A Positive Feedback Loop Between Gastric Cancer Cells and Tumor-associated Macrophage Induces Malignancy Progression

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Abstract

Background: Hypoxia and inflammation tumor microenvironment (TME) play a crucial role in tumor development and progression. Although increased understanding of TME contributed to gastric cancer (GC) progression and prognosis, the direct interaction between macrophage and GC cells was not fully understood.

Methods: Hypoxia and normoxia macrophage microarrays of GEO database was analyzed. The peripheral blood mononuclear cell acquired from the healthy volunteers.

The expression of CXCL8 in GC tissues and cell lines was detected by quantitative reverse transcription PCR (qRT-PCR), western-blot, Elisa and immunofluorescence. Cell proliferation, migration, and invasion were evaluated by cell counting kit 8 (CCK8), colony formation, real-time imaging of cell migration and transwell. Luciferase reporter assays and chromatin immunoprecipitation were used to identify the interaction between transcription factor and target gene. Especially, a series of truncated and mutation reporter genes were applied to identify precise binding sites. The corresponding functions were verified in the complementation test and *in vivo* animal experiment.

Results: Our results revealed that Hypoxia triggered macrophage secreted C-X-C Motif Chemokine Ligand 8 (CXCL8), which induced GC invasion and proliferation. This macrophage-induced GC progression was CXCL8 activated C-X-C Motif Chemokine Receptor 1/2 (CXCR1/2) on the GC cell membrane subsequently hyperactivated Janus kinase 1/ Signal transducer and activator of transcription 1 (JAK/STAT1) signaling pathway. Then, the transcription factor STAT1 directly led to the overexpression and secretion of Interleukin 10 (IL-10). Correspondingly, IL-10 induced the M2-type polarization of macrophages through the Nuclear Factor kappa B (NF-κB) pathway-dependent mechanism and continued to increase the expression and secretion of CXCL8 through the transcription factor Nuclear Factor Kappa B Subunit 1 (NFKB1, p50). It suggested a positive feedback loop between macrophage and GC. In clinical GC samples, increased CXCL8 predicted a patient's pessimistic outcome.

Conclusion: Our work identified a positive feedback loop governing cancer cells and macrophage in GC that contributed to tumor progression and patient outcome.

1. Background

Gastric cancer (GC) is a prevalent yet incurable malignancy of the digestive system. It is the 3rd leading cause of cancer-related mortalities across the globe ¹. China has a high GC incidence and a heavy disease burden, with an estimated 320,000 annual deaths, accounting for 45% of all GC deaths globally ². Despite the constant iterations of comprehensive therapy based on surgical resection, GC prognosis remains poor, with a 5-year survival rate of not more than 30% ³. On one hand, this is attributed to the malignant phenotype of the tumor; while on the other hand, the tumor microenvironment plays an essential role as an "accomplice" ⁴. Hypoxia and inflammation are essential components of the tumor

microenvironment (TME) ^{5 6}. Previous studies confirmed that hypoxia promotes GC progression ⁷. Moreover, the hypoxia-related core element, hypoxia-inducible factor-1 α (HIF-1 α) were confirmed as transcription factors that could broadly regulate the transcription of downstream genes, promoting GC proliferation ⁸ and invasion ^{9 10}. Therefore, it is essential to further explore the crosstalk between hypoxia and inflammation in the GC-TME.

Macrophages are the most abundant inflammatory cells in the tumor microenvironment. They exhibit significant plasticity and can freely switch from one phenotype to another; however, this depends on the signals received from their surrounding microenvironment ^{11 12}. The process is called macrophage polarization. Based on the activation state, macrophages can be classified into classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) ¹³. Although this dichotomy is somewhat arbitrary, it remains the most popular macrophage definition ^{14 15}. It is generally believed that the M1 type has an anti-tumor property, whereas the M2 type is a pro-cancer factor. Also, the cancer cell is an "educator" that converts M1 to M2 ¹⁶. M2 macrophages promote tumor metastasis and angiogenesis by secreting various cytokines and exerting immunosuppressive effects ¹⁷. Macrophages recruited into TME by cytokines are referred to as tumor-associated macrophages (TAMs) ¹⁸. In TME, TAMs predominantly exhibit an M2 phenotype, indicating a poor prognosis of solid tumors ¹⁹ ²⁰. Since hypoxia could promote immune evasion, angiogenesis, proliferation, and metastasis, research suggests that it can as well facilitate TAMs in tumor development. Additionally, the TAMs in tumor hypoxic regions mediate treatment resistance and promote cancer recurrence ²¹.

This paper focuses on the effect of hypoxia on macrophages then analyzes the role of hypoxic microenvironment and inflammatory microenvironment in GC development. *CXCL8* (C-X-C Motif Chemokine Ligand 8, also called *IL-8*) was overexpressed in hypoxic macrophages. Moreover, we discovered that the macrophage-derived *CXCL8* could activate the *JAK/STAT1* signaling pathway and promote GC invasion as well as proliferation. Besides, as a transcription factor, *STAT1* could upregulate *IL-10* expression (Interleukin 10) in GC; the latter could naturally trigger macrophage M2-type polarization. The M2 macrophages increased the release of *CXCL8*. The hypoxia-activated positive feedback loop *CXCL8/CXCR1/2* (C-X-C Motif Chemokine Receptor 1/2)/*STAT1/IL-10/NFKB1/CXCL8* could cascade and amplify the interaction between GC and macrophages leading to uncontrolled progressive signaling in GC.

2. Materials And Methods

2.1 Bioinformatics

Hypoxic and normoxic cultured human monocyte-derived macrophage dataset GSE4630 was downloaded from the GEO (Gene Expression Omnibus) database (<https://www.ncbi.nlm.nih.gov/geo/>). They included two hypoxic-cultured samples and two normoxic-cultured samples. The GC RNA sequencing data were downloaded from the TCGA (The Cancer Genome Atlas, TCGA-STAD, 375 tumor

samples vs. 31 normal samples, <https://portal.gdc.cancer.gov/>). The GC and normal sample microarray data were also obtained from the GEO (GSE54129, 111 tumor samples vs. 21 normal samples). The differentially expressed genes were identified by the "limma" package ($P < 0.05$, and $|\text{FoldChange}| \geq 2$). JASPAR (<http://jaspar.genereg.net/>) and UCSC (<http://genome.ucsc.edu/>) were performed to predict the transcription factor.

