Human umbilical cord mesenchymal stem cells derived Exosomes attenuate injury of myocardial infarction by miR-24-3p-promoted M2 macrophage polarization

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Research

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Abstract

Background-

Exosomes derived from human umbilical cord mesenchymal stem cells (UMSCs-Exo) were recommended as ideal substitutes for cell-free cardiac regenerative medicine, which had presented encouraging effects in regulating inflammation and attenuating myocardial injury. The phenotype of macrophages resident in myocardium were regulated dynamically in response to environmental cues, which may either protect against injury or promote maladaptive remodeling. However, the underlying mechanisms about UMSCs-Exo regulating macrophage polarization are still not well understood. Herein, we aimed to explore the effects of UMSCs-Exo on macrophage polarization and their roles in cardiac repair after myocardial infarction (MI).

Methods and Results-

Exosomes were isolated from the supernatant of human umbilical cord mesenchymal stem cells (UMSCs) and transplanted by intramyocardial injection after MI. Our results showed that UMSCs-Exo improved cardiac function by increasing M2 macrophage polarization and reducing excessive inflammation. After depletion of macrophages with clodronate liposomes, the therapeutic effects of UMSCs-Exo were disrupted. Administrated with UMSC-Exo, macrophages are inclined to polarize towards M2 phenotype in inflammatory environment in vitro. The results of RNA-sequencing indicated Plcb3 was a key gene concerned in UMSCs-Exo facilitated M2 macrophage polarization. Further bioinformatics analysis revealed exosomal miR-24-3p as a potential effector mediated Plcb3 down regulation in macrophages. Increasing miR-24-3p expression in macrophages effectively enhanced M2 macrophage polarization by suppressing Plcb3 expression and NF-κB pathway activation in inflammatory environment. Furthermore, diminishing miR-24-3p expression in UMSCs-Exo attenuated the effects of UMSCs-Exo on M2 macrophage polarization.

Conclusions-

Our study demonstrated that macrophages, as important inflammatory regulators, participated in UMSCs-Exo mediated myocardial repair after MI. And the therapeutical effects were at least partially carried out by UMSCs-Exo promoting M2 macrophage polarization in an inflammatory microenvironment. Mechanically, exosomal miR-24-3p inhibits the expression of Plcb3 and NF-κB pathway activation to promote M2 macrophage polarization.

1. Background

Acute myocardial infarction (AMI), is one of the leading causes of death worldwide [1]. After ischemia occurred, inflammatory response is triggered in the heart, which is closely related to the prognosis of MI

[2]. In fact, inflammatory responses are necessary for cardiac repair, but excessive inflammatory responses often aggravate myocardial injury. Ameliorating the over-activated inflammatory response is important for cardiac repair. Cardiac macrophages exhibit proinflammatory M1 phenotype or anti-inflammatory M2 phenotype in response to the alterations of myocardial microenvironment, and subsequently aggravate or alleviate the inflammation in infarcted hearts. Increasing attention was paid to the modulation of macrophage polarization to reduce the excessive inflammatory reaction and improve the quality and outcome of cardiac healing [3–5].

UMSCs have been used in numerous studies about the biological therapy of cardiovascular diseases, due to their function in tissue regeneration, repair and immunoregulation [6, 7]. Stem cell transplantation takes effects in the MI treatment mainly through paracrine function, such as releasing exosomes and various cytokines [8]. Multiple stem cells derived exosomes are effective therapeutic agents for cardioprotection post-MI. Exosomes (Exo) are miniature vesicles with a diameter of about 30–150 nm and secreted by almost all types of cells [9]. Compared with stem cells, exosomes possess many advantages including low immunogenicity, easy modification of contents, and long-term preservation after extraction [10]. However, there are few studies investigated the effect of MSCs-Exo, especially UMSCs-Exo, on macrophage polarization after MI. In this study, we demonstrated that UMSCs-Exo alleviated MI injury by promoting M2 macrophage polarization through miR-24-3p mediated reduction of Plcb3/NF-κB pathway activation.

2. Methods

2.1 Cell Culture and Identification

UMSCs were cultured with DMEM/F12 containing 10% fetal bovine serum (FBS, BD) and 1% Penicillin/Streptomycin (P/S, Beyotime), and passage 3 to 6 cells were used for further experiments. The identification of UMSCs was carried out by flow cytometry analysis with CD29, CD31, CD44, CD45, CD73 and HLA-DR expression (BioLegend). To evaluate the multilineage differentiation potency, UMSCs were cultured with inducing medium (Cyagen). Alizarin red staining, Oil red O staining and Alcian blue staining were performed to detect osteoblast, adipocyte and chondrocyte differentiation respectively.

Murine macrophage RAW264.7 cells were cultured in RPMI 1640 medium (Gibco) containing 10% FBS and 1% P/S. The methods of isolation and culture of peritoneal macrophages were supplied in supplementary materials.

2.2 UMSCs-Exo Isolation and identification

When UMSCs reached 70–80% confluency, the supernatant was replaced with fresh culture medium containing 10% exosome-free FBS. After cultured for another 48 hours, the supernatant was collected and UMSCs-Exo were isolated by serial differential centrifugation plus ultracentrifugation as described previously [11]. The identification of exosomes was performed as follows: the size distribution and morphology were recorded by Nanoparticle Tracking Analysis (NTA) and Transmission Electron

Microscopy (TEM) respectively; Exosomal protein markers CD63 and TSG101 were detected by Western blot analysis.

2.3 Cell experimental protocol

The uptake of DiI-labeled MSC-Exo was determined by fluorescence microscope. To assess the effects of UMSCs-Exo on macrophage polarization in an inflammatory microenvironment, macrophages were pretreated with UMSCs-Exo and simulated with LPS according to the method described previously [12]. RAW264.7 cells or peritoneal macrophages were treated with 40 μg/mL UMSCs-Exo for 24 hours, and then the cells were further incubated with fresh medium containing 100 ng/mL lipopolysaccharide (LPS, Sigma) for an additional 24 hours (Exo + LPS group). LPS group only cultured with LPS, untreated cells as control (Con group). The cells, RNAs or proteins were collected for the subsequent analysis.

2.4 Flow cytometry analysis

To detect the polarization of macrophages in tissues, the infarcted myocardium was minced and digested with collagenase buffer containing 500 U/mL collagenase II (Sigma), 0.5 U/mL Dispase, and 1 U/mL DNase I for 1 hour at 37°C. After centrifugation (500 g, 10 min, 4°C), cells were resuspended in PBS and filtered through a 70 μm mesh, lysed with ACK buffer (Beyotime). The single-cell suspensions were stained with FITC labelled anti-F4/80 antibody (eBioscience) and APC labelled anti-CD206 antibody (eBioscience) for 30 minutes protected from light. For intracellular staining, cells were fixed and permeabilized with intracellular Fixation and Permeabilization kit (eBioscience), and then stained with PE labelled anti-iNOS antibody (eBioscience) for another 30 minutes. Finally, cells were detected by flow cytometry.

