

M1-like tumor-associated macrophages enhance proliferation and anti-apoptotic ability of liver cancer cells via activating the NF- κ B signaling pathway

GAOWA SHAREN^{1,2}, HAIDONG CHENG³, XIUHUA HU³, JIE MIAO³ and DEFANG ZHAO⁴

¹Department of Pathology, The Affiliated Hospital of Inner Mongolia Medical University;

²Department of Pathological Anatomy, College of Basic Medicine of Inner Mongolia Medical University;

Departments of ³Gastroenterology and ⁴Hepatobiliary Surgery, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia Autonomous Region 010059, P.R. China

Received December 29, 2020; Accepted February 8, 2022

DOI: 10.3892/mmr.2022.12847

Abstract. Liver cancer is the second leading cause of cancer-associated death worldwide. The present study aimed to evaluate the infiltration of M1-like tumor-associated macrophages (TAMs) and explore the role of infiltration of M1-like TAMs in the proliferation and apoptosis evasion of liver cancer cells. Furthermore, the association between M1-like TAM and the efficacy of postoperative transcatheter arterial chemoembolization (TACE) for patients with liver cancer was investigated. The levels of CD68, human leukocyte antigen-DR and phosphorylated NF- κ B (p)-p65 were detected by western blot analysis and immunohistochemistry. Cell cycle analysis, MTT and clonogenic assays were utilized to investigate the proliferation of liver cancer cells. It was indicated that M1-like TAM increased the p-p65/p65 ratio in liver cancer cells and promoted cell proliferation. Furthermore, JSH-23, an inhibitor that prevents p65 from entering the nucleus, decreased the proliferation of liver cancer cells in M1-like TAM-conditioned medium. In addition, M1-like TAM increased the number of liver cancer cells in the S and G2/M phases of the cell cycle and also upregulated the expression levels of cyclin-dependent kinase (CDK)1, CDK2 and cyclin D1. By contrast, M1-like TAM decreased the expression level of p21. Through these effects, the anti-apoptotic ability of liver cancer cells was enhanced. Of note, JSH-23 reversed these changes related to the cell cycle, anti-apoptotic ability and the expression levels of proteins induced by M1-like TAM in liver cancer cells. In conclusion, the infiltration of M1-like TAM in liver tissue

negatively influenced the efficacy of postoperative TACE for patients with liver cancer.

Introduction

Liver cancer is the second leading cause of cancer-related death worldwide. Currently, the number of diagnosed cases of liver cancer in China accounts for ~45% of the global incidence, and the disease ranks first and second among diverse types of cancer in rural and urban areas, respectively. At present, China has the highest incidence of primary liver cancer in the world (1-4). Although the one-year survival rate after radical resection of liver cancer has increased from 39 to 87% in recent decades in China, the five-year survival rate after curative resection has been reported to be 15-40% (5,6). The lack of effective targeted therapies and high frequency of metastatic tumors are the main causes of poor prognosis for patients with primary liver cancer (7,8).

Epidemiological evidence indicated that inflammation may lead to tumorigenesis and cancer development. It has been estimated that 20% of malignant tumors were induced or promoted by inflammation (9,10). Furthermore, for liver cancer, numerous studies have reported that inflammation is closely associated with the molecular mechanisms underlying liver cancer. In addition, inflammation has been indicated to facilitate the development and metastasis of liver cancer (11,12). Although the molecular mechanisms of how inflammation may cause malignancies have remained to be fully elucidated, inflammation is well known to promote the proliferation, invasion and migration of cancer cells. It has been demonstrated that overexpression of nuclear factor- κ B (NF- κ B)-p50 accounts for the inability of tumor-associated macrophages (TAMs) to mount an effective M1 antitumor response capable of inhibiting tumor growth (13). Furthermore, PI3K- γ is able to control the transformation of M1-type to M2-type macrophages, thereby promoting tumor immunosuppression and progression.

Macrophages may polarize into pro- or anti-inflammatory phenotypes (M1-like and M2-like macrophages, respectively). Cancer-related inflammation in the tumor

Correspondence to: Dr Defang Zhao, Department of Hepatobiliary Surgery, The Affiliated Hospital of Inner Mongolia Medical University, 1 Tongdao North Street, Huimin, Hohhot, Inner Mongolia Autonomous Region 010059, P.R. China
E-mail: defang2010314@163.com

Key words: liver cancer, NF- κ B signaling, M1-like tumor-associated macrophages, proliferation, evasion of apoptosis

microenvironment (TME) is a hallmark of cancer. A study reported the association of the incidence of liver cancer with TAM. The M2-type macrophages polarized from TAM were indicated to promote the migration and epithelial-mesenchymal transition of liver cancer cells through the Toll-like receptor 4/STAT3 signaling pathway (14). In addition, microRNA-101 inhibited macrophage-induced growth of liver cancer by targeting dual-specificity phosphatase 1 (15). In addition, the importance of M1-like TAM was reported, as they promoted the migration and invasion of liver cancer cells (16,17). However, the effects of M1-like TAM on the proliferation and anti-apoptotic ability of liver cancer cells have remained elusive. Therefore, the present study aimed to evaluate the role of infiltration of M1-like TAM in the proliferation and anti-apoptotic ability of liver cancer cells. Furthermore, it was attempted to investigate the relationship between M1-like TAM and the efficacy of postoperative transcatheter arterial chemoembolization (TACE) in patients with liver cancer.

Materials and methods

Sample collection and ethical statement. All pathologically confirmed liver cancer samples were collected from 79 patients who underwent curative resection of primary liver cancer. The patients consisted of 64 males and 15 females, aged 22-84 years (median, 57 years). The surgical procedures were undertaken at the Affiliated Hospital of Inner Mongolia Medical University (Hohhot, China) between January and December 2018. All patients underwent TACE at 1-2 months post-operation. After TACE, in accordance with the Guidelines for the Diagnosis and Treatment of Primary Liver Cancer in China from 2017 (18), a total of 45 patients with liver cancer were assessed for their eligibility to be included in the study and were assigned into groups of complete remission (CR) or partial remission (PR). In the CR group, all visible features of the tumor disappeared after less than one month. In the PR group, a >50% decrease in the cross-product of the two largest diameters of the tumor was observed, which lasted >1 month. In addition, 34 patients with liver cancer were allocated to the no remission (NR) group, where the cross-product of the two largest perpendicular diameters of the tumor decreased by <50.0% or increased after TACE. The study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University (Hohhot, China; approval no. YJ 2020001) and the study was performed in accordance with the Declaration of Helsinki. In addition, all participants provided written informed consent prior to the study commencing.

Immunohistochemistry (IHC). Paraffin-embedded sections of adjacent non-tumor liver tissues and tumor tissues were analyzed by IHC. IHC was used to detect the expression levels of CD68, human leukocyte antigen (HLA)-DR and phosphorylated (p)-p65 of NF- κ B proteins. The sections were washed with PBS and the proteins were detected with the Vectastain Elite ABC kit (Vector Laboratories, Inc.). Staining for CD68 and HLA-DR was performed with a double-staining kit (catalog no. SP-900; ZSGB-Bio, Inc.) according to the manufacturer's protocol. In brief, 3.5- μ m paraffin-embedded sections were placed in an autoclave,

the contained distilled water was heated to the boil and the samples were maintained for 3 min for antigen retrieval. The paraffin-embedded sections were incubated at 60°C for 2 h, deparaffinized and hydrated with xylene and ethanol. This was followed by washing with PBS and double-distilled water to retrieve the nuclear antigen, and finally by staining. Primary antibodies were as follows: Anti-NF- κ B p65 (dilution, 1:500; cat. no. ab86299; Abcam), anti-p-NF- κ B p65 (dilution, 1:100; cat. no. ab86299; Abcam), anti-CD68 (dilution, 1:400; cat. no. ZM-0464; ZSGB-Bio) and anti-HLA-DR α (dilution, 1:200; cat. no. 2741-1; Epitomics, Inc.). Secondary antibodies were as follows: Goat anti-mouse IgG (H&L; dilution, 1:2,000; cat. no. ab205719; Abcam), goat F(ab')₂ anti-rabbit IgG H&L (AP) (dilution, 1:500; cat. no. ab6015; Abcam) and goat anti-rabbit IgG (H&L; dilution, 1:2,000; cat. no. ab6702; Abcam). Primary antibodies were incubated overnight at 4°C and secondary antibodies were incubated for 2 h at 37°C. Finally, the samples were observed under a TCS SP5 microscope (Leica Microsystems GmbH) and the images acquired with 512x512 pixels were processed by LAS AF Lite 2.6.0 software (Leica Microsystems GmbH). In the present study, CD68-, HLA-DR α - and p-p65-positive cells were stained red, brown and brown, respectively.

