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# HMGB1 recruits myeloid derived suppressor cells to promote peritoneal dissemination of colon cancer after resection

Wei Li [a], Ke Wu [a], Ende Zhao [a], Liang Shi [b], Ruidong Li [a], Peng Zhang [a], Yuping Yin [a], Xiaoming Shuai [a], Guobin Wang [a], Kaixiong Tao [a,*]

[a] Department of General Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[b] Department of Clinical Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

Peritoneal metastasis of colorectal cancer is a major clinical issue and results in poor prognosis for patients after surgical resection. Here, we found that abdominal surgery trauma induced high release of high-mobility group box 1 (HMGB1) in the peritoneal cavity of mice. Recombinant HMGB1 injected in the peritoneal cavity recruited abundant myeloid derived suppressor cells (MDSCs) after the surgical trauma. HMGB1 Box-A and gemcitabine reduced the recruitment of MDSCs in the peritoneal cavity after the operation and ameliorated the peritoneal metastasis burden of colon cancer in mouse model. These results showed that abdominal surgery trauma leads to a large amount of HMGB1 released in the peritoneal cavity which recruits numerous MDSCs to promote peritoneal metastasis of colon cancer after curative surgery.



## 1. Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer death for both males and females worldwide and results in half million death each year. Up to now, surgical removal is the only curative treatment for CRC, but numerous animal studies have clearly demonstrated that surgical trauma promotes the development of local peritoneal metastasis [9,22,24] which is the main cause of the mortality of CRC and affects more than fifty percent of patients [10,15]. However, the precise mechanisms by which surgical trauma promotes dissemination of CRC in the abdominal cavity remain to be understood.

High-mobility group box 1 (HMGB1) was originally recognized as a chromatin-binding factor that facilitates the transcription factors to assemble on specific DNA targets [3] and later discovered to serve as an extracellular signaling molecule during inflammation, cell differentiation, cell migration and tumor metastasis after it is released from cells [5,16]. HMGB1 contributes to all crucial hallmarks of cancer such as unlimited proliferation, angiogenesis, invasion and metastasis [23]. HMGB1 is actively secreted by stimulated leukocytes [8] or passively released from stressed and necrotic cells [18]. Interestingly, it was reported traumatic injury leaded to significant elevation of HMGB1 in circulation within first 6 h post injury [17]. Extracellular HMGB1 could recruit myeloid derived cells including polymorphonuclear neutrophils, macrophages, dendritic cells and immature myeloid cells [4,19,27] which negatively regulate immune responses in the microenvironment of tumor and promote tumor progression [7,29,30].

Myeloid derived suppressor cells (MDSCs), characterized by the coexpression of Gr-1 and CD11b cell surface molecules, are a heterogeneous population of the myeloid immature cells including precursors of dendritic cells, macrophages and granulocytes. MDSCs are significantly over-produced in tumor-bearing mice and cancer patients [1,20] and emerge as an independent prognostic indicator in various cancers including pancreatic, breast, esophageal, gastric and colonic cancers [14].

Here, we hypothesized that abdominal surgery trauma may induce the release of HMGB1 in the peritoneal cavity of mice. Then HMGB1 might recruit MDSCs towards the peritoneal cavity of tumor-bearing mice and promote the peritoneal metastasis during the postoperative period.

In current study, we showed that HMGB1 induced by surgical trauma recruits MDSCs into the peritoneal cavity which promotes dissemination of colon cancer after surgical resection.

## 2. Materials and methods

### 2.1. Animals

Male Balb/c mice (8 wk of age) were obtained from the Experimental Animal Center of Wuhan University (Wuhan, China) and

* Corresponding author. Address: Department of General Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Road, Wuhan 430022, China. Fax: +86 027 85776343.
*E-mail address:* tao_kaixiongg@yahoo.com (K. Tao).

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fed in Experimental Animal Center of Tongji Medical College (Wuhan, China). All animal studies were done in accordance with the guideline of the National Institutes of Health and approved by the Ethic Committee of Tongji Medical College, Huazhong University of Science and Technology.

### 2.2. Cell culture

CT26 murine colon cancer cell line was purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were kept in RPMI-1640 medium (Thermo scientific, Waltham, MA) containing 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, NY) at 37 °C in a 5% $CO_2$ atmosphere.

