Immunity, Volume *51*

Supplemental Information

Stellate Cells, Hepatocytes, and Endothelial Cells Imprint the Kupffer Cell Identity on Monocytes Colonizing the Liver Macrophage Niche

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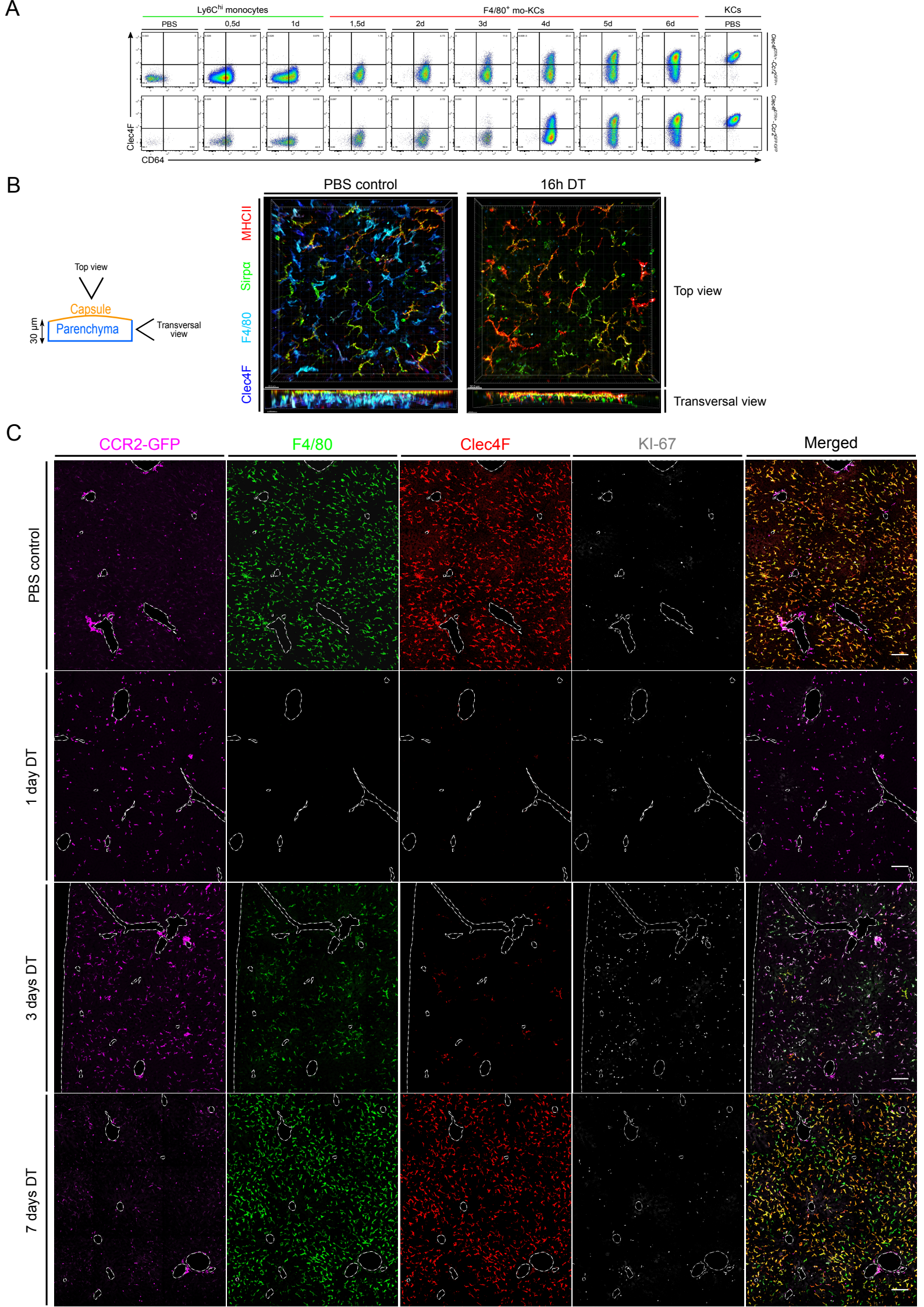
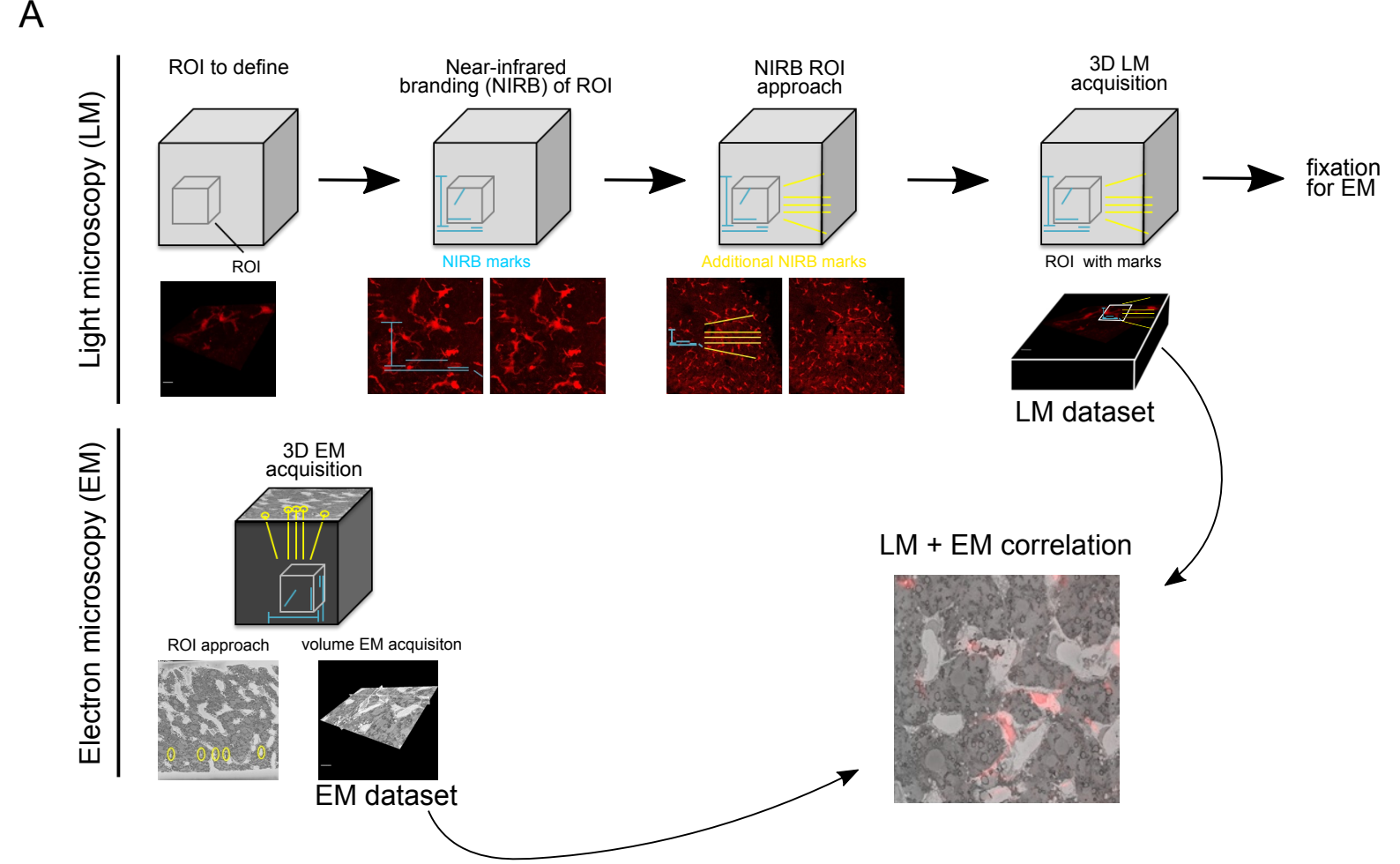

