

Critical roles of adenosine A2A receptor in regulating the balance of Treg/Th17 cells in allergic asthma

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Linlin Wang performed research, participated in the collection of data and wrote the paper. Huanying Wan and Wei Tang participated in collecting specimens, reviewed the manuscript. Yingmeng Ni, Xiaoxia Hou, Lina Pan participated in the collection of data and performed the statistical analyses. Guochao Shi participated in the design of the study. Guochao Shi and Yuanlin Song helped write the paper and revised the manuscript. All authors read and approved the final manuscript.

Ethics

This study was approved by the Research Ethics Board of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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ABSTRACT

Introduction: Deficiency of Treg cells and hyperactivity of Th17 cells together are involved in the immunological pathogenesis of asthma. The adenosine A2AR plays a critical role in the increased Foxp3 expression of Treg cells and the decreased Th17 generation.

Objective: The study aimed to investigate A2AR expression in peripheral blood and its regulatory effect on balance of Treg/Th17 cells in asthma.

Methods: 31 patients with chronic persistent asthma were recruited and divided into 18 intermittent to mild asthma patients, 13 moderate to severe asthma patients. A2AR, Foxp3 and ROR- γ t mRNA expression levels in PBMCs were measured by qPCR. TGF- β , IL-17 and IgE in plasma were detected with ELISA. 42 BALB/c mice were randomly, equally assigned to control group, OVA group and OVA+CGS (CGS21680, A2AR agonist) group. The infiltration of lung inflammation cells were evaluated by HE, A2AR, Foxp3 and ROR- γ t mRNA in lung tissues measured by qPCR, TGF- β , IL-17 and IgE in plasma measured with ELISA, and IL-17 and TGF- β protein in lung tissues analyzed with IHC.

Results: Our results showed that expression A2AR mRNA in PBMCs was associated with asthma severity. Foxp3 mRNA, TGF- β and FEV1%pred positively correlated with A2AR mRNA in asthma. ROR- γ t mRNA and IL-17 negatively correlated with A2AR mRNA in asthma. CGS could promote Foxp3 mRNA expression, TGF- β and improve lung function while inhibit ROR- γ t mRNA expression, IL-17 and the infiltration of lung inflammation cells.

Conclusion: A2AR could regulate the balance of Treg/Th17 cells in asthma.

Key words: asthma; A2AR; CGS21680; Treg cells; Th17 cells

Introduction

Asthma is a chronic inflammatory disorder of the airways, which involves several inflammatory cells, structural cells and multiple mediators (1, 2). Regulatory T cells (Treg cells) have a broad immunosuppressive capacity. Th17 cells contribute to the inflammatory response. The differentiation and development of Treg cells is dependent on TGF- β and Foxp3 while Th17 cells require IL-17 and ROR- γ t. Studies have demonstrated that Treg cells and Th17 cells play an essential role in the pathogenesis of asthma (3, 4). Deficiency of Treg cells and hyperactivity of Th17 cells together are involved in the immunological pathogenesis of asthma (4-6). Our previous studies confirmed that the imbalance of Treg/Th17 cells existed in asthma, but only limited to in peripheral blood. Moreover, the mechanisms of imbalance of Treg/Th17 cells in asthma need further to be explored.

Adenosine is released by various cells, including epithelial cells, fibroblasts, endothelial cells, platelets, and muscle cells (7-9) and also derived from the extracellular metabolism of released purine nucleotides (10). Extracellular adenosine initiates transmembrane signaling through four subtypes of G protein-coupled adenosine receptors (A1, A2A, A2B, and A3) (11). Following the cloning of adenosine receptors, investigators have gained significant insight into their roles. A2A receptor (A2AR) is considered as the main anti-inflammatory receptor which increase Foxp3 expression and inhibit Th17 cells generation (12, 13). A2AR stimulation enhances Foxp3 transcription by decreasing IL-6 expression and enhancing TGF- β expression (14). TCR engagement can lead to the development of CD4⁺ adaptive effector cells including Th17 cells. A2AR stimulation inhibits Th17 cells generation by inhibiting TCR-induced ZAP70 phosphorylation (15). But the expression pattern of A2AR and its regulatory effect on the balance of Treg/Th17 cells involved in chronic persistent asthma remain unclear.

Material and methods

Study population

31 patients with chronic persistent asthma were enrolled. Based on the GINA (13), asthmatic patients were further divided into 18 intermittent to mild asthma patients, 13 moderate to severe asthma patients. Brief lung function tests, total IgE in plasma and skin prick test were performed, and Asthma Control Questionnaire (ACQ) was reviewed. None of the patients had been treated with systemic glucocorticoids or other immunosuppressive agents within 1 month before the study. The patients with acute infection within 1 week or other chronic diseases were excluded. 19 age-and gender-matched healthy donors with normal pulmonary function and negative allergen tests were selected as normal controls. Participant details showed in Table1. This study was approved by the Research Ethics Board of Ruijin hospital, Shanghai Jiao Tong University School of Medicine. Written informed consent was obtained from all individuals.

