

Effect of oral feeding with *Clostridium leptum* on regulatory T-cell responses and allergic airway inflammation in mice

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ABSTRACT

Background: Allergic lung inflammation is mediated by allergen-specific T responses, which are negatively regulated by regulatory T cells (Tregs). Previous studies have reported that inoculation of indigenous *Clostridium* species in the early lives of mice can induce Tregs that colonize the colon. However, whether inoculation of *C leptum* alone in adult mice could induce systemic Treg responses and inhibit allergic airway inflammation remains unclear.

Objective: To investigate the effect of oral administration of *C leptum* on systemic Treg responses and allergic airway inflammation in a mouse model of asthma.

Methods: Adult BABL/c mice were injected with ovalbumin to induce asthma and treated orally with *C leptum* or vehicle daily for 2 weeks. The numbers of Foxp3⁺CD4⁺CD25⁺ Tregs in both the spleen and mediastinal lymph nodes were examined by flow cytometry. After allergen challenge, the airway hyperresponsiveness of individual mice was measured, and the numbers of inflammatory infiltrates and the levels of cytokines in bronchoalveolar lavage fluids were determined.

Results: Oral feeding with *C leptum* increased the percentage and total number of Tregs in the spleens and mediastinal lymph nodes at 14 days after inoculation and attenuated allergen-induced airway hyperresponsiveness and inflammation by inhibiting inflammatory cytokine production but enhancing interleukin 10 and transforming growth factor β 1 production in the lungs.

Conclusion: Oral treatment with *C leptum* can attenuate induced allergic airway inflammation in adult mice.

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Introduction

Asthma is an inflammatory disease caused by allergen-specific T cells, such as T_H2, T_H1, T_H17, and T_H9, but negatively regulated by regulatory T cells (Tregs). T_H2 cells can secrete interleukin (IL) 4, IL-5, and IL-13 and other cytokines that recruit and activate eosinophils and neutrophils and cause pulmonary inflammation, mucous hypersecretion, and airway hyperresponsiveness.¹ T_H17 with IL-17, T_H1 with interferon γ (IFN- γ), and T_H9 with IL-9 are involved in asthmatic pathogenesis.^{2–9} During the past several decades, the incidence of asthma has been increasing significantly in the world. Notably, Tregs can suppress T_H2, T_H1, T_H17, and T_H9 responses by secreting inhibitory cytokines, such as IL-10 and transforming growth factor β (TGF- β).¹⁰ Previous studies^{11,12} have reported that the number and function of Tregs is impaired or altered in allergic patients compared with that in healthy individuals. Therefore, development of therapeutic strategies to increase the numbers and

function of Tregs may be highly promising for the intervention of allergic disease.¹³

Interestingly, previous studies have shown that modulation of the gastrointestinal microbiota composition and oral administration of certain organisms can modulate immune responses in the airway.^{14–18} Treatment with heat-killed nonpathogenic *Mycobacterium vaccae* can induce Tregs that protect against allergic airway inflammation in vivo.¹⁹ Colonization of mice with human *Bacteroides fragilis* facilitates Treg cell differentiation and IL-10 production.²⁰ Other selective bacteria have also been shown to induce IL-10-producing Tregs by modulating dendritic cells in vitro.²¹ *Clostridium* species is one of the most prominent gram-positive and spore-forming bacteria, and increased colonization of *Clostridium* is associated with the protection against wheezing.²² Recent studies have found that inoculation of indigenous *Clostridium* species in the early lives of mice can induce Tregs that colonize the colon and subsequently migrate into the lung, liver, spleen, and other tissues, inhibiting toxicant-induced intestinal inflammation and allergen-specific IgE responses.^{23–25} *Clostridium leptum* and *Clostridium cocoides* can promote anti-inflammatory immune responses by expanding and activating Tregs, in accord with up-regulating Foxp3

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expression, a key transcription factor in programming the differentiation of Tregs.¹¹ *C leptum* is the highest population in the intestinal tract and accounts for a mean (SD) of approximately 16% (7%) of the total number of intestinal bacteria.²⁶ However, whether oral inoculation with *C leptum* alone in adult mice could induce systemic Treg response and modulate allergic airway inflammation has not been explored.

In this study, we used a well-known asthmatic mouse model to examine the effect of oral administration of *C leptum* on inducing Treg response and allergic inflammation and to determine the potential mechanisms by which this therapeutic strategy modulates airway inflammation. We found that oral administration of *C leptum* alone in adult mice induced vigorous Treg responses and inhibited airway hyperresponsiveness.

