

A Positive Feedback Loop between Mesenchymal-like Cancer Cells and Macrophages Is Essential to Breast Cancer Metastasis

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SUMMARY

The close vicinity of cancer cells undergoing epithelial-mesenchymal transition (EMT) and tumor-associated macrophages (TAMs) at the invasive front of tumors suggests that these two cell type may mutually interact. We show that mesenchymal-like breast cancer cells activate macrophages to a TAM-like phenotype by GM-CSF. Reciprocally, CCL18 from TAMs induces cancer cell EMT, forming a positive feedback loop, in coculture systems and humanized mice. Inhibition of GM-CSF or CCL18 breaks this loop and reduces cancer metastasis. High GM-CSF expression in breast cancer samples is associated with more CCL18⁺ macrophages, cancer cell EMT, enhanced metastasis, and reduced patient survival. These findings suggest that a positive feedback loop between GM-CSF and CCL18 is important in breast cancer metastasis.

INTRODUCTION

It is well established that tumor microenvironment plays an important role in cancer development and metastasis (Allavena et al., 2008; Condeelis and Pollard, 2006). Tumor-associated macrophages (TAMs), the most abundant immune-related stromal cells in tumor microenvironment (Allavena et al., 2008), are key orchestrators of tumor microenvironment, directly affecting neoplastic cell growth, neoangiogenesis, and extracellular matrix remodeling (Solinas et al., 2010). TAMs of breast cancer often display an alternatively activated phenotype, promoting tumor invasion and metastasis, and are associated with poor prognosis in cancer patients (Chen et al., 2011; DeNardo et al.,

2009). In addition, cancer cells can actively modulate nonmalignant stromal cells, including macrophages (Pollard, 2004), in tumor microenvironment to enhance cancer development and metastasis (Joyce and Pollard, 2009). Cancer is a disease of heterogeneity and plasticity; however, it is not yet known whether different kinds of cancer cells have different ability to modulate tumor microenvironment.

Epithelial-mesenchymal transition (EMT) is a process whereby cancer cells lose their epithelial properties to acquire a mesenchymal phenotype and become motile and invasive, which is closely associated with metastasis (Thiery, 2002). EMT has also been connected to induction of cancer stem cells (Mani et al., 2008), drug resistance (Thiery et al., 2009), and

Significance

Cancer cell epithelial-mesenchymal transition (EMT) and tumor-associated macrophages (TAMs) are associated with increased metastasis and are frequently observed at the invasive front of advanced tumors. However, the interaction between cancer cells undergoing EMT and TAMs remains unknown. In this study, mesenchymal-like cancer cells activate macrophages to a TAM-like phenotype via GM-CSF, suggesting that mesenchymal-like cancer cells may be a previously unappreciated source of TAM activators. In turn, TAMs induce EMT of breast cancer cells through CCL18, forming a positive feedback loop. Furthermore, the interaction between cancer cells and macrophages is essential to cancer metastasis in a humanized mouse model of breast cancer, suggesting that the feedback loop between GM-CSF and CCL18 could be a therapeutic target for cancer metastasis.

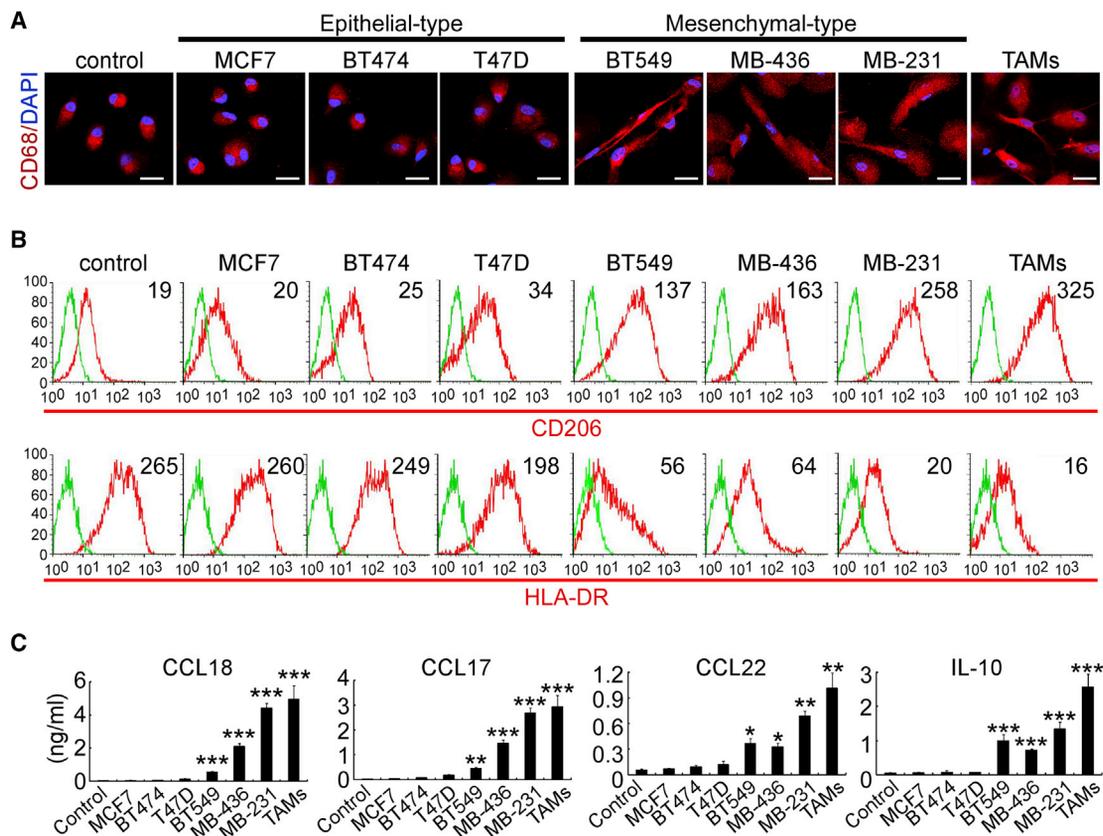


Figure 1. Mesenchymal-like Breast Cancer Cells Activate Macrophages to a TAM-like Phenotype

(A–C) Macrophages were obtained by culture of monocytes in DMEM medium supplemented with 10% heat-inactivated human AB serum in the presence or absence of 30% CM from indicated cell lines for 6 days. The primary TAMs were isolated from fresh human breast cancer tissue as positive controls.

(A) Fluorescent CD68/DAPI staining in macrophages. The scale bar represents 20 μ m.

(B) Expression of CD206/HLA-DR in macrophages. The histograms are representatives of five independent experiments of macrophages from five different donors. Numerical values denote the mean fluorescence intensity (MFI).

(C) Cytokine levels in the media of macrophages (mean \pm SEM, n = 5 independent experiments; *p < 0.05, **p < 0.01, and ***p < 0.001 compared with control). See also Figure S1.

immunosuppression (Kudo-Saito et al., 2009), suggesting that EMT may underlie many biological processes related to cancer development. It has been reported that EMT of cancer cells is frequently observed at the invasive front of advanced tumors (Christofori, 2006; Francí et al., 2006), where TAMs are usually found (Biswas and Mantovani, 2010; Wyckoff et al., 2004). These results suggest that cancer cells that undergo EMT may have a selective advantage in modulating tumor microenvironment. In this study, we investigated the interaction between cancer cells with EMT and TAMs and explored the underlying mechanisms that link these two metastasis-promoting phenomena.

RESULTS

Mesenchymal-like Breast Cancer Cells Activate Macrophages to a TAM-like Phenotype

To investigate whether cancer cells with distinct epithelial or mesenchymal-like phenotypes have different ability to activate macrophages, we cultured freshly isolated human monocytes in culture medium with 30% conditioned medium (CM) from six breast cancer cell lines, including three typical epithelial-like

lines (MCF-7, BT-474, and T47D) and three mesenchymal-like lines that express increased vimentin and decreased E-cadherin (BT-549, MDA-MB-436, and MDA-MB-231) (Figure S1A available online), for 6 days to obtain macrophages. As a positive control, we isolated primary TAMs from breast cancer tissue and found that primary TAMs produced high amounts of protumor cytokines (chemokine [C-C motif] ligand [CCL] 18, CCL17, CCL22, and interleukin-10 [IL-10]) (Chen et al., 2011; Dangaj et al., 2011; Mizukami et al., 2008) and exhibited a cluster of differentiation (CD) 206^{high}/human leukocyte antigen (HLA)-DR^{low} phenotype that is associated with immunosuppression (Dangaj et al., 2011; Kuang et al., 2007). Macrophages treated with CM from mesenchymal-like lines, but not CM from epithelial-like lines, became stretched and elongated (Figure 1A) and exhibited a CD206^{high}/HLA-DR^{low} phenotype (Figure 1B), similar to primary TAMs. Consistent with the changes in morphology and surface markers, macrophages treated with CM from mesenchymal-like lines produced significantly more tumor-promoting cytokines than untreated macrophages or those treated with CM from epithelial lines (Figure 1C), indicating functional activation of TAMs. Moreover, the level of activation by CM from

mesenchymal-like cancer cells, measured by cytokine production and CD206/HLA-DR expression, was comparable with that of primary TAMs, suggesting that CM from mesenchymal-like cancer cells is a strong macrophage activator.