2.2 Cell culture and human PBMCs isolation

The GC (HGC-27, NUGC3) cells, human normal gastric epithelial cell line (GES-1) were acquired from China Medical University (Shenyang, China). Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors in Liaoning Cancer Hospital & Institute. CD14⁺ cells were enriched by depleting CD8⁺, CD19⁺, CD56⁺ and CD14⁺ cells by MACS (magnetic-activated cell sorting, EasySep, STEMCELL Technologies, 19059) columns with negative selection. PBMCs were differentiated into M0 macrophages using 50 ng/mL human macrophage colony-stimulating factor (hM-CSF, Sigma, 81627-83-0) for 7 days (Fig. 1C). All cells were cultured in RPMI-1640 (FUJIFILM Wako Pure Chemical Corporation, 189-02145) supplemented with 10% fetal bovine serum (FBS, Gibco 26140-079) under 1% O₂ or 20% O₂ conditions as described previously^{8 10 22}.

2.3 GC tissues and ethical approval

Between January 2009 and December 2012, gastric cancer and adjacent non-cancerous tissue samples were obtained from 526 patients subjected to adequate complete surgical resection (R0) of locally advanced GC surgical resection in Liaoning Cancer Hospital & Institute. All recruited patients had not received preoperative chemotherapy or radiotherapy. They signed a written informed consent before surgery. The follow-up of patients was closed on December 31, 2020. The study was approved by the Ethics Committee of Liaoning Cancer Hospital and Research Institute (20181226).

2.4 Immunohistochemistry

The immunohistochemistry (IHC) was performed based on previously published protocols²³. Immunohistochemistry was scored based on the intensity of staining and the proportion of positive cells. The blinded review was performed by two pathologists. *Ki67* (1:1000, Abcam, ab15580), *Caspase-3* (1:1000, Abcam, ab184787) *CXCL8* (5 µg/mL, R&D Systems, AF-208-NA).

2.5 Immunofluorescence

The slices were overnight incubated with primary antibody at 4°C. Then, they were incubated with species-appropriate rabbit/mouse secondary antibodies coupled with AlexaFluor dyes (488, 594, 1:200, Invitrogen, A32814, A32723, A32740), DAPI (1:1000, Dojindo, KT013) at room temperature for 1 hour. *CXCL8* (5 µg/mL, R&D Systems, AF-208-NA), *CD68*, *CD163*, *CD86* (1:200, Abcam, ab213363, ab182422, ab270719). Olympus Fluoview FL1200 confocal microscope was used to capture 20x and 60x images.

2.6 ELISA test

ELISA assay was used to measure the *CXCL8* in the supernatant of macrophages cultured in hypoxic or normoxic conditions and *IL-10* in the medium of GC cells. The procedure followed the instructions of the Human *CXCL8* kit (R&D Systems, D8000C) and Human IL-10 ELISA Kit (Abcam, ab185986).

2.7 qRT-PCR

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed as previously documented⁸. Primer sequences were designed by Sangon (China, Supplementary Table 1).

2.8 Western blot analysis

The RIPA lysis buffer was used to extract total proteins from cells and tissues (Beyotime, Shanghai, China). Then, 10% SDS-PAGE gel was used to separate the proteins and transferred using the PVDF membrane. The membranes were overnight incubated with the primary antibodies (Supplementary Table 2) at 4°C. Subsequently, they were co-cultured with the secondary antibody for 1 h. The ECL system (Amersham Imager 600) and ImageJ software were applied to observe and calculate grayscale values⁸.

2.9 Chromatin immunoprecipitation (ChIP)

ChIP enzymatic chromatin IP kit protocol (Cell Signaling Technology, China) was used to perform the ChIP assay, where 1×10^7 logarithmic phase cells were subjected to ChIP. The chromatin was immunoprecipitated with an anti-STAT1 (Santa Cruz Biotechnology, USA, 1:50) and mouse IgG (Santa Cruz Biotechnology, USA, 1:100) on rotators at 4 °C for 16 h.

2.10 Promoter-Luciferase reporter assay

Promoter-Luciferase reporter assay confirmed the combination of *STAT1* and *IL-10* promoter sequence, *NFKB1*, and *CXCL8* promoter sequence, respectively. The recombinant *pGL-3* basic-plasmid contained truncated human *IL-10* (or *CXCL8*) promoter (wild type, wt, -2000~ +99) or mutant *IL-10* (or *CXCL8*) promoter (mutant type, mut). The plasmids were transfected with the POL03000 transfection reagent (Research and science, China). After 48 h, the luciferase activity of each group was evaluated following the manufacturer's instructions.

2.11 Colony formation assay

A total of 1×10^5 GC cells/each group were seeded into 6-well plates for 2 weeks. The colonies were washed three times using PBS, fixed with 4% paraformaldehyde, and stained with Diff-Quik III Kit (Muto Chemical, ZS0003). The stained colonies were observed under a light microscope (IX81 Olympus).

2.12 Cell counting assay

Cell proliferation was evaluated using the Cell counting kit (Cell counting kit 8, CCK-8). The cells were incubated at 37 °C for 24, 48, 72, and 96 h. Subsequently, the medium was discarded then the chromogenic solution at 10:1 was prepared. For incubation, a color-developing solution (10 µl) was added to the 96 well plates at 37 °C for 2 h. The optical density (OD) was detected using the UV spectrophotometer (BioTek Synergy H1) at 450 nm.

2.13 Transwell

Gastric cancer cells were seeded in Transwell upper chambers coated with gelatin. The lower chambers with 600 µL RPMI-1640 with 20% FBS. After 24h, the cells were fixed and stained using methanol, hematoxylin, and eosin (Sigma-Aldrich, St. Louis, MO, USA). The upper chamber was removed and the lower layer migrating cells counted under the microscope (IX81 Olympus).

2.14 Real-time imaging of cell migration

A total of 200 µl Matrigel (BD Biosciences, 356235) pre-coated the bottom of the six-well plates, the cells were then inoculated for 24h. Thereafter, a 6-well plate was cultured and imaged using KEYENCE BZ-X700 (KEYENCE, Japan), equipped with CO₂ and temperature control chamber as well as a time-lapse tracking system. BZ-X Viewer software (KEYENCE) was used to capture phase-contrast images at intervals of every 10 minutes for 48 hours, while the BZ-X Analyzer software (KEYENCE) was used to convert the continuous images into movie files. Furthermore, KEYENCE video editing and analysis software was used to analyze cell migration in the movies. Microsoft Excel 2010 was used to process the trace data to create XY coordinate graphs and distance measurements.

