

Probiotic extracts ameliorate nasal allergy by inducing interleukin-35-producing dendritic cells in mice

Jin-Mei Xue, MD, PhD^{1,*}, Fei Ma, MD, PhD^{2,3,*}, Yun-Fang An, MD, PhD^{1,*}, Li-Min Suo, MD, PhD^{1,*},
Xiao-Rui Geng, MD¹, Yan-Nan Song, MS², Li-Hua Mo, MS⁴, Xiang-Qian Luo, MD, PhD⁴,
Xiao-Wen Zhang, MD, PhD³, Da-Bo Liu, MD⁴, Chang-Qing Zhao, MD, PhD¹ and Ping-Chang Yang, MD,
PhD² 

Background: The therapeutic efficacy of allergic rhinitis (AR) needs to be improved. Probiotics have immunoregulatory functions. In this study we evaluated the effects of protein extracts of probiotics in the amelioration of AR.

Methods: Extracts of *Bifidobacterium infantis* (EBI) were prepared by lysing the live probiotics. AR mice were developed to be used to evaluate the therapeutic efficacy of EBI.

Results: The results show that EBI induced interleukin (IL)-10-producing dendritic cells (DCs) via increasing IL-35 and signal transducer and activator of transcription 3 (STAT3) phosphorylation. IL-10-expressing DCs induced IL-10-producing B cells (B10 cells), with the latter showing immunosuppressive functions. After challenge with specific antigens, AR mice showed sneezing, nasal itch, and increases in serum-specific immunoglobulin E (IgE) and mouse mast cell protease-1; higher levels of T helper 2 (Th2) cytokines (IL-4, 67.17 ± 10.66; IL-5, 62.83 ± 9.70; IL-13, 51.00

± 6.69, before treatment) in nasal mucosal protein extracts, which were significantly suppressed (IL-4, 27.00 ± 6.66; IL-5, 23.86 ± 4.53; IL-13, 25.67 ± 4.93, after treatment ($p < 0.001$) by administration with EBI nasal drops.

Conclusion: EBI can suppress AR via inducing B10 cells. Thus, after carrying out required preclinical experiments and tests, EBI has the translational potential to be used in the treatment of AR and other allergic diseases. © 2019 ARS-AAOA, LLC.

Key Words:

allergic rhinitis; probiotics; nasal mucosa; B cell; dendritic cell

How to Cite this Article:

Xue J-M, Ma F, An Y-F, et al. Probiotic extracts ameliorate nasal allergy by inducing interleukin-35-producing dendritic cells in mice. *Int Forum Allergy Rhinol.* 2019;00:1-8.

Allergy (AR) is an adverse response to food antigens by the immune system in the airway mucosa. The under-

lying mechanisms of AR are not clear. The prevalence of AR is >10% in worldwide.^{1,2} AR clinical symptoms include nasal itch, sneezing, nasal congestion, and profound nasal secretions. Besides, AR is often complicated with nasal polyposis and rhinosinusitis or allergic asthma.^{3,4} The therapeutic efficacy in AR needs to be further improved.⁵ In fact, AR has had a major negative impact on human health and the economy. Therefore, it is necessary to investigate its mechanisms and establish novel and effective treatments for treatment of AR.

AR is an immune disease featured as T helper 2 (Th2)-dominant immune inflammation in the nasal mucosa. AR nasal tissues are overpopulated by Th2 cells, mast cells, and eosinophils.⁶ Th2 cells produce large amounts of Th2 cytokines, including interleukin (IL)-4, IL-5, and IL-13. Th2 cytokines induce plasma cells and promote plasma cells to produce immunoglobulin E (IgE). IgE binds to the high-affinity receptors of IgE on the surface of mast cells to make mast cells sensitized. Re-exposure to specific antigens activates sensitized mast cells to evoke AR response.⁶

Under physiologic conditions, immune responses in the body are tightly regulated by the immunoregulatory

¹Department of Otolaryngology, Head & Neck Surgery, Second Hospital, Shanxi Medical University, Taiyuan, China; ²Research Center of Allergy & Immunology, Shenzhen University School of Medicine, Shenzhen, China; ³Department of Otolaryngology, Head & Neck Surgery, First Affiliated Hospital, Guangzhou Medical University, Guangzhou, China; ⁴Department of Pediatrics, Shenzhen Hospital, Southern Medical University, Shenzhen, China

Correspondence to: Ping-Chang Yang, Room A7-509 at Xili Campus, Shenzhen University School of Medicine, 1066 Xueyuan Boulevard, Shenzhen 518055, China; e-mail: pcy2356@163.com

Additional supporting information may be found online in the Supporting Information section at the end of the article.

*J.-M.X., F.M., Y.-F.A., and L.-M.S. contributed equally to this work.

Funding sources for the study: National Nature and Science Foundation of China (81870706, 31570932, 81700888, 81701589, 81670914, 81870707, 81970865); Key Research and Development Projects of Shanxi Province (201703D321008); Shenzhen Science, Technology and Innovation Committee (JCYJ20170307162827158, KQTD20170331145453160).

Potential conflict of interest: None provided.

Received: 26 April 2019; Revised: 22 August 2019; Accepted: 3 September 2019

DOI: 10.1002/alr.22438

View this article online at wileyonlinelibrary.com.

systems. Regulatory T cells (Tregs) and regulatory B cells (Bregs) are the cardinal cellular components of the immunoregulatory system.⁷ Upon activation, Tregs or/and Bregs release immunoregulatory cytokines, such as IL-10 and transforming growth factor-beta (TGF- β), to suppress other immune cell activities to avoid inducing tissue injury.⁸ Overproduction of Th2 cytokines and profound infiltration of allergy-related proinflammatory cells in the airway tissues mirror the dysfunctional immunoregulatory system. The pathogenesis requires further investigation.

It is recognized that probiotics have immunoregulatory functions.⁹ Probiotics are commensal bacteria in the body. Administration of *Bifidobacterium infantis* has helped to enhance the therapeutic efficacy of specific immunotherapy in allergic diseases, including AR.¹⁰ In general, oral administration or gavage-feeding with live probiotics is used in the clinic or laboratory. Because of the extremely low pH in the stomach and differing living environment in the intestine, the ingested probiotics may die sooner or later. In this regard, the dead probiotic bacteria are degraded in the digestive tract. It cannot be ruled out that the components of the probiotic bacteria may be the effective part and at least partially associated with the immunoregulatory effects they deliver. Therefore, we hypothesized that the extracts of probiotics have immunoregulatory functions that can suppress immune inflammation. Thus, in this study, we prepared protein extracts from *B. infantis* (EBI) and an AR mouse model was developed for study. The effects of EBI on suppression of AR response were evaluated, and the underlying mechanism was investigated.

