



Effects of ceftriaxone-induced intestinal dysbacteriosis on regulatory T cells validated by anaphylactic mice

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ABSTRACT

Both probiotics and pathogens in the human gut express pathogen-associated molecular patterns (PAMPs) and die with the release of endotoxin and bacterial DNA, which can stimulate our immune system and cause immune reaction. However, it's interesting and fascinating to address why the normal intestinal flora will not generate immunological rejection like the pathogen does. By investigating the changes in cells and molecules relevant to immune tolerance in mice with ceftriaxone-induced dysbacteriosis, our study discovered that both the Evenness indexes and Shannon Wiener index of intestinal flora showed a decrease in dysbacteriosis mice. Moreover, the proportion of $\alpha\beta^+ \text{TCR}^+ \text{CD3}^+ \text{CD4}^- \text{CD8}^-$ cells, $\text{CD3}^+ \gamma\delta \text{TCR}^+$ cells and $\text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ cells in the Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and spleen (SP) and the level of TGF- β 1, IL-2, IL-4 and IL-10 in the serum also changed. Intestinal dysbacteriosis in an asthma murine model resulted in enhancement of immunologic response to the allergen ovalbumin (OVA), which was an agent that aggravates asthma symptoms. In summary, it is integral to maintain a certain amount or variety of intestinal microflora for regulatory T cells to act in averting hypersensitivity.

1. Introduction

The intestinal mucosa is an area frequently exposed to foreign antigens. When encountering a large number of foreign antigens, it has to make a distinction between the harmful antigen (invading microbes, for example) and harmless substances (such as food antigen and intestinal symbiotic bacteria), to make an appropriate immune response to either remove the harmful antigen or initiate an immunotolerance to preserve the body from damage.

Consequently, it is essential for intestinal mucosal immune system to evolve two sets of immune defense mechanisms at the same time: one is for immunization to defend against harmful antigens, and the other is to induce immunotolerance for harmless antigens. Related research has confirmed that intestinal bacteria, as antigens, can maintain a low-intensity stimulation to keep a state of low-level activation of immunity, which contributes to monitoring or removing foreign antigens [1]. At the same time, the host maintains a certain immunotolerance to the intestinal bacteria itself. Does this mean that there is a certain relationship between the intestinal flora and immunotolerance?

One significant function of intestinal probiotics is hydrolyzing foods, including dietary protein. Moderate amounts of hydrolytic protein not only can activate mucosal immunity but also induce oral immunotolerance (decomposition of antigen is necessary for mucosal tolerance) [2]. Some researchers have indicated that frequent use of antibiotics during the early stages of life would increase the risks of childhood allergic diseases, such as eczema and asthma, or augment the disease conditions [3]. Alternatively, studies also found that people with inflammatory enteritis and other diseases associated with food intolerance also belong to the category of flora disorder [4]. It has been concluded that there is a certain relationship between intestinal flora and allergic disorders. Moreover, antibiotics induce the intestinal dysbacteriosis, which affects the effectiveness of mucosal immunity and ultimately oral immunotolerance [5,6].

Definitely, immunotolerance originates from suppressor T cells, now preferably called regulatory T cells, such as $\alpha\beta^+ \text{TCR}^+ \text{CD3}^+ \text{CD4}^- \text{CD8}^-$ cells and $\text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ T cells, which are closely related to immune associated diseases such as allergy and inflammatory bowel disease [7]. Previous studies have shown that

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the intestinal flora is relevant to the occurrence of allergy and the loss or inhibition of regulatory T cells. This loss or inhibition leads to a decrease in the body immune tolerance to the allergens. However, few studies have been referenced whether the occurrence of anaphylaxis is directly related to the functions of regulatory T cells caused by intestinal dysbacteriosis.

