The Chemokine Receptor CX3CR1 Is Involved in the Neural Tropism and Malignant Behavior of Pancreatic Ductal Adenocarcinoma

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Abstract

Tumor perineural dissemination is a hallmark of human pancreatic ductal adenocarcinoma (PDAC) and represents a major source of local tumor recurrence after surgery. In this study, we provide *in vitro* and *in vivo* evidence that the chemokine receptor CX3CR1 may be involved in the neurotropism of PDAC cells to local peripheral nerves. Neoplastic cells from PDAC cell lines and surgical specimens express the chemokine receptor CX3CR1, absent in normal pancreatic ducts. Its unique ligand, the transmembrane chemokine CX3CL1, is expressed by neurons and nerve fibers. CX3CR1 + PDAC cell lines migrated in response to human recombinant CX3CL1 and specifically adhered to CX3CL1-expressing cells of neural origin via mechanisms involving activation of G proteins, β1 integrins, and focal adhesion kinase. *In vivo* experiments with transplanted PDAC showed that only CX3CR1-transfected tumor cells infiltrated the local peripheral nerves. Immunohistochemistry of CX3CR1 in PDAC specimens revealed that 90% of the samples were positive with a heterogeneous pattern of expression. High receptor score was significantly associated with more prominent tumor perineural infiltration evaluated histologically (P = 0.026). Regression analyses (univariate and multivariate) showed that high CX3CR1 expression and perineural invasion were strongly associated with local and earlier tumor recurrence (P = 0.007). Collectively, this study shows that the CX3CR1 receptor may be involved in PDAC tumor neurotropism and is a relevant and independent risk factor to predict an early local tumor relapse in resected patients. Thus, the CX3CR1-CX3CL1 axis could represent a valuable therapeutic target to prevent tumor perineural dissemination in pancreatic cancer. [Cancer Res 2008;68(21):9060–9]

Introduction

Pancreatic ductal adenocarcinoma (PDAC), the fourth leading cause of cancer-related deaths in the Western world, is a highly aggressive and chemoresistant disease, with a 5-year survival rate of <5%. This is improved to 20% in the small percentage of patients eligible to surgery (1, 2). PDAC extensively infiltrate surrounding structures; indeed, certain sites that pancreatic cancer may spread to eliminate surgery as treatment option. Depending on the localization of the primary tumor, preferential sites of metastasis are the lymph nodes, big vessels as portal vein, liver, and the celiac plexus. Characteristic of this neoplasia is its peculiar neurotropism: the growth of tumor foci along intrapancreatic and extrapancreatic nerves is a common pathologic finding and a major cause of local tumor recurrence (3–6).

Several studies have addressed the molecular mechanisms of pancreatic tumor cell adhesion to nerve fibers. A number of reports have investigated the importance of neurotrophic factors expressed by peripheral nerves in pancreatic tumor cell adhesion. The expression of various neurotrophins (BDNF, NT-3, NT-4, NT-5) and their receptors (Trk A, B, C) in the perineural microenvironment suggested their possible involvement in tumor invasion (7–9). More recently, members of the glial cell–derived neurotrophic factor family, including Artemin, were also shown to favor pancreatic cancer invasion of peripheral nerves (10) and promote the growth and survival of neoplastic cells (11).

Chemokines and their receptors have been implicated in tumor growth and tumor cell invasion of surrounding organs. In particular, chemokine receptors have been suggested to elicit cancer cell mobilization and promote distant metastasis with a certain degree of organ selectivity (12–15). Chemokine receptors expressed by PDAC include CCR6 (16), CXCR5 (17), and CXCR4 (18, 19). We reported that, in this neoplasia, CXCR4 not only has chemotactic function but also sustains tumor cell survival and proliferation (20).

In the context of a transcriptional profiling aimed at exploring the chemokine system in pancreatic cancer, we found that the chemokine receptor CX3CR1 is up-regulated in selected PDAC cell lines. CX3CR1 is normally expressed by hematopoetic cells (21) and poorly studied in tumors, with few exceptions (22–24). The only ligand for CX3CR1 is the chemokine CX3CL1, also known as Fractalkine (25) or Neurotactin (26). CX3CL1 was originally described as a membrane, as well as a soluble chemokine expressed by neurons, nerve terminations, in addition to activated endothelial cells (25–28). The interest in this chemokine has recently gained a new impetus, resulting from the accumulation of data about its expression in several human diseases, including atherosclerosis (29, 30), allograft rejection (31), and age-related macular degeneration (32). CX3CL1 is one of the most expressed chemokines in the nervous system; neurons and astrocytes are major producers of the ligand, and microglia express the receptor (33, 34). A recent study highlighted the role of CX3CL1 in attenuating glial-induced

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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inflammation in the brain (34), thus indicating that the CX3CR1/CX3CL1 axis is a major player in the cross-talk between neurons and microglia, possibly contributing to the maintenance of homeostasis in the brain.

The constitutive expression and function of CX3CL1 by neural cells (28) and the high frequency of perineural invasion in pancreatic adenocarcinoma prompted us to test the hypothesis that this chemokine/receptor pair is involved in the neurotropism of PDAC. Here, we report that pancreatic tumor cells from PDAC patients strongly express CX3CR1. The receptor mediates chemotactic migration toward CX3CL1 and adhesive interaction with neural cells expressing the ligand CX3CL1. In PDAC patients, high receptor expression is associated with a marked perineural invasion and with earlier local tumor recurrence, supporting the concept that CX3CR1 is an important determinant of tumor neurotropism and malignant behavior in pancreatic cancer.

Materials and Methods

Cell lines and tissues. Human pancreatic carcinoma cell lines, the immortalized epithelial cell line HPDE6 and primary tumors from surgical specimens were cultured as previously described (20). Neuroblastoma cell lines (SKN-BE and SY5Y), purchased from American Type Culture Collection, were cultured in RPMI with 10% fetal bovine serum (FBS). To collect conditioned supernatants, cells were seeded at 10^6 cells/mL in six-well plates in RPMI with 10% FBS and stimulated with tumor necrosis factor α (TNFα) (20 ng/mL) and IFNγ (500 units/mL). After overnight incubation, medium was replaced with serum-free medium, and conditioned supernatants were collected after 24 h.

Chemotaxis. Cell migration was evaluated using a 48-well modified Boyden chamber, as previously described (20). Recombinant CX3CL1 (Peprotech) or SKN-BE conditioned supernatants were used as chemoattractants in the lower compartment. Results are expressed as the mean number of net migrated cells over control cells, counted in 10 microscope high power fields (magnification, ×1,000). Each experiment was performed in triplicate. Where indicated, cells were incubated with a blocking anti-CX3CR1 monoclonal antibody (mAb) (Torrey Pines). *P* value was calculated by Student's *t* test.

Adhesion to neuroblastoma cells. Neuroblastoma cells were grown to confluence in flat-bottomed six-well plates and stimulated with TNFα (20 ng/mL) and IFNγ (500 units/mL), where indicated. In some experiments, only IFNγ (500 units/mL) was used to prevent the TNF-induced shedding of membrane CX3CL1. ^{51}Cr (Amersham UK)–labeled tumor cells were coincubated with neuroblastoma monolayers at 37°C for 1 h in DMEM with 1% FBS, under slow agitation, to prevent nonspecific attachment. Nonadherent cells were washed away, adherent cells were solubilized with 1 mL of 0·1% SDS, and radioactivity was counted in a gamma counter. Results represent the percentage of adherent cells ± SE of three replicates per group. *P* value was calculated by Student's *t* test. Where indicated, cells were incubated with a blocking anti–β1 integrin mAb (R&D Systems, clone P5D2), pertussis toxin (PTX; Calbiochem, 500 ng/mL, 3-h incubation), or anti-CX3CL1 antibody (R&D Systems). *P* value was calculated by Student's *t* test.