2.5 Animal experimental protocol

All procedures with animals were approved by the Ethics Committee of Soochow University. MI was established as described previously [13]. Briefly, male C57BL/6 mice (8-week-old) were anesthetized by intraperitoneal injection with 80 mg/kg pentobarbital, and were mechanically ventilated using a rodent ventilator. The chest was opened at the 4th intercostal space to expose the heart, and the left anterior descending coronary artery (LAD) was ligated with a 6 − 0 polyester suture. Then, 50 μg UMSCs-Exo dissolved in 25 μL PBS was injected at three different points around the infarct border zone. Echocardiographic measurements were performed to evaluate cardiac function by using Vevo 2100 system (VisualSonics). To deplete macrophages, mice were intraperitoneally injected with 3μL/g (weight) clodronate liposomes (Cl_2MDP, Liposoma) 1 day before and post MI respectively.

2.6 Histological and Immunofluorescence analysis

At 3 days or 28 days after MI, the hearts were collected and fixed in 4% paraformaldehyde overnight, and embedded in paraffin. The tissue sectioned at 5 μm and stained with hematoxylin-eosin (HE) to quantify inflammatory cell infiltration. Masson's trichrome staining and Sirius red staining were performed to quantify fibrosis area. Capillary density in the border was quantified with immunofluorescence staining of

anti-CD31 antibody (Abcam). To evaluate macrophage polarization or macrophage depletion, tissue sections of hearts or spleens were performed to immunofluorescence staining for F4/80, iNOS or CD206.

2.7 Exosomal RNAs or proteins obtain

To obtain the exosomal RNA or protein components, the protocol was performed following the method described previously [14]. UMSCs-Exo suspension was disposed by five freeze-thaw cycles (-170°C ~ 37°C) and then incubated with RNase A (Takara) or proteinase (Sigma) at 37℃ respectively, finally the Exo-protein or Exo-Nucleic Acid were obtained. Detailed descriptions are provided in Supplementary Material.

2.8 Transfection of miRNA mimics and inhibitors

According to the manufacturer's instructions, macrophages were transfected with miRNA mimics (Ribobio) using Lipofectamine 2000 (Invitrogen) and cultured for 24 hours and then treated with 100 ng/mL LPS for another 24 hours. The cells were harvested for subsequent analysis.

To knock down the miR-24-3p expression in UMSCs-Exo, UMSCs were transfected with miR-24-3p inhibitor (Ribobio). After transfection, the culture supernatant was collected to isolate miR-24-3p low-expressed exosomes (miR-24 inhib Exo).

2.9 Luciferase reporter assays

Bioinformatics predicted miR-24-3p could bind to Plcb3 mRNA and inhibit it expression. The binding sequence of the Plcb3 was amplified by PCR and inserted into the firefly luciferase reporter psiCHECK™-2 vector (Promega). HEK293T cells were transfected with 50 pmol miR-24-3p mimics and 200 ng psiCHECK™-Plcb3 vector (Ribobio). 48 hours later, the activity of firefly and Renilla luciferase was analyzed with a dual-luciferase reporter assay kit (Meilunbio).

2.10 Statistical analysis

All data were expressed as mean ± SD. Statistical significance between 2 groups was assessed by the *Student t test*, and multiple comparisons were analyzed by ANOVA analysis, $p < 0.05$ was considered as statistical significance. All statistical analysis was carried out using the statistical software GraphPad Prism 8.

3. Results

3.1 Characterization of UMSCs and UMSCs-Exo

UMSCs displayed as long spindle-shaped morphology, and expressed with CD29, CD44 and CD73, and without CD31, CD45 and HLA-DR on the membrane by flow cytometry analysis (Fig. 1A). Meanwhile, UMSCs also could differentiate into multiple lineages including osteoblasts (positive staining of Alizarin Red), adipocytes (positive staining of Oil red O) and chondrocytes (positive staining of Alcian Blue) (Fig. 1B). UMSCs-Exo exhibited the characteristic saucer-shaped morphology by TEM (Fig. 1C) and

showed the size was distributed about 70–150 nm by NTA (Fig. 1D). Western blot analysis confirmed the positive expression of CD63 and TSG101 (Fig. 1E). All data above indicated UMSCs-derived particles collected in our experiments were exosomes.

3.2 UMSCs-Exo preserved cardiac function and alleviated inflammation after MI

According to the data of echocardiographic parameters, we found with UMSCs-Exo administration, the extent of LV dysfunction was not different between PBS and Exo groups at 3 days after MI. However, after 7 days, especially 28 days, treatment with UMSCs-Exo, cardiac function was better preserved, infarct size and interstitial fibrosis fraction were reduced relative to PBS group (Fig. 2A-C; Supplemental Fig. 1A). Further investigations for cardiac pathology and function showed the levels of VEGFA and VEGFR2, which were closely related to neovascularization, were significantly higher in the Exo group in the border zone at 28 days after MI (Fig. 2D). Meanwhile, the expression of MMP2 and MMP9 in myocardium were also reduced in Exo group (Fig. 2D). The histological assessment of heart tissues also confirmed more angiogenesis (Supplemental Fig. 1B) in the Exo group. These results together demonstrated UMSCs-Exo facilitated cardiac function restore after MI as we previous reported [15].

The prognosis of myocardial infarction is intimately associated with the intensity and duration of inflammatory reaction. The residence of exosomes in tissues after transplantation is the basis of their function. The results of immunofluorescent staining indicated that DiI (red)-labelled UMSCs-Exo were still localized in the myocardium at 3 days after transplantation (Supplemental Fig. 2); HE staining showed there was relatively less inflammatory cells infiltration in the Exo group (Fig. 2E). Further we detected the expression of several injury-related cytokines to investigate the effects of UMSCs-Exo on myocardial repair. The results showed that the protein levels of NFκB-P65 and p-P65 (proinflammatory cytokines) were reduced, while heme oxygenase-1 (HO-1), an anti-inflammatory, anti-apoptosis, anti-oxidative cytokine, was significantly upregulated in the border zone with UMSCs-Exo transplantation (Fig. 2F). These data suggested that UMSCs-Exo alleviated overactive cardiac inflammation following MI. It is well-known that the activation of NF-κB pathway favors M1 macrophage polarization whereas the upregulation of HO-1 promotes M2 macrophage polarization [16]. Thus, we speculated UMSC-Exo alleviates inflammatory action by regulating macrophage polarization.

3.3 Depletion of macrophages reduced the therapeutic effects of UMSCs-Exo after MI

Considerable evidence revealed macrophages play pivotal roles in tissue repair and regeneration [17]. Our aforementioned results have demonstrated UMSCs-Exo promote myocardial repair, however, these benefits whether through regulating macrophage polarization remains to be confirmed. Cl_2MDP was administered to deplete macrophages at 1 day before and post MI respectively as previous described [13, 18]. As expected, macrophages ($F4/80^+$) in hearts and spleens were reduced significantly by flow cytometry and immunofluorescence analysis (Fig. 3A, B). The result of echocardiography showed LV

function was slightly improved in Exo + Cl_2MDP group compared with PBS + Cl_2MDP group, but there was no significant different (Fig. 3C, D). This implied that macrophage depletion weakened the therapeutic benefits of UMSCs-Exo, that indicated macrophages are essential for the cardioprotective effects of exosomes therapy.

