

Effect of low-level laser therapy on allergic asthma in rats

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Abstract Asthma is a complex chronic inflammatory disease of the airways that involves the activation of many inflammatory and other types of cells. We investigated the effect of low-level laser therapy (LLLT) on allergic asthma in rats and compared its effect with that of the glucocorticoid budesonide. Asthma was induced by challenge and repeated exposure to ovalbumin. Asthmatic rats were then treated with LLLT or budesonide suspension. LLLT at 8 J/cm² once daily for 21 days could relieve pathological damage and airway inflammation in asthmatic rats. LLLT could decrease the total numbers of cells and eosinophils in bronchoalveolar lavage fluid. LLLT could reduce levels of IL-4 and increase IFN- γ levels in bronchoalveolar lavage fluid and serum, meanwhile reduce serum IgE levels. Flow cytometry assay showed that LLLT can regulate the Th1/Th2 imbalance of asthmatic rats. LLLT had a similar effect to that of budesonide. These findings suggest that the mechanism of LLLT treatment of asthma is by adjustment of Th1/Th2 imbalance. Thus, LLLT could take over some of the effects of budesonide for the treatment of asthma, thereby reducing some of the side effects of budesonide.

Keywords Asthma · LLLT · IL-4 · IFN- γ

Introduction

Asthma is a chronic airway inflammatory disease associated with bronchial hyper-responsiveness and reversible airflow limitation. The prevalence of asthma has been increasing at an alarming rate in developing countries. *The Global Burden of Asthma* report published by the Global Initiative for Asthma (GINA) in 2006 stated that ~300 million people worldwide have asthma and that 100 million new asthma patients will be present worldwide by 2025 [1].

Glucocorticoids are the drugs of choice for long-term treatment of patients with asthma. Glucocorticoids can effectively control asthma symptoms, improve lung function, and reduce airway hyper-responsiveness. However, glucocorticoids can be absorbed into the blood circulation through the gastrointestinal tract and terminal lung tissue. The systemic effects of glucocorticoids (especially the potential inhibition of adrenal function and body growth) have been the focus of attention. Studies have shown that long-term inhalation of high-dose glucocorticoids may have significant systemic adverse effects: adrenal suppression, osteoporosis, and an increased risk of fracture [2]. Hence, exploring new asthma treatments is very important.

Asthma is characterized by chronic inflammation of the airways, participation of various cell types (eosinophils, mast cells, T lymphocytes, neutrophils, airway epithelial cells) and various cell components (e.g., interleukin (IL)-4, interferon (IFN)- γ , and immunoglobulin(Ig) E. All of these factors have important roles in asthma [3–5].

Low-level laser therapy (LLLT) has been applied for treating musculoskeletal aches and pains, wound healing, chronic inflammation, and acute inflammation. Several studies have suggested the benefits of using LLLT. Pires et al. noted that LLLT induced an anti-inflammatory effect through the regulation of transcription factors linked to cyclooxygenase (Cox)-2 and through the regulation of the

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expression of pro-inflammatory micro-ribonucleic acids (mRNAs) [6]. In addition, Aimbire et al. reported that LLLT reduced neutrophil influx and the expression of IL-1 β mRNA [7]. However, few studies have focused on the effect of LLLT on allergic asthma. The purpose of the present study was to evaluate the effect of LLLT on ovalbumin (OVA)-induced allergic asthma and provide basic information on the clinical application of LLLT.

Materials and methods

The study protocol was approved by the Animal Care Committee of Tianjin Medical University (Tianjin, China).

Animals

Male Wistar rats (200–220 g; Laboratory Animal Center of the Academy of Military Medical Sciences, Beijing, China) were kept at an ambient temperature of 22 $\pm$ 2 °C and with a 12-h light–dark cycle. Drinking water and food pellets were freely available.

