Mesenchymal stem cell-derived CCL-9 and CCL-5 promote mammary tumor cell invasion and the activation of matrix metalloproteinases

Muthulekha Swamydas, Krista Ricci, Stephen L. Rego, and Didier Dréau*

Cell and Molecular Division; Department of Biology; University of North Carolina, Charlotte; Charlotte, NC USA

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Stromal chemokine gradients within the breast tissue microenvironment play a critical role in breast cancer cell invasion, a prerequisite to metastasis. To elucidate which chemokines and mechanisms are involved in mammary cell migration we determined whether mesenchymal D1 stem cells secreted specific chemokines that differentially promoted the invasion of mammary tumor cells in vitro. Results indicate that mesenchymal D1 cells produced concentrations of CCL5 and CCL9 4- to 5-fold higher than the concentrations secreted by 4T1 tumor cells (P < 0.01). Moreover, 4T1 tumor cell invasion toward D1 mesenchymal stem cell conditioned media (D1CM), CCL5 alone, CCL9 alone or a combination CCL5 and CCL9 was observed. The invasion of 4T1 cells toward D1 mesenchymal stem CM was dose-dependently suppressed by pre-incubation with the CCR1/CCR5 antagonist met-CCL5 (P < 0.01). Furthermore, the invasion of 4T1 cells toward these chemokines was prevented by incubation with the broad-spectrum MMP inhibitor GM6001. Additionally, the addition of specific MMP9/MMP13 and MMP14 inhibitors prevented the MMP activities of supernatants collected from 4T1 cells incubated with D1CM, CCL5 or CCL9. Taken together these data highlight the role of CCL5 and CCL9 produced by mesenchymal stem cells in mammary tumor cell invasion.

Introduction

In addition to tumor cells, solid tumors are composed of multiple stroma cell types that modulate the density and composition of a complex extracellular matrix (ECM) and of a large array of soluble and bound molecules creating a unique microenvironment that influence tumor progression.1-3 Within the breast tumor microenvironment mesenchymal-derived cells, including adipose cells, the most abundant of the stromal cells of the breast, and fibroblasts secrete a variety of chemokines, growth factors, and cytokines that modulate cancer progression and metastasis.4-6 In particular, the chemokine network can greatly affect the progression and metastasis of breast cancer through the modulation of tumor cell death, proliferation, ECM attachment, proteolysis of the basement membrane, locomotion, and colony formation, but also cell trafficking and adhesion, the local inflammatory response and the redirection to the tumor site of immune effector and stem cells.7-12

Among chemokines that modulate breast cancer progression, CCL5 (RANTES), which binds to mostly to CCR5 but also to CCR1 has been extensively studied.13-16 Increased expressions of CCL5 have been correlated with breast cancer progression, particularly with triple negative breast cancer.17-19 Indeed, CCL5 overexpressed in tumors promote cancer cell proliferation, migration, invasion, and survival in a dose-dependent manner mostly through functional CCR5 receptors.20-24 In particular, CCL5 is also produced by tissue resident stem cells under the influence of cancer cells.25-27 The secretion of CCL9 (MIP-1γ) is increased in colorectal carcinoma cells along with the heightened expression of its the cognate receptor CCR1 onto myeloid cells.28,29 These interactions between tumor cells and mesenchymal stem cells highlight a feedback mechanism that promotes tumor cell motility, invasion, growth, survival, and the overall metastatic potency.14,16 Furthermore, the concomitant expression of multiple chemokines in the human breast cancer microenvironment may contribute independently to breast malignancy.30

Chemokines, CCL5 and CCL9 especially, modulates changes in the adhesion of the tumor cells in part through stimulating the release of matrix metalloproteinases (MMPs) leading to proteolytic degradation of the extracellular matrix.31,32 CCL-5 promoted tumor metastasis through stimulation of MMP-2 and MMP-9 secretions in both leukocytes and tumor cells,33-35 and in 4T1 tumor mass in vivo.36 Mostly, stroma-derived MMP-9 and MMP-13 and also tumor-derived MMP-1, MMP-2, and MMP-14 have been shown to contribute to invasive tumor growth and metastasis.37,39