Cell lines and culture. HepG2 [HB-8065; American Type Culture Collection (ATCC)], SNU-182 (CRL-2235; ATCC) and THP-1 (TIB-202; ATCC) cells were authenticated by short tandem repeat profiling and were cultured in a Dulbecco's modified Eagle's medium (DMEM; 12491-15; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with constant 5% CO₂. Subsequently, 5 μ M JSH-23 (HY-P13982; MCE) was added to the medium, followed by incubation for 6 h. The medium was then replaced with a conditioned medium (CM) (M1-TAM-CM). Next, Adriamycin (ADM; 10 mg/ml) was added to the medium, followed by incubation for 24 h. In another experiment, to assess the effects of radiation, the medium was exposed to 4 Gy for 24 h without adding ADM using a 43885D X-ray machine (Faxitron Biophysics, LLC) with the energy of 50 keV and a distance of ~0.3 m from the surface of the sample. The cells were irradiated with a dose rate of 1.084 Gy/min at room temperature and the final dose of irradiation reached 4.0 Gy.

Preparation of M1-TAM-CM. M1 macrophages were derived from THP-1 (TIB-202; ATCC) as previously described (19,20). M1 macrophages were cultured in regular medium (RM) to obtain a CM. In brief, 5x10⁵ cells were seeded into 12-well plates and were incubated with 320 nM phorbol myristate acetate (PMA; cat. no. 16561-29-8; MilliporeSigma) for 6 h. Samples were then cultured with the addition of 100 ng/ml lipopolysaccharide (LPS; 297-473-0; MilliporeSigma) and 20 ng/ml interferon- γ (IFN- γ ; SRP3058; MilliporeSigma) for another 18 h. Next, THP-1 cells were washed thrice with PBS and cultured in RM at 37°C with 5% CO₂ for 24 h. Finally, the media were collected to obtain the CM.

Cell proliferation assay. The HepG2 or SNU-182 cells were seeded into 96-(5,000 cells/well) or 6-(200 cells/well) well plates. In the 96-well plates, the MTT Cell Proliferation and

Cytotoxicity Assay Kit (cat. no. C0009; Beyotime Institute of Biotechnology) was used to evaluate the cell proliferation ability following the manufacturer's protocol. In the 6-well plates, the culture medium was changed every 3 days and the cultivation was stopped when visible clones were observed. After that, the cells were washed thrice with PBS and fixed with 4% formaldehyde for 15 min at room temperature. Supernatants were removed, stained with 0.25% crystal violet for 25 min at room temperature and were then slowly rinsed with ultrapure water. The cell proliferation ability was assessed by measuring absorbance at a wavelength of 595 nm (Aurora-600; Hangzhou Haipei Instrument Co., Ltd.).

Cell cycle analysis. Liver cancer cells were cultured in RM or CM for 24 h and were then collected. The Cell Cycle and Apoptosis Analysis Kit (cat. no. 40301ES50; Yeasen Biotechnology Co., Ltd.) was used to assess the cell cycle with the MACSQuant® Analyzer 10 (Miltenyi Biotec GmbH) in accordance with the manufacturer's instructions.

Cell apoptosis assay. The Annexin V-FITC/PI kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to prepare cells for flow cytometry. The CytoFLEX flow cytometer (Beckman Coulter, Inc.) was utilized to analyze the apoptosis of cells.

Western blot analysis. RIPA lysis buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) was used to extract the total protein via a BCA Protein Assay Kit (cat. no. P0010; Beyotime Institute of Biotechnology). A standardized amount of 40 µg total protein per sample was used for separation of proteins by 10% SDS-PAGE at 90 mA for 2 h. Samples were then transferred onto polyvinylidene fluoride (PVDF) membranes (IB24001; Invitrogen; Thermo Fisher Scientific, Inc.) at 400 mA for 1 h. The PVDF membranes were then blocked with 5% fat-free milk powder dissolved in PBS (blocking solution) for 1 h at room temperature. Subsequently, the membranes were incubated with the primary antibodies diluted in the blocking solution at 4°C overnight. Next, the membranes were incubated with the secondary antibodies at room temperature for 1 h after three washes with PBS. Finally, Western Lightning Plus-ECL (PerkinElmer, Inc.) was used to visualize the protein bands and ImageJ 1.8.0 software (National Institutes of Health) was employed for image processing. Primary antibodies were as follows: Anti-NF-κB p65 (dilution, 1:1500; cat. no. ab86299; Abcam), anti-p-NF-κB p65 (dilution, 1:1,000; cat. no. ab86299; Abcam), anti-CDK1 (dilution, 1:2,000; cat. no. ab201008; Abcam), anti-CDK2 (dilution, 1:1,000; cat. no. ab33147; Abcam), anti-cyclin D1 (dilution, 1:1,000; cat. no. ab1663; Abcam), anti-p21 (dilution, 1:1,500; cat. no. ab109199; Abcam), anti-Bax (dilution, 1:1,000; cat. no. ab32503; Abcam), anti-Bcl-2 (dilution, 1:1,000; cat. no. ab32124; Abcam), anti-caspase-3 (dilution, 1:1,000; cat. no. ab184787; Abcam) and anti-cleaved caspase-3 (dilution, 1:1,000; cat. no. ab32042; Abcam). Secondary antibodies were as follows: Goat anti-mouse IgG (H&L; dilution, 1:5,000; cat. no. ab205719; Abcam) and goat anti-rabbit IgG (H&L; dilution, 1:5,000; cat. no. ab6702; Abcam). Primary antibodies were incubated overnight at 4°C and secondary antibodies were incubated for 2 h at 37°C.

Statistical analysis. SPSS 20.0 software (IBM Corporation) was utilized for data analysis. Differences between the two treatment groups were analyzed by the Student's t-test or the Chi-square test. Differences among multiple groups were analyzed using one-way ANOVA and Duncan's post-hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Infiltration of M1-TAM negatively influences the efficacy of postoperative TACE for patients with liver cancer. Liver cancer tissues derived from 79 patients were pathologically examined prior to the patients undergoing TACE to evaluate the significance of the infiltration of M1-TAM. The significance of the infiltration of M1-TAM was assessed by detecting the expression levels of CD68 and HLA-DR via IHC (Fig. 1A-C). As presented in Fig. 1B and C, CD68 and HLA-DR were expressed on the same cell, as is clearly indicated by a red and brown overlap in Fig. 1C. As for Fig. 1B, due to the low expression level, individual expression was observed on certain cells without any overlap. All patients underwent TACE at 1-2 months post-operation. The abundance of M1-TAM in liver cancer tissues in the CR+PR group was significantly lower than that in the NR group (Fig. 1D).

