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CCR5 Antagonism by Maraviroc Reduces the Potential for Gastric Cancer Cell Dissemination

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Abstract

The chemokine (C-C motif) receptor 5 (CCR5) that belongs to the family of G protein–coupled receptors is exploited by macrophage tropic (R5) human immunodeficiency virus type 1 (HIV-1) to enter cells. Maraviroc, a small molecule CCR antagonist, is used as a part of combination antiretroviral therapy to treat persons infected by R5 HIV-1. CCR5 is expressed in various cancers, and its level of expression is a negative predictor of patients' survival in gastric cancers. Here, we report MKN45, MKN74, and KATOIII cells, three human gastric cancer cell lines with different stages of differentiation, which express CCR5 as detected by flow cytometry and reverse transcription–polymerase chain reaction (RT-PCR), and its ligand RANTES. *In vitro* experiments demonstrate that CCR5 antagonism reduces gastric cancer cell migration induced by macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and RANTES and adhesion to the explanted murine peritoneum. Administration of maraviroc from days 3 to 10 after MKN45 cell inoculation to severe combined immunodeficient (SCID) mice effectively reduced the extent of peritoneal disease and increased survival. Maraviroc treatment also reduced the tumor burden in a xenograft model. Gene expression and RT-PCR analyses revealed that CCR5 antagonism *in vivo* modulates the expression of genes known for their role in cancer growth including interleukin-10 receptor B; hepatocyte growth factor receptor (MET); the homolog of the atypical cadherin gene, FAT1; Nm23-H1; and lymphotoxin β receptor. In summary, we have shown that CCR5 is mechanistically involved in dissemination of gastric cancer cells, suggesting that small molecule inhibitors of CCR5 might be exploited for their anticancer potential.

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Introduction

Chemokines and their receptors are essential players in the immune defense by directing and controlling the migration, activation, differentiation, and survival of leukocytes. Chemokines exert their function by binding and activating seven-transmembrane receptors belonging to the superfamily of G protein-coupled receptors (GPCRs) [1]. Inappropriate or prolonged expression of chemokines and/or chemokine receptors results in an excessive infiltration of leukocytes into inflamed tissues, resulting in chronic inflammation, autoimmune diseases, tumor growth, survival, and metastasis [1].

Gastric cancer is a leading cause of death for malignancies worldwide. Despite diagnostic and therapeutic advances, the prognosis of patients with advanced gastric carcinoma is poor and chemotherapy holds risk for major toxicity [2]. Thus, understanding the molecular mechanisms underlying gastric cancer growth and dissemination might help in finding new specific and less toxic therapeutic approaches.

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Previous studies support a role for the chemokine (C-C motif) receptor 5 (CCR5) and one of its ligand CCL5 (RANTES) in gastric cancer growth and dissemination [2]. This contention arises from the observation that tumor-infiltrating cells express CCL5, whereas tumor cells express the CCL5 receptors and serum CCL5 levels correlate with tumor progression and prognosis in patients with gastric and colorectal cancers [2,3]. Further, stimulation with CCL5 promotes proliferation of cancer cell lines [4].

In addition to its role in orchestrating leukocyte trafficking, CCR5 is the major co-receptor molecule used by human immunodeficiency virus type 1 (HIV-1) to enter cells. The CCR5Δ32 allele, which produces a prematurely truncated form of CCR5, is associated with profound resistance to HIV-1 infection in homozygotes and a better disease outcome in heterozygotes [5]. These observations have driven the development of anti-HIV drugs that disrupt the virus-CCR5 interaction, including the first-in-class–approved drug maraviroc [5]. Maraviroc is a noncompetitive, slowly reversible small-molecule antagonist of CCR5 that prevents signaling by all three ligands of CCR5 [i.e., CCL5/RANTES, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β] and is used as a part of combination antiretroviral therapy for patients infected with CCR5-tropic HIV (i.e., a subtype that uses only the CCR5 co-receptor to enter cells) [6–8].

Because maraviroc is clinically available [6] but its potential relevance to gastric cancer therapy is unknown, we have designed a study to investigate the effect of this agent in rodent models of gastric cancer dissemination. Using maraviroc as a tool, we demonstrate that the CCR5 pathway plays a mechanistic role on metastatic dissemination of three gastric cancer cell lines and identified a novel therapeutic target for this agent [7,8]. This study suggests that small molecule inhibitors of CCR5 can be exploited for their anticancer potential.

Materials and Methods

Cell Lines

Three human gastric cancer cell lines THP1, KATOIII, and MCF7 were from American Type Culture Collection (ATCC, Promochem, Milan, Italy), whereas MKN74 and MKN45 were from the Japanese Collection of Research Bioresources (Human Science Research Resources Bank, Osaka, Japan) [9–11]. Gastric cell lines were maintained in RPMI medium with 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were regularly passaged to maintain exponential growth.

Animal Studies

Eight-week-old male nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice (supplied by the animal center of the University of Perugia, Perugia, Italy) were housed under pathogen-free conditions. The care and use of the animals were approved by the Institutional Animal Care and Use Committee of the University of Perugia and were in accordance to European guidelines for care of experimental animals. Protocols were approved by the Istituto Superiore di Sanità (Rome, Italy), and the approval was granted to Prof Annibale Donini (No. 208/2009/b). To generate models of experimental peritoneal carcinomatosis and xenograft model, gastric cancer human cell lines were injected intraperitoneally (i.p.) or subcutaneously, respectively, in NOD-SCID mice. To examine maraviroc anticancer potential in peritoneal carcinomatosis, a single cell suspension of 1×10^7 MKN45 cells in a total volume of 0.2 ml of medium without serum was injected into the peritoneal cavity (i.p.) of each mouse using a 23-gauge needle, and animals were randomized in control group 1 (n = 12) and group 2 (n = 12) treated with maraviroc (10 mg/kg, i.p.) for 7 days starting from day 3 after cell inoculation. The extent of peritoneal carcinogenesis was evaluated on day 10 by necroscopy; both mesenteric and peritoneal nodules were counted. In another set of experiments, the two groups of animals mentioned above were observed until death, and overall survival was evaluated. To evaluate the role of anti-CCR5 molecule in tumor growth, NOD-SCID mice were inoculated subcutaneously with MKN74 or MKN45 (1×10^7 per mouse) cells. Mice were randomized into the following two groups (n = 8-10): control group and the other treated with maraviroc *per os* at a dose of 50 mg/kg twice a day. At sacrifice, all tissues were immediately snap frozen in liquid nitrogen and stored at −80°C until used or fixed in formalin.

