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Targeting Tumor-Associated Macrophages and Granulocytic-Myeloid-Derived Suppressor Cells Augments PD-1 Blockade in Cholangiocarcinoma

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Abbreviations: α-SMA, alpha smooth muscle actin; ApoE, Apolipoprotein E; Arg 1, arginase 1; BMDM, bone marrow-derived macrophages; CCA, cholangiocarcinoma; CK, cytokeratin; CSF1R, colony stimulating factor 1 receptor; CTL, cytotoxic T lymphocyte; G-MDSCs, granulocytic-MDSCs; GTEx, Genotype-Tissue Expression; ICB, immune checkpoint blockade; LXR, liver-X receptors; MDSCs, myeloid-derived suppressor cells; M-MDSCs, monocyte-MDSCs; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; TAMs, tumor-associated macrophages; TCGA, The Cancer Genome Atlas; TIME, tumor immune microenvironment.
Abstract

Immune checkpoint blockade (ICB) has revolutionized cancer therapeutics. Desmoplastic malignancies such as cholangiocarcinoma (CCA) have an abundant tumor immune microenvironment (TIME). However, to date ICB monotherapy in such malignancies has been ineffective. Herein, we identify that tumor-associated macrophages (TAMs) are the primary source of PD-L1 in human and murine CCA. In a murine model of CCA, recruited PD-L1+ TAMs facilitate CCA progression. However, TAM blockade failed to decrease tumor progression due to a compensatory emergence of granulocytic-myeloid-derived suppressor cells (G-MDSCs) that mediated immune escape by impairing T-cell response. Single-cell RNA sequencing (scRNA-seq) of murine tumor G-MDSCs highlighted a unique ApoE G-MDSC subset enriched with TAM blockade; further analysis of a human scRNA-seq dataset demonstrated the presence of a similar G-MDSC subset in human CCA. Finally, dual inhibition of TAMs and G-MDSCs potentiated ICB. In summary, our findings highlight the therapeutic potential of coupling ICB with immunotherapies targeting immunosuppressive myeloid cells in CCA.
**Brief Summary**

TAM blockade in cholangiocarcinoma results in compensatory emergence of G-MDSCs including a unique ApoE G-MDSC subset. Dual blockade of TAMs and G-MDSCs augments anti-PD-1 therapy.
Introduction

Cholangiocarcinoma (CCA) is the most common biliary malignancy and the second most common primary hepatic malignancy (1). CCAs are highly lethal neoplasms with limited therapeutic options and a dismal 5-year survival rate of less than 10% (2, 3). The majority of patients present with advanced disease not amenable to potentially curative options such as surgical resection or neoadjuvant chemoradiation followed by liver transplantation (1). The available systemic therapies for patients with advanced disease are of limited efficacy with an overall survival rate of <1 year in patients treated with gemcitabine and cisplatin, the current standard of care (4). Hence, there is a critical need for the development of highly effective medical therapies for patients with advanced stage CCA.

The advent of immune-directed therapies has revolutionized the treatment of human cancers. Induction of immune checkpoints such as programmed death-1 (PD-1) and its ligand PD-L1 mediates tumor immune evasion (5, 6). Immune checkpoint blockade (ICB) employs antibody-based therapies targeting these checkpoints in an effort to unleash pre-existing adaptive immunity. Desmoplastic malignancies such as CCA have an abundant tumor immune microenvironment (TIME). However, to date ICB monotherapy in such malignancies has been disappointing with a response rate of less than 10% (7), implying that these cancers have a poorly immunogenic or ‘cold’ TIME and require further strategies for effective immunotherapy (8). The suboptimal response to ICB monotherapy may be due to immunosuppressive mechanisms involving the innate immune system. Indeed, the TIME of highly lethal, difficult-to-treat, desmoplastic malignancies such as CCA or pancreatic ductal adenocarcinoma has an abundance of immunosuppressive myeloid cells including tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) (9, 10). Macrophage differentiation
towards a pro-tumor phenotype is driven, in part, by a paracrine signaling loop involving tumor-CSF1 (11, 12). Consequently, inhibition of the CSF1 receptor (CSF1R) has been utilized as an approach to deplete pro-tumor macrophages in preclinical models of cancer (13-15). MDSCs are immature myeloid cells that have morphologic and phenotypic features of neutrophils and monocytes (16). TAMs and MDSCs facilitate tumor progression via suppression of cytotoxic T lymphocytes (CTLs), enhanced angiogenesis, tumor invasion and metastasis (16, 17).

Expression of PD-L1 is an important mechanism implicated in tumor immune escape (18). Although the role of PD-L1 in tumor biology is under intense investigation, the majority of the existing body of work has focused on PD-L1 expression on cancer cells and its role in suppression of CTL function. However, PD-L1 can also be expressed by TAMs; and in some cancers PD-L1 expression by host myeloid cells is more effective than cancer cell expression of PD-L1 in suppressing CTL function (19-21). Moreover, MDSCs may also suppress CTL activity by PD-L1 dependent and independent mechanisms (22).

We have developed an oncogene-driven murine model of CCA (23). In this genetic model, the biliary tree of C57BL/6 mice is directly transduced with constitutively active human yes associated protein (YAP) along with murine myristoylated Akt as a permissive factor. Phenotypic characterization of these tumors harvested 10 weeks after biliary instillation demonstrated that they recapitulate the human disease. RNA sequencing analysis identified substantive overlap in gene expression of the mouse tumors with human CCA. Malignant murine cell lines derived from this model can be implanted in an orthotopic fashion into livers of immunocompetent mice (C57BL/6 background), generating a syngeneic mouse model, which we will simply term as SB tumors (24). The mice develop large tumors with typically one dominant nodule that can be easily separated from the adjacent liver. This enables study of the immune
microenvironment of the tumor as well as the adjacent liver, and thus is an ideal model to study the immunobiology of this desmoplastic malignancy.

Herein, we employ our orthotopic murine CCA model to uncover the interplay between immunosuppressive myeloid cells and their response to immunotherapies. We demonstrate that PD-L1+ TAMs are abundant in human CCA as well as in our murine model of CCA. However, TAM blockade failed to reduce CCA tumor burden. This observation is likely due to compensatory accumulation of immunosuppressive granulocytic-MDSCs (G-MDSCs). Dual inhibition of TAMs and G-MDSCs was necessary to potentiate ICB in our murine CCA model. These data suggest that combination immune therapies targeting macrophages and MDSCs are a promising therapeutic approach for desmoplastic cancers.
Results

*TAMs are the Predominant Source of PD-L1 in CCA.* PD-L1 expression, on both the host myeloid cells and tumor cells, has been implicated in suppression of the antitumor immune response (20, 21). As macrophages are abundant in desmoplastic malignancies such as CCA, we assessed PD-L1 expression on macrophages and tumor cells in resected human CCA specimens. Using co-staining for PD-L1 plus CD68 (a macrophage marker) and PD-L1 plus cytokeratin (CK)-19 (a CCA marker), we demonstrated that PD-L1 is expressed predominantly on macrophages rather than cancer cells in human CCA (Figure 1A; Supplemental Figure 1A).

Next, we assessed for the presence of PD-L1+ macrophages in the tumors of five patients undergoing surgical resection; two patients had received neoadjuvant chemoradiation with gemcitabine and cisplatin. Flow cytometry was conducted on human CCA tumor immediately following surgical resection. CD11b+CD68+PD-L1+ macrophages were detected in all five patients (Figure 1B).

Approximately 60% of all macrophages (CD11b+F4/80+) in the SB tumors express PD-L1 (Figure 1C). Macrophage PD-L1 expression is also increased in the tumor adjacent liver compared to WT baseline liver (from normal WT mice without tumor) (Figure 1C). Further characterization of intratumoral macrophage phenotypes in these tumors identified F4/80high and F4/80int macrophage (CD45+CD11b+F4/80+) subsets (Supplemental Figure S1, B-D). As the preponderance of macrophages in the murine CCA tumors were F4/80int, with F4/80high only comprising a minor proportion of the CD45+CD11b+F4/80+ population, we elected to focus on F4/80int macrophages for the remainder of our studies. The percentage of F4/80int macrophages that express CD206, a scavenger receptor expressed by TAMs (25), and those that are PD-L1+ (CD206+PD-L1+) is significantly increased in murine CCA tumors compared to normal liver (Figure 1D). Moreover, the majority of the macrophages that express PD-L1 are CD206-positive.
indicating that PD-L1 is expressed on TAMs (Figure 1E). MDSCs and DCs (CD45^+CD11chigh) are also significantly increased in murine CCA tumors compared to normal liver (Supplemental Figure S1, E and F). Although we identified PD-L1 expression on DCs and MDSCs, the preponderance of PD-L1 in SB tumors was expressed on TAMs (Figure 1F). In aggregate, these data indicate that PD-L1 is predominantly expressed on macrophages in CCA.

