CLINICAL & EXPERIMENTAL ALLERGY

Food anaphylaxis induces oral dysbiosis and IL-33– mediated exacerbation in the intestine

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Food anaphylaxis induces oral dysbiosis and IL-33–mediated exacerbation in the intestine

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Conflict of interest

The authors have no conflicts of interest to declare.

Abstract

Background: Food allergy is a life-threatening response to specific foods, and

gastrointestinal microbiota imbalance is considered a cause of this disease. However,

the effects of an imbalanced oral microbiota on food allergy are unknown. The host

immune response also plays an important role in the disease. Notably, interleukin

(IL)-33 released from damaged or necrotic intestinal epithelial cells facilitates Th2

responses. However, causal relationships among the gastrointestinal and oral

microbiota, food allergy, and IL-33 release remain unknown.

Objective: To analyze the effects of gastrointestinal and oral microbiota on the

development of food allergy.

Methods: A murine model of food allergy was established via ovalbumin (OVA)

injection in BALB/c mice. Viable bacteria in feces were identified using matrix-assisted

laser desorption ionization-time of flight mass spectrometry. *Il33* expression in colon-26

mouse colon cells stimulated by isolated fecal bacteria was quantified by real-time PCR.

Intestinal T lymphocytes from the mice were analyzed by flow cytometry. Hydrogen

peroxide-producing bacteria in mouse saliva were detected using Prussian blue plates.

Salivary IgA levels were quantified using ELISA, and IgA-bound oral bacteria were

detected using flow cytometry.

Results: Among fecal bacteria, *Citrobacter* sp. accumulated at a significantly higher level in the feces of allergic mice, and these bacteria induced *il33* expression in colon-26 cells. Orally administered *Citrobacter koseri* JCM1658 exacerbated systemic allergic symptoms and reduced Th17 cell counts in the gastrointestinal tract. Hydrogen peroxide-producing bacterium counts were lower in the oral cavities of allergic mice, whereas salivary IgA levels and IgA-bound oral bacterium counts were increased.

Conclusions & clinical relevance: Food allergy changed the bacterial compositions of both the gastrointestinal and oral microbiota. *Citrobacter* sp. counts were higher in the gastrointestinal tract of mice with food allergy, and they aggravated symptoms by inducing IL-33 release from intestinal epithelial cells.

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1 Introduction

Food allergy is a life-threatening anaphylactic response to foods. Its prevalence is

increasing, particularly in developed countries.[1] Gastrointestinal immune system dysfunction

is considered an inducer of food allergy, and the intestinal microbiota plays important roles in

this dysfunction.[2] A recent report revealed that clostridia, commensal bacteria indigenous to

the murine gastrointestinal tract, induce regulatory T cell accumulation in the mouse colonic

lamina propria while decreasing ovalbumin (OVA)-specific IgE production in the sera of

OVA-sensitized mice.[3] Clostridia alter the composition of the commensal gastrointestinal

microbiota, and the clostridia-containing gastrointestinal microbiota suppresses Th2 cytokine

production and allergen accession by inducing interleukin (IL)-22 production in mouse

serum.[4] In addition, orally administered *Lactobacillus brevis* HY7401 induces Th1 cytokine

production and inhibits Th2 cytokine production.[5] These reports demonstrate that the

intestinal bacteria comprising the gastrointestinal microbiota are capable of affecting host

Th2 responses. However, the detailed relationships between food allergies and the

gastrointestinal microbiota remain to be elucidated. Conversely, the relationship of the oral

microbiota composition, which influences that of the gastrointestinal microbiota, and food

allergy symptoms is completely unknown even though the effects of orally administered

bacteria have been investigated.[5]

IL-33, a member of the IL-1 family of cytokines, is produced by epithelial cells,

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keratinocytes, fibroblasts, and other immune cells.[6] IL-33 induces the expression of

cytokine-encoding genes in Th2 cells and activates MAP kinases in mast cells by binding to

cthe unique IL-33-specific receptor ST2.[6] Upon the infiltration of damaged epithelial cells,

bacterial pathogens and commensal bacteria induce host production of IL-33, which in turn

activates Th2 cells, basophils, mast cells, and group-2 innate lymphoid cells.[6] These results

illustrate the crucial role of IL-33 in inducing Th2 responses in the gastrointestinal tract,

suggesting a causal relationship between IL-33 induction and food allergy. Indeed, a recent

report revealed that IL-33 released from damaged epithelial cells enhances IgE-mediated

degranulation in mast cells and that blockade of ST2 attenuates food-induced anaphylaxis.[7] In

addition, another report found that $il33^{-/-}$ mice were completely protected against oral

challenge-induced core body temperature lowering, one of the typical symptoms of the

anaphylactic response.[8] Therefore, the IL-33–inducing activity of intestinal bacteria

influences the severity of food anaphylaxis.

In the present study, we identified an intestinal bacterium that specifically

accumulates in the gastrointestinal tract in a mouse model of food allergy. We then examined

the $il33$-inducing activity of this bacterium. Concurrently, we also compared the

compositions of the oral microbiota between food-allergic mice and control mice. Our results

demonstrated that intestinal dysbiosis exacerbates food anaphylaxis, confirming the food

allergy-inducing ability of gastrointestinal commensal bacteria. Interestingly, our results

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suggest that food allergy also induces oral dysbiosis.

2 Methods

2.1 Mouse model of food allergy

Animal experiments were approved by The Institutional Animal Care and Use

Committee of Showa University, Japan (approval number: 18008). Female BALB/c mice (8

weeks old; Japan CLEA, Tokyo, Japan) were maintained under specific pathogen-free

conditions. These mice were housed in sterilized cages, and they received a standard mouse

diet (Certified Diet MF, Oriental Yeast, Tokyo, Japan) and autoclaved water. These mice

were sensitized via two intraperitoneal (IP) injections (at a 1-week interval) of 4 μg of

ovalbumin (OVA, Wako, Osaka, Japan) mixed with 100 μl of aluminum hydroxide gel

adjuvant (alum; Invivogen, San Diego, CA, USA). Starting 1 week after the second injection,

food allergy was induced via oral (PO) administration of four doses (over the course of 1

week) of 10 mg of OVA dissolved in phosphate-buffered saline (PBS) using a feeding

needle. To elicit allergic responses, mice were administered PO 50 mg of OVA dissolved in

PBS 1 week after the last 10-mg OVA administration. To evaluate whether a food allergy

was elicited, OVA-specific IgE levels in the sera of the mice were measured using a mouse

OVA-IgE ELISA kit (AKRIE-30, Shibayagi, Gumma, Japan). Systemic anaphylaxis in the

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mice was evaluated by monitoring for decreased rectal temperature using a weighing

environment logger (AD-1687; A and D, Tokyo, Japan).