Infiltration of M1-TAM activates the NF-κB pathway in liver cancer tissues. The level of p-p65 was upregulated as the infiltration of M1-TAM increased in the liver cancer tissues (Fig. 2A). In addition, the p-p65/p65 ratio in both HepG2 (Fig. 2B and C) and SNU-182 (Fig. 2B and D) cells was significantly higher in the CM than that in the RM group. Furthermore, JSH-23, an inhibitor that prevents p65 from entering the nucleus, significantly decreased the p-p65/p65 ratio in both HepG2 (Fig. 2B and C) and SNU-182 cells (Fig. 2B and D) grown in CM compared to those cultured in RM.

M1 macrophages increase the proliferation of liver cancer cells. Liver cancer cells were cultured in different media and after the third day of culture, the number of liver cancer cells in the CM was markedly higher than that in the RM (Fig. 3A and B). Of note, JSH-23 significantly decreased the number of liver cancer cells in the CM. In addition, the number of cell clones in the CM group was significantly higher than that in the RM group and JSH-23 markedly decreased the number of cell clones in the CM compared with that in the RM group (Fig. 3C and D). Furthermore, M1-TAM-CM significantly decreased the ratio of liver cancer cells in the G1/G0 cell cycle phase, while it markedly increased the ratio of liver cancer cells in the S and G2/M phases. However, JSH-23 was able to reverse these changes (Fig. 4). The expression levels of cell cycle-related proteins, such as CDK1, CDK2, Cyclin D1 and p21, were different in liver cancer cells in different media. Of note, the expression levels of CDK1, CDK2 and cyclin D1 in the RM were markedly lower than those in the CM; however, the expression level of p21 was significantly higher in the RM than that in the CM. Collectively, M1 macrophages stimulated liver cancer cell proliferation, which was reversed by the administration

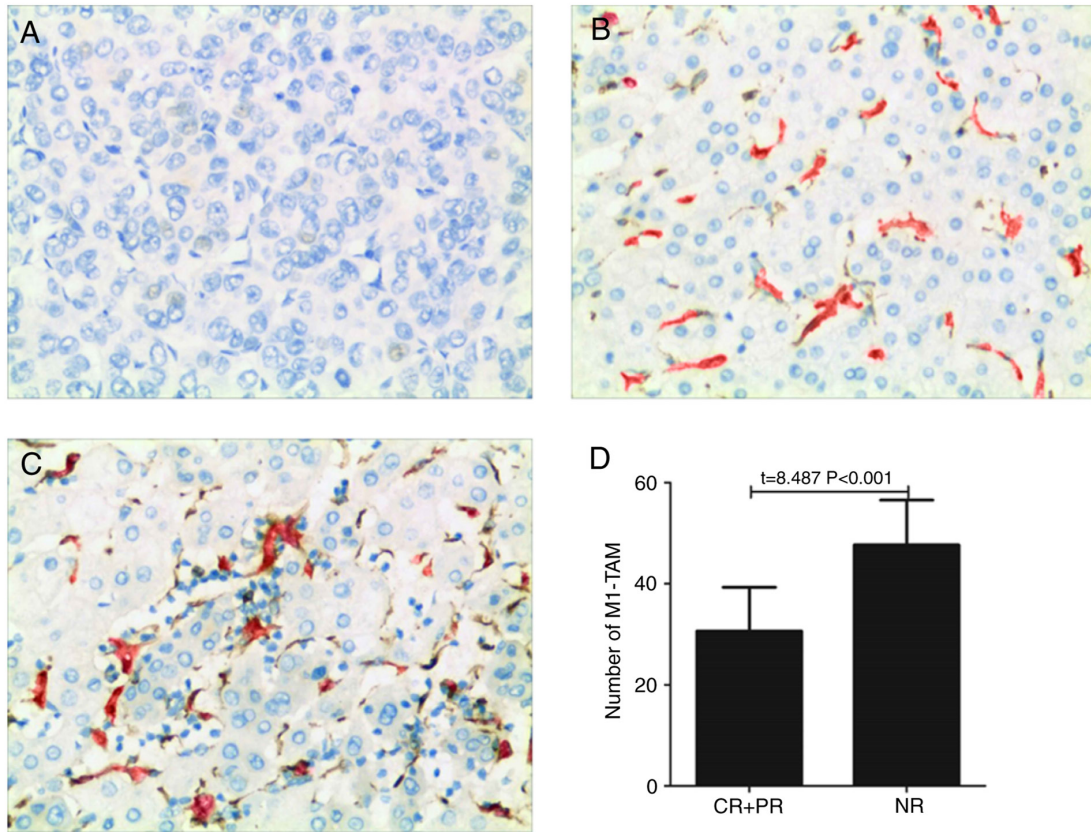


Figure 1. Infiltration of M1-TAM in human hepatoma tissue is associated with the therapeutic effects in patients with liver cancer. (A-C) Immunohistochemistry was used to detect the expression levels of CD68 (red) and HLA-DR (brown) in a hepatoma tissue, and representative images of immunohistochemical detection of CD68+HLA-DR+-labeled M1-like TAM in case of (A) no expression in a male patient (age, 53 years) with therapeutic efficacy rated as CR; (B) low expression in a male patient (age, 49 years) with therapeutic efficacy rated as PR; and (C) high expression in a male patient (age, 58 years) with therapeutic efficacy rated as NR (magnification, x200). (D) Number of CD68+HLA-DR+ (M1-TAM) cells in the hepatoma tissue of patients with liver cancer. HLA, human leukocyte antigen; TAM, tumor-associated macrophages; CR, complete remission; PR, partial remission; NR, no remission.

