

Regulation of Ferroptosis and ACSL4-15LO1 pathway Contributed to the Anti-Asthma Effect of Acupuncture

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ABSTRACT

Acupuncture has been frequently used in China for the treatment of asthma for thousands of years. Ferroptosis was recently revealed to be involved in several pathological conditions including asthma. However, the detailed links between ferroptosis and airway inflammation in asthma, as well as the detailed regulation of acupuncture on these disorders remains unclear. Our results demonstrated that the non-haem Fe^{2+} level increased markedly in the lung tissue of mouse asthma model, and positively correlated with R_L and IL-4 level in BALF. Furthermore, lipid peroxidation markers MDA and GSSG increased remarkably in OVA-induced experimental asthma mice. Up-regulation of lipid peroxidation associated proteins ACSL4 and 15-LO1 was also observed in OVA-induced experimental asthma mice. To demonstrate the role of ferroptosis in asthma and the effect of acupuncture on these disorders, ferroptosis-induction agent erastin and ferroptosis-inhibition agent Fer-1 were used, and our data demonstrated that erastin could augment lung inflammation and lipid peroxidation in OVA induced asthma model. Fer-1 was able to relieve AHR, lung inflammation, non-haem Fe^{2+} level, lipid peroxidation and ferroptosis related pathway ACSL4-15-LO1 in OVA-induced experimental asthma mice. Acupuncture treatment alleviated R_L , lung inflammation as well as type 2 cytokines IL-4 and IL-13 levels of mouse experimental asthma model induced by OVA inhalation. What's more, acupuncture significantly reduced the MDA and GSSG levels, the non-haem Fe^{2+} level and ACSL4-15-LO1 proteins expression. Acupuncture also relieved erastin-induced exacerbation in lung inflammation and lipid peroxidation in ferroptosis. Acupuncture treatment could relieve ferroptosis related exacerbation in airway inflammation and the mechanism was associated with down regulation of ferroptosis related pathway ACSL4-15-LO1. Our study provided insights into the underlying mechanisms for the protective effects of acupuncture and highlighted a therapeutic potential of acupuncture treatment in the attenuation of lipid peroxidation and ferroptosis in asthma.

Key words: asthma, airway inflammation, lipid peroxidation, ferroptosis, acupuncture

Abbreviations: OVA: ovalbumin; Mch: methacholine; AHR: airway hyperresponsiveness; MDA: malondialdehyde; GSH: glutathione; R_L: Airway resistance; BALF: bronchoalveolar lavage fluid; ROS: reactive oxygen species; HDM: house dust mite; TCM: traditional Chinese medicine; WHO: World Health Organization; Fer-1: ferrostatin; ELISA: enzyme linked immunosorbent assay; GSSG: oxidized GSH; GPX4: glutathione peroxidase 4; 15-LO1: 15 Lipxygenase 1; ACSL4: acyl-CoA synthetase long-chain family member 4; SLC7A11: catalytic subunit solute carrier family 7 member 11; N: normal control group; A: OVA-induced asthma model group; AA: the acupuncture treatment group; ASA: sham acupuncture group; AF: asthma model combined with ferroptosis-inhibition agent Fer-1 treatment group; PBS: phosphate-buffered saline; AE: asthma model combined with ferroptosis-inducing agent erastin; AEA: an asthma model combined with erastin and acupuncture treatment group; EDTA: Ethylene Diamine Tetraacetic Acid; H&E: hematoxylin and eosin; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; HRP: horseradish peroxidase; ANOVA: analysis of variance; PLOOH: phospholipid hydroperoxides; WBC: leukocytes; Neu: neutrophils; Lym: lymphocytes; Mon: monocytes; Eos: eosinophils; Bas: Basophils.

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1. Introduction

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. Asthma is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation[1][1]. Asthma is a serious global health problem affecting 1-18% of the populations in different countries, with its prevalence increasing worldwide, especially in densely industrialized urban regions. Asthma is the second leading cause of death among chronic respiratory diseases, and the Global Burden of Disease estimated that 420,000 people in the world died from asthma in 2016, more than 1000 per day[2]. It is reported that asthma can be triggered by factors such as allergen or irritant exposure in the environment, exercise, change in weather, et al. Studies reveal that numerous agents in the environment have a clear relationship to asthma outcomes[3-5]. Environmental pollution not only plays a role in asthma mortality, but also in hospitalizations, emergency department visits and exacerbations. The Asthma Global Burden Report estimated that asthma-related emergency room visits occurred between 9–23 million and 5–10 million of attributable to ozone and PM_{2.5}, respectively[6].

Air pollutants cause oxidative injury to the airways that leads to inflammation and remodeling, indicating the activation of oxidative stress and oxidant injury participated in the pathological progress of asthma. Ferroptosis is an iron-dependent, nonapoptotic cell death modality characterized by aberrant accumulation of lipid hydroperoxides and oxidative phospholipid damage[7]. Previous data suggested that altered level of lung iron is associated with lung inflammation and asthma[8, 9]. Furthermore, plasma iron and malondialdehyde (MDA) levels, a marker of lipid peroxidation and oxidative stress are significantly elevated in asthmatic patients compared to healthy controls[10]. Ferroptosis was recently revealed to be involved in several pathological conditions including asthma[11-13]. Our previous study revealed that the airway hyperresponsiveness (AHR), airway inflammation, lipid peroxidation and reactive oxygen species (ROS) levels increased in environmental allergens house dust mite (HDM)-induced asthma, and HDM inhalation induced ferroptosis in the lungs[14].

Therefore, ferroptosis prevention represents an attractive therapeutic avenue for asthma treatment.

Acupuncture has been frequently used in China for the treatment of a variety of conditions including asthma for thousands of years. As one of the most popular therapies of traditional Chinese medicine (TCM), the therapeutic effects of acupuncture on asthma is well claimed with high safety and no side effects, therefore, the World Health Organization (WHO) recommends the use of acupuncture for 64 conditions including asthma. Previous studies have shown that acupuncture was effective for improving the asthmatic symptoms [15-17] with high safety and no side effects, and 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 [15]. Our previous data demonstrated that acupuncture was effective in treating asthma by attenuation of AHR, mucus secretion and airway inflammation, and therapeutic effect is related to regulation of ferroptosis and oxidative stress [18]. However, the detailed links between ferroptosis and airway inflammation in asthma, as well as the effect of acupuncture on these disorders is further to be clarified. We established ovalbumin (OVA) induced mouse asthma model and investigated the changes in the lung function, inflammation, lung iron levels, lipid peroxidation and pathways associated with ferroptosis. We used ferroptosis-induction agent erastin and ferroptosis-inhibition agent Fer-1 to further ascertain whether ferroptosis is involved in the airway inflammation of asthma and the role of acupuncture in these disorders. Our data demonstrated that erastin could augment lung inflammation and lipid peroxidation, and Fer-1 was able to relieve AHR, lung inflammation, non-haem Fe^{2+} level, lipid peroxidation and ferroptosis-related pathway in OVA-induced experimental asthma mice. Acupuncture treatment significantly reduced the MDA and GSSG levels, the non-haem Fe^{2+} level as well as ferroptosis-related pathway ACSL4-15-LO1 expression in the lung tissue. Acupuncture treatment also relieved ferroptosis-induction agent erastin-induced exacerbation in lung inflammation and lipid peroxidation in ferroptosis. These results in all indicated that ferroptosis is involved in airway inflammation in OVA induced mouse asthma model.

Acupuncture treatment could relieve ferroptosis-related exacerbation in airway inflammation and the mechanism was associated with down regulation of ferroptosis related pathway ACSL4-15-LO1. Our study provided insights into the underlying mechanisms for the protective effects of acupuncture and highlighted a therapeutic potential of acupuncture treatment in the attenuation of lipid peroxidation and ferroptosis in asthma.

