Major surgery-induced expansion of myeloid-derived suppressor cells promotes breast cancer cell bone metastasis though PD-1

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Primary research

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Abstract

Background

Bones metastasis is a serious clinical complication and increases morbidity and mortality. Surgery remains a main therapy of most solid tumors, but itself may accelerate tumor metastasis. Myeloid-derived suppressor cells (MDSCs) have been reported to be contribute to tumor growth. Little is known about the presence and function of surgery-induced MDSCs during bones metastasis.

Methods

In the present study, implantation of 4T-1 breast cancer into the mouse was used as a bone metastasis model. We treated 4T1 bearing mice with surgical procedures and mice were divided into Control group, Surgery group and Surgery-hemorrhage group randomly. The femur and tibia fragments from three groups were isolated and bone resorption was identified by hematoxylin-eosin (HE) staining seven days after surgery. The tumor volumes, metastasis and invasion among three groups were also assessed. We detected the expression of ALP and TRAP to quantify the activities of osteoblasts and osteoclasts respectively. The percentage of MDSCs in the bones and spleens among three groups were detected though flow cytometry analysis. We analyzed the programmed cell death-1 (PD-1) and programmed cell death-Ligand 1 (PD-L1) expression on the MDSCs isolated from mice by western blot. We also detected inducible nitric oxide synthase (iNOS), indoleamine2, 3-dioxygenase (IDO) and arginase-1 (Arg-1) expression in MDSCs by Real time-polymerase chain reaction (RT-PCR). In vitro, we isolated MDSCs in bone myeloid cells (BMCs) of surgical mice and co-cultured with 4T-1 cells. The effects of MDSCs on tumor cell proliferation migration and invasion were further assessed. We also compared the migration, invasion and proliferation of 4T-1 cells co-cultured with MDSCs pretreatment with anti-mouse PD-1 or isotype antibodies.

Results

In the present study, we found more severe bone resorption and destruction in Surgery-hemorrhage group ($p < 0.05$). We did not find that there was difference about the tumor volumes among three groups. The total percentages of tumor metastasis and the invasion were more larger and deeper in surgery-hemorrhage mice. We found that mice accepted major surgery showed more serious bone lesions and increased activity of osteoblasts and osteoclasts ($p < 0.05$). Meanwhile, we also observed a striking increase of MDSCs in the bones ($p < 0.01$) and spleens obtained from surgical mice ($p < 0.05$). MDSCs derived from surgical mice at 72 hr after surgery expressed high levels of PD-1 ($p < 0.05$), IDO ($p < 0.05$), Arg-1 ($p < 0.05$) and iNOS ($p < 0.01$). The protein level of PD-L1 did not show difference among three groups ($p > 0.05$). In addition, the data showed that the proliferation rate were significantly higher when breast cancer cells co-cultued with MDSCs compared with these co-cultued with MDSC-depleted BMCs ($p < 0.05$) in vitro. The migration ($p < 0.05$) and invasion ability ($p < 0.05$ at 48h and $p < 0.01$ at 72h,
respectively) were significantly increased in MDSCs-treated 4T-1 cells. Furthermore, blockade of PD-1 weakened MDSC-mediated promotion of 4T-1 cells migration \( (p < 0.05) \), invasion \( (p < 0.05) \) and proliferation \( (p < 0.05 \text{ at 24h}, p < 0.01 \text{ at 48h and 72h, respectively}) \).

**Conclusions**

Taken together, our results suggest that surgery-induced MDSCs showed tumor-promotive ability and elimination or reduction of MDSCs may significantly delay and limit bone metastasis.

