Resolving the fibrotic niche of human liver cirrhosis at single cell level

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1 Abstract

Currently there are no effective antifibrotic therapies for liver cirrhosis, a major killer worldwide. To obtain a cellular resolution of directly-relevant pathogenesis and to inform therapeutic design, we profile the transcriptomes of over 100,000 human single cells, yielding molecular definitions for non-parenchymal cell types present in healthy and cirrhotic human liver. We uncover a novel scar-associated TREM2$^+$CD9$^+$ macrophage subpopulation, which expands in liver fibrosis, differentiates from circulating monocytes and is pro-fibrogenic. We also define novel ACKR1$^+$ and PLVAP$^+$ endothelial cells which expand in cirrhosis, are topographically scar-restricted and enhance leucocyte transmigration. Multi-lineage ligand-receptor modelling of interactions between the novel scar-associated macrophages, endothelial cells and PDGFRα$^+$ collagen-producing mesenchymal cells reveals intra-scar activity of several pro-fibrogenic pathways including TNFRSF12A, PDGFR and NOTCH signalling. Our work dissects unanticipated aspects of the cellular and molecular basis of human organ fibrosis at a single-cell level, and provides the conceptual framework required to discover rational therapeutic targets in liver cirrhosis.

Main

Recent estimates suggest that 844 million people worldwide have chronic liver disease, with two million deaths per year and a rising incidence[1]. Iterative liver injury secondary to any cause leads to progressive fibrosis ultimately resulting in liver cirrhosis. Importantly, the degree of liver fibrosis predicts adverse patient outcomes[2]. Hence, effective antifibrotic therapies for patients with chronic liver disease are urgently required[3,4].

Liver fibrosis involves a complex interplay between multiple non-parenchymal cell (NPC) lineages including immune, endothelial and mesenchymal cells spatially located within areas of scarring, termed the fibrotic niche. Despite progress in our understanding of liver fibrogenesis accrued using rodent models, there remains a significant 'translational gap' between putative targets and effective patient therapies[3,4]. This is in part due to limited definition of the functional heterogeneity and interactome of cell lineages that contribute to the fibrotic niche of human liver cirrhosis, which is imperfectly recapitulated by rodent models[3].

Single-cell RNA sequencing (scRNA-seq) is delivering a step change in our understanding of disease pathogenesis, allowing the interrogation of individual cell populations at unprecedented resolution[5]. Here, we studied the mechanisms regulating human liver fibrosis using scRNA-seq.

Results

Single-cell atlas of human liver NPC

Hepatic NPC were isolated from healthy and cirrhotic human livers spanning a range of aetiologies of cirrhosis (Fig. 1a, Extended Data Fig. 1a). Leucocytes (CD45^{+}) or other NPC (CD45^{-}) fractions (Extended Data Fig. 1b) were FACS-sorted prior to scRNA-seq. To discriminate between liver-resident and circulating leucocytes, we also performed scRNA-seq on CD45^{+}CD66b^{-} peripheral blood mononuclear cells (PBMC) (Extended Data Fig. 1c, g-i). The combined tissue and PBMC dataset was partitioned into clusters (Extended Data Fig. 1d) and annotated using signatures of known lineage

markers (Extended Data Fig. 1d-e; Supplementary Table 2). To generate an atlas of liver-resident cells, contaminating circulating cells were removed from the liver tissue datasets, by excluding cells from the tissue samples which mapped transcriptionally to blood-derived clusters 1 and 13 (Extended Data Fig. 1d). Liver-resident cells expressed higher levels of tissue-residency markers such as CXCR4 compared to PBMC (Extended Data Fig. 1f).

Re-clustering the 66,135 liver-resident cells from 10 livers (n=5 healthy and n=5 cirrhotic) revealed 21 populations (Fig. 1b), each containing cells from both healthy and cirrhotic livers (Fig. 1c; Extended Data Fig. 2), across 10 cell lineages (Fig. 1d, Extended Data Fig. 2a, b). Subpopulation markers were identified across all clusters and lineages (Fig. 1e; Supplementary Tables 3, 4). QC metrics were highly reproducible between individual samples and condition (Extended Data Fig. 2c-f, Supplementary Table 1). Expression of collagens type I and III, the main fibrillar collagens within the fibrotic niche, was restricted to cells of the mesenchymal lineage (Fig. 1e).

We proceeded to annotate all human liver NPC lineages (below, Supplementary Notes 1-3, Extended Data Fig. 3), and provide an open-access gene browser (http://www.livercellatlas.mvm.ed.ac.uk) allowing assessment of NPC gene expression between healthy and cirrhotic livers.

Distinct macrophage subpopulations inhabit the fibrotic niche

Previous studies in rodents have highlighted macrophage subpopulations orchestrating both liver fibrosis progression and regression[6–8]. Clustering of mononuclear phagocytes (MP) identified ten clusters; annotated as scar-associated macrophages (SAMΦ), Kupffer cells (KC), tissue monocytes (TMo), conventional dendritic cells (cDC) and cycling (proliferating) cells (Fig. 2a, Extended Data Fig. 4a, Supplementary Note 2). Clusters MP(4) and MP(5), named SAMΦ(1) and SAMΦ(2) respectively, were expanded in cirrhotic livers (Fig. 2b), as confirmed by quantification of the MP cell composition of each liver individually (Fig. 2c).

Clusters MP(6) and MP(7) were enriched in *CD163*, *MARCO* and *TIMD4* (Extended Data Fig. 4b); tissue staining confirmed these as Kupffer cells (KC; resident liver

macrophages), facilitating annotation of these clusters as KC(1) and KC(2) respectively (Extended Data Fig. 4c). A lack of *TIMD4* expression distinguished KC(2) from KC(1) (Extended Data Fig. 4b); cell counting demonstrated $TIMD4^+$ cell numbers to be equivalent between healthy and cirrhotic livers, but showed a loss of $MARCO^+$ cells, consistent with selective reduction in $MARCO^+TIMD4^-$ KC(2) in liver fibrosis (Fig. 2c, Extended Data Fig. 4d, e).

Scar-associated clusters SAMΦ(1) and SAMΦ(2) expressed the unique markers *TREM2* and *CD9* (Fig. 2d, e). These macrophages displayed a hybrid phenotype, with features of both tissue monocytes and KC (Fig. 2d, e), analogous to monocyte-derived macrophages in murine liver injury models[7]. Flow cytometry confirmed expansion of $TREM2^+CD9^+$ macrophages in human fibrotic livers (Fig. 2f, Extended Data Fig. 4f). Conditioned media from FACS-sorted SAMΦ promoted fibrillar collagen expression by primary human hepatic stellate cells (HSC) (Fig. 2g), indicating that SAMΦ have a pro-fibrogenic phenotype. Tissue staining demonstrated the presence of $TREM2^+CD9^+MNDA^+$ SAMΦ topographically localised in collagen-positive scar regions (Fig. 2h, Extended Data Fig. 4g-i), and significantly expanded in cirrhotic livers (Extended Data Fig. 4j, k). Cell counting of stained cirrhotic livers morphologically segmented into regions of fibrotic septae and parenchymal nodules, confirmed SAMΦ accumulation within the fibrotic niche (Extended Data Fig. 4l).

Local proliferation plays a significant role in macrophage expansion at sites of fibrosis in rodent models[7,9]. Cycling MP cells (Fig. 2a) subclustered into cDC1, cDC2, KC and SAMΦ subpopulations (Extended Data Fig. 4m, Supplementary Table 8). Cycling SAMΦ expanded in cirrhosis (Extended Data Fig. 4m), highlighting the potential role of macrophage proliferation in promoting SAMΦ accumulation in the fibrotic niche.

Pro-fibrogenic phenotype of scar-associated macrophages

To delineate the functional profile of SAMΦ we visualised co-ordinately expressed gene groups across the MP subpopulations using self-organising maps (Extended Data Fig. 5a). We identified six optimally-differentiating metagene signatures, denoted as A-F (Extended Data Fig. 5a, Supplementary Table 9). Signatures A and B defined SAMΦ and were enriched for ontology terms relevant to tissue fibrosis (Extended Data Fig.

4

104 5b). These SAMΦ-defining signatures included genes such as *TREM2*, *IL1B*, *SPP1*,

105 *LGALS3*, *CCR2*, and *TNFSF12*, some of which are known to regulate the function of

106 scar-producing myofibroblasts in fibrotic liver diseases[10–13]. The remaining MP

107 subpopulations were defined by signature C (KC), signatures D, E (TMo) and signature

108 F (cDC1); ontology terms matched known functions for the associated cell type

109 (Extended Data Fig. 5b, Supplementary Table 9).

110 In mice, under homeostatic conditions, embryologically-derived self-renewing tissue-

111 resident KC predominate[14–16]. However, following injury, macrophages derived from

112 circulating monocytes accumulate in the liver and regulate fibrosis[7,8]. The ontogeny of

113 human hepatic macrophage subpopulations is unknown. TREM2$^+$CD9$^+$ SAMΦ

114 demonstrated a monocyte-like morphology (Fig. 2h, Extended Data Fig. 4g-i) and a

115 distinct topographical distribution from KC (Extended Data Fig. 4l). To assess SAMΦ

116 origin, we performed *in silico* trajectory analysis on a combined dataset of peripheral

117 blood monocytes and liver-resident MPs. We visualised the transcriptional profile of

118 these cells (Fig. 3a, Extended Data Fig. 5c), mapped them along a pseudotemporal

119 trajectory and interrogated their directionality via spliced and unspliced mRNA ratios

120 (RNA velocity[17]). These analyses suggested a differentiation trajectory from peripheral

121 blood monocytes into either SAMΦ or cDC, with no differentiation from KC to SAMΦ,

122 and no progression from SAMΦ to KC (Fig. 3a; Extended Data Fig. 5c). Additional

123 RNA velocity analyses[17] showed downregulation (negative velocity) of the monocyte

124 gene *MNDA* in SAMΦ, upregulation (positive velocity) of the SAMΦ marker gene *CD9*

125 in tissue monocytes, and a lack of KC gene *TIMD4* velocity in SAMΦ (Extended Data

126 Fig. 5d). Furthermore, assessment of the probabilities of cells in this dataset

127 transitioning into SAMΦ, indicated a higher likelihood of tissue monocytes than KC

128 differentiating into SAMΦ (Fig. 3b). Overall, these data suggest that SAMΦ are

129 monocyte-derived, and represent a terminally-differentiated cell state within the fibrotic

130 niche.

131 To further characterise SAMΦ phenotype, we identified differentially expressed genes

132 along monocyte differentiation trajectories. We defined three gene co-expression

133 modules, with module 1 representing upregulated genes during blood monocyte-to-

134 SAMΦ differentiation (Fig. 3c). Module 1 contained multiple pro-fibrogenic genes

including *SPP1*, *LGALS3*, *CCL2*, *CXCL8*, *PDGFB* and *VEGFA*[10–13], and displayed ontology terms consistent with promoting tissue fibrosis and angiogenesis (Fig. 3c, d; Supplementary Table 10). Module 2 contained genes that were downregulated during monocyte-to-SAMΦ differentiation (Fig. 3c; Extended Data Fig. 5e), whilst Module 3 encompassed a group of upregulated genes during monocyte-to-cDC differentiation (Fig. 3c; Extended Data Fig. 5f; Supplementary Table 10). SAMΦ isolated from cirrhotic human livers (Fig. 2f, Extended Data Fig. 4f) demonstrated enhanced protein secretion of several of the mediators identified by transcriptional analysis (Extended Data Fig. 5g) and promoted fibrillar collagen expression by primary human HSC (Fig. 2g), confirming that SAMΦ have a pro-fibrogenic phenotype.

To enable cross-species comparison, we performed scRNA-seq on liver MP cells isolated from control mice or mice treated with chronic carbon tetrachloride (CCl_4), a mouse model of liver fibrosis[7]. MP cells from fibrotic livers were isolated 24 hours after the final CCl_4 injection, a time of active fibrogenesis[7]. Five MP cell clusters were defined (Extended Data Fig. 6a-d, Supplementary Table 11), and injury-specific cluster mMP(2) was differentiated by high expression of *Cd9*, *Trem2*, *Spp1* and *Lgals3* (Extended Data Fig. 6a-d). We confirmed expansion of this CD9+ SAMΦ (mSAMΦ) population in liver fibrosis (Extended Data Fig. 6e, f) and co-culture of mSAMΦ with quiescent primary murine HSC promoted fibrillar collagen expression in HSC (Extended Data Fig. 6g). Canonical correlation analysis between human and mouse MP datasets[18] demonstrated that human SAMΦ (hSAMΦ) and mSAMΦ clustered together (h&mMP(2); Extended Data Fig. 6h, i) and that this cluster was enriched for SAMΦ markers *CD9*, *TREM2* and *SPP1* (Extended Data Fig. 6j), confirming that mSAMΦ represent a corollary population to human SAMΦ (hSAMΦ).

To identify potential transcriptional regulators of human SAMΦ we defined sets of genes co-expressed with known transcription factors (regulons) along the tissue monocyte-macrophage pseudotemporal trajectory and in KC (Extended Data Fig. 5g, h, Supplementary Table 12). This identified regulons and corresponding transcription factors associated with distinct macrophage phenotypes, highlighting *HES1* and *EGR2* activity in SAMΦ.

6

165   To determine whether SAMΦ expand in earlier stage human liver disease, we analysed

166   cohorts of patients with non-alcoholic fatty liver disease (NAFLD). Application of

167   differential gene expression signatures of human SAMΦ, KC and TMo to a

168   deconvolution algorithm[19] enabled assessment of hepatic monocyte-macrophage

169   composition in whole liver microarray data across the spectrum of early-stage

170   NAFLD[20] (Extended Data Fig. 7a). This demonstrated SAMΦ expansion in patients

171   with non-alcoholic steatohepatitis (NASH) (Extended Data Fig. 7a, b), an increased

172   frequency of SAMΦ with worsening histological NASH activity (NAS) and fibrosis

173   scores (Extended Data Fig. 7c), but no association with other patient demographics

174   (Extended Data Fig. 7d). In a separate NASH biopsy cohort, SAMΦ expansion

175   increased with NASH activity (Extended Data Fig. 7e) and positively correlated with

176   the degree of fibrosis across the full severity spectrum of NAFLD-induced liver fibrosis

177   (Extended Data Fig. 7f).

178   In summary, these data demonstrate that TREM2$^+$CD9$^+$ SAMΦ derive from the

179   recruitment and differentiation of circulating monocytes, are conserved across species,

180   display a pro-fibrogenic phenotype and expand early in the course of liver disease

181   progression.

182   **Distinct endothelial subpopulations inhabit the fibrotic niche**

183   In rodent models, hepatic endothelial cells are known to regulate fibrogenesis.

184   Clustering of human liver endothelial cells identified seven subpopulations (Fig. 4a).

185   Classical endothelial cell markers did not discriminate between the seven clusters,

186   although Endo(1) was distinct in lacking *CD34* expression (Extended Data Fig. 8a). To

187   fully annotate endothelial subpopulations (Supplementary Note 3, Extended Data Fig.

188   8k), we identified differentially expressed markers (Fig. 4c, Supplementary Table 13),

189   determined functional expression profiles (Extended Data Fig. 8g, Supplementary

190   Table 14), performed transcription factor regulon analysis (Extended Data Fig. 8h,

191   Supplementary Table 15) and assessed spatial distribution via tissue staining (Fig. 4d,

192   Extended Data Fig. 8j).

193   Disease-specific endothelial cells Endo(6) and Endo(7), CD34$^+$PLVAP$^+$VWA1$^+$ and

194   CD34$^+$PLVAP$^+$ACKR1$^+$ respectively (Fig. 4a-c, Extended Data Fig. 8b), expanded in

195 cirrhotic liver tissue (Fig. 4e) and were restricted to the fibrotic niche (Fig. 4d, e,

196 Extended Data Fig. 8c), allowing annotation as scar-associated endothelia SAEndo(1)

197 and SAEndo(2) respectively. In contrast, CD34⁻CLEC4M⁺ Endo(1) (annotated as liver

198 sinusoidal endothelial cells), were reduced in fibrotic livers (Fig. 4b, e). Metagene

199 signature analysis demonstrated that Endo(6) (SAEndo(1)) cells expressed pro-

200 fibrogenic genes including *PDGFD*, *PDGFB*, *LOX*, *LOXL2*; associated ontology terms

201 included extracellular matrix organization (signature A; Extended Data Fig. 8g).

202 Endo(7) (SAEndo(2)) cells displayed an immunomodulatory phenotype (signature B;

203 Extended Data Fig. 8g). The most discriminatory marker for this cluster, *ACKR1*, has

204 a role in regulating leucocyte recruitment[21]. We confirmed increased expression of

205 PLVAP, CD34 and ACKR1 on endothelial cells isolated from cirrhotic livers

206 (Extended Data Fig. 8d). Flow-based adhesion assays[22] demonstrated that cirrhotic

207 endothelial cells display enhanced leucocyte transmigration (Extended Data Fig. 8e),

208 which was attenuated by ACKR1 knockdown (Extended Data Fig. 8f).

209 **PDGFRA expression defines scar-associated mesenchymal cells**

210 Clustering of human liver mesenchymal cells identified four populations (Fig. 5a, b,

211 Extended Data Fig. 9a, Supplementary Table 16). Cluster Mes(1), distinguished by

212 *MYH11* expression (Fig. 5b, Extended Data Fig. 9a), was identified as vascular smooth

213 muscle cells (VSMC) (Fig. 5c). Mes(4) demonstrated expression of mesothelial

214 markers (Fig. 5b, Extended Data Fig. 9a). Cluster Mes(2) expressed high levels of *RGS5*

215 (Fig. 5b, Extended Data Fig. 9a), and RGS5 staining identified this population as

216 hepatic stellate cells (HSC) (Fig. 5c) . RGS5⁺ cells were absent from the fibrotic niche

217 (Fig. 5c). Cluster Mes(3) (distinguished by *PDGFRA* expression) expressed high levels

218 of fibrillar collagens and pro-fibrogenic genes (Fig. 5b, d, Extended Data Fig. 9a).

