Targeting Tumor-Infiltrating Macrophages Decreases Tumor-Initiating Cells, Relieves Immunosuppression, and Improves Chemotherapeutic Responses

Jonathan B. Mitchem1, Donal J. Brennan10, Brett L. Knolhoff2, Brian A. Belt1, Yu Zhu2, Dominic E. Sanford1, Larisa Belaygorod2, Danielle Carpenter3, Lynne Collins4,5, David Piwnica-Worms4,5,6, Stephen Hewitt8, Girish Mallya Udupi11, William M. Gallagher11, Craig Wegner7, Brian L. West9, Andrea Wang-Gillam2, Peter Goedegebure1,6, David C. Linehan1,6, and David G. DeNardo2,3,6

Abstract

Tumor-infiltrating immune cells can promote chemoresistance and metastatic spread in aggressive tumors. Consequently, the type and quality of immune responses present in the neoplastic stroma are highly predictive of patient outcome in several cancer types. In addition to host immune responses, intrinsic tumor cell activities that mimic stem cell properties have been linked to chemoresistance, metastatic dissemination, and the induction of immune suppression. Cancer stem cells are far from a static cell population; rather, their presence seems to be controlled by highly dynamic processes that are dependent on cues from the tumor stroma. However, the impact immune responses have on tumor stem cell differentiation or expansion is not well understood. In this study, we show that targeting tumor-infiltrating macrophages (TAM) and inflammatory monocytes by inhibiting either the myeloid cell receptors colony-stimulating factor-1 receptor (CSF1R) or chemokine (C–C motif) receptor 2 (CCR2) decreases the number of tumor-initiating cells (TIC) in pancreatic tumors. Targeting CCR2 or CSF1R improves chemotherapeutic efficacy, inhibits metastasis, and increases antitumor T-cell responses. Tumor-educated macrophages also directly enhanced the tumor-initiating capacity of pancreatic tumor cells by activating the transcription factor STAT3, thereby facilitating macrophage-mediated suppression of CD8+ T lymphocytes. Together, our findings show how targeting TAMs can effectively overcome therapeutic resistance mediated by TICs. Cancer Res; 73(3); 1128–41. ©2012 AACR.

Introduction

Tumor-infiltrating immune cells are a hallmark of most solid tumors, and the presence of varied immune populations significantly affects clinical outcomes for patients with cancer (1, 2). Historically, tumor-infiltrating immune cells have been viewed as restraining tumor progression (3), but in recent years, it has become more widely appreciated that chronic immune responses play critical roles in promoting tumor progression, metastasis, and resistance to cytotoxic therapies (1). Therefore, understanding the molecular mechanisms by which malignant cells derail antitumor immune responses to favor disease progression is critical to identify potential therapeutic targets. Recently, we reported that selective depletion of tumor-infiltrating macrophages (TAM) by neutralizing colony-stimulating factor-1 (CSF1) or inhibiting CSF1 receptor (CSF1R) activity improves the efficacy of chemotherapy in mammary tumors, in part by instigating antitumor responses by CD8+ T lymphocytes (4). Similarly, deficiency in the CSF1 gene in op/op mice leads to decreased mammary tumor metastasis and slows pancreatic neuroendocrine tumor development (5, 6). Although the potent capacity of macrophages to induce tumor progression has been well established, the mechanisms by which macrophage affect chemoresistance are not well defined.

In addition to immune regulation of cancer progression and chemoresistance, tumor cells that acquire stem-like or tumor-initiating properties (often called ‘cancer stem cells’) exhibit enhanced resistance to cytotoxic therapy and increased propensity for metastatic dissemination (7, 8). Several lines of evidence suggest that the tumor-initiating capacity of malignant cells is rooted in inflammatory signals (9). However, the mechanisms by which different populations of leukocytes might support the expansion of tumor-initiating cells (TIC) are unknown. One possibility is that reciprocal

Note:

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

Corresponding Author: David G. DeNardo, Department of Medicine, Washington University School of Medicine, 660 South Euclid Ave, Box 8069, St Louis, MO 63110. Phone: 314-362-9524; Fax: 314-747-2797; E-mail: ddenardo@dom.wustl.edu

doi: 10.1158/0008-5472.CAN-12-2731
©2012 American Association for Cancer Research.
cross-talk between tumor-infiltrating leukocytes and malignant cells regulates the development of cells with stem-like properties, which in turn facilitates resistance to therapeutic interventions. A recent study showed that macrophages can induce tumor stem-like properties in vitro in murine lung and colon cancer cell lines (10). However, it is unclear whether this interaction can be exploited pharmacologically, and if so, whether it also affects tumor-derived immunosuppression.

In this study, we investigated the mechanisms by which macrophages and TICs collaborate to regulate pancreatic ductal adenocarcinoma (PDAC) progression, immunosuppression, and responses to chemotherapy. We show that targeting TAMs by inhibiting either CSF1R or chemokine (C–C motif) receptor 2 (CCR2) decreases the numbers of pancreatic TICs and improves chemotherapeutic efficacy in vivo. We also found that TAMs directly induce TIC properties in pancreatic cancer cells by activating STAT3. In turn, TICs induce immunosuppressive behavior in TAMs, and thus block antitumor CD8+ T-lymphocyte responses during chemotherapeutic treatment.

