

# Tumor-associated macrophages mediate immune suppression by secreting PD-L1+ exosomes in epithelial ovarian cancer

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## Research article

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## Abstract

Purpose: To study the role of programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) signaling pathway in patients with epithelial ovarian cancer (EOC). Methods: A total of 10 EOC specimens and 10 benign ovarian tumor were obtained from surgery and the pathological type. We used the methods of immunofluorescence confocal microscopy, western blot, MTT assay, apoptosis detection and co-culture to verify the aim of the research. Results: In the present study, it was validated that the number of PD-L1+ tumor-associated macrophages (TAMs) per field was significantly increased in EOC tissues compared with benign ovarian tumor tissues. Furthermore, it was demonstrated that PD-L1 was expressed on the membrane of TAM-derived exosomes, which may inhibit the proliferation and induce the apoptosis of T cells by activating the caspase 3 signaling pathway. The analysis of the supernatant of T cells co-cultured with TAM driven exosome revealed that the levels of pro-inflammatory cytokines and tumor necrosis factor  $\alpha$  decreased compared with those T cells co-cultured with monocyte driven exosome. However, the expression of the immuno-suppressive cytokine, interleukin 10 and markers of T cell exhaustion (the inhibitory molecule lymphocyte activated gene-3, T-cell immunoglobulin and mucin domain-containing protein-3 and PD-1) increased. Conclusions: The present study demonstrated that the M2-derived exosomes regulate immune suppression in the EOC microenvironment. The findings of the present study provide a theoretical basis for future target therapy on exosomes from immune cells to treat EOC.

## Background

Epithelial ovarian cancer (EOC) is the most common cause of gynecologic cancer-associated mortality with >2,000,000 novel cases per year worldwide (1). Debulking surgery and platinum-based chemotherapy are the principal treatments(2). However, the initial response rate varies between 40 and 80%. Furthermore, the majority of patients who initially respond to chemotherapy eventually develop chemo-resistance (2). Therapeutic strategies involving immune modulators have attracted increasing attention due to promising results from clinical trials (3,4). Many studies focus on the therapeutic blockade of inhibitory signals primarily through programmed death-1 (PD-1) and its ligand programmed death-ligand 1 (PD-L1/B7-H1).

PD-1 is expressed by activated T cells and triggers an inhibitory signal via the Src homology 2 domain-containing phosphatase-1 (5,6). PD-1 has two known ligands, PD-L1 (7,8) and PD-L2 (9,10). A number of studies have demonstrated that PD-L1 is selectively expressed on a number of tumor cells and also other cells within the tumor microenvironment in response to inflammatory stimuli (11,12). In addition, PD-L1 is upregulated in solid tumors, therefore it is able to inhibit cytokine production and the cytolytic activity of PD-1<sup>+</sup>(CD4<sup>+</sup> and CD8<sup>+</sup>) T cells in the tumor microenvironment (11,13,14). In 2012, in a study involving an antibody that specifically binds to PD-L1, Brahmer *et al* (9) demonstrated that 12-41% of 207 patients with advanced cancer and who received antibody treatment resulted in a prolonged stabilization of disease in non-small-cell lung cancer (NSCLC), melanoma and renal-cell cancer. However, the efficacy of the antibody was not sufficient for ovarian cancer (9).

An interesting question is the difference between the efficacy of the antibody between EOC and other tumors. A previous study supported the existence of metastasis-supportive microenvironments, termed pre-metastatic niches, which are potential metastatic sites that allow the colonization and growth of disseminated tumor cells (15). Furthermore, our previous study revealed that the peritoneum of patients with EOC contained a large number of immune cells, 70% of which were macrophages (16). These macrophages, termed tumor associated-macrophages (TAMs), serve an important function in the regulation of T cell differentiation and function in the tumor microenvironment by delivering microRNAs (miRNAs) through exosomes (unpublished data).

Exosomes are vesicles of endocytic origin, which range between 30 and 100 nm in size (17-19), and may mediate the intercellular communication by horizontal transfer of information via their cargo. The cargo includes proteins on the membrane, DNA, mRNA and miRNAs (20-23). Exosomes may arise from numerous cell types, but the biological functions of exosomes remain unknown, as they serve complex and diverse functions in immunobiology. Previous studies demonstrated that exosomes secreted by dendritic cells may stimulate the immune system by antigen presentation, while the tumor-associated exosomes may either promote or inhibit tumor immunity, depending on the pathophysiological context (24-26). Herbst *et al* (12) indicated that PD-L1-positive tumor-infiltrating immune cells (macrophages, dendritic cells and T cells) were more common compared with PD-L1-positive tumor cells. Since the exosomes secreted by TAMs exhibit specific markers from the TAMs, it was hypothesized in the present study that the PD-1<sup>+</sup> TAM-exosomes may regulate T cell dysfunction and apoptosis in the tumor microenvironment and participate in the formation of pre-metastatic niches, therefore contributing to immune evasion and metastasis of EOC cells. In the present study, it was demonstrated that exosomes that were released from TAMs express increased levels of PD-L1 which inhibit the proliferation and induce the apoptosis of T cells by activating the caspase 3 signaling pathway, thus regulating T cell dysfunction in the EOC microenvironment.

## Methods

*Preparation of clinical tissues.* A total of 10 EOC specimens (mean age:  $47.8 \pm 4.0$  (25-62) years old) and 10 benign ovarian tumor (mean age:  $49.8 \pm 5.4$  (25-66) years old) specimens were obtained from the Shanghai First Maternity and Infant Hospital, Tongji University (Shanghai, China) from January 2012 to December 2014. The benign/tumor specimens were obtained from surgery and the pathological type was confirmed by two pathologists. Inclusion criteria: Epithelial ovarian cancer, none of the patients with cancer received any chemotherapy prior to the present study. Exclusion criteria: those who have received any chemotherapy prior to the present study or don't want to participate in this study. The objectives and implications of the results were explained, and institutionally approved written informed consent was obtained from each participant. The study protocol was approved by the Institutional Review Board of Shanghai First Maternity and Infant Hospital (Shanghai, China).

