


Endothelial Microparticles Derived from Primary Pulmonary Microvascular Endothelial Cells Mediate Lung Inflammation in Chronic Obstructive Pulmonary Disease by Transferring microRNA-126

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Background: Extracellular vesicles (EVs) are considered to new types of intercellular communication media, and microRNA is one of the most common transferring components of EVs. This study aimed to explore the potential role of endothelial microparticles (EMPs) derived from primary pulmonary microvascular endothelial cells in regulating lung inflammation of chronic obstructive pulmonary disease (COPD) through transferring microRNA-126 (miR-126).

Methods: EMPs generated from primary pulmonary microvascular endothelial cells were isolated by gradient centrifugation and characterized by transmission electron microscopy, flow cytometry and Western blotting. EMPs were treated to in vitro and in vivo COPD models induced by cigarette smoke extract (CSE). miR-126 mimics or inhibitors were transfected into EMPs by calcium chloride. Pathological changes of lung tissue, mRNA and protein levels of inflammation-related factors were measured to explore the effect of EMPs transferring miR-126 on CSE-induced inflammation.

Results: Both in vitro and in vivo studies demonstrated that mRNA and protein levels of inflammation-related factors were significantly increased in COPD group, while EMPs could dramatically reverse these increases. In vitro, overexpression of miR-126 in EMPs decreased HMGB1 expression and magnified the decreasing effect of EMPs on inflammation-related factors.

Conclusion: The present study reveals that EMPs are capable of alleviating lung inflammation and transferring miR-126 can magnify the anti-inflammatory effect of EMPs, which may provide a novel therapeutic alternative for COPD.

Keywords: chronic obstructive pulmonary disease, endothelial microparticles, primary pulmonary microvascular endothelial cell, inflammation, microRNA-126

Introduction

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2021 report,¹ chronic obstructive pulmonary disease (COPD) is a preventable and treatable respiratory disease usually caused by exposure to toxic particles or gases, and it is characterized by persistent respiratory symptoms and airflow limitation due to respiratory and/or alveolar abnormalities. COPD has become a public health problem all over the world, and it caused 2.6% of global disability-adjusted life years (DALYs) during 1990 to 2015.² The China Pulmonary Health (CPH) study demonstrated that the overall prevalence of spirometry-defined COPD in Chinese adult population was 8.6%.³

Several mechanisms are involved in the development of COPD including inflammation, imbalance between proteolytic and anti-proteolytic activity, oxidative stress and apoptosis, and there exists interaction among different mechanisms.⁴ Chronic inflammation of the airways plays a central role in the pathophysiology of COPD.^{5,6} The risk

factors of COPD patients are heterogeneous in the population. Existing studies show that the risk factors of COPD mainly include genetic factors, environmental factors (smoking, air pollution, occupational exposure, infection, etc.) and bad lifestyles (eating habits and daily exercise).⁷ Cigarette smoke exposure is considered to be the primary risk factor for the decline of lung function and the incidence of COPD.^{8–10} In addition, previous studies have also proved that cigarette smoke exposure participates in the occurrence and development of COPD by inducing airway inflammatory response, oxidative stress injury, lung structural cell aging and cell death.^{11,12}

Endothelial microparticles (EMPs) are extracellular vesicles (EVs) less than 1 μm in diameter released from the surface of endothelial cells when they are activated or apoptotic. EMPs have some antigenic properties of endothelial cells and are considered to play important biological roles in inflammation, vascular injury, endothelial dysfunction and thrombosis.¹³ Recently, evidence has proved that EVs can be new types of intercellular communication media, which can carry nucleic acid, protein and other components to transport between cells and participate in the pathogenesis of chronic lung diseases.¹⁴

As one of the most common transferring components of extracellular vesicle, microRNAs (miRNAs) are involved in the pathogenesis of a variety of inflammatory diseases.^{15–17} miRNA is a small noncoding RNA, which plays an important role in post transcriptional gene regulation, and the regulation of gene expression by miRNA may occur in three stages of transcription/translation process, including pre-translational, post-translational or co translational silencing.^{18,19} miRNAs participate in a variety of physiological and pathological processes in human body, including cell maturation, differentiation, and immune regulation.^{20,21} A recent study found that exosomal microRNA-21 (miR-21) derived from bronchial epithelial cells is involved in aberrant epithelium-fibroblast cross-talk, which suggested an alternative therapeutic target for COPD.²² However, the cellular communication between endothelial cells and bronchial epithelial cells remains unclear.

Previous studies have demonstrated that miR-126 is an endothelial “specific” microRNA, and miR-126 was found to be the most abundant microRNA in endothelial cells by RNA sequencing.^{23,24} Van Balkom et al²⁵ found that exosomes derived from human microvascular endothelium were loaded with abundant miR-126 by gene sequencing. Through gene chip analysis, miR-126 was found to be enriched in human pulmonary microvascular endothelial cells and human pulmonary artery endothelial cell derived microparticles.²⁶ Interestingly, previous studies reported miR-126 could suppress inflammation responses in different disease models.^{27–32} Nevertheless, there have been no studies investigating the relationship between miR-126 and CSE-induced inflammation. In the present study, we explored the potential role of EMPs derived from primary pulmonary microvascular endothelial cells in regulating lung inflammation of COPD through transferring miR-126.

Methods

Cell Lines and Cell Culture

Human pulmonary microvascular endothelial cells (HPMVECs), the primary microvascular endothelial cells derived from the lungs of the human foetus lungs, were purchased from ScienCell Research Laboratories (San Diego, USA). Mouse pulmonary microvascular endothelial cells (MPMVECs), the primary microvascular endothelial cells derived from mouse foetus lungs, were purchased from Procell Research Laboratories (Wuhan, China). Human bronchial epithelial cells (HBECs) were provided by Institute of Respiratory Diseases, The Second Affiliated Hospital of Zhejiang University. The use of HBECs was approved by ethics committee of Second Xiangya Hospital of Central South University. Both HPMVECs and MPMVECs were cultured with endothelial cell medium (ScienCell, San Diego, USA) under standard cell culture conditions (37°C, 5% CO₂). And HBECs cultured in DMEM (Hyclone, Logan, UT, USA) in a 5% CO₂ humidified incubator at 37°C.

