

PM_{2.5} exposure and cold stress exacerbates asthma in mice by increasing histone acetylation in *IL-4* gene promoter in CD4⁺ T cells

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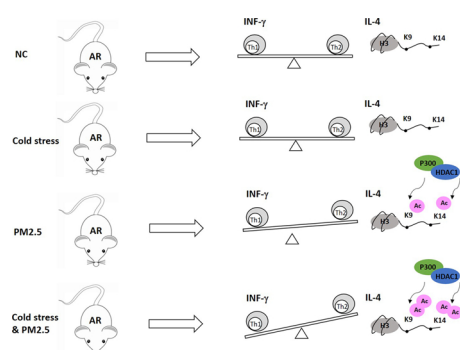
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GRAPHICAL ABSTRACT



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ABSTRACT

Asthma is a common chronic inflammatory disease which severely reduces the quality of life in patients. Studies have demonstrated that both PM_{2.5} and cold stress contribute to the development of asthma. However, the combined effects of these two risking factors are unknown. In this study, we investigated the combined effects of PM_{2.5} exposure and cold stress (PMCS) on asthma, as well as the underlying mechanisms by using a murine model. After different exposures, the immune-pathological changes and redox states in groups were evaluated. Besides, the balance of T_H1/T_H2 cells and the acetylation levels of H3K9 and H3K14 in *IL-4* gene promoter were detected. Our results showed that, compared with other exposures, PMCS led to an increased inflammation and redox levels in mice. It also significantly increased the percentage of T_H2 T cells, which was correlated with hyperacetylation of H3K9 and H3K14 in *IL-4* gene promoter in CD4⁺ T cells. Furthermore, a significantly increased P300 and decreased HDAC1 were detected in CD4⁺ T cells in PMCS group. In conclusion, our findings

Abbreviations: PM_{2.5}, particulate matter ≤ 2.5 μm; PM, PM_{2.5} exposure; CS, cold stress; PMCS, PM_{2.5} exposure and cold stress; NC, negative control; OVA, ovalbumin; Shanghai-METAS, Shanghai Meteorological and Environmental Animal Exposure System; BALF, bronchoalveolar lavage fluid; CAT, catalase; GSH, glutathione; MDA, malonaldehyde; SOD, superoxide dismutase; CHIP, chromatin immunoprecipitation assay; HAT, histone acetylase; HDAC, histone deacetylase

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demonstrated that PMCS exacerbated asthma in mice by increasing H3K9 and H3K14 acetylation in *IL-4* gene promoter in CD4⁺ T cells, and P300 and HDAC1 might contribute to their combined effects.

1. Introduction

Asthma is a heterogeneous chronic inflammatory disorder of the airways which is characterized by recurring episodes of airflow obstruction, coughing, wheezing, and shortness of breath (Ho, 2010). According to a global burden of disease study in 2015, an estimated 358 million people worldwide were affected by asthma, and its prevalence was still increasing (Abajobir et al., 2017; Collaborators, 2017). Asthma severely affects patients' quality of life and even threatens their lives. Moreover, it brings a lot of social-economic burdens to the patients and their families (James and Lyttle, 2016; Lin, 2003). Studying related factors to asthma and their underlying mechanisms that contribute to the development of asthma may offer new insights into this disease, further a better understanding of it.

Asthma has been reported to be associated with multiple factors, which include genetics, sex, age, family history, socioeconomic status, area of residence, race, maternal smoking, and many out-house and in-house environmental factors (Bijanazadeh et al., 2011; Mukherjee and Zhongjian, 2011; Richard et al., 2015). Immune cell dysfunction and airway hypersensitization are thought to be closely associated with the pathogenesis of allergic asthma (Liu et al., 2013). Asthma has classically been considered as the typical T_H2 disease, with increased circulating IgE level and eosinophilic inflammation. However, other effector T cell subsets (i.e. T_H17, T_H9 T_H22 and Treg) were reported to play a role in different asthmatic phenotypes recently (Clare and Edith, 2010; Lloyd and Saglani, 2013). Besides, a diverse range of factors, including oxidative stress and nutritional factors (such as vitamin D), were also been reported to be involved in the development of asthma (Ahmad et al., 2003; Moskwa et al., 2018; Shuk-Mei, 2010). In recent years, numerous studies have addressed the role of epigenetic regulation following environmental exposures in asthma (Feng et al., 2016; Ji and Hershey, 2012; Liu et al., 2013). Many genes related to immunology and associated with asthma, including interferon (IFN)- γ , interleukin (IL)4, IL17, and forkhead box P3 (FOXP3), have been reported to be susceptible to epigenetic regulation following environmental exposures (Stephanie and Miller, 2012). Epigenetic regulations, including DNA methylation and histone modification, at T cell cytokine locus control regions largely determine the commitment of T cell toward an allergic phenotype during the activation of T cells.

