

ORIGINAL ARTICLE

Association between histone hyperacetylation status in memory T lymphocytes and allergen-induced eosinophilic airway inflammation

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ABSTRACT

Background and objective: T lymphocytes, which are characterized by longevity and immune memory, play an important role in airway inflammation in asthma. Here, we assessed the association between immune memory and histone deacetylation and/or acetylation status.

Methods: CD4⁺CD45RB^{low} cells (memory T (T_m)) obtained from the spleens of asthma mice models were co-cultured with glucocorticoids (GCs), trichostatin A (TSA) or anacardic acid (AA) and adoptively transferred to naïve mice. Interleukin (IL)-4, 5 and 13 and IFN- γ concentrations were measured in culture supernatants and bronchoalveolar lavage fluid (BALF). Histone deacetylase (HDAC) and histone acetyltransferase (HAT) activities and the expression of T-bet, GATA-3, HDACs 1–11 and alveolar eosinophilic inflammation index (AEII) were determined in lung tissues.

Results: Culture supernatants and the BALF showed similar cytokine profiles. AA and GCs significantly inhibited HAT activity ($P=0.002$ and $P=0.018$), whereas TSA inhibited and GCs promoted HDAC activity ($P=0.004$ and $P=0.025$). HDACs 7, 9 and 10 were upregulated by AA and GCs (all $P<0.032$), while HDAC11 was upregulated by GCs ($P=0.028$). GC-induced inhibition of T_m histone acetylation alleviated AEII by downregulating IL-4, 5 and 13, similar to the effect of AA.

Conclusion: Histone hyperacetylation status induced by low expression of HDACs 7, 9 and 10 in allergen-specific T_m cells contributes to eosinophilic airway inflammation. The mechanism by which GCs improve airway inflammation involves the upregulation of HDACs 7, 9, 10 and 11 and especially HDAC-10. The role of individual HDACs and AA as novel therapeutic agents for allergic asthma needs to be explored in the future.

Key words: airway inflammation, asthma, glucocorticoids, histone deacetylation, memory T lymphocytes

SUMMARY AT A GLANCE

Upregulation of histone deacetylase (HDACs 7, 9, 10 and 11) plays a role in the epigenetic mechanism by which glucocorticoids improve allergen-driven airway inflammation in mice. The therapeutic potential of individual HDACs and anacardic acid for the treatment of allergic asthma needs to be explored in the future.

Abbreviations: AA, anacardic acid; AEII, alveolar eosinophilic inflammation index; BALF, bronchoalveolar lavage fluid; EOS, eosinophils; FBS, fetal bovine serum; GCs, glucocorticoids; HAT, histone acetyltransferase; HDAC, histone deacetylase; IL, interleukin; OVA, ovalbumin; T_H1, T helper 1; T_H2, T helper 2; T_m, memory T; TSA, trichostatin A.

INTRODUCTION

Asthma is a public health problem worldwide and particularly in developed countries.¹ It is a complex genetic disease that is induced by exposure to a variety of environmental triggers.² Asthma is a heterogeneous disease with different phenotypes characterized by various inflammation patterns, clinical features and treatment responses; allergic asthma is the most common asthma phenotype.³ The allergen-specific response is initiated by CD4 lymphocytes, especially T helper 2 (T_H2) cells, which generate cytokines involved in the recruitment, activation and survival of airway-infiltrating cells.⁴ The transcription factors T-bet and GATA-3 are mutually inhibitory for T helper 1 (T_H1) and T_H2 cell differentiation, respectively.^{5,6} T_H2 cells can adoptively transfer allergic airway inflammation to naïve mice, and elimination of T_H2 cells or T_H2 cytokines prevents induction and abolishes allergic airway inflammation in naïve mice.⁷ In clinical practice, patients have recurrent episodes of asthma associated with allergen exposure, suggesting that immunological T_H2 memory plays a crucial role in the pathogenesis of the disease. Immunological memory mediates a more

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effective response to later allergen exposure. Although the acquisition of immunological memory is important for protecting the host against pathogens, it does not seemingly benefit the host in allergic asthma.⁸ This indicates that memory T (T_m) cells likely persist in the lungs during asthma remission and, on allergen exposure, become activated effectors in patients with poor asthma control and exacerbations.⁹ Furthermore, the immunological memory response to allergens leads to marked relapses in allergic asthma. T_m cells are therefore an important potential therapeutic target for the treatment of allergic asthma.¹⁰ During the priming of T cells, T-cell receptor (TCR) signals, which are secondary signals provided by costimulatory molecules and cytokines, determine the differentiation pathway of T_m cells.¹¹ CD4⁺CD45RB^{low} T cells in mice and CD4⁺CD45⁺RO cells in human are usually identified as T_m cells,¹² and they produce a characteristic spectrum of T_H2 cytokines, including the interleukins (ILs) 4, 5 and 13. However, the mechanism underlying the development and maintenance of the immunological memory response to allergens in T_m cells remains unclear.

Epigenetic regulation (heritable changes in gene expression that occur in the absence of alterations in DNA sequences) was recently suggested to mediate the complex gene-by-environment interactions that can lead to asthma.¹³ Histone acetylation is a major mechanism of epigenetic regulation. The extent of acetylation of core histones is regulated by the balance between the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs).¹⁴ HDACs catalyse the hydrolysis of acetyl groups on lysine residues of histones, causing the condensation and coiling of chromosomal DNA around histones, and therefore regulating gene expression.¹⁵ HDACs are divided into four classes that contain HDACs 1–11. Trichostatin A (TSA), an organic compound with antifungal activity, is one of the most effective and best studied HDAC inhibitors and selectively inhibits class I and II, but not class III, mammalian HDACs.¹⁶ Anacardic acid (AA), which is a bioactive phytochemical found in the nutshell of *Anacardium occidentale*, inhibits HAT activity.¹⁷ Few studies have explored the molecular and epigenetic mechanisms underlying the aberrant differentiation of T_m cells, which could lead to the development of new approaches to tackle allergic asthma.

