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Biological Sciences

Stabilin-1 expression defines a subset of macrophages which mediate tissue homeostasis and prevent fibrosis in chronic liver injury

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Abstract

Macrophages are key regulators of fibrosis development and resolution. Elucidating the mechanisms by which they mediate this process is crucial for establishing their therapeutic potential. Here, we use experimental models of liver fibrosis to show that deficiency of the scavenger receptor, stabilin-1, exacerbates fibrosis and delays resolution during the recovery phase. We detected a subset of stabilin-1^{+} macrophages which were induced at sites of cellular injury close to the hepatic scar in mouse models of liver fibrosis and in human liver disease. Stabilin-1 deficiency abrogated malondialdehyde-LDL (MDA-LDL) uptake by hepatic macrophages and was associated with excess collagen III deposition. Mechanistically, the lack of stabilin-1 led to elevated intrahepatic levels of the pro-fibrogenic chemokine CCL3 and an increase in $GFAP^{+}$ fibrogenic cells. Stabilin-$1^{-/-}$ macrophages demonstrated a pro-inflammatory phenotype during liver injury and the normal induction of $Ly6C^{lo}$ monocytes during resolution was absent in stabilin-1 knockouts leading to persistence of fibrosis. Human stabilin-1^{+} monocytes efficiently internalised MDA-LDL and this suppressed their ability to secrete CCL3 suggesting that loss of stabilin-1 removes a brake to CCL3 secretion. In support of this, studies with cell lineage specific knockouts revealed that stabilin-1 expression in myeloid cells is required for the induction of this novel subset of macrophages and that increased fibrosis occurs in their absence. This study demonstrates a new regulatory pathway in fibrogenesis in which a macrophage scavenger receptor protects against organ fibrosis by removing fibrogenic products of lipid peroxidation. Thus stabilin-1^{+} macrophages shape the tissue microenvironment during liver injury and healing.

Significance Statement

Organ fibrosis is a major cause of global morbidity and mortality. It is driven by chronic inflammation and associated oxidative stress with depletion of cellular antioxidant defenses. We demonstrate a novel mechanism in which the evolutionarily conserved receptor stabilin-1 on tissue-infiltrating macrophages provides a second-line defense to prevent tissue damage from oxidative stress. Stabilin-1^+ monocytes take up malondialdehyde-LDL (MDA-LDL), a major product of oxidative lipid peroxidation, to form ceroid-laden macrophages. Through the uptake of MDA-LDL, stabilin-1 suppresses production of the pro-fibrogenic chemokine CCL3 and prevents excessive collagen deposition in experimental models of liver fibrosis. We propose that macrophage stabilin-1 is a critical defense against oxidative tissue damage and thereby maintains tissue homeostasis.

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Introduction

Liver fibrosis is driven by extracellular matrix (ECM) deposition by activated myofibroblasts, the majority of which arise from the hepatic stellate cell (HSC) (1). The accumulation of an ECM rich in fibrillar collagens I and III, causes architectural distortion which leads to organ dysfunction and portal hypertension. Consequently, identifying pathways which regulate the deposition and resolution of fibrillar collagen is vital to developing new treatments for chronic liver disease. Products of oxidative stress, such as oxidised-low density lipoproteins (oxLDLs), can directly activate HSC to an ECM-producing state thereby driving fibrogenesis (2, 3). In addition to HSCs, other non-parenchymal cells, contribute to the regulation of liver fibrosis. Hepatic sinusoidal endothelial cells (HSEC), which lie in close proximity to HSCs,

play an important role in maintaining HSC quiescence (4), and macrophages play a critical role in fibrosis progression, tissue remodelling and resolution (5).

Stabilin-1, also known as CLEVER-1 (gene name *Stab1*), is a highly conserved transmembrane glycoprotein that is expressed by sinusoidal endothelium, and sub-populations of macrophages (6). Previous studies have demonstrated multiple stabilin-1 ligands including oxLDL (7), the ECM glycoprotein osteonectin (8) and placental lactogen (9) suggesting that stabilin-1 functions as a homeostatic scavenger receptor. We have previously reported stabilin-1 expression by HSEC, but not by resident liver macrophages (known as Kupffer cells), in normal human liver (10). Our observation of increased expression in a range of chronic inflammatory liver diseases led us to study whether stabilin-1 contributes to the progression of chronic liver disease and fibrogenesis.

Results

Stabilin-1 deficiency exacerbates liver fibrosis

To investigate the functional role of stabilin-1 in liver injury and fibrosis we used two mouse models. In carbon tetrachloride (CCl_4)-induced chronic liver injury a fibrogenic phase is followed by a spontaneous resolution phase (11) and in mice fed a methionine choline deficient (MCD) diet hepatic steatosis is followed by inflammation and early fibrosis (12).

Consistent with previous reports in stabilin-$1^{-/-}$ mice, sirius red positive fibres consistent with increased fibrogenesis were seen within the liver parenchyma which was otherwise normal (Fig.1A,B) (13). Following CCl_4 administration, stabilin-$1^{-/-}$ mice developed a marked increase in hepatic scar formation compared with WT mice with features of bridging fibrosis (Fig.1A,B) and increased sirius red staining (SI Appendix Fig.S1A). Greater activation of fibrogenic pathways in stabilin-$1^{-/-}$ mice was confirmed by significantly increased accumulation of collagen I and III (Fig.1C,D, SI Appendix Fig.S1B,C).). In order to study

the number and activation of fibrogenic cells at baseline and injury we compared the expression of glial fibrillary acidic protein (GFAP) as a constitutive marker of stellate cells to alpha smooth muscle actin (αSMA) and matrix metalloproteinase-2 (MMP-2) as markers of activated myofibroblasts (14, 15). At baseline and throughout injury and resolution we detected increased GFAP expression in livers of stabilin-1$^{-/-}$ mice (Fig.1E-H). In contrast αSMA and MMP-2 expression was significantly higher only during injury (Fig.1E-H, SI Appendix Fig.S1D,E) compared to WT mice.

The role of stabilin-1 in the resolution of fibrosis was studied in mice during recovery after CCl_4 injury. After four weeks of recovery, we noted persistent bridging fibrosis and increased collagen I and III transcription in stabilin-1$^{-/-}$ mice when compared to WT mice (Fig.1I,J). Hydroxyproline quantification demonstrated an increase in collagen content in stabilin-1$^{-/-}$ mice at baseline comparable to that seen in WT mice after CCl_4 administration (Fig.1K). After four weeks of resolution, the hydroxyproline had reduced in WT mice but increased further in stabilin-1$^{-/-}$ mice (Fig.1K). We also noted that stabilin-1$^{-/-}$ mice demonstrated significantly increased ALT levels during liver injury (Fig.1L). To assess if elevated ALT levels were due to increased hepatocyte death or inefficient scavenging we co-stained for ALT and F4/80 and assessed markers of cell death. We found increased co-expression of ALT and F4/80 in WT mice compared to stabilin-1$^{-/-}$ mice, but no significant difference in levels of apoptosis and autophagy (SI Appendix Fig. S2 A-I). Histological assessment demonstrated minimal necrosis with no difference between WT and stabilin-1$^{-/-}$ mice at baseline or after 8 weeks of carbon tetrachloride (SI Appendix Fig. S2 J). These findings suggest inefficient scavenging in stabilin-1$^{-/-}$ mice rather than increased cellular death.