PM_{2.5} (particulate matter of diameter <2.5 μ m) has been reported to be one of the most important asthma related environmental factors (D'Amato et al., 2010; Feng et al., 2016). Cold temperature was also considered as a risk factor for asthma. Of note, it has been showed that season and temperature had some influence on the relationship of particulate matter to asthma emergency room visits (Lisa Buettner et al., 2008), suggesting that PM_{2.5} exposure and cold stress (PMCS) might have combined effects on asthma.

In the present study, by using a murine asthma model, we aimed to determine the combined effects of PMCS on asthma, as well as its underlying mechanism.

2. Material and methods

2.1. Mice

Eighty male BALB/c mice of approximately 8-week-old with body weight of 20 to 25 g, which were purchased from Shanghai Laboratory Animal Center, Shanghai, China, were maintained in a specific pathogen-free (SPF) environment and given ovalbumin (OVA)-free food and water in this study. The study was approved by the animal research

ethics board of the Shanghai Meteorological Bureau, Shanghai, China.

These mice were randomly divided into four groups (n = 20), which were exposed to different conditions: (i) Negative control (NC) group: filtered air exposure and room temperature (21–23 °C), (ii) Cold stress (CS) group: filtered air exposure and cold stress (2–4 °C), (iii) PM_{2.5} exposure (PM) group: 400 μ g/m³ ambient PM_{2.5} and room temperature (21–23 °C), (iiii) PM_{2.5} exposure and cold stress (PMCS) group: 400 μ g/m³ ambient PM_{2.5} and cold stress (2–4 °C).

2.2. OVA sensitization, antigen challenge, PM_{2.5} exposure and cold stress

Asthma model was established as previously described (Sook et al., 2004). Briefly, on day 1, mice were given intraperitoneal injection of 200 μ l phosphate-buffered saline (PBS) containing 20 μ g ovalbumin (OVA, Sigma Chemical Co, St Louis, Mo) and 2 mg Al(OH)₃ to induce primary immunity. On day 7 and 14, these mice were given two identical booster injections. On day 28–30, these mice were given aerosolized antigen challenge with 5% OVA (wt/vol in PBS) for 30 min daily.

The animal exposures to ambient PM_{2.5} and cold stress were conducted in the “Shanghai Meteorological and Environmental Animal Exposure System (Shanghai-METAS)” (patent #201510453600.8), which is located in the School of Public Health at Fudan University in Xujiahui District in Shanghai, where most of ambient PM_{2.5} is attributed to traffic exhausts. The animal housing parameters, such as temperature, 60% humidity and 12-h light/dark cycle, were automatically adjusted in the “Shanghai-METAS”. The concentrated PM_{2.5} in the exposure system of “Shanghai-METAS” was generated using a versatile aerosol concentration enrichment system (VACES) which has been published previously (Li et al., 2019). Following the drug sensitization and challenge, mice were exposed in different conditions for 8 h per day, 7 days per week for 4 weeks.

2.3. Bronchoalveolar lavage (BAL)

Twenty-four hours after the last OVA/PBS challenge, all mice were anesthetized with pentobarbital. BAL was performed as previously described (Sook et al., 2004; Yang et al., 2018), and bronchoalveolar lavage fluid (BALF) specimens were collected, centrifuged at 1500g for 10 min at 4 °C, and the supernatants were immediately frozen at –80 °C for biochemical examinations later on, while the cell sediments were processed immediately.

