Inflammatory Monocyte Mobilization Decreases Patient Survival in Pancreatic Cancer: A Role for Targeting the CCL2/CCR2 Axis

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Abstract

Purpose: To determine the role of the CCL2/CCR2 axis and inflammatory monocytes (CCR2⁺/CD14⁺) as immunotherapeutic targets in the treatment of pancreatic cancer.

Experimental Design: Survival analysis was conducted to determine if the prevalence of preoperative blood monocytes correlates with survival in patients with pancreatic cancer following tumor resection. Inflammatory monocyte prevalence in the blood and bone marrow of patients with pancreatic cancer and controls was compared. The immunosuppressive properties of inflammatory monocytes and macrophages in the blood and tumors, respectively, of patients with pancreatic cancer were assessed. CCL2 expression by human pancreatic cancer tumors was compared with normal pancreas. A novel CCR2 inhibitor (PF-04136309) was tested in an orthotopic model of murine pancreatic cancer.

Results: Monocyte prevalence in the peripheral blood correlates inversely with survival, and low monocyte prevalence is an independent predictor of increased survival in patients with pancreatic cancer with resected tumors. Inflammatory monocytes are increased in the blood and decreased in the bone marrow of patients with pancreatic cancer compared with controls. An increased ratio of inflammatory monocytes in the blood versus the bone marrow is a novel predictor of decreased patient survival following tumor resection. Human pancreatic cancer produces CCL2, and immunosuppressive CCR2⁺ macrophages infiltrate these tumors. Patients with tumors that exhibit high CCL2 expression/low CD8 T-cell infiltrate have significantly decreased survival. In mice, CCR2 blockade depletes inflammatory monocytes and macrophages from the primary tumor and premetastatic liver resulting in enhanced antitumor immunity, decreased tumor growth, and reduced metastasis.

Conclusions: Inflammatory monocyte recruitment is critical to pancreatic cancer progression, and targeting CCR2 may be an effective immunotherapeutic strategy in this disease. Clin Cancer Res; 19(13); 3404–15. ©2013 AACR.

Introduction

Pancreatic ductal adenocarcinoma, often called pancreatic cancer, is an aggressive malignancy with a death rate nearly equal to its incidence and a 5-year survival of less than 5% (1). Pancreatic cancer is characterized by a unique-
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Translational Relevance
Pancreatic cancer is an aggressive malignancy with a dismal prognosis, due in part to a high rate of metastatic dissemination and chemoresistance. Macrophages are predominant in the uniquely dense pancreatic cancer stroma, and these cells enhance tumor growth and metastasis. Inflammatory monocytes (CD14+/CCR2−) depend on CCL2/CCR2 signaling for mobilization from the bone marrow to the blood as well as recruitment to sites of inflammation where they extravasate into tissues and become macrophages. The role of inflammatory monocytes in pancreatic cancer as well as the effect of targeting these cells through CCR2 blockade has not been studied. These studies show that inflammatory monocyte recruitment from the bone marrow is prognostically and therapeutically important in pancreatic cancer. This study has laid the foundation for an ongoing phase 1b trial using CCR2 blockade in combination with chemotherapy in patients with pancreatic cancer with locally advanced, nonmetastatic disease (NCT01413022).

CX3CR1hi/CD14−/CCR2− in humans and mice (7). Resident monocytes represent approximately 15% of circulating monocytes in normal healthy humans and 40% to 50% in mice (9, 10). These cells arise from the differentiation of inflammatory monocytes in the periphery where they play a role in steady-state immunosurveillance and inflammation resolution (7, 9). In contrast, inflammatory monocytes are CD14+/CCR2+/CD16−/CX3CR1low in humans and Ly6Chi/CCR2+/CD16+/CX3CR1low in mice. These cells are the predominant monocytes in circulation representing approximately 85% of circulating monocytes in normal healthy humans and 50% to 60% in mice (8, 9). Under physiologic conditions, the CCL2/CCR2 chemokine axis is critical to the mobilization of inflammatory monocytes from the bone marrow to the blood as well as their recruitment to sites of inflammation where they extravasate into tissues and differentiate into macrophages or dendritic cells (7). Macrophages can be critical regulators of tumor progression (6); however, these cells are not highly prevalent in normal pancreas and must be selectively recruited during malignant progression (2). As such, monocyte mobilization from the bone marrow is a vital pathway for macrophages to infiltrate tumors in the periphery. Once assimilated, macrophages acquire an immunosuppressive, trophic (alternatively activated or M2) phenotype in the tumor microenvironment and at the premetastatic site (6).