A
Ly6C^hi monocytes
F4/80+ mo-KCs
KCs
PBS
0,5d
1d
1,5d
2d
3d
4d
5d
6d
PBS
Clec4F
CD64
B
PBS control
16h DT
MHCII
Sirpα
F4/80
Clec4F
Top view
Transversal view
Top view
Capsule
Parenchyma
30 µm
Transversal view
C
CCR2-GFP
F4/80
Clec4F
KI-67
Merged
PBS control
1 day DT
3 days DT
7 days DT

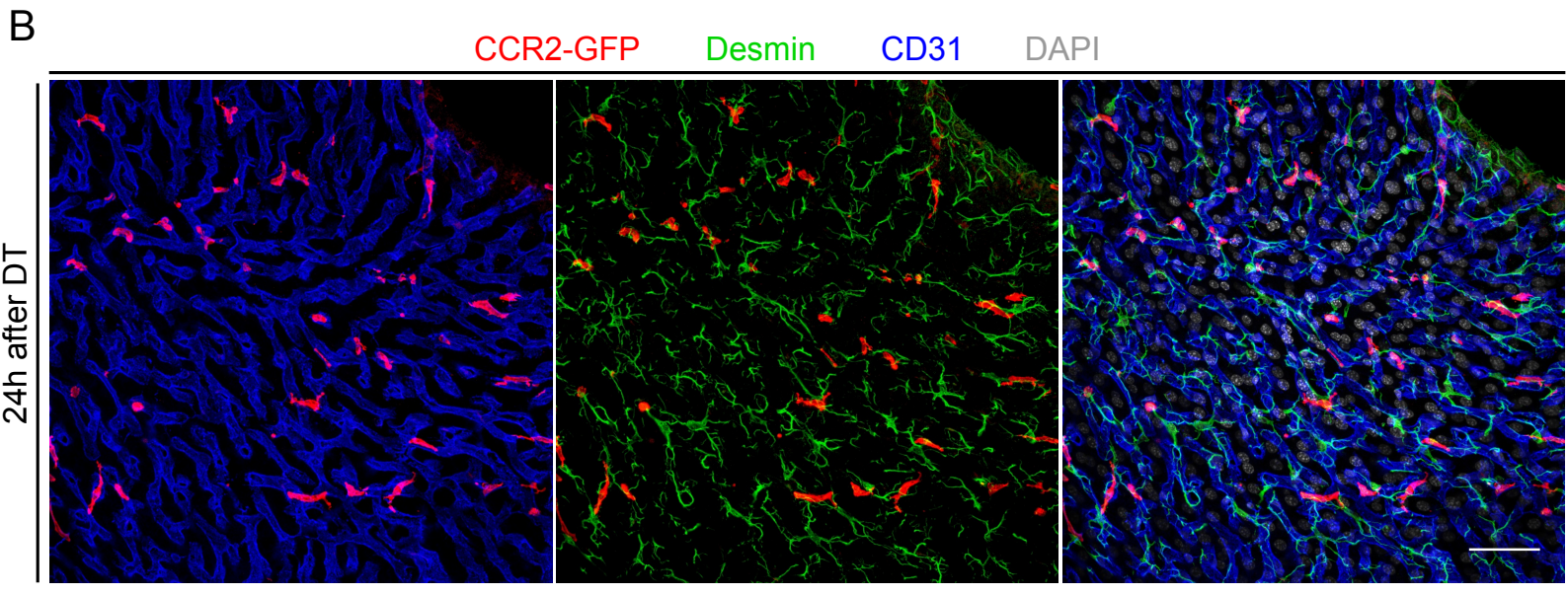
Figure S1, Related to figure 1. Differentiation and proliferation of mo-KCs.

(A) CD64 and Clec4F expression after DT injection in (top row) *Clec4f*$^{DTR/+}$-*Ccr2*$^{GFP/+}$ mice or (bottom row) *Clec4f*$^{DTR/+}$-*Ccr2*$^{GFP/GFP}$ mice. Flow cytometry-plots are pre-gated on live CD45^{+}CD11b^{+}Lyve-1^{-}SiglecF^{-}Ly6G^{-} single cells. Ly6C^{hi} monocytes, mo-KCs and em-KCs are gated as shown in Figure 1A. Data are representative of 2 to 3 experiments.

(B) MIP of *Clec4f*$^{DTR/+}$ mouse liver sections stained for Clec4F (blue), F4/80 (cyan), Sirpα (green) and MHCII (red) in PBS control mice or 16h after DT injection. Two different views are depicted showing that capsule liver macrophages (Sirpα^{hi} MHCII^{+} F4/80^{low} Clec4F^{-}) were not depleted following DT injection, on contrary to KCs (Sirpα^{low} MHCII^{low} F4/80^{hi} Clec4F^{hi}). Data are representative of 2 experiments. Scale bar = 30 µm.

(C) MIP of a confocal composite dataset (3 by 4) of *Clec4f*$^{DTR/+}$-*Ccr2*$^{GFP/+}$ mouse liver sections stained for GFP (magenta) F4/80 (green), Clec4F (red) and KI-67 (grey) in PBS control mice or 1, 3 and 7 days after DT administration. Data are representative of 2 experiments. Scale bar = 100 µm.


A
Light microscopy (LM)
ROI to define
ROI
Near-infrared branding (NIRB) of ROI
NIRB marks
NIRB ROI approach
Additional NIRB marks
3D LM acquisition
ROI with marks
fixation for EM
LM dataset
Electron microscopy (EM)
3D EM acquisition
ROI approach
volume EM acquisiton
EM dataset
LM + EM correlation


