ABSTRACT

Purpose: Numerous studies have shown that the frequency of myeloid-derived suppressor cells (MDSCs) is associated with tumor progression, metastasis, and recurrence. Chemokine (C-C motif) ligand 3 (CCL3) may be secreted by tumor cells and attract MDSCs into the tumor microenvironment. In the present study, we aimed to explore the molecular mechanisms whereby CCL3 is involved in the interaction of breast cancer cells and MDSCs.

Methods: The expression of CCL3 and its receptors was investigated using real-time polymerase chain reaction, western blotting, and enzyme-linked immunosorbent assay. The cell counting Kit-8, wound healing, and transwell assays were performed to study cell growth, migration, and invasion. Cell cycling, apoptosis, and the frequency of MDSCs were investigated through flow cytometry. Transwell assays were used for co-culture and chemotaxis detection. Markers of the epithelial-mesenchymal transition (EMT) were determined with western blotting. The role of CCL3 in vivo was studied via tumor xenograft experiments.

Results: CCL3 promoted cell proliferation, migration, invasion, and cycling, and inhibited apoptosis of breast cancer cells in vitro. Blocking CCL3 in vivo inhibited tumor growth and metastases. The frequency of MDSCs in patients with breast cancer was higher than that in healthy donors. Additionally, MDSCs might be recruited by CCL3. Co-culture with MDSCs activated the phosphoinositide 3-kinase-protein kinase B-mammalian target of rapamycin (PI3K-AKT-mTOR) pathway and promoted the EMT in breast cancer cells, and their proliferation, migration, and invasion significantly increased. These changes were not observed when breast cancer cells with CCL3 knockdown were co-cultured with MDSCs.

Conclusion: CCL3 promoted the growth of breast cancer cells, and MDSCs recruited by CCL3 interacted with these cells and then activated the PI3K-AKT-mTOR pathway, which led to EMT and promoted the migration and invasion of the cells.
Funding
This study was supported by the National Natural Science Foundation of China (No. 8167102406).

Conflict of Interest
The authors declare that they have no competing interests.

Author Contributions
Conceptualization: Luo A; Data curation: Luo A, Han R; Formal analysis: Meng M; Investigation: Wang G; Software: Jing X; Supervision: Zhang Y, Zhao L; Validation: Gu S; Writing - original draft: Luo A; Writing - review & editing: Zhao X.

Keywords: Breast neoplasms; Chemokine CCL3; Epithelial-mesenchymal transition; Myeloid-derived suppressor cells; Tumor microenvironment

INTRODUCTION

Tumor growth, invasion, and metastasis are all inseparable from the tumor microenvironment. The stability of the tumor microenvironment provides the basis for cell metabolism, proliferation, and differentiation. The tumor microenvironment is composed of tumor parenchymal cells, fibroblasts, immune cells, adipocytes, blood vessels, and lymphatic vessels [1]. As an important part of the tumor microenvironment, immune cells play a unique role in immunotherapy. These include T, B, antigen-presenting, natural killer, and myeloid suppressor cells. The interactions between tumors and the immune system are inseparable from the multiple types of immune cell.

Myeloid-derived suppressor cells (MDSCs) are an important class of immunosuppressive cells found in a variety of tumors that form a group of primitive myeloid cells with no differentiation potential. MDSCs in humans and mice are divided into 2 types, namely, monocyte-type MDSCs (Mo-MDSCs) and polymorphonuclear-type MDSCs (PMN-MDSCs). MDSCs accumulate in most malignant tumors in mice and humans, and promote cancer progression by inhibiting antitumor immune responses, promoting angiogenesis, and creating a pre-metastatic environment. Through this mechanism tumors may evade the control by the immune system and impair the efficacy of the anticancer immunotherapy [2,3].

Tumor cells recruit MDSCs via chemokines such as CCL2, CCL3, and chemokine C-X-C motif 12 (CXCL12), and immune factors such as the granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-1β, and transforming growth factor (TGF)-β. MDSCs have many functions in tumors, such as immunosuppression and promoting tumor angiogenesis, growth, metastasis, and matrix deposition. Many studies have indicated that the proportion of MDSCs in the peripheral blood is significantly increased in a variety of tumors such as breast, pancreatic, gastric, and esophageal cancers, and melanoma; a high number of MDSCs are associated with poor prognosis [4-6]. When MDSCs are recruited into the tumor microenvironment, they interact with tumor cells. Studies have shown that MDSCs may promote the epithelial-mesenchymal transition (EMT) and activate multiple signaling pathways in tumor cells to promote tumor cell migration and distant metastasis [7-9]. The phosphoinositide 3-kinase (PI3K) pathway is involved in chemotaxis of leukocytes in vivo and regulates the function of MDSCs. A downstream component of the PI3K pathway, namely mammalian target of rapamycin (mTOR), affects the production of myeloid cells, which may be related to the production of MDSCs [10,11]. In addition, the activation of the PI3K pathway is closely related to the occurrence and development of tumors and affects the prognosis and therapeutic effects in patients with cancer [12,13]. However, the role of CCL3 in the interaction between breast cancer cells and MDSCs, the specific mechanism, as well as, which signaling pathway is activated are still unclear.