### 2.3. Tumor bearing mice surgery model

One million tumor cells were resuspended in 100 μl RPMI-1640 medium without FBS and injected subcutaneously into the right flanks of Balb/c mice. After two weeks, mice were anesthetized using isoflurane inhalation and the tumors were removed with wounds sutured with 4–0 suture line. The abdominal surgery trauma was made as follows. An incision about 1.5 cm at upper midline was made on the abdominal wall of mice and a caustic damage was made on the omentum, the parietal peritoneum and the mesentery respectively without injuring mesenteric vessels. The incision was closed with stitches. Twenty-four hours after surgical trauma, peritoneal fluids were collected from mice in different groups.

### 2.4. Elisa

The levels of HMGB1 in exudation from the peritoneal cavity after surgical trauma were measured using ELISA kit following the instruction. HMGB1 ELISA kit was obtained from Westang Biotech Company (Shanghai, China).

### 2.5. HMGB1 chemotaxis assay

Mice were intraperitoneally injected with different concentrations of HMGB1 (HMGBiotech Srl, Milano, Italy) diluted in 100 μl sterile phosphate-buffered saline (PBS) once a day for three days. Cells from peritoneal lavage fluids were collected and stained for MDSCs detection by flow cytometer.

### 2.6. Flow cytometry

Cells from the peritoneal lavage fluids were collected and stained by PerCP/cy5.5 labeled anti-mouse CD45 (Biolegend, San Diego, CA), PE labeled anti-mouse CD11b (eBioscience, San Diego, CA) and APC labeled anti-mouse Gr-1 (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were analyzed with FACSCanto II flow cytometer (BD Biosciences, San Jose, CA).

### 2.7. HMGB1 blockade and MDSCs deletion

BALB/C mice were subcutaneously injected with $1 \times 10^6$ CT26 cells on the right flanks. After two weeks, the transplanted tumors were removed and the abdominal surgery trauma was made at the same time as mentioned above. MDSCs were deleted by i.p. injection with a single dose of 100 mg/kg of gemcitabine (Sigma–Aldrich, St. Louis, MO) in 100 μl PBS two days before surgery as previously described [21]. HMGB1 was blocked by i.p. administration with 100 μg/mouse of HMGB1 Box-A (HMGBiotech Srl, Milano, Italy) immediately after surgical operation. Mice from control group were subjected to tumor removal without abdominal surgery trauma.

### 2.8. Peritoneal metastasis burden score

CT26 cells were i.p. injected at $1 \times 10^5$ in 500 μl PBS immediately after surgery. HMGB1 Box-A group mice were injected i.p. with 100 μg of HMGB1 Box-A in the suspension of CT26 cells. Two weeks later, all the mice were euthanized and the peritoneal cavities were checked. Metastasis burden in the overall peritoneal cavity was evaluated by the modified Simplified Peritoneal Cancer Index System according to in Kyu Lee's report [13]. This indexing system rated the metastasis burden based on the volume scale of tumors in different regions of abdominal cavity (Supplementary Table 1).

### 2.9. Statistical analyses

The data were analyzed by the Student's *t* test. $P < 0.05$ was considered statistically significant. Each experiment was repeated at least three times.

## 3. Results

### 3.1. The level of HMGB1 was elevated by surgical trauma in peritoneal cavity

Peritoneal fluid was collected from control mice and mice received surgical trauma with or without tumor. The level of HMGB1 in peritoneal fluid was measured by ELISA. The concentration of HMGB1 in the peritoneal fluid from mice with surgical trauma was significantly higher (4237.67 ng/ml for mice without tumor, 3300.67 ng/ml for tumor-bearing mice) than that from control mice (53.97 ng/ml) (Fig. 1), indicating that surgical trauma might induce peritoneal HMGB1 release after abdominal surgery.

