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RS 504393 inhibits M-MDSCs recruiting in immune microenvironment of bladder cancer after gemcitabine treatment

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ABSTRACT

Bladder cancer (BC) is a malignant tumor of urinary epithelium. Gemcitabine is an introduced treatment for BC and also has immunomodulatory function, but the immunoregulation mechanism is not clear. In this study, we found that gemcitabine-treated BC cell recruited more monocyte-myeloid-derived suppressed cells (M-MDSCs), which played a significant role in immune suppression and contributed to cancer progression. We found that this phenomenon was induced by Chemokine (C-C motif) ligand 2 (CCL2), an M-MDSCs recruitment related monomeric polypeptide. Gemcitabine treatment promotes the generation of CCL2 and CCL2 could attach to C-C chemokine receptor type 2 (CCR2) to recruit M-MDSCs. We used RS 504393, a selective CCR2 antagonist, to inhibit the recruitment of M-MDSCs. RS 504393 improved the prognosis by blocking chemotaxis of M-MDSCs, and this finding sheds lights on how to prevent and alleviate the side effects occurred on the gemcitabine-treated BC patients.

1. Introduction

Bladder cancer (BC) is regarded as one of the most common malignant tumors found in the urinary system and ranks the secondary most frequent cause of death among genitourinary tumors (Pasin et al., 2008). About 300,000 new BC cases are discovered each year around the world (Voutsinas and Stravopodis, 2009). The leading treatment method for BC is surgery, but its morbidity rate has been observed increasing. BC sees a high recurrence rate with invasions and metastases, resulting in poor prognoses. Even worse, the pathogenesis of BC remains unclear at present. Although chemotherapy has been used to treat BC, the results are not quite satisfying (Matsubara et al., 2013). Thus it is worth studying the reasons behind the poor treatment results and its biological mechanisms.

Gemcitabine is a nucleoside analogue with active anti-tumor effect on different human solid tumors, including ovarian, pancreatic, non-small cell lung and breast cancer (Shewach and Lawrence, 1996). As a prodrug, gemcitabine travels into the cell by nucleoside transporters and is converted to its active forms. It has a more favorable efficacy and acceptable toxicity profile and thus is introduced for the treatment of bladder cancer. Gemcitabine (60–120 mg/kg) also has been developed as an immunomodulatory agent in mice tumor models beside its use in conventional cancer chemotherapy (Sasso et al., 2016). The interaction among BC cells, gemcitabine and immune cells require deeper explotation and their potential interactive mechanism in cancer immunotherapy is largely unclear. Here we investigated the interaction between gemcitabine and immune cells, especially MDSCs.

MDSCs, a heterogeneous group of immune cells from the myeloid lineage, are critical to tumor-related immunosuppression in both cancer patients and tested mice. MDSCs accumulate in neoplasms and spleen of tumor-bearing hosts and suppress activation, proliferation and cytotoxic function of T cell. In mice, MDSCs include a granulocytic (G-MDSCs) and a monocytic (M-MDSCs) fraction, phenotypically defined as $CD11b^+Ly6G^+Ly6C^{int/low}$and $CD11b^+Ly6G^-Ly6C^{high}$cells respectively (Damuzzo et al., 2014). Similarly, human MDSCs comprise monocytic ($CD45^+CD11b^+CD33^+CD14^+$) and granulocytic ($CD45^+CD11b^+CD33^+CD15^+$) subsets and other immature populations lacking lineage marker expression. In mouse tumor models, although G-MDSCs were often more abundant, M-MDSCs were found to have more potent suppressors functioning on T cell response when compared to G-MDSCs on a per cell basis (Je-In and Gabrilovich, 2010). Based on these observations, M-MDSCs appear to be an attractive strategy to effectively relief tumor-related immunosuppression and promote the efficacy of cancer immunotherapies. Accumulation,

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Table 1
The Differences of M-MDSCs and CD8 + CD28 + T Cells in Bladder tissues.

	Controls	BC			
Variables	Adjacent tissues	BC (ⅠⅡ)	BC (ⅢⅣ)	M-MDSCs	$CD8^+CD28^+$T cells
Age(years)	62.28 ± 9.84	63.16 ± 14.77	65.20 ± 7.66		
≤60	N = 20 (1.26 ± 0.73/18.06 ± 4.51#)	N = 24 (2.07 ± 1.07/13.12 ± 3.44)	N = 9 (5.47 ± 1.57/1.46 ± 1.34)	***/***/***&	***/***/***
>60	N = 19 (1.10 ± 0.50/17.67 ± 4.21)	N = 32 (1.81 ± 0.74/10.41 ± 4.07)	N = 16 (4.91 ± 1.11/1.41 ± 0.70)	***/***/***	***/***/***
Gender					
Male	N = 28 (1.26 ± 0.61/17.64 ± 4.21)	N = 36 (1.89 ± 0.80/12.68 ± 3.54)	N = 23 (5.21 ± 1.19/1.43 ± 0.99)	***/***/***	***/***/***
Female	N = 11 (0.97 ± 0.64/18.45 ± 4.74)	N = 20 (1.97 ± 1.07/9.58 ± 4.13)	N = 2(3.93 ± 2.47/1.43 ± 0.21)	***/0.34/0.04	***/***/0.01

#: Percentage of M-MDSCs/Percentage of $CD8^+CD28^+$ T cells ; .
&: BC (ⅠⅡ) *vs* Adjacent tissues/ BC (ⅢⅣ) *vs* Adjacent tissues/ BC (ⅠⅡ) *vs* BC (ⅢⅣ);
BC: Bladder Cancer; M-MDSCs: Monocyte-Myeloid Derived Suppressed Cells.
*** : $p < 0.001$.

expansion and differentiation of MDSCs rely on various of cytokines (Chiodoni et al., 2017). Chemokine (C-C motif) ligand 2 (CCL2), a small cytokine that belongs to the CC chemokine family, plays an important role in recruiting M-MDSCs to the sites of cancer. Meanwhile the previous study found that CCL2 receptor, CCR2, up-regulated M-MDSCs in cancer patients (O'Connor et al., 2015).