**Background**

More than 25% of people in worldwide will ultimately be affected by tumor. Surgery excision remains a mainstay in the therapy and control of most solid tumors. Although surgical removal of primary tumors can extend or save life, it is well known that surgery itself may accelerate tumor metastasis [1]. Bone is the most common organ of metastasis especially in patients with breast and prostate cancer [2]. Metastasis of tumor cells to the bones induced a series of clinical complications such as pain, bone fracture, hypercalcemia, and is associated with increased morbidity and mortality [3]. While current therapies have been made in treating the bone metastases, no clinically approved interventions have been shown significant efficacy at treating advanced, metastatic cancers. At present the mechanisms, especially in surgery-induced tumor progression and metastasis, are various and remains unclear. A better understanding of the factors that play important roles in bone metastases may provide new insights for therapy and extended survival.

As demonstrated by previous experiments, microenvironment of distant organ can exert inhibitory effects on aggressive malignant cells at the early stage of tumor development [2]. However, the protective restraints of the microenvironment are overridden by pathological conditions such as stress, chronic inflammation [4]. Then the local microenvironment shifts to a growth-promoting state. It has been well demonstrated that surgery caused severe stress and systemic inflammation, which subsequently influenced the bone marrow microenvironment [2]. Whether these factors modulate metastatic tumor growth in bone microenvironment has yet to be fully elucidated.

It is well known that major surgery induces suppressed cellular immunity as evidenced by decreased circulating levels of anti-tumor immune cells, such as natural killer (NK) cells, T-helper (Th) cells and cytotoxic T lymphocytes (CTLs) [2]. Moreover, surgery also increased the amount of immunosuppressive cells such as myeloid-derived suppressor cells (MDSCs) [2]. MDSCs is a heterogeneous population of immature myeloid cells in bone marrow which play a pivotal role in cancer progression by suppressing both innate and adaptive immunity [5]. Accumulation of MDSCs has been demonstrated in almost all types of cancer, both in animal models and clinical patients [6]. These cells correlate with tumor growth, clinical grade, the extent of metastatic burden, as well as response to anti-tumor therapy [2]. A recently study from Jun, et al demonstrates that circulating M-MDSCs were significantly increased after surgery in
lung cancer patients [2]. Whether MDSCs induced by major surgery play direct or indirect roles in promoting bone metastasis is rarely be elucidated.

The aim of the present study was to evaluate the association between surgery and bone metastasis. Whether MDSCs participated in this process was also mainly detected. Understanding the mechanism of bone metastasis may have a great impact in finding effective therapies to prevent and treat bone metastasis.

Material And Methods

Animals

This study was approved by Ethics Committee of Harbin Medical University. Female wild-type BALB/c mice with 6-8 weeks old were used in the present study. Animals were fed under humidity-, temperature-, and light-controlled conditions with free water and food.

Cell Lines and Culture Conditions

The breast cancer cell line 4T-1 was obtained from the Harbin Medical University Cancer Hospital Lab, and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific, USA), supplemented with 10% (v/v) fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) at 37°C in incubator with 5% CO₂. The cells were mycoplasma free and showed appropriate morphology during experiments.

Surgery and Hemorrhage Procedure

Mice were divided into three groups randomly: Control group, Surgery group and Surgery-hemorrhage group (n=7 in each group). All mice were deprived of food overnight but free access to water for 24 hr preoperatively. For surgery and hemorrhage, animals continuously inhaled isoflurane and fixed in a supine position on warm pat to maintain rectal temperature at 37 ± 0.5 °C throughout the operation. A length of 2.0-cm incision along the middle line of the abdominal wall were made under sterile conditions. The intestine was isolated outside for 30 mins. For hemorrhage, the 27-gauge needles (Becton Dickinson, MD) cannulated into the left lateral of femoral artery and vein of surgical mice. Blood pressure was measured via arterial catheter using a blood pressure analyzer (SurgiVet, USA). Nearly 20% of total blood volume was withdrawn in 30 mins through the vein to induce hemorrhage. Ringer’s lactate solution was infused slowly for resuscitation through the vein. After ensuring there was no bleeding, closed the incision aseptically and sterily. Animals in Surgery group accepted the same procedure but with no hemorrhagic shock. In Control group, mice received same anesthesia without any procedures else.

