

C-C Chemokine Receptor 5 on Stromal Cells Promotes Pulmonary Metastasis

Hendrik W. van Deventer,^{1,4} William O'Connor, Jr.,^{3,4} W. June Brickey,^{3,4} Robert M. Aris,² Jenny P.Y. Ting,^{3,4} and Jonathan S. Serody^{1,3,4}

Divisions of ¹Hematology/Oncology and ²Pulmonary and Critical Care Medicine, Department of Medicine; ³Department of Microbiology and Immunology; and ⁴Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Abstract

We have shown that mice that express the C-C chemokine receptor 5 (CCR5) have enhanced local tumor growth and an impaired response to vaccine therapy compared with CCR5 knockout (CCR5^{-/-}) mice. Here, we extend these observations to evaluate the function of CCR5 in pulmonary metastasis and the mechanism underlying the diminished tumor growth in CCR5^{-/-} mice. Lung metastases were counted in wild-type (WT) and CCR5^{-/-} mice following the injection of 1×10^6 B16-F10 melanoma cells. These results were compared with those from syngeneic bone marrow chimeric mice formed by the transfer of WT bone marrow into irradiated CCR5^{-/-} and CCR5^{-/-} marrow into irradiated WT mice. Intact CCR5^{-/-} mice developed fewer metastases than WT mice (40.2 versus 70.6; $P < 0.05$). Bone marrow chimeras formed by the transfer of WT bone marrow into CCR5^{-/-} hosts had fewer metastases than WT hosts injected with knockout marrow (46.6 versus 98.6; $P < 0.01$). Adoptive transfer of CCR5-expressing leukocytes also failed to promote metastasis in CCR5^{-/-} mice. However, the i.v. transfer of WT pulmonary stromal cells into CCR5^{-/-} mice increased the number of metastases compared with transfer of CCR5^{-/-} stromal cells (102.8 versus 26.0; $P < 0.05$). These results show for the first time that CCR5 expression on stromal and not hematopoietic cells contributes to tumor metastasis. Therefore, recently developed CCR5 inhibitors may have a novel benefit in cancer therapy. (Cancer Res 2005; 65(8): 3374-9)

Introduction

Chemokines form a family of structurally related proteins that were initially recognized as mediators of chemotaxis and cellular homing (1). This family is loosely divided into three groups: homeostatic or constitutive chemokines, inflammatory or inducible chemokines (2, 3), and those with dual functions. The ligands for C-C chemokine receptor 5 (CCR5) belong to the inflammatory group (4). As a result, surface expression of CCR5 facilitates the migration of a cell toward inflammatory stimuli. This mechanism exposes macrophages (5), immature dendritic cells, and Langerhans cells (4) to potentially new antigens found in such areas. At the same time, CCR5 advances the effector

response by promoting the chemotaxis of Th1 CD4 T cells (6), CD8 T cells (7), and other lymphoid cells (8, 9).

Despite the appealing simplicity of this paradigm, CCR5 likely has a broader array of biological functions. One clue to this breadth is the diversity of cells that express it. In addition to leukocytes, CCR5 can also be found on vascular cells and fibroblasts (4). The functional heterogeneity for CCR5 is consistent with the broader view of chemokine function that has emerged from investigators evaluating other chemokine receptors. In addition to chemotaxis, chemokine receptors have been implicated in immune modulation (10) and promotion of angiogenesis (11, 12). Because these processes are intimately involved with cancer progression, we began to focus on the role of CCR5 in cancer biology.

Several recent observations have begun to link CCR5 with the progression of cancer. Expression of CCL5, a ligand that binds CCR5, correlates with breast cancer stage (13) and is associated with enhanced melanoma formation in nude mice (14). Furthermore, treatment with a CCL5 antagonist decreases tumor growth in a breast cancer model (15). We have provided direct evidence by showing that CCR5 knockout (CCR5^{-/-}) mice have slower-growing local tumors and improved responses to cancer vaccines (16).

Two important questions remain. First, there is no explicit evidence connecting CCR5 and cancer metastasis. Murine studies have concentrated on local tumor models and human studies have been correlative. Second, the mechanism by which CCR5 promotes cancer progression is not known.

In response to these questions, we hypothesized that CCR5 would promote metastasis by recruiting monocyte/macrophage cells to the site of metastasis. This lineage was considered the most likely candidate because recruitment of these cells (17) has been associated with progression of a variety of cancers, including breast cancer (18), renal cell cancer (19), and cervical cancer (20). Furthermore, CCR5 expression on these cells has been associated with processes that promote metastasis, including angiogenesis (21) and extravasation (22).

