Med Oncol (2016) 33:17
DOI 10.1007/s12032-016-0729-9

ORIGINAL PAPER

Tumor-associated macrophage or chemokine ligand CCL17 positively regulates the tumorigenesis of hepatocellular carcinoma

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Received: 29 August 2015 / Accepted: 4 January 2016 / Published online: 19 January 2016

Abstract Alternatively activated macrophages (M2) can secrete chemokines, such as chemokine ligand 17 (CCL17), and are associated with promoting tumorigenesis of hepatocellular carcinoma (HCC). This study aimed at investigating the potential role of M2 and CCL17 in progression of HCC. The levels of CCL17 expression in 90 HCC samples were characterized by tissue microarray and stratified for the postsurgical survival. MHCC97L cells were co-cultured with classically activated M1, M2 or CCL17-silencing $M2^{ccl17mute}$ or treated with conditional medium (CM) from these cells or CCL17 in vitro. The wound healing, invasion, viability and apoptosis of MHCC97L cells in vitro and tumor growth in vivo were determined. The stemness of MHCC97L cells was examined by sphere formation, flow cytometry and Western blot. The relative expression levels of epithelial–mesenchymal transition (EMT) factors and the Wnt/β-catenin signaling were determined. Higher levels of intratumoral CCL17 expression were significantly associated with clinical pathological characteristics of HCC and with poorer overall survival rates in HCC patients ($P < 0.05$). High levels of CCR4 were detected in MHCC97L cells. Treatment with the CM from M2 or with CCL17 significantly enhanced the wound healing process, invasion and proliferation of MHCC97L cells in vitro. Co-implantation MHCC97L cells with M2 significantly promoted the growth of MHCC97L tumors in vivo. Co-culture with M2 or treatment with CCL17 enhanced the stemness, EMT process, the TGF-β1 and Wnt/β-catenin signaling in MHCC97L cells. CCL17 promotes the tumorigenesis of HCC and may be a potential biomarker and target for HCC prognosis and therapy.

Keywords Hepatocellular carcinoma · Tumor-associated macrophage · Epithelial–mesenchymal transition · Wnt/β-catenin signaling

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in humans with high mortality in the world [1]. HCC is associated with chronic liver diseases, such as chronic hepatitis B (CHB) and chronic hepatitis C (CHC) [2]. During the process of CHB and CHC, alternatively activated M2 cells may contribute to the development and progression of HCC [3]. Indeed, therapy with trabectedin, an anti-DNA repair reagent, is a promising strategy in cancer treatment [4]. However, the precise mechanisms by which M2 cells contribute to tumorigenesis of HCC have not been clarified.

It is well known that chemokines are secreted by M2 cells, while the C–C chemokine receptor, such as CCR4 and CCR5, is expressed by HCC cells [5–7]. Chemokines, through their receptors, not only are responsible for chemotaxis of their receptor expressing HCC, but also may regulate the tumorigenesis of HCC. CCL17, also known as thymus and activation-regulated chemokine (TARC) [8], is a ligand of CCR4 and expressed constitutively in the thymus and on M2 cells [9]. CCL22, also a ligand of CCR4,

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can promote the intratumoral migration of HCC and recruit regulatory T cells [6]. Although it was reported CCL17 could induce trophoblast migration and invasion [10], the role of CCL17 in the development and progression of liver cancer remains largely unknown.

In this study, we characterized the levels of intratumoral and peritumoral CCL17 expression in HCC and analyzed its prognostic values. Furthermore, we examined the impact of conditional medium (CM) from classically activated M1, M2, or CCL17-silencing M2 or treatment with CCL17 on the proliferation, migration, invasion and apoptosis of human HCC MHCC97L cells in vitro and the growth of implanted MHCC97L tumors in vivo. Finally, we employed cell co-culture system to test the effect of co-culture with M1, M2, CCL17-silencing M2 or treatment with CCL17 on the stemness, epithelial–mesenchymal transition (EMT), and the Wnt/β-catenin and TGF-β1 signaling in MHCC97L cells to explore the potential mechanisms underlying the action of CCL17 in regulating the tumorigenesis of HCC.

Materials and methods

Analysis of CCL17 expression in human HCC samples

The CCL17 expression in the primary HCC tissue samples was analyzed by tissue microarray. Nighty HCC intratumoral tissues and the corresponding peritumoral tissue samples were collected from primary HCC patients undergoing surgery from 2010 to 2011. Individual patients with HCC were diagnosed, according to the biopsied tissue pathology.

The tissue array chips were deparaffinized, rehydrated, subjected to antigen retrieval and blocked by 10 % goat serum. The chips were incubated with rabbit anti-CCL17 primary antibodies or control rabbit IgG (1:300, Abcam) at 4 °C overnight. After being washed, the bound antibodies were detected with HRP-conjugated goat anti-rabbit secondary antibodies (1:1000, CST) and visualized using DAB reagent (Dako). Images were analyzed using Aperio ImageScope software (Leica) by two pathologists independently. The staining intensity was graded using semi-quantitative and subjective grading system and the following criteria: 0, negative staining; 1, light yellow or light brown staining; 2, yellow or brown staining; and 3, dark yellow or dark brown staining. The percentages of positive staining were graded as follows: 0, <1 % of positive cells; 1, 1–25 % of positive cells; 2, 26–50 % of positive cells; 3, 51–75 % of positive cells; and 4, >75 % of positive cells. The staining index was calculated by the staining intensity score multiplied by the positive cell score. A staining index score of <6 was considered as a low level of CCL17 expression, while a score of 6–12 was identified as a high level of CCL17 expression.

Cell stimulation and co-culture

Human monocytic leukemia THP-1 cells (ATCC) and low metastatic HCC cell line HMCC97L were from the Third Military Medical University and cultured in RPMI1640 medium with 10 % fetal bovine serum (FBS). THP-1 cells were stimulated with 200 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) for 24 h, and after being washed, the cells were cultured for another 24 h. Subsequently, the cells were treated with 150 ng/ml of lipopolysaccharide (LPS, Sigma) and 20 ng/ml of IFN-γ (Peprotech) or 20 ng/ml of IL-4 and 100 ng/ml of MCSF (Peprotech) for 24 to induce M1 and M2 cells, respectively. The induced macrophages were verified by flow cytometry (FCM). Briefly, the cells were blocked with anti-CD16/anti-CD32 and stained with PE-antihuman IL-12/IL-23 (p40), PerCP/Cy5.5-antihuman CCR9, APC-antihuman CD163, FITC-antihuman CD11b antibodies, or the corresponding isotype controls (BioLegend). The frequency of M1 and M2 cells was determined. In addition, the relative levels of p40, CCR9, CD163, CD206 and CCL17 mRNA transcripts were determined by quantitative RT-PCR using specific primers (Table 3), as described [11]. Finally, the relative levels of arginase-1 and CCL17 expression were determined by Western blot assay using rabbit antihuman arginase-1, (1:1000, CST) and anti-CCL17 (1:1000, Abcam).

