The Promotion of Breast Cancer Metastasis Caused by Inhibition of CSF-1R/CSF-1 Signaling Is Blocked by Targeting the G-CSF Receptor

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Abstract

Treatment options are limited for patients with breast cancer presenting with metastatic disease. Targeting of tumor-associated macrophages through the inhibition of colony-stimulating factor-1 receptor (CSF-1R), a key macrophage signaling pathway, has been reported to reduce tumor growth and metastasis, and these treatments are now in clinical trials. Here, we report that, surprisingly, treatment with neutralizing anti–CSF-1R and anti–CSF-1 antibodies, or with two different small-molecule inhibitors of CSF-1R, could actually increase spontaneous metastasis without altering primary tumor growth in mice bearing two independently derived mammary tumors. The blockade of CSF-1R or CSF-1 led to increased levels of serum G-CSF, increased frequency of neutrophils in the primary tumor and in the metastasis-associated lung, as well as increased numbers of neutrophils and Ly6C+ monocytes in the peripheral blood. Neutralizing antibody against the G-CSF receptor, which regulates neutrophil development and function, reduced the enhanced metastasis and neutrophil numbers that resulted from CSF-1R blockade. These results indicate that the role of the CSF-1R/CSF-1 system in breast cancer is far more complex than originally proposed, and requires further investigation as a therapeutic target. Cancer Immunol Res 2(8); 765–76. ©2014 AACR.

Introduction

The link between chronic inflammation and the onset and progression of many cancers is well established (1). Tumor-associated macrophages (TAM), which have been subjected to extensive study, can execute several key roles required for tumor growth and progression (1, 2). Colony-stimulating factor-1 (CSF-1/M-CSF) regulates the recruitment, differentiation, survival, and proliferation of macrophages (3). CSF-1 activity is mediated through its interaction with the receptor tyrosine kinase, CSF-1 receptor (CSF-1R/c-Fms/CD115; ref. 3), which is expressed primarily on mononuclear phagocytic cells and their precursors (4). Interleukin-34 (IL34) is a second ligand for CSF-1R; it controls the development of specific macrophage lineages, such as microglia and Langerhans cells (5). IL34 and CSF-1 have distinct expression patterns (6). The association between CSF-1/CSF-1R expression and prognosis in breast cancer is still unclear. A study of invasive breast adenocarcinomas (grades 1–3) revealed that, although nuclear staining of CSF-1 in tumor cells was associated with poor outcome, it was more strongly predictive of increased numbers of infiltrating monocytes (7). In a study of noninvasive and invasive breast adenocarcinomas (grades 1–3), elevated expression of CSF-1 was found predominantly in invasive tumor cells (8). In a study of early-stage 1/2 breast cancer, the authors reported a correlation between high levels of CSF-1R in tumors and local recurrence (9). Distant metastasis and survival were not assessed in the latter two reports. Assessment of node-negative and node-positive breast cancer tissues revealed that high CSF-1R expression was associated with a shorter overall survival for node-negative, but not node-positive patients (10). However, this study also found that CSF-1R expression alone was not an independent predictor of survival (10). Beck and colleagues (11) investigated a "CSF-1 response signature" in whole breast tumor datasets and found that the signature was associated with survival in only one of five datasets. The authors concluded that the CSF-1 response signature has a complex relationship with patient survival (11).

The association between CSF-1/CSF-1R expression and prognosis in breast cancer is still unclear. A study of invasive breast adenocarcinomas (grades 1–3) revealed that, although nuclear staining of CSF-1 in tumor cells was associated with poor outcome, it was more strongly predictive of increased numbers of infiltrating monocytes (7). In a study of noninvasive and invasive breast adenocarcinomas (grades 1–3), elevated expression of CSF-1 was found predominantly in invasive tumor cells (8). In a study of early-stage 1/2 breast cancer, the authors reported a correlation between high levels of CSF-1R in tumors and local recurrence (9). Distant metastasis and survival were not assessed in the latter two reports. Assessment of node-negative and node-positive breast cancer tissues revealed that high CSF-1R expression was associated with a shorter overall survival for node-negative, but not node-positive patients (10). However, this study also found that CSF-1R expression alone was not an independent predictor of survival (10). Beck and colleagues (11) investigated a "CSF-1 response signature" in whole breast tumor datasets and found that the signature was associated with survival in only one of five datasets. The authors concluded that the CSF-1 response signature has a complex relationship with patient survival (11).

