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Original Article

CCL16 is a pro-tumor chemokine that recruits monocytes and macrophages to promote hepatocellular carcinoma progression

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Abstract: Intricate signaling cascades involving chemokines and their cognate receptors on neoplastic and immune constituents within tumor microenvironment have garnered substantial research interest. Our investigation delineates the contribution of Chemokine (C-C motif) ligand 16 (CCL16) to the clinico-pathological features and tumorigenesis of hepatocellular carcinoma (HCC). Analysis of 237 pairs of HCC specimens unraveled a significant association between CCL16 expression and vascular invasion, early-stage clinicopathological features, and diminished recurrence-free survival among HCC patients. Immunohistochemical (IHC) assays of the clinical HCC specimens indicated elevated CCL16 in tumorous versus normal hepatic tissues. Our *in vivo* experiments demonstrated CCL16 overexpression fostered tumor proliferation, whereas *in vitro* assays elucidated that CCL16-mediated chemotactic recruitment of monocytes and M2 macrophages was orchestrated via CCR1 and CCR5. In contrast to previous claims that CCL16 is physiologically irrelevant and has minimal affinity for its receptors (CCR1, CCR2, CCR5, CCR8), our findings unravel that inhibition of CCL16/CCR1 and CCL16/CCR5 interactions through receptor-specific antagonists markedly impeded CCL16-directed chemotaxis, migration, adhesion, and leukocyte recruitment. Moreover, CCL16-overexpression in HCCs significantly augmented levels of several cytokines implicated in tumor progression, namely IL-6, IL-10 and VEGFA. IHC analysis of CCL16-overexpressing xenografts elicited greatly enhanced levels of VEGFA and IL-6, while assessments of HCC specimens confirmed a positive correlation between CCL16 expression and IL-6 and VEGFA levels. Collectively, our study highlights oncogenic role of CCL16 in hepatocarcinogenesis and provides a foundational basis for novel therapeutic interventions targeting the CCL16/CCR1/CCR5 axis.

Keywords: HCC, CCL16, chemotaxis, macrophage

Introduction

Liver pathology encompasses a continuum of disease stages, including acute liver injury, chronic hepatitis, fibrosis/cirrhosis, and tumor progression [1]. Hepatocellular carcinomas (HCCs), the predominant histological variant of liver malignancy, arises from cirrhotic liver and accounts for 75 to 90% of HCC cases reported [2, 3]. Chronic inflammation, a hallmark of liver cirrhosis, entails the recruitment of diverse immune cell populations to sites of inflammation and neoplasia. The ability of malignant neoplasms to utilize local immune mechanisms within the tumor microenvironment (TME) to circumvent immunological effector functions, thereby evading immune surveillance, has gained increasing research recognition [4, 5].

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Macrophages, the most prevalent immune constituents within the TME, originate from monocytes and undergo differentiation into either M1- or M2-polarized macrophages, each possessing distinct functional roles in modulating tumor progression outcomes. Tumor-associated macrophages (TAMs), derived from monocytic precursors, are recruited by tumor-secreted chemokines from CXC, CC, C and CX3C subfamilies, which engage with corresponding receptors (CXCR, CCR, XCR and CX3CR) on immune cells [6-8]. Chemokines and cytokines play intricate roles in the regulation of immune responses, influencing pro- or anti-inflammatory states and neoplastic processes variably according to the TME dynamics [9, 10]. Within the TME, M1 macrophages are characterized by low IL-10 expression, elevated levels of cytokines (IL-12, IL-1β, TNFα, IL-6), along with chemokine interactions (CXCL9, CXCL10) that attract Th1 lymphocytes [11, 12]. Conversely, M2 macrophages exhibit low IL-12 and high IL-10 levels, engaging with chemokines (e.g. CCL2, 7, 8, 13, 16) that recruit regulatory T cells (Treg), Th2 cells, eosinophils, and basophils, thereby suppressing effector T cell activity and facilitating tumor proliferation and progression [13, 14].

CC motif chemokine receptors, predominantly expressed on the surface of monocytes/macrophages and lymphocytes, serve as chemotactic receptors for chemokines, directly their migration to inflammatory sites to mediate intricate intracellular signaling cascades including chemotaxis [15]. Chemokine ligand 16 (CCL16), a member from the chemokine (C-C motif) subfamily comprising 27 chemotactic cytokines, is a liver-expressed chemokine that interacts with CCR1, CCR2, CCR5, and CCR8 [16, 17]. CCL16 has been implicated in the activation of angiogenesis in vascular endothelium via CCR1 and, when released by engineered tumor cells, triggers rejection through a $CD8^+$/neutrophil-dependent mechanism, eliciting a tumor-specific systemic immune response [18, 19]. Analogous to the broader CCL/CCR axis implicated in driving tumor progression through various signaling pathways, recent findings associate CCL16 with hepatocarcinogenesis and the regulation of stemness in breast cancer via the STAT3 pathway [20, 21]. Although the pathology atlas suggests a favorable prognosis for HCC patients exhibiting elevated CCL16 [22, 23], another study identified CCL16 as a significant plasma biomarker beyond hepatic tissue, predictive of the onset and development of liver cirrhosis [24]. In this investigation, we elucidate the clinicopathological correlation of CCL16 with HCC progression through the analysis of 237 clinical specimen pairs, unveiling the mechanistic basis by which CCL16 mediates the directed migration of monocytes and macrophages during hepatocarcinogenesis.

Materials and methods

Cell culture and treatment

The human hepatoma cell lines J7, SK-Hep-1, and Mahlavu were routinely cultured in DMEM (Invitrogen), while THP-1 cells were cultured in RPMI-1640 (Invitrogen). All cells were supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. CCR antagonists (BX471, Sigma-Aldrich, for CCR1 and Maraviroc, Sigma-Aldrich for CCR5) were administered to THP-1, J7, and SK-Hep-1 cells with or without CCL16 overexpression at concentrations of 100 nM and 1 μM, respectively. Differentiation of macrophages from THP-1 cells or peripheral blood mononuclear cells (PBMCs) obtained from healthy donors, who provided written informed consent for the use of their blood for research purposes, was conducted as previously described [25]. The consent was specifically for the donation of serum for research use. SK-Hep-1 and THP-1 cell lines were authenticated using short tandem repeat (STR)-based assay with the Promega StemElite ID System. The cells were maintained in culture for no longer than 3 months and routinely examined for mycoplasma contamination.

