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Inhibition of 5-hydroxyindoleacetic acid to reduce neutrophil extracellular trap production improves lung condition in chronic obstructive pulmonary disease mice

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ABSTRACT

Background: Neutrophil extracellular trap (NET) correlate with chronic obstructive pulmonary disease (COPD) severity. Platelets can promote NET generation. However, serotonin alone or serotonin-deficient platelets do not adequately promote NET production. The metabolism of serotonin to 5-hydroxyindoleacetic acid (5-HIAA) in platelets may be the key to this difference.

Objective: The study aimed to determine whether 5-HIAA can influence NET production and thus play a role in COPD.

Methods: After a 4-hour co-incubation with lipopolysaccharide (LPS) and 5-HIAA, NET and ROS levels in the culture medium were measured by ELISA, and NET production with aryl hydrocarbon receptor (AHR) expression in adherent cells were analyzed by immunofluorescence.A COPD model was established in C57BL/6 mice through smoke exposure combined with LPS tracheal administration, followed by selegiline or 5-HIAA treatment. Post-intervention, lung function tests and sample collection were performed. The levels of 5-HIAA, ROS, NET, IL-6, and AHR in the samples were quantified by ELISA, pathological changes were assessed by HE staining, and NET/AHR expression was detected by immunofluorescence.

Results: 5-HIAA promoted NET production *in vitro*, and the nuclei of neutrophils secreting NET-like structures express AHR. In animal experiments, 5-HIAA levels were higher in both the plasma and lung tissues of COPD mice compared with normal mice. Inhibition of 5-HIAA in COPD mice down-regulated AHR expression, reduced reactive oxygen species and NET generation, elevated lung function indices (FEV0.1, FVC, PEF, and FEV0.1/FVC), decreased interleukin-6 levels, and improved lung tissue condition.

Conclusion: Inhibiting 5-HIAA reduces NET generation, thereby improving lung conditions in COPD mice, which is associated with the 5-HIAA/AHR pathway.

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5-HIAA; COPD; NET; AHR

1. Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most common diseases worldwide; it is characterized by persistent airflow limitation and respiratory symptoms, which severely affect patients' quality of life and lung function. According to statistics, COPD has become the third leading cause of death globally. With the increasing trend of an aging population, the number of patients with COPD continues to increase [1]. Cigarette smoking is a major risk factor for COPD and leads to an abnormal increase in reactive oxygen species (ROS) levels in the body, triggering a harmful response in the lungs [2]. When ROS levels overwhelm antioxidant defenses, ROS induces oxidative stress and inflammatory reactions, which are central drivers of COPD exacerbation, with neutrophils as crucial effector cells [3–6].

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Activated neutrophils release a network called neutrophil extracellular trap (NET), which primarily comprise chromatin and surface-attached histones, granulins, and proteases that help neutrophils capture and eliminate pathogens, such as bacteria, fungi, and viruses, in an intrinsic immune response. ROS are closely associated with NET formation [7], which is involved in the regulation of cytoskeletal rearrangements and chromatin depolymerization in neutrophils, prompting the release of NET from neutrophils. Simultaneously, NET formation by activated neutrophils is accompanied by a high level of ROS production. Since NET were first described, the understanding of their functions has expanded from their initial beneficial roles in capturing and killing pathogens to their deleterious roles in tissue injury, promotion of inflammation, and thrombosis. It has become a significant research focus across various areas, including infection, autoimmune diseases, and neoplasia [8,9]. In the context of COPD, NET are associated with exacerbation and severity of disease in patients with COPD [10,11]. Evidence has shown a large number of NET in the sputum of patient with COPD, and components such as neutrophil elastase (NE) and myeloperoxidase (MPO) are believed to cause inflammation and tissue damage [12,13]. Additionally, limiting the production of NET or eliminating excess NET can reduce inflammation, improve lung function and mitigate tissue damage [14,15].

Platelets induce neutrophil recruitment, migration, and NET production [16]. Serotonin from activated platelets promotes NET generation; however, its exact mechanism remains unclear. Notably, neither serotonin alone nor platelets without serotonin adequately induce NET production [17], suggesting that serotonin is involved in the reaction within platelets, influencing neutrophils. The serotonin transporter on the surface of the platelet absorbs serotonin into platelets and is subsequently metabolized by monoamine oxidase B (MAO-B) on the outer mitochondrial membrane to 5-hydroxyindoleacetic acid (5-HIAA). Interestingly, 5-HIAA is involved in neutrophil recruitment as a ligand for G protein-coupled receptor 35 (GPR35) [18]. Therefore, 5-HIAA may be critical for the response. In addition, we are concerned that 5-HIAA is also a ligand for the aryl hydrocarbon receptor (AHR) [19], which plays an important role in immunity and inflammation [20] and regulates multiple pathways involved in ROS generation [21]. Lung tissue contains a large number of AHR, and many studies have shown that AHR activation is associated with the pathogenesis of COPD [22–24]. Therefore, it is worth exploring whether 5-HIAA in platelets can promote NET production, whether it is associated with AHR, and whether it can influence the condition of COPD.

Hence, this study aimed to design cellular and animal experiments to investigate the role and effects of 5-HIAA on NET and COPD mouse model from different perspectives.

2. Materials and methods

2.1. Neutrophil extraction and purification

Mice were killed by cervical dislocation, tibia and femur were isolated, and bone marrow was rinsed with sterile 4 °C phosphate-buffered saline (PBS) and filtered through a 70-um cell filter to obtain bone marrow cell suspension. The samples were purified into neutrophil suspensions using the Mice Bone Marrow Neutrophil Isolate Kit (Beijing Solarbio Science & Technology Co., Ltd.) according to the instructions.

2.2. Cell culture and treatment

Extracted peripheral blood neutrophils were added to well plates containing 1% penicillin-streptomycin and Roswell Park Memorial Institute 1640 medium (Gibco, United States), with approximately 2×105 cells per group. The experimental groups were as follows: control (without any intervention), lipopolysaccharide (LPS, 1 μg/mL; Sigma-Aldrich, United States), 5-HIAA (100 nm; Sigma-Aldrich, CAS No. 54-16-0), and LPS + 5-HIAA The plates were then incubated at 37 °C and 5% CO2 under saturated humidity for 4 h. The supernatant was aspirated and stored at −80 °C for spare use, and the adherent cells were used for immunofluorescence detection.

2.3. Animal modelling and grouping

Experimental procedures for animal experiments were approved by Laboratory Animal Welfare and Ethics Committee Of the Army Medical University (approval number: AMUWEC20237054), and in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines. One hundred 7-week-old male C57BL/6 mice from the Animal House of the Army Medical University, each weighing approximately 20 g, were used in the experiments. The mice were housed at a constant temperature of 25 °C, under a 12-h controlled alternating cycle of bright and dark environments, and fed pellet mice food and water ad libitum. The mice were kept for 1 week to eliminate stress and acclimatize them to the environment. A COPD model

was established using smoke exposure and LPS tracheal administration [25]. The mice were exposed to smoke for 90 days by placing them in several homemade smoke inhalation containers (70 cm × 50 cm × 50 cm, relatively airtight Plexiglas boxes) and exposing them to cigarette smoke (10 mg of tar, 1.0 mg of nicotine, and 9 mg of carbon monoxide in the smoke from Chongqing Tobacco Industry Co. Ltd., China) twice daily each time lasting for 1 h and received an intratracheal injection of LPS (2 mg/kg) on days 1 and 14, with smoke exposure suspended on the days of administration.

