Anti-Bv8 Antibody and Metronomic Gemcitabine Improve Pancreatic Adenocarcinoma Treatment Outcome Following Weekly Gemcitabine Therapy[1,2]

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Abstract

Weekly gemcitabine therapy is the major treatment offered for patients with pancreatic adenocarcinoma cancer; however, relative resistance of tumor cells to chemotherapy, rapid regrowth, and metastasis are the main causes of death within a year. Recently, the daily continuous administration of chemotherapy in low doses – called metronomic chemotherapy (MC) – has been shown to inhibit primary tumor growth and delay metastases in several tumor types; however, its use as a single therapy is still in question due to its moderate therapeutic benefit. Here, we show that the combination of weekly gemcitabine with MC of the same drug delays tumor regrowth and inhibits metastasis in mice implanted orthotopically with pancreatic tumors. We further demonstrate that weekly gemcitabine, but not continuous MC gemcitabine or the combination of the two drug regimens, promotes rebound myeloid-derived suppressor cell (MDSC) mobilization and increases angiogenesis in this tumor model. Furthermore, Bv8 is highly expressed in MDSCs colonizing pancreatic tumors in mice treated with weekly gemcitabine compared to MC gemcitabine or the combination of the two regimens. Blocking Bv8 with antibodies in weekly gemcitabine-treated mice results in a significant reduction in tumor regrowth, angiogenesis, and metastasis. Overall, our results suggest that pro-tumorigenic effects induced by weekly gemcitabine are mediated in part by MDSCs expressing Bv8. Therefore, both Bv8 inhibition and MC can be used as legitimate 'add-on' treatments for preventing post-chemotherapy pancreatic cancer recurrence, progression, and metastasis following weekly gemcitabine therapy.

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Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive human neoplasms exhibiting extremely poor prognosis with a 5-year survival rate of <5% in an unresectable disease [1]. In contrast to several other malignancies, pancreatic cancer is highly resistant to chemotherapy and targeted therapy. The molecular mechanisms that determine treatment resistance are poorly understood, but it is clear that microenvironmental elements such as fibrosis and decreased blood supply with relative hypoxia play a role in treatment failure [2].

The administration of certain chemotherapy drugs at the maximum tolerated dose (MTD) may result in an acute mobilization of bone marrow–derived proangiogenic cells to the treated tumor site [3]. Such a mobilization may promote tumor regrowth, further refractoriness to therapy, induce angiogenesis, and even accelerate metastasis [4–6]. However, metronomic chemotherapy (MC)

scheduling, i.e., the continuous infusion of low-dose chemotherapy (sometimes even on a daily basis) has been shown to inhibit metastases and primary tumor growth of several tumor types including pancreatic cancer [7,8]. In addition, MC has been shown to limit toxicity, chemoresistance effects, and poor long-term efficacy sometimes seen after MTD chemotherapy alone [9]. Initially, the mechanistic basis for the activity of MC was believed to be antiangiogenic by nature, through a direct killing of endothelial cells in the tumor vasculature [10], the suppression of bone marrow–derived endothelial progenitors [11], or the release of endogenous antiangiogenic factors [12]. However, it seems that additional therapeutic effects of MC in the microenvironment of the poorly vascularized PDA is not fully understood, especially when antiangiogenic drug therapy failed to improve therapy in this malignancy [13]. Thus, other mechanisms may account for the activity of MC in PDAs.

One of the major contributors to PDA growth is the presence of CD11b+Gr1+ myeloid-derived suppressor cells (MDSCs) in the complex tumor microenvironment [14]. MDSCs secrete many factors that directly contribute to tumor growth, among them prokineticin 2 (PK2/Bv8) that binds to the two highly related G protein–coupled receptors referred to as PKR1 and PKR2. PK2/Bv8 production by CD11b+Gr1+ myeloid cells can lead to a positive feedback loop, with enhanced differentiation of MDSCs into macrophages, as well as increased mobilization of these cells from the bone marrow into the blood stream [14]. These macrophages infiltrating the tumor microenvironment secrete PK2/Bv8, leading to increased proliferation and migration of endothelial cells, increased pro-inflammatory cytokines interleukin (IL)-1β and IL-12, and decreased anti-inflammatory cytokines IL-4 and IL-10 [15]. Interestingly, the changes in the cytokine profile of the tumor microenvironment were found also following MTD chemotherapy and are probably ameliorated with the use of metronomic scheduling [16]. In addition, our previous studies indicated that bone marrow–derived proangiogenic cells homing to the MTD chemotherapy–treated tumor site promote angiogenesis and accelerate metastasis due in part to the up-regulation of several growth factors and cytokines [4,5]. This pro-tumorigenic effect found after MTD chemotherapy was abrogated when bolus injection of chemotherapy was followed by MC chemotherapy of the same drug [17]. This raises the question of whether the concomitant administration of MTD chemotherapy followed by MC may increase the therapeutic efficacy of PDA treatment.

In this study, we investigated the hypothesis that MDSC-derived Bv8 plays a critical role in the resistance of PDA to MTD gemcitabine. Our results show that MTD gemcitabine markedly increased the mobilization and homing of MDSC-derived Bv8 to the tumor site. The elimination of such cells by MC or anti-Bv8 antibodies markedly increased the therapeutic efficacy of gemcitabine treatment in orthotopic metastatic models of PDA.

Materials and Methods

Tumor Models and Cell Lines

Human Panc-1 and murine Panc-02 pancreatic adenocarcinoma cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) were passed in culture for no more than 4 months after being thawed from authentic stocks. Cells (5×10^6) stably transfected with luciferase were mixed in Matrigel (BD Biosciences, San Jose, CA) and were injected transperitoneally into the head of the pancreas of 6-week-old female Severe Combined Immunodeficiency (SCID) (for Panc-1) or C57Bl/6 (for Panc-02) mice (Harlan, Jerusalem, Israel) after incision of the skin and fixation of the pancreas. Tumor size was assessed

regularly with in vivo imaging system (IVIS) as indicated below. One week after tumor implantation, treatment was initiated according to the schedule and regimen indicated in Supplemental Figure 1, unless indicated otherwise. Mice were randomly grouped before therapy (n = 3-5 per group, unless indicated otherwise).

Drugs and Concentrations

Gemcitabine hydrochloride (Lilly France S.A.S., Fegersheim, France) was administered intraperitoneally as a single bolus injection on a weekly basis at a dose of 500 mg/kg (representing an MTD regimen) or daily at a dose of 3.3 mg/kg representing an MC regimen. In the weekly and MC combined therapy regimens, a reduced dose of weekly gemcitabine was used (375 mg/kg) to avoid major toxicities. Anti-Bv8, a hamster anti-mouse Bv8 monoclonal antibody (mAb 2D3, kindly provided by Genentech, San Francisco, CA), was administered twice weekly at a dose of 5 mg/kg for 2 weeks in C57Bl/6 mice (for Panc-02) to avoid neutralization of the antibodies by the immune system as previously documented [18] and for 4 weeks in SCID mice (for Panc-1). In some experiments, after 21 days of therapy, some of the mice in each group were sacrificed and tumor, bone marrow, and peripheral blood were analyzed. Animal weight was monitored twice a week during the course of treatment. In some experiments, as indicated in the text, a hamster anti-mouse monoclonal antibody was used as a control antibody (Jackson ImmunoResearch Europe, Newmarket, UK), administered in a dose of 5 mg/kg every 2 weeks.

