CCL26 Participates in the PRL-3-Induced Promotion of Colorectal Cancer Invasion by Stimulating Tumor-Associated Macrophage Infiltration

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Abstract

Both phosphatase of regenerating liver-3 (PRL-3) and tumor-associated macrophages (TAM) influence cancer progression. Whether PRL-3 plays a critical role in colorectal cancer invasion and metastasis by inducing TAM infiltration remains unclear. In the current study, we investigated the effects of chemokine ligand 26 (CCL26) on TAM infiltration and colorectal cancer invasion and the underlying mechanism in colorectal cancer cells by overexpressing or silencing PRL-3. We found that PRL-3 upregulated CCL26 expression correlatively and participated in cell migration, according to the results of gene ontology analysis. In addition, IHC analysis results indicated that the PRL-3 and CCL26 levels were positively correlated and elevated in stage III and IV colorectal cancer tissues and were associated with a worse prognosis in colorectal cancer patients. Furthermore, we demonstrated that CCL26 induced TAM infiltration by CCL26 binding to the CCR3 receptor. When LoVo-P and HT29-C cells were cocultured with TAMs, CCL26 binding to the CCR3 receptor enhanced the invasiveness of LoVo-P and HT29-C cells by mobilizing intracellular Ca²⁺ of TAMs to increase the expression of IL6 and IL8. In addition, IHC results indicated that protein levels of CCR3 and TAMs counts were higher in stage III and IV colorectal cancer tissues and correlated with CCL26. Moreover, similar results were observed in vivo using mice injected with LoVo-P and HT29-C cells. These data indicate that PRL-3 may represent a potential prognostic marker that promotes colorectal cancer invasion and metastasis by upregulating CCL26 to induce TAM infiltration.

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Introduction

Recent reports have indicated that colorectal cancer metastasizes in approximately 50% to 60% of patients (1, 2). The liver is the first organ for the distant metastasis of colorectal cancer (3). Approximately 90% of liver metastases express phosphatase of regenerating liver-3 (PRL-3), an atypical tyrosine phosphatase of the liver regeneration phosphatase family that is minimally expressed in normal colon tissues (4, 5). Investigations have found that PRL-3 expression predicts colorectal cancer development (6). Therefore, PRL-3 closely correlates with colorectal cancer progression, suggesting that this enzyme is involved in liver metastasis.

In a review of the associations between cancer and inflammation, Balkwill suggested that the inflammatory cells and cytokines found in tumors are more likely to contribute to tumor progression (7). Many studies have demonstrated that the tumor microenvironment is an essential participant in the promotion of tumor cell proliferation, survival, and metastasis (8, 9). Notably, a high number of tumor-associated macrophages (TAM) are observed in tumors (10–12). TAMs are a significant component of inflammatory infiltrates in tumor tissues and can produce a large number of lymphangiogenic growth factors, cytokines, and proteases, all of which are mediators that promote tumor cell proliferation and metastasis (13).

Increasing evidence has revealed that tumor cells establish contact with stromal cells through a complicated intercellular signaling network (14). Song found that a positive feedback loop between GM-CSF and CCL18 is important in breast cancer metastasis (15). Moreover, we previously confirmed that both IL6 and IL8, secreted by TAMs, promote the PRL-3–induced invasiveness of LoVo cells through the activation of the intermediate conductance Ca²⁺-activated K⁺ (KCNN4) channel (16). We also showed that PRL-3 upregulated the expression of phosphorylated glycogen synthase kinase-3 β (p-GSK-3 β) via the KCNN4 pathway, leading to increased intracellular calcium levels (17). Furthermore, Gaspar and colleagues found that CCL26 mobilized intracellular Ca²⁺ by binding to C-C...
chemokine receptor type 3 (CCR3) to promote migration (18). Therefore, we proposed that elevated PRL-3 expression in colorectal cancer can influence TAM activity through cytokines or growth factors, which could induce TAM infiltration into tumor tissues to establish the tumor microenvironment.

In the current study, we used gene arrays and gene ontology (GO) analyses to investigate the effects of PRL-3 on TAM infiltration and the tumor microenvironment in colorectal cancer. The findings suggested that PRL-3 mediates the upregulation of CCL26 in colorectal cancer cells to induce TAM infiltration into tumor tissues. Furthermore, we showed that CCL26 mobilized the intracellular Ca²⁺ of TAMs to increase the expression of IL6 and IL8 to promote colorectal cancer invasion by binding to the CCR3 receptor. Importantly, PRL-3 correlates with high CCL26 expression in colorectal cancer stage III/IV tissue samples, and positive PRL-3 and CCL26 staining and increased TAM numbers are associated with a shorter postsurgical overall survival (OS) for colorectal cancer patients.

Materials and Methods

Clinical colorectal cancer tissue samples
Colorectal cancer tissue samples were obtained from 83 patients from 2006 to 2008, including 20 stage I patients, 21 stage II patients, 23 stage III patients, and 19 stage IV patients, at the Department of Gastrointestinal Surgery of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University (Guangzhou, China). The selection of colorectal cancer patients was based on pathologic diagnosis and follow-up data. All tissues were collected within 20 minutes after tumor removal after obtaining permission from the patients. All samples and related procedures were approved by the Institute Research Ethics Committee of Sun Yat-Sen Memorial Hospital at Sun Yat-Sen University.