Confocal microscopy. To assess integrins, CX3CR1 and focal adhesion kinase (FAK) distribution, MiaPaCa2-CX3CR1-GFP, MiaPaCa2-GFP, and SKN-BE cells were seeded on glass slides pretreated with poly-L-lysine. After 2 h, cells were washed once with PBS and fixed with 4% PFA for 15 min at room temperature. After blocking with 2% bovine serum albumin and normal goat serum in PBS, cells were stained with anti-CX3CR1 (AbCam, polyclonal, 1:350), anti-CX3CL1 (R&D Systems, clone 81513, 1:200), anti-CD29 (R&D Systems, clone P5D2, 1:500), and anti-FAK (Santa Cruz, goat polyclonal, 1:500). Before FAK staining, cells were permeabilized with 0.3% Triton-X in PBS for 5 min.

Mouse model of nerve invasion. Eight-week-old nude mice (Charles River) were used for the mouse model. Procedures involving animals and their care conformed to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 86/609, OJ L 358,1,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All efforts were made to minimize the number of animals used and their suffering. Mice (six per group) received an s.c. injection of 7×10^6 viable MiaPaCa2-GFP-CX3CR1or MiaPaCa2-GFPcells on the midline of the mouse back. Three weeks later, when the tumor grew to ~1 cm in long diameter, mice were sacrificed and the s.c. tissue was removed, fixed in 10% formalin, and embedded in paraffin. The samples were processed for histologic examination and stained with polyclonal (1:2,500) anti-S100 antibody and Envision as secondary antibody, both from Dako. The number of intact intratumoral and peritumoral nerves was evaluated by counting at low-power magnification (×200). The pattern of S100 positivity was evaluated at higher magnification. *P* value was calculated by Student's *t* test.

Patients and tissue specimens. Pancreatic tumor specimens were obtained from 59 patients (ages, 36–74 y) with histologic diagnosis of PDAC admitted at the San Raffaele Hospital between July 1997 and April 2004. Patient clinical characteristics are shown in Table 1 and in the supplementary data. The progression-free survival and the site of tumor relapse of the first documented observation (local versus metastatic recurrence) were evaluated. Assessment of disease, including spiral computed tomography of the abdomen and chest, was done at baseline, every 8 wk during chemotherapy and then every 3 mo or in the case of clinical suspicion of disease failure.

Immunohistochemistry of CX3CR1. Preparation of the tissue array is described in the supplementary data. Paraffin sections of pancreatic cancer patients were deparaffinized in xylene, and antigen retrieval was performed thrice using sodium citrate buffer (pH 6.0) in a microwave oven at 5 min each. Sections were stained with the rabbit polyclonal anti-human CX3CR1 antibody (Abcam; 1:350 dilution, overnight at 4°C) followed by a goat anti-rabbit secondary antibody (EnVision horseradish peroxidase rabbit/mouse, DakoCytomation). After a diaminobenzidine reaction (Liquid DAB + Substrate Chromogen System, DakoCytomation), sections were counterstained with hematoxylin (Mayer, DIAPATH). Two independent observers particularly experienced in immunohistochemistry evaluated the slides; all cases with discrepant interpretations were discussed using a double-headed microscope, and a consensus was reached. Both readers were blinded to clinicopathologic data and patient outcomes. Immunoreactivity was scored semiquantitatively according to the estimated percentage of positive tumor cells (1, 1° terzile <70% reacting cells; 2, 2° terzile 70–91% reacting cells; 3, 3° terzile >91% reacting cells) and intensity (0, no; 1, weak; 2, moderate; 3, intense). For statistical analysis, a total immunohistochemical score was calculated by summing the intensity score and the percentage score, producing a total range of 1 through 6.

Determination of neural invasion in patients. All the 59 cases of formalin-fixed, paraffin-embedded samples included in the tissue array were retrieved; H&E slides were reviewed to evaluate the degree of neural invasion, defined as the presence of cancer cells in the perineurium of nerve fascicles. Two variables were considered: number of total nerve fascicles present and number of nerve fascicles involved. An average number per case of 7.4 ± 3 slides representing the most extensively involved tumor areas was examined. Counting was performed at low-power magnification (×100), and only intrapancreatic and peripancreatic nerves in close proximity (within 0.5 cm) to the parenchymal edge were included. An average number of 92 ± 74 nerves per patient was examined. The degree of neural invasion was expressed as percentage of nerve fascicles with tumoral invasion over the total number of nerves. Both readers were blinded to positivity of CX3CR1 expression.

Statistical analysis. All the clinical characteristics of patients were compared by the χ^2 test for categorical variables. Student's *t* test or ANOVA test for unpaired data was used to compare mean values. Mann-Whitney *U* test or Kruskal-Wallis *H* test was used to compare data showing non-Gaussian distribution. The Cox proportional hazard model and the logistic regression estimated the association of the investigated risk factors with nonlocal recurrence after surgery. Odds ratio, hazard ratio, and 95% confidence interval (95% CI) are presented. All probability values were two-sided. Analyses were performed using the SPSS statistical analysis software.

Table 1. CX3CR1 expression is a predictor of local recurrence in resected pancreatic cancer patients

Variables	Univariate analysis			
	Local recurrence of pancreatic carcinoma			
	Logistic regression		Cox regression	
	Odds ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Age (y)	1.01 (0.94–1.08)	0.68	0.98 (0.93–1.04)	0.60
Sex (male)	0.97 (0.3–3.1)	0.96	1.06 (0.42–2.63)	0.89
Presurgical CA19.9 (units/mL)	1 (0.99–1)	0.8	1 (0.99–1)	0.90
Size (cm)	1.19 (0.85–1.67)	0.302	1.12 (0.89–1.4)	0.33
Head localization	0.38 (0.05–2.5)	0.31	0.61 (0.17–2.13)	0.44
T (1–4)	0.50 (0.14–1.72)	0.27	0.6 (0.24–1.44)	0.25
N1	3.41 (0.2–40.4)	0.33	1.27 (0.29–5.5)	0.74
Grading (1–3)	0.94 (0.37–2.39)	0.89	0.81 (0.38–1.7)	0.57
Stage (1–4)	0.36 (0.10– 1.23)	0.105	0.73 (0.39–1.35)	0.32
Radicality (R1–2 versus R0)	1.45 (0.45–4.6)	0.52	2.3 (0.8–6.7)	0.121
Therapy				
PEFG versus gemcytabine	1.33 (0.34–5.2)	0.68	1.32 (0.43–4.03)	0.62
Intraoperative radiation therapy	2.00 (0.19–20.7)	0.56	1.41 (0.18–10.6)	0.74
CX3CR1 expression				
% positive cells	1.04 (1.01–1.08)	0.044	1.022 (0.99–1.04)	0.08
Intensity (0–3)	2.56 (1.2–5.6)	0.018	2.01 (1.07–3.7)	0.028
Score (1–6)	2.03 (1.26–3.2)	0.003	1.61 (1.13–2.27)	0.007
Perineural invasion (% of involved nerves)	1.03 (0.99–1.06)	0.069	1.03 (1.007–1.06)	0.012

Variables	Multivariate analysis			
	Local recurrence of pancreatic carcinoma			
	Logistic regression		Cox regression	
	Odds ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
CX3CR1 score (1–6)	4.02 (1.2–12.9)	0.019	1.55 (1.02–2.3)	0.04
Perineural invasion (% of involved nerves)	1.025 (0.98–1.06)	0.22	1.02 (0.99-1.05)	0.204
Stage (1–4)	0.32 (0.08– 1.33)	0.11	0.66 (0.36–1.2)	0.42
Radicality (R1–2 versus R0)	0.74 (0.17–3.11)	0.68	1.07 (0.39–1.2)	0.89

NOTE: Univariate and multivariate logistic and Cox regression analyses of pancreatic cancer risk factors for local recurrence in the population of 49 relapsed patients. Among all the factors analyzed, only CX3CR1 expression and perineural invasion are significantly associated with local recurrence (univariate analysis of both logistic and Cox regression). The multivariate analysis, performed using variables significant at $P < 0.2$ in univariate analysis (stage and radicality), confirms the relevant role of CX3CR1 expression as independent factor for local recurrence in resected patients. For the univariate analysis, the variable CX3CR1 expression was evaluated as percentage of positive cells (the population divided in terziles), intensity of expression (0–3), and score (1–6; see Supplementary Fig. S5).

