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Myeloid-derived Suppressor Cells Elimination by 5-Fluorouracil Increased Dendritic Cell-based Vaccine Function and Improved Immunity in Tumor Mice

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) are capable of suppressing the immune response. 5-Fluorouracil (5-FU) compared to other chemotherapy drugs have shown considerable decreases in the number of MDSCs without visible effects on T, B and natural killer cells, as well as dendritic cells (DCs). DC-based vaccines considered to be appropriate candidates for cancer immunotherapy. However, due to the presence of various factors like MDSCs in tumor microenvironment, DC vaccine cannot effectively perform its function. The purpose of this study was to evaluate the effect of low doses of 5-FU on the efficacy of DC-based vaccines in preventing and treating of melanoma tumor model.

This research was performed on 28 melanoma tumor bearing C57BL/6 female mice. The mice were randomly divided to 4 groups, group 1 is control population while group 2 and 3 were treated with DC vaccine and 5-FU respectively and group 4 was treated with both DC Vaccine and 5-FU. The mice survival, tumor growth rate, number of MDSC and CD8+/ CD107a+ T cells in mice spleen were evaluated in each group with maximum result in group 4.

Our results revealed that combination of DC vaccine and 5-FU reduced number of MDSCs (3%) and also tumor growth rate(10%)(p<0.05) and increased mice survival (70%) and increased CD8+ /CD107a+ T cells (25%).

This study have shown that combinational therapy with DC vaccine improved immunity in tumor mice compared to the therapy consisting of DC vaccine or 5-FU only.

Keywords: Dendritic cell; 5- Fluorouracil, Myeloid-derived suppressor cell; Tumor

INTRODUCTION

Various factors are present in tumor micro environment,which activate immunosuppressive pathways. Myeloid-derived suppressor cell (MDSCs) play an important role in the suppression of anti-tumor

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immune responses.[1] MDSCs are a population of immature myeloid cells that suppress T-cell activation in mice and humans.[2,3] In mice, identification of MDSCs is performed through cell surface antigens Gr-1 and CD11b.[4] The human counterpart of MDSCs is recognized by the expression of CD11b+CD33+CD14−HLA-DR−markers. Previous studies have shown that DCs generated in the presence of MDSCs in-vitro, lose their ability of up taking antigen, maturation, migration and immune cells stimulatory capacity.[2] Therefore, elimination of MDSCs has shown to improve antitumor responses, with reducing tumor growth.[6]

DC vaccination is an active immunization approach which is considered as a promising method for treatment of cancers.5-FU which has been used as a cytotoxic drug in the treatment of cancers is a uracil analogue that has a destructive effect on the proliferation of immune cells.[7] On the other hand, in low concentrations or suboptimal doses, this drug also has been reported to be capable of inducing tumor immune responses by selectively decreasing MDSCs number without known side effects.[8]

Former studies have shown that elimination of MDSCs by 5-FUnoticeably increased the cytotoxic T cell's function.[1]Also in other study, a combination therapy of DC vaccine and 5-FU resulted in an increase in NK cells function via stimulation of TNF producing pathway and so promoted splenocyte cytotoxicity and treated tumor.[9] In this study we investigated whether 5-FU can considerably increase the efficacy of DC-based vaccine by elimination of MDSCs. For the first time, we found that administration of DC vaccine along with 5-FU could improve antitumor response via dramatically reducing tumor growth rate and considerably increasing mice overall survival. We also observed that depletion of MDSCs by 5-FU led to accumulation of immune cells such as tumor specificCD8+/ CD107a+ T cells in the tumor bearing mice's spleen.

MATERIALS AND METHODS

Animals and Cell Line

6 to 8 weeks old C57BL/6 Female mice were purchased from the Pasteur Institute of Iran. All practical works with animals were performed according to the local ethical committee protocol. C57BL/6 derived melanoma (F10-B16) cell line (purchased from the Pasteur Institute of Iran)were kept in in-vitro culture in RPMI 1640 medium (Biosera, Korea) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 2 mML-glutamine (Sigma, USA), 100 µg/mL streptomycin, and 100 U/mL penicillin.

