

Colony-Stimulating Factor-1 Antibody Reverses Chemoresistance in Human MCF-7 Breast Cancer Xenografts

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Abstract

Overexpression of colony-stimulating factor-1 (CSF-1) and its receptor in breast cancer is correlated with poor prognosis. Based on the hypothesis that blockade of CSF-1 would be beneficial in breast cancer treatment, we developed a murinized, polyethylene glycol-linked antigen-binding fragment (Fab) against mouse (host) CSF-1 (anti-CSF-1 Fab). Mice bearing human, chemoresistant MCF-7 breast cancer xenografts were treated with combination chemotherapy (CMF: cyclophosphamide, methotrexate, 5-fluorouracil; cycled twice i.p.), anti-CSF-1 Fab (i.p., cycled every 3 days for 14 days), combined CMF and anti-CSF-1 Fab, or with Ringer's solution as a control. Anti-CSF-1 Fab alone suppressed tissue CSF-1 and retarded tumor growth by 40%. Importantly, in combination with CMF, anti-CSF-1 Fab reversed chemoresistance of MCF-7 xenografts, suppressing tumor development by 56%, down-regulating expression of the chemoresistance genes *breast cancer-related protein*, *multidrug resistance gene 1*, and *glucosylceramide synthase*, and prolonging survival significantly. Combined treatment also reduced angiogenesis and macrophage recruitment and down-regulated tumor matrix metalloproteinase-2 (MMP-2) and MMP-12 expression. These studies support the paradigm of CSF-1 blockade in the treatment of solid tumors and show that anti-CSF-1 antibodies are potential therapeutic agents for the treatment of mammary cancer. (Cancer Res 2006; 66(8): 4349-56)

Introduction

Breast cancer remains a serious health care concern with an incidence that has continued to increase over the last two decades (1). Much research has, therefore, focused on the mechanisms by which mammary epithelial cells undergo the genotypic changes towards malignancy. Existing evidence suggests that the generation of genetically altered mammary cancer cells is a complex, multistep process (2). However, the mammary gland comprises stromal and epithelial cells that communicate with each other through the extracellular matrix (ECM). Although the mammary epithelium has the largest tumorigenic potential, the mammary stroma, comprising adipocytes, fibroblasts, vessels, inflammatory

cells, and ECM, contributes both instructive and permissive signals to tumor growth and progression (3). Thus, targeting the normal cells that surround the cancer cells might also be beneficial in breast cancer therapy.

Colony-stimulating factor-1 (CSF-1) is produced by a variety of cells (4–6), and we have shown that CSF-1 stimulates the proliferation, differentiation, and survival of cells of the mononuclear phagocytic lineage (6–8). CSF-1 plays a unique role in mammary gland physiology because it is synthesized by the mammary ductal epithelium (4), and macrophages recruited by CSF-1 promote both mammary ductal invasion during puberty (9) and lobulo-alveolar differentiation during pregnancy (10). A paracrine CSF-1 loop, therefore, exists in the normal mammary gland. Enhanced recruitment of macrophages to mammary tumors on one hand (11, 12) and the poor prognosis associated with elevated tumor-associated macrophages (13) on the other suggested a role for CSF-1 and CSF-1-regulated macrophages in breast cancer (7). In line with this, metastatic progression of mammary gland tumors in CSF-1-deficient mice with decreased tumor-associated macrophages is profoundly reduced (14). In addition, our earlier work indicated that CSF-1 accelerates angiogenesis *in vivo* (15). Relevant to this, recent reports suggest that CSF-1 stimulates macrophage progenitor cells, monocytes (16), and tumor macrophages (17) to secrete vascular endothelial growth factor (VEGF).

Based on these biological properties of CSF-1, we have used antisense constructs to block CSF-1 transcription in solid tumors, including mammary cancer (18, 19), and showed that this treatment resulted in significant suppression of tumor growth. In the present article, we extend this therapeutic approach to the use of neutralizing anti-CSF-1 antibodies. Using a murinized, polyethylene glycol (PEG)-linked antigen-binding fragment (Fab) directed against mouse CSF-1 (anti-CSF-1 Fab), we show that five injections of anti-CSF-1 Fab in mice bearing human breast cancer xenografts suppressed CSF-1 tissue expression and retarded tumor growth by 40%. More importantly, anti-CSF-1 Fab reversed the human breast cancer xenograft's chemoresistance so that in combination with combination chemotherapy, tumor development was suppressed by 56%, and long-term survival was significantly prolonged.

Materials and Methods

Anti-CSF-1 Fab-PEG antibody. A recombinant Fab fragment of the neutralizing rabbit anti-murine CSF-1 antibody (MCSF1-033) was constructed by VH and VL fusion to murine CH1 γ 1 and C κ constant regions and modified by site-specific attachment of 40-kDa PEG to increase protein half-life (20). The anti-CSF-1 Fab was purified and shown to specifically bind and neutralize murine CSF-1 as described previously (20).

Tumor models, anti-CSF-1 Fab treatment, and chemotherapy. The experiments done in this study were approved by the Institutional Animal Care and Use Committee at the Vienna Medical University. Human breast

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cancer cells (MCF-7; American Type Culture Collection, Manassas, VA) were grown in immune-deficient mice as described (19). Pathogen-free female athymic *nu/nu* (nude) mice (Harlan-Winkelmann, Borchon, Germany), 5 weeks of age, were weighed and coded and randomly assigned to experimental groups of $n = 10$. Mice were anesthetized (ketamine hydrochloride/xylazine at 55/7.5 mg/kg, s.c.), and 2×10^7 MCF-7 cells per 100 μ L of culture medium were grafted s.c. into their left flank (19). Twenty-four days after cell injection, one group of animals was killed and evaluated for tumor weight (342 ± 78 mg). Animals in an earlier study had developed tumors of similar size at 24 days (19), and treatment was initiated on the same day. The anti-CSF-1 Fab was injected i.p. at 4 mg/kg and cycled every 3 days for 14 days. The selected dosage of 4 mg/kg was based upon pilot pharmacokinetic studies in 20 adult mice comprising five groups ($n = 4$ /group that received 0.02, 0.2, 2, 4, and 8 mg/kg, respectively). Subsequently, adult mice were shown to tolerate repeated injections with 10 mg/kg anti-CSF-1 fab cycled every 3 days over an 8-week period. This dose was sufficient to induce decreases in tissue macrophage density and osteoclast numbers in adult mice.³ However, in another study involving postnatal administration, 10 mg/kg was not quite optimal because circulating antibody could only be detected in 40% of 2-month-old mice (20). Combination chemotherapy [CMF: 100 mg/kg cyclophosphamide, 50 mg/kg methotrexate, 100 mg/kg 5-fluorouracil (5-FU)] was cycled twice i.p. at days 24 and 34. The selected dosage of CMF therapy was based upon pilot studies (21–23) in 12 mice (comprising four groups of $n = 3$). The dosage of cyclophosphamide/methotrexate/5-FU in the pilot study was 25:12.5:25, 50:25:50, 100:50:100, and 150:75:150 mg/kg for groups 1 to 4, respectively. The end point of the chemotherapy pilot study was the highest tolerable CMF dose at which 100% of animals survived. Controls received Ringer's solution. All animals were killed at 38 days.

