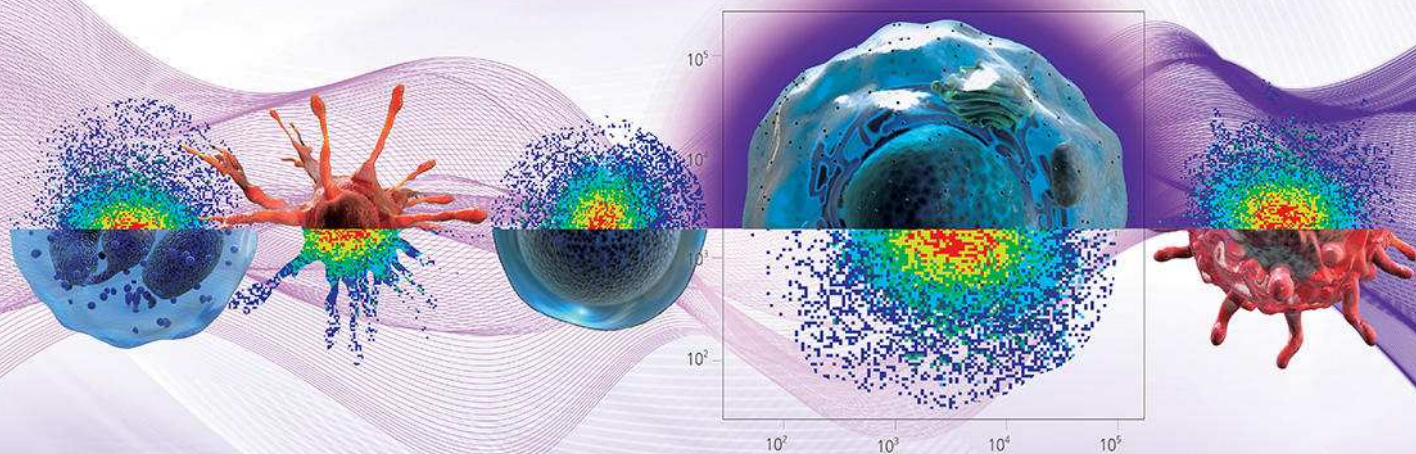




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Influences of PON1 on Airway Inflammation and Remodeling in Bronchial Asthma[†]

Running title: PON1 and bronchial asthma

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Abstract

This study aims to explore the influences of Paraoxonase-1 (PON1) involved in airway inflammation and remodeling in asthma. Mice were divided into control, asthma, asthma + PON1 and asthma + NC groups, and asthma models were established via aerosol inhalation of ovalbumin (OVA). HE, Masson and PAS stains were used to observe airway inflammation and remodeling, Giemsa staining to assess inflammatory cells in bronchoalveolar lavage fluid (BALF), qRT-PCR and western blot to detect PON1 expression, lipid peroxidation and glutathione assays to quantify malondialdehyde (MDA) activity and glutathione peroxidase (GSH) levels, ELISA to determine inflammatory cytokines and immunoglobulin, and colorimetry to detect PON1 activities. Additionally, mice lung macrophages and fibroblasts were transfected with PON1 plasmid *in vitro*; ELISA and qRT-PCR were performed to understand the effects of PON1 on inflammatory cytokines secreted by lung macrophages, MTT assay for lung fibroblasts proliferation and qRT-PCR and western blot for the expressions of PON1, COL1A1 and fibronectin. After overexpression of PON1, the asthma mice had decreased inflammatory cell infiltration, fibrosis degree, and airway wall thickness; inflammatory cells and inflammatory cytokines in BALF were also reduced, expressions of OVA-IgE and IgG1, and MDA activity were decreased, but the expressions of OVA-IgG2a and INF- γ and GSH levels were increased. Besides, *PON1* significantly inhibited microphage expression of LPS-induced inflammatory cytokines, lung fibroblast proliferation, and COL1A1 and fibronectin expression. Thus, PON1 could relieve airway inflammation and airway remodeling in asthmatic mice and inhibit the secretion of LPS-induced macrophage inflammatory cytokines and the proliferation of lung fibroblasts. This article is protected by copyright. All rights reserved

Keywords: PARAOXONASE-1; ASTHMA; AIRWAY INFLAMMATION; AIRWAY REMODELING

Introduction

Bronchial asthma (hereafter referred to as asthma) is a chronic inflammatory airway disease involving multiple cells and cellular components, with the major characteristic of persistent airway inflammation, airway hyperresponsiveness (AHR) and airway remodeling [Lee et al., 2017]. According to the global asthma report in 2014 issued by the World Health Organization (WHO), almost 334 million people all over the world suffer from asthma, with continuously rising incidence and mortality worldwide; asthma patients will exceed 400 million by 2025 (http://www.globalasthmareport.org/resources/Global_Asthma_Report_2014.pdf). In asthma, the activation of airway inflammation is closely related with T cells, particularly T helper (TH) 1 and TH2 that function as important factors in multiple clinical manifestations [Li et al., 2016]. Recently, airway remodeling has been considered to be the pathological basis for irreversible AHR and airway obstruction, explaining the intractable root of asthma [Gabehart et al., 2013]. Therefore, it has been a popular topic to investigate the pathogenesis of airway inflammation and airway remodeling in asthma.

Paraoxonase 1 (PON1), secreted by the liver, is composed of 9 exons and 8 introns and is located at chromosome 7 (q21~22) with a molecular weight of 43 kDa and a length of 25.9 kb [Li et al., 1997]. Additionally, PON1 is a major antioxidant enzyme of high density lipoprotein (HDL), which can hydrolyze oxidized phospholipids and protect low density lipoprotein (LDL) from oxidization [Kappelle et al., 2012]. A number of studies have revealed that PON1 is involved in the occurrence and development of various diseases along with oxidative stress and inflammation, including rheumatoid arthritis [Isik et al., 2007; Tanimoto et al., 2003], diabetes [van den Berg et al., 2008], cardiovascular disease [Gur et al., 2006] and lung cancer [Elkiran et al., 2007]. Moreover, current studies have reported that the expression and activity of PON1 were evidently decreased in

asthma and may have potential effects on diagnosing asthma [Emin et al., 2015; Sarioglu et al., 2015; Tolgyesi et al., 2009].

However, the mechanism of PON1 in asthma is still unknown, especially the effects of PON1 on airway inflammation and airway remodeling. Therefore, in this study, BALB/c mice were selected to establish an OVA-induced asthma model and were then injected intraperitoneally with overexpressed PON1 plasmid to observe the effects of PON1 on airway inflammation and airway remodeling. In addition, overexpressed PON1 plasmids were transfected into mouse lung macrophages and lung fibroblasts to observe the effects of PON1 on the secretion of inflammation cytokines by lung macrophages and the proliferation of lung fibroblasts *in vitro*.

Materials and methods

Ethics statement

The animal experimental design obtained permission from the experimental animal ethics committee of No. 2 People's Hospital of Kunming, and all animal experiments were in strict accordance with animal protocols and guidance made by the International Association for the Study of Pain (IASP). Every effort contributes to minimize animals' suffering [Orlans, 1997].

Animals

The animal study population consisted of 40 BALB/c mice of clean grade including male and female halves with weights of 18-22 g. All mice were fed with food and water freely at room temperature (22-25°C) in a normal circadian rhythm in a clean animal room. All mice were purchased from SLAC Laboratory Animal Co., Ltd., Shanghai, China.

