

Trichostatin A attenuates airway inflammation in mouse asthma model

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Summary

Background Histone deacetylase (HDAC) inhibition has been demonstrated to change the expression of a restricted set of cellular genes. T cells are essential in the pathogenesis of allergen-induced airway inflammation. It was recently reported that treatment with HDAC inhibitors induces a T cell-suppressive effect.

Objective The purpose of this study was to determine whether treatment with trichostatin A (TSA), a representative HDAC inhibitor, would reduce allergen-induced airway inflammation in a mouse asthma model.

Methods BALB/c mice were intraperitoneally sensitized to ovalbumin (OVA) and challenged with an aerosol of OVA. TSA (1 mg/kg body weight) was injected intraperitoneally every 2 days beginning on day 1. Mouse lungs were assayed immunohistochemically for HDAC1, a major HDAC subtype, and for infiltration of CD4⁺ cells. The effect of TSA on airway hyper-responsiveness (AHR) was determined, and the bronchoalveolar lavage fluid (BALF) of these mice was assayed for the number and types of inflammatory cells, and for the concentrations of IL-4, IL-5, and IgE.

Results HDAC1 was localized within most airway cells and infiltrating inflammatory cells of asthmatic lungs. Treatment with TSA significantly attenuated AHR, as well as the numbers of eosinophils and lymphocytes in BALF. TSA also reduced infiltration of CD4⁺ and inflammatory cells and mucus occlusions in lung tissue, and decreased the concentrations of IL-4, IL-5, and IgE in BALF.

Conclusion TSA attenuated the development of allergic airway inflammation by decreasing expression of the Th2 cytokines, IL-4 and IL-5, and IgE, which resulted from reduced T cell infiltration. Our results suggest that HDAC inhibition may attenuate the development of asthma by a T cell suppressive effect.

Keywords allergic airway inflammation, asthma, histone deacetylase, mouse, T lymphocyte, trichostatin A

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Asthma is an inflammatory disease of the airways, characterized by lung eosinophilia, mucus hypersecretion by goblet cells, and hyper-responsiveness to inhaled allergens. T cells play an essential role in the pathogenesis of asthma [1, 2]. Th2 cells, including CD4⁺ T cells, participate in asthma pathogenesis by stimulating B cells that produce allergen-specific IgE, as well as by inducing the infiltration of eosinophils and other inflammatory cells into the airways [3, 4]. In addition, cytokines produced by Th2 cells, including IL-4, IL-5, IL-9, and IL-13, exacerbate airway inflammation [5, 6]. IL-4 is an autocrine inducer of the growth and development of Th2 cells, as well as a paracrine inducer of B cell and mast cell development and IgE secretion [5]. IL-5 induces eosinophil

generation in the bone marrow and lung eosinophilia [5]. Thus, inhibition of T cell proliferation and activation may prevent or attenuate asthma.

The status of histone acetylation is associated with the transcriptional regulation of genes and is controlled by the reversible covalent modifying enzymes, histone acetyltransferase (HAT), and histone deacetylase (HDAC) [7]. Unwinding of the condensed DNA structure by HAT permits accessibility to transcription factors, allowing them to initiate transcription of the target genes. In contrast, HDAC causes the DNA structure to condense, leading to gene silencing [8]. Histone acetylation has been shown to regulate the expression of a restricted set of cellular genes [9, 10]. Using specific reversible HDAC inhibitors, including trichostatin A (TSA), trapoxin, and suberoylanilide hydroxamic acid (SAHA), the relationship between histone acetylation status and the control of gene expression has been elucidated [11, 12], with histone hyperacetylation due to HDAC inhibition shown to regulate various genes controlling the cell cycle and apoptosis, such as p21^{waf1}, c-myc, plasminogen activator, gelsolin, and p53 [13–16].

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The finding that these HDAC inhibitors reduce tumour formation, without overt toxicity, in mouse models of cancer [17] suggested that they may be novel anticancer agents. In addition, these HDAC inhibitors were recently found to alter the expression of several genes related to the inflammatory process, including inducible nitric oxide synthase (iNOS), macrophage inflammatory protein (MIP)-2, and IL-8 [18–20]. In lipopolysaccharide (LPS)-treated mice, SAHA was shown to reduce dose-dependently serum levels of tumour necrosis factor (TNF)- α , IL-1 β , IL-6, and IFN- γ , as well as to attenuate Con A-induced hepatic injury [21]. In addition, the HDAC inhibitors FR901228 and TSA were shown to have an immunosuppressive effect on T cell proliferation, with a reduction in IL-2 secretion and CD40L expression [22], and TSA was observed to reverse the aberrant expression of CD154, IL-10, and IFN- γ by T cells from patients with systemic lupus erythematosus (SLE) [23]. Taken together, these findings suggested to us that inhibition of HDAC may reduce T cell-dependent diseases, such as asthma, by modulation of T cell functions.

