Xiaoyi Qu

COVER SHEET

TITLE: Antitumor effects of anti-CD40/CpG immunotherapy combined with gemcitabine or 5-

AUTHOR'S NAME: Xiaoyi Qu

MAJOR: Biology

DEPARTMENT: Human Oncology

MENTOR: Paul Sondel

DEPARTMENT: Human Oncology

MENTOR(2): Alexander Rakhmilevich

DEPARTMENT(2): Human Oncology

YEAR: Senior

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ABSTRACT

ANTITUMOR EFFECTS OF ANTI-CD40/CPG IMMUNOTHERAPY COMBINED WITH GEMCITABINE OR 5-FLUOROURACIL CHEMOTHERAPY IN THE B16 MELANOMA MODEL

Our previous studies demonstrated that anti-CD40 mAb (anti-CD40) can synergize with CpG oligodeoxynucleotides (CpG) to mediate antitumor effects by activating myeloid cells such as macrophages in tumor-bearing mice. Separate teams have shown that chemotherapy with Gemcitabine (GEM) or 5-fluorouracil (5-FU) can reduce tumor-induced myeloid-derived suppressor cells (MDSC) in mice. We asked if the same chemotherapy regimens with GEM or 5-FU will interfere with, or enhance, the antitumor effect of anti-CD40/CpG. Using the model of B16 melanoma growing intraperitoneally in syngeneic C57BL/6 mice, we show that GEM or 5-FU treatment regiments either did not change or reduced, respectively, the number of MDSC in the peritoneal cavity of tumor-bearing mice. In *vivo,* GEM or 5-FU chemotherapy regimens did not substantially affect antitumor effects induced by anti-CD40/CpG immunotherapy.

Xiaoyi Qu/ Biology
Author Name/Major

Author Signature

2/27/2013
Date

Paul Sondel/ Human Oncology
Mentor Name/Department

Mentor Signature

Antitumor effects of anti-CD40/CpG immunotherapy combined with gemcitabine or 5-fluorouracil chemotherapy in the B16 melanoma model

Xiaoyi Qu

Mentors: Alexander Rakhmilevich, Zulmarie Perez Horta, Paul Sondel

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Abstract

Our previous studies demonstrated that anti-CD40 mAb (anti-CD40) can synergize with CpG oligodeoxynucleotides (CpG) to mediate antitumor effects by activating myeloid cells such as macrophages in tumor-bearing mice. Separate teams have shown that chemotherapy with Gemcitabine (GEM) or 5-fluorouracil (5-FU) can reduce tumor-induced myeloid-derived suppressor cells (MDSC) in mice. In this study we asked if the same chemotherapy regimens with GEM or 5-FU will interfere with, or enhance, the antitumor effect of anti-CD40 and CpG. Using the model of B16 melanoma growing intraperitoneally (i.p.) in syngeneic C57BL/6 mice, we show that these GEM or 5-FU treatment regiments did not change or reduced, respectively, the number of MDSC in the peritoneal cavity of tumor-bearing mice. Treatment of mice with the GEM or 5-FU did not significantly affect the antitumor function of macrophages as assessed *in vitro*. *In vivo*, treatment with these GEM or 5-FU regimens followed by anti-CD40/CpG resulted in antitumor effects similar to those of anti-CD40/CpG, in the absence of GEM or 5-FU. Likewise, reduction of MDSC by *in vivo* anti-Gr1 mAb treatment did not significantly affect anti-CD40/CpG antitumor responses. Together, the results show that the GEM or 5-FU chemotherapy regimens did not substantially affect the antitumor effects induced by anti-CD40/CpG immunotherapy.

Introduction

Chemotherapy is one of the leading methods of cancer treatments; however, tumor cells often become drug-resistant and cancer recurs or progresses. Combining chemotherapy with immunotherapy has been increasingly used in the clinical practice to improve the clinical outcome [1]. Many experimental studies showed that certain chemotherapeutic drugs such as cyclophosphamide (CY) given at low doses facilitate activation of antitumor T cells via depleting T suppressor (regulatory) cells [2, 3]. Although many chemotherapeutics are immunosuppressive, the cells of innate immune system like monocytes and macrophages (Mϕ) were found to be more resistant to chemotherapy than T cells in cancer patients [4]. Therefore, it appears that immunotherapy designed to activate innate immune cells including Mϕ may be beneficial for patients receiving chemotherapy.

Agonistic anti-CD40 mAb (anti-CD40) interacts with the CD40 molecule expressed on the surface of dendritic cells and Mϕ which leads to immune activation. Anti-CD40 has been shown to induce T cell-dependent [5, 6] and independent [7] antitumor responses in mice, which led to its clinical trials for cancer treatment [8, 9, 10, 11]. We have previously showed that anti-CD40 induced antitumor effects via Mϕ activation [12]. Combining anti-CD40 with CpG-oligodeoxynucleotides (CpG) *in vivo* resulted in synergistic activation of Mϕ and induction of potent antitumor effects even in the absence of T- and NK-cells[13]. Combined treatment with CY and anti-CD40/CpG resulted in a greater reduction in tumor growth in B16 melanoma-bearing mice than either CY alone or anti-CD40/CpG [14]. Even multidrug chemotherapy consisting of vincristine, CY and doxorubicin suppressed the functions of T cells and NK cells, but primed Mϕ

to secrete NO, IFN-γ and IL-12, and synergized with anti-CD40/CpG in inducing antitumor effects [15].