We also detected histologically more severe scarring in stabilin-1$^{-/-}$ MCD diet fed mice and significantly increased hydroxyproline levels compared to WT MCD diet fed mice, as well as,

higher, but not statistically significant, ALT levels (SI Appendix Fig.S3 A-E). Collectively these data show that *in vivo* there is an increased baseline fibrogenic response in stabilin-1$^{-/-}$ mice, which is due to increased numbers of fibrogenic cells. Fibrosis and scarring are enhanced upon injury, and the resolution phase is impaired in the absence of stabilin-1.

Injury-dependent induction of stabilin-1 in hepatic macrophages

We proceeded to study the cellular expression of stabilin-1 in normal and injured murine livers. In uninjured WT mice, stabilin-1 was restricted to CD31-positive sinusoidal endothelial cells and was absent from F4/80-positive Kupffer cells (SI Appendix Fig. S4A,B). In response to liver injury from either CCl_4 or MCD diet we detected a subset of stabilin-1$^+$ F4/80$^+$ intrahepatic macrophages (SI Appendix Fig.S4C, S5A). Using αSMA as a cell lineage marker, we clearly demonstrated that myofibroblasts, which play a central role in liver fibrosis, do not express stabilin-1 during liver injury (SI appendix Fig. S4D, S5B).

Analysis of chronic human liver disease demonstrated that CD31-positive sinusoidal endothelial cells expressed stabilin-1 whereas CD68-positive Kupffer cells within the sinusoids did not (SI Appendix Fig. S4E,F). In contrast, stabilin-1$^+$ macrophages were readily detected within the fibrous septa associated with the hepatic scar (SI Appendix Fig.S4G). Stabilin-1 was not detected on αSMA-positive myofibroblasts (SI Appendix Fig. S4H). These studies demonstrate for the first time an intrahepatic subpopulation of stabilin-1$^+$ macrophages in liver injury.

Stabilin-1 deficiency leads to a reduction of ceroid macrophages in liver injury.

The presence of a population of stabilin-1$^+$ macrophages in liver injury and previous findings that macrophages are critical to the development of fibrosis (16), led us to investigate whether an alteration in macrophage distribution or function contributes to the increased fibrosis in stabilin-1$^{-/-}$ mice. Intrahepatic macrophage distribution and peripheral

158 blood monocyte numbers were comparable between uninjured WT and stabilin-$1^{-/-}$ mice
159 (Fig.2A,B, SI Appendix Fig S9A). After CCl_4 injury, WT mice demonstrated prominent
160 aggregates of macrophages, which were not seen in the stabilin-$1^{-/-}$ animals (Fig.2A-C).
161 These macrophage aggregates resembled ceroid-laden macrophages. Ceroid-laden
162 macrophages contain lipid-like pigment deposits and have been previously described in
163 experimental liver fibrosis but their pathological significance is unknown (17, 18).
164 We hypothesized that stabilin-1 deficiency was associated with a reduction in ceroid-laden
165 macrophage formation during liver injury and fibrosis. To test this hypothesis, we used
166 periodic acid-schiff diastase (PAS-D) staining to detect cytoplasmic ceroid accumulation.
167 This confirmed the presence of large ceroid macrophages in WT mice (Fig.2D) that were
168 largely absent in stabilin-$1^{-/-}$ animals (Fig.2D). Interestingly, PAS-D also stains the fibrous
169 scar and in the stabilin-$1^{-/-}$ mice the absence of ceroid-laden macrophages was associated
170 with prominent fibrosis (Fig.2D).
171 MDA is the most abundant aldehyde produced by lipid peroxidation and is highly reactive
172 leading to the formation of MDA-modified LDL. This product of oxidative stress is taken up
173 by macrophages and contributes to cytoplasmic ceroid accumulation (19). In WT mice,
174 prominent MDA-positive cells were clearly visible (Fig.2E), whereas minimal staining was
175 detected in stabilin-$1^{-/-}$ mice (Fig.2E,F).
176 A further well established property of ceroid accumulation is that the pigment, including
177 MDA adducts, is autofluorescent (20). We identified prominent autofluorescent $F4/80^{+}$
178 aggregates in WT mice which were greatly reduced in stabilin-$1^{-/-}$ mice in both CCl_4- and
179 MCD diet-induced liver injury (Fig.2G,H). We then showed that $F4/80^{+}$ ceroid-laden
180 macrophages in experimental liver injury in WT mice co-express stabilin-1 (Fig.2I).
181 We used a thiobarbituric acid reactive substances (TBARS) assay to detect serum MDA
182 and found similar levels in WT and stabilin-$1^{-/-}$ mice after CCl_4 injury and MCD diet

suggesting that production of MDA is not reduced in stabilin-1 deficiency (Fig.2J). Proteomic analysis of baseline serum samples demonstrated increased levels of several proteins in the serum of stabilin-$1^{-/-}$ mice compared to WT mice (SI Appendix Table S1) several of which are related to oxidative stress. However we did not detect increased circulatory levels of pro-inflammatory or pro-fibrogenic cytokines such as TGFβ, PDGF A and B, in keeping with previous studies (13).

Our findings prompted us to study the distribution of ceroid-laden macrophages in relation to sites and stage of fibrosis. We detected ceroid-laden macrophages along the hepatic scar in WT mice (Fig.3A) whereas in stabilin-1 deficient animals, the absence of ceroid macrophages was associated with a dense network of collagen III in the hepatic scar (Fig.3B). Further analysis of livers after 4 weeks resolution demonstrated the persistence of ceroid-laden macrophages in close proximity to residual hepatic scar in WT mice (Fig.3C). In contrast, ceroid-laden macrophages were not seen in stabilin-$1^{-/-}$ mice in which extensive collagen-rich scars persisted after 4 weeks of resolution (Fig.3D). Thus, collectively our data suggest that stabilin-1 on macrophages protects against liver fibrosis by taking up fibrogenic modified lipids.

Stabilin-1 deficiency is associated with excess CCL3 production in liver tissue and stabilin-1 suppresses CCL3 expression in macrophages.