HDACs are expressed in all cells of the human asthmatic airway, including the bronchial epithelium and infiltrating inflammatory cells [24]. Compared with normal airways, however, the amounts and activities of HDAC1 and HDAC2 proteins are considerably reduced in asthmatic airways [24]. In addition, low-dose theophylline exerts an anti-asthma effect by increasing the activation of HDAC, which is subsequently recruited by corticosteroids to suppress inflammatory genes [25]. These results suggest that histone acetylation may play a role in asthma pathogenesis. In this study, we examined the effect of HDAC inhibition by TSA on allergen-induced airway inflammation in a mouse model of asthma.

Materials and methods

Mice

Female BALB/c mice, aged 6–8 weeks, were purchased from Charles River Japan (Hino, Japan). All animal study protocols were approved by the animal care committee of Seoul National University. The mice were divided into three groups. Six mice were treated with saline (SAL), eight were sensitized and challenged with ovalbumin (OVA), and eight were sensitized and challenged with OVA and treated with TSA (TSA+OVA).

Allergen sensitization/challenge protocol

Mice underwent OVA sensitization and challenge as previously described [26]. OVA (Pierce, Rockford, IL, USA; 5000 μ g/mL in PBS) was mixed with an equal volume of 10% (w/v) aqueous aluminium potassium sulphate (alum; Sigma, St Louis, MO, USA), and its pH was adjusted to pH 6.5 with 10 N NaOH. After 60 min incubation at room temperature, the mixture was centrifuged at 750 g for 5 min, and the OVA/alum pellet was resuspended to its original volume in distilled water. Each mouse was injected intraperitoneally (i.p.) with 1000 μ g OVA complexed with alum on day 1.

Intratracheal (i.t.) challenges were performed as described previously [27]. Briefly, each mouse was anesthetized by an i.p.

injection of 0.2 mL of a mixture of ketamine (6.3 mg/mL) and xylazine (0.44 mg/mL) in normal SAL and placed on a board in the supine position. On the back of the tongue was placed 2500 μ g OVA (1000 μ L of a 2.5 mg/mL solution) on day 8 or 1250 μ g OVA (500 μ L of a 2.5 mg/mL solution) on days 15, 18, and 21. The deposition pattern of OVA was examined by mixing OVA (2.5 mg/mL) with toluidine blue and administering 1000 μ L i.t. The majority of the toluidine blue dye was detected in the lumen and interstitium of the tracheal wall, with the remainder in the lumen and interstitium of the small airways. No staining of the esophagus or stomach was observed. Control mice received SAL in alum i.p. SAL on day 1 and i.t. challenges with SAL on days 8, 15, 18, and 21.

TSA treatment

TSA was purchased from Sigma, dissolved in SAL containing 1% dimethyl sulphoxide (DMSO), and administered i.p. (1 mg/kg) every other day beginning on day 1 to the mice in the TSA+OVA group. Mice in the SAL and OVA groups were administered SAL containing 1% DMSO. The dose of TSA was similar to those demonstrating significant inhibition of tumor progression in mice [28, 29].

Airway hyper-responsiveness (AHR)

In vivo airway responsiveness to methacholine was measured 24 h after the last OVA challenge in conscious, freely moving, spontaneously breathing mice. Mice were challenged with aerosolized SAL or methacholine (6.25 and 25 mg/mL in SAL), generated by an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA, USA), for 2 min. The degree of bronchoconstriction was measured using whole-body plethysmography (model PLY 3211; Buxco Electronics, Sharon, CT, USA) and was expressed as enhanced pause (P_{enh}), a calculated dimensionless value that correlates with measurements of airway resistance, impedance and intrapleural pressure in the same mouse. After each nebulization challenge, P_{enh} readings were taken and averaged for 4 min, and were calculated as $P_{\text{enh}} = [(T_e/T_r - 1) \times (\text{PEF}/\text{PIF})]$, where T_e is the expiration time, T_r the relaxation time, PEF the peak expiratory flow and PIF the peak inspiratory flow, $\times 0.67$, a coefficient. The time for the box pressure to change from a maximum to a user-defined percentage of the maximum represents the relaxation time. The T_r measurement was begun at the maximum box pressure and ended at 40%.

Bronchoalveolar lavage fluid (BALF)

After measurement of the AHR, the mice were exsanguinated by cardiac puncture, and BALF was collected (0.4 mL SAL, three times) from the right lung, after tying off the left lung at the main stem bronchus. The total number of cells in a 0.05 mL aliquot of BALF was counted, and the remaining fluid was centrifuged at 200 g and 4 °C for 10 min. The cell pellets were resuspended in SAL containing 10% BSA, and smears were made on glass slides. Eosinophils were stained for 5 min with 0.05% aqueous eosin and 5% acetone in distilled water, rinsed with distilled water, and counterstained with 0.07% methylene blue.