To establish cell co-culture system, HMCC97L cells (1×10^5/well) were cultured in the bottom chamber and co-cultured with 5×10^4 M1 or M2 macrophage in the top chamber of transwell plates (Corning) for another 72 h, respectively. The MHCC97L cells were then collected for furtherer studies.

CCL17 chemokine stimulation

The expression of CCR4 on MHCC97L cells was examined by FCM using PE-antihuman CD194 (CCR4) antibody. The levels of secreted CCL17 in the supernatants of cultured MHCC97L cells or M2 macrophages were tested by ELISA using a Human CCL17/TARC Quantikine ELISA Kit (R&D System). In addition, MHCC97L cells (5×10^5/well) were cultured in six-well plates for 6 h and treated with varying concentrations (100–200 ng/ml) human recombinant CCL17 (Peprotech) for 24 h and the cells were harvested for further studies.

Cell migration and invasion assay

The impact of CCL17 on the migration and invasion of MHCC97L cells was analyzed by wound healing and

transwell invasion assays. MHCC97L cells (5×10^5/well) were cultured in complete 1640 medium, and when the cells reached 90 % confluence, the cells were wounded using a plastic tip. After being washed, the cells were exposed to the conditioned mediums (CM) prepared by harvesting the supernatants of cultured macrophages and the wound healing process was observed at 24 and 48 h post-treatment.

Furthermore, HMCC97L cells (2×10^5/well) were cultured in the top chamber of 24-well transwell plates that had been coated with Matrigel. The different diluted CM and concentrations (100–200 ng/ml) of CCL17 in RPMI 1640 medium containing 10 % FBS were added to the bottom chamber, separately. After a 48-h culture, the Matrigel was removed. The invaded cells were stained with crystal violet, and the numbers of invaded cells were counted under a microscope. Five fields (200× magnification) were randomly selected, and the average number of invaded cells per field was calculated.

Transduction of TAM with ccl17 shRNA

Lentiviral constructs expressing ccl17 shRNA (LV-CCL17-RNAi) or control LV-GFP-RNAi were from Shanghai Genechem, China. The target sequences are CTT AGAAAGCTGAAGACGT for CCL17 and TTCTCCGAA CGTGTCACGT for the control, respectively. The induced M2 macrophages (5×10^4/well) were cultured overnight in six-well plates and infected with LV-CCL17-RNAi or control LV-GFP-RNAi at a multiplicity of infection (MOI) of 60 in Enhanced Infection Solution (Genechem) containing 8 μg/ml polybrene (Santa Cruz Biotechnology). After incubation for 12 h, the cells were exposed to fresh RPMI1640 medium and cultured for another 72 h. The infection efficacy was examined under a fluorescence microscope. The ccl17-silencing M2 ($M2^{ccl17mute}$) macrophages were used for functional study with MHCC97L.

Cell proliferation assay

The impact of M2 or CCL17 on the proliferation of MHCC97L cells was determined by CCK8 assay using the specific reagent, according to the manufacturers' instruction (Beyotime Biotech). Briefly, MHCC97L cells (2×10^3 cells/well) were treated in triplicate with the CM from M2 cells, from the co-culture of $M2^{ccl17mute}$ with MHCC97L cells, control CM of cultured MHCC97L cells as well as 200 ng/ml of CCL17 for 9 h. During the last 3-h culture, the cells were exposed to 10 μl WST-8 reagent and the absorbance at 450 nm was measured using a microplate reader (xMark^{TM} Microplate Spectrophotometer System, Bio-Rad, CA, USA).

Cell pluripotency analysis

The impact of M2 or CCL17 on the stemness of MHCC97L cells was determined by sphere formation and side population using flow cytometry. Briefly, HMCC97L cells (2000 cells/well) were cultured in the CM from M1, M2 cells or $M2^{ccl17mute}$ or treated with 100 or 200 ng/ml of CCL17 for 72 h. After being washed, the cells were harvested and cultured for 7 days in the ultra-low attachment cell culture flasks (50 ml, NEST) in DMEM/F12 medium supplemented with 50 μl 1× B27 (life), 20 ng/ml recombinant human FGF-1, 20 ng/ml human EGF (Peprotech) and 5 μg/ml insulin. The sizes of 20 sphere selected randomly were evaluated in a blinded manner.

The impact of M2 or CCL17 on the stem-like side population (SP) in MHCC97L cells was determined by FCM, as described previously [12]. Briefly, MHCC97L cells (1×10^6/well) were stained with 20 μg/ml Hochest 33342 (Sigma) at 37 °C for 90 min and analyzed by FCM. The viable cells without Hochest33342 staining were considered as the SP cells.

Western blotting

MHCC97L cells were cultured in control medium, the CM from M1, M2 or $M2^{ccl17mute}$, or treated with, or without, 100 or 200 ng/ml of CCL17 for 48 h. The cells were harvested and lyzed in whole cell lysis buffer (KeyGEN). The nuclear proteins from some cells in individual groups were extracted using the Nuclear Cytoplasmic Protein Extraction Kit (KeyGEN). The cell lysates (40 μg/lane) or the extracted nuclear proteins (20 μg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12 % gels and transferred onto PVDF membranes. After being blocked with 5 % fat-free milk, the membranes were incubated with rabbit monoclonal antibody (mAb) against TGF-β1 (1:1000), phospho-Smad3 (Ser423/425,1:1000), E-cadherin (1:1000), N-cadherin (1:1000), vimentin (1:1000), Snail (1:1000), Nanog (1:1000), Oct-4 (1:1000), Sox-2(1:1000), β-catenin (1:1000), phospho-β-catenin (Ser33/37/Thr41, 1:1000), Wnt3a (3A6, abcam, 1:1000), survivin (abcam,1:10,000), control β-actin (1:10,000) or nuclear protein control lamin B1 (D9V6H, CST, 1:1000) overnight at 4 °C. Subsequently, the bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000) at 37 °C for 1 h and visualized using enhanced chemiluminescence (ECL) kit (Beyotime Biotech). All primary and secondary antibodies were from Cell Signaling Technology (Danvers, USA) unless indicated. The relative levels of target proteins to the control were determined by densitometric scanning using Imaging J software.

Immunofluorescence

The distribution of β-catenin expression in MHCC97L cells was determined by immunofluorescence assay. Briefly, MHCC97L cells that had been co-cultured with M2 or $M2^{ccl17mute}$ were fixed with 4 % paraformaldehyde for 30 min, permeabilized with 0.1 % Triton X-100 (Sigma) for 5 min and blocked with 10 % goat serum (Thermo) for 60 min at room temperature, followed by incubation of anti-β-catenin (1:100) at 4 °C overnight. After being washed, the cells were stained with Alexa Fluor 555 goat anti-rabbit IgG (H + L) antibody (Life Technology, 1:500) at 37 °C for 1 h and co-stained with DAPI (Beyotime, 1:1000). The cells were examined under a fluorescent microscope.

