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Juglone eliminates MDSCs accumulation and enhances antitumor immunity

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) contribute to immune activity suppression and promote the tumor progression. Elimination of MDSCs is a promising cancer therapeutic strategy, and some chemotherapeutic agents have been reported to hamper tumor progression by suppressing MDSCs. Juglone has been showed to exert a direct cytotoxic effect on tumor cells. However, the effect of juglone on MDSCs and anti-tumor immune statue has remained unexplored. In our study, we observed that juglone suppressed tumor growth and metastasis markedly, and the tumor growth suppression in immunocompetent mice was more drastic than that in immunodeficient mice. Juglone reduced the accumulation of MDSCs and increased IFN-γ production by $CD8^+$ T cells. Consistently, juglone affected myeloid cells differentiation and maturation, impairing the immunosuppressive functions of MDSCs. Moreover, juglone down-regulated the level of IL-1β which was mediating accumulation of MDSCs. In addition, juglone inhibited 5FU-induced liver injury in a colorectal carcinoma-bearing mice model. Thus, our work suggests that the anti-tumor effect of juglone is mediated, at least in part, by eliminating accumulation of MDSCs.

1. Introduction

Cancer remains one of the highest mortality malignant diseases on worldwide [1]. Effective therapeutic strategies to control cancer are needed urgently. As the treatment of immune checkpoint inhibitors is successful in cancer treatment, immunotherapy provides a promising approach for cancer treatment [2]. Although immunotherapies are effective for multiple common cancer types, the majority of patients do not respond to immunotherapies as a result of immunosuppressive tumor microenvironment [3].

Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of immature myeloid cells that accumulate in spleens and at tumor sites and play a crucial role in immunosuppressive activity during malignancy [4–6]. Patients harboring malignancies who responded poorly to immunotherapy have been showed to have high levels of MDSCs [7]. MDSCs level is upregulated by tumor-derived multiple cytokines in spleens and in tumors of murine tumor models. It has been reported that accumulation of MDSCs was regulated by two sets of signals [8]. One signal contributes to the expansion of immature myeloid cells and is mediated by macrophage CSF (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and other growth factors [9–12]. Another signal supports the immunosuppressive capacity by different proinflammatory cytokines, such as IL-6, IL-1β, TNF-α, IFN-γ [13–15].

Ample evidence supports the contribution of MDSCs in promoting tumor growth and metastasis. Elimination of MDSCs has showed to restore the immunological activity resulting in inhibited tumor progression. Several chemotherapeutic strategies have been shown to regulate the level of MDSCs in different types of cancer. 5-Fluorouracil has been shown to enhance immune response by its depletion of MDSCs and increase antitumor response [16,17]. It was shown that all-trans-retinoic (ATRA) promoted the differentiation of MDSCs into DCs and improved anti-tumor immunity [18,19]. These studies indicate that targeting MDSCs may be a promising strategy for antitumor therapies. Therefore, the effect of agents on immune activity is necessary to investigate for selecting optimal anti-tumor agents.

Juglone is a nature component isolated from *Juglandaceae* walnut, a Chinese herbal medicine, used for cancer therapy [20,21]. It has been reported that juglone inhibits tumor proliferation and metastasis, induces cancer cells apoptosis and differentiation in different types of

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cancer [22–24]. A recent research suggested that juglone plays an immunomodulatory effect by increasing the population of $CD8^+$ T cells in BCG-vaccinated mice [25]. However, the effects of juglone on the function of MDSCs and the immunosuppressive tumor microenvironment have not been explored.

In this study, we investigated the effect of juglone on tumor immunosuppressive environment. Here, we showed that juglone significantly inhibited subcutaneous tumor growth and reduced hepatic colorectal cancer metastasis. Juglone treatment downregulated the level of MDSCs in spleen and at tumor sites of tumor-bearing mice, resulting in a marked increased population of tumor infiltrating T cells. In addition, $CD11c^+CD11b^+$ dendritic cells and $F4/80^+CD11b^+$ macrophages population were showed to increase with juglone treatment. Moreover, we found that juglone decreased the level of IL-1β at tumor site. Thus, we presented a novel antitumor mechanism of juglone by which the inhibition of MDSCs could enhance anti-tumor immune activity.

2. Materials and methods

2.1. Cell culture

CT26 cells were cultured in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS, BI). 4T1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, BI).

2.2. Tumor growth model

All animal studies were pre-approved by the Institute Animal Care and Use Committee of Harbin Medical University, and carried out in accordance with national and international guidelines for care and maintenance of laboratory animals. BALB/c mice and BALB/c nude mice (five to six-week-old) were purchased from Beijing Vital River Laboratory. The mice were standardized by 12 hours light/12 hours dark cycle for 7 days in our animal facility (Harbin Medical University, Harbin).

The mice were six to seven weeks old before inoculation with tumor cells. CT26 cells or 4T1 cells were harvested and resuspended in RMPI-1640 or DMED medium. To establish tumor growth model, 1×10^6 cells/100 μl were delivered into abdomen of mice by subcutaneous injections. The tumor volumes were measured by calipers once every two days and were calculated using the formula $(A \times B^2) / 2$ (A: the greatest diameter, B: the diameter perpendicular to A).

2.3. Colorectal cancer liver metastatic model

Each tumor injection was prepared by 50 μl suspension of 1×10^6 cells. The CT26 suspension was injected into spleen of 6–8 weeks old BALB/c mice. After 2 weeks juglone treatment, the mice were injected luciferin (in vivo grade, Promega) for imaging in vivo. Then, the mice were sacrificed and autopsy and liver metastases were isolated.

2.4. Chemotherapeutic treatment

For tumor growth and liver metastasis models, mice received injection of juglone (sigma) 1 mg/kg or 2 mg/kg body weight by intraperitoneal injection every other day. And 5-FU was administrated at dosage of 50 mg/kg body weight intraperitoneally every week.

2.5. Isolation of leukocytes

To prepare the splenocytes, mouse spleens were pressed through 70 μm cell strainer. Erythrocytes were removed by incubating with lysis buffer (BD Bioscience) for 15 min at room temperature.

Tumors were minced into fragments and incubated in HBSS (Gibco) supplemented with Collagenase IV (Sigma) at 50 μg/ml, DNase I (Sigma) at 10 mg/ml, and Hyaluronidase V (Sigma) at 100 μg/ml at 37 °C for 40 min. The solution was then passed through a 70 μm cell strainer. Tumor infiltrating leukocytes were subsequently isolated with Percoll density gradient supplied by a tumor-infiltrating immune cells isolation kit (Solarbio).

