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Mouse forestomach carcinoma cells immunosuppress macrophages through TGF-β1

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Background/aims: *Peritoneal implantation metastasis of gastric cancer cells is associated with poor prognosis. Peritoneal macrophages are the most important immune cells in the abdominal cavity to control tumor metastasis. In the present study, the immunosuppressive effects of mouse forestomach cells on macrophages were examined.* ***Materials and Methods:*** *Conditioned medium from mouse forestomach cell cultures were used to treat isolated peritoneal macrophages. A colorimetry-based phagocytosis assay was performed to investigate the functional change of macrophages. The alternation in cytokine secretion by macrophages was measured by enzyme-linked immunosorbent assay. Specific markers of macrophage polarization were analyzed by real-time reverse transcription polymerase chain reaction. The transforming growth factor-β1 signaling was evaluated by Western blotting. Neutralization experiments were performed by using transforming growth factor-β1 antibody.* ***Results:*** *The conditioned medium reduced the phagocytotic capability of macrophages. Lower tumor necrosis factor-α and interleukin-1β levels and higher interleukin-10 and vascular endothelial growth factor levels were observed. Real-time reverse transcription polymerase chain reaction showed increased mRNA levels of M2 macrophage markers. Further study revealed that transforming growth factor-β1 was significantly elevated in the conditioned medium and transforming growth factor-β1 signaling was activated in the macrophages with treatment with conditioned medium. Neutralization of transforming growth factor-β1 reversed the immunosuppressive effects on macrophages.* ***Conclusions:*** *Immunosuppressive macrophages can be induced by conditioned medium from mouse forestomach cell cultures. These effects seemed to be through the production of transforming growth factor-β1 by the tumor cells. Targeting transforming growth factor-β1 intervention might help in the control of peritoneal metastasis of gastric cancers.*

Key words: Mouse forestomach carcinoma cells, macrophages, transforming growth factor-β1, immunosuppression

Fare önmide karsinom hücreleri makrofajları TGF-β1 aracılığıyla immunosüprese etmektedir

Giriş ve Amaç: *Mide kanser hücrelerinin peritoneal dökülme metastazı kötü prognozla ilişkilidir. Abdominal kavitede tümör metastazını önlemede en önemli immune hücreler peritoneal makrofajlardır. Bu çalışmada, fare önmide hücrelerinin makrofajlar üzerindeki immünosupresif etkisi araştırılmıştır.* ***Gereç ve Yöntem:*** *Fare önmide hücre kültürlerinden elde edilen şartlı ortam, izole peritoneal makrofajların üzerinde uygulandı. Makrofajların fonksiyonel değişikliklerini değerlendirmek için kolormetrik tabanlı fagositoz testi uygulandı. Makrofajlardan salınan sitokinlerin değişimi enzim bağlı immun test yöntemiyle test edildi. Makrofaj polarizasyonunun özgün belirteçleri, gerçek zamanlı polimeraz zincir reaksiyonu ile analiz edildi. Değiştirici büyüme faktörü-β1 sinyali, Western blot ile değerlendirildi. Nötralizasyon deneyleri, değiştirici büyüme faktörü-β1 antikoru kullanılarak yapıldı.* ***Bulgular:*** *Şartlı ortam, makrofajların fagositik kapasitesini azaltmıştı. Daha az tümör nekrozu faktörü-α ve interlökin-1β seviyeleri ve daha yüksek interlökin-10 ve vasküler endotelyal büyüme faktörü değerleri gözlenmişti. Gerçek zamanlı polimeraz zincir reaksiyonu, M2 makrofaj belirteçlerinin mRNA seviyelerinde artış gösterdi. İleri analizde, şartlı ortamda değiştirici büyüme faktörü-β1'in anlamlı derecede yüksek olduğu ve şartlı ortamla muamele edilen makrofajlarda değiştirici büyüme faktörü-β1 sinyalizasyonunun arttığı gösterildi. Değiştirici büyüme faktörü-β1'in nötralize edilmesi, makrofajların üzerindeki immünosupresif etkiyi geri döndürmekteydi.* ***Sonuç:*** *İmmünosupresif makrofajlar, fare önmide hücre kültürlerinden elde edilen şartlı ortam ile uyarılabilir. Bu etkiler, tümör hücrelerinden değiştirici büyüme faktörü-β1 üretilmesiyle ilgili görülmektedir. Değiştirici büyüme faktörü-β1'in hedeflenmesi, mide kanserlerinin peritoneal metastazının kontrol edilmesine yardımcı olabilir.*

