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Targeting TNF-α producing macrophages activate antitumor immunity in pancreatic cancer through IL33 signaling

Ajay Dixit1,2,3#, Aaron Sarver3, Jon Zettervall1,2, Huocong Huang4, Kexin Zheng1, Rolf A. Brekken4, Paolo Provenzano1,2,3,5,6,7#

Affiliations:
1 Department of Biomedical Engineering, University of Minnesota
2 University of Minnesota Physical Sciences in Oncology Center
3 Masonic Cancer Center, University of Minnesota
4 Hamon Center for Therapeutic Oncology Research and Department of Surgery, UT Southwestern, Dallas, TX
5 Department of Hematology, Oncology, and Transplantation, University of Minnesota
6 Institute for Engineering in Medicine, University of Minnesota
7 Stem Cell Institute, University of Minnesota
#To whom correspondence should be addressed:

Dr. Ajay Dixit
Department of Biomedical Engineering
University of Minnesota
7-120 NHH
312 Church St SE
Minneapolis, MN, 55455
e-mail: akdixit@umn.edu

Dr. Paolo Provenzano
Department of Biomedical Engineering
University of Minnesota
7-120 NHH
312 Church St SE
Minneapolis, MN, 55455
e-mail: pprovenz@umn.edu

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Key findings:

● PDA macrophages are heterogeneous populations with bone marrow-derived TAMs largely making up the immunosuppressive populations
● Chemokine CCL2 is overexpressed in both primary and metastatic tumor lesions with myCAFs and to a greater degree iCAFs being the major sources of CCL2
● Deletion of CCR2 reduces tumor TAMs, metastasis, and prolongs survival in KPC mice
● Pharmacological inhibition of monocyte-derived macrophages through CCR2 blockade disrupts immunosuppression to enhance T cell activation and confer sensitivity to ICB therapy
● Macrophages are the primary source of TNF-α in PDA, which suppresses carcinoma cell expression of the alarmin IL33 that is capable of orchestrating a strong anti-tumor immune response
Abstract
Pancreatic ductal adenocarcinoma (PDA) remains resistant to immune therapies, largely due to robustly fibrotic and immunosuppressive tumor microenvironments. It has been postulated that excessive accumulation of immunosuppressive myeloid cells influences immunotherapy resistance and recent studies targeting macrophages in combination with checkpoint blockade have demonstrated promising preclinical results. Yet, our understanding of tumor-associated macrophage (TAM) function, complexity, and diversity in PDA remains limited. Here, analysis reveals significant macrophage heterogeneity, with bone marrow-derived monocytes serving as the primary source for immunosuppressive TAMs. These cells also serve as a primary source of TNF-α, which suppresses expression of the alarmin IL33 in carcinoma cells. Deletion of Ccr2 in genetically engineered mice decreases monocyte recruitment resulting in profoundly decreased TNF-α and increased IL33 expression, decreased metastasis, and increased survival. Moreover, intervention studies targeting CCR2 with a new orthosteric inhibitor (CCX598) renders PDA susceptible to checkpoint blockade resulting in reduced metastatic burden and increased survival. Our data indicate that this shift in anti-tumor immunity is influenced by increased levels of IL-33, which increases dendritic cell and cytotoxic T cell activity. These data demonstrate that interventions to disrupt infiltration of immunosuppressive macrophages, or their signaling, have the potential to overcome barriers to effective immunotherapeutics for PDA.
Introduction
Pancreatic cancer (PDA) is one of the most lethal forms of cancer (1, 2). This is due, in part, to robust metastatic behavior and multiple mechanisms of resistance to molecular, immune, and radiation therapy interventions (3, 4). Importantly, PDA is characterized by a robust stromal fibrotic and immunosuppressive response that creates drug-free and anti-tumor immunity-free sanctuaries in primary and metastatic disease (5-9). While immune therapy with immune checkpoint blockade (ICB) has been successful in rare cases (10), most pancreatic cancers are resistant to ICB (11). Yet, overcoming stromal barriers found in PDA can render the disease susceptible to ICB (12-16), demonstrating that a robust anti-tumor immune response can take place in PDA under the right therapeutic conditions.

In addition to dense extracellular matrix (ECM) and the immunosuppressive behavior of carcinoma-associated fibroblasts, one of the primary barriers to an effective anti-tumor immune response in PDA is thought to be the abundance and activity of immunosuppressive tumor associated macrophages (TAMs) populations (16-19). Indeed, myeloid-derived suppressor cells (MDSCs) and macrophages are often the most abundant stromal populations in PDA (14, 20). This has strong implications for disease progression and resistance to therapy since distinctly polarized macrophages are capable of promoting all steps of tumor progression including carcinoma cell proliferation, invasion, and colonization of metastatic sites as well as having robust inflammatory and immunosuppressive functions (21-24). Furthermore, in addition to a spectrum of polarization states, distinct behaviors can emerge from tissue-resident macrophages versus infiltrating macrophages that are derived from bone marrow progenitor cells, where the latter are thought to play a larger role in immune regulation (23, 24). In this context, macrophages can not only prevent efficient T cell infiltration into the tumor (17, 24) but also decrease the ability of intra-tumoral T cells to recognize and kill tumor cells (23, 25, 26), ultimately posing a major obstacle to effective immunotherapy. Given their importance in tumor dynamics, therapeutic approaches that either deplete or reprogram macrophages have been proposed in order to improve immune therapy outcomes. However, a comprehensive characterization of macrophage heterogeneity and the mechanisms that cause a weak anti-tumor immune response in PDA still remains elusive. In this study using single-cell RNA sequencing (scRNA-seq) data, we further define the macrophage landscape of murine and human PDA. We identify unique and targetable features of infiltrated immunosuppressive macrophages that are derived from bone marrow progenitor cells. Notably, monocytes utilize CCR2 to infiltrate into PDA, where CCL2 is secreted by carcinoma cells and to a larger degree by multiple CAF phenotypes. Notably, genetic depletion of CCR2 in genetically engineered murine models of PDA results in a reduction of infiltrated macrophages leading to decreased metastasis and increased survival. Consistent with these findings, focused inhibition of CCR2 with a novel inhibitor renders primary and metastatic PDA tumors susceptible to ICB, a behavior that is augmented, in part, through increased anti-tumor immunity
from elevated levels of IL33 in cancer cells. Mechanistically, TNF-α, predominantly secreted by TAMs, decreases the expression of the alarmin, IL33, in carcinoma cells. Once IL33 is released it helps regulate the attraction of CD103+ dendritic cells and ultimately a more robust cytotoxic T cell response. Thus, this data collectively expands our knowledge of TAM dynamics in PDA and presents an effective strategy to reduce immunosuppression to achieve effective anti-tumor immunity.

Results

Diverse tumor-associated macrophages co-exist in human PDA.