2. Material and methods

2.1 Reagents and kits

Ovalbumin (OVA, grade V or II), aluminum hydroxide, methacholine (Mch) and pentobarbital sodium were purchased from Sigma-Aldrich (Merck KGaA). Isoflurane was purchased from Shenzhen Ruiwode Life Technology Co. Ltd. Ferrostatin (Fer-1) and erastin were purchased from Selleck Chemicals. Sterile stainless steel acupuncture needles (Hwato brand, 13 mm length, 0.25 mm diameter) were purchased from Suzhou Medical Supplies Co. Ltd. IL-4 and IL-13 Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd. The MDA, glutathione (GSH) and oxidized GSH (glutathione disulfide, GSSG) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. The non-haem Fe^{2+} detection kit was purchased from Dojin Do, Japan. Anti-glutathione peroxidase 4 (GPX4) and anti-15 Lipoygenase 1 (15-LO1) antibodies were purchased from Abcam, and anti-acyl-CoA synthetase long-chain family member 4 (ACSL4) and anti-catalytic subunit solute carrier family 7 member 11 (SLC7A11) antibodies were purchased from Novus.

2.2 Experimental animals and study protocol

BALB/c mice (female, 6-8 weeks, special pathogen-free) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd, and placed in the animal holding unit building (School of Pharmacy, Fudan University). Mice were housed under pathogen free conditions with air conditioning and a 12h light/dark cycle with food and water freely available. All animal experiments in this study were approved by the Ethics Committee of Animal Experiments of Fudan University (2021JS-HSYY-531).

2.3 Asthma model establishment and treatment

For experiment one, mice were randomly divided into 5 groups, including normal control group (N, n=8), an OVA induced asthma group (A, n=8), an asthma model combined with acupuncture treatment group (AA, n=8), an asthma model combined with sham acupuncture treatment group (ASA, n=8) and asthma model combined with ferroptosis-inhibition agent Fer-1 treatment group (AF, n=8). As one of the lipophilic radical traps, Fer-1 could neutralize the deleterious effects of lipid peroxidation in ferroptosis execution [7, 19], so it was selected for usage in our study. As shown in Figure 1A, on day 0 and day 7, mice were sensitized with 0.2 ml normal saline containing 20 µg of OVA and 2 mg of aluminum hydroxide. From days 14 to 16, mice were put into plexiglas chambers and challenged with 3% OVA for 30 min every day using ultrasonic nebulizers (402AI, Yuyue Medical Equipment Company, Jiangsu, China). Mice in group A were challenged with 3% OVA, and mice in groups AA and ASA were administrated with acupuncture or sham acupuncture combined with 3% OVA inhalation, and mice in AF group were intraperitoneally injected with Fer-1 (1mg/kg body weight) combined with 3% OVA inhalation from days 21 to 23. Mice in N group were managed with phosphate-buffered saline (PBS) with the same method.

To perform the acupuncture treatment, the acupoints of Dazhui (GV 14), bilateral Fengmen (BL12) and Feishu (BL13) were selected according to the theory of acupuncture. The irrelevant distal acupoints of bilateral Huantiao (GB30, Figure 1B), which has been shown to be neuroanatomic unrelated to the lungs and does not have therapeutic effect on asthma according to TCM theory, was selected for sham acupuncture administration. From day 21, both acupuncture and sham acupuncture procedures were performed 1 h before each challenge. Mildly anesthetized mice with isoflurane were administrated with acupuncture, and sterile needles were inserted to a depth of 3mm into the acupoints (GV14, BL12 and BL13) and withdrawn after 30 min. For bilateral acupoints of GB30, acupuncture needles were inserted to a depth of 5mm. 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.

For experiment two, mice were randomly divided into 4 groups, including normal

control group (N, n=8), an OVA induced asthma group (A, n=8), an asthma model combined with ferroptosis-inducing agent erastin (an inhibitor of system x_c^- -cystine/glutamate antiporter) group (AE, n=8) and an asthma model combined with erastin and acupuncture treatment group (AEA, n=8). Erastin is considered as class I ferroptosis inducer[20], so it was selected in our experiment to induce ferroptosis in mice. Mice in N and A groups received the same administrations as in experiment one. From days 14 to 16, mice were inhaled with 3% OVA solution by ultrasonic nebulizer for 30 minutes each time. On days 21 to 23, mice in AE group received intraperitoneal injection of erastin (30mg/kg body weight) one hour before the OVA challenge, and mice in AEA group were administered with acupuncture under mild anesthesia with isoflurane one hour before the intraperitoneal injection of erastin and OVA challenge. Mice in N group were challenged with PBS.

2.4 Measurement of AHR

The measurements of airway responsiveness were performed after the last challenge. Mice were inhaled with gradient doses of Mch under anaesthesia with pentobarbital sodium using Buxco Pulmonary System (DSI Buxco Electronics, NY, USA) to assess pulmonary resistance (R_L). Mice were weighed, intraperitoneally anesthetized with pentobarbital sodium (50 mg/kg), incised and intubated via the trachea. Afterwards, mice were placed in the chamber and the tracheal intubation was connected to the ventilator. After the reach of a stable baseline airway pressure (<5% variation over 2.5 min), PBS and Mch were administered to mice via nebulisation. Results were expressed as the percentage change in baseline following Mch challenge.

2.5 Histological analysis

The middle lobe of the right lung was fixed in 4% paraformaldehyde and embedded in paraffin, and then cut into thickness of 4 μ m. Afterwards, lung tissue sections were stained with hematoxylin and eosin (H&E) to observe the inflammatory cell infiltration around the bronchus and vessels.

2.6 Leukocyte differential counts in Bronchoalveolar Lavage Fluid (BALF)

BALF was collected by flushing the right lungs three times with 0.3 ml PBS using a 1-ml syringe inserted into a cannula. The samples were centrifuged, and the

supernatant was collected for BALF cytokines analysis. Afterwards, the pellet containing the cells was resuspended in 100 µl PBS, and the inflammatory cell counts were determined using the BC-5000 Vet auto hematology analyzer (MINDRAY Medical International Co., Ltd.).

2.7 ELISA assay

The IL-4 and IL-13 levels in BALF collected from mice in each group were assayed using ELISA kits (Multi Science, China) according to the manufacturer's instructions.

2.8 MDA, GSH and GSSG assay

After AHR detection, the intact left and both upper and lower lobes of the right lung were removed and snap frozen in liquid nitrogen and then stored at -80°C for further usage. The lung tissue homogenates were prepared to assay the production of MDA, which is a degradation product of polyunsaturated-lipid hydroperoxides and biomarker for lipid peroxidation [21] as well as GSH and GSSG according to the manufacturer's instructions.

2.9 Iron assay

The non-haem Fe^{2+} level in the lung tissues from mice in each group was assessed using Iron Assay Kit according to the manufacturer's instructions.

2.10 Western blot analysis

Lipid peroxidation is influenced by several lipids and enzymes including ACSL4 and 15LO1. Furthermore, GPX4 is the major protective mechanism of biomembranes against peroxidation damage in ferroptosis, and SLC7A11 is required for GSH, which is the substrate of GPX4 [22]. These results demonstrated that ACSL4, 15-LO1, GPX4, and SLC7A11 were mediators of lipid peroxidation and ferroptosis, and had been reported to be involved in the ferroptosis pathway [23-25]. The expression levels of these proteins were assayed using western blotting. Proteins were separated on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (15 µg per lane), and transferred onto polyvinylidene fluoride (PVDF) membranes. After blockage in 5% non-fat milk, the PVDF membranes were incubated with GPX4 (11000), ACSL4 (11000), 15-LO1 (11000) and SLC7A11 (11000) antibodies at 4°C overnight. Afterwards, the PVDF membranes were incubated with anti-mouse or anti-

rabbit horseradish peroxidase(HRP)-conjugated secondary antibodies for 1 h at room temperature. Actin was used as the loading Control. Quantitative analysis was performed from three independent experiments with Image J.

2.11 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. AHR data were analysed using two-way repeated measures analysis of variance(ANOVA) with Dunnett's post-hoc test. Comparisons between multiple groups were performed using one-way ANOVA with uncorrected Tukey's post-hoc test. Correlation analyses were performed using Spearman rank correlation. A p value<0.05 means statistically significant.