219 PDGFRα⁺ cells expanded in cirrhotic livers (Fig. 5a, e, f) and were mapped to the

220 fibrotic niche (Fig. 5f), enabling annotation as scar-associated mesenchymal cells

221 (SAMes).

222 To study SAMes heterogeneity, further clustering (Extended Data Fig. 9b) identified

223 two populations of SAMes (Extended Data Fig. 9c, d, Supplementary Table 17). *OSR1*

224 expression distinguished cluster SAMesB (Extended Data Fig. 9c), and labelled a

225 subpopulation of periportal cells in healthy liver and scar-associated cells in the fibrotic

226 niche (Extended Data Fig. 9e, f). Cluster SAMesB also expressed other known portal

227 fibroblast markers[23] (Extended Data Fig. 9g).

228 In rodent liver fibrosis models, HSC differentiate into scar-producing myofibroblasts

229 [24–26]. Pseudotemporal ordering and RNA velocity analyses demonstrated a trajectory

230 from human HSC to SAMes (Extended Data Fig. 9h). Assessment of gene co-

231 expression modules along the HSC-to-SAMes differentiation continuum indicated

232 upregulation of fibrogenic genes including *COL1A1*, *COL1A2*, *COL3A1, TIMP1* and

233 downregulation of genes including *RGS5*, *IGFBP5*, *ADAMTS1* and *GEM,* which are

234 known to be downregulated in murine HSC in response to liver injury[25] (Extended Data

235 Fig. 9i).

236 **Resolving the multi-lineage interactome in the fibrotic niche**

237 Having defined the scar-associated macrophage, endothelial and mesenchymal

238 populations, we confirmed the close topographical association of these cells within the

239 fibrotic niche (Extended Data Fig. 10a, b), and used CellPhoneDB[27] to perform an

240 unbiased ligand-receptor interaction analysis between these populations.

241 Numerous statistically significant paracrine and autocrine interactions were detected

242 between ligands and cognate receptors expressed by SAMΦ, SAEndo and SAMes

243 (Supplementary Table 18, Extended Data Fig. 10f-m). To interrogate how scar-

244 associated NPC regulate fibrosis and to identify tractable therapeutic targets, we

245 focused functional analyses on interactions with SAMes (Fig. 6a, e, Extended Data Fig.

246 10d). In keeping with our data demonstrating that SAMΦ promote fibrillar collagen

247 expression in HSC (Fig. 2g), SAMΦ expressed epidermal growth factor receptor

248 (EGFR) ligands which are known to regulate mesenchymal cell activation[28] (Fig. 6a).

249 Additionally, SAMΦ expressed mesenchymal cell mitogens *TNFSF12* and *PDGFB*,

250 signaling to cognate receptors *TNFRSF12A* and *PDGFRA* on SAMes (Fig. 6a). We

251 confirmed localization of these ligand-receptor pairs within the fibrotic niche (Fig. 6b).

252 Both TNFSF12 and PDGF-BB induced primary human HSC proliferation, which was

253 inhibited by blockade of TNFSF12A and PDGFRA respectively (Fig. 6c, d).

254 Conditioned media from primary human SAMΦ promoted primary human HSC

255   proliferation *ex vivo* (Extended Data Fig. 10c), demonstrating a functional role for

256   SAMΦ in regulating SAMes expansion.

257   SAEndo expressed high levels of non-canonical Notch ligands *JAG1*, *JAG2* and *DLL4*

258   interacting with Notch receptor *NOTCH3* on SAMes (Fig. 6e). NOTCH3 was identified

259   on PDGFRα[+] SAMes within the fibrotic niche (Fig. 6f), whilst primary endothelial cells

260   from cirrhotic human liver demonstrated increased expression of JAG1 (Fig. 6g). Co-

261   culture of primary human HSC and endothelial cells from cirrhotic livers promoted

262   fibrillar collagen production by HSC, which was inhibited by addition of the Notch-

263   signalling inhibitor Dibenzazepine (DBZ) (Fig. 6h). Furthermore, knockdown of

264   *NOTCH3* expression in primary human HSC resulted in reduced fibrillar collagen

265   expression (Fig. 6i).

266   In summary, our unbiased dissection of the key ligand-receptor interactions between

267   scar-associated NPC highlights TNFRSF12A, PDGFRA and Notch signaling as

268   important regulators of mesenchymal cell function within the human liver fibrotic

269   niche.

270   **Discussion**

271   Here, using scRNA-seq and spatial mapping, we resolve the fibrotic niche of human

272   liver cirrhosis, identifying pathogenic subpopulations of TREM2^{+}CD9^{+} macrophages,

273   ACKR1^{+} and PLVAP^{+} endothelial cells and PDGFRα^{+} collagen-producing

274   myofibroblasts. We dissect a complex, pro-fibrotic interactome between multiple scar-

275   associated cell lineages and identify highly relevant intra-scar pathways that are

276   potentially druggable. In this era of precision medicine, this unbiased multi-lineage

277   approach should inform the design of highly-targeted combination therapies which will

278   very likely be necessary to achieve effective antifibrotic potency[3,4].

279   Application of our novel scar-associated cell markers could also potentially inform

280   molecular pathology-based patient stratification, which is fundamental to the

281   prosecution of successful antifibrotic clinical trials. Our work illustrates the power of

282   single-cell transcriptomics to decode the cellular and molecular basis of human organ

283   fibrosis, providing a conceptual framework for the discovery of relevant therapeutic

284   targets to treat patients with a broad range of fibrotic diseases.
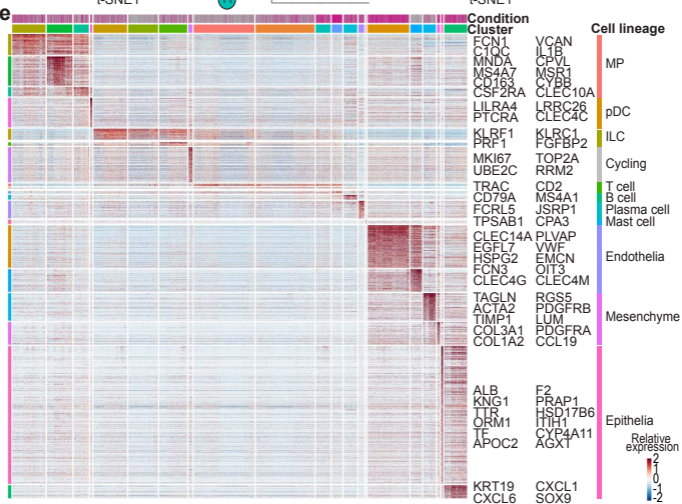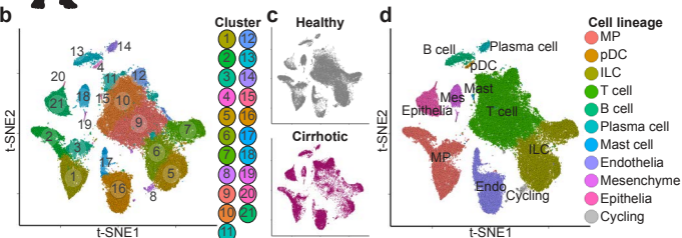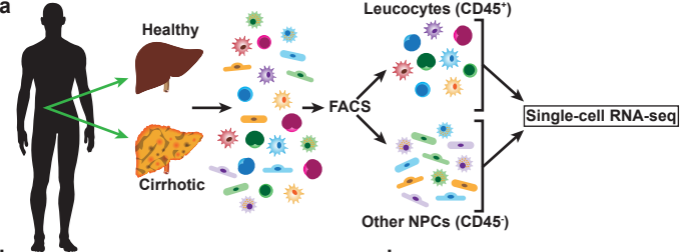
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347   pathway that regulates fibrosis in several organs. *Nat. Med.* **19**, 1617–1624
348   (2013).

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350   Activation in Culture and In Vivo. *Gastroenterology* **132**, 1937–1946 (2007).

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357   via Amphiregulin-Induced Transforming Growth Factor Beta Activation.
358   *Immunity* **50**, 645-654.e6 (2019).

359

360

Figure 1: Single cell atlas of human liver NPC.

a, Overview: isolation, FACS-sorting and sc-RNASeq of leucocytes (CD45$^+$) and other NPC fractions (CD45$^-$). **b**, Clustering 66,135 cells from 5 healthy and 5 cirrhotic human livers. **c**, Annotation by injury condition. **d**, Cell lineage inferred from expression of marker gene signatures. Endo, endothelial cell; ILC, innate lymphoid cell; Mast, mast cell; Mes, mesenchymal cell; MP, mononuclear phagocyte; pDC, plasmacytoid dendritic cell. **e**, Heatmap: cluster marker genes (top, colour coded by cluster and colour coded by condition) and exemplar genes and lineage annotation labelled (right). Cells columns, genes rows.

b

t-SNE1 / t-SNE2

Cluster: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

c

Healthy

Cirrhotic

d

Cell lineage
- MP
- pDC
- ILC
- T cell
- B cell
- Plasma cell
- Mast cell
- Endothelia
- Mesenchyme
- Epithelia
- Cycling

B cell, Plasma cell, pDC, Mast, Mes, Epithelia, T cell, MP, Endo, Cycling, ILC

e

Condition / Cluster / Cell lineage

MP: CLQN, VCAN, C1QL, IL1B, MNDA, CPVL, MS4A7, MSR1, CSF1R3, CYBB, CSF2RA, CLEC10A

pDC: LILRA4, LRRC26, PTCRA, CLEC4C

ILC: KLRF1, KLRC1, PRF1, FGFBP2

Cycling: MKI67, TOP2A, UBE2C, RRM2

T cell: TRAC, CD2
B cell: CD79A, MS4A1
Plasma cell: FCRLS, JSRP1
Mast cell: TPSAB1, CPA3

Endothelia: CLEC14A, PLVAP, EGFL7, VWF, HSPG2, EMCN, FCN3, OIT3, CLEC4G, CLEC4M

Mesenchyme: TAGLN, RGS5, ACTA2, PDGFRB, TIMP1, LUM, COL3A1, PDGFRA, COL1A2, CCL19

Epithelia: ALB, F2, KNG1, PRAP1, TTR, HSD17B6, ORM1, ITIH1, IF, CYP4A11, APOC2, AGXT

KRT19, CXCL1, CXCL6, SOX9

Relative expression: 2 1 0 -1 -2

370 **Figure 2: Identifying scar-associated macrophage subpopulations.**

371 **a**, Clustering 10,737 mononuclear phagocytes (MP) from 5 healthy and 5 cirrhotic

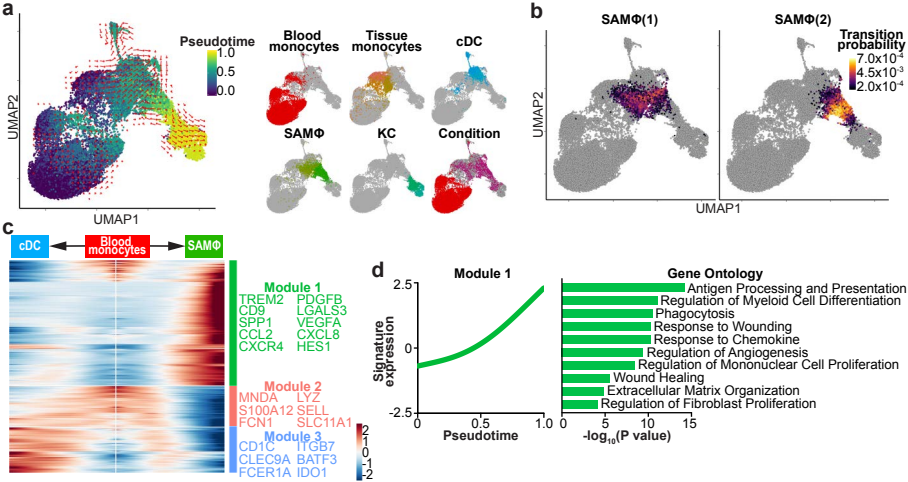372 human livers. TMo, tissue monocyte; SAMΦ, scar-associated macrophage; KC,

373 Kupffer cell; cDC, conventional dendritic cell. **b**, Annotation by injury condition. **c**,

374 Fractions of MP subpopulations in 5 healthy *versus* 5 cirrhotic livers, Mean±SEM,

375 Wald test. **d**, Heatmap: MP cluster marker genes (top, colour coded by cluster and

376 condition), exemplar genes labelled (right). Cells columns, genes rows. **e**, Scaled gene

377 expression of SAMΦ and TMo cluster markers across MP cells from healthy (n=5) and

378 cirrhotic (n=5) livers. **f**, Flow cytometry: TREM2$^+$CD9$^+$ MP fraction in healthy (n=2)

379 *versus* cirrhotic (n=3) liver, Mean±SEM. **g**, Primary human hepatic stellate cells (HSC)

380 treated with conditioned media from SAMΦ (n=3) or TMo (n=3); qPCR of stated genes,

381 expression relative to mean expression of control HSC (n=6), Mean±SEM, Kruskal-

382 Wallis and Dunn. **h**, Representative immunofluorescence image, cirrhotic liver:

383 TREM2 (red), CD9 (white), collagen 1 (green), DAPI (blue), scale bar 50μm.

384

a

Cluster	Identity
1 MP(1)	TMo(1)
2 MP(2)	TMo(2)
3 MP(3)	TMo(3)
4 MP(4)	SAMΦ(1)
5 MP(5)	SAMΦ(2)
6 MP(6)	KC(1)
7 MP(7)	KC(2)
8 MP(8)	cDC2
9 MP(9)	cDC1
10	Cycling

b Healthy / Cirrhotic

c Fraction of MP cells — Healthy / Cirrhotic; P<0.001, P<0.001, P<0.001. Clusters: TMo(1), TMo(2), TMo(3), SAMΦ(1), SAMΦ(2), KC(1), KC(2), cDC2, cDC1

d Heatmap. Condition / Cluster. Genes: S100A12, FCN1, MNDA, NBEAL1, IFITM2, APOBEC3A, RGS2, C1QC, TREM2, CD9, CD5L, CD163, VCAM1, MARCO, SIGLEC1, TIMD4, MERTK, NR1H3, ARL4C, FCER1A, CD1E, LGALS2

e Gene expression violin plots: S100A12, MNDA, C1QC, CD9, TREM2. Clusters: TMo(1), TMo(2), TMo(3), SAMΦ(1), SAMΦ(2), KC(1), KC(2), cDC2, cDC1

f CD9-PE-Vio770 vs TREM2-APC. Healthy 5.9±1.5%, Cirrhotic 19.7±3.2%. % of MPs — Healthy / Cirrhotic

g COL1A1 (P=0.022), COL3A1 (P=0.01). Relative HSC gene expression. Conditioned media: Control, SAMΦ, TMo

h TREM2 (red), CD9 (white), Collagen 1 (green), DAPI (blue)

Figure 3: Fibrogenic phenotype of scar-associated macrophages.

a, UMAP visualisation, 23,075 cells from liver-resident MP (5 healthy and 5 cirrhotic) and blood monocytes (5 PBMC), annotating *monocle* pseudotemporal dynamics (purple to ye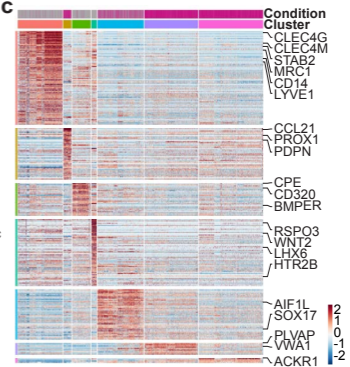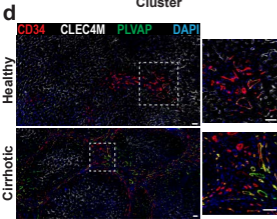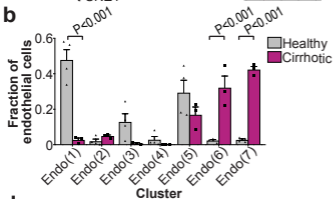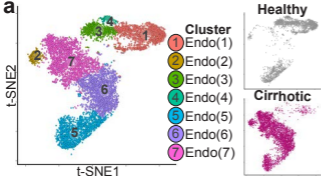llow). RNA velocity field (red arrows) visualised using Gaussian smoothing on regular grid. Right: Annotation of MP subpopulation, injury condition. **b**, Transition probabilities per SAMΦ subpopulation, indicating for each cell the likelihood of transition into either SAMΦ(1) or SAMΦ(2), calculated using RNA velocity (yellow high; purple low; grey below threshold of 2×10^{-4}). **c**, Heatmap: spline curves fitted to genes differentially expressed across blood monocyte-to-SAMΦ (right arrow) and blood monocyte-to-cDC (left arrow) pseudotemporal trajectories, grouped by hierarchical clustering (k=3). Gene co-expression modules (colour) labelled right with exemplar genes from each module. **d**, Spline curve fitted to averaged expression of all genes in module 1, along monocyte-to-SAMΦ pseudotemporal trajectory, selected GO enrichment (right), Fisher's exact test.

a

Pseudotime

Blood monocytes · Tissue monocytes · cDC · SAMΦ · KC · Condition

b

SAMΦ(1) SAMΦ(2) Transition probability
7.0×10⁻⁴ / 4.5×10⁻³ / 2.0×10⁻⁴

c

cDC ← Blood monocytes → SAMΦ

Module 1
TREM2   PDGFB
CD9   LGALS3
SPP1   VEGFA
CCL2   CXCL8
CXCR4   HES1

Module 2
MNDA   LYZ
S100A12 SELL
FCN1   SLC11A1

Module 3
CD1C   ITGB7
CLEC9A   BATF3
FCER1A   IDO1

d

Module 1

Gene Ontology

Antigen Processing and Presentation
Regulation of Myeloid Cell Differentiation
Phagocytosis
Response to Wounding
Response to Chemokine
Regulation of Angiogenesis
Regulation of Mononuclear Cell Proliferation
Wound Healing
Extracellular Matrix Organization
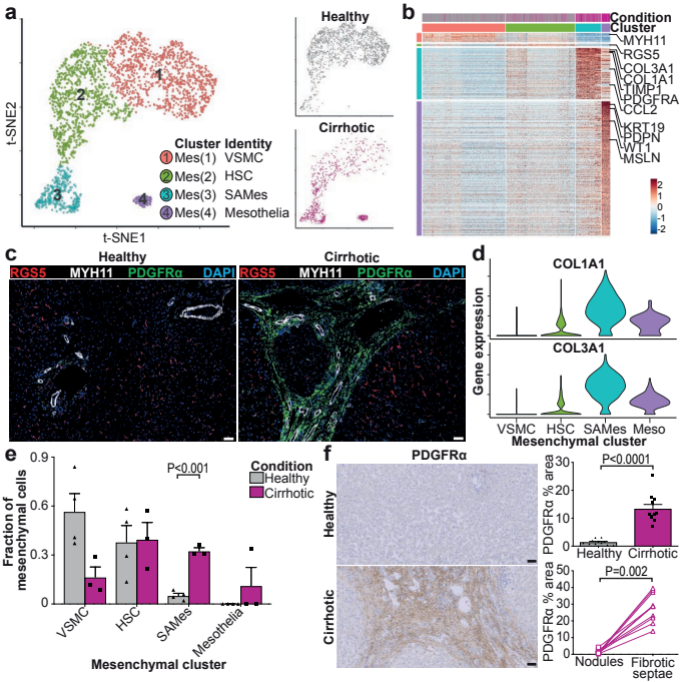Regulation of Fibroblast Proliferation

-log₁₀(P value)

399   **Figure 4: Identifying scar-associated endothelial subpopulations.**

400   **a**, Clustering 8,020 endothelial cells from 4 healthy and 3 cirrhotic human livers,

401   annotating injury condition (right). **b**, Fractions of endothelial subpopulations in

402   healthy (n=4) *versus* cirrhotic (n=3) livers, Mean±SEM, Wald. **c**, Heatmap: endothelial

403   cluster marker genes (colour coded top by cluster and condition), exemplar genes

404   labelled right. Cells columns, genes rows. **d**, Representative immunofluorescence

405   images: CD34 (red), CLEC4M (white), PLVAP (green), DAPI (blue), scale bar 50μm.

