## SUPPLEMENTARY METHODS

*YAP Signature Differential Expression Analysis.* We examined the RNA-Seq by Expectation Maximization (RSEM) transformed normalized gene expression values for a previously published YAP expression signature (1) in The Cancer Genome Atlas (TCGA)-CHOL primary tumor samples (n=36) and compared them to TCGA adjacent liver samples (n=50) as well as to Genotype-Tissue Expression (GTEx) database normal liver samples (n=110). The data was downloaded from the University of California Santa Cruz's Xenabrowser (2). These samples were uniformly processed together using University of Santa Cruz's TOIL software. Plots were created using ClustVis software (3).

*Human immunohistochemical staining (IHC)*. Tissue sectioning and IHC staining was performed at the Pathology Research Core (Mayo Clinic, Rochester, MN) using the Leica Bond RX stainer (Leica). FFPE tissues were sectioned at 5 microns and IHC staining was performed on-line. Slides for the PD-L1/Keratin 19 and PD-L1/CD68 stain were retrieved for 20 minutes using Epitope Retrieval 2 (EDTA; Leica) and incubated in Protein Block (Dako) for 5 minutes. The PD-L1 primary antibody (rabbit monoclonal, clone E1L3N, 13684, Cell Signaling) was diluted to 1:400 in Background Reducing Diluent (Dako) and incubated for 15 minutes. Post primary and polymer reagents from the Bond Polymer Refine DAB Detection Kit (Leica) were applied and DAB detection used for PD-L1. The second antibody Keratin 19 (mouse monoclonal, clone RCK108, M0888, Dako) or CD68 (clone PGM1, Dako) were diluted to 1:200 in Background Reducing Diluent (Dako) and incubated for 15 minutes. Post primary and polymer reagents from the Bond Polymer Refine AP-Red Detection Kit (Leica) were applied and AP-Red detection used for CK-19 or CD68. Slides were rinsed between steps with 1X Bond Wash Buffer (Leica) and were counterstained for five minutes using Schmidt hematoxylin and molecular biology grade water (1:1 mixture). Slides were removed from the strainer and rinsed in tap water for five

minutes. Slides were dehydrated in increasing concentrations of ethyl alcohol and cleared in 3 changes of xylene prior to permanent coverslipping in xylene-based medium. Images were acquired with Olympus BX 53 microscope at 20x and 40x.

*Cell Culture*. Mouse CCA cell line SB cells were maintained in DMEM supplemented with 10% FBS, 0.2% primocin and 0.01% insulin under standard conditions. This cell line was isolated from our murine model of YAP-associated CCA and has been described in detail (4). The murine nonmalignant, immortalized cholangiocyte cell line normal mouse cholangiocyte (NMC) was cultured in Eagle's minimum essential medium supplemented with 10% FBS, penicillin (1,000 U/mL), and streptomycin (100 μg/mL) (5). All cell lines underwent *Mycoplasma* contamination test using Mycoalert™ Mycoplasma Detection Kit (Lonza).

*Mice.* Eight-week-old male C57BL/6 mice, BL6 SJL mice (CD45.1 allele) or C57BL mice (CD45.2 allele) were purchased from Jackson Laboratories. Six-week-old male and female C57BL/6 *Ccr2*$^{-/-}$ mice were purchased from Charles River Laboratory and bred at Mayo Clinic. C57BL/6 mice *Pd-l1*$^{-/-}$ mice (47) were bred at Mayo Clinic.

*Isolation of liver, spleen, or tumor-infiltrating immune cells and flow analysis.* Upon excision, tumor, liver and spleen were dissociated with gentleMACS™ Octo Dissociator (Miltenyi) according to the manufacturer's protocol. CD45$^{+}$ cells were isolated by CD45 (TIL) mouse microbeads (Miltenyi). Cells were incubated with Fixable Viability Stain 510 (BD Horizon™) for 15 minutes followed by 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 and Fluorescence Minus One controls were used for each independent experiment to establish gates. For subsequent intracellular staining of granzyme B, cells were

stained using the intracellular staining kit (Miltenyi). 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: F4/80-PE (REA126, Miltenyi), CD11b-PE-Cy5 (M1/70, eBioscience), CD206-PE-Cy7 (C068C2, BioLegend®), CCR2-APC-Vio®770 (REA538, Miltenyi), PD-L1-BV421 (10F.9G2, BioLegend®), F4/80-PE-Vio®770 (REA126, Miltenyi), CD11c-APC (REA754, Miltenyi), Gr-1-APC-Vio®770 (REA810, Miltenyi), Ly6G-PE (Rat 1A8, Miltenyi), Ly6C-APC-Vio®770 (REA796, Miltenyi) CD3-APC-Vio®770 (REA641, Miltenyi), CD8-BV421 (53-6.7, BD Horizon™), CD11a-PE-Vio®770 (REA880, Miltenyi), PD-1-PerCP-Vio®700 (REA802, Miltenyi), granzyme B-PE (REA226, Miltenyi), CD45.1-Vioblue (A20, Miltenyi), CD45.2-PE-Vio®770 (104-2, Miltenyi), 7AAD-annexinV-APC kit (640930, BioLegend®) and Clec4F (MAB2784, R&D System). Clec4F antibody was conjugated with AlexaFluor® 647 antibody labeling kit (Invitrogen®).

*Isolation of MDSCs*. MDSCs were isolated from tumor tissue using gentleMACS™ Octo Dissociator (Miltenyi) per the manufacturer's protocol. $CD45^+$ cells were isolated by CD45 (TIL) mouse microbeads (Miltenyi), stained with Fixable Viability Stain 510 (BD Horizon™), CD11b-PE-Cy5 and Ly6G-PE and immediately separated by cells sorting. MDSCs were sorted as $CD11b^+$ $Ly6G^+$ on the live cells population.

*Isolation of T cells*. $CD8^+$ T cells were isolated from a single cell suspension of WT mouse spleens. Single cell suspension was filtered through 70μm mesh, centrifuged at 400 g for 10 minutes and re-suspended at $1x10^8$ cell/mL. $CD8^+$ T cells were isolated using easy step mouse $CD8^+$ T cell isolation kit (#19856, stemcell) per the manufacturer's protocol.

*Immunohistochemistry in mouse liver specimens*. Liver tissue from euthanized mice was fixed in 4% paraformaldehyde for 48h, embedded in paraffin, and sectioned into 3–5 μm slices.

Paraformaldehyde fixed, paraffin-embedded mouse tumor and adjacent liver sections were deparaffinized, hydrated and incubated with primary antibody overnight at 4°C. Sections were stained with antibody for SOX9 (ab5535; Millipore). Primary antibody was detected with HRP-conjugated secondary antibody and diaminobenzidine (Dako). Liver tissue sections were counterstained with hematoxylin. Images were acquire with a Leica AXIO scope A1 at 10x, 20x and 40x.

*Extracellular vesicle (EV) isolation*. EVs were isolated from SB cell conditioned medium. SB cell culture was maintained in EV-free culture medium for 24h. The conditioned medium was subsequently collected and EVs were purified by serial ultracentrifugation (2000 RPM for 20 minutes to remove cell debris; 9600 RPM for 20 minutes to remove cell debris; 25500 RPM for 2 hour precipitate EV; 28500 RPM for 2 hour to wash EV with PBS).

*BMDM isolation and in vitro culture*. BMDM were isolated from the leg of WT or *Pd-l1*$^{-/-}$ mice. Bone marrow was extracted and maintained in a BMDM medium (RPMI 1640 + 10% low endotoxin FBS + L929 condition medium) for 7 days to isolate BMDM. *Pd-l1*$^{-/-}$ BMDM were plated in 6-well plate at 2 x $10^5$ cell and co-cultured with SB cells at a 1:1 ratio for 72h. BMDM were separated by CD45 (TIL) mouse microbeads (Miltenyi), stained with Fixable Viability Stain 510 (BD Horizon™), F4/80-PE and PD-L1-BV421 and analyzed by flow cytometry. For the conditioned medium and EV experiments, BMDM were plated in a 12-well plate at 2 x $10^5$ cell. 24 hours later, BMDM were incubated with 1 mL of SB cell conditioned medium or $1.5x10^{10}$ particle of SB EVs for 24h. BMDM were separated by CD45 (TIL) mouse microbeads (Miltenyi), stained with Fixable Viability Stain 510 (BD Horizon™), F4/80-PE and PD-L1-BV421 and analyzed by flow cytometry.

*Immunoblot Analysis*. EVs were purified from SB cells and lysed using RIPA buffer. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The following primary antibodies were used for immunoblot analysis: PD-L1 (AF1019, R&D System), CD81 (sc-9158, Santa Cruz) and Alix (2171, Cell Signaling) Membranes were blotted with primary antibody overnight at 4°C at a dilution of 1:500 (PD-L1 and CD81) or 1:1000 (Alix). Proteins were visualized with enhanced chemiluminescence reagents (ECL/ECL Plus, Amersham GE) and Kodak X-OMAT film.

*Soluble PD-L1 ELISA*. SB cells were plated on a 150mm dish, and once 90%-100% confluence was reached the cell culture medium was changed and collected 24h later. Culture medium was centrifuged at 1,500 RPM for 10 min at 4°C and proteins were concentrated using Amicon Ultra-4 10K. ELISA was performed on SB conditioned medium using DuoSet® Ancillary Reagent Kit 2 (DY008, R&D systems) and Mouse PD-L1/B7-H1, (DY1019-05, R&D systems).

*T cell incubation with conditioned medium from murine CCA cells.* To neutralize PD-L1 in SB1 conditioned medium, 2 μg/ml anti-PD-L1 antibody (AF1019, R&D) or 2 μg/ml goat IgG isotype (31245, Invitrogen®) was added to the SB conditioned medium before T cell culture. T cells were subsequently isolated from spleen single cell suspension and cultured in SB conditioned medium with PD-L1 neutralized for 24h with CD3/CD28 beads (Gibco Dynabead). T cell INFγ transporters were inhibited 5 hours before flow staining with 5 μg/mL of brefeldin A (1000x) (420601, Biolegend) and Monensin (420701, Biolegend). 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.

