

Original article

TMT-based quantitative proteomics reveals suppression of SLC3A2 and ATP1A3 expression contributes to the inhibitory role of acupuncture on airway inflammation in an OVA-induced mouse asthma model

Weifeng Tang^{a,b,1}, Ming Dong^{c,1}, Fangzhou Teng^{a,b}, Jie Cui^{a,b}, Xueyi Zhu^{a,b}, Wenqian Wang^{a,b}, Tulake Wuniqiemu^{a,b}, Jingjing Qin^{a,b}, La Yi^{a,b}, Shiyuan Wang^{a,b}, Jingcheng Dong^{a,b,*}, Ying Wei^{a,b,*}

^a Department of Integrative Medicine, Huashan Hospital, Fudan University, Shanghai, China

^b Institutes of Integrative Medicine, Fudan University, Shanghai, China

^c Gumei community Health center of Minhang district of Shanghai, Shanghai, China



ARTICLE INFO

Keywords:

asthma
airway inflammation
quantitative proteomics
bioinformatics analysis
acupuncture
mechanism

ABSTRACT

Asthma is a chronic airway inflammatory disease and acupuncture is frequently used in patients suffering from asthma in clinic. However, the regulatory mechanism of acupuncture treatment in asthma is not fully elucidated. We sought to investigate the effectiveness of acupuncture on asthma and the associated regulatory mechanism. An ovalbumin (OVA)-induced mouse asthma model was established and the effect of acupuncture on airway hyperresponsiveness (AHR), mucus hypersecretion and inflammation was assessed. Tandem mass tag (TMT)-based quantitative proteomics analysis of lung tissue and bioinformatics analysis were performed. Our results revealed that the OVA-induced mouse asthma model was successfully established with the significantly elevated AHR to methacholine (Mch), and acupuncture was effective in attenuation of AHR to Mch, peribronchial and perivascular inflammation and mucus production. The inflammatory cells around the airways, mucous secretion as well as levels of IgE, CCL5, CCL11, IL-17A in bronchoalveolar lavage fluid (BALF) and IL-4, IL-5 and IL-13 levels in serum were significantly inhibited by acupuncture. TMT-based quantitative proteomics analysis found that a total of 6078 quantifiable proteins were identified, and 564 (334 up-regulated and 230 down regulated) differentially expressed proteins (DEPs) were identified in OVA-induced asthma model group (A) versus normal control group (NC). Acupuncture treatment resulted in 667 DEPs (416 up-regulated and 251 down regulated) compared with A group, and 86 overlapping DEPs were identified in NC, A and AA groups. Among the 86 overlapping DEPs, we identified 41 DEPs regulated by acupuncture. Based on the above data, we performed a systematic bioinformatics analysis of the 41 DEPs, and results showed that these 41 DEPs were predominantly related to 4 KEGG pathways including SNARE interactions in vesicular transport, ferroptosis, endocrine and other factor-regulated calcium reabsorption, and protein digestion and absorption. DEPs of SLC3A2 and ATP1A3 expression levels were verified by immunohistochemical staining. Mice in OVA-induced asthma model group had elevated SLC3A2 and ATP1A3 expression and acupuncture had the ability to downregulate SLC3A2 and ATP1A3 protein expression. Furthermore, acupuncture reduced the MDA level and increased the GSH and SOD

Abbreviations: GINA, Global Initiative for Asthma; WHO, World Health Organization; ICS, inhaled corticosteroids; TCM, traditional Chinese medicine; TMT, Tandem mass tag-based; iTRAQ, isobaric tags for relative and absolute quantification; OVA, ovalbumin; Mch, methacholine; AHR, airway hyperresponsiveness; R_L, Airway resistance; C_{dyn}, lung dynamic compliance; BALF, bronchoalveolar lavage fluid; EOS, eosinophils; Ig, immunoglobulin; IL, interleukin; ELISA, enzyme linked immunosorbent assay; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; AGC, automatic gain control; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEPs, differentially expressed proteins; BSA, bovine serum albumin; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione; PAS, periodic acid-Schiff; H&E, hematoxylin and eosin; NC, normal control group; A, OVA-induced asthma model group; AA, the acupuncture treatment group; ASA, sham acupuncture group; CCL5, C-C motif chemokine ligand 5; CCL11, C-C motif chemokine ligand 11; ROS, reactive oxygen species; ATP1A3, Sodium/potassium-transporting ATPase subunit alpha-3; SLC3A2, 4F2 cell-surface antigen heavy chain; ANOVA, one-way analysis of variance; Th, T helper cell; FDR, false discovery rate.

* Corresponding authors at: Department of Integrative Medicine, Huashan Hospital, Fudan University, 12 Middle Urumqi Road, Shanghai 200040, China.

E-mail addresses: jcdong2004@126.com (J. Dong), weiyiing_acup@126.com (Y. Wei).

¹ Contributed equally.

<https://doi.org/10.1016/j.bioph.2020.111001>

Received 26 September 2020; Received in revised form 29 October 2020; Accepted 8 November 2020

Available online 16 December 2020

0753-3322/© 2020 The Author(s).

Published by Elsevier Masson SAS. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

levels in the lung tissue. Taken together, our data suggested that acupuncture was effective in treating asthma by attenuation of AHR, mucus secretion and airway inflammation, and the mechanism was associated with regulation of ferroptosis, SLC3A2 and ATP1A3 protein expression as well as oxidative stress. Results from our experiments revealed the anti-inflammatory effect of acupuncture in OVA-induced mouse asthma model, leading to a more effective approach to be chosen by patients in clinic.

1. Introduction

Asthma is a serious global health problem affecting 1-18% of the population in different countries, and the prevalence is increasing in many countries according to the Global Initiative for Asthma (GINA) report. Asthma is a heterogeneous respiratory disease, usually characterized by airway hyperresponsiveness (AHR) to direct or indirect stimuli and chronic airway inflammation, even when symptoms are absent or lung function is normal [1]. Asthma is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, cough and variable expiratory airflow limitation over time and in intensity. Asthma exacerbations may be triggered by factors such as irritant exposure, and imposes a significant burden to patients as well as on health care systems and society due to loss of productivity in the workplace. Inhaled corticosteroids (ICS) and bronchodilators are the preferred first-line treatment for asthma to control symptoms, improve lung function, and reduce morbidity and mortality. However, there are some asthmatics do not respond adequately to these therapies, and systemic steroids and/or hospitalization are required. Moreover, long-term use of ICS has been associated with obvious side effects [2]. In addition to the first-line therapies, some complementary and alternative medical treatments proved to have obvious advantages.

Acupuncture is one of a widely used complementary and alternative therapies in clinic. National Institutes of Health recognized acupuncture as an effective treatment for a variety of conditions in 1997. Furthermore, the World Health Organization (WHO) recommends the use of acupuncture for 64 conditions including asthma. Due to advantages in regulation of multiple biologic processes and pathways, acupuncture is widely used in the treatment of heterogeneous and complex diseases, such as asthma. Previous studies have shown that acupuncture was effective for improving the asthmatic symptoms [3–6] with high safety and no side effects. Previous studies reveal that the use of acupuncture as adjunct to the routine care of allergic bronchial asthma was superior to routine care alone in improving both specific symptoms and general quality of life [3]. However, the mechanism of acupuncture treatment in asthma is still unclear.

Quantitative proteomics technique is a precise method to reveal the differentially expressed proteins (DEPs) of life processes or diseases, and to predict the therapeutic targets of drugs and underlying mechanism [7, 8]. Due to the advantages of proteomics technology, it has been widely used in the study of traditional Chinese medicine (TCM) to explore the complex regulatory mechanisms [9–13]. Quantitative isobaric tags for relative and absolute quantification (iTRAQ) proteomics is frequently used in acupuncture research to clarify the underlying mechanism for treating diseases [14–19]. However, quantitative proteomics is seldom used in the mechanism study of acupuncture treatment in asthma. In this study, tandem mass tag-based (TMT) quantitative proteomics technology is used to further investigate the effect of acupuncture on asthma and the underlying regulatory mechanism.

2. Material and methods

2.1. Experimental animals

Female BALB/c mice (6–8 weeks), special pathogen-free, were purchased from Shanghai Jiesijie Laboratory Animal Co. Ltd. The mice were placed in the animal holding unit building (School of Pharmacy, Fudan University), with a pathogen-free facility and were regularly fed

sufficient water and food. All animal experiments in this study were approved by the Ethics Committee of Animal Experiments of Fudan University (authorization number: 2019-07-HSYW-WY-01).

2.2. Asthma model establishment

Forty female mice were randomly divided into 4 groups, including normal control group (NC, $n = 10$), an ovalbumin (OVA) induced asthma group (A, $n = 10$), an asthma model combined with acupuncture treatment group (AA, $n = 10$), and an asthma model combined with sham acupuncture treatment group (ASA, $n = 10$). As shown in Fig. 1A, mice were challenged by intraperitoneal injection of 0.2 ml of saline solution containing 20 μg of OVA (grade V, sigma, Taufkirchen, Germany) and 2 mg of aluminum hydroxide (Sigma-Aldrich, St Louis, MO, USA) on day 0 and day 7. From days 14 to 16, mice were inhaled with 3% OVA solution by ultrasonic nebulizer (402AI, Yuyue Medical Equipment Co Ltd, Jiangsu, China) for 30 minutes each time. On days 21 to 23, mice in group A were challenged with OVA, and mice in groups AA and ASA were challenged with OVA and administered with acupuncture or sham acupuncture. Mice in NC group were challenged with phosphate-buffered saline.

2.3. Acupuncture treatment

Acupoints of GV14 (Dazhui), BL12 (Fengmen) and BL13 (Feishu) (Fig. 1B) were selected for acupuncture treatment based on acupuncture theory. GB30 (Huantiao, Fig. 1C), which has been shown to be neuro-anatomic unrelated to the lungs and does not have therapeutic effect on asthma according to TCM theory, was selected for sham acupuncture administration. GV14 is located between C7 and T1 vertebrae. Bilateral BL12 are located laterally between the T2 and T3 vertebrae, and bilateral BL13 are located laterally between the T3 and T4 vertebrae (Fig. 1B). From day 21, mice were subjected to acupuncture intervention 1 hour before the OVA attack. The conscious mice were fixed on the plate, and sterile stainless steel acupuncture needles (Hwato brand, 13 mm length, 0.25 mm diameter, Suzhou Medical Supplies Co Ltd, Jiangsu, China) were inserted into the acupoints (GV14, BL12 and BL13) to a depth of 3 mm. For bilateral acupoints of GB30, acupuncture needles were inserted to a depth of 5 mm. Acupuncture needles were rotated at a frequency of 60 times per minute for 20 seconds, operated every five minutes, and the needle was pulled out after 20 minutes.

2.4. Measurement of AHR

Within 24 hours of the last OVA challenge, AHR was accurately assessed using Fine Pointe Resistance and Compliance system (DSI Buxco Electronics, Troy, NY). Mouse in each group was weighed, intraperitoneally anesthetized with pentobarbital sodium (Sigma-Aldrich, St Louis, MO, USA) at a dose of 50 mg/kg, and incised and tracheal intubated. Mouse was placed in the chamber, and the tracheal intubation is connected to the ventilator. Airway resistance (R_L) and lung dynamic compliance (C_{dyn}) were recorded with methacholine (Mch, Sigma-Aldrich, St Louis, MO, USA) dose changing (0, 3.125, 6.25 and 12.5 mg/ml).