Establishment of CCL16 knockdown and overexpression cell lines

The shRNA of CCL16 was purchased from RNAi core lab, Academia Sinica. The plasmid and lentiviral package plasmids were co-transfected in HEK-293T cell via Turbofect reagent Kit (Fermentas Life science) to produce viral particles. After 24 hours, viral supernatant was collected to infect Malahvu cell lines. After 48 hours of incubation with puromycin-containing culture medium, selected cells were harvested and resulting cellular lysates were subject to protein expression level analyses by western

Table 1. Characterization of *CCL16* mRNA expression in HCC patients determined by Q-RT-PCR

Clinical parameters		No[b]	Mean ± S.E.	*P*-values
Gender	Male	130	1.93±0.17	0.537
	Female	107	1.76±0.23	
Age	<65	145	1.18±0.18	0.775
	>65	92	1.90±0.20	
Cirrhosis	No	142	1.90±0.19	0.644
	Yes	95	1.77±0.20	
Tumor size	<5 cm	89	2.05±0.28	0.245
	>5 cm	14S	1.73±0.14	
Grading[a]	I	11	1.92±0.23	0.993
	II	164	1.85±0.17	
	III	62	1.85±0.26	
Pathological stages[a]	I	115	1.58±0.13	0.017*
	II	71	2.44±0.27	
	III+IV	51	1.64±0.39	
Tumor type	Solitary	179	1.87±0.14	0.833
	Multiple	57	1.81±0.37	
Vascular invasion	No	127	1.59±0.13	0.040*
	Yes	110	2.15±0.25	

The mRNA expression level of *CCL16* was determined by qRT-PCR. T/N (tumor/Non-tumor) ratios are presented as mean ± standard error of the mean. *p*-values present unpaired t test; *P<0.05; [a]One-Way ANOVA analyses.

blots. CCL16 coding sequence was cloned into pcDNA3.1 expression vector. This plasmid was utilized for CCL16 overexpression by transient transfection into SK-Hep and J7 cells using the Turbofect reagent.

Human HCC specimens

The liver specimens from HCC patients were collected from the National Health Research Institute Biobank in Taiwan. We obtained written consent from these HCC subjects. Both HCC and adjacent non-tumorous liver tissues were verified by H&E staining. Approval for the research protocol was issued by the Medical Ethics and Human Clinical Trial Committee at Chang Gung Memorial Hospital (Approval No: 201900860B0), in accordance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the 2013 Declaration of Helsinki and the 2018 Declaration of Istanbul. Briefly, the frozen clinical tissues analyzed in our study were derived from 237 HCC patients. Pairs of specimens refer to HCC tissue (tumor part) and the surrounding non-HCC normal liver tissue (adjacent normal part) from the same patient. Clinical information for these HCC tissues, including gender, age, cirrhosis status, tumor size, grading, pathological stages, tumor type and the presence of vascular invasion are fully listed in **Table 1**. In addition, RNA from these 237 pairs of frozen specimens were extracted using the TRIzol reagent (Life Technologies Inc., Carlsbad, CA) according to the manufacturer's protocol. The extracted RNA was reverse-transcribed to cDNA using Superscript II kit (Life Technologies, Karlsruhe, Germany), followed by real-time PCR analysis for the detection of CCL16 mRNA levels within the specimens. The tumor/normal (T/N) values of CCL16 in the 237 HCC specimens were next statistically analyzed as per clinical parameters of the HCC patients (**Table 1**).

Cell proliferation assay

The proliferation capacities of different hepatoma cell lines that were CCL16-overexpressing or -knockdown were assessed. Briefly, the cell number seeded was consistently 3×10^4 cells, and viable cell number count was conducted by trypsinization at 24, 48, 72 and 96 hours using hemacytometer.

In vitro co-culture model

Co-culturing of THP-1 cells or macrophages differentiated from THP-1 or PBMC with HCC cancer cells was performed in RPMI 1640 medium supplemented with 10% FBS, in 6-well cell culture inserts equipped with a permeable PET membrane (pore size of 0.4 μm). Tumor cells were seeded at a density of 2×10^5 cells in the upper chamber in 1.5 mL of medium. Similarly, THP-1 or macrophage cells were seeded at a density of 2×10^5 cells in the lower compartment in 3 mL of medium. After a co-culture period of 5 days, both THP-1 and HCC cells were collected for RNA extraction and subsequent qRT-PCR analysis.

Immunoblot and immunohistochemical (IHC) staining analysis

Immunoblotting and IHC were conducted as previously described [26]. Briefly, paraffin-

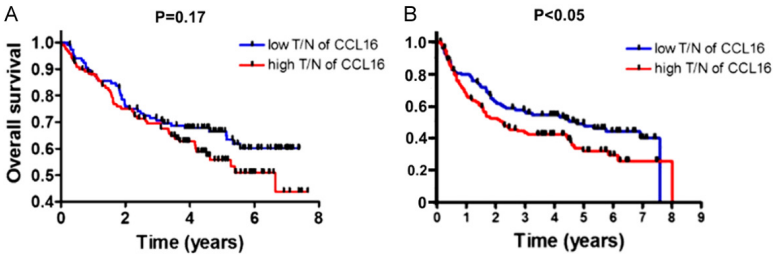

Figure 1. Expression of *CCL16* is significantly correlated to clinical recurrence and survival rate. Correlation of CCL16 expression to overall survival (A) or recurrent survival (B) of 237 pairs of clinical HCC patients was determined using Kaplan-Meier analysis based on an CCL16 expression cutoff value of 118 (median) established from the T/N ratio.

embedded clinical HCC specimens (LV809b, TissueArray.Com) were utilized to assess the expression levels of CCL16, IL-6 and VEGFA. The following primary antibodies were used at dilutions recommended by the manufacturers: anti-CCL16 (Abcam, ab199162), anti-ACTIN (Chemicon, MAB1501), IL-6 (Abcam, ab2907-35) and VEGFA (Abcam, ab52917).

Chemotaxis and cell migration assay

The migration capacity of HCC, THP-1 cells or THP-1 differentiated macrophages were assessed using transwell assays of 0.4 μm or 8 μm pore sizes depending on experimental designs that prevent or facilitate migration of cells cultured in the upper chamber, respectively. In this transwell system, J7 cells (5×10^4) or SK-Hep-1 cells (2×10^4) were co-cultured with THP-1 monocytes or THP-1 differentiated macrophages (2×10^5) to assess influences of CCL16 on HCC, monocytes and macrophages. Cells were stained with crystal violet after 22 hours of co-culture, and images were acquired under 20× objective using Olympus light microscopy.

Animal model

CCL16-overexpressed or control J7 cells were resuspended in fresh PBS to a concentration of 2×10^6 cells per 200 μl for inoculation into flank of nude mice. Tumor volumes were measured twice weekly, and mice were sacrificed a month after initial inoculations to harvest xenografted tumors, which were dissected and fixed with 3.7% formaldehyde before subjecting to IHC staining with antibodies specifically against IL-6 (Abcam, ab290735) or VEGFA (Abcam, ab52917). Animal experiments were performed in accordance with the United States National Institutes of Health guidelines and the Chang-Gung Institutional Animal Care and Use Committee Guide (CGU-106-142).

Statistical analysis

One-way ANOVA was used to compare the results obtained for more than one treatment. Data were analyzed using medians, standard deviations, one-way ANOVA, and Tukey's Honest Significant Difference post hoc test. Student's t-test was employed to analyze differences between groups. Data are presented as mean ± SD from at least three independent experiments.