Anahtar kelimeler: Fare önmide karsinom hücreleri, makrofajlar, değiştirici büyüme faktörü-β1, immunosupresyon

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INTRODUCTION

Gastric cancer is the most common malignant tumor of the digestive tract. It has a poor prognosis and results in frequent death caused by postoperative relapse and metastasis (1,2). Peritoneal implantation metastasis of gastric cancers constitutes nearly 50% of postoperative relapses and is a leading cause of death in patients with gastric cancer (3,4). However, the detailed mechanisms of peritoneal metastasis of gastric cancer have not been fully understood. A better strategy to prevent and treat peritoneal metastasis is also required.

Peritoneal immune cells, including T lymphocytes, neutrophils, natural killer cells, and macrophages, are cellular components of innate immunity that protect against tumor cells (5). Peritoneal macrophages are the most important immune cells in the abdominal cavity, and the function of the macrophage is critical to prevent the peritoneal implantation metastasis of gastric cancer cells (6). The classically activated M1 macrophages are capable of phagocytosing microorganisms and tumor cells, antigen processing and presentation, and producing proinflammatory cytokines (7). Therefore, they are involved in the peritoneal immunity against infection and tumor cell invasion, and play a critical role in the cellular immunity against gastric cancer. On the contrary, the alternatively activated M2 macrophages display distinct function from M1 macrophages. M2 macrophages cannot prevent tumor progression, but rather facilitate tumor cell proliferation, angiogenesis and tissue remodeling (7,8). It has been reported that tumor cells can secrete inhibitory cytokines to evade immune surveillance (9). Peritumoral macrophages exhibit alternative activation possibly through the action of some cytokines secreted by the tumor cells.

In this study, mouse forestomach carcinoma (MFC) cells (10) and isolated peritoneal macrophages were recruited to investigate the immunosuppressive effects of gastric carcinoma cells on macrophages. MFC cell conditioned medium (CM) was collected and used to treat peritoneal macrophages. The phagocytotic ability, cytokine secretion and M1/M2 macrophage markers were analyzed. Further examination disclosed that transforming growth factor (TGF)-β1 might be the key cytokine through which MFC cells modify macrophage functions.

MATERIALS AND METHODS

Isolation of Peritoneal Macrophages and Cell Culture

Eight-week-old male C57BL6 mice were purchased from the Institute of Laboratory Animal Science of the Chinese Academy of Medical Science. Mice were sacrificed by cervical dislocation. Five milliliters of precooled RPMI 1640 medium was injected into the abdominal cavity using a syringe. After injection, gentle massage was performed on the peritoneum to dislodge attached cells. The peritoneal fluid was collected into another syringe, and the peritoneal cavity was washed twice. The fluid and wash solution were centrifuged, and the macrophages were purified using the adherence method. All the protocols were reviewed and approved by the Animal Care and Use Committee of the Third Military Medical University.

Both macrophages and MFC cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified, 5% CO_2 atmosphere. 1 x 10^6 MFC cells were plated into a 6-well plate. After culture for two days, the supernatant of MFC cells was collected as MFC cell CM and stored -20°C until use. Macrophages were treated with CM alone or together with 1 µg/ml TGF-β1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for two days and underwent subsequent analysis.

