Science Advances
AAAS

Supplementary Materials for

Cancer cells produce liver metastasis via gap formation in sinusoidal endothelial cells through proinflammatory paracrine mechanisms

Truong Huu Hoang *et al.*

Corresponding author: Norifumi Kawada, kawadanori@omu.ac.jp

Sci. Adv. **8**, eabo5525 (2022)
DOI: 10.1126/sciadv.abo5525

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Methods

Immunofluorescence staining

Primary LSECs were plated onto 12 mm micro cover glass in the bottom of each well of a 24 well-plate. The cells were washed twice for 5 min with PBS and fixed with 4% paraformaldehyde solution for 30 min at room temperature. The cells were permeabilized with 0.25% Triton X-100 in PBS at room temperature for 10 min, blocked with 10% BSA in PBS for 1 h at room temperature, incubated with primary antibodies (list of antibodies in **table S5**) diluted in blocking solution overnight at 4°C, and then incubated with secondary antibody at room temperature for 1 h. The cells were carefully rinsed three times with PBS between each step. The cover glasses were mounted in ProLong Gold Antifade reagent (Thermo Fisher Scientific, Invitrogen).

Primary mouse LSECs and hepatocyte isolation

Primary mouse LSECs and hepatocytes were isolated in our laboratory from C57BL/6J mice using a modified version of a previously published protocol. Briefly, normal livers were perfused for 5 min with SC-1 solution consisting of 8,000 mg/l NaCl; 400 mg/l KCl; 88.17 mg/l $NaH_2PO_4 \cdot 2H_2O$; 120.45 mg/l Na_2HPO_4; 2,380 mg/l HEPES; 350 mg/l $NaHCO_3$; 190 mg/l EGTA; and 900 mg/l glucose (pH 7.3). The livers were then digested at 42°C for 5 min with 0.2% collagenase (Fujifilm Wako) and 0.056% $CaCl_2$ dissolved in SC-2 solution consisting of 8,000 mg/l NaCl; 400 mg/l KCl; 88.17 mg/l $NaH_2PO_4 \cdot 2H_2O$; 120.45 mg/l Na_2HPO_4; 2,380 mg/l HEPES; 350 mg/l $NaHCO_3$; and 560 mg/l $CaCl_2 \cdot 2H_2O$ (pH 7.3). The digested livers were then suspended in SC-1 solution. The resulting suspension was filtered through a 70 μm nylon cell strainer (Corning, Glendale, AZ, USA) and then centrifuged at 100 × *g* and 4°C for 5 min, which produced an LSEC-enriched fraction in the supernatant and a hepatocyte fraction in the pellet. To collect hepatocytes, we suspended the pellet in 5.5 ml DF-10, gently pipetted the suspension, and then added 4.5 ml Percoll solution (Cytiva) and softly inverted the tube. The suspension was centrifuged at 1,000 rpm and 4°C for 10 min, and the resulting pellet was then washed once with DF-10. The LSEC-enriched supernatant was transferred to a new tube and incubated at room temperature for 5 min to precipitate contaminating hepatocytes at the bottom of the tube. The LSECs were pelleted by centrifugation at 2,500 rpm and 4°C for 10 min and then suspended in 3 ml DF-10. The resulting cell suspension was subjected to isopycnic centrifugation with Percoll solution to produce gradients (50%, 25% in SC-1 solution). The LSECs were separated from Kupffer cells by placing the supernatant in

a polystyrene petri dish and incubating the dish in a humidified tissue culture incubator at 37°C for 8 min, during which the Kupffer cells attached to the plate. The medium was then collected and centrifuged at 2,500 rpm and 4°C for 10 min to enrich the LSECs in the pellet. Red blood cells that accompanied the LSECs in the pellet were removed by pipetting with a blood-cell removal medium consisting of $KHCO_3$ 10 mM, NH_4Cl 155 mM, and EDTA 0.1 mM, followed by incubation on ice for 5 min. Finally, the LSECs were extracted by centrifugation at 2,500 rpm and 4°C for 10 min and suspended using an EGM™-2 MV Microvascular Endothelial Cell Growth Medium-2 SingleQuots™ kit (LSEC medium, Lonza, Basel, Switzerland).

Western blot analyses

Conditioned medium collected from LSECs and Hepa1-6 cells in mono-culture, direct co-culture, or indirect co-culture using inserts was conducted similar as shown in zymography assay. Cells were lysed in radioimmunoprecipitation buffer containing protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (Sigma-Aldrich). Cellular protein samples 7 (1-3 μg) were separated by 5–20% SDS-polyacrylamide gel electrophoresis (PAGE, (DRC, Tokyo, Japan) using Precision Plus Protein™ Dual Colour standards (Bio-Rad, Hercules, CA, USA) and transferred to 0.45 μm polyvinylidene difluoride membranes (Sartorius, Göttingen, Germany). After blocking with 5% skim milk, the membranes were incubated with the primary antibodies overnight at 4°C (**table S5**). The membranes were then labeled with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:2,000, Dako, Agilent Technologies, Santa Clara, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence using L-012 substrate (ImmunoStar LD, Fujifilm Wako) and documented with a Fujifilm Image Reader LAS-3000 (Fujifilm, Tokyo, Japan) coupled to image analysis software (Multi-Gauge, Fujifilm). GAPDH was used as the loading control. ImageJ was used to evaluate the band intensities for western blot analysis (NIH).

Quantitative RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Thermo Fisher Scientific) and the Direct-zol™ RNA miniPrep kit (Zymo Research, Irvine, CA, USA). The extracted

RNAs were quantified using a NanoDrop™ 2000c Spectrophotometer (Thermo Fischer Scientific). SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific) was then used to generate cDNA. Quantitative RT-PCR assays were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and the CFX96 Real-time PCR detection system (Bio-Rad) with the primers described in **table S6**. The relative expression levels were normalized to *18s* or *Gapdh* RNA expression, and fold expression changes were calculated using the $2^{-\Delta\Delta CT}$ method.

Sirius red staining

Tissue sections were stained with 0.1% (w/v) Sirius Red (Direct Red 80; Sigma-Aldrich) in a saturated aqueous picric acid solution for 1 h at room temperature to visualize collagen fibres. After staining, the sections were washed twice in 0.01 N HCl and mounted with NEW MX (Matsunami Glass) [2]. Images were captured using a BZ-X700-All-in-One fluorescence microscope (Keyence Co., Osaka, Japan).

Cytokine array

Following incubation with a detection antibody cocktail, antibody conjugation, and recommended washes, immunoblots were developed with a chemiluminescent substrate reagent kit (Fujifilm Wako), and signal was obtained using a Fusion Solo S machine (VILBER, Marne-la-Vallée Cedex 3, France). In the next step, spot findings were used to determine signal and background intensities using ImageJ software. For background correction, median background intensities were subtracted from mean signal intensities. To further calculate the differential expression, the mean signal intensity of the cytokine/chemokine spots on the co-culture membrane was divided by the mean signal intensity of the spot on the control membrane.