Results

CCL16 expression is pathologically pertinent to HCC survival and progression

While the involvement of CCL16 in various biological processes, including inflammation, immune response and bone metabolism, has been extensively documented [27-29], the role of CCL16 in cancer progression has only recently been uncovered. To assess the clinicopathological relevance of *CCL16* expression in HCC, we initiated our investigation with Kaplan-Meier analyses on *CCL16* gene expressions in 237 paired clinical specimens, categorized by tumor to normal ratios (T/N ratios). These analyses revealed no significant differences in overall survival rates for patients exhibiting elevated *CCL16* expression within tumor compared to normal parts (high T/N of *CCL16*) (**Figure 1A**). Nonetheless, a significant association was observed between higher *CCL16* expression and reduced recurrence survival rates (**Figure 1B**). To strengthen this observation, we conducted a systematic evaluation of 237 HCC specimen pairs, considering clinical parameters such as gender, age, cirrhosis, tumor size, grading, pathological stage, tumor type, and vascular invasion (**Table 1**). This prompted our investigation into the mechanism through which CCL16 promotes HCC tumorigenicity. HCC xenograft models were first established using CCL16-overexpressing J7 cells using immunodeficient nude mice, with in vivo tumor growth monitored over four weeks. This in vivo

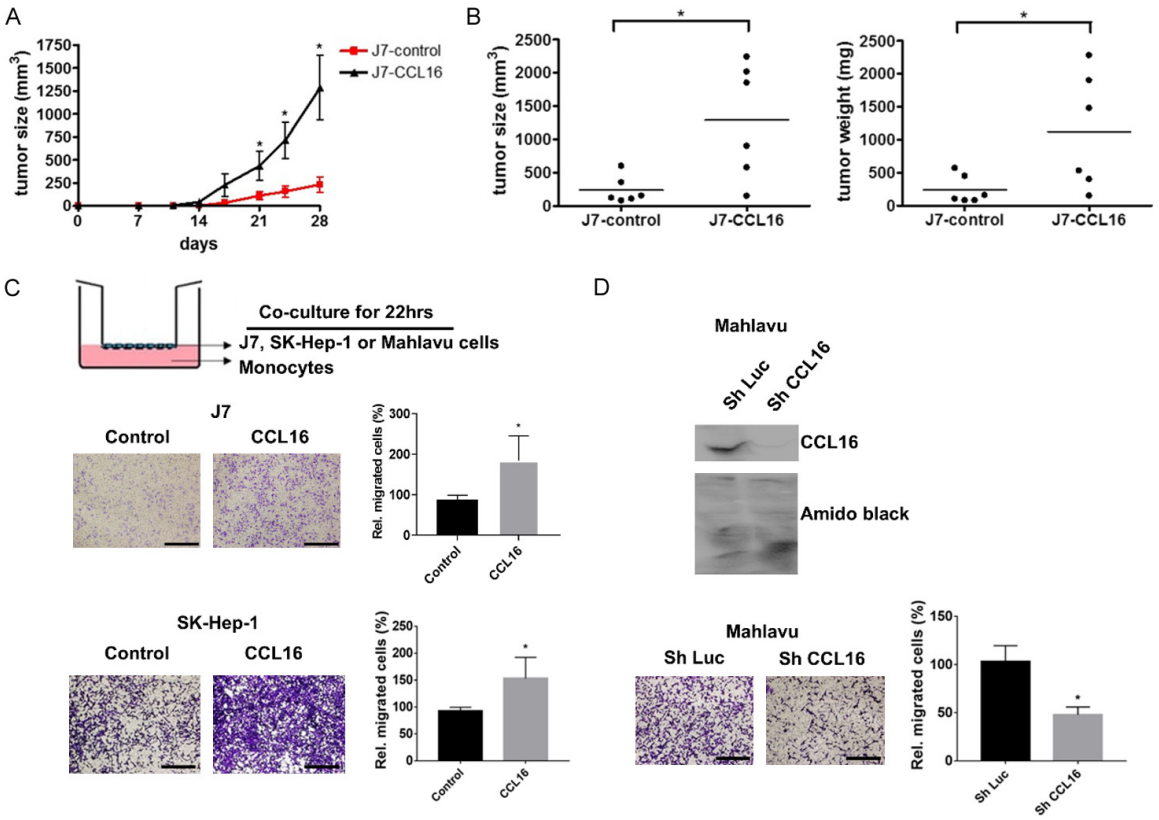

Figure 2. Overexpression of CCL16 significantly promotes J7 tumor growth capacity and cellular migration in presence of monocyte. Nude mice were subcutaneously injected with cells overexpressing CCL16 (J7-CCL16) or control cells (J7-control) at a dose of 2 × 10^6 cells (n = 6 per group) for monitoring tumor growth for four weeks (A) prior to sacrifice for determinations of tumor size and tumor weight (B) (*P<0.05). (C) A schematic diagram that illustrates the co-culture system employed for assaying influences of monocytic THP-1 on chemotaxis of HCC cells (SK-Hep-1 or Mahlavu cells). SK-Hep-1 cells that are CCL16 low-expressing were overexpressed with CCL16 were co-cultured (upper chamber) with THP-1 monocytic cells (lower chamber) for cellular migration ability evaluations (*P<0.05). Scale bar: 100 μm. (D) Mahlavu cells that are CCL16 high-expressing were infected with lentivirus expressing shRNA specifically target *CCL16,* and resulting CCL16-stable knockdown cells (upper chamber) were used to co-culture with THP-1 (lower chamber) for migration assays (*P<0.05). Scale bar: 100 μm.

analysis demonstrated significant increases in tumor growth in mice implanted with J7-CCL16-overexpressing xenografts compared to control J7 xenografts (**Figure 2A**), with excised xenograft weights further validating these findings (**Figure 2B**). However, CCL16 expression exhibited minimal impact on the in vitro cellular proliferation of HCC cells with lower endogenous CCL16 expression levels, such as J7 and SK-Hep-1 cells (Supplementary Figure 1A-C), and did not significantly affect migration (Supplementary Figure 2).

These observations suggest that while CCL16 is a critical factor in promoting HCC tumor growth, the physiological context including the availability of cellular cytokines, chemokines and growth factors must be adequately represented to recapitulate the *in vivo* TME for CCL16 to exert its prominent tumorigenic role. Hence, we next conducted co-culture experiments of J7, SK-Hep-1 or Mahlavu cells with THP-1 cells using a transwell system to examine the effects of manipulated CCL16 expression on migratory capacity of HCC cells (**Figure 2C**). Our findings indicated that CCL16 overexpression indeed significantly enhanced cellular migration in both J7 and SK-Hep-1 cells when co-cultured with THP-1 monocytes (**Figure 2C**). To validate, we utilized Mahlavu cells, an HCC cell line with endogenously high CCL16 expression (Supplementary Figure 1A), and generated *CCL16*-knockdown cells via shRNA that specifically target *CCL16*. Conversely, **Figure 2D** demonstrates that Mahlavu cells with CCL16 knockdown, when co-cultured with THP-1 cells, exhib-