Live Animal Imaging

Bioluminescent imaging of the luciferase-expressing tumors was performed with a highly sensitive, cooled charge coupled device (CCD) camera mounted in a light-tight specimen box (IVIS; Xenogen Corp., Waltham, MA). Briefly, mice were injected intraperitoneally with substrate D-luciferin at 150 mg/kg, and after an 8-minute interval, they were anesthetized using isoflurane. Mice were then placed onto the warmed stage inside the light-tight camera box, with continuous exposure to isoflurane (EZAnesthesia, Palmer, PA) for maintenance of anesthesia. The mice were imaged for 2 minutes. Light emitted from the bioluminescent cells was detected by the IVIS camera system with images quantified for tumor burden using a log-scale color range set at 5×10^4 to 1×10^7 and measurement of total photon counts per second (photons per second) using Living Image software (Xenogen).

Animals were also imaged by magnetic resonance imaging (MRI) using a dedicated whole body mouse coil in a high-field (1 T) small animal scanner (M2 Compact High-Performance MRI Platform; Aspect, Toronto, Canada). The mice were continuously supplied with 2% isoflurane in air for anesthetization. Animals' respiration rate and body temperature were monitored throughout the entire imaging procedure. Gadolinium enhancement was used by intraperitoneal injection of 50 μl of 0.5 M MultiHance (Bracco S.p.A., Milan, Italy).

Flow Cytometry Acquisition and Analysis

Blood or cell suspensions were quantitated for MDSCs, as described previously [19]. Briefly, blood was obtained from mice by retro-orbital sinus bleed and was collected in EDTA tubes. Tumors were removed from the mice and prepared as single-cell suspensions as previously described [4]. Cells were immunostained for MDSCs using Gr1+/CD11b+ surface markers. All antibodies were purchased from BD Biosciences or BioLegend (San Diego, CA). The experiments were performed on Cyan-ADP flow cytometer

(Beckman Coulter, Nyon, Switzerland) and analyzed with Summit Version 4.3 (Beckman Coulter).

Quantitation and Visualization of Tissue Hypoxia, Vessel Perfusion, and Microvessel Density

Tissue processing and immunohistochemistry were performed as described previously [31]. Briefly, blood vessel perfusion and hypoxia were analyzed in tumor cryosections (10 to 12 μm) using the DNA-binding dye Hoechst 33342 (40 mg/kg; Sigma-Aldrich, Rehovot, Israel) and pimonidazole hydrochloride (60 mg/kg; Natural Pharmacia International Inc., Burlington, MA), respectively, according to the manufacturers' instructions. Blood vessels were immunostained with anti-CD31 antibodies, an endothelial cell–specific antibody (1:200 ratio; BD Biosciences), and the number of vessel structures per field was counted and plotted (at least five fields per tumor, $n > 20$ fields per group). Hypoxia and perfusion were quantitated from micrographs by analyzing the number of positive pixels from the total pixels using Photoshop CS2 Version 2 software (Adobe Systems, San Jose, CA). Tumor sections were visualized under Leica CTR 6000 microscope (Leica Microsystems, Wetzlar, Germany) using the Leica Application Suite Version 3.

Bv8 mRNA Expression Analysis by Reverse Transcription–Polymerase Chain Reaction

RNA was extracted from tumors using the RNeasy Mini Kit (Quagen Inc., Valencia, CA) and subsequently converted into cDNA by reverse transcriptase (Promega Corporation, Madison, WI). A total volume of 20 μl containing 300 ng of total RNA template and 10 μl of Power CYBR Green Master Mix (Applied Biosystems, Modiin, Israel) was prepared. Reactions were run on a 7000 Real-Time PCR System (Applied Biosystems). The amounts of target genes were determined from the comparative threshold cycle (C_T) method. Expression level of Bv8 gene was further quantified against the housekeeping HPRT gene using the same treatment and expressed as $2–\Delta C_T (\Delta C_T$ of target gene $– C_T$ of HPRT). The primer sequences were given as follows: for mouse Bv8 forward, GCATGACAGGAGTCATCATTTT; reverse, AAATGGCAGGATATCAGGAAA; for mouse HPRT forward, ATGTTCCAGTATGACTCCACTCACG; reverse, GAAGACACCAGTAGACTCCACGACA.

Invasion and Migration Assays

The invasion and migration properties of Panc-1 and Panc-02 cells in response to plasma of mice treated with all treatment regimens or treatment combinations, as indicated above, were evaluated in fibronectin and Matrigel-coated Boyden chambers for migration and invasion, respectively, using a previously described protocol [4]. Briefly, serum-starved cells (2×10^5 cells in 0.2 ml medium) were added to a filter coated with 10 μl of fibronectin or 50 μl of Matrigel (Biological Industries, Beit Haemek, Israel and BD Biosciences, Israel, respectively). The lower compartment was filled with Dulbecco's modified Eagle's medium that contained 10% plasma obtained from mice 24 hours or 7 days after they were treated with gemcitabine and/or anti-Bv8 antibodies, as indicated in the text. After 4 and 12 hours, respectively, the cells that migrated to the bottom filter were stained with crystal violet and counted under an inverted microscope (Leica DM IL LED) per ×100 objective field. All experiments were performed in triplicate.

Statistical Analysis

Data are expressed as means ± SD. The statistical significance of differences was assessed by one-way analysis of variance, followed by Newman-Keuls ad hoc statistical test using GraphPad Prism 4 software (La Jolla, CA). Differences between all groups were compared with each other and were considered significant at values below 0.05.

Results

Pancreatic Tumor-Bearing Mice Treated with MC Regimen Exhibit Controlled Tumor Growth and Reduced Metastatic Spread

The potential role of MC to delay tumor growth has been shown in several preclinical models as well as in clinical studies [8,20–22]. Here, we sought to evaluate the possible therapeutic effect of MC in mice bearing orthotopic metastatic pancreatic adenocarcinoma, in comparison to the conventional weekly gemcitabine therapy at the MTD. To this end, Panc-02 tumor cells tagged with luciferase were implanted orthotopically into the pancreas of 6-week-old C57Bl/6 mice. A week later, treatment with weekly gemcitabine, MC gemcitabine, or the combination of the two regimens was initiated according to the schedule and doses described in Supplemental Figure 1 and Materials and Methods section. Tumor growth was assessed using IVIS as described previously [23]. The results in Figure 1 (A–C) demonstrate that while treatment with weekly gemcitabine improved therapy outcome following 1 week of therapy, rapid tumor regrowth was subsequently observed, and no therapeutic benefit was observed over time despite the continuous treatment protocol. In contrast, treatment with either MC gemcitabine or the combination of weekly and MC gemcitabine resulted in suppressed tumor growth during the treatment period. Consistently, mice treated with the combined regimen survived longer than all of the other treatment regimens (Figure 1C). Similar results were observed in Panc-1–bearing SCID mice (Supplemental Figure 2). However, we must note the high toxicity profile in the combination therapy–treated mice (data not shown).