2.5. Lung histopathology detection

The middle lobe of left lung from each mouse was removed, fixed in

4% paraformaldehyde and embedded in paraffin. Lung tissue sections were prepared with thickness of 4 μm . Lung sections were stained with hematoxylin and eosin (H&E) to evaluate the degree of inflammatory cell infiltration around the bronchus, and stained with periodic acid Schiff (PAS) to evaluate the degree of goblet cell metaplasia and mucus secretion. According to previous studies[20], we used a semi-quantitative grading method to evaluate the degree of peribronchial inflammation and expressed it as a subjective score from 0 to 4 (normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of 4 cells deep). PAS-positive cells in the airway were counted and their percentage was calculated to evaluate the degree of goblet cell metaplasia in the airway epithelial cells as previous reported[21]. In brief, purple goblet cells were determined as PAS-positive. The numbers of PAS-negative and PAS-positive cells were counted around the internal perimeter of the airway. Percentage of PAS-positive cells in total goblet cells was calculated as goblet cell metaplasia using Image J software. The data was collected by three independent blinded researchers.

2.6. Flow cytometric analysis

Eosinophil (EOS) has been implicated in the pathogenesis of asthma. Flow cytometry assay was performed to identify proportion of CD45+CD11b + Ly6C-SiglecF + EOS in the lung tissue of mice in each group. The upper right lobe of each lung was dissociated to single cells using lung dissociation kit (Miltenyi Biotec Technology & Trading Co. Ltd), and the single cells were further stained with the fluorescently labeled antibodies (Live Dye-APC-eF780 and anti-CD45 eF506, Thermo; anti-CD11b BV421, anti-Ly6C PE, anti-Siglec F APC, anti-CD3 FITC and anti-CD4 APC, BioLegend) and detected by an Attune NxT instrument (Thermo Fisher Scientific).

2.7. Inflammatory cytokines, chemokines and IgE detection

After the detection of AHR, blood was collected from the eyeballs of the mice. Then mice were sacrificed by cervical dislocation and the

bronchoalveolar lavage fluid (BALF) and lung tissue were collected. Serum and BALF were prepared by centrifugation and stored in a refrigerator at -80°C after aliquoting. The levels of IgE, IL-17A, CC motif chemokine ligand 11 (CCL11) and CCL5 in BALF and concentrations of IL-4, IL-5 and IL-13 in serum of mice in different groups were detected by enzyme linked immunosorbent assay (ELISA) (Multi-Sciences, Hangzhou, China).

2.8. TMT-based quantitative proteomics analysis

Proteomic analysis of lung tissue samples was performed by TMT-based quantitative approach. The whole TMT-based quantitative proteomics analysis process includes protein sample preparation, TMT labeling, high performance liquid chromatography (HPLC) fractionation, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and proteomics data analysis. Briefly, 12 lung tissues (3 lung tissues/group) were grinded by liquid nitrogen into cell powder, and lysis buffer including protease inhibitor cocktail was added, followed by sonication on ice using a high intensity ultrasonic processor (Scientz). Centrifugation was performed to remove the remaining debris by centrifugation at 12000 g at 4°C for 10 min and the supernatant was collected for protein concentration detection using BCA kit (Thermo Scientific, USA) according to the manufacturer's instructions. The protein solutions were then reduced, alkylated, acetone precipitated, resuspended and digested with trypsin, and further desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptides were reconstituted and processed according to the manufacturer's protocol for TMT labeling using TMT kit (Thermo Scientific, USA). For HPLC fractionation, the tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 8% to 32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into 9 fractions and dried by vacuum centrifuging. For LC-MS/MS analysis, the tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column. The gradient was comprised of an

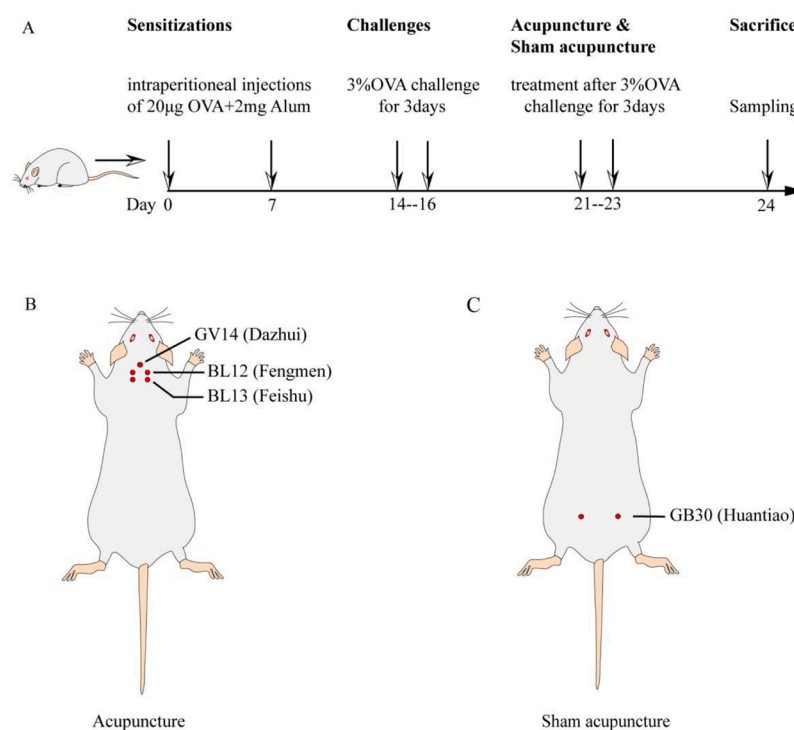


Fig. 1. Protocol of asthma model establishment, treatment and localization of acupoints. A. Protocol of the asthma model establishment and acupuncture/sham acupuncture treatment. B. The localization of acupoints selected for acupuncture. C. The localization of acupoints selected for sham acupuncture.

increase from 6% to 23% solvent B over 26 min, 23% to 35% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 400 nL/min on an EASY-nLC 1000 UPLC system. The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTMPlus (Thermo) coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. Fixed first mass was set as 100 m/z. The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against human uniprot database concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to 4 missing cleavages. The mass tolerance for precursor ions was set as 20 ppm in first search and 5 ppm in main search, and the mass tolerance for fragment ions was set as 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification and acetylation modification and oxidation on Met were specified as variable modifications. False discovery rate (FDR) was adjusted to <1% and minimum score for modified peptides was set >40. The DEPs were satisfied the following conditions: unique peptides ≥ 1 with average ratio-fold change > 1.2 (up-regulation) and < 0.83 (down-regulation), as well as p-value < 0.05.

2.9. Bioinformatics analysis

Bioinformatics tools were used to further analyse proteins with significant differential abundances observed by proteomic analysis. Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/> (<http://www.ebi.ac.uk/GOA/>)). Proteins were classified by GO annotation based on three categories: biological process, cellular component and molecular function. For each category, a two-tailed Fisher's exact test was employed to test the enrichment of the DEP against all identified proteins. The GO with a corrected p-value < 0.05 is considered significant. Encyclopedia of Genes and Genomes (KEGG) database was used to identify enriched pathways by a two-tailed Fisher's exact test to test the enrichment of the differentially expressed protein against all identified proteins. The pathway with a corrected p-value < 0.05 was considered significant. These pathways were classified into hierarchical categories according to the KEGG website.

2.10. Immunohistochemical staining

Lung sections were deparaffinized with xylene and ethanol, subjected to antigen retrieval, blocked with 3% bovine serum albumin (BSA), and stained with mouse anti-4F2 cell-surface antigen heavy chain (SLC3A2) antibody (Thermo Fisher scientific) and anti-Sodium/potassium-transporting ATPase subunit alpha-3(ATP1A3) antibody (Thermo Fisher scientific). Mouse serum at the same protein concentration as the antibody solution was used as a control. The positive tissue sections appeared as brown or tawny-brown under the microscope and tissue images were captured under an optical microscope ($\times 100$). The integrated optical density (IOD) values of tissue sections were measured by Image-Pro Plus 6.0 software. Five views in each tissue section and three tissue sections in each group were randomly selected to determine the positive IOD values, and the mean IOD values were considered as the relative expressions of SLC3A2 and ATP1A3.

2.11. Evaluation of oxidative stress biomarkers MDA, GSH and SOD

The lung tissue homogenates were prepared to assay the concentration of oxidative stress biomarkers malondialdehyde (MDA),

glutathione (GSH) and superoxide dismutase (SOD) using commercial kits (MultiSciences, Hangzhou, China).

2.12. Statistical analysis

All data were analyzed and graphs by GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) and were expressed as mean $\pm$ SD. All data were analyzed by one-way analysis of variance (ANOVA) to calculate the statistical significance of differences between more than two groups. A p value < 0.05 means statistically significant.

3. Results

3.1. Acupuncture improved lung function in OVA-induced mouse asthma model

AHR to increasing doses of Mch was assessed within 24 h after the last OVA challenge. Compared with NC group, mice in the A group demonstrated an enhanced increase in R_L (Mch = 6.25 mg/ml and Mch = 12.5 mg/ml, $p < 0.01$ or $p < 0.001$, Fig. 2B) and decrease in Cdyn ($p < 0.001$ in all dose of Mch, Fig. 2C), indicating the successful establishment of mouse asthma model. Treatment with acupuncture induced a significant decrease in R_L (Mch = 6.25 mg/ml and Mch = 12.5 mg/ml, $p < 0.01$ or $p < 0.001$, Fig. 2D-F) and an increase in Cdyn (all dose of Mch, $p < 0.05$ or $p < 0.001$, Fig. 2G-I). As shown in Fig. 2D-I, sham acupuncture administration did not improve the AHR compared with the A group ($p > 0.05$).

3.2. Acupuncture therapy attenuated airway inflammation in OVA-induced mouse asthma model

In order to evaluate the inhibitory effect of acupuncture on airway inflammation and mucus production, the lung tissues sections were analyzed with H&E and PAS staining. The inflammation scores and PAS + bronchial cell counts were measured. As shown in Fig. 3, compared with the NC group, a large amount of inflammatory cells infiltration around the peribronchial and mucus secretion were observed in A group ($p < 0.001$, Fig. 3B-C). Acupuncture induced a significant decrease in the inflammatory cell counts around the peribronchial compared with the A group ($p < 0.001$, Fig. 3B-C). Sham acupuncture did not remarkably influence the severe inflammation around the airways. The mucus secretion level and the percentage of goblet cells were significantly reduced in mice treated with acupuncture compared with A group ($p < 0.001$, Fig. 3D-E). In contrast to the AA group, the ASA group showed no histological differences compared with the A group ($p > 0.05$, Fig. 3).

