MicroRNA-223 attenuates hepatocarcinogenesis by blocking hypoxia-driven angiogenesis and immunosuppression

Yaojie Fu[1], Bryan Mackowiak[1], Dechun Feng[1], Hongkun Lu[1], Yukun Guan[1], Taylor Lehner[1], Hongna Pan[1], Xinwei Wang[2,3], Yong He[1*], Bin Gao[1*]

Supporting materials and methods

DEN-induced mouse HCC model

Male miR-223KO and littermate WT mice were administered with a single dose of N-nitrosodiethylamine (DEN) (Sigma-Aldrich, St. Louis, MO) (i.p. injection of 25 mg/kg at 15 days of age). Mice will be sacrificed 9 months after single injection of DEN, and tumor size and number will be analyzed.

RNA isolation and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from HCC tissues, non-tumor liver tissues, and cell samples by using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. 1µg total RNA was reverse transcribed into cDNA by using High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). For miR-223 measurement, total RNA was reverse-transcribed to cDNA by TaqMan MicroRNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA). RT-qPCR was performed by SYBR Green Realtime PCR master mix. The mRNA level was measured by QuantStudioTM 6 Real-Time PCR System (278861830; Thermo Fisher Scientific). The expression levels of target genes were normalized to 18S rRNA expression. Comparative Ct ($2^{-\Delta\Delta Ct}$) method was performed to quantify the mRNA expression level. All primers used for RT-qPCR are listed in **Supporting Table S1**.

RT-qPCR analysis for miR-223 expression measurement was performed by using TaqMan MicroRNA Assays (Introgen) and TaqMan Universal PCR Master Mix (Introgen) following the manufacturer's instruction. The fold-change for miR-223 relative to snoRNA202 was quantified by the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Western Blot

Tumor or tumor adjacent liver tissues, and cell lysates were homogenized in RIPA lysis buffer containing cocktail of protease inhibitors (Santa Cruz, CA) according to the manufacturer's instruction on ice (0°C), and centrifuged the samples at 12,000 *rpm* for 12 minutes. Protein extracts were then quantified by using BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA), mixed with loading buffer, and then were subjected to 4-12% Bis-Tris protein gels (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes (Thermo Fisher, Waltham, MA, USA). Protein bands were

visualized by using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher, Waltham, MA, USA). Primary antibodies against proteins of interest are listed in **Supporting Table S2**. Secondary horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were used for analysis. The results were determined with ImageJ software (National Institutes of Health, Bethesda, MD).

Cell culture

Hepa1-6 cell (murine hepatoma cell line) and mouse macrophage cell line Raw264.7 cell (ATCC) were cultured in DMEM medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS), 0.5% penicilin-streptomycin, and was incubated at 37°C with atmosphere of 5% CO_2. Hypoxic environment was created by adding 100 μM CoCl2 (Sigma-Aldrich) in the cell culture media.[1]

Transient transfection of miR-223 mimics

Hepa1-6 cell or Raw264.7 cell was transfected with negative control miRNA mimics (NC-mimics) or miR-223 mimics (Ambion, CA, USA) by using Lipofectamine RNAiMAX Reagent (Introgen) and OptiMEM medium (Gibco, NY, USA) according to the manufacturer's instruction at a final concentration of 20nM for 48h (1). After that, medium was replaced by fresh DMEM medium with 10% FBS. The efficiency of transfection was confirmed by RT-qPCR analysis.

Tumor size calculation in Hepa1-6 cell orthotopic and DEN+CCl_4 HCC models

The method for building Hepa1-6 cell derived orthotopic HCC model was discussed in Methods and materials and figure legends of the main text. Tumor volume was calculated by using the following formula: volume $=3.14/6\times(\text{length}\times\text{width}^2)$. For DEN+$CCl_4$ induced HCC model, tumor number, largest diameter of tumor mass was analyzed.

Luciferase reporter assay

Hepa1-6 cell was cultured 24h prior to transfection, and then co-transfected with 250 ng Control luciferase vector or Hif1a 3′-UTR luciferase vector plasmid (GeneCopoeia, USA) by using Lipofectamine™ 3000 (Invitrogen), 20 nM miR-223 mimics or the negative controls by using Lipofectamine RNAiMAX Reagent 4 (Invitrogen) following the manufacturer's instructions. As previously described, the Firefly and Renilla luciferase activities were measured by using the Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (GeneCopoeia, USA) 48h after transfection as described by the manufacturer. The ratio of luminescence from the Firefly luciferase to the Renilla luciferase was calculated as the relative luciferase activity.[1]

Flow cytometry analysis

Mouse livers were ground passing through a 70 μm cell strainer in PBS and the cell suspension was centrifuged at 50g for 5 minutes to pellet the cells. The supernatant enriched liver leukocytes were centrifuged at 1600 rmp for 10 minutes. The pellet was re-suspended in 15 ml of 40% Percoll (GE Healthcare, Pittsburgh, PA) and centrifuged at 2200 rmp for 18 minutes. The resulting pellet containing leukocytes was resuspended in 2 ml of ACK lysing buffer (BioWhittaker, Walker sville, MD) for 30 seconds. Cells were then stained with antibodies of interest for 30 minutes at 4°C in the dark. The following antibodies were used: anti-CD45 (BD Biosciences), anti-CD3 (BD Biosciences), anti-CD4 (BD Biosciences), anti-CD8 (BD Biosciences), anti-CD11b (BD Biosciences), and antiNK1.1 (BD Biosciences), anti-CCR2 (BD Biosciences), anti-F4/80(BD Biosciences), anti-PD-1(BD Biosciences), anti-PD-L1(BD Biosciences). Flow cytometry analysis was performed by using FACS Calibur (Beckman). Percentage of PD-1$^+$ T cells and mean fluorescence intensity (MFI) of PD-L1$^+$ macrophage in CD45$^+$ lymphocytes were calculated.

Immunofluorescent staining for HCC sections and adherent cells

Hepa1-6 cell adherent sections were fixed in 4% paraformaldehyde (4% PFA, pH7.4) for 10 minutes at room temperature and washed with PBS for three times. Paraffin-embedded HCC sections were incubated in 3% H_2O_2 for 20 mins at RT. All slides were blocked in blocking solution (3% bovine serum albumin in PBS) for 1 hour at RT and were incubated with targeted primary antibodies (**Supporting Table S2**) overnight at 4°C. After PBS washing, slides were incubated with the fluorescence conjugated antibodies (**Supporting Table S2**) for 1 hour at RT. Nuclear staining was obtained by incubation with 1 mg/mL 4', 6'-diamino-2-phenylindole (DAPI) for 5 min at room temperature. Frozen HCC tissues from DEN+CCl_4 mouse model were cryostat sectioned at 10 μm thick and incubated with anti-CA9, anti-PCNA, anti-Ki-67 and anti-CD31 antibodies for staining. Images were acquired using LSM 710 confocal microscope (Zeiss, Thornwood, NY, USA). Images were analyzed by the con-focal microscopic system from Zeiss (Thornwood, NY).

Tissue processing and Histological analysis

Formalin fixed mouse HCC samples were processed (Leica TP1020), and 4-μm–thick paraffin sections were stained with hematoxylin and eosin (H&E) for histological analysis, the images were taken with an Olympus camera DP72.

Immunohistochemistry (IHC) staining

The paraffin-embedded slides for immunohistochemistry staining were applied for heat-induced epitope retrieval first. For PD-L1 staining, 0.5M EDTA pH 8.0 buffer (Quality biological. INC.) was used for antigen retrieval, for other markers, citrate buffer (Thermo Fisher, USA) was used for antigen retrieval. Then, the slides were incubated in 3% H_2O_2, and followed by another 60 mins incubation in 1% BSA.

Sections were incubated with primary antibodies overnight at 4°C. ImmPACT AEC kit (Vector Laboratories, Inc., Burlingame, CA) and DAB Peroxidase Substrate Kit (Vector Laboratories, Inc., Burlingame, CA) were used to visualize the staining according to the manufacturer's instructions. For the multiple makers staining for the same section (CD4/CD8/PD-1 and IBA-1/CLEC4F/PD-L1), the slides were incubated in stripping buffer (62.5mM Tris-HCl pH6.8(Bio-Rad, Hercules, USA), 2%w/v SDS (Rockland Immunochemicals, Limerick, PA,USA) and 114.4mM beta-mercaptoethanol (Sigma-Aldrich) prepared in distilled water) for 30min at+56°C. afterwards, slides were washed for 3 times 20mins in PBS containing 1% Tween-20. The $CD31^+$ area, Sirius red positive area, a-SMA positive area and in 10 randomly selected high-power fields were calculated and analyzed. The percentage of positive area was determined with ImageJ software (National Institutes of Health, Bethesda, MD). The number of $PD\text{-}1^+/PD\text{-}L1^+$ cell in specific area was counted for statistics.

Double staining of IBA-1 and PD-L1

Formalin-fixed, paraffin-embedded tissue sections were de-paraffinized and re-hydrated with PBS, followed by antigen retrieval as the protocol described above. Incubate sections for 20 minutes with Normal Horse Serum (2.5%). Incubate the sections with rabbit (PD-L1) and mouse (IBA-1) primary antibody, and then followed the staining procedure of ImmPRESS Duet Double Staining Polymer Kit (Vector Laboratories, Inc., Burlingame, CA).

Bioinformatic analysis on TCGA cohort and miR-223 target gene

Bioinformatic analyses were performed based on transcriptomic data from publicly available dataset of HCC cohort, including: The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/) and UCSC Xena (https://xena.ucsc.edu/). In our study, gene expression file data and miR-223 expression data of TCGA hepatocellular carcinoma cohort (TCGA-LIHC cohort) were used for differentially expressed genes (DEGs) analysis, correlation analysis and Gene ontology (GO) enrichment analysis. The statistical analysis was performed by using R software (3.2.2). All data acquirement and application were in accordance with TCGA publication guidelines and policies. To search for the potential target of miR-223, a public bioinformatics approach (microRNA.org database: http://www.microrna.org) was used in the study.[1]

Treatment of mice with adenovirus-miR-223

For adenovirus-mediated miR-223 overexpression in two HCC mouse models, the mice were administered by tail vein injection (once per 2 weeks) with adenovirus loaded mouse miR-223 (Ad-miR-223) (Vector Biolabs, Malvern, PA) or green fluorescence protein (GFP) (Ad-GFP) (Vector Biolabs) as a control.

Hif1α short hairpin RNA (shRNA) knockdown

Hepa1-6 cells were transfected with Hif1α shRNA constructs against Mus musculus

Hif1a (catalog# TR517255, Origene, Technologies, Inc., MD, USA) or its negative control (NC) shRNA (Origene, Technologies, Inc.). The medium was changed 48h later, and the cells were treated with puromycin (Santa Cruz Biotechnology) for selection. Western blot and RT-qPCR were used to monitor the efficiency of transfection of Hif1α shRNA.

***In vitro* co-culture of Hepa1-6 cell and T cells or macrophages**

The Transwell plates with 0.4 μm pore size polycarbonate membrane inserts (Corning, Inc.) were used to set up the co-culture experiments according to the manufacturer's instructions. Hepa1-6 cells were seeded in the lower chamber before co-culture. Hypoxic environment was created by adding 100 μM $CoCl_2$ (Sigma-Aldrich) in culture medium. Cell culture medium was changed to a new medium after $CoCl_2$ stimulation for 24 hours. Primary T cells or macrophages (in upper chamber) were co-cultured with Hepa1-6 cells at a 1:1 ratio for 24 hours, followed by western blot and flow cytometry analyses.

