Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of anti-angiogenic therapy

Saul J. Priceman1, James L. Sung1, Zory Shaposhnik5, Jeremy B. Burton1*, Antoni X. Torres-Collado6, Diana L. Moughon1, Mai Johnson1, Aldons J. Lusis7, Donald A. Cohen8, M. Luisa Iruela-Arispe3,6, Lily Wu1-4‡

1Department of Molecular and Medical Pharmacology, 2Institute for Molecular Medicine (IMED), 3Jonsson Comprehensive Cancer Center, 4David Geffen School of Medicine, 5California Nanosystems Institute, 6Molecular, Cell, and Developmental Biology, 7Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA

8Microbiology, Immunology, and Molecular Genetics, University of Kentucky, College of Medicine, Lexington, KY

*Genentech Inc., 1 DNA Way, South San Francisco, CA

Key words: TIMs, MDSCs, CSF1R Signaling, Anti-angiogenic therapy

Running title: Targeting MDSCs by Inhibiting CSF-1 Receptor Signaling

‡Requests for reprints: Lily Wu, Department of Molecular and Medical Pharmacology, CHS 33-142, Box 951735, University of California, Los Angeles, California 90095, USA. Phone (310)-825-8511; Fax (310)-794-4759; E-mail: LWu@mednet.ucla.edu
Abstract

Tumor-infiltrating myeloid cells (TIMs) support tumor growth by promoting angiogenesis and suppressing anti-tumor immune responses. CSF-1 receptor (CSF1R) signaling is important for the recruitment of CD11b+F4/80+ tumor-associated macrophages (TAMs) and contributes to myeloid cell-mediated angiogenesis. However, the impact of the CSF1R signaling pathway on other TIM subsets, including CD11b+Gr-1+ myeloid-derived suppressor cells (MDSCs), is unknown. Tumor-infiltrating MDSCs have also been shown to contribute to tumor angiogenesis and have recently been implicated in tumor resistance to anti-angiogenic therapy, yet their precise involvement in these processes is not well understood. Here, we utilize the selective pharmacologic inhibitor of CSF1R signaling, GW2580, to demonstrate that CSF-1 regulates the tumor recruitment of CD11b+Gr-1^hiLy6C^hi mononuclear MDSCs. Targeting these TIM subsets inhibits tumor angiogenesis associated with reduced expression of pro-angiogenic and immunosuppressive genes. Combination therapy utilizing GW2580 with an anti-VEGFR-2 antibody synergistically suppresses tumor growth and severely impairs tumor angiogenesis along with reverting at least one TIM-mediated anti-angiogenic compensatory mechanism involving MMP-9. These data highlight the importance of CSF1R signaling in the recruitment and function of distinct TIM subsets, including MDSCs, and validate the benefits of targeting CSF1R signaling in combination with anti-angiogenic drugs for the treatment of solid cancers.
**Introduction**

Solid tumors contain a significant population of infiltrating myeloid cells that support tumor progression by promoting angiogenesis and suppressing anti-tumor immune responses. Clinical data and experimental studies have established the protumorigenic potential of tumor-associated macrophages (TAMs)[1,2]. TAMs are typically characterized as either “classically” activated tumoricidal macrophages (termed M1) or “alternatively” activated pro-tumorigenic macrophages (termed M2)[3]. Recently, infiltrating myeloid cells have been further classified by their surface marker expression profiles into distinct subsets of tumor-infiltrating myeloid cells (TIMs), including the aforementioned TAMs, along with neutrophils, CD11b^+Gr-1^loLy6C^hi^ mononuclear and CD11b^+Gr-1^hiLy6C^lo^ polymorphonuclear myeloid-derived suppressor cells (MO-MDSCs and PMN-MDSCs, respectively) with diverse functions within the tumor and in other tissues[4-7]. A more detailed characterization of these TIMs will be important for understanding their relevance in tumor progression and for developing novel anti-cancer therapies.

The mechanisms underlying the recruitment and function of TIMs have been an area of intense research. Several cytokines and chemokines are clearly implicated in the recruitment of TAMs, including macrophage colony-stimulating factor-1 (M-CSF, CSF-1) and monocyte chemotactic protein-1 (MCP-1, CCL2)[5,8]. CSF-1 signaling through its receptor CSF1R (CD115, c-fms) is a critical regulator of the survival, differentiation, and proliferation of monocytes and macrophages[9]. Early studies by Lin et al. using CSF-1 op/op mice provided the first evidence for the critical role of CSF-1 in TAM infiltration of spontaneous MMTV-PyMT breast tumors[8]. These TAM-depleted tumors
exhibited reduced angiogenesis and delayed tumor progression to metastasis. More recent studies using therapeutic antibodies directed against human CSF-1 showed similar anti-tumor effects on human breast cancer xenografts[10]. CSF-1 has also been shown to stimulate VEGF-A production in monocytes, demonstrating its direct role in myeloid cell-mediated angiogenesis[11]. Recently, CSF1R expression was observed on MDSCs[12,13], although the consequence of this expression was not experimentally evaluated.

GW2580, a selective small molecule kinase inhibitor of CSF1R was recently described[14]. This orally bioavailable competitive inhibitor of ATP-binding completely prevented CSF1R-dependent growth of macrophages in vitro and in vivo at therapeutically relevant doses[14]. Subsequent in vitro kinase assays demonstrated at least 100-fold selectivity for its target compared with approximately 300 other structurally related and unrelated kinases[15]. The selectivity and specificity of GW2580 makes it a powerful pharmacological tool for determining the contributions of CSF1R signaling to the recruitment and function of different TIM subsets in vivo.

In the current study, we evaluate the impact of CSF1R signaling on the recruitment of various TIM subsets and their contributions to tumor progression. Using GW2580, we show that recruitment of TAMs as well as MO-MDSCs to lung, melanoma, and prostate tumors is regulated by CSF1R signaling. These TIM subsets modulate the expression of VEGF-A, MMP-9, and ARG1, thus contributing to the pro-angiogenic and immunosuppressive environment within the tumor. Moreover, we demonstrate that targeting CSF1R signaling in combination with a specific blocking antibody against VEGFR-2 results in greater inhibition of tumor angiogenesis along with synergistic
tumor growth reduction as compared with anti-angiogenic therapy alone. This work also highlights a TIM-mediated compensatory mechanism involving MMP-9, which may underlie tumor evasion of anti-angiogenic therapy. Overall, these data demonstrate the importance of CSF1R signaling for the recruitment and function of distinct TIM subsets, including MDSCs, in solid tumors.
Materials and Methods

Cell culture: Murine macrophage RAW264.7 cells (ATCC), 3LL Lewis lung carcinoma cells (ATCC), B16F1 melanoma cells (ATCC), and ras + myc-transformed RM-1 prostate tumor cells (a kind gift from Dr. Timothy C. Thompson, Baylor College of Medicine) were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete DMEM) at 37°C with 5% CO₂.