3.4 UMSCs-Exo regulated macrophage polarization after MI

Subsequently, we aimed to figure out the role of macrophages in UMSCs-Exo mediated cardiac repair. Among macrophage subpopulations, M2 macrophages could promote injury repair. To further investigate the influence of UMSCs-Exo on macrophage behaviors, we detected the subpopulations of macrophages in the infarcted myocardium. As showed in Fig. 4A and Fig. 4B, the M2 ($F4/80^+CD206^+$) phenotype ratio of total macrophages ($F4/80^+$) were increased in Exo group (PBS group: 46.7 ± 0.95%; Exo group: 58.8 ± 2.66%). And the ratio of M1 ($F4/80^+iNOS^+$) macrophages were reduced (PBS group: 63.0 ± 3.27%; Exo group: 46.0 ± 2.42%), as showed in Fig. 4A and Fig. 4C. Furthermore, western blotting showed that M2 makers (CD206 and Arg1) were also significantly increased and M1 maker (iNOS) was reduced in Exo group (Fig. 4D), which was consistent with immunofluorescence (Fig. 4E, F). All these data demonstrated that UMSCs-Exo could enhance macrophages polarize towards M2 phenotype after MI.

3.5 UMSCs-Exo promotes macrophages polarize towards M2 phenotype in vitro

As showed in Fig. 5A, the DiI (red)-labelled UMSCs-Exo were internalized into RAW264.7 macrophages within 24 hours. Flow cytometry analysis showed that with UMSCs-Exo pretreated, the ratio of M2 macrophages ($CD206^+$) were significantly increased whereas M1 macrophages ($iNOS^+$) were markedly reduced with LPS inducing (Fig. 5B-D). Further, qPCR results also showed the relative expression of CD206 and Arg1 (M2 phenotype markers) were increased while iNOS and TNFα (M1phenotype markers) were remarkably reduced in Exo + LPS group (Fig. 5E, F). Consistently, the results of western blot analysis revealed a uniform trend (Fig. 5G).

In addition, peritoneal macrophages were isolated from mice to explore the effects of UMSCS-Exo on macrophage polarization (Supplemental Fig. 3A, B). And as expectation, similar results of macrophage polarization were observed (Supplemental Fig. 3C-H). All data above indicated that UMSCs-Exo could promote macrophages to polarize towards M2 phenotype.

3.6 Exosomal RNAs played pivotal roles in macrophage polarization

Exosomes mediate intercellular communication by transferring different biomolecules, including proteins, RNAs, and lipids from one cell to another. To date, several important exosomal protein and nucleic acid cargos responsible for regulating macrophage polarization have been identified, but many remain undiscovered. To identify whether protein or RNA components are responsible for the UMSCs-Exo-induced macrophage polarization, we got exosomal proteins or RNAs as previous reported (Fig. 6A) [14]. The TEM

images showed that intact exosomes were ruptured and aggregated following the freeze–thaw operation (Fig. 6B), and RNase A or proteinase would be enforced completely. Then we used exosomal proteins or nucleic acid compenent respectively to treat RAW264.7 macrophages and LPS induced them polarization. Flow cytometry and qPCR analysis indicated that exosomal nucleic acid play dominant roles in promoting macrophages polarize towards M2 phenotype, rather than exosomal proteins (Fig. 6C-E).

3.7 Exosomal miR-24-3p mediate M2 macrophage polarization by suppressing Plcb3 expression and NF-κB signaling activation

MiRNAs are main components of exosomal RNA, regulates the gene expression of recipient cells by pairing and binding to the mRNA and promoting mRNA degradation [19]. To explore the candidate genes and associated pathways for UMSCs-Exo mediated M2 polarization, RNA-sequencing analysis of macrophages was performed. Lots of differentially expressed genes were identified, and further analysis showed 14 genes were closely related to inflammation or macrophage polarization. Combined with our previous study about UMSC-Exo miRNA profile analysis, we speculated exosomes transfer of miR-24-3p to macrophages and targeted to Plcb3, which might be a key target gene concerned with macrophage polarization (Fig. 7A). MiRWalk revealed Plcb3 is a potential target of miR-24-3p (Fig. 7B), therefore, we cloned the Plcb3 binding sequence fragment into a luciferase reporter plasmid. Subsequently, transfected the reporter plasmid, and miR-24-3p mimics into HEK293T cells. Luciferase assays revealed that Plcb3 transcriptional activity decreased markedly in the presence of miR-24-3p mimic (Fig. 7C). Further, we transfected miR-24-3p mimic into RAW264.7 macrophages and western blotting analysis showed the protein level of Plcb3 were significantly reduced (Fig. 7D). The flow cytometry analysis also showed that miR-24-3p mimic obviously facilitated the macrophages to polarize towards M2 phenotype in vitro (Fig. 7E).

According to the loss of function strategy, we reduced miR-24-3p expression in exosomes as we previously reported and treated macrophages (miR-24 inhib Exo group) (Fig. 7F). The results showed the up-regulation of M2 marker (CD206) was induced as well as the down-regulation of M1 marker (iNOS) was partially negated (Fig. 7G).

NF-κB signal pathway is important for the inflammation of macrophages [12, 13, 20], previous findings showed Plcb3 was relevant to inflammatory reaction by activating NF-κB signal pathway [21, 22]. With miR-24-3p mimic transfection, Plcb3, iNOS, P65 and p-P65 were markedly down-regulated while CD206 and Arg1 were significantly up-regulated (Fig. 7H). Further, the result of the Exo group was similar with miR-24-3p group, and the trend was partially reversed after knocking down the miR-24-3p expression in UMSCs-Exo (Fig. 7I). These data confirmed that exosomal miR-24-3p participated in the UMSCs-Exo mediated macrophage polarization by targeting the Plcb3/NF-κB signal pathway in the inflammatory microenvironment.

4. Discussion

In the past few years, UMSCs have been proved to be beneficial in cardiac repair after ischemic injury by paracrine secreted exosomes [23–25]. Teng *et al.* demonstrated that MSCs derived exosomes could regulate the inflammatory microenvironment after myocardial infarction [26], however, the mechanisms have not been fully understood. M2 Macrophage polarization is an important endogenous process for promoting post-infarction regenration and aliviating adverse cardiac remodeling in myocardial infarction. The subtypes of macrophages are regulated by many different stimuli, and 'chimeric' M1–M2 macrophages with mixed phenotypic features, such as surface and genetic markers that often appear in the inflammatory environment [27].

In this study, we isolated and characterized the exosomes derived from UMSCs successfully. Our data demonstrated that UMSCs-Exo administration promoted cardiac repair and restored heart function as measured by echocardiographic. Pathological analysis showed that UMSCs-Exo reduced the area of MI induced myocardial fibrosis and downregulated MI-induced inflammatory factor expression as reported previously [23, 25, 28]. Furthermore, we found those cardioprotection effects were disrupted by macrophage depletion, which revealed macrophages are necessary in UMSCs-Exo mediated myocardial injury repair. Our previous investigation has proved UMSCs derived exosomal miR-24-3p prevents cardiomyocytes apoptosis by inhibiting Bim expression after MI [15]. Our present work also confirmed UMSCs-Exo could effectively attenuate injury of myocardial infarction by promoting macrophages towards M2 phenotype. The anti-inflammatory M2 macrophages provided a kind of microenvironment and decreased the secondary apoptosis induced by inflammation, ultimately promoting the regeneration of ischemic injured myocardium in the therapy with UMSCs-Exo.

UMSCs-Exo transplantation upregulated the level of HO-1 and inhibited NF-kappaB P65 expression. It is well-known that the activation of NF-κB pathway favors M1 macrophage polarization whereas the upregulation of HO-1 promotes M2 macrophage polarization. Therefore, it is not a surprise that UMSCs-Exo can improve the severe inflammation microenvironment after myocardial infarction via modulating macrophage polarization. Our results suggested that UMSCs-Exo might regulate macrophage polarization between the following mechanisms: 1) attenuating the level of NF-κB P65 and inhibiting NF-κB pathway activation; 2) inducing HO-1 expression and favoring an M2 phenotype as reported [29].