In the present study, we conducted in vitro and in vivo experiments to analyze the effect of CCL3 on breast cancer cells and their interaction with MDSCs, and investigated the potential underlying mechanisms. Results demonstrated that the CCL3–C-C chemokine receptor 5 (CCR5) axis is essential for the growth of breast cancer cells, and CCL3 plays a vital role in promoting EMT via the PI3K-protein kinase B (Akt)-mTOR signaling pathway in breast cancer cells when co-cultured with MDSCs.
METHODS

Patients and samples
Peripheral blood sample was collected from 48 patients with breast cancer and 44 healthy donors. All patients were diagnosed from June 2017 to May 2019 at the Department of Breast Surgery, First Affiliated Hospital of Medical School of Xi’an Jiaotong University. The patients included in this study received no treatment such as surgery or chemotherapy. Meanwhile, these patients had no other malignant tumor along with breast cancer and their record data were complete. The experimental protocol was approved by the Human Ethics Review Board of the First Affiliated Hospital of Medical School of Xi’an Jiaotong University and written informed consent was obtained from all subjects (Institutional Review Board approval number: XJTUIAF2019LSK-035).

Cell culture
Human breast cancer MDA-MB-231, MCF-7, T47D, and SK-BR-3 cell lines, or mouse breast cancer 4T1 cell line at passages 3 to 15 were obtained from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in the following media: DMEM, Leibovitz’s L15, or RPMI1640 supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, USA) in a humidified 5% CO2 incubator at 37°C.

MDSCs analysis and isolation
Peripheral blood of patients with breast cancer was collected in tubes with ethylenediaminetetraacetic acid anticoagulant and transported at 4°C. Peripheral blood mononuclear cells (PBMCs) were isolated from blood with the Ficoll-Paque plus (Amersham Biosciences, Piscataway, USA) density gradient centrifugation. PBMCs were stained with CD33 (BioLegend Inc., San Diego, USA), HLA-DR (BD Biosciences, Franklin Lakes, USA), CD14 (BioLegend Inc.), and CD15 (BD Biosciences) for 2 hours. Then, MDSCs stained with CD33 and HLA-DR were sorted on a FACS Aria cell sorter (BD, San Diego, USA). Finally, different subsets of MDSCs were analyzed through flow cytometry. This process yielded a cell-type suspension with purity > 95%.

Real-time polymerase chain reaction (RT-PCR)
Total RNA was extracted from tissues using TRIzol (Invitrogen; Thermo Fisher Scientific Inc., Waltham, USA) according to the manufacturer’s protocol. Synthesis of cDNA was performed using the TaKaRa PrimeScript® RT reagent Kit (Takara Bio Inc., Shiga, Japan). Quantitative RT-PCR was performed using the SYBR Premix Ex Taq TM system (TaKaRa Bio Inc.); primer sequences are listed in Table 1. The target gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase and calculated using the comparative threshold cycle (2^−ΔΔCT) method.

Western blot analysis
Cells were lysed in RIPA buffer in the presence of protease and phosphatase inhibitor. Samples were separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked for 2 hours at 30°C and then with primary antibodies diluted in Tris buffer saline-Triton X at 4°C overnight. The membranes were washed and incubated with the appropriate secondary antibodies (1:10,000; Cat. No. 93702; Cell Signaling Technology Inc., Danvers, USA). The primary antibodies included were: E-cadherin (1:1,000; Cat. No. 3195), N-cadherin (1:1,000; Cat. No. 13116), vimentin (1:1,000; Cat. No. 5741), Akt (1:1,000; Cat. No. 4685), p-Akt
(1:1,000; Cat. No. 4060), mTOR (1:1,000; Cat. No. 2983), p-mTOR (1:1,000; Cat. No. 5536), proliferating cell nuclear antigen (PCNA; 1:1,000; Cat. No. 5536) (Cell Signaling Technology Inc.), occludin (1:1,000; Cat. No. ab167161), CCR5 (1:1,000; Cat. No. ab65850), Snail (1:1,000; Cat. No. ab53519), matrix metalloproteinase-1 (MMP1) (1:1,000; Cat. No. ab137332), MMP2 (1:1,000; Cat. No. ab37150), and MMP9 (1:1,000; Cat. No. ab38898; Abcam, Cambridge, USA). The pathway inhibitors used in the assay were PI3K inhibitor-LY294002 (20 μM, pretreated for 12 hours; Cat. No. S1105; Selleck Chemicals, Houston, USA) and Akt inhibitor-MK2206 (1 μM, pretreated for 12 hours; Cat. No. S1078; Selleck Chemicals).

**Enzyme-linked immunosorbent assay (ELISA)**

The supernatants of 4 types of breast cancer cells were collected after 24, 48, and 72 hours. The supernatants of the breast cancer cells with CCL3-knockdown were collected after 24 hours. The CCL3 levels of these supernatants were detected with the CCL3 ELISA kit (Cat. No. PDMA00; R&D Systems Inc., Minneapolis, USA) according to the manufacturer’s instructions. Three wells per condition were tested and all experiments were repeated in triplicates.

**SiRNA transfection**

The transient transfection with small interfering RNAs (siRNAs) specific for CCL3 was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Negative control siRNAs and siRNA targeting CCL3 (Si-CCL3: 5′-CGCGAAGAAUCCACAGAUAUUTT-3′, 5′-AAUACUGGGAUAUCGCGCGG-3′) were synthesized by Qiagen (Cat. No. SI03078439; Hilden, Germany). CCL3 protein and mRNA levels were analyzed through RT-PCR and ELISA.