Materials and methods

Preparation of protein extracts of EBI

B. infantis (American Type Culture Collection strain 15697) cells were lysed by incubation with the RIPA lysis buffer (50 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl-sulfate, 2 mmol/L sodium pyrophosphate, 25 mmol/L β -glycerophosphate, 1 mmol/L ethylene-diamine tetraacetic acid, 1 mmol/L Na₃VO₄, and protease inhibitor cocktail [Solarbio Life Science, Beijing, China]) following the manufacturer's instructions. The lysates were centrifuged at 10,000g for 10 minutes. The supernatant was collected. The protein levels of supernatant were determined using a Bio-Rad protein assay. EBI was used in further experiments. Lipopolysaccharide (LPS) in EBI was eliminated by passing the extracts through a polymyxin column (Pierce Biotech).

Development of an AR mouse model

BALB/c mice were sensitized by subcutaneous injection with ovalbumin (OVA; 0.2 mg/mouse mixed in 0.1 mL alum). OVA is a model antigen that is commonly used to develop an allergic animal model.¹¹ Injections were administered on the back skin of each mouse on day 0, day 3,

and day 7, respectively. Mice were challenged with nasal drops (50 μ L per nostril, containing 5 mg/mL OVA) daily from day 9 to day 15. Mice were rechallenged with OVA by nasal drops on day 16 and killed 30 minutes later by decapitation. The truncated blood was collected for serum isolation. The nasal mucosa and lung tissue were collected for further experiments.

Evaluation of therapeutic efficacy of EBI in AR

After completing sensitization, AR mice were treated daily with nasal drops containing EBI (1 mg/mouse in 0.1 mL saline; control mice were received saline alone) from day 16 to day 22. AR response (sneezing and nasal scratches) were recorded over 30 minutes after the last challenge with OVA. The nasal mucosal tissues were collected from mice immediately after the animals were killed. Proteins were extracted from the tissue in a homogenizer containing protein extraction buffer. Levels of Th2 cytokines, specific IgE, and mouse mast cell protease-1 (mMCP1) were determined by enzyme-linked immunoassay with commercial reagent kits following the manufacturer's instructions.

Isolation of mononuclear cells from the airway mucosa

The nasal mucosa and lungs were extracted from mice upon killing. The tissue was cut into small pieces and incubated in RPMI 1640 medium containing collagenase IV (1 mg/mL) at 37°C for 2 hours with mild agitation. Single cells were collected by filtering cells through a cell strainer (70 μ m initially, then 40 μ m) and Percoll gradient density centrifugation.

Statistics

The difference between the 2 groups was determined by Student *t* test. Analysis of variance and Dunnett's test or Bonferroni test were carried out for multiple comparisons. *p* < 0.05 was considered statistically significant.

Results

EBI converts naive DCs to IL-10⁺ DCs

Peripheral blood mononuclear cells were isolated from blood samples collected from AR patients. The cells were cultured in the presence of EBI for 7 days. As analyzed by flow cytometry, exposure to EBI induced IL-10⁺ DCs (Fig. 1A and B). The data suggest that EBI can induce tolerogenic DCs (TolDCs). The results were verified in an in-vivo experiment. Naive BALB/c mice were treated daily with nasal drops containing EBI for 7 days. The mice were killed on day 8. Airway tissue mononuclear cells (AMCs) were isolated from the nasal mucosa and lungs and analyzed by flow cytometry. The results showed that nasal administration of EBI markedly induced IL-10⁺ DCs in the airway (Fig. 1C and D). The DCs were also CD25⁺ (Fig. 1E and F). We analyzed the CD25⁺ DCs; none of the CD25⁺ DCs were IL-10⁺ (Fig. 1G and H). The results demonstrate that nasal