Values are presented as median (minimum − maximum) or averages ± SD, unless otherwise stated.

Results

Human pancreatic adenocarcinoma cells express the chemokine receptor CX3CR1. Ten human pancreatic tumor cell lines were tested for the expression of CX3CR1 mRNA. Six cell lines showed substantially higher expression compared with the immortalized cell line HPDE6 derived from normal pancreatic ducts (Fig. 1*A*). CX3CR1 mRNA was strongly up-regulated also in seven of seven surgical samples from which purified tumor cells (>95% cytokeratin 7 positive) were isolated. The expression levels were much higher compared with both HPDE6 cells (10-fold to 500-fold) and to a preparation of freshly isolated normal pancreatic ducts (4-fold to 100-fold; Fig. 1*B*).

CX3CR1 protein expression was confirmed in PDAC tumor samples by immunohistochemistry (Fig. 1*C*). Whereas the epithelial cells from exocrine normal pancreas were negative (*a*; note that only endocrine β-islets stained positive), CX3CR1 was highly expressed in ductal cancer cells (*b–f*). Interestingly, tumor cells adjacent to intrapancreatic nerves were strongly positive for the receptor (*d* and *e*). Comparison of the pattern of CX3CR1 expression with that of neurotrophin receptors, reported to be involved in the neurotropism of PDAC, was investigated with

antibodies to TRKA, one of the most expressed receptors (9). Both TRKA and CX3CR1 were expressed by virtually all PDAC, although with a distinct pattern of expression. While CX3CR1 was homogeneously expressed (*f*), TRKA accumulated in the apical part of the cells (*g*). Expression of the ligand CX3CL1 was detected in intrapancreatic nerves (*h*). Tumor cells were also faintly positive.

CX3CR1 on pancreatic tumor cells mediates chemotactic migration and enhanced survival. To confirm that the CX3CR1 receptor expressed by tumor cells was functional, we tested the cell lines with the highest expression (A8184 and AsPC-1) in classical chemotaxis assays. Recombinant CX3CL1 elicited significant migration of both tumor cell lines in a dose-response manner (Fig. 2*A*), which was strongly inhibited by a blocking anti-CX3CR1 mAb (Fig. 2*B*). A stable trasfectant expressing the CX3CR1 receptor and GFP was obtained from the cell line MiaPaCa2 (Supplementary Fig. S1). MiaPaCa2-GFP-CX3CR1 dose-dependently migrated to recombinant CX3CL1 gradients *in vitro* in contrast to MiaPaCa2-GFP cells (Fig. 2*C*). In addition, PDAC cell lines migrated to the conditioned supernatant obtained from cells of neural origin (neuroblastoma cell line SKN-BE), which produces CX3CL1 upon stimulation with TNFα/IFNγ (Supplementary Fig. S2*A* and *B*). Most of the enhanced migration of A8184 PDAC cells in response to the supernatant of activated neuroblastoma was inhibited by anti-CX3CR1, indicating that it was indeed mediated by CX3CL1 (Fig. 2*D*).

Because chemokines have been implicated in the protection of tumor cells from apoptosis (14, 17, 20), we tested whether CX3CL1 could deliver survival signals in PDAC. MiaPaCa2 tumor cells, transfected or not with CX3CR1, were exposed *in vitro* to proapoptotic conditions [treatment with interleukin 1β (IL-1β)/TNFα 20 ng/mL for 72 h]. When recombinant CX3CL1 (300 ng/mL) was added, only MiaPaCa2-GFP-CX3CR1 cells were partially rescued from apoptosis (Supplementary Fig. S3*A*).

Pancreatic tumor cells adhere *in vitro* to neural cells via CX3CR1/CX3CL1. To test the hypothesis that the CX3CR1/CX3CL1 pair is involved in the perineural invasion of pancreatic cancer cells, we investigated *in vitro* the interaction between CX3CR1-positive PDAC and the SKN-BE neuroblastoma expressing the membrane form of CX3CL1 after activation with TNFα/IFNγ. Adhesion of A8184 cells to TNFα/IFNγ-stimulated neuroblastoma was twice that of cells adhering to unstimulated cells (Supplementary Fig. S3*B*). This increased adhesion was inhibited by pretreatment of pancreatic cells with a blocking CX3CR1 antibody. In the same assay, another neuroblastoma cell line (SY5Y), unable to express the ligand CX3CL1 even after TNFα/IFNγ treatment, did not stimulate the adhesion of A8184 cells. The involvement of CX3CR1 in pancreatic cancer cell adhesion to neural cells was further confirmed by using CX3CR1-transfected cells. Only MiaPaCa2-GFP-CX3CR1 showed high adhesion to SKN-BE neuroblastoma cells stimulated to express the chemokine (Fig. 3*A*). Overall, the results indicate that CX3CR1 functions as an important adhesion molecule for PDAC tumor cells adhering to neural cells expressing the ligand.

Besides cells of neural origin, we also tested human endothelial cells, which are strong producers of CX3CL1 upon TNFα/IFNγ activation (refs. 25, 35; Supplementary Fig. S4*A*). The