In vivo tumor formation

Male Balb/c nude mice at 4 weeks old were purchased from the Laboratory Animal Center of Chongqing Medical University and housed in a specific pathogen-free (SPF) facility. The mice were randomized and injected subcutaneously with 2×10^6 MHCC97L cells plus 1×10^6 M2; 2×10^6 MHCC97L plus 1×10^6 $M2^{ccl17mute}$; 2×10^6 MHCC97L cells plus 1×10^6 M1; 2×10^6 MHCC97L cells plus 1×10^6 M0; or 2×10^6 MHCC97L cells alone ($n = 5$ per group) to the left flank of mice. The development and progression of formed tumors were monitored by measuring the tumor size every 6 days using a vernier calipers and calculating the tumor volume (V) according to the following equation [13]: $V(mm^3) = \text{length} \times \text{width}^2/2$. All tumors were harvested at sixth week post-inoculation.

Statistical analysis

Data are presented as mean ± SD. The difference among the groups was analyzed using one-way analysis of variance (ANOVA), student's t test, Chi-square analysis, log-rank test and COX regressive analysis using the SPSS21.0 software (IBM) and GraphPad Prism 5 (GraphPad Software). A P value of <0.05 was considered statistically significant.

Results

Higher levels of intratumoral CCL17 expression are associated with poorer survival of HCC patients

To determine the potential importance of CCL17 in the pathogenesis of HCC, 90 surgically intratumoral and peritumoral HCC tissues were examined for CCL17 expression by immunohistochemistry (Fig. 1a). Because most peritumoral HCC tissues displayed negative or low CCL17 expression, we considered them as negative or positive. In contrast, varying levels of positive anti-CCL17 staining were detected in intratumoral tissues and were counted as high or low expression. The overall median follow-up period was 35 months for those patients with a median overall survival of 36 months and a median disease-free survival of 30 months. Stratification analysis indicated that high levels of intratumoral CCL17 expression were associated with aging, tumor sizes, pathologic grades and TNM stages and positive metastasis (Table 1). Multiple variate analysis revealed that high levels of intratumoral CCL17 expression were associated with pathologic grades (HR = 1.655, $P = 0.027$) and high levels of AST in HCC patients of this population (Table 2). Analysis of the survival of 90 patients indicated that the periods of patients with high intratumoral CCL17 expression were significantly shorter than those with low intratumoral CCL17 expression. As a result, higher levels of intratumoral CCL17 expression were associated significantly with poorer survival of patients with HCC. These data suggest that CCL17 may be a pathogenic factor for the development and progression of HCC (Table 3).

CCL17 is expressed predominantly by M2 cells

CCL17 is a chemokine expressed by macrophages. To characterize the expression of CCL17 and its receptor of CCR4, monocyte-like THP-1 cells were activated by PMA and their differentiation was induced by LPS/IFN-γ and IL-4/MCSF stimulation, respectively. FCM analysis indicated that LPS/IFN-γ stimulation induced $p40^+$ M1 cells, while IL-4/MCSF stimulation induced $CD163^+CD11b^+$ M2 cells (Fig. 2a). Similarly, the relatively higher levels of p40, CCR9, but lower CCL17 mRNA transcripts were detected in M1 cells, while higher levels of CD163, CD206 and CCL17 mRNA transcripts were observed in M2 cells (Fig. 2b) Furthermore, higher levels of CCL17 and arginase-1 were detected in M2 cells (Fig. 2c). In addition, higher levels of CCL17 were detected in the supernatants of cultured M2 and following co-culture with MHCC97L cells, but no CCL17 was observed in the supernatants of MHCC97L cells (Fig. 2d). Moreover, FCM analysis revealed high levels of CCR4 on the surface of MHCC97L cells.

CCL17 enhances the survival, migration and tumorigenesis of MHCC97L cells

To investigate the role of CCL17 in the tumorigenesis of MHCC97L cells, we generated stable CCL17-silencing MHCC97L cells. We tested the impact of CM from M2 or $M2^{ccl17mute}$ or CCL17 on the wound healing and invasion