Sensitization and challenges

Forty Wistar rats were divided randomly into four groups: normal control, asthmatic, LLLT, and budesonide. OVA sensitization was carried out by concurrent subcutaneous injection of 2 mg OVA (grade V; Sigma–Aldrich, St. Louis, MO, USA) adsorbed onto 200 mg/mL aluminum hydroxide in 1 mL of phosphate-buffered saline (PBS) and intraperitoneal injection of 6 $\times$ 10⁹ heat-killed *Bordetella pertussis* bacilli (Chinese national institute for the control of pharmaceutical and biological products, Beijing, China) as adjuvant on day 0 and day 7. Rats in the normal control group were treated with 1 mL of PBS containing only 200 mg/mL aluminum hydroxide.

Two weeks later, the rats were placed in a transparent glass chamber (approximately 40 cm $\times$ 40 cm $\times$ 20 cm in volume) connected to an Ultrasonic Nebulizer (YC-Y800B; Yadu, Shanghai, China). They were then subjected to repeated bronchial challenge via OVA (2 % w/v, grade II; Sigma–Aldrich, USA) inhalation for 20 min/day for 21 days [8]. Rats in the control group were challenged with PBS.

Laser irradiation and budesonide treatment

After OVA inhalation, the LLLT group underwent treatment with LLLT. A continuous-wave Semiconductor Laser (Laser Medicine Laboratory, Biomedical Engineering Institute, Chinese Academy of Medical Science, Beijing, China) with an output power of 80 mW and a wavelength of 810 nm was used. The focus was the rat chest. The diameter of the spot size

was $\sim$ 4 cm, the exposure time $\sim$ 20 min. LLLT at 8 J/cm² was applied once daily lasting 21 days. Simultaneously, rats from the budesonide group were placed in the transparent glass chamber connected to the pump and given 1 mg of budesonide (AstraZeneca, Luton, UK) suspension for inhalation by nebulizer (air compression pump NE-C28, Omron, Japan). All animals were sacrificed via pentobarbital overdose 24 h after the final antigen challenge. We repeated the experiments twice in succession to observe the effects of LLLT on rat asthma model. Except changes of cytokines in the BLAF and Th1/Th2 cellular populations were only measured at the second experiment, all the other detections were conducted in both experiments.

Histopathology of lung tissue

Lung tissues were removed rapidly from freshly killed rats. They were fixed in 10 % (v/v) neutral buffered formalin for 24 h at 4 °C. Lung tissues were embedded in paraffin, cut into sections of 4- μ m thickness, and stained with hematoxylin and eosin (H&E). Slices were evaluated via light microscopy under identical conditions. The degrees of peribronchial and perivascular inflammation were scored in a double-blind screen with two independent investigators blinded to the study protocol using a subjective scale of 0–3 (0, no inflammation; 1, occasional cuffing with inflammatory cells; 2, most bronchi or vessels surrounded by a thin layer of 1–5 inflammatory cells; 3, most bronchi or vessels surrounded by a thick layer of >5 inflammatory cells) [9].

Bronchoalveolar lavage fluid and cellular analyses

Rats were killed 24 h after the final challenge. The trachea was exposed and cannulated, accompanied by ligation of the right main bronchus, allowing the left lung to be lavaged with 3 mL of sterile saline. The lung was aspirated gently and the process repeated thrice (9 mL in total). Bronchoalveolar lavage fluid (BALF) was centrifuged at 400 $\times$ g for 10 min at 4 °C. The cell pellets from all BALF specimens were suspended in 1 mL physiological (0.9 %) saline. To carry out counts for the total number of cells and eosinophils, 0.1 mL of the cell suspension was transferred to a glass slide and stained with Wright–Giemsa stain. A microscope was used to examine 300 nucleated cells. The total numbers of cells and eosinophils were calculated. Enzyme-linked immunosorbent assay (ELISA) was employed for measurement of the levels of IL-4 and INF- γ in BALF according to manufacturer instructions (R&D Systems, Minneapolis, MN, USA).

Hematological assays

Blood was taken from the inferior caval vein and was centrifuged at 1,000 $\times$ g for 10 min at room temperature to isolate

serum. The levels of IL-4, INF- γ , and IgE in the serum were measured by ELISA.