In these studies, we show that both the prevalence of monocytes in the peripheral blood and the mobilization of inflammatory monocytes from the bone marrow are predictive of survival in patients with pancreatic cancer. Furthermore, we show that pancreatic cancer uses the CCL2/CCR2 axis to favor the mobilization and recruitment of inflammatory monocytes from the bone marrow to the primary tumor and premetastatic liver where these cells facilitate tumor growth and metastasis. We identify CCR2 inhibition (CCR2i) using a novel agent (PI-04136309) as an adjunct to standard chemotherapy in pancreatic cancer, which blocks inflammatory monocyte recruitment resulting in the reduction of tumor growth and metastasis.

Materials and Methods
Analysis of peripheral blood monocyte prevalence and survival with multivariate analysis
All patients (n = 483) with pancreatic cancer undergoing pancreaticoduodenectomy at Barnes-Jewish Hospital (St. Louis, MO) between 1997 and 2011 were followed for survival in a prospectively maintained database under an Internal Review Board (IRB)-approved protocol. We excluded patients with elevated preoperative leukocyte counts (>11,000 cells/dL; n = 50), patients who did not have preoperative complete blood counts (CBC) obtained at our institution (n = 49), and patients who died within 30 days of surgery (n = 7). Patients were stratified into 3 groups based on the percentage of blood leukocytes, which were monocytes (monocyte prevalence) on preoperative CBC: low (<6%), normal (≥6% to <11%), and high (≥11%) monocyte groups. Ranges were established such that all patients in the mid group fell within 1 SD of the mean; thus, patients in the low and high monocyte groups were greater than 1 SD below and above the mean, respectively. Multivariate analysis was conducted using pancreatic cancer patient demographic and pathologic data. Further analytic details are described in the Supplementary Methods.

Isolation of blood, bone marrow, and tumor from pancreatic cancer patients
Informed consent was obtained from all patients in accordance with institutional Human Studies Committee Protocol. Peripheral blood and bone marrow mononuclear cells (BMMC) were isolated from healthy volunteers and patients with pancreatic cancer before chemotherapy, radiotherapy, or surgery, as has been previously described (11). Human pancreatic adenocarcinomas and normal pancreas were snap-frozen in liquid nitrogen or minced, mechanically dissociated, digested in enzyme buffer for 30 minutes, and filtered.

Mice, cell lines, and murine pancreatic cancer model
C57BL/6 and Ccr2−/− mice (B6.129S4-Cr2tm1Hls/J) were purchased from The Jackson Laboratory. The murine pancreatic adenocarcinoma cell line KCKO, a metastatic tumor line, was the kind gift of Dr. Pinku Mukherjee (University of North Carolina at Charlotte, Charlotte, NC; ref. 12). Eight-to 10-week-old mice were anesthetized and injected in the tail of the pancreas with 1 × 10^5 KCKO cells suspended in a 1:1 PBS:Matrigel mixture. After mice were sacrificed at indicated times, bone marrow was extracted from the femurs and blood collected in heparinized capillary tubes. Blood and bone marrow cells were subjected to red blood cell (RBC) lysis (BioLegend) as per manufacturer’s protocol. Orthotopic tumor burden was measured by the gross wet...
weight of the pancreas. Metastatic and disseminated tumors were scored by serial sectioning and gross evaluation, which was validated by tissue pathology.

Chemotherapy and CCR2 inhibitor
PF-04136309 (Pfizer) is a CCR2 kinase antagonist and the details have been published previously (13). Mice were injected subcutaneously with 100 mg/kg of PF-04136309 twice-daily beginning 2 days after tumor implantation. Mice were also injected intravenously with 50 mg/kg of gemcitabine (GEM; Hospira) into the retro-orbital sinus every 4 days. When indicated gemcitabine and PF-04136309 were given in combination without altering dose or schedule of either agent separately.

Flow cytometry
Human and mouse single-cell suspensions were blocked with TrueStain FeX or anti-CD16/32 antibody, respectively (BioLegend) and stained with fluorescent antibodies using standard protocols for flow cytometry. Cells undergoing intracellular staining were permeabilized with eBioscience Permeabilization Buffer according to the manufacturer’s protocol. Antibodies used for human staining are listed in Supplementary Methods.