2.2 Identification of fecal bacteria

The feces of both OVA/alum mice and PBS/alum mice were suspended in 20 volumes

of PBS, inoculated onto plates containing Columbia agar with 5% sheep blood, and cultured

anaerobically for 2 days at 37°C. To identify viable bacteria in the feces of the mice, a

portion of the fresh colonies that developed on each plate was smeared onto a target slide as

samples. These samples were covered with α-cyano-4-hydroxycinnamic acid matrix solution

and loaded into a VITEK® MS MALDI-TOF MS system (BioMérieux, Lyon, France). The

spectra of the samples were analyzed using the Myla® database to identify the species of

origin.[9] Forty-eight fresh colonies from each group of mice were analyzed.

2.3 mRNA expression

Colon-26 RCB2657, a mouse colon epithelial cell line, was purchased from the Cell

Engineering Division of RIKEN BioResource Center (Tsukuba, Japan). The cells were

cultured in RPMI 1640 medium (Wako, Tokyo, Japan) supplemented with 10%

heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. The

cells were stimulated by each of four species of fecal-extracted bacteria (*Citrobacter* sp.,

Enterococcus sp., *Lactobacillus* sp. 1, and *Lactobacillus* sp. 2), each at multiplicities of

infection of 1, 5, and 10, for 4 h. Total RNA from the cells was isolated using TRIzol®

reagent (Thermo Fisher Scientific, MA, USA) and quantified spectrophotometrically. Then,

200 ng of total RNA were converted into cDNA using a ReverTra Ace® qPCR RT kit

(Toyobo, Osaka Japan). The primer pairs for mouse *il33* (sense,

5′-GATGGGAAGAAGGTGA-3′; anti-sense, 5′-TTGTGAAGGACGAAGA-3′) and *gapdh*

(sense, 5′-GCACAGTCAAGGCCGAGAAT-3′; anti-sense,

5′-GCCTTCTCCATGGTGGTGAA-3′) transcripts were used for real-time PCR.

Amplification was performed using a Fast SYBR™ Green Master Mix (Applied Biosystems,

CA, USA) and the Step One Plus Real-Time PCR System (Applied Biosystems). *Il33*

expression in each sample was normalized to that of *gapdh* (as internal control) and a

non-stimulated cell sample (as a calibrator) using the comparative Ct method.

2.4 Oral administration of bacteria

For antibiotic-mediated depletion of the gastrointestinal microbiota, mice were

permitted *ad libitum* access to drinking water containing an antibiotic cocktail consisting of

ampicillin (0.1 g/ml; Wako), kanamycin (0.2 g/ml; Wako), and enrofloxacin (120 mg/ml;

Bayer, Tokyo, Japan) for 1 week. *Citrobacter koseri* JCM1658, purchased from the Cell

Engineering Division of RIKEN BioResource Center, was cultured at 37°C under anaerobic

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conditions in BHI broth. After depleting the gastrointestinal microbiota, mice were

administered PO 200 μl of 5% NaHCO3 in PBS followed by 1×10^9 colony-forming units of

live *C. koseri* JCM1658 suspended in 400 μl of PBS using a feeding needle. Changes in the

bacterial composition of the gastrointestinal microbiota were evaluated via speciation of fecal

bacteria using the aforementioned plating and VITEK® methods.

2.5 Flow cytometry

Mice were anesthetized via IP injections of ketamine–xylazine (ketamine, 100 mg/kg

body weight; xylazine, 10 mg/kg body weight) and perfused with Hanks' Balanced Salt

Solution (HBSS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) to blood

removal. The small intestines of mice were longitudinally opened, washed with PBS, and

shaken in HBSS containing 2 mM EDTA for 70 s. The intestines were shredded and shaken

in RPMI 1640 containing 4% FBS, 0.2 mg/ml collagenase type 1 (Wako), 0.4 mg/ml dispase

1 (Wako) and 10 μg/ml DNase I (Nippon Gene, Japan) for 30 min at 37°C. Cells in the

digested tissue were passed through a cell strainer, and single-cell suspensions were

pretreated with brefeldin A (100 ng/ml) for 1 h. The cells then were stained with

allophycocyanin-conjugated anti-mouse CD3ε (Becton Dickinson [BD], NJ, USA), Brilliant

Violet™-421 (BV-421)-conjugated anti-mouse CD4 (BD), phycoerythrin (PE)-conjugated

anti-mouse IL-17A (BD), PE-conjugated anti-mouse IL-17F (BD), and fluorescein

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isothiocyanate-conjugated anti mouse CD4 (BD) antibodies. These cells were analyzed using

FACS Verse (BD).

2.6 Histological analysis

Small intestinal tissues were fixed in 4% paraformaldehyde and embedded in paraffin.

Sections were stained via May-Grünwald-Giemsa staining. The numbers of eosinophils were

counted under a high-power field at ×400 magnification. The numbers of eosinophils were

expressed as the means of three high-power fields.

2.7 Quantification of hydrogen peroxide-producing bacteria in the oral cavity

Saliva from both OVA/alum and PBS/alum mice was obtained using cotton swabs

from the oral cavity, and these cotton swabs were each immersed in 500 μl of PBS. Fifty

microliters of the swab-immersed PBS were inoculated onto a Prussian blue (PB) agar plate

to measure hydrogen peroxide production. PB plates were prepared by dissolving 1 g of

FeCl3 ・6H2O, 1 g of potassium hexacyanoferrate (Ⅲ), 36 g of BHI broth, and 15 g of agar in

deionized water.[10] Hydrogen peroxide-producing bacteria were visualized by the formation of

colonies with a blue halo on the PB plates after anaerobic incubation.

2.8 16S rRNA sequencing

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Saliva was obtained from OVA/alum and PBS/alum mice as described previously.

Total RNA was isolated from swab-immersed PBS samples using an RNeasy Mini Kit

(Qiagen, Hilden, Germany). All RNA samples were sent to Hokkaido System Science

(Sapporo, Japan), which performed 16S rRNA sequencing.

2.9 Quantification of salivary IgA levels and analysis of IgA-bound oral bacteria

OVA/alum and PBS/alum mice were anesthetized as described previously. Five

minutes after the ketamine–xylazine injection, mice were IP injected with 1 mg/kg body

weight of pilocarpine hydrochloride (Santen, Osaka, Japan) to stimulate salivary secretion.

