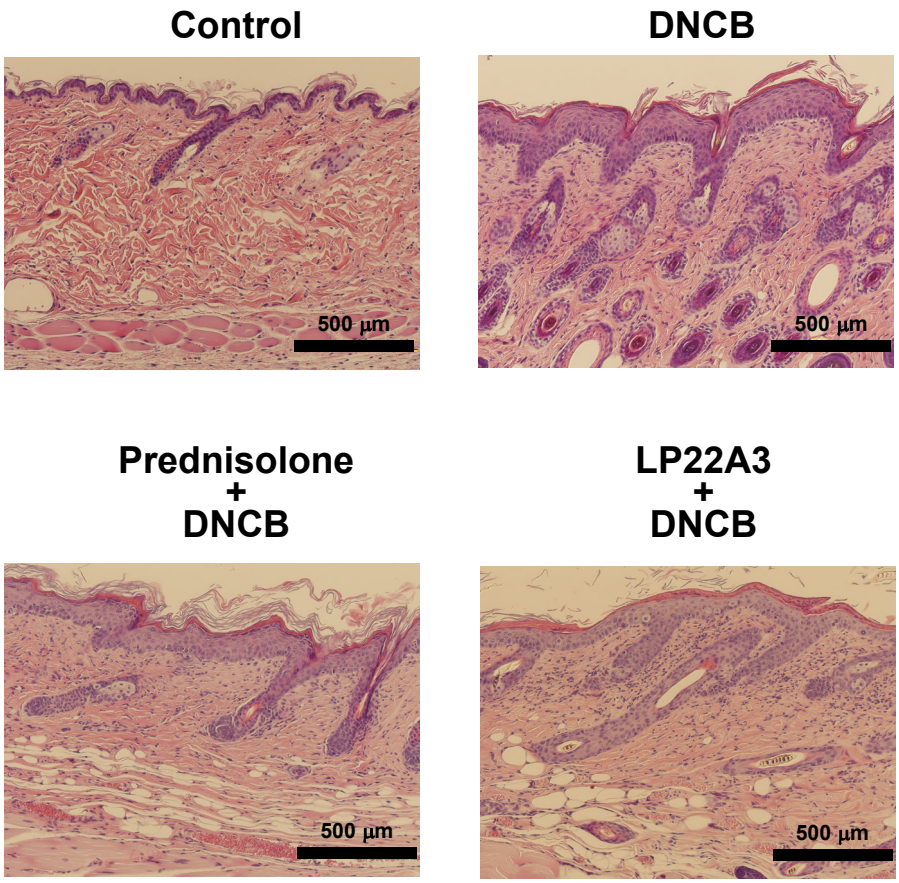

A
TEWL measurement
Hair removal on the back of mice
Tail vain sampling
-1
0
1
2
3
4
5
6
7
(Weeks)
Nc/Nga (♂)
4 weeks old
Applying 1% DNCB in dorsal skin
Applying 0.5% DNCB in dorsal skin once every two days
Cardiac puncture
Tissue sampling
LP22A (1 x 10^8 cfu/mouse/day)