Next, we used MRI technology to evaluate metastatic spread in live mice 28 days after receiving the different therapy regimens. Metastatic spread was found in the abdominal cavity, digestive system, and sometimes in the liver, in control untreated mice, as well as in all treatment regimens although not to the same extent (Figure 1D). Mice were sacrificed at end point, and metastases were spotted and counted in different anatomic areas including the gastrointestinal (GI) tract, stomach, greater omentum, hepatic port, and liver. As seen in Table 1, mice treated with the combination therapy regimen and, to a lesser extent, with MC regimen exhibited a lower number of metastases in the different organs, when compared to weekly gemcitabine-treated or untreated control mice. Overall, these results indicate that the combination of weekly and MC gemcitabine improves therapy outcome by delaying primary tumor regrowth and reducing metastatic spread.

Host Response to Weekly Gemcitabine Promotes Tumor Cell Migration and Rebound MDSC Mobilization

Our previous studies have shown that plasma obtained from mice treated with chemotherapy induces tumor cell invasion indicating a pro-tumorigenic host effect in response to chemotherapy [4]. The increased metastatic spread found following weekly gemcitabine therapy prompted us to investigate the host response in the different treatment regimens. Specifically, we asked whether plasma from mice treated with gemcitabine in the different regimens differentially affect tumor cell invasion. To this end, Panc-1 and Panc-02 tumor cells were placed on a Boyden chamber coated with Matrigel. Dulbecco's modified Eagle's medium supplemented with 10% plasma from non–
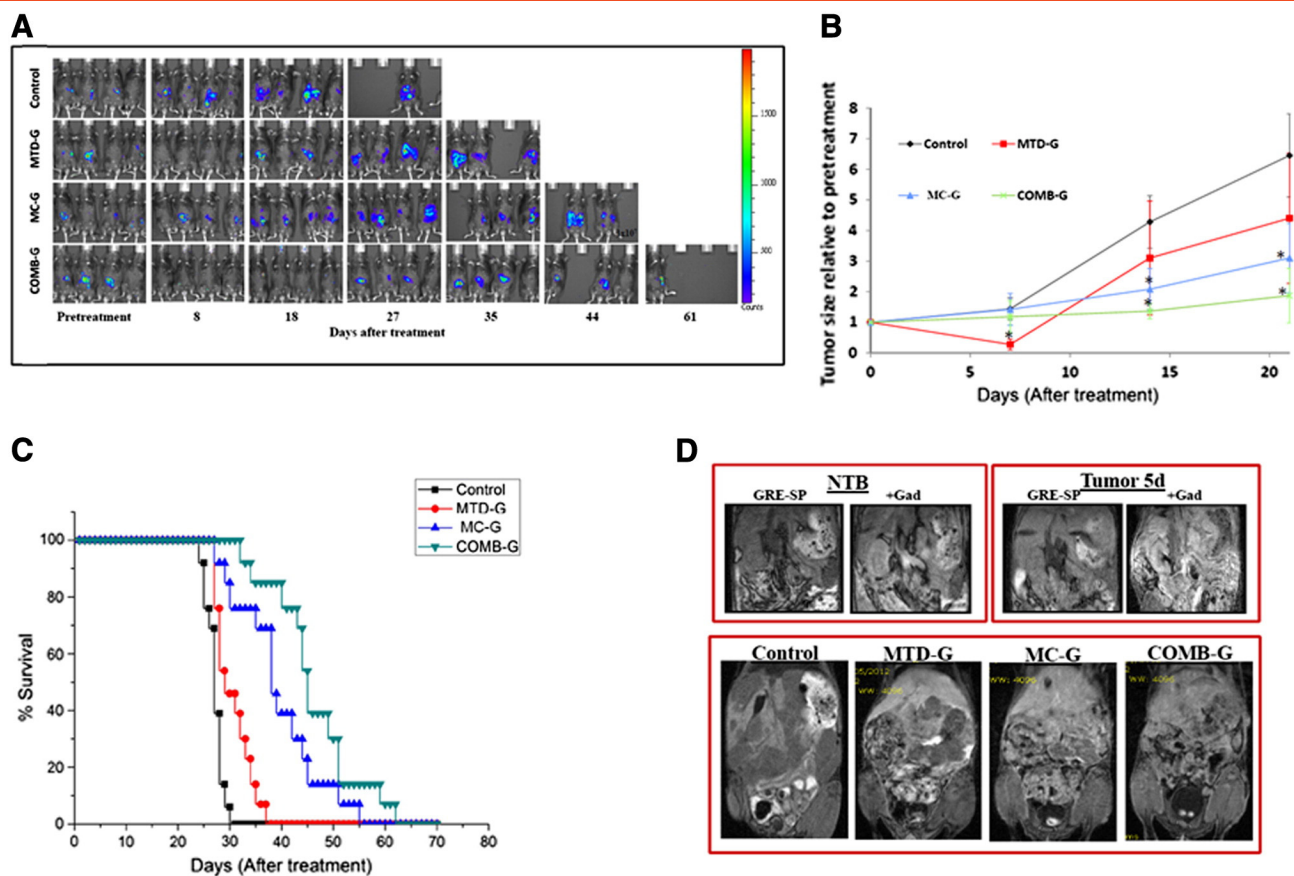
Figure 1. The combination of weekly and daily gemcitabine therapy improves therapeutic outcome in mice bearing pancreatic tumors. Eight-week-old C57Bl/6 mice were implanted orthotopically with 5×10^6 Panc-02 cells stably transfected with luciferase. After 7 days, they were either left untreated (Control) or treated with gemcitabine in the following regimens: maximum tolerated dose administered weekly (MTD-G), metronomic dose administered daily (MC-G), or the combination of the two regimens (COMB-G) as described in Figure W1. (A) Tumor growth was evaluated over time using IVIS following injection with luciferin as previously described [23]. (B) Tumor growth was quantified by counting the number of photons collected at 8 minutes after luciferin injection when compared to their pre-treatment values. *, $P < .05$ when compared to pre-treated control. (C) Survival of mice from all groups was plotted using the Kaplan-Meier curve. (D) In a parallel experiment, 28 days following the different gemcitabine regimens, mice were imaged by MRI Spectra 1T. Non–tumor-bearing mice (NBT) or mice bearing tumors on day 5 pre-treatment (Tumor 5d) were also imaged for comparison. Representative images are shown.

tumor-bearing mice 1 week after they were treated with the different regimens was placed at the bottom of the chamber. As shown in Figure 2*A*, invasion of Panc-1 and Panc-02 cells to the bottom of the chamber was significantly increased in the presence of plasma from mice

Table 1. Spread of macroscopic tumor lesions in Panc-02–bearing mice following treatment with gemcitabine in different drug regimens. Mice were implanted orthotopically with Panc-02 tumors and subsequently treated with gemcitabine in the following regimens: maximum tolerated dose administered weekly (MTD-G), metronomic dose administered daily (MC-G), or the combination of the two regimens (COMB-G) as described in Figure W1. At end point, the mice were sacrificed and macrometastases in the, GI track, stomach, greater omentum, hepatic ports, and liver were counted ($n = 13$ per group).