406   **e**, Digital pixel quantification: CLEC4M staining healthy (n=5) *versus* cirrhotic liver

407   (n=8), PLVAP staining healthy (n=11) *versus* cirrhotic liver (n=11), ACKR1 staining

408   healthy (n=10) *versus* cirrhotic liver (n=10), scale bars 50μm, Mean±SEM, Mann-

409   Whitney two-tailed.

a

t-SNE2 / t-SNE1

Cluster
1. Endo(1)
2. Endo(2)
3. Endo(3)
4. Endo(4)
5. Endo(5)
6. Endo(6)
7. Endo(7)

Healthy

Cirrhotic

b

Fraction of endothelial cells

P<0.001   *P*<0.001 *P*<0.001

Healthy
Cirrhotic

Endo(1) Endo(2) Endo(3) Endo(4) Endo(5) Endo(6) Endo(7)

c

Condition
Cluster

CLEC4G
CLEC4M
STAB2
MRC1
CD14
LYVE1

CCL21
PROX1
PDPN

CPE
CD320
BMPER

RSPO3
WNT2
LHX6
HTR2B

AIF1L
SOX17
PLVAP
VWA1
ACKR1

2
1
0
-1
-2

d

CD34 CLEC4M PLVAP DAPI

Healthy

Cirrhotic

e

Healthy   Cirrhotic

CLEC4M

PLVAP

ACKR1

CLEC4M % area
P=0.011
Healthy Cirrhotic

PLVAP % area
P=0.0006
Healthy Cirrhotic

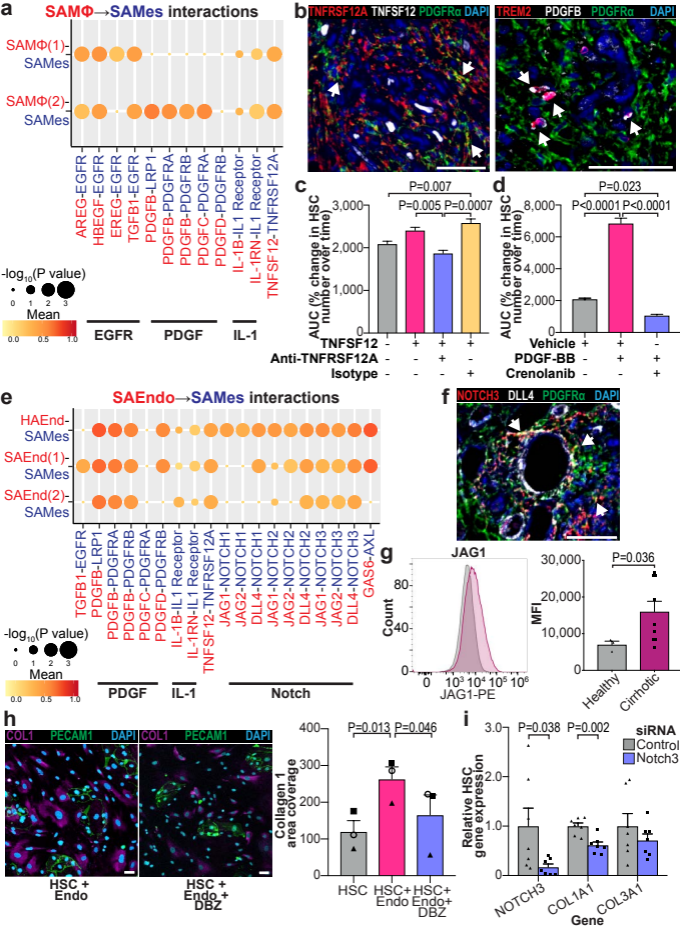ACKR1 % area
P=0.005
Healthy Cirrhotic

410 **Figure 5: Identifying scar-associated mesenchymal cell populations.**

411 **a**, Clustering 2,318 mesenchymal cells (Mes) from 4 healthy and 3 cirrhotic human

412 livers, annotating injury condition (right). VSMC, vascular smooth muscle cell; HSC,

413 hepatic stellate cell; SAMes, scar-associated mesenchymal cell. **b**, Heatmap:

414 Mesenchymal cluster marker genes (top, colour coded by cluster and condition),

415 exemplar genes labelled (right). Cells columns, genes rows. **c**, Representative

416 immunofluorescence images: RGS5 (red), MYH11 (white), PDGFRα (green), DAPI

417 (blue), scale bars 50μm **d**, Scaled gene expression of fibrillar collagens across

418 mesenchymal cells from healthy (n=4) and cirrhotic (n=3) livers. Meso, mesothelial

419 cell. **e**, Fraction of mesenchymal subpopulations in healthy (n=4) *versus* cirrhotic (n=3)

420 livers, Mean±SEM, Wald test. **f**, PDGFRα immunohistochemistry, digital pixel

421 quantification of healthy (n=11) *versus* cirrhotic (n=11) liver (top right), Mean±SEM,

422 Mann-Whitney two-tailed. PDGFRα pixel quantification in fibrotic septae and

423 parenchymal nodules in 10 cirrhotic livers (bottom right), Wilcoxon two-tailed, scale

424 bars 50μm.

a

t-SNE2 / t-SNE1

Cluster Identity
- Mes(1) VSMC
- Mes(2) HSC
- Mes(3) SAMes
- Mes(4) Mesothelia

Healthy

Cirrhotic

b

Condition
Cluster

MYH11
RGS5
COL3A1
COL1A1
TIMP1
PDGFRA
CCL2
KRT19
PDPN
WT1
MSLN

c

Healthy — RGS5 MYH11 PDGFRα DAPI

Cirrhotic — RGS5 MYH11 PDGFRα DAPI

d

COL1A1

COL3A1

Gene expression

VSMC   HSC   SAMes   Meso
Mesenchymal cluster

e

Fraction of mesenchymal cells

P<0.001

Condition
- Healthy
- Cirrhotic

VSMC   HSC   SAMes   Mesothelia
Mesenchymal cluster

f

PDGFRα

Healthy

Cirrhotic

PDGFRα % area — P<0.0001

Healthy   Cirrhotic

PDGFRα % area — P=0.002

Nodules   Fibrotic septae

Figure 6: Multi-lineage interactions in the fibrotic niche.

a, Dotplot: ligand-receptor interactions between SAMΦ (n=10 human livers) and SAMes (n=7 human livers). X-axis, ligand (red) and cognate receptor (blue); y-axis, cell populations expressing ligand (red) and receptor (blue); circle size, P value (permutation test); colour (red, high; yellow, low), means of average ligand and receptor expression levels in interacting subpopulations. **b**, Representative immunofluorescence images, fibrotic niche. Left, TREM2 (red), PDGFB (white), PDGFRα (green), DAPI (blue), arrows TREM2$^+$PDGFB$^+$ cells. Right, TNFRSF12A (red), TNFSF12 (white), PDGFRα (green), DAPI (blue), arrows TNFRSF12A$^+$PDGFRα$^+$ cells, scale bars 50μm. **c to d**, HSC proliferation assay: y-axis, area under curve (AUC) of % change in HSC number over time (hours), Mean±SEM, one-way ANOVA and Tukey. **c**, Control, TNFSF12, anti-TNFRSF12A, isotype control, all n=3. **d**, Vehicle, PDGF-BB, Crenolanib, all n=3. **e**, Dotplot: ligand-receptor interactions between SAEndo (n=7 human livers) and SAMes (n=7 human livers). X-axis, ligand (red) and cognate receptor (blue); y-axis, cell populations expressing ligand (red) and receptor (blue); circle size, P value (permutation test); colour (red, high; yellow, low), means of average ligand and receptor expression levels in interacting subpopulations. **f**, Representative immunofluorescence image, fibrotic niche. NOTCH3 (red), DLL4 (white), PDGFRα (green), DAPI (blue), arrows NOTCH3$^+$PDGFRα$^+$ cells, scale bar 50μm. **g**, Endothelial cell JAG1 flow cytometry: healthy (n=3) or cirrhotic (n=9) liver, representative histogram (left), mean fluorescence intensity (MFI, right), Mean±SEM, Mann-Whitney two-tailed. **h**, Cirrhotic endothelial cell and HSC co-culture, Notch inhibitor Dibenzazepine (DBZ). Representative immunofluorescence images (left), Collagen 1 (magenta), PECAM1 (green), DAPI (blue). Digital pixel analysis (right); collagen 1 area, n=3, Mean±SEM, RM one-way ANOVA and Tukey. **n**, HSC gene knockdown: control (n=7) or *NOTCH3* (n=7) siRNA, qPCR of stated gene, expression relative to mean expression of control siRNA, Mean±SEM, Mann-Whitney two-tailed.

a SAMΦ→SAMes interactions

b

c
TNFSF12 — + — +
Anti-TNFRSF12A — — + +
Isotype — — — —
P=0.007
P=0.005 P=0.0007

d
Vehicle + — —
PDGF-BB — + +
Crenolanib — — +
P=0.023
P<0.0001 P<0.0001

e SAEndo→SAMes interactions

f
NOTCH3 DLL4 PDGFRα DAPI

g JAG1
P=0.036
Healthy Cirrhotic

h
COL1 PECAM1 DAPI
HSC + Endo | HSC + Endo + DBZ
Collagen 1 area coverage
P=0.013 P=0.046
HSC | HSC+ Endo | HSC+ Endo+ DBZ

i
siRNA
Control
Notch3
P=0.038 P=0.002
NOTCH3 COL1A1 COL3A1
Gene
Relative HSC gene expression

453   **Methods**

454   **Study subjects**

455   Local approval for procuring human liver tissue and blood samples for scRNA-seq,

456   flow cytometry and histological analysis was obtained from the NRS BioResource and

457   Tissue Governance Unit (Study Number SR574), following review at the East of

458   Scotland Research Ethics Service (Reference 15/ES/0094). All subjects provided

459   written informed consent. Healthy background non-lesional liver tissue was obtained

460   intraoperatively from patients undergoing surgical liver resection for solitary colorectal

461   metastasis at the Hepatobiliary and Pancreatic Unit, Department of Clinical Surgery,

462   Royal Infirmary of Edinburgh. Patients with a known history of chronic liver disease,

463   abnormal liver function tests or those who had received systemic chemotherapy within

464   the last four months were excluded from this cohort. Cirrhotic liver tissue was obtained

465   intraoperatively from patients undergoing orthotopic liver transplantation at the

466   Scottish Liver Transplant Unit, Royal Infirmary of Edinburgh. Blood from patients with

467   a confirmed diagnosis of liver cirrhosis were obtained from patients attending the

468   Scottish Liver Transplant Unit, Royal Infirmary of Edinburgh. Patients with liver

469   cirrhosis due to viral hepatitis were excluded from the study. Patient demographics are

470   summarised in Extended Data Fig. 1a. Isolation of primary hepatic macrophage

471   subpopulations and endothelial cells from healthy and cirrhotic livers for cell culture

472   and analysis of secreted mediators was performed at the University of Birmingham,

473   UK. Local ethical approval was obtained (Reference 06/Q2708/11, 06/Q2702/61) and

474   all patients provided written, informed consent. Liver tissue was acquired from

475   explanted diseased livers from patients undergoing orthotopic liver transplantation,

476   resected liver specimens or donor livers rejected for transplant at the Queen Elizabeth

477   Hospital, Birmingham. For histological assessment of NAFLD biopsies, anonymised

478   unstained formalin-fixed paraffin-embedded liver biopsy sections encompassing the

479   complete NAFLD spectrum were provided by the Lothian NRS Human Annotated

480   Bioresource under authority from the East of Scotland Research Ethics Service REC 1,

481   reference 15/ES/0094.

482   **Human tissue processing**

483   Importantly, to minimise artefacts[29], we developed a rapid tissue processing pipeline,

484   obtaining fresh non-ischaemic liver tissue taken by wedge biopsy prior to the

485   interruption of the hepatic vascular inflow during liver surgery or transplantation, and

486   immediately processing this for FACS. This enabled a workflow time of under three

487   hours from patient to single-cell droplet encapsulation.

488   For human liver scRNA-seq and flow cytometry analysis, a wedge biopsy of non-

489   ischaemic fresh liver tissue (2-3 grams) was obtained by the operating surgeon, prior to

490   interruption of the hepatic vascular inflow. This was immediately placed in HBSS

491   (Gibco) on ice. The tissue was then transported directly to the laboratory and

492   dissociation routinely commenced within 20 minutes of the liver biopsy. To enable

493   paired histological assessment, a segment of each liver specimen was also fixed in 4%

494   neutral-buffered formalin for 24 hours followed by paraffin-embedding. Additional

495   liver samples, obtained via the same method, were fixed in an identical manner and

496   used for further histological analysis. For human macrophage cell sorting and

497   endothelial cell isolation, liver tissue (40 grams) was used from cirrhotic patients

498   undergoing orthotopic liver transplantation or control samples from donor liver or liver

499   resection specimens.

500   **Animals**

501   Adult male C57BL/6JCrl mice aged 8-10 weeks were purchased from Charles River.

502   Mice were housed under specific pathogen-free conditions at the University of

503   Edinburgh. All experimental protocols were approved by the University of Edinburgh

504   Animal Welfare and Ethics Board in accordance with UK Home Office regulations.

505   Liver fibrosis was induced with 4 weeks (9 injections) of twice-weekly intraperitoneal

506   carbon tetrachloride (CCl_4) at a dose of 0.4 μl/g body weight, diluted 1:3 in olive oil as

507   previously described[7]. Mice were randomly assigned to receive CCl_4 or to serve as

508   healthy controls. No sample size calculation or blinding was performed. Liver tissue

509   was harvested 24 hours following the final CCl_4 injection, a time of active fibrogenesis[7].

510   Comparison was made to age-matched uninjured mice.

511   **Preparation of single-cell suspensions**

512 For human liver scRNA-seq, liver tissue was minced with scissors and digested in
513 5mg/ml pronase (Sigma-Aldrich, P5147-5G), 2.93mg/ml collagenase B (Roche,
514 11088815001) and 1.9mg/ml DNase (Roche, 10104159001) at 37°C for 30 minutes
515 with agitation (200–250 r.p.m.), then strained through a 120μm nybolt mesh along with
516 PEB buffer (PBS, 0.1% BSA, and 2mM EDTA) including DNase (0.02mg/ml).
517 Thereafter all processing was done at 4°C. The cell suspension was centrifuged at 400g
518 for 7 minutes, supernatant removed, cell pellet resuspended in PEB buffer and DNase
519 added (0.02mg/ml), followed by additional centrifugation (400g, 7 minutes). Red blood
520 cell lysis was performed (BioLegend, 420301), followed by centrifugation (400g, 7
521 minutes), resuspension in PEB buffer and straining through a 35μm filter. Following
522 another centrifugation at 400g for 7 minutes, cells were blocked in 10% human serum
523 (Sigma-Aldrich, H4522) for 10 minutes at 4°C prior to antibody staining.

524 For both human liver macrophage flow cytometry analysis and cell sorting and mouse
525 liver macrophage flow cytometry, cell sorting and scRNA-seq, single-cell suspensions
526 were prepared as previously described, with minor modifications[30]. In brief, liver tissue
527 was minced and digested in an enzyme cocktail 0.625 mg/ml collagenase D (Roche,
528 11088882001), 0.85 mg/ml collagenase V (Sigma-Aldrich, C9263-1G), 1 mg/ml
529 dispase (Gibco, Invitrogen, 17105-041), and 30 U/ml DNase (Roche, 10104159001) in
530 RPMI-1640 at 37°C for 20 minutes (mouse) or 45 minutes (human) with agitation
531 (200–250 r.p.m.), before being passed through a 100μm filter. Following red blood cell
532 lysis (BioLegend, 420301), cells were washed in PEB buffer and passed through a
533 35μm filter. Before the addition of antibodies, cells from human samples were blocked
534 in 10% human serum (Sigma-Aldrich, H4522) and mouse samples were blocked in
535 anti-mouse CD16/32 antibody (1:100; Biolegend, 101302) and 10% normal mouse
536 serum (Sigma, M5905) for 10 minutes at 4 °C.

