



Involvement of macrophage-derived exosomes in abdominal aortic aneurysms development



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HIGHLIGHTS

- A large amount of exosomes were present in adventitia of aneurysmal tissues, and mainly from macrophages.
- GW4869, an inhibitor of exosome biogenesis, significantly reduced dilation of calcium phosphate (CaPO₄)-induced AAA.
- Macrophage-derived exosomes induced MMP-2 expression in vascular smooth muscle cells by activating JNK and p38 pathways.

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ABSTRACT

Background and aims: Abdominal aortic aneurysm (AAA) is characterized by infiltration of inflammatory cells, extracellular matrix (ECM) degradation, and dysfunction of vascular smooth muscle cells (VSMCs). Recent studies reported that exosomes mediate intercellular communication and are involved in different diseases. Whether exosomes play a role in AAA is poorly understood. Hence, this study evaluated the function of exosomes in AAA development.

Methods: The presence of exosomes in human and calcium phosphate (CaPO₄)-induced AAA tissues was determined by immunofluorescence staining of CD63 and Alix. GW4869, an inhibitor of exosome biogenesis, was intraperitoneally injected into CaPO₄-induced AAA tissues to evaluate the effects of exosomal inhibition on AAA development. To explore the underlying mechanisms, the human monocytic cell line THP-1 was differentiated into macrophages, and exosomes were collected from macrophages. VSMCs were treated with macrophage-derived exosomes, and the expression of matrix metalloproteinase-2 (MMP-2) was evaluated. The activation of mitogen-activated protein kinases (MAPKs) pathways was also investigated *in vitro* and *in vivo*.

Results: Exosomes were detected in the adventitia of aneurysmal tissues obtained from humans and mice. They were mainly expressed in clusters of macrophages. Intraperitoneal injection of GW4869 for two weeks significantly attenuated the progression of CaPO₄-induced AAA, preserved elastin integrity and decreased MMP-2 expression. Similarly, administration of GW4869 suppressed the systemic and aneurysmal exosome generation. *In vitro*, treatment with macrophage-derived exosomes elevated MMP-2 expression in human VSMCs, while pretreatment with GW4869 abolished these effects. It was also found that JNK and p38 pathways mediated the production of MMP-2 in VSMCs following treatment with macrophage-derived exosomes.

Conclusions: This study suggests that exosomes derived from macrophages are involved in the pathogenesis of AAA. Macrophage-derived exosomes trigger MMP-2 expression in VSMC via JNK and p38 pathways. GW4869 supplementation attenuates CaPO₄-induced AAA in mice.

1. Introduction

Abdominal aortic aneurysm (AAA) is a chronic inflammatory disease caused by various etiological factors. Currently, there are no

effective pharmacological treatments or preventive strategies for AAA [1]. The pathological features of AAA include inflammatory cells aggregation, vascular smooth muscle cells (VSMCs) dysfunction, and extracellular matrix (ECM) degradation. During AAA progression, VSMCs

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display an aberrant phenotype, produce more MMPs, and undergo apoptosis [2]. A variety of inflammatory cells have been detected in aneurysmal tissues, which play critical roles in AAA development [3]. Macrophages are dominant players in the inflammatory environments. Besides the production of matrix metalloproteinases (MMPs) to degrade ECM, macrophages are also the major source of pro-inflammatory cytokines, which mediate aortic inflammation [3,4]. Several studies suggested that communication between macrophages and VSMCs is a possible cause of AAA progression, thus being a promising target for AAA treatments [5,6].

Exosomes are 30–100 nm lipid bilayer particles [7]. Several studies indicated that exosomes contain a myriad of RNAs and proteins, mediate cell-cell communication, and participate in the pathogenesis of cardiovascular diseases [7–9]. GW4869, a neutral sphingomyelinase 2 (nSMase2) inhibitor, is a widely used blocker of exosome secretion, which works by reducing ceramide-dependent exosome production [10]. Recent studies demonstrated that administration of GW4869 attenuated the progression of several diseases, suggesting it is a potential pharmacological agent [10,11]. The majority of circulating exosomes are macrophage-derived exosomes [12]. In inflammatory environments, activated macrophages mediated inflammation by producing more exosomes, which are uptaken by other cells [10,13]. A study by Ismail et al. demonstrated that macrophage-derived exosomes facilitated adjacent macrophage differentiation [12]. In hypertensive conditions, macrophage-derived exosomes promote the production of proinflammatory factors in endothelial cells [14]. Furthermore, exosomes produced by foam cells increased the migration of VSMCs, and this was associated with phosphorylation of ERK and Akt in VSMCs [13]. These findings suggested that macrophage-derived exosomes participate in cardiovascular diseases. Previous studies found that exogenous human mesenchymal stromal cell (MSC)-derived exosomes mitigated elastase-induced AAA development [15]. However, whether exosomes take part in AAA pathogenesis remains unclear. We hypothesized that macrophage-derived exosomes mediate AAA development by crosstalk with VSMCs. Based on the experimental results, we found that exosomes were mainly released in macrophages in human and CaPO₄-induced mouse AAA samples. Global administration of GW4869 alleviated CaPO₄-induced AAA development in mice, accompanied by decreased MMP-2 level. *In vitro* results suggested that macrophage-derived exosomes promoted MMP-2 expression in VSMCs, and these effects were mediated by the JNK and p38 signaling pathway. These findings indicate that exosomes play a role in the pathogenesis of AAA.

2. Materials and methods

2.1. Human AAA samples

Human AAA specimens were collected from patients who underwent open AAA repair surgery. Normal aortic tissues were harvested from organ transplantation donors. All the procedures were performed in accordance with the Human Research Ethics Committee of the Second Affiliated Hospital, Zhejiang University School of Medicine. The study was performed according to the Guidelines of the World Medical Association Declaration of Helsinki. Samples were embedded with paraffin and sectioned to 5 μm thickness.

