Microscopic polyangiitis plasma-derived exosomal miR-1287-5p induces endothelial inflammatory injury and neutrophil adhesion by targeting CBL

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Abstract

Background: An inflammatory environment around the vessel wall caused by leukocyte infiltration is one of the characteristic histopathological features of Microscopic polyangiitis (MPA); however, the pathogenic mechanisms are not fully understood. Studies have found that circulating microRNA (miRNA) can be used as potential biomarkers for the diagnosis and classification of anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV), and the E3 ubiquitin ligase casitas B-lineage lymphoma (CBL) seems to be associated with inflammation. In addition, evidence indicates that miRNA can be tracked into exosomes and transferred into recipient cells to mediate the process of vascular endothelial injury. Herein, we aimed to identify the profiles of exosomal miRNA, and determine the effect of exosomal miR-1287-5p and its target gene CBL on vascular endothelial cells in MPA.

Method: We isolated plasma exosomes from patients with MPA (MPA-exo) and healthy controls (HC-exo) by ultracentrifugation and conducted exosome small-RNA sequencing to screen differential miRNA expression in MPA-exo (n=3) compared to HC-exo (n=3). We measured the expression levels of miR-1303, miR-1287-5p, and miR-129-1-3p using quantitative reverse transcription-polymerase chain reaction (qRT-PCR, n=6) and performed dual luciferase reporter gene assays to confirm the downstream target gene of miR-1287-5p. In addition, we treated human umbilical vein endothelial cell (HUVEC) with MPA-exo, or transfected them with miR-1287-5p mimic/inhibitor or with CBL-siRNA/CBL-siRNA+ miR-1287-5p inhibitor. After cell culture, we evaluated the effects on vascular endothelial cells by examining the mRNA levels of IL-6, IL-8, MCP-1, ICAM-1 and E-selectin using qRT-PCR and performed neutrophil adhesion assay with haematoxylin staining.

Result: Transmission electron microscopy, Western blot and nanoparticle tracking analysis showed that we successfully purified exosomes and MPA-exo could be absorbed into HUVEC. We screened a total of 1077 miRNA by sequencing and observed a high abundance of miR-1287-5p in the exosomes obtained from MPA plasma. The dual luciferase reporter assay identified CBL as a downstream target gene of miR-1287-5p, and the results revealed that MPA-exo decreased CBL protein expression in HUVEC. In addition, treatment with MPA-exo, up-regulating miR-1287-5p or silencing of CBL in HUVEC significantly increased the mRNA expression of inflammatory factors (including IL-6, IL-8, and MCP-1) and adhesion molecules (including ICAM-1 and E-selection) and promoted the adhesion of neutrophils to HUVEC. However, down-regulating miR-1287-5p had the opposite effect.

Conclusion: Our study revealed that MPA-exo was involved in the intercellular transfer of miR-1287-5p and subsequently promote the development of acute endothelial injury in MPA. MiR-1287-5p and CBL agonists may be promising therapeutic approach for MPA-induced vascular inflammatory injury.

Introduction

Anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) are disorders characterized by inflammation and destruction of small-sized vessels that lead to endothelial injury and tissue damage accompanied by the presence of ANCA in serum. It is well established that myeloperoxidase (MPO) and proteinase 3 (PR3) are two major target antigens of ANCA (Nakazawa et al. 2019). AAV is divided into three clinical phenotypes: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA) (Jennette & Nachman 2017). AAV is an uncommon disease with notable ethnicity-based differences, where MPO-ANCA positive MPA predominates in East Asian countries, including China (Geetha & Jefferson 2020). Massive inflammatory injury to vascular endothelial cells can result in necrotizing vasculitis, which is one

79 of the defining histopathological features of MPA. Neutrophils are recognized as effector cells
80 responsible for endothelial damage in acute AAV-related injury (Al-Hussain et al. 2017). Neutrophils
81 primed by inflammatory cytokines interact with activated vascular endothelial cells, resulting in tissue
82 inflammation and injury of affected organs through degranulation and the release of free radicals and
83 proteases (Haubitz et al. 2009). Therefore, considering that MPA is the most common clinical AAV
84 subtype in China, further exploration of the molecular mechanism underlying inflammatory injury of
85 vascular endothelial cells in MPA is particularly crucial to understand the occurrence of acute injury,
86 which will be of great clinical significance reducing mortality.

87   Exosomes are nanometre-sized microvesicles secreted by almost all mammalian cell types and range
88 in size from 30 to 150 nm. Increasing evidence suggests that exosomes are abundant in biological fluids,
89 including serum, and circulate with the fluids, facilitating communication between various cells and their
90 microenvironment (Prikryl et al. 2020; Surmiak et al. 2021; Wang et al. 2020). Exosomes deliver
91 bioactive molecules, such as mRNA, microRNA (miRNA), long noncoding RNA, proteins, and lipids,
92 from parental cells to recipient cells, playing an important role in many diseases, including cancer, AAV,
93 and acute kidney injury (Dong et al. 2019; Huang et al. 2020; Oh & Kwon 2021). MiRNA, which is small
94 noncoding RNA with a length of approximately 20-22 nucleotides, mediates the suppression of target
95 mRNA translation and affects cellular metabolism, proliferation, and inflammatory reactions (Lara-Barba
96 et al. 2021). Growing evidence suggests that the quality and quantity of circulating miRNA in AAV
97 patients has changed compared with healthy individuals and is considered as reliable markers for diseases
98 diagnosis and classification (Skoglund et al. 2015). A recent study revealed significant correlations
99 between exosomal miR-185-3p and miR-125a-3p and MPA disease activity (Wang et al. 2020). In
100 addition, exosomal miRNA derived from neutrophils preincubation with anti-PR3 IgG induces
101 endothelial cells to release adhesion molecules and chemokines. However, the relationship between
102 plasma-derived exosomal miRNA and the inflammatory injury of vascular endothelial cells in MPA
103 remains unclear.

104   In this study, we investigated the characteristics of miRNA expression profiles in circulating
105 exosomes from MPA patients. We first isolated exosomes from plasma for small-RNA sequencing
106 analysis and then identified the candidate miRNA responsible for MPA by comparing the miRNA
107 expression profiles between MPA patients and healthy controls. MiR-1287-5p was highly expressed in
108 exosomes derived from active MPA. Considering the important role of inflammatory injury of endothelial
109 cell in the pathogenesis of MPA, we speculated that miR-1287-5p from circulating exosomes from active
110 MPA may be absorbed by vascular endothelial cells and contribute to inducing endothelial cell
111 inflammation and neutrophil adhesion.

112

Materials & Methods

113
114 **Subjects and sampling**
115   We obtained blood samples from nine MPA patients in the Department of Nephrology of the Second
116 Affiliated Hospital at Guangxi Medical University. All of the MPA patients fulfilled the criteria in the
117 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitis (Jennette et al.
118 2013) and were positive for IgG anti-MPO antibodies. We excluded patients with serious infections and
119 patients who were positive for anti–PR3 and anti–glomerular basement membrane IgG antibodies. The
120 blood samples were collected before MPA patients received glucocorticoid or immunosuppressants
121 therapy. Nine healthy control individuals (HC) matching the MPA group with respect to age and sex were
122 selected as HC group. The demographic characteristics of the MPA patients and HC in this study are

123   shown in Table 1. The Ethical Committee of the Second Affiliated Hospital at Guangxi Medical
124   University approved this study (Number: KY-0018) in accordance with the Declaration of Helsinki, and
125   we obtained written informed consent from all participants.
126

Isolation and purification of exosomes

128   We purified exosomes from the plasma of HC (HC-exo) and MPA patients (MPA-exo) as previously
129   described with minor changes (Zhang et al. 2020a). Briefly, we collected peripheral blood samples in
130   anticoagulant tubes containing EDTA and centrifuged them at 3,000×g for 15 min at 4℃ to obtain
131   plasma. We then centrifuged the plasma at 12,000×g for 45 min before filtering it through a 0.22-μm
132   filter. Next, we subjected the collected supernatant to ultracentrifugation at 120,000×g for 70 min at 4℃.
133   We washed the exosome pallet with ice-cold phosphate-buffered saline (PBS), centrifuged it at
134   120,000×g for 70 min at 4℃, and finally resuspended it in 100 μL of PBS.
135

Identification of exosomes

137   After washing the exosome suspension in PBS, we adsorbed the exosomes onto Formvar/carbon
138   support film copper-mesh grids and fixed them with 3% uranyl acetate for negative staining. We observed
139   the ultrastructure of the exosomes using transmission electron microscopy (TEM), detected the expression
140   of exosomal markers via Western blot, and analyzed the size distribution of the exosomes via nanoparticle
141   tracking analysis (NTA) using a Zetasizer Nano-ZS. We diluted the exosome samples 10,000× with
142   sterilized PBS, injected it into the chamber and quantified the exosomes using NTA software.
143

Western blot

145   We harvested the collected exosomes and the cells with RIPA lysis buffer (Beyotime Biotechnology,
146   Shanghai, China) containing 1% PMSF (a protease inhibitor) for protein extraction and examined the
147   protein concentrations using a BCA protein assay kit (Beyotime Biotechnology, China). We then mixed
148   the proteins in the different groups with loading buffer, denatured the proteins at 100℃ for 5 min and then
149   subjected the proteins to SDS/PAGE. After electrophoresis, we incubated the polyvinylidene difluoride
150   membranes with the following antibodies: anti-CD9 (1:2000, Abmart, Shanghai, China), anti-CD63
151   (1:2000, Abmart), anti-TSG101 (1:2000, Abmart), anti-CBL (1:2000, Abmart), and anti β-tubulin (1:2000,
152   Abmart). After incubating the membranes overnight at 4℃, we incubated the membranes with conjugated
153   secondary antibodies (1:10000, Abmart) for 1 hour at room temperature. We visualized the images with
154   enhanced chemiluminescence reagent (Epizyme, Shanghai, China) and quantitated them using ImageJ.
155   We compared the grayscale value of each protein signal to that of β-tubulin.
156