To confirm whether the ability of cancer cells to activate TAMs is determined by their mesenchymal state, we induced EMT of MCF-7 cells with transforming growth factor β (TGF- β), tumor necrosis factor α (TNF- α) or by mammosphere culture (Guttilla et al., 2012) (Figure S1B). CM from MCF-7 cells that had undergone EMT also induced both morphological and functional TAM-like changes in macrophages, with CM from mammosphere-cultured MCF-7 (MCF-7_M) having the strongest effects (Figures S1C–S1E). BT-474 cells in which EMT was induced also showed similar effects (data not shown). Together, these data indicate that mesenchymal-like cancer cells or cancer cells that have undergone EMT can directly activate macrophages to a TAM-like phenotype and may serve as a previously unappreciated source of TAM activators as important as interleukin-4 (IL-4)-producing CD4⁺ T cells (DeNardo et al., 2009).

Granulocyte-Macrophage Colony-Stimulating Factor Mediates the Activation of Macrophages by Mesenchymal-like Breast Cancer Cells

To identify the cytokines secreted by mesenchymal-like cancer cells that activate macrophages, the cytokine profiles of CM from MCF-7, MCF-7 induced to undergo EMT by treatment of TGF- β , TNF- α , and prolonged mammosphere culture, and MDA-MB-231 cells were analyzed using RayBio Human Cytokine Antibody Array. Five cytokines were significantly increased in the CM of MCF-7 cells under EMT and MDA-MB-231 cells compared with CM of MCF-7 cells, which are granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-8 (IL-8), CCL2, growth-regulated oncogene (GRO; includes GRO α , GRO β , and GRO γ), and GRO α (Figure 2A). ELISA further confirmed the increases in GM-CSF, IL-8, CCL2, GRO α , GRO β , and GRO γ in the CM of mesenchymal-like cells (Figure S2). We then evaluated which of these cytokines is responsible for activating TAMs. We found that only GM-CSF, but not IL-8, CCL2, or GROs, significantly induced the production of TAM-related cytokines to levels comparable with the CM from MDA-MB-231 cells (Figure 2B). Addition of IL-8, CCL2, or GROs to GM-CSF did not further enhance the activation (data not shown), suggesting that GM-CSF alone is sufficient to activate macrophages. Furthermore, the addition of a neutralizing anti-GM-CSF antibody to the CM of MCF-7_M, MDA-MB-231, and MDA-MB-436 significantly suppressed the induction of the TAM-related surface marker expression (Figure 2C) and cytokines (Figure 2D), indicating that GM-CSF is necessary for mesenchymal-like cancer cells to induce macrophage activation to a TAM-like phenotype.

Tumor-Derived Lactate Abrogates the Proinflammatory Cytokine Production from GM-CSF-Activated Macrophages

It has been reported that GM-CSF can exhibit proinflammatory or anti-inflammatory activity in different disease models, and it has been hypothesized that conflicting roles of GM-CSF may be due to the concomitant stimulation factors (Hamilton, 2002). We compared the cytokine profiles of macrophages treated

with interferon- γ (IFN- γ) (a standard activator of classically activated macrophages), GM-CSF, or IL-4 (a standard activator of alternatively activated macrophages). We found that GM-CSF alone induced the production of both proinflammatory and anti-inflammatory cytokines. As previously reported (Hamilton, 2008), the addition of lipopolysaccharide (LPS) further increased the production of proinflammatory cytokines (Figure S3A). However, LPS is not a common factor found in tumor microenvironment. We then explored the role of lactate, one of the most abundant products of aerobic glycolysis and a widespread concomitant factor in tumor microenvironment (Hirschhaeuser et al., 2011). Breast cancer cell lines produced significantly more lactate (4.8–15.6 mM) than macrophages or immortalized breast epithelial cell lines (MCF-10A and HMLE) (0.8–1.7 mM) (Figure 3A). The addition of 5 mM lactate abrogated the production of proinflammatory cytokines but did not inhibit or even promoted that of anti-inflammatory cytokines from GM-CSF-activated macrophages (Figure 3B).

To further demonstrate the effect of tumor-derived lactate, we treated macrophages with CM of MDA-MB-231 and found that proinflammatory cytokines were significantly decreased, while anti-inflammatory cytokines were significantly increased. The suppression of proinflammatory cytokine production by 231 CM was completely reversed if the 231 cells were pretreated by oxamic acid, an inhibitor of lactate dehydrogenase that blocked the lactate production. The addition of exogenous lactate in oxamic acid-pretreated 231 CM restored the suppression of the proinflammatory cytokines (Figure 3C; Figure S3B), indicating that lactate is responsible for the skew of macrophages toward an anti-inflammatory phenotype.

Furthermore, we found that the higher the lactate level, the lower the pH value measured in the cell culture media (Figure 3A). To investigate whether the lactate or the drop in pH level was responsible for the suppression of proinflammatory cytokine production, we adjusted the pH value of the oxamic acid-pretreated 231 CM to the level of untreated 231 CM by 1% hydrochloric acid (HCl) (Figure S3B). We found the acidification by HCl did not suppress the production of proinflammatory cytokines (Figure 3C), suggesting that it is the lactate, not the drop in pH, that is accountable for the immunosuppressive effects by CM of cancer cells.

GM-CSF-Activated Macrophages Induce EMT in Breast Cancer Cells via CCL18

Previously, we have reported that TAMs can enhance metastasis of breast cancer cells (Chen et al., 2011; DeNardo et al., 2009), which is often associated with EMT. Thus, we examined whether activated macrophages can induce EMT of cancer cells. We found that MCF-7 cells, when cocultured with GM-CSF-activated macrophages, changed from a rounded shape to an elongated one, with loss of cell-cell contact, decrease of E-cadherin, increase of vimentin, and enhancements of migration and invasiveness (Figures 4A and 4B; Figure S4A), suggesting that GM-CSF-activated macrophages induced EMT of breast cancer cells. Further, anti-CCL18 neutralizing antibody inhibited EMT induced by GM-CSF-activated macrophages, and CCL18 alone induced EMT in MCF-7 (Figures 4A and 4B; Figure S4A), indicating that CCL18 is the cytokine responsible for the EMT of breast cancer cells induced by GM-CSF-activated macrophages.

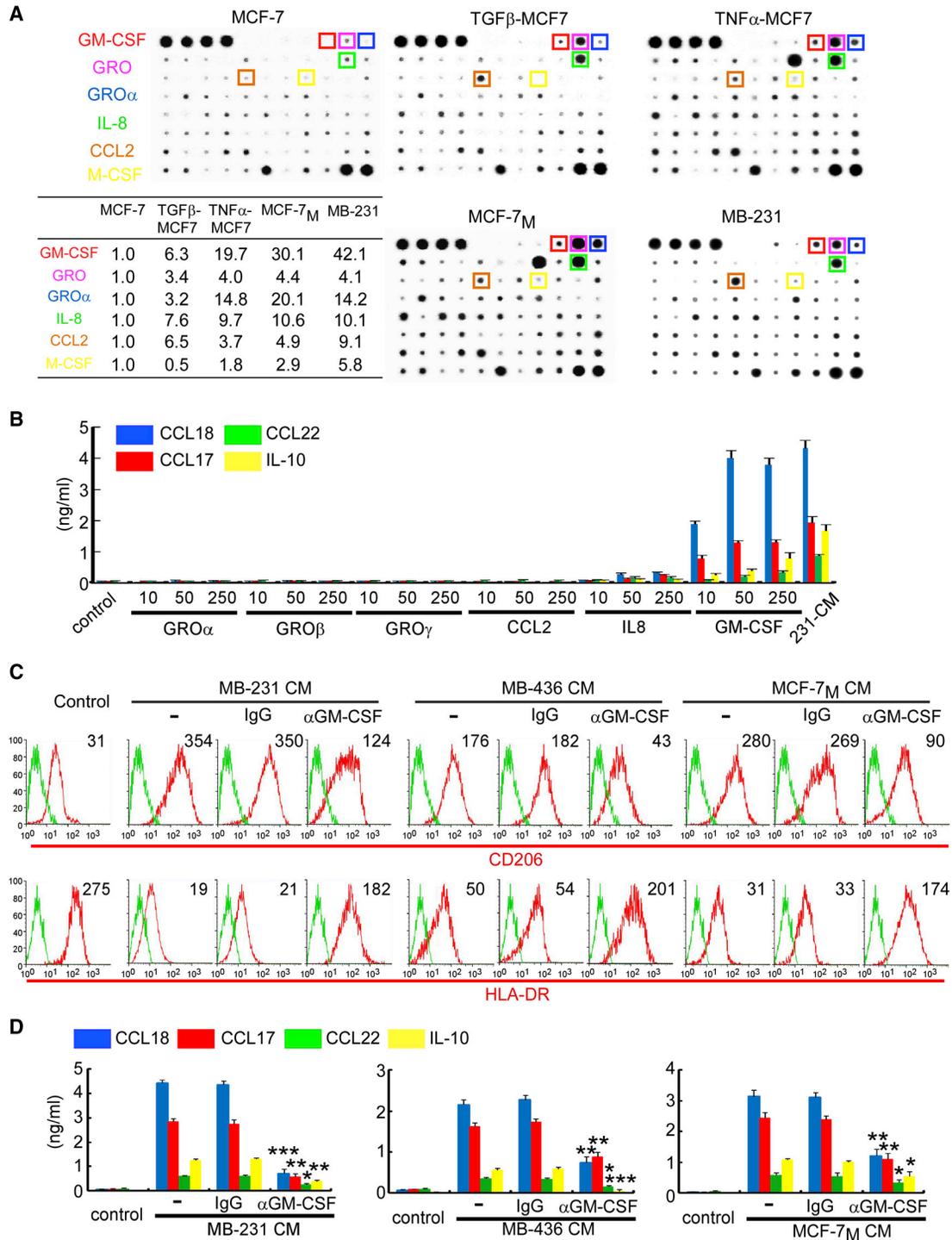


Figure 2. GM-CSF Mediates the Activation of Macrophages by Mesenchymal-like Breast Cancer Cells

(A) Cytokine array of the CM of MCF-7; MCF-7 induced to undergo EMT by treatment of TGF- β , TNF- α , and prolong mammosphere culture; and MDA-MB-231 cells. A table summarizing the relative signal intensity of indicated cytokines is presented in the lower left corner.