Treatment Group	No. of Mice (n)	Primary Tumor				Liver Metastasis (n)
		Weight (mg)	Spread to Anatomic Location			
			GI	Stomach and Greater Omentum	Porta Hepatis	
Control	13	1074 ± 662	11/13 (85%)	13/13 (100%)	7/13 (54%)	2/13 (15%)
MTD-G	13	785 ± 544	9/13 (69%)	13/13 (100%)	5/13 (38%)	5/13 (38%)
MC-G	13	619 ± 490	6/13 (46%)	11/13 (84%)	3/13 (23%)	0/13 (0%)
COMB-G	13	245 ± 189	4/13 (30%)	8/13 (61%)	1/13 (8%)	0/13 (0%)

treated with weekly gemcitabine when compared to control, MC, or the combination of the two regimens. It should be noted that the tumor cells exhibited similar migration properties in the presence of plasma from all treatment regimens (data not shown). We next assessed whether rebound angiogenesis sometimes seen between successive drug intervals [6] may also account for tumor regrowth and metastatic spread found following weekly gemcitabine. To test this, we focused on MDSCs because they have been shown to promote angiogenesis in pancreatic tumors [24]. In addition, gemcitabine has already been reported as a drug that does not affect circulating endothelial progenitor cells known to directly support tumor angiogenesis [3,25]. To test MDSC mobilization, non–tumor-bearing mice were treated with the drug regimens indicated above and the level of MDSCs in daily drawn blood samples was evaluated by flow cytometry. Weekly gemcitabine caused an immediate suppression in MDSC levels followed by a rapid and significant mobilization that returned back to normal levels by day 10. In contrast, no significant changes in the number of MDSCs were observed with the other treatment regimens (Figure 2*B*).

Next, we assessed the number of MDSCs in the peripheral blood, bone marrow, and tumors of pancreatic tumor-bearing mice treated
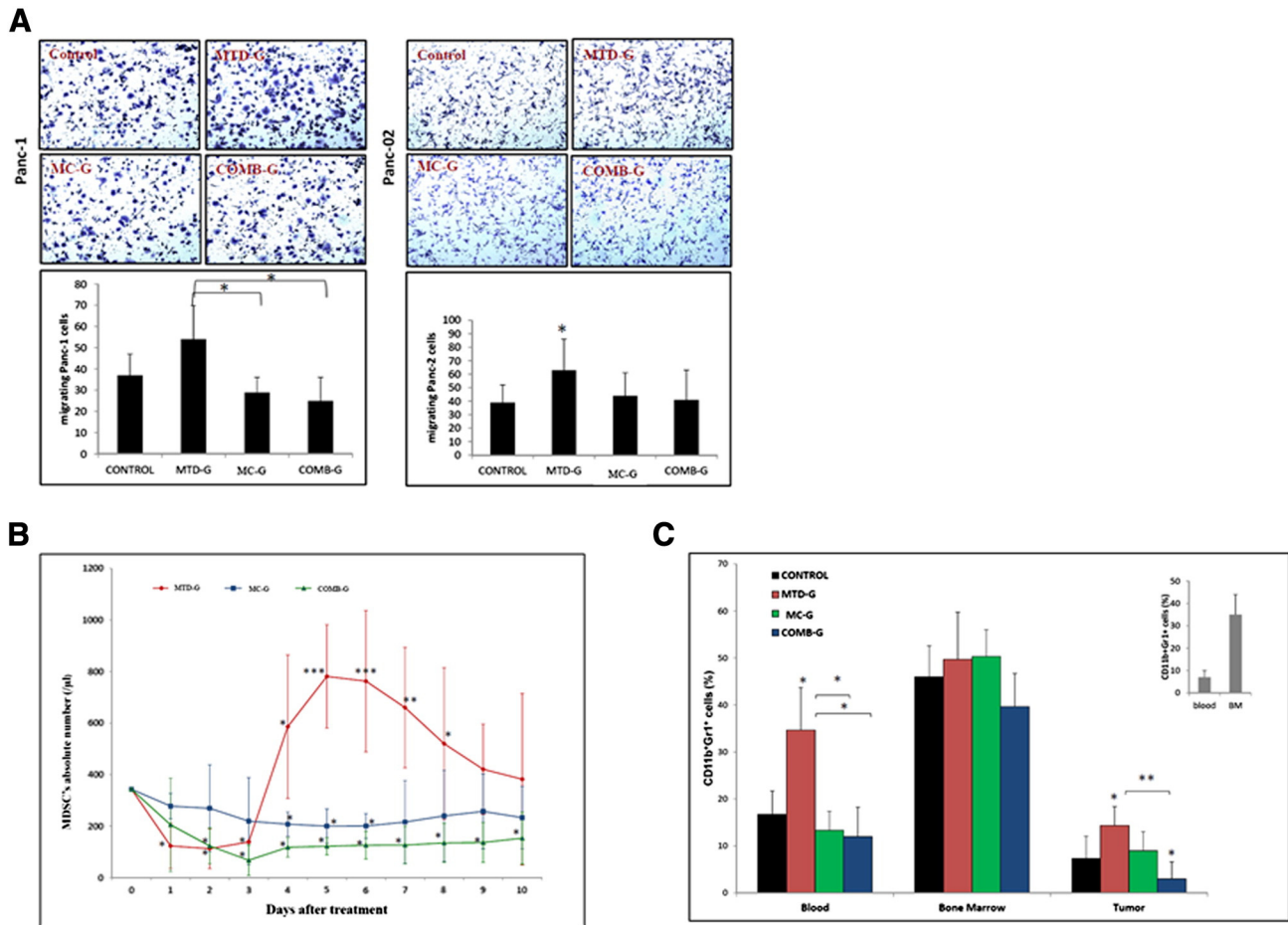
Figure 2. Weekly gemcitabine induces host pro-tumorigenic effects that promote tumor cell invasion and increase the number of MDSCs. Non–tumor-bearing C57Bl/6 mice were either untreated (Control) or treated with gemcitabine administered weekly at the maximum tolerated dose (MTD-G), daily at a metronomic dose (MC-G), or in a combination of the two regimens (COMB-G). (A) Plasma was drawn after 7 days of therapy and used in a Boyden chamber assay for the assessment of Panc-1 and Panc-02 tumor cell invasion as described in the Materials and Methods section. Cells migrating to the bottom of the filter were counted and plotted. (B) In a parallel experiment, blood from mice treated with the different gemcitabine regimens was sampled on a daily basis for 10 sequential days. The number of MDSCs in the peripheral blood was evaluated by flow cytometry. (C) In a separate experiment, mice bearing orthotopic pancreatic tumors were treated with the different gemcitabine treatment regimens as indicated above. After 21 days, blood was drawn by cardiac puncture, bone marrow cells were flushed from the bone marrow, and tumors were removed. The percentage of MDSCs in the blood, bone marrow, and tumor samples was assessed by flow cytometry. The MDSC count in non–tumor-bearing mice is shown in the inset. *, $P < .05$; **, $.01 > P > .001$; ***, $P < .001$.

with the various regimens. The number of MDSCs was significantly increased in the peripheral blood and tumors of pancreatic tumor-bearing mice on day 21 after weekly gemcitabine therapy. Interestingly, a significant decrease in the number of MDSCs was observed in mice treated with the combination of the two drug regimens in the blood (Figure 2C). Overall, these results suggest that, in response to weekly gemcitabine treatment, the host promotes tumor cell invasion as well as MDSC mobilization and tumor homing, which could account for rapid regrowth and metastasis. In contrast, the addition of MC to weekly gemcitabine could inhibit these pro-tumorigenic properties.