Viability assay: Cell viability studies were performed using murine bone marrow-derived macrophages (BMDMs). Briefly, mouse bone marrow cells were harvested by flushing the tibias and femurs of adult C57BL/6 mice followed by red blood cell (RBC) lysis (Sigma-Aldrich). Cells were cultured for 5-7 days in complete DMEM with 10 ng/mL mouse CSF-1 (RnD Systems). BMDMs (1.5 x 10⁴ cells/well in 96-well plates) were incubated with 10 or 50 ng/mL CSF-1 in the presence or absence of GW2580. GW2580 was dissolved in DMSO at 25 mM and stored at –20°C. Cell viability was measured at indicated timepoints using the CCK-8 assay (Dojindo).

In vitro migration assay: BMDMs (2 x 10⁵ cells) were seeded in cell culture inserts (8-μm pore size; BD Falcon) in DMEM containing 0.1% FBS with or without 1000 nM GW2580. Inserts were placed in 24-well plates with DMEM containing 0.1% FBS in the presence or absence of 10 ng/mL CSF-1 and/or 1000 nM GW2580. After 6 hours, migrated cells were immediately fixed in 3% paraformaldehyde (PFA) stained with DAPI. At least ten fields/well at 4x magnification were quantified using ImageJ.
**Western blot (WB) analysis:** Tissues were minced to a fine powder in liquid nitrogen and lysed in 1% NP-40 lysis buffer containing protease inhibitor cocktail (1:1000, Sigma-Aldrich) and 2 mM sodium orthovanadate (Sigma-Aldrich). Protein lysates (50-100 μg) were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were probed and re-probed using antibodies specific for mouse MMP-9 or α-Tubulin (1:1000, Abcam), and detected using an enhanced chemiluminescence kit (Amersham).

**Immunoprecipitation (IP):** RAW264.7 cells (5 x 10^5 cells/well in 6-well plates) were cultured in DMEM containing 0.1% FBS overnight and then incubated with GW2580 for 1.5 hrs followed by stimulation with CSF-1 in the presence or absence of GW2580 for 20 min. Protein lysates (500 μg) were incubated with anti-mouse CSF1R antibody (1:250, Upstate) for 1 hr at 4°C prior to adding protein A/G PLUS-Agarose (Santa Cruz Biotechnology) overnight. Samples were separated as described above and membranes were probed using antibodies specific for mouse CSF1R (1:2000, Upstate), mouse phosphotyrosine (1:1000, PY99, Santa Cruz Biotechnology), or β-Actin (1:5000, Sigma-Aldrich).

**Gelatin Zymography:** Protein lysates (200 μg) were purified with Gelatin Sepharose pre-equilibrated in PBS (GE Healthcare) for 2 h at 4°C with constant rotation, washed twice in PBS, and separated through a 0.1% gelatin-containing SDS-PAGE. Gels were washed with renaturing buffer (2.5% Triton X-100 in water) for 1 h, and then incubated for 24 hrs at 37°C in reaction buffer (50mM Tris-HCl pH 7.5, 10mM CaCl2, 0.02%...
sodium azide). Gels were stained in 0.1% amido black and destained with destaining solution (10% acetic acid, 30% methanol, 60% H$_2$O).

**Real-time RT-PCR analysis:** Real-time quantitative PCR was carried out as previously described[16]. Primer sequences and product sizes are listed in Table S1.

**In vivo tumor models:** C57BL6 male mice (4-8 week old) were purchased from Taconic Farms, Inc. 3LL cells (2 x 10$^5$ cells) and B16F1 cells (1 x 10$^5$ cells) were implanted subcutaneously above the right shoulder. All animal experiments were approved by the Animal Research Committee of the University of California, Los Angeles. Tumor size was measured by digital calipers as previously described[16]. Mice were sacrificed and tissues were analyzed at the ethical tumor size limit of 1 cm in diameter. For orthotopic implants, RM-1 cells (5 x 10$^3$ cells in 50% matrigel) were surgically implanted in the dorsolateral prostate of C57BL/6 male mice as previously described[16]. Mice were treated with control diluent (0.5% hydroxypropyl methylcellulose (Sigma-Aldrich), 0.1% Tween20 in distilled H$_2$O) or GW2580 by oral gavage beginning one day following tumor implantation. For clodronate liposome (Clodrolip) studies, mice were treated with PBS or Clodrolip at 0.2 mL per 25 g total body weight every third day beginning two days following tumor implantation. Clodronate was purchased from Sigma-Aldrich. Liposomes-encapsulated clodronate was prepared as previously described[17]. For DC101 studies, mice were treated with PBS control or 800 μg DC101 every other day (i.p.). For combination studies, mice were treated with 160 mg/kg GW2580 daily and DC101 every other day beginning one day following tumor implantation.
MaFIA transgenic mice were bred in-house (originally obtained from Dr. Donald Cohen, University of Kentucky). To generate MaFIA chimeras, bone marrow cells were harvested from donor MaFIA mice and intravenously injected into lethally irradiated (950 rad) recipient C57BL/6 mice and transplanted mice recovered for at least 4 weeks. To ablate CSF1R+ cells, mice were treated i.v. or i.p. with 4% ethanol (Control) or AP20187 (in 4% ethanol, ARIAD Pharmaceuticals) as previously described[18].

**Immunohistochemistry:** Tissues were harvested and fixed in 3% PFA overnight. Sections (5 μm) were stained with the following antibodies: anti-F4/80 (1:500, Serotec), anti-Gr-1 (1:100, eBioscience), anti-MMP-9 (1:1000, Abcam), anti-GFP (1:100, Invitrogen), anti-Lyve-1 (1:300, RELIATech) or anti-CD31 (1:300, BD Biosciences) antibodies. Histology was performed, processed, and quantified as previously described[19].

