

Oral Administration of Heat-Killed *Lactobacillus gasseri* OLL2809 Reduces Cedar Pollen Antigen-Induced Peritoneal Eosinophilia in Mice

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ABSTRACT

Background: *Lactobacillus gasseri* OLL2809 strongly stimulates the production of interleukin (IL)-12 (p70) by innate immune cells. Thus, it is expected to ameliorate allergic diseases. We investigated whether the oral administration of heat-killed *L. gasseri* OLL2809 suppressed eosinophilia in cedar pollen antigen-challenged mice.

Methods: BALB/c mice sensitized with Japanese cedar pollen extract were intraperitoneally challenged with the same extract. The mice were orally given heat-killed *L. gasseri* OLL2809 at doses of 0.5, 1, or 2 mg/day throughout the experimental period (21 d). After 24 hours of the challenge, the eosinophil number and cytokine levels in the peritoneal lavage fluid and the serum antigen-specific IgG levels were determined.

Results: On administering varying amounts of heat-killed *L. gasseri* OLL2809, the number of eosinophils among the total number of cells was significantly reduced in all groups. In addition, the eosinophil number significantly decreased, and the eosinophil-suppression rate significantly increased by 44% in the 2-mg group. Although the serum immunoglobulin (Ig) G2a and IgG1 levels were not affected, the IgG2a/IgG1 ratio increased significantly in the 2-mg group compared with that of the control group. Furthermore, the administration of heat-killed *L. gasseri* OLL2809 resulted in the induction of IL-2 and reduction in granulocyte-macrophage colony-stimulating factor levels in peritoneal lavage fluid.

Conclusions: We demonstrated that the oral administration of heat-killed *L. gasseri* OLL2809 suppresses eosinophilia via the modulation of Th1/Th2 balance. These observations suggested that heat-killed *L. gasseri* OLL2809 might potentially ameliorate the increased number of eosinophils in patients with Japanese cedar pollinosis.

KEY WORDS

eosinophil, *Lactobacillus gasseri*, probiotics, Th1/Th2 balance

INTRODUCTION

In the past decade, the number of patients suffering from allergic diseases such as atopic dermatitis, allergic asthma, and allergic rhinitis has increased in many countries. In Japan, in particular, approximately

15% of the population is reported to suffer from cedar pollinosis.¹ Cedar pollinosis significantly reduces the quality of life of patients and is therefore becoming a matter of serious concern in Japan.² While immediate hypersensitivity, including pollinosis, is generally characterized by an elevation in the immunoglobulin

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(Ig) E levels,³ local or systemic increase in the number of eosinophils rather than increased IgE levels is related to the severity of the symptoms.^{4,6} Eosinophils act as the effector cells, not only by functioning as antigen-presenting cells⁷ but also by producing various cytokines/chemokines^{8,9}; they penetrate and injure the local mucosa and produce chemical mediators such as major basic proteins, eosinophilic cationic proteins, and leukotrienes.^{10,11}

Local and systemic increase in the eosinophil number has been attributed to a shift in the balance between type I helper T (Th1) cells and type II helper T (Th2) cells.¹²⁻¹⁴ Th1 cells produce cytokines such as interferon-gamma (IFN- γ) to promote cellular immunity, while Th2 cells produce cytokines such as interleukin (IL)-4 and IL-5 to promote humoral immunity through IgE production.¹⁵ IL-5, which is produced by Th2 cells as well as by eosinophils in an autocrine fashion,¹⁶ contributes to cell differentiation, proliferation, and maturation of eosinophils, thereby stimulating eosinophilic inflammation.¹⁷ It could therefore be possible to suppress not only IgE production but also eosinophilic inflammation by shifting the Th1/Th2 balance from Th2-dominant immunity toward Th1-dominant immunity.

Probiotics are functional foods containing potentially beneficial microorganisms.^{18,19} Because they exert various health-promoting effects, including the amelioration of irritable bowel disease, constipation, and diarrhea, animal and clinical studies on probiotics have been conducted extensively.²⁰ For example, some strains of lactic acid bacteria, often utilized as probiotics, exhibit immunostimulatory effects in the host even when administered as heat-killed organisms.^{21,22} *Lactobacillus gasseri* OLL2809 is one of the probiotic lactobacilli that have been selected from 273 strains isolated from humans on the basis of their immunostimulatory activity. We previously reported that the oral administration of heat-killed *L. gasseri* OLL2809 effectively reduces serum antigen-specific IgE levels in mice via the stimulation of IL-12 (p70) production in splenocytes.^{23,24} Moreover, it shifts the Th1/Th2 balance from Th2-dominant immunity toward Th1-dominant immunity. These effects are more prominent in the case of *L. gasseri* OLL2809 even when compared with other lactobacilli, including their type strains.²³ Therefore, it is expected that heat-killed *L. gasseri* OLL2809 suppressed the increase in the accumulation of eosinophils stimulated by antigen challenge.