***In vitro* treatment of cultured cells with CPI-444 (A2AR antagonist) and adenosine**

Isolated primary $CD3^+$ T cell (2×10^5) from the mouse spleen were cultured in complete medium (DMEM + 10% FBS + 1% penicillin/streptomycin) with T-activator CD3/CD28 (Gibco^{TM},11452D). Beads were removed by using magnetic selection after T cell activation. The A2AR antagonist CPI-444 (MedChemExpress, Cat. No.: HY-101978) was reconstituted according to the manufacturer's instruction. T cells were treated with CPI-444 at 1μmol/L final concentration for 24 hours. Primary peritoneal macrophages were incubated with CPI-444 with the same method as mentioned above. T cells or macrophages were treated with adenosine (Sigma-Aldrich) at 500μM, and the cells were harvested for *Pdcd1/Cd274* (PD-1/PD-L1) expression analysis by using RT-qPCR and flow cytometry.

***In vivo* treatment of Hepa1-6 derived HCC model with CPI-444**

A 10 mg/mL stock solution was reconstituted and further diluted into lower concentration following the protocol that has been reported.[2] By using the chronic inflammation related orthotopic Hepa1-6 HCC model that was mentioned in the main text, 10- to 12-week-old male C57BL/6 mice were challenged with CCl_4 (*i.p.*) once a week. Hepa1-6 cell derived HCC orthotopic implantation was performed two weeks later with CCl_4 injection once per week for 2 weeks; vehicle control solution or CPI-444 (10mg/Kg) was administered daily via oral gavage for consecutive 2 weeks after HCC model establishment.

Adenosine measurement

Convenient microplate-based method was used to measure the amount of adenosine in cell culture medium or mouse HCC tissues. Adenosine Assay Kit (Cell Biolabs, Cat.

No.: MET-5090) was used by following the manufacturer's instruction. Detection was conducted in a fluorescence-based microplate reader (SpectraMax® iD3).

In vitro incubation with CD39/CD73 inhibitors

In vitro experiment with CD39 inhibitor (POM-1; 100uM; sc-203205) and CD73 antagonist (α,β-Methyleneadenosine 5′-diphosphate sodium salt; also known as 'AMP-CP'; 100uM; sc-214457) (San Cruz Technology) were reconstituted following the protocol, which were then incubated with co-culture of Hepa1-6 cell and primary T cell and macrophage for 48h.

Anti-CD73 and isotype IgG *in vivo* treatment in mouse HCC model

Anti-mouse CD73 mAb (clone TY/23; Cat. No.:BE0209) and Rat IgG2a isotype control (clone 2A3; Cat. No.:BE0089) were purchased from BioXCell (Lebanon, NH). Antibodies were diluted with Pure pH6.5 Dilution Buffer (Cat. No.: IP0065) (BioXCell) according to the manufacturer's instruction. Mice of Hepa1-6 orthotopic HCC model as described above were treated with intraperitoneal injection (*i.p.*) of 100 μg anti-CD73 mAb or Rat IgG2a isotype control 3 times per week for 2 weeks as described previously.[3]

In vitro treatment of Hepa1-6 cells with neutrophil derived extracellular vesicles (EVs)

Hepa1-6 cells were pretreated with palmitic acid (PA) (0.3 mM) for 18 hours, and then were incubated with DiD-labeled neutrophil derived extracellular vesicles (EVs) for 24 hours. More details about neutrophils-derived EVs were previously described.[4]

Chromatin Immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assays were performed using the EZ-ChIP Assay kit (Sigma-Aldrich; Cat. NO.:17-295) according to the manufacturer's protocol. Briefly, Hepa1-6 cells were transfected with *Hif1a* shRNA or NC shRNA for 48h, and then were cultured under normoxia or hypoxia (exposed to $CoCl_2$ at 100 μM) for 20h. Transfected cells were cross-linked with 1% formaldehyde for 15min at room temperature. The reaction was then stopped by adding glycine to a final concentration at 0.125mM. Then, the cells were washed with cold PBS twice, harvested by using scraper and applied for lysis and sonication to shear chromatin into DNA fragments of 0.5-1 kb. Lysates were centrifuged, and an aliquot of supernatant was served as the input sample. Anti-HIF1a antibody (HIF-1a [D1S7W] XP® Rabbit mAb; Cat. NO.: 36169S) was then used for immunoprecipitation or with goat immunoglobulin G (Control for the non-specific binding). The purified DNA fragments were analyzed by using qRT-PCR for the presence of mouse *Cd39/Cd73* promoter region using primers: (*Cd39*): Forward: ACAGACCCAAAACAATAGCGT

Reverse: TTTGGTCTGTTTACGTGCGA

(*Cd73*): Forward: ATCCTTCGCCTAGGACGTGT

Reverse: GGTGGTTCTCCACTCCATGT

The results were normalized by input and were presented as fold enrichment over those of controls.

References

1. He Y, Hwang S, Cai Y, et al. MicroRNA-223 Ameliorates Nonalcoholic Steatohepatitis and Cancer by Targeting Multiple Inflammatory and Oncogenic Genes in Hepatocytes. Hepatology 2019;70:1150-1167.
2. Willingham SB, Ho PY, Hotson A, et al. A2AR Antagonism with CPI-444 Induces Antitumor Responses and Augments Efficacy to Anti-PD-(L)1 and Anti-CTLA-4 in Preclinical Models. Cancer Immunol Res 2018;6:1136-1149.
3. Tsukui H, Horie H, Koinuma K, et al. CD73 blockade enhances the local and abscopal effects of radiotherapy in a murine rectal cancer model. BMC Cancer 2020;20:411.
4. He Y, Rodrigues RM, Wang X, et al. Neutrophil-to-hepatocyte communication via LDLR-dependent miR-223-enriched extracellular vesicle transfer ameliorates nonalcoholic steatohepatitis. J Clin Invest 2021;131.

Supporting Table S1. Primer sequences of mouse genes for RT-qPCR

Gene	**Forward Primer/ Reverse Primer (5'-3')**
18s	ACGGAAGGGCACCACCAGGA CACCACCACCCACGGAATCG
Ly6g	TGCGTTGCTCTGGAGATAGA CAGAGTAGTGGGGCAGATGG
F4/80	CTTTGGCTATGGGCTTCCAGTC GCAAGGAGGACAGAGTTTATCGTG
Tnf-α	AGGCTGCCCCGACTACGT GACTTTCTCCTGGTATGAGATAGCAAA
Il1β	TCGCTCAGGGTCACAAGAAA CATCAGAGGCAAGGAGGAAAAC
Il6	TCCATCCAGTTGCCTTCTTG TTCCACGATTTCCCAGAGAAC
Mcp1	TCTGGACCCATTCCTTCTTGG TCAGCCAGATGCAGTTAACGC
Mip1β	AACACCATGAAGCTCTGCGT AGAAACAGCAGGAAGTGGGA
Mip2	TCCAGGTCAGTTAGCCTTGC CGGTCAAAAAGTTTGCCTTG
Icam1	CAATTTCTCATGCCGCACAG AGCTGGAAGATCGAAAGTCCG
α-sma	TCCTGACGCTGAAGTATCCGATA GGTGCCAGATCTTTTCCATGTC
Afp	CAGCAGCCTGAGAGTCCATA GGCGATGGGTGTTTAGAAAG
Golm1	GCAGGTCTCGAATGAGCTTC CCAGTCTAGCCACAGCTTCC
Gpc3	TGGTGTAGTTCTTGGCATGG TGCTCCAGTCTGCGAGTATG
Tff3	TTGCTGGGTCCTCTGGGATAG TACACTGCTCCGATGTGACAG
Endoglin	CCCTCTGCCCATTACCCTG GTAAACGTCACCTCACCCCTT
Vegfa	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGGCT
Egfr	GCCATCTGGGCCAAAGATACC GTCTTCGCATGAATAGGCCAAT
Havcr2	TCAGGTCTTACCCTCAACTGTG GGGCAGATAGGCATTTTTACCA
Lgals9	TCAGTGCCCAGTCTCCATACA

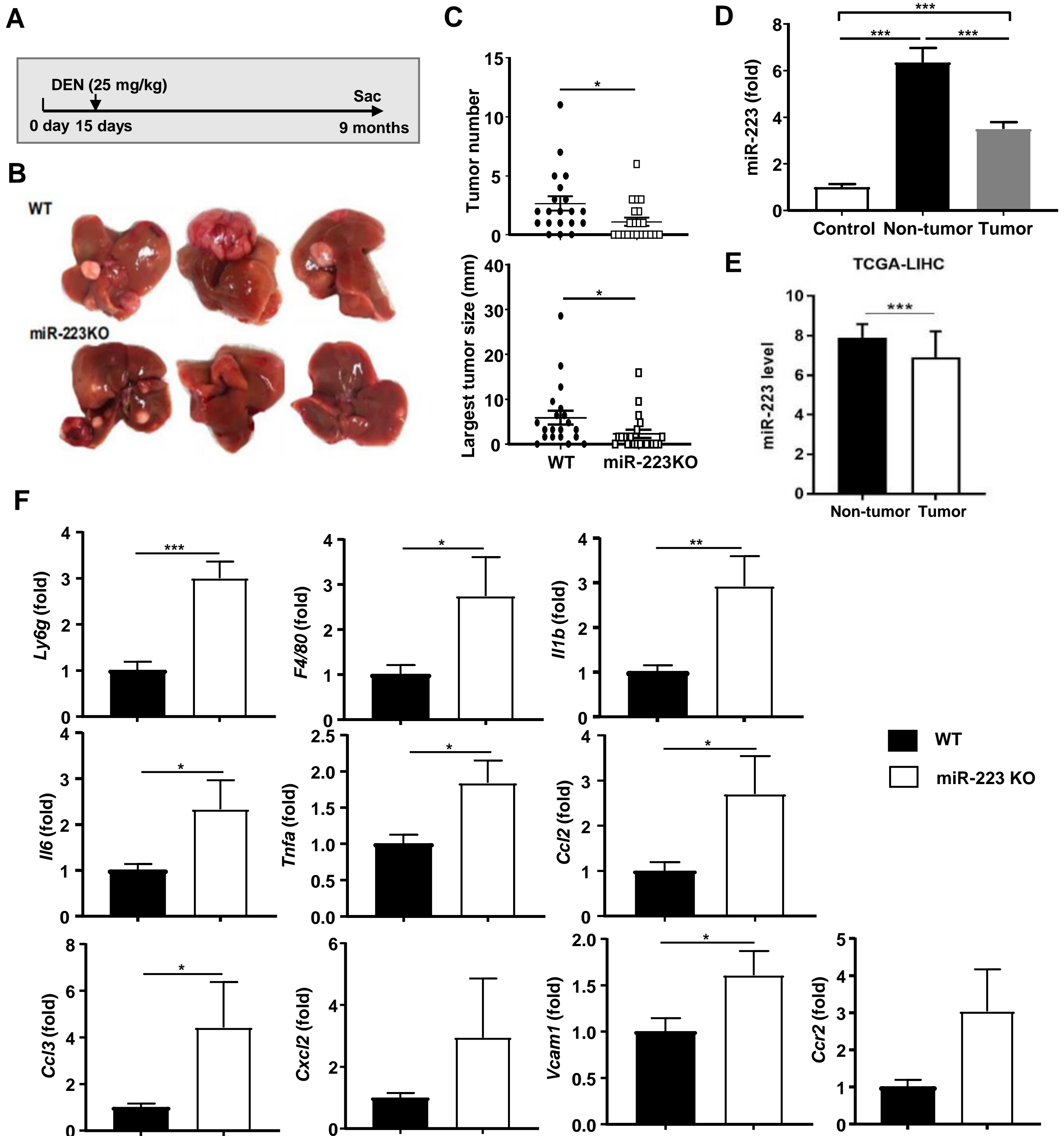
	CTCCTTGGATTGGTCCAGTAAAG
Tigit	GAATGGAACCTGAGGAGTCTCT AGCAATGAAGCTCTCTAGGCT
Pdcd1	ACCCTGGTCATTCACTTGGG CATTTGCTCCCTCTGACACTG
Cd274	GCTCCAAAGGACTTGTACGTG TGATCTGAAGGGCAGCATTTC
Ctla4	TTTTGTAGCCCTGCTCACTCT CTGAAGGTTGGGTCACCTGTA
Lag3	CTGGGACTGCTTTGGGAAG GGTTGATGTTGCCAGATAACCC
Pvrl1	GACTCCATGTATGGCTTCATCG CACTCGTTTCTCGTAGGGAGG
Vista	GGAACCCTGCTCCTTGCTATT TTGTAGATGGTCACATCGTGC
Btla (Cd272)	TGCTTGGGACTCCTCGGTTAT ACACAGATTGTTCCATTGTGCT
Cd96	ATAGAGACAAATCGGACTCTGGA CCAACCATGAAAAGGTGAACCTA
Cd39(Entpd1)	AAGGTGAAGAGATTTTGCTCCAA TTTGTTCTGGGTCAGTCCCAC
Cd73(Nt5e)	GGACATTTGACCTCGTCCAAT GGGCACTCGACACTTGGTG
Hif1a	ACCTTCATCGGAAACTCCAAAG ACTGTTAGGCTCAGGTGAACT
Ccr2	TGTGATTGACAAGCACTTAGACC TGGAGAGATACCTTCGGAACTT
Col3α1	TAGGACTGACCAAGGTGGCT GGAACCTGGTTTCTTCTCACC
Mip1α	TGAGAGTCTTGGAGGCAGCGA TGTGGCTACTTGGCAGCAAACA
Mmp13	CTTTGGCTTAGAGGTGACTGG AGGCACTCCACATCTTGGTTT
Col4a1	CTGGCACAAAAGGGACGAG ACGTGGCCGAGAATTTCACC
Col5a2	TTGGAAACCTTCTCCATGTCAGA TCCCCAGTGGGTGTTATAGGA
Col3a1	TAGGACTGACCAAGGTGGCT GGAACCTGGTTTCTTCTCACC
Mmp3	ACATGGAGACTTTGTCCCTTTTG TTGGCTGAGTGGTAGAGTCCC
Mip1α	TGAGAGTCTTGGAGGCAGCGA TGTGGCTACTTGGCAGCAAACA

