

MPO Mediated Precise Inflammation Targeting of Antioxidant Melanin Nanomedicines as an Effective Therapeutic Strategy for Chronic Inflammation

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Abstract

Inflammation is the greatest threat to human health. Anti-inflammatory treatments are not always effective and can cause serious side effects. There is a critical need for anti-inflammatory drugs that are selective for inflammatory tissue over normal tissue, as well as for single inflammatory factor (or pathological factor) over broad-spectrum anti-inflammatories. Here we report a strategy for targeting inflammation by myeloperoxidase (MPO) mediated melanin nanomedicines (MNMNs). Elevated MPO levels in blood and inflammatory tissues are significant pathological markers of numerous inflammatory diseases. We found for the first time that MPO could bind to MNMNs with high specificity and mediate the specific entry of MNMNs into inflammatory tissues. MNMNs were composed of eumelanin, a natural antioxidant substance in the human body that eliminates a variety of reactive oxygen species (ROS). MNMNs inhibited oxidative stress damage mediated by a variety of inflammatory factors or pathology, breaking the vicious cycle of inflammation and oxidative stress in inflammatory tissues. With hepatopulmonary syndrome (HPS, a complex inflammatory syndrome driven by multiple pathological factors) as an example, the powerful anti-inflammatory therapeutic effect of this strategy was verified, which could significantly inhibit the progression of HPS. This highly targeted strategy can serve as a blueprint for the development of other highly specific targeted anti-inflammatory treatments.

1. Introduction

Almost all diseases are accompanied by inflammation¹. The World Health Organization (WHO) classifies inflammation as the greatest threat to human health and the most important cause of death in the world. Worldwide, 3 out of 5 deaths are caused by chronic inflammation, such as cerebrovascular disease, chronic respiratory disease, heart disease, obesity and diabetes^{2,3}. In 2014, nearly 60% of Americans had at least one chronic inflammation, 42% had more than one, and 12% of adults had five or more chronic inflammatory diseases⁴. More seriously, aging of the population is anticipated to contribute to a sustained rise in the prevalence of chronic inflammatory diseases⁵. Corticosteroids, immunosuppressants, and drugs targeting inflammatory factors (such as anti-TNF- α Infliximab) are commonly employed in the treatment of various chronic inflammatory diseases^{6,7}. However, the therapeutic effect of anti-inflammatory drugs is still unsatisfactory due to the lack of precise targeting inflammatory tissues, leading to numerous side effects. Precision treatment of specifically and highly targeting inflammatory tissues remains a clinically unmet need.

The inflammatory response triggers a series of alterations in blood vessels of inflammatory tissues, including abnormal morphology and function of endothelial cells, increased vascular permeability⁸. In the early stage of inflammation, the vascular endothelium is the first to be damaged, leading to pathological changes in endothelial cells⁹. Among them, loss of glycocalyx on the surface of vascular endothelial cells is one of the characteristic changes in vascular injury induced by inflammation¹⁰. Physiologically, the glycocalyx layer is between 500 and 2000 nm thick, which exceeds the size of intercellular adhesion molecules (such as intercellular adhesion molecule 1, vascular cell adhesion

molecule 1, P-selectin and L-selectin), thereby weakening the interaction of blood cells with these molecules^{11,12}. At the site of inflammation, various proteases and heparinase induced by inflammatory factors can cause detachment and degradation of the glycocalyx^{11,13}. After the glycocalyx falls off and degrades, the adhesion molecules on the vascular wall are exposed, allowing immune cells or cytokines to adhere to the vascular wall of the inflammatory site and achieving a high degree of targeting to the inflammatory tissue^{11,14}. This natural targeting mechanism in the body provides us with clues to explore the targeting of inflammation. MPO belongs to the peroxidase subfamily and is highly expressed in immune cells, like neutrophils, monocytes, and some other cell types¹⁵. In inflammatory conditions, activated neutrophils, monocytes, and certain tissue resident macrophages release MPO into the blood circulation. Increased MPO levels have been detected in the plasma of patients with various acute and chronic inflammatory conditions^{16,17}. Interestingly, MPO has a natural targeting property that is enriched at sites of inflammation. MPO can specifically bind to the phosphatidylserine domain (PS) exposed on the surface of damaged endothelial cells or immune cells¹⁸. Moreover, MPO can adhere to the surface of endothelial cells and transport through the blood vessel wall¹⁹.

Inspired by MPO targeting inflammatory sites, we have pioneered a strategy for the efficient treatment of inflammatory diseases with MPO as a hitchhiker to transport MNMs with potent antioxidant properties to the inflammatory sites. In our previous work, we developed a new method for preparing MNMs and confirmed that the artificially prepared MNMs have a broad-spectrum ROS scavenging effect similar to human eumelanin²⁰. Here, we revealed for the first time that MNMs can be highly targeted to inflammatory sites through MPO-mediated inflammatory pathways with HPS as an example. HPS is a chronic inflammatory complication of the lungs secondary to severe liver inflammation²¹. HPS occurs in 5–32% of cirrhotic patients awaiting liver transplantation and in 3–20% of children with cirrhosis^{22,23}. HPS significantly increases the mortality rate of patients, reaching 41–63% 2.5 years after HPS diagnosis²⁴. Although HPS is identified as a highly inflammatory disease, anti-inflammatory drugs are ineffective for HPS due to the lack of liver- and lung-specific targeting, and liver transplantation is currently the only effective treatment option²⁵. In this study, we found that MNMs had low binding energy to MPO (as low as -4.91 Kcal/mol), which means strong binding force between MPO and MNMs. After intravenous injection, MNMs combined with high levels of MPO in the blood and were specifically enriched in highly inflammatory lung and liver tissues of HPS rats. MNMs had demonstrated a strong therapeutic effect on HPS and can significantly reduce the mortality of HPS rats. The therapeutic mechanism of MNMs had been systematically revealed in HPS. In the lungs, MNMs could reduce macrophage accumulation and the secretion of NO, TNF- α , TGF- β 1, and VEGFA by scavenging ROS, subsequently inhibiting vasodilation, reducing angiogenesis and decreasing fibrosis. In the livers, MNMs entered the intrahepatic cholangiocytes and stellate cells (HSCs) under the mediation of MPO. MNMs inhibited the activation of HSCs to reduce liver fibrosis, and suppressed cholangiocytes proliferation to inhibit the secretion of endothelin-1 (ET-1) into the blood. This study provides a feasible targeting strategy with broad application prospects in many other inflammatory diseases because almost all inflammatory diseases can cause an increase in MPO in the blood and inflammatory tissues. More

importantly, this study explored a new strategy for HPS treatment from the perspective of oxidative stress with nanomedicines, and demonstrating the effectiveness and safety of MNMs in the treatment of serious and complex chronic inflammatory HPS.

2. Results

2.1 MNMs target inflammatory tissues via MPO

The MNMs were synthesized as our previously established method and subsequently characterized by X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS) (**Figure S1**). The size of MNMs was about 100nm with transmission electron microscope (TEM) (**Figure 1A**), and the zeta potential measurement further confirmed the presence of a highly negative charge on the surface of MNMs (**Figure 1B**). The binding energy between MNMs and the glycocalyx, and between MNMs and serum albumin only were -0.32 Kcal/mol and 0.50 Kcal/mol respectively with Autodock 4.2 and MGL tools (**Figure 1C and S2**). In contrast, the binding energy with MPO could be as low as -4.91 Kcal/mol, which was 15 times lower than that of the glycocalyx, indicated the binding force was strong between MNMs and MPO, but were negligible with glycocalyx or serum albumin. Their relatively low affinity (glycocalyx and serum albumin) with MNMs can be attributed to the strong negative charge in glycocalyx (rich in sulfonic acid groups) or serum albumin, which leads to mutual repulsion with negatively charged MNMs. Specifically, MNMs contained abundant phenolic hydroxyl groups, which formed hydrogen bonds with the lysine(D308) and leucine(D485) in the MPO. Subsequently, the MPO, glycocalyx and rat serum albumin were coated onto a plate *in vitro*, and submerged in a solution containing FITC-labeled MNMs (MNMs-FITC) to investigate the interaction with MPO, serum albumin and glycocalyx. As shown in **Figure 1D**, very small amount of MNMs were adsorbed on the serum albumin and glycocalyx plate, while a large amount of MNMs were adsorbed on MPO plate, whose fluorescence intensity was nearly 30 times that of the serum albumin and glycocalyx plate. The above results fully demonstrated that MNM can specifically bind to MPO in the circulatory system.

To validate the specific targeting ability of MNMs towards inflammatory tissues *in vivo*, a rat model of HPS were further conducted through common bile duct ligation (CBDL) (**Figure 1E**), which induced concurrent chronic inflammation in the lungs and livers^{26,27}. We initially administered MNMs-FITC via the rats' tail veins in both the CBDL and Sham groups, and then collected the lung, liver, heart, spleen, kidney, and intestine tissues of rats in both groups after 24 hours. The fluorescence intensity of MNMs-FITC in the lung and liver tissues of the CBDL group was found to be significantly higher than the Sham group through the fluorescence stereomicroscope (**Figure 1F**), exhibiting a 6.4-fold increase in the lungs and a 7.1-fold increase in the livers. The heart, spleen, kidney, and intestinal tissues showed low levels of fluorescence intensity without significant differences between the groups (**Figure S3**). We also observed that the fluorescence in the lung and liver tissue sections of the CBDL rats was stronger than the Sham group (**Figure S4**). Furthermore, we directly observed the accumulation of MNMs in pulmonary macrophages and hepatic cholangiocytes in CBDL rats through TEM (**Figure S5**), thereby confirming the successful delivery of MNMs to the lungs and livers of CBDL rats. Given that the MPO levels in the blood,

livers, and lungs of CBDL rats were 8.91, 4.65, and 6.26 times higher than those in Sham group rats (**Figure S6**), respectively, it was further investigated whether MPO mediated the delivery of MNMs to the inflammatory livers and lungs. There was a significant co-localization of MPO and MNMs through immunofluorescence (IF) (**Figure 1G**). Additionally, we observed a significant co-localization of MPO with MNMs in LPS-activated bone marrow derived macrophage (BMDM) with fluorescence confocal microscopy (Pearson's $R=0.981$, **Figure S7**). We further validated the targeting mechanism of MNMs by reducing MPO levels in CBDL rats through *in vivo* siRNA. The levels of MPO in plasma, lung, and liver tissues were reduced to 14.16%, 20.57%, and 23.67% of the CBDL group by *in vivo* siRNA^{MPO} in CBDL rats, respectively (**Figure S8**). Consequently, the MNMs in lung and liver tissues of the siRNA^{MPO} group was reduced to 20.57% and 21.25% of CBDL group, respectively (**Figure 1 H-I**). Interestingly, after intravenous re-administration of the MPO recombinant protein (the siRNA^{MPO}+MPO group, **Figure S8**), the fluorescence intensities of MNMs accumulated in the lungs and livers of CBDL group rats effectively recovered to 76.47% and 86.78% of the CBDL group (**Figure 1 H-I**). The residing duration time of MNMs was further investigated in inflammatory cells and tissues. The uptake and excretion of MNMs were assessed by activated BMDMs. As shown in **Figure S9**, MNMs were efficiently taken up by activated BMDMs and persisted for up to 96 hours. In CBDL rats, the accumulation of MNMs in the livers and lungs peaked at 48 hours and persisted for 120 hours (**Figure S10**). As the above results, MNMs targeting the inflammatory lung and liver tissues of CBDL rats mediated by MPO. Moreover, MNMs could remain in these inflammatory tissues for up to 5 days, providing a prerequisite basis to exert the antioxidant stress effects.