Analysis of transcriptome data demonstrates that human PDA is characterized by excessive accumulation of heterogenous macrophages (Figure 1A and S1A), consistent with previous reports (14, 20). This macrophage expansion and infiltration starts at the early stages of disease (8, 20) (Figure S1B), and increased levels of macrophages correlate with poor prognosis in PDA patients (Figure 1B). To further characterize human PDA macrophage populations, we utilized single-cell RNA sequencing data (scRNA-Seq) from human PDA patients (n=6) and two cancer adjacent-normal pancreas samples (n=2); data from ref.(27) where it is noted that more than 96% of cells in this grouping were identified as monocytes or macrophages. Overall all the macrophages showed patient specific heterogeneity (Figure S1C). We observed distinct clusters and distribution of genes associated with processes such as proinflammation, matrix remodeling, metabolism and immunosuppression using dimensionality reduction with t-distributed stochastic neighbor embedding (t-SNE). Overall, we found that, and consistent with reports (23, 28), PDA TAMs display a more alternatively activated (“M2”-like) polarization with the expression of genes associated with the M2 phenotype and immunosuppression such as SPP1, CD163, CXCR4, HIF1, TGF-β1 and multiple MCHII molecules (Figure 1C). Likewise, the vast majority of PDA TAMs robustly expressed the M2 marker CLEC7A (Dectin-1), which in concert with its ligand galectin 9 promotes strong immune suppression from macrophages that can be blocked to promote anti-tumor immunity in PDA (29). Further, PDA TAMs also display shifts in metabolism (Figure S1D). The majority of TAMs display a heterogeneous expression of transcripts associated with higher immunosuppressive metabolism and signaling such as KYNU (tryptophan metabolism), NLRP3 (inflammasome/IL18), ADK (Adenosine metabolism), and STAT3 (Figure S1D). Notably, tryptophan and kynurenine productions are known to inhibit T cell proliferation and cytotoxic activity (30). Likewise, TAMs also expressed high levels of ADK, which is involved in adenosine metabolism, where adenosine metabolites promote anti-inflammatory macrophage phenotype. In contrast, transcripts suggesting a classically activated “M1”-like polarization such as CCR7, IL-6, and IL-2RA were low while NOS2 (iNOS), a robust M1 marker, was extremely low (Figure 1C), further supporting the conclusion that the majority of TAMs in PDA possess a more alternatively activated phenotype. Collectively, this culminates with strong statistical enrichment for immunosuppressive TAM function (Figures 1D) and also high enrichment
for TNF-α production pathways (Figure 1E), which is consistent with high TNF-α levels in the PDA stroma (Figure S1E), further suggesting that PDA TAMs could be a major source of TNF-α in PDA and are predominantly immunosuppressive.

Further analysis of the human PDA macrophage transcriptome supports findings from mice demonstrating that macrophages are either tissue-resident or bone marrow-derived (20, 27), where pancreatic resident macrophages originate from the fetal derived yolk sac and are maintained independently of circulating macrophages while infiltrating macrophages arise from circulating CD14+ monocytes. Indeed, PDA TAMs are a mixed population displaying markers of both populations. However, the majority of TAMs across all patients display high gene expression of CD14, C1QB, and MHCII (e.g. HLA-DR, Figure 1C) typically associated with bone marrow-derived macrophages (20, 28). For instance, ~80% of TAMs that originate from infiltrated HSC-derived monocytes are MHCIIhi (20) and monocytes that infiltrate tissue and differentiate into macrophages can be distinguished by high expression of C1QB (31)(Figure 1C). In contrast, genes associated with resident macrophages such as CX3CR1 or MerTK were also expressed but noticeably less abundant, suggesting that despite their heterogeneity the majority of TAMs originated from bone marrow.

**Immunosuppressive TAMs in genetically engineered murine models of PDA**

To examine how well human PDA TAMs are represented in various murine models of PDA we extended our investigation to the analysis of sc-RNAseq data from three different genetically engineered murine models of PDA, namely the KIC (KrasLSL-G12D/+ Ink4aββPtf1aCre/+), KPC (KrasLSL-G12D/+Trp53LSL−R172H/+Ptf1aCre/+), and KPfC (KrasLSL-G12D/+Trp53ββpdx1-Cre) systems (Figure 2), as described previously (32). Overall, TAMs populations observed in human PDA are also well represented in these murine models. Consistent with human data, murine PDA TAMs display high expression of M2-associated and immunosuppressive transcripts such as Spp1, C1qb, Arg1, Tgfb1, and multiple MCHII molecules (e.g. H2-Aa, H1-Ab1, H2-Dma, H2-Dmb1, H2-Dmb2, and H2-Eb1; Figure 2 and S2). Furthermore, similar to human data, M1 markers (e.g. Nos2, Ccr5, Il-6) were found at a lower frequency (Figure S2), consistent with previous data showing low levels of iNos+ TAMs in murine PDA (15). Further, Cd14, C1qb and Cxcr4 expressing macrophages are present in lower numbers in the normal pancreas but expand in early disease and are maintained throughout late stage disease. Likewise, the presence of Spp1+ and Arg1+ are extremely low in normal the pancreas but their number increases in PDA. Together, this data demonstrates robust TAM heterogeneity in murine PDA similar to human with dominant immunosuppressive features and the majority showing high expression of markers indicating they are bone marrow-derived in origin.
CCL2 is overexpressed by distinct cell populations at primary and metastasis sites

Our data analysis in both human and murine PDAs suggests that the majority of immunosuppressive TAMs originate from bone marrow-derived monocytes. Loss or inhibition of CCR2 is known to significantly reduce levels of circulating monocytes, and as a result, numbers of bone marrow-derived TAMs (20, 33), and reducing TAMs may be beneficial as a therapeutic strategy against PDA, e.g. (14, 17, 18, 34). Therefore, to examine the expression of the CCR2 ligand CCL2 in PDA we first performed correlation analysis with human patient data and observed a significant correlation between CCL2 and CD14 (Figure 3A), as well as other macrophage markers such as CXCR4, CD206, CD163 and MHCII transcripts (not shown), further linking expression of CCL2 in tumors with myeloid cell infiltration. Furthermore, examination of CCL2 levels using IHC demonstrated overexpression in the primary tumor and as well as in a metastatic lesion of human PDA (Figure 3B), where CCL2 overexpression starts from early disease and continues to be expressed highly throughout disease progression (Figure S3A). Similarly, elevated expression was seen in PanINs and primary and metastatic PDA in KPC mouse samples (Figure 3C). In murine and human PDA, IHC revealed CCL2 expression in carcinoma cells and the stromal compartment (Figure 3B, C), suggesting that multiple cell populations in PDA can recruit circulating monocytes. To confirm this, we performed immunofluorescent staining for CCL2 and αSMA, a marker for a dominant subtype of carcinoma-associated fibroblasts, or myCAFs, in human PDA and observed CCL2 localization with carcinoma cells, αSMA+ CAFs, and αSMAlow or αSMA-negative cells in the stroma (Figure 3D). Therefore, we sought to further evaluate the relative CCL2 contribution of carcinoma cells and CAFs. Interestingly, while primary carcinoma cells secrete CCL2, matched metastatic lines (i.e. lines from metastatic lesions in the same animals) showed greater mRNA (Figure S3B) and CCL2 secretion (Figure 3E). Yet, primary CAFs from KPC tumors secrete substantially higher CCL2 protein when compared to carcinoma cells (Figure 3F).

