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Oral *Bifidobacterium* modulates intestinal immune inflammation in mice with food allergyLi-Li Zhang,* Xiao Chen,[†] Peng-Yuan Zheng,* Yu Luo,[‡] Gao-Feng Lu,* Zhi-Qiang Liu,* Huang Huang* and Ping-Chang Yang[†]

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Abstract

Background and Aims: It is proposed that probiotics have a therapeutic effect on the treatment of immune disorders. However, the underlying mechanisms require clarification. The present study aimed to evaluate the effect of gavage-feeding *Bifidobacteria* on suppression of T helper 2 (Th2) pattern inflammation in the intestines of mice with food allergy.

Methods: Mice were sensitized to ovalbumin to induce the intestinal Th2 pattern inflammation. Allergic mice were treated with or without *Bifidobacteria* via gavage-feeding. Th2 response, number of regulatory T cells (Treg) in the spleen and intestine, intestinal epithelial barrier function and bifidobacterial translocation were examined.

Results: The results showed that serum-specific immunoglobulin E antibody and interleukin 4 (IL-4) were increased in allergic mice. Intestinal epithelial barrier function was impaired in allergic mice as shown by the increase in epithelial ion secretion and permeability to macromolecular protein horseradish peroxidase in Ussing chambers. Number of Treg was decreased in both spleen and intestines of allergic mice. Gavage-feeding *Bifidobacteria* significantly suppressed the skewed Th2 response and increased the number of Treg. Transient bifidobacterial translocation was observed in allergic mice.

Conclusions: Oral administration of *Bifidobacteria* has the capacity to suppress the skewed Th2 response in allergic mice, increasing the number of Treg and IL-10-positive cells and improve the impaired intestinal epithelial barrier function.

Introduction

Despite rapid progress in the research of allergies, the prevalence of allergic disorders still remains high worldwide.¹ The symptoms of food allergy, one of the common allergic diseases, vary from mild abdominal discomfort to life-threatening allergic shock.² In fact, food allergy has emerged as one of the health problems that have a great impact on human quality of life and social economy. The treatment for food allergy is currently not satisfactory. Thus, it is imperative to develop novel therapeutic remedies for the treatment of food allergy.

Food allergy is seen as skewed T helper 2 (Th2) responses in the body, especially in the intestine. The causative factors are not clear. It is proposed that intestinal epithelial barrier dysfunction plays a role in the pathogenesis of food allergy.³ The impaired epithelial barrier allows intact antigens or other noxious substances to pass the barrier to reach the subepithelial region⁴ where the antigens have an opportunity to contact immune cells and initiate inappropriate immune responses, such as skewed Th2 polarization.

Currently, we do not have any satisfactory remedies to cure food allergy. Avoiding taking in the offending food antigen is the only effective therapy.

Regulatory T cells (Treg) include several subtypes such as CD4⁺ Foxp3⁺ Treg, Tr1 cells, Th3 cells and CD8⁺ Foxp3⁺ Treg.⁵ Treg play a critical role in the maintenance of immune homeostasis in the body. Reduction of Treg number and impairment of Treg function have been noted in patients with allergic diseases by an unknown mechanism.⁶ Interleukin (IL)-10 and transforming growth factor- β (TGF- β) are required in the development of Treg. Tolerogenic dendritic cells (DC) can be the source of IL-10 and TGF- β for the induction of Treg.⁷ However, our knowledge of the production of IL-10 and TGF- β in DC is still limited.

It is reported that oral administration of probiotics regulates immune responses in the body. Accumulated evidence indicates that probiotics have the ability to prevent and ameliorate allergic diseases to a certain extent.^{1,8} It is proposed that probiotics promote the production of IL-10;⁹ but the cellular source of the IL-10 has not yet been determined. Oral probiotics are also

beneficial in the development of Treg;¹⁰ however, cells involved in the process need to be further elucidated. Based on the evidence aforementioned, we hypothesized that oral probiotics might modulate DC function and further promote Treg development in the body and thus suppress the skewed immune responses. The present data show that oral probiotics favor the generation of IL-10⁺ DC in the intestine, increase the number of Treg and suppress the Th2 pattern of inflammation.

