

Tumor-derived exosomes induced M2 macrophage polarization and promoted the metastasis of osteosarcoma cells through Tim-3

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Abstract

Background: Osteosarcomas, the second most prevalent primary malignancy of the bone, are often presented with high-grade subclinical metastatic disease that metastasizes at very early stages. Exosomes, as molecular information carriers, may play a potent role in the occurrence and development of tumors through oncogenic molecular reprogramming of tumor-associated macrophage (TAM). In this study, we will investigate the effect of osteosarcoma-derived exosomes on the polarization of TAM and decipher its underlying molecular mechanism. Material and

Methods: Osteosarcoma-derived exosomes from MG63 cells were isolated and characterized by transmission electron microscopy, and nano-particle size analysis. Double fluorescence staining was performed to confirm the macrophages phagocytosis of exosomes. Western blot, qRT-PCR, and transwell assays were conducted to assess the effect of exosomes on migration, invasion, and macrophage differentiation. The mouse model of osteosarcoma was established to evaluate the effects of exosomes on lung metastasis in vivo.

Results: MG63 exosomes were successfully isolated and verified to be phagocytized by macrophages through fluorescence confocal microscopy. The results revealed that osteosarcoma cells could induce M2 type differentiation of macrophages largely through Tim-3 mediated by exosomes, which in turn could promote the migration, invasion, EMT, and lung metastasis of osteosarcoma cells through the secretion of cytokines including IL-10, TGF β , and VEGF.

Conclusions: Our results demonstrated that osteosarcoma-derived exosomes induced M2 polarization of macrophages and promoted the invasion and metastasis of tumors through Tim-3; besides, the study also suggests a novel therapeutic target for future studies.

Background

Osteosarcoma represents the fourth most frequent *malignant* primary neoplasm of the bone, constituting approximately 35% of all the primary malignant bone tumors. Predominantly, osteosarcomas originate in the metaphyseal region of tubular long bone during adolescence and early adulthood and are histologically characterized by the presence of malignant mesenchymal cells producing osteoid or immature bone. Approximately 15%~20% of osteosarcoma patients are presented with high-grade subclinical metastatic disease that metastasizes at *very early stages*[1,2]. Although chemotherapy combined with surgery is the standard of care for osteosarcoma, the prognosis in patients with metastatic osteosarcoma remains *poor*; besides, 20–40% of patients still experience tumor recurrence or distant metastasis following this treatment[2].

TAM, a dominant population of infiltrating inflammatory cells in a majority of malignant tumors, plays an important role in tumor progression through the production of soluble mediators that possibly promotes proliferation, matrix remodeling, invasion, immune evasion, and chemoresistance. M2 macrophages are known to have strong tumor-supporting functions as they contribute to the establishment of a local

immunosuppressive microenvironment[3–6]. Phenotypically and functionally, macrophages can be polarized in two main directions, activated pro-inflammatory M1 macrophages with antitumor properties and alternatively, activated anti-inflammatory M2 macrophages with tumor-supporting functions. The polarization of macrophages have been more recently demonstrated by Low et al.[7–10], wherein, the co-culture of nasopharyngeal carcinoma (NPC) cells and macrophages exerted pro-tumoral functions by inducing various inflammatory cytokines during the early stage; however, up-regulated the induction of cancer-related genes in macrophages in the later stages, suggesting that recruited macrophages into the NPC tumor microenvironment was tuned to promote tumor progression. In the gastric cancer tumor microenvironment, crosstalk between tumor cells and TAMs induced the secretion of inflammatory factors including Il-6, TNF- α , and CCL2, *via* the NF- κ B signaling cascade to promote the development of gastric cancer[11–14]. Besides, the effect of TAMs on tumor progression possibly depends on the tumor type, TME, and the localization of TAMs. Given the increasing evidence supporting active crosstalk between TAMs and EMT in tumor metastasis and progression, investigating the signaling pathways involved in this process might provide an insight into new strategies for the treatment of invasive cancer including osteosarcoma.

Exosomes are lipid bilayer-enclosed nanovesicles with a size range of 40–200 nm that are secreted by nearly all cell types, including tumor cells, and are extensively found in all body fluids[15–17]. Exosomes enclose various types of molecules including lipids, proteins, DNA, messenger RNA (mRNA), and non-coding RNAs, microRNA and long non-coding RNA (lncRNA). Thus, exosomes may mediate specific intercellular communication, exchange of products with other cells, eliminate undesired products from cells, and activate signaling pathways in cells they fuse or interact with. Increasing evidence also suggested that exosomes may participate in the formation and progression of different cancer processes, including remodeling of the tumor microenvironment (TME), angiogenesis, immune escape, dissemination and metastasis[18]. A plethora of evidence indicates that tumor cell-derived exosomes could interact with immune cells in the TME to assist the immune escape of tumor cells and promote tumor progression and therapy resistance in cancer cells. Furthermore, exosomes rich in miR-21 secreted by head and neck tumors could induce M2 phenotype differentiation, thus promoting tumor progression and metastasis[19]. Recent studies have demonstrated that macrophages, particularly M2 macrophages, play an extremely crucial role in the occurrence and development of tumors by inducing immunosuppression, promoting tumor metastasis, and tumor drug resistance[20–24].