Host PD-L1 Contributes to CCA Progression. The relative contribution of PD-L1 expression in the host immune cell and tumor compartments in CCA is unknown. To examine the relative roles of PD-L1 expression on tumor versus myeloid cells, we employed our syngeneic CCA model and implanted murine CCA cells (SB cells) orthotopically into livers of WT mice and Pd-l1^-/- mice. Interestingly, although the SB cells have abundant PD-L1 expression (Figure 2A), implantation of these cells in Pd-l1^-/- mice resulted in significant reduction in the CCA tumor burden compared to WT mice, suggesting that tumor-derived PD-L1 was not the driver of immune escape in these tumors (Figure 2, B and C; Supplemental Figure S2, A and B). Moreover, CD206^+ and CD206^+PD-L1^+TAMs were significantly decreased in SB tumors in Pd-l1^-/- deficient hosts (Figure 2D; Supplemental Figure S2C). Although SB tumors in WT hosts display suppression of CD8^+ T cell infiltration, SB tumors in Pd-l1^-/- deficient hosts manifest enhanced CD8^+ T cell infiltration and increased reactive CTLs as demonstrated by CD11a expression (Figure 2, E and F; Supplemental Figure S2C). CD11a, an integrin which is upregulated in effector and memory CD8^+ T cells, mediates conjugation between CTLs and target cells and can be used to identify and monitor endogenous tumor-reactive CTLs (26). These results suggest that host myeloid cell PD-L1 is requisite for CCA progression.

Soluble Non-functional PD-L1 is Transferred from Murine CCA Cells to TAMs. Although host immune cells in Pd-l1^-/- mice are devoid of PD-L1, we consistently observed the presence of PD-
L1+ TAMs in Pd-ll− mice tumors, implying direct transfer of PD-L1 from the cancer cells to TAMs (Figure 2D). Hence, we postulated that SB cells which have abundant PD-L1 expression (Figure 2A) transfer PD-L1 to TAMs. Accordingly, we assessed PD-L1 levels on bone marrow-derived macrophages (BMDM) isolated from Pd-ll− mice and subsequently co-cultured with SB cells. Using flow cytometry analysis, we observed a significant increase in PD-L1 levels on Pd-ll−-BMDM co-cultured with SB cells compared to control Pd-ll−-BMDM (Figure 2G). We also tested whether direct cell contact was required for accumulation of PD-L1 on BMDM. Addition of conditioned medium from SB cells to BMDM isolated from WT C57BL/6 mice significantly increased BMDM PD-L1 protein levels as assessed by flow cytometry, implying that direct cell contact is not required for PD-L1 accumulation on BMDM (Figure 2H). As tumor derived extracellular vesicles (EVs) can induce a protumor immune microenvironment (27), we next assessed PD-L1 expression on SB cell-derived EVs. Although SB cell-derived EVs expressed PD-L1 (Supplemental Figure S2, D and E), incubating BMDM with SB EVs did not alter macrophage associated PD-L1 (Supplemental Figure S2F). PD-L1 may also be present in a circulating or soluble form with immunosuppressive properties that facilitate cancer progression (28, 29). Indeed, we identified soluble PD-L1 in conditioned medium from SB cells (Figure 2I), implying that soluble PD-L1 from CCA cells is transferred to TAMs. However, the percentage of soluble PD-L1 transferred to TAMs in our murine model appears to be trivial (Figure 2D). To assess whether soluble PD-L1 is functional, SB cell conditioned medium was incubated with anti-PD-L1 antibody to neutralize any soluble PD-L1, and conditioned medium was subsequently added to T cells. Neutralization of PD-L1 from the conditioned medium did not have an effect on T cell proliferation as assessed by Ki67, or T cell activation as assessed by IFN-γ production (Figure 2J). Taken together, these results suggest that although cancer cells
transfer a small amount of PD-L1 to TAMs, this transferred soluble PD-L1 is not immunosuppressive based on its impact on T cells. As it is not functional, soluble PD-L1 likely does not contribute to CCA tumor biology in a meaningful manner.

**PD-L1⁺ TAMs are Recruited from the Bone Marrow in CCA.** Hepatic macrophages may be categorized as resident Kupffer cells (yolk-sac-derived) or recruited (from circulating monocytes differentiating into macrophages) (30, 31). The distinction between the two is important because strategies for blocking their protumor activity are different as chemokine inhibitors may block macrophage recruitment to CCA but would not alter resident Kupffer cell abundance or function.

Although WT SB tumors had a significantly higher ratio of recruited macrophages (CD45⁺CD11b⁺F4/80⁺CCR2⁺) than resident Kupffer cells (CD45⁺CD11b⁺F4/80⁺Clec4F⁺), PD-L1 was significantly increased in both subsets (Figure 3, A-C; Supplemental Figure S3, A and B). Notably, the preponderance of resident macrophages was F4/80^{high}, whereas recruited macrophages were F4/80^{int} (Figure S3, C and D). Next, we sought to ascertain whether PD-L1⁺ macrophages recruited from the bone marrow promote CCA progression. We irradiated WT and *Pd-l1⁻/⁻* mice and transplanted them with bone marrow (BM) from *Pd-l1⁻/⁻* and WT mice, respectively (Figure 3D; Supplemental Figure S3E). WT BM was transplanted into irradiated WT mice and *Pd-l1⁻/⁻* BM was transplanted into irradiated *Pd-l1⁻/⁻* mice as a control (Supplemental Figure S3, E-G). *Pd-l1⁻/⁻* mice with WT BM (WT-*Pd-l1⁻/⁻*) had a significant increase in tumor burden compared to WT mice with *Pd-l1⁻/⁻* BM (*Pd-l1⁻/⁻*-WT) (Figure 3, E and F). Accordingly, WT-*Pd-l1⁻/⁻* had an increase in recruited macrophages and decreased CD8⁺ T cell infiltration. In contrast, *Pd-l1⁻/⁻*-WT mice had a significant decrease in recruited macrophages and increased CD8⁺ T cell activation and effector function (Figure 3, G-I).
together, these data suggest that recruited macrophages are the primary source of PD-L1+ macrophages, and facilitate CCA progression.

*TAM Blockade Promotes a Compensatory Accumulation of G-MDSCs.* Recruitment of infiltrating monocyte-derived macrophages to the tumor bed in malignancies is dependent on CCR2 (32). Accordingly, *Ccr2* deficient mice have defective monocyte recruitment (33). As we had observed an increase in recruited TAMs in WT tumors, we hypothesized that the *Ccr2*−/− mice would have reduced tumor burden. Unexpectedly, *Ccr2*−/− mice had a similar tumor burden and CD8+ T cell infiltration compared to WT mice following SB cell implantation (Figure 4A; Supplemental Figure S4, A-C). As expected, *Ccr2*−/− mice did not have PD-L1+ recruited TAMs (Supplemental Figure S4D). However, an increase in PD-L1+ resident TAMs was not observed either (Figure 4B), suggesting compensatory emergence of another immunosuppressive cell population. MDSCs, immature myeloid cells with robust immunosuppressive properties, mediate tumor immune evasion and immunotherapy resistance (34, 35). Using flow cytometry, we demonstrated an increase in CD11b+Gr-1+ MDSCs in *Ccr2*−/− mice compared to WT mice (Figure 4C). In mice, MDSCs encompass CD11b+Gr-1+ high (Ly6C lowLy6G high) granulocytic or G-MDSCs and CD11b+Gr-1 int (Ly6C highLy6G low) monocytic MDSCs (M-MDSC) (36). Further characterization of MDSCs revealed that G-MDSCs were the dominant subset in *Ccr2*−/− mice (Figure 4D).