B
CCR2-GFP
Desmin
CD31
DAPI
24h after DT

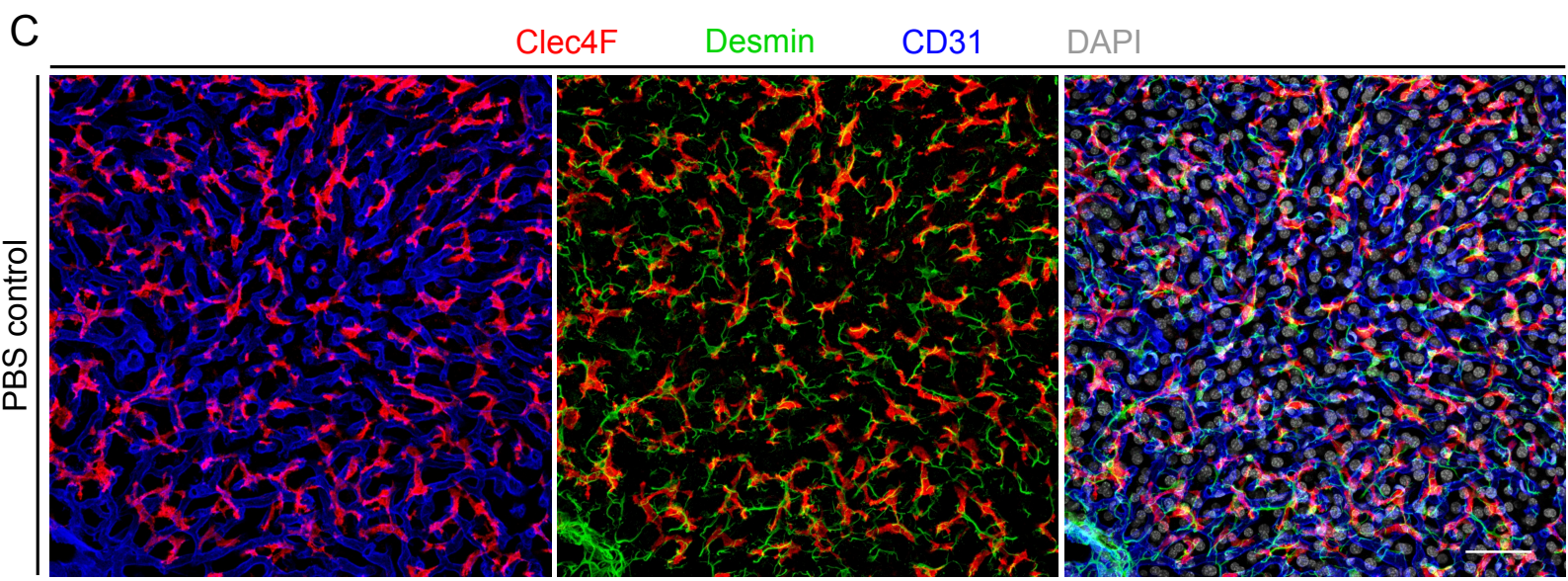
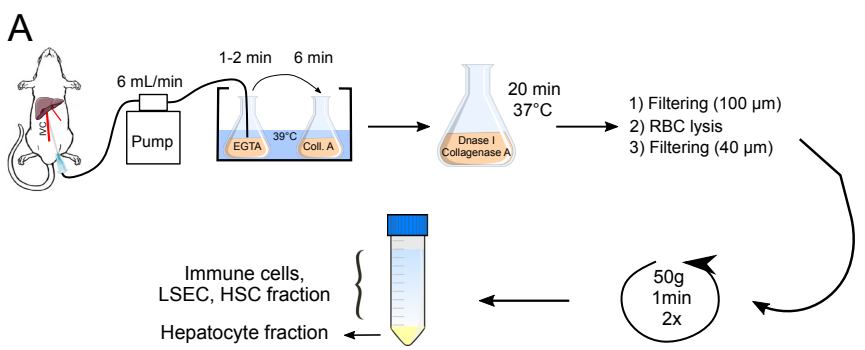

C
Clec4F
Desmin
CD31
DAPI
PBS control

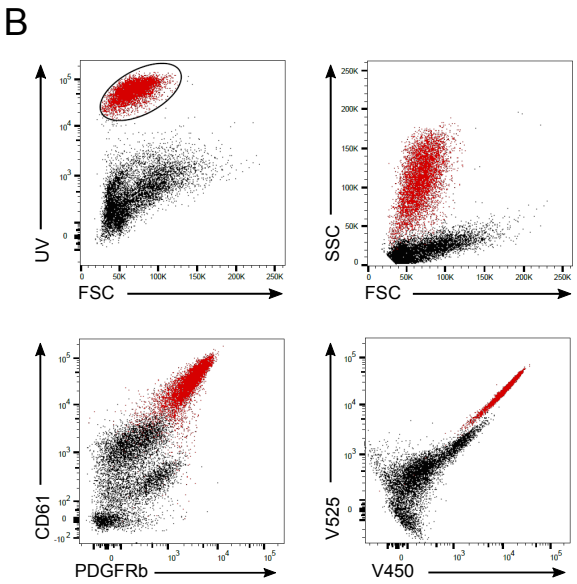
Figure S2, Related to figure 3. Correlative light-electron microscopy and interaction between HSC and monocytes or KCs.

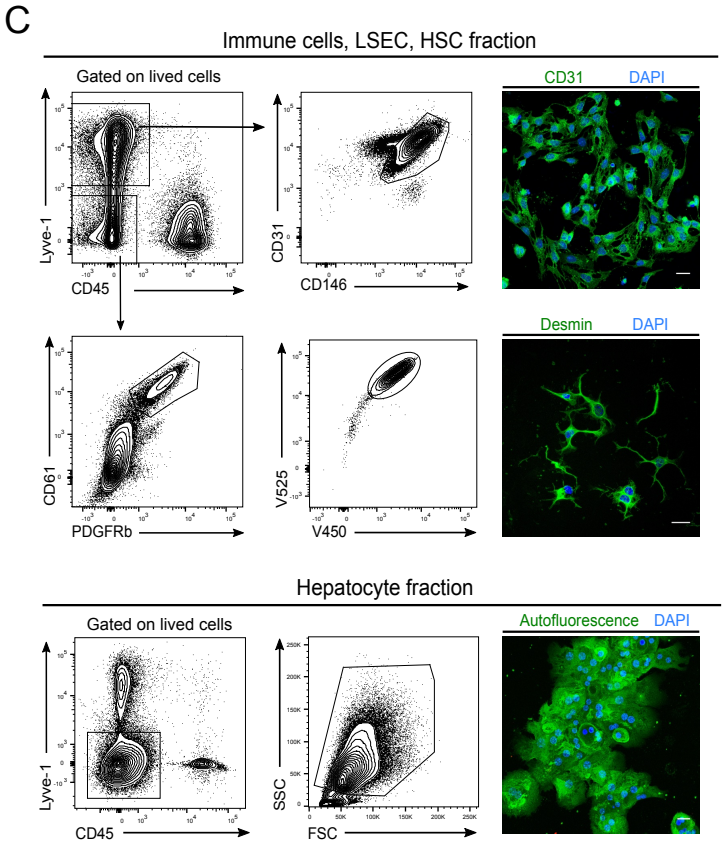
(A) Schematic representation of the protocol used to performed the correlative light-electron microscopy.

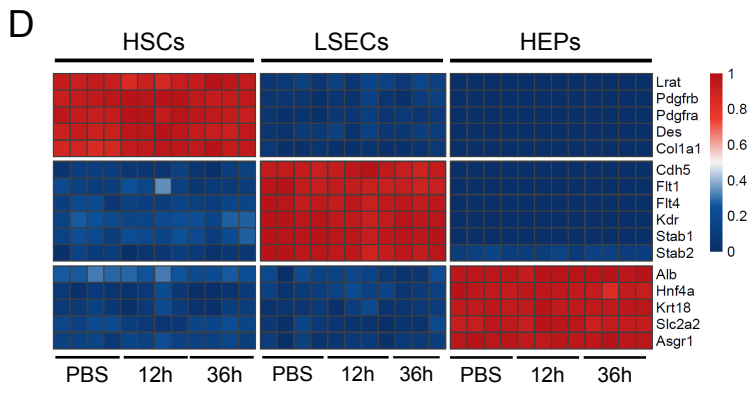
(B) MIP of *Clec4f*$^{DTR/+}$-*Ccr2*$^{GFP/+}$ mouse liver sections stained for GFP (red), Desmin (green), CD31 (blue) and DAPI (grey) 24h after DT administration. Data are representative of 3 experiments. Scale bar = 50µm.

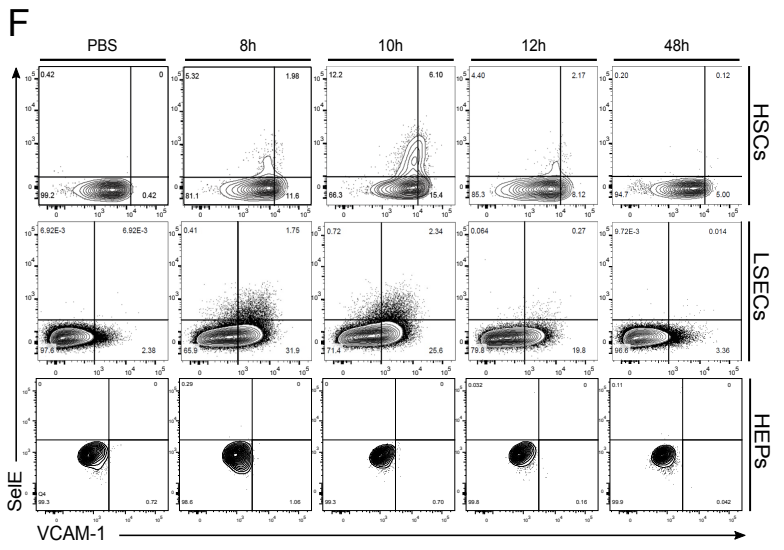
(C) MIP of *Clec4f*$^{DTR/+}$ mouse liver sections stained for Clec4F (red), Desmin (green), CD31 (blue) and DAPI (grey) in PBS control. Data are representative of 5 experiments. Scale bar = 50µm.


