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A C-X-C chemokine receptor type 2-dominated crosstalk between tumor cells and macrophages drives gastric cancer metastasis

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Abstract

Purpose: C-X-C chemokine receptor type 2 (CXCR2) is a key regulator that drives immune suppression and inflammation in tumor microenvironment. CXCR2-targeted therapy has shown promising results in several solid tumors. However, the underlying mechanism of CXCR2-mediated crosstalk between gastric cancer (GC) cells and macrophages still remains unclear.

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Conflict of interest: None.

Experimental Design: The expression of CXCR2 and its ligands in 155 human GC tissues was analyzed via immunohistochemistry, and the correlations with clinical characteristics were evaluated. A co-culture system was established, and functional assays, including ELISA, transwell, MTT, and qPCR, were performed to determine the role of the CXCR2 signaling axis in promoting GC growth and metastasis. A xenograft GC model and a lymph node metastasis model were established to study the function of CXCR2 *in vivo*.

Results: CXCR2 expression is associated with prognosis of GC patients (P=0.002). Of all the CXCR2 ligands, CXCL1 and CXCL5 can significantly promote migration of GC cells. Macrophages are the major sources of CXCL1 and CXCL5 in the GC microenvironment, and promote migration of GC cells through activating a CXCR2/STAT3 feed-forward loop. GC cells secrete TNF-α to induce release of CXCL1 and CXCL5 from macrophages. Inhibiting CXCR2 pathway of GC cells can suppress migration and metastasis of GC *in vitro* and *in vivo*.

Conclusion: Our study suggested a previously uncharacterized mechanism through which GC cells interact with macrophages to promote tumor growth and metastasis, suggesting that CXCR2 may serve as a promising therapeutic target to treat GC.

Keywords

chemokine; tumor microenvironment; carcinogenesis

Introduction

Gastric cancer (GC) is the second leading cause of cancer-related deaths worldwide (1). Most GC-related deaths result from cancer recurrence and metastasis (2, 3). Novel therapeutic targets for the treatment of GC metastasis are urgently needed. C-X-C Motif Chemokine Receptor 2 (CXCR2) is a potent pro-tumorigenic chemokine receptor that can induce inflammation in the tumor microenvironment (4, 5). CXCR2-targeted therapies can enhance the efficacy of immunotherapy in several solid tumors (4, 5), and increase the sensitivity of chemotherapy (6). Meanwhile, CXCR2-mediated recruitment of different stromal cells promotes progression of cancer cells (4–7). These studies hinted that the interaction between CXCR2 and tumor microenvironment was of critical importance for tumor progression.

As key regulators of the tumor microenvironment (8), macrophages comprise a large part of primary tumor mass (9). Macrophages can drive the metastasis of GC cells by increasing the activity of RhoA and Cdc42 in a p-Akt and p-ERK1/2 dependent manner (10). There is a robust correlation between CD163-positive macrophages infiltration and a prognostic gene signature named stromal-response cluster (SRC), which can predict the survival of GC and ovarian cancer (11). Tumor-associated macrophages (TAMs) promote proliferation, invasion, and metastasis of tumor cells by producing a plethora of cytokines (12–15). The chemokines that interact with CXCR2 consist of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8. Studies have showed that CXCR2 ligands derived from tumor cells can promote metastasis and chemoresistance in breast cancer (6), pancreatic cancer (5) and rhabdomyosarcoma (4). CXCR2 ligands can also drive carcinogenesis and progression of GC (16–18). Mice that infected with H. pylori had higher level of CXCL1 and CXCL2,

which would induce the development of dysplasia and GC (16). CXCL8-transgenic mice (IL-8Tg) presented with earlier initiation and progression of inflammation-associated gastric carcinogenesis and CXCL8 can induce metastasis of GC (17, 18). Previously, we found that lymphatic endothelial cells stimulated lymphangiogenesis, angiogenesis, and growth of GC by secreting CXCL1 (19, 20). CXCL1 expression is an independent prognostic factor in pancreatic ductal adenocarcinoma (21). However, whether chemokines from macrophages can promote the metastasis of GC remains unclear.

Epithelial to mesenchymal transition (EMT) is the initial step of metastasis in epithelial tumors (22–24). During EMT, epithelial cells acquire the ability to efficiently invade and disseminate (25–27). EMT is often recognized as the result of gene mutation or as a response to the alteration of tumor microenvironment (28, 29). CXCR2 on cancer cells can induce EMT and metastasis of hepatic cancer (30). However, whether chemokines from macrophages contribute to GC metastasis via CXCR2-mediated EMT remains unknown.

In the current study, we constructed the co-culture model to imitate the interaction between tumor cells and macrophages in the tumor microenvironment. We found that CXCR2 expression in cancer cells is a prognostic factor for GC. CXCL1 and CXCL5 can promote migration of GC cells by activating CXCR2. Interestingly, we discovered that macrophages are the major sources of CXCL1 and CXCL5 in the GC microenvironment. Macrophages contribute to tumor migration by activating a CXCR2/STAT3 feed-forward loop in GC cells. GC cells secrete TNF-α to induce release of CXCL1 and CXCL5 from macrophages. Inhibiting the CXCR2 pathway of GC cells can suppress metastasis of GC *in vivo*. These findings indicate that CXCR2 may be a promising therapeutic target to suppress GC metastasis.

Materials and Methods

Patients and tissue samples

A total of 155 archived human GC specimens were obtained from the GC database and tissue bank in the First Affiliated Hospital of Sun Yat-sen University. These tissues are from patients who underwent radical dissection for GC between 2006 and 2007 without preoperative chemotherapy or radiation therapy. The follow-up was maintained until December 2013. The studies were conducted in accordance with International Ethical Guidelines for Biomedical Research Involving Human Subjects (CIOMS). The studies were performed after approval by the institutional review board (IRB). We have obtained written consent from the subjects before the study.

Cell culture and reagents

Human GC cell lines (AGS, BGC-823, MGC-803, SGC-7901, HGC-27 and MKN-45), normal gastric epithelial cell line (GES-1) and human acute monocytic leukemia cell line (THP-1) were purchased from ATCC in 2016. Cells were maintained and cultured according to routine procedures. CXCR2 inhibitor, CXCL1 specific neutralizing antibody and CXCL5 specific neutralizing antibody were purchased from R&D Systems (Minneapolis, MN).

CXCR2 ligands (CXCL1, 2, 3, 5, 6, 7 and 8) were purchased as recombinant proteins from PeproTech Inc. (Rocky Hill, NJ).

Cell co-culture model

A co-culture model of macrophages and GC cells was established. A transwell apparatus with a 0.4μm pore membrane six-well plate was used for cancer cell/macrophage co-culture. THP-1 differentiated into macrophages after stimulation with phorbol 12-myristate 13-acetate (PMA) for 24 hours. 10^6 cells were seeded to the lower compartment of each well, and an equal number of co-culture cells were added onto the upper compartment. After 24 hours, cells in the lower compartment were collected for RNA and protein measurement.

RNA interference

Human CXCR2 siRNA sequences were purchased from RiboBio (Guangzhou) as shown in Supplementary Table S1. AGS and BGC-823 cells were transfected with either 5 μM of scrambled oligo control, si1, si2, or si3 using Lipofectamine 2000 (Invitrogen, CA), according to manufacturer's protocols. The expression of CXCR2 was evaluated by Western blot assay (Abcam, Cambridge).

