



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

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**Regular paper**

**Title:**

*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

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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  $\beta$ -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  $\beta$ -hexosaminidase release from RBL-2H3. Its treatment before the sensitization decreased  $\beta$ -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;  $\beta$ -hexosaminidase; *Lactiplantibacillus plantarum* 22A-3; transforming growth factor- $\beta$

#### Abbreviation

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

## 63    **Introduction**

64            Allergic disorders are one of the major health problems in developed countries  
65    (1,2). Allergy is defined as an excessive immune response against normally harmless  
66    substances such as food, pollen or metals. Allergy is classified into four groups, from  
67    type I to type IV, on the basis of mediators, antigen, and effector mechanism. In  
68    particular, type I allergy patients are increasing world-wide (3). Although there are  
69    many steps in the development of type I allergy, antigen-specific immunoglobulin E  
70    (IgE) production, with subsequent fixation of IgE to FcεRI receptors on mast cells, is  
71    central to the initiation and propagation of immediate hypersensitivity reactions (4).

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

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

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

anaphylaxis (PCA) reaction and increased transforming growth factor (TGF)- $\beta$  contents in blood (18). Moreover, LP22A3 treatment directly to RBL-2H3 cells shows no effect on  $\beta$ -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- $\beta$ -D-glucosaminide were purchased from Sigma-Aldrich (St Louis, MO). Trypsin, RPMI 1640 medium, MEM non-essential amino acids (NEAA) were purchased from Gibco



BRL (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA). DNP-bovine serum albumin (DNP-BSA) was purchased from Cosmo Bio (Tokyo, Japan). Mouse anti-2,4,6-trinitrophenyl (TNP) monoclonal IgE was purchased from BD Biosciences (San Jose, CA). Recombinant mouse TGF- $\beta$  was purchased from BioLegend (San Diego, CA). Blocking One, and Chemi-Lumi One L and picrylchloride (PiCl) were purchased from nacalai tesque, Inc. (Kyoto, Japan). Anti-mouse IgE and anti-goat IgG-HRP conjugate antibodies were purchased from Bethyl Laboratories (Montgomery, AL). Other chemicals and reagents were ordinary commercial and guaranteed products.

#### **Preparation of UV-inactivated LP22A3 and oral administration**

LP22A3 was cultured in Mann Rogosa Sharp (MRS) broth and incubated overnight at 30°C in anaerobic chamber. After incubation, bacterial cells were obtained by centrifugation (4°C, 10,000  $\times$ g, 5 min) and was resuspended in PBS and washed three 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<sup>2</sup> cfu/mL indicated more than 9-log reduction of viability. Samples were stored at -80°C until use. The inactivated LP22A3 (1 x 10<sup>11</sup>

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 Ethics 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( $1 \times 10^8$  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)<sub>3</sub> 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

dissolved in 100 µl PBS to trigger allergy response. One day before each OVA sensitization, blood samples were collected from caudal vein. On the final day, blood samples were collected by cardiac puncture.

#### **PCA reaction in mice**

An IgE-PCA reaction was performed in accordance with a previous study (19). BALB/C mice (5 weeks old, female) were divided into 3 groups (3 per group). Mice were intravenously administered 100 µl of anti-TNP IgE (20 µg/ml) in 0.1% BSA-PBS in the tail vein and then were injected TGF-β (40 ng/mouse) in the same manner. After 30 min, the ear thickness was measured using an upright dial thickness gauge (Ozaki MFG Co., Ltd., Tokyo, Japan). Immediately after that, 10 µl of 1.6% PiCl solution in olive oil/acetone was applied on ventral side of the right ear, and the ear thickness was measured again 2 h later. The ear edema was determined as the difference in the ear thickness before and after the challenge. The blood samples were collected by cardiac puncture to measure TGF-β contents.