Methods

Materials

Horseradish peroxidase, *O*-dianisidine, cadaverine dihydrochloride, diamine oxidase standard substance and ovalbumin were purchased from Sigma Aldrich (St Louis, MO, USA). Staphylococcal enterotoxin B (SEB) was synthesized by the Department of Biotechnology, Zhengzhou University. ATCC (15700, *Bifidobacterium breve*, mouse immunoglobulin (Ig)E, IL-4 and gamma-interferon (IFN- γ) enzyme-linked immunosorbent assay (ELISA) assay kit were purchased from Alpha Diagnostic International, (ADI, San Antonio, TX, USA). Antibodies used for flow cytometry were purchased from BD Bioscience (Mississauga, ON, Canada). Anti-IL-10 receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co. (Beijing, China). The experimental procedures were approved by the Animal Care Committee at Zhengzhou University.

Murine model of intestinal allergy

Female BALB/c mice weighing 18–22 g were sensitized to ovalbumin (OVA) with our established protocol.¹¹ Briefly, mice were intraperitoneally injected with OVA (20 μ g/mouse) or/and SEB (10 μ g/mouse) on day 0. The mice were boosted twice with the same reagents on days 3 and 9, respectively. Mice were challenged with OVA (100 μ g/mouse) in 0.25 mL saline intragastrically twice on days 7 and 14. Mice were killed 1 day after last intragastric challenge (day 15). Mice demonstrating profuse liquid stool were used as allergic and diarrhea-positive animals. Two days after the last challenge, mice were killed by decapitation. The blood samples were collected; the sera were separated and stored at -70°C for further analyses. Diarrhea was also noted by direct observation of the colon and cecum; the liquid stool observed following OVA challenge-induced diarrhea in the sensitized mice contrasted with the solid pellets seen in the distal colon of mice in control groups.

Probiotics intervention

Mice were gavage-fed with 0.4 mL normal saline containing *Bifidobacterium* (10^8 cfu/mL) seven times, 24 h apart. To determine the bifidobacterial translocation in the intestine, a batch of *Bifidobacteria* was transfected with green fluorescent protein (GFP)-tagged plasmid according to the reported procedures.¹² These *Bifidobacteria* could be visualized by confocal microscopy.

Determination of IgE, IL-4, IFN- γ and diamine oxidase in the sera

Levels of IgE, IL-4 and IFN- γ in the sera were quantified using ELISA kits in accordance with the manufacturer's instructions. The level of diamine oxidase (DAO) was determined by the method of absorption spectrometry.¹³

Flow cytometry

CD4⁺ T cells were isolated from the spleen and intestine by magnetic cell sorting.¹⁴ The cells were fixed with 1% paraformaldehyde and permeabilized for 30 min on ice. After washing with phosphate-buffered saline, cells were stained with fluorescence-labeled antibodies (or isotype IgG used as control) for 30 min on ice. The cells then were analyzed by flow cytometry. The acquired data were processed with software FLOWJO (Ashland, OR, USA).

Ussing chamber studies

The proximal jejunum was removed, placed in 37°C oxygenated Krebs, stripped of longitudinal muscle and myenteric plexus, and opened along the mesenteric border. Two to four adjacent pieces from each mouse were mounted in Ussing chambers (World Precision Instruments, Sarasota, FL, USA). The chamber opening exposed 0.6 cm² of tissue surface area to 8 mL circulating oxygenated Krebs buffer at 37°C . The experimental procedures were the same as those we have reported previously.⁴ Short circuit current (Isc; $\mu\text{A}/\text{cm}^2$) was recorded by a computer connected to the voltage-clamp system.

Horseradish peroxidase flux

Mucosal-to-serosal transport of macromolecules was assessed by measuring transepithelial flux of horseradish peroxidase (HRP). HRP (type II) was added to the luminal buffer 15 min after the tissues were mounted at a final concentration of 10^{-5} mol/L and allowed to equilibrate for 30 min. Serosal samples (0.5 mL) were obtained at 30-min intervals for 2 h and were replaced with Krebs' buffer to maintain a constant volume in the chambers. HRP activity was determined by a modified Worthington method as previously described.⁴ Mucosal-to-serosal fluxes of HRP were calculated according to standard formulas⁴ and expressed as picomoles per hour per square centimeter.