Materials and Methods

Pancreatic cancer tissue microarray cohort and analysis

Tissue microarray (TMA) studies were conducted on a patient cohort constructed from 60 cases of invasive PDAC.
Figure 2. Depletion of TAMs results in reduced ALDH<sup>bright</sup> TICs. A, analysis of leukocyte and ALDH<sup>bright</sup> TIC frequency in KCM tumors from mice treated for 21 days with vehicle, CSF1Ri1, CCR2i, or in CCR2<sup>−/−</sup> hosts. (i) The presence of CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>F4/80<sup>−</sup>MHCII<sup>−</sup> macrophage, CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>F4/80<sup>−</sup>MHCII<sup>−</sup> monocyte (mono), CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>MHCII<sup>−</sup> (M-DC), CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>MHCII<sup>−</sup> (basophil), and CD11b<sup>−</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>CD11c<sup>−</sup>MHCII<sup>−</sup> (lymph-DC) subsets is depicted as the mean% of total live cells. (ii) ALDH<sup>bright</sup> TICs are depicted as the mean% of total live CD45<sup>−</sup>mCherry<sup>+</sup> cells. B, analysis of macrophage subsets following CCR2 or CSF1R inhibition. (i) CD11b<sup>+</sup>CD3/49b<sup>−</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>F4/80<sup>−</sup> macrophages were subdivided by MHCII and CD11c expression, and (ii) the mean frequency of each subset is displayed for all treatment groups. (iii) Relative expression of F4/80, CD206, and mCherry (indicator of phagocytosis) is depicted. C, flow-cytometry analysis of TAMs and M-MDSCs in infiltrating PAN02 tumors in mice treated for 4 or 8 days with vehicle, anti-CSF1, CCR2i, and/or CSF1Ri2 is depicted. D, the mean frequency of macrophages and CD45<sup>+</sup>mCherry<sup>−</sup>ALDH<sup>bright</sup> TICs in Kras-INK tumors following 8 days of CSF1Ri treatment is depicted. Representative flow-cytometry plots of mCherry<sup>−</sup> ALDH<sup>bright</sup> tumor cells (blue gate) are shown. E, quantitative real-time PCR (qRT-PCR) analysis in orthotopic Kras-INK tumor tissue following treatment with CSF1Ri for 14 days. Graph depicts the mean fold change compared vehicle. Flow cytometry and qRT-PCR data depict the mean values from 5 to 10 mice ± SEM. *, Statistically significant differences at P < 0.05 (Mann–Whitney U test).
diagnosed at the Department of Pathology at Washington University (St Louis, MO). Patients had not received neoadjuvant therapy and underwent pancreaticoduodenectomy, typically followed by adjuvant chemotherapy. To assemble TAMs, clearly defined areas of tumor tissue were demarcated and 2 biopsies (1.0-mm diameter) taken from each donor block. We used 4.0-mm paraffin sections for immunohistochemical (IHC) analyses. The Washington University School of Medicine (St Louis, MO) ethical committee approved this study. Fully automated image acquisition was used using Aperio ScanScope XT Slide Scanner (Aperio Technologies) system to capture whole-slide digital images with a ×20 objective. A tumor-specific nuclear algorithm (IHC-MARK) developed in-house was modified to quantify CD8 and CD68 expression as previously published (4, 11).

**CCR2 kinase, CSF1R kinase, and STAT3 inhibitors**

CCR2 inhibitor PF-04136309 was provided by Pfizer and administered to mice at a concentration of 100 mg/kg in twice-daily subcutaneous injections. PF-04136309 details have been previously published (12). Inhibitors of CSF1R1 and CSF1R2 were provided by Plexikon Inc. CSF1R1 is PLX6134 that contains the GW2580 compound, which was described in detail elsewhere (13). CSF1R2 is PLX3397, a selective bio-specific inhibitor for c-Fms and the c-Kit receptor tyrosine kinases, with biochemical IC50 values of 0.02 and 0.01 μmol/L, respectively. PLX3397 was used as a confirmatory compound for PLX6134/GW2580 with better specificity for CSF1R, and details were presented elsewhere (4, 14, 15). Both CSF1R inhibitors were administered to mice in a formulated diet at a concentration of 800 mg/kg Chow. STAT3 was obtained from Calbiochem/EMD, used at doses less than the reported IC50 (<10 μmol/L) in vitro, and handled according to manufacturer instructions.