A
6 mL/min
Pump
1-2 min
6 min
EGTA
39°C
Coll. A
20 min
37°C
Dnase I
Collagenase A
1) Filtering (100 µm)
2) RBC lysis
3) Filtering (40 µm)
50g
1min
2x
Immune cells,
LSEC, HSC fraction
Hepatocyte fraction


B
UV
FSC
SSC
FSC
CD61
PDGFRb
V525
V450


C
Immune cells, LSEC, HSC fraction
Gated on lived cells
Lyve-1
CD45
CD31
CD146
CD31
DAPI
CD61
PDGFRb
V525
V450
Desmin
DAPI
Hepatocyte fraction
Gated on lived cells
Lyve-1
CD45
SSC
FSC
Autofluorescence
DAPI


D
HSCs
LSECs
HEPs
Lrat
Pdgfrb
Pdgfra
Des
Col1a1
Cdh5
Flt1
Flt4
Kdr
Stab1
Stab2
Alb
Hnf4a
Krt18
Slc2a2
Asgr1
1
0.8
0.6
0.4
0.2
0
PBS
12h
36h
PBS
12h
36h
PBS
12h
36h


F
PBS
8h
10h
12h
48h
HSCs
LSECs
HEPs
SelE
VCAM-1

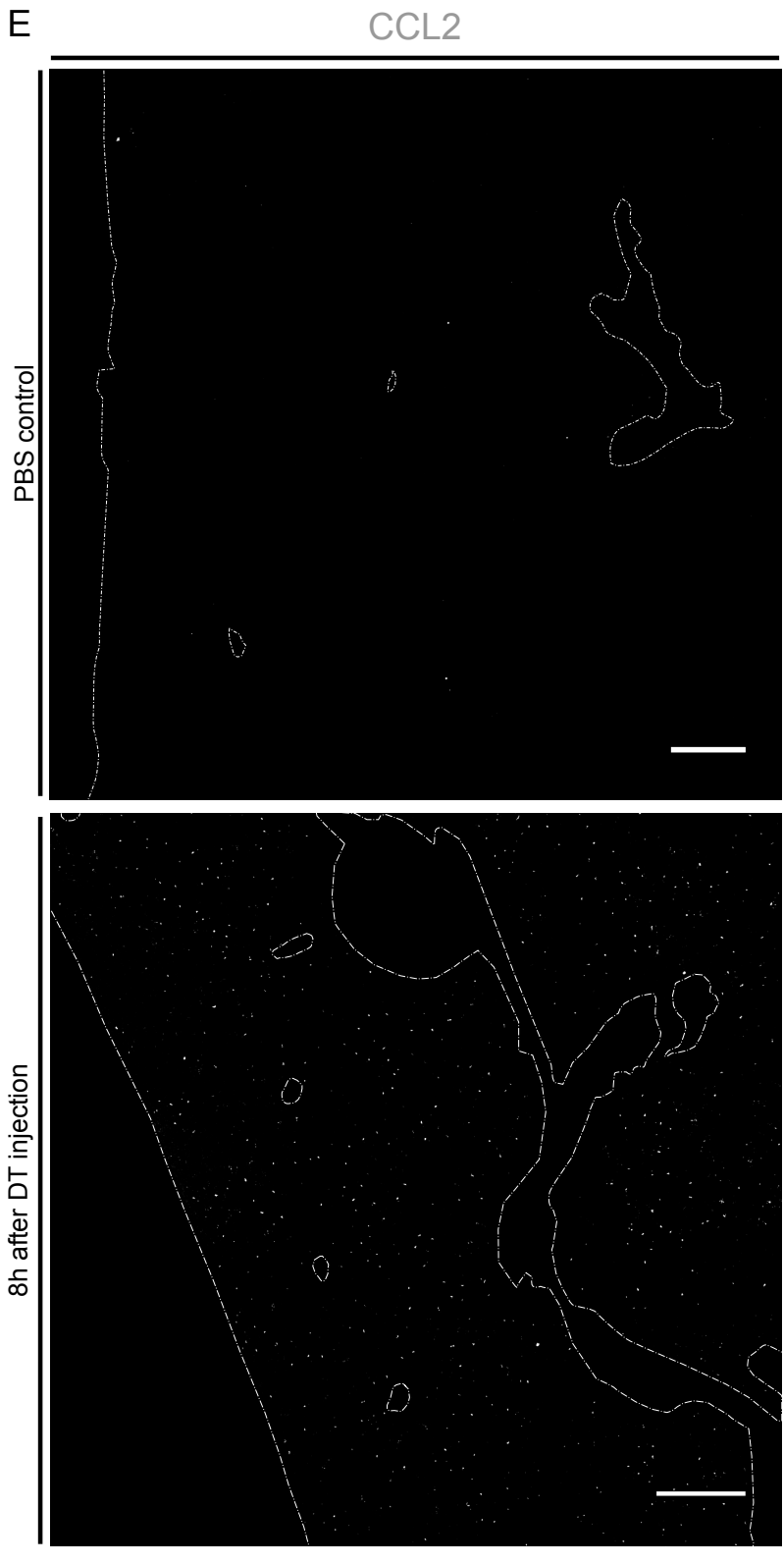

E
CCL2
PBS control
8h after DT injection

Figure S3, Related to figure 4. Isolation protocol and gating strategy of HSCs, LSECs and hepatocytes.

(A) Schematic representation of the HSC, LSEC and hepatocyte isolation procedure.

(B) Flow cytometry-plots shows the endogenous autofluorescence of HSCs (red) in the UV channel (395nm channel and 450/50 band-pass filter), V450 and V525 channel as well as their expression of CD61 and PDGFRβ and their SSC and HSC characteristics. Cells were pre-gated on live cells.

(C) Flow cytometry-plots shows the gating strategy used to isolate HSCs ($CD45^-Lyve\text{-}1^-CD61^+PDGFR\beta^+Autofluorescence^+$ in the V525 and V450 channel) LSECs ($CD45^-Lyve\text{-}1^+CD31^+CD146^+$) and hepatocytes ($CD45^-Lyve\text{-}1^-SSC^{hi}FSC^{hi}$). Cells were sorted, stained for DAPI and CD31 (LSECs), Desmin (HSCs) or autofluorescence (hepatocytes) and imaged by confocal microscopy in order to confirmed their identity by morphology.

