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

Xiaoyi Qu[*], **Mildred A.R. Felder**[†], **Zulmarie Perez Horta**[*], **Paul M. Sondel**[*,§,‡], and **Alexander L. Rakhmilevich**[*,‡]

[*]University of Wisconsin, Department of Human Oncology, Madison, WI, USA

[†]University of Wisconsin, Department of Obstetrics and Gynecology, Madison, WI, USA

[§]University of Wisconsin, Department of Pediatrics, Madison, WI, USA

[‡]Paul P. Carbone Comprehensive Cancer Center, Madison, WI, USA

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 enhance the antitumor effect of anti-CD40 and CpG. Using the model of B16 melanoma growing intraperitoneally in syngeneic C57BL/6 mice, we show that these GEM or 5-FU treatment regimens reduced MDSC in the peritoneal cavity of tumor-bearing mice. Treatment of mice with 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-Gr-1 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.

Keywords

anti-CD40; CpG; gemcitabine; 5-fluorouracil; immunotherapy

Introduction

Cytotoxic chemotherapy is effective treatment for some types of cancers; however, tumor cells often become drug-resistant, enabling many cancers to recur or progress. Combining chemotherapy with immunotherapy has been increasingly used in clinical practice to

Address correspondence and reprint requests to: Dr. Alexander L. Rakhmilevich, University of Wisconsin-Madison, Department of Human Oncology, 4136 WIMR, 1111 Highland Avenue, Madison, WI 53705. Phone: (608)263-5193; Fax: (608)263-4226; rakhmil@humonc.wisc.edu.

improve the clinical outcome [1]. Many experimental studies have shown that certain chemotherapeutic drugs such as cyclophosphamide (CY) given at low doses facilitate activation of antitumor T cells by depleting T suppressor (regulatory) cells [2,3]. Although many chemotherapeutics are immunosuppressive, certain cells of the innate immune system, particularly monocytes and macrophages (Mϕ), are more resistant to chemotherapy than T cells in cancer patients [4]. Therefore, we hypothesize that chemotherapy will not prevent the efficacy of an antitumor immunotherapy designed to activate monocytes or Mϕ and may even enhance it.

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 testing as a cancer treatment [8–11]. We have previously shown in mouse models 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 was observed with either CY alone or anti-CD40/CpG [14]. Even multidrug chemotherapy consisting of vincristine, CY and doxorubicin, while suppressing the functions of T cells and NK cells, 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 [12–15], CY alone and in combination with vincristine and doxorubicin induced expansion of myeloid cells and synergized with anti-CD40/CpG [14, 15]. In contrast, other chemotherapeutic drugs, such as gemcitabine (GEM) and 5-fluorouracil (5-FU), with different mechanisms 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) and inhibit aspects of immune function [20], in this study we asked whether the reduction of myeloid cells with the same GEM or 5-FU therapy regimens would enhance the antitumor effects of anti-CD40/CpG.

Material and 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-FU was dissolved in DMSO (both from Sigma Chemical, St Louis, MO) at 50 mg/ml. GEM-HCl (Eli Lilly and Company, Indianapolis, IN) was obtained from the UWHospital 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 on day 7–9 after tumor implantation; 50μg CpG were injected i.p. three days after anti-CD40 injection (all i.p. injections were given in 0.5 PBS). Anti-Gr-1 (clone RB6-8C5) was injected intratumorally (i.t.) (0.2 mg in 0.1 PBS) on the same days as anti-CD40 (days 7 and 14) and CpG (days 10 and 17). 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 (following tumor implantation) 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 length x tumor $width^2$)/2.

Activation of Mφ

Peritoneal cells (PEC) were obtained via 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 PEC were lysed by hypotonic shock. Collected PEC 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 DNA synthesis in tumor cells. Briefly, adherent PEC 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 ^{3}H-TdR (1 μCi/well) during the last 6 h of incubation. ^{3}H-TdR-incorporation was determined by β-scintillation of total cells harvested from the cell cultures onto glass fiber filters (Packard, Meriden, CT), using the Packard Matrix 9600 Direct β-counter (Packard, Meriden, CT). In this assay proliferation of B16 cells is >100 fold higher than that of PEC [12]. 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.