**Cell lines and constructs**

PAN02 cells were obtained from Dr. David C. Linehan (Washington University School of Medicine, St. Louis, MO), KCM cells from Dr. Pinku Mukherjee (University of North Carolina, Charlotte, NC), and Kras-INK from Dr. Douglas Hanahan’s laboratory (Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland) and have been published elsewhere (16–18). Briefly, KCM and Kras-INK were derived from pancreatic adenocarcinomas from p48-CRE/LSL-KrasG12D/Muc1.Tg (17, 18) and p48-CRE/LSL-KrasG12D/INK4afox/wt or Pdx-CRE/LSL-Kras/Tg-Muc1) or tumors arising from 3-methylcholanthrene carcinogenesis (PAN02; refs. 16, 17, 22). To identify potential TIC cellular subsets, these cell lines were labeled with mCherry and Click-beetle Red (CBR) luciferase and analyzed for EpCAM, CD24, CD44, CD29, CD49f, CD133, and cMet expression and Aldefluor activity (a measure of aldehyde dehydrogenase activity) using mCherry to identify implanted tumor cells in vivo. Our analysis revealed a distinct population of tumor cells with high Aldefluor activity (ALDHBright; Fig. 1A). No distinct populations of CD133+ or cMet+ cells were observed. Analysis of cell sorted from orthotopic Kras-INK and KCM PDAC tumors illustrated that ALDHBright cells express higher levels of CD29, CD44, and CD49f, display increased tumor spheroid formation in vitro, and have increased tumorigenic potential in nude mice (Fig. 1A–C, data not shown). Analysis of fresh human PDAC tissue also revealed an identifiable population of ALDHBright tumor cells with a frequency ranging from 2% to 15% of the total CD45+ EpCAM+ cells (Fig. 1E). IHC analysis of ALDH1A1 revealed a significant increase in positive cells in PDAC tissue compared with the normal pancreas (Fig. 1D). These results are consistent with previous reports showing the tumorigenic potential of ALDH1+ cells in human tumors (23, 24).

**Depletion of TAMs decreased the presence of ALDHBright TICs**

To determine whether targeting TAMs alters the frequency of TICs, we treated mice bearing orthotopic KCM tumors with 2 CSF1R tyrosine kinase antagonists and a CCR2 antagonist and used CCR2 knockout mice (CCR2−/−). The CSF1R kinase inhibitors used were PLX6134, a preparation of the GW2580 compound (CSF1Ri1), and PLX3397 (CSF1Ri2). To test CCR2 antagonism, we used PF04136309 (CCR2i). Additional details and structures for these compounds are in the Materials and Methods and published elsewhere (12–15, 25). Analysis of tumor tissue after 21 days of treatment revealed significant reductions at day 21 (21) or gross palpation of the pancreas. Gemcitabine (GEM; Hospira) was obtained from the Washington University School of Medicine pharmacy and diluted in PBS. Mice were treated with 50 mg/kg gemcitabine by intravenous injection into the right retro-orbital sinus every 4 to 5 days. Preclinical studies were conducted with 10 to 15 age-matched 10-week-old female mice/group. In survival studies, a death event was classified as a loss of 15% of body weight or poor body conditioning score. Disease and tumor burden were measured by the gross wet weight of the pancreas. Metastatic and disseminated tumors were scored by gross evaluation, which was validated by either bioluminescence or tissue pathology.

Additional methodologic detail is in the Supplementary Data.

**Results**

**ALDH1+ PDAC cells have high tumor-initiating capacity**

To investigate how macrophages impact the presence of TICs, we used 3 distinct murine pancreatic tumor cell lines, denoted as Kras-INK, KCM, and PAN02, derived from PDACs arising from genetic models (p48-CRE/LSL-Kras/INK4Afox/wt or Pdx-CRE/LSL-Kras/Tg-Muc1) or tumors arising from 3-methylcholanthrene carcinogenesis (PAN02; refs. 16, 17, 22). To identify potential TIC cellular subsets, these cell lines were labeled with mCherry and Click-beetle Red (CBR) luciferase and analyzed for EpCAM, CD24, CD44, CD29, CD49f, CD133, and cMet expression and Aldefluor activity (a measure of aldehyde dehydrogenase activity) using mCherry to identify implanted tumor cells in vivo. Our analysis revealed a distinct population of tumor cells with high Aldefluor activity (ALDHBright; Fig. 1A). No distinct populations of CD133+ or cMet+ cells were observed. Analysis of cell sorted from orthotopic Kras-INK and KCM PDAC tumors illustrated that ALDHBright cells express higher levels of CD29, CD44, and CD49f, display increased tumor spheroid formation in vitro, and have increased tumorigenic potential in nude mice (Fig. 1A–C, data not shown). Analysis of fresh human PDAC tissue also revealed an identifiable population of ALDHBright tumor cells with a frequency ranging from 2% to 15% of the total CD45+ EpCAM+ cells (Fig. 1E). IHC analysis of ALDH1A1 revealed a significant increase in positive cells in PDAC tissue compared with the normal pancreas (Fig. 1D). These results are consistent with previous reports showing the tumorigenic potential of ALDH1+ cells in human tumors (23, 24).
in numbers of tumor-infiltrating CD11b+Ly6G−Ly6C+\textsuperscript{hi}F4/80\textsuperscript{hi}MHCII\textsuperscript{hi} macrophages, CD11b+Ly6G−Ly6C+\textsuperscript{hi}F4/80\textsuperscript{mid}MHCII\textsuperscript{+} inflammatory monocytes, and CD11b+Ly6G−Ly6C+CD11c+MHCII\textsuperscript{hi} dendritic cells (DC; presumably myeloid-derived dendritic cells) in response to blockade of CCR2 or CSFIR signaling. In contrast, we observed no alteration in the number of CD11b+Ly6G−Ly6C+CD11c−MHCII\textsuperscript{hi} dendritic cells (presumably lymphoid-derived dendritic cells), and the numbers of CD11b+Ly6G\textsuperscript{hi}Ly6C\textsuperscript{+} immature granulocytes/neutrophils were modestly increased (~30%; Fig 2A). Despite possible cellular diversity, we will use the terms granulocytic myeloid-derived suppressor cells (G-MDSC) for CD11b+Ly6G\textsuperscript{hi}Ly6C\textsuperscript{+} cells and monocytic MDSCs (M-MDSC) for CD11b+Ly6G\textsuperscript{hi}Ly6C\textsuperscript{hi}F4/80\textsuperscript{mid}MHCII\textsuperscript{hi} cells.