(B) Cytokines levels in the media of macrophages treated with indicated recombinant human cytokines at indicated concentrations (ng/ml) (mean \pm SEM, n = 4 independent experiments of macrophages from four different donors).

(C) Expression of CD206/HLA-DR in macrophages treated with 30% indicated tumor-CM in the presence or absence of control IgG or a GM-CSF neutralizing antibody. The histograms are representative of five independent experiments of macrophages from five different donors. Numerical values denote the MFI.

(D) Cytokine levels in the media of macrophages treated as in (C) (mean \pm SEM, n = 5 independent experiments; *p < 0.05, **p < 0.01, and ***p < 0.001 compared with macrophages treated with cancer cell CM alone; p values were obtained using two-tailed Student's t tests).

See also Figure S2.

It was recently shown that CCL18 induced EMT in breast cancer cells via the phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase 3 β (GSK3 β)/Snail signaling pathway (Zhang et al., 2013). We also observed that CCL18 activated PI3K/Akt pathways (data not shown). NF- κ B is an important transcription factor that links the activation of the PI3K/Akt pathway with EMT (Julien et al., 2007). Thus, we explored the role of NF- κ B and found that CCL18 induced p65 nuclear translocation and enhanced NF- κ B transcriptional activity through membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3) (Figure 4C; Figures S4B and S4C), indicating the activation of the NF- κ B pathway. Inhibition of NF- κ B by BAY-117082 (an IKK inhibitor), 4-methyl-N1-(3-phenyl-propyl)-benzene-1,2-diamine (JSH-23) (an inhibitor of NF- κ B nuclear translocation) or small interfering RNAs (siRNAs) to p65 reversed the CCL18-induced EMT in MCF-7 cells (Figure 4D), suggesting that CCL18 activates NF- κ B pathway to induce EMT.

NF- κ B is known to be important in EMT, and it also regulates the expression of various cytokines, including GM-CSF, IL-8, CCL2, and GRO (Barnes and Karin, 1997). We found that inhibition of NF- κ B by pharmacological inhibitors or siRNA targeting p65 not only led to decreased vimentin and increased E-cadherin in mesenchymal-like breast cancer cell lines (Figure 4D; Figure S4D) but also inhibited their cytokine production in CM (Figure 4E; Figure S4E), suggesting that high NF- κ B activity in cancer cells is not only essential to their mesenchymal properties but also important to the ability of secreting cytokines to induce TAMs.

A Positive Feedback Loop between GM-CSF and CCL18 In Vitro

Because mesenchymal-like cancer cells activate tumor-promoting macrophages that can in turn induce EMT of cancer cells, we investigated the possibility of a positive feedback loop between TAMs and cancer cells with EMT. CCL18-treated MCF-7 cells (MCF-7_{CCL18}) displayed an elongated shape and mesenchymal markers with increased GM-CSF in media. When these cells were replated without CCL18, the cells gradually reversed to their original round shape, with reexpression of E-cadherin and loss of vimentin by day 5 (Figure 5B; Figure S5A). The GM-CSF in the media also decreased after an initial increase within 48 hr (Figure 5A). However, coculture with macrophages maintained the mesenchymal state of MCF-7_{CCL18} cells, and the levels of GM-CSF and CCL18 in media kept increasing through day 5 (Figures 5A and 5B; Figure S5A). The cocultured macrophages became stretched and elongated and displayed a CD206^{high}/HLA-DR^{low} TAM-like phenotype (Figure S5B). Furthermore, the addition of anti-GM-CSF or anti-CCL18 neutralizing antibodies led to regression of the mesenchymal state of MCF-7_{CCL18} cells and TAM-like phenotype of macrophages, while decreasing the levels of both GM-CSF and CCL18 in media (Figures 5A and 5B; Figures S5A and S5B), suggesting mutually dependent increases of GM-CSF and CCL18 in the coculture system of MCF-7_{CCL18} cells and macrophages.

On the other hand, GM-CSF-activated macrophages, when replated without GM-CSF, slowly reversed to a CD206^{low}/HLA-DR^{high} phenotype (Figure 5D), and the production of CCL18 decreased after the peak at day 4 (Figure 5C). Nevertheless, coculture of GM-CSF-activated macrophages with MCF-7

not only induced EMT of MCF-7 cells (Figure S5C) but also maintained the TAM-like phenotype of the macrophages and increased the levels of GM-CSF and CCL18 through day 20 (Figure 5C and 5D). The addition of anti-GM-CSF or anti-CCL18 neutralizing antibodies, but not control immunoglobulin G (IgG), again led to the reversal of the mesenchymal state of MCF-7 cells and the TAM-like phenotype of macrophages, as well as decreasing GM-CSF and CCL18 in media (Figures 5C and 5D; Figure S5C). Collectively, these data suggest that a positive feedback loop between GM-CSF from mesenchymal-like cancer cells and CCL18 from TAMs sustains or promotes the mesenchymal state of cancer cells and the TAM-like phenotype of macrophages.

Furthermore, similar results were observed when we cocultured MCF-7 cells with macrophages activated by CM of MDA-MB-231, or cocultured macrophages with MDA-MB-231 cells or MCF-7 cells that were induced to EMT by TGF- β or TNF- α (data not shown), corroborating the existence of a GM-CSF-CCL18 positive feedback loop between TAMs and mesenchymal-like cancer cells.

The GM-CSF-CCL18 Loop Promotes EMT and Metastasis in a Humanized Mouse Model of Breast Cancer

It has been reported that chemokines released by human macrophages are very different from those released by mouse macrophages. Many of the chemokines upregulated in human alternatively activated macrophages either lack murine orthologs or are not upregulated in mice (Martinez et al., 2009). CCL18, the key cytokine released by human macrophages to promote metastasis (Chen et al., 2011), does not yet have a mouse counterpart. Therefore, to study the interaction between human macrophages and cancer cells in vivo, it is necessary to use a humanized mouse model with human hematopoietic cells. To establish such a model, nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice were treated with anti-asialo-GM1 antibody to deplete host natural killer (NK) cells, irradiated, transplanted with enriched CD34⁺ hematopoietic stem cells (HSCs) isolated from fresh human umbilical cord blood (CB) and injected with lentiviral vectors expressing human CSF1 to improve human monocyte reconstitution. This model, modified from a recent protocol (Wang et al., 2012), was reported to increase the engraftment of human monocytes and macrophages (Chen et al., 2009).

Engraftment of human HSCs into the mice resulted in the detection of human CD45⁺ leukocytes, CD14⁺ monocytes and macrophages, and CD19⁺ B cells in the peripheral blood and xenografts of humanized mice. Few human CD3⁺ T cells and CD56⁺ NK cells were detected (Figures S6A, S6B, and S6E), which is consistent with previous reports (Ito et al., 2012; Wang et al., 2012).

Consistent with the findings in vitro, injection of MCF-7_{CCL18} cells into the fat pads of humanized mice activated many CCL18⁺ macrophages nearby, maintained the GM-CSF expression and EMT state of cancer cells (Figure 6A), and significantly increased lung and liver metastasis (Figures 6B–6D), compared with the injection of MCF-7_{CCL18} cells into the irradiated mice without HSC transplantation or injection of MCF-7 cells without CCL18 induction into the humanized mice. Anti-GM-CSF