2.4. Biochemical assay

100 μ l Peripheral blood from caudal vein was collected for blood routine examination via a biochemical analyzer (Beckman coulter, USA). 200 μ l BALF was used to detect the levels of catalase (CAT), glutathione (GSH), malonaldehyde (MDA) and superoxide dismutase (SOD) by using enzyme-linked immunosorbent assay (ELISA) kits (R&D, USA) in accordance with the given instructions.

2.5. Determination of OVA-specific IgE

Serum OVA-specific IgE levels were measured by ELISA (Mouse OVA-sIgE ELISA Kit, AMEKO Co., Shanghai, China) according to the manufacture's instruction.

2.6. Measures of T_H1/T_H2 cells

CD4 + T cells, which were isolated from BALF by AutoMACS (Miltenyi Biotec), were used to determine T_H1/T_H2 cells. Intracellular staining was performed according to the manufacturer's instructions. Briefly, following 6 h stimulation (Cell Stimulation Cocktail, eBioscience), cells were stained with FITC-conjugated anti-CD4 antibody for surface marker staining. After that, the cells were fixed, permeabilized, and stained with APC-conjugated anti-IFN- γ and PE-conjugated anti-IL-4 antibodies (ebioscience) for intercellular cytokine staining. FlowJo 10 was used for further image processing and data analysis.

2.7. Analysis of BALF and hematoxylin and eosin (HE) staining

BALF cell pellet was resuspended in RPMI 1640 medium, then the total number of cells was counted. Slides for differential cell counts were prepared with cytospin and stained with Diff-Quik. After that, the cell number was counted by 2 independent investigators, and the results were averaged.

For HE staining, lung tissues after lavage performance were fixed with 4% paraformaldehyde at room temperature, sedimentated within 20% and 30% sucrose solution 24 h for dehydration, then embedded in optimal cutting temperature (OCT) compound for prechilling, followed by serial histological sections at 8 μ m. The slides were dipped in hematoxylin for 10 min, 0.1% HCl-ethanol for 3 min, 0.5% NH_3H_2O for 30 s, and 0.5% eosin-ethanol for another 30 s. Each step was separated by rinsing with tap water for 5 s. Finally, the sections were treated with 80%–100% ethanol for 5 s, xylene for 5 s twice and neutral balsam for mounting, followed by observation under microscope.

2.8. Chromatin immunoprecipitation assay (CHIP)

Purified CD4 + T cells from lung single cell preparations were treated with 1% formaldehyde for 10 min at room temperature. The cells were then harvested and CHIP assays were performed as previously described (Jing et al., 2012). The antibodies used in CHIP are anti-H3K9 acetylation antibody and anti-H3K14 acetylation antibody (Abcam), and the Primers sequences used for ChIP are as below: 5' primer: 5'-TTGGTCTGATTTCACAGG-3'; 3' primer: 5'-AACATGCAATGCTGGC-3'.

2.9. Western blot

Total proteins were extracted using RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, 0.1% SDS). The total proteins were then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked, then incubated with anti-P300, GCN5 and HDAC1 antibody (Abcam). Finally, immunoreactive bands were detected by ECL reagent (Millipore).

2.10. Statistical analysis

The experimental data was processed with SPSS 20 software, and the results were presented as mean $\pm$ standard error of the mean (SEM) unless indicated otherwise. Statistical analysis was performed by Student's unpaired *t* test. *p* value less than 0.05 was considered statistically significant.

