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(Citation)

Food Bioscience, 47:101785

(Issue Date)

2022-06

(Resource Type)

journal article

(Version)

Version of Record

(Rights)

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(URL)

<https://hdl.handle.net/20.500.14094/0100476297>





# Oral administration of *Lactiplantibacillus plantarum* 22A-3 exerts anti-allergic activity against intestinal food allergy mouse models sensitized and challenged with ovalbumin

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## ARTICLE INFO

### Keywords:

Anti-allergic activity

Food allergy

*Lactiplantibacillus plantarum* 22A-3

Ovalbumin

OX40L

## ABSTRACT

In recent years, researches on food components with anti-allergic effects have been gathering attention, because of the expectation for the establishment of a safe and effective treatment for food allergy. Previous studies have reported that *Lactiplantibacillus plantarum* 22A-3 (LP22A3) inhibited degranulation of mast cells and reduced IgE production. We have developed a gastrointestinal allergy system in which mice are sensitized by intraperitoneal and oral administration of OVA and then challenged by oral administration of high doses of OVA. As a result, an increase in the amount of IgE in the blood and a decrease in the temperature of the colon were confirmed, and it was clarified that food allergy was induced by oral administration of high dose of OVA as the challenge. Oral administration of LP22A3 ameliorated allergic responses significantly by reducing the amount of IgE in the blood and to recovered the decrease of rectal temperature. However, LP22A3 did not affect the intestinal barrier function. Administered LP22A3 significantly suppressed mRNA expression of OX40L and IL-4. These results suggested that LP22A3 suppressed Th2 differentiation and IL-4 production via downregulation of OX40L, and consequently suppressed IgE production. LP22A3 might provide a safe and effective treatment for allergic diseases due to ability modulating intestinal immune system.

## 1. Introduction

In recent years, lactic acid bacteria (LAB) have gathered attention for their ability to modulate immune function and intestinal barrier function. Various strains of LAB have been reported to modulate the intestinal immune system and successfully ameliorate allergic response in mouse model of food allergy. It is known that the effect of LAB is strain-specific, and that different strains of LAB exert distinct activities. Mechanisms reported for the improvement of allergic symptoms include induction of immunosuppressive Treg cells (Karimi et al., 2009), a decrease in the level of antigen-specific IgE in the blood (Shida et al., 2002), and modulation of Th1 and Th2 immune balance (Yeom et al., 2015). There are several reports on the effect of LAB on intestinal barrier

function, such as preventing the reduction of tight junction associated protein induced by antigen exposure (Tulyeu et al., 2019). Recently, not only living LAB, which is known as probiotics, but also killed-LAB has been reported to show these effects (Liu et al., 2014; Murosaki et al., 1998).

Food allergy is a potentially life-threatening condition. However, there is still no effective therapy. The primary way to manage food allergies is to avoid taking food that causes allergic reaction. For fulminant allergic reaction, adrenaline medicine called epinephrine will be injected to control the symptoms immediately (Sicherer & Simons, 2017). For moderate treatment, oral immunotherapy in which patients suffering from food allergy consume allergy-triggering food gradually to increase their threshold for an allergic reaction. This therapy is meant to

**Abbreviations:** CMC, carboxymethyl cellulose sodium; IL, Interleukin, IL; LAB, lactic acid bacteria; LP22A3, *Lactiplantibacillus plantarum* 22A-3; FcεRI, Fcε Receptor I; OVA, Ovalbumin; OX40L, OX40 ligand; TNF, tumor necrosis factor; ILC, innate lymphoid cell; Th2, type 2 helper T.

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<https://doi.org/10.1016/j.fbio.2022.101785>

Received 5 February 2022; Received in revised form 2 May 2022; Accepted 10 May 2022

Available online 13 May 2022

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completely relieve food allergies, but it sometimes carries a significant risk of causing severe allergic reactions (Chu et al., 2019; Romantsik et al., 2018). Therefore, the establishment of safe and effective treatment for food allergy is expected. Food allergy is a result of an abnormal immune response towards harmless food antigens. It is known as an IgE-mediated immediate reaction and there are two phases: sensitization phase and effector phase. The sensitization phase is characterized by increased IgE production and type 2 helper T (Th2) responses, mainly occurs in the gastrointestinal tract, oral cavity and skin (Ahmad et al., 2018). The mechanisms are as follows. After the initial exposure, food allergen is recognized by antigen presenting cells and presented antigen to naive CD4<sup>+</sup> T cells. Naive CD4<sup>+</sup> T cells are activated to differentiate into Th2 cells, which secrete Th2 cytokines such as Interleukin (IL) -4, IL-5 and IL-13. Th2 cytokines promote B cell proliferation and immunoglobulin isotype class-switch recombination toward IgE. IgE binds to Fcε Receptor I (FcεRI) on the surface of the mast cells. In the effector phase, subsequent antigen exposure induces cross-linking of the IgE bound FcεRI, which results in degranulation of mast cells. The mediators such as histamine and prostaglandins released by mast cells cause typical allergic symptoms.

