# Upregulation of MELK promotes chemoresistance and induces macrophage M2 polarization via CSF-1/JAK2/STAT3 pathway in gastric cancer

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## Abstract

**Background**  Gastric cancer (GC) stands out as one of the most prevalent malignancies affecting the digestive system, characterized by a substantial incidence rate and mortality. Maternal embryonic leucine zipper kinase (MELK) has been implicated in the advancement of various cancer types and the modulation of the tumor microenvironment. This study aims to delve into the involvement of MELK in chemoresistance and the tumor microenvironment of GC.

**Methods**  The MELK expression was detected using quantitative real-time polymerase chain reaction (qRT-PCR), western blotting and immunohistochemistry. Lentiviral transfection was employed to establish stable cell lines with either overexpressed or silenced MELK. The impact of MELK on the chemoresistance of GC cells and the polarization of macrophages was investigated through in vitro and in vivo functional assays. Additionally, the correlation between MELK and the cytokines colony-stimulating factor 1 (CSF-1), as well as stromal macrophages, was analysed. The prognostic significance of MELK, CSF-1, and CD206 expression levels in clinical samples was further investigated.

**Results**  MELK was found to be highly expressed in chemoresistant GC cells and tissues. Furthermore, both in vitro and in vivo assays indicated that MELK overexpression conferred chemoresistance in GC cells. Additionally, MELK overexpression was observed to induce M2 macrophage polarization via the CSF-1/JAK2/STAT3 pathway, thereby contributing to chemoresistance within the tumor microenvironment. The expression of MELK in GC tissues from neoadjuvant chemotherapy patients correlated positively with CSF-1 and CD206. Moreover, patients with higher expression levels of MELK, CSF-1, or CD206 exhibited significantly shorter OS and DFS rates.

**Conclusions**  Our investigation underscores the critical role of MELK in promoting chemoresistance and inducing M2 macrophage polarization in GC. It proposes novel targets and methods for the treatment of GC, as well as prognostic factors for neoadjuvant chemotherapy.

**Keywords**  Gastric cancer, MELK, Chemoresistance, Tumor-associated macrophages, Polarization, Prognosis

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## Background

While combining surgery and perioperative chemotherapy has partially enhanced the therapeutic outcomes for gastric cancer (GC), it remains the fifth most prevalent malignancy and the fourth most common cause of cancer-related mortality worldwide [1]. 5-fluorouracil (5-Fu) is one of the most commonly prescribed antitumor drugs in perioperative chemotherapy for GC patients. However, chemoresistance stands out as a pivotal factor contributing to treatment failure and dismal prognoses among patients with GC [2]. Therefore, it is necessary to explore molecular targets of chemotherapy resistance in order to enhance the efficacy of treatment strategies.

Maternal embryonic leucine zipper kinase (MELK) is a serine/threonine kinase belonging to the AMP-activated protein kinase (AMP)/sucrose-non-fermenting kinase 1 (SNF1) family [3]. Studies have shown that MELK has vital function in multiple cellular processes, including cell cycle regulation, cell proliferation, cell apoptosis, mitotic progression, and RNA processing [4–6]. In addition, upregulation of MELK has been found in various human tumors, including breast cancer, glioblastomas, prostate cancer, colorectal cancer and GC [7–11]. It has been shown that higher MELK expression was closely related to poorer clinical outcomes, such as more advanced stages, vascular invasion and distant metastasis [3, 12]. Moreover, abnormal expression of MELK could confer therapy resistance in multiple types of cancers. For instance, MELK-dependent activation of FOXM1 regulates DNA repair and mediates resistance to doxorubicin in breast cancer [13, 14] In glioblastoma multiforme, JNK-driven MELK/c-JUN signaling maintains glioma stem cells in an immature state to facilitate therapy resistance in a p53-dependent manner [15]. Du et al. identified that MELK promoted cell migration and invasion through activating the FAK/paxillin signaling pathway and played a vital role in the development and occurrence of GC [16]. However, to date, the function of MELK in the development of chemoresistance of GC remains unexplored.

Tumor progression is influenced not only by the malignant biological characteristics of tumor cells, but also by the regulation and support provided by tumor microenvironment (TME) [17]. As one of the major cellular components of TME, tumor-associated macrophages (TAMs) exhibit various phenotypes and functional features, constituting the dominant immune cells involved in tumor growth, angiogenesis, metastasis and therapy resistance [18]. TAMs can be broadly separated into the classically activated (M1 phenotype) and the alternatively activated phenotype (M2 phenotype), depending on the stimulating signals from various cytokine or pathogen in the TME [19, 20]. As tumor progress, TAMs often shift towards the M2 phenotype, promoting tumor malignancy and contributing to poor prognosis in the majority of cases. In our previous study, we found that chemoresistant GC cells induced macrophages polarization towards M2 phenotype more effectively than parental GC cells. M2 macrophages, in turn, further promoted the chemoresistance of GC cells by activating the CXCL5/PI3K/AKT/mTOR signaling axis [21]. However, the mechanism underlying the M2 polarization of TAMs induced by chemoresistant GC cells remains unclear.

The present study illustrated that MELK expression was upregulated in chemoresistant GC cells and tissues. Both in vitro and in vivo experiments revealed that heightened MELK expression could increase resistance to 5-Fu in GC cells, whereas knocking down MELK could enhance sensitivity to 5-Fu. Then we found that MELK overexpression could induce macrophage polarization towards M2 phenotype through activating the colony-stimulating factor 1 (CSF-1)/Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway, thereby contributing to chemoresistance within the TME. MELK expression in GC tissue following neoadjuvant chemotherapy correlated positively with CSF-1 and CD206. Patients with elevated expression levels of MELK, CSF-1, or CD206 exhibited significantly poorer prognoses. Our study delved into the interplay between MELK expression, GC chemoresistance and M2 polarization of TAMs. These findings suggest the potential utility of MELK and CSF-1 as predictive markers for chemotherapeutic effective and the prognosis, offering a novel avenue for precision therapy in patients with GC.

## Materials and methods

### Clinical samples

GC tissues were collected from 106 patients who underwent neoadjuvant chemotherapy with the 5-Fu-based regimen and radical gastrectomy between 2016 and 2018 at Peking Union Medical College Hospital. The clinical and demographic characteristics of patients were listed in Supplementary Table S1. Patients were divided into two groups based on the degree of pathological response, as evaluated according to the guidelines of the College of American Pathologists (CAP) [22]. CAP 0, CAP 1 and CAP 2 were defined as exhibiting pathological response (n = 63), whereas CAP 3 indicated no pathological response (n = 43). Additionally, fifteen normal gastric mucosal tissue samples were obtained via endoscopic biopsy. Tumor samples were collected with patients' written informed consent, and the study was reviewed and approved by the Institutional Review Board of Peking Union Medical College Hospital.

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## Immunohistochemistry staining

A total of 106 collected tumor specimens were fixed in 10% formaldehyde solution, embedded in paraffin, and then serially severed into slices (4 μm). After being deparaffinized and rehydrated by xylene and graded ethanol, antigen was retrieved using microwave heating with sodium citrate retrieval buffer (pH 6.0). The endogenous peroxidase was inactivated by treatment with 3% $H_2O_2$ for 10 min. Following treatment with blocking buffer to obstruct nonspecific bindings, the samples were incubated with primary antibodies: Anti-MELK (1:100, #11403-1-AP, Proteintech Group), Anti-CSF-1 (1:100, #ab233387, Abcam), and Anti-CD206 (1:100, #18704-1-AP, Proteintech Group) at 4 °C overnight. The next day, after PBS washing, horseradish peroxidase (HRP)-conjugated antibody anti-Rabbit IgG (1:100, #7074, Cell Signaling Technology) was added for 30 min' incubation at room temperature. 3, 3′-diaminobenzidine (DAB) regent was taken for brown staining subsequent to PBS washing, then all slices were re-dyed with hematoxylin staining solution, dehydrated, and sealed for microscopic examination. At least three slices were taken from each specimen and five independent fields were randomly chosen from each slice for microscopic examination. The immunoreactivity of MELK and CSF-1 was scored by multiplying the scores of percentage of positive cells (< 5% scores 0, 5–25% scores 1, 25–50% scores 2, 50–75% scores 3, and 75–100% scores 4) with the scores of staining intensity (no staining scores 0, weak staining scores 1, moderate staining scores 2, and strong staining scores 3). The overall scores of 0–3 were defined as low expression, while scores above 3 were considered high expression. For CD206 evaluation, the Olympus microscope was used to count the number of positive cells in five random 400-fold fields.

