Published in final edited form as:

Cancer Lett. 2013 April 28; 330(2): 241–249. doi:10.1016/j.canlet.2012.11.055.

Thrombospondin-1 and Pigment Epithelium-Derived Factor Enhance Responsiveness of KM12 Colon Tumor to Metronomic Cyclophosphamide but have Disparate Effects on Tumor Metastasis

Li Jia and David J. Waxman

Division of Cell and Molecular Biology, Department of Biology, Boston University, Boston, MA 02215

Abstract

The anti-tumor activity, metronomic chemotherapy sensitization potential and metastatic effects of the endogenous angiogenesis inhibitors thrombospondin-1 and PEDF were investigated in KM12 colon adenocarcinoma xenografts. Thrombospondin-1 and PEDF decreased KM12 tumor microvessel density, increased macrophage infiltration, and improved responsiveness to metronomic-cyclophosphamide (CPA) treatment, but did not activate the anti-tumor innate immunity stimulated by metronomic-CPA in other tumor models. Moreover, thrombospondin-1, but not PEDF, significantly increased KM12 metastasis to the lung, while PEDF augmented the anti-metastatic activity of metronomic-CPA. Thus, while thrombospondin-1 and PEDF both increase the KM12 tumor responsiveness to metronomic-CPA, they have disparate effects on tumor metastasis.

Keywords

thrombospondin-1; pigment epithelial derived factor; angiogenesis; metronomic chemotherapy; innate anti-tumor immunity

1. Introduction

Angiogenesis plays a critical role in tumor growth and metastasis and is a promising target for cancer therapy [1]. Thrombospondin-1 (TSP1) is a secreted glycoprotein with endogenous angiogenesis inhibitory activity. It is most abundant in α -granules of platelets and is essential for wound healing and anti-inflammatory processes [2]. TSP1 inhibits angiogenesis by several mechanisms. TSP1 binds to CD36 on the surface of endothelial cells, thereby inhibiting endothelial cell migration and inducing endothelial cell apoptosis via the Fas/FasL pathway [3]. TSP1 also binds to CD47, interrupting the association of CD47 and its receptors, namely, VEGFR-2 and SIRP α , thereby inhibiting VEGFR-2

© 2012 Elsevier Ireland Ltd. All rights reserved.

Corresponding Author: Dr. David J. Waxman, Department of Biology, Boston University, 5 Cummington Mall, Boston, MA 02215, Fax: 617-353-7404, djw@bu.edu.

Conflict of Interest Statement

None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

stimulated angiogenesis [4] and promoting phagocytosis of target cells [5]. TSP1 can also bind directly to VEGF, leading to internalization and degradation of VEGF via low density lipoprotein receptor-related protein-1 [6]. Consistent with these findings, decreased TSP1 expression correlates with tumor progression in colon carcinoma, penile squamous cell carcinoma, cervical carcinoma, melanoma, prostate cancer, neuroblastoma, and breast ductal carcinoma in human patients [2], while treatment with TSP1-mimetic peptides or overexpression of TSP1 inhibits tumor growth in ovarian cancer, glioma, and melanoma [7; 8; 9; 10]. Additionally, TSP1 overexpression in melanoma leads to enhanced recruitment and activation of anti-tumor (M1) macrophages in tumor xenografts, indicating a role for TSP1 in the activation of antitumor innate immunity [7]. Other studies report, however, acceleration of tumor growth and metastasis stimulated by TSP1 when overexpressed in several cancers [11; 12; 13].

Pigment epithelium-derived factor (PEDF) is a 50 kD secreted glycoprotein that belongs to the non-inhibitory serpin family [14]. PEDF has potent angiogenesis inhibitory activity [15]; it blocks endothelial cell migration and tube formation even in the presence of VEGF, and it selectively induces endothelial cell apoptosis in actively remodeling vessels via Fas/FasL death receptor signaling [16; 17]. PEDF also up regulates TSP1/TSP2 and down regulates pro-angiogenic factors such as bFGF, VEGF, and MMP-9 [18]. PEDF can suppress tumor growth and metastasis, and down regulation or loss of PEDF is correlated with increased metastases and poor prognosis in a variety of cancers, including breast cancer [19], melanoma [20; 21], prostate cancer [22], pancreatic cancer [23] and osteosarcoma [24]. PEDF exerts antitumor activity by inhibiting tumor angiogenesis or by direct suppression of tumor cell migration and induction of tumor cell differentiation and apoptosis [25]. In addition to anti-angiogenesis, PEDF and TSP1 have both been reported to recruit cytotoxic macrophages into human melanoma and prostate tumors, with increased tumor cell killing [7; 26].

Metronomic chemotherapy refers to the administration of chemotherapeutic agents at relatively low, minimally toxic doses, without prolonged drug-free breaks, and can be more effective and have fewer toxic side-effects than traditional, maximum-tolerated dose chemotherapy [27; 28; 29]. Metronomic chemotherapy is widely understood to inhibit tumor growth primarily by inhibiting tumor angiogenesis, resulting in indirect tumor cell killing by inducing hypoxia and starvation of nutrients [29; 30]. Recent studies have shown, however, that metronomic chemotherapy can also enhance tumor cell killing by activating anti-tumor innate and adaptive immune responses [31; 32; 33].

TSP1 is proposed to be required for the enhanced anti-tumor activity of metronomic chemotherapeutic schedules. Tumor xenografts are unresponsive to metronomic chemotherapy in TSP1 knockout mice [34; 35], and TSP1 expression is frequently increased by metronomic chemotherapy, both in animal models and in patients (e.g. [36; 37; 38; 39]). Treatment of brain tumor xenografts with a metronomic schedule of cyclophosphamide (CPA) induces marked tumor regression associated with substantial increases in TSP1 and PEDF expression. Moreover, these responses are accompanied by a striking recruitment of innate immune cells, including macrophages and natural killer (NK) cells, which are essential for the observed tumor regression [33]. In contrast, in preliminary studies of KM12 human colon tumor xenografts, we found that metronomic CPA treatment did not induce TSP1 or PEDF expression, and only moderate tumor growth delay was observed. Conceivably, the absence of TSP1, or PEDF, may underlie the absence of a strong anti-tumor response in KM12 tumors. Presently, we use the KM12 tumor model to investigate the relationship between TSP1 and PEDF expression, anti-angiogenesis, innate immune cell recruitment, and responsiveness to metronomic chemotherapy. Our findings show that TSP1 and PEDF are anti-angiogenic when expressed by KM12 tumors, and that their expression

increases macrophage recruitment, and responsiveness to metronomic CPA treatment; however, it does not facilitate NK cell recruitment or tumor regression. Moreover, we observed significant effects on metastasis, with TSP1 increasing metastasis of KM12 tumors to the lung, and PEDF synergizing with metronomic CPA to decrease lung metastatic activity.

