

# Early-phase Treatment by Low-dose 5-Fluorouracil or Primary Tumor Resection Inhibits MDSC-mediated Lung Metastasis Formation

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**Abstract.** *Background/Aim: The outcome of patients with malignant tumors is poor if they suffer from lung metastases. Myeloid-derived suppressor cells (MDSCs), a major player for tumor-induced immunosuppression, can be suppressed by certain chemotherapeutic agents, such as low-dose 5-fluorouracil (5-FU) or surgical treatment. Based on these findings, we hypothesized that early-phase treatment by low-dose 5-FU or surgical resection of primary tumors would prevent lung metastasis formation by inhibiting MDSCs. Materials and Methods: B16F10 melanoma-bearing C57BL/5 mice with lung metastases were treated with low-dose 5-FU or surgical resection of primary tumors. Results: Low-dose 5-FU chemotherapy inhibited systemic and lung-accumulating MDSCs in tumor-bearing mice. The therapy inhibited lung metastasis formation and prolonged the survival of the animals. Consistently, early-phase resection of primary tumors improved survival, which was concomitant with a reduction of lung-accumulating MDSCs and lung metastases. Conclusion: Early-phase treatment may provide therapeutic values to prevent MDSC-mediated lung metastasis formation in tumor-bearing hosts.*

The presence of metastases in patients with malignant tumors is a sign of advanced systemic disease (1). Consequently, few

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patients survive more than one year after diagnosis of metastasis. In particular, over 20% of patients with solid tumors possess metastases exclusively in the lung with no detectable involvement of other organs. Therefore, it is clinically important to control lung metastasis of malignant tumors.

One of the hallmarks of tumor progression is tumor immune escape (2) that allows tumor cells to escape from and suppress antitumor immune responses. As a consequence, the establishment of primary tumors and subsequent metastases is achieved. Among various factors, myeloid-derived suppressor cells (MDSCs) are thought to play central roles in the tumor immune escape (3). MDSCs are a heterogeneous population comprising of immature myeloid cells (IMCs). Under normal conditions, IMCs differentiate into mature macrophages, dendritic cells and granulocytes (4). However, in pathological conditions, such as malignant tumors, IMC differentiation is inhibited and immunosuppressive MDSCs are generated instead. In mice, MDSCs are identified mainly by the presence of CD11b and Gr-1, which further comprise of two distinct subsets: monocytic MDSCs (mMDSC; CD11b<sup>hi</sup>Gr-1<sup>mid</sup>Ly6C<sup>hi</sup>Ly6G<sup>lo</sup>) and granulocytic MDSCs (gMDSC; CD11b<sup>hi</sup>Gr-1<sup>mid</sup>Ly6C<sup>lo</sup>Ly6G<sup>hi</sup>) (5, 6). In humans, MDSCs are characterized mainly as CD11b<sup>+</sup> CD33<sup>+</sup>HLA-DR<sup>-</sup> cells. MDSCs expressing CD14 are mMDSCs, whereas CD14<sup>-</sup> cells are gMDSCs (5, 6).

Recent studies have demonstrated that certain chemotherapeutic agents can directly eliminate MDSCs (7-9). In particular, 5-fluorouracil (5-FU) has been shown to induce selective MDSC apoptosis when given to mice bearing established subcutaneous (*s.c.*) tumors at a size of around 100 mm<sup>2</sup> (10). Given that 5-FU exhibits its toxic effects on tumor cells, as well as MDSCs, the addition of 5-FU to standard chemotherapy would be a promising strategy to enhance anticancer immune responses (11). However, it

remains unclear how 5-FU treatment would affect the kinetics of tumor-associated MDSCs if the therapy is initiated at earlier time points.

Based on these findings, we hypothesized that early-phase administration of low-dose 5-FU would prevent lung metastasis formation by inhibiting the generation and/or tumor accumulation of MDSCs. To elucidate this question, we established a lung metastasis animal model using B16F10 melanoma cells; the tumor-bearing mice were treated with low-dose 5-FU at an early phase. Herein, we first showed that early-phase administration of low-dose 5-FU inhibits MDSCs systemically and subsequently lung-accumulating MDSCs in tumor-bearing mice. We then showed that low-dose 5-FU chemotherapy inhibits lung metastasis formation and prolongs the survival of the mice. Then, we performed surgical resection of primary tumors as an alternative strategy of 5-FU chemotherapy. We showed that early-phase resection of primary tumors improves the survival of tumor-bearing mice, which was concomitant with a reduction of lung-accumulating MDSCs, as well as lung metastasis formation. Taken together, the data in this study suggest that early-phase treatment may provide therapeutic values to prevent MDSC-mediated lung metastasis formation in tumor-bearing hosts.