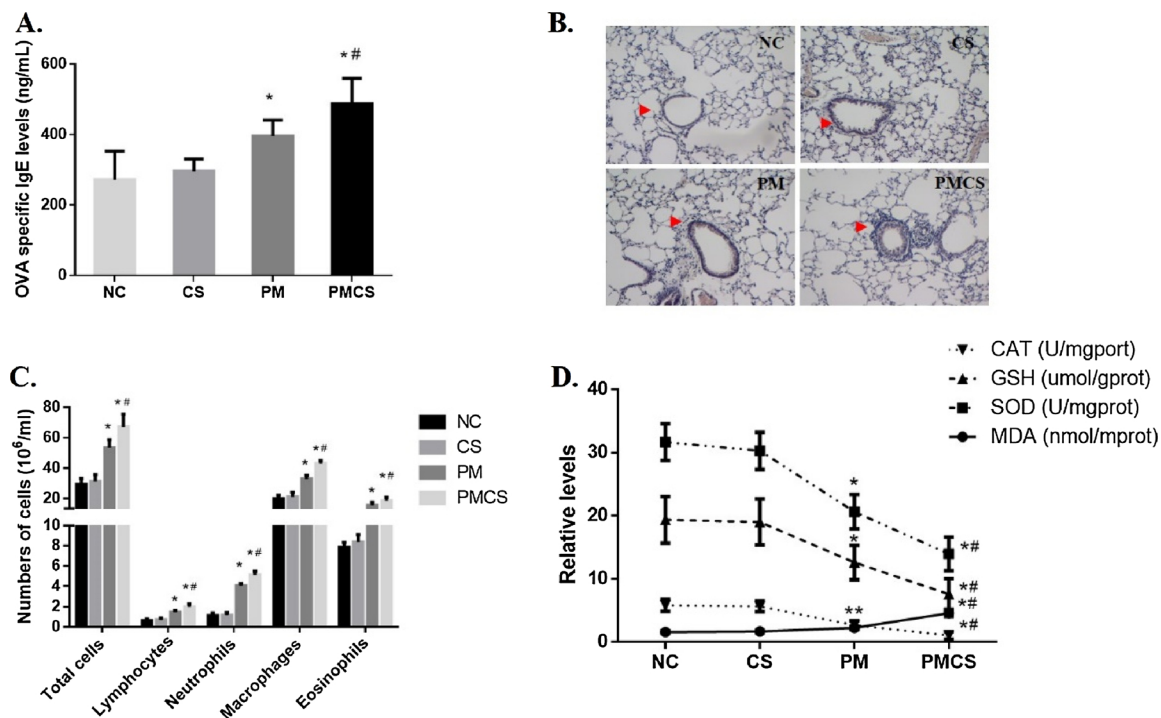


Fig. 1. PM_{2.5} exposure and cold stress (PMCS) exacerbates asthma in mice. (A) Detection of serum ovalbumin (OVA)-specific IgE in the groups by enzyme-linked immunosorbent assay (ELISA). (B) Representative photomicrographs of hematoxylin and eosin (HE) staining of lung tissue in groups. Arrows point to infiltration of inflammatory cells. (C) Detection of the number of total cells, lymphocytes, neutrophils, macrophages and eosinophils in bronchoalveolar lavage fluid (BALF) in groups. (D) Detection of catalase (CAT), glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) in BALF in groups (n = 5). Values are expressed as mean $\pm$ SEM (n = 5). * *P* < 0.05 vs NC group, ** *P* < 0.01 vs NC group, and # *P* < 0.05 vs PM group. n = 5. NC, negative control; CS, cold stress; PM, PM_{2.5} exposure.

3. Results

3.1. PM_{2.5} exposure and cold stress (PMCS) exacerbates asthma in mice

To determine the individual and combined effect of PM_{2.5} exposure and cold stress on asthma, an experimental murine model of asthma was used in this study. The mean concentration of PM_{2.5} was 120–125 µg/m³ in the PM_{2.5} exposure groups, while 1–2 µg/m³ in the filtered air exposure groups. The mean temperature was 22–24 °C in the room temperature groups, while 2–4 °C in the cold stress groups.

As shown in Fig. 1A, compared to NC group, the OVA-specific IgE levels were higher in mice in the PM and PMCS groups, while there was no significant difference between NC and CS group. Furthermore, mice in the PMCS group had significantly higher OVA-specific IgE levels than that in the PM group. Consistently, compared with other groups, increased infiltration of inflammatory cells and more thickened alveolar walls were observed in mice in the PMCS group (Fig. 1B).

We further examined the number of inflammatory cells obtained from BALF. The results showed that, compared with NC group, mice in the PM and PMCS groups had significantly increased total cell numbers, as well as lymphocytes, neutrophils, macrophages and eosinophils. Furthermore, compared with PM group, mice in the PMCS group had significantly increased number of total cells and cell subsets mentioned above (Fig. 1C).