Bv8 Is Highly Expressed in MDSCs in Mice Treated with Weekly Gemcitabine

Bv8 has been shown to promote angiogenesis and tumor growth [14]. Because it is abundantly expressed in MDSCs [26], which we showed were mobilized by weekly gemcitabine, we asked whether blocking Bv8 could improve weekly gemcitabine therapy outcome. We assessed the relative expression of Bv8 in peripheral blood, bone marrow, and

MDSCs in mice treated with the different treatment regimens to identify whether other cell types besides MDSCs express Bv8 and whether the expression of Bv8 is related to the host effects in response to the therapy. We found that the levels of Bv8 were highly elevated specifically in MDSCs of non–tumor-bearing mice treated with weekly gemcitabine when compared to all other treatment regimens (Figure 3A). Then, we asked whether mice bearing pancreatic tumors also exhibit elevated levels of Bv8 in MDSCs both in the peripheral blood and in the tumor, following any of the treatment regimens. The results in Figure 3B show that Bv8 is highly expressed specifically in MDSCs isolated from the peripheral blood or tumors only in mice treated with weekly gemcitabine when compared to all other treatment regimens. Overall, these results suggest that the host response to weekly gemcitabine therapy induces Bv8 expression specifically in MDSCs.

Next, because MDSCs and Bv8 have been shown to promote angiogenesis and tumor growth, especially in mice treated with antiangiogenic drugs [27], we investigated the effect of MDSCs on angiogenesis in pancreatic tumors following treatment with
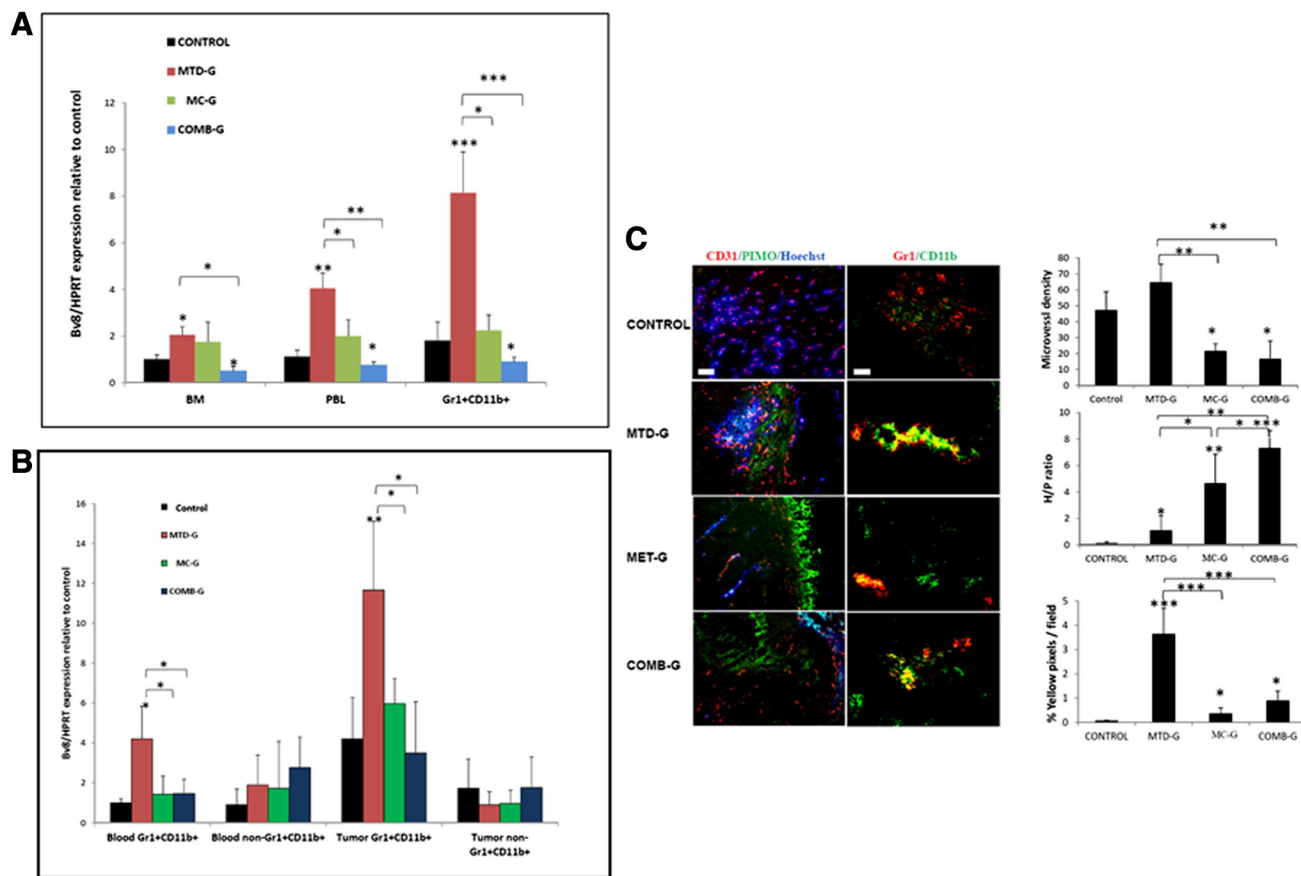
Figure 3. Weekly gemcitabine therapy increases Bv8 expression and tumor angiogenesis. (A) Non–tumor-bearing C57Bl/6 mice were either untreated (Control) or treated with gemcitabine administered weekly at the maximum tolerated dose (MTD-G), daily at a metronomic dose (MC-G), or in a combination of the two regimens (COMB-G). Seven days after treatment initiation, mice were sacrificed. The level of Bv8 mRNA was assessed by reverse transcription–polymerase chain reaction in the peripheral blood (PBL), bone marrow cells (BM), and MDSCs (Gr1+CD11b+) 7 days after treatment initiation. (B) Pancreatic tumor (Panc-02) bearing mice were treated with the different gemcitabine regimens as indicated above. After 21 days of treatment, blood was drawn by cardiac puncture, mice were sacrificed, and tumors were removed. The relative expression of Bv8 mRNA was assessed by reverse transcription–polymerase chain reaction on sorted MDSCs from both peripheral blood and tumor cells that were prepared as single cell suspensions. (C) Left panel: Tumor sections from all treatment groups were stained for CD31 representing endothelial cells (in red), hypoxia using hypoxic probe (in green), and Hoechst for assessment of perfusion (in blue). Right panel: To visualize MDSCs within the tumor, the sections were immunostained with anti-Gr1 (red) and anti-CD11b (green) antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue); scale bar, 200 μm. Microvessel density, the ratio of hypoxia over perfusion, and cells expressing both Gr1 and CD11b were quantified from the stained tumor sections from all groups ($n > 20$ fields per group) and subsequently plotted. *, $P < .05$; **, $.01 > P > .001$; ***, $P < .001$.

gemcitabine in the different treatment regimens. To do so, mice bearing Panc-02 tumors were treated with weekly gemcitabine, MC daily gemcitabine, or the combination of the two regimens. On day 21 post treatment, the mice were sacrificed and the pancreas was analyzed for hypoxia, perfusion, and microvessel density to assess the nature of angiogenesis. The results in Figure 3C show that treatment with weekly gemcitabine resulted in increased colonization of MDSCs in the tumor, consistent with the flow cytometry results shown in Figure 2C. In addition, tumors from mice treated with weekly gemcitabine were relatively more perfused and less hypoxic than tumors treated with MC gemcitabine or the combination of the two regimens. Overall, these results further suggest that the increased colonization of Bv8-expressing MDSCs in pancreatic tumors results in highly perfused tumors that could explain their rapid regrowth following weekly gemcitabine therapy. These effects can be blocked by the addition of MC gemcitabine to weekly MTD gemcitabine, improving therapy outcome.