CCR5 Expression

To assess CCR5 expression in MKN45, MKN74, and KATOIII cell lines, cells were cultured at 37°C, in an atmosphere of 5% CO_2, in RPMI medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin in a six-well plate at 5×10^5 cells per well. To evaluate CCR5 surface expression, cells were trypsinized, transferred to a collection tube, and suspended in medium with 10% FBS. After centrifugation at 1300 rpm for 10 minutes, cells were suspended in 500 μl of phosphate-buffered saline (PBS), stained with 5 μl of CCR5 antibody (Novus Biologicals, Cambridge, United Kingdom), and incubated on ice for 30 minutes. Samples were washed with PBS, centrifuged at 1300 rpm for 10 minutes, and stained with a fluorescein isothiocyanate–conjugated goat anti-rabbit secondary antibody (20 minutes at +4°C). Cells were washed twice with PBS and finally suspended in 500 μl of PBS plus 1% formaldehyde; CCR5-positive cells were assessed by flow cytometry. To evaluate CCR5 intracellular expression, MKN45, MKN74, and KATOIII cells [10] were resuspended in 250 μl of Cytofix/Cytoperm solution (BD Biosciences), incubated at +4°C for 25 minutes, and washed twice with 1× Perm/Wash solution (BD Biosciences, Milan, Italy). After centrifugation at 1300 rpm for 10 minutes, 5 μl of CCR antibody (Novus Biologicals) were added to each tube and cells were incubated on ice for 30 minutes. Samples were washed with 1× Perm/Wash solution, centrifuged at 1300 rpm for 10 minutes, and stained with a fluorescein isothiocyanate–conjugated goat anti-rabbit secondary antibody (20 minutes at +4°C). Cells were washed two times with 1× Perm/Wash solution and finally suspended in 500 μl of PBS plus 0.04% formaldehyde and analyzed by flow cytometry. Experiments were conducted in triplicate. MKN45 and MKN74 (3×10^7 per mouse) and KATOIII (1×10^7 per mouse) cells were inoculated in the peritoneum of C57BL/6 mice (three animals per group); after 4 hours, mice were killed and peritoneal washings were collected to evaluate CCR5 surface expression on gastric cancer cell lines. Retrieved cells were stained as described above, and CCR5 expression was evaluated by flow cytometry using forward scatter (FSC) and side scatter (SSC) discrimination gate to avoid mouse immune cell contamination.

Proliferation Assay

MKN45, MKN74, and KATOIII cells (1×10^6) were plated onto 10-cm culture dish in RPMI medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. On day 2, cells were left untreated or stimulated with 5 μM maraviroc for 72 hours. After treatment, cells were trypsinized and resuspended in complete medium; proliferation was assessed by trypan blue viability measurements. The

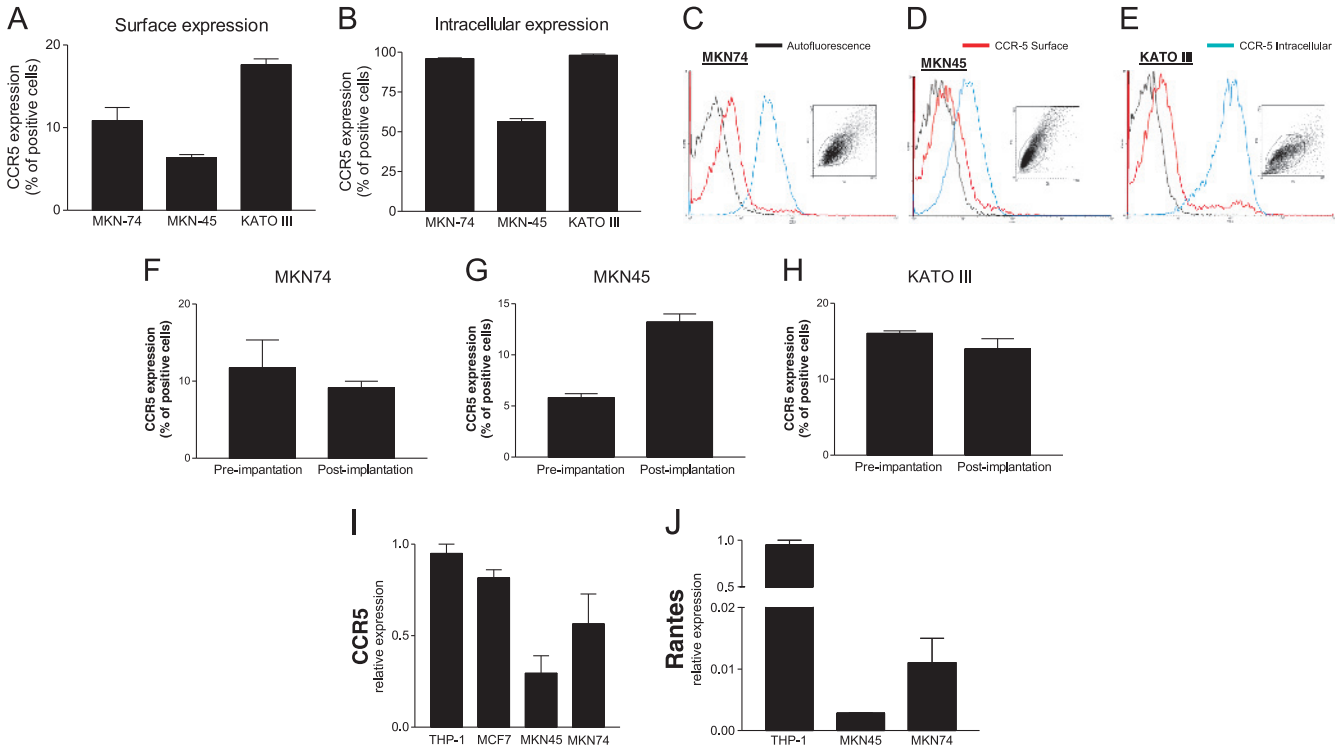

Figure 1. CCR5 is expressed by gastric cancer cell lines. (A and B) MKN45, MKN74, and KATOIII cells were evaluated by cytofluorometry analysis. Data are means ± SE of six experiments. (C–E) Representative flow cytometry histograms of surface and intracellular CCR5 expression in MKN45, MKN74, and KATOIII cells. (F–H) CCR5 on MKN45, MKN74, and KATOIII implanted i.p. in mice. Data are means ± SE of three experiments. (I and J) RT-PCR analysis confirmed the expression of CCR5 and RANTES on MKN35 and MKN74. Data are means ± SE of four experiments.

values were expressed as percentage of proliferation inhibition compared to nontreated cells. Experiments were conducted in triplicate.

