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Research article

Astragaloside IV inhibits colorectal cancer metastasis by reducing extracellular vesicles release and suppressing M2-type TAMs activation

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ABSTRACT

Ethnopharmacological relevance: Tumour-derived extracellular vesicles (TEVs) have been confirmed to facilitate colorectal cancer (CRC) metastasis by remodelling the tumour microenvironment (TME). Drugs targeted TEVs is considered as a promising therapeutic strategy for cancer treatment. Traditional Chinese medicine (TCM) plays a vital role in improving the prognosis of CRC patients and eventually CRC patients with distant metastasis. Although the antitumour effects of active compounds from TCM prescriptions are observed widely, the molecular mechanisms remain unknown.

Aim of the study: This study aims to investigate the effects of active compounds in our library of TCM on preventing CRC metastasis, and also explore the potential mechanisms from the perspective of TEVs. *Materials and methods:* The effects of active compounds on the proliferation of CRC cells were determined by CCK-8 assay. TEVs were extracted from MC38 cells by ultracentrifugation and characterized by electron microscopy, Nanosight NS300 and western blotting. The TEV particles were quantified by Nanosight NS300. The potential mechanism by which astragaloside IV (ASIV) reduced TEV secretion was determined by western blotting. RAW264.7 cells were cocultured with the conditioned medium (CM) of MC38 cells treated with or without

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ASIV, and the activation of tumour-associated macrophages (TAMs) was assessed by immunofluorescence and quantitative polymerase chain reaction (qPCR). The migration of CRC cells was measured by wound healing and Transwell assay. A spleen-to-liver metastasis model of colorectal cancer was used to confirm the efficiency of ASIV *in vivo*. Liver metastatic tumours of the mice were used for liver weight measures and H&E staining. Immunofluorescence was applied to observe the infiltration of TAMs, the expression of neutral sphingomyelinase 2 (nSMase2) and Rab27a.

Results: By screening our TCM monomer library, we found that ASIV, which is mainly extracted from *Radix Astragali*, reduced the release of TEVs from CRC cells in a time- and concentration-dependent manner. Mechanistically, ASIV inhibited the production and secretion of TEVs by downregulating nSMase2 and Rab27a expression in CRC cells. CM from ASIV-treated CRC cells reshaped the polarization of TAMs by decreasing M2-type polarization, increasing M1-type polarization. Consequently, the repolarization of M2-type to M1-type macrophages led to reduced invasion and migration of CRC cells. Moreover, we confirmed that ASIV inhibited the liver metastasis of CRC, reduced M2-type macrophage infiltration and decreased the expression of nSMase2 and Rab27a in liver metastases.

Conclusions: ASIV inhibited CRC metastasis by reducing EVs release and suppressing M2-type TAMs activation. All these findings reveal a new insight into the mechanisms of ASIV in preventing CRC progression and provide a promising approach for anti-tumour therapy.

Glossary

ASIV	Astragaloside IV
CRC	Colorectal cancer
TAMs	Tumour associated macrophages
EVs	Extracellular vesicles
TEVs	Tumour-derived extracellular vesicles
TME	Tumour microenvironment
EMT	Epithelial mesenchymal transformation
MMPs	Matrix metal proteins
CM	Conditional medium
FBS	Fetal bovine serum
DMEM	Dulbecco's Modified Eagle Medium
TGF-β	Transforming growth factor-β
IL-4	Interleukin-4
CXCL13	C-X-C Motif Chemokine 13
PD-L1	Programmed cell death 1
VISTA	V-set immunoregulatory receptor
ATG5	Autophagy-related proteins 5
nSMase2	Neutral sphingomyelinase 2
PLD2	Phospholipase D2
RAC1	Ras-related C3 botulinum toxin substrate 1
ESCRT	Endosomal sorting complex required for transport
H2-Ab1	Histocompatibility 2, class II antigen A, beta 1
iNOS	Inducible nitric-oxide synthase
Arg-1	Arginase-1
qPCR	Quantitative polymerase chain reaction

1. Introduction

Colorectal cancer (CRC) is the third most common malignant tumour worldwide, with an increasing incidence and mortality rate [1]. The five-year survival rate of CRC patients with distant metastasis is only approximately 15 %, which is much lower than that of patients in early stages [2]. Approximately 10 % of stage II CRC patients and 15%–30 % of stage III CRC patients will develop recurrence and metastasis within 3 years after 6-month oxaliplatin and fluoropyrimidine standard chemotherapy [3,4].

Liver metastasis is the leading cause of CRC-related death and is generally considered to be the result of the interaction between tumour cells themselves and the tumour microenvironment (TME) [5]. TME plays a critical role in CRC progression by forming a premetastatic niche that facilitates tumour metastasis [6,7]. Macrophages are the most dominant cellular component of the TME and

strongly contribute to the development and occurrence of tumour [8,9]. In the early stages of tumour metastasis, macrophages provide a pro-metastatic niche by expressing high levels of matrix metalloproteinases (MMPs) that disrupt the histological barrier, promote epithelial-mesenchymal transformation (EMT) and induce the differentiation of Treg cells [10]. In the rapid enlargement stages, macrophages secrete increasing amounts of transforming growth factor-β (TGF-β), interleukin-4 (IL-4), C-X-C Motif Chemokine 13 (CXCL13) and other inflammatory factors to recruit tumour cells, and macrophages express costimulatory molecules such as programmed cell death 1 (PD-L1) and V-set immunoregulatory receptor (VISTA) to inhibit T-cell function and accelerate the growth of tumour cells [10–12]. It's worth noting that extracellular vesicles (EVs), as intercellular messengers, participate in the communication between tumour cells and macrophages in the TME [13]. Noncoding RNAs within EVs, such as miR-934 and circ_0001142, have been proven to induce macrophage M2-type polarization to accelerate tumour metastasis [14,15]. Due to the vital effect of EVs in regulating the TME, therapeutic strategies that target EVs have become a new direction for the treatment of tumours.

Traditional Chinese medicine (TCM) and its active compounds have been considered as new strategies for the treatment of CRC [16]. Many clinical trials have demonstrated the advantages of TCM in enhancing the efficiency of anticancer therapies, ameliorating serious adverse effects, and prolonging the survival of CRC patients [17,18]. Evidence from experimental studies also revealed the function of TCM and its monomers in preventing CRC growth and metastasis [19,20]. Currently, the regulation of the TME is a hotspot for research on the functional mechanisms of TCM. TCM treatments, including classic traditional compound prescriptions, extracts, and monomers, are related to the regulation of T cell, dendritic cell, myeloid monocyte, and macrophage differentiation in the TME [21]. However, there are few proofs about whether TCM and its active compounds can influence the biological processes of EVs secretion. In this study, we identified that the active compound astragaloside IV (ASIV) could reduce the biogenesis and secretion of EVs. And ASIV-mediating EVs amount decreasing further prevented CRC metastasis by reducing the activation of M2-type tumour--associated macrophages (TAMs), providing novel preventive and therapeutic strategies for tumour metastasis.

