Chemokine (C-C Motif) Ligand 2 Engages CCR2+ Stromal Cells of Monocytic Origin to Promote Breast Cancer Metastasis to Lung and Bone*

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Metastatic spread of cancer to distant vital organs, including lung and bone, is the overwhelming cause of breast cancer mortality and morbidity. Effective treatment of systemic metastasis relies on the identification and functional characterization of metastasis mediators to multiple organs. Overexpression of the chemokine (C-C motif) ligand 2 (CCL2) is frequently associated with advanced tumor stage and metastatic relapse in breast cancer. However, the functional mechanism of CCL2 in promoting organ-specific metastasis of breast cancer has not been rigorously investigated. Here, we used organ-specific metastatic sublines of the MDA-MB-231 human breast cancer cell line to demonstrate that overexpression of CCL2 promotes breast cancer metastasis to both lung and bone. Conversely, blocking CCL2 function with a neutralizing antibody reduced lung and bone metastases. The enhancement of lung and bone metastases by CCL2 was associated with increased macrophage infiltration and osteoclast differentiation, respectively. By performing functional assays with primary cells isolated from the wild type, CCL2 and CCR2 knock-out mice, we showed that tumor cell-derived CCL2 depends on its receptor CCR2 (chemokine, CC motif, receptor 2) expressed on stromal cells to exert its function in promoting macrophage recruitment and osteoclast differentiation. Overall, these data demonstrated that CCL2-expressing breast tumor cells engage CCR2+ stromal cells of monocytic origin, including macrophages and preosteoclasts, to facilitate colonization in lung and bone. Therefore, CCL2 and CCR2 are promising therapeutic targets for simultaneously inhibiting lung and bone metastasis of breast cancer.

Breast cancer is the most common malignancy in women in the United States, with an estimated 182,000 new cases and 40,000 deaths in 2008 (1). Late stage breast cancer patients develop metastases in bone, lung, liver, brain, and other organs, which are responsible for most breast cancer-related mortality and morbidity (2). Severe complications from bone metastasis include debilitating bone fractures, nerve compression and bone pain, and hypercalcemia (3–5), whereas lung metastasis is accompanied by cough, bloody sputum, rib cage pain, and, eventually, failure of the respiratory functions (6). Colonization of different secondary organs by breast cancer is believed to be a complex, multigenic process that depends on productive interactions between tumor cells and stromal microenvironments through concerted actions of organ-specific metastasis genes (7, 8). Functional genomic analysis of preclinical models of breast cancer to bone, lung, and brain have identified distinct sets of organ-specific metastasis genes (9–11), providing novel mechanistic insights into key rate-limiting steps of metastasis to different organs. However, as advanced breast cancer patients often suffer from metastases at several secondary organs, identifying genes that are capable of instigating metastasis to multiple sites may provide the ideal targets for therapeutic intervention of systemic metastasis.

Chemokines are small (8–14 kDa) proteins classified into four conserved groups (CXC, CC, C, and CX3C) based on the position of the first two cysteines that are adjacent to the amino terminus (12). They are chemotactic cytokines that stimulate directed migration of leukocytes in response to inflammatory signals. Chemokines are also involved in the maintenance of hematopoietic homeostasis, regulation of cell proliferation, tissue morphogenesis, and angiogenesis (13). Chemokines bind to the seven-transmembrane domain receptors to elicit downstream molecular events that coordinate cell movement. Even though chemokines are unlikely to be a contributing factor for tumor initiation, they can have pleiotropic effects on tumor progression (13, 14). Among more than 50 human chemokines, CCL2 is of particular importance. CCL2, also called monocyte chemoattractant protein-1 (MCP-1), is a potent chemoattractant for monocytes, memory T lymphocytes, and natural killer cells (15). It is involved in a number of inflammatory conditions associated with monocyte recruitment, including delayed hypersensitivity reactions, bacterial infection, arthritis, and renal disease (15). The importance of CCL2 in cancer was manifested by its overexpression in a variety of tumor types, including glioma, ovarian, esophagus, lung, breast, and prostate cancers (15–17). In prostate cancer, CCL2 expression levels was associated with advanced pathological stage (16). Importantly, CCL2-neutralizing antibodies inhibit bone resorption in vitro and bone metastasis in vivo (18–20). In lung cancer, serum CCL2 levels were elevated in lung cancer patients with bone metastasis compared with localized diseases. Neutralizing antibodies against CCL2 also inhibited the tumor conditioned...
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media-induced osteoclast formation in vitro and bone metastasis in vivo (17). Taken together, these findings suggested a role of CCL2 in bone metastasis.