Analysis of the *in vivo* effects of CSF-1 antibody and CMF therapy. On day 38, blood samples were obtained to examine complete blood count. Tumors were then isolated and weighed, and the animals were sacrificed. The tissue was processed for paraffin embedding, real-time reverse transcription-PCR (RT-PCR), Western blotting, and RIA. Sections were stained with H&E, Ki-67 antibody (tumor proliferation assay; DAKO, Glostrup, Denmark), or double-stained with von Willebrand (DAKO) and Ki-67 antibodies to evaluate the density of proliferating endothelial cells (18, 19). Macrophages were immunostained with anti-F4/80 rat monoclonal antibody (Caltag Laboratories, Burlingame, CA; ref. 14). Digitalized images were generated, and morphometry was carried out (18, 19).

Analysis of the effects of CSF-1 blockade and combined CSF-1 blockade with CMF on survival. The survival study ($n = 48$) was set for 6 months. Mice were treated with anti-CSF-1 Fab ($n = 12$), CMF ($n = 12$), combined anti-CSF-1 Fab and CMF ($n = 12$), or Ringer's solution ($n = 12$). The animals treated with Ringer's solution served as controls.

Quantitative real-time RT-PCR. Tissue was processed for PCR as described (18, 19). The primer sequences for mouse molecules were (sense/antisense) CSF-1, 5'-CATCTCCATTCCTAAATCAAC-3'/5'-ACTTGCTGATCCCTCCTCC-3'; CSF-1R, 5'-GCGATGTGTGAGCAATGGCA-3'/5'-CGGATAATCGAACCTCGCCA-3'; VEGF-A, 5'-TTACTGCTGTACCTCCACC-3'/5'-GCTCATTCTCTATGTGCTG-3'; β -actin, 5'-GCGTGACATCAAGAGAA-3'/5'-AGGAGCCAGAGCAGTAAT-3'. The primer sequences for human molecules were (sense/antisense) CSF-1, 5'-GCTGTTGTTGGTCTGTCTC-3'/5'-CATGCTCTTCATAATCCTTG-3'; CSF-1R, 5'-TGCTGCTCC-TGCTGCTATTG-3'/5'-TCAGCATCTTCACAGCCACC-3'; VEGF-A, 5'-AGCCTTGCCGCTTGTGCTCTA-3'/5'-GTGCTGGCCTTGGTGAGG-3'; multidrug resistance gene 1 (*MDR1*; ref. 24), 5'-GCTCCTGACTATGCCAA-AGC-3'/5'-TCTTACCTCCAGGCTCAGT-3'; breast cancer related protein (*BCRP*; ref. 25), 5'-CACCTTATTGGCCTCAGGAA-3'/5'-CCTGCTTGGAGGCTCTATG-3'; glucosylceramide synthase (*GCS*; ref. 26), 5'-TGCTCAGTACATTGCCGAAG-3'/5'-CTGGCAACAAAGCATTCTGA-3'; β -actin, 5'-TGCCATCCTAAAAGCCAC-3'/5'-CAACTGGTCTCAAGTCAGAGTG-3'. The specificity of the CSF-1 and CSF-1R primers was tested by examining melting curves of the products obtained using both human- and mouse-

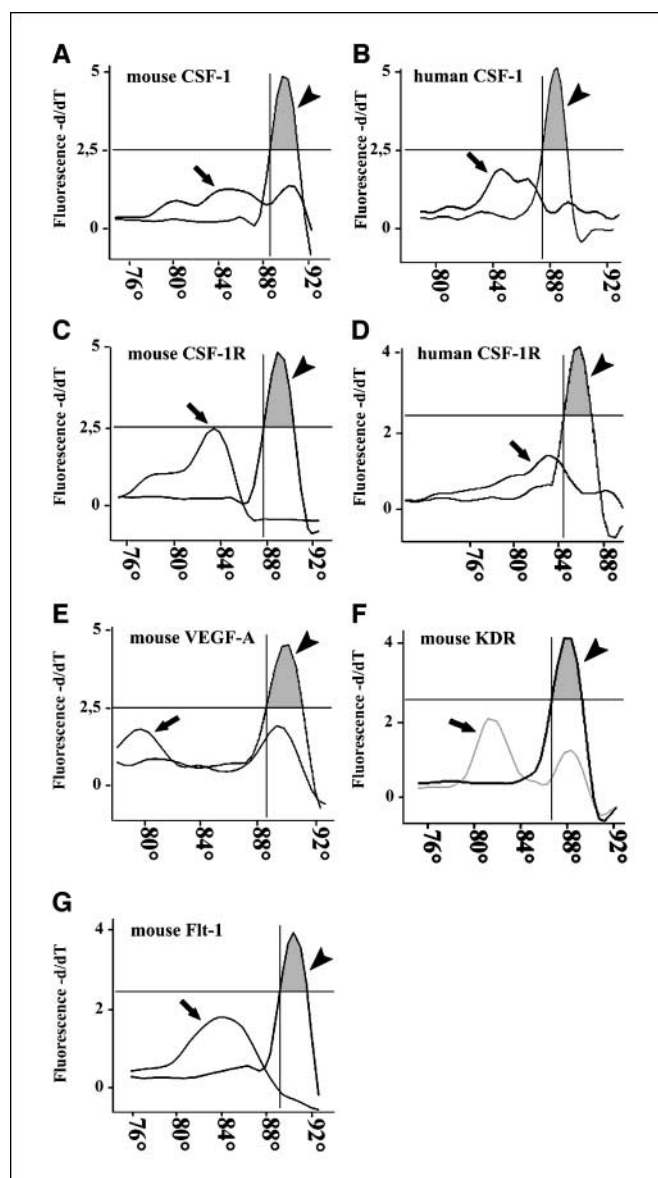


Figure 1. Species specificity of the mouse and human primers used in the real-time RT-PCR measurements. Melting curves of RT-PCR products obtained using mouse or human primers with mouse or human RNA to identify the specificity of the products by their specific melting temperature (T_m). Based on these experiments, primer dimers were excluded by setting a threshold at the specific T_m (vertical line), and the fluorescence threshold was set at 2.5 to eliminate nonspecific products (horizontal line). Gray, area measured for the species-specific product. A, mouse CSF-1 primers with mRNA from mouse macrophages (arrowhead) or human MCF-7 cells (arrow). B, human CSF-1 primers with mRNA from MCF-7 cells (arrowhead) or mouse macrophages (arrow). C, mouse CSF-1R primers with mRNA from mouse macrophages (arrowhead) or human MCF-7 cells (arrow). D, human CSF-1R primers with mRNA from MCF-7 cells (arrowhead) or mouse macrophages (arrow). E, mouse VEGF-A (E), KDR (F), and Flt-1 (G) primers with mRNA from mouse (arrowhead) or human (arrow) microvascular endothelial cells.