Preparation of Tumor Lysate

Briefly, F10 cell line was cultured in 75mL cell culture flasks (SPL), when they reached 90% confluence, the cells were collected using Trypsin/EDTA (GIBCO). Pellet of the cells was subjected to the repeated freeze-thaw cycles. After centrifugation (900 g, 10 min), the supernatants were collected and passed through a 0.22 µm filter. The protein concentration of the lysate was measured. The resulting supernatants were filtered through a 0.2 µm membrane and stored at -70°C until use.

DC preparation and Culture

Bone marrow-derived DCs (BMDCs) were generated according to protocol described by Inaba et al with slight modifications.[3] Briefly, cells were harvested from the femurs and tibias of the C57/BL6 mice. Erythrocytes content were lysed with 0.8% NH4Cl water solution. Cells (5×10^5 cells/mL) were cultured in 24-well plates in RPMI 1640b (Gibco, USA) medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA), 2 mM L-glutamine(Sigma, USA), 100 U/mL penicillin and 100 µg/mL streptomycin, 20 ng/mL of recombinant murine granulocyte-macrophage colony-stimulating factor (GMC-SF, R & D, USA), and 10 ng/mL of recombinant murine IL-4 (R & D). On the third and fifth days, half of the medium was replaced with fresh medium containing GMC-SF and IL-4. On sixth day, 100 µg/mL of tumor lysate was added to immature DC's culture. After 4 h, 2 µg/mL of lipopolysaccharide (LPS, Sigma, USA) was added to all wells. On seventh day, mature DCs were harvested. The lysate were spun at 10,000 rpm for 10 min to remove particulate cellular debris. Dendritic cell were then stimulated by adding 1µg/mL LPS (Sigma-Aldrich, USA) 14 h before harvesting cells.

Immunization

Eight days afterB16/F10 tumor inoculation, the mice were injected with two doses of5-Fluorouracil (5-FU, Sigma Aldrich, USA) within three days.

Subsequently, three days after administration of the 5-FUlast dose, 10^5 DCs were subcutaneously injected in tumor region. 5-FU alone and DC vaccine, described in the previous section entitled "DC preparation", alone were administered to other groups, including control, drug and vaccine group, respectively. When the tumor was palpable, the shortest and longest surface diameters were measured every day by digital calipers; the tumor area was calculated through multiplying the shortest and longest axes. When the tumor dimension achieved>400 mm^2, the mice were sacrificed.

Isolation of MDSC from Spleens

Spleens were mechanically dissociated and individual spleen cells obtained through passing a 70-μm cellular sieve, then centrifuged, counted and washed with phosphate buffered saline (PBS). Single-cell suspensions prepared from the spleens of tumor-bearing mice were purified by magnetic selection of MDSC, using Gr1-phycoerythrin-cyanine 7 staining followed by anti-phycoerythrin-cyanine 7 magnetic beads (Miltenyi Biotec, Germany).

Cytotoxicity Assay

100 μL of the splenocytes was added to each well of 96 well plates. Four wells were considered for each sample: one well as work solution (Activating reagent), one well containing unstimulated control, one well containing isotype control and the last well without any antibody. Then, 100 μL work solution, 5 μL CD107a antibody and isotype control were added to targeted wells. Cultures were incubated for 6h at 37°C.Samples were collected and centrifuged for 5min at 1500 rpm. Removing supernatant, diluted rat serum with a ratio of 1/100 in washing buffer was added to all samples. Incubation and centrifugation were repeated. Afterward, 1 μL CD8antibody and isotype control antibody were added to cell suspension and isotype control tube respectively. Tubes were incubated for 15 min at room temperature in the dark. After incubation, washing buffer was added to all tubes following by centrifugation in 1500 rpm. Finally, supernatant was removed and 500 μL of buffer was added to all samples. Evaluations were performed by flow cytometry.

Flow Cytometry Analysis

To evaluate DC maturation by immunofluorescence labeling, DCs cultured for six and seven days period were stained with Phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies (mAbs) against cell surface molecules CD40and major histocompatibility complex II (MHC-II) (BDP har Mingen, USA) and carefully analyzed with flowjo software (version7.6.1, flowjo. USA).