2.2 MNMs enter cells by mediation of MPO, and reduce oxidative stress by maintaining mitochondrial homeostasis.

MNMs could potentially eliminate multiple ROS (**Figure S11**), and were further investigated to eliminate oxidative stress in CBDL rats (**Figure 2A**). Excitingly, we found for the first time that MPO can mediate the entry of MNMs into activated BMDMs. After being activated by H₂O₂ or LPS, the BMDMs significantly increased the secretion level of MPO (**Figure S12A**), and could significantly enhance the uptake of MNMs (**Figure S12B, C**). We further suppressed the phagocytosis of unactivated BMDMs with a highly effective phagocytosis inhibitor (Pitstop2), and then treated with MPO recombinant protein at a concentration gradient ranging from 30-180nM. After Pitstop2 treatment, unactivated BMDMs couldn't take up MNMs, but BMDMs could promote the uptake of MNMs when the concentration of MPO was increased (**Figure 2B**). Furthermore, when activated BMDMs were treated with siRNA^{MPO} (**Figure 2C**), the uptake of MNMs by BMDMs was significantly reduced, and the addition of MPO could restore the ability of activated BMDMs to take up MNMs. The above evidences fully prove that MPO facilitated the phagocytosis-independent internalization of MNMs into macrophages.

The anti-oxidative stress effects of MNMs were further investigated in CBDL rats. The optimal therapeutic dose of MNMs was screened first (**Figure S13**). Subsequently, the CBDL rats were treated with MNMs intravenously (with 2.5 mg/kg as the therapeutic dose), and the CBDL rats treated with the

same dose of N-acetylcysteine (NAC), rats in CBDL and Sham group served as controls. A significant increase in ROS levels was observed in lung and liver tissues in the CBDL and NAC groups, compared to the Sham and MNMs groups (**Figure 2D**). Since the malondialdehyde (MDA) can reflect mitochondrial damage to a certain extent, we further detected MDA in lung and liver tissues. MDA levels of MNMs group's lung and liver tissues decreased to 76.78% and 75.65% of CBDL group, respectively, while there was no significant difference between NAC and CBDL group (**Figure S14**). Through TEM, the mitochondrial membrane structure and cristae structure of macrophages in lung and liver tissues of MNMs were intact compared with those in CBDL and NAC groups, revealing that MNMs could significantly reduce mitochondrial damage in lung (**Figure 2E**) and liver tissues (**Figure 2F**). *In vitro*, through co-labeling with TOM20 and MNMs-FITC, the red mitochondrial fluorescence had a high degree of overlap with the green fluorescence carried by MNMs-FITC (Pearson's $R = 0.810$) (**Figure 2G**). The level of ROS increased significantly in BMDMs after the intervention of H_2O_2 , and MNMs had a strong scavenging effect on ROS, which was significantly better than NAC (**Figure S15**). Further, venous blood plasma from 4 groups of rat models were extracted to interfere with BMDMs. The mitochondrial ROS (mtROS) level of BMDMs in the CBDL group was 14.30-fold of the Sham group with MitoSox as probe, while in the MNMs group greatly decreased to 20.24% of CBDL group (**Figure S16**). MNMs effectively scavenged ROS with DCFDA as probe (**Figure 2H and S17A**), and enhanced mitochondrial membrane potential with JC-1 as probe (**Figure 2I and S17B**). The cytoplasmic expression of mtDloop (**Figure 2J**), mtCytb (**Figure 2K**) and mtND4 (**Figure 2L**) in MNMs group decreased significantly to 35.17%, 28.81% and 20.24% of CBDL group by qRT-PCR, which proved that MNMs could maintain mitochondrial homeostasis and prevent mitochondrial DNA leakage by scavenging mtROS. The mechanism of MNMs was also investigated by RNA-Seq analysis (**Figure S18A-C**). Gene Ontology (GO) analysis of the differential genes in lung tissues of CBDL group and MNMs group suggested that the MNMs may play a positive role in the regulation of membrane potential, which might contribute to maintain mitochondrial homeostasis (**Figure S18D-E**, $P < 0.001$). According to the above results, we confirmed *in vivo* and *in vitro* that MNMs can maintain mitochondrial homeostasis by scavenging mtROS, and play a protective role in inflammatory tissue of CBDL rats.

2.3 MNMs effectively inhibit macrophage accumulation in the lungs of CBDL rats

Oxidative stress and inflammation are closely related in inflammatory diseases to form a vicious cycle, which are widely recognized as core pathologies in inflammatory diseases²⁸. MNMs were furtherly investigated to inhibit inflammation in the lungs of CBDL rats (**Figure 3A**). The results of GO (**Figure S17D, Table S4**) and KEGG analysis (**Figure S19, Table S5**) revealed that the MNMs group exhibited significant down-regulation of inflammatory signaling pathways associated with cytokine receptor action, TNF, leukocyte migration, and chemotaxis in comparison to CBDL group, implicated a potent anti-inflammatory efficacy of MNMs. The macrophages accumulating in lungs could actively contribute to the secretion of various inflammation-related factors. Notably, the pro-inflammatory factor TNF- α released by M1 macrophages promotes pulmonary angiogenesis and exacerbates pulmonary shunting, while the anti-inflammatory factor TGF- β 1 secreted by M2 macrophages facilitates pulmonary fibrosis. In

CBDL group, the expressions of iNOS (M1 marker), CD206(M2 marker), TGF- β 1 and TNF- α in the lung tissues increased by 3.33-, 4.27-, 6.57- and 4.09-fold respectively compared to the Sham group, and the expressions of classical inflammatory effectors COX-2 (inflammation mediator) and ZO-1 (tight junction related proteins which indicates the integrity of endothelial barrier) were increased by 2.89-fold and 4.46-fold respectively, which were similar to the NAC group. After MNMs treatment, iNOS, CD206, TGF- β 1, TNF- α , COX-2 and ZO-1 were all significantly reduced (**Figure 3B**). Through FCM, a significant increase was found in the proportion of CD11b- and CD68-marked macrophages in the lung lavage fluid of CBDL rats, reaching 34.7%. Conversely, the MNMs group reduced to 15.3%, while the NAC group was 23.5% (**Figure 3C**). IF staining revealed a significant increase in the number of CD68-labeled macrophages, iNOS-labeled M1 macrophages, and CD206-labeled M2 macrophages in lung of CBDL rats. After MNMs treatment, there was a significantly lower presence of macrophages (8.80%), M1 macrophages (3.59%), and M2 macrophages (17.98%) in lungs compared to the CBDL group (**Figure 3D**). Moreover, MNMs significantly reduced the levels of the pro-inflammatory factors (IL-1 β , IL-6 and TNF- α) and increased the anti-inflammatory factor (IL-10) in the plasma by ELISA (**Figure S20**). *In vitro*, MNMs could also significantly reduce the inflammatory factors released by LPS-activated BMDMs. After treatment with MNMs, the expression levels of iNOS, IL-1 β , and TNF- α were significantly reduced (**Figure S21**).

Through transcriptome analysis, it was found that MNM treatment resulted in a significant reduction in chemokines and VEGFA (**Figure 3E**). Further, the mRNA expression levels of CCL20, CXCL1, and CXCL10 were upregulated in the CBDL group, and these chemokines significantly reduced to 27.75%, 33.01%, and 5.19% of the CBDL group after treatment with MNMs. VEGFA also played an important role in the accumulation of macrophages²⁹⁻³¹, and the RNA-Seq results further revealed a 12-fold increase of VEGFA mRNA levels in the lungs of CBDL group compared to the Sham group, and that administration of MNMs greatly reduced VEGFA expression to 60.53% of the CBDL group. At the protein level, a significant upregulation of CCL20, CXCL1, CXCL10, and VEGFA levels were observed in the CBDL group, and treatment with MNMs resulted in a notable reduction of these chemokines and VEGFA (**Figure 3F**). *In vitro*, the expression levels of CCL20, CXCL1, and CXCL10 in the THP-1 macrophages treated with MNMs were significantly reduced to 7.10%, 8.77%, and 13.48% of the H₂O₂ group through a Proteome Profiler Chemokine Array Kit (**Figure 3G, H**). Thus, both *in vivo* and *in vitro* results suggest that MNMs reduced the accumulation of lung macrophages by inhibiting chemokines CCL20, CXCL1, and CXCL10. These chemokines and VEGFA were further adopted to individually treat with LPS-activated THP-1 macrophage by the transwell migration assay. As shown in **Figure S22**, MNMs significantly reduced THP-1 migration induced by these factors. The above results demonstrate that MNMs can effectively reduce the macrophages accumulation in lung not only by inhibiting the production of these cytokines, but also by inhibiting the macrophage migration behavior induced by these factors.

2.4 MNMs inhibit pulmonary vascular dilation and angiogenesis in CBDL rats

Pulmonary vascular dilation and angiogenesis can cause pulmonary vascular shunt and ventilation-blood flow ratio imbalance, which is the direct cause of HPS hypoxemia²². With the GO analysis of RNA-Seq results, the regulation of blood vessel size (GO:0050880) and angiogenesis (GO:0001525) in the

MNMs group showed significant down-regulation compared with the CBDL group (**Figure S18D, S23A-B and Table S4**, $P < 0.001$). The KEGG analysis also revealed significant enrichment in signaling pathways such as focal adhesion, calcium, Hippo, and PI3K-AKT, which were closely associated with angiogenesis and vascular smooth muscle contraction (**Figure S23C, Table S6**), suggesting that MNMs might inhibit vasodilation and angiogenesis in the lungs of CBDL rats. Through IF, CD34 and eNOS in the lung tissues of the CBDL group were significantly higher than those of the Sham group, indicating that angiogenesis was widely present in CBDL lungs. The diameter of blood vessels in the lung tissues of the CBDL group was also much larger than that of the Sham group. Significantly, the number of small blood vessels in the MNMs group was reduced to only 42.86% of that in the CBDL group ($P = 0.010$). In addition, MNMs effectively attenuated vasodilation in the CBDL group (39.67 ± 3.52 vs $14.67 \pm 2.16\mu\text{m}$, $P < 0.001$). However, there was no change in either angiogenesis or vasodilation in the NAC group compared with the CBDL group (**Figure 4A**).