2.6. Flow cytometry

Single cell suspension from tumors and spleens were washed and resuspended with DPBS containing 2% FCS. Cells were resuspended in DPBS containing 2% FCS with Fc blocker (anti-mouse CD16/32 Ab, Biolegend) to prevent nonspecific binding. Incubated cells with the relevant surface antibodies on ice for 30 min in darkness. Wash the stained cells with pre-cold DPBS containing 2% FCS. Cells were resuspended and filtered through 70 μm mesh prior to flow cytometry analysis. Data were processed on FlowJo software.

To analyze the population of IFN-γ positive CTL, we preformed IFN-γ intracellular staining. The T cells were isolated as reported above, and then cultured with RMPI-1640 containing 10%FBS and cell stimulation and protein transport inhibitor cocktail (Thermo Fisher Scientific). Stimulation and transport inhibitor were maintained for 10 h. Cells were harvested and stained surface antibody as reported above. IFN-γ staining was performed using Fixation/Permeabilization Solution Kit (BD GolgiPlug™) following the manufacturer's instructions.

2.7. Immunohistochemistry

For liver anatomical structure visualization, the liver tissue was fixed with 4% paraformaldehyde and embedded in paraffin. Subsequently, immunohistochemistry sections (5 μm) were performed to dewax in xylene and rehydrate in descending ethanol series and stained with hematoxylin and eosin.

$Gr1^+$ MDSC quantification from paraffin-embedded mice liver metastases were performed antigen retrieval by boiling the sample in EDTA buffer (pH 8.0) and then Anti-mouse Gr-1 (1:100 dilution, eBioscience) was incubated at 4 °C overnight. Secondary antibodies were incubated for 1 h and then added 3,3′-diaminobenzidine (DAB) hydrochloride (Zsbio). After dehydration for in ethanol series and incubation in xylene, sections were mounted.

2.8. Real-time quantitative PCR analysis

Total RNAs of tumor were extracted with TRIzol reagent (Life technologies) following the manufacturer's instructions. High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) was used to generate cDNA. Real-time PCR was performed using Platinum SYBR Green qPCR SuperMix (Roche). Each sample was detected with relative gene mRNA primer. GAPDH was used for internal crossing normalization. Detailed primer sequences for qPCR were listed in Supplementary Table 1.

2.9. Whole transcriptome sequencing (RNA-seq)

BALB/c mice were injected subcutaneously 1×10^6 cells/100 μl 4T1 cells into abdomen of mice. On day 7, the mice were randomly distributed into two groups that were treated with control or 2 mg/kg juglone by intraperitoneal injection. The tumors were removed after 14 days treatment. RNA was extracted from tumor using TRIzol reagent (Life technologies) and subjected to Illumina RNA-sequencing platform (ANOROAD (Beijing) Limited Company) to determine the global expression of mRNA.

