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

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Abstract

Endoplasmic reticulum (ER) stress promotes tumor cell escape from immunosurveillance. However, the underlying mechanisms remain unknown. We hypothesized that ER stress induces hepatocellular carcinoma (HCC) cells to release exosomes, which attenuate antitumor immunity by modulating the expression of programmed death ligand 1 (PD-L1) on macrophages. In this study, we demonstrated that expression of several ER stress markers (glucose-regulated protein 78 [GRP78], activating transcription factor 6 [ATF6], PKR-like endoplasmic reticulum kinase [PERK], inositol-requiring enzyme 1 α [IRE1 α]) was up-regulated in HCC tissues and negatively correlated with the overall survival and clinicopathological scores in HCC patients. Expression of ER stress-related proteins positively correlated with cluster of differentiation 68-positive (CD68⁺) macrophage recruitment and PD-L1 expression in HCC tissues. High-throughput sequencing analysis identified microRNA (miRNA/miR)-23a-3p as one of the most abundant miRNAs in exosomes derived from the ER stress inducer tunicamycin treated HCC cells (Exo-TM). miR-23a-3p levels in HCC tissues negatively correlated with overall survival. Treatment with Exo-TM up-regulated the expression of PD-L1 in macrophages *in vitro* and *in vivo*. Bioinformatics

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analysis suggests that miR-23a-3p regulates PD-L1 expression through the phosphatase and tensin homolog (PTEN)- phosphoinositide-4,5-bisphosphate 3-kinase (PI3K)-protein kinase B (AKT) pathway. This notion was confirmed by *in vitro* transfection and co-culture experiments, which revealed that miR-23a-3p inhibited PTEN expression and subsequently elevated phosphorylated AKT and PD-L1 expression in macrophages. Finally, co-culture of T cells with Exo-TM-stimulated macrophages decreased CD8⁺ T-cell ratio and interleukin-2 production but increased T-cell apoptosis *in vitro*.

Conclusion: ER-stressed HCC cells release exosomes to up-regulate PD-L1 expression in macrophages, which subsequently inhibits T-cell function via an exosome miR-23a-PTEN-AKT pathway. Our findings provide a new insight into the mechanism how tumor cells escape from antitumor immunity.

Keywords

hepatocellular carcinoma; endoplasmic reticulum stress; exosome; microRNA; macrophage

Hepatocellular carcinoma (HCC) is a common malignant tumor with very poor overall survival worldwide.⁽¹⁾ Surgery is the most effective treatment strategy for HCC patients, but more than 80% of patients have lost the opportunity by the time they are diagnosed.⁽²⁾ Due to insensitivity to current treatment strategies, the median overall survival of advanced-stage HCC patients is only 3 to 16 months.⁽³⁾ Thus, more effective systemic therapies for HCC patients are urgently needed.

HCC is closely associated with immune tolerance and suppression⁽⁴⁾; thus, immunotherapy may be a promising strategy against HCC. Recently, immunotherapy has been found to be effective in several types of human cancers, such as melanoma⁽⁵⁾ and lung cancer,⁽⁶⁾ but only obtained minimal beneficial effect in HCC. The majority of HCC is caused by chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, nonalcoholic fatty liver, and alcoholic liver disease.⁽⁷⁾ It is generally believed that chronic inflammation contributes to hepatocarcinogenesis and HCC progression.^(8,9) Recruitment of several types of immune cells to the tumor area has been correlated with poor outcome in HCC patients, including tumor-associated macrophages (TAMs),^(10,11) cluster of differentiation 4-positive (CD4⁺) regulatory T cells,⁽¹²⁾ and myeloid-derived suppressor cells (MDSCs).⁽¹³⁾ Furthermore, many cytokines,⁽¹⁴⁾ co-inhibitory signaling and immune inhibitory receptors,⁽¹⁵⁾ and their ligands^(15,16) also induce immunosuppression to promote HCC progression. In this setting, modulation of immune cell infiltration in the tumor microenvironment may represent a novel and effective strategy for the treatment of HCC. However, how HCC cells escape from immunosurveillance has not been fully elucidated, which is a critical barrier to developing effective immunotherapeutic intervention for the treatment of HCC.

Endoplasmic reticulum (ER) stress is critical for maintaining cell survival by activating the unfolded protein response (UPR), which is controlled by several ER stress-related proteins, including glucose-regulated protein 78 (GRP78), activating transcription factor 6 (ATF6), inositol essential enzyme 1 α (IRE1 α), and protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). Activation of ER stress has been reported in various types of tumors, including HCC, and plays an important role in promoting tumor progression.^(17,18)

We have shown that ER stress contributed to HCC chemotherapy and radiation resistance.^(19,20) Other studies have also shown that ER stress promotes tumor cell escape from immunological surveillance. Moreover, activation of ER stress in immune cells has been shown to influence the function of infiltrating immune cells.^(21,22) For example, ER stress has been reported to increase a variety of inflammatory factors, including interleukin (IL)-6 and IL-23 in macrophages.⁽²³⁾ ER-stressed tumor cells also impact immune cell functions by releasing ER stress-related molecules and subsequently facilitating tumor survival, progression, and metastasis.⁽²⁴⁾ Furthermore, cytokines secreted by infiltrating immune cells (predominantly TAMs, MDSCs, and dendritic cells [DC]) can also enhance ER stress and promote tumor cell growth.⁽²⁵⁾ These studies indicated that ER stress in tumor cells may not only involve intracellular events in controlling tumor cell proliferation but also have extracellular effects in regulating tumor microenvironment and immune responses. However, how ER-stressed tumor cells educate immune cells and dysregulate immune response remains unclear.

Exosomes are a class of 40- to 100-nm membrane vesicles that play an important role in cell-to-cell communication. Recently, microRNAs (miRNAs) were shown to be packaged in the purified exosomes, indicating that these vesicles may deliver genetic information to recipient cells.^(26,27) For example, metastatic breast cancer cell-secreted exosomes transfer miR-105 to endothelial cells and promote tumor cell metastasis by destroying tight junctions and the integrity of endothelial monolayers.⁽²⁸⁾ Furthermore, exosomes derived from HCC cells can transfer specific miRNAs to enhance recipient cell growth.⁽²⁹⁾ These results suggest that miRNAs are vital molecules in exosome-mediated intercellular communication. However, few studies have examined whether ER stress affects HCC exosomal-miRNA transfer and how these exosomal-miRNAs affect tumor progression. Thus, the purpose of the current study was to investigate whether ER-stressed HCC cells can transmit miRNA-enriched exosomes to macrophages and how these exosomal-miRNAs modulate the immunologic functions of macrophages. To answer these questions, we performed high-throughput sequencing, *in vitro* and *in vivo* analyses, and found that exosomes derived from ER-stressed HCC cells (Exo-TM) increased the expression of programmed death ligand 1 (PD-L1) and inflammatory cytokines in macrophages, thereby decreasing CD8⁺ T-cell ratio and increasing T-cell apoptosis. Mechanistically, Exo-TM up-regulates PD-L1 expression by the transfer of miR-23a-3p, which inhibits phosphatase and tensin homolog (PTEN) and subsequently activates protein kinase B (AKT). Our data suggest that ER stress promotes HCC immune escape by transferring specific miRNAs to infiltrated macrophages in tumor microenvironment. Thus, interference of HCC-macrophage crosstalk may be an effective strategy for the treatment of HCC.

Materials and methods

Patients and tissue samples.

The present protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the First Affiliated Hospital of Anhui Medical University. All enrolled patients provided informed written consent. Primary HCC tumor tissues were obtained from 169 HCC patients who were hospitalized in the First Affiliated

Hospital of Anhui Medical University between 2004 and 2010. The Edmondson-Steiner grading system was employed to define the histologic grade of tumor differentiation. Tumors were categorized based on the World Health Organization (WHO) classification and the International Union Against Cancer tumor-node-metastasis (TNM) classification system. The HCC tumor tissues were formalin-fixed and paraffin-embedded for histopathological diagnosis and construction of paraffin-embedded tissue microarrays. Freshly resected HCC tissues, paired liver tissues, and healthy donor peripheral blood were obtained in the First Affiliated Hospital of Anhui Medical University between August 2017 and October 2018.

Definition of ER stress high and low patients.

For analysis, the levels of ER stress-related proteins, 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 was qualitatively scored by the percentages of positive cells multiplied by staining intensity, and the values less than 5 and greater than or equal to 5 indicated low and high levels of GRP78, respectively, whereas values less than 3 and greater than or equal to 3 were defined as low and high levels for ATF6, PERK, and IRE1 α , respectively.

Exosome co-culture with macrophages *in vitro*.