In this study, we investigated the effects of intestinal dysbacteriosis induced by ceftriaxone sodium on regulatory T cells in different anatomical sites. In mice with oral administration of ceftriaxone, we found that the Evenness index and the Shannon Wiener index of intestinal flora decreased compared with normal controls. At the same time, the proportion of immunotolerance associated cells such as $\alpha\beta^+ \text{TCR}^+ \text{CD3}^+ \text{CD4}^- \text{CD8}^-$, $\text{CD3}^+ \gamma\delta \text{TCR}^+$ and $\text{CD4}^+ \text{CD25}^+ \text{FoxP3}^+$ cells decreased, which appeared in the mesenteric lymph nodes (MLNs) and spleen (SP). Analogously, the cytokines in serum, such as TGF- β 1, IL-2, IL-4 and IL-10, also decreased significantly. Intestinal dysbacteriosis also aggravated the anaphylaxis to ovalbumin (OVA) in OVA-induced allergy mice. In conclusion, the intestinal microflora can affect hypersensitivity by regulatory T cells and suppressive effectors, which indicates that the intestinal microflora could be a target for the treatment of anaphylactic disease.

2. Materials and methods

2.1. Animals

Male BALB/c mice (aged 6–8 weeks, weighing 18–22 g) were obtained from the Medical Experimental Animal Center of Guangdong Province (Guangdong, China), where they were maintained under specific pathogen-free (SPF) conditions. The experimental protocol was conducted under the SPF conditions in the Laboratory Animal Center of Guangzhou University of Traditional Chinese Medicine, according to the guidelines approved by the Ethics Committee of Guangzhou University of Chinese Medicine. Ceftriaxone sodium was purchased from Shanghai Roche Pharmaceuticals (Shanghai, China).

2.2. Murine intestinal dysbacteriosis model

The mice were divided into two groups ($n = 12$ per group): the dysbacteriosis group and the normal control group. The mice in the dysbacteriosis group were administered ceftriaxone orally with a dosage of 8 g/kg per day for a week, and the mice in the other group were administered sterile water intragastrically as controls.

2.3. The proportion of regulatory T cells in the Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and spleen (SP)

The mice were sacrificed by cervical dislocation in the end of ceftriaxone or water administration. To obtain single-cell suspensions, the PPs, MLNs and SP were harvested and ground gently with a glass pestle on 200-gauge steel mesh to prepare single cell suspensions. Then, isolated cells were washed twice in phosphate-buffered saline (PBS) and re-suspended. After eliminating erythrocytes with low-osmotic hemolysis method, cell samples were stained with PEcy7-CD3, CD19-PE and FITC-CD69 antibodies at room temperature for 20 min and then washed with PBS. Finally, the samples were fixed with fixation solution and analyzed on a FACS CantoII flow cytometer (BD Biosciences) with FACSDiva™ software.

2.4. Microbial diversity

Mouse excrement (50–200 mg) was collected in a germ-free environment. Total genomic DNA was extracted from the pellets using a ONE-4-ALL Genomic DNA Mini-Preps Kit, kept in a clean 0.5 ml DNase-free tube and stored at -20°C for PCR. The PCR primers were designed and synthesized by Genewiz Company (Jiangsu, China). The primer sequences were as followed:

IRS1XhoIF:5'ccgctcgag CGTAACTGGACATCAGCAGAATG 3'

IRS1NotIR:5'ataagaatgcggccgcAAACACCCACATTCTTCATTCCG 3'.

After an initial denaturation at 94°C for 5 min, the PCR mixture was subjected to 30 cycles of denaturation at 98°C for 15 s, annealing at 56°C for 15 s and extension at 72°C for 1 min, followed by a final extension step for 7 min at 68°C .