In this study, we investigated the potential association between gemcitabine treatment and recruitment of M-MDSCs in BC microenvironment and the underlying mechanism *in vitro* and *in vivo*.

2. Materials and methods

2.1. Clinical data

The present study was approved by the Medical Ethics Committee of Shanghai General Hospital, Shanghai Jiao Tong University and consent was obtained before surgery from each patient. Fresh BC tissues (ⅠⅡ:56, ⅢⅣ:25) and 39 corresponding adjacent bladder tissues were collected from the Urology Department of Shanghai General Hospital from October 2015 to August 2017 (Table 1). Blood immune cell test reports of BC patients (ⅠⅡ:193, ⅢⅣ:33) and 116 healthy people were collected (Table 2). None of the patients had undergone radio- or chemotherapeutic treatment, and all specimens were staged in accordance with the tumor-node-metastasis (TNM) classification enacted by the American Joint Committee on Cancer (AJCC).

2.2. BC cell lines

Cell lines of human BC, T24 and 5637, and cell line of mouse BC, MBT-2, were obtained from the typical culture preservation commission cell bank, Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in RPMI 1640 Medium (11875119, Gibco, NY, USA) supplemented with 1% penicillin/streptomycin (15140122, Gibco) and 10% FBS (10100147, Gibco) and under a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Flow cytometry

A total of 1×10^6 cells were re-suspended in cold PBS buffer (10010023, Gibco) and incubated for 5 min at 4 °C. Cells were additionally incubated for 30 min on ice in 50 µl of staining buffer with 1 µg/ml of relevant fluorochrome-conjugated or matched isotype control antibodies. We used $CD11b^+CD45^+CD33^+CD14^+CD15^-$ to label M-MDSCs (Table 3). Labeled cells were washed twice with cold PBS. The samples were analyzed by FACSCalibur flow cytometer and CellQuest software (ver 5.2.1) (both from BD Biosciences, NJ, USA).

2.4. Cell proliferation assays

5637 and T24 were treated with gemcitabine at various concentrations (0, 0.001, 0.01, 0.1, 1, 10) for 48 h and 1 nM gemcitabine (HY-17026, MedChemExpress, NJ, USA) treated BC cells for different periods of time (1, 2, 3, 4 days). Cell proliferation rate was determined by Cell Counting Kit – 8 (CCK-8) reagents (96992, Sigma, NJ, USA). At 37 °C, cells were incubated in 10% CCK-8 reagent that was diluted in normal culture medium until color conversion was visually visible. The absorbance was measured at 450 nm by microplate reader.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The detection of CCL2 in the supernatant was performed in strict accordance with the specifications of the corresponding ELISA kits (RAB0575, Sigma). 100 ml of each standard and sample was added into appropriate wells with gentle shaking. After the last wash, Biotinylated Detection Antibody was added to each well. After the solution was discarded, 100 ml of prepared HRP-Streptavidin solution was added into each well. Repeat the wash and add 100 ml of ELISA Colorimetric TMB Reagent (Item H) to each well. The optical density (OD) values (450 nm) of all samples were detected within 15 min. The microplate reader was from Bio-Rad (Bio-Rad Laboratories, CA, USA).

2.6. Transwell chemotaxis assay

Transwell polycarbonate membrane was used to perform chemotaxis assays (3422, Coring, NY, USA). Use 1 nM gemcitabine to treat BC cells (T24 and 5637) for 48 h, and replace cell supernatant without serum. After 48 h, we collected cell supernatant and loaded its lower chamber. PBMC were harvested and loaded onto the upper chamber

Table 2
The Difference of $CD8^+$ $CD28^+$T Cells in Peripheral Blood.

Variables	Healthy Controls	BC (ⅠⅡ)	BC (ⅢⅣ)	$CD8^+CD28^+$T cells (%)
Age(years)	60.52 ± 10.68	65.4 ± 13.12	65.00 ± 7.43	
≤60	N = 61(9.24 ± 4.26)	N = 60(8.81 ± 3.95)	N = 10(9.13 ± 3.93)	0.57/0.94/0.82&
>60	N = 55(7.39 ± 3.87)	N = 133(7.48 ± 3.93)	N = 23(8.99 ± 5.09)	0.89/0.18/0.18
Gender				
Male	N = 68 (8.69 ± 4.30)	N = 155 (7.77 ± 3.96)	N = 25 (8.72 ± 4.68)	0.12/0.98/0.24
Female	N = 48 (7.90 ± 3.98)	N = 38 (8.37 ± 4.06)	N = 8 (14.10 ± 1.41)	0.59/0.03/0.08

&: BC (ⅠⅡ) *vs* Healthy Controls / BC (ⅢⅣ) *vs* Healthy Controls / BC (ⅠⅡ) *vs* BC (ⅢⅣ); BC: Bladder Cancer.

Table 3
Monoclonal Antibodies Used for Flow Cytometric Assay.