Confocal microscopy

Jejunal segments were excised immediately after death. Cryosections were prepared and stained with fluorescence-labeled anti-IL-10 or anti-CD11c antibodies (or isotype IgG used as control) with our established protocol.¹⁴ The sections were observed with a confocal microscope (LSM510; Carl Zeiss Micro Imaging GmbH, Germany).

Experimental design

BALB/c mice were used in this study. Mice were randomly grouped (six mice each) as follows: (i) naïve; (ii) allergy; or (iii) allergy (or naïve mice) plus gavage-feeding with *Bifidobacteria*.

Mice in the allergy group were sensitized to and challenged with OVA. A group of mice with intestinal allergy and a group of naïve mice were gavage-fed with *Bifidobacteria* with or without antibody against IL-10 receptor (0.1 mg/mouse, i.p.) daily from day 7 to day 13 in the period of sensitization (see above). Mice were killed on day 14. The sera were collected for determining the levels of IL-4, IFN- γ and DAO. A portion of jejunal segments were subjected to Ussing chamber study to determine the epithelial barrier function. Jejunal extracts were prepared for determining the levels of IL-4, IFN- γ and IL-10. Jejunal cryosections were prepared to visualize the GFP-tagged *Bifidobacteria*, IL-10 $^{+}$ and CD11c $^{+}$ cells. Lamina propria mononuclear cells were prepared to assess the number of Foxp3 $^{+}$, CD11c $^{+}$ and IL-10 $^{+}$ cells by flow cytometry.

Statistical analysis

Data were expressed as the means $\pm$ SD. All statistical analyses were carried out using SPSS statistical version 13.0. Differences between groups were determined with one-way ANOVA; a *P*-value of less than 0.05 was considered to be statistically significant.

Results

Allergic reaction increases intestinal epithelial permeability

As shown by HRP flux in Ussing chambers, a significant amount of HRP passed the epithelial layer from the apical side to the serosal side in mice with intestinal allergy. The Isc was markedly higher in allergic mice than in controls. This fact indicates that allergic reaction increases intestinal epithelial permeability. Measuring levels of DAO can mirror the intestinal epithelial barrier function at an integrated physiological condition.¹⁵ Used as a comparison, we measured the serum levels of DAO in these mice. As expected, the serum level of DAO in allergic mice was markedly higher than in control mice. The DAO value was also positively correlated with HRP flux value. Treating with *Bifidobacteria* markedly improved the allergy-induced intestinal epithelial barrier dysfunction, in which IL-10 might be involved, as the effect of *Bifidobacteria* could be abolished by pretreatment with anti-IL-10 receptor antibody (Fig. 1).

Intestinal hypersensitivity to food antigens suppresses Treg

Clinical studies show a reduction of Treg in patients with food allergy,¹⁶ but this needs to be confirmed by experimental data. With an established protocol,¹¹ we sensitized mice with OVA. After challenge with specific antigen (OVA), these mice showed allergic responses in the peripheral system and intestine (Fig. 2). To elucidate whether the induced allergic environment affected the number of CD4 $^{+}$ Foxp3 $^{+}$ Treg, CD4 $^{+}$ T cells were isolated from the spleen and jejunum. The isolated CD4 $^{+}$ T cells were stained with anti-Foxp3 antibody and analyzed by flow cytometry. As shown in Figure 3, approximately 10.3% Foxp3 $^{+}$ Treg was identified in splenic CD4 $^{+}$ T cells and approximately 17.5% Foxp3 $^{+}$ Treg was identified in intestinal CD4 $^{+}$ T cells from naïve control mice. However, much less Foxp3 $^{+}$ Treg was identified in mice with