A potential role of CCL2 in breast cancer progression and metastasis has been indicated by the analysis of CCL2 expression of tumor and serum samples from breast cancer patients. Serum CCL2 levels were significantly higher in postmenopausal breast cancer patients than in age-matched controls (21). Over 50% of breast cancer tumor samples had intense staining of CCL2 in tumor cells (22). Prognostic analysis further revealed that high expression of CCL2 was correlated with advanced tumor stage, lymph node metastasis (23), and early relapse (24). CCL2 up-regulation in breast tumors was also associated with the infiltration of tissue-associated macrophages (TAMs)3 and with increased microvessel density (22, 24). TAMs have been known to contribute to primary tumor progression and metastasis of breast cancer (25), which is supported by epidemiological evidence showing that TAM infiltration portended a poor clinical outcome (26, 27). However, whether the function of CCL2 in modulating activity of macrophages and possibly other cell types is important for breast tumor organotropism has not been rigorously investigated. CCL2 may engage organ-specific cell types derived from the same bone marrow myelomonocytic progenitors. These progenitors differentiate into osteoclast precursors in bone or into blood monocytes that eventually become mature macrophages in different tissues, like alveolar macrophages in lung (28). These stromal cell types of myelomonocytic origin may contribute to different functions in different organ-specific metastases. Another unresolved question regarding the function of CCL2 in tumor-stroma interaction is the functional involvement of CCL2 receptors. CCL2 can bind to both CCR2 and CCR4 (29, 30). Loss of function studies in mice showed CCL2 and CCR2 knock-out mice displayed similar impairments in monocyte migration (31, 32), suggesting that CCR2 is the major functional receptor for CCL2. Understanding whether CCR2 deficiency in stromal cells leads to compromised monocyte engagement by CCL2-expressing tumor cells may have important implications in designing targeting therapeutics against the CCL2/CCR2 axis.

In this study, we used the recently developed organ-specific metastatic sublines of the human breast cancer cell MDA-MB-231 (9, 10, 33) and showed that overexpression of CCL2 promotes both lung and bone metastases. This function was associated with increased TAM infiltration in lung metastasis and increased osteoclast differentiation in bone metastasis, respectively. Furthermore, by using macrophages and bone marrow cells isolated from wild type, CCL2-deficient, and CCR2-deficient mice, we showed that CCR2 expression in stromal cells is essential for tumor-derived CCL2 to recruit macrophages and promote osteoclastogenesis. Targeting tumor-derived CCL2 by a neutralizing antibody significantly reduced metastasis formation in both bone and lung.

3 The abbreviations used are: TAM, tissue-associated macrophage; BLI, bioluminescence imaging; TRAP, tartrate-resistant acid phosphatase; ELISA, enzyme-linked immunosorbent assay; DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The SCP28, LM2, and 1833 cell lines were derived from the parental cell line MDA-MB-231 (American Type Culture Collection) (9, 10). These sublines, their genetically modified variants, the retroviral packing cell line H29, and the mouse monocyte/preosteoclast cell line RAW264.7 (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. The human cell line hFOB1.19 (American Type Culture Collection) was maintained as preosteoblasts in Dulbecco’s modified Eagle’s medium:F12 without phenol red supplemented with 0.3 mg/ml G418 and 10% fetal bovine serum at 34 °C. When switched to 39.5 °C for 2 days after confluence, the cells differentiated into mature osteoblasts. Recombinant human CCL2, murine receptor activator of NF-κB ligand and murine macrophage colony stimulating factor were purchased from PeproTech.

Generation of CCL2-overexpressing Cells—The human CCL2 coding sequence was inserted into the Hpal site of the retroviral vector pMSCVhyg (Clontech). pMSCVhyg-CCL2 or the vector was transfected into the packaging cell line H29. After 48 h, viruses were collected, filtered, and used to infect target cells in the presence of 5 μg/ml polybrene. The infected cells were selected with 1.5 mg/ml hygromycin. To avoid clonal variations, a pooled population of at least 500 independent colonies from each transduction was used to generate each stable cell line.

Western Blot Analysis—To detect CCL2 in conditioned medium by Western blotting, cells were grown to confluence, changed to serum-free medium for 24 h before the medium was collected, concentrated with Amicon centrifugal filter units (Millipore), and applied to immunoblotting. The primary antibody was mouse anti-human CCL2 (MAB279, R&D Systems). Anti-mouse IgG secondary antibody conjugated to horseradish peroxidase was used for chemiluminescence detection (GE Healthcare).

ELISA—To detect CCL2 in the conditioned medium by enzyme-linked immunosorbent assay (ELISA), cells were grown to confluence, changed to fresh medium and cultured for 24 h before the medium was collected. Human CCL2 ELISA kit (eBioscience) was used to measure CCL2 concentration following the manufacturer’s instructions.

Reverse Transcription (RT)-PCR and Quantitative RT-PCR—Total RNA was isolated from adherent cells using RNeasy kit (Qiagen) and reverse-transcribed with Superscript III kit (Invitrogen) according to the manufacturer’s instructions. Primers used for amplifying cDNA fragments are: human CCR2 (364 bp), 5′-gacaagccacagctgaaca-3′ (forward) and 5′-ggtggtgcttggagtca-3′ (reverse); human GAPDH (475 bp), 5′-gtcaccagacctgttgct-3′ (forward) and 5′-tcagctggtgctgctgca-3′ (reverse); mouse CCL2 (248 bp), 5′-aggtccctgcctctg-3′ (forward) and 5′-ctggtgagctgctgctg-3′ (reverse); mouse CCR2 (252 bp), 5′-ggtcaatctcagctgctg-3′ (forward) and 5′-ggttcagctgtgctgctg-3′ (reverse); mouse CCL2 (248 bp), 5′-aggtccctgcctctg-3′ (forward) and 5′-ctggtgagctgctgctg-3′ (reverse); mouse GAPDH (977 bp), 5′-gttgggtgtaagctgctg-3′ (forward) and 5′-ggttcagctgtgctgctg-3′ (reverse). Quantitative PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) with the ABI Prism 7900HT thermocycler (Applied Biosystems).
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The following primers were used: human CCL2 (66 bp): 5'-cagccagatgaatcagtgc-3' (forward) and 5'-gacctgagttctctctgtggtaa-3' (reverse); Human GAPDH (225 bp), 5'-gagatgtaagggctgttgatgc-3' (forward) and 5'-gagatggtagggattc-3' (reverse). CCL2 mRNA level was normalized with GAPDH level.