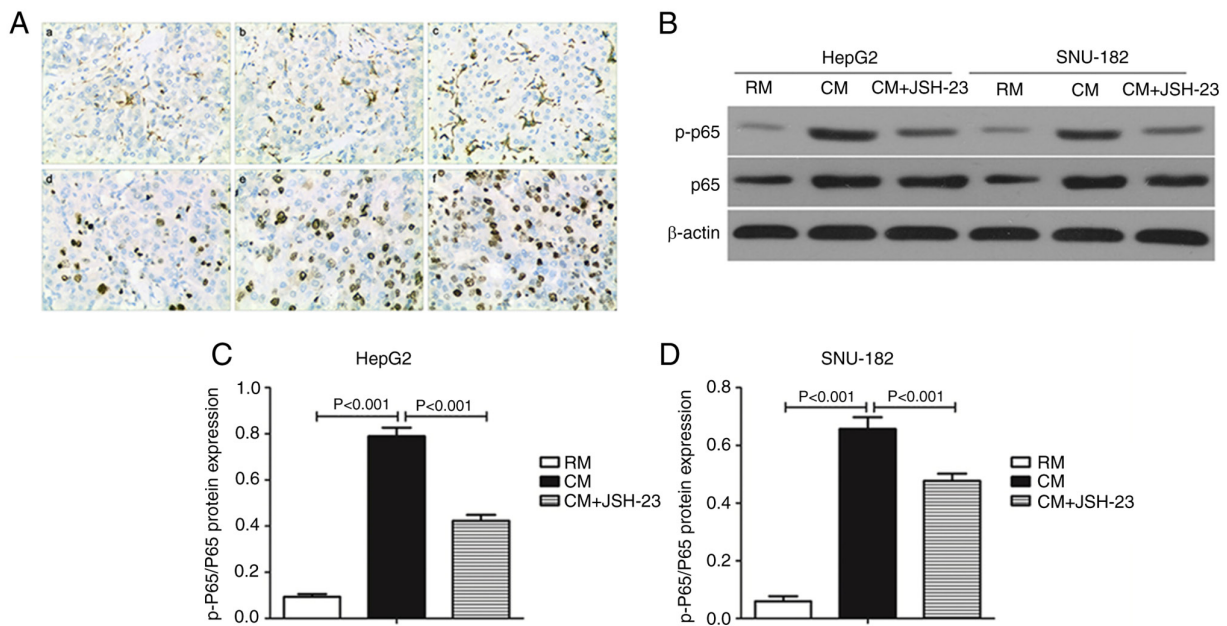


Figure 2. Infiltration of M1-tumor-associated macrophages is associated with the activation of the NF- κ B signaling pathway. (A) Immunohistochemistry was used to detect the expression levels of CD68+HLA-DR+ in hepatoma tissue samples with (a) low, (b) intermediate and (c) high macrophage infiltration and p-p65 in hepatoma tissue with (d) low, (e) intermediate and (f) high macrophage infiltration. Samples within the same column (a and d; b and e; and c and f) represent the same tissue in a patient with liver cancer (magnification, x200). (B-D) Following 6 h of treatment with JSH-23, the medium was replaced and the expression levels of p-p65 and p65 were measured by western blot analysis. (B) Representative western blot image and quantified p-p65/p65 ratio in (C) HepG2 and (D) SNU-182 cells. The experiments were performed in triplicate. RM, regular medium; CM, conditioned medium; HLA, human leukocyte antigen; p-p65, phosphorylated NF- κ B p65.

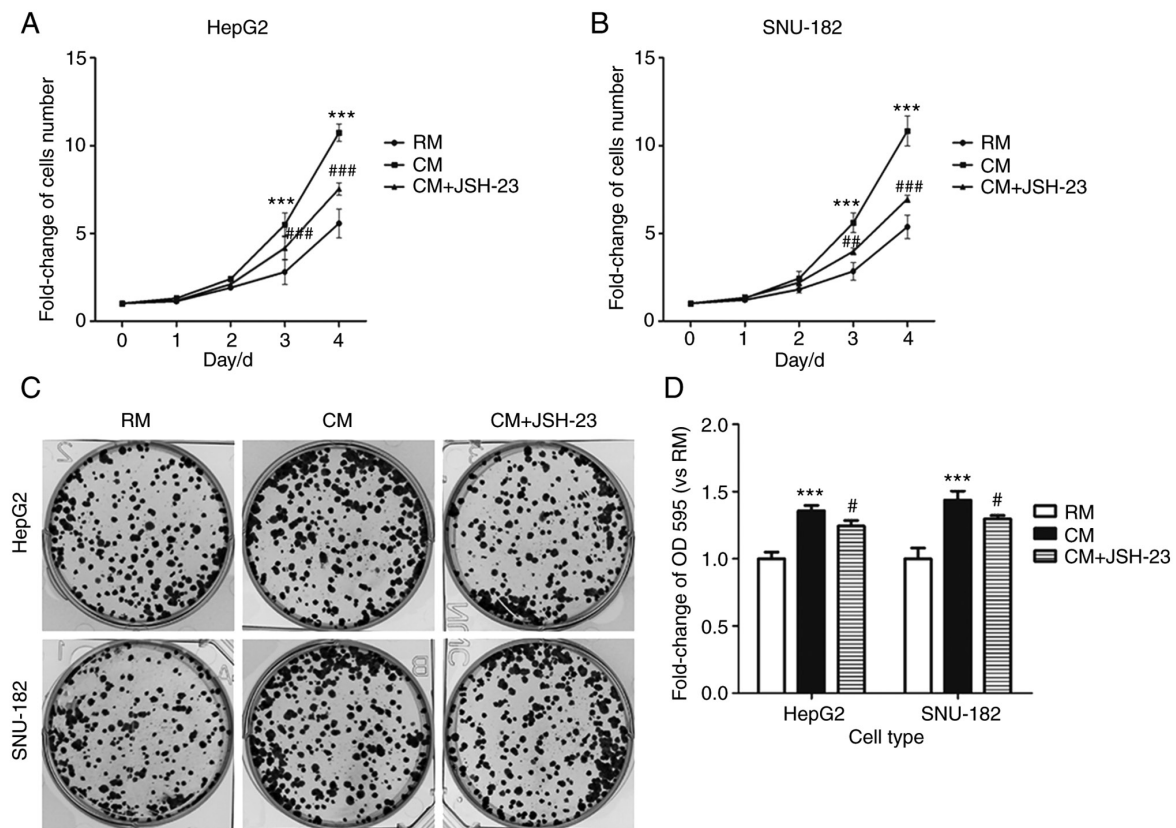


Figure 3. Effects of M1 macrophages on the proliferation of liver cancer cells via activating the NF- κ B pathway. The HepG2 and SNU-183 cells were cultured in CM or RM or JSH-23 + CM. (A and B) Following 6 h of treatment with JSH-23, the medium was replaced and the cells were cultured for another 4 days. Finally, the cell growth of (A) HepG2 and (B) SNU-182 cells was determined by the MTT assay. (C and D) A clonogenic assay was utilized to assess the proliferation of liver cancer cells (C) representative images of wells with colonies and (D) Quantitative results. All experiments were performed in triplicate. ***P<0.001 vs. RM group; #P<0.05, ##P<0.01, ###P<0.001 vs. CM group. RM, regular medium; CM, conditioned medium.

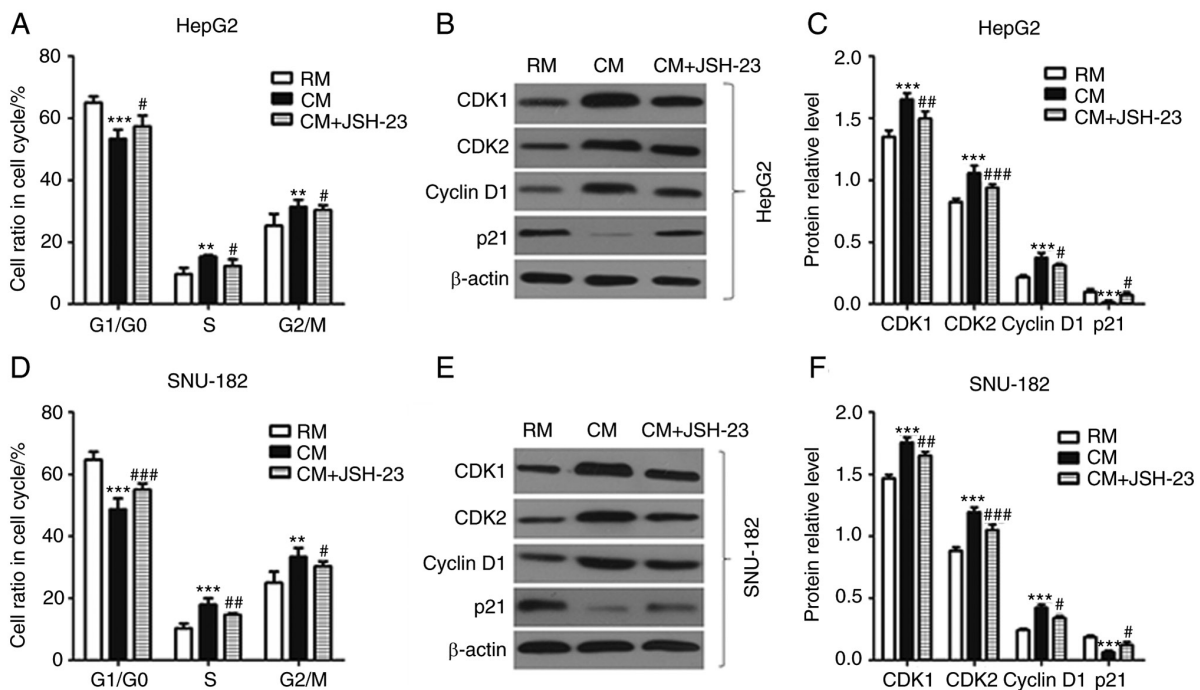


Figure 4. Effects of M1 macrophages on the cell cycle of liver cancer cells via activating the NF- κ B pathway. (A) The cell cycle of HepG2 cells was detected by flow cytometry. (B and C) The expression levels of CDK1, CDK2, cyclin D1 and P21 in HepG2 cells were detected by western blot analysis. (B) Representative western blot image and (C) quantified results. (D) The cell cycle of SNU-182 cells was detected by flow cytometry. (E and F) The expression levels of CDK1, CDK2, cyclin D1 and P21 in SNU-182 cells were detected by western blot analysis. (E) Representative western blot image and (F) quantified results. All experiments were performed in triplicate. **P<0.01, ***P<0.001 vs. RM group; #P<0.05, ##P<0.01, ###P<0.001 vs. CM group. RM, regular medium; CM, conditioned medium; CDK, cyclin-dependent kinase.