#### **Induction of AD-like skin lesions in NC/Nga mice**

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 ( $1 \times 10^8$  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  $\mu$ l) in the mixture of acetone and ethanol (2:3, v/v) was applied. After the first application, 0.5% DNCB (150  $\mu$ l) in the mixture (acetone:olive oil = 3:1) was applied once every two days for 3 weeks. Mice were orally administrated with LP22A3( $1 \times 10^8$  cfu/day) dissolved in 100  $\mu$ l 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°C overnight after placed at 22-25°C for 30 min. Serum was collected by centrifugation of blood samples in 1.5 ml microtube at 11,000 x g at 4°C 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- $\beta$  levels were

measured by using TGFβ1 Emax ImmunoAssay System (Promega, Madison, WI). All procedures were specifically following manufacturer's protocol.

### **Measurement of skin barrier function**

To evaluate the skin barrier function, TEWL values, which increase when skin barrier function is disturbed, were determined in accordance with a previous study (20). All mice had TEWL values measured using a Tewameter TM300 (Courage & Khazaka, Cologne, Germany) in standard conditions ( $23 \pm 2^{\circ}\text{C}$  and  $50 \pm 10\%$  relative humidity) every week.

### **Histopathologic Examination**

Dorsal skin tissues were isolated from each mouse and fixed with 10% formalin in 50 mM of a phosphate buffer (pH 7.0) for 24 hours at  $4^{\circ}\text{C}$ . Skin tissues were subsequently embedded in paraffin, sectioned ( $4 \mu\text{m}$ ), and stained with H&E. Epidermal thickness was measured using optical microscopy and included software (Olympus, Tokyo, Japan). A minimum of three points were analyzed from each section slide for measuring the epidermal thickness.

207     **Cell culture**

208     Rat basophilic leukemia cell line, RBL-2H3 cells, were cultured in dishes in Eagle's  
209     MEM supplemented with 10% (v/v) heat-inactivated FBS (57 °C, 30 min), 100 µg/mL  
210     streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine. Cell cultures were  
211     incubated at 37 °C in a 5% CO<sub>2</sub> incubator. Passage numbers 14-32 were used. Human  
212     intestinal epithelial cell line, Caco-2 cells, were cultured in a 75 cm<sup>2</sup> plastic flask in  
213     DMEM (high glucose) supplemented with 10% FBS, 1% MEM-NEAA, 100 µg/mL  
214     streptomycin, and 100 U/mL penicillin, and incubated at 37 °C in a 5% CO<sub>2</sub> incubator.  
215     Passage numbers 48-64 were used. When either cell line reached 80% confluence, cells  
216     were recovered from the culture dish or flask by trypsin digestion after washing with  
217     phosphate-buffered saline (PBS). The cells were replated in a new dish or flask.

218

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

220     Caco-2 cells were seeded at the concentration of  $0.6 \times 10^5$  cells/well onto 24-well  
221     Transwell insert plates (0.33 cm<sup>2</sup>, 0.4 µm pore size, Corning Costar Corp., Cambridge,  
222     MA). Cell culture medium was changed every 3 day until the cells were fully  
223     differentiated (TER value  $>300\Omega \cdot \text{cm}^2$ ). RBL-2H3 cells were seeded at  $2.0 \times 10^5$   
224     cells/500 µL/well onto 24-well tissue culture plates in Eagle's MEM and incubated

overnight with 1 µg/mL at a final concentration of anti-DNP IgE. After replacing all media with Siraganian buffer (SB; 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 40 mM NaOH, 25 mM PIPES, 5.6 mM glucose, 0.1% BSA, pH 7.2) the Transwell inserts on which Caco-2 cells had been cultured were added into the plate wells preloaded with RBL-2H3 cells. In an experiment to evaluate the anti-allergic effect of test samples, 0.2 mL of SB or test sample solution was applied into the apical side. After incubation for 6 h, the cells were challenged with 10 ng/mL final concentration of DNP albumin for 10 min at 37 °C. The plate was cooled in an ice bath for 10 min to stop degranulation responses.