	Antibody	Conjugate	Function	Source
M-MDSCs (human)	CD45	PE-cy7	Leukocyte common marker	Biolegend
	CD15	PE-cy5.5	Neutrophils marker	Biolegend
	CD11b	APC	polymorphonuclear leukocytes marker NK cells marker mononuclear phagocytes marker	Biolegend
	CD14	FITC	Monocyte–macrophage marker	Biolegend
	CD33	PE	Myeloid cells marker	Biolegend
$CD8^+$ T cells	CD3	FITC	T cell marker	Biolegend
(human)	CD28	PE	T cell marker	Biolegend
	CD8	APC	T cell marker	Biolegend
M-MDSCs (mouse)	CD11b	Violet 1	Monocytes, granulocytes and NK cells et al marker	Biolegend
	Ly6G	APC	Granulocyte marker	Biolegend
	Ly6C	PE	Monocyte marker	Biolegend

M-MDSCs: Monocyte-Myeloid Derived Suppressed Cells; NK cells: Natural Killer Cells.

subsequently at 10^6 cells in serum free media. After 48 h of incubating, cells of the bottom chamber were collected and tested by flow cytometry.

2.7. Xenograft mouse model

MBT-2 was established from urothelial carcinoma of the bladder removed from a female C3H/He mouse. 8-weeks old female C3H/He mice were obtained from Slaccas Animal Laboratory (Shanghai, China). The Animal Care Committee of Shanghai Jiao Tong University School of Medicine approved all the procedures in this study. 1 week after tumor inoculation, in the treatment model, mice bearing BC xenograft were randomly divided into three groups with eight mice in each group. For treatment groups, mice were individually treated with gemcitabine (20 mg/kg, i.p., MedChemExpress) once per week (Chong et al., 2011). In gemcitabine discontinuance group, treatment was stoped after 1-week long gemcitabine injection. In CCL2 chemokine receptor antagonist group, RS 504393 (HY-15418, 2 mg/kg, i.p., MedChemExpress) was used subcutaneously during gemcitabine withdrawal (Wenbin et al., 2014). The surviving time was recorded.

2.8. Statistics

All data are presented as the mean ± SD and were analyzed by the means of two-tailed independent sample one-way ANOVA, chi-square test and Student's *t*-test with SPSS 21.0 software (SPSS, USA). A log rank test was performed, and Kaplan-Meier survival curves were plotted. The *p*-values were two-sided, and the value of 0.05 was defined as statistically significant.

3. Results

3.1. Gemcitabine treatment promotes the recruitment of M-MDSCs

Gemcitabine with two fluorine atoms inserted into the deoxyribofuranosyl ring is a nucleoside analogue of deoxycytidine (Fig. 1A). To investigate the anti-tumor function, gemcitabine was used to treat 5637 and T24 at various concentrations and for different periods of times. As shown in this study below, the survival rate of 5637 and T24 cells significantly decreased in gemcitabine treatment group compared with control group (Fig. 1B, C). To determine the M-MDSCs recruitment ability of BC cells after being treated by gemcitabine, chemotaxis assays were performed, which confirmed that the chemotactic responses of M-MDSCs improved along with the increase of gemcitabine (Fig. 1D, E). These data indicated that gemcitabine inhibited the proliferation of BC cells, while at the same time it also promoted the M-MDSCs recruitment ability of BC cells.

3.2. M-MDSCs play an important role in BC immune environment

Combination of $CD11b^+CD45^+CD33^+CD14^+CD15^-$ can identify M-MDSCs (Fig. 2A). To evaluate the immune microenvironment of BC tissues, M-MDSCs of fresh BC tissues and corresponding adjacent bladder tissues were tested and the result indicated that M-MDSCs increased along with the BC progression (Fig. 2B, C). $CD8^+$ T cells were co-cultured with M-MDSCs at different ratio (100:1, 10:1, 1:1) and then at 1:1 ratio, the proliferation of the $CD8^+$ T cells was significantly inhibited (Fig. 2D). Blood immune cell test reports indicated that there was insignificant difference between BC patients and healthy people (Fig. 2E). However in BC tissues, $CD8^+$ T cells decreased along with the BC progression (Fig. 2F, G). These data indicated that M-MDSCs increased in BC tissues and this process suppressed the immune function of $CD8^+$ T cells.

3.3. Gemcitabine treatment induces CCL2 production of BC cells

To investigate how gemcitabine-treated BC cells recruit M-MDSCs, 5637 and T24 were treated by 1 nM gemcitabine for 48 h. Suspension Array Technology was used to analyze the cytokines in supernatant and it turned out that CCL-2 increased after gemcitabine treatment (Fig. 3A). Transcriptional level of CCL2 (Fig. 3B) and protein level of CCL2 in 5637 and T24 increased after gemcitabine treatment (Fig. 3C). MFI of CCL2 was significantly higher after gemcitabine treatment (Fig. 3D). To investigate whether gemcitabine can influence the expression of CCR2 of M-MDSCs or not, 1 nM gemcitabine was used to treat M-MDSCs for 48 h. Flow cytometry results indicated that there was insignificant difference after gemcitabine treatment (Fig. 3E, F). These data indicated that gemcitabine treatment promoted the CCL2 level of BC cells.

