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RESEARCH ARTICLE

Arsenic trioxide alleviates airway hyperresponsiveness and eosinophilia in a murine model of asthma

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Asthma is one of the most common chronic airway inflammatory diseases. The clinical hallmarks of asthma include elevated serum levels of immunoglobulin E (IgE), eosinophilic inflammation and airway hyper-responsiveness (AHR). Arsenic trioxide (As_2O_3) is considered a carcinogen; however, it has also been used to treat diseases, such as syphilis, in traditional Chinese and Western medicine. Today, As_2O_3 is used as one of the standard therapies for acute promyelocytic leukemia (APL). Previous studies have indicated that As_2O_3 can induce apoptosis in eosinophils. However, the effect of As_2O_3 on asthma has not been investigated. We used ovalbumin (OVA)-immunized mice as a model for asthma and treated mice with As_2O_3 at doses of 2.5 and 5 mg/kg. The mice were then monitored for OVA-specific IgE production, airway inflammatory cell infiltration and AHR. We found that administration of As_2O_3 in OVA-immunized mice abrogated airway eosinophil recruitment by downregulating eotaxin expression but did not alter serum IgE or IL-5 levels in bronchoalveolar lavage fluid (BALF). Furthermore, the development of AHR and cellular infiltration into the airway were reduced by treating mice with As_2O_3. *In vitro* data suggested that low concentrations of As_2O_3 could induce only a small degree of apoptosis in primary pulmonary cells but could significantly inhibit the secretion of eotaxin by these cells. These results indicate that the administration of As_2O_3 to OVA-immunized mice can suppress lung allergic inflammatory responses. As_2O_3 might therefore have therapeutic potential in treating allergic airway inflammatory diseases.

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BACKGROUND

Asthma is a chronic inflammatory disease caused by allergic airway inflammation, which is characterized by increased mucus secretion, airway remodeling and airway hyper-responsiveness (AHR).[1] Many cells, particularly mast cells, eosinophils and T lymphocytes, have been suggested to play a critical role in the pathogenesis of asthma. In addition, other parameters, such as high serum immunoglobulin E (IgE) levels and cellular infiltration of the airways by eosinophils and T lymphocytes expressing T helper 2 (Th2) cytokines, are also pathological features of asthma. IgE binds to mast cells, providing for the recognition of specific antigens. Th2 cells account for the accumulation of eosinophils, which release reactive oxygen species and toxic granular proteins when activated. The pathological symptoms of asthma appear to be correlated with the degree of airway inflammation and the level of local Th2 cytokine production.[2,3]

Arsenic has been considered a poison for a long time. Since the 1820s, many studies have shown arsenic to be a potent environmental carcinogen in human malignancies, especially in skin and lung cancers.[4–6] However, in traditional Chinese medicine, arsenous compounds, including arsenous acid and arsenic trioxide (As_2O_3), are often used to treat tooth marrow disease, psoriasis and rheumatosis.[7] Arsenic has been used as both a therapeutic agent and a poison in ancient Greece and Rome. In Western medicine, arsenic has been used more recently in the treatment of syphilis and trypanosomiasis, which affect the central nervous system.[8,9] In the 1970s, As_2O_3 was introduced as a treatment for acute promyelocytic leukemia (APL) and showed striking effectiveness. Today, As_2O_3 has become one of the standard therapies for APL and is thought to be the major factor in achieving complete remission of APL.

Previous studies have found that As_2O_3 promotes apoptosis of pulmonary eosinophils in a guinea pig model of asthma.[10] As_2O_3 also reduces eosinophil recruitment. In current studies, the ovalbumin (OVA)-induced mouse model of asthma was used to investigate the possible therapeutic effects of As_2O_3. Our data demonstrated that As_2O_3 treatment could block eosinophil infiltration into the airway by downregulating eotaxin levels and decreasing AHR. In addition, we found that low concentrations of As_2O_3 could significantly inhibit the secretion of eotaxin and regulated upon activation, normal T cell expressed and secreted (RANTES) by primary lung epithelial cells without damaging the cells. Therefore, we believe that As_2O_3 has therapeutic potential in the treatment of asthma.