#### **β-Hexosaminidase activity assay**

RBL-2H3 cells were dispensed into 96-well plates at a concentration of  $2 \times 10^5$  cells/well and incubated over-night at 37°C in 5% CO<sub>2</sub>. Cells were sensitized with anti-DNP IgE for 2 h. Cells were washed twice with Siraganian buffer before adding test sample to each well and incubated for 1 h, followed by challenge with DNP-albumin, antigen for 1 h. The reaction was stopped by cooling in an ice bath for 10 min. The supernatant (50 µL) was transferred into 96-well microplate and incubate with 50 µL of substrate 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 ( $4.0 \times 10^5$  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<sub>2</sub> 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  $\pm$  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)<sub>3</sub> 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- $\beta$  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- $\beta$  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

proinflammatory cytokines such as TNF- $\alpha$  and IL-12. Thus, the fact that the cytokines produced by different types of LAB were different is instructive when considering their bioactivities. Probably, in the OVA-induced allergic system, TGF- $\beta$  production was expected to be a major contributor to the suppression of allergy by oral administration of LP22A3.

### **The effects of exogenous TGF- $\beta$ on PCA reaction**

PCA reaction was performed to evaluate TGF- $\beta$  effect *in vivo* model. TGF- $\beta$  was administrated intravenously at concentrations of 40 ng/mouse according to the method of Masuda et al. (19). The hypersensitivity reaction was evoked by challenging PiCl on the earlobe of the mice. The independent experiments were repeated twice and typical result was shown in Fig. 2. The suppression of ear edema was observed with statistically significant improvement by exogenous TGF- $\beta$  treatment. TGF- $\beta$  contents in serum increased to be approximately 3.3 fold by its intravenous injection. It was suggested that oral administration of LP22A3 increased the TGF- $\beta$  content in the blood, and that this increase may have suppressed the onset of allergy.

### **Oral administration of LP22A3 alleviated atopic dermatitis in 2,4-**

### **dinitrochlorobenzene (DNCB)-induced atopic mice**

It has reported that exogenous TGF- $\beta$  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 vein 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 DNCB 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  $\beta$ -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,  $\beta$ -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- $\beta$ suppresses directly $\beta$ -hexosaminidase release from RBL-2H3**

It was ascertained that the oral administration of LP22A3 promotes TGF- $\beta$  production

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

Although it is certain that more research needs to be done on how oral administration of LP22A3 promotes TGF- $\beta$  production and suppresses allergy, it is very important to note that oral administration of LP22A3 suppresses both type I allergy and atopic dermatitis. In this study, we used LP22A3 inactivated by UV irradiation as in our previous report (18), but we are currently investigating whether the same effect can be 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.

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The authors declare no conflict of interest.

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Figure legends

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

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

mice were sensitized with 10 µg OVA + 1 mg Al(OH)<sub>3</sub> adjuvant dissolved in 300 µL phosphate-buffered saline (PBS) by intraperitoneal injection. The sensitizations were continued once every 5 days for 2 more times. Five days after the last intraperitoneal sensitization, mice were challenged with OVA by intravenous injection with 3 µg OVA dissolved in 100 µL PBS to trigger an allergy response. (A) Experimental design. Total IgE (B), OVA specific IgE (C), and TGF-β (D) contents were measured by ELISA. Values represent means ± SD (n = 6). Items with different letter were significantly different ( $p < 0.05$ ).

**Fig. 2.** Effect of exogenous TGF-β on PCA reaction.

Values represent means ± SD (n = 3). Items with different letter were significantly different ( $p < 0.05$ ).

**Fig. 3.** Effect of LP22A3 administration on DNCB-induced atopic mice

Mice were orally administered with LP22A3 ( $1 \times 10^8$  cfu/mouse/day) or prednisolone (3 mg/mouse/day) for 7 weeks. DNCB was applied to the dorsal skin to induce AD lesions. DNCB (1%, 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( $1 \times 10^8$  cfu/day) dissolved in 100  $\mu$ l 0.5% CMC solution. (A) Experimental design. (B) Total IgE contents were measured by ELISA. (C) The TEWL values were measured using a Tewameter TM300. (D) Changes in atopic dermatitis symptoms at the dorsal skin lesion on week 0, 3 and 7. (E) Change in skin epidermal thickness elicited by DNCB. Values represent means  $\pm$  SD (n = 4). Items with different letter were significantly different ( $p < 0.05$ ).

