

C-C motif chemokine ligand 14 inhibited colon cancer cell proliferation and invasion through suppressing M2 polarization of tumor-associated macrophages

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Summary. Background. Colon cancer is one of the most common cancers with high incidence and high mortality. Chemokines play a crucial role in the development of cancer.

Methods. Here, qRT-PCR was performed to detect gene expression. Western blot and immunohistochemistry were implemented to examine the expression of C-C motif chemokine ligand 14 (CCL14) in colon tumors. Besides, the expression of CD68 and CD206 in tumors was measured by immunohistochemistry. The percentages of M1- and M2-polarized macrophages were detected by flow cytometry. Furthermore, CCK-8 assay was performed to detect cell proliferation, and Transwell assay for cell invasion.

Results. CCL14 was decreased in both colon tumors and colon cancer cells, and many tumor-associated macrophages (TAMs) infiltrated into the tumor. An increase CCL14 inhibited colon cancer cell proliferation. Importantly, CCL14 promoted THP-1 to M1 polarization induced by LPS and IFN- γ , and inhibited THP-1 to M2 polarization induced by IL-4 and IL-13. Besides, CCL14 enhanced the inhibition of M1-polarized macrophages to colon cancer cell proliferation and invasion, and reversed the promotion of M2-polarized macrophages to cell proliferation and invasion.

Conclusion. Our data demonstrated that CCL14 inhibited the proliferation and invasion of colon cancer cells through suppressing the formation of M2-like TAMs.

Key words: Colon cancer, C-C motif chemokine ligand 14, Tumor-associated macrophages, Cell proliferation, Cell invasion

Introduction

Colon cancer is one of the most common cancers globally. According to the statistics reported by the American Cancer Society, the mortality rate of colon cancer is up to 9.2%, and the incidence rate of the disease is up to 10.2%. Colon cancer is the second leading cause of death in the world (Wen et al., 2019). Genetics, the environment, diet, host immunity and microbial exposures affect the occurrence and development of colon cancer (O'Keefe, 2016). At present, the main therapeutic method of the patients with colon cancer still is surgical ablation. However, most colon cancer patients are diagnosed at a late stage. At that stage, the cancer cells already have metastasized to other positions, so surgical ablation for patients is no longer valid (Ma et al., 2017). Hence, it is very important to find a new therapeutic target for colon cancer.

Non-resolving inflammation induced by cancer cells in the tumor microenvironment is an important characteristic of cancer. Immune cells coinhabit with cancer cells in all stages of cancer progression (Petty and Yang, 2019). In the tumor microenvironment, infiltrated macrophages, also defined as tumor-associated macrophages (TAMs), are a crucial component of tumor stroma (Lin et al., 2019). During the progression of cancer, cancer cells produce multiple types of cytokines and chemokines which stimulate the formation of TAMs from blood monocytes. TAMs are phenotypically similar

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to M2-polarized macrophages (Kovaleva et al., 2016). Generally, TAMs contribute to the progression of cancer, and are associated with a poor prognosis and outcome of cancer (Zhao et al., 2017). It was reported that TAMs could facilitate the migration and invasion of colon cancer cells through transferring exosomes to cancer cells (Lan et al., 2019). In addition, homeoprotein six1 was highly expressed in colon cancer cells, and it promoted the progression of colon cancer through recruiting TAMs (Xu et al., 2017). Ovatodiolide could effectively impede the growth of colon cancer tumors via inhibition of TAMs formation by regulating the Yes-associated protein pathway (Huang et al., 2017). More and more studies have indicated that the crosstalk between TAMs and colon cancer cells is important for the development of the disease.

Chemokines are important regulators in cancer progression, and participate in the regulation of cell apoptosis, migration, proliferation, invasion and metastasis in cancer (Zhu et al., 2019). Also, chemokines contribute to the activation of immune cells. C-C motif chemokine ligand 14 (CCL14) is a member of the chemokine family. It was reported that CCL14 is lowly expressed in many types of cancer, such as hepatocellular carcinoma, ovarian cancer, bladder cancer, gastric cancer and colorectal cancer (Cai et al., 2020; Gu et al., 2020). Importantly, the lowly expressed CCL14 was associated with the infiltration level of macrophages in 19 types of cancer (Gu et al., 2020). It was indicated in multiple myeloma that CCL14 promotes the proliferation and polarization of macrophages, and stimulates the bone marrow homing of macrophages (Li et al., 2015). However, the role and action mechanism of CCL14 in colon cancer progression is still unclear.

In the present study, we found that CCL14 was decreased in human colon cancer tissues. CCL14 effectively inhibited the proliferation and invasion of colon cancer cells by promoting macrophages to M1 polarization and inhibiting macrophages to M2 polarization. Our data demonstrated that CCL14 may be a novel target for colon cancer treatment.

Materials and methods

Clinical samples

A total of 30 cases of tumor tissues and 30 cases of matched peritumoral tissues were obtained from colon cancer patients who underwent surgical resection in the First Affiliated Hospital of Harbin Medical University. All experiments were approved by the Institutional Ethics Committee of the hospital, and all patients signed the informed consent before enrolment. All tissues were immediately frozen in liquid nitrogen until use.