Phagocytosis Assay

The phagocytotic ability of macrophages was measured by CytoSelect phagocytosis assay kit (Cell Biolabs, Inc. San Diego, CA) according to the manufacturer's instructions. Zymosan was used as substrate in this assay. The absorbance of each sample was read at 405 nm.

Measurement of Cytokine Secretion

After treatment, the supernatants of macrophages were collected and the concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10, and vascular endothelial growth factor (VEGF) were measured by ELISA kit (Invitrogen, Carlsbad, CA).

RNA Extraction and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the macrophages by TRIzol reagent (Invitrogen), and first strand cDNA was generated by AMV reverse transcriptase (Takara, Dalian, China) with oligo dT-Adaptor

primer (Takara). Gene specific primers for inducible nitric oxide synthase (iNOS) (forward, 5-TTCTGTGCTGTCCCAGTGAG-3; reverse, 5-TGAAGAAAACCCCTTGTGCT-3), chemokine (C-X-C motif) ligand (CXCL) 11 (forward, 5-CGCCCCTGTTTGAACATAAG-3; reverse, 5-CTGCTGAGATGAACAGGAAGG-3), arginase-1 (forward, 5-TTTTTCCAGCAGACCAGCTT-3; reverse, 5-AGAGATTATCGGAGCGCCTT-3), and found in inflammatory zone 1 (Fizz1) (forward, 5-CTGGATTGGCAAGAAGTTCC-3; reverse, 5-CCCTTCTCATCTGCATCTCC-3) were used for expression analysis by real-time PCR on the ABI 7500 thermocycler (Applied Biosystem, Foster City, CA) using SYBR Green mix (Applied Biosystem). β-actin (forward, 5-ATGGAGGGGAATACAGCCC-3; reverse, 5-TTCTTTGCAGCTCCTTCGTT-3) was used for normalization.