3.4. RS 504393 increased the surviving time of gemcitabine-discontinuance BC mice by blocking recruitment of M-MDSCs

RS 504393 ($C_{25}H_{27}N_3O_3$) is a selective CCL2 chemokine receptor antagonist (Fig. 4A). Thus gemcitabine plays a strong role in anti-tumor (Fig. 4B) and the combination of gemcitabine and RS 504393 can further prolong the surviving time (Fig. 4C). To investigate whether CCL2 chemokine receptor antagonist can block the recruitment of M-MDSCs or not, various concentrations (0, 1, 10, 100 nM) of RS 504393 were added into the supernatant of 5637 and T24. Chemotaxis assays were used to test the chemotactic effect of M-MDSCs. Flow cytometry showed that the recruitment of M-MDSCs decreased as the concentration increased (Fig. 4D). Furthermore, 100 nM RS 504393 could partly inhibit the strong recruitment ability of gemcitabine-treated 5637 and T24 cells (Fig. 4E). *In vivo*, M-MDSCs significantly increased in gemcitabine-treated BC models, while RS 504393 could inhibit the recruitment of M-MDSCs (Fig. 4F). These data indicated that RS 504393 could partly inhibit the M-MDSCs recruitment enhancement of gemcitabine-treated

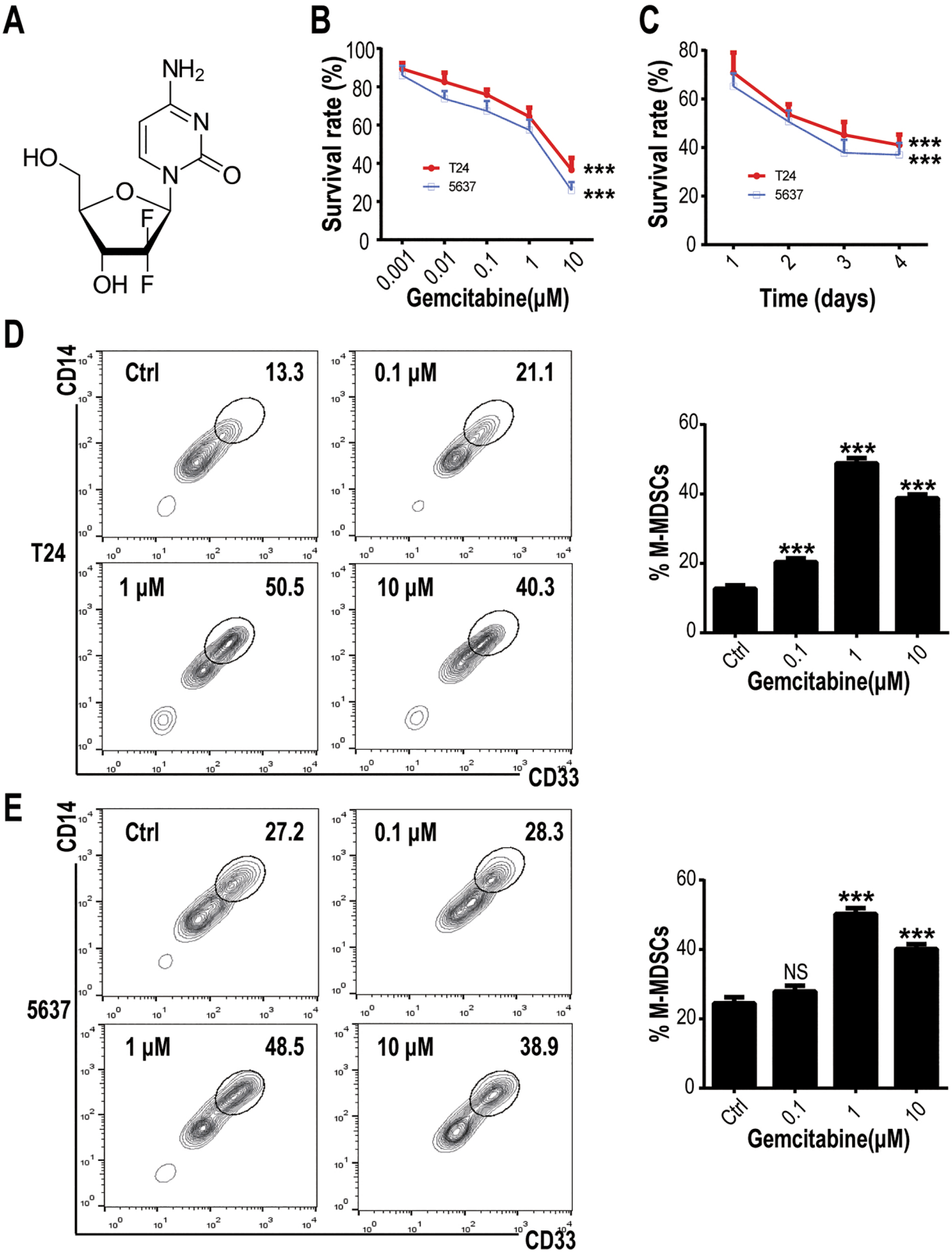

Fig. 1. Gemcitabine treatment promotes the recruitment of M-MDSCs. A. Chemical structural formula of gemcitabine. B. Various concentration of gemcitabine (0, 0.001, 0.01, 0.1, 1, 10 nM) treated 5637 and T24 for 48 h and CCK-8 assays were used to test the cell viability. C. 1 nM gemcitabine treated BC cells for different time (1, 2, 3, 4 days) and CCK-8 assays were used to test the cell viability. D, E. After various concentration of gemcitabine (0, 0.1, 1, 10 nM) treated 5637 and T24 for 96 h, cell supernatant was replaced. After another continuous culture for 48 h, cell supernatant was collected and used to M-MDSCs chemotaxis assays. NS: no significance; *** : $p < 0.001$.