Intestinal epithelial barrier dysfunction is one of the causes of excessive immune responses to harmless food antigens. Intestinal epithelial barrier is responsible for maintenance of gut homeostasis by limiting the penetration of luminal bacteria and dietary allergens (Yu, 2012). In particular, tight junctions play an important role in intestinal barrier function. Tight junctions in intestinal epithelial cells are composed of different transmembrane proteins, such as claudin and occludin, that regulate the paracellular permeability of water, ions, and macromolecules (Suzuki, 2020). In food allergy, an apparent defect in epithelial barrier was noted. For example, early clinical studies in children with cow's milk allergy demonstrated higher intestinal permeability than in non-allergic children (Jalonen, 1991). In addition, a recent study has shown that tight junctional protein, occludin, claudin-1 and ZO-1, decreased in small intestinal tissues obtained from patients with food allergy compared to those from normal subjects (Pizzuti et al., 2011). Maintaining the intestinal barrier function to prevent excessive antigen entry into gut lamina propria is thought to be important for protection against food allergy.

OX40 ligand (OX40L) and its receptor OX40 belong to the tumor necrosis factor (TNF) superfamily and TNF receptor superfamily, respectively. OX40 is expressed in activated CD4<sup>+</sup> T cells, whereas OX40L is mainly expressed in antigen presenting cells, including activated DCs, B cells, macrophages, as well as in innate lymphoid cells (ILCs). It was reported that OX40 and OX40L regulate cytokine production from T cells, antigen-presenting cells, and modulate cytokine receptor signaling. Recent study suggests that upregulation of OX40L on DCs and ILCs appears to be important for effective Th2 cell responses to antigen exposure (Halim et al., 2018; Linton et al., 2003). For example, costimulatory signals from OX40L upregulates IL-4 production at priming and promotes the differentiation of CD4<sup>+</sup> T cells into high IL-4-producing effector cells (Ohshima et al., 1991). In addition, it was revealed that blockade of OX40-OX40L interactions using OX40L-specific monoclonal antibodies resulted in substantial reductions in the amount of Th2 cytokines and antigen-specific IgE in mouse model of asthma (Seshasayee et al., 2007; Wang & Liu, 2007). Thus, OX40/OX40L has been shown to be a candidate for therapeutic immunization strategies for allergic diseases.

Previous research demonstrated that *Lactiplantibacillus plantarum* 22A-3 (LP22A3) could reduce serum IgE content in active cutaneous anaphylaxis using OVA (Mizuno et al., 2021). However, it is still unclear whether LP22A3 can alleviate allergic symptoms such as systemic anaphylaxis, as well as its effects on intestinal barrier function and the intestinal immune system. Therefore, in the present study, we investigated the effect of LP22A3 on OVA-induced food allergic reactions (anaphylaxis, diarrhea, etc.) induced by oral administration of high dose OVA as the challenge and its impact on the intestinal immune system.

## 2. Materials and methods

### 2.1. Reagents

Ovalbumin (OVA) from chicken egg was purchased from Sigma (St Louis, MO, USA). Aluminum hydroxide gel was purchased from Wako Pure Chemical Corporation (Osaka, Japan). Anti-claudin-1, claudin-2, claudin-4, occludin and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse IgG-HRP conjugate antibodies were purchased from R&D systems (Minneapolis, MN, USA). Blocking One and Chemi-Lumi One L were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). UV-inactivated LP22A3 were prepared by the method reported previously (Mizuno et al., 2021). Other chemicals and reagents were ordinary commercial and guaranteed products.

### 2.2. Mice

BALB/c mice (5 weeks, female) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained in Kobe University Life Science Laboratory with specific pathogen-free conditions, stable temperature (23 ± 2 °C) and humidity (55 ± 10%). All mice were allowed *ad libitum* access to diet and water. All animal experiments were approved and carried out in accordance with the Animal Experimental Ethics Committee of Kobe University (registration number: 30-10-03-R2).

### 2.3. Ovalbumin-induced food allergy model

Mice (5 per group) were sensitized by 4 times intraperitoneal injection of 300 μL PBS containing 10 μg OVA mixed with 1 mg Al(OH)<sub>3</sub> adjuvant once in 5 days. Mice were orally administered 10 mg OVA dissolved in PBS every day for 5 days to induce food allergy. Five days after the last administration, systemic allergic responses were induced by oral administration with 100 mg OVA dissolved in PBS. After OVA exposure, the rectal temperature was measured in 10 min intervals for 90 min as indication of the severity of systemic allergic reaction as previous method with slight modification (Mizuno et al., 2020). Mice in LP22A3 group were orally administered with LP22A3 (1 × 10<sup>8</sup> cfu/day) dissolved in 100 μL of 0.5% carboxymethyl cellulose sodium (CMC, Nacalai Tesque) solution every day during this experiment. While, blank and OVA groups were administered just CMC solution. One day before the injection of OVA/Al(OH)<sub>3</sub>, blood samples of each group were collected from tail vein to measure the total IgE level in serum. On the final day, whole blood sample was collected using cardiac puncture. Small intestines were also collected and washed with cold PBS to remove feces.