2. Materials and Methods

2.1. Reagents

Cyclophosphamide (CPA) was purchased from Sigma-Aldrich Co. (St. Louis, MO). 4-hydroperoxycyclophosphamide (4-HC) was obtained from Dr. Ulf Niemeyer (Baxter Oncology GmbH, Frankfurt, Germany). Anti-TSP1 and anti-PEDF antibodies were purchased from Santa Cruz Biotechnology (Cat. #sc-12312 and sc-25594, respectively; Santa Cruz, CA). Anti-CD31 (Cat. #557355), anti-CD68 (Cat. #MCA1957GA), and anti-human COX IV (Cat. #4850) antibodies were obtained from BD Pharmingen (San Diego, CA), AbD Serotec (Raleigh, NC), and Cell Signaling (Danvers, MA), respectively. Biotinylated rabbit anti-rat (Cat. #BA-4000), rabbit anti-goat (Cat. #BA-5000) and goat anti-rabbit (Cat. #BA-1000) secondary antibodies, Vectastain Elite ABC kit (Cat. #PK-6100), and peroxidase substrates VIP (Cat. #SK-4600) were purchased from Vector Laboratories (Burlingame, CA). RPMI 1640 and DMEM high glucose culture media, fetal bovine serum (FBS), geneticin, and TRIzol were purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, RNase inhibitor, and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Foster City, CA).

2.2. Cell culture

Human KM12 colon cancer cells (National Cancer Institute, Bethesda, MD) and rat 9L gliosarcoma cells (Neurosurgery Tissue Bank, UCSF, San Francisco, CA) were authenticated by and obtained from the indicated sources. KM12 cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin. 9L cells were cultured in DMEM high glucose medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

2.3. Retroviral plasmid construction

The retroviral vector pWZL-Neo was obtained from Dr. Thomas Gilmore (Boston University, Boston, MA). pCMV-SPORT6 plasmids carrying mouse TSP1 and mouse PEDF cDNAs were purchased from Open Biosystems (Huntsville, AL). TSP1 cDNA was excised from pCMV-SPORT6 with EcoRV and XhoI, and then ligated into pWZL-Neo vector that was digested with BamHI, polished with T4 DNA polymerase/T4 polynucleotide kinase, and digested with XhoI. PEDF cDNA was excised from pCMV-SPORT6 with HindIII, followed by polishing with T4 DNA polymerase/T4 polynucleotide kinase, and EcoRI digestion. The cDNA was then ligated into pWZL-Neo digested with EcoRI and SnaBI. The retroviral expression plasmids were validated by restriction enzyme digestion and sequencing. Generation of infectious retroviral particles in 293T cells transfected with pWZL-Neo-TSP1 and pWZL-Neo-PEDF plasmids in the presence of pCL-10A1 helper plasmid, harvesting of retroviral supernatants followed by infection of parental KM12 cells in the presence of polybrene, and selection of pools of retrovirally transduced KM12 cells for 10–14 days with geneticin (600 µg/ml) was carried out as described [40].

2.4. Cell growth inhibition assay

The sensitivity of cancer cells to the active form of CPA, 4-OH-CPA, was evaluated using the chemically activated derivative 4-HC. Cells were seeded at 1,000 or 4,000 cells/well in a

96-well plate and cultured overnight. 4-HC was dissolved in ddH₂O, sterilized by passing through a 0.2 µm syringe filter, and added to the cells at final concentrations ranging from 0.4–100 µM. Cells were cultured for 4 days, and the number of viable cells then determined by A₄₉₀ using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). IC₅₀ values were calculated by sigmoidal concentration-response analysis with a variable slope using GraphPad Prism software (GraphPad, San Diego, CA).

2.5. Tumor growth studies

Male immunodeficient Fox Chase ICR *scid* mice, 4–6 wk old, were obtained from Taconic (Germantown, NY) and housed in the Boston University Laboratory of Animal Care Facility in accordance with institutionally approved protocols and federal guidelines. Autoclaved cages containing food and water were changed once a week. Mouse body weight was measured twice weekly. On the day of tumor cell inoculation, cells at 70–80% confluence were collected by trypsinization and washed twice with serum-free RPMI 1640 (KM12 cells) or DMEM high glucose medium (9L cells). Cells were resuspended in serum-free medium at a density of 2×10^7 cells/ml. Four million cells in a total volume of 0.2 ml were injected subcutaneously into each flank of a mouse. Tumor growth was measured every 3 days using a digital caliper (VWR, West Chester, PA), and tumor volumes were calculated as $(3.14/6) \times (\text{length} \times \text{width})^2$. When the average tumor volume reached ~500 mm³, mice were assigned into control and metronomic CPA treatment groups to give similar average tumor volumes in each group. CPA solution was prepared fresh on the day of treatment by dissolving CPA powder into PBS (140 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄) followed by sterilization by passing through a 0.2 µm syringe filter (Pall Corp, Ann Arbor, MI) in a laminar flow hood. CPA was administered on a 6 day-metronomic schedule [41; 42]: 140 mg CPA monohydrate/kg body weight by intraperitoneal injection every 6 days. Tumor samples were collected at designed time points. The number of visible lung metastasis was counted upon dissection. Metastases were confirmed by staining 10 µm lung sections with anti-human COX IV antibody.

