# Effect of follicular helper T cells on the pathogenesis of asthma

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**Abstract.** Follicular helper T (TFH) cells are considered to be a separate T helper cell subset, specifically to help memory B cell participate in humoral immunity. It has been reported that there is an association between the imbalance of TFH function and certain autoimmune diseases. However, to the best of our knowledge, the effect of TFH cells on the process of bronchial asthma has not been investigated. The aim of the present study was to investigate the associated markers of TFH cells in bronchial asthma-induced mice. In the current study, sensitized and long-term challenges induced a mouse asthmatic model and were used to investigate the associated markers of TFH cells in the pathogenesis of asthma. The results demonstrated that B cell lymphoma 6, inducible T-cell costimulator (ICOS), ICOS ligand, C-X-C chemokine receptor type 5 (CXCR5) and interleukin (IL)-21 protein and mRNA expression levels were higher in the asthma group, as compared with the control group. Furthermore, the ratio of cluster of differentiation (CD) 4$^+$CXCR5$^+$/CD4$^+$ and CD4$^+$CXCR5$^+$ICOS$^+$/CD4$^+$CXCR5$^+$ was significantly increased in the asthma group. The results of the current study suggest that TFH cells and associated markers may have a role in the pathogenesis of chronic bronchial asthma.

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## Introduction

Bronchial asthma is a chronic inflammatory airway disease in which a variety of cells and cytokines are involved, including eosinophils, macrophages, neutrophils and Th2 cells, in addition to other inflammatory cytokines and chemokines (1-3). Although the mechanism of asthma remains unclear, previous studies have suggested that an imbalance of Th1/Th2 is directly involved in the inflammatory response of asthma (4,5). The treatment approaches for asthma that are used clinically include hormones, β2 receptor agonists and leukotriene receptor antagonists. However, due to the complex causes and mechanisms of bronchial asthma, these treatments are not satisfactory, and do not significantly reduce the symptoms, which include wheezing, shortness of breath, chest tightness and coughing. Studies have also identified imbalances in Th1/Th2 and Th17/Treg in patients with allergic asthma (6,7). Previous evidence (8) has suggested that follicular helper T (TFH) cells are a specific cluster of differentiation CD4$^+$ effector T cells subset, that is distinguished from T helper 1 (Th1) cells and Th2 cells in the tonsils, spleen and lymph nodes. They specifically maintain the structure and function of germinal centers (GC) and regulate memory B cell responses (9,10). There is also evidence indicating that a TFH imbalance or dysfunction is associated with autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis and autoimmune thyroiditis (11-13). To the best of our knowledge, the functions of TFH cells in the pathogenesis of asthma, however, have not been reported. The present study hypothesized that TFH cells affect the asthma pathogenesis via TFH-associated markers.

## Materials and methods

*Animals.* A total of 16 female BALB/c mice (6-8 weeks old, 20-22 g), purchased from the Animal Experiment Center, Xinjiang Medical University (Xinjiang, China), were housed in microisolator cages and received a regular diet (access to food and water *ad libitum*). The laboratory temperature was 24±1˚C, and the relative humidity was 40-80%. All experimental protocols were approved by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

*Reagents*. Ovalbumin (OVA) was used as an allergen (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Aluminum hydroxide gel was used as an adjuvant (Tianjin Pharmaceutical Group Co., Ltd., Tianjin, China). Anti-mouse inducible T-cell costimulator (ICOS)-fluorescein isothio-cyanate (FITC) (11-9942), anti-mouse C-X-C chemokine receptor type 5 (CXCR5)-phycoerythrin (PE) (85-12-1859-41), anti-mouse CD4-PE Cy5 (15-0042-82), anti-mouse CD3-PE-allophycocyanin (APC) (85-17-0032-80) antibodies were purchased from eBioscience (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Red Blood Cell Lysis Buffer was purchased from Tiandz, Inc. (Beijing, China). Lymphocyte separation medium was purchased from Westang Bio-Tech, Co., Ltd. (Shanghai, China). TRIzol, synthesize first-strand cDNA with reverse transcriptase and Platinum SYBR-Green qPCR SuperMix-UDG were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Primers were designed and synthetized by the Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). SDS-PAGE electrode buffer was purchased from BestBio (Shanghai, China), antibodies against B cell lymphoma 6 (BCL-6) (sc-365618), CXCR5 (sc-8178) and ICOS (sc-65285) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). ICOS ligand (ICOSL) antibody (bs-2471R) and interleukin (IL)-21 rabbit polyclonal antibody (bs-2621R) were purchased from Bioss (Woburn, MA, USA). All other chemicals were of reagent grade.

