Supplementary Materials for

Targeting CD93 on Monocytes revitalizes Anti-Tumor Immunity by Enhancing the Function and Infiltration of CD8$^+$ T Cells

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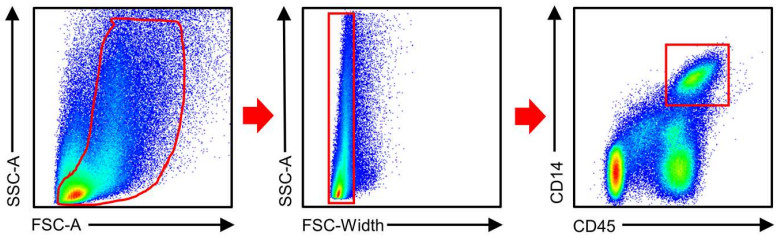
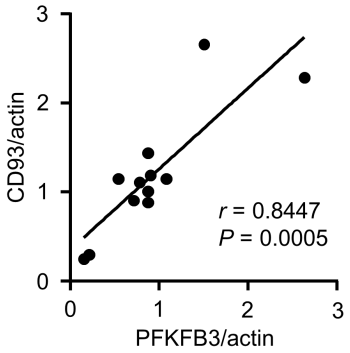
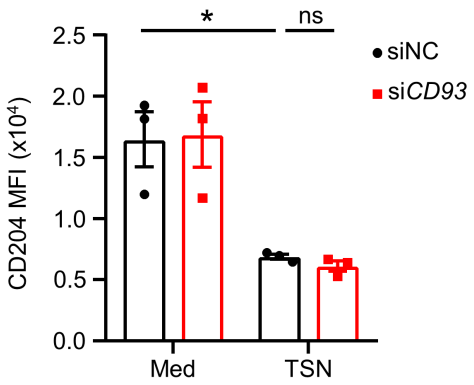


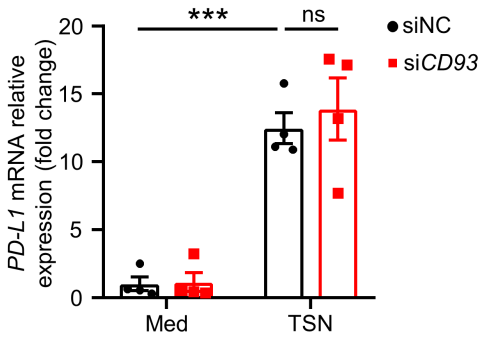
Supplementary Figure S1. Gating strategies for Fluorescence Activated Cell Sorting (FACS) of CD14$^+$ monocytes from HCC tissues.



