

# Regulation of the migration of colorectal cancer stem cells via the TLR4/MyD88 signaling pathway by the novel surface marker CD14 following LPS stimulation

YUFEI LI<sup>1</sup>, JIAYI SHI<sup>2</sup>, ZHIXIN LIU<sup>1</sup>, YONGGANG LIN<sup>3</sup>,  
AN XIE<sup>1</sup>, WENXIU SUN<sup>1</sup>, JIAQI LIU<sup>1</sup> and JUN LIANG<sup>1</sup>

<sup>1</sup>Morphology Laboratory; <sup>2</sup>School of Life Sciences, Mudanjiang Medical University;  
<sup>3</sup>Department of Extracorporeal Circulation, Mudanjiang Cardiovascular Disease Hospital,  
Mudanjiang, Heilongjiang 157011, P.R. China

Received September 7, 2023; Accepted November 24, 2023

DOI: 10.3892/ol.2023.14194

**Abstract.** Cell surface markers are most widely used in the study of cancer stem cells (CSCs). However, cell surface markers that are safely and stably expressed in CSCs have yet to be identified. Colonic CSCs express leukocyte CD14. CD14 binding to the ligand lipopolysaccharide (LPS) is involved in the inflammatory response via the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) signaling pathway. TLR4 and MyD88 have been reported to promote the proliferation, metastasis and tumorigenicity of colon cancer cells, which is consistent with the characteristics of CSCs. In the present study, the proposed experimental method to detect cell proliferation, metastasis and tumorigenesis was used to confirm that, under LPS stimulation, CD14 promoted the proliferation, migration and tumorigenesis of colonic CSCs via the TLR4/MyD88 signaling pathway. Cell Counting Kit-8 and 5-ethynyl-2'-deoxyuridine assays were used to assess the proliferation and migration of the cells. Colony formation and nude mouse xenograft assays were used to assess the capacity of cells to form tumors. Using western blotting and reverse transcription-quantitative PCR, the mRNA and protein levels of CD14, TLR4 and MyD88 were examined. It was confirmed that CD14 promoted the proliferation, metastasis and tumorigenesis of colon CSCs in response to LPS stimulation via the TLR4/MyD88 signaling pathway, and CD14<sup>+</sup> colon cancer cells were successfully isolated and sorted. According to the results of proliferation assay, it was determined that CD14 regulated the LPS-induced proliferation of colon CSCs. CD14, TLR4

and MyD88 protein and mRNA expression was upregulated in colon CSCs in response to LPS stimulation. This indicates a potential novel target for colon CSC-related studies.

## Introduction

Adenocarcinomas account for 96% of cases of colorectal cancer (CRC), which is defined as cancer of the colon or rectum (1,2). At present, therapeutic advances and improved early detection screening are available; however, CRC continues to rank among the leading causes of cancer-related mortality worldwide (1,3). The primary causes of this are post-operative cancer recurrence or metastasis, as well as cancer cell drug resistance (4), which is related to CRC stem cells (CCSCs), a subpopulation of CRC cells with the capacity to self-renew, differentiate into multiple lineages, resist therapy and develop metastasis (5). Therefore, studies are currently underway to create novel CCSC-targeted therapeutics that will enhance the isolation and differentiation of CCSCs from other types of CSCs (6,7).

The most frequently employed markers in CCSC research are cell surface markers (7). For example, targeting CD133, CD166, CD44, aldehyde dehydrogenase 1 (ALDH1), leucine rich repeat containing G protein-coupled receptor 5 (Lgr5) and epithelial cell adhesion molecule, cell surface markers present on CCSCs, with monoclonal antibodies has the potential to shrink tumors and lessen metastasis (8). The lack of a consistent and reliable marker for CCSCs restricts their use in clinical practice (7), and they are also present in varying degrees in stem cells from normal tissues or other cancer types (9,10).

To initiate pro-inflammatory reactions to invading pathogens, lipopolysaccharide (LPS) and CD14, a particular surface marker of monocytes, macrophages and neutrophils, interact via the Toll-like receptor 4 (TLR4) signaling pathway (8,11,12). Notably, LPS promotes CRC development and metastasis via the TLR4 signaling pathway (13-15). Additionally, CD14 has been linked to tumor recurrence, growth, metastasis and therapy resistance, which is consistent with CSC traits (such as recurrence, growth, metastasis and resistance to therapy),

---

*Correspondence to:* Professor Jun Liang, Morphology Laboratory, Mudanjiang Medical University, 3 Tongxiang Road, Mudanjiang, Heilongjiang 157011, P.R. China  
E-mail: 2193232585@qq.com

**Key words:** CD14, cell surface markers, colorectal cancer stem cells, Toll-like receptor 4/myeloid differentiation factor 88, lipopolysaccharide

suggesting also that CD14 may be linked to CCSCs (16-18). Furthermore, a previous study by the authors revealed that esophageal CSCs expressed CD14, a novel surface marker (19).

In the present study, CD133 and CD14 were examined by immunofluorescence double labeling to qualitatively assess CD14 expression in CCSCs in paraffin-embedded slices of CRC tissues and tissues adjacent to the tumor. Subsequently, CD14<sup>+</sup> cells were extracted from CRC tissues to examine the stemness characteristics by analyzing proliferation, tumorigenicity and treatment resistance to corroborate the phenotypic identification. Furthermore, the *in vitro* detection of migration enabled the examination of CD14 function.

## Materials and methods

**Patients and specimens.** CRC tissues were obtained from 60 patients (median age, 60.3 years; range, 45-78 years) who underwent surgical resection without radiotherapy from January, 2017 to January, 2022 at Hongqi Hospital, Mudanjiang Medical University (Heilongjiang, China). Paraffin-embedded sections of colon cancer tumor tissues [i) seven highly differentiated squamous carcinomas; ii) nine moderately differentiated squamous carcinomas; iii) six lowly differentiated squamous carcinomas; iv) 14 highly differentiated adenocarcinomas; v) 12 moderately differentiated adenocarcinomas; vi) seven lowly differentiated adenocarcinomas; and vii) seven paracarcinomatous tissues] were obtained from Mudanjiang Tumor Hospital, and five post-operative colon cancer tumor tissues of patients who have not been treated with chemotherapy and radiotherapy (two highly differentiated and three moderately differentiated) were obtained from the Hongqi Hospital of Mudanjiang Medical University (Heilongjiang, China). The clinical and pathological data of the patients are presented in Table I. All patients signed an informed consent form. The present study was approved by the Ethics Committee of Mudanjiang Medical College (approval no. 2022-MYGZR06). The study used 12 specific pathogen-free grade nu/nu immunodeficient mice (male; age, 4-5 weeks; median body weight, 20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The animal experiments in the present study were approved by the Laboratory Animal Welfare and Ethics Committee of Mudanjiang Medical College (approval no. 20220228-26).