THP-1 monocytes were differentiated into macrophages by incubation with 100 nM of phorbol 12-myristate 13-acetate (PMA) for 48 hours and were thereafter named mTHP-1 cells. RAW264.7 cells and mTHP-1 cells (1×10^6 /well) were plated in 12-well plates and co-cultured with 10 μ g/mL of Exo-con or Exo-TM. After co-incubation for 24 hours, cells were collected for flow cytometry, western blot, and quantitative polymerase chain reaction (qPCR) analysis, and the supernatants were used for cytokine measurement.

***In vivo* assay.**

Exosomes were injected intravenously through the tail vein into 6-week-old female nude mice once every other day for 10 times. Peritoneal macrophages were isolated from euthanized mice and allowed to attach at 37°C for 2 hours. The unattached cells were removed and washed with phosphate-buffered saline (PBS) twice; the remaining cells were then incubated overnight. PD-L1 levels were analyzed by flow cytometry and immunohistochemistry (Clone 10F.9G2, BioLegend) and qPCR. Cytokines secreted by peritoneal macrophages were measured by a mouse Cytometric Bead Array (CBA) inflammation kit. Four mice were used per group in the animal experiments.

Statistical Analysis.

SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was employed for statistical analysis. Two-tailed Student *t* test or the Wilcoxon-Mann-Whitney test was applied to evaluate the difference between two groups, and 1-way analysis of variance (ANOVA) multiple comparisons were employed to compare three or more groups. Associations between ER stress-related proteins and PD-L1 expression or miR-23a-3p and overall survival were assessed using Pearson correlation analysis. Representative images and flow cytometric

graphs from three independent experiments are shown. Values are presented as the mean \pm standard deviation (SD). Differences were considered to be statistically significance when P was less than 0.05.

For more detailed materials and methods information, see Supporting Data.

Results

ER stress is up-regulated and positively correlates with poor survival in HCC patients

To evaluate the role of ER stress in HCC progression, we first tested whether ER stress is up-regulated in HCC patients by measuring the expression of ER stress-related proteins in 169 cases of surgically resected HCC tissues by immunohistochemistry. These ER stress-related proteins include GRP78, ATF6, IRE1 α , and PERK. Representative images of low and high expression of GRP78, PERK, ATF6, and IRE1 α proteins are shown in Fig. 1A, in which these ER stress-related proteins were predominantly detected in the cytoplasm. Expression of these ER stress-related proteins was much stronger in tumor tissues compared with paired paracarcinoma tissues (65.68% versus 41.42% for GRP78; 63.91% versus 37.87% for ATF6; 60.36% versus 46.75% for PERK; 60.95% versus 43.79% for IRE1 α). Based on the percentage of ER stress-related protein positive cells and staining density, we divided patients into two groups with low and high ER stress (see details in Methods). Moreover, we performed western blot analyses to confirm the ER stress-related protein expression in 5 freshly resected HCC tissues, and found that the expression of these ER stress-related proteins was much higher in tumor tissues compared to paracarcinoma tissues (Fig. 1B). We also examined the levels of *GRP78*, *PERK*, *ATF6*, and *IRE1 α* messenger RNAs (mRNAs) in HCC tissues and found that the expression of these genes was significantly up-regulated in tumor tissues compared with paired paracarcinoma tissues (Fig. 1C). The clinicopathological characteristics of HCC patients are shown in Supporting Tables S1-S4, showing that the high ER stress-related patients had larger tumor size and poorly differentiated tumors compared to the low ER stress patients. Finally, the association of GRP78, ATF6, PERK, or IRE1 α expression with overall survival of 55 cases of HCC patients was analyzed. As illustrated in Fig. 2, the overall survival of low ER stress patients was significantly longer than the high ER stress patients.

Hepatic ER stress-related protein levels correlate with hepatic macrophage infiltration and PD-L1 expression in HCC patients

ER stress has been shown to play an important role in regulating immune cell functions in tumor microenvironment and antitumor immune response.^(24,30) Thus, we tested whether expression of ER stress-related proteins such as GRP78 correlates with macrophage infiltration in HCC patients. As illustrated in Fig. 3A, tumor tissues that expressed a higher level of GRP78 (GRP78^{high}) had massive CD68 staining in the stroma, whereas CD68⁺ macrophage infiltration was much lower in tumor tissues that expressed a low level of GRP78 (GRP78^{low}). Quantitative analysis revealed that CD68⁺ areas in GRP78^{high} tumor stroma were significantly higher than those in the GRP78^{low} stroma (Fig. 3A).

PD-L1 was reported to inhibit T-cell activation and contribute to tumor immune evasion.⁽³¹⁾ To examine whether ER stress activation correlates with PD-L1 expression, we measured PD-L1 levels by immunohistochemistry analysis and found in GRP78^{high} tumors, PD-L1 was often distributed in a crumby manner, whereas it sporadically existed in GRP78^{low} tumors (Fig. 3B). Quantitative analysis showed that 51.35% of the GRP78^{high} tumors expressed PD-L1, and only 27.59% of the GRP78^{low} tumors expressed PD-L1, and PD-L1 expression was positively correlated with GRP78 levels ($P = 0.003$) (Supporting Table S5). Consistently, the expression levels of ATF6 (54.63% versus 22.95%; $P < 0.001$), PERK (54.90% versus 25.37%; $P < 0.001$), and IRE1 α (55.34% versus 24.24%; $P < 0.001$) were also positively correlated with PD-L1 expression (Supporting Tables S6-S8). qPCR and western blot analyses showed that PD-L1 mRNA and protein were markedly up-regulated in tumor tissues compared with paired paracarcinoma tissues (Fig. 3C,D).

To test whether infiltrated macrophages expressed PD-L1, we performed immunofluorescence double-staining and found PD-L1 was frequently co-localized with CD68⁺ macrophages in GRP78^{high} HCC tumor stroma (Fig. 3E). Finally, Kaplan-Meier analysis showed that patients with lower PD-L1 expression had better survival time than those with higher PD-L1 expression (24.9 ± 2.0 months versus 16.4 ± 2.2 months; $P = 0.002$) (Fig. 3F). Together, these results suggest that ER stress activation is associated with higher macrophage PD-L1 expression and poor prognosis in HCC patients.

Exosomes from ER-stressed HCC cells up-regulate PD-L1 expression on macrophages *in vitro*

The above data revealed that high ER stress status is associated with up-regulated PD-L1 expression in macrophages on HCC patients, suggesting ER-stressed HCCs may promote PD-L1 expression in macrophages. Because exosomes are an important communicator between different cell types, we hypothesized that ER-stressed HCC cells may release exosomes and subsequently up-regulate PD-L1 expression on macrophages. To test this hypothesis, we first optimized the *in vitro* cell culture system to mimic the ER stress status by tunicamycin (TM) treatment and found that treatment with TM (2.5 $\mu\text{mol/L}$) for 24 hours generated significant ER stress in several HCC cell lines, including HepG2, Hep3B, Hepa1-6, and A549 cells (Supporting Fig. S1A-C). Second, exosomes derived from tunicamycin (TM) treated HCC cells (defined as Exo-TM) or without TM (defined as Exo-con). The purity of these exosomes was confirmed by transmission electron microscopy showing a homogeneous population of rounded membrane-bound vesicles with a diameter ranging from 30 to 100 nm (Supporting Fig. S2A,B) and by western blot analysis showing the expression of two exosomal-marker proteins CD63 and tumor susceptibility gene (TSG)-101 (Supporting Fig. S2C). Additionally, ER-stressed HCC cells secreted a higher number of exosomes compared with those without ER stress (Supporting Fig. S2D).

Third, to investigate whether HCC cells transmit ER stress signals to macrophages through exosomes, we performed a co-culture experiment and found that co-culture with Exo-TM derived from HepG2 cells or normal HL-7702 hepatocytes significantly up-regulated the expression of GRP78, ATF6, PERK, or IRE1 α proteins compared to co-culture with Exo-

con (Supporting Fig. S3A,B). Moreover, co-culture with Exo-con or Exo-TM increased the proliferation of HepG2 cells (Supporting Fig. S3C).

Fourth, we co-cultured macrophages with PKH67-labeled exosomes and found that mTHP-1 cells effectively engulfed exosomes (Fig. 4A). Moreover, flow cytometric analysis showed that co-culture with HepG2-derived Exo-TM significantly up-regulated PD-L1 expression in mTHP-1 cells compared with those in the Exo-con group (Fig. 4B). Such up-regulation of PD-L1 in macrophages by Exo-TM was also confirmed by western blot, immunohistochemistry, and qPCR analyses (Fig. 4C-E). Similarly, Hep3B-derived Exo-TM also significantly up-regulated PD-L1 levels in co-cultured macrophages (Supporting Fig. S4A-D). However, Exo-TM from normal HL-7702 hepatocytes or primary human hepatocytes did not up-regulate PD-L1 expression in mTHP-1 cells compared to the Exo-con group (Supporting Fig. S4E-G). Finally, HepG2-derived Exo-TM markedly up-regulated PD-L1 expression on mouse RAW264.7 macrophages (Supporting Fig. S5A,B), whereas Hep3B-derived Exo-TM only slightly increased PD-L1 expression in RAW264.7 cells (Supporting Fig. S5C,D).