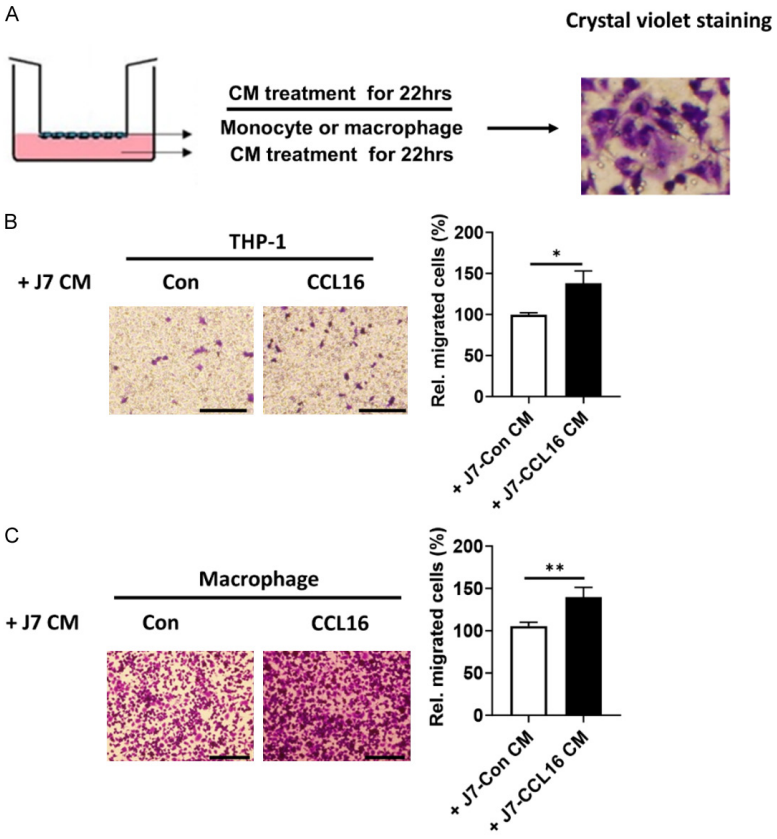

Figure 3. Overexpression of CCL16 triggers chemotaxis of monocyte and differentiated macrophage *in vitro*. (A) The ability of CCL16 in chemoattracting THP-1 monocytes and macrophages was assessed by employing similar transwell co-culture system for monitoring cellular chemotactic migration in which condition media from J7-control or J7-CCL16 stable clones were added to the lower chamber of the transwell plates, while THP-1 (B) or THP-1-differentiated macrophages (C) at 2×10^5 cells were seeded in the upper chamber. After 22 hours of incubation, the cells were stained with crystal violet and statistically quantified (*P<0.05, **P<0.01). Scale bar: 100 μm.

ited a significantly diminished migration capability compared to the control cells (**Figure 2D**).

CCL16 overexpression induces chemotaxis of monocyte and macrophage

To elucidate the mechanism through which CCL16 facilitates tumor progression by enhancing monocyte and macrophage migration, we next explored the effect of CCL16 overexpression on inducing chemotaxis of THP-1 monocytes and THP-1-differentiated macrophage. Employing a transwell co-culture system with condition media (CM) from CCL16-overexpressing J7 cells in the lower chamber, we observed a significant enhancement in the THP-1 cell chemotaxis when exposed to CM from two CCL16-overexpressing J7 clones (**Figure 3A** and **3B**). Similarly, macrophages chemotaxis was markedly increased in the presence of elevated CCL16 levels in the CM from J7 cells overexpressing CCL16 (**Figure 3C**), indicating the critical role of CCL16 abundance in mediating chemotactic migration of both monocytes and differentiated macrophages.

CCR1 and CCR5 are central to CCL16-mediated monocytic and macrophagic chemotaxis

Given the pivotal role of CC chemokines in mediating chemotaxis through interaction with C-C motif receptors (CCRs) to exert downstream intercellular communication within the TME [30], we assessed the expression of CCL16-associated CCRs, including CCR1, CCR2, CCR5 and CCR8 (previously reported to be receptors for CCL16 [17, 18]), in our HCC models. Analysis of gene expression in THP-1 cells, THP-1-derived macrophages, and J7 xenografts revealed that CCR1 and CCR5 were the CCRs predominantly expressed in THP-1 monocytes, differentiated macrophages as well as J7 xenografts, with or without CCL16 overexpression (**Figure 4A** and **4B**). To ascertain the functional relevance of CCR1 and CCR5 as the chemokine receptors mediating the protumorigenic functions of CCL16 in HCC, we treated J7- and SK-Hep-1-CCL16 overexpression models with CCR1- or CCR5-specific antagonist BX471 or Maraviroc, respectively. As illustrated in **Figure 4C** and Supplementary Figure 3, the CCL16-directed migration in both J7 and SK-Hep-1 cells, when co-cultured with THP-1 cells, was significantly inhibited upon administration of BX471 or Maraviroc. This suggests that CCR1 and CCR5 serve as specific receptors for CCL16 in these cells. In line with these observations, the chemotactic response of THP-1 cells to CCL16-overexpressing J7-CM