In vivo Antitumor Experiments

Mice were divided into 4 groups for treatment (n=7 mice each): 1) untreated (normal saline control), 2) treated by 5-FU, 3) treated by DC vaccine, 4) treated by 5-FU and DC vaccine. All mice were subcutaneously injected in the right flank with 5×10^5 DCs or normal saline nine days after B16/F10 tumor cell line inoculation.

The antitumor effects were evaluated by the tumor volume rate and overall survival time. Tumor volume was assessed by the measurement of the shortest (A) and longest (B) dimensions applying a digital caliper every other day. Then the tumor volume (V) was calculated by following the formula: V=(A×B).

To evaluate the antitumor activity of the splenocytes in vaccinated mice, we extracted these cells from immunotherapy group of mice.

Mice were received two injections of 5-FU (50 mg/kg) within 3 days. Simultaneously, bone marrow derived DCs were generated in the presence of recombinant mouse Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and IL-4. Three days after 5-FU second injection, DCs were administered around tumor sites. Tumor volume and overall survival were assayed after DCs administration.

Statistical Analysis

Statistical differences were analyzed by one-way analysis of variance (ANOVA) with a significance level of $p<0.05$. Kaplan–Meier survival curves were analyzed for statistical difference between groups using the log-rank test. Statistical analysis and graph generation were conducted using GraphPad Prism (Version 5, graphpad software, USA).

RESULTS

Combination of DC and 5-FU Treatment was Capable of Significantly Reducing MDSCs Compared with 5-FU Alone

Fourteen days after DC vaccination, 3 mice from each group were selected and the evaluation of MDSC

numbers in spleen was performed using MACS techniques. In different groups, we studied the effect of 5-FU, DC vaccine and combination of 5-FU and DC vaccine to decrease MDSC's number. In our tumor model, MDSCs considerably rose in tumor bearing mice's spleens, as formerly described.[11]

After the last injection, the number of MDSCs evaluated in all groups. We clearly observed that in two groups (5-FU and combination of 5-FU and DC vaccine) MDSCs markedly declined compared with other groups. Also we found that treatment with both DC vaccine and 5–FU had the ability to significantly reducing MDSCs compared to treatment with 5-FU alone (p<0.05).

Combination of DC and 5-FU treatment increased survival of tumor bearing mice

To determine whether combination of DC and 5-FUis able to inhibit melanoma tumor growth, mice were inoculated subcutaneously with B16-F10 cell lines in the left flank. One week after tumor inoculation, different experimental groups were treated, received two injections of 5-FU (50 mg/kg) within 3 days. Simultaneously, bone marrow derived DCs were generated in the presence of recombinant Mouse GM-CSF and IL-4. Three days after 5-FU second injection, DCs were administered around tumor site. Comparing to5-FU- treated mice only and DC- treated mice only, combination of DC and 5-FU-treated mice showed a significant decrease in tumor growth(p<0.05).Animals were then followed up for survival rate after fourteen days of treatment with 5-F only, DC only and combination of both DC and 5-FU. Results have proven that combinational therapy was associated with a significant prolongation of survival compared with other groups (p< 0.05).

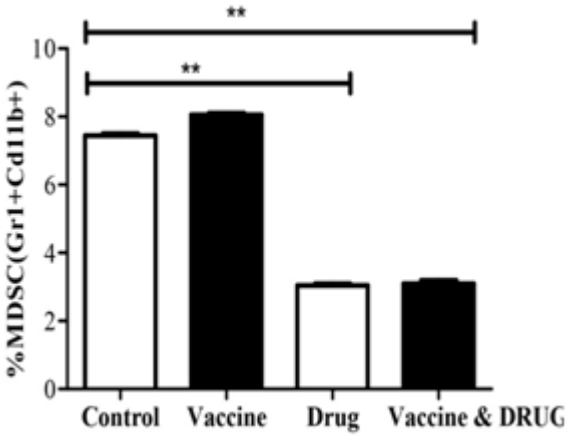


Figure1.C57BL6 mice were infected with 5×10^5 B16/F10 cell line. Mice in vaccine group were injected10^{5}dendritic cells, mice in 5-Fluorouracil group were injected two doses of 5-FU, mice in DC and 5-FU group were inoculated10^{5}DCs and two doseSof5-FU and control group were received phosphate buffered saline. Splenic MDSCs isolation was performed 7 days after treatment. Bar Graph represents the mean percentage of Gr-1+ CD11b+ MDSCs in different groups.