Finally, antioxidant enzyme activities and malondialdehyde (MDA) levels were detected in groups. The results showed that, compared with NC group, mice in the PM and PMCS groups had significantly reduced levels of catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD), but significantly elevated levels of MDA in BALF. Furthermore, compared with PM group, mice in PMCS group had significantly decreased levels of CAT, GSH and SOD, but significantly increased levels of MDA in alveolar lavage (Fig. 1D).

3.2. PMCS skews the polarization of T_H1/T_H2 balance toward T_H2 T cells

Because the polarization of T_H1/T_H2 balance toward T_H2 T cells has

been demonstrated to be tightly related with the development of asthma, the percentage of IFN-γ-producing CD4⁺ cells (T_H1) and IL-4-producing CD4⁺ cells (T_H2) from BALF in different groups were determined in this study. As shown in Fig. 2, compared with NC group, mice in the PM and PMCS groups had significantly decreased T_H1 cells, but significantly increased T_H2 cells. Furthermore, compared with PM group, mice in the PMCS group had significantly decreased T_H1 cells, but significantly increased T_H2 cells.

The concentration of IL-4 in BALF was also measured in this study. The results showed that, compared with NC group, IL-4 levels were significantly increased in PM and PMCS groups. Furthermore, compared with PM group, IL-4 levels were also significantly increased in PMCS group.

3.3. PMCS increases H3K9 and H3K14 acetylation in IL-4 gene promoter region

Histone acetylation is usually considered to be associated with active chromatin and further activation of the gene expression. Two histone markers, including acetylation of histone H3K9 (H3K9ac) and K14 (H3K14ac) in IL-4 gene promoters in lung CD4⁺ T cells, were examined in this study. As shown in Fig. 3, compared with NC group, mice in the PM and PMCS groups had significantly increased H3K9ac and H3K14ac in IL-4 gene promoter. Furthermore, compared with PM group, mice in the PMCS group had significantly increased H3K9ac and H3K14ac in IL-4 gene promoter.

3.4. PMCS increases P300, but decreases HDAC1 expression in CD4⁺ T cells

Because P300/CBP and GCN5/PCAF are major histone acetyltransferases responsible for H3K9ac and H3K14ac, we further examined the global expression of P300 and GCN5 in CD4⁺ T cells by Western blot. As shown in Fig. 4, compared with NC group, a significantly increased expression of P300 was observed in mice in the PM and PMCS groups, while GCN5 had no significantly difference between groups.

