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

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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 that 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 profibrogenic chemokine CCL3 and an increase in GFAP$^+$ fibrogenic cells. Stabilin-1$^{-/-}$ macrophages demonstrated a proinflammatory 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 internalized MDA-LDL and this suppressed their ability to secrete CCL3, suggesting that loss of stabilin-1 removes a brake to CCL3 secretion. Experiments with cell-lineage–specific knockouts revealed that stabilin-1 expression in myeloid cells is required for the induction of this subset of macrophages and that increased fibrosis occurs in their absence. This study demonstrates a previously unidentified 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.

stabilin-1 | liver | fibrosis | macrophages | CCL3

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 that leads to organ dysfunction and portal hypertension. Consequently, identifying pathways that regulate the deposition and resolution of fibrillar collagen is vital to developing new treatments for chronic liver disease. Products of oxidative stress, such as oxidized-low density lipoproteins (oxLDLs), can directly activate HSC to an ECM-producing state thereby driving fibrogenesis (2, 3). In addition to HSCs, other nonparenchymal cells contribute to the regulation of liver fibrosis. Hepatic sinusoidal endothelial cells (HSECs), 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 subpopulations 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).

Significance

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 pathway 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 profibrogenic 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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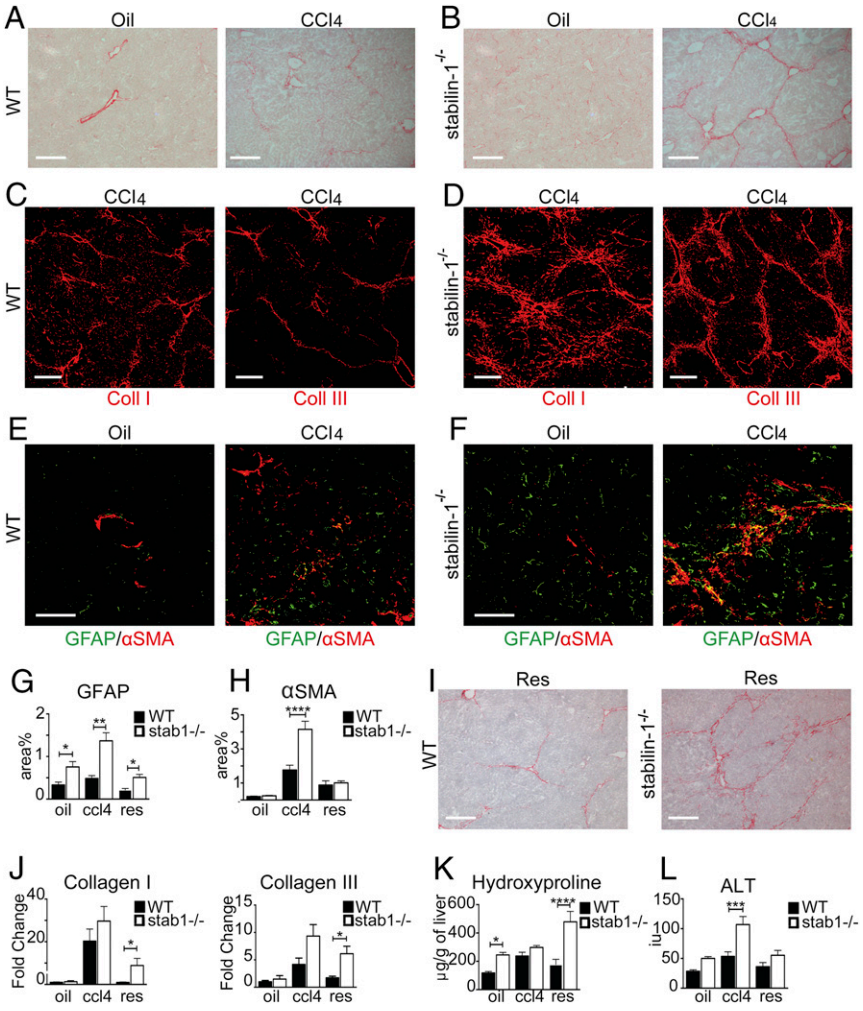