Animal grouping and establishment of asthma model

The mice were randomly divided into control, asthma group, asthma + PON1 and asthma +

negative control (NC) groups, with 10 mice in each group. Except for the control group, the mice in the other three groups had an intraperitoneal injection including 1 ml of sensitization solution containing 10 µg of OVA (1001757518, Sigma-Aldrich Technologies Inc., California, USA) and 100 µg of aluminum hydroxide gel (101284146, Sigma-Aldrich Technologies Inc., California, USA) on the 1st and 14th days. Twenty-one days after the experiment, the mice were stimulated by 2.5% OVA aerosol inhalation for 30 min every other day for the next 8 weeks in preparation of the asthma mouse model. Except for the fact that OVA was replaced by normal saline in the equivalent amount, all of the operations in the control group were consistent with the asthma groups. Thirty minutes before stimulation, the mice in the asthma + PON1 and asthma + NC groups received an intraperitoneal injection with the PON1 overexpressing plasmid (50 µg/mouse) and the blank plasmid, respectively (Gene Pharma Co., Ltd., Shanghai, China).

Specimen collection

Twenty-four hours after the last stimulation, the mice were executed, and their blood samples and peritoneal macrophages were collected. After injection of the 10% chloral hydrate (Boster Bioengineering Co. Ltd., Wuhan, China) for anesthesia, the neck skin of mice was bluntly dissected to expose the main trachea and heart in order to collect blood samples from the right auricle. The blood was centrifuged to collect the supernatant and saved at -80°C, followed by ligation of the main bronchus. Then, 4°C normal saline was used to conduct bronchoalveolar lavage 3 times. The bronchoalveolar lavage fluid (BALF) (more than 80% of the injected normal saline) was collected and centrifuged for the supernatant to determine the inflammatory factor while the rest was submitted to Wright Giemsa staining to classify the cells. With the blood taken, the left lung tissue was partly saved in liquid nitrogen, while the other part was treated via paraformaldehyde (PA) fixation, dehydration, transparency, and embedment in paraffin and then was cut into slices for

later experiments. At the same time, 70% ethanol was used to clean the mouse abdomen, and a sterile scalpel was used to make a small incision. Then, the forceps were used to remove the abdominal skin to expose the peritoneum; the isolation and culture of peritoneal macrophages was described by Pineda-Torra I et al. [Pineda-Torra et al., 2015].

Histology and immunohistochemistry

Left lung lobes were fixed with PFA, embedded in paraffin and cut into 5- μ m sections. Then, the sections were stained with HE, periodic acid-Schiff (PAS), or Masson's trichrome. Additionally, we conducted the immunostaining of fixed sections with alpha-smooth muscle actin (α -SMA) (Boster Bioengineering Co. Ltd., Wuhan, China) using Vectastain Alkaline Phosphatase Universal (Vector Laboratories Inc., Burlingame, Calif, USA) based on the manufacturer's protocols. Later, 3 independent investigators were performed to access staining intensity as follows: 0 for no reactivity; 1 for minimal staining; 2 for moderate staining; and 3 for prominent staining. MetaMorph imaging software (Molecular Devices) was applied to quantify the positive staining areas [Habibovic et al., 2016].

Measurement of airway wall thickness

Under an optical microscope, hematoxylin-eosin (HE) sections were detected and analyzed for airway wall thickness using image analysis software ImagePro Plus 6.0 (Media Cybernetics, Bethesda, USA). Under an optical microscope magnification of 20 times, the complete bronchiole was selected to determine the inner perimeter (P_i), wall area (W), smooth muscle area (S) and number (N) of smooth muscle cells in the bronchus basilar membrane. Then, the measured values were standardized by the bronchial basilar membrane P_i to calculate W/P_i , S/P_i and N/P_i . The experiment was repeated 3 times in each group.

Malondialdehyde (MDA) activity and Glutathione (GSH) assay

MDA activity and glutathione levels were detected by the lipid peroxidation assay kit and glutathione assay kit in lung tissues in each group respectively, in line with the manufacturer's instructions (Sigma, St. Louis, MO, USA). MDA activity was measured using a Multi-Mode Microplate Reader (BioTek SynergyHT, Bedfordshire, UK) as nanomolars in per milligram weight of wet tissue. The intensity of color of glutathione was determined spectrophotometrically at 412 nm in a Thermo Scientific Multiskan FC microplate reader (Waltham, MA, USA). **Wright Gimsa staining of inflammatory cells in BALF**

The cells in BALF were counted at high magnification, and the total cells were recorded. BALF was dropped at one end of a clean and no-grease slide; with the BALF slide in the left hand and the smooth edged slide in the right hand, we placed the edge of the right slide in front of the blood drop and then pulled back to launch a uniform blood smear. After Wright Gimsa staining, cells were classified at low magnification and the cell number was recorded including monocytes, lymphocytes, eosinophils and neutrophils. The total number of inflammatory cells in BALF was calculated according to the total cells and the proportion of inflammatory cells. Every experiment was repeated three times in each group.

Cell culture and grouping of mouse lung macrophages and lung fibroblasts

The C57BL/6 mouse lung macrophages and lung fibroblasts (ATCC Inc., Manassas, Virginia, USA) were inoculated in 6-well plates with 2×10^6 cells/well and cultured in PRMI1640 (Thermo Fisher Inc., Waltham, MA, USA) composed of 10 ml/L fetal bovine serum (FBS), 100 U/ml penicillin and 100 g /mL streptomycin at 37°C with 50 ml/L CO₂ for 4 h. After the medium was changed, the adherent cells were abandoned. The C57BL/6 mouse lung macrophages and lung fibroblasts were divided into the PON1 group, the NC group and the control group. In the PON1

group, 2 μg of overexpressed PON1 plasmid was added in 250 μL of Dulbecco's modified Eagle's medium (DMEM) and named solution A. Additionally, 4 μL of Lipofectamine 2000 (Invitrogen Inc., California, USA) was added to 250 μL of DMEM to get solution B. After 5 min at room temperature, we mixed solution A and solution B. After 20 min at room temperature, the mixed solution was added to 1.5 ml of medium and mixed gently. In the NC group, 2 μg of blank plasmids was transfected into cells, and the rest of the procedures were the same as those of the PON1 group, while cells in the control group were cultured with 2 ml of original medium. After 48 h of transfection, 100 $\mu\text{g}/\text{ml}$ lipopolysaccharide (LPS) was added to mouse lung macrophages for another 12 h of culture, and the supernatant was collected to detect the expression of inflammatory cytokines. Then, the proliferation of mouse lung fibroblasts and expression of COL1A1 and fibronectin were determined by an MTT assay.

MTT assay

After 24, 48, 72 and 96 h of transfection of the mouse lung fibroblasts, the supernatant was discarded, and DMEM/F12 containing 500 mg/mL MTT was added. Four hours later, centrifugation was conducted to collect the cells, followed by dissolution via 150 μL of dimethyl sulfoxide (DMSO). Then, a microplate reader (Thermo Fisher, Waltham, MA, UK) was used to detect the optical density (OD) of each well at 490 nm, and the growth curve was drawn with OD as the ordinate and time as the abscissa. Each experiment in each group was repeated 3 times.

PON1 activities in the serum and peritoneal macrophages detected by colorimetry

The activities of PON1 were measured using commercially available kits (Rel Assay Diagnostic, Gaziantep, Turkey). Arylesterase activity or lactonase activity was performed in a total reaction volume of 200 μL by using phenyl acetate and dihydrocoumarin (DHC) as the substrate, respectively [Gaidukov and Tawfik, 2005]. The serum samples were diluted 1:10 with 1 mM CaCl_2

in 50 mM Tris HCl (pH 8.0).