In this study, we investigated whether the oral administration of heat-killed *L. gasseri* OLL2809 suppressed the accumulation of eosinophils stimulated by Japanese cedar pollen extract.

METHODS

MICROORGANISM

In this study, we used *L. gasseri* OLL2809, which

was isolated from the feces of Japanese subjects and previously selected on the basis of its immunostimulatory activity.²³ The organism was cultured in Lactobacilli MRS broth (Becton Dickinson, Sparks, MD) at 37°C for 18 hours. After fermentation, the cells were harvested in a refrigerated centrifuge (10,000 \times g, 15 minutes) and washed twice with saline solution followed by 1 wash with water. The cells were resuspended in distilled water, heat-killed at 75°C for 60 minutes, and lyophilized.

MICE

Five-week-old specific-pathogen-free female BALB/c mice (9 or 10 per group) were purchased from Japan SLC (Shizuoka, Japan) and maintained on a standard diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan). The experimental protocols were approved by the Animal Care Committee of the Division of Research and Development, Meiji Dairies Corporation.

EXPERIMENTAL SCHEDULE

The animal model was prepared according to the procedure of Kaneko *et al.*,²⁵ with minor modifications. In brief, BALB/c mice were sensitized with an extract of Japanese cedar pollen which was diluted 50-fold with saline (pH 7.2). We subcutaneously injected 0.2 ml of the diluted extract, which contained a final concentration of 0.2 μ g/ml Cry j 1, one of the major antigens in Japanese cedar pollen, into the dorsal portion of the mice on days 0, 1, 4, 6, 8, and 14 (Fig. 1). On day 20, the mice were challenged with an intraperitoneal injection of 0.2 ml of the same diluted extract. Normal (non-sensitized) mice were prepared with subcutaneous injection of saline instead of the cedar pollen extract and were challenged in the same manner as described above. Throughout the experimental period (21 days), the mice were orally given heat-killed *L. gasseri* OLL2809 by gastric gavage at doses of 0.5, 1, or 2 mg/body/day (5×10^8 cells/mg) in 0.5 ml distilled water. Mice in both the normal and control (sensitized and challenged with an extract of Japanese cedar pollen) groups were given only distilled water.

The mice were anaesthetized with diethyl ether 24 hours after the challenge; sera were collected from the tail veins to determine antigen-specific IgG levels. The mice were then sacrificed by cervical dislocation, and peritoneal cells were harvested to count the accumulated eosinophils.

EOSINOPHIL COUNT

Peritoneal cells were harvested from the peritoneal lavage fluid after injecting 5 ml of phosphate-buffered saline (PBS; pH 7.2) containing 1.0% fetal calf serum (Intergen, Purchase, NY). On a microscope slide, 50 μ l peritoneal cell suspension that was adjusted to a cell concentration of approximately 2×10^5 cells/ml was smeared using Cytospin 2 apparatus (Shandon,

Lactobacillus Reduces Eosinophilia

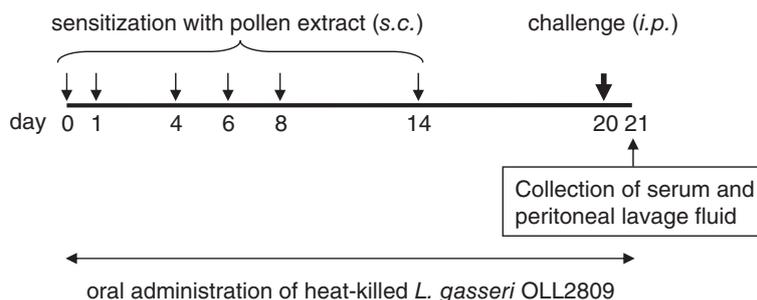


Fig. 1 Experimental schedule for the preparation of Japanese cedar pollen antigen-induced peritoneal eosinophilia in BALB/c mice. Sensitization was performed by injecting an extract of Japanese cedar pollen in the dorsal portion of mice. Normal (non-sensitized) mice were injected with saline instead of the cedar pollen extract.