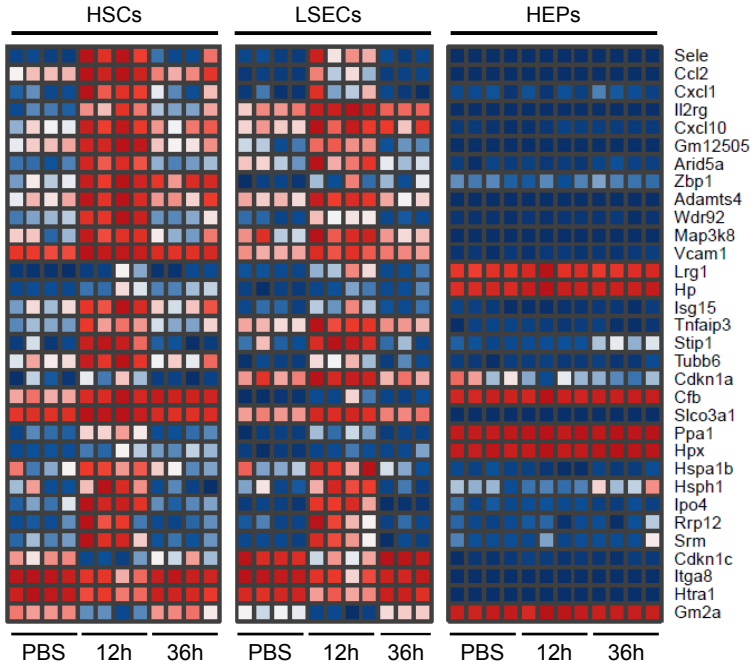
(D) Heatmap shows the expression by RNA-seq of specific genes of HSCs (*Lrat*, *Pdgfrb*, *Pdgfra*, *Des*, *Col1a1*), LSECs (*Cdh5*, *Flt1*, *Flt4*, *Kdr*, *Stab1*, *Stab2*) and Hepatocytes (*Alb*, *Hnf4a*, *Krt18*, *Asgr1, Slc2a2*) on the different sorted cell populations.

(E) MIP of a confocal dataset of $KC^{DTR/+}$-$CCR2^{GFP/+}$ mouse liver sections stained for CCL2 (Grey) 8h after DT or PBS injection.

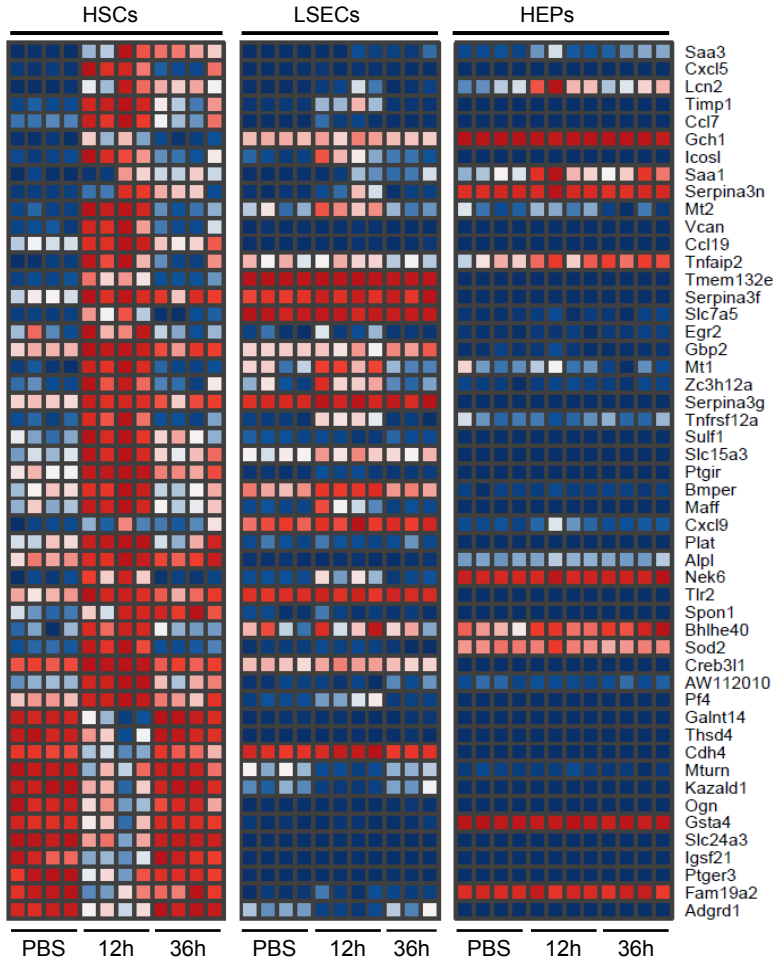
(F) Expression of VCAM-1 and Selectin E on HSCs, LSECs and Hepatocytes after DT injection. FACS-plots are pre-gated on live $CD45^-$ and further gated on $Lyve\text{-}1^+$ (LSECs), UV^+ $autofluorescence^+$ (HSCs), $SSC^{hi}FSC^{hi}$ (HEPs). Data are representative of 2-4 experiments.

A

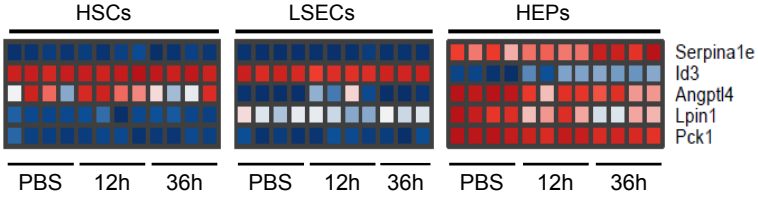
Genes differentially expressed both by HSCs and LSECs during depletion



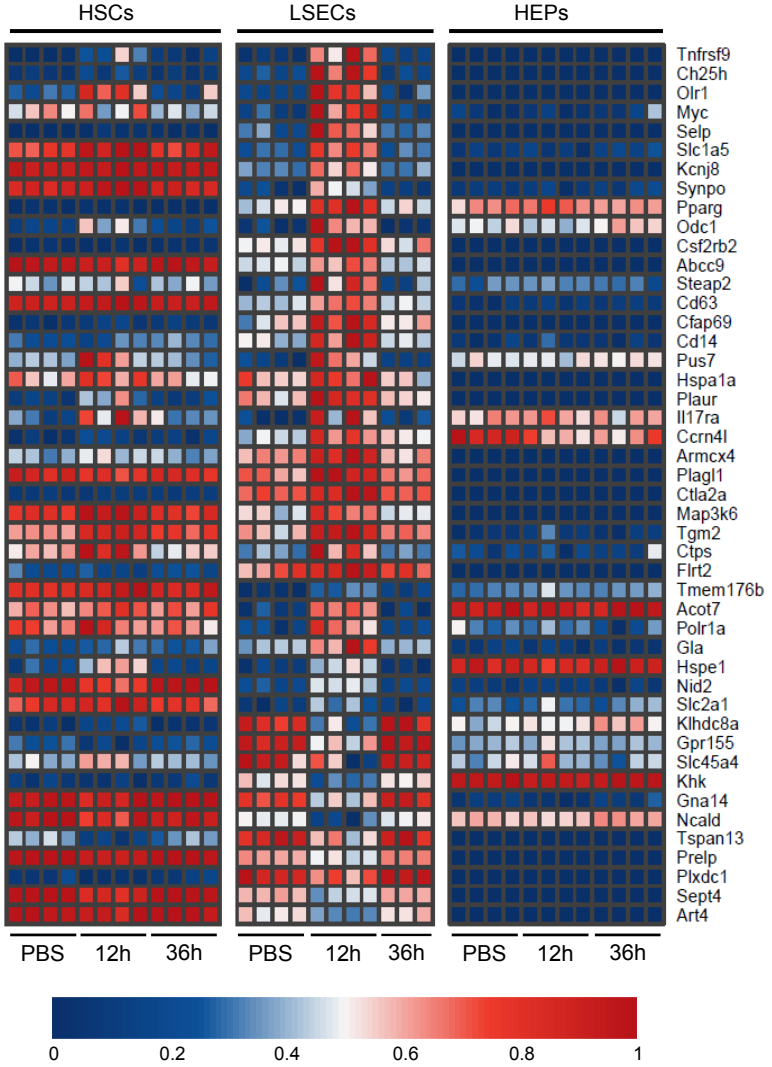
Genes specifically differentially-expressed by HSCs during depletion