QRT-PCR assay

For qRT-PCR assay, TRIzol reagent (Invitrogen,

Carlsbad, CA, USA) was used to isolate total RNA from tissues and colon cancer cells. Next, cDNA was synthesized using a Reverse ace qPCR RT Kit (Toyobo, Osaka, Japan). Next, real-time PCR was carried out using SYBR Green Real Time PCR Master Mixes (Takara, Dalian, China). All experiments were performed in accordance with the specific manufacturer's protocol. GAPDH served as the internal reference of *CCL14*, *iNOS*, *IL-1 β* , *TNF- α* , *Arg-1*, *IL-10* and *TGF- β 1*. The relative expression of mRNAs was calculated according to the method of $2^{-\Delta\Delta Ct}$.

Western blot assay

For western blotting assay, RIPA lysis buffer (Beyotime, Jiangsu, China) was utilized to isolate total protein from tissues. Next, 25 μ g of protein sample was separated by 12% SDS-PAGE, and then was transferred into PVDF membrane (Millipore, MA, USA). Subsequently, the membrane was incubated with 5% non-fat milk for one hour at room temperature, and then was incubated with primary antibody against CCL14 (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. Next day, the membrane was maintained with secondary antibody for one hour at room temperature. Finally, an ECL Western Blotting Substrate (Solarbio, Beijing, China) was used to visualize the protein bands, and Image J software was used to analyze the relative expression of CCL14.

Immunohistochemistry assay

Immunohistochemistry assay was carried out to detect the expression of CCL14, CD68 and CD206 in tissues. Firstly, human tissues were embedded into paraffin, and then were cut into 4 μ m thick sections. Next, the sections were deparaffinized and treated with 0.3% H_2O_2 . After that, sections were incubated with 0.01 M sodium citrate for 10 min, and then were incubated with primary antibodies against CCL14 (1:200), CD68 (1:200, Abcam) and CD206 (1:200, Abcam) at 4°C overnight. Next day, a streptavidin-biotin-peroxidase complex kit (Lab Vision, Fremont, CA, USA) was utilized to examine the protein in accordance with the manufacturer's protocols. Finally, the sections were developed by usage diaminobenzidine substrate (Sigma-Aldrich), and dyed with hematoxylin.

THP-1 culture and treatment

Human monocyte cell THP-1 was purchased from the American Type Culture Collection (ATCC; USA). RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, UK), 100 μ g/ml streptomycin (Sigma) and 100 U/mL penicillin (Sigma) was used to culture THP-1 cells. In our study, in order to get M1-polarized macrophages, THP-1 was treated with 20 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma), 10 ng/ml lipopolysaccharide (LPS; Sigma) and