The antitumor effects of anti-CD40 with and without CpG involved Mϕ and other myeloid cells[16, 17]. In our experiments mentioned above, CY alone and in combination with vincristine and doxorubicin induced expansion of myeloid cells and synergized with anti-CD40/CpG [14, 15]. However, the chemotherapeutic drugs such as gemcitabine (GEM) and 5-fluorouracil (5-FU) with a different mechanism of action were reported to substantially deplete tumor-induced myeloid cells, namely myeloid-derived suppressor cells (MDSC), in certain tumor models [18, 19]. As MDSC are present in large numbers in tumor-bearing mice (TBM) [20], in this study we asked whether the reduction of myeloid cells with the same GEM or 5-FU therapy regimens interferes with the antitumor effects of anti-CD40/CpG.

Methods

Mice and cell lines

Female C57BL/6 mice 6 to 10 weeks old obtained from Taconic, Germantown, NY were housed, cared for, and used in accordance with the Guide for Care and Use of Laboratory Animals (NIH publication 86-23, National Institutes of Health, Bethesda, MD, 1985). Mouse B16-F10 melanoma cell line was grown in RPMI 1640 complete medium supplemented with 10% FCS (Sigma Chemical, St Louis, MO), 2 mM L-glutamine, and 100U/ml of penicillin/streptomycin (all from Life Technologies, Inc. Grand Island, NY) at 37°C in a humidified 5% CO2 atmosphere.

Antibodies and reagents

Anti-CD40 was prepared from the FGK 45.5 hybridoma cell line as described previously[12]. Endotoxin-free CpG1826 was purchased from Coley Pharmaceuticals Group (Wellesley, MA). 5-Fluorouracil was dissolved in DMSO (both from Sigma Chemical, St Louis, MO) at 50 mg/ml. Gemctabine-HCl (GEM, Eli Lilly and Company, Indianapolis, IN) was obtained from UW-Hospital Pharmacy and prepared in phosphate-buffered saline (PBS). Bacterial LPS from Salmonella enteritidis was purchased from Sigma Chemical, St Louis, MO. Mouse recombinant IFN-γ was purchased from eBioscience, San Diego, CA.

In vivo tumor models and therapy

C57BL/6 mice were injected subcutaneously (s.c.) or intraperitoneally (i.p.) with $1x10^5$ B16 melanoma cells in 0.1 or 0.5 ml PBS, respectively (day 0). TBM were injected i.p. with 0.5 mg anti-CD40 starting days 7-9 after tumor implantation and 50μg CpG three days after anti-CD40 injection. Anti-Gr1 (clone RB6, 2mg/ml in Rat IgG) was injected i.t. into s.c. TBM on days 7, 10, 14 and 17 after anti-CD40/CpG treatment on the same days. 5-FU DMSO solution

was diluted in PBS to achieve 50mg/kg and administered i.p. into mice. GEM (120 mg/kg) was injected i.p. in 0.5 PBS. Days of injection are specified for each experiment. Antitumor effects were determined by measuring the perpendicular diameter of s.c tumors twice weekly, or extended survival of the mice in i.p. models. Tumor volumes were calculated according to the formula: (tumor lengh x tumor width^2)/2.

Activation of Mϕ

Peritoneal cells (PECs) were obtained via a peritoneal cavity lavage with 5ml of ice-cold RPMI 1640 complete medium, supplemented with 1IU/ml of heparin (American Pharmaceutical Partners, Inc., Schaumburg, IL) when collected from TBM. Erythrocytes in TBM PECs were lysed by hypotonic shock. Collected PECs were placed into 96-well flat-bottom cell culture plates (Corning Inc, Corning, NY) at a concentration of $2x10^5$ cells/well (or $1x10^5$ cells/well for sorted cell populations). The peritoneal Mϕ population was enriched by allowing PEC to adhere to plastic for 1.5-2 hrs followed by removal of non-adherent cells. For in vitro activation, total PEC or adherent Mϕ were incubated in medium alone or in the presence of 10 U/ml of IFN-γ and 1 ng/ml of LPS.

Mϕ mediated tumoristasis in vitro

Tumoristatic activity of Mϕ was determined by the inhibition of inhibition of DNA synthesis in tumor cells. Briefly, adherent Mϕ were stimulated in vitro as described above and simultaneously co-cultured with B16 tumor cells ($1x10^4$/well) for 48 h. To estimate DNA synthesis, cells were pulsed with 3H-TdR (1 μCi/well) during the last 6 h of incubation. 3H-TdR-incorporation was determined by β-scintillation of total cells harvested from the cell culture clusters onto glass fiber filters (Packard, Meriden, CT), using the Packard Matrix 9600 Direct β-

counter (Packard, Meriden, CT). Results are presented as counts per 5 min for triplicate wells ± SD.

Nitric oxide production

Peritoneal Mϕ were prepared and co-cultured with B16 cells for 48 h, as described above in the Mϕ cytostatic assay. Supernatants were collected and nitrite accumulation was determined using Griess reagent (Sigma, St. Louis, MO). Equal volumes of supernatants and Griess reagent were mixed for 10 min, and the A570 was measured by a microplate reader and compared to a standard nitrite curve ranging from 0–125 μM.

Splenocyte preparation

Splenocytes were prepared from whole spleens pooled from two C57BL/6 mice by processing the spleens to a single-cell suspension, followed by lysis of erythrocytes by hypotonic shock.