To rule out whether Exo-TM-induced ER stress in macrophages was a consequence of TM contamination, we performed the following experiments. First, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) confirmed that no TM existed in Exo-con, whereas Exo-TM contained approximately 20.25 ng/mL of TM (Supporting Fig. S6A). As the exosome used in the current study was diluted 10 times, the final concentration of TM contained in Exo-TM was very low (~2 ng/mL). Second, treatment with up to 50 ng/mL of TM did not up-regulate GRP78 levels in mTHP-1 (Supporting Fig. S6B). Finally, coincubation with TM did not affect PD-L1 expression on mTHP-1 cells (Supporting Fig. S6C). Collectively, Exo-TM-mediated up-regulation of PD-L1 in macrophages is not due to TM contamination.

Exo-TM up-regulates PD-L1 expression on macrophages *in vivo*

To determine whether Exo-TM also enhances PD-L1 expression *in vivo*, we injected nude mice with Exo-TM and Exo-con once every 2 days for 10 times, and the peritoneal macrophages were isolated 12 hours after the last injection (Supporting Fig. S7). Confocal microscopy and flow cytometric analyses confirmed that PKH67-labeled exosomes were effectively engulfed by the peritoneal macrophages *in vivo* (Fig. 5A,B). As illustrated in Fig. 5C-F, flow cytometric, immunohistochemistry, and qPCR analyses demonstrated that peritoneal macrophages isolated from Exo-TM-injected mice had significantly higher levels of PD-L1 compared with the Exo-con and PBS group. In addition, these macrophages also had higher levels of several inflammatory mediators compared to those from the Exo-con or PBS group (Supporting Fig. S8).

Exo-TM-treated macrophages decrease CD8⁺ T-cell ratio and promote T-cell apoptosis

Macrophage (predominantly M2 macrophage) infiltration in tumor microenvironment has been reported to promote tumor progression by impairing the immune responses of cytotoxic CD8⁺ T cells.⁽³²⁾ We wondered whether ER-stressed HCC cells also polarized macrophages to M2 phenotype to promote HCC progression by impairing the immune

responses of cytotoxic CD8⁺ T cells. To test this hypothesis, we first examined whether Exo-TM induces macrophage polarization and found co-culture with HepG2-derived Exo-TM markedly increased levels of M2 marker (such CD206) and M2 cytokines (IL-10) in macrophages compared with Exo-con treatment (Supporting Figs. S9-S10). Together, these results suggest that ER stress-related exosomes promote macrophages toward M2 phenotype.

Then, we examined the effects of Exo-TM-treated macrophages on T cells. As illustrated in Fig. 6A-C, Exo-TM-treated macrophages significantly decreased CD8⁺ T-cell proportion and attenuated IL-2 production in T cells compared with Exo-con-treated macrophages. Moreover, we examined the impact of Exo-TM-treated macrophages on T-cell apoptosis using Annexin V /PI apoptosis detection kit and found that the ratio of apoptotic T cells was significantly higher after incubation with Exo-TM-treated macrophages compared to that with Exo-con-treated macrophages (Fig. 6D,E).

ER-stressed HCC-derived exosomes contain high levels of miR-23a-3p, which up-regulates PD-L1 expression in macrophages by regulating the PTEN/AKT pathway

To determine whether ER-stressed HCC cells transmit specific miRNAs to macrophages and impact its immunologic functions, high-throughput sequencing analyses of exosomes were performed. As illustrated in Fig. 7A, 14 miRNAs were differentially expressed between Exo-TM and Exo-con. In comparison with Exo-con, six miRNAs (miR-486-5p, miR-486-3p, miR-1-3p, miR-433-3p, miR-432-5p, and miR-92b-5p) were down-regulated, and eight miRNAs (miR-29a-3p, miR-378a-3p, miR-193b-3p, miR-20a-5p, miR-23a-3p, miR-27a-3p, miR-184, and miR-16-5p) were up-regulated in Exo-TM (Supporting Table S9).

Subsequently, bioinformatics analysis revealed that miR-29a-3p, miR-23a-3p, miR-486-3p, and miR-486-5p were predicted to regulate PD-L1 expression through the PTEN-phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Supporting Fig. S11A). Moreover, qPCR analysis (plus cel-miR-39-3p as external control) demonstrated that miR-23a-3p had the highest fold induction (2.44-fold) in Exo-TM, followed by miR-29a-3p with a 1.95-fold induction; in contrast, miR-486-5p expression was down-regulated by 1.96-fold, and miR-486-3p had no changes (Supporting Fig. S11B). Furthermore, we explored the relationship between miR-23a-3p levels and survival times in 55 cases of HCC patients and found that miR-23a-3p levels were negatively correlated with overall survival ($r = -0.306$; $P = 0.023$) (Supporting Table S10).

According to TargetScan, microRNA Database (miRDB), and miRanda, we found *PTEN* is a putative target of miR-23a-3p, and this notion was confirmed by a dual luciferase reporter gene assay (Supporting Fig. S11C,D). Western blot analysis shows that transfection of miR-23a-3p mimics down-regulated *PTEN* expression in macrophages (Supporting Fig. S11E). *PTEN* is known to regulate PD-L1 expression^(33,34); therefore we hypothesized that miR-23a-3p may up-regulate PD-L1 expression through the down-regulation of *PTEN* expression in macrophages. To confirm whether miR-23a-3p regulates PD-L1 expression by modulating the *PTEN* pathway in macrophages, mTHP-1 cells were transfected with miR-23a-3p mimics, inhibitors, and their relative controls for 48 hours, and we found miR-23a-3p mimics significantly down-regulated *PTEN* at both protein and mRNA levels

(Fig. 7B,C) and up-regulated phosphorylated-AKT (p-AKT) protein expression (Fig. 7B). Moreover, miR-23a-3p mimics significantly up-regulated PD-L1 expression on macrophages, whereas the miR-23a-3p inhibitor and control had no influence on PD-L1 protein and mRNA expression (Fig. 7D,E). However, transfection of macrophages with either miR-23a-3p mimics or inhibitors did not influence the expression of IL-6, IL-10, and tumor necrosis factor- α (TNF- α) (data not shown).

To further confirm whether miR-23a-3p regulates PD-L1 expression through the activation of the PTEN/AKT pathway, we first co-cultured macrophages with Exo-TM or Exo-con and found that, compared to Exo-con, Exo-TM significantly down-regulated PTEN levels at both protein and mRNA levels (Fig. 8A,B) and up-regulated phosphorylated-AKT (p-AKT) protein expression (Fig. 8A). Moreover, qPCR analysis demonstrated that Exo-TM significantly increased miR-23a-3p expression in coincubated macrophages compared to Exo-con (Fig. 8C). Transfection of macrophages with miR-23a-3p inhibitors abolished Exo-TM-mediated PTEN down-regulation and p-AKT up-regulation (Fig. 8D). Next, we knocked down PTEN using RNA interference (RNAi) and found PTEN significantly reduced at both protein and mRNA levels, whereas p-AKT protein expression was up-regulated (Supporting Fig. S12A,B). Furthermore, knockdown of PTEN significantly up-regulated PD-L1 protein and mRNA expression on macrophages (Fig. 8E,F). However, knockdown of PTEN had no impact on expression of inflammatory cytokines, such as IL-6, IL-10, and TNF- α (data not shown).

Discussion

Multiple studies have shown that ER stress can dysregulate antitumor immunity through various mechanisms,^(17,21) including stimulating protumor inflammatory factors⁽³⁵⁾ and inducing MDSCs.⁽²³⁾ In the current study, we identified a novel mechanism by which ER stress promotes HCC cells to release miR-23a-3p-enriched exosomes which up-regulate PD-L1 expression on macrophages and subsequently suppress T-cell functions. We found that most of human HCC samples highly express ER stress-related proteins and genes, and their expression levels negatively correlated with patients' overall survival. Furthermore, GRP78^{high} tumors had more CD68⁺ macrophages and PD-L1⁺ cells in the stroma, and PD-L1 expression also correlated with poor overall survival in HCC patients. Finally, ER-stressed HCC cells up-regulated macrophage PD-L1 expression and consequently impaired T-cell function through the transmission of exosomal-miR-23a-3p, which inhibits *PTEN* and consequently activates the AKT pathway, resulting in elevation of PD-L1 expression. Together, our results suggest that HCC cells can transmit ER stress signals to infiltrated macrophages through miR-23a-3p-enriched exosomes and promote tumor progression, which is summarized in Supporting Fig. S13.

ER stress promotes HCC cells to release exosomes.