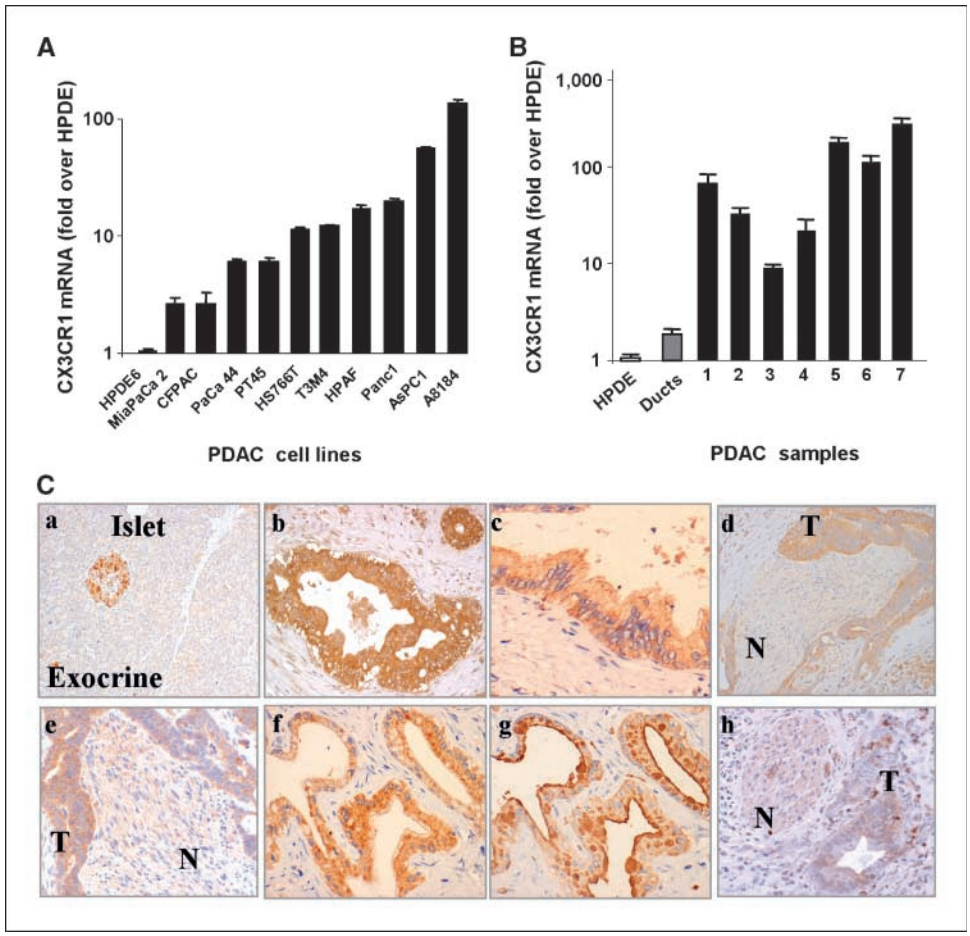

Figure 1. CX3CR1 is up-regulated in PDAC cell lines and surgical samples. *A, CX3CR1* mRNA in PDAC cell lines as assessed by semiquantitative real-time PCR (described in the supplementary data). The amount of *CX3CR1* mRNA, normalized to *β-actin*, is expressed as relative to the immortalized cell line HPDE6, derived from normal pancreatic ducts. *CX3CR1* is expressed (>10-fold over HPDE6) in 6 of 10 PDAC. *Columns,* mean of triplicates; *bars,* SD. *B, CX3CR1* mRNA expression in tumor cells from surgical samples of pancreatic cancer patients. In all samples, receptor expression is higher than HPDE6, as well as primary ducts isolated from normal pancreatic tissue (*gray column*). *Columns,* mean of triplicates; *bars,* SD. *C,* expression of CX3CR1 protein in PDAC surgical samples. *a,* immunohistochemistry with anti-CX3CR1 of normal human pancreas not expressing CX3CR1 (only endocrine islets stain positive); *b-f,* surgical samples of PDAC express CX3CR1. Tumor cells (*T*) adjacent to intrapancreatic nerves (*N*; *d, e*) are strongly positive for CX3CR1. *f, g,* consecutive slides of PDAC stained with anti-CX3CR1 (*f*) or with anti-TRKA neurotrophin receptor (*g*); differently from CX3CR1, TRKA staining accumulates in the apical part of neoplastic cells. *h,* PDAC stained with anti-CX3CL1 specific antibody. An intrapancreatic nerve (*N*) shows expression of CX3CL1; tumor cells (*T*) are also slightly positive. Magnification, ×100 (*a, b, d, f, g*) and ×200 (*c, e, h*).

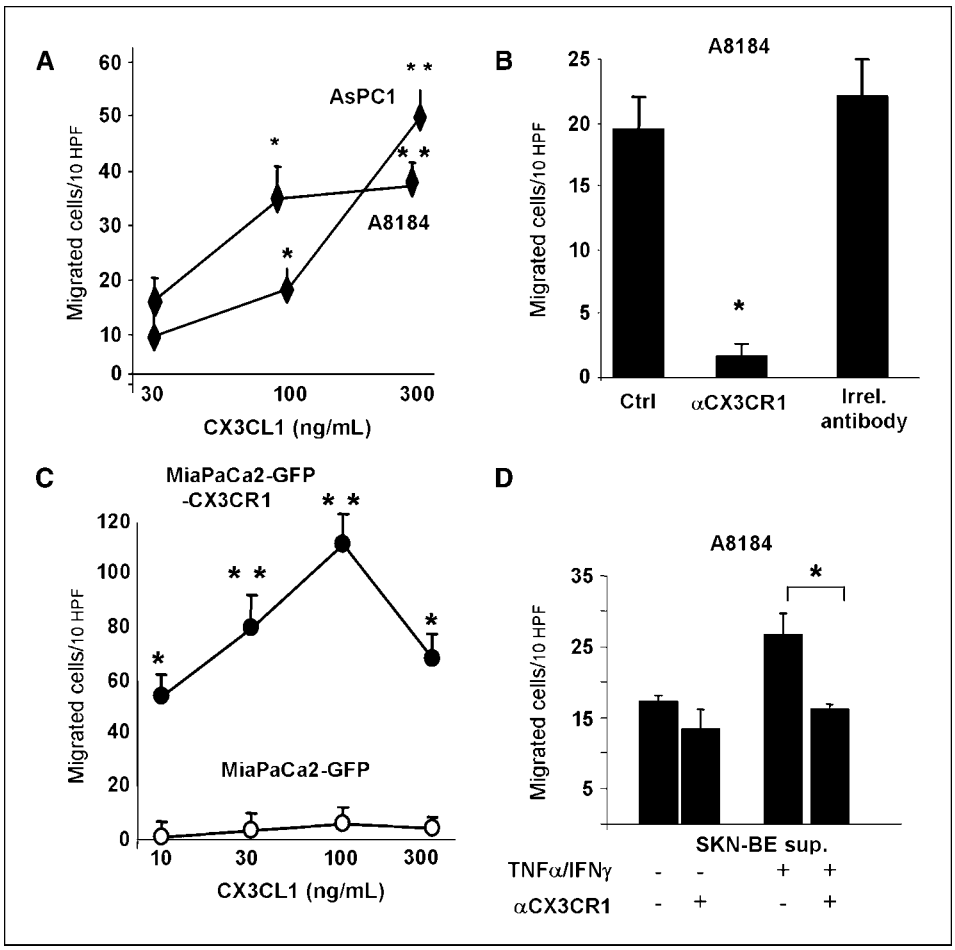

Figure 2. Chemotactic migration of CX3CR1-positive PDAC cells to recombinant and neural cell–derived CX3CL1. *A,* chemotaxis of the CX3CR1-positive PDAC cell lines, A8184 and AsPC1, to different concentrations of recombinant CX3CL1. *Points,* mean of three experiments; *bars,* SE. Net numbers of migrated cells over basal migration (medium alone). *, $P < 0.02$ versus control; **, $P < 0.001$ versus control, Student's *t* test. *B,* pretreatment of A8184 cells with a blocking anti-CX3CR1 mAb drastically reduces CX3CL1-induced chemotaxis. *, $P < 0.01$ versus control, Student's *t* test. *C,* the PDAC cell line MiaPaCa2, stably expressing the receptor (*MiaPaCa2-GFP-CX3CR1*; Supplementary Fig. S1), shows high chemotactic activity in response to CX3CL1, whereas the negative one (*MiaPaCa2-GFP*) is unresponsive. Values (net numbers of migrated cells over basal migration) are the mean ± SE of two experiments. **, $P < 0.001$ versus control, Student's *t* test. *D,* chemotaxis of PDAC cell line A8184 to the supernatant of SKN-BE neuroblastoma producing CX3CL1 upon stimulation with TNFα/IFNγ (Supplementary Fig. S2*A* and *B*). The enhanced migration, in response to CX3CL1, was inhibited in the presence of anti-CX3CR1 antibody. Values (net numbers of migrated cells over basal migration) are the mean ± SE of three experiments. *, $P < 0.02$ versus control, Student's *t* test.

receptor-positive A8184 cells showed enhanced adhesion to endothelial cells in a CX3CR1-dependent manner, in contrast to the receptor-negative PaCa44 cells (Supplementary Fig. S4*B*). These results bear relevance *in vivo*, as blood vessels are also importantly involved by the neoplastic spreading of pancreatic cancer (1, 2).

CX3CR1 mediates adhesion to neural cells via G-protein–dependent and β1 integrin–dependent mechanism. To better elucidate the mechanism by which CX3CR1-positive pancreatic tumor cells adhere to CX3CL1-expressing neural cells *in vitro*, we investigated the involvement of G protein–dependent signaling in this interaction. Preincubation with PTX, a G_i protein inhibitor, strongly reduced the adhesion of MiaPaCa2-GFP-CX3CR1 to SKN-BE neuroblastoma cells, indicating that this interaction requires PTX-sensitive G proteins (Fig. 3*A*). We then investigated the role of β1 integrins after CX3CR1 engagement. Pretreatment of pancreatic tumor cells with anti–β1 integrin blocking mAbs inhibited by 46% the CX3CL1-enhanced binding of MiaPaCa2-GFP-CX3CR1 cells to IFNγ-stimulated neuroblastoma (Fig. 3*B*). Concomitant addition of anti-CX3CL1 did not further inhibit adhesion, in line with the concept that membrane CX3CL1 initiates loose binding, followed by integrin-mediated firm adhesion.

