Supplementary Materials

Materials and Methods

Patients. Study included 25 consecutive patients with HCC not curable by locoregional therapy, who received single agent sorafenib after at least one prior dose of ICB. The ICB treatment was nivolumab in 17 patients (68%), pembrolizumab in 3 cases (12%), and other PD-1 antibodies in the other 5 (20%). The observational study was approved by the Institutional Review Board of The University of Hong Kong Hospital Authority/Hong Kong West Cluster and did not require patient's written consent. Tumor responses were assessed according to the Response Evaluation Criteria in Solid Organ Tumor 1.1 (RECIST 1.1). Objective response rate (ORR) was defined as the proportion of patients with complete (CR) or partial response (PR). In general, reassessment imaging was planned every 8-12 weeks after treatment commencement. Overall survival (OS) was calculated from the date of first dose to death or censored at last follow-up for patients alive at data cut-off. Kaplan-Meier analysis was used to estimate median OS.

Cells. The murine HCC cell lines used in this study were RIL-175 (a *p53*-null/*Hras*-mutant murine HCC cell line from C57Bl/6 mouse background), a kind gift from Dr. Tim F. Greten (National Institutes of Health), and HCA-1, established from a spontaneous HCC in a C3H mouse in our laboratory[15,16].

Orthotopic HCC mouse model with liver fibrosis and treatments. To establish orthotopic HCC mouse model with liver damage, RIL-175 cells were implanted to male C57Bl/6 mice and HCA-1 cells to male C3H mice, after 6-8 weeks of CCL4 treatment. To establish autochthonous HCC mouse model with liver damage, we induced hepatocarcinogenesis by i.v. injection of a Cre-adenovirus in 4-week-old male *Mst*1$^{-/-}$*Mst*2$^{f/-}$ mice, and then induced liver fibrosis with CCl4 treatment for 12 weeks. When largest tumors reached ~5mm in diameter (after 12-16 weeks), mice were randomly

assigned to a treatment group. Tumor growth was monitored by high-frequency ultrasound imaging. Mouse anti-PD-1 antibody (clone RMP-014) was purchased from BioXcell Therapeutics (New Haven, CT) and mouse IgG isotype control was purchased from Thermo Fischer Scientific (Waltham, MA). Anti-mouse PD1 antibody, IgG (control) and anti-CD8 depleting antibody were administrated intraperitoneally (i.p.) at doses of 10mg/kg diluted in PBS thrice weekly. For the survival studies, moribund status was used as the endpoint. Sorafenib and regorafenib (Bayer, Germany) were administered orally at a daily dose of 40mg/kg and 10mg/kg, respectively, dissolved in 12.5% cremophor, 12.5% ethanol and 75% water. Ultrasonography was performed under isoflurane induced anesthesia. Briefly, animals were induced under 5% isoflurane and maintained under 1.5% isoflurane during imaging. Surgery was performed under anesthesia using subcutaneous injection of ketamine (90mg/kg per body weight) and xylazine (9mg/kg per body weight) in PBS. For analgesia, buprenorphine 0.05-0.1 mg/kg was given by subcutaneous injection 20 min before the anesthesia/surgery and then every 8-12 hr for 72 hr. All mouse studies were performed in accord with institution guidelines after receiving prior approval for the protocol from the Massachusetts General Hospital IACUC.

Flow cytometry analysis. Prior to immunostaining, cells were washed with the buffer and fixed and permeabilized with Transcription Factor Staining Buffer Set (eBioscience/Thermo Fischer Scientific) to stain the intracellular markers. Harvested cells were incubated in RPMI1640 with cell activation cocktail with Leukocyte Activation Cocktail, with BD GolgiPlug (BD Pharmingen) for 5 hr at 37°C. Cells were stained with the antibodies of cell markers in this buffer.

Anti-mouse CD16/32 antibody (clone 93, Biolegend, San Diego, CA) was added for FcR blockade, and incubated for 5 min at room temperature. After another washing step, antibodies for cell phenotyping were added, and cells were incubated for 40 min at 4°C.