RNA isolation and real-time PCR
Total RNA was isolated by TRIzol extraction and reverse transcribed into cDNA. Quantitative real-time PCR (qRT-PCR) was conducted using predesigned TaqMan Gene Expression Assays (Life Technologies) on a 7500 Fast Thermal Cycler (Applied Biosystems). Target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HPRT1, or β-actin. The normalized expression levels of genes were analyzed using the 7500 software for 7500 RT-PCR system V2.0.6.

T-cell proliferation assays
CD14+ cells were isolated from peripheral blood mononuclear cells (PBMC) of patients with pancreatic cancer before tumor resection and single cell tumor suspensions with the EasySep Human CD14 Positive Selection Kit as per manufacturer’s instructions (STEMCELL Technologies). CD14-depleted PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) and cocultured in 96-well round-bottom plates (Corning) coated with LEAF purified anti-human CD3 (BioLegend, clone OKT3) with varying concentrations of autologous CD14+ cells in complete media supplemented with human interleukin (IL)-2 (NIH, Bethesda, MD). Cell cultures were harvested after incubating for 72 hours at 37°C and the CFSE dilution of the CD4+ and CD8+ T-cell fractions were analyzed by flow cytometry. The division index (defined as the average number of divisions that a cell present in the starting population has undergone) was calculated using Flowjo software.

Immunofluorescence and immunohistochemistry
Tissue sections from formalin-fixed paraffin-embedded tissue blocks of human pancreatic cancer and normal pancreas were stained as previously described (11). Confocal images were acquired on an Axiovert 100M microscope equipped with a LSM 510 META Confocal Laser Scanning Microscope system (Zeiss). Immunohistochemical images were acquired at ×10 magnification on an Olympus RX51 microscope with a SPOT RT Slider digital camera and software (Diagnostic Instrument, Inc.). See Supplementary Methods for further details.

Pancreatic cancer tissue microarray survival analysis
After obtaining IRB approval, tissue microarray (TMA) studies were conducted on a cohort of 60 previously untreated patients with pancreatic cancer who underwent pancreaticoduodenectomy at Barnes-Jewish Hospital. Patients did not receive neo-adjuvant therapy and were typically treated with adjuvant chemotherapy. To construct the TMA, well-defined areas of tumor were demarcated and punched (1-mm diameter) from paraffin-embedded tumor blocks. An Aperio Scan-Scope XT Slide Scanner (Aperio Technologies) system was used to acquire digital images using a ×20 objective. A tumor-specific nuclear algorithm (IHC-MARK) developed in-house (14, 15) was modified to quantify CCL2 and CD8 expression.

Statistical analysis
All data (other than multivariate analyses) were analyzed using GraphPad Prism version 5.01 (GraphPad Software Inc.). Calculating differences in numerical values was conducted using Mann–Whitney test for nonparametric data. Fisher exact test was used to compare categorical data. \( P < 0.05 \) defined statistically significant differences.

See Supplementary Methods for additional methodological data.

Results
Decreased monocytes in the peripheral blood is associated with better survival in pancreatic cancer patients
To investigate the importance of monocytes in pancreatic cancer, we examined whether there was a correlation between survival and the prevalence of monocytes in the blood of 377 patients with pancreatic cancer. All patients were chemotherapy naïve, diagnosed with local, surgically resectable disease, and underwent pancreaticoduodenectomy. Patients were stratified into low (<6% of leukocytes, >1 SD below mean), mid (≥6% to <11% of leukocytes, within 1 SD of mean), or high (≥11% of leukocytes, 1 SD above mean) monocyte groups based on the prevalence of monocytes in their preoperative CBC. There was a significant correlation between the prevalence of preoperative monocytes in the peripheral blood of patients with pancreatic cancer and overall survival (Supplementary Table S1). Patients with high blood monocytes had significantly decreased overall survival compared with the rest of the cohort with a 5-year survival of 11.0% versus 22.1% (\( P = 0.03 \); Fig. 1A). In addition, low blood monocyte count was found to be a prognostic factor for survival in patients with
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Figure 1. The prevalence of peripheral blood monocytes is prognostic in patients with pancreatic cancer. A, patients were stratified into low (<6% of leukocytes, >1 SD below mean), mid (6% to <11% of leukocytes, within 1 SD of mean), and high (>11% of leukocytes, >1 SD above mean) peripheral blood monocyte groups. Kaplan–Meier survival curves compare patients with pancreatic cancer in high (A) and low (B) blood monocyte groups to the rest of the cohort as well as patients in low, mid, and high blood monocyte groups separately (C). P values are by log-rank (Mantel-Cox) test.