Histopathology

After BALF was obtained, the trachea and upper and lower lobes of the left lung were removed and fixed for 24 h in 10% neutral buffered formalin solution. The tissues were embedded in paraffin, cut into 3 µm sections, and stained with hematoxylin and eosin, or with alcian blue at pH 2.5, and counterstained with nuclear fast red to identify the airway goblet cells and mucus. The degree of airway inflammatory cell infiltration, mucus occlusion of the airway diameter, and airway edema were determined by morphometry, with the morphometric analyses performed by individuals blinded to the protocol design [30]. Briefly, for each mouse, 8 airway sections randomly distributed throughout the left lung were analysed, and their average scores were calculated. The occlusion of the airway diameter by mucus was assessed on a semiquantitative scale, with 0 representing no mucus; 1 representing ~ 25% occlusion; 2 representing ~ 50% occlusion; 3 representing ~ 75% occlusion; and 4 representing ~ 100% occlusion. The degree of inflammatory cell infiltration around the bronchioles, peribronchial arteries, and veins was scored as 0 for no infiltration; 1 for ~ 3 layers of inflammatory cells; 2 for ~ 6 layers; 3 for ~ 10 layers; or 4 for more than 10 layers of inflammatory cells. The degree of edema was assessed as 0 for none; 1 for ~ 25% airway involvement; 2 for ~ 50% airway involvement; 3 for ~ 75% airway involvement; and 4 for ~ 100% airway involvement.

Immunohistochemistry

Lung tissues were fixed for 24 h in 10% neutral buffered formalin solution and routinely processed. Paraffin sections were placed on Probe-On slides (Fisher Scientific, Pittsburgh, PA, USA) and incubated with methanol containing 3% H₂O₂ to inhibit endogenous peroxidase activity. The tissue sections were treated with 10% normal goat serum for 1 h at room temperature to block non-specific binding. The slides were subsequently incubated overnight at 4°C with rabbit anti-HDAC1 (Upstate Biotechnology, Lake Placid, NY, USA; 1 : 200) or rat anti-CD4 (Serotec, Raleigh, NC, USA; 1 : 100). Antigens were detected by the avidin-biotin complex method, using 3, 3'-diaminobenzidine as the chromogen. Human gastric cancer tissue, which overexpresses HDAC1 [31], was used as a positive control. Negative control tissues were prepared in the same manner as described above, except for the omission of the primary antibody and the substitution of an isotype-matched, but irrelevant, antibody.

ELISA

Concentrations of IL-4, IL-5, and IgE in BALF were determined by ELISA, according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA).

Statistical analyses

The data are reported as the mean ± standard error of the mean (SEM). Between-group differences were analyzed by Student's two-tailed *t*-test, with *P* < 0.05 defined as statistically significant. To compare the pathological grade of each group, the Mann-Whitney test, a non-parametric method,

was performed using Statview software (SAS Institute Inc., Cary, NC, USA).

Results

Immunohistochemical detection of HDAC1 in murine asthmatic lung

We examined the expression patterns of HDAC1 in normal and asthmatic lungs of OVA-sensitized/challenged mice by immunohistochemistry. Since HDAC1 is normally expressed in cell nuclei [32], samples were considered positive only if strong nuclear staining was observed. We found that HDAC1 was localized within all resident lung cells of SAL-treated mice, including the pneumocytes, bronchial epithelial, endothelial, and smooth muscle cells (Fig. 1a). OVA-induced airway inflammation did not change the patterns of HDAC1 expression in these resident cells (Fig. 1b). In addition, infiltrating inflammatory cells, including eosinophils and mononuclear cells, were found to express HDAC1 in OVA-treated mice (Fig. 1b).

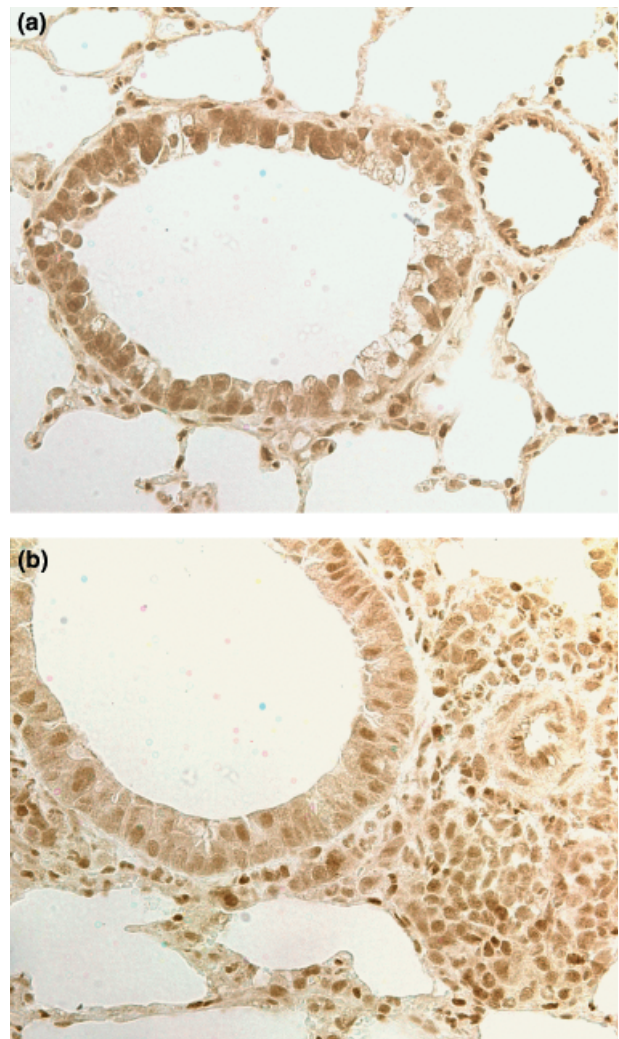


Fig. 1. HDAC1 (a major histone deacetylase subtype) expression in normal and asthmatic mouse lungs. HDAC1 expression was localized in the airways of normal (a) and asthmatic (b) mice (× 200).