specific CSF-1 and CSF-1R primers on RNA from both human MCF-7 cells and mouse macrophages that express CSF-1 and CSF-1R (6, 7, 19). Human and mouse microvascular endothelial cells were used to test the specificity of VEGF-A, KDR, and Flt-1 primers. Measurements were done thrice.

CSF-1 RIA. Preweighed tissue and cell samples were homogenized, heat-inactivated, and centrifuged, and the supernatant was saved for assay as described (27). CSF-1 was measured in duplicate on three samples from each mouse using a RIA that only detects biologically active mouse CSF-1 (27, 28).

³ S. Wei and E.R. Stanley, personal observation.

Western blotting. Tissue lysates were prepared as described (19) and were separated (50 μ g/lane) by 8% to 12% SDS-PAGE before electrophoretic transfer onto Hybond C super (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Blots were probed with antibodies against matrix metalloproteinase-2 (MMP-2), MMP-9, MMP-12 (polyclonal, Santa Cruz Biotechnology, CA), tissue inhibitor of metalloproteinase-1 (TIMP-1), TIMP-2, TIMP-3 (polyclonal, Calbiochem, Temecula, CA), and urokinase plasminogen activator (uPA; monoclonal, EMD Biosciences, San Diego, CA) before incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were immunodetected by chemiluminescence (Supersignal-West-Femto, Pierce, Rockford, IL) and quantified by Easy Plus Win 32 software (Herolab, Wiesloch, Germany).

Statistical analysis. ANOVA with Bonferroni *t* test was used to compare the data between the groups. The χ^2 test was used to compare the groups in the analysis of long-term survival (6 months). The overall survival curves after treatment were analyzed by the Kaplan-Meier survival test. Data are expressed as mean \pm SD.

Results

CSF-1, CSF-1R, VEGF-A, KDR, and Flt-1 primer specifically measure human or mouse genes. To establish the specificity of the human CSF-1 and CSF-1R primers for the corresponding human versus mouse mRNAs and of the mouse CSF-1 and CSF-1R primers for the corresponding mouse versus human mRNAs, RT-PCR products derived using human- or mouse-specific primers on either human MCF-7 cells or mouse macrophages were examined by melting curve measurements (Fig. 1A-D). To test the specificity of the mouse VEGF-A, KDR, and Flt-1 primers for identification of mouse target genes, their effectiveness on mRNA from human and mouse microvascular endothelial cell lysates was similarly compared (Fig. 1E-G). The results showed that, under the conditions used, the primers used for human and mouse CSF-1 and CSF-1R and the primers used for mouse VEGF-A, KDR, and Flt-1 exhibit a high degree of species specificity.

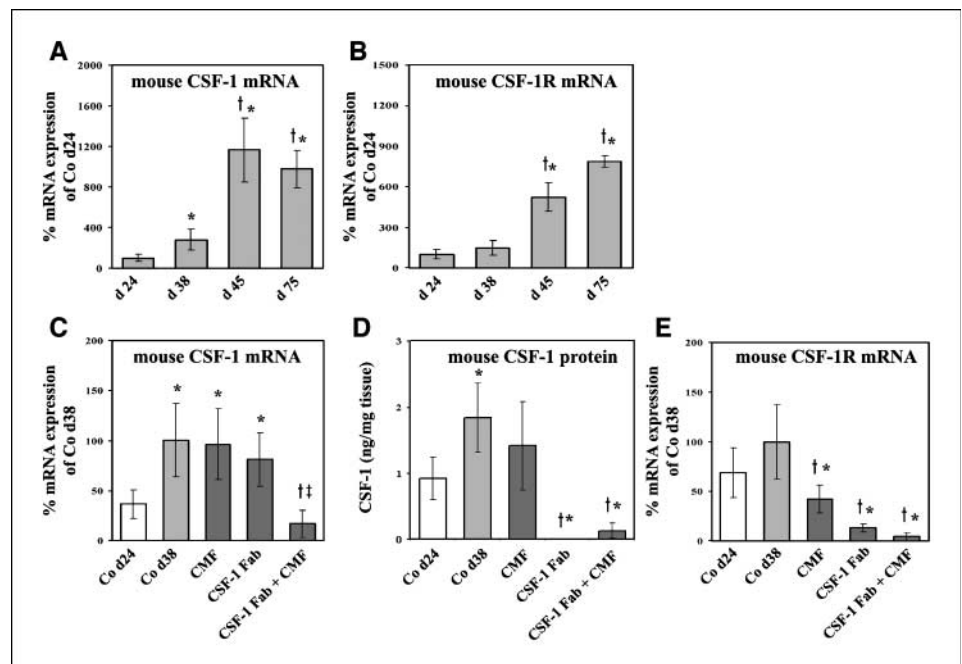
Anti-CSF-1 Fab significantly reduces tissue CSF-1 expression *in vivo*. As indicated above, our earlier experiments indicated that

human MCF-7 breast cancer cells express both mRNA and protein for human CSF-1 and its receptor, the CSF-1R proto-oncogene (*c-fms*) *in vitro* (19). When these cells were xenografted to mice, mouse tissue CSF-1 expression increased significantly with tumor progression ($P < 0.0001$; Fig. 2A), whereas MCF-7 cells lost their ability to express human CSF-1 (data not shown). Mouse CSF-1R mRNA was expressed throughout tumor development (Fig. 2B). Treatment of mice bearing human breast cancer xenografts with anti-CSF-1 Fab did not reduce tissue CSF-1 mRNA but reduced CSF-1R mRNA ($P < 0.0001$) and abolished tissue CSF-1 protein expression ($P < 0.0001$). Combined CMF and anti-CSF-1 Fab treatment resulted in significantly reduced tissue CSF-1 mRNA ($P < 0.0001$) and protein ($P < 0.0001$) as well as CSF-1R ($P < 0.0001$) expression. CMF alone did not change tissue CSF-1 but reduced CSF-1R expression ($P < 0.0001$; Fig. 2C-E). These experiments indicated that human cancer cells lose their own (human) CSF-1 expression and stimulate host (mouse) CSF-1 that can be neutralized by anti-CSF-1 Fab. They also show that anti-CSF-1 Fab treatment lowers host CSF-1R mRNA expression.