After successful modelling, the mice were subjected to group interventions: (1) normal group (normal mice by saline gavage), (2) COPD group (COPD mice by saline gavage), (3) selegiline group (COPD mice by selegiline hydrochloride gavage), (4) 5-HIAA group (COPD mice by 5-HIAA gavage), and (5) selegiline +5-HIAA group (COPD mice with selegiline hydrochloride and 5-HIAA by gavage). Selegiline and 5-HIAA were dissolved in saline, with selegiline hydrochloride (10 gm/kg, MedMol, CAS No. 14611-52-0) [26] and 5-HIAA (0.5 mg/only, Sigma-Aldrich, CAS No. 54-16-0) [27]. The gavage solution was continuously administered in equal volumes to each group for 14 days. This procedure is shown in (Figure 1A).

2.4. Lung function

The mice in each experimental group were anesthetized *via* intraperitoneal injection with 50 mg/kg pentobarbital sodium. The neck tissues were disinfected, and the trachea was exposed by tissue separation. An endotracheal tube was inserted into the exposed trachea. The endotracheal tube of the anesthetized mice was connected to a lung function tester (FlexiVent; SCIREQ, Montreal, QC, Canada) to perform lung function assays using the FlexiVent FX-Mouse NPFE mode.

2.5. Histologic detection and pathological scoring

The right lungs of the mice were fixed in 4% paraformaldehyde (Biosharp, China), dehydrated and paraffin-embedded slices were made with a section thickness of 3 µm, haematoxylin and eosin (H&E) was completed, and the sections were observed and photographed using an Olympus light microscope. A crosshair was drawn at the center of each picture, the number of alveolar intervals (Ns) intersecting the crosshair was counted, the total crosshair length (L) was measured, and the alveolar mean linear intercept (MLI) (reflecting the mean alveolar diameter) was calculated using the following formula: MLI = L/Ns.

2.6. Collection of bronchoalveolar lavage fluid and plasma

The mice were anesthetized and fixed to the operating table. A tracheotomy was performed to insert an endotracheal tube, and a 1-mL syringe was used to draw 1 mL of sterile PBS (4 °C) into the lungs for two repeated rinses, which was then aspirated and collected into 1.5-mL centrifuge tubes. Subsequently, the heart was exposed, and blood was collected from the right ventricle using a 1-mL insulin syringe, injected into an anticoagulant tube containing ethylene diamine tetraacetic acid, and shaken well. Subsequently, the blood was transferred into a 1.5-mL centrifuge tube. Blood and BALF were centrifuged (4 °C, 3000 rpm, 10 min), and the supernatant was stored at −80 °C for spare use.

2.7. Enzyme-linked immunosorbent assay

BALF, plasma, cell culture media, and tissues were collected following the manufacturer's instructions. Tissue samples were homogenized, the total protein concentration was measured *via* bicinchoninic acid assay, and buffer was added to adjust the protein concentration of the samples for consistency. The absorbance values were obtained at 450 nm using a mouse-specific kits for 5-HIAA ELISA, AHR ELISA, mouse ROS ELISA, mouse NET, and IL-6 ELISA (all kits from Beijing Huabo Deyi Biotechnology Co., Ltd., China). The concentrations were calculated using a standard linear regression equation.

2.8. Immunofluorescence

After mice were anesthetized, PBS and 4% paraformaldehyde were perfused intracardially. Lung tissues were obtained and fixed in a 4% paraformaldehyde solution for 24 h, followed by dehydration in a 30% sucrose solution for another 24 h, and sliced into 10-µm-thick sections using a frozen sectioning machine (CM1900, Leica, Germany). The sections were permeabilized using 0.5% TritonX-100 for 30 min, blocked with 5% goat serum (BOSTER, China) for 1 h, and incubated overnight at 4 °C with primary antibodies anti-CitH3 (1:200; ab5103; Abcam), anti-Ly6G (1:200; ab25377; Abcam), and AHR antibody (1:200; AF6278; Affinity) and with secondary antibodies Alexa Fluor 488 Goat Anti-Rat antibody (1:400; Thermo Fisher Scientific) and Alexa Fluor 555 goat anti-rabbit antibody (1:400; Thermo Fisher Scientific) for 2 h, and DNA was stained with 4′,6-diamidino-2-phenylindole. Cell-climbing slice immunofluorescence with primary and secondary antibodies was performed at a concentration of 1:400, and