Sample treatment

Eight millilitres of heparinized peripheral vein blood was collected. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density centrifugation. Plasma was isolated from peripheral blood. TGF- β , IL-17 and IgE in plasma were measured by ELISA. PBMCs were suspended at a density of 2×10^6 cells/mL in 1 mL Trizol reagent (Invitrogen, USA) for quantitative polymerase chain reaction (qPCR). The total mRNA in the Trizol reagent was reverse-transcribed into cDNA using cDNA kit (Promega, USA). The primers were designed by Life Technologies and synthesized by BioTNT. For amplification, the SYBR-Green I Real-Time PCR kit (BioTNT, China) was used. Each reaction was run in triplicate on the ABI ViiA 7 Real-Time PCR system (Life Technologies, USA) and was normalized to housekeeping gene β -actin transcripts. ΔCT values were recorded and converted to $2^{-\Delta CT}$. The sequence used were as follows: A2AR forward primer 5'ATC GCC ATT GAC CGC TAC AT3' and reverse primer 5'GCT GAC CGC AGT TGT TCC A3', Foxp3 forward primer 5'ATG CGA CCC CCT TTC ACC TAC3' and reverse primer 5'TGG CGG ATG GCG TTC TTC3', ROR- γ t forward primer 5'GGC TCC CTG GAT GAA TAG AAT G3' and reverse primer 5'AGG CAG AGG CAG AAA ATG TAA AG3', β -actin forward primer 5'AAG GTG ACA GCA GTC GGT T3' and reverse primer 5'TGT GTG GAC TTG GGA GAG G3'.

Mice

Allergen sensitization/challenge protocol

BALB/c mice (6–8 week of age) received an intraperitoneal injection of 100 μ g ovalbumin (OVA) (Sigma, USA) on days 0 and 14. On days 25, 26, 27, the mice inhaled 1mg/mL OVA for 30 min. Control animals received an intraperitoneal injection of 200 μ L saline with alum on days 0 and 14, and inhaled saline for 30 min on days 25, 26, 27. The A2AR agonist CGS21680 (Sigma, USA) (2 mg/kg) was given to OVA-sensitized mice on days 25, 26, 27 by intraperitoneal injection after the challenge. All mice were sacrificed on days 28. Experiments were performed in accordance with the approved institutional protocol and guidelines of the Institutional Animal Care and use Committee.

ELISA

Whole blood was collected into tubes, and centrifuged. Then the supernatant was collected for TGF- β , IL-17 and IgE measurement by ELISA.

Lung histology

Twenty-four hours after the challenge, lungs were analyzed by histology. The lung was fixed

in 10% formaline, dehydrated, mounted in paraffin, sectioned and stained with haematoxylin/eosin staining (HE) and analyzed with Leica Qwin plus software.

Lung tissue mononuclear cells isolation

Isolated the mononuclear cells using lymphocytes separation medium, adjusted the concentration to $2 \times 10^6/\text{mL}$, added 1 mL Trizol reagent, frozen at -80°C for RNA extraction. After reverse transcription reaction, qPCR was performed using SYBR-Green I Real-Time PCR kit. The sequence used were as follows: M-A2AR forward primers 5' TTC TTC GCC TGC TTT GTC CTG and 5' TTC GCC CTC ATA CCC GTC A, M-Foxp3 forward primers 5'CTA TGC CAC CCT TAT CCG A 3' and reverse primers 5' TCC TCT TCT TGC GAA ACT CA 3', M-ROR- γ t forward primers 5' CAG GAG CAA TGG AAG TCG TC 3' and reverse primers 5'CCG TGT AGA GGG CAA TCT CA 3', M- β -actin forward primers 5' CTT CTT TGC AGC TCC TTC GTT G 3' and reverse primers 5' ATG GAG GGG AAT ACA GCC CG 3'.

Immunohistochemical (IHC)

Immunohistochemistry was used to analyze the protein levels of TGF- β and IL-17 in lung tissues. Briefly, lung sections were embedded in paraffin and sectioned to $5\mu\text{m}$, followed by dewaxing in xylene and rehydration. Endogenous peroxidases were inhibited with 0.5% hydrogen peroxide in methanol for 10min, and slides were incubated overnight at 4°C with a rabbit polyclonal IgG antibody against TGF- β or IL-17. Immunodetection was performed with biotinylated goat anti-rabbit IgG reagent, HRP reagent and diaminobenzidine. Image pro plus 6.0 was used to analyze the average optical density (AOD) of TGF- β and IL-17 (the smaller AOD, the smaller the expression level of TGF- β and IL-17).

Lung function test

All mice were weighed, and anesthetized using sodium pentobarbital (50 mg/Kg) (Sigma, USA) by intraperitoneal injection, given the tracheal intubation. The anesthetized mice were placed into the body tracing box of mice. RC system and AniRes2005 (V3.0) (Bestlab technology, China) was used to collect the data.