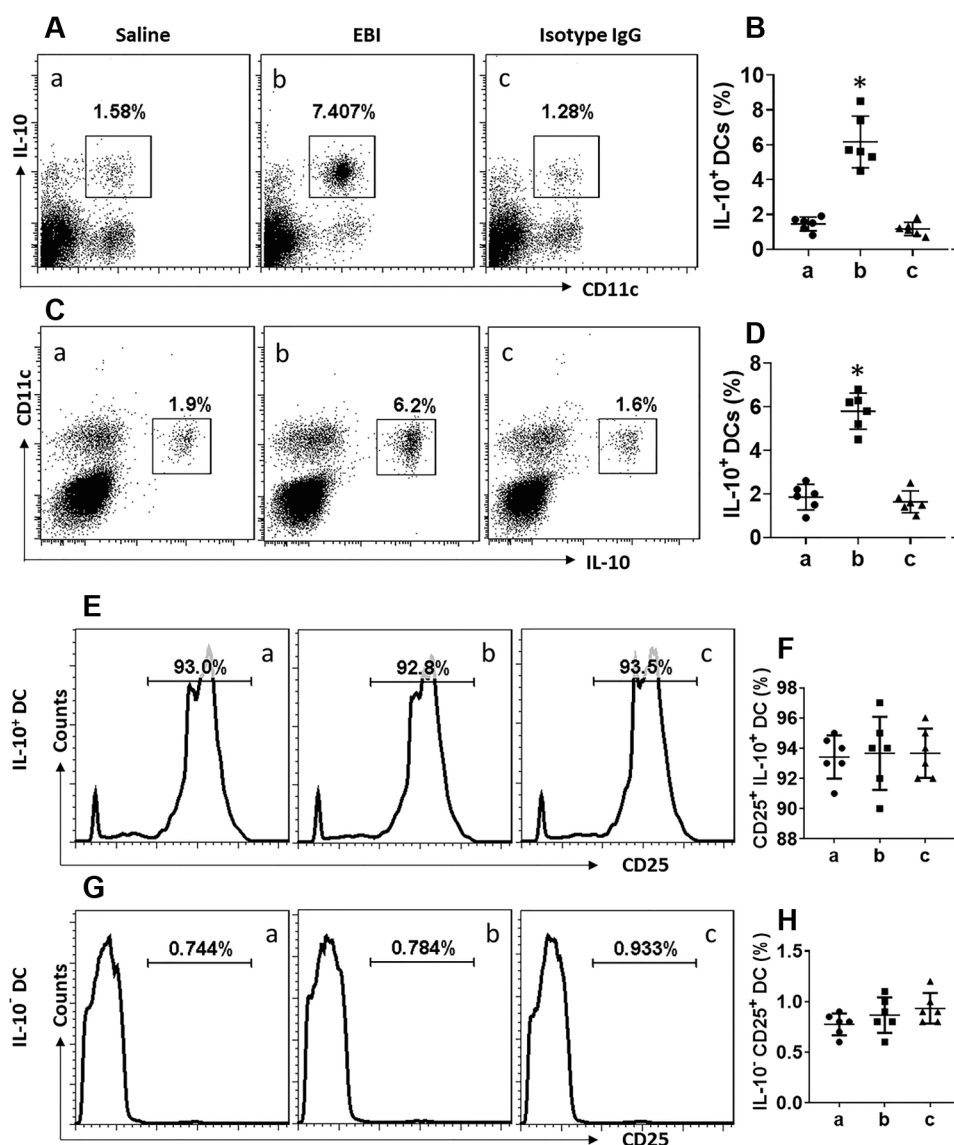


FIGURE 1. EBI generates IL-10⁺ DCs. (A) PBMCs were collected from AR patients ($n = 6$) and cultured in the presence of EBI (10 $\mu\text{g}/\text{mL}$) for 48 hours. The cells were analyzed by flow cytometry. Gated dot plots indicate frequency of IL-10⁺ CD11c⁺ DCs. (B) Summarized data of (A). (C-H) Naive BALB/c mice (6 mice per group) were treated with nasal drops (50 μL per mouse, containing with EBI 50 mg/mL) daily for 7 days. The nasal mucosal tissue and the lungs were excised postmortem. Airway mononuclear cells were isolated from the airway tissues and analyzed by flow cytometry. (C) The gated cell population demonstrates the frequency of IL-10⁺ CD11c⁺ DCs. (D) Summarized data of (C). (E) Gated histograms indicate the frequency of CD25⁺ cells in IL-10⁺ DCs. (F) Summarized data of (E). (G) Gated cells show CD25⁺ cells in IL-10⁺ DCs. (H) Summarized data of (G). Data of scatter dot plots are presented as mean $\pm$ SEM. Each dot presents data obtained from 1 mouse or 1 human subject. Bars indicate mean $\pm$ SEM. * $p < 0.01$ (t test) vs group a. Isotype IgG used as a control. AR = allergic rhinitis; DC = dendritic cell; EBI = extracts of *Bifidobacterium infantis*; IgG = immunoglobulin G; IL = interleukin; PBMC = peripheral blood mononuclear cell; SEM = standard error of the mean.

administration of EBI can induce IL-10-expressing DCs; these DCs are CD11c⁺ CD25⁺. Because IL-10-producing DCs have immune-tolerogenic properties,¹² this fraction of DCs may be TolDCs.

Induction of IL-10⁺ DCs by EBI in in-vitro experiments

To further test the effects of EBI on induction of TolDCs, an in-vitro experiment was carried out. DCs were isolated from the spleen of naive mice and cultured in the presence

of EBI for 1 week. The DCs were harvested and analyzed by flow cytometry. The results showed that exposure to EBI in the culture induced IL-10⁺ DCs in an EBI concentration-dependent manner. The cells were also analyzed by reverse transcript quantitative-polymerase chain reaction and Western blotting. The results show that the expression of IL-10 in DCs was induced by EBI in a concentration-dependent manner. Taking together the data in Figures 1 and 2, nasal administration of EBI can be seen to induce IL-10-producing IL-10⁺ DCs.

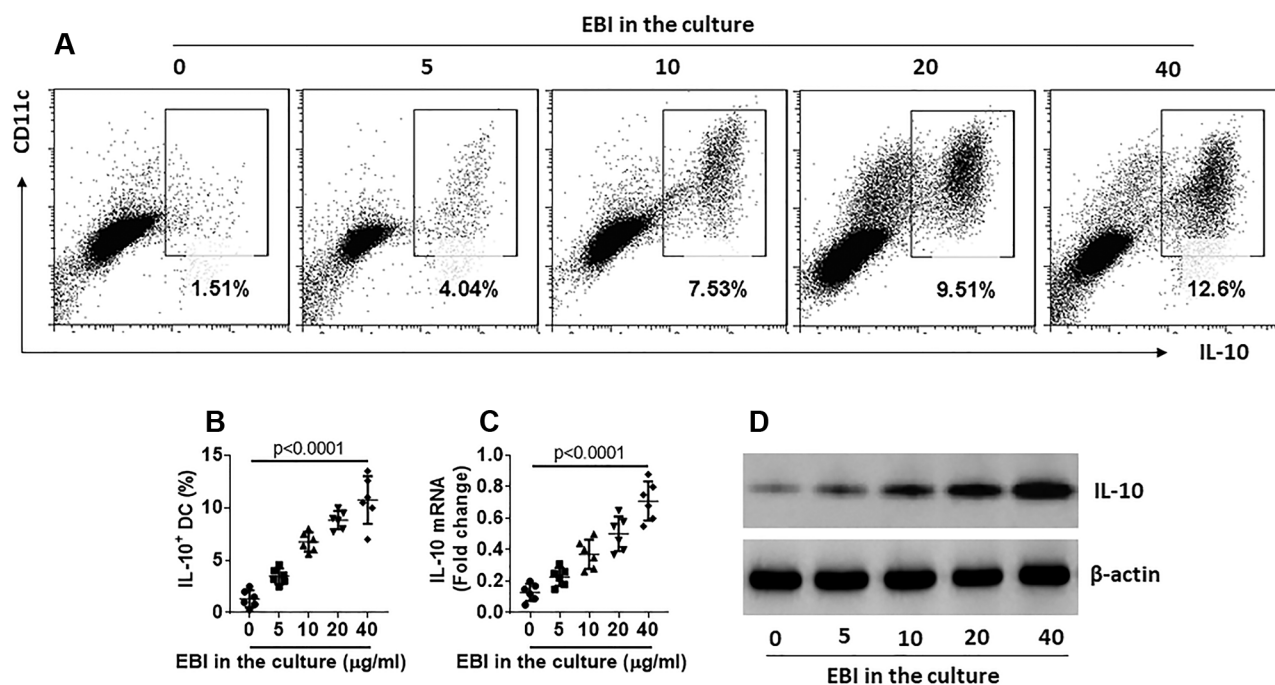


FIGURE 2. EBI induces IL-10⁺ DCs from BMDCs. BMDCs were generated and exposed to EBI at the indicated concentrations, as indicated above each subpanel of (A). One week later the cells were analyzed by flow cytometry (A). The gated dot plots indicate frequency of B10 cells. (B) Summarized data of B10 cells in (A). (C) Summarized data of IL-10 mRNA levels in DCs of (A). (D) Immunoblots show IL-10 protein levels in the DCs of (A). Each dot in (B) and (C) shows data obtained from a single experiment. Bars indicate mean $\pm$ SEM. Immunoblots in (D) are from a single experiment representing 3 independent experiments. Analysis of variance was applied (results of post-hoc tests not presented). BMDC = bone marrow dendritic cell; DC = dendritic cell; EBI = extracts of *Bifidobacterium infantis*; IL = interleukin.