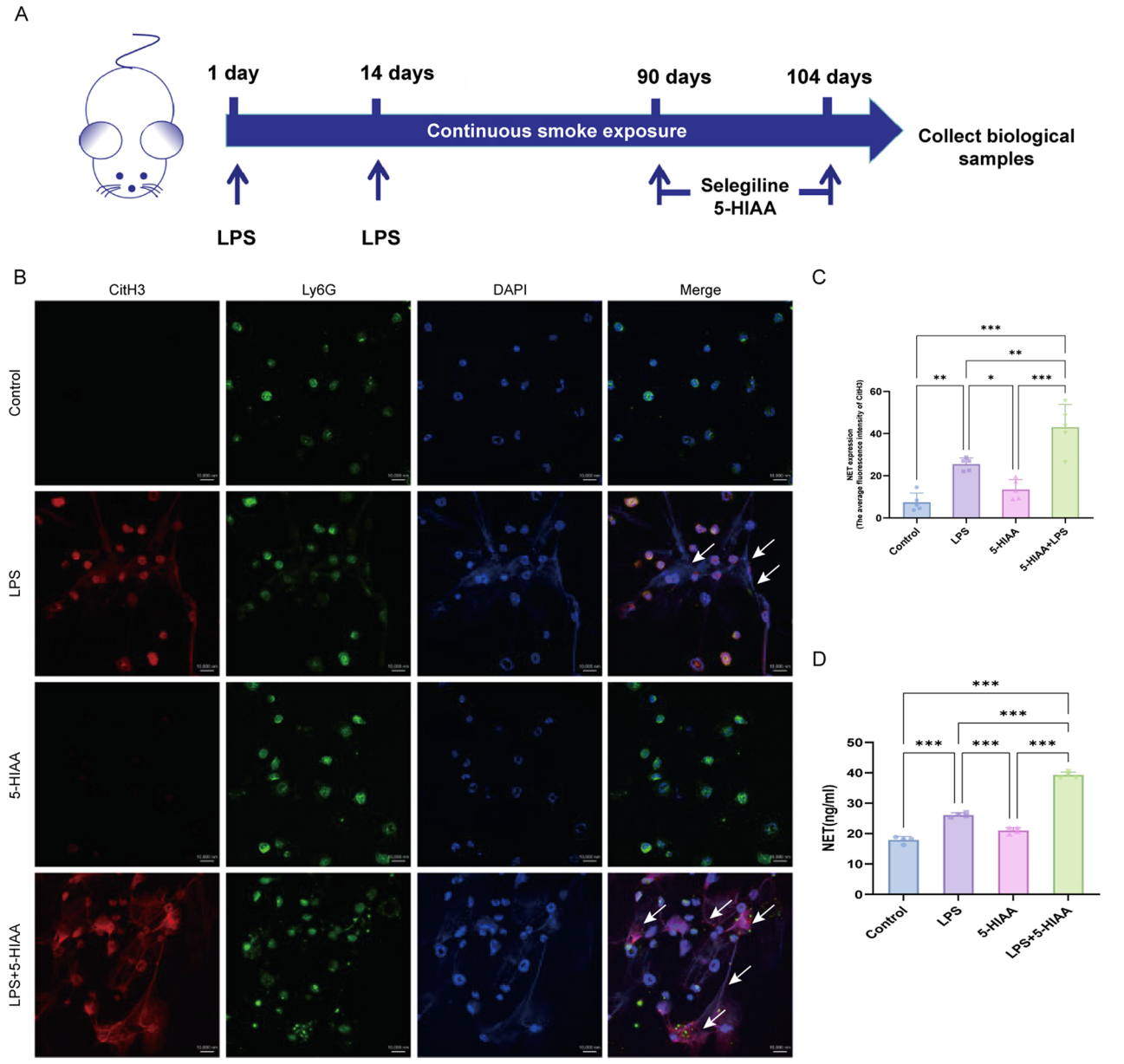

Figure 1. Chronic obstructive pulmonary disease (COPD) mice modelling process and 5-hydroxyindoleacetic acid (5-HIAA) intervention in lipopolysaccharide (LPS)-induced neutrophil extracellular trap (NET). (A) Flowchart of modelling and drug intervention in COPD mice. (B) Immunofluorescence staining of LPS-induced neutrophils in response to 5-HIAA intervention, citrullinated histones H3 (CitH3)-labeled NET in red, Ly6G-labeled neutrophils in green, and 4′,6-diamidino-2-phenylindole-labeled nuclei in blue, and all three together localize NET production by neutrophils. Arrowheads mark NET secreted by neutrophils, original magnification: ×630. (C) CitH3 mean fluorescence intensity quantified NET on cells, $n = 5$. (D) Enzyme-linked immunosorbent assay quantification of NET in culture medium, $n = 4$. Multiple comparisons of groups, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cells were observed under a confocal microscope (Zeiss 880, Germany) and photographed.

2.9. Statistical analyses

All statistical analyses were performed using GraphPad Prism 10.0 software, and experimental data are presented as means ± standard deviations. Statistical analyses were performed using one-way analysis of variance, and the significant differences between groups were analyzed using pairwise comparison and Dunnett's tests within the multiple comparisons framework. Significant differences are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

3. Results

3.1. 5-Hydroxyindoleacetic acid promotes lipopolysaccharide-induced neutrophil secretion of neutrophil extracellular trap

To verify the effect of 5-HIAA on neutrophils, we extracted mice bone marrow neutrophils and performed intervention experiments under lipopolysaccharide (LPS) stimulation (with or without 5-HIAA). We used immunofluorescence technique to examine the cell crawls and found that both the control and 5-HIAA groups produced less amount of NET. Moreover, compared with the control group, NET were significantly higher in the LPS and LPS+5-HIAA groups. Meanwhile, the LPS + 5-HIAA group had significantly higher NET compared with the LPS group (Figure 1B,C). The supernatant was tested for NET using enzyme-linked immunosorbent assay (ELISA) technique yielding similar results (Figure 1D).

3.2. Inhibition of in vivo 5-HIAA in chronic obstructive pulmonary disease mice improves lung function, reduces inflammation, and decreases lung tissue damage by decreasing NET production

To investigate whether 5-HIAA plays a role in COPD, drug (selegiline or 5-HIAA) treatment was performed after the COPD mouse model was constructed. The lung function test is the gold standard for assessing COPD in clinics, particularly with forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) % < 70% indicating airflow limitation. FEV0.1/FVC% in mice is a surrogate for FEV1/FVC% in humans (based on the conversion of human–mice physiological relationship and experimental experience) [28]. As shown in (Figure 2A), the mean lung function indices (FEV0.1, FVC, peak expiratory flow [PEF], FEV0.1/FVC%) of the COPD group were significantly lower than those of the normal group, and the mean FEV0.1/FVC% was <70%, which can be preliminarily determined that COPD modelling was successful. The mean lung function indices of mice in the selegiline group were significantly higher than those in the COPD group, whereas the changes in lung function indices in the 5-HIAA and selegiline + 5-HIAA groups were not significant.

To examine the histopathological changes in the lungs of mice, we used H&E staining to observe the lung tissue sections of mice and found that the COPD group exhibited COPD-related features, such as alveolar damage, alveoli fusion, inflammatory cell aggregation, bronchial wall thickening, and cilia shedding. The selegiline group demonstrated significantly thinner bronchial walls and lower inflammatory cell infiltration and aggregation than the COPD group. Structural damage to the alveolar wall was less severe, and the pathological condition of the lungs in the 5-HIAA and selegiline + 5-HIAA group showed lung tissue damage similar to that in the COPD group (Figure 2B). Additionally, we used the alveolar MLI to estimate alveolar size in order to assess the extent of lung tissue damage (Figure 2C). The alveolar MLI in the COPD group was significantly larger than that in the normal group. The MLI was significantly smaller in the selegiline group than in the COPD group, whereas the change in the MLI was not significant in the 5-HIAA and selegiline + 5-HIAA groups.

To assess inflammation, plasma and alveolar lavage fluid interleukin (IL)-6 levels were measured using ELISA (Figure 3A). IL-6 level was significantly higher in the plasma and bronchoalveolar lavage fluid (BALF) of the COPD group compared with the normal group. IL-6 level was significantly lower in the selegiline group compared with the COPD group, and IL-6 level significantly increased in the 5-HIAA group. Immunofluorescence detection revealed that compared with the normal group, neutrophil levels and NET in the lung tissues were significantly higher in COPD mice. Compared with the COPD group, the number of neutrophils and NET in the selegiline group were significantly lower, whereas there were no significant change in the 5-HIAA and selegiline + 5-HIAA groups (Figure 3B–D). Moreover, ELISA for NET in the plasma and BALF provided similar results (Figure 3E).