Analysis of the impact of CCR2 or CSFIR inhibition on TAM subsets found that these inhibitors significantly deplete macrophages expressing high levels of MHCII, but not MHCII\textsuperscript{lo} or Tie2\textsuperscript{−} TAMs (Fig 2B and Supplementary Fig. S1A). Analysis of the MHCII\textsuperscript{hi} macrophages showed that these cells express the highest levels of F4/80, display high tumor phagocytosis as measured by mCherry fluorescence, and express modest levels of extracellular MRC1 (features consistent with mature macrophages).

To determine whether CSFIR and CCR2 blockade have differential effects on tumor-infiltrating myeloid cells, we analyzed the impact of CSFIR and CCR2 inhibitors as single agents or in combination (Fig 2C). Mice bearing established (~1 cm) PDAC tumors were treated for 4 or 8 days with CCR2i, anti-CSF1, CSF1Ri, and CCR2i all effectively depleted mature TAMs in the first 4 days, whereas CCR2 inhibition did not effectively deplete TAMs until after 8 days of treatment. These results suggest that CSFIR and CCR2 have redundant rather than divergent activities on depleting TAMs and M-MDSCs.

To assess the effects of these inhibitors on TICs, we conducted a parallel analysis of the KCM tumor cells, identified by CD45\textsuperscript{mCherry\textsuperscript{+}}, and found a 40% to 55% reduction in CD44\textsuperscript{hi}ALDH\textsuperscript{bright} TICs following CCR2 or CSFIR blockade (Fig. 2A(i)). To determine whether macrophage depletion can rapidly alter the presence of ALDH\textsuperscript{bright} TICs, we treated mice bearing approximately 1-cm Kras-INK tumors with CSFIR inhibitors for 8 days and found a 40% to 70% decrease in CD44\textsuperscript{hi}ALDH\textsuperscript{bright} TICs (Fig. 2C). We observed similar results in orthotopic PAN02 tumors (Supplementary Fig. S1B). Correlating with these observed decreases in ALDH\textsuperscript{bright} cells, we found that OCT4, Nanog, and SOX2 mRNA expression was decreased following treatment with CSFIR or CCR2 inhibitors (Fig. 2E and Supplementary Fig. S1C). Notably, IHC and mRNA analysis of CCR2 or CSFIR expression in these PDAC tumors revealed they do not express significant levels of CCR2 or CSFIR in vivo and in vitro (Supplementary Fig. S1D and S1E). Taken together, these results suggest that targeting TAMs can rapidly reduce the numbers of ALDH\textsuperscript{bright} TICs.

**TAMs can directly enhance the tumor-initiating properties of PDAC cells**

To determine whether macrophages can directly enhance the tumor-initiating properties of pancreatic cancer cells, we cocultured macrophages with PDAC cells. Coculture with macrophages increased the frequency of ALDH\textsuperscript{bright} cells in murine and human PDAC cell lines (Fig. 3A). To determine whether soluble factors derived from tumor-educated macrophages enhanced TIC properties, we first cultured bone-marrow–derived macrophages (BM-MAC) in PDAC-conditioned medium (CM) and then used the resultant "tumor-educated" BM-MACs to create conditioned medium for tumor spheroid assays. Conditioned medium from tumor-activated BM-MACs enhanced the formation of tumor spheres in PAN02, Kras-INK, and KCM cells (Fig. 3E). Similar results were also observed in Kras-INK cells cultured in Transwells with BM-MACs (Supplementary Fig. S2A). Consistent with enhanced tumor-initiating properties, we observed that BM-MAC coculture increased CD29 and CD49f protein and OCT4, Nanog, and SOX2 mRNA expression in PDAC cells (Fig. 3C and Supplementary Fig. S2B).

Another feature commonly associated with TICs is increased resistance to chemotherapy. Fitting this, ALDH\textsuperscript{bright} cells isolated from Kras-INK tumors displayed decreased response to gemcitabine (Supplementary Fig. S2C). To elucidate whether macrophages can enhance the resistance to chemotherapy, we treated PDAC cells with gemcitabine and found reduced numbers of Annexin V\textsuperscript{+} cells when BM-MACs were present in coculture (Fig. 3D and Supplementary Fig. S2D). Similar results were observed using a Transwell system or BM-MAC-CM (Supplementary Fig. S2E and S2F). Together, these results suggest that tumor-educated macrophages produce soluble factors that can regulate both tumor-initiating capacity and chemoresistance in PDAC cells.

