

Effects and Mechanism of Arsenic Trioxide on Reversing the Asthma Pathologies Including Th17-IL-17 Axis in a Mouse Model

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Received: 19 August 2011 ; Received in revised form: 14 March 2012 ; Accepted: 17 March 2012

ABSTRACT

In traditional Chinese medicine, arsenous compounds, including arsenic trioxide (ATO), are often used to treat many diseases, which are safe and effective. Recently, studies have indicated that Th17-IL-17 involved in the pathogenesis and development of asthma. The goal of this study was to investigate the effect and mechanism of ATO on asthma, especially the Th17-IL-17 axis.

We used oval bumin (OVA)-immunized mice as a model for asthma and treated mice with ATO or dexamethasone. The mice were then monitored airway responsiveness, airway inflammation, mucus production, IL-17 levels in BALF and the positive rate of Th17 cells. In vitro, CD4⁺ T cells from splenic cell suspensions were separated and purified.

We measured the expression of IL-17 and caspase-12 protein in purified CD4⁺ T cells, and detected IL-17 levels in CD4⁺ T lymphocyte culture solution with or without ATO. Moreover, apoptosis, mitochondrial membrane potential, cytosolic calcium were analyzed.

We found that ATO could reduce airway responsiveness, airway inflammation, mucus hyperplasia, the expression of IL-17 in BALF and the positive rate of Th17 cells at a level comparable to treatment with DXM. In vitro data suggested that ATO can induce CD4⁺ T cells apoptosis, cause mitochondrial dysfunction, Ca²⁺ overload and promote caspase-12 activation. Our study suggested that ATO had potential medical value for the treatment of human asthma.

Keywords: Apoptosis; Arsenic trioxide; Asthma; IL-17, Th17

INTRODUCTION

Bronchial asthma is a chronic inflammatory disease of the airways, and CD4⁺ T cells play a crucial role in

controlling inflammation. Th17 cells, characterized by the secretion of IL-17A (also called IL-17), are a T cell lineage distinct from Th1 and Th2 cells.¹ Recent studies have suggested that Th17 cells and IL-17 were involved in the pathogenesis of asthma. Adoptive transfer of antigen-specific Th17 cells are involved not only in causing antigen-induced neutrophil recruitment into the airways but also in the enhancement of Th2-cell-mediated eosinophil recruitment into the airways.²

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Furthermore, it has been shown that IL-17 is expressed in the airways of patients with asthma³ and that its expression is correlated with the severity of asthma.⁴ These findings indicate the importance of the Th17–IL-17 axis in inflammatory responses, and Th17–IL-17 is expected to become a new therapeutic target of asthma. However, therapeutic intervention for this link is relatively little at present.

In recent years, clinicians have recognized that glucocorticoids can not always cure asthma patients, especially patients with refractory asthma, and long-term use of corticosteroids may lead to other side effects. Therefore, it is necessary to look for new drug to treat asthma. Arsenic trioxide (As₂O₃ or ATO), extracted from arsenic compound, is a powerful ancient medication for a variety of ailments, including asthma, with the principle of ‘using a toxic against another toxic’ in traditional Chinese medicine. And in Western medicine it has been used to treat chronic myeloid leukemia and was known as Fowler’s solution. A recent renaissance of ATO came about, following the discovery in China of its therapeutic effect in acute promyelocytic leukemia (APL).⁵ Today, ATO has become one of the standard therapies for APL, and this ancient drug is thought to be relatively safe.

Thousands of years of Chinese medicine clinical practice shows that ATO is effective and repeatable in the treatment of asthma, but the effect of ATO on the pathophysiology of asthma and the mechanism under it are not very clear. Previous studies have found that ATO can promote apoptosis of pulmonary eosinophils and inhibit the overexpression of NF-κB in a guinea pig model of asthma.⁶ ATO has a direct inhibitory effect on the production of eotaxin by pulmonary cells without affecting IgE levels in the serum or IL-5 levels in BALF, and can block eosinophil infiltration into the airway.⁷ However, the effects of ATO on asthmatic CD4+ T cells, especially the Th17–IL-17 axis, have not been studied.

The therapeutic effect of ATO is known to be mediated by its ability to induce cell apoptosis in a variety of cells. It is now well accepted that cell apoptosis can result from activation of three major pathways: the extrinsic, the mitochondria-mediated pathway, and the most recently identified endoplasmic reticulum (ER) stress-mediated pathway, known to generally involve caspase-12.⁸ Although previous studies suggested that the mitochondria is a key target for the toxic effect of ATO,⁹ there is increasing

evidence that ATO may also act as an ER stressor.^{10,11} To our knowledge, however, it has not been investigated whether the mitochondria and ER involved in ATO-induced apoptosis in asthmatic CD4+ T cells.

In the current study, we reproduced the ovalbumin (OVA)-induced mouse model of asthma, aimed to confirm the inhibitory effects of ATO on AHR, airway inflammation and mucus hyperplasia, and compared with the effects of dexamethasone (DXM). Moreover, we investigated whether ATO could inhibit the expression of asthmatic Th17–IL-17 axis. More importantly, the molecular basis for action of ATO was also addressed. We found that ATO can induce apoptosis in asthmatic CD4+ T cells, cause mitochondrial dysfunction, Ca²⁺ overload and promote caspase-12 activation. Our data provided evidence that ATO induced apoptosis in asthmatic CD4+ T cells, at least partly, via ER stress and mitochondrial dysfunction.

MATERIALS AND METHODS

In vivo experiments

Experimental animals

BALB/c female mice aged 6–8 weeks, SPF grade, weighed between 18–22g, were obtained from and maintained in the Animal Center of the Second Xiangya Hospital (Changsha, Hunan, China). Animals were randomly divided into 4 groups (10 mice/group): PBS/PBS [PBS sensitized/challenged mice treated with PBS], OVA/PBS [ovalbumin (OVA) sensitized/challenged mice treated with PBS], OVA/ATO [OVA sensitized/ challenged mice treated with ATO] and OVA/DXM [OVA sensitized/ challenged mice treated with DXM]. Sensitization–challenge protocols were utilized as described previously¹² and summarized in Figure 1. Briefly, mice were sensitized by intraperitoneal injection with 10 µg OVA (grade V; Sigma-Aldrich, St. Louis, MO, USA), which was adsorbed to 2 mg aluminum hydroxide gel (Sigma-Aldrich) in 200 µL of PBS on days 1 and 13. For challenge, mice inhaled aerosolized 5% OVA for 30 min on days 19–24. The PBS/PBS group was treated in the same way with PBS without OVA. All animal experimental protocols were approved by the Animal Subjects Committee of the Central South University, China.