3.3. Significantly elevated levels of 5-HIAA in mice model of COPD

To evaluate the effects of selegiline and 5-HIAA, we measured the concentration of 5-HIAA in the plasma of each experimental group. Plasma 5-HIAA levels were significantly reduced after treatment with selegiline, and 5-HIAA level in the plasma of mice after 5-HIAA gavage significantly increased (Figure 3F). Selegiline is effective in lowering plasma 5-HIAA levels, but it does not completely eliminate 5-HIAA, likely owing to the presence of monoamine oxidase (MAO)-A. Since platelets are involved in neutrophil recruitment, migration, and generation of NET; thus, the relevant 5-HIAA is secreted by MAO-B-containing platelets. Notably, we found significantly higher plasma concentrations of 5-HIAA in the COPD group compared with the normal group. Therefore, we further examined the lung tissue and obtained similar results.

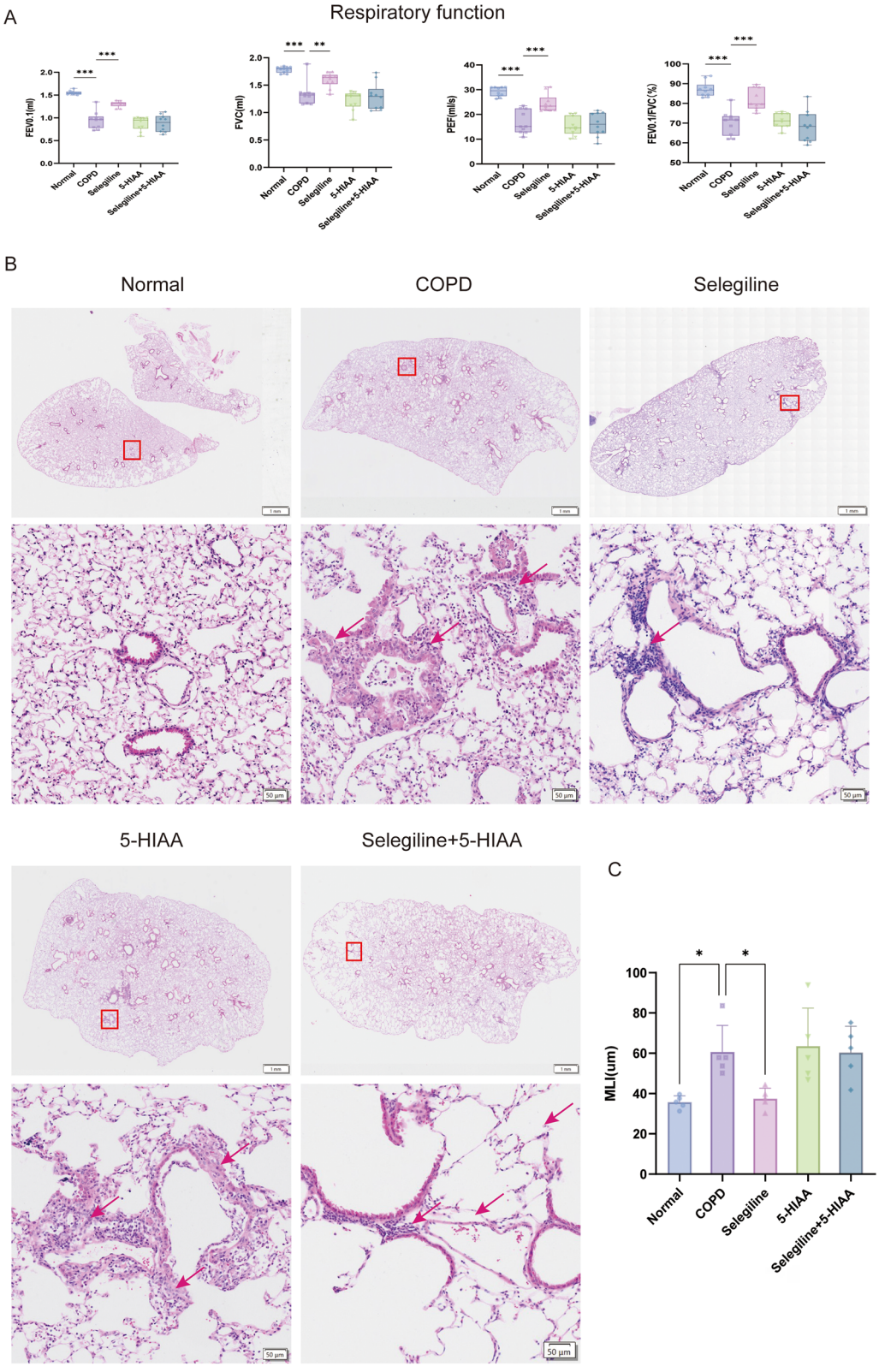

Figure 2. Respiratory function and pathological changes in chronic obstructive pulmonary disease (COPD) mice after inhibition or enhancement of 5-hydroxyindoleacetic acid. (A) Lung function assays, forced expiratory volume in 0.1 s (FEV0.1), forced vital capacity (FVC), peak expiratory flow [PEF], FEV0.1/FVC, $n = 10$. (B) Representative haematoxylin and eosin-stained sections of lung tissue. Arrowheads mark the characteristic pathological change of COPD, original magnification: ×200. (C) Alveolar mean linear intercept values were analyzed by two independent investigators, $n = 5$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with COPD mouse model.