2.6. Quantitative real-time PCR (qPCR)

Tumor tissue samples were collected, snap frozen in liquid nitrogen, and stored at –80°C. Total RNA were isolated using TRIzol reagent. RNA (1 µg) was treated with DNase I for 60 min at 37°C, followed by reverse transcription in 20 µl containing MuLV reverse transcriptase, random primers, and RNase inhibitor. The resultant cDNA was diluted 1:20 in 50 µg/µl yeast tRNA and used as the template for quantitative real-time PCR (qPCR) using the following primer sets: mouse TSP1: 5'-TGT-TCA-AGA-GGA-CCG-GGC-T-3', 5'-TGG-ATG-GGT-ACA-TCC-AGC-TCC-3'; mouse PEDF: 5'-ACG-GCC-CTC-TCT-GCC-CTT-TCT-3', 5'-CTG-TGG-ATG-TCA-GGG-TTG-GTG-ATC-AG-3'; mouse CD68: 5'-GCC-CGA-GTA-CAG-TCT-ACC-TGG-3', 5'-AGA-GAT-GAA-TTC-TGC-GCC-AT-3'. RNA primers for iNOS and Arginase 1 (M1 and M2 macrophages, respectively) and NK1.1 (NK cell recruitment) were described previously [33]. qPCR mixtures containing 8 µl of SYBR Green PCR Master Mix, 4 µl of cDNA template, 1 µl of each forward and reverse primers, and 2 µl of ddH₂O were loaded into 384-well plates in triplicate (5 µl/well) and run through 40 cycles on ABS 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Relative RNA levels were calculated from the C_t values of each gene normalized to the 18S rRNA content of each RNA sample. Data were expressed as relative RNA levels, mean ± SE for at least three individual tumors per group.

2.7. Western blot analysis

Culture medium containing secreted proteins was collected and condensed using a PM30 polyethersulfone ultrafiltration membrane (Millipore, Billerica, MA). Frozen tumor samples were homogenized on ice in cell lysis buffer containing 150 mM NaCl, 1% NP-40, 0.5%

deoxycholate, 0.1% SDS, and 50 mM Tris-HCl (pH 7.4). Samples were centrifuged at 2,000 g for 10 min, and the supernatant (50 µg protein) was analyzed on a western blot blocked in TBST (20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1% Tween 20, 5% nonfat dry milk) for 1 h at room temperature and then incubated overnight at 4°C with mouse anti-TSP1 antibody (1:500), followed by incubation with HRP-conjugated anti-mouse antibody (1:3,000, GE Healthcare) for 40 min at room temperature and detection using enhanced chemiluminescence reagents (Pierce, Rockford, IL).

2.8. Tumor cryosectioning and immunohistochemical staining

Fresh tumor and lung tissues were snap-frozen in dry ice-cold 2-methylbutane and stored at -80°C. Cryosections (6 µm and 10 µm, respectively) were prepared using a Leica 1800 cryostat and fixed with 1% paraformaldehyde for 30 min at room temperature. After two 5 min washes with PBS, the sections were incubated with permeabilization solution containing 1% Triton X-100 (v/v) and 1% sodium citrate (w/v) for 5 min on ice. Staining was performed either manually or using a BioGenex i6000 Autostainer (Fremont, CA). Tissue sections were washed twice with PBS for 5 min and blocked for 20 min at room temperature with PBS containing 2% or 3% normal serum from the species where the secondary antibody was raised. Sections were then incubated for 1 h at room temperature with primary antibody (1:1000 dilution for anti-CD31, 1:1000 dilution for COX IV, and 4 µg/ml for anti-PEDF, anti-CD68, and anti-TSP1). After two PBS washes, the sections were incubated with biotinylated secondary antibodies (7.5 µg/ml; Vector Laboratories, CA) for 1 h at room temperature. After two more washes, the sections were incubated with ABC complex for 30 min at room temperature. Peroxidase substrate VIP was added and color development was terminated by immersing the slides in running tap water for 5 min. The slides were dehydrated by sequential immersion in 95% ethanol (2 min, twice), 100% ethanol (2 min, twice), and 100% xylene (3 min, twice) and sealed with VectaMount (Vector Laboratories, CA).

2.9. Statistical analysis

Data were analyzed by one-way ANOVA using GraphPad Prism software (version 4, Graphpad, San Diego, CA), and $p < 0.05$ was considered statistically significant.

3. Results

3.1. KM12 tumor responsiveness to metronomic CPA

Treatment with CPA on a 6-day repeating metronomic schedule [41; 42] induces strong tumor regression in several tumor models, including 9L gliosarcoma (Fig. 1A) [33; 43], but only modest tumor growth delay in other models, including PC3 prostate cancer [44] and KM12 colon adenocarcinoma (Fig. 1B). The absence of KM12 tumor regression is not due to intrinsic CPA resistance, as KM12 cells show sensitivity to activated CPA ($IC_{50} = 6.2$ µM; also see below) very similar to that of 9L and U251 cells (IC_{50} : 3.7 µM and 8.5 µM, respectively) (Fig. 1C).

Metronomic CPA treatment significantly increases expression of the endogenous angiogenesis inhibitor TSP1 in both 9L and U251 tumors [33] but not in KM12 tumors (Fig. 1D). Metronomic CPA also significantly induces the expression of PEDF, a potent endogenous angiogenesis inhibitor that acts upstream of TSP1, in both 9L and U251 tumors [33] but not in KM12 tumors (Fig. 1D). Given the anti-tumor potential of TSP1 and PEDF, and the reported requirement of TSP1 for both anti-angiogenesis and for the anti-tumor activity associated with metronomic chemotherapy [34], we investigated whether the unresponsiveness of these factors in KM12 cells explains the absence of KM12 tumor regression following metronomic CPA treatment.

3.2. KM12 tumors overexpressing TSP1 and PEDF

KM12 cells were infected with retroviral vectors encoding TSP1 and PEDF cDNAs. NH₂-terminal signal peptide sequences were included, to ensure secretion of TSP1 and PEDF proteins, and mouse TSP1 and PEDF sequences were used, to allow each protein factor to interact with its mouse cell targets in the tumor stromal cell compartment. The resultant retrovirally-transduced KM12 cell lines, KM12/TSP1 and KM12/PEDF, are pools comprised of several thousand independently infected KM12 cells. These cells showed marked increases in mouse TSP1 and PEDF RNA and protein when compared to parental (wild-type) KM12 cells (KM12/WT) or empty vector-infected controls (KM12/pWZL-Neo cells) (Fig. 2A–D). As such, we employed tumors seeded with KM12/TSP1 and KM12/PEDF cells as a model for the metronomic CPA-induced expression of TSP1 and PEDF that is observed in several tumor models where metronomic CPA induces innate immune cell recruitment leading to tumor regression [33].