Culture of HUVEC and treatment with plasma-derived exosomes

158   We obtained human umbilical vein endothelial cells (HUVEC) from the cell bank of American Type
159   Culture Collection (ATCC) and cultured them at 37℃ with 5% CO_2 in endothelial cell medium (ECM)
160   containing 5% foetal bovine serum (FBS), 1% endothelial cell growth supplement, and 100 U/ml of
161   penicillin and streptomycin (HyClone, Logan, Utah, U.S.A.). We planted the cells with exosomes-
162   depleted FBS and allowed them to reach approximately 90-95% confluence before use. We prepared
163   exosomes-depleted serum by ultracentrifugation at 120,000×g at 4℃ for 18 hours followed by passage
164   through a 0.22-μm filter. We treated the HUVEC with HC–exo or MPA–exo for 24 or 48 hours. Then, we
165   subjected the treated HUVEC to the following experiments.
166

167 **Exosome uptake**
168   To trace the cellular uptake of exosomes, we stained exosomes with the green dye PKH67 (Sigma–
169 Aldrich, U.S.A.) for 5 min. After terminating the labelling reaction with 5% bovine serum albumin
170 (BSA), we harvested the exosomes and washed them with PBS by centrifugation (110,000×g for 70 min).
171 We resuspended the PKH67–labeled exosomes in FBS–free DMEM and cultured the HUVEC with 50 μg
172 of exosomes for 24 hours on confocal dishes at 37°C with 5% CO_2. After incubation, we washed the cells
173 twice with PBS, fixed them with 4% paraformaldehyde for 10 min, and counterstained them with 4′,6-
174 diamidino-2-phenylindole (DAPI) for 5 min to label the nuclei at room temperature. We then observed
175 the cellular uptake of exosomes using a laser scanning confocal microscope (Leica Microsystems, Inc.,
176 IL, U.S.A.) and analyzed the absorption efficiency between HC–exo and MPA–exo with ImageJ.
177

178 **Transfection**
179   The mimic negative control (MNC), miR-1287-5p mimic, inhibitor negative control (INC) and miR-
180 1287-5p inhibitor were synthesized by GenePharma (Shanghai, China). When the density reached
181 approximately 50-70%, we transfected HUVEC with miR-1287-5p mimic at a concentration of 50 nM
182 and miR-1287-5p inhibitor at a concentration of 100 nM. We used Lipofectamine 3000 (Invitrogen,
183 Carlsbad, CA, U.S.A.) as a transfection reagent following the manufacturer's recommendations. After
184 transfection for 6 hours, we replaced the antibiotic–free medium with complete medium and cultured the
185 HUVEC for 24 or 48 hours. Then, we transfected si–001, si–002, and si–003 (si–CBL) (RiboBio,
186 Guangzhou, China) into HUVEC to determine which siRNA most strongly decreased the mRNA
187 transcription of CBL. We performed the transfection process as described above. Subsequently, we used
188 qRT–PCR or Western blot to evaluate transfection efficiency by examining the mRNA or protein
189 expression of CBL. After determining the most efficient siRNA for CBL silencing, we transfected
190 HUVEC with siRNA negative control (si-NC), si-CBL, or si-CBL combined with miR-1287-5p inhibitor
191 for 24 or 48 hours. Table 2 displays the related sequences.
192

193 **Exosome small-RNA sequencing and bioinformatics analysis**
194   To conduct small-RNA sequencing, we lysed plasma exosomes isolated from HC (n=3) and MPA
195 patients (n=3) and extracted total RNA using a miRNeasy Micro Kit (Qiagen, Germany) according to the
196 manufacturer's instructions. We generated sequencing libraries using an NEBNext® Multiplex Small
197 RNA Library Prep Set for Illumina® (NEB, USA). We clustered the index-coded samples on a cBot
198 Cluster Generation System using a TruSeq SR Cluster Kit v3-cBot-HS (Illumina) according to the
199 manufacturer's instructions. After cluster generation, we sequenced the library preparations sequenced on
200 an Illumina HiSeq 2500/2000 platform. The Software Novomagic was used to obtain the potential
201 miRNA and volcano map. We considered each miRNA with a p value<0.05 and a $\log_2|$(fold change)$| > 2$
202 to be physiologically relevant. A volcano map showed the differential miRNA expression profiles among
203 exosome samples. The statistical power of experimental design, calculated in RNASeqPower is (depth =
204 15, cv = 0.4, effect = c (0.8,1.1), alpha = 0.05, power = c(0.8,0.9)).
205

206 **RNA extraction and qRT-PCR**
207   We extracted miRNA from the exosomes with a miRNeasy Serum/Plasma Kit (Qiagen, Germany)
208 according to the manufacturer's instructions. We reverse transcribed the extracted miRNA with a miRcute
209 Plus miRNA First-Stand cDNA kit and detected it with a miRcute Plus miRNA SYBR Green qPCR Kit
210 (Tiangen, Beijing, China). We extracted total RNA from HUVEC using TRIzol Reagent (Invitrogen) and

211 performed qRT–PCR with HiScriptII Q RT SuperMix and ChamQ Universal SYBR qPCR Master Mix
212 kits (Vazyme, Shanghai, China). We used an ABI StepOne Real-time PCR instrument (Applied
213 Biosystems, Waltham, MA, U.S.A.) for quantitative analyses. We measured the relative expression levels
214 of miRNA and mRNA using the $2^{-\Delta\Delta Ct}$ method and standardized them to the expression levels of cel-miR-
215 39 and GAPDH, respectively. Sangon (Shanghai, China) synthesized the primers for miRNA detection,
216 while Vazyme synthesized the primers for mRNA examination. The primer sequences are listed in Table
217 2.
218

219 **Neutrophil adhesion assay**
220 We performed a neutrophil adhesion experiment with minor modifications as previously described
221 (Choi et al., 2017; Nistri et al., 2003). We isolated neutrophils from the peripheral venous blood of
222 healthy volunteers by using Ficoll-Paque density gradient centrifugation (Tiangen, China) and determined
223 the purity of neutrophils by Giemsa staining. We primed freshly prepared neutrophils (2×10^5) with 10
224 μg/mL lipopolysaccharide (LPS, Sigma–Aldrich, U.S.A.) for 30 min at 37℃ and added them to HUVEC.
225 We maintained the cocultures for 120 min, rinsed them with PBS three times to remove unbound
226 neutrophils, fixed them in 4% formaldehyde and counterstained them with haematoxylin for light
227 microscopic screening. We performed each group experiment three times and counted the number of
228 adherent neutrophils and HUVEC in five randomly chosen microscopic fields at a 200× final
229 magnification. Finally, we analyzed the ratio of the neutrophils number to HUVEC number.
230

231 **Dual-luciferase reporter assay**
232 We predicted miR-1287-5p target genes using the TargetScan database available on the internet
233 (www.targetscan.org). We performed a dual-luciferase reporter assay to verify whether *CBL* is the target
234 mRNA of miR-1287-5p. Briefly, we constructed a wild-type *CBL* 3′-untranslated region (3′-UTR)
235 reporter plasmid (*CBL* Wt) and a mutated-type *CBL* 3′-UTR reporter plasmid (*CBL* Mut) with the pMIR-
236 promoter vector by OBiO Technology (Shanghai, China). We plated HEK293T cells in 96-well plates and
237 transfected them with *CBL* Wt or *CBL* Mut in the presence of miR-1287-5p mimic or negative control
238 (NC) in strict accordance to the manufacturer's specifications. At 48 hours after transfection, we
239 quantified luciferase activity in the cell lysates with a luciferase assay kit (Promega).
240

241 **Statistical analysis**
242 We performed all statistical analyses using SPSS software and GraphPad Prism 7. We express the
243 data as the mean ± SD. We performed comparisons between two groups using independent-sample *t*-tests.
244 We considered $p < 0.05$ to indicate statistical significance.
245

246 # Results
247 **Characterization and internalization of exosomes derived from plasma**
248 We collected blood samples from both MPA patients and HC. We identified plasma-derived
249 exosomes by TEM, NTA and Western blot. We observed typical cup-shaped vesicles, ~100 nm in
250 diameter, using TEM (Fig. 1A). We confirmed the expressions of exosomal marker proteins in the two
251 groups, including CD9, CD63 and TSG101, by Western blot analysis (Fig. 1B). NTA showed that the
252 particle size peaks from MPA patients and HC were 80 nm and 100 nm, respectively (Fig. 1C). These
253 results revealed that we successfully isolated exosomes from plasma.