Materials and Methods

C leptum Preparation

The *C leptum* were purchased from Jilin Baoxin Biological Technology, Changchun, China. The strain was isolated from a human feces sample and stored at -80°C . A single colony of *C leptum* was grown in chopped meat broth for 24 hours and harvested by centrifugation, followed by washing with phosphate-buffered saline (PBS). The freshly prepared bacteria in PBS were used for oral feeding.

Animals

Female BALB/c mice at 6 to 8 weeks of age and 18 to 22 g were obtained from the Animal Research Center of Jilin University and housed in a specific pathogen-free facility in a light/dark cycle with free access of water and chow ad libitum. The experimental protocols were established, according to the guidelines of the National Institutes of Health Animal Research and Care, and were approved by the ethics committee of Jilin University.

Induction of asthma and grouping

A mouse model of asthma was induced, as described previously,²⁷ with minor modifications. Briefly, BALB/c mice were sensitized by intraperitoneal injection with 20 μg of ovalbumin (OVA; grade V, Sigma, St Louis, Missouri) and 200 μg of aluminum hydroxide (Alum) in 100 μL of PBS on day 0 and boosted with the same reagent on day 13. Another group of mice were injected with PBS and used as controls. On day 14, the sensitized mice were randomized and fed with 10^9 colonies of *C leptum* in 200 μL of PBS or vehicle alone by gavage daily for 14 consecutive days. As a result, there were 24 mice in each of the *C leptum*-fed OVA-sensitized group (OVA-CL group), vehicle-fed OVA-sensitized group (OVA group), and 18 mice in each of the *C leptum*-fed PBS-sensitized group (PBS-CL group) and vehicle-fed PBS-sensitized group (PBS group), respectively. Some mice from each group were killed on days 14, 20, and 27, and their spleens, mediastinal lymph nodes, and lung tissues were collected for the experiments. On days 28, 29, and 30, the mice were challenged with 1% of aerosolized OVA or PBS for 20 minutes, respectively. On day 32, the remaining mice were killed and subjected to bronchoalveolar lavage, followed by collection of their lungs.

Measurement of airway responsiveness

Two days after the final OVA challenge, airway responsiveness to increased concentrations (6–100 mg/mL) of aerosolized methacholine in individual unrestrained and conscious mice was measured by whole-body plethysmograph (Buxco Electronics, Troy, New York) to determine changes in enhanced respiratory pause.^{28,29} The methacholine solutions were nebulized through an inlet of the main chamber for 3 minutes, and enhanced respiratory pause was measured for 5 minutes. Data are expressed as the

percentage increase of enhanced respiratory pause after challenge with each concentration of methacholine, and the baseline enhanced respiratory pause was designated as 100%.

Bronchoalveolar lavage

Two days after the final OVA challenge, individual mice were subjected to bronchoalveolar lavage with 3 aliquots of 250 μL of PBS through a tracheal cannula. The collected bronchoalveolar lavage fluid (BALF) samples were centrifuged at 4°C , 600g for 10 minutes, and the supernatants were stored at -70°C . The cells in individual BALF samples were stained with Trypan blue for determining the number of viable cells. Aliquots of BALF were applied to glass slides for Wright staining. The numbers of macrophages, eosinophils, lymphocytes, and neutrophils in 200 cells per BALF sample were counted in a masked manner.

Histologic analysis

The lung tissues were fixed in 10% formalin overnight and paraffin embedded. The tissue sections (5 μm) were stained with hematoxylin-eosin for histologic assessments under a light microscope in a masked manner.

Flow cytometry analysis

Single cell suspensions from spleen and mediastinal lymph nodes were prepared, as described by Gunzer et al.³⁰ $\text{CD}3^{+}$ T cells were enriched by nylon wool column,³⁰ and individual preparations of $\text{CD}3^{+}$ T cells with a purity of 87% or higher were used for flow cytometry analysis using the Foxp3 staining set (eBioscience, San Diego, California), according to the manufacturers' instruction. $\text{CD}3^{+}$ T cells at 2×10^6 cells/mL were stained with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated anti-CD25 or isotype controls. The cells were fixed, permeabilized, and stained with PE-Cy5-conjugated anti-Foxp3, followed by flow cytometry analysis. The frequency of $\text{CD}4^{+}\text{CD}25^{+}\text{Foxp}3^{+}$ Tregs in individual samples was calculated.