Saliva was collected from the oral cavity of each mouse using a micropipette. Total IgA in

the saliva was quantified using an IgA Mouse Uncoated ELISA kit (Thermo Fisher

Scientific). Collected salivary samples from both groups of mice were centrifuged at 8000 ×

g to pellet bacteria. The bacterial pellets were resuspended in 660 nM SYTO BC (Thermo

Fisher Scientific) in 0.25% BSA/PBS. After 30 min of incubation on ice, the suspensions

were centrifuged at 8000 × g, and bacterial pellets were resuspended in 1.3 μg/ml goat

anti-mouse IgA-Alexa Fluor® 647 (Southern Biotech, AL, USA) in 0.25% BSA/PBS and

incubated in 30 min on ice. After washing, bacterial pellets were resuspended in 0.25%

BSA/PBS and analyzed using FACS Verse (BD).

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2.10 Statistical analysis

The statistical differences were assessed using a two-tailed Student's *t*-test, and

homoscedasticity of the data was assumed. P values of ≤0.05 were considered significant.

3 Results

3.1 Establishment of a murine model of food allergy (OVA/alum mice)

A schematic of the establishment of the mouse model of food allergy is presented in

Fig. 1A. Serum concentrations of OVA-specific IgE were significantly higher in OVA/alum

mice than in PBS/alum mice, demonstrating that OVA/alum mice were sensitized to OVA

(Fig. 1B). PO administration of 50 mg of OVA elicited a significant decrease in rectal

temperature in the OVA/alum group compared with that in the control group (Fig. 1C). As

decreased rectal temperature after PO administration of antigen is a representative symptom

of food allergy,[11] our results demonstrated the establishment of a murine model of food

allergy.

3.2 Citrobacter sp. *significantly propagated in the gastrointestinal tract of OVA/alum mice*

To identify intestinal bacteria specifically accumulated in the gastrointestinal tract of

food-allergic mice, we identified viable bacteria in the feces of both groups of mice using the

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VITEK® MS MALDI-TOF MS system. This analysis revealed that the density of

Citrobacter sp. was selectively elevated in the feces of OVA/alum mice compared with the

findings in control mice (Fig. 2).

3.3 Citrobacter sp. *induces IL-33 expression in a colon cell line*

Recent reports revealed that IL-33 plays an important role in allergic responses,[12]

including the exacerbation of food-induced anaphylaxis in the gastrointestinal tract.[8] We

therefore examined whether fecal bacteria, including *Citrobacter*, induce *il33* expression in

gastrointestinal epithelial cells under *in vitro* conditions. Among the four tested species of

fecal bacteria, only *Citrobacter* sp. induced *il33* expression in a dose-dependent manner (Fig.

3).

3.4 Orally administered Citrobacter *exacerbates an allergic symptom*

To further confirm the augmentation of allergic responses by *Citrobacter*, we

examined the influence of gastrointestinal microbiota (composed predominantly of

Citrobacter) on the allergic responses induced by a food antigen. The analysis revealed that

the PO *C. koseri* survived longer in the feces of OVA/alum mice than in the feces of

PBS/alum mice (Fig. 4A). Using these two groups of mice, we next examined whether orally

administered *C. koseri* exacerbated the allergic response. Monitoring of rectal temperatures

demonstrated that PO-administered OVA elicited a significantly lower rectal temperature in

C. koseri-inoculated OVA/alum mice than in OVA/alum mice (Fig. 4B). In addition,

histological observation of small intestinal tissue demonstrated that PO administration of *C.*

koseri significantly increased the number of eosinophils in OVA/alum mice (Fig. 5).

Furthermore, an examination of the proportion of Th2 lymphocytes induced in the small

intestinal lamina propria revealed that PO administration of *C. koseri* yielded an increased

proportion of CD4+ IL-4+ lymphocytes (Th2 lymphocytes) in OVA-specific allergy mice

(Fig. 6).

Recent studies reported that several intestinal bacteria including *Citrobacter*

rodentium, which is closely related to *C. koseri*, induce the accumulation of Th17 cells in the

intestinal lamina propria.[13] Consistent with those reports, we observed that PO *C. koseri*

strongly induced Th17 cell accumulation in the intestinal lamina propria of PBS/alum mice,

but not in OVA/alum animals (Fig. 7). These results are consistent with the known role of

Th17 cells in protecting the intestinal mucosa against exogenous pathogenic bacteria,

including *C. rodentium*,[13] and they may explain why orally administered *C. koseri* is more

readily fixed in the gastrointestinal microbiota of OVA/alum mice than in that of PBS/alum

mice.

3.5 Allergic symptoms changed the composition of the oral microbiota

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Although some causal relationships between food allergy symptoms and the

gastrointestinal microbiota have been expected, the roles of the oral microbiota in food

allergy are unknown. Thus, we compared the oral microbiota compositions of OVA/alum and

PBS/alum mice. In the human oral cavity, it is well known that the oral cavity microbiota is

normally dominated by oral streptococci including *Streptococcus sanguinis* and

Streptococcus gordonii, which explicitly produce hydrogen peroxide.[14] In the mouse oral

cavity, it has been reported that *Lactobacillus* sp. also dominates the oral microbiota in

addition to *Streptococcus*.[15] *Lactobacillus* sp. is reported to produce hydrogen peroxide.[16] As

a result, fewer colonies with blue halos were formed on plates inoculated by saliva from

OVA/alum mice than on plates exposed to saliva from PBS/alum mice (Fig. 8A). In addition,

16S rRNA sequencing confirmed the reduced diversity of oral commensal bacteria in

OVA/alum mice (Fig. 8B). Furthermore, IgA-coated bacterium counts have been reported to

increase in the gastrointestinal tracts of patients with inflammatory bowel disease (IBD).[17]

The report suggested that IgA plays a key role in changing the gastrointestinal microbiota

composition. In our experiment, salivary IgA levels were significantly higher in the oral

cavities of OVA/alum mice than in PBS/alum mice (Fig. 8C). The increased salivary IgA

content was bounded by oral bacteria (Fig. 8D).