MCF-7 tumor xenografts are chemoresistant but sensitive to CSF-1 antibody. In chemotherapy dose pilot studies, MCF-7 tumor xenografts grew at the same rate even in the group treated with the highest tolerated CMF dose (100:50:100 mg/kg). The next highest CMF dose (150:75:150 mg/kg) was lethal. Anti-CSF-1 Fab treatment at 0.02, 0.2, and 2 mg/kg did not affect the MCF-7 tumor xenograft growth in pilot studies. However, anti-CSF-1 Fab at 4 and 8 mg/kg suppressed tumor growth significantly, although there was no significant difference in treatment effect between these two doses. These experiments indicated that MCF-7 tumor xenografts are resistant to CMF, and that anti-CSF-1 Fab single treatment at a dose of 4 mg/kg can significantly suppress the tumor growth.

Anti-CSF-1 Fab reverses MCF-7 cancer cell chemoresistance *in vivo* and suppresses mammary tumor growth. Mice bearing human mammary tumor xenografts were treated with CMF, anti-CSF-1 Fab, combined CMF and anti-CSF-1 Fab, or Ringer's solution (control). Both CMF and anti-CSF-1 Fab treatments were well

Figure 2. Anti-CSF-1 Fab alone and combined with CMF down-regulates CSF-1 and CSF-1R mRNA expression *in vivo*. CSF-1 and CSF-1R mRNA expression increase with tumor progression (A and B). CSF-1 mRNA measurements by real-time RT-PCR (C), CSF-1 measurements by RIA (D), and real-time RT-PCR measurements of mouse CSF-1R mRNA (E) in tumor lysates of mice xenografted with MCF-7 cells. *, significantly different from controls on day 24 (Co d24); †, significantly different from controls on day 38 (Co d38); ‡, significantly different from anti-CSF-1 Fab treated mice. Columns, mean from $n = 10$ mice per group; bars, SD.



tolerated. In line with the tissue CSF-1 and CSF-1R down-regulation, the mean tumor weight was markedly reduced in mice with human mammary tumors treated with anti-CSF-1 Fab ($1,142 \pm 292$ mg; $P < 0.0001$) or with combined CMF and anti-CSF-1 Fab (828 ± 212 mg; $P < 0.0001$) compared with mice treated with CMF ($1,711 \pm 327$ mg) or Ringer's solution ($1,871 \pm 394$ mg; Fig. 3A and C). Thus, treatment with anti-CSF-1 Fab and combined CMF suppressed tumor development by 40% and 56%, respectively. In addition, tumor proliferation as assessed by Ki-67 antibody staining was decreased after anti-CSF-1 Fab or CMF and anti-CSF-1 Fab therapy compared with control mice (Fig. 3B). Anti-CSF-1 Fab, but not CMF treatment alone, significantly reduced the expression of mRNA of resistance genes *BCRP*, *MDR1*, and *GCS*. However, the suppression effect of combined anti-CSF-1 Fab/CMF treatment was significantly stronger than the effect of anti-CSF-1 Fab treatment alone (Fig. 3D-F). These experiments indicated that blocking CSF-1 with anti-CSF-1 Fab

alone suppresses the progression of MCF-7 mammary tumor xenografts in mice, and that this treatment also reverses the chemoresistance of MCF-7 tumor xenografts.

CSF-1 antibody treatment down-regulates mouse MMP-12, MMP-9, and MMP-2 expression in MCF-7 mammary tumor xenografts. Following human MCF-7 cell xenografting in mice, macrophage invasion into the tumor xenografts was observed (Fig. 4A). In association with this, host (mouse) MMP-12, a macrophage-specific protease involved in ECM remodeling, was strongly expressed during tumor progression in control mice (Fig. 4B). Likewise, host (mouse) MMP-2 and MMP-9, key molecules in tumor metastasis and angiogenesis, were up-regulated during tumor progression in control animals ($P < 0.0001$). Combined treatment with CSF-1 antibody and CMF significantly reduced macrophage recruitment to the tumor compared with untreated control mice ($P < 0.0001$) and to mice treated with Fab alone ($P = 0.0085$; Fig. 4A). The anti-CSF-1 Fab alone and its