254   To further determine whether HUVEC absorbed the plasma-derived exosomes, we cocultured
255   exosomes labelled with PKH67 (a green fluorescent cell linker) with HUVEC. After 24 hours of
256   incubation, the HUVEC had efficiently internalized PKH67–labeled exosomes, as indicated by confocal
257   laser scanning microscopy (Fig. 1D), which indicated that HUVEC could take up plasma-derived
258   exosomes. Moreover, there was no significant difference between HC–exo and MPA–exo in the average
259   grey value (Fig. 1E).
260

261   **Treatment of HUVEC with MPA-exo induced endothelial inflammation and neutrophil adhesion**
262   To investigate whether MPA-exo induced endothelial inflammation, we cocultured HUVEC with
263   HC-exo and MPA-exo for 24 hours and measured the expression of inflammatory factors by qRT–PCR.
264   As shown in Fig. 2A, the relative mRNA levels of IL-6, IL-8, and MCP-1 (fold changes: 1.44, 1.82 and
265   2.07, respectively) and of the adhesion molecules ICAM-1 and E-selectin (fold changes: 1.81 and 1.86,
266   respectively) were significantly higher in HUVEC treated with MPA-exo than in HUVEC treated with
267   HC-exo ($p < 0.05$).
268   To assess whether MPA-exo attracted neutrophils to the endothelium, we first isolated neutrophils
269   by using Ficoll-Paque density gradient centrifugation. We observed that the cytoplasm was light red, the
270   nuclei was rod-like or 2~5 lobulated, and the purity of cells ranged from 92 to 96% (Fig. 2B). The results
271   indicated that we have successfully isolated neutrophils and the isolated neutrophils could be used for
272   experiments. Then, we cultured HUVEC in the presence of HC-exo or MPA-exo and subsequently
273   incubated the HUVEC monolayer with primed LPS-stimulated neutrophils. As shown in Fig. 2C and 2D,
274   treatment with MPA-exo enhanced the adhesion of neutrophils to HUVEC, and the number of neutrophils
275   adhering to HUVEC was increased three times that in the HC-exo group according to the ratio of
276   neutrophil/HUVCE (HC-exo: 0.12±0.03; MPA-exo: 0.24±0.01; $p = 0.002$). Our results indicated that
277   MPA-exo treatment significantly increased proinflammatory cytokine release in HUVEC and neutrophil
278   adhesion to HUVEC.
279

280   **The miRNA expression profiles of plasma exosomes in MPA patients**
281   To characterize whether the miRNA in MPA-exo play an important role in the development of MPA,
282   we used RNA sequencing to explore the miRNA spectrum between HC and MPA patients. We screened a
283   total of 1077 miRNA in exosomes purified from plasma samples. According to the criteria of a p value＜
284   0.05 and a $\log_2|$(fold change)$| > 2$, we identified 9 differentially expressed miRNA. Of these, 6 miRNA
285   were upregulated and 3 miRNA were downregulated in MPA (Table 3). The volcano map displays the
286   overall distribution of differentially expressed miRNA (Fig. 3A). Moreover, we randomly selected three
287   differentially expressed exosomal miRNA (miR-1303, miR-129-1-3p, and miR-1287-5p) for qRT–PCR
288   to validate the sequencing data. We normalized the expression levels of these miRNA to that of cel-miR-
289   39 as a control. The mRNA expressions of miR-1287-5p and miR-1303 were significantly upregulated in
290   MPA samples compared with HC samples, whereas that of miR-129-1-3p was significantly
291   downregulated ($p < 0.05$, Fig. 3B).
292

293   **MiR-1287-5p enhanced HUVEC inflammatory response and neutrophil adhesion**
294   For further study, we first examined the basal expression of miR-1303, miR-129-1-3p and miR-
295   1287-5p in HUVEC (Fig. 4A). We found that the basic expression of miR-1303 was relatively high in
296   HUVEC, which indicated that exogenous exosomal miR-1303 had difficulty affecting the endogenous
297   expression level of miR-1303 in HUVEC. The basal expression of miR-129-1-3p in HUVEC and the

298   expression of miR-129-1-3p in plasma-derived exosomes (HC-exo and MPA-exo) were not high, which
299   also suggested that exogenous exosomal miR-129-1-3p and the miR-129-1-3p inhibitor had little
300   influence on the expression of miR-129-1-3p in HUVEC. Therefore, we tested only the changes in miR-
301   1287-5p expression after the uptake of exosomes by HUVEC after 24 hours, and found that the
302   expression levels of miR-1287-5p increased in the HUVEC ($p < 0.05$, Fig. 4B). Subsequently, we
303   determined the effects of miR-1287-5p on HUVEC's inflammatory response and neutrophil adhesion.
304   The qRT–PCR results shown in Fig. 4C suggested that the miR-1287-5p mimic predominantly increased
305   the mRNA expression of IL-6, IL-8 and MCP-1 (fold changes: 2.29, 1.99, and 2.50, respectively), while
306   the miR-1287-5p inhibitor significantly inhibited the mRNA expression of these factors compared to that
307   in the control group (fold changes: 0.50, 0.36 and 0.31, respectively, $p < 0.05$); we obtained marginal
308   evidence for MCP-1 ($p = 0.081$). We also investigated the mRNA expression of adhesion molecules by
309   qRT–PCR. In comparison with the control group, ICAM-1 and E-selectin mRNA levels were markedly
310   elevated after treatment with miR-1287-5p mimic (fold changes: 2.36 and 1.17), but were reduced after
311   treatment with the miR-1287-5p inhibitor (fold changes: 0.53 and 0.26, $p < 0.05$, Fig. 4C).
312   The neutrophil adhesion assay results shown in Fig. 5A revealed that miR-1287-5p mimic promoted
313   the adhesion of primed neutrophils to the HUVEC, which was suppressed by the miR-1287-5p inhibitor
314   (Fig. 5B, $p < 0.05$). Similarly, compared with MNC group, the number of neutrophils adhering to
315   HUVEC increased 2.36 times in miR-1287-5p mimic group according to the ratio of neutrophil/HUVEC
316   (MNC: 0.5±0.14; mimic: 1.18±0.14; $p = 0.004$); while the ratio of neutrophil/HUVEC in miR-1287-5p
317   inhibitor group was decreased in contrast to INC group (INC: 0.53±0.12; inhibitor: 0.31±0.04, $p = 0.036$).
318

319   **CBL is a target gene of miR-1287-5p**
320   We next investigated the downstream mechanism by which plasma-derived exosomal miR-1287-5p
321   induces HUVEC inflammatory response and neutrophil adhesion. We predicted a binding site in CBL-
322   mRNA for miR-1287-5p using TargetScan (Fig. 6A). We constructed *CBL* Wt and *CBL* Mut in OBiO
323   Technology (Shanghai, China, Fig. 6B) and used them to transfect HEK393T cells together with miR-
324   1287-5p mimic or MNC. The results of the dual luciferase reporter assay suggested that cotransfection
325   with miR-1287-5p mimic decreased the luciferase activity of *CBL* Wt ($p < 0.001$), but not that of *CBL*
326   Mut ($p = 0.0031$, Fig. 6C). Subsequently, we determined the *CBL*-mRNA levels using qRT–PCR after
327   transfecting HUVEC with a miR-1287-5p mimic or a miR-1287-5p inhibitor for 24 hours. As shown in
328   Fig. 6D, *CBL*-mRNA was downregulated in HUVEC treated with the miR-1287-5p mimic ($p < 0.05$). The
329   *CBL*-mRNA level in HUVEC increased by 1.35 times after transfection with the miR-1287-5p inhibitor,
330   but the difference was not significant ($p = 0.058$). Western blot analysis also demonstrated that HUVEC
331   treated with miR-1287-5p mimic for 48 hours exhibited marked reductions in CBL protein expression;
332   however, HUVEC treated with miR-1287-5p inhibitor exhibited upregulation of CBL protein expression
333   (Fig. 6E). In brief, these results indicated that *CBL*-3'UTR-mRNA contains a binding site targeted by
334   miR-1287-5p.
335

336   **Downregulation of CBL promoted inflammatory response and neutrophil adhesion in HUVEC**
337   To determine whether MPA-exo affect CBL protein expression in HUVEC, we cultured HUVEC in
338   the presence of NC-exo or MPA-exo for 48 hours. As shown in Fig. 7A, treatment with MPA-exo
339   decreased CBL protein expression in HUVEC ($p < 0.05$). Subsequently, to explore the effect of *CBL* gene
340   on inflammation and neutrophil adhesion in HUVEC, we used siRNA (si-001, si-002, si-003) to silence
341   *CBL*-mRNA expression. Given the results of both qRT–PCR and Western blot, the most efficient siRNA

342   for *CBL* silencing was si-003 (Fig. 7B&C). HUVEC transfected with si-CBL (si-003) exhibited
343   significantly increased the mRNA expression of IL-6, IL-8, MCP-1, ICAM-1 and E-selectin after 48
344   hours compared with that in the si-NC group (fold changes: 1.44, 3.44, 2.14, 3.87, and 5.03, respectively,
345    $p < 0.05$, Fig. 7D). Blocking CBL protein expression also contributed to neutrophil adhesion on HUVEC,
346   the ratio of neutrophil/HUVEC was increased in si-003 group compared with si-NC group (si-NC:
347   0.55±0.06; si-003: 1.66±0.14; $p < 0.05$, Fig. 8A&B). In addition, the dysfunction induced by si-CBL was
348   reversed by the miR-1287-5p inhibitor. Compared with si-003 group, the mRNA expression of IL-6, IL-8,
349   MCP-1, ICAM-1 and E-selectin was decreased in si-003+miR-1287-5p inhibitor group (fold changes:
350   0.74, 0.43, 0.71, 0.39, and 0.28, respectively, $p < 0.05$). The ratio of neutrophil/HUVEC in si-003 group
351   and si-003+miR-1287-5p inhibitor group was 1.66 and 0.48, respectively. Taken together, the results
352   revealed that CBL protein inhibition was associated with an increased inflammatory response and
353   increased neutrophil adhesion.
354

