



Nasal instillation of probiotic extracts inhibits experimental allergic rhinitis

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Aim: Allergic rhinitis (AR) is a common disease. The therapeutic efficacy of AR needs to be improved. This study aims to evaluate the effects of local administration of probiotic extracts on inhibiting experimental AR. **Methods:** Epithelial cells (ECs) were primed by exposing to *Clostridium butyricum* extracts (CBe) in the culture to upregulate the expression of IL-10. A mouse AR model was developed to assess the therapeutic potential of CBe in AR. **Results:** CBe markedly induced the expression of IL-10 in ECs. Co-culture of naive B cells with CBe-primed ECs significantly increased IL-10 expression in the B cells (iB10 cells). The iB10 cells showed immune suppressive function in suppressing effector CD4⁺ T-cell proliferation. Treatment with nasal drops containing CBe efficiently inhibited experimental AR in mice. **Conclusion:** Local administration of CBe can efficiently inhibit experimental AR.

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Keywords: allergic rhinitis • B cells • epithelial cells • immune regulation • nasal mucosa

The prevalence of allergic rhinitis (AR) is more than 10% in the world, which raised rapidly in the last a few decades [1]. The clinical symptoms of AR include sneezing, profound nasal secretions and nasal congestions [2]. Although AR usually does not induce the life-threatening anaphylactic shock, the complications of AR are common, such as nasal polyposis, rhinosinusitis and allergic asthma [3]. The therapeutic efficacy of AR is to be further improved [4]. The pathogenesis of AR is not fully understood yet. Therefore, it is necessary to further investigate the mechanism of AR and to invent novel, effective therapeutic remedies for AR.

AR is featured as T helper (Th) 2-dominant inflammation of the nasal mucosa, in which the nasal mucosa is over populated with Th2 cells, the cells produce large amounts of Th2 cytokines, such as IL-4, IL-5 and IL-13. Th2 cytokines, especially IL-4, promote the production of immunoglobulin (Ig)E by plasma cells. Such a condition is designated as Th2-dominated inflammation [5]. In general, the immune regulatory system tightly regulates the immune response in the body. Immune regulatory cells, such as regulatory B cells (Bregs) and regulatory T cells (Tregs), immune regulatory mediators, such as TGF- β and IL-10 [5]. Immune regulatory cell-derived immune regulatory mediators can suppress other immune cell activities in order to avoid injuring tissues and cells of the hosts [6]. It has been recognized that dysregulation of the immune regulatory system is associated with many immune diseases, such as allergic diseases, autoimmune diseases, diabetes, etc [7]. Yet, the underlying mechanism of dysfunction of the immune regulatory system is not fully understood.

It is recognized that epithelial cells are associated with immune regulation [8]. Epithelial cells cover the surface of the body and the inner surface of hollow organs in the body. Besides the important barrier function, epithelial cells also involve in regulating other cells' activities. Epithelial cells produce IL-25 and IL-33 to upregulate Th2 response [9]. Epithelial cell-derived integrin $\alpha\text{v}\beta 6$ promotes immune tolerance [10]. Upon exposure to certain microbial products, such as lipopolysaccharide (LPS), epithelial cells produce ubiquitin E3 ligase A20 to facilitate the development of the homeostasis in the body [11]. It is recognized that probiotics, a portion of commensal bacteria in the human body, have immune regulatory function. Whether probiotics have the role of regulating

epithelial immune tolerogenic properties remain to be further investigated. Thus, in this study, we further observed the role of the probiotic extract-primed epithelial cells in the regulation of immune response in the nasal mucosa. We found that the probiotic extract-primed epithelial cells produced IL-10 that played an important role in the induction of Bregs and ameliorates experimental AR.

Materials & methods

Reagents

CRISPR kit of IL-10 receptor, antibodies of CD22 (48), IL-10 (3C12C12), CD3 (6A224) and CD28 (3H1179) were purchased from Santa Cruz Biotech (CA, USA). ELISA kits of IL-10, IL-4, IL-5, IL-13, specific IgE, mMCP1 were purchased from Dakewe Biomart (Beijing, China). FITC-IL-10, APC-CD19, PE-CD25 and Cy5-CD4 antibodies were purchased from BD Biosciences (NJ, USA). CD40L and FITC-dextran were purchased from Sigma Aldrich (MO, USA). Reagents and materials for real-time quantitative RT-PCR (RT-qPCR) and Western blotting were purchased from Invitrogen (CA, USA).

