

RESEARCH ARTICLE

Childhood co-exposure of cold stress and PM_{2.5} aggravates the susceptibility and severity of asthma in adulthood of miceJi Zhou^{1,2,3}  | Jianming Xu^{1,2} | Fuhai Geng^{1,2} | Li Peng^{1,2} | Xiaofang Ye^{1,2} | Dandan Yang^{1,2} | Jinzhao Zhao^{1,4}  | Qinghua Sun⁵¹Shanghai Key Laboratory of Meteorology and Health, Shanghai Meteorological Bureau, Shanghai, China²Shanghai Typhoon Institute, CMA, Shanghai, China³Department of Atmospheric and Oceanic Sciences & Institute of Atmospheric Sciences, Fudan University, Shanghai, China⁴Department of Environmental Health, School of Public Health and the Key Laboratory of Public Health Safety, Ministry of Education, Fudan University, Shanghai, China⁵Division of Environmental Health Sciences, College of Public Health, The Ohio State University, Columbus, Ohio

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Abstract

Both cold stress and ambient fine particle particulate matter (PM_{2.5}) has been reported to aggravate and induce respiratory problems like asthma, but the mechanism involved in that has not been fully understood. Therefore, the present study is to explore the mechanism involved in the increased susceptibility and severity of asthma caused by cold stress and PM_{2.5} exposure. Urban PM_{2.5} of Shanghai was concentrated to simulate a PM_{2.5}-polluted environment with an average concentration of 400 µg/m³, where 1-month young C57BL/6J mice were exposed for 2 months under cold stress (2°C). Co-exposure of cold stress and PM_{2.5} in childhood of mice led to significant infiltration of inflammatory cells in the peribronchial region or airspaces and the thickening or fibrosis of alveolar septum, increased OVA-specific IgE in serum and total cells, eosinophil cells, and the levels of inflammatory cytokines including IL-4, IL-8, IL-1β, IL-5, IL-13, and IFN-γ in bronchoalveolar lavage fluid (BALF) of asthma mice. Moreover, mice in co-exposure group presented a significantly high cough feature, reduced catalase (CAT), glutathione (GSH), superoxide dismutase (SOD), and elevated malonaldehyde (MDA) elevated in BALF; increased ratio of Th2/Th1 and the markable inhibition of Th17 differentiation toward Treg cells in the adulthood of asthma mice. Cold stress and PM_{2.5} co-exposure in childhood may promote the deterioration of asthma symptoms in adulthood of mice by increasing inflammatory cytokines, ROS formation, Th2/Th1 imbalance, and suppressing the differentiation of Th17 toward Treg cells, which will help to provide experimental references when making some therapeutic strategies in allergic diseases through focusing on some natural solutions.

KEYWORDS

asthma, cold stress, fine particulate matter, inflammation, Th2/Th1 imbalance

1 | BACKGROUND

The International Study of Asthma and Allergies in Childhood declared that around 14% children worldwide were reported to have asthmatic symptoms in 2016, leading to a great socioeconomic burden.¹ Accumulated evidences increasingly suggest that there was significant association between ambient fine particulate matter (PM_{2.5}) exposure and adverse human health impacts such as asthma symptoms, missed

school days, hospitalization, and the frequency of asthma episodes.^{2–5} Mirabelli et al even estimated that each unit increase in PM_{2.5} might be associated with an increase in the prevalence of asthma symptoms, even at levels as low as 4.00 to 7.06 µg/m³. Recent literature has well documented the nonlinear association between childhood asthma and ambient temperature,⁴ indicating that extreme temperature can be recognized as one of the underlying triggers and potential causes of asthma. Compared with high temperature, low temperature has

showed greater risk of asthma attacks, particularly in China.⁶ In addition, researches of human studies indicated there was interactive effect of cold stress and exposure to particulate matter on respiratory diseases morbidity and mortality.^{7,8} These evidences suggest that both PM_{2.5} and low temperature may also affect the asthma, but the mechanism remains unclear.

PM_{2.5}, with an aerodynamic diameter less than 2.5 μm , can be inhaled and deposit in the lung,⁹ which induce serious inflammation and oxidative stress to cause lung damage.¹⁰ Inhalation of PM_{2.5} induces eosinophilic and neutrophilic airway inflammation and even act as an adjuvant to trigger allergic asthma in a guinea pig model.¹¹ Cold stress was also reported to affect respiratory system through suppressing the macrophage phagocytic function of alveolar macrophage.¹² Particularly, cold stress may aggravate the lung injury caused by PM_{2.5}, indicated by reduced lung function and worsened pathologic change in rat lung.¹³ It is possible that PM_{2.5} exposure combined with cold stress may also trigger the asthma. Besides, potential effects of lower temperature on asthma may not occur immediately after exposure in childhood but presented in adulthood with symptomatic attacks, suggesting the contribution of childhood lower temperature exposure to the development of asthma.¹⁴ Therefore, the present study is to study the effects of cold stress and PM_{2.5} co-exposure in the childhood on asthma attacks in adulthood and explore the possible mechanism.