We tested this hypothesis by evaluating the metastasis of the B16-F10 melanoma cells in CCR5^{-/-} and wild-type (WT) mice. These experiments established a direct role for CCR5 in the promotion of metastasis. Then, we assessed the role of CCR5 on monocyte/macrophage cells through a series of adoptive transfer and bone marrow chimer experiments. Contrary to our initial hypothesis, we have shown that CCR5 promotes metastasis by its expression on stromal cells.

The practical impetus behind this investigation is that blockade of CCR5 is now a pharmaceutical reality. Clinical trials in HIV patients have shown that such antagonists are safe and well tolerated (23). Our data provide a novel insight into the mechanism by which these compounds may benefit cancer patients and further justify their use in a clinical cancer trial.

Requests for reprints: Hendrik W. van Deventer, Division of Hematology/Oncology, University of North Carolina at Chapel Hill, Room 3009, Old Clinic Building, Chapel Hill, NC 27599-7305. Phone: 919-966-3835; Fax: 919-966-6735; E-mail: hvand@med.unc.edu.

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Materials and Methods

Mice. C57BL/6 WT and enhanced green fluorescent protein (EGFP) transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CCR5^{-/-} mice have been described previously (24, 25). EGFP transgenic CCR5^{-/-} mice were generated by crossing CCR5^{-/-} mice with EGFP transgenic mice as described previously (26). All animals were housed at the University of North Carolina at Chapel Hill in specific pathogen-free conditions. All experiments were done using protocols approved by Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Metastatic tumor model. B16-F10 melanoma cells were purchased from the American Type Culture Collection (Rockland, MD). The cells were cultured at 37°C and 5% CO₂ in complete DMEM supplemented with 7.5% (v/v) FCS (Life Technologies, Grand Island, NY) and 100 µg/mL streptomycin sulfate (Life Technologies). The original stock was expanded and multiple aliquots were frozen down. For each experiment, an aliquot was thawed, expanded by two passes, and harvested in log-phase growth. One million cells were injected by the tail vein in 200 µL PBS.

The mice were euthanized at either 14 or 28 days after tumor injection. At that time, the lungs were insufflated with Fekete's destaining solution (27) and removed en bloc. Lung metastases were counted in the left upper lobe by an investigator who was blinded to the genotype. Mice that showed signs of distress before the planned end point were euthanized. The decision to euthanize was done by an investigator who was also blinded to the genotype.

Fluorescence-activated cell sorting analysis. Single-cell suspensions were formed by mincing the lung tissue and digesting it with 1 mg/mL collagenase A and 0.02 mg/mL DNase I (Boehringer Mannheim, Mannheim, Germany) for 25 minutes at 37°C in 2% RPMI. The suspension was passed through a 70 µm filter and red cells were depleted by ACK lysis. Particulate matter was removed by aspirating the supernatant after the suspension was allowed to stand for 3 minutes.

Cell surface phenotype was determined by first blocking nonspecific binding with CD16/CD32 (PharMingen, San Diego, CA) and 3% FCS and then incubating with the following antibodies: CD4 (L3T4), CD8α (Ly-2), Pan NK (DX5), CD11c (HL3), B220 (RA3-6B2), γδ TCR (GL3), NKT (U5A2-13), and CCR5 (C34-3448; all PharMingen) or F4/80 (C1: A3-1; Cedarlane, Ontario, Canada). Data were collected on FACSCalibur (Becton Dickinson, Franklin Lakes, NJ) and analyzed using WinMDI version 2.8 software (Scripps Research Institute, La Jolla, CA). These data are reported as the average of six mice from two separate experiments.

Adoptive transfer. Adoptive transfer experiments were done by isolating cells from WT mice and injecting them into CCR5^{-/-} mice 24 hours before injection of B16-F10 melanoma cells. The following doses of cells were injected: 1 × 10⁶ immature dendritic cells, 5 × 10⁶ B-cell enriched splenocytes, and 5 × 10⁵ alveolar macrophages.

