High Expression of IL-13 Receptor α2 in Colorectal Cancer Is Associated with Invasion, Liver Metastasis, and Poor Prognosis

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Abstract

Autocrine secretion of cytokines by metastatic colorectal cancer cells and their role during invasion and liver homing has been poorly characterized. In this study, we used cytokine arrays to analyze the secretomes of highly metastatic colorectal cancer cells. Compared with poorly metastatic cancer cells, highly metastatic cells expressed increased levels of the immunosuppressive cytokines interleukin (IL)-4 and IL-13 in addition to increased surface expression of the high affinity IL-13 receptor IL-13Rα2, suggesting that IL-13Rα2 mediates IL-13 effects in colorectal cancer cells. Silencing of IL-13Rα2 in highly metastatic cells led to a decrease in adhesion capacity in vitro and a reduction in liver homing and increased survival in vivo, revealing a role for this receptor in cell adhesion, migration, invasion, and metastatic colonization. In support of this, IL-13 signaling activated the oncogenic signaling molecules phosphoinositide 3-kinase, AKT, and SRC in highly metastatic cells. Clinically, high expression of IL-13Rα2 was associated with later stages of disease progression and poor outcome in patients with colorectal cancer. Our findings therefore support a critical role for IL-13Rα2 expression in colon cancer invasion and metastasis. Cancer Res; 72(11); 1–11. ©2012 AACR.

Introduction

Metastasis is the final step of the malignant transformation, being responsible for the fatal outcome in patients with cancer. However, metastasis is a complex process that involves a number of different and sequential steps. Cells must detach from the primary tumor, migrate, gain access to blood or lymphatic vessel, and colonize a new target organ (1). Therefore, metastasis cannot be considered as a single process, but as a collection of different events, all of them exhibiting different molecular traits. Migration, invasion, and survival are fundamental aspects of the process.

Dissemination and metastasis of cancer cells depend on extensive interactions within the tumor microenvironment (2–3). A number of these interactions are regulated through chemokines and their receptors, which govern many different aspects of the malignant phenotype. So, it is critical to characterize chemokines and their receptors expression in metastatic cells. Chemokine-mediated inflammation has been shown to play an important role in tumor biology by influencing tumor growth, invasion, and metastasis (4). Chemokines were initially described as regulators of leukocyte trafficking and recruitment in inflammation sites. Infiltration of white blood cells, mostly tumor-associated macrophages, and the presence of proinflammatory molecules as interleukin (IL)-8 and IL-6 are typical features of cancer-related inflammation. Cytokines and chemokines are produced by multiple cell types, including fibroblasts, endothelial, and epithelial cells (5), including cancer cells, which also express their G-protein coupled receptors. These G-proteins activate a number of signaling pathways such as phospholipase C (PLC)-β, phosphoinositide 3-kinase (PI3K), and the mitogen—activated protein kinase (MAPK) cascade, which play a critical role in adhesion, migration, and survival of cancer cells (6).

Concomitant with these effects, cytokines and chemokines also confer cancer cells with the ability to adhere to fibronectin and collagen IV, allowing the formation of micrometastases (7). Therefore, chemokine networks control critical steps of the adhesion-invasion-metastasis cascade, providing the necessary signals that facilitate cancer cell survival and growth in distant sites (3). Little is known about the role played by the autocrine secretion of chemokines by tumor epithelial cells in cancer-related inflammation, which might regulate the recruitment and maturation of macrophages and other effector cells. We used the well-known KM12 cell model for the study of late metastatic events in colorectal cancer, including liver colonization and survival of the tumor cells in the new environment. KM12C and KM12SM epithelial cells differ only in their metastatic properties (8). KM12SM, a highly metastatic

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cell line, was derived from the poorly metastatic cell line KM12C, through successive passages in nude mice. A previous proteomic characterization of these 2 colon cancer cell lines showed a preference for expression of homing molecules in KM12SM cells (9). Moreover, a decrease in the expression levels of enzymes from the glycolysis, pentose phosphate pathway, and amino acid transporters was observed.