METHODS

Reagents

As_2O_3 (1 mg/ml) was purchased from TTY Biopharm Co., Ltd (Taoyuan, Taiwan) and stored at 4 °C before use.

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Animals

Female BALB/c mice aged 6–8 weeks were obtained from and maintained in the Animal Center of the College of Medicine, National Taiwan University. Animal study protocols were approved by the Animal Research Committee, College of Medicine, National Taiwan University.

Immunization and As_2O_3 treatment

We modified a previously established murine model of airway inflammation as described.[11] For systemic immunization, mice were sensitized by intraperitoneal injection of 50 μg of OVA (Sigma, St Louis, MO, USA) mixed with 4 mg of alum on day 0; they were then given 25 μg of OVA mixed with 4 mg of alum on days 14, 21 and 28. On days 42, 43 and 44, mice were challenged with 100 μg/mouse (in a total volume of 40 μl) of OVA by intranasal administration. Four days prior to the OVA challenge, As_2O_3 was administered intraperitoneally for 7 days. For the positive control group, the sensitized mice were treated with phosphate-buffered saline (PBS) intraperitoneally prior to OVA challenge. The naive group received OVA challenge but were not sensitized.

Measurement of OVA-specific antibodies

Blood was collected from the retro-orbital venous plexus at days 0 and 47 and centrifuged to separate the sera for antibody assays. The amount of OVA-specific IgE was determined by ELISA. Briefly, 96-well plates were coated with OVA at 10 μg/well. After the plates were blocked, 100 μl/well of diluted sera was added, and the plates were incubated at room temperature for 2 h or at 4 °C overnight. After incubation, the plates were washed five times with PBS with Tween 20 buffer, the secondary antibody (biotinylated rat anti-mouse IgE; AbD Serotec, Kidlington, UK) was added and plates were incubated for 1 h at room temperature. After plates were washed, avidin-horseradish peroxidase (Pierce Chemical, Rockford, IL, USA) was added, and samples were incubated at room temperature for 30 min. The avidin-horseradish peroxidase was removed by washing with PBS with Tween 20 buffer, and the bound enzyme substrate was detected by adding tetramethylbenzidine reagent (KPL, Gaithersburg, MD, USA). After incubation at room temperature for a short time, the reaction was stopped by adding 50 μl/well of 2 N H_2SO_4. Optical density was measured at 450 nm (550 nm was used as a reference filter) in a microplate autoreader (Anthos Reader 2010; Anthos Labtec Instruments GmbH, Salzburg, Austria).

Measurement of AHR

On day 45, the airway response to aerosolized methacholine (Sigma) was measured in unrestrained, conscious mice as previously described.[12] The mice were placed in the main chamber of a whole body plethysmograph (Buxco Electronics, Inc., Sharon, CT, USA) and challenged with aerosolized 0.9% normal saline accompanied by increasing doses of methacholine (6.25–50 mg/ml). Each nebulization lasted for 3 min, and after each nebulization, recordings were taken and averaged for the 3 min. The Penh (enhanced pause 5 pause 3 (peak expiratory box flow/peak inspiratory box flow)) values were determined, and the data were expressed as Penh values.

Bronchoalveolar lavage fluid (BALF) assessment

To measure airway inflammation, we examined the accumulation of inflammatory cells in the BALF.[13] After assessment of pulmonary function, the mice were killed, and the trachea was cannulated and immediately lavaged three times with 1 ml of Hank's balanced salt solution without calcium and magnesium. The lavage fluid was kept on ice and then centrifuged (400*g*) at 4 °C for 10 min. After being washed, cell pellets were resuspended in 1 ml of Hank's balanced salt solution, and the total number of cells in the BALF was counted with a standard hemocytometer. A differential count was performed on a smear prepared with a cytocentrifuge and stained with Liu's stain solution. A minimum of 200 cells were counted and classified as macrophages, lymphocytes, neutrophils and eosinophils based on standard morphological criteria.

BALF CYTOKINE MEASUREMENT

The levels of eotaxin and IL-5 in BALF supernatants were evaluated using an appropriate ELISA Kit (R&D, Minneapolis, MN, USA). These eotaxin and IL-5 assays have a threshold of detection of 500 pg/ml and 2 ng/ml, respectively. The cytokine levels were calculated by linear regression analysis based on the values obtained from a standard curve.