Genes specifically differentially-expressed by HEPs during depletion



Genes specifically differentially-expressed by LSECs during depletion



B

Ingenuity Pathways Analysis (IPA)

HSCs

Top Canonical Pathways

Name	p-value	Overlap	
Acute Phase Response Signaling	1.16E-09	11.9 %	21/176
Granulocyte Adhesion and Diapedesis	5.38E-08	10.6 %	19/180
Interferon Signaling	1.10E-07	25.0 %	9/36
Agranulocyte Adhesion and Diapedesis	7.13E-07	9.4 %	18/192
EIF2 Signaling	2.24E-06	8.3 %	19/229

LSECs

Top Canonical Pathways

Name	p-value	Overlap	
Hepatic Fibrosis / Hepatic Stellate Cell Activation	4.74E-06	5.9 %	11/186
Atherosclerosis Signaling	8.78E-06	7.0 %	9/128
STAT3 Pathway	5.07E-04	5.3 %	7/132
Granulocyte Adhesion and Diapedesis	6.64E-04	4.4 %	8/180
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	6.66E-04	3.4 %	11/323

HEPs

Top Canonical Pathways

Name	p-value	Overlap	
PXR/RXR Activation	9.40E-05	4.6 %	3/65
LPS/IL-1 Mediated Inhibition of RXR Function	2.28E-04	1.8 %	4/223
Glutamine Biosynthesis I	1.36E-03	100.0 %	1/1
Dermatan Sulfate Biosynthesis (Late Stages)	1.79E-03	4.3 %	2/46
Chondroitin Sulfate Biosynthesis (Late Stages)	2.03E-03	4.1 %	2/49

C

DE genes for granulocyte and agranulocyte adhesion and diapedesis

HSCs

Up = *Ccl2, Ccl7, Ccl19, Cxcl1, Cxcl5, Cxcl9, Cxcl10, Cxcl16, Myl9, Hspb1, Icam1, Pf4, Sdc4, Sele, Vcam1*

Down = *Mmp2, Mmp11, Mmp17, Cxcl12, Sdc2*

LSECs

Up = *Ccl2, Cxcl1, Cxcl10, Il1a, Icam1, Vcam1, Sele, Selp*

Down = none

Figure S4, Related to figure 4. Differentially-expressed genes of HSCs, LSECs and hepatocytes 12h and 36h after KC-depletion.

(A) Heatmap shows the 31 DE genes (27 upregulated, 4 downregulated) in common between HSCs and LSECs, the top 50 of genes (38 upregulated, 12 downregulated) only DE in HSCs, the 46 genes (39 upregulated, 11 downregulated) only DE in LSECs and the 5 genes (2 upregulated, 3 downregulated) only DE in hepatocytes after KC depletion with a logFC > 1.5 or < -1.5.

(B) Ingenuity Pathways Analysis of the DE genes of each cell population.

(C) List of genes involved in “granulocyte and agranulocyte adhesion and diapedesis” upregulated by HSCs or LSECs after KC depletion according to ingenuity pathway analysis.

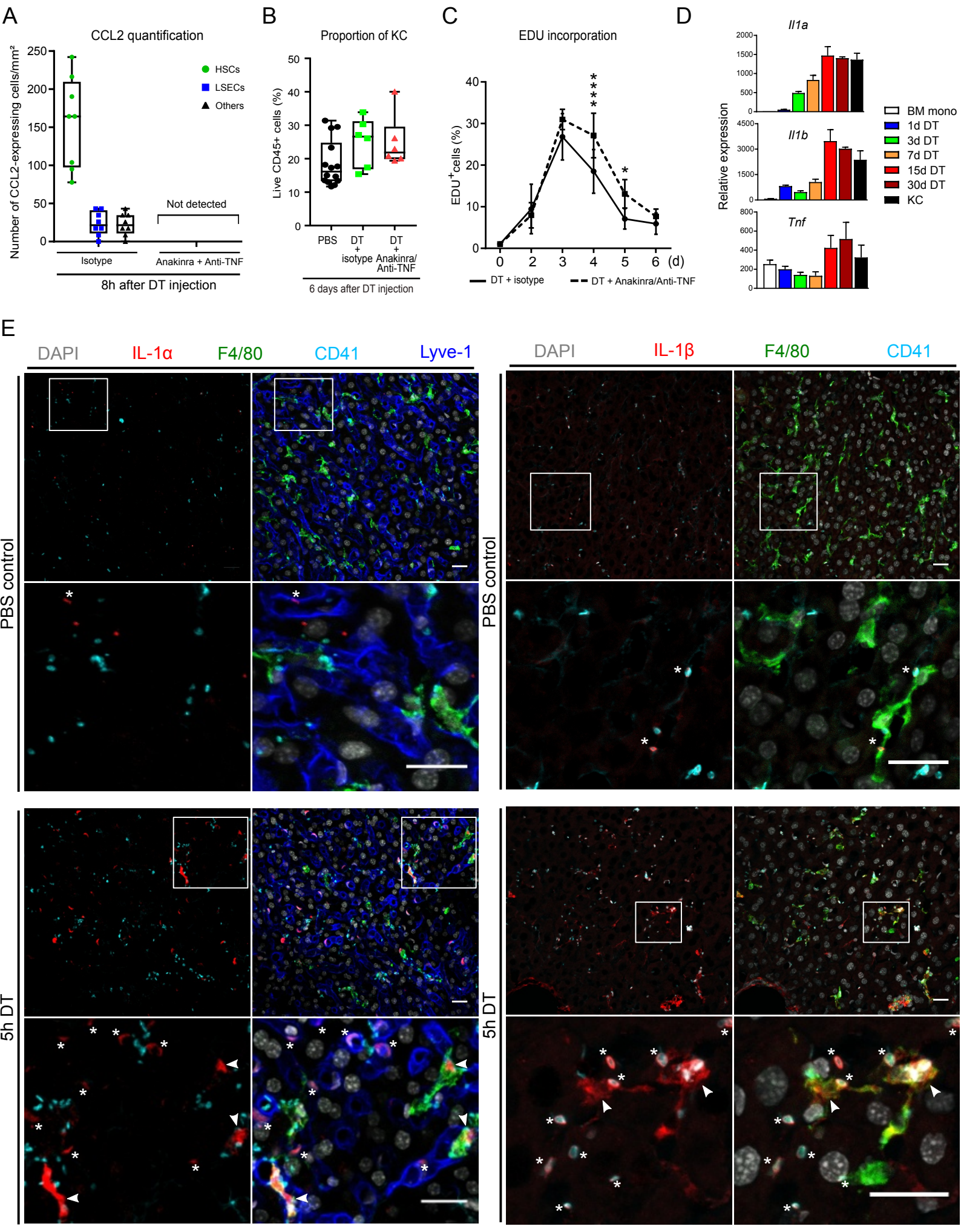

A
CCL2 quantification
Number of CCL2-expressing cells/mm²
HSCs
LSECs
Others
Not detected
Isotype
Anakinra + Anti-TNF
8h after DT injection
B
Proportion of KC
Live CD45+ cells (%)
PBS
DT + isotype
DT + Anakinra/ Anti-TNF
6 days after DT injection
C
EDU incorporation
EDU+cells (%)
(d)
DT + isotype
DT + Anakinra/Anti-TNF
D
Il1a
Il1b
Tnf
Relative expression
BM mono
1d DT
3d DT
7d DT
15d DT
30d DT
KC
E
DAPI
IL-1α
F4/80
CD41
Lyve-1
DAPI
IL-1β
F4/80
CD41
PBS control
PBS control
5h DT
5h DT

Figure S5, Related to figure 5. KCs, LSECs and platelets as a potential source of IL-1 following KCs depletion.