To characterize the metastatic process in colorectal cancer, we analyzed the cytokine/chemokine profiles released by colorectal metastatic cancer KM12SM cells using antibody microarrays. These microarrays constitute a powerful tool to get a complete overview of cytokine profiles and pathways alterations in cancer (10). We observed higher secretion of IL-13, which was associated to a more abundant expression of their receptor IL-13Rα2 in the highly metastatic KM12SM cells. Silencing of IL-13Rα2 revealed a major role for this receptor in cell adhesion, migration, invasion, and metastatic colonization of colorectal cancer cells. A clear increase of expression of IL-13Rα2 was observed in late-stage human colon cancer tissues, showing a correlation between IL-13Rα2 expression and poor outcome of patients with colon cancer metastasis.

Materials and Methods

Cell culture

KM12C and KM12SM human colon cancer cells were obtained directly from I. Fidler’s laboratory (MD Anderson Cancer Center, Houston, TX). KM12 cells were expanded in the laboratory to prepare a large batch of working aliquots that were stored in liquid nitrogen. For each experiment, cells were thawed and kept in culture for a maximum of 10 passages. These 2 cell lines were not authenticated in our laboratory. Parental cells, and their derivatives, were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco-Life Technologies) containing 10% fetal calf serum and antibiotics at 37°C in a 5% CO2-humidiﬁed atmosphere. Other cell lines were authenticated by ATCC and were passaged fewer than 6 months after purchase for all the experiments.

Human protein cytokine array

Conditioned medium from each KM12 cell type was collected after 24 and 48 hours in serum-free medium and incubated with arrays containing 79 human cytokine speciﬁc antibodies as described (10). Then, the membranes were scanned and analyzed using Redﬁn, a 2D-gel image analysis software (Ludesi). Relative cytokine intensities were normalized in comparison to control spots on the same membrane. Ratios between both cell lines were calculated for the different cytokines at the 3 experiments. Individual quantification of IL-4 and IL-13 was carried out by speciﬁc ELISA kits (RayBiotech; see Supplementary Methods).

Stable transformation of KM12 cells, cell adhesion, and invasion assays

pLKO.1 vectors containing 5 different short hairpin RNAs (shRNA) for IL-13Rα2 were purchased from Open Biosystems. As shRNA control vectors, we used a scrambled shRNA and an empty pLKO.1 vector obtained from AddGene (11, 12). Stable transformed cells were obtained by lentiviral infection. Briefly, HEK293T cells were transfected with pLKO.1 vectors and the packaging vectors pCMV-rev, psMLg-pRRE, and pNGVL-VSVG with jetPrime Transfection Reagent (Polyplus). After incubating the cells for 12 to 15 hours in serum-free medium, medium was replaced with DMEM containing 10% FBS and antibiotics. The day after, conditioned medium containing lentiviral particles was centrifuged, diluted 1:2 to 1:10 in DMEM containing 10% FBS and antibiotics and directly added to KM12C and KM12SM cells. After 3 days of incubation, infected KM12C and KM12SM CRC cells were selected using 1 µg/mL puromycin (Sigma) during 2 to 3 weeks. Then, cells were cultured with medium containing 0.5 µg/mL puromycin.

Cell adhesion and invasion using Matrigel assays were carried out according to previously published procedures (13; see Supplementary Methods for a full description). Inhibitors LY294002 and PP2 were from Sigma and U0126 was from Calbiochem. For apoptosis and proliferation assays see Supplementary Methods.

Western blotting

The following antibodies were used in the study. Anti-IL-4Rα, anti-IL-13Rα1, and anti-c2 integrin were from Abcam, whereas anti-IL-13Rα2 was from R&D Systems. AKT, p-AKT, p-ERK1/2, p-JNK, STAT3, p-STAT3, STAT6, and p-STAT6 were from Cell Signalling. Extracellular signal–regulated kinase (ERK)1/2, c-jun-NH2-kinase (JNK), and RhoGDI were from Santa Cruz Biotechnology. Tubulin was from Sigma. Anti-β-1 integrin was a gift from Dr F. Sánchez-Madrid (H. de la Princesa, Madrid, Spain).

For Western blotting, KM12C and KM12SM cells were washed twice with chilled PBS and homogenized in 0.05% SDS. Protein extracts were sonicated 3 times for 30 seconds on ice, and clarified at 10,000 × g at 4°C. Protein extracts were run in 10% SDS-PAGE and immunoblotting was carried out following standard procedures.