In Vitro Growth Curve and Anchorage-independent Growth—To establish growth curves, 2 × 10^4 cells were seeded in each well of 6-well plates with triplicate for each time point. Medium was replaced every 2 days. Cell number was determined with dead cell exclusion using trypan blue every 2 days. For soft agar growth, a bottom layer of 0.6% agar in growth medium was added to 6-well plates with triplicate for each cell line. 2 × 10^4 cells were seeded in 0.3% top agar containing growth medium to each well. Three weeks later, colonies were stained with crystal violet for visualization.

Tumor Xenografts and Bioluminescence Imaging (BLI) Analyses—All procedures involving mice, such as housing and care, and all experimental protocols were approved by Institutional Animal Care and Use Committee of Princeton University. For intracardiac injections, 10^5 cells were injected into the left cardiac ventricle of female nude mice (National Cancer Institute) as described (9). For intravenous injection, 2 × 10^5 cells were injected into the tail vein of nude mice as described (10). Development of metastases in long bones and lungs was monitored by BLI with the IVIS Imaging System (Xenogen) as described (10). Analysis was performed with Living Image software (Xenogen) by measuring photon flux of the region of interest. Data were normalized to the signal obtained immediately after injection (Day 0). X-ray radiography analysis of bone lesions was performed as described previously (9).

Histological Analysis—Hind limb bones and lungs were excised, fixed in 10% neutral-buffered formalin, decalcified (for bone only), and embedded in paraffin for hematoxylin and eosin staining (9), Goldner’s trichrome staining (34) or tartrate-resistant acid phosphatase (TRAP) staining (35) as described previously. The osteoclast number was assessed as multinucleated TRAP^+ cells along the tumor-bone interface and reported as number/mm of interface. Immunohistochemical analysis was performed with heat-induced antigen retrieval. The primary antibody used was rabbit anti-human von Willebrand Factor (DAKO), rat anti-mouse F4/80 (Abcam), mouse antibody used was rabbit anti-human von Willebrand Factor (DAKO), rat anti-mouse F4/80 (Abcam), or ER-MP58 as myeloid precursor cells antibody (Abcam). Biotinylated secondary antibody was used with Vectastain ABC kit (Vector Laboratories) and DAB detection kit (Zymed Laboratories, Inc.) to reveal the positively stained cells with nuclei counterstained with hematoxylin.

Knock-out Mice—CCL2^−/− and CCR2^−/− mice were generated previously (31, 32) and obtained from The Jackson Laboratory in C57BL/6 background. The colony was maintained by crossing homozygous siblings. Wild type C57BL/6 mice were used as control.

Macrophage Chemotaxis Assay—Resident and thioglycolate-elicited peritoneal monocytes/macrophages were harvested by peritoneal lavage as described previously (32). Chemotaxis assay was performed with 12-well cell culture inserts (BD Falcon) following previous methods (31). Migrated cells were stained with crystal violet for visualization. The agonist (lower chamber) was either RPMI 1640 medium alone or RPMI 1640 containing 100 ng/ml recombinant human CCL2 or RPMI 1640 conditioned by confluent tumor cells for 24 h.

Bone Marrow Osteoclastogenesis Assay—Bone marrow cells were flushed out from femora and tibiae from 6-week-old mice and plated in basal culture medium (minimum essential medium α supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics) overnight. The next day, the non-adherent cells were plated at 1 × 10^6/well in 24-well plates in basal culture medium supplemented with 30 ng/ml receptor activator of NF-κB ligand and 30 ng/ml macrophage colony stimulating factor to induce osteoclast differentiation. When the effect of CCL2 was tested, 100 ng/ml CCL2 was added to the medium or the medium was preconditioned with confluent tumor cells for 24 h. Medium was changed at day 3. TRAP staining was performed on day 6 using a leukocyte acid phosphatase kit (Sigma). Nuclei were stained with 1 μg/ml 4’,6-diamidino-2-phenylindole (DAPI). TRAP^+ cells with 3–10 nuclei or 10–30 nuclei were separately quantified and reported as the number per microscopic field.

RAW264.7 Osteoclastogenesis Assay—2 × 10^4 RAW264.7 cells were seeded to each well of 24-well plates. Receptor activator of NF-κB ligand (40 ng/ml) was added to the medium to induce differentiation. When the effect of CCL2 was tested, different concentrations of CCL2 was added to the medium. Medium was changed at day 2. TRAP staining, DAPI staining, and quantification were performed on day 4 similar to the bone marrow assay.

Blocking CCL2 with Neutralizing Antibody—Neutralizing antibody to human CCL2 (MAB679, R&D Systems) or control mouse IgG were injected intraperitoneally to mice every 4 days from the date of tumor cell inoculation to the end of the mouse experiment at dose 10 μg/mouse (36).

