Original Article
Effects of montelukast on $CD_4^+CD_{25}^+$ regulatory T cell expression in children with acute bronchial asthma

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Abstract: Objective: To explore the effects of montelukast on $CD_4^+CD_{25}^+$ regulatory T cells in the peripheral blood of children with acute bronchial asthma. Methods: Ninety-four children with acute bronchial asthma admitted to our hospital from March 2014 to September 2015 were selected as the subjects. They were randomized into two groups, the montelukast group (47 cases) and the conservative treatment group (47 cases). The conservative treatment group was only given inhaled and aerosolized budesonide suspensions at 0.5 mg/2 mL (one inhalation per time and once a day) for 4 weeks while the montelukast group received 5 mg of montelukast chewable tablets (once a day and orally administered before sleep) for 4 weeks. The serum levels of IFN-γ and IL-5 were detected by using ELISA, and day and night symptom scores were recorded and the pulmonary function indexes were compared before and after treatment. The expression levels of $CD_4^+CD_{25}^+$ Treg cells in the periphery blood were observed by flow cytometer before and after treatment. Results: 1) At 3 months follow up after treatment, the lung function indexes of the two groups were significantly enhanced (P<0.05); 2) At 3 months follow up after treatment, the day and night symptom scores of the two groups were significantly decreased (P<0.05) with those of the montelukast group decreased more significantly (P<0.05); The IFN-γ levels of the two groups were significantly increased (P<0.05); 3) At 3 months follow up after treatment, the IL-5 levels of the two groups were significantly decreased (P<0.05); And 4) at 3 months follow up after treatment, the percentages for the expression levels of $CD_4^+CD_{25}^+$ T and of $CD_3^+CD_4^+CD_{25}^+$ T in the two groups were significantly increased (P<0.05). Conclusion: Montelukast could improve lung functions and day, night symptom scores of the patients. Inflammation of asthma could be suppressed by up-regulating $CD_4^+CD_{25}^+$ T cells which secrete IFN-γ and IL-10. Thus, it is an effective drug for treatment of acute bronchial asthma in children.

Keywords: Montelukast, bronchial asthma, IFN-γ, IL-5, regulatory T cells

Introduction

Pediatric Bronchial asthma is one of the most common allergic disorders in children, frequently presented with such symptoms as episodic wheeze, chest tightness and coughs. Severe episodes may even lead to emphysema, respiratory failure or other complications, endangering the lives of patients [1]. A great number of clinical studies have found that the pathophysiology of asthma is that leukotrienes (LTs) may be directly involved in the interaction among asthma airway inflammation, smooth muscle spasm, airway hyper-responsiveness and airway remodeling and other inflammatory mediators, leading to airway reversible obstruction and inflammations [2]. In recent years, there is a growing incidence of bronchial asthma in children. Numerous treatment methods

for bronchial asthma have been developed, but their efficacy is poor. It is generally believed that asthma is a chronic airway inflammation in which a variety of inflammatory cells, mediators and cytokines are involved. With the further development of immunology and molecular biology, increasing evidence shows that immune dysfunction plays an important role in the pathogenesis of asthma. $CD_4^+CD_{25}^+$ regulatory T cells (Tregs) are subsets of cells which regulate or suppress the actions of other immune cells. Recently, studies have revealed that $CD_4^+CD_{25}^+$ regulatory T cells may play a crucial role in the pathogenesis of asthma [3]. New findings [4] show that montelukast, a novel kind of agent for bronchial asthma can suppress the proliferation of inflammatory cells and reduce the secretion of cytokines and inflammatory mediators while inhibiting airway remodeling or fibro-

sis. As a result, it bring new insight for the research on treatment of bronchial asthma. Currently, however, the mechanism of action of montelukast is anti-inflammation, and few studies have been conducted on the effect of montelukast on $CD_4^+CD_{25}^+$ T lymphocytes. In this study, we detected the levels of IFN-γ, IL-5 and the proportion of $CD_4^+CD_{25}^+$ lymphocytes in the peripheral blood of children with bronchial asthma, and explored the effect of leukotriene receptor antagonist montelukast Sodium on Treg cells and their secreted effectors (IFN-γ and IL-5) as well as the potential mechanisms for treating asthma.

