Multiple Low Doses of 5-Fluorouracil Diminishes Immunosuppression by Myeloid Derived Suppressor Cells in Murine Melanoma Model

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ABSTRACT

Background: Melanoma progression and metastasis is suggested to be mediated by increased accumulation of myeloid derived suppressor cells. Various chemotherapeutic drugs such as 5-Fluorouracil in single low concentration have the capacity, at least in part, to reverse tumor progression by reducing myeloid derived suppressor cells-mediated immunosuppression. **Objective:** To assess whether multiple low doses of 5-fluorouracil could repress myeloid derived suppressor cells in low frequency and, in turn, could enhance anti-tumor responses and promote a more prolonged survival in a murine melanoma model. **Methods:** Fifty milligram per kilogram body weight dose of 5-Flourouracil was administered intraperitoneally 4 times with 3-day intervals to C57BL/6 mice after B16 melanoma tumor models were established. The frequency and suppressive functions of myeloid derived suppressor cells and induction of anti-tumor $CD8^+$ T cells as well as tumor growth and survival were evaluated in drug treated and untreated mice. **Results:** Our results demonstrated that this therapeutic strategy increases the overall mice survival ($p \leq 0.01$) and induces melanoma-specific CD8+T cell immunity ($p \leq 0.01$) by reducing the frequency of myeloid derived suppressor cells ($p \leq 0.01$) as well as their immune suppressive functions ($p \leq 0.05$). **Conclusion:** Altogether, our data suggest that 5-fluorouracil in multiple low regimens might be used to overcome tumor immunosuppression and improve the efficacy and outcome of anti-tumor immune responses in a mouse model.

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INTRODUCTION

Despite the intensive biomedical research in oncology, malignant melanoma is one of the most aggressive cancers exhibiting unsatisfactory responses to various conventional therapies (1). Moreover, although highly expressed tumor antigens have made melanoma an immunogenic cancer, the immune-based treatments in experimental and clinical trials have been only marginally successful (2,3). Many studies have demonstrated that low responses to immunotherapies in melanoma mainly rely on various immunosuppression mechanisms in tumor microenvironment (4). Among all the mechanisms, myeloid derived suppressor cells (MDSCs) are one of the main cell subsets sharing immunosuppressive effects and also play critical roles in melanoma progression and metastasis (5,6).

MDSCs are defined as a heterogeneous population of immature myeloid lineages expanded in various human cancers and experimental tumor models. In mice, MDSCs express both the granulocyte differentiation marker Gr-1 and α_M-integrin CD11b and according to the expression level of Gr-1 isoforms, MDSCs are divided into granulocytic and monocytic subpopulations. Increase in MDSCs have been shown to inhibit antigen specific T cell responses and this capability is mediated mainly by secreting suppressive factors like reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS) (7,8). Therefore, eliminating MDSCs using chemotherapeutic drugs is represented as one of the therapeutic approaches to overcome immune suppression in tumors (9).

Administered in maximum tolerated doses in standard treatments, chemotherapeutic drugs are known to suppress immune system in cancer patients. However, preclinical and clinical studies have shown that chemotherapies in low noncytotoxic concentrations, among other immunomodulatory effects, can induce anti-tumor responses by targeting immune suppressor cells (10). Previous studies revealed that anticancer agents like gemcitabine, doxorubicin and 5-fluorouracil (5-FU), when administered in single low noncytotoxic dose, selectively reduce the numbers of MDSC in spleen as well as tumor tissues; however, this effect was not long lasting and after a short period of time, an increase in the number of MDSCs was observed (11-13). Among these chemotherapeutic drugs, 5-FU has received much attention related to its higher reducing effect on MDSC frequency than others which may potentially be useful adjunct in the treatment of melanoma. Therefore, the aim of the present study was to assess whether multiple low noncytotoxic doses of 5-FU might affect MDSC frequencies and their suppressive functions, specific antitumor T cell responses as well as clinical outcomes in B16 melanoma-bearing mice.

MATERIALS AND METHODS

Animal Model. Six- to eight-week-old female C57BL/6 mice (purchased from Pasteur Institute of Iran) were maintained in the animal care unit of the Department of Immunology (Tehran University of Medical Sciences) according to the local guidelines for animal welfare and ethics.