Combination of DC and 5-FU Treatment Reduced Tumor Growth Rate in B16-F10 Tumor-bearing Mice

There are growing evidences that MDSCs and chronic inflammation are participating in tumor genesis.[12] We determined the effects of DC and 5-FUcombination impact on tumor growth rate.

To calculate tumor growth rate, the dimensions difference between two consecutive measurements of tumor growth was calculated for each mouse and the mean of tumor growth in each group was measured in square millimeters for 24h. In order to obtain more reliable estimation, the rate of tumor growth, the numbers obtained from consecutive measurements attained from different groups (3 mice per group) were taken on the slope of the graph for each rat tumor growth rate and the average slope for each group was calculated and analyzed. Although, groups of mice vaccinated with dendritic cells and received drug compared to the control group showed a significant reduction, no noticeable differences were observed among the remaining groups (p<0.05).

Combination of DC and 5-FU Induced Number of CD8+ CD107a+ T Cells

MDSCs are discovered as suppressors of antigen-dependent CD8+ T cell proliferation and differentiation. At present study we examined whether combination of DC and 5-FUcould affect Th1- or T-cytotoxic polarization in tumor-bearing mice. At the end, separated leukocytes from the spleens of tumor-bearing mice treated with DC vaccine and 5-FU or PBS treated were stimulated with anti-CD8.We detected that combination of 5-FUand DC vaccine resulted in increased CD8+/CD107a+ T lymphocytes in the spleen's of tumor-bearing mice compared with

single injection of 5-FU or DC vaccine alone in contrast with other groups. Antigen-specific tumor-infiltrating CD8+on MDSC could locally increase the polarization of CD8+ T cells.

T cells did not produce detectable levels of IFN-γ, which was enhanced after DC vaccine and 5-FU treatment of F10 tumor-bearing mice. This data collectively revealed that the cytotoxic activity of 5FU on MDSC could locally increase the polarization of CD8+ T cells.

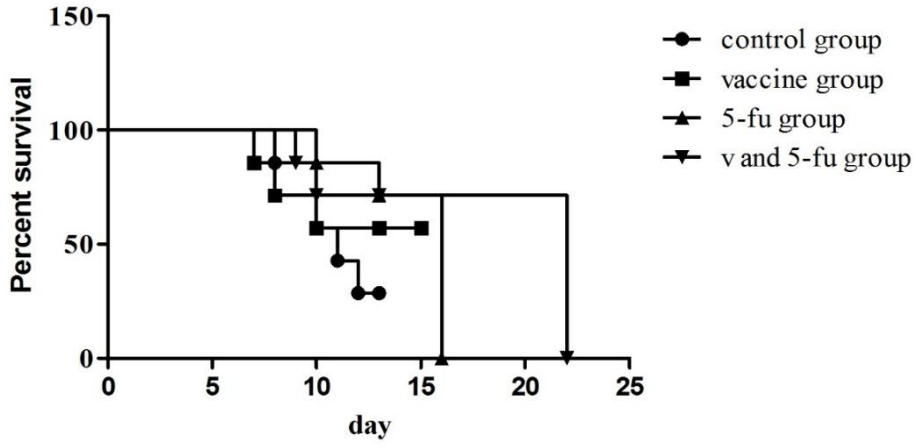


Figure2. Tumor-bearing mice were treated with phosphate buffered saline, dendritic cell vaccine, 5-Fluorouracil or combination of DC vaccine and 5-FU. Percent of survival was determined. It was shown that combination of DC vaccine and 5-FU resulted in significant increase in percent of survival.

