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Proteogenomic verifies targets underlying erythromycin alleviate neutrophil extracellular traps-induced inflammation

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Abstract

Background Neutrophil Extracellular Traps (NETs) are closely related to the progression of inflammation in Chronic Obstructive Pulmonary Disease (COPD). Erythromycin (EM) has been shown to inhibit inflammation in COPD, but its molecular mechanisms is still unclear. The aim of our study is investigate the molecular mechanisms of EM's anti-inflammatory effects in NETs-induced inflammation.

Methods Transcriptomics and proteomics data were obtained from U937 cells treated with NETs and EM. Differentially expressed genes (DEGs) and differentially expressed proteins (DEPs) were identified using R software. Pathway enrichment analyses, were employed to identify inflammation-related pathways. Cytoscape were utilized to construct network of hub targets regulated by EM which related with oxidative stress and inflammation. Additionally, Cytoscape and STRING were used to construct protein–protein interaction (PPI) network of key targets regulated by EM. The expression levels of key targets were further confirmed through WB and PCR experiments.

Results Both transcriptomics and proteomics indicate that EM decrease NETs –induced AKT1 expression. Enrichment analysis of DEGs and DEPs reveal multiple common pathways involved in EM's regulation inflammation, including the PI3K/AKT pathway, response to oxidative stress, IKK/NF-κB signaling and PTEN signaling pathway. Nine key targets in PI3K/AKT-related inflammatory pathways regulated by EM and ten targets of EM-regulated oxidative stress were identified. WB and PCR results confirmed that EM reversing the NETs-induced inflammation by modulating the activity of these targets. Furthermore, clinical samples and vitro experiments confirm that EM alleviates NETs-induced glucocorticoid resistance via inhibiting PI3K/AKT, thereby repressing inflammation.

Conclusions Our study provides a comprehensive proteogenomic characterization of how EM alleviates NET-related inflammation, and identify PI3K/AKT play a critical role in the mechanism by which EM inhibits inflammation.

Keywords Proteogenomic, Erythromycin, NETs, Inflammation, PI3K/AKT

Introduction

According to the World Health Organization (WHO), COPD was the fourth leading cause of death globally in 2021, resulting in 3.5 million deaths, which accounted for approximately 5% of all deaths globally (https://www.who.int/news-room/fact-sheets/detail/chronic-obstructive-pulmonary-disease-(copd)). Consequently, the prevention and management of COPD is an urgent task

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in public health. Persistent and aberrant inflammatory processes in the airways is the characterized of COPD [1, 2]. The pathogenesis of COPD is intricately linked to environmental factors, tobacco smoke and oxidative stress playing pivotal roles in the development of respiratory inflammation [3, 4]. NETs are complex DNA–protein structures released by neutrophils, in chronic inflammation of COPD, tobacco smoke and oxidative stress stimulation can cause the production of NETs [5–7]. Recent studies have demonstrated that increased NET formation in the airways of COPD patients correlates with disease severity and chronic inflammation [8, 9].

In patients with COPD, neutrophilic inflammation is significantly elevated compared to healthy controls, and the enhance formation of NETs in COPD is related to heightened airway inflammation, and more severe airflow limitation [10, 11]. By promoting the differentiation of Th1 and Th17 cells, NETs also play a significant role in the progression of smoking-related chronic lung inflammation [8]. In sputum and peripheral blood, NETs are associated with elevated levels of pro-inflammatory cytokines, including CXCL8, IL-1β [8, 12, 13]. Additionally, the TLR4/MAPK/NF-κB pathway can be activated by NETs, inhibiting NETs producing by mitoTEMPO, and degrading NETs-DNA with DNase-I repress tobacco smoke-stimulated NF-κB-related inflammation in COPD patients and mouse [14, 15]. All in all, these results indicate a strong association between NETs and airway inflammation in COPD, however, the molecular mechanism of NETs-induced inflammation in COPD still require comprehensive and systematic investigation.

Numerous studies have demonstrated that macrolide antibiotics, such as azithromycin and erythromycin (EM), possess not only antibacterial properties but also significant anti-inflammatory effects [16, 17]. Long-term, low-dose azithromycin has been widely utilized to treat COPD [18–21]. In a large randomized controlled trial involving 1142 COPD patients, daily administration of 250 mg of azithromycin for one year significantly reduced the frequency of exacerbations (HR for time to first episode 0.72 [95% CI 0.63–0.84]), and improved the patients' quality of life [22]. Our previous study showed long-term, low-dose EM treatment for 6 months reduced exacerbation frequency in stable COPD patients, and decreased their inflammatory cytokine levels [23]. In animal models, EM mitigated lung inflammation in mice exposed to tobacco by downregulating NF-κB protein expression [24]. Additionally, EM upregulates HDAC1, HDAC2, and HDAC3 in human macrophages, and inhibits NF-κB expression via its antioxidative effects, ultimately reduces NE-kb related inflammatory cytokine release [16]. Combine treatment with EM and inhaled corticosteroids improves glucocorticoid resistance in COPD patients by suppressing the PI3K/AKT signaling pathway in mononuclear cells [25]. In vitro experiments of U937 cell, EM reduced glucocorticoid resistance induced by tobacco smoke stimulation via inhibiting JNK/AP-1 signaling and decreasing IL-8 release [26]. However, the protective effects of EM against NETs-induced inflammation in COPD is still lacking in comprehensive and systematic studies.

In this study, we aim to investigate systematically study the protective effect of EM on NETs-induced inflammation. First, transcriptomics and proteomics were employed to observe the changes of gene and protein expression underlying EM stimulate, then enrich the pathways involved in inflammation. Next, key modules and targets of EM in regulating NETs-related inflammation were screened based on the omics analyses. Finally, the effect of EM on key targets were validated by biological experiments.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University in 2018 (number: 2016-KY-048) and performed in accordance with the latest revision of the Declaration of Helsinki. All participants provided written informed consent before enrollment, and all subjects could withdraw from the study at any time during the study.

Patients

The study included a cohort of six smokers and six healthy volunteers. Additionally, six COPD patients who fit in the GOLD guidelines 2018 [27] were enrolled.

Inclusion criteria

The inclusion criteria of COPD patients with stable phase as follows: (1) Age over 40 years with a history of long-term smoking or exposure to tobacco smoke. (2) Confirmed diagnosis of COPD requiring inhalation therapy. (3) Without the following diseases: tuberculosis, interstitial lung disease, diffuse panbronchiolitis, pulmonary cystic fibrosis, asthma, acute respiratory distress syndrome, severe systemic infection, cancer, and liver and kidney failure. (4) Ability and willingness to complete lung function tests. Prior to hospital admission, patients had not received antibiotic treatment. The patients were recruited from the respiratory department at the First Affiliated Hospital of Guangxi Medical University between January 1, 2018, and December 1, 2020, in accordance with the GOLD 2018 Guidelines [27]. All enrolled subjects provided informed consent before specimen collection.

Exclusion criteria

The exclusion criteria as follow: (1) Patients in acute exacerbation of COPD, those with a history of chest surgery, bronchial asthma, interstitial lung disease, old pulmonary tuberculosis, primary bronchiectasis, diffuse panbronchiolitis, active pulmonary tuberculosis, bronchogenic carcinoma, cystic fibrosis, and other pulmonary restrictive ventilation dysfunctions. (2) Patients with severe cardiovascular, autoimmune, renal, ulcerative colitis, hyperthyroidism, liver, nervous system, Crohn's, hematological diseases, or advanced malignant tumors. (3) Patients who were unable or unwilling to communicate or cooperate.

Preparation of NETs

Anticoagulant tubes containing EDTA were used to collect peripheral blood samples from volunteers. Approximately 25 ml of peripheral venous blood was collected from each participant and kept on ice for subsequent experiments.

Isolation of neutrophils from human peripheral blood: 5 ml of human neutrophil isolate (Histopaque-1119) was added to a sterile 15 ml centrifuge tube and 5 ml of fresh anticoagulated blood was gently added above the isolate. After centrifugation at 600*g* for 20 min at room temperature, the liquid surface can be divided into several layers, the liquid surface divided into several layers, from top to bottom: serum layer, thin white cloud-like mononuclear cell (PBMCs) layer, neutrophils layer mixed with a small amount of red blood cells, and the lowest layer is red blood cell.

Neutrophils suspension was collected and mixed with 10 ml PBS, and centrifuged at 600*g* for 10 min at room temperature to wash cells. After centrifugation, the cells were pelleted into cell suspension with 2 ml PBS. Add 3 ml of Percoll solution of 75%, 70%, 65% concentration into 15 ml centrifuge tube according to concentration from high to low. Slowly add 2 ml of neutrophil suspension to Percoll solution. Centrifuge at 600*g* for 20 min at room temperature. After centrifugation, collect the neutrophil layer, resuspend it in 10 ml of PBS, and centrifuge again at 600*g* for 10 min. The final pellet is the neutrophil fraction. Resuspend the cells in RPMI 1640 medium and adjust the concentration to 1×10^6 cells/ml.

Cigarette smoke extract (CSE)-induced NETs: Cigarette smoke extracts and NETs were prepared according to previous methods [8, 12]. First, 500 µl of neutrophil suspension (10 µ cells/ml) was added to a 24-well plate, add 0.3% CSE and incubate in a cell culture incubator for 4 h. After stimulation, the supernatant is aspirated, add 1 ml RPMI 1640 medium and AluI (final concentration 20 IU/ml), and incubate for 20 min. Finally, collect NETs from each well.

Extraction of peripheral blood mononuclear cells (PBMCs) and stimulated by NETs

For monocyte extraction, PBMCs were separated by Ficoll-sodium diatrizoate discontinuous density gradient centrifugation, following a previously established method [12]. 5 ml of human neutrophil isolate (Histopaque-1119) was added to a sterile 15 ml centrifuge tube, and 5 ml human peripheral blood lymphocyte mitosis medium was mixed with equal volume of room temperature PBS, this mixed liquor slowly added to upper layer of the preadded separation medium. After centrifugation at 600*g* for 20 min at room temperature, the liquid surface can be divided into several layers, from top to bottom: serum layer, thin white cloud-like PBMC layer, separation solution, granulocyte layer mixed with a small amount of red blood cells, and the lowest layer is red blood cell precipitate. Remove the serum and collect PBMC by pipettes. The middle mononuclear cell layer transferred into a new centrifuge tube and mixed with PBS, centrifuged this mixed liquor at 500–600×*g* for 10 min at room temperature. To remove platelets, 10 ml PBS was added to the PBMC suspension and centrifuged at 600*g* for 10 min at room temperature, repeating this procedure twice. Then add red cell lysate to remove red cells, PBMC cells were obtained after PBS washing and centrifugation. PBMCs from each group of clinical samples were stimulated with NETs extracted from the respective groups. After incubation, the cells were collected for WB.

U937 cell and BEAS-2B cell culture and treatment

U937 (human histiocytic lymphoma cell line, TCHu1593.2, Shanghai Cell Bank, Chinese Academy of Sciences) cell was cultured as described in our previous paper [26]. The concentrations of drugs and NETs used are based on CCK-8 results and previous studies, method details and CCK-8 results can be found in the supplementary documents. Experimental groups of U937 cells as follow: 1) Control group: Cells were cultured under standard conditions without any treatment. 2) NETs stimulation group (NETs): Stimulation with 50 ng/ml NETs for 24 h. 3) EM stimulation group (EM): Cells were pre-incubated with 1 µg/ml EM for 24 h, then stimulated with 50 ng/ml of NETs for an additional 24 h. 4) PI3K-δ inhibitor IC87114 group (IC87114): Cells were incubated with IC87114 (1 µmol/L) for 24 h. 5) SIRT1 inhibitor nicotinamide (NAM) group (NAM): Cells were incubated with NAM (20 mmol/L) for 24 h [28]. 6) JNK inhibitor SP600125 group (SP600125): Cells were incubated with SP600125 (10 mmol/L) for 24 h [29]. The concentrations of NETs, EM, and IC87114 used to stimulate U937 cells were based on CCK-8 results (Supplementary Material, Fig S1 A, C, D). Following the respective treatments, cells

were collected for PCR and WB analysis, and collect the supernatant for ELISA to detect inflammatory cytokines.