Statistical Analysis—Results were reported as average ± S.D. or average ± S.E., indicated in the figure legend. Two-sided independent Student’s t test without equal variance assumption or nonparametric Mann-Whitney test was performed to analyze the data with p < 0.05 considered as statistically significant.

RESULTS

CCL2 Overexpression Promotes Lung Metastasis—Two organotropic metastatic sublines of MDA-MB-231 were used for this study: LM2 for lung metastasis (10) and SCP28 for bone metastasis (9). To evaluate the function of CCL2 in metastasis, human CCL2 was stably overexpressed in LM2 and SCP28, as confirmed by quantitative RT-PCR (Fig. 1A) and Western blot analysis of conditioned media (Fig. 1B). CCL2 overexpression did not cause any significant change in the proliferation rate in vitro (Fig. 1C) and anchorage-independent growth on soft agar (Fig. 1D). These results suggest that CCL2 does not have as direct role in promoting tumor cell proliferation.

Functional involvement of CCL2 in lung metastasis was tested by using LM2 cells with or without CCL2 overexpression in lung metastasis assays. To facilitate noninvasive BLI of metastatic tumor growth, LM2 and SCP28 had been previously labeled with a firefly luciferase reporter (37). LM2-Vec (vector control) and LM2-CCL2 were intravenously injected into nude mice, and the development of lung metastases were monitored.
weekly by BLI. As early as 1 week after injection, LM2-CCL2 cells already showed >5-fold stronger signals than LM2-Vec ($p = 0.027$; Fig. 2, A and B), and this trend persisted for 5 weeks ($p < 0.05$ for all time points), up to the point when the mice were sacrificed for histological analyses. Lung metastasis nodule quantification and hematoxylin and eosin staining confirmed the increased pulmonary metastasis burden in mice injected with LM2-CCL2 (Fig. 2, C and D).

Lung contains several different types of macrophage, including alveolar, interstitial, intravascular, and airway macrophages (38). As an important function of CCL2 is to mobilize cells of monocyte lineage, we tested whether CCL2 overexpression caused an increase of infiltrating macrophages in lung metastases. We used an F4/80 antibody, which recognizes a cell surface glycoprotein specifically expressed in

FIGURE 1. Characterization of CCL2 overexpression in MDA-MB-231 sublines. A, CCL2 mRNA level in vector control (Vec) and CCL2-overexpressing (CCL2) derivatives of SCP28 and LM2, as measured by quantitative RT-PCR. B, Western blot of the conditioned medium showing the overexpressed CCL2 (doublet bands). Control denotes cells without transfection of plasmid. C and D, in vitro proliferation and anchorage-independent growth of control and CCL2-overexpressing tumor cells. Scale bar, 200 μm. In A, C, and D, data represent average ± S.D.

FIGURE 2. CCL2 overexpression promotes lung metastasis and macrophage infiltration. A, in vivo lung metastasis assay of LM2 cells with or without CCL2 overexpression. BLI data represent average ± S.E., *$p < 0.05$ by Mann-Whitney test on each time point. B, representative BLI images showing the development of lung metastasis in mice at different time points after LM2 injection. C, quantification of lung metastasis nodules with a diameter of ≥1 mm during necropsy. $p = 0.0087$ with Mann-Whitney test. D, hematoxylin and eosin staining of lung tissue from the mice injected with LM2 derivatives. T, tumor nodule; L, lung parenchyma. Scale bar, 400 μm. E and F, immunohistochemical staining of F4/80, Ly6C, ER-MP58, and von Willebrand Factor (vWF) on the lung sections for quantification of mature macrophages, immature macrophages, and vessel density. Scale bar, 200 μm. Data represent average ± S.E.; $p$ values by two-sided Student's $t$ test. Vec, vector control.
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Mature tissue macrophages (39). Indeed, CCL2 overexpression led to increased F4/80⁺ macrophage infiltration by >3-fold (Fig. 2, E and F). Immature macrophages were stained with two different myeloid precursor markers Ly6C and ER-MP58 (40), and no significant differences in their numbers were observed between two groups of samples (Fig. 2, E and F). CCL2 has also been reported to affect angiogenesis (41). However, we did not observe significant change of microvessel density between control and CCL2-overexpressing pulmonary metastases, as revealed by von Willebrand Factor staining (Fig. 2, E and F), suggesting that elevated angiogenesis is not the cause of the increased lung metastasis burden in our experimental model. Instead, the observation that CCL2 overexpression already led to more lung metastasis at early time point (week 1) suggests that the enhancement of tumor invasion and initial lung colonization mediated by CCL2-recruited macrophages may be the main reason for the overall increased lung metastasis burden.

**CCL2-overexpressing Tumor Cells Promote Macrophage Chemotaxis in a CCR2-dependent Manner**—Both of the CCL2 receptors, CCR2 (29) and CCR4 (30), are widely expressed in a variety of cell types, including monocytes and macrophages (42, 43). CCR2- and CCR4-deficient mice displayed similar defects in macrophage recruitment in experimental inflammation conditions (31, 43). Similar impairments in monocyte migration was also observed in mice lacking CCL2 (32, 44), suggesting that CCL2 may have a cell autonomous function in monocyte mobilization. These observations prompted us to investigate two related questions about the function of CCL2 in macrophage recruitment during lung metastasis. Is CCR2 essential for tumor cell-derived CCL2 to recruit macrophages? Can macrophages lacking CCL2 still be recruited? Answering these questions may have important clinical implications regarding whether targeting CCR2 and/or CCL2 in stroma may achieve the same therapeutic effects as targeting CCL2 in tumor cells.