The T cell immunoglobulin and mucin domain (Tim) family proteins are type-I transmembrane proteins expressed by diverse cell types within the immune systems of rodents and humans. In 2001, proteins of the Tim family were initially identified in mice T lymphocytes (Tim-1, Tim-2, Tim-3, and Tim-4) and subsequently in humans (Tim-1, Tim-3, and Tim-4). These proteins can regulate T cell activation, tolerance, and diverse effectors pathways of the immune response. Tim-3, also known as HAVCR2, was initially identified to be selectively expressed in the activated Th1 cells, its expression was later confirmed in diverse immune cell types, including regulatory T cells (Tregs), macrophages, dendritic cells, natural killer (NK) cells and in a variety of cells in different types of tumors[25,26]. Scientific evidence indicates that Tim-3 could influence the occurrence and development of diseases by regulating the function of

macrophages in various diseases. Moreover, Tim-3 could promote immune tolerance by inhibiting the activation of macrophage-mediated by LPS/TLR through interacting with STAT1 in infectious diseases[27]. Tim-3 could induce macrophage M2 differentiation and aggravate podocyte damage to participate in the occurrence and development of the disease through NF- κ B/TNF- α pathway in diabetic nephropathy[28,29]. In osteosarcoma, it has been documented that the expression of Tim-3 is significantly increased in tumor tissue[30], and the overexpression of Tim-3 could promote the occurrence and development of osteosarcoma. However, whether the overexpression of Tim-3 in osteosarcoma could also regulate the differentiation of tumor-associated macrophages and the underlying molecular mechanism of this regulation remains elusive. Therefore, this study was initiated to investigate the effect of osteosarcoma-derived exosomes on M2 macrophage polarization and to elucidate the underlying molecular mechanism in the occurrence and development of osteosarcoma. Furthermore, this study also provides a theoretical basis and experimental evidence for refining the therapeutical strategy and improving the prognosis of patients with osteosarcoma.

Materials And Methods

Tissue samples

Tumor tissue samples were obtained from 18 patients with primary osteosarcoma who received a histopathological diagnosis and underwent surgical resection for osteosarcoma at the department of orthopedics and pediatric surgery of our hospital from March 2018 to October 2019 were collected. We excluded patients with a prior diagnosis of tumors of other organs and patients with serious diseases of heart, lung and other organs. The tissue parts surrounding the tumor, which was not diagnosed as cancer tissue, from the same patient was collected as normal control. This study was approved by the Institutional Ethics Review Board of Huanggang Central Hospital. Written informed consent was obtained from all the patients. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored until analysis in the present study.

Cell lines and main reagents

Human osteosarcoma MG63 and normal osteoblast hFOB cell lines were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium-F12 (DMEM/F12) was purchased from Hyclone, USA; Fetal Bovine Serum (FBS), Penicillin-streptomycin (100 U/mL) were purchased from Hangzhou Sijiqing Biological Company; RIPA Lysis buffer, BCA Protein Quantification Reagent Kit were purchased from Invitrogen, USA; Opti-MEM (Gbico, UK); Anti-Tim-3 monoclonal antibody was obtained from BD Bioscience, USA; Rabbit anti-E-Cadherin, N-cadherin, Vimentin, and GAPDH monoclonal antibodies were purchased from Abcam, USA; Rabbit anti-CD9, CD81, and CD63 monoclonal antibodies were purchased from Santa Cruz, USA; Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG secondary antibody was purchased from

Guangzhou Jingcai; PKH67 green fluorescent cell linker dye, and PMA were purchased from Sigma Company, USA.

Cell culture and transfection

After resuscitation of osteosarcoma cell lines MG63 and hFOB, the cells were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% of penicillin-streptomycin at 37 °C in a humidified incubator with 5% CO₂. When the cells reached 70%–80% confluence, the cells were digested with 0.25% trypsin and centrifuged at 800 r/min for 5 min at room temperature (RT). The supernatant was discarded, and the cells were resuspended with DMEM/F12 for passage.

The MG63 cells (2×10^5 cells/well) at the logarithmic growth stage were seeded onto a 6-well plate and incubated overnight. MG63 cells were transfected with 30 ng of Tim-3 sgRNA, Cas9 plasmid or negative control (NC) sequence (Guangzhou Ruibo Biological Company) using Transfection reagent Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's instructions. After transfection for 6 h, the medium was replaced with DMEM/F12 medium containing 10% FBS and incubated in a humidified atmosphere of 5% CO₂ at 37 °C. 48 h after transfection, cells were collected for the downstream experiments. The experiments were repeated three times. The required macrophages in the experiment were obtained by treating about 1×10^6 THP-1 cells with 50 ng/mL PMA for 48 h.