ER stress has been reported to promote tumor cell immune escaping by constructing an immunosuppressive microenvironment, but the precise mechanism remains unclear. Exosomes have been reported to play an important role in intercellular communication by delivering their contents (such as miRNAs) to the "recipient" cells.^(28,36,37) However,

whether ER-stressed HCC cells influence the function of immune cells by transmitting exosomes and whether this immunomodulation is mediated by specific exosomal miRNAs is not clear. Recently, Kanemoto et al. reported that ER stress promoted exosome release in IRE1 α and PERK-dependent manners.⁽³⁸⁾ Moreover, ER stress increased the levels of cytochrome P450 2E1 (CYP2E1) and other P450 isoforms in exosomes of alcohol-exposed rodents and patients with alcoholism.⁽³⁹⁾ Our results demonstrated that ER stress significantly increased HCC cell exosome secretion, and exosome RNA sequencing found that 14 miRNAs were differentially packaged in Exo-TM, and bioinformatics analysis revealed these miRNAs are closely related to several cancer-associated pathways, such as the PTEN-PI3K-AKT pathway. Moreover, these exosomes were effectively transferred into macrophages and transmitted ER stress signals to macrophages, suggesting that ER stress influences HCC cell exosome release and transmits miRNAs-enriched exosomes to macrophages that resided in the tumor microenvironment.

ER stress-related exosomes modulate PD-L1 expression through the PTEN-AKT pathway.

Clinical and preclinical evidence showed that loss of PTEN was associated with enhanced PD-L1 expression in human glioma,⁽⁴⁰⁾ breast cancer,⁽⁴¹⁾ and colorectal cancer.⁽⁴²⁾ A recent study revealed that PTEN mutation resulted in an increase of PD-L1 protein expression through the activation of the PI3K-AKT- mammalian rapamycin target protein (mTOR)-ribosomal S6 kinase 1 (S6K1) pathway.⁽⁴⁰⁾ In the present study, we found that miR-184 is the most up-regulated miRNA in Exo-TM, however, bioinformatics analysis revealed miR-184 was not associated with PD-L1 expression while other miRNAs including miR-23a-3p, miR-29a-3p, miR-486-3p and miR-486-5p did. Moreover, qPCR assays demonstrated that miR-23a-3p was the most significantly up-regulated miRNA packaged in Exo-TM, and we provided several lines of evidence suggesting that miR-23a-3p up-regulates PD-L1 expression through the inhibition of PTEN. First, miR-23a-3p was elevated in ER stress-related exosomes, and Exo-TM significantly reduced PTEN expression but augmented PD-L1 expression in macrophages. Second, bioinformatics analysis and dual luciferase reporter assay revealed that miR-23a-3p specifically targeted *PTEN*.^(33,34) Third, overexpression of miR-23a-3p in macrophages inhibited *PTEN* expression but significantly up-regulated PD-L1 expression. Finally, knockdown of PTEN elevated PD-L1 expression in macrophages. Taken together, our results suggest that ER-stressed HCC cells down-regulate PTEN in macrophages by transmitting exosomal-miR-23a-3p to macrophages and subsequently increasing PD-L1 expression to promote immune escaping.

Macrophage expressed PD-L1 influences T-cell functions.

Macrophages are the prominent components in tumor stroma, and high macrophage density was associated with unfavorable prognosis in many types of tumors.⁽³⁵⁾ Several studies have reported that macrophages directly communicate with tumor cells, thereby promoting tumor progression.⁽⁴³⁾ We found that GRP78^{high} HCC tissues recruited more CD68⁺ macrophages in the stroma, and *in vitro* studies demonstrated that Exo-TM polarized macrophages to M2 phenotype. Moreover, there is evidence suggesting that cancer cells promote macrophage production of inflammatory mediators, such as IL-6 and TNF- α , to facilitate tumor metastasis.^(44,45) Here, we found Exo-TM not only increased macrophage IL-6 and TNF- α expression but also up-regulated protumor factor IL-10 expression, suggesting that ER-

stressed HCC cells may “educate” macrophages toward TAMs to facilitate disease progression.

PD-L1, also called B7-H1 or CD274, inhibits T-cell proliferation and promotes T-cell dysfunction by binding to programmed death 1 (PD-1).⁽³¹⁾ PD-L1 is expressed by both hematopoietic cells and nonhematopoietic cells, and up-regulation of PD-L1 has been observed in various tumors and contributes to immune evasion. High levels of PD-L1 have been observed in macrophages and been shown to play an important role in suppressing antitumor immunity.^(46–48) Macrophage infiltration in the tumor microenvironment has been reported to promote tumor progression by impairing the immune responses of cytotoxic CD8⁺ T cells.⁽³²⁾ Several studies have also demonstrated that Kupffer cells⁽¹⁵⁾ or activated monocytes/macrophages⁽¹⁶⁾ in the HCC stroma express higher levels of PD-L1 than that in the surrounding nontumor liver tissues. Furthermore, blocking PD-L1 using a specific antibody improved T-cell immune response, suggesting that increased PD-L1 expression on macrophages helps HCC cells escape from cytotoxic T cells. However, it is unclear how PD-L1 expression is up-regulated in macrophages. In this study, we found that macrophages infiltrated in ER-stressed HCC tissues expressed high levels of PDL-1, and PD-L1 levels negatively correlated with overall survival. Finally, we provided evidence that ER stress promotes HCC cells to release miR-23a-3p-enriched exosomes, and these exosome-treated macrophages significantly up-regulate PD-L1 expression that reduced the proportion of CD8⁺ T cells and IL-2 production and increased T-cell apoptosis in a co-culture system. Collectively, our data suggest that exosomes released by ER-stressed HCCs induce immunosuppression through the up-regulation of PD-L1 on macrophages.

In summary, we found ER stress is activated in HCC tissues, and activation of ER stress contributes to CD68⁺PD-L1⁺ macrophage recruitment and poor prognosis. Moreover, ER-stressed HCC cells transmit exosomal-miR-23a-3p to macrophages and activate the PI3K-AKT pathway by inhibiting PTEN, thereafter increasing PD-L1 expression and inhibiting T-cell function. Our findings suggest that the transfer of exosomal-miR-23a-3p from ER-stressed HCC cells to macrophages plays an important role in tumor cell escape from immunologic cytotoxicity, and blockade of the HCC-macrophage communication may be a novel strategy to treat HCC progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

Akt protein kinase B

APC	antigen-presenting cell
ATF6	activating transcription factor 6
CD	cluster of differentiation
Cum	cumulative
DAPI, 4'	6-diamidino-2-phenylindole
ER	endoplasmic reticulum
Exo-con	exosomes derived from untreated HCC cells
Exo-TM	exosomes derived from tunicamycin treated HCC cells
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GRP78	glucose-regulated protein 78
HCC	hepatocellular carcinoma
IRE1α	inositol-requiring enzyme 1 α
IL	interleukin
Max	maximum
MDSC	myeloid-derived suppressor cell
MIP-1	macrophage inflammatory protein 1
miRNA/miR	microRNA
mRNA	messenger RNA
p-AKT	phosphorylated protein kinase B
PBS	phosphate-buffered saline
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PERK	protein kinase R (PKR)-like endoplasmic reticulum kinase
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog
Q	quadrant
qPCR	quantitative polymerase chain reaction
TAM	tumor-associated macrophage
TNF-α	tumor necrosis factor- α