Cell Adhesion to Peritoneum

Gastric cancer cell lines were plated onto complete RPMI medium; on day 2, cells were starved and left untreated or pretreated with 5 μM maraviroc for 8 hours. Cells were subsequently triggered with chemokines MIP-1α (20 ng/ml), MIP-1β (20 ng/ml), and RANTES (10 ng/ml) for 36 hours [9]. On day 4, excised parietal peritoneum (~1.6 cm^2) was placed in a 24-well culture plate, which had been filled with 1.0 ml of 1% BSA/RPMI 1640. Gastric cancer cells were detached, fluorescently labeled with BCECF-AM (3 μM) at 37°C for 30 minutes, and washed twice with 1% BSA/RPMI 1640. After trypan blue staining, a suspension of living cells (5 × 10^5 cells/ml in 1% BSA/RPMI 1640; 0.5 ml) was overlaid on the peritoneum in a 24-well plate, and the plate was incubated at 37°C for 40 minutes. After gentle washing with PBS, the cells adherent to the peritoneum were lysed with 1.0 ml of 1% NP-40 and the fluorescence intensity was measured with a fluorescence spectrophotometer (Ex = 490 nm and Em = 520 nm). Experiments were conducted in triplicate.

Polymerase Chain Reaction Array Analysis

Total RNA was prepared from each specimen using TRIzol (Invitrogen, Milan, Italy) to derive total RNA from MKN45 xenograft nodules treated with vehicle alone or maraviroc (nodules from three to four mice per group were used). The RNA was reverse transcribed with SuperScript II

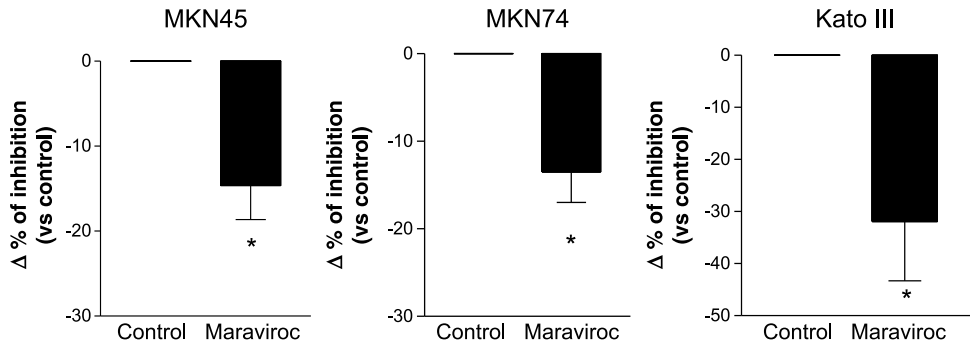

Figure 2. CCR5 antagonism by maraviroc inhibits proliferation of gastric cancer cells. Exposure of MKN45, MKN74, and KATOIII cells to maraviroc significantly inhibits cell proliferation. $N = 4$ to 6; $^*P < .05$ *versus* control.

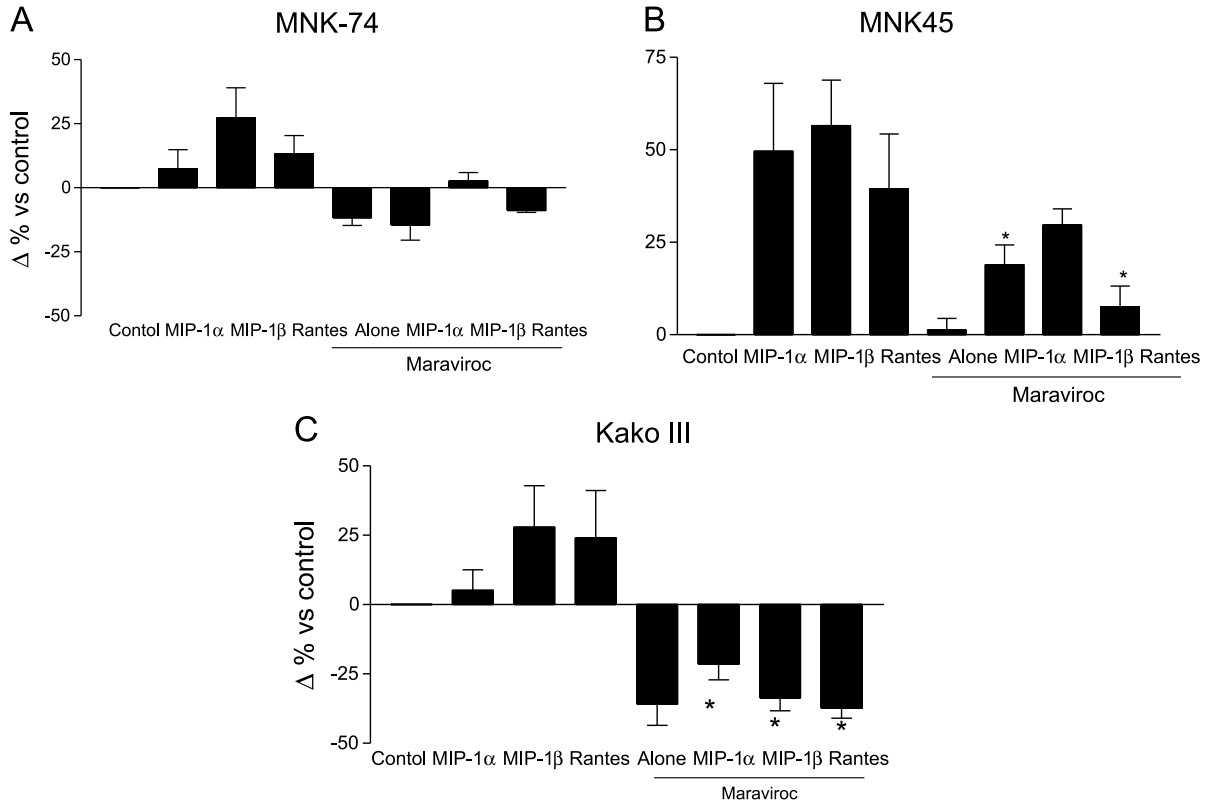

Figure 3. Promotion of gastric cancer cell adhesion to mouse peritoneum by CCR5 ligands is reversed by CCR5 antagonism. Treatment of MKN45 (B), MKN74 (A), and KATOIII (C) with MIP-1α (20 ng/ml), MIP-1β (20 ng/ml), and CCL5/RANTES (10 ng/ml) increases the adhesion of gastric tumor cells to mouse peritoneal tissue (~1.6 cm^2 placed in a 24-well culture plate). The adhesion was reversed by pretreatment with maraviroc. $N = 4$ to 6 experiments; * $P < .05$ *versus* the CCR5 ligand alone.

Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. A total of 50 ng of cDNA was pipetted into each well of a 96-well gene array plate [Human Tumor Metastasis RT^2 Profiler PCR Array (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-028Z.html) and Human Inflammatory Cytokines and Receptors RT^2 Profiler PCR Array (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-011Z.html), Superarray Biosciences, Frederick, MD] and amplified following the manufacturer's instructions. These gene arrays are designed to assess 84 genes known to be involved in metastasis and inflammation, respectively. Genes selected for tumor metastasis array encode several classes of protein factors including those for cell adhesion, extracellular matrix components, cell cycle, cell growth and proliferation, apoptosis, transcription factors and regulators, and other genes related to tumor metastasis. Genes selected for inflammatory cytokine and receptor array encode several classes of protein including chemokines, chemokine receptors, interleukins, interleukin receptors, other cytokines, and other cytokine receptors. Array analysis was carried out with the online software RT^2 Profiler PCR Array Data Analysis (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

The signal detected for each gene in the Human Inflammatory Cytokines and Receptors Array was normalized to the signal obtained for β-actin, β_2-microglobulin, hypoxanthine phosphoribosyltransferase 1, and large ribosomal protein P0 on the same gene array to derive gene expression values for each gene. The signal detected for each gene in the Human Tumor Metastasis Array was normalized to the signal obtained for β-actin, β_2-microglobulin, and large ribosomal protein P0 on the same gene array to derive gene expression values for each gene. Upregulated/downregulated genes are genes whose expressions had been altered by more than 1.8.

Quantitative Real-Time Polymerase Chain Reaction

Quantization of nodule (from four to five mice per group) gene expression was performed by quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR). One microliter of RNA was incubated with DNase I and reverse transcribed with SuperScript II (Invitrogen) according to the manufacturer's specifications. For real-time PCR, 20 ng of template was used in a 20-μl reaction containing a 0.2 μM concentration of each primer and 10 μl of KAPA SYBR FAST (Kapa Biosystems, Woburn, MA). The following PCR primers were designed using the software Primer3 Output using published sequence data obtained from the NCBI database.

hGAPDHs: gaaggtgaaggtcggagt;
hGAPDHas: catgggtggaatcatattggaa;
hIL10RBs: gggaacctgactttcacagc;
hIL10RBas: ccacaggacagaaggtgatg;
hLTBs: gaggaggagccagaaacaga;
hLTBas: agccgacgagacagtagagg;
hFAT1s: gacaatgcaccacaatttcg;
hFAT1as: gtgattacgccggacagttt;
hMETs: aagagggcattttggttgtg;
hMETas: ctcggtcagaaattgggaaa;
hNME1s: cattgcgatcaaaccagatg;
hNME1as: cggtccttcaggtcaacgta.

Statistical Analysis

Data are expressed as means ± SE. Statistical significance was determined by one-way analysis of variance followed by the Tukey test. The Mann-Whitney test was used to compare two groups of data. A value of $P < .05$ was considered significant. GraphPad Prism version 3.0 was used for graphics and statistical analyses (GraphPad Software, San Diego, CA).

Results

Expression and Function of CCR5 on Gastric Cancer Cell Lines

By flow cytometry analysis, cell surface and intracellular expression of CCR5 was detected in MKN45, MKN74, and KATOIII, three gastric cancer cell lines (Figure 1, *A–E*). We confirmed that the expression of CCR5 on gastric cancer cells was relatively stable even after these cells were implanted i.p. in mice (Figure 1, *F–H*). The expression of CCR5 and its ligand RANTES in gastric tumor cells was confirmed by RT-PCR (Figure 1, *I–J*). The expression of CCR5 in MKN45 and MKN74 cells was compared to that of Thp1 cells, a myelomonocytic leukemia cell line, and to MCF7 cells, a breast cancer cell line. The RT-PCR analysis shown in Figure 1*I* demonstrates that the CCR5 expression was higher in Thp1 cells than in MKN45 and MKN74 cells. Interestingly, the expression of CCR5 in MKN74 was close to that of MCF7 cells. Further, detectable levels of RANTES were found in both gastric cancer cell lines (Figure 1*J*), thus indicating the potential for an autoregulatory loop in gastric cancers.

To investigate whether CCR5 was functional, cells were cultured in the presence of maraviroc, a small molecule CCR5 antagonist. As shown in Figure 2, exposure to maraviroc effectively inhibited cell proliferation ($P < .05$). Furthermore, pretreating MKN45, MKN74, and KATOIII cells with maraviroc effectively inhibited the adhesion of these cancer cell lines to murine parietal peritoneum. For these experiments, gastric cancer cell lines were first preactivated by exposure to CCR5 ligands, i.e., MIP-1α (20 ng/ml), MIP-1β (20 ng/ml), and CCL5/RANTES (10 ng/ml). As shown in Figure W1, MKN45 and MKN74 cells expressed detectable levels of RANTES. In Figure 3, *A* to *C*, all three ligands effectively increased adhesiveness of gastric tumor cells to mouse peritoneal tissue (~1.6 cm^2 placed in a 24-well culture plate; $n = 4$-6 experiments; $P < .05$). Of relevance, these activities were reversed by pretreating the cells with maraviroc ($n = 4$-6 experiments; $P < .05$ *versus* the CCR5 ligand alone).

CCR5 Antagonism Attenuates Gastric Cancer Cell Dissemination In Vivo

As shown in Figure 4, *A* to *C*, treating mice with maraviroc (10 mg/kg i.p.) for 7 days starting from day 3 after MKN45 (1×10^7 cells) injection in mice significantly reduced the number of peritoneal and mesenteric nodules, from 23.0 ± 2.8 to 7.2 ± 1.4 for peritoneal nodules and from 13.7 ± 2. 4 to 2.4 ± 0.7 for mesenteric nodules ($P < .05$), detected in the peritoneum at day 10 after cell inoculation. Maraviroc also reduced the total volume of nodules (peritoneal and mesenteric nodules), from 832.0 ± 59.0 to 336.0 ± 62.0 mm^3 ($P < .05$). Treatment with maraviroc had no effect on mouse body weight