2 | METHODS

2.1 | PM_{2.5} concentration and cold stress simulation

The urban PM_{2.5} of Shanghai city was concentrated to 400 $\mu\text{g}/\text{m}^3$ in a chamber by "Shanghai Meteorological and Environmental Animal Exposure System (Shanghai-METAS)" (patent #201510453600.8-). Shanghai-METAS, well used in other studies,¹⁵ is comprised of versatile aerosol concentration enrichment system (VACES), TEOM 1405 (Thermo, Waltham, Massachusetts) and meteorological environment simulator, which can not only concentrate and monitor PM_{2.5}, but also simulate weather parameters, like temperature and humidity. In this study, the temperature of 2°C was chosen as cold stress for animal experiment.

2.2 | Animals study

Forty one-month old weaned male C57BL/6J mice (SLACCAS, China) raised in specific pathogen-free environment were randomly divided into four groups (10 mice in each group), including negative control group (NC), PM_{2.5} group (PM), cold stress group (CS), and PM_{2.5} + cold stress (PM + CS) group. Under normal temperature (22°C), the NC group was kept in a room with filtered air, while the PM group was exposed to 400 $\mu\text{g}/\text{m}^3$ of PM_{2.5}. The CS group was exposed to cold stress with filtered air, while the PM + CS group was co-treated with 400 $\mu\text{g}/\text{m}^3$ of PM_{2.5} and cold stress. All these treatments were conducted in Shanghai-METAS system for 8 hours a day

and continued for 2 months, with a persistent relative humidity of 60% and 12-hour light/dark cycle. After that, all mice were raised in normal environment with filtered air for 1 month before establishing asthma model. Asthma model was established via intraperitoneal injection of 200 μL PBS containing 10 μg ovalbumin (OVA) and 1.5 mg Al(OH)₃ once a week for 3 weeks, followed by 1% OVA/PBS nebulization stimulation for 20 minutes twice every other week as previously described (Figure 1). In order to confirm the success of asthma model establishment, three mice were randomly selected 24 hours after the last treatment. After sacrifice, the structure of lung was checked under microscope with the help of Hematoxylin eosin (HE) staining. If the surface of the lung was pale and hard, and there were obvious increase of bronchial folds, obvious proliferation and disorder of epithelial cells, stenosis of lumen, airway remodeling pathological changes of chronic asthma such as airway and vascular smooth muscle cell proliferation, the asthma animal model would have been established successfully. All procedures performed in studies involving animals were in accordance with the ethical standards of the Research Ethics Committee of Public Health, Fudan University which the studies were conducted.

2.3 | Biochemical assay

Twenty-four hours after the last OVA/PBS challenge, all mice were sacrificed under general anesthesia with pentobarbital.¹⁶ The left lung of each mouse was used for alveolar lavage with PBS to harvest bronchoalveolar lavage fluid (BALF). A 200 μL suspended lavage was used for testing the catalase, glutathione, malonaldehyde, and superoxide dismutase level using colorimetric assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) in accordance with the given instructions. The interleukin (IL)-4, IL-1 β , IL-5, IL-8, IL-13, and interferon (IFN)- γ levels in the BALF were measured using an enzyme-linked immunosorbent assay (ELISA); meanwhile OVA-specific IgE antibodies in serum were measured using a Mouse OVA-Specific IgE ELISA kit (BioLegend, Cat. No. 439807, San Diego, California). Each sample was conducted for three individual experimental duplications.

2.4 | HE staining

Briefly, the upper lobe of the right lung was fixed by 4% paraformaldehyde at RT, and sedimentated within 20% and 30% sucrose solution 24 hours for dehydration, then embedded by optimal cutting temperature (OCT) compound for prechilling and conducted serial histological sections at 8 μm . The slides were dipped in hematoxylin for 10 minutes, 0.1% HCl-ethanol for 3 minutes, 0.5% NH₃-H₂O for 30 seconds, and 0.5% eosin-ethanol for another 30 seconds. The positive staining was statistical analyzed using Image J. H-score is calculated by $\sum(\text{Pi} \times \text{I})/100$, "Pi" denotes the percentage of stained cells and "I" denotes the intensity of the staining ranging from 0 to 3, which represent 0: no staining, 1: faint yellow, 2: claybank, and 3: dark claybank. At least five individual views for each group were observed and analyzed.

2.5 | Flow cytometry assay

In brief, for each sample, the diluted lavage containing 10^6 cells were centrifuged with 800 rpm for 3 minutes at 4°C, washed by cold PBS twice, resuspended gently, and incubated within 500 μ L PBS with 1% propidium iodide, 5 μ L CD4 and 5 μ L IL-17A or 5 μ L Foxp3 antibodies (R&D, Minneapolis, Minnesota) for 10 minutes on ice. Intracellular staining was performed according to the manufacturer's instructions. Briefly, the cells were fixed, permeabilized, and stained with FITC-conjugated anti-IL-17A and PE-conjugated anti-Foxp3 antibodies (eBioscience, San Diego, California) for intercellular cytokine staining. Cells flow through the cytometer was adjusted at about 600 cells per

second. PI negative population were further separated by 488 nm (CD4) and 594 nm (IL-4 or IL-17A) channels. FlowJo 10 (TreeStar, San Carlos, California) was used for image processing and data analysis.