**Gemcitabine treatment increases macrophage infiltration into PDAC tumors**

Common chemotherapeutics have been reported to induce the recruitment of myeloid cells to regressing tumors (4, 26, 27). To assess whether gemcitabine treatment alters myeloid cell recruitment in PDAC tumors, we analyzed pancreatic tissue from normal mice or mice bearing Kras-INK tumors ± GEM treatment. The numbers of TAMs, G-MDSCs, inflammatory monocytes, and CD4\textsuperscript{+}FOXP3\textsuperscript{+} regulatory T cells (T\textsubscript{reg}) were increased by the presence of PDAC tumors, but only TAMs increased in number following gemcitabine treatment (Fig. 3E). Corresponding with increased TAM infiltration, CSF1 and CCL2 but not CCL5 were upregulated by gemcitabine treatment (Fig. 3F). Taken together, these results suggest that blockade of CCR2 and/or CSFIR would improve the response to gemcitabine in PDAC tumors.

**Targeting TAMs enhances the response to chemotherapy and reduces metastasis**

CSFIR and CCR2 inhibitors as single agents modestly slow PDAC tumor growth, similar to gemcitabine therapy (Figs. 4A–C and Supplementary Fig. S3A–S3C). Similar results were also observed in CCR2\textsuperscript{−/−} mice (Supplementary Fig. S3B), suggesting that these effects are due to alterations in the tumor
stroma. To determine whether inhibiting CSF1R or CCR2 could improve responses to chemotherapy, we treated mice bearing established orthotopic Kras-INK or Pan02 tumors with gemcitabine alone or in combination with CSF1R or CCR2 inhibitors. We found that CSF1R+ plus gemcitabine dramatically slowed tumor progression in both Pan02 and Kras-INK orthotopic tumors (Fig. 4A and Supplementary Fig. S3A and S3C).

For example, in Kras-INK tumors, gemcitabine reduced tumor growth (compared with parallel mice sacrificed at the start of treatment, Rx Start) by 32% as compared with vehicle-treated tumors, whereas CSF1Ri plus gemcitabine reduced tumor growth by 81%. Similar but somewhat less dramatic results were obtained with gemcitabine in combination with CCR2i (Fig. 4B). Similar results were observed with paclitaxel plus...
Detailed analysis of primary tumor pathology revealed a high level of necrosis in tumors from mice treated with gemcitabine plus CSF1Ri, but not either agent alone. No alterations in stromal desmoplasia were observed (Fig. 4F).

Similar to human PDAC, orthotopic Kras-INK and Pan02 tumors develop hepatic and peritoneal metastatic disease. In mice bearing Kras-INK tumors, gemcitabine or CSF1R inhibition alone reduced peritoneal metastases, and combined treatment regimens had an additive effect (Fig. 4D).
We observed similar results in the Pan02 model (Fig. 4E). Gemcitabine or CSF1R inhibition alone also decreased the frequency of hepatic metastases; however, an additive reduction in the liver metastatic frequency was only observed in the Pan02 model (Fig. 4D and E). Consistent with these results, CSF1Ri plus gemcitabine also increased...
Figure 6. Macrophage-induced chemoresistance and stem-like properties require STAT3 signaling. A, quantitation and *40 images of pSTAT3+ tumor cells are depicted. The mean frequency of positive cells in PAN02 and KCM tumors treated with either vehicle or CSF1R or CCR2 inhibitors is shown (n >5/group). B, ELISA analysis of STAT3 phosphorylation in Kras-INK cells following treatment with macrophage conditioned medium (MCM). C, normalized mean fold changes in gene expression are depicted from Kras-INK tumor cells and BM-MACs alone or in coculture for 24 hours using a Transwell chamber. D, MCM was added to adherence-free Kras-INK tumor spheroid cultures ± the STAT3 inhibitor STATIC, and the number of tumor spheroids after 14 days is depicted. E, MCM was added to adherence-free KCM-shLacZ, KCM-shSTAT3#3, or KCM-shSTAT3#5 tumor spheroid cultures ± the STAT3 inhibitors and the number of tumor spheroids after 14 days is depicted. F, analysis of Annexin V+ Kras-INK cells in direct coculture with BM-MACs ± STATIC and treated with gemcitabine for 36 hours is depicted as percentage of CD45+mCherry+ tumor cells (n = 3/group). G, analysis of Annexin V+ KCM, KCM-shLacZ, KCM-shSTAT3#3, or KCM-shSTAT3#5 cells cocultured with BM-MACs ± GEM is depicted (n = 3/group).
In the survival of Pan02 tumor-bearing mice (Supplementary Fig. S3D), the observed efficacy of CSF1Ri plus gemcitabine, although gemcitabine treatment significantly reduced the frequency of both TAMs and ALDH\textsuperscript{bright} TICs, this increase was abrogated by CSF1Ri inhibition (Fig. 4G and H). Similar but less dramatic effects were observed with CCR2i inhibition (Supplementary Fig. S3E). Taken together, these results suggest that blocking macrophage infiltration into tumors during therapy reduces the number of TICs and improves the efficacy of chemotherapy.