Western blot

Lung tissues were homogenized and lysed, and tissue lysates (50 μg per lane) were separated on 10% gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transferring onto nitrocellulose membranes. Subsequently, the membranes were blocked with 5% of fat-free milk in Tris-buffered saline and Tween 20 and incubated with goat anti-mouse Foxp3 (1:50 dilution) or goat anti-mouse β -actin antibodies (1:300 dilution; Santa Cruz Biotechnology, Santa Cruz, California) at 4°C overnight. The bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-goat or anti-mouse IgG (1:800 dilution; Santa Cruz Biotechnology) and visualized using the enhanced chemiluminescent (Santa Cruz Biotechnology), followed by optical density analysis.

Enzyme-linked immunosorbent assay

The concentrations of IL-4, IL-5, IL-13, IL-10, TGF- β 1, IFN- γ , tumor necrosis factor α (TNF- α), IL-2, IL-6, IL-9, IL-17A, IL-21, and IL-23 in BALF were measured by enzyme-linked immunosorbent assay using cytokine-specific kits, according to the manufacturer's instructions (Invitrogen, Grand Island, New York).

Statistical analysis

Data are expressed as mean (SEM). The difference between groups was statistically analyzed by the Mann-Whitney test using a statistics program (SPSS statistical software, version 13.0; SPSS Inc, Chicago, Illinois). $P < .05$ was considered statistically significant.

Results

Oral feeding with *C leptum* and Treg responses in mice

To investigate whether oral treatment with *C leptum* alone could induce systemic Treg responses, adult female BALB/c mice were fed *C leptum* or vehicle PBS for 14 days and the numbers of splenic mononuclear cells, CD3⁺ T cells, and CD4⁺CD25⁺Foxp3⁺ Tregs were evaluated at successive time points. No significant difference was found in the numbers of splenic mononuclear cells and CD3⁺ T cells between the PBS and PBS-CL groups of mice at 1, 7, and 14 days after inoculation (data not shown). Although no significant difference was found in the numbers and frequency of Tregs between the PBS and PBS-CL groups of mice at 1 and 7 days after inoculation, the numbers and frequency of Tregs in the PBS-CL group of mice at 14 days after inoculation were significantly greater than that in the PBS group of mice (Fig 1). These data clearly indicated that oral feeding with *C leptum* induced systemic Treg responses in mice.

Next, we examined whether oral feeding with *C leptum* alone could also induce Treg responses in the mice that had been sensitized with an allergen. BALB/c mice were sensitized with OVA/Alum twice and orally fed with *C leptum* or control PBS for 14 days, and the numbers of Tregs in the spleen and mediastinal lymph nodes were measured. Clearly, the numbers of Tregs in the spleen (Fig 2A) and mediastinal lymph nodes (Fig 2B) of the OVA-CL group of mice were significantly greater than that in the OVA group of mice at 14 days ($P < .05$ for both), but not at one 1 and seven 7 days post after inoculation (data not shown). Further characterization of Foxp3 expression revealed that similar levels of Foxp3 expression were detected in the lungs of both groups of mice at one 1 and seven 7 days post after inoculation (Fig 2C and 2D). In contrast, the levels of Foxp3 expression in the lungs of the OVA-CL group of mice were significantly higher than that in the OVA group of mice at 14 days post after inoculation ($P < .05$). Apparently, oral feeding with *C leptum* induced systemic Treg responses, even after allergen sensitization in mice.

Oral feeding with *C leptum* and allergic airway hyperresponsiveness

Allergic inflammation can cause airway hyperresponsiveness. To determine the effect of *C leptum* feeding on allergic airway hyperresponsiveness, BALB/c mice were sensitized with OVA/Alum twice, randomized, and fed orally with vehicle PBS alone or *C leptum* daily for 14 days. The mice were challenged with aerosolized OVA, and 2 days later, their airway responses to increased concentrations of methacholine were measured by whole-body plethysmography (Fig 3). The airway responsiveness to increased concentrations of methacholine in the OVA group of mice gradually increased and was nearly 4-fold higher than that in the PBS group of mice. In contrast, the airway responsiveness to increased concentrations of methacholine in the OVA-CL group of mice was similar to that in the PBS group of mice and was significantly reduced compared with that in the OVA group of mice. Hence, oral feeding with *C leptum* attenuated allergic airway hyperresponsiveness in asthmatic mice.