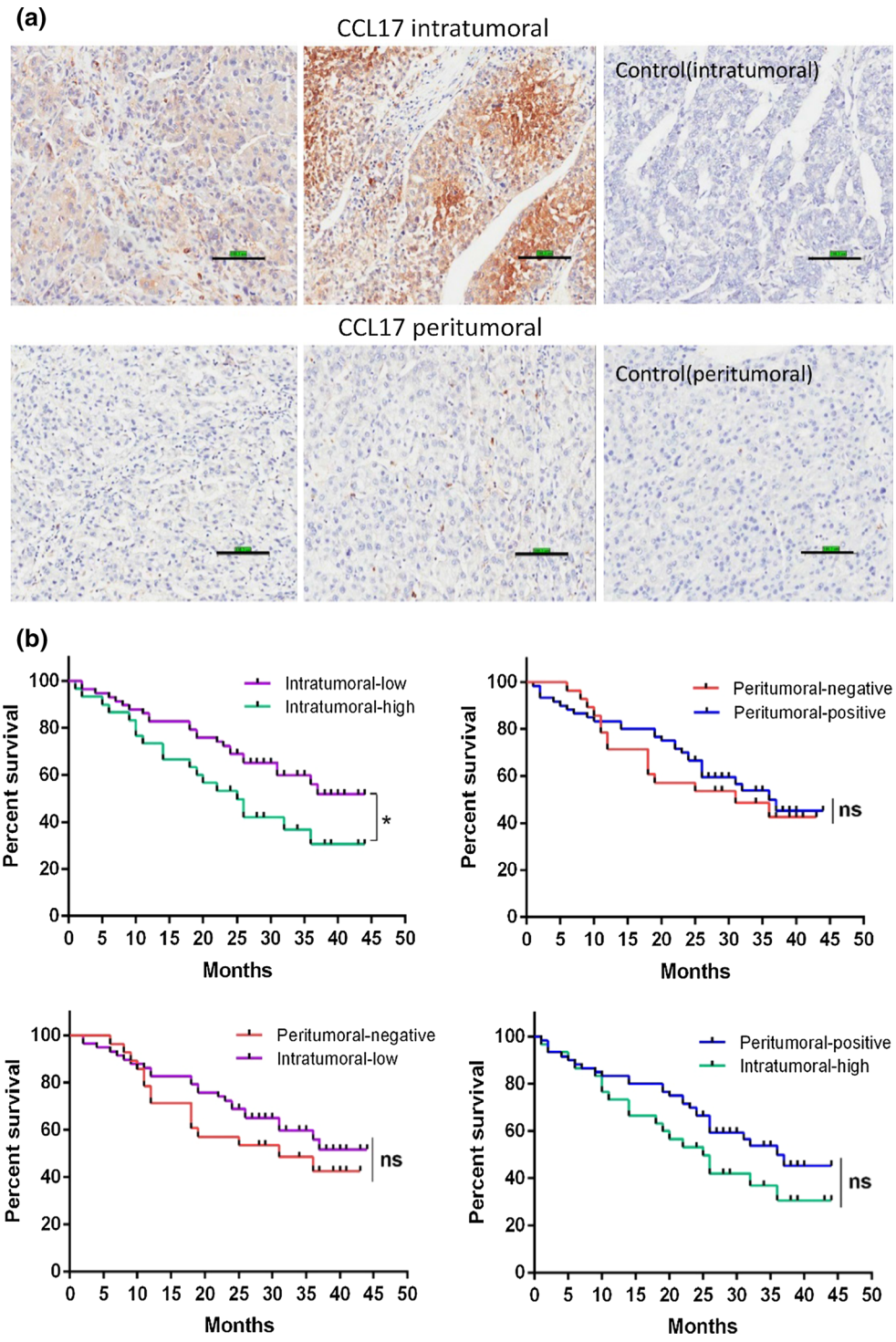

Fig. 1 CCL17 expression in HCC tissues and its association with patients' survival. The levels of CCL17 expression in 90 pairs of HCC and surrounding tissues are determined by immunohistochemistry using anti-CCL17 antibodies. The survival of those patients is stratified, according to the levels of CCL17 expression and statistically analyzed. Data are representative images or survived curves of different groups of groups of patients. **a** The immunohistochemistry staining of intratumoral and peritumoral CCL17 expression in HCC tissues ($n = 90$). Magnification, $\times 200$. *Scale bar* 100 μm. **b** The survival curves of different groups of patients with varying levels of intratumoral and peritumoral CCL17 expression. $^*P < 0.05$

in vitro, and we found that the CM from M2, M2^{ccl17mute} or CCL17 promoted the wound healing process and the effect of the CM from M2 was obviously stronger than that of the CM from M2^{ccl17mute} cells. A similar pattern of enhanced invasion of MHCC97L cells was observed different groups of cells (Fig. 3b, c). However, any treatment did not significantly increase the percentages of apoptotic MHCC97L cells in vitro (Fig. 3d). Furthermore, while treatment with CCL17 or CM from M2 cells promoted significantly, the proliferation of MHCC97L cells treatment with CM from M2^{ccl17mute} cells did not significantly affect the proliferation of MHCC97L cells (Fig. 3e). Following inoculation with MHCC97L cells alone or combination with indicated type of cells, longitudinal measurements revealed that the

Table 1 Relationship between the CCL17 expression intratumoral or peritumoral and clinical parameters of HCC

	HCC intratumoral			HCC peritumoral		
	CCL17 expression		*P*	CCL17 expression		*P*
	High (32)	Low (58)		Positive (62)	Negative (28)	
Gender						
Male	22	39	0.883	44	20	0.964
Female	10	19		18	8	
Age						
≤65	18	50	0.002**	49	19	0.253
>65	14	8		13	9	
Tumor size						
≤5 cm	5	46	0.000***	22	10	0.983
>5 cm	27	8		40	18	
Pathological grading						
I–II	12	53	0.000***	46	19	0.534
III	20	5		16	9	
TNM stage						
I/II	9	51	0.000***	36	18	0.577
III/VI	23	7		26	10	
Tumor capsular						
Present	21	44	0.299	48	17	0.101
Absent	11	14		14	11	
Metastasis						
Yes	20	17	0.002**	23	14	0.249
No	12	41		39	14	
ALT						
Normal (≤40)	12	25	0.605	26	11	0.813
Abnormal (>40)	20	33		36	17	
AST						
Normal (≤40)	15	29	0.776	31	13	0.754
Abnormal (>40)	17	29		31	15	
AFP						
Normal (≤20)	4	7	1	8	3	1
Abnormal (>20)	28	51		54	25	
HBsAg						
Normal (≤0.04)	11	27	0.263	25	13	0.587
Abnormal (>0.04)	21	31		37	15	

Tumor size, pathological grading, TNM stage, metastasis are highly associated with high CCL17 expression in HCC, intratumoral

** $P < 0.01$; *** $P < 0.001$

tumor volumes in the mice receiving HCC and M2 were significantly greater than that in other groups of mice (Fig. 3g) and the tumor size and weights from the mice receiving MHCC97L and M2 were obviously larger than that in other groups of mice (Fig. 3f, h). Collectively, these data indicated that the CM from M2 cells or CCL17 enhanced the migration, invasion and proliferation of MHCC97L cells in vitro and promoted the tumor growth of MHCC97L cells in vivo.

CCL17 enhances the stemness of MHCC97L cells

Cancer stem cells are crucial for recurrence and metastasis of cancer. To determine the potential role of CCL17 in the

Table 2 Analyses of clinical parameters associated with HCC patients overall survival by COX regression model

Parameters	HR	*P*	95 % CI	Parameters	HR	*P*	95 % CI
Gender				*ALT*			
Male versus female	0.908	0.786	(0.454–1.819)	≤40 versus >40	2.017	0.131	(0.812–5.013)
Age				*AST*			
≤65 versus >65	1.33	0.458	(0.627–2.824)	≤40 versus >40	0.342	0.016*	(0.143–0.817)
Tumor size				*AFP*			
≤5 versus >5 cm	2.428	0.075	(0.915–6.447)	≤20 versus >20	3.103	0.114	(0.763–12.613)
Pathological grading				*HBsAg*			
I–II versus III	1.655	0.027*	(1.059–2.586)	≤0.04 versus >0.04	0.682	0.334	(0.314–1.482)
TNM stage				*CCL17 intra*			
I/II versus III/VI	1.925	0.086	(0.912–4.063)	High versus low	0.521	0.189	(0.197–1.379)
Tumor capsular				*CCL17 peri*			
Present versus absent	0.775	0.508	(0.364–1.649)	Positive versus negative	0.963	0.917	(0.472–1.966)
Metastasis							
Yes versus no	1.159	0.643	(0.621–2.162)				

HR hazard ratio, *CI* confidence interval, *intra* intratumoral, *peri* peritumoral

* $P < 0.05$

Table 3 Primers used in the study

Gene	Sense (5′–3′)	Antisense (5′–3′)
p40	ACCCTGACCATCCAAGTCAAA	TTGGCCTCGCATCTTAGAAAG
ccr9	ATGTCAGGCAGTTTGCGAG	TGCAGTACCAGTAGACAAGGAT
cd163	TTTGTCAACTTGAGTCCCTTCAC	TCCCGCTACACTTGTTTTCAC
cd206	TCCGGGTGCTGTTCTCCTA	CCAGTCTGTTTTTGATGGCACT
ccl17	GAGCCATTCCCCTTAGAAAG	AGGCTTCAAGACCTCTCAAG

stemness of MHCC97L cells, we determined the impact of the CM from M1, M2, M2^{ccl17mute} or CCL17 on the sphere formation in vitro. The size of formed spheres from the M2 CM-treated MHCC97L was obviously larger than that of other groups of cells (Fig. 4a). Similarly, the stem-like side population in the cells treated with M2 CM was near threefold greater than other groups of cells determined (Fig. 4b). Following co-culture with different types of macrophages, the relative levels of Nanog, Oct-4 and Sox-2, critical transcription factors of cancer stem cells, in the MHCC97L co-cultured with M2, were obviously higher than that in other groups of cells (Fig. 4c). Treatment with CCL17 also significantly up-regulated the levels of Nanog, Oct-4 and Sox-2 expression in MHCC97L cells. In addition, up-regulated levels of TGF-β1 expression and Smad3 phosphorylation were detected in the MHCC97L following co-culture with M2 or treatment with CCL17 (Fig. 4d). The increased stem cell side population and sphere formation capacity and up-regulated transcription factor expression as well as increased levels of TGF-β1 signaling demonstrated that CCL17 or M2 cells enhanced stemness of MHCC97L cells in vitro.