Vimentin	TCCACTTTCCGTTCAAGGTC AGAGAGAGGAAGCCGAAAGC
Tgfb1	CTCCCGTGGCTTCTAGTGC GCCTTAGTTTGGACAGGATCTG
Col4a1	CTGGCACAAAAGGGACGAG ACGTGGCCGAGAATTTCACC
Vcam1	AGTTGGGGATTCGGTTGTTCT CCCCTCATTCCTTACCACCC
Vegfb	GCCAGACAGGGTTGCCATAC GGAGTGGGATGGATGATGTCAG
Lox	TCTTCTGCTGCGTGACAACC GAGAAACCAGCTTGGAACCAG
Loxl2	ATTAACCCCAACTATGAAGTGCC CTGTCTCCTCACTGAAGGCTC
Plod2	GAGAGGCGGTGATGGAATGAA ACTCGGTAAACAAGATGACCAGA
Glut1	CAGTTCGGCTATAACACTGGTG GCCCCCGACAGAGAAGATG
Hmox1	AAGCCGAGAATGCTGAGTTCA GCCGTGTAGATATGGTACAAGGA
Adm	CACCCTGATGTTATTGGGTTCA TTAGCGCCCACTTATTCCACT
Epo	ACTCTCCTTGCTACTGATTCCT ATCGTGACATTTTCTGCCTCC
Eno1	TGCGTCCACTGGCATCTAC CAGAGCAGGCGCAATAGTTTTA
Ldha	TGTCTCCAGCAAAGACTACTGT GACTGTACTTGACAATGTTGGGA
Pkm2	GCCGCCTGGACATTGACTC CCATGAGAGAAATTCAGCCGAG
Pdgfa	GAGGAAGCCGAGATACCCC TGCTGTGGATCTGACTTCGAG

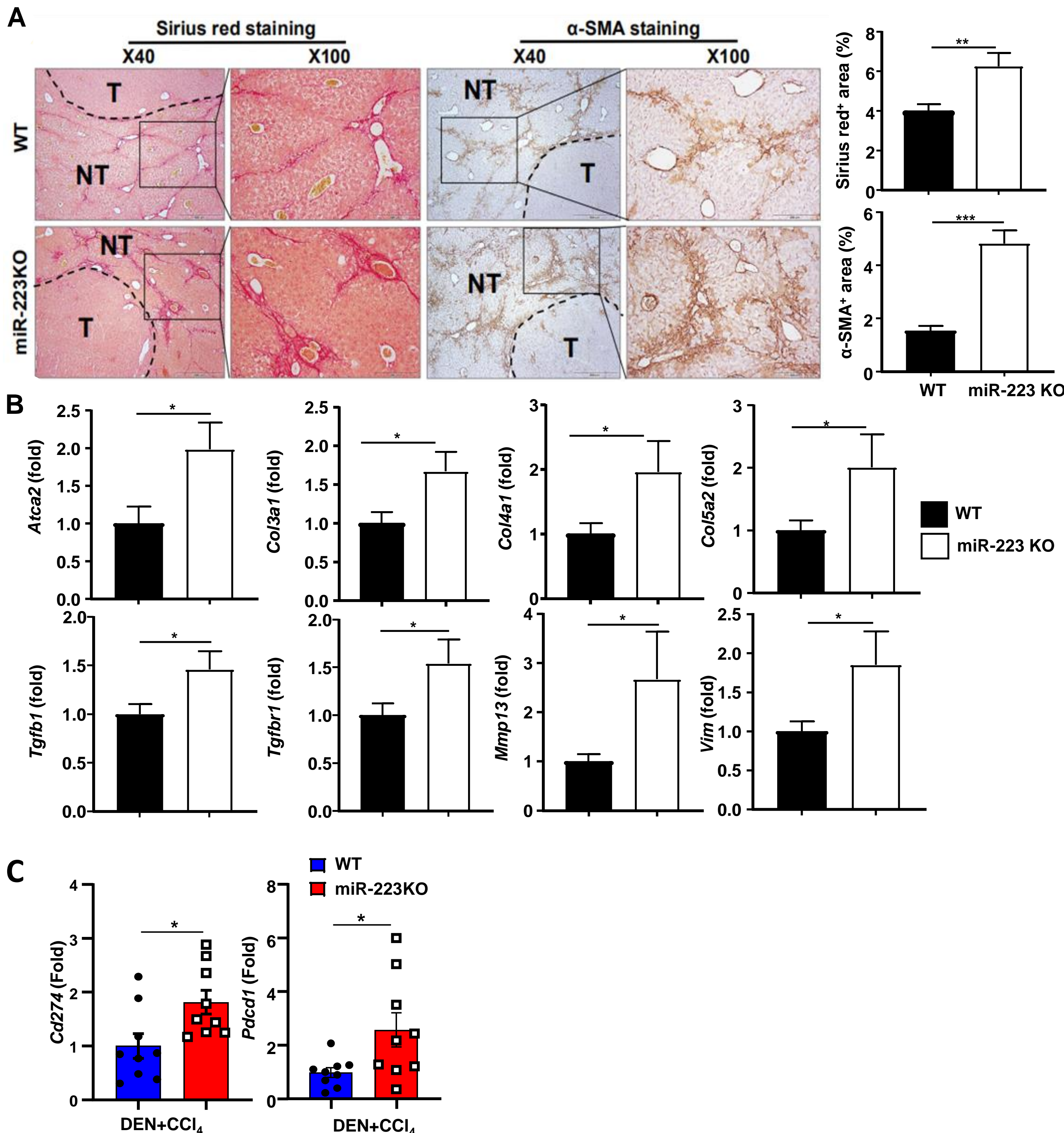
Supporting Table S2. Primary antibodies used for WB and IHC/IF staining

Reagent	Catalog No.	Manufacturer
β-Actin	A1978	Sigma-Aldrich
CD3	ab135372	Abcam
Ki-67*	12202s	Cell signaling technology
CD31	77699	Cell signaling technology
p-VEGFR2	2478s	Cell signaling technology
VEGFR2	2479s	Cell signaling technology
F4/80	70076s	Cell signaling technology
MPO	REF: PP023AA	BIOCARE MEDICAL
PD-1#	84651 PA5-20350	Cell signaling technology Thermo Fisher
PD-L1#	PA5-20343	Thermo Fisher
PD-1*	84651	Cell signaling technology
PD-L1*	64988	Cell signaling technology
HIF-1α #	36169	Cell signaling technology
HIF-1α *	NB100-105	Novus Biologicals
AFP	14550-1-AP	Proteintech
PCNA	13110	Cell signaling technology
CA-9	NB100-417	Novus Biologicals
p-Tie2	AF2720	R&D
Tie2	19157-1-AP	Proteintech
α -SMA	19245	Cell signaling technology
IBA-1	MABN92	Sigma-Aldrich
CD45	70257	Cell signaling technology
CD4	25229	Cell signaling technology
CD8	ab217344	Abcam
CLEC4F	AF2784	biotechne
GFP	2955	Cell signaling technology

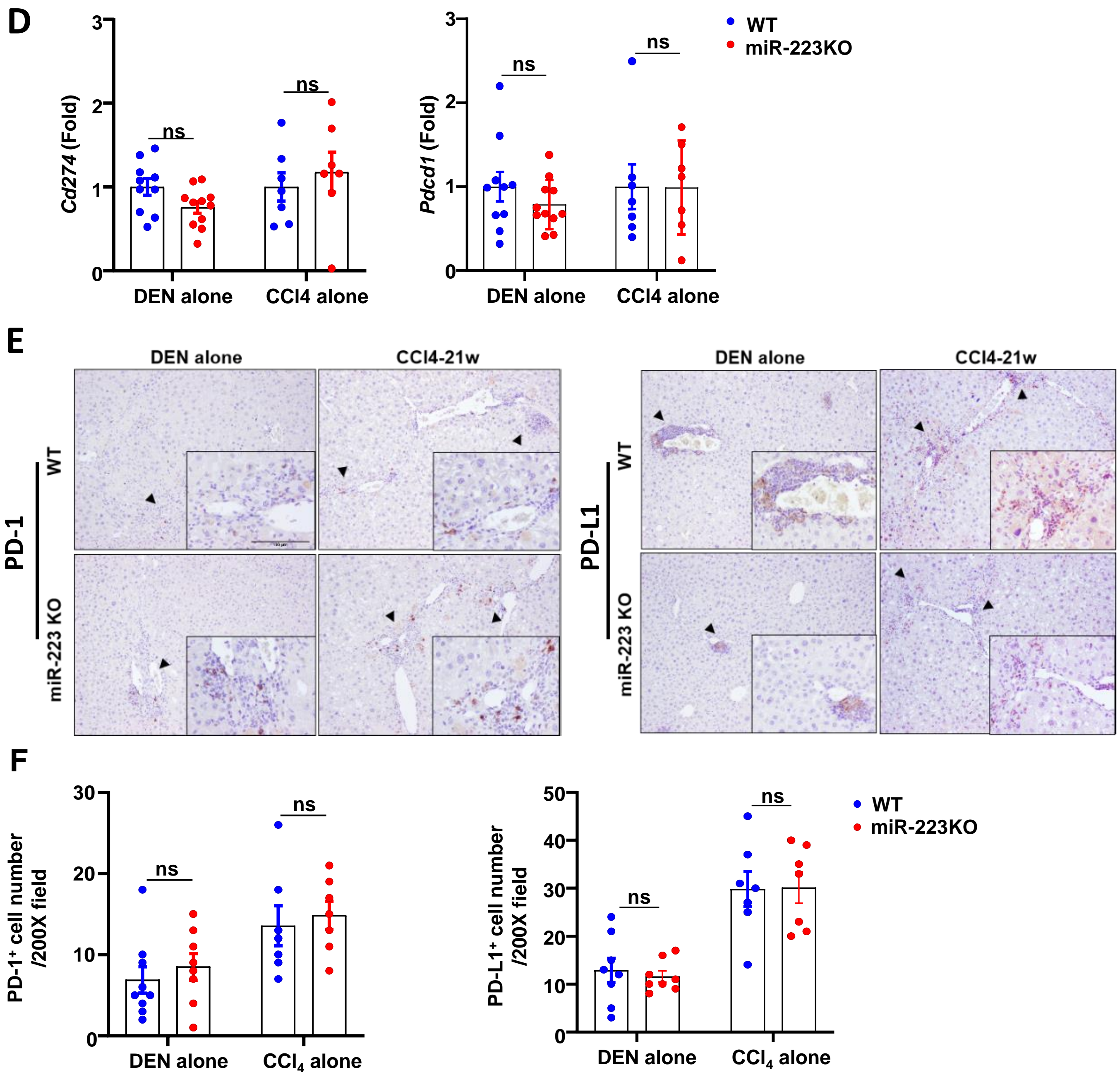
Antibodies used for western blot analysis
*Antibodies used for IHC/IF staining