*Immunofluorescence confocal microscopy.* The detection of PD-L1<sup>+</sup> TAM cells (PD-L1<sup>+</sup>CD68<sup>+</sup>) in benign ovarian tumor and EOC tissues was performed using anti-PD-L1 (ab210931, Abcam, Cambridge, Cambridge, UK5ug/ml) and anti-CD68 (MAB20401, R&D Systems, Minneapolis, MN, USA, 25ug/ml). Alexa Fluor 488-conjugated goat anti-mouse antibody (104546, Jackson, Lancaster, PA, USA, 1:200) was used for the detection of PD-L1 and Cy3-conjugated goat anti-rat antibody (99002, Jackson, 1:200) was used for the detection of CD68 . cell nuclei were counterstained with DAPI (D9542, Sigma, St Louis, MO, USA). The images were captured using a laser scanning confocal microscope (Zeiss LSM 510; Zeiss GmbH, Jena, Germany). Quantitative analysis was performed on five random fields/tumor samples by counting the number of cells (magnification, x400).

*M2 macrophage cell model and exosome isolation.* The M2 macrophages were induced from the Thp-1 cell line (Cell bank of Chinese Academy of Sciences, Shanghai, China), using PMA (P1585-1MG, Sigma-Aldrich, St Louis, MO, USA, 50 ng/ml) and interleukin (IL)-4 (204-IL-010, R&D, Minneapolis, USA, 20 ng/ml) for 24 h with a density of 10<sup>6</sup>/plate in RPMI1640 (Invitrogen, CA, USA)+10%FBS (Invitrogen, CA, USA), 37°C. In order to isolate the block mass of exosomes(exo-macs), Thp-1 cells (M0)/M2 macrophages were cultured in RPMI-1640 for 24 h at 37°C. The Thp-1 derived M2 cells were pre-stimulated into M2 macrophages using PMA and IL-4 as aforementioned for 24 h at 37°C, and subsequently the culture medium was changed to RPMI-1640 at 37°C. In order to isolate exosomes, the supernatants were centrifuged twice at 1,000 x g for 10 min and at 3,000 x g for 30 min to deplete the cell or fragments under 4 °C. Subsequently, the Total Exosome Isolation kit (Life technology) was added overnight under 4 °C. Subsequently, the exosomes were centrifuged (10,000 x g for 1 h) under 4 °C, re-suspended in PBS and stored at -80°C. The size and purity of the exosomes were validated using an Transmission electron microscope. The concentration of exosomes was determined using theBCA Protein Assay Kit (Pierce Biotechnology, USA).

*Detection of PD-L1 on M0/M2 exosomes.* The exosomes released from the M0/M2 macrophages were isolated and lysed using the Total Protein Lysis buffer (as manufacturer's instruction). The detection of PD-L1 was performed using an anti-PD-L1 antibody (ab58810, Abcam, Cambridge, UK) by western blot analysis. CD63 was used as a control reference.

*Proliferation of Jurkat T cells.* The proliferation of Jurkat T cells was observed using an MTT assay. The MTT reagent and Jurkat T cells were donated by the Central Lab of Shanghai First Maternity and Infant Hospital, Tongji University (Shanghai, China). In brief, the Jurkat T cells were cultured in 96 well-plates (3,000 cells/well) with 200 µl RPMI-1650, supplemented with 2% FBS at Invitrogen, CA, USA, for 24 h. Subsequently, M0/M2-exosomes (50 ng/ml) were added and co-cultured for 1,2 and 3 days. The control group was treated with PBS alone. Subsequently, 20 µl MTS Solution Reagent (Promega Biosciences, CA,

USA) to each well and incubated for 2 hours at 37°C, 5% CO<sub>2</sub> atmosphere. The absorbance was recorded at 490 nm using a 96-well plate reader, and calculated using the standard curve performed on the first day.

*Detection of apoptosis of Jurkat T cells following treatment with M0/M2-exosomes.* Jurkat T cells were cultured in 6-well plates (1x10<sup>6</sup> cells/well) with RPMI-1640, supplemented with 10% FBS at 37°C, 5% CO<sub>2</sub> atmosphere. Subsequently, M0/M2-exosomes (50 ng/ml) were added and cultured for 3 days. The cells were collected and stained using the annexin V Apoptosis detection kit fluorescein isothiocyanate (eBioscience; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Flow cytometric analysis was performed using a fluorescence activated cell sorter (FACS) Calibur cytometer and data was analyzed with CellQuest software.

*Detection of IL-2, IL-4, IL-6, IL-8, IL-10 and tumor necrosis factor (TNF)-α in the supernatants of Jurkat T cells.* The supernatants were selected following co-culture of T cells and exosomes for 3 days at 37°C, 5% CO<sub>2</sub> atmosphere. The cytokines were determined using ProcartaPlex® Multiplex Immunoassays (eBioscience; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol.

*Detection of the PD-1, lymphocyte activated gene (LAG)-3 and T-cell immunoglobulin and mucin domain-containing protein (TIM)-3 in Jurkat T cells following treatment with M0/M2-exosomes.* Jurkat T cells were cultured in 6-well plates (1x10<sup>6</sup> cells/well) RPMI-1640 supplemented with 10% FBS. M0/M2-exosome (50 ng/ml) was added and cultured for 3 days. Subsequently, the cells were collected and incubated with PE conjugated anti-PD-1(12-2799-41, eBioscience, Thermo Fisher Scientific, Inc.), APC conjugated anti-LAG-3 (17-2239-41, eBioscience, Thermo Fisher Scientific, Inc.) and FITC conjugated anti-TIM-3 antibodies (11-3109-41, eBioscience; Thermo Fisher Scientific, Inc.) for 30 min at 4°C, protected from light. Flow cytometric analysis was performed using a FACS Calibur cytometer, and data was analyzed with CellQuest software.

*Assessment of mRNA expression.* Following the treatment of Jurkat T cells with M0 or M2 exosomes for 3 days, the cells were collected. The total RNA in T cells was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the miScript II RT kit (catalog no. 218161; Qiagen, Inc., Valencia, CA, USA). The templates were subjected to detection by polymerase chain reaction (PCR) using the miScript SYBR Green PCR kit (catalog no. 218073; Qiagen, Inc.). For q-PCR analysis, 500 ng of total RNA was reversed transcribed to cDNA, and amplified by PCR cycling conditions: 5 s at 95 °C and 30 s at 60 °C for 40 cycles. Differences in gene expression were determined by the 2ΔΔCT method ( $\beta$ -

actin was used for calibration). The following primer oligonucleotide sequences were used: LAG-3 forward, 5'-GCAGGGACTTCTCGCTATG-3' and reverse, 5'-GGCTCTGAGAGATCCTGGGG-3'; TIM-3 forward, 5'-CTGCTGCTACTACTTACAAGGTC-3' and reverse, 5'-GCAGGGCAGATAGGCATTCT-3'; PD-1 forward, 5'-CCAGGATGGTTCTTAGACTCCC-3' and reverse, 5'-TTTAGCACGAAGCTCTCCGAT-3'.