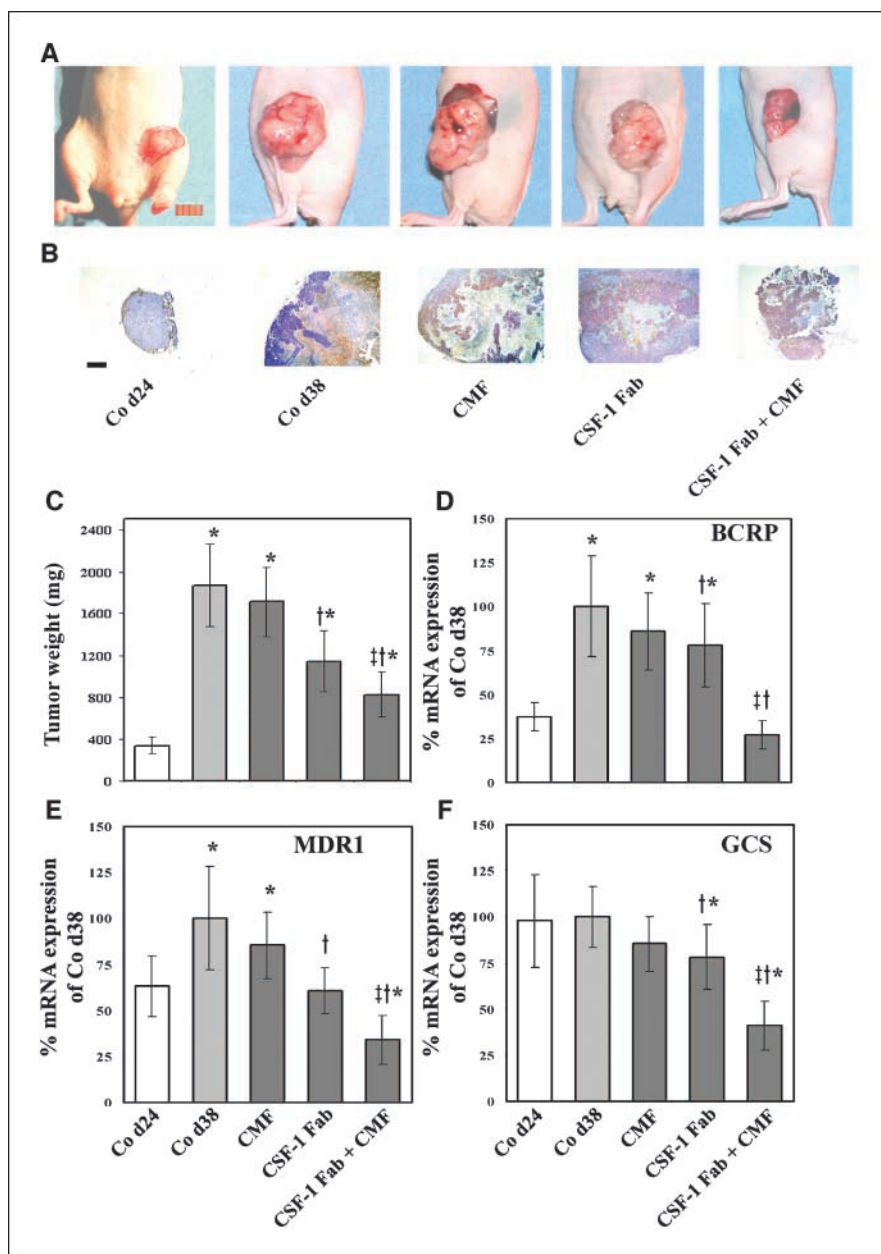
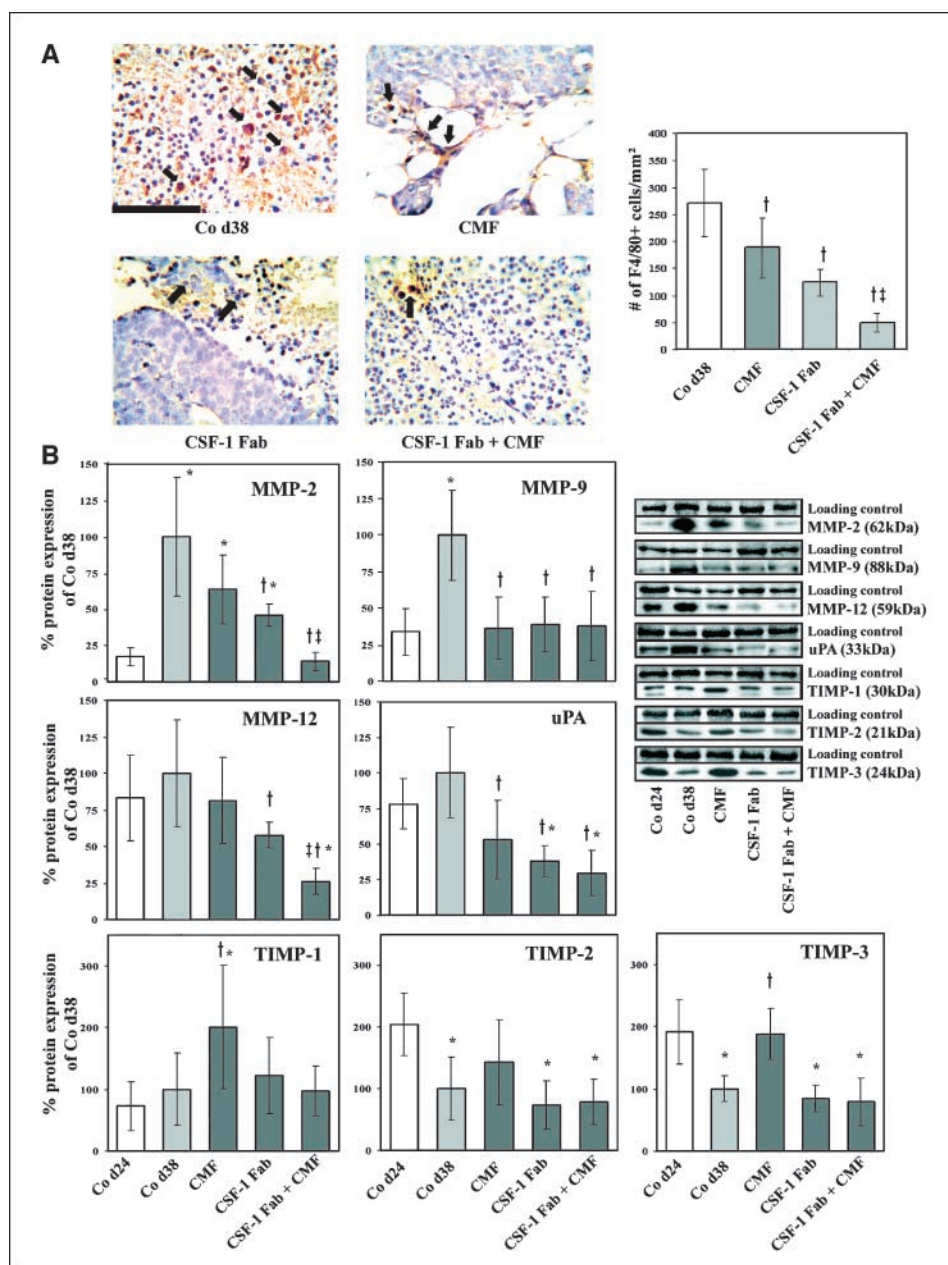


Figure 3. Anti-CSF-1 Fab or a combination of CMF with anti-CSF-1 Fab suppresses mammary tumor growth in mice and the expression of chemoresistance genes. **A**, representative images of human mammary tumor xenografts in a control mouse on day 24 (*Co d24*), in a control mouse that had been treated with Ringer's solution on day 38 (*Co d38*), and in mice treated with CMF alone, anti-CSF-1 Fab-1, or a combination of anti-CSF-1 Fab with CMF. Bar, 1 cm. **B**, representative photomicrographs of sections of mammary tumor xenografts stained with a Ki-67 antibody. Bar, 1 mm. **C**, mean mammary tumor xenograft weight. Real-time RT-PCR measurements of *BCRP* (**D**), *MDR1* (**E**), and *GCS* (**F**) in tumor xenograft lysates. *, significantly different from day 24 control; †, significantly different from day 38 control; ‡, significantly different from anti-CSF-1 Fab-treated mice. Columns, mean from $n = 10$ mice per group; bars, SD.