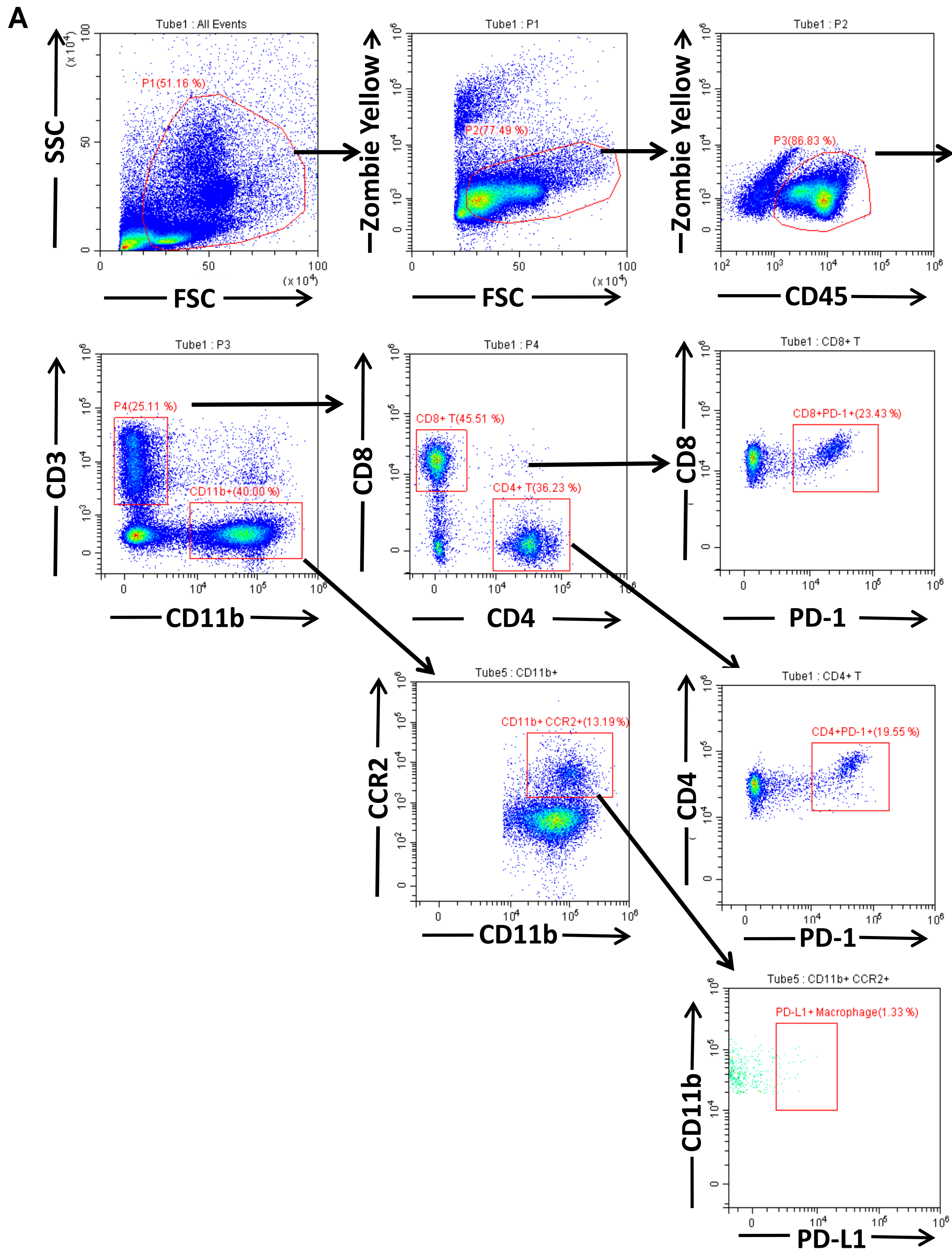
Supporting Figure S1. (A-C) Scheme of single DEN-induced HCC mouse model (see details in methods)(panel A). Representative gross images of livers from miR-223KO and WT mice post DEN treatment (panel B). The tumor number and largest tumor diameter were calculated (panel C). **(D)** RT-qPCR analysis of miR-223 levels in control liver samples from normal C57BL6J mice, adjacent non-tumor livers and tumor samples from DEN+CCl_4-induced HCC model. **(E)** miR-223 level comparison between non-tumor liver samples and HCC samples (labeled as 'tumor') from LIHC cohort in TCGA database. **(F)** RT-qPCR analysis of neutrophil marker (Ly6G), macrophage marker (F4/80) and several inflammatory mediators in non-tumor adjacent tissues from DEN+CCl_4-treated WT and miR-223KO mice. Values represent means ± SEM. *p<0.05; **p<0.01; ***p<0.001.



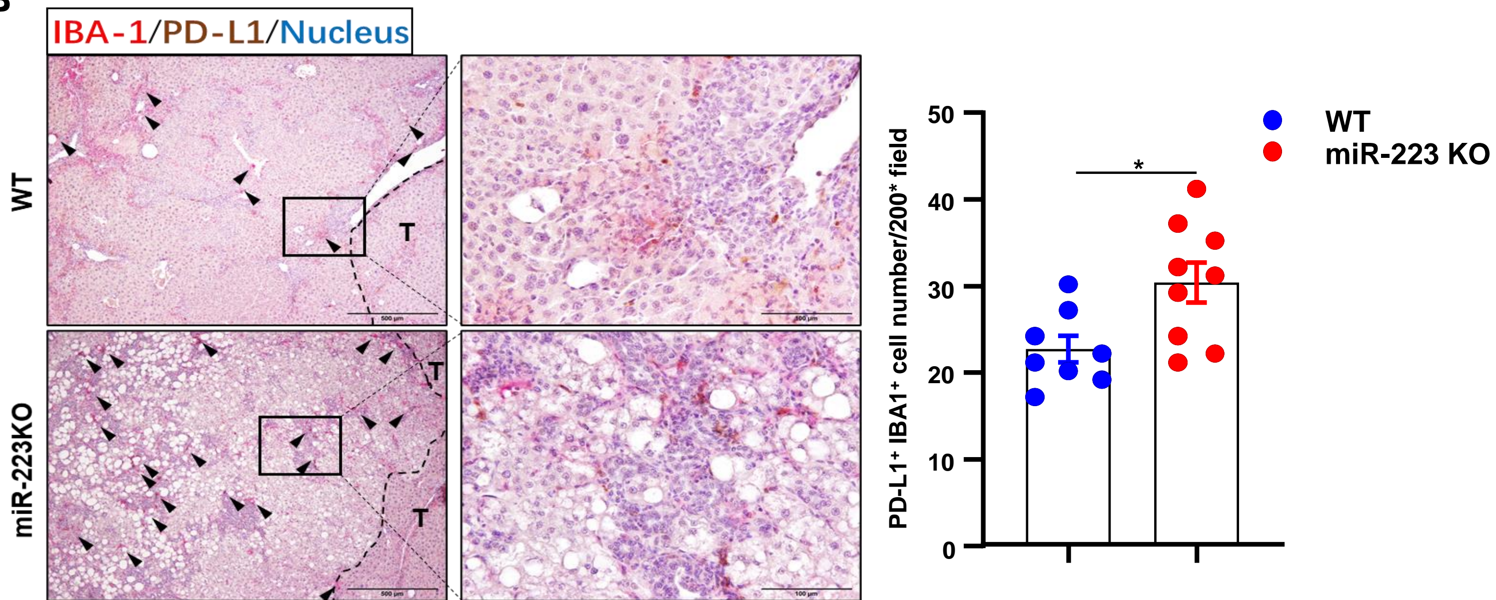
Supporting Figure S2A-C. Mir-223KO mice have greater degree of fibrosis and PD-1/PD-L1 expression than WT mice after DEN+CCl_4 treatment. WT and miR-223KO mice were subjected to DEN+CCl4 treatment, Liver tissues were collected for H&E staining and RT-qPCR. **(A)** Representative images of Sirius red staining and α-SMA staining (×40 & 100× magnifications) in HCC adjacent non-tumorous (NT) liver from WT and miR-223KO mice ('T' stands for tumor region). The percentage of Sirius red⁺ and α-SMA⁺ area per field was quantified. **(B)** Fibrosis-related genes in WT and miR-223KO mice were measured by using RT-qPCR. **(C)** PD-1 (*Pdcd1*) and PD-L1 (*Cd274*) levels in non-tumor regions were determined by RT-qPCR. Values represent means ± SEM. *p<0.05; **p<0.01; ***p<0.001.

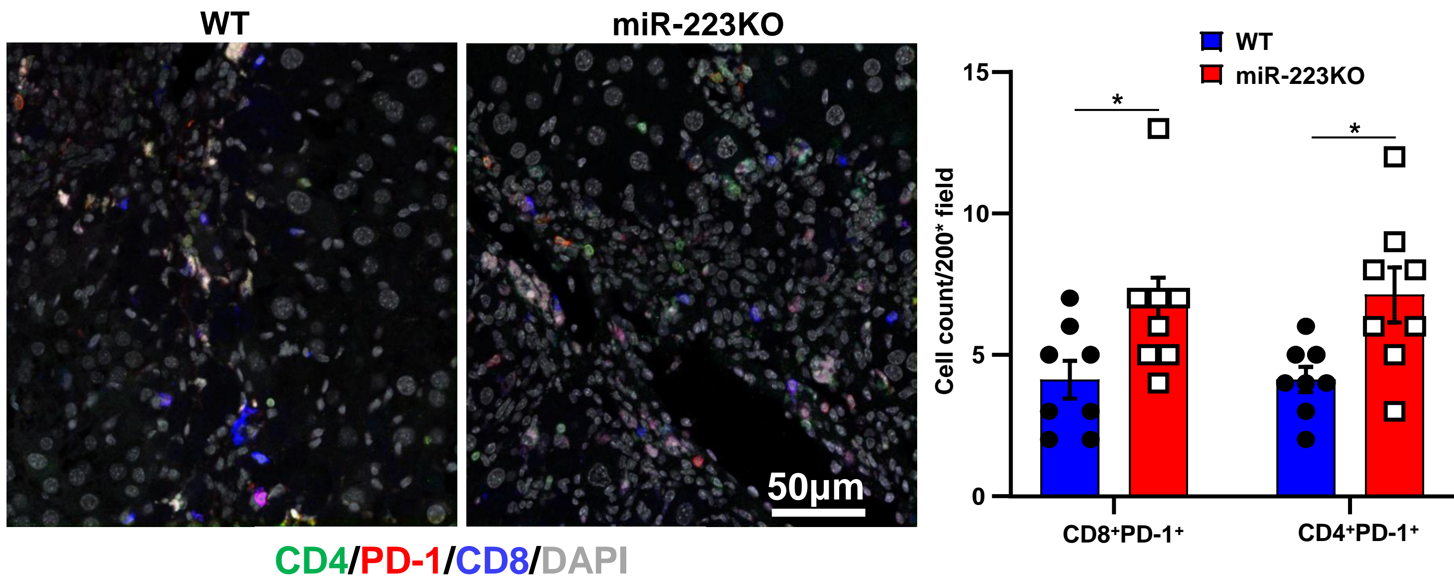


Supporting Figure S2D-F. Upregulated PD-1/PD-L1 expression in miR-223KO mice than those in WT mice is not observed in DEN or CCl_4 injection alone model. WT and miR-223KO mice were subjected to single DEN-induced HCC model or chronic CCl_4 injection (25% dissolved in olive oil; 2 ul/g; once per week via i.p injection, continuous 21 weeks). **(D)** RT-qPCR was subjected to *Cd274* and *Pdcd1* analyses. **(E)** Representative images of PD-1 and PD-L1 staining in liver tissues from DEN model or CCl4 alone are shown. **(F)** PD-1$^+$ and PD-L1$^+$ cells per field from panel E were quantified. Values represent means ± SEM. ns: no statistical differences.