KM12/TSP1 and KM12/PEDF tumors grew at rates similar to KM12/WT tumors (Fig. 2E). Moreover, they retained the elevated expression of TSP1 and PEDF RNA observed in cell culture, as seen in tumors collected up to 38 days after tumor cell implantation (Fig. 3A, 3B). TSP1 and PEDF protein levels were also considerably higher in KM12/TSP1 and KM12/PEDF tumors than in KM12/WT tumors (Fig 3C–E). Notably, KM12/PEDF tumors also showed an increase in TSP1 expression (Fig. 3A, 3C), indicating that ectopically expressed PEDF can up regulate TSP1, consistent with an earlier report in a human glioblastoma cell line [18].

Metronomic CPA markedly inhibited the growth of KM12/TSP1 and KM12/PEDF tumors (Fig. 4A). This strong anti-tumor response contrasts with the more modest growth delay seen in metronomic CPA-treated KM12/WT tumors (tumor growth inhibition: 33% in KM12/WT vs. 62% and 56% in KM12/TSP1 and KM12/PEDF tumors, respectively; Fig. 4B). The increased responsiveness of KM12/TSP1 and KM12/PEDF tumors to metronomic CPA treatment is not due to a higher intrinsic tumor cell chemosensitivity, as the IC₅₀ values for activated CPA (4-HC) were similar between KM12/TSP1 cells (4.8 μM), KM12/PEDF cells (5.2 μM) and wild type KM12 cells (6.2 μM) (Fig. 4C).

Further analysis showed that visible lung metastasis counts were significantly greater in KM12/TSP1 tumor-bearing mice than in mice with KM12/WT tumors or KM12/PEDF tumors (Fig. 4D). Moreover, metronomic CPA treatment significantly decreased lung metastases in mice bearing KM12/PEDF tumors, but not in mice bearing KM12/WT or KM12/TSP1 tumors (Fig. 4D). This finding was confirmed by immunostaining of lung sections for human COX IV, used as a marker for KM12 tumor-derived human tumor metastases in the mouse lung tissues, as validated with non-tumor-bearing mouse lung as a negative control (Fig. 4E). In agreement with the visible metastasis counts, the intensity of COX IV staining was significantly higher in lungs of the KM12/TSP1-tumor bearing mice than in mice bearing KM12/WT or KM12/PEDF tumors. Mouse lung COX IV staining was also significantly decreased by metronomic CPA treatment of the mice bearing KM12/PEDF tumors (Fig. 4E and 4F).

3.3. Impact of TSP1 and PEDF on KM12 tumor microvessel density and innate immune cell recruitment

KM12/TSP1 and KM12/PEDF tumors contained significantly lower levels of microvessel density than KM12/WT tumors (CD31 immunostaining; Fig. 5A, B), indicating that both factors induce anti-angiogenesis, which may contribute to the enhanced responsiveness of these tumors to metronomic CPA. Notably, CD31 immunostaining was less extensive in KM12/PEDF tumors than in KM12/TSP1 tumors, indicating more effective angiogenesis

inhibition by PEDF (Fig. 5A, B). This may, in part, result from the associated increase in TSP1 levels in the PEDF-expressing KM12 tumors.

Next, we considered whether the elevated expression of TSP1 and PEDF in KM12 tumors increases recruitment of cytotoxic macrophages, as occurs in prostate tumors expressing these factors, leading to increased tumor cell killing [7; 26]. Consistent with this possibility, KM12/TSP1 and KM12/PEDF tumors showed significant increases in the macrophage marker CD68 compared to KM12/WT tumors, at both the RNA and protein levels (Fig. 5C, 5D), with further significant increases seen following metronomic CPA treatment (Fig. 5C). However, neither TSP1 nor PEDF increased the ratio of cytotoxic macrophages (M1) to tumor growth-promoting macrophages (M2), as determined using the macrophage markers iNOS (M1) and arginase 1 (M2) (data not shown). This contrasts to the preferential recruitment of cytotoxic (M1) macrophages in TSP1-expressing melanoma and prostate tumors [7; 26]. Finally, other studies have shown that metronomic CPA dramatically increases tumor recruitment of macrophages and also NK cells, leading to increased tumor cell killing and substantial regression of brain tumor xenografts [33]. However, no NK cell infiltration was seen following metronomic CPA treatment of the KM12/WT, KM12/TSP1, or KM12/PEDF tumors (data not shown).

4. Discussion

TSP1 and PEDF are anti-angiogenic and can inhibit tumor growth and metastasis in a variety of cancers [7; 24; 45; 46; 47; 48]. However, TSP1 and PEDF did not alter tumor growth rates when overexpressed in KM12 colon tumor xenografts, despite a marked reduction in tumor microvessel density. This suggests that KM12 tumors tolerate the decreased availability of oxygen and nutrients associated with anti-angiogenesis; alternatively, TSP1 and PEDF may activate pathways and mechanisms that enable KM12 tumors to maintain a normal growth rate under conditions of anti-angiogenesis. Nevertheless, KM12/TSP1 and KM12/PEDF tumors both showed improved responses to metronomic CPA treatment compared to wild type KM12 tumors (near growth stasis vs. modest growth delay). This increase in response could result from increased drug exposure due to anti-angiogenesis-induced blood vessel normalization [49; 50], or perhaps anti-angiogenesis induced drug retention [51]. Other possibilities include the increased infiltration of tumor-associated macrophages when KM12 tumors express TSP1 or PEDF, which may lead to increased phagocytosis [5]. Importantly, although the anti-tumor activity of metronomic CPA was improved by overexpression of TSP1 or PEDF, the improvement was not sufficient to induce tumor regression. KM12 tumors thus differ from 9L gliosarcoma and other tumor models where the same 6 day-repeating metronomic CPA treatment schedule used here not only induces large increases in TSP1 and PEDF expression, and macrophage recruitment, but induces tumor infiltration by NK cells leading to major tumor regression (Fig. 1A) [33], neither of which was seen here in KM12/TSP1 or KM12/PEDF tumors.