Splenic T-cell proliferation assay

Splenocytes were prepared from the spleens pooled from two C57BL/6 mice by processing the spleens to a single-cell suspension, followed by lysis of erythrocytes by hypotonic shock. Two hundred thousand spleen cells were stimulated with 0.5μg/ml of monoclonal anti-CD3 and 5μg/ml of anti-CD28 (both from eBioscience, San Diego, CA) and cultured with sorted PEC in complete media in flat-bottomed 96-well plates for 72 hr. Cells were pulsed with ^{3}H-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-Gr-1, APC-conjugated anti-CD11b, or FITC-conjugated anti-B220 (all from eBioscience, San Diego, CA) for 40 min at 4 °C. Isotype-matched control rat IgG FITC, IgG APC and IgG PE, purchased from eBioscience or BD Pharmingen, were used as background controls. Cells were washed in ice-cold PBS supplemented with 0.5% FCS (flow buffer), subjected to flow cytometry using either a FACSCalibur flow cytometer or a MACSQuant Analyzer, and analyzed with FlowJo software (Ashland, OR). To calculate the absolute number of a PEC subset, the absolute number of total PEC (obtained via counting the viable cells on a haemocytometer) was multiplied by the percentage of that subset (%) obtained via flow cytometry analysis. Data were collected for 10,000 live events per sample.

Statistical Analysis

A two-tailed Student's t-test was used to determine significant differences between groups within one experiment. Differences in the mean tumor growth rate of treatment groups were determined by fitting the tumor growth curve of each mouse to an exponential curve using the equation for exponential growth ($V_t = V_o e^{rt}$), where V_o is tumor volume, t is time and the rate constant r is the parameter taken to describe the overall tumor growth rate for each mouse. The parameter V_o was calculated to be the same for all mice within an experiment. The group mean r was compared between groups using a 2-tailed Student t-test. Survival data were analyzed with the Log-rank test. For all tests, $P < 0.05$ was considered statistically significant. Statistical analyses of nonlinear fit curves and Log-rank tests were performed using the GraphPad Prism 5.01 software. For all figures, * = $P<0.05$, ** = $P < 0.01$, *** = $P<0.001$, NS: =Non-significant.

Results

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

It has been reported that GEM treatment (120 mg/kg) reduced the number of tumor-induced MDSC in several tumor models [18, 19, 21]. To determine whether GEM is effective in reducing the number of MDSC 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. Peritoneal cells (PEC) were collected on day 14 and evaluated by flow cytometry for $CD11b^+$ $Gr\text{-}1^+$ cells to assess the number of MDSC. We recognize that different criteria and different markers have been used by different labs, and in different models, to characterize MDSC. As most analyses of MDSC in TBM show the MDSC are $CD11b^+$ $Gr\text{-}1^+$ cells [18–21], we are using this phenotypic definition of MDSC in this report; namely, we will refer to $CD11b^+$ $Gr\text{-}1^+$ cells as MDSC while recognizing that not all cells with this phenotype will necessarily function as MDSC. The results in Figs. 1A and 1B show that the percentage and number of $CD11b^+$ $Gr\text{-}1^+$ PEC were increased in TBM compared to naïve mice ($p = 0.0005$ and 0.0325, respectively). When the GEM treatment regimen was given to TBM mice, the percentage of $CD11b^+$ $Gr\text{-}1^+$ cells in the peritoneal cavity was significantly reduced compared to the TBM mice not receiving GEM ($p=0.0037$, Fig. 1A), whereas the reduction in absolute number of MDSC was noticeable, but did not reach statistical significance ($p=0.089$, Fig. 1B).

In parallel to GEM we tested another chemotherapeutic drug, 5-FU, reported to be more effective than GEM in depleting MDSC [19]. The results in Figure 1C and 1D show that treatment of B16 TBM with 5-FU resulted in statistically significant reduction of the relative and absolute numbers of $CD11b^+$ $Gr\text{-}1^+$ PEC. Neither GEM nor 5-FU treatment affected number of $CD11b^+$ $Gr\text{-}1^+$ spleen cells in mice bearing i.p. B16 tumors (data not shown). In a separate experiment, MDSC were similarly reduced when 5-FU was given to TBM once, 2 days before collecting PEC, or twice, 9 days and 2 days before collecting PEC (data not shown).