**Cell counting kit-8 (CCK-8) assay**

Cells were cultured in a 96-well plate with 5,000 cells/well. CCK-8 solution (10 μL; CCK-8; Dojindo Molecular Technologies Inc., Kumamoto, Japan) was added to each well for different amounts of time (24 and 48 hours) and incubated for 2 hours at 37°C. Cell proliferation was determined measuring the absorbance at 450 nm.

**Cell cycle analysis**

Some groups of cells were cultured at different CCL3 concentrations (1 and 10 nM; Cat. No. 300-08; Peprotech, Rocky Hill, USA), whereas another group of cells was pretreated with the CCR5 inhibitor (DAPTA; 1 nM; Cat. No. S8501; Selleck Chemicals) for 4 hours and then treated with CCL3. Otherwise, cells were transfected with specific siRNA targeting CCL3. After 24 hours, cells were treated with 75% ethanol at 4°C overnight, washed with phosphate buffer saline (PBS), and resuspended. The cell cycle was analyzed through flow cytometry.
after staining with propidium iodide (BD Pharmingen, Franklin Lakes, USA) and RNaseA (BD Pharmingen) according to the manufacturer's instructions.

**Apoptotic analysis**
Cells were cultured with different CCL3 concentrations (1 and 10 nM) or pretreated with DAPTA for 4 hours and then treated with CCL3. Otherwise, cells were transfected with specific siRNA targeting CCL3. After 24 hours, cells were stained with Annexin-V (BD Biosciences) and 7-AAD (BD Biosciences), and analyzed through flow cytometry.

**Wound healing assay**
Cells pretreated with CCL3 or pretreated with DAPTA for 4 hours and then treated with CCL3 were plated in 6-well plates, cultured in serum-free medium for 24 hours, and then scratched using a sterile 20-µL pipette tip. Cell cultures were washed with PBS, serum-free medium was added to the cells, and the wound size recorded at 0 and 24 hours using an inverted light microscope (Nikon Eclipse TS 100; Nikon Corporation, Tokyo, Japan). The migration rate was measured according to the changes of the wound width.

**Transwell migration and invasion assay**
Breast cancer cells pretreated with CCL3, DAPTA for 4 hours and then CCL3, or co-cultured with MDSCs were seeded on the upper chambers of 24-well transwell inserts (8 µm; Millipore, Billerica, USA). For the invasion assay, the underside of the insert membrane was coated with Matrigel (BD Biosciences). The bottom well contained 500 µL of RPMI-1640 medium containing 10% FBS. After 24 hours (migration assay) or 36 hours (invasion assay) at 37°C and 5% CO₂, the filtered cells were fixed in 4% paraformaldehyde solution and stained with 1% crystal violet. The stained cells from 5 selected views were observed under a light microscope at a × 200 magnification. In all experiments, each data point was performed in triplicates.

**Conditioned medium**
Breast cancer cells were cultured in serum-free medium for 24 hours. Supernatants were centrifuged and collected, and further purified through a 0.2 µm filter (Millipore).

**Chemotaxis**
MDSCs isolated from the peripheral blood of patients with breast cancer were resuspended and placed on the upper chambers of 24-well transwell inserts. The lower chamber contained 1 nM or 10 nM CCL3 in 500 µL of RPMI-1640 medium, or 500 µL of breast cancer conditioned medium. After 12 hours, the number of cells migrating into each bottom compartment was counted using a hemocytometer. Experiments were performed at least in triplicates.

**Co-culture of breast cancer cells with MDSCs**
A transwell insert (0.4 µm; Millipore) was used for the co-culture of breast cancer cells with MDSCs. MDSCs were added on top of the transwell insert and breast cancer cells were harvested in the bottom chambers. After 24 hours, breast cancer cells were isolated for RT-PCR, western blot, and the rest of the assays.

**Construction of 4T1 breast cancer-bearing murine models**
Four-week-old female BALB/c mice were purchased from Silaike Laboratory Animal (Shanghai, China) and housed in a pathogen-free animal facility supplied with autoclaved water and food. The mammary fat pads of BALB/c mice (n = 5 per group) were injected with 1 × 10^5 4T1 cells. Mice were randomly divided into control and anti-CCL3 group. The
CCL3 neutralizing antibody (Cat. No. MAB4502; R&D Systems Inc.) was intraperitoneally injected (1.6 μg per mouse) to mice every 2 days. The tumor volume was calculated using the formula: \( V = a \times b^2 / 2 \), where “a” is the length and “b” is the width of the tumor. The metastatic tumor nodules on lungs were counted and stained with hematoxylin and eosin (H&E). All procedures were conducted according to the guidelines approved by the Animal Ethics Review Board of Xi’an Jiaotong University.

**Immunohistochemistry (IHC)**

Sections were deparaffinized, rehydrated, and incubated with anti-E-cadherin (1:400; Cat. No. 3195; Cell Signaling Technology Inc.), anti-N-cadherin (1:125; Cat. No. 13116; Cell Signaling Technology Inc.), anti-PCNA (1:8,000; Cat. No. 13110; Cell Signaling Technology Inc.), or anti-Gr-1 (1:50; Cat. No. 553123; BD Biosciences) at 4°C overnight. Sections were further treated with horseradish peroxidase-conjugated secondary antibody (1:2,000; Cat. No. sc-2005; Santa Cruz Biotechnology Inc., Dallas, USA) for 30 minutes at 30°C. The visualization was performed with the DAB substrate (Dako; Agilent Technologies Inc., Santa Clara, USA) for 2 minutes and counterstained using hematoxylin. The tissue sections were examined under a light microscope and 5 high-power fields (magnification × 400) for each section were randomly selected for histological evaluation. The staining intensity was categorized into 4 grades: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The percentage of positively stained cells was categorized into 5 grades: 0 (no staining), 1 (≤ 10%), 2 (11%–50%), 3 (51%–80%) and 4 (≥ 81%). The IHC score was the sum of the multiplication of the staining intensity grade and the percentage of positive cells.