### 2.4. Clinical score

Allergic responses to the antigen exposure were evaluated by scoring symptoms 30 min after the antigen challenge. The severity of anaphylaxis was evaluated according to scoring system of Xu et al. (2008). Scores were as follows: 0 = no symptoms, 1 = mild shock including itching, ruffling of fur, dyspnea, and decrease in spontaneous activity, 2 = moderate shock including prostration, sluggish gait, and slight activity after prodding, 3 = severe shock including complete paresis and no activity following prodding with or without convulsions, 4 = death within 30 min. The severity of the diarrhea was scored according to as follows: 0 = normal stool or absent, 1 = slightly wet and soft stool, 2 = unforned stool, and 3 = watery stool.

### 2.5. Measurement of total IgE in serum

Blood samples were stored at 4 °C overnight after placed at 22–25 °C for 1 h. Serum was collected by centrifugation of blood samples in 1.5

mL eppendorf tube at 11,000×g (10,000 rpm in a AF-2724A rotor, Kubota 6200 centrifuge, Kubota Corp., Tokyo, Japan) at 4 °C for 10 min. The supernatant was collected to obtain the serum for total IgE determination. The amount of total IgE in serum was measured by using BD OptEIA Mouse IgE ELISA Set (BD Bioscience, San Jose, CA, USA) according to the manufacturer's protocol.

## 2.6. Protein extraction and Western blotting

Proteins were extracted with 500 µL RIPA buffer and quantified by Lowry assay. RIPA buffer contains 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 50 mM glycerophosphate, 1 mM DTT, 5 mg/mL leupeptin, aprotinin and 100 µM PMSF. Total protein (30 µg) was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (General Electric, Fairfield, CT, USA). Membranes were blocked with Blocking One at 20–25 °C for 30 min. After washing four times with Tris-buffered saline containing Tween-20 (pH 7.5), membranes were incubated at 4 °C overnight with anti-claudin-1, anti-claudin-2, anti-claudin-4, anti-occludin, and anti-β-actin antibodies as primary antibodies, respectively, followed by anti-mouse IgG-HRP conjugate antibodies as the secondary antibody at 4 °C for 2 h. All signals were detected with an enhanced chemiluminescence kit, Chemi-Lumi One L, and the intensity of the band was quantified using the Image J program.

## 2.7. RNA isolation and real-time PCR

Total RNA was extracted from small intestine using Sepasol RNA I super (Nacalai Tesque). cDNA synthesis was performed by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA) and T100™ Thermal Cycler (Bio-Rad Laboratories, California, USA) according to the manufacturer's protocol. The quantitative PCR was performed using a 7500 Fast Real Time PCR system (Life Technologies, Carlsbad, USA) and FastStart Universal Probe Master (ROX) (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. TaqMan gene expression assay were purchased from Applied Biosystems; their assay identifications (IDs) involve are as follow: mouse β-actin (Mm00607939\_s1), mouse GATA-3 (Mm00484683\_mH), mouse IL-4 (Mm00445259\_mH), mouse TBX21 (Mm00450960\_m1), mouse IFNG (Mm01168134\_mH), mouse Foxp3 (Mm00475162\_m1), mouse TNFSF4 (Mm00437214\_m1). β-actin was used as endogenous control.