Depletion of TAMs results in increased CTL response during chemotherapy

Previous studies showed that TAMs have significant immunosuppressive capacity (4, 28, 29). To determine whether macrophage depletion would restore antitumor T-cell activity in PDAC tumors, we analyzed tumor-infiltrating T lymphocytes in mice treated with CCR2i or CSF1Ri ± GEM. Analysis of tumor-infiltrating lymphocytes revealed significantly increased CD\textsuperscript{+} and CD\textsuperscript{8} T cell and reduced FOXP3\textsuperscript{+} Treg infiltration when CSF1Ri or CCR2i was given in combination with gemcitabine (Fig. 5A–C). Thus, despite eliciting decreased tumor growth, single-agent gemcitabine, CCR2i, or CSF1Ri did not alter T-cell infiltration, suggesting that both tumor cell destruction by chemotherapy and macrophage depletion are necessary to sustain CTL infiltration. Consistent with elevated CTL responses, we also observed increased IFN-\gamma, IFN-\beta1, granzyme B, perforin, and interleukin (IL)-12 p35 and decreased TGF-\beta and arginine-1 mRNA expression in tumors (Fig. 5D). To determine the role of CD\textsuperscript{8} T lymphocytes in the efficacy of combined therapy, we used CD8-depleting antibodies (clone 2.43) in the context of CSF1Ri plus gemcitabine treatment. While treatment with CSF1Ri plus GEM significantly blunted tumor growth compared with the effects of gemcitabine alone (\(-80\%\)), this therapeutic efficacy was largely dependent on CD\textsuperscript{8} T lymphocytes (Fig. 5E).

TAMs and TICs cross-talk to repress CD\textsuperscript{8} T lymphocytes

Immunosuppression in the tumor microenvironment can be mediated by TAMs, M-MDSCs, G-MDSCs, T\textsuperscript{reg}, and immature dendritic cells. Although TAMs are the most prevalent of these cells, 2 recent reports showed that G-MDSCs mobilized by tumor-derived granulocyte macrophage colony-stimulating factor (GM-CSF) can also suppress CTL activation in PDAC tumors (30, 31). To assess the immunosuppressive capacity of these leukocytes, we isolated tumor-infiltrating TAMs and G-MDSCs from Kras-INK tumors and compared their ability to suppress CD\textsuperscript{8} T lymphocyte proliferation (by Carboxyfluorescein succinimidyl ester (CFSE) dilution) following polyclonal anti-CD3/CD28 activation (Fig. 5F). Enhanced T-cell–suppressive activity in TICs has been reported in glioblastoma (32). To test if PDAC TICs are highly immunosuppressive, we analyzed the suppressive capacity of ALDH\textsuperscript{bright} and ALDH\textsuperscript{dim} KCM cells and found that although both subsets inhibit CD\textsuperscript{8} T-cell proliferation at high concentrations, ALDH\textsuperscript{bright} cells exhibited modestly greater suppressive activity (Fig. 5G).

Activation of the immunosuppressive properties in innate immune cells by tumor cells is a common feature of aggressive tumors. To determine whether PDAC TICs increase the immunosuppressive capacity of macrophages, we cocultured small numbers of ALDH\textsuperscript{bright} and ALDH\textsuperscript{dim} KCM cells with BM-MACs (Fig. 5H). Although tumor-naïve BM-MACs exhibited decreased CD8-suppressive capacity compared with that of TAMs, CD8 suppression by BM-MACs was significantly elevated by the presence of ALDH\textsuperscript{bright} but not ALDH\textsuperscript{dim} KCM cells. Taken together, these data suggest that inhibiting CSF1R or CCR2 in the context of chemotherapy allows productive CD8\textsuperscript{+} T-cell responses via the combined action of the (i) reduced presence of immunosuppressive ALDH\textsuperscript{bright} TICs, (ii) reduced presence of immunosuppressive TAMs (as well as M-MDSC), and (iii) disruption of TIC-induced macrophage immunosuppression.

STAT3 activation is necessary for macrophage-dependent increases in TIC numbers and chemoresistance

STAT3 is a key mediator of proinflammatory cytokines and immune suppression in both leukocytes and neoplastic cells, and has also been linked to TIC survival and chemoresistance in several cancer types (33–36). IHC analysis of tumor tissue from mice treated with CSF1R or CCR2 inhibitors revealed significantly reduced levels of phospho-STAT3 (pSTAT3, Ser205) in malignant cells, but not adjacent normal or leukocytes (Fig. 6A). Correlating with these results, macrophage conditioned medium increased pSTAT3 levels in Kras-INK cells in vitro (Fig. 6B). Corresponding with the activation of STAT3-mediated transcription, we observed increased IL-6, GPR130, and STAT3 mRNA expression in PDAC cells and increased IL-1β, IL-6, and ARG1 mRNA expression in macrophages when cocultured in a Transwell (Fig. 6C). To determine if STAT3 was necessary for TAM-mediated regulation of PDAC TICs, we used small-molecule inhibitors of STAT3 signaling [STATIC (37) and WP1066] and STAT3 short hairpin RNA (shRNA) constructs. Treatment with STATIC or WP1066 abrogated the formation of tumor spheroids in the presence or absence of BM-MAC-CM (Fig. 6D and E). In addition, partial suppression of STAT3 expression (\(-50\%–60\%\)) using shRNA reduced the induction of tumor spheroid formation by BM-MAC-CM (Fig. 6E). Inhibition of STAT3 by either STATIC or shRNA was sufficient to overcome the chemoprotective effects of macrophage coculture (Fig. 6F and G). These data suggest that TAMs induce TIC properties and chemoresistance through the activation of STAT3.