Reagents and antibodies
FBS was purchased from Biolind. G418 was purchased from MP Biomedicals. PMA was purchased from Sigma. Lipofectamine 2000 and RIPA buffer were purchased from Thermo Fisher Scientific. The siRNA molecules were purchased from GenePharma. RPMI was purchased from Gibco. TRizol and Prime Script RT were purchased from Takara. Recombinant CCL26 (rCCL26) was purchased from PeproTech. Matrigel matrix was purchased from BD Biosciences. Anti–PRL-3, anti–CCL26, anti–CCR3, anti–KCNN4, anti–p-GSK-3β, anti–GSK-3β, anti–IL6, and anti–IL8 antibodies were purchased from Abcam. Anti–CD68, anti–S100A14, and anti–GAPDH antibodies were purchased from Santa Cruz Biotechnology. The catalog number has been mentioned in Supplementary Table S1.

Cell culture and treatments
LoVo, HT29, and THP-1 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) in 2013. The cell lines were authenticated in July 2017 by Guangzhou Cellcook Biotech who compared their STR profiles with ATCC and DSMZ databases. All cells were cultured in RPMI1640 supplemented with 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin. PRL-3 expression was detected in colorectal cancer cell lines by Western blot analysis, and the results showed that LoVo cells expressed low levels of PRL-3, while HT29 cells expressed high levels of PRL-3 (Supplementary Fig. S1A). LoVo cells were selected for constructing PRL-3 overexpression lines, and HT29 cells were selected for the knockdown of endogenous PRL-3 expression. The stable transfection of pacGFP-PRL-3 (LoVo-P) and pacGFP (LoVo-C) vectors was achieved as described previously, resulting in the overexpression of PRL-3 and control vector (19). To knock down PRL-3, HT29 cells were stably transfected with PRL-3 short hairpin RNA (HT29-shPRL-3), and the shRNA-control group was stably transfected into HT29 (HT29-control) cells as a negative control group (Supplementary Fig. S1, P < 0.05). LoVo-P and LoVo-C cells were cultured in RPMI1640 supplemented with 10% FBS and 600 μg/mL G418. THP-1 cells were transformed into M1 and M2 macrophages as described previously (16). THP-1 cells differentiate into M2 macrophages, a type of TAM, and subsequently treated with PMA for 6 hours, followed by the addition of IL4 for 18 hours (total of 24 hours). M1 macrophages were generated using the same method but without IL4 addition. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

RNA extraction, array platform and analysis, and qRT-PCR
Total RNA was extracted using TRizol reagent and reverse transcribed using PrimeScript Reverse Transcriptase from 500 ng RNA according to the manufacturer’s instructions. qRT-PCR was performed using the LightCycler 480 (Roche) and SYBR Assays (Takara) according to the manufacturers’ instructions. Primers were designed to detect SLIT2, MMP28, LAMA4, F3, LGALS3, S100A14, CCL12, CCL17, CCL18, CCL22, CCL26, CXC12, EGF, IL1, IL6, IL8, VEGFA, TGFß, PRL3, CCR3, and GAPDH. The sequences of the qRT-PCR primers are shown in Supplementary Table S2.

Arraystar Human mRNA Array V4.0 was used for the global profiling of 20,730 human mRNA and 40,173 long noncoding RNA (LncRNA) transcripts according to the manufacturer’s instructions. The raw data have been released on the Gene Expression Omnibus (GEO: www.ncbi.nlm.nih.gov/geo/index. cgi, ID: GSE103989, accessed September 20, 2014). Sample labeling and array hybridization were performed using the Agilent One-Color Microarray-Based Gene Expression Analysis (Agilent Technology) according to the manufacturer’s instructions, with minor modifications. Briefly, mRNA was purified from total RNA after the removal of rRNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3’ bias using a random priming method. The labeled cRNAs were purified using the RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using the NanoDrop ND-1000. The hybridized arrays were washed, fixed, and scanned using the Agilent DNA Microarray Scanner (part number G2505C).

ELISA
CCL26 was assayed in the culture supernatant of LoVo-P, LoVo-C, HT29-C, and HT29-shPRL-3 cells using the Quantikine Kit (CUSABIO Life Science) according to the manufacturer’s protocol.

Transfections of HT29 with shRNA lentiviruses
Short hairpin RNA-PRL-3 lentiviruses were packaged according to Chen and colleagues (20). We further generated lentivirus...
vectors encoding shRNA-PRL-3 and shRNA-control. The sequence of shRNA-PRL-3 is as follows:

5’-CTTACAACTTCTACACAGCGCATGTGTTTGTAGCTTTTTTCCTCGAG-3’. The shRNA-PRL-3 lentivirus vectors and shRNA-control lentivirus vectors were transfected into HT29 cells using Lipofectamine 2000 according to the manufacturer's protocol.

**Coculture of M2 macrophages with colorectal cancer cells**

Approximately 1 × 10⁵ M2 macrophage cells were seeded onto 6-well plates, and LoVo-P, LoVo-C, HT29-C, and HT29-shPRL-3 cells were cocultured at a ratio of 1:1 with M2 macrophage cells in the upper transwell inserts for 48 hours. After coculturing, the LoVo-P, LoVo-C, HT29-C, HT29-shPRL-3, and M2 macrophage cells were washed and used for subsequent experiments.

**Cell invasion and migration assays**

Transwell inserts were used to detect cell invasion. After spreading 10% Matrigel onto the upper chamber, 10⁵ of either LoVo-P or LoVo-C cells (HT29-C or HT29-shPRL-3 cells) in 0.2 mL of serum-free RPMI1640 medium was added to the chamber, followed by the addition of 10% FBS to the lower chamber. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 hours, the invasive cells located on the lower side of the chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet in methanol, air dried, and counted under a microscope.