Oral feeding with *C leptum* and allergic lung inflammation in mice

We further examined the effect of oral administration of *C leptum* on allergic lung inflammation in mice. Two days after allergen challenge, the lung tissue sections from individual mice were examined by hematoxylin-eosin staining. As shown in Figure 4A, airway epithelium hyperemia and edema, many pulmonary goblet cells, and inflammatory infiltrates were observed in the lungs of the OVA group of mice but not in the PBS group of mice. There were obvious lower degrees of airway epithelium hyperemia and edema, fewer pulmonary goblet cells, and inflammatory infiltrates in the lungs of the OVA-CL group of mice.

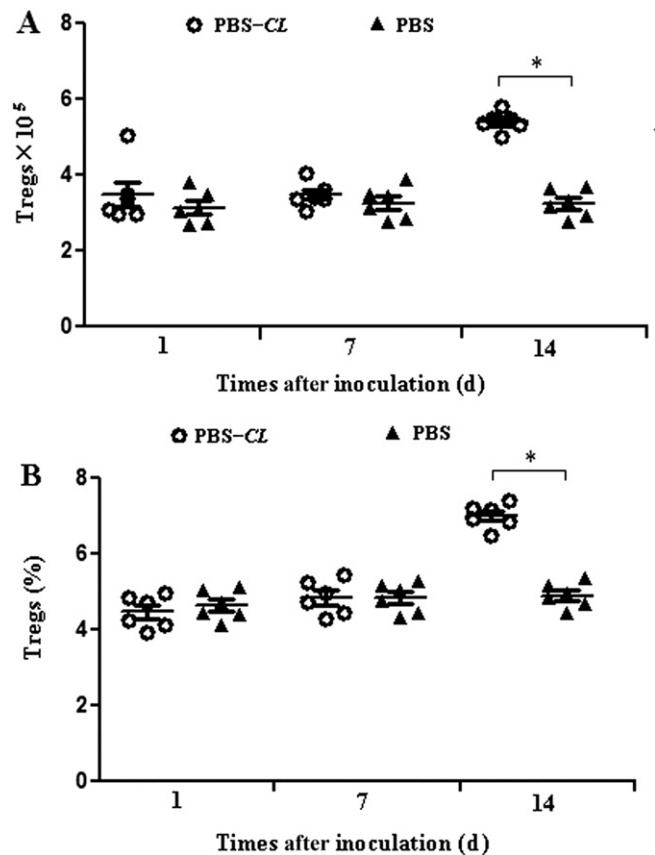


Fig. 1. Oral feeding with *Clostridium leptum* induces regulatory T-cell (Treg) responses in adult mice. BALB/c mice at 6 to 8 weeks of age were fed with *C leptum* (PBS-CL group) or control phosphate-buffered saline (PBS) daily (PBS group) for 14 consecutive days. The total numbers of mononuclear, CD3⁺, and CD4⁺CD25⁺Foxp3⁺ cells and the frequency of Tregs in CD3⁺ T cells in individual groups of mice were evaluated at successive time points. A, The total numbers of Tregs in the spleen. B, The percentages of Tregs in CD3⁺ T cells. Data are expressed as individual values and mean (SEM) of each group (n = 6 per group at each time point) of mice from 3 separate experiments. * $P < .05$ vs the PBS group of mice.

Characterizations of inflammatory infiltrates indicated that although there were a few inflammatory infiltrates in BALF from the PBS group of mice, the numbers of total inflammatory infiltrates in BALF from the OVA-CL group of mice only moderately increased and were significantly less than that in the OVA group of mice ($P < .01$, Figure 4B). A similar pattern of eosinophils, neutrophils, lymphocytes, and macrophages in BALF from different groups of mice was observed. The significantly less extent of lung epithelial damages and fewer numbers of inflammatory infiltrates in the lungs of *C leptum*-fed asthmatic mice clearly indicated that oral administration of *C leptum* mitigated allergic lung inflammation in mice.