pancreatic cancer compared with the rest of the cohort with a 5-year survival of 28.8% versus 20.2% (P = 0.03; Fig. 1B). When comparing patients in the low, mid, and high monocyte groups separately, there was an incremental decrease in survival as blood monocytes increased; 5-year survival was 28.8% versus 20.9% versus 11.0% (mean survival = 35.6 months vs. 27.6 months vs. 21.1 months; P = 0.02) in the low, mid, and high groups, respectively (Fig. 1C).

As monocyte prevalence in the blood correlated inversely with survival on univariate analysis, we conducted a multivariate analysis using patient demographic and pathologic data to determine if monocyte count was an independent predictor of survival in patients with pancreatic cancer. Indeed, low blood monocyte prevalence was independently associated with increased survival on multivariate analysis [HR, 0.58; 95% confidence interval (CI), 0.40–0.86]. However, the association between high monocyte prevalence and survival was not independent of other pathologic factors. Compared with the rest of the cohort, patients in the high monocyte group had a strong trend toward having a higher incidence of lymph node–positive tumors (81.5% vs. 68.1%; P = 0.066), which is a strong predictor of decreased survival after tumor resection (16). These findings indicate that monocyte prevalence in the peripheral blood is a prognostic indicator for patient survival, and thus targeting monocytes might represent an attractive therapeutic strategy.

Inflammatory monocyte mobilization from the bone marrow is prognostic in pancreatic cancer patients

As with other chronic inflammatory conditions, monocytes are elevated in the peripheral blood of patients with solid organ malignancy (17), including pancreatic cancer (Supplementary Fig. S1A). Given the fact that the bone marrow acts as a storage reservoir for monocytes, we hypothesized that inflammatory monocytes are being mobilized from the bone marrow to the peripheral blood in human pancreatic cancer (7). Therefore, we analyzed the prevalence of inflammatory monocytes and resident monocytes in PBMCs and BMMCs from patients with nonmetastatic pancreatic cancer before any treatment (i.e., chemotherapy, radiotherapy, or surgical resection) and compared these with normal controls (Fig. 2A). We found that inflammatory monocytes were significantly more prevalent in the blood of patients with pancreatic cancer (Fig. 2B), however, the prevalence of resident monocytes was unchanged (Supplementary Fig. S1B). Consistent with previous reports, inflammatory monocytes made up 85% of blood monocytes in healthy individuals (8), and were increased to 92% in patients with pancreatic cancer (Supplementary Fig. S1C). Analysis of the bone marrow revealed that the prevalence of inflammatory monocytes were significantly decreased in patients with pancreatic cancer compared with controls, whereas resident monocytes were unchanged, suggesting that there is a shift in inflammatory monocyte equilibrium from the bone marrow to the peripheral blood during pancreatic cancer (Fig. 2C).

Given the finding that patients with elevated peripheral blood monocytes have worse overall survival, we hypothesized that the ratio of inflammatory monocytes in the blood versus the bone marrow (blood:bone marrow inflammatory monocyte ratio) may serve as a surrogate for inflammatory monocyte mobilization which could contribute to pancreatic cancer progression and ultimately decrease survival. This ratio was increased in patients with pancreatic cancer compared with normal controls (Supplementary Fig. S1D). Importantly, patients with pancreatic cancer who experienced rapid treatment refractory recurrence and death within the first year following tumor resection had significantly higher blood:bone marrow inflammatory monocyte ratios compared with 1-year pancreatic cancer survivors (Fig. 2D). Similarly, patients with pancreatic cancer with blood:bone marrow inflammatory monocyte ratios $\geq 1.5$ had significantly decreased survival (Supplementary Fig. S1D). This suggests that inflammatory monocyte mobilization plays a key role in pancreatic cancer patient outcome.
Human pancreatic tumors express CCL2 and are infiltrated by immunosuppressive CCR2+ macrophages

The recruitment of inflammatory monocytes in various human inflammatory diseases, such as rheumatoid arthritis (18), type I diabetes (19), and atherosclerosis (20) is mediated by the chemokine CCL2 and its receptor CCR2. Gene expression analysis found that human pancreatic cancer tissue has elevated CCL2 mRNA compared with normal pancreas by both qRT-PCR analysis of surgical specimens and by retrospective analysis of published datasets (ref. 21; Fig. 3A; Supplementary Fig. S2A). Upon further analysis of tumors, CCL2 protein was highly expressed by malignant ducts as well as cells apparently within the stroma, which could represent stromal cells and/or cancer cells in epithelial–mesenchymal transition (Fig. 3A and Supplementary Fig. S2B).