### 3.2. HMGB1 recruited MDSCs into peritoneal cavity in a dose-dependent manner

As HMGB1 could recruit myeloid derived cells, in order to test whether HMGB1 also recruits MDSCs, we performed chemotactic assay in vivo using injection of recombinant HMGB1 into the peritoneal cavity of mice. The results showed that there were limited

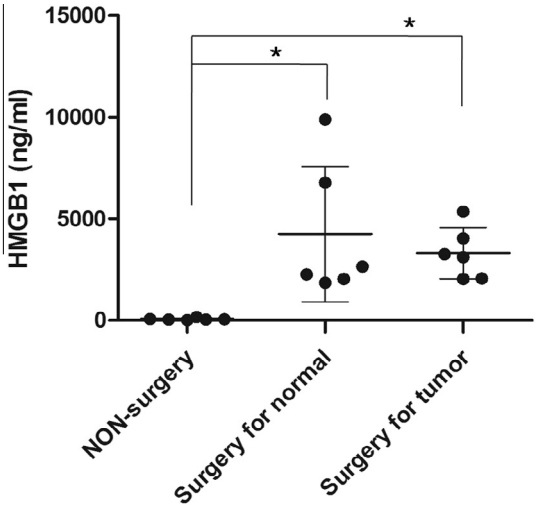


**Fig. 1.** The average levels of HMGB1 in the peritoneal cavity of three groups. Tumor bearing mice were made by subcutaneous injection with syngeneic CT26 cells for two weeks. Twenty-four hours after surgical trauma, the peritoneal fluids were collected and measured with HMGB1 ELISA Kit, Bars: means ± SEM ($n = 4–6$). Student's *t* test, $^*p < 0.05$ compared with Ctrl.

amount of Gr-1$^+$CD11b$^+$ MDSCs (less than 2% in CD45$^+$ cells) in the peritoneal cavity of control mice. However, among CD45$^+$ cells, there were 10% MDSCs (about $3.9 \times 10^4$ MDSCs in 1 ml peritoneal lavage fluid) recruited by 100 ng/ml HMGB1 and 30% MDSCs (about $1.15 \times 10^5$ MDSCs in 1 ml peritoneal lavage fluid) recruited by 1000 ng/ml HMGB1 (Fig. 2). The results indicated that intraperitoneal administration of HMGB1 can recruit MDSCs towards peritoneal cavity in a dose-dependent manner.

### 3.3. Blockade of HMGB1 prevented the accumulation of MDSCs in peritoneal cavity

To confirm HMGB1 recruits MDSCs towards the peritoneal cavity of tumor-bearing mice after abdominal operation, we made abdominal surgery trauma in tumor-bearing mice immediately after tumor resection and measured MDSCs in the peritoneal cavity of mice in HMGB1 Box-A group (*Surgery plus HMGB1 Box-A*), MDSCs deleted group (*Surgery plus Gemcitabine*), the surgical group *(Surgery)* and the control group (*No Surgery*). As demonstrated in Fig. 3, blockade of HMGB1 by HMGB1 Box-A inhibited MDSCs migration to the peritoneal cavity of mice in *Surgery plus HMGB1 Box-A group*, and gemcitabine treatment also resulted in reduced MDSCs accumulation in the peritoneal cavity of mice in *Surgery plus Gemcitabine* group compared with that in *Surgery* group. It indicated that blockade of HMGB1 abolished the recruitment of MDSCs towards the peitoneal cavity of tumor-bearing mice after abdominal surgery.

### 3.4. Peritoneal metastasis burden after operation

To determine the contribution of MDSCs to the development of peritoneal metastasis after abdominal trauma caused by tumor resection surgery, we made abdominal surgery trauma in CRC-bearing mice immediately after tumor removal and injected CT26 cells into the peritoneal cavity of mice in *No Surgery, Surgery plus HMGB1 Box-A, Surgery plus Gemcitabine and Surgery groups*. The distribution and the implantation size of tumor in eight abdominal areas were showed in Supplementary Table 1. The quantitative analysis showed that mice received surgical trauma (*Surgery group*) had highest metastasis score and the biggest tumor size among all the groups. Blockade of HMGB1 by HMGB1 Box-A significantly reduced metastasis score and tumor size *as showed in Surgery plus HMGB1 Box-A group*. Similar result was observed by depleting MDSCs with gemcitabine *as showed in Surgery plus Gemcitabine group* (Fig. 4). Taken together, those data indicated inhibition of HMGB1 or MDSCs depletion reduced the peritoneal metastasis of tumor after surgical trauma.

## 4. Discussion

In order to study the mechanism by which surgical trauma promotes the development of peritoneal metastasis after curative surgery for CRC, we set up the tumor-bearing murine model by subcutaneously inoculating syngenetic CT26 murine colon cancer cells, then we made abdominal surgery trauma immediately after tumor removal, mimicking abdominal trauma caused by CRC resection surgery. CT26 was i.p. injected immediately after operation to mimic peritoneal implantation of the loss cancer cells before and/or during resection surgery.