2. Materials and methods

2.1. Cell culture

Mouse colon cancer cell line MC38 cell (Chinese Academy of Sciences, Shanghai Cell Bank) and RAW264.7 cell (ATCC, United States) were cultured in Dulbecco's Modified Eagle Medium (DMEM) which contains 10 % fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. All the cells were cultured in a 5 % CO_2 incubator at 37 Celsius ° (°C).

2.2. EVs extraction and purification

EVs extraction by ultracentrifugation are performed as before [19]. In brief, the cell supernatant was centrifuged by 300 g at 4 °C for 10 min. Take the supernatant and centrifuge by 2000 g at 4 °C for 10 min. Then, the supernatant was taken and centrifuged by 10000 g at4 °Cfor 60 min. Take the supernatant and continue to ultra-centrifuge at 4 °C, 100000 g, for 90 min. Remove the supernatant and add 10 mL of PBS suspension granules. Centrifuge at 4 °C, 100000 g, for 90 min. Finally, the sediment in the tube is EVs after removing PBS. 20–50 μL of PBS was added for re-suspension precipitation and stored at −80 °C for further experiments.

2.3. EVs characterization

Add prepared 2 μg/10 μL EV to the carbon-coated copper grids and adsorb for 90 s. Filter paper sucks up excess liquid. Each copper mesh was dyed with 1 % PBS and stained with 10 μL of tungstic solution in the dark for 10 s. Then the excess liquid is absorbed with filter paper and stored in the dark after drying under an electron microscope (model: TECNAI G2 S-TWIN, Thermo Fisher Scientific China Co, Ltd). The size and density of EVs were characterized using NS300 system (NanoSight, Malvern Instruments), and the test was repeated three times for each sample. Amount of EVs (Particles/cell) = EVs density (Particles/mL) ÷ cell concentration (cell/mL) × 100 %; Relative inhibition rate EVs (%) = (EVs amount in control group - EVs amount in ASIV group) ÷ EVs amount in control group × 100 %.

2.4. Western blotting assay

Total protein was extracted from the whole cell or tissue lysates using RIPA lysis buffer (Beyotime, P0013B) containing 1 % PMSF. The concentration of protein was detected by the BCA assay kit (Beyotime, P0010). 15–30 μg protein samples were separated via 10 % SDS-PAG. The resolved proteins were subsequently transferred to the PVDF membranes (Millipore Sigma, IPVH00010) and blocked with 5 % BSA for 1 h at room temperature. Then, the membranes were incubated with the primary antibodies at 4 °C overnight. Antibodies for Western blot analysis are as follow: TSG101 (ab125011, 1:1000), CD81 (ab155760, 1:1000), Rab27a (95394,1:2000), neutral sphingomyelinase 2 (nSMase2) (abs133841,1:2000), β-actin (93473, 1:5000) was used as the internal control. Following primary antibody incubation, the goat anti-rabbit (7074, 1:5000) and goat anti-mouse (7076, 1:5000) horseradish peroxidase secondary antibodies were incubated at room temperature for 2 h. The enhanced chemiluminescence was used to visualize the signals (ECL, Millipore, CA, United States).

2.5. Quantitative real time PCR (qPCR)

The total RNA was extracted according to the manufacturer's instructions. The concentration of RNA was detected by NanoDrop ND-1000. 500–1000 ng total RNA was used to synthesize cDNA with PrimeScript RT kit. QPCR analysis was performed to quantitate the relative mRNA expression of targeted genes, and GAPDH was used as a control. The expression level of mRNA was calculated by $2^{-\triangle\triangle Ct}$. Here were the primers: CD206, Forward: 5-CTCTGTTCAGCTATTGGACGC-3, Reverse: 5- CGGAATTTCTGGGATTCAGCTTC-3; CD274, Forward: 5-GGAATTGTCTCAGAATGGTC-3, Reverse: 5-GTAGTTGCTTCTAGGAAGGAG-3; Inducible nitric-oxide synthase (iNOS): Forward: 5-GGGACTGAGCTGTTAGAGACAC-3, Reverse: 5-CCAAATCCAACGTTCTCCGT-3; Histocompatibility 2, class II antigen A, beta 1 (H2-Ab1), Forward: 5-CACTCTG GTCTGTTCGGTGAC-3, Reverse: 5-CCTCTCCCTGATGAGGGG-3; GAPDH, Forward: 5-GGTGGTCTCCTCTGACTTCAACA-3, Reverse: 5-CCAAATTCGTTGTC ATACCAGGAAATG-3.

2.6. Transwell assay

The density of MC38 cells was adjusted to 4×10^4/mL during logarithmic growth. Then 100 μL of cell suspension with DMEM containing 0.5 % FBS was added in the upper chamber of Transwell. 600 μL of conditional medium (CM) was added to the lower chamber, which prepared with 300 μL of DMEM medium with 20 % FBS and 300 μL of RAW264.7 cell supernatant with or without ASIV treatment. After incubation for 48 h, the chambers were fixed with 4 % paraformaldehyde for 15 min, and then stained with crystal violet for 10 min. Finally, erase the upper chamber cells with a cotton swab. Five fields of view were randomly selected under an inverted microscope (DMI3000B, Leica), and the number of cells in each field was counted by ImageJ. All the experiments were performed three times independently.

2.7. Wound healing assay

When MC38 cells grew to 80 % of the 6-well plate, 2 mL of CM was added. Then, use a 100 μL pipette to draw a straight scratch on the bottom of the 6-well plate. Serum-free DMEM medium was added to the control group. And CM prepared with 300 μL serum-free DMEM and 300 μL RAW264.7 cell supernatant was added to the experimental group. Images were collected at 0 h, 12 h, 24 h by an inverted microscope.