Flow cytometry analysis

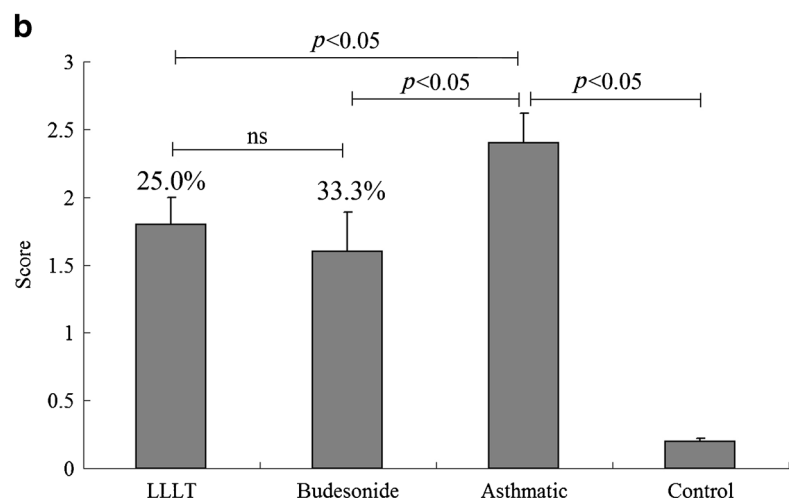
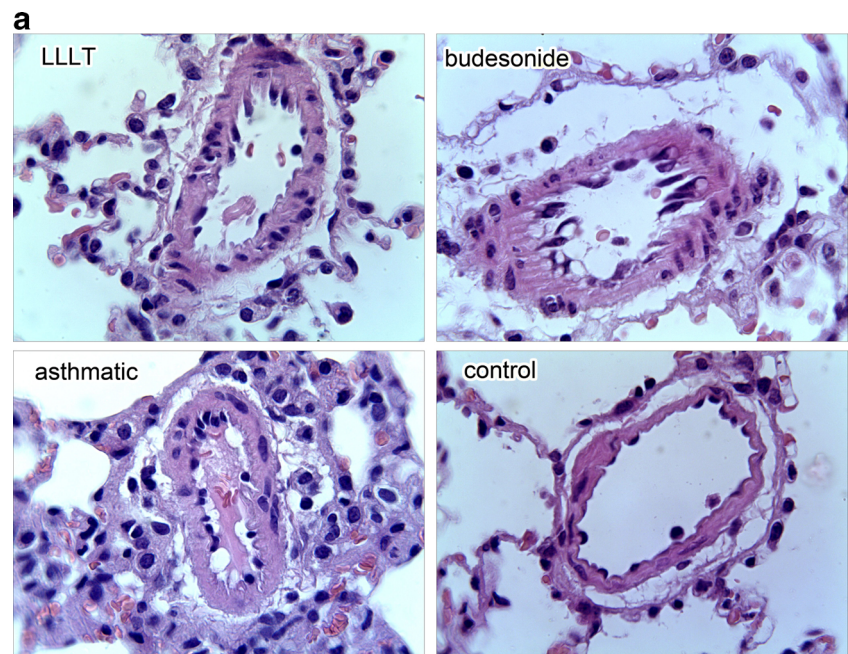
The spleen of rat was minced using 200 purpose nylon nets. Splenic cell was collected by centrifuged suspension at $380\times g$ for 3 min. The red blood cells were lysed with red blood cell lysing solution. Finally, the cells were suspended at 2×10^6 cells per milliliter in RPMI-FCS. Phytohemagglutinin (Sigma–Aldrich, USA) at 10 $\mu\text{g/mL}$ and BFA/Monensin (4 $\mu\text{g/mL}$) mixture (MultiScience, Hangzhou, China) were added. The cells were cultured in an incubator at 37 °C with 5 % CO_2 for 8 h. Then, APC-CD4, FITC-IFN- γ , and PE-IL-4 antibodies (BD Pharmingen, Franklin Lakes, NJ, USA) were added

according to the manufacturer's protocol for flow cytometry analysis. In flow cytometry analysis, APC-CD4 antibody and FITC-IFN- γ were used to mark Th1 cells, while APC-CD4 antibody and PE-IL-4 antibody were used to mark Th2 cells. Analysis was carried out on the flow cytometer (LSR, BD, USA) using Cell Quest Pro software.