BEAS-2b cells (Human bronchial epithelial cell, ATCC® CRL-9609™, Shanghai Cell Bank, Chinese Academy of Sciences) cell was cultured as described in our previous paper [30]. The concentrations of drugs and NETs used are based on CCK-8 results and previous studies, method details and CCK-8 results can be found in the supplementary documents. Experimental groups of BEAS-2b cells as follow: 1) Control group: Cells were cultured under standard conditions without any treatment. 2) NETs stimulation group (NETs): Stimulation with 50 ng/ml NETs for 24 h. 3) EM stimulation group (EM): Cells were pre-incubated with 10 μg/ml EM for 24 h [31], then stimulated with 50 ng/ml of NETs for an additional 24 h. 4) PI3K-δ inhibitor IC87114 group (IC87114): Cells were incubated with IC87114 (1 μmol/L) for 24 h. 5) SIRT1 inhibitor NAM group (NAM): Cells were incubated with NAM (10 mmol/L) for 24 h. The concentrations of NETs, NAM, and IC87114 used to stimulate BEAS-2B cells were based on CCK-8 results (Supplementary Material, Fig S1 B, E, F). Following the respective treatments, cells were collected for PCR and WB analysis, and collect the supernatant for ELISA to detect inflammatory cytokines.

Transcriptome sequencing and enrichment analysis

Transcriptome sequencing was performed by transcriptomics and analyzed using bioinformatics methods. Method details can be found in supplementary documents.

Proteomics and enrichment analysis

U937 cell samples were analyzed by liquid chromatography (LC)-MS/MS for proteomics profiling, and method details can be found in the supplementary documents.

Gene set enrichment analysis

Java Gene Set Enrichment Analysis (GSEA) software was used to functionally annotate differentially expressed genes and proteins, and hallmark gene sets were obtained from MsigDB. A false discovery rate (FDR) < 0.1 and a nominal P value < 0.05 were used to determine statistically significant genes and proteins.

Protein–protein interaction (PPI) network analysis of EM-regulated AKT-related inflammatory pathways and identification of key targets

To predict the potential targets related with the effect of EM, a comprehensive network pharmacology approach was employed. After screening AKT-related inflammatory pathways via the enrichment analysis of differentially expressed genes (DEGs) and differentially expressed proteins (DEPs), DEGs and DEPs involved in these pathways were used to construct PPI network and identify key targets. At first, genes of AKT-related inflammatory pathway which enriched in the DEGs between NETs group and control group were collected, these DEGs uploaded to Search Tool for the Retrieval of Interacting Genes (STRING) for constructing PPI network. A similar approach was performed to build PPI network of DEGs between the NETs and EM groups. Cytoscape was used to merge these two PPI networks and extract the key module of the merged PPI network. AKT-related inflammatory pathway PPI networks were constructed for DEPs using the same methodology, and key modules within the combined PPI network were analyzed with Cytoscape. At last, to identify common targets of EM regulating AKT-associated inflammation in proteogenomics, the key modules of PPI networks derived from both transcriptomic and proteomic were integrated using Cytoscape, allowing for analysing key targets within the combined PPI network.

PPI network of EM regulate oxidative stress targets in proteogenomics and key targets

Our transcriptome and proteome enrichment analyses showed that inflammation induced by NETs is related to oxidative stress. Based on this, we hypothesized that oxidative stress is a critical molecular mechanism by which NETs activate AKT. To further investigate the mechanism of EM regulating oxidative stress, we constructed a PPI network and identified key targets for the DEGs and DEPs related to oxidative stress pathway, using the same methodology as described in the previous step.

Validation of the results of multiomics analysis via quantitative real-time polymerase chain reaction (PCR) and westernblot (WB)

Method details of PCR and WB can be found in the supplementary documents.

PCR: Total RNA was extracted by using the RNAeasyTM Isolation Reagent (Vazyme Biotech, Nanjing, China). Method details of PCR and the primer sequences of targets in PI3K/AKT pathway are detailed in Table S1 of supplementary material.

WB: The cell were centrifuged and lysed using radioimmunoprecipitation assay (RIPA) buffer to extract proteins, proteins in the PI3K/AKT/mTOR pathway associated with glucocorticoid resistance were analyzed by Western blot, and the details antibodies can be found in the supplementary material.

Correlation analysis between clinical information and protein expression

Pearson correlation coefficient analysis was performed on clinical information and protein expression

levels of clinical samples using DataExplorer package in R language.

Enzyme linked immunosorbent assay (ELISA) detect inflammatory cytokines

According to the ELISA kit (ABclonal, Wuhan, China) protocols, inflammatory cytokines in the supernatant of each group were detected, including IL-6, IL-8 and TNF-α.

Detection of ROS

U937 cells were grouped as defined above, and 2',7'-dichlorofluorescin diacetate (DCFH-DA, 10 mm) was used as a fluorescent probe to observe the ROS release level of each group. The fluorescence intensity of the cells was observed through fluorescence microscopy.

Statistical analysis

The PCR, WB and fluorescence microscopy data are expressed as the mean ± SD, and statistical analyses of these data were performed with SPSS for Windows (version 16.0.0; SPSS, Chicago, IL, USA). Significant differences between multiple groups were tested using one-way ANOVA. The clinical data is expressed as the number of cases and percentage (%) and the data were analyzed by χ^2 test. Statistical significance was assigned at a P value < 0.05.

Results

Identification of DEPs and DEGs

To investigate the mechanisms and potential targets of EM on U937 cells stimulated by NETs, transcriptional and proteomic analyses were performed on the treated cells (Fig. 1A). A total of 5590 DEGs including 2789 downregulated and 2801 upregulated genes were identified between NETs and control groups. Additionally, 6996 DEGs including 3410 downregulated and 3586 upregulated genes were identified between NETs and EM groups. The distribution of DEGs among each groups is shown in the volcano plot (Fig. 1B, $P < 0.05$). Furthermore, 1881 DEPs, consisting of 912 upregulated and 969 downregulated proteins were identified between NETs and control groups (Fig. 1C, $P < 0.05$). Similarly, 1843 DEPs including 961 upregulated and 882 downregulated were identified between NETs and EM groups (Fig. 1C, $P < 0.05$). Analysis of both the transcriptome and proteome revealed that the expression of AKT1 was significantly increase under NETs stimulated, and treatment with EM inhibited AKT1 expression (Fig. 1B, C, $P < 0.05$). These results suggest that down-regulating AKT1 activity induced by NETs is one of the molecular mechanisms by which EM relieves inflammation.

Functional enrichment of DEGs

GO enrichment analysis revealed that DEGs induced by NETs were primarily related to neutrophil mediated immunity, autophagy, response to oxidative stress, steroid hormone receptor binding, histone binding, etc. (Fig. 2A). The DEGs regulated by EM were predominantly linked to negative regulation of immune system process, response to oxidative stress, IKK/NF − κB signaling, cellular response to oxidative stress, response to reactive oxygen species, and histone acetylation (Fig. 2C).

KEGG enrichment demonstrated that DEGs induced by NETs were associated with PI3K/AKT signaling pathway, mTor signaling pathway, NF-κB signaling pathway, PTEN signaling pathway, oxidative stress response, etc. (Fig. 2B). In comparison, the DEGs regulated by EM were associated with PI3K/AKT signaling pathway, mTor signaling pathway, NF-κB signaling pathway, cytokine Signaling in Immune system, signaling by interleukins, inflammation mediated by chemokine and cytokine signaling pathway, and oxidative stress response (Fig. 2D).

The results of GSEA showed that the DEGs induced by NETs were primarily enriched in IL-6/JAK/STAT3 pathway, mTorc1, inflammatory response, ROS pathway, TGF-β via NF-κB pathways, and apoptosis-related pathways (Fig. 3A). In the comparison between the NETs and EM groups, DEGs were enriched in PI3K/AKT/mTor pathway, the IL-6/JAK/STAT3 pathway, mTORC1, the inflammatory response, the P53 pathway, TGF-β via NF-κB pathways, and apoptosis-related pathways. The gene sets with FDR < 0.1 and $p < 0.05$ were regarded as significantly enriched (Fig. 3B).

The results of the DEGs enrichment analysis suggest that NETs induced inflammation primarily through the PI3K/AKT pathway. Moreover, the molecular mechanisms underlying EM's anti-inflammatory effects are correlate to the modulation of PI3K/AKT pathway and oxidative stress pathways.

Functional enrichment of DEPs

GO enrichment analysis revealed DEPs induced by NETs were primarily related to cytoplasmic translation, response to oxidative stress, production of molecular mediator involved in inflammatory response, and mitochondrial matrix (Fig. 4A). In contrast, the DEPs regulated by EM were predominantly enriched in histone modification, I-κB kinase/NF-κB signaling, neutrophil activation involved in immune response, and histone deacetylase complex (Fig. 4C).

KEGG enrichment showed the DEPs induced by NETs were primarily associated with cytokine signaling in immune system, signaling by interleukins, chemokine signaling pathway, PI3K/AKT signaling

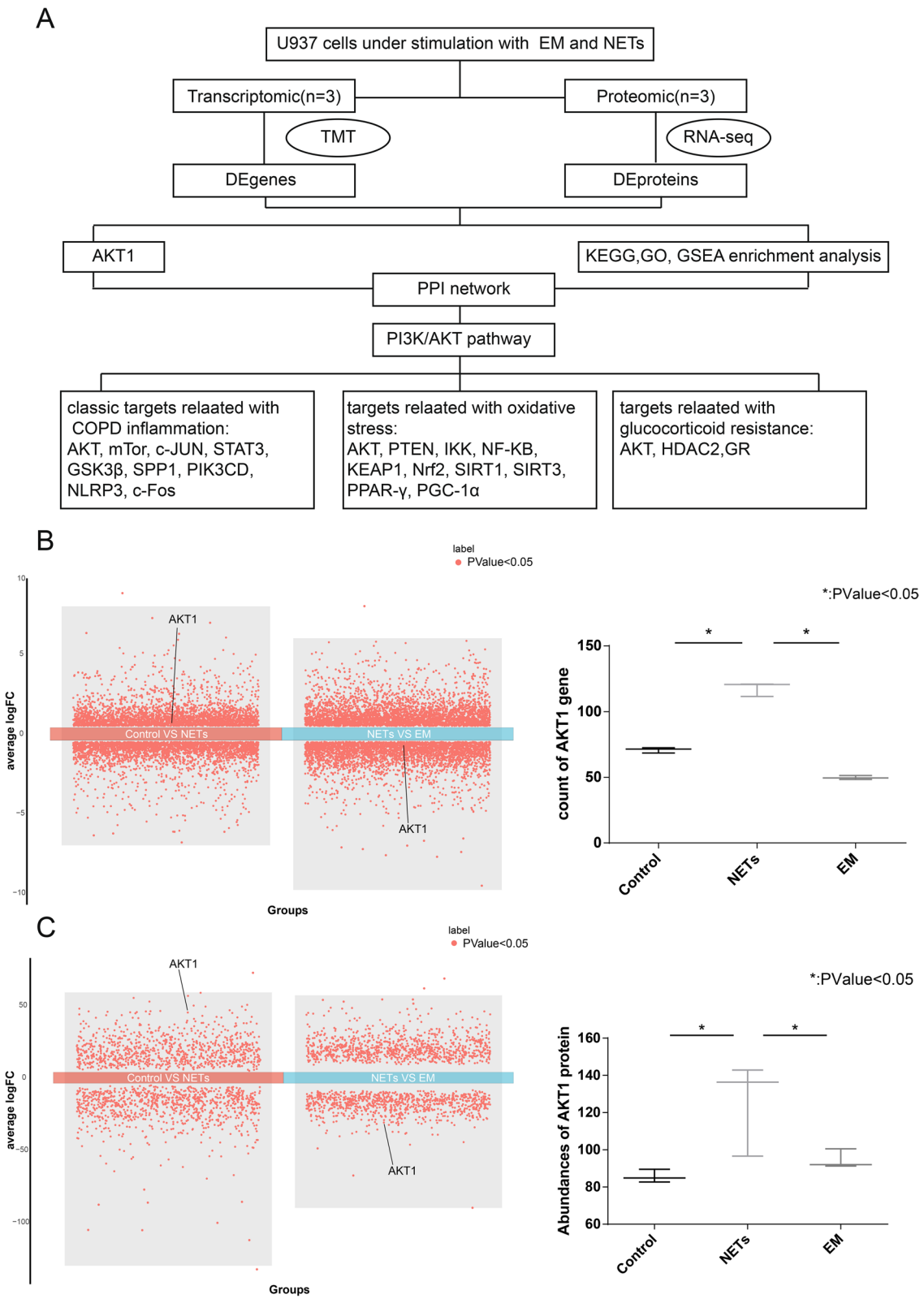

Fig. 1 Identify DEGs and DEPs in each group. **A** The flow chart show analysis of proteomic and transcriptomic. **B** Volcano map of DEGs between each group, and AKT1 gene expression between each group ($P < 0.05$). **C** Volcano map of DEPs between each group, and AKT1 protein expression between each group ($P < 0.05$)