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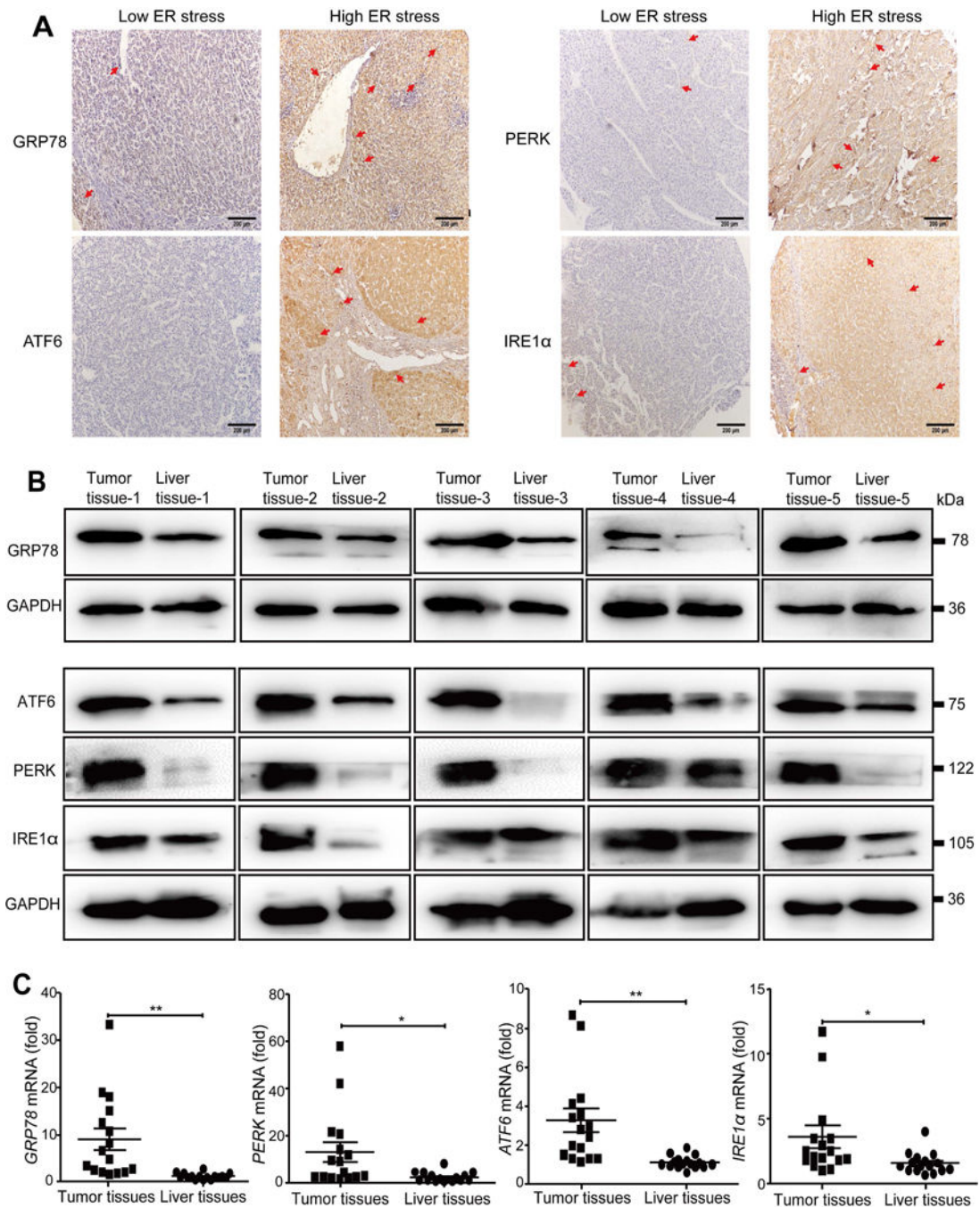


FIG. 1. ER stress is activated in human HCC.

(A) Representative low and high expression of ER stress markers GRP78, PERK, ATF6, and IRE1α in HCC tissue samples (scale bar = 200 μm). (B) Representative western blot analyses of GRP78, PERK, ATF6, and IRE1α protein expression in human HCC tissues. (C) qPCR-detected the mRNA levels of *GRP78*, *PERK*, *ATF6*, and *IRE1α* in 16 cases of freshly resected human HCC tissues. * $P < 0.05$, ** $P < 0.01$.

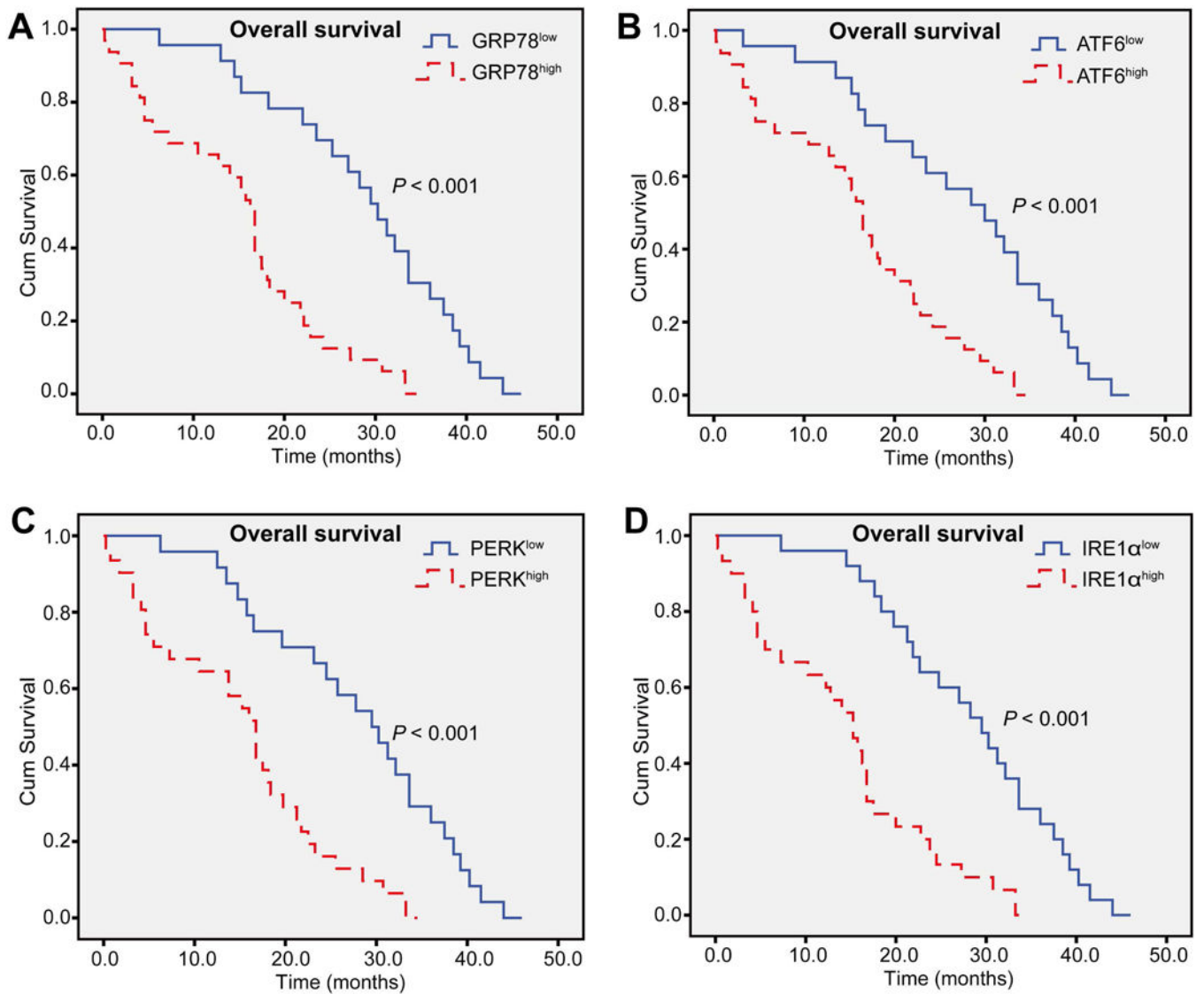


FIG. 2. Activation of ER stress correlates with poor survival in human HCC.

Kaplan-Meier curves demonstrated that the overall survival in patients with overexpression of (A) GRP78, (B) ATF6, (C) PERK, and (D) IRE1 α was significantly shorter than those with low expression levels (n = 55). Log-rank test was used to evaluate statistical significance. ** $P < 0.01$ as indicated.