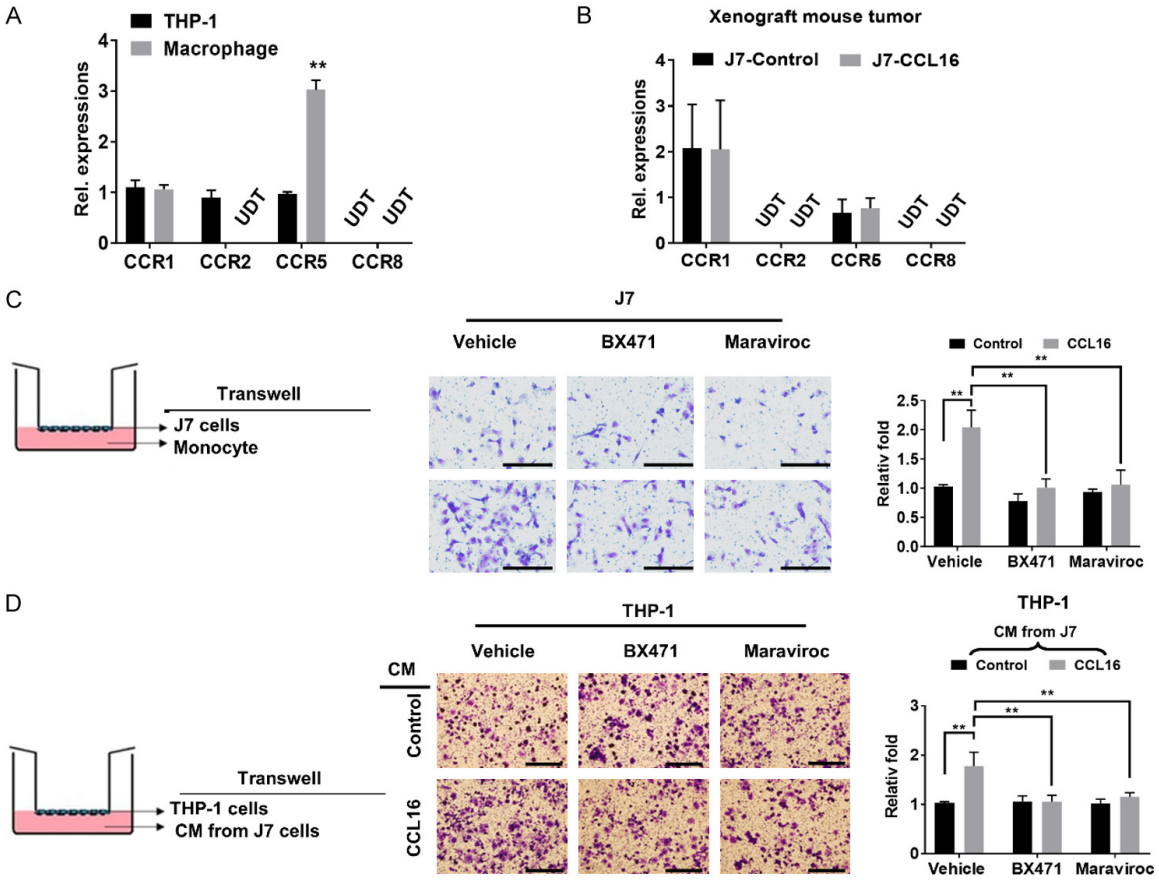

Figure 4. CCR1 and CCR5 are critical CCRs for CCL16-mediated chemotaxis in monocyte and macrophage *in vitro*. A, B. Chemokine receptors CCR1, 2, 5 and 8 were assessed for their expression in THP-1, differentiated macrophages and control or CCL16-overexpressed J7 xenografts (**P<0.01). C. Transwell system of 0.4 μm pores were utilized to assess the roles of CCR1/5 in mediating differentiation of THP-1 monocytes cultured on the lower chamber with CCL16-overexpressed or control J7 cells on the upper chamber. Antagonists against CCR1 or CCR5 were employed to determine whether CCR1/5 could act as CCL16-specific chemokine receptors (**P<0.01). Scale bar: 100 μm. D. CCL16-specific chemotactic activities through CCR1 or CCR5 were assessed using THP-1 migration assays, where condition media from J7 were added to the lower chamber while differentiated macrophages were seeded on the upper chamber of the transwell plates (**P<0.01). Scale bar: 100 μm.

was also significantly diminished following treatment with CCR1/5-specific antagonists BX471 and Maraviroc (**Figure 4D**), confirming the essential role of CCR1 and CCR5 in CCL16-induced chemotaxis.

CCL16 triggers M2 polarization of THP-1 cells and release of inflammatory cytokines in HCC

Although our data in **Figure 3** indicates that CCL16 can enhance the chemotaxis of both THP-1 monocytes and macrophages differentiated from THP-1, the relevance of CCL16 expression to monocytic differentiation remained uncertain. Consequently, THP-1 cells were stimulated with conditioned media (CM) from control or CCL16-overexpressing J7 cells to observe the effects of CCL16 on the differentiation of THP-1 cells. Our data showed that THP-1 cells could be induced and differentiated into adherent macrophage-like cells by CM released from CCL16-overexpressing J7 cells but not by that from control J7 cells (**Figure 5A**). Given the growing recognition of M2 macrophage activation as exhibiting anti-inflammatory and tumorigenic properties [31, 32], we conducted experiments involving co-culture of THP-1 cells or human macrophages isolated from PBMCs with either control or CCL16-overexpressing J7 cells. Subsequently, we evaluated the expression of M2 markers, including ARG1, IL-10, CD206, CD163, and CLEC7A, in both THP-1 cells and human macrophages. In THP-1 cells, the canonical M2 surface marker

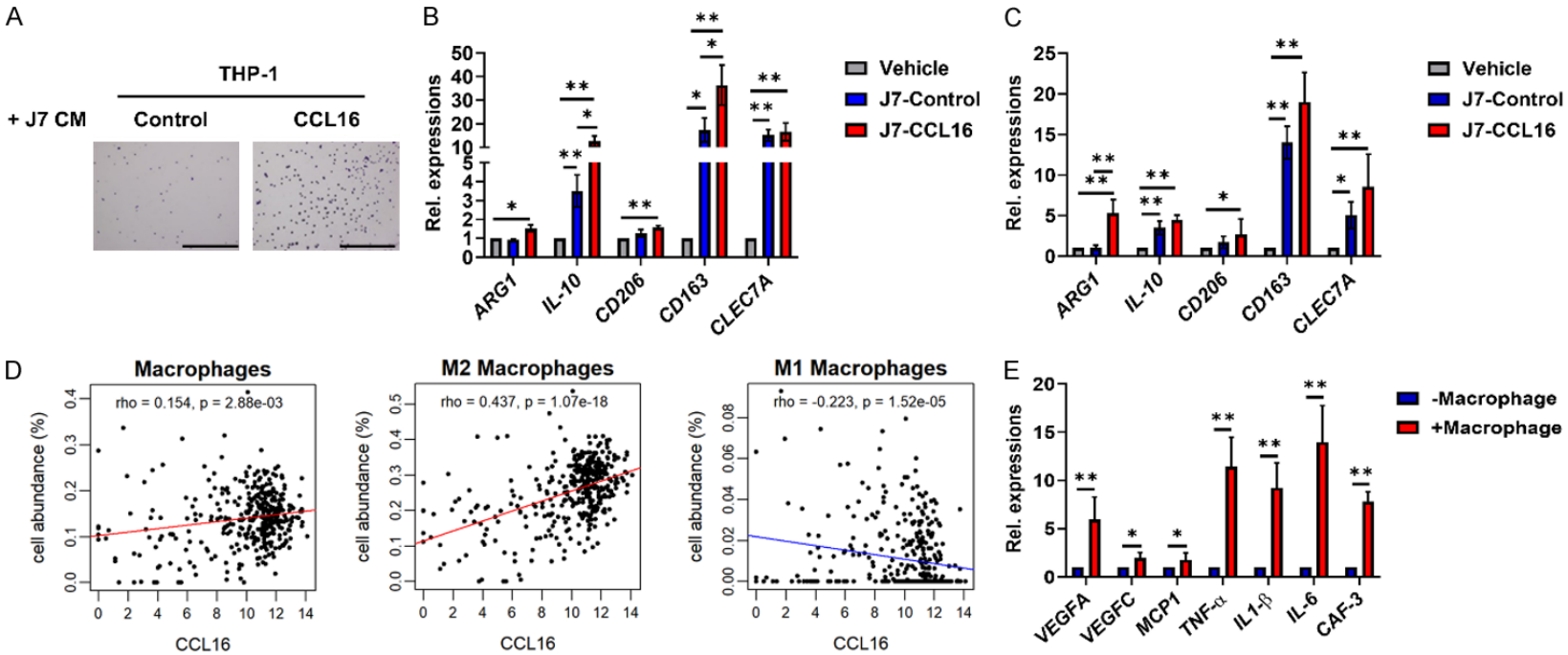

Figure 5. CCL16 triggers M2 polarization of THP-1 cells and elevates inflammatory cytokine expression. A. Effects of CCL16 on THP-1 differentiation were examined by seeding THP-1 cells onto the bottom while J7 cells were seeded on upper chamber of transwell plates. Scale bar: 100 μm. B. THP-1 cells were cultured or co-cultured with control or CCL16-overexpressing J7 cells prior to qPCR analysis on expression of markers known to associate with M2 macrophage activation including ARG1, IL-10, CD206, CD163 and CLEC7A (**P*<0.05, ***P*<0.01). C. Human macrophages derived from peripheral blood mononuclear cells were cultured alone or co-cultured with either control or CCL16-overexpressing J7 cells before undergoing qPCR analysis to assess the expression of markers associated with M2 macrophage activation, including ARG1, IL-10, CD206, CD163, and CLEC7A (*P<0.05, **P<0.01). D. xCell analysis was conducted to explore the co-expression network and calculate the Spearman correlation coefficient between CCL16 and the abundance of macrophages, M2 macrophages, and M1 macrophages within HCC specimens from the TCGA database. E. Effects of macrophages derived from THP-1 on CCL16-overexpressing J7 cells were evaluated by examining gene expression of a panel of cytokines including VEGFA/C, MCP1, TNF-α, IL-1β, IL-6 and CAF-3 (**P*<0.05, ***P*<0.01).