Mice

Male BALB/c mice (6–8-week old; to use male mice was to avoid the possible influence of the menstrual circle on experiments) were obtained from the Guangdong Experimental Animal Center (Guangzhou, China). In a specific pathogen free facility at Shenzhen University, the mice were maintained. These mice could access water and food freely. The animal experimental protocol was reviewed and approved by the Animal Ethics Committee at Shenzhen University.

Epithelial cell culture

A mouse airway epithelial cell line, MLE-15 cells (hereafter epithelial cell [EC], in short), was purchased from the abm (BC, Canada). The cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. The medium was changed in 1 or 2 days. Cell viability was more than 99% as assessed by Trypan exclusion assay.

Real-time quantitative RT-PCR

Total RNAs were extracted from cells collected from relevant experiments and converted to cDNA with a reverse transcription kit. The samples were amplified in a qPCR device with the SYBR Green Master Mix and the presence of IL-10 primers, ataactgcaccacttccca and ggcatcactctaccaggt. The results were presented as fold changes against the housekeeping gene β -actin.

Western blotting

Cells were collected from relevant experiments. The proteins were extracted from the cells and separated by SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated with 5% skim milk for 30 min to block nonspecific binding, incubated with primary antibodies (1:500) overnight at 4°C, washed with TBST (Tris-buffered saline containing 0.1 triton-x100) three-times, stained with the peroxidase-conjugated secondary antibodies for 2 h at room temperature and washed with TBST three-times. The immunoblots on the membrane were developed with the enhanced chemiluminescence and photographed with an imaging station (UVI, Cambridge, UK).

Flow cytometry

In the surface staining, cells were incubated with antibodies of interest (labeled with fluorochrome) or isotype IgG for 30 min at 4°C. In the intracellular staining, cells were treated with the fixative/permeable reagent first, then incubated with antibodies of interest (labeled with fluorochrome) or isotype IgG for 30 min at 4°C. The cells were then washed with saline three-times and analyzed with a flow cytometer. With the data of isotype IgG staining as a gating reference, the data were analyzed with a software package flowjo.

Isolation of mononuclear cells from the nasal mucosa tissues & the lung tissues

Immediately after the sacrifice, the nasal mucosa tissues and lung tissues were excised from mice. The tissues were cut into small pieces (2 × 2 × 2 mm). The samples were incubated with collagenase IV (1 mg/ml) at 37°C with mild agitation for 2 h. The single cells in the lysates were filtered through a cell strainer (70 μ m diameter first,

followed by 40 μm). Mononuclear cells were further isolated from the single cells by the Percoll gradient density centrifugation and cultured for further experiments. Cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Isolation of immune cells

Single cells were prepared from the BALB/c mouse spleen, stained with fluorescence-labeled antibodies and sorted out by flow cytometry cell sorting (FCCS). The cell purity was checked by flow cytometry. If the purity did not reach or over 95%, FCCS was repeated with the cells. The cells were cultured in RPMI1640 medium. The medium was supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine. The medium was changed in 2–3 days. Cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Preparation of *Clostridium butyricum* extracts (CBe)

Probiotics (*Clostridium butyricum*) were harvested from the culture and centrifuged at $5000\times g$ for 10 min. The pellets were lysed by incubation with the RIPA lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4 and protease inhibitor cocktail; Solarbio Life Science, Beijing, China) following the manufacturer's instructions. The lysates were centrifuged at $10,000\times g$ for 10 min at 4°C . The supernatants were collected. Proteins were quantified by using the BCA method.

Priming ECs by CBe

ECs were cultured at 5×10^6 cells/ml in the presence of CBe (10 $\mu\text{g}/\text{ml}$) for 48 h. The ECs were collected, washed with fresh medium and used as the primed ECs.

Induction of IL-10-producing B cells (iB10 cells)

B cells were isolated from naive mouse spleen by FCCS and cultured in basal chambers of transwells (5×10^6 cells/ml) with primed ECs in apical chambers (5×10^6 cells/ml). 6 days later, $\text{CD}25^+$ B cells were isolated from the B cells. As analyzed by flow cytometry, more than 90% $\text{CD}19^+ \text{CD}25^+$ B cells show IL-10 $^+$ (data not shown).