## Materials and Methods

**Mice and cell lines.** The procedure has been described previously (12). Briefly, pathogen-free C57BL/6 female mice (6-8 weeks-old) were purchased from CLEA Japan (Tokyo, Japan). Mice were maintained under specific pathogen-free conditions and used in compliance with the Institutional guidelines of Kobe University (approval numbers: P141208 and P150107). The B16F10 melanoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640 medium supplemented with 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Waltham, MA, USA).

**Cell preparation.** The procedure has been described previously (13). Briefly, to purify splenocytes, spleens were harvested, grounded and filtered using 70-µm cell strainers (BD Bioscience, San Diego, CA, USA). Red blood cells were lysed with lysis buffer (155 mM NH<sub>4</sub>Cl in 10 mM Tris-HCl buffer, pH 7.5, 25°C) to obtain single-cell suspensions. Regarding immune cells in the lung and *s.c.* tumors, these organs were harvested, minced and suspended by using 2% collagenase A and 0.75% DNase I (Roche, Mannheim, Germany) in RPMI 1640 medium supplemented with 10% FBS at 20°C for 60 min. The cell suspension was passed through the cell strainers to obtain single cell suspensions. The purified single cells were stained with 7-aminoactinomycin D (7-AAD) viability staining solution (Bioscience, San Diego, CA, USA) to label dead cells. The cells were then stained with the antibodies described below.

**Antibodies and flow cytometry.** The procedure has been described previously (14). The following monoclonal antibodies (mAbs) were purchased from BD Biosciences: fluorescein isothiocyanate (FITC)-

conjugated anti-mouse CD11b, phycoerythrin (PE)-conjugated anti-Gr-1, and FITC-conjugated anti-Ly6C. The following mAbs were purchased from BioLegend: PE-conjugated anti-mouse CD11b and peridinin chlorophyll protein complex (PerCP)-Cy5.5-conjugated anti-CD45. Cells were stained in a 96-round-bottom-well plate for 20-30 min at 4°C and washed with PBS containing 5% FBS. Flow cytometry data were obtained using a FACSVerse (Becton Dickinson, San Jose, CA, USA) and analyzed using the FlowJo software, version 7.6.5 (TreeStar, Ashland, OR, USA). All the cells were gated with CD45.

**Tumor inoculation and measurement.** The procedure has been described previously (15). Briefly, mice received *s.c.* inoculation of B16F10 cells ( $5 \times 10^5$  per mouse) into the right flank on Day 0. Tumor sizes were measured every 3 days.

**Chemotherapeutic treatment and surgical removal.** A previously described procedure was applied with minor modifications (10). Briefly, in some experiments, the tumor-bearing mice received a single injection of cyclophosphamide (CYP) or 5-FU at a dose of 50 mg/kg of body weight intraperitoneally (*i.p.*) on Day 3. In other experiments, we performed *s.c.* tumor resection. The tumor-bearing mice received isoflurane for anesthesia and ketoprofen (6 mg/kg of body weight) for analgesia on Day 7, 14 and 21. Once the mice became immobilized, their skin covering the *s.c.* tumors was shaved and sterilized with isopropanol. Then, tumors were resected *en bloc* using sterilized surgical instruments. Wounds were closed with silk suture. The mice were transferred to a warm dry area and monitored until recovery. Skin closures were removed on Days 30-35.

**Lung metastatic assay.** To induce lung metastases, the tumor-bearing mice further received intravenous (*i.v.*) injection of B16F10 cells ( $1 \times 10^5$  or  $5 \times 10^5$  per mouse depending on the study design) through the tail vein on Day 10. Some mice were sacrificed on Day 21; lung metastatic colonies were extracted, fixed with methanol and stained with 0.03% (w/v) methylene blue solution. Then, two independent observers evaluated the number of blue colonies.