**H&E staining**

Sections were deparaffinized, rehydrated, stained with hematoxylin for 10 minutes, and then treated with hydrochloric acid alcohol for several seconds. Sections were washed, placed in eosin for 5 minutes, and dehydrated in 70% and 100% alcohol for 10 minutes each step. The sections were then placed in xylene for 5 minutes until they became transparent. Five fields (magnification, × 200 and × 400) for each tissue section were randomly selected for analysis.

**Statistical analysis**

Data were analyzed using the SPSS software (version 22.0; IBM., Armonk, USA). Results were expressed as mean ± standard deviation /standard error of the mean from 3 independent experiments. The 2-tailed Student’s t-test and analysis of variance were used to assess differences between experimental groups. The \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**The CCL3–CCR5 axis promotes cell proliferation, migration, invasion, and cycling of breast cancer cells in vitro**

We examined CCL3 and its receptors in 4 breast cancer cell lines. MDA-MB-231 cells exhibited the highest mRNA level of CCL3 and the expression of CCR5 was higher in MDA-MB-231 and MCF-7 cells. Lower levels of CCL3 and CCR5 were observed in T47D and SK-BR-3 cells (Figure 1A and B). The ELISA analysis indicated that MDA-MB-231 cells exhibited the highest protein level of CCL3, which increased as the culture period was prolonged (Figure 1C). MDA-MB-231 and MCF-7 cells were used for further studies. Further experiments were performed to investigate how the CCL3–CCR5 axis affected the biological functions of breast cancer cells. The CCK-8 analysis showed that CCL3 was able to promote cell...
proliferation (Figure 1D). Flow cytometry was used to determine the cell cycle distribution of breast cancer cells and identify changes in their apoptosis rate. The cell cycling analysis indicated that the percentage of cells in the S-phase increased when breast cancer cells were treated with CCL3. In contrast, the percentage of cells in the S-phase decreased when the cells were treated with DAPTA, which is a potent and specific antagonist of CCR5 (Figure 1E). Moreover, the results indicated that the apoptosis rate of breast cancer cells decreased when the cells were treated with CCL3 but increased when DAPTA was added. However, this result had no practical significance because the total apoptotic rate was too low (below 10%) (Figure 1F). The effect of the CCL3–CCR5 axis on the migration and invasion of breast cancer cells was determined with the wound healing and transwell assays, which showed that cancer cells treated with CCL3, were more motile than control cancer cells (p < 0.05; Figure 1G and H). However, breast cancer cells exhibited a significant decrease in migration when pretreated with DAPTA. The number of invading breast cancer cells significantly increased when the cells were treated with CCL3, whereas the number was significantly lower than that of cancer cells treated with CCL3 alone when the cells were treated with DAPTA and then CCL3 (p < 0.001; Figure 1H). Hence, the CCL3–CCR5 axis is crucial for the proliferation, migration, invasion, and survival of breast cancer cells in vitro.

**Knockdown of CCL3 in breast cancer cells inhibits cell proliferation, migration, invasion, and cycling, and promotes apoptosis of breast cancer cells in vitro**

We investigated the effects of CCL3 knockdown on the biological behavior of MDA-MB-231 and MCF-7 cells. Fluorescence microscope, RT-PCR and ELISA showed the transfection and knockdown effect of CCL3 in breast cancer cells (Supplementary Figure 1). Compared with

![Figure 1](https://ejbc.kr)
siNC cells, the expression level of CCL3 were inhibited up to 80–90% in both siCCL3 cell lines (p < 0.001). The treatment with siRNA targeting CCL3 significantly decreased the levels of cell proliferation (p < 0.001; Figure 2A). The cell cycling analysis indicated that CCL3 knockdown in breast cancer cells decreased the percentage of cells in the S-phase (p < 0.01 and p < 0.001; Figure 2B). The flow cytometry analysis revealed that CCL3 knockdown increased the rate of apoptotic breast cancer cells (Figure 2C). The wound healing and transwell assays
showed that the migration and invasion abilities of both cell lines were reduced after CCL3 knockdown ($p < 0.001$; Figure 2D-I). Collectively, these data showed that CCL3 knockdown in breast cancer cells exhibited strong effects in terms of inhibiting cell proliferation, migration, invasion, and cycling, while promoting apoptosis.

The frequency of MDSCs increases in peripheral blood of patients with breast cancer

We found a significant increase in MDSCs levels in breast cancer patients compared with healthy donors when the MDSCs population was calculated as a percentage of the total PBMCs (8.08% ± 3.31% vs. 3.11% ± 1.36%, $p < 0.001$; Figure 3A). We also identified the 2 subtypes of MDSCs and found that the percentage of PMN-MDSCs was higher than that of Mo-MDSCs ($p < 0.001$; Figure 3B). Moreover, we observed that the frequency of MDSCs was correlated with some clinical characteristics of patients with breast cancer. The percentage of MDSCs in patients in the advanced stages (III–IV) was higher than that in patients in the...
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CCL = chemokine (C-C motif) ligand; CCK-8 = cell counting kit-8; si = short interfering; NC = negative control.

