Supporting Materials

Endoplasmic Reticulum Stress Promotes Liver Cancer Cells to Release Exosomal miR-23a-3p and Up-regulate PD-L1 Expression in Macrophages

Jiatao Liu,[1,2#] Lulu Fan,[1#] Hanqing Yu,[1#] Ju Zhang,[1#] Yong He,[3] Dechun Feng,[3] Fang Wang,[1] Xiaoqiu Li,[1] Qingqing Liu,[1] Yuhuan Li,[1] Zhenli Guo,[1] Bin Gao,[3] Wei Wei,[4] Hua Wang,[1,5*] and Guoping Sun[1*]

[1]Department of Oncology, [2]Department of Pharmacy, the First Affiliated Hospital of Anhui Medical University, Hefei, China; [3]Laboratory of Liver Diseases, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD; [4]Institute of Clinical Pharmacology, Anhui Medical University, Hefei, China; [5]Institute for Liver Diseases of Anhui Medical University, Hefei, China

Contents of the Supporting Data

Supporting Materials and Methods

Mice, cell culture, and reagents. Female BALB/c nude mice (6-8 weeks old) were purchased (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), housed, and fed under pathogen-free conditions. All mice protocols were consistent with the guidelines of the Animal Research: Reporting of *In Vivo* Experiments (https://www.nc3rs.org.uk/arrive-guidelines) and approved by the Animal Research Committee at the First Affiliated Hospital of Anhui Medical University. The primary human hepatocytes were obtained from Lonza (Walkersville, MD) and the Liver Tissue Cell Distribution System at the University of Minnesota, which was funded by NIH contract HHSN276201200017C. The human HCC cell lines HepG2 and Hep3B, normal hepatocytes HL-7702 cells, mouse HCC cell lines Hepa1-6, and human lung cancer cell line A549 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, NY, USA) plus 10% fetal bovine serum (Gibco, NY, USA). The human promonocytic leukemia cell line THP-1 cells and mouse RAW264.7 macrophage cells were maintained in Roswell Park Memorial Institute (RPMI) medium (1640; Gibco, NY, USA) containing 10% fetal bovine serum.

Tunicamycin (TM) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (MO, USA), resuspended in dimethyl sulfoxide (DMSO), and stocked at −20°C. Interleukin-2 was obtained from Sigma-Aldrich and reconstituted in phosphate-buffered saline (PBS). The PKH67 Green Fluorescent Cell Linker Mini Kit was from Sigma-Aldrich. The exosome isolation kit ExoQuick-TC, exosome-depleted FBS media, and anti-CD63 antibody were purchased from System Bioscience (Mountain View, CA). FITC-conjugated anti-mouse F4/80 and control IgG2a,κ, APC-conjugated anti-mouse F4/80 and control IgG2a,κ, APC-conjugated anti-mouse CD274 antibodies and control IgG2b,κ, PE-conjugated anti-mouse CD206 and control

IgG2 a,κ, FITC-conjugated anti-human CD3, PE-conjugated anti-human CD8，APC-conjugated anti-human CD4, and anti-mouse CD16/32 antibodies were purchased from BioLegend (San Diego, CA). PE-conjugated anti-human CD274 and control IgG1,κ and human FC block were from BD Biosciences (Mississauga, CA). Rabbit-anti Calnexin (#2679), PD-L1 (#13684), GAPDH (#5174), PTEN (#9188), AKT (#4685), and phospho-AKT (#4060) and mouse anti-β-actin (#3700) were purchased from Cell Signaling Technology (Danvers, USA). Rabbit-anti TSG101 (ab125011), GRP78 (ab108615), CD274 (ab205921), ATF6 (ab37149), PERK (ab65142), IRE1α (ab48187), and mouse-anti CD68 (ab955) were purchased from Abcam (Cambridge, UK). Anti-rabbit and anti-mouse IgG peroxidase-conjugated secondary antibodies were obtained from Bioworld (Nanjing, China). BD IMag Human T Lymphocyte Enrichment Set-DM (Cat. #557874), mouse and human Cytokine Bead Array (CBA) inflammation kits, and Cell Counting kit-8 were obtained from BD Biosciences (Mississauga, CA). BCA Protein Assay Kit was obtained from Beyotime Biotechnology (Shanghai, China). Ficoll-Paque Plus was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). The antibodies used in the current study are listed in Supporting Table S11.

Immunohistochemistry. HCC samples were analyzed immunohistochemically using antibodies against GRP78, ATF6, PERK, IRE1α, CD68, and PD-L1. Briefly, 4-μm sections were deparaffinized in xylene and dehydrated in an ethanol diluent serial. Then, 0.3% hydrogen peroxidase was used to inhibit the activity of endogenous peroxidase at room temperature for 30 minutes. Next, antigen retrieval was carried out using a microwave oven before nonspecific binding was blocked using 5% normal goat serum at 37°C for 15 minutes. The sections were incubated with the primary antibody at 4°C overnight in a humidified box. After three washes with PBS, biotinylated anti-mouse immunoglobulin was used to incubate the sections at 37°C for 30 minutes. The samples were visualized using 3, 3-diaminobenzidine

tetrahydrochloride and counterstained with hematoxylin.

For analysis, the level of ER stress-related proteins and PD-L1 protein, the percentage of positive cells (0 for negative, 1 for ≤10%, 2 for 11%-50%, 3 for 51%-75%, 4 for >75%) and their staining intensity (0 for negative staining, 1 for light yellow, 2 for claybank, 3 for brown) were analyzed in five random fields of each sample. The expression of ER stress-related proteins and PD-L1 protein was qualitatively scored by positive cell percentage multiplied by staining intensity, and the product less than 5 and greater than or equal to 5 indicated low and high levels of GRP78, whereas values less than 3 and greater than or equal to 3 were defined as low and high levels for ATF6, PERK, IRE1α, and PD-L1, respectively. The distribution of CD68 was scored by counting the mean percentage of positive cells in five random fields of each tumor sample.

Immunofluorescence. The sections were processed as per the protocol of immunohistochemistry and incubated with the desired primary antibodies against mouse anti-CD68 (Clone KP1, Abcam) and rabbit anti-CD274 (Clone 28-8, Abcam) at 4°C overnight. Then, the goat-anti-mouse IgG (H+L) AF488 and goat-anti-rabbit IgG (H+L) AF555 secondary antibodies were employed for 1 hour before counterstaining the nuclei with DAPI (Sigma-Aldrich, MO, USA) for 10 minutes at 37°C. Finally, the images were examined and photographed with a fluorescence microscope system (Olympus IX71, Japan).

Exosome isolation and characterization. ExoQuick-TC Precipitation Solution was used to collect exosomes from the supernatants of normal cultured HCC cells or normal hepatocyte (Exo-con) and cells treated with 2.5 μmol/L TM (Exo-TM) as reported.[1] Briefly, the cultured supernatants were collected, and the cell debris was removed by centrifugation at 3000*g* for 15 minutes. Subsequently, the supernatants were transferred to a 15-mL sterile vessel, and 1 mL ExoQuick-TC solution was added to 5-mL supernatants,

mixed well by inverting, and coincubated overnight at 4°C. Then, the supernatant was discarded after centrifuging the mixture at 1500*g* for 30 minutes. The pellets were centrifuged again for 5 minutes at 1500*g,* and the remaining fluid was carefully removed and washed with sterile PBS twice. Finally, the pellets were resuspended in PBS and stored at −80°C. The exosome biomarkers were analyzed by western blot, and the typical morphology of the exosomes was assessed by transmission electron microscopy (TEM). The concentration of total exosomal proteins was quantified using a BCA protein assay kit.

Cell Proliferation Assays. The effect of ER stress-related exosomes on HepG2 cells proliferation was measured using a Cell Counting Kit-8 according to the manufacturer's protocol. Briefly, HepG2 cells were coincubated with Exo-con and Exo-TM (10 μg/mL) for 24, 48, and 72 hours, respectively. Then, the optical density (OD) was measured at 490 nm. Cell viability = average OD value of the experimental group/average OD value of the control group × 100%.

Determination of TM concentration in Exo-TM by LC-ESI-MS/MS. The supposed TM packaged in Exo-TM was characterized by SPE-based TM extraction and LC-ESI-MS/MS analysis. Briefly, the supposed TM in Exo-TM was dissolved in methanol by ultrasonic; then these exosomes were dissolved in 1 mL of 10% methanol (0.1% formic acid), and the supposed TM in Exo-TM was extracted and purified using a Solid Phase extraction (SPE) system (ProElut PLS, Dikma Technologies, Inc.). The eluted fraction was evaporated under a gentle flow of nitrogen gas and dissolved in 400 μL of 50% methanol solution (0.1% formic acid) for analysis. Mobile phase consisted of methanol and 0.1% formic acid aqueous solution using isocratic elution. Separation was performed in Poroshell 120 column (100 mm × 2.1 mm, 2.7 μm) at a flow rate of 0.3 mL/minute, and 5 μL was injected for analysis. Multiple reaction monitor

was used to quantify these compounds. The Multiple reaction monitoring (MRM) transitions were optimized as Supporting Table S12..

Macrophages engulf exosome *in vitro* and *in vivo*. PKH67, a green fluorescent dye, was used to label the purified exosomes according to the manufacturer's recommendations. *In vitro,* macrophages were incubated with the PKH67-labeled exosomes at 37°C for 12 hours, and confocal microscopy (Leica, Germany) was employed to assess macrophage uptake of exosomes. *In vivo*, the PKH67-labeled exosomes were injected intravenously through the tail vein, and macrophages were harvested from peritoneal irrigation fluid and detected using flow cytometry (FCM) and confocal microscopy. Peritoneal macrophages uptake PKH67-labeled exosomes was detected by confocal microscopy analysis. Briefly, cells were allowed to attach on a coverslip overnight and washed with cold PBS three times and fixed with 4% paraformaldehyde at room temperature for 20 minutes.

Exosome RNA sequencing and miRNA profiling. The expression profiling of exosomal miRNA and data analysis were conducted by a commercial service (RiboBio, Guangzhou, China). In brief, total exosomal RNA was extracted from Exo-con and Exo-TM. Then, 5′ and 3′ adaptors were ligated, and the adaptor-ligated small RNAs were reverse transcribed into cDNA and amplified using PCR. cDNA fragments were purified by electrophoresis and then sequenced using an Illumina HiSeq 2500 platform according to the manufacturer's instructions. The differentially expressed miRNAs were compared between the Exo-con and Exo-TM groups.

Quantitative real-time PCR (qPCR). The mRNA levels of four differentially expressed exosomal-miRNAs, ER stress-related genes, *PD-L*1*, PTEN,* and other genes were evaluated using qPCR. Briefly, total RNA was extracted, and cDNA was synthesized using an RT kit (TaKaRa). qPCR was performed using

the CFX96 real-time PCR detection system (Bio-Rad, USA). The specificity of the amplification reactions was confirmed by melting curve analysis. The relative expression level of the selected genes was quantitatively analyzed using a qPCR kit. The PCR primers are listed in Supporting Table S12.

Bioinformatics analysis of the differentially expressed miRNAs. Three well-known public web-based prediction tools (TargetScan, miRDB, and miRanda) were used to predict the target genes of the differentially expressed miRNAs. The DAVID bioinformatics database analysis tools were employed to analyze the Gene Ontology (GO) biological processes feature and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of different miRNAs. For each analysis, the threshold was set at 0.05.

Luciferase Reporter Assay. Dual-luciferase reporter assays were performed using the GP-miRGLO vector. Cells were cotransfected with GP-miRGLO (wild type or mutant) or empty vector plus miR-23a-3p mimics or negative control using Lipofectamine 2000 (Invitrogen) when growing to approximately 70% confluence in 12-well plates. After coincubation for 24 hours, the Dual-Luciferase Reporter Assay system (Promega) was employed to determinate Firefly and Renilla luciferase activities.