CD163 showed a significant increase in the presence of CCL16-overexpressing J7 cells. Similarly, the M2 macrophage-produced cytokine IL-10 was substantially elevated when co-cultured with CCL16-overexpressing J7 cells compared to controls (**Figure 5B**). Importantly, the M2 markers exhibited significant increases in THP-1 cells co-cultured with CCL16-overexpressing J7 cells, suggesting a potential role for CCL16 in promoting M2 macrophage differentiation (**Figure 5B**). In macrophages derived from PBMCs of healthy donors, co-culturing with CCL16-overexpressing J7 cells, as opposed to control cells, led to the induction of M2 markers ARG1 and CD163. This finding underscores the significant role of CCL16 overexpression in HCC cells in facilitating macrophage differentiation into the M2 phenotype (**Figure 5C**).

To further validate the role of CCL16 in enhancing M2 macrophage differentiation within HCC, we employed xCell, a gene signature-based tool designed to infer immune and stromal cell types within tissue samples [33]. This approach enabled us to assess the impact of genes correlated with CCL16 on the abundance of macrophages in HCC specimens derived from the TCGA database. Our analysis of the clinical HCC cohort from TCGA revealed a notable positive correlation between CCL16-associated gene expression and the abundance of macrophages and M2 macrophages, while demonstrating a negative correlation with the abundance of M1 macrophages. This evidence supports the pivotal role of CCL16 in modulating macrophage polarization within the HCC microenvironment (**Figure 5D**).

To further extrapolate these findings, a panel of inflammatory cytokines known to promote tumor growth was examined. The data revealed that cytokines including VEGFA/C, MCP1, TNF-α, IL-1β, IL-6 and CAF-3 were significantly elevated only in CCL16-overexpressing J7 cells when co-cultured with macrophages (**Figure 5E**). These data together implicated CCL16 as a pro-tumorigenic chemokine that not only activated but also directed M2 macrophage migration, thereby promoting cancer progression in HCC.

VEGFA and IL-6 are potential markers for CCL16-mediated tumorigenicity in HCC

As a crucial component of the HCC TME, tumor-associated macrophages (TAMs) exert significant influence on tumor development and progression. TAMs engage in intricate interactions with tumor cells, inducing them to express oncogenes and inflammatory factors such as IL-1, IL-6, and VEGF, which play pivotal roles in cancer progression [34-36]. Our findings indicated that co-culturing with macrophages resulted in significantly elevated cytokines, including VEGFA/C, MCP1, TNF-α, IL-1β, IL-6, and CAF-3, in CCL16-overexpressing J7 cells (**Figure 5C**). Building upon these observations, we further validated these *in vitro* findings using a xenograft model and human HCC specimens. Immunohistochemistry (IHC) results revealed notable enhancements in both IL-6 and VEGFA protein levels in CCL16-overexpressing xenografts compared to J7 controls (**Figure 6A**, **6B**). Additionally, in human HCC tissues, CCL16 expression was found to be elevated in HCC tissue compared to normal tissue (**Figure 6C**). Spearman's analysis of the expressions of CCL16, IL-6, and VEGFA, as determined by IHC staining, confirmed a positive correlation between the levels of CCL16 expression and those of IL-6 and VEGFA (**Figure 6D**, **6E**). These results collectively underscore the role of CCL16 as a pro-tumorigenic chemokine that not only facilitates but also directs M2 macrophage migration, thereby advancing cancer progression in HCC through the upregulation of IL-6 and VEGFA expression.

Discussion

CCL16, recognized as a liver-expressed chemokine (LEC), exhibits chemotactic activity through its interaction with CCR1, CCR2 and CCR5 in mouse L1.2 and pre-B cells, facilitating leukocyte migration [16]. In addition, the binding of LEC to CCR1 and CCR8 identified in HEK-293 has been shown to induce chemotaxis and adhesion of THP-1 monocytes [17]. These initial studies, nevertheless, questioned the *in vivo* relevance of chemotactic activity for CCL16 due to its high plasma levels and the lack of discrete regulation thereof. Indeed, the role of CCL16 in clinicopathogenesis and tumorigenesis of HCC was not clarified until the present study. Curated 237 HCC specimen pairs and the CCL16 characterization unveiled significant associations between CCL16 expression and both early-stage disease progression and vascular invasion in HCC patients (**Table 1**). In line with this observation, a significant correlation