Statistical analysis

SPSS17.0 was used for statistical analysis. Homogeneity of variance was tested first. If each group showed homogeneity, further analysis was done using Student Newman Keuls test and the data are presented as means $\pm$ SD. When heteroscedasticity was present in each group, the data were analyzed using Kruskal-Wallis H test and were expressed as medians (interquartile range). Spearman's rank correlation test was used to analyze the relationship between variables. Statistical significance was defined as P value less than 0.05.

Results

Expression A2AR mRNA in PBMCs was associated with asthma severity

Many studies reported that A2AR could be expressed by macrophages, dendritic cells and T cells including Treg cells and effector T cells and so on. We accessed the A2AR mRNA expression in PBMCs by qPCR, and found that the A2AR mRNA level in the asthma group slightly higher than in the control group, but there was no difference ($P > 0.05$) (Fig.1A). But when the patients with asthma were divided into intermittent to mild group and moderate to severe group, we found that the A2AR mRNA level in the intermittent to mild group higher than in the moderate to severe group ($0.30(0.22-0.33) \times 10^{-3}$ vs. $0.16(0.13-0.19) \times 10^{-3}$, $P < 0.001$) (Fig.1B).

A2AR might regulate the expression Foxp3 and ROR- γ t mRNA in PBMCs and TGF- β and IL-17 in plasma

We also detected the levels of Foxp3 and ROR- γ t mRNA expression in PBMCs by qPCR and TGF- β and IL-17 in plasma by ELISA. As we expected, Foxp3 mRNA ($0.35(0.27-0.37) \times 10^{-3}$ vs. $0.64(0.50-0.74) \times 10^{-3}$, $P < 0.001$) and TGF- β were significantly decreased while ROR- γ t mRNA ($10.67(9.58-11.99) \times 10^{-3}$ vs. $8.83(8.11-9.34) \times 10^{-3}$, $P < 0.001$) and IL-17 were highly elevated in patients with asthma compared with the controls. Then we investigated the correlations. We found that Foxp3 mRNA, TGF- β and FEV1%pred positively correlated with A2AR mRNA in asthma ($r = 0.522$, $p = 0.003$; $r = 0.642$, $p = 0.001$; $r = 0.391$, $p = 0.029$). ROR- γ t mRNA and IL-17 negatively correlated with A2AR mRNA in asthma ($r = -0.430$, $p = 0.016$; $r = -0.507$, $p = 0.019$). There was no significant correlation between A2AR mRNA and serum IgE ($r = -0.158$, $p = 0.460$). We also analyzed the correlations in different severity of asthma. But no significant correlations were found.

CGS could up-regulate Treg cells-related Foxp3 while down-regulate Th17 cells-related ROR- γ t in mouse model of asthma

Based on the above data, we hypothesized that A2AR might regulate the balance of Treg/Th17 cells. We firstly detected A2AR mRNA expression level in lung tissue of mice, and found that there was significant difference between control group and OVA group ($0.27(0.24-0.26) \times 10^{-3}$ vs. $0.16(0.14-0.20) \times 10^{-3}$, $P < 0.01$) (Fig.2A). Then CGS was given to OVA-sensitized mice by intraperitoneal injection after challenge. We found that CGS could up-regulate Foxp3 mRNA ($0.48(0.39-0.60) \times 10^{-3}$ vs. $0.33(0.26-0.39) \times 10^{-3}$, $P < 0.05$) while significantly down-regulate ROR- γ t mRNA ($6.23(4.94-7.21) \times 10^{-3}$ vs. $8.86(7.17-10.23) \times 10^{-3}$, $P < 0.01$) in mouse model of asthma (Fig.2B, C).

CGS could increase Treg cells-related cytokine TGF- β while decrease Th17 cells-related cytokine IL-17 in mouse model of asthma

The data above showed that the enhanced activity of A2AR could maintain Foxp3 expression and reduce ROR- γ t expression. It is obvious that the balance of Treg/Th17 cells connects with A2AR tightly. After CGS treatment, we detected Th17 cells-related cytokine IL-17 and Treg cells-related cytokine TGF- β in plasma. Control group was TGF- β (112.63 ± 85.68) pg/mL, IL-17 (207.74 ± 140.14) pg/mL. OVA group was TGF- β (28.20 ± 20.01) pg/mL, IL-17 (338.33 ± 48.90) pg/mL. OVA+CGS group was TGF- β (91.33 ± 65.81) pg/mL, IL-17 (59.77 ± 75.74) pg/mL. TGF- β level in the OVA+CGS group was higher than in the OVA group ($P < 0.05$), and IL-17 level in the OVA+CGS group was lower than in the OVA group ($P < 0.01$). The data showed that CGS could elevate TGF- β concentration while decrease IL-17 concentration (Fig.3A, B). We also examined the protein expression of TGF- β and IL-17 in lung tissues, and found that TGF- β and IL-17 mainly expressed in membranes. Average optical density (AOD) of TGF- β and IL-17 in control group was $0.34(0.32-0.36)$ and $0.22(0.13-0.29)$, OVA group $0.28(0.25-0.31)$ and $0.33(0.31-0.35)$, OVA+CGS group $0.37(0.35-0.39)$ and $0.26(0.34-0.29)$ (Fig.4). As we expected, TGF- β were significantly elevated while IL-17 highly decreased in mouse model of asthma after CGS treatment ($P < 0.001$, $P < 0.01$) (Fig.4).