**Statistical analysis.** The procedure has been described previously (16). Briefly, the Student's *t*-test was performed to analyze differences between two groups; one-way analysis of variance (ANOVA) with Holm's post hoc test was performed for multiple groups. The log-rank test was performed to analyze survival of mice. All data were analyzed using R Environment, version 3.1.3 (R Development Core Team, Vienna, Austria).  $p < 0.05$  was considered statistically significant.

## Results

**Early-phase administration of low-dose 5-FU inhibits MDSCs systemically and subsequently affects lung-accumulating MDSCs in B16F10 tumor-bearing mice.** Recent studies have demonstrated that low-dose chemotherapy affects tumor-associated immune responses (9) and that the chemotherapeutic agent 5-FU directly eliminates MDSCs (10). Based on these previous reports, we sought to investigate the effects of low-dose 5-FU on MDSCs' kinetics in B16F10 tumor-bearing mice. To this end, we first administered 5-FU (50 mg/kg, *i.p.*) on Day 3 after *s.c.* inoculation of B16F10

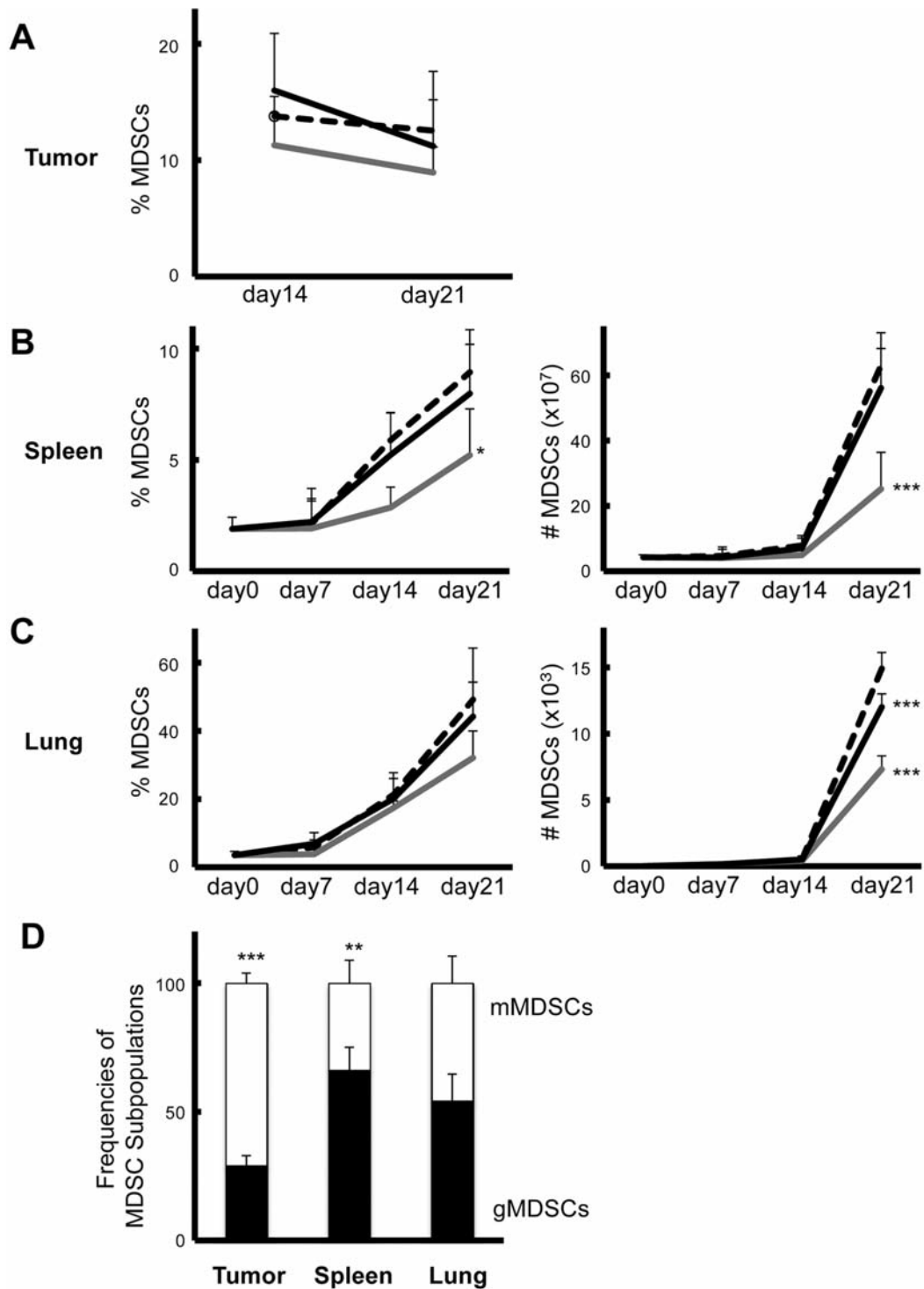


Figure 1. Early-phase administration of low-dose 5-FU inhibits MDSCs systemically and, subsequently, lung-accumulating MDSCs in B16F10 tumor-bearing mice. Wild-type C57BL/6 mice received subcutaneous (s.c.) inoculation of B16F10 syngeneic melanoma cells ( $5 \times 10^5$ /mouse) on Day 0. On Day 3, the following chemotherapeutic agents