In order to identify the functional exosome components related to M2 macrophage polarization, we successful isolated the nucleic acids and proteins of UMSCs-Exo respectively. The subsequent results found UMSCs-Exo promoted macrophages to polarize towards M2 phenotype by exosomal RNA rather than proteins in vitro. MiRNAs are main components of exosomal RNA, with important immunomodulatory properties by regulating the gene expression of recipient cells [30–35]. RNA-sequencing showed miR-24-3p was enriched in UMSCs-Exo. Recent studies about miR-24-3p have demonstrated miR-24-3p could repress inflammation and protect cardiomyocytes from ischemia/reperfusion injury [36, 37]. After co-cultured with UMSCs-Exo, miR-24-3p expression was increased in macrophages (Fig. 7F), which indicated miR-24-3p is responsible, at least in part, for the macrophage M2 polarization.

Plcb3 is a key regulator of amplitude of inflammatory response, implicated in the activation of the nuclear transcription factor NF-κB P65 [21, 22]. Our results of the RNA sequencing revealed that Plcb3 was down-regulated after treating with UMSCs-Exo in LPS-stimulated macrophages. In addition, numerous studies have revealed that NF-κB signal pathway could mediate the activation and polarization of macrophages [38–40]. Therefore, we speculated Plcb3 as the candidate gene concerned with macrophage polarization through NF-κB pathway.

For more intensive research, we performed and demonstrated miR-24-3p was involved in the UMSCs-Exo mediated macrophage polarization by targeting the Plcb3/NF-κB signal pathway. It was worth noting that Plcb3 was high expressed, however P65 and p-P65 were low expressed in the macrophages of Con group (Fig. 7H, I), which implied the Plcb3/NF-κB signal pathway might only be activated in inflammatory environment.

Exosomes are miniature vesicles containing complex bioactive components. Although our data suggested that exosomal miR-24-3p had a significant role in macrophage polarization and cardioprotection effects, we have not rule out the function of many other exosomal cargoes, that undoubtedly exerted the multiple functional benefits as an ensemble.

5. Conclusions

In summary, our study elucidated that UMSCs-Exo attenuated injury of MI via enhancing M2 macrophage polarization. Especially, the exosomal miR-24-3p played the pivotal role in the modulation of macrophage polarization by targeting Plcb3/NF-κB signal pathway, which further explained the molecular mechanism of UMSCs-Exo therapy for MI from a new perspective (Fig. 8). Meanwhile, Plcb3 maybe a therapeutic target for the development of novel drugs against myocardial injury.

Abbreviations

AMI
Acute myocardial infarction
Cl_2MDP
Clodronate liposomes
EF
Left ventricle ejection fraction
Exo
Exosomes
FCM
Flow cytometry
FS
Left ventricle fractional shortening
HO-1

Heme Oxygenase-1
ICF
Immunofluorescence
LAD
Left anterior descending coronary artery
LV
Left ventricle
MSCs
Mesenchymal stem cells
NTA
Nanoparticle Tracking Analysis
P/S
Penicillin/Streptomycin
TEM
Transmission Electron Microscopy
UMSCs
Human umbilical cord mesenchymal stem cells
UMSCs-Exo
Exosomes derived from human umbilical cord mesenchymal stem cells

Declarations

Ethics approval and consent to participate

All animal experiments in this study were approved by the Ethics Committee of Soochow University (Ref: SZUM2008031233), and all experimental procedures were conducted according to institutional animal ethics guidelines for the Care and Use of Research Animals established by Soochow University, Suzhou, China.

Consent for publication

Not applicable.

Availability of data and materials

All datasets supporting this study are included within the article.

Competing interests

The authors declare that there was no conflict of interest, financial relationships or otherwise that could be construed as a potential conflict of interest.

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Authors' contributions

Feng Zhu, Yihuan Chen and Jingjing Li contribute equally in this study. Feng Zhu, Jingjing Li, Yang Lin and Boxuan Jiang performed the experiments; Yihuan Chen and Ziying Yang analyzed the data; Lianbo Shao, Shengshou Hu and Zhenya Shen designed the investigation, reviewed and edited the manuscript. All authors read and approved the manuscript.

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Figures

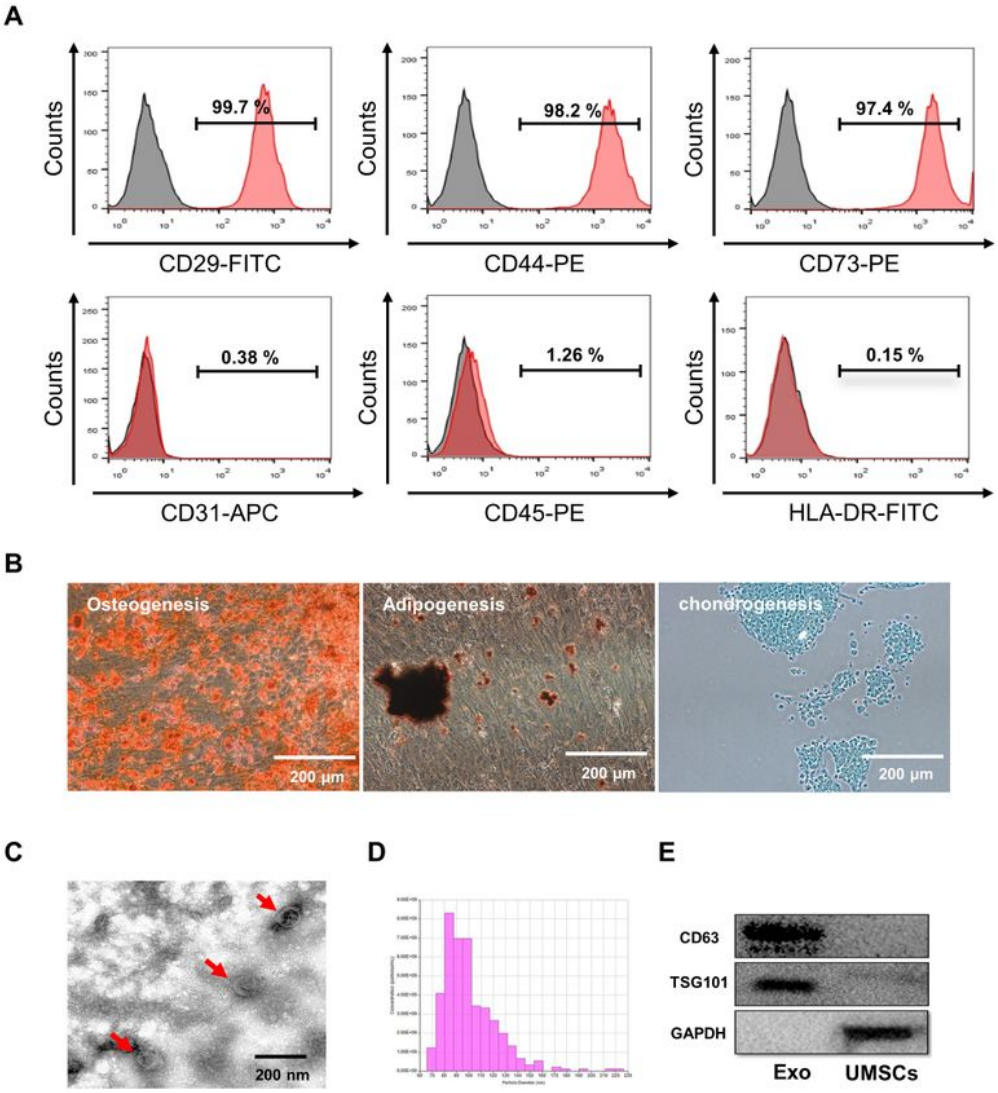

Figure 1

Characterization of UMSCs and UMSCs-Exo. (A) Flow cytometry analysis of UMSCs. (B) Alizarin red staining, Oil red O staining and Alcian blue staining of UMSCs to identify osteogenesis differentiation, adipogenesis differentiation and chondrogenesis differentiation respectively. Scale bar: 200 μm. (C) Electron microscopy image of UMSCs-Exo, as shown by the arrow. Scale bar: 200 nm. (D) The size