As recent studies have highlighted CAF heterogeneity in PDA (27, 32, 35, 36), we further dissected CCL2 secretion from two prominent CAFs populations, namely myCAFs and iCAFs, that we generated from KPC tumors as described (35). Remarkably, both CAFs phenotypes showed significantly higher expression of CCL2 compared to primary or metastatic carcinoma cells, which is consistent with sc-RNAseq data and moderate correlations between iCAF or myCAF transcript markers and CCL2 in human PDA (Figure 3I). However, iCAFs expressed significantly higher levels of CCL2 than myCAFs (Figures 3H and S3D), suggesting a hierarchy for recruiting monocytes derived macrophages to immunosuppressive niches in order of iCAF>myCAF>metastatic cells>primary carcinoma cells. However, it is notable that both iCAFs and myCAFs express profoundly more CCL2 cytokine than other cell populations from PDA tumors (Figures 3F-H and S2D), suggesting they both have a robust capacity to recruit monocytes via CCL2. In fact, this behavior was observed for a number of factors known
to promote PDA progression and/or therapeutic resistance (e.g. collagens, proteoglycans, hyaluronan synthesis, lysyl oxidase, IGF1, interleukin 6, CSF1 etc.; Figure S4), while others such as CXCL12 appear more phenotype specific. Thus, compared to the broader cell populations in PDA (vs. comparing levels solely between iCAFs and myCAFs), it is clear that many key factors are elevated in both iCAFs and myCAFs relative to other cells types but sometimes to different degrees (i.e. both are significant sources of key ECMs, cytokines, and growth factors), adding further complexity to dissecting distinct roles of CAF phenotype, particularly spatially and temporally within complex TMEs.

**Ccr2 deletion delays PDA progression and reduces metastasis to improve overall survival.**

To better understand the influence of CCL2-CCR2 signaling and the impact of bone marrow derived TAMs in PDA, we generated *KPC* mice lacking *Ccr2* genes. Ccr2 deleted mice have been reported to have reduced capacity for monocytes recruitment from bone marrow (37). To generate *KPC-CCR2* knockout mice, *KC* mice (*Kras<sup>LSL-G12D/+</sup>; *Pdx1-Cre*) were crossed with *Ccr2* global knockout mice and then bred with *Trp53<sup>LSL-R172H/LSL-R172H</sup>* mice (Figure 4A). The progenies were born in the expected Mendelian ratio, with no obvious functional defects. The deletion of *Ccr2* in *KPC-CCR2*<sup>-/-</sup> was confirmed by PCR (Figure S5A). As expected, the loss of CCR2 led to a profound reduction of circulating CD11b-positive myeloid cells (>80% reduction; Figure S5B), which results in a concomitant decrease in the PDA stroma (Figure S5C). Interestingly, examination of full survival data demonstrates that *KPC-CCR2*<sup>-/-</sup> mice survived significantly longer compared to *KPC* littermates (median survival 168 vs. 120 days, respectively; Figure 4B). Histological examination of pancreatic tumors from early-stage (10-11 weeks old) *KPC* and *KPC-CCR2*<sup>-/-</sup> mice showed that *KPC-CCR2*<sup>-/-</sup> mice have more disease-free normal pancreatic tissue and lower grade PanINs than the *KPC* group (Figure 4C), consistent with the right shifted survival curve (Figure 4B) suggesting a slower progression of disease. Consistent with this finding, depletion of circulating myeloid cells also reduced the proliferation of carcinoma cells in early and late stage disease (Figure S5D). Examination of PDA in both cohorts showed regions of well, moderate, poorly differentiated, and necrotic regions, however, *KPC-CCR2*<sup>-/-</sup> mice again showed less high-grade tumor as well as significantly less metastatic burden, but no differences in local invasion, α-SMA<sup>+</sup>-CAF frequency or fibrillar collagen deposition, when compared to *KPC* mice (Figures 4C and S5D-G), suggesting that reduced levels of CCL2-recruited TAMs in PDA slows disease progression and reduces metastatic burden, resulting in longer survival of *KPC* mice.

Examination of the metastatic distribution and burden in *KPC* mice demonstrates that while 91% of *KPC* mice display metastatic dissemination only 40% of *KPC-CCR2*<sup>-/-</sup> present with metastasis (Figure 4D, E, Tables 1 and S1). In the liver, 57% of mice in the *KPC* cohort displayed metastatic lesions, in contrast to 25% in *KPC-CCR2*<sup>-/-</sup> mice (Figure 4E). *KPC-CCR2*<sup>-/-</sup> mice also exhibited a lower percentage of diaphragm metastasis (38% vs. 20%),
and a non-significant trend of decreasing lung metastasis (41% vs. 30%). Lastly, we note that recent studies have established a role for elevated fibronectin (FN) in promoting the pre-metastatic niche, in part through the recruitment of bone marrow-derived macrophages (38, 39). Therefore, to determine if our observed reduction in metastasis is due, at least in part, to a decrease in pre-metastatic niche formation, we measured the levels of fibronectin in the 8-11 week old mice. No differences in FN levels were observed in the liver or lungs (Figure S5H) suggesting the observed decrease is not due to a difference in key ECM in the pre-metastatic niche formation but rather could be due to decreased macrophages. Taken together, this data suggests that the depletion of CCR2 mediated macrophage infiltration profoundly decreases metastatic disease.

**CCR2 inhibition mitigates the immunosuppressive environment and renders PDA susceptible to immune checkpoint blockade**

Given collective findings by us and others showing a predominate immunosuppressive TAM phenotype in PDA and our data from *KPC-CCR2−/−* mice, we sought to deplete bone marrow-derived monocytes to test the hypothesis that depletion of infiltrated TAMs is an avenue for the development of new combination immunotherapies for PDA. To test our hypothesis, we enrolled the *KPC* mice with 4-8 mm tumors in the longest axis by high-resolution small animal ultrasound into treatment cohorts using a rolling enrollment model. The enrolled mice were treated with 1) standard-of-care chemotherapy with Gemcitabine (Gem), 2) Gemcitabine in combination with ICB in the form of anti-PD-1 and anti-CTLA-4 (Gem+ICB), or 3) a combination of Gemcitabine, ICB, and CCR2 inhibition (Gem+ICB+CCR2i) (Figure 5A). Note, to our knowledge CCR2i in combination with ICB immune therapies has not been previously tested in PDA, especially using autochthonous disease that arises in the *KPC* model, and we focused on preclinical drug combinations that could be clinically viable (i.e. include a standard-of-care chemotherapy). In order to inhibit CCR2, we tested a new orthosteric inhibitor CCX598 (a potent third generation CCR2 antagonist from ChemoCentryx). CCR2i was given orally every day, a dosing scheme that achieves the desired concentration in the circulation to obtain receptor coverage (Figure S6A). Importantly, and consistent with our hypothesis, the Gem+ICB+CCR2i combination therapy significantly increased the survival of *KPC* mice compared to Gem or Gem+ICB treatments (Figure 5B), while the addition of ICB did not improve outcomes from gemcitabine alone, consistent with other reports showing that without a stroma targeting approach chemotherapy plus ICB is not impactful in PDA (12, 40). Concomitantly with this behavior, in primary tumors, total myeloid cells, F4/80+ macrophages, CD206+ immunosuppressive TAMs, MDSCs, which are robustly immunosuppressive in PDA (18), and neutrophils are all significantly decreased, while numbers of CD8+ T cells are concurrently significantly increased (Figure 5C-F and Figure 6A,B). We note that the observed decrease in neutrophils in autochthonous disease is in contrast to findings observed with grafted tumor systems using a distinct CCR2 inhibitor (41). Interestingly, Gem+ICB+CCR2i combination treatment also increased the frequency of iNOS+
cells, a marker of classically activated macrophages, in the TME (Figure S6B). Along with these shifts in the immune landscape, we also identified decreased tumor cell proliferation as evidenced by reduced Ki67+ staining and enhanced apoptosis in the Gem+ICB+CCR2i compared to the Gem or Gem+ICB groups (Figure 6C,D). Combination therapy also resulted in increased vascular patency (Figure S6C) without decreasing general fibrosis (as discerned from levels of α-SMA+ CAFs and fibrillar collagen in the PDA stroma; Figure S6D,E), demonstrating that reducing TAMs derived from bone marrow not only renders PDA susceptible to ICB, but can also surmount aspects of the vascular collapse phenotype that play a key role in driving drug-free sanctuaries in PDA(42).