Bone metastasis model

In the present study, implantation of 4T-1 breast cancer into the mouse was used as bone metastasis model [2]. Briefly, 1×10⁵ 4T-1 cells injected orthotopically into the left mammary fat pad and the other
3×10^5 4T-1 cells mixed with matrigel (Sigma, USA) injected into the proximal end of the left tibia. The surgical procedure was performed at 24 hr post 4T1 cells challenge.

**Histopathological analysis**

For histopathological studies, the femoras and tibias implanted within tumor cells were fixed within 4% paraformaldehyde and embedded in a paraffin block. The sections with 5 um-thick were performed from each fragment. Histology slides were stained with hematoxylin and eosin to assess histological changes. Percent of bone resorption was calculated from the laser scanning confocal microscope images using Adobe Photoshop software. Then chose at least three images of each slides to get a mean value.

**Tartrate-resistant Acid Phosphatase (TRAP) and Alkaline Phosphatase (ALP) Staining**

Immunohistochemical (IHC) staining was used to assess the activity of osteoclasts. Briefly, slides were stained for rat anti-mouse TRAP (Abcam, UK), antibodies for 3 hr at 37°C, followed by blocked for 1 hr using the biotin-streptavidin per-oxidase kit, rat primary antibody. The stained slides were then developed using DAB kits (Vector Laboratories, Burlingame, CA, USA). To assess the activity of osteoblasts, ALP staining was carried out by ALP staining Kits according to manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Jiangsu Sheng, China). Images were captured with a Nikon Digital Sight DS-Fi1 camera and NIS-Element software (Nikon Instruments, Melville, NY, USA) and observed under an optical microscope at 200×.

**Isolation of Bone Myeloid Cells (BMCs) and MDSCs**

In mice, the BMCs were collected from mice in Control group, Surgery group and Surgery-hemorrhage group at day 3 and day 7. In tumor bearing mice, BMCs in femurs and tibias were isolated at day 3 and day 7 after surgery. Then filter the cell suspension through a 70-μm cell strainer. The erythrocytes were lysed with lysis buffer (BD Biosciences, San Jose, CA), and then the cells were cultured in RPMI1640 medium with 10% FBS and 1% penicillin and streptomycin.

MDSCs from surgery-hemorrhage mice at day 7 were separated using Myeloid-Derived Suppressor Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer’s instructions.

**Flow cytometry analysis (FACS)**

Single cell suspensions from spleens and bone marrow were filtered through a 70 μm cell strainer (BD Biosciences, USA) and resuspended in FACS buffer at a concentration of 1×10^6 cell/100ul. Then cells were respectively stained with FITC conjugated anti-mouse CD11b, APC conjugated anti-mouse Gr-1 antibodies (BD Biosciences, USA) respectively at room temperature for 30 minutes. Finally cells were fixed in 2% paraformaldehyde until detected using the FACS Scan II (BD Biosciences, USA) and analyzed with FlowJo software (Treestar, USA).

**Western blot**
MDSCs were lysed in RIPA buffer and the protein concentration was analysed by BCA protein assay kit (Beyotime Biotechnology, China). Samples were separated by SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked for 60 mins in TBS/5% no fat milk, and then incubated with the anti-PD-1, anti-PD-L1 (1:1000) and GAPDH (1:10000) antibodies (Abclonal, China) at 4°C temperature for 12 h. Then the HRP-conjugated secondary antibody was incubated for 30 mins at room temperature. At the end the membrane was exposed by ABI western blot imaging system.

**Real time-polymerase chain reaction (RT-PCR)**

RT-PCR analysis was used to detected the mRNA levels of inducible nitric oxide synthase (iNOS), arginine (Arg)-1 and indoleamine-2,3-dioxygenase (IDO) which is associated with the immunosuppressive activity of MDSCs. The procedures were performed as our previously described [2]. The primer sequences (Invitrogen™, USA) were as previously described. iNOS forward, 5′-CCGAAGCAAACT-CACATTCA-3′; reverse, 5′-GGTCTAAGGCTCCGGCT-3′. IDO forward 5′-TGTG GCTAGAATCTGCCTGT-3′, reverse 5′-CTGCCATTTCACCAATAGAG-3′; Arg-1 forward 5′-CTCCAGAACAGGAGTCCTTAGAG-3′; reverse, 5′-AGAGGCTGTCATTAGGGACATC-3′. β-actin forward 5′-AGAGGGAATCTGAAGTAC-3′; reverse 5′-CAATAGTGATGACCTGGCGT-3′.