*Model*. A total of 16 female BALB/c mice were randomly divided into two equal groups: Control and asthma group (n=8). As previously described (14), mice in the asthma group received an intraperitoneal injection of sensitizing agent 0.2 ml (containing 100 $\mu$g OVA and 1 mg aluminum hydroxide gel) on days 1 and 15. The asthma group breathed in 1% OVA for 30 min, starting on day 22, three times per week for 8 weeks. The control group was treated with phosphate-buffered saline (PBS) instead of OVA. Mice were sacrificed by cervical dislocation following the last challenge, and blood, lung and spleen specimens were collected.

*Determination of flow cytometry*. During the last 2 h of the final challenge, a blood sample was collected from all mice. The spleen was removed following sacrifice and 4 ml red blood cell lysis buffer was added prior to the tissues being ground. The mixtures were filtered and collected by a 75-$\mu$m sieve. Red blood cell lysis buffer was added, centrifuged (2,120 x g, 5 min, room temperature) and the supernatant was decanted. Subsequently, 10% RPMI-1640 (1 ml) (Thermo Fisher Scientific Inc.) was added and mixed with lymphocyte separation medium. The lymphocyte layer was aspirated, diluted to 10 ml by PBS and washed it to form a cell suspension. The cell concentration was adjusted with PBS to 1-5x10$^6$ cells/ml. Samples were then stained with 0.5 $\mu$l antibodies: Anti-mouse ICOS-FITC (1:200), anti-mouse CXCR5-PE (1:200), anti-mouse CD4-PE Cy5 (1:200) and anti-mouse CD3 PE APC (1:100) (away from light at 4˚C, 20 min). Cell pellets were resuspended in 500 $\mu$l FACS-PBS and maintained on ice prior to flow cytometry analysis. Flow cytometry was conducted on a BD FACSCalibur flow cytometer and analyzed using Cell Quest Pro software version 5.1 (both from BD Biosciences, Franklin Lakes, NJ, USA).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*. RNA (2 $\mu$l) was extracted from lung tissue, collected following sacrifice, using TRIzol reagent RNA isolation reagent. cDNA was synthesized from 1 $\mu$l RNA of each sample using a synthesize M-MLV first-strand reverse transcription system kit (C28025-032) according to the manu-facturer's protocol. Primers were designed as follows: 5'-GGA AACCCAGTCAGAGTATTCG-3' (forward) and 5'-CACATT TGTAGGGCTTTTCTCC-3' (reverse) for BCL-6; 5'-TCTG CCGTGTCTTTGTCTTCT-3' (forward) and 5'-GAGCAT TGGATTCTTGATGGA-3' (reverse) for ICOS; 5'-ATCTCG TGGGGATGTTCTGT-3' (forward) and 5'-GGTTTCCTG TGGGTTCTTTGT-3' (reverse) for ICOSL; 5'-GACTCCTCT CCATCCACATCA-3' (forward) and 5'-TAACACCATCCC ATCACAAGC-3' (reverse) for CXCR5; 5'-GGACCCTTGTCT GTCTGGTAG-3' (forward) and 5'-TGTGGAGCTGATAGA AGTTCAGG-3' (reverse) for IL-21; and 5'-TGTTACCAACTG GGACGACA-3' (forward) and 5'-GGGGTGTTGAAGGTC TCAAA-3' (reverse) for β-actin. RT-qPCR was performed in a 20-$\mu$l final volume containing 0.8 $\mu$l cDNA sample, 0.4 $\mu$l primer pairs and 10 $\mu$l SYBR-Green PCR Master mix. PCR was performed as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of annealing at the following temperatures: 53.5˚C (ICOS), 55˚C (CXCR5), 55.1˚C (ICOSL), 57.6˚C (BCL-6), 56˚C (IL-21) and 50˚C (β-actin) in an ABI PRISM 7000 (Applied Biosystems; Thermo Fisher Scientific, Inc.) system sequence detector. Each RNA sample was measured in triplicate. The specificity of amplified PCR products was evaluated by a comparative Cq method (15). The threshold cycle value (Cq value) was calculated from cycle number at which the PCR product crosses a threshold of detec-tion. BCL-6, CXCR5, ICOS, ICOSL, IL-21 mRNA expression levels were normalized against β-actin, as determined by the $2^{-\Delta\Delta Cq}$ method.