distribution of exosomes recorded by NTA. (E) Western blot analysis for CD63 and TSG101 expression in UMSCs-Exo.

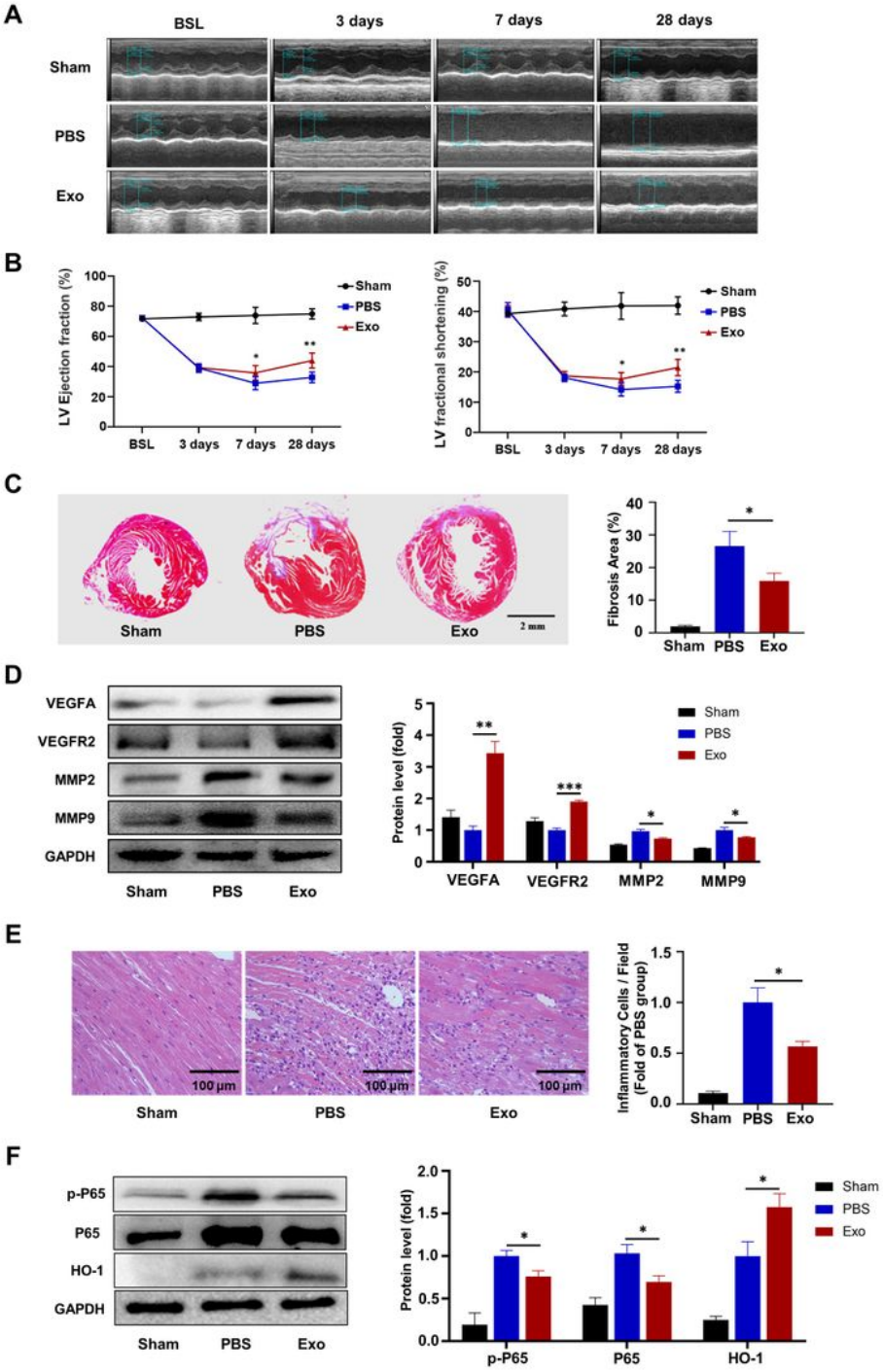

Figure 2

UMSCs-Exo preserved cardiac function and alleviated inflammation after MI. (A) Representative echocardiography recordings of infarcted hearts at different time points after MI. (B) The statistical results of left ventricular (LV) ejection fraction and LV fractional shortening parameters at different time

points after MI. n = 5. (C) The fibrosis area was showed by Masson's trichrome staining at 28 days after MI (Scale bar: 2 mm.) and relative quantification of the fibrosis area statistics (n = 4). (D) Western blot assay for VEGFA, VEGFR2, MMP2 and MMP9 expression at 28 days after MI and relative protein quantification of band intensities statistics (n = 4). (E) Inflammatory cell infiltration showed by HE staining at 3 days after MI (Scale bar: 100 μm) and relative quantification of inflammatory cell infiltration statistics (n = 4). (F) Western blot assay for p-P65, P65 and HO-1 expression at 3 days after MI. n = 4. *p < 0.05. ***p < 0.001.