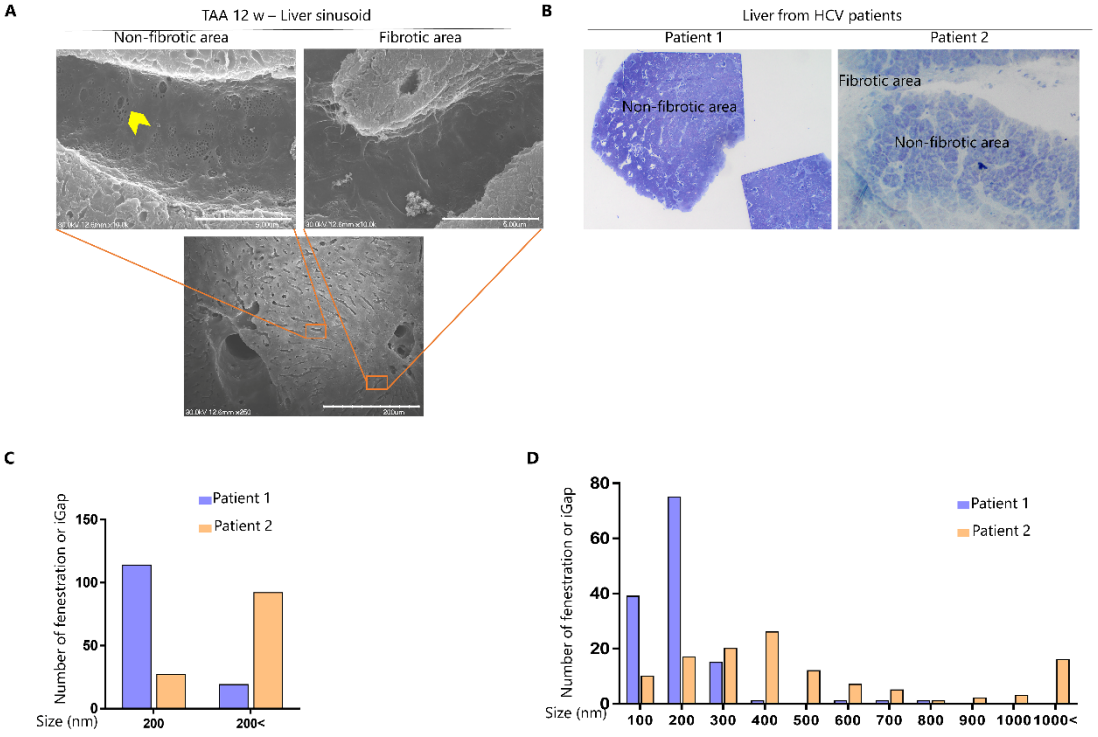

A
TAA 12 w – Liver sinusoid
Non-fibrotic area
Fibrotic area
B
Liver from HCV patients
Patient 1
Patient 2
Non-fibrotic area
Fibrotic area
Non-fibrotic area
C
Number of fenestration or iGap
Patient 1
Patient 2
150
100
50
0
Size (nm)
200
200<
D
Number of fenestration or iGap
Patient 1
Patient 2
80
60
40
20
0
Size (nm)
100
200
300
400
500
600
700
800
900
1000
1000<

Fig. S1: iGap formation in fibrotic liver in mice and human.

(A) Representative SEM images of liver sinusoid from mice after 12 weeks of TAA treatment to induce liver fibrosis. Defenestration is present in the fibrotic area (left), and iGap (yellow arrowhead) is present in the non-fibrotic area (right). (**B)** Representative images with trypan blue staining of liver biopsy specimens from two patients with HCV infection. (**C, D)** Quantification of fenestration and iGap in two patients (**C**) and the numbers of fenestrations or iGap with the indicated diameters (**D**).

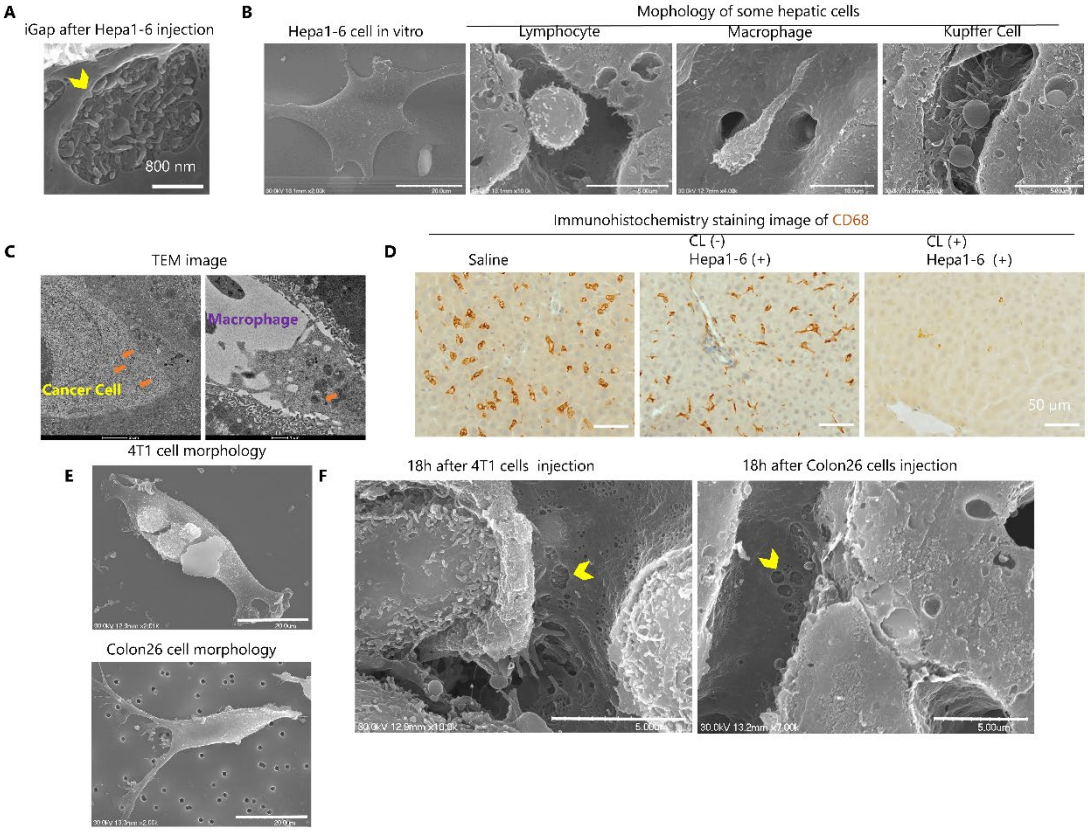

A
iGap after Hepa1-6 injection
800 nm
B
Hepa1-6 cell in vitro
Mophology of some hepatic cells
Lymphocyte
Macrophage
Kupffer Cell
C
TEM image
Cancer Cell
Macrophage
4T1 cell morphology
Colon26 cell morphology
D
Immunohistochemistry staining image of CD68
Saline
CL (-)
Hepa1-6 (+)
CL (+)
Hepa1-6 (+)
50 μm
E
F
18h after 4T1 cells injection
18h after Colon26 cells injection

Fig. S2: Morphological SEM images of cancer cells and hepatic cells.