Table 1 Effect of oral administration of heat-killed *Lactobacillus gasseri* OLL2809 on peritoneal eosinophilia

Groups	<i>n</i>	Total cells ($\times 10^5$ cells/ml)	Eosinophils ($\times 10^5$ cells/ml)	Relative proportion of Eosinophils (%)	Eosinophil- suppression rate (%)
Normal	10	8.5 \pm 1.2	0.18 \pm 0.05	1.9 \pm 0.3	100 \pm 1
Control	9	16.7 \pm 2.1**	4.30 \pm 0.70**	25.5 \pm 1.6**	0 \pm 17**
0.5 mg	10	18.7 \pm 2.1	3.29 \pm 0.54	17.2 \pm 2.0##	25 \pm 13
1 mg	10	19.4 \pm 1.6	3.35 \pm 0.35	17.6 \pm 1.9#	23 \pm 9
2 mg	10	15.8 \pm 1.7	2.48 \pm 0.35#	16.2 \pm 1.6##	44 \pm 9#

Data are expressed as mean \pm SEM.

Cell counts indicate cell numbers in the collected peritoneal lavage fluid.

Relative proportion of eosinophils represents the number of eosinophils among the total number of cells.

** : $p < 0.01$, as compared with the normal group by using Student's *t* test.

#, ##: $p < 0.05, 0.01$, respectively, as compared with the control group by using Dunnet's multiple comparison tests.

The same experiment was performed twice, producing similar results, and 1 representative result is shown.

Pittsburgh, PA) at 700 rpm for 1 minute. After fixation and staining with Microscopy Hemacolor (Merck kGaA, Darmstadt, Germany), eosinophils were counted under a microscope. The eosinophil-suppression rate was calculated as follows: eosinophil suppression rate (%) = $\{1 - (\text{number of eosinophils in individual mice in each group} - \text{mean eosinophil number in the normal group}) / (\text{mean eosinophil number in the control group} - \text{mean eosinophil number in the normal group})\} \times 100$.

MEASUREMENT OF CYTOKINES AND Cry j 1-SPECIFIC IgG1, IgG2a, AND IgE LEVELS

The concentrations of 8 cytokines in the peritoneal lavage fluid were simultaneously determined using Mouse Cytokine Th1/Th2 Panel (Bio-Rad, Hercules, CA) and Bio-Plex 200 system (Bio-Rad). Transforming growth factor (TGF)- β 1 levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Promega, Mannheim, Germany). The detection limit of TGF- β 1 was 15.6 pg/ml. We strictly followed the procedures recommended by the manufacturer.

Serum Cry j 1-specific IgG1, IgG2a, and IgE levels were measured, as described by Kozutsumi *et al.*²⁶

STATISTICAL ANALYSIS

Data were expressed as mean \pm standard error of the mean (SEM). Statistical differences between the normal and control groups were analyzed with Student's *t* test or Mann-Whitney's U test. Differences between the control group and the groups given heat-killed *L. gasseri* OLL2809 were analyzed with Dunnet's multiple comparison tests. The differences were considered significant when the *p* value was less than 0.05.

RESULTS

EFFECT OF *L. gasseri* OLL2809 ON PERITONEAL ACCUMULATION OF EOSINOPHILS

We first examined the effect of sensitization with Japanese cedar pollen extract on the accumulation of cells in the peritoneal cavity. As summarized in Table 1, the total number of cells and eosinophils in the normal group was $8.5 \pm 1.2 \times 10^5$ and $0.18 \pm 0.05 \times 10^5$ cells/ml respectively, while that in the control group was $16.7 \pm 2.1 \times 10^5$ and $4.30 \pm 0.70 \times 10^5$ cells/ml re-

Table 2 Effect of oral administration of heat-killed *Lactobacillus gasseri* OLL2809 on serum anti-Cry j 1-specific IgG1, serum anti-Cry j 1-specific IgG2a (pg/ml) and their ratio

Groups <i>n</i>	Normal 10	Control 9	<i>Lactobacillus gasseri</i> OLL2809		
			0.5 mg 10	1 mg 10	2 mg 10
IgG2a	221 ± 29	168 ± 28	197 ± 2	203 ± 3	226 ± 61
IgG1	138 ± 21	219 ± 33	140 ± 2	144 ± 2	153 ± 8
IgG2a/IgG1	1.39 ± 0.01	0.75 ± 0.02*	1.40 ± 0.01	1.42 ± 0.03	1.81 ± 0.46#

Data are expressed as mean ± SEM.