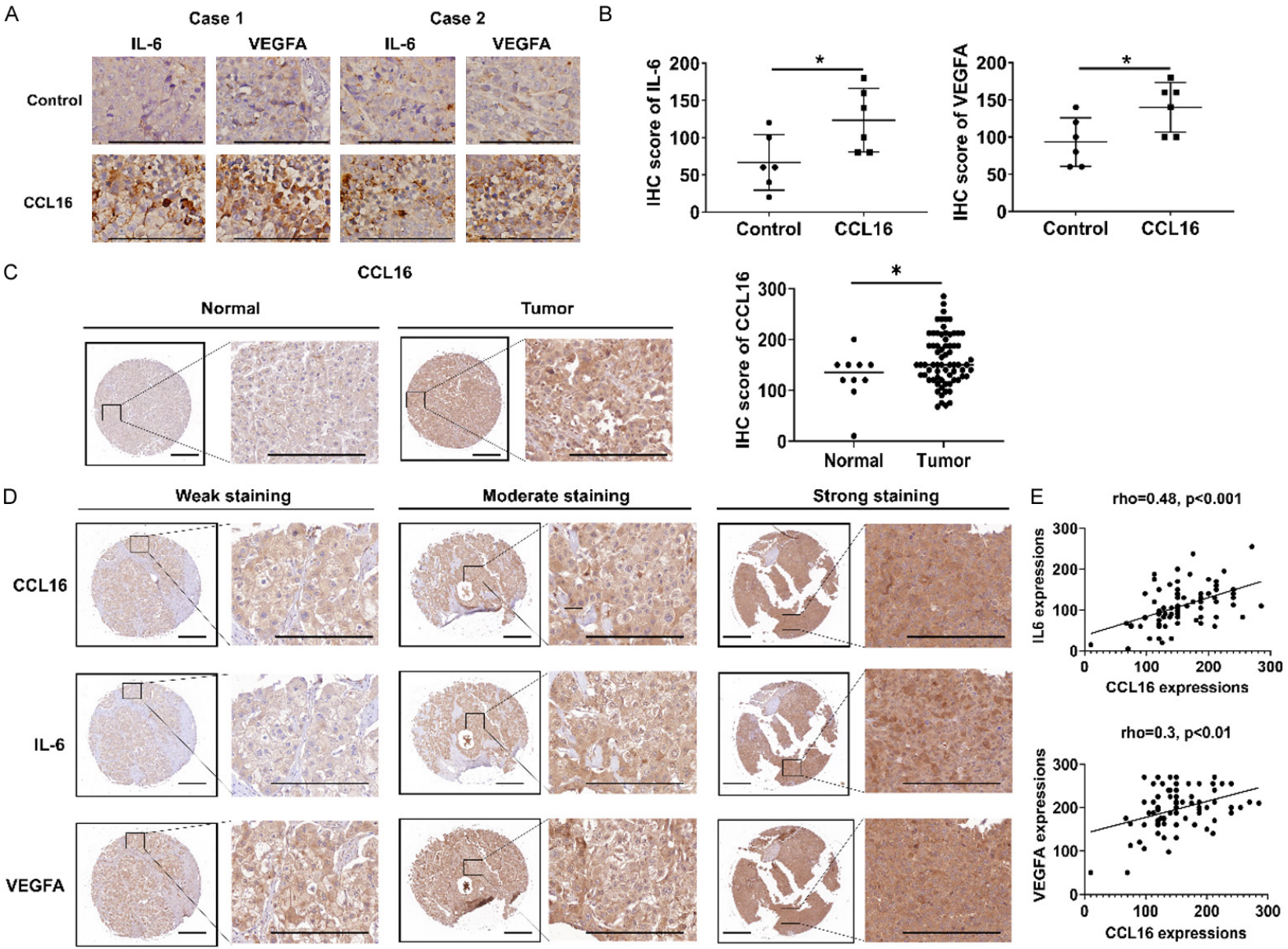

Figure 6. VEGFA and IL6 are predominantly overexpressed in CCL16-overexpressed HCC. A. The representative figures of the analysis of VEGFA and IL-6 protein levels using mmunohistochemistry (IHC) staining in two cases from the xenograft of J7-control and J7-CCL16 cells (×400). B. The expression levels of VEGF and IL-6 in

tumors from these groups were quantitatively scored, with the statistical results presented (*P<0.05). C. Representative images of normal liver and HCC specimens immunostained with a specific anti-CCL16 antibody (magnification ×400). D. The association between the expression levels of CCL16, IL-6, and VEGFA in HCC tumors was assessed, with statistical significance determined through Spearman's rank correlation analysis. E. Spearman rank correlation coefficient of IL6 and VEGFA with respect to CCL16 in 80 HCC tissue samples obtained from TissueArray.Com (LV809b). **P<0.01. Scale bar: 400 μm.

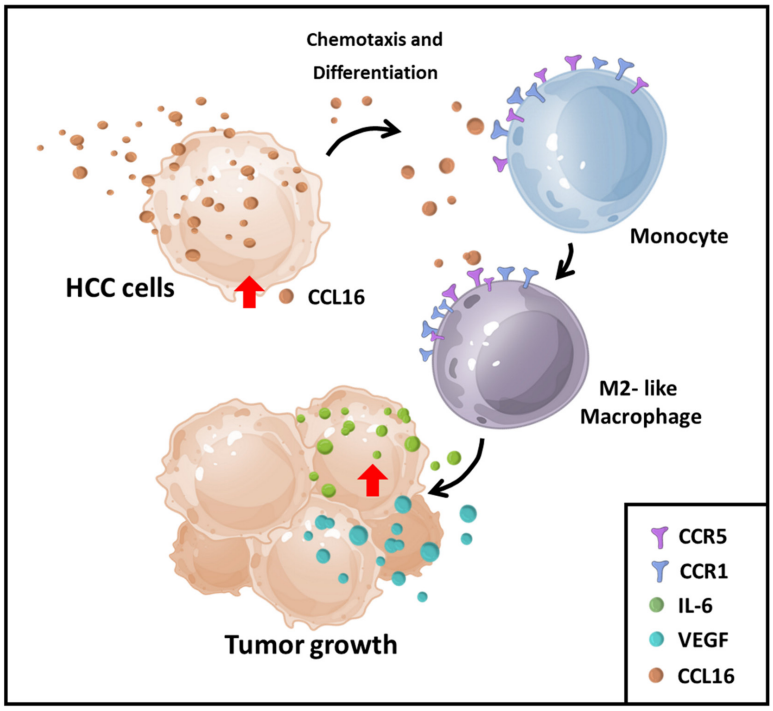

Figure 7. Overexpression of CCL16 in HCC cells induces monocyte chemotaxis and differentiation into M2-like macrophages via CCR1 and CCR5. The schematic diagram showing the role of CCL16 in promoting HCC progression through monocyte chemotaxis and M2-Like macrophage Differentiation Overexpression of CCL16 in HCC cells induces monocyte chemotaxis and differentiation into M2-like macrophages via CCR1 and CCR5. This process enhances tumor growth through elevated IL-6 and VEGF expression in the tumor microenvironment.

of CCL16 expression with reduced RFS was noted (**Figure 1B**).

CCR2, a well-characterized CCR in liver pathology, plays a pivotal role across a spectrum of liver conditions, from acute and chronic injuries to fibrosis/cirrhosis and hepatocarcinogenesis [1]. Except for CCL2 and CCL7, which share high sequence homologies and bind to CCR2 at high affinities [37-39], other CCR2 ligands like CCL8, CCL12, CCL13 and CCL16 bind to CCR2 with minimal affinities and very little information on their physiological functions [40]. Our present findings highlight CCL16-specific chemotactic activities in recruiting monocytes and M2 macrophages, mediated by CCR1 and CCR5, as demonstrated by the inhibitory effects of antagonists BX471 and Maraviroc on CCL16-directed cellular migration (**Figure 4**). In concert with our findings, elevated CCR16 expression in breast tumors has recently been identified and suggested as a novel biomarker for triple-negative breast cancer (TNBC) diagnosis and treatment [41, 42]. Beyond breast cancer, CCL16 upregulation in serum of patients with pneumonia and its role in promoting angiogenesis of vascular endothelial cells through CCR1 further underscore its tumorigenic capacity [18, 43, 44].

Contrastingly, some studies posit CCL16 as a prognostic indicator for improved outcomes. In liver fibrosis/cirrhosis, for instance, CCL16 reportedly mitigates disease progression of cirrhosis by inactivating hepatocellular stellate cells; while its downregulation by IL-30 during human pancreatic cancer (hPCa) progression stimulates tumor growth [45]. Our research aligns with these findings, eliciting greatly elevated IL-10 level in CCL16-overexpressing HCC, analogous to another TNBC study where CCL16 maintains stem cell-like properties TNBC by activating CCR2/GSK3β/β-catenin/OCT4 axis through IL-10-mediated STAT3 inhibition, thereby hindering its anti-tumor functions [20]. Moreover, the association of VEGFs with poor HCC prognosis [46] is reinforced by our cytokine analyses via qPCR and IHC, which establishes a significant correlation between VEGFA expression and CCL16 overexpression. In fact, an antibody specifically against CCL16 has been reported to abolish angiogenic responses mediated by VEGFA [19]. Consequently, our findings corroborate recent findings on the

greatly augmented IL-6 level in CCL16-overexpressed tumors, supporting the potential of IL-6/STAT3 signaling disruption by an immunosuppressive effects with IL-6 neutralizing antibody within the TME for HCC treatment [47]. In summary, findings presented in this current study highlight the pro-tumorigenic role of CCL16 in HCC pathogenesis, specifically through its regulation of migration of monocytes and M2 macrophage differentiation via CCR1 and CCR5, laying a foundational basis for future therapeutic strategies targeting the CCL16/IL-6/VEGF pathway (**Figure 7**). Further iinvestigation is warranted to elucidate the potential of CCL16 as a dual-regulatory molecule in HCC progression, particularly concerning its capacity in enhancing T cell-mediated antitumor activity.

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Informed consent was obtained from each patient involved in the study.

Disclosure of conflict of interest

None.

Abbreviations

CCL16, C-C Motif Chemokine Ligand 16; HCC, hepatocellular carcinoma; TME, tumor microenvironment; OS, overall survival; CCR, CCR1 CC motif chemokine receptor; PBMCs, peripheral blood mononuclear cells; TAM, Tumor-associated macrophages; CM, conditioned media; LEC, liver-expressed chemokine; TNBC, triple-negative breast cancer; hPCa, human pancreatic cancer.