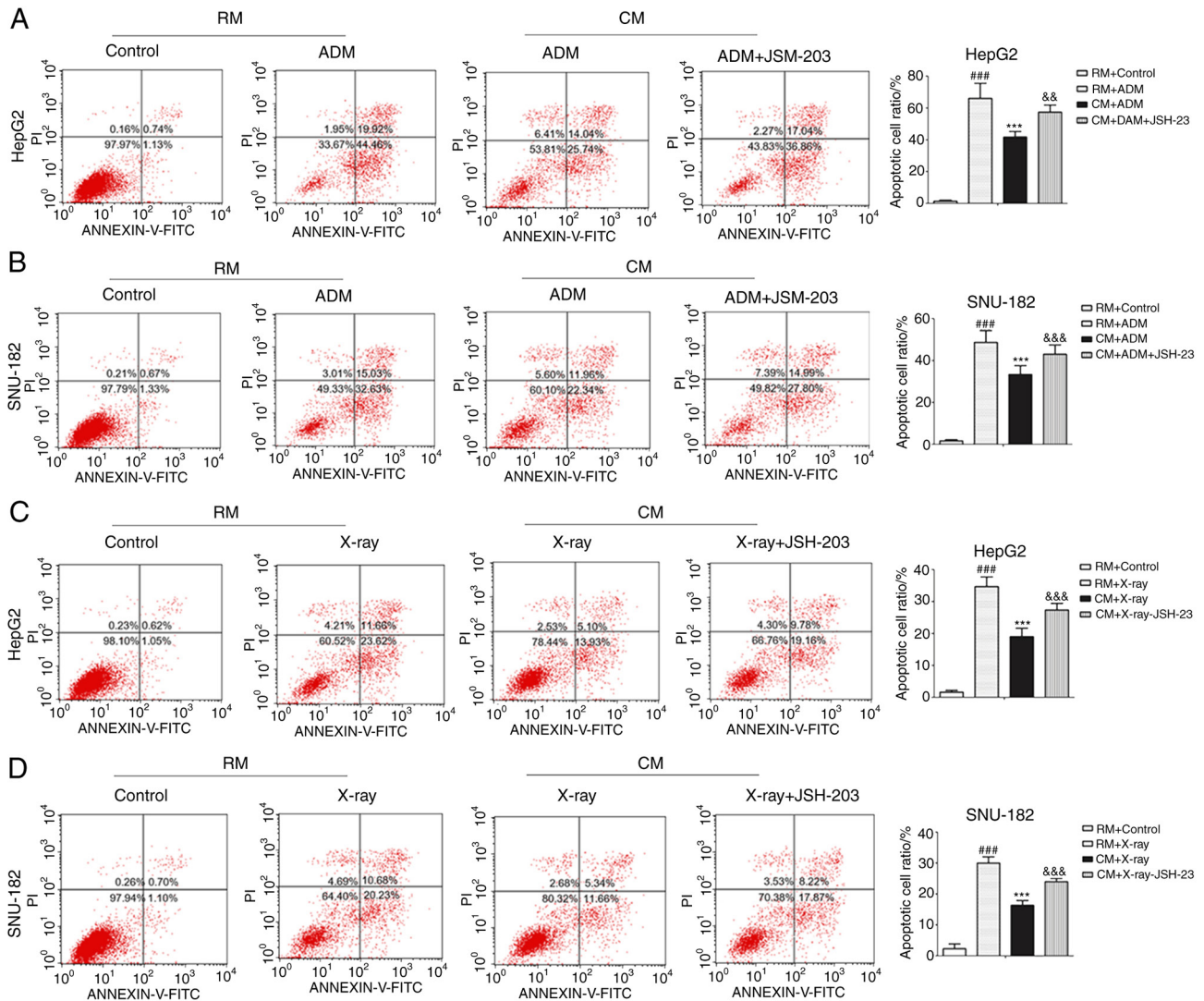


Figure 5. Effects of M1 macrophages on the anti-apoptotic ability of liver cancer cells via activating the NF- κ B pathway. (A and B) Flow cytometry was used to measure apoptosis of (A) HepG2 and (B) SNU-182 cells in RM or CM after treatment with or without ADM. (C and D) X-ray induced-apoptosis of (C) HepG2 and (D) SNU-182 cells in RM or CM. $^{###}P<0.001$ vs. RM + Control group; $^{***}P<0.001$ vs. RM+X-ray group; $^{&&}P<0.01$, $^{&&&}P<0.001$ vs. CM+X-ray or CM+ADM group. RM, regular medium; CM, conditioned medium; PI, propidium iodide; ADM, Adriamycin.

of JSH-23 (an inhibitor of NF- κ B), i.e. this effect was mediated via NF- κ B.

M1 macrophages enhance the anti-apoptotic ability of liver cancer cells. ADM is frequently used for the treatment of liver cancer and may induce apoptosis of liver cancer cells (21). In the present study, it was indicated that ADM significantly increased the number of apoptotic liver cancer cells in the RM (Fig. 5A and B). In addition, with ADM administration, the number of apoptotic liver cancer cells in the CM was significantly lower than that in the RM. The present results indicated that JSH-23 markedly increased the number of apoptotic liver cancer cells in the CM. X-ray irradiation was also used to induce apoptosis of liver cancer cells similar to ADM and the X-rays significantly increased the number of apoptotic liver cancer cells in the RM (Fig. 5C and D). However, the number of apoptotic liver cancer cells in the CM was significantly lower than that in the RM following exposure to X-rays. It was also observed that JSH-23 significantly increased the number of apoptotic liver cancer cells in samples subjected to X-ray in

the CM. Furthermore, the expression levels of apoptosis-related proteins, such as Bax, Bcl-2 and caspase-3, were detected by western blot analysis. It was observed that after AMD treatment, the expression levels of Bax and caspase-3 in the RM were significantly higher than those in the CM (Fig. 6). In addition, the expression level of Bcl-2 in the RM was significantly lower than that in the CM after treatment with AMD. Taken together, M1 macrophages had anti-apoptotic effects on liver cancer cells, which were reversed by the administration of JSH-23 (an inhibitor of NF- κ B), i.e. this effect was mediated via NF- κ B.