CCL17 promotes the epithelial–mesenchymal transition and the Wnt/β-catenin signaling in MHCC97L cells

The EMT process is important for the migration and invasion of cancer cells. To understand the role of CCL17 or M2 in regulating the migration and invasion of MHCC97L cells, MHCC97L cells were co-cultured with M1, M2, M2^{ccl17mute} or treated with CCL17 and the relative levels of E-cadherin, N-cadherin, vimentin and Snail were characterized by Western blot assay (Fig. 5a). Co-culture with M2 clearly increased the relative levels of N-cadherin, vimentin and Snail, but decreased the levels of E-cadherin expression in MHCC97L cells. Similarly, treatment with CCL17 also increased levels of N-cadherin, vimentin and Snail, but decreased the levels of E-cadherin expression in MHCC97L cells. In contrast, co-culture with M1 had no such regulatory effect and co-culture with M2^{ccl17mute} only moderately altered the expression levels of these factors in MHCC97L cells.

Aberrant activation of the Wnt/β-catenin signaling is associated with tumorigenesis and metastasis in HCC [14–

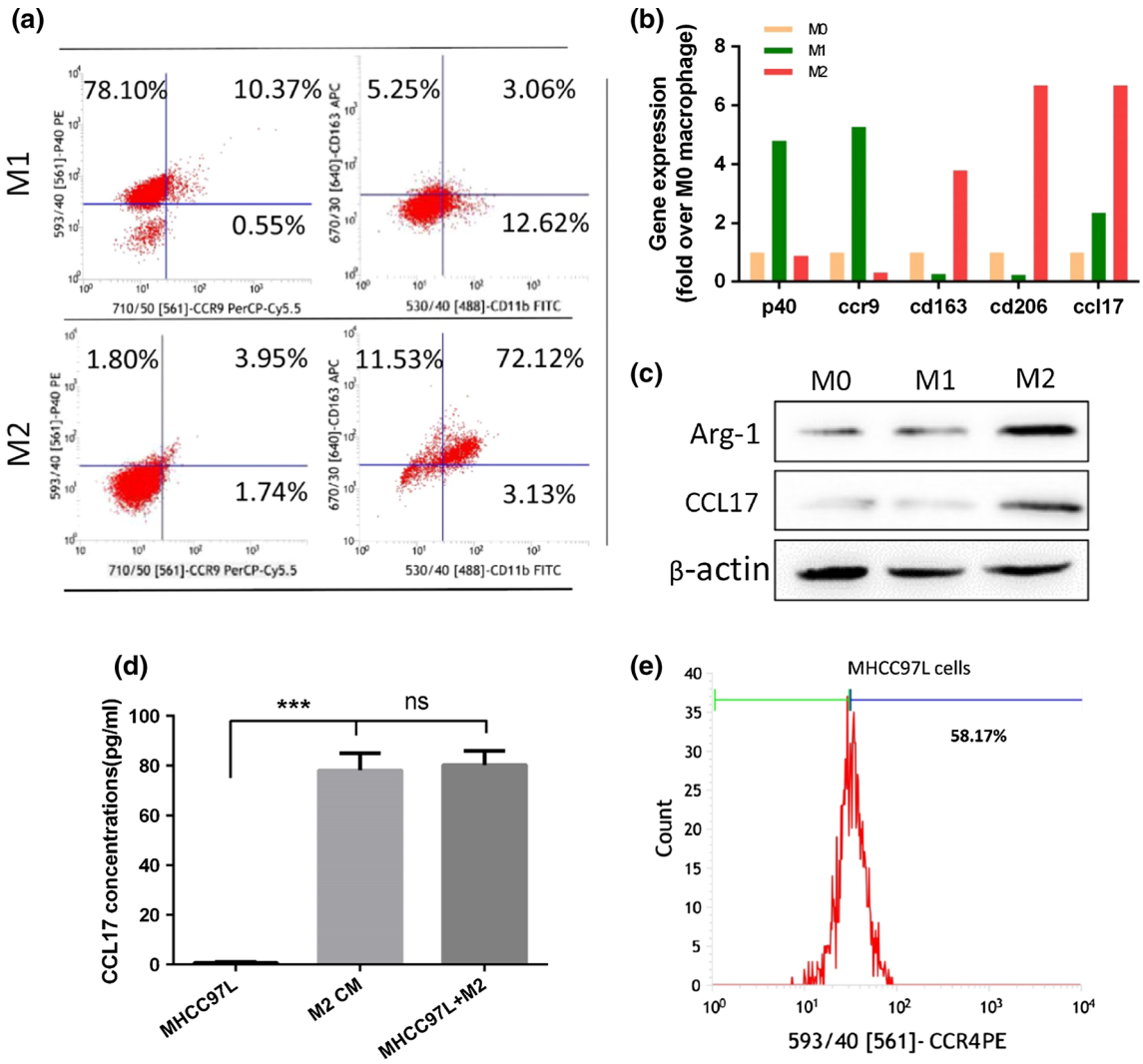

Fig. 2 Characterization of differential M1 and M2 cells. THP-1 cells are activated with PMA and stimulated with IFNγ/LPS or IL-4/MCSF for M1 or M2 differentiation, respectively. The cells are characterized by FCM, quantitative RT-PCR and Western blot assays. Furthermore, the levels of secreted CCL17 are determined by ELISA and the levels of CCR4 in MHCC97L cells are determined by FCM. Data are representative images, charts, histogram or expressed as the mean ± SEM of each group of cells from at least three separate experiments. **a** FCM analysis of M1 and M2 cells. **b** Quantitative RT-PCR analysis of the relative levels of M1- or M2-related gene mRNA transcripts. **c** Western blot analysis of the relative levels of Arg-1 (M2 marker) and CCL17 expression in M1 and M2 cells. **d** ELISA analysis of the levels of CCL17 in the supernatants of cultured M1, M2 and MHCC97L cells. In addition, **e** the $CCR4^{+}$ expression in MHCC97L cells. $^{***}P < 0.001$; *NS* no significant difference

16]. Finally, we tested the impact of M2 or CCL17 on the Wnt/β-catenin activation in MHCC97L cells. First, co-culture with M2 increased the frequency of nuclear β-$catenin^{+}$ MHCC97L cells, while co-culture with $M2^{ccl17\text{-}mute}$ only displayed a few nuclear β-$catenin^{+}$ MHCC97L cells (Fig. 5b). Western blot analysis indicated that co-culture with M2 increased the relative levels of nuclear β-catenin, but decreased the levels of phosphorylated β-catenin in MHCC97L cells (Fig. 5c). In contrast, co-culture with monocytes, M1 or $M2^{ccl17mute}$ did not alter the relative levels of nuclear β-catenin and β-catenin phosphorylation in MHCC97L cells. In addition, treatment with CCL17 up-regulated Wnt3a and survivin expression in MHCC97L cells. Therefore, CCL17 or M2 promoted the EMT process and enhanced the Wnt/β-catenin signaling in MHCC97L cells in vitro.