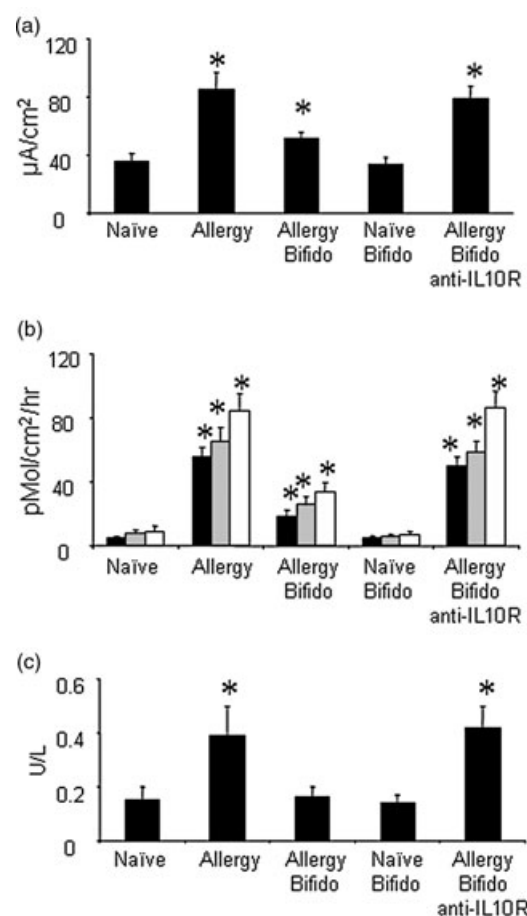


Figure 1 Assessment of intestinal epithelial barrier function. Mouse intestinal allergy model was developed. Intestinal epithelial barrier function was assessed with the Ussing chamber technique. (a) Bars indicate the intestinal ion secretion ($\mu\text{A}/\text{cm}^2$). (b) Bars indicate the horseradish peroxidase (HRP) flux from the mucosal side to the serosal side. ■, 0–30; □, 30–60; □, 60–90. (c) Bars indicate serum diamine oxidase (DAO) level. Naïve, naïve mice; Allergy, mice with intestinal allergy; Allergy Bifido, allergic mice treated with *Bifidobacteria*; Anti-IL10R, allergic mice pretreated with anti-IL-10 receptor antibody, then treated with *Bifidobacteria*. Data were collected from six mice/group and expressed as means $\pm$ SD. **P* < 0.05, compared with naïve controls.

intestinal allergy. This indicates that intestinal allergy suppresses the number of CD4 $^{+}$ Foxp3 $^{+}$ Treg in both the intestine and peripheral system.

Oral *Bifidobacteria* modulates the skewed immune responses in the intestine

Bifidobacteria are believed to possess immune regulative function.¹⁷ Thus, we treated allergic mice with *Bifidobacteria* by gavage-feeding. Mice were then killed 1 day after the last treatment. The rate of CD4 $^{+}$ Foxp3 $^{+}$ Treg in CD4 $^{+}$ T cells was increased in allergic mice after treatment with *Bifidobacterium*. The Th2 response in the intestine was suppressed after *Bifidobacterium* treatment (Fig. 2). However, the serum level of IL-10 was

Figure 2 *Bifidobacteria* modulate Th2 response. Allergic mice were treated with *Bifidobacteria* as described in the text. The serum, jejunal tissue extract and intestinal CD4⁺ T cells were prepared and subjected to the analyses of Th2 response. Cytokines were evaluated by enzyme-linked immunosorbent assay. T-cell proliferation was determined by [³H]-thymidine incorporation. (a) Bars indicate ovalbumin (OVA)-specific immunoglobulin (IgE) level in the serum. (b) Bars indicate T-cell proliferation as shown in counts per minute (cpm). (c,d) Bars indicate the levels of interleukin (IL)-4 (■) and gamma-interferon (IFN- γ) (□) in the (c) serum and (d) jejunal extracts. Data are presented as means $\pm$ SD. * P < 0.05, compared with (a) allergic mice or with (b–d) naïve mice. Each group consisted of six mice.