(A) Representative SEM images of iGap in LSECs 18 h after Hepa1-6 intrasplenic injection. Scale bar, 800 nm. (**B)** Representative SEM images of a Hepa1-6 cell *in vitro* and a lymphocyte, a macrophage, and a Kupffer cell *in vivo*. Scale bars from left to right are 20 μm, 5 μm, 10 μm, and 5 μm. (**C)** Representative TEM images of a Hepa1-6 cell (left) and a macrophage (right). Orange arrows indicate mitochondria of the cell. (**D)** Representative IHC images of CD68 staining, a marker of macrophages, in liver tissue 1 day after saline or Hepa1-6 cell injection with or without intravenous CL. (**E)** Morphology of a 4T1 breast cancer cell (upper) and a Colon 26 colon cancer cell (lower) in SEM images. Scale bar, 20 μm. **(F)** Representative SEM images of iGap (yellow arrowhead) in liver sinusoid 16 h after 4T1 cell (left) and Colon26 cell (right) intrasplenic injection. Scale bar, 5 μm.

Movie S1 and S2: 3D-RT video of the interaction between Hepa1-6 cells (yellow) and LSECs (blue) *in vivo*. Interaction between the cancer cell and LSEC (S1). Cancer cell breaks through the LSEC wall (S2).

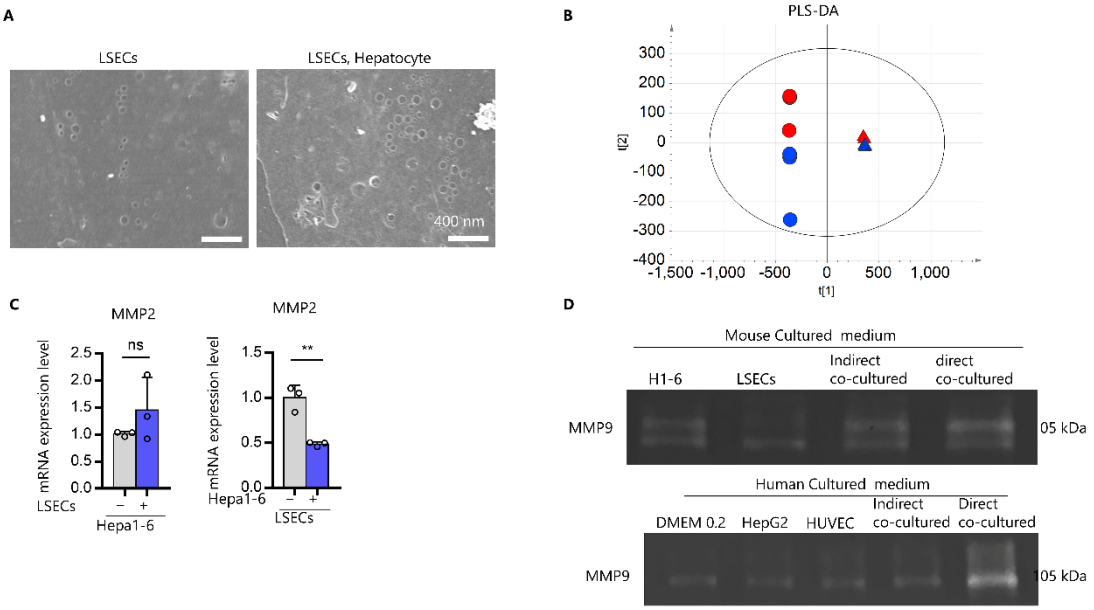
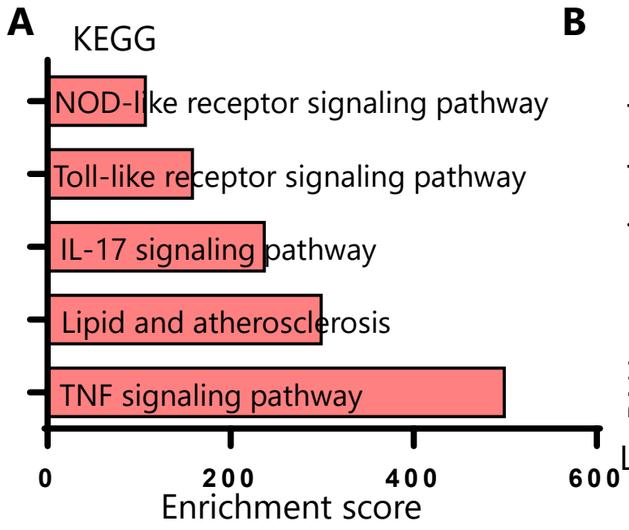
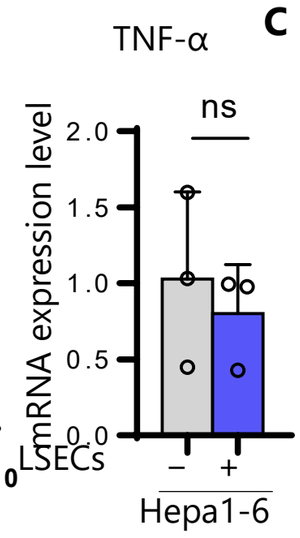
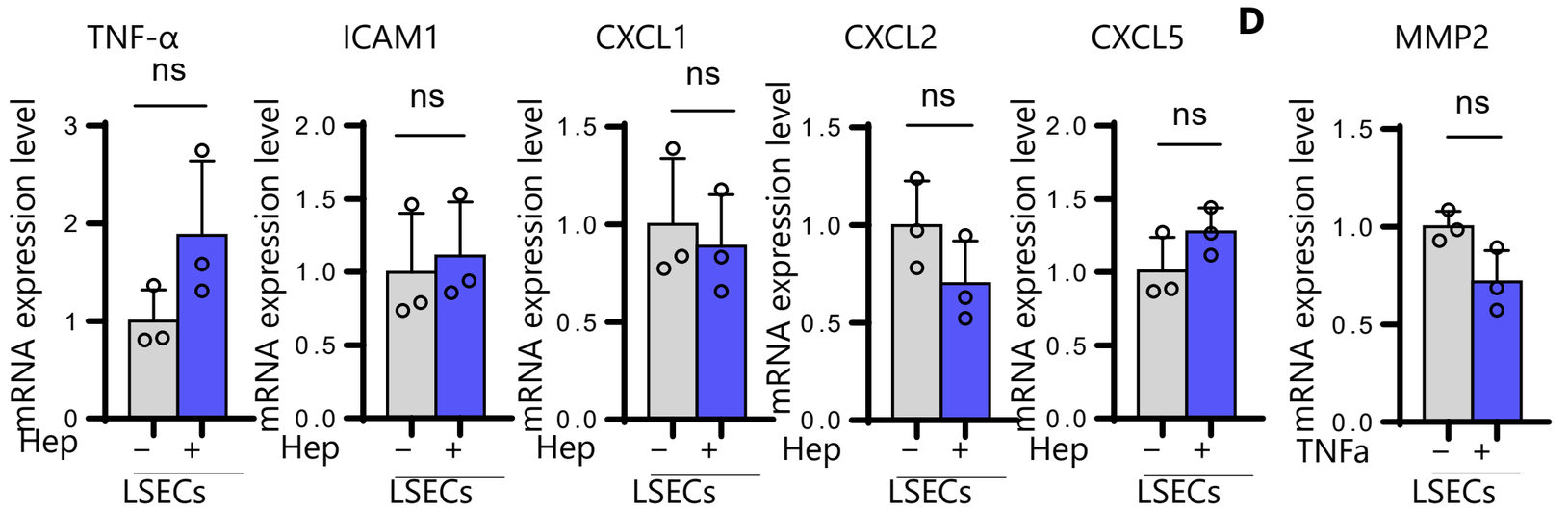