*Western blot analysis*. The lungs were removed following sacrifice. Tissue protein (200 $\mu$g) was extracted using RIPA lysis buffer (Beijing Solarbio Science and Technology Co., Ltd., Beijing, China) and the concentrations were determined using the bicinchoninic acid method. Proteins (50 $\mu$g/well) were separated by 5% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. Blocking by 5% skim milk powder (65˚C, 1 h) was performed following denaturation. Subsequently, the membrane was incubated with primary antibodies (BCL-6 1:500, ICOS 1:1,000, CXCR5 1:2,000, ICOSL 1:300; 4˚C, overnight) and secondary antibodies (Goat anti-rabbit IgG 1:10,000; room temperature, 1 h), then devel-oped with the Enhanced Chemiluminescence Plus Western Blotting Detection system (Amersham, Cambridge, UK), according to the manufacturer's instructions.

*Hematoxylin-eosin staining*. The left lung of each mouse was fixed in 10% formalin for 24 h and embedded in paraffin. Sections of 4-5 $\mu$m were cut, deparaffinized, rehydrated and stained with hematoxylin and eosin. A pathologist blind to experimental groups evaluated the changes of lung tissue by using a light microscope.

*Immunohistochemistry stain*. The left lung tissue was fixed in 10% buffered formalin for 48 h. Tissue samples, from
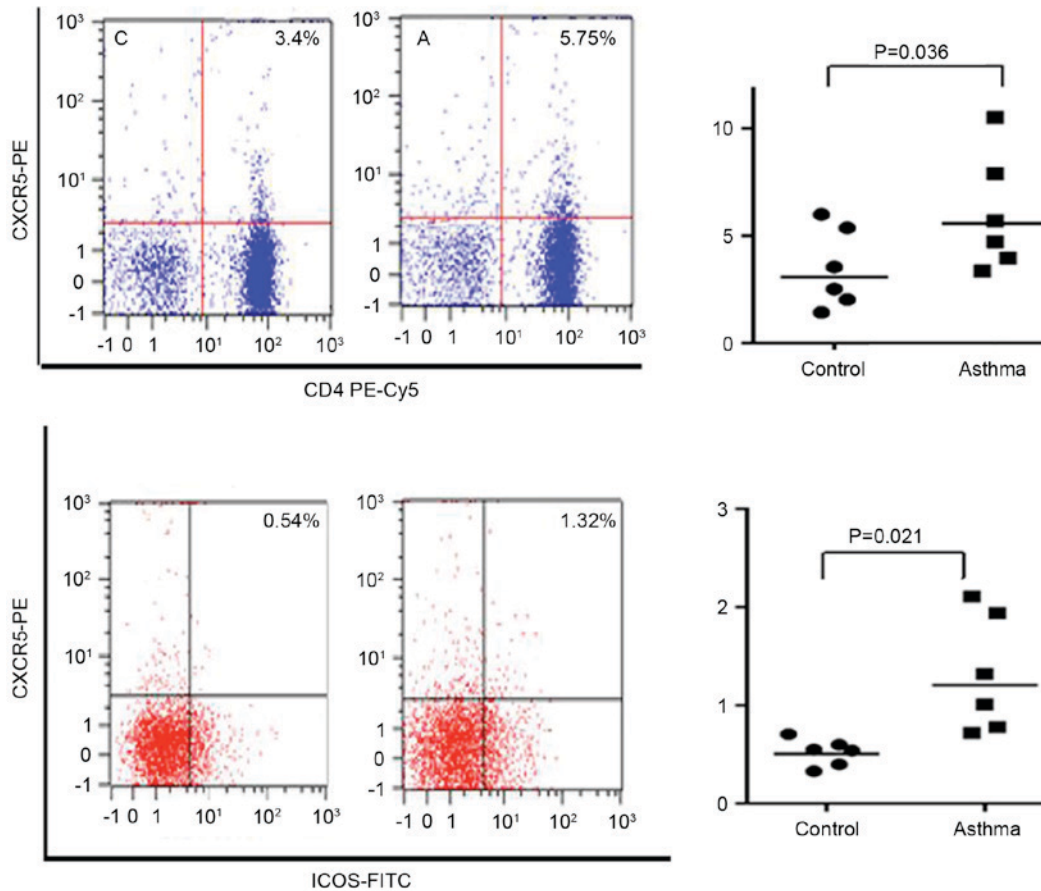
Figure 1. Flow cytometry results demonstrate that the ratio of CD4$^+$CXCR5$^+$/CD4$^+$ and CD4$^+$CXCR5$^+$ICOS$^+$/CD4$^+$ CXCR5$^+$ in the spleen tissues was significantly increased in the asthma group compared with the control group. Data are presented as the mean ± standard error of the mean. C, control group; A, asthma group; CD4, cluster of differentiation 4; CXCR5, C-X-C chemokine receptor type 5; ICOS, inducible T-cell costimulator; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