**Combination of CCR2 inhibition and checkpoint blockade decreases metastasis in KPC mice**

Consistent with decreased myeloid cells in primary tumors, CCR2 inhibition also blocked the accumulation of myeloid cells in metastatic organ sites (Figure 7A,B). Therefore, to specifically determine the impact of combination Gem+ICB+CCR2i therapy on metastatic lesions, we performed a detailed necropsy and histopathological analysis of each of the three preclinical treatment cohorts. Analysis demonstrated that Gem+ICB+CCR2i combination therapy but not Gem or the Gem+ICB combination decreased total metastasis (Figure 7C-F and Table S2). 44% of Gem+ICB+CCR2i has liver metastases compared with 66% in the Gem and 70% in the Gem+ICB cohorts (Figure 7D). Gem+ICB+CCR2i treated mice also exhibited a profoundly lower percentage of lung metastasis (from 55 % to 11%; Figure 7E). This profound decrease in lung metastasis suggests a stronger role for marrow-derived macrophages in lung metastases. Notably, we also observed a concerning trend of increased diaphragm metastasis in the Gem+ICB, but a decreasing trend in the Gem+ICB+CCR-2i cohort (Figure 7F). However, in the Gem+ICB+CCR2i cohort we again observed that decreasing myeloid cells leads to a significant increase in CD8+ T cell population (Figure 7G) and tumor cell death (Figure 7H). We note the larger increases in cytotoxic T cell levels in metastatic sites compared to increases in primary tumors, suggesting that myeloid targeting therapy has robust benefit for combating metastatic disease. Thus, overall this data shows that similar to the primary disease, combination therapy can render metastatic disease susceptible to ICB, increase anti-tumor immunity, and increase cell death in established metastatic disease resulting in an overall decrease in metastatic burden.

**TNF-α/IL33 signaling resulting in increased CD8+ T cell infiltration**

To identify the signal that led to increased anti-tumor immunity, we measured cytokine levels in treated tumors. Among all cytokines, interleukin-33 (IL33) was the most highly enriched in the Gem+ICB+CCR2i treated tumors (Figure 8A). We confirmed this result in all three treatment groups through IHC analysis that shows a heterogenous expression in both the carcinoma and stromal compartments in both the GEM and GEM+ICB groups.
with higher levels in the stroma (Figure 8B). However, in the triple therapy group we again observe expression in both compartments, but a profound increase in IL33 expression in carcinoma cells (Figure 8B). Increased levels of IL33 were also observed in KPC CCR-2-/- animals (Figure 8C). This data suggests blockade of bone marrow derived macrophages in concert with chemo and immune therapy leads to robust increases in IL33 expression in pancreatic tumors.

Macrophages also showed very high enrichment of TNF-α production pathways (Figure 1F). Indeed, dual staining of KPC tumor shows that most of the TNF-α colocalizes with F4/80+ macrophages (Figure S7A) and analysis of RNAseq data shows strong expression from TAMs (Figure S7B), validating the conclusion that macrophages are a primary source of TNF-α in PDA, consistent with previous findings (43, 44). Further, IF analysis of both tumors from KPC-CCR-2-/- mice and combination therapy treated tumors shows a profound decrease in TNF-α levels (Figure 9A,B), again supporting the conclusion that tumor infiltrating bone marrow derived TAMs are a primary source of TNF-α in PDA. Previously, TNF-α has been shown to modulate the expression of IL33 in normal and diseased fibroblasts (45), Thus next, we hypothesized that this increase in IL33 could be due to a decrease in TNF-α from macrophages. Since the major increase in IL-33 in the Gem+ICB+CCR2i group was observed from carcinoma cells, we sought to test if TNF-α can directly regulate IL33 expression in carcinoma cells. We treated primary KPC cell lines with recombinant TNF-α which led to significantly decreased IL33 at both the gene and protein levels (Figure 9C,D). Interestingly, IL33 is a member of the IL-1 family that has been shown to play a key role in innate and adaptive immunity (46, 47). IL33 is normally released by damaged or necrotic cells and can act as an alarmin capable of activating either TH1 or TH2 response(47, 48). Furthermore, IL33 was recently shown to activate tumor infiltrated group 2 innate lymphoid cells (ILC2s) to regulate CD8+ T cells responses(49). Indeed, IL33 expression correlates strongly with CD8A and granzyme B as well as genes like BATF3, IRF8, THBD, CLEC9, and XCR1 that are required for CD103+ DC cross-presenting tumor antigen functionality (Figures 9E and S7C). Therefore, to test if decreased TNF-α and increased IL33 leads do indeed lead to increased numbers of CD103+ dendritic cells in the tumor microenvironment, we stained the tumor section for both CD11c and CD103 and observed a significant increase in both dendritic cells markers (Figure S7D and 9F). Next, we subcutaneously implanted KPC cells in syngeneic mice with or without recombinant IL33 (rIL33) mixed into growth factor reduced Matrigel. Consistent with evidence suggesting that increased IL33 levels promote anti-tumor T cell responses; PDA tumor growth was profoundly inhibited in the rIL33 group compared to control conditions (Figure 9G) with concomitant and robust increases in CD8+ T cell infiltration (Figure 9H). We note that consistent with finding from Moral et al., (49) we do not observe IL33R expression of CD8+ T cells (Figure S7E-G), suggesting that IL33 signaling impacts T cells through an intermediary, such as ILC2s and CD103+ dendritic cells. These
data therefore show that the alarmin IL33 promotes CD8+ T infiltration and anti-tumor response in PDA. Thus, we demonstrate that depletion of immunosuppressive TAMs and MDSCs results in decreased TNF-α causing increased IL33 levels in carcinoma cells that results in increased cytotoxic T cell response to combat metastatic PDA.