Assessment of immune suppressive function of iB10 cells

$\text{CD}4^+ \text{CD}25^-$ T cells were isolated from naive mouse spleen by FCCS and used as effector T cells (Teffs). Teffs (labeled with CFSE) and iB10 cells were co-cultured at a ratio of 5:1. Anti-CD3 (5 $\mu\text{g}/\text{ml}$) and anti-CD28 (2 $\mu\text{g}/\text{ml}$) antibodies were added to the culture to activate Teffs. CD40L (5 $\mu\text{g}/\text{ml}$) was added to the culture to activate iB10 cells. Cells were collected 3 days later and analyzed with a flow cytometer. The effects of iB10 cells on reducing the Teff proliferation were regarded as the immune suppressive function of iB10 cells.

Development of an AR mouse model

Following our established procedures [12], an AR mouse model was developed. Briefly, mice were subcutaneously injected with ovalbumin (OVA, a specific antigen) mixed in 0.1 ml alum on the caudal back skin on day 0, day 3 and day 6, respectively. The mice were challenged by receiving nasal drops (50 μl per nostril per day; 50 mg/ml) daily from day 14 to day 20. After the last challenge with OVA, AR symptoms, including sneezing counts, nasal itch (nasal scratching counts) were recorded from each mouse during 2 h after the nasal drops. The mice were sacrificed by decapitation. The blood was collected by eye ball pulling. The serum was isolated and analyzed by ELISA. The nasal mucosa tissue was collected by scraping with a spatula, immediately after the sacrifice. The AR pathological changes were assessed with our established procedures [13].

Treatment of AR mice with CBe

One day after completing the sensitization, AR mice were received nasal drops at 50 $\mu\text{l}/\text{nostril}$ (containing CBe at 50 mg/ml saline) daily for 7 days. Control AR mice were treated with saline (the vehicle for the CBe-containing nasal drops).

Depletion of B cells

In the course of treating AR mice with CBe-nasal drops, mice were injected with anti-CD22 antibodies (160 mg/kg) on day 0 and day 2, respectively. B cells were completely depleted in mice (data not shown).

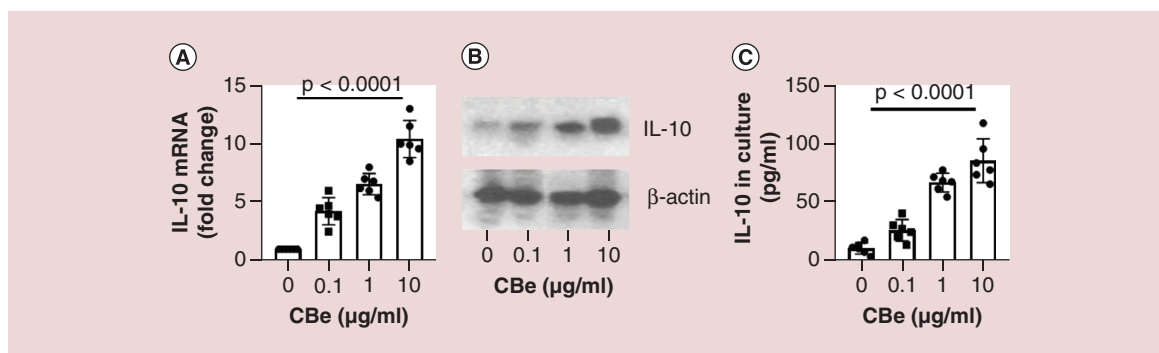


Figure 1. *Clostridium butyricum* extracts (CBe) induce epithelial cells to produce IL-10. ECs (a mouse intestinal epithelial cell line; 10^6 cells/ml) were cultured in the presence of CBe at indicated concentrations for 48 h. **(A)** Bars indicate IL-10 mRNA in ECs (by RT-qPCR). **(B)** Immunoblots indicate IL-10 protein in ECs (by Western blotting). **(C)** Bars indicate IL-10 in culture supernatant (by ELISA). Data of bars are presented as mean $\pm$ SEM. Each dot in bars present data obtained from one independent experiment. Statistics: ANOVA. The data represents independent experiments. CBe: *Clostridium butyricum* extract; EC: Epithelial cell; RT-qPCR: Real-time quantitative RT-PCR; SEM: Standard error of the mean.