**Flow cytometry:** To prepare single cell suspensions for flow cytometry, harvested tissues (tumors, lungs, and livers) were dissected into approximately 1-3 mm³ fragments and digested with 80 U/mL collagenase (Invitrogen) in DMEM containing 10% FBS for 1.5 hrs at 37°C while shaking. Spleens were gently dissociated between two glass slides for single cell isolation. Peripheral blood was isolated directly into BD Vacutainer K2 EDTA tubes (BD Biosciences). Following RBC lysis, single cell suspensions were filtered and incubated for 30 min on ice with the following FITC-, PE-, APC-, PerCP-Cy5.5-, PE-Cy7- and APC-Cy7-conjugated antibodies: CD45, CD11b, Gr-1, and F4/80 were purchased from eBioscence (1:200). Ly6C (1:200) and 7-AAD were purchased
from BD Bioscience. DAPI was purchased from Invitrogen. Cells were washed twice prior to analysis on the BD LSR-II flow cytometer (Beckman Coulter). Cell sorting was performed on the BD FACS Aria-II high-speed cell sorter using fluorophore-conjugated antibodies for CD11b, Gr-1, and Ly6C. Data was analyzed with FlowJo software (TreeStar).

**In vivo migration assay:** Bone marrow cells (BMCs) obtained from naïve C57BL/6 mice were labeled with 0.25 μM CFDA-SE (CFSE, Invitrogen) for 3 min at ART, washed and incubated with control or 1000 nM GW2580 on ice for 1 hr. 20 x 10^6 cells were then i.v. injected into 3LL tumor-bearing mice (Day 10). Mice were treated with control diluent or 160 mg/kg GW2580 4 hrs prior to BMC injection and sacrificed 4 hrs post-injection. Harvested tissues were subjected to flow cytometric analysis of infiltrating BMCs.

**Statistical analysis:** Data are presented as averages with standard error mean (SEM). Statistical comparisons between groups were performed using the Student’s *t* test.
Results

Inhibiting CSF1R signaling with the pharmacologic inhibitor GW2580. We first validated the inhibition of CSF1R phosphorylation with GW2580[14] using RAW264.7 murine macrophages stimulated with 10 ng/mL CSF-1. CSF1R phosphorylation was potently inhibited in a dose-dependent fashion (Figure 1A) with an IC\textsubscript{50} of approximately 10 nM, substantiating prior results using \textit{in vitro} kinase assays[15]. Complete phospho-inhibition was observed between 100 and 1000 nM GW2580. These experiments were repeated using 50 ng/mL CSF-1 with similar results (data not shown).

CSF1R signaling plays a critical role in the survival, differentiation, and proliferation of monocytes and macrophages[9]. As shown in Figure 1B, CSF1R blockade with GW2580 showed a dose-dependent inhibition of CSF-1-stimulated growth of murine bone marrow-derived macrophages (BMDMs), with an IC\textsubscript{50} of approximately 100 nM. Similar growth inhibition was observed with BMDMs stimulated with 50 ng/mL CSF-1 (data not shown). Of note, GW2580 showed negligible effects on BMDM growth in the absence of CSF-1 (data not shown). Moreover, BMDM migration towards CSF-1 was completely abrogated with 1000 nM GW2580 (Figure 1C, P<0.001).

Pharmacological effects of GW2580 \textit{in vivo}. Before pursuing a therapeutic study, we performed a dose titration of GW2580 at 20 mg/kg or 80 mg/kg b.i.d. or 160 mg/kg once daily in naïve mice by oral gavage. As shown in Figure S1A, 160 mg/kg once daily achieved GW2580 levels that peaked at approximately 9 μM, and remained above 1 μM for 24 hours, exceeding the estimated minimum plasma concentration to achieve a therapeutic effect[14]. We also assessed the potential systemic toxicities associated with
prolonged GW2580 treatment. Tumor-bearing mice treated for 14 days with 160 mg/kg GW2580 showed no significant differences in total body weight compared with control diluent-treated mice (Figure S1B). In addition, serum levels of ALT, AST, total protein, Bun, and LDH were not significantly different, demonstrating minimal hepatic and renal toxicities associated with the 160 mg/kg GW2580 dosing regimen (Figure S1C-G). These pharmacokinetic and toxicology studies indicate that GW2580 treatment at 160 mg/kg once daily is as effective as 80 mg/kg b.i.d.[14].

**Inhibiting CSF1R signaling targets distinct TIM subsets in vivo.** GW2580 has been shown to be an effective inhibitor of CSF1R signaling in vivo, reducing growth of the CSF-1-dependent monocytic cell line M-NSF-60 in mice[14]. However, the effects of GW2580 on infiltrating myeloid cells in a solid tumor model have yet to be investigated. In addition, the role of CSF1R signaling in the recruitment of other TIM subsets such as MDSCs remains unclear, although it has been certainly implicated in the recruitment of TAMs[8,10,20]. We demonstrated expression of CSF1R in murine macrophages but not in 3LL Lewis lung carcinoma, B16F1 melanoma, or RM-1 prostate tumor cell lines (data not shown), thus precluding direct effects of GW2580 on tumor cells.

We treated C57BL/6 mice bearing subcutaneously implanted 3LL lung carcinoma with control diluent or GW2580 at 20 mg/kg or 80 mg/kg b.i.d., or 160 mg/kg once daily by oral gavage. Flow cytometric analysis of tumors revealed that total CD45⁺CD11b⁺ myeloid cells were reduced by over 2-fold in the tumors of GW2580-treated mice compared to control (Figure S2A, P<0.05). CD11b⁺F4/80⁺ TAMs were also significantly reduced by over 2-fold (Figure S2B, P<0.05). Histological analysis revealed a similar
reduction in F4/80+ TAMs, equally reduced at the center and edges of tumors from 80 mg/kg GW2580-treated mice (Figure S2C, P<0.01), while the 20 mg/kg regimen resulted in a lesser extent of TAM reduction (P=0.07).

Interestingly, we observed a greater than 2-fold reduction in total CD11b\(^+\)Gr-1\(^+\) MDSCs in tumors from GW2580-treated mice (Figure 2A, P<0.02). Recent reports further distinguish the myeloid subsets within the MDSC population as consisting of mononuclear MDSCs (MO-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs)[5,13]. As shown in Figure 2B, Gr-1\(^{lo}\)Ly6\(^{hi}\) cells constituted a large portion of total MDSCs in the tumor (approximately 80%) as compared to a considerably smaller Gr-1\(^{hi}\)Ly6\(^{lo}\) population. Inhibition of CSF1R signaling strongly reduced the recruitment of Gr-1\(^{lo}\)Ly6\(^{hi}\) cells by approximately 4-fold (P<0.01) but had no effect on Gr-1\(^{hi}\)Ly6\(^{lo}\) cells (Figure 2B).