CCL3 attracted MDSCs and the number of MDSCs increased with the increase in CCL3 concentration. The difference in comparison with the control group was significant (p < 0.001; Figure 4A). Then we placed conditioned medium from breast cancer cells in the lower chambers and MDSCs in the upper chambers. A statistical analysis showed a decrease in chemotaxis when the conditioned medium was obtained from CCL3 knockdown breast cancer cells (p < 0.001; Figure 4B).

Figure 2. (Continued) CCL3 knockdown inhibits cell proliferation, migration, invasion, and cycling and promotes apoptosis of breast cancer cells in vitro. (A) The effect of CCL3 knockdown on the proliferation of breast cancer cells was determined with the CCK-8 assay. The effect of CCL3 knockdown on the cell cycling (B) and apoptosis (C) of breast cancer cells was determined by flow cytometry. The effect of CCL3 knockdown on the migration of breast cancer cells was determined with the wound healing (D, magnification, ×100; G) and the transwell assays (E, magnification, ×200; H). The effect of CCL3 knockdown on the invasion of breast cancer cells was determined by the transwell assay (F, magnification, ×200; I). Three experiments for each setting were performed.

CCL = chemokine (C-C motif) ligand; CCK-8 = cell counting kit-8; si = short interfering; NC = negative control.

*p < 0.01, †p < 0.001 vs. controls.

early stages (I–II) (p = 0.001; Figure 3C). Besides, patients with an extensive tumor burden, lymph node metastases, distant metastases, and poorly differentiated tumors had a higher percentage of MDSCs and there was a significant difference between the 2 groups (p < 0.05). Age and menopause status were not related to the percentage of MDSCs.

CCL3 recruits MDSCs in vitro

MDSCs were sorted through flow cytometry and then we used the transwell assay to investigate the chemotactic effect of CCL3 on MDSCs. First, we added recombinant human CCL3 (rhCCL3) to the lower chambers and MDSCs to the upper chambers. We found that CCL3 attracted MDSCs and the number of MDSCs increased with the increase in CCL3 concentration. The difference in comparison with the control group was significant (p < 0.001; Figure 4A). Then we placed conditioned medium from breast cancer cells in the lower chambers and MDSCs in the upper chambers. A statistical analysis showed a decrease in chemotaxis when the conditioned medium was obtained from CCL3 knockdown breast cancer cells (p < 0.001; Figure 4B).
Blockade of CCL3 inhibits the growth and metastasis of breast cancer cells in mice and reduces the recruitment of MDSCs

In vitro experiments revealed that CCL3 might promote cell proliferation, migration, and invasion, and regulate the cell cycle of breast cancer cells, which prompted us to investigate the effect of CCL3 in vivo. We injected 4T1 cells into the mammary fat pads of mice to explore the effect of CCL3 on tumor cells in vivo. We found that tumors of the control group were larger and heavier than those of the anti-CCL3 group ($p < 0.001$; Figure 5A-C); many more lung metastatic nodules were observed in mice of the control group. A quantitative analysis showed that the mean number of lung metastatic nodules in the control group was significantly higher than that in the anti-CCL3 group ($p < 0.001$; Figure 5D and E).
Histological staining clearly showed metastatic tumors in mice lungs (Figure 5F). The length and weight of the spleens in the anti-CCL3 group decreased, which may be related to the decrease in the accumulation of MDSCs (Figure 5G-I). The immunohistochemical (p < 0.001; Figure 5J) analysis revealed that the number of Gr-1+ cells in the tumors, lungs, and spleens of the control group was higher than that of the anti-CCL3 group. This result suggested that blocking CCL3 in mice might reduce the recruitment of MDSCs and CCL3 might attract MDSCs in vivo. Considering that CCL3 might promote the proliferation of breast cancer cells in vitro and CCL3 and MDSCs might affect the EMT, PCNA and EMT-related markers such as E-cadherin and N-cadherin were analyzed using IHC and western blot. The staining intensity of anti-PCNA and anti-N-cadherin in control tumors was significantly higher than that in anti-CCL3 tumors, while the E-cadherin level increased in anti-CCL3 tumors (p < 0.001; Figure 5K). Western blot analysis also showed that the level of PCNA and N-cadherin in control tumors was higher than that in anti-CCL3 tumors, while the E-cadherin level increased in anti-CCL3 tumors (p < 0.01 and p < 0.001; Supplementary Figure 2). These results indicated that CCL3 played a key role in the recruitment of MDSCs and regulated the EMT, tumor growth, and metastasis of breast cancer cells.

**CCL3 is crucial for promoting the proliferation, migration, and invasion of breast cancer cells after co-culture with MDSCs**

EMT is a crucial step in cancer invasion and metastasis. In previous studies, it was found that both CCL3 and MDSCs play important roles in the migration and invasion of breast cancer
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Figure 5. Blocking CCL3 inhibits the growth and metastasis of breast tumors and reduces the recruitment of MDSCs in mice. (A) Growth of breast tumors. Volume (B) and weight (C) of tumors in control (n = 5) and anti-CCL3 (n = 5) mice. Macroscopic image (D) and H&E staining (F, magnification, ×200 and ×400) of the lungs and livers of inoculated mice and the number of metastatic nodules in the lungs (E). (G-I) Volume and weight of the spleens in the 2 groups. Representative images of immunohistochemical staining of Gr-1 in the tumors, lungs, and spleens (J, magnification, ×200 and ×400), and E-cadherin, N-cadherin, and PCNA in the tumors (K, magnification, ×200 and ×400). Data are from 3 independent experiments and represent 3 sections from each sample and 5 fields for each tissue section of 5 mice in the control and anti-CCL3 groups. CCL = chemokine (C-C motif) ligand; MDSC = myeloid-derived suppressor cell; PCNA = proliferating cell nuclear antigen.