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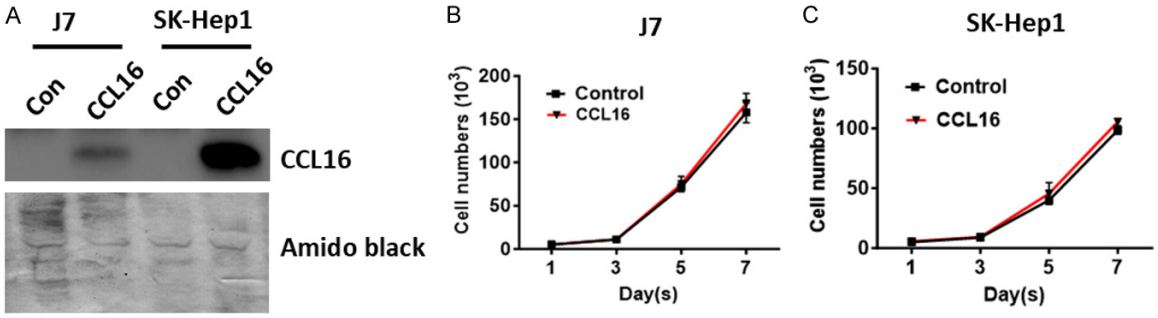
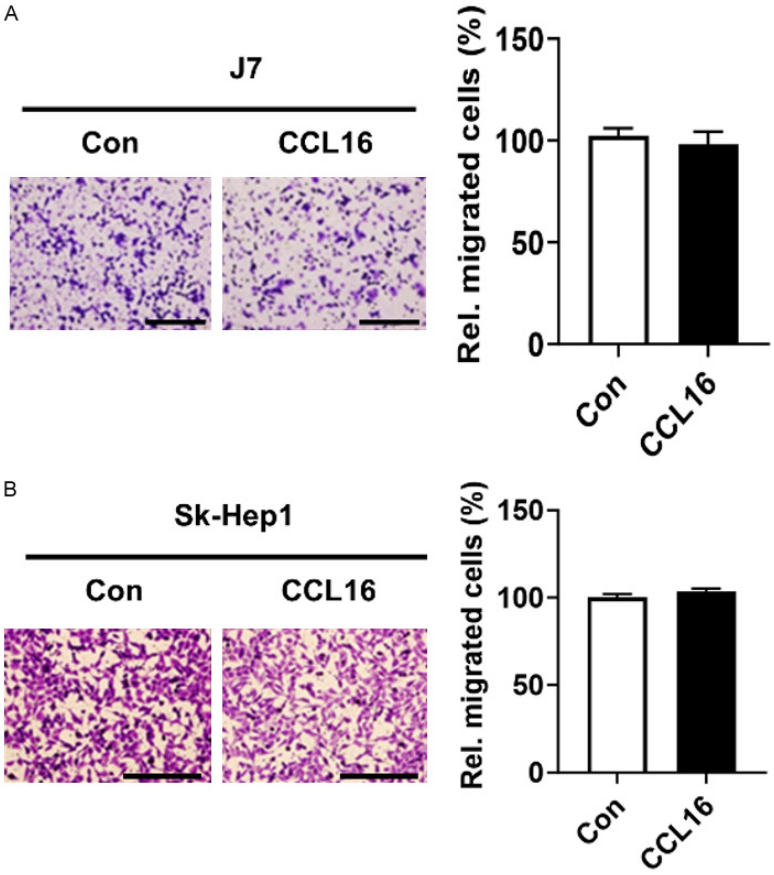
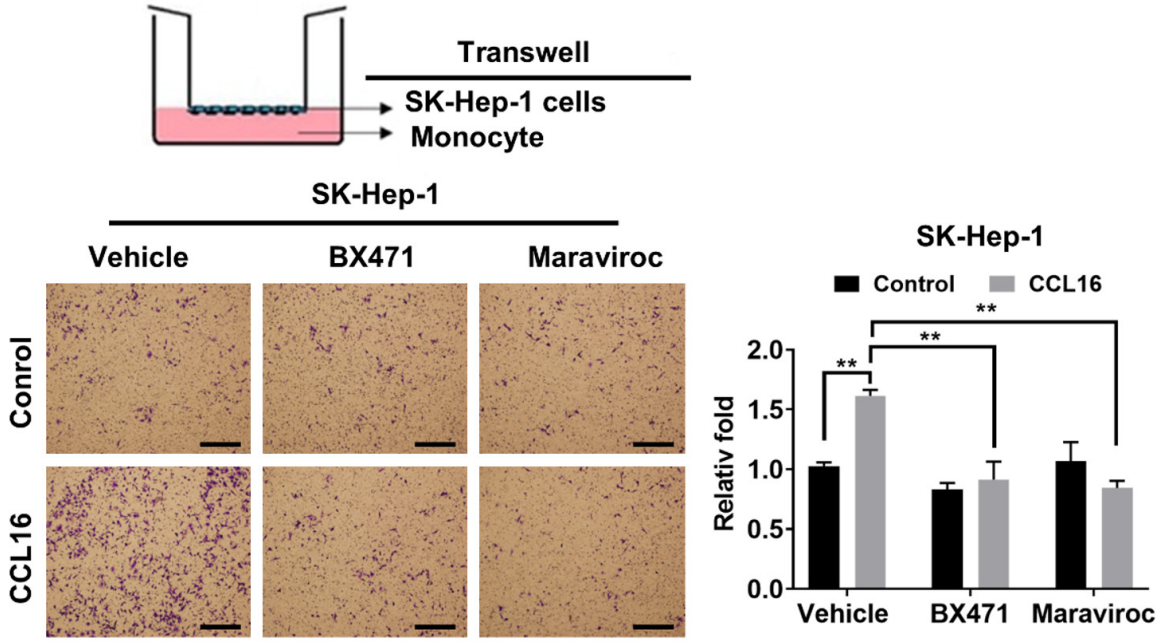


Supplementary Figure 1. CCL16 does not affect the proliferation and colony-formation ability of HCC cell lines *in vitro*. A. CCL16-overexpressed stable clones from J7 or SK-Hep-1 cells were confirmed using Western blot. B, C. Cellular proliferation of CCL16-overexpressed J7 and SK-Hep-1 cells was measured by comparison to control cells.



Supplementary Figure 2. CCL16 does not affect cellular migration ability of HCC cell lines *in vitro*. CCL16-overexpressed stable clones from J7 (A) or SK-Hep-1 cells (B) were used to assess cellular migration ability when CCL16 was overexpressed by comparisons to control stable clones without CCL16 overexpression. Scale bar: 100 μm.



Supplementary Figure 3. BX471 and Maraviroc inhibit CCL16 mediated chemotaxis of SK-Hep-1 cells towards THP-1. Transwell system of 0.4 μm pores were utilized to assess the roles of CCR1/5 in mediating differentiation of THP-1 monocytes cultured on the lower chamber with CCL16-overexpressed or control SK-Hep-1 cells on the upper chamber. Antagonists against CCR1 or CCR5 were employed to determine whether CCR1/5 could act as CCL16-specific chemokine receptors (**P<0.01). Scale bar: 100 μm.