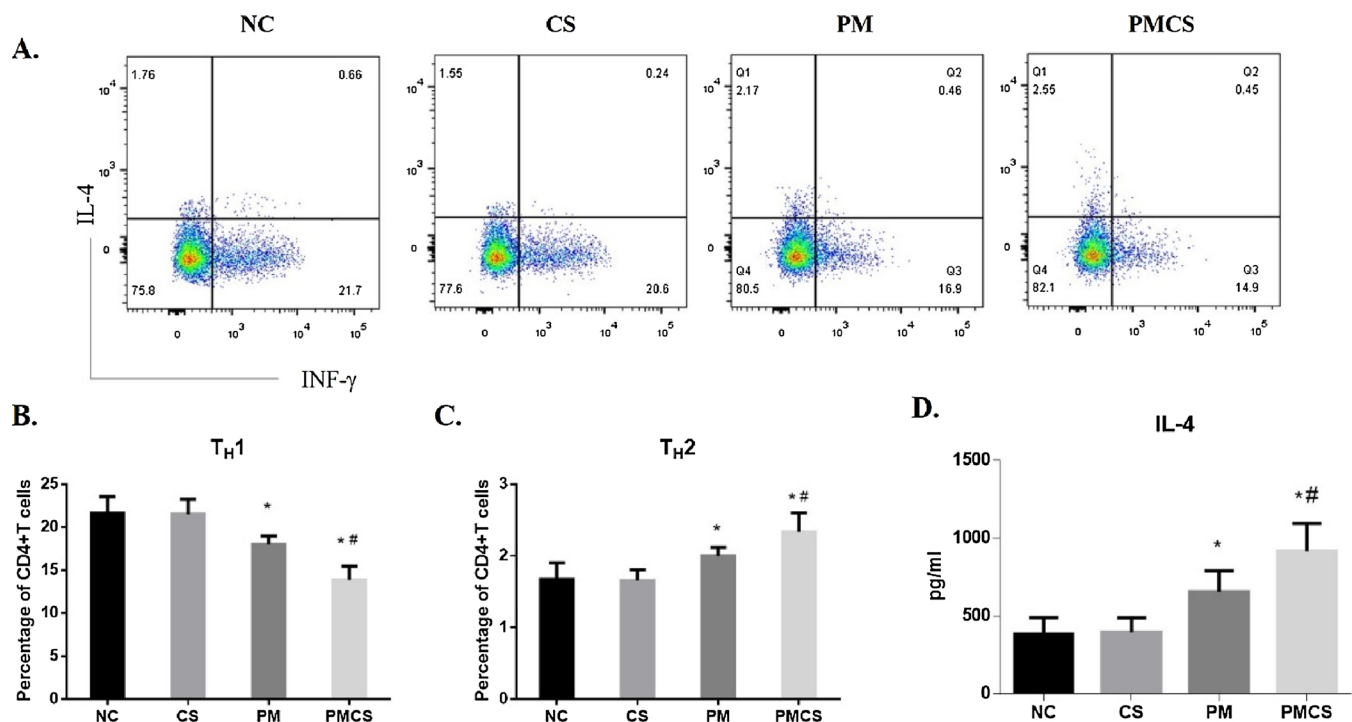


Fig. 2. Effect of PMCS in regulating T_H2 response in asthmatic murine model. (A) Representative flow cytometric plots were shown. Lung cells were stained with intracellular IFN-γ (T_H1) and IL-4 (T_H2) in the groups. (B & C) Percentage of T_H1 and T_H2 in CD4⁺ T cells were expressed as the mean ± SEM (n = 5). (D) Levels of IL-4 in BALF were detected in groups, and results are expressed as the mean ± SEM (n = 5). * *P* < 0.05 vs NC group, and # *P* < 0.05 vs PM group.

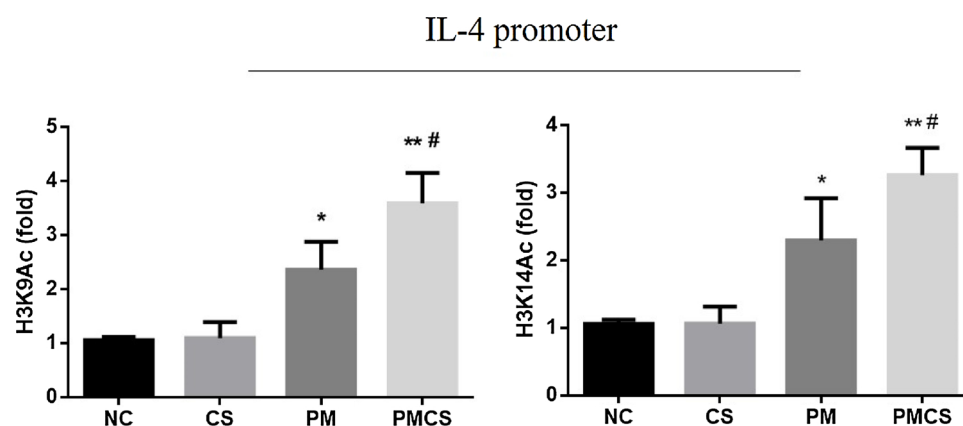


Fig. 3. Enrichment of H3K9 and H3K14 acetylation in *IL-4* gene promoter was detected by Chromatin immunoprecipitation assay (CHIP). * $P < 0.05$ vs NC group, ** $P < 0.01$ vs NC group, and # $P < 0.05$ vs PM group.

Furthermore, compared with PM group, a significantly increased expression of P300 was observed in mice in the PMCS group.

The global expression of HDAC1 in CD4 + T cells was also detected in groups. As shown in Fig. 4, compared with NC group, a significantly decreased expression of HDAC1 was observed in mice in the PM and PMCS groups. Furthermore, compared with PM group, a significantly decreased expression of HDAC1 was observed in mice in the PMCS group.