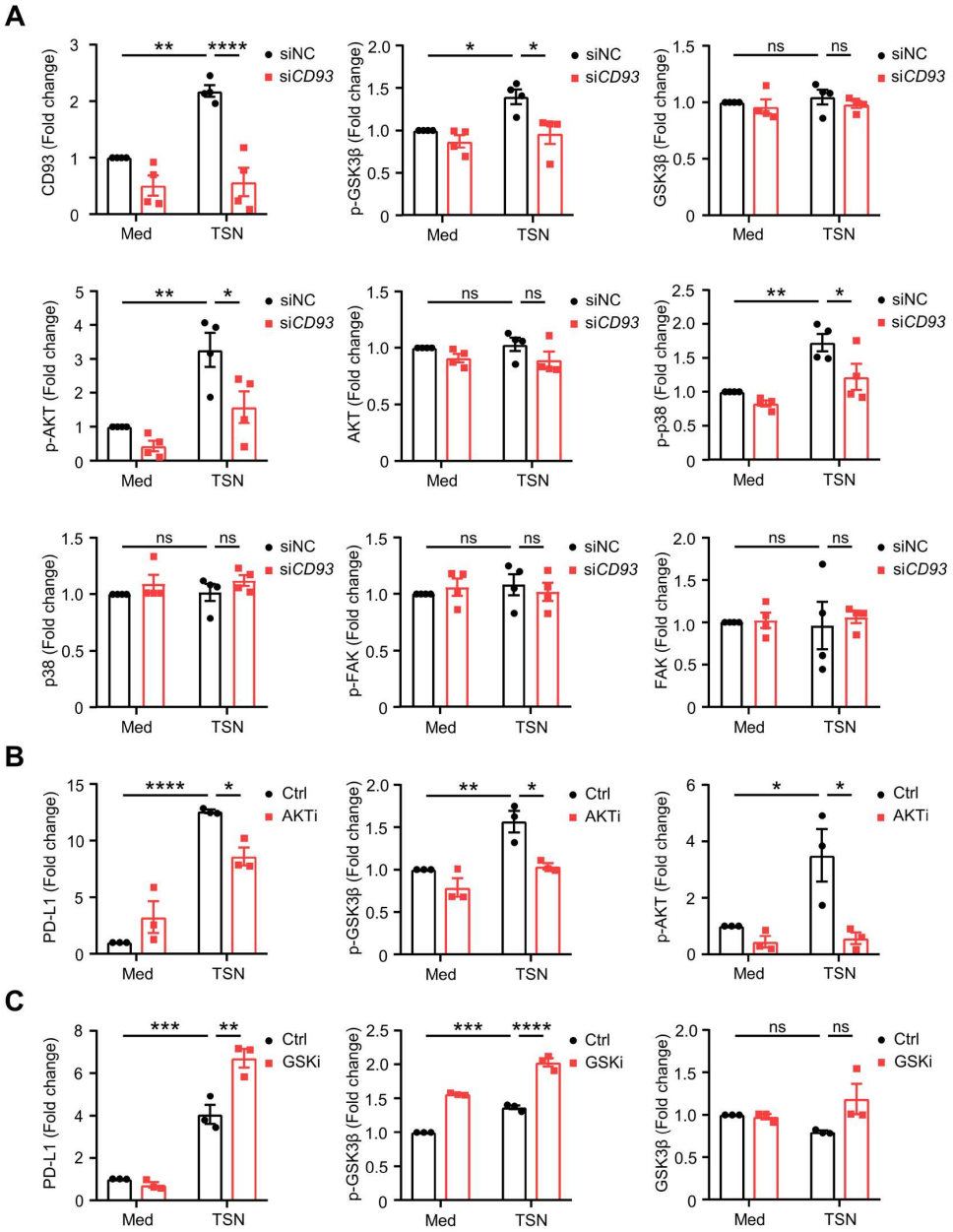
Supplementary Figure S2. Correlation between CD93 and PFKFB3 expression in HCC tumor purified-monocytes. $CD14^+$ cells were purified from tumor tissues of 12 patients with HCC. Correlations between the expression levels of CD93 and PFKFB3 in these cells were analyzed via Western blotting. The following statistical analyses were performed: Pearson's correlation and linear regression analysis.



Supplementary Figure S3. si*CD93* does not impact the expression of CD204 by monocytes. $CD14^+$ cells were purified from the peripheral blood of healthy donors. Cells were transfected with control siRNA (siNC) or si*CD93* before being treated with medium (Med) or HepG2 TSN for 24 hours. Their levels of CD204 expression were determined by flow cytometry (n = 3). Results are expressed as mean ± SEM. **P* < 0.05. The following statistical analyses were performed: two-way ANOVA.

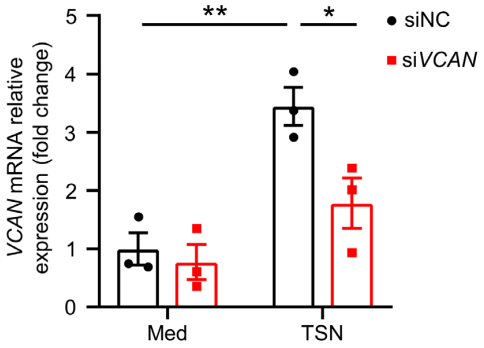


Supplementary Figure S4. si*CD93* does not impact the mRNA levels of PD-L1 in monocytes. $CD14^+$ cells were purified from the peripheral blood of healthy donors. Cells were transfected with control siRNA (siNC) or si*CD93* before being treated with medium (Med) or HepG2 TSN for 24 hours. Their mRNA levels of *PD-L1* expression were determined by Q-PCR (n = 4). Results are expressed as mean ± SEM. ****P* < 0.001. The following statistical analyses were performed: two-way ANOVA.