**Reagents.** The following reagents were used in the present study: Collagenase I (Coolaber), DMEM/F12 powder (Gibco; Thermo Fisher Scientific, Inc.); basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), leukemia inhibitory factor (LIF) (all from PeproTech, Inc.); CD133 polyclonal antibodies (cat. no. 18495-1-AP, Proteintech Group, Inc.); CD14 polyclonal antibodies (cat. no. CL647-65056; 1:1,000, Proteintech Group, Inc.); Protein RIPA Lysis Solution (cat. no. abs9229, Absin); SDS-PAGE Gel Rapid Preparation Kit (cat. no. abs9367-1Kit, Absin); ECL luminescent solution (cat. no. abs920-2, Absin); TLR4 primary antibody (cat. no. 132000; 1:1,000, Absin); myeloid differentiation factor 88 (MyD88) primary antibody (cat. no. abs135682; 1:1,000, Absin); human CD14 antibody (MAB3832; 1:1,000); LPS (cat. no. abs47014848, Absin); Cell Counting Kit-8 (CCK-8; cat. no. abs50003, Absin) and 5-ethynyl-2'-deoxyuridine

Table I. Clinicopathological characteristics of the 60 patients with colon cancer.

Characteristic	Value
Sex, n (%)	
Male	32 (53.3)
Female	28 (46.7)
Age, years, n (%)	
≥60	36 (60.0)
<60	24 (40.0)
Median age (range), years	60.3 (45-78)
Differentiation, n (%)	
Squamous carcinoma	22 (36.7)
Well	7 (11.7)
Moderate	9 (15.0)
Poor	6 (10.0)
Adenocarcinoma	38 (63.3)
Well	16 (26.7)
Moderate	15 (25.0)
Poor	7 (11.7)

(EdU) Assay/EdU Staining Proliferation Kit (Abcam); penicillin and streptomycin (Millipore, Sigma); β-actin antibody (AB0035, 1:1,000, Shanghai Abways Biotechnology Co., Ltd.); horseradish enzyme labeled goat anti-rabbit IgG (ZB-2301, 1:10,000, Beijing Zhongsui Jinqiao Biotechnology Co.); the EasySep™ Human CD14 Positive Selection Kit II (EasySep™; Stemcell Technologies, Inc.); Cellular Rapid RNA Extraction Kit (abs60027, Absin); SYBR Premix Ex Taq (Takara Bio, Inc.); Prime Script™ RT kit (RR047A, Takara Bio, Inc.).

**Immunofluorescence staining.** CD133 and CD14 co-expression in CRC was examined using tissue slices of the patient-derived paraffin-embedded tumor samples. Dewaxed paraffin sections (5 μm thickness) were placed in water, permeabilized with 0.3% Triton X-100 in PBS for 15 min at 37°C and then subjected to 3% peroxide in PBS for 10 min. In order to label CCSCs, the sections were incubated with CD133 antibody (1:100 dilution) at 37°C for 120 min and stained with the secondary antibody IgG Texas Red (cat. no. ab6800; 1:100 dilution; Abcam) at 37°C for 40 min. For the analysis of CD14, the sections were incubated again with CD14 antibody (1:100 dilution) at 37°C for 120 min and stained with another secondary antibody IgG FITC (cat. no. abs20004; 1:100 dilution; Absin Bioscience Inc.) at 37°C for 40 min. To facilitate cell counting, the sections were counterstained with DAPI (1:100 dilution, MilliporeSigma) at 37°C for 30 min. The results were observed under a fluorescence microscope (Nikon Corporation) and images were captured (scale bar, 50 μm).

**Primary culture of human CRC.** The surgically removed CRC tissues were transported to the laboratory as fast as possible. The tissues were washed three times with PBS, cut into 3-mm-thick sections and then incubated in serum-free DMEM/F12, 37°C, humidified atmosphere with 5% CO<sub>2</sub>. This was followed by the addition of 10 ng/l bFGF, 20 ng/l EGF, 20 ng/l LIF, 100,000 units/l penicillin and 100 mg/l

streptomycin. The medium was changed every other day until no new cells could proliferate 'crawl' out of the tissue block.

To mimic the process of inflammation promoting tumor development, CD14<sup>+</sup> cell activation was achieved using LPS. The inhibition of CD14 by CD14-neutralizing antibodies in the presence of LPS clarified the effect of CD14 on the migration of colonic CSCs.

**CD14<sup>+</sup> cell activation by LPS.** A total of three groups of CD14<sup>+</sup> cells were randomly formed: i) CD14 group (control group; untreated); ii) CD14 + LPS group (1 mg/l LPS for 24 h at 37°C) and CD14 neutralizing antibody + LPS group (10 µg/ml neutralizing antibody + 1 mg/l LPS for 24 h at 37°C). At the end of the treatment period, the cell supernatant of each group was collected and cell proteins were extracted as samples for subsequent experimental testing.

**Transwell migration assay.** Cell migration was detected using Nunc<sup>TM</sup> polycarbonate (cat. no. 140644; 8 µm pore size; six-well plates; Thermo Fisher Scientific, Inc.). Cells were inoculated in the upper chamber of the cell culture inserts in multiculture dishes at 1x10<sup>4</sup> cells/well in 500 µl DMEM/12, and 2 ml DMEM/F12 containing 10 ng/l bFGF, 20 ng/l EGF and 20 ng/l LIF as a chemotactic incubator was added to the lower chamber. Cells were placed in the CO<sub>2</sub> incubator at 37°C for 24 h. The waste solution was discarded, 0.1% crystal violet (MilliporeSigma) staining solution was added for 20 min at 37°C and cells were washed three times with PBS. The number of migrated cells was observed and counted under an inverted biological microscope (Olympus Corporation).

**Wound healing assay.** Cell migration was examined using a wound healing assay. Cells were seeded into a six-well plate and cultured until they reached 80% confluency. Following one PBS wash, the cells at the bottom of the six-well plate were directly scraped with a 100-µl pipette tip. The cells were washed twice with PBS. After capturing images under a microscope and measuring the scratch width, the sample was incubated at 37°C with 5% CO<sub>2</sub> for 24 h. An inverted biological microscope (Olympus Corporation) was used to capture images of the affected area.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted using the Cellular Rapid RNA Extraction Kit (abs60027, Absin Bioscience Inc.) and reverse transcribed to cDNA using the Prime Script<sup>TM</sup> RT kit (RR047A, Japan) and amplified using SYBR Premix Ex Taq (Takara Bio Inc.) according to the manufacturer's instructions. The reverse transcription products were used for RT-qPCR analysis, and the following primer sequences were used for RT-qPCR (Shenggong Bioengineering Co.): human GAPDH forward, 5'-CAACAGCCTCAAGATCATCAGC-3', reverse, 5'-ATG AGTCCTTCCACGATACCA-3'; human TLR4 forward, 5'-TGTGCAACACCTTCAGATAAGCA-3', reverse, 5'-ACA ACAGATACTACAAGCACAC-3'; and human MyD88 forward, 5'-CTGGCTGCTCTCTCAACATGCG-3', reverse, 5'-CCAGTTGCCGGGATCTCCA-3'. 15 min at 95°C, 10 sec at 95°C, 10 sec at 60°C annealed for 40 cycles. The relative quantification of mRNA of target genes was calculated using the 2<sup>-ΔΔC<sub>q</sub></sup> (20) method with GAPDH as an internal reference.