Isolation and Characterization of EMPs

As previously reported by Pan et al,³³ EMPs generated from endothelial cells were isolated with minor changes. Briefly, confluent cells were placed and starved in basic medium (without endothelial growth factor or fetal bovine serum) for 24 hours to induce apoptosis. After starvation, the culture medium was collected and centrifuged at 300g for 5 minutes to remove cells. Next, the supernatant was centrifuged at 2000g for 10 minutes to remove debris. Then the collected supernatant

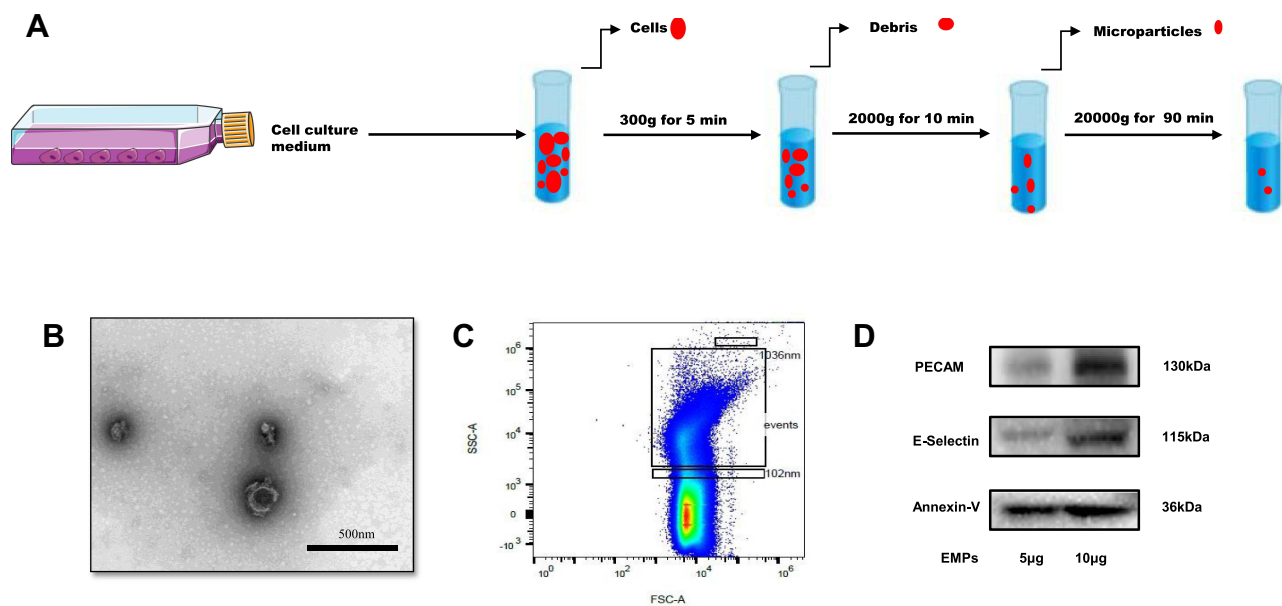


Figure 1 Identification of endothelial microparticles (EMPs). **(A)** Flowchart of EMPs isolation. **(B)** Transmission electron microscopy (TEM) of EMPs released from human pulmonary microvascular endothelial cells (HPMVECs), scale bar: 500nm; **(C)** Flow cytometry (FCM) of EMPs, standard fluorescent beads of 0.1µm and 1µm were used to define the gate; **(D)** Western blotting for specific biomarkers of EMPs (PECAM, E-selectin and Annexin V).

was centrifuged at 20000g for 90minutes to pellet EMPs. Flowchart of EMPs isolation is demonstrated in [Figure 1A](#). The pelleted EMPs were resuspended in filtered phosphate-buffered saline (PBS). EMPs were further identified by transmission electron microscopy (TEM), flow cytometry (FCM) and Western blotting. For flow cytometry, EMPs gate was defined as microparticles with a diameter of 0.1 µm–1µm using 0.1 µm and 1µm calibration beads (Invitrogen, USA).

Preparation of microRNA-126 Mimics/Inhibitors Loaded-EMPs

As stated by Zhang et al,³⁴ a method of calcium chloride (CaCl_2) transfection was used to transfect miR-126 mimics/inhibitors into EMPs. In brief, 200 pmol of microRNA mimics or inhibitors were mixed with 20 µg (protein) EMPs in filtered PBS, and then CaCl_2 was added to achieve a concentration of 0.1 M. Filtered PBS was used to adjust the final volume of mixture to 300 µL. The mixture was placed on ice for 30 min. After thermal shock at 42 °C for 1 min, the mixture was placed on ice for an additional 5 min. For cell treatment, 20 µg (protein) EMPs were added to a 60-mm cell culture dish (50–70% HBECs confluency) with 5 mL EMPs-free cell culture media.

Preparation of CSE

CSE solution was prepared as previously described by Ma et al.³⁵ Firstly, a modified syringe-driven apparatus was added with 20 mL phosphate buffered saline (PBS) or serum-free cell culture medium. Secondly, cigarettes (Furong, China Tobacco Hunan Industrial co. LTD, Hunan, China) were burned, and a vacuum pump with a pressure at –300 mmHg was used to bubble the cigarette smoke into the modified syringe-driven apparatus; of note, 10 cigarettes were burned for animal experiment and one cigarette was burned for cell experiments Thirdly, the cigarette smoke and PBS (or serum-free cell culture medium) were fully mixed to get 100% CSE solution. Fourthly, the CSE solution was filtered by the microfilter with a pore size of 0.2 µm. 100% CSE solution was used to intraperitoneally inject to mice, while 5% CSE solution was used in the in-vitro experiments.

Animal Experiment

COPD mice models were established referring to the protocol by He et al³⁶ with minor revisions. A total of 24 BALB/C mice (six weeks old) were purchased from Hunan Slyke Jingda Laboratory Animal co. LTD. All mice were randomly divided into three groups: 1) control group, 2) COPD group, 3) COPD + EMPs group, and there were eight mice in each

group. In short, the COPD mice were intraperitoneally injected with 0.3mL 100% CSE solution on days 0, 11, and 22, and they were exposed to cigarette smoke in a sealed box with ventilation holes twice a day for 28 consecutive days, except for days 0, 11, 22. For mice in control group, they were maintained in fresh air; on days 0, 11, and 22, an intraperitoneal injection of 0.3 mL PBS was given to them. Mice in COPD + EMPs group were treated with 100 μ g (protein) EMPs (released from primary MPMECs) in 30 μ L filtered PBS by intratracheal instillation on days 13 and 26, while mice in control and COPD group were intratracheally instilled with 30 μ L filtered PBS on days 13 and 26. The study was reviewed and approved by the Ethics Committee of Second Xiangya Hospital of Central South University. The animal experiments followed guidelines for the welfare of the laboratory animals in Second Xiangya Hospital of Central South University, and conformed to internationally accepted ethical standards. A summarized figure of the animal experiment protocol is demonstrated in the [Supplementary Figure 1](#).