Metastasis experiments in nude mice

Swiss nude mice (Charles River) were used for intrapleural or intratral inoculation. The Ethical Committee of the Consejo Superior de Investigaciones Cientíﬁcas (Madrid, Spain) approved the protocols used for experimental work with mice. Mice were injected with 10⁶ KM12 cells in 0.1 mL PBS. Mice were daily inspected for signs of disease, such as abdominal distension, locomotive deﬁcit, or tumor detectable by palpation. Metastasis nodes in liver were determined by counting the number of visible nodules in dissected livers.

Immunohistochemical analysis

A total of 80 patients diagnosed and treated of colorectal adenocarcinoma between 2001 and 2006 in Fundación Jiménez Díaz (Madrid, Spain) were used for the study. We have reviewed the clinical records of the patients to determine tumor stage at the time of diagnosis and outcome (Supplementary Table S1). Hematoxylin and eosin–stained sections of the colectomy...
specimens were reviewed to select representative areas of the tumor to carry out immunohistochemical detection of IL-13 and IL-13Rα2. The working dilution was 1:100 for IL-13 and 1:500 for IL-13Rα2. Immunohistochemistry was carried out on a tissue microarray (TMA) using an automated system for immunostaining (Dako Autostainer), with antigen retrieval at high pH. The immunostained sections were counterstained with hematoxylin and the intensity of the membrane and cytoplasmic staining was graded as absent, weak, moderate, or intense, although for subsequent statistical analysis the cases were reclassified as positive (either moderate or intense) or negative (either complete absence of positivity or weak staining similar to control areas of normal colonic mucosa). In all cases, sections from normal colonic mucosa distant from the tumor site were used as negative controls.

Statistical analyses

Data from the patients were recorded in an excel file. Descriptive statistics included mean (and SD) for quantitative Gaussian variables and percentages for qualitative ones. Bivariate descriptive statistics included mean (and SD) for quantitative variables and chi-square test for qualitative ones. In both analyses, the minimum acceptable level of significance was P < 0.05. Survival curves were plotted with Kaplan–Meier technique and compared with the log-rank test. The level of significance for survival was also settled at a P value less than 0.05.

Results

Chemokine profiling in the secretome of KM12 cells

We investigated the production and release of 79 different chemokines, growth factors, and other immunomodulators in conditioned culture media of colorectal cancer KM12 cells by using a chemokine array. Results after 24 and 48 hours of culture are shown in Fig. 1A. Several chemokines, proangiogenic, and growth factors were highly expressed in both cell lines, among them GROα (CXCL1), IL-8 (CXCL8), Rantes (CCL5), macrophage inflammatory protein (MIP)-1β (CCL4), eotaxin-2 (CCL24), angiogenin, MIF, osteoprotegerin, or insulin-like growth factor–binding protein 2 (IGFBP2; Fig. 1B). Regarding differential expression, TIMP2, IL-4, and IL-13 were upregulated in the highly metastatic cells (Fig. 1A and B). IL-4 and IL-13 differences were confirmed by using a specific ELISA, with fold-changes between 1.5 and 2.13, in the presence or absence of serum, respectively (Fig. 1C).

Most of the biologic effects of IL-4 and IL-13 are mediated by STAT transcription factors (14). We analyzed KM12 cells by Western blotting using antibodies specific for total and phosphorylated forms of STAT3 and STAT6 (Fig. 1D). Total and activated STAT6 were more abundant in KM12SM cells, with no significant differences in STAT3. High levels of STAT6 in colon cancer cells have been associated to high invasiveness/metastasis (15). These results support a potential action of IL-4 and IL-13 on KM12 cells.

IL-13Rα2 mediates IL-13 action on KM12 cells: effects on cell adhesion

IL-4 and IL-13 usually regulate biologic activity through the common type II receptor IL-4Rα/IL-13Rα1 complex (16), which mediates signal transduction through the JAK/STAT6 pathway (17). In addition, IL-13 binds a high-affinity receptor called IL-13Rα2 (18), which was initially assigned as a decoy receptor (19). We tested KM12 cells for the presence of these receptors. IL-4Rα was not detected on the surface of KM12