3.3. Acupuncture therapy reduced EOS proportion and related chemokines in lung tissue of mouse asthma model induced by OVA inhalation

Airway eosinophilia is a characteristic feature of asthma. To determine whether acupuncture could reduce the EOS levels in the lung tissue, we performed flow cytometry assay. Elevation of CD45+CD11b + Ly6C-SiglecF + EOS percentage was observed in lung tissue of mouse asthma model induced by OVA inhalation ($p < 0.001$, Fig. 4B). Acupuncture therapy, but not sham acupuncture, inhibited the EOS percentage markedly compared with A group ($p < 0.001$, Fig. 4B). We also determined levels of chemotactic factors for EOS in BALF. A significant increase in CCL5 and CCL11 levels were observed in BALF of A group compared with mice in NC group ($p < 0.01$ or $p < 0.001$, Fig. 4C-D). Acupuncture treated mice demonstrated an obvious decline in CCL5 and CCL11 levels compared with mice in A group ($p < 0.01$ or $p < 0.001$, Fig. 4C-D). However, sham acupuncture did not exert such reduction in these chemokine levels.

3.4 Acupuncture therapy diminished inflammatory cytokine levels in

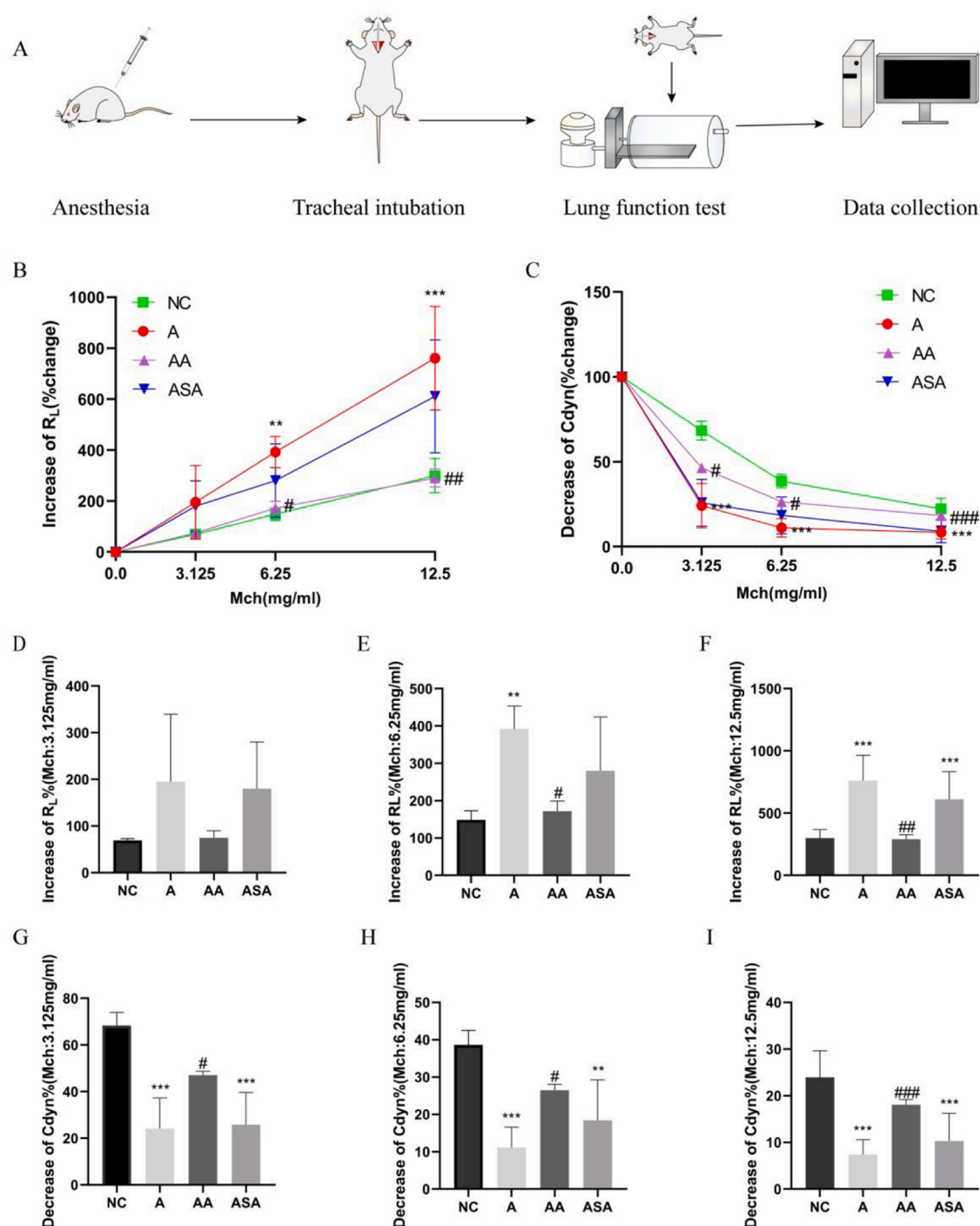


Fig. 2. Effect of acupuncture on lung function in OVA-induced mouse asthma model. A. Flow chart of the lung function detection. B. Changes in R_L to increasing doses of Mch. C. Changes in Cdyn to increasing doses of Mch. D-F. Changes in R_L to Mch dose of 3.125, 6.25 and 12.5 mg/ml. G-I. Changes in Cdyn to Mch dose of 3.125, 6.25 and 12.5 mg/ml. ** $p < 0.01$, *** $p < 0.001$ vs NC group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs A group. N = 5 mice/group.

serum and BALF of mouse asthma model induced by OVA inhalation

IgE, T helper cell (Th) 2 and Th17 associated cytokines involved in the inflammatory process of asthma. We detected IgE and IL-17A levels in BALF and IL-4, IL-5 and IL-13 levels in serum in each group. IgE and IL-17A levels were significantly increased in BALF of OVA-induced mouse asthma model compared with NC group ($p < 0.01$ or $p < 0.05$, Fig. 5C-D). Acupuncture resulted in a marked reduction in IgE and IL-17A levels ($p < 0.01$, Fig. 5C-D) and sham acupuncture did not induce such changes ($p > 0.05$). Mice in A group had elevated IL-4, IL-5 and IL-13 levels in serum compared with NC group ($p < 0.01$ or $p < 0.001$,

Fig. 5E-G), and acupuncture therapy demonstrated significant inhibitory effect on these cytokines ($p < 0.01$ or $p < 0.001$, Fig. 5E-G).

3.4. TMT-based quantitative proteomics analysis of the lung tissue

In order to further clarify the mechanism underlying the regulatory role of acupuncture treatment in asthma, we performed TMT-based quantitative proteomics assay and bioinformatics analysis. Proteomics data revealed that a total of 6078 quantifiable proteins were identified (Fig. 6C), and 564 (334 up-regulated and 230 down regulated) DEPs

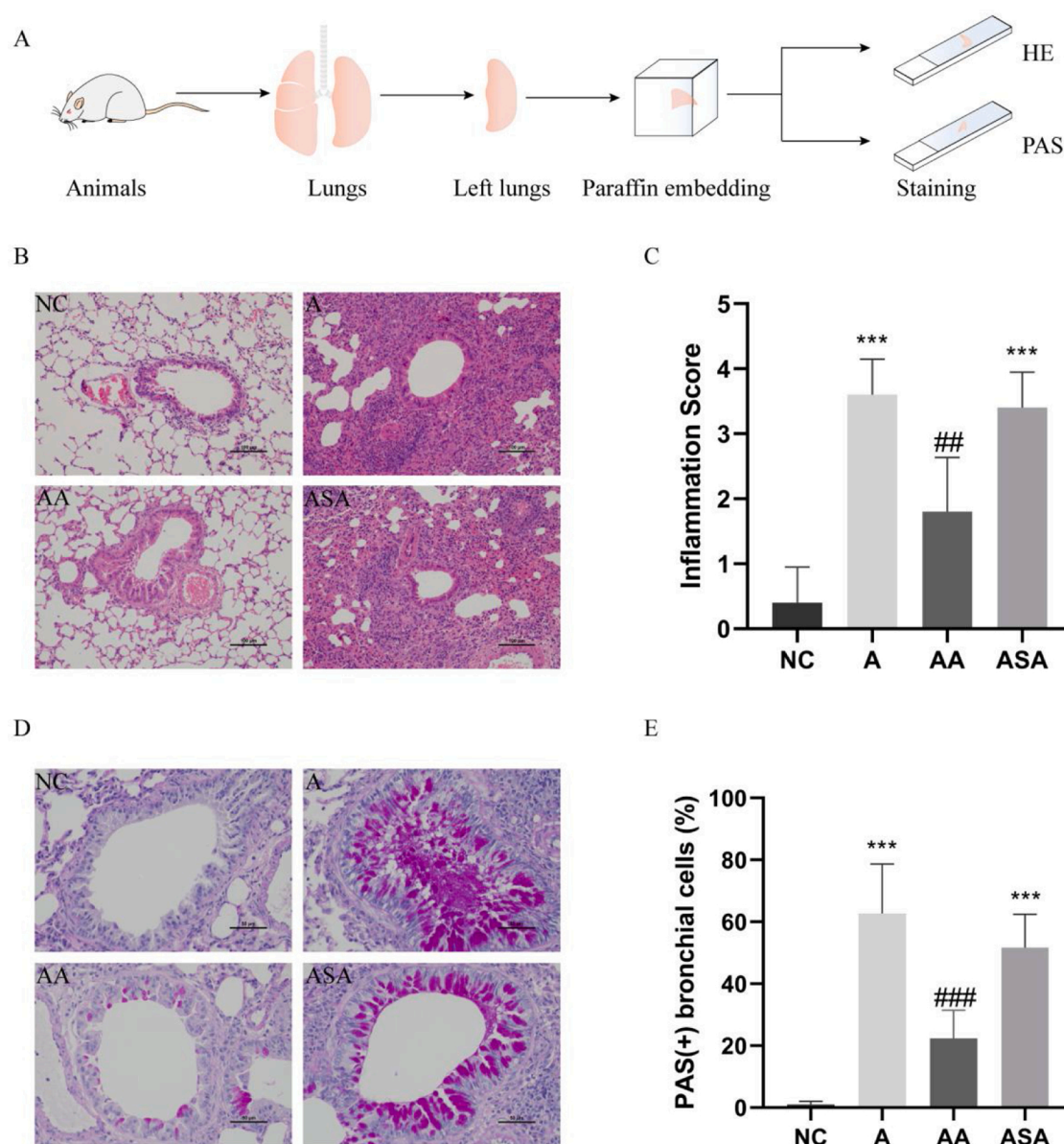


Fig. 3. Effect of acupuncture on airway inflammation in OVA-induced mouse asthma model. A. Flow chart of the lung histopathology detection. B. H&E-staining of the lung slices in each group, Bar = 100 μ m. C. Inflammatory scores of the H&E-staining results. D. PAS-staining of the lung slices in each group, Bar = 50 μ m. E. Percentage of PAS + epithelial cells. *** $p < 0.001$ vs NC group. ## $p < 0.01$, ### $p < 0.001$ vs A group. N = 5 mice/group.

were identified in A group versus NC group (Fig. 6G). Acupuncture treatment resulted in 667 DEPs (416 up-regulated and 251 down-regulated) compared to A group, and sham acupuncture administration induced 241 DEPs (154 up-regulated and 87 down-regulated) compared to A group (Fig. 6G). There were 116 DEPs (60 up-regulated and 56 down-regulated) between AA and ASA group. 86 overlapping DEPs were identified in NC, A and AA groups (Fig. 6H), and 49 overlapping DEPs were identified in NC, A and ASA groups. Among the 86 and 49 overlapping DEPs, we identified 41 DEPs (Table 1) regulated by acupuncture and 9 DEPs regulated by sham acupuncture. Based on the above data, we performed a systematic bioinformatics analysis, and performed functional classification, functional enrichment and cluster analysis based on functional enrichment of the 41 DEPs regulated by acupuncture. DEPs were enriched in the biological process (Fig. 7A), cellular component (Fig. 7B) and molecular function (Fig. 7C). Further analysis revealed that top 4 KEGG pathways were enriched ($p < 0.05$, Fig. 7D), which included SNARE interactions in vesicular transport, ferroptosis, endocrine and other factor-regulated calcium reabsorption,

and protein digestion and absorption. We focused on DEPs of SLC3A2 and ATP1A3 and enriched pathways of ferroptosis. Taken together, these findings suggested the regulation effect of acupuncture on asthma was mainly reflected in the regulation of the 41 DEPs.