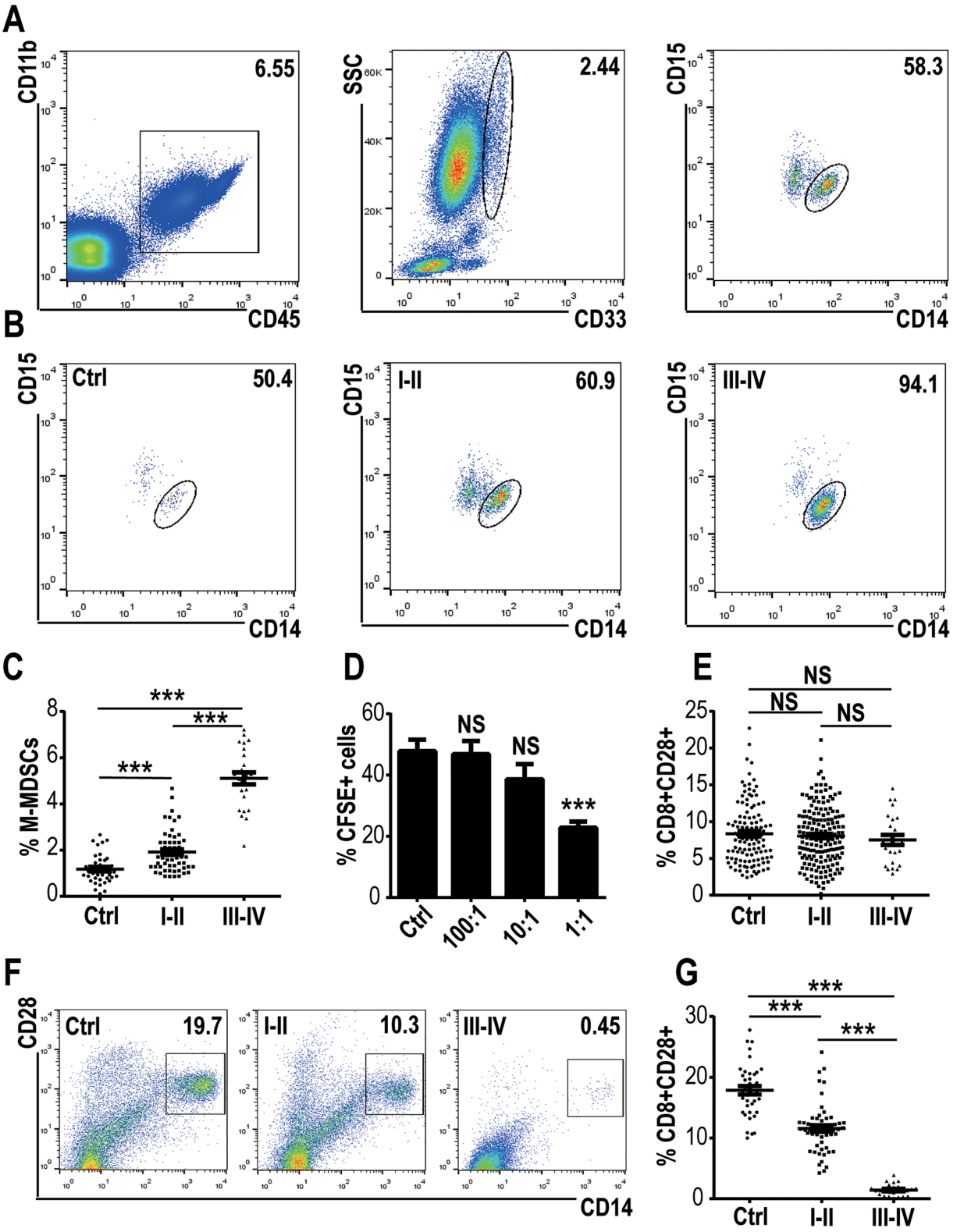

Fig. 2. M-MDSCs play an important role in BC immune environment. Adjacent bladder tissues are from normal bladder tissues and function as controls to bladder cancer tissues. Blood from healthy people acts as control to blood from bladder cancer patients. A. Tissues were disaggregated and stained with fluorochome-labeled antibodies against CD15, CD11b, CD33, CD45, and CD14 to identify M-MDSCs. B, C. The percentage of G-MDSCs in the BC tissues and adjacent bladder tissues were tested by flow cytometry. D. The isolated M-MDSCs were co-cultured with $CFSE^+$ $CD8^+$ T cells at three ratios (1:1, 10:1 and 1:100) for 24 h. Flow cytometry was used to test the proliferation of $CD8^+$ T cells. **E.** Hospital laboratory test results of $CD8^+$ T lymphocytes in the peripheral blood of BC patients and of healthy people. F, G. Tissues were disaggregated and stained with fluorochome-labeled antibodies against CD3, CD8 and CD28. The percentage of $CD8^+CD28^+$T cells in BC tissues and paired adjacent tissues were tested by flow cytometry. NS: no significance; *** : $p < 0.001$.