## Cell lines and culture

Human gastric cancer cell lines MKN45 and HGC27, human mononuclear cells.

THP-1, and human gastric epithelial cell line GES-1 were obtained from the Cell Resource Center of Peking Union Medical College (Beijing, China). Parental gastric cancer cells were regarded as 5-Fu-sensitive (MKN45-S and HGC27-S) and 5-Fu-resistant cells (MKN45-R and HGC27-R) were generated as previously reported [21]. All cells were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) at 37 °C in a humidified incubator with a volume fraction of 5% $CO_2$. For the generation of M0 macrophages, THP-1 cells were induced by 100 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

## Co-culture of cancer cells and macrophages

Transwell chambers (6-well plates, 0.4-μm pore size; Corning, NY, USA) were used for co-culture experiment. GC cells were seeded onto the membrane of upper chambers with 0.4-μm pore, while M0 macrophages were seeded onto the lower chambers of 6-well plates for 48 h co-culture. Macrophages were then harvested for subsequent experimental analysis.

## Cell viability assays

GC Cell (MKN45-S LV-NC, MKN45-S LV-MELK, HGC27-S LV-NC, HGC27-S LV-MELK, MKN45-R sh-NC, MKN45-R sh-MELK, HGC27-R sh-NC, HGC27-R sh-MELK) viability was measured using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, GC cells were planted in 96-well plates at a density of $5 \times 10^3$ cells per well and cultured at 37 °C in a humidified incubator with 5% $CO_2$ for 24 h. Then cells were incubated with culture medium containing varying concentrations of 5-Fu (0, 5, 10, 20, 40, 80, 120, 160, 200 μg/ml) for another 24 h. 10 μl of CCK-8 reagent was added to each well for 2 h incubation at 37 °C. The absorbance value at 450 nm was measured by microplate reader (Termo Scientific, Rockford, IL, USA).

## Colony formation assay

GC cells (MKN45-S LV-NC, MKN45-S LV-MELK, HGC27-S LV-NC, HGC27-S LV-MELK, MKN45-R sh-NC, MKN45-R sh-MELK, HGC27-R sh-NC, HGC27-R sh-MELK) were seeded into 6-well plates at a density of 500 cells per well. After incubation for 24 h, the cells were treated with 5-Fu at a final concentration of 15 μg/ml, and the culture medium was replenished every 3–4 days. Following a two-week incubation period, visible colonies were rinsed with PBS twice and fixed with 4% paraformaldehyde for 15 min. 0.1% crystal violet solution was applied to staining the fixed colonies for 10 min. Colonies containing a minimum of 50 cells were later photographed and counted. The reason for using 15 μg/ml of 5-FU and the method for counting cell colonies were stated and uploaded as Supplementary Text S1.

## Cell transfection

Lentiviral transfection was used to establish stable cell lines with overexpressed or silenced MELK. Lentiviral vectors were packaged with full-length MELK gene or shRNA targeting MELK by Genechem (Shanghai). MKN-45-S and HGC-27-S cells were transfected with overexpression lentivirus (LV-MELK) or its negative control (LV-NC), while MKN-45-R and HGC-27-R cells were transfected with shRNA targeting MELK lentivirus (sh-MELK) or its negative control (sh-NC). The full-length MELK gene sequence and three sequences of shRNA

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targeting MELK were listed in Supplementary Table S2 and S3. Multiplicity of infection (MOI) value was set at 10 based on our preliminary experiments. Cells were chosen for 2 weeks in a medium containing 2 μg/mL puromycin (Sangon Biotech, Shanghai, China), and the alive cells were defined as stable expression cells.

### RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA samples were reversed to cDNA using 1 μg of total RNA and 5×PrimeScript RT reagent Kit (Takara, Dalian, China). Then qPCR was carried out by using TB Green Premix Ex Taq II (Takara, Dalian, China). The primer sequences of targeted genes are listed in Supplementary Table S4. The relative expression was calculated with GAPDH using $2^{-\Delta\Delta Ct}$ method and experiment was conducted in triplicate.

### Western blot analysis

Total protein was isolated out of cells and tissues using RIPA buffer (Termo Scientifc, Rockford, IL, USA) containing Halt Protease and Phosphatase inhibitor Cocktail (Termo Scientifc, Rockford, IL, USA). The lysates were sonicated followed by centrifugation at 12,000*g* for 15 min at 4 °C, and then the concentration of extracted protein was measured by BCA Protein Assay Kit (Beyotime, Shanghai, China). Approximately 30 μg of protein was transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were blocked with TBST solution incorporating 5% non-fat milk for 1 h at room temperature and then immunoblotted overnight at 4 °C with the primary antibodies against MELK (1:1000, #11403-1-AP, Proteintech Group), Jak2 (1:1000, #3230, Cell Signaling Technology), p-Jak2 (1:1000, #3771, Cell Signaling Technology), STAT3 (1:1000, #9139, Cell Signaling Technology), p-STAT3 (1:1000, #9145, Cell Signaling Technology), GAPDH (1:1000, #5174, Cell Signaling Technology). On the following day, the membranes were incubated with corresponding HRP-labeled secondary antibody (1:5000, #7074, Cell Signaling Technology) at room temperature for 1 h. The antibody-antigen complex was visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (Termo Scientific, Rockford, IL, USA) in a Kodak Image station (Tanon, China).

### Enzyme-linked immunosorbent assay (ELISA)

The concentrations of CSF-1 in the cultured media of GC cells were measured by CSF-1 ELISA kits (Cell Signaling Technology, MA, USA) in accordance with the manufacturer's instructions. The immunoreactive was evaluated by a microplate reader (Termo Scientific, Rockford, IL, USA), and the concentrations were estimated from the standard curve.

### Apoptosis assay

Cell apoptosis was measured using Annexin-V-FITC Apoptosis Detection Kit (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. Cells were harvested and washed twice with ice-cold PBS and incubated in 100 μl binding buffer in the presence of 5 μl Annexin V-FITC and 5 μl propidium iodide for 15 min in dark conditions. Then apoptosis was quantified using BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA). The experiment was conducted in triplicate and data were analyzed using FlowJo software (Tree Star, Oregon, OR, USA).

### Flow cytometry analysis of macrophages

Macrophages were washed twice using PBS and filtered through a 100 μm cell strainer for flow cytometry analysis. Then $1 \times 10^6$ cells in 100 μl staining buffer were stained with FITC-CD11b (BioLegend, San Diego, CA, USA) and PE-CD206 antibodies (BioLegend, San Diego, CA, USA) in darkness for 15 min at 4 °C. Finally, the stained cells were analyzed by BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA). The process was conducted in triplicate and data were transferred and analyzed using FlowJo software (Tree Star, Oregon, OR, USA).

### Mouse tumor xenograft and drug resistance assay in vivo

Male BALB/c nude mice (Sinogenetic Biotechnology, Beijing), aged 4–5 weeks, were randomly divided into four groups (five mice each group). Approximately $1 \times 10^7$ cells were resuspended in 200 μL of PBS and then inoculated subcutaneously into the armpit of each mouse. 10 days after the inoculation, the tumor-bearing mice were administrated intraperitoneally with 5-Fu (30 mg/kg) every 3 days. Tumor lengths and widths were measured using calipers and tumor volumes were calculated according to the formula: length×width$^2$/2. Mice were sacrificed at the end of 3 weeks after the administration. Tumors were carefully separated and weighed. The procedure abided by the National Institutes of Health guide for the care and use of Laboratory animals and approved by the Ethics Committee of Animal Experiments of Peking Union Medical College Hospital.