Materials and methods

Subjects

A total of 94 cases of children with bronchial asthma admitted to our hospital from March 2014 to September 2015 were enrolled in this study as subjects. They included 60 males and 34 females, aged 4 to 14 (mean, 6.24±1.36) years, and suffered the disease for 6-25 (mean, 22.34±9.07) days. Inclusion criteria were: 1) All the children met the diagnostic criteria for bronchial asthma in Guidelines for the Prevention and Treatment of Pediatric Bronchial Asthma released by the Respiratory Group, Pediatric Branch of Chinese Medical Association in China in 2008 [5]; 2) No one had received leukotriene receptor antagonism therapy; 3) No one had received glucocorticoid therapy within one month; And 4) each patient voluntarily participated in the study and provided the informed consent signed by their legal guardians. Exclusion criteria were: 1) Patients with respiratory tract infection and systemic infection; 2) Patients complicated with inhaled corticosteroid allergy; 3) Patients complicated with cardiopulmonary or other important organ diseases; or 4) Patients complicated with severe systemic diseases or mental disorders. A total of 94 children with acute exacerbation of bronchial asthma were randomized into the montelukast group (n=47) and the conservative treatment group (n=47). The montelukast group consisted of 28 males and 19 females with an average age of 8.82±1.18 years. The patients in the conservative treatment group were treated with budesonide aerosol (trade name: Pulmicort Respules produced by Glaxo-SmithKline, 500 μg per day) for a period up to 4 weeks. In addition to the conservative treat-

ment, the subjects in montelukast group were also given montelukast chewable tablets (trade name: Schnellen produced by Merck, 5 mg×6 tablets/box, Qd) daily for a period up to 4 weeks. No statistically significant differences were shown in the general information (age, gender, asthma severity) between the two groups (P>0.05), so it is comparable, as shown in **Table 1**. Our study was approved by the Ethical Committee of our hospital and the guardians of the patients provided informed consents for participation in the study.

Main reagents and instruments

Main reagents and instruments were IL-5, IFN-γ Detection Kit (Shanghai Jingmei Bioengineering Co., Ltd.); CD3-PC5, CD4-FITC, CD25-PE, CD19-FITC and CD23-PE monoclonal antibodies (Immunotech, France); Thermo Scientific Plate Washer and ELISA Reader (DYNEX MRX, US); Spirometer (WellKang LLC ,US); ELISA PLATE (Nunc-immuno Plate, Denmark); hemolysis preparation apparatus and Flow Cytometer (Beckman Coulter, US).

Methods

Treatment protocol

After admission into our hospital, the two groups of pediatric patients received all the necessary physical examinations and symptomatic treatment including oxygen inhalation, dissipate phlegm, anti-infection, spasmolysis and light diets. The conservative treatment group was given budesonide aerosol (Glaxo-SmithKline (GSK), approval document: H200-80283) with daily spray in each nostril (500 μg) for 4 weeks while the montelukast group was given montelukast chewable tablets (Merck & Co., approval document: J20070068, UK) (4 mg, qd) for 4 weeks before bedtime in addition to the conservative treatment. The two groups of patients underwent regular outpatient physical examination before and after treatment and the changes in their asthma symptoms were recorded in details.

Pulmonary function evaluation

The professionals demonstrated the patients specific operation and told them the principles and operating procedures about how to use the pulmonary function machine. Then the patients measured their own pulmonary functions in

Table 1. Comparison of general information between the two groups

Groups	Cases	Severity		Gender		Age	Duration
		Moderate	Severe	Male	Female		
Montelukast group	47	14	33	28	19	6.49±1.02	23.79±6.17
Conservative treatment group	47	15	32	32	15	6.74±1.46	22.15±5.08
X^2		0.42		0.79		0.44	0.57
P		>0.05		>0.05		>0.05	>0.05

Table 2. Comparison of pulmonary ventilation function indexes between the two groups before and after treatment ($\overline{x}$±s)

Groups	Cases	FVC (L)		FEV$_1$ (L)		FEV1/FVC	
		Pretreatment	Post-treatment	Pretreatment	Post-treatment	Pretreatment	Post-treatment
Montelukast group	47	2.0±0.4	2.4±0.3*	1.4±0.5	1.8±0.5*	60.2±13.4	70.2±13.5*
Conservative treatment group	47	2.0±0.4	2.1±0.4	1.5±0.6	1.5±0.5	61.3±12.5	62.8±13.2

Note: *was expressed as compared with the control group, P<0.05.