Confocal microscopy showed that upon binding of MiaPaCa2-GFP-CX3CR1 cells to CX3CL1-expressing neuroblastoma, β1 integrins redistribute on the cell membrane of PDAC cells and cluster at the contact site, colocalizing with CX3CR1 (Fig. 3*C, a* and *b*). This clustering is absent in MiaPaCa2-GFP cells cocultured with stimulated IFNγ-neuroblastoma (Fig. 3*C, c*). We next investigated the activation of FAK, a signal transducer downstream of integrins. FAK was redistributed on the cell membrane upon coculture with CX3CL1-expressing neuroblastoma (Fig. 3*D, d*). The same redistribution of FAK and colocalization with β1 integrins were observed when MiaPaCa2-GFP-CX3CR1 cells were stimulated with recombinant CX3CL1 (Fig. 3*D, e*). Activation of FAK and β1 integrins did not occur in MiaPaCa2-GFP cells lacking the CX3CR1 receptor (Fig. 3*D, f*). Overall, the results indicate that the chemokine CX3CL1, expressed on the surface of neural cells, engages the CX3CR1 receptor on pancreatic tumor cells and activates downstream a G protein–dependent signaling, leading to the activation of β1 integrins and FAK, which stabilize the adhesion.

Involvement of CX3CR1 in the neurotropism of pancreatic cancer cells *in vivo*. To investigate *in vivo* the role of CX3CR1 in pancreatic cancer nerve tropism, MiaPaCa2-GFP-CX3CR1 cells and MiaPaCa2-GFP cells were transplanted in nude mice. In a first experiment, tumor cells were implanted orthotopically in the pancreas. Both cells lines grew in 3 to 4 weeks with a similar kinetics. Tumors were analyzed to detect the presence of intratumoral or peritumoral nerves, identified with an anti-S100 antibody. However, probably due to the different anatomy of the murine pancreas, no nerves were detected inside or around the tumor mass (not shown).

We then performed an experiment with PDAC cell lines transplanted s.c. After 3 weeks, tumors were excised and analyzed. Nerve terminations interspersed in the tumor mass and were easily

identified by S100 positivity in the peritumoral space. In tumors derived from MiaPaCa2-GFP cells, nerve terminations showed an intact morphology and were surrounded by neoplastic cells (Fig. 4*A, d–f*). In MiaPaCa2-GFP-CX3CR1 tumors, nerves were frequently infiltrated by neoplastic cells, as visualized by the spotted pattern of S100 positivity (Fig. 4*A, a–c*). By analyzing several tumor sections from distinct mice, the mean number of tumor-infiltrated nerves in MiaPaCa2-GFP-CX3CR1 tumors was 42 ± 11% (mean of 12 sections). Nerve infiltration was never observed in tumors derived from MiaPaCa2-GFP cells lacking the receptor (mean of 14 sections). The number of intact nerves (intratumoral +peritumoral) in MiaPaCa2-GFP-CX3CR1 tumors was significantly lower compared with MiaPaCa2-GFP tumors (mean ± SE, 9 ± 3 versus 16 ± 2, $P < 0.05$; Fig. 4*B*). The results suggest that expression of the CX3CR1 receptor confers to PDAC tumor cells an adhesive advantage to bind to nerve terminations. Proliferation of tumor cells neighboring peripheral nerves eventually leads to nerve invasion and subversion of nerve fibers.

Correlation between high CX3CR1 expression and grade of perineural invasion in pancreatic adenocarcinoma patients. To assess the actual relevance of CX3CR1 expression in pancreatic cancer, we evaluated a series of PDAC patients in a prospective study. All patients were recruited in the same center and presented similar characteristics of tumor stage and surgical and medical treatments (Supplementary Table S1). A tissue array with PDAC surgical specimens from 59 patients was prepared and tested by immunohistochemistry. CX3CR1 was expressed by cancer cells in 90% of the samples, with a heterogeneous pattern of positivity that was scored from 1 to 6 (percentage of positive cells + staining intensity), with 1 being the lowest expression (Supplementary Fig. S5). Patients' distribution according to immunohistochemistry results was as follows: score 1, 5 cases (8.4%); score 2, 8 cases (13.5%); score 3, 13 cases (22%); score 4, 12 cases (20.3%); score 5, 8 cases (13.5%); score 6, 13 cases (22%). CX3CR1 score was not significantly associated with tumor stage (tumor-node metastasis), grading, tumor localization, and size (Supplementary Table S1).

To test the hypothesis that high CX3CR1 expression was associated with perineural invasion, we performed a systematic evaluation of the grade of perineural invasion in H&E-stained surgical specimens retrieved from the same 59 patients used in the tissue array. An average of 7.4 ± 3 slides for each patient was carefully reviewed by two independent observers blinded to CX3CR1 expression values. A highly significant correlation between CX3CR1 expression score and perineural infiltration was observed ($P = 0.026$; Fig. 5*A*). Hence, high receptor expression is strongly associated with more prominent perineural tropism of neoplastic cells in pancreatic patients.

Correlation between high CX3CR1 expression and site and time of tumor recurrence in pancreatic adenocarcinoma patients. Because tumor cells hidden in local peripheral nerves and ganglia are a major cause of local recurrence (3–6), we analyzed the association between CX3CR1 expression, site of relapse, and disease-free survival after surgery. Patients were monitored every 8 weeks during chemotherapy and, thereafter, every 3 months (or in the case of suspected relapse). The relapse occurred in 49 of 59 patients (83.1%), with a median disease-free survival time of 394 days (337–450 days). Of the 49 patients with relapse, 19 (38.7%) had local recurrence. Notably, patients with high expression of CX3CR1 preferentially relapsed locally rather than at distant sites, with 72.7% of local recurrence in score 6 patients ($P = 0.037$; Fig. 5*B*). Moreover, local recurrence occurred earlier in patients with scores 5 and 6 compared with patients with scores 4 to 3 and scores 1 to 2 ($P = 0.007$; Fig. 5*C*). On the other hand, overall survival was not different in the patient groups (Fig. 5*D*).

Univariate logistic and Cox regression analyses were performed for the local recurrence after surgery. Both CX3CR1 expression (positive cell percentage, intensity, and score) and perineural invasion were strongly associated with local recurrence, whereas no other factors considered reached statistical significance (Table 1). The multivariate analysis, performed using variables significant at $P < 0.2$ in univariate analysis (stage and radicality), confirmed the relevant role of CX3CR1 expression as independent factor for local recurrence in resected patients (Table 1). Importantly, the high percentage of local recurrence was not ascribed to a different minimal residual disease (R0–R3) after surgical resection nor to different therapy (Supplementary Table S1).

Discussion

Although frequently underestimated, tumor perineural dissemination occurs in diverse human malignancies, such as those arising from bladder, prostate, head and neck, and colorectal cancers, and is usually associated with poor prognosis (3, 11, 36, 37). This peculiar pattern of invasion and metastasis is much more frequently observed in PDAC: intrapancreatic nerves can be involved in up to 90% of the patients (69% for extrapancreatic nerves; ref. 3). Such a high frequency may be due to anatomic reasons. Indeed, the human pancreas hosts a large amount of neural tissue, including ganglia, and it is in close proximity to abundant neural plexi in the retroperitoneum. Neoplastic cells hidden within intrapancreatic and extrapancreatic nerve terminations and ganglia are believed to represent an important source of local tumor recurrence (3–6).