*Single cell RNA-seq*. Whole live cells were washed twice in 1x PBS + 0.04% BSA and immediately submitted to the Mayo Clinic Core for Single Cell sorting (Mayo Clinic, Rochester,

MN). The cells were first counted and measured for viability using either the Vi-Cell XR Cell Viability Analyzer (Beckman-Coulter) or a basic hemocytometer and light microscope. The barcoded Gel Beads were thawed from -80 C and the cDNA master mix was prepared according to the manufacturer's instruction for Chromium Single Cell 3' v3 library kit (10x Genomics). Based on the desired number of cells to be captured for each sample, a volume of live cells was mixed with the cDNA master mix. A per sample concentration of 500,000 cells per milliliter or better is required for the standard targeted cell recovery of 2000 cells. The stock concentration requirements would not change for higher cell recovery numbers. The cell suspension and master mix, thawed Gel Beads and partitioning oil were added to a Chromium Single Cell B chip. The filled chip was loaded into the Chromium Controller, where each sample was processed and the individual cells within the sample were captured into uniquely labeled GEMs (Gel Beads-In-Emulsion). The GEMs were collected from the chip and taken to the bench for reverse transcription, GEM dissolution, and cDNA clean-up. The resulting cDNA contained a pool of uniquely barcoded molecules. A portion of the cleaned and measured pooled cDNA continued on to library construction, where standard Illumina sequencing primers and a unique i7 Sample index were added to each cDNA pool. All cDNA pools and resulting libraries were measured using Qubit High Sensitivity assays (Thermo Fisher Scientific), Agilent Bioanalyzer High Sensitivity chips (Agilent) and Kapa DNA Quantification reagents (Kapa Biosystems). Libraries are sequenced at 60,000 fragment reads per cell following Illumina's standard protocol using the Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. The flow cells are sequenced as 100 X 2 paired end reads on an Illumina HiSeq 4000 using HiSeq 3000/4000 sequencing kit and HCS v3.3.52 collection software. Base-calling is performed using Illumina's RTA version 2.7.3.

*Immune electron microscopy*. Isolated EV were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C and then placed on a Formvar-carbon-coated grid and air dried for 20 min. After a PBS wash, grids were transferred to 1% glutaraldehyde for 5 minutes and washed with distilled water. The grids were first contrasted with uranyl-oxalate solution and then contrasted and embedded in a mixture of 4% uranyl acetate and 2% methylcellulose (1:9 ratio). The grids were air dried and visualized with a JEOL 1400 electron microscope (JEOL USA, Peabody, MA) at 80 kV. For immunogold staining, grids were fixed as described above and blocked with 10% FBS in PBS for 20 minutes followed by overnight incubation at 4 °C with a primary anti-PD-L1 antibody (R&D system, Minneapolis, MN) in blocking solution. Next, grids were incubated with secondary antibody anti-Goat-12 nm gold or with antibody rabbit anti-goat IgG-Protein A (10 nm) gold (Jackson ImmunoResearch lab, West Grove, PA) for 1h. The grids were contrasted and embedded with a mixture of 4% uranyl acetate and 2% methylcellulose (1:9 ratio), air dried, and subsequently visualized with a JEOL 1400 electron microscope.

*Cytokine array*. mRNA was extracted from 30 mg of murine tumor tissue using RNeasy plus mini kit (Qiagen). Reverse transcription (RT) was performed using Invitrogen first strand synthesis system (Thermo Fisher Scientific). Real-time polymerase chain reaction (qPCR) was performed using the RT2 Profiler PCR Array (Qiagen) according to the manufacturer's instructions. The threshold cycle (CT) for each well was calculated using the real-time cycler software.

*Quantitative Real-Time PCR*. RNA was extracted from 20 mg of murine tumor tissue using the RNeasy plus mini kit (Qiagen). RT was performed using the Invitrogen first strand synthesis system (Thermo Fisher Scientific). Real-time PCR (Light Cycler, Roche Diagnostics) for

quantification of the cDNA template was performed using SYBR Green (Roche Diagnostics) as the fluorophore. Target gene expression was calculated using the Δ-Δ CT method. The following primers were used: *Ccl2,* forward primer TTAAAAACCTGGATCGGAACCAA, and reverse primer GCATTAGCTTCAGATTTACGGGT. *Ccl3*, forward primer TTCTCTGTACCATGACACTCTGC, and reverse primer CGTGGAATCTTCCGGCTGTAG. *Ccl4*, forward primer TTCCTGCTGTTTCTCTTACACCT, and reverse primer CTGTCTGCCTCTTTTGGTCAG. *Ccr1*, forward primer CTCATGCAGCATAGGAGGCTT, and reverse primer ACATGGCATCACCAAAAATCCA. *Ccl5*, forward primer GCTGCTTTGCCTACCTCTCC, and reverse primer TCGAGTGACAAACACGACTGC. *Ccr1*, forward primer CTCATGCAGCATAGGAGGCTT, and reverse primer ACATGGCATCACCAAAAATCCA. *Cx3cr1*, forward primer GAGTATGACGATTCTGCTGAGG, and reverse primer CAGACCGAACGTGAAGACGAG. *Cxcl2*, forward primer CATCCAGAGCTTGAGTGTGACG, and reverse primer GGCTTCAGGGTCAAGGCAAACT. *Cxcl5*, forward primer CCGCTGGCATTTCTGTTGCTGT, and reverse primer CAGGGATCACCTCCAAATTAGCG. *Cxcr2*, forward primer TGGCTGGGATTCACCTCAAGAACA, and reverse primer TGTGGCTATGACTTCGGTTTGGGT. *Gapdh*, forward primer CCACCCCAGCAAGGAGACT, and reverse primer GAAATTGTGAGGGAGATGCT.

## References


1. Yuan WC, Pepe-Mooney B, Galli GG, Dill MT, Huang HT, Hao M, et al. NUAK2 is a critical YAP target in liver cancer. *Nat Commun.* 2018;9(1):4834.
2. Goldman MJ, Craft B, Hastie M, Repecka K, McDade F, Kamath A, et al. Visualizing and interpreting cancer genomics data via the Xena platform. *Nat Biotechnol.* 2020.
3. Metsalu T, and Vilo J. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res.* 2015;43(W1):W566-70.
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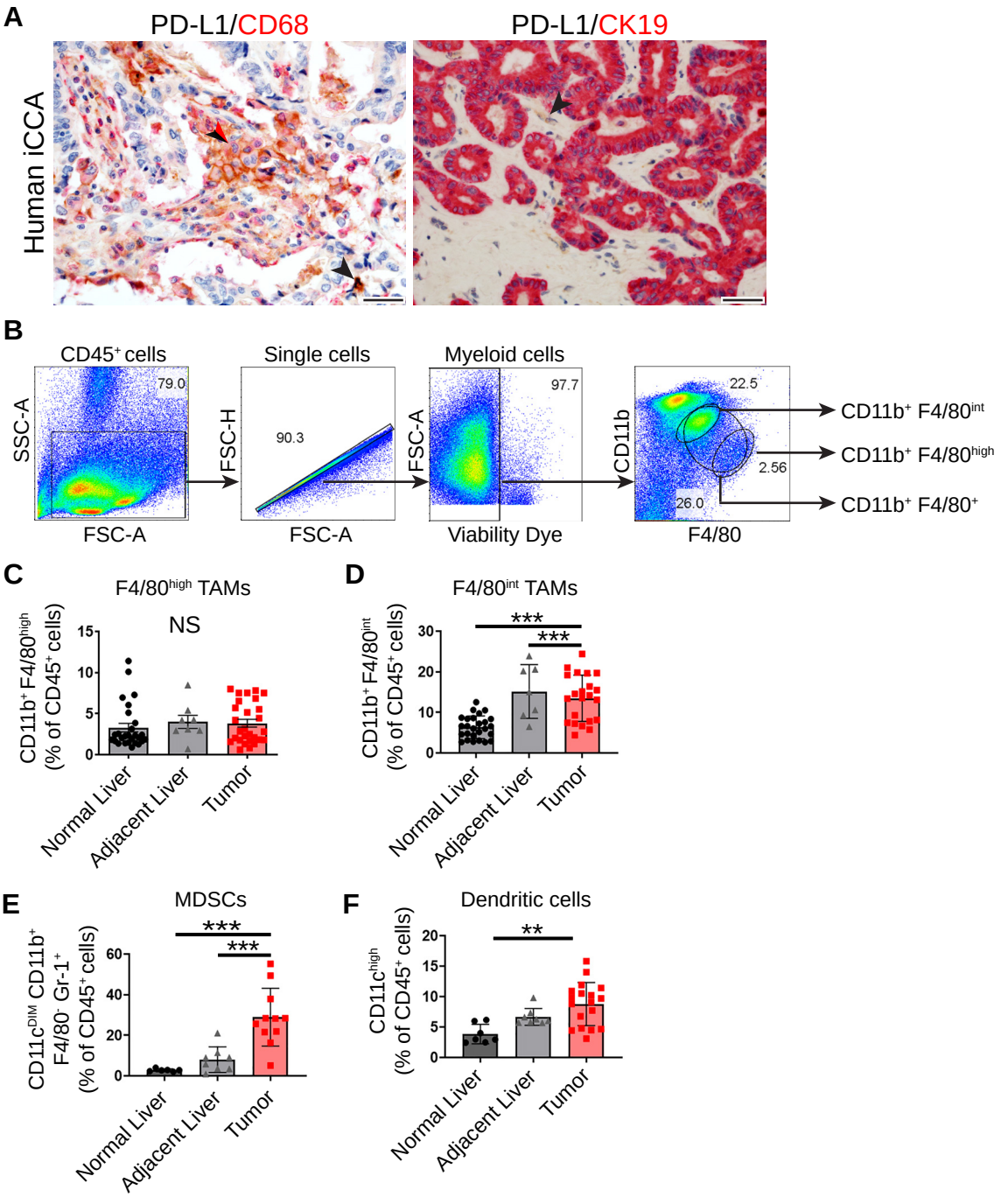
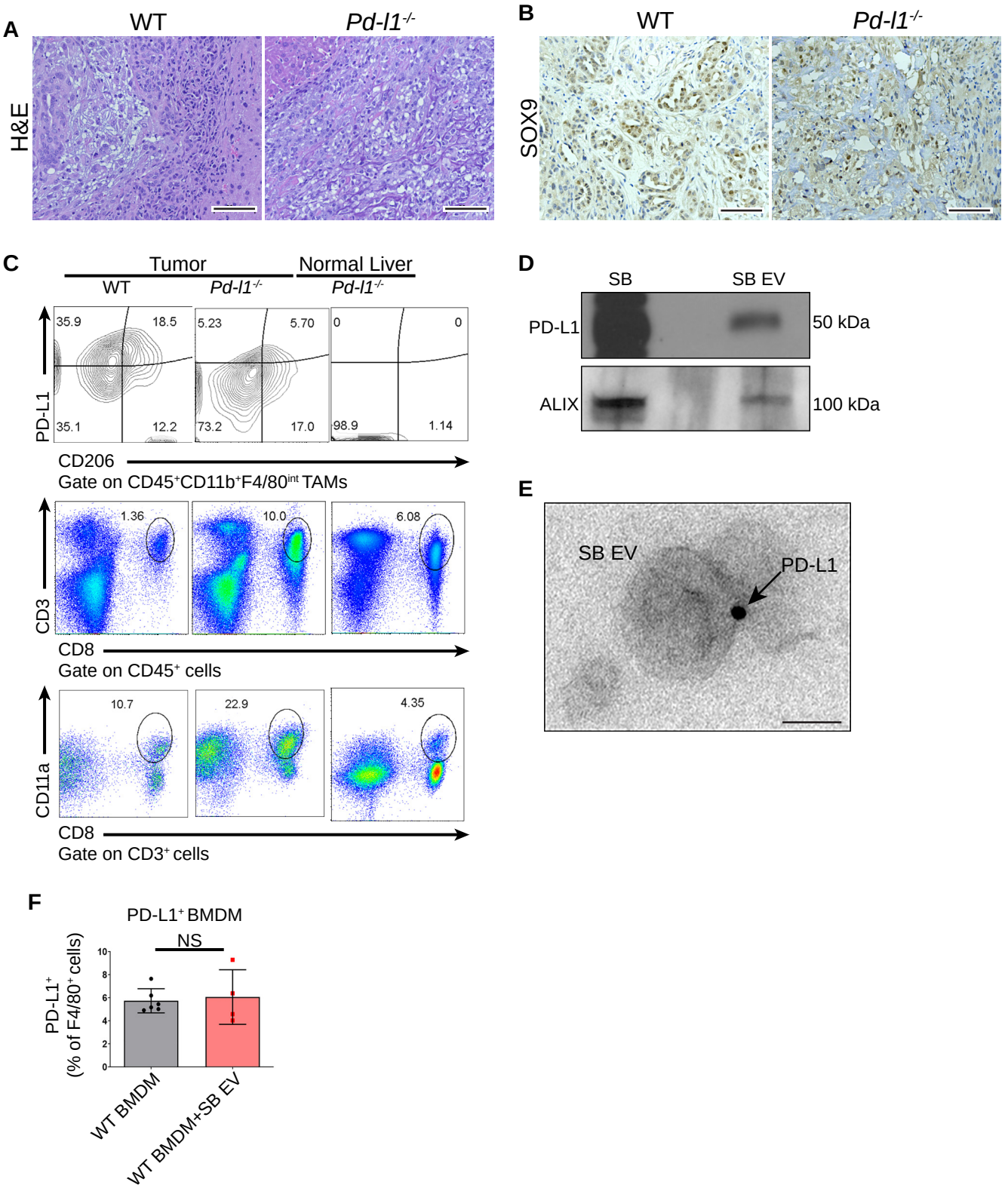
**Figure S1**