Cell transfections. To explore the role of PTEN on PD-L1 expression, mTHP-1 cells (2×10^5/well) were transfected with siRNA specific for PTEN (Genepharma, Shanghai, China). For gain-of-function assays, mTHP-1 cells were transfected with miR-23a-3p mimics (Cat. #4464066, ID: MC10644, Applied Biosystems, CA, USA) or its negative controls (Cat. #4464058). For loss-of-function experiments, mTHP-1 cells were transfected with miR-23a-3p inhibitor (Cat. #4464084, ID: MH10644, Applied Biosystems, CA, USA) or its negative controls (Cat. #4464058) using RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Subsequently, cells were collected to detect

PD-L1 and PTEN-AKT pathway protein expression by FCM or western blot analysis and detect mRNA levels by qPCR. The RNA sequences are listed in Supporting Table S13.

Flow cytometry. RAW264.7 or mTHP-1 macrophages (1 × 10^6/well) were co-cultured with 10 μg/mL of Exo-con or Exo-TM. After coincubation for 24 hours, cells were washed with PBS and collected. Nonspecific and false-positive staining was blocked using Fc-block (for mTHP-1 cells) or CD16/32 (for RAW264.7 cells) at ambient temperature for 10 minutes. For detection of PD-L1, RAW264.7 cells and peritoneal macrophages were washed and stained with FITC-conjugated anti-mouse F4/80 (Clone BM8, BioLegend) and APC-conjugated anti-mouse CD274 antibodies (Clone 10F.9G2, BioLegend), and mTHP-1 cells were stained with PE-conjugated anti-human CD274 antibodies. For detection of macrophage phenotypes, RAW264.7 cells were stained with APC-conjugated anti-mouse F4/80 (Clone BM8, BioLegend) and PE-conjugated anti-mouse CD206 antibodies (Clone C068C2, BioLegend). Then, the stained cells were washed three times with PBS solution containing 1% BSA. Data were acquired by FCM (FACSCalibur, BD Biosciences) and analyzed using FlowJo7.6.1 software (Tree Star, Inc.). The supernatants were collected, and cytokines were measured by a CBA inflammation kit.

Western blot assay. Western blot assay was performed as described.[1] Briefly, proteins were extracted from human liver cancer tissues and paired paracarcinoma tissues and macrophages or related exosomes, and approximately 20 μg of total proteins was loaded and electrophoresed by 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Proteins were detected by incubating with the indicated antibody at 4°C overnight. The immunoreactive bands were visualized using the enhanced chemiluminescence reagent (Thermo Fisher, USA), and signals were

examined by an Image Quant LAS-4000 Mini Imager (Fuji, Japan).

Exosome-stimulated macrophages co-cultured with mononuclear leucocyte. Primary human $CD3^+$ T cells were enriched from the peripheral blood of healthy donors by using a human T lymphocyte enrichment set-DM following the manufacturer's protocols. Enriched T cells were seeded at 2×10^6 cells/mL in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, and cells were fed with 50 IU/mL interleukin (IL)-2.

mTHP-1 cells (1×10^5/well) were co-cultured with 10 μg/mL of Exo-con or Exo-TM for 48 hours, and then these stimulated macrophages were coincubated with $CD3^+$ mononuclear leucocytes (at a ratio of 1:20) for another 48 hours in the presence of 10 ng/mL IL-2. Then mononuclear leucocytes were collected and coincubated with mouse anti-human CD3, mouse anti-human CD4, and mouse anti-human CD8 antibody according to its specification. Finally, the ratio of $CD4^+$ T cells and $CD8^+$ T cells was measured by FCM, cytokines were measured by a human CBA inflammation kit, and the apoptotic cells were measured by Annexin V/PI kit.

Reference

1) **Li XQ, Liu JT,** Fan LL, Liu Y, Cheng L, Wang F, et al. Exosomes derived from gefitinib-treated EGFR-mutant lung cancer cells alter cisplatin sensitivity via up-regulating autophagy. Oncotarget 2016;7:24585-24595.

Supporting Tables

Supporting TABLE S1. Clinicopathologic features of HCC patients depending on GRP78 expression

Clinicopathologic Features	Case (n)	GRP78 Expression		χ^2/Z	*P*
		Low (n)	High (n)		
Sex				2.543	0.116
Female	35	16	19		
Male	134	42	92		
Age (years)				0.485	0.504
＜60	108	35	73		
⩾60	61	23	38		
Hepatitis				7.684	0.008
No	40	21	19		
Yes	129	37	92		
Cirrhosis				3.920	0.052
No	87	39	57		
Yes	82	19	54		
AFP value (U/mL)				−0.009	0.993
＜20	66	22	44		
⩾20,＜400	40	15	25		
⩾400	63	21	42		
Clinical stages				1.052	0.355
I/II	126	46	80		
III/IV	43	12	31		
Tumor size (cm)				−2.333	0.020
＜5	52	23	29		
⩾5,＜10	85	29	56		
⩾10	32	6	26		
Differentiated degree				−3.818	＜0.001
High	50	27	23		
Middle	90	27	63		
Low	29	4	25		

Supporting TABLE S2. Clinicopathologic features of HCC patients depending on ATF6 expression

Clinicopathologic Features	Case (n)	ATF6 Expression		χ^2/Z	*P*
		Low (n)	High (n)		
Sex				8.478	0.005
Female	35	20	15		
Male	134	41	93		
Age (years)				0.437	0.510
＜60	108	37	71		
⩾60	61	24	37		
Hepatitis				10.409	0.002
No	40	23	17		
Yes	129	38	91		
Cirrhosis				1.173	0.333
No	96	38	58		
Yes	73	23	50		
AFP value (U/mL)				−0.532	0.595
＜20	66	25	41		
⩾20,＜400	40	15	25		
⩾400	63	21	42		
Clinical stages				0.037	1.000
I/II	126	46	80		
III/IV	43	15	28		
Tumor size (cm)				−2.087	0.037
＜5	52	24	28		
⩾5,＜10	84	29	55		
⩾10	33	8	25		
Differentiated degree				−3.483	＜0.001
High	50	28	22		
Middle	90	27	63		
Low	29	6	23		

Supporting TABLE S3. Clinicopathologic features of HCC patients depending on PERK expression

Clinicopathologic Features	Case (n)	PERK Expression		χ^2/Z	*P*
		Low (n)	High (n)		
Sex				3.954	0.054
Female	35	19	16		
Male	134	48	86		
Age (years)				0.850	0.414
＜60	108	40	68		
⩾60	61	27	34		
Hepatitis				9.074	0.003
No	40	24	16		
Yes	129	43	86		
Cirrhosis				1.565	0.267
No	96	42	54		
Yes	73	25	48		
AFP value (U/mL)				−0.934	0.350
＜20	66	23	43		
⩾20,＜400	40	17	23		
⩾400	63	27	36		
Clinical stages				0.546	0.477
I/II	126	52	74		
III/IV	43	15	28		
Tumor size (cm)				−2.631	0.009
＜5	52	29	23		
⩾5,＜10	84	28	56		
⩾10	33	10	23		
Differentiated degree				−4.365	＜0.001
High	50	31	19		
Middle	90	32	58		
Low	29	4	25		

Supporting TABLE S4. Clinicopathologic features of HCC patients depending on IRE1α expression

Clinicopathologic Features	Case (n)	IRE1α Expression		χ^2/Z	*P*
		Low (n)	High (n)		
Sex				0.268	0.698
Female	35	15	20		
Male	134	51	83		
Age (years)				0.511	0.514
＜60	108	40	68		
⩾60	61	26	35		
Hepatitis				5.599	0.025
No	40	22	18		
Yes	129	44	85		
Cirrhosis				0.231	0.750
No	96	39	57		
Yes	73	27	46		
AFP value (U/mL)				−0.212	0.832
＜20	66	25	41		
⩾20,＜400	40	16	24		
⩾400	63	25	38		
Clinical stages				1.022	0.367
I/II	126	52	74		
III/IV	43	14	29		
Tumor size (cm)				−3.527	＜0.001
＜5	52	30	22		
⩾5,＜10	84	29	55		
⩾10	33	7	26		
Differentiated degree				−5.624	＜0.001
High	50	35	15		
Middle	90	28	62		
Low	29	3	26		

Supporting TABLE S5. Correlations between GRP78 and PD-L1

	GRP78		*r*	*P*
PDL-1	Low	High		
Negative	42	54	0.228	0.003
Positive	16	57		

Supporting TABLE S6. Correlations between ATF6 and PD-L1

	ATF6		*r*	*P*
PDL-1	Low	High		
Negative	47	49	0.307	＜0.001
Positive	14	59		

Supporting TABLE S7. Correlations between PERK and PD-L1

	PERK		*r*	*P*
PDL-1	Low	High		
Negative	50	46	0.292	＜0.001
Positive	17	56		

Supporting TABLE S8. Correlations between IRE1α and PD-L1

	IRE1α		*r*	*P*
PDL-1	Low	High		
Negative	50	46	0.306	＜0.001
Positive	16	57		

Supporting TABLE S9. Dysregulated miRNAs in Exo-TM compared with Exo-con

Transcript	Regulation	Log2	*P* Value	Sequence
hsa-miR-29a-3p	Up	1.56	0.005	UAGCACCAUCUGAAAUCGGUUA
hsa-miR-16-5p	Up	1.13	0.022	UAGCAGCACGUAAAUAUUGGCG
hsa-miR-20a-5p	Up	1.09	0.035	UAAAGUGCUUAUAGUGCAGGUAG
hsa-miR-23a-3p	Up	1.35	0.014	AUCACAUUGCCAGGGAUUUCC
hsa-miR-27a-3p	Up	1.03	0.036	UUCACAGUGGCUAAGUUCCGC
hsa-miR-184	Up	2.42	0.031	UGGACGGAGAACUGAUAAGGGU
hsa-miR-378a-3p	Up	1.09	0.038	ACUGGACUUGGAGUCAGAAGGC
hsa-miR-193b-3p	Up	1.36	0.030	AACUGGCCCUCAAAGUCCCGCU
hsa-miR-1-3p	Down	14.21	0.039	UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-433-3p	Down	2.08	0.029	AUCAUGAUGGGCUCCUCGGUGU
hsa-miR-432-5p	Down	1.73	0.033	UCUUGGAGUAGGUCAUUGGGUGG
hsa-miR-92b-5p	Down	1.81	0.016	AGGGACGGGACGCGGUGCAGUG
hsa-miR-486-5p	Down	2.32	0.006	UCCUGUACUGAGCUGCCCCGAG
hsa-miR-486-3p	Down	2.01	0.009	CGGGGCAGCUCAGUACAGGAU