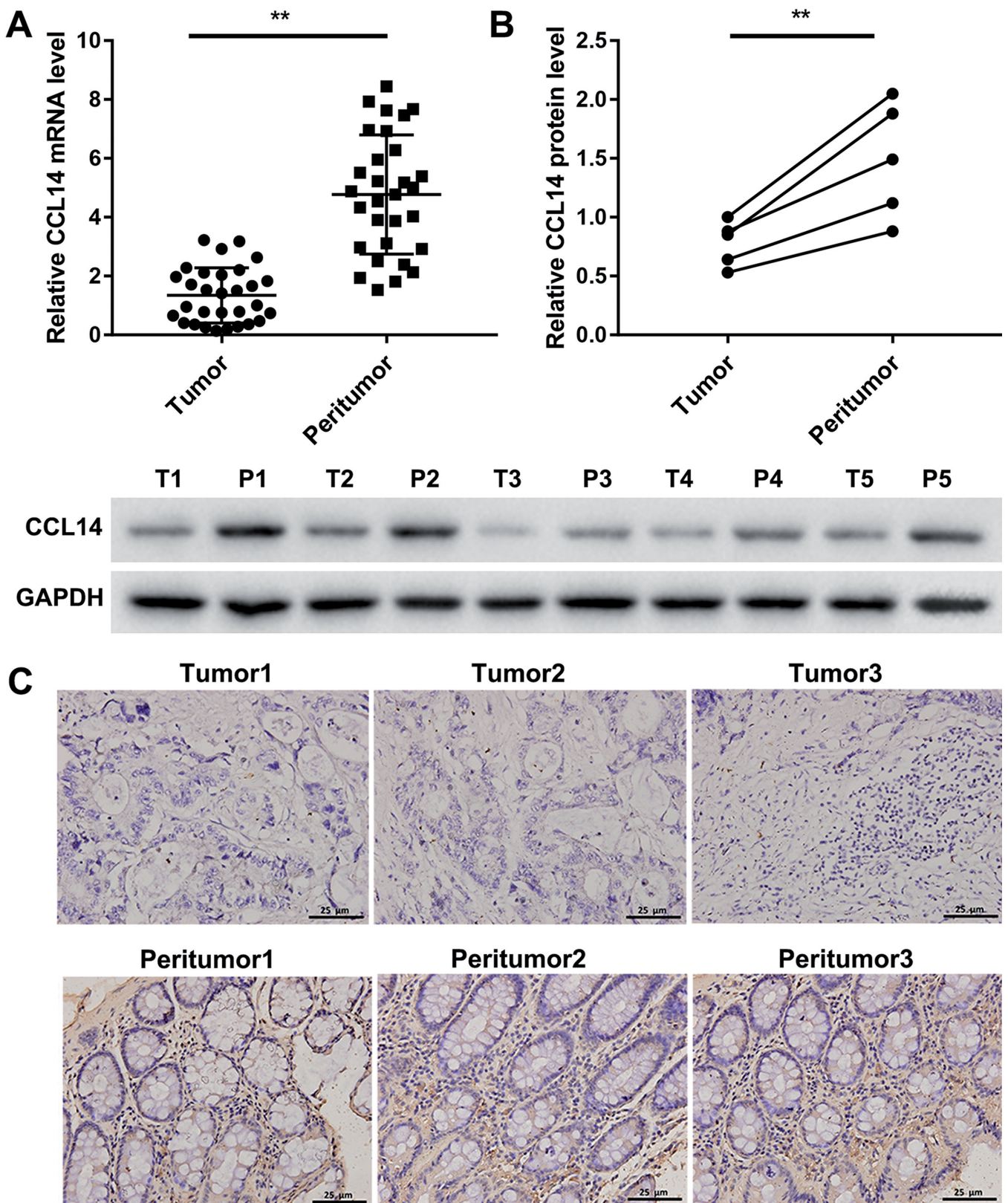


Fig. 1. Detection of CCL14 expression in colon cancer tissues and matched normal tissues. **A.** The expression of CCL14 mRNA in colon cancer tissues and matched peritumoral tissues was measured by using qRT-PCR. $n=30$. $**P<0.01$ compared with Tumor group. **B.** The expression of CCL14 protein in 5 cases of tumor tissues and peritumoral tissues was detected by using western blotting assay. $n=5$. $**P<0.01$ compared with Tumor group. **C.** Three cases of tumor tissues and peritumoral tissues were selected, and were then used to examine CCL14 expression by immunohistochemistry assay. $n=3$.

20 ng/ml IFN- γ (PeproTech, Offenbach, Germany) for 48 hours. In order to get M2-polarized macrophages, THP-1 was treated with 20 ng/ml PMA, 20 ng/ml IL-4 (PeproTech) and 20 ng/ml IL-13 (PeproTech) for 48 hours. 1 μ g/ml of CCL14 (Human) recombinant protein (AmyJet Scientific, Wuhan, China) was used to stimulate THP-1.

Flow cytometry assay

Flow cytometry was performed to examine the percentages of M1- and M2-polarized macrophages. THP-1 was incubated with monoclonal antibodies (FITC-labeled CD86 and PE-labeled CD206) in the dark for 30 min at 4°C. Then, the stained cells were analyzed by using a FACSCalibur flow cytometer (BD Biosciences).

Treatment of colon cancer cells

Human intestinal epithelial cell line FHC and colon cancer cell lines (HCT116 and SE480) were obtained from ATCC. A 1:1 mixture of DMEM and F12 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin was utilized to culture the colon cancer cells. To explore the effect of CCL14 on colon cancer cells, M1- and M2-polarized macrophages were treated with CCL14 recombinant protein. Next, the conditional cell culture medium of each group of macrophages were collected, and then were used to culture colon cancer cells for 24 hours.

CCK-8 assay

CCK-8 assay was carried out to examine the proliferation of colon cancer cells. Colon cancer cells at a density of 5×10^3 cells/well were planted into 96-well plates, and macrophage-conditional medium was added into the well. At 24 hours after conditional incubation, 10 μ l of CCK-8 solution (Dojindo Molecular Technologies, Inc., Shanghai, China) was added into the well. Next, the cells were maintained with CCK-8 solution for 2 hours at 37°C. Finally, the absorbance values at 450 nm were examined by using a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.).

Transwell assay

Transwell assay was carried out to detect the invasion of colon cancer cells. Colon cancer cells at a density of 1×10^5 cells/well were planted into the upper chamber of a transwell. A 1:1 mixture of the conditional medium of macrophages and serum-free DMEM/F12 medium was used to culture colon cancer cells. At the same time, DMEM/F12 medium containing 10% FBS was added into the lower chamber, 24 hours later, invaded cancer cells were stained with crystal violet, and then the number of invaded cancer cells were counted.

Statistical analysis

All data were analyzed using GraphPad Prism 7.0 software (GraphPad Software, Inc.), and are presented as mean \pm standard deviation (SD). The differences between the two groups were examined using Student's t-test, and one-way analysis of variance (ANOVA) was used for the analysis of multiple groups. A value of P lower than 0.05 was considered as statistically significant.

Results

CCL14 was decreased in human colon cancer tissues

Here, 30 cases of tumor tissues and matched peritumoral tissues were detected in our study. Our results demonstrated that expression of CCL14 was decreased in tumor tissues when compared with peritumoral tissues (Fig. 1A). Consistently, western blotting assay showed that expression of CCL14 also was decreased in tumor tissues when contrasted with peritumoral tissues (Fig. 1B). Besides, we also measured the expression of CCL14 in tissues using immunohistochemistry assay. The results also indicated a lowly expressed CCL14 in colon cancer tissues (Fig. 1C). Moreover, our results showed that expression level of CCL14 mRNA was lower in colon cancer cells than that in control cells (Fig. 2A). An increase of CCL14 in colon cancer cells suppressed cell proliferation of both SW480 (Fig. 2B) and HCT116 (Fig. 2C). Overall, CCL14 was notably downregulated in colon cancer tissues and cell lines.