KM12 and 9L cells have similar sensitivities to activated CPA in cell culture, but as noted, only 9L tumors regress following metronomic CPA treatment *in vivo*. This difference in response could, in part, be explained by the higher vascularity of 9L tumors [36], which presumably leads to increased intratumoral drug exposure. More important, however, 9L tumor regression induced by metronomic CPA treatment is associated with recruitment and activation of innate immune cells, including NK cells, which are required for the observed strong regression response [33]. In contrast, no NK cell recruitment was seen in metronomic CPA-treated KM12 tumors. Moreover, this deficiency was not rectified by retroviral-induced expression of TSP1 or PEDF. Thus, the resistance of KM12 tumors to metronomic CPA-induced tumor regression appears to reflect the absence of a mechanism to activate

anti-tumor innate immunity. Consistent with this proposal, NK cells are rare in human colorectal carcinoma tissues, even in the presence of high levels of chemokines that activate and recruit these cells, with the capacity for NK cell migration into colorectal carcinoma being impaired early during colorectal carcinoma development [52].

In the present study, we used a 6-day metronomic CPA schedule [41; 42], which we have found is more effective at eliciting an innate immune response than other low-dose CPA schedules including daily CPA treatment (unpublished data). A 6-day repeating metronomic schedule may be sufficiently frequent so as to induce strong tumor cytotoxicity as well as repeated waves of cytokine/chemokine responses leading to the activation of anti-tumor immunity, while including breaks in treatment that are sufficiently long to minimize the killing of immune cells recruited to the tumor. Notably, following metronomic CPA treatment of 9L tumors, there is a sustained period of continued tumor growth (~10 days) during which the tumors almost double in size before regression begins (Fig. 1A). This finding is consistent with our earlier findings [42; 53] and with the mechanism that we proposed for the striking tumor regression seen with the 6-day CPA schedule in this and several other tumor models, namely, activation of an innate immune response, rather than direct cytotoxicity to the tumor cells and to tumor-associated endothelial cells.

TSP1 and PEDF had disparate effects on metastasis of KM12 tumors to the lung. TSP1 increased KM12 lung metastasis, which persisted despite metronomic CPA treatment. In contrast, no increase in lung metastasis was seen in mice bearing KM12/PEDF tumors, and furthermore, metronomic CPA treatment significantly decreased lung metastasis in these mice. A pro-invasive, metastatic response to TSP1 has been reported in other tumor models [11; 12; 13] and is associated with an increase in hypoxia, which triggers cell migration to distant organs [12]. In contrast, PEDF blocks tumor cell extravasation and decreases the frequency of metastasis [21; 54]. Although KM12/PEDF tumors also show increased expression of TSP1, the level of TSP1 is lower than that in KM12/TSP1 tumors, and may not be sufficient to induce metastasis. Additionally, the ability of TSP1 to induce metastasis may be countered by the presence of PEDF. Although metronomic CPA alone did not inhibit metastasis in the KM12 colon tumor model, a daily schedule of metronomic topotecan treatment was reported to significantly reduce liver metastasis and prolong survival in a colon cancer model [55]. Thus, results may vary between drugs, tumor models and metronomic schedules.

In conclusion, we find that overexpression of TSP1 or PEDF in anti-angiogenic, activates macrophage recruitment, and increases metronomic CPA anti-tumor activity in a KM12 colon tumor model. However, neither TSP1 nor PEDF increased tumor infiltration by NK cells or conferred the tumor regression response that follows metronomic CPA treatment in several other tumor models. Finally, TSP1 overexpression led to a significant increase in lung metastasis, while PEDF enhanced the metastasis inhibitory activity of metronomic CPA.

Acknowledgments

This work was supported in part by CA49248 grant (to DJW).

References

1. Weis SM, Cheresh DA. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med.* 2011; 17:1359–1370. [PubMed: 22064426]
2. Isenberg JS, Martin-Manso G, Maxhimer JB, Roberts DD. Regulation of nitric oxide signalling by thrombospondin 1: implications for anti-angiogenic therapies. *Nat Rev Cancer.* 2009; 9:182–194. [PubMed: 19194382]