Isolation and identification of exosomes

MG63 and hFOB cells at 80%~90% confluence were pelleted and washed thrice with DMEM/F12 media, followed by replacement of media with fresh DMEM/F12 containing 10% FBS and the cells were cultured for additional 72 h at 37 °C in a 5% CO₂ atmosphere before harvesting exosomes. The cell culture medium was collected and intact cells and cell debris were removed and exosomes were isolated by differential centrifugation at 300×g for 10 min, and 2,000×g for 10 min, 1,000×g for 30 min, respectively. The resulting pellet was resuspended, washed once in ice-cold PBS, and filtered through sterile 0.22 μm (pore-size) syringe filter and repelleted at 1,000,000 g for 70 min at 4 °C. The pellet containing exosomes was washed and resuspended in ice-cold PBS and stored at –80 °C.

For Transmission Electron Microscopy (TEM), freshly prepared osteosarcoma-exosomes (2 μL) were diluted in 20 μL PBS, fixed with 3.5% paraformaldehyde, then, exosome preparation was allowed to adsorb in the mesh copper grid. The resulting grids were rinsed twice with wash buffer and contrasted by uranyl-oxalate solution at pH 7 for 5 min. The TEM visualizations of exosomes morphology were performed using a Hitachi H-9000 transmission electron microscope at 300 kV and images were acquired using a slow-scan CCD camera. Size profiles of isolated exosomes were determined by the Nanosight size analyzer. Briefly, 50 μL of exosomes was diluted in PBS to a final volume of 1 ml and filtered through a 0.22 μm filter membrane. Size curves were determined by nanoparticle tracking analysis using a Nanosight NS300TM. Three video recordings of 40 sec were carried out for each preparation.

NTA 3.2 software version was used to record and analyze the videos. The expression of exosome characteristic proteins including CD9, CD81, and CD63 was detected using Western blot assay.

Labeling and tracking of exosomes

The extracted and purified exosomes were collected and labeled KPH67 reagent according to the manufacturer's instructions. The nuclei of macrophages were counter-stained with DAPI dye following the manufacturer's instructions. KPH67 labeled exosomes were added to DAPI stained macrophages and co-cultured at 37 °C in a humidified atmosphere of 5% CO₂ for 12 h. After incubation, the exosomes were analyzed by a fluorescence confocal microscope.

Cell migration and invasion

The cell invasion assay was carried out using 24-well transwell chambers with 6.5 mm diameter polycarbonate filters (8 μm pore size, Corning, NY, USA) coated with 35 μL Matrigel (Becton, Dickinson and Company, USA). Cells were seeded in the upper chambers at a density of 5×10⁴ cells/well and then treated with different concentrations of Anlotinib (0, 2, 4, 8 μmol/L), and allowed to invade for 24 h toward the lower chamber containing 10% FBS or serum-free media. After washing twice with PBS, cells invading through the Matrigel layer were fixed with methanol followed by mounting and staining with Giemsa stain for 15 min. The inner side of the chambers was wiped with a cotton swab. The number of invading cells from at least five fields of each of three separate membranes was counted under an inverted microscope (Olympus Cor., Tokyo, Japan). Three independent experiments were carried out in triplicate.

The MG63 cells were routinely digested and resuspended in DMEM/F12 medium without FBS and seeded at a density of 5×10⁴ cells/well in the upper chamber of Transwell containing 200 μL serum-free medium allowed to invade for 24 h toward the lower chamber containing 1×10⁴ cells/mL macrophages and 800 μL of conventional DMEM/F12 medium supplemented with 10% FBS. After washing twice with PBS, cells invading through the Matrigel layer were fixed with absolute alcohol for 15 min, dried at RT, followed by mounting and staining with 0.1% crystal violet for 15 min at RT. The inner side of the chambers was wiped with a cotton swab. The number of invading cells from at least five fields of each of three separate membranes was counted under an inverted microscope (Olympus Cor., Tokyo, Japan). Three independent experiments were carried out in triplicate.