Effect of TSA on AHR

The AHR to inhaled methacholine at 25 mg/mL was significantly higher in the OVA group than in the SAL group ($P < 0.05$). Treatment with TSA, however, significantly reduced AHR compared with that in the OVA group ($P < 0.05$) (Fig. 2).

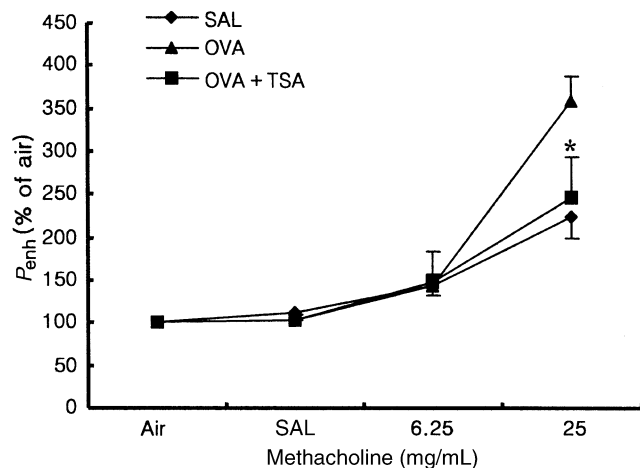


Fig. 2. Effect of trichostatin A (TSA) on airway hyper-responsiveness to methacholine in ovalbumin (OVA)-sensitized mice. The degree of bronchoconstriction, expressed as P_{enh} (percentage of air), to inhaled methacholine (6.25 and 25 mg/mL) was determined in the saline (SAL)-, OVA-, and OVA+TSA-treated groups.

Effect of TSA on allergic airway inflammation

In OVA-sensitized mice, intratracheal OVA challenge caused a marked influx of leucocytes into the BALF. Compared with the SAL group, the OVA group had a 17.6-fold increase in total cells recovered from BALF (Fig. 3a). In the SAL group, 1.4% of the BALF leucocytes were eosinophils (Fig. 3b), with the mean number of eosinophils in BALF being $(0.013 \pm 0.012) \times 10^5$ (Fig. 3c). In the OVA group, however, 76.1% of the total leucocytes from BALF were eosinophils (Fig. 3b), with the mean number of eosinophils in BALF being $(1.20 \pm 0.28) \times 10^6$ (Fig. 3c). Treatment with TSA decreased the influx of leucocytes into the BALF by 25.2% compared with the OVA group ($P < 0.05$) (Fig. 3a). In the OVA+TSA group, the number of eosinophils in BALF was 34.2% lower than in the OVA group, and the number of lymphocytes was 41.5% lower ($P < 0.05$ for each) (Fig. 3c, d). By light microscopy, the lungs of SAL-treated mice were of normal appearance, without inflammatory cells in the airways, whereas the lungs of the mice in the OVA group showed a marked infiltration of inflammatory cells, including eosinophils and mononuclear cells, into the peribronchial and perivascular spaces and alveolar walls. TSA treatment markedly reduced the inflammatory changes in OVA-sensitized/challenged mice (Fig. 4). Morphometric analysis showed that TSA reduced the infiltration of inflammatory cells by 24%, compared with the OVA group ($P < 0.05$). In the OVA-treated mice, airway edema and mucus occlusions were also