We first isolated resident peritoneal monocytes/macrophages from wild type, CCL2⁻/⁻, and CCR2⁻/⁻ mice and assessed the expression of CCL2 and CCR2 using RT-PCR (Fig. 3A). Wild type macrophages express both genes, whereas those isolated from knock-out mice lack the expression of the corresponding gene. Next, we performed chemotaxis assay of isolated resident macrophages with recombinant human CCL2 or tumor cell-conditioned medium as attractants (Fig. 3B). For macrophages isolated from wild type mice, both recombinant CCL2 and conditioned medium from LM2-CCL2 significantly increased the migratory activity (2.7-fold and 2.8-fold, respectively) compared with CCL2-free medium or medium conditioned by LM2-Vec. This indicates that CCL2 secreted by LM2-CCL2 cells elicits similar macrophage-mobilizing potency as recombinant CCL2. Similar macrophage chemotaxis-promoting effect was observed when macrophages isolated from CCL2⁻/⁻ mice were used in the experiment (Fig. 3B, CCL2-KO), suggesting that endogenous CCL2 expression is not required for macrophage migration in response to CCL2. In contrast, CCR2 deficiency led to complete loss of CCL2-induced macrophage mobility (Fig. 3B, CCR2-KO). This result suggests that CCR2 is a receptor essential for increased macrophage migration induced by CCL2-overexpressing tumor cells.

Resident macrophage behavior may not necessarily reflect the behavior of TAMs as the latter is usually associated with inflammation. Therefore, we performed the same assays using macrophages elicited by thioglycollate-induced nonspecific inflammation. Monocytes/macrophages, which constitute most of the elicited leukocyte population 72 h after the intraperitoneal thioglycollate instillation (32), were harvested from wild type, CCL2⁻/⁻, and CCR2⁻/⁻ mice. Consistent with previous reports, the recruitment of macrophages was significantly reduced in the two mutant mice (data not shown). Nevertheless, equal numbers of cells were collected and applied to the chemotaxis assays. Similar to resident macrophages, thioglycollate-elicited macrophages were mobilized by recombinant CCL2 or tumor cell-derived CCL2, and such an effect was again found to be dependent on the intact expression of CCR2, but not CCL2, on macrophages (Fig. 3, C and D). Taken together,
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CCL2 Overexpression Promotes Osteolytic Bone Metastasis—To test whether CCL2 overexpression could also increase bone metastasis formation, vector control and CCL2-overexpressing derivatives of SCP28 were injected into the left ventricle of nude mice, and bone metastases development in the hind limbs were quantitatively monitored by BLI (Fig. 4, A and B). Similar BLI intensities were found in both groups 1 week after injection. However, starting from week 2, bone metastasis burden in the CCL2 overexpression group became significantly higher than that in the vector group until the end of the experiment. Consistently, the tumor-bone interface in metastatic lesions formed by SCP28-CCL2 as compared with bone metastasis of SCP28-Vec (Fig. 4D). Because osteoclast activity is critical for the establishment of osteolytic bone metastasis (3–5), we hypothesize that CCL2-overexpressing breast cancer cells become more efficient in colonizing the bone through increased activation of osteoclasts.

CCL2-overexpressing Tumor Cells Promote Osteoclast Differentiation in a CCR2-dependent Manner—Previous studies have shown that recombinant CCL2 protein can promote osteoclast fusion and differentiation in vitro (45–48). Here, we wanted to test whether tumor cell-derived CCL2 could similarly elicit osteoclast activation and whether this is dependent on CCR2 expressed on osteoclast progenitor cells. Primary bone marrow cells isolated from wild type mice expressed both CCL2 and CCR2, which were lost in CCL2 and CCR2 knock-out mice, respectively, as confirmed with RT-PCR (Fig. 5A, left panel). Bone marrow cells isolated from different genotypes were applied to an in vitro osteoclastogenesis assay. Bone marrow growth medium preconditioned with SCP28-Vec or SCP28-CCL2 cells for 24 h were added to the bone marrow culture to compare their ability to induce the formation of multinucleated TRAP+ osteoclasts. Recombinant CCL2 was used as the positive control. The number of nuclei in the osteoclasts indicates the efficiency of cell fusion during differentiation. Therefore, we classified osteoclasts into two types: cells with 3–10 nuclei (less efficient fusion) and cells with 10–30 nuclei (more efficient fusion) by counting DAPI-stained nuclei. In the wild type bone marrow, we found the number of TRAP+ osteoclasts with 10–30 nuclei was expanded significantly by recombinant CCL2, whereas the number of osteoclasts with 3–10 nuclei was unaffected (Fig. 5, A and B, wild type). When bone marrow cells from mutant mice were used, both CCR2- and CCL2-deficient osteoclast progenitor cells could be induced to form TRAP+ osteoclasts with 3–10 nuclei. However, bone marrow cells deficient in CCR2, unlike wild type and CCL2-deficient cells, significantly lost the responsiveness to recombinant CCL2 to form larger osteoclasts with 10–30 nuclei, indicating the critical role of CCR2 as the receptor for the function of CCL2 in stimulating osteoclast fusion. Importantly, we observed that CCL2 derived from SCP28-CCL2 medium was equally able to promote osteoclast differentiation in a CCR2-dependent manner, supporting our hypothesis that CCL2-overexpressing tumor cells promote directional migration of monocyte/macrophages in a CCR2-dependent manner, which may be responsible for the elevated infiltration of macrophage in the lung metastases formed by LM2-CCL2 cells.

these results suggest that tumor cell-derived CCL2 induces directional migration of monocyte/macrophages in a CCR2-dependent manner, which may be responsible for the elevated infiltration of macrophage in the lung metastases formed by LM2-CCL2 cells.