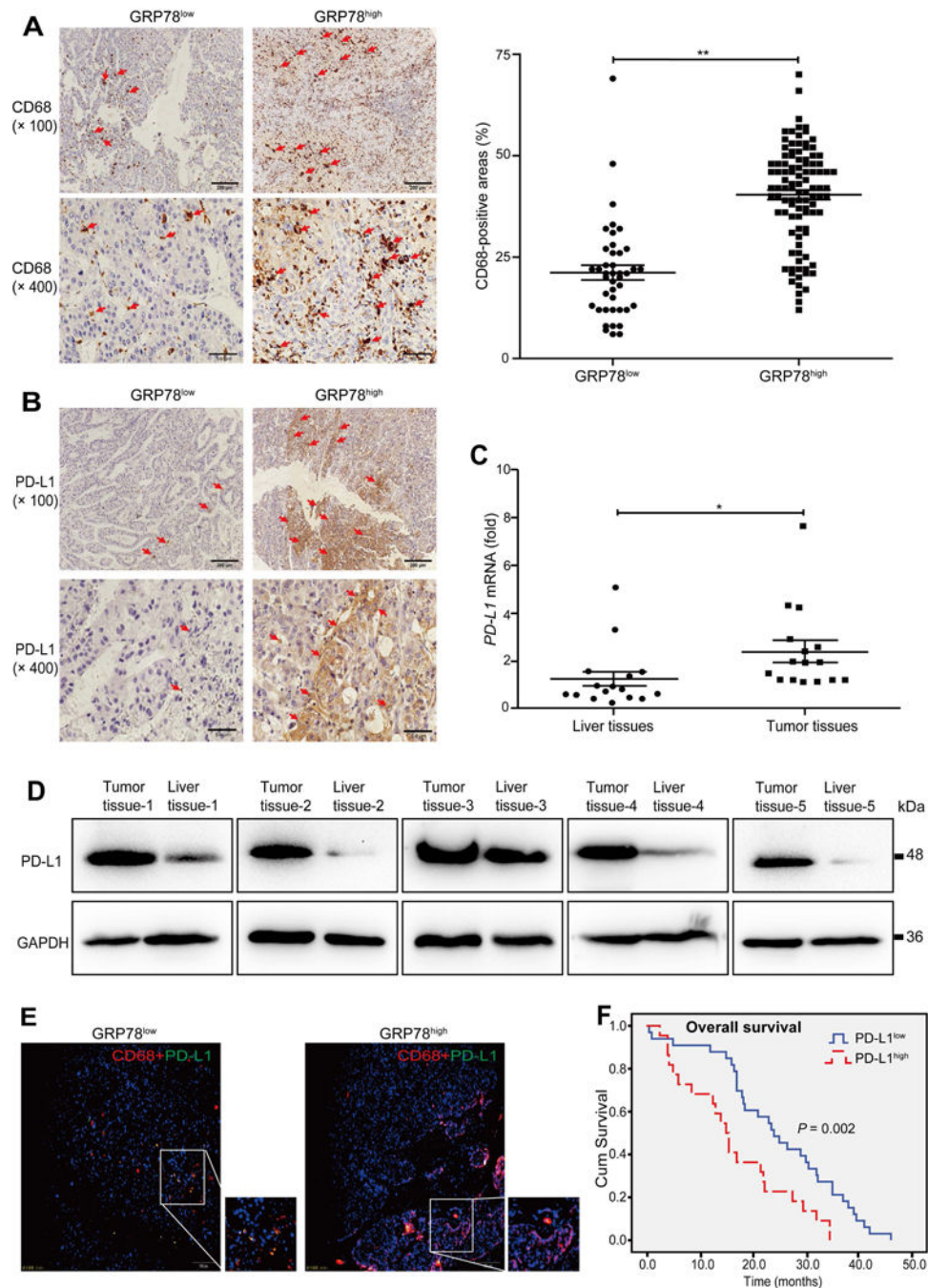


FIG. 3. ER stress is associated with macrophage infiltration and PD-L1 expression in HCC patients.

(A) Representative images of the expression pattern of CD68 protein in HCC tissues with GRP78^{high} and GRP78^{low} groups (scale bar = 200 μ m for the upper panel; scale bar = 50 μ m for the lower panel). Quantitative analysis of CD68-positive areas in the GRP78^{high} and GRP78^{low} group is shown in the right panel. (B) Representative images of the expression pattern of PD-L1 protein in HCC tissues with GRP78^{high} and GRP78^{low} groups (scale bar = 200 μ m for the upper panel; scale bar = 50 μ m for the lower panel). (C) qPCR analyses of *PD-L1* mRNA levels in 16 cases of freshly resected HCC tissues. (D) Western blot analyses

of PD-L1 protein levels in human HCC tissues. (E) The expression pattern of PD-L1 on CD68⁺ cells in GRP78^{low} and GRP78^{high} HCC tissues was measured by immunofluorescence (scale bar = 100 μ m). (F) Log-rank test evaluated overall survival and PD-L1 expression in 55 HCC patients. Data are presented as the means \pm SD (error bar). * P < 0.05, ** P < 0.01 as indicated.

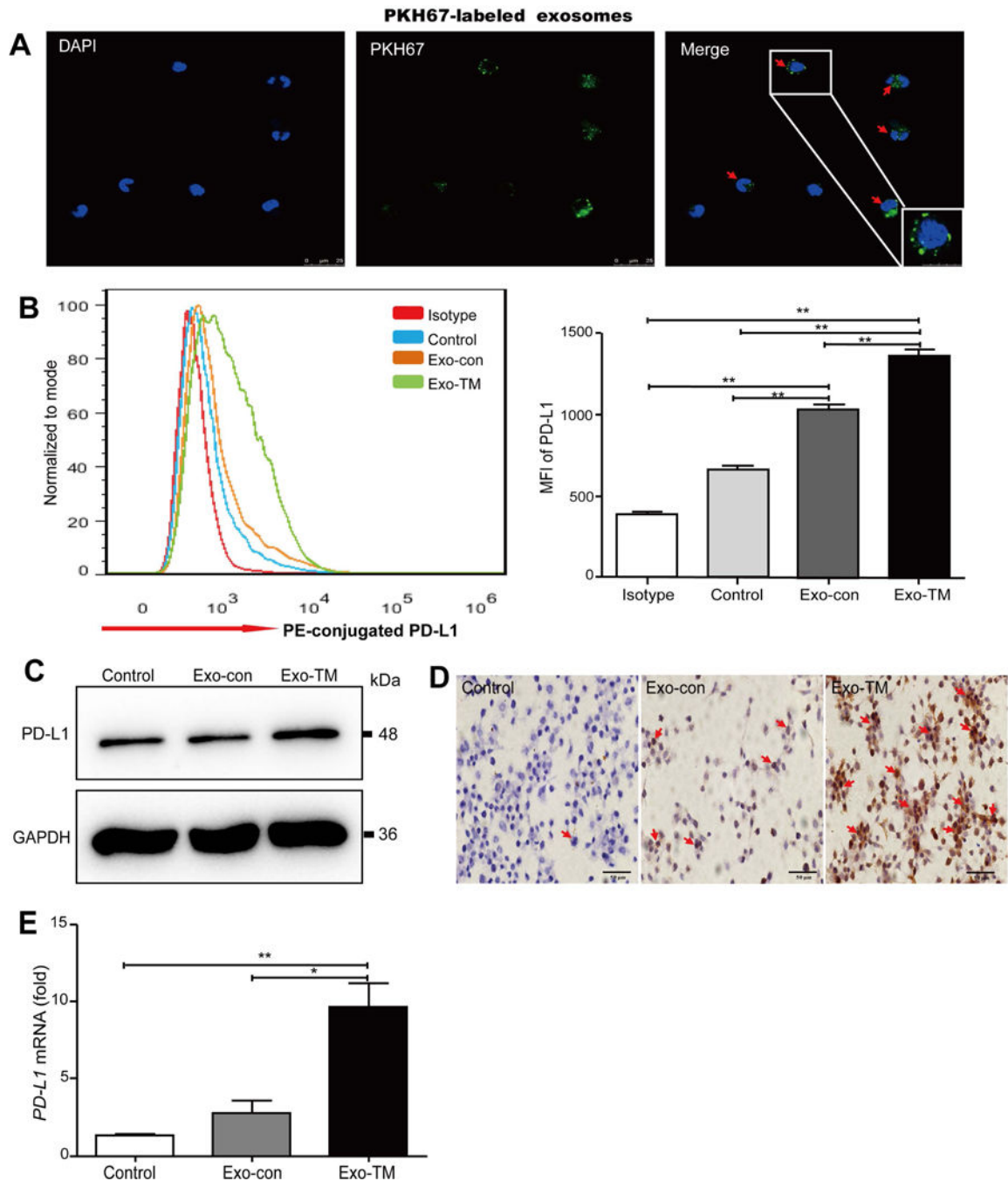


FIG. 4. Exo-TM increases macrophage PD-L1 expression *in vitro*.

(A) PKH67-labeled exosomes were coincubated with mTHP-1 cells and examined by confocal microscopy (scale bar = 25 μ m). Representative images are shown. Red arrows indicate PKH67-labeled exosomes. (B) Macrophages were coincubated with HepG2-derived Exo-con and Exo-TM, the protein levels of PD-L1 were determined by flow cytometry, and the mean fluorescence intensity (MFI) was statistically analyzed. Moreover, the impact of Exo-TM on macrophage PD-L1 protein expression was also determined by (C) western blot and (D) immunohistochemical analysis. *PD-L1* mRNA was detected by (E) qPCR. Data are

shown as the means \pm SD of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$ with indicated groups.