The effects of MNMs on HPS pulmonary vascular were further explored. The main vasodilatory substance is nitric oxide (NO) in the lungs of HPS. As shown in the **Figure 4B**, MNMs treatment could restore the high NO level of the CBDL group to normal. NO were mainly derived from eNOS activated by phosphorylation in endothelial cells and iNOS in macrophages in the lungs of HPS. By WB, the p-eNOS, eNOS (**Figure 4C**) and iNOS (**Figure 3B**) in the lung tissues of the CBDL group were significantly higher than those of the Sham group, respectively. Similarly, IHC analysis showed that p-eNOS and iNOS in lung tissues of the CBDL group were significantly higher than those of the Sham group (**Figure S24**). Interestingly, MNMs strongly reduced the levels of p-eNOS and iNOS, whereas NAC had a negligible effect. In HPS, vasodilation in lung tissue is mainly caused by the increase of p-eNOS in endothelial cells induced by angiogenic factors such as VEGFA and Ang-2. As shown in **Figure 3F** and **Figure 4C**, MNMs treatment could significantly reduce the protein levels of VEGFA and Ang-2 in CBDL lung tissues. *In vitro*, we initially used the plasma from rats of 4 groups to treat pulmonary microvascular endothelial cells (PMVECs) and BMDMs to validate the anti-vasodilator effect of MNMs. MNMs significantly reduced the NO level (**Figure 4D-E**) and the expression of iNOS in BMDMs (**Figure 4F**) and p-eNOS in PMVECs (**Figure 4G**) induced by CBDL plasma. The endothelial tube-forming ability of PMVECs was significantly enhanced after CBDL group plasma intervention (**Figure 4H**), and was further enhanced when PMVECs were co-cultured with BMDMs (**Figure 4I**) because activated BMDMs promoted endothelial cell tube formation by secreting VEGFA and Ang-2^{32,33}. As expected, MNMs significantly reduced the tube-forming ability of PMVECs treated with CBDL plasma and CBDL plasma + macrophages (**Figure 4J-K**). The above findings demonstrated that MNMs could exert a significant role in suppressing vascular dilation and angiogenesis by inhibiting the synthesis of vasodilator and angiogenic factors in lungs of HPS rats (**Figure 4G**).

2.5 MNMs inhibit liver inflammation, the ET-1 secretion and fibrosis progression in the HPS livers

Liver inflammatory disease is the cause of HPS, and pulmonary symptoms are improved after liver transplantation in HPS patients²². In view of the high targeting of MNMs to inflammatory livers, we further explored the treatment mechanism of MNMs in liver tissues of CBDL rats. GO analysis of RNA-

Seq data showed that the potential functions of MNMs in the liver including regulation of cytokine production, regulation of cell killing, mesenchyme development, extracellular structure organization, and regulation of epithelial cell proliferation (**Figure 5A**), suggesting that the therapeutic mechanism of MNMs is to reduce liver inflammation, inhibit fibrosis, reduce cholangiocytes proliferation and ET-1 secretion in the liver.

First, we investigated the anti-inflammatory effects of MNMs on HPS livers. The liver tissues of the CBDL, Sham, MNMs, and NAC group were digested for FCM examination. As shown in **Figure 5B**, the proportion of inflammatory macrophages in the CBDL group was very high (25.0%), while the proportion in the MNMs group was significantly reduced to only 4.01%. Moreover, MNMs could significantly reduce the levels of inflammatory cytokines in liver tissues of CBDL rats (**Figure S25**). NAC treatment had a weak effect in reducing the inflammatory macrophage ratio and level of inflammatory factors. In HPS, increased ET-1 levels in the circulation caused by cholangiocytes proliferation are a key factor causing pulmonary inflammation and functional damage³⁴. MNMs effectively reduced the secretion of ET-1 into the circulation (5.07 ± 0.83 vs 3.14 ± 0.78 , $P=0.002$, **Figure 5C**). Through IHC (**Figure 5D**) and WB (**Figure 5E**), the expression levels of CK19 (cholangiocyte markers), PCNA (proliferation marker), ET-1, and Apelin were significantly upregulated in the livers of CBDL rats and predominantly localized within bile duct regions. MNMs could significantly reduce the levels of these proteins, and the effect was much better than NAC. In HPS, bile duct proliferation and liver fibrosis promote each other^{35,36}. As expected, MNMs also significantly reduced the levels of α -SMA (activated hepatic stellate cells marker) in liver tissues as shown by IHC (**Figure 5D**) and WB (**Figure 5F**), indicating that MNMs could effectively inhibit HPS liver fibrosis.

The mechanism by which MNMs inhibit bile duct proliferation and liver fibrosis was further investigated. The apelin/NOX4/ROS axis is a key pathway leading to HSCs activation and cholangiocytes proliferation³⁷. Through IHC (**Figure 5D**) and WB (**Figure 5E**), MNMs treatment significantly reduced the level of apelin in CBDL liver tissue. Apelin/NOX4 stimulation resulted in an elevation of ROS in cholangiocytes (**Figure 5G**) and HSCs (**Figure S26**). Treatment with GLX351322 (a selective NOX4 inhibitor) effectively reduced ROS levels induced by apelin by 52.68% in cholangiocytes, while showing no efficacy against exogenously added H₂O₂. Interestingly, when MNMs were introduced to either apelin- or H₂O₂-induced cholangiocytes, a remarkable reduction of ROS by 92.07% and 96.54% respectively was observed, surpassing the scavenging effect achieved by GLX35132 and NAC (**Figure 5G-H**, **Figure S26A-B**). Furthermore, MNMs effectively decreased the expression levels of PCNA (22.56%, 20.02%), and ET-1 (6.74%, 5.52%) in comparison to apelin- and H₂O₂-induced cholangiocytes (**Figure 5I**) by WB, confirming that MNMs could inhibit cholangiocytes proliferation and ET-1 synthesis through the apelin/NOX4/ROS signaling pathway axis. Similarly, MNMs reduced the expression levels of COL1A, α -SMA, PCNA, TGF- β 1, and RHOA in HSCs (**Figure S26C**), indicated their inhibitory effects on HSCs activation and fibrosis induction. These findings validated that MNMs could safeguard liver function in CBDL rats by suppressing liver inflammation, fibrosis progression as well as cholangiocytes proliferation while reducing ET-1 expression and secretion from a hepatic perspective (**Figure 5J**).

2.6 MNMs inhibit HPS progression in CBDL rat models and safety verification

We validated the therapeutic effects of MNMs on HPS, as shown in **Figure 6A**. Five weeks after CBDL, the survival rate of rats in the MNMs group was 72.22%, significantly higher than that of the CBDL group (33.33%) and the NAC group (44.34%), suggesting that MNMs significantly prolonged the survival time of CBDL rats (**Figure 6B**). Consequently, the weight of rats in the MNMs group was significantly heavier than that of the CBDL group ($P < 0.0001$), while the survival time and weight of rats in the NAC group were not significantly different from the CBDL group (**Figure 6C**). From the morphological point of view, the lung tissues of the CBDL and NAC groups exhibited increased firmness, a yellowish hue, and multiple hemorrhagic spots. In contrast, the lung tissues of the MNMs and Sham groups were comparatively softer with no evident hemorrhagic spots (**Figure 6D**). Livers in CBDL group and NAC group were apparently cirrhotic, hard, yellow and nodular hyperplasia, while the livers of MNMs group were softer than CBDL and NAC groups, and nodular hyperplasia was less obvious (**Figure 6E**). Further through HE staining of lung and liver tissues, compared with CBDL and NAC groups, the corresponding interstitial inflammatory exudation, emphysema and atelectasis of lung tissues in MNMs group were significantly decreased (**Figure 6F**), and the hepatic fibrosis areas and bile duct proliferation were significantly reduced (**Figure 6G**). Notably, the lungs of both the CBDL and NAC groups exhibited significant fibrosis and collagen hyperplasia by Masson staining (**Figure 6H-I**) and Sirius scarlet staining (**Figure 6J-K**). The lungs of MNMs group showed a significant reduction to only 45.62% of the fibrotic areas in the CBDL group (**Figure 6L**). In terms of liver tissues, both the CBDL and NAC groups exhibited widespread distribution of fibrotic areas, resulting in disruption of hepatic lobular structure (**Figure 6M**). Conversely, in the MNMs group, these fibrotic areas were reduced to only 69.77% compared to the CBDL group and were primarily confined to portal areas. According to the above results, we demonstrated that MNMs effectively alleviated the progression of HPS in CBDL rats, inhibited pulmonary and liver fibrosis, and improved pulmonary ventilation, and the therapeutic effect was significantly better than the traditional ROS scavenger NAC.

The pulmonary and liver function was further measured at the 4 groups of rats. Arterial oxygen saturation (SpO_2) is the gold standard for measuring lung function. Normal SpO_2 is 97-100%, and SpO_2 below 90% is considered hypoxemia. As shown in **Figure 6N**, the SpO_2 of CBDL rats was only 88.67%, while MNMs were able to restore SpO_2 to 92.17%, indicating that MNMs effectively alleviated HPS-induced hypoxemia. In addition, MNMs could also effectively restore other indicators reflecting lung function, such as alveolar-arterial oxygen pressure difference (AaPO_2 , **Figure 6O**), arterial carbon dioxide pressure (PCO_2 , **Figure 6P**) and arterial oxygen pressure (PO_2 , **Figure 6Q**), and its effect was much better than NAC. We further evaluated the effect of MNMs treatment on liver function in CBDL rats. As shown in **Figure 6 R-S**, MNMs could effectively reduce the levels of alanine transaminase (ALT) (**Figure 6R**), aspartate transaminase (AST) (**Figure 6S**) and total bilirubin (**Figure S27**) in the serum, indicating that MNMs could restore liver function to a certain extent, and its effect was far better than NAC.

Finally, we tested the safety of MNMs by tail intravenous injection into rats. After 7 days of administration, lung, liver, heart, spleen, kidney and intestine sections were collected from rats for HE

staining. Compared with the control group, no damage to these vital organs caused by the two doses of MNMs was observed (**Figure S28A**). Inflammatory cytokines IL-1 β , IL-6, TNF- α , and anti-inflammatory cytokine IL-10 in plasma were measured to assess inflammation response, and neither of the two doses of MNMs resulted in an elevation of inflammatory cytokines compared to the control group (**Figure S28B**). These results confirmed that MNMs demonstrated an excellent safety profile in rats.

3. Discussion

Chronic inflammation has a high incidence and imposes a huge medical burden on the world. Drug development for chronic inflammation is a hot area of medical research. However, chronic inflammation has complex pathogenic mechanisms and complications, and traditional drugs do not target inflammatory lesions, resulting in serious off-target effects and side effects³⁸. Our study elucidated for the first time the exact mechanism by which MNMs targeted inflammatory lesions *in vivo*, and confirmed that MPO mediated MNMs enter into inflammatory tissues. MNMs are mainly composed of endogenous antioxidant eumelanin in the human body, which effectively reduces inflammation by efficiently eliminating ROS to break the vicious cycle of oxidative stress and inflammation. This highly targeted therapy and high biocompatibility of MNMs ensure excellent therapeutic effects while avoiding side effects.

We first confirmed the strong binding force of MNMs and MPO, and then verified the inflammatory targeting of MNMs mediated by MPO *in vivo* and *in vitro*. Upregulation of MPO expression is a common manifestation of multiple inflammatory diseases, therefore MPO-mediated targeting can also be applied to other inflammatory disease. Moreover, the expression level of MPO may be positively correlated with the severity of inflammation³⁹, and the delivery capacity of MPO may be enhanced dynamically as the severity of inflammation. The previous approach to drug development typically focused on a single disease. However, the shared characteristics of MPO in various inflammatory diseases and the understanding mechanism of MPO-mediated MNMs targeting inflammation offer an opportunity to explore treatments for a range of chronic inflammatory conditions. This strategy holds significant value and presents promising application prospects. Additionally, the composition of MNMs can facilitate the loading of other drugs, potentially enhancing MPO-mediated targeting and improving efficacy.