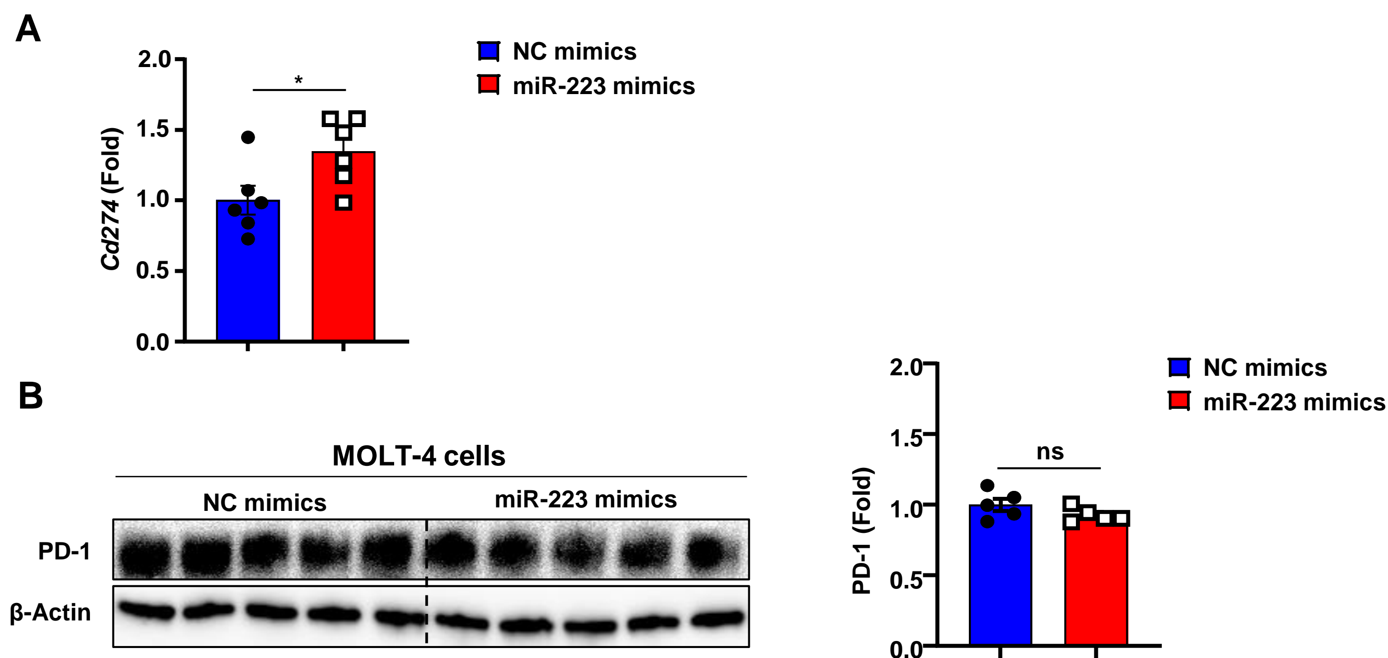


Supporting Figure S3A. Identification of PD-1⁺ and PD-L1⁺ cells in tumor microenvironment in DEN+CCl_4 model. Gating strategy used in the FACS analyses to determine PD-1⁺ and PD-L1⁺ cells infiltrating in HCC adjacent region. PD-1 was expressed by both CD4⁺ T cell and CD8⁺ T cell. PD-L1 was expressed by CD11b⁺ CCR2⁺ infiltrating macrophage.

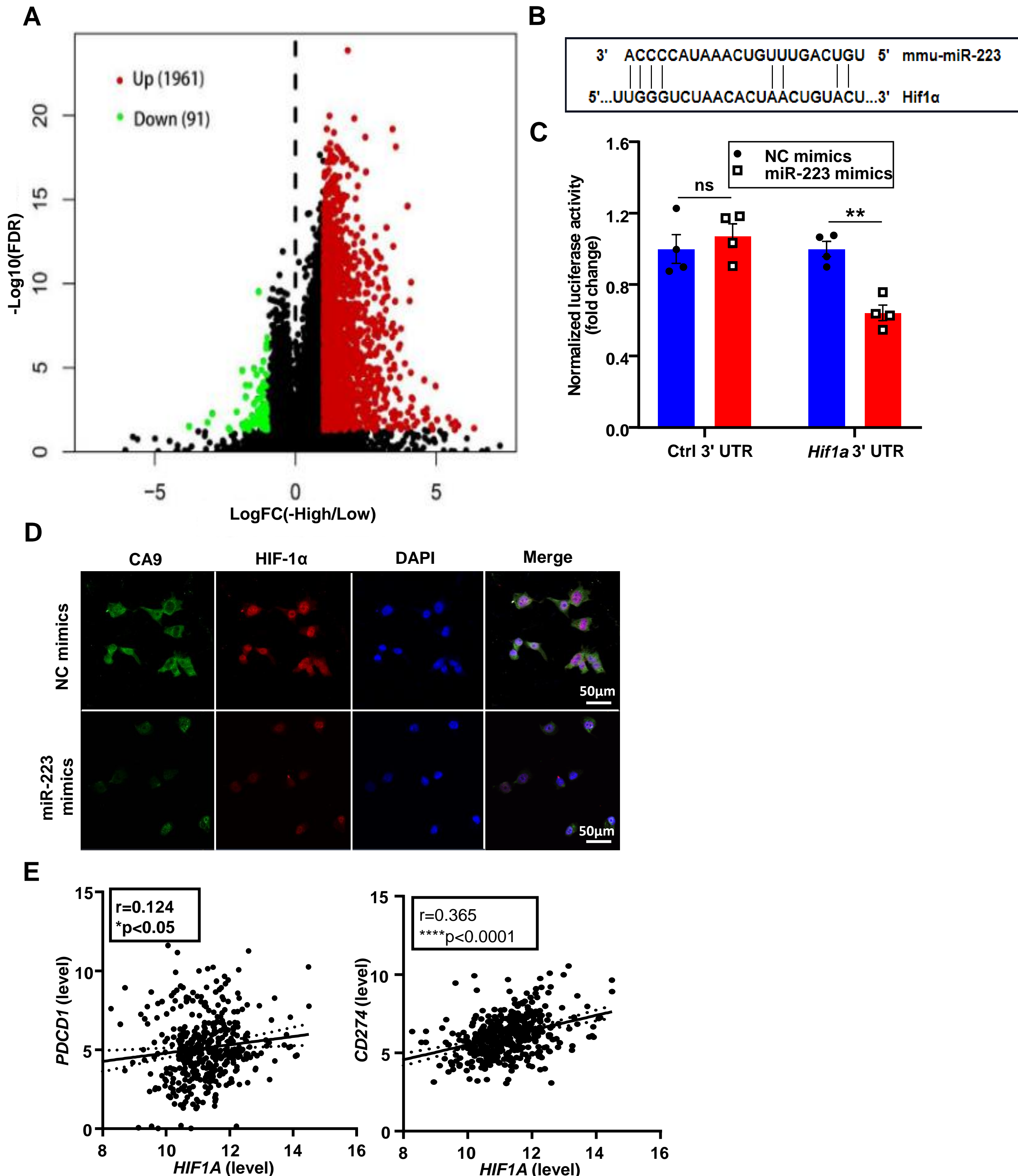




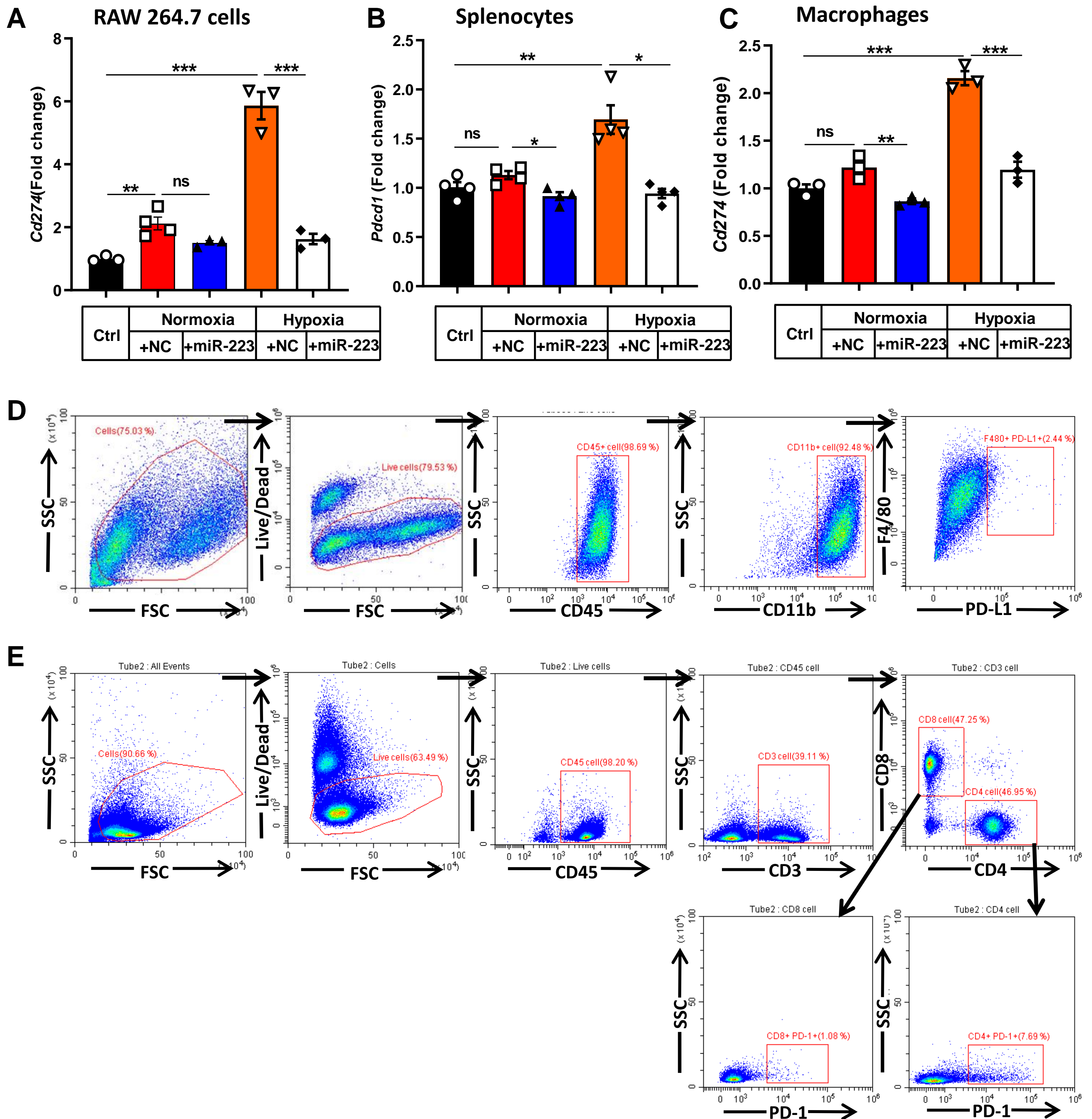
Supporting Figure S3B-C. Identification of PD-1⁺ and PD-L1⁺ cells in tumor microenvironment in DEN+CCl_4 model. **(B)** Double staining confirmed that PD-L1 was expressed in macrophages (IBA1⁺ PD-L1⁺; Scale bar:100μm). The number of PD-L1⁺IBA1⁺ cells per field was quantified. **(C)** Immunofluorescence staining confirmed that PD-1 was expressed in T cells (CD8⁺PD-1⁺ and CD4⁺PD-1⁺; Scale bar: 50 μm). The numbers of CD8⁺PD-1⁺ and CD4⁺PD-1⁺ cells per field were quantified. Values represent means ± SEM. *$p<0.05$.