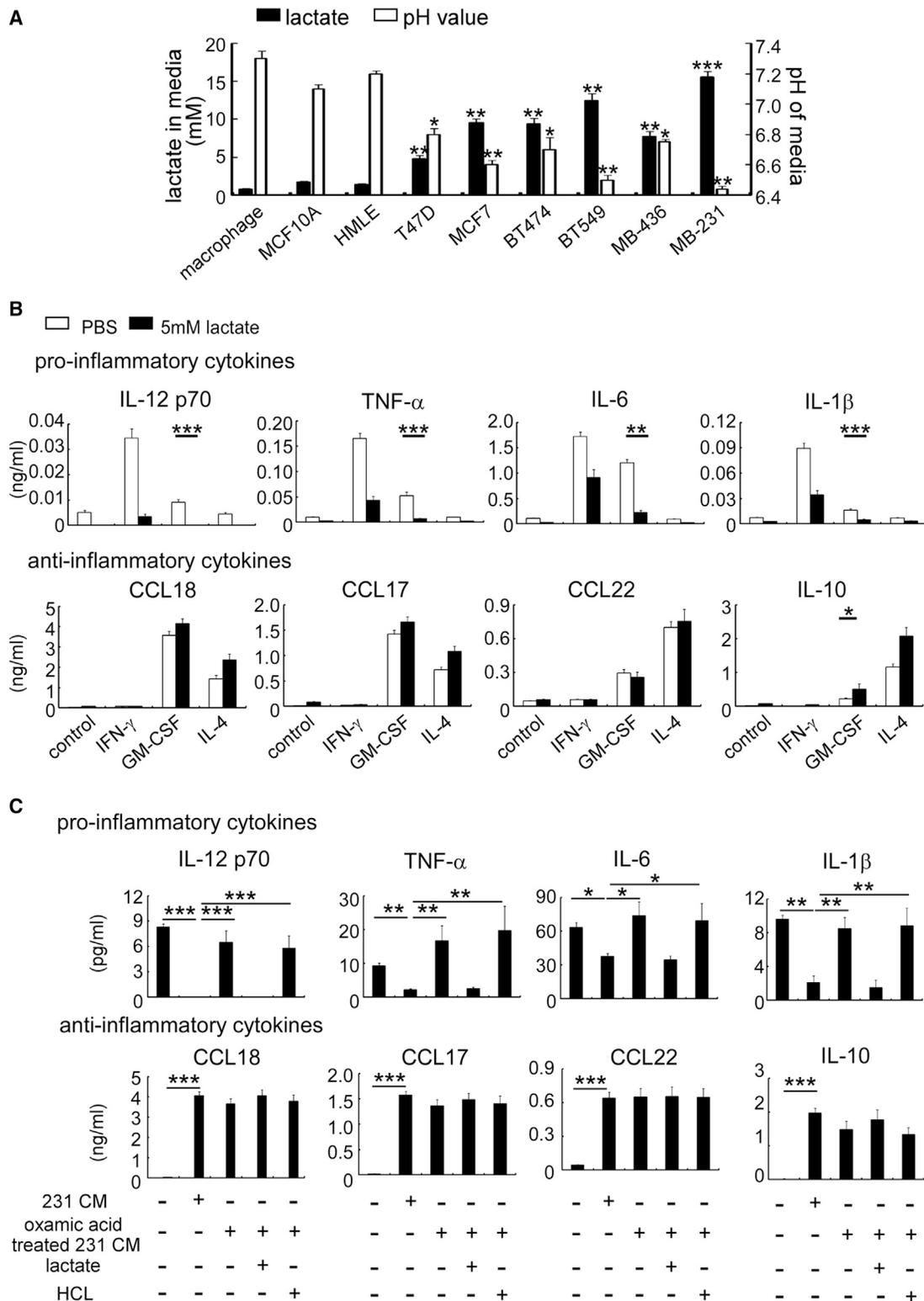


Figure 3. Tumor-Derived Lactate Abrogates the Proinflammatory Cytokine Production from GM-CSF-Activated Macrophages

(A) The indicated cells were plated in a 24-well plate at a density of 2×10^5 /ml. After 24 hr, lactate production and pH values were measured in the media (mean \pm SEM, n = 3; *p < 0.05 and **p < 0.01 compared with MCF10A; p values were obtained using two-tailed Student's t tests).

(B) Macrophages were obtained by culture of monocytes in culture medium in the presence or absence of 500 U/ml IFN- γ , 50 ng/ml GM-CSF, or 20 ng/ml IL-4 for 6 days. Afterward, macrophages were cultured in culture medium with or without 5 mM L-(+) lactic acid (lactate) for 24 hr. Cytokine levels in the media were

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neutralizing antibody not only decreased the number of CCL18⁺ cells adjacent to the tumor but also reversed the EMT state of cancer cells and inhibited their metastasis. Furthermore, anti-CCL18 antibody also decreased the expression of GM-CSF in tumor cells, reversed the EMT state of cancer cells, and inhibited metastasis (Figures 6A–6D). Neither GM-CSF nor CCL18 blockade altered human leukocyte reconstitution, the percentages of monocytes and macrophages (Figures S6A–S6D), and primary tumor size (Figure 6E) in humanized mice. These results indicate that GM-CSF from cancer cells and CCL18 from TAMs form a positive feedback loop and promote metastasis of cancer cells *in vivo*.

In addition, either anti-GM-CSF or anti-CCL18 antibody prolonged the metastasis-free survival of humanized mice injected with MCF-7_{CCL18} cells (Figure 6F), suggesting that the GM-CSF-CCL18 loop could be a therapeutic target to inhibit breast cancer metastasis. Similar results were observed in humanized mice injected with MDA-MB-231, a mesenchymal breast cancer cell line, or when GM-CSF was silenced in MCF-7_{CCL18} cells or CCL18 was silenced in HSC-derived macrophages (data not shown) in humanized mice injected with MCF-7_{CCL18} cells, substantiating the findings using MCF-7_{CCL18} cells with neutralizing antibodies.

The GM-CSF-CCL18 Loop Is Associated with EMT, Triple-Negative Subtype, Metastasis, and Poor Prognosis in Breast Cancer Patients

To determine whether our findings are clinically relevant, we examined 1,017 breast cancer cases from three independent breast cancer centers for the expression of GM-CSF. We found that GM-CSF was highly expressed in 293 (28.8%) cancer samples, often observed at the invasive front, with no to weak expression in normal or nonmalignant lesions of the breast (Figure S7A). Double immunostaining of GM-CSF and CCL18 showed significantly more stromal CCL18⁺ cells in cancers with high GM-CSF expression than in cancers with low GM-CSF expression (Figure 7A). The levels of GM-CSF and CCL18 were also highly correlated in patients' serum (Figure S7B). High GM-CSF expression was significantly correlated with lymph node and distant metastasis, more advanced histological grade, triple-negative breast cancer subtype (Table S1). Either GM-CSF or CCL18 was an independent prognostic factor associated with poor prognosis (Figure 7B; Tables S2 and S3).

Furthermore, the cancer samples with high GM-CSF expression or more stromal CCL18⁺ cells nearby expressed significantly less E-cadherin and more vimentin than the cancer samples with less GM-CSF or CCL18 expression (Figures S7C–S7E), an indication of EMT. More interestingly, the infiltration of CCL18⁺ macrophages was often observed at the invasive

front of tumors, where cancer cells displayed more mesenchymal features and expressed high levels of GM-CSF (Figure 7C). These data are consistent with the notion that mesenchymal-like cancer cells secrete more GM-CSF to activate TAMs that produce CCL18 at the invasive front.

To further validate these findings, we searched the OncoPrint database for the prognostic value of GM-CSF and CCL18 in human breast cancer. Four data sets showed a correlation between GM-CSF expression and high-grade or triple-negative subtype breast cancer, while one and four data sets showed associations between CCL18 expression and high-grade breast cancer and between CCL18 expression and triple-negative subtype breast cancer, respectively. In addition, four and three data sets confirmed that high levels of GM-CSF and of CCL18 are significantly correlated with poor prognosis in breast cancer patients, respectively. Furthermore, four and two data sets showed significantly inverse correlations between GM-CSF and CCL18 expression and E-cadherin expression, respectively. More important, in The Cancer Genome Atlas data set, with 593 breast cancer cases, GM-CSF was significantly correlated with CCL18 in breast cancer samples ($p < 0.0001$) (Table 1). Collectively, these data suggest that the GM-CSF-CCL18 loop plays an important role in EMT and metastasis of human breast cancer.

DISCUSSION

In this study, we show that cancer cells that have undergone EMT secrete GM-CSF to activate macrophages to a TAM-like phenotype. In turn, activated macrophages produce CCL18 to induce EMT of cancer cells, forming a positive feedback loop. This GM-CSF-CCL18 loop is essential to promoting the metastasis of breast cancer cells and is associated with poor prognosis in breast cancer patients.

EMT of cancer cells results in increased cell motility and is associated with metastasis. Our results indicate that cancer cells that undergo EMT, often located at the invasive front of tumors, have a superior function in activating macrophages to support tumor expansion. Besides metastasis, EMT of cancer cells is also linked with cancer stem cells, drug resistance, and immunosuppression (Kudo-Saito et al., 2009; Mani et al., 2008; Thiery et al., 2009). We report here that the change of secretory cytokine profile is also an important feature for cancer cells that undergo EMT. We previously described that CCL18 is one of the major cytokines released by TAMs and enhances breast cancer cell metastasis (Chen et al., 2011). In line with these results, CCL18 from activated macrophages induced EMT of breast cancer cells through the activation of NF- κ B. In addition, a panel of inflammatory cytokines, including GM-CSF, CCL2, IL-8, and GROs, were significantly induced as NF- κ B target genes (Barnes

measured by ELISA (mean \pm SEM, $n = 4$ independent experiments of macrophages from four different donors; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; p values were obtained using two-tailed Student's t tests).

(C) 231 cells were cultured in the presence or absence of 90 mM oxamic acid for 5 days. CM were collected after the cells were cultured for another 24 hr without oxamic acid. In addition, the lactate or pH level in 231 cell CM treated with oxamic acid were adjusted to the levels in untreated 231 cell CM by the addition of lactate or 1% HCl, respectively. Macrophages were obtained by culture of monocytes in culture medium in the presence or absence of indicated 231 CM for 6 days. The cytokine concentrations of the macrophages were measured afterward (mean \pm SEM, $n = 4$ independent experiments of macrophages from four different donors; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; p values were obtained using two-tailed Student's t tests).