Lung Tissue Morphometry

Hematoxylin and eosin (HE) staining was applied to measure pathological changes of mouse lung tissue. Left lungs were inflated with 4% paraformaldehyde for 24 hours and then embedded in paraffin. Embedded lung tissue was sectioned (5 μ m) and stained with HE for light microscopy examination. To assess emphysematous changes of lung tissue, mean linear intercept (MLI) and destructive index (DI) were calculated as stated by He et al.³⁷ A semi-quantitative scoring system with a grading scale ranging from 0 to 3 was used to evaluate inflammation in the light of the method reported by Jang et al.³⁸

Real-Time Quantitative Polymerase Chain Reaction Analysis (RT-qPCR)

Total RNA from HBECs was extracted using the TRIzol reagent (Invitrogen, USA). mRNA was reversely transcribed into cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). RT-qPCR was performed using an All-in-One qPCR Mix kit (GeneCopoeia, Guangzhou, China) based on general SYBR Green fluorescence detection. In accordance to the comparative Ct ($\Delta\Delta$ Ct) method, relative mRNA levels were calculated after normalization to GAPDH.

Western Blot Analysis

To extract protein from cells and lung tissues, RIPA lysis buffer (Beyotime, China) was applied. Protein concentrations were measured by a Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific, USA). Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate total protein, and separated protein was transferred to polyvinylidene fluoride (PVDF) membranes activated by isopropanol. Sequentially, PVDF membranes were blocked in 5% non-fat milk dissolved in Tris-buffered saline with Tween (TBST) for 1 hour at room temperature. Next, PVDF membranes were incubated with primary antibodies at 4 °C for 12–16 hours. Primary antibodies included nuclear factor kappa B p65 (NF- κ B p65) (Cell Signaling Technology, USA, 1:1000 dilution), phosphorylated nuclear factor kappa B p65 (p-p65) (Cell Signaling Technology, USA, 1:1000 dilution), high mobility group box 1 (HMGB1) (Abcam, USA, 1:2000 dilution), platelet endothelial cell adhesion molecule-1 (PECAM-1) (Santa Cruz, USA, 1:500 dilution), E-selectin (Santa Cruz, USA, 1:500 dilution), Annexin V (Santa Cruz, USA, 1:500 dilution), and GAPDH (Proteintech, China, 1:1000 dilution). After washing primary antibodies, PVDF members were incubated with HRP-labeled IgG secondary antibodies (Proteintech, China, 1:5000 dilution). Lastly, the ECL plus Western blotting detection system (Bio-Rad, USA) was employed to visualize labeled protein bands. And ImageJ software was used for the quantitative analysis of protein band densities.

Immunohistochemistry (IHC)

Lung tissue fixed in 4% formaldehyde for more than 24 hours were embedded in paraffin, and cut into 3.5- μ m-thick sections. 0.3% hydrogen peroxide was used to fixed lung sections for 10 min after antigen retrieval in citrate buffer (pH 6.0) for 10 min in microwave. Then lung sections were incubated with anti-HMGB1 (Abcam, USA, 1:200 dilution) at 4 °C overnight. Next, lung sections were incubated with goat anti-rabbit IgG antibody conjugated with peroxidase for 30 min at room temperature. Finally, diaminobenzidine (DAB) was added and hematoxylin was applied for counterstaining.

Statistical Analysis

Data were presented as mean \pm standard error (SEM) and analyzed by GraphPad Prism (GraphPad Prism 7.04, San Diego, CA, USA). One-way analysis of variance (ANOVA) combined with post hoc test was used to perform statistical comparisons. P values less than 0.05 were considered statistically significant.

Results

Identification of EMPs

Figure 1A demonstrated the flowchart for isolating EMPs from HPMVECs. As shown in Figure 1B, TEM analysis showed that EMPs released from HPMVECs ranged from 100 to 500 nm in diameter. As the size of released EMPs varied, FCM analyses were conducted to further measure the size of isolated vesicles. (Figure 1C) FCM revealed that the size of isolated vesicles majorly ranged from 0.1 μ m to 1 μ m. Furthermore, Western blotting results confirmed the expression of specific endothelial markers (PECAM-1, E-selectin, and Annexin V) in EMPs. (Figure 1D) Above results suggest a proper isolation of EMPs.

Effect of EMPs on Inflammation in Human HBECs Induced by CSE

The flowchart of using EMPs to treat HBECs was illustrated in Figure 2A. In order to demonstrate EMPs involving in HBECs, we used DAPI to dye the nucleus of HBECs and CFSE to dye EMPs. Figure 2B illustrated that EMPs distributed in or around the nucleus of HBECs. RT-qPCR results indicated that pro-inflammatory gene (TNF- α , IL-6, and IL-1 β) mRNA levels in CSE group significantly increased in contrast with control group, and EMPs treatment markedly reversed increased pro-inflammatory gene (TNF- α , IL-6, and IL-1 β) mRNA levels induced by CSE (Figure 2C). Furthermore, the relative p-p65 protein level to p65 in CSE group significantly increased compared with control group, and treatment of EMPs could significantly diminish the protein level of p-p65 to p65 (Figure 2D and E). Figure 2F and G showed protein levels of TNF- α and IL-1 β in cell supernatant of HBECs measured by ELISA. CSE dramatically increased TNF- α and IL-1 β protein levels in cell supernatant, and elevated TNF- α and IL-1 β protein levels were significantly reversed by EMPs. These results reveal that EMPs released from primary human microvascular endothelial cells are able to inhibit CSE-induced inflammation in vitro.