Immunohistochemistry. For analyses of endothelial and perivascular cells, we used antibodies against CD31 (Armenian Hamster monoclonal antibody, clone 2H8, Millipore) and α-SMA (Cy3-conjugated, mouse monoclonal antibody, clone 1A4, Sigma), respectively. $CD4^+$ or $CD8^+$ T-cells were detected by immunostaining using anti-CD4 antibody (rat monoclonal, clone RM4-5, BD Pharmingen) and anti-CD8 antibody (rabbit monoclonal, clone YTS 105.18, Biorbyt), respectively. Positive cells were counted from five random fields for each HCC tissue section.

Supplementary Figure Legends

Supplementary Figure 1. Overall survival (OS) and objective response rate (ORR) in a cohort of patients who received tyrosine kinase inhibitors after prior anti-PD-1 therapy and preclinical study design. (**A**) Median OS and ORR after multikinase therapy in ICB-primed cohort; data are compared to results for the sorafenib treatment arm in the randomized phase III trials Asia-Pacific and SHARP. (**B**) Median OS distribution in HCC patients treated with sorafenib after initially receiving off-label treatment with nivolumab (n=17), pembrolizumab (n=3) or other anti-PD-1 therapy (n=5). All 25 patients had Child-Pugh A cirrhosis and were treated at Hong Kong University. (**C**) Mice were randomized into 4 treatment groups. (**D**) Timeline of treatments: After 7 days of anti-PD1 antibody (aPD-1) therapy or IgG (i.p.), oral treatment with a tyrosine kinase inhibitor (sorafenib or regorafenib) or control was initiated from day 8. For time matched studies, all mice were sacrificed on day 13. (**E**) Mice were randomized into 3 treatment groups. (**F**) Timeline of treatments: After 7 days of aPD-1 therapy (i.p.), sorafenib treatment or control was initiated from day 8 (PS and C groups). Anti-CD8 depleting antibodies (i.p., on days 8, 11, 14, 17) were administrated concomitantly with sorafenib in PDS group. For time matched studies, all mice were sacrificed on day 13.

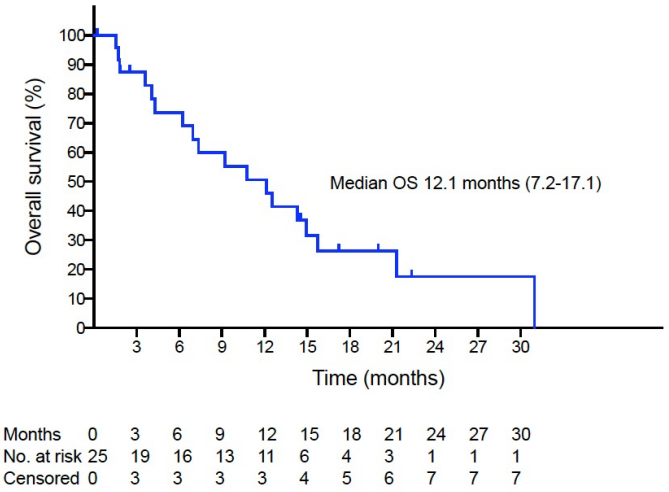
Supplementary Figure 2. Changes in overall survival and tumor growth kinetics in grafted HCC-bearing mice receiving regorafenib treatment after prior anti-PD-1 therapy, and intratumoral lymphocyte infiltration and microvascular density in autochthonous HCC model in mice receiving sorafenib after initial anti-PD-1 priming therapy. (**A**, **B**) Kaplan-Meier distributions of overall survival (**A**) and tumor growth kinetics (**B**) in orthotopic HCC-bearing mice receiving regorafenib after initial anti-PD-1 therapy (PR) compared to IgG control followed by PBS gavage (CC), IgG control followed by sorafenib (CR), and anti-PD-1 therapy followed by PBS