CSF-1 is a potent macrophage chemoattractant, and it polarizes macrophages in the tumor microenvironment toward tumor-promoting TAMs (12). Preclinical studies have reported a role for CSF-1 in metastasis via the regulation of TAMs. In macrophage-deficient MMTV-PyMT mice carrying a null mutation in the Csf1 gene, TAMs were unable to accumulate in primary tumors, and metastasis to the lung was reduced (13). In breast cancer xenografts in immunocompromised mice,
depletion of TAMs by CSF-1 antisense oligonucleotides or neutralizing antibody resulted in reduced primary tumor growth (14, 15). On the basis of the results from these studies, CSF-1R/CSF-1 signaling is considered a potential target for adjuvant therapy of breast cancer. A phase I clinical trial has been initiated using a monoclonal antibody (mAb) against CSF-1R—IMC-CS4—as a therapy for solid tumors (16). PLX3397, a small-molecule inhibitor of CSF-1R, is also in phase I trials for solid tumors (17) and for metastatic breast cancer in combination with chemotherapy (18).

Little is known about the role of tissue macrophage precursors, blood monocytes, in metastasis. CD11b+ Gr-1+ myeloid cells facilitate tumor growth (19), and their human homologs are reported to be elevated in patients with cancer (20). These cells are also found in blood, the spleen, and in the premetastatic lung of tumor-bearing mice (19). Because Gr-1 recognizes both the Ly6C and Ly6G antigens (21), Gr-1+ cells can be monocytes, macrophages, or neutrophils. Two major blood monocyte subpopulations have been defined, based primarily on surface marker expression. In the mouse, these are "classical" monocytes (Ly6C(Gr-1)hiCX3CR1loCCR2hi) and "nonclassical" monocytes (Ly6C(Gr-1)lo CX3CR1hiCCR2lo CD62Lhi) and by its expression of CD11b, CD115 (c-Fms: AFS-98-APC) and F4/80 (BM8-FITC) on bone marrow-derived macrophages (22, 23). However, the relationship between monocyte subpopulations and tissue macrophages is still unclear, particularly during inflammatory/immune reactions (24).

Neutrophils are found in breast tumors and have been implicated in promoting metastasis (19, 25, 26). Elevated levels of neutrophils in tumors are associated with decreased survival in a number of cancer types, including pulmonary and gastric adenocarcinomas (27, 28). Whether there is an association between neutrophil numbers and prognosis in human breast cancer is not known, although increased levels of the neutrophil-specific protease, neutrophil elastase, have been associated with reduced disease-free survival (29). G-CSF regulates neutrophil production by inducing proliferation and maturation of myeloid progenitors and by promoting neutrophil release from the bone marrow (30). A recent study in tumor-bearing mice found that blocking G-CSF reduced neutrophil numbers and metastasis to the lung, whereas treatment with rG-CSF had the opposite effect (25).

Given the association between CSF-1, TAMs, and breast cancer metastasis, we anticipated that blockade of CSF-1R or CSF-1 would deplete TAMs and subsequently limit metastasis and possibly primary tumor growth as well. However, unexpectedly, we found that such neutralization actually increases metastasis to the lung and bone in two independent mammary tumor models, and is associated with an increase in neutrophils at multiple sites. Blockade of G-CSF overrides the increase in metastasis and neutrophil numbers that results from CSF-1R neutralization, indicating that this enhanced metastasis is driven by G-CSF.