Supporting TABLE S10. Correlations between miR23a-3p and OS

	Mean	SD	*r*	*P*
miR-23a-3p	8.23	20.12	−0.306	0.023
OS (months)	21.13	12.13		

Supporting TABLE S11. Antibodies used in this study

Antibody	Clone	Source	Dilution
Anti-human GRP78	EPR4041(2)	Abcam	1:200
Anti-human ATF6		Abcam	1:200,
Anti-human PERK		Abcam	1:1000
Anti-human IRE1α		Abcam	1:200,
Anti-human CD68	KP1	Abcam	1:1000
Anti-human PD-L1	28-8	Abcam	1:200,
Anti-mouse F4/80 (FITC)	BM8	BioLegend	1:1000
Anti-mouse F4/80 (APC)	BM8	BioLegend	1:200
Anti-mouse CD274	10F.9G2	BioLegend	1:100
Anti-mouse CD16/32	93	BioLegend	-
Anti-mouse CD206	C068C2	BioLegend	-
Anti-human CD274	MIH1	BD	-
Anti-human CD3	HIT3a	BioLegend	-
Anti-human CD4	A161A1	BioLegend	-
Anti-human CD8	SK1	BioLegend	-
Anti-human CD63	-	SBI	1:1000
Anti-human Calnexin	C5C9	Cell Signaling Technology	1:1000
Anti-human PTEN	D4.3	Cell Signaling Technology	1:1000
Anti-human AKT	11E7	Cell Signaling Technology	1:1000
Anti-human phospho-AKT	D9E	Cell Signaling Technology	1:1000
Anti-human TSG101	EPR7130 (B)	Abcam	1:1000
Anti-human GRP78	MB0050 (catalog)	Bioworld	1:1000
Anti-human PD-L1	E1L3N	Cell Signaling Technology	1:1000
mouse anti-human β-actin	8H10D10	Cell Signaling Technology	1:1000
Anti-human GAPDH	D16H11	Cell Signaling Technology	1:1000

Supporting TABLE S12. The optimized MRM transition conditions

Compound	MRM Transition		DP	CE
	$(M+H)^+$	$(M+H-221)^+$		
TM-14:1	817.5	596.5	200	27
	817.5	614.1	200	22
TM -15:1	831.5	610.4	200	27
	831.5	628.3	200	22
TM-16:1	845.3	624.5	220	28
	845.3	642.5	220	22

Supporting TABLE S13. RNA sequence used in this paper

RNA	Sequence
cel-miR-39	RT: ssD1083145001
	FP: ssD1083145002
hsa-miR-486-3p	FP: ssD809231081
	RT: ssD809230389
hsa-miR-486-5p	FP: ssD809231082
	RT: ssD809230390
hsa-miR-23a-3p	FP: ssD809230952
	RT: ssD809230260
hsa-miR-29a-3p	FP: ssD809230968
	RT: ssD809230276
hsa-miR-23a-3p mimics	Forward primer：AUCACAUUGCCAGGGAUUUCC
	Reverse primer：AAAUCCCUGGCAAUGUGAUUU
hsa-miR-23a-3p NC	Forward primer：UUCUCCGAACGUGUCACGUTT
	Reverse primer：ACGUGACACGUUCGGAGAATT
PTEN^{wt}	TACTATTGTAAAGCTAATGTGAA
PTEN^{mut}	TACTATTGTAAAGCTTTACACTA
PTEN siRNA NC	Forward primer：UGACCUCAACUACAUGGUUTT
	Reverse primer：AACCAUGUAGUUGAGGUCATT
PTEN siRNA	Forward primer：GCCAGCUAAAGGUGAAGAUTT
	Reverse primer：AUCUUCACCUUUAGCUGGCT
Mouse *Pd-l1*	Forward primer：GGAGCCTTGTTTGTGTCTCC
	Reverse primer：AGGCGTCTGTGTTTGAGAGA
Mouse *β-actin*	Forward primer：AGTGTGACGTTGACATCCGT
	Reverse primer：TGCTAGGAGCCAGAGCAGTA
Mouse *Il-10*	Forward primer：TGCACTACCAAAGCCACAAG
	Reverse primer：TCAGTAAGAGCAGGCAGCAT
Mouse *Il-6*	Forward primer：AGTCCGGAGAGGAGACTTCA
	Reverse primer：ATTTCCACGATTTCCCAGAG
Mouse *Mcp-1*	Forward primer：AACTGCATCTGCCCTAAGGT
	Reverse primer：CTGTCACACTGGTCACTCCT
Mouse *Tnf-a*	Forward primer：GACAGTGACCTGGACTGTGG
	Reverse primer：TGAGACAGAGGCAACCTGAC
Human *β-actin*	Forward primer：GGGAAATCGTGCGTGACATTAAGG
	Reverse primer：CAGGAAGGAAGGCTGGAAGAGTG
Human *IRE1a*	Forward primer：CAGAACAGCTCTGCACCATC
	Reverse primer：ACAGATGTGACCAAGCTCCA
Human *PERK*	Forward primer：ATCCGGTTCCTTGGTGTCAT
	Reverse primer：GTCTTGGTCCCACTGGAAGA
Human *ATF6*	Forward primer：GCCAACTCCTCAGATGCAAG
	Reverse primer：CCTTCCTGCTCTTCAGTGGA

Human *GRP78*	Forward primer：CCGAGAACACGGTCTTTGAC
	Reverse primer：CACCTTGAACGGCAAGAACT
Human *PD-L1*	Forward primer：GGAAATTCCGGCAGTGTACC
	Reverse primer：GAAACCTCCAGGAAGCCTCT
Human *IL-10*	Forward primer：GCCCCTTGAGAAACCTTATTGT
	Reverse primer：GGCTTCTTTCTAAATCGTTCACAG
Human *IL-6*	Forward primer：AGTAGTGAGGAACAAGCCAGAG
	Reverse primer：ATGCTACATTTGCCGAAGAG
Human *TNF-a*	Forward primer：CCCTGGTATGAGCCCATCTAT
	Reverse primer：CGAAGTGGTGGTCTTGTTGC
Human *PTEN*	Forward primer：ACCATAACCCACCACAGC
	Reverse primer：CAGTTCGTCCCTTTCCAG

Supporting Figure legends:

Supporting FIG. S1. TM induces ER stress in HCC cells. (A) The GRP78 protein expression in HepG2 cells was dose dependently increased following coincubation with TM for 24 hours. (B) TM (2.5 μmol/L) was coadministered with HepG2 cells for different times (12, 24, and 48 hours), and the GRP78 protein level was time dependently increased. (C) TM (2.5 μmol/L for 24 hours) also induced Hep3B, Hepa1-6, and A549 cell ER stress and activation of three UPR branches (PERK, ATF6, and IRE1α). Data are presented as the means ± SD (error bar) of at least three independent experiments.

Supporting FIG. S2. Characteristics of purified exosomes. Transmission electron microscopic image of exosomes derived from (A) normal cultured HepG2 cells (Exo-con) or (B) HepG2 cells co-cultured with TM (Exo-TM) for 24 hours (scale bar = 100 nm). (C) Western blot analysis of CD63, TSG101, and Calnexin using cell lysates derived from HepG2 cells or purified exosomes. (D) The effects of ER stress on HepG2 and Hep3B cell exosome secretion were measured by a BCA protein assay kit. Data are presented as the means ± SD (error bar) of at least three independent experiments. $^{**}P < 0.01$ with indicated groups.

Supporting FIG. S3. Exosomes transmit ER stress signals to macrophages. Western blot analysis was used to investigate whether exosomes could transmit ER stress signals to macrophages. Both human (A) hepatoma cell line HepG2 cells and (B) normal hepatocyte HL-7702 cells released exosomes coincubated with macrophages for 24 hours increased GRP78, PERK, ATF6, and IRE1α protein expression. (C) Exo-con and Exo-TM (derived from HepG2 cells) promoted HepG2 cell proliferation after coincubation for 24, 48, or 72 hours. Data are presented as the means ± SD

(error bar) of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ with indicated groups.

Supporting FIG. S4. Exo-TM increases macrophage PD-L1 expression *in vitro*. (A) Macrophages were coincubated with Exo-con and Exo-TM derived from Hep3B cells, and the protein level of PD-L1 was determined by (A) FCM, (B) western blot, and (C) immunohistochemical analysis; scale bar = 50 μm. *PD-L1* mRNA was also measured by (D) qPCR. The impact of hepatocyte HL-7702 cells' released exosomes on PD-L1 protein expression was detected by (E) FCM and (F) western blot analysis. (G) Moreover, the impact of primary human hepatocytes' released exosomes on PD-L1 expression in mTHP-1 was detected by FCM. Data are shown as the means ± SD of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ with indicated groups.

Supporting FIG. S5. Exo-TM increases RAW264.7 cells' PD-L1 expression. (A) RAW264.7 cells were coincubated with HepG2 cells' released exosomes, and the expression level of PD-L1 was determined by FCM and (B) statistically analyzed. Moreover, exosomes derived from Hep3B cells were coincubated with RAW264.7 cells for 24 hours, the expression level of PD-L1 was measured by (C) FCM, and (D) MFI was statistically analyzed. Data are shown as the means ± SD of at least three independent experiments. *$P < 0.05$, **$P < 0.01$ with indicated groups.

Supporting FIG. S6. Tunicamycins contained in Exo-TM did not influence PD-L1 expression on macrophages. (A) LC-ESI-MS/MS analysis was used to detect TM in PBS, Exo-con, Exo-TM, and TM standards (32 ng/mL). (B) TM (500, 400, 300, 200, 100, 50 ng/mL) was coadministered with mTHP-1 cells for 24 hours, respectively, and GRP78 protein was measured by western blot. (C) PD-L1 expression on mTHP-1 cells after coincubation with TM directly was measured using FCM, and MFI was statistically analyzed. Data are presented

as the means ± SD (error bar) of at least three independent experiments.

Supporting FIG. S7. The protocol for *in vivo* experiments. Exosomes were purified, and 10 μg/100 μL of Exo-con or Exo-TM was injected intravenously once every other day for 10 times. The expression level of PD-L1, inflammatory factors, and related genes expressed in peritoneal macrophages was analyzed by FCM, western blot, immunohistochemistry, and qPCR.

Supporting FIG. S8. Exo-TM influences the expression of inflammatory factors in peritoneal macrophages. PBS, Exo-con, or Exo-TM was injected intravenously through the tail vein, and macrophages were isolated from the peritoneal lavage fluids. The expression of (A) IL-6, (B) TNF-α, (C) monocyte chemotactic protein-1 (MCP-1), and (D) IL-10 secreted by peritoneal macrophages was determined by a CBA inflammatory factor kit. And the mRNA levels of (E) *Il-6*, (F) *Tnf-α*, (G) *Mcp-1,* and (H) *Il-10* were measured by qPCR. Data are shown as the means ± SD (error bar) of at least three independent experiments. **P* < 0.05, ***P* < 0.01 with indicated groups.

Supporting FIG. S9. Exo-TM polarizes macrophages to M2 phenotype. (A) RAW264.7 cells were coincubated with HepG2 cells' released exosomes, and CD206 expression was determined by (A) FCM and (B) statistically analyzed. Data are shown as the means ± SD of at least three independent experiments. **P* < 0.05, ***P* < 0.01 with indicated groups.