537 For human PBMC scRNA-seq, 4.9ml peripheral venous blood samples were collected
538 in EDTA-coated tubes (Sarstedt, S-Monovette® 4.9ml K3E) and placed on ice. Blood
539 samples were transferred into a 50ml Falcon tube. Following red cell lysis (Biolegend,
540 420301), blood samples were then centrifuged at 500g for 5 minutes and supernatant
541 was removed. Pelleted samples were then resuspended in staining buffer (PBS plus 2%
542 BSA; Sigma-Aldrich) and centrifugation was repeated. Samples were then blocked in

543   10% human serum (Sigma-Aldrich, H4522) in staining buffer on ice for 30 minutes.

544   Cells were then resuspended in staining buffer and passed through a 35μm filter prior

545   to antibody staining.

Flow cytometry and cell sorting

547   Incubation with primary antibodies was performed for 20 minutes at 4°C. All

548   antibodies, conjugates, lot numbers and dilutions used in this study are presented in

549   Supplementary Table 19. Following antibody staining, cells were washed with PEB

550   buffer. For both human macrophage flow cytometry analysis and cell sorting, cells were

551   then incubated with streptavidin-BV711 for 20 minutes at 4°C (Biolegend 405241;

552   Dilution 1:200). For human and mouse cell sorting (FACS) and mouse flow cytometry

553   analysis, cell viability staining (DAPI; 1:1000 dilution) was then performed,

554   immediately prior to acquiring the samples.

555   Human cell sorting for scRNA-seq was performed on a BD Influx (Becton Dickinson,

556   Basel, Switzerland). Viable single CD45$^+$ (leucocytes) or CD45$^-$ (other non-

557   parenchymal cells) cells were sorted from human liver tissue (Extended Data Fig. 1b)

558   and viable CD45$^+$ CD66b$^-$ (PBMC) cells were sorted from peripheral blood (Extended

559   Data Fig. 1c) and processed for droplet-based scRNA-seq.

560   To generate conditioned media from cirrhotic liver macrophage subpopulations, cells

561   were sorted on a BD FACSAria^{TM} Fusion (Becton Dickinson, Basel, Switzerland).

562   Sorted SAMΦ (viable CD45$^+$Lin$^-$HLA-DR$^+$CD14$^+$CD16$^+$CD163$^-$TREM2$^+$CD9$^+$),

563   TMo (viable CD45$^+$Lin$^-$ HLA-DR$^+$CD14$^+$CD16$^+$CD163$^-$TREM2$^-$CD9$^-$) and KC (viable

564   CD45$^+$Lin$^-$HLA-DR$^+$CD14$^+$CD16$^+$CD163$^+$CD9$^-$) were plated in 12-well plates

565   (Corning, 3513) in DMEM (Gibco, 41965039) containing 2% FBS (Gibco, 10500056)

566   at 1x10^6 cells/ml for 24 hours at 37°C 5%CO$_2$. Control wells contained media alone.

567   Conditioned media was collected, centrifuged at 400g for 10 minutes and supernatant

568   stored at -80°C.

569   For human macrophage flow cytometry analysis, following surface antibody staining,

570   cells were stained with Zombie NIR fixable viability dye (Biolegend, 423105)

571   according to manufacturers' instructions. Cells were washed in PEB then fixed in IC

fixation buffer (Thermo-Fisher, 00-8222-49) for 20 minutes at 4°C. Fixed samples were stored in PEB at 4°C until acquisition. Flow cytometry acquisition was performed on 6-laser Fortessa flow cytometer (Becton Dickinson, Basel, Switzerland). The gating strategy is shown (Extended Data Fig. 4f, Fig. 2f).

Mouse macrophage cell sorting for scRNA-seq and co-culture experiments was performed on a BD FACSAriaII (Becton Dickinson, Basel, Switzerland). For scRNA-seq, viable $CD45^+$ Lin(CD3, NK1.1, Ly6G, CD19)$^-$ cells were sorted from healthy (n=3) and CCl_4-treated (n=3) mice and processed for droplet-based scRNA-seq. For transwell co-culture, viable $CD45^+$ Lin$^-$ $CD11b^+$ $F4/80^+$ $TIMD4^-$ $CD9^+$ (mSAMΦ) or $CD9^-$ (mTMo) cells were sorted from CCl_4-treated mice (Extended Data Fig. 6e). Flow cytometry analysis on macrophages from healthy and CCl_4-treated mice was also performed on a BD FACSAriaII (Becton Dickinson, Basel, Switzerland), using the same gating strategy (Extended Data Fig. 6e). All flow cytometry data was analysed using Flowjo software (Treestar, Ashland, TN).

Luminex Assay

Detection of CCL2, Galectin-3, IL-1β, CXCL8 and Osteopontin (SPP1) and CD163 proteins in conditioned media from human liver macrophage subpopulations was performed using a custom human luminex assay (R&D systems), according to the manufacturers protocol. Data was acquired using a Bio-Plex® 200 (Bio-Rad, UK) and is presented a median fluorescence intensity (MFI) for each analyte.

Cell Culture

Human hepatic stellate cell activation
Primary human hepatic stellate cells (HSC) were purchased (ScienCell, 5300) and cultured in stellate cell medium (SteCM, ScienCell, 5301) on Poly-L-Lysine (Sigma, P4832) coated T75 tissue culture flasks, according to the suppliers protocol. All experiments were performed using cells between passage 3 and 5. For assessment of fibrillar collagen gene expression, HSC were plated at 75,000 cells per well in 24 well-plates (Costar, 3524) in HSC media consisting of DMEM (Gibco, 21969-035) supplemented with 20 μM HEPES (Sigma, H3375,), 2 mM L-Glutamine (Gibco ,

25

601   25030-024), 1% Penicillin Streptomycin (Gibco, 15140-122, Gibco) and 2% Foetal

602   Bovine Serum (FBS, Gibco, 10270). HSC were serum starved overnight (in HSC media

603   without FBS), washed with PBS, then 250µl of conditioned media from primary human

604   macrophage subpopulations added for 24 hours. HSC were harvested for RNA.

605   *Human hepatic stellate cell proliferation*

606   For proliferation assays, following serum starvation HSC were harvested using TrypLE

607   Express (Gibco, Cat. no. 12604013), re-suspended in HSC media at 2.5×10^4/ml with

608   Incucyte NucLight Rapid Red (Essen Biosciences, 4717) at a dilution of 1 in 500 and

609   seeded into 384-well plates (Greiner Bio-One, 781090) at 25µl per well. HSC were then

610   treated with control media or (i) PDGF-BB (10ng/ml; Peprotech, 100-14B) or

611   TNFSF12 (100ng/ml; Peprotech, 310-06-5) with or without the PDGFRα inhibitor

612   Crenolanib[31] (1µM; Cayman chemicals, CAY1873), anti-TNRSF12A (2µg/ml; Life

613   Technologies, 16-9018-82, clone ITEM-4), mouse IgG2b kappa isotype control

614   antibody (2µg/ml; Life Technologies, 16-4732-82, clone eBMG2b) or vehicle control

615   as indicated or (ii) conditioned media from human hepatic macrophage subpopulations

616   as indicated. The final volume was 50µl for all conditions. Cells were then incubated

617   in an Incucyte ZOOM live cell analysis system (Essen Biosciences) humidified at 37°C

618   with 5% CO_2 with imaging every 3 hours using the 10x optic for either 87 hours

619   (recombinant cytokines/inhibitors) or 39 hours (macrophage conditioned media).

620   Analysis was performed with the Incucyte proprietary analysis software (version

621   2018A) by using machine learning to distinguish the individual nuclei (stained red by

622   the NucLight Rapid Red dye) and perform nuclear counts of the images at each 3 hour

623   time point over the period of culture. Data are expressed as area under curve (AUC)

624   for % change in nuclear number from baseline *versus* time (hours), calculated in

625   GraphPad Prism (GraphPad Software, USA).

626   *Gene knockdown in human hepatic stellate cells*

627   Knockdown of NOTCH3 in human HSC was performed using siRNA. HSC were plated

628   at 75,000 cells per well in a 12 well plate (Costar, 3513) followed by serum starvation

629   overnight (in HSC medium without FBS). siRNA duplexes with Lipofectamine

630   RNAiMAX Transfection Reagent (ThermoFisher, 13778075) were prepared in

631   OptiMEM (ThermoFisher, 31985070) according to the manufacturer's

recommendations, and used at a concentration of 50nM. Cells were exposed to the

duplex for 48 hours, in HSC media containing 2% FBS. Cells were harvested for RNA

and RT-qPCR. Knockdown efficiency was assessed by NOTCH3 RT-qPCR. The best

siRNA for knockdown was determined empirically using the FlexiTube GeneSolution

kit (Qiagen, GS4854). HSC treated with control siRNA (Qiagen, 1027280) and siRNA

for NOTCH3 (Qiagen, Hs_NOTCH3_3, SI00009513; knockdown 83%) were then

assessed for fibrillar collagen gene expression.

Mouse hepatic stellate cell activation

Primary murine HSC culture were isolated from healthy mice as described[26]. Briefly,

after cannulation of the inferior vena cava, the portal vein was cut to allow retrograde

step-wise perfusion with pronase (Sigma, P5147) and collagenase D (Roche,

11088882001) containing solutions, before *ex vivo* digestion in a solution containing

pronase, collagenase D and Dnase1 (Roche, 10104159001). HSC were isolated from

the digest solution by Histodenz (Sigma, D2158-100G) gradient centrifugation. HSC

were plated at a density of 400,000 cells per well in a 24 well plate (Costar, 3524) in

HSC media containing 10% FBS. Following overnight culture, cells were washed with

PBS and cultured in HSC media containing 2% FBS. For macrophage co-culture,

transwell inserts (0.4μm polyester membrane; Costar, 3470) were then placed above

adherent HSC. FACS-sorted CD9+ mSAMΦ or CD9- mTMo from CCl$_4$-treated mice

were resuspended in HSC media containing 2% FBS at 400,000 cells/ml and 200,000

cells added to the top of the transwell insert. Co-culture proceeded for 48 hours and

HSC were harvested for RNA. Quiescent HSC (harvested at start of co-culture) were

used as a control population.

Human liver endothelial cell isolation

Human liver endothelial cells (LEC) were isolated from cirrhotic explant livers and

non-fibrotic control donor liver as previously described[32]. Endothelial cells were

cultured on plasticware coated with rat-tail collagen (Sigma, C3867) in complete LEC

medium consisting of endothelial basal media (ThermoFisher, 11111044) containing

10% heat inactivated human serum (tcsBiosciences, CS100-500), 100U penicillin, 100

μg/mL streptomycin, 2mM glutamine (Sigma, G6784), VEGF (10 ng/mL; Peprotech,

662 100-20) and 10 ng/mL HGF (10 ng/mL; Peprotech, 100-39). LEC expression of

663 PLVAP, CD34, ACKR1 and JAG1 was assessed using flow cytometry.

664 *Flow-based adhesion assays*

665 Flow-based adhesion assays were performed as described[22,32]. Briefly, LEC from

666 healthy and cirrhotic liver were seeded into a rat-tail collagen coated Ibidi slide VI[0.4]

667 (Ibidi, 80606) at a density to give a monolayer and incubated overnight. Peripheral

668 blood was collected from healthy donors in EDTA-coated tubes. Peripheral blood

669 monocuclear cells (PBMC) were isolated using a lympholyte density gradient

670 (Cedarlane laboratories) then washed in PBS containing 1mM Ca^{2+}, 0.5 mM Mg^{2+} and

671 0.15% bovine serum albumin (PBS/BSA). Monocytes were enriched from PBMC using

672 pan-monocyte isolation kit (Miltenyi biotech, 130-096-537) according to the

673 manufacturer's protocol. For flow-based adhesion assay, cells were resuspended at 10^6

674 cells per millilitre in endothelial basal media (ThermoFisher, 11111044) containing

675 0.15% BSA, then perfused over the LEC monolayer for 5min at 0.28ml/min. Non-

676 adherent cells were washed off during 5min perfusion of 0.15% BSA human basal

677 endothelial medium and 10 random non-overlapping images were randomly recorded

678 from each channel. Total adherent (bright-phase; expressed as cell number/mm^2/1

679 million cells perfused) and transmigrating cells (dark-phase; expressed as percentage

680 total adherent cells) on an LEC monolayer from each patient were counted and

681 quantified as previously described[22].

682 *Gene knockdown in endothelial cells*

683 Knockdown of ACKR1 and PLVAP gene expression in human cirrhotic LEC was

684 performed using siRNA as previously described[32]. In brief, siRNA duplexes for

685 PLVAP, ACKR1, or negative control (Qiagen, 1027280) with Lipofectamine

686 RNAiMAX Transfection Reagent (ThermoFisher, 13778075) were prepared in

687 OptiMEM (ThermoFisher, 31985070) according to the manufacturer's

688 recommendations, and used at a concentration of 25nM. Cells were exposed to the

689 duplex for 4 hours at 37°C after which time the media was replaced with endothelial

690 basal media containing 10% heat-inactivated human serum for 24 hours. The media

691 was then replaced with complete LEC media and incubated at 37°C with 5% CO_2 for a

692 further 24 hours. Knockdown efficacy was assessed by flow cytometry and mean

28

693 fluorescence intensity (Extended Data Fig. 8f). The best siRNA for knockdown was

694 determined empirically using the FlexiTube GeneSolution kit (Qiagen, GS83483

695 (PLVAP) and GS2532 (ACKR1)). For flow-based adhesion assays, siRNAs for

696 PLVAP (Qiagen, Hs_PLVAP_1, SI00687547; knockdown 50.6%), ACKR1 (Qiagen,

697 Hs_Fy_5, SI02627667; knockdown 37.7%) or control siRNA were selected. 90,000

698 LEC from cirrhotic patients (n=6) were seeded into channels of a rat-tail collagen

699 coated Ibidi slide VI$^{0.4}$, gene knockdown performed, followed by flow-based adhesion

700 assay as described above.

701 *Endothelial and hepatic stellate cell co-culture*

702 HSC (15,000 cells) were seeded into an Ibidi slide VI$^{0.4}$ with and without primary

703 human LEC (15,000 cells) from individual cirrhotic patients (n=3) in LEC complete

704 medium. After 2h, all growth factor supplements were removed and cells were cultured

705 for a further 72 hours in endothelial basal media containing 10% heat-inactivated

706 human serum ± Notch-signalling inhibitor Dibenzazepine (10μM, Bio-Techne,

707 4489/10) or vehicle (DMSO) control. Cells were fixed in 4% PFA for 30 minutes,

708 permeabilised with 0.3% Triton PBS for 5 minutes, blocked with 10% goat serum in

709 PBS for 30 minutes followed by primary antibody incubation (mouse anti-PECAM1

710 and rabbit anti-collagen 1; see Supplementary Table 19) for 1 hour. Cells were washed

711 in 0.1% Triton PBS followed by addition of fluorescently-conjugated secondary

712 antibodies (1:500 dilution) for 1 hour. Cells were mounted with Pro-long Gold anti-

713 fade DAPI, images were taken on the Confocal Microscope Zeiss LSM780, and

714 collagen 1 area staining quantified using IMARIS.

715 **RNA extraction and RT-qPCR**

716 RNA was isolated from HSC using the RNeasy Plus Micro Kit (Qiagen, 74034) and

717 cDNA synthesis performed using QuantiTect Reverse Transcription Kit (Qiagen,

718 205313) according to the manufacturers' protocol. Reactions were performed in

719 triplicate in 384-well plate format and were assembled using the QIAgility automated

720 pipetting system (Qiagen). RT-qPCR for human HSC was performed using PowerUp

721 SYBR Green Master Mix (ThermoFisher, A25777) with the following primers (all

722 Qiagen): *GAPDH* (QT00079247), *COL1A1* (QT00037793), *COL3A1* (QT00058233),

723   *NOTCH3* (QT00003374). RT-qPCR for mouse HSC was performed using TaqMan

724   Fast Advanced Master Mix (ThermoFisher, 4444557) with the following primers:

725   *Gapdh* (ThermoFisher, Mm99999915_g1) and *Col3a1* (ThermoFisher,

726   Mm00802300_m1). Samples were amplified on an ABI 7900HT FAST PCR system

727   (Applied Biosystems, ThermoFisher Scientific). Data was analysed using

728   ThermoFisher Connect cloud qPCR analysis software (ThermoFisher Scientific). The

729     $2^{-\Delta\Delta Ct}$ quantification method, using *GAPDH* for normalization, was used to estimate

730   the amount of target mRNA in samples, and expression calculated relative to average

731   mRNA expression levels from control samples.

732   **Immunohistochemistry, immunofluorescence, smFISH**

733   Formalin-fixed paraffin-embedded human liver tissue was cut into 4 μm sections,

734   dewaxed, rehydrated, then incubated in 4% neutral-buffered formalin for 20 minutes.

735   Following heat-mediated antigen retrieval in pH6 sodium citrate (microwave; 15

736   minutes), slides were washed in PBS and incubated in 4% hydrogen peroxide for 10

737   minutes. Slides were then washed in PBS, blocked using protein block (GeneTex,

738   GTX30963) for 1 hour at room temperature before incubation with primary antibodies

739   for 1 hour at room temperature. A full list of primary antibodies and conditions are

740   shown in Supplementary Table 19. Slides were washed in PBST (PBS plus 0.1%

741   Tween20; Sigma-Aldrich, P1379) then incubated with ImmPress HRP Polymer

742   Detection Reagents (depending on species of primary; rabbit, MP-7401; mouse, MP-

743   6402-15; goat, MP-7405; all Vector Laboratories) for 30 minutes at room temperature.

744   Slides were washed in PBS followed by detection. For DAB staining, sections were

745   incubated with DAB (DAKO, K3468) for 5 minutes and washed in PBS before a

746   haematoxylin (Vector Laboratories, H3404) counterstain. For multiplex

747   immunofluorescence staining, following the incubation with ImmPress and PBS wash,

748   initial staining was detected using either Cy3, Cy5, or Fluorescein tyramide (Perkin-

749   Elmer, NEL741B001KT) at a 1:1000 dilution. Slides were then washed in PBST

750   followed by further heat treatment with pH6 sodium citrate (15 minutes), washes in

751   PBS, protein block, incubation with the second primary antibody (incubated overnight

752   at 4°C), ImmPress Polymer and tyramide as before. This sequence was repeated for the

753 third primary antibody (incubated at room temperature for 1 hour) and a DAPI-
754 containing mountant was then applied (ThermoFisher Scientific, P36931).