To identify specific mediators of fibrogenesis in stabilin-1 deficiency, we used RNA-seq to compare the gene expression profile of liver tissue from WT and stabilin-$1^{-/-}$ mice. A number of genes showed increased expression in stabilin-$1^{-/-}$ mice (SI Appendix Table S2) but the only established fibrogenic mediator which was increased was the chemokine CCL3 (21, 22). We validated our findings with qPCR of liver tissue and critically demonstrated that CCL3 was persistently elevated after four weeks of resolution in stabilin-$1^{-/-}$ mice

208 (Fig.4A). In support of these findings we detected increased CCL3 protein in stabilin-1$^{-/-}$
209 mice which colocalised with hepatic macrophages (Fig. 4B-D).
210 We investigated whether the pro-fibrogenic phenotype in livers of stabilin-1$^{-/-}$ mice was
211 linked to ox-LDL uptake to form ceroid-laden macrophages. We initially confirmed the
212 presence of autofluorescent stabilin-1^{+} ceroid-laden macrophages in livers from patients
213 with chronic liver diseases (SI Appendix Fig. S6A,B).
214 As MDA-LDL is one of the most abundant products of lipid peroxidation and contributes to
215 ceroid formation (23), we investigated whether stabilin-1 on human macrophages is
216 involved in its uptake and degradation. We isolated monocytes from human peripheral
217 blood and stimulated their stabilin-1 expression *in vitro* (24). After 2 hours of MDA-LDL
218 incubation we were able to detect intracellular deposits of MDA-LDL completely co-
219 localised with stabilin-1 (SI Appendix Fig. S7A). Uptake of MDA-LDL was minimal in
220 unstimulated monocytes which had low expression of stabilin-1 (SI Appendix Fig. S7B). A
221 function blocking antibody to stabilin-1 led to the internalisation of stabilin-1 thereby
222 inhibiting MDA-LDL uptake (Fig. 5A,B). We also linked the scavenging function of stabilin-
223 1 positive macrophages to CCL3 expression by demonstrating a significant reduction in
224 macrophage CCL3 transcription after MDA-LDL uptake compared to control (Fig. 5C). We
225 could reverse this effect using the function blocking antibody against stabilin-1 (Fig. 5D).
226 These results suggest that stabilin-1 expression is critical for the uptake of MDA-LDL by
227 macrophages, a process which also modulates macrophage-derived CCL3 secretion.
228
229 **Stabilin-1 deficiency is associated with a pro-inflammatory hepatic macrophage**
230 **phenotype.**
231 To assess how stabilin-1 deficiency influenced macrophage phenotype we sorted
232 macrophages from CCl_4 treated livers. Analysis of WT hepatic macrophage populations

during liver injury demonstrated higher transcript expression of stabilin-1 in F4/80^{hi}/CD11b^{lo} compared to the F4/80^{lo}/CD11b^{hi} population (SI Appendix Fig S8A). The loss of stabilin-1 led to higher expression of M1 markers CCL3 and TNFα in both F4/80^{hi}/CD11b^{lo} and F4/80 ^{lo}/CD11b^{hi} subsets. M2 marker MMP-9 was also elevated but Arginase-1 was reduced in the F4/80^{hi}/CD11b^{lo} subset (SI Appendix Fig. S8B-E). This is in keeping with previous studies where defining macrophages through their polarisation to classical M1 or M2 macrophages is unreliable in liver injury as M1/M2 markers can be expressed simultaneously by liver macrophages (25).

Ramachandran *et al.* demonstrated that the CD11b^{hi}Ly6C^{lo} monocyte-derived macrophage population increases significantly during fibrosis resolution and functions as the 'restorative' macrophage (25) leading us to compare populations of 'pro-fibrotic' CD11b^{hi}Ly6C^{hi} and 'restorative' CD11b^{hi} Ly6C^{lo} macrophages. Liver injury in both WT and stabilin-1$^{-/-}$ mice resulted in a predominance of Ly6C^{hi} macrophages in the liver (SI Appendix Fig. S8F,G). During resolution there was a shift towards Ly6C^{lo} macrophages in the WT group which was not seen in the stabilin-1$^{-/-}$ mice (SI Appendix Fig.S8H). Intrahepatic lymphocyte populations did not differ between WT and stabilin-1$^{-/-}$ mice after the injury or during resolution (SI Appendix Fig. S9B-K). Collectively, these results demonstrate that the profibrogenic response in stabilin-1 deficiency is associated with a pro-inflammatory macrophage phenotype during injury.

Deletion of stabilin-1 in myeloid cells is associated with a loss of ceroid-laden macrophages and exacerbated fibrosis.

The preceding results suggest that stabilin-1 protects the liver from fibrosis by allowing macrophages to take up and remove pro-fibrogenic lipid peroxidation products and at the same time suppressing CCL3 production. To test this hypothesis *in vivo* we used Tie-2 Cre

and Lys2 Cre strains to generate cell-selective knockouts. We have previously confirmed their selectivity and efficiency in knocking down stabilin-1 (26). We have shown that in our Tie-2 Cre model stabilin-1 is absent from endothelium (ENDO stab-$1^{-/-}$) but macrophage expression is maintained. In our Lys2 Cre model stabilin-1 is absent from the myeloid population (MACRO stab-$1^{-/-}$)**.** In practice this model is selective for macrophages, since neutrophils do not express stabilin-1 and therefore are unaffected by this knockout. We confirmed this specificity in the livers of our cell selective strains at baseline and during liver injury (SI Appendix Fig. S10A,B).

Fibrosis in ENDO stab-$1^{-/-}$ mice was comparable to WT mice in both CCl_4 liver injury and MCD diet (SI Appendix Fig. S11A-C), whereas fibrogenesis was increased in MACRO stab-$1^{-/-}$ animals (Fig.6A,B) with significantly more accumulation of collagen III associated with increased αSMA staining in MACRO stab-$1^{-/-}$ mice versus WT after CCl_4 injury (Fig.6C-F). Fibrosis resolution was delayed in the MACRO stab-$1^{-/-}$ animals as demonstrated by persistently elevated transcript levels of αSMA and increased hydroxyproline staining and critically for our hypothesis we found persistent elevation of CCL3 compared to WT animals (Fig.6G-I) and increased serum ALT levels in response to CCl_4 as seen in the full knockout (Fig.6J). We also detected significantly elevated hydroxyproline content in MACRO stab-$1^{-/-}$ mice compared to WT mice after MCD diet (SI Appendix Fig.S11D). Thus the increased fibrosis in response to liver injury seen in the absence of stabilin-1 is predominantly mediated through stabilin-1 expressing macrophages.

There were no detectable differences in the numbers of ceroid-laden macrophages in the livers of WT and ENDO stab-$1^{-/-}$ mice (SI Appendix Fig. S11E,F). In contrast, very few ceroid-laden macrophages were seen in the livers of CCl_4 treated MACRO stab-$1^{-/-}$ animals (SI Appendix Fig. S11E,F). To further confirm the role of macrophage stabilin-1 in fibrosis resolution we undertook experiments similar in design to those described by

Ramachandran *et al.* (25). This consisted of a 4 week model of CCl_4 liver injury performed in MACRO stab-$1^{-/-}$ and WT mice followed by adoptive transfer of wild type myeloid cell elements or vehicle control at 24 and 72 hours after the final injection of CCl_4 followed by an analysis of fibrogenesis at 120 hours. Transcription of fibrogenic markers and collagen III expression in liver tissue showed no differences between WT mice receiving myeloid cells or vehicle without cells (SI Appendix Fig S12A,D,F), whereas in the MACRO stab-$1^{-/-}$ there was a trend of reduced transcription in nearly all fibrogenic markers in mice receiving wild type myeloid cells (SI Appendix Fig.S12B). We confirmed the presence of adoptively transferred Dsred+ myeloid cells within liver tissue at sites of collagen deposition (SI Appendix Fig. S12C). Finally, we demonstrated a significant reduction in hepatic collagen III expression in MACRO stabilin-$1^{-/-}$ mice receiving wild type myeloid cells compared to vehicle control (SI Appendix Fig. 12E,F).