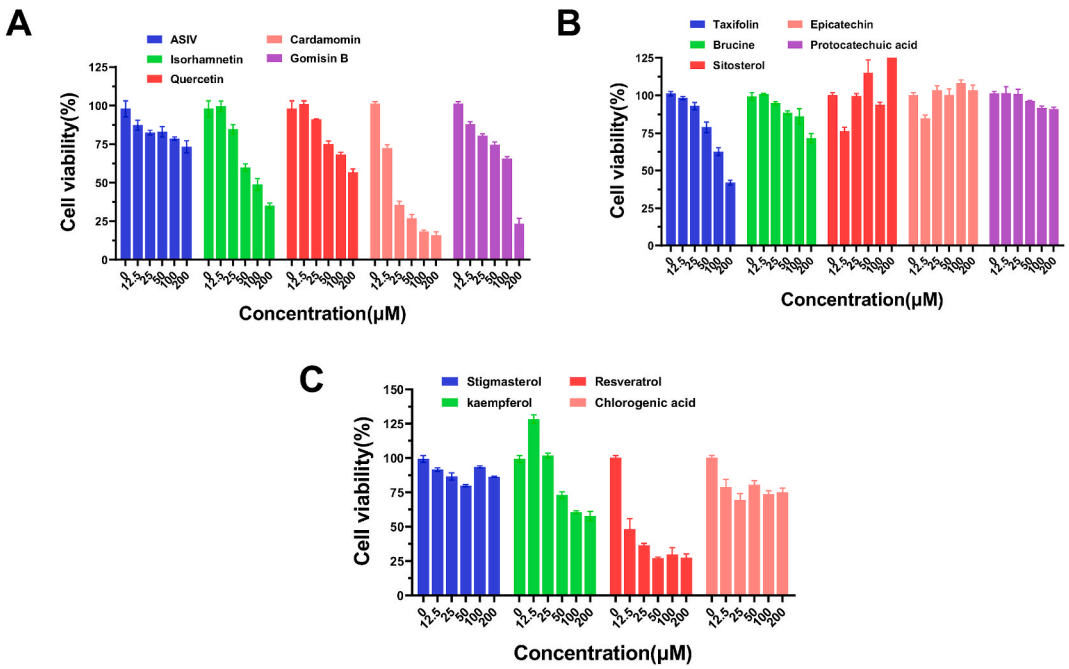

Fig. 1. Effect of the active compounds in monomer library of TCM on the proliferation of CRC cells. MC38 cells were seeded into 96-well palate and treated with different-concentration of compounds (0, 12.5 μM, 25 μM, 50 μM, 100 μM, 200 μM) for 48 h. CCK-8 was used to detect the cell viability. (A) The cell viability of MC38 cells treated with ASIV, Ishorhamnetin, Quercetin, Cardamomin and Gomisin B (n = 5). (B) The cell viability of MC38 cells treated with Taxifolin, Brucine, Sitosterol, Epicatechin, and Protocatechuic acid (n = 5). (C) The cell viability of MC38 cells treated with Stigmasterol, Kaempferol, Resveratrol and chlorogenic acid (n = 5). TCM: Traditional Chinese medicine; CRC: colorectal cancer; CCK-8: cell counting-8 assay; ASIV: Astragaloside IV.

2.8. Tumour mouse model

CRC liver metastasis model of spleen to liver was conducted to confirm the efficiency of ASIV [19]. 6-8-week old C57BL/6J mice were randomly divided into model group (control), MC38-EVs group, GW4869 group and ASIV group. 5×10^5/50 μL MC38 cells were injected into the spleen of mice, and the mice were sacrificed after 3–4 weeks to obtain the liver tissues. The model group received PBS by tail vein injection, every other day for 3 weeks from 1 week before spleen injection; The MC38-EVs group received 10 μg/100 μL of MC38-EVs by tail vein injection, every other day for 3 weeks from 1 week before spleen injection; The GW4869 group received 2.5 μg/g of GW4869 intraperitoneal injection, every other day for 2 weeks after spleen injection; The ASIV group received 50 mg/kg of ASIV intragastric administration, every day from 1 week before spleen injection. The animal experiments were carried out in accordance with animal ethics guidelines and permitted by the Animal Care and Use Committee at Shanghai University of Traditional Chinese Medicine. Animal ethics number is PZSHUTCM210723003, July 23, 2021.

2.9. Hematoxylin & eosin and immunofluorescence staining

The liver tissue specimens were fixed with paraffin wax, then dewaxed and dehydrated before staining. Hematoxylin and eosin (H&E, Merck) was used to stain the tissue specimens. For immunofluorescence staining, the slides were permeabilized by 0.5 % Triton X-100 for 15 min, blocked with 5 % BSA for 1 h and incubated with CD11B (1:1000) or CD206 (1:1000) for 1 h. Then, the secondary antibodies were used for incubation for 1 h at room temperature. Finally, nucleus was stained with DAPI for 5 min.

2.10. Statistic analysis

All data were statistically analyzed using SPSS 26.0 software. The measurement data were expressed as mean ± standard deviation, and one-way ANOVA was used for multi-group comparison, and least significant difference was used for pairwise comparisons within groups. For data that did not conform to normal distribution and uneven variance, the H rank sum test was performed. Test standard was $\alpha = 0.05$, $P < 0.05$ was considered statistically significant.

3. Results

3.1. Astragaloside IV reduces the release of EVs from CRC cells

Intervention in the biological processes of EVs production and secretion has been confirmed to inhibit tumour metastasis [22,23]. Herein, to identify active compounds that can reduce EVs production at nontoxic doses, we first investigated the effect of active compounds in our TCM monomer library on the proliferation of CRC cells (Fig. 1A–C). 9 active compounds, including ASIV, Ishorhamnetin, Quercetin, Cardamomin, Gomisin B, Taxifolin, Brucine, Kaempferol, and Resveratrol showed little effect on CRC cell proliferation (Table 1) and were selected as candidate drugs for further research. Before the formal experiment, we repeatedly confirmed our technology for EVs extraction. EVs were extracted from MC38 cells by ultracentrifugation and characterized by electron microscopy, Nanosight NS300 and western blotting. The data in Fig. 2A–C showed that we successfully extracted EVs. Next, to investigate the effects of these 9 active compounds on the release of EVs from CRC cells, MC38 cells were treated with the above 9 compounds for 48 h, and cell supernatants were collected for EVs extraction. GW4869, the inhibitor of EVs biogenesis by targeting nSMase2 was used to compare the efficiency of the monomers [24]. The results showed that ASIV, Ishorhamnetin, Quercetin, Gomisin B, Taxifolin, and Brucine effectively inhibited EVs release from MC38 cells ($P < 0.05$) (Fig. 2D, Fig. S1). Moreover, among these active compounds, only ASIV could significantly inhibit the release of EVs in a dose- and time-dependent manner ($P < 0.05$) (Fig. 2E–F, Fig. S2, Table 2).

3.2. Astragaloside IV inhibits the biogenesis and secretion of EVs from CRC cells

The biogenesis and secretion of EV depend on a variety of proteins, including nSMase2, Ras-related C3 botulinum toxin substrate 1 (RAC1), phospholipase D2 (PLD2), autophagy related 5 (Atg5) and Rab27a/Rab27b [25]. Therefore, we measured the mRNA and protein expression levels of nSMase2, PLD2, Rab27a, Rab27b, RAC1, and ATG5 to screen potential targets of ASIV. The results demonstrated that the mRNA expression levels of nSMase2, Rab27a and ATG5 in MC38 cells were significantly reduced after treatment

Table 1
The IC10 of monomers for the inhibition of MC38 viability.

Monomer	IC10(μM)	Monomer	IC10(μM)	Monomer	IC10(μM)
ASIV	12.5	Taxifolin	50	Stigmasterol	/
Ishorhamnetin	20	Brucine	50	Kaempferol	20
Quercetin,	25	Sitosterol	/	Resveratrol	10
Cardamomin	10	Epicatechin	/	chlorogenic acid	/
Gomisin B	25	Protocatechuic acid	/		

IC10 represents the concentration of drugs with a 10 % inhibition rate of cell proliferation. IC: inhibitory concentration; ASIV:Astragaloside IV.