*: $p < 0.05$, as compared with the normal group by using Student's *t* test.

#: $p < 0.05$, as compared with the control group by using Dunnett's multiple comparison tests.

The same experiment was performed twice, producing similar results, and 1 representative result is shown.

spectively. Thus, the total number of cells, the number of eosinophils, and the relative proportion of eosinophils in the control group increased significantly ($p < 0.01$) as compared with the findings in the normal group. The relative proportion of eosinophils increased from $1.9 \pm 0.3\%$ in the normal group to $25.5 \pm 1.6\%$ in the control group. It could therefore be confirmed that the eosinophil-accumulation model was successfully prepared.

When varying amounts of heat-killed *L. gasseri* OLL2809 were given to the eosinophilia-induced, the relative proportion of eosinophils reduced significantly ($p < 0.05$ or 0.01) in all groups compared with that of the control group. While there was no difference in the total number of cells in the groups subjected to allergen sensitization and challenge, the eosinophil number decreased significantly in the 2-mg group compared with that of the control group, and the eosinophil-suppression rate was significantly increased by 44%.

These results indicated that the oral administration of heat-killed *L. gasseri* OLL2809 to the cedar pollen-challenged mice reduced eosinophil accumulation in the peritoneal cavity.

EFFECT OF *L. gasseri* OLL2809 ON SERUM ANTI-Cry j 1-SPECIFIC IgG2a/IgG1 RATIO

To further investigate the effects administering of heat-killed *L. gasseri* OLL2809, the levels of serum anti-Cry j 1-specific IgG1 and anti-Cry j 1-specific IgG2a were measured. No distinct differences could be observed in these levels even between the normal and control groups (Table 2). However, the ratio between the IgG2a and IgG1 levels was significantly ($p < 0.05$) lower in the control group than in the normal group. On the other hand, this ratio was significantly higher ($p < 0.05$) in the 2-mg group. These data clearly demonstrated that in the eosinophilia-induced mice the administration of heat-killed *L. gasseri* OLL2809 promoted a shift in the systemic Th1/Th2 balance from Th2-dominant immunity toward Th1-dominant immunity. The serum anti-Cry j 1-specific

IgE levels were under the detection limit in all groups.

EFFECT OF *L. gasseri* OLL2809 ON CYTOKINE LEVELS IN PERITONEAL LAVAGE FLUID

We determined the serum levels of cytokines related to Th1/Th2 balance, such as those of IL-2, IL-4, IL-5, IL-10, IL-12 (p70), IFN- γ , TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF), and TGF- β 1. Although IL-5 and GM-CSF levels significantly ($p < 0.05$) increased in the control group compared with those in the normal group, no significant changes were found in the groups given heat-killed *L. gasseri* OLL2809 when compared with the control group (data not shown).

We have therefore analyzed cytokine levels in peritoneal lavage fluid. In contrast to serum cytokine levels, while most cytokine levels in the peritoneal lavage fluid in the normal group were under the detection limit, they were significantly increased in the control group except for TGF- β 1 (Table 3). This observation suggested that not only eosinophils but also other mononuclear cells, including both Th1 and Th2 cells, migrated to local inflammatory sites and were activated by the intraperitoneally injected antigens.

Oral administration of heat-killed *L. gasseri* OLL2809 resulted in the induction of a Th1-derived cytokine, specifically, IL-2 which was observed in the 0.5- and 1-mg groups. On the other hand, the levels of GM-CSF, which enhances the proliferation of eosinophils,²⁷ were lower in the 1- and 2-mg groups than in the control group. In particular, the GM-CSF level showed a positive correlation to the number of eosinophils in the peritoneal lavage fluid; and the correlation was statistically significant (Fig. 2A, $p < 0.01$). Although the change in IL-5 levels was not statistically significant, it was comparable to that of the GM-CSF levels since the IL-5 levels decreased as the amount of the administered *L. gasseri* OLL2809 increased. Moreover, these levels were positively correlated with the eosinophil number as well as the GM-CSF level (Fig. 2B, $p < 0.01$). TGF- β 1 was not de-

Table 3 Effect of oral administration of heat-killed *Lactobacillus gasseri* OLL2809 on the production of cytokines in the peritoneal lavage fluid