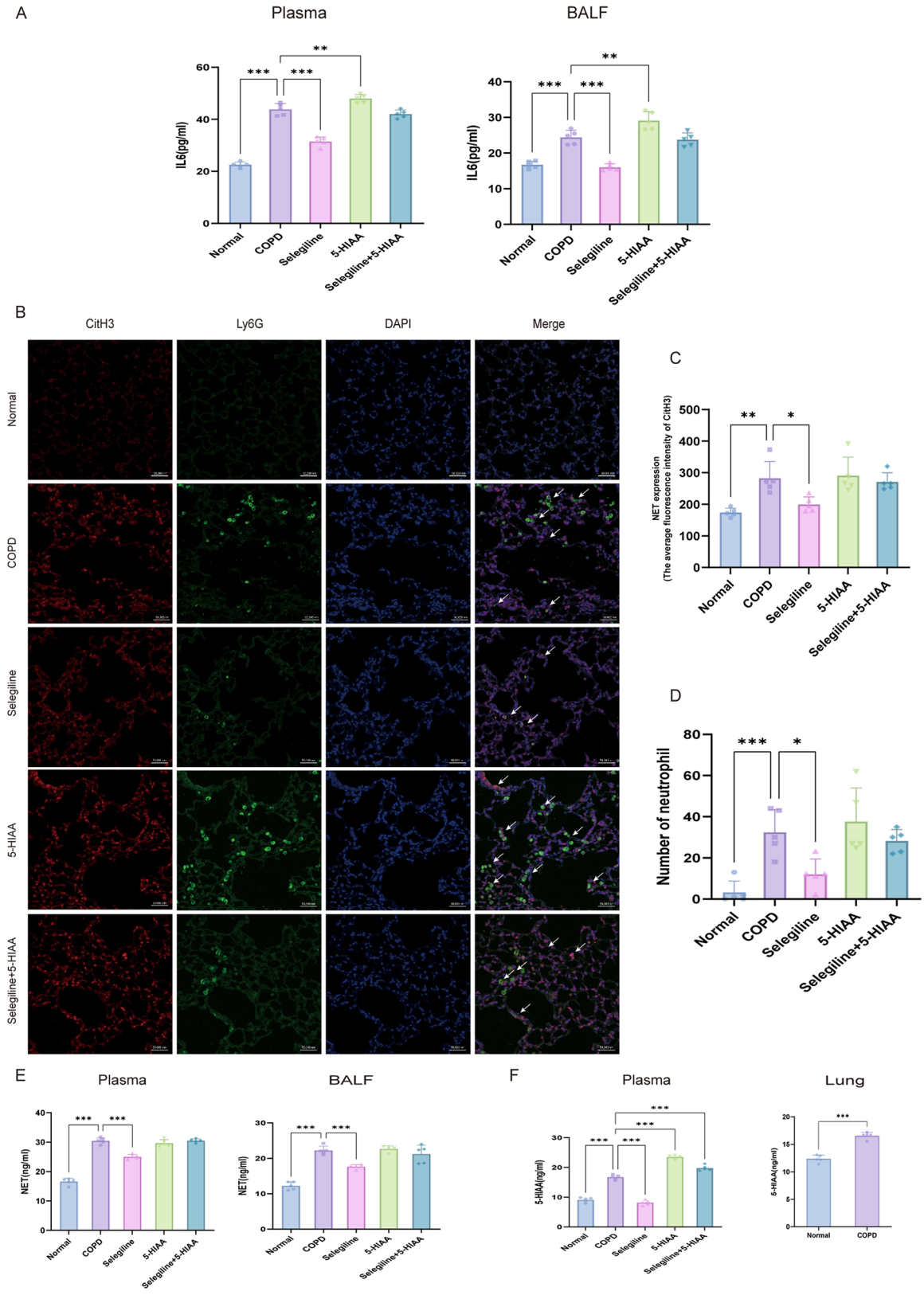

Figure 3. Inflammation and neutrophil extracellular trap (NET) changes in chronic obstructive pulmonary disease (COPD) mice after inhibition or enhancement of 5-hydroxyindoleacetic acid. (A) Interleukin-6 assay in mice plasma and bronchoalveolar lavage fluid (BALF), n=5. (B) Immunofluorescence staining of lung tissues in the animal test group, citrullinated histones H3 (CitH3)-labeled NET in red, Ly6G-labeled neutrophils in green, and 4′,6-diamidino-2-phenylindole-labeled nuclei in blue. Arrowheads mark NET in the lung tissue of mice, original magnification: ×200. (C) Quantification of NET by mean fluorescence intensity of CitH3 in lung tissues, n=5. (D) Neutrophil count based on Ly6G antibody labeling. (E) Detection of NET in plasma and BALF of mice, n = sample points per bar. (F) Concentration of 5-HIAA in mice plasma and lung tissues, n=5. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with COPD mouse model.

3.4. 5-HIAA promotes aryl hydrocarbon receptor nuclear expression and reactive oxygen species production

5-HIAA regulates AHR expression in B cells to inhibit arthritis [27]. However, it remains unclear whether 5-HIAA regulates neutrophils to promote NET production by acting on AHR in neutrophils. To verify our conjecture, we also assessed AHR expression in neutrophils *via* immunofluorescence (Figure 4A). We found that the nuclei and cytoplasm of cells in the control group expressed AHR, but the overall expression was low. AHR expression was higher and more evident in the nucleus in the LPS, 5-HIAA, and LPS + 5-HIAA groups than in the control group. In addition, we have observed that the expression of AHR in the nuclei of neutrophils secreting NET-like structures is often evident. ROS is an essential part of NET generation [29]. Therefore, we collected the culture medium supernatant to assess ROS levels using ELISA (Figure 4B). Compared with the control group, the LPS, 5-HIAA, and LPS + 5-HIAA groups showed higher ROS levels, and the increased ROS levels in the LPS + 5-HIAA group had a significant advantage.

3.5. Inhibition of in vivo 5-HIAA downregulates AHR expression and ROS production in lung tissues of COPD mice

AHR is expressed in various immune cell types (including neutrophils) and human tissues (including lungs) [30], and its level and activity are regulated by exogenous or endogenous ligands [31]. Increased AHR expression in COPD rats may be related to the higher amount of AHR ligands in cigarettes or airborne hazardous particles [32], although the possibility that COPD causes endogenous ligand changes cannot be excluded. We found elevated 5-HIAA levels in COPD mice (Figure 3A). To investigate the impact of 5-HIAA changes on AHR, we examined AHR expression levels in lung tissues (Figure 5B). Compared with the normal group, AHR expression was higher in the COPD group. Additionally, we found that inhibiting 5-HIAA decreased AHR expression, whereas exogenously increasing 5-HIAA increased AHR expression. To examine the site of AHR expression, immunofluorescence was used to detect AHR expression in the lung tissues. As shown in Figure 5A, AHR in the normal group was expressed in most cells of lung tissues, mainly in the cytoplasm, with relatively minimal expression in the nucleus. AHR expression was significantly enhanced in the nuclei of lung cells and neutrophils in the COPD mouse model. Compared with the COPD group, there was a significantly lower expression of AHR in the nuclei in the selegiline group and a higher expression of AHR in the nuclei of the cells in the 5-HIAA group, which was particularly evident in the neutrophils. ROS levels in plasma and BALF were assessed, and significant increases were observed in the COPD group compared with the normal group. Compared with the COPD group, ROS was significantly lower in the selegiline group and was found to increase but not significantly in the 5-HIAA group, whereas in the selegiline + 5-HIAA group, the changes were not significant (Figure 5C).

4. Discussion

COPD is a complex and cryptic chronic respiratory disease, and its pathology involves narrowing of the small airways and damage to lung tissues. Currently, there are no specific drugs for COPD treatment, and treatment strategies primarily focus on palliative therapies to improve symptoms, such as airflow limitation and dyspnoea. Treatment modalities mainly include medications, oxygen therapy, and rehabilitation, and the research and development of targeted medications are advancing [33]. Current research suggests that the main mechanisms of COPD pathogenesis include the following: repeated inflammatory stimuli, protease/antiprotease imbalance, and oxidative stress. As COPD progresses, inflammatory cells infiltrate lung tissues to produce long-term inflammatory stimuli, causing airway remodelling and alveolar damage, particularly through neutrophil secretion of ROS, elastase, and NET, causing cytoarchitectural damage, further activating inflammatory cells, aggravating oxidative stress, disrupting the activity of antiproteases (α1-antitrypsin), and causing damage, such as impairment of the function of the lung parenchymal elastin fibres and alveolar barriers [34]. Additionally, 20–30% of patients with COPD exhibit eosinophilia [35], and eosinophilic inflammation is associated with an increased risk of worsening COPD [36]. COPD is attacked in a multidimensional way, and targeting a single mechanism to improve it may have limited effect.