The M1 or M2 macrophage cell migration assays were also performed using transwell inserts without Matrigel. The number of migrated cells was counted after 24 hours.

**RNAi**

The siRNA sequences of CCL26, CCR3, and negative control are listed in Supplementary Table S3. siRNA against CCL26 was transfected into LoVo-P and HT29-C cells using Lipofectamine 2000 according to the manufacturer's protocol. siRNA against CCR3 was transfected into M2 macrophages. Confirmation of target gene silencing was measured using Western blotting at 48 hours after transfection. Scrambled siRNA (negative control) and Lipofectamine 2000 (Lipo2000) were used as control.

**Western blot analysis**

The cells were lysed on ice in RIPA buffer containing protease and phosphatase inhibitors. The protein concentration was determined using a Bradford assay. Equal amounts of protein were separated on either 10% or 12% SDS-PAGE gels, followed by transfer to PVDF membranes. The membranes were blocked in either 5% nonfat milk or 5% BSA. After blocking, the membranes were incubated with the appropriate primary antibodies overnight. After washing, the membranes were then incubated with a horseradish peroxidase-labeled secondary antibody for 1 hour at room temperature. The labeled proteins were visualized using chemiluminescence.

**IHC assay**

IHC was performed to detect PRL-3, CCL26, CCR3, and CD68 using the UltraSensitive SP IHC Kit (Maxim Biotech) according to the manufacturer’s instructions. Normal IgG replaced the primary antibody as a negative control. The staining was evaluated as described previously (21). The following extensional standard was used: (i) The number of cells at that intensity ≤5% of the total, 0; 6%–25%, 1; 26%–50%, 2; 51%–75%, 3; and >75%, 4; (ii) intensity of stain: colorless, 0; light yellow, 1; yellow, 2; and brown; (iii) the scores for 1 and 2 were multiplied to obtain the final score (range, 0–12).

**Selection of cut-off score**

The ROC curve analysis was used to determine the cut-off value for delineating between negative and positive sections. The sensitivity and specificity for each outcome under study was plotted on the basis of the PRL-3 score, generating various ROC curves. The score was selected as the cut-off value, which was closest to the point with both maximum sensitivity and specificity. For the ROC curve analysis, the clinicopathologic features were dichotomized: TNM (I + II or III + IV); tumor site (colon or rectum); tumor grade (well-moderate or poor differentiation); and lymph node metastasis (negative or positive). According to the ROC curve analysis, scores for PRL-3 expression in colorectal cancer tissues above the cut-off value of 2 were defined as positive, and the corresponding AUC is described in Supplementary Fig. S2A. The staining grade was stratified as –, score of 0–1; +, score of 2–6; and +++, score of 7–12. Similarly, colorectal cancer tumors with scores above 2, 3, and 1 were considered positive for CCL26, CCR3, and CD68, respectively.

**Immunofluorescence analysis**

Cells were grown in conflocal dishes (Nest) to 40% to 60% confluence, washed with PBST, and fixed in 4% paraformaldehyde for immunofluorescence staining. The cells were incubated with mouse anti-CCL26 antibody (1:100; Bioss) and rabbit anti-CCR3 antibody (1:100) overnight at 4°C. The slides were subsequently incubated with Alexa Fluor 594–conjugated donkey anti-rabbit and Alexa Fluor 488–conjugated goat anti-mouse secondary antibodies at room temperature for 1 hour. The labeled cells were visualized using a laser confocal microscope (Zeiss LSM710) after washing with PBST.

**Mouse tumor model**

Athymic nude mice (BALB/c-nude, 6-week-old males) were purchased from the Laboratory Animal Science Center of Guangdong Province and maintained under the defined conditions at the Animal Experiment Center of Sun Yat-Sen University. All animal protocols were approved by the Institutional Animal Care and Use Committee and Welfare Committee of Sun Yat-Sen University.

Nude mice were randomly divided into four groups of 6 animals. One group of mice was injected with 5 × 10⁶ LoVo-P cells into the subcutaneous tissue in the left flank of nude mice to establish tumor xenografts, and the other group was injected with LoVo-C cells. In the other two groups, mice were injected with HT29-C or HT29-shPRL-3 cells. The mice were maintained in pathogen-free environments, and the tumor volume was measured every 7 days. All nude mice were sacrificed on day 30, and the volume and weight of the tumors were recorded, and the xenografted tumors were excised from the animals for further studies. One half of the xenograft tumors was embedded in paraffin for IHC assay, while the other half was stored at −80°C for Western blot analysis.
Statistical analyses

All analyses were performed using statistical software package (SAS 8 for Windows, SAS Institute). All data are presented as the mean ± SD. The differences between two groups and three or more groups were determined using Student t test and one-way ANOVA, respectively. Survival curves were calculated using the Kaplan–Meier analysis. The log-rank test was used to evaluate differences in survival distributions. Spearman rank test was used to assess the correlation between PRL-3 and CCL26 expression levels. The Cox proportional hazards model was performed to estimate the significance of various factors for survival in a multivariate analysis. All experiments were performed independently at least three times. In all cases, P < 0.05 was considered statistically significant.