Quantitative real-time fluorescence polymerase chain reaction (qRT-PCR)

According to the miRNeasy mini kit instructions (217004, Qiagen Bioinformatics Inc., Dusseldorf, Germany), total RNA was extracted using a Trizol kit (15596026, Invitrogen Inc., California, USA), and the RNA concentration and purity was detected using a NanoDrop 2000 (Thermo Fisher Inc., Waltham, MA, USA), followed by storage at - 80°C. Based on gene sequences published in the GenBank database, Primer 5.0 software was used to design PCR primers (Table 1) that were synthesized after primers were compared and confirmed. The PRISM 7500 real-time PCR System (Applied Biosystems by Life Technologies Corporation, Foster city, California, USA) was adopted, and the reaction system included 12.5 ul of SYBR Premix Ex Taq II (Takara Biotechnology Ltd., Dalian, China), 1 ul of forward primer, 1 ul of reverse primer, 2 ul of the template and 8.5 ul of sterile water, with reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) as the internal reference. PCR conditions: 3-min pre-denaturation at 94°C followed by 40 cycles of 30-s denaturation at 94°C, 30-s annealing at 54°C and 60-s extension at 72°C. Then, the solubility curve was determined to evaluate the reliability required to obtain the Ct value (the inflection point of amplification curve). Here, $\Delta Ct = Ct(\text{target gene}) - Ct(\text{internal reference})$, $\Delta \Delta Ct = \Delta Ct(\text{treatment group}) - \Delta Ct(\text{control group})$, as the relative expression of the target gene [Tuo et al., 2015]. Every experiment was repeated 3 times.

Western blot assay

A BCA kit (Boster Biological Technology Co. Ltd., Wuhan, China) was used to extract the cellular and tissue proteins for later determination of the protein concentration based on the manufacturer's directions. Then, the loading buffer was added in the extracted proteins, followed by 10-min of boiling at 95°C and loading in each well to run 10% polyacrylamide gel (Boster

Biological Technology Co. Ltd., Wuhan, China) electrophoresis for protein isolation at 80 V and 120 V. Next, at 100 mV, the wet transference to polyvinylidene fluoride (PVDF) membranes was carried out for 45-70 min. After sealing with 5% bull serum albumin (BSA) at room temperature for 1 h, PVDF membranes were added with the primary antibodies PON1 (1:10000, ab126597), COL1A1 (1:500, ab208638), FN (1 µg/ml, ab2413) and β-actin (1 µg/ml, ab16039) at 4°C overnight. Following washing with tris buffered saline tween (TBST) 3 times (5 min per wash), the secondary antibody (1 µg/ml, ab191866) was added for 1 h of incubation at room temperature, followed by a TBST wash 3 times (5 min per wash). All primary and secondary antibodies were purchased from Abcam Inc., Cambridge, UK. With β-actin as the internal reference, chemiluminescence reagent was added to develop the membrane using a Gel Dol EZ imager (Bio-rad, California, USA). The gray value of the target band was analyzed using ImageJ software. The ratios of the target band and the β-actin band were taken as the relative protein expression levels. Every experiment in each group was repeated 3 times.

Enzyme-linked immune-sorbent assay (ELISA)

A double antibody sandwich ELISA kit (Thermo Fisher Inc., Waltham, MA, USA) was applied to detect expressions of Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-13 (IL-13), Interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), Interleukin-8 (IL-8) and the specific immunoglobulins (Ig) E, IgG1 and IgG2a of serum OVA following the manufacturer's instructions. Then, IL-4, IL-5, IL-6, IL-13, IFN-γ, TNF-α, IL-8, and OVA-IgE were developed using 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate, and their OD values were detected by a microplate reader at 450 nm. OVA-IgG1 and OVA-IgG2a were developed by an OPD substrate, and their OD values were detected by a microplate reader at 492 nm. The experiment was repeated 3 times in each group.

Statistical analysis

The statistical analysis was performed using SPSS 21.0 software (SPSS Inc, Chicago, USA).

The measurement data following a normal distribution in two groups were compared and expressed as the mean $\pm$ standard deviation (SD). The *t*-test was used to analyze pairwise comparisons, and a comparison among groups was analyzed using one-way analysis of variance (ANOVA). Values of *P* < 0.05 were statistically significant.

Results

Behavioral observations of mice

In the asthma and asthma + NC groups, the mice showed different degrees of clinical symptoms, such as irritability, nasal itching, tachypnea and lip cyanosis, as well as urinary and bowel incontinence. After repeated stimulation, the mice suffered from dull hair, a poor spirit, less activity, and a slow weight gain. Moreover, some mice even lost activity along with decreased respiratory rate or irregular respiratory rhythm. The mice were generally healthy in the control group and the asthma + PON1 group because the mice demonstrated no changes in tachypnea, diet, behavior or respiratory rhythm.

Pathologic observation of lung tissue

As shown by HE staining in Figure 1A, the mice had a complete lung structure, a regular bronchial lumen, no thickening of the bronchial smooth muscle, a full epithelial mucosa and no inflammatory cell infiltration around blood vessels or bronchia in the control group. The mice had obvious changes, including an evidently thickening airway wall and smooth muscle, distinct airway mucous edema, widened submucosa, narrowed lumen, desquamation of epithelial cells, mucous plug and a large quantity of inflammatory cell infiltration in the submucosa and around the airway

in the asthma and asthma + NC groups. The thickness of the airway wall and smooth muscle was decreased, and the lung inflammation was relieved in the asthma + PON1 group compared to that in the asthma group ($P < 0.05$). PAS staining shown in Figure 1A-B revealed that in the control group, the mice had a small quantity of PAS-positive cells. In both the asthma and asthma + NC groups, the PAS-positive cells were greatly increased, as were goblet cells. Additionally, the proliferation of goblet cells was clearly decreased in the asthma + PON1 group compared to that in the asthma group ($P < 0.05$). The Masson staining results in Figure 1A-C demonstrated that there was very little collagen fibrosis in the control group, while a large area of collagen fibrosis in the airway wall was found in both the asthma and asthma + NC groups. In comparison with the asthma group, the collagen staining area around the airway wall was evidently reduced in the asthma + PON1 group ($P < 0.05$). The immunohistochemistry results showed that the positive expression of α -SMA was distinctly enhanced in every asthma group; the positive expression of α -SMA was notably decreased in the asthma + PON1 group compared with that in the asthma and asthma + NC groups ($P < 0.05$, Figure 1A-D).

Comparisons of bronchial wall area, bronchial smooth muscle area, smooth muscle cell nuclei

The mouse airway wall thickness in each group showed that in comparison with the control group, W/Pi, S/Pi and N/Pi post-HE staining were significantly increased (all $P < 0.05$), but the wall thickness was mostly lower the control group than in the asthma group (all $P < 0.05$). There were no significant differences in the asthma and asthma + NC groups (all $P > 0.05$, Table 2).

Expression of PON1 in lung tissues, peritoneal macrophages and serum of mice in each group

As shown in Figure 2, the mRNA and protein expression of PON1 in peritoneal macrophages and lung tissues were substantially lower in both the asthma and asthma + NC groups than in the control group (all $P < 0.05$) but were greatly increased in the asthma + PON1 group compared with

those in the asthma group (both $P < 0.05$). Additionally, the arylesterase and lactonase activities of PON1 in peritoneal macrophages and serum were consistent with the mRNA and protein expressions of PON1 in peritoneal macrophages and lung tissues.

Effects of PON1 on MDA and GSH activity in the lung tissues

As shown in Figure 3, the mice in asthma and asthma + NC groups had significantly elevated MDA activity and reduced GSH levels in lung tissues compared to the normal mice in control group. However, PON1 markedly suppressed MDA activity and induced GSH production in lung tissues as compared to the levels in asthmatic mice.