Immunohistochemical analysis

A total of 155 archived GC tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and then sectioned (5 μm), deparaffinized, rehydrated and subjected to antigen-retrieval. Tissue sections were incubated with CAS blocking buffer and subsequently incubated with primary antibodies (Supplementary Table S2) at 4 °C overnight. Reactivity was detected using DAKO EnVision-HRP (Dako, Denmark). The degree of immunostaining was scored by multiplying the percentage of positive cells (P) with intensity (I), according to the formula: H = P × I. The range for P is 0–4 (~5% scores 0; 5% ~ 25% scores 1; 25% ~ 50% scores 2; 50% ~ 75% scores 3; 75% ~100% scores 4). The range for I is 0–3 (0, no staining; 1, weak; 2, moderate; 3, strong.). We calculated the staining index score by multiplying P with I, and obtained a range from 0–12. A staining index score of 0–3 was defined as negative and a staining index score of 4–12 was defined as positive. The number of TAMs was calculated from the mean number of CD163 positive cells in 5 random fields of 400× magnification. Two independent pathologists observed the 5 random fields of each tissue and scored each sample without knowledge of the patient outcome. The average value of these two scores was presented. A cut-off value of TAMs relating to the prognosis of GC patients was calculated according to established procedures. The cut off number for TAM (CD163 positive) is 14.9.

RNA isolation and qPCR

Human macrophages differentiated from THP-1 cells were cultured alone or in combination with GC cells for 24 hours. Total RNA was extracted using RNAplus reagent (Takara, Japan), according to manufacturer's instructions. After treatment with RNase-free DNase, 1 μg of total RNA from each sample was used for cDNA synthesis using a Reverse Transcription kit (Takara, Japan). Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed with the 7900 HT (Applied Biosystems, Foster City)

using SYBRVR Green qPCR SuperMix (Invitrogen). Primers were designed and synthesized by Sangon Biotech. Sequences are shown in Supplementary Table S1. The expression of mRNA was normalized to the geometric mean of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control variability in expression levels. Results were expressed as the fold change using the $2^{-\Delta\Delta Ct}$ method.

Cell viability assay

In vitro cell viability was evaluated by the Cell Counting Kit-8 (CCK-8) assay. Four thousand cells were seeded into the wells of a 96-well plate. After 16 hours of culture, the medium was replaced with a low serum medium. Then, cells were cultured with or without conditioned medium for 72 hours. Subsequently, the medium in each well was replaced with 100 μl RPMI-1640 medium containing 10 μl CCK-8 reagent (Dojindo laboratories, Kyoto, Japan), and the absorbance was measured after 3 hours using a microplate reader at 450 nm.

Western blot

Cell lysates and tumor lysates were collected using the whole protein extraction kit (Keygen, Nanjing). Supernatants were recovered by centrifugation at 13,000 rpm for 15 minutes at 4 °C. Protein concentrations were measured, and equal amounts of total protein were separated by SDS-PAGE. Proteins were transferred onto PVDF membranes (Merck Millipore, Darmstadt, Germany) and the membranes were blocked for 1 hour in TBST. Then, the membranes were incubated overnight at 4 °C with primary antibodies (Supplementary Table S2). After washing with TBST, the membranes were incubated with corresponding peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Specific bands were detected using an enhanced chemiluminescence reagent (ECL; Perkin Elmer Life Sciences, Boston) on auto-radiographic film.

Lentivirus and transfection

The shCXCR2 RNAi lentivirus was constructed by Laura Biotech (Guangzhou). BGC-823 cells (5×10^5) were cultured in 25-cm^2 dishes. After culturing for 24 hours, cells were incubated with 5 ml of medium containing lentivirus and 5 μl polybrene. This procedure was repeated twice a day for two days. Infected cells were treated with 0.5 μg/ml of puromycin for one week. Cells stably expressing the shCXCR2 were isolated and CXCR2 expression was determined by Western blot and q-PCR. The shCXCR2 sequence is shown in Supplementary Table S1.

Enzyme-linked immunosorbent assay (ELISA)

Supernatant was collected from control cells (1×10^6 AGS or BGC-823 or THP-1 cells) cultured in serum free media in the presence or absence of cells (1×10^6 AGS or BGC-823 or THP-1 cells). The concentration of CXCL1 and CXCL5 was measured by ELISA (R&D Systems).

Cell migration assay

A transwell migration was used to assess cell migration. AGS or BGC-823 cells (1×10^5) were treated with mitomycin C (10 μg/ml, Sigma) for 2 h before being seeded onto the upper

compartment of Matrigel-coated transwell chambers (24-well insert, 8-μm pore size; BD Biosciences, Bedford). Macrophages (1×10^5) were cultured in the lower chamber. Tumor cells were allowed to migrate for pre-specified times at 37 °C. The number of cells that migrated to the underside of the transwell was counted in five high-power fields at 200× magnification. Data were normalized using a migration index.

Xenograft tumor growth and metastasis assays

Animal experiments were approved by Institutional Animal Care and Use Committee of the First Affiliated Hospital of Sun Yat-sen University. Six-week-old female Balb/c athymic mice were purchased from the Guangdong Medical Laboratory Animal Center. Mice were randomized into four groups (n=5 *per* group). We constructed subcutaneous xenagraft model by injecting GC cells alone (3×10^6 cells/mouse) or combined with macrophages (1×10^6 cells/mouse). Therefore, we have four groups: Group A is shV GC cells, Group B is shV GC cells plus macrophages, Group C is shCXCR2 GC cells, and Group D is shCXCR2 GC cells plus macrophages. Tumor size was measured twice a week, and tumor volume (V) was calculated by using the formula: $V = 0.5 \times (\text{length} \times \text{width}^2)$. After 19 days, all mice were euthanized with CO_2; and tumors were removed, weighed and processed for IHC and Western blot. In the tumor metastasis experiment, nude mice (4 weeks, male) were obtained from the Shanghai Public Health Clinical Center (Shanghai). Mice were randomly assigned to two different groups (n=6 *per* group). BGC-823-shCXCR2 or BGC-823 shV cells (3×10^6/mouse) were injected subcutaneously into the footpad of the left hind limb of each mouse. Both of these two cell lines were stable cell lines which would express firefly mCherry and GFP. The mice were euthanized on day 28, and the primary tumors and popliteal lymph nodes were collected. Popliteal lymph nodes were evaluated for metastatic lesions by hematoxylin and eosin staining and IHC staining with anti-mCherry or anti-GFP antibodies.

Statistical analysis

SPSS 17.0 (SPSS Inc., Chicago, IL) software was used for statistical analysis. Quantitative data were presented as mean ± SD, and Student's t-test was applied. Chi-square test was utilized to assess the associations of the expression of CD163, CXCR2, CXCL1 and CXCL5 with clinicopathological features. The relationship among the expression of proteins was analyzed by Fisher's exact test. Survival curves and overall survival rates were determined by Kaplan–Meier and log-rank methods. Two-sided P-values <0.05 were considered statistically significant.

Results

CXCR2 is overexpressed in GC and is inversely correlated with overall survival.

IHC analyses were performed on GC tissues. Among the 155 GC tissues, 56.1% (87/155) displayed strong expression of CXCR2 (Fig. S1A, Supplementary Table 3). Positive CXCR2 expression was correlated to inferior prognosis in GC (Fig. 1A). We found that CXCR2 expression was higher in human GC tissue than that in paired normal gastric tissue by western blot (Fig. S1B). Accordingly, CXCR2 expression was higher in six different GC cell lines compared to that in a normal gastric epithelium cell line (GES-1) (Fig. S1C). Then we

examined whether knocking down CXCR2 can suppress the progression of GC. We found that knocking down CXCR2 can suppress the migration of GC cells (Fig. 1B). Meanwhile, knocking down CXCR2 can significantly suppress cell proliferation (Fig. 1C).