### Statistical analysis

Data were shown as mean±standard deviation (SD) of more than three separate experiments to ensure

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accuracy. Statistical comparisons between groups were performed by Student's t-test or one-way analysis of variance (ANOVA). Correlations were assessed using Spearman rank-order correlation. Actuarial rates of survival were analyzed and compared using Kaplan–Meier methods and log-rank tests. All statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA) in conjunction with GraphPad Prism 8 (GraphPad Prism Software, Inc., San Diego, CA, USA) and $p$-value $< 0.05$ indicated a statistically significant difference ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

## Results

### MELK was upregulated in chemoresistant GC cells and tissues

To investigate the potential role of MELK in GC progression, we initially assessed MELK expression in GES-1 cells, as well as the parental GC cells MKN45-S and HGC27-S, along with their chemoresistant counterparts MKN45-R and HGC27-R. Our findings revealed significantly higher levels of MELK expression in GC cells compared to GES-1 cells (Fig. 1A, B). In addition, the chemoresistant GC cells showed higher MELK expression than their parental cells (Fig. 1A, B). We further examined MELK expression in human gastric tissues, including samples from 15 individuals with normal gastric mucosa and samples from patients with or without response to neoadjuvant chemotherapy (n = 63 and n = 43, respectively). As depicted in Fig. 1C and D, MELK expression in tumor tissues was notably higher compared to that in normal gastric mucosal tissues. Particularly noteworthy was the significantly higher MELK expression observed in tumor tissues from patients who did not exhibit a pathological response to neoadjuvant chemotherapy, compared to those who did. These results indicated that MELK expression was upregulated in both GC cells and tumor tissues, particularly in chemoresistant contexts, highlighting its potential role in mediating resistance mechanisms.

### MELK could lead to 5-Fu-resistance in GC cells

Stably transfected GC cells with MELK overexpression, MELK knockdown, and their corresponding negative control cells were established and then validated using qRT-PCR and western blotting. The results showed that the knockdown efficiency was higher using sh-MELK#2 compared with sh-MELK#1 and sh-MELK#3 (Fig. 2A), therefore, sh-MELK#2 was selected for further experiments. GC cells with MELK overexpression, MELK knockdown and their corresponding negative control cells were respectively incubated with gradient concentrations of 5-Fu (0, 5, 10, 20, 40, 80, 120, 160, 200 μg/ml) for 24 h. CCK-8 assay indicated that MELK overexpression significantly increased 5-Fu-resistance of parental GC cells, while MELK knockdown significantly reduced 5-Fu-resistance in chemoresistant GC cells (Fig. 2B). Similarly, MELK overexpression remarkably increased colony formation of parental GC cells in the presence of 15 μg/ml 5-Fu, and conversely MELK knockdown decreased colony formation of chemoresistant GC cells (Fig. 2C). Moreover, the apoptotic flow cytometry assay demonstrated that MELK overexpression inhibited apoptosis of parental GC cells induced by 5-Fu (Fig. 2D). In contrast, MELK suppression significantly increased apoptosis in chemoresistant GC cells.

Since our in vitro study revealed that MELK promotes the chemoresistance of GC cells, we conducted further investigation to explore its role in vivo. MKN45-S cells overexpressing MELK, MKN45-R cells silencing MELK, and their corresponding negative control cells were subcutaneously implanted into the armpits of mice (n = 5) followed by the administration of 5-Fu. The results demonstrated that tumors originating from MKN45-S cells overexpressing MELK exhibited accelerated growth, resulting in larger tumor volume and weight compared to those in the control group (Fig. 3A–C). Conversely, MELK knockdown significantly attenuated the chemoresistance of MKN45-R cells, manifesting in slower tumor growth and smaller tumor volume and weight compared to those in the control group (Fig. 3D–F). Therefore, our finding suggest that MELK contributes to 5-Fu-resistance in GC cells.

### MELK could induce M2 polarization of macrophages

Our previous study demonstrated that chemoresistant GC cells could induce macrophages polarization towards the M2 phenotype, further promoting the chemoresistance of GC cells [21]. The present study indicated that MELK increased the 5-Fu-resistance of GC cells, promoting us to investigate whether MELK could induce M2 polarization of macrophage. TPH-1 cells-derived macrophages were co-cultured with parental GC cells overexpressing MELK, chemoresistant GC cells with MELK knockdown, or their corresponding negative control cells in a non-contacting transwell system for 48 h. The expression of M2 macrophages markers CD163 and CD206 was detected by qRT-PCR. Compared to the negative control cells, co-culturing with parental GC cells overexpressing MELK markedly increased CD163 and CD206 expression in macrophages (Fig. 4A, B). In contrast, co-culturing with chemoresistant GC cells silencing MELK significantly decreased CD163 and CD206 expression in macrophages (Fig. 4C, D). Furthermore, the percentages of CD11b$^+$CD206$^+$ macrophages (M2 macrophages) after co-culturing with GC cells were measured by flow cytometry. Compared to the negative control group, the

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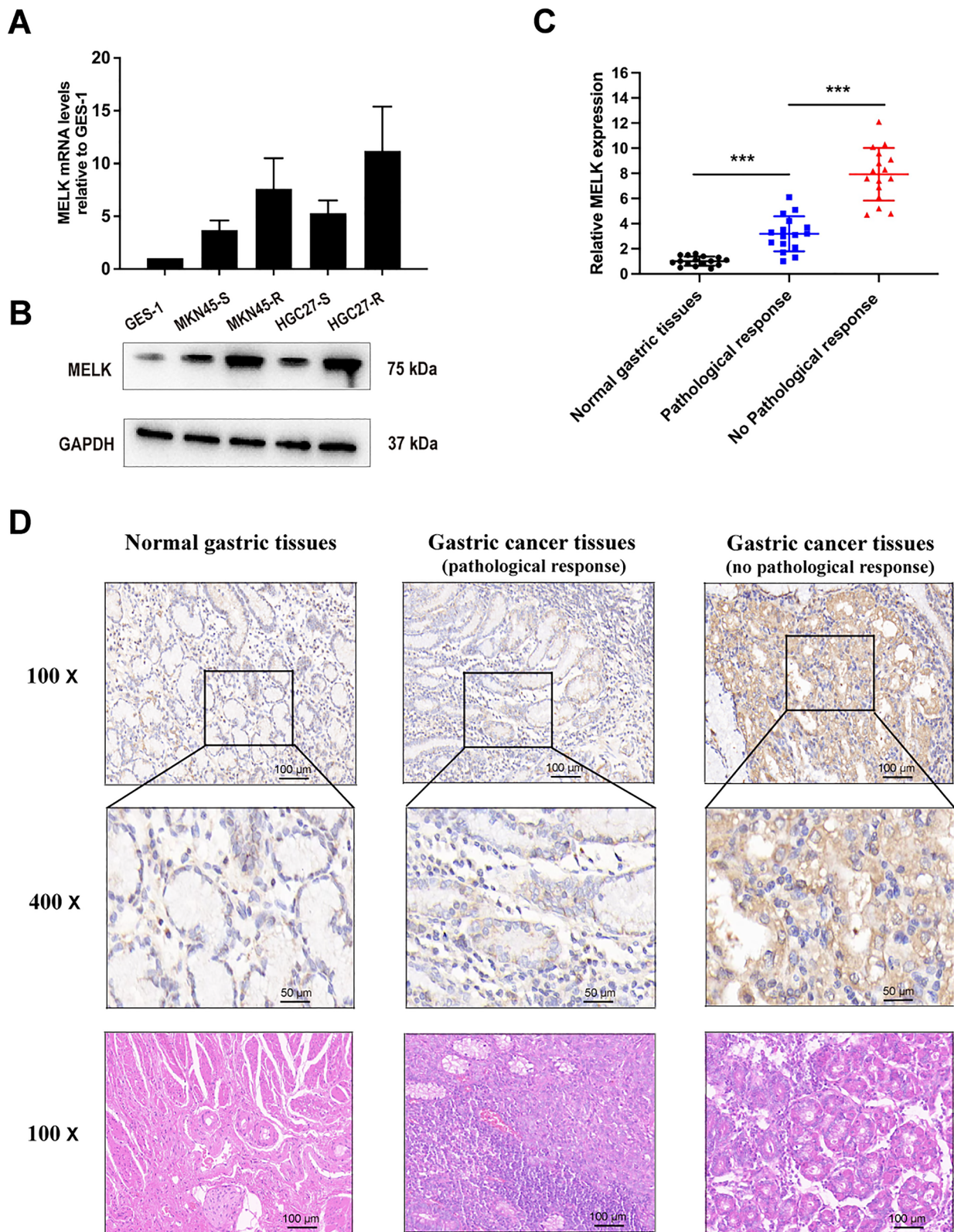


**Fig. 1** MELK was highly expressed in chemoresistant GC cells and tissues. **A**, **B** MELK expression in gastric epithelial cells, parental and chemoresistant GC cells were evaluated by qRT-PCR and western blot assay. **C**, **D** MELK expression in normal gastric tissues and GC tissues was evaluated by qRT-PCR and IHC. Data were presented as mean ± SD of at least three independent experiments. ***$p < 0.001$

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percentages of CD11b$^+$CD206$^+$ macrophages increased after co-culturing with parental GC cells overexpressing MELK (Fig. 4E, F), whereas the percentages decreased after co-culturing with chemoresistant GC cells that silenced MELK (Fig. 4G, H). These results indicate that MELK induces macrophages polarization towards M2 phenotype.