2.15 Xenograft mouse model

For subcutaneous tumor xenografts, an estimated 1×10^6 GC cells/0.2 ml PBS (with or without Recombinant Human CXCL8) were subcutaneously injected into the right axillary region of a 5-week-old female BALB/ C mouse. After 28 days of injection, tumors were collected, measured volumes weighed, and photographed. The volume was calculated using the following formula (tumor volume = $L \times W \times W/2$).

For the intraperitoneally model, approximately 5×10^6 GC cells/0.1 ml PBS (with or without Recombinant Human CXCL8) were injected into the mouse as mentioned above. Similarly, the mice were euthanized 28 days after injection, then the volume of ascites and the number of visible (>0.1 cm) metastatic nodules in the peritoneal cavity were measured.

2.16 Other reagent or resource

Recombinant Human *IL-8* (CXCL8, PeproTech, 200-08), Recombinant Human *IL-10* (PeproTech, 200-10), Recombinant Human M-CSF (PeproTech, 300-25), Repertaxin (Sigma-Aldrich, 266359), Fludarabine (Tocris, 3495/10), Sarsasapogenin (Selleck, S3607).

2.17 Statistical analysis

GraphPad Prism 9.0 (GraphPad Software Inc) and SPSS 24.0 statistical software (IBM) were used for statistical analyses of data. The student's *t*-test was utilized to perform statistical analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Hypoxia promotes macrophage-derived CXCL8 secretion

Relevant expression profile data were downloaded from the GEO database (GSE4630) to identify the reflection of macrophages under hypoxic conditions. The dataset included four PBMCs-derived macrophages samples. Two of them were cultured in 0% O₂, while the other two were cultured in 20% O₂. The "limma" package was used to identify the differentially expressed genes (DEGs, Supplementary Table 3). As a type of immune cell, macrophages could disrupt the microenvironment through cytokines. Thus, this work assessed the effect of hypoxia on cytokine-related gene expression. Among them, *CXCL8* was significantly upregulated in the hypoxic-cultured macrophage (Fig. 1A, 1B). This implies that hypoxic TME might stimulate *CXCL8* secretion by macrophages; nevertheless, this warrants further validation. CD14+ positive monocytes by MACS (magnetic-activated cell sorting) negative selection were obtained from healthy volunteers to accurately reflect the *in vivo* features of macrophages. The PBMCs were induced into M0 macrophage (M0) for 7 days (Fig. 1C). Subsequently, incubation of M0 for 72 h was continued under hypoxic (1% O₂) and normoxic (20% O₂) conditions, respectively. Both qRT-PCR and Western-blot showed that *CXCL8* was overexpressed in hypoxic-cultured M0 (Fig. 1D, 1E). Similarly, the upregulated *CXCL8* in the supernatant of hypoxic-culture M0 was confirmed through the ELISA test (Fig. 1F). Immunofluorescence analysis in human GC tissues showed extensive co-localization of *CD68* and *CXCL8*, suggesting that macrophages could secrete *CXCL8* (Fig. 1G).

3.2 CXCL8 correlates with poor prognosis and tumor progression of GC

CXCL8 expression in TCGA and GEO (GSE54129) was assessed to further elucidate its role in GC. *CXCL8* expression was significantly upregulated in GC tissues compared to that in normal tissues (Fig. 2A, 2B). Subsequently, we assessed the clinical significance of *CXCL8* based on the Liaoning cancer hospital cohort. *CXCL8* in cancerous tissues was remarkably higher than that in paired paracancerous tissues (Fig. 2C). Figure 2D reveals the representative images of high expression and low expression *CXCL8* in GC tissue. Moreover, high *CXCL8* expression promoted poor DFS (disease-free survival, Fig. 2E) and OS (overall survival, Fig. 2F). The cohort information is listed in Exhibit Supplementary Table 4.

3.3 CXCL8 activates the CXCL8-CXCR1/2 axis of GC and exacerbates the malignant phenotype

In vitro and *in vivo* assays were performed to assess the function of *CXCL8* on GC cells. The GC cells were seeded on the six-well plates coated with Matrigel. The real-time imaging recorded the motion state of the cells. Migration of GC cells was significantly improved when co-culture with recombinant human *CXCL8*. *CXCL8* accelerated cells migration (50ng/ml, Fig. 3A-3C, Sup movie 1, 2). Nonetheless, the enhanced motility was inhibited by adding *CXCL8-CXCR1/2* inhibitors (Repertaxin, 50 µg/ml, Fig. 3A-3C,

Sup movie 1, 2). The promoting impact of *CXCL8* on GC invasion ability was achieved by the *CXCL8-CXCR1/2* axis. In keeping with this result, the Transwell assay also exhibiting *CXCL8* caused an increase in the number of cells penetrating the membrane, but was blocked by repertaxin (Fig. 3D). On cell proliferation, the CCK-8 and colony formation assay demonstrated the promotion of cell proliferation by *CXCL8* (Fig. 3E, 3F); this was also achieved through the *CXCL8-CXCR1/2* axis (Fig. 3E, 3F). In the *in vivo* assays, we also confirmed that *CXCL8* promoted the formation of the subcutaneous tumor. After injection of GC cells stimulated by *CXCL8*, the subcutaneous tumors had a larger size and heavier weight than the control. In contrast, no significant difference was noted in tumor size between the control and the inhibitor-added groups (Fig. 3G).

Furthermore, unlike other digestive system cancers metastasizing primarily through the vasculature, GC cells had a greater tendency to develop peritoneal metastases. Therefore, we evaluated the effect of *CXCL8* on these types of metastases. After intraperitoneal GC cells co-culturing with *CXCL8*, the mice evolved peritoneal metastases, but it did not appear in both control and inhibitor groups (Fig. 3H). This implies that *CXCL8* could promote GC malignant phenotype; this was achieved through the *CXCL8-CXCR1/2* axis.