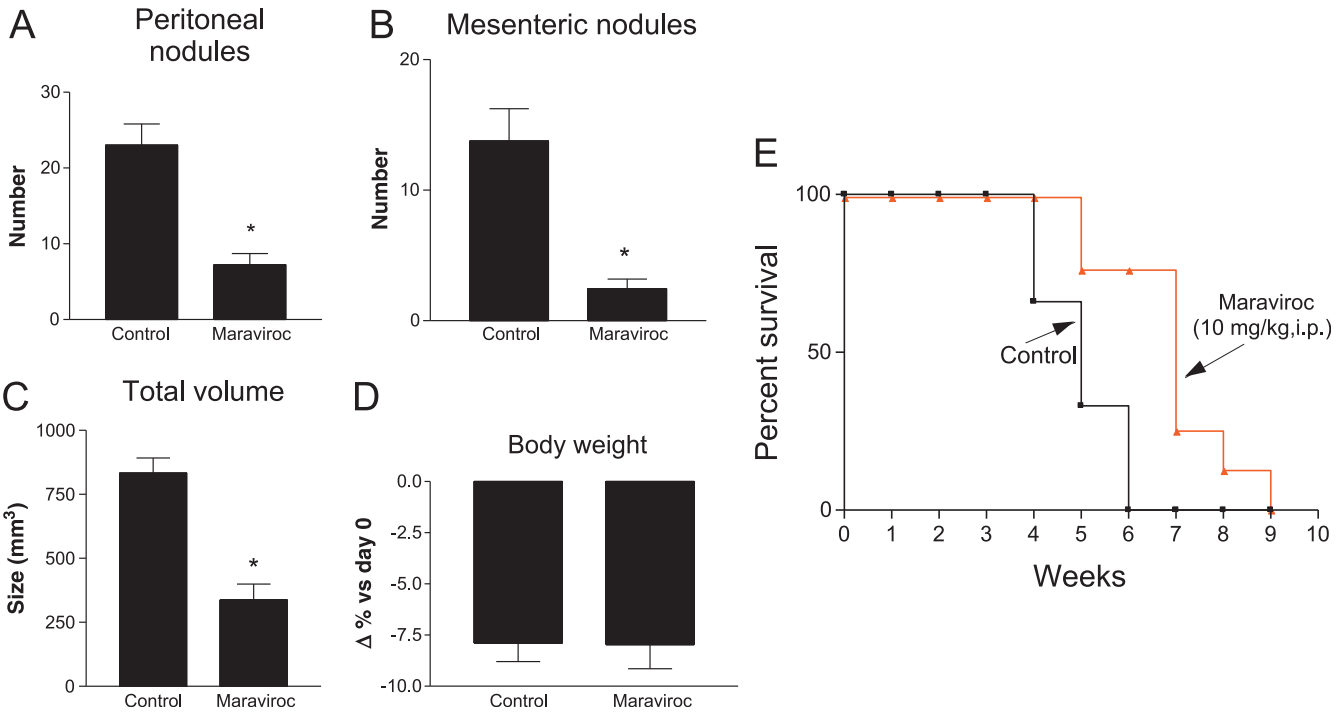

Figure 4. *In vivo* effect of CC5 antagonism: maraviroc protects against gastric cancer cell implantation in the peritoneal cavity. (A–C) Treating NOD-SCID mice with maraviroc (10 mg/kg i.p.) for 7 days starting from day 3 inhibits MKN45 (1×10^7 cells) implantation into the peritoneum as measured by counting the number of peritoneal and mesenteric nodules at the time of autopsy. Maraviroc also reduces significantly the total volume of nodules (peritoneal and mesenteric nodules), from 832 ± 59 to 336 ± 62 mm^3. The extent of peritoneal carcinogenesis was evaluated on day 10. (D) Treatment with maraviroc had no effect on mice body weight. Data are from 10 mice per group. (E) CCR5 antagonism by maraviroc increases animal survival. In another experimental set, animals were observed until death, and overall survival was evaluated. Maraviroc administration to mice implanted with MKN45 cells enhances the survival. Data are from 10 mice per group.

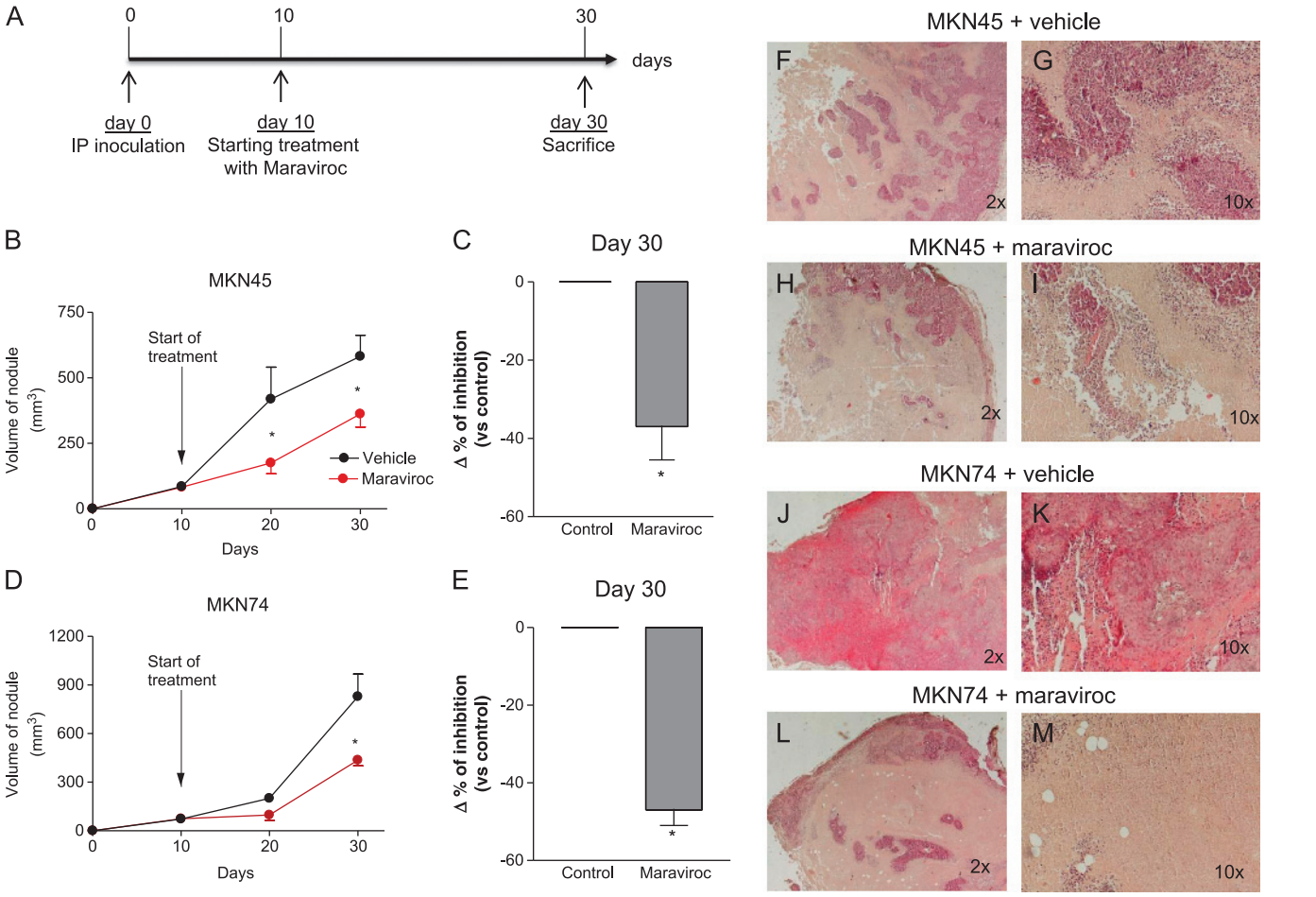

Figure 5. CCR5 antagonism by maraviroc reduces tumor burden in the xenograft model. (A–E) MKN74 or MKN45 (1×10^7/mouse) cells were implanted subcutaneously, and mice were treated with maraviroc (50 mg/kg twice a day) for 30 days. Data are from 8 to 10 mice per group. (F–M) Histopathology analysis of xenograft nodules obtained at day 30. Treating animals with maraviroc (H, I and L, M in comparison with vehicle treatment F, G and J, K) increased significantly the extent of tumor necrosis. Original magnifications, ×2 and ×10 (as indicated). Each image shown is representative of one nodule.