Discussion

This study reports a novel mechanism which involves a subset of stabilin-1^{+} macrophages found in both experimental and human liver injury which play a critical role in protecting against excessive fibrosis in response to oxidative stress. Stabilin-1 deficiency led to an increase in hepatic CCL3 associated with the recruitment of $GFAP^{+}$ fibroblasts and an increase in baseline hepatic fibrosis. Using cell-specific knockout animals we were able to show that stabilin-1 mediates its effects by enabling macrophages to take up and clear fibrogenic oxidised lipids generated in response to liver injury. Stabilin-1 deficiency was associated with a marked reduction of ceroid-laden macrophages during liver injury. Ceroid-laden macrophages are a well recognised pathological feature of liver injury but, to our knowledge, their contribution to liver fibrosis is unknown. Ceroid contains modified lipoproteins (such as MDA-LDL) which are generated as a consequence of chronic

oxidative stress. We found in both humans and mice that they are derived from a subset of macrophages, that upregulate stabilin-1. The expression of stabilin-1 allows macrophages to take up and clear modified LDLs and in addition we show that this uptake suppresses the secretion of the pro-fibrogenic chemokine CCL3 resulting in reduced fibrosis and the promotion of scar resolution. The highest levels of stabilin-1 were detected in the F4/80^{hi} CD11b^{lo} population suggesting that tissue resident macrophages may upregulate stabilin-1 during liver injury. Interestingly, stabilin-1 deficiency was associated with an inflammatory phenotype in both infiltrating monocytes and mature tissue resident macrophages. The therapeutic potential of stabilin-1^{+} macrophages was demonstrated by our finding that the adoptive transfer of wild type myeloid cells can promote resolution of fibrosis in stabilin-1 deficiency. These results suggest that macrophages which are stimulated or engineered to express high levels of stabilin-1 could be a potential cell therapy in fibrotic liver disease. In addition to its potential role in liver disease we suggest that stabilin-1 plays a role in tissue homeostasis by removing local products of low level oxidative stress. This explains why increased fibrosis is seen in stabilin-1 deficient mice even in the absence of exogenous injury. The liver is constantly exposed to bacterial products and xenobiotics from both the portal and systemic circulation and under normal conditions stabilin-1 on endothelium as well as on macrophages may allow the rapid removal of products of oxidative stress thereby preventing low level continuous injury and scarring. Whereas in response to liver injury, protection and resolution requires the involvement of stabilin-1^{+} macrophages. These findings describe a novel mechanism involved in the regulation of tissue fibrosis that allows efficient wound healing without destructive scarring in response to liver injury.

Methods

Animals

Stabilin-1 knockout mice and cell-specific mice were generated as previously described (26). CAG-Dsred*MST$^{1Nagy/J}$ (stock 005441) were from the Jackson Laboratory. All animal studies were done in adherence with the rules and regulations of The Finnish Act on Animal Experimentation (62/2006), and accepted by the local Committee for Animal Experimentation (Animal licence number 5587/04.10.07/2014).

Liver injury models

Carbon tetrachloride (CCl_4) injury model

8-week-old mice were injected twice weekly for 8 weeks with either CCl_4 (1.0 ml/kg CCl_4 diluted 1:3 in mineral oil, Sigma-Aldrich) or a mineral oil vehicle control. Animals were sacrificed 72 h after the final dose of CCl_4 (27) or after a 4-week recovery period.

Methionine Choline Deficient (MCD) Diet

Mice were fed an MCD diet (Harlan laboratories TD90262) for 6 weeks *ad libitum*. Control animals received normal chow for 6 weeks (27).

Human tissue

Tissue and blood samples from patients were obtained with written informed consent and with local ethics committee approval (LREC reference 06/Q2702/61 South Birmingham, Birmingham, UK and 04/Q2708/41 South Birmingham, Birmingham, UK).

Statistical analysis , see SI Appendix Supplementary methods.

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Figure Legends

Figure 1

Stabilin-1 deficiency exacerbates fibrosis after CCl_4 liver injury. Wild-type (WT) and stabilin-1-deficient (stabilin-$1^{-/-}$) mice were subjected to CCl_4-induced liver injury: control (Oil); 8 week CCl_4 injury (CCl_4) and 4 week resolution after the CCl_4 injury (Res). (A,B) Sirius red stainings in livers (n=4-5 in each group). (C-H) Collagen I, Collagen III

immunostainings stainings and GFAP/αSMA co-immunostainings and quantifications of liver sections (n=3-5 in each group). (I) Sirius red staining from livers after resolution. (J) mRNA expression of Collagen I and Collagen III in livers. (K) Hydroxyproline determinations in liver samples and (L) serum alanine transaminase (ALT) levels. (for J-L n=5-6 mice in each group). Statistical significance was determined by unpaired *t*-test (G,H,J)) and 1-way ANOVA analysis, with a Tukey's *post-hoc* multiple comparison test (K, L). * P<0.05, ** P<0.005, ***P<0.001, ****P<0.0005. Bars 200μm (A-D, I) Bars 50 μm (E, F)

Figure 2

Stabilin-1 deficiency is associated with a reduction of ceroid-laden macrophages during liver injury. (A,B) Staining of livers for F4/80 from WT and stabilin-1$^{-/-}$ mice in oil controls and CCl_4 injury. Arrows indicate aggregates of F4/80^{+} cells. (C) Quantification of F4/80 positive area staining in WT and stabilin-1$^{-/-}$ mice in oil controls and CCl_4 injury. (D) Periodic acid schiff-diastase (PAS-D) staining of livers from WT and stabilin-1$^{-/-}$ mice after CCl_4 injury (black arrows highlight PAS-D positive cells and white arrows highlight areas of increased scar formation. (E) Staining of livers for Malondialdehyde (MDA) from WT and stabilin-1$^{-/-}$ mice after CCl_4 injury (F) Quantification of MDA positive area staining in WT and stabilin-1$^{-/-}$ mice after CCl_4 injury. (n=3-4 mice in each group) Representative high magnification fields in black box areas. (G) Autofluorescent ceroid aggregates (red) co-stained with F4/80 (green) in WT and stabilin-1$^{-/-}$ mice after CCl_4 injury. (H) Quantification of ceroid staining within F4/80^{+} cells in livers from WT and stabilin-1 $^{-/-}$ mice after CCl_4 injury and MCD diet. (n=5 mice in each group). (I) Immunofluorescent staining of livers from WT mice after CCl_4 injury double stained for F4/80 (green), stabilin-1 (orange) and autofluorescent ceroid (red). (J) Measurement of serum MDA levels using TBARS assay in WT and stabilin-1$^{-/-}$ mice after CCl_4 injury and MCD diet-induced injury (n=4 mice in each

group). Statistical significance was determined by unpaired *t*-test (C,F,H,J) * P<0.05, ** P<0.005, ***P<0.001. Bars 100 μm (A,B), 200 μm (D,E), 50 μm (G), 10 μm (I).