Effect of GEM and 5-FU on Mφ function

We determined next whether the GEM and 5-FU treatments *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 (p=0.56, Fig. 2A) or produce NO (p=0.95, Fig. 2B) *in vitro*. Although it reduced the number of myeloid cells in TBM as shown in Fig. 1C, 5-FU did not significantly affect the ability of adherent PEC to produce NO (p=0.1, Fig. 2C). Similarly, in vivo administration of GEM to TBM did not cause any significant change in NO production by PEC (stimulated with IFNγ + LPS) (p=0.101, Fig. 2D).

To more precisely determine the effect of 5-FU on the function of myeloid cells, $CD11b^+$ $Gr\text{-}1^+$ 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. In two experiments, sorted myeloid cells from both untreated and 5-FU treated TBM substantially suppressed B16 proliferation (99.4 and 95.0% suppression, respectively) and induced similar moderate levels of NO production. As a control, sorted $B220^+$ (B) cells from these same preparations were added to the tumor cells instead of the sorted myeloid cells, and, as expected, showed no significant inhibition of tumor cell proliferation (P= 0.071, Fig. 3A) and no production of NO (Fig. 3B). Purified $CD11b^+$ $Gr\text{-}1^+$ PEC from TBM caused substantial suppression of anti-CD3/CD28-induced proliferation of splenic T cells (Fig. 3C). The comparable MDSC from 5-FU treated TBM also showed substantial suppression of CD3/CD28 induced T cell proliferation ($p<0.01$), and were slightly more suppressive than those from the TBM not receiving 5-FU (97.4 vs. 94.9 % suppression, respectively, $p < 0.05$). These data would suggest that *in vivo* 5-FU treatment reduces the numbers of $CD11b^+$ $Gr\text{-}1^+$ cells (Fig. 1C–D), but does not keep the remaining $CD11b^+$ $Gr\text{-}1^+$ cells from suppressing the T cell proliferative response (Fig. 3C)

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

To determine if GEM or 5-FU affects the immune mediated antitumor responses of anti-CD40/CpG *in vivo*, we first determined the sensitivity of B16 melanoma cells to GEM 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 (Fig. 4A). Combined treatment with GEM followed by anti-CD40/CpG resulted in an antitumor effect similar to that of anti-CD40/CpG alone (Fig. 4A). Similarly, combining 5-FU with anti-CD40/CpG did not significantly modify the antitumor effect of anti-CD40/CpG alone against s.c. B16 tumors (Fig. 4B); treatment with 5-FU alone showed a trend toward tumor growth inhibition, but this was not statistically significant (p=0.11, Fig. 4B). In addition, combined treatment with 5-FU and anti-CD40/CpG was not significantly different from the immunotherapy alone in the i.p. B16 melanoma model (Fig. 4C).

To further test the potential influence of MDSC depletion on anti-CD40/CpG therapy, mice bearing s.c. B16 melanoma were treated i.p. with anti-Gr-1 mAb, RB6, an approach shown previously to deplete Gr-1$^+$ cells *in vivo* [22–24]. Flow cytometric analysis confirmed a significant depletion of CD11b$^+$Gr-1$^+$ PEC in RB6-injected TBM compared with Rat IgG-treated TBM (3.14 ± 0.7% vs. 40.13 ± 4.08%, respectively; P<0.005). Reduction of MDSC by anti-Gr-1 mAb treatment did not significantly inhibit B16 tumor growth, and did not significantly affect anti-CD40/CpG antitumor responses (Fig. 4D); this was comparable to the results of our studies in mice receiving 5-FU (Figs. 4B–C) or GEM treatments (Fig. 4A). Similar to i.p injections, intra-tumoral injections of anti-Gr-1 mAb did not influence the antitumor effect of anti-CD40/CpG against s.c. B16 melanoma (data not shown).