## Materials and Methods

### Antibodies, reagents, and cell lines

The 4T1.2, 66cl4, 67NR, and EMT6.5 murine tumor cell lines (31–34) expressing mCherry fluorescent protein were maintained in α Minimal Essential Medium (αMEM), penicillin/streptomycin at 100 U/mL (Invitrogen), and 5% fetal calf serum (CSL Biosciences). All cell lines were tested and validated to be Mycoplasma-free. No other authentication was completed. The 4T1, 66cl4, and 67NR lines were obtained directly from Dr. F. Miller, Karmanos Cancer Institute, Wayne State University, Detroit, MI (32), and the 4T1.2 line was derived in-house from the 4T1 line. There are no universal standards from which authentication of these lines can be obtained. The following were gifts: neutralizing anti-mouse CSF-1R (AFS-98: Dr. S.-I. Nishikawa, RIKEN Center for Developmental Biology, Kobe, Japan), IgG2a isotype control (GL17.41: Dr. J. Abrams, DNAx, Palo Alto, CA), neutralizing anti-mouse CSF-1 and IgG2a isotype control (Dr. F. Dodeller, MorphoSys, Munich, Germany), neutralizing anti-mouse G-CSFR and IgG1 isotype control (Dr. A. Andrews, CSL Biosciences, Parkville, VIC, Australia). GW2580 was provided by Cancer Therapeutics CRC, and CYC11645 was provided by Dr. C. Burns (Walter and Eliza Hall Institute, Parkville, VIC, Australia). mAbs were used against the following antigens: CD11b (Mac-1 α-chain, M1/70-APC-Cy7), Ly6C (AL-21-PerCP-Cy5.5, and Ly6G (1A8-PE-Cy7) all from BD Pharmingen, CD115 (c-Fms: AFS-98-APC) and F4/80 (BM8-FITC) both from eBioscience.

### Tumor growth

Female Balb/c mice (6–8 weeks; Walter and Eliza Hall Institute) were injected into the fourth mammary gland with 1 × 10^5 tumor cells in PBS or saline alone. Tumor volume, (L × W/2), was measured using electronic calipers. For analysis of circulating monocyte and neutrophil numbers, peripheral blood was collected from the tail vein after tumor cell inoculation as indicated. In some experiments, the primary tumor was resected at 0.5 g (700 mm^3), and treatment was commenced 6 days later. Primary tumors (if not resected), lungs, and spines were collected at endpoint. Primary tumors and lungs were disaggregated (see below), spines were snap-frozen in liquid nitrogen, and homogenized. All procedures involving mice were conducted in accordance with the National Health and Medical Research Council of Australia guidelines and approved by the University of Melbourne Animal Ethics Committee and the Peter MacCallum Animal Experimentation Ethics Committee.

### Antibody and inhibitor treatment

Tumor-bearing mice were treated i.p. every 4 days from days 7 to 25 after tumor cell inoculation with anti–CSF-1R (250 μg), anti–CSF-1 (200 μg), or IgG2a isotype control antibody, as indicated. In some experiments, mice were treated i.p. 3 days per week with 50 μg of anti–G-CSF or IgG1 isotype control antibody. These doses and treatment regimens have been determined previously to be optimal for mouse studies (ref. 35; and unpublished data). For the anti-CSF-1R and anti–G-CSF antibody combination treatment, the same protocols were used as described above with the 4T1.2 tumor-bearing mice receiving either both experimental antibodies or both isotype controls. For the kinase inhibitors, mice were treated by oral gavage with 160 mg/kg of...
GW2580 or vehicle. This dose has been shown previously to be optimal for inhibition of CSF-1R (36, 37). CYC11645 is another potent inhibitor of CSF-1R (38), described previously by Burns and colleagues as compound 15 (38). CYC11645 (100 mg/kg) was administered twice daily by oral gavage, from days 1 to 30 after tumor cell inoculation.

**Flow cytometry**

Cells or 20 μL of blood were washed, counted, incubated in mouse serum, washed, and incubated with primary antibodies or appropriate isotype controls. Cells were analyzed using the Beckman Coulter CyAn ADP Flow Cytometer (Beckman Coulter) or the BD LSR II Flow Cytometer (Becton/Dickinson) and FlowJo software (TreeStar; Supplementary Fig. S1). See Supplementary Materials and Methods for detailed procedure.

**Tumor burden analysis**

Tumor burden for each tissue was measured by qPCR (StepOne System; Applied Biosystems) and TaqMan chemistry (33). Lung tumor burden was visualized by India ink staining. See Supplementary Materials and Methods for detailed procedure.

**Kaplan–Meier plotter and BreastMark online survival analysis**

Kaplan–Meier plotter (39) and BreastMark (40) were used to assess high and low expression of tumor CSF-1R in relation to relapse-free survival in all breast cancers using default settings. See Supplementary Materials and Methods for a more detailed description of these platforms.

**Measurement of serum CSF-1 and G-CSF**

Serum was harvested at endpoint via cardiac puncture. CSF-1 levels were measured using a murine M-CSF standard ELISA development kit (Peprotech), and G-CSF levels were measured using a mouse G-CSF DuoSet kit (R&D Systems) as per the manufacturer’s instructions.