In this study, we hypothesized that the histone deacetylation status of T_m cells was related to airway inflammation-produced T_H2 cytokines. Furthermore, we explored the role of individual HDACs in allergen-induced airway inflammation and investigated whether the mechanism underlying the effect of glucocorticoids (GCs) on reducing airway inflammation in the asthma mouse model involved T_m cell histone acetylation/deacetylation.

METHODS

Mice, allergen sensitization, and challenge

Twenty male specific-pathogen-free BALB/c mice, aged 8–10 weeks, were obtained and maintained in

the Laboratory Animal Center of the West China School of Medicine. All protocols for animal use were approved by the Institutional Animal Care Committee of the West China Hospital, Sichuan University, China. Mice were sensitized with 20 µg of OVA (Grade V, Sigma-Aldrich, St Louis, MO) emulsified in 50 µg of aluminium hydroxide in a total volume of 200 µL by means of intraperitoneal injection on days 1 and 14, as previously described.^{18,19} On days 21, 22 and 23, mice were challenged for 30 min with an aerosol of 30 µg/mL of ovalbumin (OVA) in PBS with an ultrasonic nebulizer (Berry Co. Ltd., Germany).

Isolation and purification of CD4 + CD45RB^{low} T cells

CD4⁺ T lymphocytes were obtained from the spleen using magnetic beads, and CD4⁺CD45RB^{low} T cells were isolated and purified by flow cytometry (Supplementary Figure S1) as described previously.^{18,19} For details, see Supplementary Appendix S1.

Cell culture, treatments and cytokine assays

Isolated CD4⁺CD45RB^{low} cells were cultured in triplicate in RPMI-1640 medium containing 0.5% FBS (GIBCO, USA) at a density of 1.5×10^5 cells/mL. The cells were treated with 5 µg/mL of concanavalin A (ConA, BD Company, New Jersey, USA) and 100 U/mL of penicillin and streptomycin (Shandong Lukang Pharmaceutical Company, China), and then treated at 37°C with 5% CO₂ for 36 h as follows: (1) GCs hydrocortisone 1000 nM (Sigma); (2) TSA: trichostatin A 50 nM (Sigma); (3) AA: anacardic acid 50 µM (Merck, Germany); (4) sham intervention as the control group (Sham); asthma mice models without reagent drug treatment; and (5) naïve: healthy mice without reagent or drug treatment (naïve). After culture, the supernatants were kept at –80°C and IL-4, IL-5, IL-13 and IFN-γ concentrations were measured by ELISA (Senxiong Biotech, Shanghai, China). CD4⁺CD45RB^{low} cells were used for adoptive transfer to naïve mice.

Adoptive transfer and challenge, bronchoalveolar lavage and cytokine assays

Cultured CD4⁺CD45RB^{low} cells from each group were resuspended in 250 µL of PBS, divided into five equivalent parts, and then adoptively transferred to five naïve mice at approximately 0.8×10^5 cells per mouse. Adoptive transfer was performed as previously described.^{18,19} For details, see Supplementary Appendix S1.

Lung tissue pathology

Right lower lung tissues were used for pathology. Eosinophilic inflammation intensity in the alveolar area was calculated according to the number of eosinophils (EOS) per alveolar area. The alveolar eosinophilic inflammation index (AEII) was calculated as the inflammation intensity ratio of each group to that of naïve mice.¹⁸ For details, see Supplementary Appendix S1.

HDAC and HAT activities

Histone deacetylase and histone acetyltransferase activities in right upper lung tissues after adoptive transfer were measured using colorimetric activity assay kits (BioVision Inc., Mountain View, CA, USA) according to the manufacturer's protocol.

Western blot analysis for T-bet, GATA-3 and HDACs

Right upper lung homogenates were boiled, separated by SDS-PAGE (12% SDS tricine gel) and transferred to polyvinylidene difluoride membranes. Membranes were blocked and then incubated with anti-GATA-3 mAb, anti-T-bet mAb (Santa Cruz Biotechnology, Inc., Dallas, TX, California, USA) or anti-HDAC (1–11) mAbs (HDAC Family Antibody Set, BioVision, USA). The procedures are described in Supplementary Appendix S1. Results were expressed as the mean band density normalized against β -actin as an internal control.

Statistical analysis

Data are presented as the mean $\pm$ SD. Data were analysed by one-way analysis of variance (one-way ANOVA) and correlation analysis (Spearman) with Stata version 11.0 (Stata Corp LP, College Station, TX, USA). A double-sided P -value ≤ 0.05 was considered statistically significant.

RESULTS

Characterization of Tm cells by histone hyperacetylation status

The concentrations of IL-4, IL-5 and IL-13 (pg/mL) and HAT activity ($\mu\text{M}/10\mu\text{g}$) were significantly higher in the ovalbumin (OVA)-challenged group than in the naïve group, whereas HDAC activity ($\mu\text{M}/\mu\text{g}$) and the ratio of HDAC/HAT activity were decreased. The level of IFN- γ did not differ significantly between the two groups (Supplementary Figure S2). These data indicated that Tm cells were characterized by histone hyperacetylation status.

Cytokines in cell culture supernatants and in bronchoalveolar lavage fluid

Interleukin-4, IL-5, IL-13 and IFN- γ concentrations (pg/mL) are shown in Figure 1. In cell supernatants, IL-4, IL-5 and IL-13 concentrations were significantly lower in the glucocorticoids (GCs) and AA groups than in the sham group ($P < 0.05$), whereas they were higher in the TSA group than in the sham group ($P < 0.05$). IL-4 and IL-5 concentrations were lower in the GCs and AA groups than in the TSA group (all $P < 0.05$), whereas differences between the GCs and AA groups were not statistically significant ($P = 0.274$). IFN- γ concentrations in the GCs and AA group were higher than those in the TSA group (both $P < 0.05$).