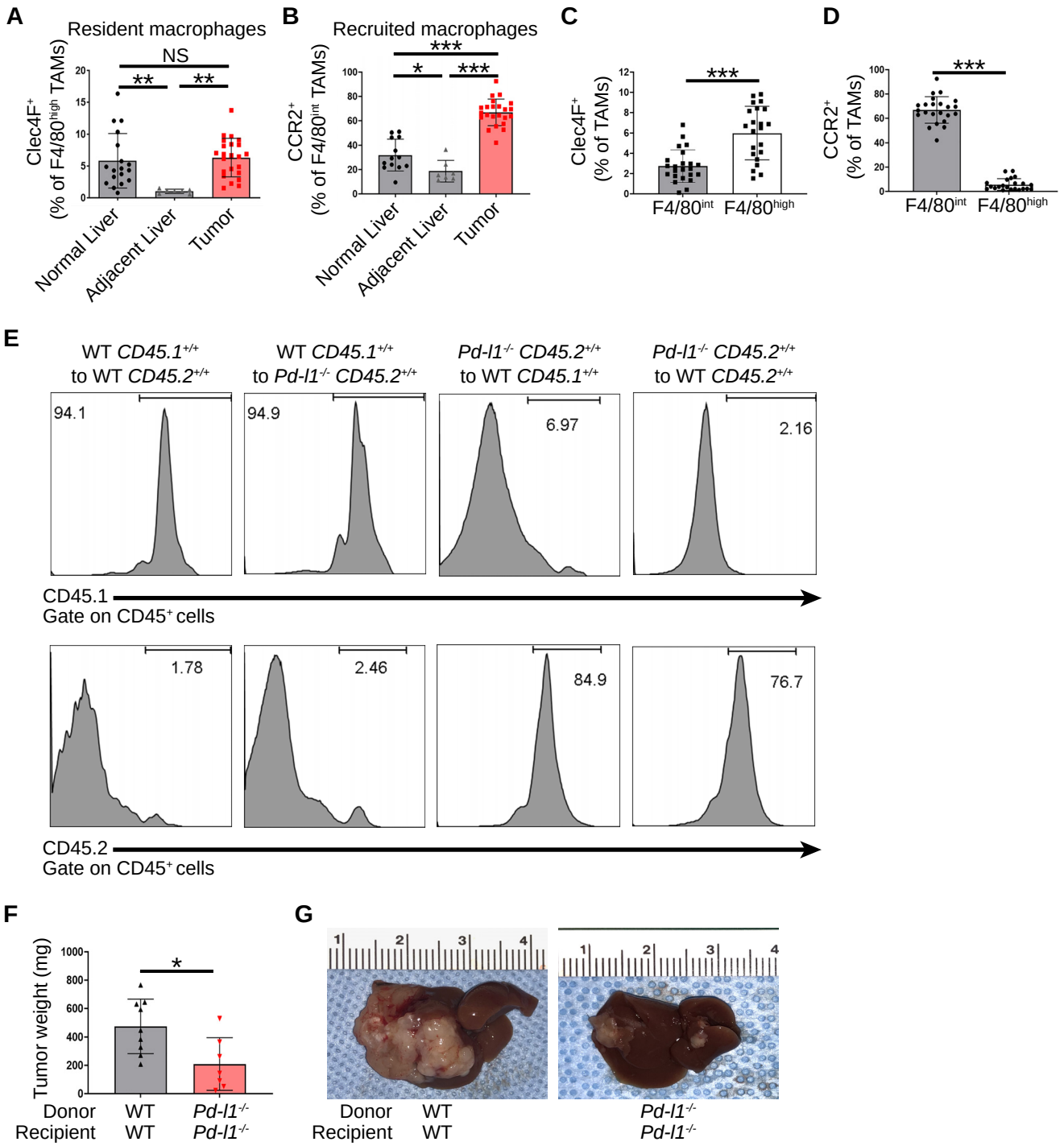

**Supplementary Figure S1.** TAMs are the predominant source of PD-L1 in CCA. **(A)** Representative images (left and right panels) of PD-L1 (brown staining, black arrow) plus CD68 (red staining, red arrow) co-immunostaining (n=33) and PD-L1 (brown staining) plus cytokeratin-19 (CK-19) (red staining) co-immunostaining (n=18) in human resected CCA specimens. Scale bar, 40 µm. **(B)** Gating strategy for TAMs is shown in a WT mouse tumor (28 days after orthotopic implantation of $1x10^6$ SB cells). **(C-F)** Tumor growth of 28 days after orthotopic implantation of $1x10^6$ SB (murine CCA) cells in WT mice. **(C)** Percentage of $F4/80^{high}$ TAMs of total $CD45^+$ cells in WT mouse liver (from mice without tumor), tumor adjacent liver or tumor, ($n \geq 8$). **(D)** Percentage of $F4/80^{int}$ TAMs of total $CD45^+$ cells in WT mouse liver, tumor adjacent liver or tumor, ($n \geq 7$). **(E)** Percentage of $CD11c^{Dim}F4/80^-CD11b^+Gr\text{-}1^+$ G-MDSCs of $CD45^+$ cells in WT mouse liver, tumor adjacent liver or tumor, ($n \geq 6$). **(F)** Percentage of $CD11c^+$ Dendritic cells of $CD45^+$ cells in WT mouse liver, tumor adjacent liver or tumor, ($n \geq 6$). Data represent mean ± SD. One-way ANOVA with Bonferroni *post hoc* test (B-E) was used. $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$; NS, non-significant.