**Co-culture system in vitro**

To determine the influences of MDSCs on the breast cancer cells, the MDSCs were added into 4T-1 tumor cells cultures at a ratio of 100 : 1 in RPMI 1640 medium with 10% FBS, 1% penicillin and streptomycin, as well as 50 ng/ml GM-CSF [2]. Anti-mouse PD-1 (2 ug/ml, eBioscience, USA), and isotype (5 ug/ml, Biolegend, USA) antibodies were added to detected in some cases.

**Proliferation assay**

Total 4×10³ tumor cells were seeded in 96-well plates and allowed to attach for 24 hr. MDSCs or MDSCs-depleted BMCs were seeded into plate and co-cultured with 4T1 cells at a ratio of 100:1. The plates were washed three time to discard the BMCs and 10% CCK solution were added into plated at 12 hr, 24 hr, 48 hr, and 72 hr respectively. Then detected the OD value of each well The proliferation of breast cancer cells with or without BMCs was evaluated with. Each experiment repeated at least in triplicate.

**Cell invasion and migration assays**

The transwell assays with matrigel was used for cell invasion assays. Permeable supports (Corning Incorporated Life Sciences, USA) were precoated with 40 ul of 1 mg/ml matrigel. Then freshly isolated BMCs 4×10⁶ cells suspended in 200 ul medium without FBS were placed on the top chamber of each well (Millipore, MA, USA). For migration assays, freshly isolated BMCs 5×10⁶ and 4×10⁴ 4T1 cells suspended in 200 ul medium without FBS were placed on the upper chamber without matrigel (Millipore, MA, USA). The lower chamber was filled with 800 ul of medium with 10% FBS as the nutritional attractant.
Then the cells were fixed with 4% polyformaldehyde for 1 h and then stained with 0.1% crystal violet. Cleaned off the cells that not invaded to the lower surface of membrane. The membranes were then visualized under an optical microscope at 200 ×. Images were captured with a Nikon Digital Sight DS-Fi1 camera and NIS-Element software. Cells in three different visual fields were counted.

Statistics

Statistical analysis was carried out by SPSS 19.0 software. Results were represented as means ± standard deviations (SD). One-way ANOVA was used to assess the significance of intergroup differences. The two-tailed t test was performed to compare the difference between two groups. Statistical significance was accepted for p values < 0.05. In vitro, each experiment was repeated at least three times.

Results

Surgery-hemorrhage promoted bone resorption and destruction

To confirm whether surgical trauma has influences on bone metastasis, we treated 4T1 bearing mice with surgical procedures. Seven days after surgery, the femur and tibia fragments from three groups were isolated and bone resorption was identified by HE staining. We did not find that there was difference about the tumor volumes among three groups. However, as expected we found more severe bone resorption and destruction in Surgery-hemorrhage group (Fig 1; p<0.05). As shown in the figs, the total percentages of tumor metastasis and the invasion were more larger and deeper in surgery-hemorrhage mice.

Surgery-hemorrhage increased the activity of osteoclast and osteoblasts

The activities of osteoclasts and osteoblasts reflect the degree of bone destruction and resorption. Here we detected the expression of ALP and TRAP to quantify the activities of osteoblasts and osteoclasts respectively. Compared with normal mice, we found a higher score of TRAP positive cells in bones isolated from tumor-bearing mice underwent surgery or surgery-hemorrhage, which suggested the activity of osteoclasts was enhanced (Fig 2A; p<0.05). An increase in the activity of osteoblasts in mice accepted surgery-hemorrhage was also observed compared with normal mice (Fig 2B; p<0.05), as evidenced by increased staining scores of ALP in bones.