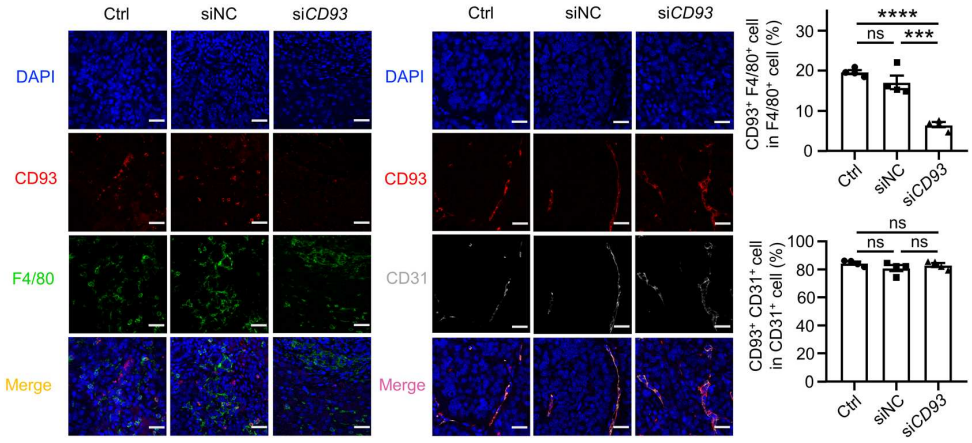


Supplementary Figure S5. CD93 increases levels of PD-L1 via the AKT-GSK3β signaling pathway. CD14^{+} cells were purified from the peripheral blood of healthy donors. **A**, Cells were transfected with siNC or si*CD93*, and left untreated or treated with HepG2 TSN for 24 hours. Their levels of CD93, p-GSK3β, GSK3β, p-AKT, AKT, p-p38, p38, p-FAK, and FAK expression, analyzed by western blotting, were quantified. n = 4. **B-C**, Cells were left untreated or treated with HepG2 TSN in the presence or

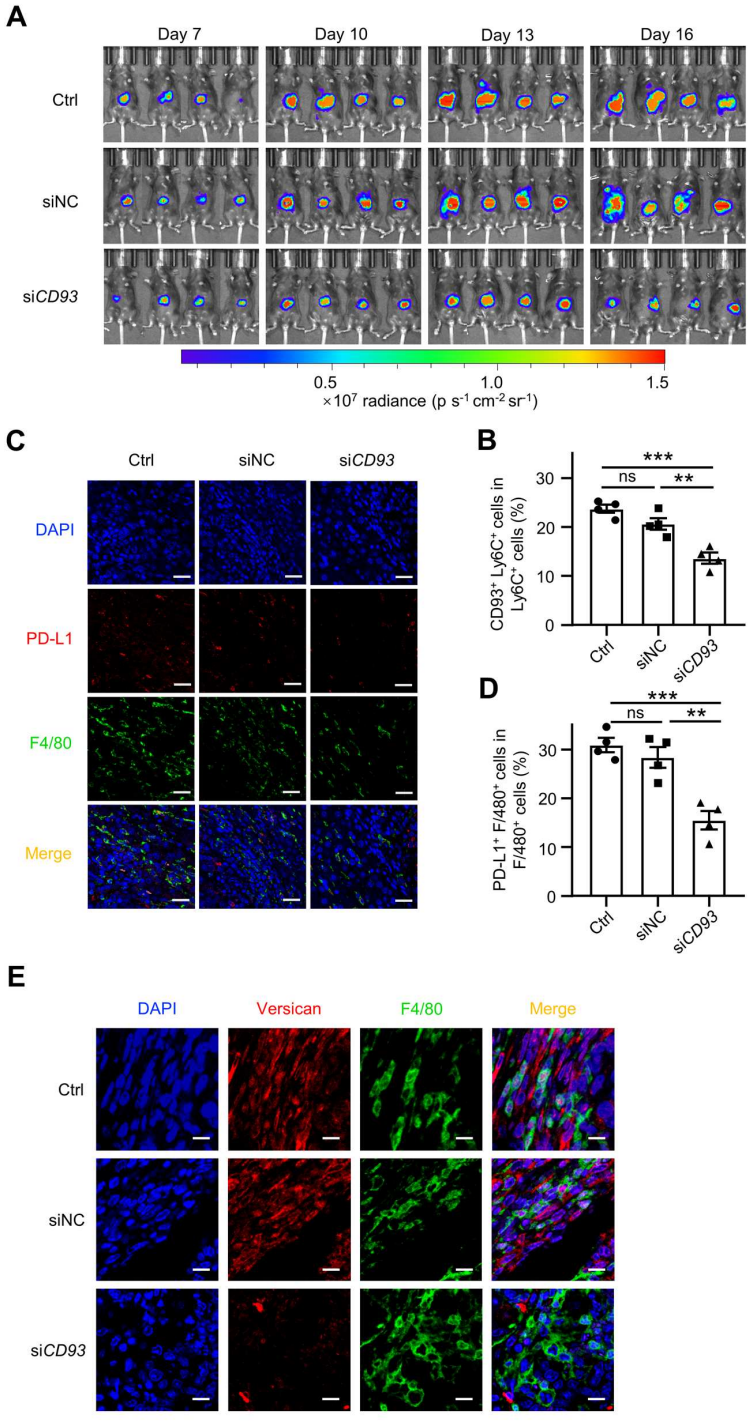
absence of AKTi (5 μM) for 24 hours, or LiCl (20 mM) for 24 hours. Their levels of PD-L1, p-GSK3β, GSK3β, and p-AKT expression, as determined by western blotting, were quantified. n = 3. Results are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. The following statistical analyses were performed: two-way ANOVA.