The inflammatory targeting properties of MNMs are derived from its unique physicochemical properties. Although some studies have suggested that positively charged nanoparticles preferred to targeting in normal livers and lungs, they also have obvious cytotoxicity, which may aggravate the inflammatory response of tissue⁴⁰. The MNMs, a negatively charged nanoparticle, exhibited significantly enhanced safety and biocompatibility compared to their positively charged counterparts. Moreover, they demonstrated prolonged retention in the circulation and tissues owing to their repulsion to the negatively charged glycocalyx of endothelial cells. Inflammation in the lungs and livers of CBDL rats lead to the disconnection of endothelial cells in the blood vessel wall and the releasement of inflammatory factors, resulting in enhanced vascular permeability⁴¹. Glycocalyx shed and degrade in response to inflammatory factors and related enzymes, exposing P-selectin and MPO binding sites, making MNMs easily pass

through the blood vessel walls of the lungs and livers and accumulate in the organs under the mediation of MPO. In general, the clearance of nanoparticles *in vivo* is mainly dependent on cytophagocytosis in the livers, and extending the half-life of nanomaterials *in vivo* is more conducive to the accumulation of drugs in target organs⁴². MNMs is exhausted in lung and liver tissues 7 days after injection, giving it sufficient time to perform function *in vivo*.

As a serious chronic inflammation, the pathogenesis of HPS is complex. It is generally believed that the pulmonary vasodilation and angiogenesis are the characteristic pathological changes, which are the direct cause of the decreasing of blood oxygen saturation^{43,44}, while the aggregation of mononuclear-macrophages in the lung is its core pathological change^{45,46}. Main causes of HPS including increased circulation ET-1 from cholangiocyte and selective activation of ETB receptors in the lung^{47,48}, portal hypertension-induced loss of BMP-9 signaling contributes to pulmonary vascular damage⁴⁹, macrophages accumulation in lung and activation of inflammatory factors such as TNF- α ⁵⁰, the high expression of eNOS and iNOS increasing the vasodilator NO, etc⁵¹. A number of previous studies have explored medical treatment regimens for HPS, such as inhibition of TNF- α synthesis with pentoxifylline⁵², blocking the vasodilatation function of NO by Fingolimod⁵³, or inhibition of the pro-angiogenesis pathway with sorafenib^{54,55}. However, these treatments are still limited in their effectiveness, and liver transplantation is still the only treatment strategy for HPS. The predicament may be due to the complexity of HPS occurrence and development mechanism. It's difficult to achieve satisfactory therapeutic effect with drugs targeting one single pathological link or single inflammatory organ, as the effective dose is large or the side effects are unbearable, and lack of targeting.

In this study, we confirmed that MNMs achieved good efficacy in CBDL rat models, and showed several advantages over previous drug exploration strategies: 1. MNMs can act on several key pathological links in the progression of HPS by scavenging ROS, including maintaining mitochondrial homeostasis, inhibiting the accumulation of macrophages in the lung, and inhibiting angiogenesis and vasodilation. More importantly, MNMs can reduce the degree of fibrosis and inflammation of the liver, and reduce the secretion of ET-1, the initiating factor of HPS pathological changes. To alleviate symptoms in lung, it is worth noting that the multiple functions of MNMs in the rat model of CBDL render it a unique therapeutic effect, which is superior to previously studied drugs in terms of mechanism of function. 2. The distribution of MNMs has a unique targeting property in the HPS. This drug has high-affinity for inflammatory tissues and cells, which is the basis of its function, and can also ensure that other tissues and organs are not affected. 3. MNMs has good biocompatibility. Even if injected into the body in large doses, it still has no significant toxic and side effects on the organs. The above characters make MNMs have unique advantages and prospects.

4. Conclusion

Our research has confirmed that MPO mediates MNMs to specifically target inflammatory tissues. MNMs can specifically target the lung and liver tissues with chronic inflammation, maintain

mitochondrial homeostasis by scavenging ROS, inhibit pulmonary macrophage accumulation, reduce pulmonary angiogenesis and vasodilation, alleviate liver fibrosis and secretion of ET-1, and thus effectively inhibit the progression of HPS. As an inflammatory targeting agent, MPO has potential applications in other chronic inflammation. In addition, MNMs have significant application prospects as a new HPS drug with targeting ability.

5. Materials and Methods

5.1 Materials

The primary antibodies are shown in **Table S1** and the reagents are shown in **Table S2**.

5.2 Characterization test of MNMs

MNMs TEM images were captured by TECNAI G2 high-resolution transmission electron microscopy. The valence states of MNMs were analyzed by X-ray photoelectron spectroscopy (XPS) instrument (VG ESCALAB MK II). XRD measurements were made using the Bruker D8 Discover X-ray diffraction system.

5.3 ROS scavenging experiment

The ability of MNMs to remove $O_2^{\bullet-}$ was detected by NBT method. Different concentrations of MNMs (0, 1, 2, 4, 8, and 16 $\mu\text{g/mL}$) were mixed with methionine (50 μM), riboflavin (2 μM), NBT (1 mM), PBS (100 mM, pH 7.4), and deionized water, and irradiated with ultraviolet light for 5 min. $O_2^{\bullet-}$ combines with NBT to form blue methylhydrazine, with maximum absorption at 560 nm. The ability of MNMs to remove $O_2^{\bullet-}$ was determined by inhibiting the photochemical reduction of NBT by MNMs.

The $OH^{\bullet}$ removal efficiency was determined by calculating the inhibition effect of MNMs on TMB oxidation. TMB (400 μL , 10 mM), FeSO_4 (40 μL , 50 mM), H_2O_2 (20 μL , 100 mM) and MNMs (final concentration 0, 0.2, 0.4, 0.8, 1.6 and 3.2 $\mu\text{g/mL}$, respectively) were added into PBS (0.01 mol/L, pH = 7.4) solution with a final volume of 3 mL, and the absorbance change at 652 nm was measured after 3 min of light shielding reaction.

The scavenging ability of MNMs to ONOO^- was detected by ultraviolet/visible spectroscopy using pyrogallol red as indicator. MNMs (20 $\mu\text{g/mL}$) was mixed with ONOO^- (777.6 μM) in different volumes (0, 62.5, 125, 250, 500, and 1000 μL) at a final volume of 3 mL for 15 min, then ONOO^- clearance was determined by detecting UV absorption.

The scavenging activity of MNMs on H_2O_2 was determined by ultraviolet/visible spectrophotometry. MNMs (0, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 μM) was mixed with 0.4 mM H_2O_2 , incubated in the dark for 12 h, ultraviolet absorbance of 425 nm was determined, and the clearance rate of H_2O_2 was calculated.

5.4 Animal experiment

As previously mentioned, we successfully established an experimental HPS rat model using CBDL in male Sprague-Dawley rats. 72 rats were randomly divided into 4 groups. Under isoflurane inhalation anesthesia, CBDL group, MNMs group and NAC group underwent CBDL, and the control group (Sham group) underwent common bile duct exposure without ligation. From the 7th day after CBDL, the rats in MNMs and NAC groups were injected with saline, 2.5mg/kg MNMs and 2.5mg/kg N-acetylcysteine (NAC) via tail vein once every 7 days for a total of 4 weeks. Sham group rats were injected with drug-free saline. The weight of the rats was measured weekly, and the rats were sacrificed 5 weeks after surgery. All rats were raised in standard laboratory living conditions (22–24°C, 12H light /12H dark cycle) and fed standard laboratory feed.

The other 12 rats were divided into Sham group and CBDL group, and 2.5mg/kg MNMs -FITC was injected into the tail vein 4 weeks after the operation of CBDL, and samples were collected after the tail vein injection at 12H, 24H, 48H, 72H, 120H, 168H and 240H, respectively. The fluorescence intensity of liver and lung tissues was measured by Leica Fluorescence stereomicroscope (Leica M165 FC). For determine the proper therapy concentration of MNMs, other 15 SD rats divided into 5 groups, and the Sham group as the negative control did not perform CBDL, CBDL group as the positive control group, other 3 groups were performed tail vein injection with MNMs at concentration of 1.25mg/kg, 2.5mg/kg and 5.0mg/kg respectively, once every 7 days for a total of 4 weeks. The plasma was obtained for TNF- α and ET-1 examination 5weeks after CBDL. All procedures were performed on the animals according to National Institutes of Health guidelines. All experimental protocols were approved by the Ethics Committee of Xiangya Hospital, Central South University (approval number:201808213).

5.5 Cell experiments

In this study, rat microvascular endothelial cells PMVEC, and human mononuclear macrophage THP-1 cells were purchased from CellCook. Rat BMDM cells were extracted from rat bone marrow tissue, human primary cholangiocytes, and human primary stella cells were extracted from liver tissues of benign liver mass patients undergoing partial hepatectomy. In the experiments about BMDM, THP-1, PMVEC, human primary cholangiocytes, DMEM medium (Biological Industries, Kibbutz Beit Haemek, Israel) were used. 10% fetal bovine serum (Biological Industries) and 1% penicillin-streptomycin dual anti-solution (Hyclone, Logan, UT) were added; Human primary stellate cells were treated with DMEM medium + 20% fetal bovine serum + 1% penicillin-streptomycin dual antibiotic solution. Human primary cholangiocytes and human primary stellate cell culture bottles were coated with rat tail collagen. The use of human liver tissue samples was approved by the Ethics Committee of Xiangya Hospital, Central South University (approval number:201703568).

5.6 Tail intravenous injection of *in vivo* siRNA

The 15 SD rats were divided into 5 groups. The Sham group received common bile duct exposure without ligation, and the other 4 groups received CBDL and were injected with PBS, siRNA^{Ctr}, siRNA^{MPO}, and siRNA^{MPO}+MPO recombinant protein through the tail vein, respectively. Lipid-encapsulated siRNA^{MPO} and blank control siRNA were purchased from RioBio corporation. According to the supplier's

instructions, before CBDL operation, 40nM/piece, twice/week, 3 pieces/group, the reagents were injected through the tail vein. The siRNA^{MPO}+MPO group was injected with MPO recombinant protein (50μg/kg, twice/week) through the tail vein at the 4th week after CBDL, and the rats in each group were injected with 2.5mg/kg MNMs-FITC through the tail vein at the 4th week after CBDL. The rats were sacrificed at 48H after injection of MNMs-FITC, and the lung and liver tissues of the rats were detected by Fluorescence stereomicroscope.

5.7 TOM20 staining Assay

TOM20 staining was used to label mitochondrial by IF. Rat BMDM cells were cultured in confocal culture dish with DMEM medium for 24H, LPS and MNMs-FITC were added, and washed with PBS at 4°C for 3 times after 24H, and then the cells were fixed with paraformaldehyde, incubated with TOM20 primary antibody at 37°C for 2 hours. After incubation and washing, Fluor 594 Secondary antibody was added to incubate for 30min and DAPI for 10min, and PBS was washed 3 times. Fluorescence images were captured using a high-resolution confocal microscope (Nikon C1, Japan) with 100-fold objective lens, and fluorescence Colocalization analysis was performed using Image-J Colocalization analysis.

5.8 MitoSOX staining Assay

MitoSOX red staining was used to detect the production of mitochondrial superoxide in BMDM cells. BMDM cells were cultured in 6-well plates for 24H and then added into 200μL/2mL plasma to medium of the 4 groups of rats, respectively. After 48H, it was washed 3 times with ice cold PBS, and then incubated with 1μM of MitoSOX red at 37°C for 30 min. After incubation and washing, DAPI was added and incubated for 10min. After PBS washing for 3 times, fluorescence images were captured using a fluorescence microscope with 20× objective lens (Leica DMI4000, Germany), and fluorescence intensity analysis was performed using Image-J.

5.9 Pathological examination of paraffin section of tissue

The lung, liver, and other organs tissues fixed with paraformaldehyde were embedded in paraffin wax, and the 5μm sections were stained with HE, Masson or Sirius scarlet.