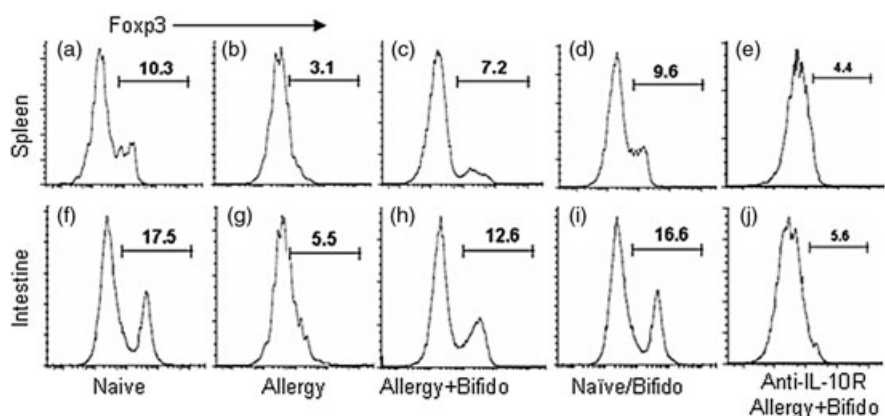
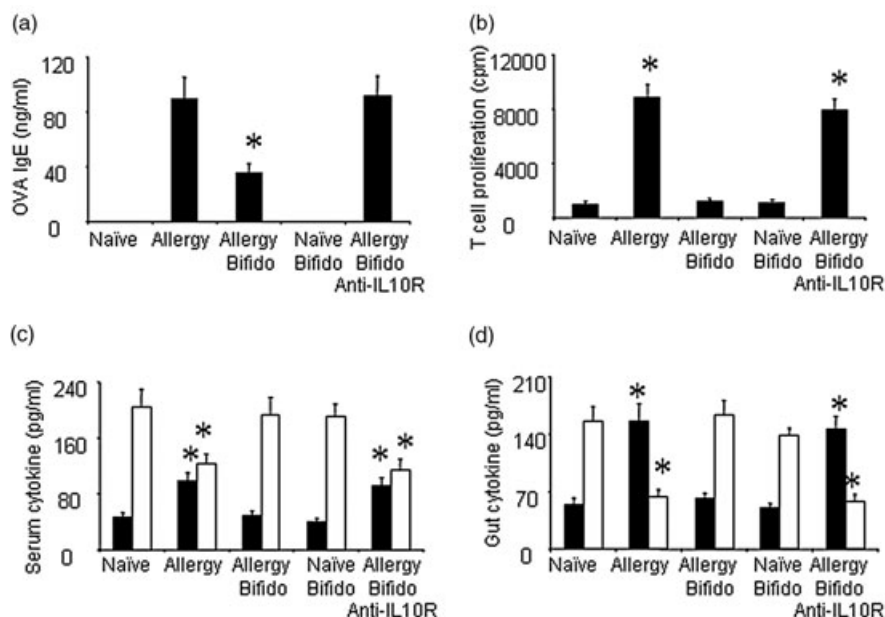
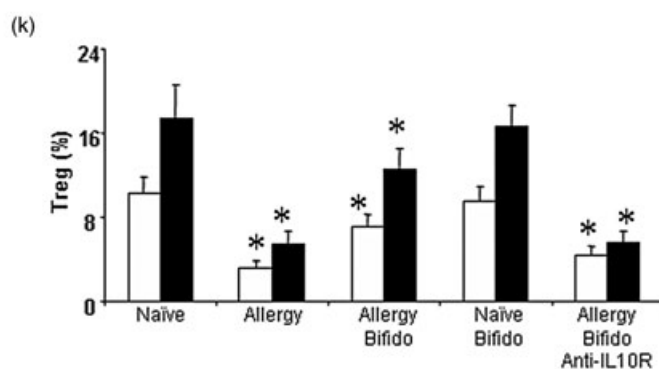


Figure 3 Allergic response suppresses the number of Treg. Splenic and jejunal CD4⁺ T cells were isolated by magnetic cell sorting and stained with fluorescence-labeled anti-Foxp3 antibody. The stained cells were analyzed by flow cytometry. Flow cytometry histograms show Foxp3⁺ T cells in the (a–e) spleen and (f–j) jejunum. Anti-IL-10R, pretreatment with anti-IL-10 receptor antibody. Isotype IgG staining did not show any positive staining (data not shown). (k) Bars indicate the percentage of Treg in isolated lamina propria mononuclear cells. * P < 0.05, compared with the naïve group. □, Spleen; ■, gut.