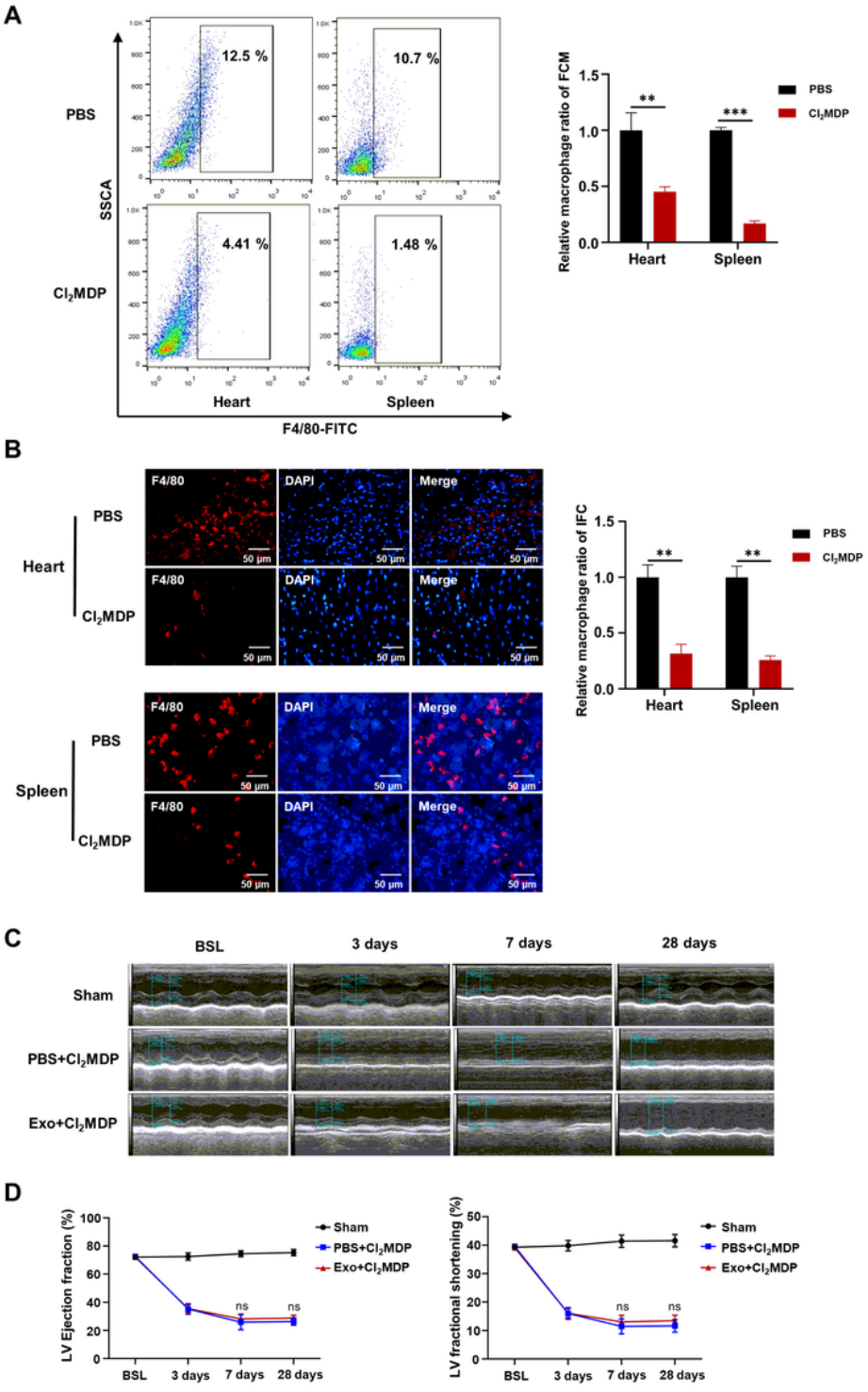

Figure 3

Depletion of endogenous macrophages weakened the efficacy of UMSCs-Exo therapy for MI in mice. (A) Flow cytometric analysis of macrophage population (F4/80+) in the hearts and spleens with and without Cl2MDP administration at 3 days after MI; and the relative quantification of macrophage depletion ratio (n = 4). (B) Macrophage population in the hearts and spleens showed by F4/80 immunofluorescence staining and the relative quantification statistics (n = 4). (C) Representative echocardiography recordings of infarcted hearts at different time points after MI. (D) The statistical results of left ventricular (LV) ejection fraction and LV fractional shortening parameters at different time points after MI. n = 5. ns $p > 0.05$. **$p < 0.01$. ***$p < 0.001$. Cl2MDP: clodronate liposomes. FCM: flow cytometry. ICF: immunofluorescence.

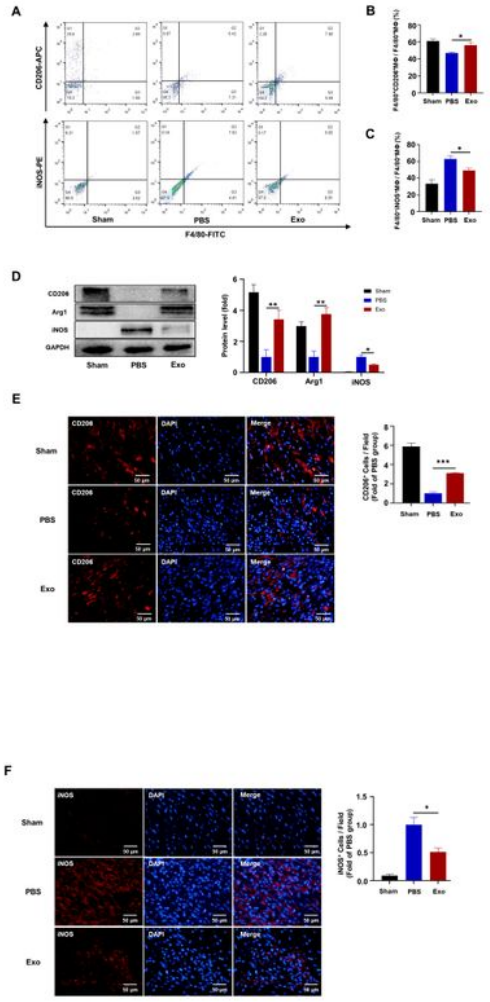

Figure 4

Effects of UMSCs-Exo on macrophage polarization at 3 days after MI. (A) Flow cytometric analysis of M2 (F4/80+CD206+) macrophages and M1 (F4/80+ iNOS+) macrophages in different groups. (B) The percentages of M2 (F4/80+CD206+) phenotype in the total macrophages (F4/80+). (C) The percentages of M1 phenotype (F4/80+iNOS+) in the total macrophages. (D) Western blot assay for CD206, Arg1 and iNOS expression and relative protein quantification. (E) Immunofluorescence assay for CD206+ cells and

relative quantification statistics. Scale bar: 50 μm. (F) Immunofluorescence assay for iNOS+ cells and relative quantification statistics. Scale bar: 50 μm. The number of statistical samples (n = 4); *p < 0.05, **p < 0.01, ***p < 0.001. MΦ: macrophages.