Splenic T-cell proliferation assay

Flat bottom 96-microwell cell-culture clusters were precoated with 0.5μg/ml (0.05ml/well) of monoclonal anti-CD3 and 5μg/ml (0.05ml/well) of anti-CD28 (both from eBioscience, San Diego, CA). Two hundred thousand of spleen cells were cultured with sorted PECs in triplicate for 72 hr in complete medium in plates precoated with anti-CD3/CD28. Cells were pulsed with 3H-TdR (1 μCi/well) during the last 6 h of incubation, and retained radioactivity was counted by β-scintillation of total cells harvested from the cell culture clusters onto glass fiber filters, using the Packard Matrix 9600 Direct β-counter. Results are presented as counts per 5 min for triplicate wells ± SD.

Flow cytometric analysis and sorting

PEC from treated and control C57BL/6 mice were harvested and stained with FITC-conjugated anti-CD45, PE-conjugated anti-Gr1, APC-conjugated anti-CD11b, or FITC-conjugated anti-B220 (all from eBioscience, San Diego, CA) for 40 min at 4 °C. Isotype-matched irrelevant rat IgG FITC, IgG APC and IgG PE, purchased from eBioscience or BDPharmingen, were used as background controls. Cells were washed in ice-cold PBS supplemented with 0.5% FCS (flow buffer), and the cell pellet was resuspended in 0.3 ml of flow buffer. The cell suspension was subjected to flow cytometry using a FACSCalibur flow cytometer or MACSQuant Analyzer and analyzed by FlowJo software (Ashland, OR). To calculate the absolute number of a PEC subset, the absolute number of total PECs (obtained via counting the cells on a haemocytometer) was multiplied by the relative prevalence of that subset (%) obtained via flow cytometry analysis. Data were collected for 10,000 live events expressing the antigen of interest per sample.

Statistical Analysis

A two-tailed Student's t-test was used to determine significant differences between experimental and relevant control values within one experiment. Nonlinear regression (curve fit) was analyzed under exponential growth model for tumor volumes of each individual mouse over time, and best-fit k-values were obtained for t-tests between two groups of mice. Statistical analysis of inhibition of the tumor cell proliferation under different experimental conditions was performed on original [3H]TdR-incorporation values or nitrite concentrations.

Results

Effect of GEM and 5-FU on B16 melanoma-induced MDSC

It has been reported that GEM treatment reduced the number of tumor-induced MDSC in several tumor models [18, 19, 21]. To determine whether GEM is effective in reducing MDSC ($CD11b^+$ $Gr\text{-}1^+$) number in the i.p. B16 melanoma model, C57BL/6 mice were injected i.p. with B16 cells and treated with 120 mg/kg of GEM 11 days later. The results in Fig. 1A and 1B show that the percentage and number of $CD11b^+$ $Gr\text{-}1^+$ PEC were increased in TBM compared to naïve mice. However, this GEM treatment regimen did not significantly affect the percentage (Fig. 1A) or number (Fig. 1B) of $CD11b^+$ $Gr\text{-}1^+$ cells in the peritoneal cavity or spleen (data not shown) in B16 TBM.

Because GEM did not reduce the number of $CD11b^+$ $Gr\text{-}1^+$ myeloid cells in TBM, we used another chemotherapeutic drug, 5-FU, which was reported to be more effective than GEM in depleting MDSC [19]. The results in Figure 1C and 1D show that indeed, in contrast to GEM, treatment of B16 TBM with 5-FU resulted in statistically significant reduction of the relative and absolute numbers of $CD11b^+$ $Gr\text{-}1^+$ PEC but not spleen cells (not shown) compared to PBS-treated TBM.