Oral feeding with *C leptum* and cytokine responses in the lungs

Allergen-specific T_H2 cells (secreting IL-4, IL-5, IL-9, and IL-13), T_H17 cells (secreting IL-17A, IL-17F, and IL-22), T_H1 cells (secreting IFN- γ), and T_H9 cells (secreting IL-9) are crucial for the development of allergic inflammation. In contrast, Tregs secrete IL-10 and TGF- β , which negatively regulate allergic lung inflammation. To further understand the mechanisms underlying the effect of oral feeding with *C leptum* on allergic lung inflammation, we measured the levels of these cytokines in BALF from different groups of mice by enzyme-linked immunosorbent assay (Fig 5A and 5B). The levels of IL-4, IL-5, IL-13, IL-6, IL-9, IL-17A, IL-21, IL-23, IFN- γ , TNF- α , and IL-2 in the BALF from the OVA-CL group of mice were significantly

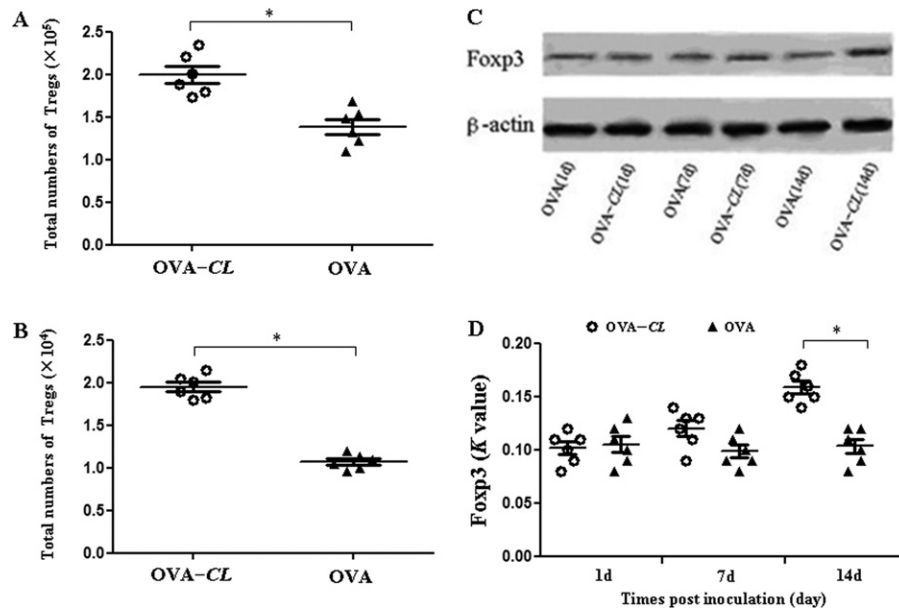


Fig. 2. Oral feeding with *Clostridium leptum* induces regulatory T-cell (Treg) responses in the ovalbumin (OVA)-sensitized mice. BALB/c mice were sensitized with OVA/aluminum hydroxide (Alum) twice (OVA group) and fed with phosphate-buffered saline (PBS) as control or with *C. leptum* daily (OVA-CL group) for consecutive 14 days. The total numbers of Tregs in the spleens and mediastinal lymph nodes and the relative levels of Foxp3 expression in the lungs of different groups of mice were measured by flow cytometry and Western blot assays, respectively. **A**, The total numbers of Tregs in the spleens. **B**, The total numbers of Tregs in the mediastinal lymph nodes. **C**, Western blot analysis of the relative levels of Foxp3 to β-actin expression in the lungs. **D**, Quantitative analysis of Foxp3 expression in the lungs. Data are representative images or expressed as individual values and mean (SEM) of each group (n = 6 per group at each time point) of mice from 3 separate experiments. *P < .05 vs. the OVA group of mice.

lower than that in the BALF from the OVA group of mice, but the levels of IL-10 and TGFβ1 in BALF from the OVA-CL group of mice were significantly higher than that in the BALF from the OVA group of mice ($P < .05$ for all). The levels of IL-4, IL-5, IL-13, IL-23, IFN-γ, TNF-α, IL-2, IL-10, and TGF-β1 in the BALF from the OVA-CL group of mice were not significantly different from that in the PBS group of mice; however, the levels of IL-6, IL-9, IL-21, and IL-17A in the BALF from the OVA-CL group of mice remained significantly higher than that in the PBS group of mice. Apparently, oral feeding with *C. leptum* modulated cytokine responses in the lungs, contributing to the inhibition of allergic airway inflammation in mice.