Statistics

The difference between two groups was determined by Student *t* test. ANOVA followed by Dunnett's test or Bonferroni test was performed for multiple comparisons. The $p < 0.05$ was considered statistical significance.

Results

Extracts of *C. butyricum* (CBe) induces ECs to produce IL-10

ECs were cultured in the presence of CBe for 48 h. The cells were analyzed by RT-qPCR and Western blotting. The results showed that exposure to CBe induced the expression of IL-10 by ECs in a CBe concentration-dependent manner (Figure 1A & B). IL-10 was also detected in culture supernatant that was in parallel to the expression of IL-10 in ECs (Figure 1C). The results indicate that exposure to CBe can induce ECs to produce IL-10. The IL-10 can be released to the microenvironment.

CBe-primed ECs induce iB10 cells (inducible B10 cells) in the culture

Since IL-10 is an immune regulatory mediator, IL-10 producing B cells have the immune suppressive function and called B10 cells [14], we inferred that CBe-primed ECs could induce B10 cells. To test this, ECs were primed by exposing to CBe in the culture for 48 h. The ECs were washed with fresh medium and cultured with naive B cells (isolated from the naive mouse spleen) in a transwell system for 1 week (ECs in apical chambers and B cells in basal chambers. We employed such a model to differentiate the induction of iB10 cells is not by the cell–cell contact manner). The B cells were analyzed by flow cytometry, RT-qPCR and Western blotting. As shown by Figure 2A and B, co-culture with CBe-primed ECs induced IL-10-expression B cells (hereafter, iB10 cells), while naive ECs could not. To verify the role of IL-10 in the induction of iB10 cells, AS101, an IL-10 inhibitor, was added to the culture. Indeed, the induction of iB10 cells was abolished. The results indicate that CBe-primed ECs can induce iB10 cells, in which IL-10 plays a critical role.

Local application of CBe induces iB10 cells in the airway tissues

To verify the effects of CBe on inducing iB10 cells, an *in vivo* experiment was carried out. Mice were treated with nasal drops containing CBe daily for 1 week. Markedly increase in iB10 cells in the airway tissues was detected, that was abolished by concomitantly application of AS101. However, application of IL-10-containing nasal drops did not induce iB10 cells in the airway mucosa (Figure 3). The results demonstrate that CBe can induce iB10 cells in the airway mucosa.

CBe-induced iB10 cells have immune suppressive functions

We then tested the immune suppressive functions of the iB10 cells. Effector $CD4^+$ $CD25^-$ T cells (Teffs, labeled with CFSE) were isolated from naive mouse spleens. iB10 cells and Teffs were co-cultured at a ratio of 1:5 in the presence of activators. The cells were harvested 3 days later and analyzed by flow cytometry. As shown by