Immature dendritic cells were prepared from bone marrow precursors using the method described by Lutz et al. (28). In brief, 2 × 10⁶ bone marrow cells were cultured on 100 mm bacterial culture plates (Fisher Scientific, Pittsburgh, PA) in 10 mL RPMI 1640 supplemented with 10% FCS and 20 ng/mL murine granulocyte macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ). Another 10 mL RPMI with 10 ng/mL GM-CSF was added on day 3. Loosely adherent immature dendritic cells were harvested on day 6. These cells expressed low levels of surface MHC class II and CD80/CD86 at harvest and high levels with the addition of lipopolysaccharide or tumor necrosis factor-α (data not shown).

Splenocytes were enriched for B cells using adherence and complement depletion. Single-cell suspensions from whole spleens were plated in RPMI with 10% FCS following erythrocyte depletion. Two hours later, the nonadherent cells were harvested and resuspended in cytotoxicity medium (Cedarlane). To this suspension, the following hybridoma supernatants were added: anti-CD4 (GK1.5), anti-CD8 (2.43), and anti-Gr-1 (RB6-C5/1; American Type Culture Collection). Following complement-mediated lysis, the cells were washed and resuspended in PBS.

Alveolar macrophages were harvested from WT mice using bronchial alveolar lavage. Following anesthesia with Avertin, the mice were perfused with PBS via the right ventricle. A 22 gauge i.v. catheter was introduced into the trachea, and PBS (800 µL) was insufflated and withdrawn five times.

Pulmonary stromal cells were isolated by plating a cell suspension derived from either WT or CCR5^{-/-} lung in 10% DMEM (29). The medium was replaced after 1 hour and on days 3 and 10. The cells were split 5:1 on day 7 and harvested on day 14.

Bone marrow chimeras. Recipient mice were irradiated with 950 rads from a ¹³⁷Cs source at a dose rate of 86.3 cGy/min the evening before transplantation. Donor cells were prepared by depleting red cells from whole bone marrow of EGFP transgenic mice. Three million donor cells were injected via the tail vein into recipient mice. Recipient mice were injected with B16-F10 melanoma cells 5 weeks after transplant.

To assess engraftment at the time of tumor injection, fluorescence-activated cell sorting analysis was done on the lungs of a subset of mice 5 weeks after transplant. Single-cell suspensions were incubated with phycoerythrin-labeled antibodies for CD3, CD4, CD8, CD11c, and CD19 (1D3; PharMingen) and F4/80 (Cedarlane). Engraftment was measured as the percentage of EGFP⁺ cells within each PE⁺ subpopulation.

Engraftment was also verified at the time of lung harvest. This was done by collecting 1.5 mL blood by cardiac puncture, depleting the red cells, and measuring the percentage of EGFP-expressing cells by flow cytometry for each recipient.

Angiogenesis assay. B16-F10 melanoma cells were resuspended at 1.5 × 10⁶ cells/mL in a 1.5% sodium alginate/PBS solution (Chem Service, West Chester, PA) as described previously (30). This solution was added dropwise to a sterile 250 mmol/L solution of calcium chloride to form 30 to 35 µL beads. Six beads were s.c. implanted on the dorsal side of CCR5^{-/-} and WT control mice. Twelve days later, 200 µL of a 100 mg/kg FITC-dextran solution (MP Biomedicals, Irvine, CA) were i.v. injected. Twenty minutes after injection, the beads were harvested, weighed, and added to 1 mL of 1 mmol/L Tris-HCl. After an overnight incubation, the beads were ground between two microscope slides and vortexed. The amount of fluorescence in the resulting supernatant was measured using the FluStar fluorometer. Results were expressed as the concentration of FITC normalized to the weight of the beads.

Reverse transcription-PCR. Total RNA was isolated from cultured stromal cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers. PCR was done using Taq DNA polymerase (Promega, Madison, WI) and a PCR 9700 System GeneAmp thermocycler (Applied Biosystems, Foster City, CA). Touchdown PCR conditions were used starting with an annealing temperature of 61°C for 10 cycles. The annealing temperature was decreased by increments of 0.5°C for subsequent groups of 10 cycles. The final 25 cycles was completed at 56°C. The primer used was CCR5: 5'-GACTCTGGCTCTTGCAAGAT-3' (sense) and 5'-GCCGCAA-TTTGTTTCACAT-3' (antisense). The PCR products were visualized using a 1.5% agarose gel.

Statistics. Unless otherwise stated, data are presented as the mean of measurements taken from at least two separate experiments. Statistical error for these means is presented as ±1 SE. Significance was determined by *P* > 0.05 using a homoscedastic *t* test assuming equal variance.