Figure 3

Loss of ceroid-laden macrophages is associated with exacerbation of hepatic scarring.

(A,B) Immunofluorescent staining of WT and stabilin-$1^{-/-}$ mice after CCl_4 injury and (C,D) 4 weeks resolution for $F4/80^+$ cells (green) and Collagen III (red). Right hand panels are magnification of inset boxes in left hand panels (arrows highlight ceroid-laden macrophages). Bars 200 μm (A-D).

Figure 4

Stabilin-1 deficiency is associated with increased intrahepatic CCL3. Wild-type (WT) and stabilin-1-deficient (stab-$1^{-/-}$) mice were subjected to CCl_4-induced liver injury: control (Oil); 8 week CCl_4 injury (CCl_4) and 4 week resolution after the CCl_4 injury (Res). (A) mRNA expression of CCL3 in livers (n=5-7 mice in each group). (B) Immunofluorescent staining of WT and stabilin-$1^{-/-}$ mice in control (Oil) livers of $F4/80^+$ cells (red) and CCL3 (green) and (C) quantification of CCL3 staining (n=3 in each group for B,C). (D) High magnification image of co-staining for $F4/80^+$ cells (red) and CCL3 (green) from stabilin-1-deficient (stabilin-$1^{-/-}$) liver. Statistical significance was determined by unpaired *t*-test (A,C) *P<0.05, ** P<0.005, Bars 50 μm (B), 5 μm (D).

Figure 5

Stabilin-1 suppresses CCL3 expression during Malondialdehyde-LDL uptake by macrophages.

(A) Immunofluorescent staining of IL-4/Dex cultured human monocytes pre-treated with isotype control and or (B) Stabilin-1 function blocking antibody (3-372) followed by incubation with MDA-LDL (10 μg/ml) for 2 h. Representative images from three separate cell isolates. (C) Comparison of mRNA expression of CCL3 in IL-4/Dex cultured human monocytes (control) and those exposed to MDA-LDL for 24h. (D) Comparison of mRNA expression of CCL3 in IL-4/Dex cultured human monocytes exposed to MDA-LDL for 24hours, pre incubated with IgG1 antibody (control; 10 μg/ml) or 3-372 antibody (Stab-1 blocking antibody; 10 μg/ml) (n=3 independent experiments). Statistical significance was determined by a paired t-test. *P<0.05, ***P<0.005. Bar 10 μm (A,B),

Figure 6

Stabilin-1 deficiency on macrophages leads to increased ECM deposition. Wild-type (WT) and macrophage stabilin-1-deficient (MACRO stab-$1^{-/-}$) mice were subjected to (A-H) CCl_4-induced liver injury: control (Oil); 8 week CCl_4 injury (CCl_4) and 4 week resolution after the CCl_4 injury (Res). (A,B) Sirius red stainings in livers. (C-F) Collagen I, Collagen III and αSMA immunostainings and quantifications of liver sections. (G) mRNA expression of αSMA and CCL3 in livers, (for A-G n=4-6 mice in each group). (H) Hydroxyproline determinations in liver samples (n=5-6 mice in each group) and serum alanine transaminase (ALT) levels (n=4-5 mice in each group). Statistical significance was determined by unpaired *t*-test (E) and 1-way ANOVA analysis, with a Tukey's *post-hoc* multiple comparison test (G, H). * P<0.05, ***P<0.001. Bars 200 μm (A-D). Bars 50 μm (F).