4 Discussion

In this study, we demonstrated that intestinal dysbiosis can exacerbate food

anaphylaxis. Intestinal commensal bacteria affect the development of the host gastrointestinal

immune system, and antibiotic-induced intestinal dysbiosis increases host susceptibility to

colonization by pathogenic bacteria.[18] Several commensal bacteria that protect or induce IBD

have been reported.[18] Indeed, fecal microbiota transplantation has been proposed as an

efficient method for treating IBD.[19] Commensal microbiota or specific bacteria have been

revealed to provide protection against food allergy.[4,20]

We used a VITEK® system to identify intestinal bacteria specifically enriched in a

murine model of food allergy. This system uses MALDI-TOF-MS to speciate bacterial

colonies that form on blood agar plates. This analysis made it possible to compare the

quantities of viable bacteria between food-allergic mice and healthy control mice. This

method revealed the allergy-inducing ability of intestinal bacteria more clearly than other

methods that use samples containing dead bacteria or fragments of bacterial components

because host cells respond to bacterial infiltration or bacterial metabolites to elicit immune

responses. The validity of bacterial identification obtained by VITEK as established by

Dubois et al.[21] However, causal relationships between food allergy and the composition of

the intestinal microbiota remain to be elucidated. In the present study, we identified a

Citrobacter bacterium that was specifically enriched in the gastrointestinal tract in a mouse

model of food allergy. Although the virulence and pathogenicity of *C. rodentium* in the

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gastrointestinal tract have been investigated,[22] the food allergy-inducing abilities of

Citrobacter sp. have not been demonstrated. *C. rodentium* is reported to employ a pathogenic

mechanism of epithelial "attachment and effacement" (A/E),[23] whereby this species attaches

to the surface of intestinal epithelial cells and induces local destruction ("effacement") of the

epithelial cells. This mechanism triggers reactive inflammation and the induction of

IL-17-producing CD4 helper T (Th17) cells and IL-22-producing type-3 innate lymphoid

cells.[23] IL-33 is an alarmin cytokine that is released after cell or tissue damage.[6] As

Citrobacter sp. was isolated from the feces of food-allergic mice, this species is presumed to

employ some attachment and infiltration mechanisms similar to A/E in *C. rodentium*. The

Citrobacter sp. isolated in the present work is thus expected to be capable of disrupting host

intestinal epithelial cells, thereby triggering IL-33 production. In the present work, we

observed the *il33*-inducing activity of *Citrobacter* sp. isolated from OVA/alum mice. IL-33 is

a Th2 response-driving cytokine. We further demonstrated that another *Citrobacter* species,

C. koseri JCM1658, exacerbated the anaphylactic response when administered PO to

OVA/alum mice. These results clearly demonstrated a causal relationship between

Citrobacter species and food anaphylaxis.

It has reported that *C. rodentium* induces Th17 cells in the gastrointestinal tract and

that Th17 cells in turn facilitate the elimination of exogenous pathogenic bacteria, including

Citrobacter.[13] We postulate that attenuation of Th17 cell levels observed in this study can be

attributed to Th2-biased responses in OVA/alum mice. Presumably, a trade-off occurred

between Th2 and Th17 responses, such that (in the gastrointestinal tracts of OVA/alum mice)

the Th2 response predominated over the weakened induction of Th17 cells, permitting the

increased induction of Th2 cells. Apparently, the depleted pool of Th17 cells was not

sufficient to eliminate *C. koseri* within a week of PO administration. The surviving *C. koseri*

presumably then stimulated the intestinal epithelial cells to produce IL-33, which in turn

further reinforced the Th2 response in the gastrointestinal tracts of OVA/alum mice.

Furthermore, IL-33 can stimulate naïve T cells to exhibit Th2 responses but not Th17

responses.[24] Such a circular feedback loop is expected to be an exacerbating factor for food

allergy symptoms, facilitating further dysbiosis in the gastrointestinal tract. The resulting

dynamic, imbalanced composition of the gastrointestinal microbiota has a causal relationship

with food allergy symptoms. Thus, in the present study, we demonstrated that intestinal

dysbiosis exacerbates food anaphylaxis and the food allergy-inducing ability of intestinal

commensal bacteria.

In addition to intestinal dysbiosis, we demonstrated that the oral microbiota is also

affected by food allergy. Although no detailed molecular mechanisms have been uncovered,

an extended duration of food allergy symptoms changed the composition of the oral

microbiota. Repeated stimulation by allergens derived from food can induce Th2 responses in

the gastrointestinal tract, and intestinal microbes affected by Th2 responses trigger a vicious

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circle of systemic dysbiosis and allergic symptoms. This "oral dysbiosis" is considered the

result of Th2 conversion. It is supposed that atopic B cells activated in the gastrointestinal

tract migrate through common mucosal immune systems and affect the oral microbiota

composition.[25] *Lactobacillus* sp. is reported to be one of the dominant bacteria in the oral

cavities of mice,[15] these bacteria are known to produce hydrogen peroxide.[16] The study

results suggested that hydrogen peroxide-producing *Lactobacillus* counts are decreased in the

oral cavities of allergic mice, with a similar finding noted in the feces of OVA/alum mice.

The similar compositional change in both the oral and gastrointestinal microbiota indicated

that intestinal dysbiosis induced in food allergy affects the oral microbiota. The importance of

intestinal IgA for maintaining the commensal bacteria in the gastrointestinal tract was

recently reported.[17] In the feces of patients with IBD, colitogenic members of the intestinal

microbiota were coated with IgA.[17] Oral supplementation of high-affinity and poly-reactive

IgA improved the pathological colon phenotype and increased regulatory T cell counts in the

colon.[26] The results of the current study suggest that IgA induction in the gastrointestinal tract

affects the composition of both the gastrointestinal and oral microbiota and indicate existence

of causal relationships between food allergy as a systemic disease and oral dysbiosis.

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Figure legends

Fig. 1 Establishment of food allergy model mice.

Time line of ovalbumin (OVA) sensitization and the induction of food allergy in mice (A).

The production of OVA-specific IgE antibodies in the sera of both groups of mice were

measured by ELISA (B). OVA-specific food allergy symptoms were assessed by measuring

the maximum change in rectal temperature after the oral administration of 50 mg of OVA.

Percentage changes after the oral administration of 50 mg of OVA based on before treatment

are indicated. (C). Results are presented as the mean ± SD (n = 3), and they are representative

of three independent experiments. N.D., not detected, *P < 0.05.

Fig. 2 Identification of fecal bacteria of ovalbumin (OVA)-specific food-allergic mice.

Feces from food-allergic (OVA/alum) and control (phosphate-buffered saline [PBS]/alum)

mice were collected and analyzed using a VITEK MALDI-TOF-MS system. The numbers of

identified fecal bacteria derived from each group of mice (OVA/alum group;

OVA/alum#1-OVA/alum#5, PBS/alum group; PBS/alum#1-PBS/alum#5) were indicated in

the left bar graph. Results are presented as the mean ± SE (n = 5). *P < 0.05. The presence of

Citrobacter sp. in all fecal samples from both groups of mice is indicated in the circle charts

on the right.