A
LSECs
LSECs, Hepatocyte
400 nm
B
PLS-DA
300
200
100
0
-100
-200
-300
-400
t[2]
-1,500 -1,000 -500 0 500 1,000
t[1]
C
MMP2
ns
mRNA expression level
2.5
2.0
1.5
1.0
0.5
0.0
LSECs
− +
Hepa1-6
MMP2
**
mRNA expression level
1.5
1.0
0.5
0.0
Hepa1-6
− +
LSECs
D
Mouse Cultured medium
H1-6
LSECs
Indirect co-cultured
direct co-cultured
MMP9
105 kDa
Human Cultured medium
DMEM 0.2
HepG2
HUVEC
Indirect co-cultured
Direct co-cultured
MMP9
105 kDa

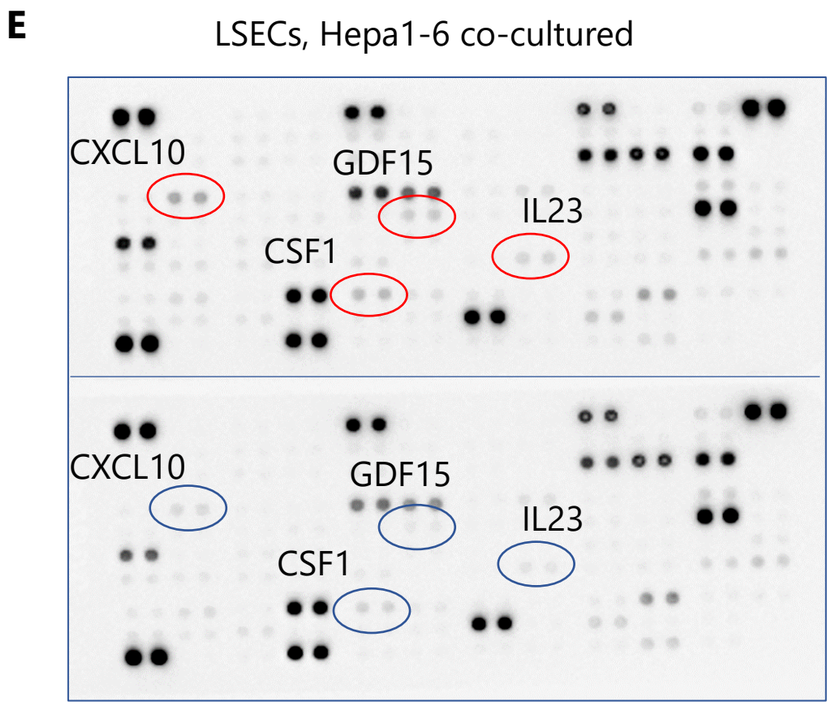
Fig. S3: MMPs activity in cell-cell interaction.

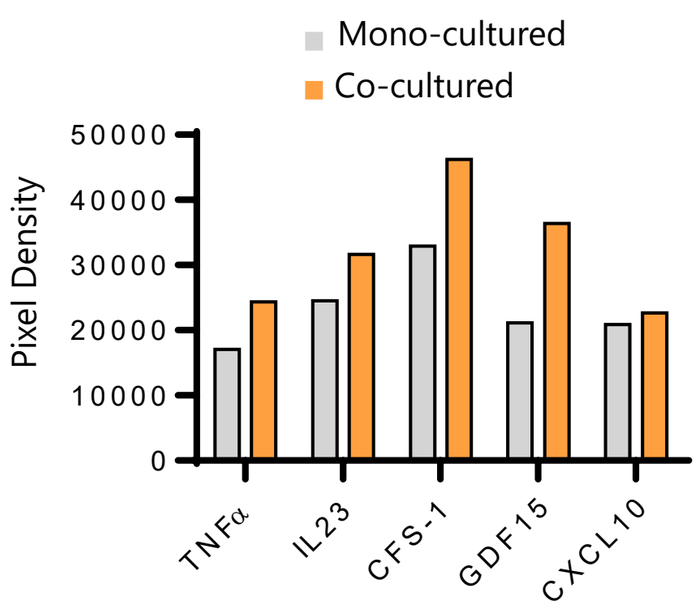
(A) Representative SEM images of iGap in LSECs after 24 h of mono-culture or co-culture with hepatocytes. Scale bars, 400 nm. (**B)** Partial least squares-discriminant analysis (PLS-DA) score plot using FPKM values for 13,525 genes shows the segregation of gene clusters between co-cultured and mono-cultured LSECs. Red, co-cultured; blue, mono-cultured; circle LSECs; triangle, Hepa1-6 cells. (**C)** Validation of MMP2 mRNA expression by qRT-PCR in Hepa1-6 cells (left) and LSECs (right). (**D)** Gelatin zymography for MMP9 activity in conditioned medium from Hepa1-6 cell and LSEC mono-cultures, Hepa1-6 cell/LSEC indirect (*via* insert) and direct co-cultures (upper blot), HepG2 cell and HUVEC mono-cultures, and HepG2/HUVEC indirect (*via* insert) and direct co-cultures (lower blot). Data are from three independent experiments performed in triplicate and presented as mean values ± s.d.; ns, not significant; ** $P< 0.01$, based on two-tailed unpaired Student's *t*-test for panel **C**.