**Discussion**

Recent studies across a range of cancer have greatly advanced our understanding of the suppression of host immunity by TAMs (22, 50-52). However, macrophage diversity, complexity and regulatory mechanisms in many tumors, including those of the pancreas, remain poorly defined. Our findings highlight the inter-patient and intra-tumor TAM heterogeneity present in PDA patients and its faithful recapitulation in preclinical murine models. While additional studies are needed to parse out and define the impact of this heterogeneity, it likely contributes to the patient-specific tumor biology and therapeutic responses. Indeed, while analysis shows that most PDA TAMs display a more M2-like polarization and high immunosuppressive capacity, there exists significant variability within and across tumors, suggesting that a better understanding of both the spatial and temporal TAM dynamics is needed. However, we do note that most of the immunosuppressive and pro-tumor TAM genes overlapped with markers suggestive of a bone marrow origin and that limiting large portions of this heterogeneous population by disrupting monocyte homing to PDA is therapeutically beneficial. In fact, blocking infiltration bone marrow derived TAMs significantly relieves immune suppression and augments checkpoint blockade therapy. Previously, in grafted tumor models targeting CCR2 resulted in a compensatory influx of neutrophils (41), something we did not observe here using a different CCR2 inhibitor against autochthonous disease. Furthermore, we observed strong TNF-α signaling from bone marrow-derived TAMs. Interestingly, TNF-α is an abundant cytokines in PDA TME (53), yet the role of TNF-α in tumor development remains paradoxical and has been shown to be anti-, and pro-tumor in different contexts. For instance, treatment of PDA cells with recombinant TNF-α increased EGFR expression from carcinoma cells (54) while in another study recombinant TNF-α promoted the growth of Panc02 tumors in mice but inhibited KPC cells growth (55). Furthermore, a recent study suggests that higher expression of TNF-α forces classical neoplastic cells into an aggressive basal like states suggesting a pro-tumorigenic role for TNF-α in PDA (43). Then there are additional roles in regulating tumor stromal dynamics and immunity. Our study suggests that TNF-α directly regulates the expression of IL33 in carcinoma cells, thus supporting the conclusion that TNF-α plays a role in immune suppression in PDA that can be overcome through blockade of bone marrow-derived macrophages in concert with immune therapy. Thus, targeting broad and diverse collections of pro-tumor immunosuppressive TNF-α producing TAMs appears to be part of a viable strategy to break down one of the key barriers to effective anti-tumor immunity in PDA.
In recent years preclinical and clinical studies have sought to disrupt myeloid cell levels and/or function to improve outcomes. For instance, inhibiting CSF1R signaling functionally reprograms macrophage responses to enhance antigen presentation and productive antitumor T-cell responses in orthotopic grafted tumor PDA models. Unfortunately, the CSFRI blocking monoclonal antibody cabiralizumab combined with nivolumab and chemotherapy in advanced PDA did not improve progression-free survival in phase II clinical trial (NCT03336216). Further, in another recent study, CSF1R inhibition by PLX5622 caused long-term changes in bone marrow-derived macrophages and also reduced the population of resident and interstitial macrophages of peritoneum, lung, and liver raising concerns about the potential long-term consequences of CSF-1 inhibition.

Yet, targeting MDSCs and TAMs remains a viable strategy to overcome one of the major obstacles to effective anti-tumor immune responses in PDA. For instance, targeted depletion of granulocytic MDSCs in autochthonous PDA in KPC mice increases the intratumoral accumulation of activated CD8 T cells. While partial activation of CD11b leads to TAM repolarization, a reduction in immunosuppressive myeloid cells, and enhanced dendritic cell responses to improve antitumor T cell immunity Moreover, targeting CCR2 has also emerged as a strategy to improve outcomes since it has been hypothesized to reduce infiltration of myeloid cells. Indeed, in a grafted tumor model, CCR2 inhibition was effective at reducing TAMs, which is consistent with our finding here in autochthonous primary and metastatic disease where we find that TAM reductions render PDA susceptible to ICB. In two different clinical trials, CCR2 antagonists have been combined with chemotherapy in an attempt to overcome chemoresistance in pancreatic cancer patients, yet to our knowledge, CCR2i in concert with immune therapy has not yet been trialed in PDA. However, the data from these trials suggest that CCR2i inhibitors are well tolerated in patients and thus could likely be safely combined with immune checkpoint inhibitors to overcome immune resistance. As such, novel ways to target myeloid cell infiltration such as the new CCR2 inhibitor used here, or through manipulation of key signaling pathways such as IL33 signaling appears to be viable strategies for improving outcomes in PDA. Likewise, we note that our analysis demonstrates that both myCAFs and iCAF produce significant amounts of CCL2, suggesting that targeting CAFs has the potential to also improve anti-tumor immunity, consistent with reports targeting CXCR4 or FAK signaling to increase susceptibility to ICB.

However, care must be taken to overcome barriers from CAF signaling as manipulation of the stroma can also lead to more aggressive disease in some contexts, particularly when altering the stroma prior to the onset of disease, but can be very beneficial in other cases, including increased influx of CD8 T cells and M1 macrophages after antifibrotic therapy(15) highlighting the complex role of the TME in disease pathogenesis and therapeutic response. Yet, is clear that we must find ways to overcome these barriers to effective distribution of therapies and anti-tumor immunity. Indeed, it appears likely that combinations of stroma targeting therapies (targeting CAFs, ECM etc.), myeloid suppression, perhaps with immune priming, in concert with molecular or cellular immune
therapies will be needed to overcome patient specific stromal and immunosuppressive barriers in PDA to improve patient outcomes.

Lastly, our data also suggest that blocking bone marrow-derived TAMs leads to a profound decrease in TNF-α and increased IL33 in PDA. IL33 is a Th1 and Th2 promoter cytokine and an alarmin that can be released from cells during cell death\(^ \text{(60, 61)} \). IL33 has also been suggested to have both protumor\(^ \text{(62, 63)} \) and antitumor\(^ \text{(49, 64, 65)} \) roles. In our study increased IL33 due to decreased immunosuppressive myeloid cells/TNF-α resulted in increased CD103+ DCs and CD8+ cytotoxic T cells, particularly in metastatic sites, supporting the previous finding that PDA patients with higher IL33 survive longer and that IL33 can promote anti-tumor immune responses by actively ILC2s that can indirectly prime CD8+ T cells, likely through recruitment of CD103+ DCs that promote T cell recruitment and priming\(^ \text{(49)} \). In the same study, intraperitoneal injection of rIL33 activated ILC2 in orthotopic tumors but this was not the case for subcutaneous tumors murine grafted tumor model. However, in our study, a direct implant of rIL33 in the developing tumor, which we believe well represents elevated IL33 levels in the tumor microenvironment, led to increased T cell response and decreased tumor growth suggestive of a global role of L33 in activating anti-tumor immunity. Thus, put all together, our study suggests a novel therapeutic strategy to decrease TNF-α and/or increase IL-33 in PDA patients which could lead to better outcomes. Indeed, these collective data suggest a potential for multiple therapeutic avenues, including targeting TAMs and MDSCs, anti-TNF-α therapy, manipulating IL-33 levels directly, or by altering the behavior of ILC2s and DCs. Thus, in summary, our study reports heterogeneous TAM populations that originate from bone marrow that are the primary source of TNF-α. \( \text{Ccr2} \) deficiency decreases bone marrow derived TAMs and thus TNF-α that led to increases IL33 which increases survival and decreases metastasis in \( \text{KPC} \) mice. Furthermore, blocking marrow-derived macrophages in combination with gemcitabine and immune checkpoint blockade increases survival and decreases metastasis, suggesting a rational combination strategy to activate anti-tumor immunity in PDA patients.
Materials and Methods

Single-cell data analysis
Human single-cell data (phs001840.v1.p) processed by Cell Ranger version 1.3.1 (10x Genomics)(27) were loaded to the R package 2.3.1. Cell clusters were identified via the FindClusters function using a resolution of 0.6 for all samples, based on a graph-based clustering algorithm. Functional enrichment of Gene Ontology (GO) and KEGG pathways analyses were performed using ToppGene (https://toppgene.cchmc.org). p-value < 0.05 was considered to be significant enrichment. Mouse single-cell data (GSE125588) processed by Cell Ranger version 1.3.1 (10x Genomics)(32) were loaded to the R package Seurat version 2.3.1. Cell clusters were identified via the FindClusters function using a resolution of 0.6 for all samples, based on a graph-based clustering algorithm. A likelihood ratio-based test or an AUC-based scoring algorithm was used to compute marker genes for each cluster, and expression levels of several known marker genes were examined. Different clusters expressing known marker genes for a given cell type were selected and combined as 1 for each cell type. Macrophage subclusters were then further identified in macrophage clusters using the SetAllIdent function.