M2-like TAMs infiltrated into tumor tissues

It was reported that M2-like TAMs contribute to the progression of cancer (Yin et al., 2019). Here, we measured the infiltrated TAMs in colon cancer tissues and matched peritumoral tissues. Our data indicated that a large number of TAMs infiltrated in tumor tissues when compared with the matched peritumoral tissues. Importantly, a lot of M2-like TAMs infiltrated in tumor tissues when contrasted with the match peritumoral tissues (Fig. 3).

CCL14 promoted macrophages to M1 polarization and inhibited M2 polarization

To explore the role of CCL14 in TAMs polarization, PMA was used to stimulate the differentiation of THP-1 into macrophages. Meanwhile, LPS combined with IFN- γ were used to induce M1 polarization of macrophages, and IL-4 combined with IL-13 were used to induce M2 polarization of macrophages. In the present study, we found that LPS and IFN- γ increased the expression of iNOS, IL-1 β and TNF- α mRNAs in macrophages, and the promotion of LPS and IFN- γ to these factors expression was further enhanced by CCL14 treatment

(Fig. 4A). In IL-4 combined with IL-13-stimulated macrophages, the expression of Arg-1, IL-10 and TGF- β 1 mRNAs was obviously upregulated, while the promotion of IL-4 and IL-13 to these factors expression was partly reversed by CCL14 treatment (Fig. 4B). In addition, flow cytometry results showed that LPS and IFN- γ significantly increased the cell number of CD86-positive macrophages, and CCL14 treatment contributed to the formation of CD86-positive macrophages (Fig. 4C,E). Furthermore, IL-4 and IL-13 obviously upregulated the cell number of CD206-positive macrophages, which was then partly rescued by CCL14 treatment (Fig. 4D,F). In summary, CCL14 promoted M1 polarization of macrophages, while it inhibited M2 polarization.

CCL14 inhibited colon cancer cell proliferation and invasion

Next, to investigate whether CCL14 suppresses colon cancer cell proliferation and invasion via promoting TAMs to M1 polarization and inhibiting TAMs to M2 polarization, we collected the cell culture supernatant of M1- and M2-polarized macrophages, which treated with or without CCL14. Subsequently, colon cancer cells were conditional cultured with the cell culture supernatant of macrophages. Our data showed that the cell culture supernatant of M1-polarized macrophages notably inhibited proliferation of HCT116 and SW480, and CCL14 treatment further enhanced the effect of M1-polarized macrophage (Fig. 5A,E).