Pancreatic tumors possess a highly immunosuppressive microenvironment in which myeloid cells are crucial (2, 3, 11, 22). There is evidence that inflammatory monocytes are recruited to tumors where they differentiate into immunosuppressive TAM (23–25). CCR2+ TAM (CD45+/CD11b+/CD115+/HLA-DR+/CD14+/CCR2+) reside in human pancreatic tumors, and make up roughly 28% of tumor-infiltrating leukocytes (Fig. 3B and Supplementary Fig. S2C). However, effector T cells are significantly outnumbered by immunosuppressive TAM as CD8 T cells make up only around 7% of tumor-infiltrating leukocytes (Fig. 3B and Supplementary Fig. S2C). We sought to determine if inflammatory monocytes are immunosuppressive in the peripheral blood of patients with pancreatic cancer or whether tumor infiltration is a prerequisite. We isolated peripheral blood of patients with pancreatic cancer or inflammatory monocytes are immunosuppressive in the peripheral blood and bone marrow of patients with pancreatic cancer (n = 21) and healthy controls (n = 11). Graphs compare the prevalence of inflammatory monocytes in the blood in peripheral blood and bone marrow of patients with pancreatic cancer (n = 21) and healthy controls (n = 11). Graphs show means ± SEM, and P values are by Mann–Whitney test.

Figure 2. Inflammatory monocyte (IM) equilibrium in the peripheral blood and bone marrow is prognostic in patients with pancreatic cancer (PC). A, flow cytometry gating strategies used to define inflammatory monocytes (CD115+/CD16+ CX3CR1hi/M CCR2+/CD14+/CCR2+) and residents monocytes (RM) (CD115+/CD16–/CX3CR1low/CD14–/CCR2low) in peripheral blood and bone marrow (C) of patients with pancreatic cancer and controls. D, graph compares the blood:bone marrow inflammatory monocyte ratios of patients with pancreatic cancer with <1 year survival to >1 year survivors following tumor resection. All graphs show means ± SEM, and P values are by Mann–Whitney test.

**Human pancreatic tumors express CCL2 and are infiltrated by immunosuppressive CCR2+ macrophages**

Both inflammatory monocytes and TAM from the fresh blood and tumors, respectively, of individual patients with pancreatic cancer (Supplementary Fig. S2D), and compared their abilities to suppress autologous effector T-cell proliferation. We observed that while TAMs were markedly immunosuppressive, inflammatory monocytes were not (Fig. 3C). This suggests that the tumor microenvironment changes the phenotype of inflammatory monocytes following infiltration.

Patients with pancreatic cancer having tumors with high TAM:CD8 T-cell ratios have a poor prognosis (15). Similarly, patients having tumors which express high CCL2 with a low CD8 T-cell infiltrate (upon univariate analysis of a TMA) also have significantly reduced survival compared with patients with tumors expressing low CCL2 and having high CD8 T cells (Fig. 3D). This further shows the critical tumor cell–stroma interplay that is characteristic of pancreatic cancer (5).