Supplementary Figure S6. Confirmation of the effects of si*VCAN* RNA. $CD14^+$ cells were purified from the peripheral blood of healthy donors. Cells were transfected with control siRNA (siNC) or si*VCAN* before being treated with medium (Med) or HepG2 TSN for 48 hours. Their levels of versican expression were determined by Q-PCR (n = 3). Results are expressed as mean ± SEM. **P* < 0.05, ***P* < 0.01. The following statistical analyses were performed: two-way ANOVA.

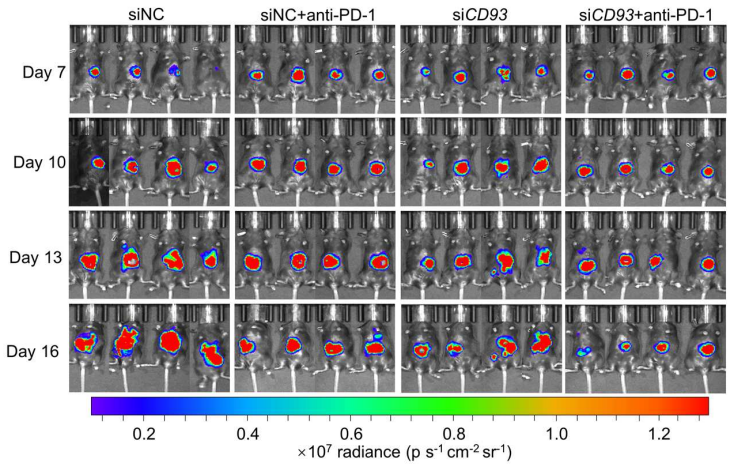


Supplementary Figure S7. si*CD93* preferentially targeted CD93 expression on monocytes/macrophages instead of endothelial cells in mice in vivo. C57BL/6 mice with established orthotopic, Luminescence-positive, Hepa1-6 tumors were injected with siNC-containing liposome, or si*CD93*-containing liposome via tail vein. Levels of CD93 expression on F4/80$^+$ cells or CD31$^+$ cells were evaluated through IF staining (n = 4). Scale bar = 25 μm. Results were expressed as mean ± SEM. ***P < 0.001, ****P < 0.0001. The following statistical analyses were performed: one-way ANOVA.