See also Figure S3.

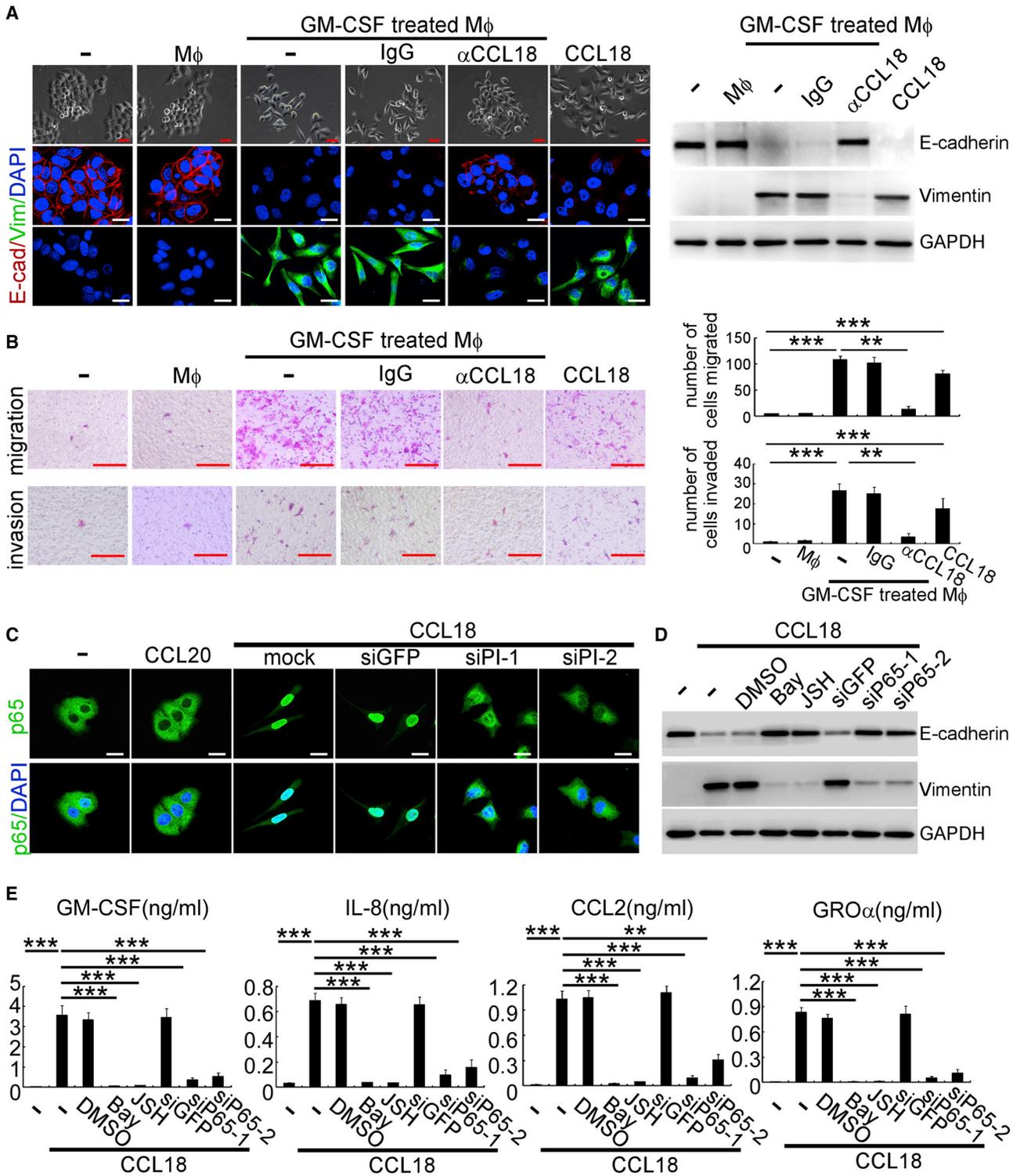


Figure 4. GM-CSF-Activated Macrophages Induce EMT of Cancer Cells through CCL18

(A) Morphology and E-cadherin and vimentin expression of MCF-7 cells without or with the coculture of control macrophages (M ϕ), GM-CSF-activated macrophages alone or with control IgG, anti-CCL18 neutralizing antibody, or recombinant human CCL18. The scale bar represents 20 μ m. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(B) Migration and invasion assays of MCF-7 cells treated as in (A). The scale bar represents 200 μ m (mean \pm SEM, n = 3; **p < 0.01 and ***p < 0.001; p values were obtained using two-tailed Student's t tests).

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and Karin, 1997) by CCL18. Among the cytokines induced during EMT, GM-CSF is the one responsible for macrophage activation. Other increased cytokines are also known to be important in other aspects of tumor microenvironment, for example, CCL2 in recruiting monocytes (Qian et al., 2011), GROs in neutrophil recruitment (Jinquan et al., 1995), and IL-8 in angiogenesis (Lin et al., 2004). It is possible that the inflammatory factors induced during EMT work together to provide a permissive environment for tumor progression.

It has been reported that both breast cancer cells and macrophages comigrate and depend upon each other to be invasive in vivo (Wyckoff et al., 2004). Our findings may extend knowledge of interactions between cancer cells and macrophages by showing that GM-CSF from cancer cells and CCL18 from TAMs form a positive feedback loop to sustain or promote EMT and metastasis of cancer cells. The levels of these two cytokines are mutually dependent, such that neutralizing either of them broke the vicious circle and suppressed breast cancer metastasis in a humanized mouse model. These results, together with the analysis of clinical samples, provide an explanation for the colocalization of TAMs and cancer cells that have undergone EMT at the invasive front of tumors.

Our data suggest a protumor role of GM-CSF in cancer. However, GM-CSF has been shown to elicit powerful immune responses and is often used as an immune adjuvant to cancer vaccines (Dranoff, 2002). However, the effect of exogenous GM-CSF on cancer remains controversial. Some recent clinical studies reported that GM-CSF as an adjuvant to different cancer vaccines can lead to adverse outcome in terms of immune response as well as relapse-free and overall survival (Faries et al., 2009; Filipazzi et al., 2007; Slingluff et al., 2009). Careful analysis suggested that GM-CSF may increase the vaccine-induced immune response when administered repeatedly at relatively low doses (range 40–80 μg for 1–5 days), whereas an opposite effect was often reported at higher dosages of 100–500 μg (Parmiani et al., 2007). Similar findings were also reported in animal models (Serafini et al., 2004). Furthermore, it has been consistently reported that endogenous GM-CSF from cancer cells is capable of suppressing the immune response (Bronte et al., 1999; Marigo et al., 2010; Tsuchiya et al., 1988) and correlated with increased metastasis (Park et al., 2007; Takeda et al., 1991). The analysis of our clinical samples as well as independent Oncomine data sets also showed that GM-CSF is associated with poor prognosis in breast cancer patients. We observed that LPS, a common factor of infectious disease, skewed GM-CSF-activated macrophages to a proinflammatory phenotype, while lactate, one of the most abundant tumor metabolites, changed GM-CSF-activated macrophages to an anti-inflammatory phenotype. It is possible that long-term proximal exposure to endogenous GM-CSF from cancer cells together with tumor metabolites, unlike the short-term sys-

temic use of low doses of GM-CSF in cancer vaccines, leads to cancer progression.

Compared with immortalized breast epithelial cell lines, both epithelial-like and mesenchymal-like breast cancer cell lines produced significantly more lactate. Further, the addition of 5 mM lactate, which was equivalent to or lower than the amount produced by both epithelial-like and mesenchymal-like breast cancer cell lines (4.8–15.6 mM), abrogated the production of proinflammatory cytokines but did not inhibit that of anti-inflammatory cytokines from GM-CSF-activated macrophages. These results indicate that lactate produced by epithelial-like breast cancer cells is sufficient to affect GM-CSF-activated macrophage. However, our data also demonstrated that GM-CSF produced by epithelial-like breast cancer cell lines was significantly less than that by mesenchymal-like breast cancer cells and was insufficient to activate macrophages. Thus, both increased lactate level and enhanced GM-CSF production are needed for breast cancer cells to activate macrophages to an anti-inflammatory phenotype.

Previous studies have identified the interaction between cancer cells and macrophages, including the colony-stimulating factor 1 (CSF-1)(macrophage colony-stimulating factor [M-CSF])-epidermal growth factor (EGF) loop by using a rat mammary cancer cell line (MTLn3) and mouse macrophages (Goswami et al., 2005; Hernandez et al., 2009). When human breast cancer line MDA-MB-231 was used in an in vivo mouse xenograft model, the proportion of macrophages among the invasive cells was only 6%, much less than the 25% observed with rodent tumors (Patsialou et al., 2009). In addition, while Iressa (an EGF receptor inhibitor) completely inhibited the EGF or CSF-1-driven invasion in rodent tumor, it failed to do so in the MDA-MB-231 xenografts (Patsialou et al., 2009). It is known that the chemokine profiles of human and mouse macrophages are very different. At least five chemokines that are upregulated in human alternatively activated macrophages either lack murine orthologs or are not upregulated in mice (Martinez et al., 2009). We find that GM-CSF is significantly induced in human mesenchymal-like cancer cells and is responsible for the macrophage activation, which then promotes the EMT of cancer cells to form the self-reinforcing loop. We also found that CSF-1 is much lower than GM-CSF and is only mildly increased in human mesenchymal-like cancer cells (data not shown). Moreover, blocking the CSF1-EGF loop has no significant effect on mesenchymal-like cancer cell-induced macrophage activation in vitro and metastasis of cancer cell in vivo (data not shown). More important, our findings using human breast cancer cell lines and human macrophages were validated in a humanized mouse model of breast cancer in vivo and further corroborated in 1,017 clinical samples as well as Oncomine data sets. Nevertheless, it is possible that GM-CSF plays an indispensable role in initiating the positive feedback loop at

(C) Confocal fluorescent microscopy of p65/DAPI staining in MCF-7 cells treated without or with rhCCL20 or rhCCL18 together with siRNAs targeting GFP or PITPNM3. The scale bar represents 20 μm .