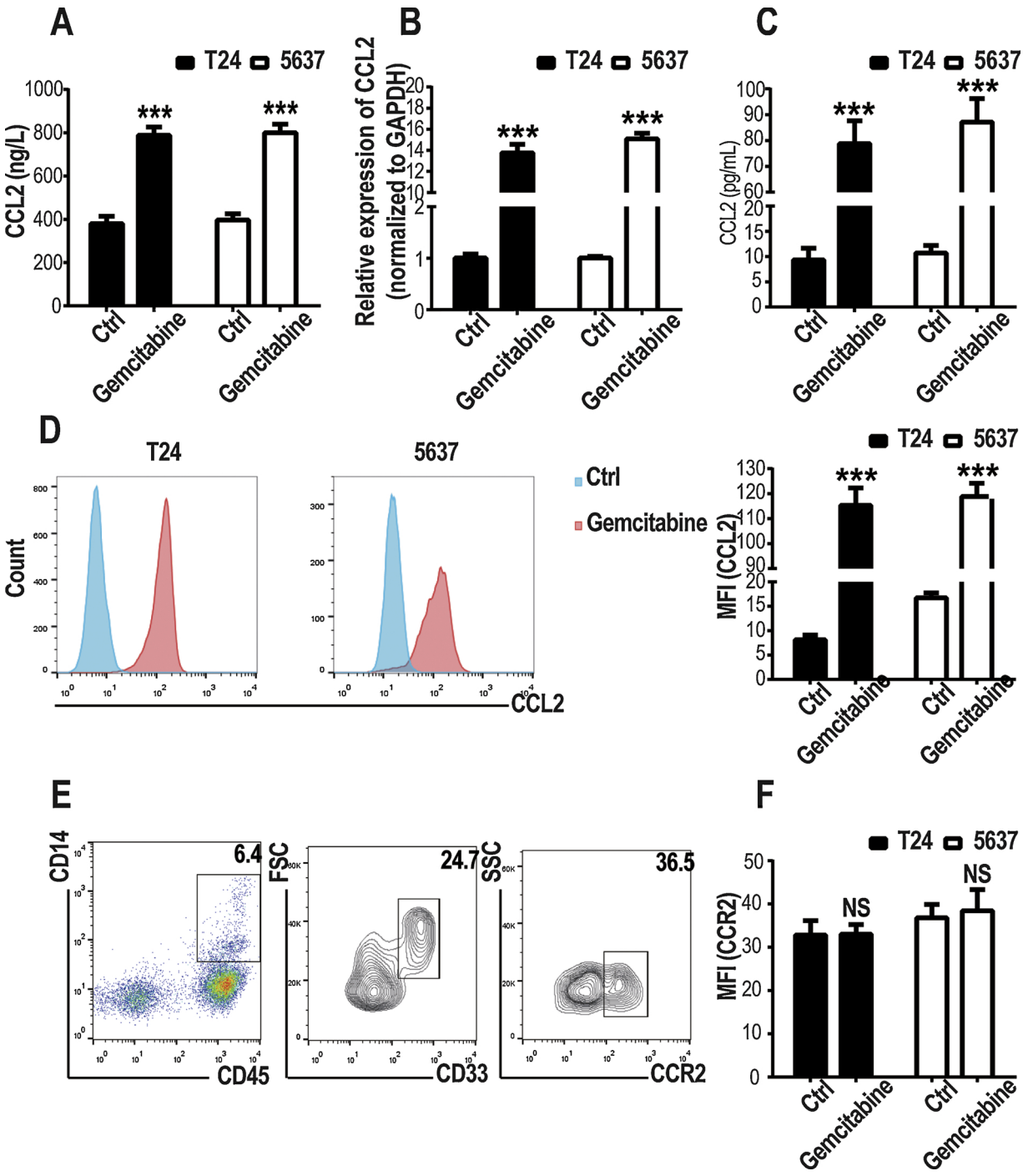

Fig. 3. Gemcitabine treatment induces CCL2 production of BC cells. A. Supernatant was collected after gemcitabine treated and Suspension Array Technology was used to analyze the M-MDSCs recruiting related cytokines. B. Transcriptional level of CCL2 was tested by qRT-PCR after Gemcitabine treatment. C. After the gemcitabine treated cells were subcultured, cell supernatant was analyzed by Elisa assays. D. The MFI of CCL2 was tested by flow cytometry after gemcitabine treated. E. Flow cytometry was used to test the CCR2 level of M-MDSCs after gemcitabine treated. NS: no significance; *** : $p < 0.001$.

BC cells, in which way prolonging the surviving time of discontinuously-treated mice by blocking the chemotaxis of M-MDSCs.

4. Discussion

There is a strong demand for further development of therapeutic options in the platinum-refractory setting. While targeted therapies have become standard in other malignancies, there are limited acknowledged targeted agents in BC, either due to unsatisfactory efficacy or significant treatment-related toxicities. The current standard first-line treatment of BC is the combined therapy of gemcitabine and cisplatin (GC), primarily due to an acceptable toxicity profile over methotrexate, vinblastine, doxorubicin and cisplatin (MVAC).