Tumor Cell Lines, Lysate Preparation and Protein Quantification. B16F10 melanoma and 4T1 mouse mammary tumor cell lines were purchased from Cell Bank of Iran (Pasteur Institute of Iran), and maintained in complete RPMI 1640 medium

(cRPMI, Gibco, USA), supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine, 100 µg streptomycin and 100 U/ml penicillin (all were purchased from Biosera, South Korea). To prepare tumor cell lysate, the suspended cells in phosphate buffer saline (PBS, 10^7/ml) were frozen in liquid nitrogen and then thawed by incubating in 37°C water bath for 7 cycles. After centrifugation and removal of the cell debris, the protein concentration in supernatant was measured with bicinchoninic acid assay (Thermo scientific, USA).

***In Vivo* Therapy Protocol.** B16F10 cells (5×10^5) were injected subcutaneously into the right flank of C57BL/6 mice. Eight days after tumor cell injection, when the tumors were palpable, mice were injected intraperitoneally with single or multiple low non-cytotoxic dose (four doses with 3-day intervals) of 50 mg/kg of 5-FU (Sigma-Aldrich, USA). Suboptimal dose selection of 5-FU was based on dose comparison and response in the previous study (13). Mice in corresponding control groups received PBS alone. Tumor surfaces (mm^2) were measured using the formula (long axis × short axis) by digital calipers every 2 days. No signs of drug toxicity and adverse side effects during the treatment with 5-FU were observed. Three days after single dose and the last multiple dose injections of 5-FU, the mice in treated and corresponding untreated groups were sacrificed and the spleens and tumors were removed.

Cell Isolation from Spleen and Tumor Tissues. Spleens were harvested and dissociated mechanically and red blood cells were lysed using 4% ammonium chloride lysis buffer. In order to prepare single-cell suspension, dissected fresh tumor tissues were minced and digested with 1 mg/ml of collagenase IV and 500 µg/ml of DNase (Roche, Germany) at 37°C for 30 minutes followed by passing through 50 µm nylon mesh. Dead cells were removed by centrifugation over Ficoll-Hypaque gradients (Lymphodex, InnoTrain, Germany).

MDSC Isolation and Suppression Assay. MDSCs were isolated from splenocytes of both multiple low doses of 5-FU treated and untreated mice using anti-Gr-1 antibody and streptavidin-magnetic beads according to the manufacturer's instructions (Miltenyi Biotech, Germany). Purity of isolated cells was determined by FACS Calibur flowcytometry (BD Biosciences, USA) and was routinely above 95%.

MDSC suppression assay was performed as previously described with slight modifications (12). Briefly, mononuclear cells were separated from splenocytes of a naïve 6-8 week-old C57BL/6 mouse by centrifugation over Ficoll Hypaque (Lymphodex, InnoTrain, Germany). Naïve T cells were then isolated by incubating the cells in a nylon wool column (Amersham, USA) for 45 minutes at 37°C. The purity of nylon wool isolated naïve T cells was above 90%, as determined by flowcytometry.

To track cell proliferation using carboxy-fluorescein diacetate succinimidyl diester dye (CFSE, Life Technologies, USA), naïve T cells (1.2×10^7/ml) were stained with 2.5 µM CFSE at 37°C for 15 minutes. The reaction was terminated by adding of 5 ml of FBS. After washing with cRPMI 1640 medium, the CFSE labeled T cells (10^5/well) were co-cultured with various numbers of MDSC ($0.25\text{-}1\times10^5$) isolated from 5-FU treated and untreated tumor-bearing mice in 96-well round bottom plates, in cRPMI medium containing 3 µl/ml of phytohemagglutinin (PHA, Sigma-Aldrich, USA) for 3 days at 37°C in humidified 5% Co_2 incubator. Unstimulated naïve T cells served as negative controls, and stimulated naïve T cells with PHA as positive controls. Cells were then harvested and incubated with APC conjugated anti-CD3 antibody (Biolegend, USA) to analyze CFSE dilution in gated $CD3^+$cells using flowcytometry.

Measurement of ROS Level and MDSC Frequency. The intracellular ROS level of Gr-1$^+$CD11b$^+$cells in splenocytes and tumor infiltrated cells was determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) dye (Sigma-Aldrich, USA). Cell suspensions (5×10^5/1 ml PBS) were incubated in the presence of 5 μM DCFDA with or without 50 μM H_2O_2 (as control positive) at 37°C for 30 minutes. Samples were then washed in cold wash buffer (PBS containing 1% bovine serum albumin and 0.1% sodium azide) followed by incubation with APC-conjugated anti-Gr-1 and PE-conjugated anti-CD11b antibody (Biolegend, USA) at room temperature for 20 minutes. The frequency of Gr-1$^+$CD11b$^+$ cells and the level of ROS production were then detected using flowcytometry.