**Western blotting.** Total protein was extracted in the treated group (Primary cell extracts derived from previous steps) using RIPA (cat. no. abs9229, Absin Bioscience Inc.) lysate buffer, quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.), electrophoresed on an SDS-PAGE (cat. no. abs9367-1Kit) gel and transferred to a PVDF membrane (Immobilon05317). The membrane was blocked with 5% skimmed milk, incubated with primary antibody (Abways AB0035, 1:1,000); TLR4 primary antibody (Absin cat. no. 132000; 1:1,000); MyD88 primary antibody (Absin cat. no. abs135682; 1:1,000), overnight at 4°C, and washed three times with TBS with Tween-20 (TBST) for 10 min each. Membranes were incubated with Horseradish enzyme labeled goat anti-rabbit IgG (ZB-2301, 1:10,000) at room temperature for 2 h. The membrane was washed three times with TBST for 10 min each. Quantification was performed using Quantity-One software (Bio-Rad Laboratories, Inc.) using the ECL chemiluminescence kit (cat. no. BL161A) to detect protein expression. Protein band signals were semi-quantified using ImageJ software (version 1.46r) for Windows (National Institutes of Health).

**Statistical analysis.** Origin 2021b SR1 v9.8.5.204 (OriginLab) and SPSS 14.0 (SPSS, Inc.) were used to conduct the statistical analysis. The data are presented as the mean and standard deviation. One-way ANOVA and the Least Significant Difference post hoc test were used to compare the means of several groups, and the Student's t-test (paired or unpaired, where appropriate) was performed to compare the means of only two groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**CD133-labeled CCSCs express CD14.** The presence of CD14 in CRC tissues was detected using immunofluorescence double staining. Under an orthogonal fluorescence microscope, CD14<sup>+</sup> cells exhibiting green fluorescence in immunofluorescence staining, CCSC surface marker CD133<sup>+</sup> cells exhibiting red fluorescence, non-specific DAPI-stained nuclei exhibiting blue fluorescence, and cells co-expressing CD14 and CD133 exhibiting yellow fluorescence were observed in CRC. CD133-labeled CSCs exhibited CD14 expression. Relative to the nuclei of cancer nests, CD14 is small and the nuclei may present a split image, located between the relatively sparsely structured and vascularized cancer nests. The positive rate of CD14 and CD133 co-expression was significantly increased in all CRC tissues compared with paraneoplastic tissues (Fig. 1).

**Primary cell culture.** During the initial extraction, cells slowly proliferate and 'crawl' out of the tissue, and several cells with odd shapes and numerous colonies were observed. Cells gradually adhered to the plate wall and manifested into a spindle shape. When the cell fragments were removed from the culture after 14 days, it was observed that the cells varied in size and shape (Fig. 2).

**Magnetic bead sorting analysis.** Following immunomagnetic bead sorting, CD14<sup>+</sup> (Fig. 3A-b) cells exhibited spindle cell characteristics and decreased nuclear division, whereas the CD14<sup>-</sup> (Fig. 3A-a) cells primarily presented with polyhedral or



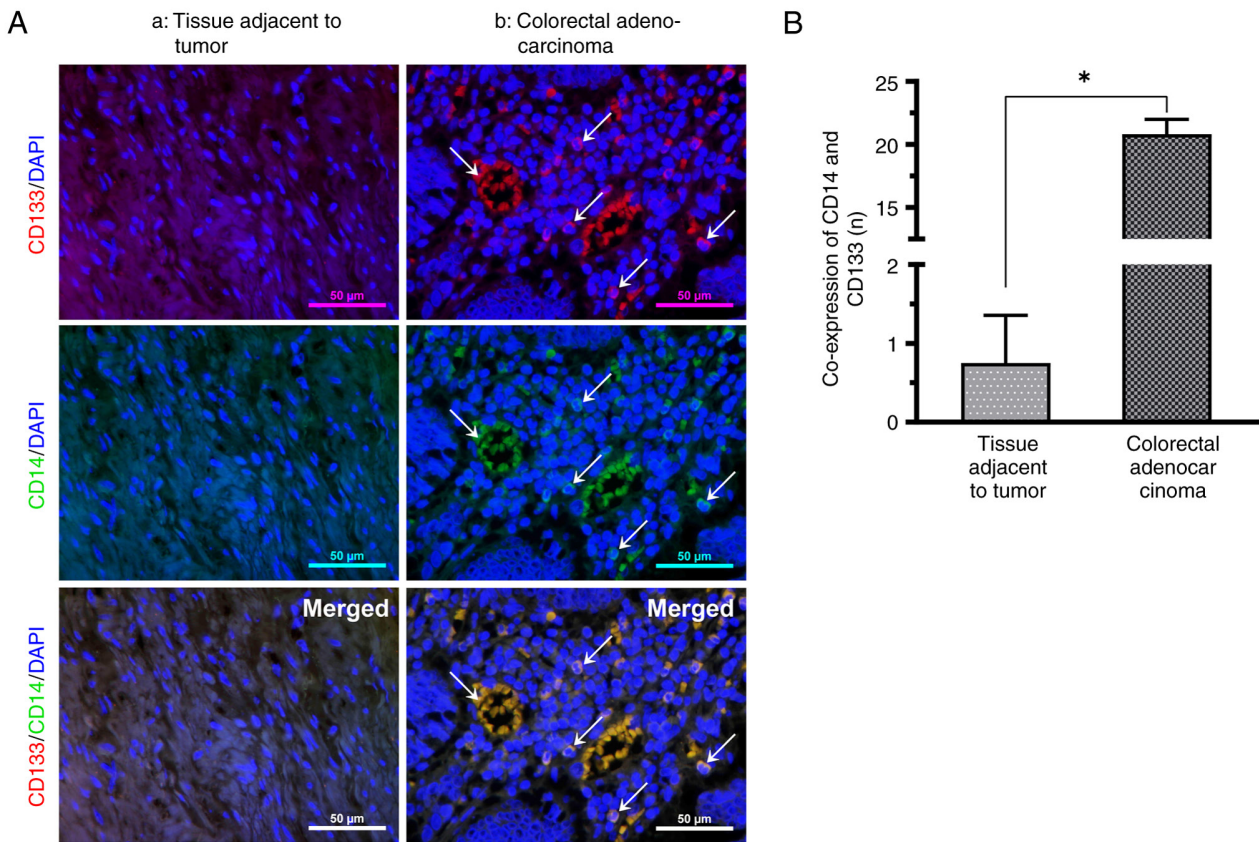


Figure 1. CD14 is expressed in CD133-labeled colorectal CSCs. (A) CD14 was expressed in CCSCs that had been CD133-labeled. Results of the analysis of CD14 and CD133 expression in CRC (Fig. 1Ab) and nearby tissues (Fig. 1Aa). (B) CD133- and CD14-coexpression in CCSCs. \* $P < 0.05$  vs. tissue adjacent to tumor. CD133 is also referred to as leukocyte differentiation antigen. CSC subtype. CRC, colorectal cancer; CCSC, CRC stem cell.