Results

CCR5^{-/-} mice form fewer metastases than WT mice in a dose-dependent fashion. We have shown previously that the local growth of B16-F10 melanoma cells is inhibited in CCR5^{-/-} mice. For this study, we evaluated the effect of CCR5 on the metastatic potential of melanoma by injecting CCR5^{-/-} and WT mice i.v. with 1 × 10⁶ B16-F10 cells. Fourteen days later, the WT mice developed a mean of 70.6 ± 9.3 metastatic colonies compared with 40.2 ± 6.88 colonies in the CCR5^{-/-} mice (*P* = 0.018; Fig. 1A). Tumor dose

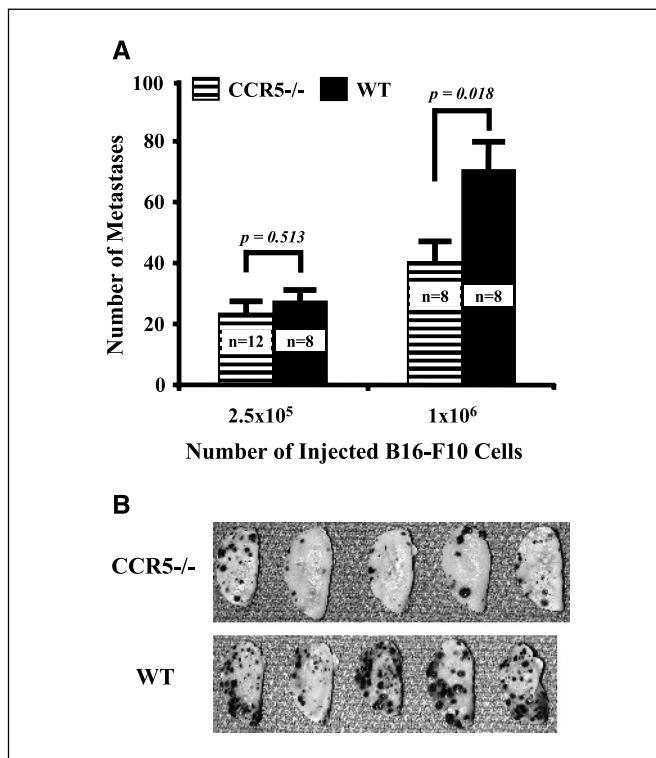


Figure 1. CCR5 promotes metastasis of B16-F10 melanoma cells in a dose-dependent manner. *A*, 2.5×10^5 (right) or 1×10^6 (left) B16-F10 melanoma cells were injected into CCR5^{-/-} (hatched columns) or WT (solid columns) mice. The lungs were harvested 14 days after tumor injection and insufflated with Fekete's solution and the number of metastatic colonies was counted. CCR5^{-/-} mice developed fewer metastases at both doses, but this was only significant for an injection with 1×10^6 cells. *B*, representative lungs from CCR5^{-/-} and WT mice 14 days after injection with 1×10^6 B16-F10 melanoma cells.

dependence was suggested by the injection of 2.5×10^5 melanoma cells, which produced a nonsignificant decrease in metastatic colonies in CCR5^{-/-} compared with WT mice. Although metastatic colonies in CCR5^{-/-} mice were reduced in number, they did not seem grossly different in size or distribution when visually compared with colonies from WT mice (Fig. 1*B*).

To assess the impact of CCR5 after day 14, we compared the survival of CCR5^{-/-} mice with WT mice after injection with 1×10^6 melanoma cells. Again, there was a highly significant difference favoring the CCR5^{-/-} compared with WT (24.7 ± 0.58 versus 21.9 ± 0.62 days; $P = 0.007$). These observations support our assertion that CCR5 promotes tumor metastasis.

CCR5 on hematopoietic cells does not promote metastasis.

Our next objective was to identify the cells responsible for promoting metastasis via CCR5. CCR5 is principally expressed on hematopoietic cells; however, there are a variety of CCR5⁺ nonhematopoietic cells (31–33). To isolate the contribution of CCR5 on hematopoietic cells from CCR5 on nonhematopoietic cells, we created bone marrow chimeric mice in which the leukocytes were CCR5⁺ and the stroma was CCR5 deficient. This was done by lethally irradiating CCR5^{-/-} mice and injecting them with EGFP WT bone marrow. Five weeks after irradiation, fluorescence-activated cell sorting analysis confirmed that $\geq 70\%$ of each leukocyte population within the lung were of donor origin. Consequently, we injected B16-F10 cells 5 weeks after irradiation and counted the number of metastases 14 days later.