CGS could alleviate the infiltration of lung inflammation cells and bronchioles plasticity of mouse model of asthma

Total IgE of control group was (79.78 ± 12.64) ng/mL, OVA group (102.23 ± 1.92) ng/mL, OVA+CGS group (88.01 ± 11.53) ng/mL. IgE in OVA+CGS group was lower than OVA group ($P < 0.05$) (Fig.3C). Based on these data, we concluded that CGS could inhibit the IgE secretion of mouse model of asthma. Lung tissue was stained with HE and analyzed with a microscope to

observe the infiltration of inflammation cells. We found that a plenty of inflammation cells including mononuclear macrophages, lymphocytes, eosinophils and neutrophils infiltrated around the bronchioles and the vessels in lung tissues of OVA group compared with control group (Fig.5). However, CGS could suppress the infiltration of lung inflammation cells (Fig.6A). The ratio of ID (inner diameter) and OD (outer diameter) of bronchioles in the control group (0.78 ± 0.96 vs. 0.70 ± 0.72 , $P < 0.01$) and OVA+CGS group (0.80 ± 0.05 vs. 0.70 ± 0.72 , $P < 0.01$) was much higher than in the OVA group (Fig.6B).

CGS could improve the lung function of mouse model of asthma

We further investigated the influence of CGS to lung function of mouse model of asthma, and found that the percentage of FEV0.1/FVC (35.08 ± 5.39 vs. 21.48 ± 2.09 , $P < 0.001$), FEV0.3/FVC (76.92 ± 9.82 vs. 63.22 ± 5.32 , $P < 0.05$), FVC (1.26 ± 0.07 vs. 0.60 ± 0.17 , $P < 0.001$), flow of 25%FVC (3.13 ± 0.68 vs. 3.95 ± 0.67 , $P < 0.05$), flow of 50%FVC (2.46 ± 2.29 vs. 1.72 ± 0.64 , $P < 0.05$) and Cdyn (dynamic compliance) (0.033 ± 0.056 vs. 0.016 ± 0.037 , $P < 0.001$) in the control group were obviously higher than in the OVA group (Fig.7), and CGS could improve the percentages of FEV0.1/FVC (29.92 ± 2.23 vs. 21.48 ± 2.09 , $P < 0.01$), FVC (1.09 ± 0.19 vs. 0.60 ± 0.17 , $P < 0.001$) and Cdyn (0.028 ± 0.056 vs. 0.016 ± 0.037 , $P < 0.001$) of OVA group (Fig.7A, B, D). However, CGS could slightly improve the percentage of FEV0.3/FVC, the flow of 25%FVC and 50%FVC and 75%FVC of OVA group, but there was no significant difference between two groups ($P > 0.05$) (Fig.7A, C).

Discussion

It is known that the imbalance of Treg/Th17 cells existed in asthma (16). But previous studies only found that this imbalance existed in peripheral blood of asthma. In the present study, we found that the imbalance also occurred in lung tissue of mouse model of mice.

In the present study, the A2AR mRNA level was associated with asthma severity. It is known that adenosine is produced in asthmatic airway and plays an active role in airway inflammatory responses and hyperactivity in asthma (17, 18). But adenosine acts on A2AR, negative feedback of inflammation, namely, endogenous protective mechanisms aiming at the restoration of homeostasis will be activated (19, 20). The reason why the A2AR mRNA level in intermittent to mild group was obviously higher than in the control group, we speculated that the body of intermittent to mild asthmatic patients enhanced the negative feedback by elevating the expression of A2AR to antagonize the inflammation. However, the body of moderate to severe asthmatics might lose this powerfully antagonistic ability.

Haskó et al reported that adenosine activates A2AR on T-effector cells to inhibit T-cell receptor (TCR)-mediated signal to decrease T effector cell proliferation and Th17 cells generation, and A2A receptor stimulation on Treg cells augments Foxp3 expression in these cells (21). Zarek et al confirmed that the activation of A2AR could induce the generation of Treg cells (22). In our population study, we found that Foxp3 mRNA, TGF- β and FEV1%pred positively correlated with A2AR mRNA in asthma. ROR- γ t mRNA and IL-17 negatively correlated with A2AR mRNA in asthma. The data indicated that A2AR might regulate the balance of Treg/Th17 cells in asthma. To further confirm this hypothesis, we used the A2AR agonist CGS (moderate affinity and high selectivity) (23) in a mouse model of asthma.

Moreover, we found that CGS significantly alleviated accumulation of lung inflammatory cells, decreased IgE in plasma and improved lung function of mouse model of asthma. Some studies found that monocytes, lymphocytes, neutrophils, basophils, mast cells express A2AR (24).

The lung inflammation in mouse model of mice mediated by these inflammatory cells could be inhibited when CGS acted on the A2AR expressed by these cells.

