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*Original Article*

# Effect of tumor-associated macrophages on gastric cancer stem cell in omental milky spots and lymph node micrometastasis

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**Abstract:** We observed whether the effect of tumor-associated macrophages on gastric cancer stem cell in omental milky spots and lymph nodes micrometastasis and research its possible mechanism. Macrophage THP-1 cells and Human gastric adenocarcinoma SGC-7901 cells were collectively cultivated in vivo. We found macrophage could suppress the proliferation and accelerated cell death of MFC cell. Meanwhile, these effects may be concerned with many signaling pathways, and we detected MCP-1 and COX-2 miRNA expressions, PGE-2 release levels, IL-4 and IL-10 activities, and TGF-β, IFN-γ, VEGF, MMP-2 and MMP-9 protein expressions in collectively cultivated cell. We found that MCP-1 and COX-2 miRNA expressions, and PGE-2 release levels were suppressed, IL-4 activity was inhibited and IL-10 activity was activated in collectively cultivated cell. Meanwhile, TGF-β, MMP-2 and MMP-9 protein expressions were inhibited and IFN-γ and VEGF protein expressions were activated in collectively cultivated cell. Taken together, these results suggest that the effect of tumor-associated macrophages on gastric cancer stem cell in omental milky spots and lymph nodes micrometastasis via COX-2/PGE-2/TGF-β/VEGF signal pathways.

**Keywords:** Tumor-associated macrophages, gastric cancer stem cell, COX-2, VEGF

## Introduction

Cancer Gastric is one of the most common malignant tumors in China, and the incidence rate ranks first in the digestive tract tumor [1]. At present, the clinical diagnosis and treatment of gastric cancer are mostly in its advanced stage and the postoperative relapse and metastasis rate is high especially the peritoneal metastasis even if through the radical surgical treatment [2]. Adjuvant chemotherapy improve the gastric cancer patients' 5-year survival rate and life quality, but the current chemotherapy drugs is mainly for the tumor cells in division stage and cannot eliminate the tumor cells in dormant state which are the key to postoperative tumor relapse and metastasis [3].

The tumor cells are usually in a complex environment consist of inflammatory immune cells, stromal cells, endothelial cells, cytokines and chemokines and etc., and this micro environment is called "tumor microenvironment" [4]. A large number of studies show that the tumor microenvironment plays an important role in tumor development, while the vicious circle of "inflammatory destruction-tissue repair" plays an important role in promoting the relapse and development of tumor [5]. In tumor tissues, many macrophages infiltrate, these cells are called tumor-associated macrophages (TAM) [6]. TAMs accounting for about 10%-65% of inflammatory cells are involved in the tumorigenesis, growth, invasion and metastasis of the tumor and their high infiltrations is closely related to the poor prognosis of the tumor [7]. According to the recent tumor immunology researches, the mononuclear cells reaching the tumor environment have 2 different types of polarization stimulated by different tumor mi-

**Table 1.** The specific primers used in the reactions

| | Sense primer | Reverse primer |
|---|---|---|
| MCP-1 | 5'-CCCAATGAGTAGGCTGGAGA-3' | 5'-TCTGGACCCATTCCTTCTTG-3' |
| Cox-2 | 5'-GGAGAGACTATCAAGATAGTGATC- 3' | 5'-ATGGTCAGTAGACTTTTACA-GCTC-3' |
| GAPDH | 5'-ATTGTCAGCAATGCATCCTG-3' | 5'-ATGGACTGTGGtcATGAGCC-3' |

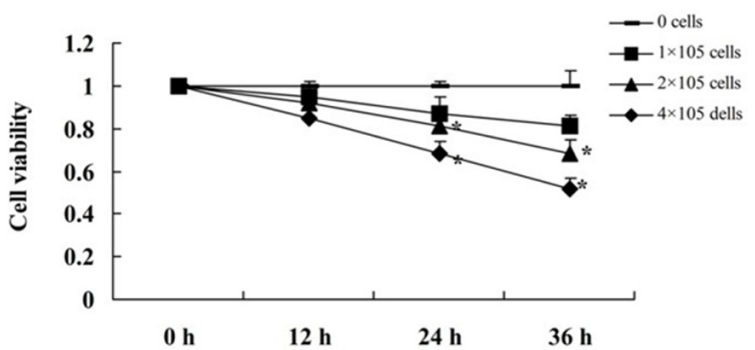


**Figure 1.** Effect of tumor-associated macrophages on gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

croenvironment, namely, classically activated macrophages (M1) and alternatively activated macrophages (M2) [6].