CD107a Degranulation Assay. The expression level of CD107a on the cell surface of splenic CD8$^+$T cells was analyzed as described previously (14) with some modifications. Spleen cells (10^6/ml) were incubated with 100 μg/ml of B16F10 cell lysate, in the presence of APC conjugated anti-mouse CD107a and monensin (2 μl/ml) for 6 hours at 37°C. Besides, in order to understand whether melanoma-specific CD8$^+$T cells are activated, 4T1 tumor lysate was concomitantly used as irrelevant inducer of T cell activation. Cells without lysate stimulation were also used as control. Cells were then washed with cold PBS and allowed to stain with FITC labeled anti-mouse CD8 for 20 minutes prior to assessment of CD8$^+$CD107a$^+$ T-cells by flowcytometry. All antibodies and reagents for CD107a degranulation assay were purchased from Biolegend (USA).

Intracellular IFN-γ Staining. Intracellular cytokine staining for IFN-γ was performed as described (15) with minor modifications. Briefly, splenocytes (1×10^6/200 μl) were cultured and stimulated in 96-well round bottom plates in cRPMI medium with 40 ng/ml of phorbol myristate acetate (PMA) and 4 μg/ml of Ionomycin (Sigma-Aldrich, USA) in the presence of 2 μl/ml monensin and 2 μl/ml brefeldin for 6 hours at 37°C. After surface staining with FITC conjugated anti-mouse CD8, the cells were fixed and permeabilized with fix/perm buffer. Cells were then intracellularly stained with APC conjugated anti-mouse IFN-γ for 30 minutes followed by flowcytometry analysis. All antibodies and reagents for intracellular IFN-γ staining were purchased from Biolegend (USA).

Statistical Analysis. Statistical analyses were performed using the Graph Pad Prism 5 software package. Nonparametric means comparisons were conducted using Mann-Whitney test (two-group comparisons) and the existence of statistical differences between groups was then established using a one way ANOVA followed by Tukey's post- test. Survival data were analyzed by Log-rank test. Flowcytometry results were analyzed using Flow Jo software version 7.6.1 (Ashland, OR, USA). Significant p values were marked as follows: single asterisk for $p\leq 0.05$ and double asterisk for $p\leq0.01$.

RESULTS

Multiple Low Dose of 5-FU Reduces MDSC. We initially examined the modulatory effects of single or multiple low dose of 5-FU on the frequency and function of MDSCs in spleen and tumor tissue. The frequency of MDSCs was evaluated by analyzing cell surface markers Gr-1 and CD11b as depicted in Figure 1A.