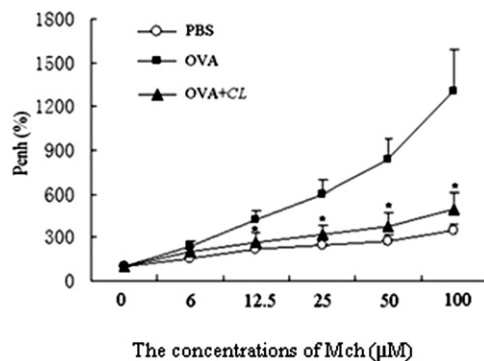


Fig. 3. The effect of treatment with *Clostridium leptum* on allergic airway responsiveness to methacholine. BALB/c mice were sensitized with ovalbumin (OVA)/aluminum hydroxide (Alum) twice (OVA group) and fed with phosphate-buffered saline (PBS) as control (PBS group) or with *C. leptum* daily (OVA-CL group) for consecutive 14 days. The mice were challenged with aerosolized OVA 3 times. Another group of mice were treated with PBS as healthy controls. Two days after the last challenge, their airway responses to increased doses of methacholine (Mch) were measured by whole-body plethysmography to determine changes in enhanced respiratory pause (Penh). Data are expressed as mean (SEM) of the values from different groups (n = 6 per group) of mice from 3 separate experiments. *P < .05 vs the OVA group of mice.

Discussion

Allergic airway inflammation results from allergen-specific T-cell responses. T_H2, T_H9, and T_H17 cells secrete IL-4, IL-17A, IL-9, and other cytokines, which contribute to pulmonary inflammation and airway hyperresponsiveness.⁴⁻⁹ T_H1 cells secrete IFN-γ and TNF-α, which suppress T_H2 responses and cytokine secretion, and induce epithelial and smooth muscle cells apoptosis.^{2,3} Tregs are important for the maintenance of peripheral immune tolerance and can inhibit different effector T-cell-mediated inflammation. Hence, induction of Treg responses will be of great significance. Notably, recent studies have found that oral administration of young mice with *Clostridium* species (mainly with *C. leptum* and *C. coccoides*) increases Treg clones in the colon, which can migrate into other organs, inhibiting inflammation in rodents.^{23,24} Given that *C. leptum* (cluster IV) is the predominant strain among different *Clostridium* species in the intestinal tract,¹⁹ in this study, we tested the effect of oral administration of *C. leptum* alone on systemic Treg responses in mice. We found that oral feeding with *C. leptum* for 14 days did not affect the total splenic mononuclear and T cells but did increase the total numbers and frequency of Tregs in the spleens of mice. These data indicate that oral administration with *C. leptum* alone can induce systemic Treg responses in adult mice.

It is well known that allergic airway inflammation is mediated by allergen-specific T_H2 and other effector T cells, including T_H1, T_H17, and T_H9 cells.^{31,32} Previous studies have shown that the pre-existed immune responses can affect subsequently primed immune T-cell responses.³³⁻³⁵ We tested whether administration with *C. leptum* could induce Treg responses in the lungs of the allergen-sensitized mice and found that oral feeding with *C. leptum* induced stronger Treg responses in the spleens and the target tissues. Evidentially, the total numbers and frequency of Tregs in the spleens and mediastinal lymph nodes of the OVA-CL group of mice were significantly greater than that in the OVA group of mice. Furthermore, the relative levels of Foxp3 expression in the lungs of the OVA-CL group of mice were significantly higher than that in the