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

LP22A3 ( $1 \times 10^8$  cfu/mL) was applied into apical side of Caco-2/RBL-2H3 cells after (A) and before (B) the sensitization of anti DNP-IgE antibody. Values represent means  $\pm$  SD (n = 3). \*\* $P < 0.01$ , \* $P < 0.05$  versus anti DNP-IgE/DNP-BSA group.

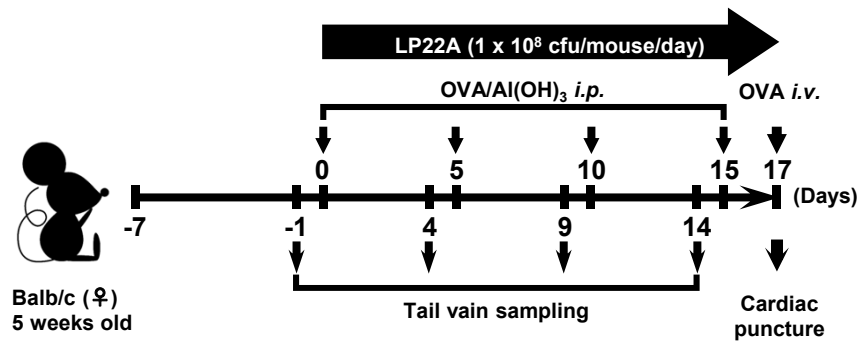
**Fig. 5.** Effect of exogenous TGF- $\beta$  on  $\beta$ -hexosaminidase release from RBL-2H3 cells.

\*\* $P < 0.01$ , \* $P < 0.05$  versus anti DNP-IgE/DNP-BSA group.

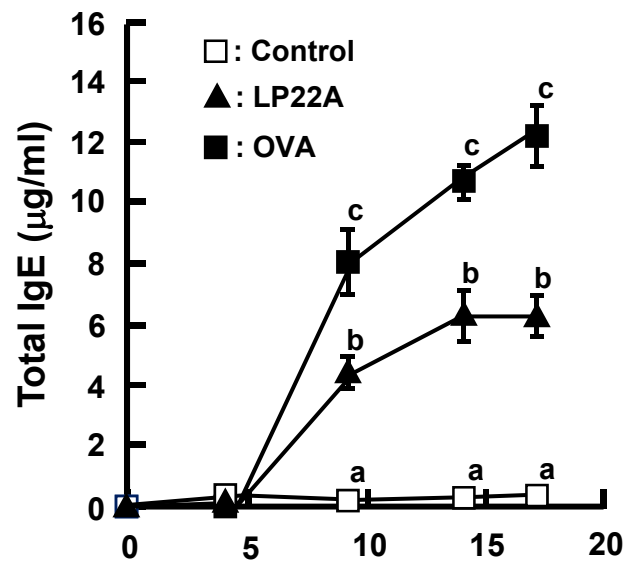
**Fig. 6.** Effect of exogenous TGF- $\beta$  on IgE contents binding RBL-2H3 cells.

516 RBL-2H3 cells were sensitized with anti DNP-IgE (1  $\mu$ g/mL) for 2 h, and then TGF- $\beta$   
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  $\pm$  SD (n = 3).