2.6 | Sequencing and analysis

Total RNA was extracted from BALF using Trizol reagent (Life Technologies, Inc., Carlsbad, California) and the resulting lysate was used for affinity RNA purification in silica columns following manufacturer's instructions (Norgen Biotek, Thorold, Ontario, Canada). RNA quality was assessed by measuring 260/280 absorbance ratio and by

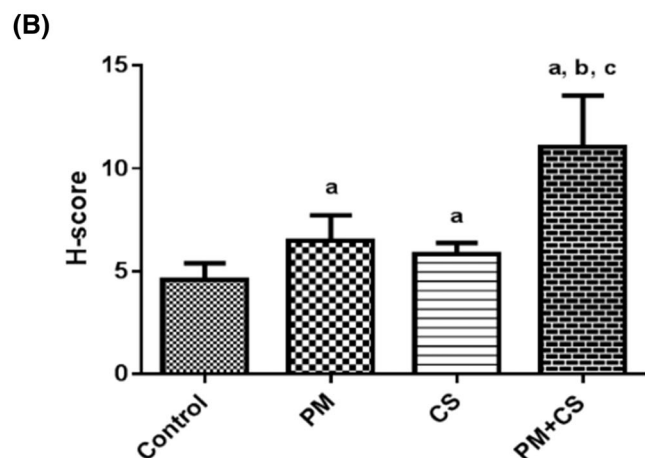
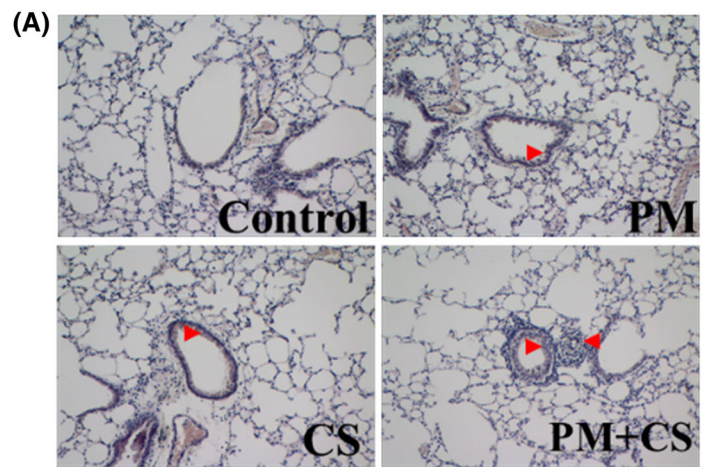
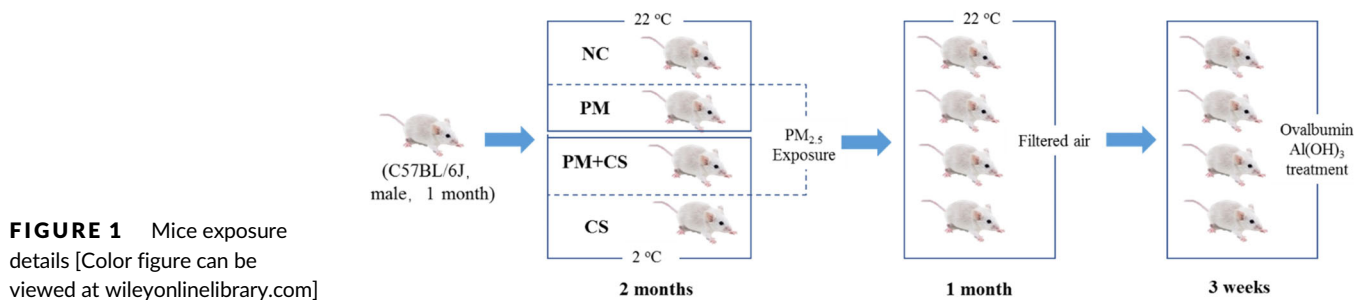


FIGURE 2 Histopathological evaluation of lung tissue from different groups of asthma mice. The morphology (A) and score (B) of pulmonary alveoli in different groups using HE staining with $\times 200$ amplification and analyzed using Image J. Note: Red triangles in A represent infiltration of inflammatory cells, the thickening or fibrosis of alveolar septum. a $P < .05$ vs negative control group; b $P < .05$ vs $PM_{2.5}$ group (PM); c $P < .05$ vs cold stress group (CS) [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com/doi/10.1002/ox.23023)]

integrating proportions of RNA using microchip electrophoresis on Agilent Bioanalyzer 2100 RNA 6000 nanochips (Agilent, Palo Alto, California). Reads were preprocessed using PRINSEQ-lite 0.20.4 (and FastQC 0.10.1. Exploratory analysis and differential species abundance testing were performed in R 3.1.2 and Bioconductor 3.0. For gene differential expression analysis, the dataset was aligned to the human genome using TopHat v2.0.6 and estimated the expressed gene abundance using Cufflinks v2.1.1 represented as fragments per kilobase of exon per million fragments mapped.