Given the complexity of the interactions between stroma and tumor cells within the breast tumor microenvironment, in vitro analyses of cell–cell interactions are essential models to provide a detailed understanding of the role of chemokines. Despite their
cells did not.46 murine D1 mesenchymal stem cells whereas 4T1 murine tumorMary gland (NMuMG) cells formed acini only in the presence of
sion in in vitro models. 43-45 Furthermore, normal murine mam-
oxygen concentrations, the density of the ECM to the formation
We, and others, have also investigated parameters from the local
stroma tumor cell interactions and anticancer drug efficacy.40-42
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experiments repeated at least 3 times, the cells moving
toward different control and CMs were counted. Data were analyzed by one-way ANOVA and differ-
captured following the 5-d incubation. In experiments repeated at least 3 times, the cells moving
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Figure 1. 3D invasions of NMuMG and 4T1 cells are increased by conditioned media from both mammary epithelial cells and D1 mesenchymal stem cells. Migration of NMuMG (A) and 4T1 cells (B) following a 5-d incubation with CMs derived from 4T1, NMuMG, and the mesenchymal stem D1 cells were quantified using 3D migration assays (see Materials and Methods section for details). Serum-free and 10% FBS–DMEM media were used as negative and positive controls, respectively. Representative microphotographs of cells migrating out of the wells for each condition were captured following the 5-d incubation. In experiments repeated at least 3 times, the cells moving toward different control and CMs were counted. Data were analyzed by one-way ANOVA and differences between treatment groups tested using the Student Newman–Keuls post-hoc test. *P < 0.05 compared with negative control (0% FBS).
limitations, in vitro models using cell lines have been utilized extensively and are invaluable. Recently, various 3D models of breast tissues have been described and used to investigate the stroma tumor cell interactions and anticancer drug efficacy.40-42
To determine whether chemokines present within the breast tumor microenvironment, especially CCL5 and CCL19, promoted the invasion of mammary tumor cells, we first determined the effects of molecules secreted by epithelial (NMuMG), tumor (4T1) cells, and mesenchymal D1 stem cells on NMuMG and 4T1 cell invasions using 2D and 3D culture conditions. Further, we identified the predominant role of CCL5 and CCL9 in the invasion of 4T1 tumor cells through CCR1 and CCR5 receptor activation and the secretion of MMPs.

Results
Conditioned media from mammary epithelial NMuMG cells and D1 mesenchymal stem cells differentially affects the invasion of NMuMG and 4T1 cells. The 3D invasions of NMuMG and 4T1 cells were significantly different in response to various CMs tested in 3D cultures. As shown in Figure 1, cells migrated within a 0.8% agarose 3D matrix toward the different CMs tested. Both NMuMG and 4T1 cells migrated toward mammary epithelial NMuMG cell and mesenchymal stem D1 cell CMs. However, CMs derived from mammary tumor 4T1 cells did not have any effect on the invasion of either NMuMG or 4T1 cells. In contrast, using wound healing assays (data not shown) no changes were noticed in the migration speed of 4T1 or NMuMG cells compared with the negative control (0% FBS) in monolayer repair over time measured following a 6 h incubation.
Conditioned media collected from D1 mesenchymal stem cells increases the invasion of 4T1 cells but not of NMuMG cells. To further define the effects of D1CM on cell migrations, the invasion of both NMuMG and 4T1 cells toward various CMs were determined using transwell migration assays. The D1 mesenchymal stem cell CM significantly increased the invasion of 4T1 cells (P < 0.05, Fig. 2). No significant change in invasion toward any of the D1CM tested was observed with NMuMG cells (P > 0.05, Fig. 2).

Chemokines CCL-5 and CCL-9 concentrations were higher in conditioned media derived from D1 cells. To determine the specific molecules differentially present in mesenchymal stem D1 cell CM compared with NMuMG and 4T1 CMs, the expression of chemokines and cytokines was evaluated using antibody arrays. Expressions of both CCL-5 and CCL-9 chemokines were significantly increased in mesenchymal stem D1 cell CM compared with the CMs derived from 4T1 cells (P < 0.05, Fig. 3A and C). Additionally, CXCL-16, MIP-1α, and soluble TNFα receptor 2 were also decreased in CM derived from D1 cells (not shown).