3.5. Acupuncture therapy suppressed SLC3A2 and ATP1A3 protein expression and regulated oxidative stress of OVA-induced mouse asthma model

Based on literature and detailed information of our selected two enriched pathways, we verified DEPs of SLC3A2 and ATP1A3. SLC3A2 and ATP1A3 were measured in the lung tissues from mice in each group by means of immunohistochemical staining. An obvious upregulation of SLC3A2 and ATP1A3 was observed in OVA-induced mouse asthma model ($p < 0.001$ or $p < 0.01$, Fig. 8B-E), and acupuncture resulted in a significant downregulation of SLC3A2 and ATP1A3 protein expression ($p < 0.001$ or $p < 0.01$, Fig. 8B-E). SOD, MDA and GSH were involved in the process of oxidative stress. Experiments were carried out to detect

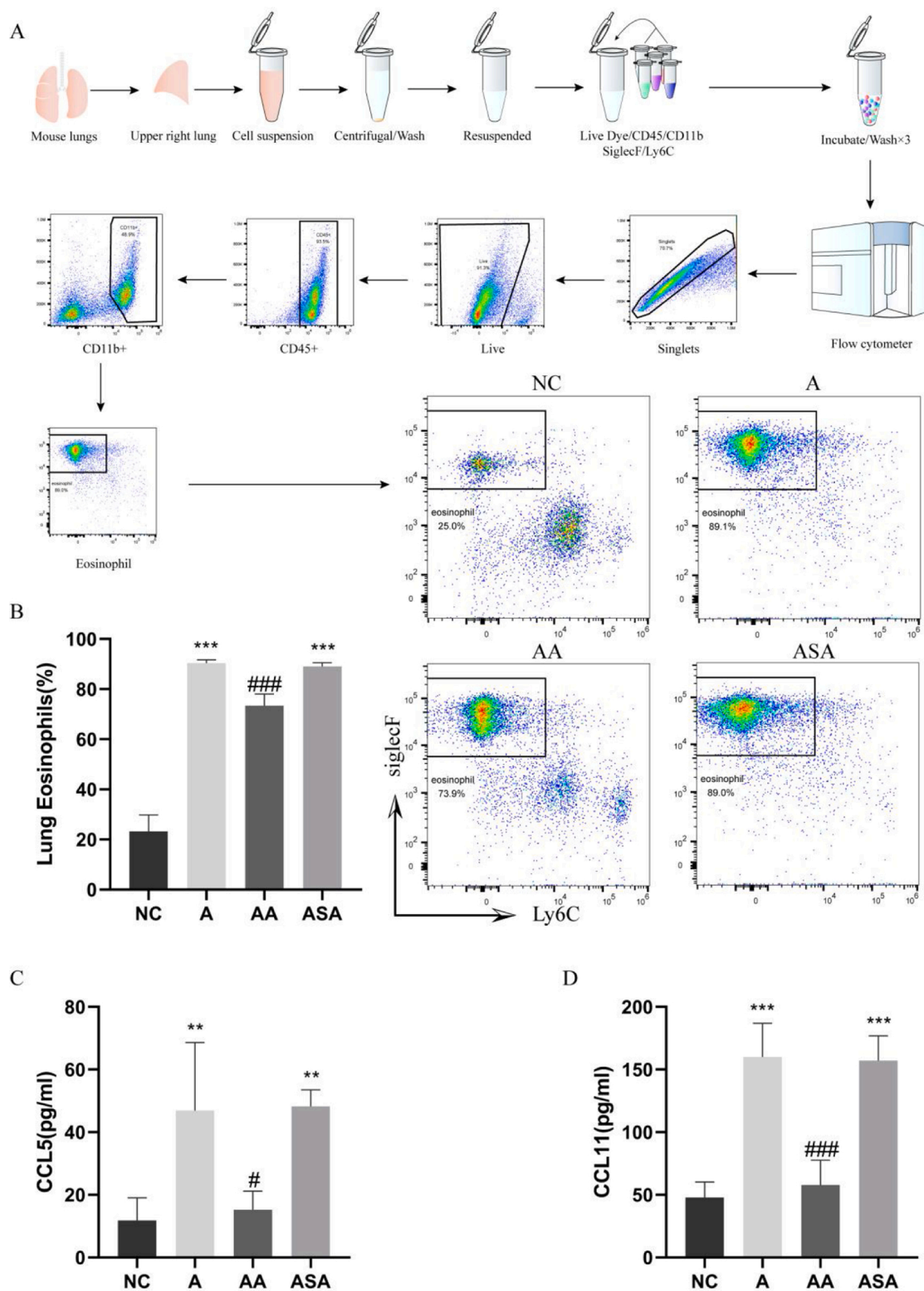


Fig. 4. Effect of acupuncture on EOS proportions and related chemokines in lung tissue of OVA-induced mouse asthma model. A. Flow chart of flow cytometric analysis. B. EOS proportions in the lung tissue. C. CCL5 level in BALF. D. CCL11 level in BALF. ** $p < 0.01$, *** $p < 0.001$ vs NC group. # $p < 0.05$, ### $p < 0.001$ vs A group. N = 5 mice/group.

the levels of SOD, MDA and GSH in the lung tissue homogenate of mice from each group. OVA inhalation induced a remarkably increase of MDA ($p < 0.001$, Fig. 8F) and acupuncture significantly reduced the MDA level ($p < 0.001$, Fig. 8F). Compared with NC group, mice in A group had a marked decrease in GSH and SOD ($p < 0.001$, Fig. 8G-H), and

acupuncture therapy resulted in remarkable elevation in GSH and SOD ($p < 0.001$, Fig. 8G-H). However, sham acupuncture did not remarkably influence the SOD, MDA and GSH levels compared with A group ($p > 0.05$).

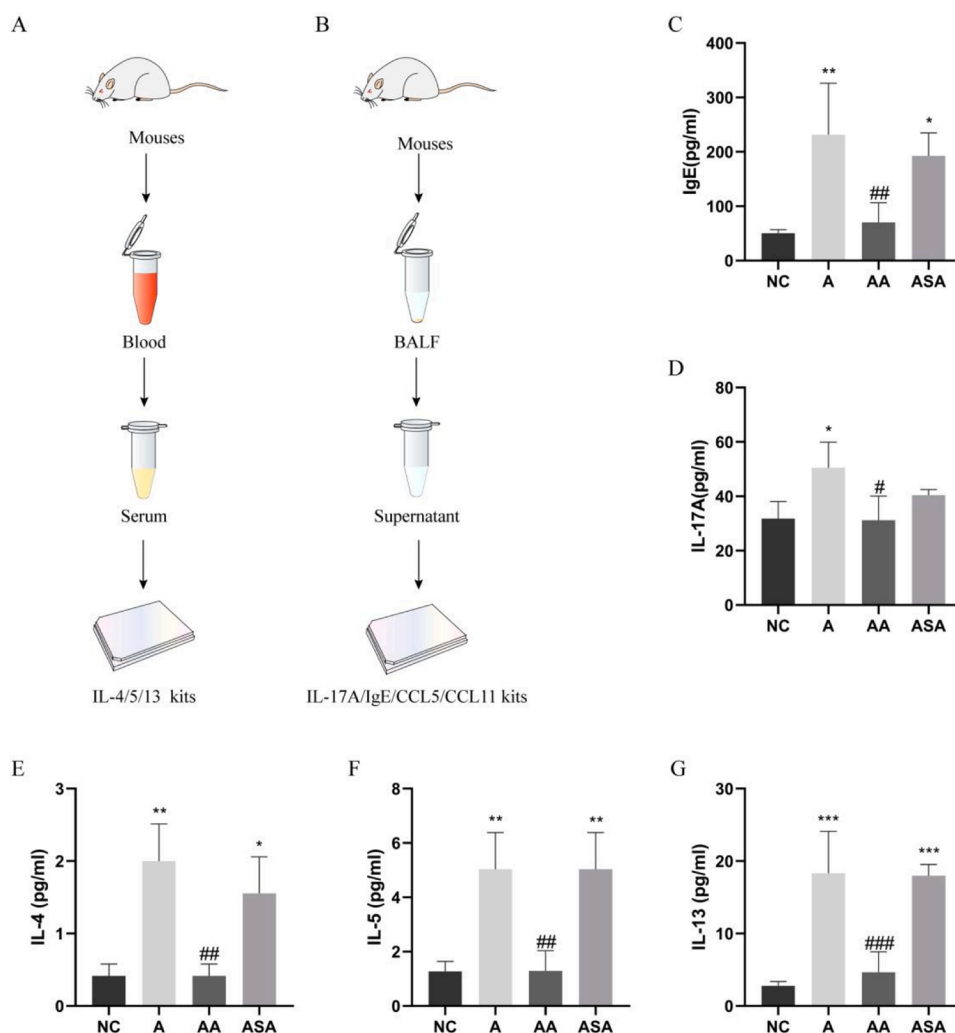


Fig. 5. Effect of acupuncture on inflammatory cytokines in OVA-induced mouse asthma model. A. Flowchart of cytokine levels of IL-4, IL-5, IL-13 in serum by ELISA assay. B. Flow chart of levels of IL-17A, IgE, CCL5, CCL11 in BALF by ELISA assay. C-D. IgE and IL-17A levels in BALF. E-G. IL-4, IL-5, IL-13 levels in serum. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs NC group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs A group. $N = 5$ mice/group.

4. Discussion

Asthma is a chronic inflammatory disease of the airways affecting 1-18% of the population in different countries [1]. Due to the highly heterogeneous of presentation and pathobiology, asthma treatment is challenging for clinicians. Acupuncture has been proven to be effective for the treatment of asthma [6]. Based on the efficacy of acupuncture in treating asthma, the WHO recommends asthma as an indication for acupuncture treatment. What's more, patients receiving acupuncture treatment often have improved quality of life and reduction in health-care cost. For decades, the progress made in improving our understanding of asthma pathogenesis and treatment has focused on airway inflammation. Herein, we reported that acupuncture was effective in treating asthma by attenuation of AHR, mucus secretion and airway inflammation.