(Figure 4*D*). Importantly, as shown in Figure 4*E*, treating NOD-SCID mice implanted with MKN45 cells with maraviroc also increased the animal survival. Thus, median survival time increased from 30 to 46 days in mice administered maraviroc.

To evaluate the role of maraviroc in tumor growth in another model, NOD-SCID mice were inoculated subcutaneously with MKN74 or MKN45 (1×10^7/mouse) cells and followed for 30 days. A group of animals was treated with maraviroc (50 mg/kg twice a day) for 20 days starting on day 10. As illustrated in Figure 5, *A* to *E*, treating mice with maraviroc resulted in a significant reduction of tumor burden in both MKN45 (Figure 5, *B* and *C*) and MKN74 (Figure 5, *D* and *E*) transplanted mice. The histopathology analysis of xenograft nodules demonstrates that with both cell types the administration of maraviroc resulted in extensive intratumoral necrosis (Figure 5, *F–M*), which is consistent with our observation that maraviroc reduces proliferation of MKN45 and MKN74 cells (Figure 2).

PCR Array Analysis of Cancer Nodules from MKN45 Xenografts

Because maraviroc reduces the gastric cancer cell line proliferation *in vitro* and subcutaneous nodule volumes *in vivo*, we have then investigated the molecular mechanisms involved in these effects. For these purposes, two gene arrays designed to analyze the expression of genes related to tumor metastasis and inflammatory cytokines (chemokines and their receptors) were used. RNA from MKN45 xenografts was used for these studies. For the tumor metastasis array, the expression of 15 genes was undetected, the expression of 6 genes [hepatocyte growth factor receptor (*MET*), *FAT1*, *MDM2*, *PNN*, *CTSK*, and *APC*] was downregulated by less than 1.8-fold, whereas 3 genes [*NME1*, *vascular endothelial growth factor A*, and *PLAUR*] were upregulated ≥+1.8 ($P \leq .05$) in response to maraviroc treatment *in vivo* (Figure 6, *A–C*). For the inflammatory cytokines/chemokines and receptors, the expression of 39 genes was undetected, the expression of 8 genes [*NAMPT*, *interleukin-10 receptor B* (*IL-10RB*), *IL-16*, *IL-1RN*, *lymphotoxin β receptor (LTB)*, *IL-15*, *IL-5RA*, and *IL-17c*] was downregulated ≤−1.8, whereas only 1 gene (*IL-27*) was upregulated ≥+1.8 ($P \leq .05$) in response to maraviroc treatment *in vivo* (Figure 7, *A–C*). As shown in Figure 8, the modulatory effect of maraviroc on IL-10RB, MET, FAT1, NME1, and LTB was confirmed with RT-PCR ($n = 4/5$; $P < .05$ *versus* control group).

Discussion

The results shown in this report demonstrate that CCR5 is expressed on gastric cancer cell lines and that its activation by the CCR5 ligands

MIP-1α, MIP-1β, and RANTES triggers an adhesion of these cells to the peritoneum. In addition, we have provided compelling evidence that maraviroc, a drug approved for the treatment of R5, CCR5-trophic, HIV-1 reduces the proliferation of gastric cancer cell lines *in vitro* and attenuates the cancer burden *in vivo*.

Previous studies have shown that RANTES and CCR5 are highly expressed in gastric cancers [8] with lymph node metastasis and that the expression level of RANTES in the lymph nodes with cancer invasion was substantially increased, suggesting that RANTES and its receptor CCR5 were associated with the metastatic potential of this tumor [9,11]. Further, it has been reported that protein extracts from lymph nodes harboring metastasis from gastric cancers yield significant chemotaxis toward either primary gastric cancer cells or AGS cells [9]. Importantly, these effects could be reversed by RANTES antagonism, which indicates that RANTES and its receptor participate in the migration of gastric cancer cells from primary to metastatic sites [9].

Given the aggressive clinical behavior of gastric cancer and the lack of targeted therapies, we have investigated the effects of CCR5 antagonism in regulating the invasiveness and metastatic potential of MKN45, MKN75, and KATOIII cells, three human gastric cancer cell lines [10]. These cell lines reflect clinicopathologic features of gastric cancer subtypes in that they show different phenotypes and stages of differentiation [10]. The results of *in vitro* studies demonstrate that all three cell lines express CCR5 as evaluated by flow cytometry analysis. Further, CCR5 antagonism effectively counteracts the proinvasive potential of these cells, attenuating the proliferation potential and migration and adhesion to explanted murine peritoneum. Taken together, these data support a previously unrecognized role for CCR5 antagonism in regulating gastric cancer cell proliferation and tendency toward diffusion.

Because previous studies have shown that CCR5-deficient mice develop less lung metastases than their wild-type counterparts [12] and had a reduction in intratumoral accumulation of macrophages, granulocytes, and fibroblasts, resulting in less angiogenesis [12], we have examined whether CCR5 antagonism modulate the tendency toward gastric cancer diffusion *in vivo*.

In vivo results demonstrate that treating SCID mice with maraviroc reduces the peritoneal colonization by gastric cancer cells. This result is consistent with previous studies in which inhibition of CCR5 expression or administration of anti-CCL5/RANTES neutralizing antibody to tumor-bearing mice reduced metastatic capability induced by coinjection of breast cancer cells and mesenchymal stem cells [11,12].