CD11b+Gr-1+ MDSCs increased in mice underwent surgery

As depicted in Figs, at postoperative days 3 and 7 we observed much higher percentage of MDSCs in bones marrow from both Surgery and Surgery-hemorrhage groups than those in Control group (Fig. 3A; p<0.01). The percentage of MDSCs in spleens from mice accepted surgery and hemorrhage significantly also significantly increased compared with those in Control group (Fig. 3B; p<0.05).

We also compared the percentage of MDSCs isolated from mice which accepted tumor implantation and then underwent surgery. The data showed that the percentage of MDSCs in 4T-1 bearing mice increased
at day 3 after surgery-hemorrhage (Fig 3C; $p<0.05$). But there were no statistical significance at day 7 (Fig 3D; $p>0.05$).

**Surgery-induced MDSCs showed immunosuppressive activity**

We analyzed the PD-1 and PD-L1 expression on the MDSCs isolated from mice. We found that the protein level of PD-1 in MDSCs isolated from surgery-hemorrhage mice were significantly increased compared with those isolated from control mice (Fig. 4A, B; $p<0.05$). The protein level of PD-L1 did not show difference among three groups (Fig. 4A, C; $p>0.05$). We also detected iNOS, IDO and Arg-1 expression in MDSCs by RT-PCR. As shown in figures, the much higher expression of iNOS (Fig. 4D; $p<0.01$), IDO (Fig. 4E; $p<0.05$), and Arg-1 (Fig. 4F; $p<0.05$) were observed in surgical mice.

**Surgery-induced MDSCs enhanced the metastatic capacity of 4T-1 breast cancer cells in vitro**

To further evaluate surgery-induced MDSCs was responsible for the tumor metastasis, we isolated MDSCs in BMCs of surgical mice and co-cultured with 4T-1 cells. The data showed that the proliferation rate were significantly higher when breast cancer cells co-cultued with MDSCs compared with these co-cultued with MDSC-depleted BMCs (Fig. 5C; $p<0.05$). The effects of MDSCs on tumor cell migration and invasion were further assessed. By transwell assay, the migration ability were significantly increased in MDSCs-treated 4T-1 cells (Fig 5A; $p<0.05$). Furthermore, the invasiveness of 4T-1 cells were also dramatically increased (Fig 5B; $p<0.05$ at 48h and $p<0.01$ at 72h, respectively).

**Anti-PD-1 pretreatment repressed MDSCs induced 4T-1 metastasis in vitro**

Our data showed that PD-1 was up-regulated on MDSCs under surgical trauma condition. To detect whether inhibition of PD-1 influenced MDSC function, we compared the migration, invasion and proliferation of 4T-1 cells co-cultured with MDSCs pretreatment with anti-mouse PD-1 or isotype antibodies. As shown in Figs, blockade of PD-1 weakened MDSC-mediated promotion of 4T-1 cells migration (Fig 6A; $p<0.05$), invasion Fig 6B; $p<0.05$) and proliferation (Fig 6C; $p<0.05$ at 24h, $p<0.01$ at 48h and 72h, respectively).

**Discussion**

Surgery excision of primary tumor is still an effective therapy in cancers. But emerging experimental and clinical researches suggested that surgical intervention may promote the spreading and metastasis of malignant cells [7]. In our study, we first evaluated the influence of surgical procedure on bone metastasis in 4T1 bearing mice. As expected, mice accepted surgery and hemorrhage showed more serious bone lesions and destruction. The activity of osteoblasts and osteoclasts is coordinated in time and space through the process of bone remodeling, which could reflected the progression of bone destruction and bone-resorption [2]. During the process of bone metastasis, increased activity of osteoblasts, characterized by high expression of ALP, was found in many researches, which reflect activated and continuous remodeling of the skeleton [2]. Osteoclasts, characterized by high expression of TRAP, are
highly specialized multinucleated cells, and the only cells capable of bone resorption [2]. In this research, we observed more numbers of TRAP and ALP positive cells in bone slides from mice undergone surgery with hemorrhage. Collectively, these data suggested that major surgery accelerated the bone metastasis.