Discussion

Tumor-associated macrophages are a well-known component of the TME and may have pivotal roles in the onset and development of liver cancer, as well as in the regulation of inflammation in the TME (22). However, the association between the infiltration of macrophages and their effects on the effectiveness of liver cancer treatment has remained

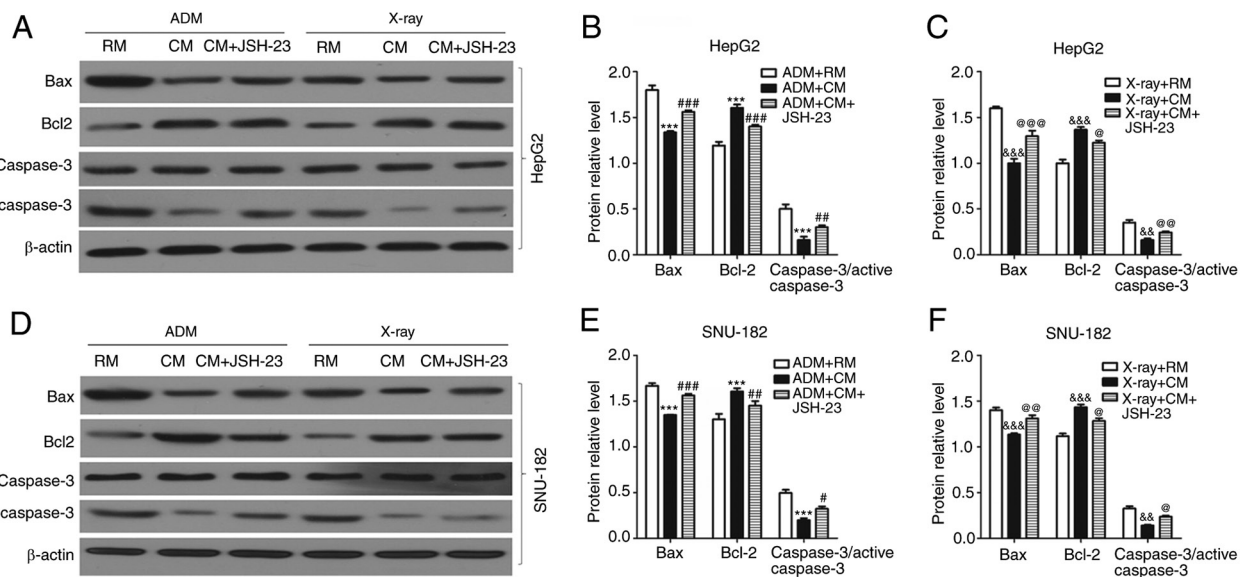


Figure 6. Effects of M1 macrophages on the expression levels of apoptosis-related proteins in liver cancer cells via activating the NF- κ B signaling pathway. (A-C) Western blot analysis was used to detect the expression levels of Bax, Bcl-2 and caspase-3 in HepG2 cells in RM or CM after treating with or without ADM or treating with or without X-rays. (A) Representative western blot image and quantified expression levels in (B) the ADM experiment and (C) the X-ray experiment. (D-F) Western blot analysis was employed to detect the expression levels of Bax, Bcl-2 and caspase-3 in SNU-182 cells in RM or CM after treating with or without ADM or treating with or without X-rays. (D) Representative western blot image and quantified expression levels in (E) the ADM experiment and (F) the X-ray experiment. All experiments were performed in triplicate. *** $P < 0.001$ vs. ADM+RM group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. CM+ADM group; && $P < 0.01$, &&& $P < 0.001$ vs. X-ray + RM group; @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$ vs. X-ray + CM group. RM, regular medium; CM, conditioned medium; ADM, Adriamycin.

elusive. Concerning post-resection therapy for patients with liver cancer, TACE is able to limit blood supply to tumorous tissues and significantly inhibit tumor progression in comparison with other popular treatments (23,24). Thus, the approach of the present study is highly significant, as it was observed that a high number of CD68+HLA-DR+ M1-like TAM in tumorous tissues was negatively associated with the therapeutic efficacy of TACE. It should also be pointed out that the therapeutic effect of TACE on liver cancer depends on not only the sensitivity to a drug (ADM) but also on the degree of embolization by lipiodol and gelatin sponges. Consistently with this, in the present study, it was determined that infiltration of M1-like TAM was associated with the level of p-p65 in liver cancer tissues. In general, TAMs are reported to closely resemble M2 macrophages (25,26), but in the present study, M2 macrophages were not detected. In addition, it was observed that M1-like TAM were able to activate the NF- κ B signaling pathway in liver cancer cells. Therefore, it was concluded that the infiltration of M1-like TAM in liver cancer tissues may activate the NF- κ B signaling pathway in liver cancer cells.

The NF- κ B signaling pathway is also important, as it is a crucial mediator of inflammation-associated tumors (27,28). The continuous activation of the NF- κ B pathway has been confirmed to have roles in the initiation, development and progression of inflammation-associated liver cancer and it is an important documented link between hepatitis and liver cancer (29,30). The activation of the NF- κ B pathway is associated with the onset of liver cancer, increased expression of the related genes in the liver, as well as the regulation of the expression of anti-apoptotic and pro-apoptotic genes [e.g., Bcl-2/Bax and Bcl-2/Bcl-2-associated

death promoter (Bad)] (31). In addition, several studies have concluded that the NF- κ B signaling pathway mainly participates in the proliferation and anti-apoptotic regulation of liver cancer cells through the following pathways: i) Promoting transformation of cells from G1/G0 to S phase via regulating the expression of cyclin D1, thereby leading to excessive cell proliferation (32,33); ii) inducing the expression of various inhibitors of apoptosis, such as cellular inhibitor of apoptosis protein 1 (c-IAP1) and c-IAP2, thereby increasing the resistance of cancer cells to apoptosis (34,35); iii) inhibiting cell autophagy (36,37); and iv) inducing the expression of genes essential for survival (38,39). Consequently, the infiltration of M1-like TAM in liver cancer tissues may participate in the molecular mechanisms underlying the regulation of proliferation and apoptosis of liver cancer cells via stimulating the NF- κ B signaling pathway.

Cell proliferation assays were employed in the present study to indicate whether infiltration of M1-like TAM into liver cancer tissues was able to promote the proliferation of liver cancer cells by activating the NF- κ B signaling pathway. As expected, M1-like TAM promoted the proliferation of liver cancer cells *in vitro*. However, JSH-23 attenuated the proliferation of liver cancer cells. Notably, the present study indicated that M1-like TAM promoted the transition of liver cancer cells from G1/G0 to S and G2/M phases, while JSH-23 reversed these effects. Regarding the molecular mechanisms, it was determined that M1-like TAM upregulated the expression levels of CDK1, CDK2 and cyclin D1, whereas it decreased the expression levels of p21 in liver cancer cells. CDKs and CDK inhibitors (CDKIs) have substantial roles in regulating the transition from G1 to S phase of the cell cycle (40,41). Furthermore,

p21 is a member of the CDKI family and is a negative regulator of the cell cycle with diverse effects on tumorigenesis according to its localization within different subcellular compartments. The CDKs, through binding to cyclin, CDK or cyclin-CDK, consequently cause cell cycle arrest and block cell proliferation (42,43). The present results regarding the role of p21 are significant, as p21 is an important downstream gene of the p53 gene and it has a specific binding site for the p53 protein. When cells are subjected to various types of damage *in vivo* and *in vitro*, p53 protein acts upon the p21 gene to promote the expression level of p21 in a relatively fast manner. Consequently, the p21 protein binds to almost all cyclin-CDK complexes and inhibits them. This includes cyclin D1-CDK4, cyclin E-CDK2 and cyclin A-CDK2, while p21 is able to weakly inhibit cyclin B-related complexes. Furthermore, p21 inhibits the activities of cyclin D1-CDK4 and cyclin E-CDK2 to block the phosphorylation of the Rb protein and cause the release of E2F protein, thereby causing cell cycle arrest in G1 phase. It may be concluded that M1-like TAM regulate the expression of cell cycle-associated proteins by activating the NF- κ B signaling pathway. Thus, M1-like TAM may stimulate the transformation of liver cancer cells from G1/G0 to S phase and promote the proliferation of liver cancer cells.