In BALF, IL-4, IL-5 and IL-13 concentrations were significantly lower in the GCs and AA groups than in the sham group (all $P < 0.05$), whereas they were higher in the TSA group than in the sham group (all

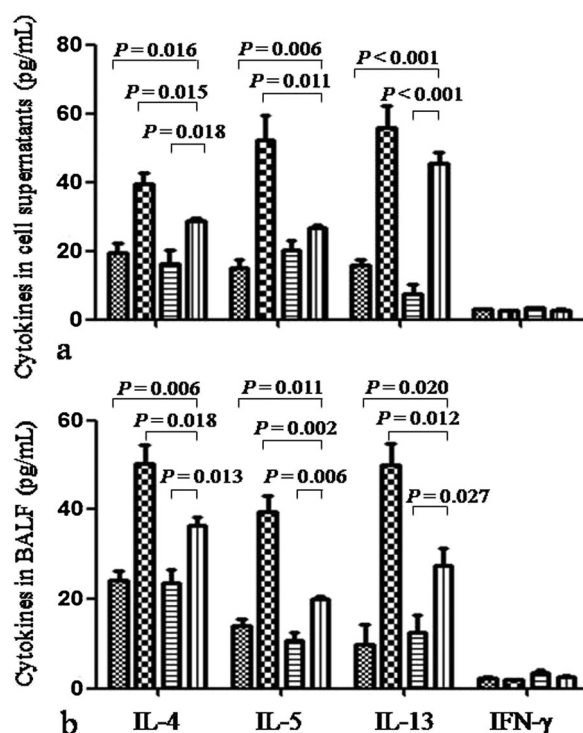


Figure 1 Interleukin (IL)-4, IL-5, IL-13 and interferon gamma (IFN- γ) concentrations (pg/mL) detected by enzyme-linked immunosorbent assay in cell culture supernatants (a) and bronchoalveolar lavage fluid (BALF) (b). The Student–Newman–Keuls (SNK) test was used for multiple group comparisons. (■) GCs, (□) TSA, (▒) AA, and (░) Sham. AA, anacardic acid; GCs, glucocorticoids; TSA, trichostatin A.

$P < 0.05$). The IFN- γ concentration was higher in the AA group than in the sham group ($P = 0.037$) and lower in the TSA group than in the sham group ($P = 0.01$).

HDAC and HAT activities

Histone deacetylase ($\mu\text{M}/\mu\text{g}$) and histone acetyltransferase ($\mu\text{M}/10\mu\text{g}$) activities in the lung tissues of adoptively transferred mice are shown in Figure 2. HDAC activity in the GCs group was significantly higher than that in the TSA, AA and sham groups (all $P < 0.05$). HDAC activity was significantly lower in the AA group than in the TSA group ($P = 0.004$). HAT activity in the GCs, TSA and AA groups was significantly lower than that in the sham group (all $P < 0.01$). The ratio of HDAC to HAT (HDAC/HAT) activity in the GCs group was higher than that in the TSA and sham groups (both $P < 0.001$), and the HDAC/HAT activity ratio in the AA group was higher than that in the sham group ($P = 0.001$).

GATA-3, T-bet and HDACs 1–11 expression

The protein expression of GATA-3 and T-bet is shown in Figure 3. GATA-3 expression did not differ significantly between groups (all $P > 0.05$), whereas T-bet expression in the AA group was higher than that in the sham group ($P = 0.049$).

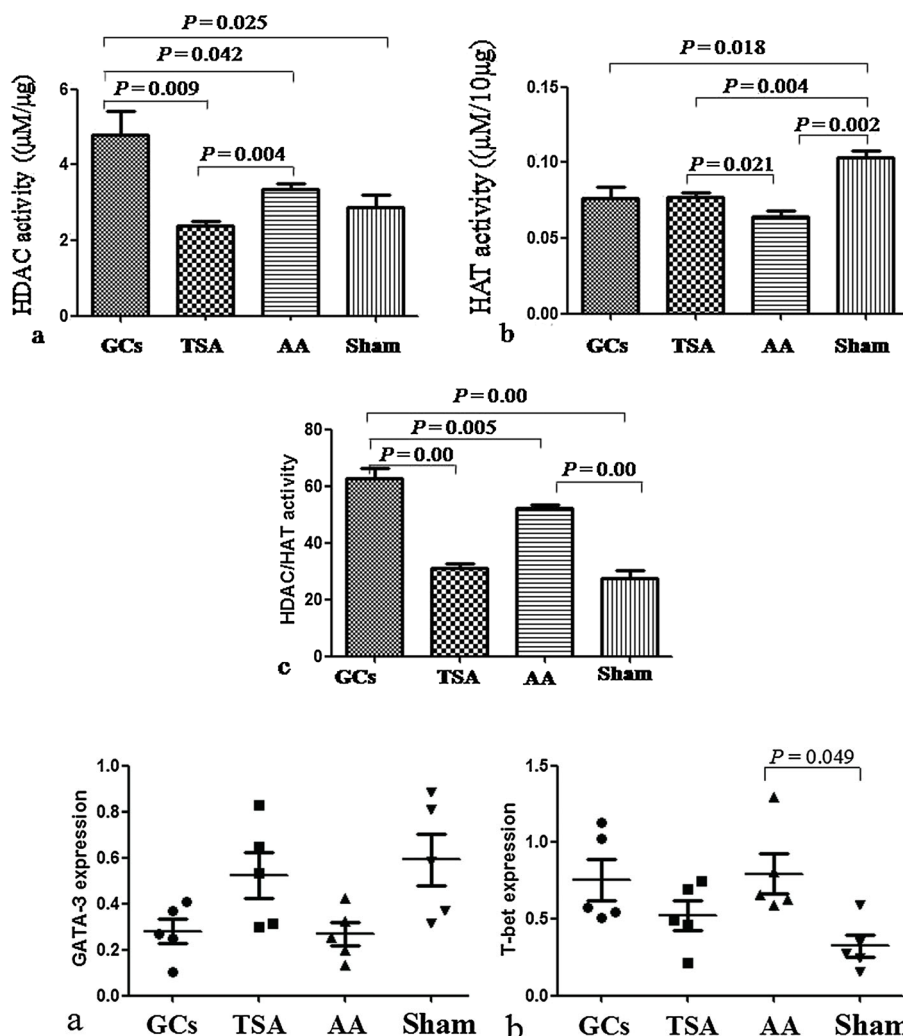


Figure 2 Total histone deacetylase (HDAC) activity ($\mu\text{M}/\mu\text{g}$) (a) and histone acetyltransferase (HAT) activity ($\mu\text{M}/10\mu\text{g}$) (b) detected using colorimetric activity assay kits in lung tissues of adoptive transfer mice. The Student–Newman–Keuls test was used for multiple group comparisons. AA, anacardic acid; GCs, glucocorticoids; TSA, trichostatin A.