3. Results

3.1 Acupuncture and Fer-1attenuated AHR of OVA-induced experimental asthma mice

We assessed AHR of mice in each group within 24h after the last OVA challenge. After OVA sensitization and challenge, mice demonstrated an obvious increase in R_L with increasing Mch concentration compared with mice in N group ($p<0.05$ or $p<0.001$ or $p<0.0001$, Figure 1C-F). Acupuncture treatment significantly decreased R_L (Mch=6.25 mg/ml, Mch=12.5 mg/ml and Mch=25 mg/ml, $p<0.05$ or $p<0.01$ or $p<0.0001$, Figure 1C-F). Sham acupuncture administration did not suppress the R_L compared with OVA-induced experimental asthma mice ($p>0.05$). Furthermore, intraperitoneal injection of ferroptosis-inhibition agent Fer-1 induced an obvious decrease of R_L compared with OVA-induced experimental asthma mice (Mch=25 mg/ml, $p<0.01$, Figure 1C, 1F), partly indicating the involvement of ferroptosis in the pathogenesis of AHR in asthma.

3.2 Acupuncture and Fer-1relieved airway inflammation and type 2 cytokines secretion of OVA-induced experimental asthma mice

We further evaluated the inhibitory effect of acupuncture on airway inflammation. Inflammatory cells infiltration around the peribronchial were observed in OVA-induced experimental asthma mice demonstrated by H&E assay (Figure 2A). Acupuncture treatment significantly decreased the inflammatory cell counts around the

peribronchial compared with the OVA-induced experimental asthma mice. Sham acupuncture did not alleviate the inflammation around the airways. Mice administered with Fer-1 induced significantly remission in lung inflammation, which was comparable to that of the acupuncture treatment.

Significant increase in the total leukocyte (Figure 2B, $p < 0.01$) along with a remarkable enhancement of eosinophil counts (Figure 2B, $p < 0.001$) were observed in the OVA-induced experimental asthma mice group tested by the leukocyte differential counts, and acupuncture treatment obviously inhibited the total leukocyte and eosinophil counts in BALF (Figure 2B, $p < 0.05$ or $p < 0.01$). In contrast, sham acupuncture-treated mice exhibited much higher total leukocyte (Figure 2B, $p < 0.01$), monocyte (Figure 2B, $p < 0.05$) and eosinophil counts (Figure 2B, $p < 0.01$) compared to mice in the N group, and no decrease in these cell counts compared with mice in the A group (Figure 2B, $p > 0.05$), clearly suggesting the invalid function of sham acupuncture in attenuation of inflammatory cell counts. On the contrary, inhibition of total leukocyte (Figure 2B, $p < 0.05$) and eosinophil (Figure 2B, $p < 0.01$) counts by Fer-1 were observed, indicating the involvement of ferroptosis in inflammation in asthma.

We next analyzed type 2 cytokine levels in BALF, and our results revealed that IL-4 and IL-13 levels were significantly increased in BALF of OVA-induced mouse asthma model compared with N group ($p < 0.01$, Figure 2C-D). After acupuncture treatment, IL-4 and IL-13 levels reduced markedly ($p < 0.05$) and sham acupuncture did not induce such changes ($p > 0.05$) compared with OVA-induced experimental asthma mice. Mice treated with Fer-1 had decreased IL-4 and IL-13 levels in BALF compared with mice in A group ($p < 0.001$ or $p < 0.05$). These results demonstrated that acupuncture and Fer-1 could relieve lung inflammation and type 2 cytokines secretion, and ferroptosis might be involved in airway inflammation in asthma.

3.3 Acupuncture and Fer-1 suppressed lung lipid peroxidation

Since lipid peroxidation is one hallmark of ferroptosis, we firstly assessed whether lipid peroxidation was increased in the lungs of OVA-induced experimental asthma mice. MDA, GSH and GSSG were involved in the process of lipid peroxidation, and

their levels in the lung tissue homogenate were detected. MDA and GSSG increased remarkably in OVA-induced experimental asthma mice ($p < 0.001$ or $p < 0.0001$, Figure 3A, 3C), which were consistent with previous results [25]. To determine whether acupuncture had the ability to suppress lipid peroxidation in asthma, we investigated changes in MDA, GSH and GSSG in the lung. Acupuncture significantly reduced the MDA and GSSG levels ($p < 0.05$ or $p < 0.0001$, Figure 3A, 3C) compared with mice in A group. There was no significant difference in GSH levels among all groups ($p > 0.05$, Figure 3B). Fer-1 administration also induced decrease in MDA and GSSG levels ($p < 0.01$ or $p < 0.0001$, Figure 3A, 3C). However, sham acupuncture did not remarkably influence the MDA and GSSG levels compared with mice in A group ($p > 0.05$). Collectively, these data indicated that acupuncture and Fer-1 were effective in suppression of lipid peroxidation in ferroptosis.

3.4 Acupuncture and Fer-1 lowered lung iron level

Ferroptosis is an iron-dependent, lipid peroxidation enhanced cell death modality. To further determine the change in the non-haem iron level, we assayed the non-haem iron level in the lung tissue of mice in each group. The non-haem Fe^{2+} level was found to increase in OVA-induced experimental asthma mice (Figure 4A, $p < 0.001$). Acupuncture and Fer-1 administration markedly reduced the non-haem Fe^{2+} level in the lung tissue compared with mice in A group (Figure 4A, $p < 0.01$ or $p < 0.05$).

Correlation analysis between the Fe^{2+} level and the AHR to Mch was performed, and the results demonstrated that the non-haem Fe^{2+} level positively correlates with R_L (Figure 4B, $r = 0.639$ or $r = 0.696$ or $r = 0.741$, $p < 0.05$ or $p < 0.01$). We further performed the correlation analysis between the Fe^{2+} level and the type 2 cytokine levels in BALF. A positive relationship was observed between Fe^{2+} level and IL-4 level in BALF (Figure 4C, $r = 0.633$, $p < 0.05$). However, we did not observe a positive relationship between the Fe^{2+} level and IL-13 level in BALF (Figure 4C, $r = 0.433$, $p > 0.05$). Our findings revealed that elevated Fe^{2+} level correlated with increase in AHR and IL-4 level in BALF.

3.5 Acupuncture and Fer-1 inhibited ferroptosis associated pathways

To evaluate the regulatory role of acupuncture in ferroptosis associated pathways, we performed western blotting assay with specific antibodies. Up-regulation of ACSL4 and 15-LO1 proteins expression were observed in OVA-induced experimental asthma mice (Figure 5A, C-D, $p < 0.001$ or $p < 0.0001$). Moreover, Up-regulation of GPX4 and SLC7A11 proteins expression demonstrated in western blotting bands were also observed in OVA-induced experimental asthma mice (Figure 5A), nevertheless with no statistical difference revealed by semi-quantitative analysis of WB bands (Figure 5B, E, $p > 0.05$). Treatment with acupuncture and Fer-1 significantly decreased ACSL4 and 15-LO1 proteins expression (Figure 5A, C-D, $p < 0.05$ or $p < 0.01$) compared with mice in A group. However, acupuncture and Fer-1 treatment induced no obvious differences compared with mice in A group revealed by semi-quantitative analysis of WB bands of GPX4 and SLC7A11 (Figure 5B, E, $p > 0.05$). Sham Acupuncture did not revert the ACSL4, 15LO1, GPX4 and SLC7A11 expression pattern compared with OVA-induced experimental asthma mice (Figure 5A-E, $p > 0.05$). Taken together, these findings demonstrated that OVA inhalation induced significant up-regulation of ferroptosis related pathway ACSL4-15LO1, and acupuncture could serve as an efficient treatment for inhibition of ferroptosis related pathway ACSL4-15LO1.