**Statistical analysis**

Tumor growth and peripheral blood data were analyzed using two-way ANOVA with Bonferroni correction. For all other data, a preliminary statistical analysis (D’Agostino–Pearson omnibus normality test) was used to determine whether the data followed a normal distribution. If the data were normally distributed, an unpaired, parametric $t$ test was used; if not, the Mann–Whitney $U$ test was used. $P \leq 0.05$ was considered significant.

**Results**

**Peripheral blood monocyte and neutrophil numbers increase in mice bearing metastatic tumors**

Previously, we described an isogenic series of spontaneous mammary tumors, including 67NR (nonmetastatic), 66cl4 (weakly metastatic), and the highly metastatic 4T1.2, that mimics the course of human breast cancer, with primary tumor formation from a small inoculum, invasion of cells through the stroma into the circulation, and colonization at distant organs, including the lung and bone (31, 32).

4T1.2 tumors contain approximately 8% CD4 T cells, 0.3% CD8 T cells, 4% B cells, 10% TAMs, and 15% neutrophils (data not shown), similar to the proportions of infiltrating immune cells or 20 μL of blood were washed, counted, incubated in mouse serum, washed, and incubated with primary antibodies or appropriate isotype controls. Cells were analyzed using the Beckman Coulter CyAn ADP Flow Cytometer (Beckman Coulter) or the BD LSR II Flow Cytometer (Becton/Dickinson) and FlowJo software (TreeStar; Supplementary Fig. S1). See Supplementary Materials and Methods for detailed procedure.

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cells in human breast tumors (41, 42). Given that TAMs and neutrophils are the predominant immune cells in 4T1.2 primary tumors, we assessed whether any changes took place in peripheral blood monocytes and neutrophils during mammary tumor growth. Monocytes were defined as CD11b+CD115+Ly6G−Ly6C−, CD11b+CD115+Ly6G+Ly6C−, or CD11b+CD115+Ly6G−Ly6C+ (23, 35, 43), and neutrophils as CD11b+Ly6G+ (21, 25, 35, 43). Compared with 66cH or 67NR tumor-bearing mice, those bearing highly metastatic 4T1.2 tumors had significant increases in circulating Ly6G−Ly6C− monocytes, as well as neutrophils, from day 20 onwards (Fig. 1A–C). It is important to note that this large increase in myeloid cells is not regularly reported in other tumor models (25, 44, 45). This is due to reporting of the percentage of monocytes as opposed to the actual number in circulation as shown in Fig. 1. The difference in interpretation of the results is revealed in Supplementary Fig. S2, in which the two methods are compared.

Although primary tumor growth was the same in all three tumor types (Fig. 1D), end-stage lung and spleen weights were increased in 4T1.2 tumor-bearing mice (Fig. 1E and F), indicating an enhanced metastatic burden (46). The extensive lung metastatic burden from 4T1.2 compared with 67NR tumor-bearing mice is evident in Supplementary Fig. S3.

Neutralizing anti–CSF-1R antibody and CSF-1R inhibitors increase metastasis to the lung and spine and the levels of blood neutrophil and Ly6C− monocytes

Given the data linking CSF-1R/CSF-1 and TAMs to breast cancer metastasis (1, 2, 7, 45), we anticipated reduced TAMs and reduced metastasis upon treatment with the widely used neutralizing anti–CSF-1R antibody, AFS-98, or the small-molecule inhibitors, GW2580 and CYC11645. Although there was no change in the growth of the 4T1.2 primary tumor following treatment with any of these inhibitors (Fig. 2A and E and Supplementary Fig. S4A), AFS-98 increased metastasis to the lung and spine in the 4T1.2 tumor-bearing mice (Fig. 2B and C). This result was unexpected. We next tested whether the increased metastasis also occurred with other mammary tumors. Indeed, treatment with any of these inhibitors (Fig. 2A and E and Supplementary Fig. S4B). The 4T1.2 and EMT6.5 tumor cell lines were used as controls (Fig. 2D). Treatment of 4T1.2 tumor-bearing mice with either AFS-98 or GW2580 increased serum CSF-1 levels (35), most likely as a result of TAM death and subsequent inflammatory cytokine release (46). The extensive lung metastatic burden from 4T1.2 compared with 67NR tumor-bearing mice is evident in Supplementary Fig. S3. The anti–CSF-1R antibody further enhanced this tumor-mediated expansion of neutrophils and Ly6C− monocytes (Fig. 3A and B), but not the Ly6C+ monocytes (Fig. 3C). For the less metastatic EMT6.5 tumor-bearing mice, with blood collected before the first and third treatments, only neutrophils increased in the control group (Fig. 3D). Antibody treatment led to an expansion of neutrophils (Fig. 3D), Ly6C− monocytes (Fig. 3E), and the Ly6C+ subpopulation (Fig. 3F). For GW2580 treatment of 4T1.2 tumor-bearing mice, where blood was collected before the first and 12th dose (days 6 and 17 after inoculation), tumor-mediated expansion of neutrophils and Ly6C+ monocytes was further enhanced, but not of the Ly6C− monocytes (Fig. 3G–I).