2.2. Mouse model of calcium phosphate-induced AAA

12-week-old male C57BL/6 mice were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). The CaPO₄-induced mouse AAA model was generated as previously described [16]. Briefly, the infrarenal segment of the abdominal aorta was isolated. A small piece of gauze soaked in 0.5 mol/L CaCl₂ was perivascularly applied for 10 min, and was subsequently replaced by another piece of PBS-soaked gauze for 5 min. The control group received PBS-soaked gauze for

15 min. Mice were sacrificed two weeks later, and tissues were perfused with 4% paraformaldehyde (PFA) and embedded in paraffin. Using a caliper, the maximum external diameter of infrarenal aorta was measured before CaPO₄ administration (initial measurement) and at the time of sacrifice (final measurement). Aortic dilation was defined as aortic expansion relative to initial diameter ((Final measurement – initial measurement)/Initial measurement)*100, according to a previous report [17]. Mice were randomized into the following groups: (1) PBS group (n = 5) and (2) CaPO₄ group (n = 10). All procedures performed were in accordance with guidelines of the Institutional Animal Care and Use Committee at Zhejiang University College of Medicine.

2.3. GW4869 treatment in calcium phosphate-induced AAA

Male C57BL/6 mice were randomly assigned to four groups: PBS + DMSO, PBS + GW4869, CaPO₄+DMSO, and CaPO₄ + GW4869 (N = 9–10). In GW4869 groups, each mouse was intraperitoneally injected with GW4869 every 48 h for two weeks [11]. GW4869 was dissolved in DMSO (0.005%) as previously described, and the injection dose was 2.5 μg/g body weight [10,11]. The other groups received an equal amount of DMSO.

2.4. Histological studies

Samples were cut into 5 μm thickness, mounted on microscope slides and stained with hematoxylin and eosin. Van Gieson staining was applied using Van Gieson kit (Sigma) as detailed by the manufacturer. Elastin degradation scores were evaluated as previously described (1, no elastin degradation or mild elastin degradation; 2, moderate; 3, moderate to severe; and 4, severe elastin degradation) [18]. For immunofluorescence staining, paraffin-embedded sections were deparaffinized and rehydrated, and incubated in 10 mmol/L citrate buffer for antigen retrieval. Primary antibodies against CD68 (BioRad, MCA1957GA), CD63 (Santa Cruz, sc-5275), Alix (Santa Cruz, sc-53540), MMP-2 (Abcam, ab37150), CD31 (BD, 550274), α-SMA (Sigma, A 2547), CD3 (BioRad, MCA500GA), and ER-TR7 (Abcam, ab51824) were used. Secondary antibodies were anti-rat Alexa Fluor 488 (Thermo Fisher, A-21208), anti-mouse Alexa Fluor 594 (Thermo Fisher, A-21203) and anti-rabbit Alexa Fluor 488 (Thermo Fisher, A32790). Four high-power random fields per section, for three sections per mouse were analyzed and quantified.

2.5. Cell culture

Human monocytic leukemia (THP-1) cells were cultured in RPMI1640 medium with 10% fetal bovine serum. THP-1 cells were differentiated into macrophages with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 24 h. Then, the medium was replaced by a serum-free medium containing 10 ng/ml TNF-α (Peprotech). After 48 h, supernatants were collected for exosomes isolation. Human aortic VSMCs were purchased from ScienCell, and cultured in smooth muscle cell medium (ScienCell). Cells between passages three and seven were used in this study. THP-1-derived macrophages were cocultured with VSMCs in a transwell system (Corning). A subset of THP-1 derived macrophages were incubated with GW4869 20 μM for 24 h before coculture. VSMCs were prepared for qPCR and Western blot analysis. SP600125, SB203580 and FR 180204, inhibitors of JNK, p38, and ERK, respectively, were purchased from Beyotime (China).

2.6. Exosome isolation, characterization, and uptake

The cell medium was centrifuged at 2000g for 10 min and 12,000g for 30 min, and passed through a 0.22 μm filter to remove cellular debris. Subsequently, the filtered medium was centrifuged at 110,000g for 1 h, and the supernatant was discarded. The pellet was resuspended in phosphate-buffered saline (PBS), and then centrifuged at 110,000g