Various studies have demonstrated that HMGB1 can be released from stressed, necrotic and mechanically injured cells [17,18,26]. In our experiment, the level of HMGB1 in peritoneal fluid of surgical groups was significantly higher than that of non-surgery group, although there was no significant difference between tumor-free and tumor-bearing mice. Clinical researches have showed most of tumors express high level of HMGB1 and the serum levels of HMGB1 are also higher in tumor patients as compared with those of patients without tumors [23] which seems to be inconsistent

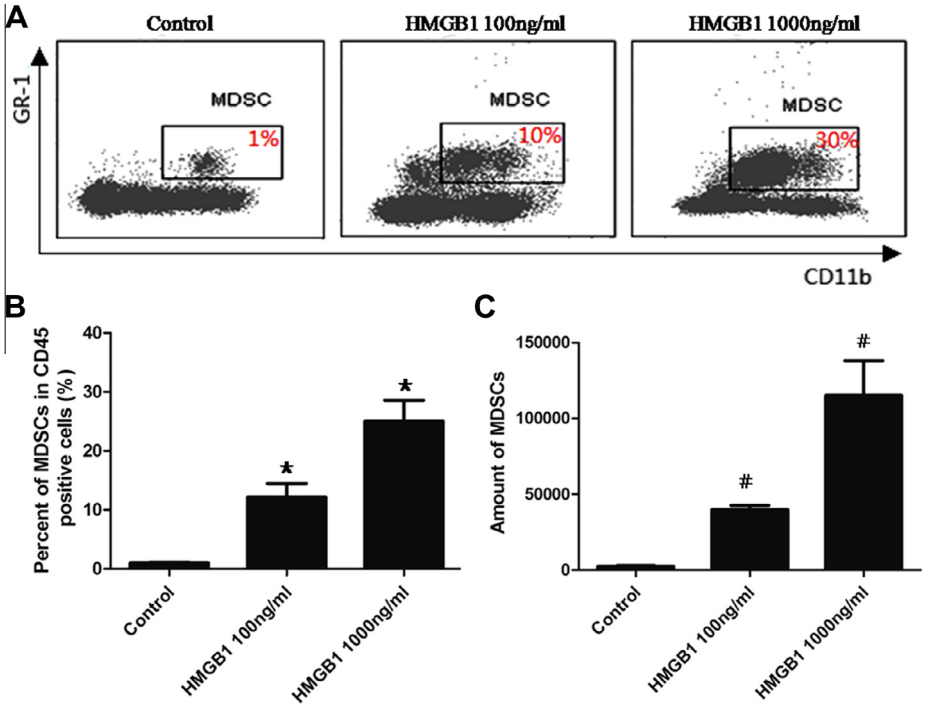


**Fig. 2.** Recombinant HMGB1 recruited MDSCs to the peritoneal cavity. (A) Different concentrations of HMGB1 were intraperitoneally injected once a day for 3 days and PBS was injected as control, and MDSCs in the peritoneal cavity of mice were measured by FACS. (B) Quantitative analysis of the percentages of MDSCs in CD45$^+$ cells in the peritoneal cavity of mice from different groups. (C) Quantitative analysis of amount of MDSCs in 1 ml of the peritoneal lavage fluids. Bars: means ± SEM ($n$ = 4–6). Student's test, $^*p < 0.05$ compared with Ctrl, $^\#p < 0.05$ compared with Ctrl.