Fig. 1. Stabilin-1 deficiency exacerbates fibrosis after CCl_4 liver injury. WT and stabilin-1–deficient (stabilin-$1^{-/-}$) mice were subjected to CCl_4-induced liver injury: control (oil); 8 wk CCl_4 injury (CCl_4) and 4-wk resolution after the CCl_4 injury (Res). (*A* and *B*) Sirius red stainings in livers (n = 4–5 in each group). (*C–H*) Collagens I and III immunostainings and GFAP/α-SMA coimmunostainings and quantifications of liver sections (n = 3–5 in each group). (*I*) Sirius red staining from livers after resolution. (*J*) mRNA expression of collagens I and III in livers. (*K*) Hydroxyproline determinations in liver samples and (*L*) serum ALT levels (for *J–L*, n = 5–6 mice in each group). Statistical significance was determined by unpaired t test (*G*, *H*, and *J*) and one-way ANOVA analysis, with Tukey's post hoc multiple comparison test (*K* and *L*). $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$, $^{****}P < 0.0005$. [Scale bars, 200 μm (*A–D* and *I*) and 50 μm (*E* and *F*).]

Consistent with previous reports in stabilin-$1^{-/-}$ mice, sirius red positive fibers consistent with increased fibrogenesis were seen within the liver parenchyma, which was otherwise normal (Fig. 1 *A* and *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. 1 *A* and *B*) and increased sirius red staining (*SI Appendix*, Fig. S1*A*). Greater activation of fibrogenic pathways in stabilin-$1^{-/-}$ mice was confirmed by significantly increased accumulation of collagen I and III (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S1 *B* and *C*). 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 (α-SMA) 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. 1 *E–H*). In contrast α-SMA and MMP-2 expression was significantly higher only during injury (Fig. 1 *E–H* and *SI Appendix*, Fig. S1 *D* and *E*) compared with WT mice.

The role of stabilin-1 in the resolution of fibrosis was studied in mice during recovery after CCl_4 injury. After 4 wk of recovery, we noted persistent bridging fibrosis and increased collagens I and III transcription in stabilin-$1^{-/-}$ mice compared with WT mice (Fig. 1 *I* and *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. 1*K*). After 4 wk of resolution, the hydroxyproline had reduced in WT mice but increased further in stabilin-$1^{-/-}$ mice (Fig. 1*K*). We also noted that stabilin-$1^{-/-}$ mice demonstrated significantly increased alanine transaminase (ALT) levels during liver injury (Fig. 1*L*). To assess whether elevated ALT levels were due to increased hepatocyte death or inefficient scavenging, we costained for ALT and F4/80 and assessed markers of cell death. We found increased coexpression of ALT and F4/80 in WT mice compared with 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 wk of CCl_4 (*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 with 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 $CD31^+$ sinusoidal endothelial cells and was absent from $F4/80^+$ Kupffer cells (*SI Appendix*, Fig. S4 *A* and *B*). In response to liver injury from either CCl_4 or MCD diet, we detected a subset of stabilin-1^+ $F4/80^+$ intrahepatic macrophages

