Supplemental information

Single tumor-initiating cells evade immune clearance by recruiting type II macrophages

Xiaocan Guo, Yang Zhao, Huan Yan, Yingcheng Yang, Shuying Shen，Xiaoming Dai, Xinyan Ji, Fubo Ji, Xing-Guo Gong, Li Li, Xueli Bai, Xin-Hua Feng, Tingbo Liang, Junfang Ji, Lei Chen, Hongyang Wang, Bin Zhao

Supplemental materials provided:

Supplemental Figures (5)

- Supplemental Figure S1. Related to Figure 1. Efficiency of hydrodynamic tail-vein injection delivery.
- Supplemental Figure S2. Related to Figure 1. The time course of liver tumorigenesis induced by hydrodynamic injection of active YAP.
- Supplemental Figure S3. Related to Figure 2. Validation of the active YAP-specific antibody.
- Supplemental Figure S4. Related to Figure 3. Depletion of $Ccr2^{+}$ cells eliminates YAP-recruited TICAMs.
- Supplemental Figure S5. Related to Figure 5. YAP-induced TICAMs protect TICs from immune clearance.

Supplemental materials and methods

Supplemental figure legends

Supplemental Figure S1. Related to Figure 1. Efficiency of hydrodynamic tail-vein injection delivery. (**A**) Hydrodynamic tail-vein injection transfects hepatocytes *in vivo*. Mice injected with *piggyBac* (*PB*) transposon plasmids expressing human YAP were sacrificed at post-injection day 1. Liver sections were stained for human YAP. A representative image of more than five mice was shown. (**B**) Hydrodynamic tail-vein injection transiently transfects about 20% of hepatocytes. Eight views similar to that in (A) were quantified by ImageJ for the ratio of human YAP positive cells in all liver cells. According to previous reports that hepatocytes constitutes about 60% of cells in the liver (Kmiec 2001), the efficiency of hepatocyte transfection was estimated.

Supplemental Figure S2. Related to Figure 1. The time course of liver tumorigenesis induced by hydrodynamic injection of active YAP. Representative livers of mice from 30 to 130 days after hydrodynamic injection of active YAP were shown. From 60 days after injection small tumors could be observed, which becomes obvious at day 90. All mice develop large tumors by 4 months.

Supplemental Figure S3. Related to Figure 2. Validation of the active YAP-specific antibody. (**A**) The active YAP-specific antibody specifically detects YAP in its dephosphorylated form. 293T cells cultured in the presence or absence of serum and 293T cells with *YAP* knockout were lysed and analysed by western blotting as indicated. The lysates were treated with lambda protein phosphatase as indicated. The experiments have been duplicated in the lab. (**B**) The active YAP-specific antibody specifically

detects active YAP in IHC applications. MCF10A cells transfected with the indicated siRNAs were fixed 72 hours after transfection. The cells were paraffin embedded and IHC stained with anti-active YAP antibody. The experiments have been duplicated in the lab. (**C**) Clonal Yap activation induced by *Lats1/2* knockout. Post-injection day 30 liver sections were stained for active Yap. A representative image was shown.

Supplemental Figure S4. Related to Figure 3. Depletion of Ccr2$^+$ cells eliminates YAP-recruited TICAMs. (**A**) Injection of DT into Ccr2 depleter mice eliminates *Ccr2*-expressing cells in a dose-dependent manner. DT of the indicated dose was intraperitoneally injected for three consecutive days. On the fourth day, the samples were collected, and the mRNAs were extracted. The *Ccr2* mRNA levels were determined by RT-PCR. Three technical replicates representative of two independent experiments. Values represent mean + s.d.. (**B**) DT injection eliminates TICAMs in Ccr2 depleter mice. Non-transgenic and Ccr2 depleter mice were hydrodynamically injected with YAP and DT treated according to the scheme in Fig. 3J. The liver sections were stained for human YAP and CD45. Images are representative for three independent experiments. (**C**) Depletion of Ccr2$^+$ cells leads to massive expansion of human YAP-expressing cells. Non-transgenic and Ccr2 depleter mice were hydrodynamically injected with YAP and DT treated. Liver sections at post-injection day 10 were stained for human YAP. Images are representative for two independent experiments.

Supplemental Figure S5. Related to Figure 5. YAP-induced TICAMs protect TICs from immune clearance. (**A**) Quantification of YAP-expressing cells in clones at

post-injection day 10 in *Rag1* knockout mice. YAP^+ cell number in clones in 3 random fields of stained liver sections were quantified. Data is presented as mean ± s.e.m. *P* value is calculated by unpaired *t*-test with Welch's correction. ***$P<0.001$ (**B**) *p53* knockout rescues TICAM-negative YAP-expressing clones. The liver sections of injected $p53^{-/-}$ mice at post-injection day 10 were stained for human YAP. Images are representative of images from five mice.