were intraperitoneally (i.p.) administered to the tumor-bearing mice: 5-fluorouracil (5-FU), cyclophosphamide (CYP) and PBS (for control). MDSCs were extracted from the spleen (A), the lung (B) and the s.c. tumors (C) on the following time points: Days 7, 14 and 21. Their frequency and absolute number were evaluated by flow cytometry. —, 5-FU; —, CYP; - - -, PBS. Each data point shows the mean value  $\pm$ SD obtained from 5 mice. (D) The tumor-bearing mice were sacrificed on Day 21. The ratio of monocytic MDSCs (CD11bhiGr-1midLy6CloLy6Ghi; black bars) and granulocytic MDSCs (CD11bhiGr-1midLy6CloLy6Ghi; open bars) in the indicated organs were enumerated on Day 21. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

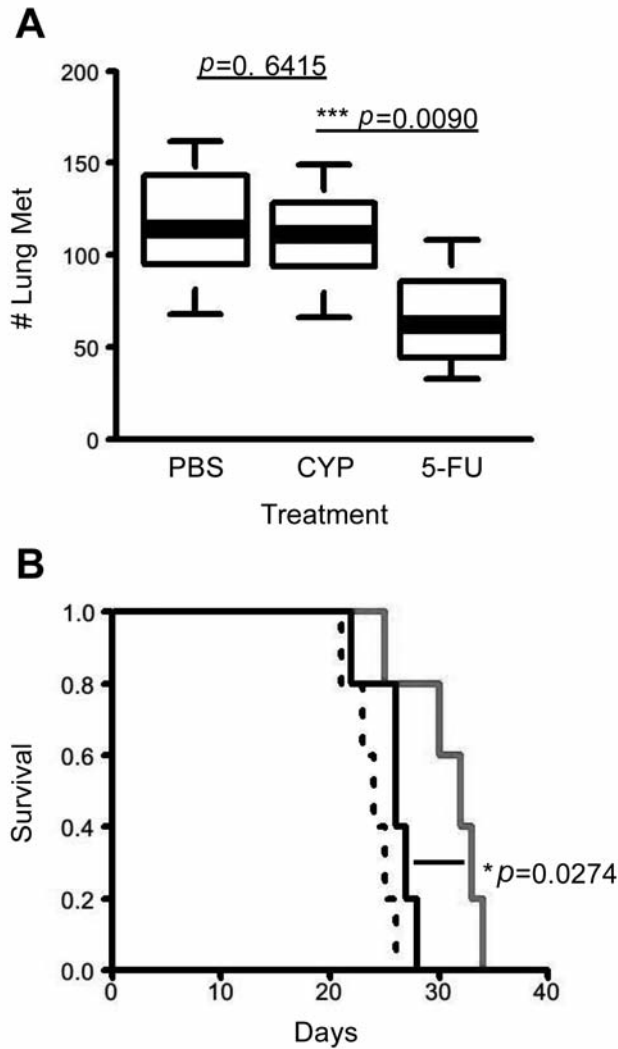


Figure 2. Early-phase administration of low-dose 5-FU improves the survival of B16F10 lung metastatic model. (A) On Day 10, B16F10 cells ( $1 \times 10^6$ /mouse) were intravenously (i.v.) injected into the mice that had received s.c. inoculation of B16F10 cells and subsequent i.p. administration of the chemotherapeutic agents according to Figure 1. On Day 21, the numbers of lung metastatic colonies were evaluated. The experiments were repeated three times.  $n=8$  per group.  $*p<0.05$ ;  $**p<0.01$ . (B) Survival of the tumor-bearing mice was monitored. —, 5-FU; — —, CYP; - - -, PBS. The experiments were repeated 3 times with similar results.  $n=5$  per group.