Discussion

In this study, we have investigated the potential pro-tumor function of CCL17 in HCC. We found high levels of CCL17 were predominantly expressed in intratumoral regions, but not in peritumroal areas in HCC. Stratification

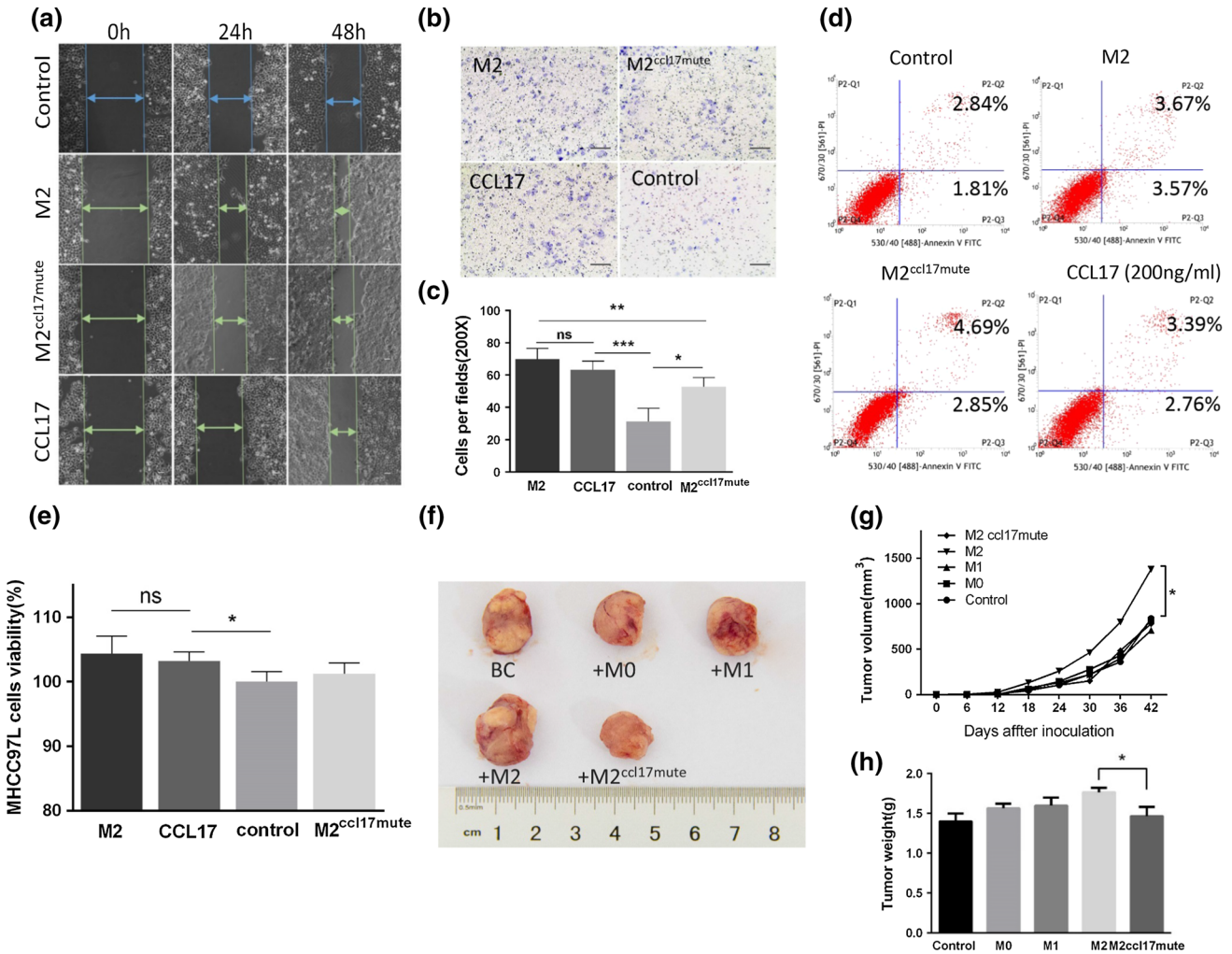

Fig. 3 M2 and CCL17 promotes the proliferation, migration and invasion of MHCC97L cells. MHCC97L cells are treated with the CM from M1, M2 or M2^{ccl17mute} or with CCL17, and their wound healing process, invasion, proliferation and apoptosis are determined. In addition, MHCC97L cells are co-implanted with M0, M1, M2, or M2^{ccl17mute} into male BALB/c nude mice and the growth of tumors is monitored longitudinally. At the end of the experiment, the tumors are dissected out and weighed. Data are representative images or FCM charts or expressed as the mean ± SEM of each group of cells or mice ($n = 5$ per group) from three separate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; *NS* no significant difference. **a** Wound healing assays. Magnification, ×100. *Scale bar* 100 μm. **b**, **c** Transwell-Matrigel invasion assay. Magnification, ×200. *Scale bar* 100 μm. **d** Apoptosis of MHCC97L cells. **e** CCK8 assay for the proliferation of MHCC97L cells. **f–h** In vivo tumor growth and weights

analysis indicated that higher levels of intratumoral CCL17 expression were associated significantly with aging, pathologic grades, TNM stages, tumor sizes and metastasis in this population. More importantly, high levels of intratumoral CCL17 expression were associated with poorer overall survival of HCC patients. Besides, we found that in HCC intratumoral, higher CCL17 expression is in associated with larger tumor size, higher pathological grading, higher TNM stage and metastasis; all those factors may contribute to the liver impairment. Given that CCR4 is expressed by HCC cells besides activated Th2 and regulates the proliferation of T cell leukemia and lymphoma [17, 18], it is possible that CCL17 through CCR4 may contribute to the development and progression of HCC. Our finding extended previous observations in other types of solid tumors [6, 19–21] and suggests that the CCL17/CCR4 signaling may promote the development and progression of HCC. Hence, the CCL17/CCR4 signaling may be a promising biomarker for the prognosis of HCC and a target for design of new therapies of HCC.