### MELK induced macrophages polarization to M2 phenotype via CSF-1/JAK2/STAT3 pathway

After observing that non-contacting co-culture with GC cells overexpressing or silencing MELK influenced M2 polarization of macrophage, we hypothesized that the distinct abilities of GC cells to induce M2 polarization were attributable to variations in secreted cytokines levels. Following a comprehensive review of relevant literature, thirteen cytokines (IL-1α, IL-6, IL-9, CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, TNF-α, CSF-1, GM-CSF and LIF) were selected for analysis via qRT-PCR. As indicated in Fig. 5A–F, among the thirteen cytokines, only the transcriptional level of CSF-1 remained consistent with the trend observed in previous results (Fig. 4). The mRNA levels of CSF-1 elevated in chemoresistant GC cells compared to parental cells (Fig. 5A, D), and they increased in parental GC cells overexpressing MELK compared to negative control cells (Fig. 5B, E). Conversely, they decreased in chemoresistant GC cells with MELK knockdown compared to negative control cells (Fig. 5C, F). CSF-1 levels in the culture supernatants of GC cells were measured using ELISA. Similarly, CSF-1 levels in the culture supernatants aligned with the results of qRT-PCR. Specifically, CSF-1 levels in the culture supernatants of chemoresistant GC cells were significantly higher than those in parental GC cells. Overexpression of MELK augmented CSF-1 protein levels in parental GC cells, whereas silencing of MELK in chemoresistant GC cells diminished CSF-1 protein levels (Fig. 5G, H).

Flow cytometry analysis indicated that incubation in the presence of CSF-1 (100 ng/ml) or co-culture with parental GC cells overexpressing MELK significantly increased the percentages of CD11b$^+$CD206$^+$ cells in macrophages. Additionally, the presence of anti-CSF-1R (0.5 μg/ml) mitigated the effect of MELK overexpression on M2 polarization (Fig. 6A–D). Several studies have

verified that the JAK2/STAT3 signaling axis was involved in the process of macrophages M2 polarization [23–26]. Based on these findings, we further investigated whether MELK induced macrophages polarization towards M2 phenotype through the JAK2/STAT3 pathway. As depicted in Fig. 6E and F, the expression of phosphorylated JAK2 and STAT3 significantly increased in macrophages when incubated with CSF-1, co-cultured with chemoresistant GC cells, or in parental GC cells overexpressing MELK. Treatment with anti-CSF-1R markedly weakened the ability of chemoresistant GC cells or parental GC cells overexpressing MELK to activate this signaling pathway. Furthermore, the JAK2/STAT3 signal pathway inhibitor, AG490, significantly attenuated the effect of parental GC cells with MELK overexpression on inducing M2 polarization (Fig. 6A, B).

### Clinical relevance of the MELK with CSF-1 and CD206

To explore the correlation between MELK and the cytokine CSF-1, as well as stromal macrophages, we conducted staining on tumor tissues using antibodies against CSF-1 and the M2 macrophage marker CD206. As depicted in Fig. 7A, there was a correlation observed between high or low expression levels of MELK and corresponding levels of CSF-1 expression, along with varying infiltration degree of CD206 positive macrophages. Spearman rank-order correlation analysis demonstrated a statistically significant relationship (Fig. 7B). These results further supported the notion that MELK promotes M2 polarization of macrophages through the cytokine CSF-1.

### Expression of MELK, CSF-1 and CD206 were correlated with the prognosis of GC patients

The immunoreactive intensity of MELK, CSF-1 and CD206 was individually categorized as high and low. Patients were followed up until death or more than 5 years, and the average period of follow-up was 36.97 ± 22.37 months (mean ± SD; range, 4 to 84 months). The overall survival (OS) rate and the disease-free survival (DFS) rate were calculated based on the expression of MELK, CSF-1 and CD206. As shown in Fig. 8, patients with high expression of MELK, CSF-1 or CD206 experienced a worse outcome of OS and DFS compared with those with low expression of the three molecules.
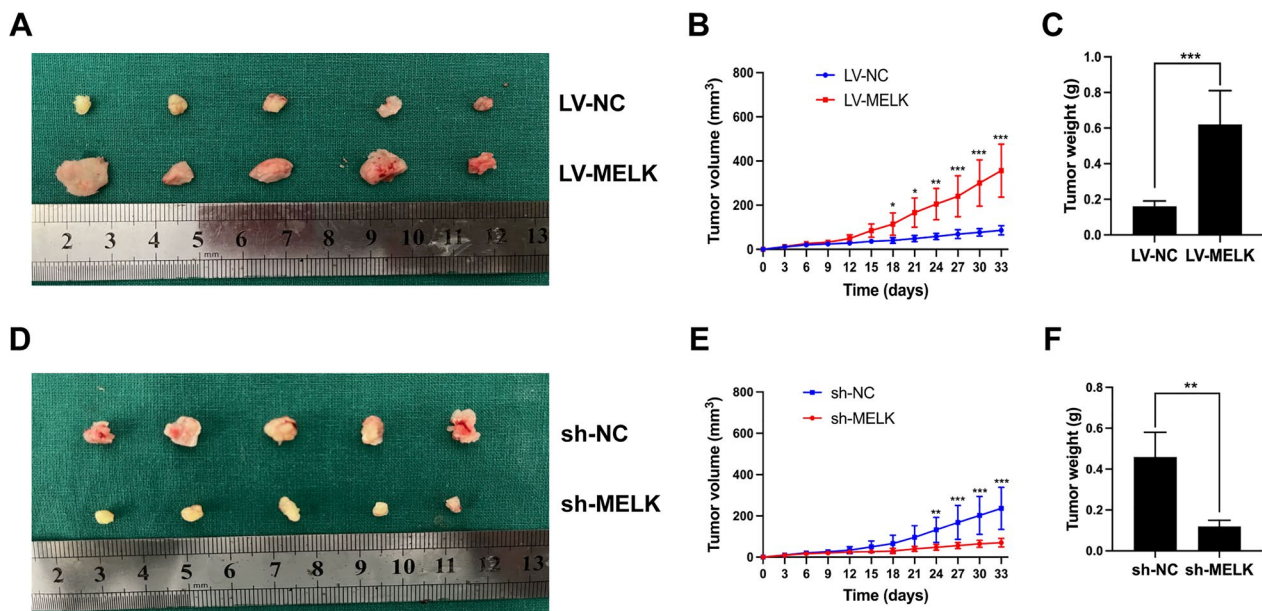
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**Fig. 2** Effect of MELK on 5-Fu-resistance of GC cells in vitro. **A** MELK expressions in GC cells with MELK overexpression or knockdown were detected and validated by qRT-PCR and western blot. **B** GC cells with MELK overexpression or knockdown were incubated with different concentrations of 5-Fu (0, 5, 10, 20, 40, 80, 120, 160, 200 μg/ml) for 24 h, and then cell viability was detected by CCK-8 assay. **C** GC cells treated with or without 5-Fu for two weeks, the numbers of colonies were counted. **D** Apoptotic flow cytometry assay was performed to verify that MELK inhibited 5-Fu-induced apoptosis. Data were presented as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