3.4 JAK/STAT1 pathway is downstream of CXCL8

After clarifying the oncogenic effect of *CXCL8* on GC, it is essential to explore the possible mechanisms involved. Since *JAK/STAT1* pathway is a canonical downstream of the Chemokine signaling pathway²⁴²⁵, the effect of the *CXCL8-CXCR1/2* axis on the *JAK/STAT1* pathway activation was assessed. The protein expression of *p-JAK*, *p-STAT1* was significantly upregulated after stimulation of GC with *CXCL8*, while the expression of *JAK*, *STAT1* did not significantly change (Fig. 4A). Similarly, the *CXCL8-CXCR1/2* inhibitors and repertaxin inhibited the activation of the *JAK/STAT1* pathway by *CXCL8* (Fig. 4A). *JAK/STAT1* pathway might be the downstream effector of the *CXCL8-CXCR1/2* axis. To validate the result, *CXCL8*-induced GC cells were co-cultured with *STAT1* specific inhibitor (Fludarabine). As expected, the activation effect of *CXCL8* on *JAK/STAT1* disappeared when cells were treated with Fludarabine (50μM, Fig. 4A). This confirmed that the *JAK/STAT1* is the downstream effector of *CXCL8*. Unsurprisingly, the *JAK/STAT1* pathway inhibitors also limited the effects of *CXCL8* on invasion (Fig. 4B-4E, Sup movie 3, 4) and proliferation (Fig. 4F, 4G). Thus, a preliminary conclusion would be exogenous *CXCL8* continuity activated the *CXCL8/CXCR1/2-JAK/STAT1* pathway and triggered invasion and proliferation of GC.

3.5 STAT1 regulates the expression of IL-10 directly but not HIF-1α

STAT1 belongs to the *STAT* protein family that forms homo- or hetero-dimers and translocates to the nucleus to exert regulatory effects as a transcriptional activator. Naturally, we sought to identify the downstream regulatory molecules of *STAT1*. Through the JAPAR database, the target genes of *STAT1* were predicted. Among them, *HIF-1α* and *IL-10* attracted our attention. The former was the hypoxic associated core effector molecule, while the latter was closely related to the polarization of macrophages. It should assess the influence of *STAT1* on *HIF-1α* and *IL-10*. The shRNA and OE-vector of *STAT1* could

up-or down-regulated *STAT1* expression (Fig. 5A). *STAT1* inhibition was followed by a downregulation in *HIF-1α* and *IL-10* expressions both at the transcriptional and protein levels (Fig. 5B-5E). In contrast, both were upregulated with *STAT1* overexpression (Fig. 5B-5E). With the promising results, luciferase reporter assay and ChIP were performed to assess the interaction of *STAT1* with *HIF-1α* and *IL-10* promoters. Therefore, full-length *HIF-1α* and *IL-10* promoters were cloned into luciferase reporter plasmids, respectively. The luciferase reporter assay revealed that the up-regulated *STAT1* could enhance the luciferase activity of *IL-10-wt*, but not *IL-10-mut* (Fig. 5F). Unfortunately, *STAT1* did not affect the fluorescence intensity of *HIF-1α*, whether *HIF-1α-wt* or *HIF-1α-mut* (Fig. 5G). This suggests that as a transcription factor, *STAT1* could directly activate the transcription of *IL-10*, however, *HIF-1α* promotion might rely on other indirect pathways. Subsequently, the ChIP assay confirmed that the *STAT1* antibody could dismantle the *IL-10* sequence but not the control IgG (Fig. 5H). A series of reporter genes comprising truncated *IL-10* promoter sequences were constructed to further refine the target sequence of the promoter. The luciferase reporter assay showed that deleting the region between -1811 and -1821 bases of *IL-10* promoter severely abolished *IL-10* by *STAT1* activation (Fig. 5I).

3.6 IL-10 induces M2 polarization through the NF-κB pathway and promotes CXCL8 expression

As evidenced above, hypoxic-macrophage-derived *CXCL-8* could promote *IL-10* expression by activating *JAK/STTA1* in GC while the cytokine *IL-10* can induce M2 polarization. Therefore, the effect of *IL-10* on macrophages and the potential underlying mechanisms should be confirmed. Unsurprisingly, immunofluorescence confirmed that *IL-10* significantly promoted *CD163* expression in PBMCs induced macrophage, and M2 polarization (Fig. 6A). Of note, the inhibition of the *NF-κB* pathway is an essential factor in driving macrophage M1 polarization²⁶. Thus, it is necessary to evaluate the effect of *IL-10* on the *NF-κB* pathway in macrophages. The addition of *IL-10* upregulated p50 and p65 expression in the nucleus and promoted p65 phosphorylation (Fig. 6B). Subsequently, *IL-10* and Sarsasapogenin (50μM), an inhibitor, specifically targeting *IκB* were used to inhibit the *NF-κB* pathway and co-cultured macrophages. Sarsasapogenin attenuated *CD163* expression (Fig. 6C). It inhibited the M2 polarization. This demonstrates that the effect of *IL-10* on macrophage M2 polarization was partly achieved through the *NF-κB* pathway.

Besides, we analyzed the effects of *IL-10* and *NF-κB* pathways on *CXCL8* expression. *IL-10* upregulated *CXCL8* expression at the transcriptional and protein levels (Fig. 6D, 6E) and intensified the secretion of the latter (Fig. 6F). Correspondingly, the *NF-κB* pathway could antagonize this effect (Fig. 6D-6F). This implies that the *IL-10-NF-κB* axis was implicated in *CXCL8* regulation at the transcriptional level. The subsequent transcription factor luciferase reporter assay and ChIP confirmed our suspicions. The gene (*p50*) was bound to the promoter of *CXCL8* (Fig. 6G, 6H). A series of truncated *CXCL8* promoter sequences were constructed, which included at least one potential binding site (in red). Nevertheless, every sequence could promote *CXCL8* expression. Mutated the mut1 (in blue, -1829~ -1819) and mut2 (in green, -91~ -81) sequences simultaneously, the fluorescence intensity significantly decreased. This confirmed the presence of two *NFKB1* binding sites in the promoter sequence and up-regulated *CXCL8* expression.

In this way, a positive feedback loop was formed between macrophage and GC cells that exacerbated tumor deterioration under the catalysis of hypoxic TME.