*Detection of active caspase 3 and total caspase 3 in co-cultured T cells.* Following 3 days of co-cultured with M0/M2-exosome, the Jurkat T cells were collected and lysed using the Total Protein Lysis buffer (C500001-0010, Sangon Biotech, Shanghai) and concentration of protein was quantified using a BCA Protein Assay Kit (Pierce Biotechnology, USA). 20ug/lane protein was loaded in a 10% gel and then transferred with PVDF membrane. The membrane was blocked with BSA (HZB0148, sigma, USA) under room temperature for 1 hour. The primary antibody was incubated under 4°C overnight. The detection of the levels of active caspase 3 and the total levels of caspase 3 was performed using the rabbit anti human-anti-active-caspase 3 antibody(ab2302, abcam, 1ug/ml) and rabbit anti human-anti-caspase3 antibody (ab32351, Abcam, 1:5000) The second antibody was using the goat anti-rabbit IgG(HRP) antibody(ab6721, abcam) and incubated under room temperature for 1 hour. The valuation of the protein concentration was using the Pierce™ ECL Western Blotting Substrate (32106, Thermo Scientific™, USA).

*Statistical analysis.* Statistical analyses were performed using the SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). The data are expressed as the mean ± standard deviation. The Mann-Whitney test, univariate analysis of variance with post-hoc LSD test and Kruskal-Wallis test were used to determine the P-values. The continuous variables in the figures are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Number of PD-L1<sup>+</sup> TAMs is significantly increased in EOC tissues.* The frequency of PD-L1<sup>+</sup> TAM (PD-L1<sup>+</sup>CD68<sup>+</sup>) cells in 10 EOC specimens and 10 benign ovarian tumor specimens were evaluated using immunofluorescence confocal microscopy (Fig. 1A). The number of PD-L1<sup>+</sup> TAMs per field was significantly increased in EOC tissues compared with benign ovarian tumor tissues (17.80±1.8 vs. 4.60±0.5, Mann-Whitney test, P=0.01) (Fig. 1B). This result was consistent with Herbst *et al* (12).

*PD-L1 expression is significantly increased in M2-exosomes.* The expression of PD-L1 was increased in the exosomes that were released by M2 macrophages. A previous study by the authors revealed that the exosomes released by M2 macrophages might regulate the differentiation of T cells via miRNAs (data in submission). Since exosomes carry specific proteins from the host cell, exosomes from M2 macrophages were purified and examined using an electron microscope (Fig. 2A). Subsequently, PD-L1 expression in

the exosomes was determined. The expression of PD-L1 was significantly increased in the M2-exosome group compared with the control group (Mann-Whitney test, P=0.002) (Fig. 2B and C).

*Exosomes released from M2 macrophage are able to reduce the proliferation of Jurkat T cells.* The exosomes from M2 macrophages were collected and co-cultured with Jurkat T cells. Following co-culture with M2 exosomes for 2 days, the proliferation of Jurkat T cells was significantly decreased compared with the M0-exo group and the control group (M2: 5348.70±41.13 cells/well vs. control: 7112.26±441.09 cells/well; M0: 6146.35±237.27 cells/well; M2 vs control: P=0.005, M2 vs M0: P=0.1. ANOVA). A similar result was observed after 3 days of co-culture (control, 10718.54±346.1 cells/well; M0, 10079.23±137.32 cells/well; M2, 7245.52±80.32 cells/well; M2 vs control: P<0.001, M2 vs M0: P<0.001. ANOVA) (Fig. 3).

*Exosome released from M2 macrophages regulate the apoptosis of Jurkat T cells.* Mediation of T cell apoptosis by PD-L1 is a novel mechanism for tumor immune escape (4,5). In the present study, the apoptosis of Jurkat T cells was determined following co-culture with M2-exosome/M0 (Thp-1)-exosome for 3 days. The exosome released from M2 macrophages (early apoptosis, 36.43±1.44%; late apoptosis, 22.13±0.49%;) significantly induced the apoptosis of Jurkat T cells compared with the M0-exosome (early apoptosis, 9.52±4.03%; late apoptosis, 6.82±2.74%) and the control group (early apoptosis, 13.06±2.69%; late apoptosis, 4.62±0.15%) (early apoptosis, LSD, M2 vs control: P=0.001; M2 vs M0: P=0.001; late apoptosis, Tamhane: M2 vs control: P=0.001; M2 vs M0: P=0.08) (Fig. 4A-C)

*Exosomes released from M2 macrophage upregulate the secretion of IL-10 from Jurkat T cells.* The aim of the next part of the study was to determine if the M2-exosome affect the function of Jurkat T cells. The ProcartaPlex® Multiplex Immunoassays were used to determine the release of IL-2, IL-4, IL-6, IL-8, IL-10 and TNF- $\alpha$  from the supernatants of Jurkat T cells following co-culture with M2-exosome/M0-exosome for 3 days. The pro-inflammatory cytokines, IL-2, IL-8, IL-4, IL-6 and TNF- $\alpha$ , were significantly decreased in the M2-exo group (IL-2: 2.16±2.16 pg/ml; IL-8: 0 pg/ml; IL-4: 0 pg/ml; IL-6: 0 pg/ml; TNF- $\alpha$ : 0 pg/ml) compared with the M0-exo group (IL-2: 16.48±0.56 pg/ml; IL-8: 1258.36±24.59 pg/ml; IL-4: 16.52±0.31 pg/ml; IL-6: 25.69±0.62 pg/ml; TNF- $\alpha$ : 18.58±0.94 pg/ml) and the control group (IL-2: 9.35±0.50 pg/ml; IL-8: 588.4±2.70 pg/ml; IL-4: 34.13±1.13 pg/ml; IL-6: 0 pg/ml; TNF- $\alpha$ : 10.71±0.51 pg/ml) (IL-2: M2 vs M0: P=0.05, M2 vs control: P=0.046, Mann-Whitney; IL-8: M2 vs M0: P=0.046, M2 vs control: P=0.034, Mann-Whitney; IL-4: M2 vs M0: P=0.05, M2 vs control: P=0.037, Mann-Whitney; IL-6: M2 vs M0: P=0.037, M2 vs control: P=1, Mann-Whitney; TNF- $\alpha$ : M2 vs M0: P<0.001, M2 vs control: P<0.001, ANOVA,) (Fig. 5). Whereas, IL-10 was significantly increased in M2-exo group (5.57±0.38 pg/ml;) compared with the other two groups (M0: 3.04±0.07 pg/ml; control: 0 pg/ml, M2 vs control: P=0.037, M2 vs M0: P=0.037, Mann-Whitney) (Fig. 5E). IL-8, TNF- $\alpha$ , IL-4 and IL-6 were undetected in the M2-exo group.