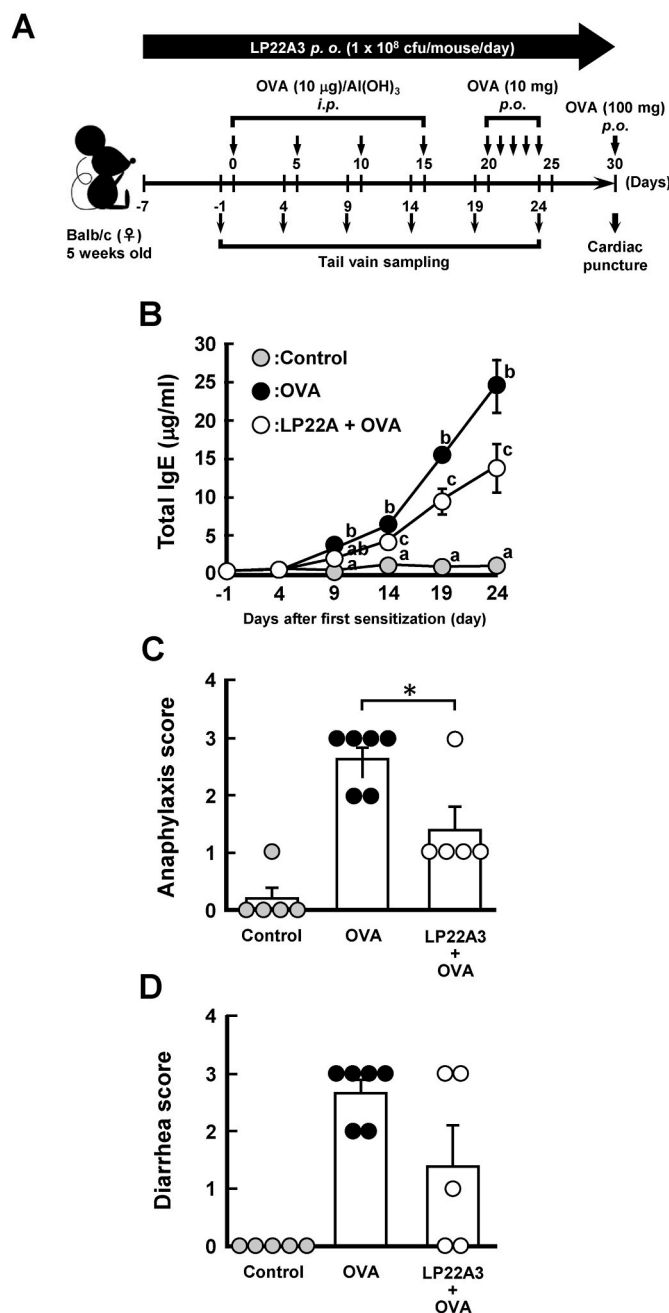
## 2.8. Statistical analysis

All the data were shown as mean ± standard deviation (M ± SD). To compare with any two groups, statistical significance was analyzed using the Student's t-test. To compare with more than two groups, statistical significance was analyzed using the Tukey-Kramer test. Statistical significance was defined as  $p < 0.05$ .

## 3. Results

### 3.1. Anti-allergic effect of LP22A3 on OVA-induced food allergy model

The anti-allergic effects of LP22A3 were tested in the OVA-induced food allergy mouse model. In this model (Fig. 1A), mice were exposed to OVA through the gastrointestinal tract to mimic exposure of food antigens in humans. IgE is known as a major mediator for triggering allergic response. In order to confirm whether the LP22A3 can reduce IgE production in allergic state, total IgE content was monitored during this experiment. As shown in Fig. 1B, total IgE content in the blood of mice that were sensitized with OVA and Al(OH)<sub>3</sub> adjuvant increased gradually up to day 14, and then rapidly increased to day 24 upon subsequent challenge with 10 mg OVA orally administration. Mice orally administered with LP22A3 showed significantly the tendency of



**Fig. 1.** LP22A3 attenuates food allergy induced by OVA sensitization and challenge. A: Experimental design. Mice were sensitized by 4 times intraperitoneal injection of 300 µL PBS containing 10 µg OVA mixed with 1 mg Al(OH)<sub>3</sub> adjuvant once in 5 days. Mice were orally administered 10 mg OVA dissolved in PBS every day for 5 days starting on day 20 to induce food allergy. Five days after the last administration (day 30), systemic allergic responses were induced by oral administration with 100 mg OVA dissolved in PBS. Mice in LP22A3 group were orally administered with LP22A3 ( $1 \times 10^8$  cfu/day) dissolved in 100 µL of 0.5% carboxymethyl cellulose sodium (CMC) solution every day during this experiment. B: Total IgE (until day 24). C and E: Anaphylaxis and diarrhea score (on day 24). Values represent the means ± SE (n = 5). The different letter means significant difference ( $p < 0.05$ ).

suppression in total IgE production compared to OVA group from day 14. Total IgE contents on day 24 in the group administered with LP22A3 was decreased to approximately 56% of the control group. To further confirm the protective effect of LP22A3 against OVA exposure, the severity of allergic reaction and diarrhea was evaluated by a scoring system. The LP22A-3 group showed significantly lower anaphylaxis

score than the OVA group (Fig. 1C) and showed a trend of protective effect against diarrhea (Fig. 1D). These results indicate that LP22A-3 can inhibit systemic allergic reactions in the OVA-induced food allergy model.

### 3.2. Suppression of rectal temperature and IgE contents by LP22A3 oral administration

Exposure to food allergens causes a rapid increase in vascular permeability due to mediators released from mast cells, resulting in symptoms such as decreased body temperature and blood pressure (Brandt et al., 2003; Makabe-Kobayashi et al., 2002). Therefore, the transition of rectal temperature was monitored as another indicator of

the severity of systemic allergic reaction induced by oral administration of large doses (100 mg) of OVA. As shown in Fig. 2A, both the OVA group and the LP22A3 group challenged with OVA showed the lowest rectal temperature after 30 min (OVA group:  $33.4 \pm 0.4$  °C, LP22A3 group:  $35.3 \pm 0.2$  °C). In the LP22A3 group, the decrease in rectal temperature was significantly suppressed from 20 min to 80 min compared to the OVA group. Moreover, total IgE contents in blood was significantly decreased to approximately 28 µg/mL by LP22A3 administration, compared with approximately 41 µg/mL in the control group (Fig. 2B).