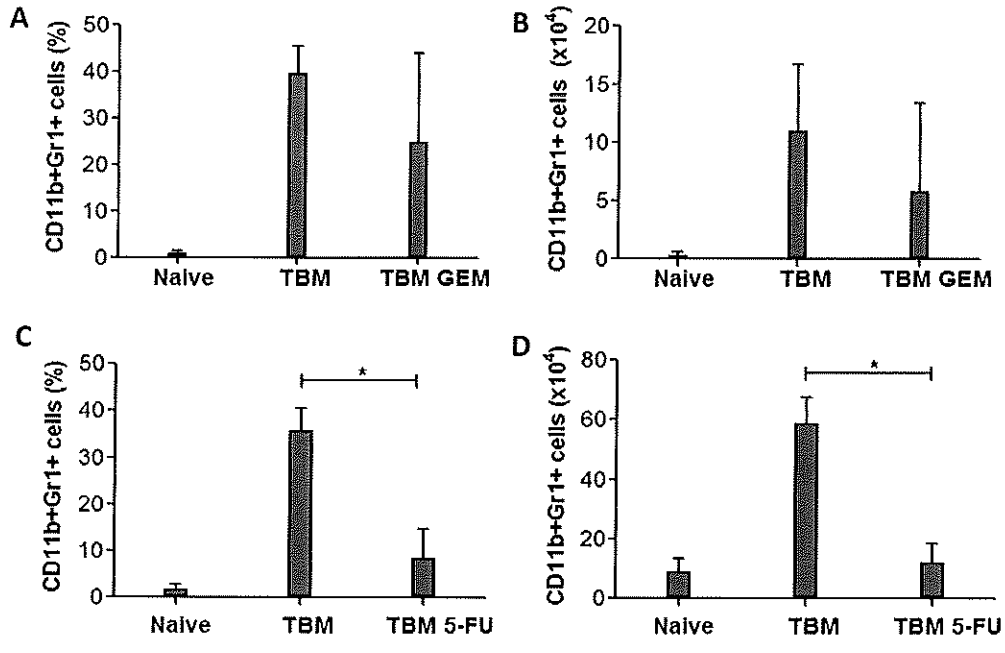

Figure 1. Effect of GEM or 5-FU on i.p. B16 melanoma-induced MDSC. **A, B**: C57BL/6 mice were injected i.p. with 10^5 B16 cells on day 0. TBM (n= 6 per group) received either 120 mg/kg GEM (TBM GEM) or PBS (TBM) i.p. on day 11. PECs were collected on day 14 from naïve mice (n=7), TBM and TBM GEM, stained with FITC-conjugated anti-CD45, PE-conjugated anti-Gr1 and APC-conjugated anti-CD11b, and subjected to flow cytometry. Graphs represent the combined results from two similar experiments. **C, D**: B16 TBM (n=3 or 4 per group) received either 50 mg/kg of 5-FU (TBM 5-FU) or DMSO control (TBM) i.p. on day 7 and 14. PECs were collected on day 16 from naïve mice (n=4), TBM, TBM 5-FU, and subjected to flow cytometry using similar protocol as A, B. The percentage (A, C) and absolute number (B, D) of $CD45^+$ $CD11b^+Gr1^+$cells out of total PECs were calculated. The data are shown as Mean ± SD. * $P < 0.01$.

Effect of GEM and 5-FU on Mϕ function.

We determined next whether GEM and 5-FU treatment *in vivo* affected the functions of Mϕ. Treatment of naïve mice with 5-FU did not significantly affect the ability of Mϕ to suppress proliferation of B16 tumor cells (Fig. 2A) and produce NO (Fig. 2B) *in vitro*. In spite of reducing the number of myeloid cells in TBM as shown in Fig. 1C, 5-FU did not affect the ability of adherent PEC to produce NO (Fig. 2C), and GEM had a similar effect (2D).

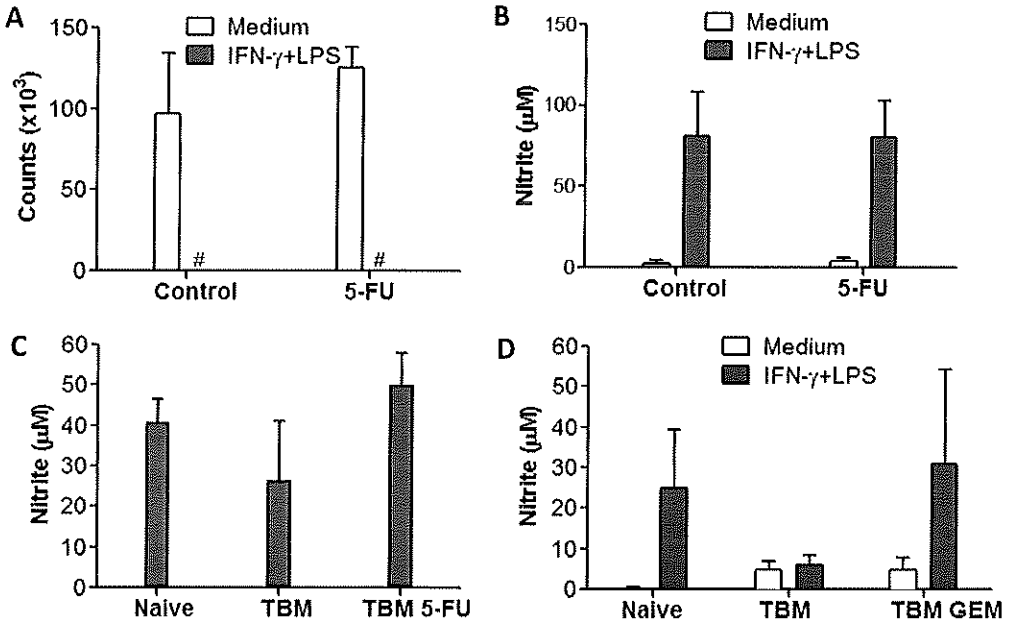

Figure 2. Effect of GEM or 5-FU on Mϕ function. **A, B**: Two groups of mice (n=2 per group) were injected with either 50 mg/kg of 5-FU (5-FU) or DMSO control (Control) i.p. , and PECs were collected 5 days later. Total PECs ($2x10^5$/well) and B16 tumor cells (10^4/well) were placed in 96-well plate with medium or stimulated with IFN-γ (10 U/ml) and LPS (1 ng/ml). **C**: B16 i.p. TBM were injected with either 50 mg/kg of 5-FU (TBM 5-FU) or DMSO control (TBM) i.p. on day 5 and 10. PECs from naïve, TBM and TBM 5-FU (n=3 or 4 per group) were collected on day 14. Total PECs were stimulated with IFN-γ (10 U/ml) and LPS (1 ng/ml). **D**: B16 i.p. TBM were injected with 120 mg/kg of GEM (TBM GEM) or PBS (TBM) i.p. on day 11. Total PECs were collected on day 14 and placed in 96-well plate with medium or IFN-γ (10 U/ml) and LPS (1 ng/ml). All plates were incubated for 48 hours. Counts of B16 cells (A) inhibited by PECs were measured based on thymidine incorporation in tumor cells, and NO activity was determined by nitrite level (B, C, D) in the supernatant. The data are shown as Mean ± SD. # Counts <150.