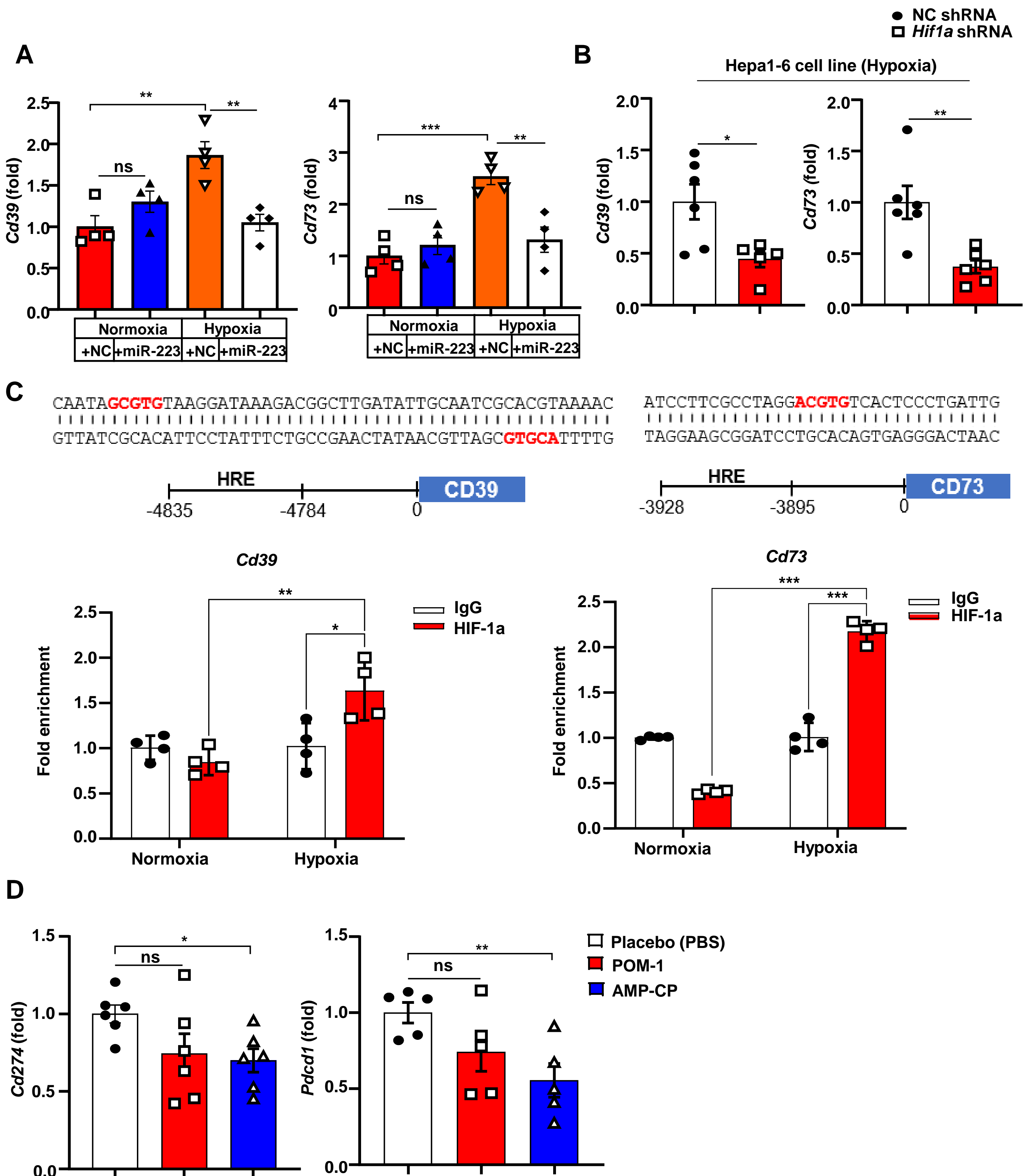
Supporting Figure S4 PD-1 and PD-L1 are not direct targets of miR-223. **(A)** RT-qPCR analysis of *Cd274* mRNA level post transfection of miR-223 mimics in mouse macrophage cell line RAW 264.7 cells. **(B)** Western blot analysis of PD-1 expression post transfection of miR-223 mimics in MOLT-4 cells (T cell line). Values represent means ± SEM. $^*P<0.05$



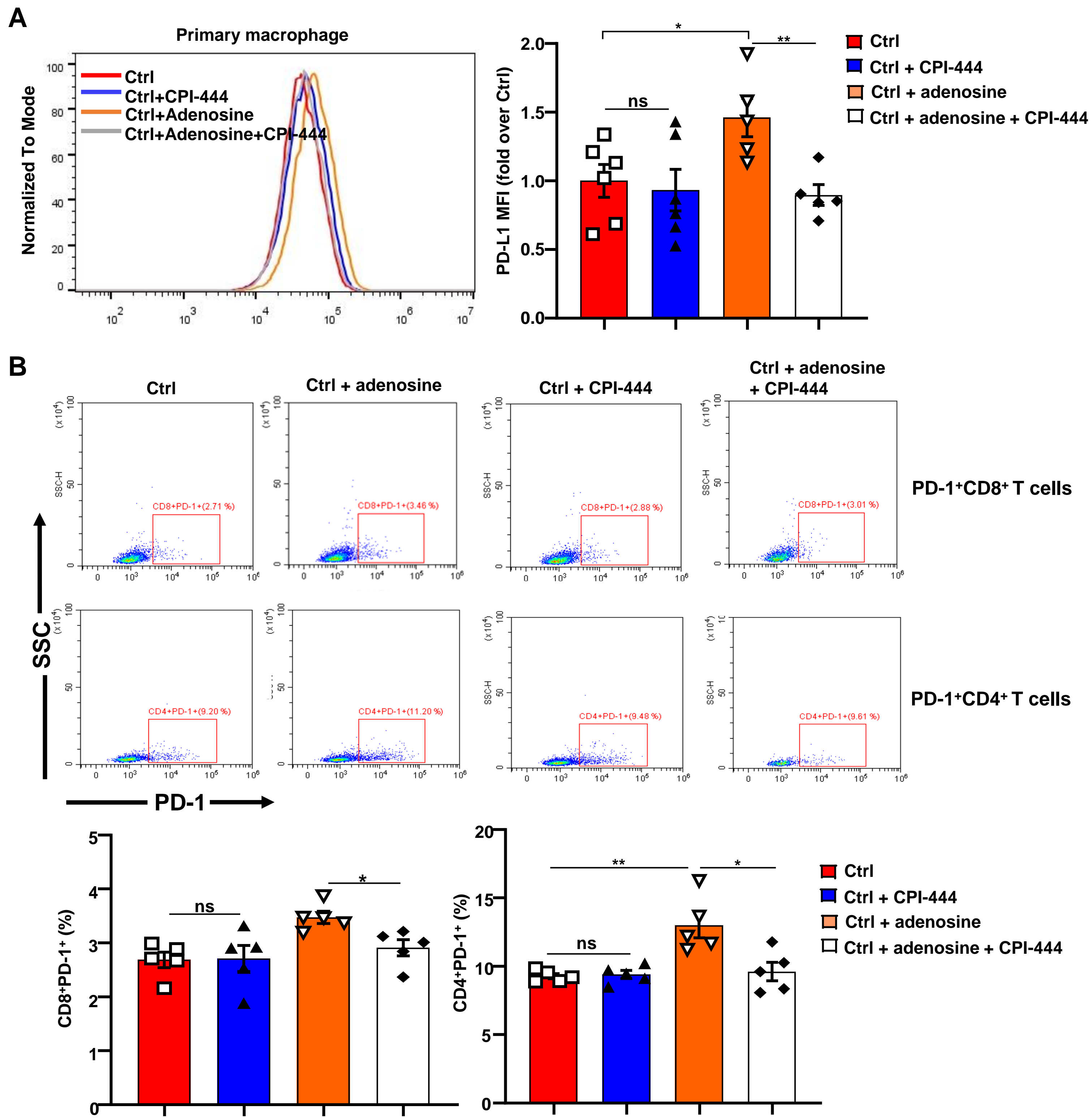
Supporting Figure S5. miR-223 targets HIF-1α in HCC cells. **(A)** Volcano plot analysis with data from TCGA database presented the differentially expressed genes in HCC patients with high- *vs.* low-miR-223 expression. Red dots: the up-regulated genes in patients with high-miR-223 expression (1961 in total); Green dots: down-regulated genes in patients with high-miR-223 expression (91 in total). **(B)** Bioinformatic analysis predicted that HIF-1α is a potential target of miR-223 (microRNA.org database:http://www.microrna.org). **(C)** Luciferase reporter assay was used to verify that *Hif1α* in Hepa1-6 cell is a direct target of miR-223. **(D)** Representative immunofluorescent staining images of CA9 (green), HIF-1α (red), and DAPI (blue) in miR-223 mimics/negative control mimics treated Hepa1-6 cell are shown. Scale bar: 50μm. **(E)** Correlation analysis of *HIF1A* expression and PD-1(*PDCD1*)/PD-L1(*CD274*) in TCGA-LIHC cohort. Values represent means ± SEM. **p<0.01.