Table 3. Comparison of the day and night symptom scores between the two groups before and after treatment ($\overline{x}$±s)

Groups	Cases	Day symptom scores		Night symptom scores	
		Pretreatment	Post-treatment	Pretreatment	Post-treatment
Montelukast group	47	2.92±0.33	0.81±0.16*,#	3.98±0.32	0.70±0.15*,#
Conventional treatment group	47	3.08±0.34	2.06±0.23*	3.75±0.29	2.02±0.44*
T		1.07	14.04	1.69	9.05
P	-	>0.05	<0.05	>0.05	<0.05

Note: *was expressed as compared with the control group, P<0.05; #was expressed as compared with the conservative treatment group, P<0.05.

Table 4. Changes in IFN-γ and IL-5 levels in the two groups before and after treatment ($\overline{x}$±s)

Groups	Cases	IFN-γ (ng/L)		IL-5 (pmol/L)	
		Pretreatment	Post-treatment	Pretreatment	Post-treatment
Montelukast group	47	15.38±1.82	17.05±1.95*	305.70±91.80	162.34±38.56*
Conventional treatment group	47	15.43±1.77	17.73±1.87*	307.52±87.62	182.82±37.48*
T		0.89	2.39	1.69	6.15
P	-	>0.05	>0.05	>0.05	>0.05

Note: *was expressed as compared with the conservative treatment group before treatment, P<0.05.

such aspects as forced expiratory rate in one second (FEV1/FVC%), vital capacity (VC) and forced expiratory volume in one second (FEV1).

IL-5 and IFN-γ levels in the serum samples determined by ELISA

Five ml of peripheral blood was collected from the forearms of the subjects before treatment and at three months follow-up after the treatment, respectively. The collected peripheral blood was centrifuged at 3000 r/min for 15 min to obtain the supernatant which was then stored at -80°C for detection. The enzyme labeling solution (50 μl) and the standard or sample (100 μl) were respectively poured into the corresponding sites on the ELISA plate, reacted at the temperatures (18 to 25°C) for 90 min, and washed with the washer for 5 times (20 s interval between times). A and B base solutions (50 μl, respectively) were poured into each well of the ELISA plate and then incubated at 18-25°C for 15 min, and reacted away from light at the temperatures (18 to 25°C) for 15

Table 5. Comparison of the results by flow cytometer between the two groups before and after treatment ($\overline{x} \pm s$)

Groups	Cases	$CD_4^+CD_{25}^+$ T (%)		$CD_3^+CD_4^+CD_{25}^+$ T (%)	
		Pretreatment	Post-treatment	Pretreatment	Post-treatment
Montelukast group	47	9.62±2.28	13.13±1.97*	3.53±1.57	6.74±1.66*
Conventional treatment group	47	9.53±2.37	13.05±2.15*	3.49±1.31	6.17±1.33*
T		0.91	4.29	1.07	5.11
P	-	>0.05	>0.05	>0.05	>0.05