3. Lawler PR, Lawler J. Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. *Cold Spring Harb Perspect Med.* 2012; 2:a006627. [PubMed: 22553494]
4. Kaur S, Martin-Manso G, Pendrak ML, Garfield SH, Isenberg JS, Roberts DD. Thrombospondin-1 inhibits VEGF receptor-2 signaling by disrupting its association with CD47. *J Biol Chem.* 2010; 285:38923–38932. [PubMed: 20923780]
5. Willingham SB, Volkmer JP, Gentles AJ, Sahoo D, Dalerba P, Mitra SS, Wang J, Contreras-Trujillo H, Martin R, Cohen JD, Lovelace P, Scheeren FA, Chao MP, Weiskopf K, Tang C, Volkmer AK, Naik TJ, Storm TA, Mosley AR, Edris B, Schmid SM, Sun CK, Chua MS, Murillo O, Rajendran P, Cha AC, Chin RK, Kim D, Adorno M, Raveh T, Tseng D, Jaiswal S, Enger PO, Steinberg GK, Li G, So SK, Majeti R, Harsh GR, van de Rijn M, Teng NN, Sunwoo JB, Alizadeh AA, Clarke MF, Weissman IL. The CD47-signal regulatory protein alpha (SIRPα) interaction is a therapeutic target for human solid tumors. *Proc Natl Acad Sci U S A.* 2012; 109:6662–6667. [PubMed: 22451913]
6. Greenaway J, Lawler J, Moorehead R, Bornstein P, Lamarre J, Petrik J. Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1). *J Cell Physiol.* 2007; 210:807–818. [PubMed: 17154366]
7. Martin-Manso G, Galli S, Ridnour LA, Tsokos M, Wink DA, Roberts DD. Thrombospondin 1 promotes tumor macrophage recruitment and enhances tumor cell cytotoxicity of differentiated U937 cells. *Cancer Res.* 2008; 68:7090–7099. [PubMed: 18757424]
8. Campbell N, Greenaway J, Henkin J, Petrik J. ABT-898 induces tumor regression and prolongs survival in a mouse model of epithelial ovarian cancer. *Mol Cancer Ther.* 2011; 10:1876–1885. [PubMed: 21844212]
9. Greenaway J, Henkin J, Lawler J, Moorehead R, Petrik J. ABT-510 induces tumor cell apoptosis and inhibits ovarian tumor growth in an orthotopic, syngeneic model of epithelial ovarian cancer. *Mol Cancer Ther.* 2009; 8:64–74. [PubMed: 19139114]
10. Nabors LB, Fiveash JB, Markert JM, Kekan MS, Gillespie GY, Huang Z, Johnson MJ, Meleth S, Kuo H, Gladson CL, Fathallah-Shaykh HM. A phase I trial of ABT-510 concurrent with standard chemoradiation for patients with newly diagnosed glioblastoma. *Arch Neurol.* 2010; 67:313–319. [PubMed: 20212229]
11. Lin XD, Chen SQ, Qi YL, Zhu JW, Tang Y, Lin JY. Overexpression of thrombospondin-1 in stromal myofibroblasts is associated with tumor growth and nodal metastasis in gastric carcinoma. *J Surg Oncol.* 2012
12. Firlej V, Mathieu JR, Gilbert C, Lemonnier L, Nakhle J, Gallou-Kabani C, Guarmit B, Morin A, Prevarskaya N, Delongchamps NB, Cabon F. Thrombospondin-1 triggers cell migration and development of advanced prostate tumors. *Cancer Res.* 2011; 71:7649–7658. [PubMed: 22037878]
13. Nucera C, Porrello A, Antonello ZA, Mekel M, Nehs MA, Giordano TJ, Gerald D, Benjamin LE, Priolo C, Puxeddu E, Finn S, Jarzab B, Hodin RA, Pontecorvi A, Nose V, Lawler J, Parangi S. B-Raf(V600E) and thrombospondin-1 promote thyroid cancer progression. *Proc Natl Acad Sci U S A.* 2010; 107:10649–10654. [PubMed: 20498063]
14. Filleur S, Nelius T, de Riese W, Kennedy RC. Characterization of PEDF: a multi-functional serpin family protein. *J Cell Biochem.* 2009; 106:769–775. [PubMed: 19180572]
15. Manalo KB, Choong PFM, Dass CR. Pigment epithelium-derived factor as an impending therapeutic agent against vascular epithelial growth factor-driven tumor-angiogenesis. *Mol Carcinog.* 2011; 50:67–72. [PubMed: 21229603]
16. Zaichuk TA, Shroff EH, Emmanuel R, Filleur S, Nelius T, Volpert OV. Nuclear factor of activated T cells balances angiogenesis activation and inhibition. *J Exp Med.* 2004; 199:1513–1522. [PubMed: 15184502]
17. Elayappan B, Ravinarayannan H, Pasha SP, Lee KJ, Gurunathan S. PEDF inhibits VEGF- and EPO- induced angiogenesis in retinal endothelial cells through interruption of PI3K/Akt phosphorylation. *Angiogenesis.* 2009; 12:313–324. [PubMed: 19657716]
18. Guan M, Pang CP, Yam HF, Cheung KF, Liu WW, Lu Y. Inhibition of glioma invasion by overexpression of pigment epithelium-derived factor. *Cancer Gene Ther.* 2004; 11:325–332. [PubMed: 15044958]

19. Cai J, Parr C, Watkins G, Jiang WG, Boulton M. Decreased pigment epithelium-derived factor expression in human breast cancer progression. *Clin Cancer Res.* 2006; 12:3510–3517. [PubMed: 16740777]
20. Orgaz JL, Ladhani O, Hoek KS, Fernandez-Barral A, Mihic D, Aguilera O, Seftor EA, Bernad A, Rodriguez-Peralto JL, Hendrix MJ, Volpert OV, Jimenez B. Loss of pigment epithelium-derived factor enables migration, invasion and metastatic spread of human melanoma. *Oncogene.* 2009; 28:4147–4161. [PubMed: 19767774]
21. Ladhani O, Sanchez-Martinez C, Orgaz JL, Jimenez B, Volpert OV. Pigment epithelium-derived factor blocks tumor extravasation by suppressing amoeboid morphology and mesenchymal proteolysis. *Neoplasia.* 2011; 13:633–642. [PubMed: 21750657]
22. Halin S, Wikstrom P, Rudolfsson SH, Stattin P, Doll JA, Crawford SE, Bergh A. Decreased pigment epithelium-derived factor is associated with metastatic phenotype in human and rat prostate tumors. *Cancer Res.* 2004; 64:5664–5671. [PubMed: 15313905]
23. Uehara H, Miyamoto M, Kato K, Ebihara Y, Kaneko H, Hashimoto H, Murakami Y, Hase R, Takahashi R, Mega S, Shichinohe T, Kawarada Y, Itoh T, Okushiba S, Kondo S, Katoh H. Expression of pigment epithelium-derived factor decreases liver metastasis and correlates with favorable prognosis for patients with ductal pancreatic adenocarcinoma. *Cancer Res.* 2004; 64:3533–3537. [PubMed: 15150108]
24. Ek ET, Dass CR, Contreras KG, Choong PF. Pigment epithelium-derived factor overexpression inhibits orthotopic osteosarcoma growth, angiogenesis and metastasis. *Cancer Gene Ther.* 2007; 14:616–626. [PubMed: 17479108]
25. Fernandez-Garcia NI, Volpert OV, Jimenez B. Pigment epithelium-derived factor as a multifunctional antitumor factor. *J Mol Med (Berl).* 2007; 85:15–22. [PubMed: 17106733]
26. Halin S, Rudolfsson SH, Doll JA, Crawford SE, Wikstrom P, Bergh A. Pigment epithelium-derived factor stimulates tumor macrophage recruitment and is downregulated by the prostate tumor microenvironment. *Neoplasia.* 2010; 12:336–345. [PubMed: 20360944]
27. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *J Clin Invest.* 2000; 105:1045–1047. [PubMed: 10772648]
28. Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest.* 2000; 105:R15–24. [PubMed: 10772661]
29. Pasquier E, Kavallaris M, Andre N. Metronomic chemotherapy: new rationale for new directions. *Nat Rev Clin Oncol.* 2010; 7:455–465. [PubMed: 20531380]
30. Andre N, Padovani L, Verschuur A. Metronomic chemotherapy: Back to the future! *Drug News Perspect.* 2010; 23:143–151. [PubMed: 20369080]
31. Sistigu A, Viaud S, Chaput N, Bracci L, Proietti E, Zitvogel L. Immunomodulatory effects of cyclophosphamide and implementations for vaccine design. *Semin Immunopathol.* 2011; 33:369–383. [PubMed: 21611872]
32. Rozados VR, Mainetti LE, Rico MJ, Zacarias Fluck MF, Matar P, Scharovsky OG. The immune response and the therapeutic effect of metronomic chemotherapy with cyclophosphamide. *Oncol Res.* 2010; 18:601–605. [PubMed: 20939436]
33. Doloff JC, Waxman DJ. VEGF receptor inhibitors block the ability of metronomically dosed cyclophosphamide to activate innate immunity-induced tumor regression. *Cancer Res.* 2012
34. Bocci G, Francia G, Man S, Lawler J, Kerbel RS. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. *Proc Natl Acad Sci U S A.* 2003; 100:12917–12922. [PubMed: 14561896]
35. Hamano Y, Sugimoto H, Soubasakos MA, Kieran M, Olsen BR, Lawler J, Sudhakar A, Kalluri R. Thrombospondin-1 associated with tumor microenvironment contributes to low-dose cyclophosphamide-mediated endothelial cell apoptosis and tumor growth suppression. *Cancer Res.* 2004; 64:1570–1574. [PubMed: 14996710]
36. Ma J, Waxman DJ. Collaboration between hepatic and intratumoral prodrug activation in a P450 prodrug-activation gene therapy model for cancer treatment. *Mol Cancer Ther.* 2007; 6:2879–2890. [PubMed: 17989319]