**CCR2 mediates inflammatory monocyte mobilization in murine pancreatic cancer**

To determine if targeting inflammatory monocyte recruitment through CCR2 signaling inhibition could prevent inflammatory monocyte mobilization from the bone marrow in pancreatic cancer, we used a murine model. KCKO is a metastatic cell line derived from a genetically engineered, spontaneous murine pancreatic cancer model (LSL-KRAS<sup>C12D</sup> × p48-Cre; ref. 12). Like human pancreatic cancer, KCKO tumors express increased CCL2 compared with normal murine pancreas (Supplementary Fig. S3A). Also, KCKO does not express CCR2 in vivo. We injected KCKO orthotopically into the pancreas of wild-type (WT)
mice to study the effects of pancreatic cancer on inflammatory monocyte mobilization from the bone marrow. Mimicking our observations in patients with pancreatic cancer, tumor-bearing mice displayed significant increases in circulating inflammatory monocytes, whereas these cells were decreased in the bone marrow (Fig. 4A). Signaling through CCR2 is crucial to monocyte egress from the bone marrow (26, 27). We observed that tumor-bearing WT mice treated with a CCR2 antagonist (PF-04136309) and tumor-bearing CCR2−/− mice exhibit a significant decrease in circulating inflammatory monocytes (Fig. 4A and B and Supplementary Fig. S3B). In addition, these mice had an increased prevalence of inflammatory monocytes in the bone marrow compared with vehicle-treated tumor-bearing mice, suggesting that these cells are retained in the bone marrow (Fig. 4A and C and Supplementary Fig. S3C). Furthermore, CCR2 blockade reverses the blood:bone marrow inflammatory monocyte ratio in tumor-bearing mice, which we have shown to be prognostically important in patients with pancreatic cancer (Fig. 4D).

**CCR2 inhibition promotes antitumor immunity in murine pancreatic cancer**

Like human pancreatic cancer, KCKO implanted in the pancreas of mice is characterized by a dense stromal infiltrate with a predominance of immunosuppressive myeloid cells (Supplementary Fig. S4A). We found that tumor-bearing WT mice treated with CCR2i and tumor-bearing...
CCR2−/− mice displayed marked decreases in tumor-infiltrating inflammatory monocytes and macrophages (CD45+/CD11b+/F4/80+/Ly6C<sup>−</sup>/Ly6C<sup>+</sup>/MHCII+; Fig. 5A). In contrast, tumor-infiltrating effector T cells were increased with a concomitant decrease in regulatory T cells (CD45+/CD4+/CD25+/FoxP3+; Fig. 5B) suggesting an enhanced antitumor immune response. Tumors from WT mice treated with CCR2i and from CCR2−/−/C0 mice exhibited a shift from a T-helper cell (TH)2 to a TH1 gene expression profile, characterized by decreased Arg1, TGF-β, IL-1β, IL-6, and IL-10 with an increase in IFN-γ (Fig. 5C; ref. 28). In addition, mRNA expression of the macrophage recruitment mediators, CCL2, and macrophage colony-stimulating factor (M-CSF), were both increased in tumors from CCR2i-treated mice and from CCR2−/− mice by qRT-PCR (Supplementary Figs. S4B and S4C). This may suggest a regulatory feedback mechanism between tumors and infiltrating macrophages; specifically, tumors deprived of TAM may attempt to recruit additional macrophages by both upregulating CCL2 production and adapting to use alternative mechanisms, such as M-CSF production.

During tumorigenesis, granulocytes acquire immunosuppressive properties and promote tumor growth (22, 29, 30). These granulocytes are often referred to as granulocytic myeloid-derived suppressor cells (G-MDSC), and their importance has previously been shown in human and murine pancreatic cancer (11, 31). Gemcitabine is a standard chemotherapeutic agent used in human pancreatic
CD11b recruitment of metastasis-associated macrophages (MAM; CCL2 has been shown to play an important role in the establishment of metastasis (6). Pancreatic cancer (Fig. 5D).

Additive decrease in tumor growth was also observed when gemcitabine was given in combination with CCR2i (Fig. 5A). Alternatively, gemcitabine alone led to an increase in granulocytes (CD45+/CD11b+/Gr1hi/Ly6G+; ref. 33). In contrast, monocytes/macrophages are more resistant to the effects of gemcitabine, and we have found that inflammatory monocytes persist in the blood of patients with pancreatic cancer treated with chemotherapy (Supplementary Fig. S5). We observed a complementary effect of gemcitabine on granulocyte depletion in the tumors of CCR2i-treated mice, as CCR2i alone resulted in a slight increase in granulocytes (Fig. 5A). Alternatively, gemcitabine alone led to an increase in TAM, which CCR2i effectively depleted in the tumors of mice treated with the combination of these 2 agents (Fig. 5A). CCR2i-/- mice and CCR2i-treated WT mice displayed significantly reduced tumor growth of both subcutaneous and orthotopic tumors (Fig. 5D). An additive decrease in tumor growth was also observed when gemcitabine was given in combination with CCR2i (Fig. 5D).