2.10. Statistical analysis

Each experiment was performed at least three times. Statistical tests

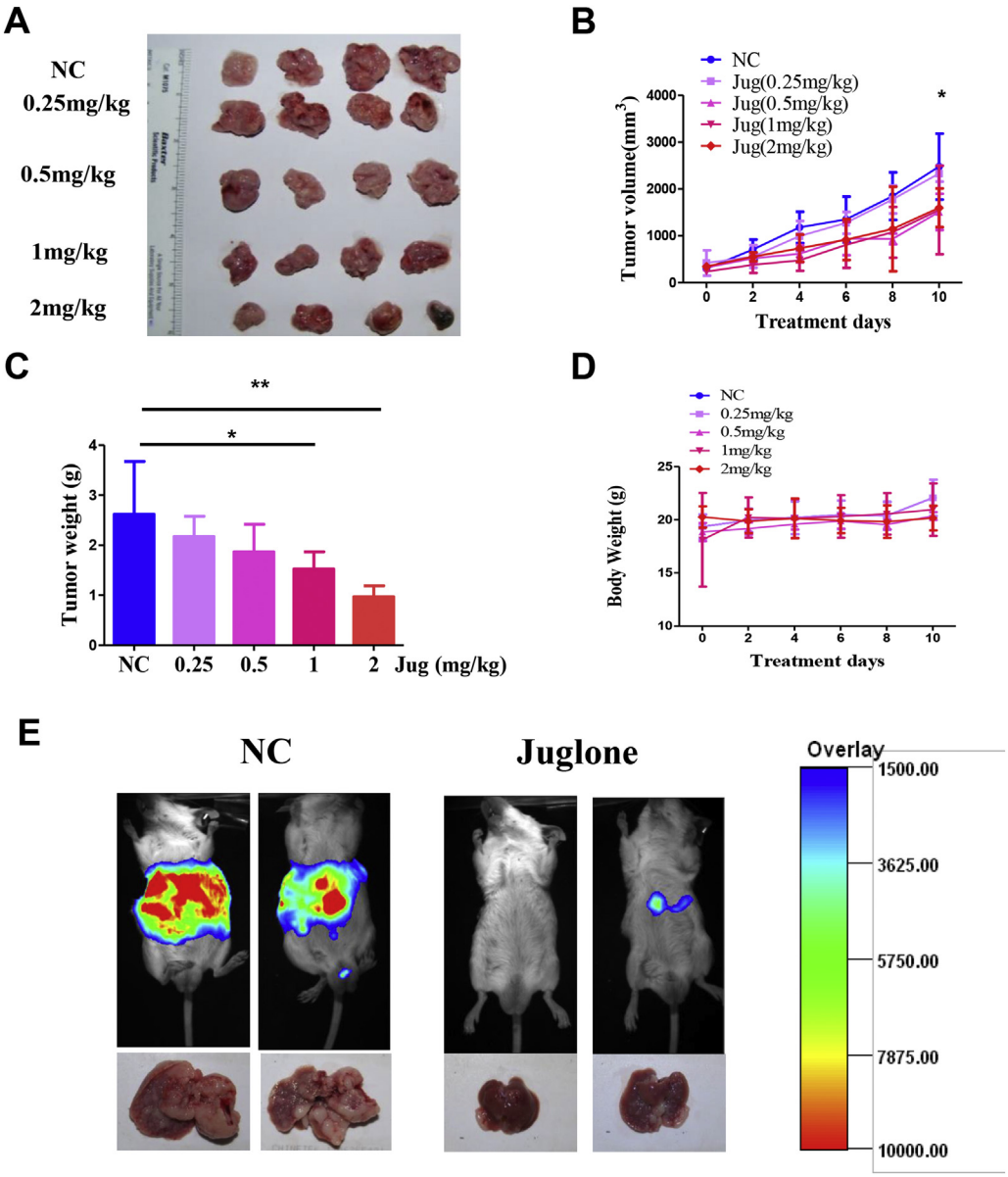

Fig. 1. Juglone inhibits tumor growth and colorectal cancer liver metastasis. Five tumor bearing mice were treated with control or different dosage juglone in each group by intraperitoneal injection every other day for 10 days. (A) Tumors were removed from tumor bearing mice and the representative images of tumor were photographed. (B) Tumor volume was measured every other day with Vernier calipers. (C) The dissected tumors weights were measured. (D) The body weights were monitored every other day. (E) Bioluminescence of liver metastasis of 1×10^6 CT26 colorectal cancer cells injected into spleen of BALB/c mice. Mice were intraperitoneal injected juglone (2 mg/kg) every other day 14. Exemplary images of bioluminescence were taken on day 14. All data are presented as the mean ± S.D. (*$p < 0.05$, **$p < 0.01$).

were performed using the SPSS Statistical 25.0. A statistical comparison between two groups was performed by the unpaired Student's *t*-test. For multiple comparisons, one-way ANOVA test, followed by Least Significant Difference test was used. The Kruskal-Wallis test was used to perform a statistical comparison with non-normal distribution. All reported data were expressed as the mean ± standard error (SD). A p value < 0.05 was considered statistically significant (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results

3.1. Juglone inhibits tumor growth and metastasis in BALB/c mouse model

To evaluate the effectiveness and toxicity of juglone, we employed a transplanted tumor model. BALB/c mice were inoculated subcutaneously with 1×10^6 CT26 cells and treated with different dosages of juglone by intraperitoneal injection every other day. We observed that juglone suppressed tumor growth with the increasing dosage (Fig. 1A). The tumor volume (Fig. 1B) and tumor weight (Fig. 1C) were marked reduced in 1 mg/kg and 2 mg/kg treated group. In addition, there was no significant difference in body weight between control group and juglone-treated groups (Fig. 1D). These results indicated that juglone inhibited tumor growth meanwhile induced scarcely toxicity in transplanted tumor. Thus, subsequent studies were carried out at 1 mg/kg or 2 mg/kg.

To identify the effect of juglone on tumor metastasis, we employed a colorectal cancer liver metastasis model by injecting 1×10^6 CT26 cells to spleen. Juglone treatment reduced hepatic colorectal cancer metastasis compared with the control (Fig. 1E). These findings indicated that juglone reduced hepatic colorectal cancer metastasis.

3.2. Immune activity contribute to antitumor effects of juglone

Juglone has been reported to act as immunomodulatory [25]. We hypothesized that immune contributes to the antitumor effect of juglone. To identify this hypothesis, we compared the juglone-induced tumor suppression of a syngeneic 4T1 tumor in a fully immunocompetent BALB/c model to an immunodeficient BALB/c nude model. We observed that the anti-tumor effect of juglone is more marked in a fully immunocompetent BALB/c model (Fig. 2A) than that in an immunodeficient BALB/c nude model (Fig. 2B). It suggested the immunological role of juglone in tumor growth suppression.