**Figure S2**



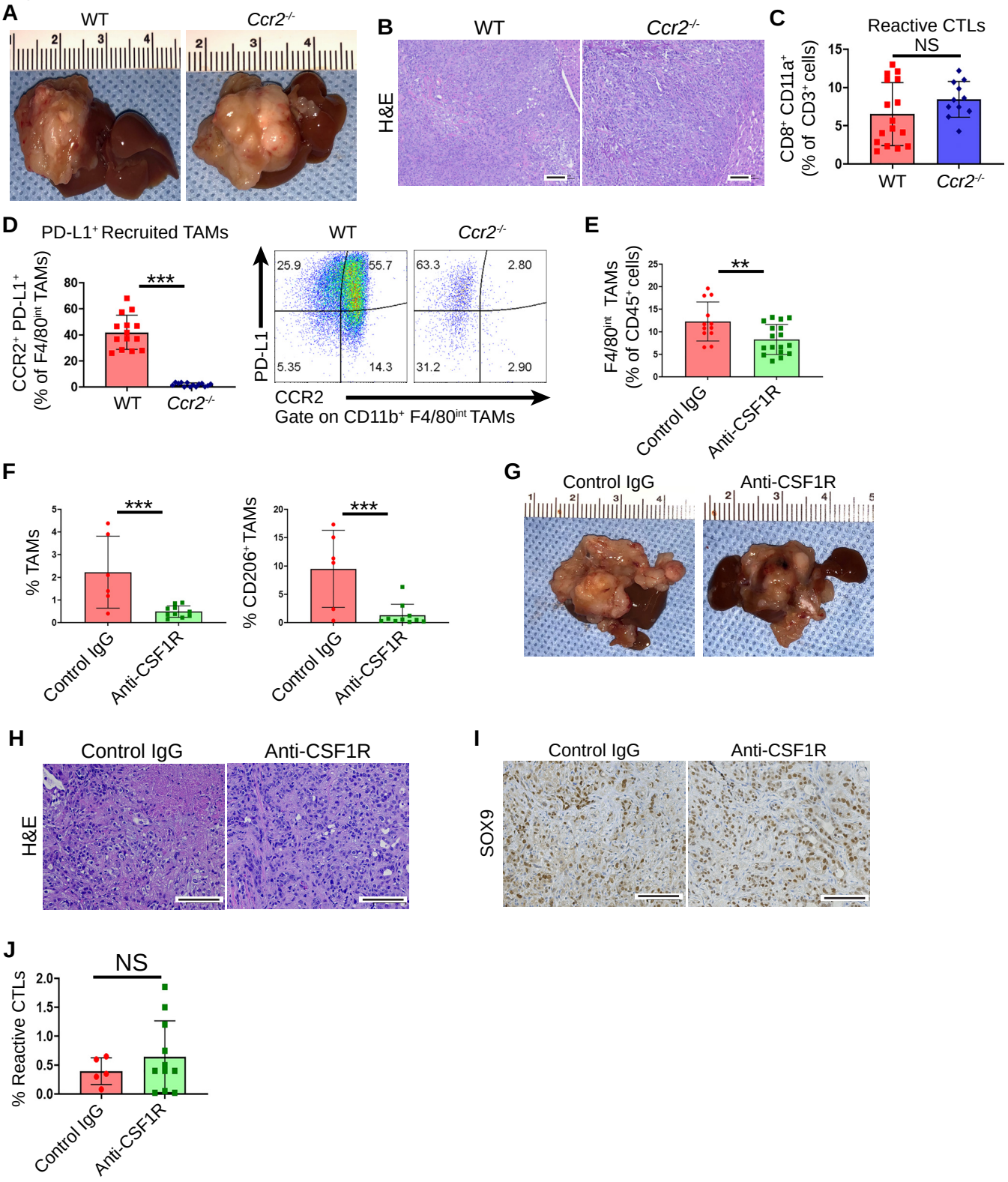

**Supplementary Figure S2.** Extracellular vesicles do not modulate PD-L1 expression on TAMs. **(A-C)** Tumor growth of 28 days after orthotopic implantation of $1x10^6$ SB (murine CCA) cells in WT or *Pd-l1*$^{-/-}$ mouse livers. **(A)** Representative photomicrographs of hematoxylin and eosin-stained SB tumor sections from WT or *Pd-l1*$^{-/-}$ mice. Scale bar, 50 µm. **(B)** Representative photomicrographs of SOX9 IHC of SB tumor sections from WT or *Pd-l1*$^{-/-}$ mice. Scale bar, 50 µm. **(C)** Representative flow plots (upper panel) show CD206 and PD-L1 expression of F4/80$^{int}$ TAMs, ($n \geq 8$). Representative flow plots (middle panel) show the population of CD8$^+$CD3$^+$ CTLs in CD45$^+$ cells, ($n \geq 12$). Representative flow plots (lower panel) show the population of CD8$^+$CD11a$^+$ reactive CTLs of CD45$^+$CD3$^+$ cells, ($n \geq 12$). **(D)** Immunoblot analysis of PD-L1 in mouse CCA cells (SB cells) and in purified extracellular vesicles (EV) from SB cells. **(E)** Immunogold-labeled PD-L1 in purified EVs from SB cells. **(F)** Percentage of PD-L1$^+$F4/80$^+$ BMDM after 24h of incubation with $1.5x10^{10}$ extracellular vesicles from SB cells. BMDM were isolated from WT mice, ($n \geq 4$). Data represent mean ± SD. Unpaired Student's t test was used. NS, non-significant.

**Figure S3**



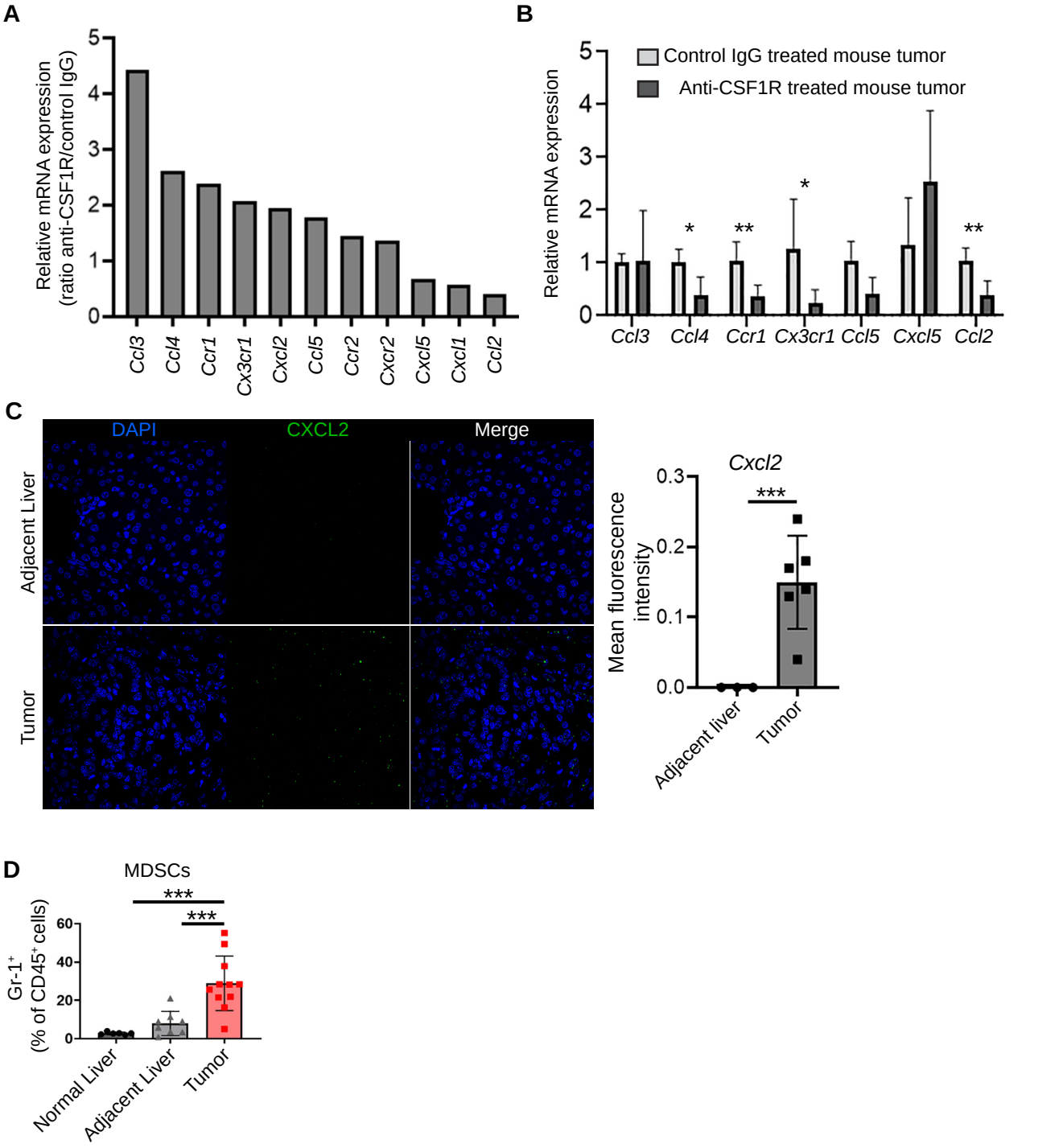

**Supplementary Figure S3.** PD-L1$^{+}$ TAMs are recruited from the bone marrow in CCA. **(A-G)** Tumor growth of 28 days after orthotopic implantation of 1x10$^{6}$ SB (murine CCA) cells in WT mouse livers. **(A)** Percentage of Clec4F$^{+}$ resident TAMs of F4/80$^{high}$ TAMs (CD45$^{+}$CD11b$^{+}$F4/80$^{high}$) in WT mouse liver, tumor adjacent liver or tumor, ($n \geq 7$). **(B)** Percentage of CCR2$^{+}$ recruited TAMs of F4/80$^{int}$ TAMs (CD45$^{+}$CD11b$^{+}$F4/80$^{int}$) in WT mouse liver, tumor adjacent liver or tumor, ($n \geq 7$). **(C)** Percentage of Clec4F expression in F4/80$^{high}$ TAMs or F4/80$^{int}$ TAMs (CD45$^{+}$ CD11b$^{+}$F4/80$^{+}$) in WT SB tumors, ($n \geq 22$). **(D)** Percentage of CCR2 expression in F4/80$^{high}$ TAMs or F4/80$^{int}$ TAMs (CD45$^{+}$CD11b$^{+}$F4/80$^{+}$) in WT SB tumors, ($n \geq 22$). **(E)** Representative flow histograms show expression of CD45.1, CD45.2, and PD-L1 in CD45$^{+}$ cells after bone marrow transplantation. **(F)** Average tumor weights in milligrams (mg) of WT mice transplanted with WT bone marrow (WT-WT) or *Pd-l1*$^{-/-}$mice transplanted with *Pd-l1*$^{-/-}$ bone marrow (*Pd-l1*$^{-/-}$-*Pd-l1*$^{-/-}$), ($n \geq 7$). **(G)** Representative pictures of livers from **E**. Data represent mean ± SD. Unpaired Student's t test (C-D, F) and one-way ANOVA with Bonferroni *post hoc* test (A-B) were used. $^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$; NS, non-significant.