* p < 0.05, † p < 0.01, ‡ p < 0.001 vs. controls.
to understand better their crosstalk, in vitro co-cultures of breast cancer cells and MDSCs were established. MCF-7 cells, which initially exhibited a typical cobblestone-shaped phenotype with less aggressive invasion behavior, when co-cultured with MDSCs underwent a notable morphological change to a spindle phenotype whereby the cell gap was increased and the cell polarity was lost. However, this failed to occur when MCF-7 cells underwent CCL3 knockdown. MDA-MB-231 cells showed no remarkable morphological change when co-cultured with MDSCs (Figure 6A). Then we used the CCK-8 assay to detect whether the proliferation capacity of breast cancer cells would change after co-culture with MDSCs. As shown in Figure 6B, co-culture with MDSCs promoted cell proliferation. However, there were no remarkable differences in the proliferation capacity after CCL3 knockdown in breast cancer cells when co-cultured with MDSCs. We found that the morphology of breast cancer cells changed when they were co-cultured with MDSCs. Hence, we hypothesized that the migration and invasion abilities of these cells might change as well. As shown in Figure 6C-F, the number of migrating and invading breast cancer cells increased.

Figure 6. Co-culture with MDSCs promotes cell proliferation, migration, invasion, and the EMT in breast cancer cells. (A, magnification, ×200) Morphological changes in MDA-MB-231 and MCF-7 cells. (B) The effect of co-culture with MDSCs on the proliferation of breast cancer cells was determined with the CCK-8 assay. The effect of co-culture with MDSCs on the migration (C, magnification, ×200; E) and invasion (D, magnification, ×200; F) of breast cancer cells was determined with the transwell assay. The effect of CCL3 knockdown on the invasion of breast cancer cells was determined with the transwell assay (F). Western blot analysis of EMT markers (G), MMPs (H), and Akt and p-Akt (I) in breast cancer cells with or without CCL3 knockdown after the cells were co-cultured with MDSCs. (J) The protein levels of p-Akt, Akt, p-mTOR, mTOR, E-cadherin, N-cadherin, MMP2, and MMP9 in different groups of breast cancer cells after treatment with LY294002 or MK2206 were determined through western blot. Images and data are representative of 3 independent experiments.

MDSC = myeloid-derived suppressor cell; EMT = epithelial-mesenchymal transition; CCK-8 = cell counting kit-8; CCL = chemokine (C-C motif) ligand; si = short interfering; NC = negative control; NS = no significance; MMP = matrix metalloproteinase; Akt = protein kinase B; p-Akt = phospho-protein kinase B; mTOR = mammalian target of rapamycin; p-mTOR = phospho-mammalian target of rapamycin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

*p < 0.001.
after the cells were co-cultured with MDSCs ($p < 0.001$). However, a small difference was observed in migration and invasion abilities between cancer cells alone and co-cultured cancer cells after these cells were treated with siRNA targeting CCL3 ($p > 0.05$). Therefore, co-culture with MDSCs promoted the proliferation, migration, and invasion of breast cancer cells in vitro and CCL3 played an important role in this process.

MDSCs promote the EMT in breast cancer cells via activation of Akt and mTOR phosphorylation in breast cancer cells

To investigate whether MDSCs promote the migration and invasion of breast cancer cells via EMT, we used the transwell system to co-culture breast cancer cells with MDSCs and determined the expression levels of EMT markers by western blot. The results showed that co-culture with MDSCs caused an increase in the expression of the mesenchymal markers N-cadherin, vimentin, and Snail and a decrease in the expression of the epithelial markers E-cadherin and occludin. However, even when the cells were co-cultured with MDSCs, the treatment of breast cancer cells with siRNA that targeted CCL3 did not significantly change the relative expression levels of E-cadherin, occludin, N-cadherin, vimentin and Snail (Figure 6G).

Moreover, we found that the expression levels of MMP2, MMP9, and p-Akt increased when breast cancer cells were co-cultured with MDSCs, but their expression did not change when the cells with CCL3 knockdown were co-cultured with MDSCs (Figure 6H-I).
To further confirm that MDSCs promote EMT in breast cancer cells via activating the PI3K-Akt-mTOR signaling pathway, we treated breast cancer cells with the PI3K inhibitor LY294002 and the Akt inhibitor MK2206. The protein levels of Akt and mTOR did not change when breast cancer cells were co-cultured with MDSCs or treated with the inhibitors. The co-culture with MDSCs increased the levels of phosphorylation of Akt and mTOR compared to the levels in control cells. When co-cultured breast cancer cells were treated with LY294002 or MK2206, the expression of p-Akt and p-mTOR was significantly inhibited, and a similar change was observed in the expression of MMP2 and MMP9. In addition, co-culture with MDSCs increased the expression of N-cadherin and decreased the expression of E-cadherin compared to the expression in control cells; however, the treatment with LY294002 or MK2206 reversed this phenomenon. There were no significant changes among the control cells and the breast cancer cells upon treatment with LY294002 or MK2206 (Figure 6J).

Collectively, these data indicated that MDSCs induced the EMT and enhanced the expression of MMPs in cancer cells via activating the PI3K-Akt-mTOR signaling pathway, which might increase the invasion and metastasis ability of breast cancer cells.