Supplementary Figure S8. Targeting CD93⁺ monocytes inhibits tumor progression in mice in vivo. C57BL/6 mice with established orthotopic, Luminescence-positive, Hepa1-6 tumors were injected with siNC-containing liposome, or si*CD93*-containing

liposome via tail vein. **A**, Tumor growth was monitored by bioluminescence imaging. **B**, Levels of $CD93^{+}CD11b^{+}Ly6C^{+}$ monocytes in tumor tissues were measured on day 16 via flow cytometry (n = 4). **C-D**, Levels of $PD\text{-}L1^{+}F4/80^{+}$ cells infiltration in peritumoral tissues were measured by IF staining (n = 4). Scale bar = 25 μm. **E**, Levels of versican expression in peritumoral monocytes/macrophages were measured by IF staining (n = 4). Scale bar = 12.5 μm. Results show in **B**, **D** were expressed as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$. The following statistical analyses were performed: one-way ANOVA.



Supplementary Figure S9. Combination of si*CD93* liposome and anti-PD-1 antibodies exhibits more significant effects in prohibiting tumor growth in mice in vivo. C57BL/6 mice with established orthotopic, Luminescence-positive, Hepa1-6 tumors were injected with siNC-containing liposome or si*CD93*-containing liposome via tail vein in the presence or absence of anti-PD-1 antibodies. Tumor growth was monitored by bioluminescence imaging (n = 4).

Supplementary Table S1. Clinical characteristics of HCC patients

Patients characteristics	Cohort 1	Cohort 2
No. of patients	99	39
Age, years (median, range)	53, 20-81	52, 35-72
Gender (male/female)	86/14	37/2
HBsAg (negative/positive)	14/70	4/35
Cirrhosis (absent/present)	60/38	15/22
ALT, U/L (median, range)	34.3, 13.1-119.8	36.4, 6.4-863.7
AFP, ng/ml (≤25/>25)	39/49	14/25
Tumor size, cm (≤5/>5)	43/55	17/19
Tumor multiplicity (solitary/multiple)	79/20	11/28
Vascular invasion (absent/present)	57/42	18/20
Intrahepatic metastasis (no/yes)	37/61	9/24
TNM stage (I/II + III)	49/50	16/21
Tumor differentiation (I+II/III + IV)	56/41	18/20

Supplementary Table S2. Sequence of primers and siRNAs

Genes		Sequences
Human *CD93* (qPCR)	Forward	GGCAGACAGTTACTCCTGGGTT
	Reverse	GGAGTTCAAAGCTCTGAGGATGG
Human *VCAN* (qPCR)	Forward	TAGGTGGAGTGTATGTGTTG
	Reverse	AGTGTTGTTCTGTGCTCTAA
Human *CD274* (qPCR)	Forward	TGTCAGTGCTACACCAAGGC
	Reverse	ACAGCTGAATTGGTCATCCC
Human *ACTB* (qPCR)	Forward	GGATGCAGAAGGAGATCACT
	Reverse	CGATCCACACGGAGTACTTG
Mouse *Cd93* (qPCR)	Forward	GAGAATCAGTACAGCCCAACAC
	Reverse	ATGTGGCTTCCCCCTCATCTA
Mouse *Actb* (qPCR)	Forward	CCAGGTCATCACTATTGGCAAC
	Reverse	ATGTGGCTTCCCCCTCATCTA
Human *CD93* (siRNA)	Sense	GCGAAUGCUGGGUUGGCUAdTdT
	Antisense	UAGCCAACCCAGCAUUCGCdAdG
Human *VCAN* (siRNA)	Sense	GGAAAAGAUUUGAAAGAGAdTdT
	Antisense	UCUCUUUCAAAUCUUUUCCdTdT
Mouse *Cd93* (siRNA)	Sense	CCAAGAAGGAGGAGAUAAAUUdTdT
	Antisense	UUUAUCUCCUCCUUCUUGGUUdTdT
Negative control (siRNA)	Sense	UUCUCCGAACGUGUCACGUdTdT
	Antisense	ACGUGACACGUUCGGAGAAdTdT

Supplementary Table S3. Antibodies used in studies.