However, there are several limitations that should be considered when interpreting these results. First, we need to further group, and eliminate the interference of ICS on the altered expression of A2AR, Foxp3 and ROR- γ t mRNA in PBMCs and lung tissues, and TGF- β and IL-17 in plasma and lung tissues. Second, although some studies had found that the expressions of A1R, A2AB and A3 have elevated in lungs of asthmatic subjects [25-27], we need to look at the expression of these adenosine receptors and the effect of CGS on adenosine receptors. Third, to further confirm our hypothesis, we still need to explore the effect of A2AR on the proportions of Treg cells and Th17 cells, the mRNA or protein levels of Treg cells related Foxp3 and Th17 cells related ROR- γ in lungs of mice. In addition, TGF- β and IL-17 could be secreted by many other cells including B cells and NKT cells and so on. Thus, we also need to evaluate the levels of Treg cells related TGF- β and Th17 cells related IL-17. Four, CGS was given to OVA-sensitized mice after the challenge. Our founding indicated that CGS as prophylactic drugs might prevent the development of asthma. But the therapeutic effects of CGS on inflammation, AHR and Treg/Th17 balance need to be explored. Lastly, we missed the effect of CGS alone in the absence of OVA.

In conclusion, A2AR could regulate the balance of Treg/Th17 cells in asthma. But the further mechanism still needs to be explored in the future.

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Table1 General Characteristics of Subjects

Index	Intermittent to mild asthma	Moderate to severe asthma	Control	P
Number	18	13	19	---

age (years)	37.56±11.54	35.62±10.813	38.11±11.37	>0.05
gender (M/F)	12/6	9/3	13/6	>0.05
FEV1%pred	80.35±16.63	66.20±10.05	92.27±6.54	<0.01
IgE (ng/ml)	28.77±18.54	44.34±27.19	0.95±1.67	<0.01

Figure 1. A2AR mRNA level in PBMCs. The total RNA was isolated from PBMCs and followed by reverse transcription and real-time PCR. A2AR mRNA level of asthma group (n=31) slightly higher than control group (n=19), but there was no difference ($P > 0.05$). When the patients with asthma divided into intermittent to mild group and moderate to severe group, intermittent to mild group (n=18) showed significant high level compared with control group (n=19) ($P < 0.05$) and moderate to severe group (n=13) ($P < 0.001$). A2AR mRNA level was associated with the illness severity. The experiment was repeated three times. Data are represented as median (interquartile range), *indicates p-values < 0.05 , ***indicates p-values < 0.001 .

Figure 2. A2AR, Foxp3 and ROR- γ t mRNA level in lung tissues of mice. The total RNA was isolated from lung tissues and followed by reverse transcription and real-time PCR. The difference of A2AR mRNA between control group and OVA group was significant ($P < 0.01$). The difference of Foxp3 ($P < 0.05$) and ROR- γ t ($P < 0.01$) mRNA between OVA group and OVA+CGS group was significant. CGS could enhance Treg cells-related Foxp3 and decrease Th17 cells-related ROR- γ t in mouse model of asthma. The experiment was repeated three times. Data are represented as median (interquartile range), *indicates p-values < 0.05 , **indicates p-values < 0.01 .

Figure 3. TGF- β , IL-17 and IgE levels in plasma of mice. The plasma was assayed by ELISA. OVA+CGS group showed significant high level of TGF- β ($P < 0.05$) and low levels of IL-17 ($P < 0.01$) and IgE ($P < 0.05$) compared with OVA group. CGS could elevate TGF- β concentration while decrease IL-17 and IgE concentration in plasma. The experiment was repeated three times. Data are represented as means $\pm$ SD, *indicates p-values < 0.05 , **indicates p-values < 0.01 .

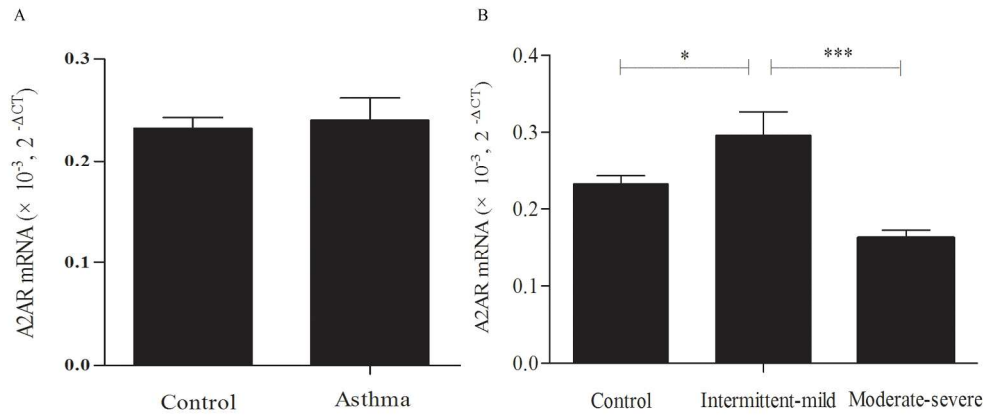
Figure 4. Immunohistochemistry analysis of the expression of TGF- β and IL-17 in lung tissues of mice (Magnification: 200 $\times$). Representative images of immunohistochemistry for TGF- β in control group (A), OVA group (B), OVA+CGS group(C) and for IL-17 in control group (D), OVA group (E), OVA+CGS group (F). C and F show TGF- β were significantly elevated while IL-17 highly decreased in OVA group after CGS treatment.