To more precisely determine the effect of 5-FU on the function of myeloid cells, CD11b+ Gr-1+ PEC from 5-FU or PBS-treated B16 TBM were sorted by Flow cytometry, and their ability to suppress tumor cells and secrete NO following activation *in vitro*, as well as to suppress proliferation of T cells, was compared. Sorted myeloid cells from 5-FU-treated TBM stimulated with IFN-γ and LPS were comparable to the myeloid cells from PBS-treated mice in the ability to suppress tumor cell proliferation (Fig. 3A) and produce NO (Fig. 3B) in vitro. Likewise, MDSC from 5-FU- and PBS-treated TBM suppressed anti-CD3/CD28-induced proliferation of splenic T cells to a similar extend (Fig. 3C), suggesting that 5-FU treatment affected neither effector nor suppressive abilities of B16 tumor-associated MDSC on a cellular basis.

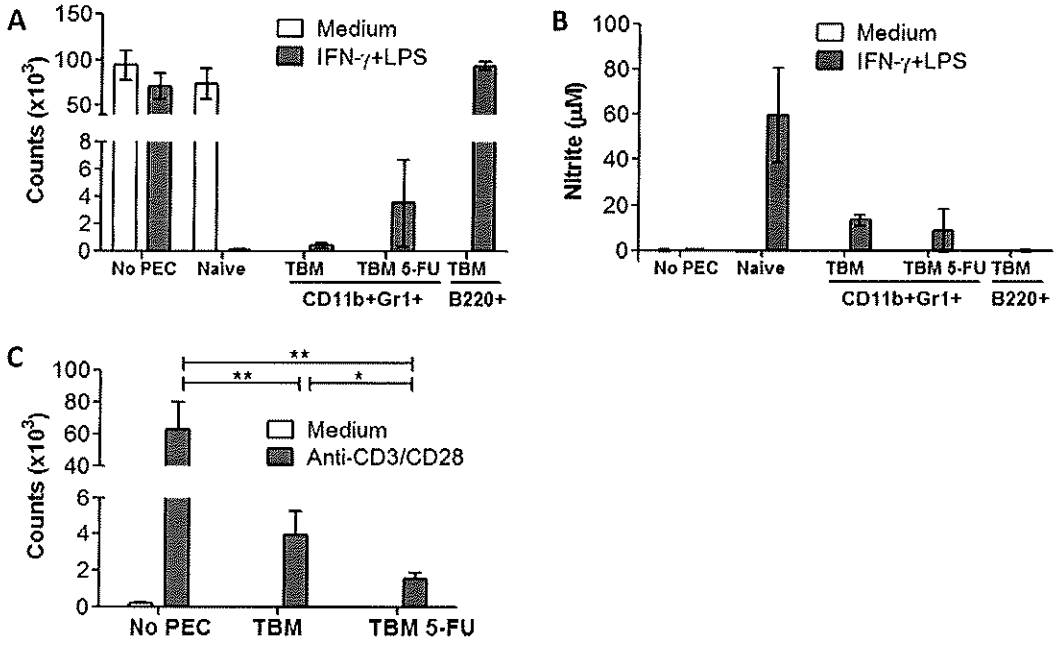

Figure 3.Sorting CD11b+Gr1+ Cells from 5-FU treated mice. C57BL/6 mice were injected i.p. with 10^5 B16 cells on day 0. TBM received either 50 mg/kg of 5-FU (n=9 per group, TBM 5-FU) or DMSO control (n=5 per group, TBM) i.p. on day 5 and 11. PECs were collected on day 14 from all TBM and two naïve mice. **A, B**: PECs were stained with FITC-conjugated anti-B220, PE-conjugated anti-Gr1 and APC-conjugated anti-CD11b. CD11b+Gr1+ cells from TBM 5-FU and TBM, as well as B220+ cells TBM were sorted. In 96-well plate, adherent naïve PECs or sorted PECs (10^5/well) were incubated with B16 cells (10^4/well) in medium or IFN-γ (10 U/ml) and LPS (1 ng/ml) for 48 hours. Counts of B16 cells (A) inhibited by PECs were measured based on thymidine incorporation in tumor cells, and NO activity was determined by nitrite level (B) in the supernatant. Graphs represent the combined results from two similar experiments. **C**: Spleen cells pooled from two naïve mice were placed $2x10^5$/well with sorted PECs ($1x10^5$/well) in medium or anti-CD3 (0.5μg/ml)/anti-CD28 (5μg/ml). Counts of spleen and B16 cells were measure by thymidine incorporation assay 48 hours later. The data are shown as Mean ± SD. * $P < 0.05$; ** $P < 0.01$.

Antitumor effects of GEM and 5-FU in combination with CD40/CpG

To determine if GEM or 5-FU affect the antitumor responses of anti-CD40/CpG *in vivo*, we first determined the sensitivity of B16 melanoma cells to GEN and 5-FU *in vitro*. Each of these chemotherapeutic drugs mediated dose-dependent inhibition of B16 cell proliferation *in vitro* (data not shown).

Then we tested the effects of chemotherapy with GEM and 5-FU *in vivo*, separately and in combination with anti-CD40/CpG immunotherapy. In the subcutaneous B16 tumor model, GEM alone was not effective, whereas anti-CD40/CpG treatment reduced tumor growth. A combined treatment with GEM followed by anti-CD40/CpG resulted in the antitumor effect similar to this of anti-CD40/CpG alone (Fig. 4A). Similarly, there was no beneficial effect of combining 5-FU with anti-CD40/CpG, although 5-FU alone, in contrast to GEM, slowed tumor growth (Fig. 4B). This combined treatment also was as effective as the immunotherapy alone in the i.p. B16 melanoma model (Fig. 4C).