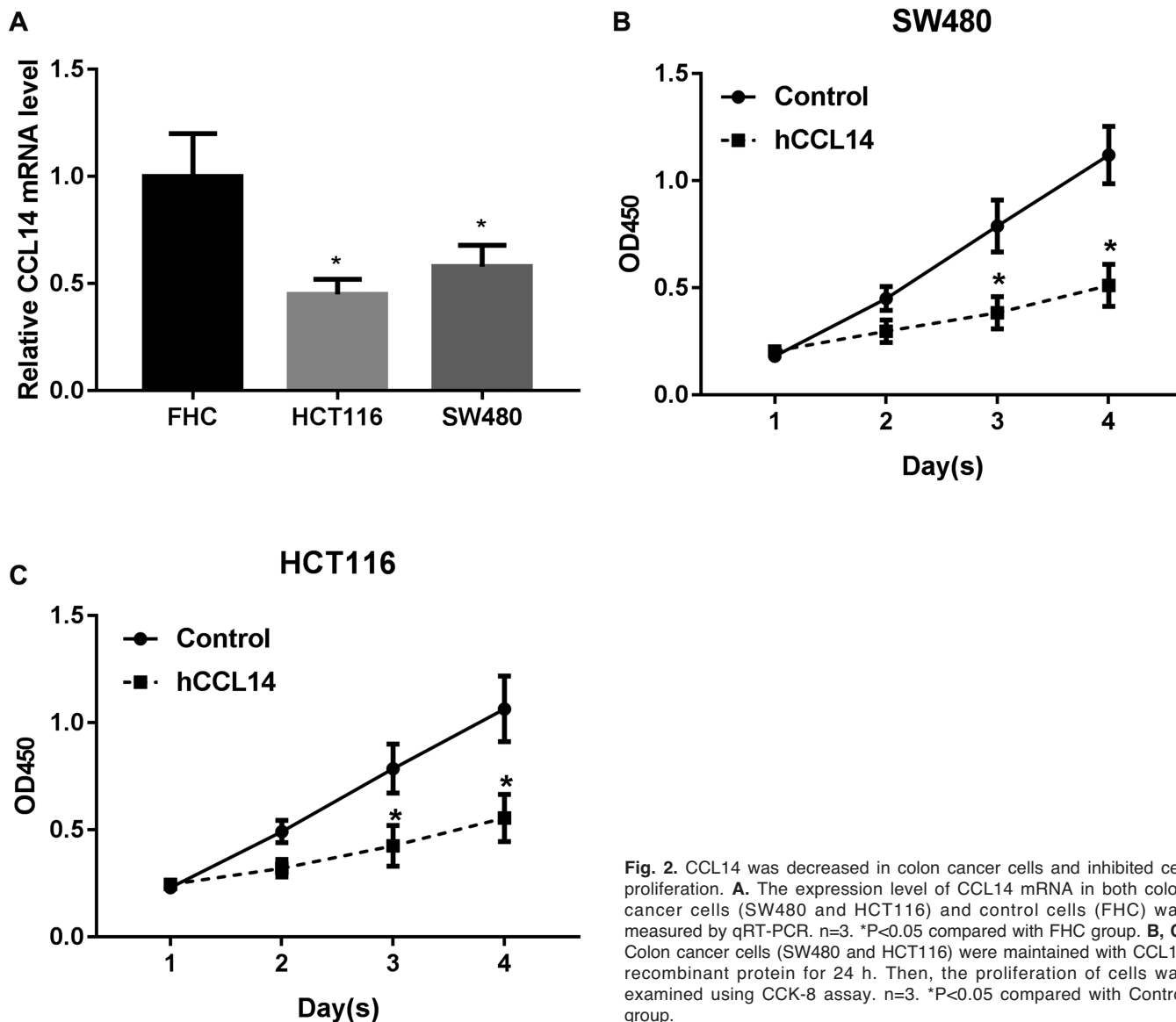


Fig. 2. CCL14 was decreased in colon cancer cells and inhibited cell proliferation. **A.** The expression level of CCL14 mRNA in both colon cancer cells (SW480 and HCT116) and control cells (FHC) was measured by qRT-PCR. $n=3$. * $P<0.05$ compared with FHC group. **B, C.** Colon cancer cells (SW480 and HCT116) were maintained with CCL14 recombinant protein for 24 h. Then, the proliferation of cells was examined using CCK-8 assay. $n=3$. * $P<0.05$ compared with Control group.