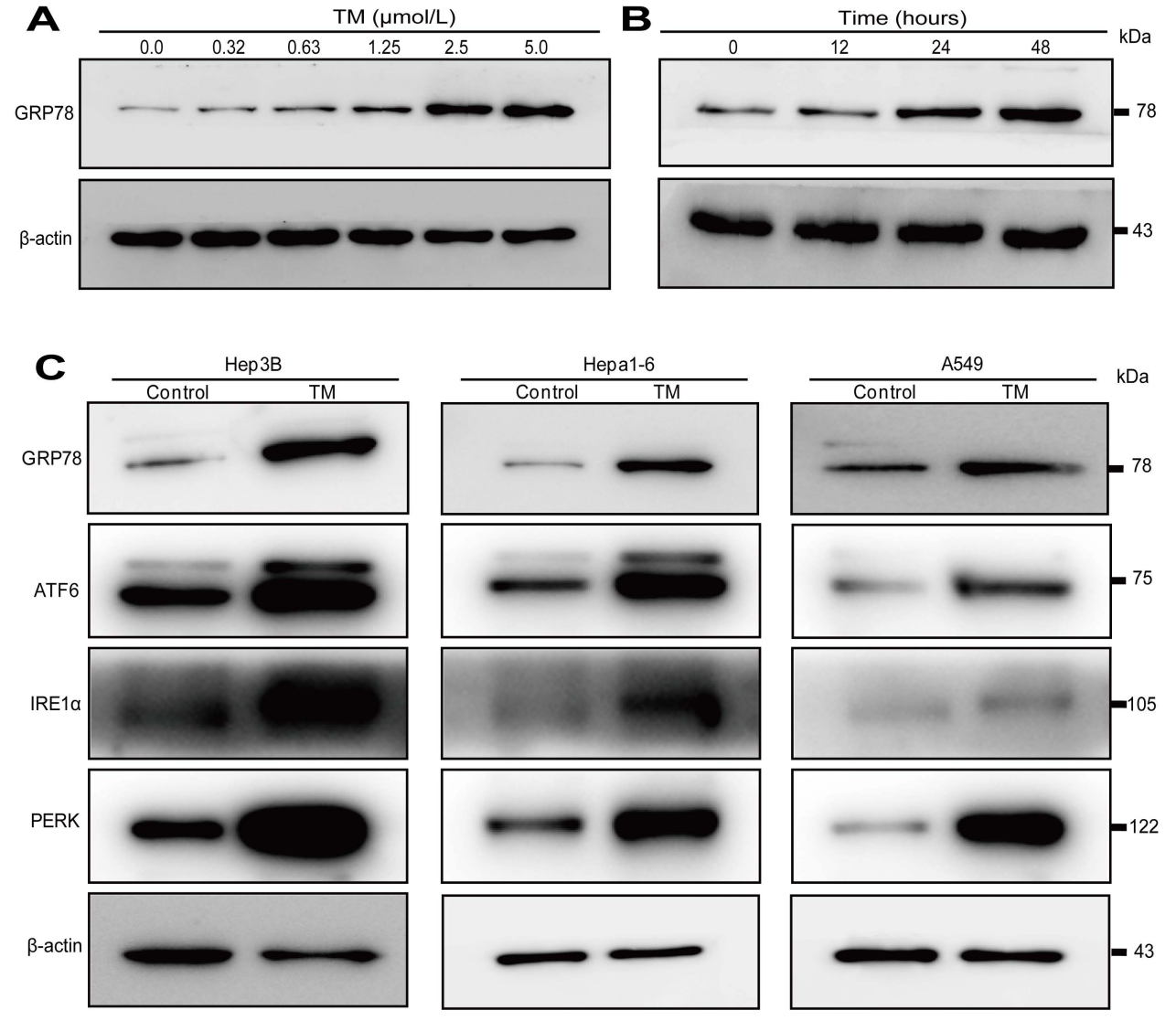
Supporting FIG. S10. Exo-TM increases macrophage inflammatory factor expression *in vitro*. Exo-TM increases the expression of both (A,B) proinflammatory factor TNF-α and (C,D) anti-inflammatory factor IL-10 at both the protein and mRNA level. Exo-TM only slightly increases (E) IL-6 protein levels but (F) significantly increases mRNA level. Data are shown as the means ± SD (error bar) of at least three independent experiments. **P* < 0.05,

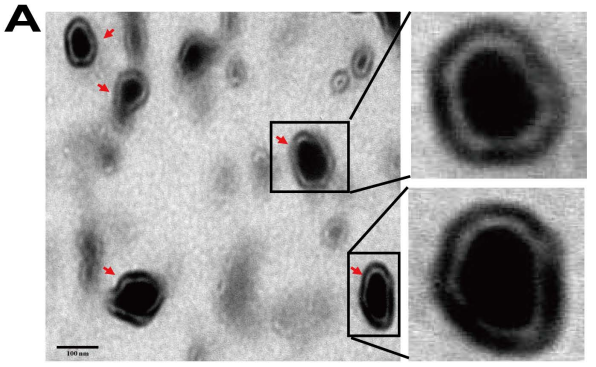
**P < 0.01 with indicated groups.

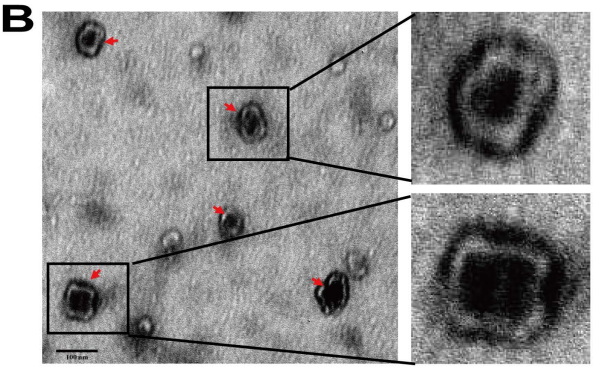
Supporting FIG. S11. PTEN is the downstream target of miR-23a-3p. (A) The pipeline of bioinformatics analysis found that four miRNAs (miR-29a-3p, miR-23a-3p, miR-486-3p, and miR-486-5p) were associated with the regulation of PD-L1 expression. (B) qPCR was employed to assess the amount of predicted miRNAs in Exo-con and Exo-TM. (C) Putative binding site for miR-23a-3p in the 3-untranslational region (3-UTR) of *PTEN*. (D) Dual luciferase reporter assay showed the effect of miR-23a-3p on the activity of *PTEN* 3′UTR reporter. (E) Transfection of miR-23a-3p mimics significantly down-regulated the expression level of PTEN in macrophages. Data are presented as the means ± SD (error bar) of at least 3 independent experiments. *P < 0.05 with indicated groups. $PTEN^{wt}$ = wild type PTEN; $PTEN^{mut}$ = mutant PTEN

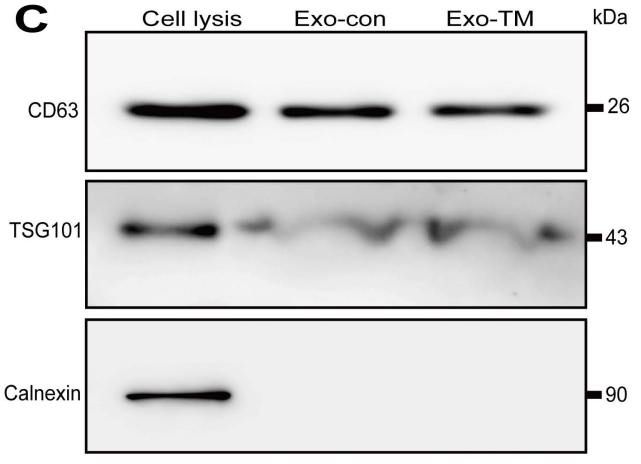
Supporting FIG. S12. Down-regulation PTEN increases p-AKT expression in macrophages. (A) Transfection of mTHP-1 cells with a specific *PTEN* siRNA (si-PTEN) significantly reduces PTEN protein and increases p-AKT protein expression and (B) reduces *PTEN* mRNA levels. Data are presented as the means ± SD (error bar) of at least 3 independent experiments. **P < 0.01 with indicated groups.

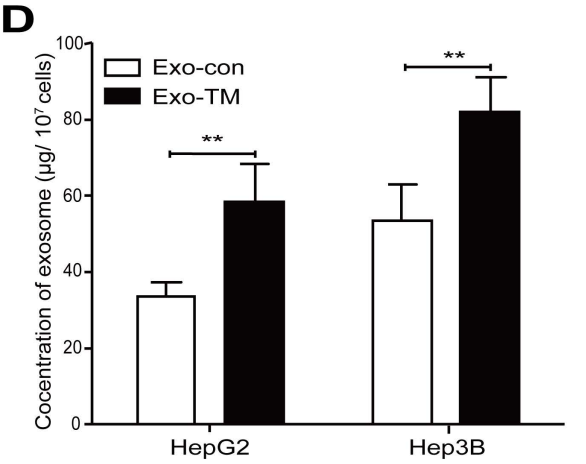
Supporting FIG. S13. A working model for the ER-stressed HCC cells release exosomal miR-23a-3p and up-regulate PD-L1 in macrophages. Exosomes secreted by ER-stressed HCC cells could specifically sort some types of miRNAs, such as miR-23a-3p. These miR-23a-3p-enriched exosomes are subsequently internalized by macrophages and then target *PTEN*. When PTEN is inhibited in macrophages, AKT is activated, thereby promoting the expression of PD-L1, which further inhibits T-cell function and induces T-cell apoptosis to facilitate HCC cells escaping from immunologic surveillance.

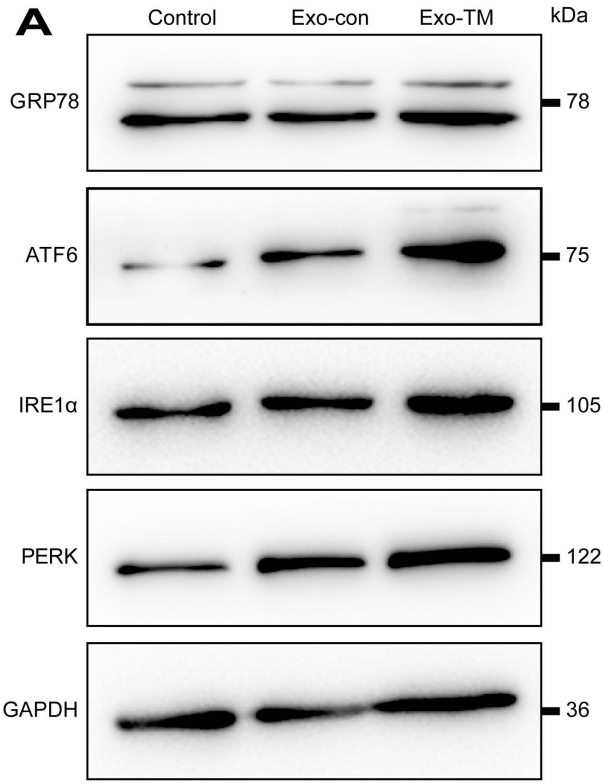

A
TM (μmol/L)
0.0
0.32
0.63
1.25
2.5
5.0
GRP78
β-actin
B
Time (hours)
kDa
0
12
24
48
78
43
C
Hep3B
Hepa1-6
A549
Control
TM
kDa
GRP78
ATF6
IRE1α
PERK
β-actin
78
75
105
122
43