AHR, inflammation and goblet cell hyperplasia are key features of asthma. Our data demonstrated that acupuncture could attenuate AHR to Mch provocation, inflammatory cell infiltration and mucus secretion in the lung tissue of OVA-induced asthma model, indicating the alleviation of characteristic pathological progress of asthma by acupuncture. Both the innate and adaptive immune responses are recognized to be critical mediators underlying allergic inflammation in asthma. Increased secretion of the Th2-associated cytokines IL-4, IL-5 and IL-13 were reported in asthma, participating in goblet cell metaplasia, airway

eosinophilia and progression of chronic inflammation of the airway [22, 23]. Consistent with previous reports, our results revealed the involvement of Th2 cytokines IL-4, IL-5 and IL-13 in the pathogenesis of inflammation in asthma, and acupuncture resulted in an obvious reduction in these inflammatory cytokine levels. The 2019 GINA guidelines suggest that patients inadequately controlled by high doses of ICS are likely to have residual type 2 inflammation and be candidates for an anti-type 2 biologic therapy [24]. Our results demonstrated a remarkable suppressive effect on Th2-associated cytokines by acupuncture, which in part explained the efficacy of acupuncture in treating asthma. EOS infiltration in the airways is a key feature of allergic asthma, contributing to asthma pathogenesis, inflammation, AHR and tissue remodeling. Th2-associated cytokine IL-5 is the most specific cytokine for the EOS lineage and is responsible for its expansion and survival [25,26]. In addition to being involved in Th2-associated inflammation, IL-13 has been ascribed a central pathogenic role in mucus production and bronchoconstriction in experimental models of asthma. The role of EOS is not limited to the effector phase of Th2 responses but also important for the induction of adaptive immunity [27] and EOS was shown to promote Th2 immunity [28,29]. Recent studies focused on the development of new agents to block EOS recruitment to inflammatory foci decrease EOS activation and survival [30]. The EOS levels increased significantly in our experiment, demonstrating that our observations in mice were consistent with previous data. The EOS

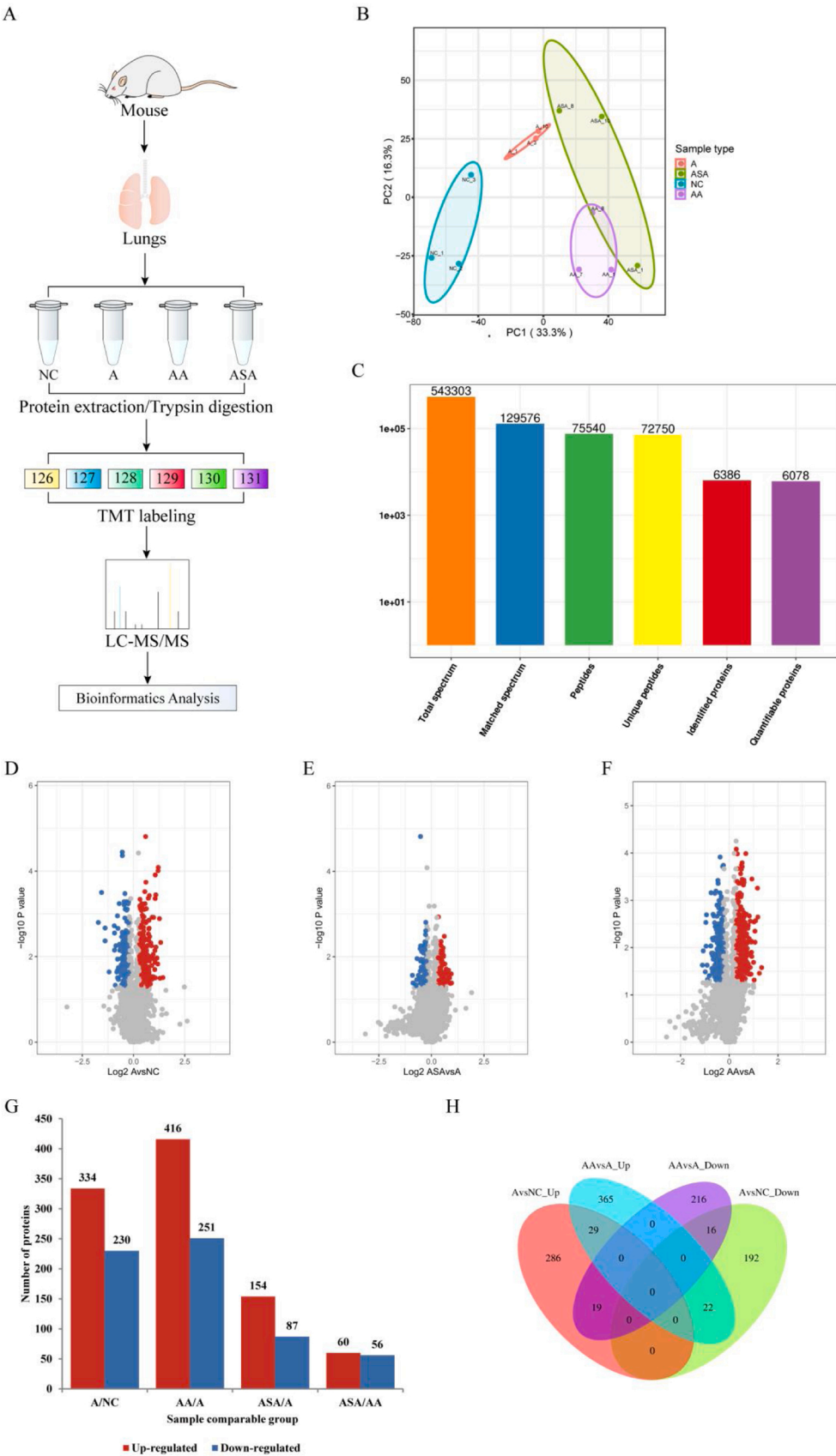


Fig. 6. Identification and quantitative analysis of proteins. A. Flow chart for the quantitative TMT proteomics approach. B. Principal component analysis (PCA) of samples. C. Basic statistics of MS results. D-F. Volcano plots showing the distribution of significance and fold change of identified proteins between the NC and A groups, A and AA groups, ASA and AA groups. The blue spots and red spots indicate significantly downregulated and upregulated proteins. G. The number of DEPs among each group. H. Venn diagrams showing the distribution of overlapping proteins among NC, A and AA groups. N = 3 mice/group.

Table 1
41DEPs regulated by acupuncture.

Protein accession	Gene name	Protein description	A/NC Ratio	A/NC Regulated Type	AA/A Ratio	AA/A Regulated Type	Subcellular localization	KEGG Gene
O09172	Gclm	Glutamate–cysteine ligase regulatory subunit OS = Musmusculus OX = 10090 GN = Gclm PE = 1 SV = 1	0.82	Down	1.3	Up	mitochondria	GCLM; glutamate–cysteine ligase regulatory subunit
O35295	Purb	Transcriptional activator protein Pur-beta OS = Musmusculus OX = 10090 GN = Purb PE = 1 SV = 3	0.803	Down	1.214	Up	extracellular	PURB; transcriptional activator protein Pur-beta
O70480	Vamp4	Vesicle-associated membrane protein 4 OS = Musmusculus OX = 10090 GN = Vamp4 PE = 1 SV = 1	1.323	Up	0.822	Down	cytoplasm	VAMP4; vesicle-associated membrane protein 4
O88983	Stx8	Syntaxin-8 OS = Musmusculus OX = 10090 GN = Stx8 PE = 1 SV = 1	1.448	Up	0.646	Down	cytoplasm	STX8; syntaxin 8
P01823	—	Ig heavy chain V region 36-60 OS = Musmusculus OX = 10090 PE = 1 SV = 1	1.654	Up	0.658	Down	cytoplasm	
P07759	Serpina3k	Serine protease inhibitor A3K OS = Musmusculus OX = 10090 GN = Serpina3k PE = 1 SV = 2	0.657	Down	1.407	Up	endoplasmic reticulum	SERPINA; serpin peptidase inhibitor, clade A
P10852	Slc3a2	4F2 cell-surface antigen heavy chain OS = Musmusculus OX = 10090 GN = Slc3a2 PE = 1 SV = 1	1.253	Up	0.787	Down	cytoplasm	SLC3A2, MDU1, CD98; solute carrier family 3, member 2
P56695	Wfs1	Wolframin OS = Musmusculus OX = 10090 GN = Wfs1 PE = 1 SV = 1	1.373	Up	0.77	Down	plasma membrane	WFS1; wolframin
P58058	Nadk	NAD kinase OS = Musmusculus OX = 10090 GN = Nadk PE = 1 SV = 2	1.362	Up	0.787	Down	nucleus	ppnK, NADK; NAD + kinase [EC:2.7.1.23]
Q3TNL8	Itprp	"Inositol 1,4,5-trisphosphate receptor-interacting protein OS = Musmusculus OX = 10090 GN = Itprp PE = 1 SV = 1"	0.655	Down	1.85	Up	plasma membrane	
Q3UL36	Arglu1	Arginine and glutamate-rich protein 1 OS = Musmusculus OX = 10090 GN = Arglu1 PE = 1 SV = 2	1.257	Up	0.818	Down	nucleus	ARGLU1; arginine and glutamate-rich protein 1
Q61247	Serpinf2	Alpha-2-antiplasmin OS = Musmusculus OX = 10090 GN = Serpinf2 PE = 1 SV = 1	0.691	Down	1.292	Up	extracellular	SERPINF2, AAP; alpha-2-antiplasmin
Q61559	Fcgrt	IgG receptor FcRn large subunit p51 OS = Musmusculus OX = 10090 GN = Fcgrt PE = 1 SV = 1	1.52	Up	0.693	Down	extracellular	
Q61838	Pzp	Pregnancy zone protein OS = Musmusculus OX = 10090 GN = Pzp PE = 1 SV = 3	0.738	Down	1.248	Up	extracellular	
Q6PIC6	Atp1a3	Sodium/potassium-transporting ATPase subunit alpha-3 OS = Musmusculus OX = 10090 GN = Atp1a3 PE = 1 SV = 1	2.047	Up	0.458	Down	plasma membrane	ATP1A; sodium/potassium-transporting ATPase subunit alpha [EC:3.6.3.9]
Q80TM9	Nisch	Nischarin OS = Musmusculus OX = 10090 GN = Nisch PE = 1 SV = 2	0.751	Down	1.495	Up	plasma membrane	
Q80UW2	Fbxo2	F-box only protein 2 OS = Musmusculus OX = 10090 GN = Fbxo2 PE = 1 SV = 1	1.653	Up	0.722	Down	cytoplasm	FBXO2, NFB42; F-box protein 2
Q8BH70	Fbxl4	F-box/LRR-repeat protein 4 OS = Musmusculus OX = 10090 GN = Fbxl4 PE = 1 SV = 1	1.961	Up	0.508	Down	mitochondria	FBXL4; F-box and leucine-rich repeat protein 4
Q8BZ98	Dnm3	Dynamin-3 OS = Musmusculus OX = 10090 GN = Dnm3 PE = 1 SV = 1	0.792	Down	1.403	Up	cytoplasm	DNM; dynaminGTPase [EC:3.6.5.5]
Q8C1B7	Septin11	Septin-11 OS = Musmusculus OX = 10090 GN = Septin11 PE = 1 SV=4	0.605	Down	1.213	Up	nucleus	SEPT6_8_11; septin 6/8/11
Q8C7V3	Utp15	U3 small nucleolar RNA-associated protein 15 homolog OS = Musmusculus OX = 10090 GN = Utp15 PE = 1 SV = 1	1.533	Up	0.788	Down	cytoplasm	UTP15; U3 small nucleolar RNA-associated protein 15
Q8CCS6	Pabpn1	Polyadenylate-binding protein 2 OS = Musmusculus OX = 10090 GN = Pabpn1 PE = 1 SV = 3	0.822	Down	1.342	Up	nucleus	PABPN1, PABP2; polyadenylate-binding protein 2
Q8CE50	Snx30	Sorting nexin-30 OS = Musmusculus OX = 10090 GN = Snx30 PE = 1 SV = 1	0.753	Down	1.523	Up	cytoplasm	SNX7_30; sorting nexin-7/30
Q8K1E0	Stx5	Syntaxin-5 OS = Musmusculus OX = 10090 GN = Stx5 PE = 1 SV = 3	1.518	Up	0.776	Down	plasma membrane	STX5; syntaxin 5
Q8VHQ9	Acot11	Acyl-coenzyme A thioesterase 11 OS = Musmusculus OX = 10090 GN = Acot11 PE = 1 SV = 1	1.761	Up	0.621	Down	nucleus	ACOT11; acyl-CoA thioesterases 11 [EC:3.1.2.-]
Q91V64	Isoc1	Isochorismatase domain-containing protein 1 OS = Musmusculus OX = 10090 GN = Isoc1 PE = 1 SV = 1	0.728	Down	1.432	Up	cytoplasm	
Q91VN6	Ddx41	Probable ATP-dependent RNA helicase DDX41 OS = Musmusculus OX = 10090 GN = Ddx41 PE = 1 SV = 2	1.436	Up	0.801	Down	cytoplasm	DDX41, ABS; ATP-dependent RNA helicase DDX41 [EC:3.6.4.13]
Q91ZU6	Dst		0.813	Down	1.244	Up	cytoplasm	DST; dystonin