We have shown previously that blockade of CSF-1R with AFS-98 increases serum CSF-1 levels (35), most likely

![Image](image.png)

Figure 2. Effect of CSF-1R inhibition on metastasis. A–C, 4T1.2 tumor-bearing mice were administered 250 μg of anti–CSF-1R or isotype antibody twice weekly from days 7 to 25 after tumor cell inoculation. D, EMT6.5 tumor-bearing mice were administered 250 μg of anti–CSF-1R or isotype antibody twice weekly for 2 weeks after primary tumor resection. E and F, 4T1.2 tumor-bearing mice were administered 160 mg/kg of GW2580 or vehicle daily from days 7 to 28 after inoculation (A) 4T1.2 primary tumor growth. Tumor burden in lungs (B) and spines (C) of the 4T1.2, and lungs (D) of the EMT6.5 mice. Primary tumor growth (E) and tumor burden in lungs (F) of 4T1.2 mice after GW2580 treatment. Data, mean ± SEM; n = 15 mice per group for antibody treatment and n = 9 mice per group for GW2580 treatment. *, P < 0.05; **, P < 0.01 anti–CSF-1R or GW2580 versus controls. Mann–Whitney U test (B, C, and F). An unpaired parametric t test (D).
due to a reduction in CSF-1R-mediated CSF-1 internalization by macrophage lineage cells (47, 48). Treatment of 4T1.2 and EMT6.5 tumor-bearing mice with AFS-98 significantly elevated CSF-1 levels in the serum (Fig. 3J and K), indicating that the antibody was also blocking CSF-1R in these experiments.

Neutralizing anti–CSF-1 antibody also increases metastasis to the lung and spine and further increases blood neutrophil and monocyte numbers

Recently, a second CSF-1R ligand, IL34, was identified (5, 6, 49). We therefore examined the impact of an anti–CSF-1 antibody on metastasis. As with the anti–CSF-1R antibody, there was no change in the growth of the 4T1.2 primary tumor (Fig. 4A). Metastasis was increased to the lung (Fig. 4B), but was not altered in the spine (data not shown). Blood was collected before the first and fourth antibody treatments. Expansion of neutrophils, Ly6C\(^{hi}\), and Ly6C\(^{lo}\) monocytes was seen in the isotype control-treated 4T1.2 tumor-bearing mice. Treatment with the anti–CSF-1 antibody significantly enhanced the tumor-mediated expansion of all three populations (Fig. 4C–E).

Neutralizing anti–CSF-1R and anti–CSF-1 antibodies, as well as GW2580, increase neutrophil numbers in the primary tumor and lung

We have reported previously that anti–CSF-1R antibody (AFS-98) reduces tissue macrophages in the steady state and during inflammation (35). We therefore measured macrophage and neutrophil numbers in 4T1.2 primary tumors and lungs, following anti–CSF-1R (AFS-98), anti–CSF-1 antibody, or GW2580 administration. Macrophages/TAMs were characterized as CD11b\(^{+}\)CD11c\(^{+}\)Ly6G\(^{−}\)Ly6C\(^{hi}\) or CD11b\(^{+}\)CD11c\(^{−}\)Ly6G\(^{−}\)Ly6C\(^{lo}\) (23), and neutrophils/tumor-associated neutrophils (TAN) as CD11b\(^{+}\)Ly6G\(^{+}\) (21, 25). At endpoint, treatment with anti–CSF-1R antibody did not reduce TAMs in primary tumors (Fig. 5A) or macrophages in the lung (Fig. 5C). Surprisingly, TANs in primary tumors were elevated...
after anti–CSF-1R treatment (Fig. 5B), as were neutrophils in the lung (Fig. 5D). Treatment with anti–CSF-1 antibody also did not alter TAMs (Fig. 5E), but increased TANs in the primary tumor (Fig. 5F). Treatment with GW2580 reduced Ly6C<sup>hi</sup> TAMs in the primary tumor but also increased TANs (Fig. 5G and H), whereas in the lung, GW2580 did not alter macrophages but again increased neutrophil numbers (Fig. 5I and J).