To confirm the effect of MDSC depletion obtained with 5-FU on the antitumor effects of anti-CD40/CpG , mice bearing s.c. B16 melanoma were treated with anti-Gr-1 mAb, RB6, which were shown to deplete Gr-1^+ cells *in vivo* [22, 23, 24]. The flow cytometric analysis confirmed the depletion of $CD11b^+Gr\text{-}1^+Ly6G^+$ cells in RB6-injected mice (data not show). Reduction of MDSC by anti-GR-1 mAb treatment did not significantly inhibit B16 tumor growth, in contrast to 5-FU, and did not significantly affect anti-CD40/CpG antitumor responses similar to the effects of 5-FU and GEM treatments (Fig. 4D).

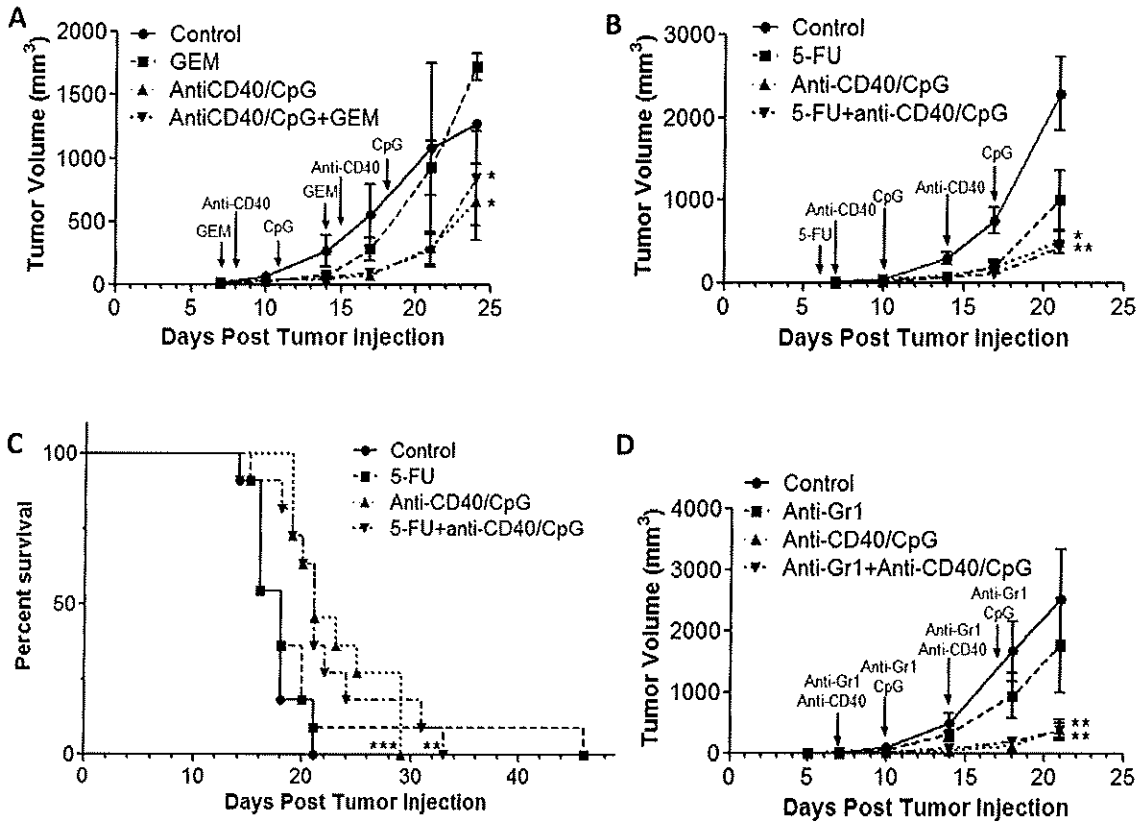

Figure 4. Antitumor effect of GEM, 5-FU or Gr-1+ cell depletion in combination with anti-CD40/CpG *in vivo*. C57BL/6 mice were injected s.c. (A, B, D) or i.p. (C) with 10^5 B16 cells on day 0. **A:** TBM (n=4 or 5 per group) had no treatment or were treated with GEM i.p. on days 7 and 14, anti-CD40/CpG i.p. on days 8/11 and 15/18, or a combination of GEM and anti-CD40/CpG. **B**: TBM (n=6 or 7 per group) had no treatment or were treated with 5-FU i.p. on day 6, anti-CD40/CpG i.p. on days 7/10 and 14/17, or a combination of 5-FU and anti-CD40/CpG. **C**: TBM (n=11 per group) had no treatment or were treated with 5-FU i.p. on days 5 and 15, anti-CD40/CpG i.p. on day 9/12, or a combination of 5-FU and anti-CD40/CpG. The graph represents combination of two similar experiments. **D**: C57BL/6 mice were injected s.c. with 10^5 B16 cells on day 0. TBM (n=6 or 7 per group) had no treatment or were treated with 0.2mg of anti-Gr-1 i.t. on days 7, 10, 14 and 17, anti-CD40/CpG i.p. on days 7/10 and 14/17, or a combination of anti-Gr-1 and anti-CD40/CpG. Means ± SE of tumors volumes (A, B, D) are presented. Control mice received DMSO control for 5-FU, PBS, or Rat IgG. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for control group versus any other group. Arrows indicate treatment schedule.