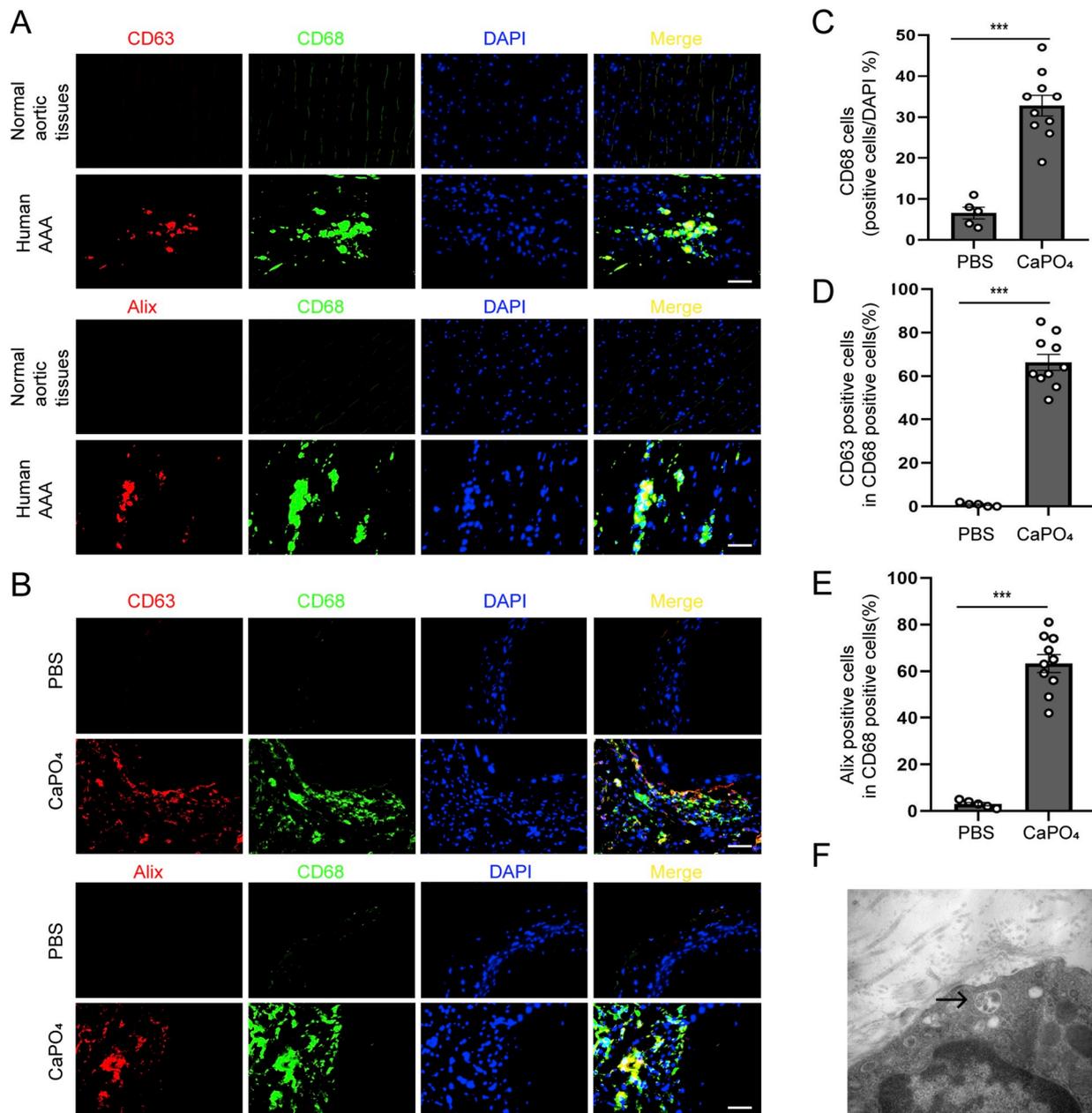


Fig. 1. The production of exosomes is increased in the human aneurysm and CaPO₄-induced abdominal aortic aneurysm.

(A) Representative images of immunofluorescence staining of CD63 and Alix with CD68 in human normal aortic tissues and human aneurysmal samples. Scale bar: 50 μ m. (B) Co-staining of CD63 and Alix with CD68 in CaPO₄-induced AAA. Scale bar: 50 μ m. (C) Quantitative analysis of infiltrated macrophages in CaPO₄-induced AAA. (D) Percentage of CD63 positive cells among CD68 cells in CaPO₄-induced AAA. (E) Percentage of Alix positive cells among CD68 cells in CaPO₄-induced AAA. (F) TEM of CaPO₄-induced AAA samples. Intracellular MVBs (arrowed) containing exosomes were present in the vicinity of the plasma membrane of macrophages. Scale bar: 0.5 μ m (n = 5 in the PBS group; n = 10 in the CaPO₄ group. ***p < 0.001).

for 1 h again. The supernatant was discarded, and the pellet was re-suspended in PBS and stored at -80°C . Exosome concentrations were measured by BCA assay (Invitrogen). Exosomes were determined by transmission electron microscopy (TEM) and Western blot. To test whether VSMCs can take up exosomes, macrophage-derived exosomes (10 μ g) were incubated with PKH-26 (Sigma) at room temperature for 15 min. A PKH-26-labelled control was prepared by adding PKH-26 dye into tubes without exosomes, as previously suggested [19]. 5% bovine serum albumin was added to stop the labeling, after which exosomes were centrifuged at 110,000 g for 1 h and cocultured with VSMCs for 24 h. Isolation of exosomes from plasma was carried out using the commercially available kit (Invitrogen), following the manufacturer's instructions.

2.7. Measurement of acetylcholinesterases (AChE) activity

AChE is regarded as an indirect index reflecting the number of exosomes [10]. AChE activity was determined with a commercial AChE activity assay kit (Sigma). According to manufacturer protocol, plasma samples were incubated in working reagent in 96-well plates. Then, the initial absorbance was measured at 412 nm, and AChE activity was evaluated as described by the manufacturer.

2.8. Western blot analysis

Protein from aortic tissues or cells were obtained by treating the samples with radio immunoprecipitation assay lysis buffer (RIPA,