4. Discussion

TME of most solid tumors is characterized by hypoxia and inflammation²⁷. The crosstalk between TME and cancer cells promotes tumor growth and progression²⁸. Cytokines are the critical bridge that maintains this intercellular communication; besides, they can favorably shape the TME for tumor cells²⁹. Gastric cancer cells rely on this complex environment to achieve sustainable development, invasion, and metastasis. In our previous study, we continuously focused on the effect of hypoxia on the progression of GC cells³⁰. Nevertheless, this effect on TME stromal cells and inflammatory cells has been overlooked.

Of note, tumor-infiltrating macrophages release the strongest signal in inflammatory TME³¹. Thus, we concentrated on the effect of hypoxia on macrophage cytokines i.e., the communication tools. Among them, *CXCL8* exhibited the most potent response to the hypoxic signal. *CXCL8* is one of the earliest and most comprehensively studied chemokines. It was discovered, purified, and sequenced in monocytes in the 1980s³². *CXCL8* is secreted by different cells, including monocytes, alveolar macrophages, fibroblasts, endothelial cells, and epithelial cells^{33 34}. Additionally, cytokines (interleukin-1, interleukin-6, *CXCL12*, *TNFα*), hypoxia, reactive oxygen species (ROS), bacterial particles could stimulate *CXCL8* expression. The transcription factor *NF-κB* and activator protein-1 (*AP-1*) were also implicated in *CXCL8* activation^{35 36}. Notably, *CXCL8* was nearly undetectable in unstimulated cells. In contrast, the above stimulation would upregulate *CXCL8* expression by 10 to 100 times³⁷. Besides, this type of explosive growth provides a basis for its role in cancers. *CXCL8* binds G protein-coupled receptors or CXCR1/2 to transmit its signal. *CXCL8*-CXCR1/2 axis stimulates endothelial cells and promotes tumor angiogenesis³⁸. Blocked *CXCL8*-CXCR2 signal transmission between TAMs and cancer cells could improve the effect of anti-PD1 treatment^{39 40}. This study found that the *CXCL8*-CXCR1/2 axis activated the *JAK/STAT1* pathway in GC, thereby promoting the malignant phenotype. Further, *STAT1* acted as a transcription factor directly regulating *IL-10* expression, this was closely associated with macrophages. In addition, it could ignore that *STAT1* also affected the *HIF-1α* expression, which was the most crucial element for the organism responding to hypoxia. The indirect regulatory pattern of hypoxia/*CXCL8*/CXCR1/2/*JAK/STAT1/HIF-1α* might be a non-classical adaptation pattern of GC cells to the hypoxic microenvironment.

IL-10 is one of the most famous molecules causing macrophage polarization⁴¹. It triggers M2 polarization by regulating the *NF-κB* and *JAK/STAT* pathway⁴². Generally speaking, *NF-κB* pathway activation is often regarded as a marker of M1 macrophages because of its pro-inflammatory effects. However, with the introduction of the subclassification theory of M2 macrophages, the M2d subtype is the closest to TAMs, and the activation of *NF-κB* pathway is also a sign event of M2d activation^{43 44}. This means that the activation of the *NF-κB* pathway in the complex TME is still a double-edged sword. Subsequently, it was natural for us to further evaluate its effect on macrophage and macrophage-derived

CXCL8. Our results demonstrated that *IL-10* regulated M2 polarization via the *NF-κB* pathway and affected *CXCL8* expression. Surprisingly, among the macrophage added with *IL-10* and *NF-κB* inhibitors, a few macrophages expressed both M1 and M2 type markers, presenting a hybrid state. Although the simple dichotomy of macrophage is widely used, researchers continue to express doubts. With the advancement of single-cell technology, further sub-classification of macrophage by markers on the surface of the macrophage membrane deepen the understanding of the TME⁴⁵. Specifically, tissue-resident macrophages and monocyte-derived macrophages have entirely different biological functions, however, the two co-exist in TME¹⁹. Subsequent luciferase reporter assays and ChIP assays confirmed the regulatory role of the transcription factor *NFKB1* (*p50*) on *CXCL8*. This was also consistent with previous findings^{46 47}. Thus, macrophage-derived *CXCL8* could further promote the deterioration of GC and cause *IL-10* expression, recurrence of the cycle.

The above results illustrate a novel paradigm of GC-TAMs' interaction: Hypoxia spurs the release of overexpressed *CXCL8* by macrophages which then activates the *CXCL8/CXCR1/2-JAK/STAT1* pathway and promotes GC progression. Moreover, this pathway directly promotes the expression of GC-derived *IL-10*, further accelerating the release of macrophage-derived *CXCL8* under the *IL-10/NF-κB/CXCL8* axis. In this process, the M2-type polarization is simultaneously induced. This causes ordinary macrophages involved in immune surveillance to "betray" into TAMs implicated in immune escape and suppression. Hypoxia/*CXCL8/CXCR1/2-JAK/STAT1/IL-10/NF-κB/CXCL8* forms a positive feedback loop between GC and macrophage (or TAMs). As the core molecule of this circuit, *CXCL8* is a high-risk factor for GC prognosis. This suggests that the signaling loop is a potential therapeutic target. Also, research on anti-*CXCL-8* therapy in solid tumors recruits or collates data (NCT01831310, NCT02536469, NCT04347226), and we have reason to expect these corresponding results.

5. Conclusion

In conclusion, our work identifies a hypoxic-activated, inflammation-associated molecular network; which involve *CXCL8*, *CXCR1/2*, *JAK/STAT1*, *IL-10*, and *NF-κB* signaling pathways that regulate a positive feedback loop between GC cells and TAMs, tumor progression, macrophage polarization, as well as patient outcomes (Fig. 7). These results indicate the potential of this feedback loop as a therapeutic target for GC.

Abbreviations

GC: Gastric cancer

TME: Tumor microenvironment

HIF-1α: Hypoxia-inducible factor-1α

TAMs: Tumor-associated macrophages

CXCL8/IL-8: C-X-C Motif Chemokine Ligand 8

IL-10: Interleukin 10

CXCR1/2: C-X-C Motif Chemokine Receptor 1/2

JAK: Janus Kinase 1

STAT1: Signal Transducer And Activator Of Transcription 1

GEO: Gene Expression Omnibus

TCGA: The Cancer Genome Atlas

PBMC: Peripheral blood mononuclear cell

FBS: Fetal bovine serum

IHC: Immunohistochemistry

qRT-PCR: quantitative real-time polymerase chain reaction

ChIP: Chromatin immunoprecipitation

CCK8: Cell counting kit 8

OD: Optical density

DEGs: Differentially expressed genes

MACS: Magnetic-activated cell sorting

DFS: Disease-free survival

OS: Overall survival

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the Faculty of Science Ethics Committee at Liaoning Cancer Hospital & Institute (Cancer Hospital of China Medical University, 20181226).