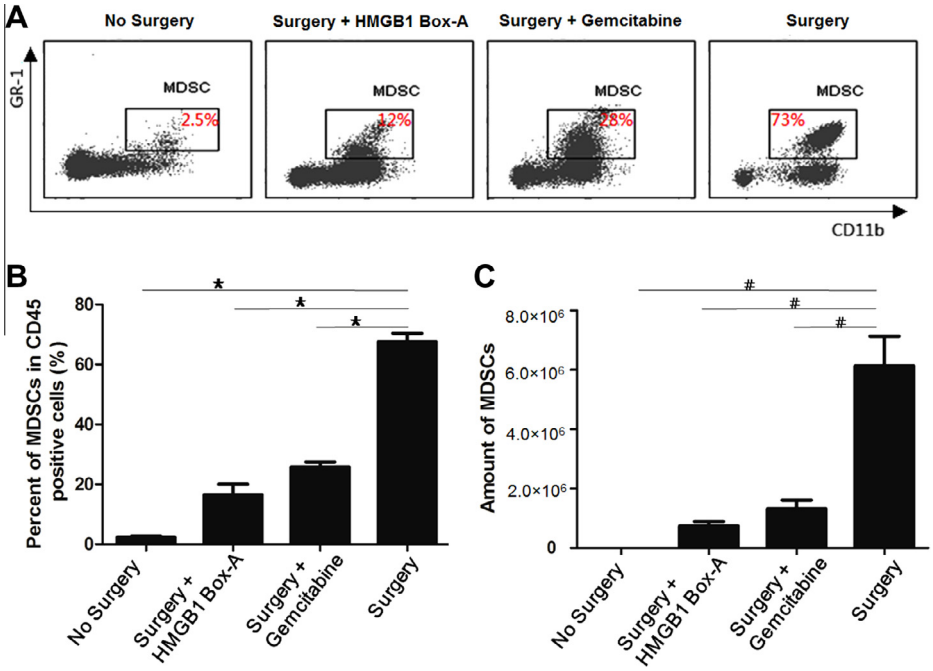


**Fig. 3.** MDSCs accumulate in the peritoneal cavity of mice treated with surgical trauma. (A) Mice from *No Surgery*, *Surgery plus HMGB1 Box-A*, *Surgery plus Gemcitabine and Surgery groups* were sacrificed at indicated time and peritoneal cavities were rinsed with 1 ml of PBS. The lavage fluids were collected and MDSCs in the lavage fluids were measured by FACS. (B) Quantitative analysis of the percentage of MDSCs in $CD45^+$ cells in the peritoneal cavity of mice from different groups. (C) Quantitative analysis of amount of MDSCs in 1 ml of the peritoneal lavage fluids. Bars: means ± SEM ($n$ = 4–6). Student's $t$ test, $^*p < 0.05$ compared with Surgery group, $^\#p < 0.05$ compared with Surgery group.