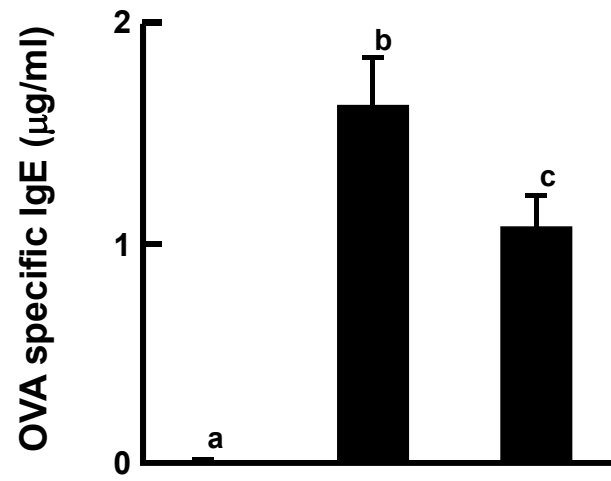
**A**



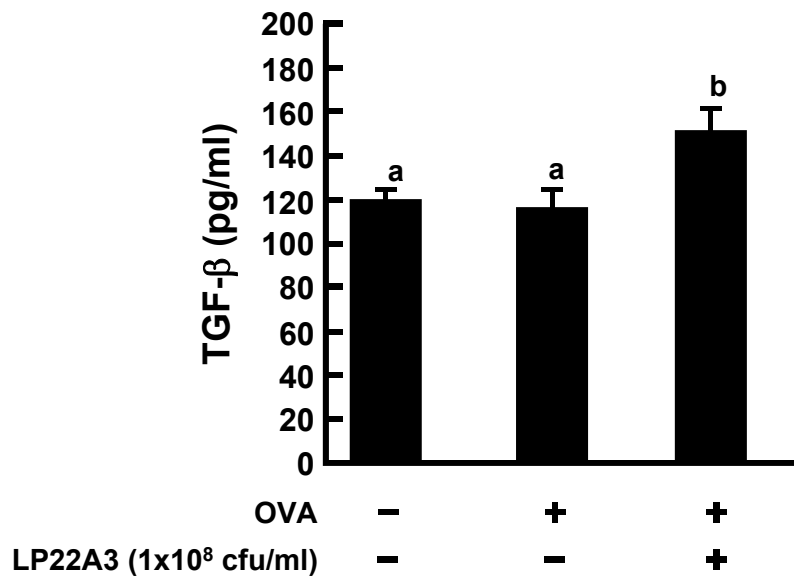
**B**



**C**

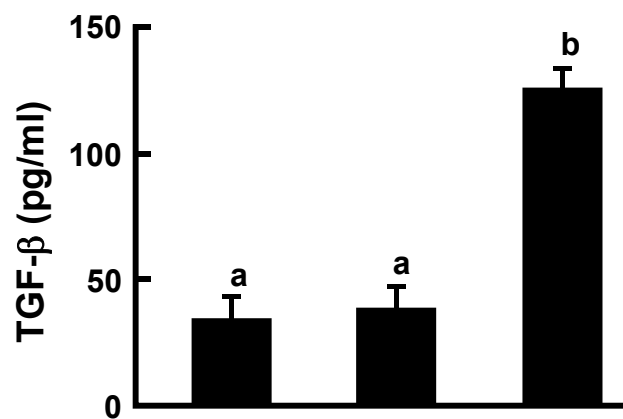
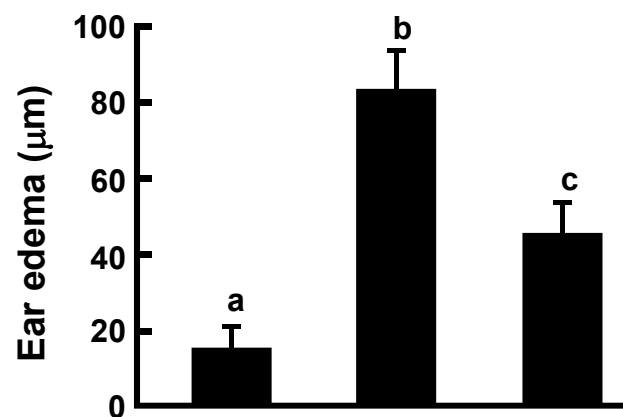
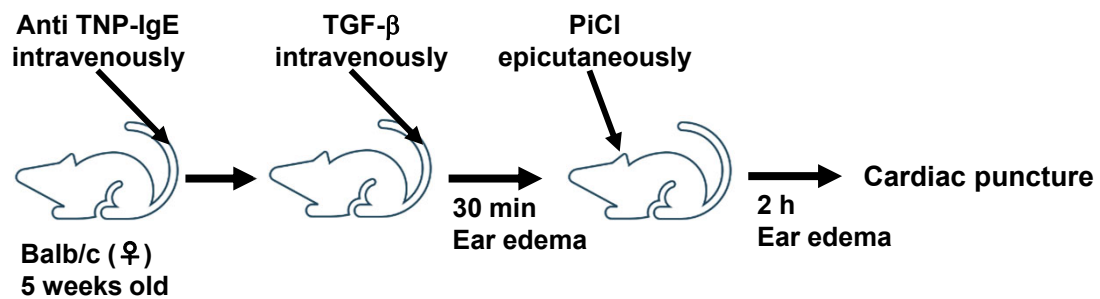