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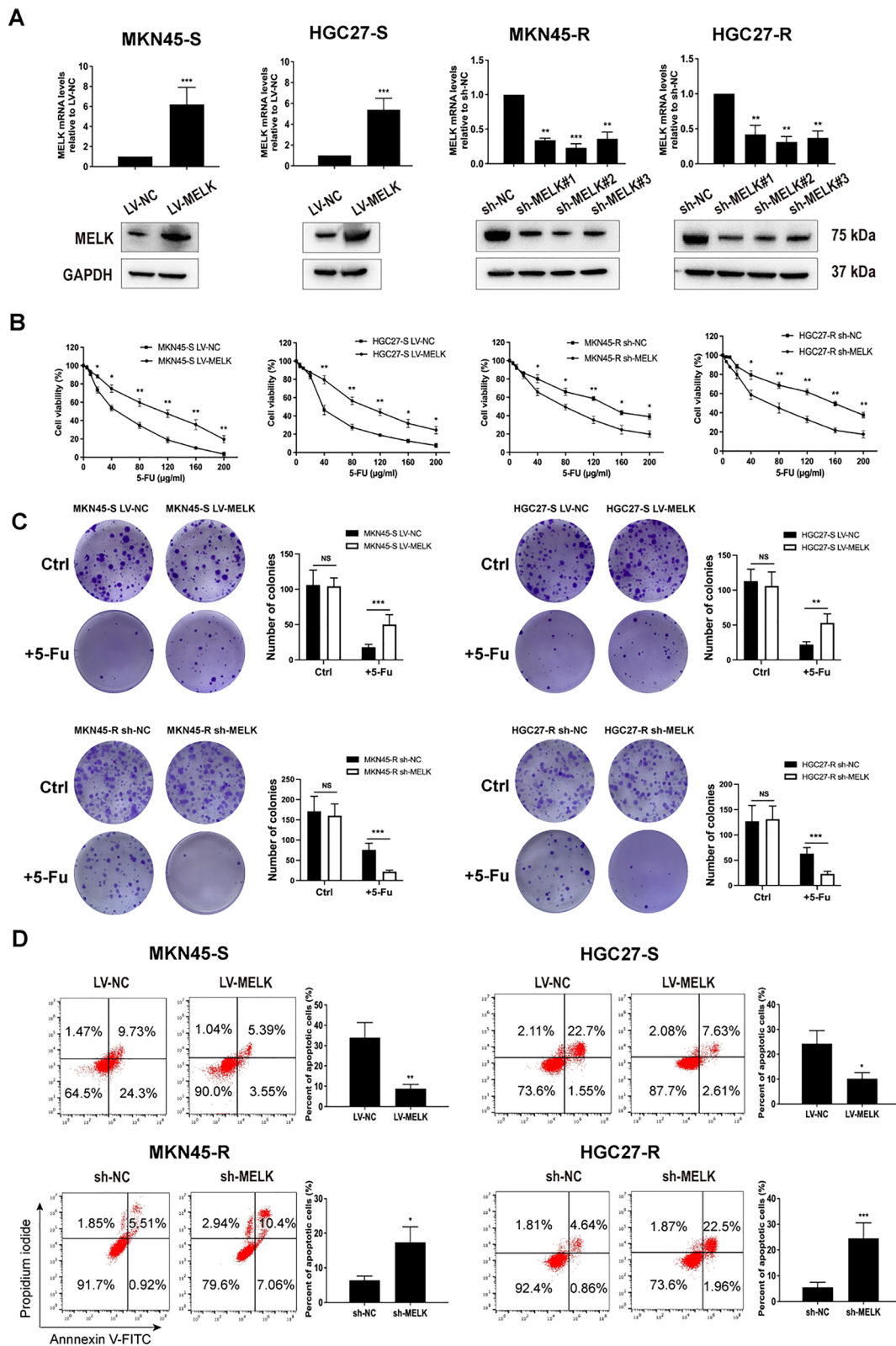


**Fig. 2** (See legend on previous page.)

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**Fig. 3** Effect of MELK on 5-Fu-resistance of MKN45 cells in vivo. **A**–**C** The comparison of tumor volume and weight in nude mice inoculated with MKN45-S cells overexpressing MELK and negative control cells. **D**–**F** The comparison of tumor volume and weight in nude mice inoculated with MKN45-R cells silencing MELK and negative control cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

The data above indicated that MELK, CSF-1 and CD206 expression were correlated with the prognosis of GC patients who underwent 5-Fu based neoadjuvant chemotherapy followed by radical gastrectomy.

## Discussion

Over the past decade, upregulation of MELK has been observed in various types of malignant carcinomas and has been correlated with the occurrence and progression through diverse signaling pathways and cellular functions [3, 7–11]. As the roles of MELK in tumorigenesis and tumor progression have been elucidated, it has been increasingly emerged as a potential therapeutic target for tumor therapy. In human GC, elevated MELK expression has been found in cancer tissues and cell lines, with higher expression often associated with poorer clinical outcomes, including advanced stages, distant metastasis, and peritoneal spreading [3, 11, 16, 27]. Consistently, the present study confirms upregulated MELK expression in two GC cell lines and clinical specimens from patients with GC compared to that in normal gastric mucosal epithelial cell line and normal gastric mucosal tissues. Notably, we observed higher MELK expression levels in chemoresistant GC cells and tumor specimens compared to their parental counterparts and non-chemoresistant tumor specimens. Given the unclear function of MELK in the development of chemoresistance in GC, we hypothesized that MELK might contribute to the chemoresistance of GC. Subsequent in vitro and in vivo

*experiments* demonstrated that MELK overexpression significantly increased 5-Fu-resistance in parental GC cells, while MELK knockdown dramatically suppressed the chemoresistance. These findings suggest that MELK can induce 5-Fu-resistance in GC cells.

Patients with GC frequently experience recurrence, mainly due to the development of chemoresistance [2, 28, 29]. Numerous studies have attempted to elucidate the molecular mechanisms of chemoresistance in various cancer cells. These mechanisms include epigenetic alterations, epithelial-mesenchymal transition, drug efflux, enhanced DNA repair and impaired apoptosis [30–32]. In recent years, emerging research has illustrated the interaction between cancer cells and immune cells within the TME, which contributes to tumor progression, metastasis and chemoresistance [33, 34]. Accumulating evidence indicated that tumor associated macrophages (TAMs), especially those exhibiting alternative activation (M2 phenotype), play a significant role in dedicating tumor chemoresistance [20, 30]. An analysis of 7135 patients with breast cancer from multiple independent large cohorts, revealed a positive correlation between high MELK expression and immune cells infiltration, including TAMs [35]. Another study demonstrated that MELK could function as an important component of immune response genes and predict the efficacy of immune checkpoint inhibitors in lung cancer [36]. In addition, high expression of MELK could induce macrophages polarization towards M2 phenotype

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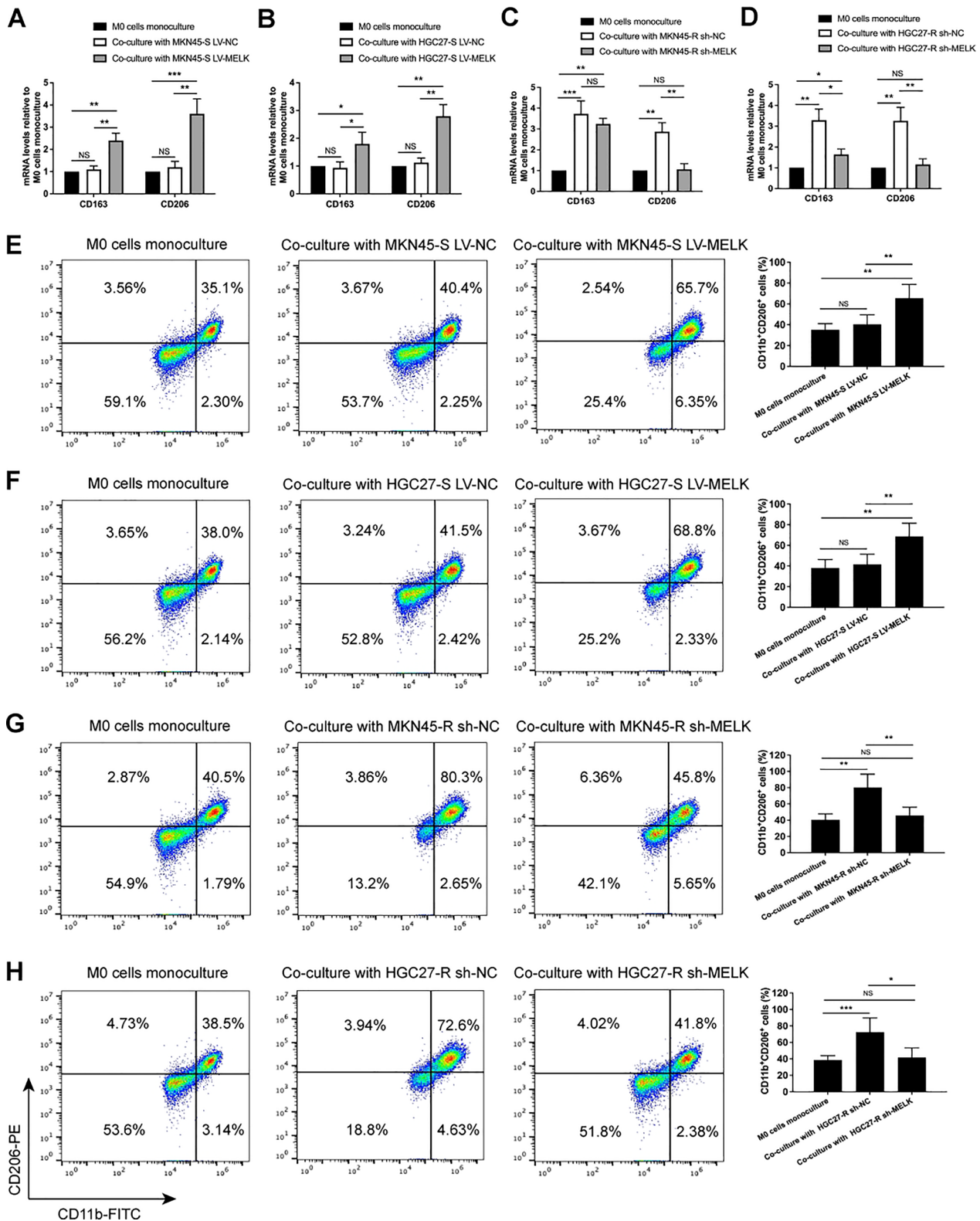