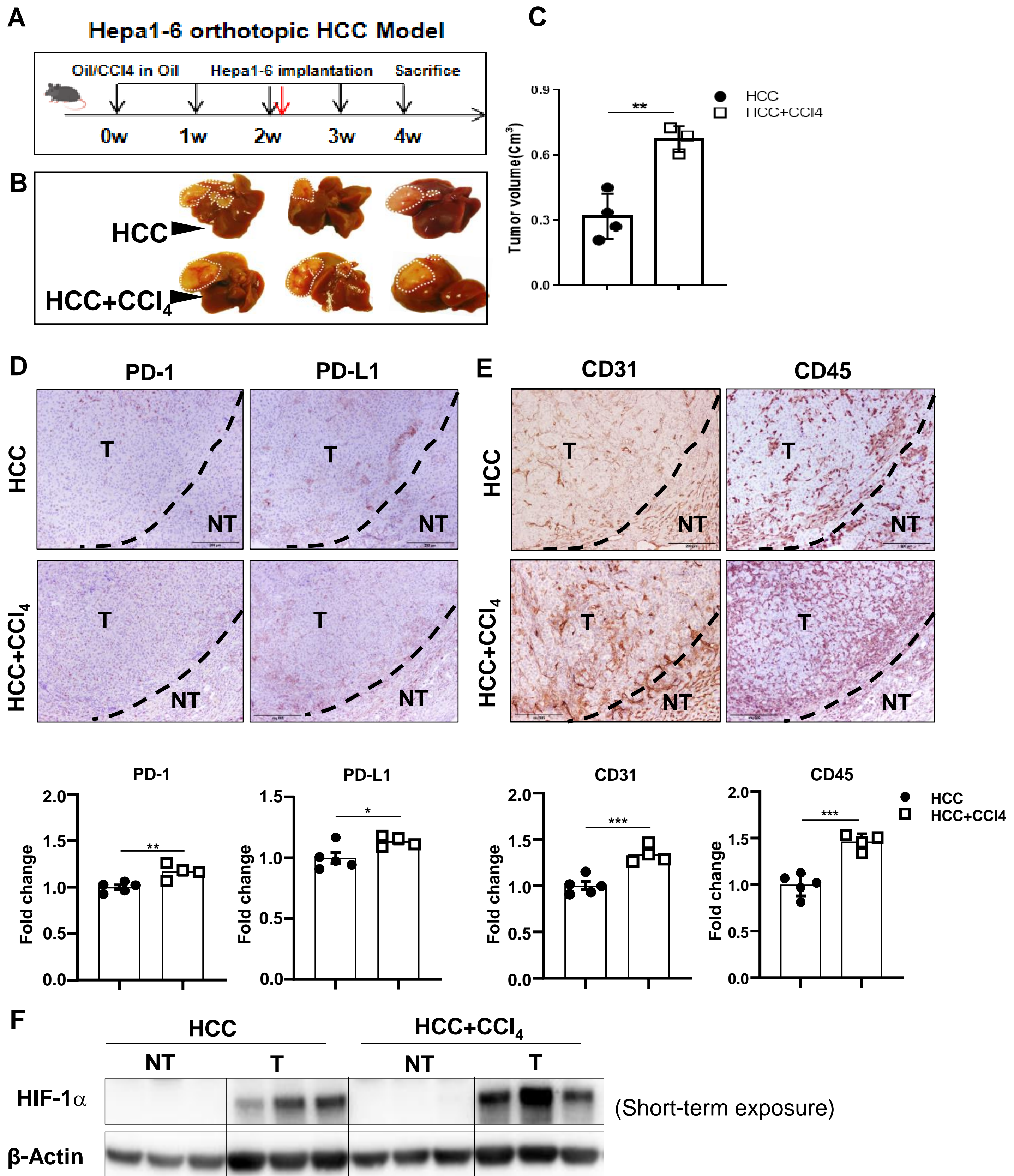
Supporting Figure S6. miR-223 inhibits PD-1/PD-L1 activation under hypoxic condition. **(A)** RT-qPCR analysis of *Cd274* mRNA in RAW 264.7 macrophages after miR-223 mimics transfection in HCC cells under normoxia or hypoxia. Notes: 'Ctrl' means the cell without any stimuli; '+NC' means 'transfected with negative control mimics'; '+miR-223' means 'transfected with miR-223 mimics'. **(B, C)** *Pdcd1* and *Cd274* expression in primary mouse splenocytes and macrophages, representatively, after miR-223 mimics/NC mimics transfection in HCC cells under normoxia or hypoxia. **(D, E)** Gating strategy used in the FACS analyses to determine mean fluorescence intensity (MFI) of PD-L1 in primary mouse macrophages, and PD-1⁺ percentage of CD3⁺ T cell derived from mouse spleen. Values represent means ± SEM. *p<0.05; **p<0.01; ***p<0.001.



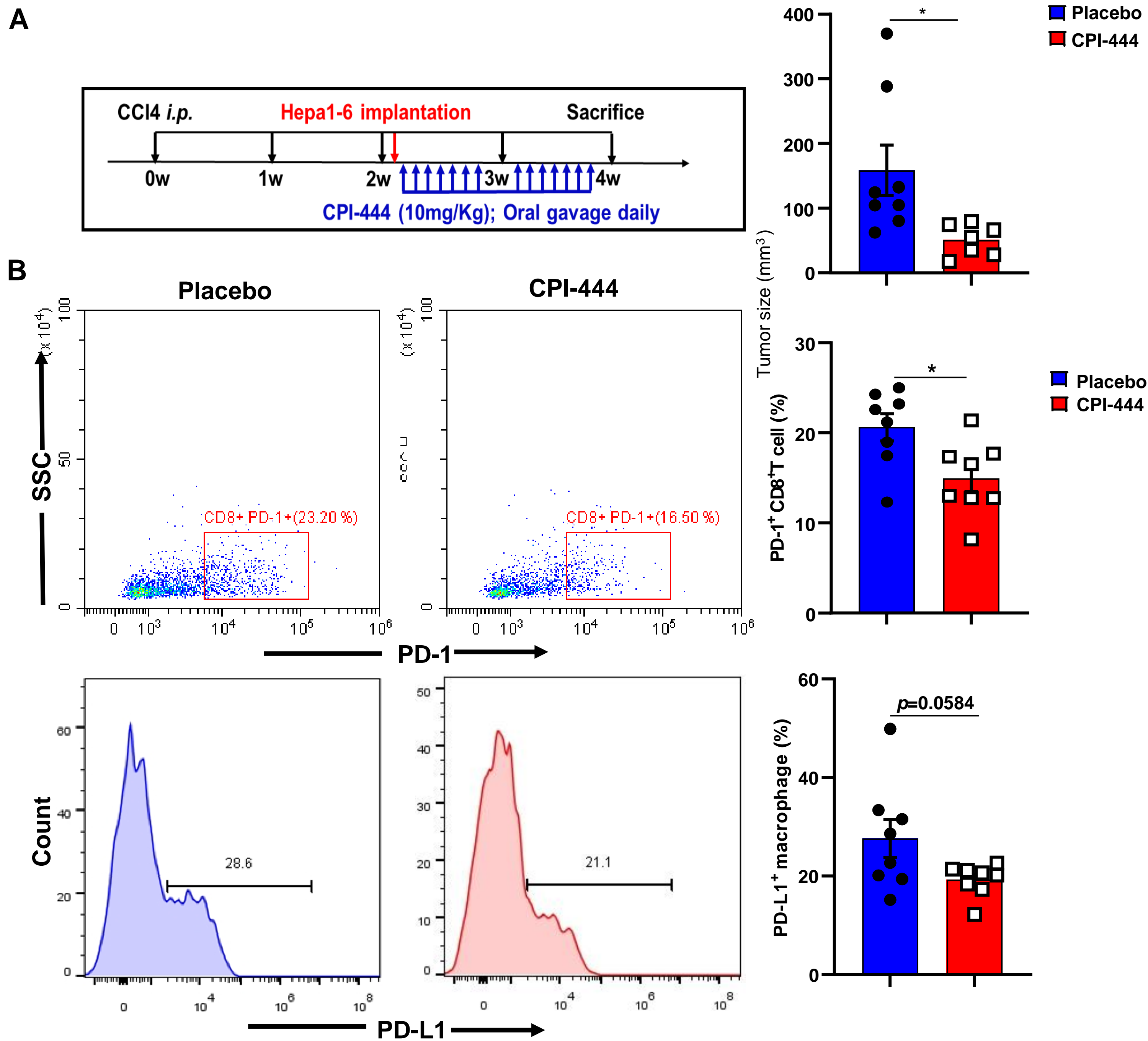
Supporting Figure S7. Hypoxia-driven CD39/CD73 signaling contributes to PD-1/PD-L1 activation. **(A)** RT-qPCR was used to determine the expression of extracellular adenosine ectonucleotidases *Cd39* and *Cd73* in Hepa1-6 cells after miR-223/NC mimics transfection under normoxia and hypoxia. **(B)** *Hif1a* shRNA transfection reduced *Cd39* and *Cd73* mRNA levels in Hepa1-6 cells under CoCl2-induced hypoxia. **(C)** Hepa1-6 cells were exposed to CoCl2 (100 μM) to induce hypoxia. Cell extracts were incubated with IgG, or HIF-1α antibodies, followed by performing ChIP assay. Fold enrichment was normalized to IgG controls (n=4) under normoxia and hypoxia. **(D)** RT-qPCR analyses of *Cd274* and *Pdcd1* mRNA levels in primary T cells and macrophages co-cultured with Hepa1-6 cells after treatment with CD39 inhibitor POM-1 (100 μM) and CD73 inhibitor AMP-CP (100 μM). Values represent means ± SEM. *p<0.05; **p<0.01; ***p<0.001.



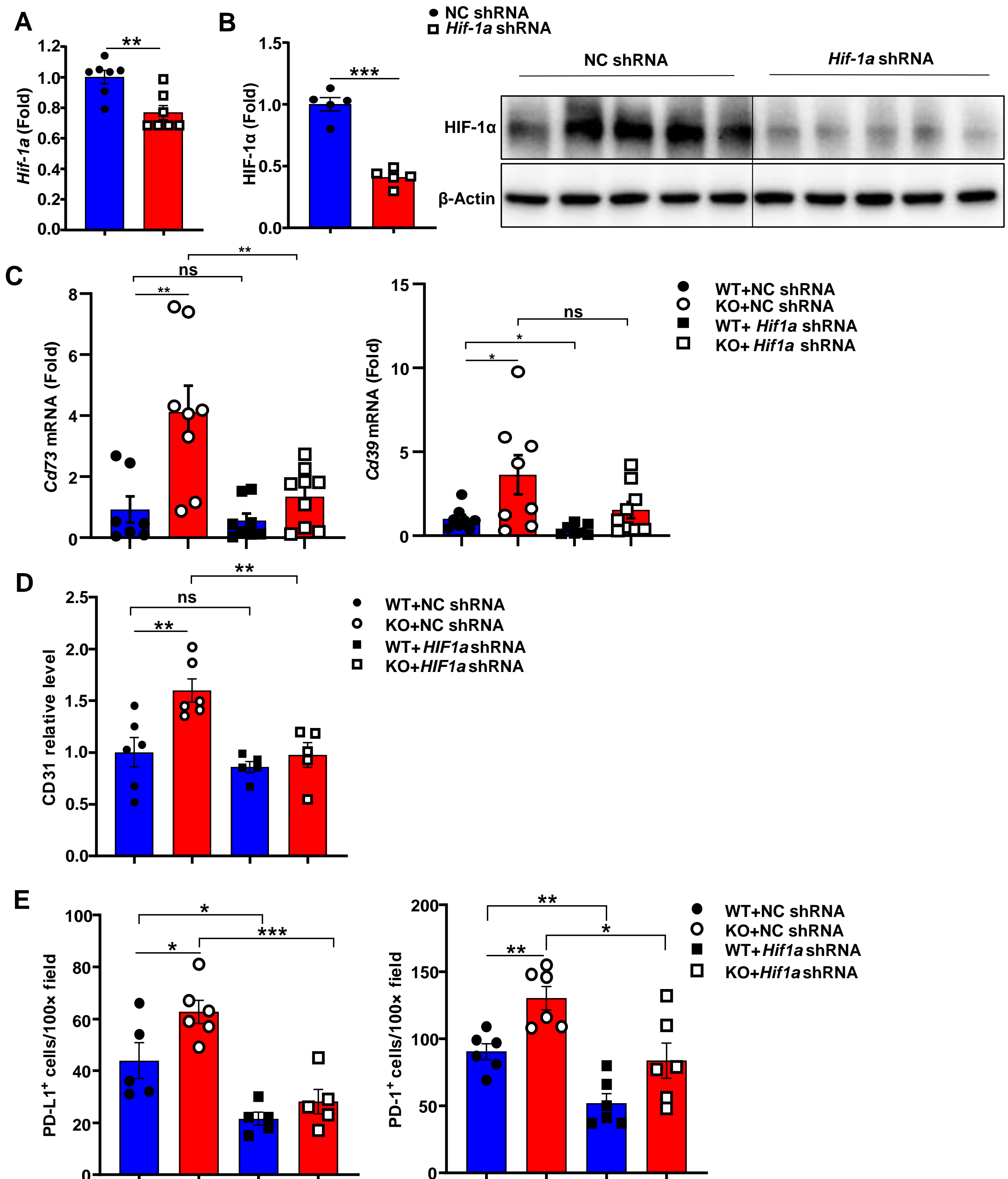
Supporting Figure S8. Inhibition of adenosine A2A receptor suppresses PD-1/PD-L1 activation *in vitro*. Primary macrophages or primary T cells were incubated with adenosine or its A2A receptor inhibitor CPI-444. **(A)** The percentage of PD-L1⁺ macrophages was analyzed by flow cytometry. **(B)** The percentage of PD-1⁺CD8⁺ T cells and CD4⁺PD-1⁺ T cells were analyzed by flow cytometry. Values represent means ± SEM. *$p<0.05$; **$p<0.01$.