FIGURE 4. CCL2 overexpression enhances osteolytic bone metastasis and associated osteoclasts. A, in vivo bone metastasis assay of SCP28 cells with or without CCL2 overexpression. BLI data represent average ± S.E., *, p < 0.05; **, p < 0.01, and *** p < 0.001 by Mann-Whitney test on each time point. B, representative BLI images showing the development of bone metastasis in mice at different time points after SCP28 injection. Arrows, bone metastases in the hind limbs. C, representative x-ray radiographs of the hind limbs at week 6 after injection showing the osteolytic lesions (arrows) in proximal tibia. D, Goldner’s trichrome and TRAP staining (with quantification) of proximal tibia from the mice injected with SCP28 derivatives. B, bone matrix; T, tumor area. Scale bar, 200 μm. Data represent average ± S.E., p values by two-sided Student’s t test. Vec, vector control.
osteoclast activation during the formation of osteolytic bone metastasis.

CCL2 may exert its function in promoting osteoclast differentiation directly through preosteoclasts or indirectly through osteoblasts, which is often the cell type required for paracrine activation of osteoclast differentiation. To differentiate these two possible mechanisms, we determined the levels of CCR2 mRNA expression in osteoblast cell line hFOB1.19 and preosteoclast cell line RAW264.7 with RT-PCR (Fig. 5C). Expression of CCR2 was not detected in hFOB1.19, consistent with the previous observation in another osteoblast line, UMR 106–01 (46). In contrast, CCR2 is expressed in RAW264.7 cells. This result indicates that CCL2 should function directly through CCR2 on preosteoclasts to promote its cell fusion and differentiation. Indeed, when we treated RAW264.7 cells under the differentiation culture condition with an increasing dose of recombinant CCL2 and profiled the number of nuclei in the resultant multinucleated TRAP$^{+}$ osteoclasts (Fig. 5D), we observed a clear dose-dependent increase of nuclear number in the induced osteoclasts by CCL2, supporting its direct role in promoting cell fusion during osteoclast differentiation.

Inhibition of CCL2 Reduces Bone and Lung Metastases—We wished to evaluate whether inhibiting CCL2 function could reduce metastasis to bone and lung. To this end, we used a neutralizing antibody for human CCL2 to block the function of tumor-derived CCL2 in metastasis assays in vivo. Two strongly metastatic sublines derived from in vivo selection of MDA-MB-231 (the lung-tropic LM2 and the bone-tropic 1833 (9)) were used in the experiment. The 1833 subline has a much stronger bone metastasis ability than SCP28 and is therefore more appropriate to be used for testing the possible effect of CCL2 inhibition on reducing bone metastasis. 1833 and LM2 sublines express considerable amount of CCL2 at the basal level, as measured by ELISA of the conditioned media (2.41 ± 0.42...
ng/ml for 1833 and 0.77 ± 0.01 ng/ml for LM2), whereas fresh medium does not contain any detectable level of human CCL2. 1833 and LM2 were inoculated into nude mice by intracardiac or intravenous injections, respectively, to generate bone and lung metastases. Antibodies or isotype IgG controls were injected into mice from the date of tumor inoculation until the end of the experiment. Both 1833-generated bone metastasis and LM2-generated lung metastasis were reduced significantly by antibody treatment (Fig. 6, A and B). These results indicate that inhibition of CCL2 is effective in suppressing metastasis formation in both bone and lung.

Overexpression of CCL2 Alone in Weakly Metastatic Cells Is Sufficient to Promote Lung but Not Bone Metastasis Potential—Although overexpression of CCL2 promotes metastasis to lung or bone in cell lines that already display significant basal metastatic abilities to the respective organs (e.g. SCP28 to bone and LM2 and lung, Figs. 2A and 4A), we wished to test whether CCL2 overexpression can render an otherwise weakly metastatic or non-metastatic cell capable of metastasizing to bone or lung. We injected vector control or CCL2-overexpressing variants of SCP28 (weakly lung metastatic) to the tail vein (Fig. 6C) and found a significant increase of lung metastasis potential by CCL2 overexpression. However, CCL2 overexpression was not sufficient to enhance bone metastasis for the otherwise weakly bone metastatic subline LM2 (Fig. 6D). Because metastasis ability is known to rely on concerted function of multiple genes (9, 10), it is not surprising to observe variable results in this test. The ability of CCL2 to confer organotropic metastasis potential may depend on the genetic background of the cell lines (i.e. the existence of cooperating organotropic metastasis genes).