To test the effect of pharmacologic TAM inhibition on CCA tumor growth, CSF1R inhibition was employed (Figure 4E). The efficiency of TAM inhibition with anti-CSF1R was confirmed (Supplemental Figure S4, E and F). However, CSF1R inhibition also did not reduce CCA growth in mice (Figure 4F; Supplemental Figure S4, G-I). Mass cytometry analysis of the CSF1R treated SB tumors revealed a compensatory infiltration of Ly6C lowLy6G high G-MDSCs.
Accordingly, reactive CD8\(^+\) T cells were unchanged between the vehicle and CSF1R treated tumors (Supplemental Figure S4J). These observations highlight that prevention of TAM recruitment or pharmacologic TAM inhibition promotes a compensatory infiltration of G-MDSCs, and counteracts the potential antitumor effect of eliminating protumor macrophages in murine CCA.

**CAF-derived CXCL2 is increased in the Context of TAM Blockade**

To examine the potential mechanism of TAM blockade-mediated G-MDSC accumulation in tumors, we assessed the expression of various chemokines in control and anti-CSF1R treated tumors using an unbiased screen. A significant upregulation of several chemokines implicated in MDSC recruitment was observed in the anti-CSF1R treated tumors compared to control treated tumors (Supplemental Figure S5A). Expression of potential chemokines identified by the chemokine array was further assessed using quantitative PCR in multiple samples from vehicle and anti-CSF1R treated tumors (Figure 5A, Supplemental Figure S5B). Cxcl2 was significantly upregulated in the anti-CSF1R treated tumors compared to vehicle treated tumors (Figure 5A). As Cxcl2 is a known chemoattractant for MDSCs (37), we conducted additional studies to investigate the cellular source of this chemokine. Using FISH, we demonstrated a significant increase in Cxcl2 in SB tumors compared to adjacent liver (Supplemental Figure S5C). Consistent with this observation, MDSCs are increased in SB tumors compared to adjacent liver (Supplemental Figure S5D). Notably, anti-CSF1R treated murine tumors had a significantly higher expression of Cxcl2 compared to vehicle treated murine tumors (Figure 5B). Alpha-smooth muscle actin (\(\alpha\)-SMA) positive cancer associated fibroblasts (CAFs), rather than the CCA cells, appeared to the primary source of Cxcl2 in anti-CSF1R treated tumors (Figure 5B). Accordingly, anti-CSF1R treated tumors had an increased abundance of CAFs compared to
vehicle treated tumors (Figure 5C). These observations are consistent with prior work demonstrating CAFs increase recruitment of MDSCs to anti-CSF1R treated tumors via the CXCL1-CXCR2 axis in murine lung carcinoma and lymphoma (38). In aggregate, these results suggest that TAM blockade employing CSF1R inhibition is associated with increased production of CXCL2 by CAFs with consequent enhanced G-MDSC accumulation in tumors.

**Single Cell Transcriptomics Demonstrates Accumulation of G-MDSC Subsets with an Immunosuppressive Signature in the Context of TAM Blockade.** To characterize the phenotype of anti-CSF1R induced G-MDSC infiltration in CCA, we isolated CD11b^+^Ly6C^low^Ly6G^High^ G-MDSCs from SB tumors of vehicle or anti-CSF1R treated mice via FACS (Figure 6A). We then employed a droplet-mediated scRNA-Seq platform to profile FACS-purified live G-MDSCs. We profiled two tumor samples from vehicle treated mice (4,757 cells) and anti-CSF1R treated mice (6,480 cells), respectively, for a total of 11,237 cells. We initially identified eight clusters in the vehicle and anti-CSF1R treated samples with high resolution (resolution = 0.5) (Figure 6B). However, after further review of expression profiles for each cluster of cells, clusters with similar gene expressed profiles were combined and ultimately there were two distinct clusters (cluster 0 and 1, resolution = 0.01) of G-MDSCs in the murine tumors with substantial differences in transcriptomic activity (Figure 6C; Supplemental Table S1 and S2). Although cluster 0 slightly decreased in cell abundance with anti-CSF1R treatment, cluster 1 was significantly enriched in the anti-CSF1R treated tumor sample (Figure 6C; Supplemental Figure S6A). Cluster 0 was characterized by higher expression of MDSC-related genes, and did not have significant expression of neutrophil- or monocyte-related genes (Supplemental Figure S6B; Supplemental Table S1). Cluster 1 was associated with expression of MDSC-related genes but also displayed expression of a few monocyte-related genes and to a lesser extent
neutrophil-related genes, suggesting that this cluster may be less well-differentiated or more immature than cluster 0 (39) (Supplemental Figure S6B; Supplemental Table S2).

*scRNA-Seq Analysis Uncovers a Distinct ApoE G-MDSC Subset.* Apolipoprotein E (*Apoe*), a transcriptional target of liver-X receptors (LXR), was the top conserved gene in cluster 1 and was also significantly downregulated with anti-CSF1R treatment (Figure 6D; Supplemental Figure S7B). In contrast, cluster 0 or “Classic G-MDSCs” did not display expression of ApoE (Figure 6D). ApoE is a secreted protein implicated in lipoprotein metabolism and inhibition of metastatic progression in melanoma (40). More recently, the LXR/ApoE axis has been linked to MDSC depletion via enhanced apoptosis across several tumor types (36). Notably, there was significant cell enrichment of cluster 1 or “ApoE G-MDSCs” with anti-CSF1R treatment, suggesting that perhaps this cluster has survival capabilities as evidenced by significant downregulation of *Apoe*. Moreover, ApoE G-MDSCs displayed an immunosuppressive gene signature (Figure 6D) with enhanced expression of several genes implicated in MDSC-related immunosuppression, tumorigenesis and metastasis including Arginase 1 (*Arg1*), S100 calcium binding protein A4 (*S100a4*), CD74 antigen (*Cd74*), and Peroxiredoxin 1 (*Prdx1*) (16, 41).

To determine the human relevance of the ApoE G-MDSC subpopulation, we compared murine ApoE G-MDSC signature genes with a human scRNA-Seq dataset, comprised of 10 human CCAs and 9 hepatocellular carcinomas (HCCs) (GSE125449) (42). Analysis of the single cell transcriptomic profile of the CCA patients in this dataset revealed 8 broad predicted cell types including TAMs, cancer-associated fibroblasts, malignant cells, and T cells (Supplemental Figure S6C) (42). The cell population predicted as TAMs likely included other myeloid cells such as MDSCs, as MDSCs were not distinctly categorized in the original cell type prediction (42). Accordingly, we conducted an AUCell gene set enrichment analysis to assess for the
presence of MDSCs in human CCA. A set of known human MDSC signature genes (43, 44) was enriched in the cell population predicted as ‘TAMs’ and to a lesser extent in the cell population predicted as T cells by Ma et al. (42) (Figure 6E; Supplemental Table S3). Next, we conducted a second AUCell gene set enrichment analysis to assess for the presence of a cell population similar to ApoE G-MDSCs in human CCA. ApoE G-MDSC signature genes from our murine model (Supplemental Table S4) were significantly enriched in the subset of CCA cells predicted as ‘TAMs’ (Figure 6F). Moreover, a subset of these cells had abundant ApoE expression (Supplemental Figure S6D). These results imply that ApoE G-MDSCs comprise a unique MDSC subset that is present in human CCA.

**TAM Blockade Facilitates Emergence of G-MDSC Subsets with Immunosuppressive Properties.** The accumulation of G-MDSCs in the context of TAM blockade may be due to enhanced survival and/or increased recruitment. ApoE G-MDSCs in the anti-CSF1R treated tumors had downregulation of genes implicated in MDSC cell death. Apoe as well as cathepsin D (ctsd) and cathepsin B (ctsb), which mediate MDSC death via interrupted autophagy and endoplasmic reticulum stress, were downregulated (36, 45). Conversely, S100a4, which is essential for MDSC survival, was significantly upregulated (41) (Figure 7A; Supplemental Figure S7, A and B).

To ascertain the functional relevance of these observations, we assessed the impact of TAM blockade on G-MDSC apoptosis. We observed a significant decrease in the percentage of G-MDSCs from anti-CSF1R treated tumors staining positive for Annexin V and 7-amino-actinomycin D compared to G-MDSCs from vehicle treated tumors (Figure 7B).