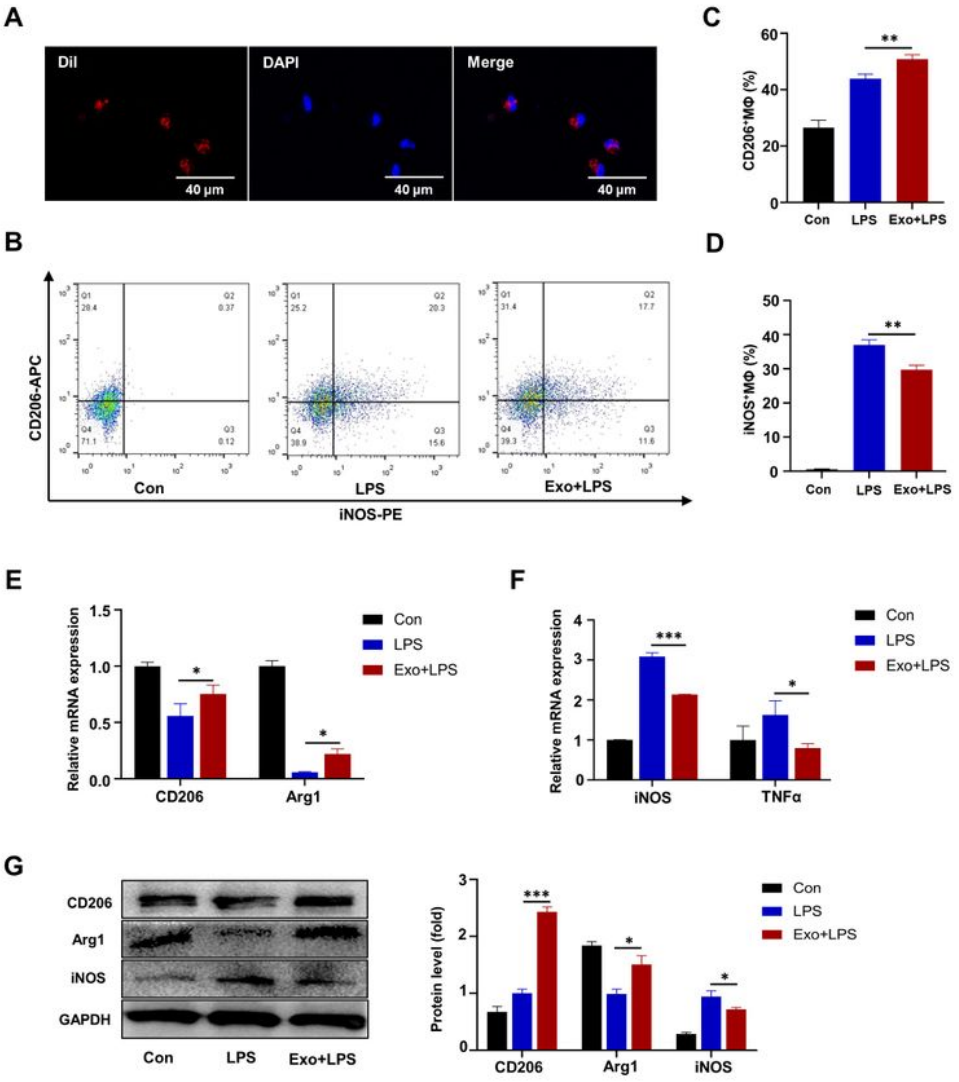

Figure 5

UMSCs-Exo Promoted M2 macrophages polarization in vitro. (A) DiI (red)-labelled UMSCs-Exo were endocytosed by RAW264.7 cells (DAPI blue). Scale bar: 40 μm. (B) Flow cytometric analysis showed the

percentages of M2 (CD206+) phenotype and M1 (iNOS+) phenotype of RAW264.7 cells. (C) Relative quantification of M2 (CD206+) macrophages. (D) Relative quantification of M1 (iNOS+) macrophages. (E) Gene expression of M2 markers (CD206 and Arg1) of RAW264.7 cells. (F) Gene expression of M1 markers (iNOS and TNFα) of RAW264.7 cells. (G) Western blot assay for CD206, Arg1 and iNOS expression of RAW264.7 cells and relative protein quantification. *p < 0.05. **p < 0.01. ***p < 0.001. MΦ: macrophages.

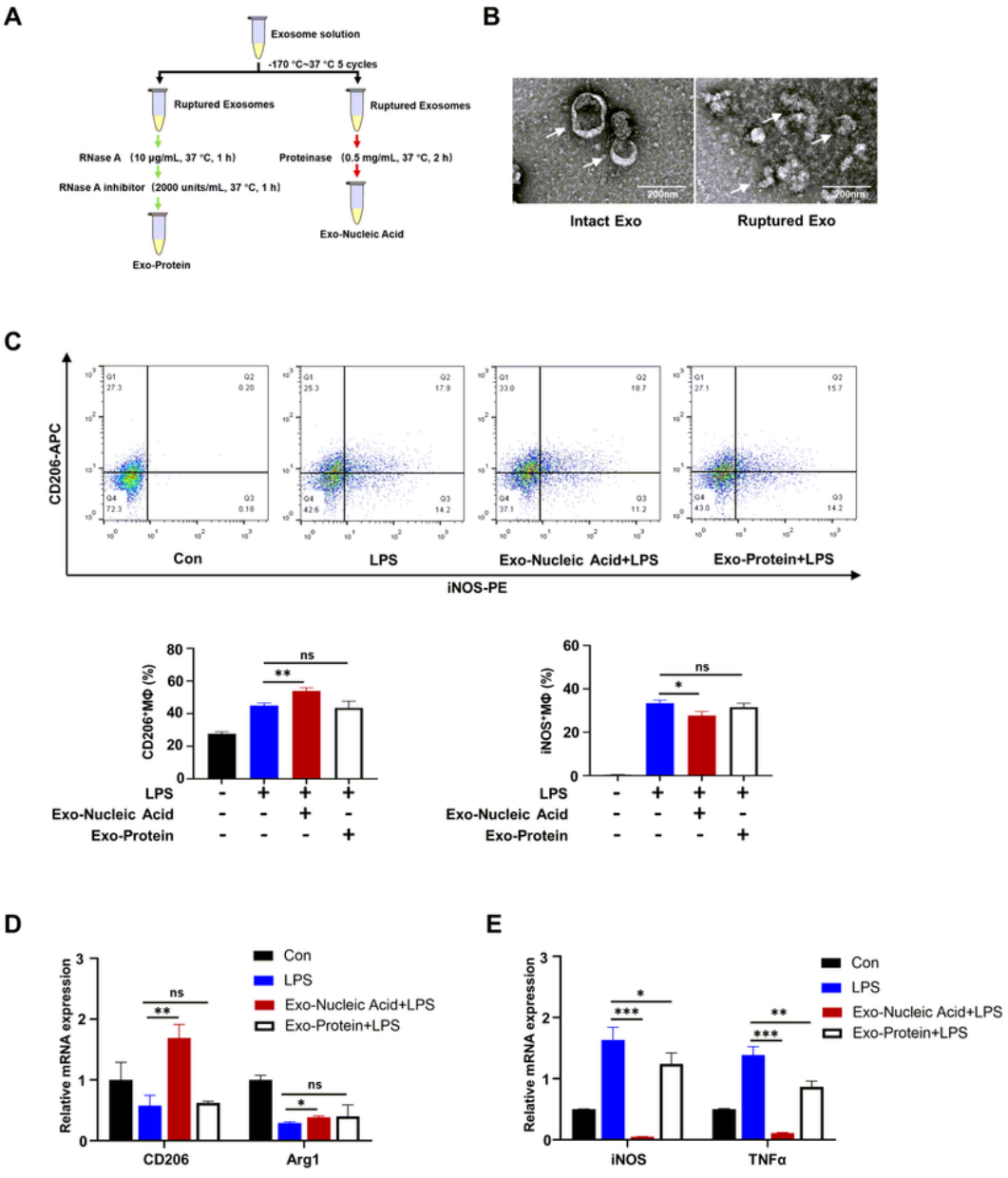

Figure 6

Nucleic acid components of UMSCs-Exo play a pivotal role in macrophage polarization. (A) The flowchart of the procedures to isolate exosomal proteins or nucleic acid. (B) TEM images of intact Exo before (Left) and ruptured Exo. Scale bar: 200 nm. (C) Flow cytometric analysis showed the percentages of M2 (CD206+) MΦ and M1 (iNOS+) MΦ of RAW264.7 cells. (D) Gene expression of M2 markers (CD206 and Arg1) of RAW264.7 cells. (E) Gene expression of M1 markers (iNOS and TNFα) of RAW264.7 cells. ns $p > 0.05$. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. MΦ: macrophages.