After PCR amplification, the product was analyzed using denatured gradient gel electrophoresis (DGGE). For assessing bacterial diversity, an 8% polyacrylamide gradient was used. Electrophoresis was then performed for 2 h at 100 V. After electrophoresis, gels were stained with 1% nitric acid and dyed with 0.2% silver nitrate and 3% carbonate containing 2% formaldehyde. Finally, the results were analyzed with ImageJ software. The Richness (S) was the count of each lane on the DGGE map. Shannon-Wiener (H'): $H' = -\sum P_i \ln P_i$, where P_i was the ratio of absorbance from the i band to that of all the bands in the lane. Evenness (E): $E = H'/H'_{\max}$, where $H'_{\max} = \ln S$ [8];

2.5. Murine allergy with dysbacteriosis model

On days 1, 8 and 15, the mice were sensitized by intraperitoneal injection of 25 μg of ovalbumin (OVA) and 1 mg of alum in 300 μl of phosphate-buffered saline (PBS). One week after the last OVA sensitization (Day 22), the mice received a series of seven daily intranasal OVA (500 μg) challenges [9]. Ceftriaxone was administered orally at a dose of 8 g/kg from day 15 to day 22.

2.6. Allergic symptoms

After the final nasal challenge with OVA, sneezing frequency events were recorded for each mouse by blinded observers over a 10-min interval. The mice were then sacrificed 24 h after the last nasal challenge for further analyses.

2.7. Serum collection and ELISA

Twenty-four hours after the final OVA nasal challenge, the mice were sacrificed, and the blood samples were harvested by cardiac puncture and collected in 1.5 ml germ-free tubes. Then the collected blood was centrifuged at $1000 \times g$ at 4°C for 10 min to obtain the serum after standing 30 min at 4°C . Serum OVA-specific IgE (Biolegend, USA), IL-2, IL-4, IL-10 and TGF- β (Multi Sciences Company, China) levels were assayed using a specific ELISA kit. Each sample was analyzed in triplicate.

2.8. Peripheral blood cytometry

Twenty-four hours after the final OVA nasal challenge, the mice were sacrificed, and the blood samples were harvested by cardiac puncture. The 50 μl blood samples were placed into a 0.5 ml EP tube containing 5 μl of 1% heparin, and the blood cytometry was performed by a BC-2800Vet automatic blood cell analyzer (Mindray, China).

2.9. Nasal and lung histology

Twenty-four hours after the final OVA nasal challenge, nasal and lung tissues were surgically removed from the mice, fixed in 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Under high magnification, infiltrating eosinophils were counted in the nasal septal mucosa and lung.

2.10. Statistical analysis

The experimental data between two groups were analyzed using Student's t -test, and data among several groups were analyzed by one-factor ANOVA in SPSS (version 17.0). A P -value ≤ 0.05 was considered significantly different.

3. Results

3.1. Ceftriaxone induced mouse intestinal dysbacteriosis

Using DGGE to analyze the diversity of intestinal microbial, we discovered that the Evenness and Shannon Wiener indexes of intestinal flora decreased noticeably in the ceftriaxone sodium-fed mice compared to the normal control mice (Fig. 1). This difference attained statistical significance (Table 1).

3.2. The change in cells relevant to immune tolerance in mice with dysbacteriosis

We adopted flow cytometry (FCM) to detect the regulatory T cells in PPS, MLNs and SP tissues. We found that the proportion of $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$, $CD3^+\gamma\delta TCR^+$ and $CD4^+CD25^+FoxP3^+$ cells was lower in dysbacteriosis mice compared with the control. (Fig. 2).

3.3. IL-2, IL-4, IL-10 and TGF- β decrease in dysbacteriosis mouse serum

The suppressors of cytokines were analyzed after being treated with ceftriaxone. The levels of IL-2, IL-4, IL-10 and TGF- β in the serum of dysbacteriosis mice decreased significantly compared with those in the normal control. (Fig. 3).

3.4. Intestinal dysbacteriosis enhances mouse sensibility to allergen OVA

The sneezing counts in allergy mice with dysbacteriosis were more than those in normal control group and allergy mice (Fig. 4A). The level of serum OVA-specific IgE in allergy mice with dysbacteriosis also exceeded those of the other group mice (Fig. 4B).