As shown in Fig. 2, chimeras formed from WT bone marrow and irradiated CCR5^{-/-} mice developed significantly fewer metastatic colonies than those formed from CCR5^{-/-} bone marrow and irradiated WT mice (46.6 ± 8.4 versus 98.6 ± 9.4 ; $P = 0.0021$). If CCR5 on bone marrow cells were responsible for the enhanced metastases seen in Fig. 1, the opposite would be expected. In fact, chimeras using WT hosts developed tumor metastases in equivalent numbers whether they were injected with WT or CCR5^{-/-} bone marrow (103.7 ± 12 versus 98.6 ± 9.4 ; $P = 0.77$).

The inability of CCR5⁺ hematopoietic cells to promote tumor metastasis was further confirmed by a series of adoptive transfer experiments. For these tests, WT B cells, immature dendritic cells, or alveolar macrophages were injected into CCR5^{-/-} mice 24 hours before injection with 1×10^6 B16-F10 cells. Two weeks later, the number of metastases in the injected mice remained fewer than the number of metastases in CCR5^{-/-} mice injected with CCR5^{-/-} cells (Table 1). Thus, these data indicate that the presence of CCR5 on nonhematopoietic cells enhanced tumor metastasis.

CCR5 on pulmonary stromal cells promotes pulmonary metastasis. Given the general inability of CCR5⁺ hematopoietic cells to promote metastasis, we evaluated the role of non-hematopoietic cells on this process. Our first hypothesis was that CCR5 increased angiogenesis by recruiting CCR5⁺ endothelial cells

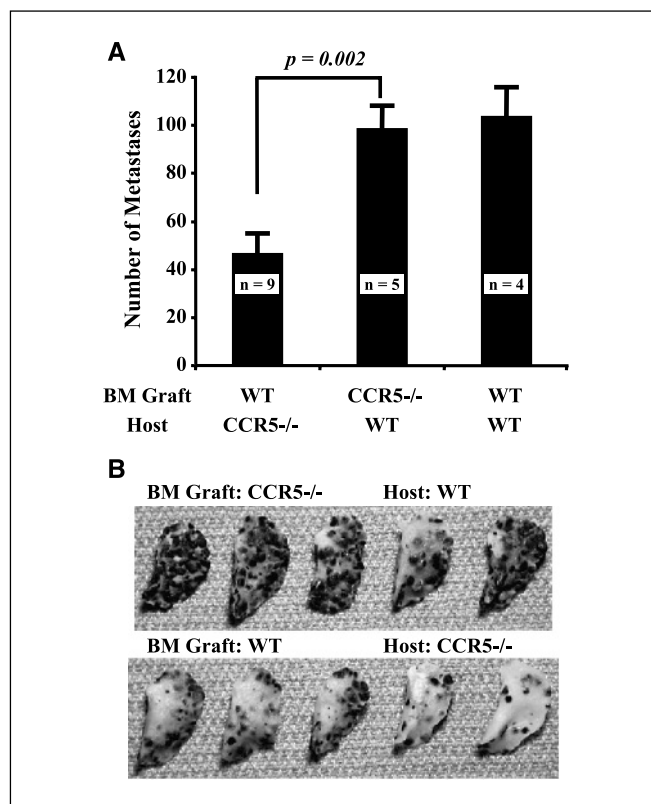


Figure 2. CCR5 on non-bone marrow (BM)-derived cells promotes tumor metastasis. *A*, bone marrow chimeras were formed from WT and CCR5^{-/-} hosts injected with CCR5^{-/-} or WT bone marrow. At 5 weeks, 1×10^6 B16-F10 cells were injected and the number of metastases was counted. CCR5^{-/-} hosts with WT bone marrow (left) had significantly fewer metastases than WT hosts with CCR5^{-/-} marrow (center). WT hosts had the same number of metastasis whether injected with CCR5^{-/-} bone marrow or WT marrow (right). *B*, representative lungs from WT hosts with CCR5^{-/-} bone marrow (top) and CCR5^{-/-} hosts with WT bone marrow (bottom).