524 **Figure 1**

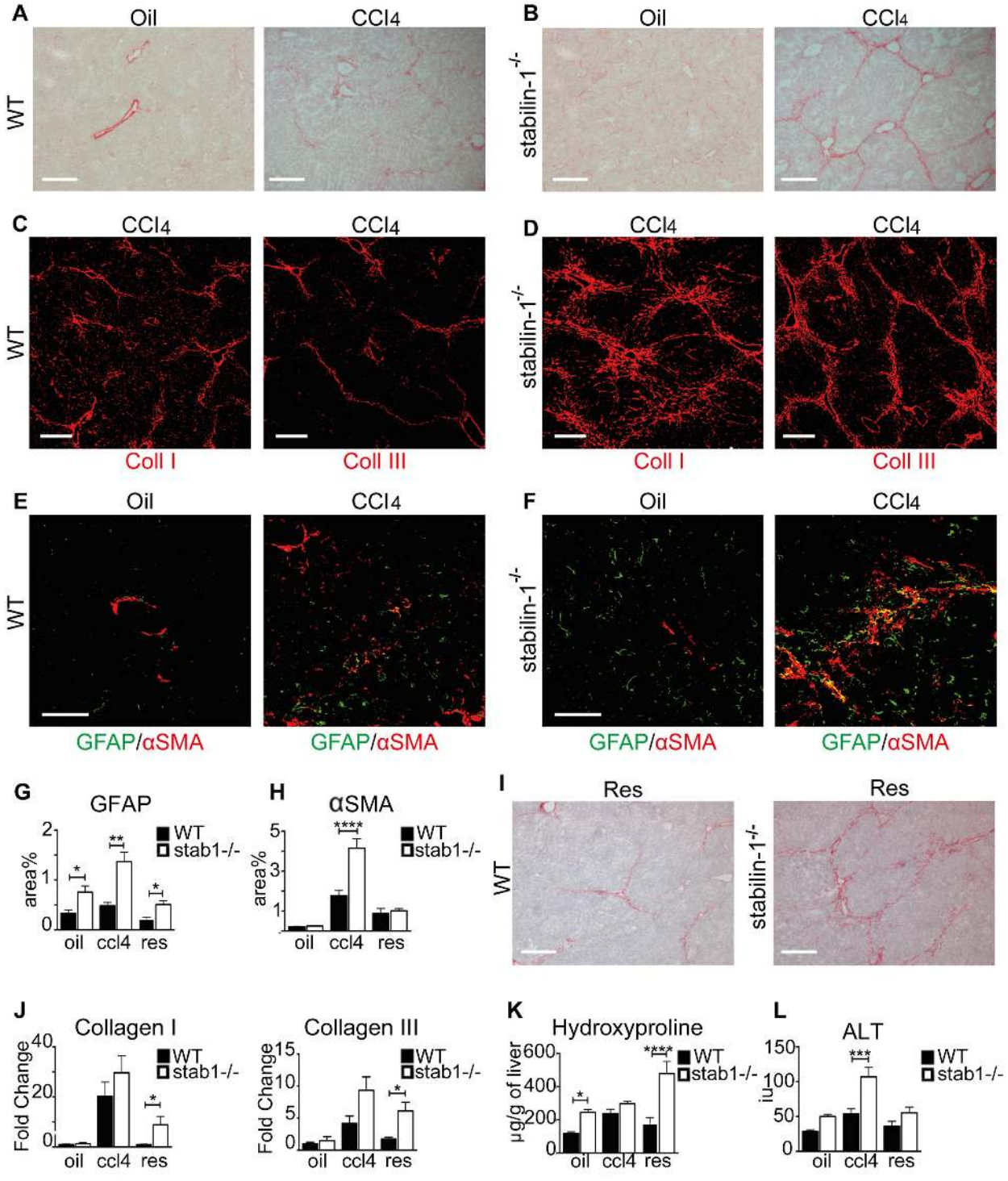

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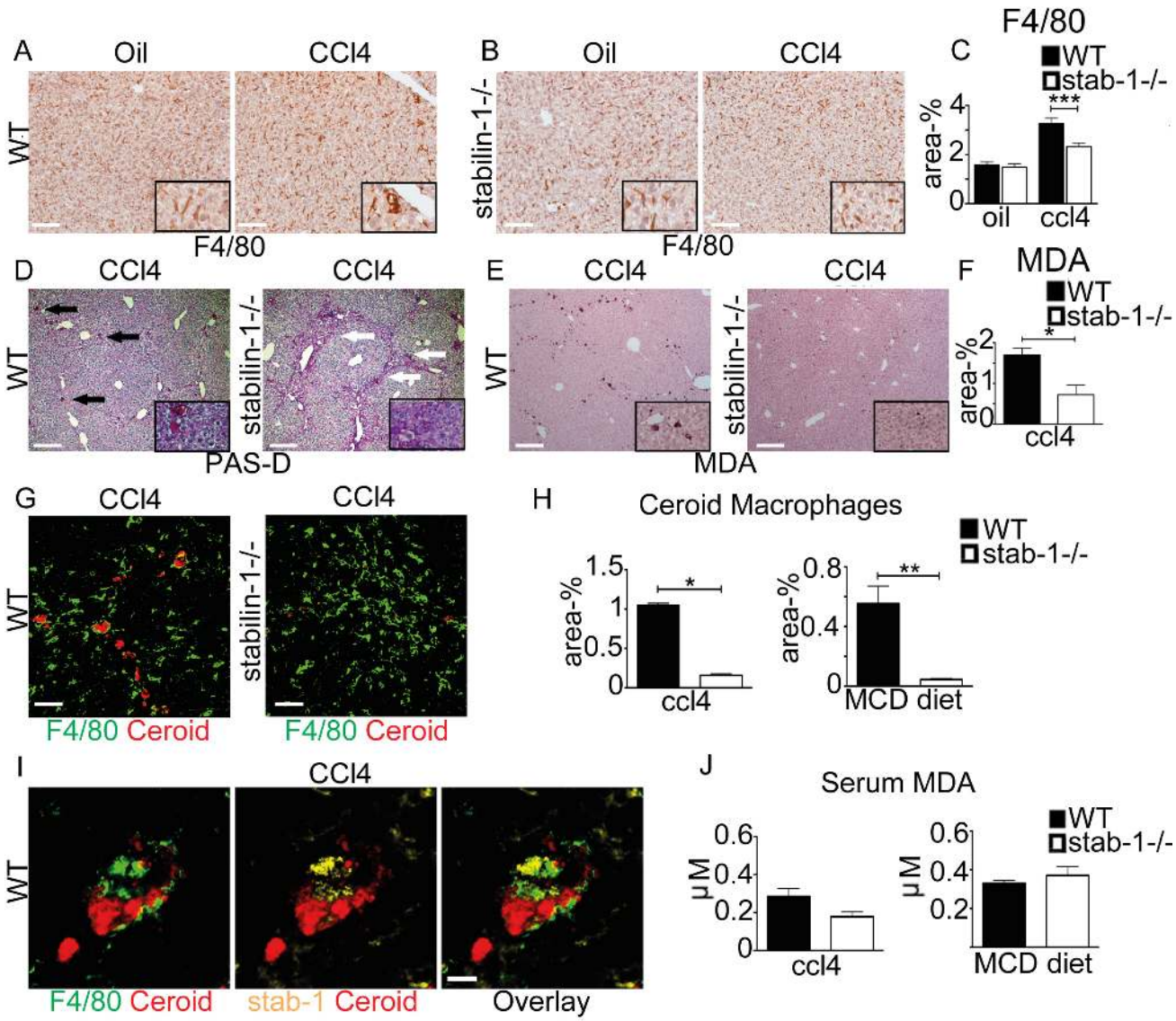
Figure 2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stab-1-/-
***
area-%
oil
ccl4
D
CCl4
CCl4
WT
stabilin-1-/-
PAS-D
E
CCl4
CCl4
WT
stabilin-1-/-
MDA
F
MDA
WT
stab-1-/-
*
area-%
ccl4
G
CCl4
CCl4
WT
stabilin-1-/-
F4/80 Ceroid
F4/80 Ceroid
H
Ceroid Macrophages
WT
stab-1-/-
*
**
area-%
ccl4
MCD diet
I
CCl4
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Overlay
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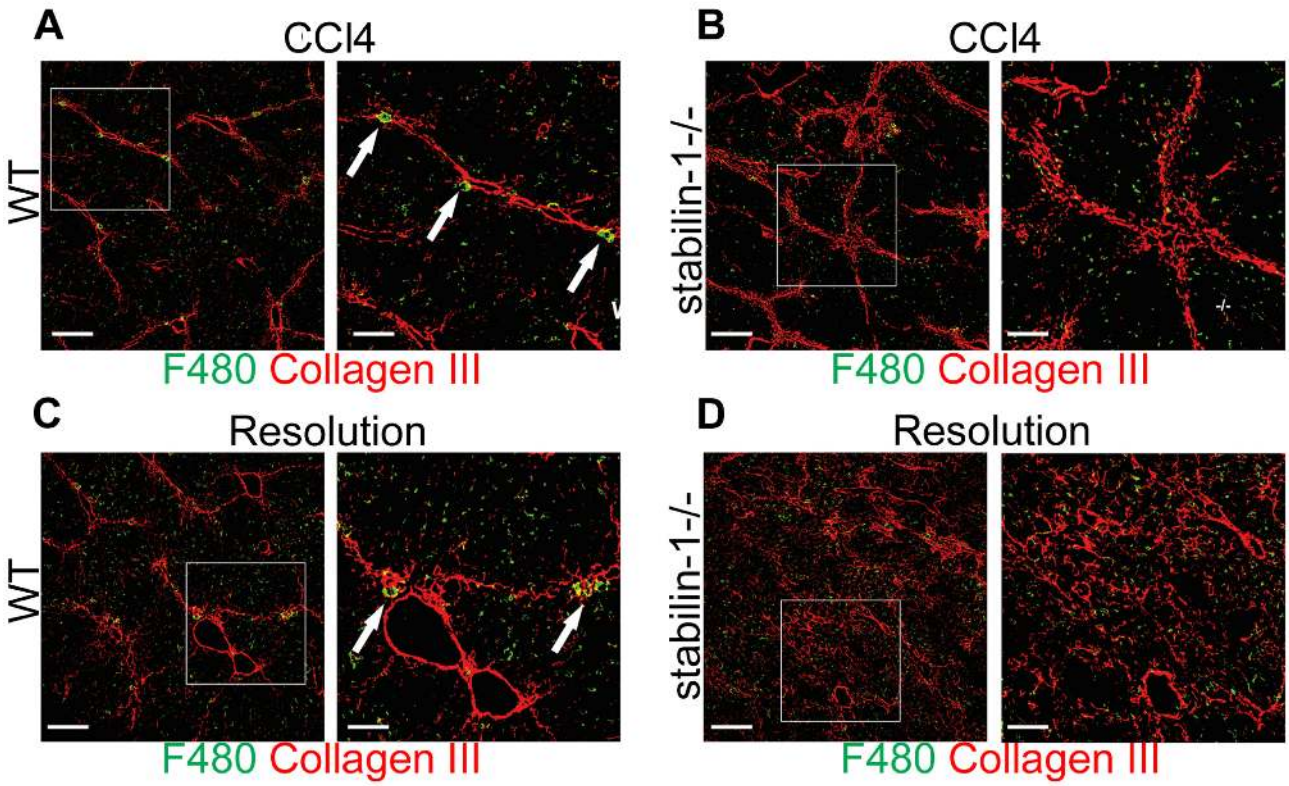
Serum MDA
WT
stab-1-/-
μM
ccl4
MCD diet