ADM is widely used for the treatment of liver cancer, as it may induce apoptosis of liver cancer cells. Radiotherapies are frequently combined with TACE to treat patients with liver cancer, as they may induce apoptosis of cancer cells. The sensitivity of tumors to radiotherapies has been indicated to be consistent with the sensitivity of tumor cells to apoptosis (44,45). In the present study, ADM and X-ray irradiation were used as external factors to induce apoptosis of liver cancer cells. It was indicated that the number of apoptotic liver cancer cells in the CM was significantly lower than that in the RM after treatment with either ADM or X-rays. However, the addition of JSH-23 to CM significantly increased the amount of ADM and X-ray-induced apoptosis of liver cancer cells. Furthermore, the results of the present study revealed that M1-like TAM significantly decreased both ADM- and X-ray-induced apoptosis of liver cancer cells. In addition, M1-like TAM increased the expression levels of Bax and caspase-3 proteins, while it decreased Bcl-2 expression in liver cancer cells. However, JSH-23 was observed to reverse the above-mentioned effects. It is also noteworthy that M1-TAM may be directly affected by ADM and irradiation. Previous studies reported that the anti-apoptotic function of NF- κ B family proteins may be a result of consequential unbalancing of the expression levels of apoptotic and anti-apoptotic proteins, as well as their direct interactions with tumor suppressor proteins (35,46). In an animal-based study, increased activity of the NF- κ B signaling pathway promoted the incidence of hepatocarcinoma and stimulated the survival of liver cancer cells by significantly enhancing the levels of anti-apoptotic proteins, such as Bcl-2/Bax and Bcl-2/Bad, compared with those of pro-apoptotic proteins (31). In the present study, the CM of THP-1 cells induced by PMA, LPS and IFN- γ was used to evaluate the effects of M1-like TAM on liver cancer cells. Although the constituents of the CM were not determined, previous studies suggested that the constituents accounting for the regulatory effects of M1-like TAM on the proliferation and

apoptosis of liver cancer cells may be inflammatory cytokines and growth factors (47,48). However, further studies are still required to verify the present results.

Regarding limitations of the present study, it should be pointed out that only CD68 and HLA-DR were assessed to detect M1 macrophages. The present results did not fully reflect the polarization of diverse macrophages in the TME *in vivo*. In addition, Ki-67 expression was not detected in clinical specimens.

In conclusion, the present study suggested that infiltration of CD68+HLA-DR+ M1-like TAM were negatively associated with the efficacy of postoperative TACE for patients with liver cancer. M1-like TAM may promote the proliferation of liver cancer cells and enhance their anti-apoptotic ability by activating the NF- κ B signaling pathway.

Acknowledgements

Not applicable.

Funding

This study was financially supported by the Doctoral Program of Inner Mongolia Natural Science Foundation (grant no. 2018BS08004), the Natural Science Foundation of Inner Mongolia (grant no. 2019LH08028) and the Science and Technology Million Project of Inner Mongolia Medical University [grant no. YKD2017KJBW(LH)002].