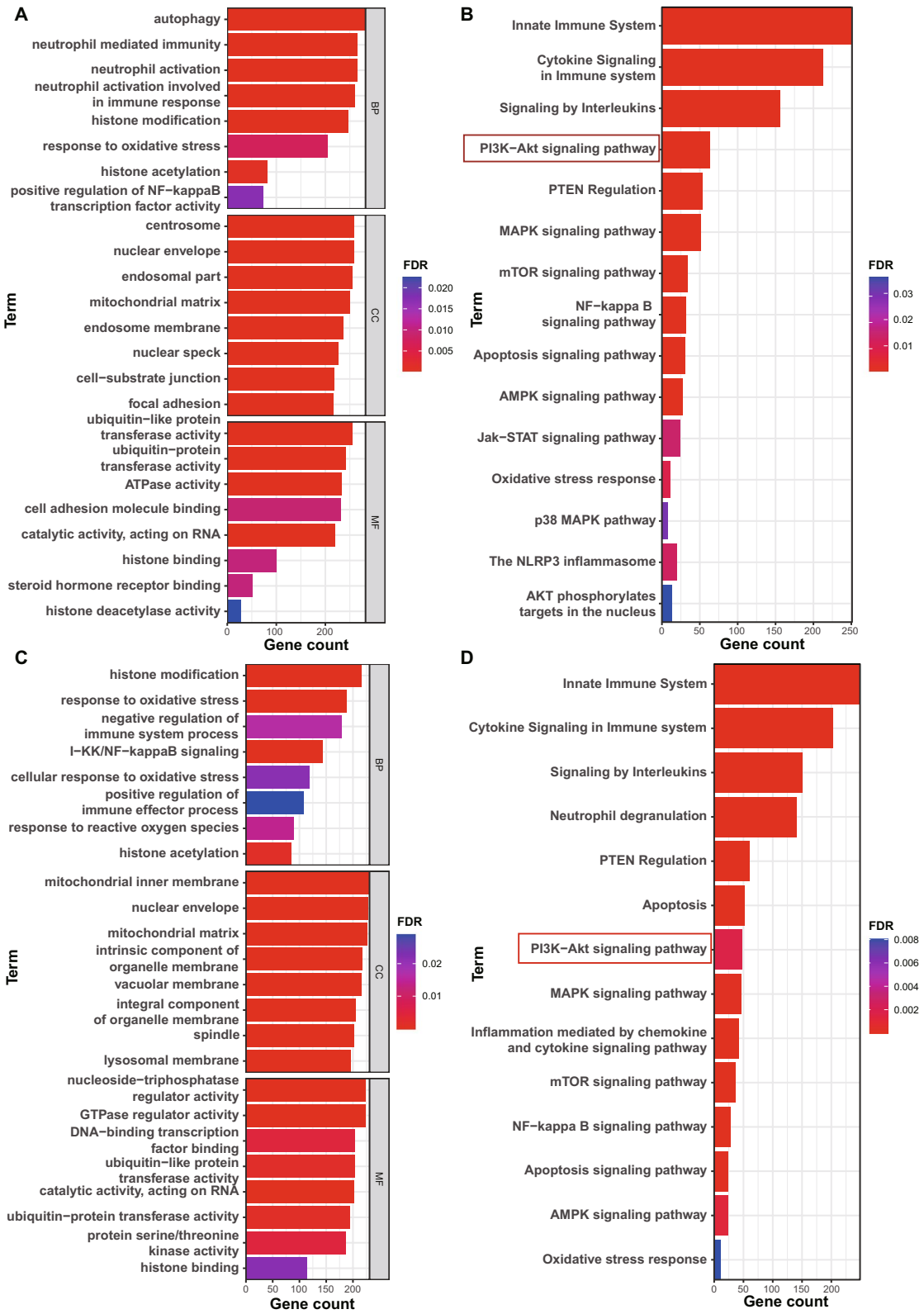

Fig. 2 GO and KEGG enrichment of DEGs. **A** The results of GO enrichment of DEGs induced by NETs ($P < 0.05$). **B** KEGG enrichment results of DEGs induced by NETs ($P < 0.05$). **C** GO enrichment of DEGs regulated by EM ($P < 0.05$). **D** The KEGG enrichment analysis results of DEGs regulated by EM ($P < 0.05$)

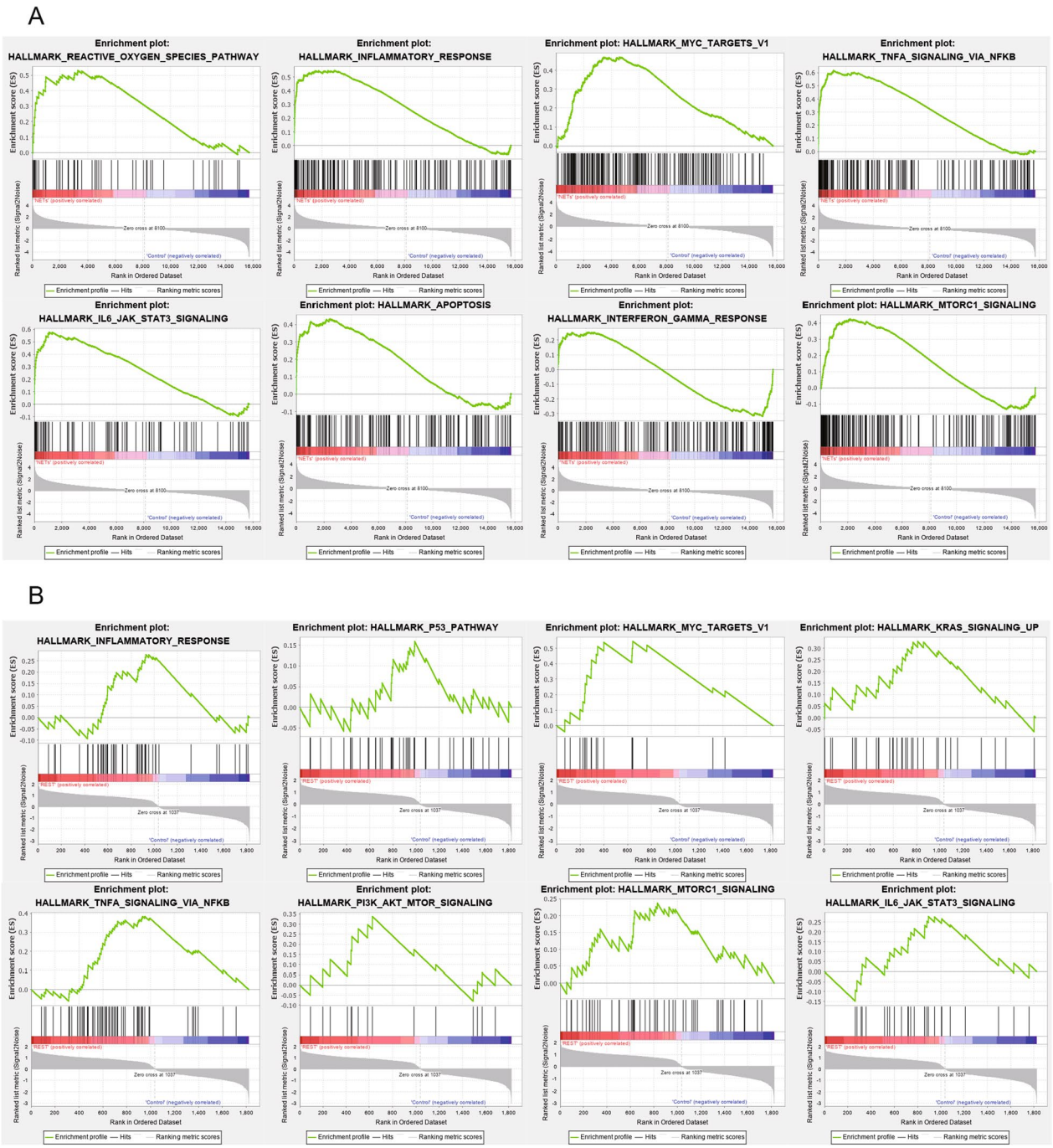

Fig. 3 The results of GSEA. **A** The GSEA results of DEGs induced by NETs ($P < 0.05$). **B** The GSEA results of DEGs between NETs and EM groups ($P < 0.05$)

pathway, inflammation mediated by chemokine, and cytokine signaling pathway (Fig. 4B). In comparison, the DEPs between the EM group and NETs group were enriched in PI3K/AKT pathway, mTor pathway, NF-κB pathway, PTEN pathway, oxidative stress response, PI3 kinase pathway, and cytokine signaling in immune system (Fig. 4D).

GSEA analysis revealed that the DEPs between NETs group and control group were mainly enriched in PI3K/AKT/mTor pathway, MYC target, inflammatory

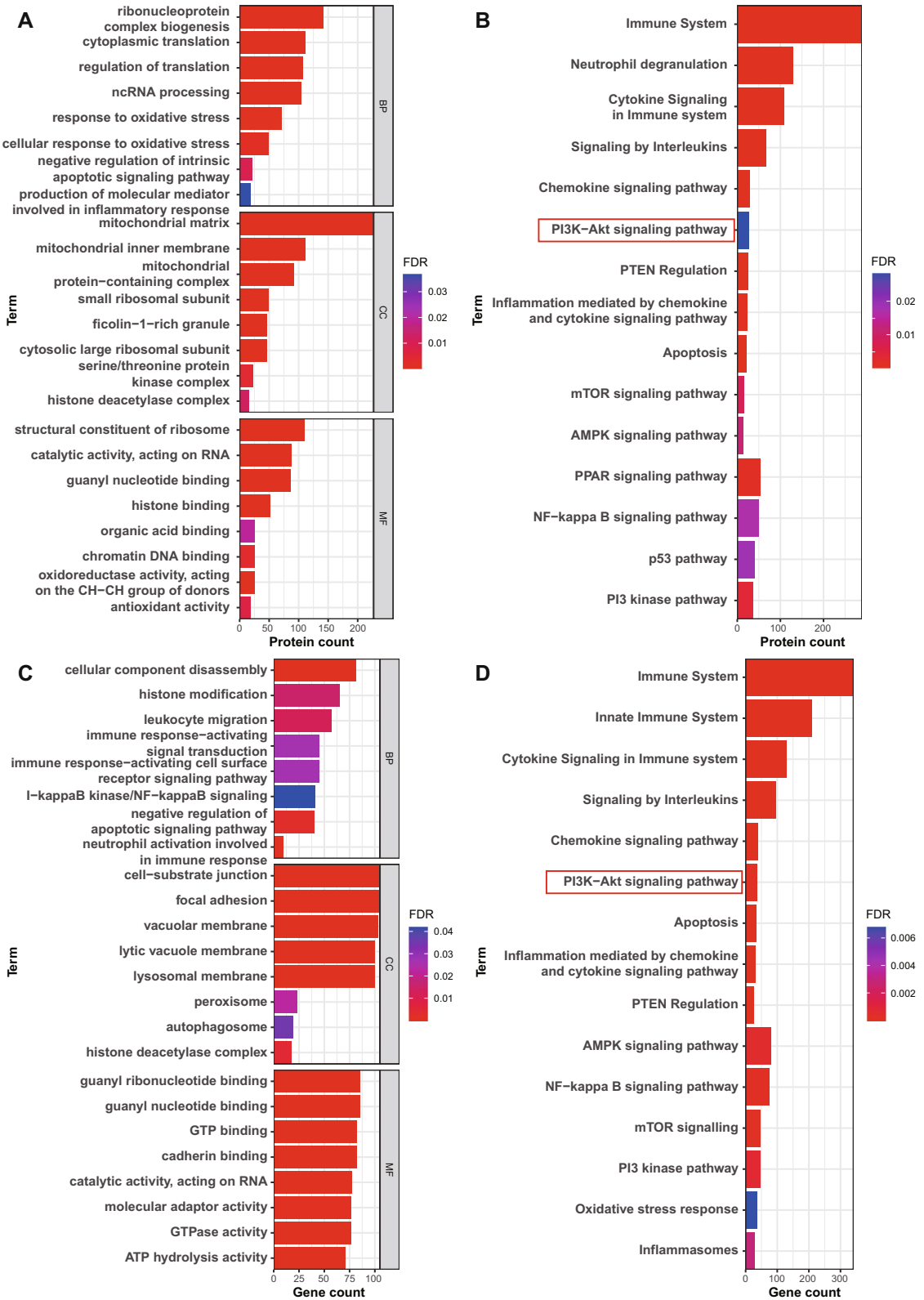

Fig. 4 GO and KEGG enrichment of DEPs. **A** The results of GO enrichment analysis of DEPs induced by NETs ($P < 0.05$). **B** KEGG enrichment of DEPs induced by NETs ($P < 0.05$). **C** GO enrichment of DEPs regulated by EM ($P < 0.05$). **D** KEGG enrichment of DEPs regulated by EM ($P < 0.05$)