Name	Supplier	Cat no.	Clone no.
AF700-conjugated anti-mouse CD11b	Biolegend	101222	M1/70
BV570-conjugated anti-mouse CD45	Biolegend	103136	30-F11
FITC-conjugated anti-mouse Gr-1	eBioscience	11-5931-85	RB6-8C5
BV421-conjugated anti-mouse Ly6C	Biolegend	128031	HK1.4
PE-CF594-conjugated anti-mouse Ly6G	BD Biosciences	562700	1A8
PE/Cyanine7-conjugated anti-mouse F4/80	Biolegend	123113	BM8
APC-conjugated anti-mouse CD93	Biolegend	136509	AA4.1
PE-conjugated anti-mouse CD3	Biolegend	100206	17A2
eFluor 450-conjugated anti-mouse CD8	eBioscience	48-0081-82	53-6.7
PE-CF594-conjugated anti-mouse TNF-α	Biolegend	506346	MP6-XT22
FITC-conjugated anti-mouse IFN-γ	Thermo Fisher Scientific	53-7311-82	XMG1.2
AF700-conjugated anti-human CD14	BD Biosciences	557923	M5E2
APC-conjugated anti-human CD86	BD Biosciences	555660	2331(FUN-1)
BV421-conjugated anti-human HLA-DR	BD Biosciences	562804	G46-6
PE/Cyanine7-conjugated anti-human PD-L1	BD Biosciences	558017	MIH1
PE/Cyanine7-conjugated anti-human CD204	Biolegend	371908	7C9C20
APC-conjugated anti-human CD93	Biolegend	336119	VIMD2
Alexa Fluor 488 donkey anti-mouse IgG	Thermo Fisher Scientific	A-21202	Polyclonal
Alexa Fluor 555 donkey anti-rabbit IgG	Thermo Fisher Scientific	A-31572	Polyclonal
*InVivo*Mab anti-mouse PD-1 antibody	Bio X Cell	BE0146	RMP1-14
*InVivo*Mab rat IgG2a isotype control antibody	Bio X Cell	BE0089	2A3
Rabbit anti-mouse Versican	Abcam	ab177480	EPR12277
Rabbit anti-mouse F4/80	Cell Signaling Technology	70076	D2S9R
Rat anti-mouse Ly6C	Abcam	ab15627	ER-MP20
Rabbit anti-mouse CD31	Cell Signaling Technology	77699	D8V9E
Rabbit anti-mouse CD93	Abmart	PK40533	polyclonal
Rabbit anti-mouse PD-L1	Cell Signaling Technology	13684	E1L3N

Rabbit anti-human CD93	Atlas antibodies	HPA009300	polyclonal
Mouse anti-human CD31	Zsbio	ZM-0044	UMAB30
Mouse anti-human CD68	DakoCytomation	M0876	PG-M1
Rabbit anti-human CD8	Zsbio	ZA-0508	SP16
Mouse anti-human α-SMA	Zsbio	ZM-0003	UMAB237
Rabbit anti-human phospho-p38	Cell Signaling Technology	4511	D3F9
Rabbit anti-human p38	Cell Signaling Technology	9212	polyclonal
Rabbit anti-human phospho-GSK-3β	Cell Signaling Technology	5558	D85E12
Rabbit anti-human GSK-3β	Cell Signaling Technology	9315	27C10
Rabbit anti-human phospho-FAK	Cell Signaling Technology	3283	polyclonal
Rabbit anti-human FAK	Cell Signaling Technology	71433	D5O7U
Rabbit anti-human phospho-Akt	Cell Signaling Technology	4691	C67E7
Rabbit anti-human Akt	Cell Signaling Technology	4060	D9E
Rabbit anti-human phospho-Erk1/2	Cell Signaling Technology	4370	D3F9
Rabbit anti-human Erk1/2	Cell Signaling Technology	4695	137F5
Rabbit anti-human PD-L1	Cell Signaling Technology	13684	E1L3N
Rabbit anti-human Versican	Abcam	ab177480	EPR12277
Rabbit anti-human PFKFB3	Abcam	ab181861	EPR12594
HRP-linked goat anti-rabbit IgG antibodies	Cell Signaling Technology	7074	polyclonal
HRP-linked goat anti-mouse IgG antibodies	Cell Signaling Technology	7076	polyclonal
Mouse anti-human β-actin	Boster	BM0627	AC-15