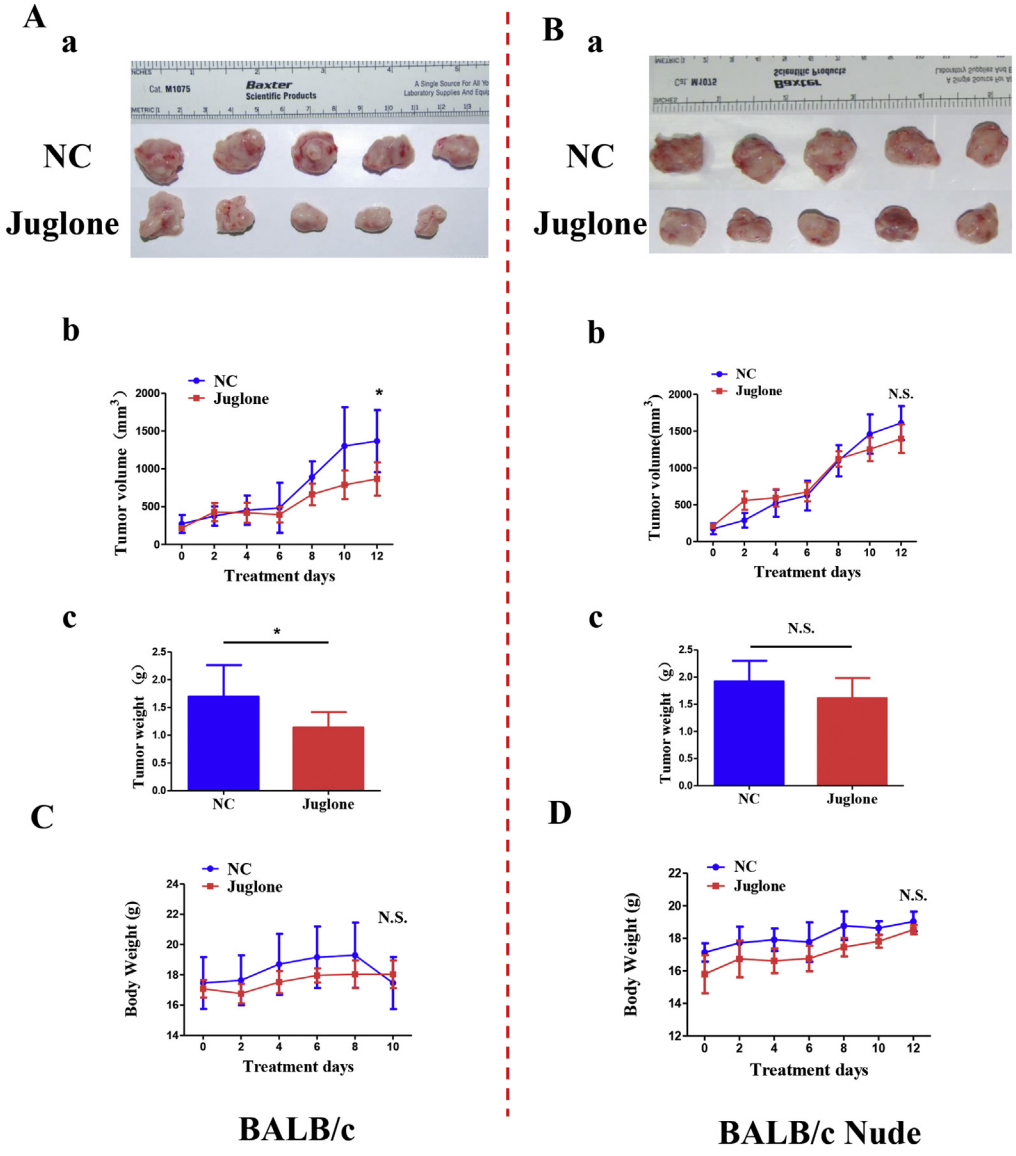

Fig. 2. Immune-mediated antitumor effect of juglone.
BALB/c mice or BALB/c nude mice were subcutaneously inoculated with 5×10^5 4T1 cells. The mice were treated with vehicle or juglone (1 mg/kg) by intraperitoneal injection every other day for 12 days. Tumor were removed from BALB/c mice (A) or BALB/c nude mice (B) were shown (a); tumor growth curves were monitored during the experimental period (b); the dissected tumor weights were measured (c). The body weights of BALB/c mice (C) or BALB/c nude mice (D) were monitored and plotted versus time. All data were presented as the mean ± S.D. (*$p < 0.05$).

Meanwhile, there was no change in body weight of immunocompetent mice and immunodeficient mice by juglone treatment (Fig. 2C–D). These results implied that immune activation is responsible for the antitumor effect of juglone.

3.3. Juglone reduces MDSCs frequencies in spleen and at tumor site

Moreover, we observed that juglone reduced the size of spleen from 4T1 tumor bearing mice (Fig. 3A), which indicated that juglone decreased systemic inflammatory response. We hypothesized that juglone may affect anti-tumor immune activity by regulating immunosuppressive cells in tumor microenvironment. Thus, we focus on MDSCs frequency in spleen and tumor. To explore this, we tested the frequency of $CD11b^+Gr1^+$ MDSCs in spleen and tumor of tumor-bearing mice after juglone treatment. Administration of juglone caused a significant reduction in $CD11b^+Gr1^+$MDSCs frequency in both spleen (Fig. 3B) and tumor (Fig. 3C) as compared with the control-treated group. As shown in Fig. 1E, juglone eradicated hepatic colorectal metastasis. Furthermore, reduction of $Gr1^+$ cells in metastatic tumors was confirmed by immunohistochemical analyses (Fig. 3D). At this stage, we concluded that MDSCs rejection was attributable to immunogenic antitumor effect of juglone.

3.4. Juglone promotes the CTLs expansion and activation

Previous findings showed MDSCs could inhibit cytotoxic T cell proliferation. Therefore, we next determined whether MDSCs depletion caused by juglone affected tumor infiltrating T cells. The flow cytometry analyses of spleens from mice bearing 4T1 tumor demonstrated systemic $CD8^+$ T cells were increased in juglone treatment group as compared with control group (Fig. 4A). Moreover, a marked increased tumor infiltrating $CD8^+$ cytotoxic T cells was observed in juglone treatment group using histologic analysis (Fig. 4B). In addition, MDSCs are known to suppress T cell activation. Consistent with this, we observed an increased production of IFN-γ in CD8 + T cells from tumor of juglone treatment group (Fig. 4C). Together, these findings revealed