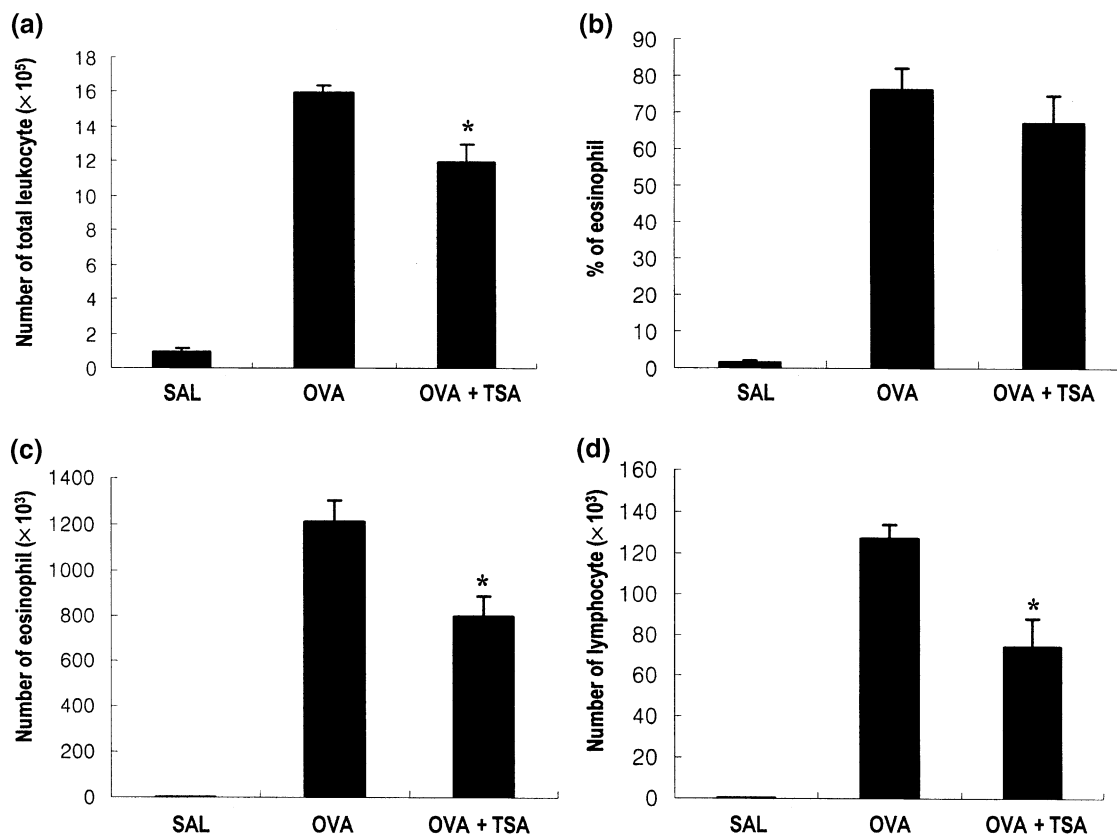


Fig. 3. Effect of trichostatin A (TSA) on numbers of inflammatory cells in bronchoalveolar lavage fluid (BALF). BALF was obtained from saline (SAL)-, ovalbumin (OVA)-, and OVA+TSA-treated groups, and the number of total cells (a), the percentage (b) and number (c) of eosinophils, and the number of lymphocytes (d) present were determined. Results are shown as mean $\pm$ SEM. * $P < 0.05$ compared with the OVA group.