D1 mesenchymal stem cell conditioned media promoted the CCR1 and CCR5 mRNA in 4T1 cells but not NMuMG cells. We next determined whether high concentrations of CCL5 and CCL9 present in D1 mesenchymal stem cell CM influenced the mRNA and protein expression of CCL5 and CCL9 receptors by mammary epithelial NMuMG and mammary tumor 4T1 cells. The mRNA expression of CCR 1 and 5 receptors for CCL5, and CCR1, 3 and 5 receptors for CCL9, respectively, in 4T1 and NMuMG cells were evaluated. As shown Figure 4A and B, CCR3 mRNA expression was not altered regardless of the CMs or the cells tested. In contrast, while the expression of CCR1 and CCR5 receptors in the NMuMG cells remained unchanged following D1CM treatment, the expression of CCR1 and CCR5 was significantly increased in the 4T1 cells treated with D1CM compared with the 4T1 cells treated with either NMuMG CM or 4T1 CM and control conditions (P < 0.05, Fig. 4).

D1 mesenchymal stem cell conditioned media increased the CCR5 cell surface expression in 4T1 cells. The cell surface expressions of CCR1 and CCR5 receptor on 4T1 cells in serum-free media or following incubations with D1CM were determined by flow-cytometry (Fig. 5). Compared with control conditions, the percentage of 4T1 cells positive for CCR1 receptors following incubation with D1CM appeared to remain unchanged (Fig. 5A). In contrast, incubation with D1CM led to an increase in the percentage of 4T1 cells expressing CCR5 on
their cell surface compared with 4T1 cells incubated in control conditions (Fig. 5B).

The antagonist to CCR5 and CCR1 Met-CCL5 inhibited D1CM-driven 4T1 cell invasion. To confirm the effects of CCL5 and CCL9 on the migration of 4T1 cells, we measured the invasion of 4T1 cells in response to CM obtained from D1 cells in the presence of different concentrations of Met-CCL5, an antagonist to both chemokine receptors CCR1 and CCR5 (Fig. 6). Treatment with Met-CCL5 reduced the invasion of 4T1 cells in response to D1CM in a dose dependent manner (P < 0.05, Fig. 6). Met-CCL5 above 0.01 ng/ml caused significant reductions in 4T1 cell invasion (Fig. 6).

4T1 cell invasion was promoted by CCL5 and CCL9 alone or combined and inhibited by MMP inhibitors. 4T1 cell invasion toward CCL5 or CCL9 alone or in combination increased (P < 0.01 compared with media alone, Fig. 7) but remained lower than the invasion observed with D1CM. Interestingly, the 4T1 invasion toward the combination CCL5 and CCL9 was not different from the 4T1 invasion toward CCL5 or CCL9 alone (Fig. 7).

Next we investigated the role of MMPs in 4T1 cells invasions. First, in 4T1 cells incubated with D1CM, the addition of the chemokine receptor antagonist Met-CCL5 lead decreased MMP-9, MMP-13, and MMP-14 expression as detected by western blots analyses (not shown). To further assess the role of MMPs, 4T1 cell invasion in the presence of the wide-spectrum MMP inhibitor GM6001 were conducted. As shown Figure 7 (hachured bars), the pre-incubation of 4T1 cells with GM6001 (1 μM) led to the inhibition of the invasion toward CCL5, CCL9, or the combination CCL5–CCL9. Only, 4T1 invasion toward D1CM was observed (Fig. 7). However, GM6001 treatment significantly inhibited 4T1 cell invasion even toward D1CM (Fig. 7).

Changes in the expressions of specific MMPs were further assessed using functional assays (Fig. 8). No detectable amount of any of the MMP’s tested was measured in D1CMs (not shown). The concentrated supernatants collected from 4T1 cells incubated with D1CM, CCL5, CCL9 or the combination CCL5 and CCL9 were tested for their abilities to cleave the fluorescent MMP substrate. Supernatants of 4T1 cells incubated with D1CM, CCL5, CCL9, or the combination CCL5 and CCL9 had significantly higher MMP activities (darken bars) compared with media alone (P < 0.001, Fig. 7). The addition of GM6001 (10 nM) to the enzymatic reaction decreased MMP activities to levels similar to those observed following incubation with control media (Fig. 8A, ns). The addition of the MMP9/MMP13 inhibitor was associated with decreases in MMP activities of 4T1 cell supernatants collected following treatments with CCL9, the combination CCL5/CCL9 and D1CM but not with CCL5 alone (P < 0.001, Fig. 8B). The addition of the MMP14 inhibitor led to decrease in MMP activities of 4T1 supernatants collected following treatments with CCL5, the combination CCL5–CCL9 and D1CMs but not with CCL9 alone (P < 0.001, Fig. 8C).