Discussion

356   The pathophysiological mechanisms associated with MPA are complex and are not entirely
357   understood. An increasing number of studies have confirmed an inflammatory damage to the vascular
358   endothelium caused by leukocyte infiltration, a pathological hallmark of MPA (Nagao et al. 2011;
359   Rymarz et al. 2021). In the present study, we aimed to determine whether circulating exosomes from
360   MPA are involved in vascular endothelial inflammatory damage and to explore the potential mechanisms.
361   The results showed that MPA-exo increased the release of inflammatory factors and adhesion molecules
362   in HUVEC, and promoted the adhesion of neutrophils to HUVEC. Furthermore, they suggested that
363   exosomal miR-1287-5p might regulate vascular endothelial inflammatory damage by targeting CBL.
364   Exosomes are potent intercellular communicators that mediate cell injury or repair by carrying
365   certain cargo (such as miRNA) into recipient cells and have attracted extensive research attention in
366   recent years (Xu et al. 2019). Previous studies have indicated that exosomes participate in immune
367   modulation and are related to the immune pathogenesis of autoimmune diseases, such as systemic lupus
368   erythematosus (SLE) and rheumatoid arthritis (RA) (Tan et al. 2016). Further investigations have shown
369   that exosomal miRNA participate in the progression of various diseases by affecting the inflammatory
370   response (Miao et al. 2021; Wang et al. 2021). Exosomes are cup-shaped membrane vesicles with a
371   diameter between 30 and 150 nm that are visible under electron microscopy. Researchers can detect the
372   unique proteins exposed to exosomal membranes to further characterize exosomes. Exosomes from
373   different sources can express proteins that participated in multivesicular body biogenesis (such as Alix
374   and TSG101), integrins and tetraspanins (such as CD9, CD63, CD81 and CD82) (Shao et al. 2018). In
375   this study, the extracellular vesicles we isolated from plasma were cup-shaped membrane vesicles with
376   particle size peaks of 80 nm and 100 nm that contained unique exosomal proteins, including CD9, CD63
377   and TSG101. The findings revealed that we successfully purified exosomes from the plasma of MPA
378   patients and HC. Currently, the commonly used exosome isolation methods include ultracentrifugation,
379   gradient ultracentrifugation, and commercial assays. Royo *et al*. analysed the expression of exosomal
380   miRNA using miRNA profiling and found that the approach use to isolate exosomes had little effect on
381   exosome-associated miRNA (Royo et al. 2016). To reduce the influences of the solvent in the commercial
382   kits on the subsequent cell experiments, we used the most commonly conventional ultracentrifugation
383   method for exosome isolation.
384   Exosomal entry into recipient cells occurs via a variety of mechanisms, such as
385   endocytosis/phagocytosis, fusion, and receptor/ligand signalling (McKelvey et al. 2015; Svensson et al.

386   2013). Similar to other types of cells, vascular endothelial cells have the ability to produce and take up
387   exosomes. Therefore, circulating exosomes may be captured by vascular endothelial cells throughout the
388   body and cause cell injury, which may contribute to the various pathophysiologies of organs in the above-
389   described diseases and MPA (Gao et al. 2021; Roig-Carles et al. 2021; Wang et al. 2020). In the current
390   study, we observed that exosomes isolated from the plasma of MPA patients and HC could be absorbed
391   into HUVEC via endocytosis. The results further showed that MPA-derived exosomes induced an
392   inflammatory response in HUVEC with neutrophil adhesion. To fully explore the molecular mechanism
393   by which MPA-exo promote inflammation, we performed RNA sequencing to identify the exosomal
394   miRNA profile. Overall, we screened a total of 1077 miRNA, of which 6 miRNA were significantly
395   upregulated and 3 miRNA were significantly downregulated in MPA. As discussed in a previous study,
396   our work did not produce the same differential miRNA profiles as those reported by Wang *et al*. (Wang et
397   al. 2020). This could be attributable to minor technical differences or sample storage procedures. Te *et al*.
398   reported that miRNA profiles are differentially expressed in lupus nephritis in different racial populations.
399   (Te et al. 2010). Furthermore, we identified by qRT–PCR that miR-1287-5p was significantly enriched in
400   MPA plasma–derived exosomes compared exosomes from HC. We showed a significant uptake of
401   exosomal miR-1287-5p by HUCEC. The results might indirectly indicate that plasma exosomes in MPA
402   may transfer pro-inflammatory miRNA such as miR-1287-5p to the site of vasculitis. A previous review
403   has reported that exosomes play a critical role in the development of autoimmune vasculitis (Wu et al.
404   2019). In addition, circulating exosome levels are correlated with disease activity in SLE (Lee et al.
405   2016). Therefore, we hypothesize that circulating exosomes might be absorbed by endothelial cells in
406   MPA and participate in vasculitis progression through a proinflammatory effect of miR-1287-5p.
407   MiR-1287-5p was first identified in tumors as a significant suppressor in different cancer, including
408   breast cancer (Schwarzenbacher et al. 2019), Cervical Cancer (Ji et al. 2020), pancreatic cancer (Zhang et
409   al. 2020b), and non-small cell lung cancer (Shanshan et al. 2021). MiR-1287-5p regulates cell growth,
410   apoptosis, ferroptosis, and invasion by targeting phosphoinositide 3-kinase CB, glutathione peroxidase 4,
411   and Y-box binding protein 1 (Cui et al. 2020; Schwarzenbacher et al. 2019; Shanshan et al. 2021). Recent
412   studies have proposed that miR-1287-5p also participates in other biological functions. Transfecting miR-
413   1287-5p–mimic,increases caspase-3 activity and inhibits angiogenesis in HUVEC by targeting
414   angiopoietin-1 (Sanchez et al. 2019). According to Zhang *et al*., miR-1287-5p is a target of circ_0004812,
415   and participates in HBV-induced immune suppression by targeting follistatin-related protein (FSTL1)
416   (Zhang & Wang 2020). In a late diabetic kidney disease cohort, Satake *et al*. demonstrated that circulating
417   miR-1287-5p was strongly associated with the 10-year risk of end-stage kidney disease. In addition, miR-
418   1287-5p mimic indirectly upregulated the concentrations of EPH receptor A2 (EPHA2) in HUVEC lysate,
419   supernatants, or both by targeting CBL (Satake et al. 2021). EPHA2 activation has been shown to induce
420   proinflammatory gene expression and stimulate monocyte adhesion in endothelial cells, which indicates
421   that miR-287-5p plays a role in promoting the endothelial cell inflammatory response (Funk et al. 2012).
422   Based on these findings, we speculated that miR-1287-5p carried by plasma-derived exosomes is related
423   to the impact of MPA on vascular inflammation. The results of our study may support this hypothesis. We
424   observed elevated production of proinflammatory genes, activation of endothelial cells and increased
425   adhesion of neutrophils to HUVEC in the miR-1287-5p mimic group; conversely, these effects were
426   reversed with miR-1287-5p inhibitor treatment. The results of this study as well as previous research have
427   demonstrated that the microenvironment of exosomal parent cells affects the cargo and function of
428   exosomes and that exosomes derived from pathological conditions may mediate body injury (Chang et al.
429   2018).

430   MiRNA induce posttranscriptional gene silencing by binding to the 3′UTR of target genes and
431   mostly interrupting mRNA expression and/or inhibiting protein synthesis. To elucidate the molecular
432   mechanism by which miR-1287-5p influences the HUVEC inflammatory response, we predicted possible
433   target genes of miR-1287-5p using the TargetScan algorithm. Among the targets, CBL was reported to be
434   associated with the inflammatory response (Duan et al. 2021), so we selected CBL as the potential target
435   of miR-1287-5p. The dual-luciferase reporter assay supported this hypothesis, as miR-1287-5p reduced
436   the activity of the luciferase/CBL-3′UTR construct. In addition, Western blot indicated that the miR-
437   1287-5p mimic downregulated CBL protein expression in HUVEC, while the miR-1287-5p inhibitor
438   enhanced CBL expression. Previous research has identified CBL, a E3 ubiquitin ligase, as a negative
439   regulator of receptor tyrosine kinase (RTK) signalling, and the function of CBL is dependent on the
440   ubiquitylation of active RTKs (Wang et al. 2002). CBL participates in controlling immature helper T-cell
441   development, immune tolerance, and angiogenesis. It has been reported that CBL might alleviate
442   endothelial dysfunction in patients with diabetes mellitus by inactivating the JAK2/STAT4 signalling
443   pathway (Jin et al. 2021). In addition, a study by Duan *et al*. demonstrated the role of CBL in regulating
444   intestinal inflammation via inhibition of fungus-induced noncanonical NF-κB activation (Duan et al.
445   2021). The above findings reveal the endothelial protective function and anti-inflammatory function of
446   CBL. In addition, Chiou *et al*. reported that CBL is a critical mediator in Toll-like receptor stimulation,
447   which plays a key role in the pathogenesis of AAV (O'Sullivan et al. 2018). Here, we showed that CBL
448   expression was decreased in HUVEC when exposed to MPA-exo. We also found that the reduction in
449   CBL expression caused by transfection of HUVEC with siRNA had an effect as similar to that of the
450   miR-1287-5p mimic on the function of HUVEC. These findings suggest that CBL deficiency promotes
451   the inflammatory response and neutrophil adhesion of HUVEC, which are pathological features of MPA.
452   Alveolar capillary inflammation can cause pulmonary haemorrhage followed by hypoxia (Hayes-Bradley
453   2004). Hypoxia is a typical microenvironmental feature of most autoimmune diseases, affecting a variety
454   of physiological processes (Deng et al. 2022; Li et al. 2022b; Lohrasbi et al. 2022). One study has shown
455   that sustained hypoxia stress can repress the CBL expression and disrupt ubiquitin degradation, leading to
456   impaired regulation of human trophoblasts, which is the main mechanism of preeclampsia (Li et al.
457   2022a). However, it has also been shown that hypoxia induces an increase in CBL expression in H9c2
458   cells. Thus far, there have been few studies on the impacts of hypoxia on MPA, and the relationship
459   between hypoxia and CBL in MPA also needs further exploration.
460   Nevertheless, this study encountered a limitation. We only measured cytokine mRNA and not
461   protein that promotes the inflammatory environment. It is well known that mRNA would be translated to
462   protein. However, we did not prove this and further investigation is expected.