Figure 1. Expression of chemokines, growth factors, and immunomodulators in conditioned medium from KM12SM and KM12C cells using cytokine arrays. A, representative image of cytokine antibody arrays results after screening of conditioned medium from KM12 cells. The experiment was carried out in triplicate. B, cytokines highly expressed in KM12 cells at 48 hours. Bar graph was calculated for each cytokine with the median value in arbitrary units of 3 independent assays. Inset, signal intensity for IL-4 and IL-13 obtained from 3 independent arrays. C, expression ratios for IL-4 and IL-13 in conditioned medium of KM12 cells in the presence or absence of serum at 48 hours after ELISA quantification. D, STAT signaling alterations in KM12 cells were tested by immunoblotting. Experiments were carried out in triplicate and quantified by densitometry.
cells by flow cytometry (Supplementary Fig. S1A) or intracellular by Western blotting (Supplementary Fig. S1D). IL-13Rα1 was not accessible on the cell surface (Supplementary Fig. S1B), although some intracellular expression was detected using permeabilized cells in flow cytometry (Supplementary Fig. S1C) or Western blotting (Supplementary Fig. S1E). In contrast, a higher surface expression of IL-13Rα2 was observed on the surface of KM12SM than in KM12C cells by flow cytometry, 73.9% versus 33.9% (Fig. 2A). Western blot analyses confirmed the increased expression of IL-13Rα2 in KM12SM cells respect KM12C, as well as a high expression in other metastatic colon cancer cells such as SW48 or HT29 (Fig. 2B).

Because the IL-4Rα heterodimer for IL-13 signaling was not available on the cell surface, we hypothesized that IL-13Rα2 could mediate IL-13 action in KM12 cells.

To examine the potential effect of IL-13Rα2 on colorectal cancer metastasis, IL-13Rα2 was silenced on KM12C and KM12SM cells by preparing stable shRNA transfectants. The lack of expression of IL-13Rα2 was confirmed by Western blotting (Fig. 2C) and semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) (data not shown). Both analyses showed that less than 10% of the original protein expression levels were detected after knockdown with shRNA#23. This silencing did not affect the expression of IL-13Rα1, which showed similar intracellular levels (Supplementary Fig. S1E). To analyze the effect of IL-13Rα2 silencing on autocrine IL-13 production we quantified the levels of IL-13 by ELISA. We observed a decrease in the expression of IL-13 in IL-13Rα2–silenced cells. The reduction was more dramatic for KM12SM cells (Supplementary Fig. S2).

Then, we analyzed the adhesive properties of the cell lines before and after IL-13Rα2 silencing using Matrigel. In basal conditions, adhesion capacity of scrambled KM12SM overexpressing IL-13Rα2 duplicated that of KM12C cells. The decrease in adhesion capacity after IL-13Rα2 silencing was particularly significant for KM12SM cells (>50%) and negligible for KM12C cells (Fig. 2D). Addition of IL-13 increased significantly cell adhesion in scrambled control cells, but not in the

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**Figure 2.** IL-13Rα2 is overexpressed on KM12SM cells and increases cell adhesion. A, flow cytometric analysis of KM12C and KM12SM cells. The percentage of positive cell is shown inside each panel. B, twenty-five micrograms of protein from lysates of the indicated colorectal carcinoma cell lines were resolved in PAGE and subjected to Western blotting using anti-IL-13Rα2 or, as a control, anti-RhoGDI antibodies. C, KM12C and KM12SM cells were infected with retroviral vectors containing different shRNAs targeting IL-13Rα2, scrambled shRNA, or empty vectors, and IL-13Rα2 expression was assessed by Western blotting. Bands were quantitated with MultiGauge software. Tubulin was used as loading control. D, cell adhesion to Matrigel of IL-13Rα2–silenced or control KM12 cells, pretreated for 5 hours with or without IL-13 plus the indicated antibodies. Adhesion was significantly upregulated by incubation with IL-13 (*, P < 0.01; **, P < 0.001) and significantly decreased by incubation with anti-IL-13Rα2 (ΔΔ, P < 0.01; ΔΔΔ, P < 0.001). Data represent the mean ± SD of 3 independent experiments.
siliated cells. The use of anti-IL-13Rα2 antibody blocked cell adhesion for IL-13–treated scrambled cells, being most relevant for KM12SM (Fig. 2D). Still, antibody blocking was not as effective as IL-13Rα2 silencing. These results suggest that IL-13Rα2 also contributes to constitutive cell adhesion in the absence of IL-13. As a control, IL-13Rα1–silenced cells (Supplementary Fig. S3A) did not show alterations in cell adhesion (Supplementary Fig. S3B).