In Masson staining, the paraffin sections were roasted at 65°C for 1H, dewaxed in xylene for 10min×2 times, and then soaked in 100%, 95%, 90%, 75%, 50% alcohol and deionized water in gradient for 5min for hydration. Bouin solution was added and mordant at 37°C for 2H, then rinsed with azure blue solution for 3min, then rinsed with water, Mayer hematoxylin solution stained for 3min, then rinsed with water, soaked in differentiation solution for 3s, rinsed with water for 10min to return to blue, ponceau magenta solution for 3min, then rinsed with water. After soaking in phosphomolybdate solution for 10min, dyeing with aniline blue solution for 5min, then washing, soaking in weak acid solution for 2min. The slices were soaked in deionized water, 50%, 75%, 90%, 95%, 100% alcohol for 5min for dehydration, then soaked in xylene for 10min for transparency, and then sealed with neutral resin. The collagen fibers are blue in color, the cytoplasm, muscle fibers and red blood cells are red, and the nucleus is black. After the images were collected, the proportion of blue collagen fibers was analyzed using ImageJ.

The dewaxing and hydration processes of HE and Sirius scarlet staining are the same as those of Masson staining. After HE stained sections were hydrated, they were dyed with Harris hematoxylin for 5min, then washed with water; differentiated with 1% hydrochloric acid solution for 5s; washed with deionized water for 10min to return to blue; dyed with 0.5% eosin dye for 10min; dehydrated and sealed after washing. In Sirius scarlet staining, after the completion of hydration, the sections were dyed with Sirius scarlet staining solution for 1h, rinsed with running water, soaked in 0.5% acetic acid for 20s, washed with deionized water, soaked in anhydrous ethanol for 1min×3 times, then soaked with xylene for 10min to be transparent, and then sealed with neutral resin. ImageJ was used to analyze the proportion of red fibrotic areas after the images were collected.

5.10 RNA-Seq of lung and liver tissues

Three rats in Sham, CBDL and MNMs groups were randomly selected to extract RNA from 500mg lung tissue and liver tissues. Total RNA was extracted using standard TRIzol protocol, and cDNA library was synthesized through mRNA enrichment. Sequencing was performed on NovaSeq high-throughput sequencing platform (Personalbio Inc.), and quantitative gene analysis and differential gene expression analysis were performed on the resulting data. Further gene function enrichment analysis (GO) and pathway enrichment analysis (KEGG) were performed with differentially expressed genes.

5.11 Detecting ROS with DCFDA staining

The ability of MNMs and NAC to scavenge ROS in BMDM cells was detected by labeling ROS with DCFDA. 1×10^6 BMDM cells were cultured in 6-well plates for 24h, and BMDM cells were treated with $1 \mu\text{g/mL}$ LPS for 6h, and no LPS was added to the negative control group. After 6h, the culture medium containing LPS was removed and washed twice with PBS. 2mL culture medium containing PBS, $10 \mu\text{g/mL}$ MNMs and $10 \mu\text{g/mL}$ NAC was added, respectively. After 24h, the culture solution was removed and washed twice with PBS. Add $0.1 \mu\text{M/mL}$ DCFDA and incubate at 37°C for 30min. Remove DCFDA and add DAPI for 10min. After washing PBS for 2 times, the green fluorescence intensity was detected by fluorescence microscope (Leica DMI4000, Germany).

5.12 Mitochondrial membrane potential detection

The protective effect of MNMs on mitochondria was verified by detecting mitochondrial membrane potential with JC-1. First, 1×10^5 BMDM cells were inoculated in each 24-well plate, and $0.1 \mu\text{g/mL}$ LPS was added into DMEM culture medium to induce cells. After 24h, the culture medium was removed and washed twice with PBS. Culture in DMEM medium for 12h, and add the plasma ($200 \mu\text{L}/2\text{mL}$) of the four groups rats to culture medium. After 48h of culture, PBS washed twice, and 1mL cell culture medium and 1mL JC-1 staining solution were added. Incubate at 37°C for 20min. After washing the cells with PBS for 2 times, DAPI was added to stain the nucleus for 10min. After washing the cells for 2 times, 2mL culture medium was added to detect the cells under a fluorescence microscope (Leica DMI4000, Germany) and pictures were captured.

5.13 Flow cytometry

Flow cytometry was used to detect DCFDA labeled ROS levels in lung and liver tissues of rats. The lung or liver tissues of each group was rinsed with 50mL PBS and 100mg of tissue was taken. The Bronchoalveolar lavage fluid (BALF) were conducted with 5mL Hanks' Balanced Salt solution added Collagenase I (0.1mg/mL) and Collagenase II (0.1mg/mL), 20 U/mL DNase I (Roche, 04716728) to lavage dissociate cells from pulmonary mesenchyme, in water bath at 37°C for 1H. Add 1 times PBS to stop digestion. The digested tissue was mechanically dissociated, filtered through a 100 μm cell filter, and then centrifuged at 4°C at 495g for 10 min. After the supernatant was discarded, 2×10^6 cells were taken and added with 0.5mL 10 μM DCFDA, incubated at 37°C for 30min in dark light, washed with serum free DMEM twice, and the cells were suspended with 0.5mL PBS. The number of green fluorescence positive cells was detected by flow cytometry FL1 channel excitation at 488nm.

The number of macrophages in lung tissue and inflammatory macrophages in liver tissues were detected by flow cytometry. The BALF were conducted as previous described; 100mg of liver tissue was chopped and digested with DMEM containing 0.25% pancreatin 1mL, 150 $\mu\text{g/mL}$ collagenase II , 150 $\mu\text{g/mL}$ collagenase III and 20 U/mL DNase I, digesting in water bath at 37°C for 1H. The digested tissue was mechanically dissociated by filtered through a 100 μm cell filter, and then centrifuged at 4°C at 495g for 10 min. After the supernatant was discarded, 2×10^7 cells were obtained and prepared for Flow cytometry. 10 mL 1 $\times$ PBS was added to the microspheres for cell count. After centrifugation, the microspheres were re-suspended in the FACS buffer (Multi Science, S1001) at a concentration of 2×10^7 cells /mL. Incubate with surface antibodies in FACS buffer containing 10% normal goat serum (NGS) at 4°C for 1H. The antibody information is shown in TableS2. Antibodies were used: rat APC anti-CD11b (Biolegend, 1:100), rat PE anti-CD68 (Biolegend, 1:100), RatAlexa FLUOR 647 anti-iNOS (Bioss, 1:100). After washing with the FACS buffer, the cell microspheres were permeated in a 500 μL 1 $\times$ fixed/permeable buffer (eBioscience, 005123-43) overnight at 4°C. The cells were rinsed with FACS buffer and filtered through a 100 μm cell filter. FlowJo software (FlowJo, LLC) was used for data analysis after flow cytometry.

5.14 mtDNA was detected by qRT-PCR

The BMDM cells of the 4 groups of rats with 48H plasma intervention were re-suspended in 150 mM NaCl + 50mM HEPES (pH 7.4) + 15-25mg/mL digitonin solution, and incubated for 10min to make the cell membrane selectively permeable. Centrifuge 980 g for 3min to make the cells settle into a mass, take the supernatant (cytoplasm), centrifuge 17,000 g for 10min to remove the remaining cell debris, and obtain the cytoplasm without nuclei, mitochondria and endoplasmic reticulum. DNA purification kit was used to extract cytoplasmic DNA, and qRT-PCR was used to determine the nuclear DNA (for detection of nuclear fragmentation) and cytoplasmic mtDNA abundance in 4 groups of samples by nuclear DNA primer (Tert) and mtDNA primer (Dloop, Cytb, Nd4).

5.15 Immunohistochemistry (IHC) and immunofluorescence (IF)

The iNOS, eNOS, chemokines CCL20, CXCL1, CXCL10, VEGFA, cholangiocyte marker CK-19, active hepatic stellate cell marker α -SMA, cell proliferation marker PCNA, Endothelin-1, and Apelin were detected by IHC. After the paraffin sections were dewaxed and hydrated according to the previously described steps, the sections were boiled with 1L sodium citrate antigen repair solution for 10min $\times$ 2 times. After natural cooling, antiperoxidase was added and incubated for 30min. After 3 times of PBS cleaning, normal goat serum was added for blocking antibody at room temperature for 30min. Remove the blocking solution and add a suitable concentration of primary antibody and incubate overnight at 4°C (see TableS2 for antibody information). Then the slices were washed three times with PBS, the corresponding secondary antibody was added, incubated at room temperature for 30min, washed three times with PBS, the color development through added with DAB for 30s, the hematoxylin was added for 10s, and the slices were rinsed under water for 5min. After dehydrated and neutral gum seal, observe with 10 $\times$ and 40 $\times$ objective lens.

The main steps of IF dehydration, antigen repair, anti-peroxidase and sealing are the same as above steps of IHC. After incubating the primary antibody overnight, wash it with PBS for 3 times, add the corresponding fluorescent secondary antibody drops, and incubate it at room temperature for 30min away from light. washed 3 times with PBS, DAPI solution was added to stain the nucleus for 15s, and immediately placed in PBS solution for 3 times. An anti-fluorescence attenuation sealer (Solarbio, Beijing, China) was added and the slices were sealed using a cover glass. Finally, the acquired images were observed under a fluorescence microscope or confocal microscopy.

5.16 Western blot

Cells and tissues were lysed with RIPA lysate. Specifically, 1mL RIPA lysate (NCM Biotech, Suzhou, China) + 10 μ L protease inhibitor was added into 200mg rat lung or liver tissues, or 5 $\times$ 10⁶ cells. A hand-held tissue homogenizer (Biospec, Bartlesville) was used to homogenize on the ice. The pyrolysis ended after 30min and was centrifuged at 4°C and 13000 $\times$ g for 15min. The supernatant was absorbed into the new centrifuge tube, the protein was quantified by BCA method, and then stored in the - 80°C refrigerator (Thermo Scientific). The extracted proteins were separated by SDS-PAGE. The protein was then transferred to PVDF membrane by wet transfer. The protein was sealed with 5% buttermilk at room temperature for 1H and incubated with the corresponding primary antibody at 4°C overnight (seen in TableS2 for antibody information). Then it was washed with TBST for 3 times, incubated with the corresponding secondary antibody for 30min at room temperature, and developed by chemiluminescence method.

5.17 Chemokine Array Kit

First, THP-1 cells induced by Phorbol-12-myristate-13-acetate (PMA, 0.1mg/mL) activating for 12 hours and then treated with H₂O₂ and H₂O₂ + MNMs for 24h, respectively, and the total protein of the cells was extracted. Chemokine array kit ARY017 (R&D Systems, Minneapolis, MN, USA) was used to detect chemokine expression, and the sample/antibody mixture was added to the blocking membrane containing different capture antibodies according to the provided instructions. After rinsing, the

membrane was incubated with diluted streptavidin and horseradish peroxidase and developed by chemiluminescence.

5.18 Plasma inflammatory cytokines were detected by ELISA

ELISA was used to detect the inflammatory cytokines IL1- β , IL-6, TNF- α , IL-10 in the plasma of rats in each group. 3mL of venous blood was collected from rats using anticoagulant collection vessels, mixed well, left for 30min, centrifuged at 2000 g for 3min, and the upper plasma was collected and stored at -20°C for detection. The extraction of protein in liver tissue follows the steps previously described in WB. The levels of inflammatory cytokines in rat plasma were detected according to ELISA kit instructions, and the absorbance at 450nm was detected by spectrophotometer. Linear regression method was used to calculate the level of inflammatory factors by standard curve.