3.6 Acupuncture relieved erastin-induced exacerbation in lung inflammation

To demonstrate further the specific role of ferroptosis in asthma, we used ferroptosis-induction agent erastin to ascertain whether erastin could augment AHR and lung inflammation in asthma, we further investigated the AHR and lung inflammatory changes in mice received OVA inhalation combined with ferroptosis-induction agent erastin administration. Increase in R_L were observed in mice of A and AE group compared with mice in N group (Figure 6A-D, $p < 0.05$ or $p < 0.01$ or $p < 0.001$). However, we did not observe an obvious enhancement of R_L in mice received OVA inhalation combined with erastin administration compared with mice only received OVA inhalation (Figure 6A-D, $p > 0.05$). Acupuncture treatment was able to attenuate R_L compared with mice received OVA inhalation combined with erastin administration (Figure 6A, C, D, $p < 0.01$). On the contrary, mice received OVA inhalation combined with erastin administration displayed an enhancement of lung

inflammation compared with mice only received OVA inhalation, and treatment with acupuncture effectively inhibit the exacerbation in lung inflammation compared with mice received OVA inhalation combined with erastin administration (Figure 6E).

OVA inhalation and administration with erastin led to elevation of total leucocytes and eosinophils as well as IL-4 and IL-13 levels (Figure 6G-H, $p < 0.05$ or $p < 0.01$) compared with mice in N group (Figure 6F, $p < 0.05$ or $p < 0.01$). However, we did not observe an obvious increase in these cell counts (Figure 6F, $p > 0.05$) and IL-4 and IL-13 levels in BALF (Figure 6G-H, $p > 0.05$) of mice received OVA inhalation combined with erastin administration compared with mice only received OVA inhalation. Acupuncture treatment suppressed the total leukocyte (Figure 6F, $p < 0.05$) and eosinophil (Figure 6F, $p < 0.05$) counts as well as IL-4 and IL-13 levels (Figure 6G-H, $p < 0.01$) in BALF compared with mice received OVA inhalation combined with erastin administration. These data indicated that acupuncture was effective in attenuation of erastin induced augmentation of AHR and lung inflammation.

3.7 Acupuncture alleviated erastin-induced aggravation in lipid peroxidation and ferroptosis associated pathways

To demonstrate further the specific role of ferroptosis in asthma, we used ferroptosis-induction agent erastin to explore whether erastin could aggravate lipid peroxidation and ferroptosis associated pathways in OVA-induced experimental asthma mice. Compared to mice in A group, the MDA (Figure 7A, $p < 0.01$) and GSSG (Figure 7C, $p < 0.01$) levels in lung were observed significantly increased in the erastin administration combined with OVA inhalation group. To validate the anti-ferroptosis role of acupuncture treatment, we treated mice with acupuncture, and our results revealed that acupuncture could attenuate erastin-induced aggravation in lipid peroxidation which included MDA and GSSG levels in lung tissue (Figure 7A,C, $p < 0.01$).

Ferroptosis associated pathways were also investigated, and up-regulation of 15LO1 and ACSL4 were observed both in A and AE group (Figure 8A,C,D, $p < 0.05$ or $p < 0.01$). Moreover, administration with erastin up-regulated the 15LO1 and ACSL4 and protein expression demonstrated in the western blot bands (Figure 8A),

however, semi-quantitative analysis of WB bands showed no statistical difference in erastin-induced up-regulation of 15LO1 and ACSL4 protein expression compared with mice in A group (Figure 8C,D, $p>0.05$). Ferroptosis-induction agent erastin also led to down-regulation of SLC7A11 and GPX4 protein expression demonstrated in the western blot bands (Figure 8A), however, semi-quantitative analysis of WB bands showed no statistical difference in erastin-induced down-regulation of SLC7A11 and GPX4 protein expression compared with mice in A group (Figure 8B,E, $p>0.05$). Acupuncture treatment down-regulated 15LO1 and ACSL4 and up-regulated SLC7A11 and GPX4 protein expression compared with mice in erastin administration group (Figure 8A), however, there were no statistical difference demonstrated in results from semi-quantitative analysis (Figure B-E, $p>0.05$).

4. Discussion

Asthma is a chronic inflammatory disorder of the airways in which gene-environment interactions involving a variety of cell types play an important role [26, 27]. Asthma is usually associated with AHR and airway inflammation in which type 2 inflammation plays significant roles [28]. Exposure to harmful stimuli, including internal, environmental, and/or allergenic components, may all hamper epithelial barrier function, leading to an increased type 2 inflammation [29, 30]. In our study, OVA inhalation led to increase in AHR, lung inflammation as well as type 2 cytokines elevation in BALF. Moreover, elevation in the total leukocyte and eosinophils were observed in the OVA-induced experimental asthma mice group, which were consistent with previous reports. Acupuncture treatment significantly decreased AHR and type 2 cytokines including IL-4 and IL-13 levels. Furthermore, acupuncture treatment induced decreased inflammatory cell counts around the peribronchial demonstrated in H&E results. A variety of inflammatory cells participated in pathogenesis of asthma, and we showed that acupuncture treatment also obviously inhibited the total leukocyte and eosinophil counts in BALF. These data in all indicated the involvement of airway inflammation and AHR in the pathogenesis of asthma as well as the efficacy of acupuncture treatment in the attenuation of inflammation and AHR in asthma.

The lung is a vital organ for gas exchange and is constantly exposed to harmful airborne, which usually induces lipid peroxidation and oxidative stress damage to lung tissue. The lipid peroxidation and oxidative stress-mediated cellular processes drive the development of asthma pathogenesis[31]. The lung inflammation in asthma is associated with increased lipid peroxidation and oxidative stress in the airways[25]. Since lipid peroxidation is one hallmark of ferroptosis, to confirm whether lipid peroxidation was increased in the lungs of OVA-induced experimental asthma mice, we examined the lipid peroxidation markers including MDA and GSSG in the lung tissue. Previous study revealed that fine particulate matter (PM_{2.5}) and ozone (O₃) might exert oxidative damage in the nose, which was hypothesized to be associated with worsened asthma symptoms and MDA level in nasal fluid could be used as a biomarker for monitoring asthma control in relation to air pollution exposure[32]. Another study revealed that MDA combined with the total antioxidant capacity, hydrogen peroxide and total nitric oxide concentrations could be used as new markers to monitor the omalizumab treatment efficacy in severe allergic asthma patients[33]. Additionally, patients with asthma also had significantly higher GSSG levels in erythrocyte hemolysates compared with healthy controls[34]. Consistent with previous reports, our results demonstrated that MDA and GSSG in the lung tissue increased remarkably in OVA-induced experimental asthma mice, indicating the involvement of lipid peroxidation in asthma.

Ferroptosis occurs via an iron-catalyzed process of lipid peroxidation initiated through non-enzymatic Fenton reactions and enzymatic mechanisms[22, 35]. Ferroptosis is determined by the balance between iron accumulation-induced oxidation and the antioxidant system that avoids lipid peroxidation[22]. Previous reports have shown that lipid peroxidation and ferroptosis play a critical role in regulating airway inflammation and driving the key features of asthma[14, 25, 36]. We first investigated the non-haem iron level in the lung tissue of mice in each group. The non-haem Fe²⁺ level was found to be increased in OVA-induced experimental asthma mice, which was consistent with previous reports that asthma is associated with altered iron homeostasis[8]. Acupuncture markedly reduced the non-haem

Fe²⁺ level in the lung tissue of asthmatic mice. Furthermore, the non-haem Fe²⁺ level positively correlates with AHR as well as type 2 cytokine IL-4 level in BALF. To better understand the specific role of ferroptosis in asthma, small-molecule-mediated ferroptosis was induced, and the results indicated that administration with erastin induced an enhancement of lung inflammation demonstrated by H&E analysis. Aggravation in lipid peroxidation was also observed in erastin administration. We also used ferroptosis-inhibition agent Fer-1 to further ascertain whether ferroptosis is involved in the pathogenesis of asthma. Our results revealed that Fer-1 administration alleviated AHR, lung inflammation, IL-4 and IL-13 levels as well as the total leukocytes and eosinophils in BALF, non-haem Fe²⁺ level and lipid peroxidation in OVA-induced experimental asthma mice. To better understand the regulatory role of acupuncture in lipid peroxidation and ferroptosis, we performed several investigations, and our data demonstrated that acupuncture significantly reduced the MDA and GSSG levels as well as the non-haem Fe²⁺ level in the lung tissue. Lipid peroxidation damage contributed to diminished lung function and frequent exacerbations despite the often and prolonged use of systemic, high dose corticosteroids, promoted corticosteroid insensitivity in asthma and COPD[37]. It was very noteworthy that our results demonstrated the effectiveness of acupuncture in inhibiting lipid peroxidation as well as inflammation in asthma, suggesting the better potential of acupuncture treatment of asthma. GSH is a ubiquitous antioxidant, and is required for proper functioning of GPX4. However, there was no significant change in GSH levels in the lung revealed by our data.