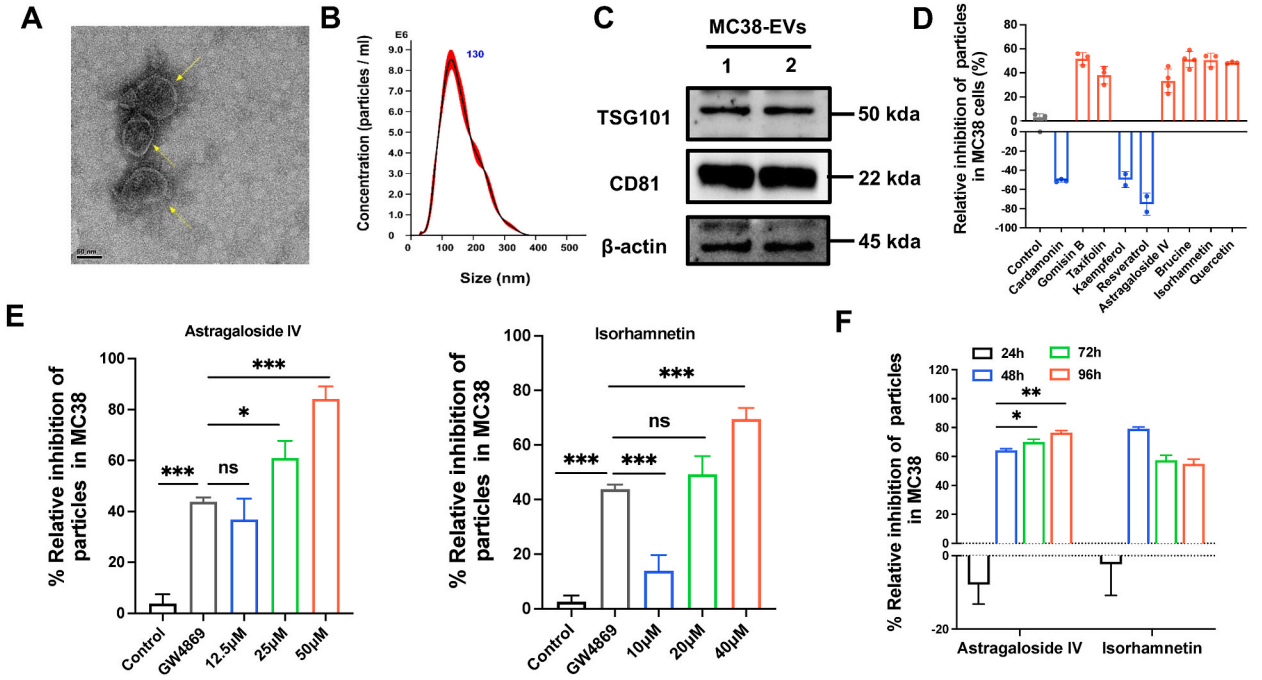

Fig. 2. ASIV inhibits EVs release from CRC cells in a dose- and time-dependent manner. (A-B) Characterization of EVs derived from MC38 cells by electron microscopy and Nanosight NS300 system. Yellow arrows indicate representative EVs. Scale bar, 50 nm. (C) Western blotting detection the expression of EVs markers TSG101, CD81 in the EVs derived from MC38 cells. 1 and 2 represent repeated experiment. (D) MC38 cells were treated with 25 μM ASIV, 20 μM Ishorhamnetin, 25 μM Quercetin, 10 μM Cardamomin, 2 5 μM Gomisin B, 50 μM Taxifolin, 50 μM Brucine, 20 μM Kaempferol and 10 μM Resveratrol for 48 h. The relative inhibition of EVs release was detected by Nanosight NS300 system (n = 3). (E) MC38 cells were treated with GW4869 (10 μM), ASIV (12.5 μM, 25 μM, 50 μM), Ishorhamnetin (10 μM, 20 μM, 40 μM), Gomisin B (12.5 μM, 25 μM, 50 μM), Taxifolin (25 μM, 50 μM, 100 μM), Quercetin (12.5 μM, 25 μM, 50 μM) and Brucine (25 μM, 5 0 μM, 100 μM) for 48 h (n = 3). The relative inhibition of EVs release was detected by Nanosight NS300 system. ns means $P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$, compared with GW4869 group. (F) MC38 cells were treated with GW4869, ASIV and Ishorhamnetin for 24 h, 48 h and 72 h. The relative inhibition of EVs release was detected by Nanosight NS300 system (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with 24 h group. EVs: Extracellular vesicles; ASIV: Astragaloside IV.

Table 2
The density of EVs derived from MC38 cells treated with active monomers.

Time (h)	Monomer	Density (10^8 particles/10^7 cells)	Inhibition rate (%)
24	Control	63.68 ± 7.74	/
	ASIV	70.62 ± 5.71	−10.90
	Ishorhamnetin	67.19 ± 4.40	−6.19
48	Control	29.71 ± 1.81	/
	ASIV	10.60 ± 0.32	64.25
	Ishorhamnetin	6.25 ± 0.46	78.96
72	Control	113.79 ± 14.27	/
	ASIV	37.53 ± 7.04	67.02
	Ishorhamnetin	48.54 ± 3.91	57.34
96	Control	105.70 ± 6.39	/
	ASIV	24.88 ± 1.67	76.46
	Ishorhamnetin	47.61 ± 3.40	54.96

ASIV:Astragaloside IV.

with 25 μM ASIV ($P < 0.05$) (Fig. 3A). Moreover, we found that ASIV could inhibit nSMase2 and Rab27a mRNA expression in a dose-dependent manner ($P < 0.05$) (Fig. 3B and C). Western blotting results further confirmed that ASIV was able to inhibit nSMase2 and Rab27a protein expression in a dose-dependent manner ($P < 0.05$) (Fig. 3D–F), suggesting that ASIV could reduce TEVs release by simultaneously blocking the biogenesis and secretion processes.

3.3. Astragaloside IV regulates macrophage M2-type polarization by inhibiting EVs release

Tumour-derived EVs (TEVs) can promote CRC metastasis by activating TAMs and reshaping the tumour microenvironment. Next, we investigated whether ASIV could affect macrophage activation by reducing EVs release *in vitro*. The flow cytometry results showed