3.5. Intestinal dysbacteriosis increases allergy inflammation cells

We found that inflammatory cells, such as white blood cells (WBCs), monocytes (Mon) and granulocyte (Gran), had higher number in the allergy group than the other groups (Fig. 5A). At the same time, the inflammatory cell infiltration in lung and nasal mucosa detected by HE staining revealed that allergic mice with dysbacteriosis were more severe than the mice in other groups (Fig. 5B). This difference was not found in nasal mucosa (Fig. 5C).

4. Discussion

Numerous studies indicate that the intestinal microflora contributes to the mucosal immunity for host. Moreover, the occurrence of some diseases, such as inflammatory bowel disease (IBD), and intestinal dysfunction are accompanied by intestinal flora disturbance [10,11]. It is interesting that, for the immune system, intestinal flora acts as a large and complex foreign antigen group. However, our immune system shows a state of tolerance to them without strong immune rejection. In addition, studies have found that human food intolerance is associated with flora disorder. Does this mean that there is an inevitable relation between the immune system and the intestinal flora?

Human immunotolerance to foreign antigens can be maintained by suppressor T cells. Early initial studies indicate that an important mediator of immune tolerance is regulatory T cells [12], such as $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$, $CD3^+\gamma\delta TCR^+$ and $CD4^+CD25^+FoxP3^+$ T cells. Although majority of researches on regulatory T cells have focused on $CD4^+CD25^+FoxP3^+$ T cells, the immunoregulatory functions of $CD3^+\gamma\delta TCR^+$ have also been confirmed [13]. And the $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$ T cells are also known as double negative T cells, which can induce the antigen-specific immune tolerance by targeted clearance of activated $CD8^+T$ cells [7,14]. They can also

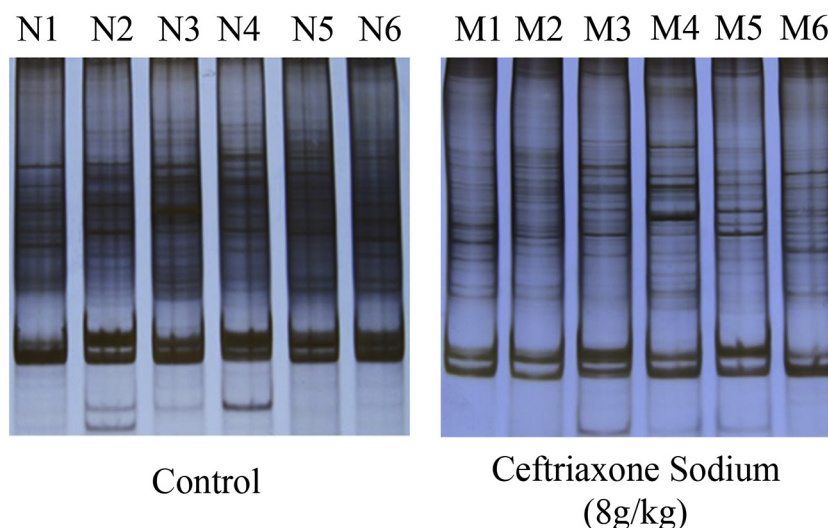


Fig. 1. The single band in DGGE gels of different mouse excrement ($n = 6$).

Table 1

The Richness (S), Shannon Wiener (H), H max and Evenness (E) values in different groups.

Group	Richness(S)	Shannon.Wiener (H)	H max	Evenness (E)
Control	21.50 $\pm$ 2.14	2.92 $\pm$ 0.09	3.06 $\pm$ 0.10	0.95 $\pm$ 0.02
Ceftriaxone sodium (8 g/kg)	16.17 $\pm$ 1.07***	2.31 $\pm$ 0.23***	2.78 $\pm$ 0.07***	0.83 $\pm$ 0.09*

Compared with the normal group, * $P < 0.05$, *** $P < 0.001$.