STAT3 signaling is necessary for TIC-induced immunosuppression

We next sought to understand how STAT3 activation in TICs regulates the immunosuppressive capacity of TICs. To accomplish this, we analyzed the effects of shRNA against STAT3 on PDAC-induced immunosuppression. Corresponding to our previous results, ALDH\textsuperscript{bright} KCM cells, but not KCM ALDH\textsuperscript{dim} cells, stably expressing shRNA against LacZ robustly
induced BM-MAC–mediated suppression of CD8⁺ T-cell proliferation. In contrast, ALDH⁹ʰigh KCM cells expressing STAT3 shRNA were unable to enhance BM-MAC–mediated CD8⁺ T-cell suppression (Fig. 7A).

To understand the clinical implications of these interactions, we analyzed a TMA containing specimens from 59 patients with PDAC. Tumors were scored for the presence of CD68⁺ and CD8⁺ leukocytes and stratified into 2 groups (CD68⁺/CD8⁻ and CD68⁻/CD8⁺). We observed that in patients in whom CD68⁺ macrophages were the dominant tumor-infiltrating leukocyte (CD68⁺/CD8⁻), overall survival was significantly reduced compared with all other groups (denoted as CD68⁻/CD8⁺, Fig. 7B). Similarly, stratification of patients by epithelial pSTAT3 revealed that high pSTAT3 indicated reduced survival (Fig. 7C). Combined analysis found that CD68⁺ leukocytes, but not CD8⁺ T cells, correlated with epithelial pSTAT3 intensity (Spearman r = 0.32, P = 0.024) and tumors classified as CD68⁺/CD8⁻ had increased epithelial pSTAT3 (Fig. 7D). Taken together, these results suggest that epithelial STAT3 signaling is high in tumors in which macrophages likely play an immunosuppressive role (e.g., CD68⁺/CD8⁻ tumors) and targeting TAMS in these patients could result in improved survival.

### Discussion

Our results show that inhibiting CSF1R or CCR2 signaling can increase chemotherapeutic efficacy and block metastasis by the combined action of reducing TIC numbers, and overcome macrophage-induced CD8⁺ CTL suppression. We illustrate that macrophages can directly induce TIC properties in PDAC cells by enhancing STAT3 activation and that STAT3 TICs enhance TAM-mediated immunosuppression. Thus, cross-talk between TAMs and TICs through STAT3 regulates the chemotherapeutic response by repressing antitumor CTL activity (Fig. 7E).

### CCR2 and CSF1R as regulators of myeloid responses

Tumor-infiltrating innate immune cells are composed of diverse cellular populations including G-MDSCs, M-MDSCs, tie-2⁺ angiogenic monocytes and myeloid-derived dendritic cells. We found that blockade of CSF1R or CCR2 result in a very similar spectrum of alterations in tumor-infiltrating myeloid cells both reducing mature CD11b⁺Ly6G⁻Ly6C⁻⁹⁰ MHCII⁻FcγRI⁺ macrophages and CD11b⁺Ly6G⁻Ly6C⁺⁹⁰ MHCII⁺FcγRI⁺ monocytes. Although the effects of CCR2 and CSF1R blockade on tumor-infiltrating leukocytes are similar, their mechanisms of action are likely divergent. Studies have
suggested that CCR2 mediates the trafficking of circulating Ly6G<sup>−</sup>Ly6C<sup>+</sup> TAMs to target tissues (38, 39), whereas CSF1R is more likely involved in maturation and/or survival of these cells at inflamed sites. Both receptors can affect bone marrow mobilization. In this and previous studies, significantly reduced macrophage infiltration was observed as early as 2 to 4 days after CSF1R inhibition (4). In contrast, CCR2 inhibition reduced monocyte numbers in 4 days but only depleted TAMs after 8 days of treatment. Neither CSF1R nor CCR2 blockade resulted in dramatic alterations in circulating monocyte numbers over these periods. Thus, these results suggest that CCR2 inhibition may affect recruitment of inflammatory monocytes from the circulation, whereas CSF1R inhibition may affect survival of monocytes/macrophages at the tumor site.

Tumor-infiltrating immature granulocytes delineated as CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>+</sup> are often composed of highly heterogeneous populations, which can include tumor-activated neutrophils and G-MDSCs (40). GM-CSF–dependent mobilization and activation of these cells have been shown to mediate suppression of CTL responses in PDAC tumors (30, 31). However, in this study, targeting either CCR2 or CSF1R did not reduce the presence of CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>+</sup> cells (Fig. 2). These findings suggest that in the context of chemotherapy, CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>+</sup> cells cannot overcome the loss of TAMs and M-MDSC. While not observed in this model, gemcitabine treatment has been shown to reduce the numbers of G-MDSCs in mammary tumors (41) and may affect the immunosuppressive capacity of G-MDSCs in PDAC. Alternatively, the depletion of TAMs may alter the cellular activity CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>+</sup> cells, pushing them toward more mature, less immunosuppressive phenotypes. These differences in immune responses following CCR2 or CSF1R inhibition are likely important considerations for their clinical application.