**IL-13Rα2 expression promotes cell migration and invasion in KM12 cells through PI3K and SRC activation**

To determine the effect on cell migration, we used wound-healing assays and different amounts of IL-13 for 22 hours. IL-13 was sufficient to promote migration in KM12 cells, with an optimum at 10 ng/mL (Fig. 3A), KM12SM cells displayed twice more migratory capacity than KM12C cells in presence of serum, which might contain IL-13 and other promigratory factors. KM12 cells showed a much lower cell migration in medium alone or with 10% serum. KM12SM cells incubated in medium with IL-13 showed a pronounced increase of cell migration compared to medium alone, while the increase in medium with IL-13 was strong for KM12C cells. Addition of IL-13 caused a significant increase in the migration speed of scrambled cells (Fig. 3C). In contrast, silenced cells were insensitive to IL-13. This inhibition was also confirmed by the use of anti-IL-13Rα2 antibodies. To study the pathways involved in the increase of cell migration we tested different inhibitors. The increase in migration induced by IL-13 was strongly reduced by LY294002, a PI3K inhibitor, partially reduced by PP2 (SRC inhibitor), and not affected by UO126 (MEK1/2 inhibitor) in KM12 cells. The inhibition was more pronounced on KM12SM cells.

Then, we tested the ability of the cells for invasion across Matrigel (Fig. 3D). In medium alone, the invasion was low, but twice higher for KM12SM cells. Addition of IL-13 caused a large increase of invasion in the control cells, but not in the silenced cells, which showed between 6 and 10 times less invasion capacity, similar to basal levels. As before, the use of PI3K inhibitor or anti-IL-13Rα2 decreased the invasive

![Figure 3. IL-13 increases cell migration and invasion through IL-13Rα2.](image-url)

- **A**: KM12C shRNA scramble vs. KM12C shRNA IL-13Rα2
- **B**: KM12C shRNA scramble vs. KM12C shRNA IL-13Rα2
- **C**: KM12SM shRNA scramble vs. KM12SM shRNA IL-13Rα2
- **D**: KM12C shRNA scramble vs. KM12C shRNA IL-13Rα2
- **E**: KM12C shRNA scramble vs. KM12C shRNA IL-13Rα2

For migration, the distance covered was calculated as the mean ± SD of 3 independent experiments. For increase in migration (†, P < 0.05; ††, P < 0.01; †††, P < 0.001) and for reduction (△, P < 0.05; △△, P < 0.01; △△△, P < 0.001). E, KM12 cells were starved in medium, treated with IL-13 (10 ng/mL) or 10% serum for 5 hours and lysed. The extracts were analyzed by Western blotting using the indicated antibodies. *, observed changes in expression.
capacity of the scrambled cells to basal level. SRC inhibitor was also quite effective to decrease the invasive capacity of KM12SM, but not so much on KM12C cells. No effect was observed for MAP–ERK kinase (MEK1/2 inhibitor). As a control, IL-13Rα2 silencing in KM12SM cells did not alter cell invasion capacity (Supplementary Fig. S3C).

To confirm the role of IL-13Rα2 in cell migration and invasion, we generated KM12 cells overexpressing this receptor, which were tested in migration and invasion assays. Both KM12C and KM12SM transfectants showed an increase in migration and invasion in response to IL-13 compared with control transfectants (Supplementary Fig. S4A and S4B). Collectively, these results support that IL-13 action was mediated by IL-13Rα2 for migration and invasion in colorectal cancer metastasis.