paraffin-wax-embedded blocks, were sectioned to be 4-5 $\mu$m thick. Following the process of dewaxing, hydration, closed endogenous peroxidase and antigen repair, polyclonal IL-21 antibody (rabbit anti-mouse, 1:300) and universal secondary antibody (PV6000, ZSGB-BIO, Beijing, China) were applied (1-2 h at room temperature). Slides were observed using a light microscope at a magnification of x200 and x400. Sections were considered positive according to the color observation, which is an indication of the antibody-antigen reaction, and manifested as an intracytoplasm brown coloration in different areas of the stained tissue section.

*Statistical analysis.* SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) was used to analyse data. Data were presented as the mean ± standard error of the mean. Comparison between groups was made with analysis of variance followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*Behavioral changes.* Mice in the asthma group exhibited symptoms following the challenge with OVA, including anxiety, frequent nose scratching, cough, nodding breathing, shortness of breath, retardation, piloerection and cyanosis. These behavioural activities were decreased following continuous challenge.

*Results of flow cytometry.* CXCR5 has been identified as an important transport molecule in the process of migration and positioning of TFH cells (16). While ICOS has a role in the differentiation and maturation of TFH cells. As presented in Fig. 1, the ratio of CD4$^+$CXCR5$^+$/CD4$^+$ and CD4$^+$CXCR5$^+$ICOS$^+$/CD4$^+$CXCR5$^+$ in the spleen tissues was significantly increased in the asthma group compared with the control group (P<0.05).

*Results of RT-qPCR.* Expression levels of BCL-6, ICOS, ICOSL, CXCR5 and IL-21 mRNA in lung tissue are presented in Fig. 2. A significant increase of BCL-6, ICOS, ICOSL, CXCR5 and IL-21 mRNA expression was detected in lung tissue from the asthma group compared with the control group (P<0.05).

*Results of western blot analysis.* Proteins within the lung tissues of mice were extracted and assayed by western blot analysis. As presented in Fig. 3, the results indicate that BCL-6, CXCR5, ICOS and ICOSL exhibited positive expression. At the same time, the amount of protein was increased in the asthma group compared with the control group.