Results

PRL-3 upregulates CCL26 expression

Increased macrophage infiltration has been detected in many different solid tumors (22). Whether colorectal cancer cells with higher PRL-3 expression exhibit increased macrophage infiltration remains unclear. The differential expression of mRNA in LoVo-P cells compared with LoVo-C cells was detected using the Arraystar Human LncRNA/mRNA Array V4.0 (Fig. 1A). The results were subjected to GO analysis to infer the involved biological processes and cellular components. Because the direction of research is cell invasion, we selected three biological processes and one cellular component of mRNA from the GO analysis results and identified pleiotropic genes using a Venn diagram (sets for 'cell motility,' 'cell migration,' 'regulation of localization,' and 'extracellular region part'). The results showed 20 mRNAs that were more highly expressed in LoVo-P cells than in LoVo-C cells (fold change cutoff, 2.0; Fig. 1B and C). A total of 7 mRNAs showing more than 5-fold changes in expression were selected for qRT-PCR analysis to verify the expression of genes in LoVo-P and LoVo-C cells. The results showed that CCL26 gene expression was more than 50-fold higher in LoVo-P cells and that of another gene, S100A14, was also significantly higher (Fig. 1D; Supplementary Fig. S2B, P < 0.01), suggesting that CCL26 or S100A14 expression is associated with PRL-3 expression.

In addition, the protein expression levels of CCL26 and S100A14 in LoVo-P and LoVo-C cells were examined using Western blotting, and the results showed that only CCL26 protein levels were significantly higher in LoVo-P cells (Fig. 1E, P < 0.01). In addition, we detected CCL26 protein in the culture medium using ELISA. Similarly, CCL26 was highly expressed in the culture medium (Fig. 1F, P < 0.01). We also selected another cell line with low PRL-3 expression to verify these results. The same results were examined in HT29-control and HT29-shPRL-3 cells, showing that PRL-3 regulated the expression of CCL26 by Western blotting and ELISA (Supplementary Fig. S3A–S3C).

CCL26 expression in colorectal cancer tissues correlates with PRL-3

CCL26 expression was upregulated by PRL-3 in colorectal cancer cells. Whether CCL26 expression correlates with PRL-3 in colorectal cancer tissue is unknown. To explore the expression of PRL-3 and CCL26 in tissue samples, we analyzed PRL-3 and CCL26 levels in colorectal cancer tissue samples from 83 patients using IHC and found that the expression levels of PRL-3 and CCL26 were gradually increased from stage I to IV and were strongly expressed in liver metastasis samples (Fig. 2A). Compared with stage I and II tissue samples, the positive expression of PRL-3 and CCL26 was significantly higher in stage III, stage IV, and liver metastasis tissue samples (Fig. 2B and C; Table 1, *P < 0.05; **P < 0.01). To show a correlation between PRL-3 and CCL26, Spearman ρ was used to analyze the relationship between these proteins, showing that a total of 85% (34/40) of the colorectal cancer samples positive for PRL-3 expression overlapped with 89.5% (34/38) of colorectal cancer samples positive for CCL26 expression. The correlation analysis revealed a significant positive correlation between PRL-3 and CCL26 (Fig. 2D and E, P < 0.001), suggesting that PRL-3 expression correlates with CCL26 expression in colorectal cancer.

High protein expression of PRL-3 and CCL26 in colorectal cancer is associated with a worse prognosis

We investigated the prognostic role using a cut-off value to delineate between the negative and positive staining of PRL-3 and CCL26. The Kaplan–Meier survival analysis of PRL-3 and CCL26 indicated that compared with positive staining, negative staining was significantly correlated with longer survival in colorectal cancer patients (Fig. 2F and G, P < 0.01). In addition, multivariate Cox regression analysis revealed (Table 1) that the increased expression of PRL-3 and CCL26 significantly correlated with lymph node metastasis, distant metastasis, poorly differentiated tumor, and a high tumor–node–metastasis (TNM) stage (P < 0.01).

CCL26 induces M2 macrophage infiltration by interacting with the CCR3 receptor

CCL26 can mobilize intracellular Ca2+ by binding to the chemokine receptor CCR3 on the cell surface to promote migration (18). However, whether CCL26 promotes M2 macrophage migration is unclear. First, the transwell analysis of the lower chamber containing 10% FBS and either LoVo-P or LoVo-C cells showed that the migratory activity of M2 macrophages in the LoVo-P cell group was more pronounced than that in the other two groups after 24 hours (Fig. 3A, P < 0.01). Another LoVo-P cell group, using M1 macrophage cells instead of M2 macrophage cells, showed that the amount of M1 macrophage cell migration was approximately 20 ± 4 less than that of the M2 macrophage group. The same results were observed in HT29-C cells but not in HT29-shPRL-3 cells (Fig. 3B, P < 0.01). Moreover, the lower chamber was replaced with culture medium containing different concentrations of rCCL26 (0, 1, 10, and 100 ng) for 24 hours and 10 ng of rCCL26 at different times (12, 24, and 36 hours). TAM migration was gradually increased in a dose- and time-dependent manner (Fig. 3C, P < 0.01).