The morphology of sorted MDSC populations was further assessed by May-Grunwald-Giemsa (M-GG) staining. CD11b\(^+\)Gr-1\(^{lo}\)Ly6\(^{hi}\) cells were mononuclear with heterogeneous size and nuclear shape, resembling inflammatory monocytes/macrophages (Figure 2Bi). The majority of CD11b\(^+\)Gr-1\(^{hi}\)Ly6\(^{lo}\) cells were polymorphonuclear, with neutrophil-like morphology (Figure 2Bii). Therefore, we hereafter consider all tumor-infiltrating CD11b\(^+\)Gr-1\(^{lo}\)Ly6\(^{hi}\) cells as MO-MDSCs, and all tumor-infiltrating CD11b\(^+\)Gr-1\(^{hi}\)Ly6\(^{lo}\) cells as PMN-MDSCs. To evaluate short-term inhibition of CSF1R signaling in tumor-bearing mice, we treated mice bearing established tumors (approximately 1-cm in diameter) with a single dose of GW2580. This 1-day treatment resulted in a significant reduction in MO-MDSCs (Figure 2C, P<0.05), indicating a
critical role for CSF1R signaling in MDSC infiltration. We observed similar inhibition of MDSCs using GW2580 in the B16F1 melanoma model (data not shown).

Recent studies suggest that ARG1 and iNOS expression mediate the immunosuppressive properties of MDSCs[5]. By RT-PCR, we found that ARG1 expression (Figure 2D, P<0.05), but not iNOS (Figure 2E), was significantly reduced in tumors of GW2580-treated mice. These data suggest that MO-MDSCs primarily regulate intratumoral levels of ARG1, but not iNOS. Collectively, we demonstrate the profound inhibition of TIMs by GW2580 and the requirement of CSF1R signaling for the tumor recruitment of MO-MDSCs.

**CSF1R signaling regulates tumor recruitment of myeloid cells from peripheral blood.** We sought to determine whether the reduction in TIMs was due to systemic myeloid cell depletion or a specific effect on tumor recruitment. As shown in Table 1, we analyzed MO levels in the bone marrow, peripheral blood, and spleens of tumor-bearing mice after GW2580 treatment for 14 days, observing significant reductions in percentages and absolute numbers in bone marrow (P<0.001) and spleens (P<0.05), and a reducing trend in the peripheral blood (P=0.22). The reductions in MOs in each of these tissues approached levels observed in naïve mice. Spleen weights (Control: 86 mg ± 2.2 vs. GW2580: 68.4 mg ± 6.1, P<0.05, vs. Naïve: 68.5 mg ± 4.9) and total splenocyte numbers (Control: 98.3e6 ± 5.0e6 vs. GW2580: 79e6 ± 5.6e6, P<0.05) were also reduced to naïve levels. Naïve mice treated with GW2580 for 14 days revealed no changes in MOs in the bone marrow and peripheral blood. However, we did observe a significant 25% reduction in total MOs in the spleens of GW2580-treated naïve mice, suggesting a
partial requirement for CSF1R signaling in this tissue. Collectively, these results suggest that the induction of MDSCs is a tumor-induced systemic effect, which can be reversed to a large extent by blockade of CSF1R signaling.

To better delineate the kinetics of CSF1R blockade on the tumor recruitment of myeloid cells, we intravenously injected CFDA-SE-labeled bone marrow cells (BMCs) into 3LL tumor-bearing mice. In order to ensure adequate inhibition of CSF1R signaling in vivo, mice were pretreated with GW2580 for 4 hours prior to BMC injection. Organs were harvested 4 hours following BMC injection and subjected to flow cytometry. Short-term CSF1R inhibition showed a dramatic reduction in BMC recruitment to the tumor (Figure 3A, B, P<0.001), while minimal changes were observed in the bone marrow and peripheral blood (Figure 3D, E). We did observe a significant reduction in BMCs in the spleen (Figure 3F, P<0.05). We confirmed that approximately 70% of tumor-recruited BMCs were CD11b^Gr-1^ myeloid cells with Ly6C^hi^ expression (Figure 3C). Overall, we have demonstrated the impact of CSF1R signaling in the migration of MO-MDSCs from the blood to the tumor. In addition to the known functions of CSF1R signaling in normal tissue recruitment of myeloid cells, the involvement of this pathway is greatly accentuated in pathological accumulation in the tumor.

**Reduced tumor angiogenesis by selectively inhibiting CSF1R signaling.** Since TAMs and MDSCs have been shown to play important roles in tumor vasculature and tissue remodeling, we evaluated angiogenic and lymphangiogenic factors that are modulated by GW2580 treatment. Expression levels of VEGF-A and MMP-9 were reduced significantly by approximately 35% and 70% in treated tumors, respectively (Figure 4A,
D, P<0.05). Expression levels of VEGF-C, MMP-2, and other MMPs and TIMPs were unaffected (Figure 4B, C, and data not shown). The reduction of MMP-9 was verified at the protein level in GW2580-treated tumors (Figure 4E, F). Next, we evaluated the impact of GW2580 treatment on tumor angiogenesis. Vascular density, as assessed by staining of the endothelial marker CD31, showed a dose-dependent reduction with GW2580 treatment (Figure 4G, H, P<0.01). Unexpectedly, the growth of subcutaneous 3LL tumors was not impaired by the suppression of tumor angiogenesis with GW2580 treatment (Figure 4I, J). Likewise, negligible tumor growth suppression was also observed in mice bearing B16F1 melanoma (Figure 4K).

In an attempt to better understand the impact of macrophage ablation on these tumors, we used the MaFIA model[18]. These mice express an inducible Fas-based suicide gene and the EGFP gene under the control of the macrophage-specific c-fms promoter. Upon treatment with the dimerizing drug AP20187, cells expressing CSF1R rapidly undergo apoptosis. We generated MaFIA chimeras by transplanting MaFIA donor bone marrow into naïve mice. Figure S3A demonstrates efficient bone marrow transplantation, with the greater than 80% of CD11b+ myeloid cells in the peripheral blood co-expressing EGFP. Treatment of 3LL tumor-bearing mice with AP20187 resulted in a substantial depletion of CSF1R+ myeloid cells in the peripheral blood and spleens (Figure S3B and C), and a significant reduction in F4/80+ and GFP+ TIMs in the 3LL tumors (Figure S3D, E, P<0.01). Recapitulating the results of GW2580 treatment, we observed a significant reduction in angiogenesis in these macrophage-ablated tumors (Figure S3F, P<0.01) without a concomitant decrease in tumor growth (Figure S3G).
These data suggest that CSF1R+ TIMs, including TAMs and MO-MDSCs, contribute significantly to tumor angiogenesis.