EBI promotes IL-10 gene transcription in DCs by facilitating IL-35 expression and STAT3 phosphorylation

Previous reports showed that IL-35 plays a role in the generation of TolDCs.¹³ We sought to determine whether EBI also promoted IL-35 expression in DCs. For testing, bone marrow–derived dendritic cells (BMDCs) were exposed to EBI in the culture for 48 hours, which indeed increased the expression of IL-35 in BMDCs (Fig. 3A and B). In line with previous studies,¹⁴ we found that IL-35 promoted STAT3 phosphorylation (Fig. 3C), which was abolished by depletion of IL-35 (Fig. 3C and D). Because STAT3 is involved in the IL-10 expression,¹⁵ we then assessed the role of STAT3 in the EBI-induced IL-10 expression in DCs. After exposure to EBI with or without the presence of STAT3 inhibitors in the culture, we found that STAT3 was required in the EBI-induced IL-10 expression in DCs (Fig. 3E–G). The results demonstrate that EBI increases the expression of IL-35; the latter facilitates STAT3 phosphorylation and then increases IL-10 expression in DCs.

EBI induces IL-10–producing B cells (B10 cells)

Because TolDCs can induce immunoregulatory cells, we next tested the role of EBI in the induction of B10 cells. By treating mice with nasal drops (containing EBI) for 7 days, AMCs were isolated from the airway tissues. The cells were analyzed by flow cytometry. The results show that

EBI could induce B10 cells in the airway mucosa (Fig. 4). Because EBI-induced DCs also express CD25 as shown by Figure 1, mice were treated with EBI as well as depletion of CD25⁺ cells. The induction of B10 cells was abolished (Fig. 4). The results demonstrate that EBI can induce B10 cells in the airway tissue by inducing IL-10⁺ DCs.

EBI-induced B10 cells have an immunosuppressive function

Based on the fact that IL-10 is an immunosuppressive mediator, we inferred that the EBI-induced B10 cells had an immunosuppressive function. To test the inference, effector CD4⁺ T cells (Teffs) were isolated from the spleen of naive mice. B10 cells were prepared by EBI. Teffs (labeled with carboxyfluorescein succinimidyl ester) were cocultured with B10 cells at a ratio of 5:1 in the presence of activators for 3 days. The cells were analyzed by flow cytometry. The results show that, compared with naive B cells, B10 cells efficiently suppressed the proliferation of Teffs (Fig. 5). The results demonstrate that EBI-induced B10 cells have immunosuppressive functions.

EBI inhibits AR by inducing B10 cells

To test the immunosuppressive function of EBI in vivo, we developed an AR mouse model. The AR mice were treated with EBI or saline (control) from day 21 to day 27 in the course of sensitization. After challenging with specific

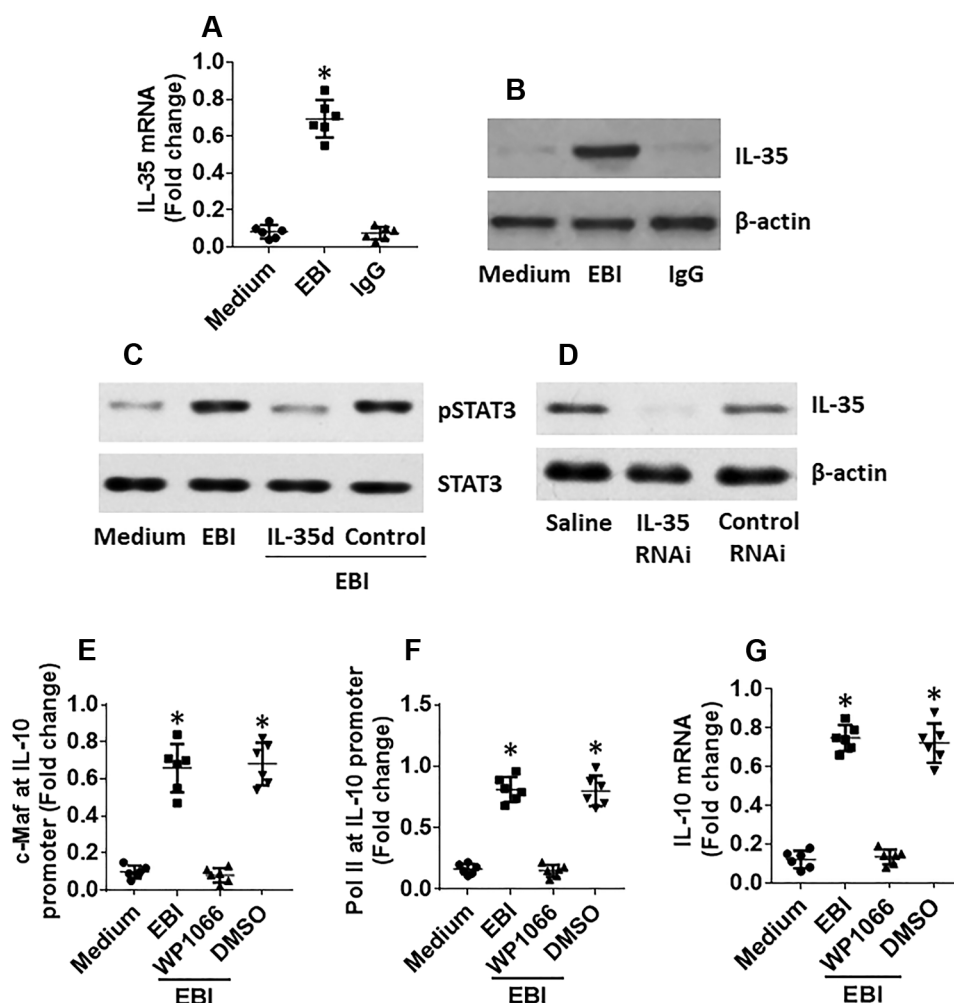


FIGURE 3. EBI increases IL-35 expression and STAT3 phosphorylation to upregulate IL-10 gene transcription in DCs. BMDCs were exposed to EBI (40 μ g/mL) or IgG (naive mouse IgG) for 48 hours. (A, B) Expression of IL-35 in BMDCs. (C) STAT3 phosphorylation in BMDCs after exposure to EBI. For control, BMDCs were treated with control CRISPR (Clustered regularly interspaced short palindromic repeats). (D) Results of IL-35 RNAi (RNA interference) in BMDCs. (E, F) IL-10 gene transcription activity, as indicated by levels of c-Maf and Pol II at the IL-10 promoter locus in BMDCs. (G) IL-10 mRNA levels in BMDCs. Each dot represents data obtained from a single experiment. Bars indicate mean $\pm$ SEM. Immunoblots were from a single experiment representing 3 independent experiments. BMDC = bone marrow dendritic cell; c-Maf = interleukin-10 gene transcription factor; CRISPR = Clustered regularly interspaced short palindromic repeats; DC = dendritic cell; DMSO = dimethylsulfoxide; EBI = extracts of *Bifidobacterium infantis*; IgG = immunoglobulin G; IL = interleukin; IL-35d = IL-35-deficient bone marrow dendritic cell; Pol II = RNA polymerase II; SEM = standard error of the mean.