Inflammation and TICs

STAT3 activity has been shown to be required for the expansion and maintenance of cancer stem cells in several cancers (33, 34). In pancreatic cancer, epithelial deletion of STAT3 results in significantly reduced Kras-induced tumor formation in part through alterations in matrix metalloproteinase 7 (MMP7) expression and decreased immune infiltration (35). These data are consistent with the idea that reciprocal cross-talk between leukocytes and cancer cells sustain tumor progression. Work by Jinushi and colleagues showed that macrophage-derived MGF-E8 and IL-6 enhance stem-like properties in lung and colon cancer cells by activating STAT3 (10). Similarly, we revealed that depletion of TAMs by CSF1R or CCR2 inhibition leads to reduced STAT3 phosphorylation (Fig. 6) and decreased numbers of ALDHB<sup>+</sup> TICs. However, regulation of STAT3 activation may be one of several pathways regulating this process. TAMs are potent producers of WNTs and Sonic hedgehog ligands as well as growth factors, such as EGF, basic fibroblast growth factor, platelet-derived growth factor, and hepatocyte growth factor, many of which have been implicated in stimulating cancer stem-like properties (42, 43). In addition, factors such as VEGF, MMP9, and Bv8 emanating from tumor-infiltrating myeloid cells can significantly alter the quality of tumor vasculature, and endothelial cell/tumor cell interactions may be a critical component of the cancer stem cell niche (44–46). Thus, multiple pathways are likely regulated by tumor inflammation to influence the prevalence of TICs.

Macrophages and chemosensitivity

Several recent studies revealed that macrophages can directly regulate tumor cell chemoresistance. Previous work by Shree and colleagues (27) and Gocheva and colleagues (47) has illustrated that macrophage-derived cathepsins regulate pancreatic neuroendocrine tumor progression and mammary tumor response to paclitaxel. Intriguingly, cathepsin activity has also been revealed as necessary for the secretion and processing of proinflammatory cytokines. However, the manner in which these activities affect mammary TICs or STAT3 activation is not known. In addition, targeting CCR2 in the tumor microenvironment has been shown to improve the delivery of chemotherapeutic regimens by regulating the tumor vasculature (48). These observations further stress the importance of TAMs and the complexity of their roles in the tumor microenvironment.

Clinical prospective

Therapeutic resistance and metastatic spread define the lethality of aggressive cancers. Thus, understanding and targeting the mechanisms responsible is critical to improving therapeutic outcomes. Several studies have indicated that targeting key pathways regulating TIC survival and/or differentiation can overcome therapeutic resistance. However, the durability of such therapies remains unproven, as such targeting the tumor stromal responses that support tumor “stemness” is an attractive alternative.

Disclosure of Potential Conflicts of Interest

D.J. Brennan has ownership interest (including patents) in patent for CD8:CD68 signature. D.C. Linehan has other commercial research support from Pfizer Oncology and Novartis Oncology. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: J.B. Mitchem, D.J. Brennan, B.A. Belt, C. Wegner, P. Goedegebuure, D.C. Linehan, D.G. DeNardo

Development of methodology: J.B. Mitchem, B.A. Belt, L. Belayevrod, S. Hewitt, W.M. Gallagher, B.L. West, P. Goedegebuure, D.C. Linehan, D.G. DeNardo

 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.B. Mitchem, D.J. Brennan, B.A. Belt, D. E. Sanford, L. Collins, D. Pownica-Worms, S. Hewitt, W.M. Gallagher, A. Wang-Gillam, D.C. Linehan, D.G. DeNardo

 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.B. Mitchem, D.J. Brennan, B.A. Belt, L. Collins, D. Pownica-Worms, G.M. Udupi, D.C. Linehan, D.G. DeNardo

Writing, review, and/or revision of the manuscript: J.B. Mitchem, D.J. Brennan, B.A. Belt, Y. Zhu, D. Pownica-Worms, C. Wegner, B.L. West, P. Goedegebuure, D.C. Linehan, D.G. DeNardo

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.B. Mitchem, D. Carpenter, G.M. Udupi, A. Wang-Gillam, D.C. Linehan, D.G. DeNardo

Study supervision: D.G. DeNardo

Acknowledgments

The authors thank the Siteman Frontier Funds Team Science Award, Drs. Mukherjee and Hanahan for providing cell lines, and the efforts of the Bright Imaging Center. The authors also thank Drs. Hawkins, Stewart, and Webber for discussions and input. The authors also thank generous support from the Lastgarten Foundation, V Foundation, Edward Mallinckrodt Jr. Award, the...
Cancer Research Foundation, and Siteman Cancer Center Career Development Award.

Grant Support

This study was supported by WU/Pfizer Biomedical Research Grant PW0457 (D.C. Linehan), NIH P50 CA 94056 (D. Piwnica-Worms), and NCI grant T32 CA 009621 (J.B. Mitchem and D.E. Sanford).

References

Macrophages Regulate Tumor-Initiating Cells

Targeting Tumor-Infiltrating Macrophages Decreases Tumor-Initiating Cells, Relieves Immunosuppression, and Improves Chemotherapeutic Responses


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-2731

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2012/12/04/0008-5472.CAN-12-2731.DC1

Cited articles
This article cites 47 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/3/1128.full#ref-list-1

Citing articles
This article has been cited by 50 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/73/3/1128.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/73/3/1128.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.