CXCL1 and CXCL5 promote migration and progression of GC.

Since CXCR2 has several ligands, then we further investigated which ligands can promote the progression of GC. The expression of all CXCR2 ligands (including CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) in GC was analyzed in TCGA datasets (http://cancergenome.nih.gov) and two public datasets from GEO databases (GSE27342 and GSE29272). Gene expression was calculated by subtracting expression values of adjacent normal gastric epithelial tissue from that of the paired GC tissue. The results showed that most of GC tissue had higher level of CXCR2 ligands than the adjacent normal tissue (Fig. S1D). We also analyzed the mRNA level of CXCR2 ligands in 8 pairs of GC tissue and adjacent tissue. The result showed that the level of CXCR2 ligands was higher than in GC tissue (Fig. 1D, S2A), which was consistent with the data in the public databases (Fig. S1D). To address the relative contribution of different CXCR2 ligands with respect to migration and EMT in GC, cells were treated with human recombinant proteins of CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, or CXCL8. Among all the CXCR2 ligands, we observed that CXCL1 and CXCL5 significantly increased GC cell migration (Fig. 1E, S2B). We examined whether expression of CXCL1 or CXCL5 in tumor tissue are associated with prognosis. The result showed that positive expression of CXCL1 can predict inferior prognosis in GC (5-year OS: 65.9% vs. 40.6%, P=0.002). Similar result was also found in CXCL5 (5-year OS: 71.8% vs. 26.9%, P<0.001) (Fig. 1F). Further study showed that CXCL1 and CXCL5 expression was positively associated with tumor metastasis and TNM stage (Supplementary Table S4). We also analyzed data from public databases (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). The results also showed that high expression of CXCL1 and CXCL5 in tumor tissue were associated with poor prognosis in lung cancer, neuroblastoma and PDAC (Fig. S3).

Macrophages are the major sources of CXCL1 and CXCL5 in GC microenvironment.

Next we explored the major sources of CXCR2 ligands in GC tissue. We detected the mRNA level of CXCR2 ligands in GC cell lines and GES-1 cells by qPCR. The results showed that the mRNA level of most CXCR2 ligands did not increase in GC cell lines significantly (Fig. S4A). These findings indicated that increased CXCR2 activity in GC cells was more likely the result of paracrine activation by ligands secreted from stromal cells in the GC microenvironment. However, whether macrophages can promote tumor progression via secreting CXCR2 ligands remains elusive. The clinical relevance of these findings was evaluated by determining the expression of CXCL1 and CXCL5 in cancer tissues in a cohort of 155 GC patients. CXCL1 expression was high in 56.1% (87/155) GC patients, while CXCL5 expression was high in 45.2% (70/155) GC patients (Fig. 2A, Supplementary Table 4). CD163 expression positively correlated with CXCL1 expression (r=0.340, P<0.001) and CXCL5 expression (r=0.300, P <0.001) (Fig. 2B). We detected the mRNA level of CXCL1 and CXCL5 in macrophages co-cultured with normal gastric epithelial cells or GC cells by qPCR. To our surprise, the mRNA levels of CXCL1 and CXCL5 in macrophages increased dramatically when co-cultured with GC cells (Fig. 2C).

Meanwhile, the mRNA level of other CXCR2 ligands besides CXCL1 and CXCL5 also increased dramatically in macrophages co-cultured with GC cells (Fig. S4B). These results indicated that macrophages may also secret high levels of CXCL1 and CXCL5 when co-cultured with GC cells. To validate this hypothesis, we performed ELISA analysis to detect the levels of CXCL1 and CXCL5 in the supernatant of GC cells and macrophages when they were cultured alone or in the co-culture system. We found that the levels of CXCL1 and CXCL5 in the supernatant of GC cells and macrophages increased in the co-culture system (Fig. 2D, 2E). Intriguingly, we found that the level of CXCL1 and CXCL5 in the supernatant of macrophages increased more dramatically than that of GC cells. These results indicate that tumor associated macrophages have higher level of CXCL1 and CXCL5 than that in GC cells.

Macrophages can promote migration of GC cells via activating CXCR2/STAT3 feed forward loop.

Then we examined whether macrophages can promote the progression of GC via CXCL1 and CXCL5. The migration of GC cells increased when co-cultured with macrophages (Fig. 3A). CXCR2 inhibitor SB225002 and CXCL1 or CXCL5 neutralizing antibodies can inhibit macrophages induced migration of GC cells (Fig. 3B, 3C, S6A). We investigated how CXCL1 and CXCL5 promote the migration and progression of GC. Previously, we found that CXCL1 promoted the migration and progression of GC via activating STAT3/VEGF pathway (20). So we detected the phosphorylation of STAT3 in GC cells treated with CXCL1 and CXCL5. As expected, CXCL1 and CXCL5 can increase the phosphorylation of STAT3 (Fig. 3D). Interestingly, when we knocked down STAT3, CXCR2 expression decreased (Fig. S6E), and when we overexpressed CXCR2, phosphorylation of STAT3 increased (Fig. 3F). Furthermore, knocking down STAT3 can reverse CXCL1 induced upregulation of CXCR2 (Fig. 3E). These results indicated that there is a feed forward loop between CXCR2 and STAT3. On the one hand, CXCR2 can increase the phosphorylation of STAT3. On the other hand, STAT3 can induce the upregulation of CXCR2.

Macrophages derived CXCL1 and CXCL5 can promote migration of GC cells via CXCR2/STAT3 mediated EMT.

Next we determined how CXCR2/STAT3 activation can promote the migration and progression of GC. We performed gene set enrichment analysis (GSEA) using data from The Cancer Genome Atlas (http://cancergenome.nih.gov), and found that CXCR2 levels correlate with STAT3-activated gene signatures and EMT-associated gene signatures (Fig. S5). These observations indicate that CXCR2/STAT3 signaling may induce EMT in GC. We examined whether CXCL1 and CXCL5 can induce EMT of GC via activating CXCR2/STAT3. We detected the expression of EMT biomarkers via western blot. The results showed that knocking down CXCR2 in GC cells decreased phosphorylation of STAT3 and suppressed epithelial to mesenchymal transition (Fig. 4A). Consistently, we found that co-cultured with macrophages can increase the phosphorylation of STAT3 and induce epithelial to mesenchymal transition in GC cells, while CXCR2 inhibitor and CXCL1 or CXCL5 neutralizing antibodies can reverse it (Fig. 4B, 4C). Later, we found that knocking down CXCR2 or pretreating GC cells with STAT3 pathway inhibitor AG490 (2uM) for 4 hours can reverse macrophages induced proliferation and migration of GC cells (Fig. 4D, S6B,

S8B). These results indicate that macrophages derived CXCL1 and CXCL5 can activate CXCR2/STAT3 pathway in GC cells, resulting in the progression of GC cells.

Inhibiting CXCR2 pathway of GC cells can suppress the migration and progression of GC *in vivo*.