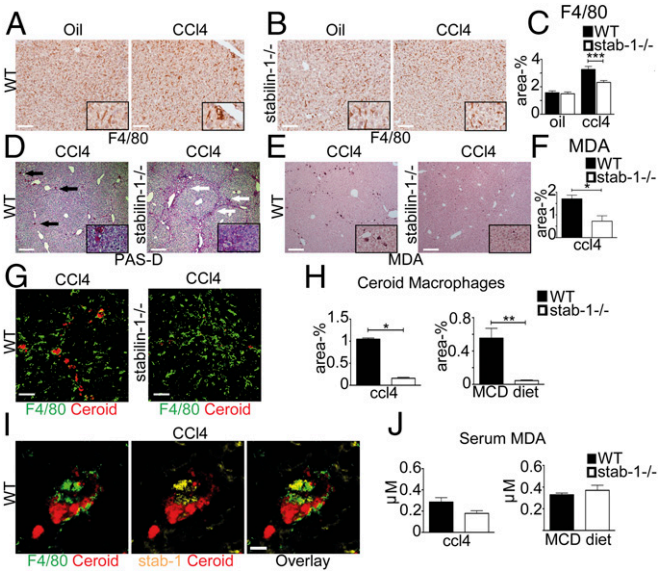

Fig. 2. Stabilin-1 deficiency is associated with a reduction of ceroid-laden macrophages during liver injury. (*A* and *B*) Staining of livers for F4/80 from WT and stabilin-1$^{-/-}$ mice in oil controls and CCl_4 injury. Arrows indicate aggregates of F4/80$^+$ cells. (*C*) Quantification of F4/80$^+$ area staining in WT and stabilin-1$^{-/-}$ mice in oil controls and CCl_4 injury. (*D*) PAS-D staining of livers from WT and stabilin-1$^{-/-}$ mice after CCl_4 injury (black arrows highlight PAS-D$^+$ cells and white arrows highlight areas of increased scar formation. (*E*) Staining of livers for MDA from WT and stabilin-1$^{-/-}$ mice after CCl_4 injury. (*F*) Quantification of MDA$^+$ 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) costained 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*, and *J*) $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$. [Scale bars, 100 μm (*A* and *B*), 200 μm (*D* and *E*), 50 μm (*G*), and 10 μm (*I*).]

(*SI Appendix*, Figs. S4*C* and S5*A*). Using α-SMA 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*, Figs. S4*D* and S5*B*).

Analysis of chronic human liver disease demonstrated that CD31$^+$ sinusoidal endothelial cells expressed stabilin-1, whereas CD68$^+$ Kupffer cells within the sinusoids did not (*SI Appendix*, Fig. S4 *E* and *F*). In contrast, stabilin-1$^+$ macrophages were readily detected within the fibrous septa associated with the hepatic scar (*SI Appendix*, Fig. S4*G*). Stabilin-1 was not detected on α-SMA$^+$ myofibroblasts (*SI Appendix*, Fig. S4*H*). These studies demonstrate for the first time to our knowledge 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 blood monocyte numbers were comparable between uninjured WT and stabilin-1$^{-/-}$ mice (Fig. 2 *A* and *B* and *SI Appendix*, Fig. S9*A*). After CCl_4 injury, WT mice demonstrated prominent aggregates of macrophages, which were not seen in the stabilin-1$^{-/-}$ animals (Fig. 2 *A*–*C*). These macrophage aggregates resembled ceroid-laden macrophages. Ceroid-laden macrophages contain lipid-like pigment deposits and have been previously described in experimental liver fibrosis but their pathological significance is unknown (17, 18).

We hypothesized that stabilin-1 deficiency was associated with a reduction in ceroid-laden macrophage formation during liver injury and fibrosis. To test this hypothesis, we used periodic acid-schiff diastase (PAS-D) staining to detect cytoplasmic ceroid accumulation. This confirmed the presence of large ceroid macrophages in WT mice (Fig. 2*D*) that were largely absent in stabilin-1$^{-/-}$ animals (Fig. 2*D*). Interestingly, PAS-D also stains the fibrous scar, and in the stabilin-1$^{-/-}$ mice the absence of ceroid-laden macrophages was associated with prominent fibrosis (Fig. 2*D*).

MDA is the most abundant aldehyde produced by lipid peroxidation and is highly reactive, leading to the formation of MDA-modified LDL. This product of oxidative stress is taken up by macrophages and contributes to cytoplasmic ceroid accumulation (19). In WT mice, prominent MDA$^+$ cells were clearly visible (Fig. 2*E*), whereas minimal staining was detected in stabilin-1$^{-/-}$ mice (Fig. 2 *E* and *F*).

A further well-established property of ceroid accumulation is that the pigment, including MDA adducts, is autofluorescent (20). We identified prominent autofluorescent F4/80$^+$ aggregates in WT mice, which were greatly reduced in stabilin-1$^{-/-}$ mice in both CCl_4- and MCD diet-induced liver injury (Fig. 2 *G* and *H*). We then showed that F4/80$^+$ ceroid-laden macrophages in experimental liver injury in WT mice coexpress stabilin-1 (Fig. 2*I*).