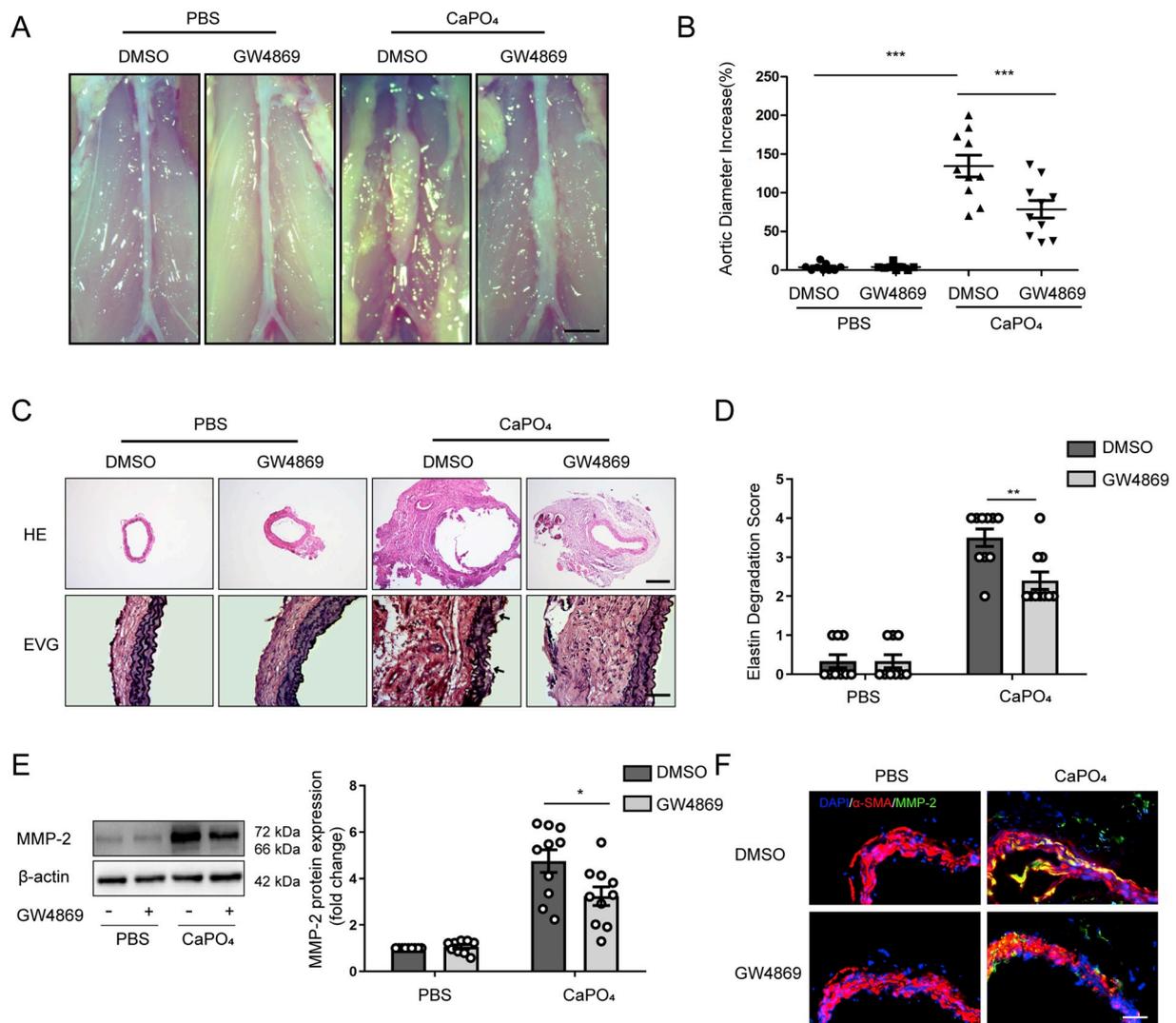


Fig. 2. Administration of GW4869 reduces CaPO₄-induced mice AAA expansion and MMP-2 expression. (A) Representative images of PBS and CaPO₄ primed arteries 14 days after treatment with DMSO or GW4869. Scale bar: 2 mm. (B) The aortic dilation. (C) Representative images of HE and EVG staining. HE scale bar: 200 μm; EVG scale bar: 50 μm. (D) Quantification of elastin degradation. (E) Western blot analysis of the expression of MMP-2 of arteries from mice receiving different treatments. (F) Co-staining of MMP-2 and α-SMA in samples from four groups. Scale bar: 50 μm. (n = 9 in PBS + DMSO group, n = 9 in PBS + GW4869 group, n = 10 in CaPO₄ + DMSO group, n = 10 CaPO₄ + GW4869 group. *p < 0.05, **p < 0.01, ***p < 0.001).

beyotime) containing protease inhibitors and phosphatase inhibitors. Protein concentration was determined by bicinchoninic acid assay (BCA assay, Invitrogen). Equal amounts of proteins from each group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Specifically, exosome samples from 20 μl plasma were loaded onto each lane. The membranes were incubated with primary antibodies at 4 °C overnight and secondary antibodies for 1 h. Finally, the bands were visualized using the ECL solution. Data were normalized to the expression of the internal control. The expression of CD63 and CD68 was normalized with the first sample in the group. The following primary antibodies were used: CD68 (BioRad, MCA1957GA), CD63 (Santa Cruz, sc-5275), MMP-2 (Abcam, ab37150), Alix (CST, 2171), p-JNK (CST, 4668), JNK (CST, 9252), p-p38 (CST, 4511), p38 (CST, 8690), p-ERK (CST, 4370), ERK (CST, 4695), GAPDH (CST, 3683), β-actin (CST, 12262).

2.9. Quantitative real-time PCR

Total RNA was extracted from cells by Trizol reagent (Invitrogen). Next, 500 ng of total RNA per sample was reverse transcribed into

complementary DNA (cDNA) by PrimeScript RT reagent Kit (Takara). Amplification of cDNA was conducted using SYBR kits (Takara) on an Applied Biosystems 7500 Fast Real-Time PCR System (ABI). The expression of GAPDH was used as the internal control. The primer sequences of MMP-2 were: 5'-CGACCACAGCCAACACTACGAT-3', 5'-GTCA GGAGAGGCCCATAGA-3'.

2.10. Statistical analysis

All values are presented as the mean ± SEM of three experimental repeats. Statistical differences between two groups were determined by Student's t-test, while ANOVA followed by Bonferroni multiple comparisons were performed for three or more groups. p < 0.05 was considered statistically significant. The statistical calculations were performed using GraphPad Prism 8.0.

3. Results

3.1. Production of exosomes are enhanced in AAA samples

To evaluate the role of exosomes in the pathogenesis of AAA, we

detected exosomes in human AAA samples by immunofluorescence staining of exosomal markers, CD63 and Alix, according to a previous study [20]. In normal aortic tissues, we failed to detect CD63 and Alix expression (Fig. 1A). However, CD63 was highly expressed in AAA tissues, especially in the adventitia of the aortic wall (Fig. 1A and Supplementary Fig. 1). Considering the pivotal role of macrophages in AAA dilation, we co-immunostained CD63 and Alix with the macrophage marker CD68. The results revealed that CD63 and Alix-positive regions were also simultaneously positive for CD68, suggesting that macrophages were the major sources of exosome production in AAA tissues (Fig. 1A). Furthermore, we successfully established a CaPO₄-induced mouse model of AAA. As expected, macrophages were intensively infiltrated in CaPO₄-induced mice AAA (Fig. 1B and C). Dual immunofluorescence staining confirmed that CD63 and Alix colocalized with CD68 positive regions (Fig. 1B, D, and E). TEM examination of mouse aneurysmal samples revealed that there were intracellular multivesicular bodies (MVB) and enclosing exosomes in the infiltrated macrophages (Fig. 1F). A small portion of CD63 overlapped with T cells (CD3), endothelial cells (CD31) and fibroblasts marker ER-TR7 in the adventitia of aneurysm (Supplementary Fig. 2). Taken together, these results demonstrate that the number of exosomes is increased in human and murine AAA tissues, and macrophages are predominantly responsible for exosome generation.

3.2. GW4869 ameliorates AAA development mediated by MMP-2 in mice

To further determine whether exosomes play a role in AAA, mice underwent surgery to induce aneurysm and subsequently received intraperitoneal injection with either GW4869 or DMSO every 48 h for two weeks [16]. Aortas treated with CaPO₄ showed a significant increase in aortic expansion after 14 days, compared with those treated with PBS (diameter increases 134.57 ± 44.18 vs. $3.69 \pm 4.67\%$; Fig. 2A and B). Injection of GW4869 significantly mitigated CaPO₄-induced AAA expansion (diameter increases 78.66 ± 35.86 vs. $134.57 \pm 44.18\%$; Fig. 2A and B). H&E staining revealed that GW4869 alleviated the expansion of the luminal region, thinning of the media, and thickening of the adventitia in CaPO₄-induced aortas (Fig. 2C). Additionally, EVG staining showed that the severity of elastin degradation was lower in GW4869 groups (Fig. 2C and D). MMP-2, mostly expressed by VSMCs, is a well-recognized cause of AAA [4]. Therefore, we examined the level of MMP-2 in different groups. The results demonstrated that MMP-2 was mainly distributed in aortic media and elevated in aortas treated with CaPO₄ (Fig. 2E and F). GW4869 attenuated MMP-2 abundance, compared with established AAA mice treated with DMSO (Fig. 2E and F). Taken together, these data imply that GW4869 weakened CaPO₄-induced AAA progression and simultaneously limited MMP-2 production in pathological VSMCs.