Supplementary Table S4. Reagents used in studies

Name	Supplier	Cat no.
DMEM	Thermo Fisher Scientific	C11995500BT
RPMI 1640 medium	Thermo Fisher Scientific	C11875500BT
FBS	Gibco	10099-141
BSA	Biofroxx	4240GR250
Penicillin	GENVIEW	AP231
Streptomycin	GENVIEW	AS325
Hepes	Sigma-Aldrich	H4034
DNase I	Sigma-Aldrich	DN25
Hyaluronidase	Sigma-Aldrich	H1136
Collagenase IV	Sigma-Aldrich	C5138
Collagenase XI	Sigma-Aldrich	C7657
DMSO	Merck Millipore	317275
Cultrex Basement Membrane Extract	R&D Systems	3432-005-01
DAPI	Roche	10236276001
Human $CD14^+$ MicroBeads	Miltenyi Biotec	130-050-201
Human $CD8^+$ MicroBeads	Miltenyi Biotec	130-045-201
TRIzol reagent	Thermo Fisher Scientific	AM9738
5X All-In-One RT MasterMix	abm	G492
SYBR Green Real-Time PCR Master Mix	TOYOBO	QPS-201
Leukocyte Activation Cocktail, with BD GolgiPlug	BD Biosciences	550583
IntraPrep Permeabilization Reagent	Beckman Coulter	A07803
Tyramide signal amplification kit	Panovue	PPK007100100
P3 primary cell 4D-Nucleofector X kit	Lonza	V4XP-3024
Human TNFα ELISA kit	eBioscience	88-7346-86
Human IL6 ELISA kit	eBioscience	88-7066-88
Human IL1β ELISA kit	eBioscience	88-7261-88

Recombinant Human Versican	Raybiotech	230-00833
Collagen I	Corning	354236
Hyaluronan	R&D Systems	GLR004
2-Deoxy-D-glucose (2DG)	Sigma-Aldrich	D8375
Hyaluronan	R&D Systems	GLR004
3PO	Sigma-Aldrich	SML1343
AG490	MCE	HY-12000
JSH-23	Merk Millipore	481408
SP600125	MCE	HY-12041
U0126	MCE	HY-12031A
MK-2206	Selleck	S1078
C12-200	MCE	HY-145405
cholesterol	Sigma-Aldrich	C8503
DSPC	Avanti	850365P
DMG-PEG2000	Avanti	880151P
LiCl	Sigma-Aldrich	L9650

Supplementary Table S5. Univariate and multivariate analysis of factors associated with overall survival and tumor recurrence of patients with HCC.

	OS				TR			
	Univariate	Multivariate			Univariate	Multivariate		
Variables	*P* value	HR	95% CI	*P* value	*P* value	HR	95% CI	*P* value
Age, years (>53 vs ≤53)	0.939			n.a.	0.294			n.a.
Gender (male vs female)	0.132			n.a.	0.495			n.a.
HBsAg (positive vs negative)	0.642			n.a.	0.090			n.a.
Cirrhosis (present vs absent)	0.600			n.a.	0.192			n.a.
ALT, U/L (>40 vs ≤40)	0.146			n.a.	0.415			n.a.
AFP, ng/ml (>25 vs ≤25)	0.155			n.a.	**0.007**	1.984	0.791-5.513	0.161
Tumor size, cm (>5 vs ≤5)	0.189			n.a.	0.197			n.a.
Tumor multiplicity (multiple vs solitary)	0.512			n.a.	0.144			n.a.
Vascular invasion (present vs absent)	0.126			n.a.	**0.005**	0.284	0.046-1.297	0.137
Intrahepatic metastasis (no/yes)	0.467			n.a.	0.736			n.a.
TNM stage (II + III vs I)	0.074			n.a.	**0.018**	1.695	0.348-10.300	0.539
Tumor differentiation (III + IV vs I + II)	**0.002**	3.055	1.246-8.121	**0.018**	0.122			n.a.
Peritumoral $CD93^{+}CD68^{+}$cells (high vs low)	**0.004**	0.321	0.128-0.733	**0.010**	**0.019**	0.396	0.168-0.871	**0.026**

Cox proportional hazards regression model. Variables used in multivariate analysis were adopted by univariate analysis. The bold terms represent statistical significance (< 0.05).

Abbreviations: HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AFP, α-fetoprotein; TNM, tumor-node-metastasis; OS, overall survival; TR, tumor recurrence; HR, hazard ratio; CI, confidence interval; n.a., not adopted.