2.7 | Statistical analysis

The experimental data were processed with SPSS 20 software. H-score was used to calculate the positive staining of HE and immunohistochemistry. The Chi-square test was used for comparing the ratio of asthma occurring, and

t test was used for comparison of the continuous data. *P*-value less than .05 was considered as statistical significance.

3 | RESULTS

3.1 | Effects of PM_{2.5} and cold stress co-exposure on lung histopathological analysis and inflammatory cytokine patterns of mice

There were significant infiltration of inflammatory cells in the peri-bronchial region or airspaces and the thickening or fibrosis of alveolar septum in PM and CS groups, compared to control group. Notably, these observations above were exacerbated in PM + CS group (Figure 2).

Furthermore, we measured the serum ovalbumin (OVA)-specific IgE levels in mice exposed to PM_{2.5} and found the level of serum

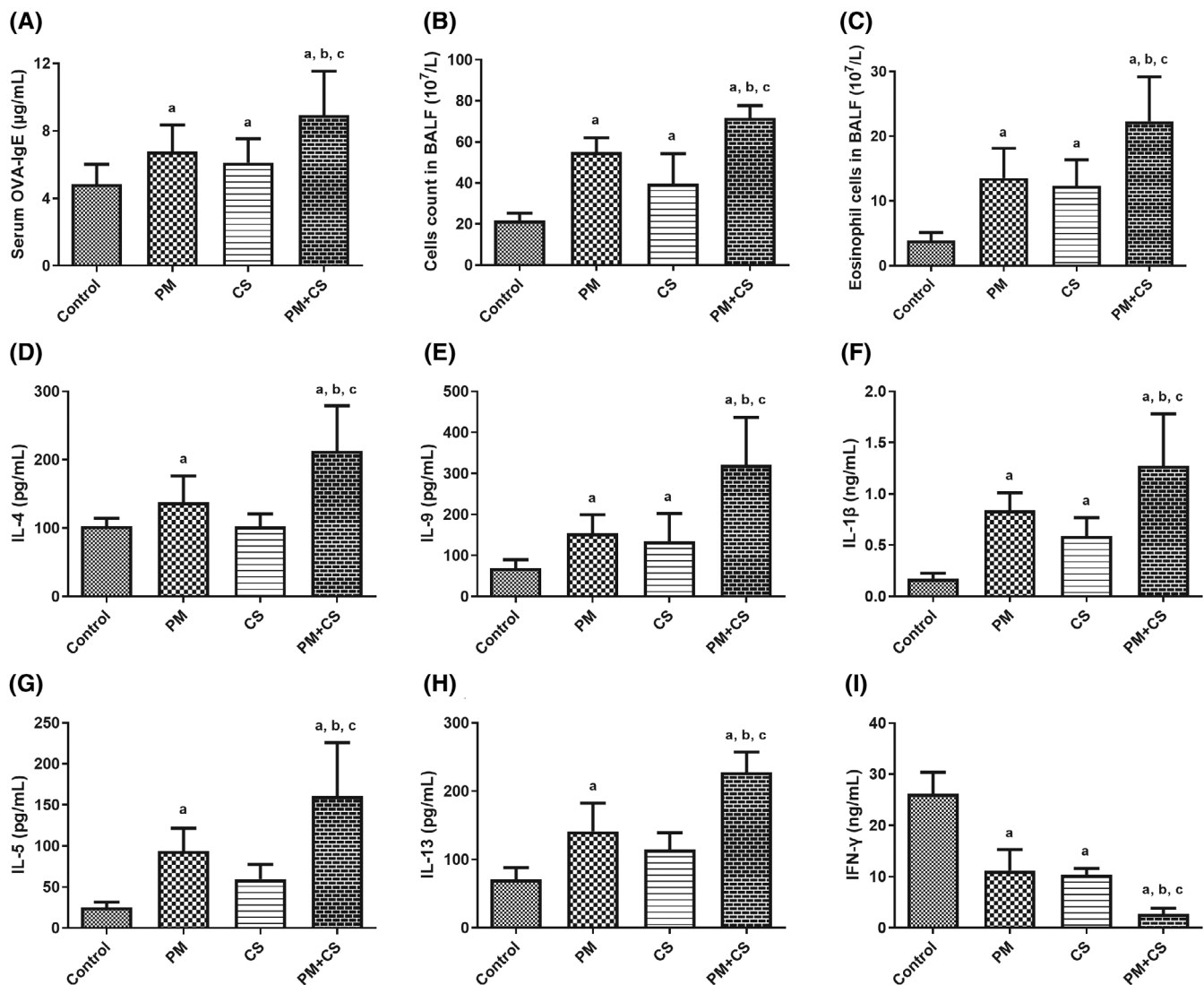


FIGURE 3 Levels of cytokines and inflammatory factors from different groups of asthma mice. Serum concentrations of OVA-IgE (A) and total cells (B), eosinophil cells (C) and the levels of inflammatory cytokines including IL-4 (D), IL-8 (E), IL-1β (F), IL-5 (G), IL-13 (H), and IFN-γ (I) in BALF was measured by ELISA assays. Data are expressed as mean ± SD (n = 8). Note: a *P* < .05 vs negative control group; b *P* < .05 vs PM_{2.5} group (PM); c *P* < .05 vs cold stress group (CS)