Quantitative Real-time PCR (qRT-PCR)

The total RNA was extracted from exosomes or MG63, hFOB cells, and transfected MG63 cells extracted using Trizol Reagent (Invitrogen). The RNA quality and concentration were measured using a spectrophotometer. The reverse transcription reaction was performed to reverse transcribe RNA into cDNA

using RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocols. qRT-PCR was performed with the SYBR Green Master Mix Kit (Shanghai Solarbio Biological Company) on an ABI Prism 7000 sequence detection system (Applied Biosystems). Expression of target genes was normalized to the expression of GAPDH relative gene expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method. Three independent experiments were performed in triplicates. The following primers were synthesized (Shanghai Biotech) and used for RT-PCR: Tim-3-F: 5'-

ACTCTACTCTACCTACATCTGGGACACT-3', Tim-3-R: 5'-GTAGGTCCCATGGTCATCCAG-3'; Arg-1-F: 5'-TTGGGTGGATGCTCACACTG-3', Arg-1-R: 5'-TTGCCCATGCAGATTCCC-3'; CD206-F: 5'-CAGGTGTGGGCTCAGGTAGT-3', CD206-R: 5'-TGTGGTGAGCTGAAAGGTGA-3'; CD163-F: 5'-GCCGGCTATCCAGACAATTA-3', CD163-R: 5'-GGCCAAACTGAGGTGGTTTA-3'; iNOS-F: 5'-CAGCTGGGCTGTACAAACC TT-3'; iNOS-R: 5'-CAGCTGGGCTGTACAAACCTT-3'; IL-10-F: 5'-TGCTATGCTGCCTGCTCTTA-3', IL-10-R: 5'-TCATTTCCGATAAGGCTTGG-3'; GAPD-F: 5'-GGAGAAACCTGCCAA GTATG-3', GAPD-R: 5'-TTACTCCTTGGAGGCCATGTAG-3'; TGF- β -F: 5'-GGGATGAGCTAGTGCTGATCTGG-3', TGF- β -R: 5'-AACTTGCTCCATGCTCCTGCTCTA-3'; VEGF-F: 5'-TGCAGGTGATGCTGACAGAGG-3', VEGF-R: 5'-GGATGAGCTAGTGCTGATCTGG-3'

Western blot Assay

Total proteins were extracted from the osteosarcoma and para-carcinoma tissues or exosomes or MG63, hFOB cells or transfected MG63 cells on ice with radio-immunoprecipitation assay (RIPA) cell lysis buffer supplemented with protease inhibitors. Protein concentrations were quantified by the BCA method. Cell lysates were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred the proteins onto the polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 for 2 h at RT. Subsequently, the membrane was incubated with primary antibodies including rabbit anti-mouse/human Tim-3 (1: 500), E-cadherin (1: 800), N-cadherin (1: 800), Vimentin (1: 800), and CD9 (1: 600), CD81 (1: 600), CD63 (1: 600), GAPDH (1: 1000) in 5% blocking buffer overnight at 4 °C. Then, the membranes were washed twice with TBST solution for 5 min/time, incubated with horseradish enzyme-labeled secondary antibody (1: 5000) for 1 h at RT. The target bands were visualized using the Enhanced Chemiluminescence Kit and the protein bands were quantified using Image J software. Using GAPDH as the internal reference, the ratio of gray value between the target band and internal reference band was considered as the relative expression of the target proteins. Three independent experiments were performed in triplicates.

Pulmonary metastases of osteosarcoma

The animals were cared for in accordance with the Guide for the care and use of laboratory animals in China. All procedures and animal experiments were approved by the Animal Protection and application committee of Huanggang Central Hospital. Fifteen male BALB/c mice (age 4–5 weeks, weight 200 to 250

g) were obtained from the Institute of Zoology, Chinese Academy of Sciences. Mice were housed under specific-pathogen-free (SPF) controlled conditions of $(22 \pm 1)^\circ\text{C}$ temperature, $60\% \pm 10$ humidity with a 12 h light/dark cycle and *ad libitum* access to water and sterile food pellets. Mice were randomly divided into 3 groups (5 in each group). MG63 and macrophages induced by MG63-Exo or NC-Exo or Tim-3 KO-Exo were administered via tail vein to observe the lung metastasis of osteosarcoma. After 5 weeks, mice in each group were anesthetized and sacrificed. All the suspected lesions of lung metastasis collected and fixed with 10% buffered formalin for (12–24) h at RT, then paraffin-embedded, and sectioned into 4- μm -thick sections. The *sections* were prepared for *histologic examination* following staining with hematoxylin and eosin (H&E).

Statistical analysis

Statistical analyses were performed using the SPSS software package (version 19.0; IBM, Chicago, IL, USA). All graphs were produced by GraphPad Prism 5.0 for Windows software (GraphPad Software Inc., La Jolla, CA, USA). Data were expressed as mean \pm standard deviation (SD). The comparison between the two groups was performed using the independent sample *t*-test, and the comparison among multiple groups was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's correction post hoc test for multiple comparisons. All tests were 2-sided, and $P < 0.05$ was considered statistically significant.