The greater omentum is one of the important immune organs in the abdomen. It will firstly carry out envelopment and devouring when the foreign body, bacteria and antigenic substances invade the abdominal cavity [8]. In the abdominal cavity metastasis of malignant tumor or the implantation of tumor cells in the abdominal cavity, the tumor cells are firstly implanted on the greater omentum and part of the transfer cell began to proliferate and form metastases [9]. These phenomena show that greater omentum has special defensive characteristics [10]. At present, it is believed that the greater omentum Milky spot plays a very important role in a series of defense mechanisms in greater omentum [11].

Milky spots in the greater omentum is a special anatomical structure which is an anatomical unit composed of omental capillaries and the macrophage gather-ed around, lymphocyteand mesenchymal cells [12]. It is an anatomical structure under-microscope and has the function of absorbing and dealing with the foreign body in abdominal cavity [13]. The current studies indicate that during the peritoneal metastasis of the gastric cancer cells, the cancer cells selectively invade the milky spot of greater omentum during the early stage of metastasis which is the first place of the adhesion infiltration metastasis of the gastric cancer cells [14]. At present for the mechanism, it is believed that the vascular endothelial cells, macropha-ges and other cells forming milky spots as a lymphoid tissue can secrete chemokines, while the gastric cancer cell surface have chemokine receptor [14]. With the regulation by chemokines, cancer cells selectively and specifically transfer into milky spots. In this process, the tumor cells home and aggregate in some way under the influence of some chemokines factor, which lay the foundation and conditions for metastasis.

## Materials and methods

### *Cell culture, tumor cell line and animals*

The study protocols were approved by the Committee or Animal Research of the first Affiliated Hospital, Dalian Medical University of China according to national guidelines. Macrophage THP-1 cells and Human gastric adenocarcinoma SGC-7901 cells were obtained from the central laboratory of the first Affiliated Hospital, Dalian Medical University (Dalian, China). All experiment cells were cultured in RPMI-1640 (Gibco Life Technologies, Los Angeles, CA, USA) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA) in an incubator of 5% $CO_2$ at 37°C. Six-week-old C57-/6J (B6) and SPF-BALB/C mice were obtained from the central laboratory of the first Affiliated Hospital, Dalian Medical University (Dalian, China) and maintained under standard laboratory conditions (22-23°C, 55% humidness) and had free access to standard laboratory food and water.

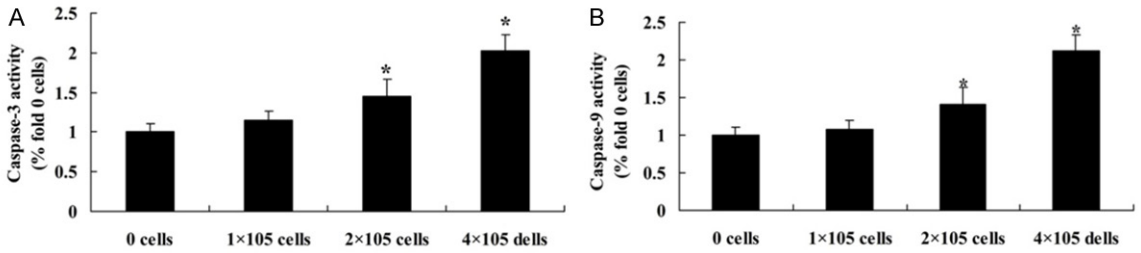


**Figure 2.** Effect of tumor-associated macrophages on caspase-3/9 activities in gastric cancer stem cell. Effect of tumor-associated macrophages on caspase-3 (A) and caspase-9 (B) activities in gastric cancer stem cell. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