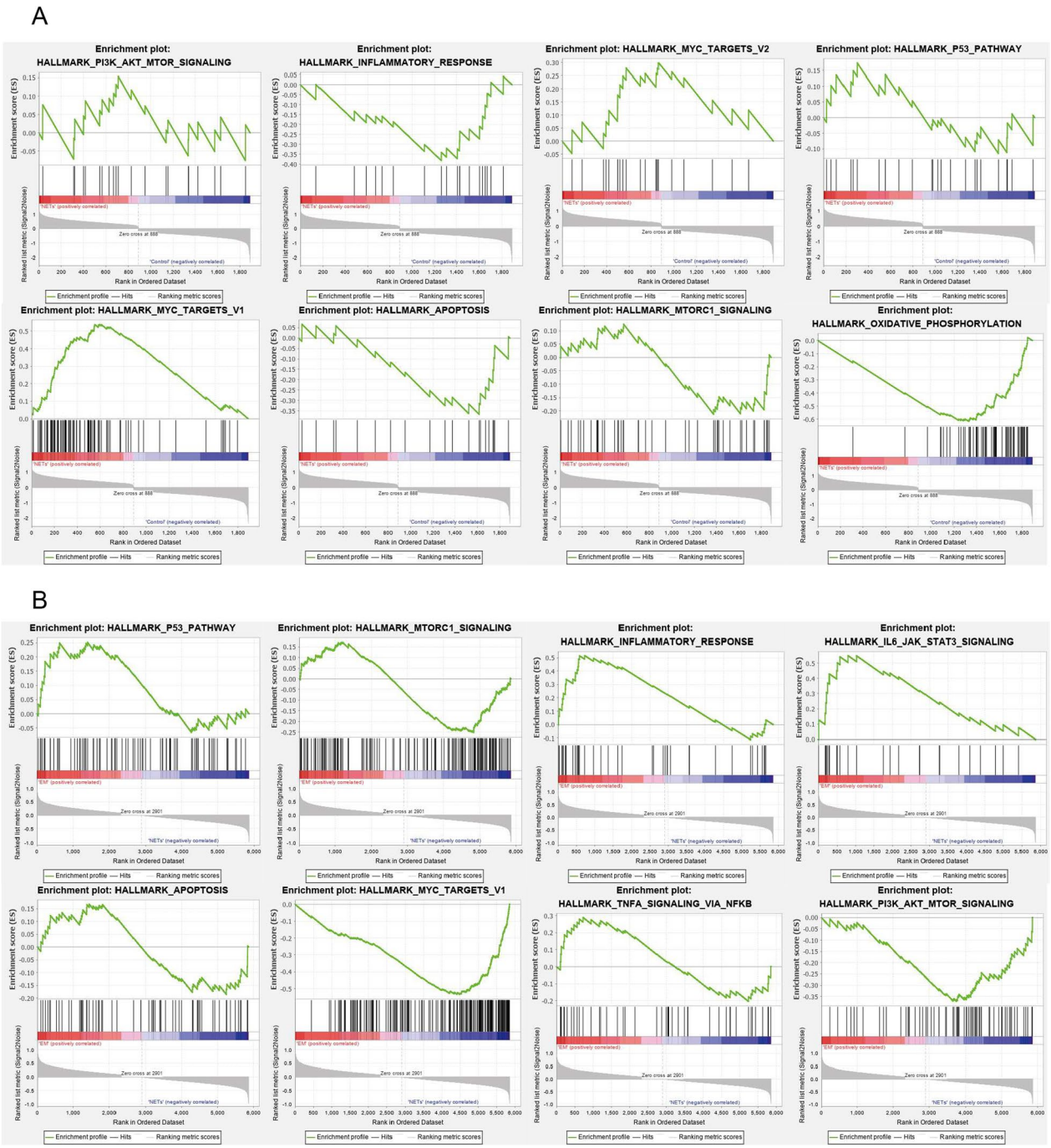

Fig. 5 GSEA result of the DEPs. **A** GSEA results of DEPs between NETs and control group ($P < 0.05$). **B** GSEA results of DEPs between NETs and EM group ($P < 0.05$)

response, apoptosis, mTorc1 pathway, P53 pathway, and oxidative phosphorylation pathway (Fig. 5A). In contrast, the DEPs between the NETs and EM were predominantly associated with PI3K/AKT/mTor pathway, MYC target, inflammatory response, apoptosis, mTorc1 pathway, P53 pathway, TNF-α/NF-κB pathway, and IL-6/JAK/STAT3 pathway(Fig. 5B). The protein sets which FDR < 0.1 and $P < 0.05$ were were considered significantly enriched.

Proteomic enrichment analysis further supported the findings from transcriptomic analyses, showing that NETs-induced inflammation was associated with the NF-κB and PI3K/AKT pathways. Importantly, EM

treatment inhibited NETs-related inflammation, particularly through modulation of the PI3K/AKT pathway.

Functional enrichment of co-expressed DEGs-DEPs

To investigate the correlation between DEGs and DEPs, we conducted enrichment analyses of DEGs and DEPs that exhibited similar expression patterns. Our enrichment analysis showed that co-expressed DEPs-DEGs inuced by NETs were primarily associated with the small molecule catabolic process, fatty acid metabolic process, and carboxylic acid catabolic process (Fig. 6A). Additionally, these co-expressed genes and proteins were linked to Metabolic pathways, Inflammation mediated by chemokine and cytokine signaling pathway, PI3K/AKT signaling pathway, PPAR signaling pathway, and JAK/STAT signaling pathway (Fig. 6B).

In contrast, co-expressed DEPs-DEGs in NETs group and EM group were predominantly related to protein folding, protein localization to nucleus, and ncRNA processing (Fig. 6C). Moreover, they involved in Metabolic pathways, apoptosis, MAPK signaling pathway, inflammation mediated by chemokine and cytokine signaling pathway, NF-κB signaling pathway, PI3K/AKT signaling pathway, and mTOR signaling pathway (Fig. 6D).

Taken together, the enrichment analyses of both proteomic and transcriptomic data highlight the pivotal role of PI3K/AKT pathway in EM-mediated inhibited NETs-related inflammation.

Functional enrichment of DEGs-DEPs with different expression patterns

Considering the mRNA and protein expression are affected by post-translational modifications, we further investigated differentially expressed pathways in mRNAs and proteins exhibiting different expression patterns via GO and KEGG enrichment analyses.

Between control group and NETs group, enrichment analysis revealed that commonly downregulated DEGs-DEPs were related with translation factor activity, RNA binding, translation initiation factor activity, protein processing in endoplasmic reticulum, spliceosome, and proteasome. Conversely, commonly upregulated DEPs-DEGs were associated with guanyl nucleotide binding, carbon metabolism, and lysosome. Proteins expression unchanged but decreased in mRNA expression were associated with single-stranded DNA binding, structural constituent of ribosome, biosynthesis of amino acids, and HIF-1 signaling pathway. Proteins expression unchanged but increased in mRNA expression were related with spinocerebellar ataxia, steroid biosynthesis, isomerase activity, intramolecular oxidoreductase activity, MHC class II protein complex binding, DNA-binding transcription factor binding, and histone demethylase activity. mRNA expression unchanged but increased protein expression were related with MAP kinase kinase activity, transcription coactivator activity, peroxisome and morphine addiction. Finally, mRNA expression unchanged but protein expression decreased were related with cell cycle, mRNA surveillance pathway, cholesterol binding, and O-acyltransferase activity (Fig. 7A–B, $P < 0.05$).

Between EM and NETs group, enrichment revealed that commonly downregulated DEGs-DEPs were involved in translation factor activity, RNA binding, translation regulator activity, nucleic acid binding, catalytic activity, acting on RNA, molecular carrier activity, salmonella infection, and nucleocytoplasmic transport. On the other hand, commonly upregulated DEPs-DEGs were associated with lysosome, cell adhesion molecules, hematopoietic cell lineage, carbohydrate binding, hydrolase activity, acting on glycosyl bonds, and carboxylic acid binding. Protein expression unchanged but decreased in mRNA expression were involved in histone binding, carbon metabolism and spliceosome. Protein expression unchanged but mRNA expression increased were related with Glycolysis/Gluconeogenesis, RNA degradation, nucleotide metabolism, organic anion transmembrane transporter activity, and active transmembrane transporter activity. mRNA expression unchanged but protein expression increased were related with GTPase activity, GDP binding, NAD binding, T cell receptor signaling pathway, and B cell receptor signaling pathway. mRNA expression unchanged but protein expression decreased were related with apelin signaling pathway, relaxin signaling pathway, and structural constituent of ribosome (Fig. 7C–D, $p < 0.05$).

Identification of key targets in EM regulation of AKT-related inflammatory pathways and oxidative stress in proteogenomics

Building upon previous studies of COPD-related inflammation, key genes enriched in inflammation-related pathways contain PI3K/AKT, NF-κB, and others were identified from the NETs and control groups. Similarly, key genes enriched in these pathways were extracted from the NETs and EM treatment groups. To visualize the interactions of these co-existing key genes, Cytoscape was used to construct PPI networks and combining the two gene sets. The PPI network of genes be interrelated to EM regulates AKT-related inflammation is shown in Fig. 8A.

Similarly, key proteins of DEPs involved in AKT-related inflammatory pathways were enriched. The corresponding PPI network of AKT-related inflammatory proteins regulated by EM is shown in Fig. 8C. To identify common targets of EM regulation in both transcriptomic and proteomic data, we merged the PPI networks from both

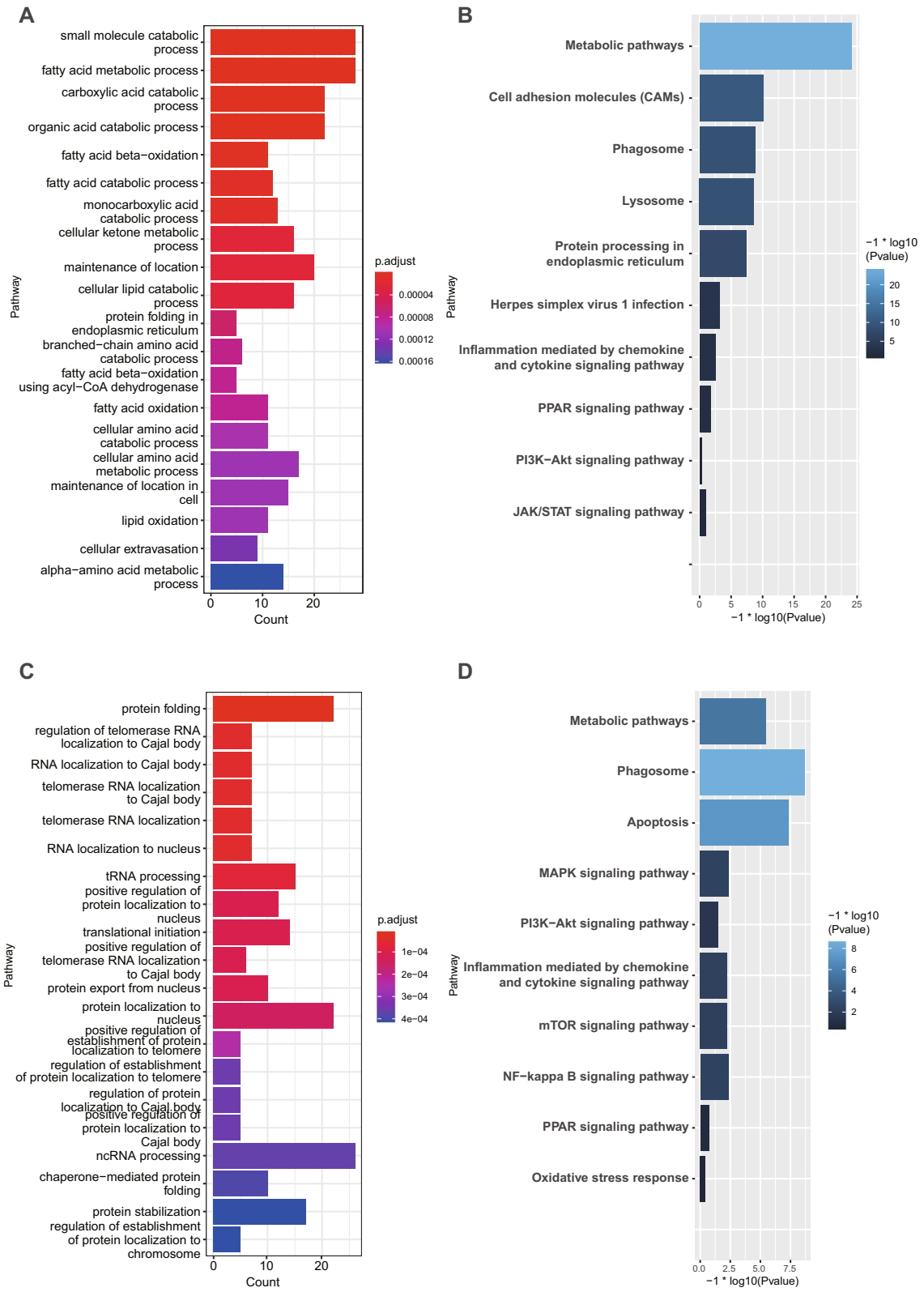

Fig. 6 Enrichment of co-expressed DEGs-DEPs. **A** GO enrichment of co-expressed DEGs-DEPs between NETs and control group. **B** KEGG enrichment of co-expressed DEGs-DEPs between NETs and control group. **C** GO enrichment of co-expressed DEGs-DEPs between NETs and EM group. **D** KEGG enrichment of co-expressed DEGs-DEPs between NETs and EM group