Discussion

Within the breast tumor microenvironment, stromal cells including mesenchymal stem cells contribute to the development of breast cancer. For example, D1 mesenchymal stem cells in culture with NMuMG cells promoted the generation of 3D acini and duct-like structures whereas NMuMG mammary epithelial cells alone did not. Here we investigated the effects of stroma mesenchymal D1 stem cells’ secretions especially the chemokines...
CCL5 and CCL9 on 4T1 invasion. Our results indicate that both CCL5 and CCL9 chemokines were produced at high concentrations by D1 mesenchymal stem cells and promoted tumor cell invasion likely through CCR1 and CCR5 receptors present on mammary 4T1 tumor cells. The invasion of 4T1 cells was dependent on MMP activities including MMP9 and/or MMP13 and MMP14 stimulated by CCL5 and CCL9 through the activation of CCR1 and/or CCR5 receptors.

In vivo, the role of stroma cells in the promotion of breast tumor progression has been demonstrated in immune-compromised mice.14,16 Indeed, mammary carcinoma co-implanted with mesenchymal stem cells in immune compromised mice led to significant increase in metastatic lesions.14,16 Stroma cell including mesenchymal stem cells and tumor cell interactions through cell-to-cell contacts or soluble mediators control most aspects of tumor formation and progression.8,14 Our observations also indicate that D1 mesenchymal stem cells express high concentrations of chemokines that promoted the invasion of the 4T1 mammary tumor cells. Furthermore, D1 cell secretions tended to differently stimulate NMuMG epithelial mammary cells and 4T1 tumor cells, highlighting the role of mesenchymal stem cell secretion in malignant cells dissemination.14,49,50 These observations confirmed previously published in vivo data13,14 and thus support the use in vitro models systems to study the invasion steps of the breast cancer progression using mammary tumor cells and tumor microenvironment. CCL9 has been shown to promote colon cancer progression,29 and our data suggest that CCL9 may also have a role in the invasion of breast cancer cells.

Furthermore, since the combination CCL5–CCL9 was not associated with significant increase in 4T1 cells migration when compared with each chemokine alone may indicate that both chemokine share similar signaling pathways.3 Moreover, CCL5 or CCL9 alone or in combination only partially generated the invasion observed in the presence of D1CM, suggesting that the secretion and shedding of multiple molecules in addition to CCL5 and CCL9 may explain the higher 4T1 cell invasion observed in the presence of D1CM compared with CCL5 and CCL9 alone or in combination. In addition to CCL5 and CCL9 chemokines, D1 mesenchymal stem cells like other stromal cells also produce other molecules including chemokines and mechanisms are likely involved in the promotion of 4T1 cell invasion. Whether physical interactions through the formation of heterodimers may also modulate the breast tumor cell responses to CCL5 and CCL9 combinations remains to be defined.34 The secretion and shedding of multiple molecules in addition to CCL5 and CCL9 may explain the higher 4T1 cell invasion observed in the presence of D1CM compared with CCL5 and CCL9 alone or in combination. In addition to CCL5 and CCL9 chemokines, D1 mesenchymal stem cells like other stromal cells also produce other molecules including interleukin 6, that also promote migration and invasion of breast cancer cells.4,6 And some of those molecules including the chemokines such as CCL5 and CCL2 have been shown to contribute, independently, to breast malignancy.30

Here treatments with Met-CCL5 an antagonist to primarily CCR5, CCR1 lead to a dose-dependent inhibition of 4T1 cell