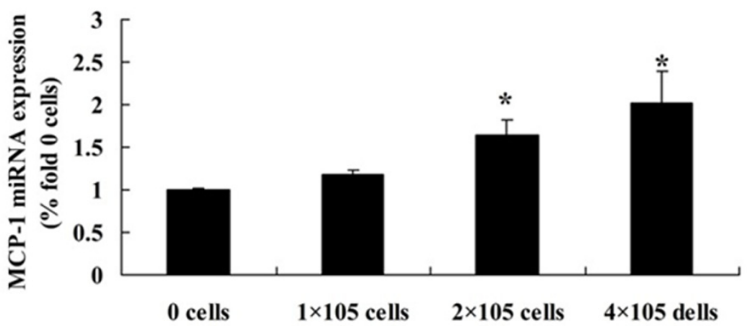


**Figure 3.** Effect of tumor-associated macrophages on MCP-1 miRNA expressions in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

*The cultivation of the gastric cancer stem cells*

SGC-7901 cells (2000/well) were seeded in 6-well plates and cultured with DMEM/F12 containing B27 (2%), N-2 (1%), EGF (20 ng/ml) and bFGF (10 ng/ml) (Gibco Life Technologies, Los Angeles, CA, USA) in an incubator of 5% $CO_2$ at 37°C. After 14-15 days, cells were collected and SGC-7901 cells (2000/well) were cultured with DMEM/F12 culture medium.

*Transwell experiments*

Transwell culture plates (Corning, Corning, NY, United States) composed upper and lower chambers separated and 0.4 mm polycarbonate membrane. Different concentrations of SGC7901 cells ($2\times10^4$/mL) were prepared in the lower chamber and pretreated with EP (Sigma-Aldrich Co. CA, USA) for 1 h. (0, $1\times10^5$/mL, $2\times10^5$/mL, $4\times10^5$/mL) THP-1 cells were prepared in the upper chamber.

*Cell culture and growth assay*

Cell growth of co-culture cells was determined by the 3-(4,5-methylthiazol-2yl)-2, 5-diaphenyl-tertrazolium bromide (MTT, Sigma-Aldrich Co. CA, USA) colorimetric assay. MTT solution was added into every well and its final concentration was 0.625 μg MTT/mL. Co-culture cells were in an incubator of 5% $CO_2$ at 37°C for 4 h. Then, cells were dissolved with 20 μL of DMSO solution and the optical density was determined with an ELISA plate reader.

*ELISA of caspase-3/9, IL-4 and IL-10 activities*

Co-culture cells were lysed in Radio Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher, Rockford, IL, USA) containing 1% phenylmethy lsulfonyl fluoride and protein concentration was measured using the BCA Protein Assay kit (Beyotime, Shanghai, China). In according with the manufacturer's protocol, caspase-3/9, IL-4 and IL-10 activities of co-culture cells was determined using ELISA assay kit (Sangon Biotech, Shanghai, China). The optical density was determined with an ELISA plate reader.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of MCP-1 and COX-2 miRNA expressions*

Total RNA was extracted from co-culture cells using TRIzol® (Takara Bio, Inc., Dalian, China),

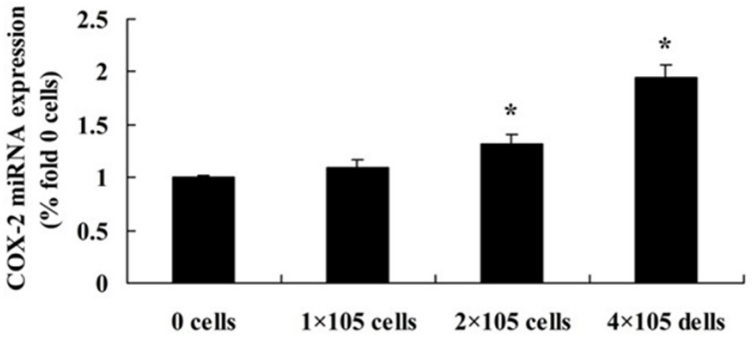


**Figure 4.** Effect of tumor-associated macrophages on COX-2 miRNA expressions in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