*Markers of Jurkat T cell exhaustion are upregulated by exosomes released from M2 macrophages.* The exosomes from M2 macrophages are able to induce apoptosis and upregulate the secretion of the anti-inflammatory cytokine, IL-10. Therefore, in the present study, the markers of T cell exhaustion, LAG-3, TIM-3 and PD-1, were determined in Jurkat T cells. The protein levels of all three markers were upregulated in the M2-exo group compared with the control (LAG-3:  $38.63\pm 5.32\%$  vs.  $2.73\pm 0.45\%$ , ANOVA,  $P=0.003$ ; TIM-3:  $7.41\pm 0.58\%$  vs.  $1.32\pm 0.09\%$ , ANOVA,  $P<0.001$ ; PD-1:  $21.25\pm 3.73\%$  vs.  $2.80\pm 0.14\%$ , ANOVA,  $P=0.008$ ) (Fig. 6A). Furthermore, the levels of LAG-3 ( $255.08\pm 94.22$  vs.  $7.45\pm 3.23$ ; Mann-Whitney,  $P>0.05$ ), TIM-3 ( $104.44\pm 4.47$  vs.  $2.39\pm 1.50$ ; ANOVA,  $P<0.001$ ) and PD-1 mRNA ( $19.45\pm 2.91$  vs.  $1.67\pm 0.56$ ; ANOVA,  $P=0.004$ ) were upregulated in the M2-exo group compared with the control (Fig. 6B). It was also validated that M2 macrophages were able to induce apoptosis and exhaustion of Jurkat T cells by activating caspase 3 ( $P=0.011$ , ANOVA) (Fig. 6C and D)

## Discussion

The PD-1/PD-L1 pathway is an important immune inhibitory signaling pathway in the tumor microenvironment (6,27). An increased PD-L1 expression in the tumor microenvironment is associated with poor prognosis in patients with a number of types of cancer, including breast, ovarian, pancreatic, gastric, kidney and bladder cancer (28-33).

Multiple monoclonal antibodies to PD-L1 are under development in clinical trials. It has been demonstrated that BMS-936559 (anti-PD-L1) exhibited therapeutic efficacy in a phase I clinical trial (ClinicalTrials.gov identifier, NCT00729664) (9). In addition, a study of metastatic urothelial bladder cancer identified MPDL3280A (an engineered anti-PD-L1 Immunoglobulin G1 monoclonal antibody) to have marker activity in controlling tumor growth; the objective response rates (ORRs) were 43% for PD-L1<sup>+</sup> tumor and 11% for PD-L1<sup>-</sup> tumors. (ClinicalTrials.gov identifier, NCT01375842) (34). Furthermore, in a phase I trial, patients with different types of cancer (melanoma, NSCLC and renal cell carcinoma) responded to MPDL3280A with an ORR of 23% total patients with NSCLC and an ORR of 85% in patients with high PD-L1 expression (NCT01375842) (12). However, ovarian cancer has not been demonstrated to respond to MPDL3280A (9). Therefore, in the present study, it was hypothesized that other types of cells, in addition to tumor cells, may express PD-L1 and contribute to tumor proliferation and metastasis. According to a previous study by the authors, the peritoneum of patients with EOC contained a high number of immune cells, 70% of which were TAM cells. In the present study, it was validated that TAM cells in the EOC tumor microenvironment expressed PD-L1, which was consistent with the results of Herbst *et al* (12), and that PD-L1 may be expressed on the membrane of exosomes (small vesicles ranging between 30 and 100 nm in size) that are released from TAM cells.

Exosomes are one of the major means of communication employed by immune cells. Exosomes contain a combination of ligands and receptors that may concurrently interact with a number of cell-surface receptors, therefore mediating the exchange of membrane and cytosolic components without cell-cell

contact. For example, antigen-presenting cells release exosomes exhibit major histocompatibility complex (MHC) class II/antigen complexes, resulting in the activation of T cells via specific T cell receptor-peptide interactions (35,36). Additionally, mast cell-derived exosomes may stimulate T cells via the MHC class II molecules (37). A previous study in 2012 revealed that the exosomes released from the first trimester and term placenta regulates immune tolerance (6). Furthermore, it was demonstrated that dendritic cells that were exposed to tumor peptides generated exosomes that were able to induce a T-cell-mediated anti-tumor immune response (26). In the present study, it was observed that the PD-L1<sup>+</sup> exosomes released from M2 macrophages, similar to TAM cells in the EOC microenvironment, may inhibit the proliferation of T cells and induce the apoptosis of T cells by activating the caspase 3 signaling pathway. According to Duraiswamy *et al* (7), it was demonstrated that the expansion of ovarian antigen-specific CD8<sup>+</sup> tumor infiltrating lymphocytes was dependent on the amount of PD-L1 signaling by tumor cells, tumor-derived myeloid cells and T regulatory cells. The present results were partly consistent with these findings. Furthermore, the present results offer a better explanation for the low efficacy of molecular targeted therapeutic drugs specific to PD-L1. As exosomes are small and are released in a huge amount, if the PD-L1 on tumor or TAM cells is inhibited, the nomadic and diffused TAM-derived PD-L1<sup>+</sup> exosomes may serve their function in immune suppression. The small size of the exosomes enables a diffuse spread of these vesicles, therefore forming pre-metastatic niches on the peritoneum for EOC tumor cells.