Fig. 3 *In vitro interleukin-33* (*il33*) expression following stimulation by fecal bacteria

isolated from ovalbumin (OVA)-induced allergic mice.

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Colon-26, a mouse colon epithelial cell line, was stimulated by four species of viable bacteria

isolated from the feces of OVA allergic mice. *il33* expression levels in each sample were

normalized to those of a housekeeping gene (*gapdh*) and expressed as fold changes relative to

that in a non-stimulated sample. Results are presented as the mean ± SD (n = 3), and they are

representative of three independent experiments. *P < 0.05.

Fig. 4 Exacerbation of allergy symptoms by oral administration of *Citrobacter*.

After 1 week of antibiotic-mediated depletion of the endogenous microbiota, ovalbumin

(OVA)/alum and phosphate-buffered saline (PBS)/alum mice were orally administered

Citrobacter koseri (1×10^9 colony-forming units per animal). Feces were collected 1 and 7

days after administration, and the resulting bacterial isolates were speciated using a VITEK

MALDI-TOF-MS system. The occupancy rate of *C. koseri* among the identified fecal

bacteria was defined as the survival rate compared to that of the inoculum (A). The severities

of OVA-specific allergic responses were compared by determining anaphylactic responses

(assessed as the maximum change in rectal temperature following OVA challenge) in

OVA/alum mice after the oral administration of *C. koseri* (B).

Fig. 5 The numbers of eosinophils infiltrated in the small intestines of ovalbumin

(OVA)/alum mice after the oral administration of *Citrobacter koseri*.

The numbers of eosinophils that infiltrated into the small intestine were visualized using

May-Grünwald-Giemsa staining and evaluated via counting in a high-power field at ×400

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magnification. Tissue images are shown on the right. Arrows indicate eosinophils. The left

graph shows the mean numbers of eosinophils calculated per three high-power fields.

Fig. 6 Induction of Th2 cells in the small intestines of ovalbumin (OVA)/alum mice after the

oral administration of *Citrobacter koseri*.

Lymphocytes were obtained from the small intestinal lamina propria of OVA/alum mice and

after the oral administration of *C. koseri*. The induction of CD4+IL-4+ (Th2) cells was

analyzed by flow cytometry. In the left graph, circles represent separate mice, and lines

denote means ± SDs (n = 3). The right histograms present the mean fluorescent intensities of

IL4+ cells in OVA/alum and *C. koseri*-administrated OVA/alum mice. *P < 0.05.

Fig. 7 Induction of Th17 cells in the small intestines of mice after the oral administration of

Citrobacter koseri.

Lymphocytes were obtained from the small intestinal lamina propria of ovalbumin

(OVA)/alum and phosphate-buffered saline (PBS)/alum mice after the oral administration of

C. koseri. Inductions of CD4+IL-17+ (Th17) cells were analyzed by flow cytometry. Right

histograms present the mean fluorescent intensities of CD4+IL17+ (Th17) cells in total

lymphocytes derived from each group of mice. In the left graph, circles represent separate

mice, and lines denote means ± SDs (n = 3). *P < 0.05.

Fig. 8 Quantification of hydrogen peroxide-producing bacteria and salivary IgA in the oral

cavity of ovalbumin (OVA) allergic mice.

Saliva was collected from OVA allergic (OVA/alum #1–#3) and control mice

(phosphate-buffered saline [PBS]/alum #1–#3) and inoculated onto three Prussian blue plates.

After an overnight anaerobic incubation, hydrogen peroxide-producing bacteria formed

colonies with blue halos. The plates of OVA/alum #1 and PBS/alum #1 are presented (left).

The right graph shows the mean number of colonies with blue halos for the OVA/alum and

PBS/alum groups (A). Percentage of detected genera of bacteria in both OVA/alum #1 and

PBS/alum #1 were indicated by a horizontal bar graph (B). Saliva was collected from both

OVA/alum and PBS/alum mice, and salivary IgA was quantified using an IgA ELISA kit (C).

Oral bacteria in the saliva of both groups of mice were stained using SYTO BC and goat

anti-mouse IgA-Alexa Fluor® 647. The histogram presents the mean fluorescent intensities of

IgA-Alexa Fluor® 647+ in SYTO BC+ oral bacteria from OVA/alum and PBS/alum mice.