Effect of EMPs on Inflammation in COPD Mice

To measure the effect of EMPs on inflammation in vivo, we intratracheally instilled EMPs to COPD mice. (Figure 3A) Histological changes in mouse lung sections stained with HE were shown in Figure 3B. Pathological changes of emphysema including enlarged alveolar space and destroyed lung parenchyma were observed in COPD mouse lung tissue. Values of MLI and DI in COPD group were significantly increased compared to control group, while treatment of EMPs dramatically decreased MLI and DI values (Figure 3C and D). The scores of lung inflammation in COPD mice were significantly increased compared with mice in control group, and the enhanced lung inflammation in COPD mice was markedly attenuated by intratracheal instillation of EMPs (Figure 3E and F). Besides, we found that pre-treatment of EMPs remarkably decreased the number of total cells (Figure 3G), neutrophils (Figure 3H), and macrophages (Figure 3I) in mouse bronchoalveolar lavage fluid (BALF). In lung tissue of COPD mice, pro-inflammatory gene (TNF- α , IL-6, and IL-1 β) mRNA levels significantly increased when comparing with control group; and EMPs could significantly diminish increased pro-inflammatory gene mRNA levels (Figure 4A). Western blotting results showed that the protein level of HMGB1 and the relative p-p65 protein level to p65 in COPD group was markedly increased compared with control group, and the increase was significantly reversed by EMPs instillation (Figure 4B–E). Also, IHC demonstrated that pre-treatment EMPs could remarkably reduce the increased protein level of HMGB1 in COPD group. (Figure 4F and G) Protein levels of TNF- α and IL-1 β in mouse BALF measured by ELISA were illustrated in Figure 4H and I. TNF- α and IL-1 β protein levels in COPD mouse BALF were significantly increased in contrast with control group, while EMPs dramatically decreased the elevated levels of TNF- α and IL-1 β . Collectively, these results indicate that EMPs released from primary mouse microvascular endothelial cells can reduce mouse lung inflammation induced by CSE.

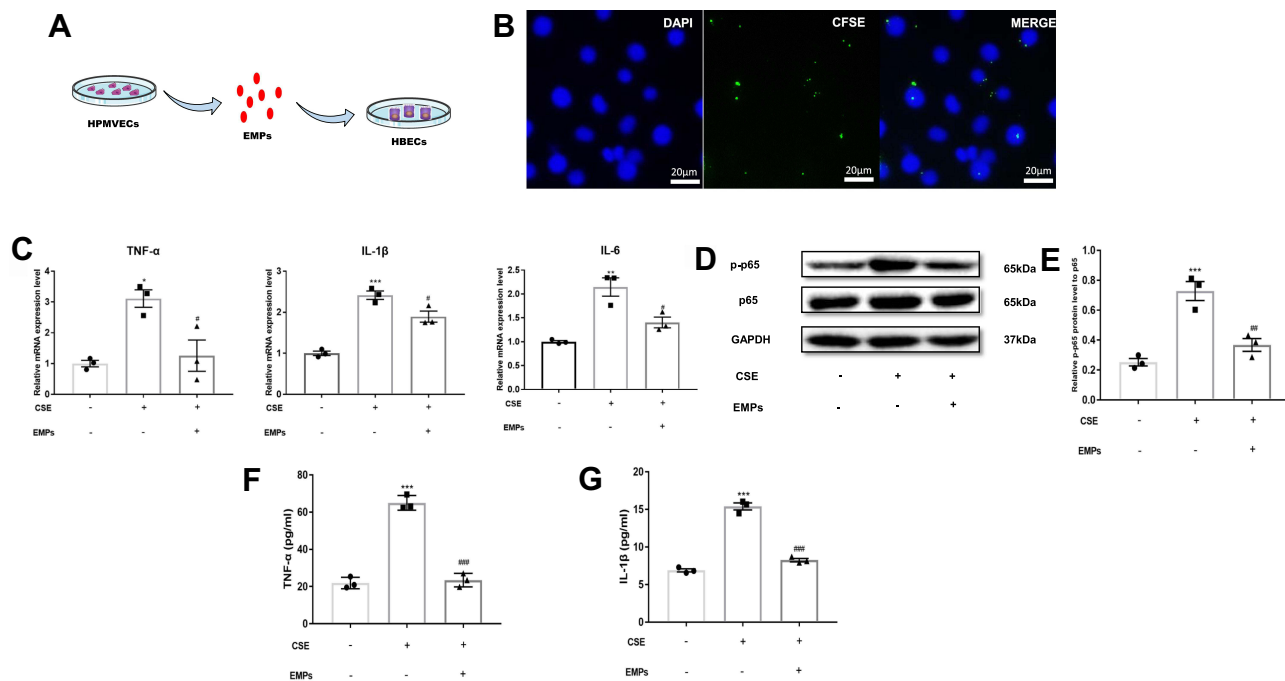


Figure 2 Effect of endothelial microparticles (EMPs) on cigarette smoke extract (CSE) induced inflammation in human bronchial epithelium cells (HBECS). **(A)** Treating diagram of EMPs from human pulmonary microvascular endothelial cells (HPMVECs) on HBECS; **(B)** Representative images of CFSE-labeled EMPs merging with HBECS (green: CFSE; blue: DAPI), scale bar: 20µm; **(C)** Levels of TNF-α, IL-1β, and IL-6 mRNA in HBECS with RT-qPCR; **(D)** Western blotting analyses of NF-κB p65 and phosphorylated-NF-κB p65 (p-p65) in HBECS; **(E)** Statistical analysis for relative p-p65 protein level in HBECS; **(F)** Levels of TNF-α in the cell supernatant of HBECS with ELISA. **(G)** Levels of IL-1β in the cell supernatant of HBECS with ELISA. Data are presented as the mean ± SEM. *p<0.05 in comparison with control group, **p<0.01 in comparison with control group, ***p<0.001 in comparison with control group, #p<0.05 in comparison with CSE group, ##p<0.01 in comparison with CSE group, ###p<0.001 in comparison with CSE group.

EMPs-Derived miR-126 Mediates CSE-Induced Inflammation in HBECS

To verify the mechanism of protecting effect of EMPs on CSE-induced inflammation, we transfected miR-126 mimics or inhibitors into EMPs and then examined the effect of EMPs-derived miR-126 on CSE-induced inflammation in HBECS. (Figure 5A) We found that EMPs^{miR-126mimic} dramatically increased miR-126 level in HBECS, while EMPs^{miR-126inhibitor} significantly reduced miR-126 level in HBECS. (Figure 5B and C) Pre-treatment of EMPs^{miR-126mimic} could significantly decrease the protein level of HMGB1 and relative protein level of p-p65 to p65 (Figure 5D–G). Moreover, the protein level of HMGB1 and relative protein level of p-p65 to p65 significantly increased after the treatment of EMPs^{miR-126inhibitor} (Figure 5H–K). Above findings suggest that the protecting effect of EMPs on CSE-induced inflammation may result from the transferred microRNA in EMPs.