Anti-Bv8 Antibodies Block Weekly Gemcitabine-Induced Rebound MDSCs and Improve Therapy Outcome

The high expression of Bv8 in MDSCs, which colonize tumors in mice treated with weekly gemcitabine, and their impact on induction of angiogenesis further prompted us to test whether blocking Bv8 in the weekly gemcitabine treatment regimen can improve therapy outcome by delaying primary tumor regrowth and metastatic spread. To this end, mice were implanted with Panc-02 cells, and a week later, treatment with weekly gemcitabine with or without anti-Bv8 antibody was initiated. As a control for Bv8 antibody administration, a hamster anti-mouse monoclonal antibody was used. Bv8 was administered only for 2 weeks to reduce the immunologic response of the host to the drug. The results in Figure 4A show that mice treated with weekly gemcitabine in combination with Bv8 antibody exhibited a significant reduction in primary tumor growth when compared to all other treatment groups. We should note that anti-
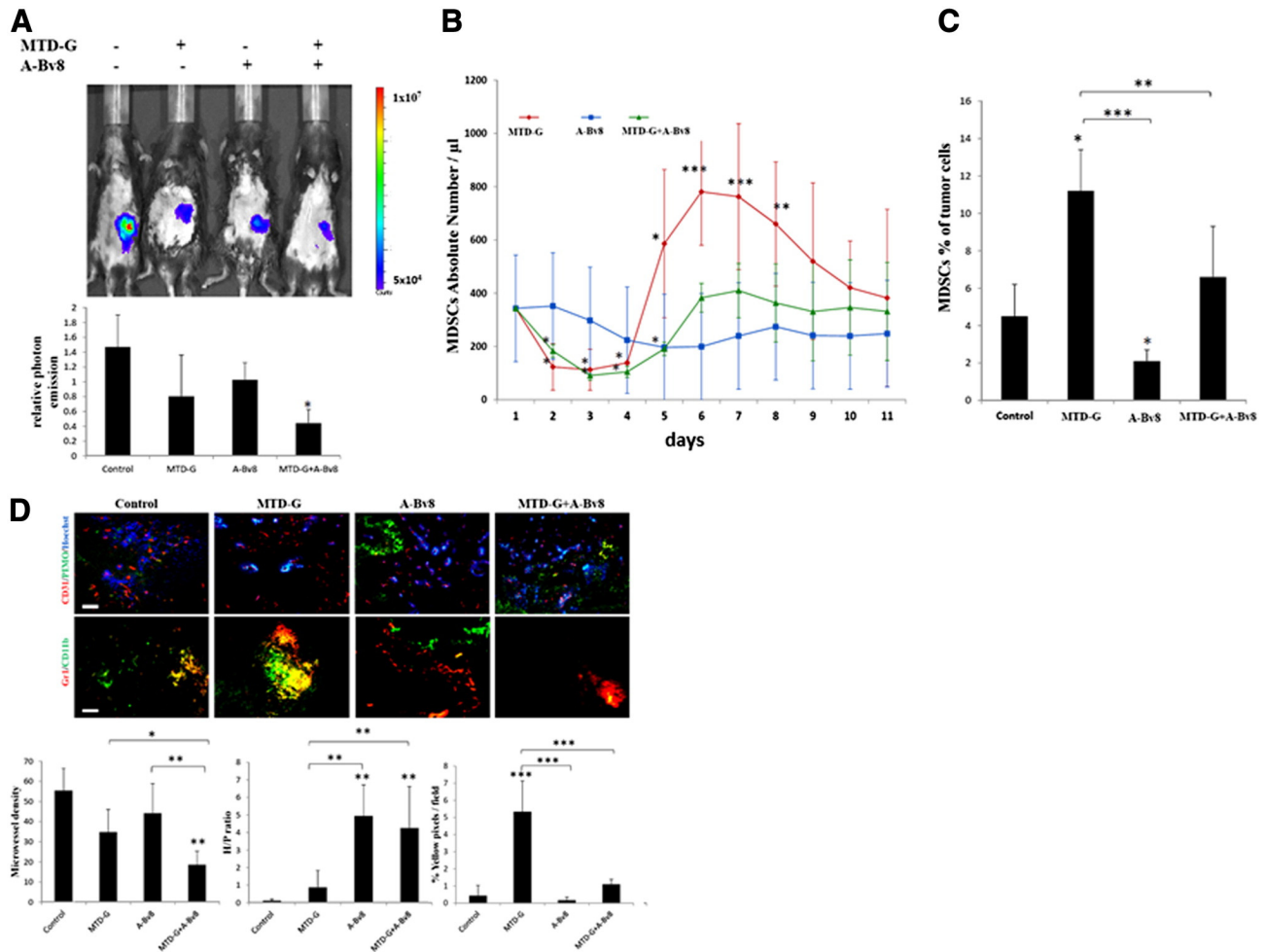
Figure 4. Anti-Bv8 antibodies block MDSC mobilization and tumor homing. Eight-week-old C57Bl/6 mice were orthotopically implanted with Panc-02 tumor cells (5×10^6 cells/100 μl). After 7 days, treatment with weekly gemcitabine (MTD-G) with or without anti-Bv8 antibody (ABv8) administered twice weekly for 2 weeks was initiated. (A) Tumor growth was assessed by IVIS. Representative images are shown in the upper panel. Tumor size was quantified by detecting photon intensity (lower panel). (B) In a different experiment, non–tumor-bearing mice were treated with the drug combinations as indicated above, and blood was sampled daily (through retro-orbital sinus bleed) for the evaluation of MDSC number in peripheral blood by flow cytometry. (C) Tumor-bearing mice from (A) were treated as indicated above, and 14 days after treatment was initiated, mice were sacrificed and tumors were removed. Half of each tumor was prepared as a single cell suspension for the assessment of the percentage of MDSCs in the tumors by flow cytometry. (D) The other half of the tumor was sectioned and stained with anti-CD31 antibodies (red) to detect endothelial cells, hypoxic probe (green) to detect hypoxia, and Hoechst (blue) to assess perfusion (upper micrographs). To visualize MDSCs within the tumor, the sections were immunostained with anti-Gr1 (red) and anti-CD11b (green) antibodies. Nuclei were stained with DAPI (blue; lower micrographs); scale bar, 200 μm. Microvessel density, the ratio of hypoxia over perfusion in tumors, and cells expressing both Gr1 and CD11b were quantified in tumor sections from all groups ($n > 20$ fields per group) and subsequently plotted. *, $P < .05$; **, $.01 > P > .001$; ***, $P < .001$.