4. Discussion

Asthma is one of the most common, complex, heterogeneous and chronic airway inflammatory diseases, which includes various clinical manifestations (Alizadeh et al., 2017). More than 100 genes located in every chromosome have been reported to be associated with asthma, and different genes have different susceptibility to environmental influences (Bijanzadeh et al., 2011). However, genetic factors could not fully explain the increasing incidence of asthma in the world in the past decades, more and more attention has been paid to epigenetic regulations in asthma. Genetic and epigenetic regulations are both considered to be involved in the development of asthma (Rosenberg et al., 2014).

Numerous environmental exposures have been reported to have an association with asthma, including maternal and paternal smoking, indoor and outdoor air pollution, house dust mite, occupational exposure, and so on (Rosenberg et al., 2014). Environmental exposure is considered to be one of the most important inducers of epigenetic changes (Ji and Hershey, 2012). These epigenetic alterations affect not only the individuals who are exposed to the environmental factors, but also their offspring. The most well-studied epigenetic mechanisms in asthma are DNA methylation, histone modifications, and small non-coding RNA (Ji and Hershey, 2012). The relationship between PM_{2.5}

and asthma has been well demonstrated over the past years. Some possible mechanisms include initiating and exacerbating cellular inflammation, eliciting oxidative stress, altering epigenetic modifications, and impairing immune responses in the lung (Feng et al., 2016; Ji and Hershey, 2012). Cold stress is also a risk factor for asthma. Cold season was reported to be associated with a higher risk of hospital admissions for asthma compared with warm season (Lisa Buettner et al., 2008), which suggested that cold stress might be associated with asthma. In fact, during a cold season, more PM_{2.5} would be generated because of fossil fuel or biomass burning. Therefore, cold air and PM_{2.5} exposure is usually co-present simultaneously.

To evaluate the combined effects of PM_{2.5} and cold stress on asthma, a classically experimental murine asthmatic model was generated and evaluated in this study. OVA-specific IgE levels and pathological changes were used to evaluate the severity of asthma. As reported before, PM alone exacerbated asthma in the mice in our study. However, CS alone had no effect on the development of asthma. Interestingly, compared with PM alone, the combination of PMCS was showed to exacerbate asthma in mice. Moreover, we examined the number of inflammatory cell numbers and the level of oxidative stress indicators in BALF in different groups. The results further demonstrated the combined effects of PMCS on asthma in mice, in which the increased inflammatory cell numbers and oxidative stress may be one of its underlying mechanisms.

T_H2 T cells, which are predominantly involved in IgE-mediated allergic inflammation, are considered to be the most important pathological contributor to the development of asthma (Lloyd and Saglani, 2013). To determine whether the polarization of T_H1/T_H2 balance towards T_H2 is involved in the combined effect of PMCS on asthma, the percentage of T_H1 and T_H2 cells in CD4 + T cells from BALF in different groups were determined in this study. We found that PM_{2.5} alone

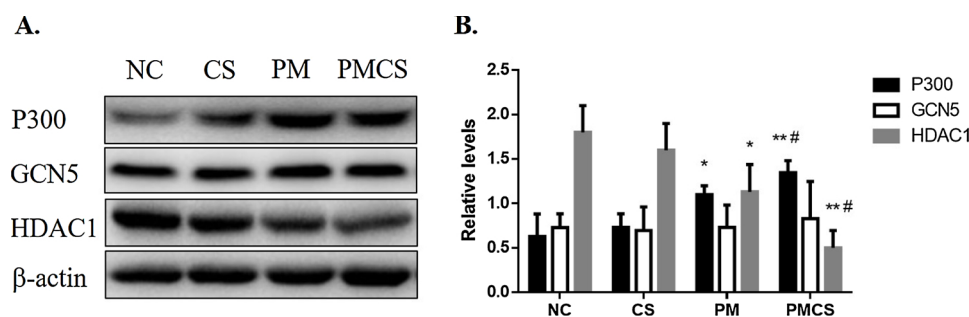


Fig. 4. Expression bands (A) and the analysis (B) of P300, GCN5 and HDAC1 by Western blot assay. * $P < 0.05$ vs NC group, ** $P < 0.01$ vs NC group, and # $P < 0.05$ vs PM group.

decreased T_H1/T_H2 cell ratio while cold stress alone had no such effect. Furthermore, compared with PM group, a more severe polarization of T_H1/T_H2 balance toward T_H2 was observed in mice in the PMCS groups, which indicated that PMCS may exacerbate asthma through skewing the T_H1/T_H2 balance.