Groups	Normal	Control	<i>Lactobacillus gasseri</i> OLL2809		
			0.5 mg	1 mg	2 mg
<i>n</i>	10	9	10	10	10
IL-2	2.2 ± 0.7	8.0 ± 0.8**	12.7 ± 0.6#	11.3 ± 0.8#	9.7 ± 1.0
IL-4	n.d.	48.4 ± 15.3**	40.9 ± 14.8	21.7 ± 3.5	29.3 ± 13.3
IL-5	n.d.	173 ± 42**	184 ± 57	114 ± 33	76 ± 8
IL-10	n.d.	12.2 ± 1.5**	14.3 ± 3.1	13.6 ± 1.3	10.2 ± 2.5
IL-12 (p70)	2.5 ± 0.9	17.5 ± 2.7**	24.1 ± 5.5	36.1 ± 14.5	20.3 ± 3.0
GM-CSF	n.d.	40.6 ± 7.0**	29.1 ± 8.5	15.7 ± 6.4#	10.5 ± 3.4#
IFN-γ	n.d.	5.04 ± 2.34**	1.34 ± 1.17	8.65 ± 8.65	n.d.
TNF-α	4 ± 2	75 ± 12**	100 ± 14	104 ± 27	54 ± 6
TGF-β1	n.d.	n.d.	n.d.	n.d.	n.d.

Data are expressed as mean ± SEM (pg/ml).

n.d.: not detected.

***p* < 0.01, as compared with the normal group by using Mann-Whitney's U test.

#*p* < 0.05, as compared with the control group by using Dunnett's multiple comparison tests.

The same experiment was performed twice, producing similar results, and 1 representative result is shown.

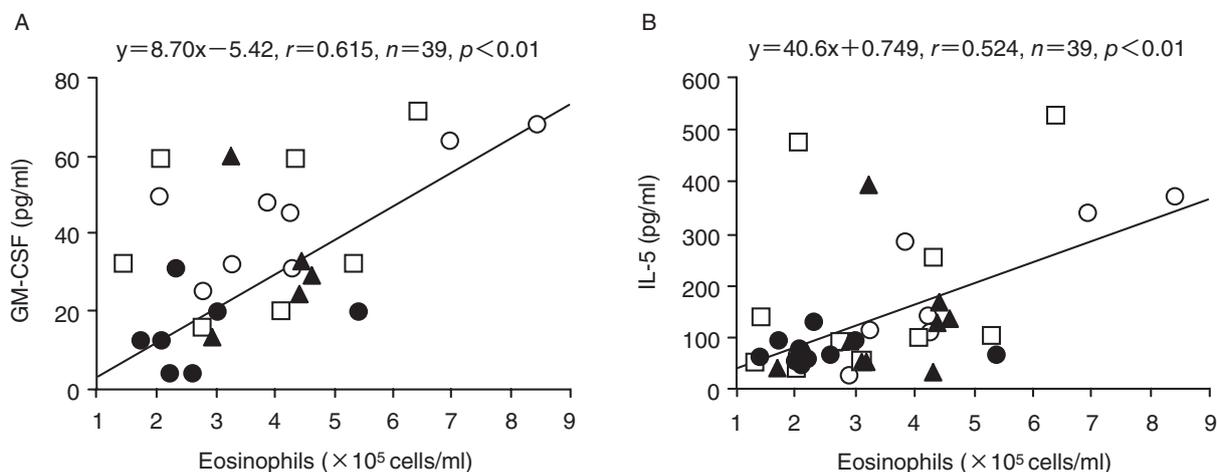


Fig. 2 Correlation between the number of eosinophils and GM-CSF (A) or IL-5 (B) levels in peritoneal lavage fluid. Symbols: ○, Control; □, 0.5 mg; ▲, 1 mg; and ●, 2 mg groups.

tected in any of the mice examined in this study.

In general, these cytokine-expression profiles strongly suggested that the suppression of eosinophilia in peritoneal lavage fluid was associated with the local Th1/Th2 balance.

DISCUSSION

We previously reported that the oral administration of heat-killed *L. gasseri* OLL2809 to ovalbumin-sensitized mice reduced the serum ovalbumin-specific IgE levels, which was associated with the enhancement of IL-12 (p70) production in splenocytes and the reduction of IL-4 production in both splenocytes and mesenteric lymph node cells.²³ In this study, we demonstrated that heat-killed *L. gasseri* OLL2809 sup-

pressed not only antigen-specific IgE levels, as reported previously,²³ but also the local accumulation of eosinophils in mice sensitized with Japanese cedar pollen extract. In addition, this effect was found to be associated with a shift in the local and systemic Th1/Th2 balance, as demonstrated by cytokine levels in the peritoneal lavage fluid and the serum-Cry j 1-specific IgG2a/IgG1 ratio, respectively.