increased sharply in *Bifidobacterium*-treated mice (Fig. 4). Considering that IL-10 might be associated with *Bifidobacterium*-increased CD4⁺ Foxp3⁺ Treg in the intestine, another batch of allergic mice were treated with anti-IL-10 receptor antibody (or isotype IgG used as control) every other day seven times during the

gavage-feeding *Bifidobacterium* period. Indeed, these mice did not show an increase in CD4⁺ Foxp3⁺ Treg in the intestine (Fig. 3e,k), which did not occur in mice treated with isotype IgG. Collectively, the results indicate that gavage-feeding *Bifidobacteria* has the capacity to reverse food allergy-induced intestinal epithelial

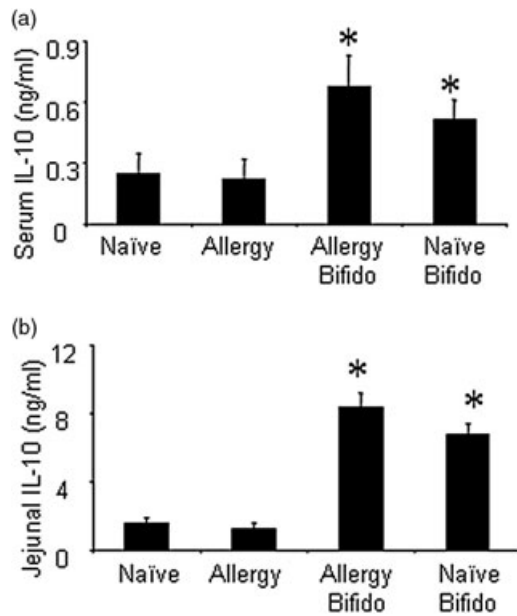


Figure 4 *Bifidobacteria* increase interleukin (IL)-10. Naïve and allergic mice were treated with *Bifidobacteria* as described in the text. Sera and jejunal segments were collected immediately after death. IL-10 level in the sera and intestinal extracts was determined by enzyme-linked immunosorbent assay. Bars indicate the level of IL-10 in the (a) sera and (b) jejunum. Data were collected from six mice per group and expressed as means $\pm$ SD. * $P < 0.05$, compared with naïve mice. Wells with added isotype IgG did not show any positive results (data not shown).

barrier dysfunction, Treg reduction and skewed Th2 responses (Figs 1–3).

Transient translocation of *Bifidobacteria*

The mechanism by which *Bifidobacteria* modulate immune function in the body is to be further understood. It is undetermined whether oral *Bifidobacteria* can translocate into intestinal tissue. If yes, how long can it stay? Thus, using GFP-tagged *Bifidobacteria* and confocal microscopy, we observed the bifidobacterial translocation in the jejunum of mice with food allergy 24 h after gavage. Five days after the gavage of *Bifidobacteria*, we rechecked the jejunum, ileum and colon in another batch of mice; no GFP-tagged *Bifidobacteria* were found (Fig. 5).

Translocated *Bifidobacteria* associate with IL-10 production

Inspired by published studies that probiotics favor the production of IL-10 and further promote the generation of Treg,¹⁸ we tried to identify direct evidence *in vivo* that giving *Bifidobacteria* was associated with IL-10 production. By confocal microscopy, *Bifidobacteria* were visualized inside the jejunal mucosa of mice treated with gavage-feeding *Bifidobacteria*. The fact indicates that these *Bifidobacteria* translocated into the jejunal mucosa. The striking finding was that most translocated *Bifidobacteria* accom-

panied or surrounded IL-10⁺ cells (Fig. 5). The *Bifidobacterium* translocation was found mainly in allergic mice, but was not seen in naïve mice. To elucidate the cell type of those IL-10⁺ cells, we stained consecutive intestinal sections. As shown in Figure 5, most of those IL-10⁺ cells were also CD11c positive. This implies that *Bifidobacteria* stimulate DC to produce IL-10 in the intestinal mucosa. Alternatively, we isolated the mononuclear cells from jejunal tissue and analyzed them by flow cytometry to further confirm and quantify the IL-10-production of DC. As shown in Figure 5d,e, IL-10⁺ cells were found in CD11c⁺ DC in both naïve and allergic mice. The ratio of IL-10⁺ CD11c⁺ DC was much lower in allergic mice than in naïve control that was recovered after administration with oral *Bifidobacteria*.