We constructed shCXCR2 stable cell line (Fig. S6C, S6D). BGC-823 cells expressing a specific shRNA for CXCR2 or shV were implanted subcutaneously with or without macrophages in nude mice. Mean tumor weight was 1.3196 ± 0.3342 g, 1.1484 ± 0.14617 g, 0.5118 ± 0.1589 g and 0.5528 ± 0.1555 g in the shV, shV+TAMs, shCXCR2 and shCXCR2+TAMs group, respectively (Fig. 5A, 5B). shCXCR2 suppressed tumor growth significantly (P <0.05 on day 19) (Fig. 5C). Furthermore, knocking down CXCR2 decreased expression of p-STAT3 and EMT markers Snail (Fig. 5D). These results revealed that the function of the CXCR2/STAT3 signaling pathway was responsible for the induction of tumor growth and EMT in GC. To further evaluate the role of CXCR2 in tumor metastasis, we established the lymph node metastasis mice model. We found that shCXCR2 decreased lymph node metastasis compared to the shV group. The lymph node metastasis rate was 83.3% in the shV group and 33.3% in the shCXCR2 group (Fig. 5E). In addition, we also constructed CXCL1 overexpressed GC cell line. CXCL1–7901 (5×10^6) and GFP-7901 cells (5×10^6) were subcutaneously injected into nude mice. Tumor growth was measured every 2 to 3 days for 25 days. The results showed that CXCL1 increased local tumor growth and the expression of Snail in xenograft nude mice (Fig. S7A, S7B).

TNF-α induced CXCL1 and CXCL5 expression in macrophages

We explored whether cytokines secreted from GC could stimulate release of CXCL1 and CXCL5 from macrophages. Firstly, we tested whether TGF-β secreted from GC cells can increase the expression of CXCL1 and CXCL5 in macrophages. However, we did not find that TGF-β increase the expression of CXCL1 and CXCL5 in macrophages (data not shown). Interestingly, we found that TNF-α levels increased in GC cells when co-cultured with macrophages (Fig. 5F). TNF-α can induce the upregulation of CXCL1 and CXCL5 in macrophages (Fig. 5G). Blocking the downstream pathway of TNF-α by p38 inhibitor and p65 inhibitor can decrease the level of CXCL1 and CXCL5 in macrophages (Fig. 5G). These results indicated that GC cells can interact with macrophages via secreting TNF-α, and macrophages promote GC progression through CXC1 and CXCL5.

Macrophages are associated with the activation of CXCR2 pathway and EMT in human GC tissue.

Expression of p-STAT3, Snail and CD163 were analyzed in 155 GC tissues by IHC (Fig. 2A, 6A). CXCL1 and CXCL5 expression positively correlated with the expression of Snail, p-STAT3, indicating CXCL1 and CXCL5 can drive EMT of GC cells (Fig. 6B, 6C). Meanwhile, the expression of CD163 and CXCR2 was also positively associated with the expression of Snail (Fig. 6D), suggesting macrophages are responsible for the activation of CXCR2 pathway in GC cells. Macrophages and the expression of Snail in GC cells predicted inferior prognosis (Fig. S8A). These results strongly suggest that macrophages can promote EMT and progression of GC via activating CXCR2 pathway (Fig. 6E). CXCR2 expression is an independent prognostic factor for GC patients (Supplementary Table S5).

Discussion

The tumor microenvironment contributes to tumorigenesis, progression and dissemination (31–33). TAMs promote EMT and metastases in several tumors including breast cancer and pancreatic cancer (13, 34, 35). CXCR2 is a key regulator for the interaction between tumor cells and stromal cells. There is emerging literature suggests that CXCR2 is fundamental for driving tumor metastasis and blockade of CXCR2 can significantly disrupt the stromal-tumor interaction in several solid tumors (5, 21). Targeting CXCR2 can suppress immune evasion of tumor cells, thus increase the sensitivity of immunotherapy (4, 5). However, the role of CXCR2 mediated interaction between macrophages and tumor cells remains uncharacterized. Previously, we have shown that CXCL1/CXCR2 signaling pathway is important for the progression of gastric cancer (20, 36). In this study, we sought to characterize the role of CXCR2 in GC metastasis and unravel the mechanism of CXCR2 mediated crosstalk between macrophages and tumor cells. In this study, we found that macrophages promote EMT and metastases of GC cells through the release of CXCL1 and CXCL5, which activates a positive feed-forward loop between CXCR2 and STAT3 in GC cells. Most importantly, the elements involved in this cascade showed a substantial prognostic correlation in a cohort of gastric cancer patients. Knocking down CXCR2 decreased EMT and metastasis *in vivo*, highlighting the clinical significance of these findings and suggesting that CXCR2 may serve as a rational target for GC treatment.

Through the analysis of chemokine profiles of TAMs, we found that CXCL1 and CXCL5 are critical for GC metastasis and associated with patient prognosis. TAMs secrete a number of cytokines like CXCL1, IL-6, TGF-β and VEGF, which promote tumor growth and metastasis (15, 37–39). Of all the cytokines secreted by macrophages, we mainly focus on CXC chemokine because there is growing evidence suggesting that CXC chemokine can drive inflammation and treatment resistance, which will result in the progression of cancer (40, 41). However, the role of CXC chemokine derived from macrophages remains uncharacterized. A recent study showed that macrophage secreted CXCL1 can promote the metastasis of breast cancer by activating SOX4 signaling pathway (42). Our previous studies have suggested that CXC chemokine receptors might serve as potential therapeutic targets in GC (36). Thus we sought to identify which CXC chemokine are responsible for driving the metastasis of GC. In prostate cancer, TAMs induce EMT through activating the CCL2/CCR2-STAT3 signaling (15). Based on the GSEA plot of the EMT process, we quantified Snail as an EMT marker for GC. We demonstrated that TAMs induced EMT of GC cells in a CXCR2-dependent manner. Then, we screened all CXCR2 ligands and found that CXCL1 and CXCL5 from TAMs can induce EMT and promote metastasis. Clinical data also showed that the expression of CXCL1 and CXCL5 was associated with tumor metastasis and TNM stage, which may serve as prognostic biomarkers for GC patients. Thus, our findings revealed a novel clinically relevant mechanism in GC metastasis, which suggested that TAMs induced EMT and metastasis of GC by activating the CXCL1/5-CXCR2 axis.

The crosstalk between tumor cells and macrophages also exists in other cancers. CCL18 released by TAMs enhanced EMT and metastasis of breast cancer cells (34). The CXCL5/CXCR2 axis contributes to the EMT of hepatic cancer cells (30). CXCL1/2 attracts CD11b(+)Gr1(+) myeloid cells to enhance breast cancer cell survival and chemoresistance

(6). In addition, cancer cells also secrete cytokines to attract macrophages into the tumor, which in turn contributes to EMT (15, 29). Here, we found that GC cells secreted TNF-α can stimulate the release of CXCL1 and CXCL5 from macrophages into the tumor microenvironment. CXCR2 can activate PI3K-Akt, NF-kB and MAPK signaling pathways (6, 30, 43). CXCL1 promoted proliferation and migration of GC cells is partially dependent on NF-kB (44). We previously demonstrated that the CXCL1/CXCR2 axis increased angiogenesis and tumor growth through activating STAT3/VEGF pathway in GC (20). Here, we found that CXCR2 mediated STAT3 pathway can be activated by CXCL1 and CXCL5, which increases expression of VIMENTIN, N-cadherin and Snail, thus leading to EMT and metastasis. STAT3 is considered to be a putative downstream effector of the CXCL1/CXCR2 pathway (45), which can induce EMT (46) and promote invasiveness (47). Previously we have demonstrated that STAT3 binds to the promoter of CXCR2 and increases CXCR2 expression (36). Thus, a positive feed-forward loop between CXCR2 and STAT3 forms a cascade to continuously induce EMT. Interestingly, a recent study showed that knocking down Snail can significantly decrease the expression of CXCR2 ligands indicating that there may be a feed forward loop between Snail and CXCR2 ligands (48). Consistent to our finding, a recent study showed that TAMs derived CXCL1 can drive metastasis of breast cancer (42). Anti-CXCR2 treatment can also increase the sensitivity of immunotherapy in several tumors (4, 5, 49). CSF-1 is a critical regulator for the infiltration and differentiation of macrophages (49). Reducing TAMs by blocking CSF-1 can enhance the anti-tumor efficacy of immune checkpoint blockade (50). Combination of CSF-1 inhibitor and CXCR2 inhibitor can dramatically increase the anti-tumor effect of PD-1 blockade in lung carcinoma and melanoma mice models (49). Targeting CXCR2 can also enhance the efficacy of anti-PD-1 therapy by disrupting the infiltration of myeloid-derived suppressor (MDSC) cells (4). Anti-CXCR2 therapy seems to be a promising strategy for those with a positive expression of CXCR2 in GC. Thus further studies to evaluate therapeutic potential of CXCR2 inhibition in GC are warranted. Meanwhile, our study may also advance our understanding on the interaction between immune cells and cancers, which may be helpful for developing novel immunotherapy strategies.