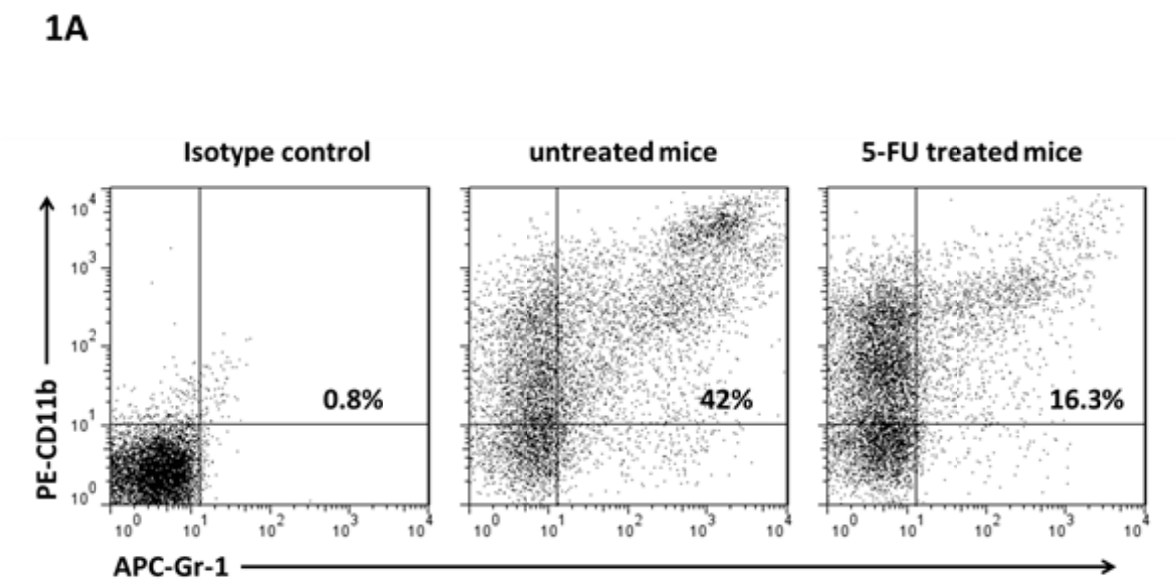

Figure 1. Multiple low dose of 5-FU reduces MDSCs in melanoma mice. Mice were injected subcutaneously with B16F10 melanoma cell line. On day 8, 50 mg/kg of 5-FU was administered intraperitoneally as single or multiple low dose (4 times with 3 day intervals) in treated groups and PBS was injected in corresponding control groups concurrently. Spleens and tumors were harvested 3 days after the last injection and the frequency of MDSC and production of ROS were analyzed using flowcytometry (n= 3 mice per group). **A**, flowcytometry plots represent the expression of Gr-1$^+$CD11b$^+$ cells in 5-FU treated vs. untreated mice. **B**, Graph shows the mean (±SD) percentage of MDSC within splenic cells and tumor infiltrated cells of mice treated or untreated with single or multiple low dose of 5-FU. **C**, The geometric mean of DCFDA expression in spleen and tumor samples of multiple low doses treated and untreated mice is shown. The data are presented as mean ± SD. Significant p values were marked as follows: Single asterisk, p≤ 0.05 and double asterisk, p≤0.01.

Analysis of the frequency of Gr-1$^+$CD11b$^+$ cell population after injection of single or multiple low doses of 5-FU revealed a decrease in the percentage of Gr-1$^+$CD11b$^+$ cells in both spleen and tumor tissue of treated mice as compared with untreated mice;

however, a significant decrease in the number of Gr-1$^+$CD11b$^+$ cell population was seen with the multiple regimen in both spleen and tumor tissue (Figure 1B).

ROS production has been recently implicated in MDSC-mediated T cell suppression. To investigate the function of MDSC in the presence of multiple low dose of 5-FU, ROS production was measured using mean fluorescent intensity (MFI) of DCFDA within the Gr1$^+$CD11b$^+$ population. Although, MDSCs of 5-FU treated mice demonstrated lower level of ROS than their control counterparts in spleen and tumor tissue, a significant decrease was only observed within splenic cells (Figure 1C).

Multiple Low Dose of 5-FU Modulates MDSC Suppressive Capability. The major activity of MDSC is to suppress the activation and proliferation of anti-tumor T cells. We sought to determine whether *in vivo* multiple low dose of 5-FU could modulate MDSC-mediated T cell suppression. To this end, MDSC were isolated from spleen of melanoma-bearing mice treated or untreated with multiple low dose of 5-FU and were then co-cultured at various ratios with naïve T cells in the presence of PHA followed by evaluation of T cell proliferation.