Ferroptosis is a form of regulated cell death regulated by ACSL4, 15LO1, GPX4 and SLC7A11. ACSL4 and 15LO1 are involved in the process of lipid peroxidation in ferroptosis[23, 38, 39] and previous study demonstrates that ACSL4 is an essential component for ferroptosis execution, and ACSL4 inhibition is a viable therapeutic approach to prevent ferroptosis-related diseases[23]. GPX4/ACSL4 double knockout cells presented an unprecedented resistance to ferroptosis. SLC7A11 is the catalytic subunit of System X_c⁻, which is the cystine/glutamate antiporter promoting the exchange of extracellular cystine and intracellular glutamate across the plasma

membrane[22].Cystine in the cell is an essential precursor for the synthesis of GSH, and the latter is required for the function of GPX4to eliminate the production of phospholipid hydroperoxides(PLOOH), the major mediator of chain reactions in lipoxygenases.In order to exploit the molecular mechanisms contributing to the alleviation of ferroptosis in asthma by acupuncture intervention, we detected the protein expression levels of ACSL4, 15LO1, GPX4 and SLC7A11. Consistent with previous studies that ACSL4 and 15LO1 are essential for ferroptosis induction, our results revealed that OVA inhalation induced up-regulation of ACSL4 and 15LO1 protein expression, and furthermore, ferroptosis-induction agent erastin resulted in augmentation in ACSL4 and 15LO1 protein expression compared with mice only receiving OVA inhalation demonstrated by western blot bands. Moreover, Fer-1 induced the down-regulation of ACSL4 and 15LO1 protein expression, which in all indicated the involvement of ferroptosis in asthma. Consistent with previous reports that pharmacological ACSL4 inhibition prevents ferroptosis[23], acupuncture treatment forasthma induced a significant decrease of ACSL4 and 15LO1 protein expression in the lung tissue, suggesting that acupuncture could serve as a promising anti-ferrotosis strategy in asthma pathogenesis.

Additionally, it should be noted thatferroptosis-induction agent erastinup-regulated the 15LO1 and ACSL4 and down-regulated SLC7A11 and GPX4 protein expression demonstrated in the western blot bands, however, with no statistical difference revealed by semi-quantitative analysis of WB bands compared with mice only receiving OVA inhalation. Acupuncture treatment down-regulated 15LO1 and ACSL4 and up-regulated SLC7A11 and GPX4 protein expression compared with mice in erastin administration group, however, there were no statistical difference demonstrated in results from semi-quantitative analysis. These data indicated that on the basis of ferroptosis in the pathologic progression of asthma induced by OVA inhalation, ferroptosis-induction agent erastin induced obvious enhancement of lung inflammation, the MDA and GSSGlevels in lung compared with mice in OVA-induced experiment asthma group, and only a marginal effect on 15LO1, ACSL4,SLC7A11 and GPX4 protein expression in the lung.

Taken together, these observations revealed that OVA inhalation promoted ferroptosis by upregulating the non-haem Fe²⁺ level, lipid peroxidation and ACSL4 and 15LO1 protein expression. Erastin stimulation caused an augmentation in inflammation and resulted in a significant increase in lipid peroxidation in ferroptosis, suggesting that ferroptosis was involved in regulating the pathological progression of asthma. Acupuncture treatment relieved erastin-induced lipid peroxidation and ferroptosis in the lung tissue, and demonstrated the potential of acupuncture as an efficient therapy for lipid peroxidation and ferroptosis in asthma.

5. Conclusion

Overall, our data showed that erastin could augment lung inflammation and lipid peroxidation in ferroptosis. Fer-1 was able to relieve AHR, lung inflammation, non-haem Fe²⁺ level, lipid peroxidation and ferroptosis pathways in OVA-induced experimental asthma mice. Acupuncture treatment significantly reduced the MDA and GSSG levels, the non-haem Fe²⁺ level as well as ferroptosis-related pathway ACSL4-15-LO1 in the lung tissue. Acupuncture treatment also relieved ferroptosis-induction agent erastin-induced exacerbation in lung inflammation and lipid peroxidation in ferroptosis. These results in all indicated that disorder of lipid peroxidation as well as ferroptosis occurred in asthma, and attenuation of lung lipid peroxidation and ferroptosis contributed to the anti-asthma effect of acupuncture (Figure 9). Our study provided insights into the underlying mechanisms for the protective effects of acupuncture and highlighted a therapeutic potential of acupuncture treatment in the attenuation of lipid peroxidation and ferroptosis in asthma.

Authors' contributions

Y. Wei and J. Dong designed the study. W. Tang, J. Qing, Y. Zhou, W. Wang, F. Teng, J. Liu, L. Yi, J. Cui, X. Zhu and S. Wang performed the experiments. W. Tang, J. Qing and Y. Zhou analyzed the data. Y. Wei wrote the draft manuscript. Y. Wei and J. Dong revised the manuscript. All authors contributed to the article and approved the submitted version.

Declaration of competing interest

The authors declared no competing interests.

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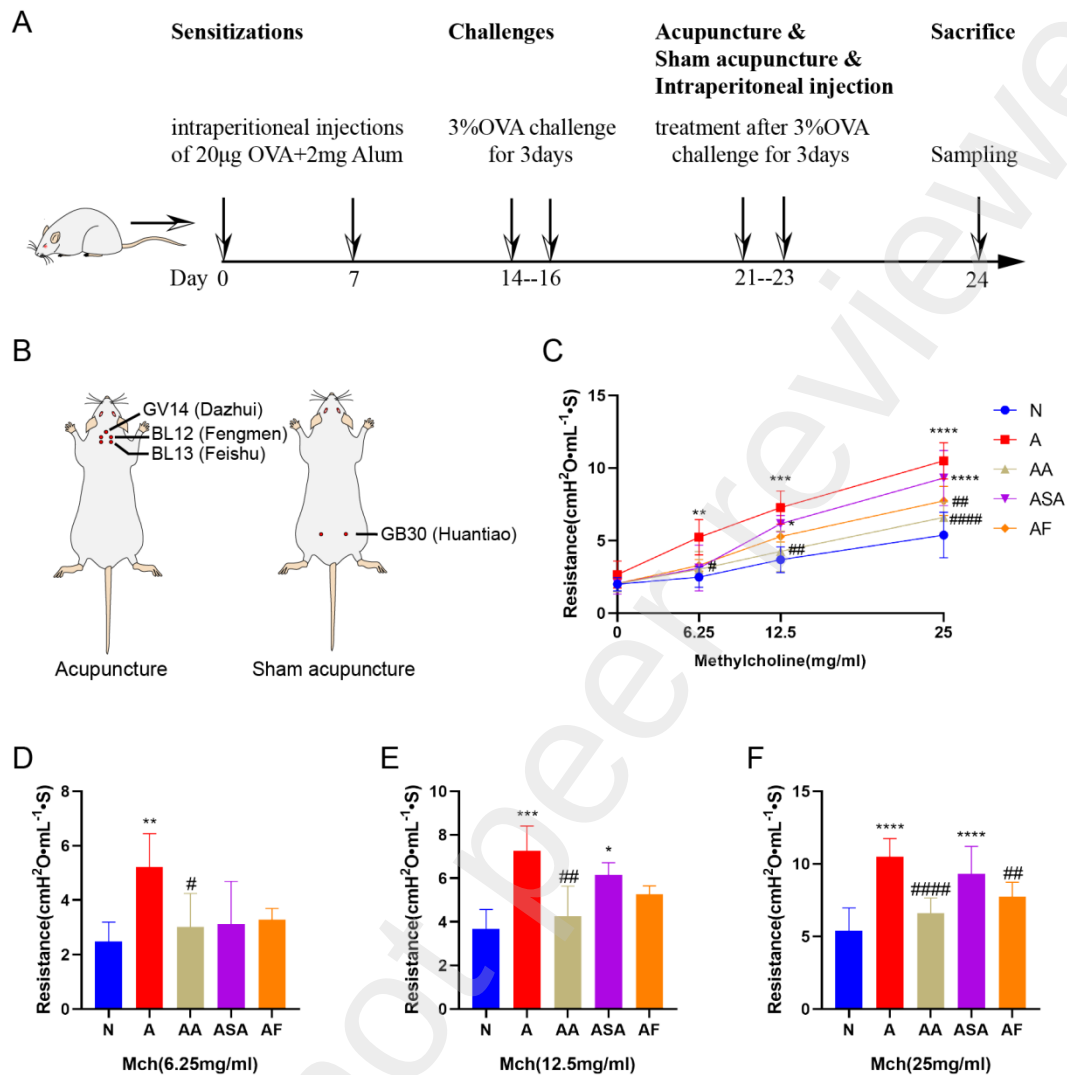
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705 Figure 1. Acupuncture and Fer-1 attenuated AHR. (A) Protocol of preparation of OVA