In this study, we show that PDAC tumor cells strongly upregulate the chemokine receptor CX3CR1 (not expressed in the normal pancreatic epithelium) and provide experimental *in vitro* and *in vivo* evidence that CX3CR1 is involved in the neurotropism of PDAC cells to local peripheral nerves. The exclusive ligand of this receptor, CX3CL1, is expressed in neural tissues, both as a soluble chemokine and a membrane molecule. Our study shows that CX3CR1-positive PDAC cells migrate *in vitro* in response to neural-derived CX3CL1 and adhere to neural cells via the membrane-bound chemokine; *in vivo* in mice, CX3CR1-positive tumor cells have the capacity to infiltrate peripheral nerves; in tumor patients with PDAC, high receptor expression is significantly associated with a more prominent neural tropism and with a local recurrence of the disease.

The chemokine receptor CX3CR1 is predominantly expressed by hematopoietic cells (monocytes, natural killer cells, and Th1 lymphocytes) and specialized macrophages, such as microglia in the nervous system (21, 32–35). Its expression by tumor cells has been poorly investigated. Fatatis and colleagues reported that prostate tumor cells express the receptor, which was involved in metastasis to bone marrow (22, 23). In breast cancer, it was found to predict the occurrence of brain metastasis (24). Expression of CX3CR1 by human pancreatic tumor cells has never been reported. The pattern of positivity/intensity, as assessed in our tissue array of PDAC samples, was heterogeneous but a frequent finding, with half of the patients having 80% to 100% CX3CR1-positive cells. Of note, CX3CR1 expression was not associated with any biological features

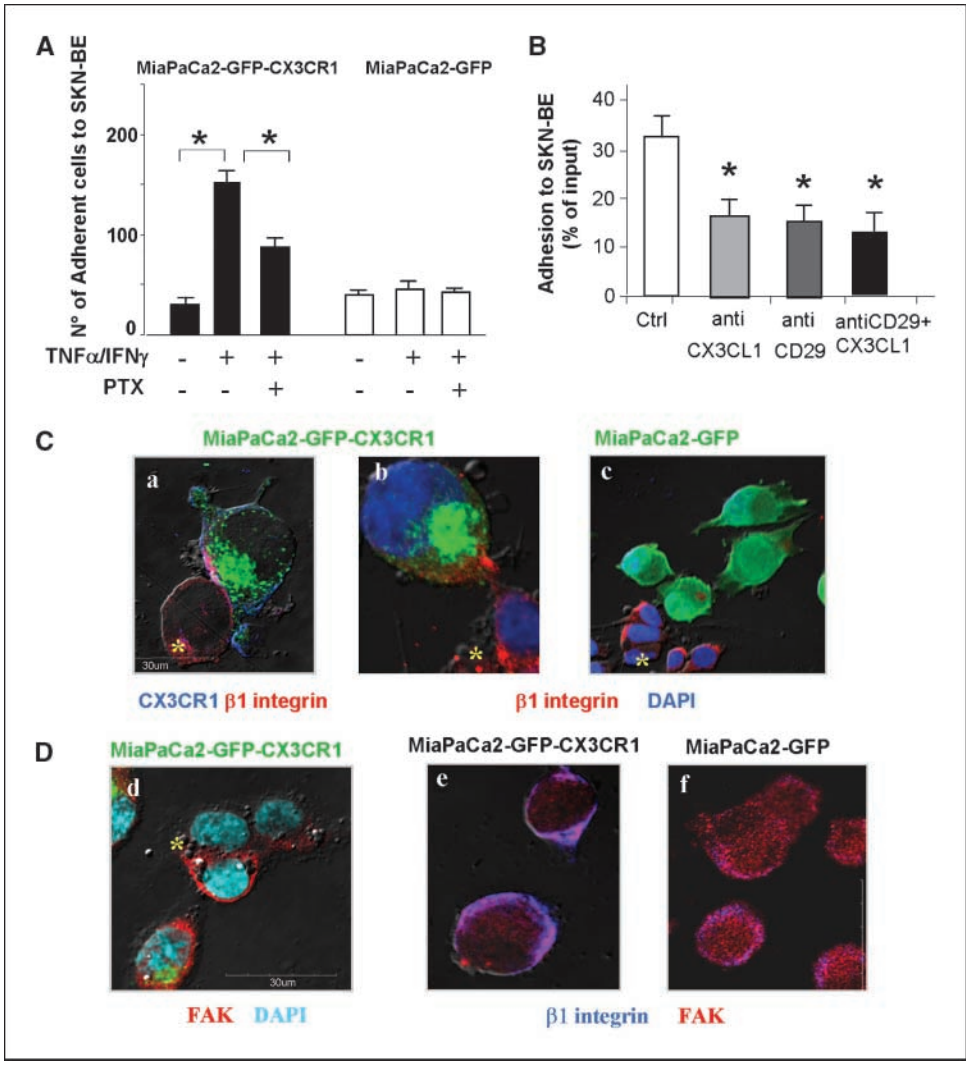

Figure 3. CX3CR1-mediated adhesion of PDAC to neural cells involves β1 integrins and activation of FAK. *A,* enhanced adhesion of MiaPaCa2-GFP-CX3CR1 cells to SKN-BE neuroblastoma cells stimulated with TNFα/IFNγ. Adhesion was inhibited by a 3-h pretreatment of PDAC with PTX. *Columns,* mean of two different experiments; *bars,* SE. *, $P < 0.02$ versus control, Student's *t* test. *B,* β1 integrin antibodies (*anti-CD29*) or anti-CX3CL1 inhibit PDAC adhesion to CX3CL1-expressing SKN-BE neuroblastoma. *Columns,* mean of three experiments; *bars,* SE. *, $P < 0.05$ versus control, Student's *t* test. *C,* confocal microscopy of β1 integrins in MiaPaCa2-GFP-CX3CR1 cells (*green*) cocultured with TNFα/IFNγ-stimulated SKN-BE neuroblastoma cells (*blue dapi, also marked with the asterisk*). β1 integrins (*red*) colocalize on the cell membrane of PDAC cells expressing CX3CR1 (*blue*) at the contact site with neuroblastoma cells (*a, b*). In MiaPaCa2-GFP cells (*green*) cocultured with TNFα/IFNγ–SKN-BE neuroblastoma (*asterisk*) β1 integrins are not clustered (*c*). *D,* FAK (*red*) accumulates at the contact site between MiaPaCa2-GFP-CX3CR1 and TNFα/IFNγ−SKN-BE neuroblastoma (*asterisk*; *d*). Recombinant CX3CL1 stimulates FAK polarization (*red*) and colocalization with β1-integrin (*blue*) in MiaPaCa2-GFP-CX3CR1 cells (*e*) but not in MiaPaCa2-GFP cells (*f*).

of the neoplasia, such as tumor grading, size, stage, and serum levels of CA19.9, nor to overall survival.

The marked up-regulation of CX3CR1 by PDAC has no clear explanation. Several chemokines and few receptors have been found to be overexpressed in tumor cells by oncogene-driven pathways (e.g., KRas, RET, BRAF; ref. 38), but no information has been reported for CX3CR1. Most pancreatic cancers express the activated form of Ras (2, 39). Of the 10 cell lines tested in this study, all expressed k-RAS but only six had detectable CX3CR1. Thus, additional genetic lesions or other factors present in the tumor microenvironment are likely to be responsible for the aberrant up-regulation of CX3CR1. We thought to gain some information by testing *in vitro* the effects of several cytokines on the modulation of CX3CR1 in PDAC cell lines and in the HPDE6 cell line derived from normal pancreatic ducts. None of the cytokines tested (i.e., IL-1β, TNFα, IFNγ, IL-6, IL-10, TGFβ) had significant effects on the transcription of CX3CR1 mRNA (data not shown). Therefore, the molecular mechanisms driving CX3CR1 up-regulation in pancreatic tumor cells remain to be elucidated.