Because preventing the homing of cancer cells to metastatic sites is a desirable characteristic in an anticancer agent, we have further investigated whether treatment with maraviroc prevented gastric cancer growth in a xenograft model. Again, results from these experiments demonstrate that CCR5 blockage for 30 days effectively reduced the burden of cancer nodules in mice implanted with MKN45 and MKN74 cells by approximately 40%. These effects on tumor volume

Tumor Metastasis Genes

A

Not changed	
gene	fold regulation
BRMS1	1,5692
CD44	-1,1487
CDH1	-1,0718
CHD4	-1,2527
COL4A2	1,6245
CST7	-1,2527
CTBP1	-1,0534
CTNNA1	-1,0353
CTSL1	-1,5157
CXCL12	1,3426
CXCR2	-1,4897
CXCR4	1,21
DENR	-1,6245
EPHB2	1,366
ETV4	-1,2968
EWSR1	1,1688
FGFR4	-1,129
FN1	-1,3195
FXYD5	1,0905
GNRH1	1,0353
HPSE	-1,5422
HRAS	-1,0534
HTATIP2	-1,5692
IGF1	-1,6818
IL18	1,0718
IL1B	1,0534
ITGA7	-1,2968
ITGB3	-1,5422
KISS1	1,0175
KISS1R	1,1688
KRAS	-1,2527
MCAM	1,0905
METAP2	1,2527
MGAT5	1,0534
MMP10	-1,366
MMP11	1,0718
MMP13	1,0905
MMP2	1,5692
MMP7	1,3195
MMP9	1,0905
MTA1	-1,1487
MTSS1	-1,3899
MYC	1,0353
MYCL1	-1,0534
NF2	1,0353
NME4	1,4897
RB1	1,0905
RPSA	1,2527
SERPINE1	-1,3899
SET	1,366
SMAD2	-1,129
SRC	-1,3426
SSTR2	-1,5157
SYK	1,2746
TCF20	-1,0534
TGFB1	1,0534
TIMP2	-1,3195
TNFSF10	1,2968
TP53	1,1487

B

Down regulated genes	
gene	fold regulation
FAT1	-3,1383
APC	-2,8779
CTSK	-2,1435
PNN	-2,1435
MDM2	-1,9319
MET	-1,834
CD82	-1,7411
Up regulated genes	
PLAUR	2,0705
VEGFA	1,834
NME1	1,8025

C

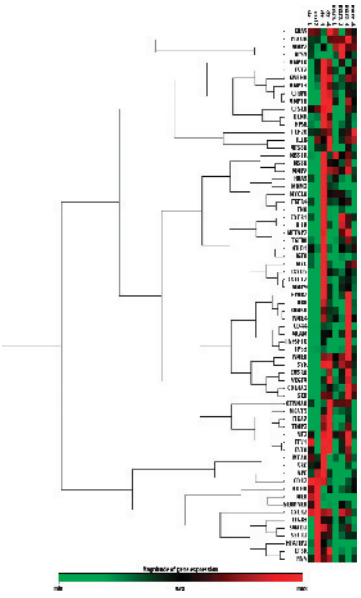

Figure 6. CCR5 antagonism modulates the expression of inflammatory genes. MKN45 xenografts treated with vehicle alone or maraviroc were used for these studies. While expression of 15 genes was undetected, the expression of 6 genes (*MET*, *FAT1*, *MDM2*, *PNN*, *CTSK*, and *APC*) was downregulated by less than 1.8-fold ($P < .05$). The expression of 3 genes (*NME1*, *vascular endothelial growth factor A*, and *PLAUR*) was upregulated by more than 1.8-fold ($P < .05$) in response to maraviroc treatment *in vivo* (A–C).

A

Immunity and Inflammatory Genes

Not changed	
gene	fold regulation
IL1B	1,3233
IL1R1	-1,6386
IL3	1,1587
IL8	-1,3939
MIF	-1,2491
AIMP1	-1,5828
BMP2	-1,2276
C5	-1,1722
CCL15	-1,5556
CCL16	-1,3621
CCL20	-1,1926
CCL24	-1,6867
CCL26	-1,3006
CCL3	-1,152
CCR5	-1,4854
CCR6	-1,0145
CSF1	-1,4683
CSF2	1,1128
CX3CL1	-1,0323
CX3CR1	-1,3157
CXCL1	-1,6769
CXCL12	-1,5289
CXCL2	-1,5737
CXCL5	-1,0443
CXCR2	1,5737
IFNG	-1,6673
IL10RA	-1,6481
IL13	-1,4101
IL1A	-1,2782
TNF	-1,3006
TNFRSF11B	-1,331
TNFSF10	-1,6867
TNFSF11	1,1064
TNFSF13	-1,0087
TNFSF4	-1,0811
VEGFA	-1,2347

B

Down regulated genes	
gene	fold regulation
IL10RB	-2,5713
NAMPT	-2,3991
LTB	-2,304
IL15	-2,2
IL16	-2,1006
IL1RN	-1,9942
IL17C	-1,9827
IL5RA	-1,8715
Up regulated genes	
IL27	3,2773

C

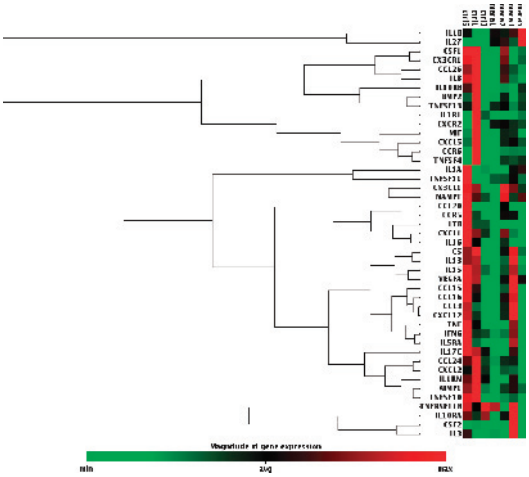

Figure 7. CCR5 antagonism modulates the expression of inflammatory genes as assessed by tumor metastasis array. MKN45 xenografts treated with vehicle alone or maraviroc were used for these studies. The expression of 39 genes was undetected, the expression of 8 genes (*NAMPT*, *IL-10RB*, *IL-16*, *IL-1RN*, *LTB*, *IL-15*, *IL-5RA*, and *IL-17c*) was downregulated by a fold change ≤ -1.8, whereas 1 gene (*IL-27*) was upregulated by $\geq +1.8$ fold ($^*P \leq .05$) in response to maraviroc treatment *in vivo* (A–C).