Conclusions

464   The current study showed that miR-1287-5p was significantly enriched in MPA exosomes. In a
465   HUVEC cell model, it mediated the expression of inflammatory factors and adhesion molecules, and
466   promoted neutrophil adhesion to HUVEC by targeting CBL, which was associated with a vascular
467   inflammatory environment. Thus, miR-1287-5p might contribute to the acute injury in MPA. As an E3
468   ubiquitin ligase, CBL is involved in inflammation and immune regulation. In this study, MPA-exo
469   reduced CBL protein expression, and CBL deficiency induced an inflammatory response in HUVEC.
470   Whether CBL agonists can protect against inflammatory injury of endothelial cells and play a therapeutic
471   role in AAV warrants further investigation. In summary, our findings demonstrate, for the first time, that
472   miR-1287-5p target *CBL* and may provide a potential new target for the treatment of MPA-induced
473   vascular inflammatory injury.

474

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641

Characteristics of study subjects

1 Table 1. Characteristics of study subjects

Demographic	MPA	HC
Number	9	9
Age (y)	59.22±12.05	50.89±6.86
Sex		
Female	7	7
Male	2	2
Hemoglobin (g/L)	73.22±26.17	-
WBC (×10⁹/L)	10.95±5.34	-
CRP (mg/L)	55.05±44.44	-
Creatinine (μmol/L)	508.67±439.68	-
MPO-ANCA	246.67±110.00	-
BVAS	16.89±3.37	-

2 MPA: microscopic polyangiitis; HC: healthy control; WBC: white blood cell; CRP: C-reactive protein; BVAS:
3 Birmingham Vasculitis Activity Score.
4
5
6 Table 2. Prime sequences for qRT-PCR

Gene	Sequences
Human IL-6	Forward: 5'-ACTCACCTCTTCAGAACGAATTG-3'
	Reverse: 5'-CCATCTTTGGAAGGTTCAGGTTG-3'
Human IL-8	Forward: 5'-GCCTTCCTGATTTCTGCAGC-3'
	Reverse: 5'-TGCACTGACATCTAAGTTCTTTAGCA-3'
Human MCP-1	Forward: 5'-AACTGAAGCTCGCACTCTCG-3'
	Reverse: 5'-TCAGCACAGATCTCCTTGGC-3'
Human ICAM-1	Forward: 5'-CAGTCACCTATGGCAACGAC-3'
	Reverse: 5'-ATTCAGCGTCACCTTGGCTC-3'
Human E-selectin	Forward: 5'-CTCTGACAGAAGAAGCCAA-3'
	Reverse: 5'-TTGAGTCCACTGAAGCCAGG-3'
Human GAPDH	Forward: 5'-ATTGTTGCCATCAATGACCC-3'
	Reverse: 5'-AGTAGAGGCAGGGATGATGT-3'
Human miR-1287-5p	5'-UGCUGGAUCAGUGGUUCGAGUC-3'
Human miR-1303	5'-UUUAGAGACGGGGUCUUGCUCU-3'
Human miR-129-1-3p	5'-AAGCCCUUACCCCAAAAAGUAU-3'
Cel-miR-39	5'-UCACCGGGUGUAAAUCAGCUUG-3'
Human miR-1287-5p mimic	Sense: 5'-UGCUGGAUCAGUGGUUCGAGUC-3'
	Antisense: 5'- CUCGAACCACUGAUCCAGCAUU-3'
Human miR-1287-5p inhibitor	5'-GACUCGAACCACUGAUCCAGCA-3'
Human si-001 (si-CBL)	5'-CTACCAGCATCTCCGTACT-3'

| Human si-002 (si-CBL) | 5'-GAGCTTTCGACAGGCTCTA-3' |
| Human si-003 (si-CBL) | 5'-TCGGATTACTAAAGCAGAT-3' |

8   Table 3. The expression of differential miRNAs in plasma derived exosomes between
9   MPA patients and healthy controls

miRNA	log2 (fold change)	Up or Down	*P* value
hsa-miR-223-3p	2.1	UP	0.044
hsa-miR-1303	3.1	UP	0.044
hsa-miR-1287-5p	3.9	UP	0.029
hsa-miR-3688-5p	5.0	UP	0.034
has-miR-200b-5p	5.1	UP	0.017
has-miR-656-3p	5.1	UP	0.026
hsa-miR-129-1-3p	5.6	DOWN	0.038
Novel_78	5.8	DOWN	0.030
Novel_310	3.6	DOWN	0.048

Figure 1

Characterization of the plasma-derived exosomes

(A) Representative micrograph of exosome was visualized by transmission electron microscopy (Scale bar = 100 nm). The red arrow indicates the exosome. (B) Marker proteins CD9, CD63, and TSG101 in exosomes were determined by Western blot. (C) Nanoparticle tracking analysis was used to measure size distribution of exosomes. (D) The uptake of PKH67-labeled exosomes by HUVEC observed using confocal laser scanning microscopy following co-culture for 24 hours (Scale bar = 25 μm). (A) Images of confocal laser scanning microscope. The nuclei of HUVEC were stained with DAPI (Blue), the exosome was labeled with PKH67 (green), Red arrow indicates the exosomes were localized in the cell nucleus. (E) Showing comparisons of average gray value between HUVEC absorbing exosomes from HC and MPA. Data are presented as mean ± SD and compared with t-test. HC-exo, exosomes derived from healthy controls. MPA-exo, exosomes derived from MPA patients; and HUVEC, human umbilical vein endothelial cells.

A

B

CD9			25KD
CD63			26KD
TSG101			44KD

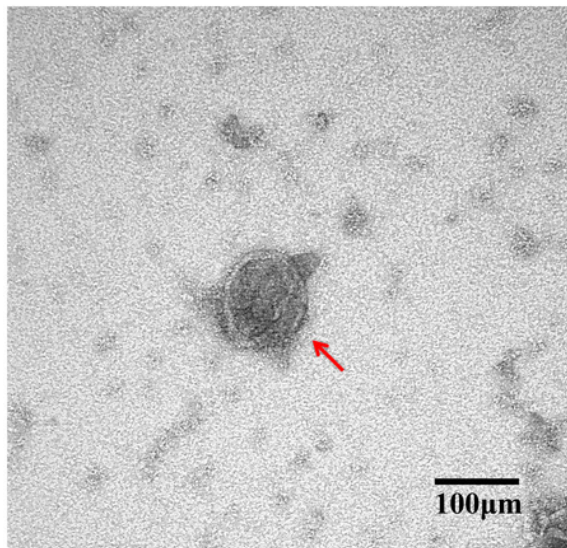
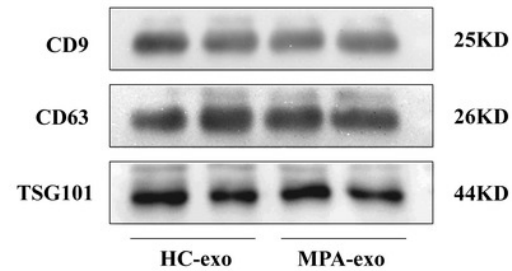
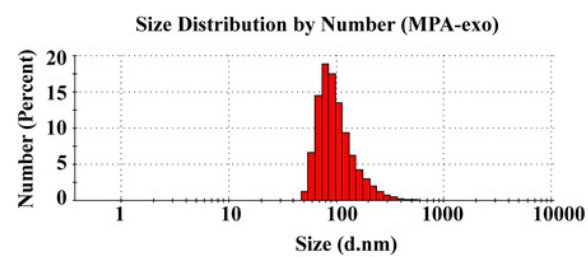
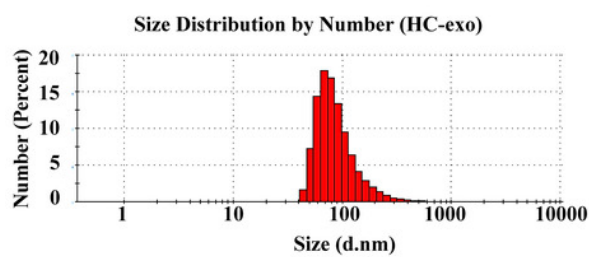
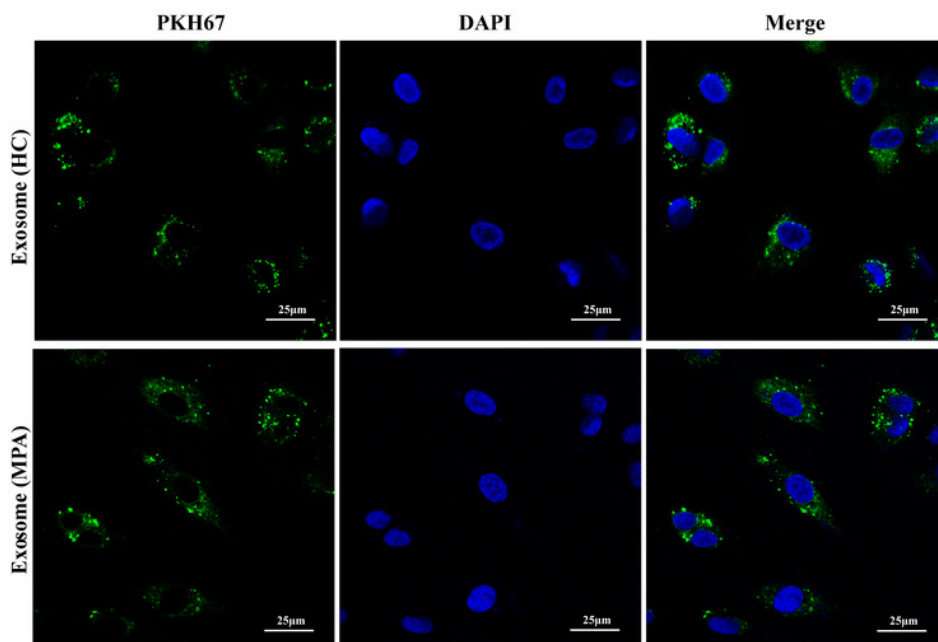
HC-exo MPA-exo