706 experimental asthma model. (B) Location of mouse acupoints. (C-F) Changes in

707 airway resistance (R_L) at different Mch dose. * $P < 0.05$ vs N, *** $P < 0.001$ vs N,

708 **** $P < 0.0001$ vs N, # $P < 0.05$ vs A, ## $P < 0.01$ vs A, ##### $P < 0.0001$ vs A.

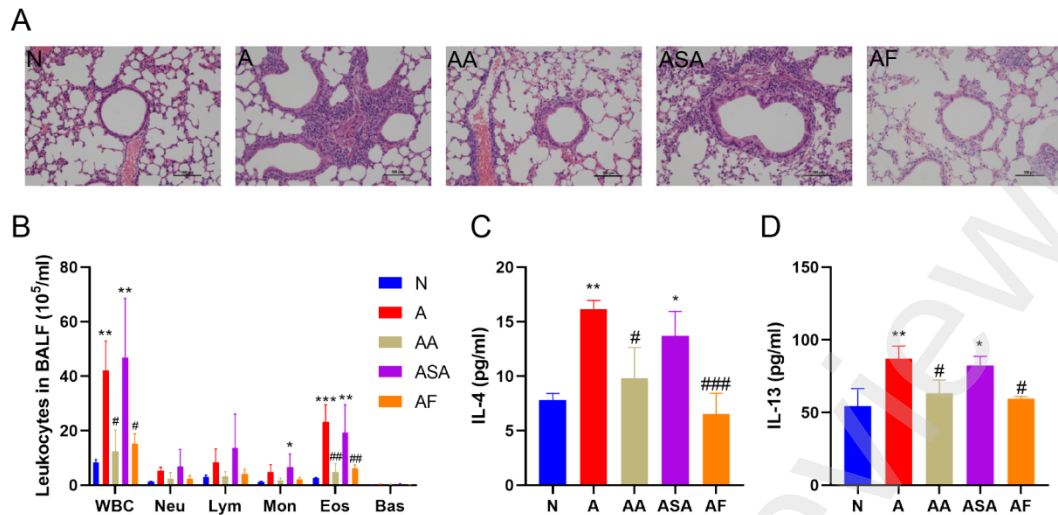
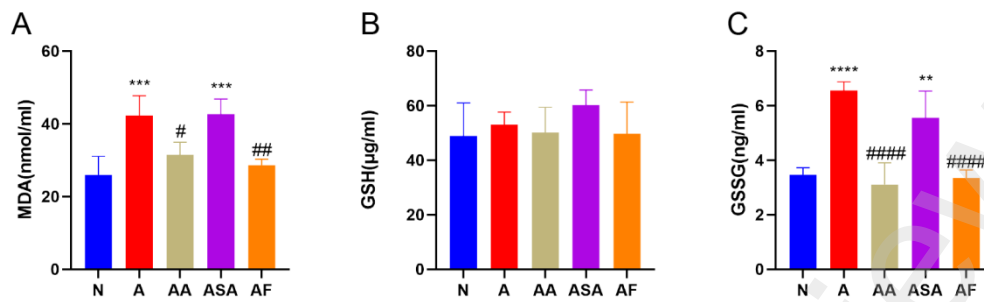


Figure 2. Acupuncture and Fer-1 relieved airway inflammation and type 2 cytokines secretion. (A) Representative images of H&E staining results. (B) Leukocytes classification in BALF. Leukocytes (WBC), Neutrophils (Neu), Lymphocytes (Lym), monocytes (Mon), Eosinophils (Eos) and Basophils (Bas). (C, D) Type 2 cytokine levels in BALF. * $P < 0.05$ vs N, ** $P < 0.01$ vs N, *** $P < 0.001$ vs N, # $P < 0.05$ vs A, ### $P < 0.01$ vs A, #### $P < 0.001$ vs A.

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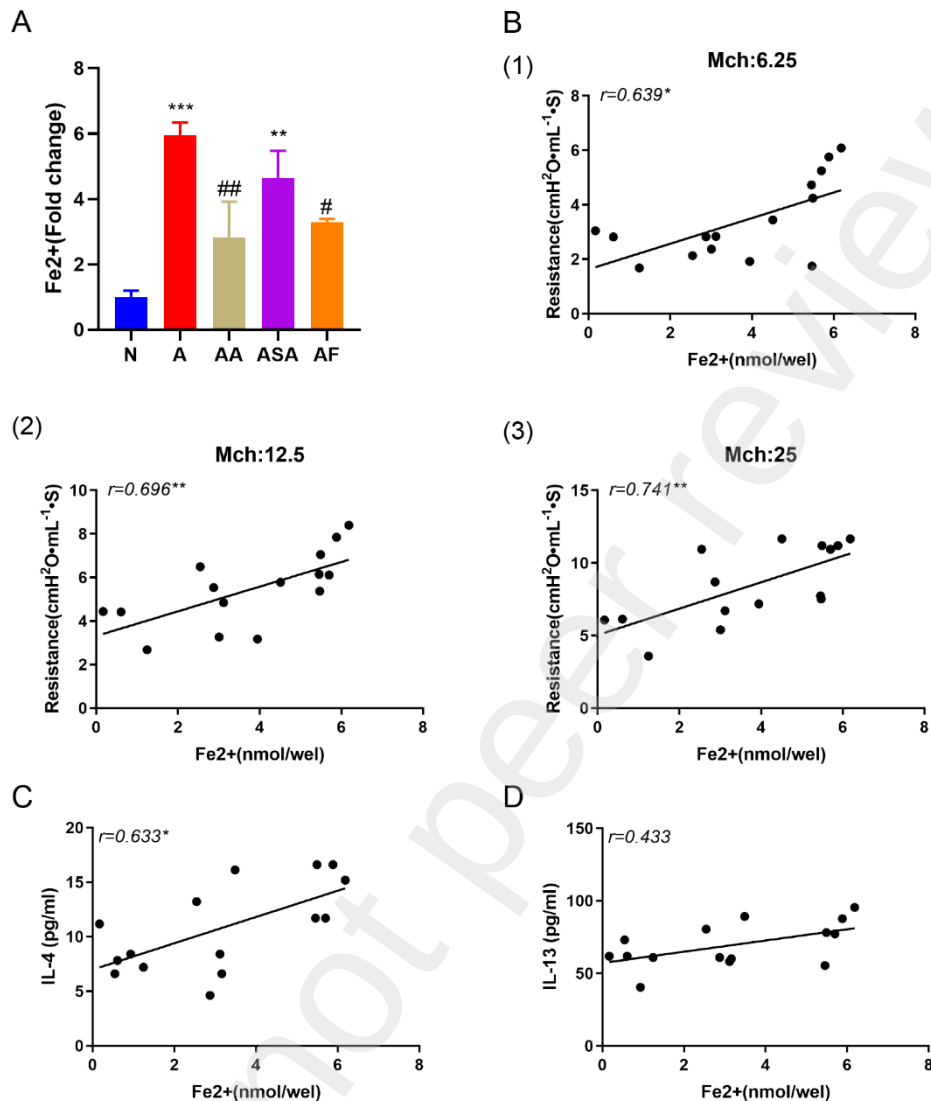
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720 Figure3. Acupuncture and Fer-1suppressed lung lipid peroxidation. (A)MDA level in
721 the lung.(B) GSH level in the lung. (C) GSSG level in the lung. **P < 0.01vs N, ***P
722 < 0.001vs N, ****P < 0.0001vs N, #P < 0.05 vs A, ##P < 0.01vs A, ####P <
723 0.0001vs A.