Results are presented as the mean ± SD (n = 3), and they are representative of three
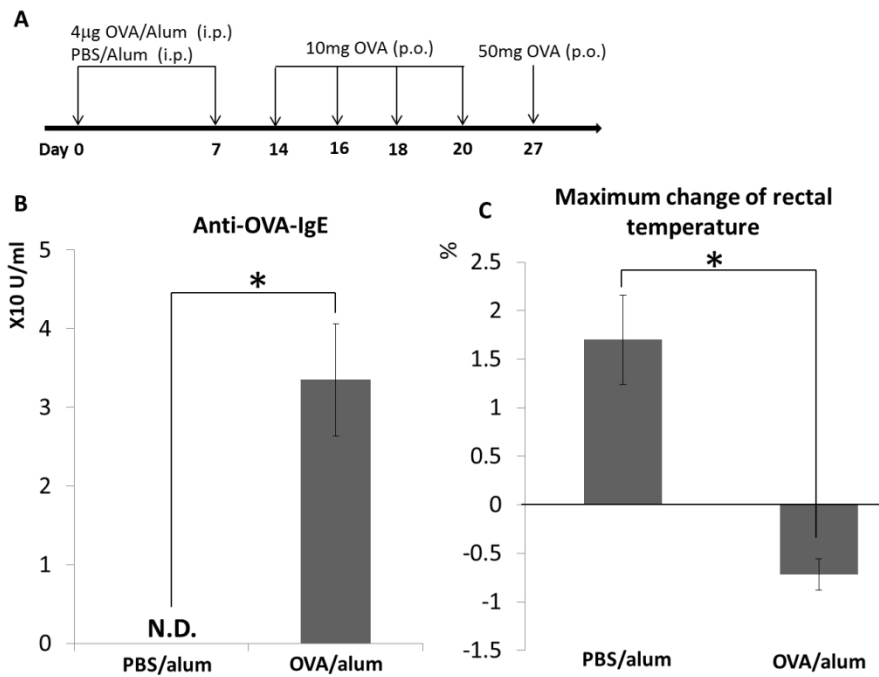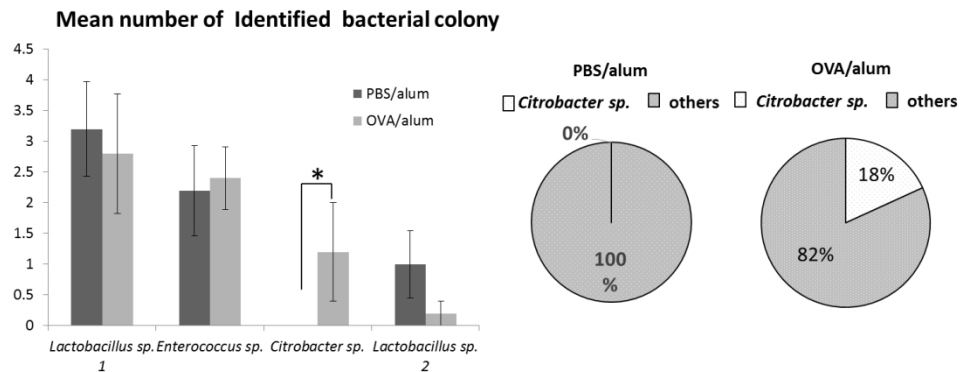
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Fig. 1 Establishment of food allergy model mice.
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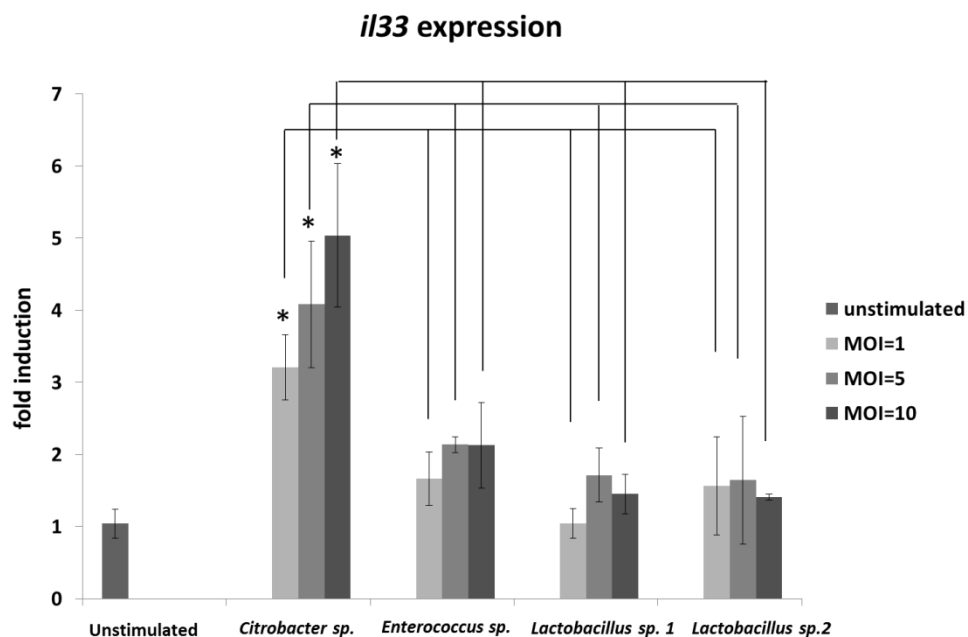the circle charts on the right.