Discussion

Some combinatory approaches using chemotherapy and immunotherapy have showed antitumor synergy in experimental studies and have been increasingly implemented in the clinic [25, 26]. These immunotherapy approaches primarily focus on the facilitation of T cell-mediated antitumor responses [25]. We have recently shown that chemotherapy and immunotherapy can also synergize in activating innate immunity to combat cancer in TBM. 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 CD11b and Gr-1 markers also characterize MDSC, which substantially increase with tumor progression in tumor-bearing hosts [20], we thought to determine the role of these CD11b$^+$Gr-1$^+$ cells in the antitumor effects induced by anti-CD40/CpG. We hypothesized two possible and opposing outcomes of depleting CD11b$^+$ Gr-1$^+$ cells. First, these immature myeloid cells might secrete NO, one of the effector molecules induced by CD40 ligation [27], and potentially be activated with anti-CD40/CpG to mediate antitumor effects. In this case, reducing the numbers of CD11b$^+$ Gr-1$^+$ cells could inhibit the antitumor effects of anti-CD40/CpG. Alternatively, CD11b$^+$ Gr-1$^+$ MDSC can polarize Mϕ to the M2 phenotype, inhibiting M1 effector responses [28], and thus could inhibit Mϕ activation with anti-CD40/CpG. If the second condition were dominant, reducing MDSC in TBM would likely promote the M1 Mϕ phenotype, resulting in better antitumor effects in response to anti-CD40/CpG. Although MDSC are known to inhibit T cell function [20], their suppression of tumor-specific T cell immunity should not play a significant role in our findings as the antitumor effect of anti-CD40/CpG against B16 melanoma was shown to be T cell-independent [7, 13–15].

To reduce MDSC *in vivo* we used three separate published approaches, namely treating with GEM, 5-FU or 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 compared with GEM and found to be more effective in depleting MDSC [19]. Anti-Gr-1 mAb has been 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) significantly reduced the percentage, and to a lesser degree the number, of MDSC in the i.p. B16 tumor model. As published data on GEM-induced MDSC reduction in TBM have not evaluated the GEM-induced MDSC reduction in the B16 melanoma model, it remains to be determined whether GEM is more effective in reducing MDSC in some murine tumor models than in others. GEM treatment did not significantly enhance or suppress the antitumor effect of anti-CD40/CpG in the s.c. B16 tumor model (Fig. 4A). This result is in

keeping with the findings from Beatty et al., who used a model of spontaneous pancreatic cancer in mice to show that GEM did not enhance the antitumor effect of anti-CD40 [11]. Similar to GEM, 5-FU (Fig. 1C–D) reduced MDSC in the i.p. B16 tumor model. Our results indicate that reducing the number of MDSC by GEM or 5-FU neither enhances nor inhibits the ability of Mϕ from these mice to be activated *in vitro* by co-culture with IFNγ and LPS (Fig. 2A–D). Furthermore, the residual $CD11b^+$ $Gr\text{-}1^+$ cells from 5-FU treated mice retain their ability to suppress T cell proliferation (Fig. 3C). Finally, reducing the number of MDSC by 5-FU or anti-Gr-1 mAb does not augment or inhibit the in vivo antitumor effects of anti-CD40/CpG (Fig. 4A–D).

Our previous findings suggest that the increased number of myeloid cells following certain chemotherapy regimens may be associated with the increased antitumor effects of the combination of these chemotherapy regimens and anti-CD40/CpG immunotherapy. Thus, since CY and/or doxorubicin increase levels of myeloid cells [29], they could enhance the efficacy of anti-CD40/CpG treatment. In our studies, chemotherapy regimens with CY alone or in combination with doxorubicin and vincristine did indeed enhance the antitumor effects of anti-CD40/CpG immunotherapy against B16 melanoma [14, 15]. It is possible, therefore, that some myeloid cells induced by the tumor, chemotherapy or both are activated by anti-CD40/CpG to induce antitumor activity. This antitumor effect by myeloid cells might potentially balance the inhibitory effects demonstrated by MDSC. In other words, we hypothesized that the depletion of MDSC might blunt some of their immunosuppressive effects, thereby enabling immunotherapy to be more effective. However, we did not observe this hypothesized result. The fact that we did not observe any detectible change in the antitumor effect of anti-CD40/CpG by reducing the number of myeloid cells in the current study might be explained by the existence of two or more concurrent independent myeloid mechanisms. That is to say, it is possible that 5-FU or anti-Gr-1 mAb depletes myeloid cells that have opposite functions: antitumor effectors as well as immunosuppressive MDSC, with the net effect being that both functions are cancelled out. Tumor-induced myeloid cells might be activated to become antitumor effectors [7], but they can also suppress M1 Mϕ [28]. Therefore, reduction of both these cell populations might not modify the resulting antitumor effect of anti-CD40/CpG immunotherapy. Another possibility may be that influencing the magnitude of the *in vivo* antitumor effect requires a more complete *in vivo* depletion of MDSC than we were able to achieve in this model with GEM, 5-FU or anti Gr-1 mAb. Alternatively, activated MDSC may kill tumor cells in parallel with other effector populations that must also be simultaneously depleted in order to inhibit the detected antitumor effect. This would be analogous to studies 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 of these cell subsets did not reduce resistance to tumor growth, while depletion of all 3 subsets interfered with the protective antitumor effect [30].