Informed consent

Informed consent was obtained from patients before surgery at the Liaoning Province Cancer Hospital & Institute.

Consent for publication

All authors agree with the content of the manuscript.

Availability of data and materials

The data are available from the sources listed in the manuscript—the TCGA and GEO data portal. The data used to support the findings of this study are available from the corresponding author upon request

Competing interests

The authors declare no conflicts of interest.

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Author Contributions

Piao HY and Fu LF performed the majority of experiments and analyzed the data and drafted the manuscript; Zhang J designed the research; Liu Y and Yang D conducted the molecular biology assays and assisted in writing the manuscript; Wang Y and Meng XY collected and analyzed the data. Xiao X contributed to data visualization.

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References

1. Siegel RL, Miller KD, Fuchs HE, et al. Cancer statistics, 2021. *CA: a cancer journal for clinicians* 2021;71(1):7-33.
2. Yang L, Zheng R, Wang N, et al. Incidence and mortality of stomach cancer in China, 2014. *Chin J Cancer Res* 2018;30(3):291-98. doi: 10.21147/j.issn.1000-9604.2018.03.01 [published Online First: 2018/07/27]
3. Galletti G, Zhang C, Gjyzezi A, et al. Microtubule Engagement with Taxane Is Altered in Taxane-Resistant Gastric Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2020;26(14):3771-83. doi: 10.1158/1078-0432.Ccr-19-3018 [published Online First: 2020/04/24]

4. Zheng ZQ, Chen JT, Zheng MC, et al. Nestin +/-CD31 + Cells in the Hypoxic Perivascular Niche Regulate Glioblastoma Chemoresistance by Upregulating JAG1 and DLL4. *Neuro-oncology* 2020 doi: 10.1093/neuonc/noaa265 [published Online First: 2020/11/30]
5. Massara M, Bonavita O, Savino B, et al. ACKR2 in hematopoietic precursors as a checkpoint of neutrophil release and anti-metastatic activity. *Nat Commun* 2018;9(1):676. doi: 10.1038/s41467-018-03080-8 [published Online First: 2018/02/16]
6. Lee KE, Spata M, Bayne LJ, et al. Hif1a Deletion Reveals Pro-Neoplastic Function of B Cells in Pancreatic Neoplasia. *Cancer Discov* 2016;6(3):256-69. doi: 10.1158/2159-8290.Cd-15-0822 [published Online First: 2015/12/31]
7. Zhang J, Wu Y, Lin YH, et al. Prognostic value of hypoxia-inducible factor-1 alpha and prolyl 4-hydroxylase beta polypeptide overexpression in gastric cancer. *World journal of gastroenterology* 2018;24(22):2381-91. doi: 10.3748/wjg.v24.i22.2381 [published Online First: 2018/06/16]
8. Piao H-y, Liu Y, Kang Y, et al. Hypoxia associated lncRNA HYPAL promotes proliferation of gastric cancer as ceRNA by sponging miR-431-5p to upregulate CDK14. *Gastric Cancer* 2021:1-20.
9. Zhang J, Guo S, Wu Y, et al. P4HB, a Novel Hypoxia Target Gene Related to Gastric Cancer Invasion and Metastasis. *BioMed research international* 2019;2019:9749751. doi: 10.1155/2019/9749751 [published Online First: 2019/08/31]
10. Zhang J, Jin HY, Wu Y, et al. Hypoxia-induced LncRNA PCGEM1 promotes invasion and metastasis of gastric cancer through regulating SNAI1. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* 2019;21(9):1142-51. doi: 10.1007/s12094-019-02035-9 [published Online First: 2019/01/29]
11. Murray PJ. Macrophage Polarization. *Annu Rev Physiol* 2017;79:541-66. doi: 10.1146/annurev-physiol-022516-034339 [published Online First: 2016/11/05]
12. Okabe Y, Medzhitov R. Tissue-specific signals control reversible program of localization and functional polarization of macrophages. *Cell* 2014;157(4):832-44. doi: 10.1016/j.cell.2014.04.016 [published Online First: 2014/05/06]
13. Hallowell RW, Collins SL, Craig JM, et al. mTORC2 signalling regulates M2 macrophage differentiation in response to helminth infection and adaptive thermogenesis. *Nat Commun* 2017;8:14208. doi: 10.1038/ncomms14208 [published Online First: 2017/01/28]
14. Stein M, Keshav S, Harris N, et al. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine* 1992;176(1):287-92.
15. Mills CD, Kincaid K, Alt JM, et al. M-1/M-2 macrophages and the Th1/Th2 paradigm. *The Journal of immunology* 2000;164(12):6166-73.
16. Elinav E, Nowarski R, Thaïss CA, et al. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. *Nature reviews Cancer* 2013;13(11):759-71. doi: 10.1038/nrc3611 [published Online First: 2013/10/25]