Comparison of inflammatory cells and cytokines in BALF in each group

The detection results of inflammatory cells and cytokines in BALF in each group (Figure 4) showed that, compared with the control group, eosinophils, mononuclear macrophages, neutrophils, lymphocytes and total cells in asthmatic groups were significantly increased as were the concentrations of IL-4, IL-5, IL-13 and TNF- α , while the content of INF- γ was sharply decreased in each asthma group (all $P < 0.05$). Compared with the asthma and asthma + NC groups, the asthma + PON1 group had increased eosinophil, mononuclear macrophage, neutrophil, lymphocyte, and total cell contents as well as increased contents of IL-4, IL-5, IL-13 and TNF- α and an obvious increase in the content of INF- γ (all $P < 0.05$). There were no significant differences between the asthma group and the asthma + NC group (all $P > 0.05$).

Expressions of immunoglobulin in peripheral blood in each group

ELISA results of immunoglobulin in the peripheral blood in each group revealed that compared with the control group, the contents of OVA-IgE and OVA-IgG1 in the serum were notably enhanced, while the content of OVA-IgG2a was greatly decreased in both the asthma group and the asthma + NC group (all $P < 0.05$). Additionally, the asthma + PON1 group had a significant

reduction in the contents of OVA-IgE and OVA-IgG1 but a distinct increase in the content of OVA-IgG2a (all $P < 0.05$). In the asthma + NC group, we found no significant differences (all $P > 0.05$, Figure 5).

Effects of PON1 on inflammatory cytokines in LPS-induced macrophages *in vitro*

qRT-PCR and western blot demonstrated that the mRNA and protein expression levels of PON1 were remarkably increased in the PON1 group compared with the control and NC groups (all $P < 0.05$) (Figure 6A-B). Additionally, the effects of PON1 on inflammatory cytokines in LPS-induced macrophages were detected using ELISA and qRT-PCR, revealing that in the PON1 group, not only the concentrations but also the mRNA expression of IL-6, IL-8 and TNF- α were decreased significantly compared with those of the control group (all $P < 0.05$). No significant differences were observed in the NC group (all $P > 0.05$, Figure 6C-D).

PON1 inhibited proliferation and collagen secretion of lung fibroblasts *in vitro*

Compared with the Control group and the NC group, the mRNA and protein expression levels in lung fibroblasts were higher in the PON1 group (Figure 7A-B). To explore the function of PON1 in airway remodeling, we detected the effects of PON1 on the proliferation of lung fibroblasts (Figure 7A). In the PON1 group, the proliferation rates (24 h, 48 h, 72 h and 96 h) were lower in the control group and the NC group (all $P < 0.05$). In addition, the effects of PON1 on collagen secretion of lung fibroblasts (Figure 7 D-F) resulted in the significant down-regulation of mRNA and protein expression of COL1A1 and fibronectin in the PON1 group compared with those in the control group and the NC group (all $P < 0.05$).

Discussion

PON1 functions as an antioxidant enzyme to metabolize proinflammatory lipid generated during oxidation of low-density lipoprotein [Mackness et al., 1993]. An apparent decrease in PON1 expression was observed in the lung tissue of OVA asthma mice to be the most striking finding in the present research. Consistently, several studies have demonstrated that PON1 is able to reduce the incidence of inflammation [Kim et al., 2014; Kotur-Stevuljevic et al., 2008]. Specifically, Gońnicka *et al.* reported that the severity of asthma in children was associated with decreased PON1 activity [Gornicka et al., 2002], further suggesting that PON1 can affect the occurrence and development of asthma via reducing the inflammation.

Asthma is a well-known chronic respiratory disease characterized by inflammatory cell infiltration, and the inflammatory cells in BALF were evidently enhanced in the allergic asthma model induced by cockroach allergen [Park et al., 2014]. Similarly, our study found that with overexpression of PON1, there was great relief of inflammatory cell infiltration in the submucosa and around the airway and an obvious decrease in eosinophils, mononuclear macrophages, neutrophils, lymphocytes and total cells. Additionally, airway mucus was mainly secreted by airway goblet cells, whose proliferation not only is one important pathologic change in airway remodeling but also contributes to the exacerbation of asthma [Davis and Dickey, 2008; Rogers, 2007]. Moreover, PAS staining results in this study showed that PON1 was effective in inhibiting OVA-induced goblet cells, indicating that PON1 is able to suppress airway mucus secretion. As another important pathologic change of airway remodeling, the increase of airway smooth muscle cells shares a close relationship with asthma severity and continuously decreasing lung function [Perry et al., 2014]. Additionally, the differentiation of fibroblasts into myofibroblasts is one important step in airway remodeling in asthma, and here α -SMA, a cytoskeletal protein expressed in

smooth muscle cells, is a key marker of myofibroblasts [Yoshida et al., 2015]. Furthermore, this study reported that PON1 can decrease airway wall thickness and smooth muscle as well as the expression of α -SMA in lung tissue, indirectly illustrating the suppressive effects of PON1 on the proliferation of airway smooth muscle cells, further confirming that PON1 plays an alleviative role in airway inflammation and remodeling in asthma.

As we know, antioxidant enzymes, such as GSH and MDA, offer protection of the deterioration of lung tissue in asthma from oxidation [Huang et al., 2017]. In *vivo* experiment, we found PON1 could suppress MDA activity with induced GSH production in lung tissues as compared to the levels in asthmatic mice, indicating the anti-asthmatic inflammation induced PON-1 in lung tissue was associated with decreased oxidative stress [Rozenberg et al., 2003].

Recently, evidences were accumulating that the lactonase activity and arylesterase activity were the most significant explanations for the antioxidant property of PON1 [Acay et al., 2013; Tavori et al., 2008]. A previous study demonstrated that PON1 could act on oxidized phosphatidyl choline, thus producing a lactone (lactonizing activity of PON1) [Gugliucci et al., 2011]. Additionally, Górnicka and his group revealed that the lower levels of arylesterase in bronchial asthma attack periods than in control subjects, which was linked to heightened systemic oxidative stress [Gornicka et al., 2002].

Consistent with the results of previous studies, our study also highlighted the significant role of lactonase activity and arylesterase activity in asthma. In our research, we found the arylesterase and lactonase activities of PON1 in peritoneal macrophages and serum were substantially lower in the asthma models, suggesting the PON1 and its enzymes (lactonase and arylesterase) could reflect the status of oxidative stress in asthma.

Recently, the mechanism of Th1 and Th2 imbalance in asthma has attracted increasing attention [Ying et al., 2011; Zhu, 2015]. As is well known, CD4⁺T cells act as the dynamic player in

the adaptive immune response including Th1 and Th2 cells [Zhu, 2015]. Wherein Th2 cells guide B cells to synthesize IgE via secreting IL-4, up-regulate the OVA-IgG1 expression by producing IL-4 and IL-13, activate eosinophils via IL-5 secretion, enhance the proliferation of fibroblasts, collagen generation and mucus secretion through IL-13 generation, finally leading to airway inflammation and airway remodeling [Cortes et al., 2009; Kwon et al., 2014]. Moreover, TNF- α could act as a vital player in the development of airway inflammation through strengthening the immune responses involving IL-23/Th17 and Th2 immune response [Lee et al., 2016]. Additionally, INF- γ could trigger the differentiation of Th1 cells to improve the serum level of OVA-IgG2a and inhibit the proliferation of Th2 cells [Lama et al., 2011; Rabe et al., 2011]. Correspondingly, this study illustrated that PON1 reduces the expressions of inflammatory cytokines (IL-4, IL-5, IL-13 and TNF- α) as well as the serum levels of OVA-IgE and OVA-IgG1 and increases the INF- γ expression and the serum level of OVA-IgG2a, indicating that PON1 up-regulates the secretion of Th1 cytokine INF- γ to inhibit the TNF- α expression and enhance the serum level of OVA-IgG2a; thus, the differentiation of Th1 cells is induced, suppressing the contents of IL-4, IL-5 and IL-13 that are secreted by Th2 cells and leading to the inhibition of the generation and activity of IgE and decreased serum level of OVA-IgG1, thus alleviating the airway inflammation and remodeling.