Supplemental reference

Kmiec Z. 2001. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol* **161**: III-XIII, 1-151.

Supplemental materials and methods

Antibodies, plasmids, and other materials

The anti-YAP antibody (sc-15407, for human) (Zhao et al. 2007) was obtained from Santa Cruz Biotechnology. Anti-HA (#3724) (von Roemeling et al. 2014), anti-YAP (#14074, for mouse), anti-pYAP (S127) (#13008) (Zhao et al. 2012), and anti-K19/17 (#12434) antibodies were obtained from Cell Signaling Technologies. Anti-Ki67 (M7249) (Tirnitz-Parker et al. 2010) and anti-CD68 (M0876) (Oehmichen et al. 2009) were obtained from DAKO. Anti-AFP (ab46799) was obtained from Abcam. Anti-HNF4α (H1415) was obtained from R&D Systems. Anti-CD45 (14-0451) (Syu et al. 2012), anti-F4/80 (14-4801) (Ibrahim et al. 2014), anti-CD11b (25-0112-82), anti-Gr-1 (11-5391-82), anti-Ly6C (12-5932-82), anti-NK1.1 (17-5941-81), anti-CD4 (11-0042-81), and anti-CD8α (17-0081-82) antibodies were obtained from eBioscience. The anti-TEAD1 antibody (610922) was from BD Biosciences. Anti-GAPDH (G8795) was from Sigma. Anti-β-actin (66009) was from Proteintech. The anti-active YAP antibody was generated by Abcam by immunization of rabbits with a non-phosphorylated peptide corresponding to a sequence flanking human YAP S127.

Diphtheria toxin was purchased from Sigma. Common chemicals were from Sigma or Sangon Biotech.

The *Ccl2* and *Csf1* promoter luciferase reporter plasmids were constructed by cloning the -3.5 to -1.6 kb *Ccl2* promoter region, the -1.6 to 0 kb *Ccl2* promoter region and the -2.7 to 0 kb *Csf1* promoter region into the pGL4.20-TATA box vector using the KpnI and NheI restriction sites. The Act-PB transposase and PB[Act-RFP]DS plasmids were previously described (Ding et al. 2005). The PB[CMV-myc-YAP]DS,

PB[CMV-myc-YAP-5SA]DS, and PB[CMV-myc-YAP-5SA-S94A]DS plasmids were constructed by excising Act-RFP from the PB[Act-RFP]DS plasmid and ligating the corresponding fragments excised from pQCXIH vectors. The PB[Act-Cas9]DS plasmid was constructed by excising Act-RFP from the PB[Act-RFP]DS plasmid and ligating the corresponding fragment excised from the pEP-330x vector. The PB[Act-^{myr}AKT-HA-IRES-RFP]DS, and the PB[Act-EGFR-L858R-HA-IRES-RFP]DS plasmids were constructed by cloning the coding regions into the SpeI and BamHI sites of the PB[Act-IRES-RFP]DS vector. To make the PB[Act-RFP-U6-Lats1/2sgRNAs]DS plasmid, sgRNAs targeting mouse *Lats1* and *Lats2* were first chosen from a published mouse gRNA library (Koike-Yusa et al. 2014) and then validated by the Zhang laboratory CRISPR Design Tool. Annealed oligonucleotides were ligated into the lentiCRISPRv2 vector digested with BsmBI. Four individual U6-sgRNA fragments, including two targeting *Lats1* and two targeting *Lats2*, were then PCR amplified from the lentiCRISPRv2 vector and ligated with the SwaI digested PB[Act-RFP]DS plasmid using Gibson assembly. To make the PB[CMV-myc-YAP-5SA-U6-shScramble]DS and PB[CMV-myc-YAP-5SA-U6-shCcl2/Csf1]DS plasmids, shRNAs targeting mouse *Ccl2* and *Csf1* were first designed by the Broad Institute Genetic Perturbation Platform web portal. Annealed oligonucleotides were ligated into the pLKO.1 vector digested with AgeI and EcoRI. Four individual U6-shRNA fragments, including two targeting *Lats1* and two targeting *Lats2*, or four tandem repeats of U6-shScramble, were then PCR amplified from the pLKO.1 vector and were ligated with the SwaI digested PB[CMV-myc-YAP-5SA]DS plasmid using Gibson assembly. sgRNA and shRNA hairpin sequences are listed as below:

sgLats1 #1: CACCGTCTGCTCTCGACGAGGGTCT

sgLats1 #2: CACCGGCAGACATCTGCTCTCGACG

sgLats2 #1: CACCGTGCCGAAGTGCCTGACTCGT

sgLats2 #2: CACCGGTGCCTGACTCGTTGGCAAA

shCcl2 #1:

CCGGCGGACTGTGATGCCTTAATTACTCGAGTAATTAAGGCATCACAGTCCGT

TTTTC

shCcl2 #2:

CCGGGGACTGTGATGCCTTAATTAACTCGAGTTAATTAAGGCATCACAGTCCT

TTTTC

shCsf1 #1:

CCGGGCACACAGGACTATCTCTTTACTCGAGTAAAGAGATAGTCCTGTGTGCT

TTTTC

shCsf1 #2:

CCGGGATGAGACCATGCGCTTTAAACTCGAGTTTAAAGCGCATGGTCTCATCT

TTTTC

shScramble:

CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGT

TTTTC

Chromatin immunoprecipitation

ChIP assays were performed using an EZ-ChIP kit from Millipore according to the manufacturer's instructions. Briefly, the mouse livers were dissociated with a two-step

collagenase perfusion at post-injection day 1. The cell suspension was then centrifuged at 50 × g for 5 min at 4°C to collect parenchymal cells. Hepatocytes were further isolated by mixture of the resuspended parenchymal cells with the same volume of Percoll, and the mixture was centrifuged at 50 × g for 20 min. The cell pellets were washed with PBS once, and the cells were cross-linked, lysed, and sonicated to generate DNA fragments with an average size of 0.5 kb. ChIP was performed using 5 μg of control IgG or antibodies against human YAP or TEAD1. The immunoprecipitates were then washed and eluted. The eluents were then de-crosslinked, and the DNA was purified for PCR analysis. PCR primers for chromatin immunoprecipitation assay

Ccl2-3.3-F: TGTGCTGATGGTAGCTGCTC

Ccl2-3.3-R: AGTGGCTTGGCTATGAGCTT

Ccl2-0.8-F: GAGCTACCTGGGTCCTGTTCC

Ccl2-0.8-R: GATGCTGTTA CTCCCCATTC TCA

Csf1-1.1-F: TTGCCACCCACAAGTTGAGAAC

Csf1-1.1-R: CCCATTCCTC CCAGTTAAGT TGGCAT

RNA isolation and real-time PCR

To extract total liver RNA, whole livers were homogenized in TRIzol reagent (Life Technologies), and RNA was extracted according to the manufacturer's instructions. To determine the RNA levels in macrophage-enriched non-parenchymal cells, the livers were perfused with the two-step collagenase technique. The cell suspension was centrifuged at 50 × g for 5 min to remove the parenchymal cell pellet. The supernatant was further centrifuged at 300 × g for 5 min. The cell pellet was resuspended in 2 ml of

PBS to generate a single-cell suspension. A centrifuge tube was layered from the bottom with 50% Percoll/PBS, 25% Percoll/PBS, and a single-cell suspension and then centrifuged at 1,300 × g for 20 min. The cell layer between 25% and 50% Percoll was collected, and total RNA was extracted with TRIzol reagent.

To extract RNA from peripheral blood mononuclear cells, bone marrow derived macrophages, and splenocytes, cells were first collected from the respective tissues. Peripheral blood was combined with 2 × volume of PBS and centrifuged at 300 × g for 5 min. To collect bone marrow derived macrophages, mouse femurs were cut at both ends and cells were flushed out with ice-cold PBS, passed through a 70-μm cell strainer, and then centrifuged at 300 × g for 5 min. To collect splenocytes, spleens were sliced into small pieces and pressed through a 70-μm cell strainer using the plunger end of a syringe. The cell strainer was washed with PBS and cell suspension centrifuged at 300 × g for 5 min. Cell pellet was resuspended in 3 ml Gey's solution and incubated at room temperature for 5 min to lyse red blood cells. RNA was then extracted using TRIzol reagent.

cDNA was synthesized by reverse transcription using random hexamers and was subjected to real-time PCR with gene-specific primers in the presence of SYBR Green (Applied Biosystems). The relative abundance of mRNA was calculated by normalization to the level of hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA. Primers are listed as below:

mCcl2-F: TGCATCTGCCCTAAGGTCTTCA

mCcl2-R: AGTGCTTGAGGTGGTTGTGGA

mCsf1-F: AGTATTGCCAAGGAGGTGTCAG

mCsf1-R: TTCCTGGTCTACAAATTCAAAGG

mCcr2-F: TCCTGTCATTTATGCCTTTGTTG

mCcr2-R: CACTCGATCTGCTGTCTCCCTAT

mFizz1-F: CCCTTCTCATCTGCATCTCC

mFizz1-R: AGGAGGCCCATCTGTTCATA

mYm1-F: CCAGCATATGGGCATACCTT

mYm1-R; CAGACCTCAGTGGCTCCTTC

mMgl2-F: TTCAAGAATTGGAGGCCACT

mMgl2-R: CAGACATCGTCATTCCAACG

mCD163-F: AATGGTTCTTCTTGGAGGTGC

mCD163-R: CCAGGAGCGTTAGTGACAGC

mHPRT-F: ACTGTAATGATCAGTCAACGGG

mHPRT-R: GGCCTGTATCCAACACTTCG

Statistical analysis

The *p*-values were determined by Student's t-tests.