3.3. GW4869 inhibits exosomes release in mouse AAA

To further elucidate the biological effects of GW4869 *in vivo*, we assessed the generation of exosomes in mice. Protein concentrations in exosomes isolated from plasma were measured. It seemed that CaPO₄-induced AAAs did not change circulating exosome levels. Nevertheless, GW4869 strikingly inhibited circulating exosome levels in PBS and CaPO₄ groups (Fig. 3A). Previous studies demonstrated that the activity of AChE was an indirect index of exosomes quantity [10]. We also detected AChE activity, and the results suggested that GW4869 markedly decreased exosomes production, compared with DMSO treatment in PBS and CaPO₄ groups (Fig. 3B). Likewise, analysis of CD63 in exosomes isolated from plasma confirmed the inhibitory effects of GW4869 on circulating exosomes (Fig. 3C). CD68 was identified in exosomes extracted from mouse plasma (Fig. 3C). Treatment of GW4869 moderately decreased CD68 expression in circulating exosomes, indicating that macrophage-derived exosomes were inhibited. Accordingly, the exosome production in aneurysmal tissues was

evaluated. In the CaPO₄ group, GW4869 significantly decreased macrophage infiltration (Supplementary Fig. 3). To verify the inhibitory effects of GW4869 on macrophage-derived exosomes in AAA, immunofluorescence staining of CD63 and Alix, together with CD68, was performed in the different groups. The results showed that the number of CD68-positive cells coexpressing CD63 or Alix was markedly decreased in CaPO₄ groups treated with GW4869 (Fig. 3D and E). Collectively, our results concluded that administration of GW4869 globally inhibited exosomes generation *in vivo*.

3.4. Macrophage-derived exosomes facilitate MMP-2 production in VSMCs

To explore the effects of macrophage-derived exosomes on aortic VSMCs *in vitro*, we used THP-1 monocyte-derived macrophages as sources of exosomes. THP-1 cells were differentiated into macrophages by PMA, and later treated with TNF- α to mimic *in vivo* inflammatory environments. Exosomes were verified by Western blotting of CD63 and Alix (Fig. 4A). TEM further observed intact exosomes, whose diameters ranged from 30 to 100 nm (Fig. 4B). To ascertain whether macrophage-derived exosomes could be internalized by VSMCs, PKH-26-labelled exosomes were added to VSMCs medium. After 24 h incubation, PKH-26 was detectable in the cytoplasm of VSMCs, implying that exosomes can be uptaken by VSMCs (Fig. 4C). We failed to observe PKH-26 in VSMCs incubated with PKH-26 labeling control. To investigate the effects of macrophage-derived exosomes on VSMCs, exosomes were cocultured with VSMCs. After 24 h, MMP-2 was upregulated at both mRNA and protein levels (Fig. 4D and E). In the presence of TNF- α , working as inflammatory environment, administration of exosomes further increased MMP-2 production (Fig. 4D and E). Moreover, we cocultured VSMCs with THP-1-differentiated macrophages in transwell systems (Fig. 4F). In TNF- α incubated medium, the presence of macrophages moderately enhanced MMP-2 expression in VSMCs, compared with groups without macrophages (Fig. 4G and H). Nevertheless, macrophages pretreated with GW4869 slightly reduced MMP-2 expression in VSMCs, implying that macrophage-derived exosomes facilitated MMP-2 generation (Fig. 4G and H). Conclusively, macrophage-derived exosomes positively regulated MMP-2 levels in VSMCs.

3.5. JNK and p38 pathways are involved in macrophage-derived exosomes induced MMP-2 production

Several studies reported that members of mitogen-activated protein kinases (MAPKs) regulate MMP production and play vital roles in AAA development [1,21,22]. The effects of macrophage-derived exosomes on MAPKs signal pathway in VSMCs were examined at different time points. As expected, phosphorylation of JNK, p38, and ERK was increased in a time-dependent manner (Fig. 5A). Phosphorylated states of JNK, p38, and ERK *in vivo* were also evaluated in different groups. In the CaPO₄-induced AAA model, results showed that JNK, p38, and ERK signaling pathway was activated (Fig. 5B and C). Administration of GW4869 in AAA mouse model attenuated JNK, p38, and ERK activation compared to the DMSO group (Fig. 5B and C). Further, specific inhibitors were used to treat VSMCs for 24 h. Subsequently, MMP-2 level was detected. Inhibition of JNK and p38 by SP600125 and SB203580, respectively, abolished exosome-induced MMP-2 generation (Fig. 5D and E). But, disruption of ERK by FR 180204 did not affect MMP-2 expression after treatment with exosomes (Fig. 5F). Overall, JNK and p38 pathways were involved in exosomes-induced MMP-2 production.

4. Discussion

Our results suggest the involvement of exosomes in the pathogenesis of AAA. We found that exosomes increased in the adventitia of AAA samples in humans and mice, and were mainly derived from macrophages. Treatment with GW4869 notably diminished CaPO₄-induced AAA dilation. Macrophage-derived exosomes stimulated MMP-2