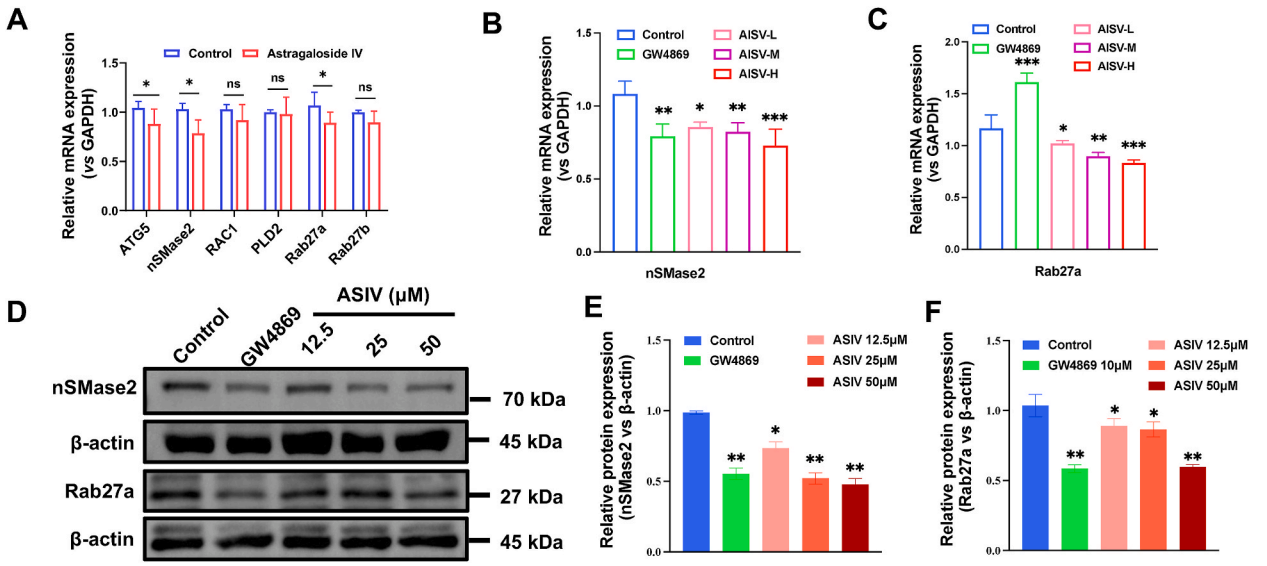

Fig. 3. Astragaloside IV inhibit the biological process of EVs biogenesis and secretion. (A) The mRNA expression of ATG5, nSMase2, RAC1, PLD2, Rab27a and Rab27b in MC38 cells by qPCR after treated with 25 μM ASIV (n = 3). The experiment was performed three times with similar results. ns means $P > 0.05$, $^{*}P < 0.05$, compared with group without treatment of ASIV. (B–C) The mRNA expression of nSMase2 and Rab27a in MC38 cells by qPCR after treated with GW4869 (10 μM) and ASIV (12.5 μM, 25 μM and 50 μM) (n = 3). The experiment was repeated three times with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, comparing to group without ASIV or GW4869 treatment. (D-F) Western blotting and quantitative assay on the protein expression of nSMase2 and Rab27a in MC38 cells after treated with GW4869 (10 μM) and ASIV (12.5 μM, 25 μM and 50 μM). The experiment was repeated three times with similar results. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, comparing to group without ASIV or GW4869 treatment. EVs: Extracellular vesicles; ATG5: Autophagy-related proteins 5; nSMase2: Neutral sphingomyelinase 2; RAC1: Ras-related C3 botulinum toxin substrate 1; PLD2: Phospholipase D2; qPCR: Quantitative polymerase chain reaction.

that CM from MC38 cells induced more than 2-fold change in the differentiation of RAW264.7 cells into macrophages compared with the control ($P < 0.01$). GW4869 and ASIV could inhibit more than 50 % of macrophages differentiation compared with the MC38-CM group ($P < 0.01$) (Fig. 4A and D), and the inhibitory effects were concentration-dependent. In addition, comparing with the control group, the number of M1-type macrophages reduced more than 50 % and the number of M2-type macrophages increased almost 3 time in MC38-CM group ($P < 0.001$). Besides, GW4869 and ASIV reduced the number of M2-type macrophages and increased the number of M1-type macrophages in a dose-dependent manner (more than a 2-fold change at the medium dose) when compared with the MC38-CM group ($P < 0.01$) (Fig. 4B and C, 4E-F). The qPCR results confirmed that the mRNA expression of iNOS and H2-Ab was increased, while the mRNA expression of arginase-1 (Arg-1) and CD274 was decreased after treatment with GW4869 and ASIV ($P < 0.05$) (Fig. 4G and H). Besides,we also confirmed that ASIV could reduce the releasing of EVs from HCT116 and SW620 cells ($P < 0.001$) (Fig. S3A). And the CM of HCT116 treated with ASIV could decrease the M2-type polarization of THP-1 cells ($P < 0.01$) (Fig. S3B), which was rescued by the stimulation of EVs in addition (Fig. S4). These results demonstrated that ASIV could regulate M2-type macrophage polarization by inhibiting EVs release *in vitro*.

3.4. Astragaloside IV inhibits CRC migration via regulating TEVs-pretreated macrophages

Tumour-derived EVs have been confirmed to activate TAMs to promote CRC metastasis [26,27]. Therefore, we detected whether ASIV could inhibit CRC metastasis of by reducing EVs biogenesis and secretion. Transwell and wound healing assays showed that the supernatant from RAW264.7 cells treated with MC38-CM increased the invasion and migration of MC38 cells at 1.5 times ($P < 0.01$), while the treatment of ASIV could impede the invasion and migration of MC38 cells in a dose-dependent manner with 20%–60 % inhibition ($P < 0.01$) (Fig. 5A–D). Meanwhile, we also confirmed that ASIV could decrease the migration of HCT116 cells by inhibiting the expression of CD206 in THP-1 cells ($P < 0.001$) (Figs. S3B–D). All the results indicated that ASIV could inhibit the invasion and migration of CRC cells by reducing the biogenesis and secretion of EVs and the activation of TAMs. Then, we further investigated the inhibitory effect of ASIV on CRC metastasis *in vivo*. A spleen-to-liver metastasis model of CRC was established, and MC38-EVs, GW4869, ASIV were administered as treatments as described (Fig. 5E). We found that the liver weight of mice treated with MC-38 EVs was increased about 2 times comparing with the control group ($P < 0.001$), while ASIV could significantly reduce the liver weight of the mice by more than 50 % compared to the liver weight in the MC-38 EVs group ($P < 0.01$) (Fig. 5F). And the H&E staining further confirmed that ASIV could significantly reduce the number and size of CRC liver metastases (Fig. 5G). Moreover, the inhibitory effect of ASIV on liver metastasis was similar to that of GW4869 (Fig. 5G).