In cellular experiments, we found that 5-HIAA promoted LPS-induced NET secretion from neutrophils, and the nuclei of neutrophils secreting NET-like structures showed expression of AHR. AHR is a ligand-activated transcription factor found in the cytoplasm. Upon ligand binding, AHR detaches from binding to the chaperone molecules Hsp90 and X-associated protein, translocates to the nucleus, and binds to the AHR nuclear translocator (ARNT) to form an AHR/ARNT heterodimer, which connects to DNA to create a site-specific xenobiotic response element [37]. LPS and 5-HIAA

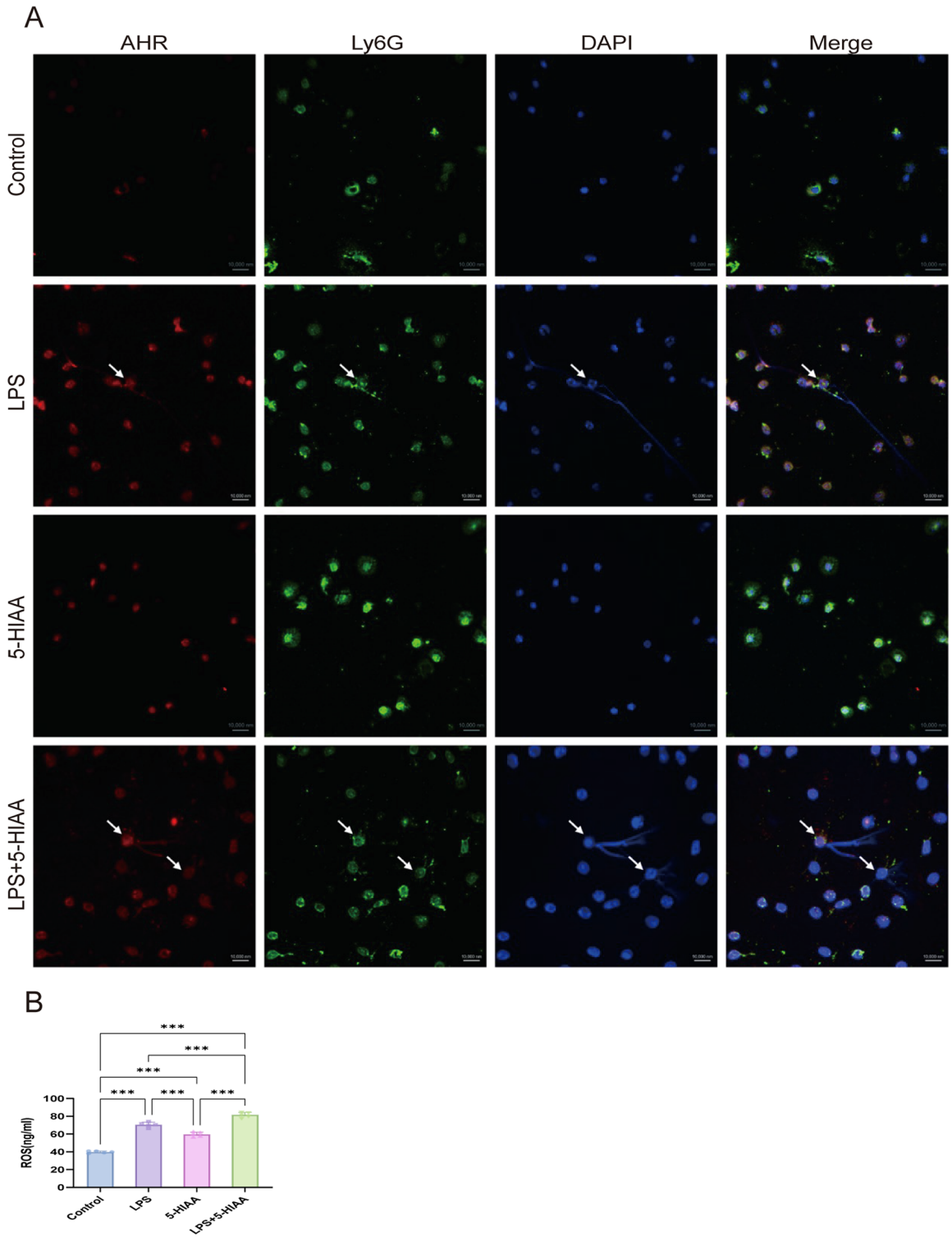

Figure 4. Changes in aryl hydrocarbon receptor (AHR) and reactive oxygen species (ROS) after 5-HIAA intervention in neutrophils. (A) Immunofluorescence staining of cell crawls of each experimental group, AHR in red, Ly6G-labeled neutrophils in green, and 4′,6-diamidino-2-phenylindole-labeled nuclei in blue. Arrowheads mark AHR nuclear expression in neutrophils secreting NET-like reticulated substances, original magnification: ×630. (B) ROS in the cell culture fluid of each group. Multiple comparisons of groups, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 5$.

promote nuclear AHR expression in neutrophils, which may promote ROS production by neutrophils. The classical AHR/CYP1 (cytochrome P450 family-1 subfamily-A polypeptide-1) pathway is accompanied by the production of ROS when metabolizing substances [38, 39]. Meanwhile, AHR activation is involved in the