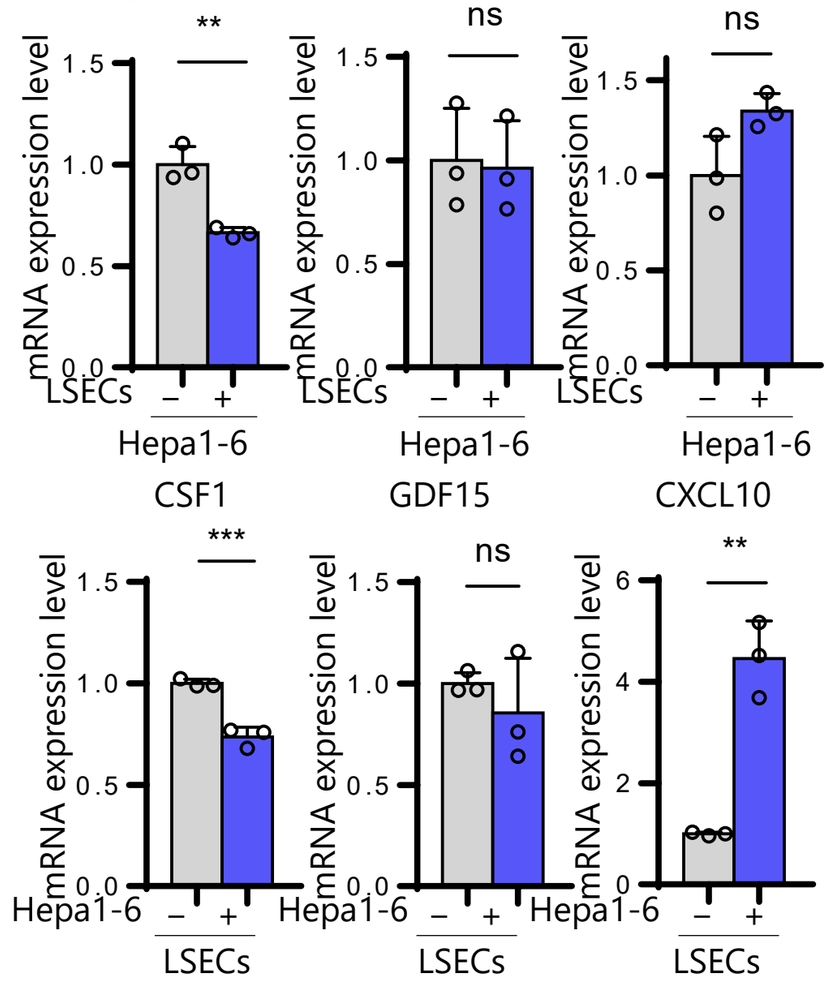

A
KEGG
NOD-like receptor signaling pathway
Toll-like receptor signaling pathway
IL-17 signaling pathway
Lipid and atherosclerosis
TNF signaling pathway
0
200
400
600
Enrichment score