The Cancer Genome Atlas human patient cohort data analysis
Transcript analysis was performed on human data sets publicly available through the Cancer Genome Atlas database. Correlation analysis was performed using the GEPIA platform(66).

Generation of murine primary KPC carcinoma cell, iCAFs and myCAFs
All murine primary cell lines were generated from freshly excised KPC tumors as described previously(15). iCAF was generated according to a previously reported protocol(36). In brief, for iCAF, the purified PSCs were plated in Matrigel and were cultured in tumor organoid condition media for 3 days, and for myCAF, the purified CAFs were plated directly on a 2D surface. The phenotype of iCAF and myCAF was confirmed by measuring the gene expression of Il-6, Cxcl-1, Acta-2, Lif and Ctgf (Figure S3C).

TNF-α treatment
KPC cells were grown on minimal media (DMEM with 1%FBS) overnight and then cells were treated with different concentrations of recombinant TNF-α for 24 hrs. After 24hr the cells were harvested for protein and RNA isolation. Western blot was done as described previously(67)
Real-Time PCR Analysis
Total RNA was extracted with the Trizol (Roche) as described by the manufacturer and cDNA synthesis, 1µg of total RNA was reverse transcribed using the Superscript II (Thermo Fisher Scientific) Reverse Transcription System. Real-time PCR was run in triplicate using Platinum SYBR green master mix (Bio-Rad Laboratories), following the manufacturer's instructions. Real-time monitoring of PCR amplification was performed using the qPCR System (Roche). Data were expressed as relative mRNA levels normalized to 18S served as endogenous normalization controls expression levels in each sample and are represented as mean ± S.E.M. between three independent experiments unless otherwise indicated in the figure legend. The primer sequences are listed in Table S3. Pre-made primers of Ccl2 primers were ordered from Singobiological (#MP200388) and 18s (#249900) from Qiagen.

Animal studies
Ccr2/- mice (#004999) were obtained from Jackson laboratory. To generate KPC-CCR2/- mice, KrasLSL-G12D;Pdx1-Cre (KC) mice were crossed with p53LSL-R17H/+ or p53LSL-R17H/LSL-R172H mice to generate KPC mice. We crossed CCR2/- mice with KC mice to generate KC-CCR2/- mice and these were then crossed with p53R17H/+ or p53LSL-R17H/LSL-R172H mice to generate KPC-CCR2/- mice. The KPC-CCR2/- mice were of C57BL/6 background. KPC mice on the C57BL/6 background were used as the control for the comparative studies. The progeny was born in an expected Mendelian ration, with no obvious functional defects. In vivo drug studies were done using KPC genetically engineered mouse model (68) on a mixed background as previously described. At the endpoint (or for the early studies 10-11weeks) full necropsies were performed on all study animals and included a gross examination of all organs for macroscopic disease as previously described (15, 67, 69).

Histologic analysis
All stained tissue samples were digitally scanned at high-resolution and viewed using Nikon Ti-3 Microscope. Using this software, we identified the PDA-positive area within hematoxylin and eosin (H&E)-stained sections. PanINs, PDA, necrotic and metastatic lesions were identified on H&E and were quantified according to previously reported protocols(67). For histopathological analysis, the quantification of high, low-grade PanIN or PDA, low grade, and high-grade tumors were quantified as a percentage relative to the total tumor. The histological invasion score of the tumor cells into the surrounding stroma was scored on a scale of 0 to 2, with 0 indicating no invasion and 2 indicating high invasion, where invasion is defined as tumor cell penetrating the adjacent organs such as the duodenum, liver, etc.
Flow cytometry

To quantitate blood monocytes, 200 µL of blood was obtained by Submandibular vein and incubated in red blood cell lysis buffer (BioLegend) for 15 minutes on ice and stained with fluorophore-conjugated antibodies for 20 minutes on ice. For ST2 receptor detection, total spleen cells were isolated from C57BL/6 mice in ice cold PBS and stained with ST2 antibodies for 15 mins on ice. Stained cells were analyzed on LSR-II.

ELISA

CCL2 levels were quantified in cell culture media using commercially available ELISA kits (Thermo Fisher Scientific, # 88-7391-22) according to the manufacturer’s instructions. Briefly, cells were seeded into 24-well plates. After 24 hr and the culture supernatant was collected, centrifuged (5,000g for 5 min) and secreted CCL2 was measured by the ELISA kit.

Western blot.

Cells were collected in ice-cold RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche; 11697498001), Protein concentration was determined by Bradford assay. Equal amounts of proteins were resolved via 4–20% (v/v) SDS–PAGE and electrophoretically transferred onto nitrocellulose membranes using a semi-dry blotting system (Bio-Rad; 10026938). Membranes were blocked with 5% (w/v) milk powder in TBST for 1 hr at RT, followed by incubation with primary antibodies overnight at 4° C. Secondary HRP-linked antibodies were incubated for 1 hr at RT. Protein was detected by a BioRad Imager using ECL substrate (Millipore). β-actin was used as a loading control.

Ultrasound for enrollment and monitoring of disease progression

Weekly ultrasound was performed on KPC mice using the Vevo 2100 Imaging System for both enrollment and disease monitoring. When tumors reached a diameter of 4 to 8 mm, mice were randomly enrolled in study groups. The Vevo 2100 software was used to reconstruct 3-D tumor volumes for quantification of tumor volume growth over time.

In Vivo Drug Treatment Experiments

For drug treatments, mice with 4-8 mm tumors in the longest direction were randomly assigned to cohorts. Treatments used were: Gemcitabine (#61745 LKT Laboratories) at 100 mg/kg by intraperitoneal (i.p.) injection every 4-5 day; CCR2 inhibitor (CCX598 compound, a CCR-2 inhibitor, was provided by ChemoCentryx LLC, California, USA) at 100 mg/kg per os (70) daily; anti-PD1 and anti-CTLA-4 (BioXcell) injection i.p. every 4-5 days at 250 and 200 µg, respectively.
IHC staining
Formalin-fixed paraffin-embedded (FFPE) tissue sections (5µ) were stained for the targets according to the previously reported protocol(15, 69, 71). In brief, For α-SMA and CD31, antigen retrieval was performed using 1× citrate buffer pH 6 (Sigma) for 20 minutes, washing with 1× TBS/0.1%Tween-20 (TBST; all washes were done in TBST), blocking with 5% goat serum for 1 hour, then incubation with primary antibody overnight at 4°C. This was followed by an endogenous peroxidase block with 3% H2O2 for 15 minutes, incubation with a polymer secondary antibody for 30 minutes (Rat Probe, BioCare Rat-on-Mouse anti-CD31 Polymer Kit), followed by Rat-HRP (BioCare) for 30 minutes, and then counterstained with freshly filtered Mayer's Hematoxylin (Fisher Scientific) for 5 minutes, followed by dehydration and clearing with increasing concentrations of ethanol and xylenes. For α-SMA, antigen retrieval was performed using a 1× Dako Tris/EDTA pH 9 solution for 20 minutes. This was followed with an endogenous peroxidase block of 3% H2O2 for 10 minutes, blocking for avidin/biotin (Vector kit), blocking with TCT buffer (0.1% trypsin, 0.1% CaCl2, 20 mmol/L Tris-Cl pH 7.8) for 10 minutes, then incubation with primary antibody for 1 hour at room temperature. Slides were then incubated in MACH2 Rabbit HRP, followed by incubation with DAB for 10 minutes, counterstaining with hematoxylin, and finally dehydration and clearing. For iNOS, IL33, S1009A, F4/80,CD206,CCL2, FN1, CD11c and cleaved caspase-3, antigen retrieval was performed using 1× citrate buffer pH6 (Sigma) for 30 minutes, washing with 1XTBS/0.1%Tween-20 (TBST), followed by an endogenous peroxidase block by 3% H2O2 for 15 minutes and then blocking with 5% goat serum for 1 hour, then incubation with primary antibody overnight at 4°C. This was followed by, incubation with anti-rabbit or anti-rat HRP polymer secondary antibody for 30 minutes (BioCare Polymer Kit), followed by incubation with DAB, and then counterstained with freshly filtered Mayer's Hematoxylin.