**Reduced MDSC infiltration and tumor angiogenesis by inhibiting CSF1R signaling in an orthotopic prostate tumor model.** We also evaluated the effects of inhibiting CSF1R signaling in the orthotopic RM-1 prostate tumor model. Flow cytometric analysis revealed negligible effects on peripheral blood total MDSC levels with GW2580, possibly due to a lack of change in the PMN-MDSC population (Figure 5A). However, we did observe a greater than 2-fold reduction in tumor-associated MDSCs, likely reflecting reductions in MO-MDSCs (Figure 5B, P<0.01). Moreover, a significant reduction in F4/80+ TAMs, particularly at the normal prostate-tumor interface regions was observed (Figure 5C). Analysis of the spleens from GW2580-treated mice revealed slight reductions in F4/80+ macrophages and Gr-1+ MDSCs (Figure S4A). GW2580 treatment also reduced blood vessel density in orthotopic RM-1 tumors by approximately 35% (Figure 5D, E, P<0.05). We observed a slight but insignificant reduction in lymphatic vascular density, as assessed by the lymphatic endothelial marker, Lyve-1 (Figure 5D, F, P=0.10). These findings suggest that TIMs contribute to angiogenesis and, to a lesser extent, lymphangiogenesis in this murine prostate tumor model.

Next, we examined the impact of a conventional pharmacological agent, clodronate liposomes (Clodrolip), on macrophage ablation in RM-1 prostate tumors [21]. Treatment of tumor-bearing mice with Clodrolip resulted in a significant reduction in peripheral blood total MDSCs (Figure 5G, P<0.001) as well as tumor-associated MDSCs (Figure 5H, P<0.05), albeit less pronounced than GW2580. Immunohistochemical
analysis also revealed substantial reductions in splenic F4/80+ macrophages and Gr-1+ MDSCs in Clodrolip-treated mice (Figure S4B). However, similar to the findings in 3LL and B16F1 tumors, neither GW2580 nor Clodrolip treatment suppressed tumor growth in this model (Figure 5I, J).

**Combination therapy with GW2580 and an anti-VEGFR-2 antibody results in synergistic tumor growth reduction.** MDSCs were recently implicated in the tumor resistance to anti-angiogenic therapy[22]. Using anti-Gr-1 monoclonal antibodies to deplete these myeloid cells in vivo in combination with anti-VEGF therapy achieved further reductions in tumor growth over anti-VEGF therapy alone. Since the heterogeneity of MDSCs in tumors is widely accepted[5,7,13], it is important to identify which TIM subsets confer this resistance. To gain further insights into this issue, we exploited the inhibitory effects of GW2580 on TIMs to evaluate pharmacologic CSF1R blockade in combination with anti-angiogenic therapy (anti-VEGFR-2 mAb, DC101).

3LL-tumor bearing mice were treated with control diluent, 160 mg/kg/day GW2580 alone, 800 μg DC101 alone every other day (i.p.), or GW2580 in combination with DC101. As shown in Figure 6A, while DC101 alone reduced tumor growth by 35% (P<0.0001), the combination of DC101 and GW2580 resulted in an apparent synergistic tumor growth reduction of approximately 70% (P<0.0001). Again, tumor growth was unaffected by GW2580 treatment alone. Histological analysis of tumors revealed a significant increase in necrotic area following DC101 and DC101/GW2580 treatment, most likely reflecting the direct effects of DC101 in both treatment groups (Figure 6B, E). Moreover, Gr-1+ MDSCs in the tumor were dramatically reduced by GW2580 in
single and combination treatments, while DC101 treatment alone showed slightly increased signals as compared to control (Figure 6C). Similar results were observed with histological analysis of F4/80^+ TAMs (data not shown). Tumor angiogenesis was incrementally reduced in each treatment group, with an overall 80% reduction in vessel density with combination treatment (Figure 6D, F).

**TIMs mediate an anti-angiogenic compensatory mechanism involving MMP-9.** The notion that tumors evade anti-angiogenic therapy has recently received heightened attention[22-24]. Several reports suggest the induction of Angiopoietin-1 (Ang-1)[25] and Fibroblast growth factor-2 (FGF-2)[26] might allow growing tumors to circumvent the absence of VEGF signaling. Moreover, induction of PlGF and MMP-9 expression following therapy with anti-VEGFR-2 blocking antibody[27] has also been implicated as a compensatory mechanism. However, the precise mechanism or the cells in the tumor microenvironment that are involved in the evasion of anti-angiogenic therapy remain undefined. The synergistic therapeutic impact of GW2580 with anti-VEGFR-2 therapy could likely provide further insight on the evasion mechanism.

To further characterize myeloid cell contributions to evasion of anti-angiogenic therapy, tumors from each treatment group were analyzed for the expression of pro-angiogenic genes. As shown in Figure 7A, levels of MMP-9 were greatly induced in tumors treated with DC101 alone (4-fold, P<0.001). Interestingly, this induction was completely abolished in tumors from the combination treatment group (P<0.01), indicating that TIMs play a critical role in the regulation of MMP-9 induction with anti-angiogenic therapy. These findings were confirmed at the protein level using Western
blot analysis and at the functional level using gelatin zymography (Figure 7C, D). Histological staining revealed more abundant MMP-9-expressing cells with heterogeneous myeloid cell morphology in viable areas of DC101-treated tumors, which were reduced in the combination group (Figure 7E). Although VEGF-A expression was modestly reduced by GW2580 alone, DC101 treatment induced its expression (1.5-fold, P<0.01), which was further enhanced by the combination treatment (Figure 7B, 2-fold, P<0.05). We also analyzed the expression levels of other growth factors suggested to be important in therapeutic resistance[25-27]. Surprisingly, the expression levels analyzed by RT-PCR of Ang-1, Ang-2, FGF-1, FGF-2, and PDGFβ, were reduced, rather than induced, by 25-50% in the DC101-treated group (data not shown). Soluble VEGFR-1 (sVEGFR-1) has also been linked to anti-angiogenic processes mediated by TIMs[28,29], however we did not observe significant differences in expression of sVEGFR-1 or granulocyte-macrophage colony-stimulating factor (GM-CSF), a potent stimulator of sVEGFR-1 expression, in these treatment groups (data not shown). Collectively, these data demonstrate that TIMs mediate at least one compensatory mechanism involving MMP-9 that contributes to tumor evasion of anti-angiogenic therapy.
Discussion