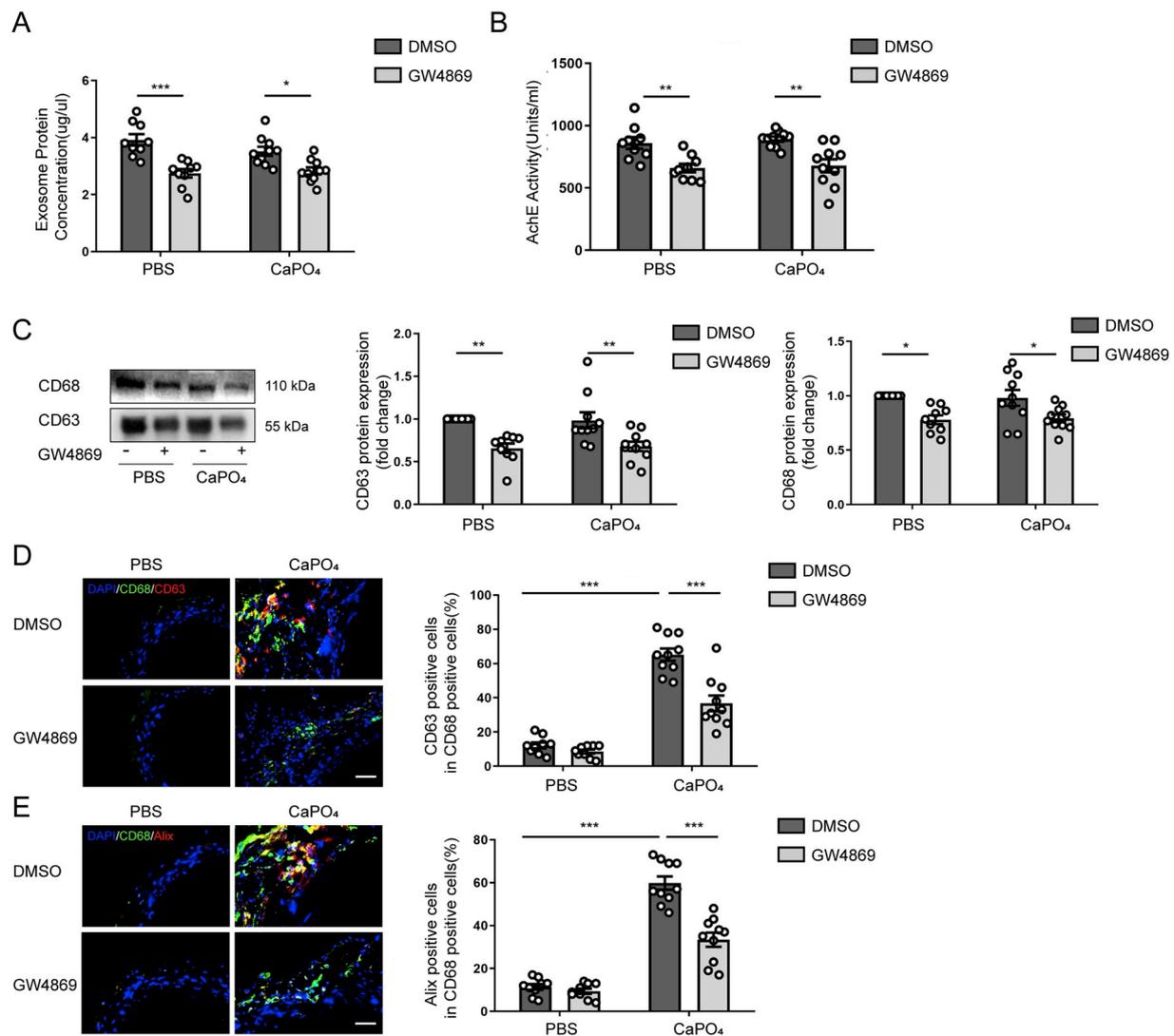


Fig. 3. GW4869 blocked exosomes secretion in CaPO₄-induced mouse AAA.

(A) Protein concentrations of exosome pellets from the plasma of mice. (B) AChE activity of mice receiving different treatments. (C) Immunoblots for CD63 and CD68 in exosomes isolated from mouse plasma. (D) Co-staining of CD63 and CD68 in aneurysmal tissues. Scale bar: 50 μ m. (E) Co-staining of Alix and CD68 in aneurysmal tissues. Scale bar: 50 μ m. (n = 9 in PBS + DMSO group, n = 9 in PBS + GW4869 group, n = 10 in CaPO₄+DMSO group, n = 10 CaPO₄+GW4869 group. * p < 0.05, ** p < 0.01, *** p < 0.001).

expression in VSMCs, one of the main causes of AAA mediated by JNK and p38 pathways, which may contribute to the development of AAA.

Exosomes have been shown to be involved in diverse pathophysiological activities [7]. Development of vascular diseases such as atherosclerosis and vascular calcification is a multi-step process, and numerous cell types are involved. Exosomes secreted from these cells influence the progression of vascular dysfunction [8,9]. In atherosclerosis, exosomes from endothelial cells regulate inflammation of endothelial cells, facilitate VSMCs differentiation, and induce macrophage apoptosis [8,23]. Macrophage-derived exosomes exacerbated endothelial inflammation by inducing leucocyte adhesion [14,24]. These findings demonstrate that exosomes modulate cell-cell crosstalk in the progression of atherosclerosis. Besides, Kapustin and colleagues found that pro-calcifying environments stimulated exosomes production in VSMCs and exosomes, which in turn, triggered calcification of adjacent VSMCs, while ablation of exosomes mitigated vascular calcification [20,25]. Intriguingly, exogenous human MSC-derived exosomes alleviated AAA development, suggesting that administration of MSC-derived exosomes may be a promising therapy [15]. This protective effect was modulated by microRNA-147 [15]. Our study, for the first time, reported that exosomes were elevated in AAA, and disruption

of exosomes dramatically alleviated AAA development. These results reveal that the involvement of exosomes in cell-cell interaction contributed to the development of vascular dysfunction.

Despite the numerous clinical trials conducted to develop therapies for AAA, there are currently no effective pharmacological treatments [1]. This is partially due to the inadequate understanding of the mechanisms of AAA. The present investigation found that GW4869 markedly retarded CaPO₄-induced AAA, implying that GW4869 is a promising agent for AAA treatment. This therapeutic effect may result from inhibition of macrophage-derived exosomes and consequent decrease in MMP-2. To date, a series of studies have explored the therapeutic effects of GW4869 [10,11,26,27]. Exosomes were strongly activated in LPS-induced sepsis in mice, and GW4869 repressed sepsis-related cardiac inflammation and myocardial dysfunction [10]. *In vitro* experiments demonstrated that exosomes from macrophages contained high levels of pro-inflammatory factors including TNF- α and IL-6 following treatment with LPS. In Alzheimer disease, GW4869 interfered with exosome-mediated amyloid beta accumulation and tau dissemination, and reduced disease progression [11,26]. Continuous injection of GW4869 significantly reduced the atherosclerotic lesion area in *Apoe*^{-/-} mice and decreased inflammation in endothelial cells and