Major surgery disturbs the immune system resulting in immunosuppression which is known as a critical factor for progression of tumor [8]. MDSCs have been well found to be enriched in tumor-bearing hosts and mediated tumor related immune suppression [2]. Numerous findings indicated that surgical factors disturbed the function, differentiation of precursor cell to mature cell subsets in bone marrow, which led to accumulation of immature cell subsets such as MDSCs [2]. Here we observed a striking increase in MDSCs in both bone and spleens from surgical mice. Especially in bone, the percentage of MDSCs in both mice accepted surgery and hemorrhage was significantly increased. Moreover, our results showed that surgery-induced MDSCs expressed more immunosuppressive receptors such as PD-1, and higher expression of Arg-1, IDO and iNOS, which all illustrated the immune inhibitory function. These data indicated that surgery-associated factors promote not only MDSCs accumulation but also their immunosuppressive activity.

Bone metastasis is common especially in breast and prostate cancer patients, and is critically relative with cancer patients’ prognosis [2]. The factors participated in this process were variable and has not been well elucidated. Bone marrow harbor many kinds of immune which mediated in metastatic progression. In this study the significant increase of MDSCs isolated from surgical mice raised the question of whether the MDSCs related to tumor metastasis. Through a co-cultured system, we found that 4T1 cells co-cultured with MDSCs isolated from surgical mice showed significantly increased proliferation, migration and invasion, compared with these co-cultured with MDSC-depleted BMCs. These data indicated that the MDSCs expanded by surgery actively contribute to tumor metastasis. Previous studies focused on the function of MDSCs in tumor microenvironment and found that MDSCs are critical determinants of cancer cell behavior during cancer progression and metastatic spread [2]. In this study we showed that MDSCs induced by surgery and hemorrhage accelerated tumor progression in vitro, which has never been reported previously.

PD-1 has been well known as a T cell checkpoint receptor, and been demonstrated to be expressed on myeloid cells. In the study of Strauss, et al, specific PD-1 ablation promotes the differentiation of suppressing myeloid cells with effector features which showed inhibited function in tumor progression [9]. As mentioned above, we found that surgical induced MDSCs expressed higher level of PD-1. Then we treated MDSCs with anti-PD-1 antibody for 24h and nextly co-cultured with 4-T1 cells. We observed that the promotion of MDSCs on tumor cells were significantly inhibited.

There are several limitations in the present study. First, we mainly detected the relationship between surgery-induced MDSCs and tumor metastasis in vitro through a co-culture system. In vivo, we only found an indirected role between major surgical trauma induced MDSCs and breast cancer bone metastasis, MDSCs-depleted mice may be more persuasive in the further study about the influence of surgery-induced MDSCs on tumor bone metastasis. Second, the bone metastasis model used in the present study was as
previous reported. However, this model cannot demonstrate the distant metastasis of whose primary tumor was not direct implantation at femur.

Collectively, this study revealed that major surgery induced accumulation of MDSCs which promoted breast cancer cells bone metastasis. Elimination or reduction of MDSCs may significantly delays and limits tumor metastasis in the bone.

**Abbreviations**

MDSCs: Myeloid-derived suppressor cells; PD-1: programmed cell death-1; IDO: indoleamine2, 3-dioxygenase; arginase-1: Arg-1; iNOS: inducible nitric oxide synthase

**Declarations**

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**Authors’ contributions**

XZ and LD conceived the designed that study. FJ and HC performed the molecular and cellular biology of the study. XZ and LD wrote the manuscript and approved the manuscript. All authors read and approved the final manuscript.

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**Ethics approval and consent to participate**

This study was reviewed and approved by the Ethics Committee of Harbin Medical University.