Histopathological analysis

To evaluate the effects of As_2O_3 treatment on allergen-induced pulmonary inflammation, each group of animals was killed for histopathological examination. After lavage, the lungs were immediately removed and fixed in 10% buffered formalin. Pulmonary tissues were subsequently sliced, embedded in paraffin and cut into 5-μμ thick sections. Sections were stained with hematoxylin–eosin and examined by light microscopy for histological changes.

Primary mouse lung cell culture

Three-week-old BALB/c female mice were killed by cervical dislocation to avoid the influence of ether or pentobarbital on lung tissue. The lungs were removed and washed with 1× PBS buffer until all blood was removed. All connective tissues and blood vessels were removed, and the lung tissue was subsequently cut into small pieces. The resulting single cell suspension was centrifuged, and the cell precipitate was collected. Lung cells were cultured with alpha-minimum essential medium (Life Technologies, Grand Island, NY, USA) complete medium including 10% fetal bovine serum, 4 mM L-glutamine, 25 mM *N*-2-hydro-xyethylpiperazine-*N*′-2-ethanesulfonic acids (pH 7.2), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin. After 10–14 days, the primary cell population was able to reach 80% confluence.[14] After confluence, cells were seeded at 2×10^5 cells/well in 24-well plates and cultured at 37 °C with 5% CO_2 until the cells again attained 80% confluence. For eotaxin detection, cells were treated with 3000 U/ml of recombinant IL-4 (R&D) and As_2O_3. For RANTES detection, cells were treated with 20 ng/ml of tumor necrosis factor-α (PeproTech, Rocky Hill, NJ, USA) and As_2O_3. After 48 h of incubation, the supernatant from each well was collected and frozen at −20 °C prior to analysis.

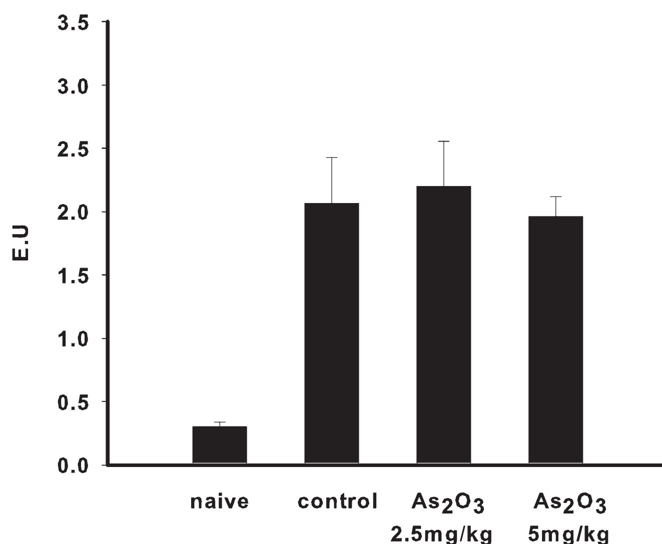

Figure 1 OVA-specific serum IgE levels. After treatment with As_2O_3, there was no significant difference in serum IgE between the control and As_2O_3 treatment groups. Each group included four to five mice. Values are expressed as mean±SEM. As_2O_3, arsenic trioxide; IgE, immunoglobulin E; OVA, ovalbumin.