(D) Western blotting for E-cadherin and vimentin of CCL18-treated MCF-7 cells pretreated with DMSO, BAY-117082, or JSH-23 or pretransfected with GFP-siRNA or P65-siRNAs.

(E) The cytokine levels in the media of cells treated as in (D) (mean \pm SEM, $n = 3$; ** $p < 0.01$ and *** $p < 0.001$; p values were obtained using two-tailed Student's t tests).

See also Figure S4.

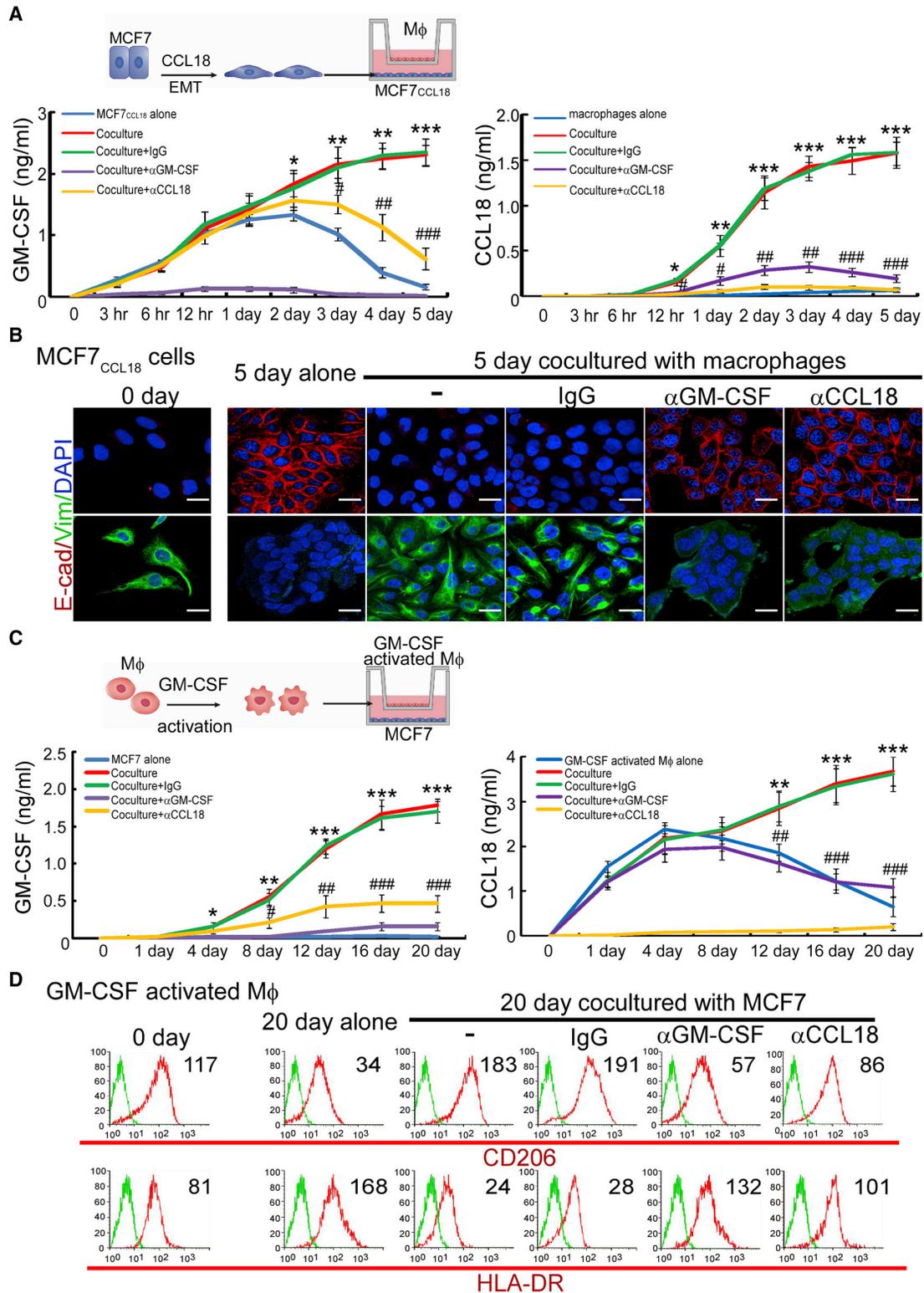


Figure 5. A Positive Feedback Loop between GM-CSF from Cancer Cells and CCL18 from Macrophages In Vitro

(A) Upper: schematic showing that MCF7_{CCL18} cells were re-plated alone or cocultured with macrophages in transwell apparatus with 0.4 μm pore size with or without control IgG, anti-GM-CSF, or anti-CCL18 neutralizing antibody for 5 days. Lower: GM-CSF and CCL18 levels in the indicated media were measured by ELISA at indicated time points. All values are mean ± SEM, n = 4 independent experiments of macrophages from four different donors; *p < 0.05, **p < 0.01, and ***p < 0.001 compared with cells cultured alone; #p < 0.05, ##p < 0.01, and ###p < 0.001 compared with cells in the coculture system without antibody addition at the same time points (p values were obtained using two-tailed Student's t tests).

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the early stage of metastasis, while other factors may be crucial at later stages.

In summary, our study suggests an important role of the positive feedback loop between cancer cells with EMT and TAMs in cancer metastasis. The GM-CSF-CCL18 loop is essential for cancer cells to maintain or promote their mesenchymal phenotype and could be a potential therapeutic target for cancer metastasis.

EXPERIMENTAL PROCEDURES

Detailed procedures are provided in [Supplemental Experimental Procedures](#).

Patients and Tissue Samples

Primary breast carcinomas were obtained from 759 patients at the Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University (Guangzhou, China), 145 patients at the First Affiliated Hospital, Shantou University Medical College (Shantou, China), and 113 patients at Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). Normal breast tissue from 9 patients undergoing breast reduction surgery and benign breast tissue samples from 23 patients with fibroadenoma and 17 patients with cystic fibrosis with or without atypical epithelial hyperplasia were collected at Sun Yat-Sen Memorial Hospital. Additionally, serum levels of GM-CSF and CCL18 were measured in peripheral blood samples from 151 patients with breast cancer before starting therapy at Sun Yat-Sen Memorial Hospital. All samples were collected with informed consent from patients, and all related procedures were performed with the approval of the internal review and ethics boards of the indicated hospitals.

Cell Culture and Treatment

MDA-MB-231, MDA-MB-436, BT-549, T47D, BT-474, and MCF-7 breast cancer cells were obtained from American Type Culture Collection and grown according to standard protocols. HMLE cells were kindly provided and cultured as recommended by Dr. R.A. Weinberg (Whitehead Institute, Cambridge, MA). When grown to 80% of confluence, cells were washed with PBS, and fresh serum-free media were added. CM were harvested 24 hr later and filtered through a 0.22 μ m filter. In some experiments, MCF-7 cells were treated with 5 ng/ml TGF- β , 10 ng/ml TNF- α , or 20 ng/ml CCL18 (PeproTech) for 2 weeks or given prolonged mammosphere culture for 5 weeks as previously described ([Guttilla et al., 2012](#)). Afterward, cells were washed in PBS and grown in adherent condition with cytokine/serum-free media, which were harvested 24 hr later. To inhibit respective pathways, cells were incubated with vehicle (DMSO), 5 μ M BAY-117082, or 6 μ M JSH-23 (Calbiochem) for 1 hr at 37°C prior to the experiments. To inhibit lactate production, cells were treated with 90 mM oxamic acid (Sigma) for 5 days.

Primary Human TAM Isolation from Breast Cancer

TAMs were isolated from five fresh breast cancer samples as previously described ([Chen et al., 2011](#); [Dangaj et al., 2011](#)), with slight modifications. Briefly, the tissues were minced into small (1 to 2 mm) pieces and digested with 5% fetal bovine serum Dulbecco's modified Eagle's medium containing 2 mg/ml collagenase I and 2 mg/ml hyaluronidase (Sigma) at 37°C for 2 hr. The cells were sequentially filtered through 500 μ m mesh, 100 μ m, and 70 μ m cell strainer. The cells were then centrifuged in a Beckman Allegra X-15R centrifuge at 2,500 rpm for 20 min with 1 ml cell suspension above

5 ml 45% Percoll (GE Healthcare) in the middle and 5 ml 60% Percoll at the bottom in a 15 ml tube. Mononuclear cells were collected from the cell layer in the interphase between 45% and 60% Percoll. CD14⁺ monocytes and macrophages were isolated by a magnetic-activated cell sorting using direct CD14 Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions.