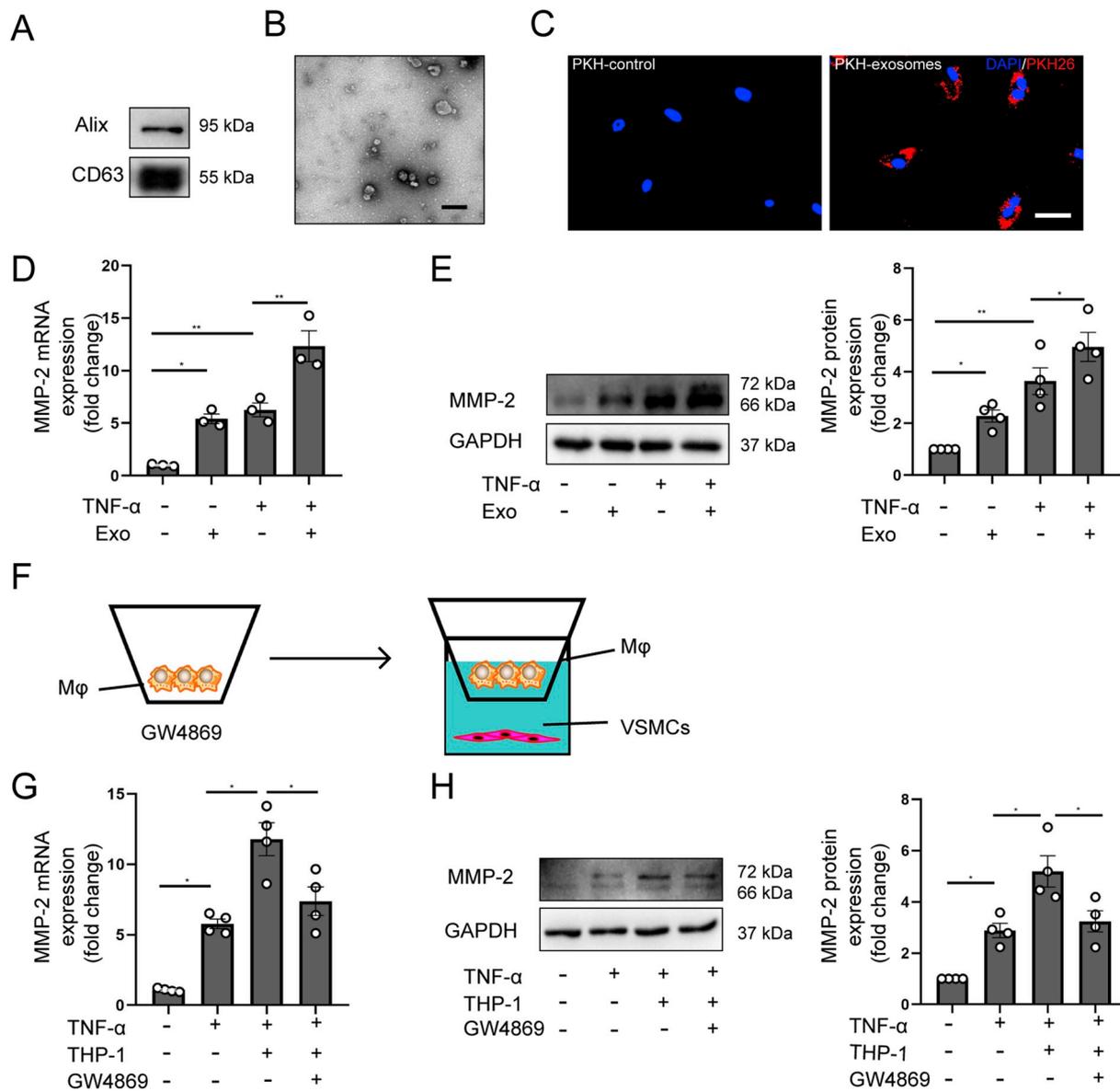


Fig. 4. Macrophage-derived exosomes enhance MMP-2 expression in VSMCs. (A) Western blot for exosomal markers CD63 and Alix. (B) TEM of exosomes isolated from cell medium of THP-1. Scale bar: 200 nm. (C) Representative images of VSMCs incubation with PKH-26 labeled control, and PKH-26 labeled exosomes. Scale bar:100 μ m. (D and E) PCR and Western blot analysis of MMP-2 levels in VSMCs treated with exosomes. (F) *In vitro* co-culture system. THP-1-differentiated macrophages were seeded in the upper chamber and VSMCs in the lower chamber. (G and H) GW4869 primed THP-1 cocultured with VSMCs reduced MMP-2 expression, compared with the group without GW4869. (* $p < 0.05$, ** $p < 0.01$).

macrophages [27]. In a nutshell, our observations and findings from previous studies point to the fact that GW4869 might be an effective approach for treating many diseases such as AAA. Indeed, further investigations are necessary prior to the clinical application of GW4896.

Intense macrophage infiltration in the aortic wall is one of the pathological features of AAA [3]. Once activated by inflammatory mediators, macrophages produce chemokines, pro-inflammatory cytokines, and MMPs, exerting pleiotropic effects on adjacent cells, such as VSMCs. VSMCs maintained aortic integrity [2]. In AAA, VSMCs acquire a secretory phenotype, and produce MMPs that degrade ECM and aggravate AAA [2,28]. Macrophage-derived exosomes were abundant, which is second only to platelet-derived exosomes in circulation [12]. The presence of stimuli increased the production of exosomes [10,13]. Previously, it was reported that macrophage-derived exosomes triggered endothelial inflammation, facilitated macrophage differentiation and induced VSMCs migration and adhesion, revealing that exosomes from macrophages were pro-inflammatory and major players in the

crosstalk between macrophages and other cells [10,13,14]. Similarly, our results showed that macrophage-derived exosomes promoted MMP-2 expression in VSMCs and vitiated aortic wall integrity, and reduction in the number of exosomes reversed these effects. This finding reveals an important effect of macrophages on VSMCs, which is mediated by exosomes in the pathogenesis of AAA. In addition to cytokines and other soluble factors, VSMCs also incorporate exosomes and receive novel messages from macrophages. These results implicate exosomes in macrophage-VSMCs interaction during AAA development. Deciphering the compositions of macrophage-derived exosomes in AAA progression would be an interesting job in the future.