In summary, the findings presented here suggest that GEM or 5-FU does not enhance the antitumor effects of anti-CD40/CpG, and 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.

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Highlights

- 5-fluorouracil (5-FU) or gemcitabine (GEM) reduced the number of tumor-associated myeloid cells.
- 5-FU or GEM did not affect macrophage functions.
- Chemotherapy with 5-FU or GEM did not affect the antitumor effect of anti-CD40 and CpG-ODN in mice.

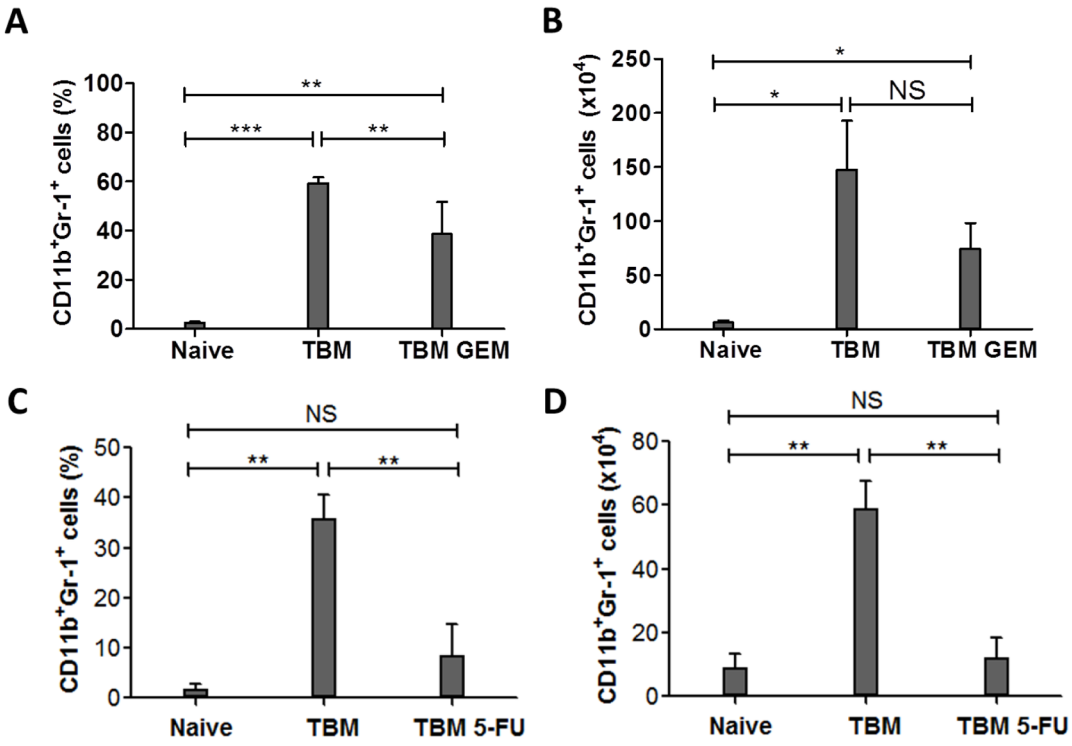

Figure 1.