Discussion

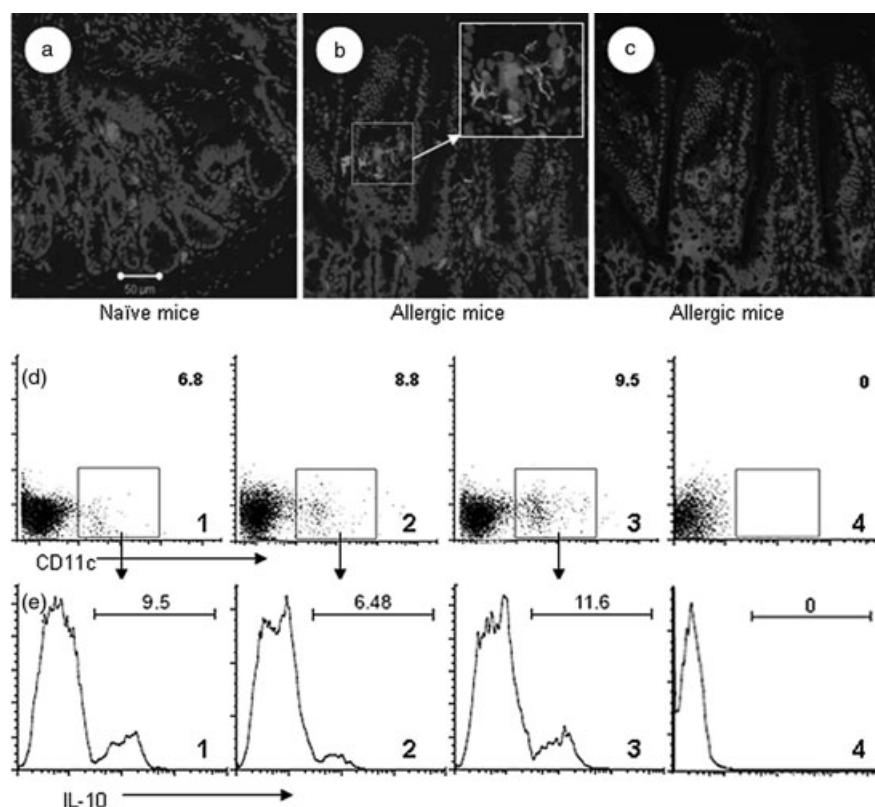
The major findings in the present study include the following. (i) Gavage-feeding *Bifidobacteria* in allergic mice induced the number of IL-10⁺ DC in the intestinal mucosa; (ii) DC-derived IL-10 played a critical role in the increase in Treg; (iii) there was improved intestinal epithelial barrier function; and (iv) gavage-feeding *Bifidobacteria* in allergic mice suppressed the skewed Th2 response.

It is accepted that Treg play a critical role in the maintenance of immune homeostasis in the body. Defect of Treg function has been associated with several immune disorders such as inflammatory bowel diseases,¹⁹ allergic diseases,⁶ rheumatoid arthritis, systemic lupus erythematosus²⁰ and psoriasis.²¹ It is natural to consider using *in vitro* generated Treg to treat immune diseases. However, there is a problem that the life span of exogenous Treg is limited. In line with previous reports, the present study provides a possible alternative to solve the problem. The data show that gavage-feeding *Bifidobacteria* induce DC to produce IL-10 and further increase Treg in the intestine. These Treg stem from the host's own cells and are expected to have the natural properties of Treg and immune regulatory function.