17. Ramesh A, Brouillard A, Kumar S, et al. Dual inhibition of CSF1R and MAPK pathways using supramolecular nanoparticles enhances macrophage immunotherapy. *Biomaterials* 2020;227:119559. doi: 10.1016/j.biomaterials.2019.119559 [published Online First: 2019/11/02]
18. Lu SW, Pan HC, Hsu YH, et al. IL-20 antagonist suppresses PD-L1 expression and prolongs survival in pancreatic cancer models. *Nat Commun* 2020;11(1):4611. doi: 10.1038/s41467-020-18244-8 [published Online First: 2020/09/16]
19. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol* 2019;19(6):369-82. doi: 10.1038/s41577-019-0127-6 [published Online First: 2019/02/06]
20. Cao Q, Yan X, Chen K, et al. Macrophages as a potential tumor-microenvironment target for noninvasive imaging of early response to anticancer therapy. *Biomaterials* 2018;152:63-76. doi: 10.1016/j.biomaterials.2017.10.036 [published Online First: 2017/11/08]
21. Henze AT, Mazzone M. The impact of hypoxia on tumor-associated macrophages. *The Journal of clinical investigation* 2016;126(10):3672-79. doi: 10.1172/jci84427 [published Online First: 2016/08/03]
22. Piao HY, Guo S, Wang Y, et al. Exosome-transmitted lncRNA PCGEM1 promotes invasive and metastasis in gastric cancer by maintaining the stability of SNAI1. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico* 2020 doi: 10.1007/s12094-020-02412-9 [published Online First: 2020/06/11]
23. Piao H-y, Guo S, Jin H, et al. LINC00184 involved in the regulatory network of ANGPT2 via ceRNA mediated miR-145 inhibition in gastric cancer. *Journal of Cancer* 2021;12(8):2336.
24. Li HS, Watowich SS. Innate immune regulation by STAT-mediated transcriptional mechanisms. *Immunol Rev* 2014;261(1):84-101. doi: 10.1111/imr.12198 [published Online First: 2014/08/16]
25. Mellado M, Rodríguez-Frade JM, Mañes S, et al. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu Rev Immunol* 2001;19:397-421. doi: 10.1146/annurev.immunol.19.1.397 [published Online First: 2001/03/13]
26. Locati M, Curtale G, Mantovani A. Diversity, Mechanisms, and Significance of Macrophage Plasticity. *Annual review of pathology* 2020;15:123-47. doi: 10.1146/annurev-pathmechdis-012418-012718 [published Online First: 2019/09/19]
27. Riera-Domingo C, Audigé A, Granja S, et al. Immunity, Hypoxia, and Metabolism-the Ménage à Trois of Cancer: Implications for Immunotherapy. *Physiol Rev* 2020;100(1):1-102. doi: 10.1152/physrev.00018.2019 [published Online First: 2019/08/16]
28. Iriando O, Liu Y, Lee G, et al. TAK1 mediates microenvironment-triggered autocrine signals and promotes triple-negative breast cancer lung metastasis. *Nat Commun* 2018;9(1):1994. doi: 10.1038/s41467-018-04460-w [published Online First: 2018/05/20]
29. Hamarsheh S, Osswald L, Saller BS, et al. Oncogenic Kras(G12D) causes myeloproliferation via NLRP3 inflammasome activation. *Nat Commun* 2020;11(1):1659. doi: 10.1038/s41467-020-15497-1 [published Online First: 2020/04/05]

30. Piao HY, Liu Y, Kang Y, et al. Hypoxia associated lncRNA HYPAL promotes proliferation of gastric cancer as ceRNA by sponging miR-431-5p to upregulate CDK14. *Gastric Cancer* 2021 doi: 10.1007/s10120-021-01213-5 [published Online First: 2021/07/12]
31. Hakimi AA, Voss MH, Kuo F, et al. Transcriptomic Profiling of the Tumor Microenvironment Reveals Distinct Subgroups of Clear Cell Renal Cell Cancer: Data from a Randomized Phase III Trial. *Cancer Discov* 2019;9(4):510-25. doi: 10.1158/2159-8290.Cd-18-0957 [published Online First: 2019/01/10]
32. Peveri P, Walz A, Dewald B, et al. A novel neutrophil-activating factor produced by human mononuclear phagocytes. *J Exp Med* 1988;167(5):1547-59. doi: 10.1084/jem.167.5.1547 [published Online First: 1988/05/01]
33. Strieter RM, Kunkel SL, Showell HJ, et al. Monokine-induced gene expression of a human endothelial cell-derived neutrophil chemotactic factor. *Biochemical and biophysical research communications* 1988;156(3):1340-5. doi: 10.1016/s0006-291x(88)80779-4 [published Online First: 1988/11/15]
34. Wanninger J, Neumeier M, Weigert J, et al. Adiponectin-stimulated CXCL8 release in primary human hepatocytes is regulated by ERK1/ERK2, p38 MAPK, NF-kappaB, and STAT3 signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 2009;297(3):G611-8. doi: 10.1152/ajpgi.90644.2008 [published Online First: 2009/07/18]
35. Brat DJ, Bellail AC, Van Meir EG. The role of interleukin-8 and its receptors in gliomagenesis and tumoral angiogenesis. *Neuro Oncol* 2005;7(2):122-33. doi: 10.1215/s1152851704001061 [published Online First: 2005/04/16]
36. Wald O, Shapira OM, Izhar U. CXCR4/CXCL12 axis in non small cell lung cancer (NSCLC) pathologic roles and therapeutic potential. *Theranostics* 2013;3(1):26-33. doi: 10.7150/thno.4922 [published Online First: 2013/02/06]
37. Podolin PL, Bolognese BJ, Foley JJ, et al. A potent and selective nonpeptide antagonist of CXCR2 inhibits acute and chronic models of arthritis in the rabbit. *J Immunol* 2002;169(11):6435-44. doi: 10.4049/jimmunol.169.11.6435 [published Online First: 2002/11/22]
38. Keane MP, Belperio JA, Xue YY, et al. Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. *J Immunol* 2004;172(5):2853-60. doi: 10.4049/jimmunol.172.5.2853 [published Online First: 2004/02/24]
39. Zhang M, Huang L, Ding G, et al. Interferon gamma inhibits CXCL8-CXCR2 axis mediated tumor-associated macrophages tumor trafficking and enhances anti-PD1 efficacy in pancreatic cancer. *J Immunother Cancer* 2020;8(1) doi: 10.1136/jitc-2019-000308 [published Online First: 2020/02/14]
40. Lin C, He H, Liu H, et al. Tumour-associated macrophages-derived CXCL8 determines immune evasion through autonomous PD-L1 expression in gastric cancer. *Gut* 2019;68(10):1764-73. doi: 10.1136/gutjnl-2018-316324 [published Online First: 2019/01/21]
41. Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 2014;41(1):14-20. doi: 10.1016/j.immuni.2014.06.008 [published Online First: 2014/07/19]

42. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. *The Journal of clinical investigation* 2007;117(5):1155-66. doi: 10.1172/jci31422 [published Online First: 2007/05/04]
43. Wang Q, Ni H, Lan L, et al. Fra-1 protooncogene regulates IL-6 expression in macrophages and promotes the generation of M2d macrophages. *Cell Res* 2010;20(6):701-12. doi: 10.1038/cr.2010.52 [published Online First: 2010/04/14]
44. Xu F, Wei Y, Tang Z, et al. Tumor-associated macrophages in lung cancer: Friend or foe? (Review). *Mol Med Rep* 2020;22(5):4107-15. doi: 10.3892/mmr.2020.11518 [published Online First: 2020/10/02]
45. Casanova-Acebes M, Dalla E, Leader AM, et al. Tissue-resident macrophages provide a pro-tumorigenic niche to early NSCLC cells. *Nature* 2021;595(7868):578-84. doi: 10.1038/s41586-021-03651-8 [published Online First: 2021/06/18]
46. Karakurum M, Shreeniwas R, Chen J, et al. Hypoxic induction of interleukin-8 gene expression in human endothelial cells. *The Journal of clinical investigation* 1994;93(4):1564-70. doi: 10.1172/jci117135 [published Online First: 1994/04/01]
47. Frey S, Derer A, Messbacher ME, et al. The novel cytokine interleukin-36 α is expressed in psoriatic and rheumatoid arthritis synovium. *Ann Rheum Dis* 2013;72(9):1569-74. doi: 10.1136/annrheumdis-2012-202264 [published Online First: 2012/12/27]

Figures

Figure 1

Hypoxia promoted macrophage-derived CXCL8 secretion

A, B. Differential genetic analysis of the transcription data of macrophages cultured under hypoxia and normoxia showed that hypoxia could promote *CXCL8* expression. (A. volcano; B. heatmap)

- C. MACS obtained human CD14⁺ monocytes and induced them into macrophages through M-CSF.
- D, E. Hypoxia promoted human-derived macrophage CXCL8 expression at both transcription and protein levels.
- F. Hypoxia promoted the secretion of macrophage-derived cytokine *CXCL8* secretion
- G. An evident co-localization of macrophages and *CXCL8* in human GC tissue specimens.

Figure 2

***CXCL8* overexpression in GC promotes poor survival.**

A, B. *CXCL8* was up-regulated in GC tissue both in TCGA-GC and GEO-GC cohort.

C. In matched GC and adjacent tissues, *CXCL8* was highly expressed in cancer tissues.

D. Representative image of *CXCL8* expression in GC tissue. (Left: high-expression; Right: weak-expression).

E, F. *CXCL8* promoted poor DFS and OS in the GC cohort. (E. DFS; F. OS.)

Figure 3

CXCL8-CXCR1/2 deteriorates the malignant phenotype of GC

A. Migration patterns of GC cells on Matrigel compared to cells cultured with *CXCL8* and/or *CXCL8-CXCR1/2* inhibitor repertaxin.

- B. Migration trail of GC cells cultured with *CXCL8* or *CXCL8* and *CXCL8-CXCR1/2* inhibitor.
- C. Migration average distances of GC cells in each group.
- D. Transwell exhibited the differences in the number of permeable cells in each group.
- E, F. CCK-8, and colony formation assay confirmed *CXCL8* could promote GC proliferation which could be inhibited by *CXCL8-CXCR1/2* inhibitor.
- G. *CXCL8* increased the subcutaneous tumor size and weight.
- H. IHC representative image of subcutaneous tumors showing that *CXCL8* could promote *Ki67* and inhibit the expression of *Capcase3*; the inhibitors could counteract this effect.
- I. *CXCL8* induced ascites formation and intra- metastasis of GC.

Figure 4

***CXCL8-CXCR1/2* activated *JAK/STAT1* signal pathway and promoted GC progression.**

A. *CXCL8* activated the *JAK/STAT1* signal pathway. The *CXCL8* inhibitor (repertaxin) and *STAT1* specific inhibitor (Fludarabine) could antagonize the effects of *CXCL8*.

B-D. Migration patterns, migration trail, and average traveled distances of GC cells treated with *CXCL8* or *CXCL8* and Fludarabine together.

E. Fludarabine limited the GC invasion.

F, G. CCK-8, and colony formation assay revealed that the Fludarabine could resist *CXCL8*'s promotion of GC proliferation

Figure 5

Transcription factor *STAT1* could directly upregulate *IL-10* expression.

A. The shRNA and plasmic regulated *STAT1* expression.

B, C. *STAT1* regulated *HIF-1 α* expression at transcription and protein levels.

D, E. *STAT1* could also influence *IL-10* expression at transcription and protein levels.

F, G. The promoter-luciferase reporter assay, showing that *STAT1* directly promoted *IL-10* expression but not *HIF-1α*.

H. The interaction of *STAT1* with *IL-10* shown using ChIP assays with control (IgG) or anti-*STAT1* antibody.

I. Deletion and selective mutation analysis identified *STAT1*-responsive regions in the *IL-10* promoter. Luciferase reporter plasmids containing serially truncated or mutated *IL-10* promoter constructs were co-transfected with OE-*STAT1* into NUGC3 cells, and relative luciferase activities were detected. The -1811~-1821 sequence was the binding site.

- B. *IL-10* activated *NF-κB* signaling pathway.
- C. The *NF-κB* pathway inhibitor (Sarsasapogenin) could limit *IL-10* induced M2 polarization.
- D, E. *IL-10* upregulated *CXCL8* expression, and it could be prevented by *NF-κB* pathway inhibitor.
- F. *IL-10* could promote *CXCL8* secretion, and Sarsasapogenin could antagonize the effect of *IL-10*.
- G. Deletion and selective mutation analysis recognized the *NFKB1*-responsive regions in the *CXCL8* promoter. Luciferase reporter plasmids containing serially truncated or mutated *CXCL8* promoter constructs were co-transfected with *OE-NFKB1* into macrophage cells, and relative luciferase activities were detected. The -1829~-1819 and -91~-81 sequences were all the binding sites.

Figure 7

The mechanism graph of the positive feedback loop between GC cells and TAMs.

Hypoxia-induced macrophage-derived *CXCL8* secretion activated *CXCL8*-*CXCR1/2*-*JAK/STAT1* signaling pathway and deteriorated the GC malignant phenotype. The transcription factor *STAT1* could directly promote *IL-10* expression. The latter activated the macrophage *IL-10*/*NF-κB* signaling pathway and induced M2 polarization. The transcription factor *NFKB1* directly upregulated *CXCL8* expression.

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