5.19 NO measurement

NO assay kit (Beyotime, S0021S) was used to detect the level of NO in tissues and cells. After 100mg of lung tissue was cut up, 1mL of cells and tissue lysate were added to the tissue homogenate for 30s, and the supernatant was taken after 12000g centrifugation for 3min. NO synthesis was detected in PMVEC cells or BMDM cells of different interventions. 5×10^6 PMVEC or BMDM cells were added to 500 μ L cell or tissue lysate, and the supernatant was taken after shaking for 30s and centrifugation for 12000g for 3min. The sample to be detected was added into the 96-well plate at the rate of 50 μ L/well, and Griess reagent I and Griess reagent II were added into the sample holes according to the kit instructions, respectively, and the absorbance of 540nm was detected by spectrophotometer.

5.20 Transwell experiment

PMA induced THP-1 cells were digested and diluted with serum-free culture to prepare single-cell suspension. After cell counting, the concentration was adjusted to 1×10^6 /mL. 200 μ L single-cell suspension was added to each chamber, 500 μ L complete medium containing 10% FBS was added to the lower chamber, and PBS, LPS (0.1 μ L/mL), chemokines, MNMs, and chemokines + MNMs were added to the lower chamber. The transwell plates were then cultured in a 5% CO₂ cell incubator at 37°C for 48H. After that, the Transwell chamber was removed, the residual culture group was gently rinsed with PBS, and the cells in the upper chamber were gently wiped with a cotton swab. Then the cells were fixed with 4% paraformaldehyde for 10min, rinsed with distilled water and soaked in crystal violet working liquid for 15min. Finally, after repeated rinsing with distilled water and wiping crystal violet and moisture on the small interior wall with a cotton swab, it was air dried naturally. The chamber was observed and photographed under a TE2000-S inverted microscope (Nikon). 5 visual fields were randomly selected from each chamber for cell count and the average value was calculated.

5.21 AST, ALT, and TBA detection

Serum ALT (Bioesn, BES2060CMT), AST (Bioesn, BES2061CMT) and TBA (HZYMES Biotech, HOEM0301-02) were detected by rate colorimetry, Lai's microplate method and enzyme cycling method. 3mL of rat venous blood was obtained using a coagulant collection vessel, mixed well, left for 30min, centrifuged at 2000 g for 3min, and the upper serum was obtained for ALT, AST and TBA detection according to the kit instructions.

5.22 Statistical analysis

SPSS 2021 and GraphPad Prism software (Version 9) were used for statistical analysis. The data were expressed as mean $\pm$ standard deviation (mean $\pm$ SD) and the sample size (n) was 3–6. The independent sample t test was used to compare the two groups when the variance was equal. When the hypothesis of equal variance is violated, the approximate T-test or non-parametric Mann-Whitney U test is used. Kaplan-Meier method was used to detect the difference of survival time in rats. RNA-Seq results were analyzed using R software. Statistical results show: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: no significance.

Declarations

Declaration of competing interest

The authors declare no conflict of interest, financial or otherwise.

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

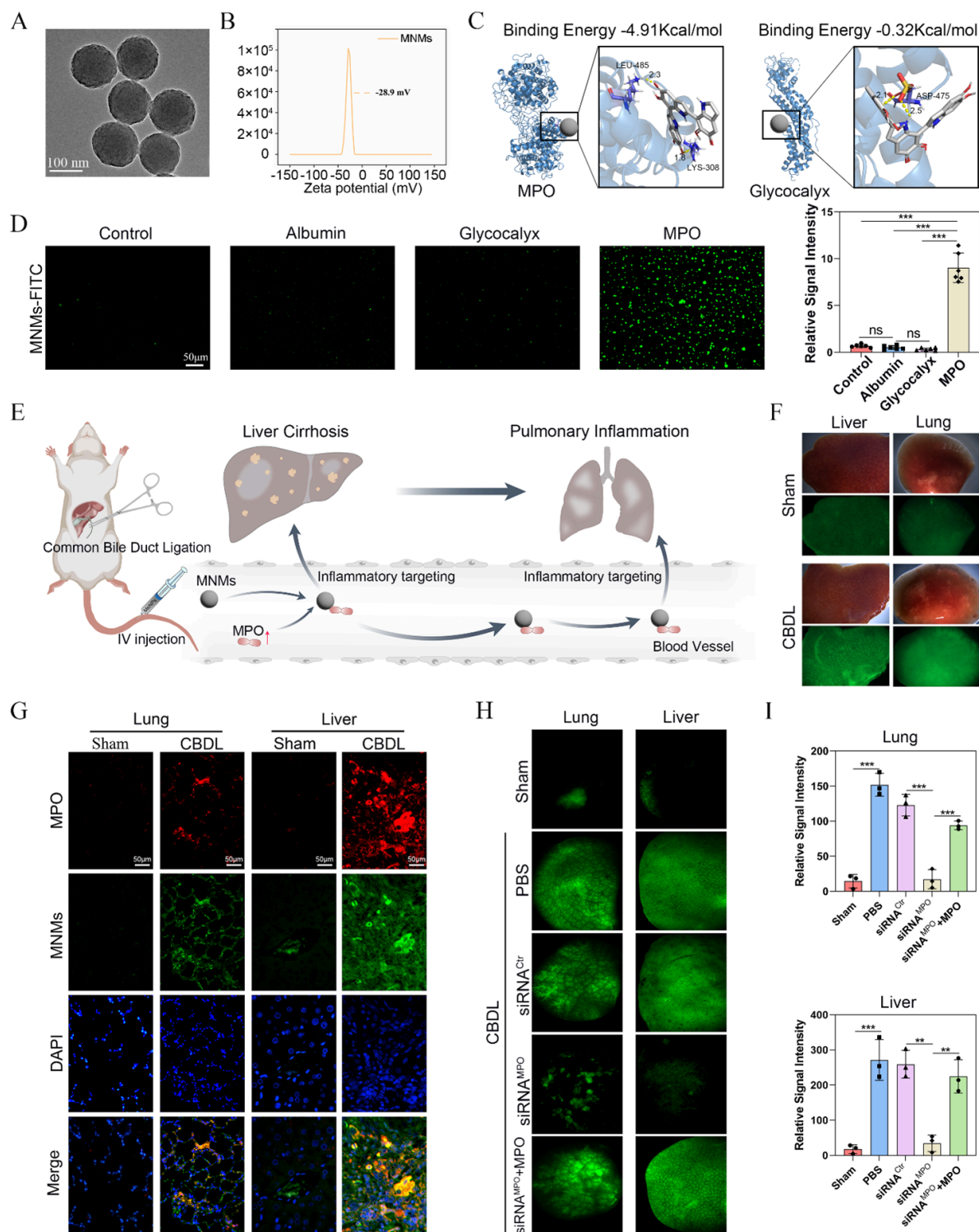


Figure 1

MNMs target to inflammatory tissues through the mediation of MPO.

A. TEM showed that MNMs were monodisperse spheres, with a diameter of approximately 100 nm. **B.** The Zeta potential measurement revealed the MNMs were negative charged (-28.9 mV). **C.** The molecular docking analysis revealed a low binding energy between the trimeric molecule constituting

MNMs and MPO protein, while with a high binding energy with Glycocalyx. **D.** The protein-coated plate experiment demonstrated MNMs could bind to MPO protein. **E.** The scheme of the mechanism of MNMs targeting inflammatory tissues mediated by MPO in CBDL induced HPS rat models. **F.** Tail intravenous injected MNMs targeting to the inflammatory lung and liver tissues under the mediation of MPO, and could not accumulate in normal liver and lung tissues. **G.** IF revealed the MPO (red) high expression and MNMs-FITC (green) accumulation in lung and liver tissues of CBDL group rats compared with Sham group. **H.** By *in vivo* siRNA interference of MPO expression in CBDL rats, or followed by intravenous injection of MPO recombinant protein, the extent of MNMs accumulation in lung and liver tissues was assessed. **I.** Histogram of fluorescence intensity in rat lung and liver tissues. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

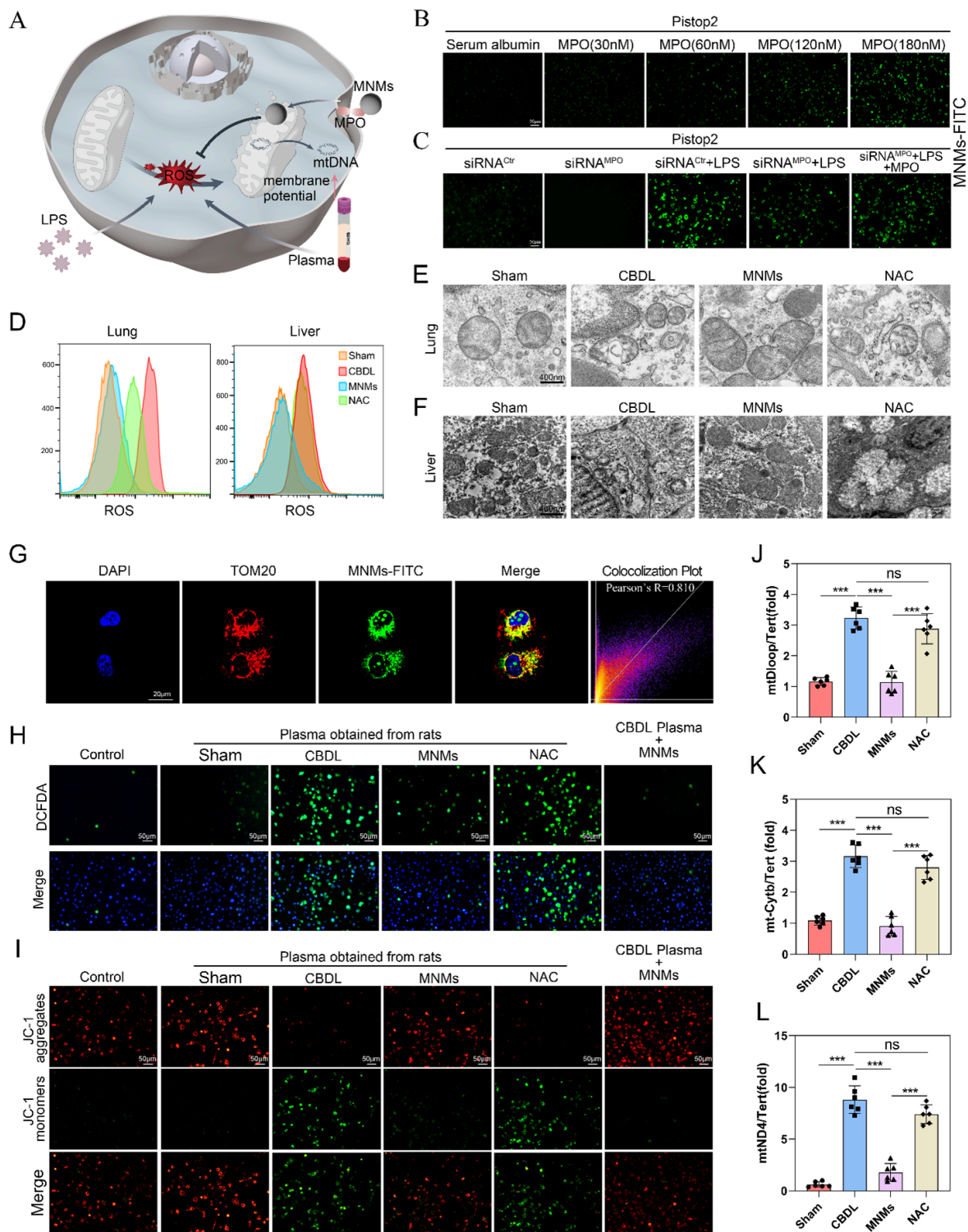


Figure 2

MNMs enter cells by mediation of MPO, and reduce oxidative stress by maintaining mitochondrial homeostasis.