OVA-specific IgE in PM_{2.5}-induced asthma mice increased respectively by 1.40- and 1.26-fold in mono-PM and mono-CS groups compared to control mice ($P < .05$ and $P < .05$, respectively) and by 1.84-fold in PM + CS group, leading to a significant difference compared to mono-PM and mono-CS groups ($P < .05$) (Figure 3A).

Meanwhile, we analyzed total cells, eosinophil cells and the levels of inflammatory cytokines including IL-4, IL-8, IL-1 β , IL-5, IL-13, and IFN- γ in BALF. It was found that, compared to control mice, total cells count, eosinophil cells count, and the levels of IL-4, IL-8, IL-1 β , IL-5, and IL-13 significantly increased but the level of IFN- γ significantly decreased in PM group, while the levels of IL-8 and IL-1 β significantly increased but IL-4, IL-5, and IL-13 nonsignificantly increased in CS group. In contrast, compared to PM or CS groups, the total cells, eosinophil cells, and the levels of inflammatory cytokines including IL-4, IL-8, IL-1 β , IL-5, IL-13 significantly increased, and IFN- γ significantly decreased in BALF in PM + CS group (Figure 3B-I).

3.2 | Cold stress in childhood enhances the susceptibility and severity of asthma recurrence in adulthood of mice

To investigate the asthma recurrence in the adulthood of mice, we fed mice for another 2 months after treatments. During the 2 months, 6 out of 10 mice in PM + CS group experienced repeatedly spontaneous asthma

attacks, while 2 and 1 out of 10 in mono-PM group and mono-CS group presented sporadic asthma attacks, no asthma attacks were observed in control group. Furthermore, we used OVA nebulization stimulation to induce asthma symptoms of mice. It was observed that mice in PM + CS group presented a significantly higher cough feature, compared to control group ($\chi^2 = 9.187$, $P = .002$), mono-PM group ($\chi^2 = 3.887$, $P = .049$), and mono-CS group ($\chi^2 = 9.187$, $P = .002$), respectively (Figure 4A,B).

Based on the recent observation of the significant association between asthma pathogenesis and ROS formation,¹⁷ we investigated the levels of ROS-related biochemical indicators of alveolar lavage after the OVA stimulation. It was observed that catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD) were obviously reduced and malonaldehyde (MDA) was significantly elevated in BALF of mice in PM + CS group, compared to control group, mono-PM group, and mono-CS group ($P < .05$) (Figure 4C-F).

3.3 | Exacerbated asthma recurrence is associated with inhibited Th17 cells differentiation in inflammatory response

The observation that PM_{2.5} and cold stress co-exposure in childhood exacerbated the susceptibility and severity of asthma recurrence in adulthood of mice encouraged us to explore the underlying molecular mechanism. Firstly, we performed an RNA-Seq data followed by