Bv8 on its own also inhibited primary tumor growth and improved therapy outcome. Next, we evaluated the number of MDSCs in the peripheral blood of these mice during the therapy and found that the rebound MDSC mobilization found following weekly gemcitabine was markedly and significantly reduced when the treatment included anti-Bv8 therapy (Figure 4B). The administration of isotype-control antibody to non–tumor-bearing mice did not significantly alter the number of MDSCs when compared to control untreated mice during the treatment period (Figure W3A). As a result, the number of MDSCs colonizing the treated tumors was also reduced when the treatment was combined with anti-Bv8 antibodies as seen by flow cytometry and immunofluorescent staining analysis (Figure 4, C–D). Parallel results of primary tumor growth were also observed in SCID mice bearing Panc-1 tumors. However, the anti-Bv8 antibody

therapy was as good as anti-Bv8 combined with weekly gemcitabine, indicating its high efficacy in blocking tumor growth in this model (Supplemental Figure 4). It should be noted that no significant differences in tumor growth, microvessel density, and MDSC colonization of Panc-02 tumors were observed between control-untreated mice and mice treated with the isotype-control monoclonal antibody (Supplemental Figure 3B-C). In terms of metastasis, we sought to determine the effect of anti-Bv8 on the invasion of tumor cells following gemcitabine therapy. To this end, 8-week-old non–tumor-bearing C57Bl/6 mice were treated with 500 mg/kg gemcitabine (MTD), 5 mg/kg anti-Bv8 antibodies, or the combination of the two drugs. After 24 hours, plasma was collected and used to assess the invasion properties of Panc-1 and Panc-02 cells. The results in Figure 5A show that plasma from mice treated with MTD gemcitabine induced tumor cell invasion
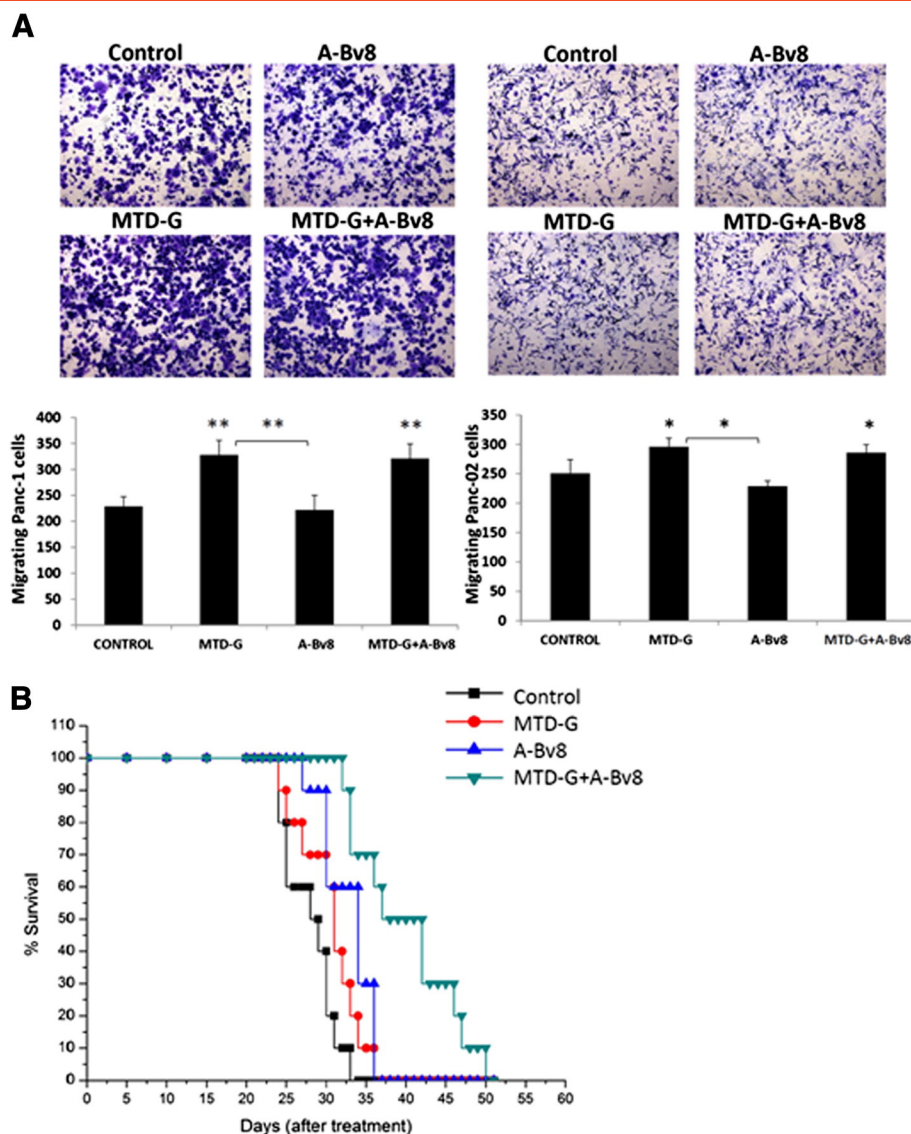
Figure 5. Anti-Bv8 antibodies improve therapy outcome. (A) Eight-week-old non–tumor-bearing C57Bl/6 mice were treated with gemcitabine (500 mg/kg, MTD-G) with or without anti-Bv8 antibody (5 mg/kg, A-Bv8). After 24 hours, blood was drawn by cardiac puncture and plasma was separated and used in a Boyden chamber assay for the assessment of Panc-1 and Panc-02 tumor cell invasion as described in the Materials and Methods section. Cells migrating to the bottom of the filter were counted and plotted. (B) Eight-week-old C57Bl/6 mice were orthotopically implanted with Panc-02 tumor cells (5×10^6 cells/100 μl). After 7 days, treatment with weekly gemcitabine (MTD-G) with or without anti-Bv8 antibody (ABv8) administered twice weekly for 2 weeks was initiated. Mouse survival was assessed. A Kaplan-Meier curve is plotted. *, $P < .05$; **, $.01 > P > .001$.

when compared to plasma from control or anti-Bv8–treated mice. Plasma from mice treated with the combination of MTD gemcitabine and anti-Bv8 therapy did not alter the invasion properties of tumor cells when compared to plasma from gemcitabine-treated mice. Of note, plasma from control-untreated or isotype-IgG treated mice did not alter the invasion properties of the tumor cells (Supplemental Figure 3D). These results further suggest that anti-Bv8 therapy does not directly affect tumor cell invasion.

Next, we evaluated the survival of mice bearing Panc-02 tumors following 2 weeks of treatment with weekly gemcitabine with or without anti-Bv8 antibodies. As seen in Figure 5B, increased survival time was observed in mice treated with the combination of MTD gemcitabine and anti-Bv8 antibodies when compared to all other treatment groups. Taken together, our results suggest that the anti-

Bv8 antibody inhibits host pro-tumorigenic and pro-metastatic effects induced by weekly gemcitabine therapy by means of delayed primary tumor growth and increased survival but not through direct effect on the tumor cell invasion.