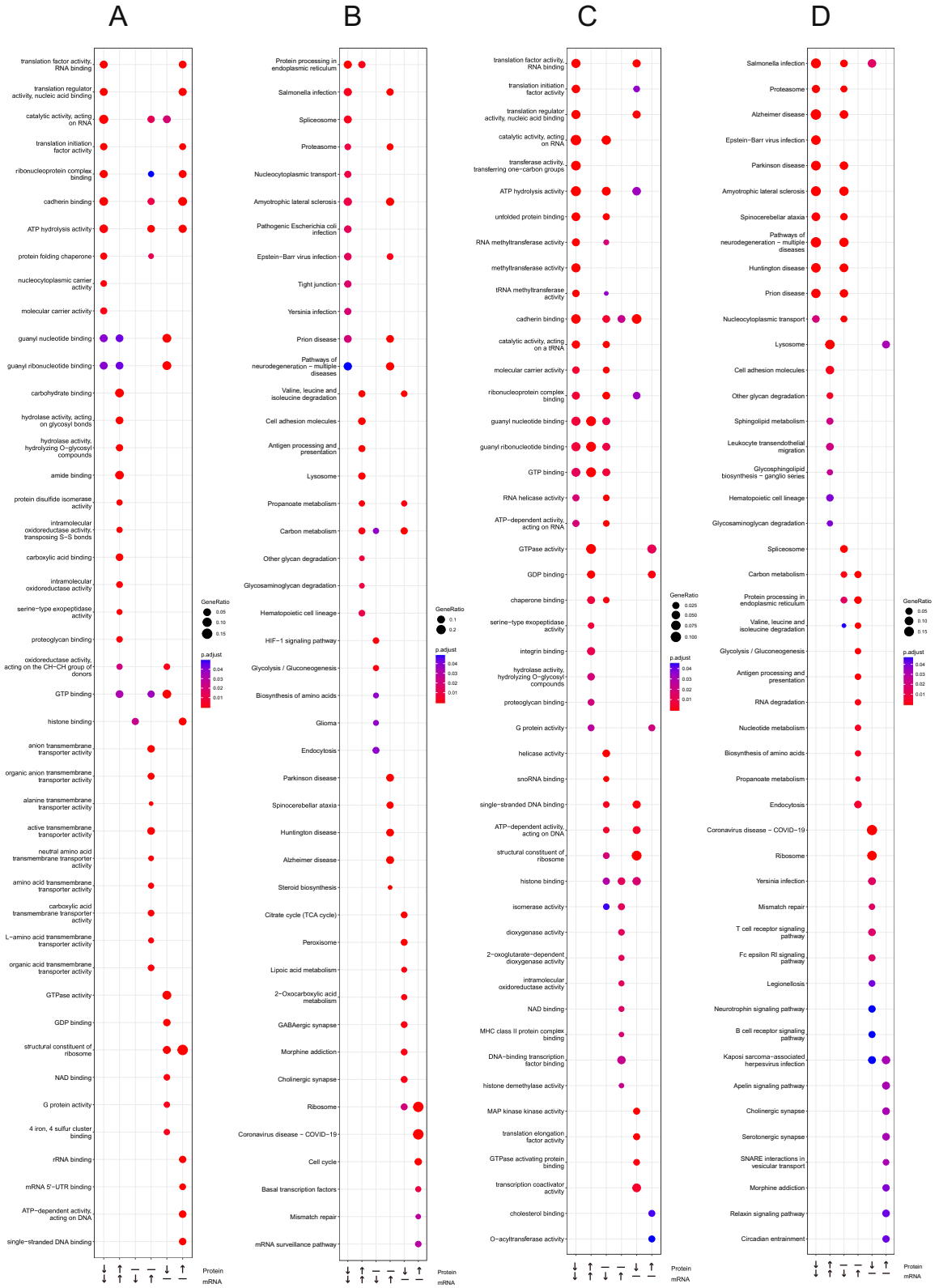

Fig. 7 Enrichment of DEGs-DEPs with different expression patterns. GO enrichment (**A**) and KEGG enrichment (**B**) of DEGs-DEPs with different expression patterns between NETs and control group; GO enrichment (**C**) and KEGG enrichment (**D**) of DEGs-DEPs with different expression patterns between NETs and EM group ($P < 0.05$)

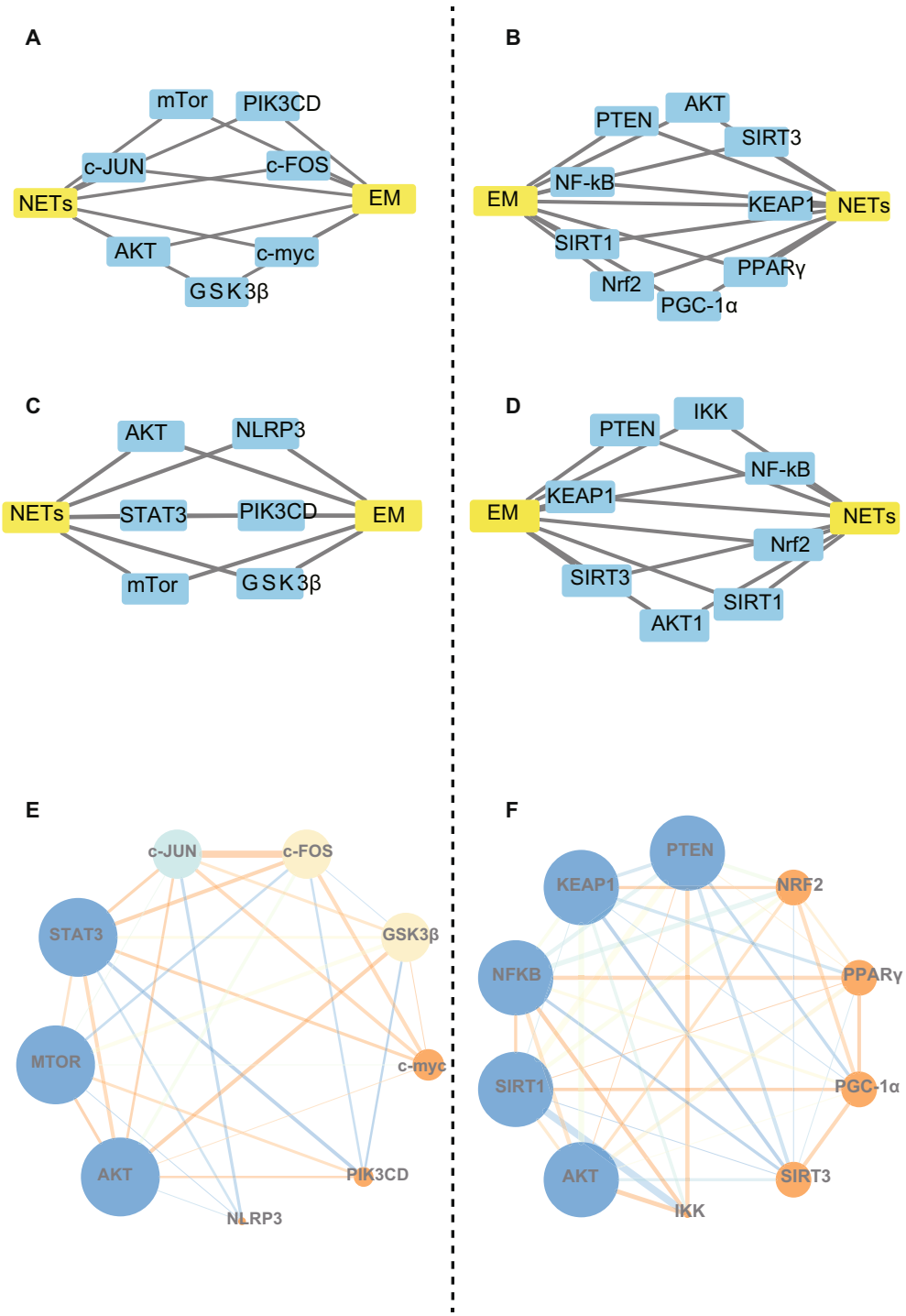

Fig. 8 Key targets in EM regulation of AKT-related inflammatory pathways and oxidative stress in proteogenomics. **A** Key genes of EM regulates AKT related inflammation in transcriptomics. **B** Key genes of EM regulates oxidative stress in transcriptomics. **C** Key proteins of EM regulates AKT related inflammation in proteomics. **D** Key proteins of EM regulates oxidative stress in proteomics. **E** Key targets of EM regulates AKT related inflammation in transcriptomics and proteomics. **F** Key targets of EM regulates oxidative stress in transcriptomics and proteomics

datasets using the STRING database (https://cn.string-db.org/) and Cytoscape. By analyzing the key modules of the merged PPI network, we identified nine key targets in the AKT-related inflammatory pathway: AKT, mTOR, c-JUN, c-FOS, STAT3, GSK3β, PIK3CD, c-myc and NLRP3 (Fig. 8E). These datas shown that these nine targets may play a key role in the suppression of NETs-related inflammation by EM in COPD.

The mechanisms through which NETs activate AKT remain complex and not fully understood. However, our transcriptomic and proteomic enrichment analysis indicates oxidative stress as a critical role in NETs-associated inflammation. To investigate the mechanism and targets of EM regulates oxidative stress, we followed a similar approach, extracting key genes enriched in oxidative stress-related pathways from the NETs and control groups and comparing them with those from the NETs and EM groups. The gene set associated with EM regulation of oxidative stress is demonstrated in Fig. 8B. We also analyzed oxidative stress-related proteins in the proteomic data and constructed a PPI network for oxidative stress proteins regulated by EM (Fig. 8D). To identify common targets in both transcriptomic and proteomic regulation of oxidative stress, we merged the corresponding PPI networks using STRING and Cytoscape. This analysis identified 10 key targets in the oxidative stress-related pathways: NF-κB, PTEN, PGC-1α, SIRT1, SIRT3, AKT, IKK, PPARγ, Nrf2, and KEAP1 (Fig. 8F).

Validating key targets of NETs-related inflammation suppresses by EM in U937 cell

Western blot were used to validate the effect of EM on key targets in NETs-related inflammation. Biological experiments showed that NETs significantly increased the expression levels of PI3K-δ, AKT, AKT 1, p-AAKT, and p-AKT1 proteins, as well as the phosphorylation of AKT and AKT1. EM treatment notably reduced the activation of PI3K-δ, AKT, AKT1, p-AKT, and p-AKT1 and decreased the phosphorylation of AKT and AKT1 induced by NETs (Fig. 9A–C, $P<0.05$).

Additionally, NETs stimulation resulted in a significant upregulation of NLRP3, mTOR and p-mTOR, accompanied by increased phosphorylation of mTOR. EM treatment downregulated NLRP3, mTOR and p-mTOR protein expression, reversing NETs-induced mTOR phosphorylation (Fig. 9D–F, $P<0.05$).

NETs were also inhibit GSK-3β phosphorylation while promoting STAT3 phosphorylation, EM reversed the effects of NETs on both STAT3 and GSK-3β phosphorylation. NETs upregulated the inflammation-related transcription factor c-myc expression, EM and IC87114 downregulated c-myc expression (Fig. 9G–I, $P<0.05$).

Moreover, NETs increased AP-1 subunits expression, including c-Fos and c-Jun, EM significantly inhibited the NETs-induced expression of AP-1. Both NETs and EM have no effect on JNK expression (Fig. 9J, K, $P<0.05$).