Figure 3. D1 mesenchymal stem cell conditioned media contains higher CCL5 and CCL9 concentrations than conditioned media collected from NMuMG mammary epithelial and 4T1 tumor cells. Higher concentrations of CCL5 and CCL9 were detected in CM collected from mesenchymal stem cells (D1) (A), than in CM obtained from mammary epithelial cells (NMuMG) (B) and mammary tumor cells (4T1) (C) using cytokine protein arrays. This increase was semi-quantified (D) in CMs collected from D1 (gray bars), and in 4T1 (black bars) cells. Both CCL5 AND CCL9 expressions were very low in NMuMG conditioned media (below the detection limit, not shown). Data were analyzed by one-way ANOVA and differences between treatment groups tested using the Student Newman–Keuls post-hoc test. *P < 0.05, **P < 0.01 compared with 4T1 conditioned media.
invasion supporting the involvement of those receptor in the signaling promoting cell invasion including MMP expression as described previously. The antagonist Met-CCL5 has been shown to inhibit multiple cellular processes in part through slower internalization and altered trafficking. CCL5 inhibitors have been shown to block metastasis of breast cancer cells, and Met-CCL5 inhibited breast tumor growth, highlighting the therapeutic potential of CC chemokine receptor antagonists.

The chemokines present in the D1CM including CCL5 and CCL9 promoted the expression and activities of MMPs essential for the invasion of 4T1 cells. MMPs through the remodeling of the extracellular matrix and of cell–ECM and cell–cell contacts, facilitate the detachment of tumor cells from the surrounding tissue within both the primary or the metastatic site. Here, inhibitions of the MMP activities led to reduced invasion. The use of specific inhibitors to MMP9/MMP13 or MMP14 indicated that these MMPs, all of which have been associated with tumor invasion and metastasis, were involved in 4T1 invasion. Possibly associated with the MMP detection methods used, the differences observed here between 4T1 cell invasion following a large-spectrum MMP inhibitor and the in vitro activities of MMPs secreted and/or shed by 4T1 cells, and in the specific in vitro activities themselves, notably following incubations with CCL5 and CCL9, remains to be fully investigated. Nonetheless, the mechanisms of 4T1 invasion observed here through chemokines, including CCL5 and CCL9 produced by mesenchymal cells that upon binding to CC receptors, led to increased MMP activities including of MMP9/MMP13 and MMP14. These observations parallel increases in CCL9 that promoted the recruitment of stroma cells expressing CCR1 and secreting MMPs in colon cancer, and the CCL5 stimulated expression of MMPs by multiple stroma cells including immune cells.

Taken together the data presented strongly suggest that 4T1 mammary tumor cell invasion in vitro is promoted by D1 mesenchymal stem cell secretions, especially the chemokines CCL5 and CCL9. They also indicate that this invasion is dose-dependent of the activation of CC chemokine receptors and result in increased MMP activities, especially of MMP9 or MMP13 and MMP14. Furthermore, they highlight the utility of in vitro models in the dissection of the complex interactions between cell types within the tumor microenvironment. These observations further emphasize the key role of stroma cell secretions in breast tumor cell invasion. Indeed, tumor cell chemotactic responses may influence the migratory traits of sub-populations within the tumor and potentially contribute to their in vivo behavior, growth, and survival as suggested earlier. Furthermore, these chemokines are also produced in large amounts by other stroma cells including adipocytes and given the role and complexity of the interactions chemokine–chemokine and chemokine–chemokine receptor in the breast tumor microenvironment, their actions on the tumor cell behavior need to be carefully assessed.

Materials and Methods

Cell cultures and media. Normal mouse mammary gland cells (NMuMG), metastatic mouse mammary cells (4T1), and mesenchymal murine stem (D1) cells were obtained from ATCC. Cells were cultured at 37 °C and 5% CO2 in DMEM media supplemented with 10% fetal bovine serum (FBS), gentamycin, and amphotericin B. Media for NMuMG cells was supplemented with 10 μg/ml of insulin (Sigma). Other media supplies were obtained from Mediatech.
Figure 5. D1 mesenchymal stem cell conditioned media increased cell surface expression of CCR1 and CCR5 receptors in 4T1 cells. Representative flow-cytometry histograms that depict the CCR1 (A) and CCR5 (B) receptors expressed on the cell surface of 4T1 cell following a 24 h incubation in the presence of control media (dotted gray line) or D1CM (solid dark line). Staining with the secondary antibody alone (Texas red conjugated anti-goat antibody) is depicted as solid black area. These results are representative of at least three separate experiments. Results indicate that incubation with D1CM led to a limited and a larger cell surface expression of CCR1 and CCR5, respectively.