Discussion

Combinatory approaches using chemotherapy and immunotherapy have showed antitumor synergy in experimental studies and have been increasingly implemented in the clinic [25, 26]. These approaches mainly focus on the facilitation of T cell-mediated antitumor responses [25]. We have recently showed that chemotherapy and immunotherapy can also synergize in activating innate immunity to combat experimental cancer. Thus, chemotherapy with CY [14] or CY in combination with doxorubicin and vincristine [15] can synergize with anti-CD40/CpG in inducing antitumor effects by activated Mϕ.

In a separate study we showed that not only Mϕ but other myeloid cells expressing CD11b and Gr-1 markers can be activated in TBM to mediate antitumor effects [17]. As these markers characterize MDSC which substantially increase with tumor progression in tumor-bearing hosts [20], we thought to determine the role of these cells in the antitumor effects induced by anti-CD40/CpG. On one hand, these immature myeloid cells could secrete NO, one of the effector molecules induced by CD40 ligation [27], and potentially be activated with anti-CD40/CpG to mediate antitumor effects. On the other hand, MDSC can polarize Mϕ to M2 phenotype inhibiting M1 effector responses [28], and thereby could inhibit Mϕ activation with anti-CD40/CpG. Therefore, it was possible that reducing MDSC in TBM would promote M1 Mϕ phenotype resulting in better antitumor effects in response to anti-CD40/CpG.

To reduce MDSC *in vivo* we used three published approaches, namely treatments with GEM, 5-FU and anti-Gr-1 mAb. GEM was found to selectively reduce the number of MDSC in several mouse tumor models [18, 21, 28]. Another chemotherapeutic agent, 5-FU, was side-by-side

compared with GEM and found to be even more effective in depleting MDSC [19]. Anti-Gr-1 mAb was shown to reduce the number of two MDSC populations: Gr-1$^+$ neutrophils and monocytes [24].

In our experiments, treatment with the GEM regimen used in other studies (120 mg/kg) did not reduce the percentage or number of MDSC in the i.p. B16 tumor model. As published data on GEM-induced MDSC reduction do not describe the B16 melanoma model, it remains to be determined if GEM is more effective in reducing MDSC in some tumor models than others. GEM treatment did not enhance or suppress the antitumor effect of anti-CD40/CpG in the s.c. B16 tumor model. This result is in keeping with the findings by Beatty et al. who showed, using a model of spontaneous pancreatic cancer in mice, that GEM did not enhance the antitumor effect of anti-CD40 [11]. Our results indicate that reducing the number of MDSC by 5-FU or anti-Gr-1 mAb neither enhances the ability of Mϕ to be activated in vitro nor augments the antitumor effects of anti-CD40/CpG.

Our results suggest that the increased numbers of myeloid cells following certain chemotherapy regimens correlate with the increased antitumor effects of the combination of these chemotherapy regimens and anti-CD40/CpG immunotherapy. Thus, CY and/or Doxorubicin increase levels of myeloid cells [29]. In our studies, chemotherapy regiments with CY alone or in combination with Doxorubicin and Vincristine enhanced the antitumor effects of anti-CD40/CpG immunotherapy [14, 15]. It is possible, therefore, that some myeloid cells induced by the tumor, chemotherapy or both are activated by anti-CD40/CpG. The lack of reduction of the antitumor effect of anti-CD40/CpG by reducing the number of myeloid cells in the current study can be explained by the existence of two or more independent effector mechanisms. Thus, in a model of spontaneous tumor regression where NK cells, Mϕ and neutrophils were each independently involved in the resistance, depletion of one or two these cell subsets did not

reduce tumor resistance [30]. It is also possible that 5-FU or anti-Gr-1 mAb depletes functionally opposing populations of myeloid cells. Thus, tumor-induced myeloid cells can be activated to become antitumor effectors [7], but also can suppress M1 Mϕ [28]. Therefore, reduction of both these cell populations does not reduce the resulting antitumor effect of anti-CD40/CpG immunotherapy.

In summary, the findings presented here suggest that partial MDSC depletion neither enhances nor interferes with the *in vivo* antitumor effects induced against B16 tumors in C57BL/6 mice by anti-CD40/CpG.

References

1. Mitchell, M. S. 2003. Combinations of anticancer drugs and immunotherapy. *Cancer Immunol Immunother.* 52: 686-692.

2. North, R. J. 1982. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. *J Exp Med.* 155: 1063-1074.

3. Lutsiak, M. E., Semnani, R. T., De, P. R., *et al.* 2005. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood.* 105: 2862-2868.

4. Solomayer, E. F., Feuerer, M., Bai, L., *et al.* 2003. Influence of adjuvant hormone therapy and chemotherapy on the immune system analysed in the bone marrow of patients with breast cancer. *Clin Cancer Res.* 9: 174-180.

5. Fonsatti, E., Maio, M., Altomonte, M. and Hersey, P. 2010. Biology and clinical application of CD40 in cancer treatment. *Semin Oncol.* 37(5): 517-523

6. Khong, A., Nelson, D. J., Nowak, A. K., Lake, R. A. and Robinson, B. W. 2012. The use of agonistic anti-CD40 therapy in treatments for cancer. *Int Rev Immunol.* 31(4): 246-266

7. Rakhmilevich A.L., Alderson K.L., Sondel P.M. T cell-independent antitumor effects of CD40 ligation. Review. *International Reviews in Immunology*, 2012, 31:267-278.