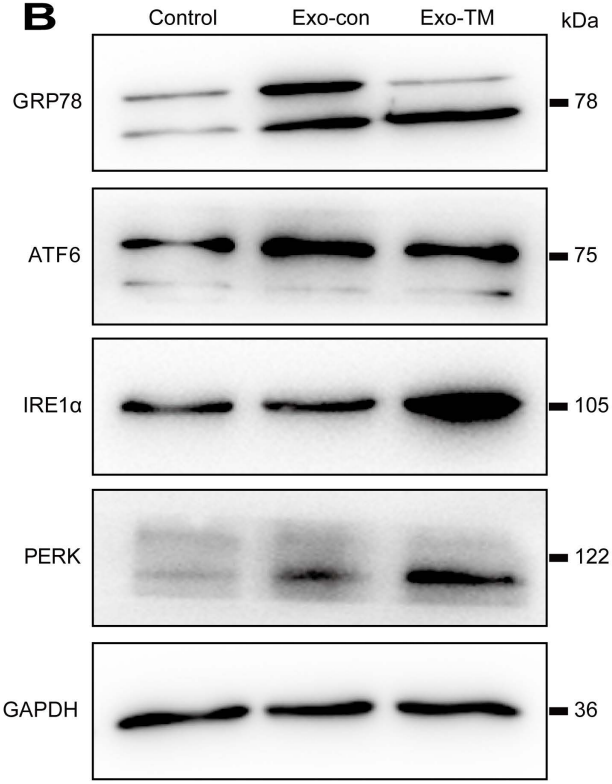

A

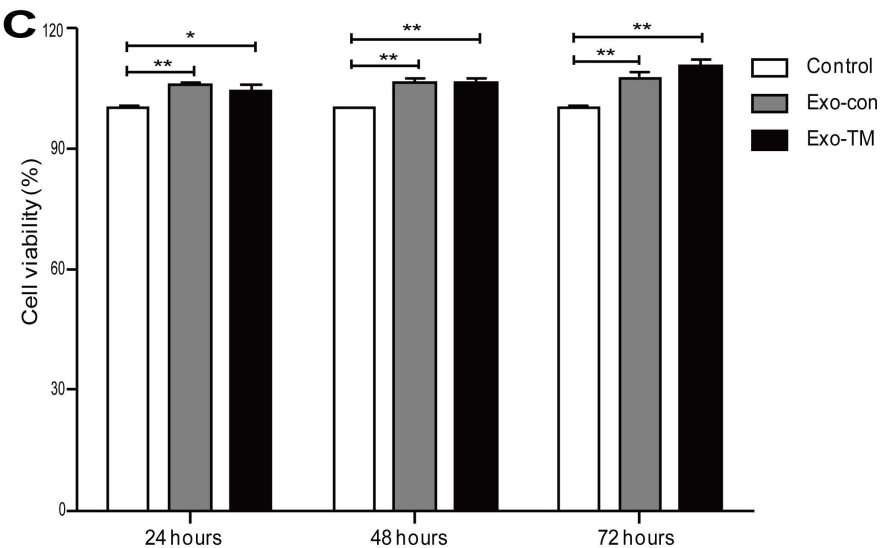

B

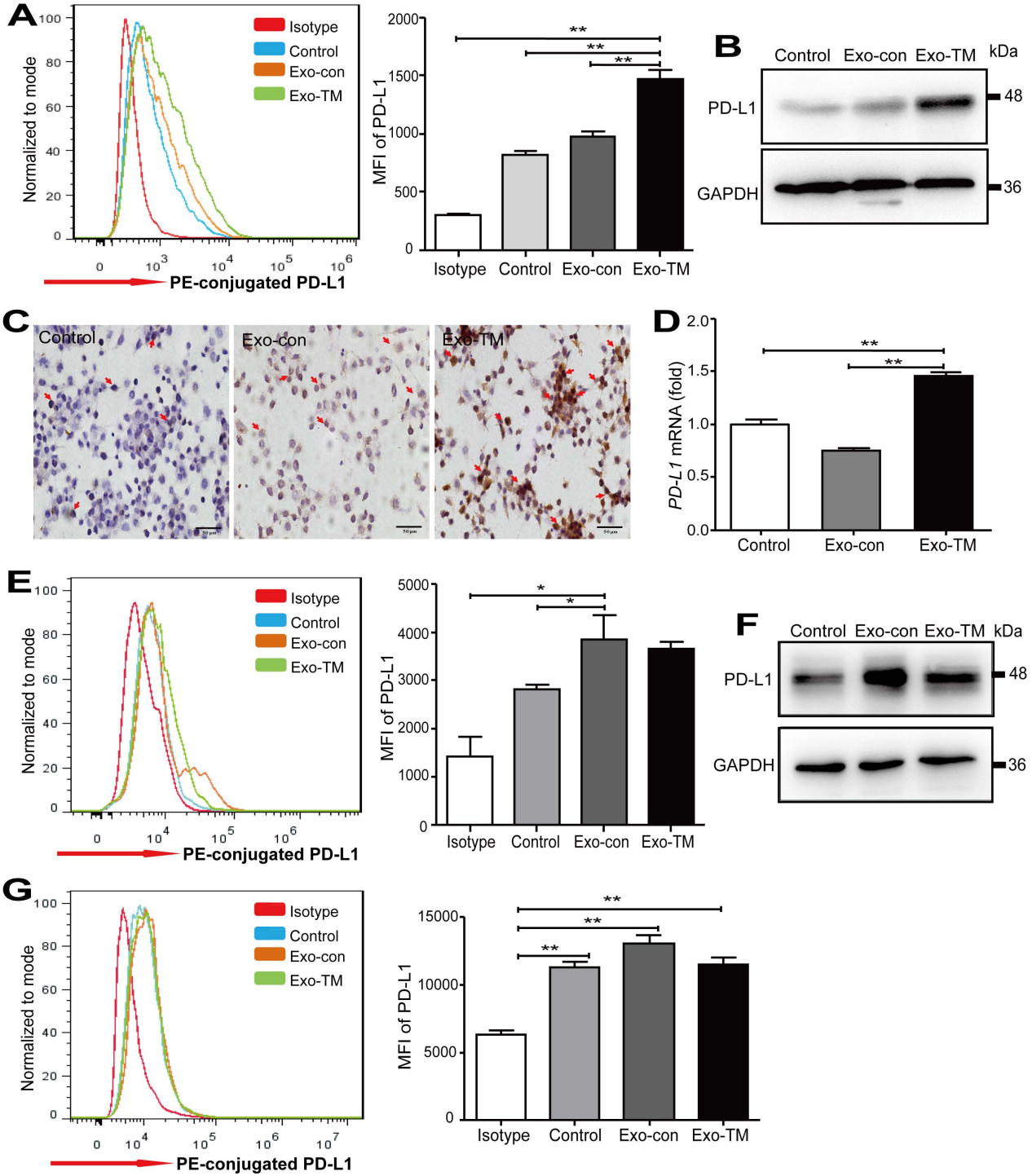

C
Cell lysis
Exo-con
Exo-TM
kDa
CD63
26
TSG101
43
Calnexin
90

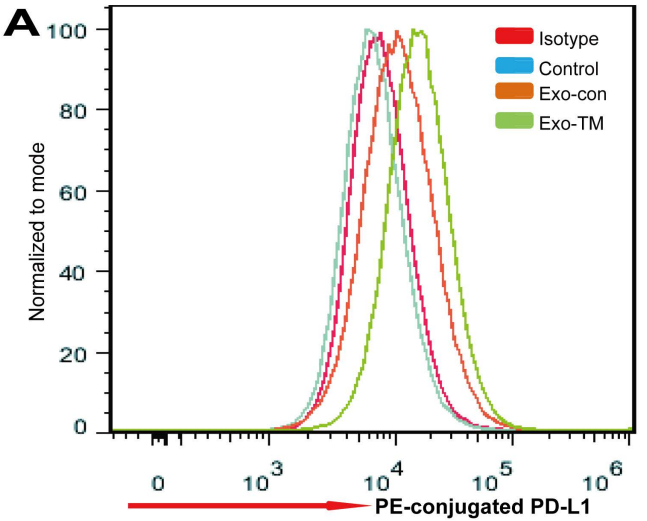

D
Exo-con
Exo-TM
Cocentration of exosome (μg/ 10^7 cells)
100
80
60
40
20
0
**
**
HepG2
Hep3B

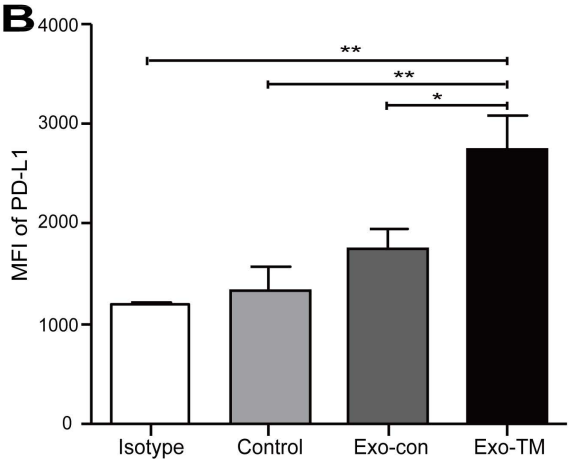

A
Control
Exo-con
Exo-TM
kDa
GRP78
78
ATF6
75
IRE1α
105
PERK
122
GAPDH
36

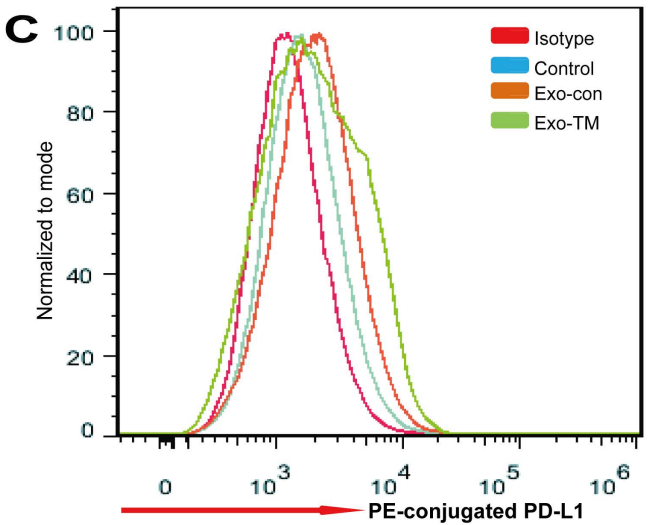

B
Control
Exo-con
Exo-TM
kDa
GRP78
78
ATF6
75
IRE1α
105
PERK
122
GAPDH
36

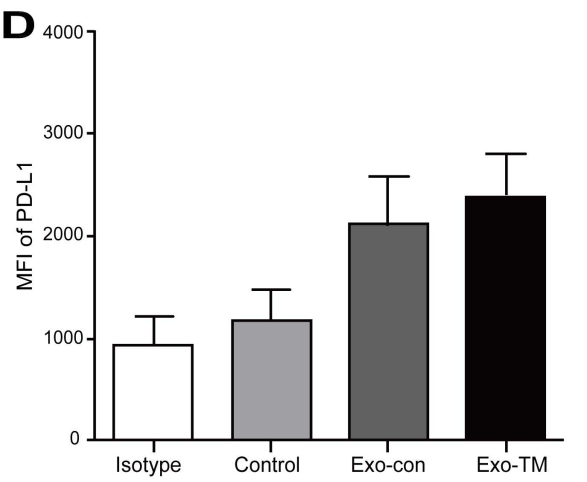

C
Cell viability (%)
120
90
60
30
0
*
**
**
**
**
**
24 hours
48 hours
72 hours
Control
Exo-con
Exo-TM

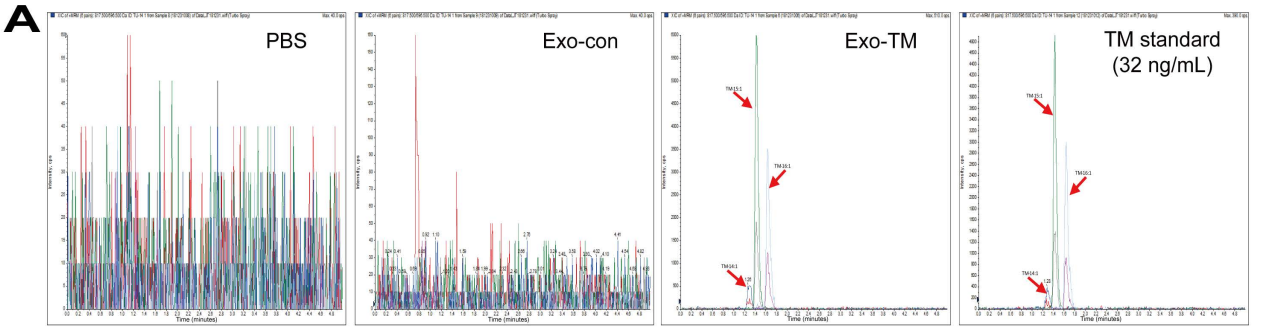

A
Normalized to mode
Isotype
Control
Exo-con
Exo-TM
PE-conjugated PD-L1
MFI of PD-L1
Isotype
Control
Exo-con
Exo-TM
B
Control
Exo-con
Exo-TM
kDa
PD-L1
GAPDH
48
36
C
Control
Exo-con
Exo-TM
D
PD-L1 mRNA (fold)
Control
Exo-con
Exo-TM
E
Normalized to mode
Isotype
Control
Exo-con
Exo-TM
PE-conjugated PD-L1
MFI of PD-L1
Isotype
Control
Exo-con
Exo-TM
F
Control
Exo-con
Exo-TM
kDa
PD-L1
GAPDH
48
36
G
Normalized to mode
Isotype
Control
Exo-con
Exo-TM
PE-conjugated PD-L1
MFI of PD-L1
Isotype
Control
Exo-con
Exo-TM