Discussion

This study demonstrates that EMPs released from primary pulmonary microvascular endothelial cells could reduce lung inflammation induced by CSE in vitro and in vivo. Moreover, we proved that the protecting effect of EMPs on CSE-induced inflammation might result from the transferred miR-126 in EMPs, as miR-126 from EMPs could target HMGB1 and inhibit its expression in HBECS. Furthermore, the inhibition of HMGB1 mitigated the activation of downstream typical inflammatory pathway (NF-κB p65). The mechanism diagram of summarized results is illustrated in Figure 6.

Chronic airway inflammation is an important pathophysiological mechanism in the pathogenesis of COPD, which is characterized by excessive mucus secretion, small airway stenosis and emphysema; and inflammatory cells and inflammatory mediators are involved in the pathogenesis of COPD.⁶ As a major risk factor for COPD, cigarette smoke augments a progressive biphasic infiltration of inflammatory cells in BALF, airways and lung parenchyma, including dendritic cells (DCs), macrophages, and lymphocytes etc.³⁹ Besides, cigarette smoke could stimulate the release of numerous pro-inflammatory cytokines and inhibit the production of anti-inflammatory cytokines.⁴⁰ Of note,

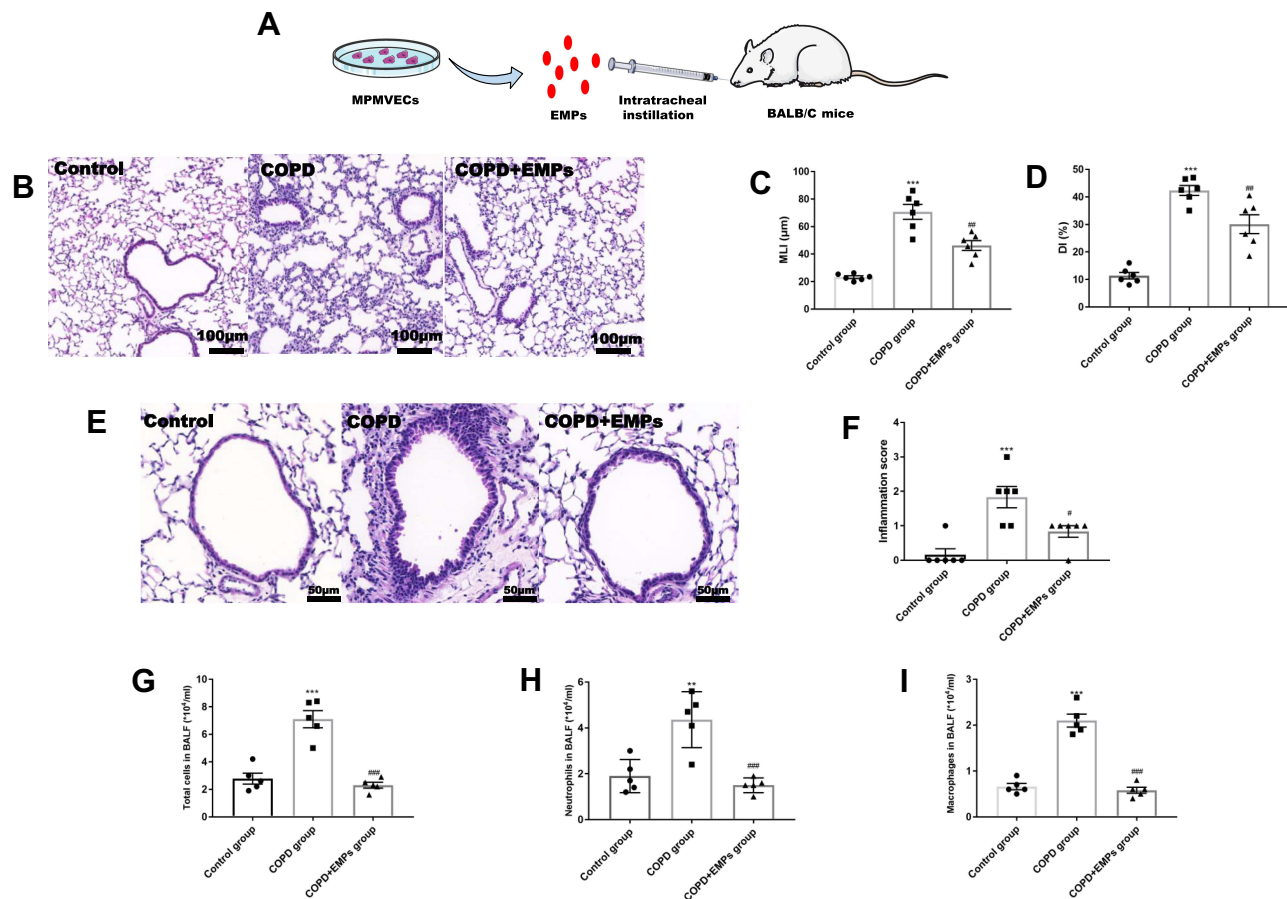


Figure 3 Effect of endothelial microparticles (EMPs) on pathological changes in COPD mice. **(A)** Treating diagram of EMPs from mouse pulmonary microvascular endothelial cells (MPMVECs) on COPD mice; **(B)** Pathological changes in mouse lung sections with hematoxylin and eosin (HE) staining; **(C)** Morphometric measurements of MLI (μm); **(D)** Morphometric measurements of DI (%); **(E)** Pathological changes in mouse airway with HE staining; **(F)** Lung inflammation scores in mice; **(G)** The number of total cells in mouse bronchoalveolar lavage fluid (BALF); **(H)** The number of neutrophils in mouse BALF; **(I)** The number of macrophages in mouse BALF. Data are presented as the mean ± SEM. ** $p < 0.01$ in comparison with control group, *** $p < 0.001$ in comparison with control group, # $p < 0.05$ in comparison with COPD group, ## $p < 0.01$ in comparison with COPD group, ### $p < 0.001$ in comparison with COPD group.

higher level of inflammatory cytokine is related to poorer prognosis of COPD patients.⁴¹ Thus, searching strategies targeting inflammatory responses seems crucial for the treatment of COPD.