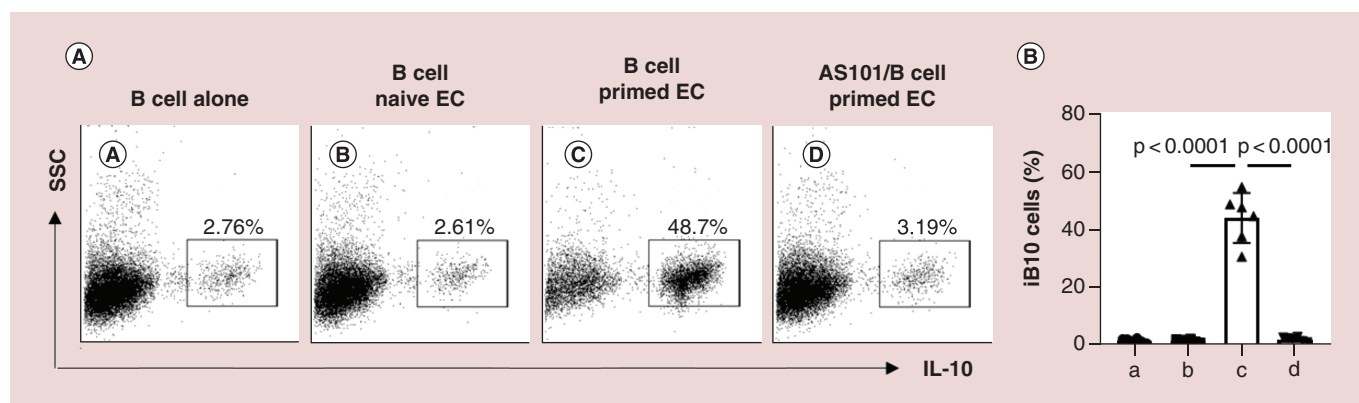


Figure 2. *Clostridium butyricum* extracts induces iB10 cells. CBe-primed ECs (10^6 cells/ml; in apical chamber) were cocultured with naive B cells (10^6 cells/ml; in basal chamber) in a transwell system at a ratio of 1:1 for 1 week. The culture conditions are denoted above each panel. (A) Gated dot plots indicate the frequency of iB10 cells. (B) Bars indicate summarized data of iB10 cells in panel A. Data of bars are presented as mean $\pm$ SEM. Each dot in bars presents data obtained from one experiment. Statistics: ANOVA/Bonferroni test. The data represent six independent experiments.

CBe: *Clostridium butyricum* extract; SEM: Standard error of the mean.

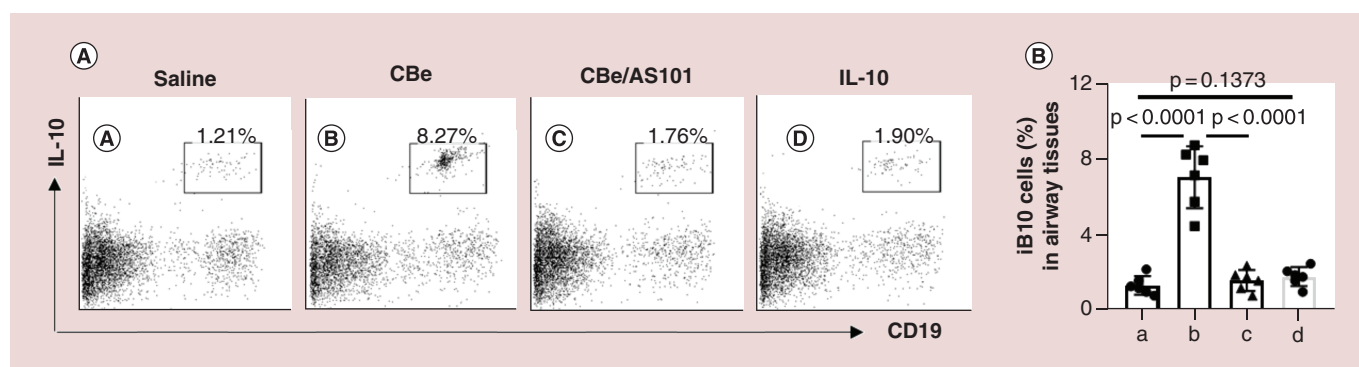


Figure 3. *Clostridium butyricum* extracts nasal drops induce iB10 cells in the airway mucosa. Naive mice (six mice per group) were treated with nasal drops (50 μ l/nostril) as denoted above each subpanel (CBe [50 mg/ml]; AS101, an inhibitor of IL-10 [0.5 μ g/ml]; IL-10 [20 μ g/ml]) daily for 1 week. Mice were sacrificed 1 day after the last nasal drops. The nasal tissues and lungs were excised. Mononuclear cells were isolated from the tissues and analyzed by flow cytometry (10^6 cells per sample). (A) Gated dot plots show iB10 cells isolated from the airway tissues. (B) Summarized data of panel C. Data of bars are presented as mean $\pm$ SEM. Each dot present data obtained from one experiment or one mouse. Statistics: ANOVA/Bonferroni test. The data represent six independent experiments.

CBe: *Clostridium butyricum* extract; SEM: Standard error of the mean.

Figure 4. iB10 cells did have the immune suppressive functions by showing the efficient suppressive effects on Teff proliferation. The results demonstrate that the iB10 cells have the immune suppressive functions.