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Colon-26, a mouse colon epithelial cell line, was stimulated by four species of viable bacteria isolated from the feces of OVA allergic mice. il33 expression levels in each sample were normalized to those of a housekeeping gene (gapdh) and expressed as fold changes relative to that in a non-stimulated sample. Results are presented as the mean ± SD (n = 3), and they are representative of three independent experiments. *P < 0.05.
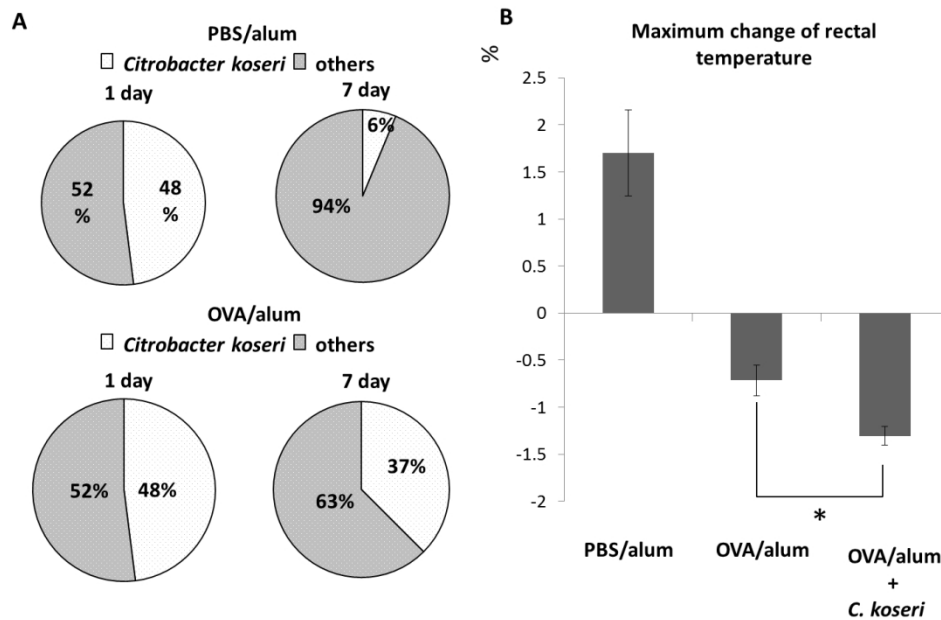
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Fig. 4 Exacerbation of allergy symptoms by oral administration of Citrobacter.
After 1 week of antibiotic-mediated depletion of the endogenous microbiota, ovalbumin (OVA)/alum and phosphate-buffered saline (PBS)/alum mice were orally administered Citrobacter koseri (1 × 109 colony-forming units per animal). Feces were collected 1 and 7 days after administration, and the resulting bacterial isolates were speciated using a VITEK MALDI-TOF-MS system. The occupancy rate of C. koseri among the identified fecal bacteria was defined as the survival rate compared to that of the inoculum (A). The severities of OVA-specific allergic responses were compared by determining anaphylactic responses (assessed as the maximum change in rectal temperature following OVA challenge) in OVA/alum mice after the oral administration of C. koseri (B).
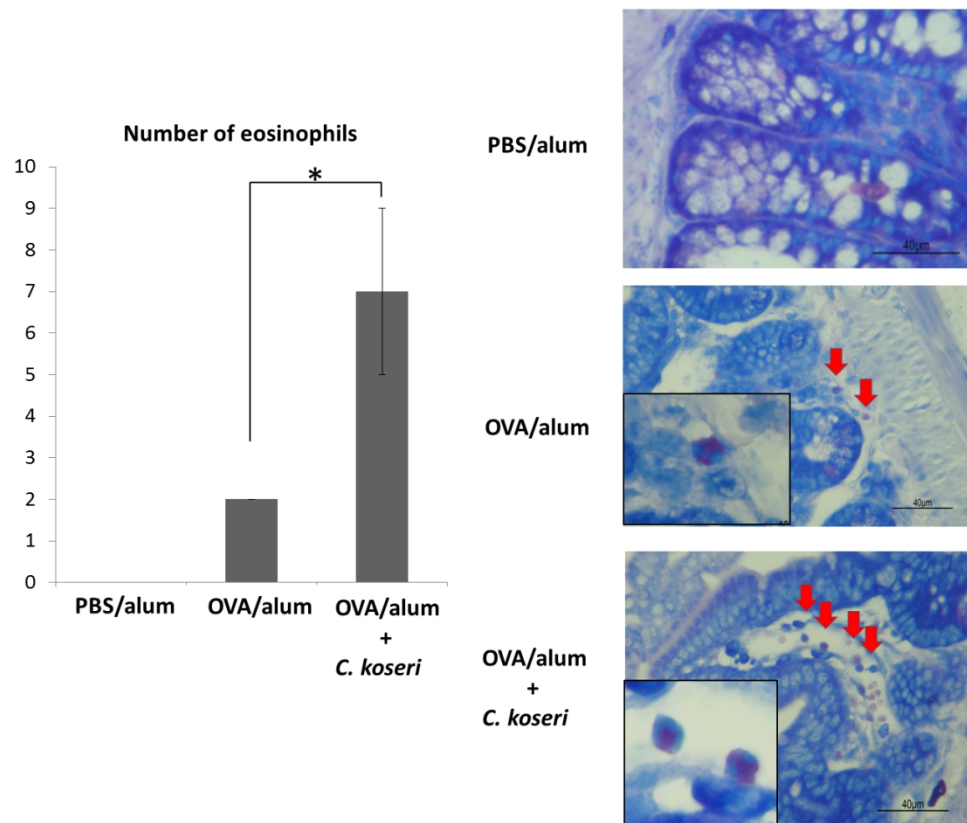
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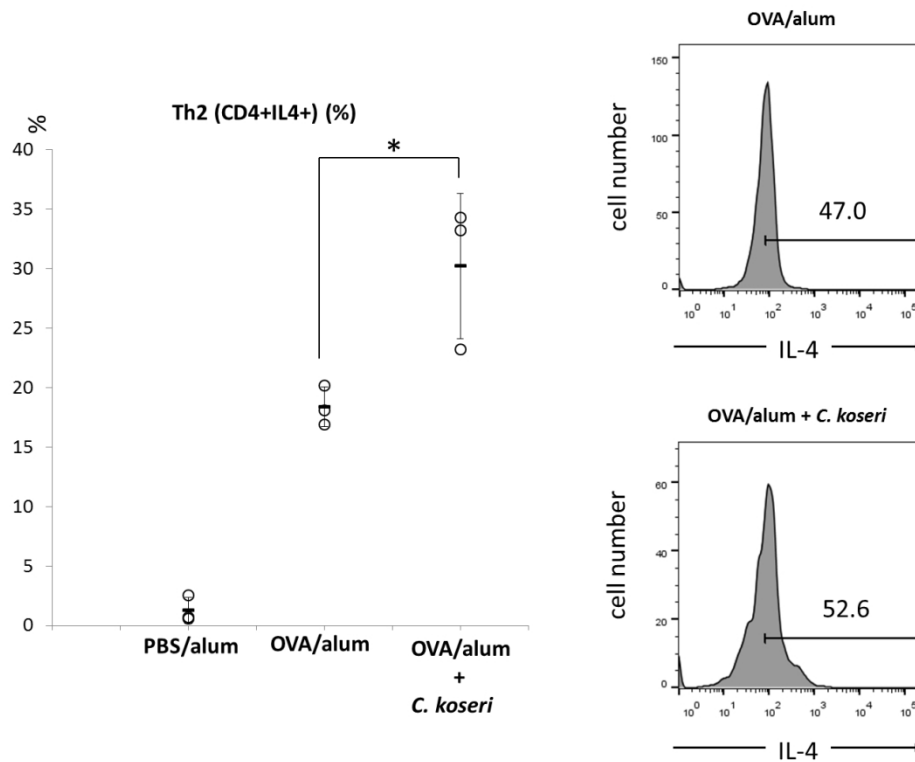
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Fig. 6 Induction of Th2 cells in the small intestines of ovalbumin (OVA)/alum mice after the oral administration of Citrobacter koseri.
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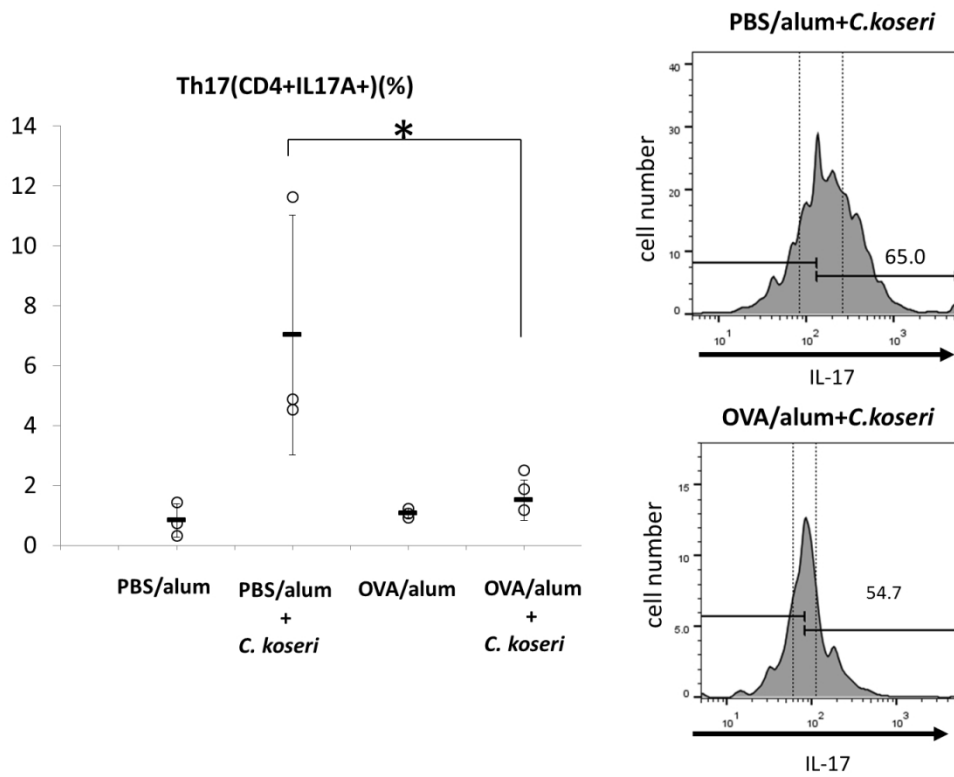
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Fig. 7 Induction of Th17 cells in the small intestines of mice after the oral administration of Citrobacter koseri.