A. CBDL could induce ROS up-regulation in the lungs and livers of rats, lead to mitochondrial damage and mtDNA leakage, and MNMs enter into macrophages mediated by MPO and maintaining

mitochondrial homeostasis. **B-C.** After treated BMDMs with Pitstop2 (phagocytosis inhibitor), the uptake of MNMs by BMDMs increased with the elevation of MPO concentration (**B**), the uptake of MNMs decreased with siRNA^{MPO} interference, and restored when MPO recombinant protein was added(**C**). **D.** Flow CytoMetry (FCM) was used to detect ROS levels in lung and liver tissues of rats in Sham, CBDL, MNMs and NAC groups. **E-F.** Morphology of mitochondria in pulmonary macrophages (**E**) and hepatic macrophages (**F**) were observed by TEM. **G.** Co-localization analysis of TOM20-labeled mitochondrial (red) and MNMs-FITC (green) fluorescence. **H-I.** BMDMs were treated with plasma of rats in 4 groups, and with the treatment of MNMs in vitro, then mitochondrial ROS was detected by DCFDA (**H**) and mitochondrial membrane potential was detected by JC-1 (**I**). **J-L.** The levels of mtDNA mtDloop (**J**), mtCytb (**K**), mtND4 (**L**) in the cytoplasm were detected after 4 groups rats' plasma intervention with BMDMs. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: no significance)

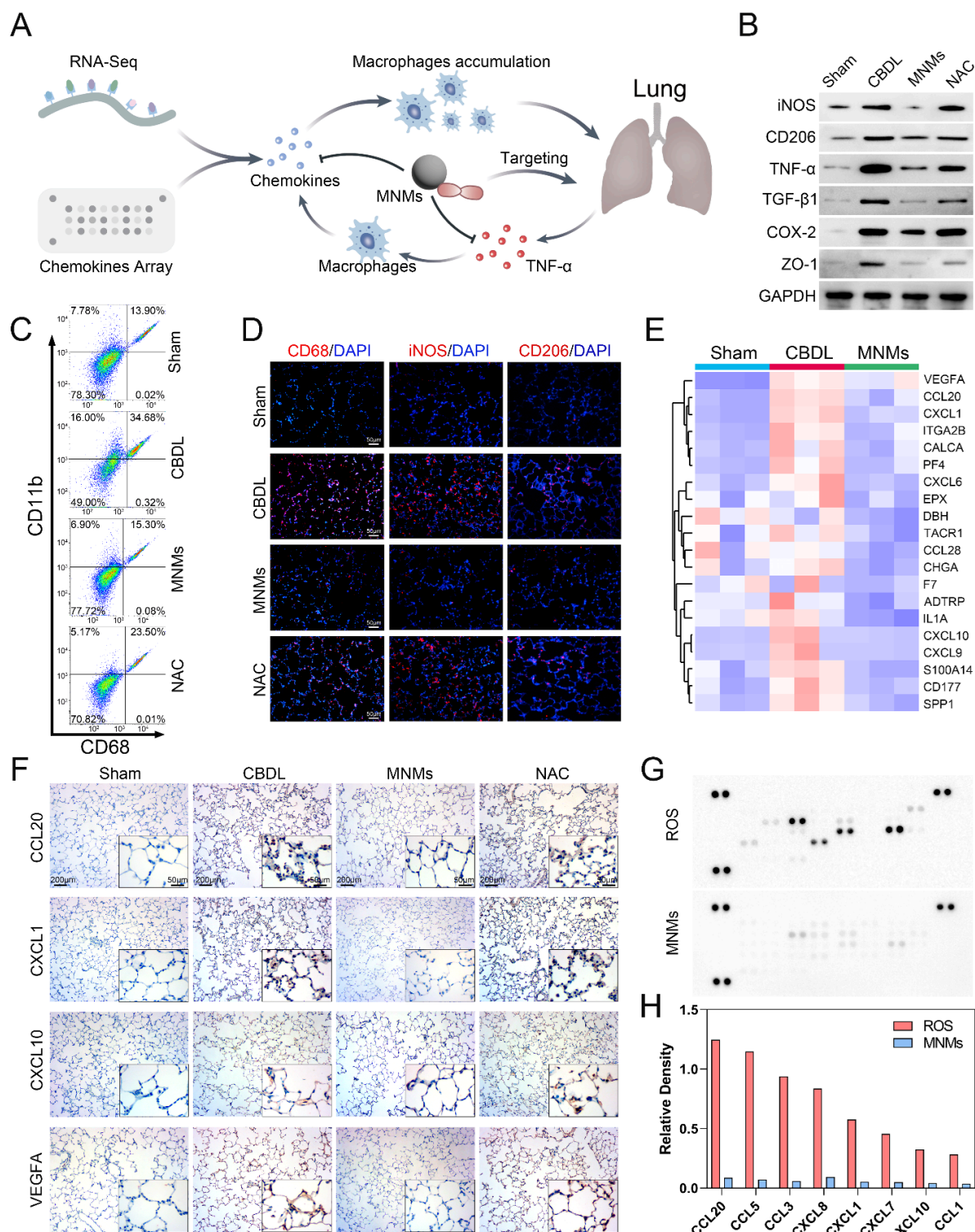


Figure 3

MNMs effectively inhibit macrophage accumulation in the lungs of CBDL rats.

A. Scheme: MNMs can reverse the phenomenon of macrophage accumulation in the lungs of CBDL rats, and we combined RNA-Seq and Chemokine Array Kit to investigate how MNMs inhibiting the expression of multiple chemokines to suppress macrophage aggregation in the lungs. **B.** The expression levels of

iNOS, CD206, TNF- α , TGF- β 1, COX-2 and ZO-1 in the lung tissues of 4 groups of rats were detected by western blot (WB). **C.** The proportions of CD11b and CD68-labeled macrophages in alveolar lavage fluid of rats in Sham, CBDL, MNMs, and NAC groups were quantified using FCM. **D.** IF staining was used to detect CD68-labeled macrophages, iNOS-labeled M1 type macrophages, and CD206-labeled M2 type macrophages in lung sections of 4 groups of rats. **E.** Heatmap of differentially expressed chemokines in the lung tissues of Sham, CBDL, and MNMs groups of rats determined by RNA-Seq. **F.** The IHC assay was employed to detect the expression level of CCL20, CXCL1, CXCL10, and VEGFA in the pulmonary tissues of rats in 4 groups. **G.** The Chemokine Array Kit suggested that MNMs might exert inhibitory effects on several chemokines by scavenging ROS. **H.** The histogram depicted the quantitative analysis result of the Chemokine Array Kit.

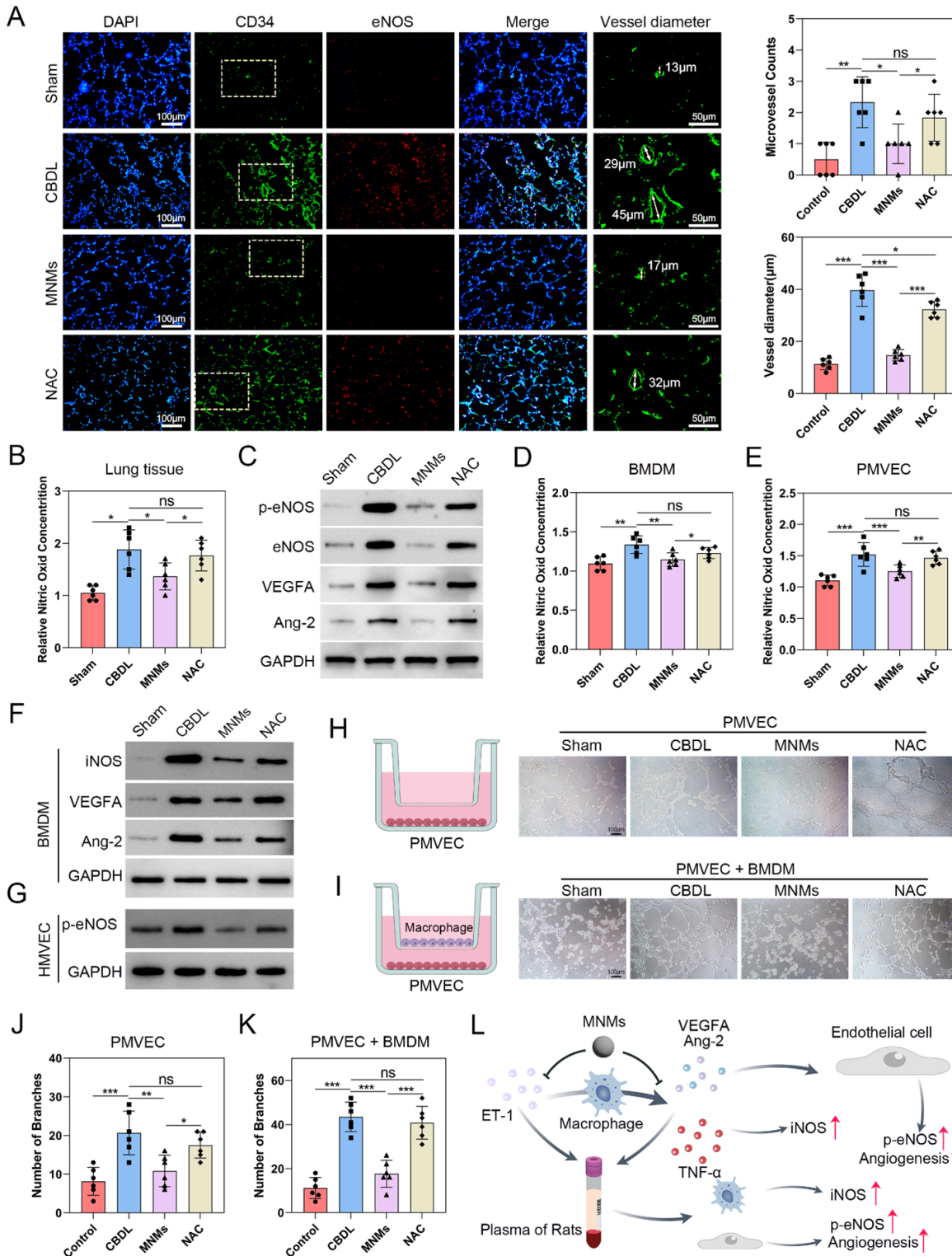


Figure 4

MNMs inhibit pulmonary vascular dilation and angiogenesis in CBDL rats

A. The expression of CD34 (green) and eNOS (red) in lungs of 4 groups of rats were detected by IF, and the microvascular diameters were measured. **B.** The NO levels in the lung tissues of 4 groups of rats were detected by spectrophotometry. **C.** The expression levels of p-eNOS, eNOS, VEGFA, and Ang-2 in

lung tissues of 4 groups of rats were detected by WB. **D-E**. By spectrophotometry, the NO levels in the BMDMs (**D**) and PMVECs (**E**) after induced by plasma of the 4 groups of rats were detected. **F-G**. After interventions with plasma from the Sham, CBDL, MNMs, and NAC groups of rats, the expression levels of iNOS, VEGFA and Ang-2 in BMDMs (**F**) and p-eNOS in PMVECs (**G**) were assessed. **H-I**. The endothelial tube-forming ability was assessed through a tube formation assay in plasma of four groups of rats treated PMVECs (**H**) and PMVEC + BMDM co-culture models (**I**). **J-K**. The number of branches formed by endothelial cells in the tube formation assay of PMVEC (**J**) and PMVEC + BMDM co-culture models (**K**) was shown in histogram. **L**. ET-1 in the circulation induced inflammation and iNOS upregulation in pulmonary macrophages, and promoted eNOS phosphorylation in endothelial cells through the VEGFA/Ang-2 pathway. MNMs exerted their anti-inflammatory effects to inhibit the above processes. (* $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, ns: no significance)