antigens, AR mice showed AR clinical symptoms (sneezing and nasal itch), allergic serologic changes (enhancement of serum-specific IgE and mMCP1), and a Th2-dominant response in the nasal mucosa (increases in Th2 cytokines in protein extracts of nasal mucosa), which were inhibited by administration of EBI; such an inhibitory effect was counteracted by depletion of B10 cells with an anti-CD22 antibody (Fig. 6). The results indicate that EBI can inhibit experimental AR, in which B10 cells play a critical role.

Discussion

In this study, we found that EBI, a protein extract from *B. infantis*, could inhibit experimental AR in mice. Exposure to EBI in the culture induced TolDCs; such an effect of EBI was reproduced in an in-vivo experiment. Treating mice

with EBI markedly induced TolDCs in the airway mucosa by induction of IL-35 and promotion of STAT3 phosphorylation. The induced TolDCs could induce B10 cells. The latter showed immunosuppressive function in the suppression of Teff proliferation.

Use of EBI to treat experimental AR achieved satisfactory therapeutic effects. This finding expands existing therapy using probiotics as a supplement in the treatment of immune diseases.¹⁶ In view of the current unsatisfactory therapeutic efficacy, studies have been investigating probiotics as a supplemental remedy in the treatment of various diseases.¹⁷ Our previous study showed that concomitant administration with specific immunotherapy and *Clostridium butyricum* results in significantly better efficacy in the treatment of experimental food allergy than with either specific immunotherapy or *C. butyricum* alone.¹⁸ Other studies

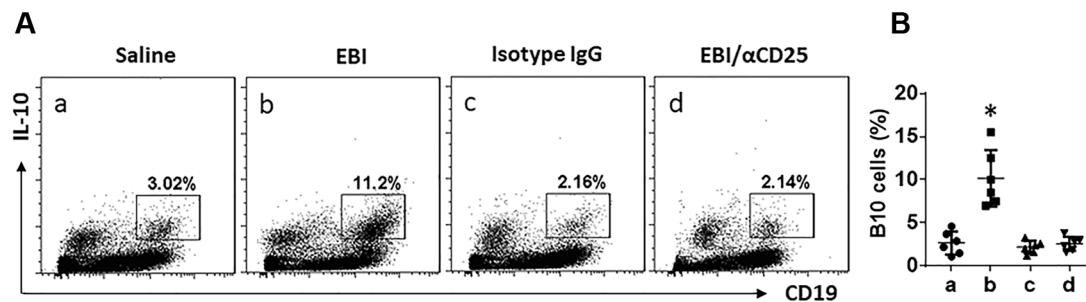


FIGURE 4. EBI induces B10 cells in airway tissues. BALB/c mice (6 mice per group) were treated with nasal drops (50 μ L per mouse, containing 50 mg/mL EBI) daily for 7 days. Nasal mucosal tissue and lungs were excised postmortem. Airway mononuclear cells were isolated from the airway tissues and analyzed by flow cytometry. (A) Gated cells are B10 cells in AMCs. (B) Summarized data of B10 cells from (A). Each dot in (B) indicates data obtained from 1 mouse. Bars indicate mean $\pm$ SEM. * p < 0.01 vs group a. = Mice were treated with α CD25 (0.25 mg per mouse intraperitoneally on day 9 in the course of sensitization). α CD25 = anti-CD25 antibody; AMC = airway tissue mononuclear cell; EBI = extracts of *Bifidobacterium infantis*; SEM = standard error of the mean.

showed probiotics had a positive effect on atopic dermatitis treatment.¹⁹ In an effort to expand the existing knowledge, in the present study we used EBI to induce TolDCs as well as B10 cells in the airway mucosa and suppressed the experimental AR.

We found that EBI-induced TolDCs expressed IL-35. Previous studies demonstrated expression of IL-35 in Treg²⁰ and Breg cells.²¹ Shamji et al reported that allergen-specific immunotherapy induced IL-35-producing type 2 innate lymphoid cells.²² Haller et al found that CD8⁺ DCs expressed IL-35, which directly suppressed CD4⁺ and CD8⁺ T-cell proliferation and contributed to immune tolerance.²³ Our data are in line with and expand those of Haller and colleagues by showing that IL-35-producing DCs can suppress experimental AR by inducing Bregs.

It was reported in a previous study that IL-35 facilitates STAT3 phosphorylation to promote IL-10 expression in B cells.¹⁴ We also found that EBI increased IL-35 in DCs and then promoted the phosphorylation of STAT3 in DCs. Thus, IL-35 is required in EBI-induced STAT3 phosphorylation in DCs. The STAT3/IL-10 axis also plays roles in other cellular activities, such as contribution to thymus-derived Treg generation²⁴ and microbial stimulus-induced IL-10 expression.¹⁵ Activation of the STAT3/IL-10 axis plays a critical role in the anti-inflammatory process.²⁵ Our data provide a practical remedy to upregulate this event to participate the anti-inflammatory activities.

The data show that EBI-primed DCs can induce B10 cells. Administration of EBI also induces B10 cells in the airway mucosa. There are several approaches to induction of IL-10 expression in B cells. One of them is that exposure to LPS in the culture or in vivo.²⁶ Because LPS was eliminated from EBI during preparation, it is clear that the effects of EBI on induction of IL-10 expression in B cells is not through LPS. Previous work has shown that TolDCs can induce B10 cells.²⁷ Our current data show that administration of EBI can induce TolDCs in the airway mucosa as well as B10 cells. Because blocking IL-10 abrogated the induction of B10 cells, IL-10 may serve as mediator in the induction of B10 cells by EBI. Of course, this does not rule out the possibility that EBI-induced immunoregulatory effects may be fully or partially due to alternatively activated macrophages²⁸ that produce IL-10 and may also induce regulatory B cell-dependent immune tolerance.