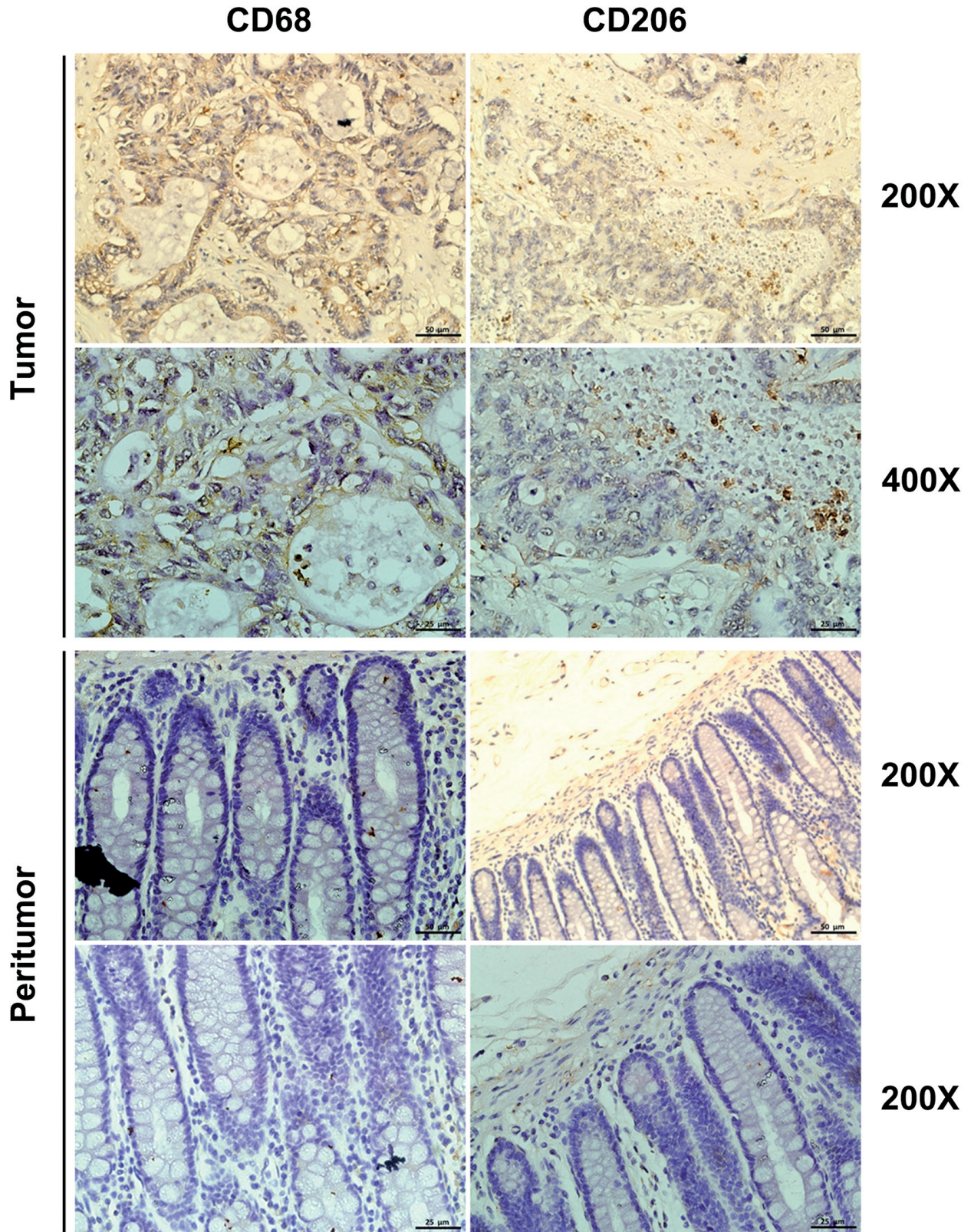
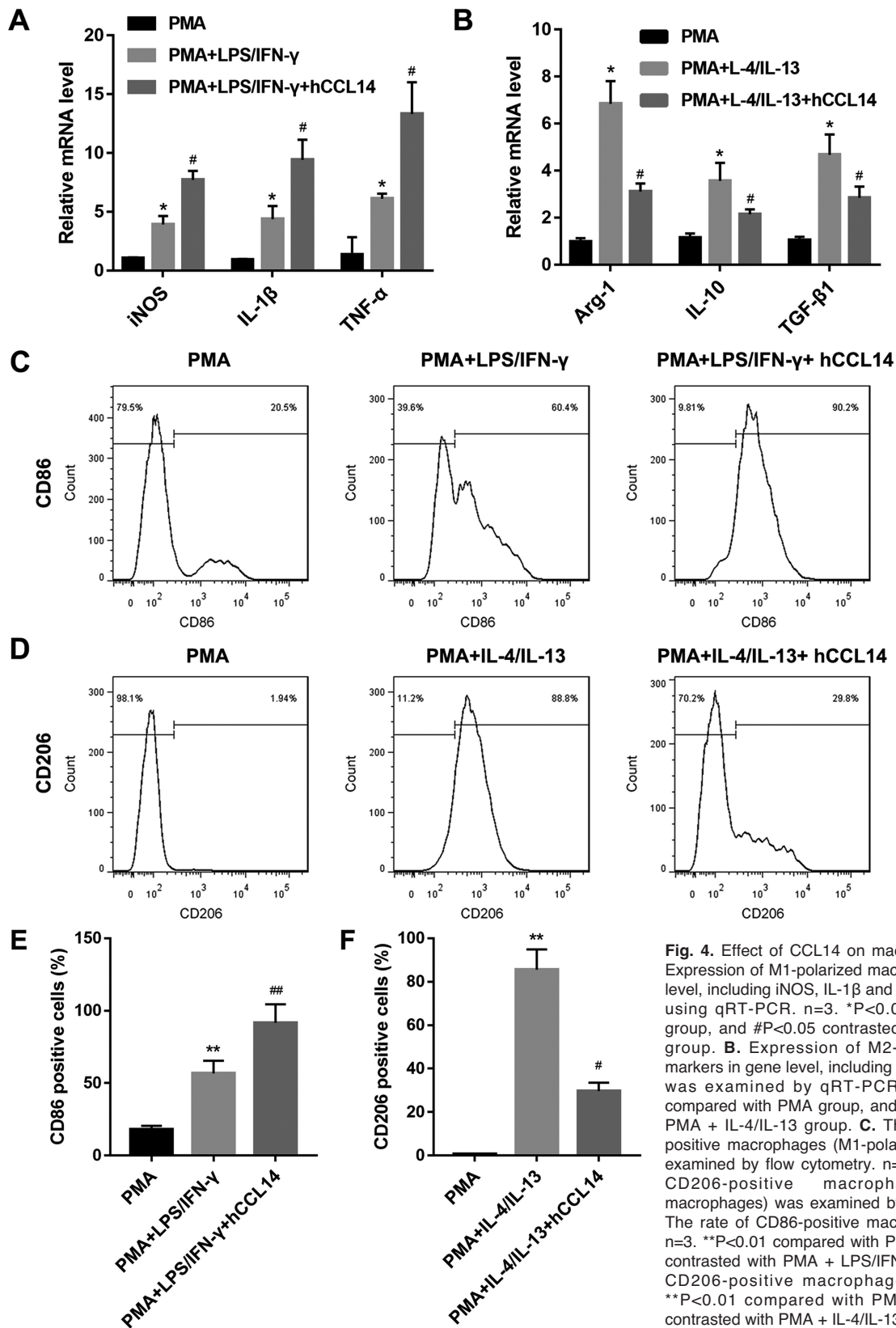


Fig. 3. M2-polarized TAMs in colon cancer tissues. Immunohistochemistry assay was performed to detect the cell number of TAMs and M2-like TAMs in colon cancer tissues and matched peritumoral tissues. CD68 was used to mark TAMs, and CD206 was utilized to mark M2-like TAMs. $n=3$. Scale bar: 50 μm .

The role of CCL14 in colon cancer



Consistently, the cell culture supernatant of M1-polarized macrophage obviously suppressed invasion of HCT116 and SW480, and CCL14 treatment further enhanced the effect of M1-polarized macrophages (Fig. 5B,F). Moreover, the cell culture supernatant of M2-polarized macrophages could significantly facilitate proliferation of HCT116 and SW168, while this effect was partly reversed by CCL14 treatment (Fig. 5C,G). In addition, the cell culture supernatant of M2-polarized macrophages promoted invasion of HCT116 and

SW168, which also was partly rescued by CCL14 treatment (Fig. 5D,H). Overall, CCL14 inhibited the proliferation and invasion of colon cancer cells through promoting macrophages to M1 polarization and inhibiting M2 macrophages.