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. On day 14, PEC were collected from TBM, TBM GEM and naïve mice (n=3–4 mice per group), stained with FITC-conjugated anti-CD45, PE-conjugated anti-Gr-1 and APC-conjugated anti-CD11b, and subjected to flow cytometry. Graphs A and B represent the results of one out of three 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 TBM, TBM 5-FU and naïve mice (n=4), and subjected to flow cytometry using a similar protocol as A, B. Graphs C and D represent the results of one out of two similar experiments. The percentage (**A**, **C**) and absolute number (**B**, **D**) of $CD45^+$ $CD11b^+Gr\text{-}1^+$ cells out of total PECs were calculated. The data are shown as Mean ± SD. * P<0.05, ** P < 0.01, *** P<0.001, NS: Non-significant.

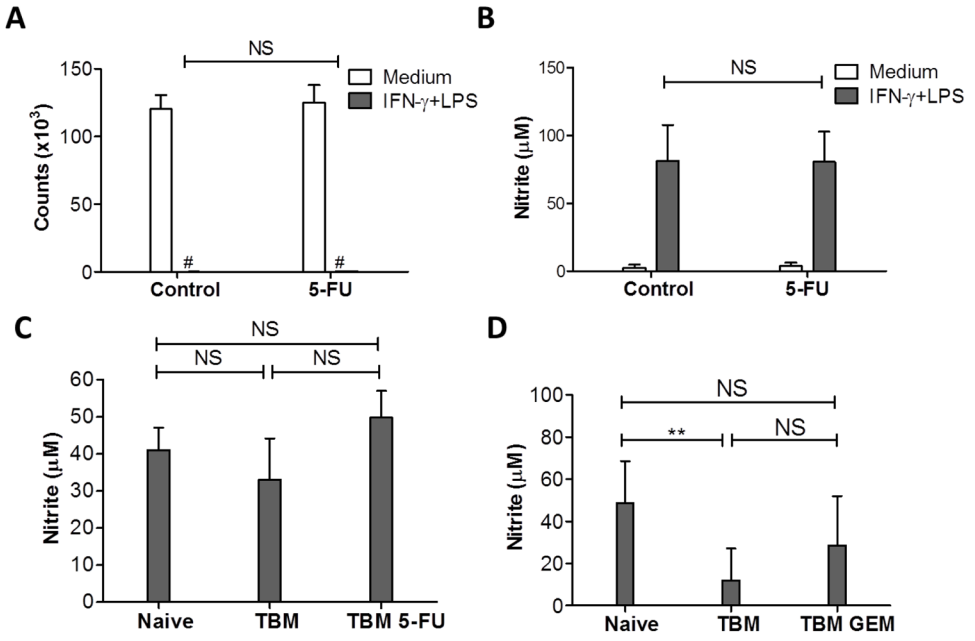

Figure 2.

Effect of GEM or 5-FU on Mϕ function. **A, B**: Two groups of naïve C57BL/6 mice (n=2 per group) were injected with either 50 mg/kg of 5-FU (5-FU) or DMSO (Control) i.p., and PEC were collected 5 days later. Total PEC ($2x10^5$/well) and B16 tumor cells (10^4/well) were placed in 96-well plates with medium or stimulated with IFN-γ (10 U/ml) and LPS (1 ng/ml). **C**: C57BL/6 mice were injected with B16 cells i.p. on day 0, and injected with either 50 mg/kg of 5-FU (TBM 5-FU) or DMSO (TBM, control) i.p. on days 5 and 10. PECs from naïve, TBM and TBM 5-FU (n=3–4 per group) were collected on day 14. Total PEC were stimulated with IFN-γ (10 U/ml) and LPS (1 ng/ml). The results of one out of two similar experiments are shown. **D:** B16 i.p. TBM were injected with 120 mg/kg of GEM (TBM GEM) or PBS (TBM) i.p. on day 11. Total PEC were collected on day 14 and placed in 96-well plates with medium or IFN-γ (10 U/ml) and LPS (1 ng/ml). All plates were incubated for 48 hours. **D** shows a combined graph of three similar experiments (8–9 mice per group). Counts of B16 cells were measured based on thymidine incorporation (**A**), and NO activity was determined by nitrite level in the supernatants (**B, C, D**). The data are shown as Mean ± SD. # Counts <150. NS: Non-significant.