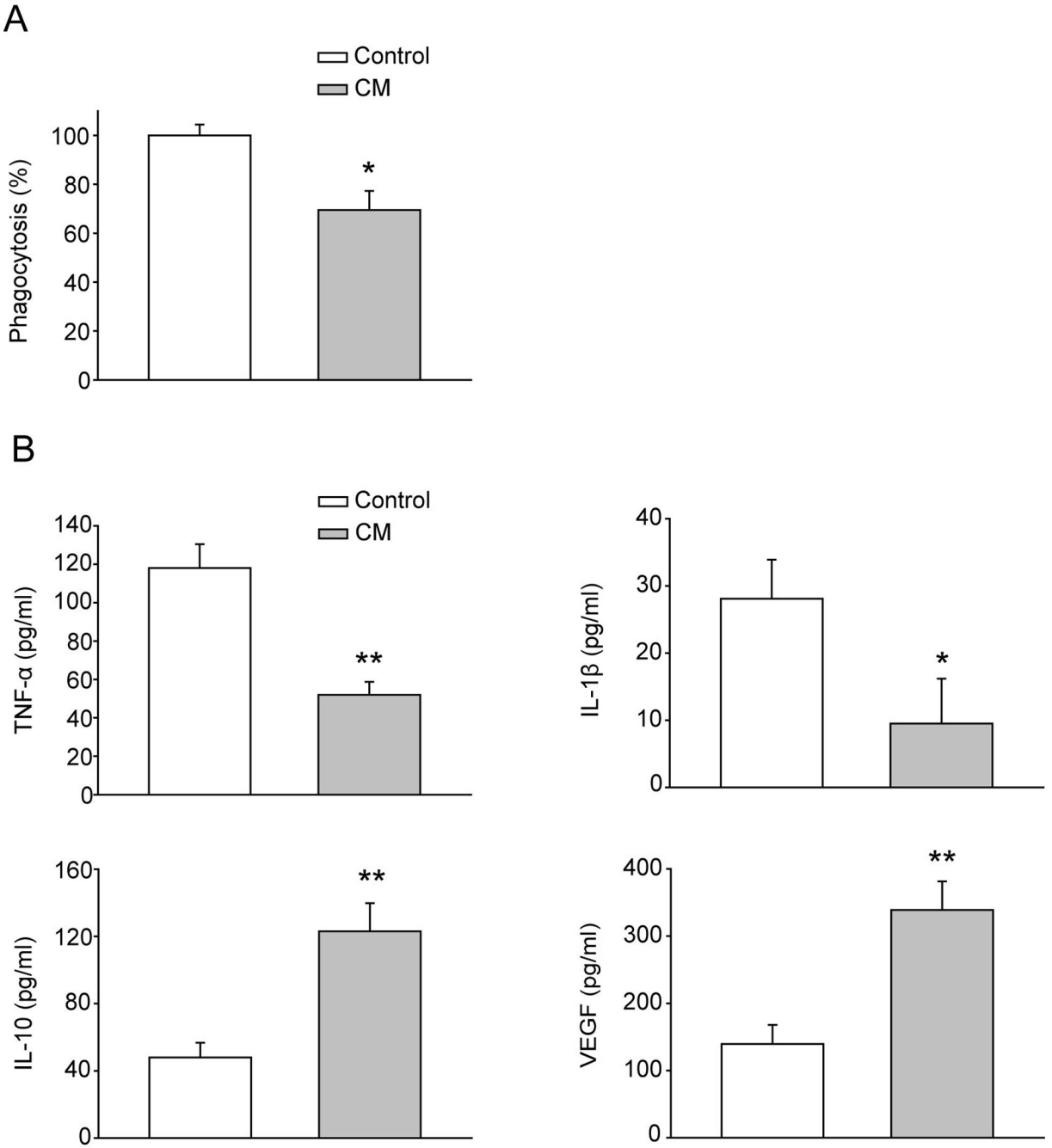

Figure 1. Conditioned medium induces immunosuppressive macrophages. (A) Isolated peritoneal macrophages were treated with conditioned medium for 2 days, and then phagocytosis assay was performed. (B) The concentration of cytokines secreted by macrophages were measured by ELISA. *, $p<0.05$; **, $p<0.01$ vs control, n = 3-5.

Western Blot Analysis

After treatment, macrophages were washed with ice-cooled phosphate-buffered saline twice and lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific Inc, Rockford, IL). Then, 20 μg of total protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). After blocking with 5% skim milk, antibodies for Smad2 or phosphorylated-Smad2 (p-Smad2) (Santa Cruz Biotechnology) were incubated with membrane overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was used to detect the protein levels.

Statistical Analysis

All values were expressed as means±SEM. Statistical analysis was performed using Student t-test. Differences with a $p<0.05$ were considered statistically significant.

RESULTS

MFC Cell CM Induces Immunosuppression of Macrophages

First, mouse peritoneal macrophages were isolated, and flow cytometer assay revealed that more than 90% of total cells were CD68-positive cells, which indicated the isolated cells were macrophages (data not shown). To determine the indirect effects of MFC cells on macrophages, the MFC cell CM was used to treat macrophages for two days. Phagocytosis assay was performed to evaluate the functional change of macrophages. As shown in Figure 1A, the CM significantly reduced the phagocytotic capability of macrophages. Macrophages also secreted much less TNF-α and IL-1β with CM treatment (Figure 1B). Meanwhile, the IL-10 secretion, which is an immunosuppressive cytokine, was elevated nearly three-fold compared to control cells. Interestingly, VEGF, which is able to promote angiogenesis and accelerate tumor growth, was also greatly increased with CM treatment. These data suggest MFC cell-derived soluble factors could generate a microenvironment that suppresses innate immunity of macrophages and induces angiogenesis, thus supporting the tumor progression.