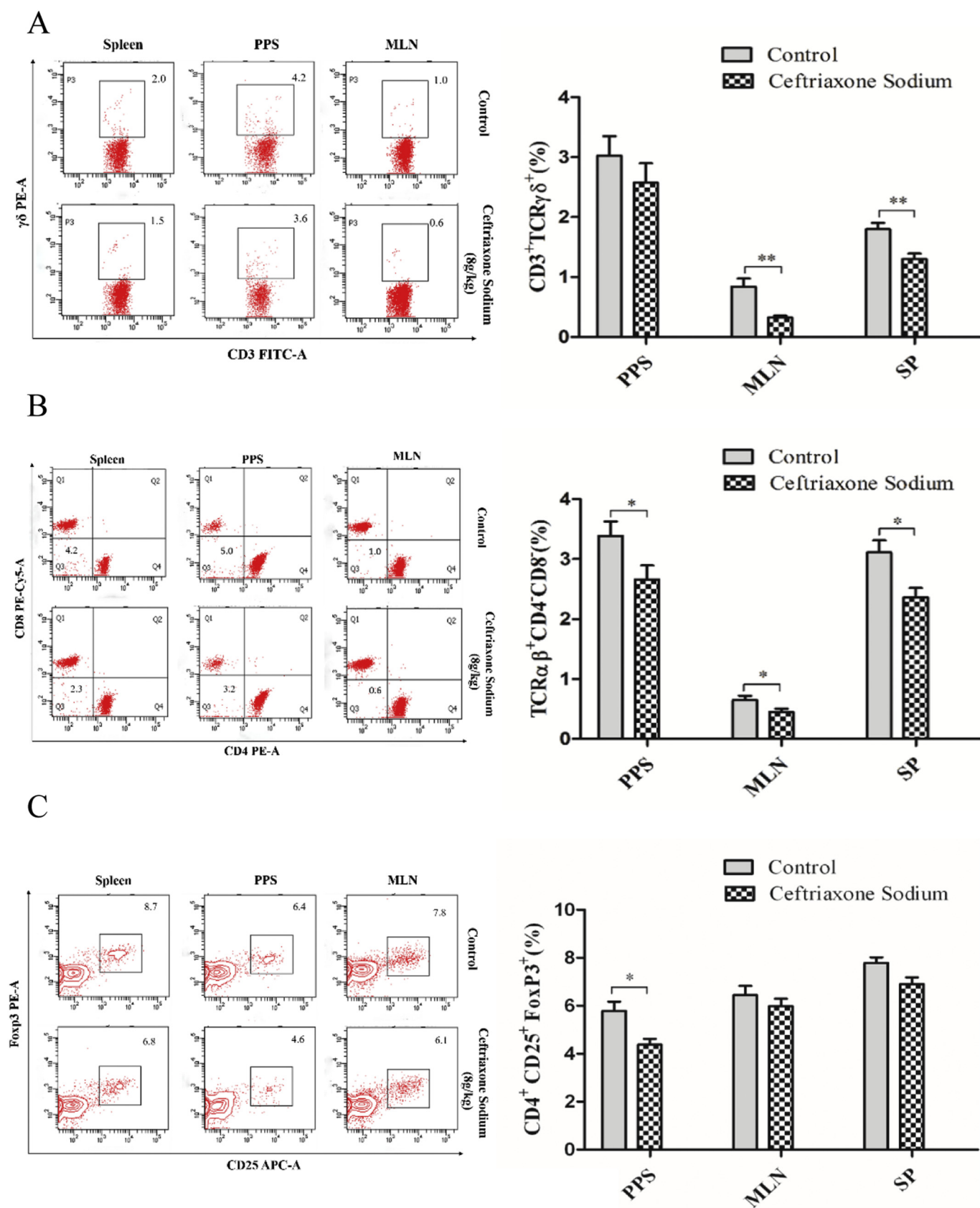


Fig. 2. The percentage of regulatory cells in MLNs, PPS and SP ($n = 8$) A. CD3⁺γδTCR⁺ T cells in gut-associated lymphatic tissue (GALT) by flow cytometry; B. The percentage of αβ⁺TCR⁺CD3⁺CD4⁻CD8⁻ T cells in different lymphoid tissues; C. The percentage of CD4⁺CD25⁺FoxP3⁺ T cells in different lymphoid tissues. Compared with the normal group, * $P < 0.05$, ** $P < 0.01$.