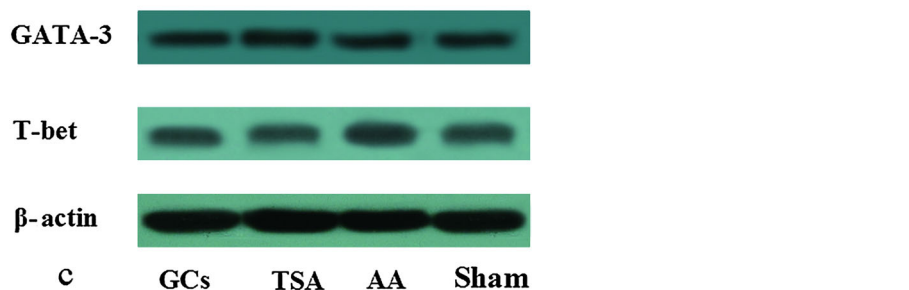


Figure 3 GATA-3 (a) and T-bet (b) expression normalized to β -actin expression (c) in lung tissues of adoptive transfer mice analysed by western blotting. The Student–Newman–Keuls test was used for multiple group comparisons. AA, anacardic acid; GCs, glucocorticoids; TSA, trichostatin A.

The protein expression of HDAC-7 and HDACs 9–11 is shown in Figure 4. HDAC-7 was significantly upregulated in the GCs and AA groups compared with the sham (both $P < 0.01$) and TSA (both $P < 0.01$) groups. Similarly, the expression of HDAC-9 was significantly higher in the GCs and AA groups than in the sham (both $P < 0.05$) and TSA (both $P < 0.05$) groups. The expression of HDAC-10 was significantly higher in the GCs and AA groups than in the sham group (both $P < 0.05$). HDAC-11 was significantly upregulated in the GCs group compared with the sham group ($P = 0.028$).

There were no statistically significant differences in the expression of HDACs 1–6 and HDAC-8 between groups (all $P > 0.05$).

AEII and lung histopathology

Alveolar eosinophilic inflammation index was significantly lower in the GCs (0.66 ± 0.11) and AA groups (0.69 ± 0.26) than in the sham group (1.02 ± 0.14) ($P = 0.003$ and $P = 0.049$, respectively), whereas it remained unchanged in the TSA group (0.95 ± 0.31) ($P > 0.05$).

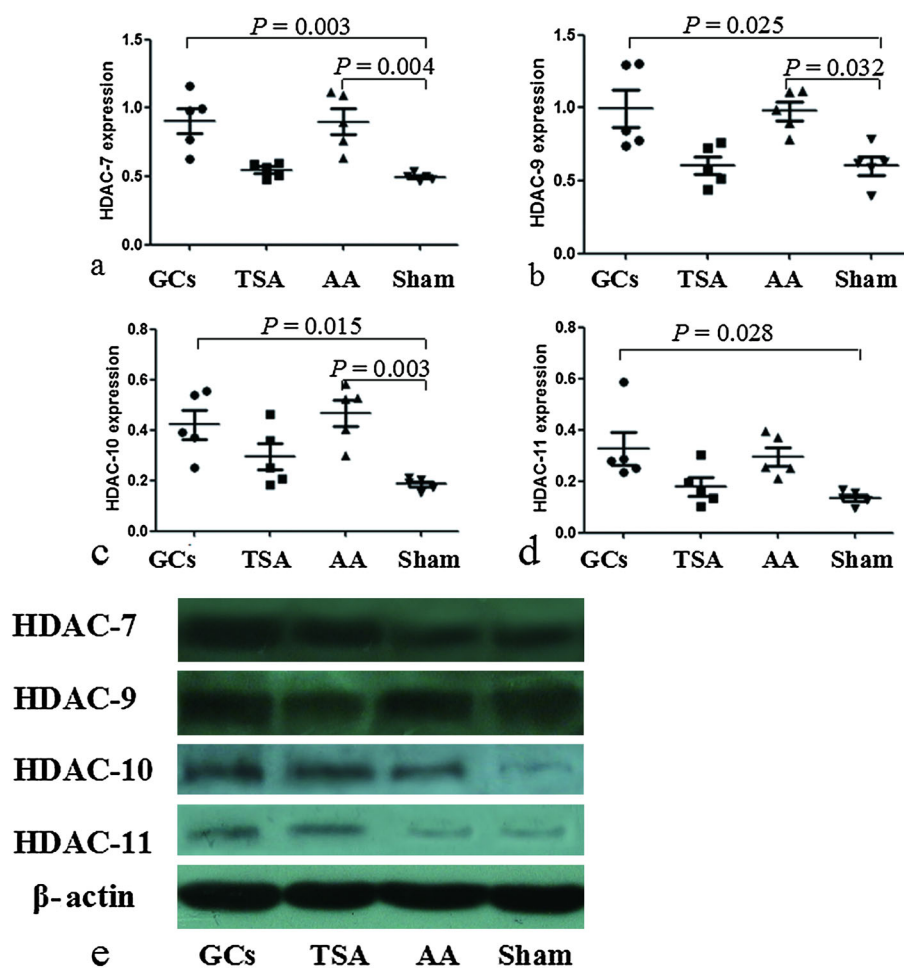


Figure 4 Expression of histone deacetylases (HDACs) 7 (a), 9 (b), 10 (c) and 11 (d) normalized to β -actin expression (e) in lung tissues of mice after adoptive transfer, as analysed by western blotting. The Student–Newman–Keuls test was used for multiple group comparisons. AA, anacardic acid; GCs, glucocorticoids; TSA, trichostatin A.

Lung inflammation in cryostat sections of lung tissues was suppressed in the GCs and AA groups. The pathological morphology of lung tissue in the sham group was characterized by wide interstitial spaces, congestion and edema, increased eosinophil infiltration and mononuclear cell and lymphocyte infiltration (Fig. 5a). In the TSA group (Fig. 5c), the pattern of lung tissue inflammation was similar to that in the sham group, whereas it was improved in the GC (Fig. 5b) and AA (Fig. 5d) groups.