DISCUSSION

Chemokines and their receptors play diverse roles in malignant tumor progression, particularly as key mediators of tumor-stroma interactions (12, 13, 49). The best characterized example is the function of SDF-1/CXCR4 axis in mediating site-specific metastasis for various cancer types (50). Data presented here demonstrate that CCL2 has dual activity to promote both lung and bone metastases of breast cancer cells through distinct functions. When CCL2 was overexpressed, lung metastasis development was accelerated with increased infiltration of F4/80<sup>+</sup> macrophages. Similarly, CCL2-overexpressing tumor cells developed more extensive osteolytic

FIGURE 6. Alteration of organotropic metastasis behavior by targeting CCL2 with neutralizing antibody in highly metastatic cells and by overexpressing CCL2 in weakly metastatic cells. A, in vivo bone metastasis assay of highly bone metastatic cell line 1833 with mice treated with control IgG or neutralizing antibody against human CCL2. B, in vivo lung metastasis assay of highly lung-seeking line LM2 with mice treated with control IgG or neutralizing antibody against human CCL2. C, in vivo lung metastasis assay of weakly lung-metastatic cell line SCP28 with or without the overexpression of CCL2. D, in vivo bone metastasis assay of weakly metastatic cell line LM2 with or without the overexpression of CCL2. In A–D, BLI data represent average ± S.E. *, p < 0.05, **, p < 0.01, and ***, p < 0.001; with no asterisk shown, p > 0.05 by Mann-Whitney test on each time point. Vec, vector control.
bone lesions with increased recruitment and activation of TRAP+ osteoclasts in the tumor-bone interface. Importantly, by using primary cells isolated from wild type, CCL2−/−, and CCR2−/− mice, we showed that tumor-derived CCL2 increases the directional migration of macrophages and promotes osteoclast differentiation in a CCR2-dependent manner. Furthermore, we demonstrated that targeting CCL2 in tumor cells with a neutralizing antibody inhibited metastasis formation in lung and bone. Overall, these results suggest that CCL2 and CCR2 as targets for therapeutic intervention of bone and lung metastasis.

CCL2 expression can be activated in advanced breast cancer through genetic or epigenetic mechanisms. An infrequent polymorphism in the CCL2 promoter region associated with increased CCL2 expression is overrepresented in metastatic breast cancer patients (49). Several pathways involved in epithelial-mesenchymal transition, including dysadherin (51) and β-catenin/TCF-4 (52), promote CCL2 up-regulation. Here, we used ectopic overexpression and blocking antibody to assess its pro-metastatic function in the MDA-MB-231 breast cancer metastasis models. We chose this model because of the unique repertoire of organ-specific metastatic sublines that was recently developed (9–11). CCL2-mediated tumor- and metastasis-promoting functions have been investigated in other preclinical models. For example, CCL2-mediated macrophage infiltration and increased tumor growth were reported in the gastric carcinoma nude mouse model (53). CCL2 inhibition by short hairpin RNA-mediated knockdown or by systemic delivery of neutralizing antibodies inhibit the development of bone metastasis by prostate cancer (19, 54). However, the functional importance and cellular mechanism of CCL2 in promoting organ-specific metastasis of breast cancer metastasis has not been rigorously investigated. In addition to using the MDA-MB-231 in vivo models to study the functional importance of CCL2 in promoting bone and lung metastasis, our current study also took advantage of the existing CCL2 and CCR2 knock-out mice and identified CCR2 as the essential receptor for mediating the function of CCL2 in macrophage recruitment and osteoclast activation by breast cancer.

Careful comparison of the vector control and CCL2-overexpressing metastasis signal curves in lung (Fig. 2A) and bone (Fig. 4A) revealed an interesting difference in the modes of metastasis enhancement by overexpressing CCL2 in two organs. Lung metastasis burdens of mice injected with LM2-Vec and LM2-CCL2 already displayed a marked difference 1 week after tumor inoculation but maintained similar rates of increase afterward, suggesting that CCL2 may provide an initial advantage in metastatic seeding in lung but did not confer further growth advantages. On the other hand, bone metastasis signals generated by SCP28-Vec and SCP28-CCL2 started to bifurcate at week 2, and the difference increased further thereafter, suggesting that CCL2 promotes bone metastasis after the initial seeding. The differences may reflect organ-specific functions of CCL2 in metastasis. In lung, the major prometastasis function of CCL2 is through recruitment of mature macrophages to promote invasion and early establishment of tumor cell clusters. On the other hand, it is likely that tumor cells arriving at the bone require the first 1–2 weeks to migrate to a suitable area in the bone microenvironment (the “metastasis niche”) (2) or to form small clusters of micrometastases. Neither of these events may be dependent on CCL2. Instead, CCL2 promotes bone metastasis expansion through inducing osteoclast differentiation and establishment of the “vicious cycle” of bone metastasis (2), which occurs after the initial adaptation. Recognizing the organ-specific modes of metastatic function of CCL2 has potential clinical implications, as treatment of lung metastasis may benefit from CCL2-targeting therapy only if the treatment is applied early, whereas macroscopic bone metastasis may still be sensitive to the inhibition of CCL2.