C

Size Distribution by Number (HC-exo)

Size Distribution by Number (MPA-exo)

D

PKH67 DAPI Merge

Exosome (HC)

Exosome (MPA)

E

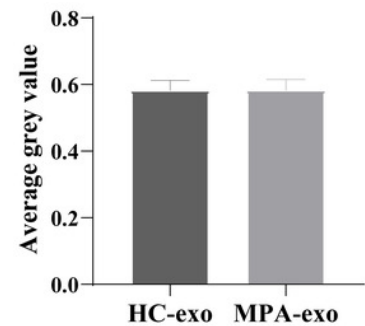



Figure 2

Plasma-derived exosomes from the MPA patients induced inflammatory response in HUVEC

HUVEC were treated with HC-exo or MPA-exo for 24 hours. (A) The inflammatory factors and adhesion molecules IL-6, IL-8, MCP-1, ICAM-1, and E-selectin levels were detected by qRT-PCR after co-culture with MPA-exo or HC-exo. (B) Neutrophils were identified by Giemasa stain (Scar bar = 10 μm). (C) Representative light micrographs of neutrophils adhering to HUVEC. Nuclei were counterstained with hematoxylin. The red arrow indicates adherent neutrophils, and the green arrow indicates HUVEC. Scale bar = 200 μm & 100μm. (D) Bar chart showing the neutrophil/HUVEC ratio. The numbers of adherent neutrophils and HUVEC were counted in five randomly chosen microscopic fields at a 200× final magnification. Data are presented as mean ± SD and compared with t-test. **$p < 0.01$. HC-exo, exosomes derived from healthy controls; MPA-exo, exosomes derived from MPA patients; and HUVEC, human umbilical vein endothelial cells.

A

IL-6

IL-8

MCP-1

ICAM-1

E-selectin

B

C

HC-exo

MPA-exo

200 µm

100 µm

200 µm

100 µm

D

Neutrophil/HUVEC ratio

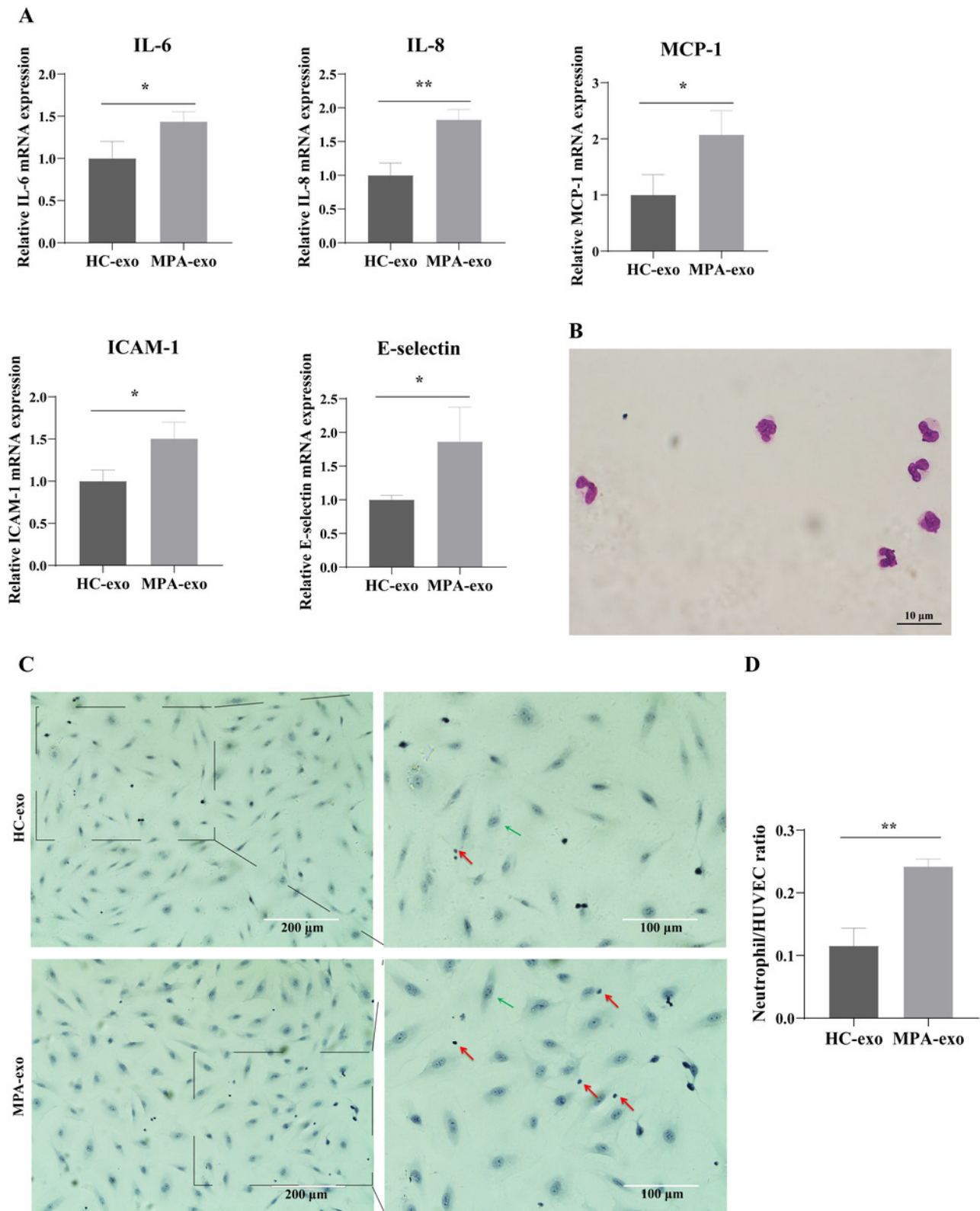



Figure 3

RNA-seq identified the differential expression of miRNA in MPA-exo

(A) Volcanic map revealed differentially expressed miRNA in HC-exo or MPA-exo (n = 3). The x-axis is $\log_2$ (fold change) and the y-axis is $-\log_{10}$ (P value). Blue dots represent miRNA with no significant differences, red dots represent up-regulated miRNA and green dots represent down-regulated miRNA. (B) The expression profiles of the randomized selected three differential exosomal miRNA were validated by qRT-PCR (n = 6). Data are presented as mean ± SD and compared with t-test. *$p < 0.05$, and **$p < 0.01$. HC-exo, exosomes derived from healthy controls; and MPA-exo, exosomes derived from MPA patients.