Discussion

Chemokines belong to secreted cytokines, which are a larger family of small molecules. Chemokines can be

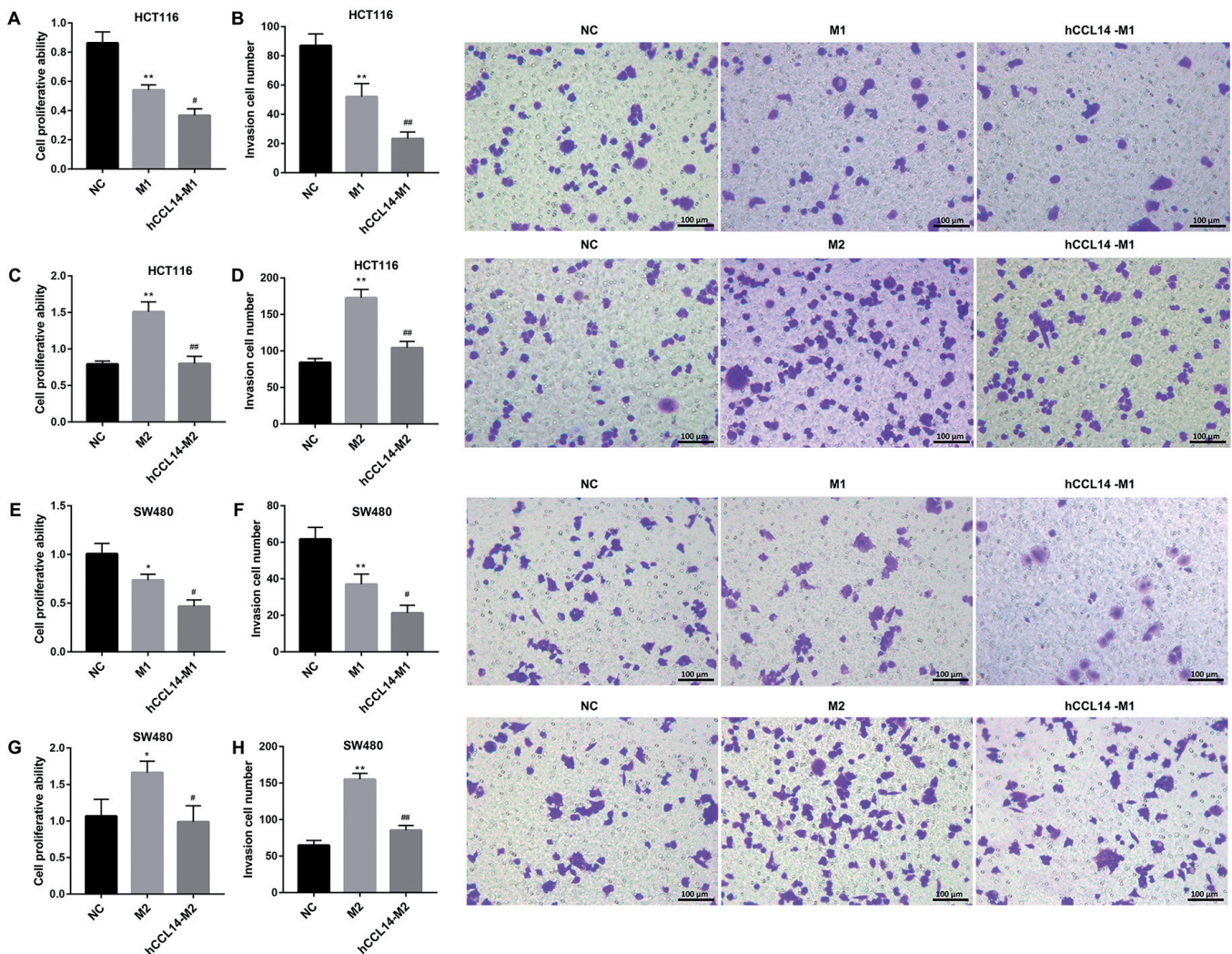


Fig. 5. Effect of CCL14 on colon cancer cell proliferation and invasion. Colon cancer cells, HCT116, were cultured with the cell culture supernatant of M1-polarized macrophages treated with or without human recombinant CCL14. **A.** Cancer cell proliferation was measured by using CCK-8 assay. **B.** Cancer cell invasion was examined by Transwell assay. $n=3$. $**P<0.01$ compared with NC group, and $\#P<0.05$ and $\#\#P<0.01$ contrasted with M1 group. Colon cancer cells, HCT116, were cultured with the cell culture supernatant of M2-polarized macrophages treated with or without human recombinant CCL14. **C.** Cell proliferation was measured by CCK-8 assay. **D.** Transwell assay was performed to examine the invasion of cancer cells. $n=3$. $**P<0.01$ compared with NC group, and $\#\#P<0.01$ contrasted with M2 group. Colon cancer cells, SW480, were cultured with the cell culture supernatant of M1-polarized macrophages treated with or without human recombinant CCL14. **E.** Cancer cell proliferation was measured by using CCK-8 assay. **F.** Cancer cell invasion was examined by Transwell assay. $n=3$. $*P<0.05$ and $**P<0.01$ compared with NC group, and $\#P<0.05$ contrasted with M1 group. Colon cancer cells, SW480, were cultured with the cell culture supernatant of M2-polarized macrophages treated with or without human recombinant CCL14. **G.** Cell proliferation was measured by CCK-8 assay. **H.** Transwell assay was performed to examine the invasion of cancer cells. $n=3$. $*P<0.05$ and $**P<0.01$ compared with NC group, and $\#P<0.05$ and $\#\#P<0.01$ contrasted with M2 group.