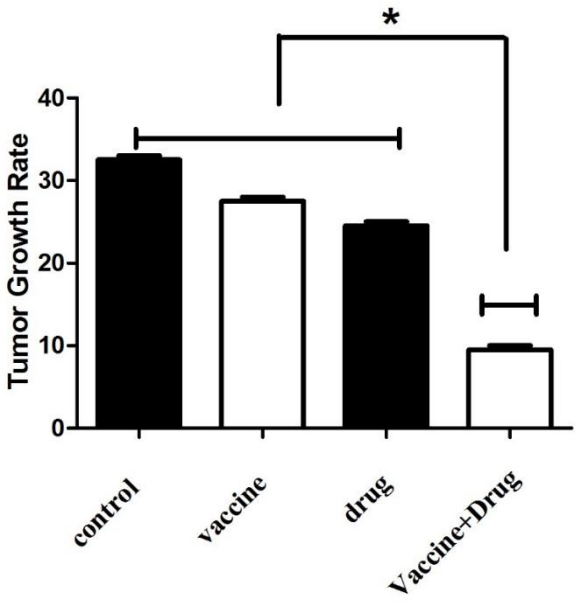
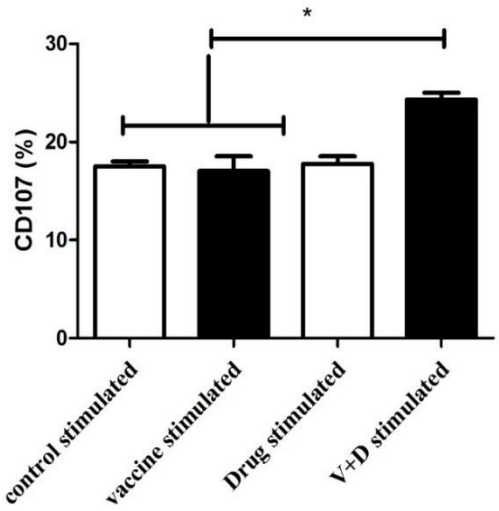
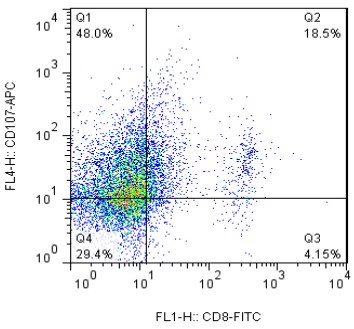


Figure3. Tumor-bearing mice were treated with phosphate buffered saline, dendritic cell vaccine, 5-Fluorouracil or combination of DC vaccine and 5-FU. One week after tumor inoculation, tumor size were measured. Tumor growth rate was evaluated .It was shown that combination of DC vaccine and 5-FU markedly decreased tumor growth rate. ($p<0.05$)





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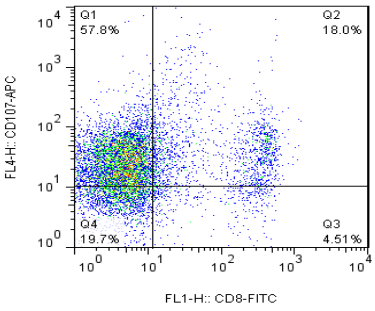

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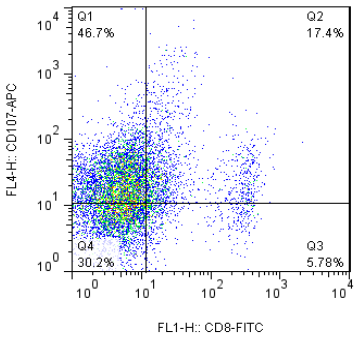
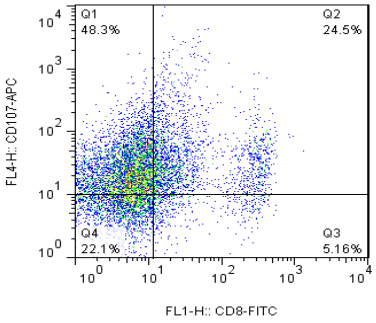