Statistical analyses

SPSS 14.0 was used for statistical analyses. Data are the mean $\pm$ standard deviation (SD). Statistical comparisons were made using one-way analysis of variance (ANOVA). Significant differences between groups were determined using the least-square difference test. $P<0.05$ was considered significant.

Fig. 1 Effect of LLLT on pulmonary inflammation in asthmatic rats. Tissues were stained with hematoxylin and eosin (magnification= $\times 400$) (a). Infiltration of inflammatory cells in lung tissues was scored (b). $N=10$. Statistically significant differences ($p<0.05$) or nonsignificant (ns) are indicated in the figure. Percentage represents the value change compared with the asthmatic group



Results

The experiments to observe the effects of LLLT on rat asthma model have been repeated twice in succession. Except changes of cytokines in the BLAF and Th1/Th2 cellular populations were only measured at the second experiment, all the other detections were conducted in both experiments. In matters of the measurements conducted in both experiments, the trends of the results were similar.

Pathologic changes

Figure 1a shows the representative histological findings. The airway wall, basement membrane, and smooth-muscle layer in asthmatic rats were significantly thicker in asthmatic rats than those in control rats. Marked infiltration with lymphocytes and a few eosinophils in the bronchial walls and peribronchial areas were found in the asthmatic group, and proliferation of goblet cells was obvious. Pathological changes and airway inflammation were significantly less in the LLLT group and budesonide group compared with the asthma group. The Inflammation Score showed that LLLT and budesonide significantly reduced the degree of infiltration of inflammatory cells compared with the asthmatic group ($p < 0.05$), but there was no significant difference between the LLLT group and budesonide group ($p > 0.05$) (Fig. 1b).

Total cell count and eosinophil count in BALF

The number of inflammatory cells in BALF between the four groups was measured and compared. Compared with the control group, the numbers of total cells and eosinophils in BALF in the asthmatic group were significantly higher

($p < 0.05$). The total numbers of cells and eosinophils in the LLLT treatment group were significantly reduced when compared with the asthmatic group ($p < 0.05$) but did not differ significantly from the numbers in the budesonide group ($p > 0.05$) (Fig. 2).

Concentration of IL-4 and IFN- γ

Cytokine detection results showed that cytokines changes in serum were consistent with that in BALF. Levels of IL-4 increased and IFN- γ levels decreased in the asthmatic group compared with the control group ($p < 0.05$). LLLT and budesonide significantly reduced the IL-4 concentration and increased the IFN- γ concentration compared with the asthmatic group ($p < 0.05$), but there was no significant difference between the LLLT group and budesonide group ($p > 0.05$) (Fig. 3).