Relationships between cytokines, T-bet/GATA-3, HDACs and AEII

The cytokine profile was similar between cell culture supernatants and the BALF of adoptive transfer mice. The HDAC/HAT activity ratio was negatively correlated with IL-4, IL-5 and IL-13 concentrations in both cell culture supernatants and BALF (all $P < 0.05$). HDAC expression in lung tissues was related to cytokines in BALF (Supplementary Figure S3), and these results are shown in Supplementary Appendix S2. AEII was positively correlated with IL-4 ($r = 0.57$, $P = 0.05$) and IL-13 in cell culture ($r = 0.61$, $P = 0.037$), IL-5 in BALF ($r = 0.59$, $P = 0.044$) and GATA-3 expression in lung tissue ($r = 0.63$, $P = 0.028$), and AEII had a significantly

negative relationship with HDACs 2, 3, 5, 6 and 10 (all $P < 0.05$).

DISCUSSION

Allergic asthma often arises early in life and persists after repeated encounters with an allergen. In the present study, we showed that histone hyperacetylation of Tm cells contributed to eosinophilic airway inflammation in a mouse model of asthma. This effect was reversed by GCs, which alleviated AEII by downregulating IL-4, 5 and 13, similar to the effect of AA. Meanwhile, upregulation of HDACs 7, 9, 10 and 11, especially HDAC-10, maybe involved in the epigenetic mechanism by which GCs improved eosinophilic airway inflammation triggered by Tm cells in mice.

Protein acetylation is a post-translational modification that is modulated by the activity of HDACs and HATs. Ito *et al.*^{20,21} found reduced HDAC and increased HAT activity in bronchial biopsies and bronchoalveolar lavage (BAL) macrophages from asthma subjects compared with those in healthy controls. Guo *et al.*²² suggested that epigenetic studies should focus on specific cell types directly affected at the epigenetic level by environmental exposure. The allergen-

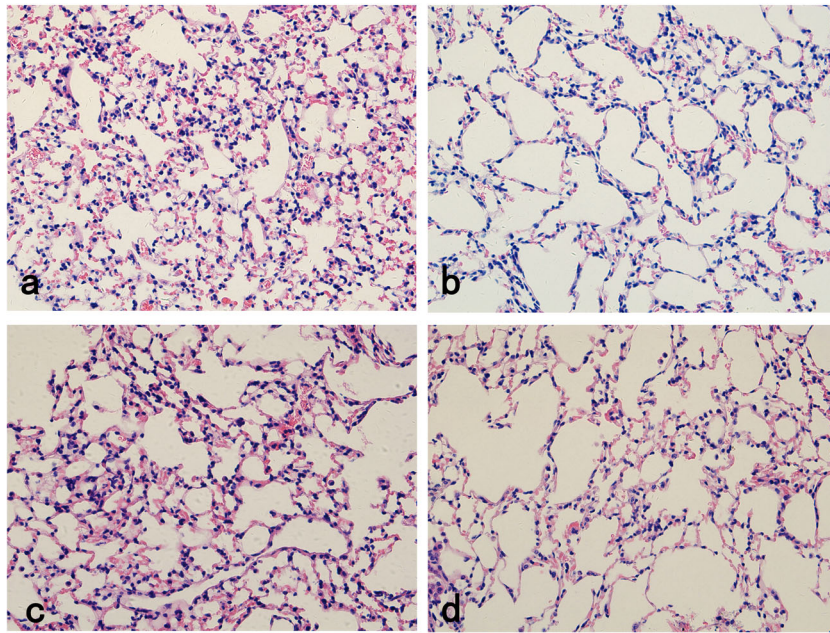


Figure 5 Pathological morphology of lung tissues in the sham (a), glucocorticoids (b), trichostatin A (c) and anacardic acid (d) groups after adoptive transfer of memory T cells to naïve mice. $n = 5$ for each group, magnification 400 $\times$.

specific Tm immune responses examined in the present study provide an illustrative example.

To examine the histone acetylation status in Tm cells, HDAC and HAT inhibitors were used. AA, at a working concentration of 50 μM ,²³ successfully reduced HAT activity by 37.9%, whereas TSA at the recommended working concentration of 50 nM²³ decreased HDAC activity by only 16.1% compared with the sham group. This suggests a histone hyperacetylation status in OVA-specific Tm cells caused by low HDAC and high HAT activity, which increases the accessibility of binding sites for transcription factors and activates gene expression.²⁴ The TSA dose and timing of administration, apoptosis induced by TSA and differences in the *in vivo* and *in vitro* interventions or specific cell types may explain the differential effects of TSA on these parameters of airway inflammation.²⁵ Although our results were in agreement with those of previous studies²⁶ showing that TSA significantly increased the concentrations of IL-4, 5 and 13 and reduced the IFN- γ level, we unexpectedly found that TSA treatment inhibited HAT activity, as shown in Figure 2. Despite limited data on the effect of TSA on HAT activity, there is a balance between HAT and HDAC activities in cells, organisms and humans that is mediated through a feedback mechanism.²⁰

The results of the present study suggest the presence of associations between individual HDACs and cytokines and T-bet/GATA-3. HDACs 7, 9 and 10 were differentially expressed in the AA and sham groups, whereas none of the HDACs showed differential expression between the TSA and sham groups. This indicates that HDACs 7, 9 and 10 may play an important role in maintaining the OVA-specific polarized status of Tm cells, although their contribution to airway inflammation differs.²⁷ Furthermore, HDACs 7, 9, 10 and 11 could be the targets of GCs and mediate their

effect on improving histone hyperacetylation triggered by allergen-specific Tm cells, which has not been reported previously. Recent studies showed that HDAC-7 is a TCR signal-dependent regulator of differentiation,^{28,29} and dephosphorylation leads to its accumulation in the nucleus and the downregulation of genes encoding key cytokines such as IL-4, 5 and 13. However, these results need to be further confirmed.