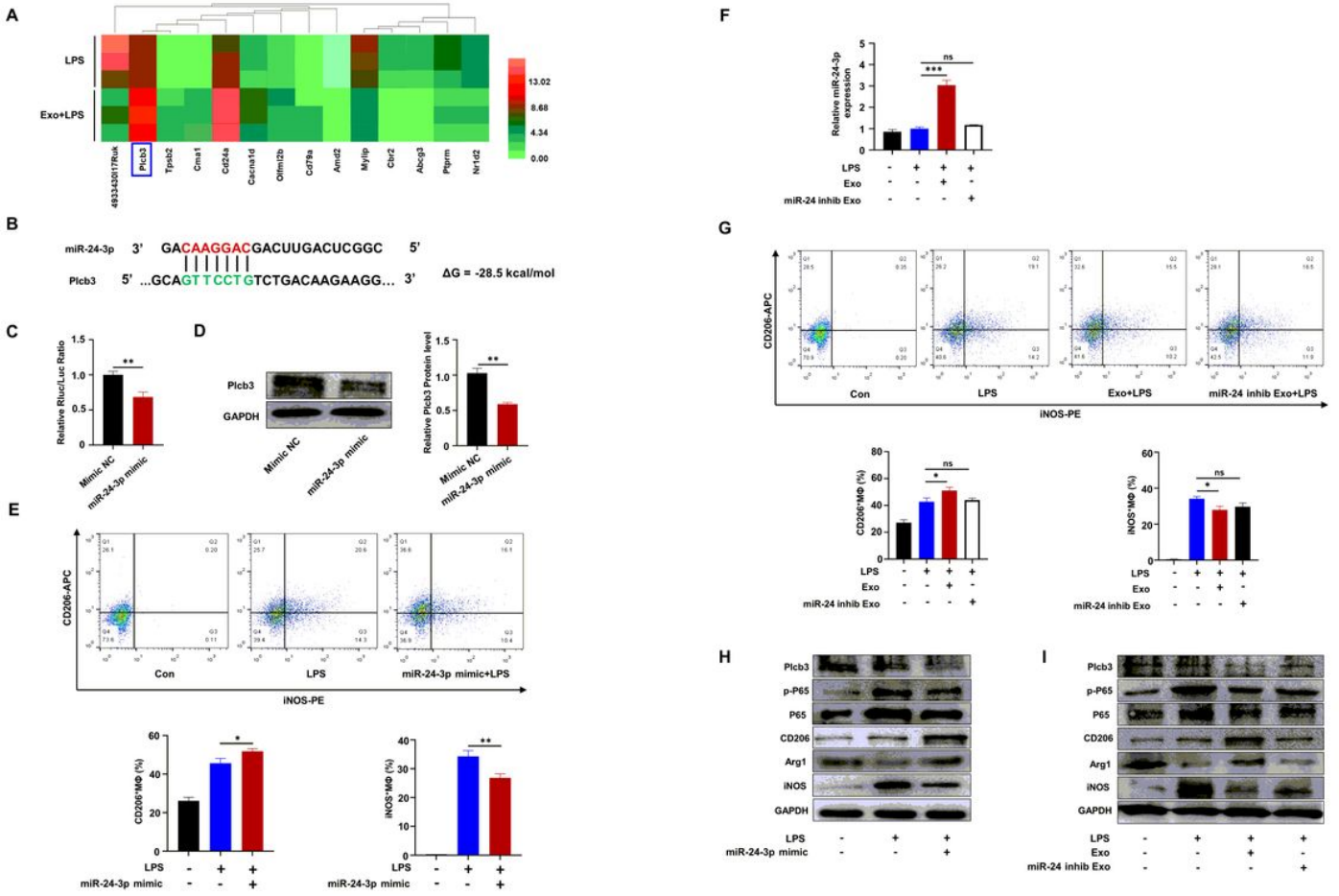

Figure 7

Plcb3 was the target gene of miR-24-3p delivered by UMSCs-Exo to mediate macrophage polarization. (A) The heat map of 14 genes related to inflammation or macrophage polarization. (B) The prediction of miR-24-3p binding sites in Plcb3. Base pairs highlighted in color (miR-24-3p in red and Plcb3 in green) are seed sequences complimentary between miRNA and target. (C) Luciferase assays of HEK293T cells co-transfected with psiCHECK™-Plcb3 vector and miR-24-3p mimic or mimic NC. (D) Western blot assay for Plcb3 expression of RAW264.7 cells after transfection of miR-24-3p mimic or NC mimic. (E) Flow cytometric analysis showed the percentages of M2 phenotype (CD206+) and M1 phenotype (iNOS+) of RAW264.7 cells after transfection of miR-24-3p mimic. (F) Relative miR-24-3p expression of RAW264.7 cells in different groups. (G) Flow cytometric analysis showed the percentages of M2 phenotype

(CD206+) and M1 phenotype (iNOS+) of RAW264.7 cells after treatment with Exo or miR-24-3p inhibitor Exo. (H) Western blot assay for Plcb3, p-P65, P65, CD206, iNOS and Arg1 expression after transfection of miR-24-3p mimic. (I) Western blot assay for Plcb3, p-P65, P65, CD206, iNOS and Arg1 expression after treatment with Exo or miR-24-3p inhibitor Exo. ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. MΦ: macrophages.

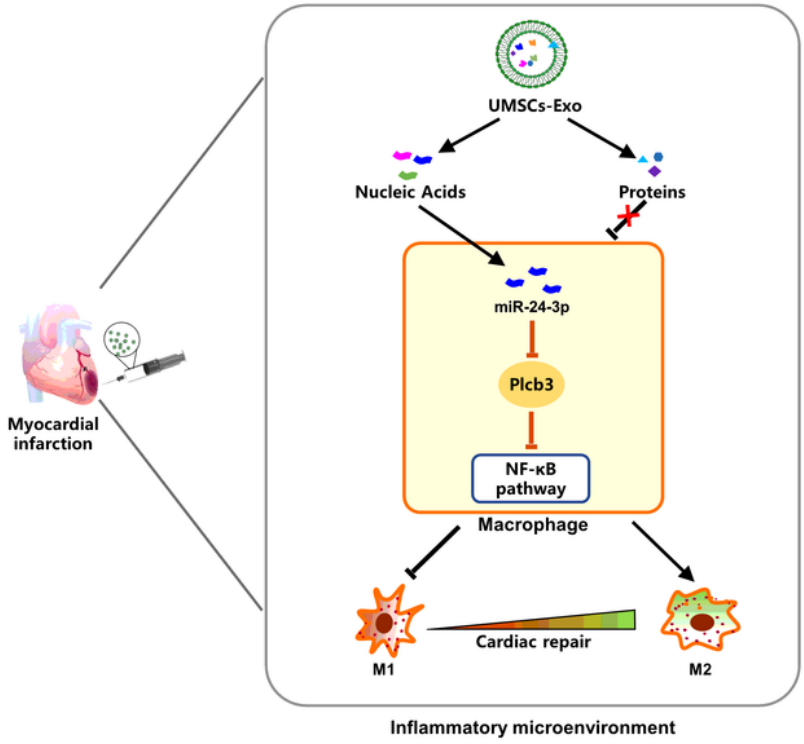

Figure 8

Mechanisms of UMSCs-Exo mediated M2 macrophage polarization. UMSCs-Exo alleviated MI injury by promoting M2 macrophage polarization through miR-24-3p mediated reduction of Plcb3/NF-κB pathway activation.

Supplementary Files

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