Figure 3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Resolution
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D
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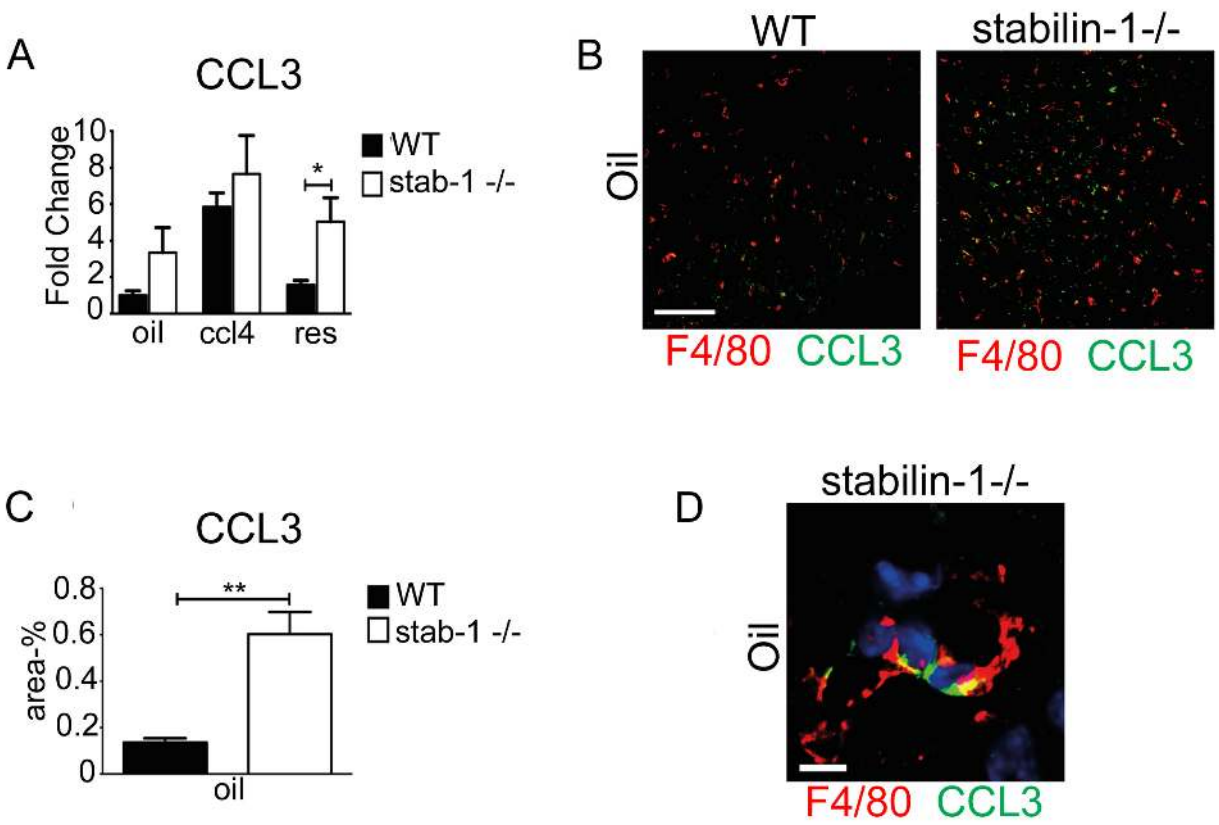
F480 Collagen III