(A) Quantification of the number of $CCL2^+$ cells/mm^2 8h after DT injection in mice injected with either isotype antibodies of a combination of anti-TNF antibodies and Anakinra. Dots represent individual pictures. Results were normalized per mm^2 of tissue. Data are pooled from 2 experiments. n= 4 per condition.

(B) Proportion of KCs in the liver of *Clec4f*$^{DTR/+}$-*Ccr2*$^{GFP/+}$ mice represented as a percentage of live $CD45^+$ cells in PBS control (black dots), or 6 days after DT administration together with isotype (green) or anti-TNF + Anakinra (red). Data are pooled from 2 to 3 experiments. n= 6 (DT + isotype; DT + Anakinra/Anti-TNF) and 14 mice (PBS).

(C) Percentage of EdU^+ cells in mo-KC population at the indicated time-points (days) in (solid line) *Clec4f*$^{DTR/+}$ injected with DT and isotype antibodies or (dash line) *Clec4f*$^{DTR/+}$ injected with DT, Anakinra and anti-TNF antibodies. Mo-KCs were pre-gated on live $CD45^+CD11b^+F4/80^+Ly6C^-Lyve\text{-}1^-SiglecF^-Ly6G^-$ cells. Data are pooled from 2-3 experiments. n= 5 (0,5d); 6 (PBS, 1d, 1,5d, 2d, 5d, 6d) and 8 mice (3d, 4d). Two-way ANOVA with Tukey post-test.*p<0.05; ***p<0.001; ****p<0.0001 .

(D) Relative micro-array expression of *Il1a*, *Il1b* and *Tnf* in BM monocytes, KC or Mo-KCs at the indicated time points (days) following KC depletion.

(E) MIP of KC$^{DTR/+}$ mouse liver sections stained for DAPI (grey), F4/80 (green), CD41 (cyan), Lyve-1 (blue) and IL-1α (left; red) or IL-1β (right; red) in PBS control mice or 5h after DT injection. For each condition a zoomed picture of a selected region is shown. 5h after depletion IL-1α showed an upregulation in KCs (arrowheads) and LSECs (asterisks) whereas IL-1β was overexpressed by both KCs (arrowheads) and platelets (asterisks). Data are representative of 2 experiments. Scale bar = 20 µm.

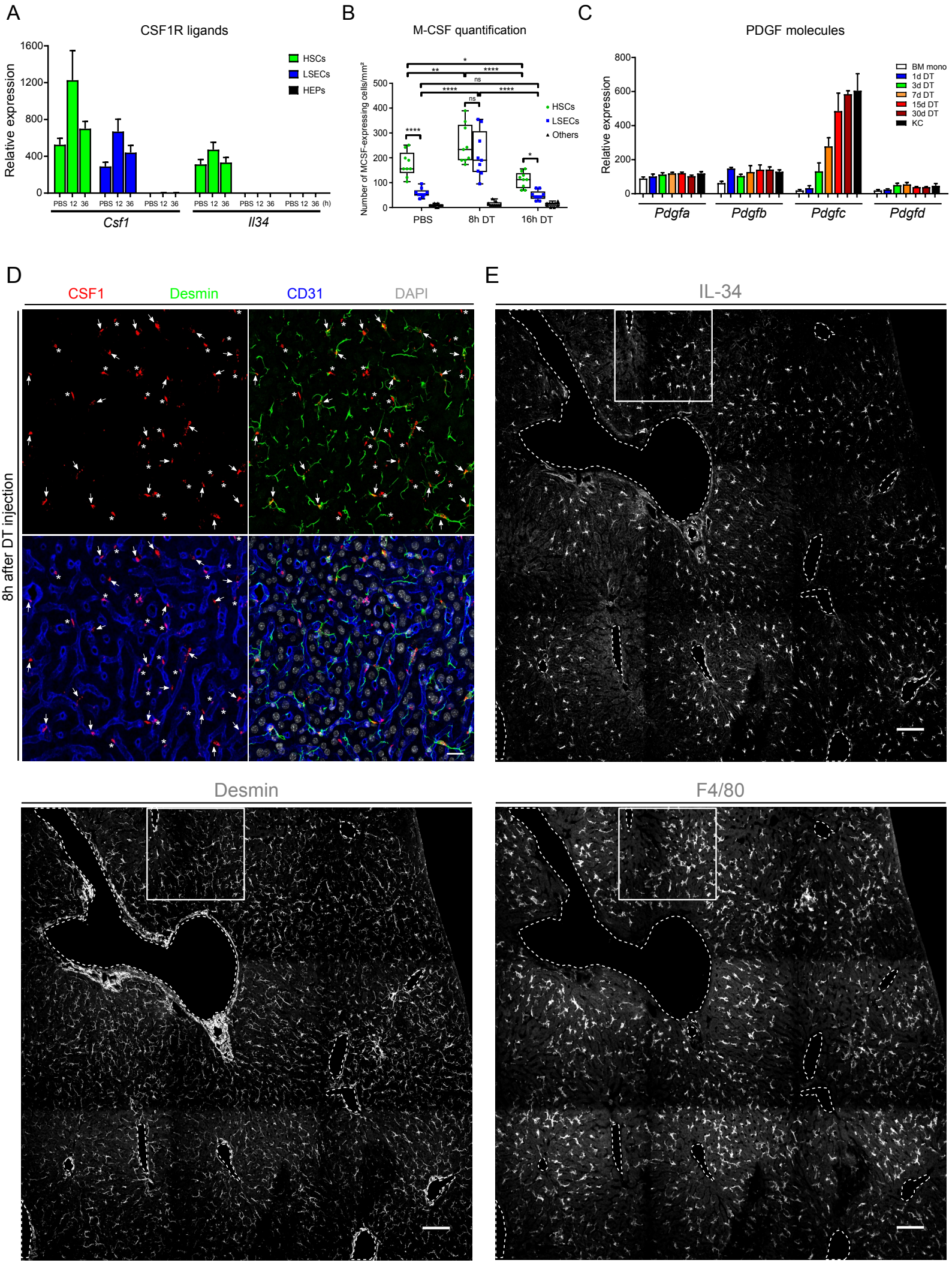

A
CSF1R ligands
Relative expression
1600
1200
800
400
0
HSCs
LSECs
HEPs
PBS 12 36 PBS 12 36 PBS 12 36 PBS 12 36 PBS 12 36 (h)
Csf1
Il34
B
M-CSF quantification
Number of MCSF-expressing cells/mm²
500
400
300
200
100
0
*
**
****
ns
****
ns
****
*
HSCs
LSECs
Others
PBS
8h DT
16h DT
C
PDGF molecules
Relative expression
800
600
400
200
0
BM mono
1d DT
3d DT
7d DT
15d DT
30d DT
KC
Pdgfa
Pdgfb
Pdgfc
Pdgfd
D
CSF1
Desmin
CD31
DAPI
8h after DT injection
E
IL-34
Desmin
F4/80

Figure S6, Related to figure 7. HSCs and KCs survival factors crosstalk.

(A) Relative RNA-sequencing expression of *Csf1* and *Il34* in HSCs, LSECs and hepatocytes at steady state or 12h and 36h after KC depletion.

(B) Quantification of the number of $CSF1^+$ cells/mm^2 in steady state mice or 8h and 16h after DT injection. Dots represent individual pictures. Results were normalized per mm^2 of tissue. Data are pooled from 2 experiments. n= 4. One way ANOVA with Bonferroni post-test. *p<0.05; **p<0.01; ****p<0.0001; ns = not significant.