We detected the expression of CCR3 in TAMs using qRT-PCR and Western blotting. Both analyses showed significantly higher CCR3 expression in M2 than in M1 macrophages (Fig. 3D and E). To determine whether CCL26 induced TAM infiltration through CCR3, CCL26-targeted siRNAs were transfected into the LoVo-P and HT29-C cells, and CCR3-targeted siRNAs were transfected into TAMs, resulting in the reduced expression of both of these proteins compared with the respective control groups (Supplementary Fig. S3D and S3E, P < 0.01). These treatments resulted in an obvious reduction in TAM migration (Fig. 3F and G, P < 0.01). To further confirm whether CCL26
Figure 1.
PRL-3 upregulates CCL26 expression. A, Heatmap showing the differential expression of mRNA in LoVo-P cells compared with LoVo-C cells. B, Venn diagram of differentially expressed genes across four categories from GO analysis. C, Heatmap showing the results of Venn diagram and the gene of CCL26 was the top gene of differential expression. D, qRT-PCR was performed to detect the expression of mRNA; CCL26 mRNA was highly expressed in LoVo-P cells, more than 50-fold higher than LoVo-C cells. S100A14 mRNA was less than 2-fold higher in LoVo-P than LoVo-C cells. E, The expression of CCL26 and S100A14 proteins in LoVo-P and LoVo-C cells detected by Western blotting, and only CCL26 was higher in LoVo-P cells. F, LoVo-P and LoVo-C cells were seeded into 6-well plates for 48 hours, and the level of CCL26 in culture medium was detected by ELISA. GAPDH was used as a loading control. Results are presented as the average of quadruplicate measurements, and the bar is the SD (n = 3). *, P < 0.05 and **, P < 0.01.
interacts with the CCR3 receptor on TAM membranes, we incubated TAMs with CCL26 (100 ng) at 4°C for 3 hours. Confocal microscopy demonstrated that CCL26 interacted with CCR3 on TAM membranes using immunofluorescence assays and efficiently abrogated the result after the siRNA silencing of CCR3 (Fig. 3H). Moreover, the results showed that CCL26 interacted with CCR3 on TAM membranes after coculturing with HT29-C cells, and this effect was reduced by siRNA silencing of CCR3 expression on TAMs (Supplementary Fig. S3f). These results suggested that CCL26 induces TAM infiltration by interacting with CCR3.

TAMs enhance the invasive activity of colorectal cancer cells induced by CCL26 binding to CCR3

Our previous study found that TAM-derived IL6 and IL8 enhance the invasive activity of LoVo cells through activating...
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Table 1. Correlation of PRL-3, CCL26, CCR3, and TAM expression levels with clinicopathologic status in 83 cases of colorectal cancer patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>PRL-3 (+)</th>
<th>CCL26 (+)</th>
<th>CCR3 (+)</th>
<th>TAMs (+)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male</td>
<td>22 (48.8%)</td>
<td>20 (44.4%)</td>
<td>18 (40.0%)</td>
<td>19 (42.2%)</td>
<td>83</td>
</tr>
<tr>
<td>Females</td>
<td>18 (47.3%)</td>
<td>18 (47.3%)</td>
<td>17 (44.7%)</td>
<td>17 (44.7%)</td>
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</tr>
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<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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<td>≥60</td>
<td>16 (44.4%)</td>
<td>17 (47.2%)</td>
<td>16 (44.4%)</td>
<td>16 (44.4%)</td>
<td>36</td>
</tr>
<tr>
<td>&lt;60</td>
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<td>21 (43.7%)</td>
<td>19 (59.6%)</td>
<td>20 (41.6%)</td>
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<td>Lymph node metastasis</td>
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<tr>
<td>Negative</td>
<td>11 (26.8%)</td>
<td>8 (19.5%)</td>
<td>6 (14.6%)</td>
<td>9 (21.9%)</td>
<td>41</td>
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<tr>
<td>Positive</td>
<td>29 (69.0%)*</td>
<td>30 (71.4%)*</td>
<td>29 (69.0%)*</td>
<td>27 (64.2%)*</td>
<td>42</td>
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<td>Differentiation status</td>
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<td>Well</td>
<td>4 (16.6%)</td>
<td>3 (12.5%)</td>
<td>3 (2.5%)</td>
<td>4 (16.6%)</td>
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<tr>
<td>Moderate</td>
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<td>8 (33.3%)*</td>
<td>7 (28.1%)*</td>
<td>8 (33.3%)*</td>
<td>24</td>
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<td>Colon</td>
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<tr>
<td>I</td>
<td>5 (25.0%)</td>
<td>4 (20.0%)</td>
<td>4 (20.0%)</td>
<td>3 (15.0%)</td>
<td>20</td>
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<tr>
<td>II</td>
<td>6 (28.5%)</td>
<td>5 (23.8%)</td>
<td>6 (28.5%)</td>
<td>5 (23.8%)</td>
<td>21</td>
</tr>
<tr>
<td>III</td>
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<td>10 (45.4%)*</td>
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<td>IV</td>
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<td>13 (73.6%)*</td>
<td>12 (63.3%)*</td>
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<tr>
<td>Metastasis</td>
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<td>17 (89.4%)*</td>
<td>15 (78.9%)*</td>
<td>15 (78.9%)*</td>
<td>19</td>
</tr>
</tbody>
</table>

NOTE: Metastasis: Liver metastasis. The percentage in bracket is positive rate.
*P < 0.01, \( ^{b}P < 0.05 \)

High protein levels of CCL26 and CCR3 as well as increased TAM numbers in colorectal cancer are associated with tumor progression and a poor prognosis

To confirm whether these findings are clinically relevant, we explored the expression of CCL26 and TAMs in the stroma of colorectal cancer from 83 clinical specimens using IHC. We found that CCL26, CCR3, and CD68 (markers of TAMs) were highly expressed in the liver metastasis specimens, stage IV, and stage III samples but weakly expressed in the stage II and stage I specimens, and the percentage of staining was upregulated in stage III and IV samples (Fig. 5A and B; Table 1, \( P < 0.05 \)). We also found that the strongly stained samples were primarily stained in the same area of the stroma, indicating its tumor-promoting role and the existence of an obvious correlation. Statistical analysis results (Table 1) show that increased CCR3 expression and TAM cell numbers significantly correlated with lymph node metastasis, distant metastasis, poorly differentiated tumor, and a high TNM stage (\( P < 0.01 \)).