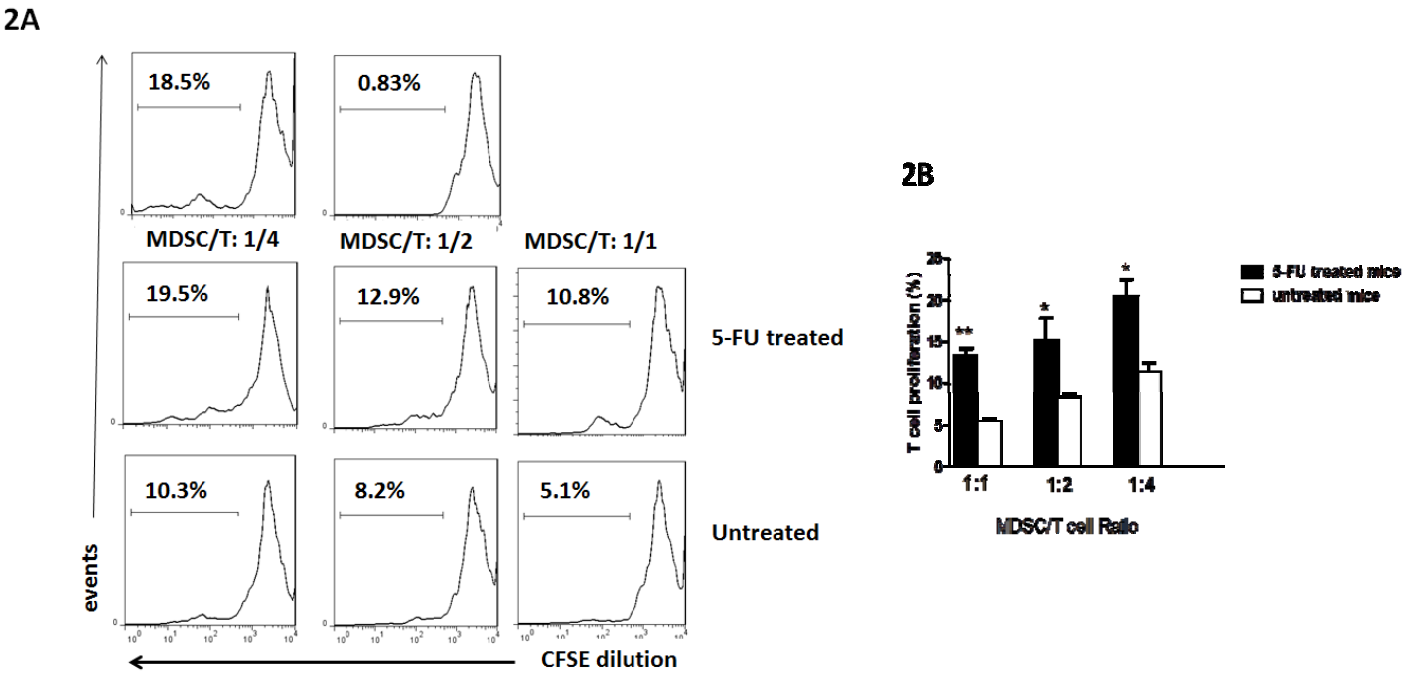

Figure 2. Multiple low doses of 5-FU modulates MDSC suppressive capability. MACS-isolated Gr-1$^+$ splenocytes from multiple low dose of 5-FU treated or untreated mice (n= 3 mice per group) were co-cultured with CFSE labeled naïve mouse T cells which were enriched using nylon wool column in the presence of PHA in various ratios. MDSC-mediated inhibition of labeled T cells was evaluated using flowcytometry. **A**, Histogram plots show CFSE intensity in PHA stimulated T cells as positive control vs. unstimulated T cells (top), PHA stimulated T cells in the presence of MDSC isolated from 5-FU treated (middle) and untreated mice(bottom) in various ratios. **B**, The mean percentage of T cell proliferation in various ratiosis shown. The data are shown as mean ± SD. Significant *P* values were marked as follows: Single asterisk, p≤ 0.05 and double asterisk, p≤0.01.

Figure 2A represents flowcytometry analysis of CFSE dilution in T cell population. Unstimulated and PHA stimulated T cells served as negative and positive controls respectively (Figure 2A top). As depicted, isolated MDSCs from 5-FU treated mice (Figure 2A middle) showed a lower suppressive impact on T cell proliferation at all ratios compared to untreated mice (Figure 2A bottom). More precisely, MDSC-mediated T cell suppression was remarkable at 1:1 ratio. This data demonstrates that multiple low doses of 5-FU could modulate MDSC-mediated T cell suppression (Figure 2B).