Neutralizing anti–G-CSFR antibody suppresses the increased metastasis to lung and spine, as well as the increased blood neutrophil and monocyte numbers following anti–CSF-1R antibody treatment

G-CSF is a major regulator of neutrophil development and mobilization but it can also mobilize other populations, such as monocytes, from the bone marrow (50), and has been implicated in Ly6G<sup>+</sup>Ly6C<sup>+</sup> granulocyte-enhanced 4T1 metastasis (25). Consistent with this, serum G-CSF levels increased 3-fold in 4T1.2 and 2-fold in EMT6.5 tumor-bearing mice compared with naïve mice (Supplementary Fig. S6). We therefore proposed a mechanism whereby the enhanced metastasis and cellular changes arising from CSF-1R/CSF-1 blockade were G-CSF-dependent. Anti–CSF-1R antibody treatment further enhanced serum G-CSF levels 5-fold in 4T1.2 and 8-fold in EMT6.5 tumor-bearing mice (Supplementary Fig. S6). We next treated 4T1.2 tumor-bearing mice with a combination of the anti–CSF-1R and anti–G-CSFR antibodies to determine whether the combined therapy could overcome the enhanced metastasis induced by anti–CSF-1R. Again, anti–CSF-1R increased metastasis to the lung and spine (Fig. 6A) without altering primary tumor growth (Fig. 6B and C) without altering primary tumor growth (Fig. 6A). In accord with our proposal, anti–G-CSFR antibody prevented the anti–CSF-1R-dependent increase in metastasis (Fig. 6B and C) and also reduced the incidence of metastasis (Fig. 6D), again without affecting primary tumor growth (Fig. 6A). Similar to the results shown in Fig. 3, CSF-1R blockade further increased blood neutrophils and Ly6C<sup>hi</sup> monocytes (Fig. 6E and F), whereas after G-CSFR blockade, neutrophils, Ly6C<sup>hi</sup>, and Ly6C<sup>lo</sup> monocytes were diminished to levels below those in the control group (Fig. 6E–G). These results further support our hypothesis that anti–G-CSFR antibody blocks the anti–CSF-1R–dependent elevation in circulating myeloid populations.

Neutralizing anti–G-CSFR antibody reduces the increased numbers of neutrophils in the primary tumor and lung following anti–CSF-1R antibody treatment

We next assessed the primary tumor and lung neutrophil numbers after G-CSF R blockade. Again, anti–CSF-1R did not alter the levels of TAMs (Fig. 7A) or lung macrophages (Fig. 7C), but increased the levels of tumor-associated neutrophils (TAN) (Fig. 7B) and lung neutrophils (Fig. 7D). Anti–G-CSFR antibody treatment prevented the TAN increase caused by the anti–CSF-1R antibody (Fig. 7B). In the lung, anti–G-CSFR antibody reduced the levels of neutrophils and prevented their anti–CSF-1R–dependent increase (Fig. 7D), and reduced the Ly6C<sup>hi</sup> and Ly6C<sup>lo</sup> macrophage numbers, either alone or in combination with anti–CSF-1R antibody (Fig. 7C).

Discussion

We have shown, using two different metastatic tumors and four different treatments, that blockade of CSF-1R/CSF-1 signaling, while not affecting primary tumor growth, can enhance spontaneous metastasis of breast carcinoma to both the lung and the bone. Our study is the first report to demonstrate that disruption of CSF-1R/CSF-1 signaling in solid tumor models enhances metastasis (13–15, 45, 51). In our study, TAMs were not depleted by anti–CSF-1R or anti–CSF-1 antibody treatment, whereas treatment with the CSF-1R kinase inhibitor (GW2580) reduced Ly6C<sup>lo</sup> TAMs in primary tumors. Unexpectedly, an increase in TANs was noted. The increase in neutrophils was also manifested in metastasis-involved lungs and in the blood. Blockade of G-CSF signaling led to a complete reversal of the anti–CSF-1R–mediated...
increase in metastasis to both the lung and the bone, in circulating neutrophil and monocyte numbers, and in neutrophil numbers in the primary tumors and lungs.