Note: *was expressed as compared with the conservative treatment group before treatment, P<0.05.
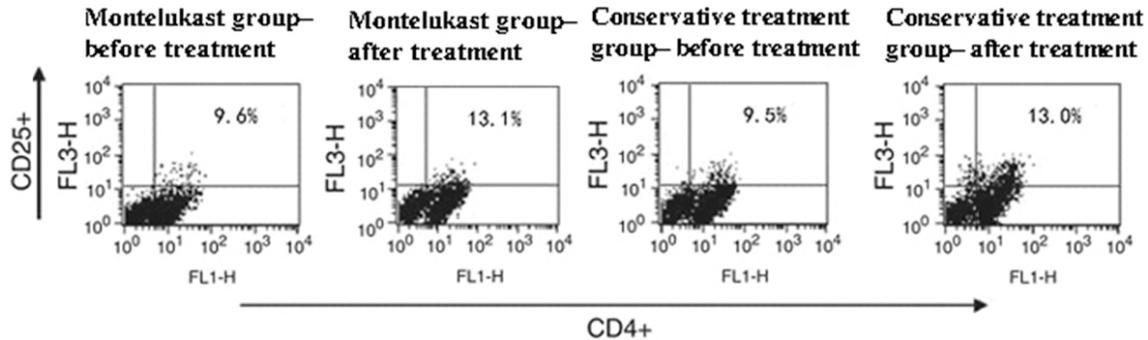
Figure 1. The percentages of $CD_4^+CD_{25}^+$ T cells in the peripheral blood of the two groups.
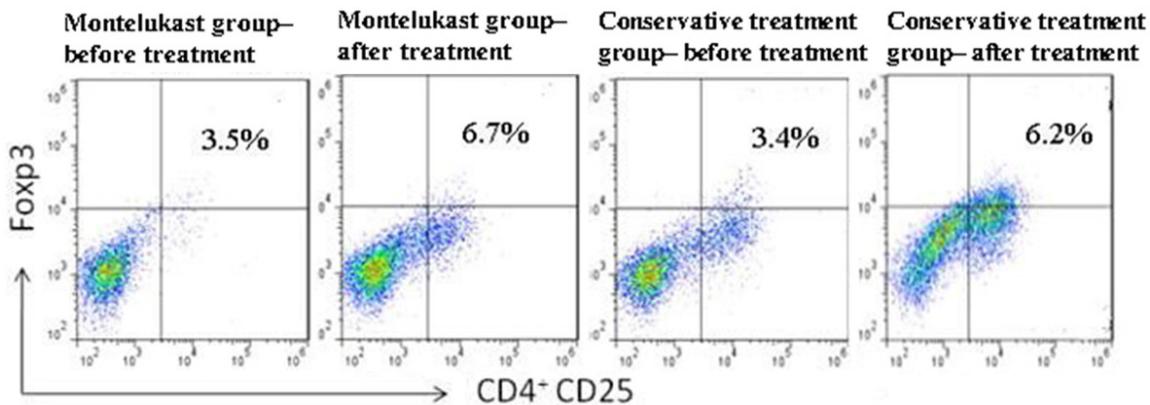
Figure 2. The percentages of $CD_3^+CD_4^+CD_{25}^+$ T cells in the peripheral blood of the two groups.

min. Finally, stop solution (50 μl) was added into each well to stop the reaction. The OD values were measured and the corresponding concentrations of IL-5, IFN-γ were calculated based on the OD values.

Flow cytometer detection

Five ml of forearm peripheral blood was collected from the forearms of the subjects before treatment and at three months follow-up after the treatment, respectively. The collected peripheral blood was centrifuged at 3000 r/min for 15 min to obtain the supernatant. CD_4, CD_{25}, and CD_3 were labeled with multicolored fluorescent antibodies, respectively. At the same time, the accordingly-labeled mouse antibodies were used as controls. The two kinds of antibodies were evenly mixed together. 100 μl of anticoagulated whole blood was incubated away from light at the temperatures (18 to 25°C) for 15 min. The Q-prep hemolysis preparation apparatus was applied to determine and calculate the positive rates of $CD_4^+CD_{25}^+$ T lym-

phocytes in the peripheral blood and the percentage of $CD_4^+CD_{25}^+$ T cells. 5000 cells taken from each sample were analyzed.

Day and night symptom scoring

The patients' asthma symptoms were scored according to their day and night symptoms [6], 1) Day symptom scoring: The patients' symptoms without asthma nor cough in the daytime were recorded as 0 point; Mild asthma symptoms were recorded as 1 point; Frequent occurrence of asthma recorded as 2 points; And persistent asthma symptoms as 3 points. 2) The patients' symptoms without asthma at night were recorded as 0 point; Only one wake-up due to asthma was recorded as 1 point; Frequent wake-ups due to asthma was recorded as 2 points; And the failure of sleep due to persistent asthma was recorded as 3 points.

Statistical methods

The SPSS21.0 statistical software was used for statistical analyses, and the quantitative data were presented as mean±standard deviation. The normal distribution test was performed and the data in compliance with the normal distribution were detected by the t test. The count data were presented as rate (%) and determined by the χ^2 test. P<0.05 was considered statistically significant.