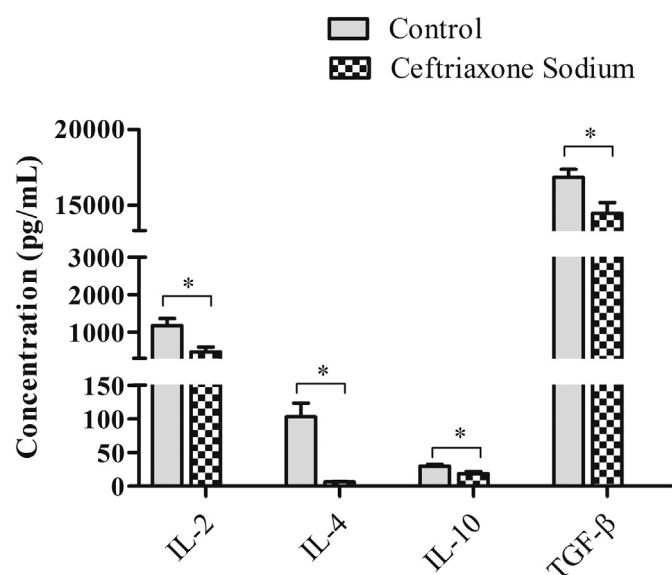


Fig. 3. The concentration of TGF-β1, IL-2, IL-4, and IL-10 in serum. Compared with the normal group ($n = 8$), * $P < 0.05$, *** $P < 0.001$.

inhibit immune response mediated by B cells [12,15]. Additionally, the $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$ T cells produce IL-4 and IL-10 upon activation in vitro. In this study, we found that in mice with reduced flora richness, the ratio of regulatory cells namely $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$, $CD3^+\gamma\delta TCR^+$ and $CD4^+CD25^+FoxP3^+$ was significantly lower than that in normal mice in PPs, MLNs and SP. Moreover, the levels of IL-2, IL-4, IL-10 and TGF-beta were less in serum of dysbacteriosis mice than that of the normal control mice. As we know, moderate interleukins are indispensable for regulatory cells to maintain their activation and function. For example, IL-2 can activate $CD4^+CD25^+FoxP3^+$ T cells [16], and survival of double negative T cells also needs the stimulation of IL-4 [17]. The less production of cytokine suppressors is consistent with the defective regulatory T cells in dysbacteriosis mice, which indicates that the function of immune tolerance in these mice was abated.

Allergies, also known as allergic diseases, are caused by hypersensitivity of the immune system to foreign antigens that usually continue to pose a major public health problem [18]. The underlying mechanism involves immunoglobulin E (IgE), an antibodies secreted by sensitized B cells, binding to an allergen and then to a receptor on mast cells or basophils where it triggers the release of inflammatory mediator such as histamine [19]. Studies have shown that the frequency use of antibiotics would increase or enhanced the subsequent childhood

allergic disease or conditions (eczema, asthma and asthma symptoms). Therefore, there may be a correlation between the intestinal flora disorder caused by antibiotics and the sensitivity to allergens in human beings. Based on this hypothesis, we developed an allergic disease model by sensitizing mice with ovalbumin, accompanying with intestinal dysbacteriosis. We found that both anaphylaxis severity and the OVA-specific IgE in serum in allergy mice with dysbacteriosis are higher than those of asthma mice with normal flora.