Previous reports of targeting CSF-1R/CSF-1 in preclinical tumor models have yielded variable results, depending on the mouse strain, the type of tumor, and its metastatic capacity. In
the more clinically relevant models using immunocompetent mice, anti–CSF-1R has been reported to reduce primary tumor growth (52), and reduce spontaneous metastasis in an osteosarcoma model (51). In the transgenic MMTV-PyMT mouse, studies targeting CSF-1R signaling showed contrasting results. In one study, there was no effect on primary tumor growth or metastasis (45); in another study, it reduced primary tumor growth, with no data reported for metastasis (52). Our data showed for the first time that CSF-1R and CSF-1 blockade actually enhanced metastasis in two different preclinical tumor models, providing evidence that such treatment can lead to different outcomes in different breast tumors. It is clear that the role of CSF-1R and CSF-1 signaling in metastasis is more complex than originally proposed.

CSF-1R or CSF-1 blockade not only increased metastasis of both 4T1.2 and EMT6.5 tumors, but also further enhanced neutrophil expansion in the circulation, in primary tumors, and in the lungs. Given the recent data linking neutrophils to metastatic events (19, 25), we hypothesize that the increase in neutrophils promotes metastasis in these models. In support of our hypothesis, blood neutrophil numbers were proportional to the metastatic capacity of the various mammary tumors (Fig. 1 and 3), and elevated in the lungs of 4T1.2 tumor-bearing mice compared with naïve mice (data not shown). Our data indicate that certain metastatic tumors may be more adept at mobilizing neutrophils, leading to increased metastasis. Consistent with this, DeNardo and colleagues noted that neutrophil numbers vary considerably between different mammary tumors, indicating a potential distinction between different tumor types (45). This could explain some of the variable results observed during CSF-1R/CSF-1 targeting.

Kowanetz and colleagues found that 66cl4 tumor metastasis was reduced after neutrophils were depleted by anti-Ly6G antibody treatment (25), and other investigators have suggested that neutrophils can acquire a “metastatic phenotype” in tumor-bearing mice (26). Results from a recent report suggested that lung neutrophils have antimetastatic activity (53). Thus, further analysis of how neutrophils are involved in metastatic events is needed.

The increase in neutrophils and circulating Ly6Chi monocytes following CSF-1R (AFS-98) or CSF-1 neutralization in 4T1.2 and EMT6.5 tumors was unexpected, although compensatory increases in neutrophils have been reported in...
infections of macrophage-deficient Csf1op/Csf1op mice (54), and after reduction of macrophages in a murine cervical cancer model (55). Using a similar protocol in non–tumor-bearing mice in the steady state or during inflammation, we and others found that AFS-98 antibody has no effect on these cell populations (35, 36). Gr1− (Ly6C− and/or Ly6G+) cells can facilitate metastasis in MMTV-PyMT transgenic mice (44). Consistent with this, we demonstrated that Ly6C+ monocytes increased with progressive tumor growth, and were further enhanced with CSF-1R and CSF-1 blockade (Fig. 3). Furthermore, the levels of circulating monocytes and lung macrophages in 4T1.2 tumor-bearing mice were reduced after G-CSFR blockade, either in the absence or presence of CSF-1R antibody (Fig. 6). For reasons unknown at present, AFS-98 treatment raised G-CSF levels in the serum of tumor-bearing mice (Supplementary Fig. S6), the activity of which would be inhibited by anti–CSF-1 antibody (Fig. 6). Thus, it is possible that in 4T1.2 and EMT6.5 tumors, G-CSFR blockade reduces the G-CSF–dependent mobilization of monocytes from the bone marrow, which is known to occur in 4T1 (25) and 4T1.2 tumors (unpublished data), whereas CSF-1R blockade promotes this G-CSF–dependent mobilization. It is also possible that the levels of Ly6C+ monocytes are reduced as a consequence of the reduced metastasis following G-CSFR blockade (44).