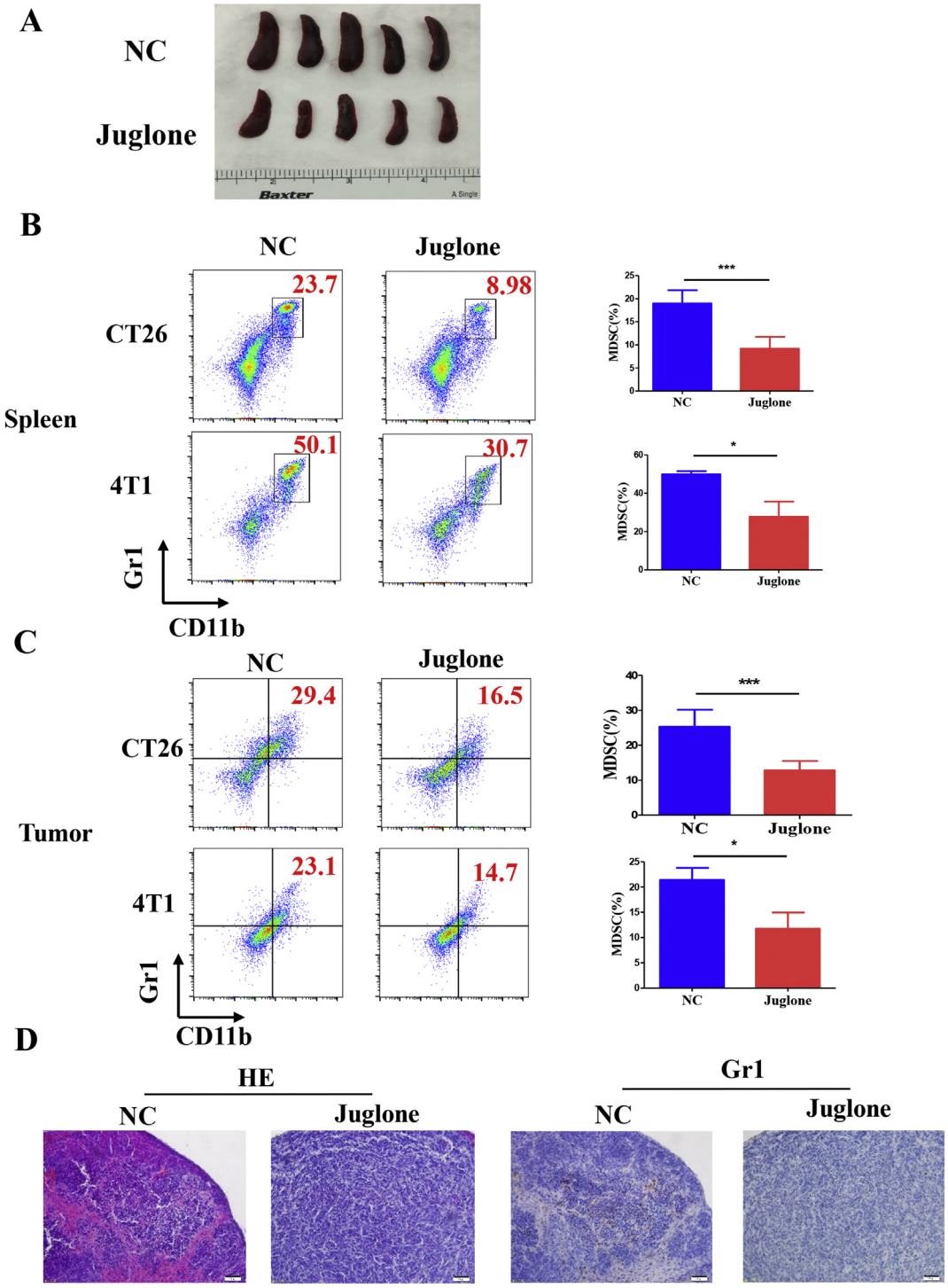

Fig. 3. Juglone depletes MDSCs in both spleen and tumor.
Five tumor bearing mice were treated with vehicle or juglone (2 mg/kg) by intraperitoneal injection every other day for 7 days. (A) Representative spleens of control or juglone (2 mg/kg) treated mice after 7 days of treatment were shown. (B) Percentage of MDSCs of splenocytes in 4T1 or CT26 tumors grown in BALB/c mice treated with control or juglone (2 mg/kg). Flow cytometry analysis was performed 7 days after tumor injection. Representative pseudocolor plots showed $CD11b^+Gr1^+$ MDSCs. (C) Percentage of MDSCs of tumor-infiltrating leukocytes in 4T1 or CT26 tumors grown in BALB/c mice treated with control or juglone (2 mg/kg). (D) Exemplary images of HE staining and $Gr1^+$ immunohistochemistry in CT26 liver metastasis sections after treatment with control or juglone (2 mg/kg). All data are presented as the mean ± S.D. ($^*p < 0.05$, $^{***}p < 0.001$).

that juglone promoted the CTLs expansion and activation.

3.5. Juglone affects myeloid cells differentiation and maturation

One of promising therapeutic strategies targeting MDSCs is promoting differentiation into nonsuppressive mature myeloid cells [26,27]. To explore the effect of juglone on maturation of myeloid cells, we tested the frequency of $CD11b^+CD11c^+$ DCs and $CD11b^+$ $F4/80^+$ macrophages that do not have suppressive function. Juglone treatment of CT26 tumor-bearing mice increased the percentage of