We used a thiobarbituric acid reactive substances (TBARS) assay to detect serum MDA 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. 2*J*). Proteomic analysis of baseline serum samples demonstrated increased levels of several proteins in the serum of stabilin-1$^{-/-}$ mice compared with WT mice (*SI Appendix*, Table S1), several of which are related to oxidative stress. However, we did not detect increased circulatory levels of proinflammatory or profibrogenic cytokines such as TGF-β and 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. 3*A*), whereas in stabilin-1$^{-/-}$ animals, the absence of ceroid macrophages was associated with a dense network of collagen III in the hepatic scar (Fig. 3*B*). Further analysis of livers after 4 wk of resolution demonstrated the persistence of ceroid-laden macrophages in close proximity to residual hepatic scar in WT mice (Fig. 3*C*). In contrast, ceroid-laden macrophages were

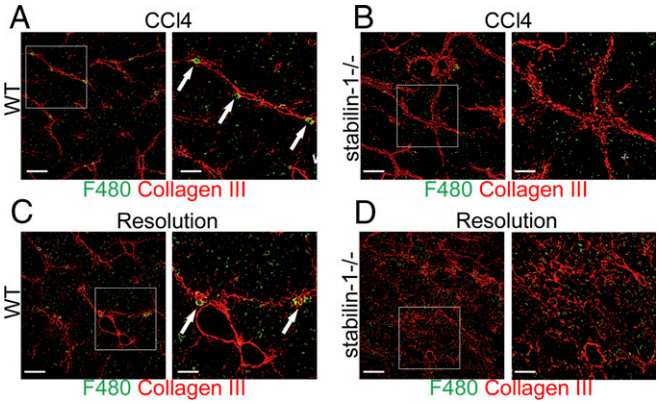

Fig. 3. Loss of ceroid-laden macrophages is associated with exacerbation of hepatic scarring. (*A* and *B*) Immunofluorescent staining of WT and stabilin-1$^{-/-}$ mice after CCl_4 injury and (*C* and *D*) 4-wk resolution for F4/80$^+$ cells (green) and collagen III (red). (*Right*) Magnification of *Inset* boxes on *Left* (arrows highlight ceroid-laden macrophages). [Scale bars, 200 μm (*A*–*D*).]

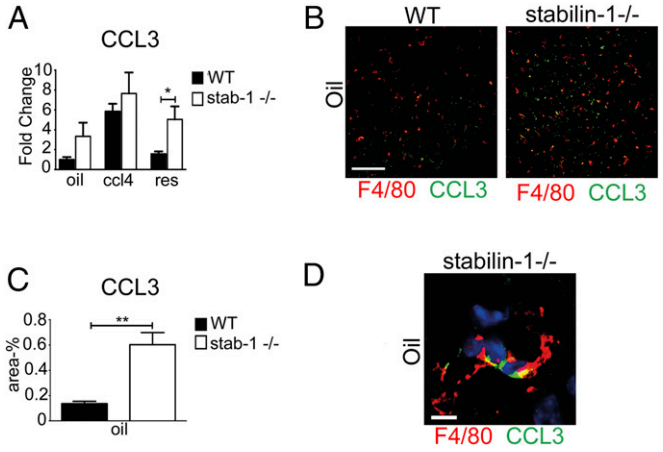

Fig. 4. Stabilin-1 deficiency is associated with increased intrahepatic CCL3. WT and stabilin-1–deficient (stab-$1^{-/-}$) mice were subjected to CCl_4-induced liver injury: control (oil); 8-wk CCl_4 injury (CCl_4) and 4-wk resolution after the CCl_4 injury (Res). (*A*) mRNA expression of CCL3 in livers (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* and *C*). (*D*) High-magnification image of costaining for F4/80$^+$ cells (red) and CCL3 (green) from stabilin-1–deficient (stabilin-$1^{-/-}$) liver. Statistical significance was determined by unpaired t test (*A* and *C*) $*P < 0.05$, $**P < 0.005$. [Scale bars, 50 μm (*B*) and 5 μm (*D*).]

not seen in stabilin-$1^{-/-}$ mice, in which extensive collagen-rich scars persisted after 4 wk of resolution (Fig. 3*D*). 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 sequencing (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 that 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 4 wk of resolution in stabilin-$1^{-/-}$ mice (Fig. 4*A*). In support of these findings we detected increased CCL3 protein in stabilin-$1^{-/-}$ mice, which colocalized with hepatic macrophages (Fig. 4 *B–D*).