The present results show that nasal administration of EBI can efficiently inhibit AR response as well as adjust the Th2-biased immune response in the airway mucosa of AR mice. To date, the therapeutic effects on allergic diseases, including AR, remain satisfactory, even though specific immunotherapy has been employed in the treatment of allergic diseases for many years. Although administration of antihistamines or steroids can satisfactorily ameliorate most allergic diseases, their effects are temporary. Administration of live probiotics is also employed in the treatment

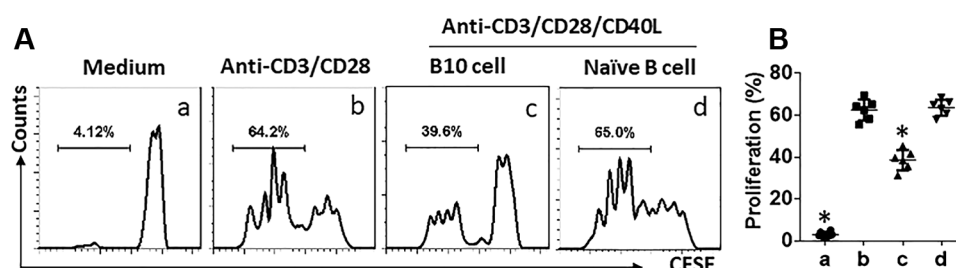


FIGURE 5. EBI-induced B10 cells have immunosuppressive functions. Teff cells were isolated from the naive mouse spleen, labeled with CFSE, and cultured with B10 cells at a ratio of 5:1 for 3 days under conditions denoted above each subpanel of (A). (A) Gated histograms show Teff proliferation frequency. (B) Dot plots show summarized data of Teff proliferation. * p < 0.01 vs group b. Bars indicate mean $\pm$ SEM. Each dot indicates data obtained from 1 mouse. CFSE = carboxyfluorescein succinimidyl ester; EBI = extracts of *Bifidobacterium infantis*; SEM = standard error of the mean; Teff = effector CD4⁺ CD25⁺ T cell.

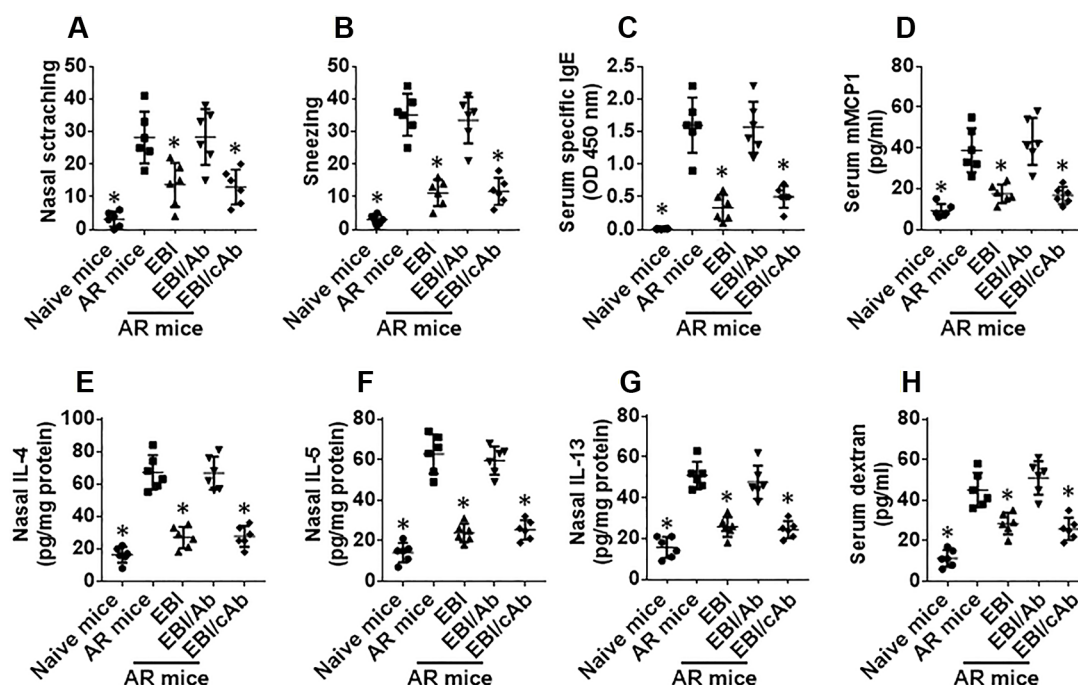


FIGURE 6. Administration of EBI inhibits experimental AR. An AR mouse model was developed. The mice were treated with procedures indicated on the x axis. (A, B) AR clinical symptoms recorded 2 hours after nasal challenge with specific antigen. (C, D) Serum levels of specific IgE and mMCP1. (E, G) Levels of IL-4, IL-5, and IL-13 in protein extracts of nasal mucosal tissue. (H) Serum dextran (labeled with TRITC) levels indicate nasal epithelial barrier function. Each group consisted of 6 mice. Each dot indicates data obtained from 1 mouse. Bars indicate mean $\pm$ SEM. * $p < 0.01$ vs AR-alone group. Ab = antibody; AR = allergic rhinitis; EBI = extracts of *Bifidobacterium infantis*; IgG = immunoglobulin G; IgE = immunoglobulin E; IL = interleukin; mMCP1 = mouse mast cell protease-1; TRITC = tetramethylrhodamine.

of allergic diseases, but their therapeutic efficacy remains uncertain. Positive suppression of serum-specific IgE or clinical symptoms of AR by probiotics have not been reported so far.²⁹ However, we found nasal administration of EBI could efficiently suppress specific IgE and clinical symptoms in experimental AR mice, and this may lead to a novel approach to treatment of AR.

In this investigation we used *B. infantis*, one of the probiotics used to alleviate experimental AR. There are many probiotics available for improving health or being used as supplementation in disease therapy. Our earlier work showed administration of *C. butyricum* markedly enhanced the therapeutic efficacy of antigen-specific immunotherapy¹⁸ by promoting immunoregulatory cell functions³⁰ and improving epithelial barrier functions.³¹ Other investigators also showed that probiotics have promise in primary prevention of allergic diseases

rather than in the treatment of established disease. Based on the present study findings, local application of probiotic extracts may further improve the effects of probiotics on prevention and/or treatment of allergic diseases.