### 3.3. Effect of LP22A3 on tight junction proteins

Intestinal epithelial barrier is responsible for maintenance of gut homeostasis by limiting the penetration of luminal bacteria and dietary allergens (Yu, 2012). Intestinal barrier defects are one of the risks of food allergy, as they lead to excessive entry of macromolecules. It was reported that *Lactobacillus reuteri* prevented IgE sensitization through the upregulation of tight junction proteins (Tulyeu et al., 2019). In order to clarify whether LP22A3 have the ability to strengthen tight junctions, the expression level of tight junction proteins was examined. Only claudin-2 expression was increased in OVA group compared to control group (Fig. 3). It was reported that claudin-2 upregulation increases tight junction permeability to  $\text{Na}^+$  and water, and it is involved in the development of diarrhea (Tsai et al., 2017). On the other hand, the expression level of claudin-1, claudin-4, and occludin, which are tight junction proteins that form a physical barrier, were not changed following 100 mg OVA administration. These results predicted that LP22A3 did not affect tight junction proteins.

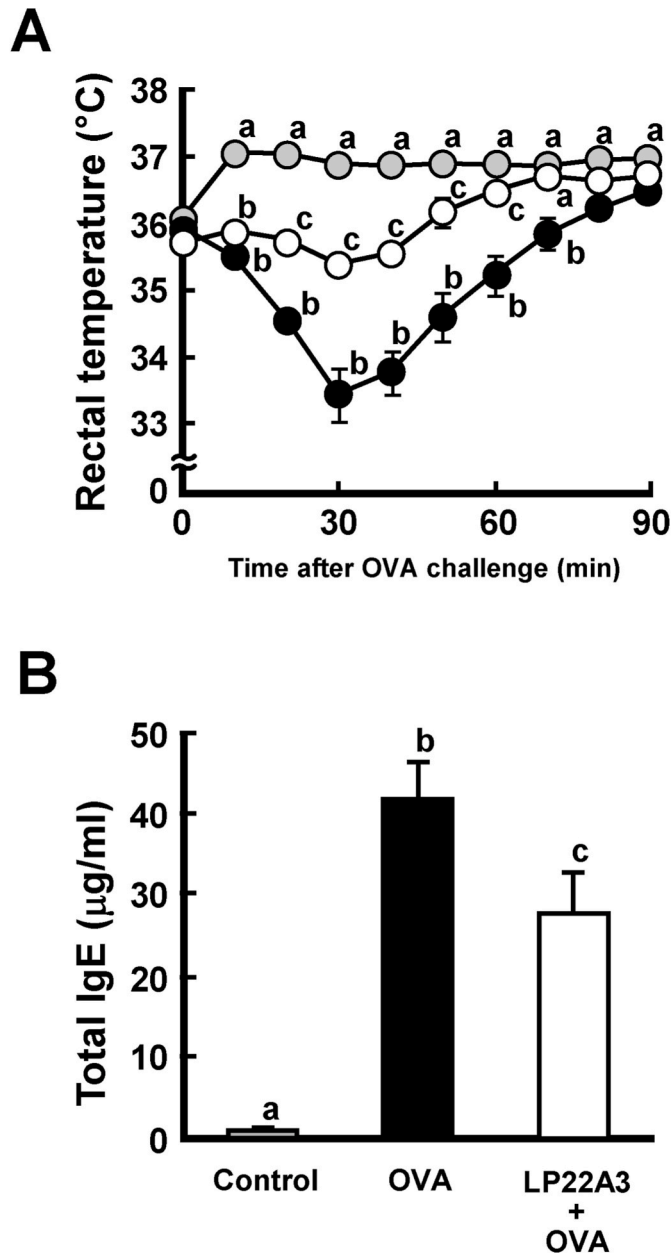


Fig. 2. Suppression of rectal temperature and IgE contents by LP22A3 oral administration. After a systemic allergic reaction was induced by oral administration of 100 mg OVA on day 30 (see Fig. 1A), rectal temperature was measured, and then whole blood samples were taken by cardiac puncture to determine the total IgE content. Values represent the means  $\pm$  SE (n = 5). The different letter means significant difference ( $p < 0.05$ ).