Results

MG63-Exo promoted the polarization of induced-macrophages into M2 phenotype

Exosomes, explicitly hFOB-Exo and MG63-Exo, were isolated from the cell culture supernatant of normal osteoblast hFOB and osteosarcoma cell line MG63 by ultracentrifugation. TEM image analysis revealed that the morphology of the exosomes was round or oval (Figure 1A). The size of exosomes was ranged from about 30 to 150 nm based on size analysis (Figure 1B). Western blot assay results indicated that exosome-specific molecular marker including CD9, D81, and CD63 was significantly highly expressed in hFOB-Exo and MG63-Exo (Figure 1C). Macrophages were the most abundantly expressed immune cells in the TME. Furthermore, the effect of osteosarcoma-derived exosomes on macrophage differentiation was also investigated. Human monocyte line THP-1 was differentiated into macrophages under PMA stimulation. As illustrated in Figure 1D, the suspension of THP-1 monocytes could be differentiated into adherent-growing macrophages and extended pseudopodia from the cell body under an optical microscope. Further, qRT-PCR results revealed that the expression of macrophage marker CD68 in THP-1 was significantly over-expressed following stimulation with PMA in comparison with the control group (Figure 1E). In order to confirm that the isolated exosomes could be phagocytized by macrophages, we labeled the exosomes with the green fluorescent dye PKH67 beforehand, which was co-cultured with macrophages for 12 h. Notably, the green fluorescence around the nuclei of macrophages was observed

under confocal fluorescence microscopy, indicating that exosomes were phagocytized by macrophages (Figure 1F). qRT-PCR data suggested that compared with the PBS control group or macrophages co-cultured with hFOB-Exo, MG63-Exo significantly promoted the expression of M2-type macrophage markers CD206, CD163, and Arginase-1; however, no alterations in the expression of iNOS, the marker of M1 macrophage, was noted. Conceivably, hFOB-Exo exhibited no apparent effect on the M1 or M2 phenotype differentiation of macrophages (Figure 1G). We further applied qRT-PCR to detect the effect of macrophages on the expression levels of M2 type cytokines, TGF- β , IL-10 and VEGF under different treatment conditions. The results indicated that compared with the control group, MG63-Exo significantly promoted the expression of TGF- β , IL-10 and VEGF; however, hFOB-Exo did not affect macrophages for the expression of the above cytokines (Figure 1H).

M2 macrophages induced by MG63-Exo promoted the migration, invasion, and EMT of osteosarcoma cells

To clarify the effects of M2 macrophages induced by MG63-Exo on the migration, invasion, and EMT of osteosarcoma cell *in vitro*, we used a co-culture system for the detection (Figure 2A). The results of migration and invasion assays revealed that compared with the control group, M2 macrophages induced by MG63-Exo could significantly promote the migration and invasion ability of osteosarcoma cells (Figure 2B). Furthermore, we investigated the effect of M2 macrophages induced by MG63-Exo on EMT of tumor cells. Western blot assays results indicated that as compared with the control group, M2 macrophages induced by MG63-Exo significantly reduced the expression of the epithelial cell marker E-cadherin in osteosarcoma cells and remarkably promoted the expression of mesenchymal cell markers N-cadherin and Vimentin (Figure 2C).

Overexpression of Tim-3 protein in MG63-Exo could be transferred to macrophages by exosomes

Previous studies have revealed that exosomes contained a large number of bioactive maternal cell proteins that could be passed on into other cells to affect their cell function. We analyzed the expression of Tim-3 in osteosarcoma and para-carcinoma tissues of 18 patients by Western blot assay. The results revealed that the positive rate of Tim-3 expression in osteosarcoma was significantly higher than that in para-carcinoma tissue (Figure 3A, B). To further clarify whether MG63-Exo could overexpress Tim-3, we used qRT-PCR and Western blot assays to detect the expression of Tim-3 mRNA and protein in MG63 cells. The results showed that compared with normal osteoblast hFOB cells, the expression of Tim-3 mRNA and protein in MG63 cells was significantly up-regulated (Figure 3C, D). Using Western blot assay, we detected the protein expression Tim-3 protein in exosomes. Furthermore, as compared with human normal osteoblast hFOB-Exo, the expression of Tim-3 protein in MG63-Exo was also significantly up-regulated (Figure 3E). To further investigate whether MG63-Exo could deliver Tim-3 into macrophages, as presented in Figure F, we found that the expression of Tim-3 protein in macrophages co-cultured with

MG63-Exo was significantly higher than that in macrophages co-cultured with hFOB-Exo or PBS. Furthermore, we knocked down the expression of Tim-3 (Tim-3 KO) in MG63 cells by CRISPR/Cas9. qRT-PCR and Western blot results indicated that compared with the cells in the NC group, the expression of Tim-3 mRNA and protein in MG63 cells in the Tim-3 KO group were significantly inhibited (Figure 3G, H). The identification and characterization of MG63 exosomes with Tim-3 knockout (Tim-3 KO-Exo) was carried out by particle size analysis and Western blot analysis (Figure 3I, J). We also detected the protein expression of Tim-3 in Tim-3 KO-Exo by Western blot assay. These results suggested that compared with MG63-Exo, the expression of Tim-3 in Tim-3 KO-Exo was significantly down-regulated (Figure 3K). At the same time, we continued to adopt Western blot assay to detect the expression of Tim-3 protein in macrophages co-cultured with Tim-3 KO-Exo, the findings indicated that the expression of Tim-3 was significantly decreased in macrophages co-cultured with Tim-3 KO-Exo compared with macrophages co-cultured with NC-Exo or MG63-Exo (Figure 3L). Collectively, these results indicated that overexpression of Tim-3 protein in MG63-Exo could be transferred into macrophages by exosomes; however, the down-regulation of Tim-3 expression in MG63 cells reduced the expression of Tim-3 in co-cultured macrophages by down-regulating the expression of Tim-3 in MG63-Exo.