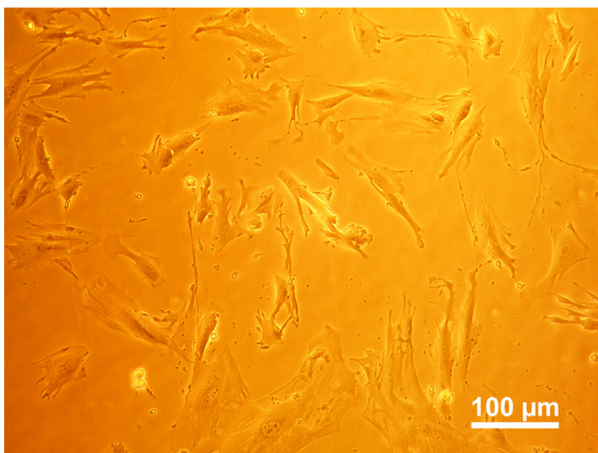


Figure 2. Colorectal cancer cell morphology in primary culture. Cells that eventually attached to the wall typically exhibited morphologies that resembled parallel spindles. After 14 days of culture, cell fragments were collected, and it was observed that the cells varied in size and appearance. Scale bar, 100 μm.

irregularly formed spindles. Both CD14<sup>+</sup> and CD14<sup>-</sup> cells could grow in multilayer cultures or form cell clusters in monolayer cultures after confluence. The growth time was ~5 days of CD14<sup>+</sup> and ~7 days for the CD14<sup>-</sup> cells (Fig. 3A).

**Proliferation analysis.** The EdU assay results revealed that CD14<sup>+</sup> (Fig. 3C-b) cell growth was faster and presenting with

a higher proliferation capability in comparison with CD14<sup>-</sup> (Fig. 3C-a) cells (Fig. 3C). This was consistent with the results of CCK-8 assay (Fig. 3B). This further demonstrated that CD14<sup>+</sup> cells exhibited an increased cell proliferative potential.

**Clonality analysis.** Cell cloning experiments demonstrated that the CD14<sup>+</sup> cells were larger, demonstrated increased nuclear division and aggregated into clusters, as compared with the CD14<sup>-</sup> cells (Fig. 4A). The CD14<sup>+</sup> cell tumorigenic ability was observed using a nude mouse xenograft assay. Following 45 days of implantation, the tumors in the CD14<sup>+</sup> cell group were considerably larger as compared with those in the CD14<sup>-</sup> cell group (Fig. 4B).

**Drug resistance analysis.** The IC<sub>50</sub> of cells in the CD14<sup>+</sup> and CD14<sup>-</sup> groups was 2.554 and 17.02 mg/l, respectively (Fig. 5), suggesting that resistance was increased in the positive group in comparison with the negative group.

**Migration via the TLR4/MyD88 pathway following LPS stimulation.** The results of Transwell assay revealed that the LPS group (Fig. 6A-c) exhibited a greater migratory ability as compared with the control group (Fig. 6A-a), and the migratory ability was greater in the LPS + neutralizing antibody group (Fig. 6A-b) compared with the control group (Fig. 6A-a). According to the results of the wound healing experiments, migration was significantly enhanced in the LPS group and slightly enhanced in the LPS + neutralizing antibody group



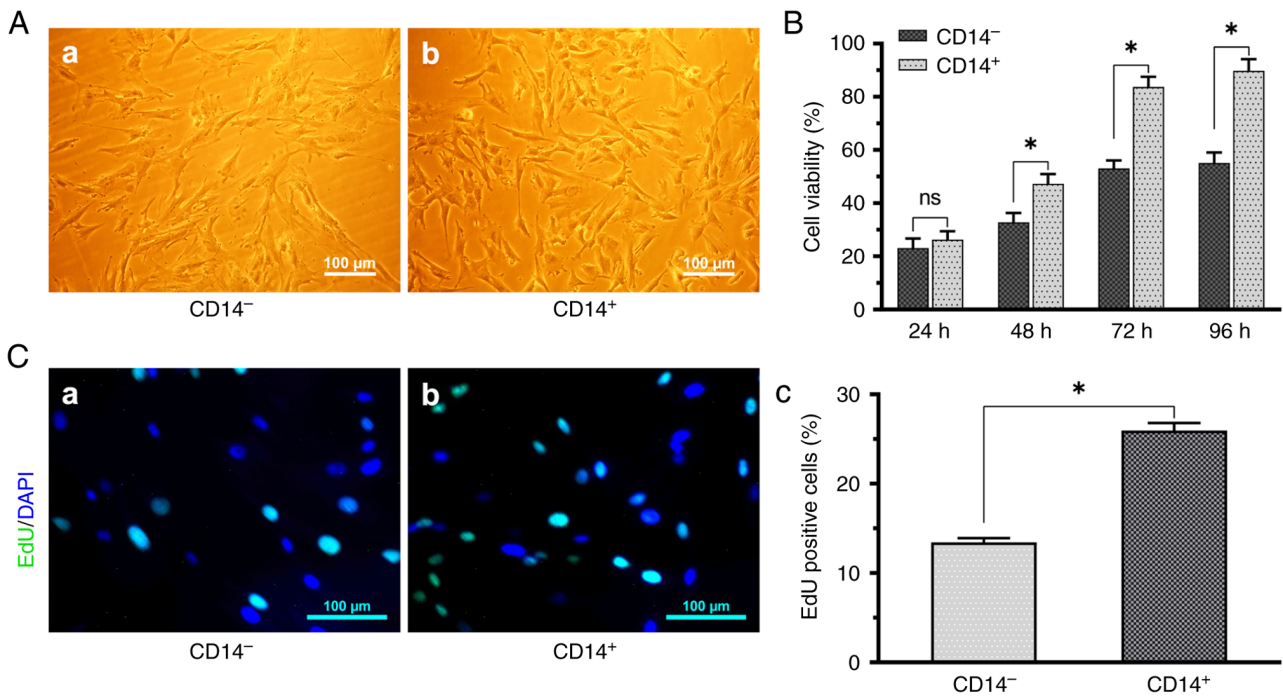


Figure 3. CD14<sup>+</sup> cells of colon carcinoma exhibit enhanced proliferative abilities. (A) As compared with (a) CD14<sup>-</sup> cells, (b) CD14<sup>+</sup> cells were spindle-shaped and smaller in size. By contrast, CD14<sup>-</sup> cells primarily exhibited polyhedral or irregular spindle shapes. Scale bar, 100  $\mu$ m. \* $P$ <0.05. (B) Results of the Cell Counting Kit-8 assay demonstrated that CD14<sup>+</sup> cells proliferated faster in comparison with CD14<sup>-</sup> cells. \* $P$ <0.05. (C) EdU assay results revealed that (b) CD14<sup>+</sup> cells exhibited increased proliferation in comparison with (a) CD14<sup>-</sup> cells; (c) EDU percentage of positive cells. Scale bar, 100  $\mu$ m. \* $P$ <0.05. EdU, 5-ethynyl-2'-deoxyuridine.

compared with the control group; the cell migratory ability was higher in the LPS group than in the LPS + neutralizing antibody group (Fig. 6B). The RT-qPCR results demonstrated that the MyD88 mRNA levels in the treatment groups were increased in comparison with the control group, with the highest mRNA levels observed in the LPS group (Fig. 6C). Western blotting also revealed that TLR4 and MyD88 protein expression was significantly elevated in both the LPS + neutralizing antibody group and the LPS group, as compared with the control group, with a greater increase observed in the LPS group (Fig. 6D).