gavage (PC); n=7-9 mice per group. (**C**) Representative immunofluorescence (IF) for $CD4^+$ and $CD8^+$ lymphocytes in tumor tissue sections from the autochthonous HCC model (using *Mst*$1^{-/-}$ *Mst*$2^{f/-}$ mice) after treatment with sorafenib after initial anti-PD-1 therapy (PS) compared to anti-PD-1 therapy followed by PBS gavage (PC). (**D, E**) The frequencies of tumor infiltrating $CD4^+$ cells (**D**) and $CD8^+$ cells (**E**), shown as mean number of cells ± standard deviation per field (320μm x 320μm square area). (**F**) Representative IF for the endothelial marker CD31 and the pericyte marker α-SMA in tumor in tumor tissue sections from the autochthonous HCC model in the 2 treatment groups. (**G, H**) Quantification of tumor microvessel density (MVD), shown as area fraction covered by vessels per field (640μm x 640μm square area) (**G**) and mature (pericyte covered) MVD (**H**), shown as fraction of tumor vessels covered by perivascular cells. Bar, 1mm (**C**, **F**). P values were from log-rank test (**A**) and Wilcoxon rank sum test (**B**, **D**, **E**, **G**, **H**). Error bars represent standard deviations. All statistical tests were 2-sided.

A

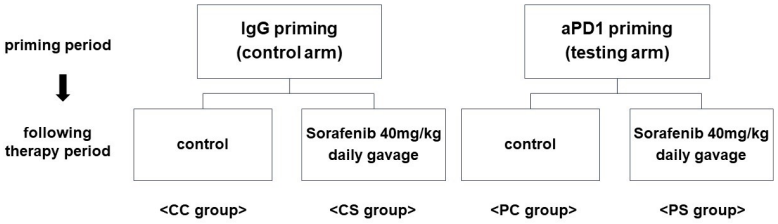
	ICB-primed Cohort (HK)	Asia-Pacific Trial	SHARP Trial
Patients (n)	25	150	299
Median OS (months, w/ 95% CI)	12.1 (7.2-17.1)	6.5 (5.6–7.6)	10.7 (9.4-13.3)
Objective Response Rate (%)	12	3	2

B



Months	0	3	6	9	12	15	18	21	24	27	30
No. at risk	25	19	16	13	11	6	4	3	1	1	1
Censored	0	3	3	3	3	4	5	6	7	7	7

C



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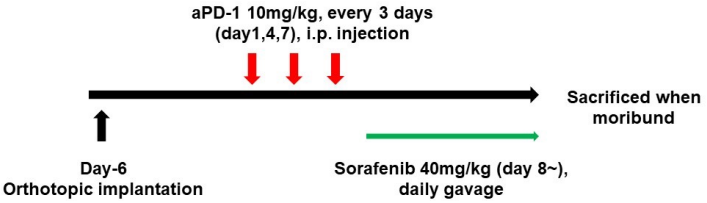

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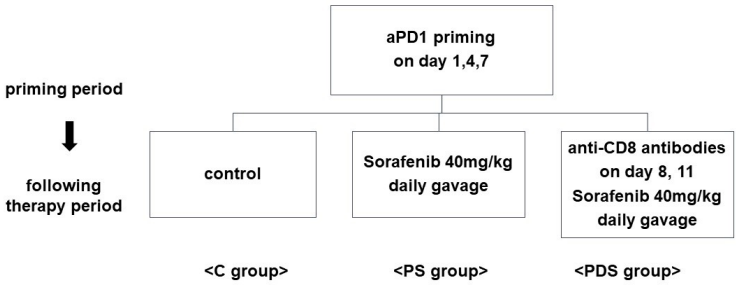