Tim-3 derived from MG63-Exo promoted the migration, invasion, and EMT of osteosarcoma cells by promoting M2 macrophage polarization

The above experimental results indicated that MG63-Exo induced the polarization of macrophages into the M2 phenotype to promote the migration, invasion, and EMT of osteosarcoma cells, while Tim-3, which was highly expressed in MG63-Exo, could be phagocytized by macrophages. Thus, we speculated that Tim-3 might play a key role in supporting this process. Tim-3 was secreted by MG63-Exo and internalized by macrophages, which could promote the metastasis, invasion, and EMT of osteosarcoma cells by promoting M2 macrophage polarization. To confirm this hypothesis, we first identified the effect of Tim-3 KO-Exo on the expression of M2-type macrophage markers CD206, CD163, arg-1, and the cytokines TGF- β , IL-10, and VEGF. The results indicated that compared with macrophages co-cultured with NC-Exo or MG63-Exo, the expression of M2 macrophage marker including CD206, CD163, Arginase-1, cytokines TGF- β and cytokines IL-10, VEGF were significantly decreased following treatment with Tim-3 KO-Exo (Figure 4A, B). The results of migration and invasion experiments revealed that compared with macrophages induced by NC-Exo or MG63-Exo co-cultured with MG63, the macrophages induced by Tim-3 KO-Exo could significantly inhibit the migration and invasion ability of MG63 cells (Figure 4C). EMT findings further also confirmed that compared with macrophages induced by NC-Exo or MG63-Exo co-cultured with MG63, macrophages induced by Tim-3 KO-Exo significantly promoted the expression of the epithelial cell marker E-cadherin and inhibited the expression of mesenchymal cell markers N-cadherin and Vimentin (Figure 4D). The findings indicated that MG63-Exo induced macrophages to differentiate into the M2 phenotype through Tim-3, which could further promote the metastasis, invasion, and EMT of

osteosarcoma cells MG63; however, the down-regulation of Tim-3 expression in MG63-Exo could reverse the above-mentioned effects of MG63-Exo.

2.5 Tim-3 derived from MG63-Exo promoted lung metastasis of osteosarcoma cells in nude mice by promoting M2 phenotype macrophage polarization

Next, to further evaluate the role of Tim-3 derived from exosome on the occurrence and development of osteosarcoma cells *in vivo* after activating macrophages to differentiate into M2-type, we administered macrophages induced with Tim-3 Ko-Exo or NC-Exo and osteosarcoma MG63 cells into nude mice *via* the tail vein. After 5 weeks, the results indicated that compared with the nude mice treated with MG63 cells and macrophages induced by Tim-3 Ko-Exo, larger and multiple metastatic tumor nodules were deposited in the lung of nude mice treated with MG63 cells and macrophages induced by NC-Exo or MG63-Exo (Figure 5). These findings indicated that Tim-3 in MG63-Exo could promote lung metastasis of osteosarcoma *in vivo* by promoting M2 macrophage polarization.

Discussion

Exosomes, as molecular information carriers, play a potent role in the mediation of cell-to-cell communication between tumor cells and immune cells to induce a pro-tumoral environment and to eventually promote the tumor progression and survival[31,32]. A plethora of evidence also indicated that exosomes derived from tumors were crucially involved in the alteration of the biological activities of their recipient cells through the transfer of their oncogenic content. TAM, as the major type of immune cell in tumor tissues, well-recognized to play a role in the occurrence, invasion, and metastasis of tumor cells[33–36]. Furthermore, accumulating studies have indicated the tumor-supporting functions of TAMs for many types of malignancies, including breast cancer, lung adenocarcinoma, esophageal cancer, cervical cancer, ovarian cancer, prostate cancer, and renal cell carcinoma. Moreover, mature macrophages could be reprogrammed into different types under different environmental conditions, such as M1 and M2, which plays differential roles in tumor tissue. In gastric cancer, studies have demonstrated that pro-inflammatory M1-type macrophages could promote apoptosis and inhibit the growth of gastric cancer cells, indicating the antitumor properties of macrophages. However, activated anti-inflammatory M2-type macrophages with tumor-supporting functions could promote the growth and invasion of tumor cells[37]. In liver cancer, M2-type macrophages could promote the malignant transformation and progression of liver cancer, through the induction of expression of vascular endothelial factors and matrix metalloproteins in the TME in comparison with M1-type macrophages, and facilitate the invasion and metastasis of liver cancer, which are important factors for poor prognosis of patients with liver cancer[33]. The tumor-supporting functions of TAMs have been demonstrated for in many types of malignancies including ovarian cancer, nasopharyngeal cancer, and melanoma, TAM was dominated mostly by M2-type macrophages; however, the reprogramming of M2 macrophages towards M1