Regulatory T cells are also related to allergic disease. $CD4^+CD25^+FoxP3^+$ T cells can inhibit the Th2 inflammation in allergic disease and increase the Type 1 helper T (Th1) cytokine expression [20]. In addition, $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$ cells can inhibit B cell activation as immune response to allergens and their capacity to secrete allergen specific IgE [21]. The proportions of peripheral blood lymphocytes, monocytes and granulocytes in dysbacteriosis mice were higher than that in other mice groups, and the level of inflammatory cell infiltration in the lung tissue was more serious than that of asthmatic mice. Among the reasons it may be the most likely that dysbacteriosis affects the condition of immune tolerance in the gastrointestinal environment, which may affect immune regulatory T cell proportions in other regions of the body, thus enhancing the immune response to allergen like OVA, and aggravating the inflammatory response.

Intestinal microflora may protect from allergy by maintaining immune tolerance in the intestinal immune homeostasis. As a foreign antigen, it can induce the host immunity by keeping it in a low activation status, simultaneously, the immunological hyporesponsiveness to the feeding antigen also can be initiated by preserving the appropriate interleukins in the body, which can maintain the immune regulatory T cell survival and its function [22].

Actually, previous study found that microbial products triggered innate immune response through pattern-recognition receptors, such as the Toll-like receptors, which might activate several signaling pathways for induction of regulatory T cells, as well as Th1 cells, which could counter-balance the predominance of Th2 cells that mediated asthmatic attacks [23,24]. Differently, we scanned the most important regulatory T cells, such as $\alpha\beta^+TCR^+CD3^+CD4^-CD8^-$ cells and $CD4^+CD25^+FoxP3^+$ cells, proving that intestinal flora can influence the development of allergic asthma by maintaining the function of regulatory T cells in host. Our study emphasized the importance of intestinal flora for maintaining regulatory T cell physiology, and that the occurrence of anaphylaxis was directly related to the dysfunction of regulatory T cells caused by intestinal flora dysbacteriosis. Therefore, it is not difficult to believe that the more likely treatment of anaphylactic diseases is to correct immune disturbance by maintaining the micro-balance of the intestinal tract as a primary prevention. Additionally, it may be possible to induce immune tolerance by adding probiotics, which may reduce the risk of anaphylactic diseases.