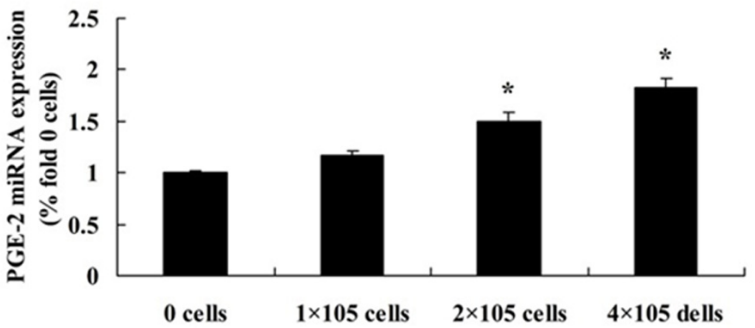


**Figure 5.** Effect of tumor-associated macrophages on PGE-2 release levels in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of RNA with a Prime Script Kit (TAKARA, Toyobo, Osaka, Japan). RT-qPCR was conducted using the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, USA). The specific primers used were listed at **Table 1**. The PCR were performed with a two-step qRT-PCR at 95°C for 15 min, then 40 cycles of 95°C for 45 s and 60°C for 45 min, followed by 95°C for 45 s.

*Western blot analysis*

Co-culture cells were lysed in Radio Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher, Rockford, IL, USA) containing 1% phenylmethy lsulfonyl fluoride and protein concentration was measured using the BCA Protein Assay kit (Beyotime, Shanghai, China). 50 μg of protein was separated on an 8-12% SDS-PAGE gel (Beyotime, Shanghai, China) and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was then blocked with 5% nonfat milk and incubated with anti-TGF-β (1:3000, Cell Signaling, Beverly, MA, USA), anti-IFN-γ (1:2000, Cell Signaling, Beverly, MA, USA), VEGF (1:2000, Cell Signaling, Beverly, MA, USA), anti-MMP-2 (1:4000, Cell Signaling, Beverly, MA, USA), anti-MMP-9 (1:2000, Cell Signaling, Beverly, MA, USA) and β-actin (1:5000, Beyotime, Shanghai, China) followed by incubation at 37°C for 1 h with horseradish peroxidase-conjugated antibody (1:2,000, Beyotime, Shanghai, China). The membrane was stained with ECL Plus (Beyotime, Shanghai, China), according to the manufacturer's instructions.

*Statistical analysis*

All data were presented as means ± S.D. Differences were assessed by one-way analysis of variance and Student's unpaired t-test, using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The *P*-value of <0.05 was considered statistically significant.

## Results

*Effect of tumor-associated macrophages on gastric cancer stem cell growth*

To examine that effect of tumor-associated macrophages on gastric cancer stem cell growth, cell viability was measured using MTT colorimetric assay. As shown in **Figure 1**, cell viability of co-culture cells was suppressed in dose-and time-dependent manner. Especially, at 24 h or 48 h after cultivation with $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells, cell viability of co-culture cells markedly suppressed was observed (**Figure 1**).

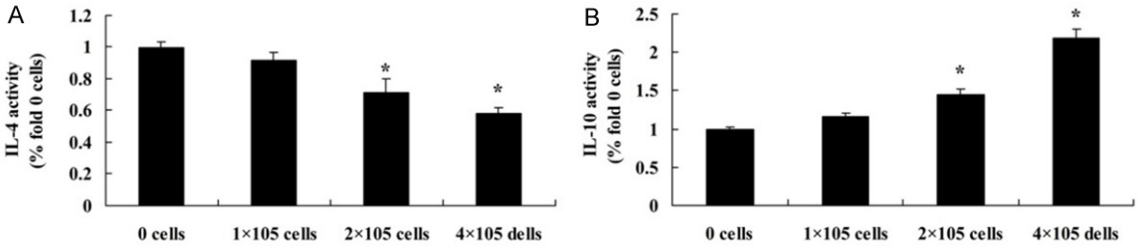


**Figure 6.** Effect of tumor-associated macrophages on IL-4 and IL-10 activities in gastric cancer stem cell growth. Effect of tumor-associated macrophages on IL-4 (A) and IL-10 (B) activities in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