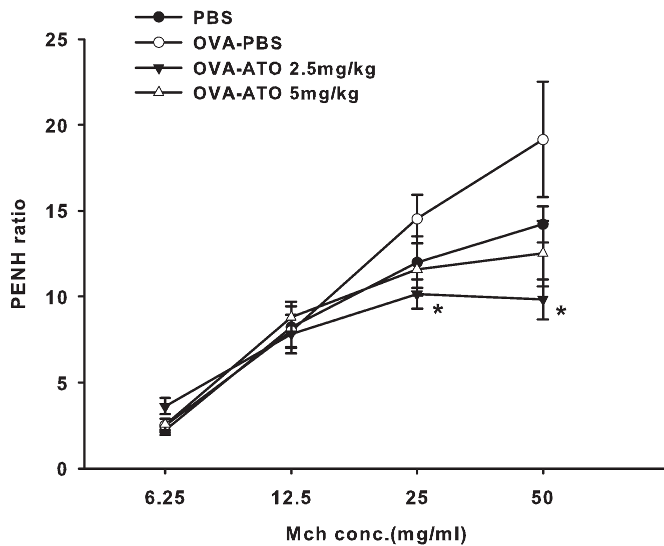

Figure 2 Airway hyperresponsiveness in mice treated with different concentrations of As_2O_3. The mice treated with 2.5 mg/kg of As_2O_3 had a significant decrease in airway hyperresponsiveness. Animals administered 5 mg/kg of As_2O_3 had a lower Penh value than the control group. Each group included four to five mice. Values are expressed as mean±SEM. The difference is statistically significant compared with the control group (*$P<0.05$). As_2O_3, arsenic trioxide; ATO, arsenic trioxide; PBS, phosphate-buffered saline; Penh, enhanced pause; OVA, ovalbumin.

Detection of apoptosis *in vitro*

Primary lung epithelial cells were seeded at 2×10^5 cells/well in 12-well plates. The cells were cultured at 37 °C with 5% CO_2 incubation until the cells attained 80% confluence and subsequently treated with 0, 0.05, 0.1, 0.5, 1 and 5 μM of As_2O_3. After 24 h, the cells were collected and analyzed for apoptotic cells by flow cytometry using 7-amino-actinomycin D and annexin V staining (Apoptosis Detection Kit, PharMingen; Becton Dickinson & Co., San Jose, CA, USA).

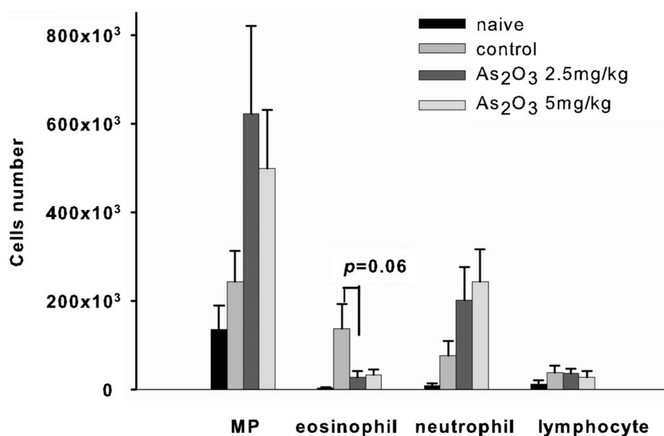

Figure 3 Cellular composition in the BALF. Mice treated with As_2O_3 had decreased numbers of eosinophils but increased numbers of inflammatory cells compared with the control group. Each group included four to five mice. Values are expressed as mean±SEM. The difference is statistically significant compared with the control group (*$P<0.05$). As_2O_3, arsenic trioxide; BALF, bronchoalveolar lavage fluid; MP, macrophages.

Statistical analysis

All data are expressed as mean±SEM. For *in vivo* experiments, each group consisted of 3–6 mice. Single pairs of the groups were compared by Student's *t*-test. Differences were considered statistically significant when $P<0.05$.

RESULTS

As_2O_3 treatment did not affect the serum IgE level

After OVA sensitization, mice were treated with As_2O_3 on days 38–44. On days 42–44, mice were challenged by intranasal administration of OVA. Figure 1 shows that there was no significant difference in serum IgE levels between OVA sensitized mice treated either with or without As_2O_3.

The effect of As_2O_3 on the AHR of mice

We next explored whether As_2O_3 treatment could protect mice from AHR. Penh, which is a measurement of airway resistance, was used as a readout to determine the degree of AHR. After the final intranasal OVA challenge, mice were subjected to a methacholine stimulation test. As shown in Figure 2, mice treated with As_2O_3 had a lower Penh