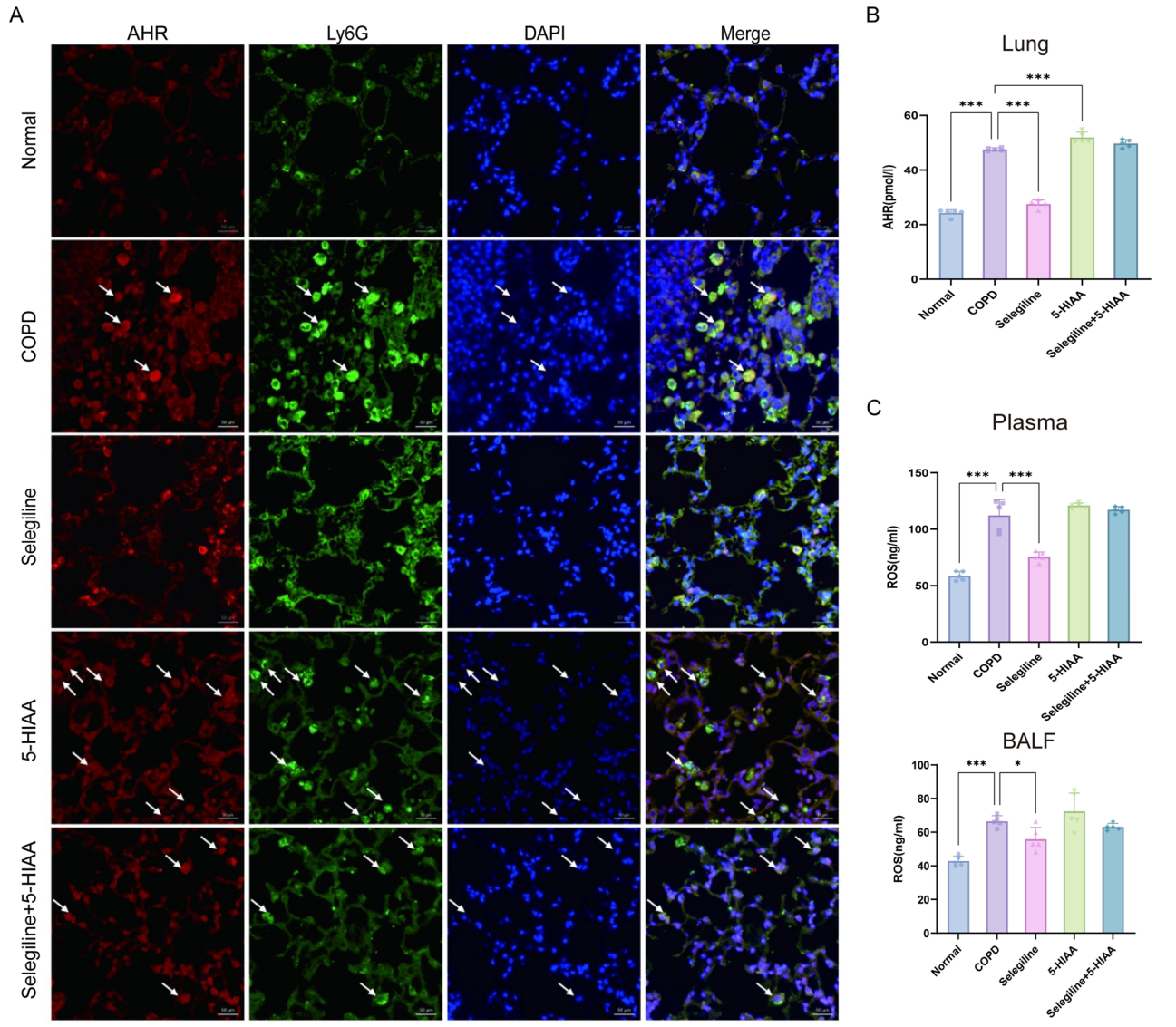

Figure 5. *In vivo* aryl hydrocarbon receptor (AHR) and reactive oxygen species (ROS) changes in chronic obstructive pulmonary disease (COPD) mice after inhibition or enhancement of 5-hydroxyindoleacetic acid. (A) Immunofluorescence staining of lung tissues in the animal test group, AHR in red, Ly6G-labeled neutrophils in green, and 4′,6-diamidino-2-phenylindole-labeled nuclei in blue. Arrowheads mark the nuclear expression of AHR in neutrophils in mouse lung tissue, original magnification: ×200. (B) Enzyme-linked immunosorbent assay quantification of AHR in the lung tissues. (C) ROS levels in plasma and bronchoalveolar lavage fluid of mice in the experimental group. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $n=5$, compared with COPD mouse model.

transcriptional regulation of NADPH oxidase (NOX) 1 and dual oxidase 1, leading to an increase in ROS [40]. A study on diabetic nephropathy found that genetic knockout or pharmacological inhibition of AHR can reduce NOX4 expression [41], and AHR is also involved in the regulation of NOX2 and p47phox expression [42,43]. These lines of evidence suggest that AHR regulation is an important pathway for physiological ROS production. ROS plays a crucial role in the generation of NET, regardless of whether it is NOX-dependent or -independent NET [44]. Sufficient amounts of ROS in neutrophils stimulate MPO to trigger the activation of NE, which translocates to the nucleus to process histone proteins to drive chromatin decondensation. Simultaneously, ROS synchronously activate peptidyl arginine deiminase 4 (PAD4) to mediate histone citrullination in concert with MPO to depolymerize chromatin [7]. Additionally, breast cancer research has confirmed that overexpression of AHR in breast cancer leads to an increase in IL-8, which is well-known to effectively stimulate neutrophils to secrete NET [45]. As an AHR ligand, 5-HIAA has a beneficial effect on AHR expression in the nucleus of neutrophils during LPS-induced neutrophil activation, which may be an important 5-HIAA-promoted NET production factor. Notably, 5-HIAA, a ligand of GPR35, can recruit neutrophils and eosinophils to

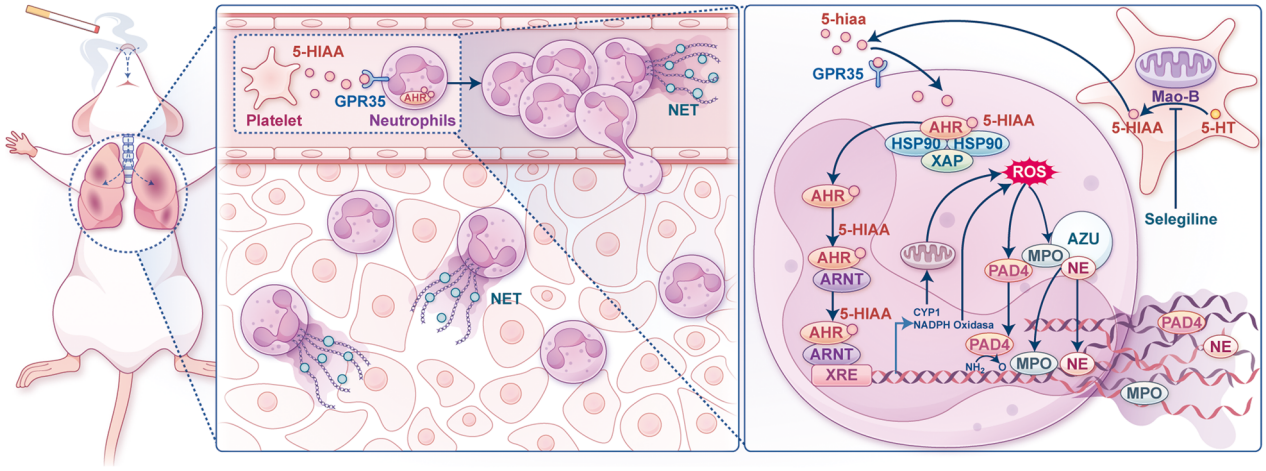

Figure 6. Mechanism diagram. Driven by inflammation, 5-hydroxyindoleacetic acid secreted by blood platelets in chronic obstructive pulmonary disease mice activates G protein-coupled receptor 35 (GPR35) and aryl hydrocarbon receptor (AHR) in neutrophils. GPR35 activation leads to neutrophil recruitment into diseased lung tissue, and AHR transfers to the nucleus after activation, where it binds to AHR nuclear translocator (ARNT) to form an AHR/ARNT heterodimer, which connects to DNA and forms a specific site of xenobiotic response element, regulating the transcriptional level and activity of cytochrome P450 family 1 subfamily B member 1 enzymes and nicotinamide adenine dinucleotide phosphate oxidase, resulting in an intracellular reactive oxygen species (ROS) burst. ROS stimulates myeloperoxidase (MPO) to trigger the activation of neutrophil elastase (NE), which then translocates to the nucleus to process histones, leading to chromatin decondensation. Concurrently, ROS also activates PAD4 to mediate histone citrullination, synergizing with MPO to disassemble the chromatin. This series of chain reactions ultimately results in the secretion of NET. In azurophilic granules(AZU), ROS stimulates myeloperoxidase (MPO) to trigger the activation of neutrophil elastase (NE), which then translocates to the nucleus to process histones, leading to chromatin decondensation. Concurrently, ROS also activates peptidyl arginine deiminase 4 (PAD4) to mediate histone citrullination, synergizing with MPO to disassemble the chromatin. This series of chain reactions ultimately results in the secretion of NET.

inflammatory sites [18,46]. These pieces of evidence suggest that inhibiting 5-HIAA may affect the inflammatory progression of COPD in multiple ways. The mechanism is shown in Figure 6.