**D**

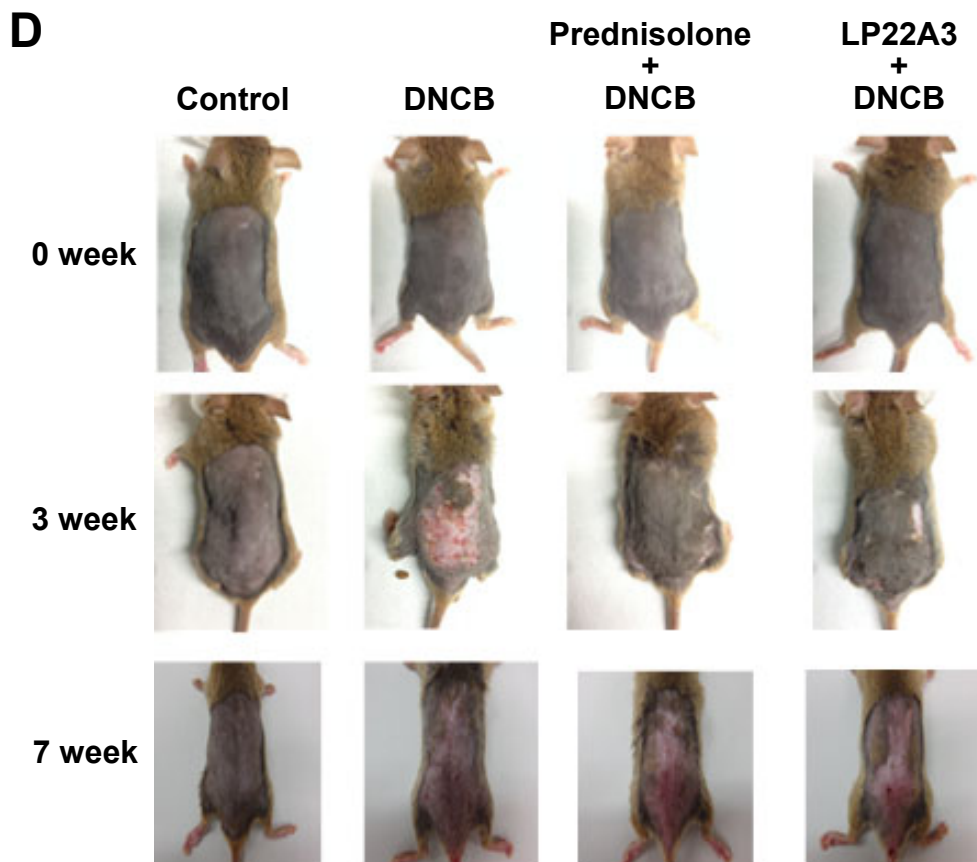
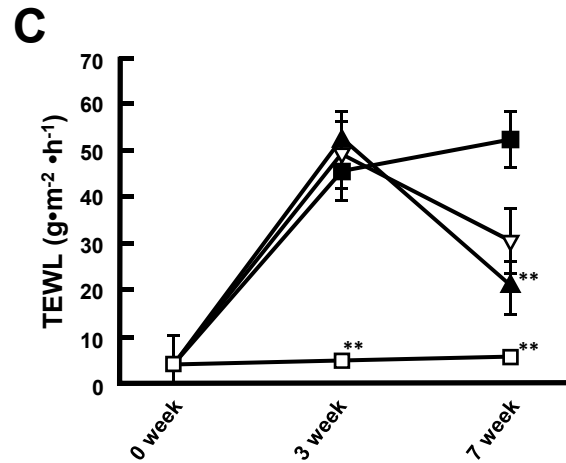
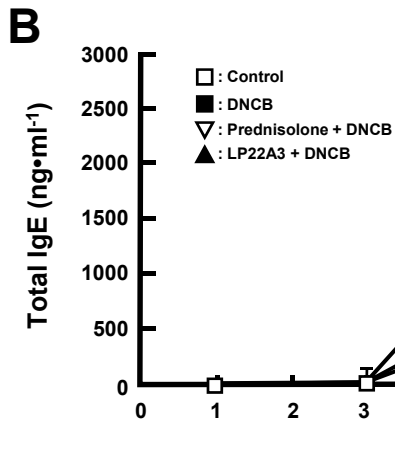
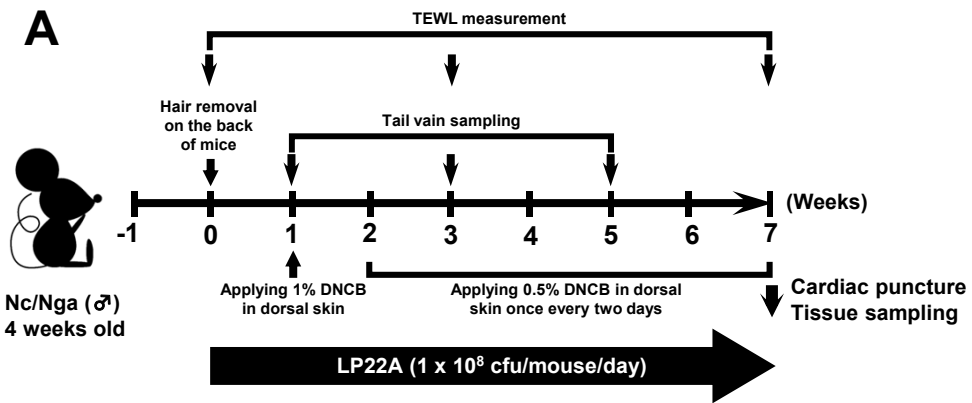