## Discussion

Specific cell surface indicators have been suggested for the identification of CCSCs (21). Excluding CD133, other cell surface markers of CCSCs have been identified, including CD44, CD166, Lgr5 and ALDH1 (8). The present study demonstrated that the cell surface marker CD14 was expressed in the CD133-labeled CCSCs. Nuclear division could also be observed in smaller-sized CCSCs as with the cancer nest cells and CCSCs were primarily distributed in the tumor around the cancer nests, which was consistent with the findings of a previous study on CSC distribution in tumor tissues (22). To validate the phenotypic detection, the assessment of the functional capacities of CCSCs by using *in vitro* and *in vivo* assays is required (23).

To identify and confirm the stemness of tumor cells, the infinite capacity for proliferation, the capacity for self-renewal and the capacity for tumorigenesis are frequently examined (24). The proliferation and/or self-renewal capacity of cancer cells can be examined using CCK-8 and EdU assays (25). Colony formation and xenograft assays are two

widely used methods for determining the features of tumorigenesis (25,26). Additionally, since CCSCs may be able to survive chemotherapy-induced toxicity, drug resistance can also be used for identification (21,27). The present study demonstrated that CD14<sup>+</sup> CRC cells possessed CSC-like stemness using the aforementioned methods.

Numerous microorganisms in the colon may activate similar receptors through their antigens (for example, the antigen LPS), which promote the development of CRC (28). The present study is one of numerous studies that have used an *in vivo* and *in vitro* experimental setup to perform research (29-31). LPS, a crucial part of the outer membrane of Gram-negative bacteria, can elicit immune system activation and acute or chronic inflammation (32). LPS has also been linked to carcinogenesis and the emergence of colon cancer in addition to its role in inflammatory reactions. In previous studies, increased LPS levels were detected in the blood and CRC tissues of patients with CRC, even including cases of early-stage adenoma (33,34), revealing also that circulating LPS could lead to systemic inflammation and a disordered coagulation system, with the ensuing chronic inflammation and active coagulation system being linked to tumorigenesis (35). By examining human CRC cell lines, it was also revealed that LPS increases CRC metastasis (13,34). The results of the present study demonstrated that LPS may have induced the proliferation and migration of CD14<sup>+</sup> CRC cells that were CSC-like.

TLR4 is a transmembrane protein that is expressed in various cancer cells and is largely involved in proliferation, migration and invasion (36,37). TLR4 stimulates two signaling pathways, the MyD88-dependent (also known as TRIF-dependent) and the MyD88-independent (also known as LPS-dependent) pathways, which are both regulated by CD14 and triggered by LPS (12,28).

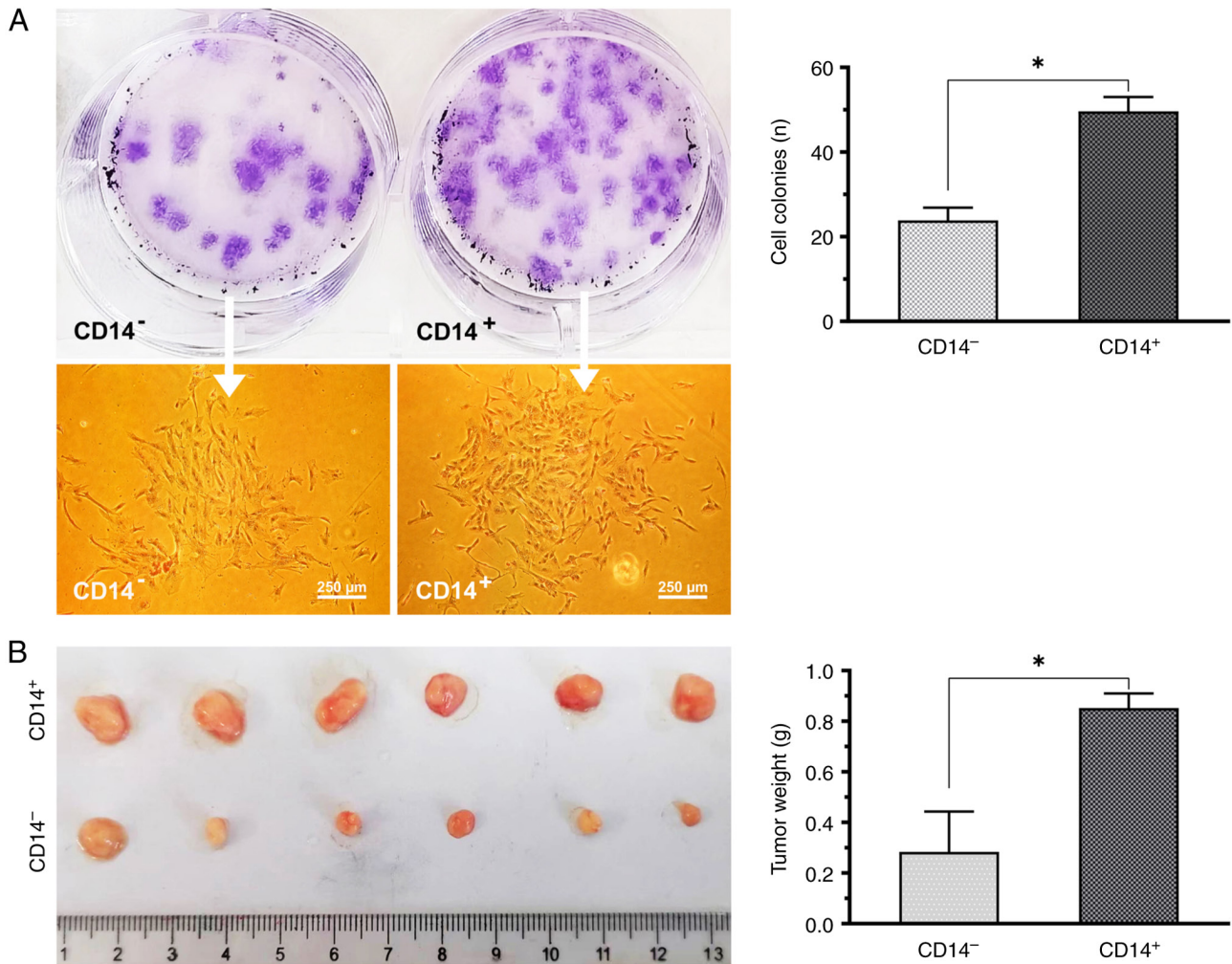


Figure 4. CD14<sup>+</sup> cells in colon cancer are tumorigenic. (A) Cell cloning experiments demonstrated that CD14<sup>+</sup> cells were larger, exhibited increased nuclear division and aggregated into clusters compared with CD14<sup>-</sup> colorectal cancer cells. Scale bar, 250  $\mu$ m. \*P<0.05. (B) CD14<sup>+</sup> cells were tumorigenic *in vivo*. Tumors in the CD14<sup>+</sup> cell group were markedly larger in comparison with the CD14<sup>-</sup> cell group 45 days after implantation in nude mice. \*P<0.05.