Few studies have assessed circulating monocytes in both human disease and animal models. In human breast cancer, CD14+CD16+ monocytes were found to increase 1.5-fold compared with that in healthy controls (57). A 2-fold increase in CD14+ cells was reported for inflammatory breast cancer compared with noninflammatory breast cancer (58). In murine models, Qian and colleagues assessed the ratio of inflammatory monocytes to resident monocytes in MMTV-PyMT transgenic mice; however, they did not report the total numbers (44). Other studies measured the numbers of macrophage and TAM, but not circulating monocytes (25, 45). Therefore, a contribution of Ly6C+ monocytes to the metastasis of 4T1.2 and EMT6.5 tumors cannot be excluded.

Consistent with a role for G-CSF in 4T1.2 metastasis, blockade of G-CSFR alone reduced metastasis of 4T1.2 tumors to the lung and spine and also suppressed the increased neutrophil numbers in the circulation, the primary tumor, and the lung. Similar results were reported previously using a neutralizing antibody against G-CSF for 4T1 tumors (25). We have extended these findings by showing that the anti–CSF-1R–dependent increase of neutrophils in the circulation, the primary tumor, and the lung, as well as the increases in metastasis to the lung and bone, was also G-CSF–dependent. Anti–Ly6G-neutrophil antibody treatment was equally effective as anti–G-CSF antibodies in reducing metastasis of 66cl4 tumors to the lung (25), supporting a role for neutrophils in the promotion of metastasis. It is highly likely that both Ly6C+ monocytes and neutrophils can promote metastasis, or that the cross-talk between these cells leads to the increases in metastasis following AFS-98 treatment.

In addition to the lack of impact of AFS-98 treatment on 4T1.2 tumor growth, we observed no change in primary tumor TAMs despite the increased metastasis. Others have shown that an oral CSF-1R inhibitor or an anti–CSF-1 antibody (45), or very high doses of AFS-98 (52), reduced TAMs in the MMTV-PyMT mice, although in the latter study there was no suppression of tumor growth or metastasis. It is possible that the development of multiple primary tumors in MMTV-PyMT mice precludes analysis of extensive metastases. In our models, TAMs could be reduced using a different protocol or a higher dose of AFS-98. However, despite a reduction in Ly6C+ TAMs after treatment with GW2580, we found that neutrophils and metastasis were still increased. It was reported recently that CSF-1R blockade in preclinical gliomas did not lead to a reduction in TAMs, although there was a decrease in M2-like markers in TAMs (59). As we found no tumor regression or increased survival but rather increased metastasis, it is unlikely that a decrease in M2-like phenotype is occurring in our system. Supporting this, no alteration in the maturation and differentiation of TAMs was noted following treatment with an
anti-CSF-1 antibody or an oral CSF-1R inhibitor in the MMTV-PyMT model (45). To account for the lack of reduction in TAMs following CSF-1R blockade, Pyonteck and colleagues suggested that TAMs were protected from cell death by survival factors produced by the tumor cells (59). A similar mechanism may be active in 4T1.2 tumors. In our study, TAM function could also be altered such that their influence on neutrophil trafficking is changed, either through G-CSF elevation or through a compromised ability to clear neutrophils by phagocytosis (60).

On the basis of our results on metastasis, we assessed the prognostic significance of CSF-1R expression in human breast tumors. In contrast with previous reported data (7–10), low levels of the CSFIR gene were found to predict worse overall survival using both Kaplan–Meier plotter (39) and BreastMark (40) online survival analyses (Supplementary Fig. S7). Likewise, a recent study by Beck and colleagues found that a “CSF-1 response signature” predicted different outcomes for patients with breast cancer depending on the tumor subtype (11). The authors concluded that the relationship between the CSF-1 response signature and survival is complex and requires further investigation. Furthermore, CSF-1 levels in the circulation may also be prognostic (61). Analysis of CSF-1R and CSF-1 in tumors and in the circulation, comparing distant metastasis versus local recurrence in different subtypes of breast cancer, may shed more light on how CSF-1R and CSF-1 are related to prognosis.

The blockade of CSF-1R or CSF-1 signaling leading to increased metastasis in 4T1.2 and EMT6 mammary tumors raises a cautionary note for the use of this type of therapy, even in an adjuvant setting, for patients at high risk of metastatic progression. Although the CSF-1/CSF-1R signaling pathway is a valid target in cancer, our data indicate that patients with progressive disease may respond better to anti-G-CSF therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): A. Swierczak, A.D. Cook, C.M. Restall, R.L. Anderson, J.A. Hamilton
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References


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