Validating the effect of EM antioxidant stress and regulation on oxidative stress-related targets in U937 cell

To study the oxidative stress caused by NETs, fluorescence microscopy was used measure the reactive oxygen species (ROS) fluorescence intensity in U937 monocytes across different experimental conditions. After 24 h of NETs stimulation, the fluorescence intensity of U937 monocytes was significantly elevated, pre-incubation with EM significantly reduced ROS fluorescence intensity, indicating EM suppressed NETs-induced oxidative stress (Fig. 10A–D, $P<0.05$).

To verify the key targets of EM in suppression oxidative stress, Western blot were use to determined their protein expression in U937 cells. Our results verified that NETs significantly decrease the expression of SIRT3 and PPARγ proteins in U937 cell, and EM treatment effectively reversed this suppression (Fig. 10H, I, $P<0.05$). Additionally, NETs stimulation enhanced the expression of NF-κB pathway proteins, including IKK, NF-κB p65, and NF-κB p50, whereas EM treatment inhibited the NETs-induced upregulation of these proteins (Fig. 10H, J, $P<0.05$).

Moreover, NETs exposure led to a downregulation of PTEN, SIRT1, Nrf2 and PGC-1α, alongside an increase in KEAP1 expression (Fig. 10K–M, $P<0.05$). EM treatment improve the reduction of PTEN, SIRT1, Nrf2 and PGC-1α expression induced by NETs, while also suppressing the NETs-induced upregulation of KEAP1 (Fig. 10K–M, $P<0.05$).

(See figure on next page.)

Fig. 9 Proteins expression of NETs-related inflammation which regulates by EM. **A** Protein expression of PI3K-δ, AKT, AKT1, p-AKT, and p-AKT1 in each group. B Relative protein expression in each group of cells (Mean ± SD, *$P<0.05$; n = 4). **C** Phosphorylation levels of AKT and AKT1 (mean ± SD, *$P<0.05$; n = 4). **D** Protein expression of NLRP3, mTor and p-mTor in each groups. **E** Relative protein expression of NLRP3, mTor and p-mTor in each group (mean ± SD, *$P<0.05$; n = 4). **F** mTor phosphorylation level in each group of cells (Mean ± SD, *$P<0.05$; n = 4). **G** Protein expression of STAT3, p-STAT3, c-myc, GSK3β and p-GSK3β in each group of cells. **H** Relative protein expression of STAT3, p-STAT3, c-myc, GSK3β and p-GSK3β in each group (mean ± SD, *$P<0.05$; n = 4). **I** STAT3 and GSK3β phosphorylation levels (mean ± SD, *$P<0.05$; n = 4). **J** Protein expression of JNK, c-Fos and c-Jun in each group. **K** Relative protein expression of JNK, c-Fos and c-Jun in each group (mean ± SD, *$P<0.05$; n = 4)

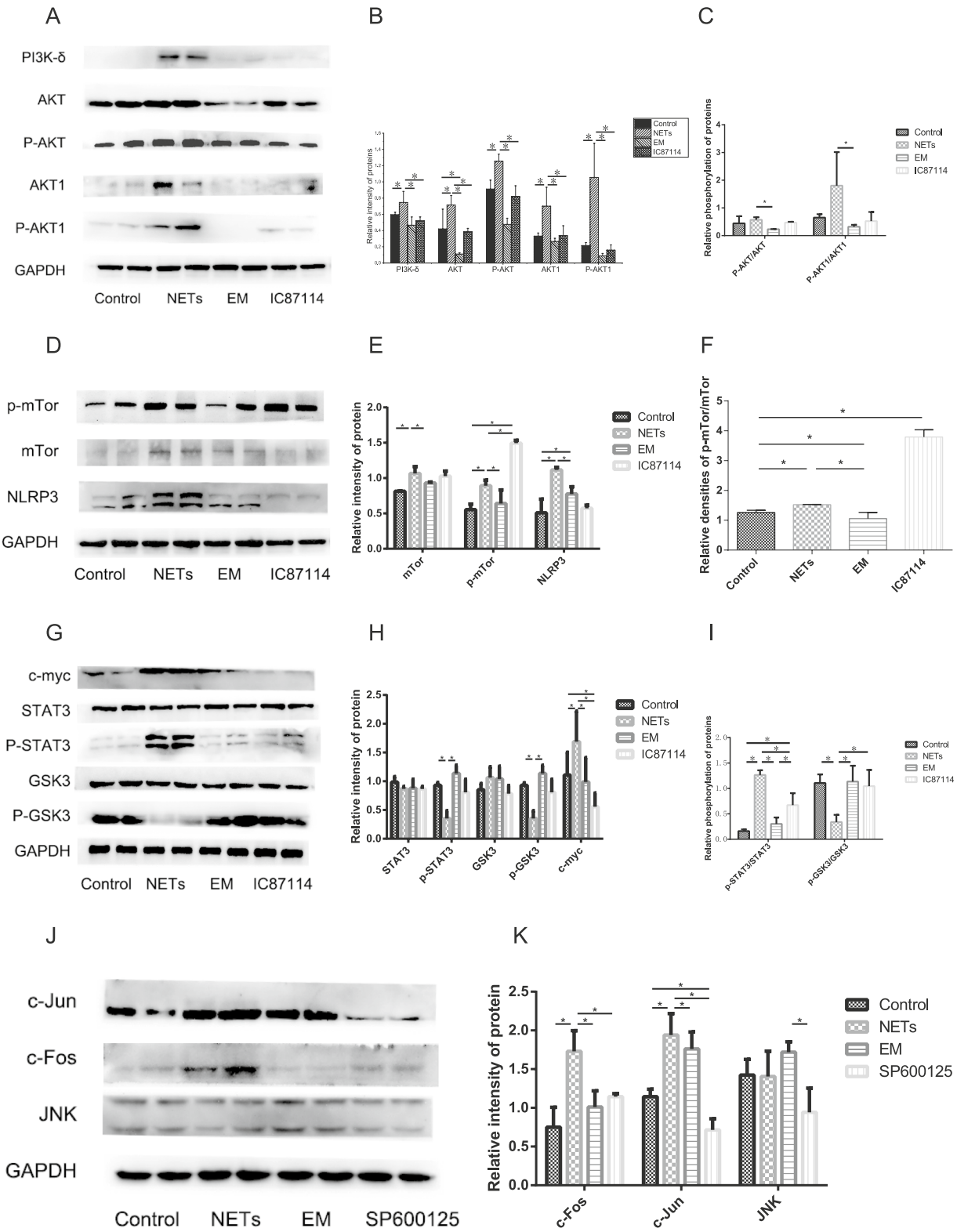

Fig. 9 (See legend on previous page.)

Effect of EM on PI3K/AKT pathway-related genes in U937 cells.

NET stimulation upregulated the expression of PI3K-δ, NRF2, NLRP3 mRNA, mTor, AKT, and AKT1 mRNA (Fig. 10E, F), which could be reversed by EM. The expression of HDAC-2 and GR was inhibited by NET stimulation, and preincubation with EM restored the expression of HDAC-2 and GR (Fig. 10E, F, $P < 0.05$).

Effect of EM on inflammatory cytokines in U937 cells

NET stimulation upregulated the expression of IL-6, IL-8 and TNF-α in U937 cell, which could be reversed by EM (Fig. 10G, $P < 0.05$).

Effect of EM on PI3K/AKT pathway-related genes in BEAS-2b cells.

NET stimulation upregulated the expression of NF-κB, mTor, and AKT mRNA (Fig. 11A, B, $P < 0.05$), which could be reversed by EM. The expression of SIRT1, HDAC-2 and GR was inhibited by NET stimulation, and preincubation with EM restored the expression of SIRT1, HDAC-2 and GR (Fig. 11A, B, $P < 0.05$).

Effect of EM on inflammatory cytokines in BEAS-2b cells

NET stimulation upregulated the expression of IL-6, IL-8 and TNF-α in BEAS-2b cell, which could be reversed by EM (Fig. 11G–I, $P < 0.05$).

Validating proteins of NETs-related inflammation regulated by EM in BEAS-2b cells

Western blot were used to validate the effect of EM on key targets in NETs-related inflammation. Biological experiments showed that NETs significantly increased the expression levels of AKT, mTor and NF-κB proteins, and decreased the expression levels of SIRT1, HDAC2 and GR proteins, EM treatment notably reversed the effect of NETs (Fig. 11C, D, E, F, $P < 0.05$).

EM improved NETs-induce glucocorticoid sensitivity by suppressed PI3K/AKT

Proteogenomic reveals that EM attenuates the increased expression of AKT1 induced by NETs, suggesting that EM ameliorate corticosteroid resistance in COPD by repressing the PI3K/AKT pathway, and reducing the inflammation. To validate these findings, we conducted biological experiments using clinical samples and U937 cells. The major clinical characteristics of the participants are provided in Table S2 of supplementary material ($P < 0.05$).

Our results show that NETs stimulation of the PBMCs isolated from COPD patients lead to the upregulation of AKT protein expression. However, AKT expression was no significant difference between smokers and healthy volunteers. Additionally, NETs stimulation in PBMCs from COPD patients caused significant downregulation of HDAC2 and GR (Fig. 12A, B, $P < 0.05$). In vitro experiments further confirmed that NETs decreased the expression of HDAC-2, GR, and phosphorylated GR, and inhibited GR phosphorylation. Notably, EM counteracted the inhibitory effects of NETs on HDAC2, GR, and P-GR, and promoted GR phosphorylation. The PI3K-δ inhibitor IC87114 also reversed the effects of NETs on HDAC2, GR, and P-GR (Fig. 11D, E, $P < 0.05$), although it did not significantly improve GR phosphorylation levels (Fig. 12F, $P < 0.05$).

Discussion

NETs are closely associated with inflammation in COPD, as they promote the production of inflammatory cytokines and sustain airway inflammation [11, 32]. Previous studies have indicated that macrolides can alleviate inflammation in COPD and reduce exacerbations [33, 34]. Previous studies have shown that azithromycin reduces the production of NETs in sputum of patients with bronchiectasis and asthma [35], meanwhile, rapamycin reduces NETs formation by inhibiting mTOR [36, 37]. However, the underlying anti-inflammatory mechanisms of macrolides on NETs-induced remain incompletely understood. In this study, transcriptomics and proteomics were employed to systematically investigate the molecular pathogenesis by which EM alleviates NETs-induced inflammation. We comprehensively presented the DEGs, DEPs, and associated pathway in involved in EM-mediated attenuation of inflammation caused by NETs. Furthermore, we

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Fig. 10 Effect of EM antioxidant stress and regulation on oxidative stress-related proteins. The level of ROS release was determined by fluorescence microscopy. **A** Control, **B** NETs, **C** EM, **D** the results of statistical analysis of fluorescence intensity (Mean ± SD, *$P < 0.05$, n = 3, 200×). **E** The expression of PI3K-δ, NRF2, NLRP3 and HDAC2 mRNA in each group of cells (Mean ± SD, *$P < 0.05$; n = 3). **F** The expression of mTor, AKT, AKT1 and GR mRNA in each group (mean ± SD, *$P < 0.05$; n = 3). **G** Inflammatory cytokines expression of IL-6, IL-8 and TNF-α in each groups (mean ± SD, *$P < 0.05$; n = 4). **H** Protein expression of IKK, NF-κB p65, NF-κB p50, SIRT3, and PPARγ in each group of cells. **I** Relative protein expression of SIRT3 and PPARγ in each group (Mean ± SD, *$P < 0.05$; n = 4). **J** Relative protein expression of IKK, NF-κB p65, and NF-κB p50 in each group of cells (mean ± SD, *$P < 0.05$; n = 4). **K** Protein expression of PTEN, SIRT1, KEAP1, Nrf2, and PGC-1α in each group of cells. **L** Relative protein expression of KEAP1 and Nrf2 in each group of cells (mean ± SD, *$P < 0.05$; n = 4). **M** Relative protein expression of SIRT1, PTEN and PGC-1α in each group of cells (mean ± SD, *$P < 0.05$; n = 4)