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Figure 4. Treatment with anti-CSF-1 Fab or CMF and anti-CSF-1 Fab reduces macrophage invasion and expression of MMP-12, MMP-9, MMP-2, and uPA proteins in mammary tumor xenografts. **A**, representative immunohistochemistry images of tumor tissue sections in a control mouse on day 38 (*Co d38*), in mice treated with CMF, anti-CSF-1 Fab, or with CMF and anti-CSF-1 Fab, stained with antibody to the mouse macrophage marker protein F4/80, and quantitative histomorphometric analysis showing the density of F4/80-positive cells per mm². *Arrows*, macrophages stained positively with F4/80 antibody inside the mammary tumor xenografts. Bar, 100 μ m. **B**, representative Western blot images of MMP-2, MMP-9, MMP-12, uPA, TIMP-1, TIMP-2, and TIMP-3 (*right*) and quantification of protein expression levels in tumor lysates. *, significantly different from control on day 24 (*Co d24*); †, significantly different from control on day 38 (*Co d38*); ‡, significantly different from anti-CSF-1 Fab treated mice. *Columns*, mean from *n* = 10 mice per group; *bars*, SD.



combination with CMF reduced MMP-2 ($P < 0.0001$), MMP-9 ($P < 0.0001$), MMP-12 ($P < 0.0001$), and uPA ($P < 0.0001$) tumor levels compared with control mice (Fig. 4B). Of note, the down-regulation of MMP-2 ($P = 0.0007$) and MMP-12 ($P = 0.0032$) levels in tumor lysates was significantly decreased with combined anti-CSF-1 Fab and CMF treatment compared with anti-CSF-1 Fab alone. CSF-1 antibody treatment or combined CMF with anti-CSF-1 Fab did not affect TIMP-1, TIMP-2, and TIMP-3 expression in tumor lysates compared with controls (Fig. 4B). CMF significantly up-regulated TIMP-1 and TIMP-3 tissue expression compared with control mice (Fig. 4B). These experiments showed that CSF-1 antibody in combination with CMF significantly reduces macrophage recruitment to the mammary tumor compared with untreated control mice and mice treated with Fab alone, and that this effect is associated with down-regulation of MMP-2 and MMP-12, consistent with the possible involvement

of macrophages and MMPs in tumor progression in this breast cancer model.

CSF-1 antibody decreases angiogenic activity in mammary tumor xenografts. With tumor progression, the density of proliferating endothelial cells increased in controls but was decreased following anti-CSF-1 Fab or combination CMF and antibody treatment compared with mice treated with CMF alone or Ringer's solution. Additionally, in mice treated with combination of anti-CSF-1 Fab and CMF, the density of proliferating endothelial cells decreased significantly when compared with mice treated with anti-CSF-1 Fab alone ($P = 0.0165$; Fig. 5A). Associated with the increased vascularity, VEGF-A mRNA levels increased during tumor progression ($P < 0.0001$) and were reduced after treatment with anti-CSF-1 Fab ($P < 0.0001$) or combined anti-CSF-1 Fab and CMF ($P < 0.0001$) but not following CMF or Ringer's solution treatment. Mice treated with anti-CSF-1 Fab and CMF combination had

significant lower VEGF-A mRNA levels in their tumor lysates versus animals treated with anti-CSF-1 Fab alone ($P = 0.0386$; Fig. 5B). The expression level of VEGF-A receptors Flt-1 and KDR was decreased by anti-CSF-1 Fab therapy ($P = 0.0076$ for Flt-1 and $P < 0.0001$ for KDR) and by combined treatment with anti-CSF-1 and CMF ($P = 0.0002$ for Flt-1 and $P < 0.0001$ for KDR) compared with controls (Fig. 5B). These data indicated that CSF-1 blockade by antibody combined with CMF decreases the expression of both VEGF-A and its receptors and reduces angiogenesis in mammary tumor xenografts.

CSF-1 antibody increases survival in mice with mammary tumor xenografts. The median time to death of mice in the control group was 56 days, and all mice died between 46 and 75 days after tumor cell grafting. Survival was significantly increased in mice following CSF-1 antibody or combined CSF-1 antibody and CMF treatment compared with mice treated with CMF alone or

control mice with median times to death of 101, 136, 65, and 56 days, respectively ($P < 0.0001$). At the time of death of the last mouse of the control group (75 days), 92% of CSF-1 antibody, 100% of CSF-1 antibody and CMF, and 42% of CMF-treated mice were alive. At the time of death of the last mouse of the anti-CSF-1 antibody group (day 139), 50% of the mice treated with the combination of CSF-1 antibody and CMF were still alive. Mice treated with anti-CSF-1 Fab and CMF combination had a significantly higher survival than those treated with anti-CSF-1 Fab alone ($P < 0.0001$; Fig. 6A). At the end of survival studies, the tumor weights were comparable in all groups at 9.53 ± 0.98 g (Fig. 6B). Importantly, the body weight of animals in the anti-CSF-1 Fab and the anti-CSF-1 Fab/CMF group was significantly higher at the end of the survival studies compared with the controls and the CMF-treated animals ($P < 0.0001$; Fig. 6B).

Discussion

CSF-1-regulated macrophages have been shown to be an important component of the tumor stromal environment. In >80% of clinical studies, an increase in tumor-associated macrophage density is correlated with poor prognosis (13). CSF-1 and the CSF-1R are expressed in tumors of the female reproductive tract (29), and in breast cancer, CSF-1 expression is correlated with leukocyte infiltration in the vast majority of cases (30). In a mouse mammary tumor model, the absence of CSF-1 accompanied by a reduction in tumor-associated macrophages caused delayed tumor progression to metastasis (14). Furthermore, we have recently shown that CSF-1 blockade with antisense oligonucleotides or small interfering RNAs slows the growth of mammary tumor xenografts, inhibiting the tumor recruitment of host-derived macrophages and the production of macrophage proteins, MMPs, and VEGF that enhance tumor progression (19). Consistent with these observations, the present findings indicate that anti-mouse CSF-1 antibody treatment reduces host CSF-1 and tumor-associated macrophages and increases the survival of human breast cancer-bearing mice.

The failure of the CSF-1 antibody treatment to lower CSF-1 mRNA is understandable as the antibody is a post-translational blocking agent. The decrease in CSF-1R mRNA is probably due to a combination of the decreased recruitment of CSF-1R-expressing F4/80-positive macrophages (Fig. 4A) and the reduced expression of the CSF-1R mRNA/cell resulting from decreased turnover of the CSF-1R due to the absence of CSF-1-induced receptor internalization and destruction, secondary to the absence of CSF-1. The reasons for the reduction in mouse CSF-1R mRNA in the mice treated with CMF alone are not clear. However, CMF treatment alone also significantly lowered the F4/80-positive macrophage density and the expression of MMP-2 and MMP-9, suggesting that the reduction in CSF-1R mRNA expression reflects the reduction in macrophage density.