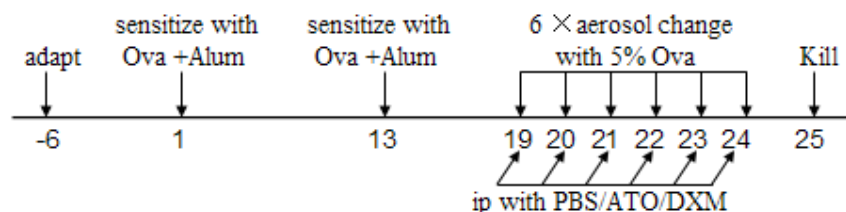


Figure 1. Sensitization and challenge protocols. 30 BALB/c mice were sensitized by intraperitoneal injection with 10 µg OVA and 2 mg aluminum hydroxide gel in 0.2 ml PBS on days 1 and 13, and then challenged by nebulization of 5% OVA for 30 min on days 19–24. 10 control mice were sensitized and challenged with 0.2 ml PBS alone. For treatment, 0.2 ml PBS or ATO or DXM was administered intraperitoneally 30 min before each OVA challenge on days 19–24 to the PBS/PBS and OVA/PBS group, the OVA/ATO group, the OVA/DXM group, respectively. PBS/PBS, OVA/PBS, OVA/ATO, OVA/DXM indicated PBS sensitized/challenged mice treated with PBS, ovalbumin(OVA) sensitized/challenged mice treated with PBS, OVA sensitized/ challenged mice treated with arsenic trioxide, OVA sensitized/challenged mice treated with dexamethasone, respectively.

Animal Treatments

For the OVA/ATO group and OVA/DMX group, 2.5 mg/kg ATO (7) or 2.5 mg/kg DXM¹³ (dissolved in 200 µL sterile PBS, respectively; Sigma-Aldrich) were administered intraperitoneally 30 min before each OVA challenge on days 19–24. For the OVA/PBS group and PBS/PBS group, 200 µL PBS was administered as the control.

Measurement of Airway Responsiveness

Twenty-four hours after the last challenge, airway responsiveness was determined by direct plethysmography (Buxco, Troy, NC, USA) referred to previous study.¹⁴ After anesthesia with 5% chloral hydrate 500 mg/kg, mice were implemented with tracheal intubation and connecting with small animal respirator (DHX-50, Chengdu Instrument Factory, China). First recorded the basis of airway resistance values for 1 min; subsequently determined airway resistance changes after NS and increasing concentration of methacholine (Mch) aerosol challenge. Each nebulization lasted for 1 min and recorded 3 min. The concentration of inspired Mch was from low to high as follows: 0.75, 1.56, 3.125 or 6.25 mg/ml in 100 µL NS. Lung resistance (RL) was obtained by measuring mouse airway flow and pressure.

Bronchoalveolar Lavage Fluid (BALF)

After the mice were anesthetized, the trachea was cannulated and immediately lavaged three times with 0.5 ml of Hank's balanced salt solution without calcium and magnesium. The total cells in BALF were counted using a hemacytometer. The BALF was cytopinned (5 min, 1 500 rpm, 4 °C) onto microscope

slides and stained with Wright–Giemsa. The percentages of macrophages, eosinophils, lymphocytes and neutrophils were obtained by counting 200 cells on randomly selected portions of the slide by light microscopy. The count of eosinophils, lymphocytes, macrophages or neutrophils in the BALF was determined by multiplying their percentage in total cells. The supernatant were stored at -80°C.

Histological Examination

After lavage, the lungs were fixed in 10% buffered formalin, and sections were stained with hematoxylin/eosin (HE) or periodic acid-Schiff (PAS). An inflammation score was assigned in a blinded fashion by a pathologist. Peribronchiolar and perivascular inflammation was scored as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells of more than four cells deep.¹⁵ PAS scores were assigned by a blinded investigator examining 10 consecutive fields per slide as follows: 0, <5% PAS positive goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; 4, >75%, with 0 being negative and 1–4 being positive for PAS-staining bronchi.¹⁶

Magnetic Activated Cell Sorting (MACS)

CD4+ T cells from splenic cell suspensions of each group of mice were purified by using a magnetic-activated cell sorter system according to manufacturer's instructions (Miltenyi Biotec, Germany). Briefly, monocytes were isolated from spleen using lymphocyte separation medium (Dakewe Biowotech Co., Ltd, Beijing, China). After two washes, cells were

resuspended in MACS buffer (90 μ l per 10^7 cells) and incubated with CD4 (L3T4) MicroBeads (10 μ l per 10^7 cells) for 15 min at 4°C. After two additional washes, bead-bound cells were isolated using a LS column (Miltenyi) placed in a strong magnetic field, and the positive fraction containing the CD4+ T cells was harvested, they could be purified to >98%. The viability of CD4+ T cells was determined by trypan blue exclusion (>95%).

Flow Cytometry Analyze the Positive Rate of Th17 Cells

Previous reports have identified IL-17-producing CD4+ T cells could represent Th17 cells. As we harvested purified CD4+ T cells by MCAS (>98%, purity), IL-17 positive cells of intracellular staining within CD4+ T cells just were Th17 cells.

Splenic CD4+ T cells from each group of mice were stimulated with 50 ng/ml of phorbol myristate acetate (PMA) (Sigma-Aldrich) and 500 ng/ml of ionomycin (Sigma-Aldrich) for 4 hours.¹⁷ For the last 2h, 2 μ M monensin (BioLegend, San Diego, CA, USA) was added to inhibit the export of cytokines. For intracellular staining, CD4+ T cells were fixed and permeabilized with fixation buffer and permeabilization wash buffer (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions and stained with FITC anti-mouse IL-17A (BioLegend). All samples were analyzed on a FACSCalibur (BD Biosciences, San Diego, CA) using CellQuest Pro software (BD Biosciences).

In vitro Experiments

Cell Culture

Splenic CD4+ T cells from asthmatic mice (1×10^6 cells per milliliter) were seeded in 12-well plates and grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin and 1% glutamine. In the absence or presence of 5 μ M ATO,¹⁸ CD4+ T cells were stimulated with ConA (5 μ g/ml) and cultured at 37°C with 5% CO₂ for 20 h. Next, the two groups of cells and their culture supernatants were collected respectively for following analysis.