Local administration of CBe inhibit AR in a mouse model

We further tested the immune regulatory effects of CBe on experimental AR. An AR mouse model was developed. The AR mice showed AR-like symptoms and Th2-dominant pathological changes, including sneezing, nasal itch and profound nasal discharge; enhanced serum specific IgE, mouse mast cell protease-1 (mMCP1), increases in Th2 cytokines in the nasal mucosa and increases in nasal epithelial barrier permeability, which were significantly attenuated by administration of nasal drop containing CBe (Figure 5). The data demonstrate that the immune suppressive effects of CBe we observed in *in vitro* can be reproduced in *in vivo* experiments. Administration of CBe nasal drop can inhibit AR in mice.

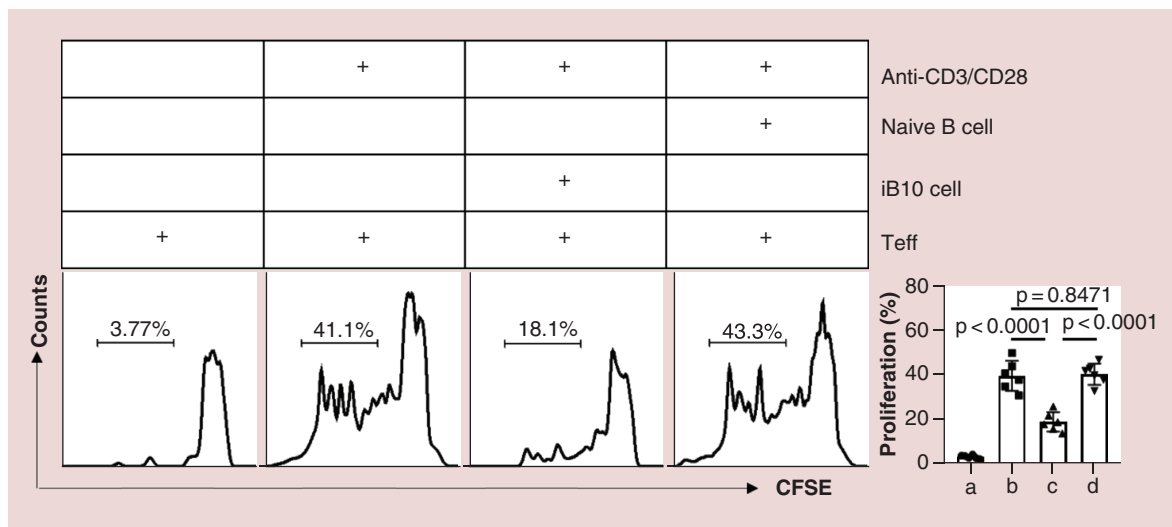


Figure 4. *Clostridium butyricum* extracts-induced iB10 cells have immune suppressive function. iB10 cells were prepared and co-cultured with Teffs (labeled with CFSE) at a ratio of 1×10^5 cells: 5×10^5 cells for 3 days and analyzed by flow cytometry. Gated histograms show frequency of proliferating Teffs. Bars indicate summarized proliferating Teffs. Data of bars are presented as mean $\pm$ SEM. Each dot in bars presents data obtained from one experiment. Statistics: ANOVA/Bonferroni. The data represent six independent experiments. SEM: Standard error of the mean.

Discussion

The present study reveals that exposure to CBe can induce ECs to produce IL-10 and then induce iB10 cells. The iB10 cells have the immune suppressive functions. Locally administration of CBe efficiently ameliorated experimental AR. Although administration of *C. butyricum* was used in the management of allergic diseases previously [15–17], using CBe in the treatment of allergic diseases as shown by the present study is the first time to report.

Probiotics have been used in clinic as supplements in the treatment of immune disorders. The therapeutic efficacy of probiotics in immune disorders varies in reports from different study groups. It may be beneficial for the prevention or treatment of allergic dermatitis [18]. However, most studies demonstrate that although probiotics used by pregnant women or breast-feeding mothers and/or given to infants may reduce the risk of eczema, no effect was observed for the prevention of other allergic conditions [19]. Because ingested via dieting, or oral administration, the effective components of probiotics may be degraded in the digestive tract. Therefore, we prepared the extracts of probiotics and locally administered in the airway by nasal drops. We got favorable results in the suppression of experimental AR. The results suggest that local administration of probiotic extracts may be an alternative approach to intervene allergic disorders.