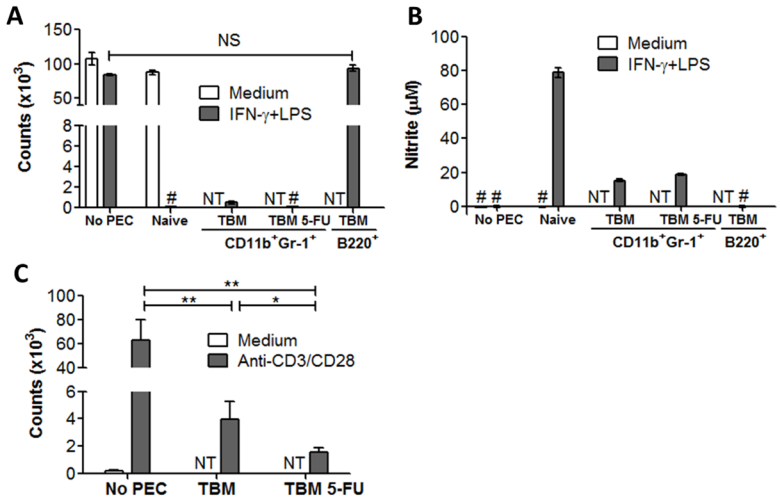

Figure 3.

Sorting CD11b$^+$Gr-1$^+$ 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 days 5 and 11. PEC were collected on day 14 from all TBM and two naïve mice. **A, B**: PEC were stained with FITC-conjugated anti-B220, PE-conjugated anti-Gr-1 and APC-conjugated anti-CD11b. CD11b$^+$Gr-1$^+$ cells from TBM 5-FU and TBM, as well as B220$^+$ cells from TBM were sorted. In 96-well plates, adherent naïve PEC or sorted PEC (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 PEC were measured based on thymidine incorporation in tumor cells, and NO activity was determined by nitrite level (B) in the supernatants. Results in panel A and B are representative of two independent experiments. **C**: Spleen cells pooled from two naïve mice were placed 2x10^5/well with sorted PEC (1x10^5/well) in medium or anti-CD3 (0.5μg/ml)/ anti-CD28 (5μg/ml). PEC were obtained from TBM treated with 5-FU (TBM-5-FU) or without treatment (TBM). Counts of spleen cells were measured by thymidine incorporation assay 48 hours later. Panel C represents the combined results from two similar experiments. The data are shown as Mean ± SD. * $P < 0.05$, ** $P < 0.01$. # counts < 150 or nitrite level < 1μM. NS: Non-significant. NT: Not tested.

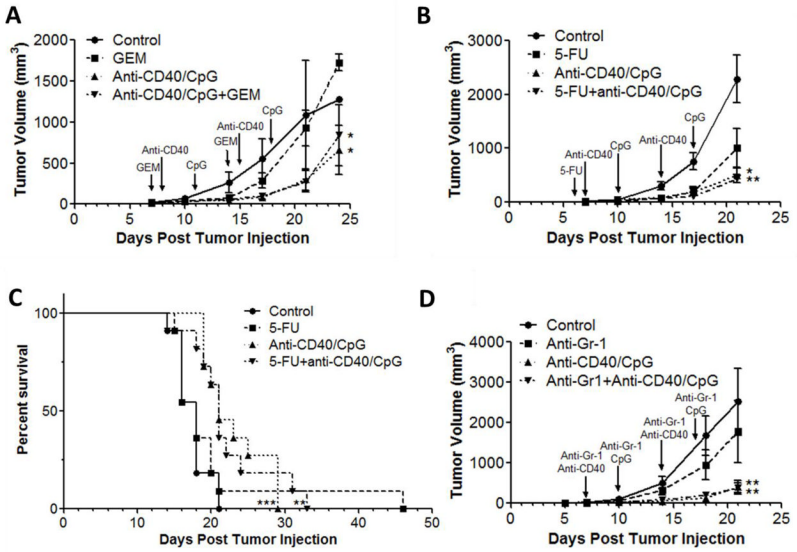

Figure 4.

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 (control) 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 the 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. Panels A, C and D represent single experiments, while B represents two similar experiments. Means ± SE of tumors volumes (A, B, D) are presented. Control mice received DMSO, PBS, or Rat IgG. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for control group versus treatment groups. There was no statistically significant difference between the anti-CD40/CpG and combined treatment groups. Arrows indicate treatment schedule.