Activation of STAT1 as well as NF-kB promotes MDSC activation with consequent upregulation of arginase 1 and inducible nitric oxide synthase (46). The IFN-γ-STAT1 axis fosters MDSC suppressive activity (16). Interestingly, anti-CSF1R treatment was associated with
a significant upregulation of Stat1 in G-MDSCs, suggesting MDSC activation and enhanced suppressive activity (Figure 7C). Moreover, G-MDSCs displayed significant downregulation of Nfkbia, which encodes the NF-kB inhibitor IkBα, with anti-CSF1R treatment (Figure 7C; Supplemental Figure S7, A and B). We next determined suppressive properties of G-MDSCs in the anti-CSF1R treated tumors. Compared to G-MDSCs isolated from vehicle tumors, G-MDSCs isolated from anti-CSF1R-treated tumors exhibited more significant suppression of CD8⁺ T cell proliferation as assessed by Ki67, as well as CD8⁺ T cell activation as assessed by IFN-γ production (Figure 7D). In summary, these findings suggest that TAM blockade is associated with emergence of G-MDSCs with immunosuppressive signatures and function.

**G-MDSCs Interact with CTLs in Human CCA.** Next, we assessed the interplay between G-MDSCs and T lymphocytes in human CCA. Using imaging mass cytometry, we identified cell-cell contact between CD11b⁺CD14⁻CD15⁺G-MDSCs and CD8⁺ T cells in resected human CCA specimens (Figure 7E; Supplemental Figure S7C). Moreover, human CCAs displayed a poorly immunogenic TIME (8) with the tumor core being relatively devoid of T cells (Supplemental Figure S7D). T cells were present along the tumor margin intermingled with CD11b⁺ myeloid cells (Supplemental Figure S7D), implying that immunosuppressive myeloid cells may prevent T lymphocyte infiltration into the tumor core. Moreover, similar to the results in our murine model, the majority of the myeloid cells expressed PD-L1, and the CD8⁺ T cells expressed PD-1 (Supplemental Figure S7, E-F). Finally, we assessed for the presence of G-MDSCs in five patients undergoing surgical resection; two patients had received neoadjuvant chemoradiation with gemcitabine and cisplatin. Flow cytometry was conducted on human CCA tumor immediately following surgical resection. CD11b⁺CD14⁻CD15⁺G-MDSCs were detected in all five patients (Figure 7F).
Dual Inhibition of G-MDSCs and TAMs Potentiates Anti-PD-1 Therapy. As G-MDSCs counteract the effect of TAM-directed therapy by mediating tumor evasion, we hypothesized that dual inhibition of G-MDSCs and TAMs may have therapeutic benefit in murine CCA. For therapeutic targeting of G-MDSCs, we employed the G-MDSC specific anti-Ly6G antibody (clone 1A8). Flow cytometry confirmed a significant reduction of tumor as well as splenic G-MDSCs in anti-Ly6G treated mice (Supplemental Figure S8, A and B). The combination of G-MDSC inhibition with anti-Ly6G and TAM inhibition using anti-CSF1R potentiated immune checkpoint blockade using anti-PD-1. Anti-PD-1+anti-CSF1R+Anti-Ly6G significantly prolonged the survival of mice bearing SB tumors compared to anti-PD-1+anti-CSF1R or anti-PD-1+anti-Ly6G (Figure 8A). Tumor bearing mice were followed with cross-sectional imaging using micro-CT while undergoing drug treatment (Figure 8C). Micro-CT imaging demonstrated significant improvement in tumor burden in anti-Ly6G+anti-CSF1R+anti-PD-1 which was confirmed at the time of sacrifice (Figure 8, B-D; Supplemental Figure S8, C and D). Thus far, ICB monotherapy in human clinical trials of cholangiocarcinoma has been disappointing (5.8% objective response rate with the anti-PD-1 antibody pembrolizumab) (7). Anti-PD-1 therapy alone failed to influence survival of mice with CCA or reduce the tumor burden, and these results in mice are consistent with the experience in the human disease (Figure 8A). Anti-Ly6G treatment alone also did not impact murine survival (Figure 8A).

The LXR/ApoE axis reduces MDSC abundance in solid tumors via enhanced apoptosis (36). Pharmacologic activation of LXR using the agonist GW3965 has been shown to reduce MDSC abundance and significantly suppress tumor growth across several malignancies (7). Accordingly, we assessed GW3965 in combination with TAM blockade and ICB, and observed a significant reduction in tumor burden in GW3965+anti-CSF1R+anti-PD-1 treated tumors.
GW3965 monotherapy has a tumor suppressive effect in several malignancies including ovarian cancer, glioblastoma, and renal cell carcinoma (36). However, in our murine CCA model GW3965 monotherapy did not reduce tumor burden, suggesting that combined inhibition of MDSCs and TAMs is required for a tumor suppressive effect in CCA (Supplemental Figure S8, E and F). Further characterization of the anti-Ly6G+anti-CSF1R+anti-PD-1 treated SB tumors and the GW3965+anti-CSF1R+anti-PD-1 treated SB tumors demonstrated a significant reduction in PD-L1+ TAMs and G-MDSCs compared to control antibody treated tumors (Figure 8, E and F). The alteration of the innate immune landscape of murine CCA tumors by anti-Ly6G+anti-CSF1R+anti-PD-1 as well as GW3965+anti-CSF1R+anti-PD-1 therapy resulted in an increase in CD8+ T cell infiltration and activation as well as increased effector function (Figure 8, G-I). In summary, we have demonstrated that dual inhibition of TAMs and G-MDSCs is necessary to potentiate ICB employing anti-PD-1 with a significant tumor suppressive effect and improvement in murine survival.
**Discussion**

In examining the immunobiology of desmoplastic malignancies such as CCA, we have identified an essential role of immunosuppressive myeloid cells in tumor progression. These data indicate that: (i) macrophages are the primary source of PD-L1 in murine and human CCA; (ii) PD-L1+ TAMs mediate a protumor immune response with consequent tumor progression; (iii) compensatory accumulation of G-MDSC subsets neutralizes the potential antitumor effect of TAM targeted therapy; (iv) a unique ApoE G-MDSC subset in murine and human CCA has immunosuppressive properties; and (v) dual inhibition of G-MDSCs and TAMs is necessary to potentiate anti-PD-1 therapy in CCA. These results are further discussed below.

Immunotherapy has revolutionized the treatment of human cancers, with a subset of patients across a variety of malignancies having durable responses to ICB targeting PD-1 or PD-L1. However, the response rate to ICB has been disappointing in other cancer types such as CCA (response rate of only 5.8% to ICB monotherapy) (7). TIME phenotype impacts ICB response with a poorly immunogenic or ‘cold’ TIME correlating with a poor response rate. This phenotype is populated by immunosuppressive innate immune cells such as TAMs and MDSCs. Our study reveals that murine CCAs have an abundance of these immunosuppressive elements, particularly TAMs. Notably, we find that TAMs are the predominant source of PD-L1 in human and murine CCA. Implantation of murine CCA cells in Pd-l1 deficient mice resulted in enhanced CD8+ T cell infiltration and activation with consequent significant reduction in tumor size compared to WT mice, indicating that PD-L1+ host myeloid cells, predominantly TAMs, foster a protumor TIME which facilitates CCA progression.

Hepatic macrophages may be categorized as resident Kupffer cells (yolk-sac-derived) (30) or recruited (30, 31). Our study has found that recruited macrophages comprise a significant
component of the protumor macrophage subset in our murine model of CCA, and foster tumor growth in established CCA. Interestingly, tumors from Ccr2−/− mice which are devoid of recruited macrophages had a similar tumor burden compared to WT mice, likely due to a compensatory increase of immunosuppressive G-MDSCs. TAM blockade in murine CCA tumors using anti-CSF1R also did not delay tumor progression despite a significant reduction in CD11b+F4/80−Gr-1− macrophages. Similar to the results in the Ccr2−/− mice, inhibition of CSF1R signaling also induced a robust accumulation of G-MDSCs in SB tumors. Expression of Cxcl2, a known G-MDSC chemoattractant, was significantly upregulated in anti-CSF1R treated tumors compared to control tumors. CAFs appeared to be the primary source of Cxcl2 in the CCA TIME. Accordingly, an increased abundance of CAFs was noted in the context of TAM blockade. These observations are consistent with a prior study that identified enhanced MDSC recruitment to anti-CSF1R treated murine lung and lymphoma tumors as a consequence of chemokine production by CAFs (38).