In conclusion, we revealed a previously uncharacterized crosstalk between GC cells and macrophages. GC cells secrete TNF-α to induce release of CXCL1 and CXCL5 from macrophages. CXCL1 and CXCL5 secreted by macrophages could activate CXCR2 mediated positive feed-forward loop to induce EMT and tumor metastasis in GC, thus representing promising therapeutic targets in GC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Chemokine is correlated with inflammation and cancer risk, however, the role of chemokine in carcinogenesis still remains unclear. Here we report a novel role for CXCR2, a chemokine receptor which drives immune escape and chemoresistance in human cancers, especially in gastric cancer progression. CXCR2 modulates gastric cancer migration and invasion by promoting the interactions between tumor-associated macrophages (TAMs) and gastric cancer cells. And macrophages promote migration of gastric cancer by activating a CXCR2/STAT3 feed-forward loop in a CXCL1/CXCL5-dependent manner. These findings unravel a previously uncharacterized role of CXCR2 in gastric cancer progression, and propose a new pathway driving cancer metastasis. Targeting CXCR2 might serve as a novel treatment strategy for gastric cancer.

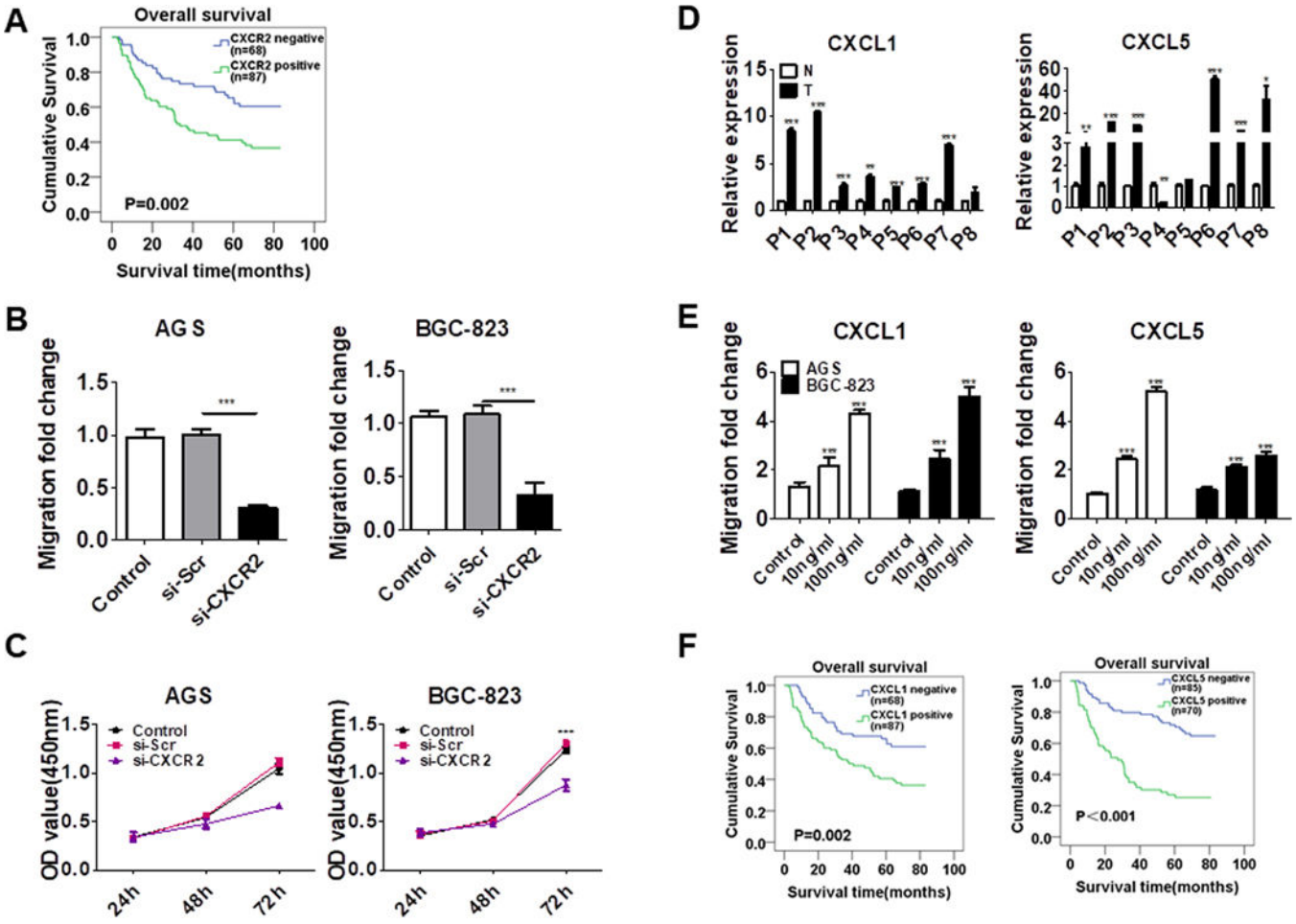

Figure 1. CXCR2 pathway is associated with prognosis in GC.

(A) Overall survival of 155 GC patients analyzed by Kaplan–Meier Method and Log-rank test according to the CXCR2 expression in GC tissue. (B) Transwell assay to evaluate the function of CXCR2 in GC cells. (C) MTT assay to evaluate the function of CXCR2 in GC cells. (D) The mRNA level of CXCL1 and CXCL5 in GC tissues (T) and paired normal gastric epithelial tissue (N) determined by qPCR. (E) Migration rates of GC cells treated with human recombinant proteins of CXCR2 ligands CXCL1 and CXCL5. (F) Overall survival analyzed by Kaplan-Meier Method and Log-rank test. Patients with positive expression of CXCL1 (P=0.002) or CXCL5 (P<0.001) in GC tissue had a worse overall survival compared to those with negative expression.