In the present study, it was demonstrated that the M2-derived exosomes were able to regulate the release of cytokines from T cells. The levels of the pro-inflammatory cytokines, IL-2, IL-8, IL4, IL6 and TNF- $\alpha$ , decreased, by contrast, levels of the immuno-suppressive cytokine, IL-10, was increased compared with control and M0 group. These results were consistent with the study by Dong *et al* (38). Furthermore, the markers of T cell exhaustion, PD-1, LAG-3 and TIM-3, were upregulated in T cells. A previous study demonstrated that anti-PD-L1 monoclonal antibody might reverse T cell exhaustion and restore the function of T cells (39). Thus, we can hypothesis that the accumulation of high levels of immune-suppressive cytokines may explain why PD-L1 blockade *in vivo* only exhibited a partial anti-tumor effect.

The results of the present study identified that TAMs mediated the dysfunction of T cells by releasing PD-L1<sup>+</sup> exosomes in the EOC tumor microenvironment, therefore promoting the proliferation of tumor cells and the formation of pre-metastatic niches on the peritoneum. Further investigation on the release of exosomes from immune cells may enable the identification of a novel treatment for EOC.

## Declarations

### Funding

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## **Authors' contributions**

Dr. Zhou X have done the experiments, Dr. Zhu Q and Dr. Liu L helped to collected the clinical tissues, Dr. Zhang Y analysed the data and Pro. Wang designed and wrote the article.

## **Ethics approval and consent to participate**

The study protocol was approved by the Institutional Review Board of Shanghai First Maternity and Infant Hospital (Shanghai, China).

## **Consent for publication**

All authors have reviewed the final version of the manuscript and approve it for submission.

## **Competing interests**

All the authors declare no conflicts of interest.

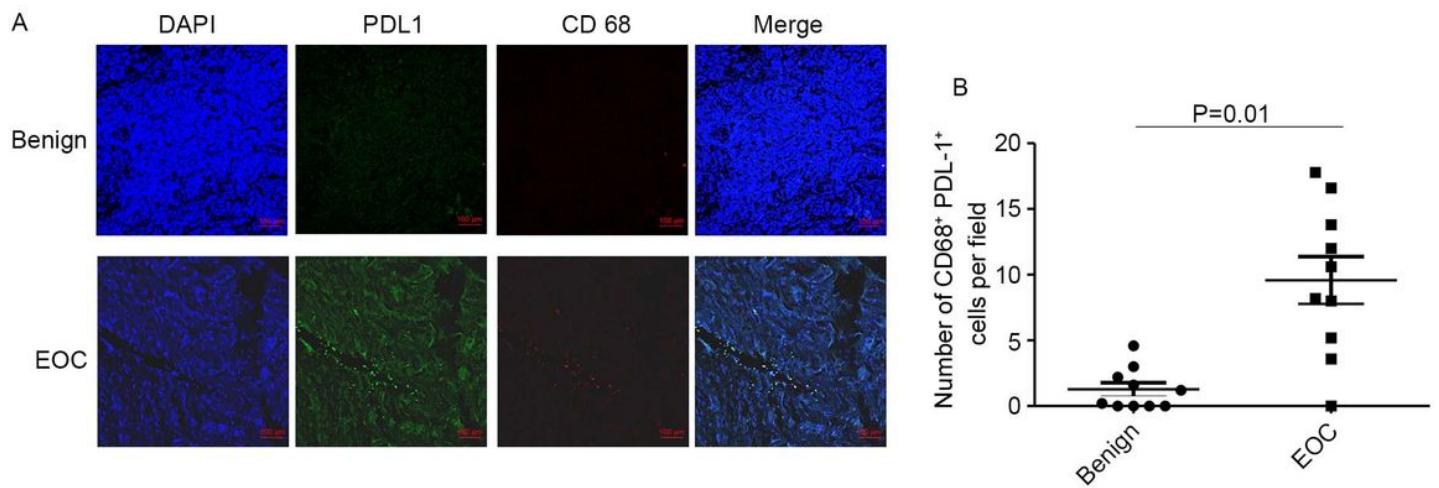
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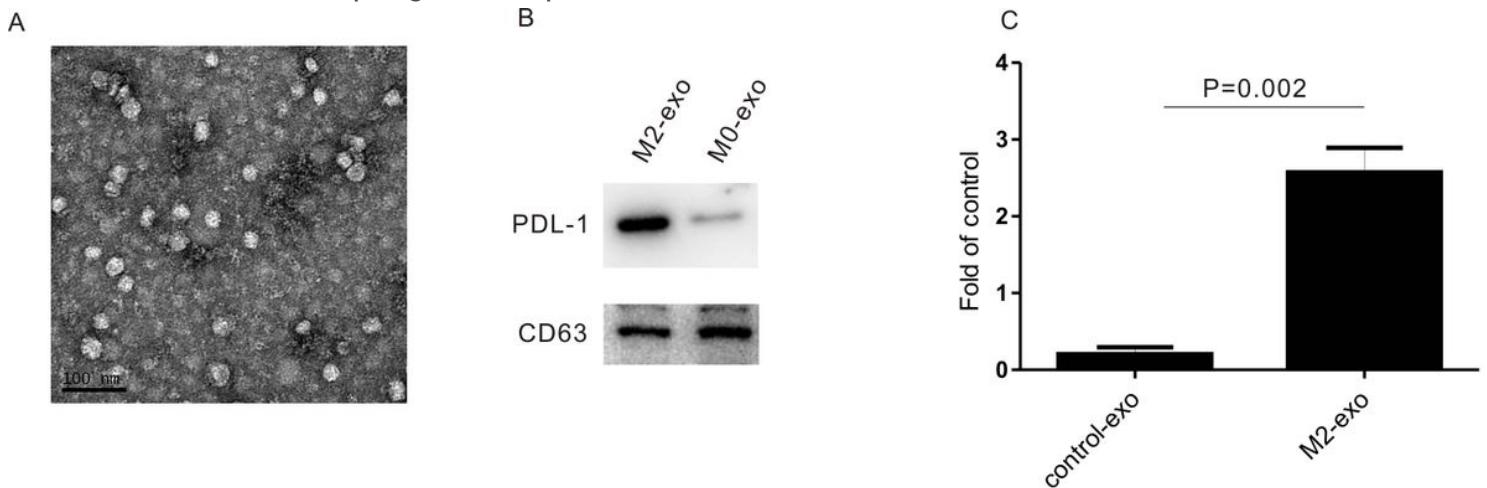
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## Figures