G-MDSCs promote tumor progression by impeding immune responses, particularly suppression of antigen-specific CD8+ T cells, and promoting tumor invasion and metastasis (22, 34). Single cell RNA sequencing highlighted unique G-MDSC subsets with gene signatures associated with enhanced STAT and NF-kB signaling. A myriad of factors released by the tumor microenvironment activate several different signaling pathways in MDSCs related to the STAT family of transcription factors (46). STAT signaling regulates MDSC expansion, promotes MDSC survival, and promotes increased production of reactive oxygen species by MDSCs. Single cell transcriptomics of vehicle and anti-CSF1R treated tumors also identified a unique G-MDSC subset, ApoE G-MDSCs with abundant expression of Apoe in the vehicle treated tumors, and significant Apoe downregulation with TAM blockade. The LXR/ApoE axis has been linked
to enhanced MDSC apoptosis and consequent tumor regression (36). Interestingly, this subset was significantly enriched in the anti-CSF1R treated tumors, suggesting that it may have enhanced survival capabilities via Apoe downregulation. Indeed, we observed a significant reduction in G-MDSC apoptosis in the anti-CSF1R treated tumors compared with vehicle treated tumors. Moreover, ApoE G-MDSCs are relevant to the human disease as we identified a similar subset in a human CCA scRNA-Seq dataset.

MDSCs also have a significant association with poor patient outcomes as well as chemotherapy and immunotherapy resistance (35, 47). There is limited information on the role of MDSCs in CCA immunobiology. A single study demonstrated an association with cancer stage and presence of circulating MDSCs in 17 patients with CCA (48). We have now demonstrated that G-MDSCs that accumulate with TAM blockade have potent immunosuppressive properties and mediate immune escape by impairing T cell activity and proliferation. Interestingly, similar to TAM blockade, G-MDSC blockade alone did not have a tumor suppressive effect. Moreover, although MDSC blockade using the LXR/ApoE agonist GW3965 has a tumor suppressive effect across several tumor types (36), GW3965 monotherapy did not reduce tumor burden in our murine CCA model. These data imply that combined inhibition of MDSCs and TAMs is required for a tumor suppressive effect in CCA. Overall, our observations suggest that multiple layers of resistance encompassing the innate and adaptive immune response contribute to tumor immune evasion in CCA. Hence, combinatorial immunotherapies targeting both the innate and adaptive immune system are more likely to be efficacious in desmoplastic malignancies such as CCA. Indeed, we observed that combined inhibition of MDSCs and TAMs reduced both populations in the CCA TIME and significantly enhances the antitumor effect of anti-PD-1 therapy in murine CCA. Blockade of either population alone failed to suppress tumor growth. Our observations
support the investigation of combination therapies targeting TAMs and MDSCs in human CCA. The toxicities of these combinatorial immune-directed therapies in humans are unclear. Targeting the CSF1-CSF1R axis has been associated with toxicity that limits dose escalation, likely related to systemic depletion of macrophages (49). An alternative therapeutic approach for TAM targeting is employing therapies that reprogram macrophages from a pro-tumor to a tumoricidal phenotype as this approach circumvents the toxicity associated with total body depletion of macrophages. Various strategies targeting TAM reprogramming are currently under investigation in preclinical and clinical studies (49). Human clinical trial data on therapeutic targeting of MDSCs is limited. In a phase I clinical trial of RGX-104, an LXR/ApoE agonist, significant decrease in MDSC levels in patients were noted (36). Notably, this agent was well tolerated without any dose-limiting toxicities. Therapeutic strategies combining TAM inhibition with MDSC inhibition need to be carefully assessed in an early phase clinical trial.

In summary, we have characterized immunosuppressive myeloid cell populations which foster CCA growth and progression. Blockade of TAMs promotes a compensatory accumulation of G-MDSCs, including a unique ApoE G-MDSC subset which is also present in human CCA. Combining TAM and G-MDSC inhibitors reduced both populations in the tumor site. Moreover, it dramatically enhanced the effect of ICB with anti-PD-1 in our preclinical model of CCA. Our findings support the role of combination immunotherapeutic strategies coupling ICB with immunotherapies targeting tumor promoting myeloid cells in CCA.
Methods

Syngeneic, orthotopic mouse model of CCA. Murine CCA cells (SB) derived from an oncogene-driven murine model of CCA (23) were maintained in culture medium as previously described (24). This model of murine CCA is a YAP-driven malignancy which has considerable overlap at the messenger RNA level with human intrahepatic CCA; employing two public databases (The Cancer Genome Atlas [TCGA]-CHOL and the Genotype-Tissue Expression [GTEx]), we demonstrated a prominent YAP signature (50) in human CCA compared to adjacent liver and normal liver samples (Supplemental Figure S9, A and B). These observations suggest that oncogenic YAP signaling is activated in human CCA, and further strengthen the validity of our murine model in regards to human disease. Mice were anesthetized using 1.5-3% isoflurane. Under deep anesthesia, the abdominal cavity was opened by a 1 cm incision below the xiphoid process. A sterile cotton tipped applicator was used to expose the superolateral aspect of the medial lobe of the liver (24). Using a 27-gauge needle, 20 µL of standard media containing 0.5 x 10^6 SB cells was injected into the lateral aspect of the medial lobe. A cotton tipped applicator was held over the injection site to prevent cell leakage and blood loss. Subsequently, the abdominal wall and skin were closed in separate layers with absorbable chromic 3–0 gut suture material. Four weeks following SB cell implantation, mice were sacrificed and tumor, adjacent liver, and spleen were collected.

Treatments in mice. All treatments were initiated 14 days after SB cell implantation. For the experiments employing anti-CSF1R, mice were randomly assigned to control isotype antibody rat IgG2b, ƙ (BioXcell) or anti-CSF1R (AFS98, BioXcell) every 3 days at a dose of 200 µg delivered via intraperitoneal (i.p.) injection (13). For the combinatorial treatment experiments, mice were randomly assigned to either control isotype antibody (rat IgG2b, ƙ, BioXcell), vehicle
consisting of olive oil (90%) and DMSO (10%), anti-CSF1R 200 µg every 3 days, anti-Ly6G (1A8, BioXcell) 400 µg i.p. loading dose followed by 200 µg three times a week, anti-PD-1 (G4, Antibody Hybridoma Core Mayo Clinic) 200 µg i.p. every other day, or GW3965 50mg/kg/day diluted in 90% of olive oil and 10% DMSO by gavage. For the survival study, treatments were administrated until animals reached approved conditional score thresholds as established by Mayo Clinic Institutional Animal Care and Use Committee (IACUC).

Isolation of human tumor-infiltrating immune cells and flow analysis. Human CCA tumor samples were collected during surgical resection in RPMI 1640 media supplemented with penicillin/streptomycin. Samples were immediately transferred for dissociation with minimal ischemic time. Following removal of fat, fibrous and necrotic areas, fresh tumor tissue specimens were cut into smaller pieces (< 3 mm). Minced sample pieces were transferred to a gentleMACS C Tube (Miltenyi #130-093-237) containing 5 mL digestion enzyme mix prepared according to manufacturer’s instructions (Human tumor dissociation kit from Miltenyi Biotech #130-095-929). Samples were subsequently dissociated with gentleMACS™ Octo Dissociator (Miltenyi) according to the manufacturer’s protocol. Following mechanical dissociation, samples were filtered using a 70-µm nylon mesh (Fisher #22363548). CD45+ cells were isolated by CD45 (TIL) human microbeads (Miltenyi). Cells were incubated with Fixable Viability Stain 510 (BD Horizon™) for 15 minutes followed by human anti-Fc blocking reagent (Miltenyi) for 10 minutes prior to surface staining. Cells were stained, followed by data acquisition on a Miltenyi MACSQuant® Analyzer 10 optical bench flow cytometer. All antibodies were used following the manufacturer recommendation. Analysis was performed using FlowJo™ (TreeStar). Forward scatter (FSC) and side scatter (SSC) were used to exclude cell debris and doublets. The following antibodies were used for flow cytometry staining: CD11b-APC-Cy7 (ICRF44, Biolegend®),

**Bone marrow transplantation.** Eight-week-old male C57BL/6 mice, BL6 SJL mice (CD45.1 allele) or C57BL mice (CD45.2 allele) received acidified water and antibiotics one week prior to irradiation. Then, mice were irradiated at 6 Gy twice within a 6 hour interval. One day following irradiation, 1 million bone marrow cells were transferred from the donor mice to the recipient mice. Donor mice had a different CD45 allele than recipient mice. Mice were maintained under pathogen free conditions, and acidified water and antibiotics were provided. Six weeks following irradiation, mice underwent SB cell implantation.