Recent reports on the role of MMPs suggest that some of the CSF-1-mediated effect on tumor progression may be transmitted through MMPs (3, 9, 31–33). Apart from regulation at the level of gene transcription, the activities of MMPs are tightly regulated by endogenous inhibitors, the TIMPs (31). In the present study, consistent with our previous results using CSF-1 antisense treatment (8), CSF-1 antibody treatment suppressed the expression of the macrophage-specific MMP-12 as well as the expression of MMP-2, MMP-9, and uPA (34) that are involved in tumorigenesis and angiogenesis but did not affect TIMP expression. Thus, MMP-2, MMP-9, MMP-12, and uPA down-regulation resulting from

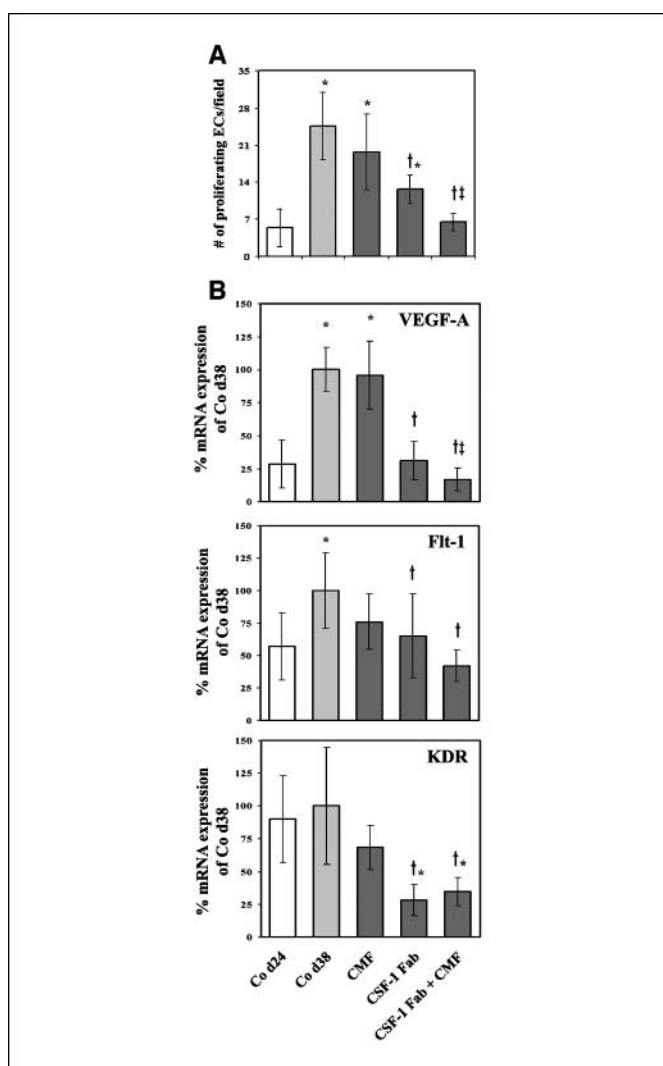


Figure 5. Angiogenic activity in mammary tumor xenografts is decreased by anti-CSF-1 Fab and combined CMF and anti-CSF-1 Fab treatment. **A**, quantitative histomorphometric analysis showing the density of capillaries with proliferating endothelial cells. **B**, real-time RT-PCR measurements of mRNA levels of VEGF-A and its receptors Flt-1 and KDR in tumor lysates. *, significantly different from control on day 24 (Co d24); †, significantly different from control on day 38 (Co d38); †, significantly different from anti-CSF-1 Fab treated mice. Columns, mean from $n = 10$ mice per group; bars, SD.

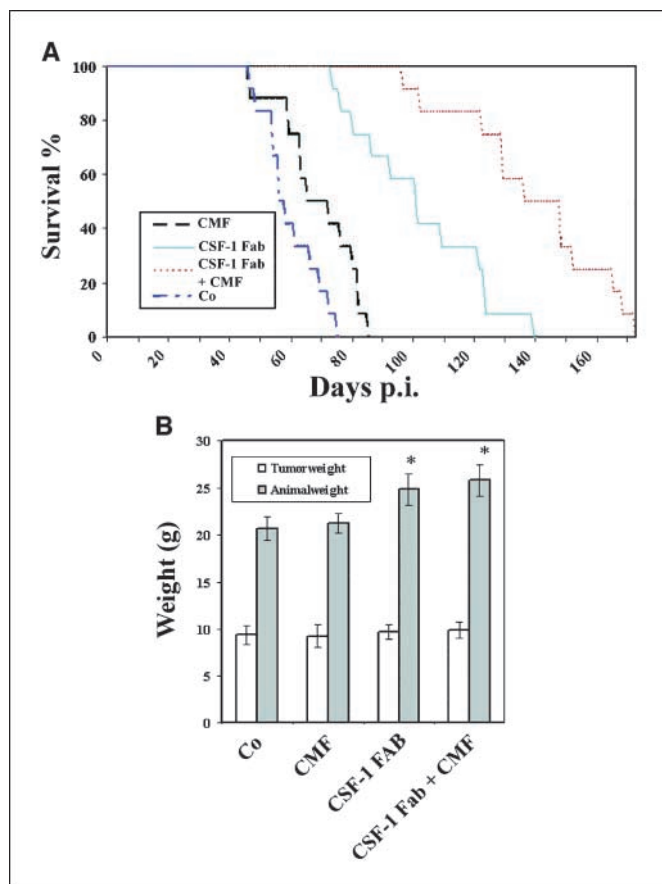


Figure 6. A, anti-CSF-1 Fab treatment increases the survival of mice with human mammary tumor xenografts. Survival was significantly increased in mice that received anti-CSF-1 Fab (*CSF-1 Fab*; $P < 0.01$) or CMF and anti-CSF-1 Fab (*CSF-1 Fab + CMF*; $P < 0.001$) but not after CMF therapy alone ($P = 1$) compared with control mice (*Co*). Mice treated with anti-CSF-1 Fab and CMF combination survived significantly longer than those treated with anti-CSF-1 Fab alone ($P < 0.001$). Points, % survival for $n = 12$ mice per group. B, animals treated with anti-CSF-1 Fab or anti-CSF-1 Fab/CMF had significantly higher body weights at the end of the survival studies compared with controls and CMF-treated animals ($P < 0.0001$). The tumor weights were not different between all groups. *, significantly different from survival controls and CMF-treated mice. Columns, mean from $n = 12$ mice per group; bars, SD.

inhibition of CSF-1 signaling could mediate part of the tumor inhibitory effect of CSF-1 antibody blockade. Consistent with this conclusion, excessive side branching and tumorigenesis occur when stromal MMPs are overexpressed in the mouse mammary gland (32, 33), and elevated stromal MMP activity is frequently seen in human breast hyperplasia, dysplasia, and carcinoma (35).