37. Damber J-E, Vallbo C, Albertsson P, Lennernas B, Norrby K. The anti-tumour effect of low-dose continuous chemotherapy may partly be mediated by thrombospondin. *Cancer Chemother Pharmacol.* 2006; 58:354–360. [PubMed: 16333676]
38. Laquente B, Lacasa C, Ginesta MM, Casanovas O, Figueras A, Galan M, Ribas IG, Germa JR, Capella G, Vinals F. Antiangiogenic effect of gemcitabine following metronomic administration in a pancreas cancer model. *Mol Cancer Ther.* 2008; 7:638–647. [PubMed: 18347150]
39. Iwamoto H, Torimura T, Nakamura T, Hashimoto O, Inoue K, Kurogi J, Niizeki T, Kuwahara R, Abe M, Koga H, Yano H, Kerbel RS, Ueno T, Sata M. Metronomic S-1 chemotherapy and vandetanib: an efficacious and nontoxic treatment for hepatocellular carcinoma. *Neoplasia.* 2011; 13:187–197. [PubMed: 21390182]
40. Jounaidi Y, Chen CS, Veal GJ, Waxman DJ. Enhanced antitumor activity of P450 prodrug-based gene therapy using the low Km cyclophosphamide 4-hydroxylase P450 2B11. *Mol Cancer Ther.* 2006; 5:541–555. [PubMed: 16546968]
41. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer research.* 2000; 60:1878–1886. [PubMed: 10766175]
42. Jounaidi Y, Waxman DJ. Frequent, moderate-dose cyclophosphamide administration improves the efficacy of cytochrome P-450/cytochrome P-450 reductase-based cancer gene therapy. *Cancer research.* 2001; 61:4437–4444. [PubMed: 11389073]
43. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. *Mol Cancer Ther.* 2008; 7:3670–3684. [PubMed: 19074844]
44. Ma J, Waxman DJ. Dominant effect of antiangiogenesis in combination therapy involving cyclophosphamide and axitinib. *Clin Cancer Res.* 2009; 15:578–588. [PubMed: 19147763]
45. Kazerounian S, Yee KO, Lawler J. Thrombospondins in cancer. *Cell Mol Life Sci.* 2008; 65:700–712. [PubMed: 18193162]
46. Yang H, Grossniklaus HE. Constitutive overexpression of pigment epithelium-derived factor inhibition of ocular melanoma growth and metastasis. *Invest Ophthalmol Vis Sci.* 2010; 51:28–34. [PubMed: 19661223]
47. Guan M, Jiang H, Xu C, Xu R, Chen Z, Lu Y. Adenovirus-mediated PEDF expression inhibits prostate cancer cell growth and results in augmented expression of PAI-2. *Cancer Biol Ther.* 2007; 6:419–425. [PubMed: 17471021]
48. Abe R, Shimizu T, Yamagishi S, Shibaki A, Amano S, Inagaki Y, Watanabe H, Sugawara H, Nakamura H, Takeuchi M, Imaizumi T, Shimizu H. Overexpression of pigment epithelium-derived factor decreases angiogenesis and inhibits the growth of human malignant melanoma cells in vivo. *Am J Pathol.* 2004; 164:1225–1232. [PubMed: 15039211]
49. Campbell NE, Greenaway J, Henkin J, Moorehead RA, Petrik J. The thrombospondin-1 mimetic ABT-510 increases the uptake and effectiveness of cisplatin and paclitaxel in a mouse model of epithelial ovarian cancer. *Neoplasia.* 2010; 12:275–283. [PubMed: 20234821]
50. Willett CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK. Direct evidence that the VEGF-specific antibody bevacizumab has antivasculature effects in human rectal cancer. *Nat Med.* 2004; 10:145–147. [PubMed: 14745444]
51. Ma J, Chen CS, Blute T, Waxman DJ. Antiangiogenesis enhances intratumoral drug retention. *Cancer Res.* 2011; 71:2675–2685. [PubMed: 21447737]
52. Halama N, Braun M, Kahlert C, Spille A, Quack C, Rahbari N, Koch M, Weitz J, Kloor M, Zoernig I, Schirmacher P, Brand K, Grabe N, Falk CS. Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. *Clin Cancer Res.* 2011; 17:678–689. [PubMed: 21325295]
53. Ma J, Waxman DJ. Modulation of the antitumor activity of metronomic cyclophosphamide by the angiogenesis inhibitor axitinib. *Mol Cancer Ther.* 2008; 7:79–89. [PubMed: 18202011]
54. Yang J, Chen S, Huang X, Han J, Wang Q, Shi D, Cheng R, Gao G, Yang X. Growth suppression of cervical carcinoma by pigment epithelium-derived factor via anti-angiogenesis. *Cancer Biol Ther.* 2010; 9:967–974. [PubMed: 20364117]