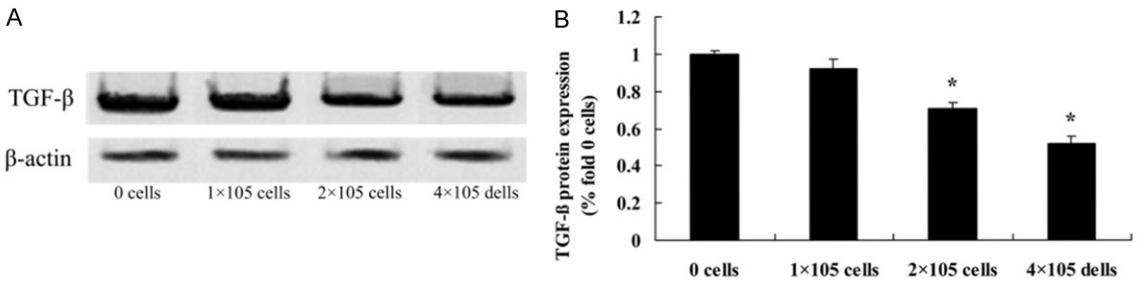


**Figure 7.** Effect of tumor-associated macrophages on TGF-β protein expression in gastric cancer stem cell growth. Effect of tumor-associated macrophages on TGF-β protein expression (A) using Western blot analysis (A) and statistical analysis of TGF-β protein expression (B) in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

*Effect of tumor-associated macrophages on caspase-3/9 activities in gastric cancer stem cell*

Next, we determined caspase-3/9 activities of co-culture cells by measuring ELISA kits. As shown in **Figure 2**, caspase-3/9 activities of co-culture cells were signally enhanced in dose-dependent manner, when cultivated with $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells.

*Effect of tumor-associated macrophages on MCP-1 miRNA expression in gastric cancer stem cell growth*

Next, we examined whether the effect of tumor-associated macrophages on MCP-1 miRNA expressions in gastric cancer stem cell growth. As shown in **Figure 3**, MCP-1 miRNA expressions of co-culture cells were dramaticlly advanced in dose-dependent manner after $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells cultivation.

*Effect of tumor-associated macrophages on COX-2 miRNA expression in gastric cancer stem cell growth*

We investigate whether the effect of tumor-associated macrophages on COX-2 miRNA expression of gastric cancer stem cell growth, RT-qPCR was used to examine COX-2 miRNA expression of co-culture cells. As shown in **Figure 4**, COX-2 miRNA expression of co-culture cells in $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells group was higher than those of gastric cancer stem cell growth-treated without THP-1 cells in dose-dependent manner.

*Effect of tumor-associated macrophages on PGE-2 release levels in gastric cancer stem cell growth*

We confirmed the effect of tumor-associated macrophages on PGE-2 release levels in gastric cancer stem cell growth. As shown in **Figure 5**, PGE-2 release levels of co-culture cells was sig-

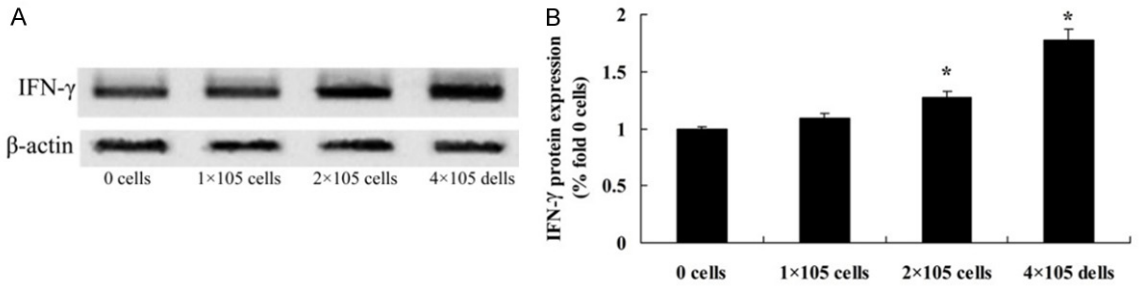


**Figure 8.** Effect of tumor-associated macrophages on IFN-γ protein expression in gastric cancer stem cell growth. Effect of tumor-associated macrophages on IFN-γ protein expression (A) using Western blot analysis (A) and statistical analysis of IFN-γ protein expression (B) in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