Tumor-infiltrating myeloid cells (TIMs), including TAMs have been implicated in tumor progression, invasion, and metastasis. Early studies have investigated the role of CSF1R signaling in TAM infiltration using CSF-1 \textit{op/op} mice\[8\]. These knockout mice display marked deficiencies in total peripheral blood monocytes and tissue macrophages, but also show various systemic effects including total body weight reductions and skeletal abnormalities\[30\]. More recent studies using antisense oligonucleotide and antibodies directed against CSF-1 showed the impact of CSF1R signaling on TAM infiltration and breast tumor xenograft growth\[10,20\]. These latter studies used immunocompromised mice, a potential limiting factor for determining the effects of host inflammation on tumor progression. In addition, the human mammary tumors used in these studies express CSF1R, raising the possibility of autocrine effects. Nevertheless, the results from \textit{op/op} mice and CSF-1 blockade studies were seminal in linking CSF1R signaling and TAM infiltration to tumor progression and metastasis.

In this study, we utilized several murine tumor models in immunocompetent mice, which allowed us to directly assess the contributions of distinct myeloid subsets to tumor progression. In addition, the tumor models used in these studies do not express detectable levels of CSF1R by RT-PCR and thus we were able to eliminate potential autocrine tumor effects when modulating this pathway. Results from this study demonstrate a significant contribution of CSF1R signaling to the recruitment of MDSCs. In addition to these implanted tumor models, preliminary studies with CSF1R blockade in the PTEN-knockout transgenic model of prostate cancer demonstrate the importance of CSF1R signaling for MDSC recruitment to spontaneous tumors (data not shown)\[31\].
However, further studies are warranted to evaluate the therapeutic effectiveness of CSF1R blockade as a single agent and in combination with other anti-cancer therapies using this model. MO-MDSCs were particularly sensitive to CSF1R blockade, while we observed no differences in PMN-MDSC infiltration. This contrasts a recent finding in CCR2-deficient mice using 3LL tumors, where severe reductions in tumor-associated MO-MDSCs was accompanied by massive accumulation of PMN-MDSCs[5]. We did not observe recruitment differences in tumor-associated PMN-MDSCs with CSF1R blockade, demonstrating marked distinction between the two myeloid recruitment pathways. Expansion of MDSCs in the bone marrow and their systemic accumulation in tumor-bearing mice correlates with primary tumor size[32]. In this study, blockade of CSF1R signaling suppressed expansion of MO-MDSCs in the bone marrow and reduced accumulation in the spleen, associated with reduced splenomegaly and total splenocyte numbers. The suppression was seen in the absence of tumor growth differences between control and GW2580-treated mice, suggesting that CSF1R signaling has direct effects on these tumor-induced systemic effects.

Blockade of the CSF1R signaling pathway, either by pharmacological inhibition of CSF1R kinase or by genetic ablation of CSF1R+ cells using the MaFIA model, did not result in appreciable changes in tumor growth despite significant reductions in tumor angiogenesis. The seminal work of Dr. Judah Folkman clearly established that growth of tumors is dependent on adequate vascular perfusion[33]. A large volume of work in the last 30 years has also demonstrated the direct correlation between blood vessel density and tumor growth. However, on the converse issue of blocking angiogenesis as cancer therapy, it might be inadequate to merely score for blood vessel density as a measure of
therapeutic effect. A recent study showed that inhibition of PDGF-B signaling using a selective aptamer AX102 resulted in dramatic reductions in tumor angiogenesis without concomitant reductions in growth of 3LL tumors[34]. Another recent study by Stockmann et al., utilizing macrophage-specific depletion of VEGF-A, reported that reduced tumor blood vasculature actually led to significant increases in tumor growth[35]. These recent findings indicate that a reduction in tumor vessel density does not necessarily result in reduced growth kinetics and further consideration of vascular integrity and perfusion is needed.

The findings of this study that combined inhibition of CSF1R axis and anti-VEGFR-2 blockade corroborated recent reports, implicating specific TIM subsets that are important for the apparent resistance to anti-angiogenic therapy. A prior study clearly implicated Gr-1+ myeloid cells in mediating resistance to anti-VEGF therapy[22], however the Gr-1 antibody treatment used systemically depletes all Gr-1+ populations of macrophage and neutrophil lineage, in addition to other cell types such as blood lymphocytes and splenic dendritic cells[36]. In the current study, we were able to further distinguish and selectively inhibit MO-MDSCs, thus implicating the involvement of this distinct MDSC subset in mediating the resistance to anti-angiogenic therapy. We also highlight the induction of MMP-9 as a compensatory mechanism put forth by the tumor to counter anti-angiogenic therapy. MMP-9 has been frequently implicated in various aspects of tumor progression, from angiogenesis, to tumor invasion and metastasis[37,38]. MMP-9 mediates the angiogenic switch by releasing ECM-bound VEGF, thereby increasing the bioavailability of VEGF[39,40]. Also, MMP-9-mediated remodeling of basement membrane is important for new blood vessel sprouting[41],
which may also be exploited by tumors in attempts to evade anti-angiogenic therapy. Our preliminary data demonstrate that CSF-1 induces MMP-9 expression in murine macrophages in vitro (data not shown), but whether CSF1R signaling directly mediates MMP-9 induction by anti-angiogenic therapy warrants further investigation.

The anti-VEGF antibody Avastin is currently approved only in combination with chemotherapy for various solid cancers, including breast and colon. Several theories describe the apparent improved response to anti-VEGF therapy when combined with chemotherapy. First, normalization of tumor vasculature by blockade of VEGF signaling may improve drug delivery, leading to an enhanced chemotherapeutic response in tumors[25,42-44]. Second, the systemic leukocyte toxicity with chemotherapy may contribute to the response when combined with anti-angiogenic therapy. However, it might be difficult to balance the systemic side effects of chemotherapy such as myelosuppression with the therapeutic benefit of combined therapy.