Figure 4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4
2
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res
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stabilin-1-/-
Oil
F4/80 CCL3
F4/80 CCL3
C
CCL3
area-%
0.8
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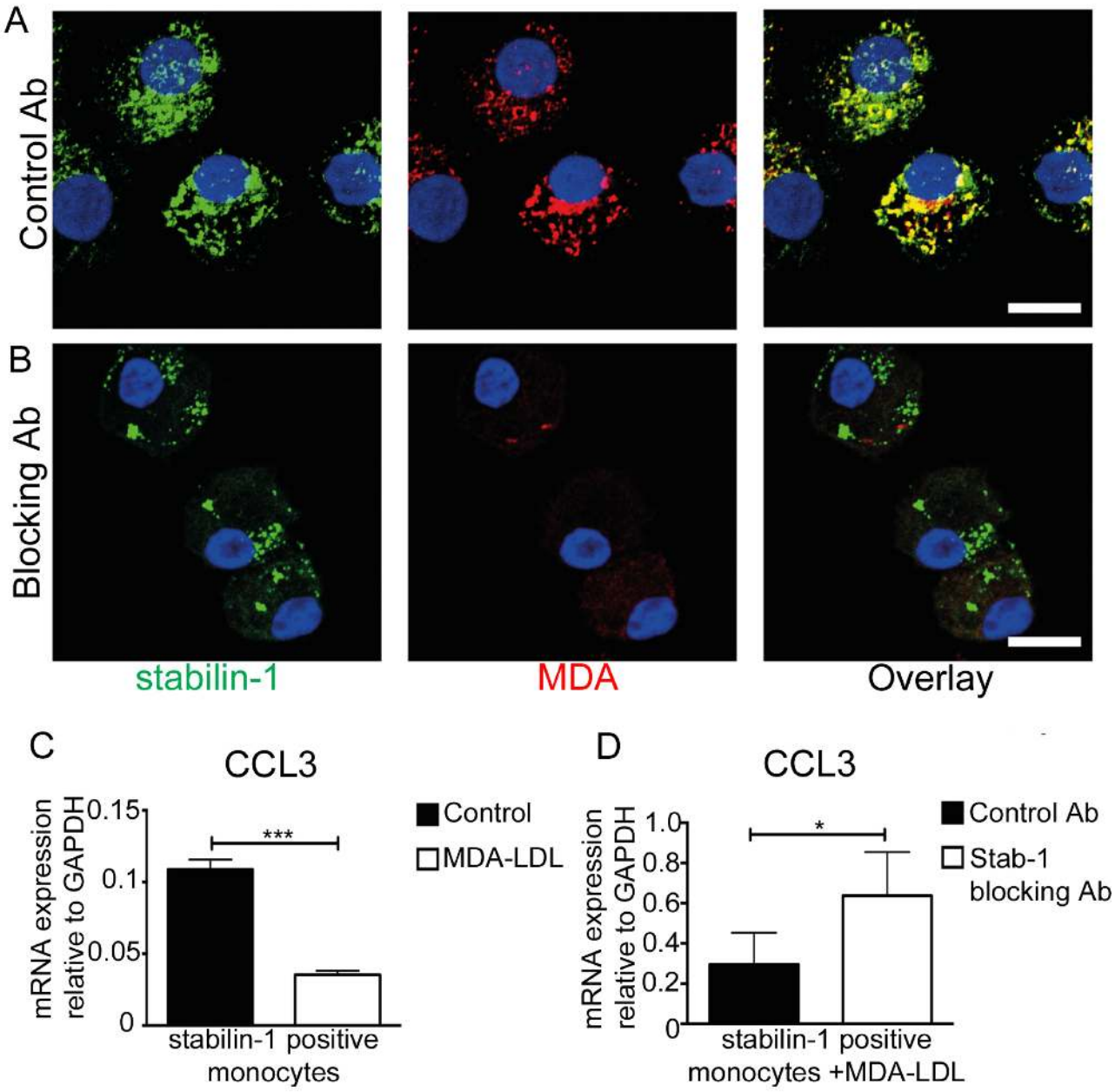
0
**
oil
WT
stab-1 -/-
D
stabilin-1-/-
Oil
F4/80 CCL3

Figure 5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0
***
Control
MDA-LDL
stabilin-1 positive monocytes
D
CCL3
mRNA expression relative to GAPDH
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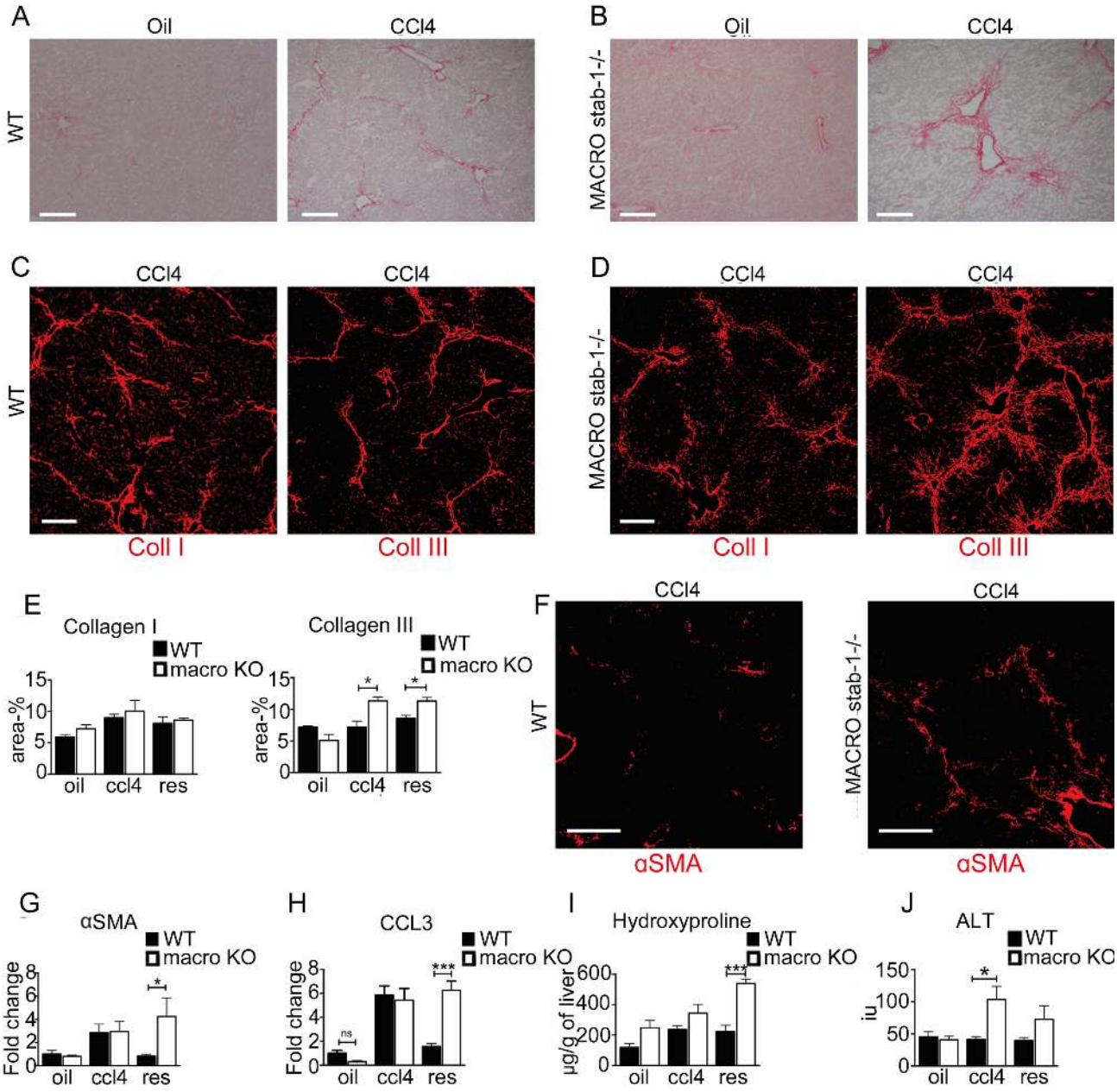
0.4
0.2
0
*
Control Ab
Stab-1 blocking Ab
stabilin-1 positive monocytes +MDA-LDL

Figure 6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CCl4
CCl4
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Coll I
Coll III
E
Collagen I
WT
macro KO
area-%
oil
ccl4
res
Collagen III
F
CCl4
CCl4
WT
MACRO stab-1-/-
αSMA
αSMA
G
αSMA
Fold change
H
CCL3
ns
I
Hydroxyproline
µg/g of liver
J
ALT
iu