Table 1. Adoptive transfer of WT leukocytes does not increase pulmonary metastasis in CCR5^{-/-} mice

Marker	% of Positive cells*	% of Dual positive cells (CCR5 ⁺)*	Dose of adoptively transferred cells (10 ⁵)	No. metastases in CCR5 ^{-/-} mice following the transfer of	
				WT cells	CCR5 ^{-/-} cells
B220	23.9	6.8	50 [†]	10.2 ± 1.76	26.7 ± 1.54
CD11c [‡]	11.5	2.3	10 [§]	17.3 ± 5.93	30.8 ± 6.35
F4/80	4.7	1.2	5	28.3 ± 2.85	
NK1.1	7.3	0.7	—	—	
CD4	6.5	0.6	—	—	
CD8	8.4	0.5	—	—	
Pan NK	9.6	0.5	—	—	
γ chain	1.9	0.2	—	—	

*Whole lung cell isolates 3 days after injection with 10⁶ B16-F10 melanoma cells.

[†]Isolated from splenocytes.

[‡]CD11c⁺ were defined as those cells in the second decade that are now recognized as pulmonary dendritic cells (52, 53).

[§]Bone marrow derived and expanded in GM-CSF.

^{||}Isolated by bronchoalveolar lavage.

(34, 35). We tested this hypothesis by comparing the ability of alginate encapsulated B16-F10 cells to recruit blood vessels in CCR5^{-/-} and WT mice (30). If CCR5 contributed to angiogenesis, then alginate beads implanted in CCR5^{-/-} mice should have reduced capacity for FITC-dextran uptake. In fact, CCR5^{-/-} mice did not exhibit a lower degree of neovascularization when compared with WT mice (13.7 ± 1.5 versus 11.2 ± 2.8 μg/mL/mL; *P* = 0.43; Fig. 3).

Stromal cells can also promote metastasis by means unrelated to angiogenesis. To examine the role of CCR5 on stromal cells, cell suspensions from CCR5^{-/-} and WT mouse lungs were cultured for 2 weeks in 10% DMEM. The resultant cells were adherent and morphologically identical to fibroblasts (Fig. 4A). Cells cultured from CCR5^{-/-} mice did not seem to be substantially different than cells derived from WT mice. The yields of cells from both strains of mice were equivalent. However,

CCR5 transcripts could be detected in the WT stromal cells but not from the knockout stromal cells by reverse transcription-PCR (Fig. 4B).

The ability of CCR5⁺ stromal cells to promote metastasis was tested by injecting 1 × 10⁶ WT stromal cells into CCR5^{-/-} mice before the injection of 1 × 10⁶ B16-F10 melanoma cells. CCR5^{-/-} mice injected with WT stromal cells yielded 102.8 ± 8.6 metastases compared with 26.0 ± 1.3 metastases when injected with CCR5^{-/-} stromal cells (*P* = 0.023; Fig. 4). There were no significant differences among any of the mice with either resident WT stromal cells (84.6 ± 10) or injected WT stromal cells (102.8 ± 8.6) or both (111.2 ± 13.8). These results are a sharp contrast to the results of the adoptive transfer experiments shown in Table 1 in which injection with WT leukocytes caused no increase in the number of metastasis. Therefore, the presence of CCR5 on stromal cells supported the growth of B16-F10 tumor cells in the lung.

Discussion

We have now shown a role for CCR5 in the progression of B16-F10 melanoma in three model systems. Previously, we have shown CCR5 increases tumor growth in a local model and inhibits the efficacy of a dendritic cell vaccine (16). This study shows that CCR5 also promotes metastasis by demonstrating more metastases in WT mice compared with CCR5^{-/-} mice.

Initially, we attributed this increase in metastasis to the recruitment of CCR5⁺ leukocytes to the tumor site. This hypothesis was tested by transferring WT bone marrow into irradiated CCR5^{-/-} mice. Had the premise been correct, we would have observed an increase in the number of metastases in the transplanted CCR5^{-/-} mice. Instead, these mice continued to have fewer metastases at levels comparable with nonmanipulated CCR5^{-/-} mice. On the other hand, WT mice continued to have increased numbers of metastases whether they received WT or knockout bone marrow. These results show that CCR5 promotes metastasis by its expression on a nonhematopoietic cell.