**CCR2 inhibition prevents liver metastasis in murine pancreatic cancer.**

Inflammatory monocytes and macrophages are believed to play a crucial role in the establishment of metastasis (6). CCL2 has been shown to play an important role in the recruitment of metastasis-associated macrophages (MAM; CD11b+/F4/80+/Ly6Clo+/-CCR2+) to the premetastatic lung in an experimental model of breast cancer (34). In pancreatic cancer, the liver is the most common site of distant metastasis (16). We observed a marked increase in CCL2 mRNA expression by qRT-PCR with a concomitant increase in inflammatory monocytes and macrophages in the premetastatic livers of WT mice bearing orthotopic pancreatic tumors (Fig. 6A–C and Supplementary Fig. S6). However, CCR2i efficiently blocked the recruitment of inflammatory monocytes and macrophages to premetastatic livers, whereas gemcitabine did not (Fig. 6B–D and Supplementary Fig. S6). Twenty-eight days after implantation, this was associated with a significant decrease in liver metastasis compared with vehicle- or gemcitabine only–treated mice (Fig. 6D). Strikingly, none of the 15 mice treated with the combination of gemcitabine and CCR2i acquired hepatic metastasis compared with 15 of 20 vehicle-treated mice that developed liver metastasis (Fig. 6D). This suggests that targeting CCR2 can prevent liver metastasis in pancreatic cancer.

**Discussion**

Monocyte mobilization from the bone marrow has been poorly characterized in human solid organ malignancy. We have identified an important pathophysiologic process in patients with pancreatic cancer that has both prognostic and therapeutic implications. Current pancreatic cancer staging
systems, such as the American Joint Committee on Cancer tumor–node–metastasis (TNM) staging, fail to identify those patients who quickly recur and die from systemic progression of their disease following removal of their tumors (pancreatectomy; ref. 16). We found that survival decreases as the prevalence of blood monocytes increases in patients with pancreatic cancer, and that a low prevalence of blood monocytes is an independent predictor of improved survival. In addition, an increased blood:bone marrow inflammatory monocyte ratio predicts which patients with pancreatic cancer experience rapid recurrence and death following pancreatectomy. Perhaps, using monocytes in the blood and bone marrow as a biomarker can assist in therapeutic decision-making by indicating which patients would benefit from early aggressive systemic treatment, such as chemotherapy or immunotherapy, rather than initial local treatment, such as surgery. Furthermore, patients with pancreatic cancer with evidence of increased monocyte mobilization from the bone marrow may be the ideal candidates in which to use antimonocyte/macrophage therapies.

Tumors are sites of chronic inflammation and monocytes/macrophages influence outcome (35). Immunosuppressive myeloid cells are the predominant tumor-infiltrating leukocytes in pancreatic cancer—not T cells (3, 11, 22). Tumors depend on the stroma for survival and spread, and monocyte/macrophages are critical. TAM arise from monocytes, and these cells suppress antitumor immunity (36) as well as directly promote tumor growth (15), invasiveness (37), angiogenesis (38), and chemoresistance (14). TAMs within the tumor microenvironment have been shown to correlate with decreased patient survival in several human malignancies, including human pancreatic cancer (39–41). However, we introduce the novel concept that there is a shift in inflammatory monocyte equilibrium from the bone marrow to the blood,

Figure 6. CCR2 inhibition reduces inflammatory monocytes and MAMs in premalignant livers and impairs hepatic metastasis in murine pancreatic cancer. A, graph compares CCL2 expression using qRT-PCR in baseline (day 0), premalignant (day 6) and metastatic (day 28) livers of WT mice. B, representative immunofluorescence images (×20) of livers 9 days postinjection from control (Matrigel only, MG) mice, vehicle-treated tumor-bearing mice, and CCR2i-treated tumor-bearing mice for F4/80 (green) expression with a nuclear (Topro) stain (blue). C, graphs depict macrophage and inflammatory monocyte prevalence in the livers of MG control or tumor-bearing mice treated with vehicle, CCR2i, gemcitabine, or CCR2i + GEM combination by flow cytometry. All flow cytometry and qRT-PCR conducted on grossly normal liver (i.e., excluded metastatic liver deposits). All graphs depict means ± SEM and horizontal bars denote statistically significant differences between groups defined as P < 0.05 by Mann–Whitney test. D, graph shows the incidence of liver metastasis after 28 days in tumor-bearing mice treated with vehicle, gemcitabine, CCR2i, and CCR2i + GEM combination as well as CCR2+/− mice. n = 13–20 mice per group. Horizontal bars denote P < 0.05 by Fisher exact test.