MFC Cell CM Induces Macrophage Polarization

The immunosuppressive state of macrophages is always accompanied by the increase in alternatively activated macrophages, also called M2 macrophages. Therefore, real-time RT-PCR was performed to examine the markers of M1 and M2 macrophages. As shown in Figure 2, CM downregulated the mRNA levels of the M1 macrophage marker, iNOS, but not CXCL11. The M2 macrophage markers, arginase-1 and Fizz1, were increased with treatment with CM for two days, indicating CM stimulated the M2 macrophage polarization.

Increased Level of TGF-β1 in CM Activates TGF-β1 Signaling in Macrophages

Many different kinds of cytokines respond for macrophage immunosuppression. We further measured the concentration of these cytokines in the CM, including macrophage inhibitory cytokine-1, soluble colony-stimulating factor, TGF-β1, IL-4, and IL-10. Among them, only TGF-β1 level was dramatically elevated in the CM (3.05±0.58 pg/ml in RPMI 1640 medium, 107.56±4.82 pg/ml in CM, $n=4$, $p<0.01$ and data not shown). Next, the downstream of TGF-β1 signaling was examined by Western blotting. As shown in Figure 3A and 3B, CM treatment increased the expression level of p-Smad2. Moreover, when macrophages were treated with CM and TGF-β1 antibody together, the increased level of p-Smad2 was significantly diminished.

Neutralization of TGF-β1 Restores Macrophage Functions

Since CM was collected two days after culturing with MFC cells, the nutrient contents and growth factors in CM might be depleted due to MFC cell consumption, subsequently interfering in macrophage functions. To further confirm the specific role of TGF-β1 in the inhibitory effects of CM on macrophages, TGF-β1 antibody was added into the CM to neutralize TGF-β1. The phagocytosis assay revealed TGF-β1 antibody reversed the suppressive effects of CM on macrophages (Figure 4A). Further, the effects of CM on cytokine secretion was also blocked by TGF-β1 antibody (Figure 4B). These data suggest TGF-β1 is the key cytokine secreted by MFC cells to induce immunosuppressive macrophages.

DISCUSSION

In present study, we demonstrated that the immune functions of isolated peritoneal macrophages were restrained by CM, which contained the inhibitory cytokines secreted by MFC cells. The mac-