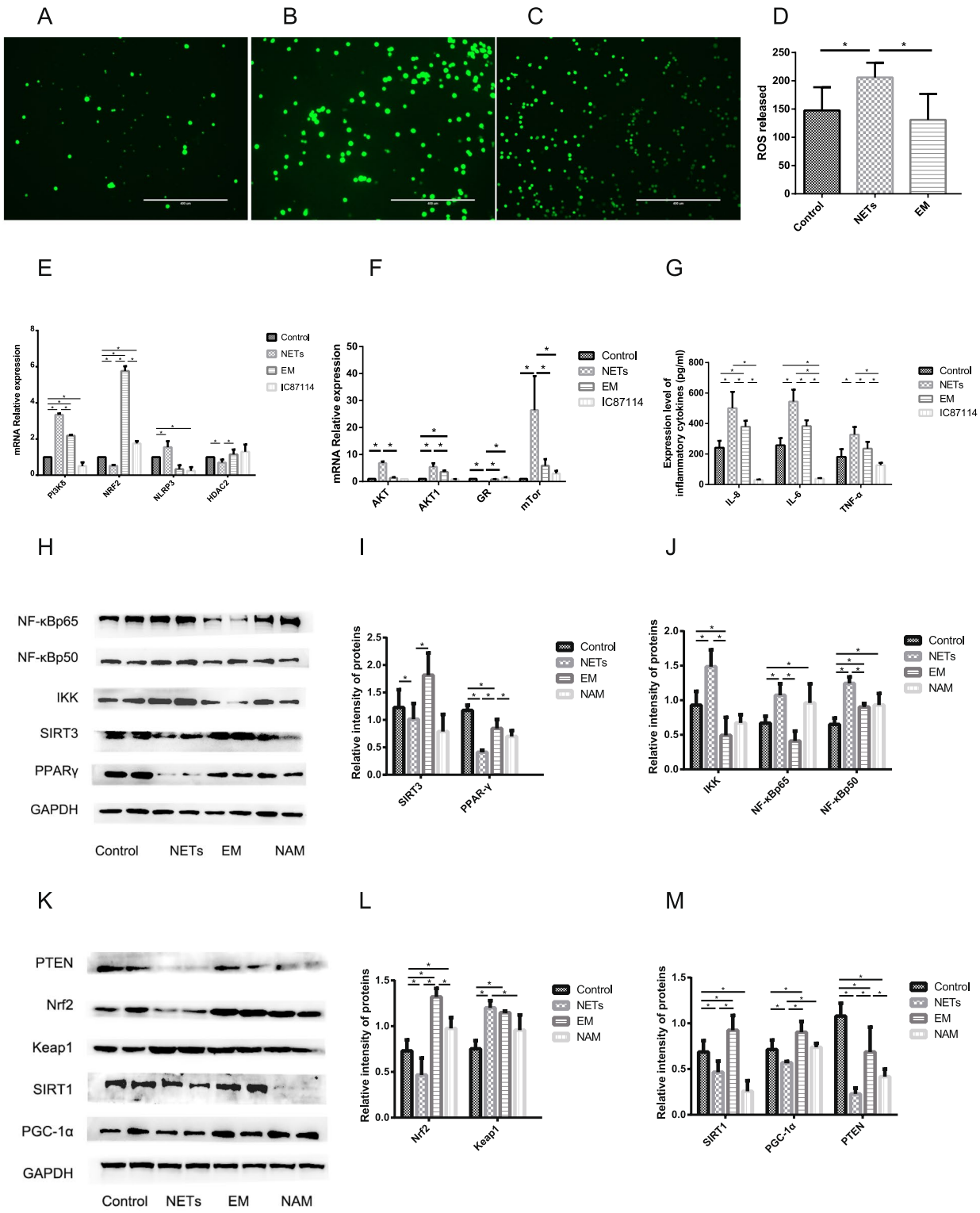

Fig. 10 (See legend on previous page.)

screened and identified the hub targets mediating the anti-inflammation activity of EM.

The DEGs and DEPs revealed that AKT1 expression significantly upregulated in U937 cells following NETs stimulation. In contrast, EM attenuated this increase, implicating AKT1 as a key target through which EM mitigates NETs-associated inflammation. Enrichment analysis of DEGs and DEPs identified several inflammatory pathways involved in this process, including the PI3K/AKT, mTOR, PTEN, NF-κB signaling pathways, cytokine signaling in the immune system, interleukin signaling, chemokine-mediated inflammation, and oxidative stress response. These findings suggest that NETs primarily promote inflammation via these pathways, while EM exerts its anti-inflammatory effects through modulation of the PI3K/AKT, mTOR, NF-κB, PTEN signaling pathways, and oxidative stress response. Enrichment of co-expressed DEPs and DEGs further supports the role of PI3K/AKT pathway in EM's inhibition of NETs-related inflammation. These data are consistent with previous studies indicating that EM reduces inflammation in COPD via PI3K/AKT pathway [25, 38]. Additionally, transcriptomic and proteomic enrichment analysis revealed the involvement of the MAPK pathway in EM's modulation of NETs-associated inflammation. However, P38, ERK and JNK expression did not change significantly in genome and proteome. We hypothesize that EM and NETs may alter the phosphorylation levels of these MAPK family members, such as p38, ERK, and JNK, thereby modulating downstream targets. Taken together, these multi-omics results suggest that PI3K/AKT pathway, NF-κB signaling pathway, PTEN signaling pathway, oxidative stress response and MAPK pathways are involved in EM's inhibition of NETs-induced inflammation.

The classic targets involve in the inflammatory pathogenesis of COPD were screened and identified based on enrichment analysis. The merged PPI network derived from proteogenomics revealed nine critical targets: AKT, mTOR, c-Jun, STAT3, GSK3β, PIK3CD, NLRP3, and c-Fos. WB and PCR were used to validate effects of EM on of these targets in vitro. These results confirmed that EM inhibits the activity of PIK3CD, NLRP3, AKT, mTor, c-Jun and c-Fos. The effects of EM on the aforementioned proteins were consistent with the findings of multi-omics analyses. We also investigated the effects of EM on the phosphorylation levels of STAT3, GSK3β, mTOR, and AKT. These data demonstrated that EM upregulates the phosphorylation of STAT3, while downregulating the phosphorylation of GSK3β, mTOR, and AKT. Effect of EM on these targets was consistent with AKT inhibitor IC87114 and JNK inhibitor SP600125. ELISA results show EM reduces NETs-induced inflammatory cytokines release, including IL-6, IL-8, and TNF-α. Taken together, these results demonstrate that erythromycin ameliorates NETs-induced inflammation by inhibiting PI3K/AKT and its downstream targets.

The PI3K/AKT pathway regulates inflammation, inflammatory cell activation, apoptosis, and oxidative stress, and is implicated in COPD progression [39]. Activation of PI3K catalyzes the conversion of Phosphatidylinositol-4,5-bisphosphate (PIP2) to Phosphatidylinositol-3,4,5-trisphosphate (PIP3), resulting in the phosphorylation and activation of AKT [40]. Activated AKT activates various downstream protein targets, contributing to the progression of inflammation [40]. Previous studies indicate that mTOR, c-Jun, STAT3, GSK3β, PIK3CD, NLRP3, and c-Fos are downstream targets regulated by the PI3K/AKT pathway. The key upstream signaling pathway activating mTOR is PI3K/AKT [41], in CSE-induced BEAS-2B cells and COPD patients, increased PI3K/AKT expression leads to mTOR activation, which promotes a pro-inflammatory senescence-associated secretory phenotype [42, 43]. NLRP3 infammasomes is one of intracellular multiprotein family that can be activated by AKT/NF-KB p65, increase the release of inflammatory factors such as IL-18 and IL-1β [44, 45]. Elevated NLRP3 expression in induced sputum from asthma and COPD patients has been associated with increased NETs formation [13]. In CSE-stimulated lung epithelial cells and lung tissues from COPD patients, STAT3 phosphorylation increase and correlate with CSE-induced inflammatory response [46, 47]. Dasatinib inhibition of AKT phosphorylation in LPS-stimulated BV2 microglial cells reduces STAT3 phosphorylation and proinflammatory cytokine release, including COX-2

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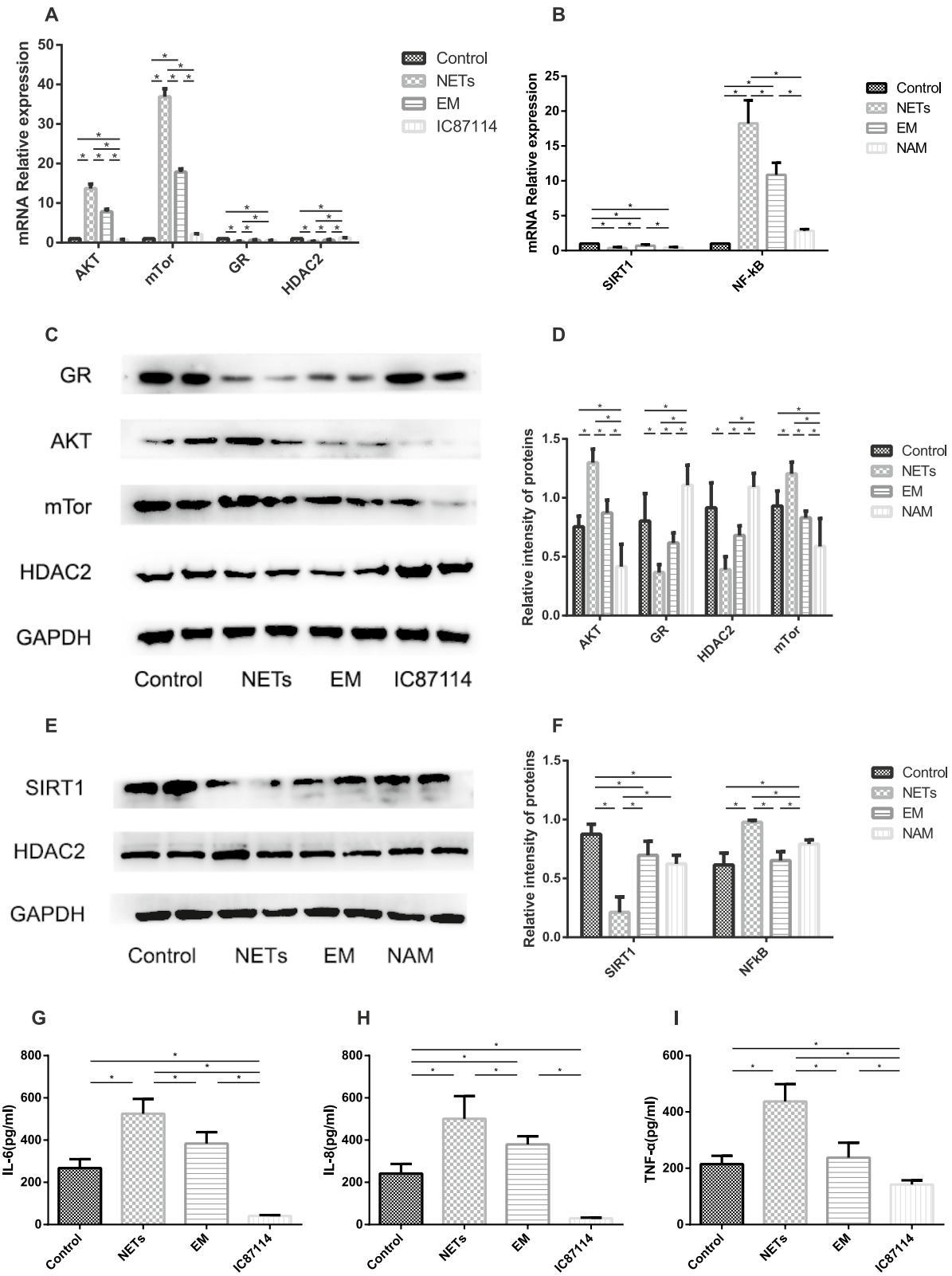
Fig. 11 Effect of EM regulates on proteins induced by NETs in inflammation. oxidative stress-related proteins. **A** The expression of AKT, mTor, GR and HDAC2 mRNA in each group of cells (Mean ± SD, *$P < 0.05$; n = 3). **B** The expression of SIRT1 and NF-κB mRNA in each group (mean ± SD, *$P < 0.05$; n = 3). **C** Protein expression of GR, AKT, mTor and HDAC2 in each group of cells. **D** Relative protein expression of GR, AKT, mTor and HDAC2 in each group of cells (mean ± SD, *$P < 0.05$; n = 4). **E** Protein expression of SIRT1 and NF-κB in each group of cells. **F** Relative protein expression of SIRT1 and NF-κB in each group of cells (mean ± SD, *$P < 0.05$; n = 4). **G** IL-6 expression in the supernatant of each group (mean ± SD, *$P < 0.05$; n = 3). **H** IL-8 expression in the supernatant of each group (mean ± SD, *$P < 0.05$; n = 3). **I** TNF-α expression in the supernatant of each group (mean ± SD, *$P < 0.05$; n = 3)