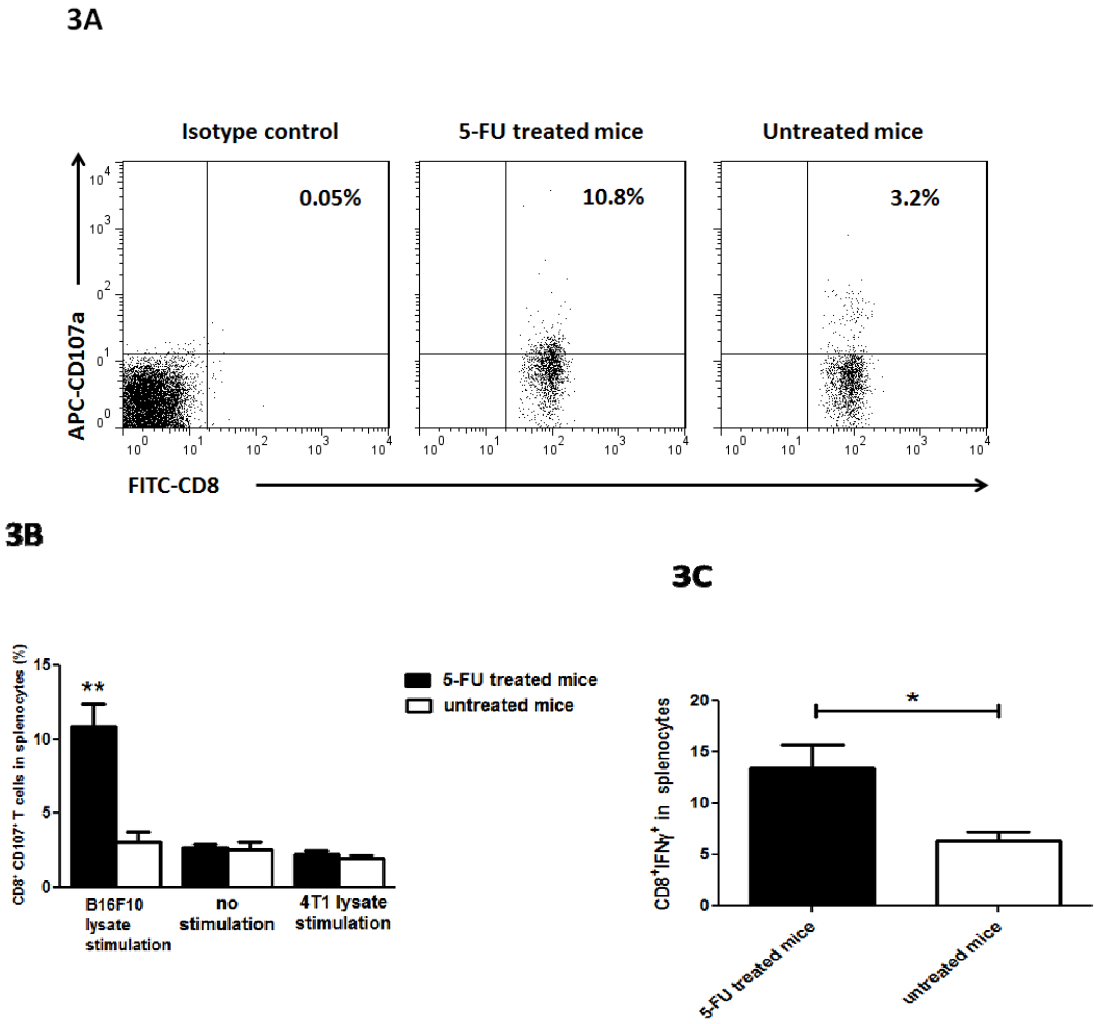

Figure 3. Multiple low dose of 5-FU increases CD8$^+$T cell activity. Isolated splenocytes from multiple low dose of 5-FU treated or untreated mice (n=3 mice per group) were cultured in 96 round bottom well plates in the presence of APC labeled anti-mouse CD107a and monensin (2µl/ml) for 6 hours at 37°C. B16F10 cell lysate (100µg/ml) was used as antigen specific stimulator, while 4T1 lysate was concomitantly used as an irrelevant stimulator. The frequency of CD8$^+$CD107a$^+$T cells was analyzed using flowcytometry. **A,** The percentage of CD8$^+$CD107a$^+$T cells in 5-FU treated and untreated mice are shown. **B**, Bar graphs represent mean percentage of CD8$^+$CD107a$^+$T cells within splenic cells in different stimulation conditions. **C**, Splenocytes were cultured and incubated in 96 round bottom well plate in the presence of PMA and Ionomycin for 6 hours at 37°C. After labeling with FITC conjugated anti-mouse CD8, cells were intracellularly stained with APC conjugated anti-mouse IFN-γ. Graphs represent the mean percentage of CD8$^+$IFN-γ$^+$ T cells in multiple low doses of 5-FU treated and untreated mice. The data are presented as mean ± SD. Significant p values were marked as follows: Single asterisk, p≤ 0.05 and double asterisk, p≤0.01.

Multiple Low Doses of 5-FU Increase CD8$^+$T Cell Activity. The transmembrane lysosomal glycoprotein CD107a (LAMP-1) is detectable on the cell surface of activated CD8$^+$ T cells after degranulation, and is likely an essential marker for cell-associated cytotoxicity. Moreover, IFN-γ production is often associated with cytotoxic T cell activity. We therefore investigated whether multiple low doses of 5-FU could induce tumor-specific CD8$^+$T cell responses. The flowcytometry plots in Figure 3A represent CD8 and CD107a expression in 5FU-treated and untreated mice. Our results demonstrated a significant increase in the expression of CD107a in tumor-specific CD8$^+$T cells of 5-FU treated mice (Figure 3B). We further investigated IFN-γ production to confirm CD8+T cells activation. We found that splenic CD8$^+$T cells isolated from 5-FU treated mice produce significantly higher level of IFN-γ compared with untreated group (Figure 3C). These data collectively suggest that multiple low dose of 5-FU could activate melanoma-specific CD8+T cells.