8. Vonderheide, R. H., Flaherty, K. T., Khalil, M. *et al.* 2007. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol.* 25(7):876-883

9. Advani, R., Forero-Torres, A., Furman, R. R. *et al.* 2009. Phase I study of the humanized anti-CD40 monoclonal antibody dacetuzumab in refractory or recurrent non-Hodgkin's lymphoma. *J Clin Oncol.* 27(26):4371-4377

10. Hussein, M., Berenson, J. R., Niesvizky, R. *et al.* 2010. A phase I multidose study of dacetuzumab (SGN-40; humanized anti-CD40 monoclonal antibody) in patients with multiple myeloma. *Haematologica.* 95(5):845-848

11. Beatty, G. L., Chiorean, E. G., Fishman, M. P., *et al.* 2011. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science.* 331(6024):1612-1616

12. Buhtoiarov I.N., Lum H., Berke G., Paulnock D., Sondel P.M., and Rakhmilevich A.L. 2005. CD40 ligation induces antitumor reactivity of murine macrophages via an IFN gamma-dependent mechanism. *Journal of Immunology*, 174: 6013-6022.

13. Buhtoiarov, I. N., Lum, H. D., Berke, G., *et al.* 2006. Synergistic Activation of Macrophages via CD40 and TLR9 results in T cell independent antitumor effects. *J Immunol.* 176: 309-318.

14. Johnson, E. E., Buhtoiarov, I. N., Baldeshwiler, M. J., *et al.* 2011. Enhanced T-cell-independent antitumor effect of cyclophosphamide combined with anti-CD40 mAb and CpG in mice. *J Immunother.* 34(1):76-84

15. Buhtoiarov, I. N., Sondel, P. M., Wigginton, J. M. *et al.* 2011. Anti-tumour synergy of cytotoxic chemotherapy and anti-CD40 plus CpG-ODN immunotherapy through repolarization of tumour-associated macrophages. *Immunology.* 132 (2): 226-39

16. Lum, H.D., Buhtoiarov, I. N., Berke, G., Paulnock, D. M., Sondel, P. M. and Rakhmilevich, A. L. 2006. In vivo CD40 ligation can induce T cell-independent antitumor effects that involve macrophages. *Journal of Leukocyte Biology.* 79:1181-1192

17. Rakhmilevich, A. L., Baldeshwiler, M. J., Van De Voort, T. J., *et al.* 2012, Tumor-associated myeloid cells can be activated *in vitro* and *in vivo* to mediate antitumor effects. *Cancer Immunology Immunotherapy.* 2012; 61:1683–1697

18. Le, H. K., Graham, L., Cha, E. *et al.* 2009. Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice. *International immunopharmacology.* 9: 900-909.

19. Vincent, J., Mignot, G., Chalmin, F. *et al.* 2010. 5-fluoruracil selectively kills tumor-associated myeloid- derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res.* 70(8): 3052-3061.

20. Gabrilovich, D. I. and Nagaraj, S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nature Reviews.* 9: 162-174.

21. Suzuki, E., Kapoor, V., Jassar, A. S., Kaiser, L. R. and Albelda, S. M. 2005. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res.* 11(18): 6713-6721

22. Rakhmilevich A.L. 1995. Neutrophils are essential for resolution of primary and secondary infection with *Listeria monocytogenes*. *J. Leuk. Biol.* 57, 827-831

23. Daley, J. M., Thomay, A. A., Connolly, M. D., Reichner, J. S. and Albina, J. E. 2008. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol.* 83(1): 64-70

24. Fortin, C., Huang, X. and Yang, Y. 2012. NK cell response to vaccinia virus is regulated by myeloid-derived suppressor cells. *J Immunol.* 189(4): 1843-1849

25. Zhang, T. and Herlyn, D. 2009. Combination of active specific immunotherapy or adoptive antibody or lymphocyte immunotherapy with chemotherapy in the treatment of cancer. *Cancer Immunology Immunotherapy.* 58(4): 475-492

26. Moschella, F., Proietti, E., Capone, I. and Belardelli, F. 2010. Combination strategies for enhancing the efficacy of immunotherapy in cancer patients. *Ann N Y Acad Sci.* 1194: 169-178

27. Lum, H. D., Buhtoiarov, I. N., Schmidt, B. E., Berke, G., Paulnock, D. M., Sondel, P. M., and Rakhmilevich, A. L. 2006. Tumoristatic effects of anti-CD40 mAb-activated macrophages involve nitric oxide and tumor-necrosis factor-α. *Immunol.* 118: 261-270

28. Sinha, P., Clements, V. K., Bunt, S. K., Albelda, S. M. and Ostrand-Rosenberg, S. 2007. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J Immunol* 179: 977-983.

29. Diaz-Montero, C. M., Salem, M. L., Nishimura, M. I., Garrett-Mayer, E., Cole, D. J. and Montero, A. J. 2009. Increased circulating myeloid-derived suppressor cells correlate with

clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunology Immunotherapy.* 58(1): 49-59

30. Hicks, A. M., Riedlinger, G., Willingham, M. C. et al. 2006. Transferable anticancer innate immunity in spontaneous regression/complete resistance mice. *Proc Natl Acad Sci U S A.* 103(20):7753-7758