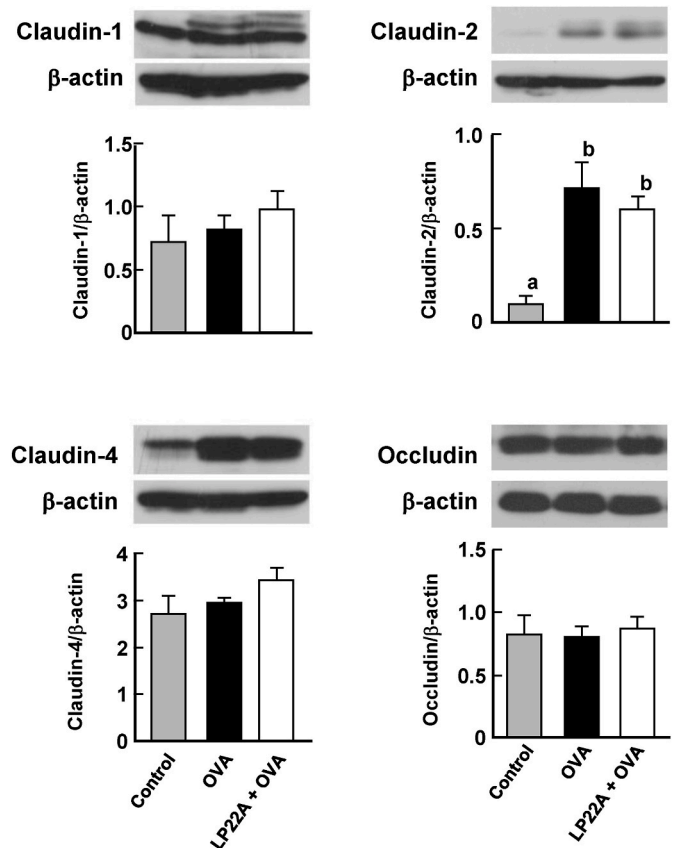


Fig. 3. mRNA expression associated with epithelial tight junction. Values represent the means  $\pm$  SE (n = 5). The different letter means significant difference ( $p < 0.05$ ).



### 3.4. Suppressive effect of LP22A3 on Th2 response mediated OX40L downregulation

Recent evidence suggests that IL-4 plays an important role in IgE class-switching and IgE production from B cells (Meli et al., 2017). It was also reported that neutralizing anti-IL-4 antibody inhibited IgE synthesis induced by anti-CD40 antibody *in vitro* (Punnonen et al., 1997). In this experiment, we investigated the effect of LP22A3 on IL-4 mRNA expression in the ileum, which is the largest immune organ. As shown in Fig. 4, IL-4 mRNA expression in the ileum was greatly increased in OVA group compared to control group. When LP22A3 was administered, IL-4 mRNA expression was significantly decreased in LP22A3 group compared to OVA group, suggesting that LP22A3 suppresses the expression of IL-4. It is widely known that Th2 immune response is increased and Th1 immune response is decreased in allergic conditions. It was assumed that LP22A3 could inhibit the differentiation to Th2 cells from naïve T cells and consequently decrease the secretion of IL-4. Therefore, in order to confirm whether LP22A3 affects CD4<sup>+</sup> T cells differentiation, it was decided to examine the expression level of T cell transcription factor in small intestine. The transcription factors, T-bet, GATA-3, and Foxp3, are master regulators of Th1, Th2, and Treg cell development, respectively. As shown in Fig. 4, the mRNA levels in the OVA group were higher in GATA-3 and lower in T-bet than in the control group. In the LP22A3 group, the mRNA expression level of GATA-3 expression tended to decrease compared to the OVA group. Furthermore, treatment with LP22A3 resulted in little change in mRNA expression of T-bet and an increase in mRNA expression of Foxp3. These results suggested that LP22A3 suppressed the differentiation of Th2 cells.

It has been suggested that upregulation of OX40L on DCs and ILCs is important for an effective Th2 cell response to antigen exposure (Linton et al., 2003). It was also revealed that anti-OX40L-specific antibody treatment resulted in reductions in the production of Th2 cytokines and antigen-specific IgE in mouse model of asthma (Seshasayee et al., 2007). Since OX40L–OX40 interaction exists upstream of the pathway that

promotes Th2 cytokine production (Ohshima et al., 1997), we examined the involvement of OX40L in the differentiation of naïve T cell into Th2 cells in a system with a slightly weakened OVA challenge (Fig. 5.). The mRNA expression level of OX40L as well as GATA-3 and IL-4 in OVA group was significantly increased compared to control group. LP22A3 treatment significantly suppressed their mRNA expression (Fig. 5). These results suggested that LP22A3 has the ability to inhibit differentiation into Th2 cells by suppressing OX40L expression.

## 4. Discussion

The prevalence of food allergy is increasing worldwide, and there is a growing need for further research into the treatments of this disease. In recent years, variety of new treatments have been developed, such as oral immunotherapy (OIT) and sublingual immunotherapy (SLIT) (Morawetz et al., 1996), but most of them reported to have undesirable side effects. Therefore, it is expected to establish safe and effective treatment for food allergy.