Epigenetic modifications, including DNA methylation, histone modification and small noncoding RNA, have been demonstrated to be involved in skewing the T_H1/T_H2 balance in asthma (Liu et al., 2013). Most studies focused on the changes in DNA methylation patterns. For example, the induction of $IFN-\gamma$ transcription was reported to be regulated primarily by DNA demethylation of CpG sites within *IFN-\gamma* gene (Miller and Ho, 2008; Nam-Hee et al., 2008). Histone modification, another important epigenetic modification, is also involved in the regulation of T_H1/T_H2 balance in asthma. Recently, H3K9 acetylation, which is an important epigenetic marker associated with transcriptionally active chromatin, was reported to be regulated by PM. Ding et al. reported that dose-and time-effect responses of DNA methylation and histone H3K9 acetylation changes were induced by traffic-related air pollution (Ding et al., 2017), suggesting that H3K9 acetylation might play a role in traffic-related air pollution-induced lung injuries. To investigate whether H3K9 acetylation is involved in regulating T_H1/T_H2 balance under PMCS, CHIP was performed using anti-H3K9 acetylation antibody in groups. The result showed that H3K9 acetylation was enriched in *IL-4* gene promoter region in PM and PMCS groups. Furthermore, mice in the PMCS group had significantly increased H3K9 acetylation levels in *IL-4* gene promoter region than that in the PM group. Moreover, H3K14 acetylation, another epigenetic marker for active chromatin which was reported to co-occur with H3K9 acetylation at many gene regulatory elements (Karmodiya, 2012), was also evaluated in our study. Our results showed that H3K14 acetylation was also enriched in *IL-4* gene promoter region in PM and PMCS groups, and mice in PMCS group had significantly increased H3K14 acetylation levels in *IL-4* gene promoter region than that in the PM group. These results suggested that PMCS might skew the T_H1/T_H2 balance towards T_H2 through regulating H3K9 and H3K14 acetylation levels in *IL-4* gene promoter region.

There are two classes of enzymes involved in determining the state of histone acetylation, including histone acetylase (HAT) and histone deacetylase (HDAC). The balance of HAT/HDAC regulates protein acetylation levels, further mediating transcriptional regulation in cells (Peserico and Simone, 2011). Because P300/CBP and GCN5/PCAF are major histone acetyltransferases responsible for H3K9ac and H3K14ac (Gajer et al., 2015), we analyzed the expression levels of P300 and GCN5 in groups. We found that P300, but not GCN5, was increased in CD4 + T cells in PM and PMCS groups. Furthermore, mice in the PMCS group had significantly increased P300 expression than that in the PM group. These results suggested that P300, but not GCN5, might play a role in PM and PMCS induced asthma in mice. In addition, the expression levels of HDAC1 were also determined in our study. We found that HDAC1 was decreased in CD4 + T cells in PM and PMCS groups, and the mice in PMCS group had significantly decreased HDAC1 expression than that in the PM group, which indicates that HDAC1 was also involved in PM and PMCS induced asthma in mice. Taken together, our results show that P300 and HDAC1 might play a role in PM and PMCS induced asthma in mice.

Our study also has some limitations. Firstly, only two histone markers were evaluated in our study. Actually, many other histone markers were also reported to be involved in regulating T_H1/T_H2 balance in asthma, which are needed to be evaluated in the further studies. In addition, we did not conduct a rescue study to determine the effect of P300 inhibition or HDAC1 enhancement on PM and PMCS induced asthma, and we plan to have this done in the near future.

In conclusion, we report that PMCS exacerbates asthma in mice by increasing H3K9 and H3K14 acetylation in *IL-4* gene promoter in CD4 + T cells, and P300 and HDAC1 may contribute to their combined effects.

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Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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All the authors have consented to make the data and results available and publish this manuscript.

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