**Fig. 4** Co-culturing with GC cells overexpressing MELK induced macrophages polarization to M2 phenotype. **A**–**D** CD163 and CD206 in macrophages were detected by qRT-PCR in mRNA levels, and the results were normalized by GAPDH mRNA levels. **E**–**H** The percentages of CD11b$^+$ CD206$^+$ cells (M2 macrophages) were measured through flow cytometry analysis. Data were presented as mean ± SD of three independent experiments. *NS* not significantly, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$

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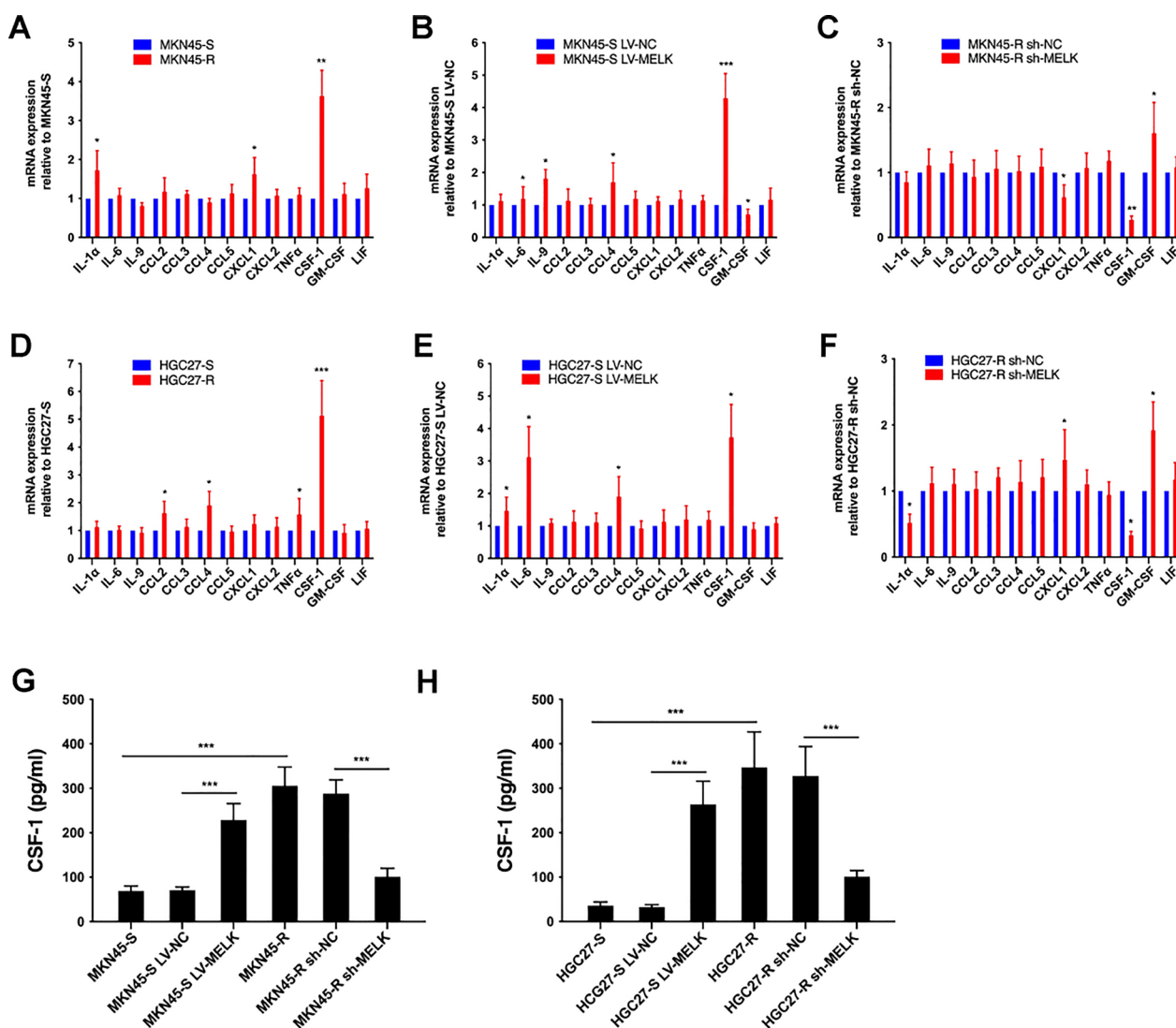


**Fig. 5** CSF-1 deriving from GC cells was associated with the expression of MELK. **A**–**F** qRT-PCR detected the mRNA levels of thirteen cytokines in GC cells, the change trend of CSF-1 was consistent with previous results and the difference was statistically significant. **G**, **H** ELISA detected CSF-1 in culture supernatants of GC cells. Data were presented as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

through the activation of miR-34a/JAK2/STAT3 signaling pathway in the TME of uterine leiomyosarcoma [37]. These findings suggests that MELK might be closely associated with the TME and serves as a critical role in tumor progression. Our previous study demonstrated that chemoresistant GC cells were more effective in inducing macrophages polarization towards M2 phenotype compared to parental GC cells [21]. In the current investigation, we revealed that co-culturing parental GC cells overexpressing MELK with macrophages in a noncontacting transwell system could induce macrophages polarization towards M2 phenotype. In contrast, MELK knockdown attenuated the ability of chemoresistant GC

cells to promote M2 polarization. Combined with our previous results, this accumulated data confirms that upregulation of MELK expression could not only promote 5-Fu-resistance in GC cells but was also accompanied by M2 macrophages polarization.