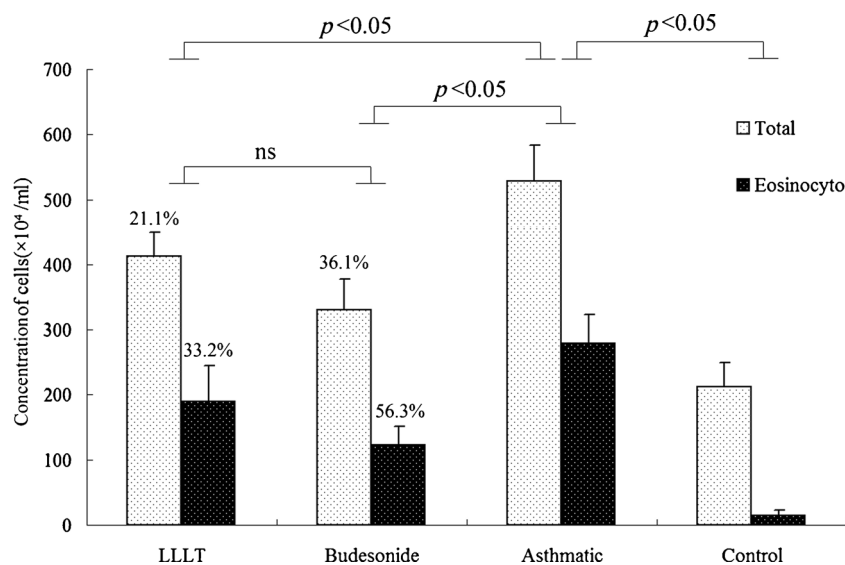
IgE level in serum

The level of IgE in the serum of rats in the asthmatic group was significantly higher than that in the normal control group ($p < 0.05$). LLLT and budesonide significantly reduced the level of IgE compared with the asthmatic group ($p < 0.05$), but there was no significant difference between the LLLT group and budesonide group ($p > 0.05$) (Fig. 4).

Th1/Th2 cellular population

Compared to the normal control, Th2 cellular population of the asthmatic group increased and Th1 cellular population

Fig. 2 Inflammatory cells in BALF of the four groups. $N=10$. Statistically significant differences ($p < 0.05$) or nonsignificant (ns) are indicated in the figure. Percentage represents the value change compared with the asthmatic group



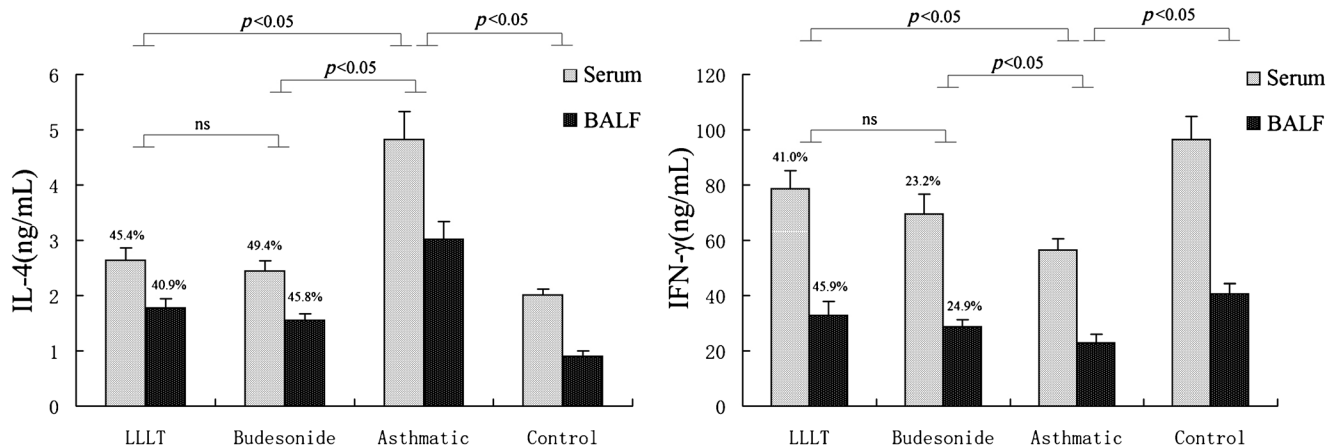


Fig. 3 Effect of LLLT on total concentrations of IL-4 and IFN- γ in the serum and BALF of asthmatic rats. $N=10$. Statistically significant differences ($p < 0.05$) or nonsignificant (ns) are indicated in the figure. Percentage represents the value change compared with the asthmatic group