There are many clinical researchers engaging in gemcitabine study, they have run some clinical trials to study the therapeutic benefits of gemcitabine treatment for bladder cancer (Gebbia et al., 1999; Pollera et al., 1994). A considerable amount of them showed that the effectiveness of gemcitabine could work as a single agent or co-function with other chemotherapeutic drugs. However that is not definite, since Lorusso et al. performed gemcitabine treatment after platin-based

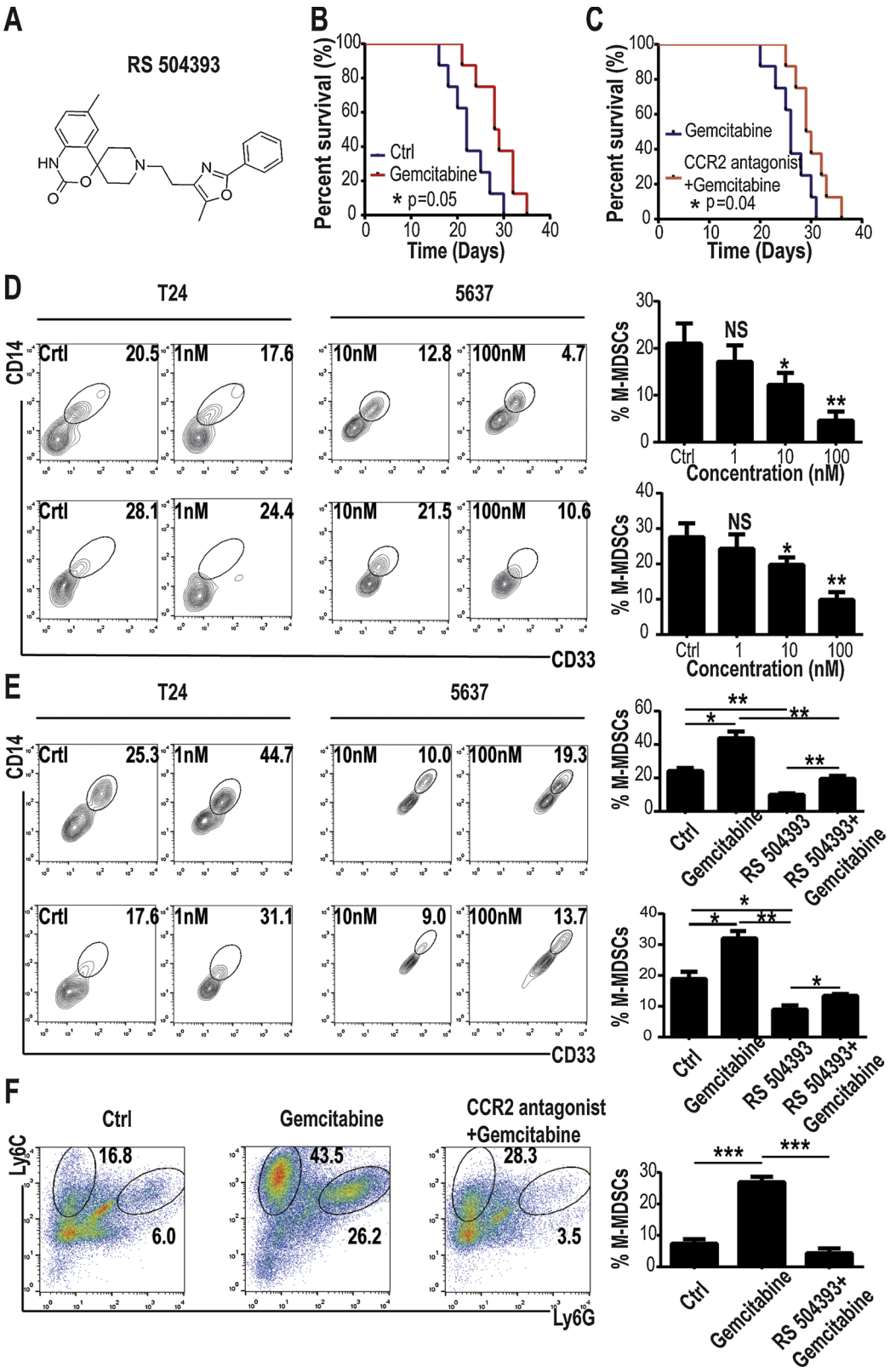

Fig. 4. RS 504393 improved the survival time of gemcitabine discontinuance BC mice by blocking recruitment of M-MDSCs. A. Chemical structural formula of RS 504393, a selective CCL2 chemokine receptor antagonist. B, C. Kaplan–Meier overall survival curve in mice with Gemcitabine treatment or Gemcitabine-RS 504393 combined treatment. D. Various concentration of RS 504393 (0, 1, 10, 100 nM) treated 5637 and T24, then recruitment of M-MDSCs were tested by flow cytometry. E. 1 nM gemcitabine treated BC cells for 48 h and replaced cell supernatant contained 100 nM RS 504393. After 48 h, cell supernatant was collected and used for chemotaxis test. F. At 21 days, mice were sacrificed and flow cytometry was used to test MDSCs of xenograft. NS: no significance; *: $p < 0.05$; **: $p < 0.01$; *** : $p < 0.001$.

cytoxan (CTX) was used. In contrast to untreated patients, survival data differed from the previous findings, showing a median progress free survival (PFS) of only 3.8 and a median overall survival (OS) of 5 months. However, in this trial, gemcitabine provided subjective relief of symptoms including pain, cystitis, dysuria, hematuria and peripheral edema, while leading to a total of 36 adverse events grade 3–4 in the 35 included patients, which consisted mainly of changes in blood cell counts (neutropenia 20%, anemia 23%, leucopenia 11.4%, thrombocytopenia 14.2%) and elevated liver enzymes (8.6%) (Lorusso et al., 1998). It indicated that side effects of gemcitabine were obvious, which might lead to unfavorable prognosis.