Figure 6. The inhibitor of CCR1 and CCR5 Met-CCL5 inhibits 4T1 cells invasion toward D1 mesenchymal stem cell conditioned media. The number of 4T1 cells migrating through uncoated and Matrigel®-coated transwell chamber was used to evaluate the effects of increasing concentrations of met-CCL5, an inhibitor of both CCR1 and CCL5 on the invasion promoted by mesenchymal stem D1 cell CM (for details see materials and Methods section). Serum-free (0% FBS) and 10% FBS media serve as negative and positive control, respectively. The numbers of migrating 4T1 cells normalized to the surface area of the transwell membrane are presented. Data were analyzed by one-way ANOVA and differences between treatment groups tested using the Student Newman–Keuls post-hoc test. *P < 0.05, **P < 0.01, ***P < 0.001 compared with 0% FBS control; #P < 0.05, ##P < 0.01, ###P < 0.001, compared with migration of 4T1 cells toward D1CM alone.

Conditioned media collection. NMuMG, 4T1 and D1 cells were cultured at 37 °C and 5% CO₂ in DMEM media supplemented with 10% FBS, gentamycin, and amphotericin B. Upon confluence, cells were cultured for 24 h in media without FBS and then incubated with RPMI media without phenol red. After a 48 h incubation, conditioned media (CMs) were collected and centrifuged to eliminate debris. Following a filtration (0.2 μm filter, BD Biosciences) step, CMs were stored at −20 °C until use. For migration and invasion assays, the CM concentration used was a 1:1 (1 part media, 1 part CM) dilution to prevent the possible masking effects of lower nutrient (e.g., amino acids and glucose) contents in CMs. For western blots, CMs were freeze-dried and reconstituted in a small volume of sterile water concentrating the proteins secreted from each cell supernatant by 40-fold.

Wound healing assays. NMuMG and 4T1 cells were plated in 6-well plates and incubated in DMEM media supplemented with 10% FBS for 24 h. The plates were then incubated in serum free media for 24 h. Cell monolayers were scratched using sterile 20 μl pipette tips, and cell cultures were washed with PBS. CMs (1:1 dilution) were added and the wound size recorded (micro-photographs) over time at 0, 3, 6, and 24 h. For each time point and treatment, migratory distances (μm) were measured at 8 different locations per condition following image acquisition using a BioChemi Camera (2/3” Cooled Monochrome CCD/High-Res/12bit) and the VisionWorks software (UVP imaging system, UVP).

3D migration assays. To study invasion in 3D conditions, NMuMG and 4T1 cells (8 × 10⁴ cells/well) were seeded in the center well made in a sterile 0.8% agarose gel composed of multiple wells prepared as described previously.¹⁸ Prior to seeding, cells were briefly incubated with the vital nuclear dye Hoechst (1:2000 dilution, Invitrogen) to enable cell visualization using fluorescent microscopes (excitation 350 nm, emission 450 nm). Cells were cultured with DMEM media without FBS and supplemented with gentamycin and amphotericin B. CMs (1:1 dilution) were placed in the wells surrounding the center well, which contained either 4T1 or NMuMG cells. Serum-free (0% FBS) and 10% FBS-DMEM media were used as negative and positive controls, respectively. At least five random representative microphotographs of the cells migrating out of the center well toward each of the conditions were recorded after 5 d of culture. The numbers of cells moving toward each chemotactic condition were numerated and the 3D chemotactic potential of each conditioned medium defined.