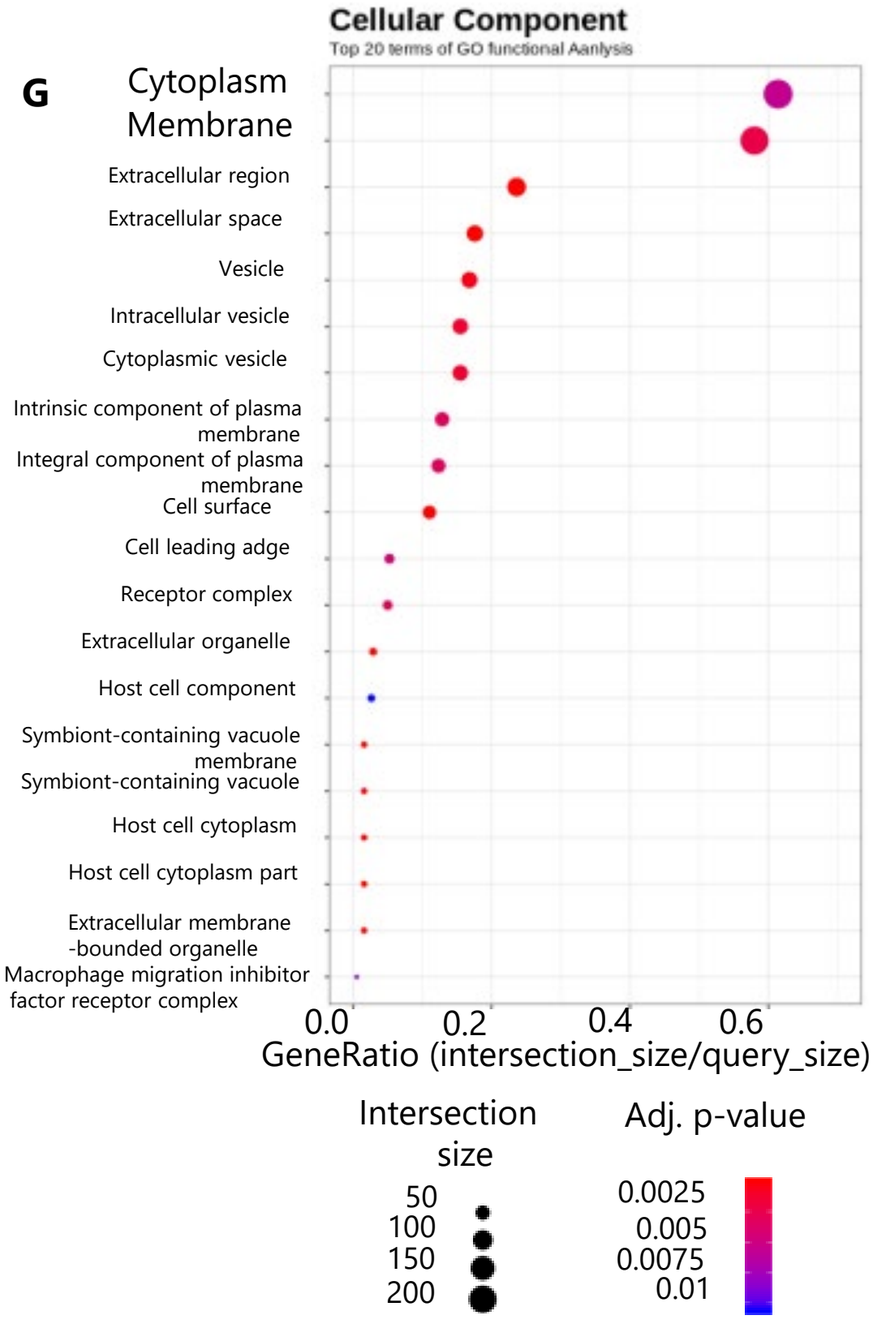

B
TNF-α
ns
mRNA expression level
LSECs
Hepa1-6


C
TNF-α
ICAM1
CXCL1
CXCL2
CXCL5
ns
mRNA expression level
Hep
LSECs
D
MMP2
TNFa


E
LSECs, Hepa1-6 co-cultured
CXCL10
GDF15
IL23
CSF1
LSECs, Hepa1-6 mono-cultured


Mono-cultured
Co-cultured
Pixel Density
TNFα
IL23
CFS-1
GDF15
CXCL10


F
CSF1
GDF15
CXCL10
**
ns
***
mRNA expression level
LSECs
Hepa1-6


G
Cellular Component
Top 20 terms of GO functional Aanlysis
Cytoplasm
Membrane
Extracellular region
Extracellular space
Vesicle
Intracellular vesicle
Cytoplasmic vesicle
Intrinsic component of plasma membrane
Integral component of plasma membrane
Cell surface
Cell leading adge
Receptor complex
Extracellular organelle
Host cell component
Symbiont-containing vacuole membrane
Symbiont-containing vacuole
Host cell cytoplasm
Host cell cytoplasm part
Extracellular membrane -bounded organelle
Macrophage migration inhibitor factor receptor complex
0.0
0.2
0.4
0.6
GeneRatio (intersection_size/query_size)
Intersection size
50
100
150
200
Adj. p-value
0.0025
0.005
0.0075
0.01

Molecular Function
Top 20 terms of GO functional Aanlysis
Binding
Protein binding
Identical protein binding
Carbohydrate derivative binding
Molecular function regulator
Signaling receptor binding
Receptor ligand activity
Signaling receptor activator activity
Receptor regulator activity
Guanyl ribonucleotide binding
Guanyl nucleotide binding
GPT binding
Cytokine receptor binding
GTPase activity
Cytokine activity
Chemokine activity
Chemokine receptor binding
CXCR chemokine receptor binding
Chemorepellent activity
Lipopeptide binding
0.00
0.25
0.50
0.75
1.00
GeneRatio (intersection_size/query_size)
Intersection size
100
200
300
Adj. p-value
0.001
0.002
0.003
0.004
0.005

H
ICAM1
***
mRNA expression level
HepG2
HUVEC
I
Hepa1-6
LSECs
ICAM1
shICAM1
T.E.M: Transendothelial migration

Fig. S4: Validation of TNF-α signal in iGap formation in LSECs.

(A) RNA-seq analysis of differentially upregulated genes in LSECs based on the Enrichr NCI-Nature pathway database for KEGG 2020. (**B)** Validation of *Tnf-α* mRNA expression by qRT-PCR in Hepa1-6 cells. (**C)** Validation by qRT-PCR of expression of *Tnf-α* and its signaling pathway-related genes *Mmp9, ICam1, Cxcl2, Cxcl5*, and *Cxcl1* in LSECs co-cultured with hepatocytes (Hep). **(D)** Validation of *Mmp2* mRNA expression by qRT-PCR in LSECs after treatment with 10 ng/ml TNF-α for 24 h. (**E)** Re-confirm cytokine array for soluble factors (circled) TNF-α, IL23, CSF1, GFD15, and CXCL10 in combined media from mono-cultured LSECs and Hepa1-6 cells and in medium from co-cultured LSECs and Hepa1-6 cells. The bottom graph shows the pixel density with grey and orange colors indicated to mono- and co-cultured, respectively. (**F)** Validation by qRT-PCR of mRNA expression of *Cfs1*, *Gdf15*, and *Cxcl10* in LSECs and Hepa1-6 cells under co-culture conditions. (**G)** GO terms related to cellular components and molecular functions for DEGs in LSECs co-cultured with Hepa1-6 cells. (**H)** Validation of *ICAM1* mRNA expression by qRT-PCR in HUVEC under co-cultured with HepG2 for 24 h. (**I**) Diagram of TEM with Hepa1-6 cells seeded on top of transwell inserts coated with shGFP-LSECs or shICAM1-LSECs. All data are from three independent experiments performed in triplicate and presented as mean values ± s.d., ns, not significant; * $P<$ 0.05, ** $P<$ 0.01, and *** $P<$ 0.001 based on two-tailed unpaired Student's *t*-test for panels **B, C,** and **E.**

A

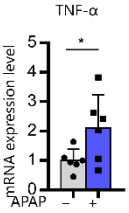


Fig. S5: *Tnf-α* mRNA expression was upregulated 6 h after APAP injection.

(A) The validation of mRNA expression of *Tnf-α* by qRT-PCR analysis in liver tissues under treatment with or without 300 mg/kg of body weight APAP dose for 6 h. Data were quantified from n = 6 mice in each group; two-sided *t-test*, mean values ± s.d., * $P<$ 0.05.