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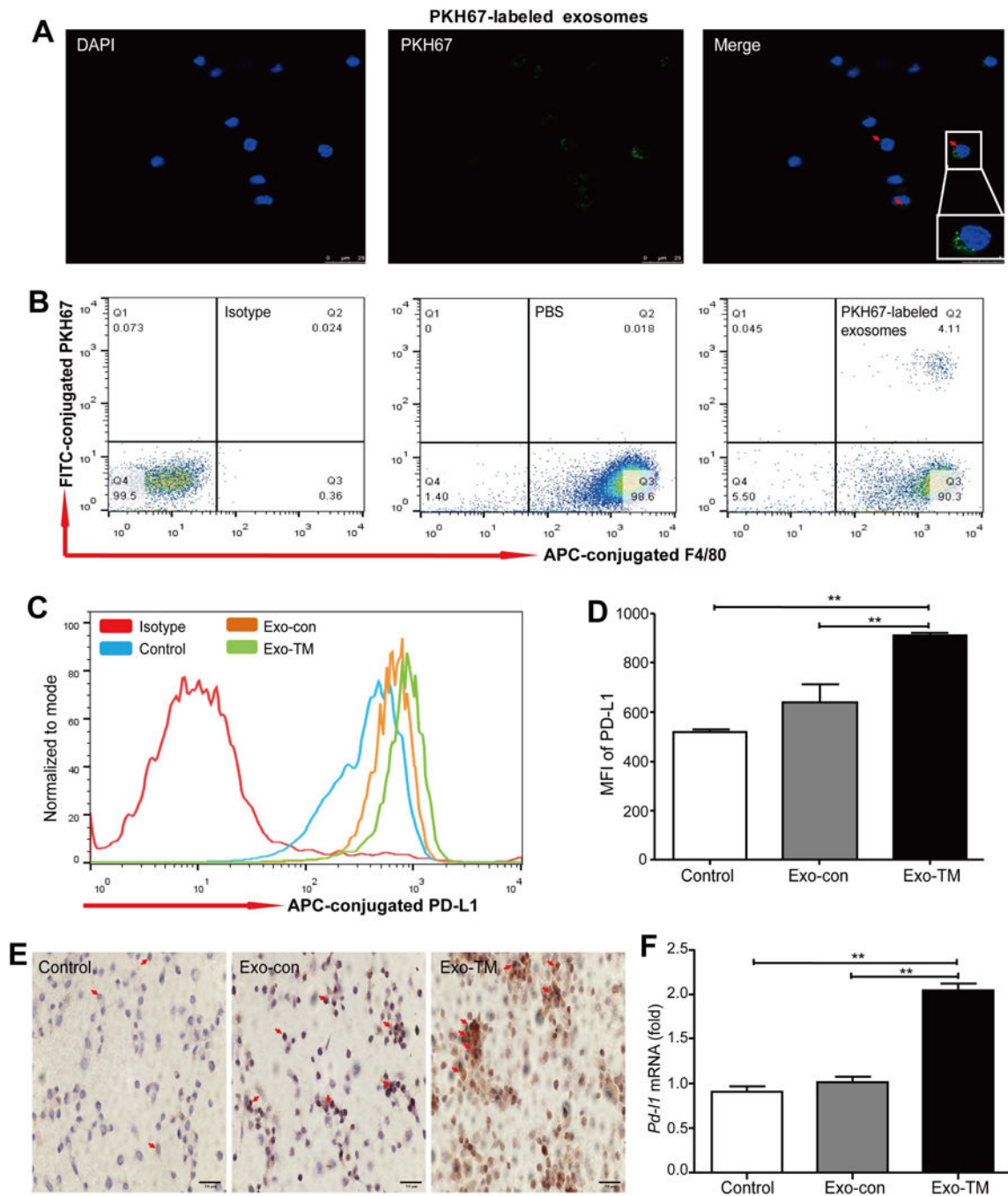


FIG. 5. Exo-TM up-regulates the expression of PD-L1 *in vivo*.

PKH67-labeled exosomes were injected to nude mice intravenously through the tail vein, and the incorporation of PKH67-labeled exosomes by peritoneal macrophages was detected by (A) confocal microscopy (scale bar = 25 μ m) and (B) flow cytometric analysis, peritoneal macrophages were isolated from mice treated with PBS, Exo-con, and Exo-TM, and the expression of PD-L1 was evaluated by (C,D) flow cytometric analysis, (E) immunohistochemical assay, and (F) qPCR. Data are shown as the means \pm SD (error bar) of at least three independent experiments. * P < 0.05, ** P < 0.01 with indicated groups.

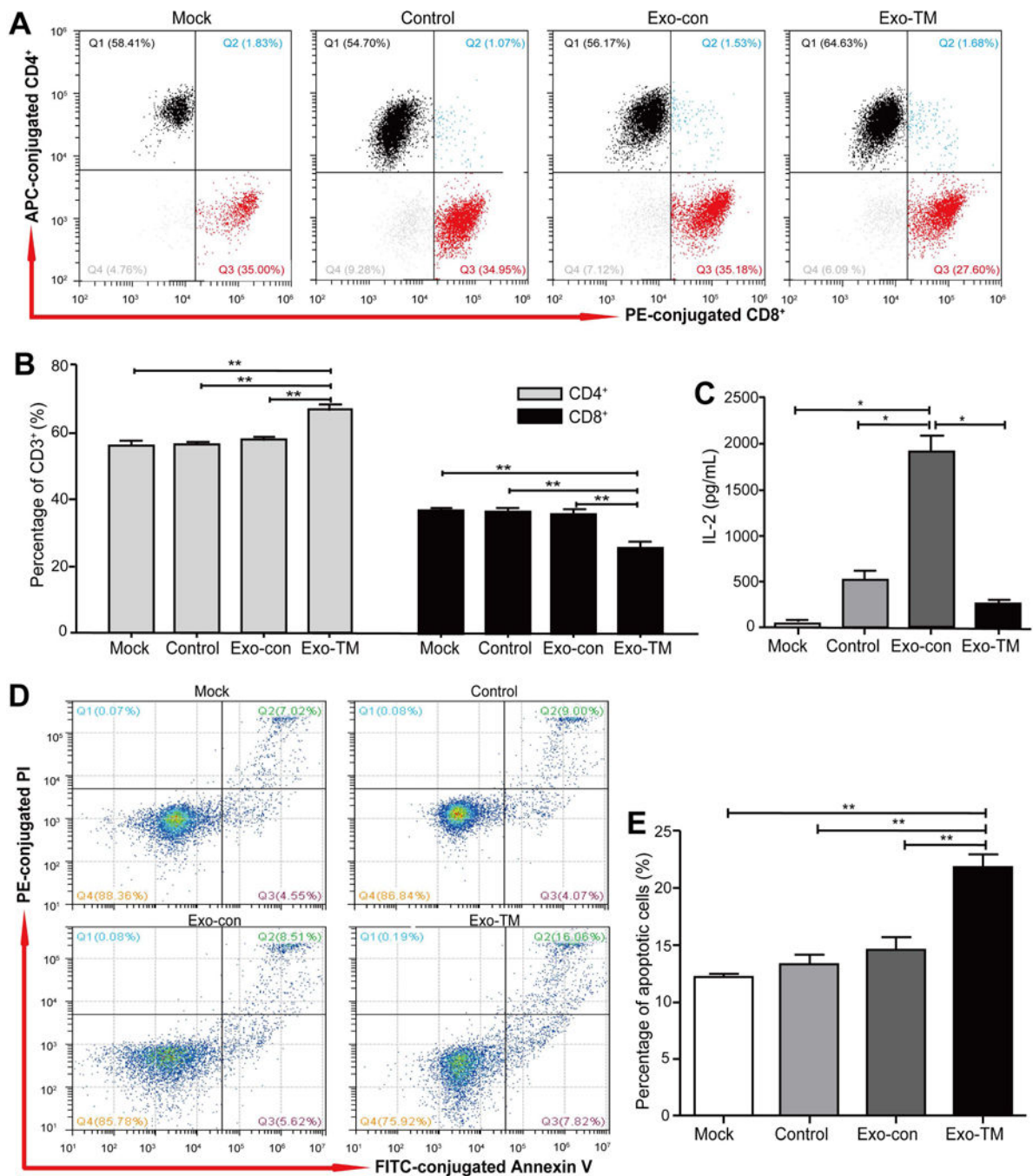


FIG. 6. Exo-TM-treated macrophages inhibit T-cell function and induce T-cell apoptosis. Macrophages were cocultured with Exo-con and Exo-TM for 48 hours, then washed with exosome-free medium, followed by coculturing with CD3⁺ T cells at a ratio of 1:20 for another 48 hours. (A) CD4⁺ and CD8⁺ T cells were measured by flow cytometry and statistically analyzed by (B) SPSS 16.0. (C) The level of IL-2 secreted by T cells was determined by a Cytometric Bead Array (CBA) inflammatory factor kit. (D) Annexin V /PI apoptosis detection kit was used to detect the ratio of apoptotic T cells and statistically analyzed by (E) SPSS 16.0. Data are shown as means \pm SD (error bar) of at least three

independent experiments. * $P < 0.05$, ** $P < 0.01$ with indicated groups. Mock= T cells; Con= macrophages + T cells; Exo-con= Exo-con-treated macrophages + T cells; Exo-TM= Exo-TM-treated macrophages + T cells.