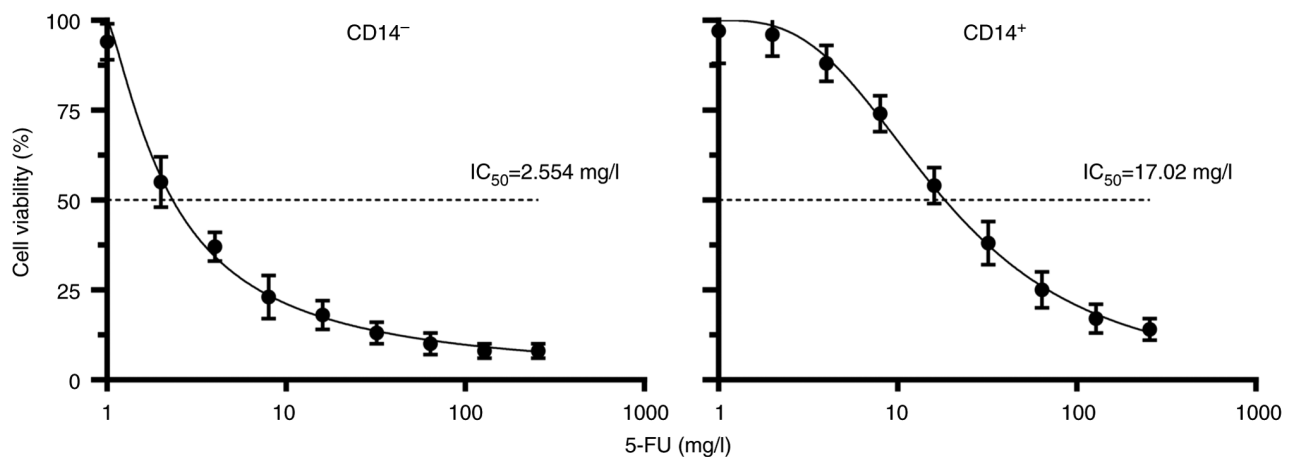


Figure 5. Cell Counting Kit-8 assay of the IC<sub>50</sub> efficacy curve of 5-FU in CD14<sup>+</sup> and CD14<sup>-</sup> cells at 48 h. 5-FU, 5-fluorouracil.

TLR4/MyD88-dependent pathway activation may encourage the development and metastasis of CRC (38,39). The findings of the present study demonstrated that following LPS stimulation, CD14 regulated the proliferation and migration of CD14<sup>+</sup> CRC cells through the TLR4/MyD88 pathway. Multiple studies

have demonstrated that the TLR4/MyD88 pathway may activate cyclooxygenase-2, the EGF receptor and -catenin-dependent pathways that promote CRC cell proliferation (14,38,40-42).

Cheah *et al* (43) reported that bladder cancer CD14-high cells expressed higher levels of numerous inflammatory