(continued on next page)

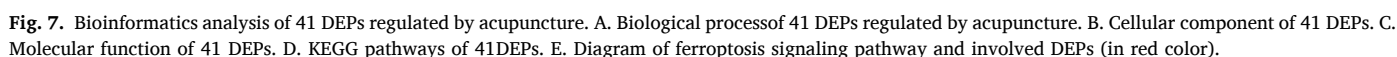
Table 1 (continued)

Protein accession	Gene name	Protein description	A/NC Ratio	A/NC Regulated Type	AA/A Ratio	AA/A Regulated Type	Subcellular localization	KEGG Gene
Q921L8	Galnt11	Dystonin OS = Musmusculus OX = 10090 GN = Dst PE = 1 SV = 2 Polypeptide N-acetylgalactosaminyltransferase 11 OS = Musmusculus OX = 10090 GN = Galnt11 PE = 1 SV = 1	0.678	Down	1.242	Up	mitochondria	GALNT1; polypeptide N-acetylgalactosaminyltransferase [EC:2.4.1.41]
Q99KN1	Arrdc1	Arrestin domain-containing protein 1 OS = Musmusculus OX = 10090 GN = Arrdc1 PE = 1 SV = 2	0.756	Down	1.241	Up	nucleus	
Q9CPQ3	Tomm22	Mitochondrial import receptor subunit TOM22 homolog OS = Musmusculus OX = 10090 GN = Tomm22 PE = 1 SV = 3	1.286	Up	0.732	Down	cytoplasm	TOM22; mitochondrial import receptor subunit TOM22
Q9CQ80	Vps25	Vacuolar protein-sorting-associated protein 25 OS = Musmusculus OX = 10090 GN = Vps25 PE = 1 SV = 1	0.817	Down	1.312	Up	cytoplasm	VPS25, EAP20; ESCRT-II complex subunit VPS25
Q9CR29	Ccdc43	Coiled-coil domain-containing protein 43 OS = Musmusculus OX = 10090 GN = Ccdc43 PE = 1 SV = 1	0.579	Down	1.785	Up	cytoplasm	
Q9D1G2	Pmvk	Phosphomevalonate kinase OS = Musmusculus OX = 10090 GN = Pmvk PE = 1 SV = 3	1.472	Up	0.665	Down	cytoplasm	PMVK; phosphomevalonate kinase [EC:2.7.4.2]
Q9D7N3	Mrps9	"28S ribosomal protein S9, mitochondrial OS = Musmusculus OX = 10090 GN = Mrps9 PE = 1 SV = 3"	1.257	Up	0.831	Down	mitochondria	RP-S9, MRPS9, rpsI; small subunit ribosomal protein S9
Q9D8T4	Tvp23b	Golgi apparatus membrane protein TVP23 homolog B OS = Musmusculus OX = 10090 GN = Tvp23b PE = 1 SV = 1	0.752	Down	1.364	Up	plasma membrane	
Q9D8 × 2	Ccdc124	Coiled-coil domain-containing protein 124 OS = Musmusculus OX = 10090 GN = Ccdc124 PE = 1 SV = 1	0.833	Down	1.337	Up	nucleus	
Q9DCB8	Isca2	"Iron-sulfur cluster assembly 2 homolog, mitochondrial OS = Musmusculus OX = 10090 GN = Isca2 PE = 1 SV = 2"	0.569	Down	1.386	Up	mitochondria	ISCA2; iron-sulfur cluster assembly 2
Q9JHQ0	Anxa9	Annexin A9 OS = Musmusculus OX = 10090 GN = Anxa9 PE = 2 SV = 2	0.714	Down	1.486	Up	cytoplasm	ANXA9; annexin A9
Q9JJQ0	Pigb	GPI mannosyltransferase 3 OS = Musmusculus OX = 10090 GN = Pigb PE = 2 SV = 2	0.785	Down	1.324	Up	plasma membrane	PIGB; phosphatidylinositol glycan, class B [EC:2.4.1.-]
Q9JMF7	Dolpp1	Dolichyldiphosphatase 1 OS = Musmusculus OX = 10090 GN = Dolpp1 PE = 2 SV = 1	1.573	Up	0.688	Down	cytoplasm	E3.6.1.43; dolichyldiphosphatase [EC:3.6.1.43]

proportion in the lungs was markedly inhibited by acupuncture therapy, indicating that acupuncture may exert anti-inflammatory effect in part by regulation of EOS. It's worth noting that, although acupuncture can reduce the percentage of EOS, the percentage of EOS in the acupuncture treatment group was still significantly higher than that in the NC group. These results indicated that acupuncture therapy had the potential to reduce EOS. However, EOS counts could not be reduced to normal levels by acupuncture. CCL5 (also called RANTES) was involved in recruitment of inflammatory cells and found to be elevated in patients with asthma [31–34]. CCL11 (also called eotaxin) played a crucial role in airway eosinophilia in asthma through modulation of the function of EOS. Patients with asthma always had elevated CCL11 levels [35]. Our results revealed a noteworthy augment in CCL5 and CCL11 levels in BALF of OVA-induced mouse asthma model, and acupuncture showed an obvious inhibitory effect on CCL5 and CCL11 levels as well as EOS proportions, which suggested that acupuncture could regulate the function of EOS as well as the associated chemokines, and further inhibited airway inflammation. IL-17A is the signature cytokine of Th17 cells, and its levels in lung biopsy specimens, sputum, and serum correlate with asthma severity [36–40]. Acupuncture induced a prominent inhibitory effect on IL-17A levels in BALF, indicating acupuncture may be effective in treating different asthma phenotypes. Several agents targeting key mediators of Th2 inflammatory cascades were developed by pharmaceutical companies, including humanized monoclonal anti-IgE, anti-IL-5, anti-IL-5R, anti-IL-4/13R antibodies. However, their efficacy in treating different processes of asthma pathogenesis is less

than satisfactory. In contrast to these agents, acupuncture therapy had showed significant anti-inflammatory effects in treating asthma, suggesting that acupuncture may have a wide range of anti-inflammatory effects in asthma.

Advances in technology have led to the detailed understanding of the pathogenesis of asthma. We used a TMT-based proteomics strategy to unravel the underlying mechanism of acupuncture in treating asthma. Proteomics data revealed that a total of 6078 quantifiable proteins were identified, and acupuncture treatment resulted in 667 DEPs (416 up-regulated and 251 down-regulated) compared to A group. Among the 667 DEPs, we identified 41 DEPs regulated by acupuncture. Systematic bioinformatics analysis revealed that top 4 KEGG pathways were enriched to be associated with the anti-inflammatory effect of acupuncture, which included SNARE interactions in vesicular transport, ferroptosis, endocrine and other factor-regulated calcium reabsorption, and protein digestion and absorption. Ferroptosis is peroxidation-induced non-apoptotic cell death in reactive oxygen species (ROS) or iron-dependent manner [41], and involves various biology processes, such as iron metabolism, lipid metabolism, oxidative stress and biosynthesis of nicotinamide adenine dinucleotide phosphate, GSH and coenzyme Q10 [42]. Effects of ferroptosis-inducing agents (FINs) on EOS death and allergic airway inflammation were investigated by researchers, and results revealed that FINs time and dose dependency induced cell death in human and mouse EOS, and antioxidants GSH and N-acetylcysteine could revert this process. Furthermore, FINs exerted a synergistic effect with dexamethasone to induce EOS death in vitro and



Asthma is a chronic inflammatory airway disease concomitant with oxidative stress, and inflammation in asthma induced enhanced oxidative stress in the pathogenesis of asthma, and the latter further induces mucus hypersecretion, AHR and activates various inflammatory signalling pathways resulting in progression of asthma pathology [48]. Oxidative stress biomarkers including lipid peroxidation marker MDA, antioxidants GSH and SOD were evaluated in the lung tissue of mice. GSH participates in eliminating ROS, and depletion of GSH induced accumulation of ROS and made cells more sensitive to ferroptosis [42]. Ferroptosis induced non-apoptotic cell death was found to result in GSH