**Availability of data and materials**

Relevant data and materials have been presented within the manuscript.

**Consent for publication**

Not applicable.
Competing interests

There is no competing interesting to declaim.

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Figures

**Figure 1**

Surgery-hemorrhage promoted bone resorption and destruction. (a) Representative hematoxylin and eosin staining images of bone metastasis. At day 0, 4T1 breast cancer cells were implanted into the right
side of fat pad and the proximal end of tibia and divided into Control group, Surgery group and Surgery-hemorrhage group. Surgical procedures were performed at day 1. Seven days after surgery, the right side of femur and tibia fragments from three groups were isolated and bone resorption was identified by Histological examination. (b) The quantitation of the bone destruction was shown. Original magnification 100 ×. * p<0.05 versus the Control group. Error bars, SD (n = 7 in each group).

Figure 2
Surgery-hemorrhage increased the activity of osteoclast and osteoblasts. (a) Immunohistochemical staining of tartrate-resistant acid phosphatase (TRAP) was used to assess the activity of osteoclasts. (b) Alkaline phosphatase (ALP) staining was used to assess the activity of osteoblasts. Upper pictures were captured at magnification 200 ×. Magnified images of the boxed regions in each picture are shown at lower panel. Osteoclast or osteoblasts bones were indicated by arrows. * p<0.05 versus the control group. Error bars, SD (n = 7 in each group).
Figure 3

Percentage of CD11b+Gr-1+ MDSCs increased in mice underwent surgery. (a) The percentage of MDSCs in bones marrow from Control group, Surgery group and Surgery-hemorrhage group. (b) The percentage of MDSCs in spleen from Control group, Surgery group and Surgery-hemorrhage group. (c) The percentage of MDSCs isolated from mice which accepted tumor implantation and then underwent surgery at day 3. (d) The percentage of MDSCs isolated from mice which accepted tumor implantation at day 7.
and then underwent surgery at day 7. *p<0.05, **p<0.01 versus the Control group. Error bars, SD (n = 7 in each group).

Figure 4

Surgery-induced MDSCs expressed higher levels of PD-1 and PD-L1, iNOS, Arg and IDO. MDSCs were isolated from mice in Control and Surgery-hemorrhage group at day 7 post surgical procedures. (a) the protein levels of PD-1 and PD-L1 were detected by western blot. (b) The relative density of PD-1. (c) The relative density of PD-L1. (d) mRNA expression of iNOS were detected by RT-PCR. (e) mRNA expression of IDO were detected by RT-PCR. (f) mRNA expression of Arg-1 were detected by RT-PCR. *p<0.05, **p<0.01 versus the Control group. Error bars, SD. Each experiment repeated independently three times.
Surgery-induced MDSCs promoted 4T-1 breast cancer cells proliferation invasion and migration in vitro. MDSCs were isolated from surgery-hemorrhage mice at day 7 post surgical procedures. Then MDSCs co-cultured with 4T-1 cells in vitro. (a) The effects of MDSCs on tumor cell migration were assessed by transwell assay. (b) The effects of MDSCs on tumor cell invasion were assessed by transwell assay. (c)
The influence of MDSCs on tumor cells proliferation rate was assessed by CCK assay. *p<0.05, **p<0.01. Error bars, SD. Each experiment repeated independently at least three times.

Figure 6

PD-1 blockade inhibits effects of MDSCs on 4T-1 cells proliferation, migration and invasion in vitro. MDSCs isolated from surgery-hemorrhage mice at day 7 post surgical procedures, and then treatment with PD-1 antibody or isotype antibody. (a) The effects of MDSCs on tumor cell migration were assessed
by transwell assay. (b) The effects of MDSCs on tumor cell invasion were assessed by transwell assay. (c) 72 h later MDSCs co-cultured with 4T-1 cells. The proliferation rate of tumor cells was assessed by CCK assay. * p<0.05, ** p<0.01, Error bars, SD. Each experiment repeated independently at least three times.