Eosinophils from patients with allergies, such as asthma, exhibit increased migratory responses, adhesiveness, and degranulation compared with those from normal subjects.²⁷ The priming of the activation of eosinophils is caused by IL-5 and GM-CSF.²⁷⁻²⁹ On the other hand, IL-2, which has been widely known to aid T cell proliferation and clonal expansion,³⁰ has an

inhibitory effect on the migration of eosinophils toward chemotactic factors.^{31,32} Therefore, the cytokine-expression profiles observed in the peritoneal lavage fluid appeared to correspond to the serum-Cry j 1-specific IgG2a/IgG1 ratio and the eosinophil number. Heat-killed *L. gasseri* OLL2809 strongly stimulate the production of IL-12 (p70) by murine macrophages and dendritic cells^{23,24} and was therefore expected to induce the proliferation of Th1 cells from naïve T cells, consequently affecting the Th1/Th2 balance. However, both IL-2 and GM-CSF are secreted by various cells other than Th1 and Th2 cells, including eosinophils, in an autocrine fashion⁹; therefore we could not determine whether the suppression of eosinophilia was caused by the modification of these cytokine levels or as a consequence of a reduction in eosinophil number due to other factors.

Recently, numerous studies have indicated that regulatory T cells are strongly associated with allergic disorders.³³ Regulatory T cells are subsets of T cells and are involved in the maintenance of peripheral self-tolerance by actively suppressing the activation and proliferation of autoreactive T cells.³³ Several types of regulatory T cells have been characterized; Th3 and Tr1 cells have been implicated in regulatory T cell function through the production of immunosuppressive cytokines such as TGF- β and IL-10.³³ As shown in this study, the levels of nearly all cytokines in the peritoneal lavage fluid were significantly higher in the control group than in the normal group. This clearly indicates that not only Th2 cells but also Th1 cells were attracted to the inflammatory site and were activated by sensitization with the Japanese cedar pollen antigens. However, since TGF- β 1 levels in the peritoneal lavage fluid were under the detection limit, and no obvious influence could be seen even in the normal and control groups, and in addition, IL-10 levels were not affected by the administration of heat-killed *L. gasseri* OLL2809, it can therefore be stated that the results of this experiment do not implicate regulatory T cells in the reduction of eosinophilia.

The effective component in *L. gasseri* OLL2809 has not been isolated and therefore much remains controversial. Nevertheless, peptidoglycan is likely to be one of the most effective components, as reported previously.^{23,24} The effectiveness of microbial peptidoglycan has been reported even in epidemiological research since it has been found to be inversely associated with the prevalence of allergic disorders.³⁴ Recently, it is becoming clear that commensal microflora are recognized by toll-like receptors (TLRs) expressed on the intestinal epithelial cells, including microfold (M) cells in Peyer's patches.^{35,36} Signaling cascades are initiated via TLRs to activate natural immune responses and induce the production of cytokines such as TNF- α and IL-12 (p70).^{37,38} It can therefore be speculated that the administered heat-killed *L. gasseri* OLL2809 was incorporated into these im-

mune cells at specific sites, inducing immunomodulatory activity, as shown in this study. Further studies are required to investigate the effective component of *L. gasseri* OLL2809 and to discuss this issue in detail.

In conclusion, we have shown that the oral administration of heat-killed *L. gasseri* OLL2809 suppresses the peritoneal accumulation of eosinophils in mice sensitized with Japanese cedar pollen extract. This effect was the result of a shift in the balance between Th1 and Th2 cells. These observations suggest that heat-killed *L. gasseri* OLL2809 can potentially ameliorate the clinical symptoms of Japanese cedar pollinosis. However, in the allergic animal model used in this study, sensitization and antigen challenge were not performed in the nasal mucosa, where both the inductive and effector phases of immune reaction occur in pollinosis; which was conducted to clearly observe the effect of *L. gasseri* OLL2809. In addition, the above mentioned methods did not induce allergic symptoms such as scratching and sneezing. In our next study, we hope to determine whether heat-killed *L. gasseri* OLL2809 ameliorates allergic symptoms in allergic rhinitis animal models and investigate in further detail the mechanism by which it affects Th1/Th2 balance.

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