CCR5 ANTAGONIST BLOCKS METASTASIS OF BASAL BREAST CANCER CELLS

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Running Head: Antimetastatic effect of CCR5 antagonist

Keywords: CCL5, CCR5, metastasis, Maraviroc, Vicriviroc, basal breast cancer

Financial support: This work was supported in part by PASPA-UNAM (M.A.V-V.), NIH grants R01CA070896, R01CA075503, R01CA132115, R01CA107382, R01CA086072 (R.G.P.), R01CA120876 (M.P.L), the Kimmel Cancer Center NIH Cancer Center Core grant P30CA056036 (R.G.P.), generous grants from the Dr. Ralph and Marian C. Falk Medical Research Trust and the Margaret Q. Landenberger Research Foundation, and a grant from Pennsylvania Department of Health (R.G.P.).

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Conflict of interests: There are no conflicts of interest associated with this manuscript.
ABSTRACT

The roles of the chemokine CCL5 and its receptor CCR5 in breast cancer progression remain unclear. Here we performed microarray analysis on 2,254 human breast cancer specimens and found increased expression of CCL5 and its receptor CCR5, but not CCR3, in the basal and HER-2 genetic subtypes. The subpopulation of human breast cancer cell lines found to express CCR5 displayed a functional response to CCL5. In addition, oncogene transformation induced CCR5 expression, and the subpopulation of cells that expressed functional CCR5 also displayed increased invasiveness. The CCR5 antagonists Maraviroc or Vicriviroc, developed to block CCR5 HIV co-receptor function, reduced in vitro invasion of basal breast cancer cells without affecting cell proliferation or viability, and Maraviroc decreased pulmonary metastasis in a preclinical mouse model of breast cancer. Taken together, our findings provide evidence for the key role of CCL5/CCR5 in the invasiveness of basal breast cancer cells and suggest that CCR5 antagonists may be used as an adjuvant therapy to reduce the risk of metastasis in patients with the basal breast cancer subtype.
INTRODUCTION

Breast cancer causes the death of 40,000 women in the USA and 410,000 women in the world annually (1). Despite advances in the treatment of the disease, 20% to 30% of patients with early breast cancers will experience relapse with distant metastatic disease (2). In those patients, metastasis is the main cause of death. Patients with basal tumors have increased risk of metastasis and lower survival rate (3, 4). Kennecke et al. studied 3,726 breast cancer patients and reported that the basal tumors have higher frequencies of metastases and reduced time from identification of metastases to death compared to that of patients with luminal A or B tumors (4). The absence of AR, ER, and HER-2 commonly found in basal breast tumors (5) means that they are unlikely to respond to hormone therapies or HER-2 targeted therapies. Currently, chemotherapy, radiation, and surgery are the only choices for patients with basal breast cancers, but all demonstrate poor outcomes (6). The need for a specific targeted therapy for basal breast cancer remains urgent.

Cancer metastasis is regulated by chemokines in the microenvironment (7). Chemokines bind to cell surface receptors that belong to the G-protein-coupled receptor family (GPCRs), controlling diverse biological and pathological processes from immune surveillance, inflammation, and cancer. Previous studies of human breast cancer and breast cancer cell lines demonstrated that the chemokine receptors CXCR4 and CCR7 are expressed in breast cancer cells, malignant breast tumors, and metastasis. Their related ligands, CXCL12 (SDF1) and CCL21, are also expressed at the site of metastasis (8). Subsequent studies identified altered expression of CCL5 (RANTES) in breast cancer patients, correlating with disease progression (9-11).

CCL5 can be expressed and secreted either by breast cancer cells (9-12) or by non-malignant stromal cells at the primary or metastatic sites (13). However, the roles of CCL5 and its receptors in breast cancer are not fully understood. CCL5 facilitates disease progression by recruiting and modulating the activity of inflammatory cells, which subsequently remodel the tumor microenvironment (14, 15). Accordingly, inhibition of CCR5 by a peptide antagonist reduced leukocyte infiltration and reduced tumor growth after subcutaneous injection of 410.4 cells into immunocompetent mice (16). Studies analyzing
the role of CCR5 in breast cancer cell proliferation have used mainly luminal MCF-7 cells and suggest a role for p53. Manes et al. reported that abrogation of cell surface CCR5 expression enhanced MCF-7 cell proliferation in vivo in the presence of p53 but did not affect proliferation in xenografts encoding a p53 mutation (17). Murooka et al. reported that in CCR5-transfected MCF-7 cells, CCL5 promotes proliferation and survival in an mTOR-dependent manner (18). Autocrine secretion of CCL5 controls migration and invasiveness of human breast cancer cells in vitro (12, 19). Forced expression of CCL5 increased tumor metastasis ~1.8-fold in one study of MDA-MB231 cells but had no effect in the 186 breast cancer cell line (13, 20). Furthermore, other authors have shown that shRNA inhibition of CCL5 expression in murine mammary cancer cells does not affect growth rate or metastatic frequency in vivo (20).