melanoma cells. We used CYP and PBS as control agents. We observed no difference in the size of the s.c. tumors in response to 5-FU (data not shown). Nevertheless, the tumor-bearing animals exhibited an increase in CD11b<sup>+</sup>Gr-1<sup>+</sup> MDSCs systemically over time (Figure 1). In details, low-dose 5-FU had a minimal effect on the frequency of MDSCs in the tumor microenvironment (Figure 1A). In contrast, the therapy decreased the frequency (and absolute number) of MDSCs in

the spleen compared to controls (Figure 1B). Concomitantly, MDSCs in the lung decreased in response to 5-FU (Figure 1C). These findings suggest that, although low-dose 5-FU promotes subtle effects on tumor-associated MDSCs, it decreases MDSCs systemically.

MDSCs consist of two major subsets: mMDSC (CD11b<sup>hi</sup>Gr-1<sup>mid</sup>Ly6C<sup>hi</sup>Ly6G<sup>lo</sup>) and gMDSC (CD11b<sup>hi</sup>Gr-1<sup>mid</sup>Ly6C<sup>lo</sup>Ly6G<sup>hi</sup>) (17). Therefore, we sought to evaluate the distribution of these MDSC subsets in our animal model (Figure 1D). MDSCs in the tumor microenvironment primarily consisted of mMDSCs (71.0%). In contrast, in the spleen (66.2%) and the lung (54.2%), gMDSCs were predominant compared to mMDSCs. These data suggest that primary tumor sites possess different immunological environments compared to potent metastatic sites, such as the lung.

Early-phase administration of low-dose 5-FU improves survival of the B16F10 lung metastatic model. The finding that low-dose 5-FU decreases MDSCs in the spleen and the lung led us to hypothesize that the same treatment would prevent lung metastasis formation by inhibiting MDSCs' accumulation in the metastatic sites. Therefore, we sought to evaluate the anti-metastatic activity of 5-FU using a B16F10-based metastasis model. As a consequence, mice treated with 5-FU exhibited a significant reduction (43.9%) in lung metastasis formation compared to those with PBS (Figure 2A). This reduction was not merely attributable to the difference in size of the primary tumors because the primary tumors grew equally among groups (data not shown). Concomitantly, 5-FU treatment prolonged the survival of tumor-bearing mice compared to CYP or PBS ( $p=0.0274$ ; Figure 2B). Taken together, these data suggest that early-phase administration of low-dose 5-FU improves the survival of the B16F10 lung metastatic model along by inhibiting MDSC-mediated lung metastasis formation.

Early-phase resection of primary tumors improves survival of the B16F10 lung metastatic model along with a reduction of MDSCs. These data further led us to hypothesize that early-phase surgical treatment would also improve the survival of tumor-bearing mice by inhibiting MDSC-mediated lung metastasis formation. To elucidate this, we evaluated the therapeutic impact of primary tumor resection at different time points (days 7, 14 and 18; Figure 3). Although the early-phase surgical resection of the primary tumor did not change the frequency of MDSCs in the lung (Figure 4A), the absolute number of MDSCs in the lung decreased on Days 7 and 14 compared to those of Day 18 ( $p=0.0283$ ; Figure 4B). Consistent with this, lung metastasis formation was significantly inhibited when surgical resection was performed on Days 7 or 14 ( $p=0.0147$ ; Figure 4C). Next, we evaluated the overall survival of these mice (Figure 4D). Day 18-resection significantly improved the survival of