*Changes to lung histopathology.* As presented in Fig. 4, alveolar samples from mice in the control group exhibited a normal structure with no histopathological changes observed under the microscope (Fig. 4). However, lung tissue obtained
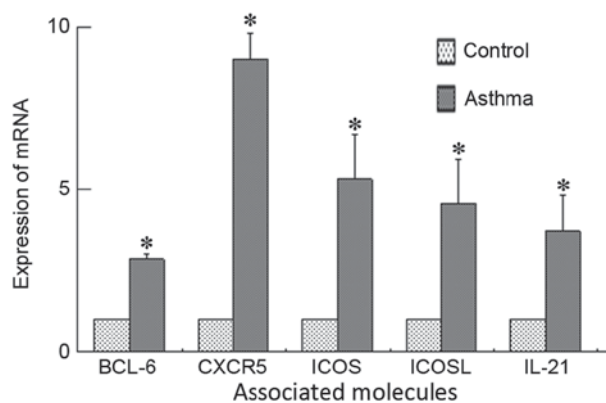
Figure 2. Comparing the mRNA levels of TFH cell-associated molecules (BCL-6, ICOS, ICOSL, CXCR5 and IL-21) in lung tissue from the control and asthma groups. *P<0.05 vs. control. Data are presented as the mean ± standard error of the mean. TFH, follicular helper T; BCL-6, B cell lymphoma 6; ICOS, inducible T-cell costimulator; ICOSL, ICOS ligand; CXCR5, C-X-C chemokine receptor type 5; IL-21, interleukin-21.
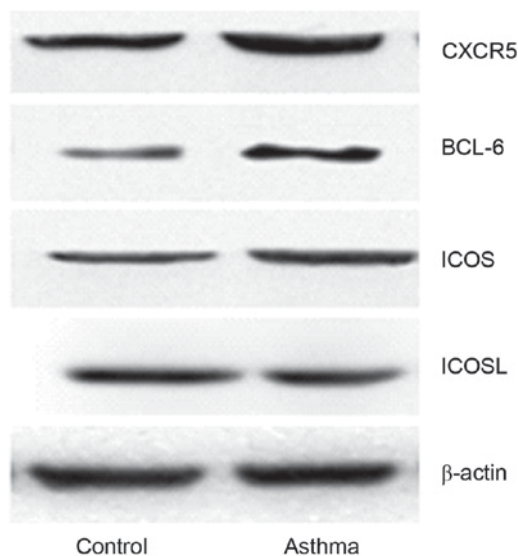


Figure 3. Western blot analysis to compare the expression levels of proteins associated with TFH cells (BCL-6, ICOS, ICOSL, CXCR5 and IL-21) in the control and asthma groups, with β-actin as the control. TFH, follicular helper T; BCL-6, B cell lymphoma 6; ICOS, inducible T-cell COStimulator; ICOSL, ICOS ligand; CXCR5, C-X-C chemokine receptor type 5; IL-21, interleukin-21.

from mice in the asthma group exhibited a marked bronchial epithelial necrosis, thickening of the bronchial walls and smooth muscle hyperplasia, bronchial lumen irregular and mucus plug within it.

*Results of immunohistochemistry staining*. Positive expression of IL-21 in the cytoplasm of false stratified columnar ciliated epithelium cells is demonstrated in Fig. 5. It was observed that there were more positive IL-21 cells in the asthma group, compared with the control group.

**Discussion**

Asthma is a type I allergic reaction mediated by immunoglobulin E. CD4+T helper cell (Th), serve a key role in the

pathogenesis of allergic inflammation (2). It has previously been suggested that Th1 and Th2 are involved in the inflammatory response of asthma directly (3). However, studies by Afshar *et al* (17) and others (10-20) have further revealed that Tregs and Th17 cells are also changed in the pathogenesis of asthma. A previous study identified novel subsets of CD4+T cells, named TFH cells, and these were demonstrated to help B cells produce antibodies (8-10). It was identified that certain patients who present with autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroid disease and immune-active chronic hepatitis B, demonstrate an association between an increased in TFH cells and injury severity of autoantibody and target organs (21,22).