Discrepancies in the reported effects of CCL5 and CCR5 in breast cancer may be due to the different models employed. To study whether CCL5/CCR5 signaling is active in specific genetic subtypes of breast cancer, we analyzed the combined expression of CCL5 and CCR5 using a microarray database comprising 2,254 human breast cancer samples from 27 independent studies (21). This report is the first to show that CCL5/CCR5 signaling is preferentially active in the basal and HER-2 subtypes. Using basal-like breast cancer cell lines, we demonstrated that only a subpopulation of cells express CCR5 and respond to CCL5. Importantly, CCR5+ cells displayed increased invasiveness, indicating that CCR5 contributes to the metastatic phenotype of basal breast cancer cells. Therefore, we examined the possibility that CCR5 inhibition may block the invasion and metastasis of basal-like breast cancer cells. We used drugs originally developed to prevent the interaction of CCR5 with the gp120 from HIV-1. The CCR5 antagonist Maraviroc (Selzentry®) received full FDA approval for use in treatment-naïve adults with CCR5-trophic HIV. Vicriviroc (SCH 417690), another CCR5 antagonist, has shown good tolerance and partial therapeutic success in phase II clinical trials for HIV (22). CCR5 inhibition blocked breast cancer cell invasiveness in vitro and efficiently reduced metastatic colonization in vivo. The antimetastatic effect produced by CCR5 inhibition was associated with reduced arrival of breast cancer cells to the target tissue but was independent of changes in cell proliferation or tumor growth. Our findings suggest that
CCR5 antagonists may be used to reduce the risk of metastasis in patients with the basal subtype of breast cancer.
**MATERIALS AND METHODS**

**Breast Cancer Patients Dataset and Statistical Analysis.** A microarray dataset that was previously compiled (21) from the public repositories Gene Expression Omnibus (23) and ArrayExpress (24) was used to evaluate CCR5 and CCL5 expression in the context of clinical samples. Samples in this dataset were assigned to five canonical breast cancer subtypes, including luminal A, luminal B, normal-like, basal, and HER-2-overexpressing disease. The classification of microarray samples among these five subtypes was achieved by computing their correlation against an expression profile centroid representative of each subtype and assigning samples to the subtype with the highest corresponding correlation coefficient (25). Samples with a maximum correlation coefficient below 0.3 were considered unclassified. Analysis of CCL5 and CCR5 transcript was then performed specifically among the luminal A, luminal B, basal, normal-like, and HER-2 subtypes. Differential expression of the averaged gene signature magnitude among these sample subsets was evaluated using two-tailed Student's *t* test. Kaplan-Meier analysis was used to evaluate survival trends within the sample subsets. Scatter plots of CCL5 versus CCR5 samples were also generated to observe co-regulation patterns specific to each subtype. For these scatter plots, gene profiles were median-centered and scaled to unitary standard deviation.

**Cell lines and Cell Culture.** MDA-MB-231, MCF-7, and Hs578T cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS). SUM-159 cells (kindly provided by Dr. Stephen Ethier, Wayne State University) were maintained in Ham’s F12 supplemented with 4 μg/ml of insulin, 1 μg/ml of hydrocortisone and 5% FBS. Oncogene transformed derivatives of MCF-10A cells (MCF10A-NeuT, MCF10A-Src, and MCF10A-Ras) (26) were maintained in DMEM/Ham’s F12 (50/50) supplemented with 4 mg/ml of insulin, 10 ng/ml of EGF and 1 mg/ml of hydrocortisone. 100 μg/ml of each penicillin and streptomycin were included in all media. Cells were cultured in 5% CO₂ at 37°C. For *in vitro* treatments, Maraviroc was dissolved in DMSO and diluted in culture medium. The final concentration of DMSO in treated and control cultures was 0.5%. Vicriviroc was dissolved in culture medium.
Fluorescence Activated Cell Sorting (FACS) Analysis. Cell labeling and FACS analysis for CCR5 were based on prior publications (27) with minor modifications. Before labeling, the cells were blocked with normal mouse IgG (1/100) and purified rat anti-mouse Fcγ III/II receptor antibody (1/100) (Pharminogen, San Diego, California) for 30 min and then incubated with allophycocyanin (APC)-labeled CCR5 antibody (R&D Systems). All experiments were conducted at 4°C. Sample analysis was performed on FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Invasion Assay. The three-dimensional invasion assay was performed as previously reported (12). Briefly, 100μl of 1.67 mg/ml Rat Tail collagen type I (BD Biosciences) was pipetted into the top chamber of a 24-well 8 μm pore transwell (Corning, Lowell, MA). The transwell was incubated at 37°C overnight to allow the collagen to solidify. 30,000 cells were then seeded on the bottom of the transwell membrane and allowed to attach. Serum-free growth medium was placed into the bottom chamber, while 15 ng/ml CCL5 or 5% FBS was used as a chemoattractant in the medium of the upper chamber. The cells were then chemoattracted across the filter through the collagen above for three days. Cells were fixed in 4% formaldehyde, permeabilized with 0.2% Triton-X in PBS and then stained with 40 μg/ml propidium iodide (PI) for 2 h. Fluorescence was analyzed by confocal z-sections (one section every 20 μm) at 10x magnification from the bottom of the filter using a Zeiss LSM 510 Meta inverted confocal microscope at the Kimmel Cancer Center Bioimaging Facility.