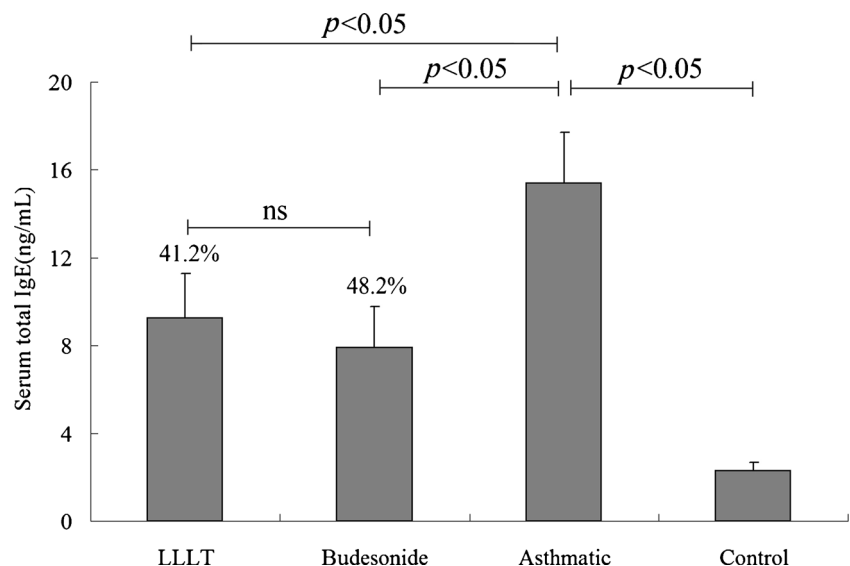
decreased, thus, the proportion of Th1/Th2 decreased significantly in the asthmatic group. Treatment with LLLT and budesonide could significantly decrease the population of Th2, increase that of Th1, thus increase the Th1/Th2 proportion significantly ($p < 0.05$). And there was no significant difference between the LLLT group and budesonide group ($p > 0.05$) (Fig. 5).

Discussion

LLL

therapeutic effect on lung inflammation. In an acute pulmonary inflammatory model in rats, animals received saline (control), LLLT, LPS, LPS+LLL

Fig. 4 Effect of LLLT on total IgE concentration in the serum of asthmatic rats. $N=10$. Statistically significant differences ($p < 0.05$) or nonsignificant (ns) are indicated in the figure. Percentage represents the value change compared with the asthmatic group