Multiple Low Dose of 5-FU have Modulatory Effects on Tumor Growth and Survival. Melanoma is a fast growing tumor which frequently exhibits a poor response to clinical treatments. We finally assessed F10 tumor growth and survival in mice either treated with single or multiple low doses of 5-FU and untreated mice. We observed that the mean of tumor growth/day in multiple low doses of 5-FU treated mice was significantly decreased (Figure 4A). Although all mice eventually died, the Kaplan-Meier plot also exhibited a prolonged survival of the mice receiving multiple low doses of 5-FU compared to single dose and corresponding untreated mice (Figure 4B).

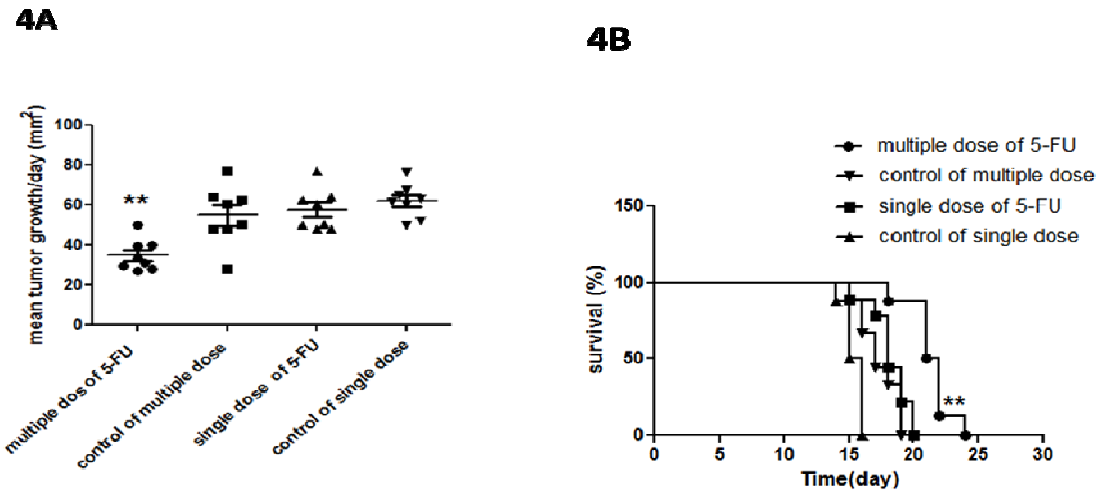

Figure 4. Antitumor activity of multiple low dose of 5-FU in B16F10 melanoma model. C57BL/6 mice were injected subcutaneously with 5×10^5 B16F10 melanoma cell line in to the right flank. On day 8, mice (n=8 mice per group) were injected intraperitoneally with 5-FU (50 mg/kg) in single or multiple low doses (4 times with 3 day intervals). Tumor size (mm^2) was measured every two days. **A**, Scatter graphs show the mean tumor growth/day in single or multiple low doses of 5-FU treated and corresponding untreated mice. **B**, Survival of mice was plotted using Log-rank test. The data are presented as mean ± SD. Significant p value was marked as follow: double asterisk, p≤0.01.

Discussion

It is well known that, apart from cytotoxic effects, chemotherapeutic drugs could exhibit immunomodulatory activities and show promise as an adjunct to cancer immunotherapy (10). Preclinical mouse models and clinical studies revealed that low dose chemotherapy regimens could induce anti-tumor immune responses through decreasing the number of tumor infiltrating immunosuppressive cells (11,16). The accumulation of MDSCs, the major immunosuppressive cell population, has been demonstrated in melanoma bearing mice and cancer patients (17). Furthermore, clinical responses in advanced melanoma patients have strongly been correlated with MDSC frequency (18). Recently, it has been reported that 5-FU in single low noncytotoxic concentration (50 mg/kg) exerts its immunomodulatory effects through reducing the numbers of MDSCs in EL4 mouse tumor model; however, MDSC numbers were returned to the untreated condition within a week (13). In addition, the modulatory effect of indicated dose of 5-FU in sarcoma persists for 4-8 days *in vivo* (19). Thus, we evaluated whether multiple low dose of 5-FU (4 doses with 3-day intervals) could repress the number of MDSCs to the low level that induce and activate specific immune response against melanoma.

This is the first report in which the immunomodulatory effects of multiple low concentrations of 5-FU were assessed in melanoma-bearing mice. We established that multiple low dose of 5-FU induced melanoma specific T cell responses through reducing MDSCs frequency as well as their suppressive functions. We also observed that mice overall survival and tumor growth rate were affected during multiple low dose 5-FU treatment.