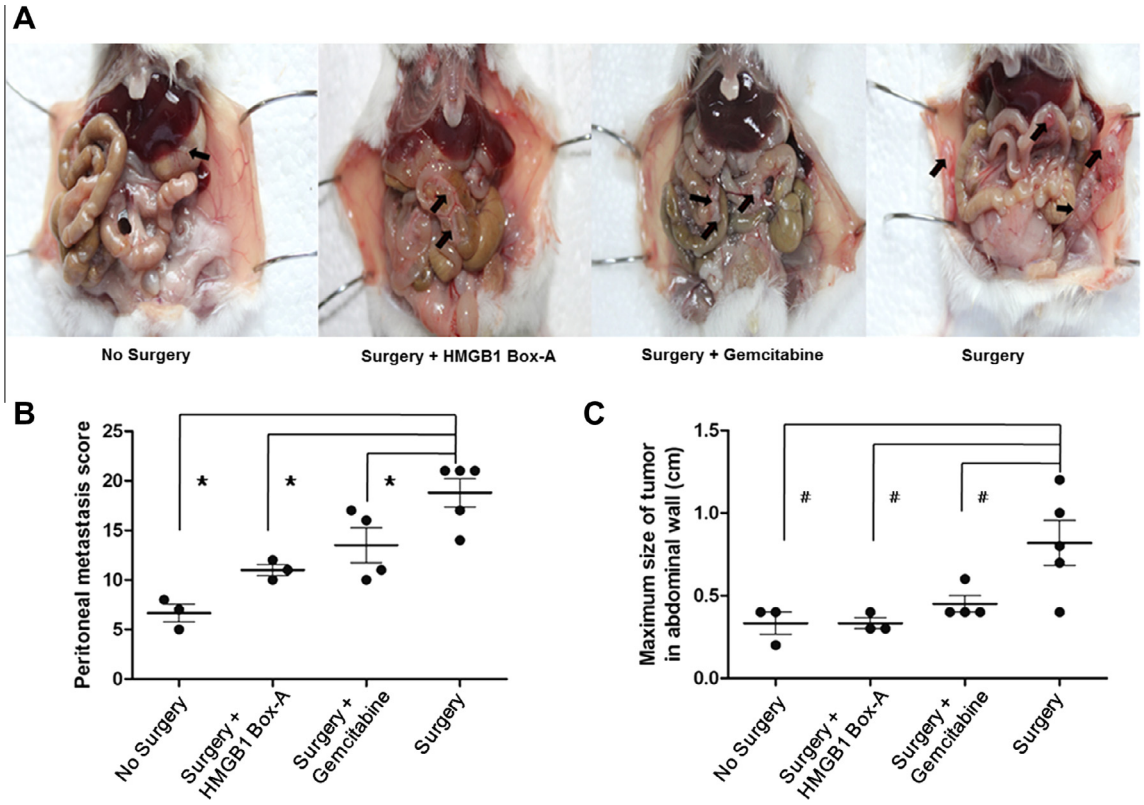


**Fig. 4.** Peritoneal metastasis burden of tumor-bearing mice after abdominal trauma. (A) Mice from *No Surgery*, *Surgery plus HMGB1 Box-A*, *Surgery plus Gemcitabine and Surgery groups* were treated by intraperitoneal implantation of CT26 cells. Mice in *Surgery plus Gemcitabine* group were treated with gemcitabine two days before surgery. Mice in *Surgery plus HMGB1 Box-A* group were treated with HMGB1 Box-A during implantation. Two weeks later, all mice were sacrificed and checked. Blank arrows indicated tumor lesions. (B), (C) Comparison of the peritoneal metastases burden by peritoneal metastasis score and maximum size of tumor of each mouse. The bold horizontal bar represented the mean of score in each group. Bars: means ± SEM ($n$ = 3–6). Student's $t$ test, $^*p < 0.05$ compared with Surgery group, $^\#p < 0.05$ compared with Surgery group.

with our observations. However, this may because HMGB1 in the peritoneal fluids was mainly released from operation-injured cells since surgical traumas were performed identically between different groups.

Extracellular HMGB1, recognized as a damage-associated molecular patter (DAMP), activates TLR (Toll-like receptor)-2, 4, 9 and/or receptor for advanced glycation end product (RAGE) to induce inflammatory responses [19] and recruits myeloid derived cells into inflammatory sites [4,19]. However, in tumor microenvironment, HMGB1 contributes to all hallmarks of cancer including proliferation, angiogenesis, invasion and metastasis [23] and is demonstrated to enhance immunosuppressive properties of Treg cells [25], induce macrophages apoptosis [11] and suppress dendritic cell functions [12]. In this experiment, we found HMGB1 efficiently recruited MDSCs towards the peritoneal cavity of mice. Both the percentage and quantity of MDSCs in the surgery-treated group (Fig. 3) were higher than those in the HMGB1 protein-treated group (Fig. 2). This may due to more MDSCs in blood and lymphoid tissue, and/or more HMGB1 in the peritoneal cavity of tumor-bearing mice, leading to more recruitment of MDSCs.

MDSCs are considered as the myeloid immature cells including precursors of dendritic cells, macrophages, and granulocytes. Elevated MDSCs are found to cause a global and profound immune suppression in many cancer patients and tumor-bearing animals [2] and be an independent prognostic indicator in various cancers [14]. MDSCs infiltration into tumor was showed to promote tumor invasion and metastasis [28]. Our results also showed reduction of MDSCs by either HMGB1 blockade or MDSCs depletion resulted in significantly low metastasis score, indicating MDSCs promote dissemination of colon cancer in the peritoneal cavity of tumor-bearing mice after abdominal surgery. MDSCs secrete a vast array of pro-tumor factors such as reactive oxygen species (ROS), nitric oxide (NO), TGF-β, IL-10, VEGF and matrix metalloproteinase 9 (MMP9), which all contribute to progression of tumor [6]. These pro-tumor factors may also favor the peritoneal metastasis of colon cancer in this murine model, which remained to be further studied in the future.

In conclusion, our results demonstrated that abdominal surgery trauma leads to release of HMGB1 in the peritoneal cavity of tumor-bearing mice, which contributes to recruitment of MDSCs towards the peritoneal cavity and the recruited MDSCs facilitate the formation of peritoneal metastasis of colon cancer in mouse model. This finding indicated that minimizing operation wound during tumor resection operation, at least for colon cancer surgery, might be beneficial to preventing postoperative peritoneal metastasis and intervention in immune microenvironment following surgical trauma might contribute to inhibiting peritoneal recurrence of colon cancer after resection surgery.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.109.

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