Figure 5. The infiltration of lung inflammation cells of mice ($\times 200$). (A) Control group. (B) OVA group. (C) OVA+CGS group. The lung was fixed in 10% formaline, dehydrated, mounted in paraffin, sectioned and stained with HE and analysed with a microscope. Plenty of inflammation cells including mononuclear macrophages, lymphocytes, eosnophils and neutrophils infiltrated around the bronchioles and the vessels in lung tissues of the OVA group. CGS could alleviate the infiltration of lung inflammation cells in mouse model of asthma. The experiment was repeated three times.

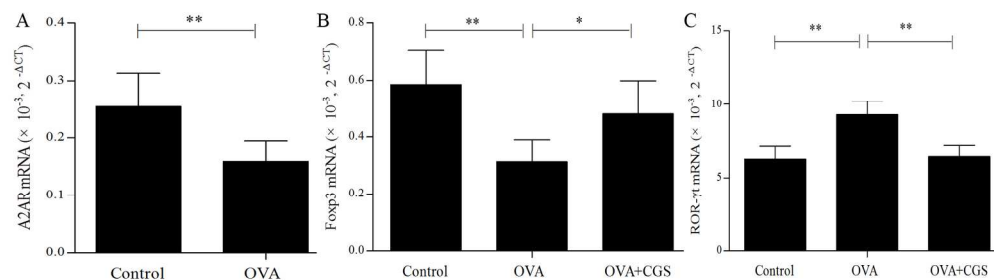
Figure 6. The infiltration of lung inflammation cells and bronchioles plasticity in mice. Mononuclear macrophages, lymphocytes, eosnophils and neutrophil were identified and counted based on cell size, morphology of cell nucleus and cell size. A plenty of mononuclear macrophages, lymphocytes, eosnophils and neutrophils infiltrated around the bronchioles and the vessels in lung tissues of the OVA group compared with control group. CGS could obviously suppress the infiltration of lung inflammation cells of mouse model of asthma (A). OVA group showed significant low level of ratio of ID and OD of bronchioles compared with control group, and CGS could improve

the ratio of ID and OD (B). The experiment was repeated three times. Data are represented as means $\pm$ SD, *indicates p-values < 0.05 , **indicates p-values < 0.01 , ***indicates p-values < 0.001 . ID: internal diameter, OD: outer diameter.

Figure 7. Lung functions of mice. OVA group showed significant low percentages of FEV0.1/FVC and FEV0.3/FVC (A), FVC (B), flow of 25%FVC and 50%FVC (C) and Cdyn (D) compared with control group. CGS could significantly improve the percentages of FEV0.1/FVC, FVC and Cdyn of in mouse model of asthma (A, B and D). The experiment was repeated three times. Data are represented as means $\pm$ SD, *indicates p-values < 0.05 , **indicates p-values < 0.01 , ***indicates p-values < 0.001 . FEV0.1/FVC: The ratio of forced expiratory volume in 0.1 second, FVC: Forced volume capacity, Cdyn: dynamic compliance.

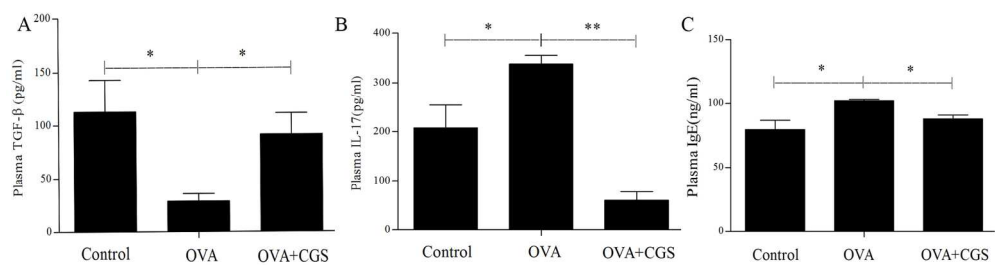


A2AR mRNA level in PBMCs. The total RNA was isolated from PBMCs and followed by reverse transcription and real-time PCR. A2AR mRNA level of asthma group (n=31) slightly higher than control group (n=19), but there was no difference ($P > 0.05$). When the patients with asthma divided into intermittent to mild group and moderate to severe group, intermittent to mild group (n=18) showed significant high level compared with control group (n=19) ($P < 0.05$) and moderate to severe group (n=13) ($P < 0.001$). A2AR mRNA level was associated with the illness severity. The experiment was repeated three times. Data are represented as median (interquartile range), *indicates p-values < 0.05 , ***indicates p-values < 0.001 .
292x127mm (300 x 300 DPI)