Detection of Apoptosis

For quantitative determination of apoptosis, the Annexin V-FITC apoptosis detection kit (Beijing 4A

Biotech Co., Ltd, Beijing, China) was used. Briefly, after treatment with or without ATO for 20 h, CD4+ T cells (1×10^6 cells) were washed twice with cold PBS and resuspended in 100 μ l Annexin V binding buffer. Cells were incubated with 5 μ l Annexin V-FITC and 10 μ l propidium iodide (PI) for 10 min at room temperature away from light before addition of 400 μ l Annexin V binding buffer. Samples were measured in the FACSCalibur using channel FL2 and channel FL1, respectively. The early apoptotic cells (Annexin-FITC positive and PI negative) were located in the lower right quadrant. Data from 10,000 gated events per sample were collected, and the results are expressed as percentage of positively stained cells in total cells.

Determination of Mitochondrial Membrane Potential ($\Delta\psi$)

Mitochondrial $\Delta\psi$ was determined using the Mitochondrial membrane potential assay kit with JC-1 (Beyotime, Haimen, China). JC-1 is a lipophilic and cationic dye, which permeates plasma and mitochondrial membranes. The dye fluoresces red when aggregates in healthy mitochondria with high membrane potential, whereas it appears in monomeric form and fluoresces green in mitochondria with diminished membrane potential. Depolarization of $\Delta\psi_m$ was expressed as an increase of green to red fluorescence ratio reflecting the transformation of JC-1 aggregates into monomers when mitochondrial membrane becomes depolarized.¹⁹ Briefly, after washing with PBS, CD4+ T cells (1×10^6 cells) were resuspended in 1 ml staining buffer and incubated with 1ml JC-1 dye (37°C; 20 min). Finally, cells were washed twice and resuspended in 1 ml PBS for Flow cytometric analysis.

Calcium Analysis

Investigations of cytosolic calcium were made with Fluo-3/acetoxymethyl ester (AM) (Beyotime, Haimen, China). Fluo-3/AM is membrane permeable. After entering the cells, the ester can be cleaved by intracellular esterases to yield the relatively cell-impermeant fluorescent indicators Fluo-3. Approximately 1×10^6 cells were harvested and resuspended in 1 ml D-Hank's solution that lacked calcium, loaded with Fluo-3/AM at a concentration of 1 μ M at 37°C for 20 min. After washing twice, CD4+ T cells were suspended in D-Hank's solution and analyzed using flow cytometry.

Western Blot Analysis of Caspase-12 and IL-17

CD4⁺ T cells (1×10^7) were lysed with RIPA lysis buffer containing protease inhibitors (Beyotime, Haimen, China). Then, Proteins were resolved on SDS-PAGE and transferred to Immobilon polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked with 5% non-fat milk for 2h at room temperature. The membrane was immunodetected with a rabbit polyclonal antibody anti-Caspase-12 (1:1000) or a rabbit polyclonal antibody anti-IL-17 (1:1000, Santa Cruz, CA, USA) overnight at 4°C, and then with corresponding HRP-conjugated secondary antibodies (Santa Cruz, CA, USA) for 1 hour at RT. Protein bands were detected and quantified using a chemiluminescence kit from pierce biotechnology. β -actin was detected as the loading control.

ELISA

The level of IL-17 in BALF and culture supernatants was quantified using the commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D systems Inc, Minneapolis, MN, USA. sensitivity <2 pg/ml), according to the manufacturer's recommendations.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software, version 17.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean $\pm$ SD. The statistical significance of differences among the

different experimental groups was analyzed using ANOVA followed by LSD or Dunnet's test. Single pairs of the groups were compared by Student's t-test. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Effects of ATO on AHR to Methacholine

To evaluate the effects of ATO on AHR in mice, their Lung resistance (RL) was assessed by direct plethysmography using methacholine (Mch) 24 h after the final challenge. As shown in Figure 2, in the OVA/PBS group, AHR to methacholine was increased more than the PBS/PBS group. Treatment with 2.5 mg/kg ATO or DXM resulted in a significant reduction of AHR compared to the OVA/PBS group, and there was no significant difference between the OVA/ATO group and the OVA/DXM group.

Effects of ATO on Airway Inflammation

To assay whether ATO could modulate the recruitment of inflammatory cells in the airway, the total cells in BALF were collected and differential cell counts were determined by Wright–Giemsa stain. The data (Figure 3) show that the numbers of total cells, eosinophils, neutrophils and lymphocytes in BALF significantly increased in the OVA/PBS group compared with those in the PBS/PBS group.

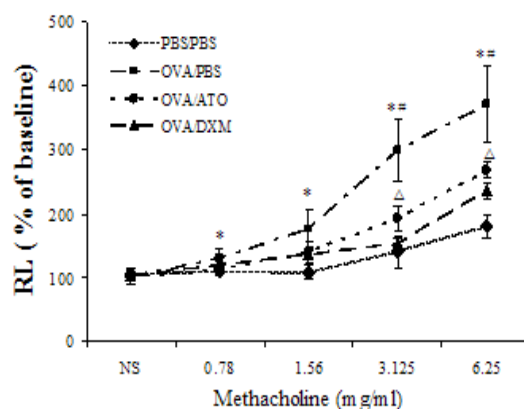


Figure 2. ATO suppressed airway hyperresponsiveness (AHR). AHR to inhaled methacholine (Mch) was measured 24 hours after the final ovalbumin (OVA) challenge. Each group included four to six mice. Values are presented as mean $\pm$ SD and expressed as the resistance change from baseline. Compared with PBS/ PBS, * $p < 0.05$; compared with OVA/ATO and OVA/DXM, [#] $p < 0.05$; compared with OVA/DXM, $\Delta p < 0.05$.

Both ATO and DXM had an inhibitory effect on those inflammatory cells. Interestingly, ATO treatment decreased neutrophils more significantly than DXM, although there were no significant differences among other cells. These data indicated that ATO treatment could inhibit airway inflammation, especially neutrophil-induced inflammation.

Effects of ATO on Airway Inflammation and Mucus Production

Twenty-four hours after the last challenge, HE staining

was performed to investigate the recruitment of inflammatory cells in lungs. Histological analysis revealed that, in the OVA/PBS group, numerous eosinophils and other inflammatory cells infiltrated the peribronchiolar and perivascular areas (Figure 4a). In contrast, ATO and DXM treatment resulted in reduced cellular infiltration. In addition, we found that ATO and DXM treated mice had significantly lower inflammation scores for the peribronchial and perivascular regions compared with asthmatic mice (Figure 4b).