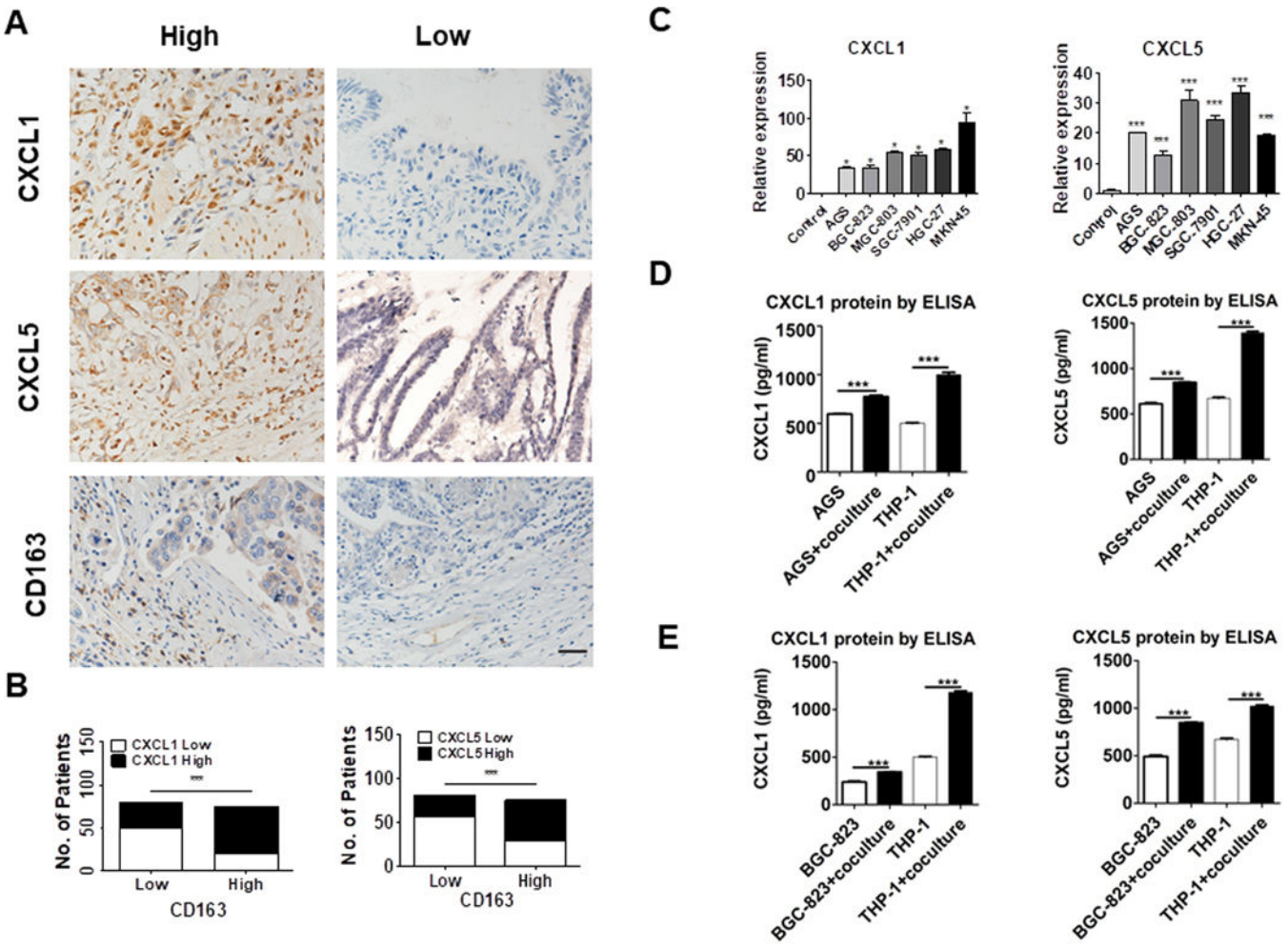

Figure 2. Macrophages are the major sources of CXCL1 and CXCL5 in GC microenvironment.

(A) IHC analysis of CXCL1, CXCL5 and macrophage marker CD163 in 155 human GC specimens (200×). The scale bar is 50μm. (B) Correlation between CD163 with CXCL1 and CXCL5 expression. (C) The mRNA level of CXCL1 and CXCL5 in macrophages co-cultured with normal gastric epithelial cells or GC cells were determined by qPCR. (D) The levels of CXCL1 and CXCL5 in the supernatant of AGS cells and macrophages (THP-1 cells) cultured alone or in the co-culture system were detected by ELISA analysis. (E) The levels of CXCL1 and CXCL5 in the supernatant of BGC-823 cells and macrophages (THP-1 cells) cultured alone or in the co-culture system were detected by ELISA analysis.

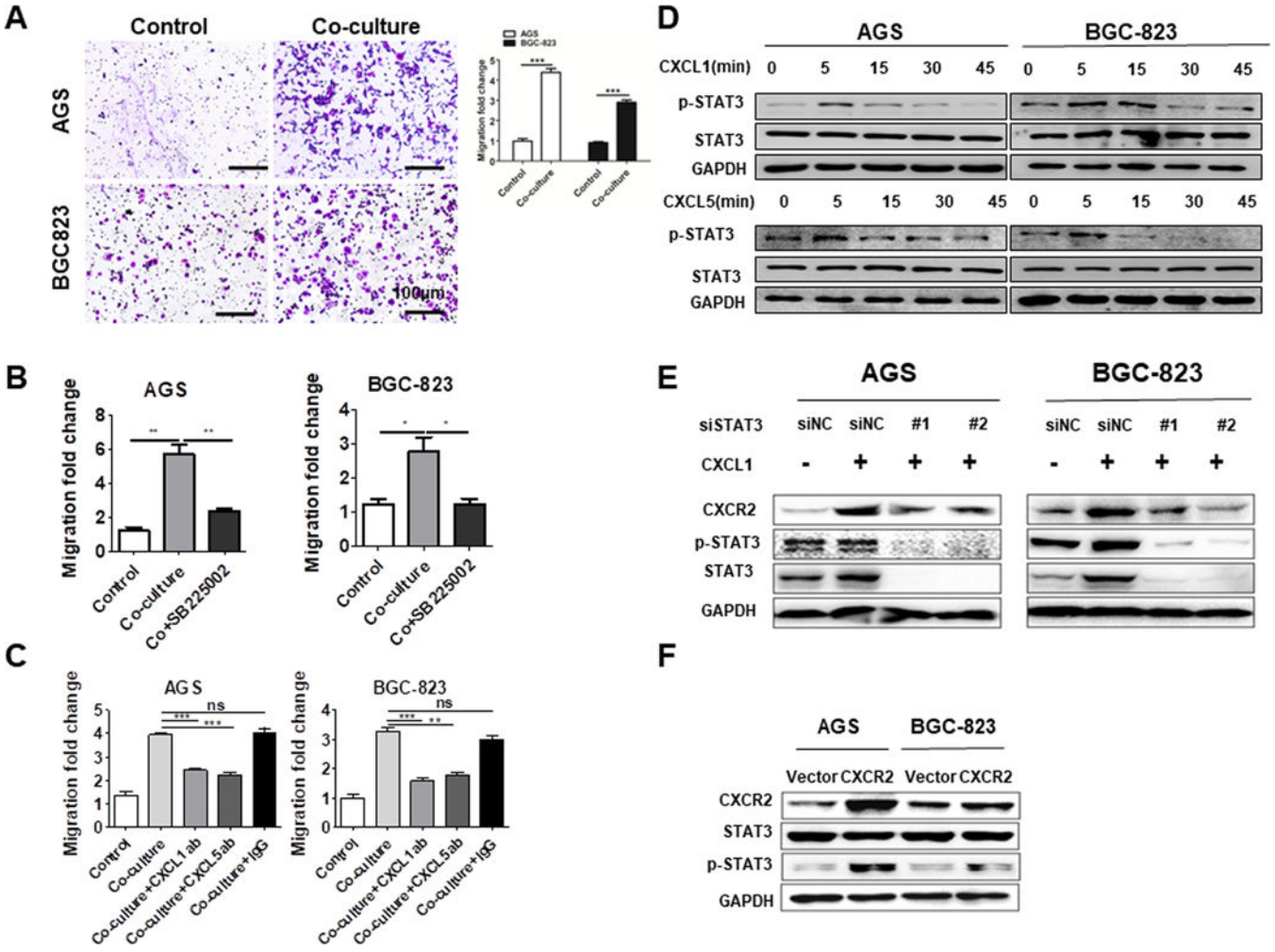

Figure 3. Macrophages can promote migration of GC cells via activating CXCR2/STAT3 feed-forward loop.