To improve oncological efficacy, multiple agents with different

modes of action are often combined to generate synergistic as well as additive effects with optimally limited additional toxicity. With the results of gemcitabine monotherapy as outlined above, the combinations of gemcitabine with different agents were tested. Considering synergistic effects, the first choice was the combination of Gemcitabine and Cisplatin (Van et al., 1999). Gemcitabine and Cisplatin showed a higher rate of thrombocytopenia (57% *vs.* 21%), which is a classic toxicity of gemcitabine (Maase et al., 2000). Severe neutropenia often requires admission of granulocyte colony-stimulating factors (G-CSFs) as prevention for the following cycles when neutropenia is overcome without loss of performance status. It indicated the pesticide effect of gemcitabine required further demonstration, and interruption of treatment occurs due to the side effect. In our study, we found that discontinuous gemcitabine treatment promoted BC cells to generate much more CCL2, which recruited more M-MDCSs into tumor environment.

M-MDSCs have much stronger immune suppressive activity than G-MDSCs do (Bogdan, 2011). Furthermore, immature myeloid cells could rapidly accumulate, become immunosuppressive and inhibit anti-tumor immune response during tumor growing and inflammation. These immature cells become part of the tumor microenvironment together with tumor-associated fibroblasts, macrophages, neutrophils, and dendritic cells that actively infiltrate the tumor and protect it from the function of the immune system. It was reported that the level of circulating M-MDSCs is correlated with the clinical stage, survival, response to therapy, and metastatic status of melanoma and NSCLC (non-small cell lung cancer) patients (Liu et al., 2010). The accumulation of MDSCs in patients with prostate cancer was detected as a prognostic factor for survival and correlated with stages of prostate cancer (Ning et al., 2014).

Apart from direct effects on MDSCs, CCL2 also recruits various immune cell subsets including macrophages (Mizutani et al., 2009) and monocytes (Qian et al., 2011) into the site of cancers, and it also mediates polarization and differentiation of these cells (Mantovani et al., 2002; Hernan et al., 2009). Immune cells' recruitment and polarization can result in the balance between tumour promotion and inhibition. For example, macrophages polarize towards an M2 phenotype and then play immunosuppressive function, thus may enhance tumor cell survival (Mantovani et al., 2002). Similarly, *in vitro* and in mouse models, CCL2 is involved in T cell polarization and differentiation (Luther and Cyster, 2001). Some studies demonstrate that CCL2 regulates Th2 polarization towards T regulatory phenotype, which is more immunosuppressive (Gu et al., 2000; Karpus et al., 1997; Matsukawa et al., 2000; Bonecchi et al., 2016).

There is a serious rearrangement of metabolism in tumor cells, which allows cells to proliferate and survive. During the development of malignant tumors, there is an imbalance between the signals of cell death and survival, proliferation and termination of division, increased production of proinflammatory cytokines and chemokines, which recruits various immune cells and in turn are suppressed by the tumor microenvironment and become part of it, and contribute to further growth of the tumor (Chow and Luster, 2014). Accumulation, expansion and differentiation of MDSCs also rely on types of cytokines. In particular, a potential target of the combined treatment for tumors and autoimmune diseases, for which a pathological role of MDSCs was shown, can be anti-cytokine cell-targeted therapy with bispecific antibodies, which inhibit proinflammatory cytokines in a particular cellular source. Therefore, the study of the effect of blocking TNF, IL-1, IL-6 and CCL2 in the development of MDSCs may be clinically relevant and important for the understanding of molecular mechanisms of their regulation (Umansky et al., 2016; Atretkhany and Drutskaya, 2016). Systemic anti-cytokine therapy is widely used in the treatment of various autoimmune diseases (Arnold, 2016). As for cancer, the use of IL-6/IL-6R blockers was also investigated in several tumor models and in clinical trials. It indicated that monoclonal antibodies to IL-6R suppressed tumor growth and MDSCs accumulation at the tumor site (Kentaro et al., 2012). A clinical trial of IL-1 blockers demonstrated their effectiveness in patients with colorectal cancer and non-small cell lung cancer (Hong et al., 2014). In our study, we found that gemcitabine treatment promoted CCL2 generation of BC cells, which indirectly recruited more M-MDSCs into tumor environment. Discontinuance of gemcitabine is against prognosis and CCR2 antagonist, RS 504393, capable of overturning the deficiency.

The main problem, however, with anti-cytokine therapy is its systemic effects, because such drugs nonspecifically inhibit cytokines that perform both physiological and pathological functions. It is not surprising that systemic anti-cytokine therapy has several side effects. Now increasing attention is given to the development of more specific inhibitors of proinflammatory cytokines for clinical use. Therefore understanding the mechanisms of myeloid suppressor cell development and the use of proinflammatory cytokine inhibitors may be beneficial for tumor therapy.

5. Conclusions

In conclusion, gemcitabine has strong killing effect of BC, but on the other hand several side effects are inevitable. We found that gemcitabine-treated BC cells produce much more CCL2, which recruits M-MDSCs into tumor microenvironment. Once gemcitabine treatment is interrupted, the remaining BC cells recruit more M-MDSCs and against prognosis. CCL2 chemokine receptor antagonist, RS 504393, inhibits progression of BC by blocking chemotaxis of M-MDSCs, which can provide a new way for patients who are discontinuously or inefficaciously treated by gemcitabine.

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Disclosure of potential conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

ZHL and KW designed and guided this research. XYM and RJW performed the research and collected the data. ZXY, ZZ, JTJ, MYT, and FS searched for relative literature and conducted animal experiments. JF, XW and JHZ analyzed the data and edited the manuscript. All authors have read and approved the manuscript and agreed to be responsible for the whole research to guarantee the accuracy and integrity of this study, every part of which was appropriately investigated.

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