Lymphocytes were obtained from the small intestinal lamina propria of ovalbumin (OVA)/alum and phosphate-buffered saline (PBS)/alum mice after the oral administration of C. koseri. Inductions of CD4+IL-17+ (Th17) cells were analyzed by flow cytometry. Right histograms present the mean fluorescent intensities of CD4+IL17+ (Th17) cells in total lymphocytes derived from each group of mice. In the left graph, circles represent separate mice, and lines denote means ± SDs (n = 3). *P < 0.05.
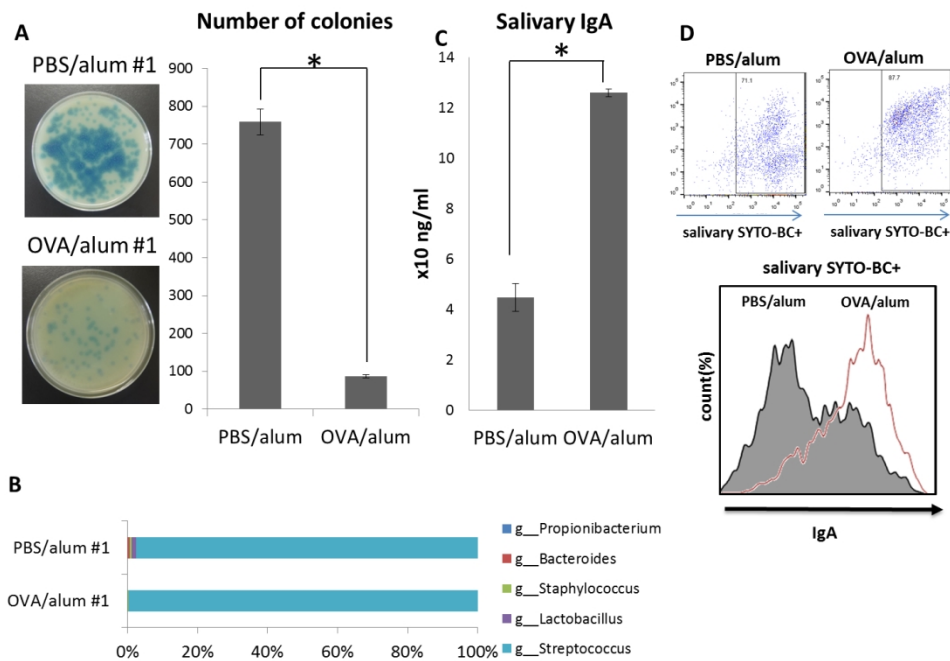
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Fig. 8 Quantification of hydrogen peroxide-producing bacteria and salivary IgA in the oral cavity of ovalbumin (OVA) allergic mice.
Saliva was collected from OVA allergic (OVA/alum #1–#3) and control mice (phosphate-buffered saline [PBS]/alum #1–#3) and inoculated onto three Prussian blue plates. After an overnight anaerobic incubation, hydrogen peroxide-producing bacteria formed colonies with blue halos. The plates of OVA/alum #1 and PBS/alum #1 are presented (left). The right graph shows the mean number of colonies with blue halos for the OVA/alum and PBS/alum groups (A). Percentage of detected genera of bacteria in both OVA/alum #1 and PBS/alum #1 were indicated by a horizontal bar graph (B). Saliva was collected from both OVA/alum and PBS/alum mice, and salivary IgA was quantified using an IgA ELISA kit (C). Oral bacteria in the saliva of both groups of mice were stained using SYTO BC and goat anti-mouse IgA-Alexa Fluor® 647. The histogram presents the mean fluorescent intensities of IgA-Alexa Fluor® 647+ in SYTO BC+ oral bacteria from OVA/alum and PBS/alum mice. Results are presented as the mean ± SD (n = 3), and they are representative of three independent experiments. *P < 0.05.
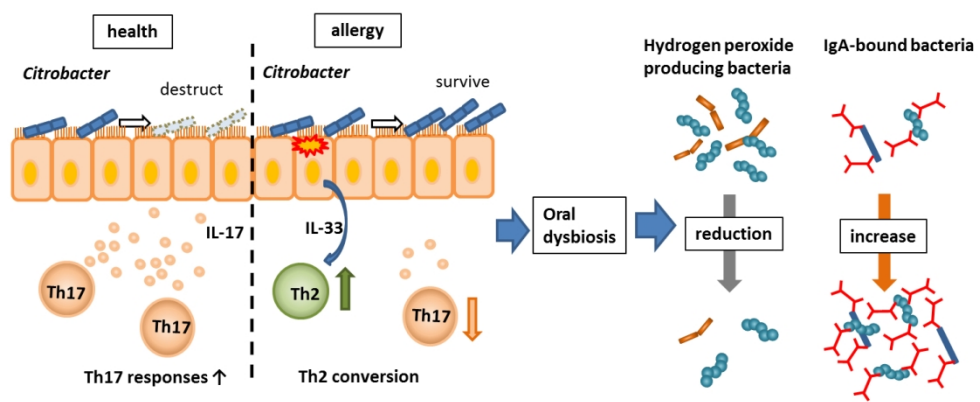
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Supporting Information

Graphical Abstract. *Citrobacter* sp. in the gastrointestinal tract is eliminated by Th17

responses. Th2 responses induced by food allergy lead to insufficient elimination of

Citrobacter sp. These responses changed the bacterial compositions of both the oral and

gastrointestinal microbiota. The induced oral dysbiosis reduced hydrogen

peroxide-producing bacterium counts and increased IgA in the oral cavity.

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