Pathways activation in IL-13Rα2–mediated cell invasion

To study the mechanism of action of IL-13Rα2 in cell invasion, we characterized the levels of activated SRC, FAK, AKT, ERK, JNK, and STAT6 in response to IL-13 or serum (Fig. 3E). Expression of p-SRC and p-AKT was more abundant in KM12SM cells treated with serum than with IL-13, whereas only p-AKT, but no p-SRC, was observed in KM12C cells. These differences in p-SRC effect between KM12SM and KM12C cells might explain previous differences in migration and invasion induced by the SRC inhibitor. The presence of a double band with different intensities in p-SRC between serum and IL-13–treated cells suggests that a different member from the SRC family might be implicated in KM12SM cells, as the antibody recognizes phosphorylation in the C-terminal tyrosine of 6 members of this family. The decrease of p-AKT in silenced KM12SM cells treated with IL-13 confirmed the relevance of PI3K and suggests a potential effect on survival. MAPK signaling was not affected by IL-13Rα2 silencing as no differences were observed for ERK1/2 or JNK. Activation of p-STAT6 was observed in KM12SM cells with serum, but not when cells were treated only with IL-13 (Fig. 3E). Therefore, IL-13–triggered activation through IL-13Rα2 is STAT6 independent. Other factors must activate STAT6 in serum-cultured KM12SM cells.

Silencing of IL-13Rα2 expression in KM12 cells decreases survival, tumorigenesis, and proliferation

The activation of the PI3K pathway has important biologic effects on cell survival and proliferation (20). To test whether IL-13 signaling via IL-13Rα2 can modulate cell survival, KM12 cells were subjected to apoptosis assays. In response to oxidative stress induced by hydrogen peroxide, scrambled KM12C and KM12SM cells showed similar levels of apoptosis (Fig. 4A). The addition of IL-13 caused a moderate effect on survival. MAPK signaling was not affected by IL-13Rα2 silencing as no differences were observed for ERK1/2 or JNK. Activation of p-STAT6 was observed in KM12SM cells with serum, but not when cells were treated only with IL-13 (Fig. 3E). Therefore, IL-13–triggered activation through IL-13Rα2 is STAT6 independent. Other factors must activate STAT6 in serum-cultured KM12SM cells.

**Figure 4.** IL-13Rα2 promotes cell survival and proliferation. A, cells were incubated with H2O2 for 16 hours in presence of medium alone or with IL-13, and in presence or absence of LY294002 inhibitor or anti-IL-13Rα2 antibodies, and subjected to apoptosis detection assays. B, colony formation assay in soft agar with the indicated cell lines. C, proliferation and cell viability was determined by MTT assays after 24 to 48 hours of incubation in medium with or without IL-13 (10 ng/mL) and anti-IL-13Rα2 antibody. Optical density was significantly increased by addition of IL-13 (*, *P < 0.05; **, **P < 0.01) and significantly inhibited by anti-IL-13Rα2 antibody (Δ, ΔΔP < 0.01).
the antibody or the silencing of the receptor abolished the increment in survival (Fig. 4A). The presence of LY294002, a PI3K inhibitor, inhibited this increase in survival induced by IL-13 through IL-13Rα2. These results indicate that IL-13 also affects the survival program in KM12 colorectal cancer cells through PI3K-AKT1 activation mediated by the IL-13Rα2 receptor. This moderate reduction in cell apoptosis may play a role during metastasis, facilitating survival of metastatic cells.

Then, we tested the effect of IL-13Rα2 silencing on tumorigenesis through the ability to form colonies in soft agar. KM12SM displayed a higher capacity to proliferate in an anchorage-independent environment than KM12C (Fig. 4B). However, IL-13Rα2-silenced cell lines showed a significant reduction in colony formation, indicating a crucial role of this receptor in tumorigenesis. Regarding proliferation, we observed only a minor effect in cells treated with IL-13 (Fig. 4C). This IL-13 effect was blocked after incubation with anti-IL-13Rα2 antibodies (Fig. 4C). These results suggest that IL-13 signaling via IL-13Rα2 does not play a major role in cell proliferation in colorectal cancer cells.

**Silencing of IL-13Rα2 in KM12SM cells provokes a decrease in liver homing and an increase in mouse survival**

As a final approach to verify the role of IL-13Rα2 in colon cancer invasion and metastasis, we carried out intrasplenic injections of control and silenced KM12 cells to analyze their ability and speed to generate metastasis in liver. IL-13Rα2-silenced KM12SM cells induced longer survival of mice than control KM12SM cells (P < 0.05; Fig. 5A). This prolonged survival was due to the slower growth of the tumor in the mice inoculated with IL-13Rα2-silenced KM12SM cells and lower ability to colonize liver and cause metastasis. Postmortem analysis showed that tumors at primary inoculation site reached similar sizes ranging between 0.7 and 1 cm³ in control and silenced KM12SM cells. Poorly metastatic KM12C cells did not cause metastasis, and only 30% of inoculated mice developed a tumor in spleen 180 days after inoculations. Therefore, IL-13Rα2-silenced KM12SM cells lost, in a significant way, the ability to colonize the liver, as few mice showed macroscopic metastasis after liver dissection (Fig. 5B).