Intracellular Calcium Assay. Calcium responses induced either by CCL5 or FBS in human cancer cell lines were monitored under fluorescence confocal microscope as previously reported (28). Briefly, breast cancer cells were seeded in 4-well labtek chambers (Nunc, Rochester, NY) at 10^4 cells/cm² and incubated for one day. After 12 h starvation, cells were labeled by incubating them with 2 mM Fluo-4-AM (Molecular Probes, Grand Island, NY) in HBSS for 30 min, washed twice, and incubated for additional 30 min before imaging under the microscope. Time-lapse images were collected using a Zeiss LSM 510 Meta inverted confocal microscope with the incubator at 37°C. Relative intracellular Ca^{2+} concentration
was determined by the changes in fluorescent intensity (FI) of Fluo-4-AM upon the addition of CCL5 (60 ng/ml) or FBS (5%), and was calculated as \((\text{FI}_t - \text{FI}_0)/\text{FI}_0\).

**MTT Assay.** The effects of CCR5 antagonists on cell viability and proliferation rate were estimated using the soluble tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (29). MTT is reduced by the mitochondria of viable cells, and the amount of reduced formazan is proportional to the number of viable cells. After 72 h of exposure to the drugs, cells were incubated with 1 mg/ml of MTT for 90 min. Then, the reduced (insoluble and colored) formazan was dissolved in dimethyl-sulfoxide and measured spectrophotometrically at 570 nm. The effect of CCR5 overexpression in breast cancer cell proliferation was studied in MDA-MB-231 cells transfected with full-length human CCR5 subcloned into pcDNA3.1+/Zeo+ vector (kindly provided by Dr. Eleanor Fish, University of Toronto) and selected with Zeocin (200 µg/ml) as previously described (18). MTT assays were performed in sextuplicate using 96-well microplates.

**Viral Cell Transduction.** A lentiviral vector encoding firefly luciferase 2 (Luc2)-eGFP fusion protein was a generous gift from Dr. Sanjiv S. Gambhir (School of Medicine, Stanford University) (30). Lentivirus propagation was performed following the protocol described by Zahler at al. (31). Breast cancer cell lines were transduced at a MOI of 20 in the presence of 8 mg/ml polybrene (Sigma, St. Louis MO) for 24 h (30, 31).

**Experimental Metastasis Assay and Bioluminescence Imaging.** MB-MDA-231 cells expressing Luc2-eGFP (called MDA.pFLUG for the rest of the paper) were detached with a non-enzymatic cell dissociation buffer [4 mM EDTA in Ca\(^{2+}\) and Mg\(^{2+}\)-free PBS], resuspended in Dulbecco’s PBS without Ca\(^{2+}\) and Mg\(^{2+}\) and immediately injected into the tail vein of 8-week old, female NOD/SCID mice (NCI, Bethesda MD). Each mouse received \(10^6\) cells. Mice were treated by oral gavage with Maraviroc (8 mg/kg every 12 h) or vehicle [5% DMSO in acidified water (32)]. Treatment was started immediately after injection or ten days later for the experiments analyzing the proliferation of established metastasis. For **in vivo** bioluminescence imaging (BLI), mice were given an intraperitoneal (i.p.) injection with 200 µL of D-luciferin (30 mg/mL). Mice were anesthetized with isoflurane (2% in 1 L/min oxygen), and
bioluminescence images were acquired 10–15 min after D-luciferin injection using the IVIS XR system (Caliper Life Sciences, Hopkinton MA). Acquisition times ranged from 10 s (for later time points) to 5 min (for early time points). Data are expressed as total photon flux and were analyzed using Living Image 3.0 software (Caliper Life Sciences). For *ex vivo* BLI, D-luciferin was diluted in PBS to a final concentration of 300 μg/mL and used to soak freshly isolated lungs for 2 to 3 min before imaging. Some lungs were stained with india ink, as previously reported (33), or processed and stained with H&E to corroborate the presence of pulmonary tumors. For homing assays, mice were euthanized 24 h after the intravenous injection of MDA.pFULG cells. Lungs were perfused with PBS, fixed with freshly prepared formaldehyde (4% in PBS), and frozen in OCT (Sakura Finetek, Torrance CA). Cryosections (10 μm) were counterstained with DAPI analyzed by confocal microscopy. Animal experiments were approved by the Thomas Jefferson University’s IACUC.