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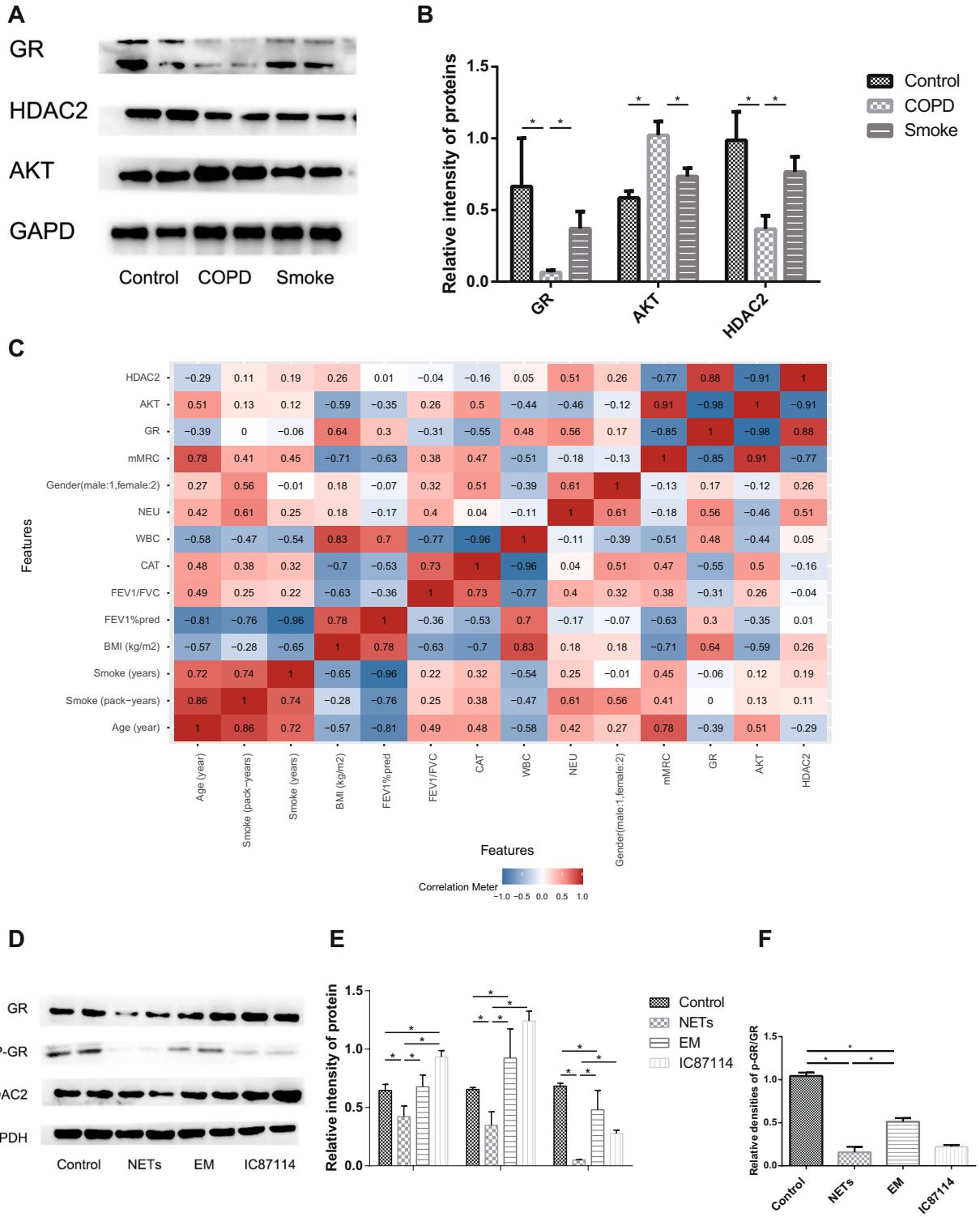

Fig. 12 EM improved NETs-induce glucocorticoid sensitivity by inhibiting PI3K/AKT activity. **A** Protein levels of AKT, HDAC2 and GR in each group. **B** Relative AKT, HDAC2, and GR protein expression in each group (mean ± SD, **P* < 0.05; n = 4). **C** Correlation heat map of clinical features and protein expression. **D** Protein expression of GR, P-GR and HDAC2 in each group of cells. **E** Relative intensity of GR, p-GR and HDAC2 protein in each group of cells (Mean ± SD, **P* < 0.05; n = 4). **F** GR phosphorylation level (Mean ± SD, **P* < 0.05; n = 4)

and IL-6 [48]. GSK3β as a AKT substrate, is inactivated by AKT through phosphorylation at Ser-9 [49]. Oxidative stress stimulation of the PI3K/AKT pathway can lead to inactivation of GSK3β, leading to glucocorticoid resistance and inflammation [50]. C-myc is one of downstream transcription factors regulated by GSK3β, promotes inflammation by upregulating cytokines including IL-1 and IL-18 [51, 52]. AP-1 is composed of c-Jun and c-Fos, as a redox sensitive proinflammatory transcription factor regulated by PI3K/AKT and MAPK in COPD [53–55], inhibition of the PI3K/AKT suppresses c-Jun activity and regulates c-Fos activation, result in impacting inflammation [54, 56, 57]. These results indicated that EM mitigates NETs-associated inflammation in COPD by regulates ten classic inflammatory targets, with the PI3K/AKT pathway acting as a critical regulatory node.

Glucocorticoid resistance critically contributes to COPD inflammation, mainly via HDAC2 and glucocorticoid receptor (GR) activity inhibition [58]. PI3K/AKT pathway closely associated with glucocorticoid resistance, it can regulate downstream target HDAC2 via GSK-3β and AP1 [26, 50]. Previous studies have showed that EM improves glucocorticoid resistance by suppressing PI3K/AKT, thereby alleviating COPD inflammation [26]. In clinical samples from COPD patients of our study, NETs upregulated AKT protein expression while downregulating HDAC2 and GR protein expression. In vitro experiments demonstrated that NETs activated the PI3K/AKT pathway, decreasing GR, p-GR, and HDAC2 protein levels and GR phosphorylation. EM reversed PI3K/AKT pathway activation, restoring GR, p-GR, and HDAC2 expression and enhancing GR phosphorylation induced by NETs. These findings indicate that that EM reduces NET-induced glucocorticoid resistance by inhibiting PI3K/AKT and its downstream targets HDAC2 and GR. This suggests that improving glucocorticoid resistance is another molecular mechanism by erythromycin alleviates COPD inflammation through the PI3K/AKT.

On the other hand, NETs are composed of a mixture of nuclear chromatin, mitochondrial DNA, and various cytoplasmic proteins associated with the DNA framework, including proteases, myeloperoxidase, histones, and other molecules [59, 60]. Given this complexity, We explore how NETs activate the PI3K/AKT pathway and EM's role in this process. Transcriptomic and proteomic enrichment analyses indicate EM regulates NETs-related inflammation via oxidative stress. Previous studies shown that NETs stimulation causes intracellular oxidative stress, and PI3K/AKT can be activated by oxidative stress [61, 62]. Results of fluorescence microscopy confirm NETs stimulation enhances ROS release in U937 cells, while EM attenuates NETs-induced ROS release. To further study the molecular mechanism of EM inhibits oxidative stress, we identified key targets associated with oxidative stress in the merged PPI network of proteogenomics. Ten key targets were identified: AKT, PTEN, IKK, NF-KB, KEAP1, Nrf2, SIRT1, SIRT3, PPAR-γ, PGC-1α. Validation confirmed EM decrease the activity of IKK, NF-KB, KEAP1, and ameliorates the decrease of PTEN, Nrf2, SIRT1, SIRT3, PPAR-γ and PGC-1α induced by NETs. EM has the opposite effect on SIRT1, SIRT3 and other targets as NAM.

Among the above targets, PTEN is an upstream target of AKT. PTEN negatively regulates the PI3K/AKT pathway [63, 64], oxidative stress inhibits PTEN activity, thereby activating the PI3K/AKT pathway and amplifying inflammation in COPD [63, 65, 66]. In COPD patients, PTEN expression is reduced in lung tissue and bronchial epithelial cells, correlating with elevated phosphorylated AKT levels [63], knockdown of PTEN promotes the phosphorylation of AKT in BEAS-2B cells, along with the release of inflammatory cytokines, including CCL5, CCL2, IL-6, CXCL8 and CXCL10 [63]. In the molecular mechanism of EM regulating oxidative stress, downstream target of AKT were IKK, NF-κB, KEAP1, Nrf2, SIRT3, PGC-1α and PPAR-γ. IKK is regulated by AKT phosphorylation, and activates NF-κB by phosphorylating IκB [67–69], then promoting the transcription of pro-inflammatory genes [67, 68]. NF-κB consists five members: c-REL, NF-κB2 (p100/p52), RelA (p65), NF-κB1 (p105/p50), RelB, and RelA [70], is a central regulator of inflammation in COPD and drives disease progression by modulating gene expression [71, 72]. KEAP1 and Nrf2 interact directly, forming an intracellular defense against oxidative stress, and are modulated by PI3K/AKT [71, 72]. Keap1 inhibits Nrf2 [73], Nrf2 is a major sensor of oxidative stress and regulates multiple antioxidant enzymes [71]. Cryptotanshinone inhibits KEAP1 and upregulates Nrf2 expression in COPD mice, thus alleviating the progression of COPD [74]. SIRT3 as a mitochondrial deacetylase regulates mitochondrial ROS production [75]. Nrf2 modulates SIRT3 activity by preventing its degradation through inhibition of its interaction with the KEAP1 ubiquitin ligase, which enhances SIRT3 transcription and its antioxidant activity [76, 77]. PPAR-γ activity is regulated by AKT [78, 79], PPAR-γ expression is downregulated in COPD animal models and the lungs of COPD patients [80, 81]. Oxidative stress induced by smoke reduces PPAR-γ expression, leading to exacerbate inflammation [82]. PGC-1α is a coactivators of PPAR-γ that involved in regulating mitochondrial function and modulating ROS production [83, 84]. PGC-1α expression decreased in the lungs of COPD model mice, upregulation of PGC-1α in BEAS-2B cells and airway smooth muscle cells is accompanied by reduced

ROS generation [85]. This study shows that AKT and SIRT1 have a synergistic effect, and both of them can affect NF-κB. SIRT1 is a redox-sensitive deacetylase that post-transcriptionally modified under oxidative, resulting in its inactivity in cells [86]. SIRT1 directly interacts with NF-κB, forming a complex that represses NF-κB activity through deacetylation, thereby reducing the release of NF-κB-related inflammatory factors [24, 87]. Collectively, these findings substantiate that EM reduce inflammatory response by regulating ten oxidative stress-related targets, and PI3K/AKT play a hub role in these targets.

This study has several limitations, at first, sample size in proteogenomic research is small and restricted the coverage of multiomics data, which also impacted the transcriptomic analysis. Second, a multiomics analysis of clinical samples was not performed, and this remains to be investigated in COPD patients treated with EM in further studies. Finally, it is remains unclear whether EM continues to exert its protective effects against NETs-induced inflammation in AKT knockout cells or mice, which warrants further exploration.

In summary, our study provides a comprehensive expression profile of inflammation-related DEGs, DEPs, and associated pathways regulated by EM through a proteogenomic approach. We highlight the critical role of PI3K/AKT in EM mitigating NETs-induced inflammation. These findings offer novel insights into the molecular mechanisms underlying EM's protective effects against NETs-related inflammation in COPD.

Abbreviations

DCFH-DA	2′,7′-Dichlorofluorescin diacetate
COPD	Chronic obstructive pulmonary disease
CSE	Cigarette smoke extract
DEGs	Differentially expressed genes
DEPs	Differentially expressed proteins
FDR	False discovery rate
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
NETs	Neutrophil extracellular traps
PPI	Protein–protein interaction
ROS	Reactive oxygen species
PCR	Real-time polymerase chain reaction
STRING	Search Tool for the Retrieval of Interacting Genes
WB	Westernblot
RIPA	Radioimmunoprecipitation assay
ELISA	Enzyme linked immunosorbent assay
NAM	Nicotinamide

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-025-03226-5.

Supplementary material 1.

Supplementary material 2.

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Author contributions
ZhiYi He the project and designed experiments. NanMa wrote the manuscript. MeiHua Li , QuanFang Chen and ZhiYi He edited the manuscript. NanMa, GuangSheng Pei, XiaoNa Liang, XiaoFei Yi, LiYan Guo and FuGang Chen performed the experiments. NanMa conducted data analysis. All authors have read and approved the final manuscript.

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Availability of data and materials
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
The project titled "Neutrophil extracellular traps (NETs) enhances the glucocorticoid resistance of mononuclear cells exposed to CSE by upregulating activity of PI3K-δ/ Akt?"has been approved by the First Affiliated Hospital of Guangxi Medical University in 2018, and in accordance with the guidelines of the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (number: 2016-KY-048).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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