Oral administration of LP22A3 significantly decreased IgE contents in the blood (Fig. 1B). Moreover, the scores of anaphylaxis and diarrhea were reduced (Fig. 1C and D), and the decreases in rectal temperature and IgE contents were significantly suppressed by high dose challenge of OVA (Fig. 2A and B), indicating that LP22A3 administration suppressed typical allergic symptoms in a mouse model exposed to OVA in the gastrointestinal tract. These results suggested that continuous intake of LP22A3 may be effective in controlling food allergy symptoms. Dysfunction of the intestinal barrier can be a promising target for food allergy treatment. It was demonstrated that LP22A3 has anti-inflammatory effects, since LP22A3 ameliorates the symptoms of DSS-induced colitis in mice, which is known to be caused by tight junction disruption (Lamubol et al., 2021). However, in the present study, the expression levels of tight junction proteins, except for claudin-2, remained unchanged after exposure to large amount of 100 mg OVA, and this phenomenon was also observed in the LP22A3 group (Fig. 3). These results suggested that LP22A3 does not attenuate allergic

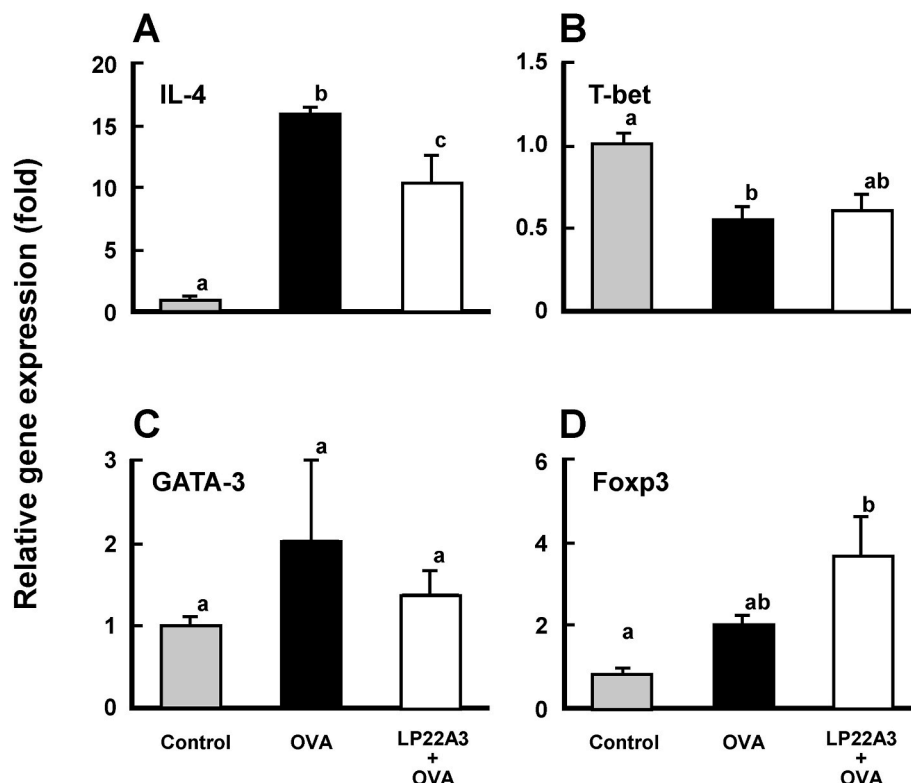
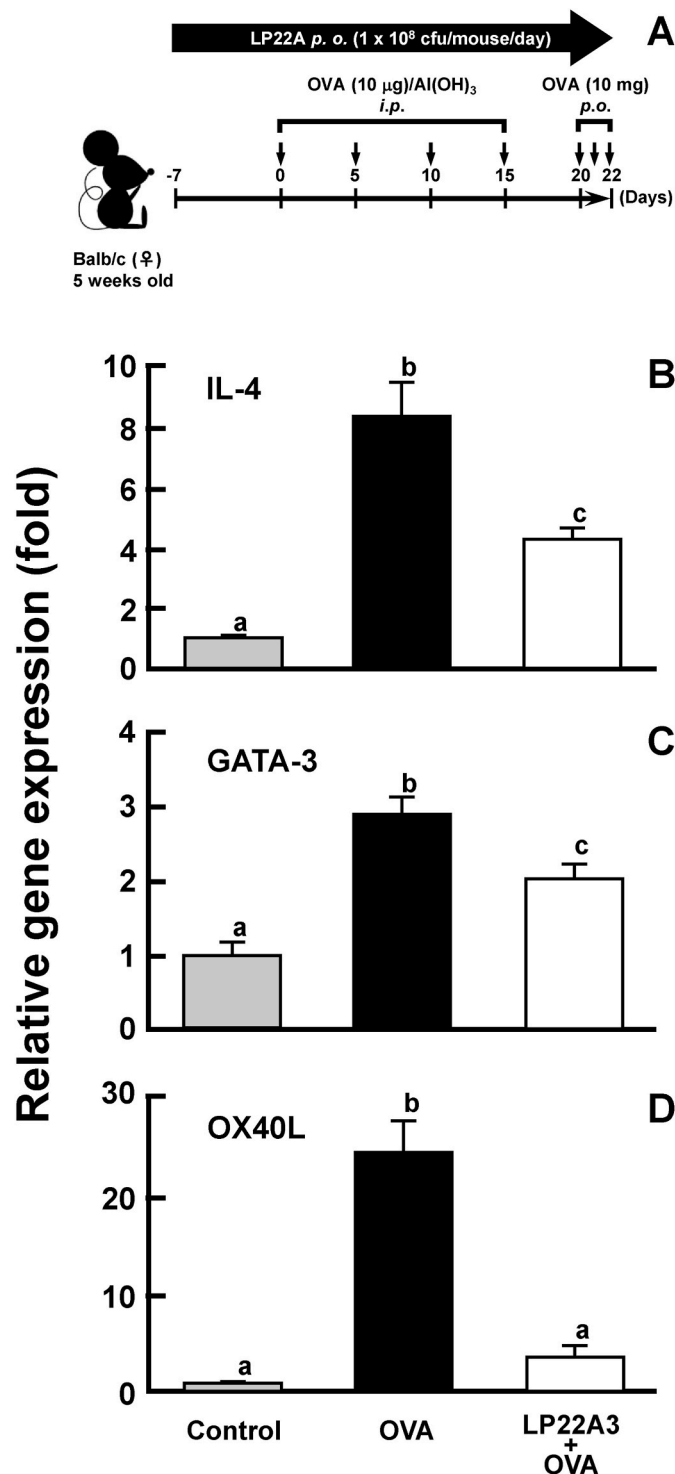