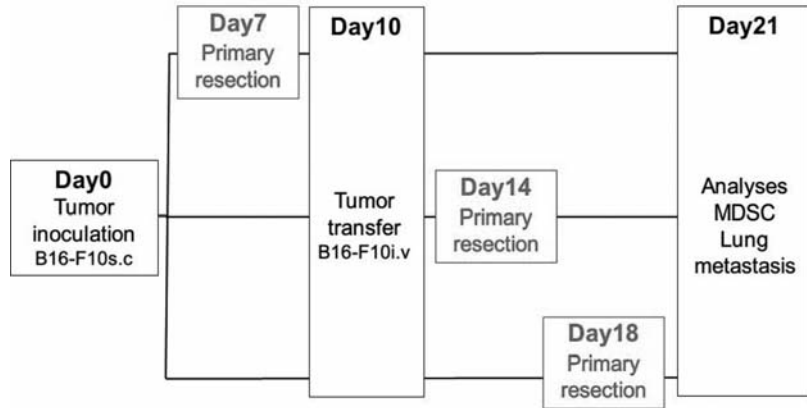


Figure 3. Scheme showing the study design of surgical resection at different time points. Mice received *s.c.* inoculation of B16F10 cells on Day 0 and *i.v.* injection of B16F10 cells on Day 10. Subsequently, the mice underwent *s.c.* tumor resection at the following time points: Days 7, 14 and 18.