Liver CCL2 Expression

Liver macrophages

Liver inflammatory monocytes

Liver metastasis by treatment
which is also predictive of survival in patients with pancreatic cancer.

CCR2+ monocytes have been shown to mediate immunosuppression (42) and metastasis (34) in murine cancer models. The CCL2/CCR2 chemokine axis plays an essential role in the recruitment of inflammatory monocytes from the bone marrow to peripheral sites of inflammation. Once recruited, the phenotype of monocytes/macrophages depends on the local immune environment (43). Tumors use this same pathway to recruit monocytes to the primary tumor where these cells acquire an alternatively activated (M2) phenotype (34, 44). CCL2 expression is also upregulated in the premetastatic liver of mice bearing orthotopic pancreatic cancer tumors. We hypothesize that inflammatory monocyte mobilization in pancreatic cancer is a surrogate for macrophage infiltration in the tumor and premetastatic liver. Our experiments in mice support this theory as the ratio of blood/bone marrow inflammatory monocytes correlated with the prevalence of macrophages in the primary tumor and premetastatic liver. Targeting TAM in pancreatic cancer is a relatively new strategy (45). We have chosen the approach of depleting macrophages in the tumor and liver by targeting inflammatory monocytes with CCR2 blockade, which acts at the level of the bone marrow (26, 27). Unlike many immunotherapeutics, CCR2i does not depend on the delivery of drug to the stroma-dense, poorly vascular tumor microenvironment. On the contrary, CCR2i depends on delivery to the highly vascular bone marrow (26).

In the present study, we revealed that circulating inflammatory monocytes were not immunosuppressive in the patients with pancreatic cancer examined. However, a monocytic subset of MDSC (Mo-MDSC; CD14+/HLA-DRlow−) with immunosuppressive properties has been described in the blood of patients with advanced malignancy (46, 47). Although we did not observe Mo-MDSC in the blood of the patients with pancreatic cancer examined, this could be due to differences in the patient populations studied. We evaluated the blood and bone marrow of surgically resectable patients with nonmetastatic pancreatic cancer without evidence of active infection (i.e., preoperative blood leukocytes < 11,000 cells/dL) before any treatment to limit confounding factors which often plague patients with advanced pancreatic cancer, such as infection, biliary obstruction, multiple courses of chemotherapy/radiation, tumor necrosis, and bowel obstruction (16). Admittedly, there is significant overlap between cells defined as Mo-MDSC and inflammatory monocytes. All CD14+/CCR2− cells in the peripheral blood of patients with pancreatic cancer in the current study were HLA-DR− and did not suppress T-cell proliferation ex vivo—thereby meeting the definition of inflammatory monocytes as opposed to Mo-MDSC (29). It is likely that inflammatory monocytes and Mo-MDSC are closely related, and Mo-MDSC may in fact represent a subset of inflammatory monocytes as has been suggested (48). There is evidence in patients with gastrointestinal malignancy that the extent of cell-mediated immune responses (mediated by monocytes/macrophages) is inversely proportional to the stage of disease (49, 50). Perhaps inflammatory monocytes in the blood of patients with more advanced malignancy may downregulate HLA-DR expression and acquire immunosuppressive properties—thereby meeting the definition of Mo-MDSC.

In summary, inflammatory monocyte mobilization from the bone marrow predicts survival in human pancreatic cancer, and the CCL2/CCR2 axis plays a critical role in the recruitment of inflammatory monocytes to the tumor microenvironment and premetastatic liver. CCR2i may be an ideal compliment to standard chemotherapeutics as this therapy had additive effect on the tumor while dramatically reducing metastasis. On the basis of the data presented here, we are proceeding with a phase Ib/II clinical trial using PF-04136309 combined with standard chemotherapy in patients with pancreatic cancer with locally advanced, nonmetastatic disease [NCT01413022; ClinicalTrials.gov]. Tumor, blood, and bone marrow are being collected pre- and posttreatment with CCR2i to determine if changing the blood/bone marrow inflammatory monocyte ratio can reduce TAM in the tumor, decrease metastasis, and improve patient survival. We believe the work presented here along with our clinical trial will make substantial contributions to the fields of cancer immunotherapy and tumor monocyte/macrophage biology.

Disclosure of Potential Conflicts of Interest

D.C. Linehan has a commercial research grant from Pfizer. No potential conflicts of interest were disclosed by the other authors.

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