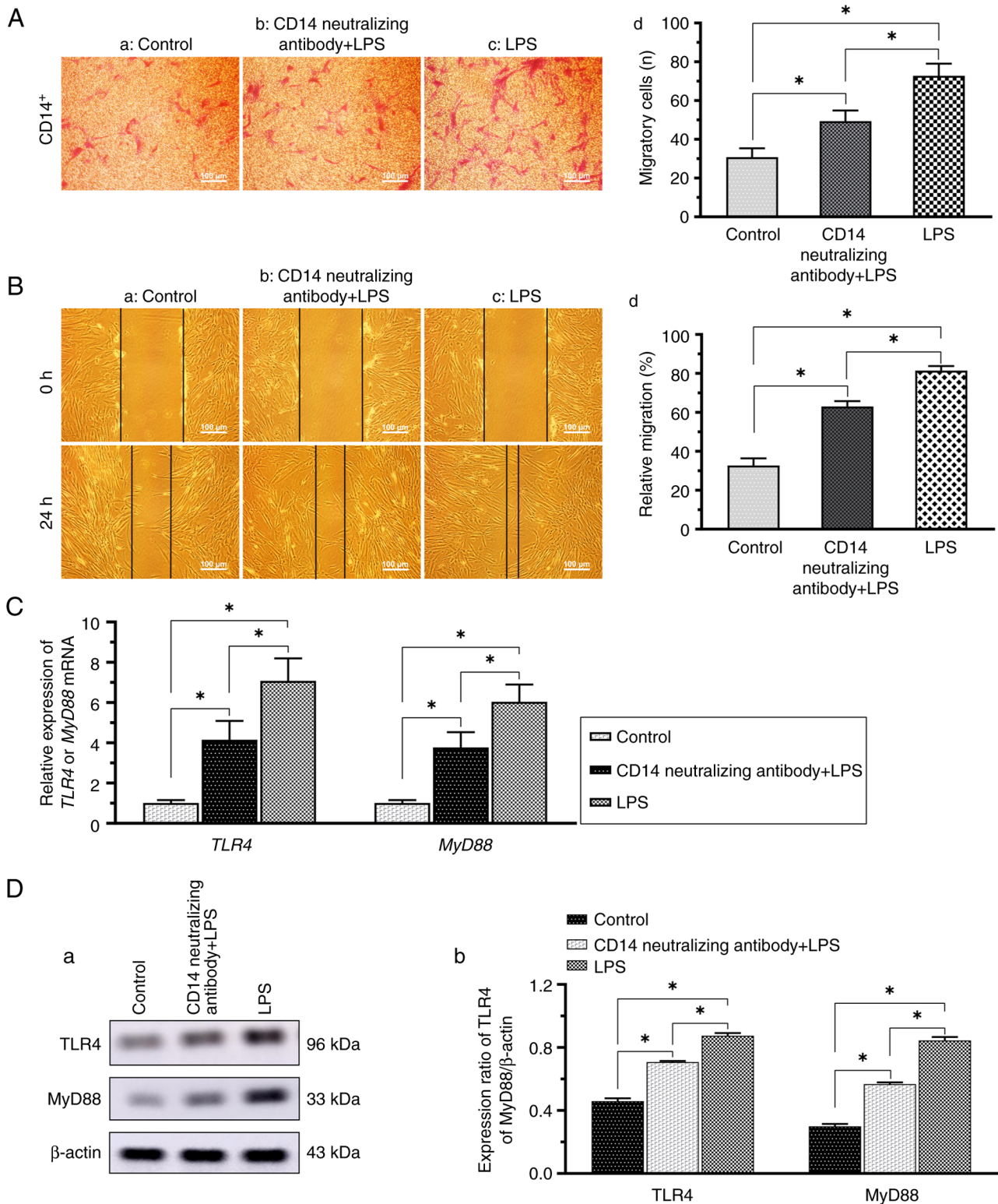


Figure 6. LPS affects cell migration. (A) Cells in the LPS group (c) exhibited the greatest migratory ability compared with those in the (a) control group, and migration was increased in the (b) LPS + neutralizing antibody group as compared with the control group. Scale bar, 100  $\mu$ m. (d) Migratory cells (n). \* $P$ <0.05. (B) Migration was significantly enhanced in the (c) LPS group and slightly enhanced in the (b) LPS-neutralized group, as compared with the (a) control group. Scale bar, 100  $\mu$ m. (d) Relative migration (%). \* $P$ <0.05. (C) mRNA levels of the cells in the treated groups were increased in comparison with the control group, the highest mRNA levels having been observed in the LPS group. Cellular mRNA levels were higher in the LPS group than in the LPS + neutralizing antibody group. \* $P$ <0.05. (D) Protein expression was significantly increased in both the LPS + neutralizing antibody group and the LPS group compared with the control group. The highest protein expression was detected in the LPS group. (a) Protein expression images; (b) Expression ratio of TLR4 of MyD88/ $\beta$ -actin. \* $P$ <0.05. LPS, lipopolysaccharide; MyD88, myeloid differentiation factor 88; TLR4, Toll-like receptor 4.

mediators (IL-6 and IL8/CXCL1) even in the absence of LPS stimulation and formed larger tumors with higher

vascularization than CD14-low cells, demonstrating that CD14 may promote tumorigenesis and development through



a variety of mechanisms, which should be investigated in further studies.

The present study revealed that CD133-labeled CCSCs expressed the surface marker CD14. *In vitro* and *in vivo* experiments demonstrated that primary CD14<sup>+</sup> cells of CRC exhibited CSC stemness, and CD14 could regulate the migration of CD14<sup>+</sup> CRC cells through the TLR4/MyD88 pathway following LPS stimulation, suggesting that CD14 may be regarded as a unique surface marker of CCSCs, potentially providing a therapeutic target against CCSCs. However, the disadvantage of the present study was the absence of CD14 expression-related rescue and knockdown tests, and the functional mechanism of CD14 was not explored in-depth. Therefore, additional research is necessary for the further elucidation of the CD14 function in CCSCs.

## Acknowledgements

Not applicable.

## Funding

The present study was supported by the Special Program for Graduate Student Supervisors' Research (grant no. YJS2X202201).

## Availability of data and materials

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

## Authors' contributions

JuL, YuL, JS and JiL conceived and designed the study. ZL, YoL and AX and WS contributed to the execution of the experiments, statistical analysis of the data and drafting of the manuscript. YuL and JuL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Mudanjiang Medical University (approval no. 2022-MYGZR06). All patients signed an informed consent form. The animal experiments were approved by the Laboratory Animal Welfare and Ethics Committee of Mudanjiang Medical University (approval no. 20220228-26).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

- Ahmad R, Singh JK, Wunnavu A, Al-Obeed O, Abdulla M and Srivastava SK: Emerging trends in colorectal cancer: Dysregulated signaling pathways (Review). *Int J Mol Med* 47: 14, 2021.
- Lewandowska A, Rudzki G, Lewandowski T, Strykowska-Góra A and Rudzki S: Title: risk factors for the diagnosis of colorectal cancer. *Cancer Control* 29: 10732748211056692, 2022.
- Salibasic M, Pusina S, Bicakcic E, Pasic A, Gavric I, Kulovic E, Rovcanin A and Beslija S: Colorectal cancer surgical treatment, our experience. *Med Arch* 73: 412-414, 2019.
- Li Y, Wu M, Xu S, Huang H, Yan L and Gu Y: Colorectal cancer stem cell-derived exosomal long intergenic noncoding RNA 01315 (LINC01315) promotes proliferation, migration, and stemness of colorectal cancer cells. *Bioengineered* 13: 10827-10842, 2022.
- Battle E and Clevers H: Cancer stem cells revisited. *Nat Med* 23: 1124-1134, 2017.
- Hervieu C, Christou N, Battu S and Mathonnet M: The role of cancer stem cells in colorectal cancer: From the basics to novel clinical trials. *Cancers (Basel)* 13: 1092, 2021.
- Kopenhaver J, Crutcher M, Waldman SA and Snook AE: The shifting paradigm of colorectal cancer treatment: A look into emerging cancer stem cell-directed therapeutics to lead the charge toward complete remission. *Expert Opin Biol Ther* 21: 1335-1345, 2021.
- Gupta R, Bhatt LK, Johnston TP and Prabhavalkar KS: Colon cancer stem cells: Potential target for the treatment of colorectal cancer. *Cancer Biol Ther* 20: 1068-1082, 2019.
- Munro MJ, Wickremesekera SK, Peng L, Tan ST and Itinteang T: Cancer stem cells in colorectal cancer: A review. *J Clin Pathol* 71: 110-116, 2018.
- Yu X, Lin Y, Yan X, Tian Q, Li L and Lin EH: CD133, stem cells, and cancer stem cells: Myth or Reality? *Curr Colorectal Cancer Rep* 7: 253-259, 2011.
- Janova H, Böttcher C, Holtman IR, Regen T, van Rossum D, Götz A, Ernst AS, Fritsche C, Gertig U, Saiepour N, *et al*: CD14 is a key organizer of microglial responses to CNS infection and injury. *Glia* 64: 635-649, 2016.
- Ciesielska A, Matyjek M and Kwiatkowska K: TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci* 78: 1233-1261, 2021.
- Hsu RY, Chan CH, Spicer JD, Rousseau MC, Giannias B, Rousseau S and Ferri LE: LPS-induced TLR4 signaling in human colorectal cancer cells increases beta1 integrin-mediated cell adhesion and liver metastasis. *Cancer Res* 71: 1989-1997, 2011.
- Santaolalla R, Sussman DA, Ruiz JR, Davies JM, Pastorini C, España CL, Sotolongo J, Burlingame O, Bejarano PA, Philip S, *et al*: TLR4 activates the  $\beta$ -catenin pathway to cause intestinal neoplasia. *PLoS One* 8: e63298, 2013.
- Song W, Tiruthani K, Wang Y, Shen L, Hu M, Dorosheva O, Qiu K, Kinghorn KA, Liu R and Huang L: Trapping of lipopolysaccharide to promote immunotherapy against colorectal cancer and attenuate liver metastasis. *Adv Mater* 30: e1805007, 2018.
- Zhao D, Sun T, Zhang X, Guo Y, Yu D, Yang M, Tan W, Wang G and Lin D: Role of CD14 promoter polymorphisms in *Helicobacter pylori* infection-related gastric carcinoma. *Clin Cancer Res* 13: 2362-2368, 2007.
- Arihara F, Mizukoshi E, Kitahara M, Takata Y, Arai K, Yamashita T, Nakamoto Y and Kaneko S: Increase in CD14+HLA-DR-/low myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis. *Cancer Immunol Immunother* 62: 1421-1430, 2013.
- Huang A, Zhang B, Wang B, Zhang F, Fan KX and Guo YJ: Increased CD14(+)-HLA-DR (-/low) myeloid-derived suppressor cells correlate with extrathoracic metastasis and poor response to chemotherapy in non-small cell lung cancer patients. *Cancer Immunol Immunother* 62: 1439-1451, 2013.
- Lin Y, Dong J, Yu W, Li Y, Liu Z, Liu J, Wang C, Qin J, Zhu L and Liang J: CD14, a novel surface marker of esophageal cancer stem cells. *Oncol Rep* 49: 13, 2023.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Zalewski A, Snook AE and Waldman SA: Stem cells as therapeutic targets in colorectal cancer. *Per Med* 18: 171-183, 2021.
- Cui G, Xu G, Zhu L, Pang Z, Zheng W, Li Z and Yuan A: Temporal and spatial changes of cells positive for stem-like markers in different compartments and stages of human colorectal adenoma-carcinoma sequence. *Oncotarget* 8: 45311-45322, 2017.
- Akbarzadeh M, Maroufi NF, Tazehkand AP, Akbarzadeh M, Bastani S, Safdari R, Farzane A, Fattahi A, Nejabati HR, Nouri M and Samadi N: Current approaches in identification and isolation of cancer stem cells. *J Cell Physiol* 234: 14759-14772, 2019.