In support of the latter theory, several tyrosine kinase inhibitors (eg. Imatinib, Sunitinib, etc) have been used to treat solid tumors by targeting both tumor- and endothelial cell-directed signaling pathways[45]. In addition to inhibiting the receptors in the VEGF, PDGF, and KIT families, these small molecule agents also exhibit significant inhibition of CSF1R kinase activity, which can contribute to the overall therapeutic effects[46]. Unfortunately, it is very challenging to decipher the specific therapeutic benefits of selective pathway blockade with multi-targeted tyrosine kinase inhibitors. Moreover, pathway-selected therapy is the Holy Grail towards minimizing potential toxic side effects. In this study, we demonstrated that of the use of the selective CSF1R inhibitor GW250 during tumor development produced minimal systemic toxicity, while
achieving significant impact on the recruitment of TAMs and MO-MDSCs. Hence, targeting this pathway enables not only a better understanding of the roles of specific TIM subsets in tumor progression, but also furthers the development of rational combined therapeutic approaches to combat tumor evasion of anti-angiogenic therapy.
Acknowledgments

We deeply appreciate Dr. Ebba Brakenhielm for valuable discussion of the manuscript. We thank Bronislaw Pytowski and ImClone Systems for their generous contribution of the mouse anti-VEGFR-2 antibody DC101. We thank Ben Van Handel for flow cytometry expertise and Nancy Nguyen for technical contributions.

This work is supported by NCI SPORE program P50 CA092131, Prostate Cancer Foundation (to L.W.), and Margaret E. Early Medical Trust award (to L.W.). S.J.P. is supported by the career developmental program of UCLA ICMIC grant (P50 CA86306 to H.R. Herschman) and a CDMRP predoctoral training award (W81XWH-09-1-0538).
Authorship

Contributions: S.J.P. designed and performed most of the research. J.L.S., Z.S., J.B.B., A.X.T-C., D.L.M., and M.J. performed additional research. D.A.C. provided the MaFIA model. A.J.L., D.A.C., and M.L.I-A. provided valuable support throughout the project. S.J.P. analyzed the data. L.W. designed the research and supervised the project. S.J.P. and L.W. wrote the manuscript with valuable contributions from all co-authors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
References


Table 1: MO levels in naïve (tumor-free) and 3LL tumor-bearing mice

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Bone marrow</th>
<th>Peripheral blood</th>
<th>Spleen</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Naïve Control</td>
<td>10.7 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Naïve GW2580</td>
<td>10.9 ± 0.4</td>
<td>2.5 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>N/A</td>
</tr>
<tr>
<td>TB Control</td>
<td>15.2 ± 0.2</td>
<td>4.1 ± 0.9</td>
<td>1.6 ± 0.1</td>
<td>11.2 ± 1.9</td>
</tr>
<tr>
<td>TB GW2580</td>
<td>11.4 ± 0.5</td>
<td>2.5 ± 0.6</td>
<td>1.2 ± 0.1</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>0.22</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>cells x 10^3/femur</td>
<td>cells x 10^3/mL</td>
<td>cells x 10^3/spleen</td>
<td>cells/mg tumor</td>
</tr>
<tr>
<td>Naïve Control</td>
<td>1858.7 ± 258.8</td>
<td>51.2 ± 7.9</td>
<td>609.2 ± 11.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Naïve GW2580</td>
<td>1816.0 ± 136.0</td>
<td>56.7 ± 8.8</td>
<td>459.7 ± 18.7</td>
<td>N/A</td>
</tr>
<tr>
<td>p-value</td>
<td>&gt;0.3</td>
<td>&gt;0.3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TB Control</td>
<td>2322.9 ± 106.8</td>
<td>86.1 ± 23.1</td>
<td>1434.1 ± 22.7</td>
<td>5662.2 ± 830.5</td>
</tr>
<tr>
<td>TB GW2580</td>
<td>1750.4 ± 100.5</td>
<td>52.7 ± 14.9</td>
<td>798.2 ± 177.1</td>
<td>1459.3 ± 247</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.01</td>
<td>0.27</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Table 1: MO levels in naïve (tumor-free) and 3LL tumor-bearing mice. Monocyte levels were measured by flow cytometry in the bone marrow, peripheral blood, spleens, and tumors of control and GW2580-treated tumor-free mice (Naïve), as well as control and GW2580-treated tumor-bearing mice (TB). Percentages and absolute numbers are represented as mean ± SEM. Monocyte levels from control and GW2580-treated mice were assessed using the Student’s t test.
Figure Legends

Figure 1: Inhibiting CSF1R signaling in macrophages with the pharmacologic inhibitor GW2580. A. Immunoprecipitation and Western blot analysis of Raw264.7 murine macrophage cells stimulated with 10 ng/mL CSF-1 for 20 min in the absence or presence of 10, 100, or 1000 nM GW2580. Blot was probed for tyrosine phosphorylation (pTyr), stripped, and reprobed for total CSF1R. B. Bone marrow-derived macrophages (BMDMs) were stimulated with CSF-1 in the absence (open squares) or presence of 10 nM (open triangles), 100 nM (open diamonds), or 1000 nM (open circles) GW2580. At the indicated timepoints, cell viability was measured using the CCK-8 assay and compared with unstimulated control. C. BMDMs were seeded on 8-μm transwell inserts and CSF-1 was added to the lower chamber in the absence or presence of 1000 nM GW2580 (added to both chambers). Cells were allowed to migrate towards CSF-1 for 6 hours, then fixed and stained with DAPI. Representative images are shown (magnification, 4x) and migrated cells were quantified using ImageJ software (white bar, Control, no CSF-1; gray bar, CSF-1; black bar, CSF-1/GW2580). Scale bar: 100 μm.

Figure 2: Targeting MO-MDSC infiltration by inhibiting CSF1R signaling. 3LL tumor cells were subcutaneously implanted in C57BL/6 mice and mice were treated with control diluent or 160 mg/kg GW2580 once daily. At day 14, single cell suspensions of tumors were analyzed by flow cytometry. Representative images and quantification of total CD11b^Gr-1^ MDSCs (A), Gr-1^LoLy6Chi MO-MDSCs and Gr-1^HiLy6Clow PMN-MDSCs (B) are shown (n=4/group). May-Grunwald-Giemsa (M-GG) staining of sorted MDSC populations within the tumor (i and ii). C. Mice were implanted with 3LL tumors
and treated with GW2580 once 24 hours prior to harvesting tumors for flow cytometric analysis. Total MDSCs, MO-MDSCs and PMN-MDSCs were quantified (n=3-4/group). RT-PCR analysis of ARG1 (D) and iNOS (E) expression in tumors from control and GW2580-treated mice. Relative mRNA expression was normalized to β-actin (n=6/group).