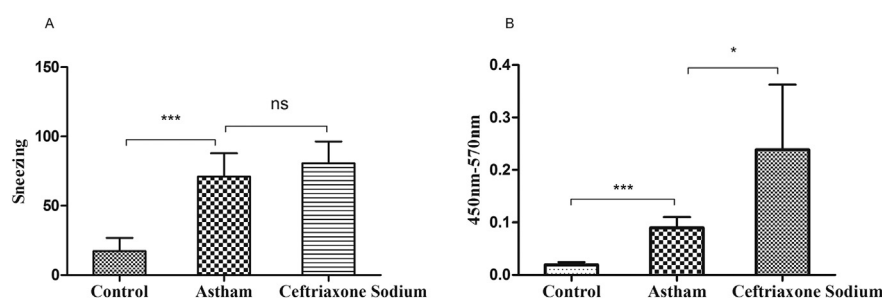
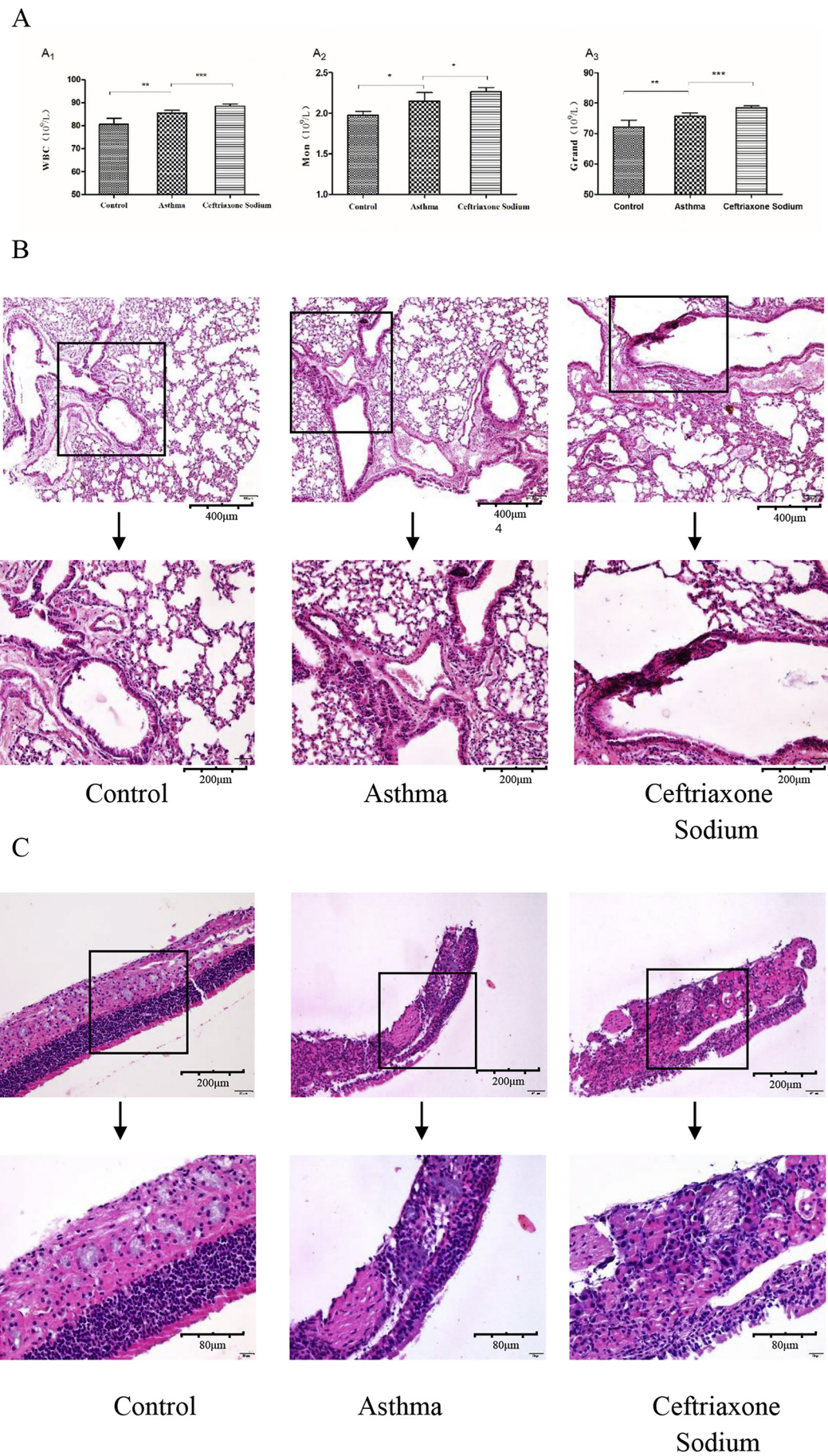


Fig. 4. Comparison of allergy symptoms and serum immunoglobulin E (IgE) levels among the control group, the asthma group and the ceftriaxone sodium group ($n = 8$) A. Sneezing; B. serum ovalbumin-specific IgE level. * $P < 0.05$, *** $P < 0.001$.



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Fig. 5. Peripheral blood cells and eosinophilic infiltration in the control group, asthma group and ceftriaxone sodium group ($n = 8$). A₁, WBC; A₂, Mon, A₃, Grand. Compared with the control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.; B. lung histology (HE, 100 $\times$); C. Nasal histology (HE, 200 $\times$).

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Conflict of interests

The authors declare that there are no conflicts of interest associated with this publication.

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