Ito *et al.*²⁰ was the first to show that HDACs 1 and 2 are downregulated in subjects with asthma. A recent study showed that mice with HDAC-1-deficient T cells develop allergic airway inflammation.³⁰ A study of cross-talk between GC receptors and HDACs suggested that HDAC-2 messenger RNA (mRNA) is decreased in BAL cells from patients with steroid-resistant asthma.³¹ However, Butler *et al.*³² found no downregulation of HDACs 1 and 2 mRNA and protein in endobronchial biopsies from a large series of patients with severe asthma compared with normal controls. Other factors such as cigarette smoke, oxidative stress, or neutrophilic airway inflammation could explain the decrease in HDAC-2.^{33–35} In the present study, HDACs 1 and 2 were downregulated after exposure to TSA, although the difference did not reach statistical significance.

In the present study, we showed that AA had a similar effect to that of GCs on eosinophilic airway inflammation triggered by OVA-specific Tm cells, and this has not been reported previously. AA suppressed HAT activity and alleviated airway inflammation and hyperacetylation, resulting in the inhibition of the production of cytokines such as IL-4, 5 and 13. However, further investigation is necessary to elucidate the mechanism underlying the effect of AA or its derivatives before it can be used as a novel therapeutic agent for asthma.

The present study had several limitations. Firstly, HAT and HDAC activities were not assessed in

adoptively transferred cells *in vitro*, although it is now evident that epigenetic changes induced by environmental exposure may alter the epigenome of the germline and persist through generations.³⁶ Secondly, airway hyper-responsiveness was not detected in the adoptive transfer mice. Thirdly, we did not perform chromatin immunoprecipitation (ChIP) assays to identify the target genes of histone acetylation/deacetylation in Tm cells. Analysis of HDAC 7, 9, 10 or 11-deficient mice or T cells is necessary to confirm our results.

In conclusion, histone hyperacetylation status induced by low expression of HDACs 7, 9 and 10 in allergen-specific Tm lymphocytes contributes to eosinophilic airway inflammation. Upregulation of HDACs 7, 9, 10 and 11, especially HDAC-10, may play a role in the epigenetic mechanisms underlying the effect of GCs on improving airway inflammation in mice. Future studies should assess the potential of HDACs and AA as novel therapeutic agents or targets for the treatment of allergic asthma.

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Supplementary Information

Additional Supplementary Information can be accessed via the *html* version of this article at the publisher's website:

Appendix S1 Methods

Appendix S2 Results

Figure S1 Isolation and purification of CD4⁺CD45RB^{low} cells (T_H cells) by magnetic beads and flow cytometry.

Figure S2 The concentrations of IL-4 (a), IL-5 (b), IL-13 (c) and IFN- γ (d) (pg/mL) in BALF, and the HDAC (e) (μ M/ μ g), HAT activity (f) (μ M/10 μ g) and HDAC/ HAT in lung tissues in the naïve group and OVA-challenged group.

Figure S3 Relationships between the expression of individual HDACs in lung tissues and cytokines (IL-4, IL-5, IL-13, and IFN- γ) in BALF.

SUPPLEMENTARY INFORMATION

Association between histone hyperacetylation status in memory T lymphocytes and allergen-induced eosinophilic airway inflammation

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APPENDIX S1-METHODS

Isolation and purification of CD4+CD45RB^{low} T cells

CD4⁺ T lymphocytes were obtained from the spleen using magnetic beads, and CD4+CD45RB^{low} T cells were isolated and purified by flow cytometry as described previously^{18,19}. In brief, mice were euthanized 24h after the last OVA challenge and the spleens were obtained under sterile conditions. Lymphocytes were isolated from the spleen by EZ-Sep™ Mouse 1× lymphocyte separation (Dakewei Biotech Company, Shenzhen, China), and CD4⁺ T lymphocytes were negatively selected using magnetic activated cell sorting (Invitrogen Dynal, USA) according to the manufacturer's instructions. CD4+CD45RB^{low} T cells were obtained by flow cytometry as previously described.^{7, 9} Antibodies for flow cytometry were FITC-conjugated anti-CD4 (Clone GK1.5; BD Pharmingen™, USA) and PE-conjugated anti-CD45RB (Clone 16A; BD Pharmingen™, USA). Cells were sorted on a FACSVantage SE cell sorter (Beckman-Coulter Company, USA) (Figure S1).

Adoptive transfer and challenge, bronchoalveolar lavage, and ELISA

Cultured CD4⁺CD45RB^{low} cells in each group were collected by centrifugation, resuspended in 250 μ L of PBS, divided into five equivalent parts, and adoptively transferred to five mice at 0.8×10^5 cells per well. Adoptive transfer was performed as previously described^{18,19}. In brief, the adoptive transfer of CD4⁺CD45RB^{low} cells into the lungs of anaesthetized and tracheotomized naïve mice was performed using a 1mL syringe, and animals were challenged for 30 min with OVA 24 h after adoptive transferring. Bronchoalveolar lavage (BAL) was performed 24 h after the last challenge. The left lungs were lavaged three times as previously described.¹⁸ BAL fluid (BALF) was collected by centrifugation and kept at -80°C until analysis by ELISA for IL-4, IL-5, IL-13, and IFN- γ levels (pg/mL).

Lung tissue pathology and alveolar eosinophilic inflammation index

Cryostat sections (4 μ m) of the tissues were cut and air-dried. Morphological observation was performed after hematoxylin and eosin staining in each group, focusing on asthma-like inflammatory features of the mice in each slice under $\times 400$ magnification. Fixed slides were air-dried, wrapped in aluminum foil, and stored at -80°C until used. The eosinophilic inflammation intensity in the alveolar area was calculated as the number of eosinophils (EOS) per alveolar area. In each group of mice, three slices were randomly selected, and three alveolar regions in each slice were analyzed under a high magnification microscope (40/0.7), trying to avoid blood vessels and bronchioles. The number of EOS in each zone was counted and the area of each alveolar region was determined using Image-Pro Plus 4.5 software (Media Cybernetics). The sum of the total area and the total amount of EOS in each group were used to calculate the average density of EOS in each group, which represented the eosinophilic inflammation intensity. The alveolar eosinophilic inflammation index (AEII) was calculated as the inflammation intensity ratio of each group to that of naïve mice. The eosinophilic inflammation index and measures of lung inflammation in the cryostat sections were obtained by a reader who was blinded to the experimental condition.