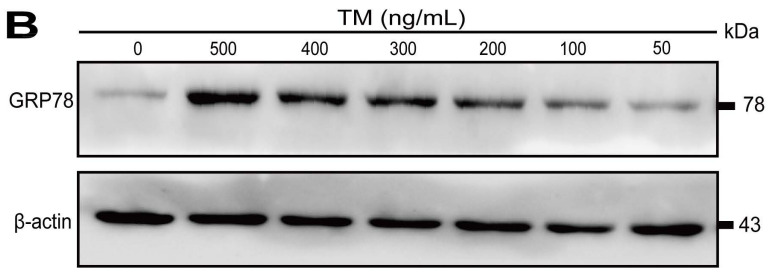

A
Normalized to mode
Isotype
Control
Exo-con
Exo-TM
PE-conjugated PD-L1

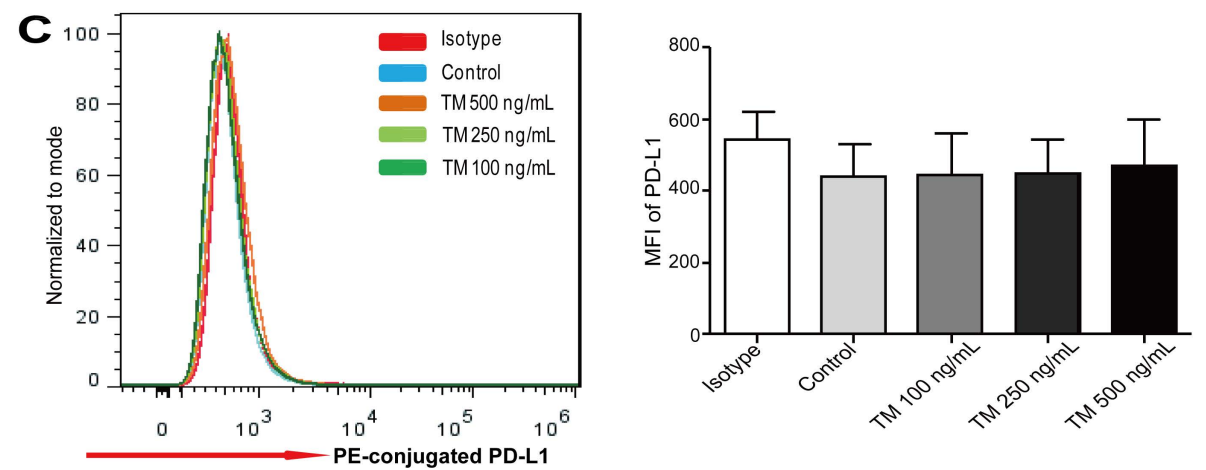

B
MFI of PD-L1
**
**
*
Isotype
Control
Exo-con
Exo-TM

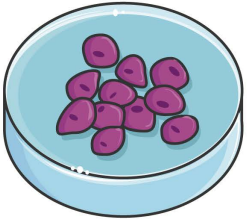

C
Normalized to mode
Isotype
Control
Exo-con
Exo-TM
PE-conjugated PD-L1


D
MFI of PD-L1
Isotype
Control
Exo-con
Exo-TM

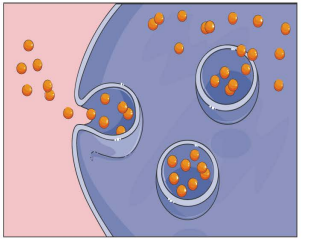

A
PBS
Exo-con
Exo-TM
TM standard
(32 ng/mL)

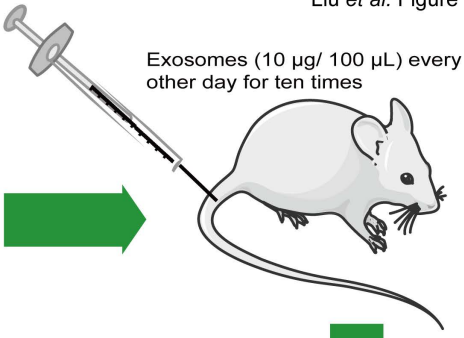

B
TM (ng/mL)
0
500
400
300
200
100
50
kDa
GRP78
78
β-actin
43


C
Isotype
Control
TM 500 ng/mL
TM 250 ng/mL
TM 100 ng/mL
Normalized to mode
PE-conjugated PD-L1
MFI of PD-L1
Isotype
Control
TM 100 ng/mL
TM 250 ng/mL
TM 500 ng/mL

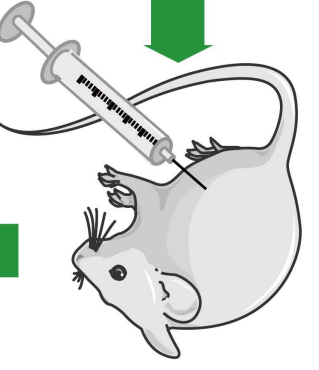


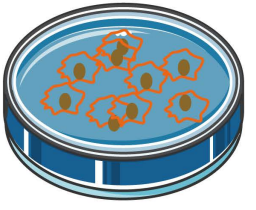
HCC cells





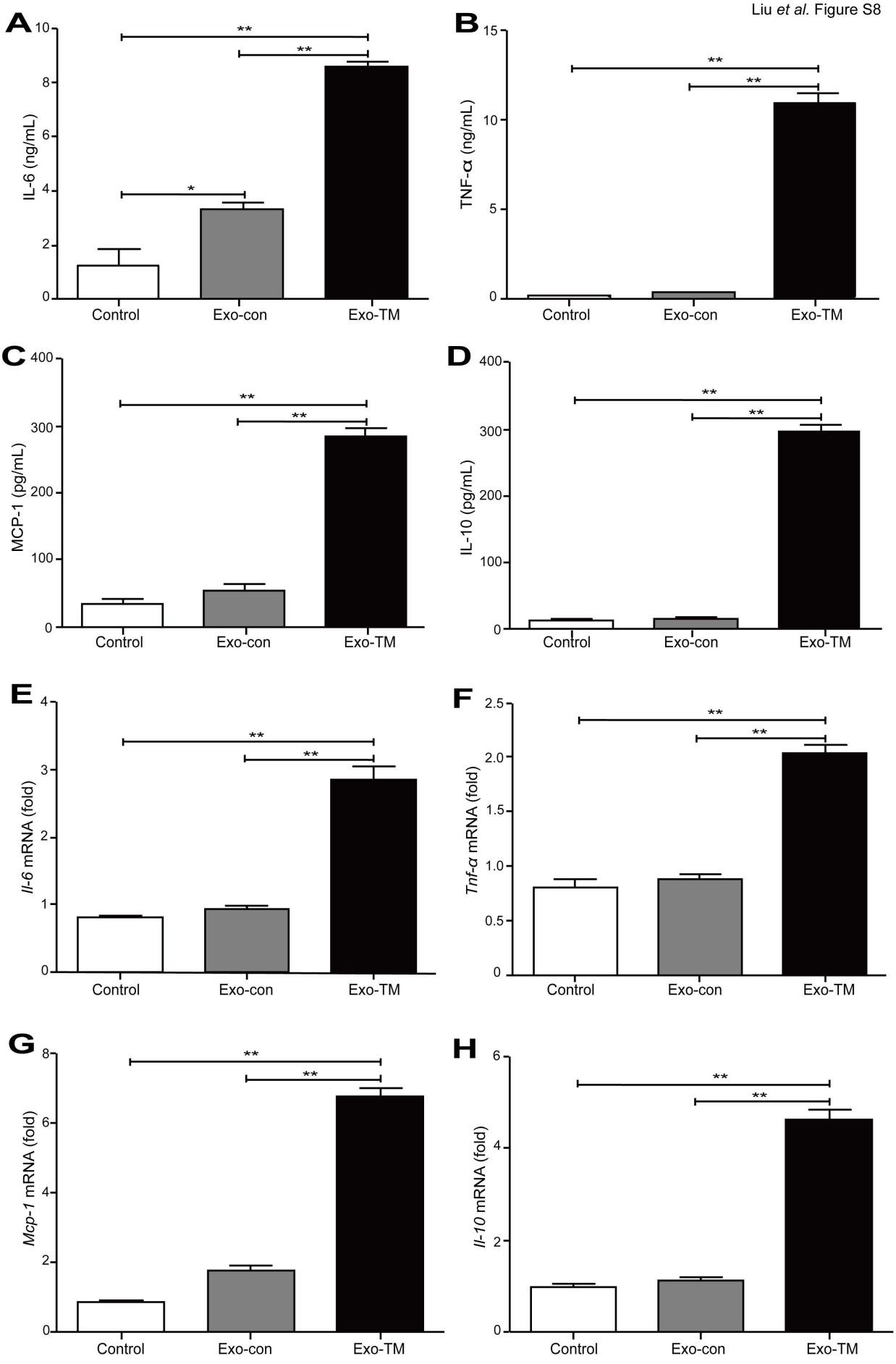
Exosomes (10 μg/ 100 μL) every other day for ten times