**Reagents and Antibodies.** CCL5 (Cat. 278-RN) and anti-CCR5 APC antibody (Cat. FAB1802A) were purchased from R&D Systems (Minneapolis, MN). A rabbit anti human CCR5 polyclonal antibody (GenScipt, Piscataway, NJ. Cat. No. is A00979) was used for IHC staining. Rat tail collagen type I was purchased from BD Biosciences (Franklin Lakes, NJ). Vicriviroc and Maraviroc were obtained from Selleck Chemicals (Houston, TX). Luciferin was obtained from Gold Biotechnology (St. Louis, MI).
RESULTS

Active CCL5/CCR5 signaling in basal breast cancer. In order to examine the relative abundance of CCL5 and its receptor CCR5 by genetic subtype, interrogation was conducted within a combined microarray database comprising 2,254 human breast cancer samples from 27 independent studies (21). The relative abundances of CCL5 and CCR5 were significantly increased in the basal and HER-2 subtypes compared with the normal-like, luminal A and luminal B subtypes (Fig. 1A). The increased expression of CCL5 and CCR5 correlated positively in individual breast cancer samples and the correlation was highly significant in the basal and HER-2 subtypes (Fig. 1B). The proportion of patients with a CCL5/CCR5 positive signature was over 58% in the basal and HER-2 subtypes (Fig. 1C). In agreement with previous reports, clinical information of the cases in this database showed that the probability to develop metastasis is increased in the basal, luminal B, and HER-2 subtypes (Fig. 1D).

In order to determine the gene expression signaling pathway associated with enrichment of CCR5 and CCL5, GSEA analysis using KEGG and GO was conducted of these tumor samples (Suppl. Fig. 1A). These studies demonstrated enrichment for gene expression of pathways including lymphocyte activation, JAK-STAT signaling, and TOLL-like receptor activation (Suppl. Fig. 1A). The receptors for CCL5 include CCR1 and CCR3. Increased expression of CCL5 associated with increased CCR1, but not CCR3, in the basal and HER-2 genetic tumor type (Suppl. Fig. 1B, C). In ER negative patients receiving chemotherapy, there was an insignificant trend towards reduced metastasis free survival and relapse free survival in the increased CCR5 population, compared with the population with reduced CCR5 expression (Suppl. Fig. 1D). A comparison of expression levels for CCL5 vs. CCR5, CCR1, and CCR3, comparing normal breast with breast cancer showed increased correlation between receptor and ligand expression levels in tumors compared with healthy breast tissue (Suppl. Fig. 2).

CCL5 promotes breast cancer Ca\(^{2+}\) signaling and cellular invasion. We chose to use as models in our studies three human breast cancer cell lines with a basal phenotype and molecular signature: MDA-MB-231, Hs578T and SUM-159 (34-37). Analysis of CCR5 expression by FACS showed that a small subpopulation of cells were positive for the receptor in all three cell lines (Fig. 2A for...
MDA-MB-231 and Suppl. Fig. 3A, C for Hs578T and SUM-159). Since CCR5 activation induces calcium flux (38, 39), we assessed the activation of calcium signaling by CCL5. Addition of CCL5 to the cultures induced immediate calcium fluxes in a subpopulation of cells (Fig. 2B for MDA-MB-231 and Suppl. Fig. 3B, D for Hs578T and SUM-159), providing evidence that CCR5 is functional in basal breast cancer cells. As a positive control the same cultures were exposed to 5% FBS (40). Calcium flux, assessed by relative fluorescence intensity, increased in more than 95% of the cells after FBS addition (Fig. 2B and Suppl. Figure. 3B, D). In order to further distinguish CCL5-dependent signaling, SUM159 cells were stably transduced with a CCR5 expression vector and the Ca\(^{2+}\) response to CCL5 vs. FBS was conducted (Supplemental Figure 4G vs. 4D). CCR5 induced Ca\(^{2+}\) signaling in the CCR5 overexpressing cells, whereas both lines responded similarly to FBS induced Ca\(^{2+}\) activation (Suppl. Fig. 4).

We next assessed the effect of CCR5 activation on breast cancer cell invasion using 3-D migration assays. CCL5 induced invasion of the basal MDA-MB-231, Hs578T, SUM-159 but not the luminal MCF-7 cells (Fig. 2C, D). CCL5 promoted invasion of MCF-10A cells engineered to express either NeuT, H-Ras or c-Src oncogenes, compared with MCF10A vector-transduced cells (Fig. 2E, F), suggesting that CCL5 responsiveness may be acquired during transformation and requires specific cooperative oncogenic signals. The finding that CCL5 induced cellular invasion led us to examine the migratory capacity of CCR5\(^+\) cells versus that of CCR5\(^-\) cells. Within the same SUM-159 breast cancer cell line, CCR5\(^+\) cells showed an approximately 40-fold greater cellular invasiveness (Fig. 2G, H), indicating that the expression of CCR5 correlates with a pro-invasive phenotype.