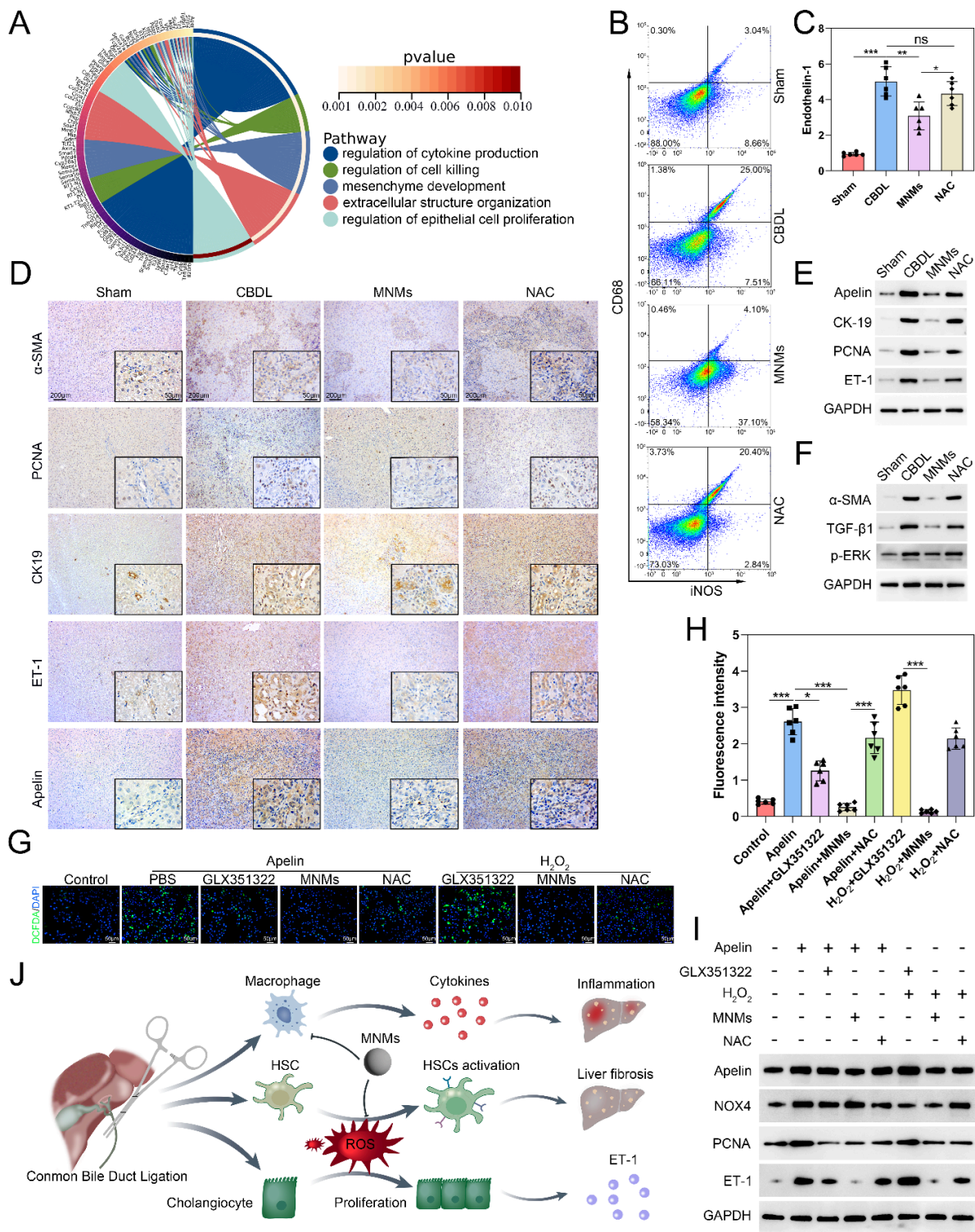


Figure 5

MNMs inhibit the synthesis of ET-1 and prevent fibrosis in the liver of CBDL rats.

A. GO analysis of differentially expressed genes in rat liver tissues of the CBDL group versus the MNMs group. **B.** FCM detected the proportion of inflammatory macrophage in 4 groups of rat livers. **C.** Plasma ET-1 levels in rats of 4 groups were detected by ELISA. **D.** IHC analysis revealed that MNMs effectively

suppressed the activation of HSCs (α -SMA), inhibited the proliferation of cholangiocytes (CK-19, PCNA), and downregulated the expression of ET-1 and Apelin. **E.** The expression levels of Apelin, CK19 (marker for cholangiocyte), PCNA (marker for proliferation), ET-1 in rat liver tissues were assessed by WB. **F.** The expression levels of α -SMA (marker for activated HSC), TGF- β , and p-ERK were assessed in rat liver tissues by WB. **G-H.** MNMs effectively eliminated the ROS generated through the Apelin/NOX4/ROS pathway in cholangiocytes as shown in DCFDA assay (**G**) and histogram (**H**). **I.** WB analysis confirmed that MNMs effectively suppressed the cholangiocytes proliferation, and inhibited synthesis of ET-1 by depressing the Apelin/NOX4/ROS pathway. **J.** MNMs can protect the liver through inhibiting liver inflammation, fibrosis, and cholangiocytes proliferation, reducing the expression and secretion of ET-1, and thus inhibiting the progression of HPS from the perspective of liver. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: no significance)

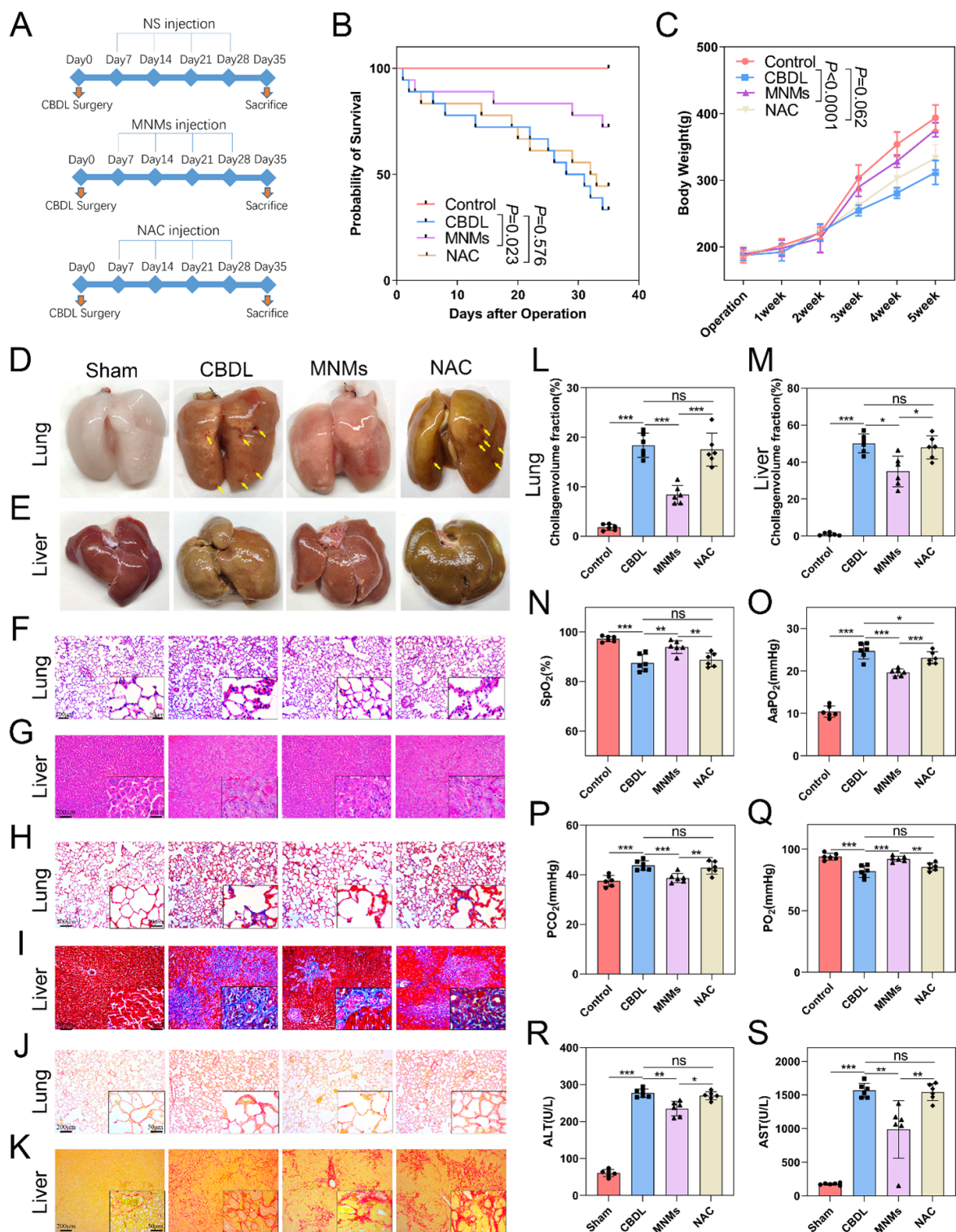


Figure 6

MNMs inhibit HPS progression in CBDL rat models

A. The rats were randomly divided into 3 groups after CBDL operation and were given normal saline, 2.5mg/kg MNMs and 2.5mg/kg NAC via tail intravenous injection, respectively. **B.** Survival rate of rats in the Sham, CBDL, MNMs, and NAC groups were analyzed at 5 weeks after CBDL operation. **C.** Growth

curves of rat body weight in four groups of rats after CBDL. **D-E.** Gross morphology of lung (**D**) and liver (**E**) tissues in 4 groups of rats, and the yellow arrows point to hemorrhagic spots. **F-G.** Pathological characteristics of lung (**F**) and liver (**G**) tissues in 4 groups of rats revealed by HE staining. **H-I.** Comparison of the collagen fibrous structure of lung (**H**) and liver (**I**) tissues in 4 groups of rats by Masson staining. **J-K.** Comparison of the collagenous fiber of lung (**J**) and liver (**K**) tissues in 4 groups of rats via Sirius scarlet staining. **L-M.** Collagen areas of lung (**L**) and liver (**M**) tissues in 4 groups of rats after Masson staining were shown in histogram. **N-Q.** Comparison of arterial blood gas analysis of SpO_2 (**N**), AaPO_2 (**O**), PCO_2 (**P**) and PO_2 (**Q**) in 4 groups of rats. **R-S.** Comparison of liver function of ALT (**R**) and AST (**S**) in 4 groups of rats (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns: no significance)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupportingInformation.docx](#)
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