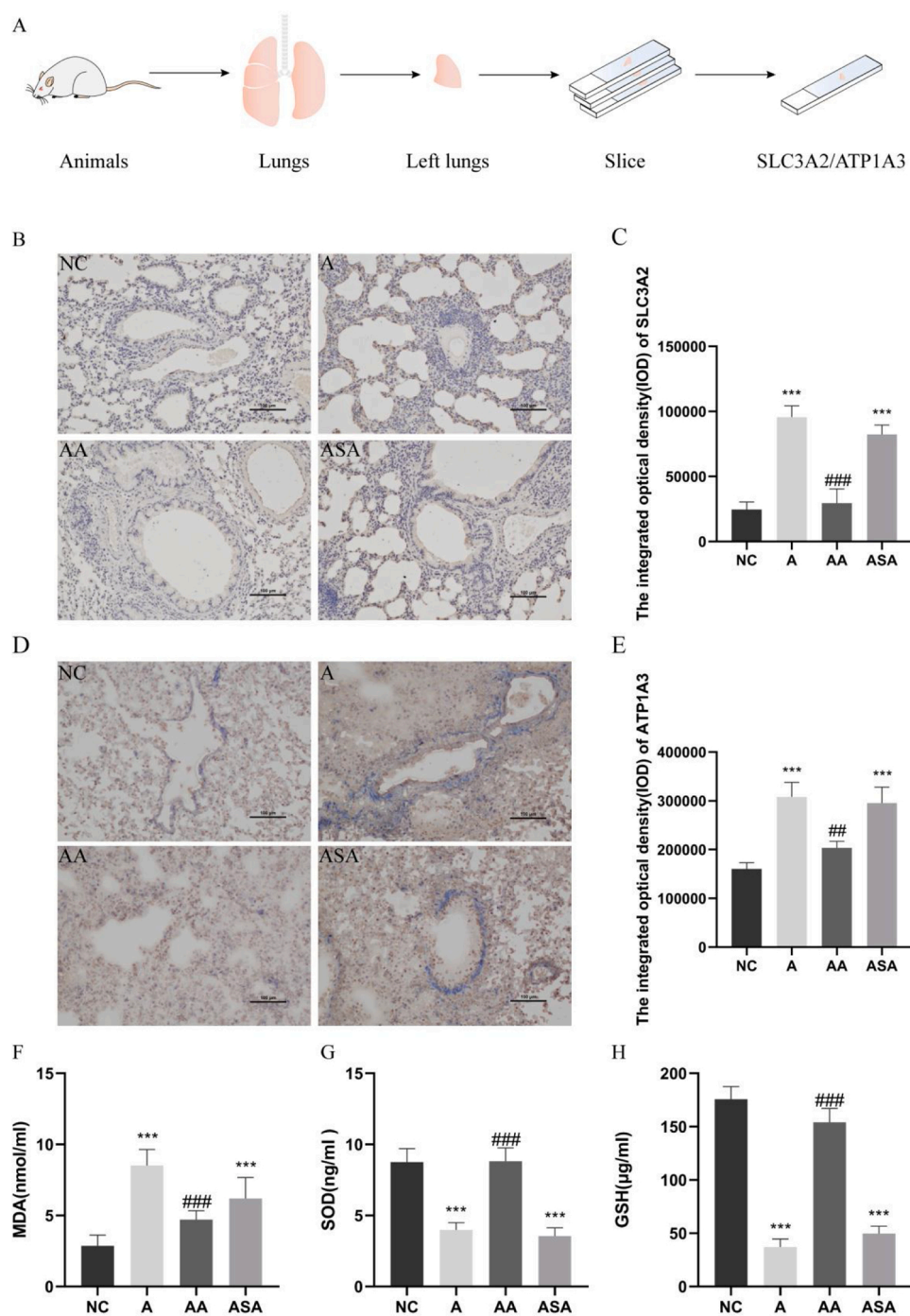


Fig. 8. Effect of acupuncture on DEPs of SLC3A2 and ATP1A3 expression and oxidative stress in OVA-induced mouse asthma model. A. Flow chart of immunohistochemical detection. B-C. Immunohistochemical analysis of SLC3A2 in the lung slices in each group, Bar = 100 μ m. D-E. Immunohistochemical analysis of ATP1A3 in the lung slices in each group, Bar = 100 μ m. F-H. Determination of SOD, MDA and GSH in the lung tissue. ** $p < 0.01$, *** $p < 0.001$ vs NC group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs A group. N = 5 mice/group.

depletion [49]. High levels of lipid peroxides were found in asthmatic airway epithelial cells, contributing to epithelial dysfunction, cell death and asthma exacerbation [50]. Consistent with previous reports that co-exposure of cold stress and PM_{2.5} in childhood of mice had reduced GSH and SOD, and elevated MDA levels in BALF [51], our data showed that OVA inhalation caused a reduction in GSH and SOD, and elevation of MDA levels in lung tissue. Furthermore, acupuncture reduced the MDA level and increased the GSH and SOD levels in the lung tissue, indicating the regulatory role of acupuncture on oxidative stress.

5. Conclusion

Collectively, our data defined acupuncture as an effective therapy for asthma, and proved that the attenuation of AHR, mucus secretion and

airway inflammation by acupuncture was associated with regulation of ferroptosis, SLC3A2 and ATP1A3 protein expression as well as oxidative stress. Results from our experiments revealed the anti-inflammatory effect of acupuncture in OVA-induced mouse asthma model, leading to a more effective approach to be chosen by patients in clinic.

Author contributions

WFT, YW and MD designed the study. WFT, MD, JC, FZT, XYZ, WQW, TW, JJQ, LY and SYW performed the experiments. WFT and MD analyzed the data and wrote the draft manuscript. YW and JCD revised the manuscript. All authors contributed to the article and approved the submitted version.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by grants from Young Elite Scientists Sponsorship Program by China Association for Science and Technology (grant no. 2018QNRC001), the National Natural Science Foundation of China (Grant No. 81704154) and Innovative research team of high-level local universities in Shanghai-Clinical and basic research on the prevention and treatment of some inflammatory diseases by integrative medicine.