Fig. 4. Effect of LP22A3 on CD4<sup>+</sup> T cell differentiation. Values represent the means  $\pm$  SE (n = 5). The different letter means significant difference ( $p < 0.05$ ).



**Fig. 5.** Change of mRNA expression related Th2 differentiation by LP22A3 treatment. A: The experimental design was almost the same as in Fig. 1, but the OVA challenge was reduced to three times to make it milder. Values represent the means  $\pm$  SE ( $n = 5$ ). The different letter means significant difference ( $p < 0.05$ ).

symptoms via intestinal barrier function.

Since Th2 cells are known to be a major cellular source of IL-4 release, Th2 cell responses are important for IgE production. As shown in Fig. 4, mRNA expression of IL-4 was significantly decreased in the ileum of mice treated with LP22A3 compared to mice treated with OVA alone. However, T-bet and GATA-3, master regulators of Th1 and Th2

cells, did show no drastic change, while Foxp3, a master regulator of Treg, trended to increase with LP22A3 administration compared to the OVA group. Assuming that the dose amount of OVA administered to induce food allergy may have been too high and thus the allergic reaction was too extreme, we decreased the OVA challenge from five to three times and examined again the factors involved in Th2 differentiation (Fig. 5). IL-4 mRNA expressions were increased by OVA challenge and significantly decreased by LP22A3 administration, similar to the results in Fig. 4. Furthermore, GATA-3 mRNA expressions were significantly decreased by LP22A3 administration compared to the increase by OVA challenge. Upregulation of OX40L expression in antigen presenting cells is known to be important for an effective Th2 cell response (Lei et al., 2014). Blocking OX40L with a neutralizing antibody inhibited the production of Th2 cytokines including IL-4 by the CD4<sup>+</sup> T cells (Lei et al., 2018). Because OX40L expression is greatly involved in the differentiation into Th2 cells, we measured OX40L mRNA expression in ileum. The results showed that OX40L expression, which was increased by OVA challenge, was significantly decreased by LP22A3 administration. Taking together with these results, it was suggested that LP22A3 has the ability to inhibit Th2 differentiation and Th2 cytokine production by suppression of OX40L, and consequently suppress IgE production.

## 5. Conclusion

It was clear that LP22A3 attenuates allergic symptoms by suppressing IgE production. One of the mechanisms of action is that LP22A3 suppresses the expression of OX40L in allergic symptoms; the decreased expression of OX40L inhibited the production of Th2 cytokines such as IL-4, which in turn reduces the production of IgE. LP22A3 might provide a safe and effective treatment for allergic diseases. However, in the present study, only mRNA expression of OX40L and IL-4, which are involved in the differentiation of Th2 cells, were confirmed. To investigate the details of the mechanism of allergy suppression by oral intake of LP22A3, it is necessary to analyze the levels of each protein involved in Th2 cell differentiation, including OX40L, and flow cytometry analysis of the cells producing them.

## Authorship contribution statement

**Mari Enokida:** Methodology, Investigation, Data curation, Writing-original draft.

**Ken-ichi Minato:** Investigation, Validation.

**Susumu Yoshino:** Investigation, Validation.

**Nobuaki Ohto:** Investigation, Validation.

**Hiroshige Kuwahara:** Validation, Resources.

**Masashi Mizuno:** Conceptualization, Writing-review & editing, Supervision.

## Declaration of competing interest

The authors have declared no conflicts of interest.

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