Appendix 1

Supplemental Experimental Procedures:

Cell culture

All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. The murine LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre (KPC) cell line derived from a pancreatic tumour of a KPC mouse was a kind gift from Dr. A. Neesse, University of Goettingen, Germany. KPC cells were cultured in DMEM (Life Technologies) containing 10% FBS (PAN). Bone marrow-derived macrophages (BMM) were isolated from femurs and tibiae of *C57BL/6* mice. Bone marrow cells were flushed with RPMI 1640 (Life Technologies), treated with erythrocyte lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) and differentiated for 6d in RPMI 1640 containing 5% FBS and 20 ng/ml of murine M-CSF (Tebu-Bio) to obtain BMM. Differentiated BMM were cultured in RPMI 1640 containing 5% FBS and 5 ng/ml murine M-CSF. Cell purity (99%) was determined by flow cytometry using CD11b and F4/80. $CD4^+CD25^+$ regulatory T cells were isolated from the spleen of *C57BL/6* mice using the $CD4^+CD25^+$ Regulatory T cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol and cultured in RPMI 1640 (Life Technologies) supplemented with 10%FCS and 2mM L-Glutamine (Life Technologies).

Stimulation of bone marrow-derived macrophages and $CD4^+CD25^+$ T cells

Bone marrow-derived macrophages (BMM) were plated at $1x10^6$ cells per well in 6-well plates and polarized either to M1 with 10 ng/ml LPS (Sigma-Aldrich), 10 ng/ml IFN-γ (Tebu-Bio) and LPS plus IFN-γ or to M2 with 10 ng/ml IL-4 (Tebu-Bio) for 4h. $CD4^+CD25^+$ purified T cells at a final concentration of 3-5 x 10^5 cells/ml were activated for 4 days in complete medium plus soluble anti-CD28 (1µg/ml; eBioscience) on anti-CD3ε (5µg/ml; eBioscience) coated 96 well flat-bottomed plates.

Cell viability assay

Differentiated BMM ($1x10^4$ cells per well of a 96 well plate, plated in triplicate) were polarized to M1 or M2 with the corresponding cytokines for 4h and subsequently exposed to Clodronate-liposomes (1mg/ml) or PBS-liposomes (equivalent volume) for 24h. Activated $CD4^+CD25^+$ T cells plated in triplicates were exposed to Clodronate-liposomes (1mg/ml) or PBS-liposomes (equivalent volume) for 24h. Viability was analyzed using CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturers instructions. Results were reported as relative light units (RLU).

Phagocytosis assay

For phagocytosis of FITC-conjugated latex beads (Sigma-Aldrich), beads were washed and dissolved in culture media. Polarized M1 and M2 macrophages ($5x10^5$/well in a 6-well plate) were incubated with FITC-conjugated latex beads (30 beads/cell) for 60 and 120 minutes at 37°C or at 4°C as negative control. After incubation cells were washed four times with cold PBS to remove non-internalized beads and harvested in PBS containing 1% fetal bovine serum. FITC-positive macrophages were analysed by flow cytometry using the LSRFortessa scanner (BD Bioscience). A total of 10.000 events per sample were acquired and analyzed using FlowJo software. Phagocytosis was normalized to macrophages incubated with FITC-conjugated latex beads at 4°C.

Western blot

Cell pellets or snap frozen tissue pieces were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate and 1% Triton X-100]

supplemented with protease inhibitor cocktail (Complete, Roche Applied Science). Serum samples were diluted in PBS. Protein concentrations were determined with Coomassie brilliant blue (Thermo Scientific). Protein samples (10-20µg of total protein per lane) were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore). After blocking immunoblots were probed with primary antibodies against VEGF (Abcam), ALB (P-20, SantaCruz), PARP (Cell Signaling) and beta-actin (Sigma-Aldrich) followed by incubation with peroxidase-conjugated secondary antibodies obtained from Amersham. Blots detected by Western ECL Substrate (BioRad) and bands were quantified by densitometry using Image J software. Relative band intensities were expressed as arbitrary units and normalized to corresponding actin density.

Lung metastasis model

C57BL/6 mice pretreated twice with clodronate- or PBS-liposomes were injected with $2x10^5$ syngeneic KPC cells in 100µl PBS in the lateral tail vein. After application, animals treated for another three weeks with clodronate- or PBS-liposomes twice per week.