HDAC and HAT activities

HDAC and HAT activities in right upper lung tissues after adoptive transfer were measured using colorimetric activity assay kits (BioVision Inc., Mountain View, CA) according to the manufacturer's protocol. Briefly, the HDAC colorimetric substrate was incubated with 80µg of total nuclear extracts from lung tissues for 60 min at 37°C. Following the incubation, the lysine-developer solution was added to generate a measurable chromophore that was analyzed using an ELISA plate reader at 405 nm. HeLa nuclear extracts (4 µg) were used as the positive control. The HDAC inhibitor (Trichostatin, TSA) was used to demonstrate the specificity of deacetylation activities. The HAT activity assay was performed using active nuclear extract as a positive control and acetyl-CoA as a cofactor. The total nuclear extracts (50 µg) were incubated with acetyl-CoA at 37°C for 120 min. Acetylation of the peptide substrate by active HAT released the free form of CoA, which served as an essential coenzyme for producing NADH. NADH can easily be detected spectrophotometrically based on its reaction with a soluble tetrazolium dye. The activity was analyzed using an ELISA plate reader at 440 nm.

Western blot analysis of T-bet, GATA-3, and HDACs

The right upper lung tissues were homogenized in the presence of protease inhibitors, and protein concentrations were determined using NE-PER[®] nuclear and cytoplasmic extraction reagents (Pierce Biotechnology, Rockford). The total proteins (50 µg) were loaded onto an SDS-PAGE gel and electrophoresed at 120 V for 90 min, and separated proteins were transferred to polyvinylidene difluoride membranes by the wet transfer method (250mA for 90 min). Nonspecific sites were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 for 1 h, and the blots were then incubated overnight at 4°C with anti-GATA-3 antibody, anti-T-bet antibody (Santa Cruz Biotechnology, Inc.), or anti-HDAC-(1-11) antibodies (HDAC Family Antibody Set, BioVision, USA). Anti-rabbit horseradish peroxidase conjugated IgG was used to detect antibody binding. Membranes were stripped and reblotted with an anti-actin antibody (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific antibody was

visualized by exposure to photographic film. The density of T-bet, GATA-3, or HDAC 1-11 stained bands relative to β -actin expression was quantified with Quantity ONE densitometry software (PDI Image ware Systems, Huntington Station, NY, USA).

APPENDIX S2-RESULTS

Tm cells were characterized by histone hyperacetylation status

The concentrations of IL-4, IL-5, and IL-13 (pg/mL), and HAT activity ($\mu\text{M}/10\mu\text{g}$) were significantly higher in the OVA-challenged group than in the naïve group whereas HDAC activity ($\mu\text{M}/\mu\text{g}$) and the ratio of HDAC to HAT activity was lower. IFN- γ levels did not differ significantly between the two groups (Figure S2). These results indicated that Tm cells were characterized by histone hyperacetylation status.

Relationships between cytokines, T-bet/GATA-3, HDACs, and AEII

The cell culture supernatants and BALF of adoptive transfer mice showed similar cytokine profiles. The HDAC/HAT activity ratio was negatively correlated with IL-4, IL-5, and IL-13 levels in both cell culture supernatants and BALF (all $P<0.05$). A relationship between HDAC expression in lung tissues and cytokines in BALF was observed (Figure S3). HDACs 3 ($r=-0.64$, $P=0.024$), 5 ($r=-0.71$, $P=0.009$), 6 ($r=-0.59$, $P=0.045$), 7 ($r=-0.80$, $P=0.002$), 9 ($r=-0.73$, $P=0.007$), 10 ($r=-0.62$, $P=0.033$), and 11 ($r=-0.59$, $P=0.045$) were negatively correlated to IL-4; HDACs 5 ($r=-0.83$, $P<0.001$), 6 ($r=-0.60$, $P=0.04$), 7 ($r=-0.72$, $P=0.009$), 9 ($r=-0.76$, $P=0.004$), 10 ($r=-0.63$, $P=0.029$), and 11 ($r=-0.72$, $P=0.008$) were negatively correlated to IL-5; and HDACs 1 ($r=-0.67$, $P=0.018$), 5 ($r=-0.68$, $P=0.014$), 7 ($r=-0.76$, $P=0.004$), 9 ($r=-0.70$, $P=0.011$), and 11 ($r=-0.72$, $P=0.008$) were negatively correlated to IL-13. HDAC-9 was positively correlated with IFN- γ ($r=0.67$, $P=0.017$).

Figure S1. Isolation and purification of CD4⁺CD45RB^{low} cells (Tm cells) by magnetic beads and flow cytometry. a. Lymphocytes (P1); b. CD4⁺ T lymphocytes (P2); c. CD4⁺CD45RB^{low} cells (P3), CD4⁺CD45RB^{high} cells (P5); d. Proportion of these cells in each group.