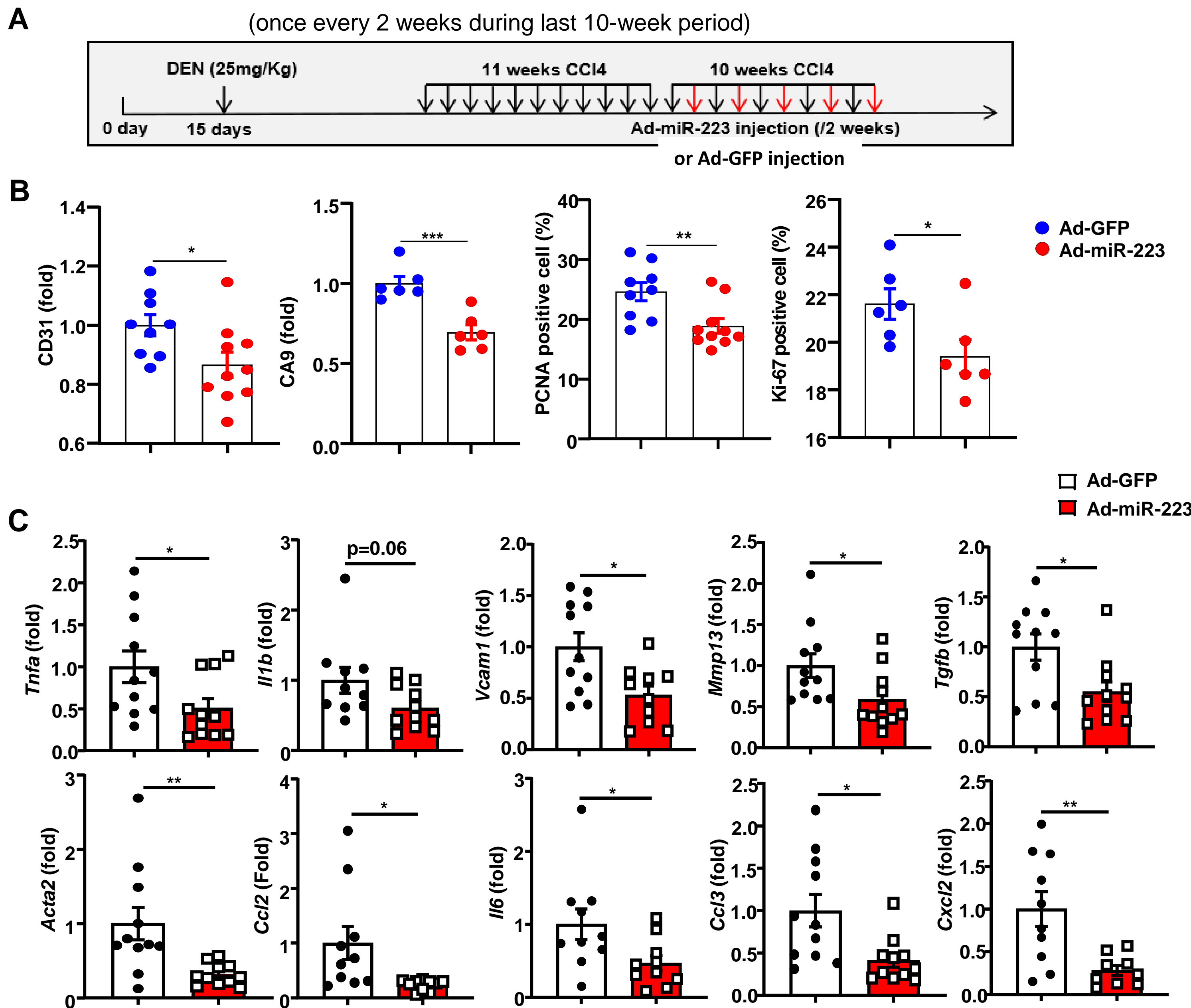
Supporting Figure S9. Establishment of Hepa1-6-derived chronic inflammation related HCC orthotopic model. **(A)** The scheme for combination of 4-week CCl_4 challenge and Hepa1-6 cell based orthotopic HCC model is shown. Black arrow: time points for CCl_4 i.p injection. Red arrow: time point for Hepa1-6 liver implantation. **(B)** Representative images for only Hepa1-6 orthotopic HCC model (written as 'HCC') and chronic inflammation associated HCC model (as shown in panel A, written as 'HCC+CCl_4') are shown. **(C)** Tumor volume between 'HCC' and 'HCC+CCl_4' models was analyzed. **(D)** Representative images and quantification of PD-1 and PD-L1 staining are shown. **(E)** Representative images and quantification for CD31 and CD45 staining are shown. Bar scale: 100μm. **(F)** Protein levels of HIF-1α in non-tumor liver samples (NT) and HCC tumor samples (T) from 'HCC' and 'HCC+CCl_4' models was determined by using western blot (short-term exposure). Values represent means ± SEM. **$p<0.01$.



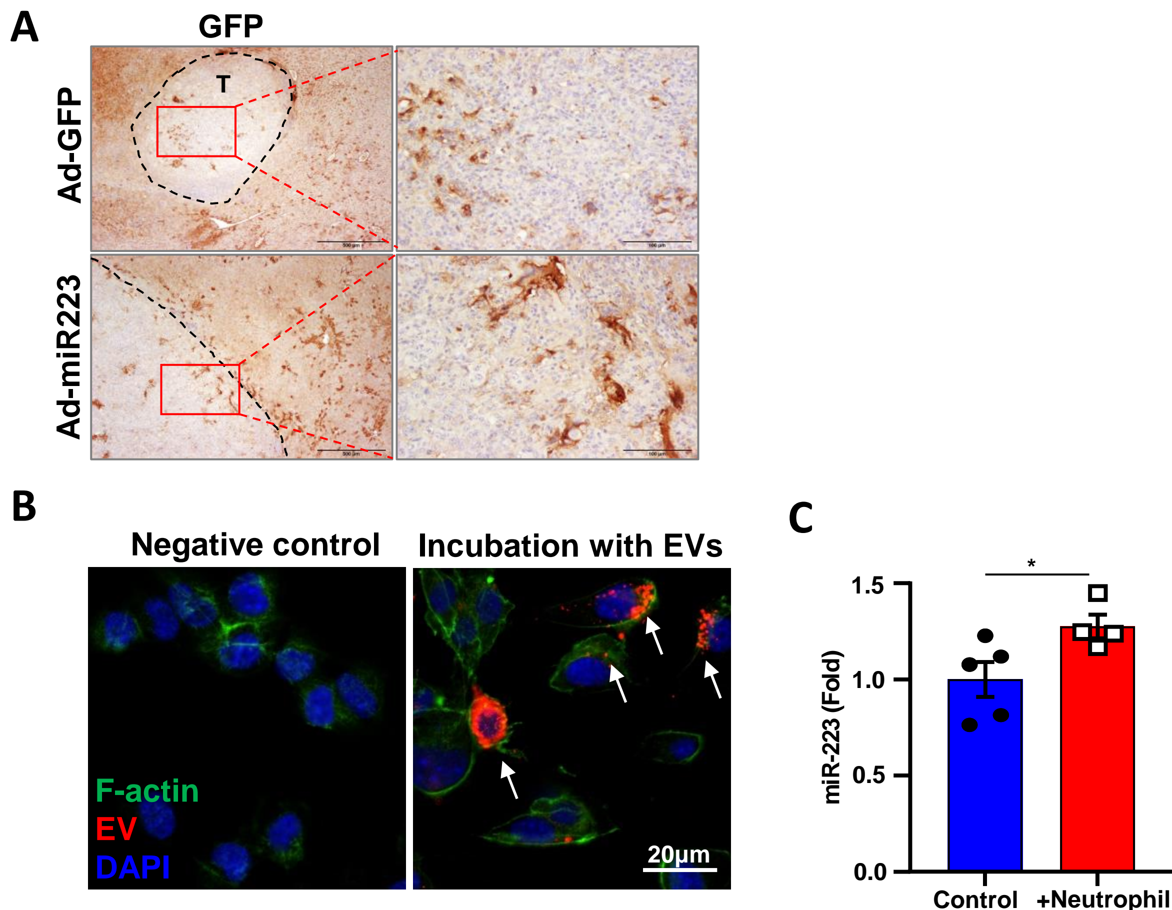
Supporting Figure S10. Suppression of adenosine pathway inhibits PD-1/PD-L1 activation in Hepa1-6-derived chronic inflammation related HCC orthotopic model. **(A)** Scheme of placebo (PBS) and A2A adenosine receptor inhibitor CPI-444 (10mg/Kg) were administrated to Hepa1-6-derived chronic inflammation related HCC orthotopic model. Right panel: tumor size measurement. **(B)** The percentage of PD-1$^+$CD8$^+$ T cells and PD-L1$^+$ macrophages were analyzed by flow cytometry. Values represent means ± SEM. *p<0.05; **p<0.01.



Supporting Figure S11. Knockdown of HIF-1α in HCC cells suppresses angiogenesis and PD-1/PD-L1 activation *in vivo*. **(A)** RT-qPCR was performed to confirm that *Hif1a* mRNA level in Hepa1-6-derived HCC orthotopic tumor samples was downregulated after *Hif1a* shRNA transfection in Hepa1-6 cells. **(B)** Measurement of HIF-1α protein levels in Hepa1-6 derived HCC orthotopic tumor samples by western blot. **(C)** RT-qPCR was performed to measure *Cd39/Cd73* mRNA level of HCC samples from *Hif1a* shRNA or negative control shRNA transfected orthotopic HCC model in WT and miR-223KO mice. **(D)** CD31 relative intensity in liver sections of *Hif1a* shRNA or negative control shRNA transfected orthotopic HCC model in WT and miR-223KO mice (100× field). **(E)** PD-L1⁺/PD-1⁺ cell number count in liver sections of *Hif1a*shRNA or negative control shRNA transfected orthotopic HCC model in WT and miR-223KO mice (100× field). Values represent means ± SEM. *p<0.05; **p<0.01; ***p<0.001.



Supporting Figure S12. Overexpression of miR-223 suppressed chronic liver inflammation and fibrosis in DEN+CCl_4 model. **(A)** The scheme for adenovirus miR-223 (Ad-miR-223) injection in DEN+CCl_4 induced HCC model. Black arrow: time points for CCl_4 i.p. injection. Red arrow: time points for Ad-miR-223 or its control Ad-GFP tail vein injection. **(B)** Relative CD31 and CA9 intensity, the percentages of PCNA and Ki67 positive cells were quantified from immunohistochemistry analyses of HCC sections in Ad-miR-223 or Ad-GFP treated mice from Figure 8C-D. **(C)** RT-qPCR was used to measure the mRNA levels of several chronic inflammation and fibrosis related genes. Values represent means ± SEM. *$p<0.05$; **$p<0.01$, ***$p<0.001$.



Supporting Figure S13. Overexpression of miR-223 in Hepa1-6 derived chronic inflammation related HCC model decreases the infiltration of PD-1/PD-L1⁺ cells. **(A)** Representative images of GFP staining in HCC sections are shown. Tumor region was labeled with 'T'; tumor border was depicted with black dash line. **(B)** Hepa1-6 cells were *in vitro* pretreated with PA (0.3 mM) for 18 hours, and then were incubated with neutrophil derived extracellular vesicles (EVs) for 24 hours; Representative images of cellular skeleton F-actin (green); EV (DiD-labeled; red); DAPI (blue). Scale bar: 20 μm. **(C)** Hepa1-6 cell was co-cultured with primary neutrophil for 6h, and miR-223 was measured by RT-PCR. Values represent means ± SEM. *p<0.05; **p<0.01.