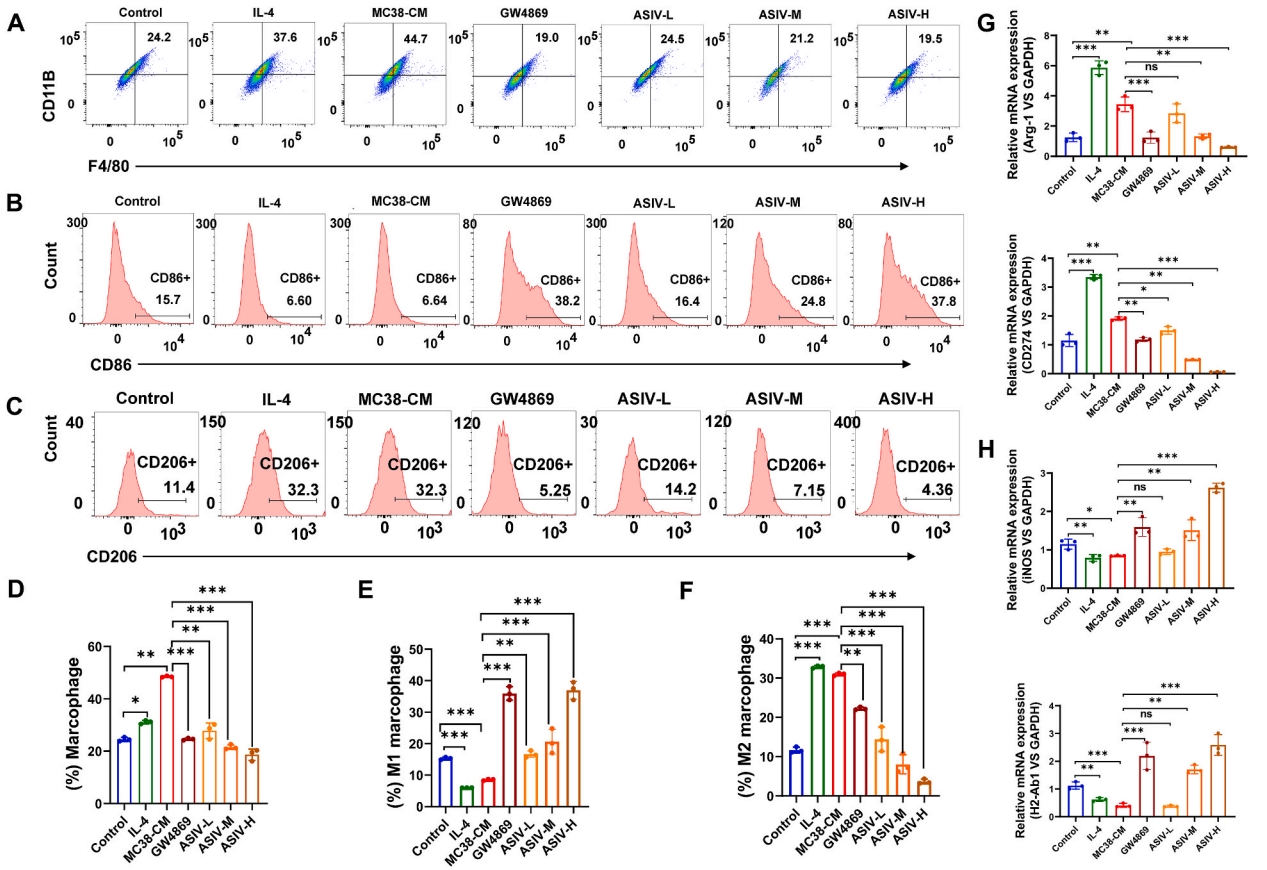

Fig. 4. Astragaloside IV regulates macrophages M2-type polarization by inhibiting EVs release. Flow cytometry detected the proportion of macrophages (A,D), M1-type macrophages (B,E) and M2-type macrophages (C,F) of RAW264.7 under induction with IL-4, MC38-CM with/without GW4869 or ASIV intervention (n = 3). Macrophages: F4/80 and CD11B; M1-type macrophages F4/80, CD11B and CD86; M2-type macrophages F4/80, CD11B and CD206. (G-H) The mRNA expression of Arg-1, CD274, iNOS and H2-Ab1 in RAW264.7 cells by qPCR (n = 3). ns means $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. EVs: Extracellular vesicles; CM: Conditional medium; Arg-1: Arginase-1; iNOS: Inducible nitric-oxide synthase; H2-Ab1: Histocompatibility 2, class II antigen A, beta 1; qPCR: Quantitative polymerase chain reaction; ASIV: Astragaloside IV.

3.5. Astragaloside IV inhibits CRC liver metastasis by suppressing TEVs-mediated M2-type TAMs activation

To confirm the function and mechanism of ASIV *in vivo*, we further measured the number of TAMs and the expression of nSMase2 and Rab27a in mouse CRC liver metastases. Flow cytometry showed that the infiltration of macrophages was almost 1.5-time higher in the MC38-EVs group than that in the control group ($P < 0.01$). However, the infiltration of macrophages in the GW4869 and ASIV groups was significantly lower than that in the MC38-EVs group (3-fold and 2-fold change, $P < 0.01$) (Fig. 6A), indicating that ASIV could prevent the infiltration of macrophages in CRC liver metastases. In addition, the numbers of M2-type macrophages were doubled in the MC38-EVs group compared with control ($P < 0.01$), and decreased by more than 50 % in the GW4869 and ASIV groups comparing with MC38-EVs group ($P < 0.01$) (Fig. 6C). In contrast, the number of M1-type macrophages was decreased by 30 % in the MC38-EVs group when compared with control ($P < 0.01$), and increased in the GW4869 (3-fold change) and ASIV (1.5-fold change) groups ($P < 0.05$) (Fig. 6B). The immunofluorescence results also confirmed that the infiltration of M2-type macrophages in the ASIV group was much lower than that in MC38-EVs group ($P < 0.05$) (Fig. 6D and E), indicating that ASIV could inhibit the infiltration of macrophages and regulate the polarization of TAMs. We also observed the expression of nSMase2 and Rab27a in mouse CRC liver metastases to verify whether ASIV could interfere with EVs *in vivo*. Western blotting analysis indicated that in contrast to the control group, the expression of nSMase2 and Rab27a in CRC liver metastases was suppressed by almost 50 % in the ASIV group ($P < 0.01$) (Fig. 6F and G). These experiments *in vitro* and *in vivo* all proved that ASIV could inhibit liver metastasis of CRC by reducing EVs release and suppressing M2-type TAMs activation.

4. Discussion

Liver metastasis is the leading cause for the death of CRC, lacking efficient treatment therapies. TCM and its active monomers have been widely used in the treatment of various cancers. ASIV, which is the most critical component of *Radix Astragali*, has been confirmed