Hematopoietic Stem/Progenitor Cell Isolation

Fresh human CB was obtained from Sun Yat-Sen Memorial Hospital, according to guidelines approved by the ethics boards and the Clinical Research Committee at Sun Yat-Sen Memorial Hospital. The HSCs were isolated as previously described ([Holt et al., 2010](#)). Briefly, CB mononuclear cells were separated by density gradient of Ficoll-Hypaque. CD34⁺ HSCs were isolated by using direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec). More than 95% of CD34⁺ cells were positively selected after two rounds of enrichment. The HSCs were transplanted within 24 hr after isolation.

Transplantation of CD34⁺ Cells and MCF-7 into NOD/SCID Mice

All animal work was conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at the medical college of Sun Yat-Sen University. Female NOD/SCID mice (3 to 4 weeks old) were injected intraperitoneally with 50 μ l of anti-asialo-GM1 antibody (Wako) 24 hr before HSC transplantation. The mice were subjected to 200 cGy total-body irradiation 12 hr before being injected with 2×10^5 HSCs in 0.2 ml of medium via the tail vein. After transplantation, treatment with anti-asialo-GM1 antibody was repeated once every 4 days for 24 days. The mice were injected with 0.1 ml of human M-CSF encoding lentiviral vectors at titers ranging from 5×10^8 to 10×10^9 total TU/ml via the tail vein 1 week after transplantation. At 8 weeks after HSC transplantation, 5×10^6 MCF-7 cells with indicated treatment were injected into the mammary fat pads of the mice. To supplement estrogen for MCF-7 growth, each mouse was implanted with a 1.5 mg 17 β -estradiol pellet (Innovative Research of America) 3 days before MCF-7 injection. For antibody treatment, mice were injected with CCL18-specific neutralizing antibody (Catalog No. ab9849; abCAM) and/or GM-CSF-specific neutralizing antibody (Catalog No. MAB215; R&D Systems) via the tail vein at 10 μ g/mouse twice weekly after the xenografts became palpable (about 0.4 cm in diameter). Tumor growth was evaluated by monitoring tumor volume (length \times width² \times 0.5) every 3 days. The whole-body metastasis burden of animals bearing xenografts that stably express luciferase was monitored using the IVIS Lumina Imaging System (Xenogen) every 2 days as previously described ([Oskarsson et al., 2011](#)). The animals were sacrificed when the xenografts reached 1.5 cm in diameter. The plasma, tumor xenografts, lungs, and livers of the sacrificed mice were harvested for further investigation. Sections (4 μ m) of the harvested organs were immunostained with antibody specific for human cytokeratin (catalog no. ab756; abCAM), and total RNA was extracted for quantitative reverse transcription polymerase chain reaction analysis of human hypoxanthine phosphoribosyltransferase (HPRT) mRNA expression.

Statistics

All statistical analysis was done using SPSS for Windows version 13.0 (SPSS). Pearson's correlation and regression analysis was performed to assess the relationship between GM-CSF and CCL18 in the serum of patients and human breast tissue mRNA expression data from Oncomine. Chi-square analysis was applied to analyze the relationship between GM-CSF expression and clinicopathological status. Kaplan-Meier survival curves were plotted, and the log-rank test was done. Groups of discrete variables were compared by means

(B) Immunofluorescence analysis of E-cadherin (E-cad) and vimentin (Vim) in MCF-7_{CCL18} cells alone or in the coculture system as described in (A). The scale bar represents 20 μ m.

(C) Upper: schematic showing that macrophages obtained by culture of monocytes in culture medium with 50 ng/ml GM-CSF for 6 days were re-plated alone or cocultured with untreated MCF-7 in the absence or presence of control IgG, anti-GM-CSF, or anti-CCL18 neutralizing antibody for 20 days. Lower: GM-CSF and CCL18 levels in the indicated media were measured by ELISA at indicated time points. All values are mean \pm SEM, n = 4 independent experiments of macrophages from four different donors; *p < 0.05, **p < 0.01, and ***p < 0.001 compared with cells cultured alone; #p < 0.05, ##p < 0.01, and ###p < 0.001 compared with cells in the coculture system without antibody addition at the same time points (p values were obtained using two-tailed Student's t tests).

(D) Surface marker detection by flow cytometry in GM-CSF-activated macrophages as described in (C). The histograms are representative of four independent experiments of macrophages from four different donors. Numerical values denote the MFI.

See also [Figure S5](#).

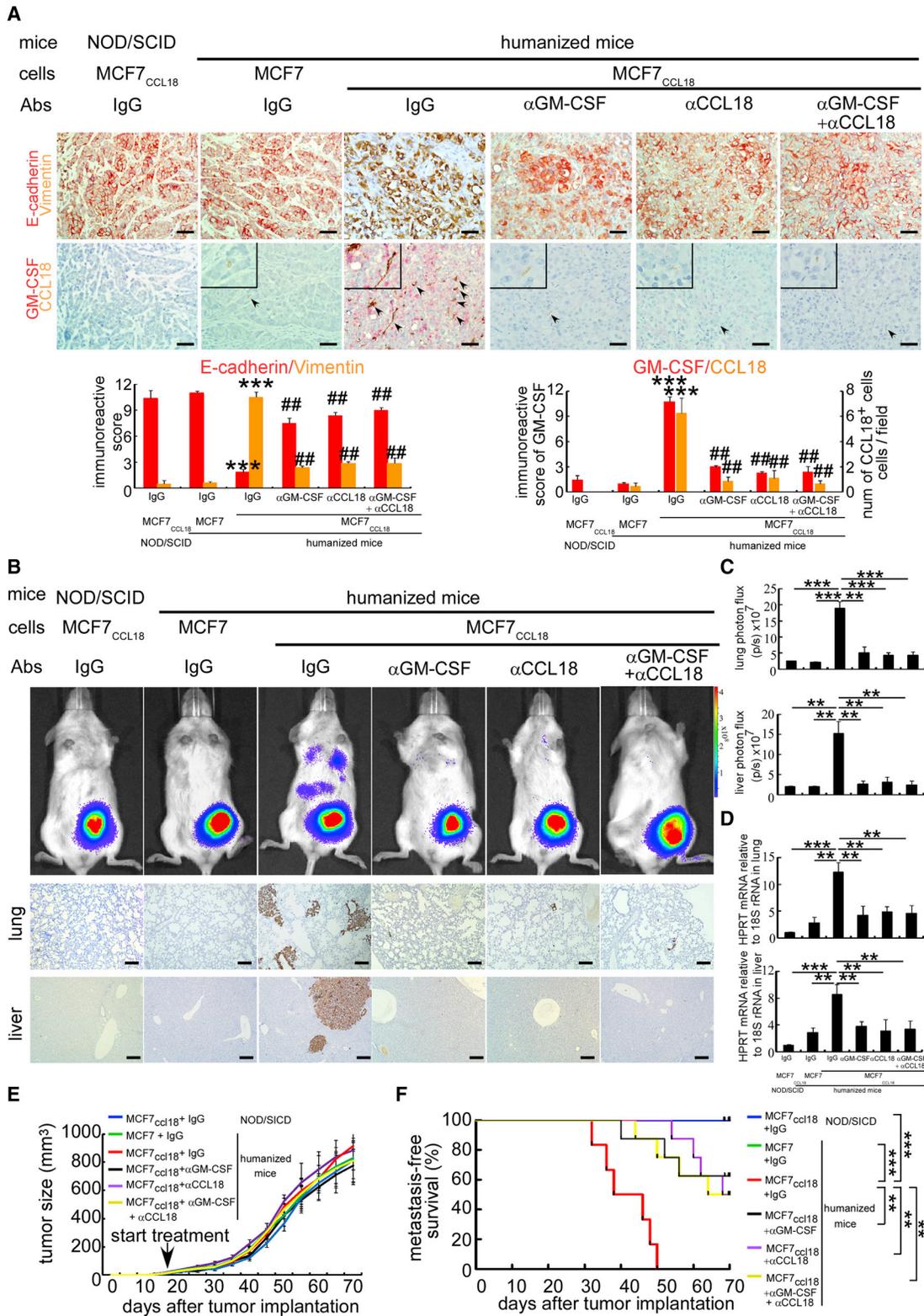


Figure 6. The GM-CSF-CCL18 Loop Promotes EMT and Metastasis in a Humanized Mouse Model

MCF-7_{CCL18} or MCF-7 cells without CCL18 treatment were injected into the mammary fat pads of NOD/SCID mice with or without human hematopoietic stem cell transplantation. Mice were injected with control IgG, anti-CCL18 neutralizing antibody, and/or anti-GM-CSF neutralizing antibody via the tail vein after the xenografts became palpable.