phenotype exhibited a marked negative impact on tumor progression and prognosis[7,38,39]. These pieces of evidence thus indicated that M1-type macrophages present an inhibitory effect on tumor immunity, while M2-type macrophages exhibit a tumor-supporting function including tumor invasion and metastasis. In this study, we isolated the exosomes from the culture supernatant of the osteosarcoma cell line MG63 and further investigated the effect overexpression of Tim-3 mediated by exosomes on the migration and invasion of osteosarcoma cells. Using TEM, morphology and size profiles of the isolated exosomes were determined and the results indicated that the obtained exosomes were round or oval in structure with a size ranging from 30 to 150 nm. The Western blot assay identified a high expression of exosomes related marker such as CD9, CD63, and CD81. Studies have demonstrated that breast cancer-derived exosomes activated NF- κ B signaling pathway through TLR2 to induce macrophage activation, while the activated macrophages regulated immunity through secretion of inflammatory factors such as IL-6/TNF- α , GCSF and CCL2 to promote distant metastasis of breast cancer[40]. Furthermore, in colon cancer, tumor-derived exosomes mediated the M2 polarization of TAM by lncRNA PPH1 to facilitate the metastasis and invasion of colon cancer cells[41]. Thus, to further clarify the effect of osteosarcoma-derived exosomes on macrophage differentiation, migration, invasion, and EMT of osteosarcoma cells, we induced THP-1 cells to polarize mature macrophages by PMA and identified the expression of macrophage marker protein CD68 by Western blot assay. Subsequently, the effect of osteosarcoma-derived exosomes on the differentiation of macrophages was evaluated by qRT-PCR. The experimental results indicated that exosomes derived from osteosarcoma could significantly promote the M2 differentiation of macrophages and secretion of cytokines IL-10, TGF β , and VEGF. Further, the Transwell co-culture assay revealed that exosomes induced macrophages significantly promoted the migration and invasion ability of osteosarcoma cells. Taken together, these systematic assays concluded that the isolated osteosarcoma-derived exosomes contained crucial factors, which were related to cellular invasion, development and cell proliferation. Tim-3, a negative regulator of immunity, is selectively induced upon the differentiation of naïve Th cells into interferon-gamma secreting Th1 effector cells. Tim-3 by binding to its ligand Galectin-9 (Gal-9) could induce apoptosis and immune tolerance of T cells. In this study, Tim-3 was also found to be expressed in macrophages, NK cells, and other natural immune cells, which may play an important role in maintaining immune homeostasis[30]. Using Western blot assay, we detected high expression of Tim-3 protein in osteosarcoma tissues and cells, which was consistent with previous studies. Further, to clarify that the overexpressed Tim-3 in osteosarcoma cells were phagocytized by macrophages through exosomes and induced the oncogenic reprogramming of macrophages into M2 type, we knocked down Tim-3 expression in osteosarcoma cells by CRISPR/Cas9 and detected the expression of Tim-3 in osteosarcoma-derived exosomes derived by Western blot analysis. The results indicated that the knocked down of Tim-3 in osteosarcoma cells could significantly down-regulated the expression of Tim-3 in exosomes, while the latter displayed no significant effect on M2 type differentiation of macrophages as well as on the expression of IL-10, TGF β and VEGF. Further, Transwell and Western blot assays also demonstrated that under-expression of Tim-3 in exosomes exhibited no apparent promoting effect on the migration, invasion, and EMT of osteosarcoma cells. The distant metastasis of osteosarcoma in nude mice further confirmed that Tim-3 expression in exosomes

promoted the lung metastasis of osteosarcoma cells by inducing macrophage differentiation; however, the down-regulation of Tim-3 expression in exosomes significantly reduced metastasis in the lung.

Conclusions

This study identified that osteosarcoma-derived exosomes induced M2 type polarization of macrophages through regulation of the expression of Tim-3. Furthermore, the differentiated macrophages promoted the migration, invasion, EMT, and distant metastasis ability of osteosarcoma cells through the secretion of IL-10, TGF β , VEGF. However, the role of exosomes in tumor microenvironment remains complex and variable and therefore the above-mentioned mechanism may represent a probable mechanism and warrant further investigations.

List Of Abbreviations

TAM: tumor-associated macrophage; NPC: nasopharyngeal carcinoma; mRNA: messenger RNA; lncRNA: long non-coding RNA; TME: tumor microenvironment; Tim: T cell immunoglobulin and mucin domain; Tregs: regulatory T cells.