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729 Figure4. Acupuncture and Fer-1 lowered lung iron level. (A)The non-haem Fe^{2+} level
 730 in the lung. $^{**}P < 0.01$ vs N, $^{***}P < 0.001$ vs N, $^{\#}P < 0.05$ vs A, $^{##}P < 0.01$ vs
 731 A.(B)Correlation analysis between the Fe^{2+} level and the AHR to Mch. (C,D)
 732 Correlation analysis between the Fe^{2+} level and the type 2 cytokine levels in BALF. $^*P$
 733 < 0.05 , $^{**}P < 0.01$.

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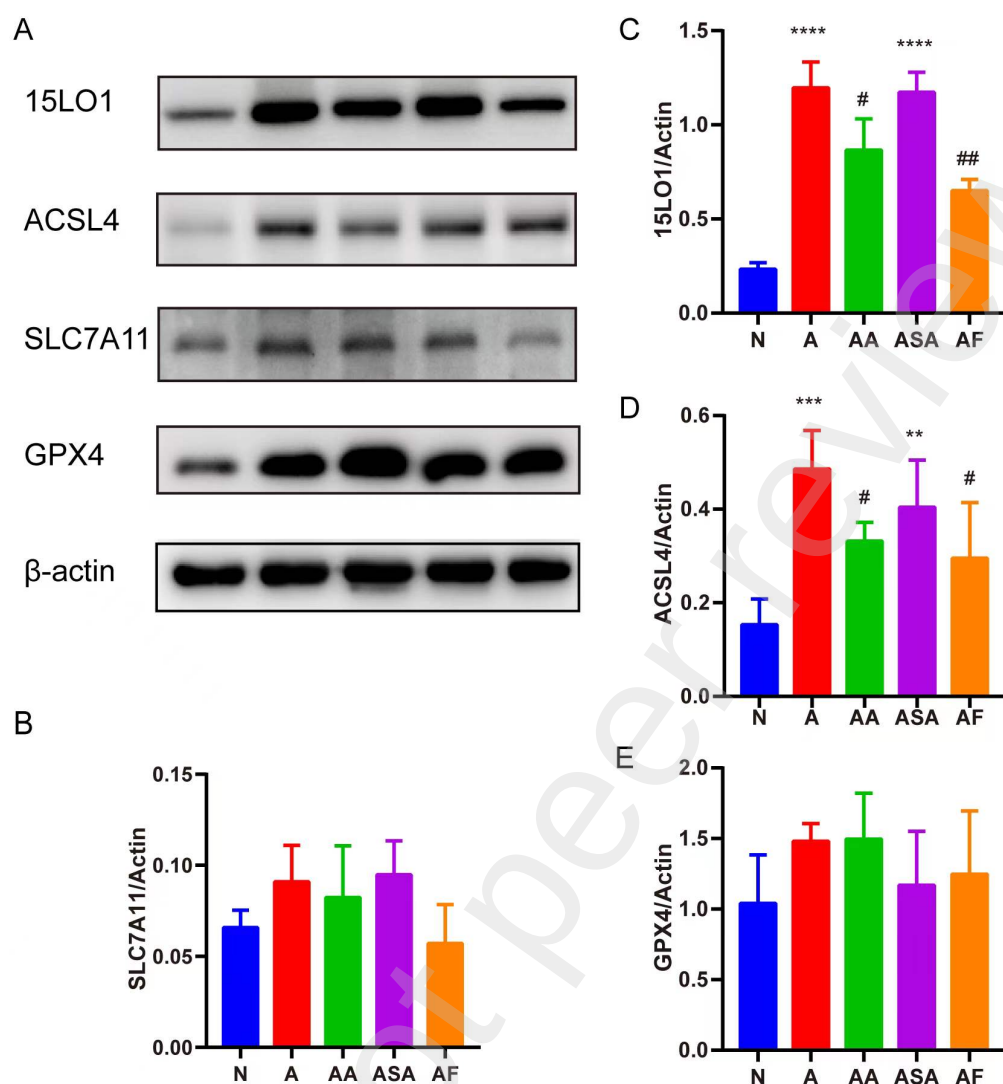


Figure 5. Acupuncture and Fer-1 inhibited ferroptosis associated pathways. (A) Representative image of western blot assay. (B-E) Bar graph of western blot assay results in (A). . ** $P < 0.01$ vs N, *** $P < 0.001$ vs N, **** $P < 0.0001$ vs N, # $P < 0.05$ vs A, ## $P < 0.01$ vs A.

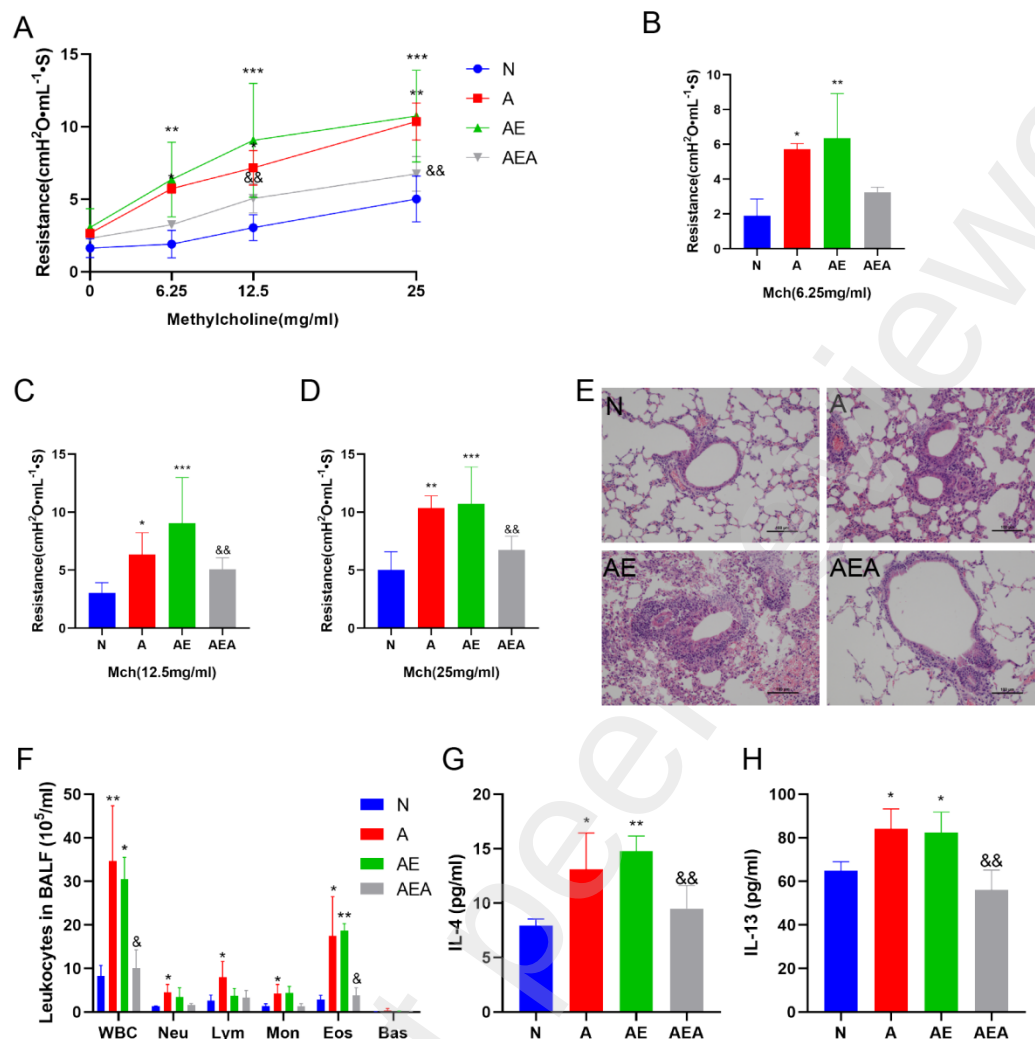
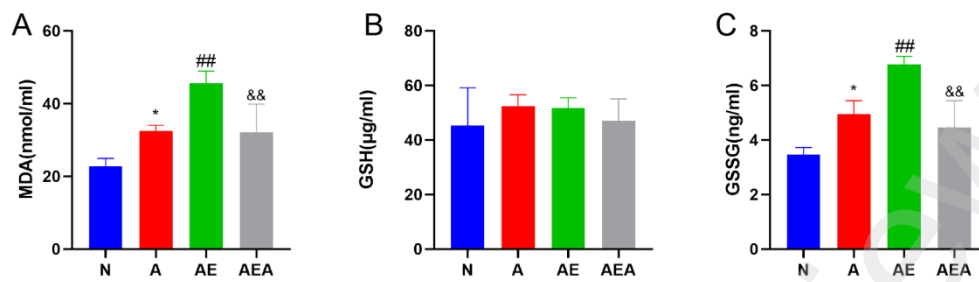