(A) The migration rates of AGS and BGC-823 cells with or without the co-culture of macrophages were determined by migration assay. (B) The effect of CXCR2 inhibitor SB225002 on inhibiting the migration rates was evaluated by migration assay in GC cells with or without the co-culture of macrophages. (C) CXCL1 or CXCL5 neutralizing antibodies can reverse macrophages induced migration of GC cells. (D) Expression of p-STAT3 and STAT3 in AGS and BGC-823 cells treated with CXCL1 and CXCL5 for indicated time. (E) GC cells were transfected with STAT3-specific or scrambled siRNA for 2 days and treated with or without 10nM CXCL1 for 12h; CXCR2 protein levels were analyzed by western-blot. (F) Western-blot showed that CXCR2 overexpression can induce phosphorylation of STAT3. *P<0.05; **P<0.01; ***P<0.001, ns: not significant.

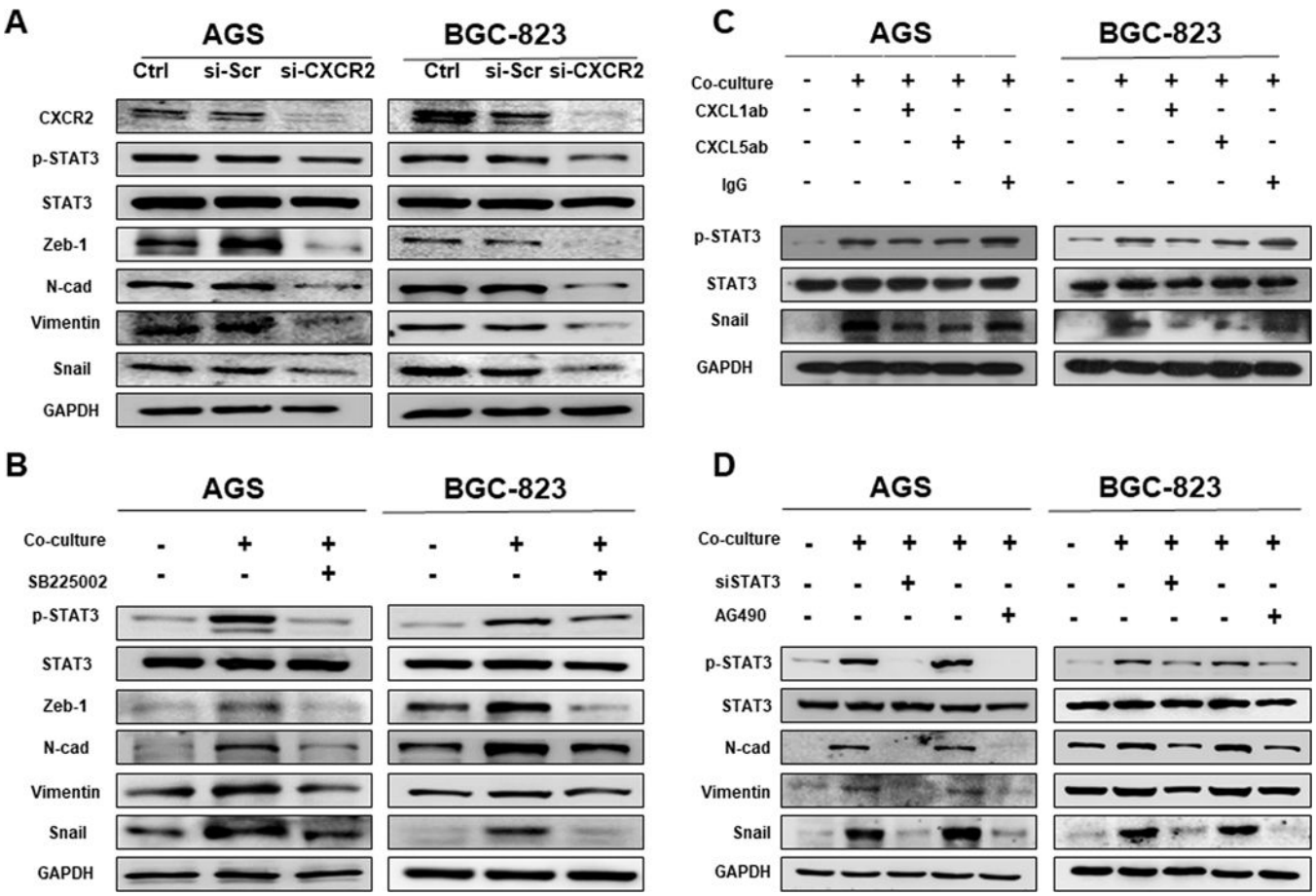

Figure 4. Macrophages derived CXCL1 and CXCL5 can promote migration of GC cells via CXCR2/STAT3 mediated EMT.

(A) Western blot analysis of p-STAT3 and EMT markers in AGS and BGC-823 cells. Knock down CXCR2 in GC cells decreased expression of p-STAT3 and EMT markers. (B) The levels of phosphorylated STAT3 and EMT markers in AGS and BGC-823 cells with or without the co-culture of macrophages and the treatment of CXCR2 inhibitor SB225002 were analyzed via western blot. (C) Macrophages can increase Snail expression and STAT3 phosphorylation in AGS and BGC-823 cells which can be reversed by neutralization antibody of CXCL1 and CXCL5. (D) Knocking down CXCR2 or pretreating GC cells with STAT3 pathway inhibitor AG490 (2uM) for 4 hours can reverse macrophages induced EMT of AGS and BGC-823 cells.