To understand the potential role of CCL17 in regulating the tumorigenesis of HCC, we first tested the impact of the CM from M1, M2, M2^{ccl17mute} or treatment with CCL17 on the proliferation, migration, invasion and apoptosis of MHCC97L cells in vitro. We found that exposure to the CM from M2, M2^{ccl17mute} or treatment with CCL17

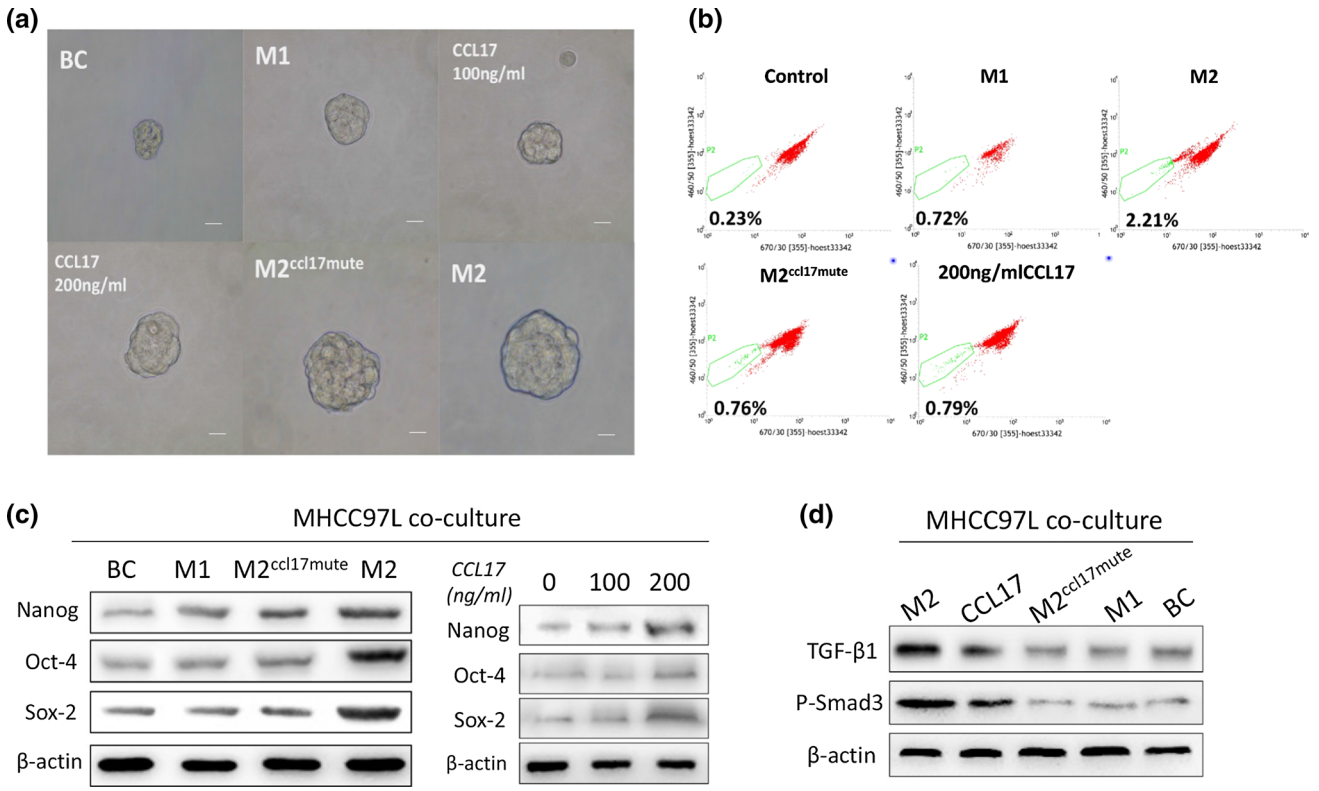

Fig. 4 M2 and CCL17 positively regulate the stemness of MHCC97L cells. MHCC97L cells are treated with the CM from M0, M1, M2 or M2^{ccl17mute} or treated with the indicated concentrations of CCL17. The sphere formation is recorded, and side population is determined by FCM. In addition, MHCC97L cells are co-cultured with M0, M1, M2 or M2^{ccl17mute} or treated with the indicated concentrations of CCL17 and the relative levels of Nanog, Oct-4 and Sox-2 and TGF-β1 expression and Smad3 phosphorylation in individual groups of cells are determined by Western blot assays. Data are representative images or FCM charts or expressed as the mean ± SEM of each group of cells from three separate experiments. **a** Sphere formation. Magnification ×200 *scare bar* 200. **b** Side populations of MHCC97L cells measured with flow cytometric. **c** Western blotting analysis of the relative levels of Nanog, Oct-4, Sox-2 in MHCC97L cells. **d** Western blotting analysis TGF-β1 and phospho-Smad3 in MHCC97L cells

significantly enhanced the proliferation, migration and invasion of MHCC97L cells, but did not affect spontaneous MHCC97L cell apoptosis and the enhanced effects of CM from M2 were greater than that from M2^{ccl17mute} cells. More importantly, co-implantation with MHCC97L cells with M2, but not with other types of cells, significantly promoted the growth of implanted MHCC97L tumors in vivo. Our findings clearly indicated that CCL17 promoted the progression and metastasis of HCC. Notably, treatment with the CM from M2^{ccl17mute} moderately promoted the proliferation, migration and invasion of MHCC97L cells. We found that CCL17 knockdown effectively reduced CCL17 expression by 95 % in M2 cells. The remaining pro-tumor activity of the CM from M2^{ccl17mute} suggests that other factors secreted by M2 cells also promote the proliferation, migration and invasion of MHCC97L cells. Indeed, IL-10 and TGF-β1 can be secreted by M2 cells and they can promote the proliferation and migration of tumor cells [22]. We are interested further investigating what types of unknown factors secreted by M2 cells can promote the proliferation and migration of HCC cells.

The EMT process is crucial for the metastasis of cancer [23]. We found that treatment with the CM from M2 significantly downregulated the expression of E-cadherin, but up-regulated the expression levels of N-cadherin, Snail and vimentin in MHCC97L cells, as compared with that in the cells treated with the CM from M2^{ccl17mute}, suggesting that factors secreted by M2 cells enhanced the EMT process in HCC. Similarly, treatment with CCL17 also enhanced the EMT process in MHCC97L cells, consistent with the findings of promoting wound healing, migration and invasion of MHCC97L cells. Furthermore, co-culture with M2 cells or treatment with CCL17 enhanced the TGF-β1/Smad signaling in MHCC97L cells. Given that the TGF-β1/Smad signaling positively regulates the EMT process and metastasis of HCC [24, 25], it is possible that CCL17/CCR4 signaling through activating the TGF-β1/Smad signaling enhances the EMT process of HCC cells and promotes the metastasis. We are interested in further