In addition, our results also revealed that the expressions of proinflammatory cytokines (IL-6, IL-8 and TNF- α) were evidently inhibited by PON1 in LPS-induced lung macrophages. LPS-stimulated overactive macrophage acts as a main component of the outer membrane of Gram-negative bacteria that could promote the expression of IL-6, IL-8 and TNF- α and activate the immune response, ultimately resulting in inflammatory-related diseases or autoimmune diseases [Li et al., 2013; Liu et al., 2012]. As shown by Dr. Eisenbarth's group, with LPS intervention in OVA-sensitized mice, inhalation of a high concentration of LPS (100 μ g/ml) could stimulate the

Th1 immune response [Eisenbarth et al., 2002]. Thus, it is possible that PON1 can regulate the expression and secretion of IL-6, IL-8 and TNF- α secreted by LPS-induced macrophages to prevent the excessive activation of macrophages, mediate the immune function of macrophages and reduce the immune damage of the body, thereby decreasing the severity of inflammation in asthma.

Basement membrane thickening is one of the major characteristics of airway remodeling in asthma because of the activation of lung fibroblasts, resulting in the deposition of collagen and the expression of fibronectin [Ruiz and Jarai, 2012]. Therefore, fibronectin acts on the whole fibrotic environment via involvement in cell adhesion and migration as well as connecting with COL1A1 and other matrix proteins [Guo et al., 2014; Reeves et al., 2014]. In addition, Guo Z et al. found that IL-33/ST2 is complicated in airway remodeling in asthma through activating the overexpression of FN1 and COL1A1 in human lung fibroblasts HLF-1 [Guo et al., 2014]. Hence, we performed an *in vitro* culture of mouse lung fibroblasts that were transfected with overexpressed PON1 plasmid, to further verify the correlation of PON1 with airway remodeling and basement membrane thickening and found that PON1 can effectively inhibit the proliferation of lung fibroblasts and the expressions of COL1A1 and fibronectin.

In summary, PON1 could inhibit TNF- α expression and promote INF- γ expression, resulting in inducing the differentiation of Th1, instructing B cells to produce IgA and IgG2a, suppressing the secretion of Th2 cytokines (IL-4, IL-5, IL-13) and inhibiting the production and activity of IgE. In this way, the prevention of overactive macrophages and the regulation of the immune function of macrophages help to alleviate the immune damage to the body, as well as airway inflammation and remodeling, showing PON1 to be a new target for the treatment of asthma. However, it remains unclear how PON1 affects the occurrence and development of asthma via the downstream pathway, so it is necessary to explore more detailed research.

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Conflicts of interest

The authors declare no conflicts of interest.

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Legends

Figure 1 Pathological observation of lung tissue and comparison of immunohistochemical staining of α -SMA in each group

Notes: A, Observation of airway inflammation and remodeling in lung tissue by HE, PAS and Masson staining and detection of α -SMA by immunohistochemistry; B, semi-quantitative statistical histogram of PAS staining in each group; C, semi-quantitative statistical histogram of Masson staining in each group; D, semi-quantitative statistical histogram of α -SMA expression detected by immunohistochemistry. **** $P < 0.001$, compared with the control group; ## $P < 0.01$, ### $P < 0.005$, #### $P < 0.001$, compared with the asthma group; && $P < 0.01$, &&& $P < 0.005$, compared with the asthma + NC group. HE, hematoxylin; PAS, periodic acid-Schiff.

Figure 2 Expression of PON1 in lung tissues, peritoneal macrophages and serum of mice in each group

Notes: A, Protein expression of PON1 in lung tissue (A) and peritoneal macrophages (C) detected by western blot; 1-3, control group; 4-6, asthma group; 7-9, asthma + NC group; 10-12, asthma + PON1 group; B-C, statistical histogram of PON1 expression in lung tissue (B) and peritoneal macrophages (C) detected by qRT-PCR and western blotting. D-E, Arylesterase activity and lactonase activity of PON1 detected by colorimetry in peritoneal macrophages (D) and serum (E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ compared with the control group; #### $P < 0.001$ compared with the asthma and asthma + NC groups. PON1, Paraoxonase 1; qRT-PCR, quantitative real-time fluorescence polymerase chain reaction.

Figure 3 Effects of PON1 on MDA (A) and GSH (B) activity in the lung tissues

Note: ** $P < 0.01$, **** $P < 0.001$, compared with the control group; # $P < 0.05$, #### $P < 0.001$, compared with the asthma and asthma + NC groups

Figure 4 Comparison of inflammatory cells and cytokines in BALF in each group

Notes: A, changes of inflammatory cells in BALF observed via Wright Giemsa staining; B, expression of inflammatory cytokines (IL-4, IL-5, IL-13, TNF- α and INF- γ) in BALF in each group by ELISA. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$, compared with the control group; #### $P < 0.001$, compared with the asthma and asthma + NC groups. BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay.

Figure 5 Expressions of immunoglobulin in peripheral blood in each group by ELISA

Notes: ** $P < 0.01$, **** $P < 0.001$, compared with the control group; # $P < 0.05$, ### $P < 0.005$, #### $P < 0.001$, compared with the asthma group; & $P < 0.05$, &&&& $P < 0.001$, compared with the asthma + NC group. ELISA, enzyme-linked immunosorbent assay.

Figure 6 Expression of PON1 and the concentrations of inflammatory cytokines in LPS- induced macrophages

Notes: A, Protein expression of PON1 in LPS-induced macrophages detected by western blot; B, statistical histogram of PON1 expression in LPS-induced macrophages detected by qRT-PCR and western blot; C, effects of PON1 on the concentrations of LPS-induced IL-6, IL-8 and TNF- α detected by ELISA; D, effects of PON1 on the mRNA expression of LPS-induced IL-6, IL-8 and TNF- α detected by qRT-PCR. *** $P < 0.005$, **** $P < 0.001$, compared with the control group; ## $P < 0.01$, #### $P < 0.001$, compared with the NC group.

Figure 7 Expression of PON1 and collagen secretion as well as the proliferation of lung fibroblasts in mice

Notes: A, Protein expression of PON1 in lung fibroblasts detected by western blot; B, statistical histogram of PON1 expression in lung fibroblasts detected by qRT-PCR and western blot; C, MTT detection of the proliferation after 24, 48, 72 and 96 h of transfection of the mouse lung fibroblasts;

D, qRT-PCR detection of the effects of PON1 on the mRNA expressions of COL1A1 and fibronectin; E-F, western blot detection of the effects of PON1 on the protein expressions of COL1A1 and fibronectin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$, compared with the control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.005$, #### $P < 0.001$, compared with the NC group.