**DISCUSSION**

A number of studies have shown that chemokines play an important role in the development of breast cancer by promoting tumor cell proliferation and angiogenesis, inducing cell migration, accelerating the spread and metastasis of tumors, and participating in the immune escape [16]. Chemokines bind to membrane surface receptors on target cells triggering the activation of a series of downstream effectors and a cascade of signal peptides and ultimately promoting cell migration and invasion. The binding of chemokines to their receptors can lead to cell migration, which may directly affect the ability of breast cancer cells to invade the extracellular matrix, and chemokines play an important role in determining the location of tumor cell metastasis [17]. CCL3 promotes tumor growth when it binds to CCR1 or CCR5 [18]. In addition, CCL3 enhances cell migration and metastasis by upregulating the expression of MMP-2 in chondrosarcoma cells [19]. Our results are consistent with those of previous studies. We pretreated breast cancer cells with rhCCL3 and found that CCL3 promoted cell proliferation, migration, and invasion. When the expression of CCL3 was inhibited, the effect was the opposite. Chemokines may also directly affect the activity of cancer cells in primary tumors and promote the migration of tumor cells to secondary organs [17]. Wu et al. [20] found that CCR5+ but not CCR1+ cells accumulate in metastases, and the number of lung metastases is significantly reduced upon CCL3 or CCR5 knockdown. The CCL3–CCR5 axis may induce the accumulation of inflammatory cells and increase the expression of MMP9 to promote neovascularization. CCL3 may enhance various functions of tumor cells, especially their motility, and thus promote tumor metastasis. Qian et al. [15] also found that the knockdown of CCL3 or the use of anti-CCL3 antibodies may reduce the metastatic potential of tumor cells in vivo and significantly reduce the metastasis of breast cancer cells to the lungs. The results of our study showed that using the anti-CCL3 neutralizing antibody might significantly slow down the growth rate of tumors in mice and reduce the tumor volume. Macroscopic images and HE staining showed that the lungs had remarkable metastases, whereas the liver did not exhibit notable metastases; the number of lung metastases in the experimental group injected with anti-CCL3 neutralizing antibodies was significantly reduced.

We used the transwell assay to study the chemotactic effect of CCL3 on MDSCs and found that CCL3 might attract MDSCs in vitro and the effect was positively correlated with the CCL3
concentration. This result is in agreement with a previous report of Hawila et al. [21]. These authors extracted MDSCs from control and CCR5 knockout mice and detected the effect of CCL3, CCL4, and CCL5 on the migration of MDSCs with the transwell assay. The mobility of MDSCs in the CCR5 knockout group was significantly lower than that in the control group, and CCL3, CCL4, and CCL5 exerted a chemotactic effect on MDSCs; this effect was related to the binding to their receptor CCR5. Chemokines exert a chemotactic effect on MDSCs not only in vitro but also in vivo. Studies have shown that CXCL5/CXCR2 recruited MDSCs, and when a CXCL5-neutralizing antibody was used, the number of MDSCs in the bone marrow and spleen significantly decreased in a 4T1 breast tumor-bearing mouse model and gradually decreased with tumor growth [22]. Hawila et al. [21] found that the number of Gr-1\(^+\) cells was significantly reduced in CCR5 knockout mice or tumor-bearing mice treated with a CCR5-neutralizing antibody. The volume and weight of tumors in mice were significantly reduced after treatment with the CCR5-neutralizing antibody, and the number of Gr-1\(^+\) cells in the bone marrow, blood, spleen and tumors decreased. MDSCs express CD11b, which is a marker of bone marrow cells of the macrophage lineage in mice, and the granulocyte marker Gr-1. Moreover, no remarkable single population of Gr-1\(^+\) cells is found in the tumor tissue [22,23]. Therefore, we used immunohistochemical staining of MDSCs with the anti-Gr-1 antibody to detect the chemotactic effect of CCL3 on MDSCs in vivo. We found that the number of MDSCs that infiltrated the tumor, lung, and liver tissues was significantly higher than that in the group treated with CCL3-neutralizing antibody. The above results indicated that CCL3 might recruit MDSCs in vitro and in vivo.

There is a dynamic interaction between MDSCs and tumor cells, which can affect each other. Culture of human PBMCs with supernatants of cholangiocarcinoma cells may promote the expansion of CD33\(^+\) CD11b\(^+\) HLA-DR\(^{low}\) MDSCs, which functionally inhibits the proliferation of CD3\(^+\) T lymphocytes. It was also found that the supernatant of cholangiocarcinoma cells contain IL-6, GM-CSF, IL-8, vascular endothelial growth factor, and other cytokines that induce the phosphorylation of Tyr705 in STAT3, which promote the amplification of MDSCs [24]. The production of MDSCs increases when PBMCs are co-cultured with colon cancer cells, which indicates that these cells may promote the expansion of MDSCs in vitro. After co-culture with MDSCs, the growth of tumor cells is promoted [25]. Yang et al. [22] performed transwell experiments in which 4T1 cells or 4T1 cells mixed with MDSCs were placed in the transwell chamber. The cells in the mixed culture group pass through the chamber to a greater extent, which indicates that the MDSCs promote the invasion of breast cancer cells. When cancer cells were co-cultured with Mo-MDSCs, their invasion ability is enhanced. Besides, the experiment showed that Mo-MDSCs isolated from tumor-bearing mice promote the invasion of breast cancer cells [9]. We co-cultured breast cancer cells with MDSCs and found that the proliferation, migration, and invasion abilities of the cells increased. When breast cancer cells in which the CCL3 gene was knocked down were co-cultured with MDSCs, the above changes were not notable. CCL3 plays an important role in promoting the proliferation, migration, and invasion of breast cancer cells when they are co-cultured with MDSCs.