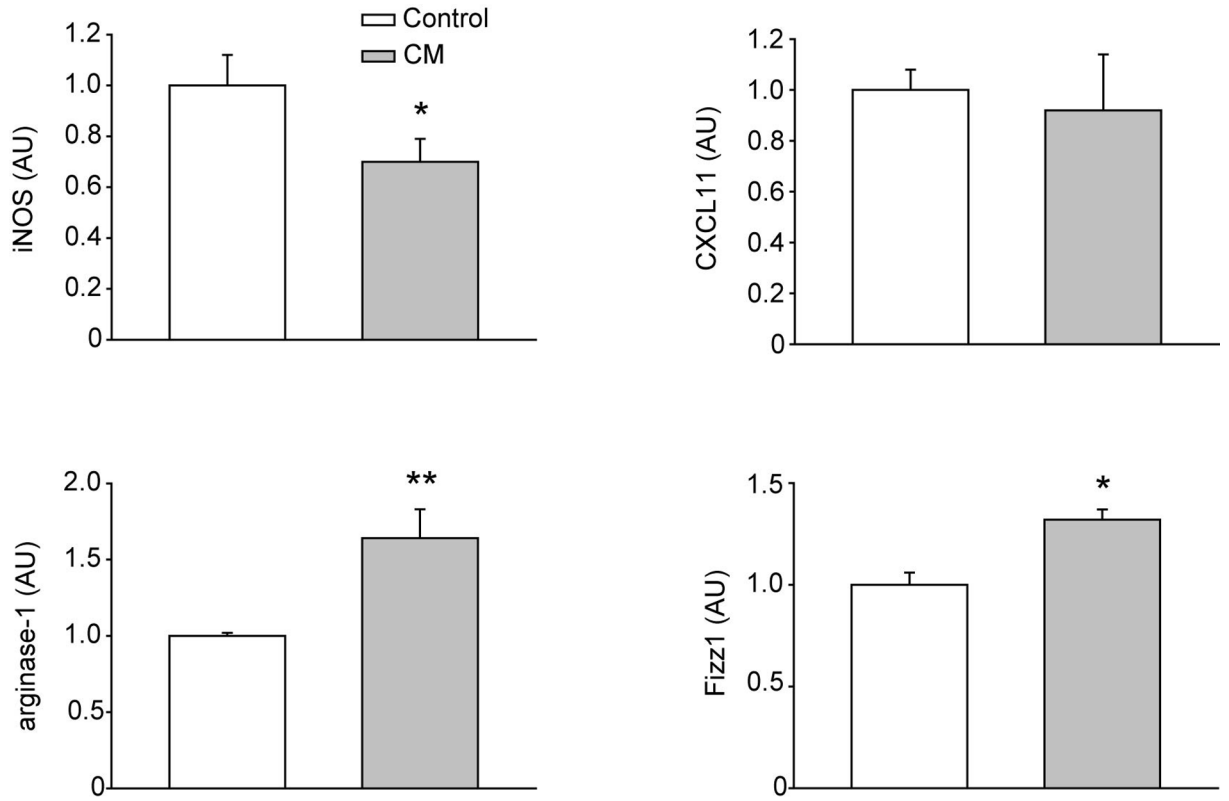

Figure 2. Conditioned medium induces M2 macrophage polarization. After treatment of conditioned medium for 2 days, RNA was extracted and gene expressions were analyzed by realtime RT-PCR with specific primers. *, $p<0.05$; **, $p<0.01$ vs control, n = 3. AU: arbitrary unit.

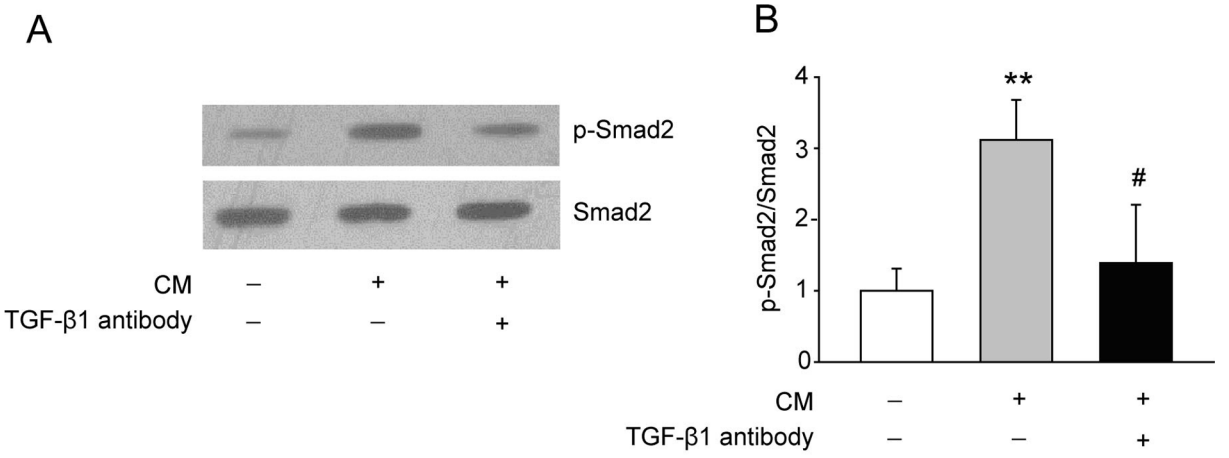

Figure 3. TGF-β1 signaling is activated in the macrophage by conditioned medium treatment. (A) The macrophages were treated with conditioned medium alone or together with TGF-β1 antibody. Then, the protein levels of Smad2 and the phosphorylation form of Smad2 were examined by Western blotting. (B) Densitometric analysis of the Western blotting data. **, $p<0.01$ vs control; #, $p<0.05$ vs CM-treated group, n = 3.

rophages treated with CM showed weaker phagocytosis, less TNF-α and IL-1β production, increased secretion of IL-10 and VEGF, and gain of M2 macrophage phenotypes. Among the different cytokines, the level of TGF-β1 in the CM was found greatly increased and the TGF-β1 signaling was activated in the macrophages, evidenced by the phosphorylation of Smad2. Neutralization of

TGF-β1 by its antibody helped macrophages retain their functions.