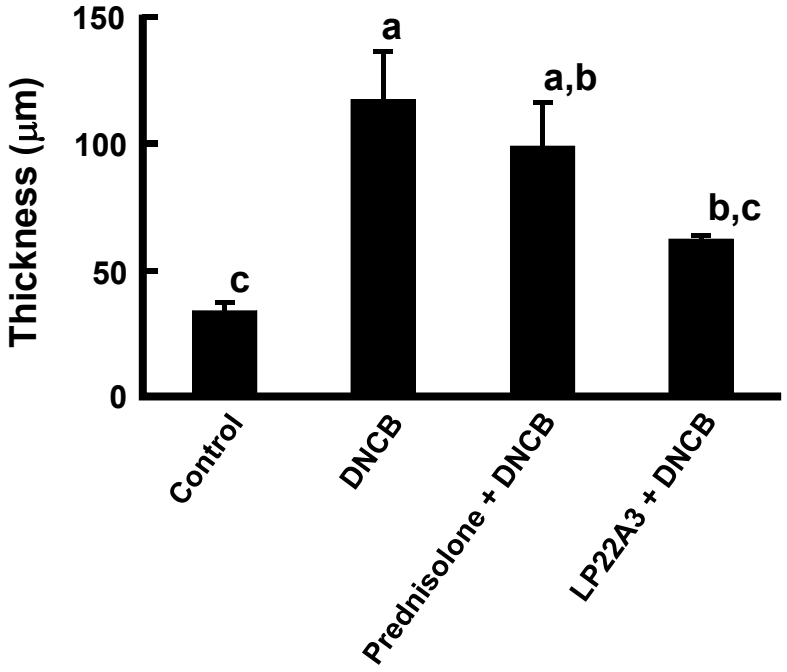

B
Total IgE (ng•ml-1)
: Control
: DNCB
: Prednisolone + DNCB
: LP22A3 + DNCB
Week