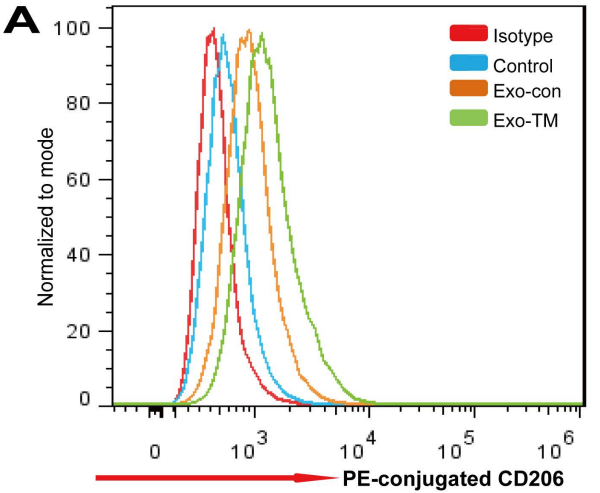


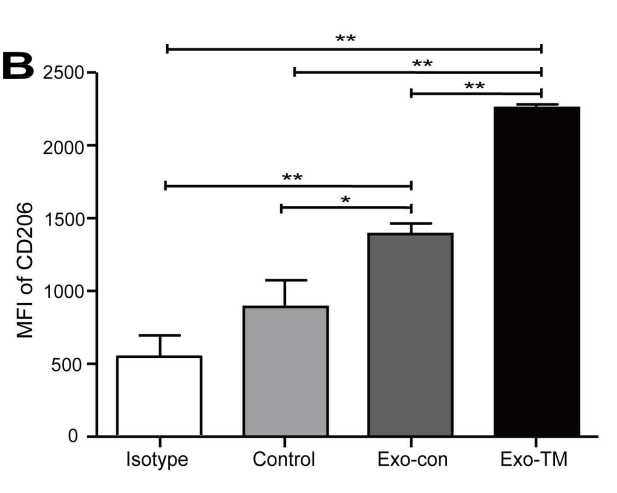


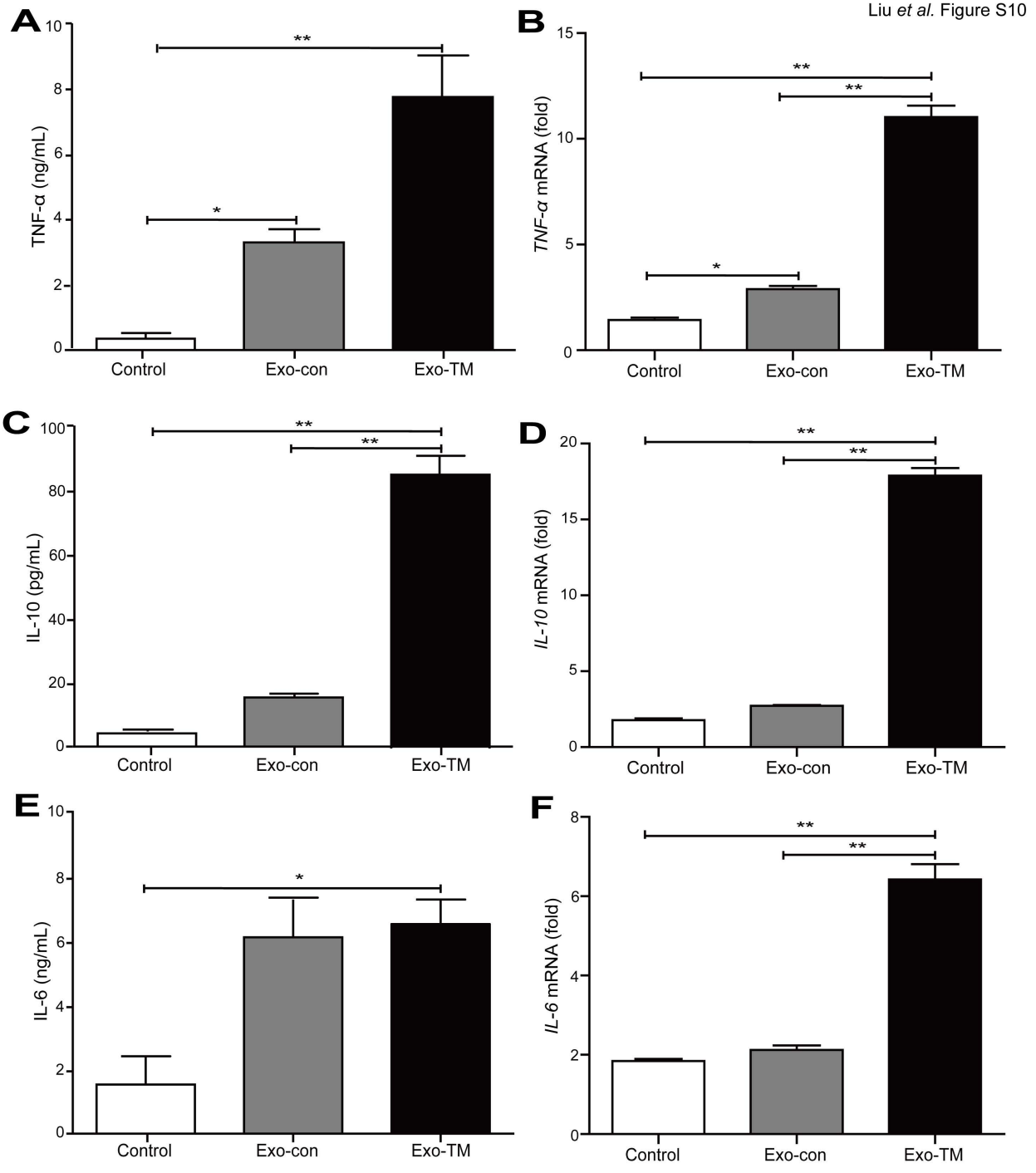
Macrophages isolated from the peritoneal lavage fluids

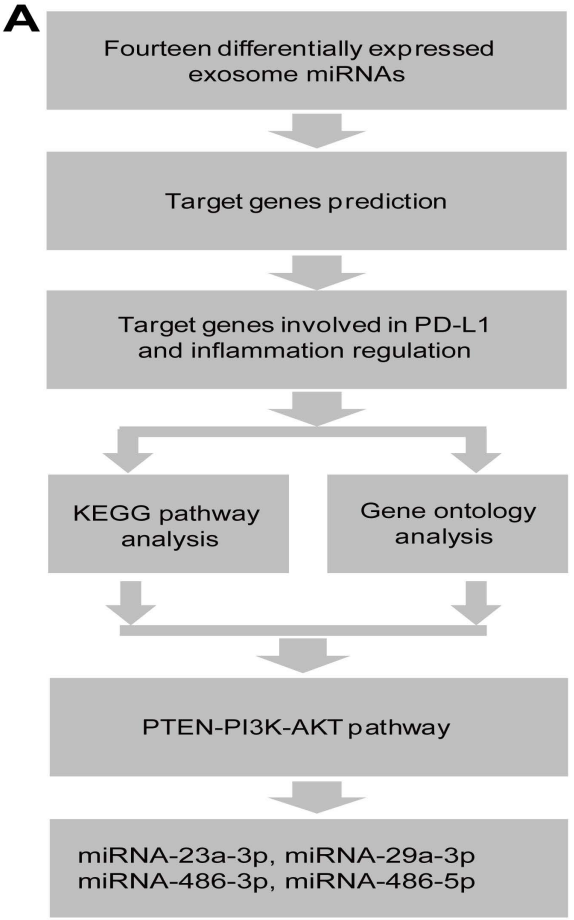
Detection of PD-L1, inflammatory factors, and related genes on macrophages

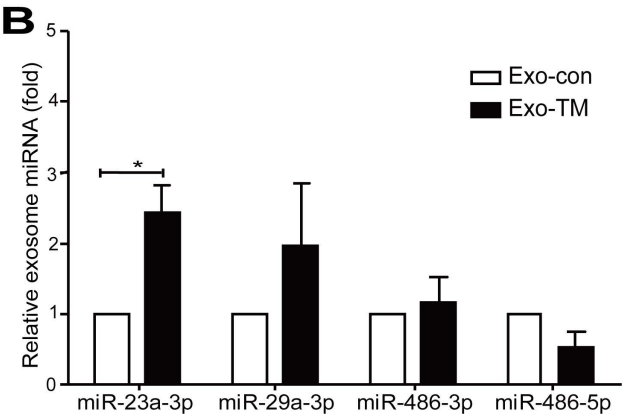

A
IL-6 (ng/mL)
10
8
6
4
2
0
**
**
*
Control
Exo-con
Exo-TM
B
TNF-α (ng/mL)
15
10
5
0
**
**
Control
Exo-con
Exo-TM
C
MCP-1 (pg/mL)
400
300
200
100
0
**
**
Control
Exo-con
Exo-TM
D
IL-10 (pg/mL)
400
300
200
100
0
**
**
Control
Exo-con
Exo-TM
E
Il-6 mRNA (fold)
4
3
2
1
0
**
**
Control
Exo-con
Exo-TM
F
Tnf-α mRNA (fold)
2.5
2.0
1.5
1.0
0.5
0
**
**
Control
Exo-con
Exo-TM
G
Mcp-1 mRNA (fold)
8
6
4
2
0
**
**
Control
Exo-con
Exo-TM
H
Il-10 mRNA (fold)
6
4
2
0
**
**
Control
Exo-con
Exo-TM

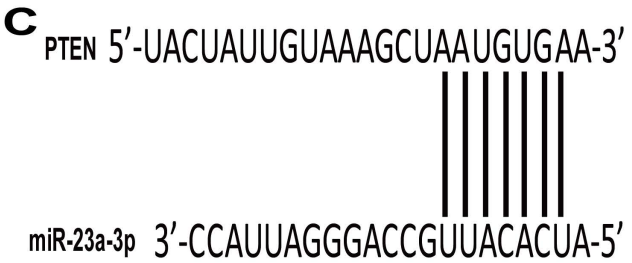

A
Isotype
Control
Exo-con
Exo-TM
Normalized to mode
100
80
60
40
20
0
0
10^3
10^4
10^5
10^6
PE-conjugated CD206

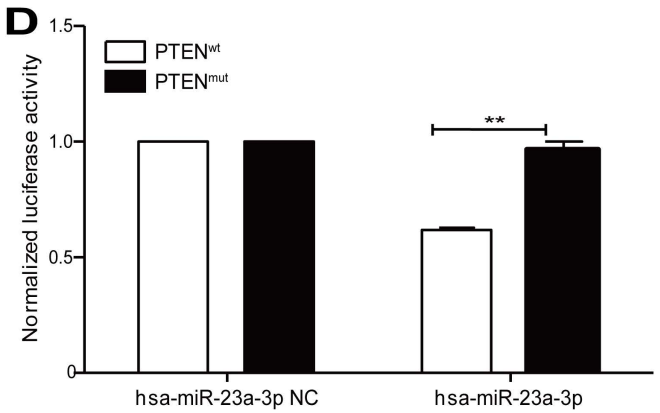

B
MFI of CD206
2500
2000
1500
1000
500
0
**
**
**
**
*
Isotype
Control
Exo-con
Exo-TM

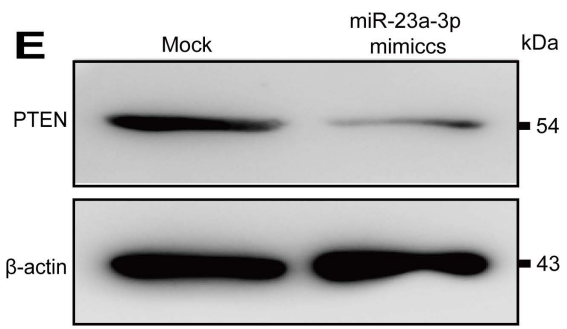

A
TNF-α (ng/mL)
Control
Exo-con
Exo-TM
B
TNF-α mRNA (fold)
C
IL-10 (pg/mL)
D
IL-10 mRNA (fold)
E
IL-6 (ng/mL)
F
IL-6 mRNA (fold)