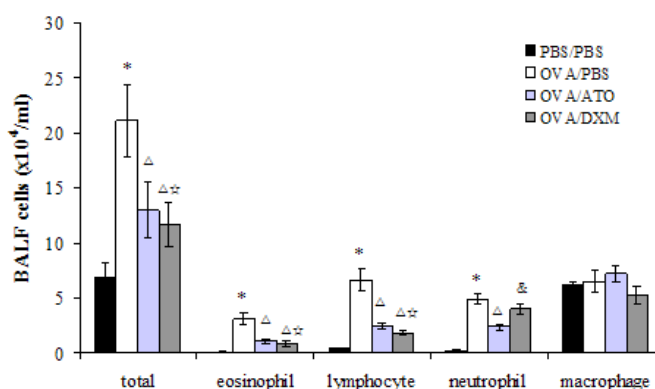


Figure 3. ATO reduced the number of inflammatory cells in BALF. Each column and bar depicted the mean $\pm$ SD, resulted from three independent experiments. * $p < 0.01$ vs. PBS/PBS, $^{\Delta}p < 0.05$ vs. OVA/PBS, $^{\star}p > 0.05$ vs. OVA/ATO, & $p < 0.01$ vs. OVA/ATO.

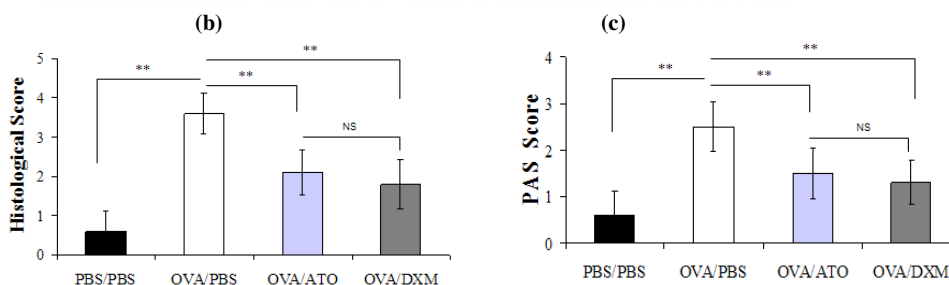
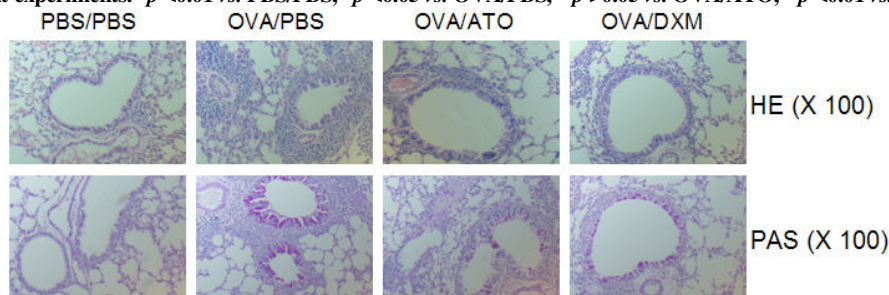


Figure 4. ATO suppressed airway cellular infiltration and mucus production. (a) Lung sections of each group were stained with HE (upper, magnification 100X) or PAS (lower, magnification 100X). Quantitative analyses of inflammatory cell infiltration (b) and mucus production (c) were evaluated in lung sections with a scoring method described in Materials and Methods. Four to five mice per group were analyzed. Each column and bar depicted the mean $\pm$ SD. **, $p < 0.01$; NS, $p > 0.05$ compared between two groups.

Increased mucus production is a common pathophysiological manifestation associated with asthma. Goblet cell metaplasia and mucus production were determined by PAS staining of histological sections at day 25. As seen in Figure 4a, PAS staining in the airways of asthmatic mice was noticeably intense, whereas very little PAS staining was seen in the airways of control mice, as well as in mice treated

with ATO or DXM. In line with the observed pathologic manifestations, the PAS score was higher in asthmatic mice and lower in mice treated with ATO or DXM. Moreover, this effect between ATO and DXM did not reach statistical significance (Figure 4c). Together, these data demonstrated that both ATO and DXM can inhibit lung inflammation and mucus hyperproduction.