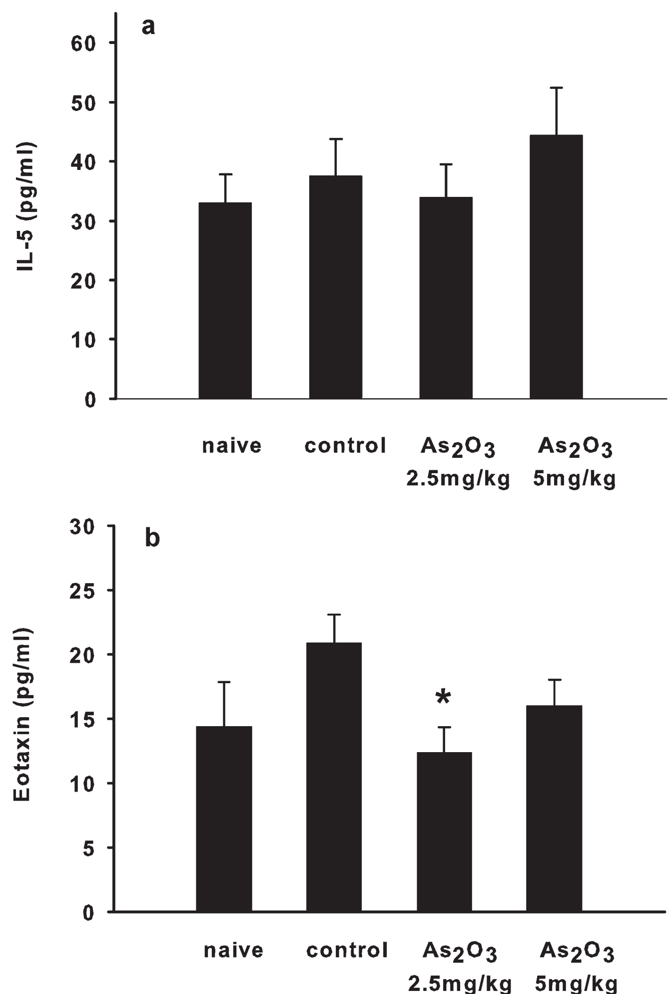

Figure 4 Cytokine levels in the BALF in each group of mice. (**a**) The IL-5 level detected in the BALF was not different between the groups of mice. (**b**) There was a significant decrease in the concentration of eotaxin in the BALF of mice treated with 2.5 mg/kg of As_2O_3. Each group included four to five mice. Values are expressed as mean±SEM. The difference is statistically significant compared with the control group (*$P<0.05$). As_2O_3, arsenic trioxide; BALF, bronchoalveolar lavage fluid.

ratio compared with mice without As_2O_3 treatment. These data indicate that As_2O_3 treatment decreased AHR in mice.

The effect of As_2O_3 on cellular composition in the BALF

To evaluate whether As_2O_3 could modulate the recruitment of inflammatory cells in the airway, differential cell counts were determined by Liu's stain. The data show that mice treated with As_2O_3 had fewer eosinophils and increased macrophages in their BALF (Figure 3). In contrast, there were a higher number of eosinophils in mice sensitized with OVA but not given As_2O_3 treatment. Thus, mice treated with As_2O_3 had significantly decreased eosinophilia in their lungs.

IL-5 and eotaxin levels in the BALF

IL-5 is a key cytokine that promotes eosinophil differentiation, maturation, recruitment and activation at sites of inflammation.[15] We thus determined the level of IL-5 in BALF by ELISA. Figure 4a shows no significant difference in IL-5 protein levels in mice either treated with As_2O_3 or left untreated. To further examine how As_2O_3 might decrease the number of eosinophils, the level of eotaxin was also determined. The data show that mice treated with 2.5 mg/kg of As_2O_3 had a decreased concentration of eotaxin in BALF (Figure 4b).

Recruitment of inflammatory cells in the airway

To determine the effect of As_2O_3 treatment on the recruitment of inflammatory cells, lungs of mice were prepared for histopathological staining. In the untreated group, the cellular infiltration consisted mainly of mononuclear cells predominantly in the peribronchial and perivascular areas (Figure 5b). In contrast, As_2O_3 treatment resulted in reduced cellular infiltration (Figure 5c and d); indeed, cellular infiltration levels were as low as that observed in the naive (unsensitized) group (Figure 5a).