The present study indicated that CSF-1 deriving from GC cells overexpressing or silencing MELK served as a pivotal bridge in the cellular interaction between cancer cells and macrophages. *CSF-1*, located on human chromosome 1 (1p13.3), encodes a 554 amino acid protein, also known as macrophage colony-stimulating factor (M-CSF). CSF-1 plays a crucial role in regulating macrophage survival, differentiation, and function. The

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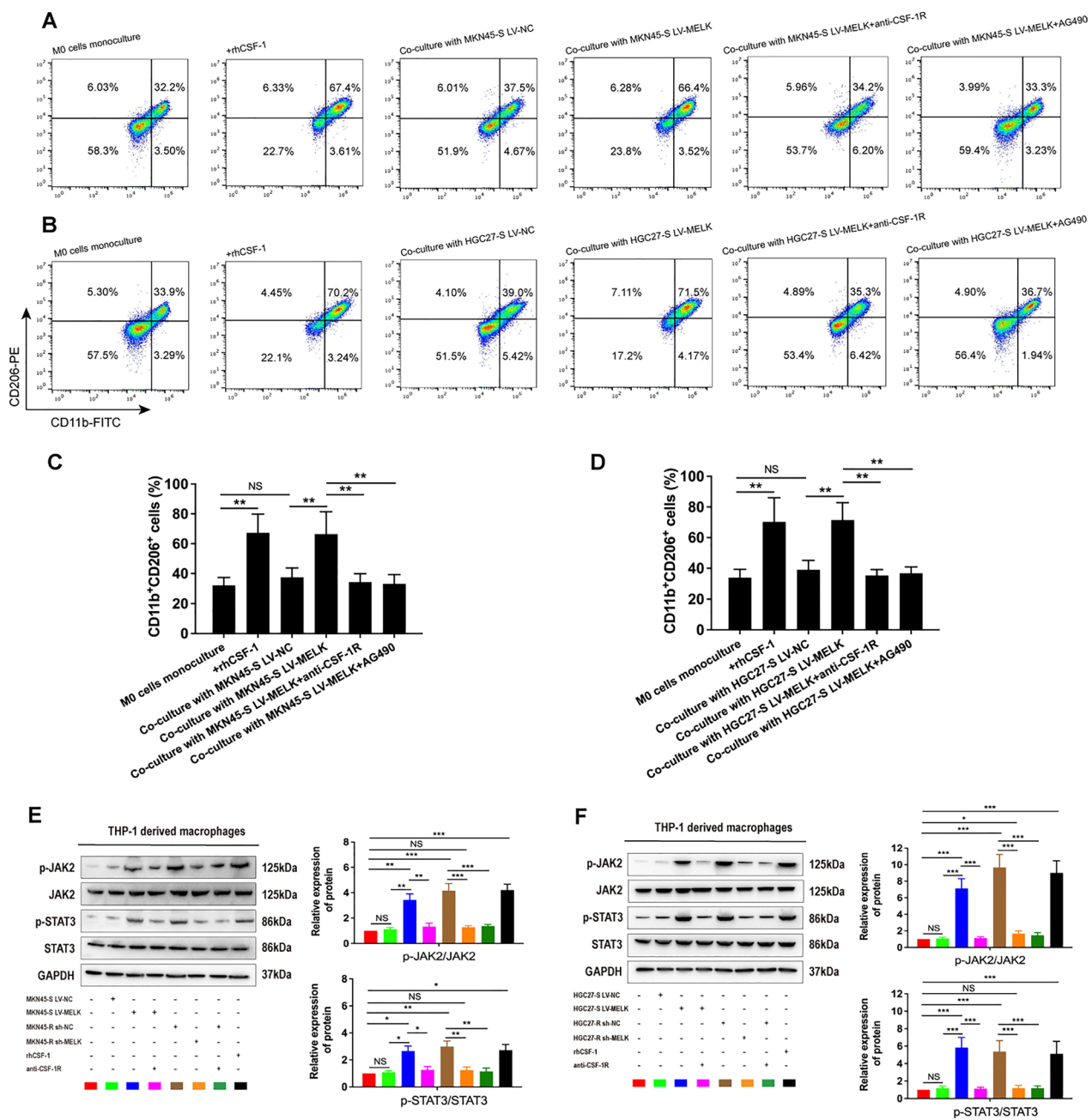


**Fig. 6** MELK induced macrophages polarization towards M2 phenotype via CSF-1/JAK2/STAT3 pathway. **A–D** Flow cytometry detected the percentages of CD11b$^+$CD206$^+$ cells in macrophages incubating with CSF-1, co-culturing with parental GC cells with MELK overexpression in the presence of anti-CSF-1R, AG490 or not. **E, F** Macrophages were incubated with CSF-1, co-cultured with parental GC cells overexpressing MELK and their negative control cells, and co-cultured with chemoresistant GC cells silencing MELK and their negative control cells in the presence of anti-CSF-1R or not, then the JAK2/STAT3 pathway proteins in these macrophages were detected by western blot. Data were presented as mean±SD of three independent experiments. *NS* not significantly, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

CSF-1 receptor, CSF-1R or CD115, is a tyrosine kinase encoded by the proto-oncogene *c-fms* and is predominantly expressed on the monocytic lineage [38]. Targeting CSF-1/CSF-1R signal transduction has garnered considerable attention from researchers. Pyonteck et al.

reported that CSF-1R blockade could reduce M2 macrophages polarization in treated gliomas and slow the intracranial growth of glioma xenografts [39]. The monoclonal antibody RG7155, an inhibitor of CSF-1R, was found to decrease M2 macrophages in patients with
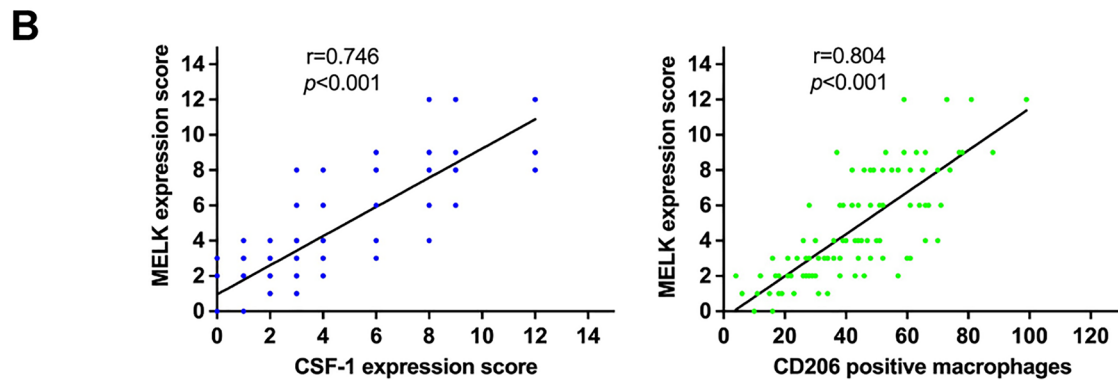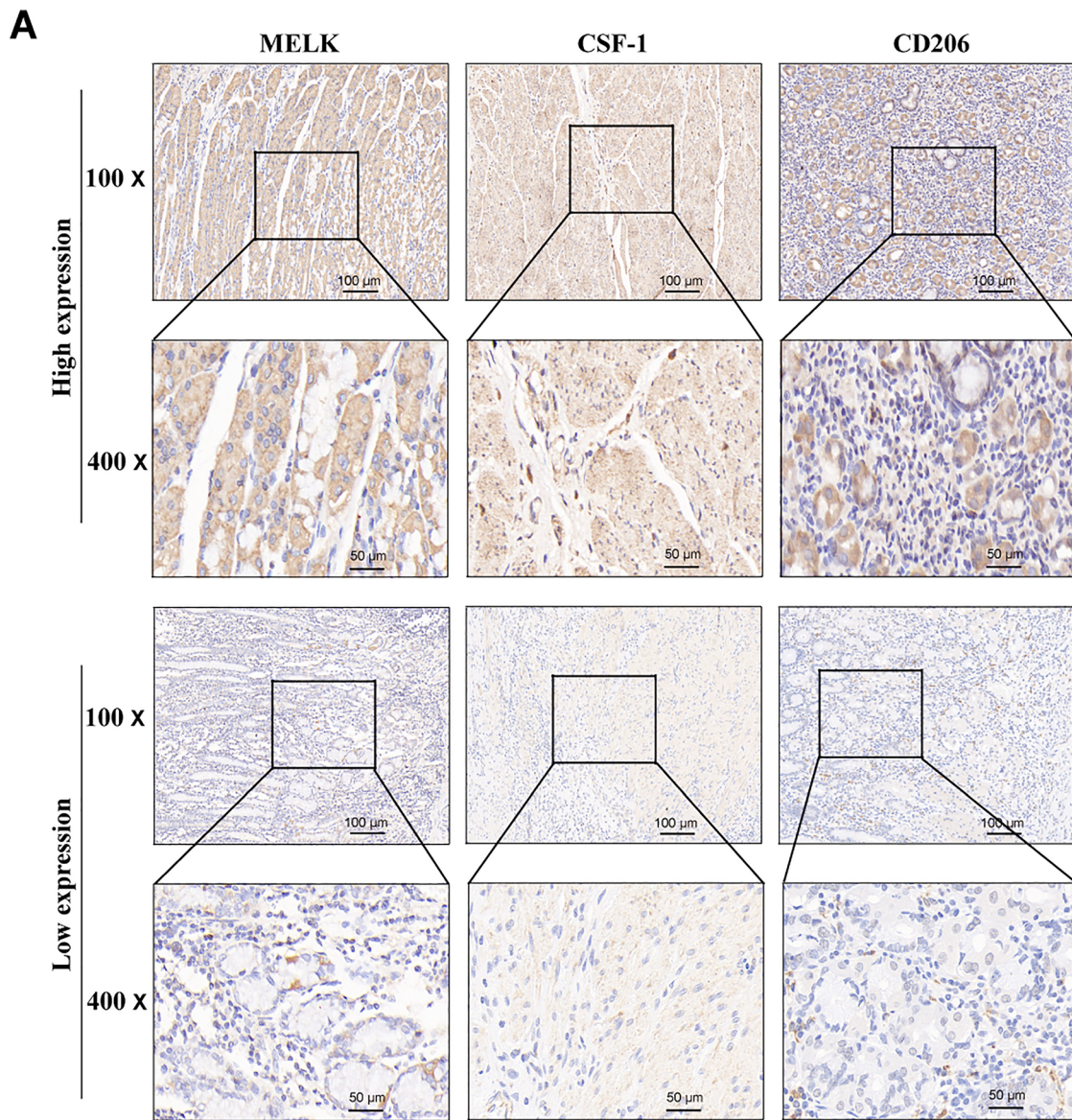
**Fig. 7** The correlation of MELK with CSF-1 and CD206. **A** Representative images of immunohistochemical staining for MELK, CSF-1 and CD206 in human GC tissues. **B** The correlation of MELK with CSF-1 and CD206 in GC tissue was analyzed through Spearman rank-order correlation