Despite these findings, there are several limitations to this study. First, global administration of GW4869 reduces exosomes production from all types of cells. Though exosomes are largely located in the clusters of macrophages near VSMCs, our observation suggests that a small portion of exosomes were present in T cells, endothelial cells and fibroblasts. The decline in exosomes in these cells may partially

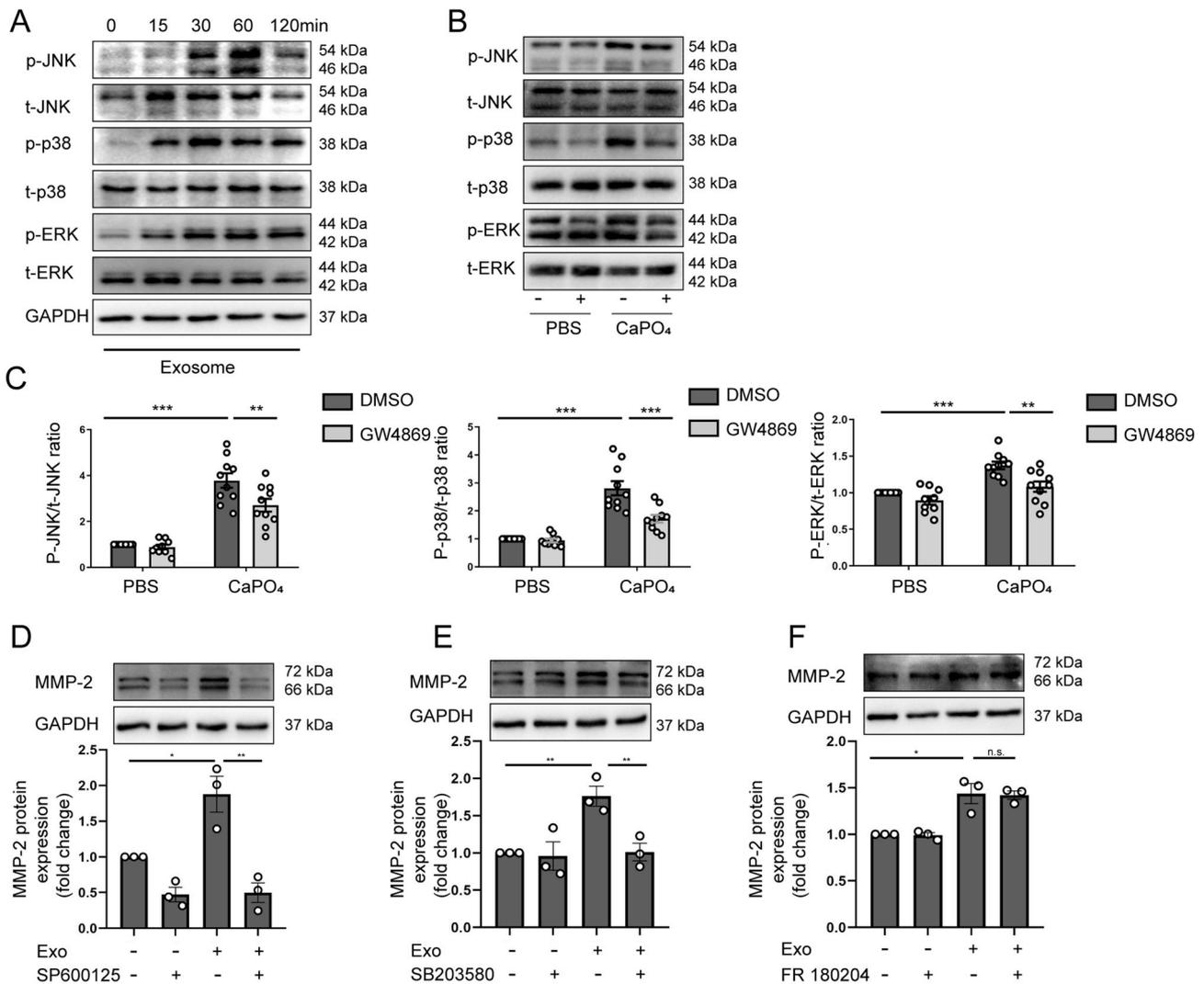


Fig. 5. JNK and p38 pathways participate in exosomes-induced MMP-2 expression. (A) Western blot analysis of the phosphorylated states of JNK, p38, and ERK and total JNK, p38 and ERK from VSMCs treated with macrophage-derived exosomes for the indicated time. (B) Western blot analysis of phosphorylation of JNK, p38, and ERK *in vivo* in different groups. (C) Quantitative analysis of phosphorylation of JNK, p38, and ERK *in vivo*. (D–F) Western blot results of MMP-2 expression from VSMCs treated with SP600125, SB203580 and FR 180204, inhibitors of JNK, p38 and ERK, respectively. (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, n.s. non-significant).

accounts for the inhibitory effects of GW4869 on AAA development, not solely due to inhibition of exosomes from macrophages. Second, this study did not address the possibility that nSMase2 inhibition could have biological effects independent of exosomes, since previous research indicated that nSMase2 inhibition strongly reduced vascular inflammation [27]. Third, to mimic *in vivo* environment, we used TNF- α as a pro-inflammatory factor in the treatment of macrophages and VSMCs. Considering the complexity of the environment *in vivo*, stimulation with only TNF- α is simplistic, to some extent. The composition of exosomes harvested *in vitro* is possibly different from that of exosomes produced by macrophages *in vivo*. Lastly, there was no gold standard method to detect exosomes until now. Recently, characterization of exosomes largely depends on morphology, particle size, and markers of exosomes [29]. It is anticipated that further methodological advancements would provide gold standard methods, and facilitate the research on exosomes.

In conclusion, this study shows that exosomes from macrophages may play a critical role in AAA development. Administration of the exosome production inhibitor GW4869 prevents CaPO₄-induced AAA development. In mechanism, macrophage-derived exosomes promote MMP-2 production via JNK and p38 pathways in VSMCs. Our study

implies that exosomes from macrophages may be a potential target of AAA treatment.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Yidong Wang was involved in literature research, study design, project administration, data analysis, manuscript writing and editing. Liangliang Jia, Yao Xie and Zhejun Cai were involved in study design, project administration and data analysis. Zhenjie Liu was involved in technical assistance. Jian Shen and Yi Lu was involved in data analysis and manuscript editing. Yaping Wang, Sheng'an Su, Yuankun Ma were

involved in manuscript review and valuable suggestions proposition. Meixiang Xiang was involved in funding acquisition, supervision, manuscript writing and editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2019.08.016>.

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