The effect of As_2O_3 on lung epithelial cells

To investigate the effect of As_2O_3 on pulmonary cell secretion of eotaxin, we isolated and cultured primary pulmonary cells from BALB/c mice. The optimal concentration of As_2O_3 in the assay was determined by assessing apoptosis. The data shown in Figure 6a indicate that there was slightly increased cell apoptosis after 24 and 72 h of treatment with 0.05 μM of As_2O_3 (14.29±0.2%, data not shown) compared with cells without treatment (10.21±0.49%). Furthermore, As_2O_3 induced pulmonary cell apoptosis in a dose-dependent manner. We thus chose the lowest concentration (0.05 μM) for further analysis, because this concentration did not affect cell viability. The secretion of eotaxin by primary pulmonary cells was stimulated by IL-4 but significantly inhibited when the cells were concurrently treated with As_2O_3 (Figure 6b, 297.8±49.2 pg/ml and 94.7±24.4 pg/ml, respectively). In addition, the RANTES level was also decreased in the As_2O_3-treated group (Figure 6c, 366.8±30.4 pg/ml and 242.4±7.9 pg/ml, respectively).

DISCUSSION

Although arsenic is often associated with environmental contamination, As_2O_3 is currently used in the clinic to treat cancer. In traditional Chinese medicine, arsenic has been used as a powerful therapeutic for various illnesses, on the principle that 'using a toxic substance against another toxic substance' could treat the illness. Therapeutic As_2O_3 has been found to induce the differentiation and apoptosis of all-trans retinoic acid-resistant APL cells *in vitro* and *in vivo*.[16–18] It has also been suggested that arsenic compounds have proinflammatory

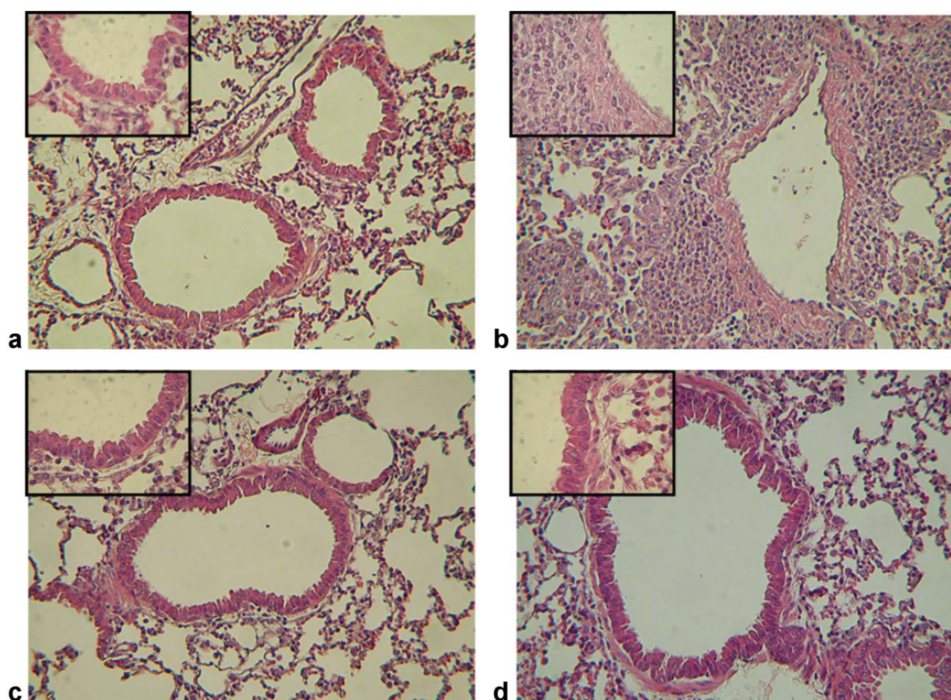

Figure 5 Histological analysis of pulmonary sections from immunized mice with or without As_2O_3 treatment. (**a**) Unsensitized mice show healthy pulmonary tissue. (**b**) Mice sensitized and challenged with OVA show cells infiltrating the airways. After As_2O_3 treatment (2.5 mg/kg (**c**) and 5 mg/kg (**d**)), cellular infiltration was reduced. (**a**–**d**) Sections are stained with H&E. Original magnification in **a**–**d**, ×100; upper left figures: ×400. As_2O_3, arsenic trioxide; H&E, hematoxylin–eosin; OVA, ovalbumin.