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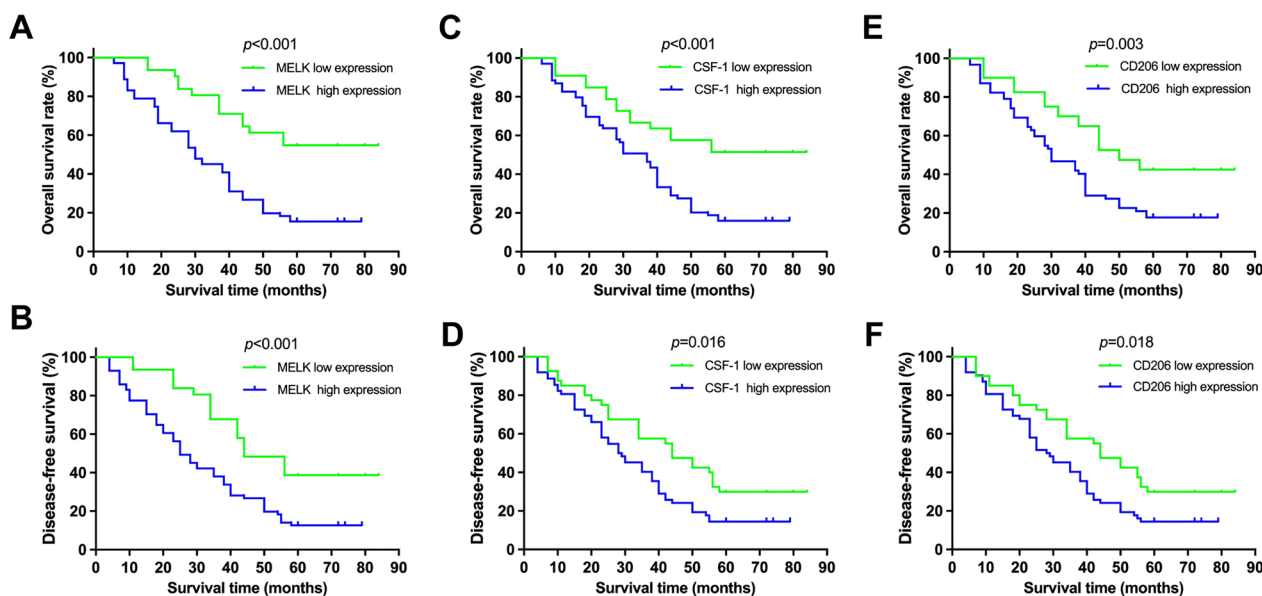


**Fig. 8** Correlation of MELK, CSF-1 and CD206 expression with the prognosis of GC patients. **A**, **B** Overall survival (OS) and disease-free survival (DFS) analysis based on MELK in our cohort. **C**, **D** OS and DFS analysis based on CSF-1. (**E** and **F**) OS and DFS analysis based on CD206 (high expression vs. low expression)

various types of solid malignancies while increasing the CD8 + /CD4 + T cell ratio [40]. Consistently, our findings indicate that the blockade of CSF-1R attenuated the effect of MELK overexpression on M2 polarization.

Supported by the literature, the polarization of M2 macrophages is closely associated with the activation or inhibition of the JAK2/STAT3 signaling pathway. IL-6 could induce macrophages differentiation into the pro-tumor M2 phenotype via activating STAT3 phosphorylation and then promote GC cells proliferation and migration [41]. The inhibitor of JAK2 suppressed the JAK2/STAT3 signaling pathway in macrophages and induced macrophages polarization towards M1 phenotype. Further investigation indicated that the JAK2 inhibitor could reverse the phenotype of macrophages from M2- to M1-type [42]. In breast cancer, the redox-active drug MnTE-2-PyP5$^+$ could decrease phospho-STAT3 levels to inhibit M2 polarization, thus stimulating the immune system [43]. In the present study, the JAK2/STAT3 signal pathway inhibitor AG490 was found to significantly attenuate the effect of parental GC cells with MELK overexpression on inducing M2 polarization. Clinically, MELK expression positively correlated with CSF-1 and the infiltration of CD206 positive macrophages. These results were confirmed by the above data from in the vitro experiments. Furthermore, survival analysis revealed that patients with high expression of MELK, CSF-1, or CD206 had worse OS and DFS than those with low expression of these three molecules.

## Conclusion

In summary, this study uncovered that the upregulation of MELK expression could confer chemoresistance of GC. More importantly, MELK overexpression induced macrophage polarization to M2 phenotype via the CSF-1/JAK2/STAT3 signal axis, which in turn further promoted the chemoresistance of GC cells. These findings suggest that targeting MELK and inhibiting the CSF-1/JAK2/STAT3 signal pathway may represent potential therapeutic strategy for patients with GC, especially chemoresistant GC.

**Abbreviations**

| | |
|---|---|
| GC | Gastric cancer |
| MELK | Maternal embryonic leucine zipper kinase |
| qRT-PCR | Quantitative real-time polymerase chain reaction |
| CSF-1 | Colony-stimulating factor 1 |
| M-CSF | Macrophage colony-stimulating factor |
| TME | Tumor microenvironment |
| TAMs | Tumor-associated macrophages |
| CAP | College of American Pathologists |
| CCK-8 | Cell counting kit-8 |
| SD | Standard deviation |
| OS | Overall survival |
| DFS | Disease-free survival |

## Supplementary Information

Supplementary Material 1. Table S1. Baseline characteristics of patients.

Supplementary Material 2. Table S2. Full-length MELK gene sequence.

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Not applicable.

## Author contributions
Conceptualization of the study concept was carried out by PS and JY. Data curation was conducted by PS, TY and YZ. Formal analysis and confirmation of the data were conducted by PS, TY and WK. Funding acquisition was achieved by JY. Research methodology was the responsibility of PS, HH and YL. Project administration was conducted by JY. Supervision was undertaken by WK, YL and JY. Visualization was conducted by PS, CC and MC. Writing of the original draft was carried out by PS and YZ. Writing, review and editing were carried out by PS, YL and JY. All authors read and approved the final version of the manuscript.

## Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate
The present study was approved by the Institutional Review Board of Peking Union Medical College Hospital and the written informed consent was obtained from all patients. The ethical approval code of this study is JS-2587. All animal studies were carried out according to the National Institute of Health Guide for the Care and Use of Laboratory Animals with the approval of Peking Union Medical College Hospital Animal Experiments Committee (ethical approval number: XHDW-2022-114).

### Consent for publication
Not applicable.

### Competing interests
The authors declare no competing interests.

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