EVs as therapeutic tools in treating lung diseases have drawn broad attention, as biologically derived EVs have a better safety profile compared with synthetic carriers.⁴² Particularly, EVs from mesenchymal stem cells (MSCs) become the research focus due to anti-inflammatory and regenerative capability of MSCs.⁴³ EVs derived from MSCs could downregulate the expression of proinflammatory cytokines IL-1 β and IL-6 in epithelial cells of cystic fibrosis.⁴⁴ Xu et al⁴⁵ reported that bone marrow mesenchymal stem cells (BMSCs)-derived exosomes can effectively rescue smoke inhalation lung injury by inhibiting the HMGB1/NF- κ B pathway, suggesting the potential therapeutic role of EVs in treating smoke inhalation lung injury. Moreover, a recent study by Ridzuan et al⁴⁶ revealed that human umbilical cord mesenchymal stem cells (HUCMSCs) derived-EVs reduced the COPD-induced inflammation in part by the expression of NF- κ B subunits p65 and p50. Recently, there have been novel findings to prove that EVs derived from non-mesenchymal stem cells also had therapeutic effects. In 2021, Kadota et al⁴⁷ confirmed that extracellular vesicles derived from normal human bronchial epithelium can inhibit TGF- β /Wnt pathway improves pulmonary fibrosis both in vitro and in vivo. Interestingly, a study previously published by Kadota and his team showed that extracellular vesicles derived from human bronchial epithelial cells stimulated by cigarette smoke can significantly promote the differentiation of pulmonary myofibroblasts through autophagy, and finally participate in the pathogenesis of COPD.¹¹ Furthermore, their team proposed that extracellular vesicles derived from normal human bronchial epithelium can “treat” diseases, while the

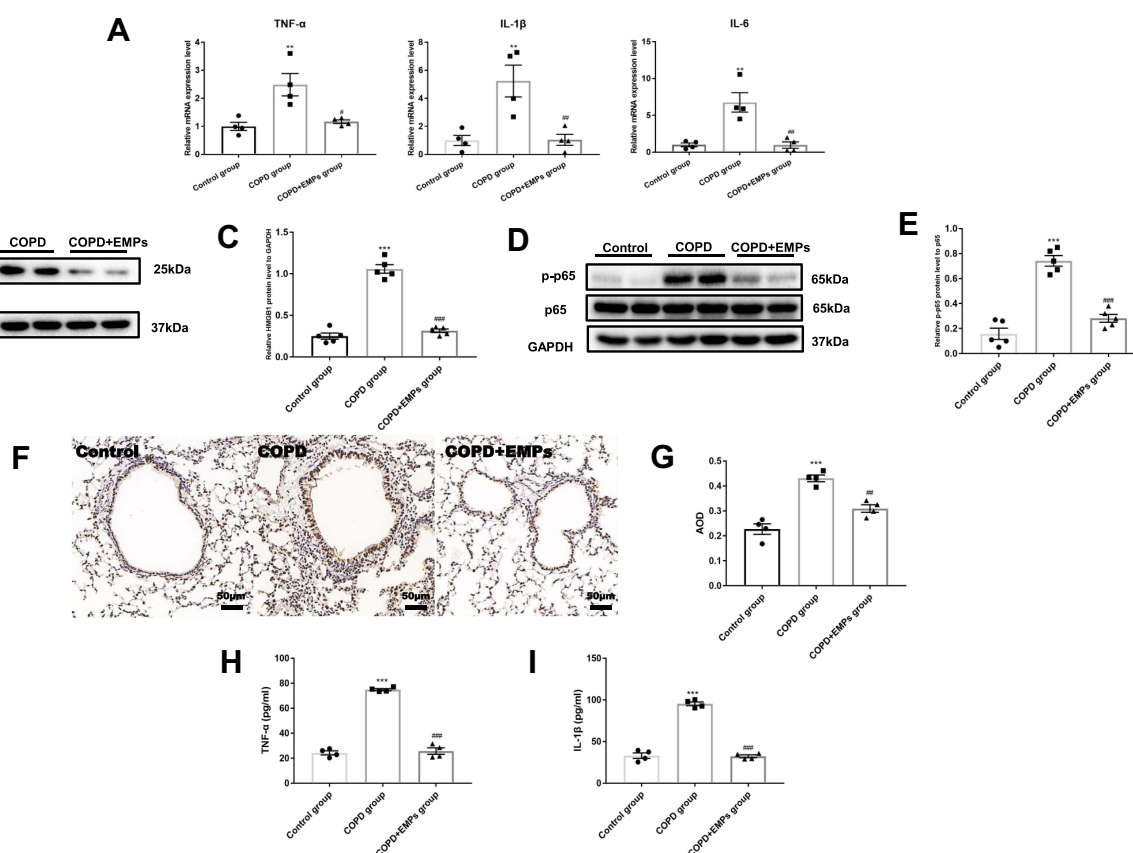


Figure 4 Effect of endothelial microparticles (EMPs) on lung inflammation in COPD mice. **(A)** Levels of TNF- α , IL-1 β , and IL-6 mRNA in lung tissue with RT-qPCR; **(B)** Western blotting analyses of HMGB1 in lung tissue; **(C)** Statistical analysis for relative HMGB1 protein level in lung tissue; **(D)** Western blotting analyses of NF- κ B p65 and phosphorylated-NF- κ B p65 (p-p65) in lung tissue; **(E)** Statistical analysis for relative p-p65 protein level in lung tissue; **(F)** Immunohistochemistry (IHC) for HMGB1 in lung tissue; **(G)** Statistical analysis for average optical density (AOD) of HMGB1 in lung tissue; **(H)** Levels of TNF- α in mouse BALF with ELISA; **(I)** Levels of IL-1 β in mouse BALF with ELISA. Data are presented as the mean \pm SEM. ** $p < 0.01$ in comparison with control group, *** $p < 0.001$ in comparison with control group, # $p < 0.05$ in comparison with COPD group, ### $p < 0.01$ in comparison with COPD group, #### $p < 0.001$ in comparison with COPD group.

extracellular vesicles derived from human bronchial epithelium stimulated by pathogenic factors (such as cigarette smoke) may participate in the “pathogenesis” of diseases. Same as their conclusion, our study proved that EVs from another kind of non-mesenchymal stem cell (endothelial cell) also have therapeutic effects. Researches also have demonstrated that EVs from endothelial progenitor cells (EPCs) are capable of reducing inflammation in lipopolysaccharide-induced acute lung injury⁴⁸ and sepsis.⁴⁹ Our study proved that primary pulmonary microvascular endothelial cells derived-EMPs were able to reduce CSE-induced inflammation responses in vitro and in vivo for the first time, which might provide a novel therapeutic choice for COPD.