The data show that local administration of CBe induces ECs to produce IL-10. Apart from the barrier functions, ECs also have immune functions. For example, our previous work showed that epithelial cell-derived exosomes can be internalized by dendritic cells (DCs) to imprint DCs to be tolerogenic phenotypes, that is essential for immune tolerance development [20]. ECs produce cellular retinol-binding protein II; this protein activates retinaldehyde dehydrogenase (ALDH1) in DCs. ALDH1 is required in RA transforming into all-trans retinoid acid, the active ingredient for Foxp3⁺ Treg conversion [21]. Our data provide novel information that probiotic extracts can drive ECs to produce immune tolerant molecules, IL-10, revealing that ECs can induce the IL-10-dependent immune regulatory activities. Of course, ECs also promote the inflammatory response by producing IL-25, IL-33 or thymic stromal lymphopoietin (TSLP), ECs also have immune tolerogenic property by inducing naive DCs to tolerogenic DCs [10], express PD-1L to induce T-cell apoptosis [22], produce corticosteroids to regulate immune responses [23]. Based on the present data, together with others' reports, we may envisage a scenario that, upon proper stimuli, epithelial cells produce IL-10 to release into the microenvironment, where the IL-10 contacts naive B cells to induce B cells to be iB10 cells. The iB10 cells suppress other immune cell activities to fulfill the immune regulatory role. We emphasize the role of iB10 cells in mediating the effects of CBe on suppressing experimental AR because depletion of B cells abolished the therapeutic effects of CBe.