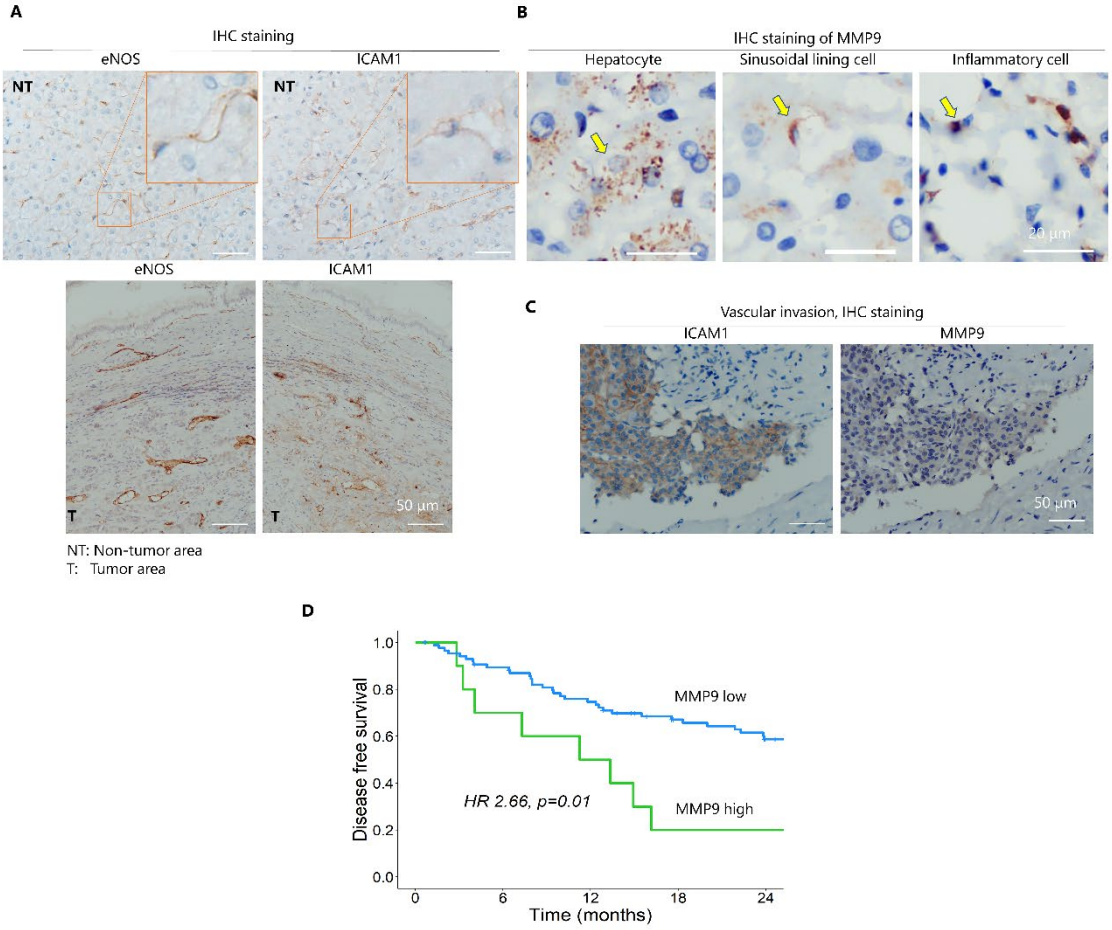

A
IHC staining
eNOS
ICAM1
NT
NT
eNOS
ICAM1
T
T
50 μm
B
IHC staining of MMP9
Hepatocyte
Sinusoidal lining cell
Inflammatory cell
20 μm
C
Vascular invasion, IHC staining
ICAM1
MMP9
50 μm
NT: Non-tumor area
T: Tumor area
D
Disease free survival
1.0
0.8
0.6
0.4
0.2
0.0
MMP9 low
HR 2.66, p=0.01
MMP9 high
0
6
12
18
24
Time (months)

Fig. S6: MMP9 and ICAM1 expression in patients with HCC.

(A) Representative images of ICAM1 and eNOS staining in HCC specimens, in tumoral and non-tumoral areas. Scale bar, 50 µm. (**B)** Representative images of MMP9 staining in hepatocytes (left), inflammatory cells in the portal tract (right), and sinusoidal lumen (middle) in human liver. Scale bar, 20 µm. (**C)** Representative images of ICAM1 and MMP9 staining in vascular invasion. Scale bar, 50 µm. (**D)** Kaplan–Meier curves show the association between low and high MMP9 staining levels and disease-free survival.

A

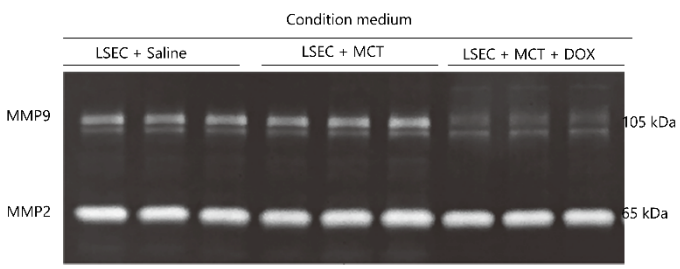

Condition medium
LSEC + Saline
LSEC + MCT
LSEC + MCT + DOX
MMP9
MMP2
105 kDa
65 kDa

Fig. S7: MMPs activity under treatment of MCT, DOX in LSECs.

(A) Gelatin zymography to detect MMP9 activity in conditioned medium from LSECs after 4 h of treatment with saline, MCT, or MCT-DOX combination.

Supplementary Tables

Table S1: Univariate and multivariate logistic regression analyses of factors were used to differentiate between patients with and those without vascular invasion.

		univariate			multivariate 1			multivariate 2		
Variable		OR	95%CI	*P* value	OR	95%CI	*P* value	OR	95%CI	*P* value
ICAM1	high	4.67	1.67–13.01	0.003**	3.36	1.13–10.00	0.03*			
MMP9	high	4.38	0.88–21.81	0.07						
ICAM1 or MMP9	high	4.62	1.80–11.82	0.001**				4.31	1.57–11.80	0.005**
Age	per 10 years	0.98	0.93–1.04	0.52						
Sex	Male	1.17	0.48–2.85	0.73						
Child-Pugh score	per 1	3.25	1.12–9.44	0.03*	2.28	0.76–6.90	0.14	2.43	0.79–7.47	0.12
ALBI score	per 1	1.74	0.65–4.63	0.27						
AFP	per 100 ng/ml	1.01	1.00–1.02	0.14						
Size of tumor	per 1 cm	1.26	1.07–1.47	0.004**	1.22	1.03–1.43	0.02*	1.25	1.05–1.48	0.01*
Number of tumors	per 1	1.59	0.85–2.98	0.15						
Fibrosis	cirrhosis	0.67	0.26–1.72	0.41						
BMI	per 1 kg/m^2	0.94	0.85–1.05	0.27						

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

Variables in multivariate 1 analysis included ICAM1, Child-Pugh score, and size of tumor, and those in multivariate 2 included ICAM1 or MMP9, Child-Pugh score, and size of tumor. Abbreviation: OR, odds ratio; CI, confidence interval; ALBI score, Albumin-Bilirubin score; AFP, α-fetoprotein; BMI, body mass index.

Table S2: Univariate and multivariate Cox regression analyses for survival.

		univariate		multivariate 1		multivariate 2	
Variable		HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
ICAM1	high	2.65 (1.15–6.12)	0.02*	2.29 (0.97–5.43)	0.06		
MMP9	high	1.73 (0.58–5.13)	0.33				
ICAM1 or MMP9	high	2.70 (1.15–6.33)	0.02*			2.46 (1.04–5.81)	0.04*
Age	per 10 years	1.63 (0.87–3.06)	0.13				
Sex	Male	0.58 (0.24–1.38)	0.22				
Child-Pugh score	per 1	1.92 (1.04–3.56)	0.04*	1.66 (0.86–3.21)	0.13	1.75 (0.92–3.33)	0.08
ALBI score	per 1	2.20 (0.87–5.55)	0.10				
AFP	per 100 ng/ml	1.00 (0.99–1.00)	0.51				
Size of tumor	per 1 cm	1.05 (0.97–1.15)	0.23				
Number of tumors	per 1	1.30 (0.80–2.11)	0.29				
Vascular invasion	with invasion	1.99 (0.77–5.19)	0.16				
Fibrosis	cirrhosis	1.59 (0.65–3.91)	0.31				
BMI	per 1 kg/m^2	0.96 (0.85–1.07)	0.46				

Variables in multivariate 1 analysis included ICAM1 and Child-Pugh score, and those in multivariate 2 included ICAM1 or MMP9 and Child-Pugh score.