Results

Pulmonary function indexes of the two groups before and after treatment

No statistically significant difference was found in indexes FVC, FEV1, FEV1/FVC between the two groups before treatment (P>0.05). After treatment, all the lung function indexes were improved in some degree and the differences between the two groups were statistically significant (t=7.32, 6.19, 10.52, all P<0.05) as shown in **Table 2**.

Comparison of symptom scores between the two groups

No significant difference was found in the day and night symptom scores between the two groups (P>0.05) before treatment; After treatment, the day and night symptom scores of the two groups were significantly lower than those before treatment (P<0.05). Furthermore, the post-treatment day-night symptom scores of the montelukast group were significantly lower than those of the conservative treatment group (t=14.04, 9.05, P<0.05), as shown in **Table 3**.

Results of serum interferon γ and IL-5 test

The differences in the levels of IFN-γ and IL-5 were not statistically significantly between the two groups before treatment (P>0.05). At 3 months follow up after treatment, the levels of IFN-γ in the two groups were significantly higher than those before treatment (P<0.05), and the levels of IFN-γ and IL-5 in the montelukast group were lower than those in the conservative treatment group (P>0.05), but the difference was not statistically significant, as shown in **Table 4**.

Comparison of the low cytometer results between the two groups before and after treatment

No statistically significant difference was found in the percentages of $CD_4^+CD_{25}^+$ T cells and $CD_3^+CD_4^+CD_{25}^+$ T cells between the two groups before treatment (P>0.05). At 3 months follow up after treatment, the percentages of $CD_4^+CD_{25}^+$ T cells and $CD_3^+CD_4^+CD_{25}^+$ T cells of the two groups were increased significantly compared to those before treatment (P<0.05), and the percentages of the montelukast group were higher than those of the conservative treatment group (P>0.05), but the difference was not statistically significant, as shown in **Table 5**; **Figures 1** and **2**.

Discussion

Bronchial asthma is a frequent bronchial inflammatory disease, mainly caused by the interaction between cytokines, growth factors, inflammatory mediators and enzymes and other factors [6]. Leukotrienes and other inflammatory mediators repeatedly stimulate the airway, resulting in the airway remodeling and fibrosis, which is mainly responsible for recurrence of bronchial asthma. With more profound research on immunology and molecular biology, increasing evidence shows that immune dysfunction plays a key role in the pathogenesis of asthma [7, 8]. A great number of studies have confirmed [7] that Th2 dominant in the imbalanced ratio of Th1 to Th2 is the internal mechanism for the development of asthma.

Clinically, the levels of IL-5 and other Th2-type cytokines were elevated with the decrease in the levels of IFN-γ and other Th-1 cytokines. Thus, how to effectively prevent against the synthesis of LTs and to regulate the balanced ratio of Th1 to Th2 has become new directions for the treatment of acute bronchial asthma, which may provide guidance for solving the problems in the conservative treatment of asthma(a treatment is prescribed to alleviate the symptoms instead of its root cause).

$CD_4^+CD_{25}^+$ T lymphocytes, the specific inhibitory cells acting as immune-regulation, which were initially discovered by Sakaguchi et al. in 1995, are involved in regulating the immunological balance of the body and have the two major properties of immune anergy and immunosuppression. Literature from China and other countries have revealed [9-12] that $CD_4^+CD_{25}^+$ T cells which account for 5%-10% of CD_4^+ T cells in the peripheral blood of a healthy person can effectively suppress and regulate the activity of other immune cells, playing a crucial role in controlling the pathogenesis of bronchial asthma. $CD_4^+CD_{25}^+$ regulatory T cells can secrete a variety of inhibitory cytokines, such as IL-4, IL-10 or TGF-β [13]. The results of the experiments varied in whether the cells take effects through the soluble cytokines. Studies show that [14] the proportion of $CD_4^+CD_{25}^+$ Treg in patients with acute asthma increased significantly; The higher the proportion of $CD_4^+CD_{25}^+$ Treg in asthmatic patients, the more severe the lung allergy was; And the proportion of $CD_4^+CD_{25}^+$ Treg was negatively correlated to the severity of the lung allergy. Another study also reported that [15], the deficiency in number and/or decline in functions of $CD_4^+CD_{25}^+$ Treg cells were closely correlated to the onset and development of asthma. The experimental study indicated that the levels of $CD_4^+CD_{25}^+$ Treg in the peripheral blood of children with acute exacerbation of asthma were significantly lower than those of healthy children, which is consistent with other reports, suggesting that children with acute exacerbation of asthma have imbalanced T regulatory cell immunity, which may suppress their immune function. That might be one of the mechanisms contributing to the onset of bronchial asthma.