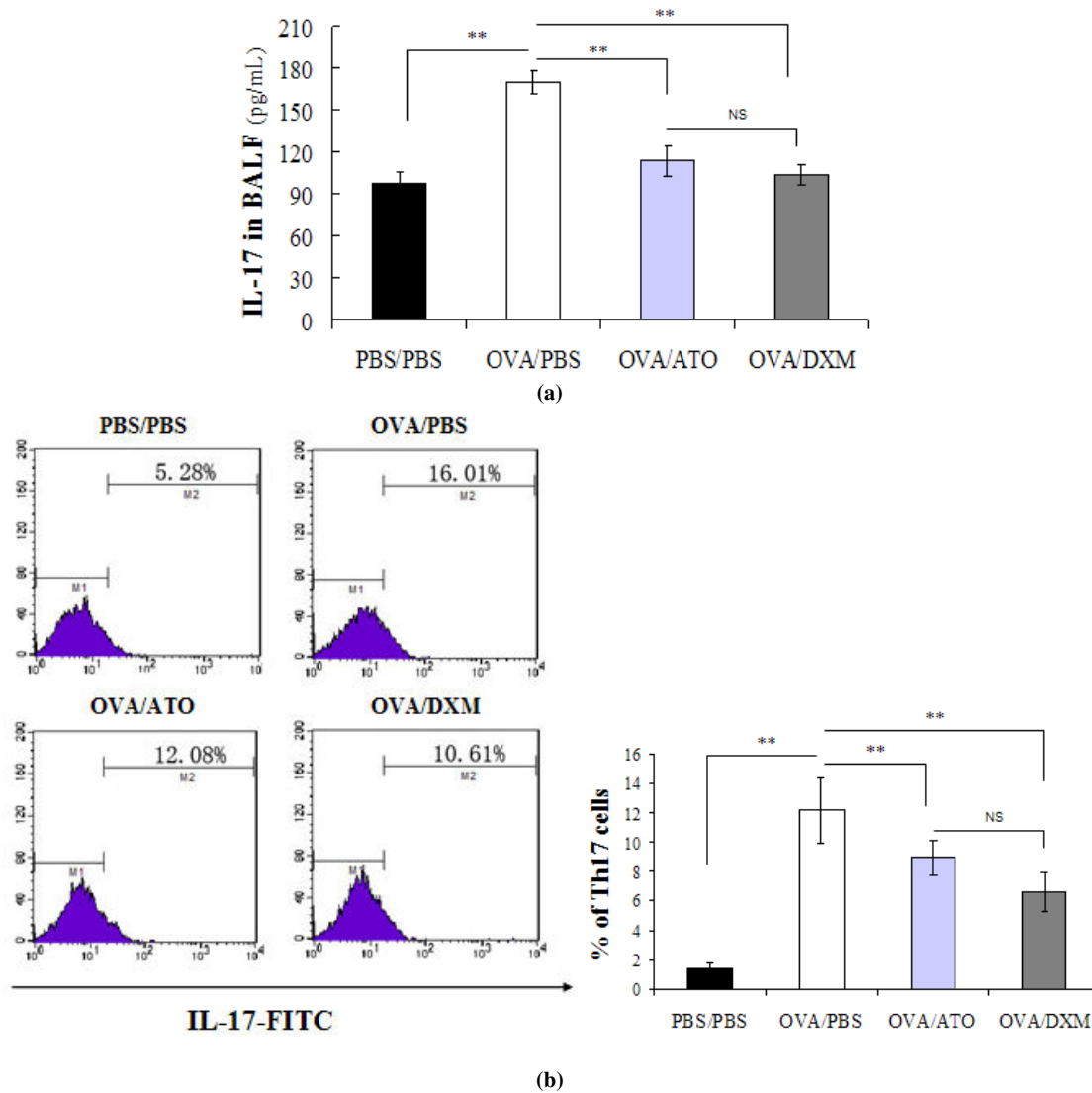


Figure 5. Effects of ATO on the Th17– IL-17 axis in vivo. (a) The levels of IL-17 in BALF were quantified with ELISA, ATO reduced the concentration of IL-17 in BALF. (b) The expression of Th17 were analyzed using flow cytometry, ATO reduced the percentage of IL-17-producing cells in purified splenic CD4+ T cells (Th17 cells). Each column and bar depicted the mean $\pm$ SD of the results of three to five mice. **, $p < 0.01$; NS, $p > 0.05$ compared between two groups.

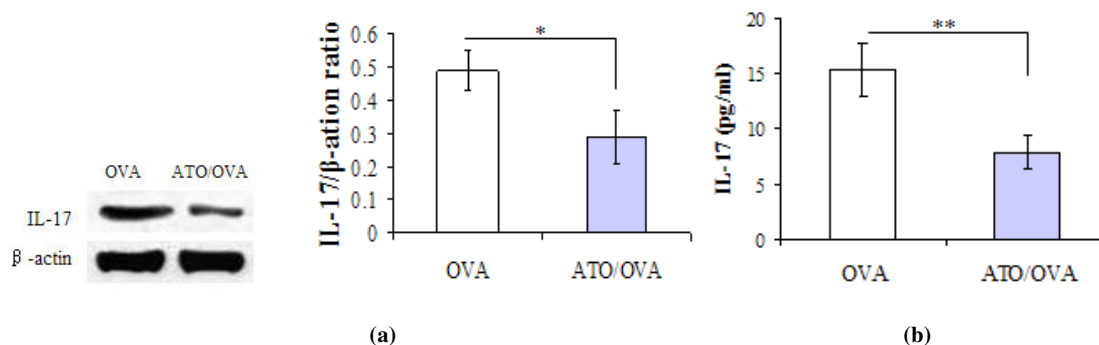


Figure 6. ATO inhibited protein synthesis and release of IL-17 *in vitro*. In all CD4⁺ T cells, only Th17 cells produce IL-17. After separation and purification of splenic CD4⁺T cells from asthmatic mice, *in vitro* experiments were carried out. After treatment with or without 5 μ M ATO for 20 h, CD4⁺T cells were harvested for following analysis. The expression of IL-17 in purified splenic CD4⁺T cells were examined by Western blot analysis (a). The levels of IL-17 in culture supernatants were examined by ELISA (b). ATO inhibited the secretion of IL-17 from Th17 cells. OVA, ATO/OVA indicated CD4⁺T cells without any treatment, CD4⁺T cells treated with 5 μ M ATO for 20 h, respectively. * p <0.05; ** p <0.01 compared between two groups.

ATO Decrease Th17 Cells and IL-17 Production *in vivo*

Recent studies have suggested Th17 cells and IL-17 were involved in the pathogenesis of allergic asthma. However, it has not been reported whether ATO can inhibit the expression of the Th17– IL-17 axis. To address this question, we collected purified splenic CD4⁺ T cells from each group of mice, and analyzed the positive rate of IL-17-producing CD4⁺ T cells (Th17 cells) in each group of mice using flow cytometry. Subsequently, we detected the change of IL-17 in BALF by ELISA. Our results showed both the concentration of IL-17 in BALF (Figure 5a) and the positive rate of splenic Th17 cells (Figure 5b) significantly increased in the OVA/PBS group compared with the PBS/PBS group, while ATO or DXM treatment significantly reduced the positive rate of splenic Th17 cells and the concentration of IL-17 in BALF.

ATO Inhibit IL-17 Synthesis and Secretion *in vitro*

To further observe the effect of ATO on Th17/IL-17, purified splenic CD4⁺ T cells from asthmatic mice were harvested after treatment with or without 5 μ M ATO for 20 h. Next, we detected the expression of IL-17 protein and the secretion of IL-17 *in vitro*. As shown in Figure 6, both the expression of IL-17 protein of CD4⁺ T cells and the levels of IL-17 in culture supernatants in the CD4⁺ T cells treated with ATO (the ATO/OVA group) were lower than that in the CD4⁺ T cells without ATO treatment (the OVA group).

ATO Induce Apoptosis in Asthmatic CD4⁺ T Cells

The therapeutic roles of ATO were exerted mainly by inducing cells apoptosis. We speculated that the inhibitory effect of ATO on asthmatic Th17– IL-17 axis was associated with increased apoptotic levels of asthmatic Th17 cells. It was our original plan to purify Th17 cells and treat them with 5 μ M ATO to investigate the role of ATO on Th17 cells directly. However, even if the naïve CD4⁺ T cells were laid under widely-recognized Th17 differentiation conditions, we would typically obtain 10 to 30% of IL-17-producing cells, 50% of IL-17F producing cells.²⁰ So we can only explore the effect of ATO on the whole CD4⁺ T cells. As shown in Figure 7a, compared with the OVA group, a high proportion of Annexin V⁺PI⁺ labeling cells were detected in the ATO/OVA group. These data suggested that ATO induced apoptosis in asthmatic CD4⁺ T cell.