Immunofluorescent staining
OCT frozen tissue sections were used to stained Ly6G, CD103 and anti-pan cytokeratin. Slides were air-dried for 15 minutes at room temperature, incubated in acetone for 15 minutes, and then air-dried for 15 minutes. Slides were rehydrated with 1× PBS for 10 minutes, blocked with 2% normal goat serum for 1 hour at room temperature, and incubated with primary antibody for 2 hours at room temperature. Following this, secondary antibody (1:1,000; Life Technologies, 1749750) and directly conjugated antibodies (PanCK) were added for 1 hour at room temperature, followed by counterstaining with Bisbenzimide (1:10,000; Sigma-Aldrich) for 10 minutes at room temperature and mounting with Prolong Gold (Life Technologies). Washes between steps were done using 1× PBS. Immunofluorescence samples were imaged on a Nikon Ti-U fluorescence microscope; CD8 primary antibody was detected with Tyramide super-boost kit (Invitrogen; B40944) according to manufactures instruction.
Heat-induced antigen retrieval was performed with Epitope Retrieval Buffer 2 for 20 minutes. Primary antibodies were incubated overnight at 4°C. The nonspecific background was blocked with 3% normal donkey serum + 3% normal goat serum in PBS-T. To quantify IHC or IF-stained tissue sections, 10-12 regions of interest were randomly selected within the PDA-positive area, defined by referencing previously identified PDA-positive areas in adjacent H&E-stained sections. The numbers of positive cells were calculated using ImageJ. For TNF-α antigen retrieval was done in citrate buffer for 20 mins and slides were incubated overnight at 4°C. The primary was detected by Goat anti-mouse Ig G (Thermo Fisher # A-21124).

**Collagen Imaging**

Second-harmonic generation (SHG) imaging was implemented to fibrillar collagen in FFPE tissue sections that were rehydrated with xylenes and serial dilutions of ethanol, then mounted with Prolong Gold (Life Technologies). Visualization of collagen was done on a custom-built multiphoton laser scanning microscope (Prairie Technologies Bruker) using a Mai Tai Ti: Sapphire laser (SpectraPhysics) that we have previously described (72) at an excitation wavelength of 880 nm as described (73, 74).

**Protein cytokines assays**

Protein cytokine array as performed according to manufactures instructions (#ARY028). In brief, tissues were homogenized in PBS with protease inhibitors. After homogenization, Triton X-100 was added to a final concentration of 1%. Samples were frozen at ≤-70 °C and thawed followed by centrifugation at 10,000 x g for 5 minutes to remove cellular debris. 200g of protein was used per assay.

**Recombinant IL33 implants.**

*KPC* cells (25K) in suspensions in Matrigel were injected s.c. into the rear right flank of C57/BL6 mice (6-8 weeks old) mix with either rIL33 (1µg; R&D; AF3626) or PBS. Tumor size was measured at every 4-5 days intervals. Tumor volumes were measured along orthogonal axes (a, b, and c) and calculated as abc/2.

**Statistical analysis**

Data were tested for the assumption of normality using the Shapiro-Wilk normality test. Normally distributed two-group data were analyzed using a t-test (for two groups) or one-way ANOVA (for multiple comparisons). The data which did not pass the normality test, the nonparametric Mann-Whitney sum rank test (for two groups) or Kruskal–Wallis (for multiple groups) followed by Dunn's multiple comparison test were performed. Kaplan-Meier survival data were analyzed using a log-rank test. Metastatic disease burden was analyzed using Fisher's exact test.
Study approval.
All animal studies were approved by the IACUC of the University of Minnesota. All human samples were deidentified and obtained either through the UMN BioNet Shared Resource program at the University of Minnesota in accordance with University of Minnesota IRB approval that includes informed consent for tissue donation or from publicly available commercial sources.

Author contributions
AD and PPP conceptualized and designed the study. AD and PPP participated in the design of experiments and AD, JZ, and HH performed experiments. AD performed mouse experiments with assistance from JZ. AD, AS, HH, KZ, RAB, and PPP participated in data analysis and interpretation, including analysis of unique datasets and quantitative metrics and algorithms. AD, PPP, and AS designed bioinformatics analysis for this study and AS and HH performed bioinformatics analysis. AD, AS, HH, and PPP performed statistical analyses of data. PPP, AS, RAB secured funding. AD, and PPP wrote the manuscript, with significant input by RAB. All authors read and contributed comments to the final manuscript. PPP oversaw all aspects of the study.