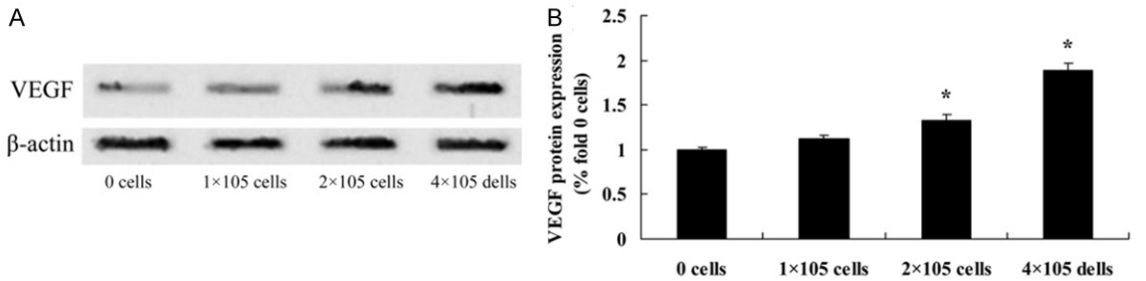


**Figure 9.** Effect of tumor-associated macrophages on VEGF protein expression in gastric cancer stem cell growth. Effect of tumor-associated macrophages on VEGF protein expression (A) using Western blot analysis (A) and statistical analysis of VEGF protein expression (B) in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

nificantly activated in SGC-7901 cells-induced by THP-1 cells ($2\times10^5$/mL or $4\times10^5$/mL).

*Effect of tumor-associated macrophages on IL-4 and IL-10 activities in gastric cancer stem cell growth*

We examine the effect of tumor-associated macrophages on IL-4 and IL-10 activities in gastric cancer stem cell growth. The IL-4 activity and IL-10 activity were significantly suppressed and increased, respectively, when $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells cultivation (**Figure 6**).

*Effect of tumor-associated macrophages on TGF-β protein expression in gastric cancer stem cell growth*

To further confirm the role of TGF-β protein expression in the effect of tumor-associated macrophages on gastric cancer stem cell growth, TGF-β protein expression was were detected. As shown in **Figure 7**, there was a significant increase in TGF-β protein expression of $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells group, compared with gastric cancer stem cell growth-treated without THP-1 cells group.

*Effect of tumor-associated macrophages on IFN-γ protein expression in gastric cancer stem cell growth*

To examine whether the effect of tumor-associated macrophages on IFN-γ protein expression in gastric cancer stem cell growth, IFN-γ protein expression was detected by Western blot analysis in SGC-7901 cells-induced by THP-1 cells. As shown in **Figure 8**, the expression of IFN-γ protein of $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells group was very higher than that of gastric cancer stem cell growth-treated without THP-1 cells in dose-dependent manner.