ATO Cause Mitochondrial Dysfunction and ER Stress

To determine whether ATO-induced apoptosis was mediated through mitochondrial dysfunction, we determined the mitochondrial membrane potential with a mitochondria-sensitive dye, JC-1, using flow cytometry. Our results (Figure 7b) showed that treatment of asthmatic CD4⁺ T cells with ATO for 20 h induced the loss of the mitochondrial membrane potential. Ca²⁺ overload is another important factor causing cells apoptosis. Subsequently, we assessed the effect of ATO on cytosolic Ca²⁺. Compared with the OVA group, the proportion of Fluo-3 positive cells significantly increased in the ATO/OVA group (Figure 7c).

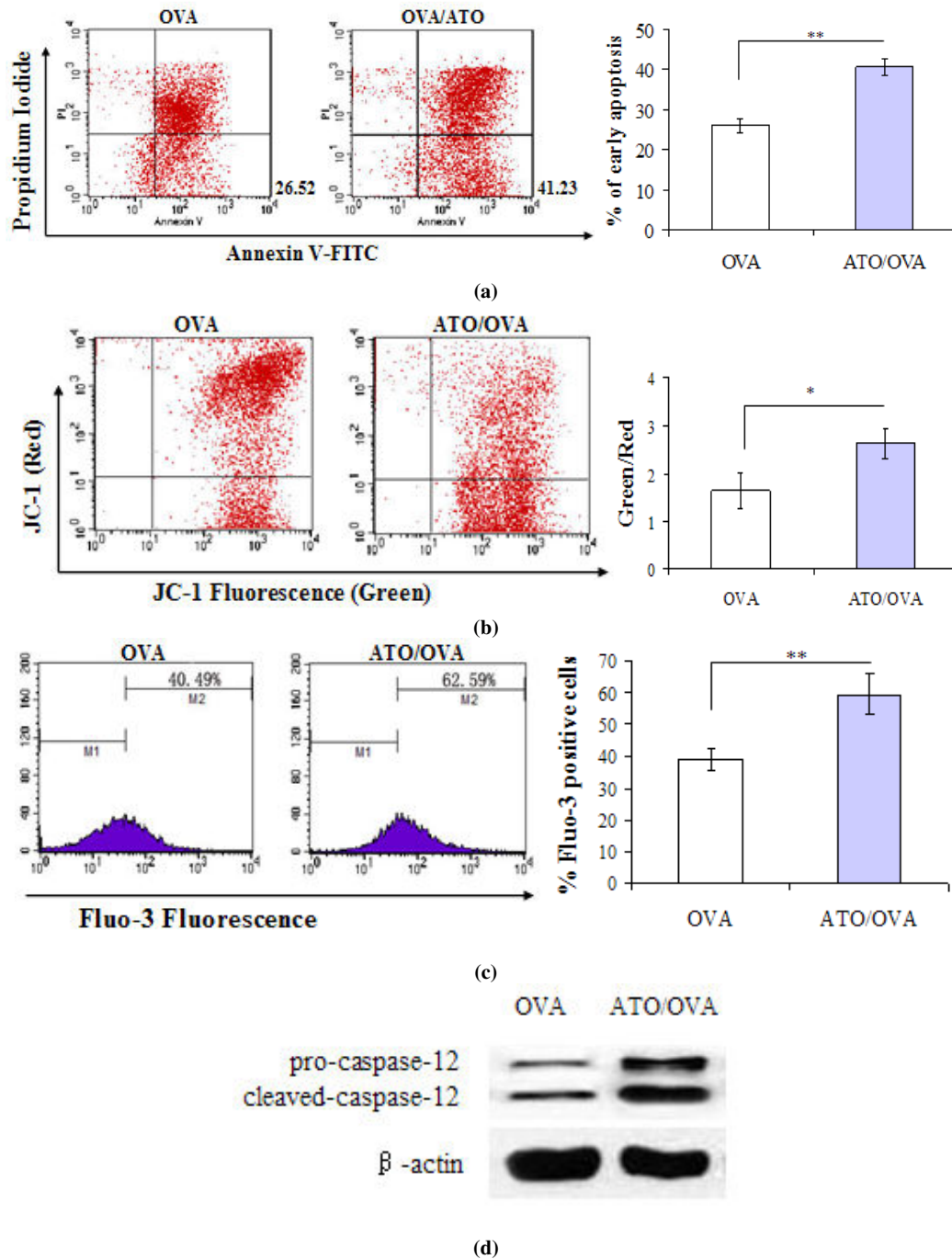


Figure 7. ATO induced asthmatic CD4⁺ T cells apoptosis involving mitochondrial dysfunction and ER stress. After treatment with or without 5 μ M ATO for 20 h, Purified CD4⁺ T cells from asthmatic mice were harvested for apoptosis analysis. ATO induced apoptosis (a) and decreased mitochondrial membrane potential (b) in asthmatic CD4⁺T cells. ATO caused Ca²⁺ release (c) and ER-localized caspase-12 activation (d). OVA, ATO/OVA indicated CD4⁺T cells without any treatment, CD4⁺ T cells treated with 5 μ M ATO, respectively. Results are expressed as the mean $\pm$ SD, experiments were repeated 3 times. *, $p < 0.05$, **, $p < 0.01$ and NS, $p > 0.05$ compared between two groups.

Previous studies indicated that Ca^{2+} overload can promote induction of ER stress.²¹ Apoptosis induced via ER stress involves the activation of ER-localized caspase-12.⁸ To identify whether caspase-12 involved in ATO-induced apoptosis, caspase-12 activity was determined in asthmatic CD4+ T cells after they were incubated with 5 μM ATO for 20 h. As shown in Figure 7d, compared to the OVA group, caspase-12 activity notably increased in the ATO/OVA group. These results suggested that mitochondrial dysfunction and ER stress apoptotic pathway participated in the process of apoptosis induced by ATO.