Unlike other chemokines, CX3CL1 possesses intrinsic cell-adhesive properties in endothelial cells (21, 40) and neurons (41). In some studies, adhesion was reported to be independent of receptor signaling (42). It was therefore important to define the mechanistic basis of the CX3CR1-driven interaction of PDAC with neural cells. We found that the CX3CR1-mediated adhesion was PTX-sensitive and, hence, G protein signaling–dependent and lead to the clustering and activation of β1 integrins and FAK at the contact site with neural cells. Thus, initial adhesion is mediated by CX3CR1 binding to the membrane form of CX3CL1 in a classical "inside-out" signaling cascade, leading to stabilization operated by β1 integrins and FAK. Also, the recently described CX3CL1-mediated adhesion of neural cells to laminin, in the brain of newborn rats, occurred through integrin-dependent, PTX-sensitive mechanisms (41).

In our *in vivo* experimental model of transplanted PDAC, we observed that tumor cells bearing the CX3CR1 receptor frequently infiltrated the local s.c. nerves and proliferated within nerve fibers. In contrast, in tumors formed by cancer cells lacking the receptor, nerve infiltration was never observed. By analyzing several tumor samples, we concluded that the number of intact nerves was significantly lower in CX3CR1-positive tumors, suggesting that the overexpression of CX3CR1 confers to tumor cells an adhesive advantage to nerve fibers.

Our *in vivo* findings in the animal model are in accordance with the pathologic findings obtained with the surgical PDAC specimens

from resected patients; high score of CX3CR1 expressed by tumor cells is strongly associated with a high percentage of tumor-involved nerves examined histologically. This finding strongly supports our initial hypothesis that CX3CR1 expression by tumor cells is involved in PDAC neural tropism.

Although this correlation was highly significant ($P = 0.026$), we noticed that nine samples among the 59 specimens analyzed had high scores of CX3CR1 expression but low perineural tropism. Two common single-nucleotide polymorphisms identified in the open reading frame of the *CX3CR1* locus, T280M and V249I, result in impaired binding to CX3CL1 and reduced cell migration (43, 44). These haplotypes, and in particular homozygosity of the M280 allele, are associated with a reduced risk of cardiovascular pathologies (43, 44). In spite of the limited number of patients in our study, we sought to investigate whether the T280M and V249I polymorphisms could explain the low neurotropism of these specimens. The *CX3CR1* genotype was characterized in 58 patients. Supplementary Table S2 shows the frequency of allele distribution in our population, which did not differ from the expected frequencies reported in other studies (32, 44). As shown in Supplementary Fig. S6, seven samples carried three or four mutated alleles; specifically, four samples were MM/II, one sample was MM/VI, and two samples were TM/II. The degree of perineural infiltration of these samples was heterogeneous and only two cases (one MM/II and one MM/VI) showed a low percentage of tumor-infiltrated nerves but high CX3CR1 (score 4). Among the other samples with peculiar behavior, four had no mutations (TT/VV) and the remaining three were TT/VI. In essence, we found no significant association between the T280M/V249I allelic polymorphisms and low nerve infiltration by tumor cells, the focus of our effort. As a matter of fact, the functional adhesive ability of polymorphic variants of CX3CR1 is controversial in the literature, as some studies have reported an enhanced adhesivity by the II and MM haplotypes (45). The genetic polymorphism at the *CX3CR1* locus in pancreatic cancer deserves to be addressed in larger case lists of patients.

An interesting characteristic of the chemokine CX3CL1 in the central nervous system is to function as a neuron-to-glia communicator. Recent animal studies have reported that CX3CL1 is implicated in the development of neuropathic pain via involvement of the microglia expressing the CX3CR1 receptor (34, 46, 47). When the chemokine was infused intrathecally, it enhanced the nociceptive response and the effect was abrogated or decreased by anti-CX3CR1 antibodies (46). Microglia involvement was also required in a model of spinal nerve ligation (48). Pancreatic cancer is a very aggressive neoplasia, and pain is not infrequent in PDAC patients, especially in the late stages. As CX3CR1 expression by tumor cells was associated with a more marked neurotropism, we asked whether patients with high receptor expression experienced a deeper perception of pain. Information on the pain felt by the patients was collected from available clinical records. A score of pain was attributed along a questionnaire filled up by the patients and on the basis of analgesic consumption and ranged from 0 to 3 (Supplementary Data). However, being surgical patients with small tumors (which usually provoke limited pain), the majority of our patients had no or mild pain (scores 0 + 1, 64.8%; score 2, 31.5%; score 3, 3.7%). No significant correlation was found with CX3CR1 receptor score; in those patients having high score pain, there was a trend to have a higher degree of tumor-infiltrated nerves, although without reaching statistical significance (not shown).

We therefore decided to investigate whether mice bearing CX3CR1-expressing tumors had enhanced nociceptive sensation. Mice were injected with tumor cells in the dorsal hind paw. Response to heat stimuli applied to paws was assessed using the classical thermal sensitivity test (hot plate test), wherein threshold