**G-MDSC/T cell in vitro co-culture.** G-MDSCs were isolated from WT mice tumor and T cells were isolated from spleen single cell suspension. T cells and G-MDSCs (ratio 4:1) (51) were co-cultured in a 96-well plate with round bottom for 48h with CD3/CD28 beads (Gibco Dynabead). T cell INFγ transporters were inhibited 5 hours before flow staining with 5 ug/mL of brefeldin A (1000x) (Biolegend 420601) and Monensin (biolegend 420701). T cells were stained with Fixable Viability Stain 510, CD3-APC-Vio770, CD4-PerCP-Vio700, CD8-BV421, Ki67-AF700 and INFγ-PE and analyzed by flow cytometry.

**Cytometry Time of Flight (CyTOF).** Cell preparation and data acquisition for mass cytometry experiments were performed by the Immune Monitoring Core, Mayo Clinic as described previously (52). Briefly, murine tumor isolates were incubated with 0.5 µm of Cisplatin solution for 5 minutes. Samples were then incubated at room temperature for 45 minutes with an antibody cocktail of the phenotyping panel (**Supplemental Table 5**) and fixed with 2% paraformaldehyde solution. After fixation, samples were resuspended in 30 nM intercalation solution and incubated
overnight at 4°C before resuspension at 0.5x10^6 cells/mL in cell acquisition solution (Fluidigm). Data were acquired with the Helios CyTOF® system at a rate of 200-400 events per second. Cell cleanup and quality control analysis was performed with FlowJo™ version 10.5.3 (Ashland, OR). Cleaned fcs files were analyzed by the R-based tool Cytofkit version 3.8 (53-55). Clustering and dimensionality reduction to 10,000 events per file was performed using the Rphenograph algorithm that included all markers used. Visualization of clusters was mapped onto a tSNE map. Antibodies for use in mass cytometry (Supplemental Table 5) Table were either purchased from the manufacturer (Fluidigm) or were purchased from the designated manufacturer and labeled with metal isotopes using Maxpar X8 antibody labeling kits (Fluidigm). All labeling was performed by the Mayo Clinic Hybridoma Core.

Single cell RNA-Seq data analysis of murine MDSCs. We used 10X Genomics Cellranger Single Cell Software Suite (v3.1.0) to generate FASTQ files, perform alignment to mm10 reference genome, filtering, barcode counting and UMI counting. For subsequent analysis, we followed the integrated analysis workflow in the Seurat package based on SCT transformation (v3.1) (https://satijalab.org/seurat/v3.1/integration.html) (56, 57). Genes that are expressed in fewer than 3 cells, cells that expressed fewer than 200 genes and >40% mitochondria genes were excluded for downstream analysis in each sample. Each dataset was normalized using SCT transform and scaled for each gene across all cells. All datasets were integrated, scaled, and clustered on the low-dimensional space. Enriched gene markers in each cluster conserved across two conditions were identified with fold change larger than 1.5, adjusted p-value smaller than 0.05 in both conditions, and at least 20% of cells with expression of a particular gene. Differentially expressed genes within each cluster between the two conditions were also detected.
with fold change larger than 1.5 in either direction and adjusted p-value smaller than 0.05. All clustering and statistical analysis were performed in R (v 3.5.2).

*Single cell RNA-Seq data analysis of human CCA dataset.* To validate the MDSC specific genes detected in our mouse data, single cell RNA-Seq data for 10 human intrahepatic cholangiocarcinoma (iCCA) samples (GSE125449) was processed using Seurat (42). After similar QC and integration steps, tSNE algorithm was applied to identify cell clusters. Then we defined two gene sets. The first gene set consisted of a published human MDSC gene signature (43). The second gene set corresponded to the 40 top genes specific to ApoE G-MDSCs (cluster 1). The assumption was that cells enriched in either of the two gene sets were more likely to be MDSCs. The enrichment was calculated for each cell based on AUCell package (v1.4.1). AUCell applies a rank-based algorithm and calculates the enrichment of a gene set (gene signatures) based on area under the recovery curve (AUC)(44). The analysis was done separately for the two gene sets.

*Tissue staining and image acquisition for imaging mass cytometry.* All tissue staining and slide preparation was performed by the Mayo Clinic Pathology Research Core. Two formalin fixed paraffin embedded tissue sections derived from human resected CCA specimens (n=3) were stained with our full antibody panel ([Supplemental Table S6](#)). The metal tagged antibodies were acquired directly from the manufacturer (Fluidigm). Briefly, slides were baked for 60 minutes in a 60°C oven and then cooled for 5 minutes before loading on to a Bond RX autostainer (Leica) for automated slide preparation prior to staining. Slides were deparaffinized with xylol and rehydrated through a graded alcohol series, subjected to antigen retrieval in Tris-EDTA for 20 minutes at 100°C, and were blocked with Superblock solution (Thermo Fisher) before a final wash with PBS supplemented with 0.05% Tween and 1% BSA (PBS-TB). Slides
were manually stained overnight in a humidity chamber at 4°C with a cocktail of the antibodies
diluted at the indicated dilution factors in Table 1. On the following day, slides were washed
with PBS-TB and then stained with an iridium nucleic acid intercalator (Fluidigm) to label cell
nuclei. Cells were washed with PBS-TB twice before a final wash with water and drying for 20
minutes at room temperature. Data acquisition was performed on a Helios time-of-flight mass
cytometer (CyTOF) connected to a Hyperion Imaging System (Fluidigm). Optical images of
slides were acquired prior to laser ablation using the Hyperion software (version 7.0.5189.0).
Ablations were performed at a resolution of 1 micron and at a frequency of 200 Hz. Ten total
regions of interest were acquired over two consecutive days. Performance stability was ensured
through daily calibration using a 3 element full coverage tuning slide embedded with the 3 metal
elements 89Y, 140Ce and 175 Lu (Fluidigm). All metals are within the mass range of the time-
of-flight detector. Images from CCA sections were generated using the MCD Viewer software
(version 1.0.560.2; Fluidigm).

Micro CT-imaging. On day 14 following SB cell implantation, a 0.1 mL single tail-vein
intravenous injection to conscious mice was performed to administer Mvivo™ Au (13210,
Medilumine). Using micro-CT (SkyScan 1276 micro-CT system, Bruker, Kontich, Belgium) at a
resolution of 20 μm, images were acquired at 85 kV, 200 μA, with a rotation step of 0.3° and in
180°. Images were acquired at day 14, 21 and 28. Beam hardening correction (30%) was applied
using a 1 mm aluminum filter. The total scanning time for each animal was approximately
5 minutes. The mice were placed supine on an animal bed with real-time visual monitoring of the
animal position, respiratory rate and chamber temperature. All micro-CT image data were
acquired in free-breathing mice under isoflurane anesthesia (1.5–2%) without respiratory or
cardiac gating. The temperature of the animal bed chamber was maintained at 28–30 °C to
prevent hypothermia. 3D imaging reconstruction was performed by the X-ray Imaging Core (Mayo Clinic, Rochester, MN).

**Cxcl2 FISH and co-staining for protein.** Detection of Cxcl2 mRNA and CK-19 or α-SMA protein in formalin-fixed paraffin embedded mouse liver tissue was performed using an in situ mRNA and protein co-staining protocol described previously (58). Briefly, tissues were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval in antigen unmasking solution (Vector Labs). Slides were subsequently incubated in pre-hybridization solution (3% BSA in 4X SSX) for 20 minutes at 54°C. Tissues were then incubated for 1 h at 54°C with a fluorescein-labeled Cxcl2 probe (Qiagen) diluted to 25 nM in hybridization buffer (10% dextran sulfate in 4X SSC). Slides were washed and subjected to a tyramide signal amplification step (Perkin Elmer). Slides were washed again, blocked in 3% BSA, and incubated overnight at 4°C with a primary antibody to CK-19 (Abcam) or α-SMA (Abcam). After overnight incubation, slides were washed and incubated with a secondary antibody (Alexa Fluor 594, Thermo Fisher Scientific), washed again, and mounted in Prolong Gold with DAPI (Thermo Fisher Scientific). Slides were analyzed on a Zeiss 710 Confocal Microscope.

**Statistics.** Experimental statistical analyses were performed using GraphPad Prism software. Student two-tailed t test, log-rank (Mantel–Cox) test, and one-way ANOVA (with Bonferroni post hoc test) were used throughout as indicated in the text. Data were considered statistically significant at $P < 0.05$.