**Figure S4**



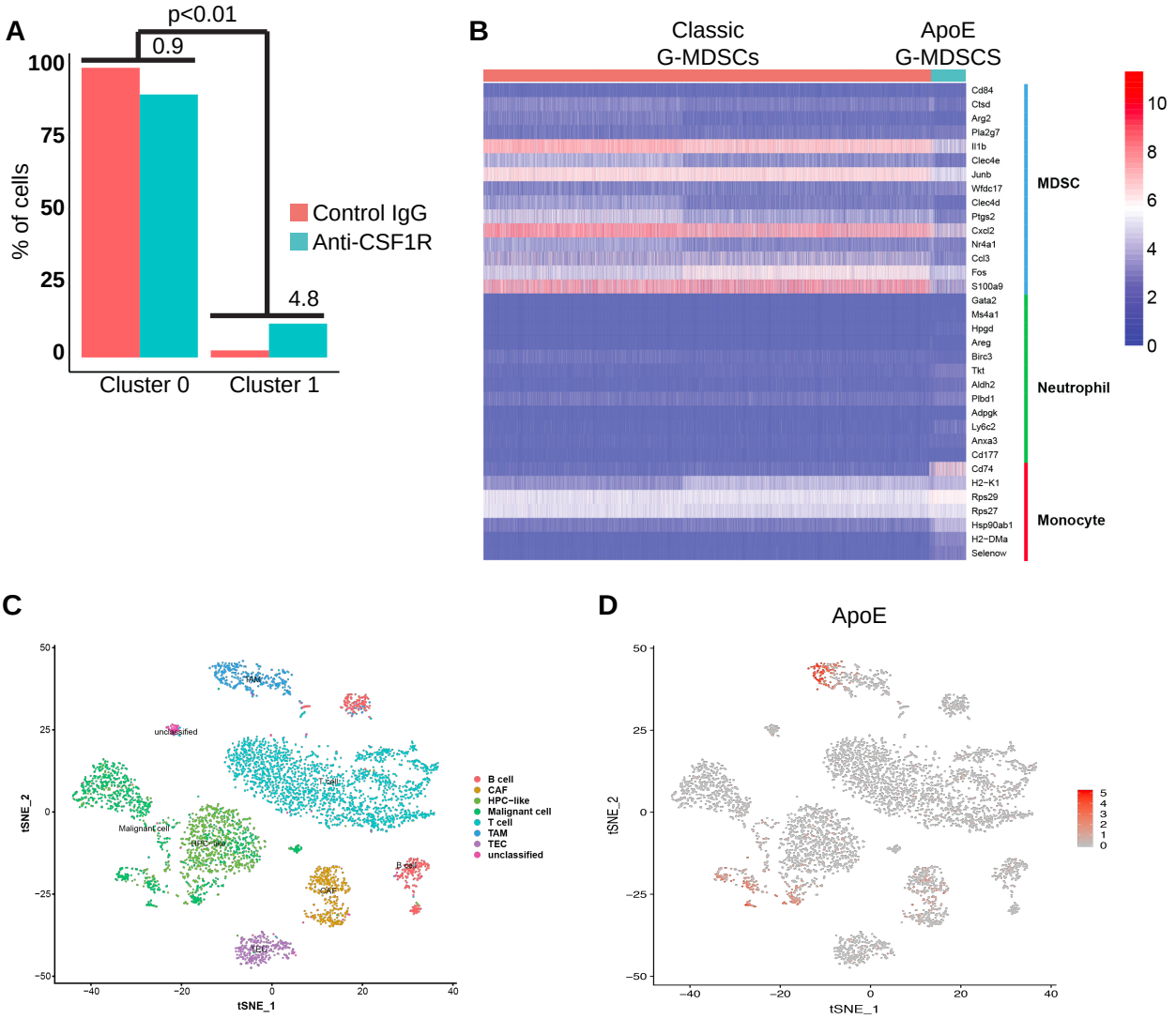

**Supplementary Figure S4.** TAM blockade does not promote an anti-tumor immune response. **(A-J)** Tumor growth of 28 days after orthotopic implantation of $1x10^6$ SB (murine CCA) cells in WT or *Ccr2*$^{-/-}$ mouse livers. **(A)** Representative pictures of livers from WT and *Ccr2*$^{-/-}$ mice. **(B)** Representative photomicrographs of hematoxylin and eosin-stained SB tumor sections from WT or *Ccr2*$^{-/-}$ mice. Scale bar, 50 µm. **(C)** Percentage of $CD8^+CD11a^+$ reactive CTLs of $CD3^+$ cells ($CD45^+CD3^+$) in WT or *Ccr2*$^{-/-}$ tumors, (n=11). **(D)** Percentage of PD-$L1^+CCR2^+$ recruited TAMs of $F4/80^{int}$ TAMs ($CD45^+CD11b^+F4/80^{int}$) in WT or *Ccr2*$^{-/-}$ tumors, ($n \geq 11$). Representative flow plots show expression of CCR2 and PD-L1 in $F4/80^{int}$ TAMs. **(E)** Percentage of $F4/80^{int}$ TAMs of $CD45^+$ cells in SB tumors from WT mice treated with a control Rat IgG isotype or anti-mouse CSF1R, ($n \geq 11$). **(F)** Percentage of TAMs and $CD206^+$ TAMs identified by CyTOF analysis of the markers expressed in CyTOF clusters 19 and 21, respectively, in SB tumors from WT mice treated with a control rat IgG isotype or anti-mouse CSF1R, ($n \geq 6$). **(G)** Representative pictures of livers from control IgG and anti-CSF1R treated mice. **(H)** Representative photomicrographs of hematoxylin and eosin-stained SB tumor sections from WT mice treated with control Rat IgG isotype or anti-mouse CSF1R. Scale bar, 50 µm. **(I)** Representative photomicrographs of SOX9 IHC SB tumor sections from WT mice treated with control rat IgG isotype or anti-mouse CSF1R. Scale bar, 50 µm. **(J)** Percentage of reactive CTLs identified by CyTOF analysis of the markers expressed in cluster 5 in SB tumors from WT mice treated with a control Rat IgG isotype or anti-mouse CSF1R, ($n \geq 6$). Data represent mean ± SD. Unpaired Student's t test was used. $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$; NS, non-significant.

**Figure S5**



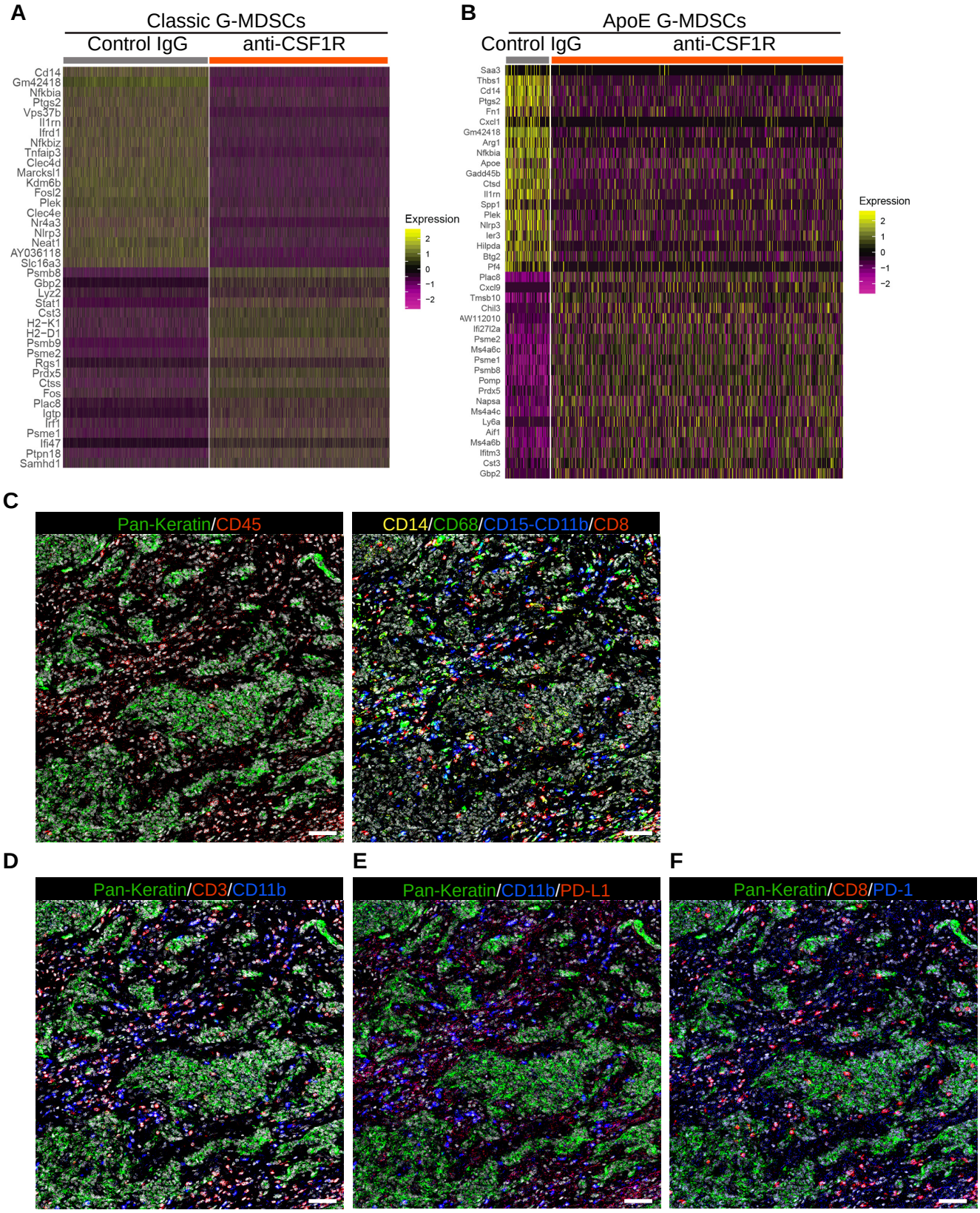

**Supplementary Figure S5.** CAF-derived CXCL2 is increased in the context of TAM blockade. **(A-D)** Tumor growth of 28 days after orthotopic implantation of $1x10^6$ SB (murine CCA) cells in WT mouse livers. **(A)** Ratio of relative mRNA expression of chemokines in anti-CSF1R to control treated tumors (mouse chemokine array). **(B)** Relative mRNA expression of chemokines in control or anti-CSF1R treated SB tumors, ($n \geq 6$). **(C)** Representative immunofluorescence images of *Cxcl2* by *in situ* hybridization in green and nuclei counterstained with DAPI in control or anti-CSF1R treated mouse tumor. Scale bar, 20 μm. Quantification of mean fluorescence intensity of *Cxcl2* in control or anti-CSF1R treated mouse liver (right panel). **(D)** Percentage of $CD11c^{Dim}F4/80^{-}CD11b^{+}Gr\text{-}1^{+}$MDSCs of $CD45^{+}$ cells in WT mouse normal liver, tumor adjacent liver or tumor, ($n \geq 8$). Data represent mean ± SD. Unpaired Student's t test (B and C) and one-way ANOVA with Bonferroni *post hoc* test (D) were used. $^{*}$, $P < 0.05$; $^{**}$, $P < 0.01$; $^{***}$, $P < 0.001$.