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Review Board of Huanggang Central Hospital. Written informed consent was obtained from all the patients. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored until analysis in the present study.

Availability of data and materials

All data are available in the included figures and tables.

Competing interests

The authors declare that they have no conflict of interests.

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None.

Consent for publication

None.

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Authors' contributions

Conceptualization: ZC, WX; Methodology: ZC, LW, CW; Software: CW, LH, YR; Supervision: WX; Writing original draft: ZC, LW; All authors read and approved the final manuscript.

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Figures

Figure 1 not provided with this version.

Figure 1

MG63-Exo promoted the M2 phenotype differentiation of induced-macrophages. A: the morphology of hFOB-Exo and MG63-Exo as observed under TEM; B: the size and density distribution of hFOB-Exo and MG63-Exo particles were detected using NanoSight size analyzer; C: the expression of exosomes specific marker such as CD9, CD81, and CD63 was detected by Western blot; D: immunofluorescence showed that PKH67-labeled exosomes were phagocytized by macrophages; E: the morphology alteration of macrophages after THP-1 induced by PMA; F: the expression of macrophage marker CD68 as detected by qRT-PCR; G: the expression of M1 and M2 macrophage markers as detected by qRT-PCR following treatment with PBS, hFOB-Exo or MG63-Exo; H: the effect of macrophages on the expression of M2 type cytokines TGF- β , IL-10, and VEGF as detected by qRT-PCR following treatment with PBS, hFOB-Exo or MG63-Exo; * P <0.05, ** P <0.01.

Figure 2 not provided with this version.

Figure 2

MG63-Exo promoted the migration, invasion, and EMT of osteosarcoma cells through induction of M2 phenotype differentiation of macrophages. A: A schematic diagram of co-culture system in vitro; B: the effects of macrophages induced by MG63-Exo on the migration and invasion of MG63 osteosarcoma cells as detected by Transwell co-culture system in vitro; C: the effect of macrophages induced by MG63-Exo on the EMT of osteosarcoma cells MG63 as detected by Western blot assay. * P <0.05.

Figure 3 not provided with this version.

Figure 3

Overexpression of Tim-3 protein in MG63-Exo could be transferred to macrophages by exosomes. A: the expression of Tim-3 protein in osteosarcoma and para-carcinoma tissues as detected by Western blot assay (n=6); B: the expression analysis of Tim-3 in 18 pairs of osteosarcoma and para-carcinoma tissues; C: the expression of Tim-3 mRNA in MG63 and hFOB as detected by qRT-PCR; D: the expression of Tim-3 protein in MG63 and hFOB as detected by Western blot assay; E: the expression of Tim-3 protein in hFOB-Exo or MG63-Exo was detected by Western blot assay; F: the expression of Tim-3 protein in macrophages following treatment with PBS, hFOB-Exo or MG63-Exo as detected by Western blot; G: RT-PCR was used to detect the expression of Tim-3 mRNA following the Tim-3 gene is knocked out in MG63 cells; H: Western blot assay was used to detect the expression of Tim-3 protein after knocking out the Tim-3 gene in MG63 cells; I: the size and density distribution of Tim-3 KO-Exo particles as detected by NanoSight size analyzer; J: the expression of exosome marker including, CD9, CD81, and CD63 in Tim-3 KO-Exo as detected by Western blot assay; K: the expression of Tim-3 protein in NC-Exo, MG63-Exo, and Tim-3 KO-Exo as detected by Western blot; L: the expression of Tim-3 protein in macrophages treated with NC-Exo, MG63-Exo and Tim-3 KO-Exo as detected by Western blot assay. * P <0.05, ** P <0.01.

Figure 4 not provided with this version.

Figure 4

Tim-3 derived from MG63-Exo promoted the migration, invasion, and EMT of osteosarcoma cells by promoting M2 macrophage polarization. A: the expression of M1, M2 macrophage marker as detected by qRT-PCR following treatment with NC-Exo, MG63-Exo or Tim-3 KO-Exo; B: the expression of M2 type cytokines TGF- β , IL-10, and VEGF in macrophage as detected by qRT-PCR following treatment with NC-Exo, MG63-Exo or Tim-3 KO-Exo; C: the effects of macrophages induced by NC-Exo, MG63-Exo or Tim-3 KO-Exo on the migration and invasion ability of osteosarcoma cells MG63 as determined by Transwell assay; D: the effect of macrophages induced by NC-Exo, MG63-Exo or Tim-3 KO-Exo on EMT of osteosarcoma cells MG63 as detected by Western blot. * P <0.05.

Figure 5 not provided with this version.

Figure 5

Tim-3 derived from MG63-Exo promoted lung metastasis of osteosarcoma cells in nude mice by promoting M2 macrophage polarization. * P<0.05.