It is generally believed that macrophages and bone marrow osteoclasts share a common monocytic hematopoietic progenitor. Several lines of evidence support this hypothesis. op/op mice (spontaneous colony-stimulating factor 1 mutation) have severe deficiency of both osteoclasts and macrophages (55). Knock-out of transcription factor PU.1 in mice caused absence of both cell types, suggesting defects at an early common myeloid differentiation stage (56). It is conceivable that common molecule(s) involved in regulating the homeostasis of both macrophages and osteoclasts can be hijacked by tumor cells to simultaneously modulate the activity of both cell types in the tumor microenvironment to promote tumor growth. Indeed, our results showed that breast tumor cells are able to recruit macrophages in the lung parenchyma as well as enhancing osteoclast differentiation in the bone marrow by engaging the same CCL2/CCR2 axis on these cells. Thus, pharmacological inhibitors targeting CCL2 or CCR2 in breast cancer patients whose tumor tissue expresses a high level of CCL2 may bring clinical benefits for controlling distant metastases in multiple organs. The CCL2/CCR2 axis may have prometastasis functions beyond the direct engagement of stromal cells of monocytic origin by tumor cells. CCL2/CCR2 may mediate a cascade of signaling events to enhance tumor malignancy. For example, MDA-MB-231 cells can induce osteoblast hFOB1.19 and MC3T3-E1 to up-regulate CCL2 (57), and CCL2 produced by osteoblasts can promote osteoclast differentiation (47, 48). Furthermore, tumor-derived CCL2 can exert function on other CCR2+ stroma cells, such as endothelial cells (41) and mesenchymal stem cells (21). Our strategy of combining xenograft models with genetically modified mice may help resolve the functional importance of these intercellular cross-talks during tumor progression.

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REFERENCES

1. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., and Thun, M. J. (2008) CA: A Cancer Journal for Clinicians 58, 71–96
2. Lu, X., and Kang, Y. (2007) J. Mammary Gland Biol. Neoplasia 12, 153–162
3. Mundy, G. R. (2002) Nat. Rev. Cancer 2, 584–593
4. Roodman, G. D. (2004) N. Engl. J. Med. 350, 1655–1664
5. Guise, T. A., Kozlow, W. M., Heras-Herzig, A., Padalecki, S. S., Yin, J. J., and Chirgwin, J. M. (2005) Clin. Breast Cancer 5, (suppl.) S46–S53
6. Schlappack, O. K., Baur, M., Steger, G., Dittrich, C., and Moser, K. (1988) Klin. Wochenschr. 66, 790–795
7. Gupta, G. P., and Massagué, J. (2006) Cell 127, 679–695
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8. Lu, X., Wang, Q., Hu, G., Van Poznak, C., Fleisher, M., Reiss, M., Mas sagué, J., and Kang, Y. (2009) *Genes Dev.* 23, 1882–1894
9. Kang, Y., Siegel, P. M., Shu, W., Drobnjak, M., Kakonen, S. M., Cordón-Cardo, C., Guise, T. A., and Massagüé, J. (2003) *Cancer Cell* 3, 537–549
10. Minn, A. J., Gupta, G. P., Siegel, P. M., Bos, P. D., Shu, W., Giri, D. D., Viale, A., Olshen, A. B., Gerald, W. L., and Massagüé, J. (2005) *Nature* 436, 518–524
11. Bos, P. D., Zhang, X. H., Nadal, C., Shu, W., Gomis, R. R., Nguyen, D. X., Minn, A. J., van de Vijver, M. J., Gerald, W. L., Foekens, J. A., and Massagüé, J. (2009) *Nature* 459, 1005–1009
12. Balkwill, F. (2004) *Nat. Rev. Cancer* 4, 540–550
13. Karnoub, A. E., and Weinberg, R. A. (2006) *Breast Dis.* 26, 75–85
14. Conti, I., and Rollins, B. J. (2004)
15. Melgarejo, E., Medina, M. A., Sánchez-Jiménez, F., and Urdiales, J. L. (2001) *Int. J. Biochem. Cell Biol.* 33, 228–236
16. Lu, Y., Cai, Z., Chen, Q., Chen, J., Lu, Y., Xiao, G., Wu, Z., Zhou, Q., and Zhang, J. (2009) *Am. J. Pathol.* 174, 228–236
17. Cai, Z., Chen, Q., Chen, J., Lu, Y., Xiao, G., Wu, Z., Zhou, Q., and Zhang, J. (2009) *Nat. Neoplasia* 3, 175–183
18. Lu, Y., Cai, Z., Galson, D. L., Xiao, G., Liu, Y., George, D. E., Melhem, M. F., Yao, Z., and Zhang, J. (2006) *Prostate* 66, 1311–1318
19. Loberg, R. D., Ying, C., Craig, M., Day, L. L., Sargent, E., Neeley, C., Wojno, K., Snyder, L. A., Yan, L., and Pienta, K. J. (2007) *Cancer Res.* 67, 9417–9424
20. Li, X., Cai, Z., and Kang, Y. (2009) *Int. J. Cancer* 124, 263–266
21. Leenen, P. J., de Bruijn, M. F., Voerman, J. S., Campbell, P. A., and van Ewijk, W. (1994) *J. Immunol. Methods* 175, 5–19
22. Saji, H., Koike, M., Yamori, T., Saji, S., Seiki, M., Matsushima, K., and Toi, M. (2001) *Cancer Res.* 61, 7176–7184
23. Lebrecht, A., Grimm, C., Lantzsch, T., Ludwig, E., Hefler, L., Ulbrich, E., M. (2001) *Semin. Cancer Biol.* 11, 1085–1091
24. Lu, Y., Xiao, G., and Wellstein, A. (2004) *Int. J. Biochem. Cell Biol.* 36, 2328–2329