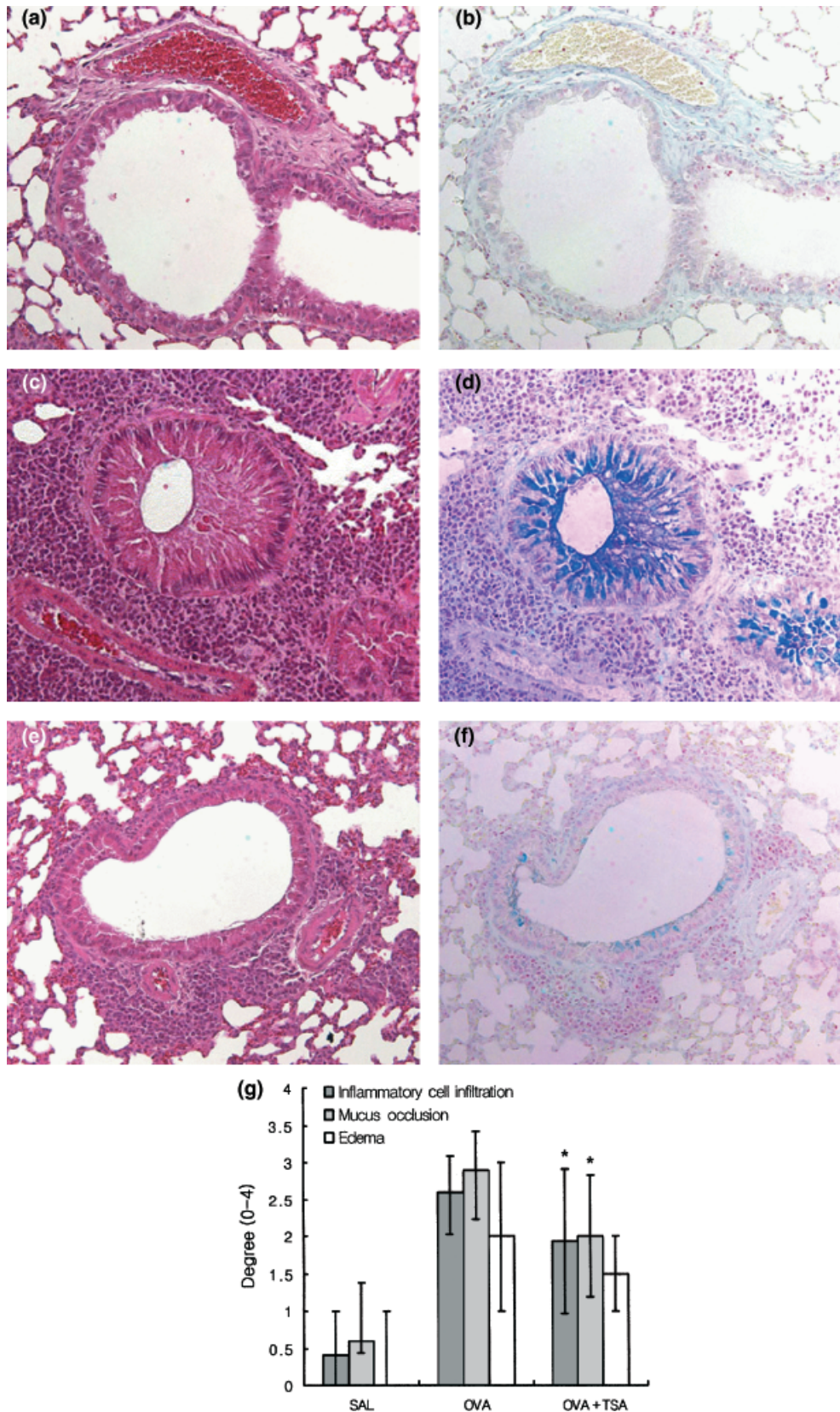


Fig. 4. Effect of trichostatin A (TSA) on airway inflammation. Representative photographs of mice in the saline (SAL)- (a, b), ovalbumin (OVA)- (c, d), and OVA+TSA-treated (e, f) groups. Lung sections were stained with hematoxylin and eosin (a, c, e), or Alcian Blue with nuclear fast red counterstaining (b, d, f), and examined by light microscopy. (g) The grades of intensity of inflammatory cell infiltration, mucus occlusion of airways, and airway edema are shown as the median values with interquartile ranges. * $P < 0.05$ compared with the OVA group.

observed. Mucus occlusions were significantly reduced in the OVA+TSA group compared with the OVA group ($P < 0.05$) (Figs 4b, d and f). There was also a tendency towards reduction of edema in the OVA+TSA group compared with the OVA group ($P = 0.17$) (Fig. 4).

Effect of TSA on recruitment of CD4⁺ T cells

To determine whether treatment with TSA reduced the recruitment of T lymphocytes into asthmatic lungs, we evaluated the number of CD4⁺ T cells in each group by immunohistochemistry. In the SAL group, there were a negligible number of CD4⁺ T cells, whereas marked expansions of CD4⁺ T cells were observed in the OVA group. The number of CD4⁺ T cells in the OVA+TSA group (67.8 ± 12.7 cells/ 0.1 mm^2), however, was significantly lower than in the OVA group (101.8 ± 10.2 cells/ 0.1 mm^2) (Fig. 5).

Effect of TSA on pulmonary cytokines and IgE

OVA sensitization and challenge induced significant elevations of IL-4, IL-5, and IgE in the BALF. The concentration of IL-4 in the BALF of OVA-treated mice was 30.4-fold higher than in the SAL-treated mice. This elevated IL-4 concentration ($85.2 \pm 10.0 \text{ pg/mL}$) was markedly reduced by TSA treatment ($34.4 \pm 4.5 \text{ pg/mL}$, $P < 0.01$) (Fig. 6a). In addition, the concentration of IL-5 in BALF was 3.9-fold higher in OVA-treated mice compared with SAL-treated mice, but the IL-5 levels in the OVA group ($15.4 \pm 2.9 \text{ pg/mL}$) were significantly reduced after treatment with TSA ($5.1 \pm 2.1 \text{ pg/mL}$, $P < 0.01$) (Fig. 6b). The IgE levels in BALF were also dramatically elevated in OVA-treated mice compared with SAL-treated mice. Treatment with TSA also reduced the IgE levels by 47.8% compared with the OVA group ($174.2 \pm 53.0 \text{ ng/mL}$, vs. $333.5 \pm 60.5 \text{ ng/mL}$, $P < 0.05$) (Fig. 6c).

Toxicity

Side-effects of TSA were monitored by measuring food intake, body weight and overall body condition of the mice. We observed no differences among the three groups. No deaths occurred during the experimental period. On the day of necropsy, body weights were $18.4 \pm 1.4 \text{ g}$ in the SAL group, $18.3 \pm 1.6 \text{ g}$ in the OVA group, and $18.4 \pm 0.7 \text{ g}$ in the OVA+TSA group (NS for each comparison). On pathological examination, no notable lesions were found in the major parenchymal organs, including the liver, kidney, heart, brain, stomach, intestine, and spleen, of any of these animals.