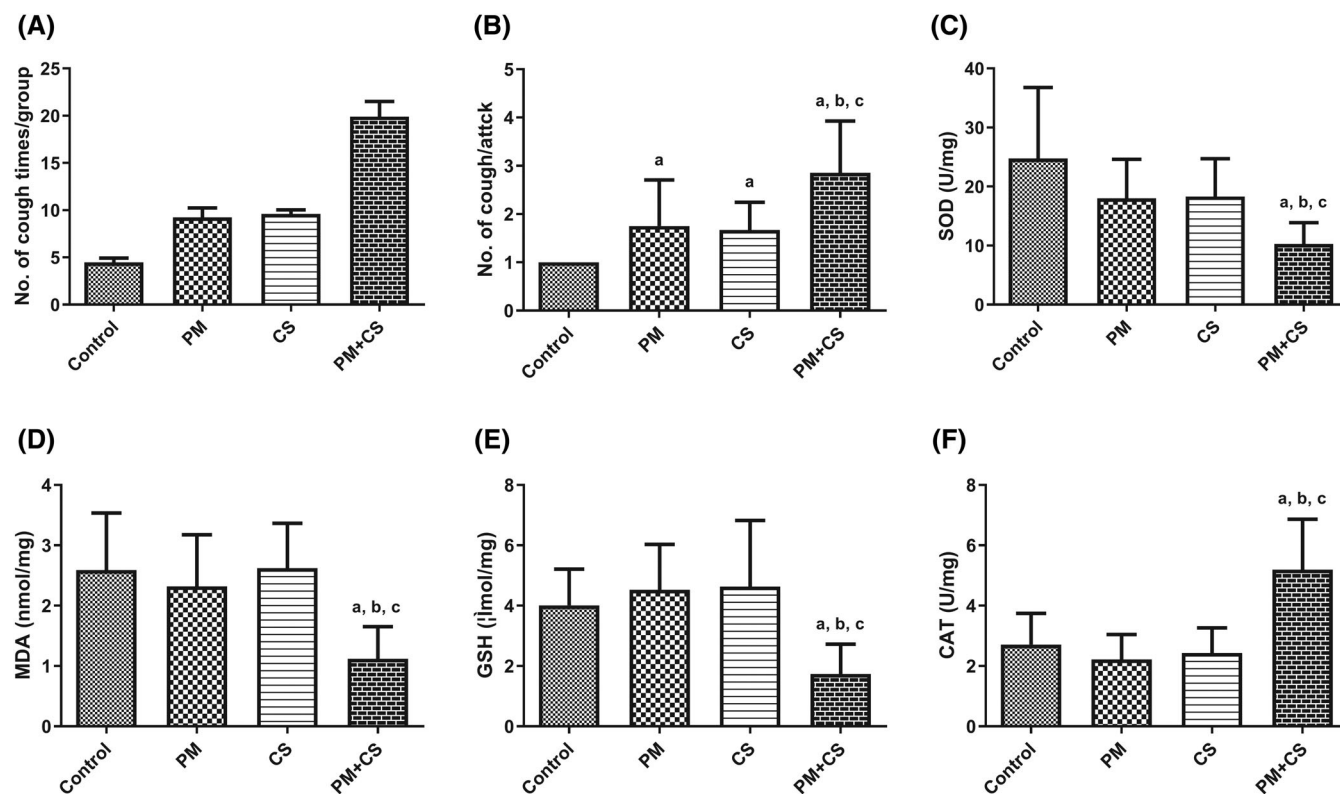


FIGURE 4 Cough feature and ROS-related biochemicals in BALF from different groups of asthma mice. A, The count of cough times in every groups. B, The number of coughs in every attack. C-F, The levels of SOD, GSH, MDA, and CAT were detected by using ELISA assays. Note: a $P < .05$ vs negative control group; b $P < .05$ vs PM_{2.5} group (PM); c $P < .05$ vs cold stress group (CS). Each experiment was performed at least three times individual duplications