Recently, microRNAs have been found to transfer from a donor cell into a recipient cell via exosomes and microparticles; emerging evidence have suggested that microRNAs are delivered with intact functionality in a paracrine fashion.⁵⁰ In addition, miRNAs regulate the translation of the target genes and the function of the target cells after being delivered by EVs.⁵¹ In particular, enrichment of certain microRNAs in EVs is able to establish a targeted cross-talk in many inflammation-related diseases. Zhang et al⁵² reported that sensory neurons transferred EVs-encapsulated miR-23a to promote inflammatory M1 macrophages by binding to A20 and enhance neuropathic pain following the peripheral nerve injury. Interestingly, a recent study manifested that inhibition of miR-10a expression in endothelial cells weakened their anti-inflammatory effects on monocytes.⁵³ Zheng et al⁵⁴ found that human umbilical vascular endothelial cells (HUVECs)-derived EVs carrying miR-129 could mitigate myocardial I/R injury by downregulating TLR4 and disrupting the NF- κ B signaling and NLRP3 inflammasome. In this study, we found that overexpression of miR-126 in EMPs was capable of decreasing HMGB1 expression in HBECs and further

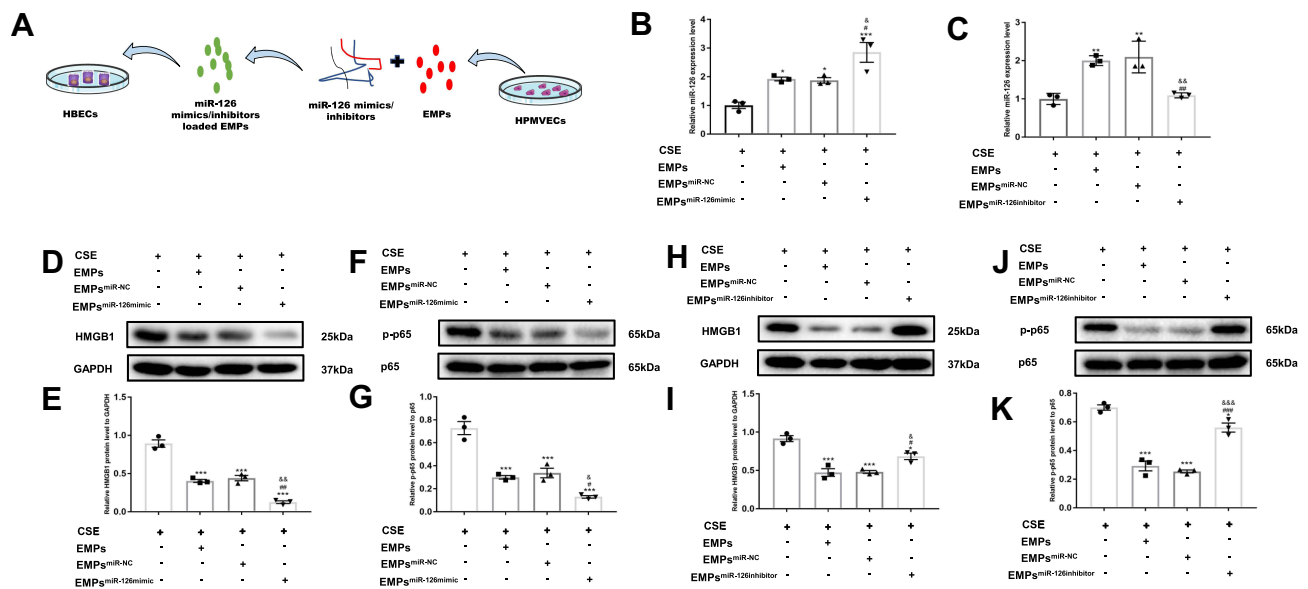


Figure 5 Effect of endothelial microparticles (EMPs)-derived microRNA-126 (miR-126) on cigarette smoke extract (CSE) induced inflammation in human bronchial epithelium cells (HBECS). (A) Treating diagram of EMPs^{miR-126} on HBECS; (B) Effect of EMPs^{miR-126mimic} on miR-126 levels in HBECS with RT-qPCR; (C) Effect of EMPs^{miR-126inhibitor} on miR-126 levels in HBECS with RT-qPCR; (D) Effect of EMPs^{miR-126mimic} on relative HMGB1 protein level in HBECS with Western blotting; (E) Statistical analysis for the effect of EMPs^{miR-126mimic} on relative HMGB1 protein level in HBECS; (F) Effect of EMPs^{miR-126mimic} on relative phosphorylated-NF- κ B p65 (p-p65) protein level in HBECS with Western blotting; (G) Statistical analysis for the effect of EMPs^{miR-126mimic} on relative p-p65 protein level in HBECS; (H) Effect of EMPs^{miR-126inhibitor} on relative HMGB1 protein level in HBECS; (I) Statistical analysis for the effect of EMPs^{miR-126inhibitor} on relative HMGB1 protein level in HBECS; (J) Effect of EMPs^{miR-126inhibitor} on relative p-p65 protein level in HBECS with Western blotting; (K) Statistical analysis for the effect of EMPs^{miR-126inhibitor} on relative p-p65 protein level in HBECS. EMPs^{miR-126mimic} represents EMPs transfected with miR-126 mimics; EMPs^{miR-126inhibitor} represents EMPs transfected with miR-126 inhibitors; EMPs^{miR-NC} represents EMPs transfected with miR-NC. Data are presented as the mean \pm SEM. * $p < 0.05$ in comparison with CSE group, ** $p < 0.01$ in comparison with CSE group, *** $p < 0.001$ in comparison with CSE group, # $p < 0.05$ in comparison with CSE+EMPs group, ### $p < 0.01$ in comparison with CSE+EMPs group, #### $p < 0.001$ in comparison with CSE+EMPs group, & $p < 0.05$ in comparison with CSE+EMPs^{miR-NC} group, && $p < 0.01$ in comparison with CSE+EMPs^{miR-NC} group, &&& $p < 0.001$ in comparison with CSE+EMPs^{miR-NC} group.