24. Han GD, Sun Y, Hui HX, Tao MY, Liu YQ and Zhu J: MiR-1224 acts as a prognostic biomarker and inhibits the progression of gastric cancer by targeting SATB1. *Front Oncol* 11: 748896, 2021.
25. Vargo-Gogola T and Rosen JM: Modelling breast cancer: One size does not fit all. *Nat Rev Cancer* 7: 659-672, 2007.
26. Tsai RY: Balancing self-renewal against genome preservation in stem cells: How do they manage to have the cake and eat it too? *Cell Mol Life Sci* 73: 1803-1823, 2016.
27. Chiodi I, Belgiovine C, Donà F, Scovassi AI and Mondello C: Drug treatment of cancer cell lines: A way to select for cancer stem cells? *Cancers (Basel)* 3: 1111-1128, 2011.
28. Fang Y, Yan C, Zhao Q, Zhao B, Liao Y, Chen Y, Wang D and Tang D: The association between gut microbiota, toll-like receptors, and colorectal cancer. *Clin Med Insights* 16: 11795549221130549, 2022.
29. Liu WT, Jing YY, Yan F, Han ZP, Lai FB, Zeng JX, Yu GF, Fan QM, Li R, Zhao QD, *et al*: LPS-induced CXCR4-dependent migratory properties and a mesenchymal-like phenotype of colorectal cancer cells. *Cell Adh Migr* 11: 13-23, 2017.
30. Jeong JW, Park C, Cha HJ, Hong SH, Park SH, Kim GY, Kim WJ, Kim CH, Song KS and Choi YH: Cordycepin inhibits lipopolysaccharide-induced cell migration and invasion in human colorectal carcinoma HCT-116 cells through down-regulation of prostaglandin E2 receptor EP4. *BMB Rep* 51: 532-537, 2018.
31. Zhu G, Cheng Z, Lin C, Hoffman RM, Huang Y, Singh SR, Zheng W, Yang S and Ye J: MyD88 Regulates LPS-induced NF- $\kappa$ B/MAPK cytokines and promotes inflammation and malignancy in colorectal cancer cells. *Cancer Genomics Proteomics* 16: 409-419, 2019.
32. d'Hennezel E, Abubucker S, Murphy LO and Cullen TW: Total Lipopolysaccharide from the human gut microbiome silences toll-like receptor signaling. *mSystems* 2: e00046-17, 2017.
33. Kang M, Edmundson P, Araujo-Perez F, McCoy AN, Galanko J and Keku TO: Association of plasma endotoxin, inflammatory cytokines and risk of colorectal adenomas. *BMC Cancer* 13: 91, 2013.
34. Zhu G, Huang Q, Huang Y, Zheng W, Hua J, Yang S, Zhuang J, Wang J and Ye J: Lipopolysaccharide increases the release of VEGF-C that enhances cell motility and promotes lymphangiogenesis and lymphatic metastasis through the TLR4- NF- $\kappa$ B/JNK pathways in colorectal cancer. *Oncotarget* 7: 73711-73724, 2016.
35. de Waal GM, de Villiers WJS, Forgan T, Roberts T and Pretorius E: Colorectal cancer is associated with increased circulating lipopolysaccharide, inflammation and hypercoagulability. *Sci Rep* 10: 8777, 2020.
36. Zhou W, Chen X, Hu Q, Chen X, Chen Y and Huang L: Galectin-3 activates TLR4/NF- $\kappa$ B signaling to promote lung adenocarcinoma cell proliferation through activating lncRNA-NEAT1 expression. *BMC Cancer* 18: 580, 2018.
37. Wang W and Wang J: Toll-Like Receptor 4 (TLR4)/Cyclooxygenase-2 (COX-2) regulates prostate cancer cell proliferation, migration, and invasion by NF- $\kappa$ B activation. *Med Sci Monit* 24: 5588-5597, 2018.
38. Rakoff-Nahoum S and Medzhitov R: Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. *Science* 317: 124-127, 2007.
39. Huang HY, Zhang ZJ, Cao CB, Wang N, Liu FF, Peng JQ, Ren XJ and Qian J: The TLR4/NF- $\kappa$ B signaling pathway mediates the growth of colon cancer. *Eur Rev Med Pharmacol Sci* 18: 3834-3843, 2014.
40. Fukata M, Chen A, Vamadevan AS, Cohen J, Breglio K, Krishnareddy S, Hsu D, Xu R, Harpaz N, Dannenberg AJ, *et al*: Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors. *Gastroenterology* 133: 1869-1881, 2007.
41. Fukata M, Chen A, Klepper A, Krishnareddy S, Vamadevan AS, Thomas LS, Xu R, Inoue H, Arditi M, Dannenberg AJ and Abreu MT: Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: Role in proliferation and apoptosis in the intestine. *Gastroenterology* 131: 862-877, 2006.
42. Fukata M, Shang L, Santaolalla R, Sotolongo J, Pastorini C, España C, Ungaro R, Harpaz N, Cooper HS, Elson G, *et al*: Constitutive activation of epithelial TLR4 augments inflammatory responses to mucosal injury and drives colitis-associated tumorigenesis. *Inflamm Bowel Dis* 17: 1464-1473, 2011.
43. Cheah MT, Chen JY, Sahoo D, Contreras-Trujillo H, Volkmer AK, Scheeren FA, Volkmer JP and Weissman IL: CD14-expressing cancer cells establish the inflammatory and proliferative tumor microenvironment in bladder cancer. *Proc Natl Acad Sci USA* 112: 4725-4730, 2015.



Copyright © 2023 Li et al. This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.