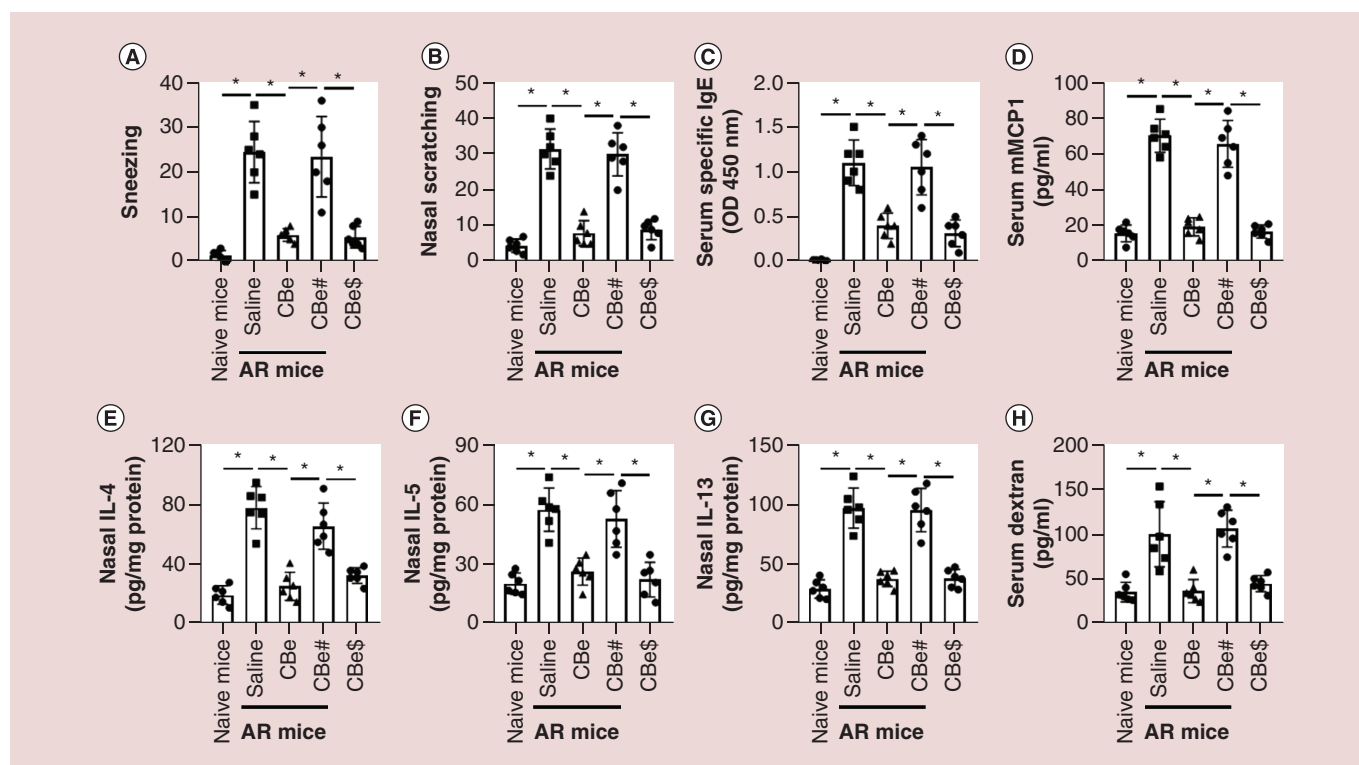


Figure 5. *Clostridium butyricum* extracts suppresses experimental allergic rhinitis. An AR mouse model was developed. The mice (six mice per group) were treated with nasal drops containing CBe (or saline) daily for 7 days. (A & B) Bars indicate AR response (sneezing [A] and nasal itch [B, scratch]) in mice recorded during 2 h after the last challenge with OVA. (C & D) Bars show serum specific IgE (sIgE, C) and mouse mast cell protease-1 (mMCP1, D) levels. (E–G) Bars show IL-4, IL-5 and IL-13 levels in mouse nasal mucosal protein extracts. (H) Bars indicate FITC-dextran levels in the serum. #, to deplete B cells, mice were treated with anti-CD22 antibody. \$, mice were treated with control Ab (isotype IgG). Each group consists of six mice. Data of bars are presented as mean ± SEM. Each dot presents data obtained from one experiment.

*p < 0.01 (ANOVA/Bonferroni).

AR: Allergic rhinitis; CBe: *Clostridium butyricum* extract; SEM: Standard error of the mean.

We observed that administration of CBe efficiently inhibited experimental AR in a mouse model. The results indicate that local administration of CBe can suppress aberrant immune responses in the airway mucosa. Currently, the therapeutic remedies of AR mainly include specific immunotherapy or immune suppressors, such as corticosteroids [4]. Although the therapeutic effects of specific immunotherapy are specific, the therapeutic course is too long, the therapeutic effects need to be improved [24]. The effects of corticosteroids are satisfactory though, the effective period is short, it only lasts for a few hours or 1 day [25]. Therefore, it is necessary to fundamentally adjust the aberrant immune response in AR. The present data shed light in this area. By local application of CBe, the AR symptoms and immune pathological changes can be attenuated. This has the translational potential for the treatment of AR or other allergic diseases.

Since EC-derived IL-10 is the cardinal mediator to induce iB10 cells, we tested the role of directly application of IL-10 in the induction of iB10 cells. It is noteworthy that direct application of IL-10 to the airway mucosa did not induce iB10 cells. This is probably that the exogenous IL-10 may be degraded within ECs of the airway mucosa.

Conclusion

The present data show that ECs can induce iB10 cells. The EC-induced iB10 cells have the immune suppressive function and inhibits experimental AR. On the other hand, local administration of probiotic extracts can efficiently inhibit AR in mice. The results demonstrate that local administration of probiotic extracts has the translational potential in the treatment of AR and other allergic diseases.

Summary points

- The therapeutic efficacy of allergic rhinitis (AR) needs to be improved.
- Probiotic extracts, *Clostridium butyricum* extract (CBe), were prepared.
- Epithelial cells produced IL-10 after stimulating with CBe.
- CBe-primed B cells differentiated into regulatory B cells (iB10 cells).
- The iB10 cells had immune regulatory function.
- A mouse AR model was developed.
- Treatment with nasal drops containing CBe inhibited AR in mice.

Acknowledgments

X-H Zeng, G Yang, J-Q Liu, X-R Geng and B-H Cheng performed experiments, analyzed data and reviewed the manuscript. P-C Yang and Z-Q Liu organized the study and supervised experiments. P-C Yang designed the project and wrote the manuscript.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved. The authors state that they have obtained verbal and written informed consent from the patient/patients for the inclusion of their medical and treatment history within this case report.

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