**CCR5 antagonists block breast cancer calcium signaling and cell invasion.** The importance of CCR5 in HIV infection led to the development of different drugs that target this receptor. We therefore examined whether the CCR5 antagonists Maraviroc and Vicriviroc were capable of blocking the CCL5/CCR5 signaling in basal breast cancer cells. Both CCR5 antagonists blocked CCL5-induced calcium mobilization. In MDA-MB-231 cells, Maraviroc and Vicriviroc inhibited calcium responses by 65% and 90% respectively (Fig. 3A, B). Similar observations were made with both drugs in Hs578T cells.
(Fig. 3C, D), indicating that CCR5 expressed in different basal breast cancer cells is sensitive to pharmacological inhibition.

To evaluate the functional relevance of CCR5 in cellular migration and invasion, we tested the effects of Maraviroc and Vicriviroc in 3-D invasion assays. Using two different cell lines, we found that both CCR5 antagonists inhibited FBS-induced breast cancer cell invasion at the clinically relevant concentration of 100 nM (Fig. 4A-D). Thus, the pro-invasive effect of CCR5 can be abrogated by using specific antagonists.

**CCR5 inhibition blocks breast cancer metastasis in vivo.** In view of the finding that CCR5 inhibition by CCR5 antagonists reduced calcium signaling and cell invasion, we determined the in vivo effect of Maraviroc on lung metastasis. We used MDA-MB-231 cells transduced within the Luc2-eGFP lentiviral vector (MDA.pFULG cells) in an experimental metastasis model. The Luc2 gene is a codon-optimized version of Luc and cells expressing this reporter were 10-100 times brighter than the unmodified Luc gene (30). After injection of MDA.pFULG cells into the tail vein of mice, noninvasive BLI enabled the early detecting of breast cancer metastasis (41). Weekly BLI was conducted for 5 weeks and the radiance ante-mortem was used as a surrogate measurement of tumor burden. Mice treated with Maraviroc (8 mg/kg twice daily) showed a significant reduction in both the number and the size of pulmonary metastases compared to vehicle-treated mice (Fig. 5A, B, Suppl. Fig. 5). To avoid the possibility that metastases were missed due to inappropriate imaging, we performed ex-vivo imaging, India ink staining (Fig. 5C) and histology (Fig. 5E) of the lungs. Histological analysis corroborated that tumor burden corresponds to bioluminescence, as previously shown (30). Metastatic tumors were still detectable in 50% of the Maraviroc-treated mice, but their mean size was reduced by 65% (Fig. 5D, F). Interestingly, analysis of CCR5 expression in lungs from control mice showed an eight-fold enrichment of the CCR5+ fraction (Suppl. Fig. 6). Collectively, these results provide evidence that CCR5 antagonists reduce breast cancer metastasis in vivo.

**CCR5 antagonist impairs lung colonization but not cell proliferation or tumor growth.** We determined whether the reduction in metastatic tumors by Maraviroc involved changes in cellular
proliferation and/or target organ colonization. We analyzed the effect of CCR5 inhibition on cell viability and proliferation both \textit{in vitro} and \textit{in vivo}. Maraviroc or Vicriviroc treatment of MDA-MB-231 cells for 48h did not affect the MTT reduction, which was used as a surrogate measurement of cancer cell number (Fig. 6A). In agreement, overexpression of CCR5 in MDA-MB-231 cells did not modify their proliferation rate compared with cells transfected with the empty vector (Fig. 6B, C). Finally, Maraviroc treatment of mice with established pulmonary metastasis did not modify tumor growth (Fig. 6D, E), indicating that CCR5 activation does not promote the proliferation of basal breast cancer cells \textit{in vitro} nor in the pulmonary microenvironment of immunocompromised mice.

On a different \textit{in vivo} experiment, we examined the effect of Maraviroc on breast cancer cell homing to lungs. In order to reach a steady state concentration in plasma and tissues, mice were given ten administrations of Maraviroc (twice a day for five days) before the intravenous injection of MDA.pFULG cells (Fig. 6F). Inoculation of equal numbers of MDA.pFULG cells in control and treated groups was corroborated by BLI immediately after injection. Maraviroc reduced the number of eGFP$^+$ cells in the lungs by 40\% (Fig 6G, H), suggesting that the \textit{in vivo} antimetastatic effect of Maraviroc is caused by a reduction in the number of cancer cells that colonize the target organ from the circulation.
DISCUSSION

The current studies demonstrate for the first time that: i) enrichment of CCL5/CCR5 expression occurs in patients with basal and Her2 positive genetic subtypes of breast cancer; ii) oncogenic transformation of immortalized human breast cells by distinct oncogenes induces CCL5 responsiveness; and iii) Maraviroc, a FDA-approved drug for the treatment of CCR5-trophic HIV infection, reduce metastatic tumor burden in vivo.