755 For AMEC Staining (only CLEC4M immunohistochemistry), all washes were carried
756 out with TBST (dH$_2$O, 2oomM Tris, 1.5M NaCl, 1% Tween20 (all Sigma-Aldrich)
757 pH7.5) and peroxidase blocking was carried out for 30mins in 0.6% hydrogen peroxide
758 in Methanol. Sections were incubated with AMEC (Vector Laboratories, SK-4285) for
759 20 minutes and washed in TBST (dH$_2$O, 200mM Tris, 1.5M NaCl, 1% Tween20 (all
760 Sigma-Aldrich)) before a haematoxylin (Vector Laboratories, SK-4285) counterstain.

761 For combined single-molecule fluorescent in situ hybridization (smFISH) and
762 immunofluorescence, detection of TREM2 was performed using the RNAscope® 2.5
763 LS Reagent Kit - BrownAssay (Advanced Cell Diagnostics (ACD)) in accordance with
764 the manufacturer's instructions. Briefly, 5 μm tissue sections were dewaxed, incubated
765 with endogenous enzyme block, boiled in pretreatment buffer and treated with protease,
766 followed by target probe hybridization using the RNAscope® LS 2.5 Hs-TREM2
767 (420498, ACD) probe. Target RNA was then detected with Cy3 tyramide (Perkin-
768 Elmer, NEL744B001KT) at 1:1000 dilution. The sections were then processed through
769 a pH6 sodium citrate heat-mediated antigen retrieval, hydrogen peroxidase treatment
770 and protein block (all as for multiplex immunofluorescence staining as above). MNDA
771 antibody was applied overnight at 4°C, completed using a secondary ImmPress HRP
772 Anti-Rabbit Peroxidase IgG (Vector Laboratories, MP7401), visualised using a
773 Flourescein tyramide (Perkin-Elmer, NEL741B001KT) at a 1:1000 dilution and stained
774 with DAPI.

775 Brightfield and fluorescently-stained sections were imaged using the slide scanner
776 AxioScan.Z1 (Zeiss) at 20X magnification (40X magnification for smFISH). Images
777 were processed and scale bars added using Zen Blue (Zeiss) and Fiji software[33].

778 **Cell counting and image analysis**

779 Automated cell counting was performed using QuPath software[34]. Briefly, DAB-
780 stained whole tissue section slide-scanned images (CZI files) were imported into
781 QuPath. Cell counts were carried out using the positive cell detection tool, detecting

31

782   haematoxylin-stained nuclei and then thresholding for positively-stained DAB cells,

783   generating DAB-positive cell counts/mm^2 tissue. Identical settings and thresholds were

784   applied to all slides for a given stain and experiment. For cell counts of fibrotic septae

785   vs parenchymal nodules, the QuPath segmentation tool was used to segment the DAB-

786   stained whole tissue section into fibrotic septae or non-fibrotic parenchymal nodule

787   regions using tissue morphological characteristics (Fig. 2j). Positive cell detection was

788   then applied to the fibrotic and non-fibrotic regions in turn, providing cell DAB-positive

789   cell counts/mm^2 in fibrotic septae and non-fibrotic parenchymal nodules for each tissue

790   section.

791   Digital morphometric pixel analysis was performed using the Trainable Weka

792   Segmentation (TWS) plugin[35] in Fiji software[33]. Briefly, each stained whole tissue

793   section slide-scanned image was converted into multiple TIFF files in Zen Blue

794   software (Zeiss). TIFF files were imported into Fiji and TWS plugin trained to produce

795   a classifier which segments images into areas of positive staining, tissue background

796   and white space[35]. The same trained classifier was then applied to all TIFF images from

797   every tissue section for a particular stain, providing a percentage area of positive

798   staining for each tissue section. For digital morphometric quantification of positive

799   staining of fibrotic septae *versus* parenchymal nodules, TIFF images were segmented

800   into fibrotic septae or non-fibrotic parenchymal nodule regions using tissue

801   morphological characteristics, followed by analysis using the TWS plugin in Fiji

802   software.

803   **Histological assessment of NASH sections**

804   Haematoxylin and eosin and picro-sirius red stained sections from each case were

805   whole-slide imaged using a NanoZoomer imager (Hamamatsu Photonics, Japan).

806   Images of stained sections were independently scored by a consultant liver transplant

807   histopathologist (T.J.K.) at the national liver transplant centre with experience in trial

808   scoring by applying the ordinal NAFLD activity score[36]. For observer-independent

809   quantification of picro-sirius red positive staining, images were split using ndpisplit[37]

810   into tiles of x5 magnification before the application of a classifier that had been trained

811 by the liver histopathologist using the machine learning WEKA plugin in FIJI[33,35], as

812 previously described[38]. All analysis was undertaken blind to all other data.

Droplet-based scRNA-seq

814 Single cells were processed through the Chromium™ Single Cell Platform using the

815 Chromium™ Single Cell 3' Library and Gel Bead Kit v2 (10X Genomics, PN-120237)

816 and the Chromium™ Single Cell A Chip Kit (10X Genomics, PN-120236) as per the

817 manufacturer's protocol. In brief, single cells were sorted into PBS + 0.1% BSA,

818 washed twice and counted using a Bio-Rad TC20. 10,769 cells were added to each lane

819 of the 10X chip. The cells were then partitioned into Gel Beads in Emulsion in the

820 Chromium™ instrument, where cell lysis and barcoded reverse transcription of RNA

821 occurred, followed by amplification, fragmentation and 5′ adaptor and sample index

822 attachment. Libraries were sequenced on an Illumina HiSeq 4000.

Computational Analysis

824 In total, we analysed 67,494 human cells from healthy (n=5) and cirrhotic (n=5) livers,

825 30,741 PBMCs from cirrhotic patients (n=4) and compared our data with a publicly-

826 available reference dataset of 8,381 PBMCs from a healthy donor.

827

828 *Pre-processing scRNA-seq data*

829 We aligned to the GRCh38 and mm10 (Ensembl 84) reference genomes as appropriate

830 for the input dataset, and estimated cell-containing partitions and associated UMIs,

831 using the Cell Ranger v2.1.0 Single-Cell Software Suite from 10X Genomics. Genes

832 expressed in fewer than three cells in a sample were excluded, as were cells that

833 expressed fewer than 300 genes or mitochondrial gene content >30% of the total UMI

834 count. We normalised by dividing the UMI count per gene by the total UMI count in

835 the corresponding cell and log-transforming. Variation in UMI counts between cells

836 was regressed according to a negative binomial model, prior to scaling and centering

837 the resulting value by subtracting the mean expression of each gene and dividing by its

838 standard deviation (E_n), then calculating $\ln(10^4 * E_n + 1)$.

839 *Dimensionality reduction, clustering, and DE analysis*

33

840   We performed unsupervised clustering and differential gene expression analyses in the

841   *Seurat* R package v2.3.0[39]. In particular we used SNN graph-based clustering, where

842   the SNN graph was constructed using from 2 to 11 principal components as determined

843   by dataset variability shown in principal components analysis (PCA); the resolution

844   parameter to determine the resulting number of clusters was also tuned accordingly. To

845   assess cluster similarity we used the *BuildClusterTree* function from *Seurat*.

846   In total, we present scRNA-seq data from ten human liver samples (named Healthy 1-

847   5 and Cirrhotic 1-5), five human blood samples (n=4 cirrhotic named Blood 1-4 and

848   n=1 healthy named PBMC8K; pbmc8k dataset sourced from single-cell gene

849   expression datasets hosted by 10X Genomics), and two mouse liver samples (n=3

850   uninjured and n=3 fibrotic). For seven human liver samples (Healthy 1-4 and Cirrhotic

851   1-3) we performed scRNA-seq on both leucocytes (CD45$^+$) and other non-parenchymal

852   cells (CD45$^-$); for the remaining three human livers (Healthy 5, Cirrhotic 4-5) we

853   performed scRNA-seq on leucocytes only (Extended Data Fig. 2e, f).

854   Initially, we combined all human scRNA-seq datasets (liver and blood) and performed

855   clustering analysis with the aim of isolating a population of liver-resident cells, by

856   identifying contaminating circulatory cells within datasets generated from liver digests

857   and removing them from downstream analysis. Specifically, we removed from our liver

858   datasets cells that fell into clusters 1 and 13 of the initial dataset in Extended Data Fig.

859   1d.

860   Using further clustering followed by signature analysis, we interrogated this post-

861   processed liver-resident cell dataset for robust cell lineages. These lineages were

862   isolated into individual datasets, and the process was iterated to identify robust lineage

863   subpopulations. At each stage of this process we removed clusters expressing more than

864   one unique lineage signature in more than 25% of their cells from the dataset as

865   probable doublets. This resulted in removal of 1,351 cells. Where the cell proliferation

866   signature identified distinct cycling subpopulations, we re-clustered these again to

867   ascertain the identity of their constituent cells.

868   The murine scRNA-seq datasets were combined, clustered, and interrogated for cell

869   lineages in a similar manner as their human counterparts.

34

870   All heatmaps, t-SNE and UMAP visualisations, violin plots, and dot plots were

871   produced using *Seurat* functions in conjunction with the *ggplot2*, *pheatmap*, and *grid*

872   R packages. t-SNE and UMAP visualisations were constructed using the same number

873   of principal components as the associated clustering, with perplexity ranging from 30

874   to 300 according to the number of cells in the dataset or lineage. We conducted

875   differential gene expression analysis in *Seurat* using the standard AUC classifier to

876   assess significance. We retained only those genes with a log-fold change of at least 0.25

877   and expression in at least 25% of cells in the cluster under comparison.

878   *Defining cell lineage signatures*

879   For each cell we obtained a signature score across a curated list of known marker genes

880   per cell lineage in the liver (Supplementary Table 2). This signature score was defined

881   as the geometric mean of the expression of the associated signature genes in that cell.

882   Lineage signature scores were scaled from 0 to 1 across the dataset, and the score for

883   each cell with signature less than a given threshold (the mean of said signature score

884   across the entire dataset) was set as 0.

885   *Batch effect and quality control*

886   To investigate agreement between samples we extracted the average expression profile

887   for a given cell lineage in each sample, and calculated the Pearson correlation

888   coefficients between all possible pairwise comparisons of samples per lineage[40].

889   *Imputing dropout in T cell and ILC clusters*

890   To impute dropout of low-abundance transcripts in our T cell and ILC clusters so that

891   we might associate them with known subpopulations, we down-sampled to 7,380 cells

892   from 36,900 and applied the *scImpute* R package v0.0.8[41], using as input both our

893   previous annotation labels and k-means spectral clustering (k=5), but otherwise default

894   parameters.

895   *Analysing functional phenotypes of scar-associated cells*

896   For further analysis of function we adopted the self-organising maps (SOM) approach

897   as implemented in the *SCRAT* R package v1.0.0[42]. For each lineage of interest we

898   constructed a SOM in *SCRAT* using default input parameters and according to its

899   clusters. We defined the signatures expressed in a cell by applying a threshold criterion

900   ($e^{thresh} = 0.95 \times e^{max}$) selecting the highest-expressed metagenes in each cell, and

901   identified for further analysis those metagene signatures defining at least 30% of cells

902   in at least one cluster within the lineage. We smoothed these SOMs using the

903   *disaggregate* function from the *raster* R package for visualisation purposes, and scaled

904   radar plots to maximum proportional expression of the signature. Gene ontology

905   enrichment analysis on the genes in these spots was performed using PANTHER 13.1

906   (pantherdb.org).

907   *Inferring injury dynamics and transcriptional regulation*

908   To generate cellular trajectories (pseudotemporal dynamics) we used the *monocle* R

909   package v2.6.1[43]. We ordered cells in a semi-supervised manner based on their *Seurat*

910   clustering, scaled the resulting pseudotime values from 0 to 1, and mapped them onto

911   either the t-SNE or UMAP visualisations generated by *Seurat* or diffusion maps as

912   implemented in the *scater* R package v1.4.0[44] using the top 500 variable genes as input.

913   We removed mitochondrial and ribosomal genes from the geneset for the purposes of

914   trajectory analysis. Differentially-expressed genes along this trajectory were identified

915   using generalised linear models via the *differentialGeneTest* function in *monocle*.

916   When determining significance for differential gene expression along the trajectory, we

917   set a q-value threshold of $1e^{-20}$. We clustered these genes using hierarchical clustering

918   in *pheatmap*, cutting the tree at k=3 to obtain gene modules with correlated gene

919   expression across pseudotime. Cubic smoothing spline curves were fitted to scaled gene

920   expression along this trajectory using the *smooth.spline* command from the *stats* R

921   package, and gene ontology enrichment analysis again performed using PANTHER

922   13.1.

923   We verified the trajectory and its directionality using the *velocyto* R package v0.6.0[17],

924   estimating cell velocities from their spliced and unspliced mRNA content. We

925   generated annotated spliced and unspliced reads from the 10X BAM files via the

926   *dropEst* pipeline, before calculating gene-relative velocity using kNN pooling with

927   k=25, determining slope gamma with the entire range of cellular expression, and fitting

928   gene offsets using spanning reads. Aggregate velocity fields (using Gaussian smoothing

929   on a regular grid) and transition probabilities per lineage subpopulations were

930   visualised on t-SNE, UMAP, or diffusion map visualisations as generated previously.

931   Gene-specific phase portraits were plotted by calculating spliced and unspliced mRNA

932   levels against steady-state inferred by a linear model; levels of unspliced mRNA above

933   and below this steady-state indicate increasing and decreasing expression of said gene,

934   respectively. Similarly we plotted unspliced count signal residual per gene, based on

935   the estimated gamma fit, with positive and negative residuals indicating expected

936   upregulation and downregulation respectively.

937   For transcription factor analysis, we obtained a list of all genes identified as acting as

938   transcription factors in humans from AnimalTFDB[45]. To further analyse transcription

939   factor regulons, we adopted the *SCENIC* v0.1.7 workflow in R[46], using default

940   parameters and the normalised data matrices from *Seurat* as input. For visualisation, we

941   mapped the regulon activity (AUC) scores thus generated to the pseudotemporal

942   trajectories from *monocle* and the clustering subpopulations from *Seurat*.

943   *Analysing inter-lineage interactions within the fibrotic niche*

944   For comprehensive systematic analysis of inter-lineage interactions within the fibrotic

945   niche we used CellPhoneDB[27]. CellPhoneDB is a manually curated repository of

946   ligands, receptors, and their interactions, integrated with a statistical framework for

947   inferring cell-cell communication networks from single-cell transcriptomic data. In

948   brief, we derived potential ligand-receptor interactions based on expression of a

949   receptor by one lineage subpopulation and a ligand by another; as input to this algorithm

950   we used cells from the fibrotic niche as well as liver sinusoidal endothelial cells and

951   Kupffer cells as control, and we considered only ligands and receptors expressed in

952   greater than 5% of the cells in any given subpopulation. Subpopulation-specific

953   interactions were identified as follows: 1) randomly permuting the cluster labels of all

954   cells 1000 times and determining the mean of the average receptor expression of a

955   subpopulation and the average ligand expression of the interacting subpopulation, thus

956   generating a null distribution for each ligand-receptor pair in each pairwise comparison

957   between subpopulations, 2) calculating the proportion of these means that were "as or

958   more extreme" than the actual mean, thus obtaining a p-value for the likelihood of

959   subpopulation specificity for a given ligand-receptor pair, 3) prioritising interactions

960   that displayed specificity to subpopulations interacting within the fibrotic niche.

961   *Canonical correlation analysis*

962   To compare human and murine populations of monocytic phagocytes, we used

963   canonical correlation analysis as implemented in *Seurat*[18]. We map the genes in the

964   human dataset to their murine orthologues using *biomaRt*, discarding any genes for

965   which no orthologues can be found. We then calculate the shared low-dimensional

966   subspace on the union of genes that are variably expressed in both datasets (n=159),

967   and align using six canonical components as determined by evaluating the biweight

968   midcorrelation. Results are visualised on t-SNEs as previously described.

969   *Deconvolution of whole liver microarray data*

970   To assess macrophage composition early-stage NAFLD, we performed deconvolution

971   analysis on publicly available microarray data from annotated liver biopsy specimens

972   taken across the NAFLD disease spectrum (GEO accession GSE48452)[20]. Tissue MP

973   cells from our human scRNA-seq data were manually clustered into the main annotated

974   MP populations. Signature gene expression profiles of SAMΦ, TMo, KC were used to

975   deconvolve the monocyte-macrophage composition of liver biopsy samples from

976   GSE48452 using Cibersort[47], as previously described[19]. The monocyte-macrophage

977   composition of each biopsy sample was then compared to the associated histological

978   and demographic features, available from the GEO database.

979   **Statistics and Reproducibility**

980   To assess whether our identified subpopulations were significantly overexpressed in

981   injury, we posited the proportion of injured cells in each cluster as a random count

982   variable using a Poisson process, as previously described[40]. We modelled the rate of

983   detection using the total number of cells in the lineage profiled in a given sample as an

984   offset, with the condition of each sample (healthy vs cirrhotic) provided as a covariate

985   factor. The model was fitted using the R command *glm* from the *stats* package. The *P*

986   value for the significance of the proportion of injured cells was assessed using a Wald

987   test on the regression coefficient.

988   Remaining statistical analyses were performed using GraphPad Prism (GraphPad

989   Software, USA). Comparison of changes between two groups was performed using a

990   Mann-Whitney test (unpaired; two-tailed) or using a Wilcoxon matched-pairs signed

991   rank test (paired; two-tailed). Comparison of changes between multiple groups was

992 performed using a Kruskal-Wallis and Dunn, one-way ANOVA and Tukey or repeated

993 measures one-way ANOVA and Tukey tests. Correlations were preformed using

994 Pearson correlation and best fit line plotted using linear regression. P values<0.05 were

995 considered statistically significant. All immunofluorescence stains were repeated in a

996 minimum of 3 patients and representative images are displayed.