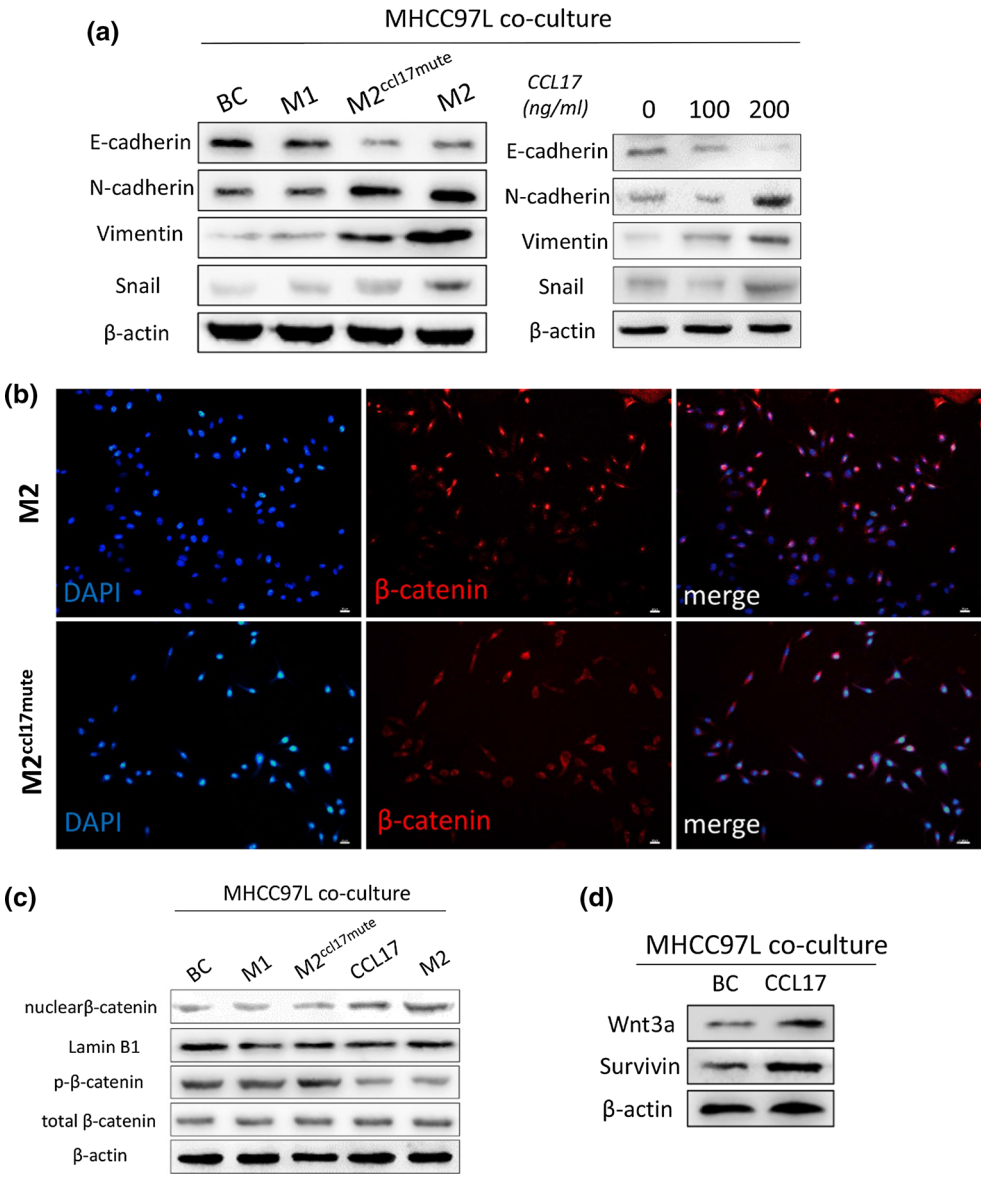

Fig. 5 M2 or CCL17 promotes the EMT process and enhances the Wnt/β-catenin signaling in MHCC97L cells. MHCC97L cells are treated with the CM from M0, M1, M2 or $M2^{ccl17mute}$ or treated with the indicated concentrations of CCL17. The relative levels of E-cadherin, N-cadherin, vimentin, Snail expression are determined by Western blot assay. In addition, MHCC97L cells are co-cultured with M0, M1, M2, $M2^{ccl17mute}$ or treated with the indicated concentrations of CCL17 and the levels of nuclear and cytosolic β-catenin are determined immunofluorescent assays. The relative levels of nuclear β-catenin and cytosolic Wnt3a, surviving expression and β-catenin phosphorylation are determined by Western blot assay. Data are representative images from each group of cells from three separate experiments. **a** Western blot analysis of the levels of EMT factor expression in MHCC97L cells. **b** Immunofluorescent analysis of nuclear and cytosolic β-catenin in MHCC97L cells. Magnification, ×100. *Scale bar* 20 μm. **c** Western blot analyze the Wnt/β-catenin signaling. **d** Western blot analysis of the levels of Wnt3a and surviving expression in MHCC97L cells

investigating the molecular mechanisms underlying regulation of CCL17/CCR4 on the TGF-β1/Smad signaling and EMT process and metastasis of HCC.

CSCs have the stemness characteristics of transcription factor Nanog, Oct-4 and Sox-2 expression, form spheres in vitro and are important for the recurrence, metastasis and drug resistance of cancer [26, 27]. The stemness of CSC is positively regulated by aberrant activation of the Wnt/β-catenin signaling [28]. In this study, we found that treatment with the CM from M2 cells or with CCL17 promoted the sphere formation and CSC cell expansion in MHCC97L cells. Furthermore, co-culture with M2 cells or treatment with CCL17 increased the relative levels of Nanog, Oct-4 and Sox-2 expression and enhanced the Wnt/β-catenin activation in MHCC97L cells. These data suggest that CCL17/CCR4 signaling or M2 cells may positively regulate the stemness of HCC CSC by enhancing the Wnt/β-catenin activation.

In summary, our data indicated that higher levels of intratumoral CCL17 expression were significantly associated with clinical pathological characteristics of HCC and with poorer overall survival in HCC patients. High levels of CCR4 were expressed by MHCC97L cells. Treatment with the CM from M2, or with CCL17 significantly enhanced the wound healing process, invasion and proliferation of MHCC97L cells. Co-implantation of MHCC97L cells with M2 significantly promoted the growth of MHCC97L tumors in vivo. Co-culture with M2 or treatment with CCL17 enhanced the stemness, the EMT process, the TGF-β1 and Wnt/β-catenin signaling in

MHCC97L cells. Our data suggest that CCL17 may be a new biomarker for prognosis of HCC patients and a target for design of new therapies for HCC.

Funding This study was funded by the grants from the National Natural Science Foundation (Grant Nos. 81071882 and 81372481) and the National Key Clinical Specialties Construction Program of China.

Compliance with ethical standards

Conflict of interest Authors declare no conflicts of interest.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All patients included in the study gave written consent themselves. For studies with animals, we declare all applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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