Several important hypotheses are refuted by the inability of CCR5⁺ leukocytes to promote metastasis in CCR5^{-/-} mice. First,

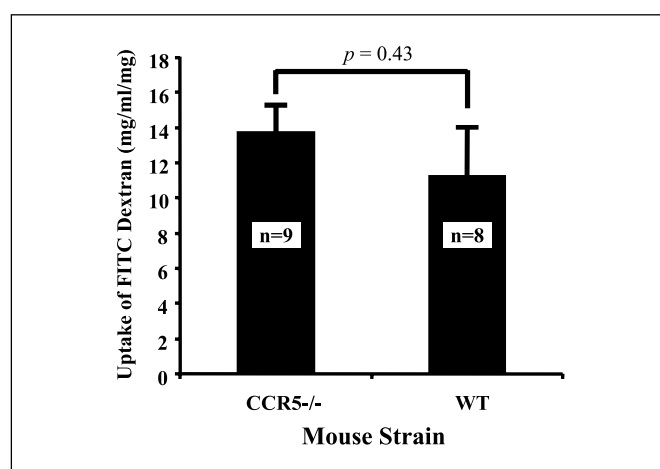


Figure 3. CCR5 does not promote tumor angiogenesis. B16-F10 tumor cells were suspended in alginate beads and placed s.c. in CCR5^{-/-} and WT mice. Twelve days later, 200 μL of 100 mg/kg FITC-dextran solution were i.v. injected. The amount of fluorescence was measured after a 24-hour incubation in 1 mmol/L Tris-HCl. Normalized to the weight of the beads. No significant difference in tumor angiogenesis can be seen.

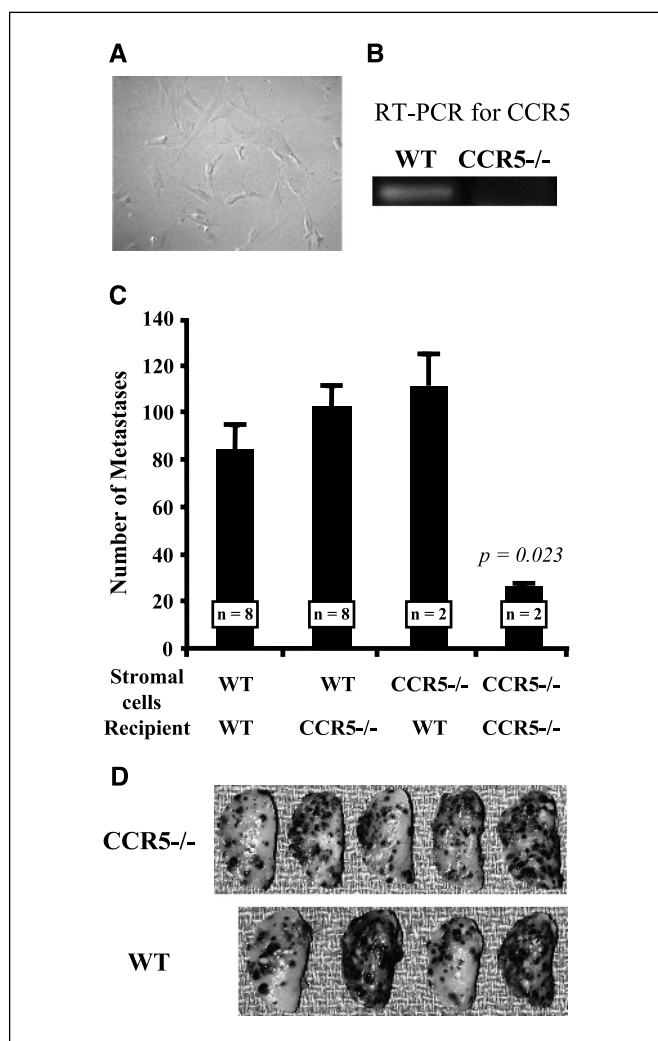


Figure 4. CCR5⁺ pulmonary stromal cells promote metastasis of B16-F10 melanoma cells. **A**, phase-contrast microscopy of pulmonary stromal cells following 2 weeks of culture. Single-cell suspensions of lungs were cultured in DMEM with 10% FCS with a 5:1 expansion after 1 week. Magnification, $\times 200$. **B**, expression of CCR5 in WT pulmonary stromal cells by reverse transcription-PCR. **C**, i.v. injection of 1×10^6 WT and CCR5^{-/-} stromal cells 24 hours before injection of 1×10^6 B16-F10 melanoma cells. The lungs were harvested 2 weeks later. There is no significant difference in the number of metastatic colonies between WT mice injected with either WT or CCR5^{-/-} stromal cells and CCR5^{-/-} mice injected with WT stromal cells. CCR5^{-/-} mice injected with CCR5^{-/-} stromal cells form significantly fewer metastases. **D**, representative lungs from CCR5^{-/-} and WT mice injected with 1×10^6 WT stromal cells followed by 1×10^6 B16-F10 cells.