**Figure 3: CSF1R signaling regulates tumor recruitment of myeloid cells from peripheral blood.** 3LL tumor-bearing mice (day 10 post-implantation) were treated with control diluent or GW2580 (for 4 hours) and subsequently i.v. injected with 20 x 10^6 CFSE-labeled bone marrow cells (BMCs). After 4 hours, animals were sacrificed and tissues were analyzed for recruitment of CSFE^+ BMCs by flow cytometry. A-B. Percentage of CFSE^+ BMCs in tumor and absolute number of cells/mg tumor are shown (n=4/group). C. The majority of tumor-recruited CFSE^+ cells were CD11b^+Gr-1^+ and Ly6C^{hi}. D-F. Percentage of CFSE^+ BMCs in the bone marrow, peripheral blood, and spleens are shown (n=4/group).

**Figure 4: Angiogenesis and growth kinetics of tumors treated with GW2580.** A-D. Tumors were harvested on day 14 after tumor implantation and GW2580 treatment, and mRNA expression was quantified using RT-PCR for the stated genes. Relative mRNA expression was normalized to β-actin (n=6/group). E. MMP-9 protein levels from tumors were analyzed by Western blot and normalized to α-Tubulin. F. Quantification of MMP-9 protein levels by ImageJ software (n=3/group). G. Representative CD31^+ vascular staining of 3LL tumors from control and GW2580-treated mice are shown. H.
Quantification of CD31+ area was performed using ImageJ software (n≥5/group). Tumor volume was monitored by caliper measurements of 3LL tumors (I, J, n=6/group) and B16F1 tumors (K, n=3/group) in mice treated with control diluent, 20 mg/kg, or 80 mg/kg GW2580 b.i.d., or 160 mg/kg GW2580 once daily. Scale bars: 100 μm.

**Figure 5: Targeting MDSC infiltration and tumor angiogenesis in orthotopic RM-1 prostate tumors.** RM-1 prostate tumor cells were implanted intraprostatically in the dorsolateral lobes of C57BL/6 males. Mice were treated with control diluent, GW2580, PBS, or clodronate liposomes (Clodrolip) at the appropriate dosing regimen. A. Peripheral blood (n=5/group) total MDSCs were analyzed by flow cytometry in control-(white bar) and GW2580- (black bar) treated mice. B. Tumor-associated MDSC levels (n=3-5/group) were also analyzed by flow cytometry in control and GW580-treated mice. C. Tumor tissue was processed and subjected to histological staining of F4/80+ TAMs at the peritumoral regions (top) and normal prostate-tumor interface (bottom). D. Representative CD31+ blood and Lyve-1+ lymphatic vascular staining of RM-1 prostate tumors from control and GW2580-treated mice are shown. Quantification of CD31+ (E) and Lyve-1+ (F) area was performed using ImageJ software (n=3-5/group). G-H. Peripheral blood (n=5/group) and tumor-associated MDSC (n=3-4/group) levels were analyzed by flow cytometry in PBS- (white bar) and Clodrolip- (grey bar) treated mice. Genitourinary tract (GUT) weight was measured at endpoint in GW2580-treated (I) and Clodrolip-treated (J) mice (n=3-5/group). Scale bars: 100 μm.
Figure 6: Synergistic tumor growth reduction and inhibition of angiogenesis by combination therapy with GW2580 and anti-VEGFR-2 antibody DC101. 3LL tumors were subcutaneously implanted and mice were treated with control diluent, GW2580 or DC101 alone, or a combination of DC101/GW2580 for 14 days. A. Tumor volume was monitored by caliper measurements (n=5-9/group). At day 14, tumors were harvested and subjected to histological analysis of H&E (B), Gr-1+ MDSCs with DAPI (C), and CD31+ vessels with DAPI (D). Necrotic tissue area (E), and CD31+ area (F) was quantified using ImageJ software (n≥5/group). Data represents combined averages from two independent animal experiments. Scale bars: 100 μm.

Figure 7: TIMs mediate MMP-9 induction by anti-VEGFR-2 therapy. Tumors were harvested at day 14 and mRNA expression was quantified using RT-PCR for MMP-9 (A) and VEGF-A (B). Relative mRNA expression was normalized to β-actin (n≥5/group). C. MMP-9 protein levels from tumors were analyzed by Western blot and normalized to α-Tubulin (n=6/group). Representative images are shown. D. The same tumor lysates were analyzed by gelatin zymography (n=6/group). Representative images are shown. Tumor tissue was subjected to histological analysis of MMP-9 expression with DAPI (E). Scale bars: 50 μm.
Figure 3

A. Control and GW2580 samples showing a decrease in CFSE+ cells.

B. BMCs in tumor (%).

C. Tumor-associated CFSE+ cells.

D. BMCs in BM (%).

E. BMCs in PB (%).

F. BMCs in spleen (%).
Figure 4

A. Relative mRNA expression of Vegf-a, $P < 0.05$

B. Relative mRNA expression of Vegf-c

C. Relative mRNA expression of Mmp2

D. Relative mRNA expression of Mmp9, $P < 0.05$

E. Western blotting for αMMP-9 and αTubulin in Control and GW2580 conditions.

F. MMP-9 protein levels (arbitrary units), $P < 0.05$

G. Confocal microscopy images of CD31 in Control, 20 mg/kg, and 80 mg/kg conditions.

H. CD31+ area (%), $P < 0.01$

I. Tumor volume of 3LL in Control, 20 mg/kg, and 80 mg/kg conditions.

J. Tumor volume of 3LL in Control and 160 mg/kg conditions.

K. Tumor volume of B16F1 in Control and 160 mg/kg conditions.
Figure 6

A

![Graph showing tumor volume over time with different treatments: Control, GW2580, DC101, DC101/GW2580. The graph indicates significant differences between groups with p-values: P < 0.0001 and P < 0.0001.]

B

![H&E stained images of control, GW2580, DC101, and DC101/GW2580 treated tissues.]

C

![Images of Gr-1 stained tissues showing different treatments.]

D

![Images of CD31 stained tissues showing control, GW2580, DC101, and DC101/GW2580 treated tissues.]

E

![Bar graph showing necrotic tissue area with significance levels: P < 0.05 and P < 0.01.]

F

![Bar graph showing CD31+ area with significance levels: P < 0.05 and P < 0.06.]

Legend:
- Control
- GW2580
- DC101
- DC101/GW2580
Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of anti-angiogenic therapy