Invasion assays. Following an incubation with the vital nuclear dye Hoechst (1:2000 dilution), NMuMG, or 4T1 cells (5 × 10⁴ cells per well) were seeded in serum free media in the upper compartment of 24 well plate transwell migration chambers (BD Biosciences). Upper compartments were coated with growth factor reduced Matrigel® (50 μl, BD-Biosciences) to evaluate the invasion associated with different conditions. The lower chamber was filled with 300 μl of either CCL5 (12.5 μM; R&D Systems) alone, CCL9 (12.5 μM; R&D Systems) alone, the combination CCL5 (12.5 μM) and CCL9 (12.5 μM), or D1CM (1:1 dilution) in serum free (0%) media. Serum-free (0% FBS) and media supplemented with 10% FBS served as negative and positive control, respectively. After a 24 h incubation at 37 °C, 5% CO₂, cells that did not migrate to the lower chamber were removed from the upper surface of the transwell membrane. Cells at the lower surface of the membrane were counted in at least 5 different random fields for each condition.
Inhibition of CCR1 and CCR5 receptors. 4T1 cells were pretreated with increasing concentrations (0–100 ng/ml) of the antagonist to CCR1 and CCR5 Met-CCL5 (R&D Systems) for 30 min. The modified CCR5 ligand Met-CCL5 has been shown to antagonize receptor activation and function in response to its natural ligands in part through a slower internalization and a trafficking independent of recycling endosomes.13,54 Although Met-CCL5 binding to CCR5 has been documented in multiple cells, binding of Met-CCL5 to CCR1 chemokine receptor has also been shown to inhibit multiple eosinophil functions including Ca++ intracellular trafficking, actin polymerization, reactive oxygen species release.64 Following an incubation with the live nuclear dye Hoechst 4T1 cells were seeded in the upper chamber of the transwell in serum-free media (0% FBS). Invasion studies were conducted in a 24-well plate in which the transwell membrane upper compartments were coated with growth factor reduced Matrigel (50 μl) to evaluate the invasion of 4T1 cells treated with Met-CCL5 associated with DICM. The lower chamber was filled with 300 μl of 1:1 dilution of DICM or control media, i.e., media with 0% FBS and media with 10% FBS for negative and positive controls, respectively. After a 24 h incubation at 37 °C, 5% CO₂, cells that did not migrate to the lower chamber were removed from the upper chamber. The fluorescent nuclear vital dye allowed the count of the cells present on the lower surface of the membrane in at least five different random high-power microscope fields for each treatment condition.

Cytokine antibody arrays. The levels of cytokines and chemokines in conditioned media derived from various cell types were determined using antibody-based arrays (RayBiotech, Inc.). Following a blocking step, cytokine array membranes were incubated with CMs (1:1 dilution). Experiments were performed following the manufacturer’s recommendations. Briefly, the different CMs were incubated with the cytokine arrays for 2 h at room temperature and the presence of cytokines or chemokines was detected (sensitivity: pg/ml) using chemiluminescence. The intensity of the signals obtained were quantified and expressed as pixels/mm² using the Quantity One software (Bio-Rad). After normalization to the number of cells present when CMs were collected, the intensity for each cytokine and chemokine tested were calculated using the formula (density of the sample – density of negative control)/(density of the positive control – density of the negative control) × 100.

CCL5 receptor and CCL9 receptor RNA expressions. NMuMG and 4T1 cells were grown to confluency and treated with 1:1 dilutions of the CMs tested for 3 h following a 24 h serum starvation. Total RNA was isolated using a one-step lysis reagent (Invitrogen Life Technologies). After quantification, mRNA expression levels of CCR1, CCR3, and CCR5 receptors for CCL9, and CCL5, respectively, were detected using specific primers (see Table 1) and the One Step RT-PCR kit per manufacturer’s instructions (Promega). The mRNA expressions of these chemokine receptors were normalized to the expression of the housekeeping gene glyceraldehyde phosphate 3 dehydrogenase (GAPDH). Amplicons were identified following electrophoresis on 2% agarose gels containing ethidium bromide. Each band was semi-quantified based on the ethidium bromide signal using Quantity One (Biorad).