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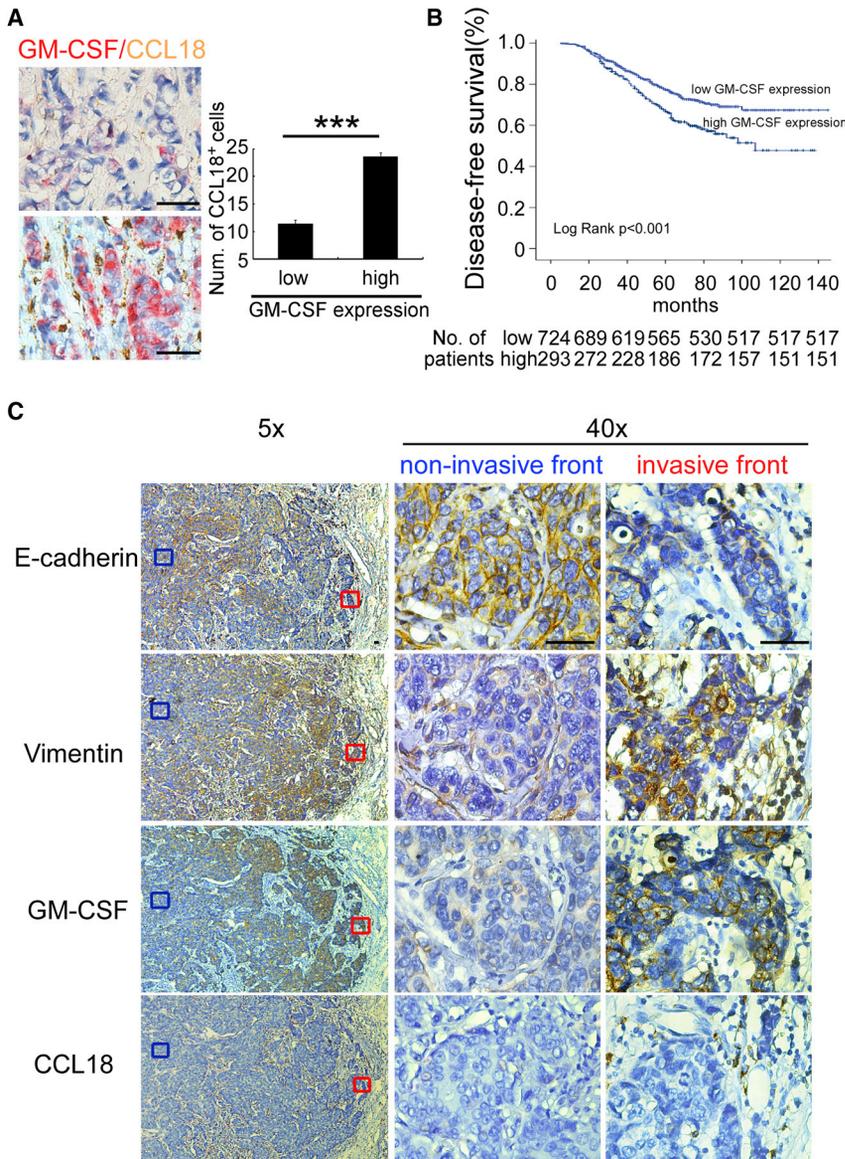


Figure 7. The GM-CSF-CCL18 Loop Is Associated with EMT and Metastasis in Breast Cancer Patients

(A) Left: representative double IHC staining of GM-CSF (red) and CCL18 (brown) in breast cancer samples with low (upper panel; n = 724) or high (lower panel; n = 293) GM-CSF expression. Right: quantification of CCL18⁺ macrophages in human breast cancer samples (mean ± SEM; ***p < 0.001; p values were determined using Student's t tests). Scale bars represent 50 μm.

(B) Kaplan-Meier survival curve of breast cancer patients with low (n = 724) and high (n = 293) GM-CSF expression (p < 0.001 by log-rank test), with a median follow-up period of 68 months. The number of surviving patients stratified to the follow-up periods is indicated below the graph.

(C) Representative IHC staining for E-cadherin, vimentin, GM-CSF, and CCL18 in the invasive front and noninvasive front of serial sections from a human breast cancer sample. The sample used was derived from 1,017 breast cancer cases from three independent breast cancer centers. Scale bars represent 50 μm.

See also Tables S1–S3 and Figure S7.

of the Mann-Whitney U test or Kruskal-Wallis nonparametric analysis of variance. All experiments for cell cultures were performed independently at least three times and in triplicate each time. In all cases, p values < 0.05 were considered statistically significant.

ACCESSION NUMBERS

The Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) accession number for the array data reported in this paper is GSE51938.

(A) Upper: representative pictures of double immunohistochemical (IHC) staining of E-cadherin (red) and vimentin (brown) or GM-CSF (red) and CCL18 (brown) in the sections from indicated tumor xenografts. Arrows indicate CCL18⁺ cells. Insets show CCL18⁺ cells at higher magnification. The scale bar represents 50 μm. Lower: immunoreactive scores of E-cadherin (red) and vimentin (brown) and immunoreactive scores of GM-CSF (red) and CCL18⁺ cell number counts (brown) within indicated xenografts (mean ± SEM, n = 8 for each group; ***p < 0.001 compared with cells in nonhumanized mice; ##p < 0.01 compared with MCF-7_{CCL18} in humanized mice treated with control antibody; p values were determined using Kruskal-Wallis tests). Abs, antibodies.

(B) Representative bioluminescence imaging (upper panel) and lung and liver sections immunostained with human cytokeratin antibody to indicate human cancer cell metastasis (lower panel) in mice treated as in (A). Scale bars represent 50 μm.

(C) Bioluminescence signal of lung and liver metastasis in mice with the indicated treatments (mean ± SEM, n = 8 for each group; **p < 0.01 and ***p < 0.001; p values were determined using Student's t tests).

(D) Expression of human HPRT mRNA relative to mouse 18S rRNA in the lungs and livers of the tumor-bearing mice. Data were normalized to the group of nonhumanized mice (mean ± SEM, n = 8 for each group; **p < 0.01 and ***p < 0.001; p values were determined using Student's t tests).

(E) Tumor size during the course of each indicated treatment (arrow indicates beginning of the treatment when tumor is palpable). Error bars show ±SEM.

(F) Kaplan-Meier analysis of metastasis-free survival in mice with the indicated treatments. Metastasis was determined by bioluminescence (n = 8 for each group; **p < 0.01 and ***p < 0.001; p values were determined using log-rank tests).

See also Figure S6.

Table 1. Correlation of CCL18 and GM-CSF Expression and Clinical Features in Breast Cancer in the OncoPrint Online Database

	Fold Change	p Value	Database (Case Number)
Upregulation of GM-CSF in Triple-Negative Breast Cancer			
Triple-negative breast carcinoma versus others	2.174	<0.0001	TCGA Breast (593)
Triple-negative breast carcinoma versus others	2.600	0.013	Stickeler Breast (57)
Triple-negative breast carcinoma versus others	1.113	0.039	Waddell Breast (85)
Triple-negative breast carcinoma versus others	1.192	0.042	Kao Breast (327)
Upregulation of GM-CSF in High-Grade Breast Cancer			
Grade III versus grades I and II	1.984	<0.0001	Loi Breast3 (77)
Grade III versus grades I and II	1.041	<0.0001	Curtis Breast (2136)
Grade III versus grades I and II	1.287	0.007	Gluck Breast (158)
Grade III versus grades I and II	1.926	0.015	Stickeler Breast (57)
Correlation of GM-CSF and Poor Prognosis in Breast Cancer			
Metastasis at 5 years versus no metastasis at 5 years	1.422	0.002	Kao Breast (327)
Metastasis at 5 years versus no metastasis at 5 years	1.424	0.015	Bos Breast (204)
Metastasis at 5 years versus no metastasis at 5 years	1.077	0.001	Hatzis Breast (508)
Recurrence at 1 year versus no recurrence at 1 year	1.022	0.043	Vandevijver Breast (295)
Upregulation of CCL18 in Triple-Negative Breast Cancer			
Triple-negative breast carcinoma versus others	2.053	<0.0001	TCGA Breast (593)
Triple-negative breast carcinoma versus others	2.695	0.006	Bild Breast (158)
Triple-negative breast carcinoma versus others	2.499	<0.0001	Bittner Breast (336)
Triple-negative breast carcinoma versus others	2.842	<0.0001	Chin Breast (118)
Upregulation of CCL18 in High-Grade Breast Cancer			
Grade III versus grades I and II	3.771	0.002	Sorlie Breast (167)
Correlation of CCL18 and Poor Prognosis in Breast Cancer			
Recurrence at 5 years versus no recurrence at 5 years	3.351	0.003	Ma Breast (60)
Dead at 5 years versus alive at 5 years	1.135	0.007	Vandevijver Breast (295)
Metastasis at 5 years versus no metastasis at 5 years	1.159	0.049	Hatzis Breast (508)
Correlation of GM-CSF and E-cadherin in Breast Cancer			
Correlation of GM-CSF and E-cadherin in breast cancer	-0.289	<0.0001	Hatzis Breast (508)
Correlation of GM-CSF and E-cadherin in breast cancer	-0.116	0.004	TCGA Breast (593)
Correlation of GM-CSF and E-cadherin in breast cancer	-0.183	0.001	Bittner Breast (336)
Correlation of GM-CSF and E-cadherin in breast cancer	-0.112	0.043	Kao Breast (327)
Correlation of CCL18 and E-cadherin in Breast Cancer			
Correlation of CCL18 and E-cadherin in breast cancer	-0.090	0.043	Hatzis Breast (508)
Correlation of CCL18 and E-cadherin in breast cancer	-0.238	0.015	TCGA Breast (593)
Correlation of CCL18 and GM-CSF in Breast Cancer			
Correlation of CCL18 and GM-CSF in breast cancer	0.533	<0.0001	TCGA Breast (593)

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2014.03.021>.

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