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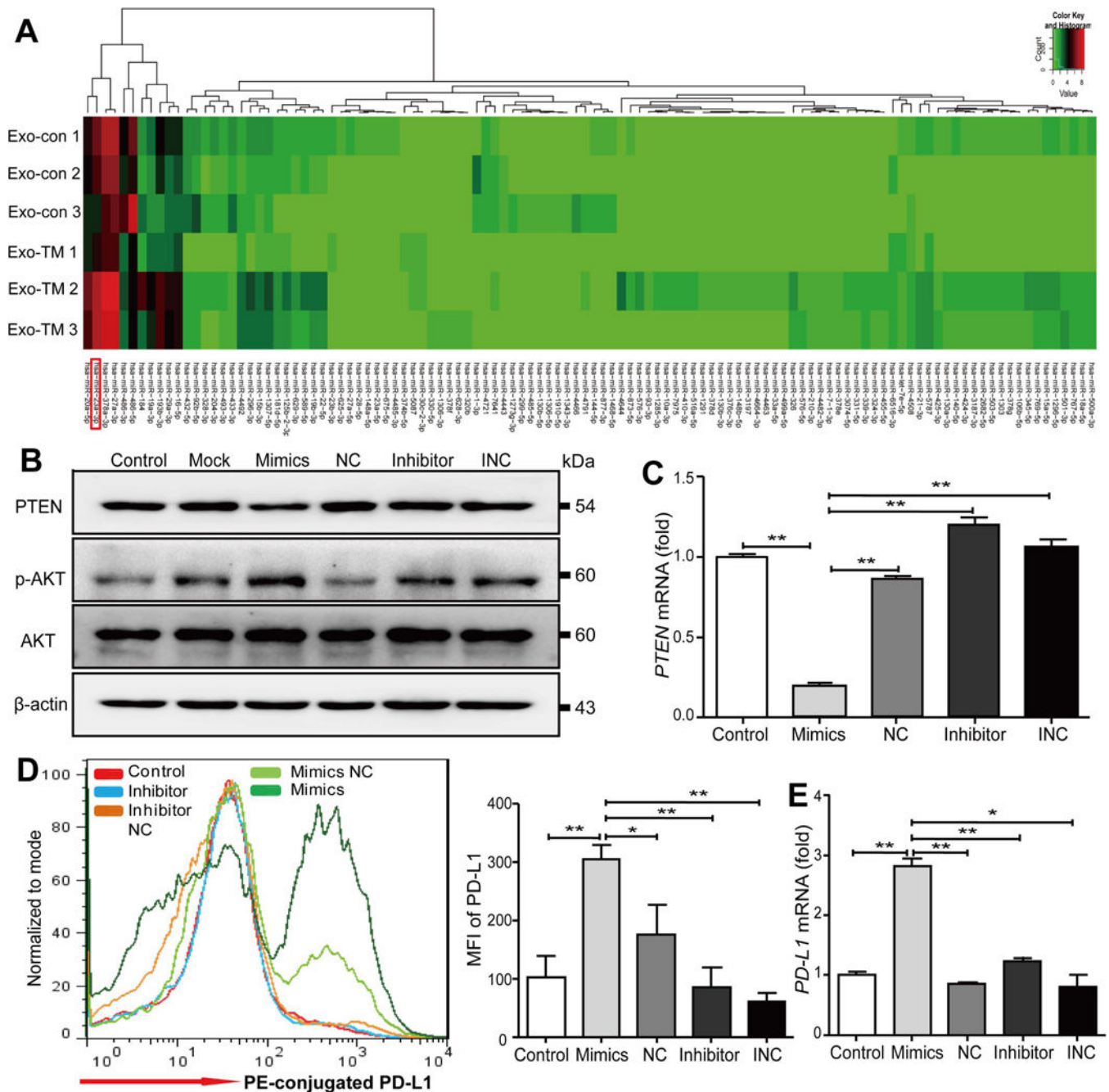


FIG. 7. miR-23a-3p up-regulates PD-L1 expression in macrophages.

(A) The heat map of miRNAs expressed in Exo-con and Exo-TM. (B-D) Macrophages were transfected with miR-23a-3p mimics, inhibitors, and their corresponding controls (NC, INC) for 48 hours. (B) Western blot and (C) qPCR assay were used to analyze the protein and mRNA levels of PTEN, respectively. The expression of PD-L1 on macrophages following miR-23a-3p transfection was analyzed by (D) flow cytometry and (E) qPCR. Data are shown as the means \pm SD of at least three independent experiments. * P < 0.05, ** P < 0.01 with indicated groups. NC=Negative control; INC= Inhibitor negative control.

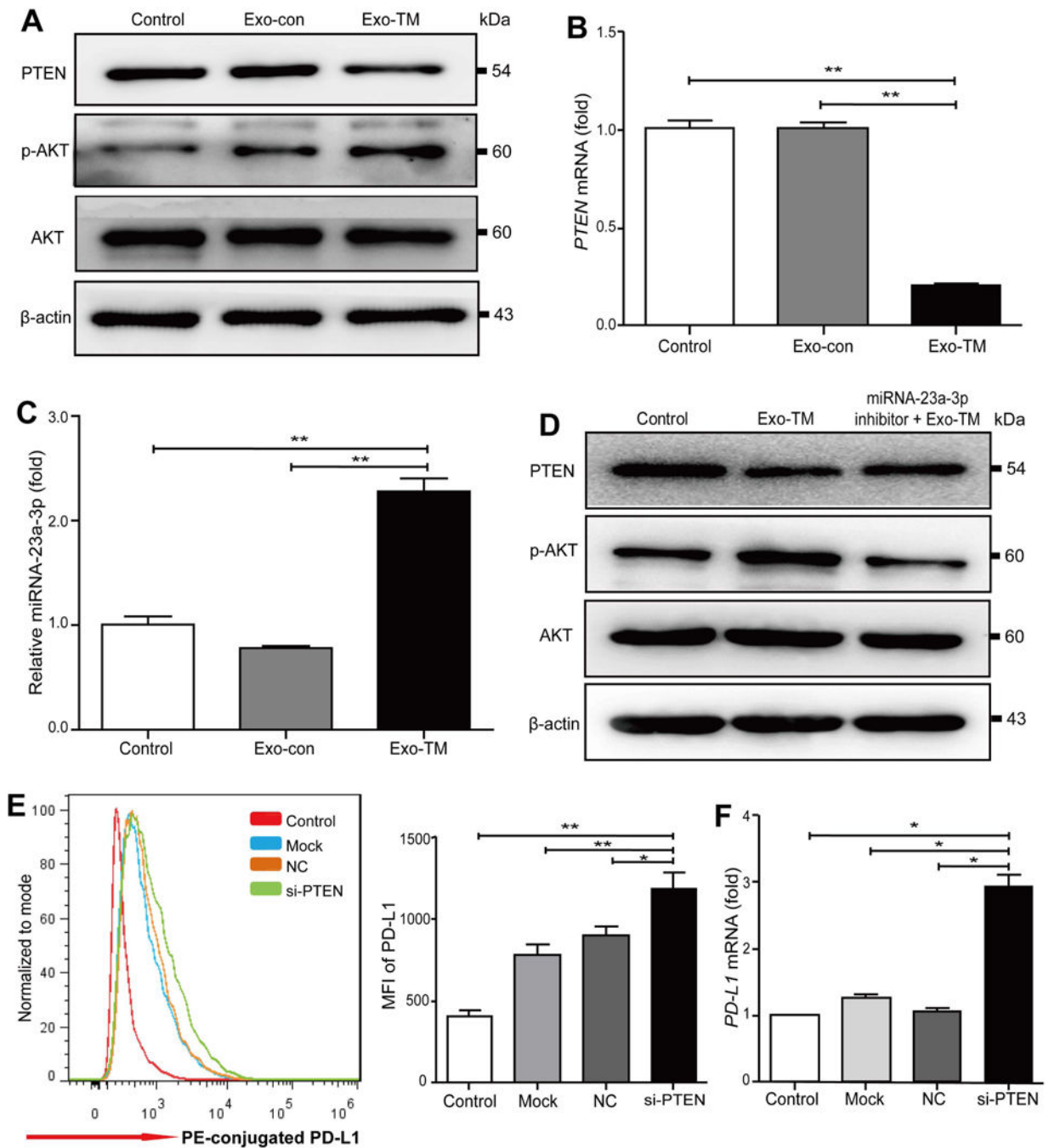


FIG. 8. Exo-TM up-regulates expression of PD-L1 in macrophages through the inhibition of PTEN and subsequent activation of AKT pathway.

mTHP-1 cells were incubated with Exo-TM or Exo-Con, and the expression of PTEN and AKT proteins (A), *PTEN* mRNA (B), miR-23a-3p (C) levels in mTHP-1 were determined. (D) Transfection of mTHP-1 cells with miR-23a-3p inhibitor significantly reversed Exo-TM-mediated down-regulation of PTEN and up-regulation of p-AKT proteins. (E,F) PTEN in mTHP cells was knocked down by transfection of *PTEN* siRNA (si-PTEN). PD-L1 protein and mRNA expression on mTHP-1 cells were measured by flow cytometry and qPCR, respectively. Data are presented as the means \pm SD of at least three independent

experiments. * $P < 0.05$, ** $P < 0.01$ as indicated. Abbreviation: siRNA, small interfering RNA.

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