Figure 6. Acupuncture-relieved ferroptosis-induction agent erastin-induced exacerbation in lung inflammation. (A-D) Changes in airway resistance (R_L) at different Mch dose. (E) Representative images of H&E staining results. (F) Leukocytes classification in BALF. (G,H) Type 2 cytokine levels in BALF. * $P < 0.05$ vs N, ** $P < 0.01$ vs N, *** $P < 0.001$ vs N, & $P < 0.05$ vs AE, && $P < 0.01$ vs AE.

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753 Figure7. Acupuncturealleviated erastin-induced aggravation in lipid peroxidation and

754 ferroptosis associated pathways. (A)MDA level in the lung.(B) GSH level in the lung.

755 (C)GSSG level in the lung. *P < 0.05 vs N, ##P < 0.01 vs A, &&P < 0.01 vs AE.

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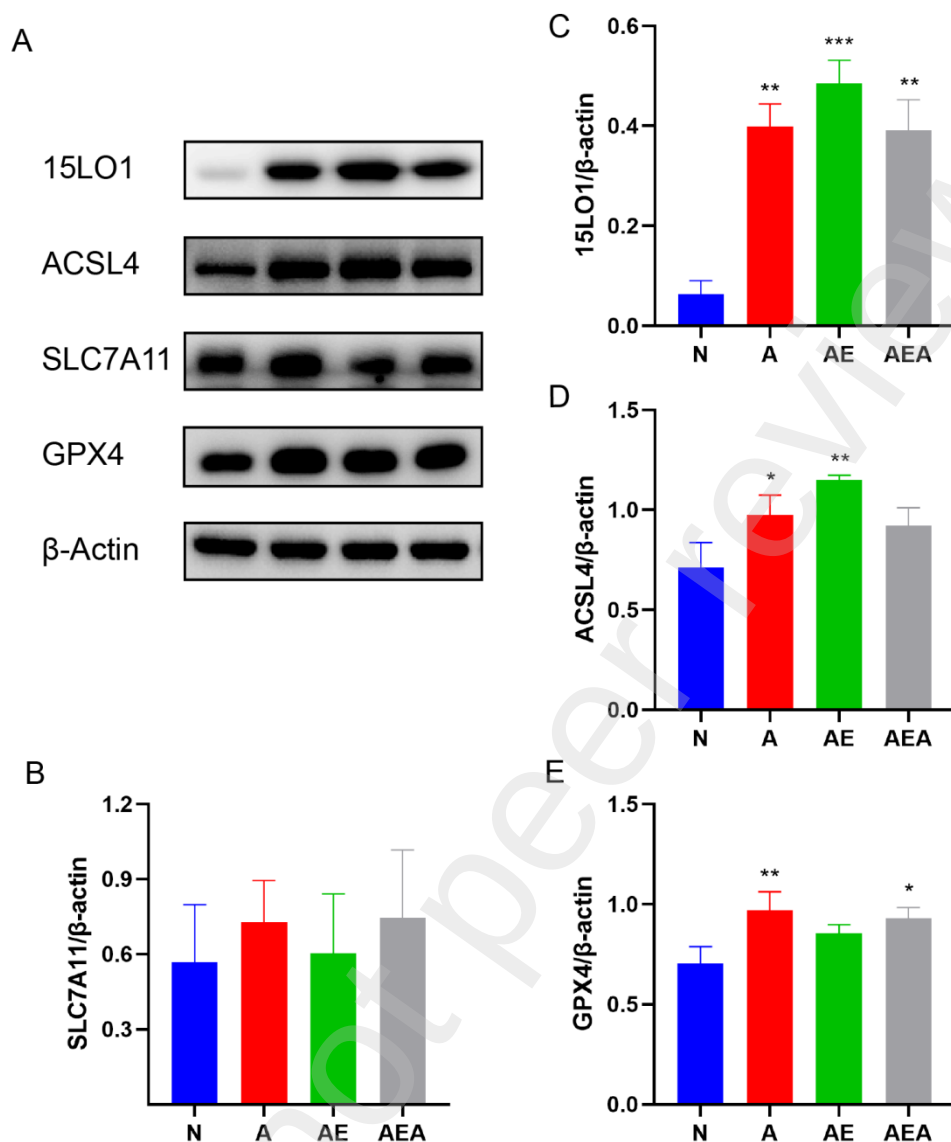


Figure 8. Effect of acupuncture on erastin-induced ferroptosis associated pathways. (A) Representative image of western blot assay. (B-E) Bar graph of western blot assay results in (A). * $P < 0.05$ vs N, ** $P < 0.01$ vs N, *** $P < 0.001$ vs N.

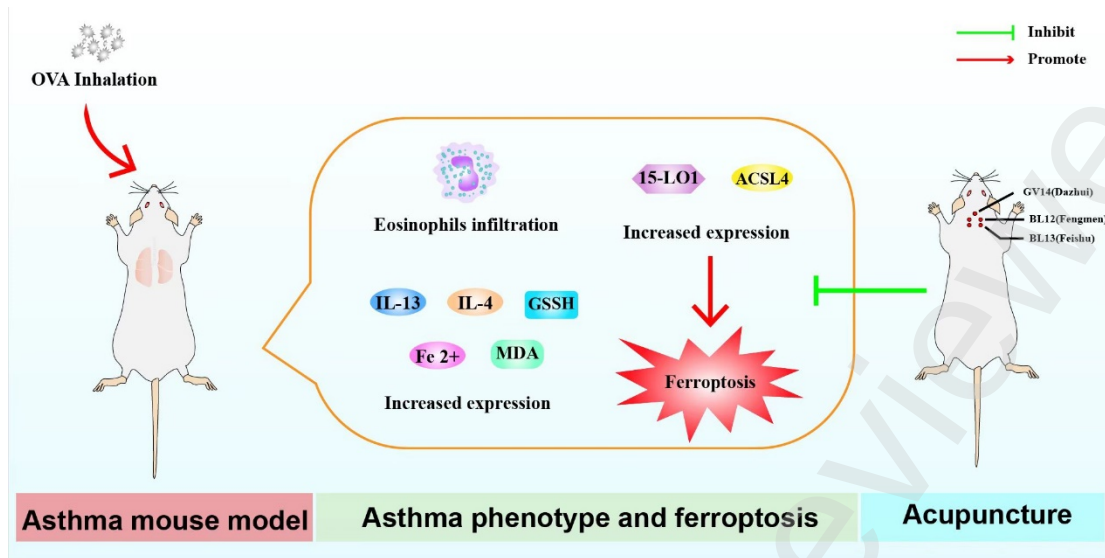


Figure9. Schematic summarising the effect of acupuncture on airway inflammation and ferroptosis in allergic asthma.