997

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1045   **Data and materials availability**

1046   Our expression data will be freely available for user-friendly interactive browsing
1047   online at http://www.livercellatlas.mvm.ed.ac.uk. CellPhoneDB is available at
1048   www.CellPhoneDB.org. All raw sequencing data will be deposited in the Gene
1049   Expression Omnibus (GEO accession GSE136103).

1050   **Code availability**

1051   R scripts enabling the main steps of the analysis are available from the corresponding
1052   authors on reasonable request.

1053   **Acknowledgements**

1082   **Author Contributions**

1083   P.R. performed experimental design, tissue procurement, data generation, data analysis

1084   and interpretation, and manuscript preparation; R.D. performed experimental design,

1085   data generation and data analysis; E.D., K.P.M., B.E.P.H., M.B., J.A.M. and N.T.L.

1086   performed data generation and analysis; J.R.P. generated the interactive online browser;

1087   M.E. and R.V-T. assisted with CellPhoneDB analyses and critically appraised the

1088   manuscript; T.J.K. performed pathological assessments and provided intellectual

1089   contribution; N.O.C., J.A.F. and P.N.N. provided intellectual contribution; C.J.W.

1090   performed tissue procurement, data generation, interpretation and intellectual

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1091 contribution; J.R.W-K. performed computational analysis with assistance from J.R.P
1092 and R.S.T. and advice from C.P.P., J.M. and S.A.T.; J.R.W-K. also helped with
1093 manuscript preparation and C.P.P., J.M. and S.A.T. critically appraised the manuscript;
1094 E.M.H., D.J.M. and S.J.W. procured human liver tissue and critically appraised the
1095 manuscript. J.P.I., F.T. and J.W.P. provided intellectual contribution and critically
1096 appraised the manuscript; N.C.H. conceived the study, designed experiments,
1097 interpreted data and prepared the manuscript.

1098 **Author Information**

1099 The authors declare no competing financial interests. Address correspondence or
1100 requests for materials to Prakash Ramachandran or Neil Henderson.

Extended Data Figure 1: Strategy for isolation of human liver non-parenchymal cells.

a, Patient demographics and clinical information, Mean±SEM. **b**, Flow cytometry gating strategy for isolating leucocytes (CD45$^+$) and other non-parenchymal cells (CD45$^-$) from human liver, representative plots from 10 livers. **c**, Flow cytometry plots: gating strategy for isolating peripheral blood mononuclear cells (PBMC), representative plots from 4 patients. **d**, Clustering 103,568 cells from 5 healthy human livers, 5 cirrhotic human livers, 1 healthy PBMC and 4 cirrhotic PBMC (left), annotating source (PBMC *versus* liver; middle) and cell lineage inferred from known marker gene signatures (right). Endo, endothelial cell; ILC, innate lymphoid cell; Mast, mast cell; Mes, mesenchymal cell; MP, mononuclear phagocyte; pDC, plasmacytoid dendritic cell. **e**, Dotplot: annotating PBMC and liver clusters by lineage signatures. Circle size indicates cell fraction expressing signature greater than mean; colour indicates mean signature expression (red, high; blue, low). **f,** CXCR4 gene expression in single cells derived from blood or liver tissue, divided by cell lineage. Representative immunofluorescence micrograph, human liver: CXCR4 (green), DAPI (blue), arrows CXCR4$^-$ cells in the lumen of a blood vessel. Scale bar 50μm. **g**, Violin plots: number of unique genes (nGene), number of total Unique Molecular Identifiers (nUMI) and mitochondrial gene fraction expressed in 5 PBMC samples; Black line, median. **g**, Pie charts: proportion of cell lineage per PBMC sample. **h**, Box and whisker plot: agreement in expression profiles across 5 PBMC samples. Pearson correlation coefficients between average expression profiles for cell in each lineage, across all pairs of samples. Black bar, median value; box edges, 25th and 75th percentiles; whiskers, full range.

a

	Healthy liver (n=5)	Cirrhotic liver (n=5)	Blood (n=4)
Age (yrs)	57.4±7.9	56.6±5.8	63.2±3.8
Gender (M:F)	4:1	3:2	3:1
Aetiology of liver disease	N/A	2xNAFLD 2xALD 1xPBC	3xNAFLD 1xHH
Haemoglobin (g/l)	145±14	106±17	131±2.1
White Cell Count (x10⁹/l)	8.2±2.2	5.9±1.7	3.7±1.5
Platelets (x10⁹/l)	300±91	137±56	73±38
Prothrombin Time (s)	11.6±1.1	19.6±3.8	16.0±3.6
Creatinine (μmol/l)	76.4±14.5	96.8±28.6	74.5±9.7
Na⁺ (mmol/l)	141±2.6	131±7.0	139±2.1
Bilirubin (μmol/l)	10±5.2	79.6±83.5	36.3±20.0
ALT (IU/l)	27.8±19.3	77.8±80.7	96.2±121.0
ALP (IU/l)	122±47	140±80	203±153
MELD Score	6.6±0.5	17.3±4.5	11.7±4.3

NAFLD:Non-alcoholic fatty liver disease; ALD:Alcohol-related liver disease; PBC:Primary biliary cholangitis; HH:Hereditary haemochromatosis; ALT:Alanine transaminase; ALP:Alkaline Phosphatase; MELD:Model for End-Stage Liver Disease
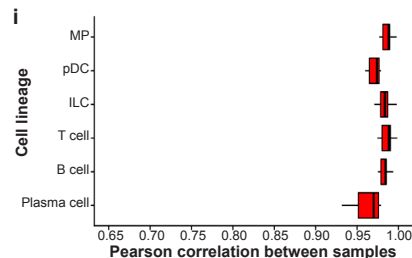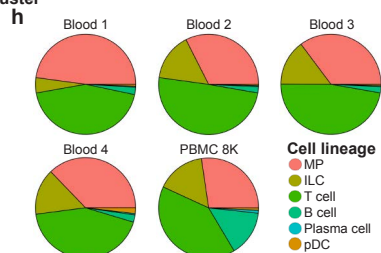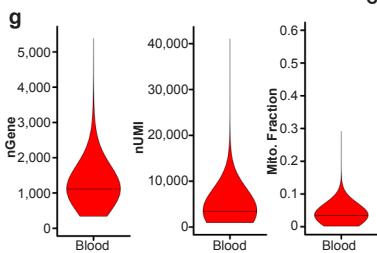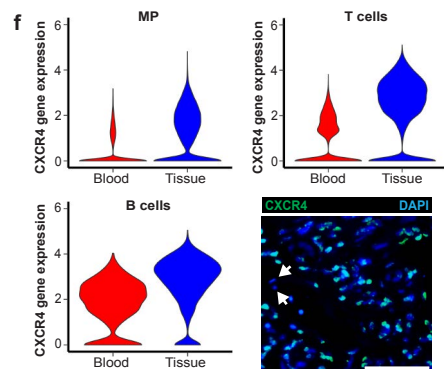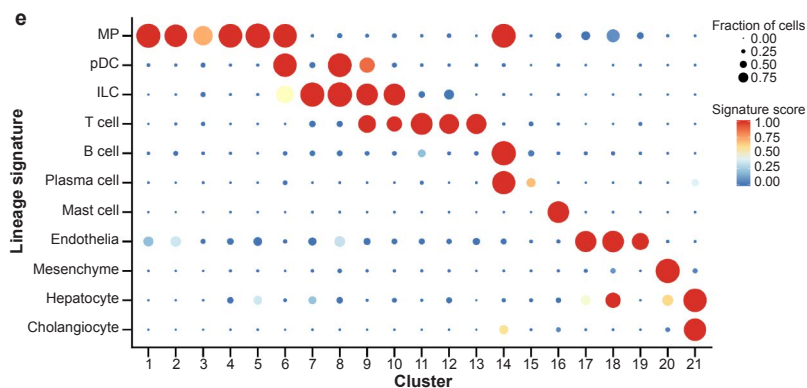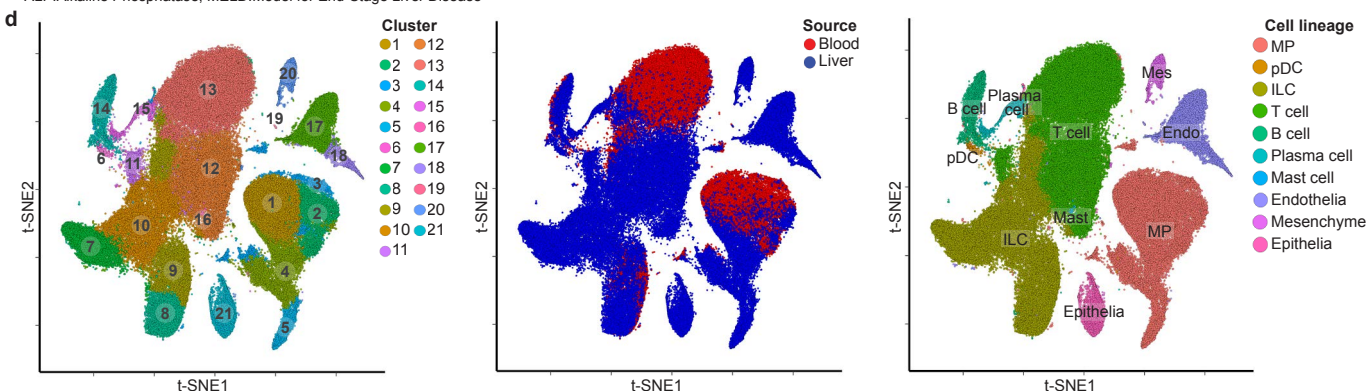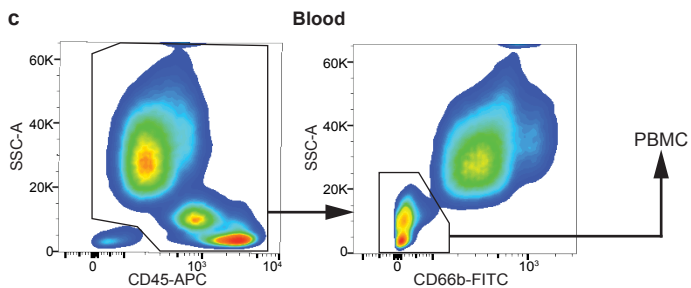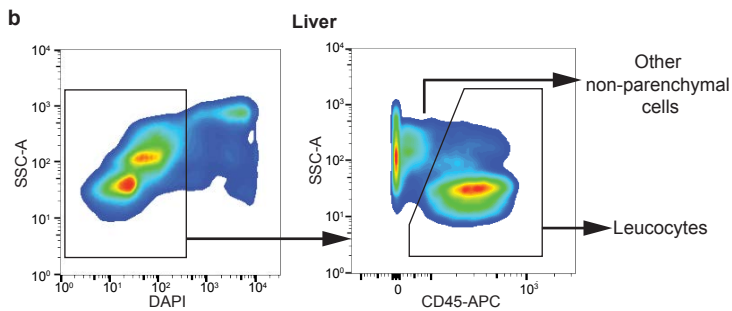
Extended Data Figure 2: Quality control and annotation of human liver-resident cells.

a, Lineage signature expression across 66,135 liver-resident cells from 5 healthy and 5 cirrhotic human livers (red, high; blue, low). **b**, Dotplot: annotating liver-resident cell clusters by lineage signature. Circle size indicates cell fraction expressing signature greater than mean; colour indicates mean signature expression (red, high; blue, low). **c**, Violin plot: number of unique genes (nGene; left), number of total Unique Molecular Identifiers (nUMI; middle) and mitochondrial gene fraction (right) across 66,135 liver-resident cells from 5 healthy *versus* 5 cirrhotic livers; Black line, median. **d**, Pie charts: proportion of cell lineage per liver sample. **e**, Box and whisker plot: agreement in expression profiles across 5 healthy and 5 cirrhotic liver samples. Pearson correlation coefficients between average expression profiles for cell in each lineage, across all pairs of samples. Black bar, median value; box edges, 25th and 75th percentiles; whiskers, range. **f**, t-SNE visualisation: liver-resident cells per liver sample; Cirrhotic samples annotated by aetiology of underlying liver disease; NAFLD, Non-alcoholic fatty liver disease; ALD, Alcohol-related liver disease; PBC, Primary biliary cholangitis.

a

MP · pDC · ILC · T cell · B cell · Plasma cell · Mast cell · Endothelia · Mesenchyme · Hepatocyte · Cholangiocyte · Cycling

Sig. score 1.00 0.75 0.50 0.25 0.00

t-SNE2 / t-SNE1

b

Lineage signature (y-axis): MP, pDC, ILC, T cell, B cell, Plasma cell, Mast cell, Endothelia, Mesenchyme, Hepatocyte, Cholangiocyte, Cycling

Cluster (x-axis): 1–21

Fraction of cells: 0.00, 0.25, 0.50, 0.75, 1.00

Sig. score: 1.00 0.75 0.50 0.25 0.00

c

nGene / nUMI / Mitochondrial Fraction violin plots

Cell lineage (x-axis): MP, pDC, ILC, T cell, B cell, Plasma cell, Mast cell, Endothelia, Mesenchyme, Epithelia, Cycling

Healthy (grey) · Cirrhotic (magenta)

d

Leucocytes and other NPCs · Leucocytes only

Healthy 1, Healthy 2, Healthy 3, Healthy 4, Healthy 5
Cirrhotic 1, Cirrhotic 2, Cirrhotic 3, Cirrhotic 4, Cirrhotic 5

Cell lineage: MP, pDC, ILC, T cell, B cell, Plasma cell, Mast cell, Endothelia, Mesenchyme, Epithelia, Cycling

e

Cell lineage (y-axis): MP, pDC, ILC, T cell, B cell, Plasma cell, Mast cell, Endothelia, Mesenchyme, Epithelia

Pearson correlation between samples (x-axis): 0.65–1.00

Healthy (grey) · Cirrhotic (magenta)

f

Healthy 1, Healthy 2, Healthy 3, Healthy 4, Healthy 5

Cirrhotic 1 NAFLD, Cirrhotic 2 ALD, Cirrhotic 3 ALD, Cirrhotic 4 NAFLD, Cirrhotic 5 PBC

t-SNE2 / t-SNE1

Extended Data Figure 3: Annotating human liver lymphoid cells.

a, Clustering and annotating 36,900 T cells and innate lymphoid cells (ILC) (left) from

5 healthy and 5 cirrhotic human livers, annotating injury condition (right). cNK,

cytotoxic NK cell. **b**, Fractions of T cell and ILC subpopulations in healthy (n=5) *versus*

cirrhotic (n=5) livers, Mean±SEM, Wald. **c**, Selected gene expression in 36,900 T cells

and ILC. **d**, Heatmap: T cell and ILC cluster marker genes (colour coded top by cluster

and condition), exemplar genes labelled right. Cells columns, genes rows. **e**, t-SNE

visualisations: downsampled T cell and ILC dataset (7,380 cells from 5 healthy and 5

cirrhotic human livers) pre- and post-imputation; annotating data used for visualisation

and clustering, inferred lineage, injury condition. No additional heterogeneity was

observed following imputation. **f**, Clustering 2,746 B cells and plasma cells (left) from

5 healthy and 5 cirrhotic human livers, annotating injury condition (right). **g**, Heatmap:

B cell and plasma cell cluster marker genes (colour coded top by cluster and condition),

exemplar genes labelled right. Cells columns, genes rows. **h**, Fractions of B cell and

plasma cell subpopulations in healthy (n=5) *versus* cirrhotic (n=5) livers, Mean±SEM.
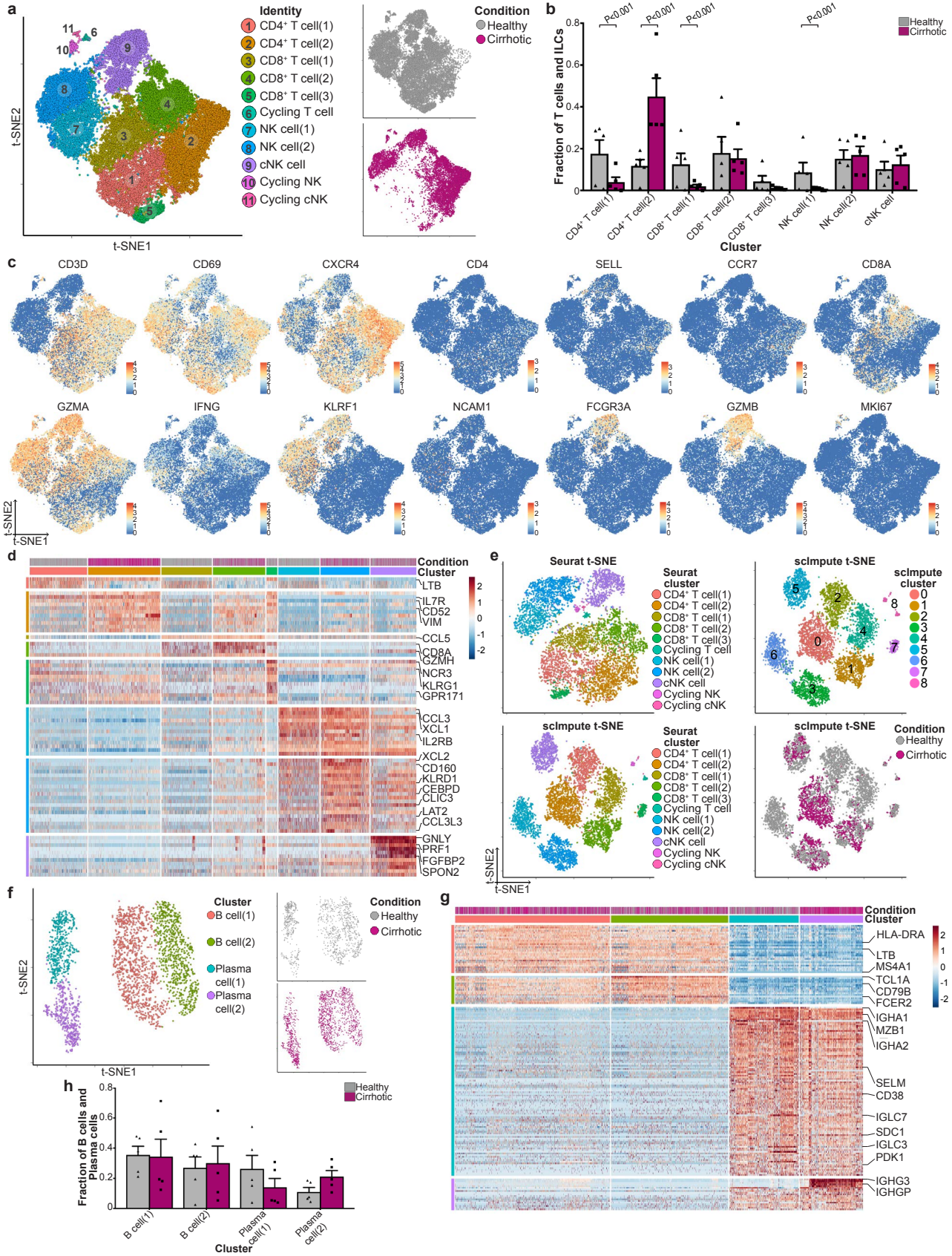
1156 **Extended Data Figure 4: Annotating human liver mononuclear phagocytes.**

1157 **a**, Clustering and selected genes expressed in 10,737 mononuclear phagocytes (MP)

1158 from 5 healthy and 5 cirrhotic human livers. **b**, Scaled gene expression of Kupffer cell

1159 (KC) cluster markers across MP cells from healthy (n=5) and cirrhotic (n=5) livers. **c**,

1160 Representative immunofluorescence images, healthy *versus* cirrhotic liver: TIMD4

1161 (red), CD163 (white), MARCO (green), DAPI (blue), arrows

1162 CD163$^+$MARCO$^+$TIMD4$^-$ cells, scale bars 50μm. **d**, TIMD4 immunohistochemistry,

1163 cell counts healthy (n=12) *versus* cirrhotic (n=9) liver, Mean±SEM, scale bars 50μm.