A variety of molecular markers are involved in the differentiation of TFH cells, including CXCR5, ICOS, ICOSL, BCL-6 and IL-21 (23). TFH cells and mature B cells are able to express CXCR5 continuously and stably (24). CXCR5 is a specific receptor of CXCR13, which is a follicular homing chemokine that induces TFH cell migration from the T zone in peripheral lymphoid organs to the center of lymphoid follicles (25). Finally, TFH cells localize to and interact with B cells. Therefore, CXCR5 is known as an important transport molecule in the process of migration and positioning of TFH cells (26). The results of the present study indicate that the mRNA and protein expression levels of CXCR5 were increased in the asthma group, as compared with the control group.

ICOS, as an induced coordinated stimulus molecule, induces the process of TH cell activation and differentiation (23). A previous study indicated that ICOS interacts with ICOSL to deliver coordination and stimulate positive signals (27). It has an important role not only for the generation and survival of TFH cells but also for the formation of GC and memory B cells (28). Choi *et al* (29) revealed that ICOS provides a critical early signal to induce differentiation and maturation of TFH cells. The present study also revealed that the expression levels of ICOS and ICOSL mRNA and protein were increased in the bronchial asthma group, as compared with the control group.

Flow cytometric analysis demonstrated that, compared with the control group, the ratio of CD4+CXCR5+/CD4+ and CD4+CXCR5+ICOS+/CD4+CXCR5+ in the spleen was higher in the asthma group. These results suggest that the increasing ratio of TFH cell subsets may be an important determinant in the pathogenesis of asthma.

Zinc finger protein, BCL-6, is a transcription receptor that was initially determined to act on B cells (30). Its expression has an important role in the formation of GC. Expression of BCL-6 was indicated at the beginning of TFH cell proliferation and the activation of T cells (28,31). The present study revealed that the expression of BCL-6 at a molecular and protein level in the asthma group was higher than in the control group. The current study demonstrated that an abundance of TFH cells proliferated in the process of asthma pathogenesis.