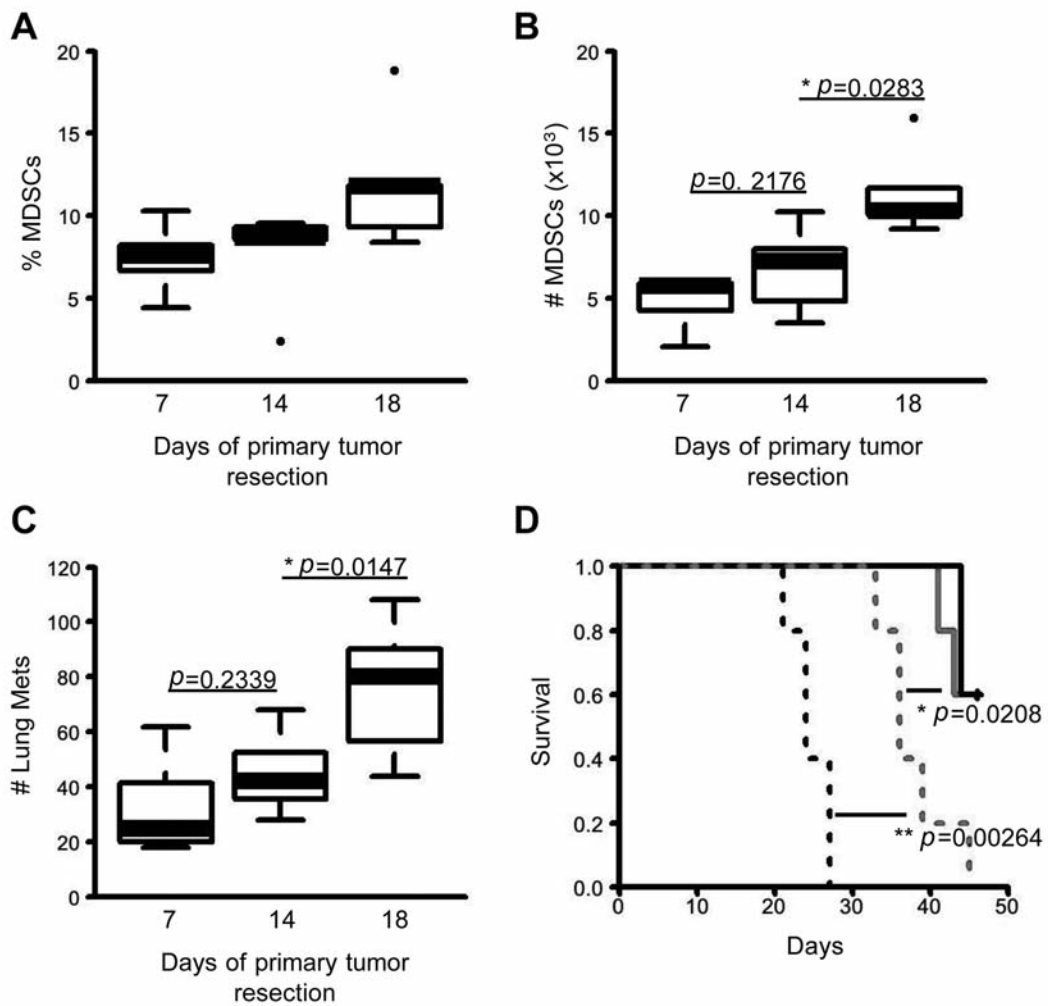


Figure 4. Early-phase resection of primary tumors improves the survival of the B16F10 lung metastatic model along with a reduction of MDSCs. Mice underwent the experimental procedure shown in Figure 3. (A) The frequency of MDSCs in the lung on Day 21 was evaluated by flow cytometry. (B) The absolute number of MDSCs in the lung was enumerated. (C) On Day 21, the number of lung metastatic colonies were evaluated.  $n=5$  per group. (D) Survival was monitored. —, Day 7-resection; —, Day 14-resection; - - -, Day 18-resection; - - -, no resection (control). \* $p<0.05$ ; \*\* $p<0.01$ .



tumor-bearing mice compared to those with no tumor resection ( $p=0.00264$ ). Furthermore, we observed a significantly improved survival when the surgical resection was performed at earlier time points, such as Days 7 or 14 ( $p=0.0208$ ). Taken together, these data suggest that early-phase resection of primary tumors improved the survival of the B16F10 lung metastatic model along with a reduction of MDSCs.

## Discussion

Recent studies have elucidated pathological roles of MDSCs in promoting malignant tumors (3). In addition, certain studies have demonstrated that low-dose 5-FU inhibits the generation and tumor accumulation of MDSCs in tumor-bearing hosts, which leads to tumor growth inhibition (10). However, it remained unclear whether 5-FU administration would also inhibit metastasis. To address this question, we established a B16F10-based lung metastasis animal model and evaluated the therapeutic impact of low-dose 5-FU administration. We first observed that early-phase administration of low-dose 5-FU inhibits MDSCs in the spleen and the lung of the tumor-bearing mice (Figures 1B-1C). Detailed analyses of MDSC subsets revealed that MDSCs in the tumor microenvironment primarily consist of mMDSCs, whereas gMDSCs are predominant in the spleen and the lung (Figure 1D). In addition, we observed that the early-phase administration of low-dose 5-FU improves survival of the mice along with inhibiting MDSC-mediated lung metastasis formation (Figure 2). Furthermore, we observed that early-phase resection of primary *s.c.* tumors, an alternative strategy for 5-FU chemotherapy, also improves survival of the tumor-bearing mice along with a reduction of MDSCs (Figures 3 and 4). Taken together, our data in this study clearly suggest that early-phase treatment has therapeutic values to prevent lung metastasis formation in tumor-bearing hosts.

Recent studies have demonstrated that 5-FU treatment directly leads to selective MDSC apoptosis and tumor regression (10). In this regard, we observed similar phenomena in that early-phase administration of low-dose 5-FU inhibited the lung metastatic colony formation and prolonged the survival of tumor-bearing mice (Figure 2) along with a reduction of MDSCs (Figure 1). Despite these favorable effects of 5-FU on MDSCs, we observed no direct effects on the primary *s.c.* tumors (data not shown). This discrepancy may be attributed to the facts that B16F10 itself is highly resistant to low-dose 5-FU and that its blood half-life is no longer than 16 min (18). To overcome this issue, we are in the process of seeking for other chemotherapeutic agents that inhibit tumors directly, as well as MDSCs.

In addition to our finding that MDSCs are associated with lung metastasis formation, as well as survival of tumor-bearing hosts (Figures 1 and 2), it has been shown that

complete resection of primary tumors improves the survival of tumor-bearing hosts by modulating tumor-associated immune responses (19). In this regard, we also observed that primary tumor resection reduced the accumulation of MDSCs in metastatic sites (Figure 4B). Moreover, this phenomenon was associated with better outcome of the tumor-bearing hosts (Figure 4D). These findings clearly indicate a great demand for developing novel modalities to detect small tumors and resect them at the earliest possible phase.

In conclusion, this study demonstrated that early-phase administration of low-dose 5-FU and early-phase resection of the primary tumor can prevent the formation of lung metastases by inhibiting MDSC generation and accumulation. These data may give a clue to develop a new strategy to control malignant tumors by modulating tumor-associated immune responses.

## Conflicts of Interest

The Authors declare no financial or commercial conflicts of interest.

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