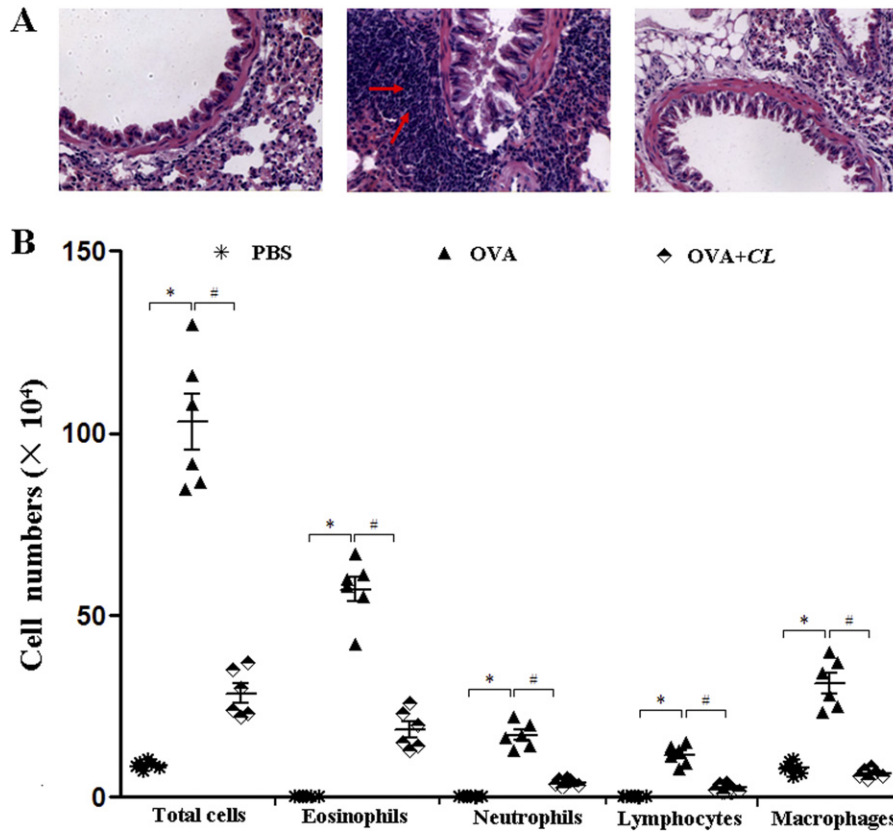


Fig. 4. Oral feeding with *Clostridium leptum* reduces allergic airway inflammation in mice. BALB/c mice were sensitized with ovalbumin (OVA)/aluminum hydroxide (Alum) twice (OVA group) and fed with phosphate-buffered saline (PBS) as control (PBS group) or with *C. leptum* daily for consecutive 14 days (OVA-CL group). The mice were challenged with aerosolized OVA 3 times. Another group of mice was treated with PBS as healthy controls. Two days after the last challenge, bronchoalveolar lavage fluid (BALF) was obtained from individual mice, and their lung tissue sections were stained with hematoxylin-eosin for the evaluation of allergic inflammation. Data are representative images or expressed as individual values and mean (SEM) of each group ($n = 6$ per group) of mice from 3 separate experiments. A, Representative images of lung tissue sections (original magnification $\times 400$). Arrows indicate inflammatory infiltrates. B, Quantitative analysis of inflammatory infiltrates in BALF. * $P < .01$ vs the PBS group of controls; # $P < .01$ vs the OVA group of mice.

OVA group of mice. These data indicated that oral feeding with *C. leptum* alone induced Treg responses in the mice with already established inflammation responses. Notably, the numbers of Tregs in the spleens from the OVA-CL group of mice (Figure 2A) were reduced by 60%, as compared with that in the PBS-CL group of mice

(Figure 1A). These data suggest that induction of Tregs by oral feeding with *C. leptum* may be negatively regulated by the established allergic inflammation. Alternatively, Tregs induced by oral feeding with *C. leptum* may migrate into the inflammation site, leading to the reduced numbers of splenic Tregs in the OVA-CL