In addition to suppressing AR in mice inducing regulatory B cells with nasal instillation, as shown here, other investigators found that gavage-feeding with probiotics could also alleviate experimental AR; for instance, Kim et al inhibited AR in mice using *Lactobacillus rhamnosus* by increasing Treg generation.³² It would be worthwhile to investigate whether nasal instillation of EBI also induces Treg generation.

In conclusion, the present data show that nasal administration of EBI can inhibit experimental AR via inducing TolDCs and B10 cells. The findings further suggest that EBI has translational potential in the treatment of allergic diseases. 

References

- Wang X, Zheng M, Lou HF, et al. An increased prevalence of self-reported allergic rhinitis in major Chinese cities from 2005 to 2011. *Allergy*. 2016;71:1170-1180.
- Mims JW. Epidemiology of allergic rhinitis. *Int Forum Allergy Rhinol*. 2014;4(Suppl 2):S18-S20.
- Khan DA. Allergic rhinitis and asthma: epidemiology and common pathophysiology. *Allergy Asthma Proc*. 2014;35:357-361.
- Sahay S, Gera K, Bhargava SK, Shah A. Occurrence and impact of sinusitis in patients with asthma and/or allergic rhinitis. *J Asthma*. 2016;53:635-643.
- Bernstein DI, Schwartz G, Bernstein JA. Allergic rhinitis: mechanisms and treatment. *Immunol Allergy Clin North Am*. 2016;36:261-278.
- Eifan AO, Durham SR. Pathogenesis of rhinitis. *Clin Exp Allergy*. 2016;46:1139-1151.
- Chien CH, Chiang BL. Regulatory T cells induced by B cells: a novel subpopulation of regulatory T cells. *J Biomed Sci*. 2017;24:86.
- Palomares O, Akdis M, Martin-Fontecha M, Akdis CA. Mechanisms of immune regulation in allergic diseases: the role of regulatory T and B cells. *Immunol Rev*. 2017;278:219-236.
- Kober MM, Bowe WP. The effect of probiotics on immune regulation, acne, and photoaging. *Int J Womens Dermatol*. 2015;1:85-89.
- Tsunemine S, Isa Y, Ohno H, et al. Longitudinal study of effects of oral dosage of *Bifidobacterium bifidum* G9-1 on Japanese cedar pollen-induced allergic nasal symptoms in guinea pigs. *Microbiol Immunol*. 2015;59:690-699.
- Sakat MS, Kilic K, Kandemir FM, et al. The ameliorative effect of berberine and coenzyme Q10 in an ovalbumin-induced allergic rhinitis model. *Eur Arch Otorhinolaryngol*. 2018;275:2495-2505.

12. Wan J, Huang F, Hao S, et al. Interleukin-10 gene-modified dendritic cell-induced type 1 regulatory T cells induce transplant-tolerance and impede graft versus host disease after allogeneic stem cell transplantation. *Cell Physiol Biochem*. 2017;43:353-366.
13. Liu X, Sun Y, Zheng Y, et al. Administration of interleukin-35-conditioned autologous tolerogenic dendritic cells prolong allograft survival after heart transplantation. *Cell Physiol Biochem*. 2018;49:1180-1196.
14. Zhu Z, Zhang Y, Ye J, et al. IL-35 promoted STAT3 phosphorylation and IL-10 production in B cells, but its production was reduced in patients with coronary artery diseases. *Hum Immunol*. 2018;79:869-875.
15. Irtegun Kandemir S, Tekin MA, Bozkurt M, Dagli AZ, Kalkanli-Tas S. LPS-induced Src family kinases activity mediates IL-10 production through activation of STAT3 in peripheral blood mononuclear cells of patients with Behcet's disease. *Cell Mol Biol (Noisy-le-grand)*. 2017;63:116-121.
16. Singh VP, Sharma J, Babu S, Rizwanulla Singla A. Role of probiotics in health and disease: a review. *J Pak Med Assoc*. 2013;63:253-257.
17. Chua KJ, Kwok WC, Aggarwal N, Sun T, Chang MW. Designer probiotics for the prevention and treatment of human diseases. *Curr Opin Chem Biol*. 2017;40:8-16.
18. Xu LZ, Yang LT, Qiu SQ, et al. Combination of specific allergen and probiotics induces specific regulatory B cells and enhances specific immunotherapy effect on allergic rhinitis. *Oncotarget*. 2016;7:54360-54369.
19. Rather IA, Bajpai VK, Kumar S, Lim J, Paek WK, Park YH. Probiotics and atopic dermatitis: an overview. *Front Microbiol*. 2016;7:507.
20. Li M, Eckl J, Geiger C, Schendel DJ, Pohla H. A novel and effective method to generate human porcine-specific regulatory T cells with high expression of IL-10, TGF-beta1 and IL-35. *Sci Rep*. 2017;7:3974.
21. Tarique M, Saini C, Naqvi RA, Khanna N, Rao DN. Increased IL-35 producing Tregs and CD19(+)IL-35(+) cells are associated with disease progression in leprosy patients. *Cytokine*. 2017;91:82-88.
22. Shamji MH, Layhadi JA, Achkova D, et al. Role of IL-35 in sublingual allergen immunotherapy. *J Allergy Clin Immunol*. 2019;143:1131-1142.e4.
23. Haller S, Duval A, Migliorini R, Stevanin M, Mack V, Acha-Orbea H. Interleukin-35-producing CD8alpha(+) dendritic cells acquire a tolerogenic state and regulate T cell function. *Front Immunol*. 2017;8:98.
24. Schmetterer KG, Pickl WF. The IL-10/STAT3 axis: contributions to immune tolerance by thymus and peripherally derived regulatory T-cells. *Eur J Immunol*. 2017;47:1256-1265.
25. Hutchins AP, Diez D, Miranda-Saavedra D. The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. *Brief Funct Genomics*. 2013;12:489-498.
26. Liu Z, Hu Y, Yu P, et al. Toll-like receptor agonists *Porphyromonas gingivalis* LPS and CpG differentially regulate IL-10 competency and frequencies of mouse B10 cells. *J Appl Oral Sci*. 2017;25:90-100.
27. Georg P, Bekeredjian-Ding I. Plasmacytoid dendritic cells control B cell-derived IL-10 production. *Autoimmunity*. 2012;45:579-583.
28. Jiang Z, Zhu L. Update on the role of alternatively activated macrophages in asthma. *J Asthma Allergy*. 2016;9:101-107.
29. Zajac AE, Adams AS, Turner JH. A systematic review and meta-analysis of probiotics for the treatment of allergic rhinitis. *Int Forum Allergy Rhinol*. 2015;5:524-532.
30. Liu J, Chen FH, Qiu SQ, et al. Probiotics enhance the effect of allergy immunotherapy on regulating antigen specific B cell activity in asthma patients. *Am J Transl Res*. 2016;8:5256-5270.
31. Song CH, Liu ZQ, Huang S, Zheng PY, Yang PC. Probiotics promote endocytic allergen degradation in gut epithelial cells. *Biochem Biophys Res Commun*. 2012;426:135-140.
32. Kim HJ, Kim YJ, Lee SH, Yu J, Jeong SK, Hong SJ. Effects of *Lactobacillus rhamnosus* on allergic march model by suppressing Th2, Th17, and TSLP responses via CD4(+)CD25(+)Foxp3(+) Tregs. *Clin Immunol*. 2014;153:178-186.