DISCUSSION

Arsenic trioxide (ATO) is considered a carcinogen; however, it has also been used to treat diseases, such as syphilis, psoriasis and rheumatosis. Today, ATO is used as one of the standard therapies for acute promyelocytic leukemia (APL). Here we demonstrated that treatment with ATO could inhibit airway inflammation, mucus hyperplasia and AHR in asthmatic mice. Moreover, the clinical hallmarks of asthma could be improved at a level comparable to treatment with DXM. These results partly explain why the administration of ATO is effective in the treatment of asthma in traditional Chinese medicine. Previous studies reported that the median lethal dose (LD50) of ATO on mice is 100 mg/kg, we just used 1/40 of the LD50 (2.5 mg/kg) to mice, which is a very small dose. During giving the used dose of ATO to mice, mice did not appear particularly abnormal symptoms, such as decreased activity degree, anorexia, lethargy, weight loss, suggesting that the small dose of ATO is relatively safe for the treatment of asthma in mice. Studies have shown that 50% of asthma cases are noneosinophilic and in these cases neutrophil inflammation in the airways is predominant.²² Apoptosis of neutrophils is inhibited by glucocorticoids^{23,24} and numerous studies have suggested noneosinophilic asthma is associated with poor response to corticosteroid treatment.^{25,26} More surprisingly, however, ATO treatment reduced neutrophils more significantly than DXM, suggesting that ATO may be more sensitive to neutrophils than DXM. ATO may therefore have bright potential in the treatment of asthma, especially neutrophil-mediated noneosinophilic asthma.

At present, almost all related reports implicated that Th17 cells and IL-17 played a critical role in the

pathogenesis of asthma. In a study by Pène et al.,²⁷ the author detected highly activated Th17 cells in bronchial biopsies of patients suffering from severe asthma, where these cells accounted for not less than 20% of all infiltrating lymphocytes. In vitro restimulation of these cells resulted in production of IL-17A and IL-17F, IL-22. Th17 transfer resulted in a primarily neutrophilic airway response, both Th17 cells transfer and IL-17 overexpression induced mucus hyperplasia and AHR.¹³ So we also investigated the effects of ATO on asthmatic Th17–IL-17 axis. To our encouraging, as expected, our *in vivo* and *in vitro* experiments together confirmed that ATO inhibited the expression of Th17 and the production of IL-17. In addition, the possibility that the therapeutic effects of ATO were related to inhibit asthmatic Th17–IL-17 axis can't be ruled out. Therefore, this study provided a new insight concerning the mechanism of action of ATO in the treatment of asthma.

Overactivation and accumulation of CD4+ T cells in the peripheral blood and airway tissues are an invariant feature of asthma,²⁸⁻³⁰ which may result from increased cell infiltration and/or prolonged cell survival. The latter represents a failure to induce programmed cell death (apoptosis). Apoptosis plays an important role in the maintenance of cellular homeostasis and the elimination of self-reactive lymphocytes.³¹ A large body of studies reported that eosinophil apoptosis could facilitate resolution of airway inflammation in asthma,³² which reminded us that inducing apoptosis in immune cells might be an important goal in suppression of allergic responses. Here we found that ATO can promote apoptosis of asthmatic CD4+ T cells, suggesting that ATO might regulate immune responses.

One of the mechanisms underlying ATO directed cell apoptosis was mitochondrial dysfunction. The loss of mitochondrial membrane potential ($\Delta\psi_m$) is an early and key event in the mitochondria-mediated apoptotic pathway.³³ In this study, we also found that ATO reduced $\Delta\psi_m$, suggesting mitochondria involved in asthmatic CD4+ T cells apoptosis induced by ATO, too. Similar depolarization of the mitochondrial membrane has been observed with ATO in human normal T cells, which involved in enhancing oxidative stress, because enhanced generation of intracellular reactive oxygen species (ROS) and decreased levels of glutathione (GSH) were confirmed.¹⁸

Endoplasmic reticulum (ER) is a major storage organelle for calcium and the site of synthesis and folding of proteins. A variety of toxic insults, including failure of protein synthesis, folding, transport or degradation, and Ca^{2+} overload, can disturb ER function and result in ER stress.^{21,34} Elevation of cytosolic-calcium levels or depletion of ER calcium stores represents typical responses of cells to various stimuli. Recent studies found that ATO could induce apoptosis in osteoblasts,¹¹ neutrophils,¹⁰ human lens epithelial cells³⁵ and chronic myeloid leukemia cells³⁶ through ER stress-mediated pathway, suggesting that the ER is a target for the toxic effect of ATO. In agreement with previous studies, our *in vitro* experiments also found that ATO could induce ER stress in asthmatic CD4+ T cells, since cytosolic calcium level was elevated after treatment with ATO. Moderate endoplasmic reticulum stress can trigger unfolded protein response (UPR) to protect cells, whereas severe endoplasmic reticulum stress disturbs endoplasmic reticulum function and threaten cell survival, and then activate endoplasmic reticulum stress-mediated apoptosis pathway.³⁷

Caspase-12, only localized on ER, is suspected to be specific to the apoptotic mechanism downstream of ER stress, because caspase-12-deficient mice are resistant to ER-stress-induced apoptosis but continue to be susceptible to apoptosis induced via other mechanisms, such as mitochondrial stimuli.⁸ Under normal conditions, caspase-12 exists in an inactive procaspase form. During ER stress, caspase-12 dissociates from the ER membrane and is cleaved to a fragment, then it is activated. Once it is activated, caspase-12 initiates downstream apoptotic pathways.⁸ So detection of caspase-12 activation is pivotal to estimate whether did ATO-induced apoptosis proceed through ER pathway. Results in our experiments showed that ATO promoted caspase-12 activation in asthmatic CD4+ T cells; therefore, ATO may also induce ER stress-related apoptosis in asthmatic CD4+ T cells.

CONCLUSION

In conclusion, to the best of our knowledge, we are the first to demonstrate the inhibitory effects of ATO on asthmatic Th17– IL-17 axis. Given that ATO could inhibit airway inflammation, mucus hyperplasia, AHR and Th17/IL-17 at a level comparable to DXM, ATO

has potential medical value for the treatment of human asthma. ATO can induce mitochondrial dysfunction in asthmatic CD4+ T cells. In addition, this study is also the first to reveal a link between ATO-induced apoptosis in asthmatic CD4+ T cells and the ER stress pathway.

ACKNOWLEDGEMENTS

This study was supported by a key project Foundation of The Project of Science and Technology of Hunan Province, China (No. 2010FJ2004). We are very grateful to professor Jun-wei Deng for technical assistance.