divided into four subfamilies, including XC, CX₃C, CXC and CC (Hughes and Nibbs, 2018). It was reported that chemokines play a crucial role in the development of multiple disorders, especially in cancer progression. In the tumor microenvironment, chemokines can be released by cancer cells, immune cells and other cells (Nagarsheth et al., 2017). Ma et al. indicated that fibroblasts were able to facilitate the proliferation and invasion of colon cancer cells via regulation of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) expression through secreting chemokine CXCL12 (Ma et al., 2019). Villarreal et al. reported that chemokine CCR8 is increased in Treg cells, and CCR8-positive Treg cells notably promote the progression of cancer. They further demonstrated that inhibition of CCR8 may be a novel treatment method of colon cancer (Villarreal et al., 2018). CCL14 is a member of the CC subfamily. It was reported that CCL14 is decreased in hepatocellular carcinoma, and it is closely associated with poor prognosis (Zhang et al., 2017). In the present study, we found that the expression of CCL14 was significantly downregulated in the tumors of human colon cancer and colon cancer cells.

Macrophages are important immune cells in cancer progression. Because they are highly responsive to the microenvironment, macrophages can differentiate into two types: M1-polarized macrophage and M2-polarized macrophages (Sica and Mantovani, 2012). It was proved that activated M1-polarized macrophages produce pro-inflammatory factors, such as IL-1 β , IL-6, IL-33 and IL-13. Oppositely, alternatively activated M2-polarized macrophages produce anti-inflammatory factors, including IL-10 and TGF- β 1 (Kim and Bae, 2016). Within different microenvironments, M1-polarized macrophages can be activated by LPS combined with IFN- γ , and M2-polarized macrophages can be activated by IL-4 combined with IL-13 (Petty and Yang, 2019). M2-like macrophages in the tumor microenvironment were also called TAMs. During cancer development, TAMs facilitated angiogenesis, proliferation, migration and invasion of cancer cells and the resistance of cancer cells to therapy (Petty and Yang, 2017). In colon cancer, it was reported that TAMs promote the growth of colon cancer tumor (Karagiannidis et al., 2020). Here, our data showed that a large number of TAMs infiltrated into the tumors from colon cancer patients.

THP-1, a human monocyte cell, is widely used in the study of macrophage. For differentiation into macrophages, THP-1 is stimulated with PMA (Gatto et al., 2017). In the present study, we utilized PMA to stimulate THP-1. At the same time, THP-1 was co-treated with LPS and IFN- γ to induce M1 polarization, or co-treated with IL-4 and IL-13 to induce M2 polarization. It was demonstrated that chemokines are pro-inflammatory or anti-inflammatory, and are involved in the regulation of the migration and residence of immune cells (Palomino and Marti, 2015). Besides, chemokines were found to regulate macrophage polarization under different physiological and

pathological conditions. For instance, in multiple myeloma, CCL14 was proved to induce macrophage polarization into multiple myeloma-associated macrophages (Ruytinx et al., 2018). Here, we found that CCL14 human recombinant protein promoted the formation of M1-polarized macrophages and inhibited M2-polarized macrophages formation. The crosstalk between TAMs and colon cancer cells plays an important role in the development of colon cancer. In the tumor microenvironment, excessive TAMs infiltration can accelerate the progression of colon cancer (Zhang et al., 2018). In recent years, Kimura et al. reported that dihydroxystilbenes could obviously inhibit the progression of colon cancer via repressing the formation of M2-like TAMs by suppressing the production of chemokine (Kimura et al., 2020). In our study, we indicated that M1-polarized macrophages obviously inhibited the proliferation and invasion of colon cancer cells, and M2-polarized macrophage promoted colon cancer cell proliferation and invasion. However, CCL14 treatment enhanced the effect of M1-polarized macrophage and mitigated the effect of M2-polarized macrophages. A previous study indicated that TAMs can protect colon cancer cell from apoptosis through targeting IL-1 β -dependent stabilization of Snail in tumor cells (Kaler et al., 2010). Lan et al. reported that TAMs can facilitate colon cancer cells migration and invasion through transferring exosomal miR-21-5p and miR-155-5p to inhibit the expression of BRG1 in cancer cells (Lan et al., 2019). However, the molecular mechanism of how CCL14-treated macrophages affect the proliferation and invasion of colon cancer cells remains unclear. We will explore this question in our further study.

Conclusion

In conclusion, our data demonstrated that CCL14 inhibited the proliferation and invasion of colon cancer cells through suppressing the formation of M2-like TAMs. Our data suggested that CCL14 may be a novel therapeutic target of colon cancer.

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Conflict of interest. The authors declare no conflict of interest.

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