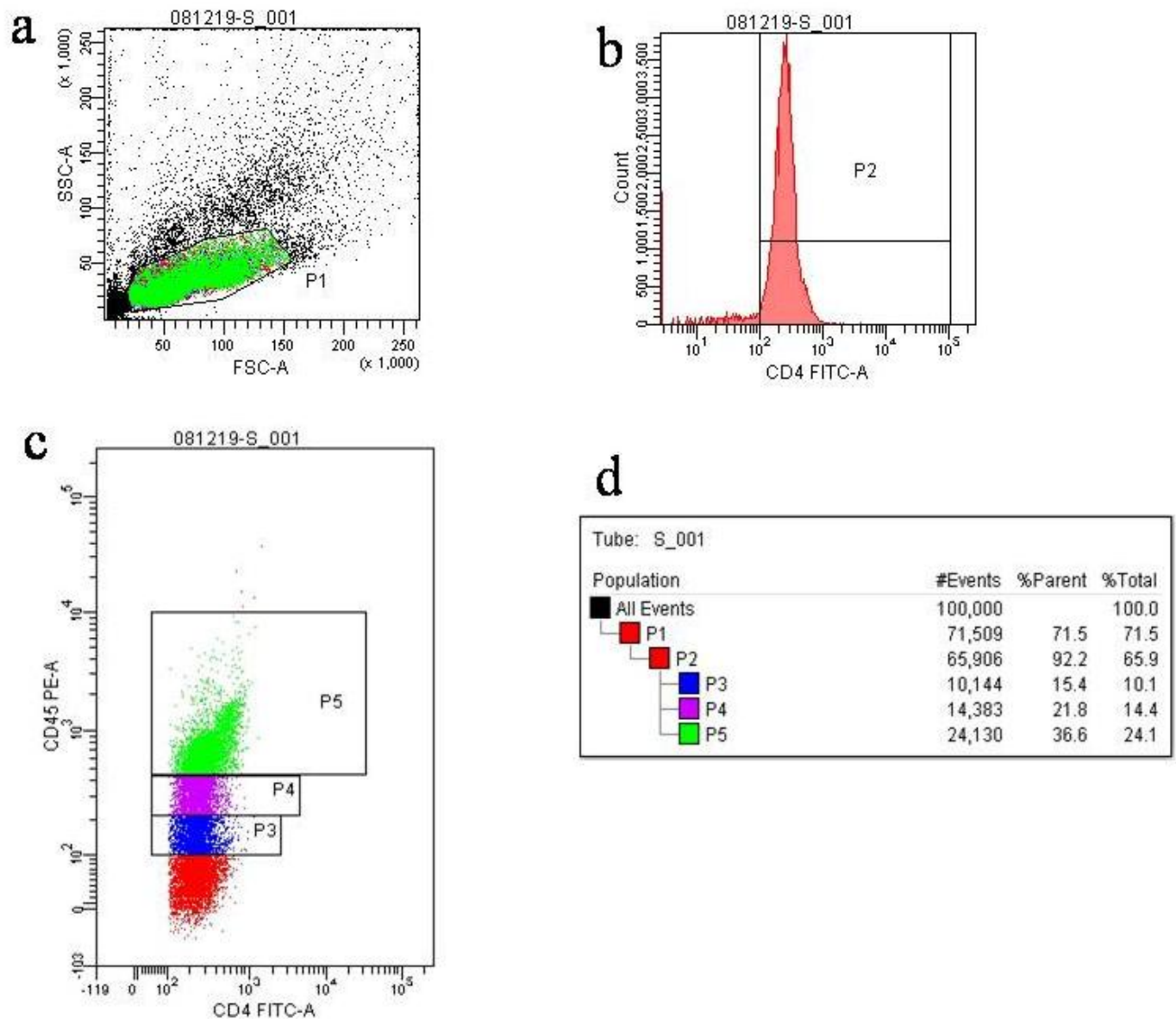


Figure S2. The concentrations of IL-4 (a), IL-5 (b), IL-13 (c) and IFN- γ (d) (pg/mL) in BALF, and the HDAC (e) ($\mu\text{M}/\mu\text{g}$), HAT activity (f) ($\mu\text{M}/10\mu\text{g}$) and HDAC/ HAT in lung tissues in the naïve group and OVA-challenged group.

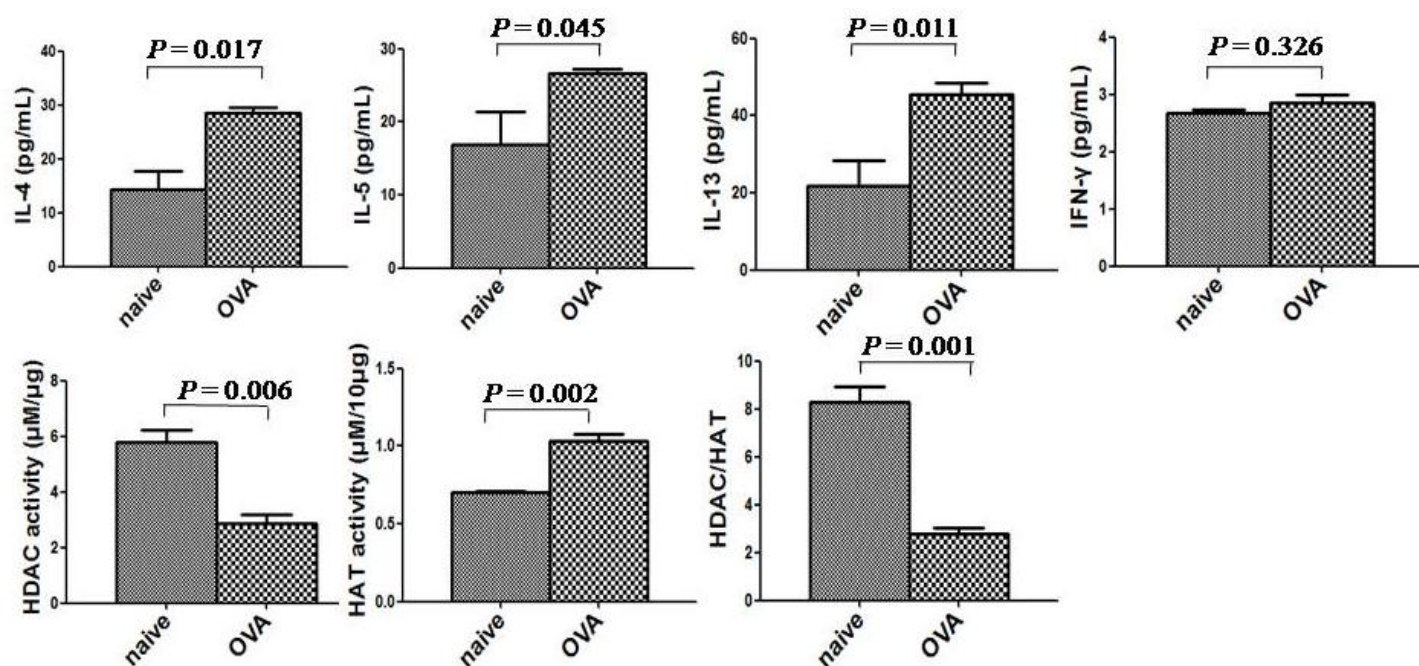


Figure S3. Relationships between the expression of individual HDACs in lung tissues and cytokines (IL-4, IL-5, IL-13, and IFN- γ) in BALF. The correlation analysis was performed by Spearman correlation.