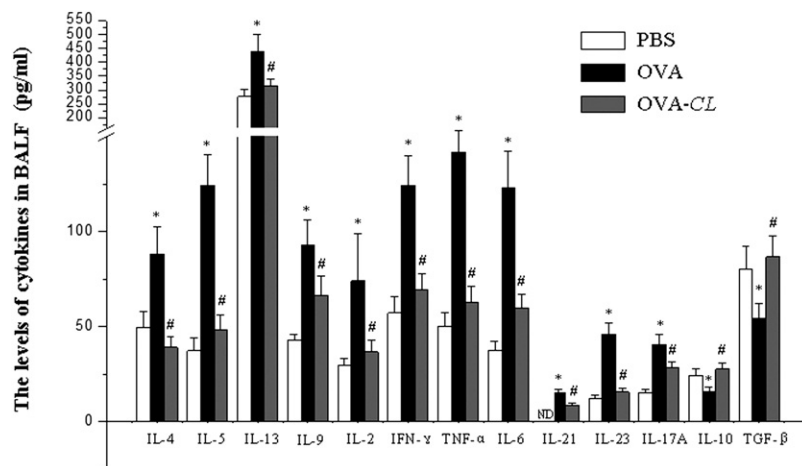


Fig. 5. Oral feeding with *Clostridium leptum* modulates the levels of cytokines in bronchoalveolar lavage fluid (BALF). BALB/c mice were sensitized with ovalbumin (OVA)/aluminum hydroxide (Alum) twice (OVA group) and fed with phosphate-buffered saline (PBS) as control (PBS group) or with *C. leptum* daily (OVA-CL group) for consecutive 14 days. The mice were challenged with aerosolized OVA 3 times. Another group of mice were treated with PBS as healthy controls. Two days after the last challenge, BALF were obtained from individual mice and the concentrations of interleukin (IL) 4, IL-5, IL-13, interferon γ (IFN- γ), tumor necrosis factor α (TNF- α), IL-2, IL-6, IL-9, IL-17A, IL-21, IL-23, IL-10, and transforming growth factor $\beta 1$ (TGF- $\beta 1$) in individual BALF samples were analyzed by enzyme-linked immunosorbent assay. Data are expressed as mean (SEM) of the values from different groups ($n = 6$ per group) of mice from 3 separate experiments. * $P < .05$ vs the PBS group of mice; # $P < .05$ vs the OVA group of mice. ND indicates not detectable.

group of mice. We are interested in further investigating how inflammation regulates *C leptum* feeding-stimulated Treg expansion in mice.

Previous studies have shown that Tregs can migrate into the lungs and associated lymph nodes and suppress allergen-induced airway inflammation, mucous hypersecretion, and hyperresponsiveness.^{36–38} Adoptive transfer of Tregs into the allergen-sensitized mice before antigen challenge suppresses the development of allergic disease, even after the establishment of inflammation.^{39–41} We found that oral feeding with *C leptum* mitigated allergic airway inflammation and hyperresponsiveness. Significantly reduced airway responses to increased doses of methacholine and markedly lower numbers of inflammatory infiltrates were detected in the lungs of the OVA-CL group of mice compared with the OVA group of mice. Furthermore, the concentrations of T_H2, T_H17, T_H1, and T_H9-related cytokines, such as IL-4, IL-5, IL-13, IL-9, IL-17A, IFN- γ , TNF- α , IL-2, IL-6, IL-21, and IL-23, in BALF from the OVA-CL group of mice were significantly lower than that in the OVA group of mice. The inhibition of airway inflammation is likely to be mediated by increased Treg responses, which are supported by the increased numbers of Tregs in the target tissue-associated lymph nodes and higher levels of Foxp3 expression and the higher levels of IL-10 and TGF- β 1 in the lungs of the OVA-CL group of mice. Our findings extend previous observations that infection with *Mycobacterium vaccae*, *Litomosoides sigmodontis*, *Nippostrongylus brasiliensis*, or *Schistosoma mansoni* induces Treg responses^{42–45} and support the notion that administration of probiotic microorganisms can induce Treg responses and suppress T_H2-, T_H17-, T_H1-, and even T_H9-mediated airway inflammation.⁴⁶ Given that IL-10 and TGF- β are inhibitory cytokines that can suppress airway inflammation,^{47,48} oral feeding with *C leptum* may result in a change in microbial environment in the gut and promote Treg activation and expansion. Subsequently, the expanded Tregs migrate into the target tissues, inhibiting pathogenic T_H1, T_H17, T_H2, and T_H9 responses and allergic airway inflammation. It is possible that oral feeding with *C leptum* changes the microbial flora in the gut that modulates dendritic cells that tend to more directly induce Treg responses.⁴⁹ Alternatively, *C leptum*, like *Lactobacillus rhamnosus*, may induce IL-10 and TGF- β production,^{50,51} which promotes Treg responses because TGF- β is an essential inducer of Tregs, and IL-10 can inhibit proinflammatory IL-6 responses.⁵² We are interested in further investigating the mechanisms by which feeding with *C leptum* induces Tregs and inhibits airway inflammation. In addition, it is worth noting that this study only focuses on female BALB/c mice and that male BALB/c mice and other strains of mice need further researches.

In summary, our data clearly demonstrate that oral feeding with *C leptum* induced systemic Treg responses in healthy and allergen-sensitized adult mice. Furthermore, oral feeding with *C leptum* inhibited allergic airway hyperresponsiveness and inflammation, accompanied by modulating cytokine responses in the lungs of mice. Although *C leptum* may be associated with the development of inflammatory bowel disease, *C leptum* is naturally colonized in human colons. *C leptum* may be safe for oral feeding in some populations. Therefore, our findings may provide a new basis for the design of novel probiotic therapies for the treatment of asthma.

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