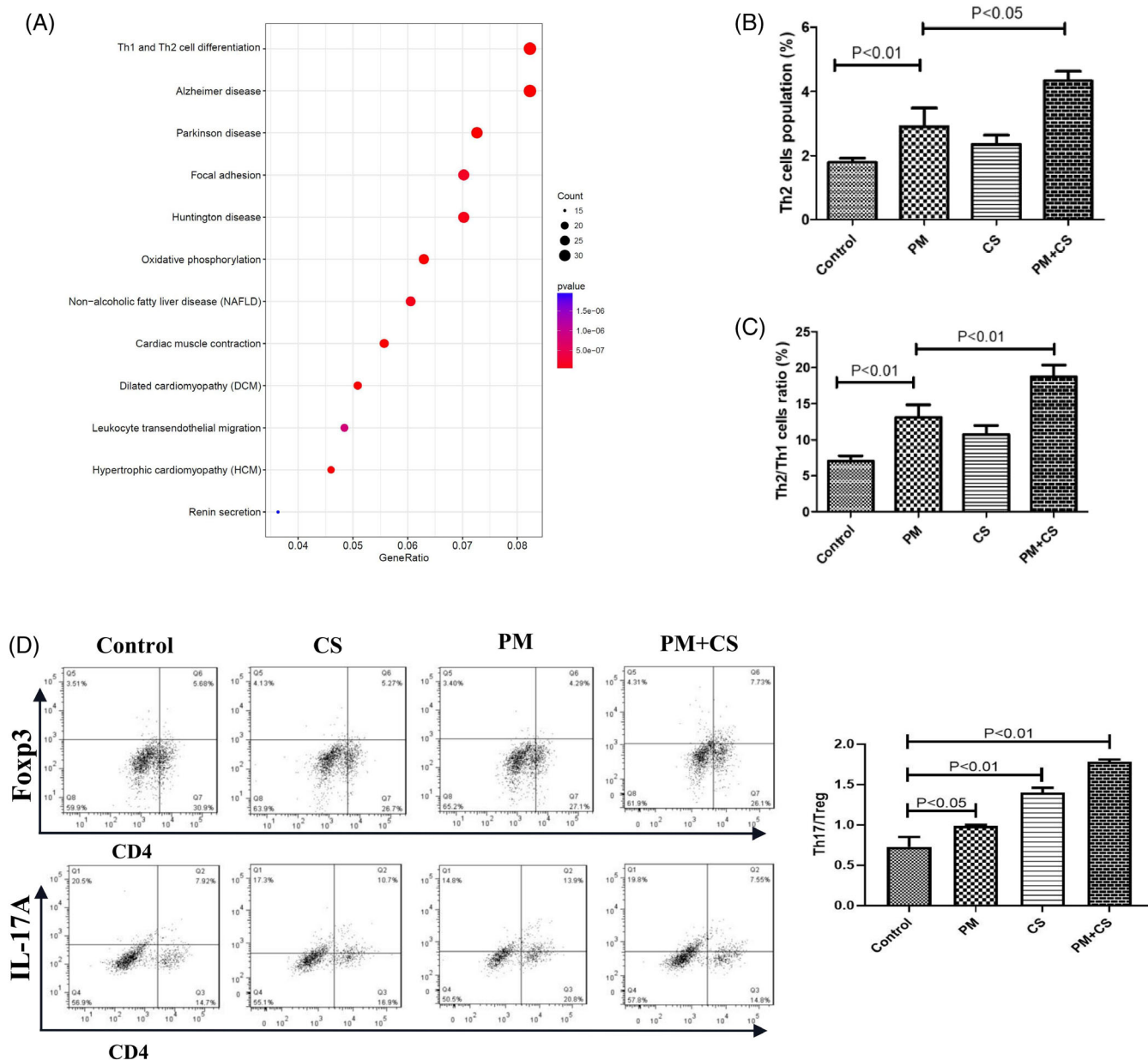


FIGURE 5 Exacerbated asthma recurrence is associated with inhibited Th17 cells differentiation. Corresponding network by sequencing analysis based on the different expression genes indicated Th1 and Th2 cell differentiation is a critical event during asthma recurrence in adulthood (A). Th2 cells population (B) and Th2/Th1 ratio (C) were determined by the cell count. In addition, IL-17A and CD4 positive are considered as Th17 cells while Fcp3 and CD4 positive are considered as Treg cells (D). Each experiment was performed at least three times individual duplications [Color figure can be viewed at wileyonlinelibrary.com]

KEGG pathway analysis from the comparison between mono-PM group and PM + CS group to further understand potential underlying mechanisms in the development of asthma, and found these genes were predominantly enriched in the pathway of Th1 and Th2 cell differentiation (Figure 5A).

Based on the increased Th2 cytokines IL-4, IL-5, and IL-13 observed above, we measured the Th2/Th1 ratio in peripheral blood mononuclear cells from various groups by flow cytometry. As shown in Figure 5B,C, the ratio of Th2 cells was increased in mono-PM or

mono-CS group, compared to control group ($P < .05$); while significantly increased in PM + CS group, compared to mono-PM or mono-CS group ($P < .05$). Moreover, Th2/Th1 ratio presented the similar trend as the changes of Th2 populations in various groups, suggesting the crucial role of imbalanced Th2/Th1 in the development of asthma. Furthermore, we investigated the changes of Th17 and Treg cells in BALF by using flow cytometric assay with Th17-related cytokines IL-17A and Fcp3 labels and observed that the differentiation of Th17 toward Treg cells was suppressed in mono-PM group, compared

to control. Meanwhile, the marked inhibition of Th17 differentiation was found in PM + CS group, compared to mono-PM group, suggesting the potential association between the susceptibility and severity of asthma in the adulthood of mice and aberrant regulation on Th17 differentiation (Figure 5D).

4 | DISCUSSION

Both cold stress and PM_{2.5} are the risk factors of causing or inducing asthma.^{3,6} Studies reported that the exposure of asthma risk factors in childhood would aggravate the asthma symptoms in adulthood.¹⁸ Here, in this study, we investigated the effects of cold stress and PM_{2.5} co-exposure in childhood on the susceptibility and severity of asthma recurrence in adulthood and found at the first time that such co-exposure in childhood exacerbated asthma recurrence in adulthood. The finding evidently suggested a potential clinical value in exploring the novel therapeutic strategies for asthma in the future.

Here, we chose firstly the natural inhalation artificial climate chamber rather than traditional way of intratracheal instillation for the treatment with cold air and/or PM_{2.5} exposure. This chamber mimics the chronic and long-term exposure process of animals to inhale pollutants, avoiding the acute pulmonary injury caused by intratracheal instillation. By this chamber, we can keep them in a relatively stable living environment from early childhood to adulthood,¹⁹ providing a feasible animal model for subsequent observation and mechanism exploration.

Recently, with the increasing motor vehicle exhaust emission, PM_{2.5} has surpassed PM₁₀ to become a public health and environmental issue in China. Previous epidemiological investigations reported the higher morbidity and mortality of PM_{2.5}-induced asthma population in some northern cities with serious air pollution compared to south region of China in cold season,^{20,21} which indicates that the seasonal characteristics is an important modifying factor to worsen the occurrence and severity of asthma. Interestingly, the immigrants to Westernized countries are found to be associated with a lower prevalence of asthma,²² which explains at some extent that the asthma status of children may be alleviated by the recent migration to places with good air quality. In other words, their asthma status may also be aggravated by worse air pollution. In addition, a high proportion of the adults with asthma in childhood were still found the hyper-reactive airways even if they displayed symptom-free. These evidences strongly suggested that higher PM_{2.5} exposure in extreme weather at childhood may be associated with an increase in the prevalence of asthma symptoms in adulthood.