Western blots for CCR1, CCR5, and MMP protein expressions. Following culture and treatments, protein extracts from NMuMG and 4T1 cells were obtained by homogenization in a lysis buffer (Tissue Protein Extraction Reagent, Thermo-Scientific) supplemented with protease inhibitors (Santa Cruz Biotechnology). For MMPs, both cell extracts and cell supernatants were collected. Cell supernatants were freeze-dried and resuspended in 40-fold lower equal volumes of DI water. Protein concentrations were evaluated using protein assays (Pierce Protein Research Products). Following denaturation, protein samples (40 μg) were run onto 8% and 10% polyacrylamide gels for cell extracts and cell supernatants, respectively, and transferred onto nitrocellulose membranes. After a blocking step, membranes were incubated overnight with specific antibodies to CCR1, CCR5, MMP9, MMP13, or MMP14 at a dilution of 1:500 (Santa Cruz Biotechnology). Following washing steps and an hour incubation with horseradish peroxidase conjugated anti-goat, anti-mouse or anti-rabbit secondary antibodies (Jackson Laboratory), the intensity and bands recognized by the specific primary antibodies were detected using chemiluminescence (Biorad) and recorded using the UVP biochemiluminescence system. All blots were stripped (Chemicon) and reprobed for β-actin (1:5000) (Sigma). For each condition, the intensity of each protein band was determined using QuantityOne (Biorad) and normalized to the β-actin intensity.

Flow-cytometry analyses of CCR1 and CCR5 receptors. Following a 24 h incubation with media alone or DICM (1:1 with media), 4T1 cells detached and fixed in 1% PFA for 30 min at room temperature. Next 4T1 cells resuspended in PBS supplemented with 1% BSA were incubated with antibodies...
Following a 24 h incubation, the number of invading cells was determined as described above. GM6001 (1 μM inhibitor of matrix metalloproteinases (MMPs) MMP inhibitor) was added to the reaction. Data are expressed in relative fluorescence unit (RFU). 

Table 1. Primer sequences used in PCR amplifications and resulting amplicon sizes

| Gene   | Forward primer                     | Reverse primer                     | Size (bp) |
|--------|------------------------------------|------------------------------------|-----------|
| CCR1 (CD191) | 5'ACT CCA ACT CCA TGC CCA AAA G3' | 5'CTA GGA CAT TGC CCA CT3'         | 161       |
| CCR3 (CD193) | 5'GAT TGC CTA CAC CCA CTG CT3'   | 5'CTG TGG AAA AAG AGC CGA AG3'     | 181       |
| CCR5 (CD195) | 5'ATT CTC CAC ACC TGT TTC G3'    | 5'GAA TCC CTG GAA GGT GGT CA3'     | 267       |
| GAPDH  | 5'AAC TTT GGC ATT GTG GAA GG3'    | 5'ACA CAT TGG GGG TAG GAA CA3'     | 223       |

CCR1, CCR3, and CCR5, C-C chemokine receptor type 1, 3 and 5, respectively; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 8. D1 mesenchymal stem cell CM, CCL5, and CCL9 promoted the activities of MMPs including MMP9/13 and MMP14. MMP activities of supernatants collected from 4T1 cells incubated 24 h with either media alone, CCL5, CCL9, or D1CM were freeze-dried and concentrated to 1/10 of their initial volume were assayed according to the manufacturer’s recommendations (see Materials and Methods for details). To determine whether the activities of MMP9/13 or MMP14 were altered by either CCL5, CCL9, the combination CCL5–CCL9 or D1CM, specific inhibitors of MMP9/13 (10 nM) and MMP14 (50 nM), along with the general MMP inhibitor GM6001 (10 nM) were added to the reaction. Data are expressed in relative fluorescence unit (RFU). Data were analyzed by one-way ANOVA and differences between treatment groups tested using the Student Newman–Keuls post-hoc test. ***P < 0.001 compared with MMP activities of 4T1 cells incubated with 0% FBS alone.

Inhibition of MMP activities. The MMP activity of 4T1 cells during invasion assays was determined. In invasion assays conducted as described above, 4T1 cells were incubated with or without the potent, cell-permeable, broad-spectrum hydroxamic acid inhibitor of matrix metalloproteinases (MMPs) MMP inhibitor GM6001 (1 μM; EMD Millipore), the MMP9/MMP13 inhibitor (10 nM; EMD Millipore, 444252), or the MMP14 inhibitor (50 nM; EMD Millipore, NSC405020). Data are presented as relative fluorescence units (RFU).

Statistical analyses. All experiments and treatments were conducted at least three times. Data are presented as mean ± SEM. Statistics for all assays were analyzed using one-way ANOVA and the post-hoc Student Newman–Keuls test or the Student t test (Sigma). Significance was set at P < 0.05 a priori.

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