Previous studies showed that CCL5 levels are elevated in breast primary and metastatic tumors (9-11), suggesting a role of CCL5 in the acquisition of malignancy. We report here that increased expression of CCL5 and CCR5 are associated and that CCL5/CCR5 expression levels are different among the different genetic subtypes of breast cancer. Increased expression of CCL5 and CCR5 is found in the basal and HER-2 subtypes. In agreement, increased CCL5 expression has been found predominantly in ER-negative patients (42). Increased CCL5 also correlated with increased CCR1 in basal and Her2 genetic subtypes of breast cancer. A trend towards reduced metastasis free survival and relapse free survival was observed amongst the CCR5 overexpressing tumors in patients who received chemotherapy.

Given the aggressive clinical behavior of basal breast cancer and the lack of targeted therapies for it, we evaluated the importance of the CCL5/CCR5 axis in invasion and metastasis in the human breast cancer cell lines MDA-MB-231, Hs578T and SUM-159. These cell lines reflect the clinicopathological features of the basal subtype of breast cancer (including the lack of HER2, ER, and PR), a basal-like molecular signature, the activation of specific signaling pathways (e.g. hypoxic or EGFR responses) and overexpression of EMT proteins (FN, VIM, and MMP2) (34-37). Only a small fraction of cells within the cell lines used in this study expressed CCR5 as evaluated by FACS analysis. Our findings are consistent with studies by Müller et al. who demonstrated CCR5 expression in MDA-MB-231 by real-time quantitative PCR (8). Our studies confirmed the expression of CCR5 in MDA-MB-23 cells by RT-PCR and showed the presence of the CCR5 protein by FACS analysis (Supplemental Figure 7), and demonstrated CCR5 immunohistochemical staining was localized primarily to the breast cancer epithelial cell, compared with normal breast tissue (Supplemental Figure 8).
We demonstrated that CCL5 activates calcium flux in basal-like human breast cancer cells, as previously described in cells of the immune system (39, 43) and CCR5-transfected cells (27, 44, 45). By using the selective CCR5-antagonists Maraviroc and Vicriviroc (both with IC$_{50}$ below 30 nM (44, 45)), we showed that CCL5-activated signaling is mediated by CCR5. However, the fraction of CCL5-responsive cells (10 and 12% for MDA-MB-231 and Hs578T cells, respectively) is higher than the percentage of CCR5-expressing cells determined by FACS. This may be due to the greater sensitivity of the Ca$^{2+}$ activation assays compared with the sensitivity of analysis by FACS. Additionally, CCL5-induced calcium redistribution is not completely blocked by CCR5 antagonists. This may be caused by the expression of other receptors to CCL5, namely CCR1 and CCR3. CCR5 has been identified as the main CCL5 receptor in MDA-MB-231 cells (13) and CCR1 and CCR3 transcripts are absent in both MDA-MB-231 or Hs578T cell lines (8) and breast tumor samples (11). We were able to detect CCR1 and CCR3 by FACS (Suppl. Fig. 7), suggesting a possible mechanism for the incomplete response to the CCR5 antagonist.

We observed that the subpopulation of CCR5$^+$ cells displayed increased invasiveness, indicating that CCR5 favors cell migration and invasion in basal-like breast cancer cells. The failure of luminal-like MCF-7 cells to respond to CCL5 is in agreement with previous publications (12). Our studies also demonstrated that CCR5 inhibition with either Maraviroc or Vicriviroc reduced in vitro FBS-induced breast cancer cellular invasion without affecting cellular viability. The finding that CCR5 antagonists block FBS-induced invasion is novel and suggested that CCR5 activation contribute to the production of metastasis in vivo where different chemotactic and growth signals are present. The mechanisms involved in CCR5 regulation of FBS-activated invasiveness are uncharacterized but they may include heterodimerization and ligand affinity regulation of other GPCRs (46), or the transactivation of growth factor receptor- (47) or integrin-mediated signaling (48), as described in non-cancerous cells.

The in vivo antimetastatic effect of Maraviroc was demonstrated by injecting MDA.pFULG cells into the circulation of immunodeficient mice and treating them with clinically relevant doses of the drug. In humans, oral doses of 300 mg produce an average C$_{max}$ of 1200 nM (49), while in mice 16 mg/kg
produce an average $C_{\text{max}}$ of 1045 nM (32). Since the drug is taken twice a day in the clinical setting, we administered 16 mg/kg/day divided into two doses during our experiments. Maraviroc significantly reduced the pulmonary tumor burden. Although it has been proposed that pharmacological CCR5 inhibition may be beneficial for breast cancer patients, to our knowledge this is the first study showing that systemic administration of a CCR5 antagonist reduces metastatic colonization of basal breast cancer cells.