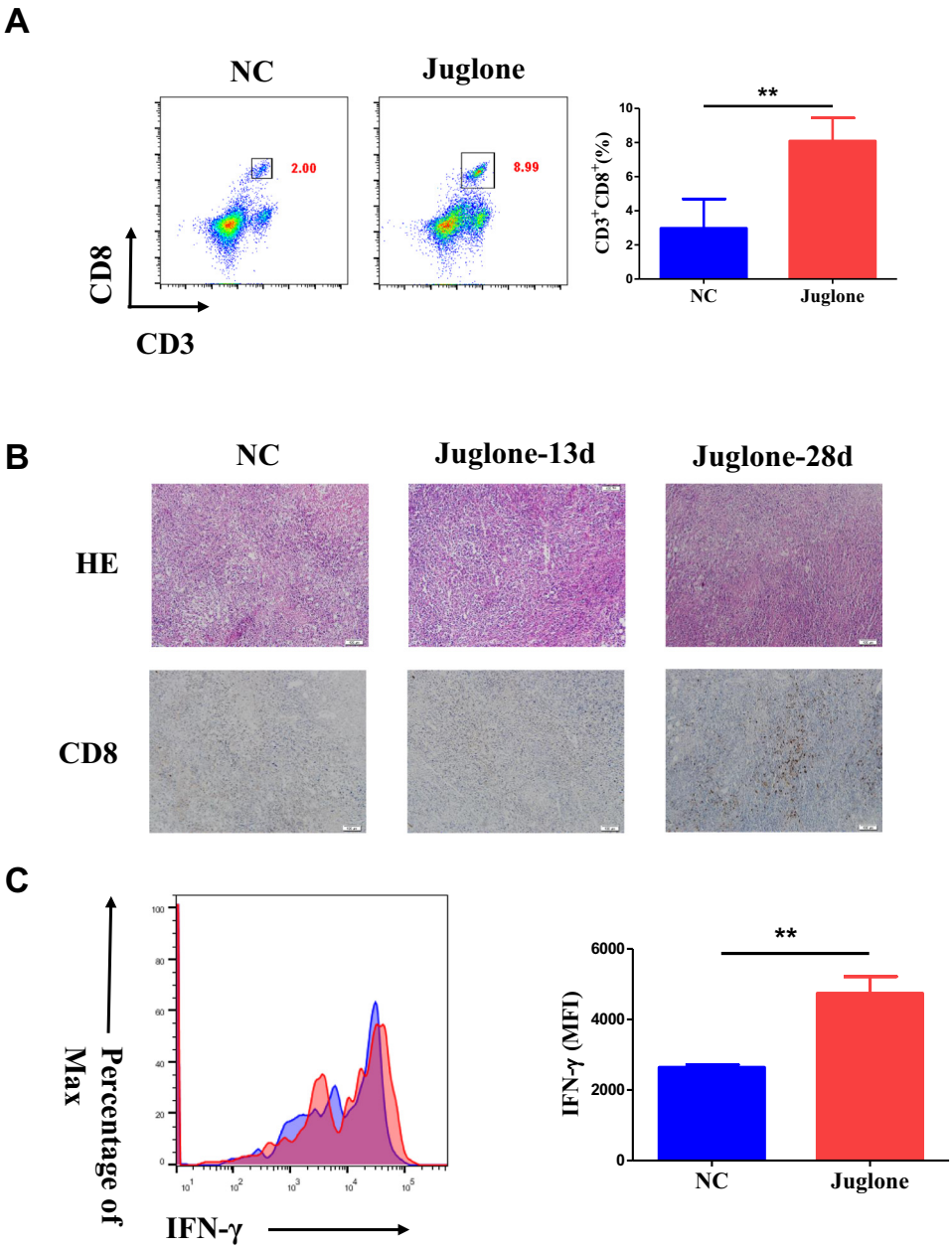

Fig. 4. Juglone increases expansion and activation of $CD8^+$ T cells.
(A) Percentage of $CD8^+$ T cells of splenocytes from 4T1 tumors of control and juglone treated mice after 14 days of treatment. Representative pseudocolor plots show $CD3^+CD8^+$ double-positive cells. (B) Exemplary images of H&E staining and $CD8^+$ immunohistochemistry in 4T1 tumors grown in BALB/c mice. The tumor sections were prepared on 0, 13 and 28 days of treatment. (C) Representative histogram showed the IFN-γ expression of tumor-infiltrating $CD8^+$ T cells of control (blue) and juglone (red) treated mice after 14 days of treatment (left). The X-axis for the histogram is the fluorescence intensity of IFN-γ (PerCP-Cy5.5). The Y-axis is the normalized percentage max of cell count number. Mean fluorescent intensity (MFI) of IFN-γ expression of tumor-infiltrating $CD8^+$ T cells. All data are presented as the mean ± S.D. ($^{**}p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$CD11b^+CD11c^+$ DCs and $CD11b^+$ $F4/80^+$ macrophages in both spleen (Fig. 5A) and tumor (Fig. 5B). These results suggested that juglone affected myeloid cells into differentiated myeloid cells, resulting in impairing immunosuppressive activity.

3.6. Juglone decreases the level of IL-1β to impair the accumulation of MDSCs

In the tumor microenvironment, many factors promote the accumulation of MDSCs. To identify the key factor mediating MDSCs accumulation, we performed RNA-seq analysis, using mRNA extracted from 4T1 tumors grown in BALB/c treated with or without juglone. Fig. 6A summarized the results for the genes have been implicated in the accumulation of MDSCs, and the difference of IL-1β expression is significant. Subsequently, we detected the mRNA level of factors using qRT-PCR shown in Fig. 6A to verify the effect of juglone on expression of factors supported MDSCs accumulation. Consistently, juglone significantly decreased the mRNA level of IL-1β in tumor, but not IL-6, TNF-α, M-CSF, G-CSF or GM-CSF (Fig. 6B). It indicated that juglone might reduce MDSCs through downregulation of IL-1β expression.

3.7. Juglone prevents from hepatic injury

Conventional chemotherapeutic reagents often cause side effects. Therefore, new reagent with lower toxicity is urgently needed. We performed synergistic analysis to explore the growth-inhibitory effects among 5FU, juglone, and 5FU + juglone treatment. Our study showed that 5FU and juglone had similar effect of tumor growth inhibition (Fig. 7A). The combination therapy showed a stronger inhibition of tumor growth than was achieved with either 5FU or juglone (Fig. 7B). The present study demonstrated the hepatoprotective effect of juglone on DMN-induced liver fibrosis [28]. Histopathologic analysis was performed to test the effect of 5FU, juglone, and 5FU + juglone treatment on liver injury. H&E staining showed that 5FU treatment caused liver injury, whereas treatment with juglone inhibited 5FU-induced liver injury (Fig. 7C). It suggested that the antitumor effect of 5FU and juglone were similar and juglone prevented the hepatocellular injury. These results suggested that juglone might be a potential