IL-21 is primarily released from TFH cells in the body. IL-21 combines with its corresponding receptor on the surface of TFH or B cells, thus the expression of IL-21 increases by autocrine function. IL-21 is involved in TFH cell proliferation, development and effectiveness (32). The present study revealed
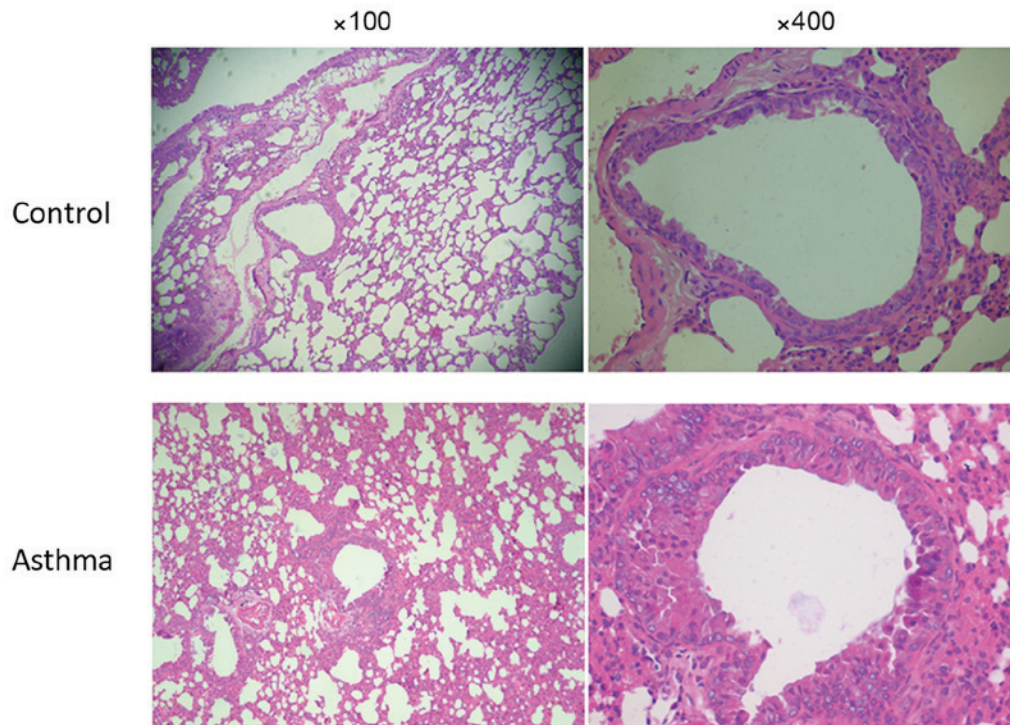
Figure 4. Histopathological changes in the lung tissue of the control and asthma groups at a magnification of x100 and x400 using a light microscope.
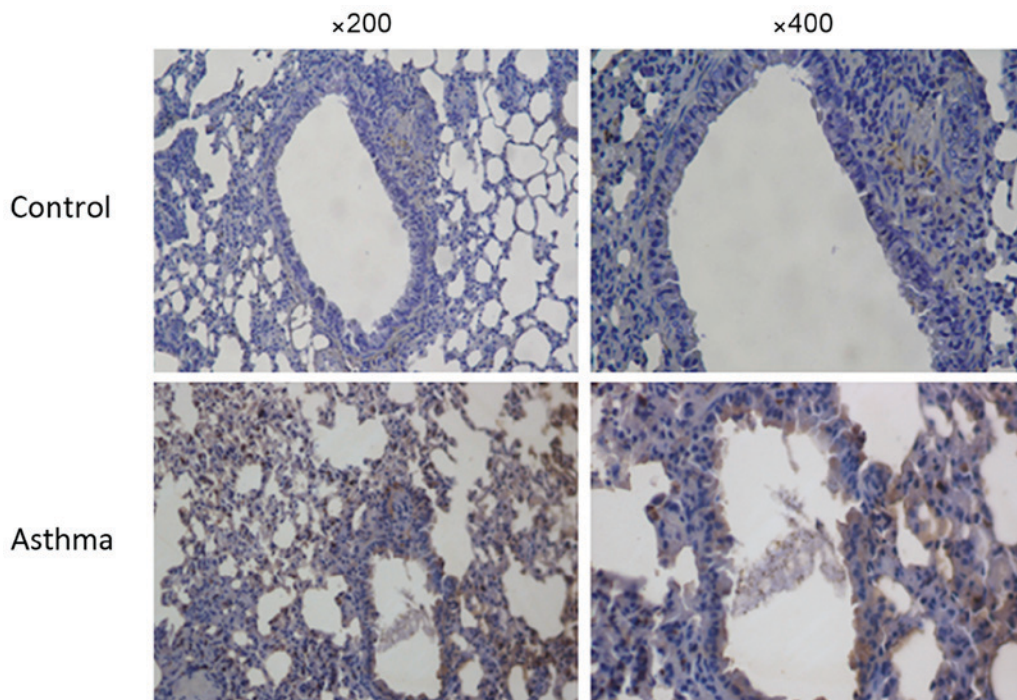


Figure 5. Immunohistochemistry staining to assess the expression of interleukin-21 in cells of the control and asthma groups at a magnification of x200 and x400 using a light microscope.

that the expression of IL-21, at the mRNA and protein level, was increased in asthmatic lung tissue.

In conclusion, the results of the current study demonstrated that TFH cells and associated markers have a role in the pathogenesis of asthma. These results suggest that regulation of the abnormal TFH cells *in vivo* may be a novel strategy for treating asthma, in the future.

## Acknowledgements

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