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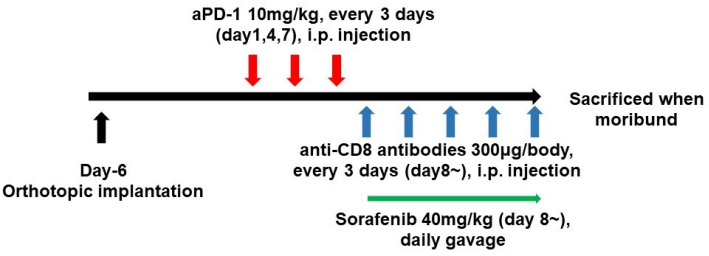
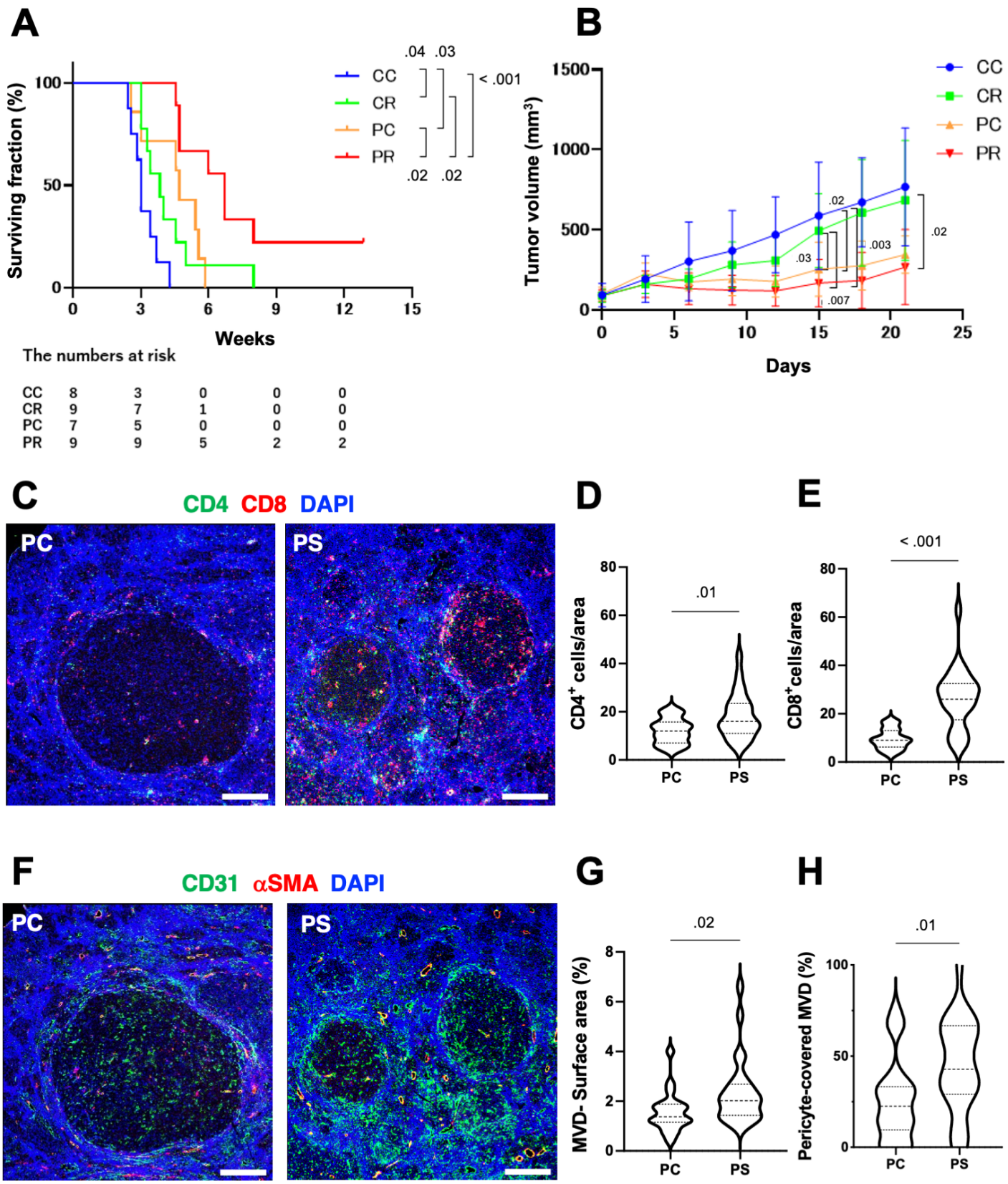


Supplementary Figure 1



Supplementary Figure 2