Discussion

We have shown here that TSA inhibition of HDAC attenuated allergen-induced airway inflammation in a mouse model of asthma. A previous study showed that another HDAC inhibitor, SAHA, has anti-inflammatory effects both *in vitro* and *in vivo* [21], prompting us to also test whether SAHA could attenuate OVA-induced airway inflammation. Although we found that SAHA also significantly reduced AHR to methacholine, it was not as effective as TSA in re-

ducing asthmatic lesions (unpublished data). These findings are in accord with the respective effects of these inhibitors on HDAC activity, in that TSA has 10-fold greater activity than SAHA [33]. Taken together with our results, these findings

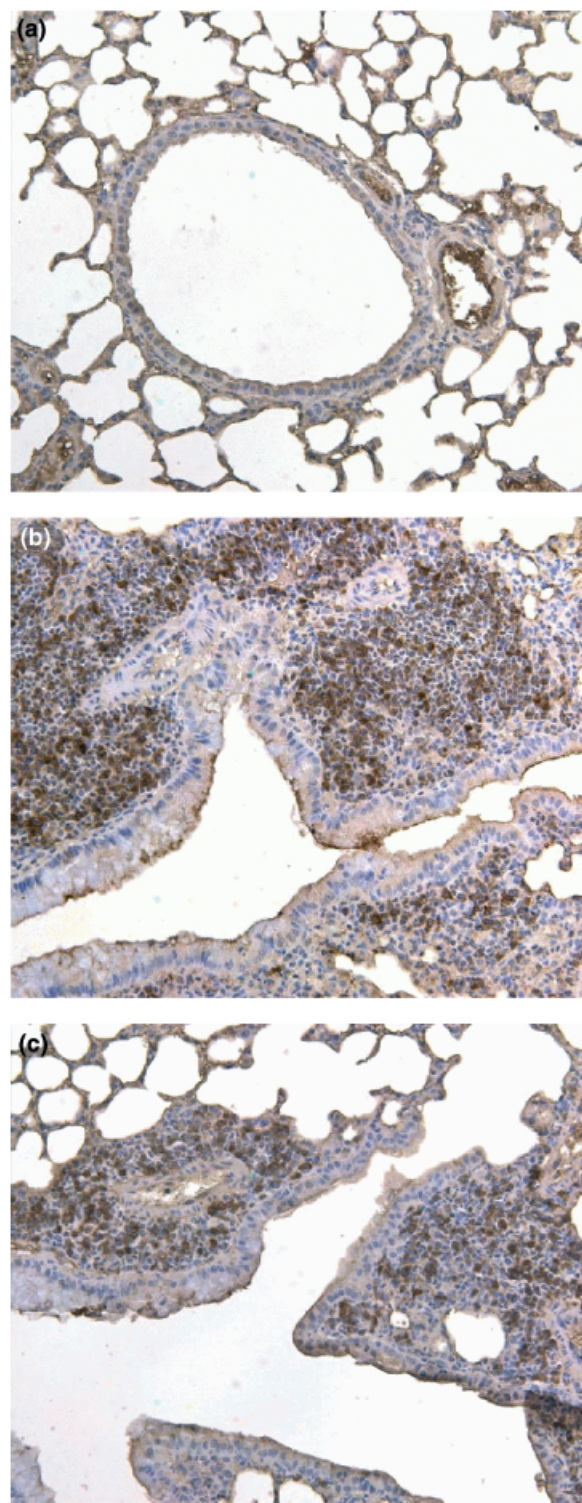


Fig. 5. Effect of trichostatin A (TSA) on infiltration of CD4⁺ T cells. Lung sections in each group were immunostained with CD4⁺ antibody. Representative photographs of mice in the saline (SAL)- (a), ovalbumin (OVA)- (b), and OVA+TSA-treated (c) groups are shown ($\times 100$).