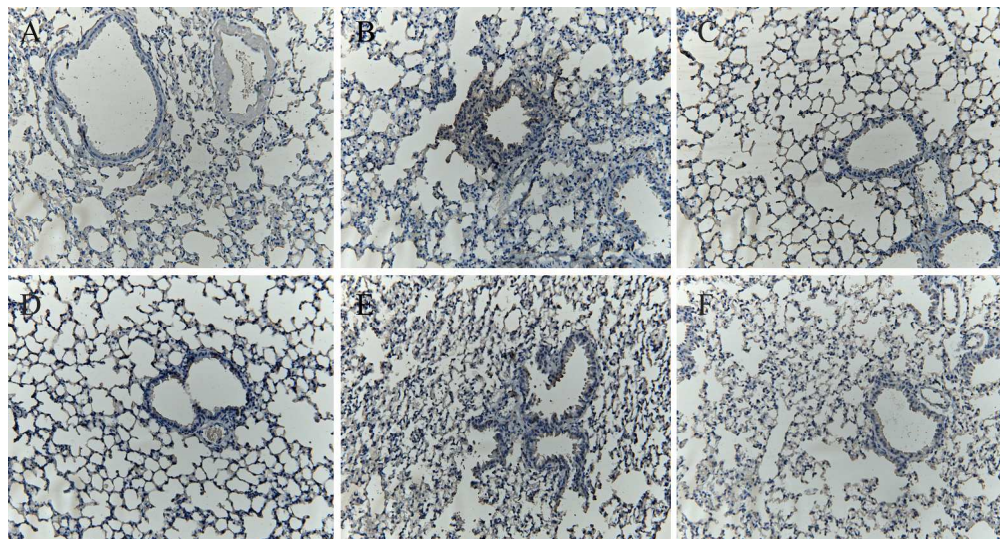


A2AR, Foxp3 and ROR- γ t mRNA level in lung tissues of mice. The total RNA was isolated from lung tissues and followed by reverse transcription and real-time PCR. The difference of A2AR mRNA between control group and OVA group was significant ($P < 0.01$). The difference of Foxp3 ($P < 0.05$) and ROR- γ t ($P < 0.01$) mRNA between OVA group and OVA+CGS group was significant. CGS could enhance Treg cells-related Foxp3 and decrease Th17 cells-related ROR- γ t in mouse model of asthma. The experiment was repeated three times. Data are represented as median (interquartile range), *indicates p-values < 0.05, **indicates p-values < 0.01.

180x50mm (300 x 300 DPI)

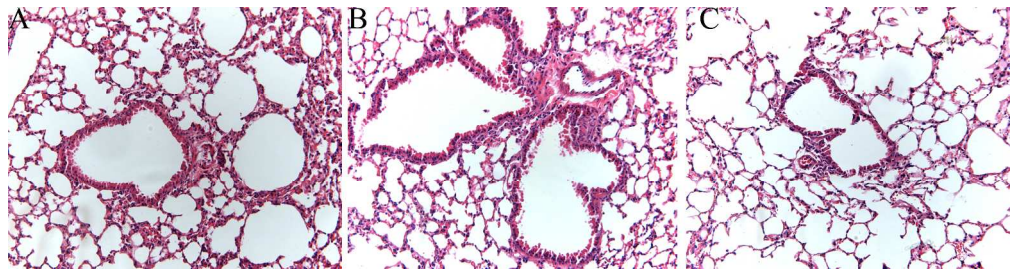


TGF-β, IL-17 and IgE levels in plasma of mice. The plasma was assayed by ELISA. OVA+CGS group showed significant high level of TGF-β ($P < 0.05$) and low levels of IL-17 ($P < 0.01$) and IgE ($P < 0.05$) compared with OVA group. CGS could elevate TGF-β concentration while decrease IL-17 and IgE concentration in plasma. The experiment was repeated three times. Data are represented as means $\pm$ SD, *indicates p-values < 0.05 , **indicates p-values < 0.01 .
180x47mm (300 x 300 DPI)



Immunohistochemistry analysis of the expression of TGF- β and IL-17 in lung tissues of mice (Magnification: 200 $\times$). Representative images of immunohistochemistry for TGF- β in control group (A), OVA group (B), OVA+CGS group (C) and for IL-17 in control group (D), OVA group (E), OVA+CGS group (F). C and F show TGF- β were significantly elevated while IL-17 highly decreased in OVA group after CGS treatment.