Table 1 Primer sequences for real-time quantitative polymerase chain reaction (qRT-PCR)

Gene	Primer sequence (5'-3')
PON1	Forward: 5'-GTCGTGGTCCAATGTTGTTTACT-3' Reverse: 5'-ACTTTCGATGACGTGCGTG-3'
IL-6	Forward: 5'-TTCACCTCTCCGGACAAAAC-3' Reverse: 5'-TCTGCCAGTACCTCCTTGCT-3'
IL-8	Forward: 5'-AGAGGTCTGCCTGGACCCCA-3' Reverse: 5'-GGGAGCCACGGAGAATGGGT-3'
TNF- α	Forward: 5'-TTCCAGCTGGCCCCCTTGAGC-3' Reverse: 5'-GAGGGCATTGGCATACCAC-3'
COL1A1	Forward: 5'-GCGGTAACGATGGTGCTGTT-3' Reverse: 5'-CTTCACCCTTAGCACCAAC-3'
Fibronectin	Forward: 5'-GAGCTGCACATGTCTTGGGAAC-3' Reverse: 5'-GGAGCAAATGGCACGGAGATA-3'
β -actin	Reverse: 5'-GATCATTGCTCCTCCTGAGC-3' Reverse: 5'-ACTCCTGCTTGCTGATCCAC-3'

Note: PON1: Paraoxonase-1; IL-6: Interleukin-6; TNF- α : tumor necrosis factor- α ; IL-8: Interleukin-8; COL1A1: type I collagen

Table 2 The mouse morphology index in each group

Groups	W/Pi ($\mu\text{m}^2/\mu\text{m}$)	S/Pi ($\mu\text{m}^2/\mu\text{m}$)	N/Pi (n/um)
control	5.47 ± 0.94	2.75 ± 0.19	0.015 ± 0.001
asthma	$11.99 \pm 0.40^{****}$	$6.71 \pm 0.22^{****}$	$0.033 \pm 0.002^{****}$
asthma+NC	$11.92 \pm 0.20^{****}$	$6.56 \pm 0.31^{****}$	$0.032 \pm 0.001^{****}$
asthma+PON1	$7.75 \pm 0.23^{****\#\#\#}$	$3.33 \pm 0.35^{****\#\#\#}$	$0.020 \pm 0.002^{****\#\#\#}$

Notes: Pi, perimeter inner; W, wall area; S, smooth muscle area; N, Number of smooth muscle cells;

*** $P < 0.005$, **** $P < 0.001$, compared with control group; #### $P < 0.001$, compared with asthma group and asthma + NC group.