5-FU only



5-FU+ DC

Figure 4. Combination of dendritic cell vaccine and 5-Fluorouracilresulted in increased cytotoxic activity of cytotoxic T lymphocyte CTLs in comparison with other groups.4b. Tumor-bearing mice were treated with phosphate buffered saline, dendritic cell vaccine, 5- Fluorouracil or combination of DC vaccine and 5-FU. Mice were sacrificed one week after treatment. Splenocytes were isolated and cells were incubated with anti-mouse CD107a FITC and anti-mouse CD8 PE. Cytotoxic activation of cytotoxic T cells (CTLs) was evaluated based on CD107a translocation.

DISCUSSION

Immunotherapy is a type of cancer treatment that attempts to improve the ability of an individual's immune response to reject the tumor immunologically.[13]However, the production of immunosuppressive cells such as MDSCs in the tumor microenvironment reduces the efficacy of this method. Therapies that target immunosuppressive mechanisms are effective tools if enhance anticancer immune responses, leading to better immune surveillance.[14,15] We combined DC immunotherapy with 5-FU as a novel combination strategy in mouse melanoma tumor model (F10-B16). It has been shown that the 5-FU and DC vaccine enforced immune system via toxicity splenocyte. Previous experiments showed that vaccines in combination with chemotherapeutic agents and/or radiotherapy to temporarily control cancer growth and/or achieve the synergistic effects.[16,17] It was recently revealed that some chemotherapeutic agents induce positive immunological reactions. Oxaliplatin and Doxorubicin enhance immunity by injuring tumor cells to release HMGB1 protein, which is a ligand of Toll-like receptor4 which activates DC.[18] Cytotoxic agents including Cisplatin, Doxorubicin, Mitomycin C, Fluorouracil and Camptothecin induce the apoptosis of cancer cells via Fas or TRAIL-dependent pathways Nowak.[19,20] Gemcitabine has multiple immune stimulatory effects, enhances antigen presentation by inducing tumor apoptosis.[21,22] Bauer et al also showed that combination therapy with Gemcitabine and DC-based vaccination had higher efficacy in a Panc02 pancreatic tumor cell line.[23] The results of immunological monitoring in pancreatic cancer patients were reported by Plate et al; concluded that Gemcitabine therapy was not immunosuppressive and may actually enhance the response to vaccine by reducing CD3+CD45RO+ memory T cells numbers.[24] However, their study encompassed a shorter period of only 21 days, and the Gemcitabine treatment schedule was different from ours. Bang et al. reported an increase in CD11c+ myeloid DC under combinational therapy with Gemcitabine and cisplatin.[25] But Bruchard et al study have shown that combination of 5-FU and Gem led to $IL_1\beta$ production in MDSCs by activating $NLRP_3$ pathway in these cells and as a consequence the drugs function impaired and therefore caused the tumor to progress.[26] Kanterman et al in their study about colorectal cancer showed that when 5-FU is used in combination with other chemotherapy drugs it can have antitumoral effect.[27] In our study, applying 5-FU and DC vaccine is able to raise mice survival. As previous studies, especially Greay et al, showed that the combination of a low-dose chemotherapeutic agent, 5-Fluorouracil, and an adenoviral tumor vaccine has a synergistic benefit on survival in a tumor model.[28] Although previous studies concluded that 5-Fu had negative effect on immune system, our study does not show this result because it seems likely we used 5-FU and DC vaccine Simultaneously and this combination inhibited the tumor growth and so increased cytotoxicity of splenocyte. Our results also demonstrated that5-FUin combination with DC vaccine decreased tumor volumes. Results revealed a rise in the numbers of $CD8^+/CD107a^+$CTL in group treated with 5-FU and DC vaccine. Also the tumor growth significantly decreased and mice survival markedly increased in this group. Moreover, DC vaccine could not decreased MDSCs expansion when were used in combination with 5-FU. This finding shows that DC vaccine does not have any effect on the number of MDSCs. Our study revealed that the combination of 5-FU and DC vaccine is more effective than using each alone.

ACKNOWLEDGEMENTS

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