**Figure S6**



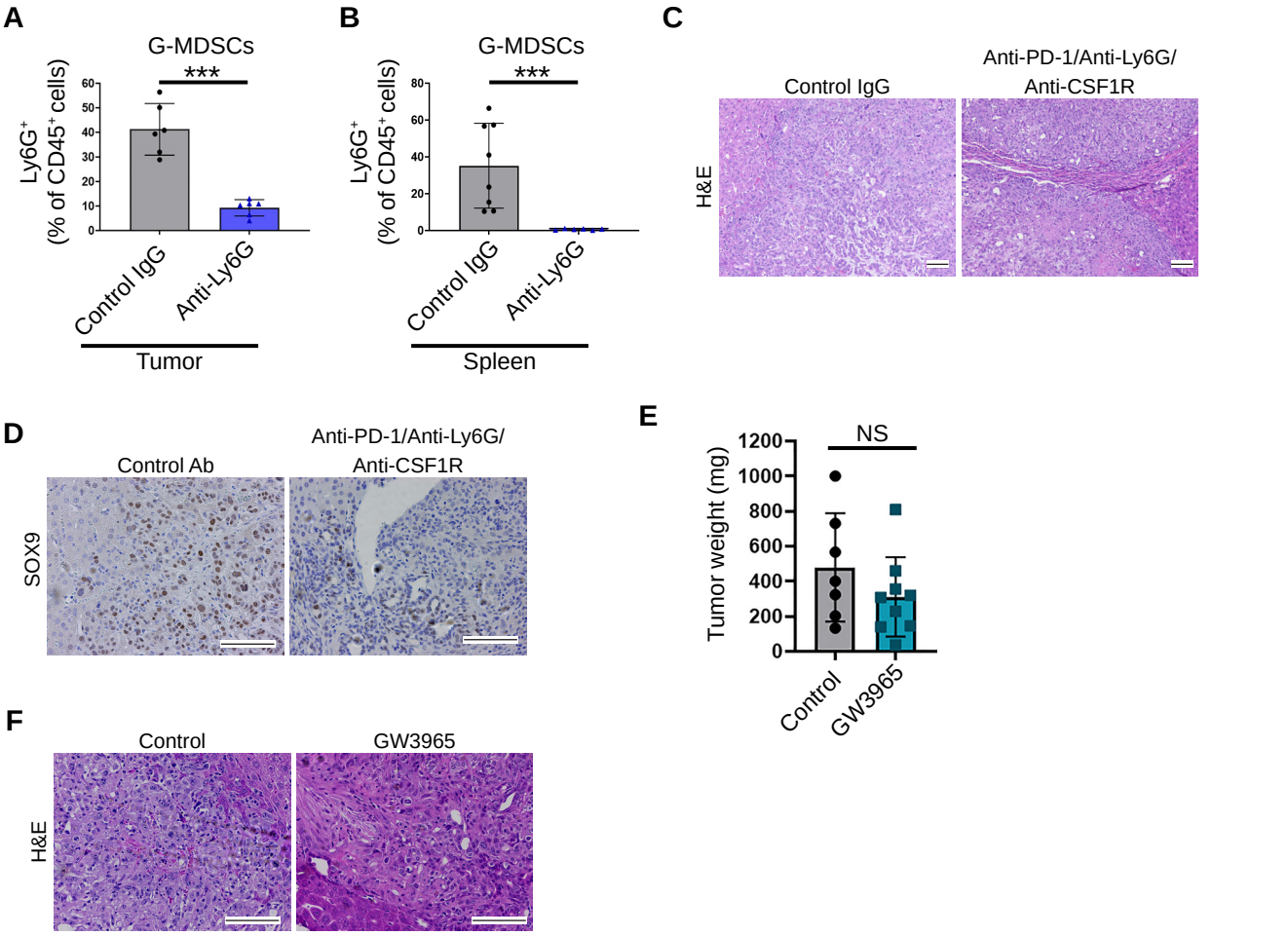

**Supplementary Figure S6.** Single cell transcriptomics analysis of murine tumors and human CCA scRNA-Seq dataset. **(A-B)** Tumor growth of 28 days after orthotopic implantation of $1x10^6$ SB (murine CCA) cells in WT mice. Mice were treated from day 14 to day 28 after implantation with control rat IgG isotype or anti-CSF1R (AFS98). **(A)** Bar plot of percentage of cells in the two clusters for WT mice samples treated with the control IgG or anti-CSF1R. Cell percentage ratio was calculated for each cluster between control and anti-CSF1R group. Fisher's exact test was used to examine the significance. **(B)** Heatmap of expression levels for selective marker genes for MDSCs, neutrophil and monocytes. **(C)** tSNE clustering results for human iCCA data (44). Cells are colored by predicted cell types from the original publication by Ma et al. **(D)** Expression abundance of *Apoe* for human CCA data. Cells with high expression level of *Apoe* are highlighted in red**.**

**Figure S7**




**Supplementary Figure S7.** G-MDSC subsets with survival and immunosuppressive properties. **(A-B)** 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)** Heatmap of gene expression profiles for top differentially expressed genes between control and anti-CSF1R sample for cells in the Classic G-MDSC subset (n=20 for up-regulated genes and n=20 for down-regulated genes in treatment sample). Expression values for each gene was z scored across all cells. **(B)** Heatmap of gene expression profiles for top differentially expressed genes between control and anti-CSF1R sample for cells in the ApoE G-MDSC subset (n=20 for up-regulated genes and n=20 for down-regulated genes in treatment sample). Expression values for each gene was z scored across all cells. **(C-F)** Multiplexed images show various immune cell populations in FFPE tissues from human CCA. Pseudo-colored raw ion images representing the markers of immune cells detected in the region of interest. **(C)** 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. Scale bar, 100 µm. **(D)** Pan-keratin (green), a CCA marker; CD3 (red), lymphocyte marker; CD11b (blue), myeloid cell marker. Scale bar, 100 µm. **(E)** Pan-keratin (green), a CCA marker; CD11b (blue), myeloid cell marker; and PD-L1 (red). Scale bar, 100 µm. **(F)** Pan-keratin (green), a CCA marker; CD8 (red), a CTL marker; and PD-1 (blue). Scale bar, 100 µm.