Quantitative real-time PCR (qPCR)

Total RNA from liver, lung and bone marrow-derived macrophages (BMM) was isolated using peqGold Total RNA Kit (Peqlab). Total RNA from $CD8^+$ cells was isolated using the Qiazol Lysis Reagent with miRNeasy Mini Kit (Qiagen) according to manufacturers protocol. RNA was reverse transcribed with the Omniscript RT Kit (Qiagen). qPCR reactions using the iTaq Universal SYBR Green Supermix (BioRad) were done in triplicate and measured with the ABI PRISM 7500 Sequence Detector System (Applied Biosystems). Relative quantitation values were normalised to

endogenous ribosomal protein RPLP0 using the comparative Ct method. The following murine qRT-PCR primer sequences were used: RPLP0 (5'-tgggcaagaacaccatgatg-3' and 5'-agtttctccagagctgggttgt-3'), F4/80 (5'-ctttggctatgggcttccagtc-3' and 5'-gcaaggaggacagagtttatcgtg-3'), Mrc1 (5'-gaatacacagcactagcgtcttaacac-3' and 5'-agtggcttacgtggttgtttctaga-3'), Ym1 (5'-caggccaatagaagggagttt-3' and 5'-tgctcatgtgtgtaagtgatct-3'), Cxcl10 (5'-agctcaggctcgtcagttctaag-3' and 5'-gggaagatggtggttaagttcgt-3'), Tnfα (5'-catcttctcaaaattcgagtgacaa-3' and 5'-tgggagtagacaaggtacaaccc-3'), Cd69 (5'-aggacatgacgtttctgaagc-3' and 5'-gctgttaaattctttgccatttg-3'), Ctla4 (5'-aatgcctcattcctgagacc-3' and 5'- tcaggaactcttgtgcatcc-3'), Perforin (5'-gagaagacctatcaggacca-3' and 5'-agcctgtggtaagcatg-3') and Gzm-B (5'-cgaccctacatggccttact-3' and 5'-cagcacaaagtcctctcgaa-3').

Flow Cytometry

Animals anesthetized and blood was collected from the *retrobulbar venous plexus* using glass capillary tubes (Fisher Scientific) and mixed with EDTA (10 mM).

For harvest of organ macrophages, mice were sacrificed and transcardially perfused with HBSS followed by 1mg/ml collagenase type IV (Sigma-Aldrich) in RPMI 1650. Cellular suspensions from EDTA-blood, livers, spleens and pancreata were prepared as follows: Spleens and pancreata washed in HBSS, injected with 2.5 ml collagenase solution, minced and digested at 37°C. Cells were suspended, passed through a 70µm strainer and treated with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Liver tissue was dissociated and cells separated by differential centrifugation using a Percoll gradient as described.[1] Blood was treated with erythrocyte lysis buffer and centrifuged. Clear pellets were pooled in PBS and 1% FBS. Single-cell suspensions from blood, liver, spleen and pancreas were

blocked with CD16/CD32 antibody (Fc Block, BD Biosciences) for 5 min at 4°C and subsequently labeled with antibodies against: CD45 (30F11), CD3ε (500A2), CD8a (53-6.7) (from BD Biosciences), CD4 (RM4-5; eBioscience), CD11b (M1/70; Miltenyi Biotec), Gr1 (RB6-8C5; AbD Serotec), CD25 (PC61) and F4/80(BM8) (from BioLegend) for 30 minutes at 4°C. Staining with DAPI (Sigma-Aldrich) was performed to exclude dead cells. Flow cytometry data were analyzed using FlowJo software.

Histology and Immunhistochemistry

Mouse pancreata, livers and lungs were fixed in 3.5% formalin solution (Fisher) and processed for paraffin embedding. Histology and metastases screening was examined on 4µm sections by H&E. Livers und lungs of treated tumour-bearing animals were screened for metastases in a blinded manner analyzing three distant sections of each organ. Whole lung sections following tail vein injection experiments were scanned using a transmitted light scanner and number and size of metastases for each section was quantified using Axio Vision 4 software (Carl Zeiss).

For CD31 and CD68 immunohistochemistry, deparaffinized sections (4µm) were rehydrated and quenched in 3% hydrogen peroxide/methanol. After antigen retrieval (10mM citric acid monohydrate (pH 6.0) in a steamer) sections were incubated with the primary antibodies CD31 (SZ31, Dianova; 1:30) or CD68 (FA-11, Abcam). After incubation with secondary biotinylated antibody and ABC solution (all from Vector Laboratories) sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Carl Roth) and counterstained with hematoxylin. For each section, staining was evaluated using 10 random fields at × 400 magnification. The number of CD31 positive vessels was counted on every single field and the average was calculated and expressed as vessels per high-power field (HPF). In case of CD68, all cells on a

single field were counted and the average of CD68 positive cells was expressed as percentage per HPF.

Statistical Analyses

Data are presented as mean ± standard deviations (SD). All statistical analyses were performed by Mann-Whitney-U-Test using GraphPad Prism software. The overall survival analysis using Kaplan-Meier curves were analysed by log rank test. Metastases incidence analysed using Fisher's exact test. For all tests, a P value of <0.05 was considered statistically significant.

Supplemental Reference:

1. Froh M, Konno A, Thurman RG. Isolation of liver Kupffer cells. Curr Protoc Toxicol 2003;Chapter 14:Unit14 4.