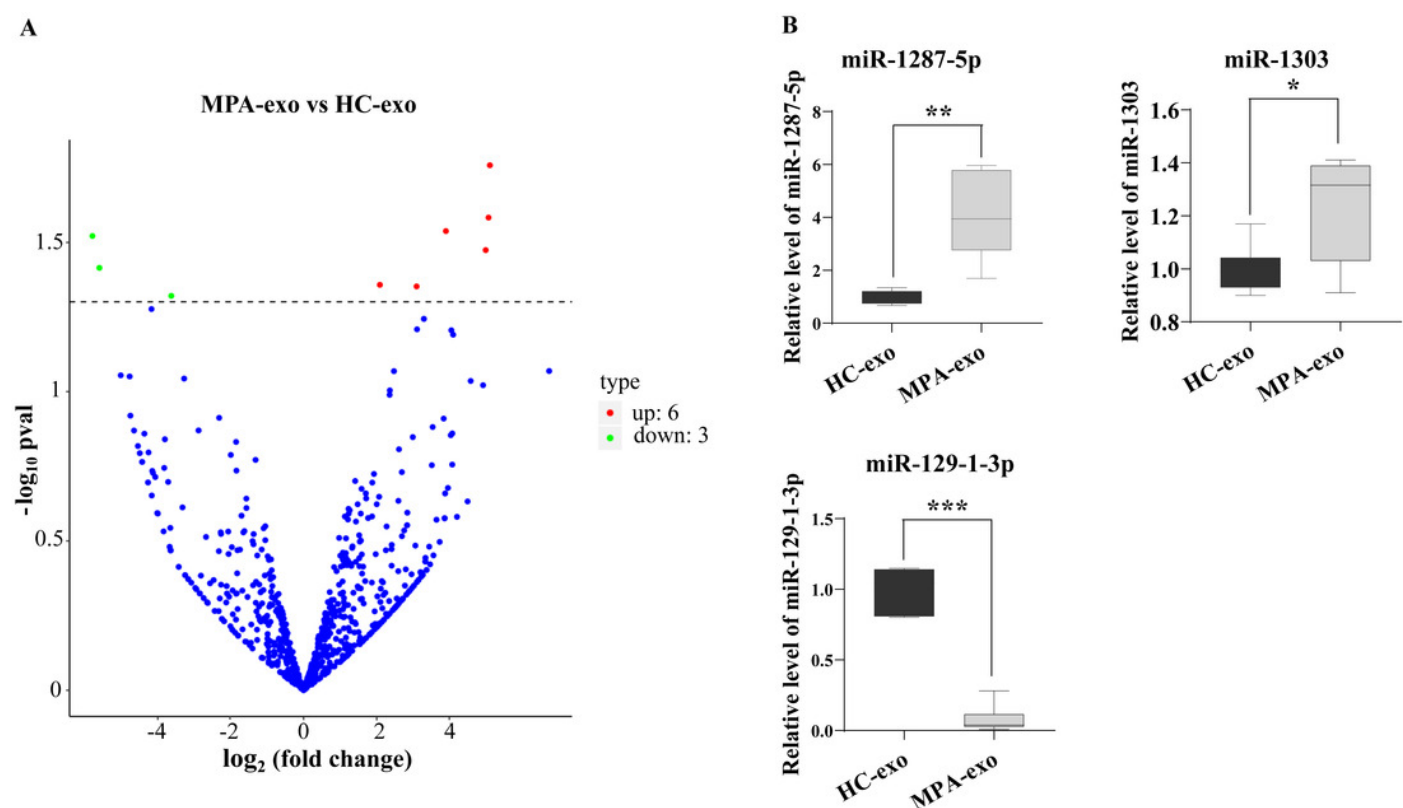



Figure 4

MiR-1287-5p induced inflammatory response in HUVEC

(A) The basal expression levels of miR-1287-5p, miR-1303, and miR-129-1-3p in HUVEC were detected by qRT-PCR. Delta CT was used to compare the levels of miRNA to the cel-miR-39. (B) The expression of miR-1287-5p in HUVEC was detected by qRT-PCR after treated with MPA-exo for 24 hours. (C) Effect of transfecting HUVEC with miR-1287-5p mimic and inhibitor on inflammatory factors and adhesion molecules. Data are presented as mean ± SD from three independent experiments and compared with t-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. HC-exo, exosomes derived from healthy controls; MPA-exo, exosomes derived from MPA patients; MNC, mimic negative control; and INC, miRNA inhibitor negative control.

A

B

C

IL-6

IL-8

MCP-1

ICAM-1

E-selectin

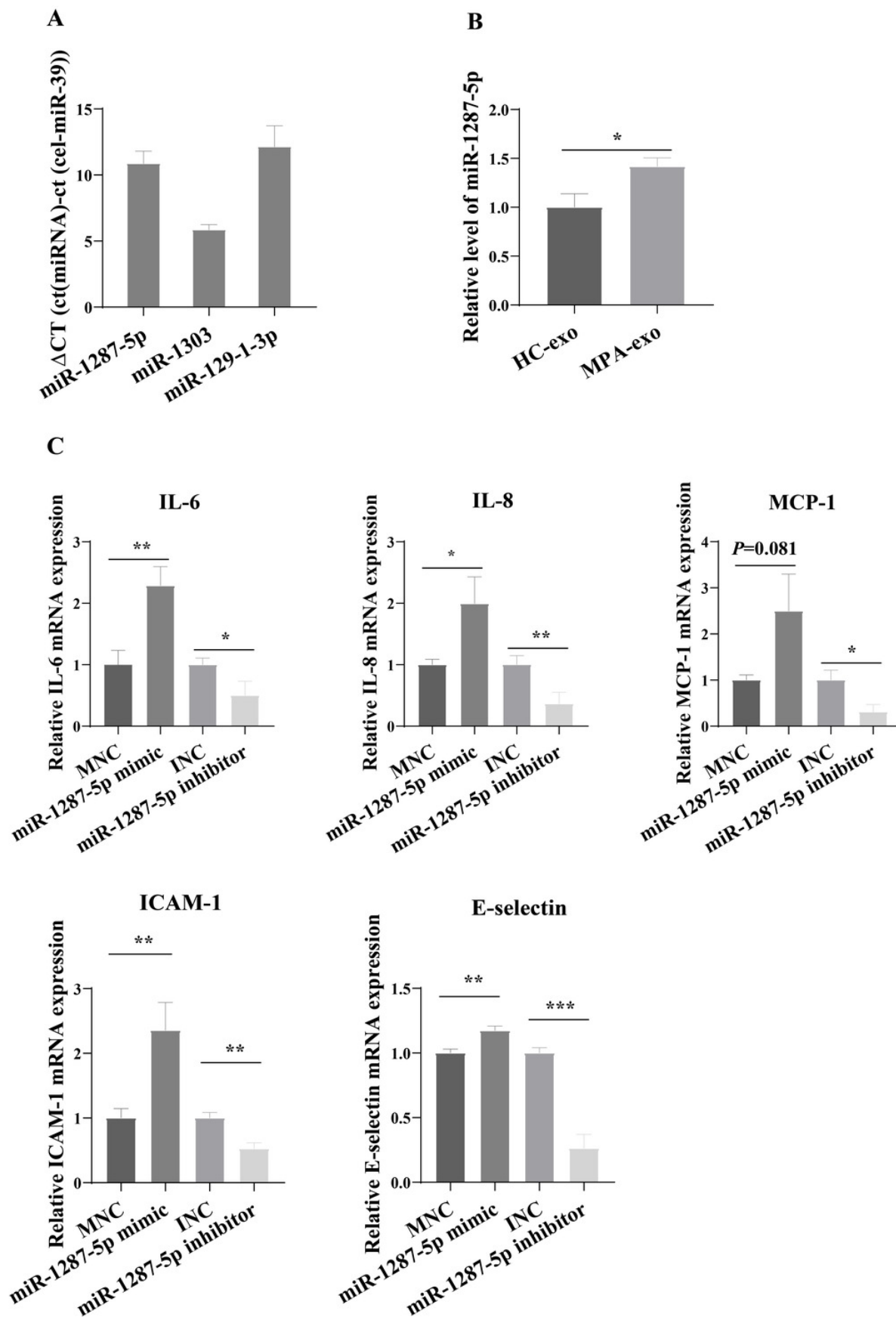



Figure 5

MiR-1287-5p promoted neutrophils adhesion to HUVEC

HUVEC were transfected with miR-1287-5p mimic, inhibitor, and negative control for 48 hours. (A&B) Representative light micrographs of neutrophils adhering to HUVEC after transfected with miR-1287-5p mimic and MNC. (C&D) Representative light micrographs of neutrophils adhering to HUVEC after transfected with miR-1287-5p inhibitor and INC. Nuclei were counterstained with hematoxylin. The red arrow indicates adherent neutrophils, and the green arrow indicates HUVEC. Scale bar = 200 μm & 100μm. (D) Bar chart showing the neutrophil/HUVEC ratio. The numbers of adherent neutrophils and HUVEC were counted in five randomly chosen microscopic fields at a 200× final magnification. Data are presented as mean ± SD from three independent experiments and compared with t-test. * $p < 0.05$, and *** $p < 0.001$. HC-exo, exosomes derived from healthy controls; MPA-exo, exosomes derived from MPA patients; MNC, mimic negative control; and INC, miRNA inhibitor negative control.