C
TEWL (g•m-2 •h-1)
0 week
3 week
7 week


D
Control
DNCB
Prednisolone + DNCB
LP22A3 + DNCB
0 week
3 week
7 week

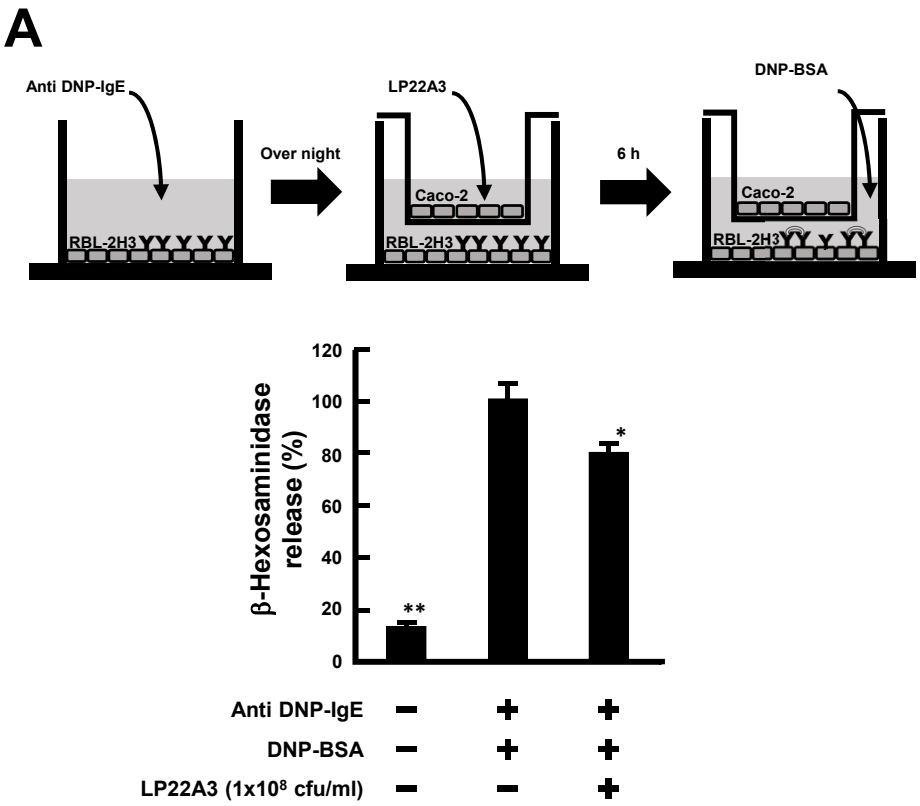
E

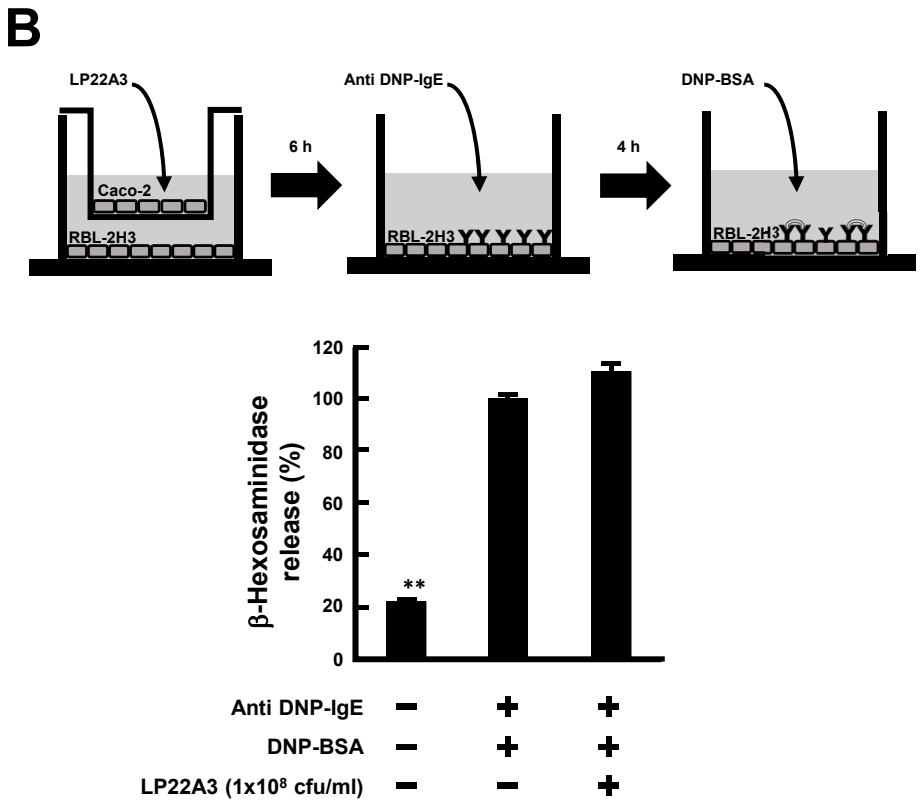




Up