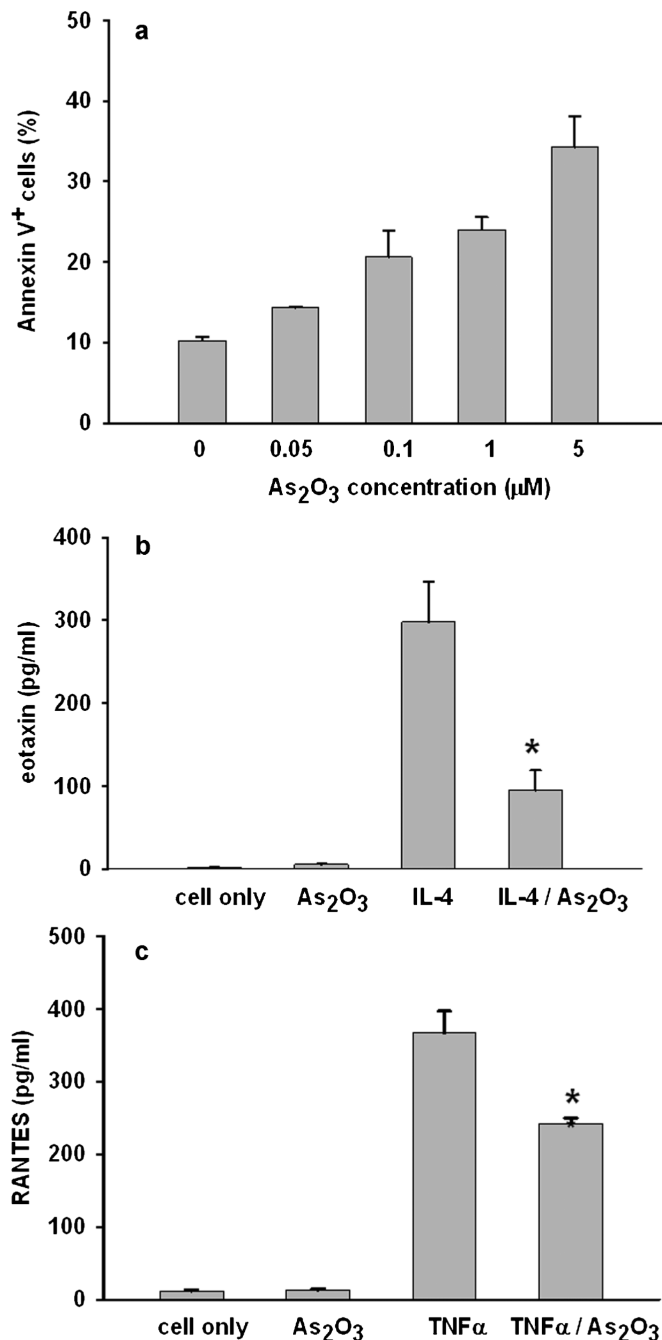

Figure 6 Twenty-four hours of treatment with As_2O_3 induces apoptosis in primary pulmonary cells in a dose-dependent manner (**a**). As_2O_3 of 0.05 μM inhibits the secretion of eotaxin by pulmonary cells stimulated with IL-4 (3000 U/ml) (**b**) and the secretion of RANTES by pulmonary cells stimulated with TNF-α (20 ng/ml) (**c**). Values are expressed as mean±SEM. The difference is statistically significant compared with the control group (*$P<0.05$). As_2O_3, arsenic trioxide; RANTES, regulated upon activation, normal T cell expressed and secreted; TNF, tumor necrosis factor.

properties when the concentration of arsenite is approximately 5 μM.[19] In contrast, another group found that As_2O_3 exerts anti-inflammatory effects through augmentation of IκB and suppression of NF-κB activation. In the current study, we investigated the function of As_2O_3 in alleviating AHR in a murine model of asthma.