REFERENCES

1. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Dong C, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005; 6(11):1133-41.
2. Wakashin H, Hirose K, Maezawa Y, Kagami S, Suto A, Watanabe N, et al. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am J Respir Crit Care Med* 2008; 178(10):1023-32.
3. Molet S, Hamid Q, Davoine F, Olivenstein R, Elias J, Chakir J, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001; 108(3):430-8.
4. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β , IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003; 111(6):1293-8.
5. Chen GQ, Zhu J, Shi XG, Chen SJ, Wang ZY, Chen Z, et al. In vivo studies on cellular and molecular mechanisms of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia: As_2O_3 induces NB4 cell apoptosis with downregulation of Bcl-2 expression and modulation of PML-RAR α /PML proteins. *Blood* 1996; 88(3):1052-61.
6. Zhou LF, Zhu Y, Cui XF, Cui XF, Hu AH, Yin KS. Arsenic trioxide, a potent inhibitor of NF- κ B, abrogates allergen-induced airway hyperresponsiveness and inflammation. *Respiratory Res* 2006; 7(1):146-58.
7. Chu KH, Lee CC, Hsin SC, Cai BC, Wang JH, Chiang BL. Arsenic trioxide alleviates airway hyperresponsiveness and eosinophilia in a murine model of asthma. *Cell Mol Immunol* 2010; 7(5):375-80.

8. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yangkner BA, et al. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000; 403(6765):98-103.
9. Cai X, Shen YL, Zhu Q, Wang ZY, Chen Z, Chen GQ, et al. Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. *Leukemia* 2000; 14(2):262-70.
10. Binet F, Chiasson S, Girard D. Arsenic trioxide induces endoplasmic reticulum stress-related events in neutrophils. *Int Immunopharmacol* 2010; 10(4):508-12.
11. Tang CH, Chiu YC, Huang CF, Chen YW, Chen PC. Arsenic induces cell apoptosis in cultured osteoblasts through endoplasmic reticulum stress. *Toxicol Appl Pharmacol* 2009; 241(2):173-81.
12. McMillan SJ, Lloyd CM. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin Exp Allergy* 2004; 34(3):497-507.
13. McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *Immunol* 2008; 181(6):4089-97.
14. Locke NR, Royce SG, Wainwright JS, Samuel CS, Tang ML. Comparison of Airway Remodeling in Acute, Subacute, and Chronic Models of Allergic Airways Disease. *Am J Respir Cell Mol Biol* 2007; 36(5):625-32.
15. Bandukwala HS, Clay BS, Tong J, Mody PD, Cannon JL, Shilling RA, et al. Signaling through Fc gamma RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation. *J Exp Med* 2007; 204(8):1875-89.
16. Mikhak Z, Fukui M, Farsidjani A, Medoff BD, Tager AM, Luster AD. Contribution of CCR4 and CCR8 to antigen-specific Th2 cell trafficking in allergic pulmonary inflammation. *J Allergy Clin Immunol* 2009; 123(1):67-73.
17. Yang XO, Panopoulos AD, Nurieva R, Wang D, Nurieva R, Dong C, et al. STAT3 Regulates Cytokine-mediated Generation of Inflammatory Helper T Cells. *J Biol Chem* 2007; 282(13):9358-63.
18. Gupta S, Yel L, Kim D, Kim C, Chiplunkar S, Gollapudi S. Arsenic trioxide induces apoptosis in peripheral blood T lymphocyte subsets by inducing oxidative stress: a role of Bcl-2. *Mol Cancer Ther* 2003; 2(8):711-9.
19. Zhu XJ, Shi Y, Peng J, Guo CS, Shan NN, Qin P, et al. The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia. *Blood* 2009; 114(26):5362-7.
20. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol* 2009; 39(1):216-24.
21. Soboloff J, Berger SA. Sustained ER Ca^{2+} depletion suppresses protein synthesis and induces activation-enhanced cell death in mast cells. *J Biol Chem* 2002; 277(16):13812-20.
22. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. *Thorax* 2002; 57(7):643-8.
23. Zhang X, Moilanen E, Adcock IM, Lindsay MA, Kankaanranta H. Divergent effect of mometasone on human eosinophil and neutrophil apoptosis. *Life Sci* 2002; 71(13):1523-34.
24. Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils: separation of survival and activation outcomes. *J Immunol* 1995; 154(9): 4719-25.
25. Pavord ID. Non-eosinophilic asthma and the innate immune response. *Thorax* 2007; 62(3):193-4.
26. Pavord ID, Brightling CE, Woltmann G, Wardlaw AJ. Non-eosinophilic corticosteroid unresponsive asthma. *Lancet* 1999; 353(9171): 2213-4.
27. Pène J, Chevalier S, Preisser L, Ravon E, Yssel H, Gascan H, et al. Chronically Inflamed Human Tissues Are Infiltrated by Highly Differentiated Th17 Lymphocytes. *J Immunol* 2008; 180(11):7423-30.
28. Corrigan CJ, Kay AB. CD4 T-lymphocyte activation in acute severe asthma: relationship to disease severity and atopic status. *Am Rev Respir Dis* 1990; 141(4):970-7.
29. Walker C, Bode E, Boer L, Hansel TT, Blaser K, Virchow JC jr. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis* 1992; 146(1):109-15.
30. Xue J, Xu Y, Zhang Z. Lymphocyte apoptosis in asthmatic patients and its molecular mechanism. *Zhonghua Jie He He Hu Xi Za Zhi* 1999; 22(9):555-7.
31. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; 407(6805):770-6.
32. Woolley KL, Gibson PG, Carty K, Wilson AJ, Twaddell SH, Wolley MJ. Eosinophil apoptosis and the resolution of airway inflammation in asthma. *Am J Respir Crit Care Med* 1996; 154(1):237-43.
33. Zamzami N, Marchetti P, Castedo M, Zanin C, Petit PX, Kroemer G, et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J Exp Med* 1995; 181(5):

- 1661-72.
34. Benali-Furet NL, Chami M, Houel L, De Giorgi F, Vernejoul F, Lagorce D, et al. Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. *Oncogene* 2005; 24(31):4921-33.
35. Zhang H, Duncan G, Wang L, Liu P, Cui H, Reddan JR, et al. Arsenic trioxide initiates ER stress responses, perturbs calciumsignalling and promotes apoptosis in human lens epithelial cells. *Exp Eye Res* 2007; 85(6):825-35.
36. Du Y, Wang K, Fang H, Li J, Xiao D, Zheng P, et al. Coordination of intrinsic, extrinsic, and endoplasmic reticulum-mediated apoptosis by imatinib mesylate combined with arsenic trioxide in chronic myeloid leukemia. *Blood* 2006; 107(4):1582-90.
37. Chen J, Qin J, Liu X, Han Y, Yang Z, Chang X, et al. Nitric oxide-mediated neuronal apoptosis in rats with recurrent febrile seizures through endoplasmic reticulum stress pathway. *Neurosci Lett* 2008; 443(3):134-9.