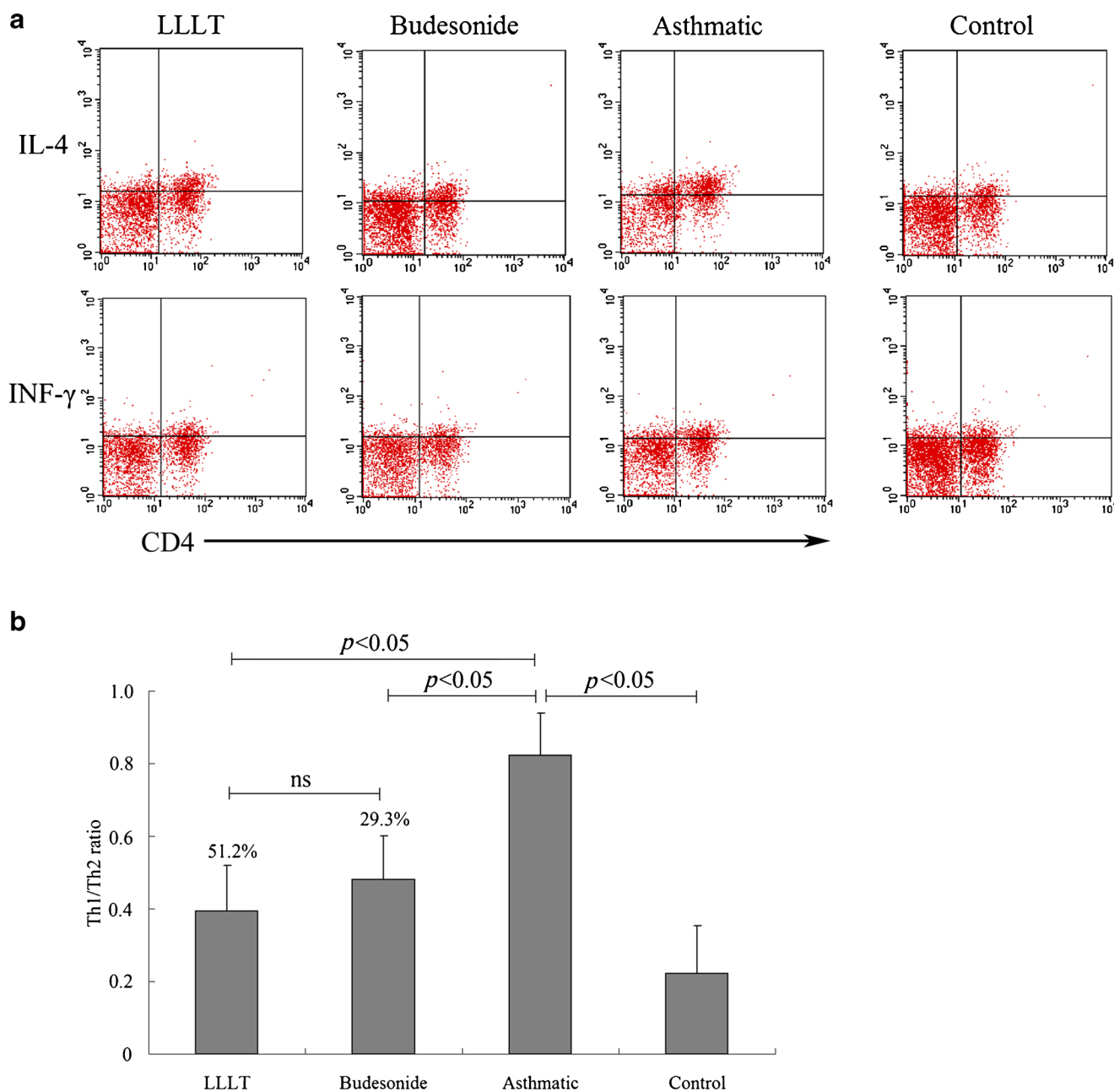


Fig. 5 Flow cytometry analysis of Th1/Th2 in the spleen. Flow cytometry analysis of Th1/Th2 markers in the spleen, APC-CD4 and FITC-IFN- γ used as markers of Th1 cells while APC-CD4 and PE-IL-4 antibody used as markers of Th2 cells (**a**). Th1/Th2 in the spleen (**b**).

$N=10$. Statistically significant differences ($p < 0.05$) or nonsignificant (ns) are indicated in the figure. Percentage represents the value change compared with the asthmatic group

GaAlAs laser. LLLT seemed to be responsible for restoring airway reactivity to cholinergic agonists and reducing inflammatory markers directly linked to pulmonary inflammation [14, 15].

We have reported that laser irradiation with a wavelength of 632 nm has anti-inflammatory effects on *Staphylococcus epidermidis* endophthalmitis in rabbits and reduced levels of TNF- α in aqueous fluid of model animals [16]. According to the World Association for Laser Therapy (WALT) dose

recommendation, doses that are frequently used in the red wavelengths for fairly superficial diseases tend to be in the region of 4 J/cm². But this doses recommendation is for human body [17, 18], the doses used in the laboratory animals may be different. In our previous study, we found that the reflection effect of animal fur at irradiation point could obstruct laser energy absorbed (data not showed). Compared with the golden standard dose 4 J/cm², we found that 8 J/cm² could get better treatment effect on asthmatic rats. In this

study, we evaluated the effect of 8 J/cm² LLLT treatment in OVA-induced asthmatic rats and compared it with budesonide treatment, which is a glucocorticoid with highly efficient local anti-inflammatory effects on asthma.

The OVA/alum model is based on common principles for sensitization; an initial intraperitoneal OVA/alum followed 2 weeks later by intranasal OVA instillations for seven consecutive days. The OVA/alum model rats exhibited signs of local airway inflammation, like an increase of inflammatory cells in BALF and pulmonary tissue, enhanced levels of serum IgE, and raised levels of traditional pro-inflammatory mediators (NO, TNF- α , and Th2 cytokines) in lung homogenate and serum [19, 20]. A histological investigation of the lung parenchyma revealed signs of eosinophils infiltration. The pathological changes of asthma have the characteristics of a general inflammatory response (vasodilation, increased vascular permeability, and edema) in addition to bronchial epithelial exfoliation and contraction of airway smooth muscle. Infiltration of macrophages, neutrophils, eosinophils, and lymphocytes in the lung has also been noted [21, 22]. Our study showed that LLLT ameliorated pathological damage in asthmatic rats and reduced the infiltration of inflammatory cells, similar to the effect of budesonide.