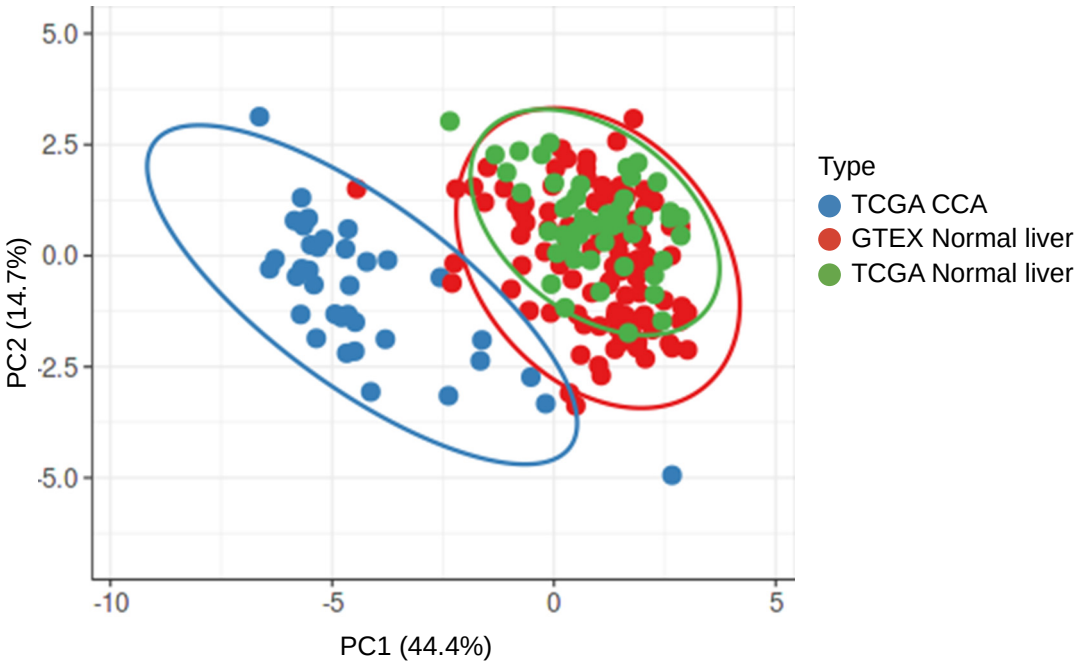
**Figure S8**



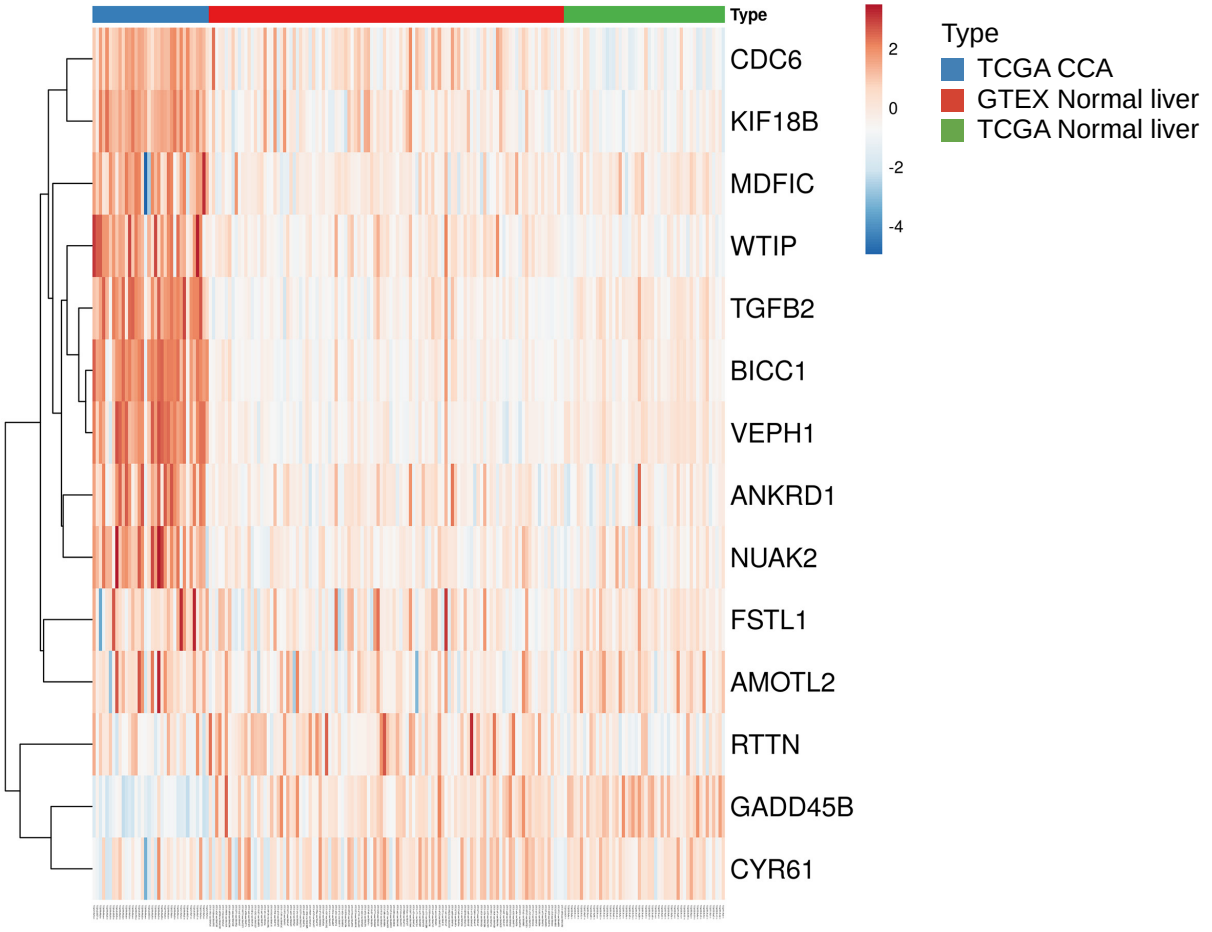

**Supplementary Figure S8.** Dual inhibition of G-MDSCs and TAMs potentiates anti-PD-1 therapy. **(A-F)** Tumor growth of 28 days after orthotopic implantation of $1x10^6$ SB (murine CCA) cells in WT mice. **(A)** Percentage of $CD11c^{Dim}F4/80^-CD11b^+Ly6G^+$ G-MDSCs of $CD45^+$ cells in tumors from WT mice treated with control rat IgG isotype or anti-Ly6G, (n=6). **(B)** Percentage of $CD11c^{Dim}F4/80^-CD11b^+Ly6G^+$ G-MDSCs of $CD45^+$ cells in spleens from WT mice treated with control rat IgG isotype or anti-Ly6G, (n=6). **(C)** Representative photomicrographs of hematoxylin and eosin-stained SB tumor sections from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G. Scale bar, 50 µm. **(D)** Representative photomicrographs of SOX9 IHC of SB tumor sections from WT mice treated with control IgG isotype or anti-PD-1+anti-CSF1R+anti-Ly6G. Scale bar, 50 µm. **(E)** Average tumor weights in milligrams (mg) of WT mice treated with control IgG isotype or GW3965. **(F)** Representative photomicrographs of hematoxylin and eosin-stained SB tumor sections from WT mice treated with control IgG isotype or GW3965. Scale bar, 50 µm. Data represent mean ± SD. Unpaired Student's t test was used. $^{***}$, $P < 0.001$; NS, non-significant.

**Figure S9**

**A**




**B**




**Supplementary Figure S9.** YAP is activated in human CCA. **(A)** Principle component analysis (PCA) comparing YAP signature genes across three cohorts: normal liver samples fromGenotype-Tissue Expression (GTEx) database, adjacent liver samples from The Cancer Genome Atlas (TCGA) database, and primary tumor samples from TCGA-CHOL. The expression data being compared are log2 RSEM normalized values. **(B)** Heatmap of the YAP signature genes for normal liver GTEx, adjacent liver TCGA, and primary tumor TCGA-CHOL samples.

## TABLES

**Supplementary Table S1. Top conserved genes in cluster 0 (Classic G-MDSCs) of murine tumor scRNA-seq dataset.**

| Gene name | Description | p value | Fold change (Log2) |
|---|---|---|---|
| S100a8 | S100 calcium binding protein A8 (calgranulin A) | 2.437E-20 | 1.976 |
| S100a9 | S100 calcium binding protein A9 (calgranulin B) | 2.571E-18 | 1.854 |
| Ccl3 | chemokine (C-C motif) ligand 3 | 4.355E-05 | 1.700 |
| Hcar2 | hydroxycarboxylic acid receptor 2 | 4.398E-15 | 1.599 |
| Ifitm1 | interferon induced transmembrane protein 1 | 3.996E-13 | 1.303 |
| Ccrl2 | chemokine (C-C motif) receptor-like 2 | 1.290E-09 | 1.237 |
| Vps37b | vacuolar protein sorting 37B | 1.306E-16 | 1.231 |
| Il1r2 | interleukin 1 receptor, type II | 1.198E-24 | 1.172 |
| Hdc | histidine decarboxylase | 2.862E-20 | 1.143 |
| S100a11 | S100 calcium binding protein A11 | 6.871E-21 | 1.086 |
| Ccr1 | chemokine (C-C motif) receptor 1 | 6.398E-24 | 1.071 |
| Cxcl2 | chemokine (C-X-C motif) ligand 2 | 7.460E-10 | 1.065 |
| Msrb1 | methionine sulfoxide reductase B1 | 1.085E-18 | 1.060 |
| Samsn1 | SAM domain, SH3 domain and nuclear localization signals, 1 | 5.472E-19 | 1.057 |
| Mxd1 | MAX dimerization protein 1 | 1.704E-15 | 1.044 |
| Tnfaip2 | tumor necrosis factor, alpha-induced protein 2 | 5.679E-14 | 1.041 |
| Acod1 | aconitate decarboxylase 1 | 2.566E-06 | 1.013 |
| Clec4d | C-type lectin domain family 4, member d | 3.165E-12 | 0.976 |
| Stfa2l1 | stefin A2 like 1 | 2.552E-03 | 0.968 |
| Pnrc1 | proline-rich nuclear receptor coactivator 1 | 7.669E-20 | 0.967 |
| Slc7a11 | solute carrier family 7, member 11 | 3.471E-06 | 0.958 |
| Btg2 | B cell translocation gene 2, anti-proliferative | 1.211E-22 | 0.955 |
| Trem1 | triggering receptor expressed on myeloid cells 1 | 5.704E-18 | 0.951 |
| Srgn | serglycin | 8.020E-26 | 0.947 |
| Clec4e | C-type lectin domain family 4, member e | 5.363E-09 | 0.937 |
| Csf3r | colony stimulating factor 3 receptor (granulocyte) | 5.669E-22 | 0.935 |
| Clec4n | C-type lectin domain family 4, member n | 2.106E-13 | 0.934 |
| Spag9 | sperm associated antigen 9 | 1.517E-18 | 0.932 |
| Ppp1r15a | protein phosphatase 1, regulatory subunit 15A | 5.213E-15 | 0.919 |
| Il1b | interleukin 1 beta | 1.195E-18 | 0.916 |

**Supplementary Table S2. Top conserved genes in cluster 1 (ApoE G-MDSCs) of murine tumor scRNA-seq dataset.**

| Gene name | Description | p value | Fold change (Log2) |
|---|---|---|---|
| Apoe | apolipoprotein E | 8.754E-121 | 4.911 |
| Cd74 | CD74 antigen | 1.103E-42 | 4.305 |
| H2-Eb1 | histocompatibility 2, class II antigen E beta | 1.144E-96 | 3.783 |
| H2-Ab1 | histocompatibility 2, class II antigen A, beta 1 | 3.992E-141 | 3.771 |
| H2-Aa | histocompatibility 2, class II antigen A, alpha | 4.421E-186 | 3.591 |
| Arg1 | arginase, liver | 0.000E+00 | 2.829 |
| S100a4 | S100 calcium binding protein A4 | 1.671E-173 | 2.470 |
| Tmsb10 | thymosin, beta 10 | 1.524E-77 | 2.441 |
| Ctss | cathepsin S | 1.542E-58 | 2.279 |
| Psap | prosaposin | 8.803E-42 | 2.239 |
| Lgmn | legumain | 6.555E-116 | 2.112 |
| Npc2 | NPC intracellular cholesterol transporter 2 | 6.319E-73 | 1.899 |
| Ctsc | cathepsin C | 1.240E-108 | 1.684 |
| Pf4 | platelet factor 4 | 8.876E-221 | 1.648 |
| Mafb | v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B | 0.000E+00 | 1.642 |
| Fcgr2b | Fc receptor, IgG, low affinity IIb | 1.009E-137 | 1.526 |
| Prdx1 | peroxiredoxin 1 | 8.202E-54 | 1.513 |
| Grn | granulin | 4.511E-48 | 1.422 |
| Fabp5 | fatty acid binding protein 5, epidermal | 5.831E-13 | 1.414 |
| Ctsb | cathepsin B | 3.282E-25 | 1.392 |
| Ccr2 | chemokine (C-C motif) receptor 2 | 0.000E+00 | 1.391 |
| Npm1 | nucleophosmin 1 | 1.464E-59 | 1.365 |
| Spp1 | secreted phosphoprotein 1 | 4.879E-08 | 1.353 |
| Tmem176b | transmembrane protein 176B | 0.000E+00 | 1.337 |
| Thbs1 | thrombospondin 1 | 2.883E-24 | 1.317 |
| Hsp90ab1 | heat shock protein 90 alpha (cytosolic), class B member 1 | 1.546E-36 | 1.296 |
| Ifi27l2a | interferon, alpha-inducible protein 27 like 2A | 2.886E-44 | 1.283 |
| Ly86 | lymphocyte antigen 86 | 0.000E+00 | 1.277 |
| Ctsh | cathepsin H | 1.910E-52 | 1.259 |
| Aif1 | allograft inflammatory factor 1 | 4.932E-269 | 1.241 |
| Ccl9 | chemokine (C-C motif) ligand 9 | 4.906E-94 | 1.211 |
| Trem2 | triggering receptor expressed on myeloid cells 2 | 1.909E-265 | 1.149 |