To corroborate the ability of KM12 cells for liver homing, we collected the livers 24 hours after spleen injection and carried out a PCR for amplification of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fig. 5C). Whereas human GAPDH could be detected in livers from mice inoculated with control cells (especially KM12SM), the PCR amplification resulted in a barely detectable band in mice inoculated with silenced cells. To examine IL-13Rα2 expression in KM12SM cells as well as their invasive properties after in vivo passage, cells were isolated from the tumors and cultured until confluence. IL-13Rα2 expression remained very low (Fig. 5D) and their invasiveness across Matrigel was not altered by in vivo passage (Fig. 5E). Collectively, these data confirm the capacity of IL-13Rα2 to mediate homing and liver metastasis in colon cancer.

**IL-13Rα2 overexpression in human patients is associated to late stages and lower overall survival**

To investigate the relevance of our results in human colon cancer, we decided to study the levels of expression of IL-13 and IL-13Rα2 in tumor and adjacent normal tissue samples. We observed IL-13 expression mainly associated to human epithelial colon cancer cells, with weak or no IL-13 expression in the stroma of the tumors (Fig. 6A). For statistical analysis of IL-13Rα2 expression, we used a TMA with representative sections of tumor and normal colonic mucosa from 80 patients diagnosed and treated of colorectal adenocarcinoma and followed on the long term (>5 years). The series was retrospectively selected. IL-13Rα2 expression was not detected in 27 cases (33.7%) and was present with either moderate or high intensity in the remaining 53 cases (66.3%; Fig. 6B). Weak or no staining was observed in all control normal samples. We found a statistically significant association between IL-13Rα2 expression and tumor progression (T-stage), with higher expression in T3 or T4 tumors as compared with T1 or T2 (P = 0.013), lymph node involvement (higher expression in tumors with lymph node involvement, P = 0.013), and metastasis at the moment of diagnosis (P = 0.038; Fig. 6C). We found no significant association with histologic grade of the tumor, but all high grade and colloid tumors showed intense IL-13Rα2 expression, as opposed to well-differentiated tumors, that were mostly negative. Finally, we wanted to know whether there was an association between IL-13Rα2 expression and survival of patients with colon cancer. Survival analysis showed a clear association with poor prognosis in terms of lower overall survival for patients with high IL-13Rα2 expression (P = 0.03; Fig. 6D).

**Discussion**

Although the KM12 cell model probably does not give a complete picture of the spontaneous metastasis in human colon cancer, actually is giving us excellent insights in the cell adhesion, invasion, and colonization of the liver in metastasis and the critical molecules involved in these processes. Here, we have described an important role for IL-13 and its receptor IL-13Rα2 in colorectal cancer invasion and metastasis. This conclusion was obtained from the following observations: (i) IL-13 was more abundant in the secretome of highly metastatic cells, (ii) IL-13Rα2 was overexpressed in highly metastatic KM12SM cells and other metastatic cell lines than poorly metastatic KM12C cells, (iii) IL-13Rα2 silencing decreased adhesion, invasion, and clonogenicity, (iv) IL-13Rα2 silencing suppressed AKT activation and promoted apoptosis, (v) mice experiments showed that removal of IL-13Rα2 reduced the homing capacity in liver of KM12SM cells and increased the survival of inoculated mice, and (vi) human colon cancer samples showed a high expression of IL-13 and IL-13Rα2 in cancer cells. IL-13Rα2 was mainly associated to T3 or T4 tumors and to a lower overall survival. These results confirmed that IL-13 and IL-13Rα2 expression were associated to colorectal cancer invasion and liver metastasis in cancer cells.