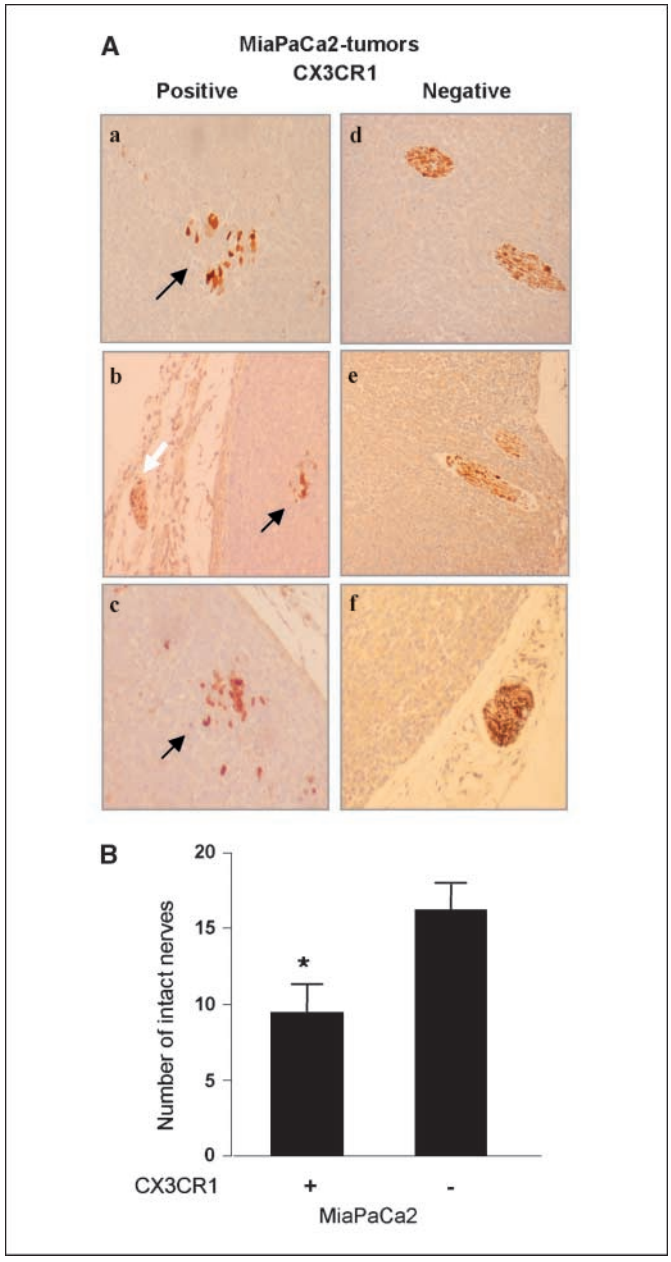

Figure 4. Expression of CX3CR1 by PDAC cells confers the ability to infiltrate *in vivo* nerve terminations. *A,* immunohistochemistry of tumors originated from transplanted MiaPaCa2-GFP-CX3CR1(*a–c*) or MiaPaCa2-GFP cells (*d–f*) injected s.c. in nude mice. Intratumoral and peritumoral nerves were stained with anti-S100 antibody. A spotted pattern of S100 staining is shown in nerves from MiaPaCa2-GFP-CX3CR1 tumors (*black arrows*), consistent with nerve infiltration by tumor cells. Nerve morphology was always unaffected in MiaPaCa2-GFP tumors. In *b*, the white arrow points to an intact nerve outside the tumor area in a MiaPaCa2-GFP-CX3CR1 tumor and the black arrow indicates an injured nerve inside the tumor mass. Magnification, ×40 (all pictures), but ×20 (*b*). *B,* quantification of the number of intact intratumoral + peritumoral nerves. The mean number of intact nerves in tumors derived from MiaPaCa2-GFP-CX3CR1 was significantly lower compared with MiaPaCa2-GFP tumors (mean of 12 and 14 sections, respectively; $P < 0.05$, Student's *t* test).

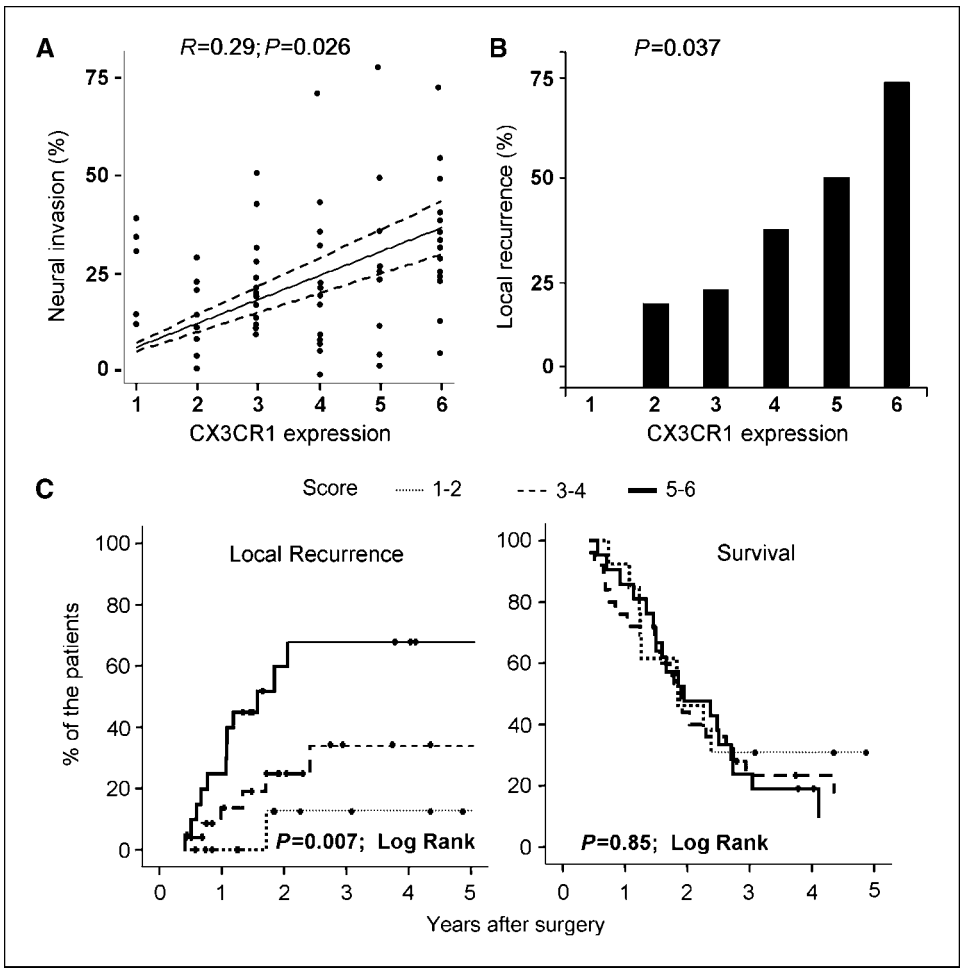

Figure 5. CX3CR1 expression by tumor cells correlates with neural invasion and local recurrence in pancreatic cancer patients. *A,* relationship between CX3CR1 expression and tumor perineural invasion. Patients were divided according to CX3CR1 expression (scores 1–6), wherein 1 is the lowest expression (Materials and Methods; Supplementary Fig. S5). Tumor perineural invasion, assessed in H&E-stained slides, was defined as the presence of cancer cells in the perineurium of nerve fascicles. Results are shown as percentage of involved nerves over total number of nerves counted (intratumor and peritumor within 0.5 cm of neoplastic edge). Each dot represents a tumor sample from an individual patient ($n = 59$). Linear regression (*solid line*) and 95% mean prediction interval (*dashed lines*) indicated that the two variables are linearly correlated ($P = 0.026$). *B,* relationship between CX3CR1 expression and site of recurrence. The percentage of patients with local recurrence (first recurrence after surgery) is reported for each group of CX3CR1 score. Patients with high CX3CR1 score experienced more frequently a local recurrence. Statistical analysis was performed by χ^2 test ($P = 0.036$). *C,* time interval between the surgery of primary tumor and local recurrence. *D,* overall survival by Kaplan-Meier estimates of event rates after cancer removal. The progression-free survival and the site of progression were assessed every 8 wk during chemotherapy and thereafter every 3 mo (or in the case of suspected relapse). Patients were divided according to CX3CR1 expression in three groups (scores 1-2, 3-4, 5-6). Patients with high score (5–6) had a statistically significant earlier local recurrence ($P = 0.007$; *C*). Statistical analysis was performed by log-rank test. Overall survival was not different in the three score groups ($P = 0.85$; *D*).

for behavioral response to heat stimuli is analyzed. The experiment showed that, throughout tumor growth, there was no significant difference in the pain threshold of mice bearing MiaPaCa2-GFP-CX3CR1 tumors compared with those bearing MiaPaCa2-GFP tumors or mice without tumors (Supplementary Fig. S7). Thus, in our experimental conditions, mice bearing CX3CR1-expressing tumors did not have an increased nociceptive sensation. Some considerations can be made to explain this lack of correlation. In the studies where CX3CL1 was reported to enhance neuropathic pain, the chemokine was infused in the central nervous system or after acute injury (46). These conditions are clearly distinct from the slow and progressive growth of tumors. Indeed, the chemokine was not implicated in experiments of chronic injury (49).

Another interesting finding of our work is that high CX3CR1 receptor expression was associated with a significantly higher frequency of local and earlier recurrence. This finding gives strong support to the assumption that tumor cells harbored in nerve fibers and ganglia are an important source of local relapse after surgery (3–6). Both univariate and multivariate analysis confirmed the relevant role of CX3CR1 expression as an independent factor.

In conclusion, this study shows that the chemokine receptor CX3CR1 is an important determinant of pancreatic cancer spreading along peripheral nerves. In PDCA patients, expression of CX3CR1 by tumor cells is a relevant risk factor to predict perineural invasion and early local tumor recurrence after surgery.

The complex network of chemokines and their receptors in the tumor microenvironment is currently the object of an intense investigation searching for innovative therapeutic approaches (50). Given the importance of perineural invasion in pancreatic tumor relapse, the CX3CR1-CX3CL1 axis could represent a valuable therapeutic target. Antagonists to CX3CR1 could specifically inhibit the spread of pancreatic tumor cells to local peripheral nerves. Novel approaches, specifically targeting the nerve tropism by tumor cells, deserve further investigation and could represent a valid complementary strategy to more conventional medical treatments.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no competing financial interests.

Acknowledgments

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