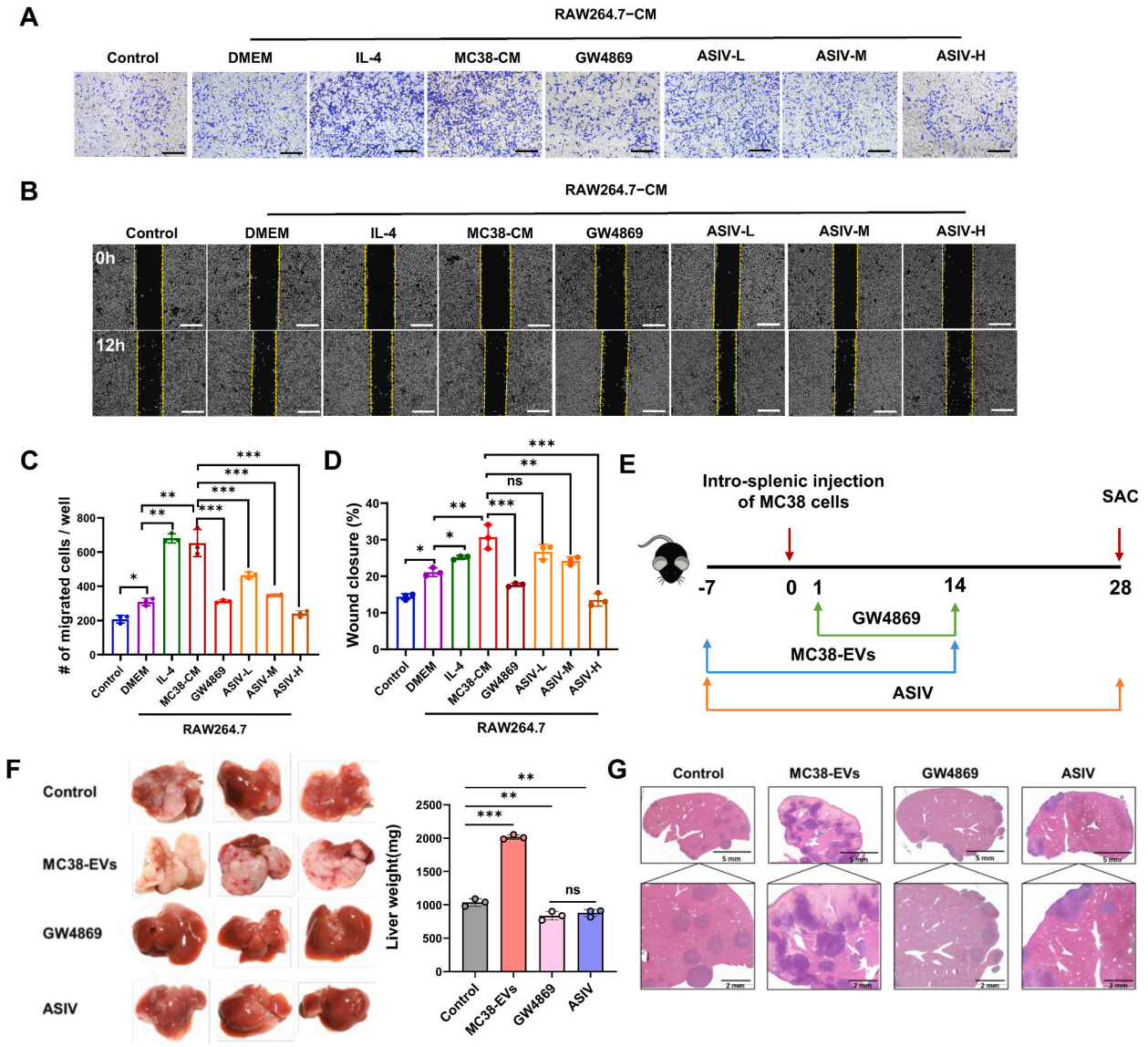

Fig. 5. Astragaloside IV inhibits CRC migration via regulating TEVs-pretreated macrophages. (A-D) MC38 cells were co-cultured with conditional medium of RAW264.7 cells induced by IL-4 and MC38 supernatant with or without ASIV treatment. Migrating cells were evaluated by Transwell and wound healing assays in three randomized microscopic fields (n = 3). The experiment was repeated three times with similar results. Scale bar, 150 μm ns means $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (E) Experimental scheme and intervention of MC38-EVs, GW4869 and ASIV in mice (n = 3 mice per group). Each group mice received intro-splenic injection with MC38 single cell suspension (5×10^5/50 μL). Control group was injected with 100 μL PBS through tail vain every other day for 3 weeks; MC38-EVs group was injected with 10 μg/100 μL MC38-EVs through tail vain every other day for 3 weeks; GW4869 group was intraperitoneally injected with 2.5 μg/g GW4869 every other day for 2 weeks; ASIV group received ASIV intragastric administration 50 mg/kg every day from 5 weeks (started from 1 week before the intro-splenic injection of MC38 cells). After 4 weeks, the mice were sacrificed. (F) Representative images and quantitative results of CRC liver metastases (n = 3). $^{**}P < 0.01$, $^{***}P <$ 0.001,compared model group; ns means $P > 0.05$, compared with GW4869 group. (G) The liver was stained by H&E and the number of metastases was counted (n = 3). Up: Scale bar, 5 mm; Down: Scale bar, 2 mm. TAMs: Tumor associated macrophages; ASIV: Astragaloside IV; EVs: Extracellular vesicles; CRC: Colorectal cancer.

to exert effects on the myocardium, endocrine system, and nervous system [28]. Recently, the anti-tumour effect of ASIV has been proven in a variety of malignant tumours, such as lung cancer, CRC and breast cancer [29–32]. Many studies have revealed that the ASIV functions in tumour treatment by preventing precancerous lesions, reversing drug resistance, and regulating immunity. However, the role of ASIV in inhibiting tumour metastasis is rarely reported. *In vitro* studies showed ASIV treatment suppressed the invasion of cervical cancer cells by reducing the expression of proteins that related to cell cytoskeletons [33]. ASIV also reduced the malignant of hepatocellular carcinoma by decreasing cell migration and EMT [34]. In the present study, we first revealed the important function of ASIV in preventing CRC metastasis both *in vitro* and vivo, providing a new strategy for the clinical prevention and treatment of CRC.