In our study, the cold living condition with PM_{2.5} in early-life significantly increased the OVA-induced asthma recurring in adult mice. However, we failed to observe the onset of asthma attack in young mice, which may be related to the fact that asthma symptoms of mice need to be induced rather than spontaneously attack, unlike human.²³ Furthermore, after OVA stimulation, we observed mice in PM + CS group presented a significantly high cough feature, compared to other groups. It was suggested that cold stress in childhood enhanced the

susceptibility and severity of asthma recurrence in adulthood of mice, the cold stress and PM_{2.5} may play a synergistic role in asthma exacerbation. As indicator of oxidative stress, CAT, GSH and SOD were obviously reduced and MDA was significantly elevated in BALF of mice in PM + CS group, indicating ROS may lead the asthma pathogenesis in the exposure of PM_{2.5} and cold stress. Accumulated evidences showed that ROS can promote the occurrence of allergic reactions through playing strong and long-lasting allergic effects or inducing the release of histamine from eosinophils and neutrophils,^{17,24} which is consistent with our observation that increased numbers of cells and eosinophils and cytokines in BALF.

It was well-known that eosinophils have been recognized as the inducer of T-cell proliferation and effector T-cell recruitment to the lung in response to allergen.²⁵ In this study, Th2 cytokines IL-4, IL-5, and IL-13, chemokines IL-8, IL-1 β in BALF and OVA-specific IgE in serum were significantly increased in the BALF of co-exposure groups, compared to other groups. These results implied the cold stress and/or PM_{2.5}-induced Th1/Th2 immunity imbalance. Furthermore, it has been widely accepted that Foxp3 and IL-17A are a pair of special biomarkers to distinguish from the Th17 and Treg cells,²⁶ and the imbalance of Th17/Treg rather than Th1/Th2 contributes to the pathogenesis of allergic asthma.^{27,28} In the study, it was observed that a high proportion of Th17/Treg in PM + CS group, which implies that Th17 are suppressed to Treg differentiation, suggesting the potential association between the susceptibility and severity of asthma in the adulthood and the treatment with PM_{2.5} and cold stress in childhood.

5 | CONCLUSION

In conclusion, cold stress and PM_{2.5} co-exposure in childhood may promote the deterioration of asthma symptoms in adulthood of mice by increasing inflammatory cytokines, ROS formation, Th2/Th1 imbalance, and suppressing the differentiation of Th17 toward Treg cells. These findings identified critical roles of living environmental factors in childhood in asthma pathobiology of adulthood and suggested novel potential intervention targets and mechanism, which will help to make some therapeutic strategies in allergic diseases through focusing on some natural solutions.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

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The authors regret to inform that they mistakenly used histopathological pictures of PM Group, CS Group and PM + CS Group in Figure 2-A. This mistake has now been corrected and the updated Figure 2 together with the previously published figure is shown below. The authors sincerely apologize for any inconvenience this error may have caused.

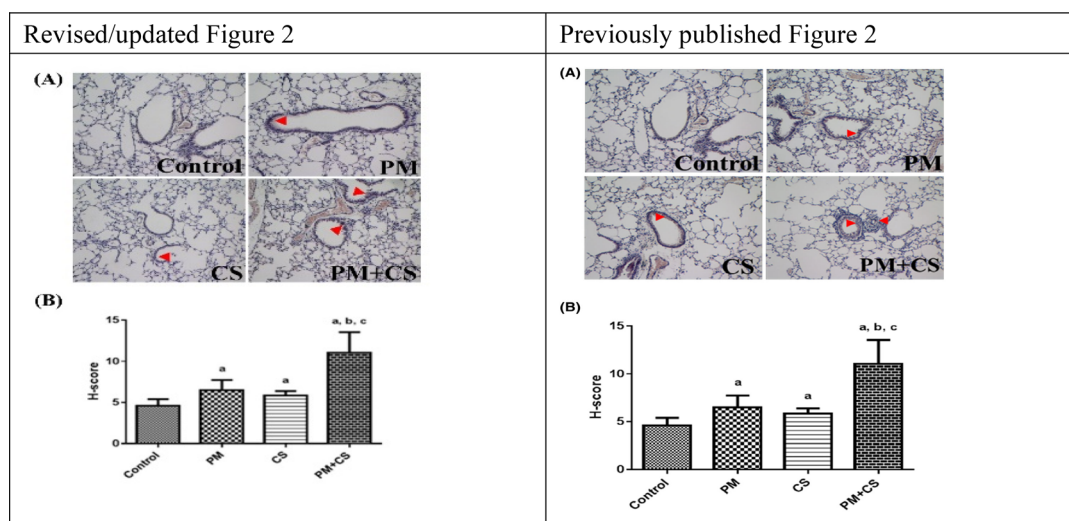


FIGURE 2 Histopathological evaluation of lung tissue from different groups of asthma mice. The morphology (A) and score (B) of pulmonary alveoli in different groups using HE staining with $\times 200$ amplification and analyzed using Image J. Note: Red triangles in A represent infiltration of inflammatory cells, the thickening or fibrosis of alveolar septum. a $P < .05$ vs negative control group; b $P < .05$ vs PM 2.5 group (PM); c $P < .05$ vs cold stress group (CS).