References

- [1] Global Initiative for asthma, Global Strategy for Asthma Management and Prevention, Available from: 2020 WWW.ginasthma.org.
- [2] E.D. Bateman, S.S. Hurd, P.J. Barnes, J. Bousquet, J.M. Drazen, J.M. FitzGerald, P. Gibson, K. Ohta, P. O'Byrne, S.E. Pedersen, E. Pizzichini, S.D. Sullivan, S. E. Wenzel, H.J. Zar, Global strategy for asthma management and prevention: GINA executive summary, *Eur Respir J* 31 (2008) 143–178. *Eur Respir J* 51(2) (2018).
- [3] B. Brinkhaus, S. Roll, S. Jena, K. Icke, D. Adam, S. Binting, F. Lotz, S.N. Willich, C. M. Witt, Acupuncture in Patients with Allergic Asthma: A Randomized Pragmatic Trial, *J Altern Complement Med* 23 (4) (2017) 268–277.
- [4] T. Reinhold, B. Brinkhaus, S.N. Willich, C. Witt, Acupuncture in patients suffering from allergic asthma: is it worth additional costs? *J Altern Complement Med* 20 (3) (2014) 169–177.
- [5] Y. Yang, H. Chen, C. Zhao, R. Wang, [Studies on regulatory effects of acupuncture on mucosal secretory IgA in patients with allergic asthma], *Zhen Ci Yan Jiu* 20 (2) (1995) 68–70.
- [6] Y.Q. Yang, H.P. Chen, Y. Wang, L.M. Yin, Y.D. Xu, J. Ran, Considerations for use of acupuncture as supplemental therapy for patients with allergic asthma, *Clin Rev Allergy Immunol* 44 (3) (2013) 254–261.
- [7] M.I. Rizal, Y. Soeroro, B. Sulijaya, B.F. Assiddiq, E.W. Bachtar, B.M. Bachtar, Proteomics approach for biomarkers and diagnosis of periodontitis: systematic review, *Heliyon* 6 (6) (2020), e04022.
- [8] Y. Ucal, A. Ozpinar, Proteomics in thyroid cancer and other thyroid-related diseases: A review of the literature, *Biochim Biophys Acta Proteins Proteom* 1868 (11) (2020), 140510.
- [9] L. Pan, Y. Tian, H. Sun, Y. Wang, G. Liu, TMT-based proteomics analysis reveals the efficacy of Jianghuo formula in improving the lipid profiles of dyslipidemia rats, *J Ethnopharmacol* (2020), 113390.
- [10] D. Zhou, J. Liu, Y. Hang, T. Li, P. Li, S. Guo, T. Liu, Z. Xia, Y. Wang, TMT-based proteomics analysis reveals the protective effects of Xuefu Zhuyu decoction in a rat model of traumatic brain injury, *J Ethnopharmacol* 258 (2020), 112826.
- [11] T. Liu, J. Zhou, H. Cui, P. Li, J. Luo, T. Li, F. He, Y. Wang, T. Tang, iTRAQ-based quantitative proteomics reveals the neuroprotection of rhubarb in experimental intracerebral hemorrhage, *J Ethnopharmacol* 232 (2019) 244–254.
- [12] K. Zhang, M. He, D. Su, X. Pan, Y. Li, H. Zhang, J. Yang, C. Wu, Quantitative proteomics reveal antidepressant potential protein targets of xiaochaihutang in corticosterone induced model of depression, *J Ethnopharmacol* 231 (2019) 438–445.
- [13] Y. Lao, X. Wang, N. Xu, H. Zhang, H. Xu, Application of proteomics to determine the mechanism of action of traditional Chinese medicine remedies, *J Ethnopharmacol* 155 (1) (2014) 1–8.
- [14] Y.H. Gao, S.P. Chen, J.Y. Wang, L.N. Qiao, F.Y. Meng, Q.L. Xu, J.L. Liu, Differential proteomics analysis of the analgesic effect of electroacupuncture intervention in the hippocampus following neuropathic pain in rats, *BMC Complement Altern Med* 12 (2012) 241.
- [15] L.H. Li, Q.M. Huang, M. Barbero, L. Liu, T.T. Nguyen, M. Beretta-Piccoli, A.L. Xu, L. J. Ji, Quantitative proteomics analysis to identify biomarkers of chronic myofascial pain and therapeutic targets of dry needling in a rat model of myofascial trigger points, *J Pain Res* 12 (2019) 283–298.
- [16] S.S. Li, Commentary—the proteomics: a new tool for Chinese medicine research, *Am J Chin Med* 35 (6) (2007) 923–928.
- [17] X. Qiu, L. Gong, X. Sun, J. Guo, A.M. Chodara, Efficacy of acupuncture and identification of tear protein expression changes using iTRAQ quantitative proteomics in rabbits, *Curr Eye Res* 36 (10) (2011) 886–894.
- [18] Y. Wu, Y. Jiang, X. Shao, X. He, Z. Shen, Y. Shi, C. Wang, J. Fang, Proteomics analysis of the amygdala in rats with CFA-induced pain aversion with electroacupuncture stimulation, *J Pain Res* 12 (2019) 3067–3078.
- [19] Y. Xu, X. Li, D. Man, X. Su, G. A, iTRAQ-based proteomics analysis on insomnia rats treated with Mongolian medical warm acupuncture, *Biosci Rep* 40 (5) (2020).
- [20] S. Myou, A.R. Leff, S. Myo, E. Boetticher, J. Tong, A.Y. Meliton, J. Liu, N.M. Munoz, X. Zhu, Blockade of inflammation and airway hyperresponsiveness in immune-sensitized mice by dominant-negative phosphoinositide 3-kinase-TAT, *J Exp Med* 198 (10) (2003) 1573–1582.
- [21] W. Bao, Y. Zhang, M. Zhang, A. Bao, X. Fei, X. Zhang, X. Zhou, Effects of ozone repeated short exposures on the airway/lung inflammation, airway hyperresponsiveness and mucus production in a mouse model of ovalbumin-induced asthma, *Biomed Pharmacother* 101 (2018) 293–303.
- [22] G.L. Chupp, R. Kaur, A. Mainardi, New Therapies for Emerging Endotypes of Asthma, *Annu Rev Med* 71 (2020) 289–302.
- [23] M. Kudo, Y. Ishigatsubo, I. Aoki, Pathology of asthma, *Front Microbiol* 4 (2013) 263.
- [24] L.P. Boulet, H.K. Reddel, E. Bateman, S. Pedersen, J.M. FitzGerald, P.M. O'Byrne, The Global Initiative for Asthma (GINA): 25 years later, *Eur Respir J* 54 (2) (2019).
- [25] A. Matucci, E. Maggi, A. Vultaggio, Eosinophils, the IL-5/IL-5Ralpha axis, and the biologic effects of benralizumab in severe asthma, *Respir Med* 160 (2019), 105819.
- [26] H.F. Rosenberg, K.D. Dyer, P.S. Foster, Eosinophils: changing perspectives in health and disease, *Nat Rev Immunol* 13 (1) (2013) 9–22.
- [27] C. Mesnil, S. Raulier, G. Paulissen, X. Xiao, M.A. Birrell, D. Pirotton, T. Janss, P. Starkl, E. Ramery, M. Henket, F.N. Schleich, M. Radermecker, K. Thielemans, L. Gillet, M. Thiry, M.G. Belvisi, R. Louis, C. Desmet, T. Marichal, F. Bureau, Lung-resident eosinophils represent a distinct regulatory eosinophil subset, *J Clin Invest* 126 (9) (2016) 3279–3295.
- [28] R. Jimenez-Saiz, V.C. Anipindi, H. Galipeau, Y. Ellenbogen, R. Chaudhary, J. F. Koenig, M.E. Gordon, T.D. Walker, T.S. Mandur, S. Abed, A. Humbles, D.K. Chu, J. Erjefalt, K. Ask, E.F. Verdu, M. Jordana, Microbial Regulation of Enteric Eosinophils and Its Impact on Tissue Remodeling and Th2 Immunity, *Front Immunol* 11 (2020) 155.
- [29] E.A. Jacobsen, A.D. Doyle, D.C. Colbert, K.R. Zellner, C.A. Protheroe, W.E. LeSuer, N.A. Lee, J.J. Lee, Differential activation of airway eosinophils induces IL-13-mediated allergic Th2 pulmonary responses in mice, *Allergy* 70 (9) (2015) 1148–1159.
- [30] C.D. Lucas, D.A. Dorward, S. Sharma, J. Rennie, J.M. Felton, A.L. Alessandri, R. Duffin, J. Schwarze, C. Haslett, A.G. Rossi, Wagonin induces eosinophil apoptosis and attenuates allergic airway inflammation, *Am J Respir Crit Care Med* 191 (6) (2015) 626–636.
- [31] W.H. Alturaki, Evaluation of C-C Chemokine Ligand 5 (CCL5) Chemokine, Interleukin 5 (IL-5) Cytokine, and Eosinophil Counts as Potential Biomarkers in Saudi Patients with Chronic Asthma During Sandstorms, *Cureus* 12 (4) (2020) e7809.
- [32] C. Hamsten, A. Haggmark, J. Grundstrom, M. Mikus, C. Lindskog, J.R. Konradsen, A. Eklund, G. Pershagen, M. Wickman, J. Grunewald, E. Melen, G. Hedlin, P. Nilsson, M. van Hage, Protein profiles of CCL5, HPGDS, and NPSR1 in plasma reveal association with childhood asthma, *Allergy* 71 (9) (2016) 1357–1361.
- [33] H. Huang, W. Nie, Y. Zang, J. Chen, Q. Xiu, Association between CC motif chemokine ligand 5 (CCL5) polymorphisms and asthma risk: an updated meta-analysis, *J Investig Allergol Clin Immunol* 25 (1) (2015) 26–33.
- [34] M. Isgro, L. Bianchetti, M.A. Marini, A. Bellini, M. Schmidt, S. Mattoli, The C-C motif chemokine ligands CCL5, CCL11, and CCL24 induce the migration of circulating fibrocytes from patients with severe asthma, *Mucosal Immunol* 6 (4) (2013) 718–727.
- [35] D. Wu, J. Zhou, H. Bi, L. Li, W. Gao, M. Huang, I.M. Adcock, P.J. Barnes, X. Yao, CCL11 as a potential diagnostic marker for asthma? *J Asthma* 51 (8) (2014) 847–854.
- [36] Q. Guan, B. Yang, R.J. Warrington, S. Mink, C. Kalicinsky, A.B. Becker, E. Simons, Z. Peng, Myeloid-derived suppressor cells: Roles and relations with Th2, Th17, and Treg cells in asthma, *Allergy* 74 (11) (2019) 2233–2237.
- [37] D. Kim, J.W. McAlees, L.J. Bischoff, D. Kaur, L.K. Houshel, J. Gray, J. Hargis, X. Davis, P.L. Dudas, H. Deshmukh, I.P. Lewkowich, Combined administration of anti-IL-13 and anti-IL-17A at individually sub-therapeutic doses limits asthma-like symptoms in a mouse model of Th2/Th17 high asthma, *Clin Exp Allergy* 49 (3) (2019) 317–330.
- [38] N. Krishnamoorthy, D.N. Douda, T.R. Bruggemann, I. Ricklefs, M.G. Duvall, R. E. Abdounour, K. Martinod, L. Tavares, X. Wang, M. Cernadas, E. Israel, D. T. Mauger, E.R. Bleeker, M. Castro, S.C. Erzurum, B.M. Gaston, N.N. Jarjour, S. Wenzel, E. Dunican, J.V. Fahy, D. Irimia, D.D. Wagner, B.D. Levy, L. National Heart, I. Blood Institute Severe Asthma Research Program, Neutrophil cytoplasts induce Th17 differentiation and skew inflammation toward neutrophilia in severe asthma, *Sci Immunol* 3 (26) (2018).
- [39] D. Liu, Y. Tan, O. Bajinka, L. Wang, Z. Tang, Th17/IL-17 Axis Regulated by Airway Microbes Get Involved in the Development of Asthma, *Curr Allergy Asthma Rep* 20 (4) (2020) 11.
- [40] X. Zhang, M. Zhang, M. Jiang, G. Nong, Effect of IL7 on Th17 cell responses in a mouse model of neutrophilic asthma, *Mol Med Res* 22 (2) (2020) 1205–1212.
- [41] S.J. Dixon, K.M. Lemberg, M.R. Lamprecht, R. Skouta, E.M. Zaitsev, C.E. Gleason, D.N. Patel, A.J. Bauer, A.M. Cantley, W.S. Yang, B. Morrison 3rd, B.R. Stockwell, Ferroptosis: an iron-dependent form of nonapoptotic cell death, *Cell* 149 (5) (2012) 1060–1072.
- [42] Y. Qiu, Y. Cao, W. Cao, Y. Jia, N. Lu, The Application of Ferroptosis in Diseases, *Pharmacol Res* 159 (2020), 104919.
- [43] Y. Wu, H. Chen, N. Xuan, L. Zhou, Y. Wu, C. Zhu, M. Li, Q. Weng, J. Shen, H. Zhang, B. Zhang, F. Lan, L. Xia, X. Xiong, Z. Li, Y. Zhao, M. Wu, S. Ying, W. Li, H. Shen, Z. Chen, Induction of ferroptosis-like cell death of eosinophils exerts synergistic effects with glucocorticoids in allergic airway inflammation, *Thorax* (2020).
- [44] G.N. Chiduzu, R.M. Johnson, G.S.A. Wright, S.V. Antonyuk, S.P. Muench, S. Hasnain, LAT1 (SLC7A5) and CD98hc (SLC3A2) complex dynamics revealed by single-particle cryo-EM, *Acta Crystallogr D Struct Biol* 75 (Pt 7) (2019) 660–669.
- [45] D. Digomann, A. Linge, A. Dubrovskaya, SLC3A2/CD98hc, autophagy and tumor radioresistance: a link confirmed, *Autophagy* 15 (10) (2019) 1850–1851.
- [46] S.J. Dixon, D.N. Patel, M. Welsch, R. Skouta, E.D. Lee, M. Hayano, A.G. Thomas, C. E. Gleason, N.P. Tatonetti, B.S. Slusher, B.R. Stockwell, Pharmacological inhibition

- of cystine-glutamate exchange induces endoplasmic reticulum stress and ferroptosis, *Elife* 3 (2014), e02523.
- [47] M.T. Sweney, T.M. Newcomb, K.J. Swoboda, The expanding spectrum of neurological phenotypes in children with ATP1A3 mutations, Alternating Hemiplegia of Childhood, Rapid-onset Dystonia-Parkinsonism, CAPOS and beyond, *Pediatr Neurol* 52 (1) (2015) 56–64.
- [48] A. Balkrishna, S.K. Solleti, H. Singh, S. Verma, N. Sharma, P. Nain, A. Varshney, Herbal decoction Divya-Swasari-Kwath attenuates airway inflammation and remodeling through Nrf-2 mediated antioxidant lung defence in mouse model of allergic asthma, *Phytomedicine* 78 (2020), 153295.
- [49] W.S. Yang, R. SriRamaratnam, M.E. Welsch, K. Shimada, R. Skouta, V. S. Viswanathan, J.H. Cheah, P.A. Clemons, A.F. Shamji, C.B. Clish, L.M. Brown, A. W. Girotti, V.W. Cornish, S.L. Schreiber, B.R. Stockwell, Regulation of ferroptotic cancer cell death by GPX4, *Cell* 156 (1–2) (2014) 317–331.
- [50] S.E. Wenzel, Y.Y. Tyurina, J. Zhao, C.M. St Croix, H.H. Dar, G. Mao, V.A. Tyurin, T. S. Anthonymuthu, A.A. Kapralov, A.A. Amoscato, K. Mikulska-Ruminska, I. H. Shrivastava, E.M. Kenny, Q. Yang, J.C. Rosenbaum, L.J. Sparvero, D.R. Emlet, X. Wen, Y. Minami, F. Qu, S.C. Watkins, T.R. Holman, A.P. VanDemark, J. A. Kellum, I. Bahar, H. Bayir, V.E. Kagan, PEBP1 Wardens Ferroptosis by Enabling Lipoxygenase Generation of Lipid Death Signals, *Cell* 171 (3) (2017), 628–641 e26.
- [51] J. Zhou, J. Xu, F. Geng, L. Peng, X. Ye, D. Yang, J. Zhao, Q. Sun, Childhood co-exposure of cold stress and PM2.5 aggravates the susceptibility and severity of asthma in adulthood of mice, *Environ Toxicol* (2020).