Availability of data and materials

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

Authors' contributions

GS and DZ designed the study, prepared the figures and drafted the manuscript. GS, HC and XH performed the experiments. GS, JM and DZ contributed to the conceptualization of the research, drafted the manuscript and revised it critically. GS and DZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University (Hohhot, China; approval no. YJ 2020001) and the study was performed in accordance with the Declaration of Helsinki. In addition, all participants provided written informed consent prior to enrolment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Chen JG and Zhang SW: Liver cancer epidemic in China: Past, present and future. *Semin Cancer Biol* 21: 59-69, 2011.
- Yu XQ and Baade P: RE: Cancer incidence and mortality in China, 2013 by Chen *et al*. *Cancer Lett* 401: 72-73, 2017.
- Shalapour S, Lin XJ, Bastian IN, Brain J, Burt AD, Aksenov AA, Vrbanc AF, Li W, Perkins A, Matsutani T, *et al*: Inflammation-induced IgA⁺ cells dismantle anti-liver cancer immunity. *Nature* 561: 340-345, 2017.
- Yang YM, Kim SY and Seki E: Inflammation and liver cancer: Molecular mechanisms and therapeutic targets. *Semin Liver Dis* 39: 26-42, 2019.
- Chen W: Cancer statistics: Updated cancer burden in China. *Chin J Cancer Res* 27: 1, 2015.
- Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J: Cancer statistics in China, 2015. *CA Cancer J Clin* 66: 115-132, 2016.
- Liu J, Ni W, Qu L, Cui X, Lin Z, Liu Q, Zhou H and Ni R: Decreased expression of EHD2 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. *Dig Dis Sci* 61: 2554-2567, 2016.
- Yokoo T, Patel AD, Levcohan N, Singal AG, Yopp AC and Pedrosa I: Extrahepatic metastasis risk of hepatocellular carcinoma based on α -fetoprotein and tumor staging parameters at cross-sectional imaging. *Cancer Manag Res* 9: 503-511, 2017.
- Grivennikov SI, Greten FR and Karin M: Immunity, inflammation, and cancer. *Cell* 140: 883-899, 2010.
- Aggarwal BB, Sung B and Gupta SC: In inflammation and cancer. *Advances in Experimental Medicine and Biology*. Vol 816. Springer LLC, New York, NY, pp5, 2014.
- Gunasekaran GR, Poongkavithai Vadevoo SM, Baek MC and Lee B: M1 macrophage exosomes engineered to foster M1 polarization and target the IL-4 receptor inhibit tumor growth by reprogramming tumor-associated macrophages into M1-like macrophages. *Biomaterials* 278: 121137, 2021.
- Barberi T, Martin A, Suresh R, Barakat DJ, Harris-Bookman S, Drake CG, Lim M and Friedman AD: Absence of host NF- κ B p50 induces murine glioblastoma tumor regression, increases survival, and decreases T-cell induction of tumor-associated macrophage M2 polarization. *Cancer Immunol Immunother* 67: 1491-1503, 2018.
- Saccani A, Schioppa T, Porta C, Biswas SK, Nebuloni M, Vago L, Bottazzi B, Colombo MP, Mantovani A and Sica A: p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res* 66: 11432-11440, 2006.
- Yao RR, Li JH, Zhang R, Chen RX and Wang YH: M2-polarized tumor-associated macrophages facilitated migration and epithelial-mesenchymal transition of HCC cells via the TLR4/STAT3 signaling pathway. *World J Surg Oncol* 16: 9, 2018.
- Wei X, Tang C, Lu X, Liu R, Zhou M, He D, Zheng D, Sun C and Wu Z: MiR-101 targets DUSP1 to regulate the TGF- β secretion in sorafenib inhibits macrophage-induced growth of hepatocarcinoma. *Oncotarget* 6: 18389-18405, 2015.
- Arvanitakis K, Koletsis T, Mitroulis I and Germanidis G: Tumor-associated macrophages in hepatocellular carcinoma pathogenesis, prognosis and therapy. *Cancers (Basel)* 14: 226, 2022.
- Zong Z, Zou J, Mao R, Ma C, Li N, Wang J, Wang X, Zhou H, Zhang L and Shi Y: M1 macrophages induce PD-L1 expression in hepatocellular carcinoma cells through IL-1 β signaling. *Front Immunol* 10: 1643, 2019.
- Medical Administration and Hospital Administration Bureau of Health and Family Planning Commission of the People's Republic of China. Standard for diagnosis and treatment of primary liver cancer (2017 edition). *Chin J Dig Surg* 16: 13, 2017.
- Tjiu JW, Chen JS, Shun CT, Lin SJ, Liao YH, Chu CY, Tsai TF, Chiu HC, Dai YS, Inoue H, *et al*: Tumor-associated macrophage-induced invasion and angiogenesis of human basal cell carcinoma cells by cyclooxygenase-2 induction. *J Invest Dermatol* 129: 1016-1025, 2009.
- Schwende H, Fitzke E, Ambs P and Dieter P: Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J Leukoc Biol* 59: 555-561, 1996.
- Yu Z, Guo J, Hu M, Gao Y and Huang L: Icaritin exacerbates mitophagy and synergizes with doxorubicin to induce immunogenic cell death in hepatocellular carcinoma. *ACS Nano* 14: 4816-4828, 2020.
- Li M, Lai X, Zhao Y, Zhang Y, Li M, Li D, Kong J, Zhang Y, Jing P, Li H, *et al*: Loss of NDRG2 in liver microenvironment inhibits cancer liver metastasis by regulating tumor associated macrophages polarization. *Cell Death Dis* 9: 248, 2018.
- Wu H, Liu S, Zheng J, Ji G, Han J and Xie Y: Transcatheter arterial chemoembolization (TACE) for lymph node metastases in patients with hepatocellular carcinoma. *J Surg Oncol* 112: 372-376, 2015.
- Barone M, Ettorre GC, Ladisa R, Schiavariello M, Santoro C, Francioso G, Vinciguerra V and Francavilla A: Transcatheter arterial chemoembolization (TACE) in treatment of hepatocellular carcinoma. *Hepatogastroenterology* 50: 183-187, 2003.
- Zeng XY, Xie H, Yuan J, Jiang XY, Yong JH, Zeng D, Dou YY and Xiao SS: M2-like tumor-associated macrophages-secreted EGF promotes epithelial ovarian cancer metastasis via activating EGFR-ERK signaling and suppressing lncRNA LIMT expression. *Cancer Biol Ther* 20: 956-966, 2019.
- Lee C, Jeong H, Bae Y, Shin K, Kang S, Kim H, Oh J and Bae H: Targeting of M2-like tumor-associated macrophages with a melittin-based pro-apoptotic peptide. *J Immunother Cancer* 7: 147, 2019.
- Dolcet X, Llobet D, Pallares J and Matias-Guiu X: NF- κ B in development and progression of human cancer. *Virchows Archiv* 446: 475-482, 2005.
- Yu H, Aravindan N, Xu J and Natarajan M: Inter- and intra-cellular mechanism of NF- κ B-dependent survival advantage and clonal expansion of radio-resistant cancer cells. *Cell Signal* 31: 105-111, 2017.
- Yang W, Yang S, Zhang M, Gao D, He T and Guo M: ZNF545 suppresses human hepatocellular carcinoma growth by inhibiting NF- κ B signaling. *Genes Cancer* 8: 528-535, 2017.
- Zhang Q, Song G, Yao L, Liu Y, Liu M, Li S and Tang H: miR-3928v is induced by HBx via NF- κ B/EGFR1 and contributes to hepatocellular carcinoma malignancy by down-regulating VDAC3. *J Exp Clin Cancer Res* 37: 14, 2018.
- Park SG, Lee T, Kang HY, Park K, Cho KH and Jung G: The influence of the signal dynamics of activated form of IKK on NF-kappaB and anti-apoptotic gene expressions: A systems biology approach. *FEBS Lett* 580: 822-830, 2006.
- Malhotra U, Hittelman WN, Wu TT, Luthra R, Swisher S, Luthra M, Correa A, Aggarwal BB, Ajani J and Izzo JG: Association of activated NF- κ B, altered cyclin D1 and poor outcome in esophageal adenocarcinoma. *Cancer Res* 65 (9 Suppl): S548, 2005.
- Ozeki M, Hamajima Y, Feng L, Ondrey FG, Schlentz E and Lin J: Id1 induces the proliferation of cochlear sensory epithelial cells via the nuclear factor- κ B/cyclin D1 pathway in vitro. *J Neurosci Res* 90: 2225, 2012.
- Varfolomeev E, Goncharov T, Fedorova AV, Dynek JN, Zobel K, Deshayes K, Fairbrother WJ and Vucic D: c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation. *J Biol Chem* 283: 24295-24299, 2008.
- Vázquez-Franco JE, Reyes-Maldonado E, Vela-Ojeda J, Domínguez-López ML and Lezama RA: Src, Akt, NF- κ B, BCL-2 and c-IAP1 may be involved in an anti-apoptotic effect in patients with BCR-ABL positive and BCR-ABL negative acute lymphoblastic leukemia. *Leuk Res* 36: 862-867, 2012.
- Wu CC, Kuo YH and Hsieh SL: 345 Sedanolid induces human liver tumor cell autophagy through regulation of NF- κ B pathway. *Eur J Cancer* 48 (Suppl 5): S84, 2012.
- Zhang H, Chen Z, Miranda RN, Medeiros LJ and McCarty N: TG2 and NF- κ B signaling coordinates the survival of mantle cell lymphoma cells via IL-6-mediated autophagy. *Cancer Res* 76: 6410-6423, 2016.
- Kim YS, Schwabe RF, Qian T, Lemasters JJ and Brenner DA: TRAIL-mediated apoptosis requires NF-kappaB inhibition and the mitochondrial permeability transition in human hepatoma cells. *Hepatology* 36: 1498-1508, 2002.
- Morotti A, Cilloni D, Pautasso M, Messa F, Arruga F, Defilippi I, Carturan S, Catalano R, Rosso V, Chiarenza A, *et al*: NF- κ B inhibition as a strategy to enhance etoposide-induced apoptosis in K562 cell line. *Am J Hematol* 81: 938-945, 2006.
- Lim S and Kaldis P: Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. *Development* 140: 3079-3093, 2013.
- Kent LN and Leone G: The broken cycle: E2F dysfunction in cancer. *Nat Rev Cancer* 19: 326-338, 2019.

42. Ocker M, Bitar SA, Monteiro AC, Gali-Muhtasib H and Schneider-Stock R: Epigenetic regulation of p21^{cip1/waf1} in human cancer. *Cancers (Basel)* 11: 1343, 2019.
43. Kim EM, Jung CH, Kim J, Hwang SG, Park J and Um HD: The p53/p21 complex regulates cancer cell invasion and apoptosis by targeting Bcl-2 family proteins. *Cancer Res* 77: 3092-3100, 2017.
44. Leszczynska KB, Foskolou IP, Abraham AG, Anbalagan S, Tellier C, Haider S, Span PN, O'Neill EE, Buffa FM and Hammond EM: Hypoxia-induced p53 modulates both apoptosis and radiosensitivity via AKT. *J Clin Invest* 125: 2385-2398, 2015.
45. Olive PL and Durand RE: Apoptosis: An indicator of radiosensitivity in vitro? *Int J Radiat Biol* 71: 695-707, 1997.
46. Papademetrio DL, Lompardía SL, Simunovich T, Costantino S, Mihalez CY, Cavaliere V and Álvarez É: Inhibition of survival pathways MAPK and NF-κB triggers apoptosis in pancreatic ductal adenocarcinoma cells via suppression of autophagy. *Target Oncol* 11: 183-195, 2016.
47. Singh R, Shankar BS and Sainis KB: TGF-β1-ROS-ATM-CREB signaling axis in macrophage mediated migration of human breast cancer MCF7 cells. *Cell Signal* 26: 1604-1615, 2014.
48. Yin Z, Ma T, Lin Y, Lu X, Zhang C, Chen S and Jian Z: IL-6/STAT3 pathway intermediates M1/M2 macrophage polarization during the development of hepatocellular carcinoma. *J Cell Biochem* 119: 9419-9432, 2018.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.