MDSCs are distributed systemically throughout the entire body of patients with cancer and accumulate in peripheral blood, lymph nodes, primary tumors, and distant organs. MDSCs may promote cancer migration by the formation of the so-called pre-metastases [26]. EMT is a process in which tumor cells change from an epithelial phenotype to a mesenchymal phenotype. It is also one of the steps involved in the spread of the cells. Cells that undergo this process exhibit a decrease in the levels of epithelial markers and adhesion molecules responsible for cell-to-cell contact, making them more easily separated from each other and spread to distant...
organs. At the same time, the expression of mesenchymal markers in tumor cells increases and cells develop invasive behavior. Eventually, tumor cells adopt a highly motile and invasive phenotype. Therefore, tumor cells that have undergone the EMT can more easily complete the multi-stage process of metastasis [27,28]. MDSCs induce the EMT in cancer cells via TGF-β, epidermal growth factor, and hepatocyte growth factor [7]. Besides, MDSCs also contribute to the mesenchymal-epithelial transition by secreting multifunctional proteins, which support the colonization of cancer cells in metastatic niches [29]. In our study, morphological changes took place in breast cancer cells when they were co-cultured with MDSCs. It was therefore speculated that the EMT was induced in cells, but when the expression of CCL3 was inhibited, no notable changes in cell morphology were observed. Then we found that the expression of epithelial markers in breast cancer cells decreased and the expression of interstitial markers increased after co-culture. This result indicated that the EMT occurred in breast cancer cells after co-culture, but not after CCL3 gene knockdown. Studies have shown that the PI3K pathway and its downstream component mTOR affect the regulation of the chemotaxis and function of MDSCs. Meanwhile, the activation of the PI3K pathway is closely related to the occurrence and development of tumors and increase the invasiveness of breast cancer cells [11,13], therefore, we wonder whether the PI3K pathway was related to the phenomenon. We further studied its mechanism and found that the PI3K-Akt-mTOR signaling pathway was activated after co-culture of breast cancer cells with MDSCs and led to EMT and the upregulation of the expression of MMP2 and MMP9, thus promoting the invasion and metastasis ability of the cells. However, when the expression of CCL3 was inhibited, the co-culture with MDSCs did not activate this signaling pathway, which indicated that CCL3 plays an important role in the interaction between breast cancer cells and MDSCs. This result was supported by recent reports. When pancreatic cancer cells were co-cultured with Mo-MDSCs, RT-PCR results showed that the levels of EMT-related transcription factors, namely, Snail, Slug and Oct-4, significantly increase. Western blot results revealed that the expression of E-cadherin in tumor cells decrease and the expression of vimentin increase. These results indicate that Mo-MDSCs promote EMT in cancer cells. When pancreatic cancer cells after co-culture with Mo-MDSCs were injected into mice, tumor growth markedly increase, which indicates that Mo-MDSCs may promote EMT in cancer cells and tumor growth [8]. In addition, MDSCs exhibit high affinity for 4T1 breast cancer cells and induce EMT. The quantitative PCR assay demonstrated that EMT-related genes are upregulated in tumor cells co-cultured with MDSCs, and an immunofluorescence assay showed enhanced expression of vimentin and cytokeratin 14. MDSCs may also activate the STAT1 and STAT3 signaling pathways to induce EMT in tumor cells, which would lead to an enhancement of the invasiveness of tumor cells and promote tumor cell migration [9].

In conclusion, our observations imply that CCL3 recruits MDSCs into the tumor microenvironment and plays an important role in the growth of breast cancer cells, as well as the interaction between MDSCs and cancer cells. The PI3K-Akt-mTOR pathway is involved in the effect of MDSCs on the biological functions of breast cancer cells and the EMT process induced by MDSCs in breast cancer cells (Supplementary Figure 3). These results show the effect of CCL3 and MDSCs on breast cancer cells and may provide new insights for the targeted therapy of breast cancer.

ACKNOWLEDGEMENTS

We would like to thank First Affiliated Hospital of Medical School of Xi’an Jiaotong University, for offering access and assistance in clinical data collection.
SUPPLEMENTARY MATERIALS

Supplementary Figure 1
The knockdown effect of CCL3 in breast cancer cells. (A) Transfection efficiency of breast cancer cells was observed under fluorescence microscope (magnification, ×100). (B) The relative mRNA expression levels of CCL3 in different groups of breast cancer cells (control, siNC and siCCL3). (C) The secretion of CCL3 in breast cancer cells. Images are representative and data are from three separate experiments.

Supplementary Figure 2
Western blot analysis of E-cadherin, N-cadherin, and PCNA levels in the tumors of control and anti-CCL3 groups (n = 5 mice per group).

Supplementary Figure 3
Schematic model showing how MDSCs affect breast cancer cells in tumor microenvironment. CCL3 from cancer cells recruits MDSCs. MDSCs migrate to the tumor microenvironment and promote the EMT in breast cancer cells via activating the PI3K-Akt-mTOR signaling pathway. Interaction with MDSCs ultimately leads to the enhanced migration and invasion ability of breast cancer cells. (Hand-drawn picture by the author: Anqi Luo).

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