References

- Weir HK, Thun MJ, Hankey BF, et al. Annual report to the nation on the status of cancer, 1975–2000, featuring the uses of surveillance data for cancer prevention and control. *J Natl Cancer Inst* 2003;95:1276–99.
- Russo J, Russo IH. The pathway of neoplastic transformation of human breast epithelial cells. *Radiat Res* 2001;155:151–4.
- Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. *Science* 2002;296:1046–9.
- Ryan GR, Dai XM, Dominguez MG, et al. Rescue of the colony-stimulating factor-1 (CSF-1)-nullizygous mouse (*Csf1^{OP}/Csf1^{OP}*) phenotype with a CSF-1 transgene and identification of sites of local CSF-1 synthesis. *Blood* 2001;98:74–84.
- Seelentag WK, Mermod JJ, Montesano R, Vassalli P. Additive effects of interleukin 1 and tumour necrosis factor-alpha on the accumulation of the three granulocyte and macrophage colony-stimulating factor mRNAs in human endothelial cells. *EMBO J* 1987;6:2261–5.
- Stanley ER. CSF-1. In: Oppenheim JJ, Feldmann M, editors. Cytokine reference: a compendium of cytokines and other mediators of host defence. London: Academic Press; 2000. p. 911–34.
- Pixley FJ, Stanley ER. CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol* 2004;14:628–38.
- Cecchini MG, Dominguez MG, Mocci S, et al. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during post-natal development of the mouse. *Development* 1994;120:1357–72.
- Gouon-Evans V, Rothenberg ME, Pollard JW. Postnatal mammary gland development requires macrophages and eosinophils. *Development* 2000;127:2269–82.
- Pollard JW, Hennighausen L. Colony stimulating factor 1 is required for mammary gland development

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⁴ S. Wei and E.R. Stanley, unpublished.

- during pregnancy. *Proc Natl Acad Sci U S A* 1994;91:9312–6.
11. Liotta LA, Kohn EC. The microenvironment of the tumor-host interface. *Nature* 2001;411:375–9.
 12. Kacinski B. Expression of CSF-1 and its receptor CSF-1R in non-hematopoietic neoplasms. *Cancer Treat Res* 2002;107:285–92.
 13. Bingle L, Brown NJ, Lewis CE. The role of tumor-associated macrophages in tumor progression: implications for new anticancer therapies. *J Pathol* 2002;196:254–65.
 14. Lin EY, Nguyen AV, Russel RG, Pollard JW. Colony-stimulating factor-1 promotes progression of mammary tumors to malignancy. *J Exp Med* 2001;193:727–39.
 15. Aharinejad S, Marks SC, Jr., Bock P, et al. CSF-1 treatment promotes angiogenesis in the metaphysics of osteopetrotic (toothless, *tl*) rats. *Bone* 1995;16:315–24.
 16. Eubank TD, Galloway M, Montague CM, Waldman WJ, Marsh CB. M-CSF induces vascular endothelial growth factor production and angiogenic activity from human monocytes. *J Immunol* 2003;171:2637–43.
 17. Barbera-Guillem E, Nyhus JK, Wolford CC, Friece CR, Sampsel JW. Vascular endothelial growth factor secretion by tumor-infiltrating macrophages essentially supports tumor angiogenesis, and IgG immune complexes potentiate the process. *Cancer Res* 2002;62:7042–9.
 18. Aharinejad S, Abraham D, Paulus P, et al. Colony-stimulating factor-1 antisense treatment suppresses growth of human tumor xenografts in mice. *Cancer Res* 2002;62:5317–24.
 19. Aharinejad S, Paulus P, Sioud M, et al. Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. *Cancer Res* 2004;64:5378–84.
 20. Wei S, Lightwood D, Ladyman H, et al. Modulation of CSF-1-regulated post-natal development with anti-CSF-1 antibody. *Immunobiol* 2005;210:109–19.
 21. Teicher BA, Chen V, Shih C, et al. Treatment regimens including the multitargeted antifolate LY231514 in human tumor xenografts. *Clin Cancer Res* 2000;6:1016–23.
 22. May C, Gunther R, McIvor RS. Protection of mice from lethal doses of methotrexate by transplantation with transgenic marrow expressing drug-resistant dihydrofolate reductase activity. *Blood* 1995;86:2439–48.
 23. James RI, Warlick CA, Diers MD, Gunther R, McIvor RS. Mild preconditioning and low-level engraftment confer methotrexate resistance in mice transplanted with marrow expressing drug-resistant dihydrofolate reductase activity. *Blood* 2000;96:1334–41.
 24. Stierle V, Laigle A, Jolles B. Modulation of MDR1 gene expression in multidrug resistant MCF7 cells by low concentrations of small interfering RNAs. *Biochem Pharmacol* 2005;70:1424–30.
 25. Choi CH. ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. *Cancer Cell Int* 2005;5:30–42.
 26. Gouaze V, Liu YY, Prickett CS, Yu JY, Giuliano AE, Cabot MC. Glucosylceramide synthase blockade down-regulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs. *Cancer Res* 2005;65:3861–7.
 27. Stanley ER. The macrophage colony stimulating factor, CSF-1. *Methods Enzymol* 1985;116:564–87.
 28. Bartocci A, Pollard JW, Stanley ER. Regulation of colony-stimulating factor 1 during pregnancy. *J Exp Med* 1986;164:956–61.
 29. Kacinski BM. CSF-1 and its receptor in breast carcinomas and neoplasms of the female reproductive tract. *Mol Reprod Dev* 1997;46:71–4.
 30. Scholl SM, Pallud C, Beuvon F, et al. Anti-colony-stimulating factor-1 antibody staining in primary breast adenocarcinomas correlates with marked inflammatory cell infiltrates and prognosis. *J Natl Cancer Inst* 1994;86:120–6.
 31. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest* 1999;103:1237–41.
 32. Sternlicht MD, Lochter A, Sympon CJ, et al. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* 1999;98:137–46.
 33. Ha HY, Moon HB, Nam MS, et al. Overexpression of membrane-type matrix metalloproteinase-1 gene induces mammary gland abnormalities and adenocarcinoma in transgenic mice. *Cancer Res* 2001;61:984–90.
 34. Frandsen TL, Holst-Hansen C, Nielsen BS, et al. Direct evidence of the importance of stromal urokinase plasminogen activator (uPA) in the growth of an experimental human breast cancer using a combined uPA gene-disrupted and immunodeficient xenograft model. *Cancer Res* 2001;61:532–7.
 35. Shiomi T, Okada Y. MT1-MMP and MMP-7 in invasion and metastasis of human cancers. *Cancer Metastasis Rev* 2003;22:145–52.