55. Hackl C, Man S, Francia G, Milsom C, Xu P, Kerbel RS. Metronomic oral topotecan prolongs survival and reduces liver metastasis in improved preclinical orthotopic and adjuvant therapy colon cancer models. *Gut*. 2012

Figure 1. Metronomic CPA regresses 9L tumors but induces growth delay of KM12 tumors

A. Metronomic CPA (140 mg/kg body weight) induces near complete 9L tumor regression after 8 treatment cycles, given 6-days apart (arrows along x-axis; mean $\pm$ SE, $n=12$ tumors). Day 0, first CPA treatment. 9L tumors continue to grow for ~ 2 –3 cycles before regressing, as seen previously [39]. **B.** Metronomic CPA treatment, as in A, caused tumor growth delay in KM12 tumors (mean $\pm$ SE, $n=10$ tumors/group). **C.** Chemosensitivity of the 3 indicated cultured cell lines to 4-HC, assayed 4 days after initiation of drug treatment. Data shown are mean values $\pm$ SD ($n=3$). IC₅₀ values were determined by sigmoidal concentration-response analysis with a variable slope using GraphPad Prism software. **D.** Relative mouse TSP1 and PEDF RNA levels in wild type KM12 tumors that were untreated (UT) or following 1, 2 or 3 cycles of metronomic CPA treatment on a 6-day repeating schedule (i.e. days 6, 12, and 18), as in A (mean $\pm$ SE, $n=4$ –6 tumors/group). Differences between groups were not significant by ANOVA ($p>0.05$).

Figure 2. Retroviral expression of TSP1 and PEDF in KM12 tumor cells

A. Relative levels of mouse TSP1 and PEDF RNA in KM12/WT, KM12/TSP1, and KM12/PEDF cells. Data shown are mean $\pm$ SD, $n=3$; ***, $p<0.001$ compared to KM12/pWZL-Neo negative control. **B.** Western blot showing secreted TSP1 protein migrating as a doublet in culture medium from two independent pools of retrovirally infected KM12/TSP1 (lanes 2, 4) and KM12/PEDF cells (lanes 3, 5), with HUVEC cell lysate serving as a positive control and pWZL-Neo (empty vector)-infected KM12 cell culture medium as a negative control. **C.** TSP1 immunostaining of KM12/WT and KM12/TSP1 cell populations. **D.** PEDF immunostaining of KM12/WT and KM12/PEDF cell populations. **E.** Growth rates of untreated KM12/WT, KM12/TSP1 and KM12/PEDF tumors (mean $\pm$ SE, $n=10-12$ tumors/group; $p>0.05$ compared to KM12/WT tumors). X-axis, days after tumor implantation.

Figure 3. Expression of TSP1 and PEDF by KM12/TSP1 and KM12/PEDF tumors
 qPCR analysis of mouse TSP1 (**A**) and PEDF (**B**) RNA levels in each of the indicated untreated KM12 tumor lines (mean $\pm$ SE, $n=4$ tumors per time point, as shown in Fig. 2E). **, $p<0.01$; ***, $p<0.001$ compared to the corresponding time point of KM12/WT tumors. **C**. TSP1 Western blot of homogenized tumor lysates showing elevated TSP1 protein levels in KM12/TSP1 tumors, and to a lesser extent in KM12/PEDF tumors, compared to KM12/WT controls. Lanes represent individual tumors. **D**. TSP1 immunostaining of untreated KM12/WT and KM12/TSP1 tumors. **E**. PEDF immunostaining of untreated KM12/WT and KM12/PEDF tumors.

Figure 4. Effect of TSP1 and PEDF expression on responsiveness of KM12 tumors to metronomic CPA treatment and on tumor metastasis

A. Metronomic CPA treatment, as in Fig. 1A, induces growth stasis in KM12/TSP1 and KM12/PEDF tumors (mean $\pm$ SE, $n=12$ tumors/group). Untreated tumor volume data is the same as shown in Fig. 3E. X-axis, days after first CPA injection. **B.** Tumor growth inhibition by metronomic CPA; * $p<0.05$ compared to KM12/WT tumors. Data are mean values $\pm$ SE, $n=10$ –12 tumors/group. **C.** Sensitivity of cultured KM12/WT, KM12/TSP1, and KM12/PEDF cells to 4-HC, assayed as in Fig. 1C. Data are mean values $\pm$ SD ($n=3$). **D.** Number of visible lung metastasis per tumor-bearing mouse. Data are mean values $\pm$ SE for $n=11$ –15 mice/group. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ for the indicated comparisons. **E.** COX IV immunostaining of mouse lung cryosections from non-tumor-bearing mice (normal) and from mice bearing the indicated KM12 tumor xenografts. Brown staining indicates positive COX IV signal and blue staining indicates nuclear counterstaining by hematoxylin. **F.** Scoring of intensity of anti-COX IV staining of lung sections on a scale of 0 (low) to 3 (high). Data are mean scores $\pm$ SE, for $n=3$ –5 mice/group, and * $p<0.05$ for the indicated comparisons.

Figure 5. TSP1 and PEDF decrease microvessel density and increase macrophage recruitment in KM12 tumors

A. CD31 staining of KM12/WT, KM12/TSP1, and KM12/PEDF tumor sections. **B.** ImageJ analysis of CD31 positive area on tumor sections, with KM12/WT tumors set to 100%. Data shown are mean $\pm$ SE, n = 5 tumors/group; * $p < 0.05$ and ** $p < 0.01$ compared to KM12/WT; †† $p < 0.01$ compared to KM12/TSP1. **C.** qPCR analysis of RNA levels of macrophage marker CD68 in metronomic CPA- treated and untreated KM12/WT, KM12/TSP1, and KM12/PEDF tumors (mean $\pm$ SE, n = 9 tumors/group). * $p < 0.05$ compared to untreated KM12/WT; ††, $p < 0.01$ and †††, $p < 0.001$ compared to CPA-treated KM12/WT; ‡, $p < 0.05$ CPA treatment compared to the corresponding untreated tumors. **D.** CD68 immunostaining of the indicated KM12 tumors.