A

MNC

miR-1287-5p mimic

B

INC

miR-1287-5p inhibitor

C

D

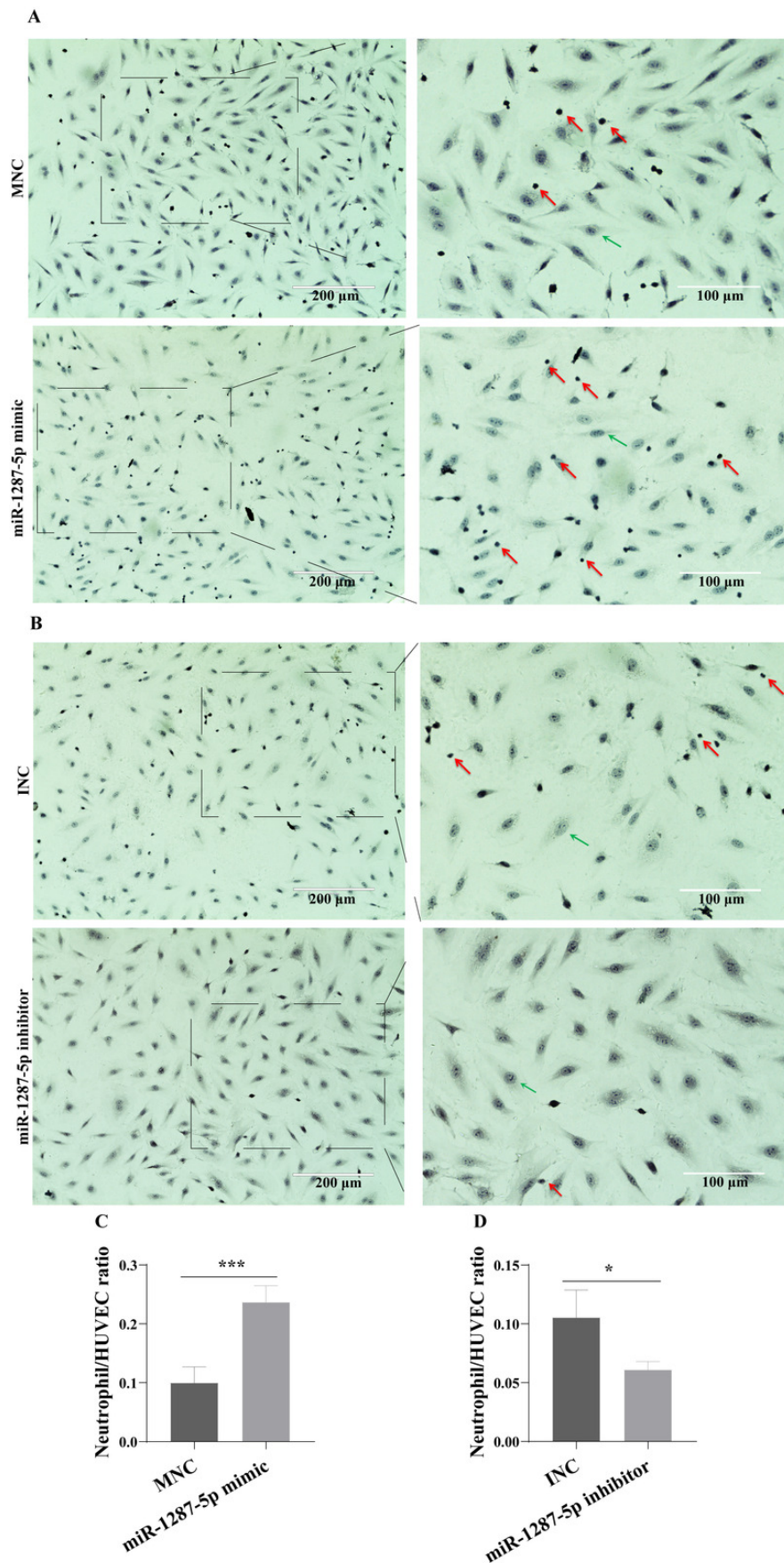



Figure 6

CBL is a direct target of miR-1287-5p

(A) TargetScan software predicted that CBL was a potential target of miR-1287-5p. (B) Binding site of CBL mRNA 3′-UTR (CBL-Wt) with miR-1287-5p and designed CBL 3′-UTR mutant (CBL-Mut) sequence. (C) Luciferase activity was significantly decreased in 293T cells cotransfected with the CBL-Wt vector and miR-1287-5p mimic but was no significance difference in cells cotransfected with CBL-Mut and miR-1287-5p mimic, when compared to the negative control. (D) qRT-PCR and (E) Western blot analysis was used to detect the mRNA and protein expression of CBL in HUVEC transfected with miR-1287-5p mimic, inhibitor, or negative control for 24 or 48 hours. Data are presented as mean ± SD from three independent experiments and compared with *t*-test. ** $p < 0.01$, and *** $p < 0.001$. MNC, mimic negative control; INC, miRNA inhibitor negative control; and NS, no significance.

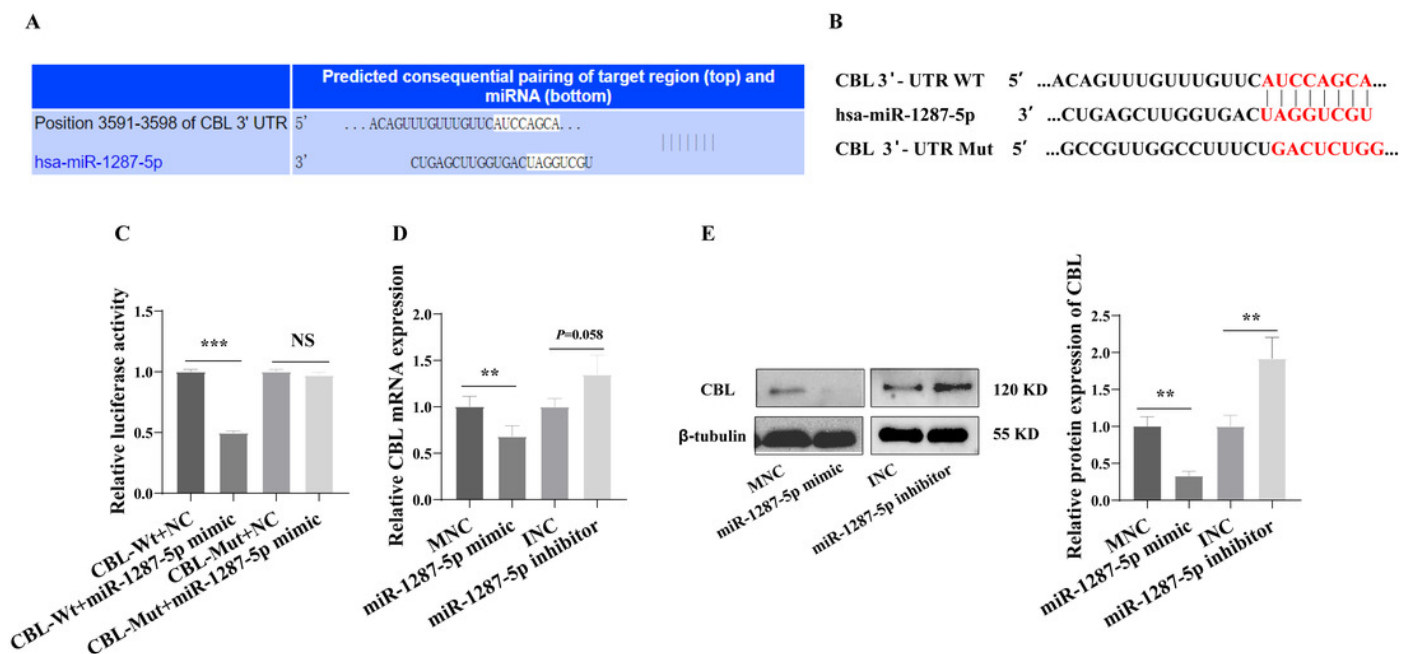



Figure 7

Silencing CBL induced inflammatory response in HUVEC

(A) CBL protein level in HUVEC was measured using Western blot after treated with HC-exo or MPA-exo for 48 hours. (B) HUVEC was transfected with CBL siRNA (si-NC, si-001, si-002, and si-003) for 48 hours, and mRNA expression of CBL was evaluated by RT-PCR to select the most efficient siRNA. (C) CBL protein level in HUVEC was measured using Western blot after siRNA transfection. (D) The effect of si-CBL or/and miR-1287-5p inhibitor treatment on HUVEC inflammatory factors and adhesion molecules. Data are presented as mean ± SD from three independent experiments and compared with t-test. * $p < 0.05$, and *** $p < 0.001$. *, si-NC compared with si-CBL; #, si-CBL compared with si-CBL + miR-1287-5p inhibitor. HC-exo, exosomes derived from healthy controls; and MPA-exo, exosomes derived from MPA patients; si-NC, negative control siRNA; and NS, no significance.

A

CBL

β- Tublin

HC-exo   MPA-exo

Protein level of CBL

*

HC-exo   MPA-exo

B

Relative CBL mRNA expression

***   ***   ***

si-NC   si-001   si-002   si-003

si-CBL

C

CBL   120 KD

β-tubulin   55 KD

si-NC   si-001   si-002   si-003

Protein level of CBL

***   ***   ***

si-NC   si-001   si-002   si-003

si-CBL

D

IL-6

Relative IL-6 mRNA expression

***   ###

si-NC   si-CBL   si-CBL+miR-1287-5p inhibitor

IL-8

Relative IL-8 mRNA expression

***   ###

si-NC   si-CBL   si-CBL+miR-1287-5p inhibitor

MCP-1

Relative MCP-1 mRNA expression

*   NS

si-NC   si-CBL   si-CBL+miR-1287-5p inhibitor

ICAM-1

Relative ICAM-1 mRNA expression

***   ###

si-NC   si-CBL   si-CBL+miR-1287-5p inhibitor

E-selectin

Relative E-selectin mRNA expression

***   ###

si-NC   si-CBL   si-CBL+miR-1287-5p inhibitor

Figure 8

Silencing CBL promoted neutrophils adhesion to HUVEC

(A) The effect of si-CBL or/and miR-1287-5p inhibitor treatment on HUVEC neutrophils adhesion, Nuclei were counterstained with hematoxylin. The red arrow indicates adherent neutrophils, and the green arrow indicates HUVEC. Scale bar = 200 μm & 100μm. (B) Bar chart showing the neutrophil/HUVEC ratio. The numbers of adherent neutrophils and HUVEC were counted in five randomly chosen microscopic fields at a 200× final magnification. Data are presented as mean ± SD from three independent experiments and compared with t-test. * $p < 0.05$, and *** $p < 0.001$. *, si-NC compared with si-CBL; #, si-CBL compared with si-CBL + miR-1287-5p inhibitor. si-NC, negative control siRNA.

A

si-NC

si-CBL

si-CBL+miR-1287-5p inhibitor

200 μm

100 μm

B