CCR5 does not increase metastasis via cells of the monocyte/macrophage lineage. These cells have been associated with cancer progression (17) and processes that promote metastasis, including angiogenesis (21) and extravasation (22). They also express CCR5. However, our adoptive transfer and bone marrow chimer experiments do not support the contention that CCR5^{-/-} mice have fewer metastases because these macrophages fail to migrate.

Second, CCR5^{-/-} mice apparently do not have fewer metastases due to an impediment of T regulatory cells. These cells have also been connected with cancer progression (36) and express CCR5 (data not shown). If such a regulatory population depended on CCR5, it would emerge in CCR5^{-/-} mice transplanted with WT

bone marrow and cause an increase in the number of metastases. Again, no such increase was seen. In fact, T regulatory cells may have only a modest effect on metastatic disease in this model because depletion of these cells yields little improvement in pulmonary metastases (37).

These experiments also imply that CCR5 does not promote metastasis by mediating apoptosis in CD8 cells as described by Mellado et al. (38). According to this hypothesis, CCR5^{-/-} CD8 cells transferred into WT mice would be less susceptible to apoptosis and more effective in controlling the growth of B16-F10 cells. However, we found no advantage in WT mice transplanted with CCR5^{-/-} marrow. This discrepancy may be due to the use of a local model by Mellado et al. rather than a metastatic one. Indeed, our article supports the important premise that the mechanism that inhibits local tumors in CCR5^{-/-} mice differs from the one that inhibits metastatic disease. Specifically, increasing the number of locally injected tumor cells overcomes the growth inhibition in CCR5^{-/-} mice (16), whereas a larger dose of i.v. injected tumor cells amplifies the benefit of CCR5 inhibition in metastatic disease.

Our data do suggest that CCR5 promotes metastasis by its expression on stromal cells. Stromal cells, including fibroblasts, can contribute to metastases by a variety of mechanisms, such as providing growth and survival signals, facilitating extravasation and migration, enhancing immune evasion, and promoting angiogenesis (39, 40). Of these processes, angiogenesis seems the least likely mechanism for CCR5 given our results. On the other hand, stromal cells can augment these other processes by the production of transforming growth factor- β (TGF- β ; refs. 41, 42). Furthermore, inhibition of TGF- β receptor II on hematopoietic cells leads to a decrease in B16-F10 melanoma metastases (43). TGF- β is released from macrophages in response to macrophage inflammatory protein-1 α (44); whether it is released from stromal cells via stimulation of CCR5 is not known.

CCR5-expressing stromal cells may be promoting metastases by a mechanism involving matrix metalloproteinases (MMP). CCR5^{-/-} mice have lower levels of MMP-3, MMP-12, and MMP-13 (45) and stimulation with eotaxin-1 leads to increases in MMP-3 and MMP-13. Furthermore, overexpression of tissue inhibitor of metalloproteinase-1 led to a decrease in the number of metastases using the B16-F10 melanoma model (46). Interestingly, the survival benefit was modest and comparable with the benefit seen in the CCR5^{-/-} mice. Survival could be substantially increased in the tissue inhibitor of metalloproteinase-1 model with the addition of interleukin-2 (47).

Finally, CCR5 could promote metastasis by increasing tissue factor. Mueller et al. initially showed that expression of tissue factor promoted the metastatic potential of melanoma cells (48). Inhibition of the tissue factor pathway leads to a reduction but not total inhibition of B16-F10 metastasis (49). This reduction is likely due to an impairment in tumor cell migration and invasion (50) and not to a decline in angiogenesis (51). Tissue factor is abundant in fibroblasts and the addition of the CCR5 agonist macrophage inflammatory protein-1 β leads to release of tissue factor from human smooth muscle cells (32). The possible connection between tissue factor and CCR5 is currently under investigation.

Our work has significant clinical implications. Because CCR5 is a G protein-coupled receptor, it is amenable to small molecule inhibition (23). Several such compounds have already been shown to be safe in clinical trials for HIV. Our preclinical work suggests

that such compounds could be useful in many cancer settings, including local and metastatic disease, and as a vaccine adjuvant. Furthermore, CCR5 inhibition for metastatic disease seems to target stromal tissue. This suggests that there may be a lower risk of developing drug resistance. Thus, we believe CCR5 is a novel cancer target that warrants continued investigation.

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