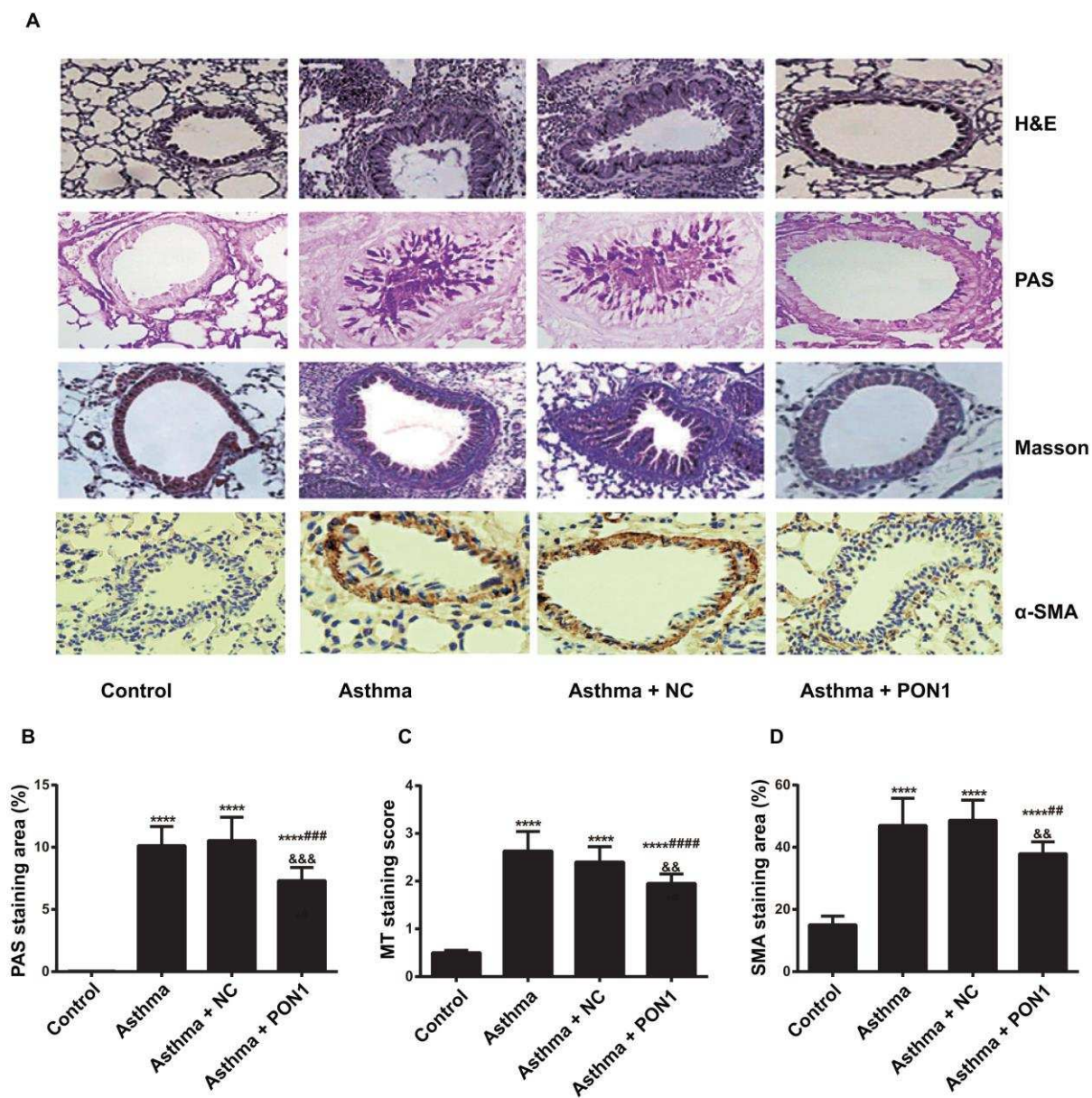


Figure 1

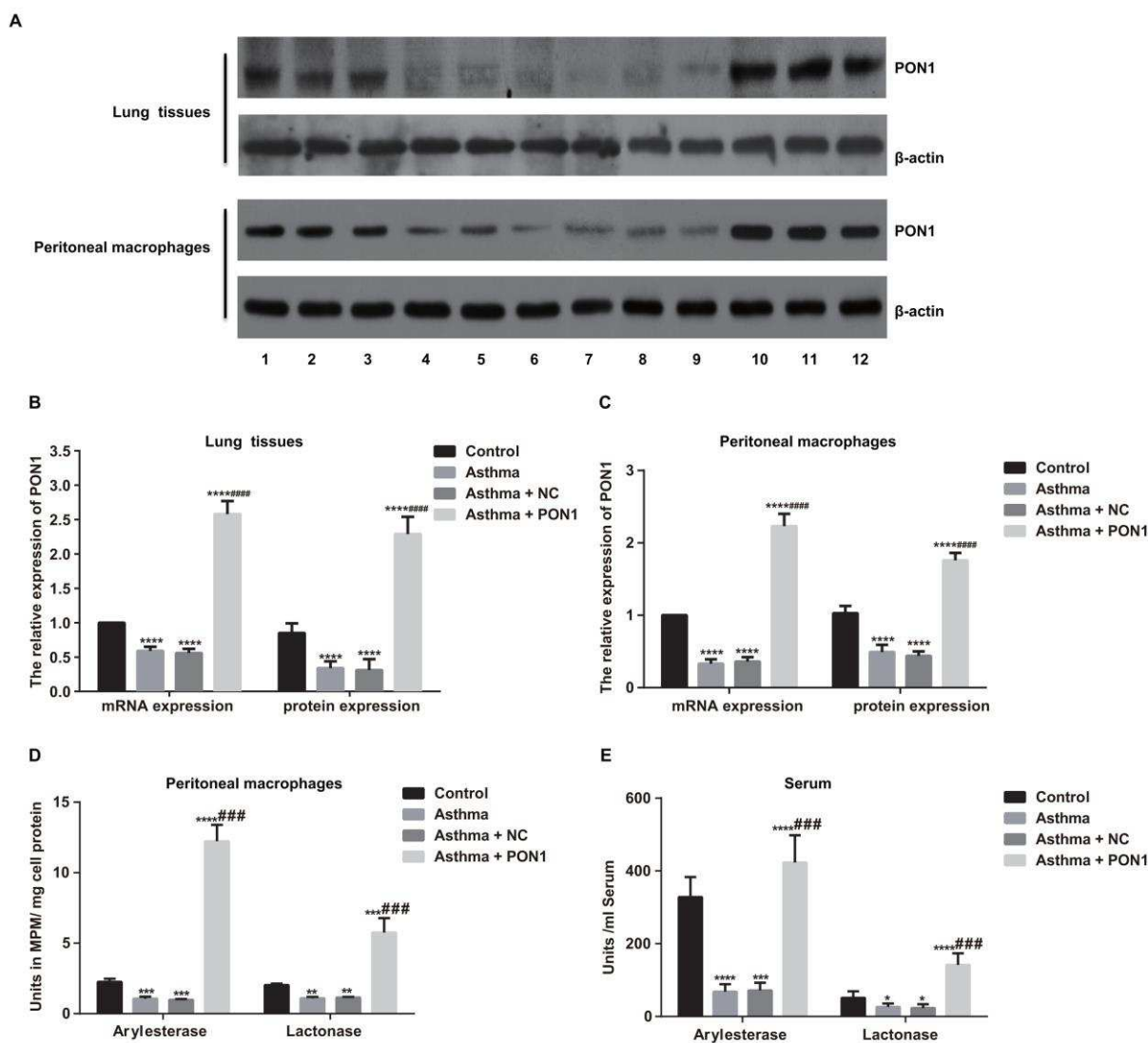


Figure 2

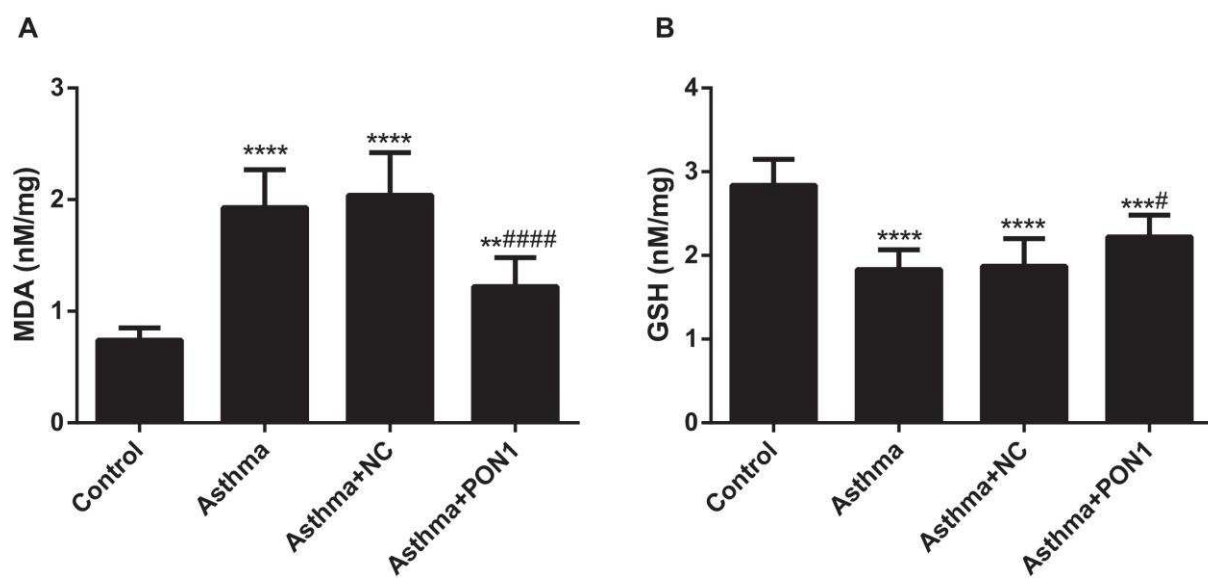


Figure 3

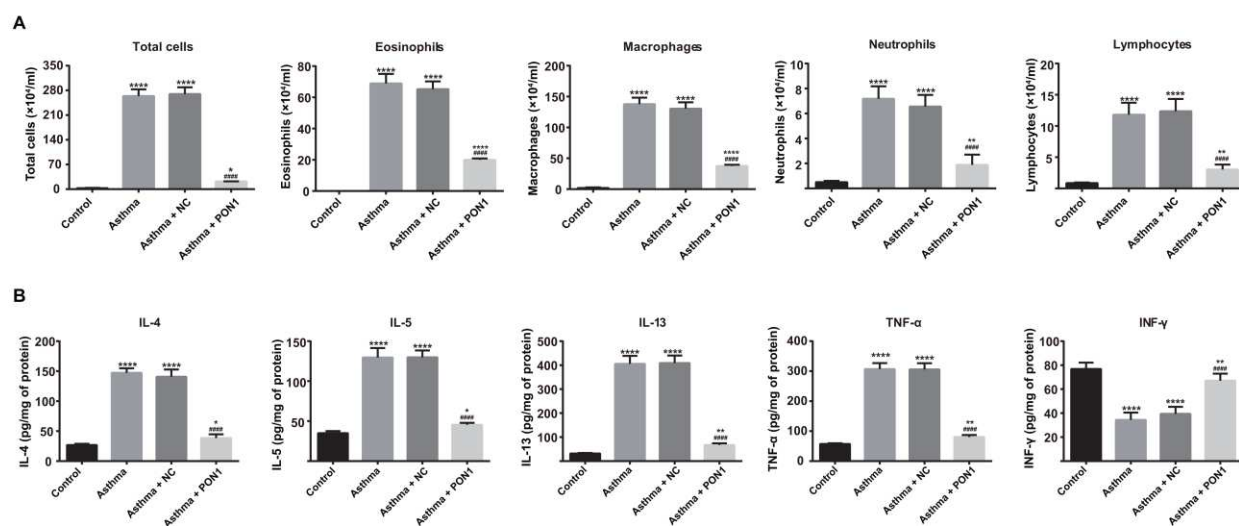