IL-13 has been previously associated to pathologic conditions such as asthma, autoimmune diseases, and
that promotes lymph node metastases (24) and is a major regulator of M2 macrophages to suppress immune surveillance in metastasis (25). This counter-surveillance activity requires the expression of IL-13Rα2 (26). IL-13Rα2 gene expression was reported in pancreatic and breast cancer metastasis (27, 28). The almost absence of IL-4Rα and IL-13Rα1 in KM12 cells (this report) and other colon cancer
cells (29) indicates that IL-13 signaling occurs through the IL-13Rα2 receptor. Surprisingly, previous reports did not detect IL-13Rα2 mRNA by RT-PCR in HT-29/B6 colon cancer cells (30).

No signaling activity was initially described for IL-13Rα2 due to its short cytoplasmic domain and the lack of JAK/STAT binding sequences (19). Therefore, IL-13Rα2 was considered as a decoy receptor for IL-13 in mouse and humans (21). However, recent studies have shown that IL-13Rα2 is internalized after IL-13 binding (31). It has been reported that IL-13Rα2 induces MAPK signal transduction in intestinal epithelial cells from ulcerative colitis or colorectal cancer (29) and pancreatic cancer (28). At low concentrations, IL-13Rα2 signaled through the MAPK pathway, but at higher concentrations worked as a decoy receptor (29). In contrast, on metastatic KM12SM cells, IL-13 action is mediated through IL-13Rα2 independent from the expression levels, as denoted by the effect on both, KM12C and KM12SM cells.

In murine macrophages, IL-13Rα2 mediates activator protein (AP-1)-dependent, STAT-6-independent signaling, resulting in inflammation and fibrosis in vivo (23). Previous reports in pancreatic cancer (28) and ulcerative colitis (29) showed STAT6-independent activation in IL-13Rα2-expressing cells, suggesting an inverse relationship between IL-13Rα2 expression and STAT6 activation. In colon cancer metastatic cells, signaling through IL-13Rα2 is also STAT6 independent, and is not mediated by JNK or ERK. The lack of ERK activation could explain the minor effect of IL-13Rα2 removal on cell proliferation and might explain why IL-13Rα2 overexpression does not provide any competitive advantage in subcutaneously implanted tumors in immunodeficient mice (32).

In our model, IL-13Rα2 signaling induced PI3K activation, as PI3K inhibitors blocked IL-13 effects on KM12 cells. SRC activation was found exclusively on IL-13–treated KM12SM cells and might be related with the higher migration and invasion of these cells. An alternative role for IL-13 in the...
increase of intestinal epithelial permeability, mediated by the PI3K pathway without STAT6 involvement, has been reported (33). Ulcerative colitis, a well-known risk factor for colon cancer development, is probably driven by IL-13 (34). Also, oxazolone-induced colitis in mice is caused by IL-13–producing natural killer T cells (35). Lack of epithelial barrier function has been reported for inflammatory bowel diseases (36, 37). This increase in permeability affected epithelial cells tight junctions, induced epithelial apoptosis and cell restitution arrest in ulcerative colitis (30). All these processes, where IL-13 is a key effector, contribute to severe inflammation and could constitute a connecting link between ulcerative colitis, colon inflammation, and colon cancer. In fact, we have observed a predominant association of IL-13 expression with colon cancer epithelial cells.

Immunohistochemical analysis of IL-13Rα2 expression in a panel of metastatic human colon cancer samples showed a clear association with late stages in human cancer and poor outcome of patients. This worst prognosis could be attributed to the increased invasiveness and homing capacity of cells overexpressing IL-13Rα2. Liver colonization by metastatic cells requires a different program of protein expression and signaling activation from early metastasis. Targeting some of the cytokines or regulatory molecules involved in metastatic invasion and homing might be a successful approach for decreasing metastasis recurrence in colorectal cancer.

In summary, we have shown a role for IL-13Rα2 in adhesion, invasion, survival, and colonization of highly metastatic colon cancer cells. Our results suggest that IL-13Rα2 does not function only as a decoy receptor of IL-13 in cancer cells but as signaling mediator. Moreover, the use of IL-13Rα2 expression in patients with colon cancer as a prognostic biomarker in metastatic colorectal cancer gives a pathophysiologic relevance to these findings and supports the interest of this molecule as therapeutic target in colon cancer.

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

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High Expression of IL-13 Receptor α2 in Colorectal Cancer Is Associated with Invasion, Liver Metastasis, and Poor Prognosis

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