Supplemental materials

Materials and methods

Reagents

IL-35 RNAi kit, antibodies of CD3, CD28, IL-10, IL-35, STAT3, pSTAT3, c-Maf, Pol II, CD25 and CD22 were purchased from Santa Cruz Biotech (Santa Cruz, CA). Fluorochrome labeled antibodies for flow cytometry (IL-10, CD11c, CD25, CD19) were purchased from BD Biosciences (Franklin Lakes, NJ). CD40L, ELISA kits of IgE, mMCP1, IL-4, IL-5 and IL-13 were purchased from R&D Systems (Minneapolis, MN). Polymyxin, protein G agarose beads, collagenase IV, TRITC-dextran and WP1066 were purchased from Sigma Aldrich (St. Louis, MO). Limulus amoebocyte lysate QCL 1000 kit was purchased from Bio Whittaker (Walkersville, MD). Materials and reagents for RT-qPCR, ChIP and Western blotting were purchased from Invitrogen (Carlsbad, CA). Immune cell isolation kits were purchased from Miltenyi Biotech (San Diego, CA).

Mice

Male BALB/c mice (6-8-week old) were purchased from the Guangdong Experimental Animal Center (Guangzhou, China). Mice were maintained in a specific pathogen free facility with access to water and food freely. The experimental procedures were approved by the Animal Ethics Committee at Shanxi Medical University.

Generation of bone marrow-derived dendritic cells (BMDC)

The femurs and tibiae were excised from naïve BALB/c mice. The bone marrow cells were prepared and cultured in RPMI1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 25 ng/ml murine GM-CSF at 37 °C. On day 3, floating cells were discarded. The remained cells were cultured with fresh medium containing 25 ng/ml GM-CSF. On day 7, the cells were used for further experiments.

Isolation of immune cells

Immune cells used in the present study was isolated from proper sources with magnetic cell sorting (MACS) using commercial reagent kits following the manufacturer's instructions. Purity of isolated cells was assessed by flow cytometry. If purity was not reach 95%, MACS was repeated with the cells.

Cell culture

Isolated cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. The medium was changed in 2-3 days. Cell viability was more than 99% as assessed by Trypan blue exclusion assay.

Flow cytometry

In the surface staining, cells were incubated with fluorochrome-labeled antibodies of

interest or isotype IgG at 4 °C for 30 min. In the intracellular staining, cells were treated with fixative and permeable reagents first, then incubated with fluorochrome-labeled antibodies of interest or isotype IgG at 4 °C for 30 min. The cells were washed with phosphate buffered saline (PBS) and analyzed with a flow cytometer (FACSCanto II, BD Bioscience). Results were analyzed with a software package flowjo. Data from isotype IgG staining were used as a gating reference.

Real-time quantitative RT-PCR (RT-qPCR)

Cells were harvested from relevant experiments. Total RNAs were extracted from cells with an RNA-extracting kit and converted to cDNA with a reverse transcription kit following the manufacturer's instructions. The samples were amplified in a qPCR device with the SYBR Green Master Mix and the presence of relevant primers, including IL-10 (ataactgcaccacttccca and ggcatcacttctaccaggt), IL-35 (gatgacatggtgaagacggc and aggcacagggtcatcatcaa) and β -actin (ggaaatcgtgcgtgacatca and gccacaggattccataccca). The results were presented as fold change against the housekeeping gene β -actin.

Western blotting

Proteins were fractioned by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk for 30 min, incubating with the primary antibodies of interest overnight at 4 °C, washed with TBST (Tris-buffered saline containing 0.1% Triton x-100) for 3 times, incubated with peroxidase-labeled secondary antibodies for 2 h at room temperature and washed with TBST for 3 times. Immunoblots

on the membrane were developed by the enhanced chemiluminescence and photographed in an imaging station.

Depletion of IL-35 expression in DCs

BMDCs were prepared and treated with IL-35 RNAi or control RNAi with commercial reagent kits following the manufacturer's instructions. The effects of RNAi were assessed 48 h later by Western blotting.

Depletion of CD25⁺ DCs and B10 cells in mice

To deplete CD25⁺ DCs and B10 cells in mice, on day 9, mice were peritoneally injected with an anti-CD22 antibody (0.25 mg/mouse). To assess the effects of B10 cell depletion, mononuclear cells were isolated from the airway tissue of mice and analyzed by flow cytometry.

Chromatin immunoprecipitation (ChIP)

Cells were collected from relevant experiments, fixed with 1% formalin for 15 min and lysed with a lysis buffer. The lysates were sonicated to shear the DNA into small pieces. The samples were precleared by incubating with protein G agarose beads for 2 h to remove pre-existing immune complexes. The beads were removed from the samples by centrifugation at 10,000 g for 10 min. The supernatant was incubated with antibodies of interest or isotype IgG overnight to form complexes. The complexes were precipitated by incubating with protein G agarose beads for 2 h. The beads were collected by

centrifugation at 10,000 g for 10 min. Complexes were eluted from the beads with an eluting buffer. DNA was recovered from the samples using a DNA recovering kit following the manufacturer's instructions. The DNA was analyzed by qPCR in the presence of primers of the IL-10 promoter (ctgtgccaacgaagatcctc and aacattcgcttagagtcctc). The results were presented as fold change against the input.

Isolation of peripheral blood mononuclear cells from human blood samples

Six AR patients (3 males, 3 females; age: 25-45 years old; median = 29.6 years) were recruited into this study. All patients were sensitized to house dust mite (HDM). Serum HDM-specific IgE levels: 38.6-144.5 IU/ml (median: 56.6 IU/ml). The diagnosis and management of AR were carried out by our doctors following the routine procedures. Blood samples (20 ml per person) were obtained from AR patients by the ulnar vein puncture. Mononuclear cells were isolated from the blood samples by Percoll density centrifugation. The cells were cultured in RPMI1640 medium and used in further experiments. The experimental procedures were approved by the Human Ethic Committee at Shanxi Medical University. A written informed consent was obtained from each patient.