We also investigated the prognostic role using a cut-off value to delineate between negative and positive staining of TAMs and CCL26. The Kaplan–Meier survival curve with a median follow-up period of 50 months and a log-rank test demonstrated that compared with positive staining, the negative staining of TAMs indicated a significantly longer survival (Fig. 5C, \( P < 0.01 \)). We subsequently divided the patients into subgroups. In the positive CCL26 subgroup, patients with positive TAMs staining had poorer survival than patients with negative staining for both proteins (Fig. 5D, \( P < 0.001 \)). These results are consistent with those of previous studies showing that PRL-3 upregulates CCL26 to induce TAM infiltration through binding with CCR3.

CCL26 induces M2 macrophage infiltration by interacting with CCR3 in vivo

To examine the effect of TAM infiltration induced by CCL26 in vivo, we inoculated LoVo-P and LoVo-C, HT29-C, and HT29-

the KCNN4 pathway (16). Gaspar and colleagues found that CCL26 could mobilize intracellular Ca\(^{2+}\) by binding to CCR3 (18). Whether CCL26 could activate the KCNN4 pathway by binding CCR3 to increase IL6 and IL8 expression is unclear. We next explored the mechanism of IL6 and IL8 secretion on enhancing colorectal cancer cell invasion. Matrigel invasion assays showed that LoVo-P and LoVo-C, HT29-C, and HT29-shPRL-3 invasion was indistinguishable (Fig. 4A, \( P > 0.05 \)). After coculturing with TAMs, LoVo-P and HT29-C cells had greater invasion activity than LoVo-C and HT29-shPRL-3 cells (Fig. 4B, \( P < 0.05 \)). In addition, protein levels of IL6, IL8, KCNN4, and p-GSK3\(^{\beta}\) levels were higher in TAMs cocultured with LoVo-P and HT29-C cells (Fig. 4C and D). After adding different concentrations of rCCL26 in the TAM culture medium for 24 hours during coculture with LoVo-C cells, the invasive activity of LoVo-C cells was enhanced in a dose-dependent manner (Fig. 4E, \( P < 0.05 \)). In addition, the protein levels of IL6, IL8, KCNN4, and p-GSK3\(^{\beta}\) were significantly increased in a dose-dependent manner in TAMs by Western blot analysis (Fig. 4F, \( P < 0.05 \)).

CCL26-targeted siRNAs were transfected into LoVo-P and HT29-C cells, an anti-CCL26 antibody was used to neutralize residual CCL26 function, and CCR3-targeted siRNAs were transfected into TAMs. Invasion assays showed that the number of invasive LoVo-P and HT29-C cells was obviously reduced (Fig. 4G Supplementary Fig. S3H P < 0.01). In addition, the protein levels of IL6, IL8, KCNN4, and p-GSK3\(^{\beta}\) were lower than those in the control group after transfection with CCR3-targeted siRNAs by Western blotting (Fig. 4H and I, \( P < 0.05 \)). These results indicate that the interaction between CCL26 and CCR3 is critical for TAMs to promote colorectal cancer cell invasion.
Figure 3.
CCL26 induced M2 macrophage cell infiltration through interacting with its receptor CCR3. **A**, Transwell chambers were used to detect the migration of M2 macrophage cells. The lower chamber contained LoVo-P cells, LoVo-C cells, and 10% FBS. After 24 hours, the number of migrated M2 macrophage cells was counted. Another LoVo-P cell group used M1 macrophage cells instead of M2 macrophage cells, and the M1 macrophages exhibited almost no migration. **B**, Transwell chambers were used to detect the migration of M2 macrophage cells in HT29-C and HT29-shPRL-3 cells. **C**, Under the same conditions, the lower chamber was filled with culture medium with different concentrations rCCL26 (0, 1, 10, and 100 ng) for 24 hours and 10 ng rCCL26 at different times (12, 24, and 36 hours). **D**, qRT-PCR was used to determine the CCR3 mRNA level in M1 and M2 macrophage cells, which is activated through THP-1 with or without IL4. **E**, The protein expression level of CCR3 in M1 and M2 macrophage cells, as determined by Western blotting. **F**, CCL26-targeted siRNAs, a CCL26 antibody, and CCR3-targeted siRNA resulted in an obvious reduction of the migration of TAMs. **G**, Both CCL26-targeted siRNAs transfected into HT29-C cells and CCR3-targeted siRNA transfected into TAMs cells resulted in an obvious reduction of TAM migration. **H**, CCL26 interacted with CCR3 on the TAM membranes, as determined by immunofluorescence assay. GAPDH used as a loading control. Results are presented as the average of quadruplicate measurements, and the bar is the SD (n = 3). *, P < 0.05 and **, P < 0.01.
Figure 4. TAM-derived IL6 and IL8 enhance the invasive activity of LoVo and HT29 cells induced by CCL26 binding to CCR3. A, LoVo-P and LoVo-C, HT29-C, and HT29-shPRL-3 invasion was indistinguishable, as determined by invasion assays. B, LoVo-P and HT29-C cells had greater invasion activity than LoVo-C and HT29-shPRL-3 cells after coculturing with TAMs for 24 hours. C and D, IL6, IL8, KCNN4, and p-GSK-3β levels were higher in TAMs after coculturing with LoVo-P or HT29-C cells, as determined by Western blotting. E, Invasive activity of LoVo-C cells was enhanced in a dose-dependent manner of rCCL26, which was added into the TAM culture medium for 24 hours during coculturing with LoVo-C cells. F, The protein levels of IL6, IL8, KCNN4, and p-GSK-3β were significantly increased in a dose-dependent manner in TAMs after adding rCCL26 into the TAM culture medium for 24 hours during coculturing with LoVo-P cells. G, CCL26-targeted siRNAs were transfected into LoVo-P cells, an anti-CCL26 antibody was used to neutralize residual CCL26 function, and CCR3-targeted siRNAs were transfected into TAMs. The invasiveness of LoVo-P cells after coculturing with TAMs for 24 hours, as determined by invasion assays. H and I, The protein levels of IL6, IL8, KCNN4, and p-GSK-3β were lower than the control group in TAMs after transfection with CCR3-targeted siRNAs during coculturing with LoVo-P and HT29-C cells. GAPDH used as a loading control. Results are presented as the average of quadruplicate measurements, and the bar is the SD (n = 3). *, P < 0.05 and **, P < 0.01.
Figure 5.
IHC staining and the prognostic value of CCL26, CCR3, and TAMs in patients with colorectal cancer. A, CCL26, CCR3, and TAM staining in colorectal cancer tissue of stroma from stage I to IV, including the tissue of liver metastasis. B, The percentage of cells containing CCL26, CCR3, and CD68 staining in stroma, and the levels of these proteins were upregulated in stage III and IV. C, Kaplan-Meier survival curve of patients with colorectal cancer with negative staining (n = 47) and positive staining (n = 36, P < 0.01) of TAMs. D, Kaplan-Meier survival curve of patients with colorectal cancer with both negative staining (n = 41) and both positive staining (n = 30, P < 0.001) of TAMs and CCL26.
Vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and transforming growth factor-β (TGF-β) have been found to induce TAMs in colorectal cancer. Many cytokines have been identified as potential mediators of TAM infiltration, including CCL26 and CCR3. In the current study, we found that CCL26 induced TAM infiltration in colorectal cancer cell lines with overexpressed (LoVo-P cells) and silenced PRL-3 (LoVo-shPRL-3). The tumor volumes and weights were both significantly higher in mice injected with LoVo-P, HT29-C cells (Supplementary Fig. S4, $P < 0.01$).