As we all know that bronchial asthma is mainly presented as IgE-mediated Type I allergic inflammatory disease. Significant increase in IgE has been observed in the peripheral blood of the patients with bronchial asthma; and IL-5, a kind of immunosuppressive factor with diverse biological activities, can suppress the activity of T Lymphocytes, macrophages and eosinophils, block the synthesis and release of various inflammatory cytokines, suppress the onset and development of inflammations and reduce airway hyperresponsiveness [15]. IFN-γ primarily secreted by Th1 cells can mediate cellular immunity, differentiation and activation of cytotoxic T cells (CTL), macrophage activation, and suppress B cells, thus playing a critical role in the removal of intracellular microbial infections [16]. The interaction between IL-5 and IFN-γ is key to maintain the balance of Th1/Th2 ratio. On one hand, Th2 cells by secreting IL-4, IL-5 and IL-13 can induce recruitment and activation of eosinophils, lung inflammations, mucus secretion, B cell subtype transformation, airway hyperresponsiveness, and even airway remodeling leading to deterioration of lung functions. On the other hand, IFN-γ can regulate Th2, Th17, NKT, B cells and other macrophages, neutrophilic granulocytes, eosinophils and other inflammatory cells, and suppress allergic inflammation, IgE release, mucual hypersecretion and airway hyperresponsiveness. The healthy people can keep a balanced Thl/Th2 ratio, but asthma patients show an imbalanced Thl/Th2 ratio due to their immune dysfunction. The normal value of IL-5 is 56.37-150.33 pmol/L and IFN-γ is 1.21~5.51 μg/L. Our present study showed that compared with the healthy population, the patients had elevated IL-5 but reduced INF-y levels, thus confirming the above theory. We presumed that at the onset of asthma, the decreased $CD_4^+CD_{25}^+$ T lymphocytes levels might result in the elevated inhibitory cytokine IL-5 and the reduced INF-γ levels, which neither blocked the synthesis and release of Th2 type cytokines nor balanced the immune response of Th1/Th2 ratio, nor suppress the dominant response of Th2 cells, thereby triggering a series of immune cascade reactions that may promote the accumulation of eosinophils, increase the activity of eosinophil cells, improve the creation of IgE. Accordingly, asthma may develop.

Our study also showed that after treatment, the day and night symptom scores were significantly improved in the montelukast group compared with those of the conservative treatment group, which might be explained by montelukast's capability of blocking the activity of leukotriene

receptors. In addition, compared with the conservative treatment group, the higher Treg cell count but lower expression levels of IL-5 and INF-γ cytokines of the montelukast group suggested that montelukast might be effective in up-regulating Treg cells and improving the chronic inflammation. The effect might be one of the mechanisms that montelukast improves bronchial asthma of the patients from innate immunity. IL-5 and INF-γ may become targeted molecules for clinical treatment of bronchial asthmas. However, we selected a too small sample size and had short follow-ups, so it is necessary to increase the number of samples. Besides, further studies on the relationship among the three are required to validate that montelukast can improve the prognosis and the exact mechanism of bronchial asthma in children, providing a reference for clinical therapy.

In conclusion, montelukast can not only selectively block the activity of LTs, effectively suppress asthma inflammations and improve lung functions but also suppress proliferation and activation of T cells, further regulate Th1/Th2 balance and control the onset of bronchial asthma through up-regulating $CD_4^+CD_{25}^+$ T cells. Therefore, the agent can completely replace the inhaled glucocorticoids and is worth promoting in clinical practice.

Disclosure of conflict of interest

None.

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