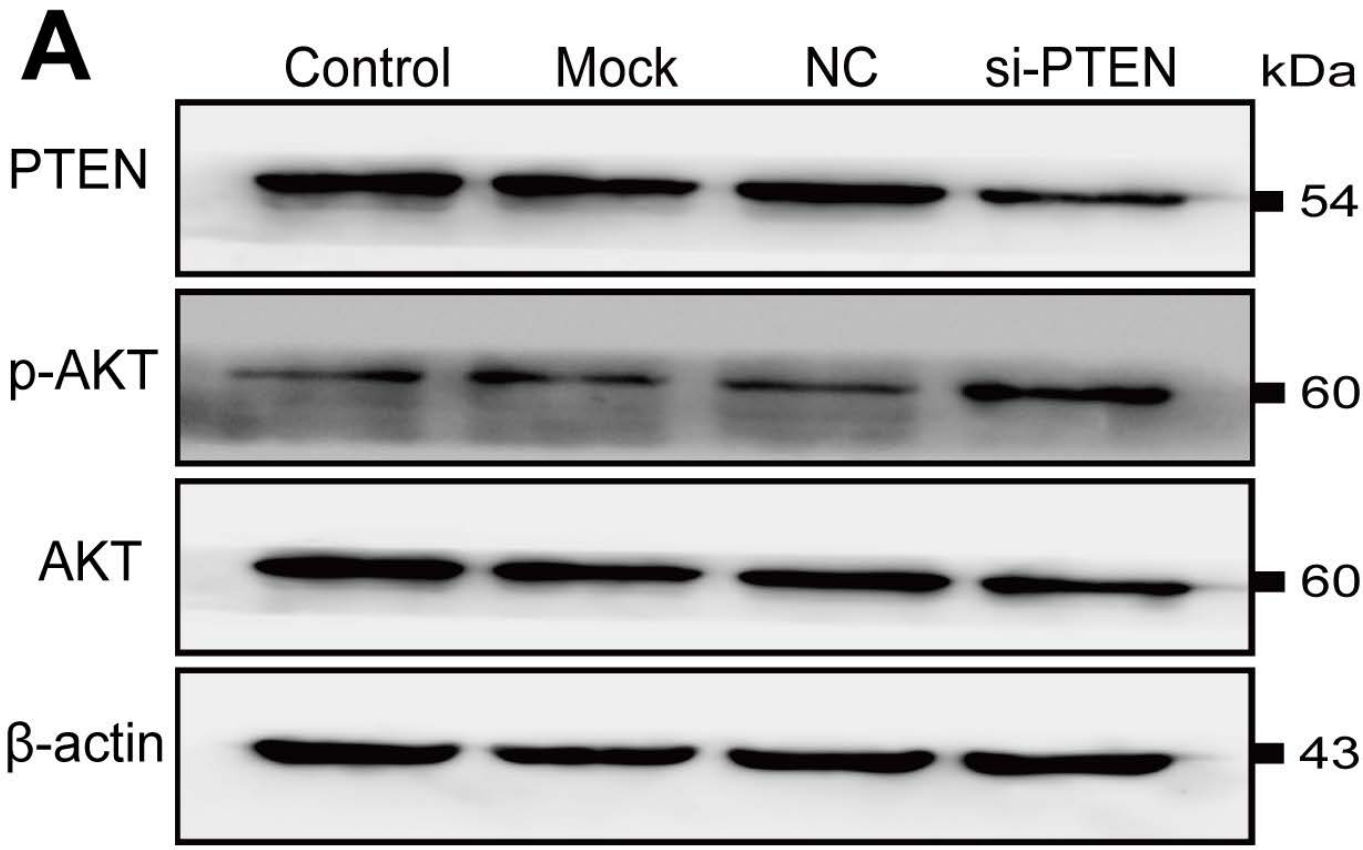

A
Fourteen differentially expressed exosome miRNAs
Target genes prediction
Target genes involved in PD-L1 and inflammation regulation
KEGG pathway analysis
Gene ontology analysis
PTEN-PI3K-AKT pathway
miRNA-23a-3p, miRNA-29a-3p miRNA-486-3p, miRNA-486-5p

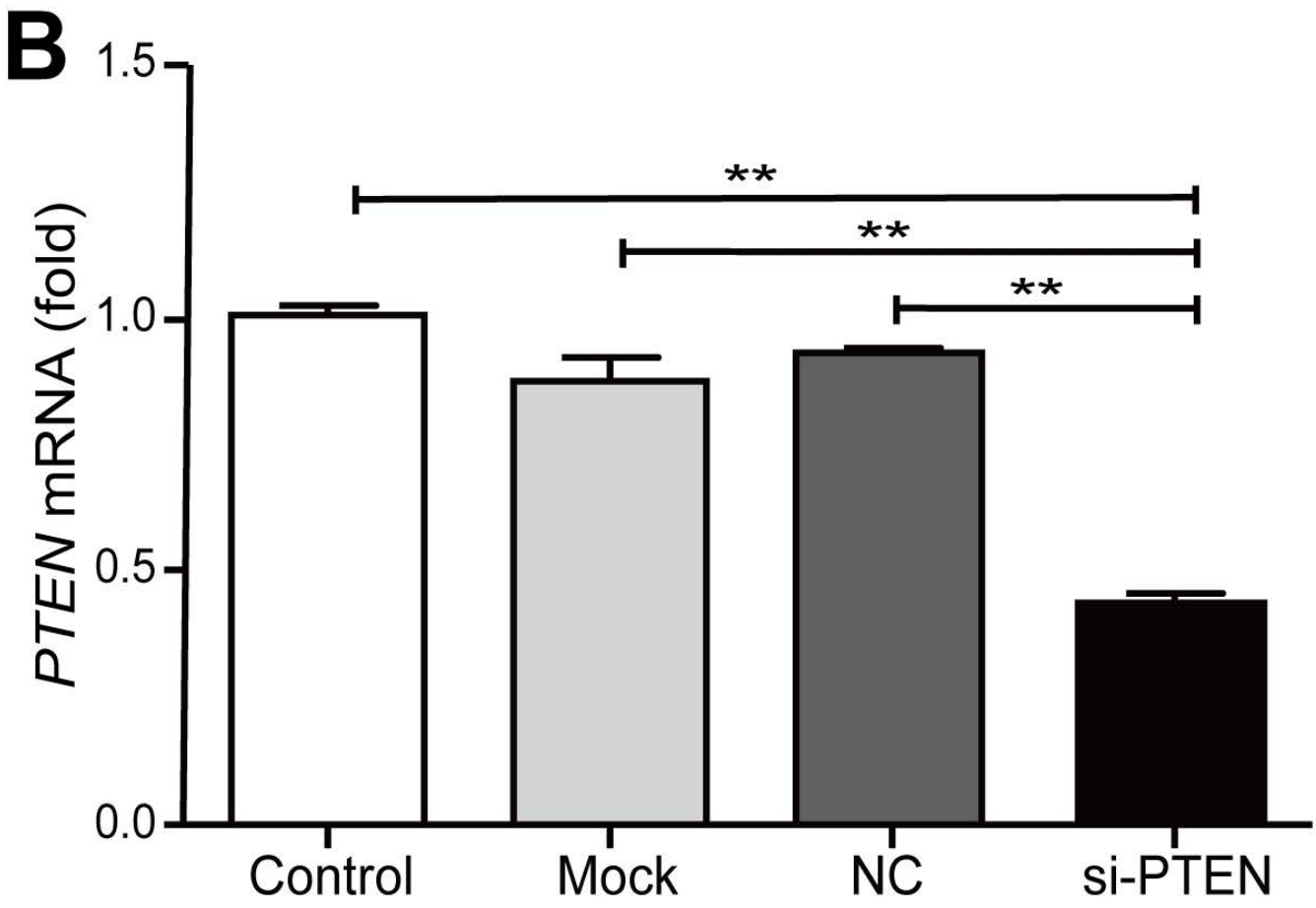

B
Relative exosome miRNA (fold)
Exo-con
Exo-TM
*
miR-23a-3p
miR-29a-3p
miR-486-3p
miR-486-5p

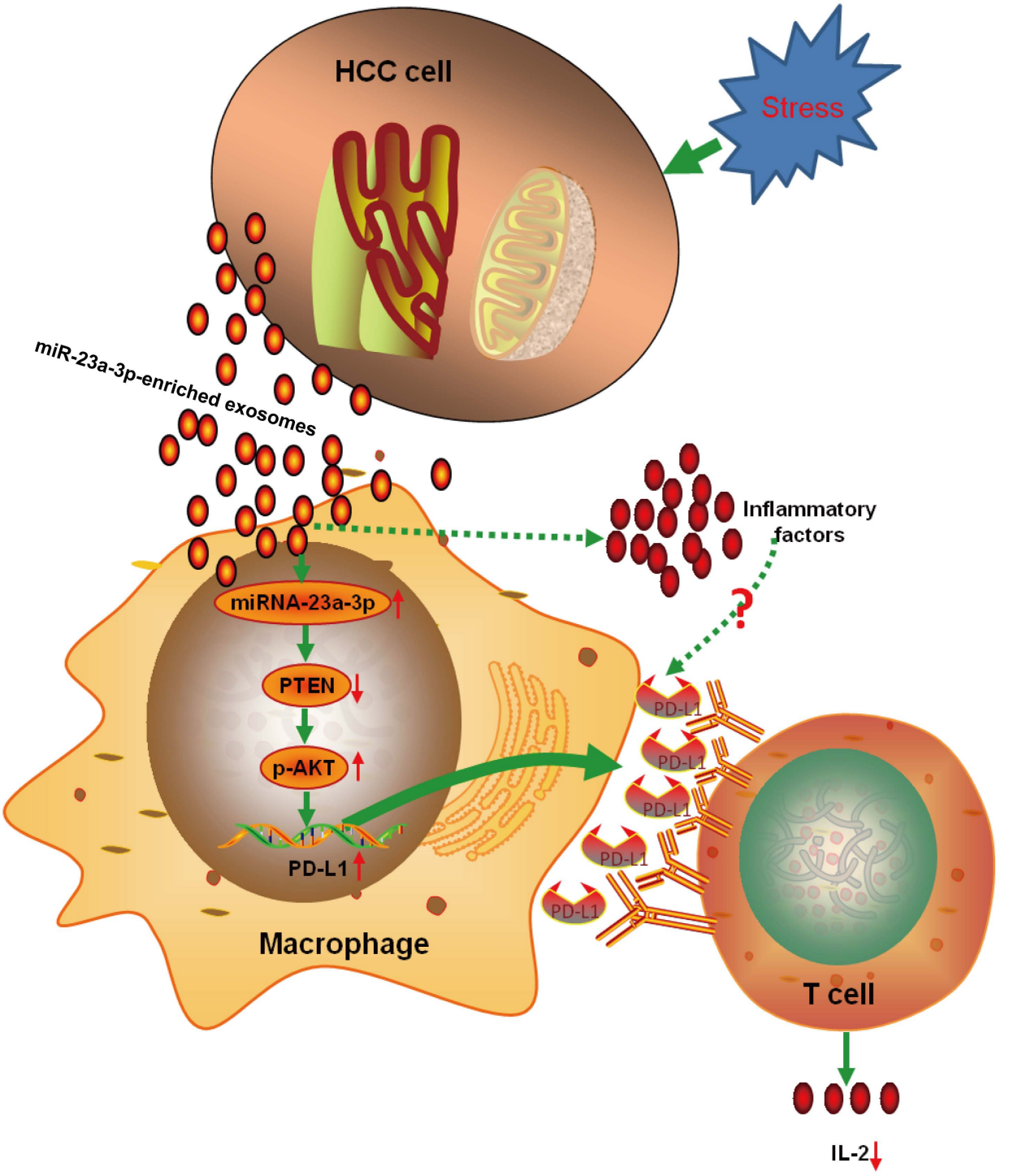

C
PTEN 5'-UACUAUUGUAAAGCUAAUGUGAA-3'
miR-23a-3p 3'-CCAUUAGGGACCGUUACACUA-5'


D
Normalized luciferase activity
PTEN^wt
PTEN^mut
**
hsa-miR-23a-3p NC
hsa-miR-23a-3p


E
Mock
miR-23a-3p mimiccs
kDa
PTEN
54
β-actin
43


A
Control
Mock
NC
si-PTEN
kDa
PTEN
54
p-AKT
60
AKT
60
β-actin
43


B
1.5
1.0
0.5
0.0
PTEN mRNA (fold)
**
**
**
Control
Mock
NC
si-PTEN


HCC cell
Stress
miR-23a-3p-enriched exosomes
Inflammatory factors
?
miRNA-23a-3p
PTEN
p-AKT
PD-L1
Macrophage
PD-L1
PD-L1
PD-L1
PD-L1
PD-L1
T cell
IL-2