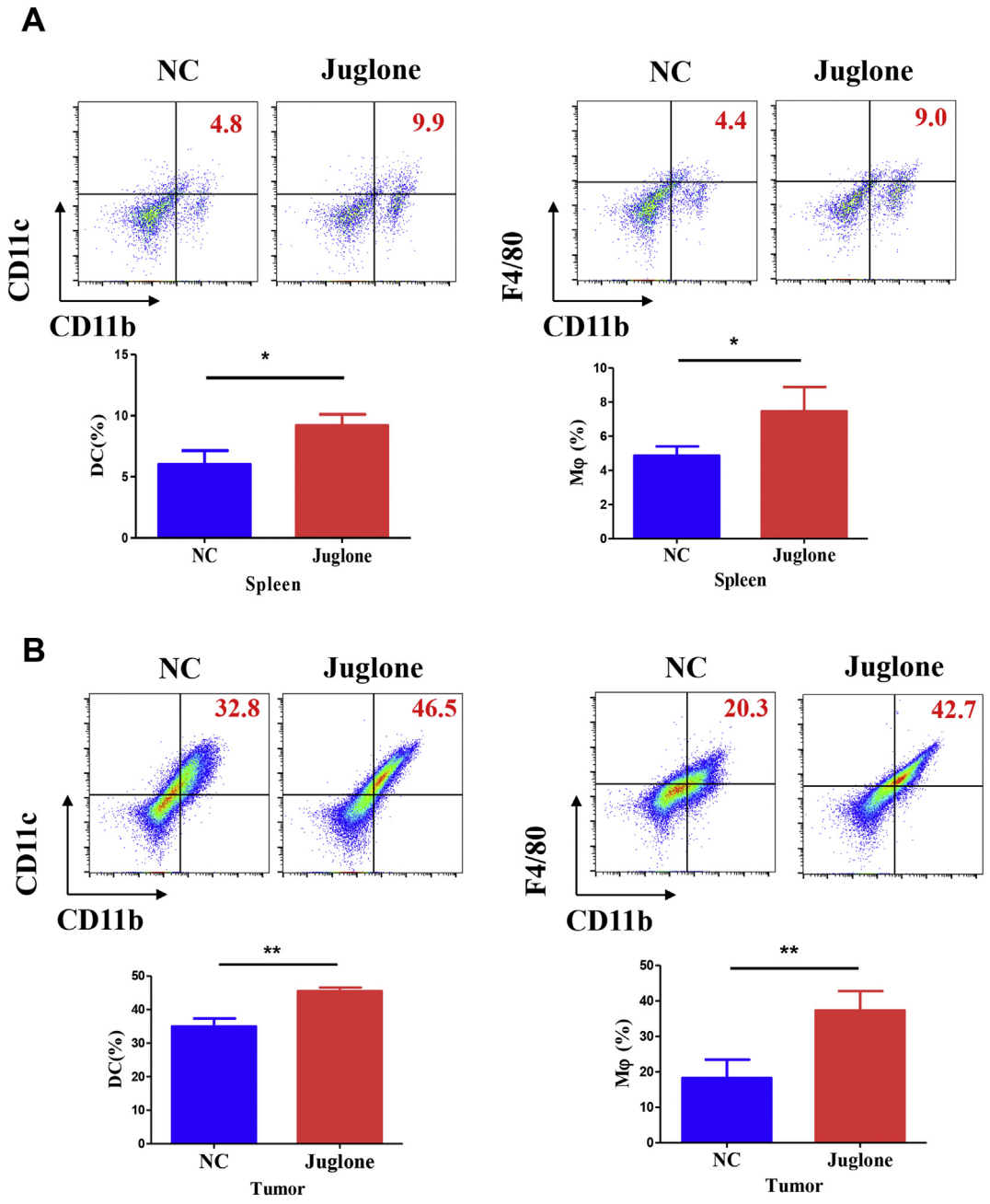

Fig. 5. Juglone promotes maturation of myeloid cells.
(A) Percentage of $CD11b^+CD11c^+$ DCs (left) and $CD11b^+$ $F4/80^+$ macrophages (right) of $CD45^+$ splenocytes in CT26 tumors grown in BALB/c mice treated with control or juglone (2 mg/kg). Flow cytometry analysis was performed 14 days after treatment. Representative pseudocolor plots were exemplary. (B) Percent $CD11b^+CD11c^+$ DCs (left) and $CD11b^+$ $F4/80^+$ macrophages (right) of TIL in CT26 tumors grown in BALB/c mice treated with control or juglone (2 mg/kg). Flow cytometry analysis was performed 14 days after treatment. All data are presented as the mean ± S.D. (*$p < 0.05$, **$p < 0.01$).

chemotherapeutic agent for tumor therapy.

4. Discussion

In this report, we demonstrated that tumor growth and metastasis are inhibited by administration of juglone, and suppression of MDSCs may mediate this effect. Consistently, the level of IFN-γ expressing $CD8^+$ T cells has been showed increased in tumor tissues, indicating that juglone treatment impairs immunosuppressive function of MDSCs. We observed similar result by immunohistochemistry that the number of tumor-infiltrating $CD8^+$ T cells was increased in juglone treatment group. Meanwhile, we found that juglone directly increased the percentage of $CD11c^+CD11b^+$ dendritic cells and $F4/80^+CD11b^+$ macrophages, suggesting the effect on myeloid cell differentiation. Furthermore, juglone treatment induced the downregulation of IL-1β expression. Taken together, our data has shown that juglone exerts anti-tumor activity by suppressing the accumulation of MDSCs. Taken together, our study presented a novel anti-tumor mechanism of juglone by which modulation of immune statue by abolishing the accumulation of MDSCs.

Immune suppression plays a crucial role in malignancy progression, resulting in poor responsiveness to cancer therapy [29]. Tregs and MDSCs are two major immunosuppressive cell types associated with tumor immunosuppressive microenvironment [30–32]. Our results showed that juglone reduced MDSCs markedly, but not affected the level of Tregs (Fig. S1). MDSCs play an important role in

A

GeneName	Gene	*p*val	Description
Il1b	ENSMUSG00000027398	**3.11E-06**	Interleukin-1 beta **(IL-1β)**
Csf3	ENSMUSG00000038067	**0.047753**	Granulocyte colony-stimulating factor **(G-CSF)**
Il6	ENSMUSG00000025746	**0.098272**	Interleukin-6 **(IL-6)**
Csf1	ENSMUSG00000014599	**0.122649**	Macrophage colony-stimulating factor 1 **(M-CSF)**
Tnf	ENSMUSG00000024401	**0.399256**	Tumor necrosis factor **(TNF-α)**
Csf2	ENSMUSG00000018916	**0.903493**	Granulocyte-macrophage colony-stimulating factor **(GM-CSF)**

B

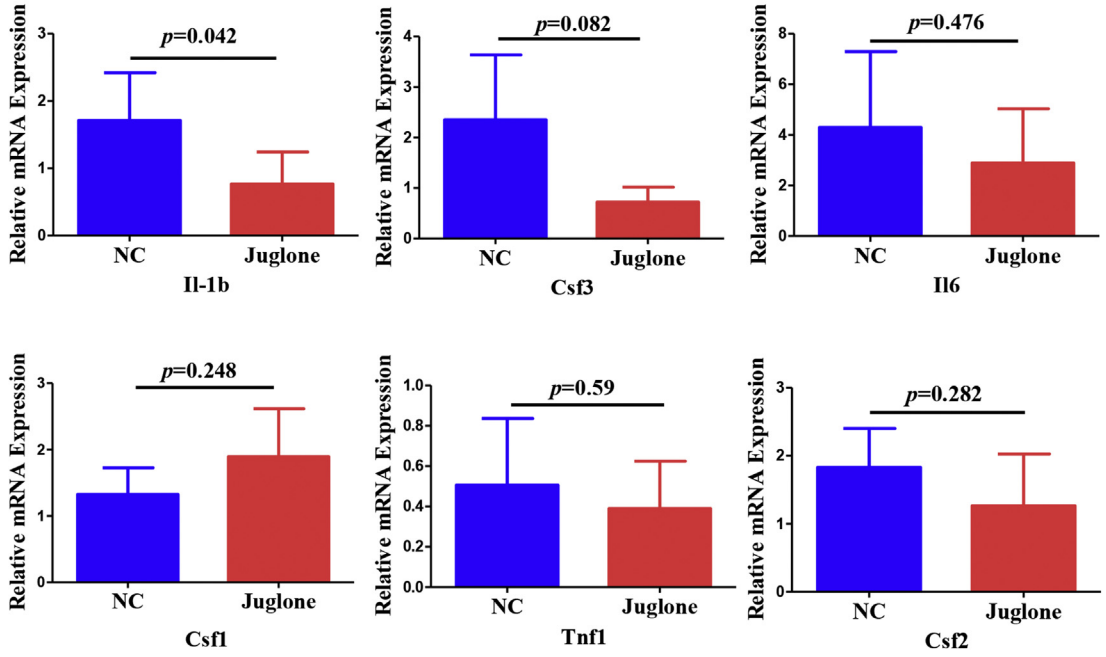

Fig. 6. The effect of juglone on the factors mediating MDSCs accumulation.
(A) *p* value for MDSCs accumulation genes detected by RNA-seq analysis of 4T1 tumor from control or juglone treated BALB/c mice. (B) The mRNA levels of IL-1β, IL-6, TNF-α, M-CSF, G-CSF and GM-CSF in 4T1 tumor treated with control or juglone were measured by real time-PCR.

immunosuppressive environment, and targeting MDSCs is a promising strategy for tumor therapy.