Up  
↑

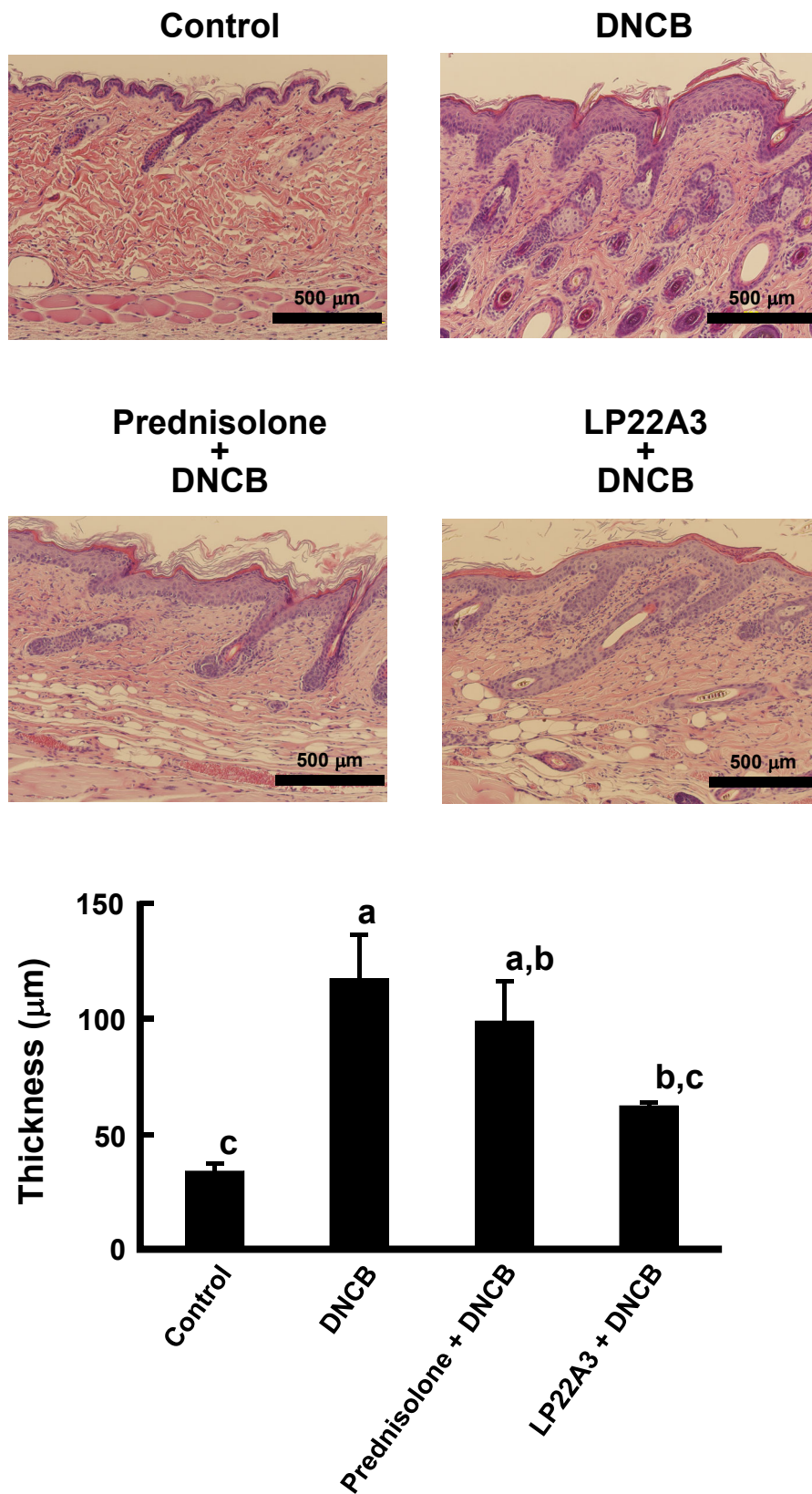




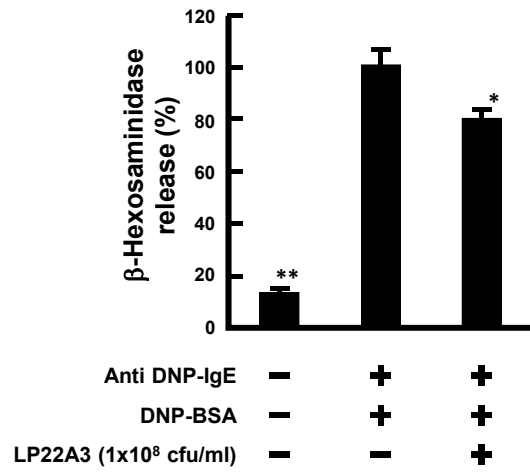
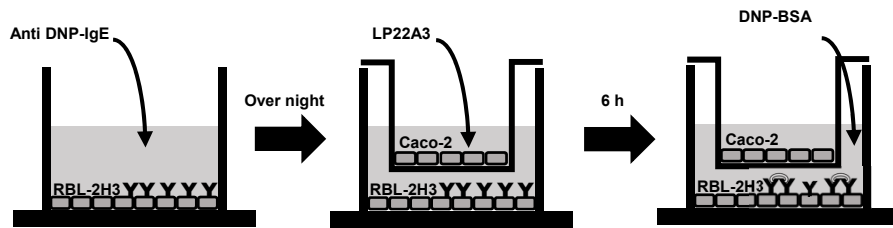
Anti TNP-IgE (2 $\mu\text{g}/\text{mouse}$ )	—	+	+
PiCl (1.6%)	—	+	+
TGF- $\beta$ (40 ng/mouse)	—	—	+



E



**A**



**B**