Tolerogenic DC are important in immune regulation in the body and are involved in the induction of Treg.⁷ Tolerogenic DC are important in the production of IL-10 or/and TGF- β . The present data show that after gavage-feeding *Bifidobacteria*, IL-10-bearing DC increased significantly in the jejunum. In other words, gavage-feeding *Bifidobacteria* has the capacity to generate tolerogenic DC. It is proposed that IL-10 can be a mediator to drive naïve CD4⁺ T cells to become Treg. The present data provide anatomical evidence that translocated *Bifidobacteria* dispersed surrounding IL-10⁺ DC in the intestinal mucosa. Those translocated *Bifidobacteria* may be associated with the development of tolerogenic DC. Results showing that blocking IL-10 abolished the increase in Treg in allergic mice supports this notion. Our data also show that *Bifidobacterium* translocation into the intestinal tissue is transient. Gavage-feeding *Bifidobacteria* were not found in the intestine 5 days later. This demonstrates that the transient translocated *Bifidobacteria* only stay in the intestinal tissue for a short time. Furthermore, as the epithelial barrier function is improved after gavage-feeding *Bifidobacteria*, no more *Bifidobacteria* can translocate into the intestinal tissue.

IL-10 is a cytokine that has multiple bioactivities involved in immune regulation.⁷ Recent reports recognize its role in the gen-

Figure 5 *Bifidobacteria* increase interleukin (IL)-10-producing DC in the intestine. Grouped BALB/c mice were gavage-fed with *Bifidobacteria* as described in the text. Jejunal sections were stained with anti-IL-10 antibody (red) and observed with confocal microscopy. The stained cells were analyzed by flow cytometry. (a,b) Confocal images show green fluorescent protein (GFP)-tagged *Bifidobacteria* (green) in the intestinal tract; some of them translocated into the jejunal mucosa. (c) Consecutive sections of panel b. Red color indicates CD11c-positive immune reactive products. (d)(1–3) Jejunal mononuclear cells were isolated by magnetic cell sorting and stained with antibodies against CD11c and IL-10. The flow cytometry dot plots show CD11c+ DC (gated cell population). (e)(1–3) Flow cytometry histograms show IL-10+ DC in gated cells in panels (d)(1–3). 1, naïve mice; 2, allergic mice; 3, allergic mice were treated with *Bifidobacteria*. Original magnification: $\times 100$ (the insert in panel b: $\times 200$). (d)(4),e(4)) Isotype IgG control staining.



eration of Treg.⁷ In line with these studies, our results indicate that *Bifidobacterium*-induced enhancement of IL-10⁺ DC is correlated with the number of Treg in the intestine of mice with allergic disorders; that fact implies a potential of an immune regulatory remedy induced by *Bifidobacteria*. The present data are a little different from one recent publication²¹ in which the authors demonstrate that oral *Bifidobacterium* did not affect the serum level of IL-10. However, the same paper also indicated that a significant increase in IL-10 levels was recognized in a culture of isolated splenocytes, which is in line with the present study.

The normal intestinal mucosa has a barrier function that prevents noxious substances and intact antigens from being absorbed into the intestinal mucosa.⁴ Epithelial barrier dysfunction is associated with many immune disorders.²² In line with those previous reports, the present data also show a marked increase in epithelial cell ion secretion and permeability to macromolecular protein HRP in allergic mice. That gavage-fed *Bifidobacteria* translocated into intestinal tissue only in allergic mice, but not in naïve control mice indicates that the impaired epithelial barrier facilitates the translocation of *Bifidobacteria*; this event may be responsible for the generation of IL-10-secreting tolerogenic DC.

Skewed Th2 response is a major feature of allergic disorders, including food allergy. Our food allergy animal model also shows Th2 polarization in the jejunum. In general, skewed Th2 response is refractory to correction. The present study sheds new light on the correction of the skewed Th2 response with the evidence that treatment with oral *Bifidobacteria* suppressed levels of serum IgE and IL-4. Therefore, gavage-feeding *Bifidobacteria* is expected to be further developed as a therapeutic remedy for allergic disorders.

In summary, the present study indicates that gavage-feeding *Bifidobacteria* has the capacity to induce tolerogenic DC IL-10⁺. DC may promote the generation of Treg in the peripheral system and jejunum. Gavage-feeding *Bifidobacteria* can suppress skewed Th2 responses by promoting Treg development.

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