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References


Figure 1: Human PDA has heterogeneous macrophage populations. A) TCGA dataset analysis demonstrating that transcripts identifying myeloid cells and TAMs in PDA (i.e. CD68, CD11b and CD14) are overexpressed in PDA. B) Transcripts associated with CD68\(^+\) macrophages correlate with poor prognosis in PDA patients (TCGA dataset analysis). C D) Distribution of factors that regulate macrophage polarization and function within the heterogeneous macrophage populations in PDA showing the strongest signal for immunosuppressive factors. E-F) GO pathway analysis of human PDA CD68\(^+\) macrophages showing decreased anti-tumor immune activation (i.e. immunosuppressive behavior) and increased pathways enriched in TNF-\(\alpha\) production.
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**Figure 2:** Cross species examination of macrophages from *KPC, KIC, and KPfC* genetically engineering murine models of PDA demonstrates similarities to human macrophages. Visualization of transcript
distributions in murine macrophage populations using t-SNE (t-distributed stochastic neighbors embedding) show that similar to human PDA, expression patterns in KPC, KIC, and KPJC macrophages suggest robust immunosuppressive behavior.
Figure 3: Both iCAF and myCAF secrete high levels of CCL2 in PDA TMEs.  A) Human TCGA data set analysis shows a strong correlation between expression levels for CCL2 and CD14.  B) CCL2 is over-expressed in human PDA. IHC analysis demonstrates high expression of CCL2 in both human primary PDA tumors and metastatic lesion (lung). C) CCL2 is also overexpressed in the genetically engineering KPC model of PDA at all stages. IHC shows high expression in PanINs, primary tumor, and metastatic lesions (Liver). D) CCL2 is co-localized with carcinoma cells and both $\alpha$-SMA low and $\alpha$-SMA high cells in the stroma of both human and murine PDA. E) Metastatic cancer cells secrete higher levels of CCL2 than carcinoma cell derived from primary tumors (CCL2 levels were measured in culture supernatant by ELISA for paired primary and metastatic cell lines derived from KPC mice). F) CAFs secrete higher CCL2 than carcinoma cells. CCL2 levels in culture supernatant of CAFs (grown on 2D culture plates with serum, i.e. myCAFs) and carcinoma cells, measured by ELISA (n=5-7 KPC cell lines, p value was derived by Mann-Whitney test). G-H) CAFs are the major CCL2 contributors in PDA. Violin plots of Ccl2 transcripts for individual cell populations in KPC PDA shows iCAF and myCAFs both express higher levels Ccl2 compared to other tumor cell populations. The expression of Ccl2 in both myCAFs and iCAFs was validated experimentally by qPCR (n=5-7 KPC cell lines, p value was derived by Mann-Whitney U test). I) Strong correlations between CCL2 and both iCAF markers (CXCL-1, LIF) and myCAF markers (ACTA, CTGF) genes in human TCGA datasets demonstrating that CCL2 is highly expressed by both iCAFs and myCAFs.
Figure 4: Genetic deletion of Ccr2 reduces the disease severity. A) Schematics of the KPC mouse model of pancreatic cancer: \( Kras^{LSL-G12D/+};p53^{LSL-R172H/+};Pdx1-Cre \). Deletion of Ccr2 was attained by crossing KPC with Ccr2\(^{-/-}\) (global) mice, referred to as KPC-CCR2\(^{-/-}\). B) Kaplan-Meier analysis comparing survival of KPC (n = 37)
and $KPC-CCR2^{-/-}$ (n = 21) mice demonstrates that $KPC-CCR2^{-/-}$ mice survive longer than KPC mice; p-value as indicated from the Logrank test. C) $KPC-CCR2^{-/-}$ animals show delayed PDA onset. Comparative hematoxylin and eosin (H&E) staining and histopathology of pancreata from $KPC$ and $KPC-CCR2^{-/-}$ mice at early (10-11 weeks) (n=3 in each group) and at the endpoint ($KPC$ n=22 and $KPC-CCR-2^{-/-}$ n=19) shows less advanced disease throughout the pancreas of $KPC-CCR2^{-/-}$ mice. D-F) $KPC-CCR2^{-/-}$ mice show decreased metastasis. D) Representative H&E stained images of the liver and lung metastatic lesions from $KPC$ and $KPC-CCR2^{-/-}$ cohorts. E) Table comparing metastatic burden in $KPC$ and $KPC-CCR2^{-/-}$ mice; p-value from Fisher's exact test. F) Percentage of metastasis in various organs of $KPC$(n=22) and $KPC-CCR2^{-/-}$ (n=19) animals, p-value from Fisher's exact test. (Scale bars = 50 µm).
Figure 5: Blocking infiltration of bone marrow-derived TAMs increases responsiveness to immune therapy in KPC mice. A) Schematics of the therapy regime. B) Kaplan-Meier curve showing that Gem+ICB+CCR2i treated animals have significantly longer survival compared to Gem or Gem+ICB cohorts. C-G) IHC/IF analysis demonstrates that Gem+ICB+CCR2i combination therapy results in significant decreases in (C) total CD11b+ myeloid cells, (D) F4/80+ macrophages, (E) CD206+ immunosuppressive macrophages, and (F) MDSCs. p-value from Kruskal–Wallis and by Dunn's multiple comparison test. n=4-6 animals in each group. (Scale bars = 50 µm).
Figure 6: Blocking infiltration of bone marrow-derived TAMs increases cytotoxic T cell levels and carcinoma cell death in KPC mice. A) IHC/IF analysis demonstrates that Gem+ICB+CCR2i combination therapy results in significant decreases in neutrophils within PDA. B) IF staining shows significant increases in CD8+ cytotoxic T cells in the Gem+ICB+CCR2i treatment group. C) Gem+ICB+CCR2i treated animals have lower numbers of Ki67+ cells. D) Gem+ICB+CCR2i therapy increases cell death as shown by IHC staining for cleaved caspase-3 (CC3+). Cell number or signal per FOV is shown p-value from Kruskal-Wallis and by Dunn's multiple comparison test. n=4-6 animals in each group. (Scale bars = 50 µm).
Figure 7: Blocking infiltration of bone marrow-derived TAMs decreases metastatic burden and increases anti-tumor immune responses in metastasis lesions.  

A) IHC staining for CD11b shows that Gem+ICB+CCR2i therapy decreases myeloid cell recruitment into metastatic liver sites. p-value from Kruskal–Wallis and by Dunn's multiple comparison test. n=4-6 animals in each group. 

B-C) Gem+ICB+CCR2i therapy significantly decreases metastatic burden. (B) Representative images of lung metastatic lesions in Gem, Gem+ICB, and Gem+ICB+CCR2i animals and (C) associated quantification of metastatic burden in the liver, lung, and diaphragm in KPC mice treated with either Gem(n=9), Gem+ICB (n=11), and Gem+ICB+CCR2i (n=13). Scale bar = 50 microns. 

D) IF staining shows that Gem+ICB+CCR2i combination increases CD8+ T cell infiltration at metastatic sites. P value was derived by Mann-Whitney test, n=4-5 animals. 

E) CC3 IHC analysis
shows that Gem+ICB+CCR2i therapy increases cell death in metastatic PDA. (p value was derived by Mann-Whitney test n=4-5 animals in each group). (Scale bars = 50 µm)

Figure 8: Blocking TNF-α producing TAMs increases alarmin IL33 levels in the TME. A) Protein cytokine array of tumor lysates and quantification of array spots showing increased IL33 expression upon Gem+ICB+CCR2i treatment (n=2 tumors in each group). B) IHC of IL33 in Gem, Gem+ICB, and Gem+ICB+CCR2i treated tumors, showing robustly elevated levels following Gem+ICB+CCR2i treatment. C) IHC and quantification show a higher IL33 levels in KPC-CCR2-/– animals compared to KPC (p value was derived by Mann-Whitney test, n=4-5 animals). Scale bar = 50 microns.
Figure 9: increases in IL33 induces increases in CD8+ cytotoxic T cell levels. A-B) IF staining shows significant decrease TNF-α levels in KPC-CCR-2 −/− and Gem+ICB+CCR2i treatment group when compared to KPC and Gem alone groups (p value was derived by Mann-Whitney test, n=4-5 animals). C-D) Treatment of KPC cells with recombinant TNF-α causes decrease in IL33 at gene and protein levels (p value from Kruskal–Wallis and by Dunn's multiple comparison test. n=3). E) Strong correlations between IL33 and markers of DC103+ cell levels and functionality (TCGA dataset analysis). F) IF staining shows significant increase in DC103+ dendritic cells in Gem+ICB+CCR2i treatment group (p value was derived by Mann-Whitney test, n=4-5 animals). G) Analysis of tumor growth curves for control and rIL33 mice shows that L33 decreases the size of subcutaneous PDA tumors and overall tumor volume at the endpoint (n=3/group). H) IF staining and
quantification demonstrates that L33 increases CD+ T cell numbers into PDA tumors (p value was derived by Mann-Whitney test, n=3 animals/group). Scale bar =10 microns.

Table 1: Table comparing metastatic burden in *KPC* and *KPC-CCR2*−/− mice; p-value from Fisher’s exact test.

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