The antimetastatic effect of Maraviroc is not caused by alterations in growth of established metastasis. CCR5 activation by CCL5 drives proliferation in CCR5-transfected MCF-7 breast cancer cells (18) and prostate cancer cells (50), but this study and others (13) showed that the CCL5/CCR5 axis does not play a role in cell proliferation or survival in the basal-like MDA-MB-231 cells. Furthermore, inhibition of CCR5 surface expression through a dominant-negative form of CCR5 (CCR5$\Delta$32) in MDA-MB-231 cells does not change in vivo proliferation or apoptotic response (17). On the other hand, we found that Maraviroc reduces lung colonization by MDA,pFULG cancer cells. This result is consistent with previous studies in which inhibition of CCR5 expression within breast cancer cells or administration of anti-CCL5 neutralizing antibody to tumor-bearing mice reduced the enhanced metastatic capability induced by co-injection of mesenchymal stem cells (MSC) (13). The authors identified cancer cell extravasation as the crucial metastatic step affected by CCL5/CCR5 inhibition (13). Together, these data support a role for CCR5 antagonists in blocking the ability of basal breast cancer cells to reach the metastatic sites instead of inhibiting their proliferation or survival after arrival to metastatic sites. Blocking the homing of cancer cells to metastatic sites is a desirable characteristic in a true antimetastatic drug (51). Therefore, CCR5 antagonists may be useful as adjuvant therapy for breast basal tumors with CCR5 overexpression or other tumor types where CCR5 promotes metastasis, such as prostate cancer (50) or gastric cancer (52). Our preclinical studies will expedite the path toward the clinical use of CCR5 antagonists as new treatments for dissemination of basal breast cancer.
ACKNOWLEDGEMENTS

This work was supported in part by the NIH grants R01CA070896, R01CA075503, R01CA132115, R01CA107382, R01CA086072 (R.G.P.), R01CA120876 (M.P.L), the Kimmel Cancer Center NIH Cancer Center Core grant P30CA056036 (R.G.P.), generous grants from the Dr. Ralph and Marian C. Falk Medical Research Trust and the Margaret Q. Landenberger Research Foundation, a grant from Pennsylvania Department of Health (R.G.P.), and by PASPA-UNAM (M.A.V-V.). The Department specifically disclaims responsibility for any analysis, interpretations or conclusions. There are no conflicts of interest associated with this manuscript. We thank Jeannine Moore and David Strahan for assistance in the preparation of this manuscript.
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FIGURE LEGENDS

Figure 1. CCL5 and CCR5 are overexpressed in the basal breast cancer subtype. (A) Heat map of the expression of CCL5 and its receptor CCR5 in samples from breast cancer patients divided by genetic subtype based in their gene expression pattern. The relative abundances of CCL5 and CCR5 are increased in patients with the basal and HER-2 subtypes. (B) Scatter plots and correlation analysis (Student’s t test) of the expression of CCL5 and CCR5 among the breast cancer molecular subtypes. (C) Quantification of the proportions of the samples overexpressing CCL5 and CCR5 (red fraction of the bar) displayed in B. The number of samples in each subtype is indicated at the top of the bar. (D) Metastasis-free Kaplan-Meier plots and log-rank analysis for the different genetic subtypes in the analyzed database (described in Materials and Methods). As reported elsewhere, patients with the basal or HER-2 subtypes display increased probability to form metastasis.

Figure 2. Human breast cancer cell lines that express CCR5 respond to CCL5. (A) Flow cytometry density plots of the CCR5 expression in MDA-MB-231 breast cancer cells identified a subpopulation of CCR5+ cells. (B) Induction of calcium signaling in cells loaded with Fluo-4-AM before the sequential addition of CCL5 (60 μg/ml) and FBS (5%). A fraction of cells responded to CCL5 (closed arrowheads in the central panel of micrographs) while the rest did not (open arrowheads). The average changes in fluorescence on 5 responsive (green line) and 5 non-responsive (red line) cells are represented in the far right graphs. Data shown are representative of 3-5 independent experiments for each cell line. Bar= 100 μm. (C) 3-D invasion into collagen gels by breast cancer cell lines, using CCL5 (15 μg/ml) as chemoattractant. (D) Mean distances of invasion ± SEM from 3 independent experiments. (E) 3-D invasion assays, and their corresponding quantification (F, mean ± SEM, n=3), for MCF-10A cells and MCF-10A-NeuT, -Ras, and -Src derivatives showing that CCL5-induced invasion is activated by oncogenic transformation. (G) CCR5+ cells display increased invasiveness. CCR5+ and CCR5− subpopulations from SUM-159 cell line were isolated by FACS and invasion into collagen gels was evaluated using FBS as chemoattractant. Quantification is shown in (H) as mean ± SEM of 2 independent experiments. Statistical analysis was conducted using the Student’s t test.
**Figure 3. CCR5 antagonists block CCL5-induced calcium signaling.** (A) Intensity vs. time analysis of Fluo-4 AM loaded MDA-MB-231 cells treated with the CCR5 antagonists Maraviroc or Vicriviroc (100 nM) for 30 min before the addition of CCL5 (60 µg/ml). Micrographs illustrate the axis (x-x’) of the pseudo-line scan plot. Those axes were employed to construct the adjacent intensity vs. time plots. (B) Comparison of the fraction of cells with increased fluorescence intensity upon addition of CCL5. (C) CCL5-induced calcium signaling was also blocked by CCR5 antagonists in Hs578T cells. The corresponding quantification is shown in (D). Data in (B) and (D) are mean ± SEM of 3-4 independent experiments. Statistical analysis was conducted using the Student's *t* test.