Macrophages are characterized by their remarkable versatility, heterogeneity and plasticity. They can respond to different cytokines and certain microbial products that are present in the microenvironment (11). The activated macrophages induced by interferon (IFN)-γ, either alone or in combination with lipopolysaccharide (LPS), produced a large amount of toxic agents, such as nit-

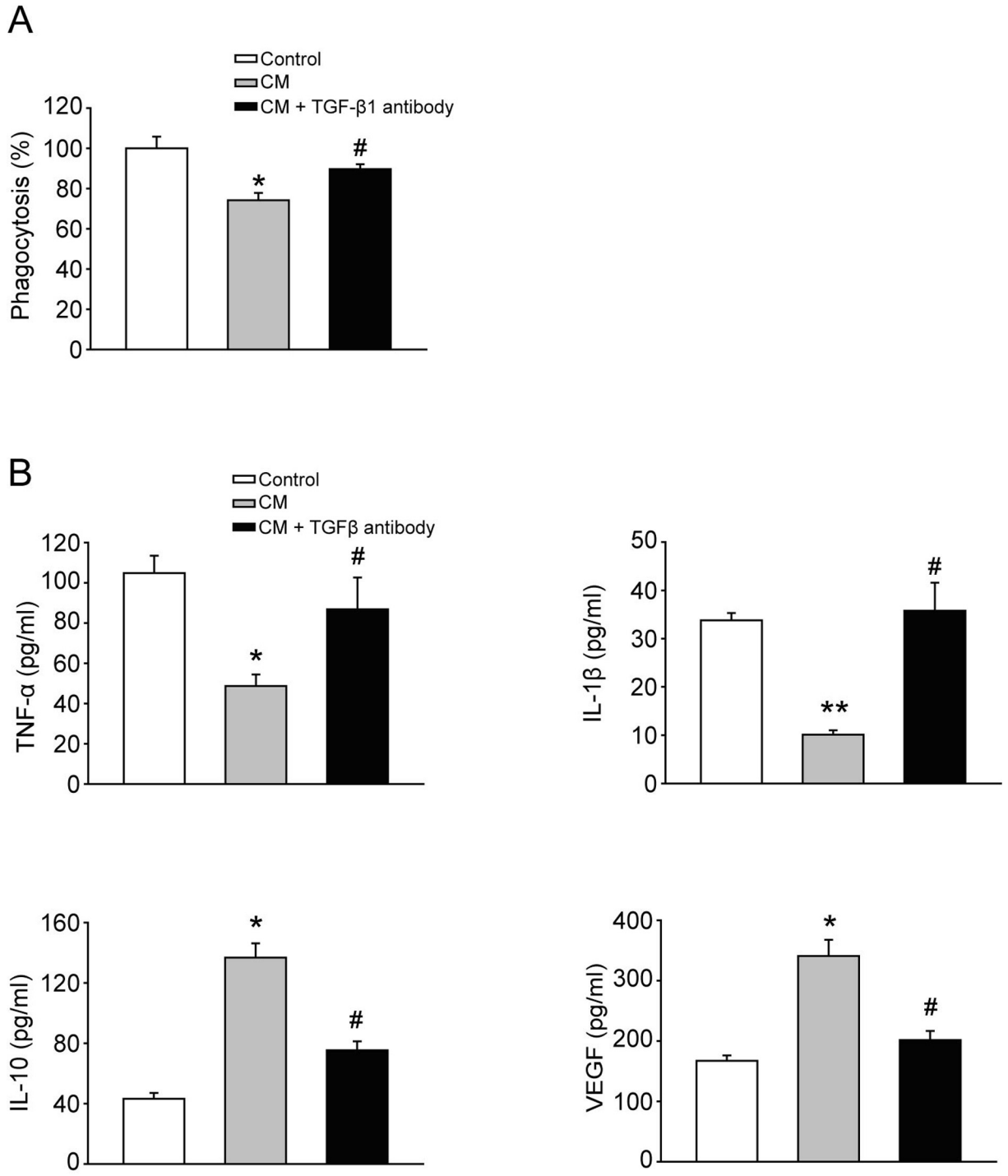

Figure 4. The inhibitory effects of conditioned medium were alleviated by TGF-β1 antibody. The macrophages were treated with conditioned medium alone or together with TGF-β1 antibody. All experimental procedures were the same as that described in Figure 1. *, $p<0.05$; **, $p<0.01$ vs control; #, $p<0.05$ vs CM-treated group, n=4-5.

ric oxide and reactive oxygen species, with strong antigen presentation capability, and they further activate type I immune response (7,8,11). Other macrophages can be alternatively activated by IL-4 and IL-10, known as M2 macrophages (12). M2 macrophages restrict inflammation and type I adaptive immunity, scavenge residues, promote angiogenesis, and participate in tissue remodeling and repair (7). The increased expression levels of arginase-1 and Fizz1, as well as reduced iNOS expression, indicated M2 polarization. However, the M1 macrophage marker CXCL11, which is an IFN-γ inducible gene (13), did not show a difference between the two groups. Several other kinds of tumors are also capable of inducing the M2 macrophages and promoting tumor progression, including glioma cancer (14), breast cancer (15) and hepatocellular carcinoma (16).

Our data suggested TGF-β1 played a central role in the CM to regulate macrophage functions. Blocking TGF-β1 signaling by its antibody decreased p-Smad2 expression and antagonized the immunosuppressive effects of CM. TGF-β1 is a class of multifunctional polypeptide growth factors that play important regulatory roles in cell proliferation and differentiation, extracellular matrix