magnifying anti-inflammatory effect of EMPs in treating airway inflammation of COPD. Our results are consistent with previous studies. Two studies by Zhou et al^{48,49} revealed that EPCs derived exosomes were beneficial in lipopolysaccharide-induced acute lung injury and sepsis potentially through the delivery of miR-126. Moreover, exosomes derived from MSCs were able to transfer miR-126 and ameliorate hyperglycemia-induced retinal inflammation.³⁰

miR-126, as an endothelial cell-restricted microRNA, were proved to be associated with the inflammatory responses. Hu et al⁵⁵ found that miR-126 promoted angiogenesis and inhibited inflammation after contusion spinal cord injury in rats. In sepsis, regulation of miR-126 could mediate inflammatory response, differentiation of T lymphocyte subsets, and apoptosis of lymphocytes.²⁸ HMGB1 is a non-histone nuclear protein, which serves as an alarmin to drive the pathogenesis of inflammatory and autoimmune diseases.⁵⁶ HMGB1 is a late inflammatory mediator that can promote NF- κ B nuclear translocation that leads to the release of inflammatory cytokines, and inflammatory cytokines further enhance the release of HMGB1, leading to a positive feedback loop that amplifies the inflammatory cascade.^{57,58} An earlier research by Ferhani et al⁵⁹ proved that elevated expression of HMGB1 existed in proximal and distal airways of smokers with COPD, and HMGB1 also showed correlations with impaired lung function and increased level of IL-1 β . A systematic review further revealed the association between HMGB1 and COPD: cigarette smoke could induce neutrophils necrosis and the necrosis of neutrophil cells resulted in HMGB1 release, suggesting that HMGB1 was an intervention target of inflammation in COPD.⁶⁰ In this study, HMGB1 in HBECS was also found to be a potential target of miR-126, which was consistent with previous studies.^{27,30,48,49,61,62}

Conclusion

In summary, our results demonstrate that EMPs released from primary pulmonary microvascular endothelial cells can reduce airway inflammation of COPD in vitro and in vivo. Moreover, overexpression of miR-126 in EMPs inhibits

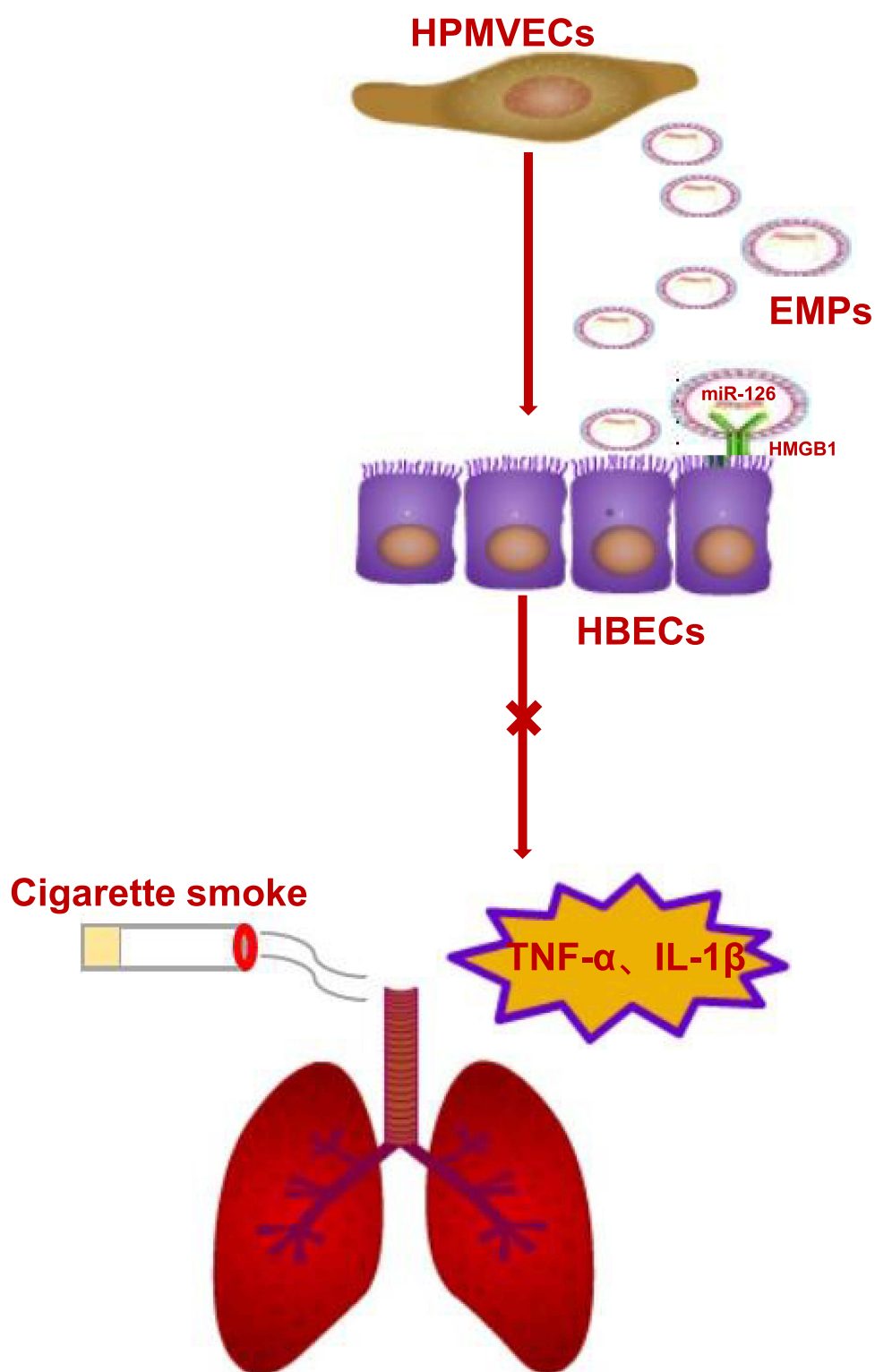


Figure 6 The mechanism diagram of summarized findings. HPMVECs-human pulmonary microvascular endothelial cells, EMPs-endothelial microparticles, HMGB1-high mobility group box 1, HBECs-human bronchial epithelium cells.

HMGB1 expression and further magnifies anti-inflammatory effect of EMPs. The present study may provide a novel therapeutic alternative for COPD.

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Disclosure

The authors report no conflicts of interest for this work and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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