Eosinophils, a prominent feature of asthma, are found in increased numbers in the circulation and sputum, usually in relation to the severity of asthma. Eosinophils, through the production and release of toxic proteins, eosinophil neurotoxic protein, and eosinophilic peroxidase, can directly damage the airway epithelium, leading to peeling of the airway epithelium and airway hyper-responsiveness [23]. We observed the effect of LLLT on the total numbers of cells and eosinophils in BALF. The results showed that LLLT, similar to budesonide, could reduce the number of eosinophils in the BALF of asthmatic rats, suggesting that LLLT may ease inflammation in asthmatic airways. This notion was consistent with pathological examinations.

CD4⁺ T cells are the predominant lymphocyte population that infiltrates the airways in asthmatics and are activated in these sites. Human and murine CD4⁺ T cells are divided into two broad functional subsets according to their profiles of cytokine secretion. CD4⁺ Th1-type cells secrete predominantly IL-2 and interferon gamma (IFN- γ) and are particularly implicated in cell-mediated immune responses against invading intracellular pathogens. CD4⁺ Th2-type cells produce a different panel of cytokines including IL-4, IL-13, and IL-5. IL-4 promotes the development of Th2 cells and together with other cytokines, promotes the growth of mast cells, basophils, and eosinophils. Th1 and Th2 cytokine secretion can promote their subtypes growth, differentiation, on the contrary it is a disincentive for other subtypes. Under physiological conditions, the innate immune function of Th1/Th2 cells is in a dynamic equilibrium, when the balance deviates, disease state may occur. Generally, the main manifestations of bronchial

asthma are the weakened Th1-type immune response and the hyper functioning of Th2-type immune response [24–26].

BALF cytokine detection results showed that LLLT can lower IL-4 secretion and increase IFN- γ secretion in asthma of rat model, similar to budesonide. Since the IL-4/IFN- γ ratio can reflect Th1/Th2 balance, adjusting Th1/Th2 imbalance may be one of the mechanisms of LLLT treatment of asthma. In order to further observe the LLLT effect on asthma Th1/Th2 balance, flow cytometry was used to detect rat Th1/Th2 cell populations, the results were consistent with cytokine detection, which suggested LLLT could regulate asthma Th1/Th2 imbalance, accordingly, improve airway inflammation, and thus relieve asthma symptoms.

IgE is an important mediator of asthma. IL-4 is the main cause of the elevated level of IgE. A high level of IL-4 promotes the proliferation and differentiation of B cells and mast cells, and promotes B cells to secrete IgE antibody as well as expression of the high-affinity IgE receptor. Cross-connections between allergens and IgE receptors result in the degranulation of mast cells and the release of inflammatory mediators (histamine, lipid mediators, enzymes, cytokines, and growth factors). These mediators can cause the dilation of blood vessels as well as an increase in vascular permeability, smooth-muscle contraction, and local inflammatory responses [27, 28]. We found that LLLT can lower the secretion of IgE in asthmatic rats in a similar way to budesonide, a result consistent with the results of cytokine detection.

In summary, LLLT treatment on asthmatic rats achieved good results. The mechanism of action of LLLT may be via adjustment of Th1/Th2 imbalances. There was no difference in the actions of LLLT and budesonide. Budesonide is a glucocorticoid and, regardless of the route of administration, side effects are inevitable. Thus, LLLT could provide some of the therapeutic effects of budesonide for the treatment of asthma, thereby reducing some of the side effects of it.

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Conflict of interest The authors have no conflicts of interest to declare.

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