Discussion

The treatment of PDA has suffered many failures, and treatment options are limited. Currently, gemcitabine is considered as the main treatment modality for PDA along with surgery and radiation. Our previous studies indicated that gemcitabine chemotherapy administered in bolus injections induces host pro-angiogenic and pro-metastatic effects, which in turn contribute to tumor regrowth and metastatic spread [5]. Indeed, in this study, we found that mice bearing orthotopic pancreatic tumor models exhibit tumor regrowth and/or resistance to therapy, as well

as an induction in metastatic spread after receiving the course of MTD gemcitabine. These tumor regrowth effects were minimized or even negated by the addition of an MC regimen using the same drug. Tran Cao et al. have previously shown that metronomic gemcitabine in combination with the antiangiogenic small molecule tyrosine kinase inhibitor, sunitinib, can also inhibit metastasis and increase survival rate of mice bearing PDAs, suggesting that such treatment modality can be used against PDA in the adjuvant setting [8]. To uncover the possible cellular mechanism(s) accounting for the tumor regrowth effects, we focused on MDSCs. Previous studies reported that the levels of MDSCs rise in peripheral blood in correlation with the development of spontaneous pancreatic tumors from EL-TFG-α/P53 –/– genetically engineered mice. It has been suggested that the colonization of MDSCs in the pancreatic tumors inhibits immune cells such as T-cells, which are responsible for the suppression of tumor growth by the secretion of interferon-γ (IFN-γ) [28]. Clinically, MDSCs were evaluated in PDA patients in both bone marrow and peripheral blood, and their levels correlated with disease stage [29]. In the current study, MDSCs were shown to mobilize and subsequently home to the treated tumor sites at baseline, an effect that was dramatically pronounced following MTD but not MC gemcitabine regimen. Thus, MDSCs may account not only for pancreatic tumor development and growth but also for tumor regrowth following acute MTD gemcitabine. In contrast, when MC was combined with MTD gemcitabine, the levels of MDSCs were markedly reduced resulting in a significant improvement in therapy outcome, both at the primary tumor level and the metastatic stage.

This study is not the first to show that the combination of MTD chemotherapy followed by MC regimen improves clinical outcome in various tumor models [17,20,30]. However, it provides a suitable explanation as to why these drug regimen combinations are effective. It was recently reported that the chemo-switch regimen, i.e., MTD followed by MC regimen, inhibited the proliferation and survival of cancer stem cells – a subpopulation of tumor cells possessing stem cell characteristics such as resistance to conventional therapy [20,31]. In addition, it has also been suggested that MC regimen blocks the rebound angiogenesis and vasculogenesis effects found at the drug-free break periods during the course of the MTD regimen. The use of maintenance MC therapy at times of drug-free breaks suppresses the levels of circulating endothelial progenitor cells and, hence, inhibits systemic angiogenesis [11]. Other studies have offered other explanations for the impact of MC gemcitabine on pancreatic tumor growth. It has been postulated that MC gemcitabine inhibits T-regulatory cell homing and retention at the tumor site, as well as suppresses CD8 + T effector cells, thereby inhibiting tumor regrowth [7,29]. We therefore assume that the suppression of MDSC colonization of tumors in the combined treatment regimen group reported in this study improved therapy outcome by increasing the immune T effector cells against the tumor. Indeed, the selective depletion of the MDSC subset, which was found to colonize a genetically engineered mouse model of PDA, has been shown to induce the accumulation of activated CD8 T cells and to remodel tumor stroma [32]. However, in our PDA orthotopic model in SCID mice, anti-Bv8 treatment also exhibited anti-tumor activity. These activities cannot be explained by the role of MDSCs as immune modulating cells but rather through their other roles as pro-angiogenic cells [15,33]. Taken together, blocking MDSCs may affect in various mechanisms on the tumor stroma, leading to inhibition in tumor growth.

Antiangiogenic drug therapy has been shown to have little or no effect in PDAs [13]. We therefore initially postulated that the use of anti-Bv8, a drug with antiangiogenic properties [34,35], would be ineffective in treating PDAs. However, on the basis of the fact that PDA regrowth following MTD gemcitabine involves MDSCs, the addition of anti-Bv8 effectively inhibited tumor growth by inhibiting the mobilization and tumor homing of MDSCs found following MTD chemotherapy. It was surprising, however, that such treatment as a single agent therapy also significantly inhibited PDA tumor growth. We assume that the inhibition of MDSCs, which may play a critical role in such tumors, accounts for the tumor growth control in anti-Bv8 monotherapy. Curtis et al. have already reported that anti-Bv8 suppresses angiogenesis in pancreatic tumors [34]. The remaining question is therefore whether anti-Bv8 in combination with MC gemcitabine regimen can also improve therapy outcome. Although we did not address this question in our study, it has been recently indicated that anti-VEGF therapy blocks the activity of MC therapy by altering the anti-tumor immune response including the activity and retention of Natural Killer (NK) cells, macrophages, and dendritic cells at the tumor site [36]. In addition, sunitinib has also been shown to be effective in combination with MC gemcitabine using orthotopic models of PDAs [8]. Thus, anti-Bv8 may counteract the MC gemcitabine regimen, an effect that is not necessarily related to the antiangiogenic activity of this drug combination. It would be of interest to use additional methodologies to comprehensively study the cross-talk between host and tumor cells and the microenvironment of pancreatic tumors following treatment with the different regimens. Hoffman et al., in several studies, have recently shown the development and utilization of a red fluorescent protein of orthotopic pancreatic cancer model in which they have evaluated the metastatic properties and the host tumor cell interactions in tumors following chemotherapy, hence allowing whole body imaging at the cellular level in living animals and fresh tissues [37–39]. It is therefore important to evaluate whether anti-Bv8 antibodies may have other therapeutic activities in tumors besides those related to angiogenesis and inhibition of MDSC activity by using additional sophisticated technologies for measuring tumor-host interactions.

Antibody-based therapy in cancer has been extensively used in the clinic, especially when such drugs are administered as add-on therapies to conventional treatments. Although therapy outcome is usually improved in many malignancies, there are some instances that the therapeutic benefits are relatively modest. This has been an ongoing discussion regarding the cost-effectiveness of such add-on drugs [40,41]. In this study, we demonstrate two different add-on treatments that could be administered in combination with MTD gemcitabine for the treatment of PDA, both of which have shown substantial preclinical therapeutic benefit. Although anti-Bv8 antibodies clearly improved the therapy outcome of MTD gemcitabine, their benefit in the clinical setting is currently unknown. However, MC gemcitabine, or its oral pro-drug, LY2334737[25], has shown therapeutic benefits when it was co-administered in combination with MTD gemcitabine. We must note, however, that the latter caused major toxicities that may or may not preclude the use of this combination regimen in the clinic. Yet, the combined regimen showed remarkable anti-tumor activity when compared to the conventional MTD gemcitabine regimen. Clinical evaluation of these two offered treatment modalities in PDAs is therefore worthy.

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2014.05.011.

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