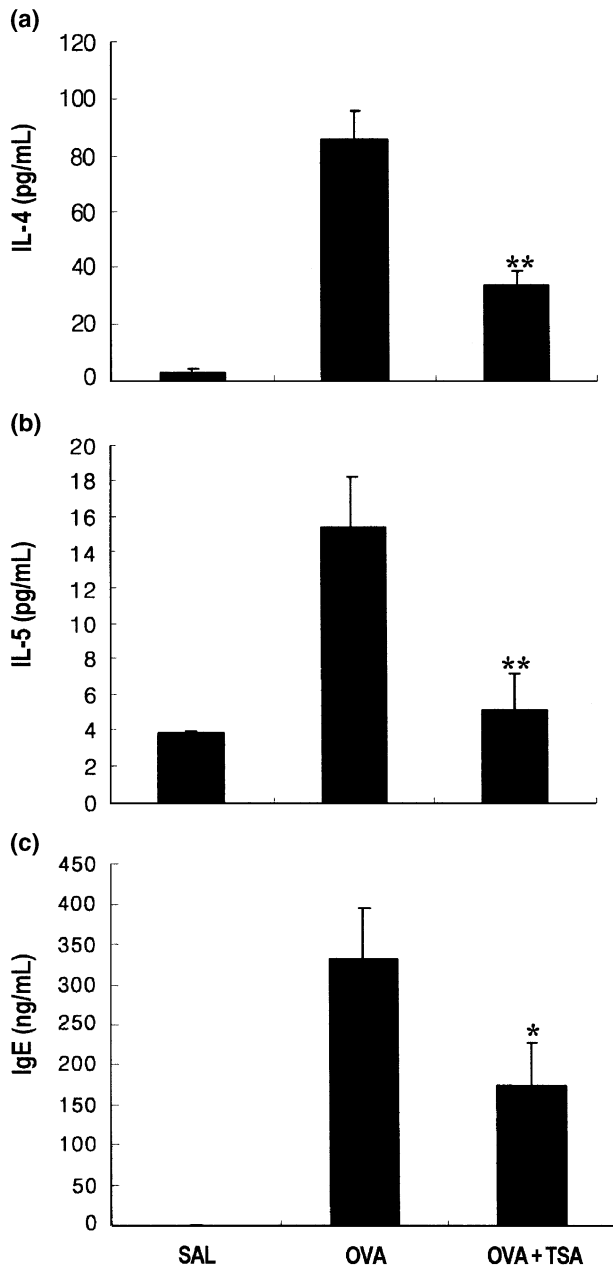


Fig. 6. Effect of trichostatin A (TSA) on IL-4 (a), IL-5 (b), and IgE (c) in bronchoalveolar lavage fluid (BALF). Protein concentrations were determined by ELISA. The concentration of each protein was calculated by comparison with standard curves, and the values are shown as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ compared with the ovalbumin (OVA) group.

indicate that control of histone acetylation can affect the development of allergic airway inflammation.

It has been suggested that the reduction in HDAC activity in asthmatic airways may stimulate the production of inflammatory molecules [24], and treatment with low doses of theophylline, with glucocorticoids, has been reported to synergistically attenuate asthma by increasing HDAC activity [25]. In addition, HDAC inhibition caused by TSA up-regulates the level of expression of NF- κ B-dependent genes, including TNF- α , IL-8, and granulocyte macrophage-colony stimulating factor (GM-CSF), by macrophages from BALF

[34]. Inhibition of HDAC also stimulates anti-inflammatory activities, including the reduced expression of cytokines such as TNF- α , IL-1 β , IL-6, IL-10, IL-12, and IFN- γ [21, 33]. Thus, although HDAC inhibition enhances NF- κ B activity, it may also stimulate the expression of other genes controlling the synthesis of inflammatory cytokines. In addition, the effect of HDAC inhibition may differ depending on cell type.

In the murine asthma model, T cell expansion and activation occur in the thoracic lymph nodes during the sensitization period. Following allergen challenge, the T cell population in the lungs markedly increases, with the bulk of the expansion due to CD4⁺ cells producing Th2 cytokines, such as IL-4, IL-5 and IL-13, which are responsible for pulmonary eosinophilia, mucus hypersecretion, and AHR [35]. Thus, a T cell inhibitory compound that controls the production of Th2 cytokines would reduce allergic airway inflammation and hyper-responsiveness [36]. Two specific inhibitors of HDAC, FR901228 and TSA, have been reported to have immunosuppressive effects by reducing CD4⁺ T cell proliferation, secretion of IL-2, and expression of CD154 [22]. In addition, the HDAC inhibitors TSA and SAHA have also been found to reduce renal disease in a murine model of lupus by down-regulating the production of Th1 and Th2 cytokines [33]. Thus, HDAC inhibitors may effectively inhibit the activity of T cells *in vivo*. In this study, we demonstrated that TSA reduced the number of lymphocytes in BALF and the infiltration of CD4⁺ T cells into the lungs, as well as the expression of representative Th2 cytokines, including IL-4 and IL-5, in BALF. These findings indicate that the inhibitory effects of TSA on T cells attenuate allergen-driven airway inflammation and AHR.

Our result that TSA reduced the level of IL-4 in BALF differs from earlier findings showing that HDAC reduced IL-4 production by T cells, whereas TSA enhanced IL-4 production by activated T cells [37]. This discrepancy may be due to the different cell types used, in that we assayed IL-4 in BALF *in vivo*, whereas the previous work assayed the effects on purified peripheral blood T cells, while excluding interactions with other cells, including monocytes. HDAC inhibition has been shown to alter the expression of T cell surface molecules involved in intercellular interactions, including CD40L and CD28 [22, 38], and the effects of HDAC inhibitors may differ depending on the presence or absence of these interacting cells. In addition, it has been shown that HDAC inhibitors, including TSA and FR901228, down-regulate the production of IL-2 and block IL-2 mediated gene expression, including the expression of *c-myc*, *LC-PTP*, and *bag-1*, which leads to the suppression of T cell proliferation [39]. This anti-proliferative effect may reduce the infiltration of CD4⁺ T cells into asthmatic lungs, thus reducing the concentration of IL-4 in BALF. Further experiments, however, are required to determine the exact mechanism behind this discrepancy.

In summary, we have shown here, using a murine asthma model, that HDAC inhibition by TSA reduces allergen-induced airway inflammation and AHR, possibly by inhibiting T cell recruitment and Th2 cytokines. These results suggest that histone acetylation is involved in the development of allergic airway inflammation and that control of histone acetylation may attenuate the development of asthma.

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