**Figure 4. CCR5 antagonists block FBS-induced breast cancer cell invasion.** 3-D reconstruction of FBS-induced invasion into collagen gels by Hs578T (A) or SUM-159 (C) breast cancer cells in presence of CCR5 antagonists (100 nM). The corresponding quantifications (mean ± SEM, n=3) and analysis (Bonferroni's *t* test) are displayed in (B) and (D).

**Figure 5. The CCR5 antagonist Maraviroc inhibits lung metastases in vivo.** (A) MDA-MB-231 cells transduced with Luc2-eGFP fusion protein were injected into the tail vein of NOD/SCID mice and the *in vivo* bioluminescent signal was quantified weekly. Representative *in vivo* images of vehicle- or Maraviroc-treated (8 mg/kg every 12 hours) mice are shown in (A). Quantification (mean ± SEM, n=6) of BLI in the control (red line) and treated groups (blue line) are portrayed in (B). Statistical comparison (* P=0.048) was performed using Student's *t* test with Welch’s correction for heterogeneous variances. (C) The presence of pulmonary tumors and the differences between treatments were corroborated by *ex vivo* imaging (left panels) and india ink staining (right panels). (D) The fraction of mice with metastatic tumors was significantly larger in the control group (P<0.0001, Fisher’s exact test). (E) Histological analysis (H&E staining, 100X) and the corresponding quantification (F) of the area covered by metastatic tumors in lung slides. Tumor area was quantified with the Nikon Elements BR 3.0 software analyzing at low magnification (40X) two random fields of two different histological sections (separated 600 µm from each other) per mouse. Statistical analysis was conducted using the Student's *t* test with Welch’s correction for heterogeneous variances (n=33 and 12 for control and treated groups, respectively).
**Figure 6. Maraviroc reduces lung colonization but does not modify cell proliferation.** (A) Effect of CCR5 antagonist on breast cancer cell viability. MDA-MB-231 cells were exposed to increasing concentrations of Maraviroc (inverted triangles) or Vicriviroc (squares) for 48h and the cell viability was evaluated by MTT assay. Graph is from a representative experiment performed by sextuplicate. No statistical differences were found (ANOVA) in three independent experiments. (B) CCR5 expression in MDA-MB-231 cells stably transfected with pcDNA3.1+/Zeo+ (MDA.Vector) or human CCR5 cloned into pCDNA3+/Zeo+ (MDA.CCR5). (C) Comparison of *in vitro* proliferation rates of MDA.Vector versus MDA.CCR5 showed no differences (ANOVA). Representative experiment form two performed by sextuplicate. (D) In order to evaluate the *in vivo* effect of Maraviroc on growth of established metastasis, treatment of mice was initiated ten days after injection of MDA.pFULG cells as illustrated. (E) Quantification (mean ± SEM, n=5) of *in vivo* BLI in the control (red) and treated groups (blue) showed no differences in the growth rate. (F) Schema of the experimental design used to evaluate CCR5 role in lung colonization. (G) Representative confocal images and quantification (H) of the number of eGFP⁺ cells in lungs 24 h after injection of MDA.pFULG cells. Cells expressing eGFP were counted in three random fields of two different histological sections (separated 700 µm from each other) per mouse (n=5 mice per group). Statistical analysis was conducted using Student's *t* test. Bar in micrographs= 100 µm.
Figure 3
Figure 4

Panel A: Images showing Hs578T cells treated with control, maraviroc 100 nM, and vicriviroc 100 nM.

Panel B: Bar graph showing the decrease in plaque area (P<0.01) with maraviroc compared to control and vicriviroc.

Panel C: Images showing SUM-159 cells treated with control, maraviroc 100 nM, and vicriviroc 100 nM.

Panel D: Bar graph showing the decrease in cell invasion (P<0.05) with maraviroc compared to control and vicriviroc.
Figure 6

A

B

C

D

E

F

G

H

[Diagram showing experimental setup and results]
CCR5 antagonist blocks metastasis of basal breast cancer cells

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Cancer Res Published OnlineFirst May 25, 2012.