In animal experiments, we treated mice with either selegiline hydrochloride or 5-HIAA to reduce or increase 5-HIAA levels *in vivo*. Selegiline is a selective MAO inhibitor that effectively inhibits MAO-B in platelets and is commonly used to treat psychiatric disorders and Parkinson's disease [47]. Our results showed that inhibition of 5-HIAA in COPD mice downregulated AHR expression, especially in the nucleus, and NET was controlled, which led to improvement of lung function and reduction of inflammation and lung tissue injury in COPD mice. However, there was an increase in AHR expression in the lungs of COPD mice after supplementation with exogenous 5-HIAA, the effect on NET was not significant, and this did not appear to be a significant exacerbation as expected. We found that 5-HIAA in plasma and lung tissue of COPD mice was already at relatively high values, and the limited effect of increasing 5-HIAA on neutrophils may be the reason for this. Additionally, the improvement of lung condition in mice in the selegiline + 5-HIAA group was not significant, suggesting that selegiline made a greater contribution to COPD improvement by inhibiting 5-HIAA production. In conclusion, we suggest that inhibition of the 5-HIAA/AHR pathway to reduce ROS in lung tissue contributes to the reduction of NET generation, which improves the lung condition of COPD mice. Certainly, the contribution made by the reduction of inflammatory cell recruitment in lung tissues induced by limiting the 5-HIAA/GPR35 pathway cannot be excluded.

5-HIAA, a metabolite of serotonin, has long been regarded as an end-product with no biological effects. However, 5-HIAA has recently been found to act as a ligand on immune cells, such as B cells, neutrophils, and eosinophils, in the immune process, and its role in immune cells and related disorders is gradually being unveiled. A previous study reported elevated 5-HIAA levels in the blood of patients with COPD complicated by depression, negatively correlating with lung function [48]. Additionally, elevated serum 5-HIAA levels have been observed in a rat model of smoke-induced chronic bronchitis [49]. We also found significantly elevated 5-HIAA in COPD mouse model, and 5-HIAA abnormality may play an important role in the pathogenesis of COPD. A preliminary study on COPD suggested that the MAO inhibitor selegiline attenuated oxidative stress and inflammatory responses in a smoke-induced lung inflammation rat model [50].

Although this mechanism may involve the direct anti-inflammatory antioxidant effect of the drug, we believe that it may also act by reducing 5-HIAA levels in rats, leading to reduced recruitment of inflammatory cells and NET production. Our data were obtained using animal models, and further clinical studies are required to clarify the role of 5-HIAA in COPD pathogenesis.

AHR plays a multifaceted role in immune system regulation, and the AHR can be either pro-inflammatory [51,52] or anti-inflammatory [53,54], depending on the ligand bound. The benefits and drawbacks of AHR expression in disease have not yet been fully recognized. The AHR has generated a large number of studies in COPD, and the results of these studies suggest that AHR expression may have favourable or unfavourable effects on COPD [55]. We believe that there is a homeostasis of AHR in the body and that when AHR is over- or under-represented, it leads to the pathological development of related diseases, and intrinsic 5-HIAA-like ligands play a regulatory role as mediators. In addition, LPS increases the expression of AHR and cytochrome P450 family-1 subfamily-A polypeptide-1 in differentiated immune cells through the activation of the nuclear factor-kappa B (NF-κB) signalling pathway, in both *in vivo* and *in vitro* experiments. Moreover, the sensitivity of AHR to ligand response was enhanced in immune cells after LPS stimulation [56]. Moreover, LPS-induced inflammatory response involves AHR expression, and in the absence of exogenous ligands for AHR, AHR is involved in regulating the transcription of pro-inflammatory genes in the LPS/Toll-like receptor 4 and NF-κB pathways *in vivo* [57]. We similarly observed increased expression of AHR in the nucleus and increased ROS production in LPS-treated neutrophils in our cell experiments, further suggesting the possibility that AHR has the potential to influence NET. In fact, we also observed that AHR in neutrophils secreting NET tended to have more pronounced expression in the nucleus. Interestingly, NET exhibit a double-edged role in inflammation. It can trap and kill exogenous microorganisms, such as bacteria, fungi, and viruses, to reduce tissue inflammation. However, excessive NET indiscriminately attack autologous cells. Therefore, there may be potential finer regulatory targets of NET in the AHR pathway, whose activation involves many complex and interesting mechanisms. Although AHR is present in most organs and tissues in the body, exploring its role in immune cells and its potential regulatory role in NET may provide new insights into the treatment of various inflammatory diseases.

This study has some limitations. First, we did not use knockout or knockdown techniques to process the samples, which may have resulted in inadequate control of the expression levels and crosstalk effects with other non-target genes. We believe that knockout or knockdown approaches yield relatively objective results but are more likely to cause uncontrollable organism damage, which is not significantly meaningful for guiding clinical practice. We selected selegiline because it is a commonly used antidepressant and treatment for Parkinson's disease, and depression is a common complication of COPD [58], and COPD and Parkinson's disease are also comorbidities in elderly patients [59]. At this point, our study is sensible. Both AHR and GPR35 are present in neutrophils as receptors for 5-HIAA and are involved in neutrophil recruitment [60,61]. We have not deeply explored the evolution of neutrophils or the disease in the presence of only one of these genes. Second, although we examined AHR expression during our study of the 5-HIAA-promoted NET pathway, we did not explore the specific mechanisms by which AHR increases ROS production. Furthermore, we did not collect clinical samples, leaving the differences between COPD mouse model and patients unexamined. This will be explored in further studies.

In conclusion, our results suggest that 5-HIAA has a role in promoting NET production, and inhibition of 5-HIAA in COPD model mice reduces NET in lung tissues, thereby improving lung function and reducing lung inflammation and lung tissue injury. The mechanism is to block the 5-HIAA/AHR pathway, which provides a new strategy and idea for the treatment of COPD.

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None.

Authors contributions

Conceptualization: W.L.; Data curation: L.X.; Formal Analysis: Q.Z.; Funding acquisition: W.X.; Investigation: Q.Z.; Methodology: L.X.; Project administration: W.Z.; Resources: W.L.; Software: C.L.; Supervision: W.L.; Validation: Q.Z. and L.X.; Visualization: Q.Z.; Writing – original draft: Q.Z.; Writing – review & editing: W.X. and X.D. All authors reviewed and agreed to the final draft.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data generated by this research can be obtained from the authors upon reasonable request.

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