1164 **e**, MARCO immunohistochemistry, cell counts healthy (n=8) *versus* cirrhotic (n=8)

1165 liver, Mean±SEM, Mann-Whitney two-tailed, scale bars 50μm. **f**, Flow cytometry:

1166 gating strategy for identifying KC, TMo and SAMΦ in healthy (n=2) and cirrhotic

1167 (n=3) liver. SAMΦ are detected as TREM2+CD9+ cells within the TMo and SAMΦ

1168 gate (see Fig. 2f). **g**, Representative immunofluorescence image, cirrhotic liver:

1169 TREM2 (red), MNDA (white), collagen 1 (green), DAPI (blue), scale bars 50μm. **h**,

1170 Representative image, cirrhotic liver: TREM2 (smFISH; red), MNDA

1171 (immunofluorescence; green), DAPI (blue), scale bars 50μm. **i**, Representative

1172 immunofluorescence image, cirrhotic liver: CD9 (red), MNDA (white), collagen 1

1173 (green), DAPI (blue), scale bars 50μm. **j**, TREM2 immunohistochemistry, cell counts

1174 healthy (n=10) *versus* cirrhotic (n=9) liver, Mann-Whitney two-tailed, scale bars 50μm.

1175 **k**, CD9 immunohistochemistry, cell counts healthy (n=12) *versus* cirrhotic (n=10) liver,

1176 Mann-Whitney two-tailed, scale bars 50μm. **l**, Exemplar tissue segmentation of

1177 cirrhotic liver section into fibrotic septae (orange) and parenchymal nodules (purple)

1178 (top); TREM2 (n=9), CD9 (n=11), TIMD4 (n=9) and MARCO (n=7)

1179 immunohistochemistry and cell counts in parenchymal nodules *versus* fibrotic septae,

1180 Wilcoxon two-tailed. **m**, Clustering and annotation of 208 cycling MP cells from

1181 healthy (n=5) and cirrhotic (n=5) livers, scaled gene expression of MP subpopulation

1182 markers across 4 clusters of cycling MP cells (top), fractions of cycling MP

1183 subpopulations in 5 healthy *versus* 5 cirrhotic livers (bottom), Mean±SEM, Wald.
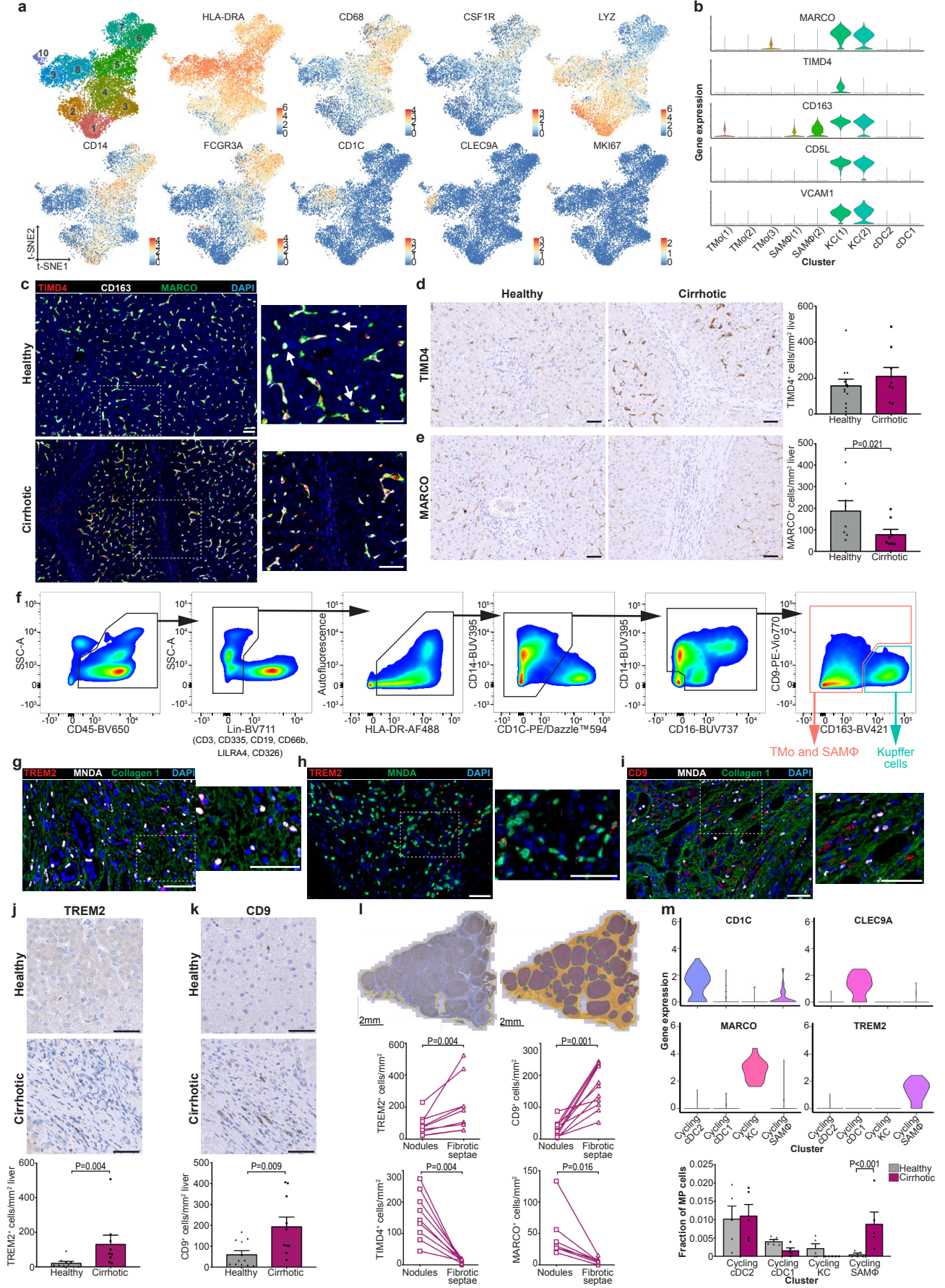
Extended Data Figure 5: Phenotypic characterisation of mononuclear phagocytes in healthy and cirrhotic human livers.

a, Self-Organising Map (SOM; 60x60 grid): smoothed scaled metagene expression of 10,737 mononuclear phagocyte (MP) cells from healthy (n=5) and cirrhotic (n=5) livers. 20,952 genes, 3,600 metagenes, 44 signatures. A-F label metagene signatures overexpressed in one or more MP subpopulations (top). Smoothed mean metagene expression profile for each MP subpopulation (bottom). **b**, Radar plots (left): metagene signatures A-F showing distribution of signature expression across MP subpopulations from 10,737 MP cells, exemplar genes (middle) and selected Gene Ontology (GO) enrichment (right), Fisher's exact test. **c**, Diffusion map visualisation, blood monocytes and liver-resident MP lineages (23,075 cells from healthy (n=5) and cirrhotic (n=5) liver samples and PBMCs (n=4), annotating *monocle* pseudotemporal dynamics (purple to yellow). RNA velocity field (red arrows) visualised using Gaussian smoothing on regular grid (top). Annotation of MP subpopulation, injury condition (bottom). **d**, Unspliced-spliced phase portraits (top row), 23,075 cells coloured and visualised as in Fig 3a, monocyte (*MNDA*), SAMΦ (*CD9*) and KC marker genes (*TIMD4*). Cells plotted above or below the steady-state (black dashed line) indicate increasing or decreasing expression of gene, respectively. Spliced expression profile for stated genes (middle row; red high, blue low). Unspliced residuals for stated genes (bottom row), positive (red) indicating expected upregulation, negative (blue) indicating expected downregulation. *MNDA* displays negative velocity in SAMΦ, *CD9* displays positive velocity in monocytes and SAMΦ, *TIMD4* velocity is restricted to KC. **e**, Cubic smoothing spline curve fitted to averaged expression of all genes in module 2 from blood monocyte-SAMΦ pseudotemporal trajectory (see Fig. 3c), selected GO enrichment (right), Fisher's exact test. **f**, Cubic smoothing spline curve fitted to averaged expression of all genes in module 3 from blood monocyte-cDC pseudotemporal trajectory (see Fig. 3c), selected GO enrichment (right), Fisher's exact test. **g**, Luminex assay: quantification of levels of stated proteins in culture medium from FACS-isolated scar-associated macrophages (SAMΦ, n=3), tissue monocytes (TMo, n=2), Kupffer cells (KC, n=2), and control (media alone, n=2), Mean±SEM, MFI median fluorescence intensity. **h**, Heatmap: transcription factor regulons across MP pseudotemporal trajectory and in KC (colour coded top by MP cluster, condition

1216   and pseudotime), selected regulons labelled right. Cells columns, regulons rows. **i**,

1217   Scaled regulon expression of selected regulons across MP clusters from healthy (n=5)

1218   and cirrhotic (n=5) livers.

a

C · B
A
F
D · E

MP cluster

TMo(1) TMo(2) TMo(3)

SAMΦ(1) SAMΦ(2) KC(1)

KC(2) cDC2 cDC1

b

	Genes		Gene Ontology

Signature A
TREM2 CST6
FN1 HTRA1
SPP1 SPARCL1
LTC4S FMNL3
CCR2 MMP14
CLEC5A TNFSF12

+ve Regulation of Inflammatory Response
Angiogenesis
Extracellular Structure Organization
Proteoglycan Biosynthetic Process
Extracellular Matrix Organization

Signature B
IL1B FCGR2A
MNDA FCGR2B
LGALS3 CD86
AREG CSTA
PLAUR IL13RA1
CXCR4 IFNGR2

Myeloid Leukocyte Activation
Regulation of T Cell Activation
Response to Interferon-gamma
Phagocytosis
Antigen Processing and Presentation

Signature C
MARCO CD302
VCAM1 NR1H3
CD5L IL18
TIMD4 SCARB2
FCGR3A MSR1
CD163 FOLR2
MERTK MRC1

Vesicle-mediated Transport
Response to Bacterium
Response to Lipid
Receptor-Mediated Endocytosis
Iron Ion Homeostasis

Signature D
HES4 PTGER3
PLOD1 S100A9
IFNLR1 S100A12
CSF3R VCAN

Inflammatory response
Response to lipopolysaccharide
NADP metabolic process
Leukocyte chemotaxis

Signature E
MAP4K4 TREM1
SLC11A1 IL3RA
PTX3 CLEC4E
BST1 CD300C
S100Z IL17RA
HRH2

Defense response
Regulation of kinase activity
Cell communication
Leukocyte migration

Signature F
BATF3 DUSP4
PLCD1 IDO1
XCR1 CLEC9A
TLR10 ITGB7
ENPP1 RHOF
CXCR3 FLT3

Positive regulation of cytokine production
Positive regulation of apoptotic process
Regulation of cell communication
T cell migration

-log₁₀(P value)

c

Pseudotime

DM2 / DM1

Blood monocytes Tissue monocytes cDC
SAMΦ KC Condition

d

MNDA CD9 TIMD4

Unspliced / Spliced

UMAP2 / UMAP1

Exp. High / Low
Residual Pos. / Neut. / Neg.

e

Module 2

Signature expression / Pseudotime

Gene Ontology

Leukocyte degranulation
Defense response
Leukocyte migration
Leukocyte chemotaxis
Response to bacterium

-log₁₀(P value)

f

Module 3

Signature expression / Pseudotime

Gene Ontology

Regulation of lymphocyte proliferation
Regulation of T cell activation
Fc receptor signaling pathway
Interleukin-12-mediated signaling pathway
Dendritic cell differentiation

-log₁₀(P value)

g

IL-1ß CCL2 SPP1 LGALS3 CXCL8 CD163

MFI

Control SAMφ TMo KC

Population

h

Cluster
Condition
Pseudotime

HOXB7
HIF1A
HES1
STAT2
SPIC
NR1H3
MAFB
EGR2
EGR3
BCL11A
E2F2
NFE2
NFKB1

i

NR1H3
SPIC
HES1
EGR2
BCL11A
NFE2

Regulon activity

TMo(1) TMo(2) TMo(3) SAMΦ(1) SAMΦ(2) KC(1) KC(2)

Cluster

1219 **Extended Data Figure 6: Characterisation of macrophages in mouse liver**

1220 **fibrosis.**

1221 **a**, Clustering and annotating 3,250 mouse mononuclear phagocytes (mMP) from

1222 healthy (n=3) and fibrotic (4 weeks carbon tetrachloride treatment, n=3) livers. mTMo,

1223 tissue monocyte; mSAMΦ, scar-associated macrophage; mKC, Kupffer cell; mcDC,

1224 conventional dendritic cell. **b**, Annotating mMP cells by injury condition. **c**, Heatmap:

1225 mMP cluster marker genes (top, colour coded by cluster and condition), exemplar genes

1226 labelled (right). Cells columns, genes rows. **d**, Selected genes expressed in 3,250 mMP

1227 **e**, Flow cytometry: gating strategy for identifying KC, TMo and SAMΦ in healthy

1228 (n=2) and cirrhotic (n=3) liver. Flow cytometry plots: gating strategy for identifying

1229 mKC, CD9- mTMo and CD9+ mSAMΦ in fibrotic mice (n=8 from 2 independent

1230 experiments). **f**, Quantifying mouse macrophage subpopulations by flow cytometry:

1231 healthy (n=6) and fibrotic (n=8) mouse livers from 2 independent experiments,

1232 macrophage subpopulation (x-axis) as a percentage of total viable CD45+ cells (y-axis),

1233 Mean±SEM, Mann-Whitney two-tailed. **g**, Hepatic stellate cell activation assay: co-

1234 culture of hepatic stellate cells (HSC) from uninjured mouse liver and FACS-isolated

1235 macrophage subpopulations (MΦ) from fibrotic mouse liver (left). Co-culture with

1236 CD9- mTMo or CD9+ mSAMΦ isolated from 8 fibrotic mice (2 independent

1237 experiments), qPCR of Col3a1 in HSC, expression relative to mean expression of

1238 quiescent HSC (right), Wilcoxon two-tailed. **h**, Clustering 3,250 mouse mononuclear

1239 phagocytes (mMP) and 10,737 human mononuclear phagocytes (hMP) into 5 clusters

1240 using canonical correlation analysis (CCA). Annotation of cross-species clusters

1241 (identity). **i**, Annotation of human and mouse macrophage subpopulations from 3,250

1242 mMP and 10,737 hMP. **j**, Selected genes expressed in 3,250 mMP and 10,737 hMP.

a Mouse

Cluster	Identity
①mMP(1)	mTMo
②mMP(2)	mSAMΦ
③mMP(3)	mKC
④mMP(4)	mcDC2
⑤mMP(5)	mcDC1

t-SNE2 / t-SNE1

b Healthy / Fibrotic

c Condition / Cluster

Lyz2, Plaur, S100a4
Trem2, Spp1, Ccl3, Lgals3
Cd5l, Timd4, Vcam1, Cd163, Marco
Cd74, Cd209a
Cst3, Xcr1, Clec9a

d Cd9, Trem2, Timd4, Lyz2, Cd209a, Xcr1

t-SNE2 / t-SNE1

e Mono-Mac / CD11b-APC-Cy7 / F4/80-PE-Cy7 / mKC / TIMD4-AF647 / CD9- / CD9+ / SSC-A / CD9-PE

f
% of CD45+ Cells
P=0.0007, P=0.0007, P=0.0007
Healthy / Fibrotic
CD9+ mSAMΦ, CD9- mTMo, mKC

g MΦ / HSC / HSC Gene Expression
Col3a1
Relative HSC Gene Expression
P=0.008
CD9-, CD9+
MΦ Subpopulation

h Human & Mouse

Cluster	Identity
①h&mMP(1)	TMo
②h&mMP(2)	SAMΦ
③h&mMP(3)	KC
④h&mMP(4)	cDC
⑤h&mMP(5)	Cycling

t-SNE2 / t-SNE1

i Human SAMΦ, Mouse SAMΦ, Human KC, Mouse KC

j TREM2, CD9, TIMD4, MARCO, SPP1, LGALS3

t-SNE2 / t-SNE1

Extended Data Figure 7: Scar-associated macrophage expansion in human

NASH

a to d, Deconvolution: publicly available whole liver microarray data (n=73) assessed

for frequency of scar-associated macrophages (SAMΦ), Kupffer cells (KC) and tissue

monocytes (TMo) using Cibersort algorithm. **a**, Macrophage composition: x-axis, GEO

accession number; y-axis, fraction of monocyte-macrophages; Top, annotated by liver

phenotype; NASH, Non-alcoholic steatohepatitis. **b**, Frequency of SAMΦ in control