In mice, IgE-mediated activation of mast cells enhanced pulmonary responsiveness to cholinergic stimulation.[20] Itami *et al.* demonstrated that IgE was not required for the induction of the late phase of immediate hypersensitivity reactions; however, IgE enhanced pulmonary inflammation and hyperresponsiveness.[21,22] We found that As_2O_3 did not alter IgE levels in the serum, indicating that the inhibition of AHR by As_2O_3 was not associated with decreased levels of IgE. As_2O_3 might therefore target the lung directly.

Eosinophil accumulation in peripheral blood and tissue is a hallmark feature of several important medical diseases, including atopic disorders (allergic rhinitis and asthma), parasitic infections and various systemic diseases (e.g., eosinophilic pneumonia).[23] IL-5 is an eosinophil-specific cytokine that can regulate eosinophil growth, differentiation and survival, and can stimulate the release of eosinophils and eosinophil precursors from the bone marrow into peripheral circulation.[24] IL-5 was originally discovered in guinea pigs and found to be responsible for allergen-induced eosinophil accumulation in the lungs.[25] Eotaxin is an eosinophil-selective chemoattractant that has been identified as a potent activator of eosinophils, inducing eosinophils to generate superoxide and release granule proteins. Early studies suggested that eosinophil recruitment in allergic reactions was regulated by Th2 lymphocytes and that eotaxin production was T cell-dependent.[23] Many cell types in the lung, including airway epithelial cells, airway smooth muscle cells, vascular endothelial cells, macrophages and eosinophils, appear to be capable of synthesizing eotaxin.[20,24,26] In our studies, we found no difference in the level of IL-5 in the BALF of mice with or without As_2O_3 treatment. However, As_2O_3 treatment decreased the level of eotaxin. These findings suggest that eosinophils can leave the bone marrow and enter the circulation in OVA-induced airway hyper-responsive mice. Furthermore, As_2O_3 directly inhibited the secretion of eotaxin by lung epithelial cells, resulting in decreased numbers of eosinophils recruited into the airway (Figure 4b). Activated eosinophils release highly toxic granule proteins and free radicals, which can result in connective tissue matrix remodeling, smooth muscle contraction, increases in vascular permeability and leukocyte activation; these processes promote AHR. Our data showed that 2.5 mg/kg of As_2O_3 decreased the Penh value in OVA-sensitized mice. We speculate that As_2O_3 inhibits AHR by downregulating eotaxin production by cells in pulmonary tissue, thus decreasing the accumulation of eosinophils. However, we cannot exclude the possibility that As_2O_3 might inhibit eosinophils from leaving the bone marrow and entering the circulation.

The pathology of asthma is thought to be mediated by $CD4^+$ T cells producing type-2 cytokines, IL-4 and IL-5, which are elevated in bronchial biopsies, bronchoalveolar lavage and peripheral blood of allergic patients.[21,27,28] These cytokines promote the accumulation and activation of eosinophils and induce IgE synthesis by B cells. We found no significant difference in either IL-5 or IgE levels between the groups, implying that the therapeutic effect of As_2O_3 is not due to blockade of eosinophils or mast cell activation. In other words, As_2O_3 alleviates the severity of asthma at the late phase of the reaction rather than at the acute stage. Therefore, As_2O_3 might be potentially beneficial for asthmatic patients.

In a study by Zhou *et al.*, eosinophils treated with As_2O_3 were found to be defective in their ability to chemoattract to eotaxin and RANTES. Herein, we treated primary pulmonary cells with much lower concentrations of As_2O_3 (0.05 μM), which would not induce pulmonary cell apoptosis, and found that As_2O_3 inhibited both eotaxin and RANTES secretion. Other studies have shown that As_2O_3 can abolish eosinophilia by downregulating chemoattractants released by pulmonary

cells.[29] Although we did not investigate the effect of As_2O_3 on dendritic cells or on the levels of other cytokines (except IL-5 and eotaxin) in BALF, the possibility that As_2O_3 might promote dendritic cell maturation and stimulate cells to produce Th1 cytokines cannot be ruled out.

In conclusion, we have shown that As_2O_3 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. Decreased eotaxin secretion due to treatment with As_2O_3 results in the ablation of eosinophilia in the lung and the alleviation of AHR in an OVA-induced asthmatic murine model. As_2O_3 may therefore have therapeutic potential in the treatment of asthma.

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