Figure 4

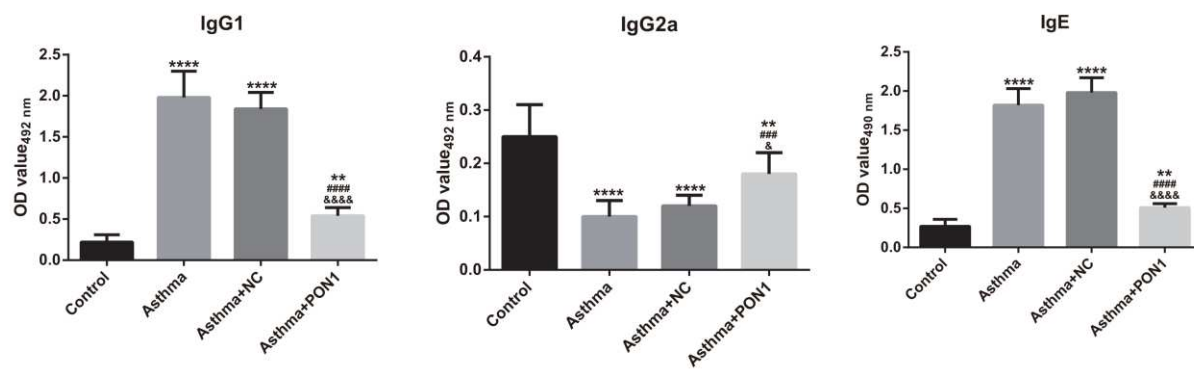


Figure 5

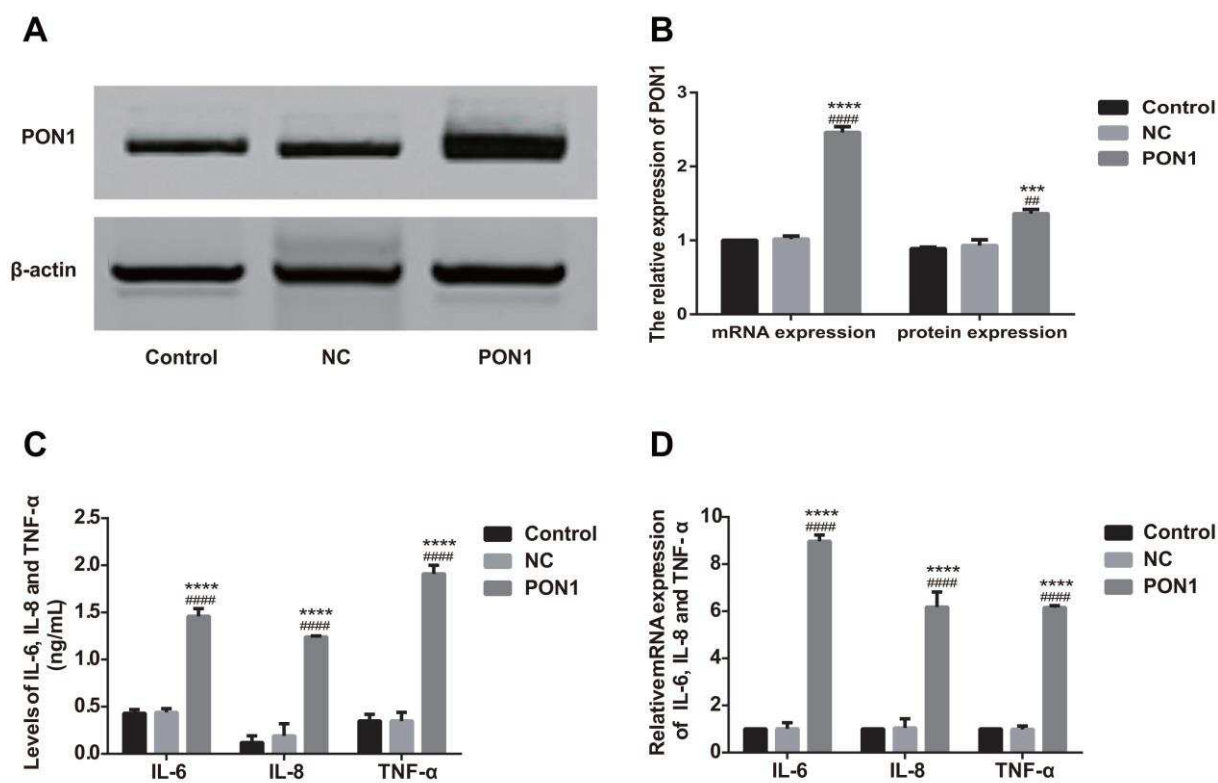


Figure 6

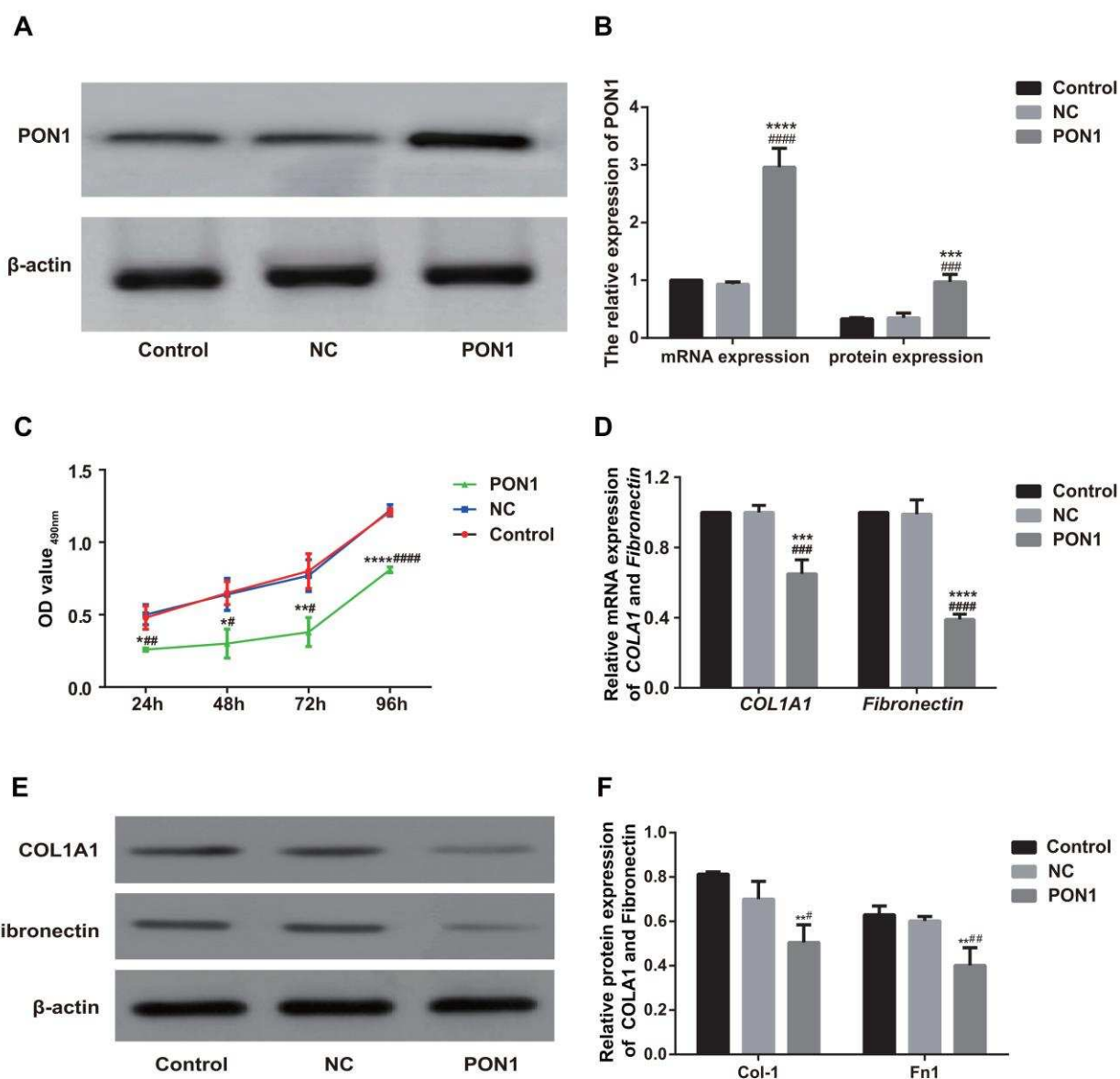


Figure 7