(n=14), heathy obese (n=27), steatosis (n=14) and NASH (n=18) livers, Mean±SEM,

Kruskal-Wallis and Dunn. **c**, Frequency of SAMΦ in patients with histological NAFLD

activity score (NAS) of 0 (n=37), 1-3 (n=19) and 4-7 (n=17) (left). Frequency of SAMΦ

in patients with histological fibrosis score of 0 (n=46), 1 (n=20) and 2-4 (n=5) (right),

Mean±SEM, Kruskal-Wallis and Dunn. **d**, Frequency of SAMΦ in female (n=58) and

male (n=15) patients (left). Frequency of SAMΦ in patients aged 23-39 (n=22), 40-49

(n=29) and 50-80 (n=22) (middle). Frequency of SAMΦ in patients with a body mass

index (BMI) of 17-30 (n=18), 31-45 (n=28) and 46-70 (n=27) (right). **e**, CD9 and

TREM2 staining in NASH liver biopsy sections (left), Scale bars, 50μm. Cell counting

(right): CD9 staining, NAS 1-3 (n=13) *versus* NAS 4-8 (n=21), Mean±SEM, Mann-

Whitney two-tailed. TREM2 staining, NAS 1-3 (n=12) *versus* NAS 4-8 (n=16),

Mean±SEM. **f**, Correlation of cell counts with picrosirius red (PSR) digital

morphometric pixel quantification in NAFLD liver biopsy tissue; CD9 staining (top;

n=39); TREM2 staining (bottom; n=32); Pearson correlation and linear regression.

a Bar chart showing fraction of monocyte-macrophages (KC, SAMΦ, TMo) across GEO accession numbers grouped by Normal, Healthy obese, Steatosis, and NASH.

b Fraction of monocyte-macrophages by Liver Phenotype (Control, Healthy obese, Steatosis, NASH), P=0.019.

c Fraction of monocyte-macrophages by NAS score (0, 1-3, 4-7), P=0.0095, P=0.0317; and by Fibrosis score (0, 1, 2-4), P=0.0409, P=0.0014.

d Fraction of monocyte-macrophages by Sex (Female, Male), Age (23-39, 40-49, 50-80), and BMI (17-30, 31-45, 46-70).

e NASH CD9 and TREM2 staining; CD9+ cells/mm² liver by NAS score (1-3, 4-8), P=0.039; TREM2+ cells/mm² liver by NAS score (1-3, 4-8).

f CD9+ cells/mm² liver vs PSR % area, p<0.0001, r=0.8199; TREM2+ cells/mm² liver vs PSR % area, p=0.0003, r=0.5952.

1264   **Extended Data Figure 8: Phenotypic characterisation of endothelial cells in**

1265   **healthy and cirrhotic human livers.**

1266   **a**, Clustering and selected genes expressed in 8,020 endothelial cells from 4 healthy and

1267   3 cirrhotic human livers. **b**, Scaled gene expression of endothelial cluster markers

1268   across endothelial cells from healthy (n=4) and cirrhotic (n=3) livers. **c**, Digital pixel

1269   quantification: PLVAP immunostaining of cirrhotic liver sections (n=10) in

1270   parenchymal nodules *versus* fibrotic septae (top), Wilcoxon two-tailed. ACKR1

1271   immunostaining of cirrhotic liver sections (n=10) in parenchymal nodules *versus*

1272   fibrotic septae (bottom), Wilcoxon two-tailed. **d**, Flow cytometry: endothelial cells

1273   from healthy (n=3, grey) or cirrhotic (n=7, red) livers, representative histogram for

1274   stated marker (top), mean fluorescence intensity (MFI, bottom), Mean±SEM, Mann-

1275   Whitney two-tailed. **e**, Flow-based adhesion assay: peripheral blood monocytes

1276   assessed for adhesion (top) and % of adherent cells which transmigrate (bottom);

1277   endothelial cells from healthy (n=5) or cirrhotic (n=4) livers, Mean±SEM, Mann-

1278   Whitney two-tailed. **f**, Endothelial cell gene knockdown: cirrhotic endothelial cells

1279   treated with siRNA to PLVAP (n=6), ACKR1 (n=5) or control siRNA (n=6).

1280   Representative flow cytometry histograms for stated marker (top); comparison to

1281   isotype control antibody. Flow-based adhesion assay (bottom), peripheral blood

1282   mononuclear cells assessed for adhesion (bottom left) and % of adherent cells which

1283   transmigrate (bottom right) following siRNA treatment of endothelial cells,

1284   Mean±SEM, Kruskal-Wallis and Dunn. **g**, Self-Organising Map (SOM; 60 x 60 grid;

1285   top left): smoothed scaled metagene expression of endothelia lineage. 21,237 genes,

1286   3,600 metagenes, 45 signatures. A-E label metagene signatures overexpressed in one

1287   or more endothelial subpopulations. SOM: smoothed mean metagene expression profile

1288   for each endothelial subpopulation (bottom left). Radar plots (middle): metagene

1289   signatures A-E showing distribution of signature expression across endothelial

1290   subpopulations, exemplar genes (middle) and Gene Ontology (GO) enrichment (right),

1291   Fisher's exact test. **h**, Heatmap: endothelial subpopulation transcription factor regulon

1292   expression (colour coded top by cluster and condition) across 8,020 endothelial cells

1293   from 4 healthy and 3 cirrhotic human livers. Exemplar regulons labelled right. Cells in

1294   columns, regulons in rows. **i**, t-SNE visualisation, endothelial lineage (8,020 cells from

1295   healthy (n=4) and cirrhotic (n=3)), annotating *monocle* pseudotemporal dynamics

1296   (purple to yellow; grey indicates lack of inferred trajectory). RNA velocities (red

1297   arrows) visualised using Gaussian smoothing on regular grid. (purple to yellow). **j**,

1298   Representative immunofluorescence images healthy *versus* cirrhotic liver: RSPO3,

1299   PDPN, AIF1L, VWA1 or ACKR1 (red), CD34 (white), PLVAP (green), DAPI (blue),

1300   scale bars, 50μm. **k**, Annotation of 8,020 endothelial cells by subpopulation and injury

1301   condition. LSEC, Liver sinusoidal endothelial cells.

a Clusters labeled Endo(1)–Endo(7) on t-SNE plots with marker genes PECAM1, ICAM2, CDH5, ENG, ERG, CD34, VWF.

b Violin plots of gene expression across Endo(1)–Endo(7) clusters for: CLEC4M, CLEC4G, CD34, PDPN, RSPO3, AIF1L, PLVAP, VWA1, ACKR1.

c PLVAP % Area: Nodules vs Fibrotic septae, P=0.002. ACKR1 % Area: Nodules vs Fibrotic septae, P=0.002.

d Flow cytometry histograms and MFI bar charts for PLVAP (PLVAP-APC, P=0.017), CD34 (CD34-BV650), ACKR1 (ACKR1-PEVio770), Healthy vs Cirrhotic.

e Adhesion: Adherent cells per mm² per 10⁶ cells perfused, Healthy vs Cirrhotic. Transmigration: % of Adherent Cells, Healthy vs Cirrhotic, P=0.016.

f Histograms for PLVAP (Control siRNA, PLVAP siRNA, Isotype) and ACKR1 (Control siRNA, ACKR1 siRNA, Isotype). Adhesion bar chart (Control, PLVAP, ACKR1 siRNA). Transmigration bar chart (Control, PLVAP, ACKR1 siRNA, P=0.045).

g Radar plots of Signatures A–E across Endo(1)–Endo(7) with Genes and Gene Ontology bar charts (−log₁₀(P value)).

Signature A Genes: LAMC1, DYSF, PGF, ITGA2, LAMB1, LOXL2, PDGFD, CX3CL1, PDGFB, LOX, COL15A1, COL4A1. GO: Vasculature development, Angiogenesis, Extracellular matrix organization, Response to wounding, Wound healing.

Signature B Genes: S100A10, HYAL2, IL3RA, IL32, ITGA6, FABP4, CTGF, ACKR1, MDK, MIF, FN1, EDN1. GO: Peptide biosynthetic process, Myeloid leukocyte activation, Antigen processing and presentation, Non-canonical Wnt signaling pathway, Activation of immune response.

Signature C Genes: AIF1L, CD200, CXCR4, SOX17, EPHA4, CXCL12, EDNRB, HBEGF, CD36, JAG2, DLL4, JAG1. GO: Endothelium development, Animal organ development, Endothelial cell differentiation, Angiogenesis, Regulation of Notch signaling pathway.

Signature D Genes: CLEC4M, FCGR2B, ITGA9, STAB1, SELP, SCARB2, CD14, MRC1, CD36, TGFBR3, GATA4, IL33. GO: Vesicle-mediated transport, Blood vessel development, Endocytosis, Immune response, Receptor-mediated endocytosis.

Signature E Genes: PROX1, PTX3, HOXA7, MMRN1, SEMA3D, IL7, IGF1, TBX1, PDPN, HOXD8, CCL21, TFF3. GO: Positive regulation of cell migration, Lymph vessel development, Regulation of cell adhesion, Regulation of cell motility, Lymphangiogenesis.

h Heatmap of transcription factors by Condition and Cluster: GATA4, NR5A2, STAT2, RARB, IRF1, ATF3, MEF2C, HOXD8, HOXD9, FOXC2, HOXA9, GATA6, PPARA, SOX17, KLF2, FOXP1, ELK3, JDP2.

i t-SNE with Pseudotime (0.0–1.0).

j Immunofluorescence: RSPO3/CD34/PLVAP/DAPI, PDPN/CD34/PLVAP/DAPI, AIF1L/CD34/PLVAP/DAPI, VWA1/CD34/PLVAP/DAPI, ACKR1/CD34/PLVAP/DAPI. Rows: Healthy, Cirrhotic.

k t-SNE plots: LSEC Cluster Endo(1); Central vein Cluster Endo(4); Lymphatic Cluster Endo(2); Hepatic artery Cluster Endo(5); Scar-associated Cluster Endo(6), Endo(7); Condition Healthy, Cirrhotic.

1302 **Extended Data Figure 9: Characterisation of mesenchymal cells in healthy and**

1303 **cirrhotic human livers.**

1304 **a**, Selected genes expressed in 2,318 mesenchymal cells from 4 healthy and 3 cirrhotic

1305 human livers. **b**, Clustering 319 scar-associated mesenchymal cells (SAMes) into 2

1306 further subclusters. **c**, Heatmap: SAMes subcluster marker genes (top, colour coded by

1307 cluster and condition), exemplar genes labelled (right). Cells columns, genes rows. **d**,

1308 Fractions of SAMes subpopulations in healthy (n=4) *versus* cirrhotic (n=3) livers,

1309 Mean±SEM, Wald. **e**, Representative immunofluorescence image, portal region of

1310 healthy liver: OSR1 (red), Collagen 1 (green), DAPI (blue), Scale bar 50μm. **f**,

1311 Representative immunofluorescence image, fibrotic niche of cirrhotic liver: OSR1

1312 (red), Collagen 1 (green), DAPI (blue), Scale bar 50μm. **g**, Scaled gene expression of

1313 selected genes across 2,318 mesenchymal cells from healthy (n=4) and cirrhotic (n=3)

1314 livers. **h**, t-SNE visualisation, 1,178 Hepatic Stellate Cells (HSC) and SAMes from

1315 healthy (n=4) and cirrhotic (n=3) livers annotated by *monocle* pseudotemporal

1316 dynamics (purple to yellow). RNA velocity field (red arrows) visualised using Gaussian

1317 smoothing on regular grid. **i**, Heatmap: cubic smoothing spline curves fitted to genes

1318 differentially expressed across HSC-to-SAMes pseudotemporal trajectories, grouped

1319 by hierarchical clustering (k=2). Colour coded by pseudotime and condition (top). Gene

1320 co-expression modules (colour) and exemplar genes labelled right.

1321

a MYH11 · RGS5 · KRT19 · PDPN · COL3A1 · COL1A1 · TIMP1 · PDGFRA

b Cluster: 3A SAMes A, 3B SAMes B

c Condition / Cluster — RBP1, IGFBP3, EDNRB, OGN, CRABP2, CTHRC1, OSR1

d Fraction of mesenchymal cells — Condition: Healthy (grey), Cirrhotic (magenta). SAMes cluster: SAMes A (P<0.001), SAMes B (P<0.001)

e Healthy — OSR1 · Collagen1 · DAPI

f Cirrhotic — OSR1 · Collagen1 · DAPI

g Gene expression — ELN, SFRP4, FBLN2, MSLN, KRT19. Mesenchymal cluster: VSMC, HSC, SAMes A, SAMes B, Mesothelia

h Pseudotime (t-SNE1 vs t-SNE2): 0.0–1.0

i HSC → SAMes. Condition / Pseudotime.

Module 1: PDGFRA, COL1A1, LOXL1, COL1A2, CCL2, COL3A1, IGF1, TIMP1

Module 2: RGS5, ID1, CSRP2, IGFBP5, FABP4, ADAMTS1, FABP5, GEM

Extended Data Figure 10: Characterisation of the cellular interactome in the fibrotic niche.

a to b, Representative immunofluorescence images, fibrotic niche cirrhotic liver. **a**, TREM2 (red), PLVAP (white), PDGFRα (green), DAPI (blue), Scale bars 50μm. **b**, TREM2 (red), ACKR1 (white), PDGFRα (green), DAPI (blue), Scale bars 50μm. **c**, Proliferation assay: Human hepatic stellate cells (HSC) treated with conditioned media from primary hepatic macrophage subpopulations SAMΦ (n=2), tissue monocytes (TMo, n=2), Kupffer cells (KC, n=2) or control media (n=2). y-axis, area under curve (AUC) of % change in HSC number over time (hours), Mean±SEM. **d**, Circle plot: potential interaction magnitude from ligands expressed by scar-associated macrophages (SAMΦ) and endothelial cells (SAEndo) to receptors expressed on scar-associated mesenchyme (SAMes). **e**, Circle plot: potential interaction magnitude from ligands expressed by SAMes to receptors expressed on SAMΦ and SAEndo. **f**, Dotplot: ligand-receptor interactions between SAMes (n=7 human livers), SAMΦ (n=10 human livers) and SAEndo (n=7 human livers). X-axis, ligand (red) and cognate receptor (blue); y-axis, ligand (red) and receptor (blue) expressing cell populations; circle size, P value (permutation test); colour (red, high; yellow, low), means of average ligand and receptor expression levels in interacting subpopulations. **g**, Representative immunofluorescence images, fibrotic niche in cirrhotic liver. Top; CCL2 (red), CCR2 (white), PDGFRα (green), DAPI (blue), arrows CCL2⁺PDGFRα⁺ cells. Bottom; ANGPT1 (red), TEK(white), PDGFRα (green), DAPI (blue), arrows ANGPT1⁺PDGFRα⁺ cells. Scale bars 50μm. **h**, Circle plot: potential interaction magnitude from ligands expressed by to receptors expressed on SAEndo. **i**, Dotplot: ligand-receptor interactions between SAMΦ (n=10 human livers) and SAEndo (n=7 human livers). X-axis, ligand (red) and cognate receptor (blue); y-axis, ligand (red) and receptor (blue) expressing cell populations; circle size, P value (permutation test); colour (red, high; yellow, low), means of average ligand and receptor expression levels in interacting subpopulations. **j**, Representative immunofluorescence image, fibrotic niche in cirrhotic liver. TREM2 (red), FLT1 (white), VEGFA (green), DAPI (blue), arrows TREM2⁺VEGFA⁺ cells, Scale bar 50μm. **k**, Circle plot: potential interaction magnitude from ligands expressed by SAEndo to receptors expressed on SAMΦ. **l**, Dotplot: ligand-receptor interactions between SAEndo (n=7 human livers) and SAMΦ

55

1354   (n=10 human livers). X-axis, ligand (red) and cognate receptor (blue); y-axis, ligand

1355   (red) and receptor (blue) expressing cell populations; circle size, P value (permutation

1356   test); colour (red, high; yellow, low), means of average ligand and receptor expression

1357   levels in interacting subpopulations. **m**, Representative immunofluorescence images,

1358   fibrotic niche in cirrhotic liver. Top; TREM2 (red), CD200 (white), CD200R (green),

1359   DAPI (blue), arrows TREM2$^+$CD200R$^+$ cells. Bottom; TREM2 (red), DLL4 (white),

1360   NOTCH2 (green), DAPI (blue), arrows TREM2$^+$NOTCH2$^+$ cells. Scale bars, 50μm.

1361

a TREM2 PLVAP PDGFRα DAPI

b TREM2 ACKR1 PDGFRα DAPI

c

d SAM (1) SAM (2) HAEnd SAEnd (1) SAEnd (2) SAMes
2.9 2.9 4.0 3.2 2.8 8.0

e SAM (1) SAM (2) HAEnd SAEnd (1) SAEnd (2) SAMes
3.0 3.0 3.1 3.3 3.6 8.7

f SAMes→SAMΦ and SAEnd interactions

g CCL2 CCR2 PDGFRα DAPI

ANGPT1 TEK PDGFRα DAPI

h SAM (1) HAEnd SAEnd (1) SAEnd (2)
2.0 2.1 1.4 5.7

SAM (2) HAEnd SAEnd (1) SAEnd (2)
2.3 2.9 2.4 7.7

i SAMΦ→SAEnd interactions

j TREM2 FLT1 VEGFA DAPI

k HAEnd SAEnd (1) SAM (2) SAEnd (2)
3.6 3.3 3.2 6.9

HAEnd SAEnd (1) SAM (1) SAEnd (2)
3.1 2.5 2.4 6.3

l SAEnd→SAMΦ interactions

m TREM2 CD200 CD200R DAPI

TREM2 DLL4 NOTCH2 DAPI