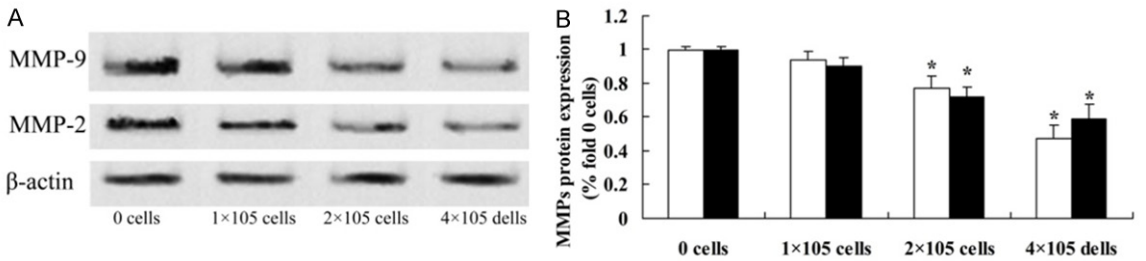


**Figure 10.** Effect of tumor-associated macrophages on MMP-2 and MMP-9 protein expression in gastric cancer stem cell growth. Effect of tumor-associated macrophages on MMP-2 and MMP-9 protein expressions (A) using Western blot analysis (A) and statistical analysis of MMP-2 and MMP-9 protein expression (B) in gastric cancer stem cell growth. 0 cell, gastric cancer stem cell growth-treated without THP-1 cells; $1\times10^5$ cell, gastric cancer stem cell growth-treated with $1\times10^5$ THP-1 cells; $2\times10^5$ cell, gastric cancer stem cell growth-treated with $2\times10^5$ THP-1 cells; $3\times10^5$ cell, gastric cancer stem cell growth-treated with $3\times10^5$ THP-1 cells; *P<0.01 compared with gastric cancer stem cell growth-treated without THP-1 cells.

*Effect of tumor-associated macrophages on VEGF protein expression in gastric cancer stem cell growth*

To investigate whether VEGF involved in the effect of tumor-associated macrophages on gastric cancer stem cell growth, VEGF protein expression was measured using Western blot analysis. As shown in **Figure 9**, VEGF protein expression of co-culture cells was observably activated by $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells cultivation.

*Effect of tumor-associated macrophages on MMP-2 and MMP-9 protein expressions in gastric cancer stem cell growth*

To confirm the roles of MMP-2 and MMP-9 protein expressions on the effect of tumor-associated macrophages on gastric cancer stem cell growth, we used Western blot analysis to analyze MMP-2 and MMP-9 protein expression. As shown in **Figure 10**, MMP-2 and MMP-9 protein expressions of co-culture cells in $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells group were lower than those of gastric cancer stem cell growth-treated without THP-1 cells in dose-dependent manner.

## Discussion

Peritoneal metastasis is the main form of the relapse of gastric cancer after operation and one of the main causes of death in gastric cancer [15]. Peritoneal metastasis of gastric cancer is a complicated process with multiple factors. It has been believed that "peritoneal seeding theory" is the formation mechanism of the gastric cancer peritoneal metastasis formation [16]. The theory says that the cancer cells fall off from gastric serosa and travel to peritoneal cavity and implant on the peritoneum for proliferation as the main way of metastasis under the influence of gravity [17]. However, this theory cannot explain all the actual situation of peritoneal metastasis of gastric cancer.

The characteristics of spot anatomy have a natural advantage in the peritoneal metastasis of the tumor. They are mainly concluded as: milky spots zone have no basement membrane; mesothelial cells appear intermittently arranged and are rich in tiny pores and the surface of have microvilli and filamentous projections. These physical anatomy characteristics contribute to the adhesion of free peritoneal cancer cells. Meanwhile, milky spots contain tortuous capillaries and the ends of lymphatic capillaries are directly open to milky spots. All these unique anatomical characteristics provide strong support and insurance for the blood supply and metastasis of the tumor cells. In our study, $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells markedly suppressed cell viability and induced caspase-3/9 activities in the gastric cancer stem cells.

Peripheral blood mononuclear cells form TAMs in tumor tissues recruited by tumor derived chemokines, including macrophage colony stimulating factor (M-CSF), vascular endothelial growth factor (VEGF) and angiogenesis factor-2 and chemokines (CCL2, CCL3, CCl4, CCL5, CCL7, CCL8) [18, 19]. In addition, hypoxia can

also promote tumor cells to release endotheliummonocyte activating polypeptide II, endothelin -2, and etc. CCL2 (MCP-1), whose main source is the secretion of tumor, is very important in the recruitment of TAMs [20]. For example, the infiltration of MCP-1 and TAMs in gastric cancer is significantly related to the malignant behavior of gastric cancer cells (angiogenesis, lymphatic infiltration and invasion) [21]. In this study, MCP-1 miRNA expressions of co-culture cells were dramaticlly advanced in dose-dependent manner after $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells cultivation.