Abbreviation: HR, hazard ratio.

Table S3: Baseline characteristics of the patients with HCC.

Variable	Without vascular invasion N = 48	With vascular invasion N = 50	*P* value
Age (y)	71 [67, 77]	72 [67, 75]	0.94
Sex			
Male	34 (70.8)	37 (74.0)	0.73
Female	14 (29.2)	13 (26.0)	
BMI (kg/m^2)	25.0 [22.2, 26.6]	23.2 [20.7, 24.9]	0.08
Child-Pugh score			
5	44 (91.7)	38 (76.0)	0.06
6	4 (8.3)	8 (16.0)	
7	0 (0.0)	4 (8.0)	
ALBI score	-2.77 [-2.96, -2.59]	-2.78 [-3.03, -2.39]	0.48
AFP (ng/mL)	8.8 [3.9, 66.1]	24.3 [4.5, 555.5]	0.10
Size of tumor (cm)	2.5 [2.0, 4.1]	4.7 [2.5, 7.2]	0.001**
Number of tumors	1.0 [1.0, 1.0]	1.0 [1.0, 2.0]	0.22
Fibrosis			
Non-cirrhosis	35 (72.9)	40 (80.0)	0.41
Cirrhosis	13 (27.1)	10 (20.0)	

Values are reported as N (%) or median [interquartile ranges].

Table S4: Univariate and multivariate Cox regression analyses for recurrence.

		univariate		multivariate 2	
Variable		HR (95%CI)	*P* value	HR (95%CI)	*P* value
ICAM1	high	1.40 (0.74–2.63)	0.30		
MMP9	high	2.66 (1.23–5.74)	0.01*	2.51 (1.11–5.68)	0.03*
ICAM1 or MMP9	high	1.57 (0.86–2.85)	0.14		
Age	per 10 years	1.17 (0.79–1.74)	0.43		
Sex	Male	1.41 (0.70–2.85)	0.34		
Child-Pugh score	per 1	1.41 (0.81–2.44)	0.23		
ALBI score	per 1	2.00 (0.98–4.07)	0.06	1.25 (0.53–2.91)	0.61
AFP	per 100 ng/ml	1.00 (1.00–1.00)	0.30		
Size of tumor	per 1 cm	1.10 (1.03–1.16)	0.003**	1.07 (1.00–1.15)	0.056
Number of tumors	per 1	1.69 (1.22–2.36)	0.002**	1.46 (0.98–2.18)	0.06
Vascular invasion	with invasion	1.81 (0.99–3.32)	0.054	1.11 (0.55–2.23)	0.77
Fibrosis	cirrhosis	1.01 (0.51–1.99)	0.98		
BMI	per 1 kg/m^2	1.02 (0.94–1.10)	0.65		

Table S5: List of antibodies used in this study.

Immunohistochemistry and Immunofluorescence Staining				
Antibody	Species		Source	Dilution
Anti-CD31	Goat	Polyclonal	R&D Systems	1/50
Anti-AFP	Mouse	Monoclonal	R&D Systems	1/50
Anti-CD68	Rabbit	Polyclonal	Abcam	1/200
Anti-ICAM1	Rabbit	Mouse Recombinant	Sino Biological	1/100
Anti-ICAM1	Rabbit	Polyclonal	Sigma	1/300
Anti-MMP9	Mouse	Monoclonal	Santa Cruz Biotechinology	1/100
Anti-eNOS	Mouse	Monoclonal	BD Transduction laboratories	1/100
Western Blot Analysis				
Anti-TNF-α	Rabbit	Polyclonal	Abcam	1/1000
Anti-pNF-kB	Rabbit	Monoclonal	Cell Signaling Technology	1/1000
Anti-tNF-kB	Rabbit	Monoclonal	Cell Signaling Technology	1/1000
Anti-ICAM1	Goat	Polyclonal	R&D Systems	1/1000
Anti-MMP9	Mouse	Monoclonal	Santa Cruz Biotechinology	1/500
Anti-IL23	Goat	Polyclonal	R&D Systems	1/2000
Anti-GAPDH (clone 6C5)	Mouse	Monoclonal	Millipore	1/10000

Table S6: List of primers used for quantitative RT-PCR.

Gene name	Primer sequences	
mTNF-α	F: 5'-TCCCAGGTTCTCTTCAAGGGA-3'	R: 5'-GGTGAGGAGCACGTAGTCGG-3'
mMMP9	F: 5'-CCCGCTGTATAGCTACCTCG-3'	R: 5'-GTGGTTCAGTTGTGGTGGTG-3'
mICAM1	F: 5'-GTGATGCTCAGGTATCCATCCA-3'	R: 5'-CACAGTTCTCAAAGCACAGCG-3'
mCXCL1	F: 5'-TGAGCTGCGCTGTCAGTGCCT-3'	R: 5'-AGAAGCCAGCGTTCACCAGA-3'
mCXCL2	F: 5'-GAGCTTGAGTGTGACGCCCCCAGG-3'	R: 5'-GTTAGCCTTGCCTTTGTTCAGTATC-3'
mCXCL5	F: 5'-GCATTTCTGTTGCTGTTCACGCTG-3'	R: 5'-CCTCCTTCTGGTTTTTCAGTTTAGC-3'
mCXCL10	F: 5'-GTGCTGCCGTCATTTTCTG-3'	R: 5'-CCCTATGGCCCTCATTCTCA-3'
mIL23	F: 5'-TGACCCACAAGGACTCAAG-3'	R: 5'-AGCAGGCTCCCCTTTGAA-3'
mCFS1	F: 5'-CCTTCTTCGACATGGCTGGG-3'	R: 5'-TCATCCAGCTGTTCCTGGTC-3'
mGDF15	F: 5'-GTGTGAGCATCCAGTCAT-3'	R: 5'-GCTTTTGAAGTCTGCCCTCC-3'
mMMP2	F: 5'-CCAGCAAGTAGATGCTGCCT-3'	R: 5'- GATGGCATTCCAGGAGTCTG -3'
m18s	F: 5'-CGGCTACCACATCCAAGGAA-3'	R: 5'-ATTGGAGCTGGAATTACCGC-3'
mGAPDH	F: 5'-TGCACCACCAACTGCTTAG-3'	R: 5'-GGATGCAGGATGATGTTC-3'
hICAM1	F: 5'-TTGGCATAGAGACCCCGTT-3'	R: 5'-GCACATTGCTCAGTTCATACACC-3'

h and m indicate human and mouse cDNA-specific primer sequences, respectively.

Supplementary Data

Data S1: PL-SDA score plot using FPKM values for 13,525 genes.
Data S2: List of up-regulated genes in RNA-seq analysis.
Data S3: Gene list for up-regulated genes in LSECs co-culture used for Enrichr analysis.