(C) Relative micro-array expression of *Pdgfa, Pdgfb, Pdgfc* and *Pdgfd* in BM monocytes, KC or Mo-KCs at the indicated time points (days) following KC depletion.

(D) MIP of *Clec4f*$^{DTR/+}$ mouse liver sections stained for CSF1 (red), Desmin (green), CD31 (blue) and DAPI (gray) 8h after DT injection. Both HSCs (arrows) and LSECs (asterisks) showed CSF1 increased expression. Data are representative of 2 experiments. Scale bar = 20 µm.

(E) MIP of a confocal composite dataset (3 by 3) of $KC^{DTR/+}$ mouse liver sections stained IL-34, Desmin, F4/80 and CD31 in PBS control mice. Data are representative of 2 experiments. Scale bar = 100 µm.

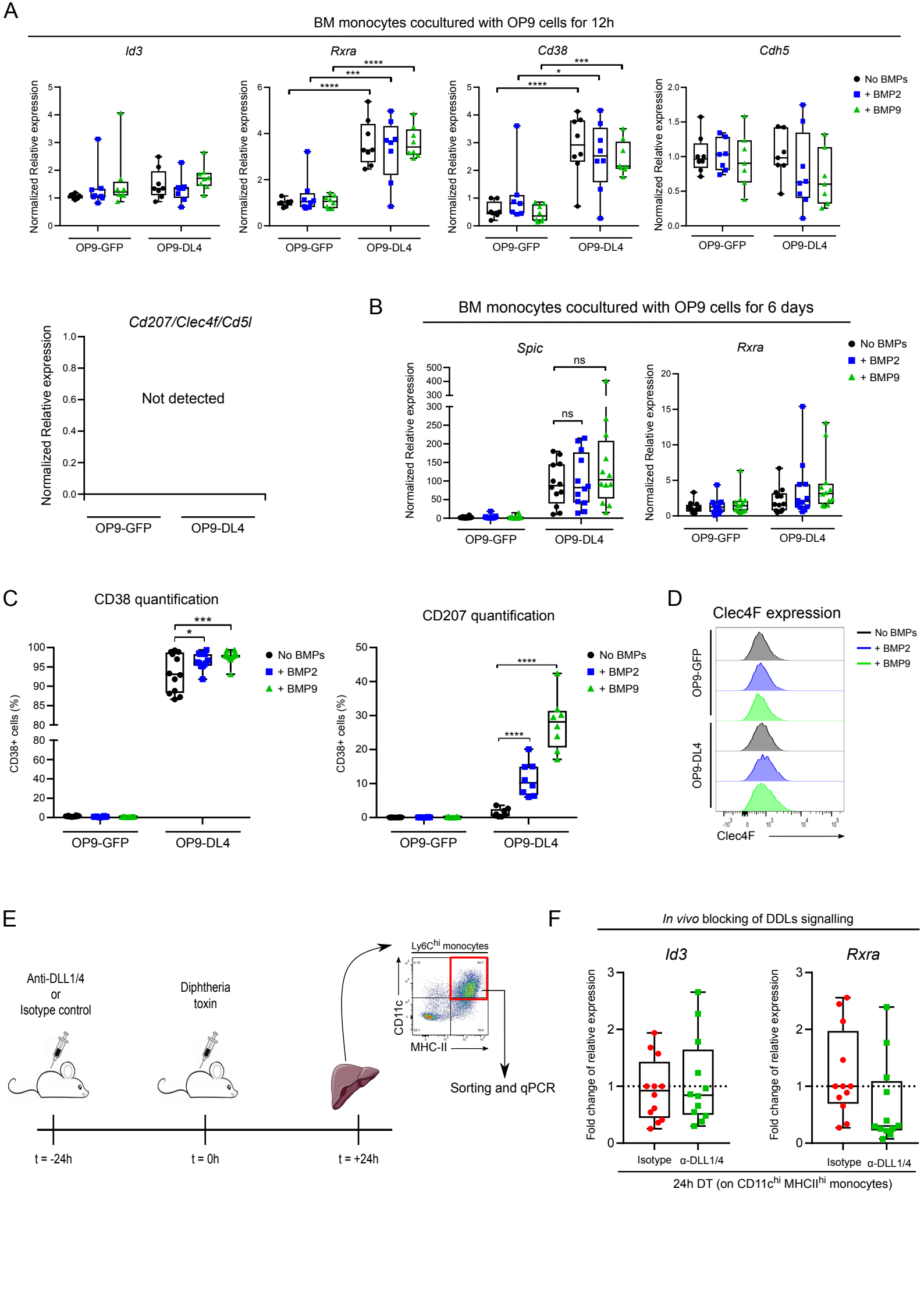

A
BM monocytes cocultured with OP9 cells for 12h
Id3
Rxra
Cd38
Cdh5
Normalized Relative expression
OP9-GFP
OP9-DL4
No BMPs
+ BMP2
+ BMP9
Cd207/Clec4f/Cd5l
Not detected
B
BM monocytes cocultured with OP9 cells for 6 days
Spic
ns
C
CD38 quantification
CD38+ cells (%)
CD207 quantification
D
Clec4F expression
Clec4F
E
Anti-DLL1/4
or
Isotype control
Diphtheria
toxin
t = -24h
t = 0h
t = +24h
Ly6C^hi monocytes
CD11c
MHC-II
Sorting and qPCR
F
In vivo blocking of DDLs signalling
Fold change of relative expression
Isotype
α-DLL1/4
24h DT (on CD11c^hi MHCII^hi monocytes)

Figure S7, Related to figure 7. Notch- and BMP-signaling induced KC identity.

(A) Relative expression of KC-associated transcription factor mRNA (*Id3*, *Rxra*) or KC-core genes (*Clec4f*, *Cd207*, *Cd38, Cdh5, Cd5l*) normalized to *B2m* in BM monocytes cultured during 12 hours on a feeder layer of DLL4-expressing OP9 cells (OP9-DL4) or control OP9 cells (OP9-GFP) with or without either recombinant BMP2 or BMP9. Data are pooled from 2 experiments, n=8 per group. Two-way ANOVA with Tukey post-test. *p<0.05, ***p<0.001, ****p<0.0001.

(B) Relative expression of *Spic* and *Rxra* normalized to *B2m* in BM monocytes cultured during 6 days on a feeder layer of OP9-DL4 cells or OP9-GFP cells with or without either recombinant BMP2 or BMP9. Data are pooled from 3 experiments, n=12 per group.

(C) Percentage of CD38 and CD207-expressing live $CD45^+$ cells. BM monocytes were cultured for 6 days on a feeder layer of OP9-DL4 cells or OP9-GFP cells with or without either recombinant BMP2 or BMP9. Data are pooled from 2-3 experiments, n=12 (CD38) or 8 (CD207). Two-way ANOVA with Tukey post-test. *p<0.05, ***p<0.001, ****p<0.0001.

(D) Representative histograms of Clec4F expression by BM monocytes cultured 6 days on a feeder layer of DLL4-expressing OP9 cells (OP9-DL4) or control OP9 cells (OP9-GFP) with or without either recombinant BMP2 or BMP9. Data are representative of 2 experiments.

(E) Schematic representation of the DLLs blocking experiment. Anti-DDL1/4 or isotype control antibodies were injected 24h before DT injection. 24h after DT administration, $CD11c^{hi}$ $MHCII^{hi}$ monocytes were sorted and mRNA was extracted to quantify KC-associated transcription factors expression.

(F) Fold change of relative expression of *Id3* and *Rxra* in $Cd11c^{hi}$ $MHCII^{hi}$ monocytes 24h after DT injection from mice pretreated 24h before DT administration with either isotype antibodies or a combination of anti-DLL1 and DLL4 antibodies. n= 12 per group.