production, angiogenesis, apoptosis, and the immune system. TGF-β1 regulates cellular processes by binding to its cell-surface receptors and initiates the intracellular signaling by phosphorylating several transcription factors known as Smads (17). TGF-β1 acts in dual roles during tumorigenesis, as both tumor suppressor and tumor promoter. In the early stage, TGF-β1 controls cell growth and cell cycle progress. As tumor cells acquire certain genetic and epigenetic changes in the genome, they switch TGF-β1 response from inhibition of proliferation to promotion of growth, motility and invasion (18). The interference in phagocytosis by MFC cell-derived TGF-β1, together with the reduced proinflammatory cytokines and raised anti-inflammatory cytokines, may contribute to the escape of immune surveillance. Moreover, the CM induced more VEGF secreted from macrophages, suggesting macrophages could ameliorate tumor growth by enhanced angiogenesis. In gastric cancer patients, elevated serum TGF-β1 was observed and correlated with venous invasion (19). In addition, TGF-β1 receptor inhibitors could downregulate the invasion, migration and epithelial-to-mesenchymal transition of scirrhous gastric cancer cells, suggesting the autocrine role of TGF-β1 during tumorigenesis (20). The approaches targeting TGF-β1 signaling may possess beneficial effects on both tumor cells and macrophages.

In summary, the secreted factors by MFC cells were able to induce immunosuppressive macrophages, thus avoiding the immune restrictions, and transform macrophages into the tumor-promoting phenotype. There effects were mostly, if not all, through TGF-β1 secretion. The present study provided preliminary *in vitro* evidence of the central role of TGF-β1 in the interaction between MFC cells and peritoneal macrophages. The components of TGF-β1 signaling might be promising candidates for the prevention and management of the peritoneal metastasis of gastric cancers.

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