It was recently shown that several cancer chemotherapeutic agents not only attributed to a direct cytotoxic effect on tumor cells, but also affected tumor immunosuppressive environment through targeting MDSCs [33]. Strategies targeting MDSCs can be placed into three categories. Firstly, MDSCs can be directly killed. 5-FU and gemcitabine selectively killed MDSCs from spleen and tumor sites, resulting in increased $CD8^+$ T cells response and decreased tumor progression [16,34,35]. The second strategy is inhibition of MDSC-suppressive function. PDE-5 inhibitors reduced the expression of MDSCs immunosuppressive effector molecules such as ROS, iNOS and arginase [36–38]. Finally, generation of MDSCs can be inhibited by promoting immature myeloid cells differentiated into more terminally differentiated. All-trans-retinoic acid (ATRA) has been shown to differentiate MDSCs into DCs and enhance the immune response [39,40]. In addition, a recent study presented that treated renal cell carcinoma patients with sunitinib reduced the level of MDSCs by affecting MDSC expansion and differentiation, resulting in increased the anti-tumor immune activity [41]. Herein, we found that the levels of DCs and macrophages were improved by juglone treatment.

Moreover, juglone reduced the expression of IL-1β that promoted MDSCs accumulation. IL-1β has been demonstrated as one of the major factors mediating accumulation of MDSCs. A pervious study demonstrated that stomach-specific expression of IL-1β recruited MDSCs to stomach and leaded gastric cancer [42]. Thus, it indicated that juglone might eliminate accumulation of MDSCs by inhibiting the expression of IL-1β.

Juglone (5-hydroxy-1,4-napthoquinone) is a natural compound isolated from *Juglans mandshurica* Maxim used in Chinese folk treatment [20]. It has been shown that juglone inhibits tumor progressions in different cancer types. Several studies show that juglone inhibits tumor growth *in vitro* and *in vivo* [43,44]. In addition, juglone has been shown to inhibit tumor metastasis and angiogenesis [24]. Xenograft

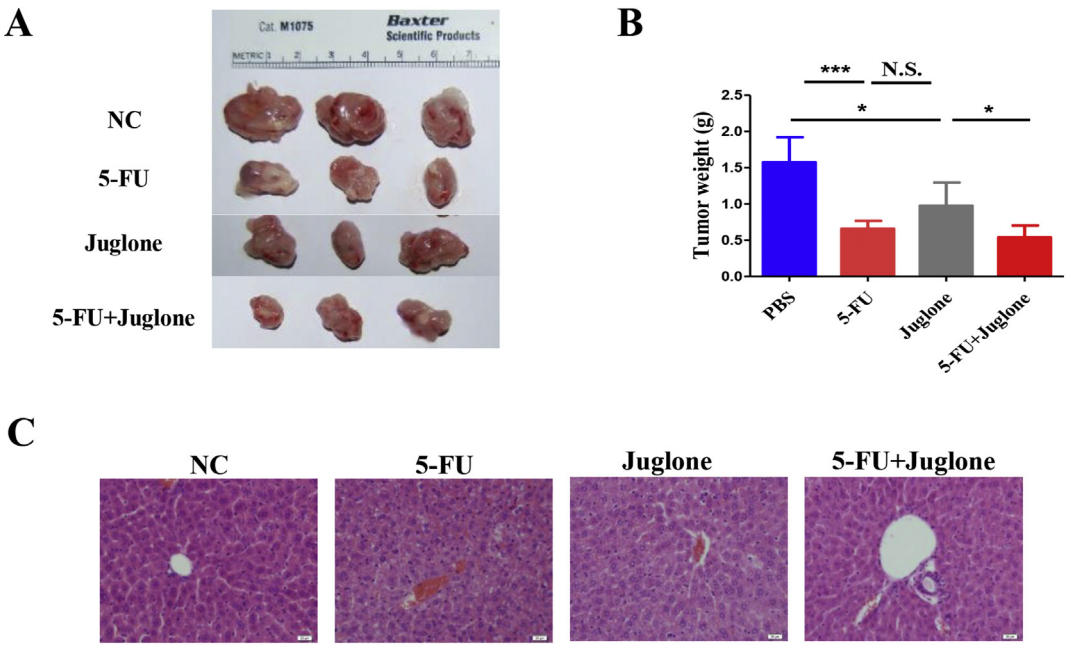

Fig. 7. Growth-inhibitory and hepatoprotective effect of juglone.
(A) BALB/c mice were treated with control, 5FU, juglone or 5FU + juglone. Tumors were removed on day 14. (B) Tumor weights were measured. (C) H&E-stained liver of 4T1 tumor-bearing mice treated with control, 5FU, juglone or 5FU + juglone. All data were presented as the mean ± S.D. (n = 5) (*$p < 0.05$, ***$p < 0.001$).

mouse model was employed for these studies. A recent study showed that juglone act as immunomodulator for increasing $CD8^+$ T cells and T-helper population in BCG-vaccinated mouse model [25]. Juglone has been reported to exert a direct cytotoxic effect on tumor cells. In our study, the anti-tumor effect of juglone is more marked in a fully immunocompetent BALB/c model than that in an immunodeficient BALB/c nude model. These results indicated that the anti-tumor effects of juglone are mediated by immune activity.

Pin1 is a major molecular target of juglone [45]. Pin1 was showed increase IL-1β in rheumatoid arthritis [46]. A previous study demonstrated that Pin1 regulates the mRNA stability of GM-CSF in activated eosinophils [47]. It is consistent with our finding that juglone decreased the level of IL-1β and GM-CSF in CT-26 tumor. It implied that juglone might inhibit the expression of IL-1β and GM-CSF through targeting Pin1. Further study on the mechanism of juglone regulating IL-1β and GM-CSF will be performed in the future.

Taken together, our results showed that juglone inhibits tumor growth and metastasis. Juglone treatment inhibited the expansion of MDSCs and increased the population of differentiated DCs and macrophage, resulting in increasing IFN-γ production $CD8^+$ T cells. Moreover, juglone suppressed the expression of IL-1β to reduce MDSCs accumulation. This report is the first to prove that juglone exerts anti-tumor activity by regulating immunosuppressive environment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.04.058.

Conflict of interest

The authors declare no conflict of interest.

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