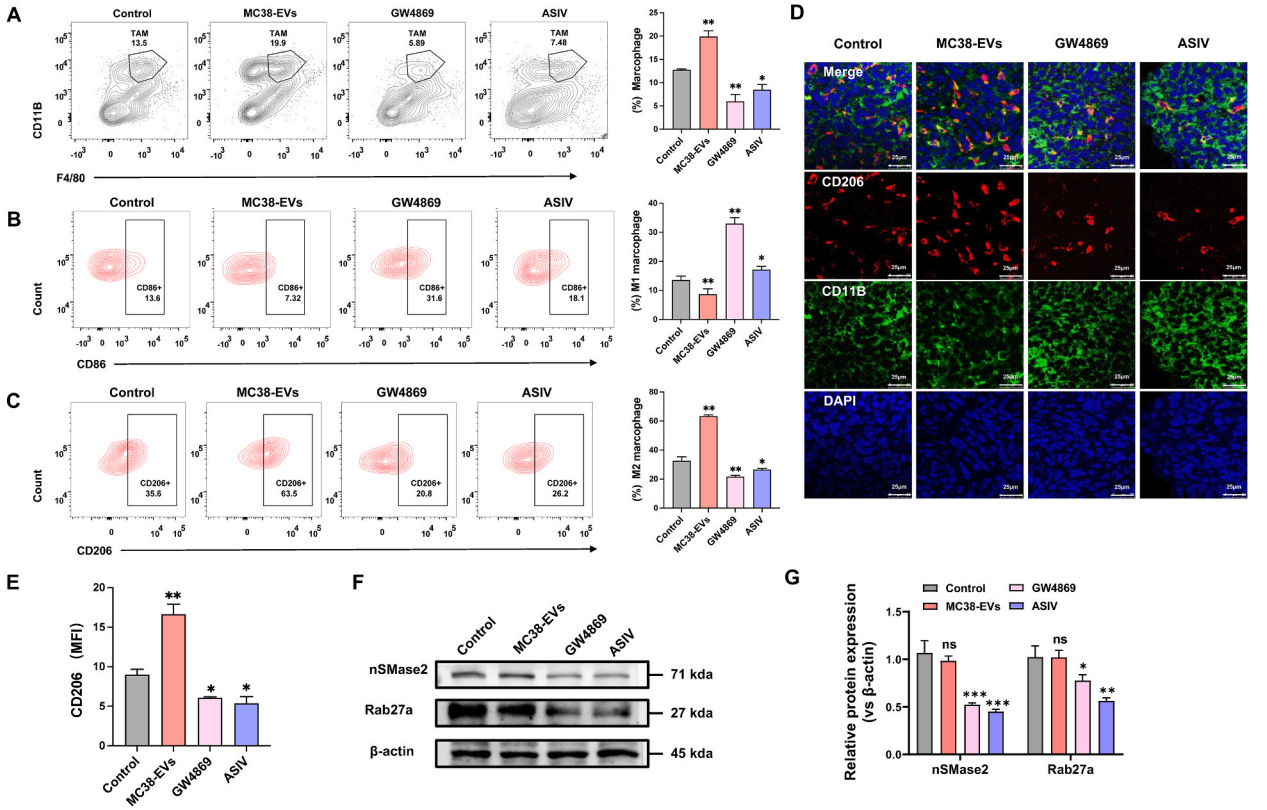

Fig. 6. Astragaloside IV inhibits CRC liver metastasis by suppressing EVs mediated M2-type TAMs activation. Flow cytometric identified the infiltration of macrophages (A), M1-type macrophages (B) and M2-type (C) macrophages in the liver metastatic tumour tissues (n = 3). Macrophages were sorted with F4/80 and CD11B staining, M1-type macrophages were sorted with F4/80, CD11B and CD86 staining, M2-type macrophages were sorted with F4/80, CD11B and CD206 staining. $*P < 0.05$, $**P < 0.01$, compared with model group. (D-E) Immunofluorescence detection and quantitative assay of TAMs in liver metastatic tumour tissues (n = 3). Red: CD206, green: CD11B, blue: DAPI. Scale bar, 25 μm $*P < 0.05$, $**P < 0.01$, compared with model group. (F-G) Western blot and quantitative assay on the expression of nSMase2 and Rab27a in liver metastatic tumour tissues. The experiment was repeated three times with similar results. ns means $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, comparing to model group. TAMs: Tumour associated macrophages; ASIV: Astragaloside IV; EVs: Extracellular vesicles; CRC: Colorectal cancer.

EVs play a critical role in promoting tumour metastasis by inducing EMT, regulating the extracellular matrix, and remodelling the TME [35]. Inhibition EVs secretion is able to suppress tumour development, thus development of drugs targeting EVs biogenesis is a new direction for the anti-tumour strategies. The production of EVs is a complex multistep process, including the endocytosis of the cell membrane, the formation of intraluminal vesicles (ILVs) in multivesicular bodies (MVBs), the transport and fusion of MVBs with the plasma membrane, which is regulated by a group of proteins [36]. Various proteins and enzymes related to phospholipid formation and protein sorting are reported to control the biogenesis of EVs [25]. Endosomal sorting complex required for transport (ESCRT) and nSMase2 promote the formation of ILVs by regulating endocytosis and cargoes sorting [37,38]. PLD2 can influence the budding of EVs vectors by producing lipid PA [39]. GW4869, the targeted inhibitor o nSMase2, is able to reduce the number and exosomal PD-L1 in TEVs derived from melanoma cells to enhance the efficiency of PD-L1 checkpoint blockade [40]. In this study, we demonstrated that ASIV could decrease EVs biogenesis by inhibiting the expression of nSMase2 in CRC cell, and the inhibitory of ASIV is similar to that of GW4869, suggesting the huge potential of ASIV to be a natural nSMase2-targeted inhibitor. Ras-GTPases, such as Rab27a, Rab27b and RAC1, are involved in the secretion of EVs [41]. The depletion of Rab27a significantly reduced TEVs secretion and reversed the liver metastasis of extrahepatic tumours by reprogramming hepatic metabolism [42]. In our work, we identified that ASIV could inhibit the expression of Rab27a in CRC cells and tumour tissues. The following investigation of biological function confirmed the ability of ASIV to prevent CRC metastasis by simultaneously reducing EVs biogenesis and secretion by inhibiting the expression of nSMase2 and Rab27a.

Macrophages are the most infiltrating immune cells in the TME, and have been confirmed to play a critical role in tumourigenesis and metastasis by supporting tumour capacity, inducing EMT, and formatting an immunosuppressive microenvironment [43]. Macrophages exhibit strong plasticity, with high expression of proinflammatory genes, such as iNOS and H2-Ab1, under inflammatory conditions and enhanced expression of protumour genes, such as Arg-1 and CD274, under tumour conditions [44,45]. As the important messenger in cell-to-cell communication, EVs are confirmed to facilitate tumour development through remodelling macrophages. Tumour-derived exosomal miR-934 accelerates the liver metastasis of CRC by inducing M2-type macrophage polarization via

activating PI3K/AKT signaling pathway [46]. Currently, the TME is a research hotspot for discussing the fundamentals of TCM. The advantages of multiple pathways and multiple targets of TCM correspond to the complex composition and dynamic changes in the TME. Our previous work indicated that JPJDR could prevent CRC metastasis by downregulating the expression of ITGBL1 within EVs derived from CRC and inhibiting the activation of cancer-associated fibroblasts [19]. In the present study, we elucidated the reducing of TEVs under ASIV treatment reshaped the polarization of TAMs by decreasing M2-type polarization and increasing M1-type polarization. Besides, both *in vivo* and *in vitro* experiments demonstrated that ASIV could inhibit the activation of M2-type TAMs and prevent CRC metastasis by regulating the release of EVs from CRC cells. We uncovered the vital function of ASIV in preventing CRC metastasis by downregulating EVs-mediated M2-type TAMs activation.

Our results clarified that active compounds which could not directly kill tumour cells might play antitumour roles by regulating the TME, such as inhibiting EVs release from tumour cells and preventing further tumour metastasis. Thus, the application of ASIV in the treatment of CRC liver metastasis maybe broad in clinical practice. Modification of the ASIV dosage form and targeting nanoparticles loaded with ASIV may improve the efficacy and utilization of ASIV and make it possible for ASIV to be translated into clinical use for CRC treatment. However, there are some limitations in our study. EVs contain a large number of active components, including proteins, lipids, RNAs, and DNA. The particular components in EVs that participate in regulating TAMs activation and how these EVs components exert their effects are not clearly understood. In addition, how ASIV affects the biogenesis and secretion of EVs is not well elucidated, especially the specific and direct targets as well as the signaling pathway involved in regulating the expression or activity of Rab27a and nSMase2. More in-depth investigation is needed tofully elucidate the mechanism of ASIV in inhibiting EVs secretion.

5. Conclusion

In summary, our study demonstrated that ASIV inhibited CRC metastasis by reducing EVs release and suppressing M2-type TAMs activation, indicating the potential clinical translation for ASIV in preventing CRC metastasis. In addition, our work makes it possible to clarify the pharmacodynamic fundamentals of TCM and its active ingredient from the perspective of EVs, and inspires more studies to develop new anti-tumour agents with clear mechanisms.

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Ethics statement

This study was reviewed and approved by the Animal Care and Use Committee at Shanghai University of Traditional Chinese Medicine, with the approval number: PZSHUTCM210723003.

Consent for publication

Not applicable.

Data availability statement

All data relevant to the study are included in the article or uploaded as online supplemental information, without undue reservation, to any qualified researcher.

CRediT authorship contribution statement

Jing Zhou: Writing – review & editing, Writing – original draft. **Ling Li:** Validation, Methodology. **Yunzhou Pu:** Validation, Methodology. **Haoze Li:** Methodology. **Xinnan Wu:** Methodology. **Ziyuan Wang:** Methodology. **Jian Sun:** Methodology. **Qing Song:** Data curation. **Lihong Zhou:** Data curation. **Xinwen Ma:** Supervision, Data curation. **Liu Yang:** Supervision, Funding acquisition. **Qing Ji:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31450.

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