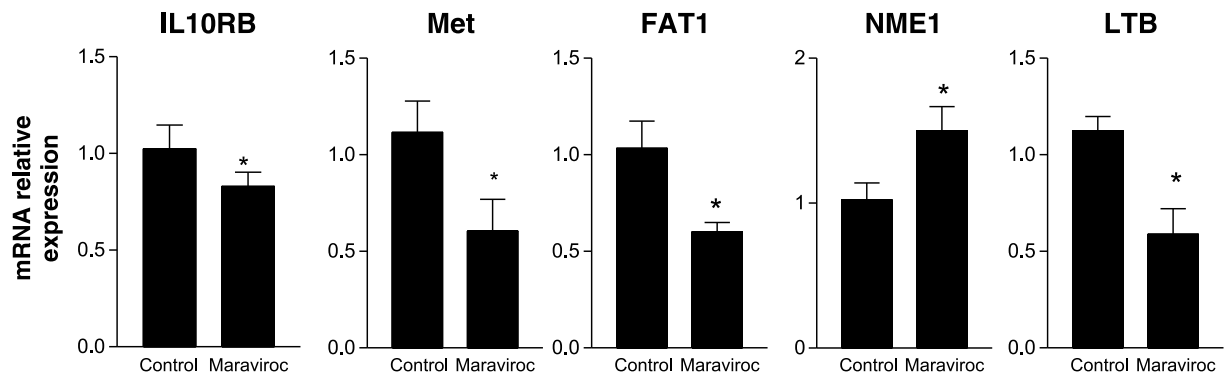

Figure 8. Validation of gene array by RT-PCR. RT-PCR validation of gene array confirmed that maraviroc regulates the expression of IL-10RB, MET, FAT1, NME1, and LTB. $N = 4/5$ per group. $^*P < .05$.

associated with a significant increase in intratumoral necrosis. This histopathology pattern associates regulation of the expression of several genes including *IL-10RB*, *MET*, *FAT1*, *NME1*, and *LTB*. The regulatory effects of maraviroc on these genes were confirmed by RT-PCR analysis. IL-10RB is upregulated in several cancer types and its activation has been linked to the development of immune tolerance. IL-10 is expressed in gastric cancer metastases, and a positive correlation exists between serum IL-10 levels and progression of cancers [13]. Previous studies have shown that expression of cytokines with a Th1 signature (IL-2 and IFN-γ) is reduced in gastric cancers with lymph node metastasis as compared with cancers without metastasis. In contrast, the expression of IL-10 is markedly increased in gastric cancers with lymph node metastasis [13]. This unbalance between Th1/Th2 cytokines has been mechanistically linked to the high level of expression of RANTES and CCR5 in gastric cancers with lymph node metastasis, suggesting that RANTES/CCR5 axis might impair the regional immunity in these cancers [9]. In the present study, we have confirmed this concept by demonstrating that CCR5 antagonism counteracts the growth of gastric cancer implants in the xenograft model and that this effect associates with a robust down-regulation of IL-10RB. Thus, the present results are a further confirmation that inhibition of IL-10/IL-10RB pathway is helpful to enhance the antitumor immune response and support a role for CCR5 in regulating immune response in the tumor environment.

MET, also known as hepatocyte growth factor receptor, is a receptor tyrosine kinase with an important role both in normal cellular function and in oncogenesis. In many cancer types, abnormal activation of MET is related to poor prognosis and various strategies to inhibit its function, including small molecule inhibitors, are currently in preclinical and clinical evaluations. *MET* is a gene involved in regulating autophagy [14,15]. Autophagy, a self-digesting recycling mechanism with cytoprotective functions, is induced by cellular stress [15]. This process is also induced on cytotoxic drug treatment of cancer cells and partially allows these cells to escape cell death. Thus, because autophagy protects different tumor cells from chemotherapy-induced cell death, current clinical trials aim at combining autophagy inhibitors with different cancer treatments. Previous studies have shown that in gastric adenocarcinoma cell lines GTL-16 and MKN45, where MET activity is deregulated because of receptor overexpression, MET inhibition leads to cell death paralleled by the induction of autophagy. A combined treatment of MET inhibitors together with the autophagy inhibitor or genetically impairing autophagy by knocking down the key autophagy gene, *ATG7*, is reported to decrease viability of gastric cancer cells [14]. It has been reported that in gastric cancer cells induction of cytoprotective autophagy in MET-expressing cells on MET inhibition and a combination of MET and autophagy inhibition result in significantly decreased cell viability [14]. Thus, despite the fact that we have not defined the mechanism by which maraviroc abrogates MET expression, regulation of this receptor tyrosine kinase might support the antitumor activity of the CCR5 antagonist.

Another gene that was negatively regulated by maraviroc in the xenograft model is *FAT1*, one of the four homologs of the atypical cadherin gene *FAT* [16]. *FAT1* plays a controversial role in cancer growth and functions as a tumor-suppressive or oncogenic gene in a context-dependent manner [16]. Thus, while from one side FAT1 loss has tumor-suppressive effect because it leads to activation of the Wnt signaling pathway and to a reduced cell-cell adhesion, it is known that FAT1 binds to ENABLE/vasodilator-stimulated phosphoprotein protein promoting actin polymerization and cell motility, thus promoting cell migration and invasion [16]. Importantly, its inhibition might help in reducing neoplastic cell attachment and motility at the metastatic sites.

The *Nm23-H1* (*NME1*) gene is a metastatic suppressor identified in a melanoma cell line and expressed in different tumors where its levels of expression associate with reduced or increased metastatic potential [17]. Nm23 is one of the more than 20 metastasis suppressor genes confirmed *in vivo*. It is highly conserved from yeast to human, implying a critical developmental function. Tumors with alteration of the *p53* gene and reduced expression of the *Nm23* gene are more prone to metastasis [17]. Reductions in Nm23 expression have been associated with aggressive behavior in various tumors including gastric carcinomas [18]. Importantly, its expression is inversely proportional to the metastatic potential of these carcinomas. Despite high levels of *Nm23* gene expression observed in other tumors (i.e., thyroid carcinoma), in gastric cancer, reinduction of NME1 expression might have a potential favorable effect on dissemination [17].

Activation of LTB initiates inflammation-induced carcinogenesis and orchestrates primary tumorigenesis [19]. LTB expression/function has been linked to tumor relapse in various cancer models through distinct mechanisms. Inhibition of LTB is of pharmacological relevance in regulating inflammation [20] and cancer progression [21].

In clinical settings, maraviroc is administered at doses ranging from 150 to 600 mg twice a day. In humans, oral doses of 300 mg produce an average C_{max} of 1200 nM [6]. Because in mice 16 mg/kg produce an average C_{max} of 1045 nM [5], the doses of 10 and 50 mg/kg per day administered during our experiments were clinically relevant and indicate that the *in vivo* effects of maraviroc in immunodeficient mice injected with gastric cancer cell lines manifest at clinically relevant doses of the drug.

In summary, we have shown that CCR5 is expressed in gastric cancer cells and that its antagonism by maraviroc, a noncompetitive, slowly reversible small molecule that is used as a part of combination antiretroviral therapy for patients infected with R5, CCR5-tropic, HIV-1, inhibits gastric cancer cell proliferation and mobility *in vitro* and effectively attenuates the tendency toward dissemination *in vivo*. While these studies support a path toward the clinical use of CCR5 antagonists as novel treatments for advanced gastric cancer and maraviroc is clinically used in the treatment of CCR5-tropic HIV with a very limited burden of side effects [6], properly designed clinical studies are required to evaluate whether the present observations extend to clinical settings.

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