It have been found that many chronic inflammatory diseases can increase the risk of cancer in situ, particularly the epithelial tumors, such as gastric cancer and lymphoma tumor associated with gastric mucosa [22]. The sites of chronic inflammation often have many macrophages and other inflammatory cells, while the differentiation of M is related to the tumor development stage [23]. Helicobacter pylori (HP) are clearly related to chronic atrophic gastritis and gastric cancer, namely, HP infect the expression of up-regulated gastric epithelial cells cyclooxygenase-2 (COX-2) /PGE-2, then the signal of PGE-2 improve the infiltration of M through the expression of up-regulated CCL2, finally, TNF-α secreted by M promote the malignant transformation of gastric epithelial tissue by Wnt signaling pathway [24, 25]. Our results showed that the gastric cancer stem cells-induced by THP-1 cells ($2\times10^5$/mL or $4\times10^5$/mL) significantly activated COX-2 and PGE-2 release levels.

IL-4, IL-10, TGF-β, pge-2 and other cytokine of inflammatory inhibition secreted from tumor cells restrain TAMs from mediated cytotoxicity effect and antigen presentation and TAMs also express [26]. TGF-β and IL-10 to further exacerbate immune suppression, such as the suppression of antitumor activity of CD8+T cells and the suppression of NK cells' expression of activating receptors NKG2-D and the suppression of the expression of ant-inflammatory cytokine IL-12 [27, 28]. In this study, IL-4 activity was significant inhibited, and IL-10 activity and TGF-β protein expression were significant improved in gastric cancer stem cell growth-treated with $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells.

The high density of TAMs infiltration in most tumors is associated with poor prognosis, such as gastric cancer, breast cancer, liver cancer, etc [29]. Studies have shown that high TAMs infiltration group in gastric cancer is related to the invasion depth of gastric cancer and lymph node metastasis but negatively correlated to the CD3-ξ expressed by tumor infiltrating lymphocytes (TILs), which indicates that TAMs may be related to the inhibition of T cell function [30]. In addition, the inhibitory molecule B7-H4 in B7 family expressed by TAMs in gastric cancer increase significantly being closely related to the gastric cancer invasion depth and invasion degree of blood vessels, lymphatic vessel which indicates that up-regulated TAMs expressed by B7-H4 inhabit the proliferation of CD4+T cells and the secretion of IFN-γ, and this is the key mechanism for the promotion of gastric cancer immune escape [31, 32]. In our study, the expression of IFN-γ protein of $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells group was very higher than that of gastric cancer stem cell growth-treated without THP-1 cells in dose-dependent manner.

Tumor cells induce the formation of lymphatic vessels by the lymphatic system metastasis through VEGF-C, VEGF-D and other cytokines [33]. The expression of VEGF-C and VEGF-D by TAMs suggest that TAMs may be involved in the generation of tumor lymphatic vessels [34]. The number of TAMs in gastric cancer was correlated with the lymph vessel density (LVD) and lymph node metastasis, which indicates that the expression of VEGF-C by TAMs may promote the generation of lymphatic vessels [33]. In hypoxic environment, gastric cancer cells are easy to recruiting TAMs and can highly express VEGF, MMP2, MMP9 to participate in the formation of tumor vessel and ECM degradation, which can promote the cell invasion and metastasis of gastric cancer [35]. EMT can induce local invasion and metastasis for tumor, and TAMs can clearly express many kinds of cytokines which can induce EMT, such as TGF-β and IL-6 [36]. It is suggested that gastric cancer cells induce EMT for the promotion of cancer cells' infiltration and metastasis and TAMs play a certain role in the induction of EMT in gastric cancer [37]. We found that VEGF protein expression was observably activated, and MMP-2 and MMP-9 protein expressions were significantly suppressed in gastric cancer stem cell growth-treated with $2\times10^5$/mL or $4\times10^5$/mL THP-1 cells.

In summary, this study demonstrates that effect of tumor-associated macrophages on gastric cancer stem cell in omental milky spots and lymph nodes micrometastasis through activation of MCP-1, COX-2, PGE-2, IL-10, IFN-γ, and VEGF, and suppression of IL-4, TGF-β, MMP-2 and MMP-9. Our results suggest that tumor-associated macrophages may be a potential new way of therapeutic strategies for gastric cancer.

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## Disclosure of conflict of interest

None.

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