Moreover, we analyzed PRL-3 and CCL26 expression in tumor tissue by Western blot analysis and IHC, and the results showed that the expression levels of PRL-3 and CCL26 were significantly higher in the LoVo-P than in the LoVo-C, and HT29-C than in the HT29-shPRL-3 xenografts (Fig. 6C–E and G). Furthermore, LoVo-P and HT29-C xenografts displayed high expression of CCL26 and CCR3 as well as an increased number of TAMs in the tumor stroma (Fig. 6F and H, $P < 0.001$). These results showed that all of these proteins were more highly expressed in the LoVo-P and HT29-C xenografts. Therefore, the infiltration of TAMs induced by CCL26 was regulated by PRL-3.

**Discussion**

Liver metastasis is a major organ for colorectal cancer metastasis, and PRL-3 is highly expressed in colorectal cancer liver metastases (23). Increasing numbers of studies have found that the complicated intercellular signaling network between tumor cells and the tumor microenvironment is essential for promoting tumor proliferation, angiogenesis, and metastasis (9, 13, 24). However, the vital role of TAMs, which regulate tumor cell proliferation and invasion, is more important (25). TAM infiltration in the tumor stroma is a negative prognostic factor that positively correlates with proliferation and invasion (26). Our previous studies found that secretion of IL6 and IL8 by TAMs enhances LoVo colon cancer cell invasion via PRL-3 (16). In the current study, we found that CCL26 mRNA was upregulated in LoVo-P cells and that CCL26 may participate in cell migration using human mRNA arrays and GO analysis. Then, we showed that CCL26 was upregulated in LoVo-P and HT29-C cells, both of which highly express PRL-3. Importantly, we found that PRL-3 expression correlates with CCL26 expression in colorectal cancer, and high-level protein expression of PRL-3 and CCL26 in colorectal cancer is associated with a worse prognosis. Moreover, we found that CCL26 induced TAM infiltration and mobilized intracellular Ca$^{2+}$ by binding to the cell surface CCR3 chemokine receptor. In turn, KCNN4 and p-GSK-3β are activated in TAMs to promote IL6 and IL8 production, which enhances the invasive activity of colon cancer cells. In addition, IHC analysis indicated that elevated CCL26, CCR3, and TAM levels in the tumor stroma were significantly associated with higher TNM stage in colorectal cancer. Finally, similar results were observed in vivo. Together, these data suggested that CCL26 participated in PRL-3 enhancing colorectal cancer invasion by induced TAM infiltration.

CCL26 is a small cytokine belonging to the CC chemokine family and is also known as eotaxin-3 (27). Many cytokines have been found to induce TAM infiltration, including CCL2, CCL5, VEGF, GM-CSF, and TGFβ (28–30). Interestingly, CCL26 mobilizes intracellular Ca$^{2+}$ by binding to the CCR3 receptor on the cell surface to promote migration (18). In the current study, we found that PRL-3 promoted colorectal cancer cell secretion of CCL26 in cell lines with overexpressed (LoVo-P) cells and silenced PRL-3 (HT29-shPRL-3). We also explored the expression of PRL-3 and CCL26 in stage I–IV colorectal cancer tissues and analyzed the correlation of PRL-3 and CCL26 expression using Spearman rank test, which indicated that PRL-3 also upregulated the expression of CCL26. Importantly, elevated PRL-3 and CCL26 levels significantly correlated with lymph node metastasis, distant metastasis, poorly differentiated tumor, and a high TNM stage. Kaplan–Meier survival analysis suggested that the increased expression of PRL-3 or/and CCL26 was associated with a short postsurgical OS for colorectal cancer patients.