**Supplementary Table S3. Human MDSC signature genes.**

| Human gene name | Description |
|---|---|
| Ccl2 | C-C Motif Chemokine Ligand 2 |
| Ccl5 | C-C Motif Chemokine Ligand 5 |
| Ccr2 | C-C Motif Chemokine Receptor 2 |
| Cd14 | CD14 Antigen |
| Cd274 | Programmed Cell Death 1 Ligand 1 |
| Cd33 | CD33 Antigen (Gp67) |
| Cd34 | Hematopoietic Progenitor Cell Antigen CD34 |
| Cd38 | CD38 Antigen (P45) |
| Cd80 | CD80 Antigen (CD28 Antigen Ligand 1, B7-1 Antigen) |
| Csf1 | Colony Stimulating Factor 1 (Macrophage) |
| Csf2 | Colony Stimulating Factor 2 (Granulocyte-Macrophage) |
| Csf3 | Colony Stimulating Factor 3 (Granulocyte) |
| Cxcl1 | C-X-C Motif Chemokine Ligand 1 |
| Cxcl12 | C-X-C Motif Chemokine Ligand 12 |
| Cxcl2 | C-X-C Motif Chemokine Ligand 2 |
| Cxcl5 | C-X-C Motif Chemokine Ligand 5 |
| Cxcr2 | C-X-C Motif Chemokine Receptor 2 |
| Cxcr4 | C-X-C Motif Chemokine Receptor 4 |
| Entpd1 | Ectonucleoside Triphosphate Diphosphohydrolase 1 |
| Foxp3 | Forkhead Box Protein P3 |
| Ido1 | Indoleamine 2,3-Dioxygenase 1 |
| Il10 | Interleukin-10 |
| Il1b | Interleukin-1 Beta |
| Il6 | Interleukin-6 |
| Il8 | Interleukin-8 |
| Itgam | Integrin, Alpha M (Complement Component 3 Receptor 3 Subunit) |
| Itgax | Integrin, Alpha X (Complement Component 3 Receptor 4 Subunit) |
| Nos2 | Nitric Oxide Synthase 2A (Inducible, Hepatocytes) |
| Pdcd1 | Programmed Cell Death 1 |
| Ptgs2 | Prostaglandin-Endoperoxide Synthase 2 |
| Ptprc | Protein Tyrosine Phosphatase Receptor Type C |
| S100a8 | S100 Calcium Binding Protein A8 |
| S100a9 | S100 Calcium Binding Protein A9 |
| Stat1 | Signal Transducer And Activator Of Transcription 1 |
| Stat3 | Signal Transducer And Activator Of Transcription 3 |
| Stat5a | Signal Transducer And Activator Of Transcription 5A |
| Tgfb1 | Transforming Growth Factor Beta 1 |
| Tlr3 | Toll-Like Receptor 3 |
| Tlr4 | Toll-Like Receptor 4 |
| Tnf | Tumor Necrosis Factor |

**Supplementary Table S4. Human equivalent of ApoE G-MDSC signature genes from murine tumor scRNA seq dataset.**

| Mouse gene name | Human gene name | Description |
|---|---|---|
| Aif1 | **Aif1** | allograft inflammatory factor 1 |
| Apoe | **Apoe** | apolipoprotein E |
| Arg1 | **Arg1** | arginase, liver |
| Ccl9 | **ccl15** | chemokine (C-C motif) ligand 9 |
| Ccl6 | **Ccl23** | chemokine (C-C motif) ligand 6 |
| Ccr2 | **Ccr2** | chemokine (C-C motif) receptor 2 |
| Cd74 | **Cd74** | CD74 antigen |
| Ctsb | **Ctsb** | cathepsin B |
| Ctsc | **Ctsc** | cathepsin C |
| Ctsh | **Ctsh** | cathepsin H |
| Ctss | **Ctss** | cathepsin S |
| Cxcl16 | **Cxcl16** | chemokine (C-X-C motif) ligand 16 |
| Emp3 | **Emp3** | epithelial membrane protein 3 |
| Fabp5 | **Fabp5** | fatty acid binding protein 5, epidermal |
| Fcgr2b | **Fcgr2b** | Fc receptor, IgG, low affinity IIb |
| Grn | **Grn** | granulin |
| H2-Aa | **HLA-DQA1** | histocompatibility 2, class II antigen A, alpha |
| H2-Ab1 | **HLA-DQB1** | histocompatibility 2, class II antigen A, beta 1 |
| H2-Eb1 | **HLA-DRB5** | histocompatibility 2, class II antigen E beta |
| Hsp90ab1 | **Hsp90ab1** | heat shock protein 90 alpha (cytosolic), class B member 1 |
| Ifi27l2a | **IFI27** | interferon, alpha-inducible protein 27 like 2A |
| Lgmn | **Lgmn** | legumain |
| Ly86 | **Ly86** | lymphocyte antigen 86 |
| Mafb | **Mafb** | v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B |
| Msr1 | **Msr1** | macrophage scavenger receptor 1 |
| Npc2 | **Npc2** | NPC intracellular cholesterol transporter 2 |
| Npm1 | **Npm1** | nucleophosmin 1 |
| Pf4 | **Pf4** | platelet factor 4 |
| Pltp | **Pltp** | phospholipid transfer protein |
| Prdx1 | **Prdx1** | peroxiredoxin 1 |
| Psap | **Psap** | prosaposin |
| Pycard | **Pycard** | PYD and CARD domain containing |
| S100a4 | **S100a4** | S100 calcium binding protein A4 |
| Sgk1 | **Sgk1** | serum/glucocorticoid regulated kinase 1 |
| Spp1 | **Spp1** | secreted phosphoprotein 1 |
| Thbs1 | **Thbs1** | thrombospondin 1 |
| Tmem176b | **Tmem176b** | transmembrane protein 176B |
| Tmsb10 | **Tmsb10** | thymosin, beta 10 |
| Tpt1 | **Tpt1** | tumor protein, translationally-controlled 1 |
| Trem2 | **Trem2** | triggering receptor expressed on myeloid cells 2 |

**Supplementary Table S5. CyTOF antibody panel for murine CCA tumor immunophenotyping.**

| No. | Species Reactivity | Label | Target | Clone | Manufacturer |
|---|---|---|---|---|---|
| 1 | Ms | 150Nd | I-A/I-E | M5/114.15.2 | Biolegend |
| 2 | Ms | 143Nd | TCRb | H57-597 | Fluidigm |
| 3 | Ms | 144Nd | MHC Class I | 28-14-8 | Fluidigm |
| 4 | Ms | 152Sm | CD3e | 145-2C11 | Fluidigm |
| 5 | Ms | 161Dy | Ly6G | 1A8 | Biolegend |
| 6 | Ms | 164Dy | CX3CR1 | SA011F11 | Fluidigm |
| 7 | Ms | 168Er | CD8a | 53-6.7 | Fluidigm |
| 8 | Ms | 172Yb | CD11b (Mac-1) | M1/70 | Fluidigm |
| 9 | Ms | 175Lu | Ly6C | HK1.4 | Biolegend |
| 10 | Ms | 089Y | CD45 | 30-F11 | Fluidigm |
| 11 | Ms | 166Er | CD19 | 6D5 | Fluidigm |
| 12 | Ms | 142Nd | CD11c | N418 | Fluidigm |
| 13 | Ms | 154Sm | TER-119 | TER-119 | Fluidigm |
| 14 | Ms | 156Gd | CCR2 | 475301 | Novus Biologicals |
| 15 | Ms | 159Tb | F4/80 | BM8 | Fluidigm |
| 16 | Ms | 170Er | CD161 (NK1.1) | PK136 | Fluidigm |
| 17 | Ms | 174Yb | CD115/CSF1R | AFS98 | Fluidigm |
| 18 | Ms | 176Yb | CD45R (B220) | RA3-6B2 | Fluidigm |
| 19 | Ms | 141Pr | Lgals3 | 202213 | Fluidigm |
| 20 | Ms | 151Eu | CD206 (MMR) | C068C2 | Biolegend |
| 21 | Ms | 155Gd | MERTK | 108928 | R&D Systems |
| 22 | Ms | 160Gd | CD64 | 290322 | R&D Systems |
| 23 | Ms | 165Ho | CD14 | Sa14-2 | Biolegend |
| 24 | Ms | 149Sm | Tim4 | 370901 | Biolegend |

**Supplementary Table S6. Hyperion imaging mass cytometry staining panel for human CCA**

| *No.* | *Target Name* | *Metal Tag* | *Clone* | *Dilution Factor* |
|---|---|---|---|---|
| **1** | Alpha-SMA | 141Pr | 1A4 | 200 |
| **2** | CD19 | 142Nd | 6OMP31 | 400 |
| **3** | Vimentin | 143Nd | D21H3 | 100 |
| **4** | CD14 | 144Nd | EPR3653 | 200 |
| **5** | CD16 | 146Nd | EPR16784 | 100 |
| **6** | Pan-Keratin | 148Nd | C11 | 200 |
| **7** | CD11b | 149Sm | EPR16784 | 100 |
| **8** | CD45 | 152Sm | 2B11 | 100 |
| **9** | CD11c | 154Sm | Polyclonal | 50 |
| **10** | FoxP3 | 155Gd | 236A/E7 | 50 |
| **11** | CD4 | 156Gd | EPR6855 | 400 |
| **12** | CD68 | 159Tb | KP1 | 100 |
| **13** | Vista | 160Gb | D1L2G | 50 |
| **14** | CD20** | 161Dy | H1 | 800 |
| **15** | CD8a | 162Dy | C8/144B | 100 |
| **16** | CD45RA | 166Er | HI100 | 100 |
| **17** | Granzyme B | 167Er | EPR20129-217 | 50 |
| **18** | Collagen type 1 | 169Tm | Polyclonal | 600 |
| **19** | CD3 | 170Er | Polyclonal | 100 |
| **20** | CD45RO | 173Yb | UCHL1 | 50 |
| **21** | HLA-DR | 174Yb | YE2/36 HLK | 50 |
| **23** | PD-1 | 165Ho | EPR4877 (2) | 50 |
| **24** | PD-L1 | 150Nd | E1L3N | 50 |
| **25** | CD15 | 149Sm | W6D3 | 50 |