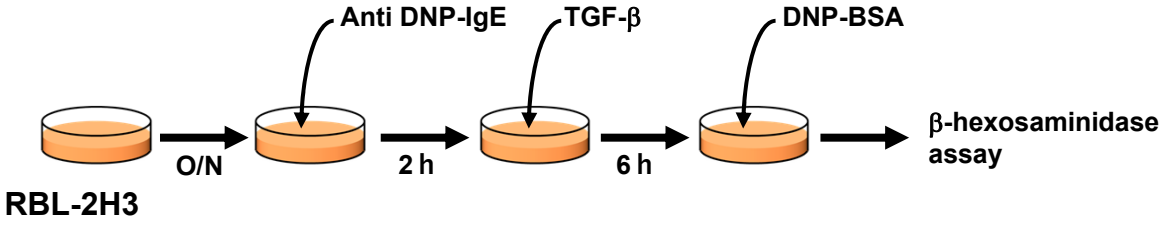
Mizuno *et al.*, Fig. 3

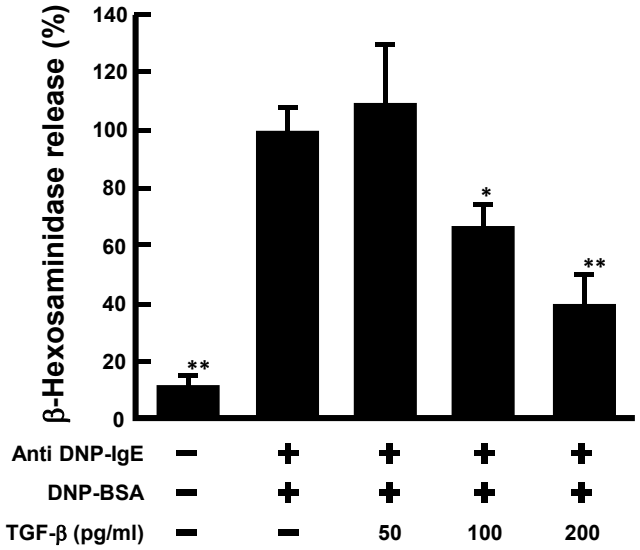




Up

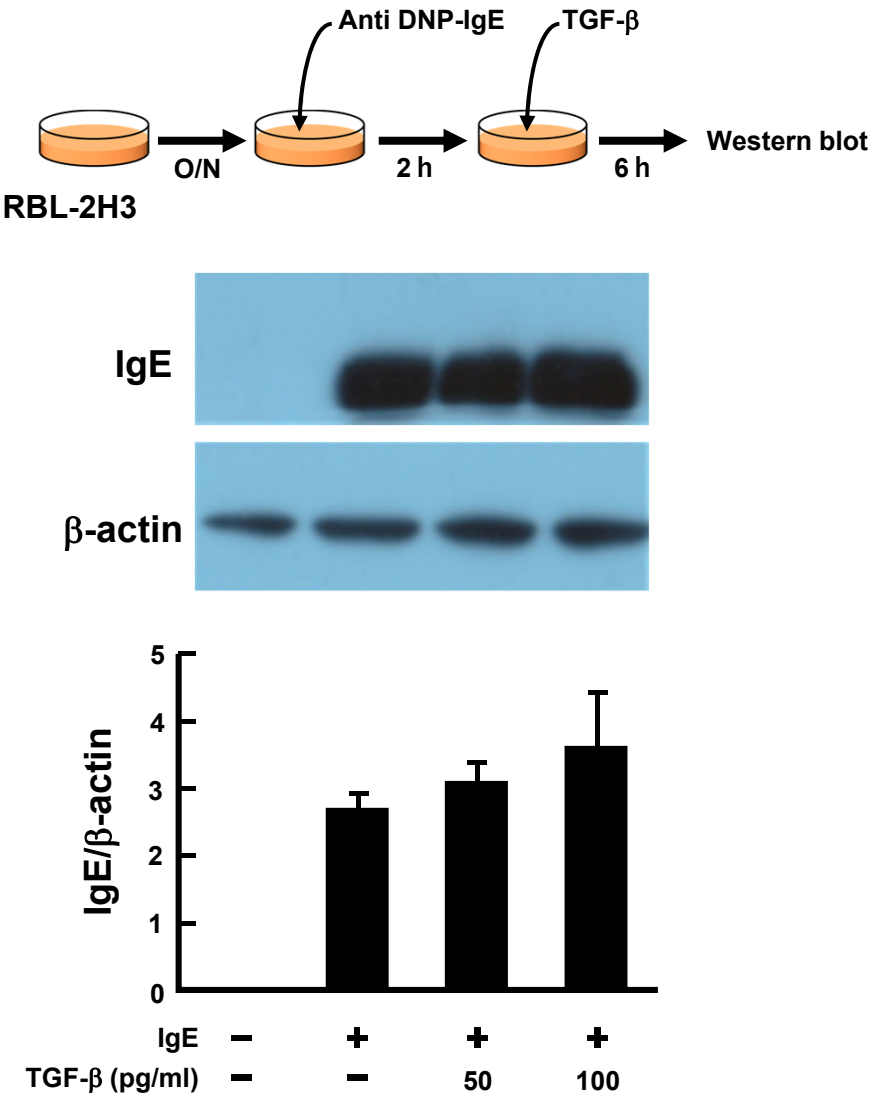
Mizuno *et al.*, Fig. 4





Up

Mizuno *et al.*, Fig. 5



Up

Mizuno *et al.*, Fig. 6