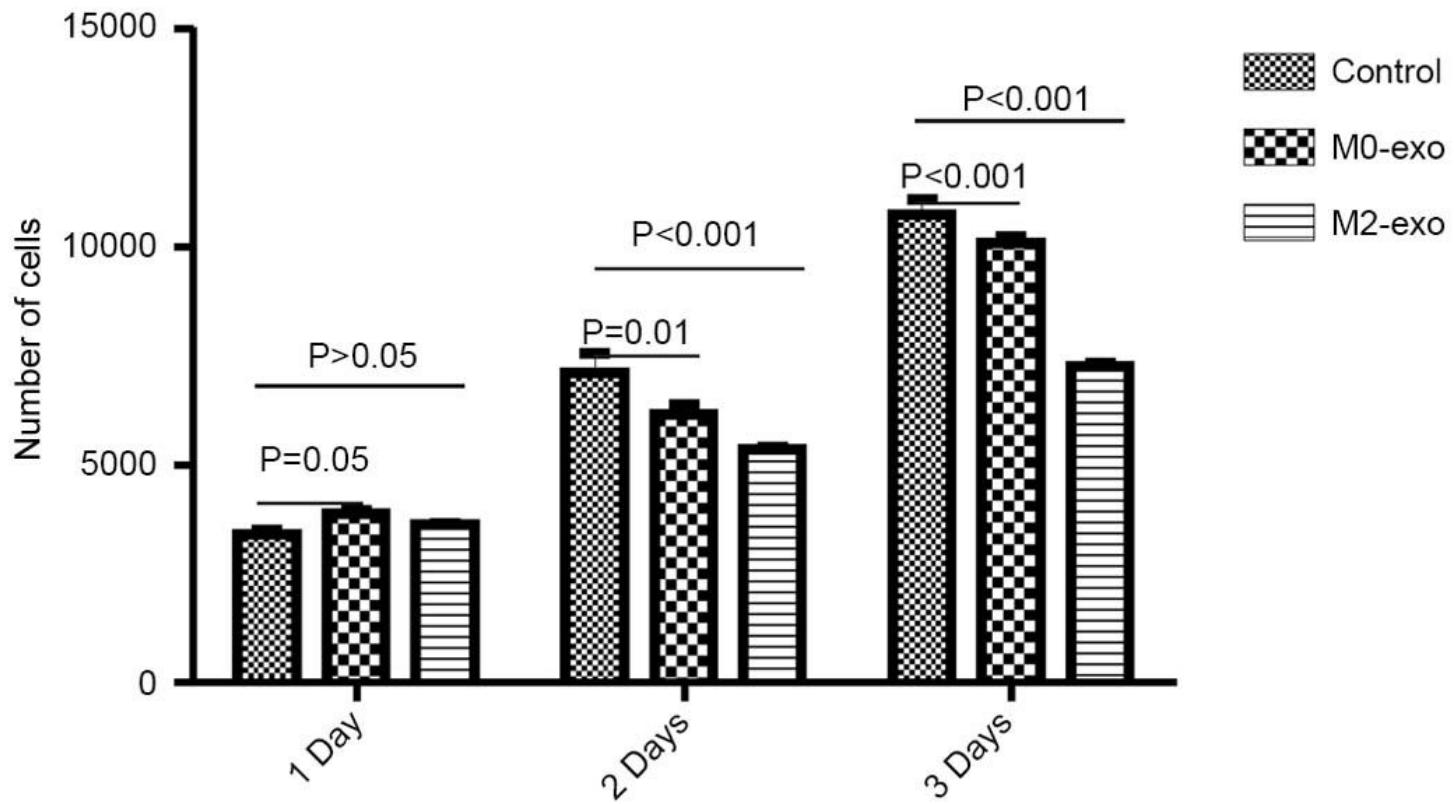
**Figure 1**

. Distribution of PD-L1+ TAM cells in EOC tissues and benign ovarian tumor samples. (A) PD-L1+ TAM cells were stained using DAPI (blue, nuclei), PD-L1 (green), and CD68 (red) in benign ovarian tumor tissues and EOC tissues. Scale bar, 100  $\mu$ m. (B) The number of PD-L1+ TAM cells in EOC tissues was higher compared with the benign ovarian tumor samples. PD-L1, programmed death-ligand 1; TAM, tumor-associated macrophage; EOC, epithelial ovarian cancer; CD, cluster of differentiation.