CCL26 is expressed in several tissues, including the heart, lung, and ovaries, and endothelial cells stimulated with IL4 (31, 32). However, little is known regarding CCL26 signaling in cancer, except for studies in liver cancer (33). In the current study, we showed that CCL26 induced TAM infiltration, and treatment with siRNA confirmed the effect of CCL26. More importantly, the proposed interaction of CCL26 and CCR3 was analyzed through confocal microscopy. We observed that CCR3, which is expressed on the membrane of TAMs, interacted well with CCL26. These results indicated that CCL26 induced TAM infiltration through interacting with CCR3 to establish the tumor microenvironment.

Additional studies have suggested that the tumor microenvironment is an essential participant in the neoplastic process, as it promotes proliferation, survival, and metastasis (34). Song et al. observed that TAMs produce CCL18 to promote breast cancer cell invasiveness (35). In a previous study, we found that IL6 and IL8 released from TAMs enhance LoVo cell invasion (16). These findings indicate that the interaction between cancer cells and TAMs via cytokines plays an important role in the neoplastic process. In further exploring the effect of the interaction between CCL26 and the CCR3 receptor on the TAM production of IL6 and IL8, the data revealed that CCL26 binds to CCR3 on the cell membrane of TAMs to upregulate KCNN4 and p-GSK-3β. The key result is the enhanced invasiveness of LoVo and HT29 cells through coculturing with TAMs, and this effect was diminished after transfection with siRNAs targeting either CCL26 in LoVo-P and HT29-C cells or CCR3 in TAMs. Meanwhile, the invasive activity of LoVo-P cells was further enhanced by the addition of rCCL26 into TAM culture medium during coculture with LoVo-P cells. Among these processes, the expression of IL6 and IL8 in TAMs was consistent with the protein levels of KCNN4 and p-GSK-3β. A previous study reported that the phosphorylation of GSK-3β was mediated by Ca$^{2+}$/calmodulin–dependent protein kinase II (36). Furthermore, CCL26 can mobilize intracellular Ca$^{2+}$ by binding to the CCR3 receptor (18). These findings suggest that the interaction between CCL26 and CCR3 mobilizes Ca$^{2+}$ in TAMs to produce IL6 and IL8, which enhances the invasive activity of LoVo and HT29 cells.

We also observed that the expression levels of CCL26 and CCR3 and the number of TAMs were increased in the stroma of advanced stages of colorectal cancer and exhibited colocalized expression. This result further illustrated that CCL26 induced TAM infiltration. Taken together, all of these markers were significantly associated with the TNM stage, lymph node metastasis, and tumor differentiation. The actual clinical significance of PRL-3, CCL26, and TAMs for patients was with regard to overall survival. Kaplan–Meier analysis suggested that positive staining of PRL-3, CCL26, and TAMs was associated with a shorter postsurgical OS for colorectal cancer patients. These data indicate that PRL-3 is an independent indicator of OS and overall survival. It is important to note that these findings suggest that targeting PRL-3 and CCL26 may provide a promising therapeutic strategy for the treatment of colorectal cancer.
Figure 6.
CCL26 induces M2 macrophage infiltration by interacting with CCR3 in vivo. A and B, The tumors from the group inoculated with LoVo-P cells, LoVo-C and HT29-C, and HT29-shPRL-3 cells. C and D, The expression levels of PRL-3 and CCL26 in LoVo-P cells and LoVo-C cells, HT-29-C, and HT29-shPRL-3 xenografts, as determined by Western blotting. E and G, The expression levels of PRL-3 and CCL26 in tumor tissue analyzed by IHC. F and H, The expression levels of CCL26, CCR3, and TAMs in tumor stroma determined by IHC. I and J, The expression status of PRL-3 and CCL26 was determined by IHC in vivo, and the percentage of cells containing CCL26, CCR3, and CD68 staining in stroma. GAPDH used as a loading control. Results are presented as the average of quadruplicate measurements, and the bar is the SD (n = 6). **, P < 0.01; *** P < 0.001.
plays a crucial role in CCL26-induced TAM infiltration in surrounding tumor tissues, likely reflecting the association of PRL-3 with colorectal cancer metastasis (23). In addition, CCL26-induced TAM infiltration was validated in vivo by introducing the LoVo-P and LoVo-C cells, HT29-C, and HT29-shPRL-3 in an *in vivo* xenograft mouse model.

The results showed that monocYTE macrophages could be polarized into different subtypes according to the signaling molecules in the environment (37). Previous studies have shown that macrophages exposed to LPS and IFNγ transform into M1 macrophages, and exposure to IL4 and IL13 results in M2 macrophages (38). The secretion of CCL26 is upregulated in intestinal epithelial cells through the IL4- and IL13-mediated activation of STAT6 signaling (39). Whether a vicious cycle in intestinal epithelial cells through the IL4- and IL13-mediated activation of STAT6 signaling (39) is crucial in the tumor microenvironment exists between TAMs and colorectal cancer is unknown and deserves further attention in future studies. In summary, these data suggest that CCL26 participate in PRL-3-induced promotion of colorectal cancer invasion by inducing TAM infiltration by binding to CCR3 in TAMs. The interaction between CCL26 and CCR3 mobilizes Ca2+ in TAMs to produce IL6 and IL8, which enhances the invasive activity of LoVo and HT29 cells. Furthermore, elevated PRL-3, CCL26, and TAMs are associated with a worse prognosis in colorectal cancer patients. Of these results, PRL-3 is the pivotal molecule. Therefore, PRL-3 may be a useful prognostic marker and/or an effective therapeutic target for colorectal cancer patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

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