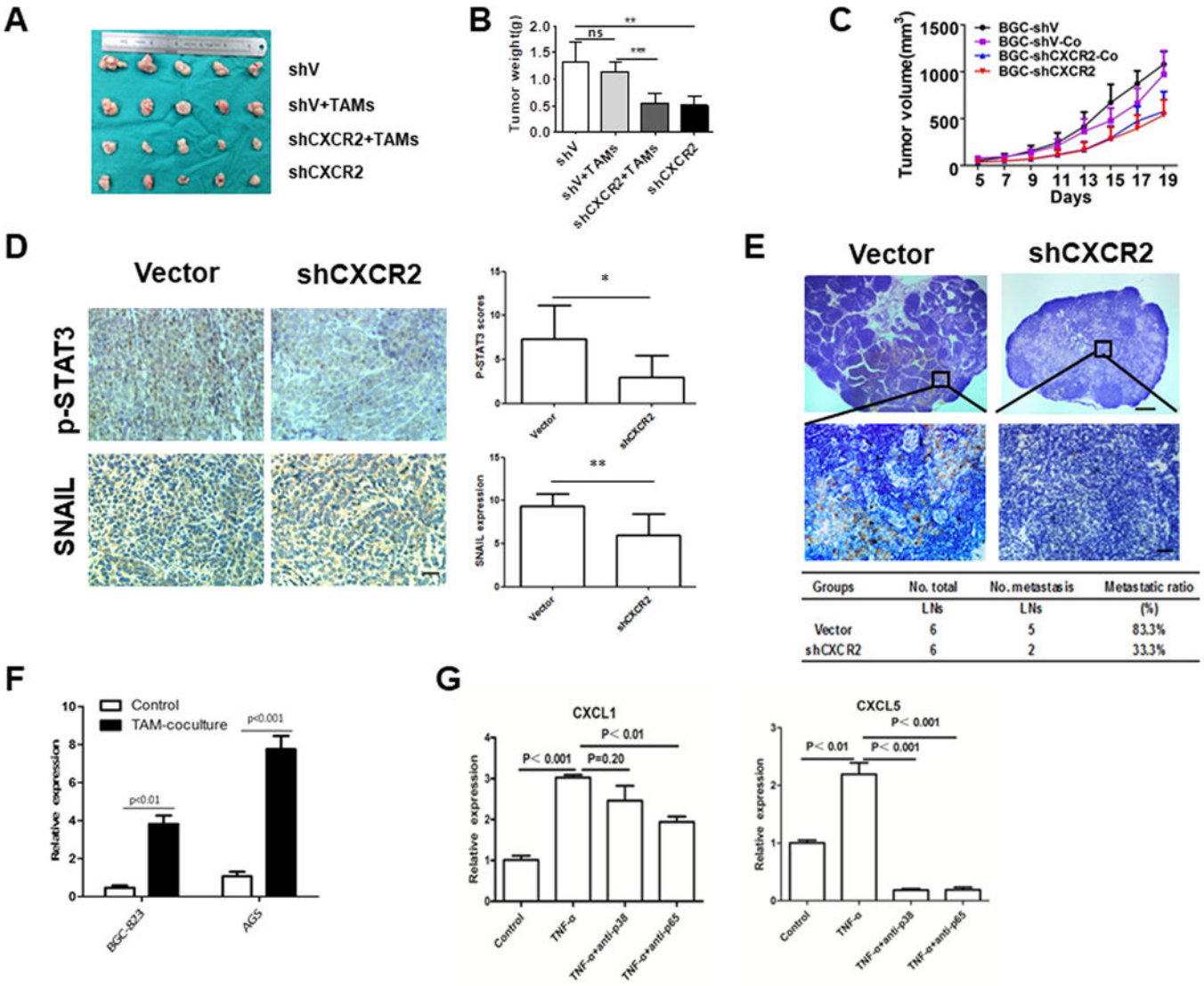

Figure 5. Inhibiting CXCR2 pathway of GC cells can suppress the migration and progression of GC *in vivo*.

(A) Subcutaneous tumor tissue from four experimental groups of nude mice. (B) Weight statistics of subcutaneous tumor tissue from four experimental groups of nude mice. (C) Growth curve of subcutaneous tumors in each experimental group. n=5 *per* group. * $P<0.05$ (D) Representative images to show that knocking down CXCR2 decreased expression of p-STAT3 and EMT marker Snail. The scale bar is 20 μm. (E) Representative images of lymph nodes metastasis from Footpad Lymph Node Metastasis mouse model, and statistics of metastatic lymph nodes. The scale bar is 100μm for above panel and 20 μm for the below panel. (F) mRNA level of TNF-α in GC cells was increased when co-cultured with macrophages (TAM). (G) Human recombinant TNF-α induced release of CXCL1 and CXCL5 from macrophages. Blockage TNF-α pathway decreased expression of CXCL1 and CXCL5 in macrophages.

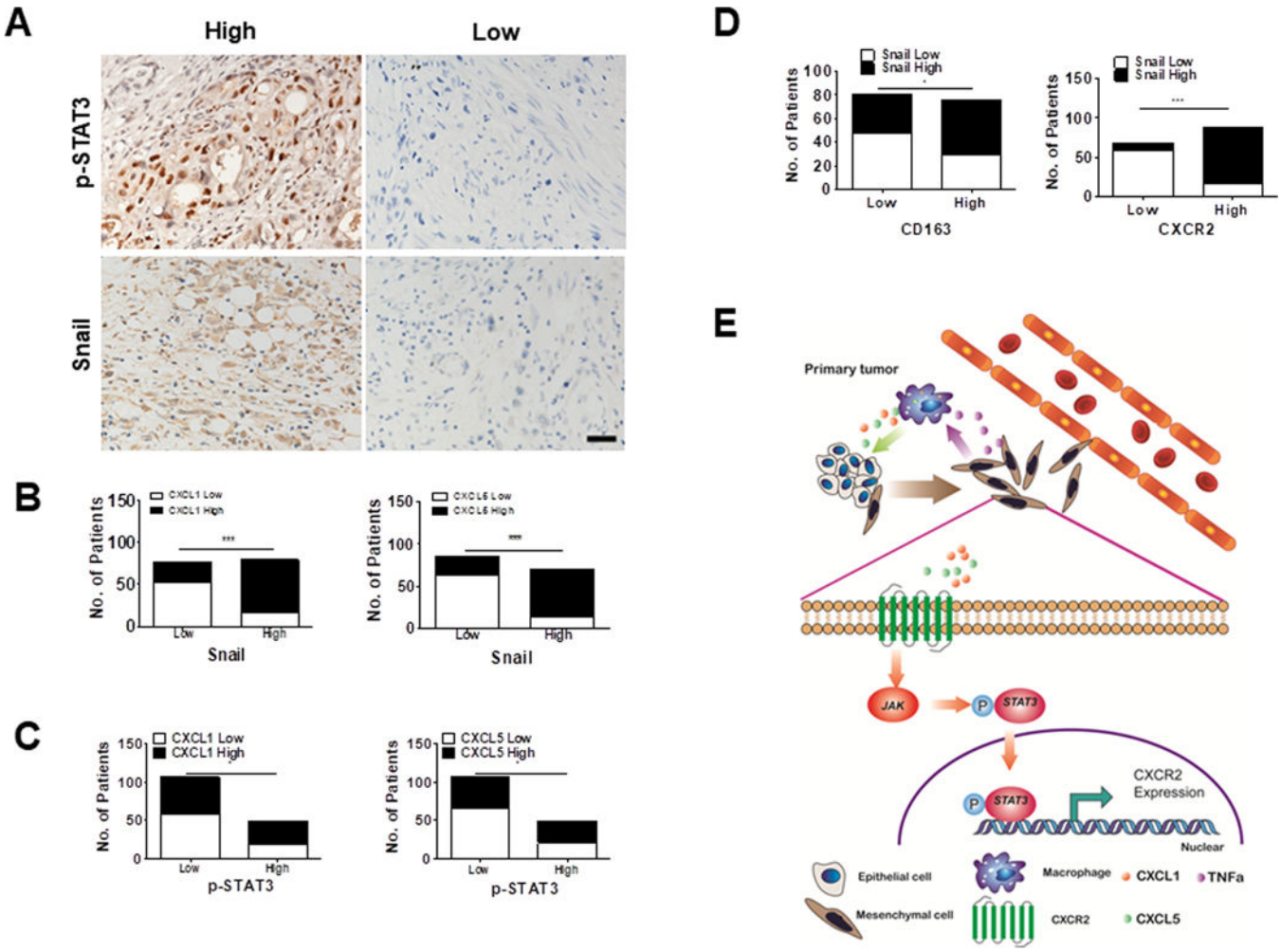

Figure 6. Macrophages are associated with the activation of CXCR2 pathway and EMT in human GC tissue.

(A) Expression of p-STAT3 and Snail were analyzed in 155 GC tissues by IHC (200×). Scale bar is 50μm. (B) Correlations between the expression of Snail and that of CXCL1/CXCL5 were analyzed. (C) Correlations between the expression of p-STAT3 and that of CXCL1/CXCL5 were analyzed. (D) Correlation between CD163, CXCR2 and Snail expression. (E) Graphical abstract to show how macrophages interact with tumor cells to promote GC metastasis. All the statistical analysis was performed with Fisher's exact test