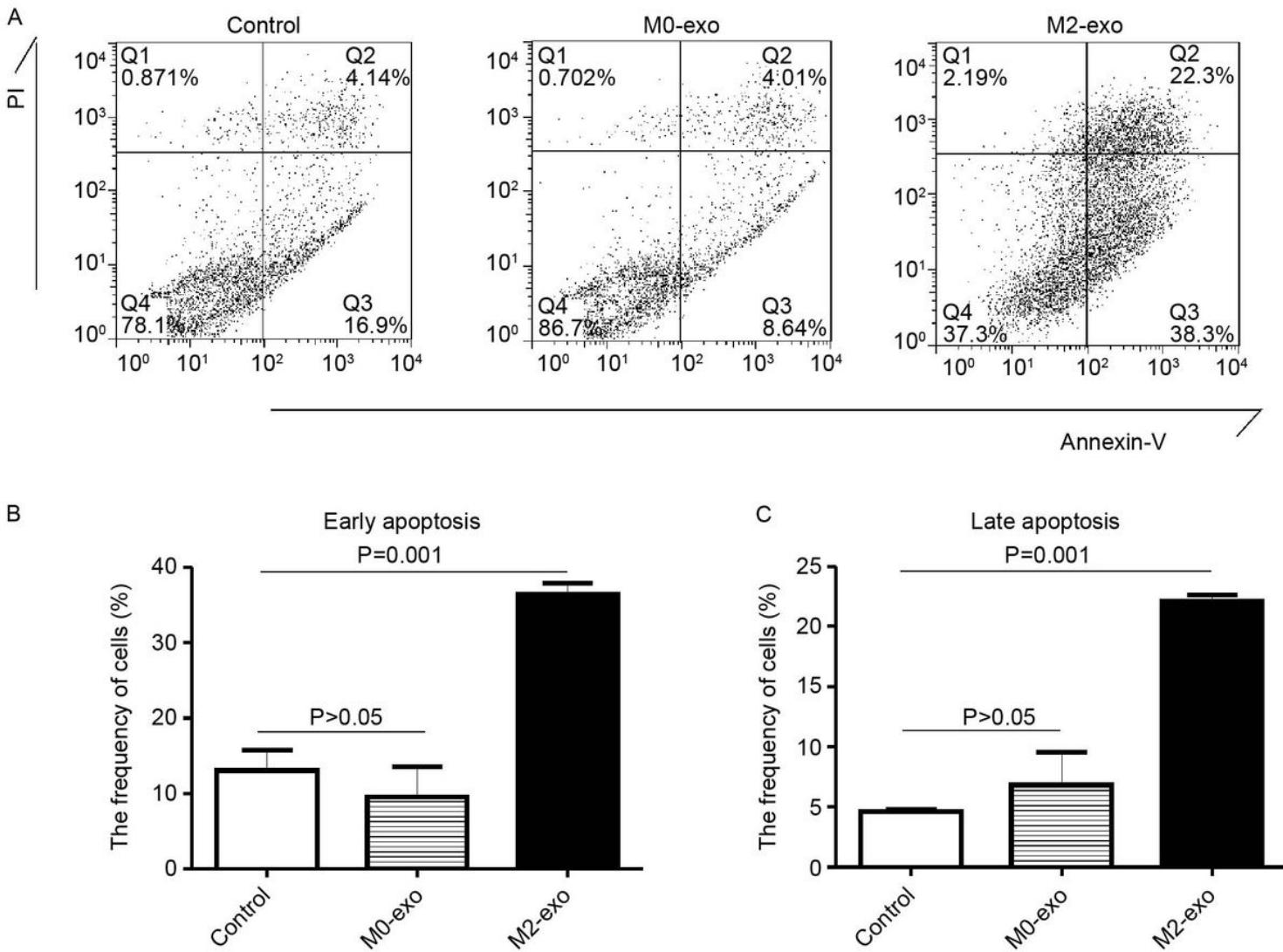
**Figure 2**

Expression of PD-L1 in the exosomes released by M2/M0 macrophages. (A) The size of the exosomes was determined to be 30-100 nm using an electron microscope. Scale bar, 100 nm. (B) PD-L1 expression in the exosomes released from M2 (Thp-1 induced)/M0 (Thp-1) macrophages as determined by western blotting. CD63 was used as the control. (C) PD-L1 expression in the exosomes released by M2/M0 macrophages. Bars represents mean  $\pm$  standard error of the mean. PD-L1, programmed death-ligand 1; CD, cluster of differentiation; Exo, exosome.



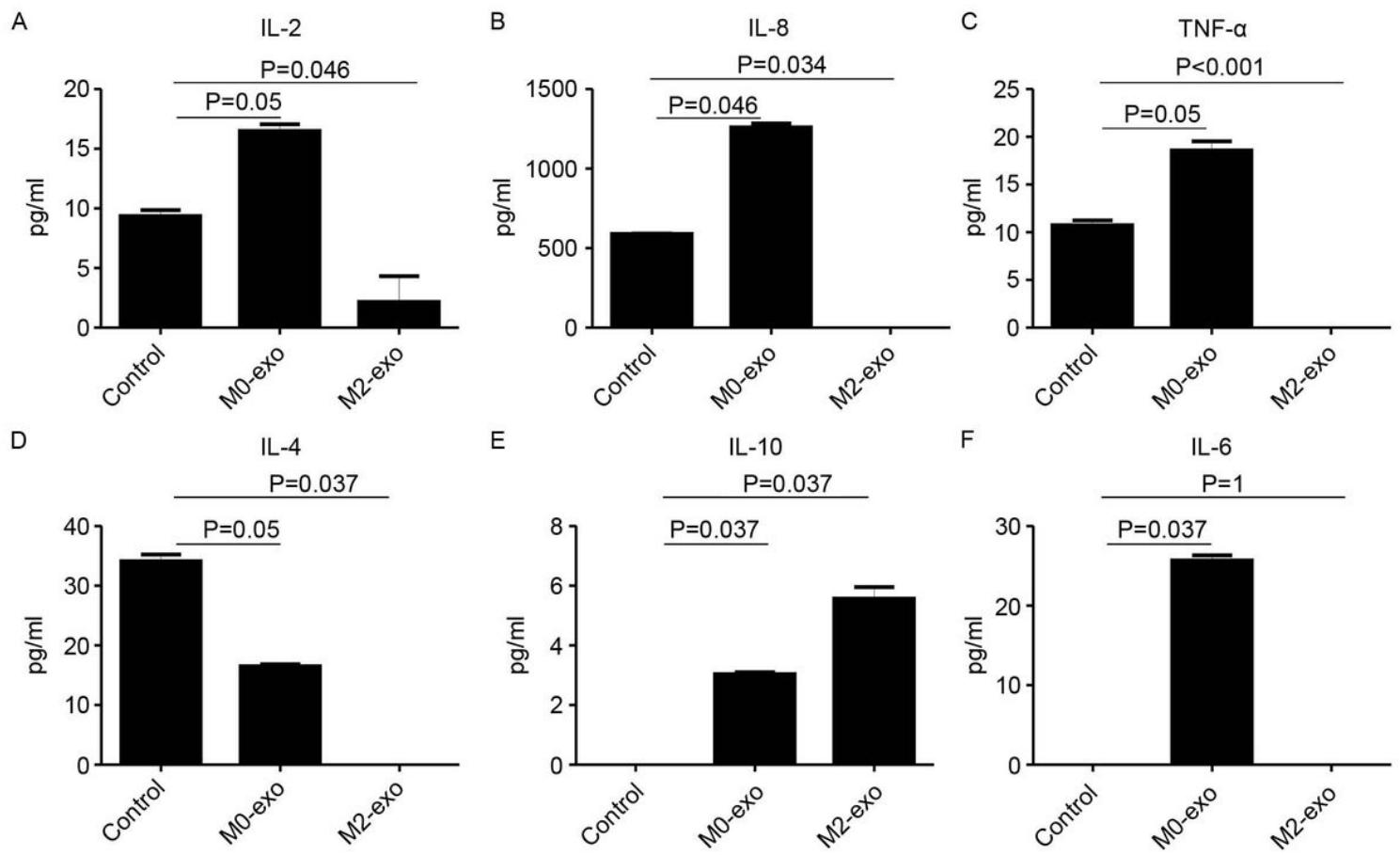
**Figure 3**

Exosomes released from M2 macrophages regulate the proliferation of Jurkat T cells. Jurkat cells were cultured with M2 or M0 exosomes in RPMI-1640 medium for 1, 2 and 3 days. The number of cells was calculated using an MTT assay. Exo, exosome. \* $P<0.05$ ; \*\* $P<0.01$ .