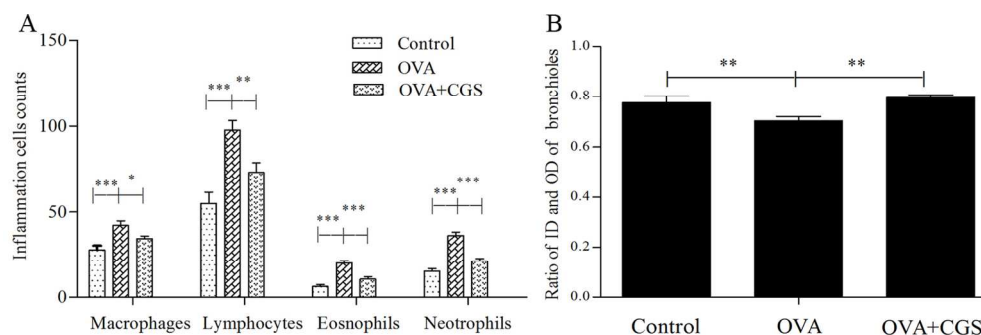
349x186mm (300 x 300 DPI)



The infiltration of lung inflammation cells of mice ($\times 200$). (A) Control group. (B) OVA group. (C) OVA+CGS group. The lung was fixed in 10% formaline, dehydrated, mounted in paraffin, sectioned and stained with HE and analysed with a microscope. Plenty of inflammation cells including mononuclear macrophages, lymphocytes, eosnophils and neutrophils infiltrated around the bronchioles and the vessels in lung tissues of the OVA group. CGS could alleviate the infiltration of lung inflammation cells in mouse model of asthma. The experiment was repeated three times.

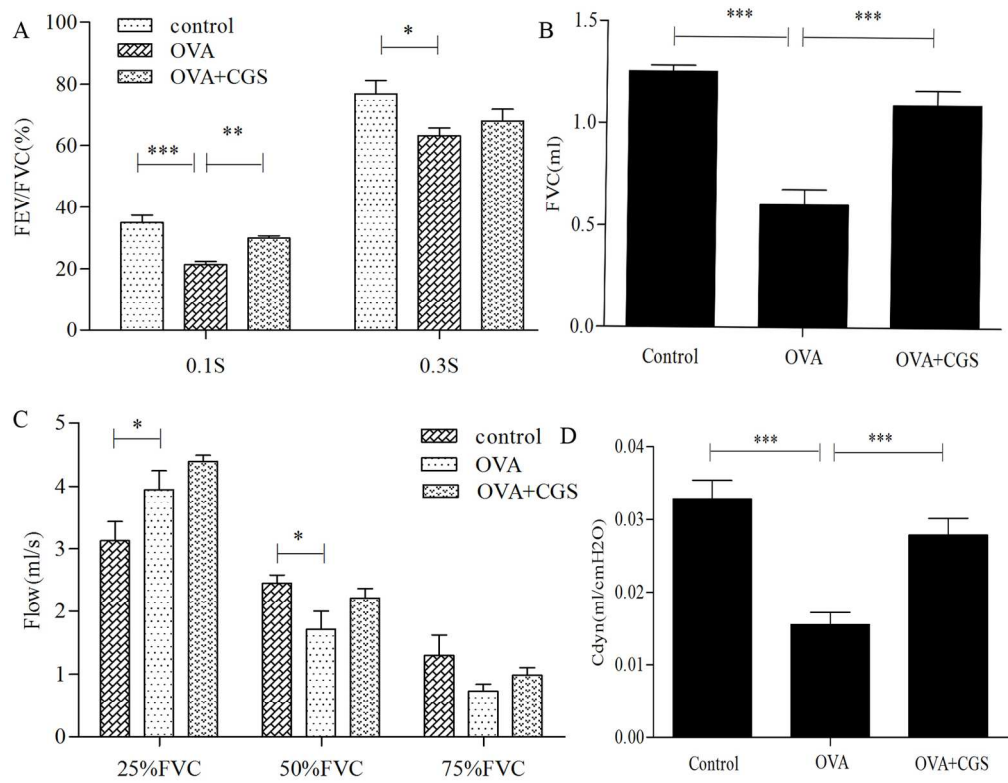
347x90mm (300 x 300 DPI)

Accepted



The infiltration of lung inflammation cells and bronchioles plasticity in mice. Mononuclear macrophages, lymphocytes, eosinophils and neutrophil were identified and counted based on cell size, morphology of cell nucleus and cell size. A plenty of mononuclear macrophages, lymphocytes, eosinophils and neutrophils infiltrated around the bronchioles and the vessels in lung tissues of the OVA group compared with control group. CGS could obviously suppress the infiltration of lung inflammation cells of mouse model of asthma (A). OVA group showed significant low level of ratio of ID and OD of bronchioles compared with control group, and CGS could improve the ratio of ID and OD (B). The experiment was repeated three times. Data are represented as means $\pm$ SD, *indicates p-values < 0.05, **indicates p-values < 0.01, ***indicates p-values < 0.001. ID: internal diameter, OD: outer diameter.

139x45mm (300 x 300 DPI)



Lung functions of mice. OVA group showed significant low percentages of FEV0.1/FVC and FEV0.3/FVC (A), FVC (B), flow of 25%FVC and 50%FVC (C) and Cdyn (D) compared with control group. CGS could significantly improve the percentages of FEV0.1/FVC, FVC and Cdyn of in mouse model of asthma (A, B and D). The experiment was repeated three times. Data are represented as means $\pm$ SD, *indicates p-values < 0.05, **indicates p-values < 0.01, ***indicates p-values < 0.001. FEV0.1/FVC: The ratio of forced expiratory volume in 0.1 second, FVC: Forced volume capacity, Cdyn: dynamic compliance.