**Study Approval.** All animal experiments were performed in accordance with protocols approved by the Mayo Clinic IACUC. Clinical information was assimilated from patient records from the Mayo Clinic. Informed consent was obtained for each patient on an ongoing research protocol approved by the Mayo Clinic Institutional Review Board (707-03).
Author Contributions

EL designed and performed experiments, analyzed data, and wrote the manuscript. JY, CC, EB, JW, KDP conducted experiments. JY, YL, JW, KDP, YL, KDP, DO, CW, RPG, RLS, HD analyzed data. CW, RLS, HD provided constructive suggestions. SR designed and supervised the study, analyzed data, and wrote the manuscript. All authors discussed the results and commented on the manuscript.
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References


Figure 1. TAMs are the predominant source of PD-L1 in CCA. (A) Representative images (left and middle panels) of PD-L1 (brown staining, black arrow) plus CD68 (red staining, red arrow) co-immunostaining (n=33) and PD-L1 (brown staining) plus cytokeratin-19 (CK-19) (red staining) co-immunostaining (n=18) in human resected CCA specimens. Percentage of patients with positive PD-L1/CD68 co-staining and PD-L1/CK19 co-staining, respectively (right panel). Scale bar, 40 μm. (B) Histograms show expression of PD-L1+ macrophages in human CCA tumors. (C-F) Flow cytometry analysis of normal WT mouse livers (from WT mice without tumors) as well as adjacent livers and tumors of mice 28 days after orthotopic implantation of 1x10^6 SB (murine CCA) cells. (C) Percentage of PD-L1+ macrophages (Mφ) of total Mφ (CD45^+ CD11b^+ F4/80^+ ) in WT mouse normal liver, tumor adjacent liver or tumor. Fluorescence Minus One (MFO) controls were used for each independent experiment to establish gates (See Fig. S1A for gating strategy), (n ≥ 8). Representative histograms show expression of PD-L1+ macrophages. (D) Percentage of CD206+ TAMs (left panel) and PD-L1+CD206+ TAMs (middle panel) of F4/80^+ macrophages (CD45^+ CD11b^+ F4/80^+ ) in WT mouse liver, tumor adjacent liver or tumor. Representative contour plots (right panel) show CD206 and PD-L1 expression of F4/80^+ macrophages, (n ≥ 7). (E) Percentage of PD-L1^+CD206^+ Mφ or PD-L1^+CD206^- Mφ (CD11b^+ F4/80^- ) of CD45^+ cells from SB tumors, (n=28). (F) Percentage of PD-L1 expression in myeloid cells from SB tumors. Mφ: Macrophages (CD45^+ CD11b^+ F4/80^+ ); MDSCs: Myeloid-derived suppressor cells (CD45^+ PD-L1^- CD11c^- CD11b^- F4/80^- GR-1^- ); DC: Dendritic cells (CD45^+ PD-L1^- CD11c^+ ), (n=11). Data represent mean ± SD. Unpaired Student’s t test (E) and one-way ANOVA with Bonferroni post hoc test (C, D and F) were used. * P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 2: Host PD-L1 contributes to CCA progression. (A) Immunoblot analysis of PD-L1 in mouse CCA cells (SB) and normal mouse cholangiocytes (NMC). (B-F) Tumor growth of 28 days after orthotopic implantation of 1x10^6 SB cells in WT or Pd-l1^-/- mice. (B) Average tumor weights in milligrams (mg) of WT or Pd-l1^-/- mice, (n ≥ 23). (C) Representative pictures of livers from b. (D) Percentage of CD206^+ TAMs (left panel) and CD206^+ PD-L1^+ TAMs (right panel) of F4/80^+ TAMs (CD45^+ CD11b^+ F4/80^+) in Pd-l1^-/- normal liver (from mice without tumors) and tumors from WT and Pd-l1^-/- mice (n≥8). (E) Percentage of CD8^+CD3^+ T cytotoxic T lymphocytes (CTLs) of CD45^+ cells in Pd-l1^-/- normal liver and tumors from WT and Pd-l1^-/- mice (n ≥ 12). (F) Percentage of CD8^+CD11a^+ reactive CTLs of CD45^+CD3^+ cells in Pd-l1^-/- normal liver and tumors from WT and Pd-l1^-/- mice (n≥12). (G) Percentage of Pd-l1^-/- TAMs bone marrow-derived macrophages (BMDM) after 72h of co-culture in vitro with SB cells (ratio 1:1). BMDM were isolated from WT mice (n=4). (H) Percentage of PD-L1^-/- TAMs BMDM after 24h of treatment with conditioned medium (CM) from SB cells (1mL). BMDM were isolated from WT mice, (n=4). (I) Concentration (pg/mL) of soluble PD-L1 in CM of SB cells after 24h of culture, (n=8). (J) Percentage of INFγ^+ T cells and Ki67^+ T cells after 24h of treatment with CM from SB cells (1mL) with IgG or anti-PD-L1 neutralizing antibody (SB-CM/IgG or SB-CM/anti-PD-L1). T cells were isolated from WT mice, (n≥5). Data represent mean ± SD. Unpaired Student’s t test (B-G-I) and one-way ANOVA with Bonferroni post hoc test (D-F and J) were used. NS, non-significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001.
**Figure 3.** PD-L1⁺ TAMs are recruited from the bone marrow in CCA. (A-C; E-I) Tumor growth of 28 days after orthotopic implantation of 1x10⁶ SB (murine CCA) cells in WT or Pd-l1⁻⁻ mouse livers. (A) Ratio of recruited TAMs (CD45⁺CD11b⁺F4/80⁺CCR2⁺) to resident TAMs (CD45⁺CD11b⁺F4/80⁺CD11c⁻⁺Clec4F⁻) in WT mouse liver (from mice without tumors) or SB tumor, (n = 11). (B) Percentage of PD-L1⁺CCR2⁺ recruited TAMs of F4/80⁺ TAMs (CD45⁺CD11b⁺F4/80⁺) in WT mouse liver, tumor adjacent liver or tumor. Representative flow plots show expression of CCR2 and PD-L1 in F4/80⁺ TAMs, (n ≥ 7). (C) Percentage of PD-L1⁺Clec4F⁻ resident TAMs of F4/80⁺high TAMs (CD45⁺CD11b⁺F4/80⁺high) in WT mouse liver, tumor adjacent liver or tumor. Representative flow plots show expression of Clec4F and PD-L1 in F4/80⁺high TAMs, (n ≥ 7). (D) Schematic of mouse bone marrow transplantation. (E) Average tumor weights in milligrams (mg) of Pd-l1⁻⁻ mice transplanted with WT bone marrow (WT-Pd-l1⁻⁻) or WT mice transplanted with Pd-l1⁻⁻ bone marrow (Pd-l1⁻⁻-WT), (n ≥ 8). (F) Representative pictures of livers from E. (G) Percentage of CCR2⁻⁺ recruited TAMs of total TAMs (CD45⁺CD11b⁺F4/80⁺) in tumors from WT-Pd-l1⁻⁻ or Pd-l1⁻⁻-WT, (n ≥ 7). (H) Percentage of CD8⁺CD11a⁺ reactive CTLs of CD45⁺CD3⁺ cells in tumors from WT-Pd-l1⁻⁻ or Pd-l1⁻⁻-WT mice, (n ≥ 7). (I) Percentage of granzyme B-expressed in CD8⁺CD11a⁺ reactive CTLs (CD45⁺CD3⁺CD8⁺CD11a⁺) in tumors from WT-Pd-l1⁻⁻ or Pd-l1⁻⁻-WT mice, (n ≥ 7). Data represent mean ± SD. Unpaired Student’s t-test (A, E, G-I) and one-way ANOVA with Bonferroni post hoc test (B-C) were used. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4. TAM blockade promotes a compensatory infiltration of G-MDSCs. (A-F; I) Tumor growth of 28 days after orthotopic implantation of 1×10^6 SB (murine CCA) cells in WT or Ccr2^-/- mouse livers. (A) Average tumor weights in milligrams (mg) of WT and Ccr2^-/- mice, (n=12). (B) Percentage of PD-L1' Clec4F' resident TAMs of F4/80^high TAMs (CD45^-CD11b^-F4/80^high) in WT or Ccr2^-/- tumors, (n=14). Representative flow plots show expression of Clec4F and PD-L1 in F4/80^high TAMs. (C) Percentage of CD11c^dim/F4/80^-CD11b^-Gr-1^-MDSCs of CD45^- cells in WT or Ccr2^-/- tumors, (n=14). (D) Percentage of CD11c^dim/F4/80^-CD11b^-Ly6G^- Ly6C^- M-MDSCs and CD11c^dim/F4/80^-CD11b^-Ly6G^- G-MDSCs of CD45^- cells in Ccr2^-/- tumors, (n=4). (E) Schematic of myeloid cell analysis in anti-CSF1R and control treated mouse tumors. (F) Average tumor weights in milligrams (mg) of WT mice treated every 3 days from day 14-28 (after orthotopic SB cell implantation) with a control Rat IgG isotype or anti-mouse CSF1R (AFS98), (n ≥ 7). (G) Heatmap showing average expression of marker expression intensity in the different CyTOF clusters. (H) iSNE plots of CyTOF datasets show different clusters of immune cell populations identified by selected markers. (I) iSNE plots of CyTOF datasets show different cluster of immune cell populations identified by selected markers in tumor from WT mice treated with the control IgG (n=6) or anti-CSF1R (n=11). Cells are color-coded and represent the mean of cell density in each cluster. Black circle outlines the G-MDSC cluster (cluster 3). Percentage of G-MDSCs identified by markers expressed in cluster 3 in tumor from WT mice treated with control IgG or anti-CSF1R (n ≥ 6). Data represent mean ± SD. One-way ANOVA with Bonferroni post hoc test was used; *, P < 0.05; **, P < 0.001; NS, non-significant.
**Figure 5.** CAF-derived CXCL2 is increased in the context of TAM blockade. (A-C) Tumor growth of 28 days after orthotopic implantation of $1 \times 10^6$ murine CCA cells in WT mouse livers. (A) Relative mRNA expression of Cxcl2 in control or anti-CSF1R treated SB tumors, ($n \geq 6$). (B) Representative immunofluorescence images of α-SMA (upper panel) or CK-19 (lower panel) in red, Cxcl2 by in situ hybridization in green, and nuclei counterstained with DAPI in control or anti-CSF1R treated mouse tumor. Scale bar, 10 μm. (C) Representative immunofluorescence images of α-SMA (left panel) in red, counterstained nuclei with DAPI in control or anti-CSF1R treated-mouse tumor. Scale bar, 20 μm. Quantification of mean fluorescence intensity of α-SMA signal in control or anti-CSF1R treated-mouse liver (right panel). Data represent mean ± SD. Unpaired Student’s t test was used. *, $P < 0.05; ~***, ~P < 0.001; ~NS, non-significant.
Figure 6. Single cell transcriptomics demonstrates accumulation of unique G-MDSC subsets with TAM blockade. (B-C) Tumor growth of 28 days after orthotopic implantation of 1x10^6 SB (murine CCA) cells in WT mice. Mice were treated from day 14 to day 28 after implantation with control rat IgG isotype or anti-CSF1R (AFS98). (A) Schematic depicting single cell RNA sequencing study of FACS sorted G-MDSCs from control and anti-CSF1R treated murine tumors. (B) Cell clustering based on tSNE algorithm for WT mice samples treated with the control IgG or anti-CSF1R. Eight clusters were initially identified with high resolution (resolution = 0.5) based on a shared nearest neighbor clustering algorithm as implemented in Seurat. (C) Cell clusters with similar expression profiles were further combined with resultant two distinct cell clusters. Percentage of cells in cluster 0 for control sample was 98%, and 86% for anti-CSF1R sample. P value < 0.01, Fisher’s exact test was used. (D) Heatmap of gene expression profiles for selected top cluster specific genes (n=25 for cluster 0 and cluster 1, respectively). Expression values for each gene was z scored across all cells. (E) Enrichment analysis for a 40 signature human MDSC’s genes using AUCell in human CCA (n=10). Significantly enriched cells are highlighted in red. (F) Enrichment analysis for 40 ApoE G-MDSC signature genes using AUCell in human CCA (n=10). Significantly enriched cells are highlighted in red.
Figure 7