Previous studies have described that during tumor progression, a substantial increase in the number of MDSCs could be easily detected in peripheral blood and spleen as well as tumor stroma; and the rate of increase correlated with tumor burden (17,20). In our study, we tested two sets of control groups in accordance with single or multiple treatments to show the correlation between MDSC expansions with tumor burden in the time of assessment. We observed a remarkable increase in MDSC frequency of the control group of multiple dose in comparison with the control group of single dose. Moreover, we found that although the number of MDSC increased during tumor development, it was profoundly kept down in low level under multiple low dose 5-FU treatment.

Single low concentration of 5-FU is shown to significantly reduce the number of MDSCs in tumor models (13,21). We also observed a decrease in MDSC frequency which was not significant. It is likely that the differences between the day of 5-FU injection and also tumor burden could make such adiscrepancy.

In addition, it has been widely reported that MDSC immune suppression was mostly mediated by biochemical factors such as ROS which was linked to the inhibition of anti-tumor T cell responses through the enhanced activity of NADPH oxidase complex (8,22). Therefore, we sought whether multiple low doses of 5-FU could down regulate ROS production in MDSC population. We found that multiple low 5-FU regimen considerably decreases ROS production which could be closely related to the decreased numbers of MDSC. This finding was more interesting when we found that multiple low doses of 5-FU subverted MDSC immune suppression and led to anti-tumor T cell activation. As described previously, MDSC subpopulation is defined by their phenotypic markers as well as suppressive activity (23). Precisely, ROS is the major product of granulocytic MDSC, while iNOS is mostly expressed by monocytic MDSC

(17,24). Marked down regulation of ROS in splenic cells in our study is in agreement with the previous studies highlighted the point that 5-FU selectively depleted granulocytic MDSC more efficiently than monocytic MDSC (13). Furthermore, our study revealed that 5-FU could not significantly decrease the ROS expression in tumor stroma. These results are consistent with the previous findings demonstrated that monocytic MDSCs mostly infiltrated tumor microenvironment (25,26).

Once treatment of melanoma-bearing mice with 5-FU in multiple low dose led to MDSC depletion, we explored whether this reduction might contribute to tumor specific immunity and finally tumor growth retardation. Assessment of the expression level of CD107a and IFN-γ in $CD8^+$T cells could directly identify activated tumor-specific CTLs. Our result revealed that removal of MDSC by multiple low concentrations of 5-FU increases CD107a expression level. This crucial finding was further confirmed by demonstrating that, in $CD8^+$T cells, IFN-γ production was also increased. In concordance with our results, Vincent *et al.* described that 5-FU in single low dose could induce $CD8^+$ T cells in EL4 tumor bearing mice to produce IFN-γ which was enhanced after antigen specific restimulation (13). Moreover, pretreatment of tumor cell lines with low non cytotoxic dose of chemotherapeutic drugs resulted in cell death by apoptosis which appeared to be mediated by CTL activation (27). On the contrary, low concentration of 5-FU caused MDSC to release catheps in B and then led to activation of inflammosome and production of inflammatory cytokine like interleukin-1β, which negatively restrained $CD8^+$ T cell anti-tumor immunity (28). Thus, it could be proposed that 5-FU in multiple low dose induces CTL activation through down regulation of inflammosome; however, the exact mechanism explaining how low dose of 5-FU induces CTL activation remains unclear. Depletion of MDSCs combined with tumor-specific T cell activation led to reduced tumor growth and increased survival in melanoma bearing mice treated with multiple low dose of 5-FU. This result has an important impact on developing more effective melanoma therapeutic strategies because this is the first report that 5-FU in multiple low dose could reduce the growth rate of melanoma through repressing the numbers of MDSC; however, our result did not find no difference in tumor growth and survival between mice treated with single dose of 5-FU and control group. Consistent with our result, Qu *et al.* indicated that 5-FU treatment did not significantly inhibited B16 tumor growth. Because melanoma is one of the highly aggressive cancers with complicated microenvironment, we reasoned that it may impede the potential effect of single low dose of 5-FU on tumor growth.

In conclusion, the present study provides novel data showing the immunomodulatory effects of multiple low doses of 5-FU in treatment of melanoma. Our results suggests rational combination of 5-FU with conventional immunotherapies used in the treatment of melanoma and/or other tumors may improve therapeutic efficacy and the outcome of immunotherapy.

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