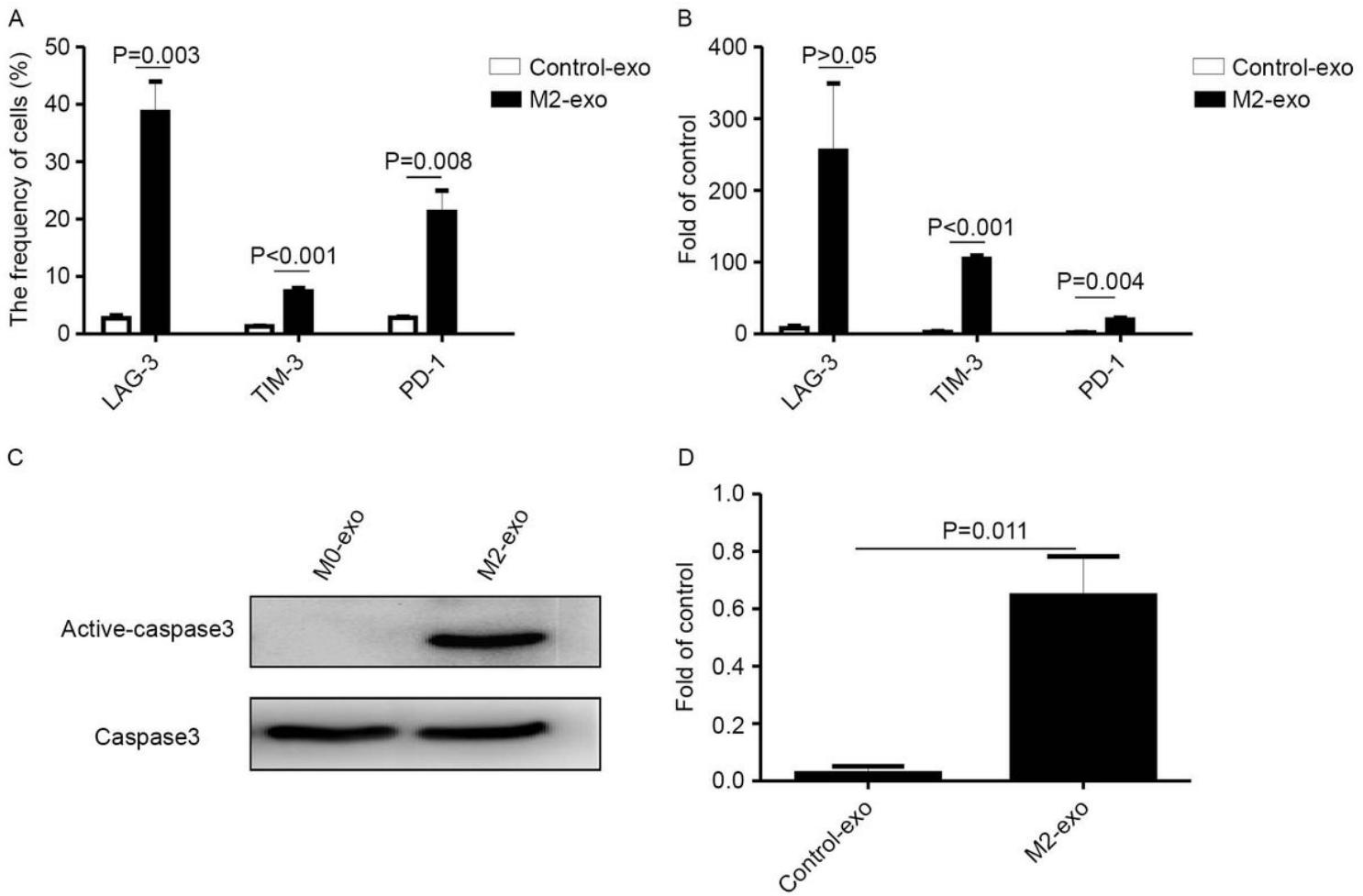
**Figure 4**

Exosomes released from M2 macrophage regulate the apoptosis of Jurkat T cells. (A) Jurkat T cells were cultured with M2 or M0 exosomes in RPMI-1640 medium for 3 days. The apoptosis of Jurkat T cells was analyzed using a fluorescence activated cell sorter with an annexin V apoptosis detection kit. The proportion of cells in (B) early apoptosis and (C) late apoptosis were determined. The exosomes released from M2 macrophage significantly induced the apoptosis of Jurkat T cells compared with the M0-exosome and control groups. PI, propidium iodide; Exo, exosome.



**Figure 5**

Detection of IL-2, -4, -6, -8 and -10 and TNF- $\alpha$  in the supernatants of Jurkat T cells. The cytokines, (A) IL-2, (B) IL-8, (C) TNF- $\alpha$ , (D) IL-4, (E) IL-10 and (F) IL-6, were determined using ProcartaPlex® Multiplex immunoassays according to the manufacturer's protocol. Pro-inflammatory cytokines, IL-2, IL-8, TNF- $\alpha$ , IL-4 and IL-6, were significantly decreased in the M2-exo group, but IL-10 was significantly increased in the M2-exo group compared with the control and M0-exo groups. IL, interleukin; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; Exo, exosome. \*P<0.05; \*\*P<0.01



**Figure 6**

Regulation of markers of Jurkat T cell exhaustion by exosomes released from M2 macrophages. Jurkat T cells were cultured with M2 or M0 exosomes in RPMI-1640 medium for 3 days. (A) Following co-culture, the cells were collected and the expression of LAG-3, TIM-3 and PD-1 was determined using a fluorescence activated cell sorter. (B) The mRNA levels of LAG-3, TIM-3 and PD-1 were determined using polymerase chain reaction. (C) The expression of active-caspase 3 was determined in Jurkat T cells by western blot analysis. (D) Quantitative analysis of active-caspase 3 expression. LAG-3, inhibitory molecule lymphocyte activated gene-3; TIM-3, T-cell immunoglobulin and mucin domain-containing protein-3; PD-1, programmed death 1; Exo, exosome.