(A-D) Tumor growth of 28 days after orthotopic implantation of 1x10^6 SB (murine CCA) cells in WT mice. Mice were treated from day 14 to day 28 after implantation with control IgG isotype or anti-CSF1R (AFS98). (A) Violin plots of expression levels for differentially expressed genes (ApoE, Ctsb, Ctsd, and S100a4) compared between control and anti-CSF1R treated tumors. Colors indicate control and anti-CSF1R treated samples. P-values indicate significance of expression differences between control and treatment. (B) Percentage of Annexin V^+ /7AAD^- G-MDSCs in control or anti-CSF1R treated tumors. Representative flow plots show expression of Annexin V and 7AAD in G-MDSCs. (C) Violin plot of expression levels for differentially expressed genes (Stat1 and Nfkbia) compared between control and anti-CSF1R treated tumors. P-values indicate significance of expression differences between control and treatment. (D) Percentage of Ki67^+ cells of CD8^+ T cells (CD3^+CD8^+) (left panel) and percentage of INFγ^+ cells of CD8^+ T cells (CD3^+CD8^+) (right panel) after 48h of co-culture with G-MDSCs. (E) Hyperion® multiplexed images show several immune cell markers using FFPE tissues from human CCA. Pseudo-colored raw ion images representing the markers of immune cells detected in the region of interest. Left panel shows pan-keratin (green), a CCA marker; CD45 (red), a leukocyte marker. Right panel shows CD14 (yellow), a monocyte marker; CD68 (green), a macrophage marker; CD8 (red), a CTL marker; CD11b-CD15 (blue) G-MDSC markers. White arrows indicate CD8^+ T cell (red) and G-MDSC (blue) interaction. Scale bar, 10 μm. (F) Flow plots show expression of CD15/CD14^+ G-MDSCs in human CCA. Data represent mean ± SD. Unpaired Student’s t test (A-C) and one-way ANOVA with Bonferroni post hoc test (D) were used.

*P < 0.05; **P < 0.01; ***P < 0.001.
Figure 8. Dual inhibition of G-MDSCs and TAMs potentiates anti-PD-1 therapy. (A) Survival curves in mice treated with control rat IgG isotype, anti-PD-1 (G4), anti-CSF1R (AFS98), anti-Ly6G (1A8), GW3965 alone or in the depicted combinations, (n ≥ 5). (B-I) Tumor growth of 28 days after orthotopic implantation of 1x10^6 SB (murine CCA) cells in WT mice. Mice were treated from day 14 to day 28 after implantation. (B) Schematic of mouse immunotherapy treatment and characterization. (C) Representative computed tomography image of liver tumor from a contrast reagent-injected mouse treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G 28 days after implantation. The liver is depicted in blue color and the tumor in red. (D) Average tumor weights in milligrams (mg) of WT mice treated with control IgG isotype, anti-PD-1+anti-CSF1R+anti-Ly6G or anti-PD-1+anti-CSF1R+GW3965, (n ≥ 6). (E) Percentage of PD-L1⁺ TAMs of F4/80⁺ TAMs (CD45⁺CD11b⁺F4/80⁺) in tumors from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G or anti-PD-1+anti-CSF1R+GW3965, (n ≥ 3). (F) Percentage of CD11c⁺CD40⁺CD11b⁺Ly6G⁺ G-MDSCs of CD45⁺ cells in tumors from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G or anti-PD-1+anti-CSF1R+GW3965, (n ≥ 3). (G) Percentage of CD8⁺ CTLs of CD45⁺ cells in tumors from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G or anti-PD-1+anti-CSF1R+GW3965, (n ≥ 3). (H) Percentage of PD-1⁺ expressed in CD8⁺CD11a⁺ reactive CTLs (CD3⁺CD8⁺CD11a⁺) in tumors from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G or anti-PD-1+anti-CSF1R+GW3965, (n ≥ 3). (I) Percentage of granzyme B expressed in CD8⁺CD11a⁺ reactive CTLs (CD45⁺CD8⁺CD11a⁺) in tumors from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G or anti-PD-1+anti-CSF1R+GW3965, (n ≥ 3). Data represent mean ± SD. Log-Rank Mantel-Cox test (A) and ANOVA with Bonferroni post hoc test (C-H) were used. *, P < 0.05; **, P < 0.01; ***, P < 0.001.