We investigated whether the profibrogenic phenotype in livers of stabilin-$1^{-/-}$ mice was linked to ox-LDL uptake to form ceroid-laden macrophages. We initially confirmed the presence of autofluorescent stabilin-1^+ ceroid-laden macrophages in livers from patients with chronic liver diseases (*SI Appendix*, Fig. S6 *A* and *B*).

As MDA-LDL is one of the most abundant products of lipid peroxidation and contributes to ceroid formation (23), we investigated whether stabilin-1 on human macrophages is involved in its uptake and degradation. We isolated monocytes from human peripheral blood and stimulated their stabilin-1 expression in vitro (24). After 2 h of MDA-LDL incubation, we were able to detect intracellular deposits of MDA-LDL completely colocalized with stabilin-1 (*SI Appendix*, Fig. S7*A*). Uptake of MDA-LDL was minimal in unstimulated monocytes that had low expression of stabilin-1 (*SI Appendix*, Fig. S7*B*). A function-blocking antibody to stabilin-1 led to the internalization of stabilin-1, thereby inhibiting MDA-LDL uptake (Fig. 5 *A* and *B*). We also linked the scavenging function of stabilin-1^+ macrophages to CCL3 expression by demonstrating a significant reduction in macrophage CCL3 transcription after MDA-LDL uptake compared with control (Fig. 5*C*). We could reverse this effect using the function-blocking antibody against stabilin-1 (Fig. 5*D*). These results suggest that stabilin-1 expression is critical for the uptake of MDA-LDL by macrophages, a process that also modulates macrophage-derived CCL3 secretion.

Stabilin-1 Deficiency Is Associated with a Proinflammatory Hepatic Macrophage Phenotype. To assess how stabilin-1 deficiency influenced macrophage phenotype, we sorted 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 with the F4/80^{lo}/CD11b^{hi} population (*SI Appendix*, Fig. S8*A*). The loss of stabilin-1 led to higher expression of M1 markers CCL3 and TNF-α 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. S8 *B–E*). This is in keeping with previous studies where defining macrophages through their polarization 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 "profibrotic" 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. S8 *F* and *H*). During resolution, there was a shift toward Ly6C^{lo} macrophages in the WT group, which was not seen in the stabilin-$1^{-/-}$ mice (*SI Appendix*, Fig. S8*G*). Intrahepatic lymphocyte populations

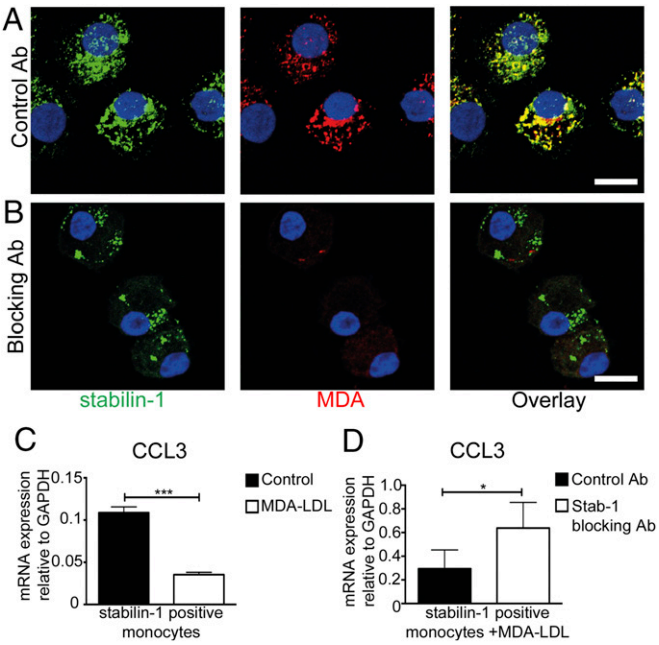

Fig. 5. Stabilin-1 suppresses CCL3 expression during MDA-LDL uptake by macrophages. (*A*) Immunofluorescent staining of IL-4/dexamethasone (Dex) cultured human monocytes pretreated with isotype control and or (*B*) stabilin-1 function-blocking antibody (clone 3-372) followed by incubation with MDA-LDL (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 24 h. (*D*) Comparison of mRNA expression of CCL3 in IL-4/Dex cultured human monocytes exposed to MDA-LDL for 24 h, preincubated 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. $*P < 0.05$, $***P < 0.005$. [Scale bar, 10 μm (*A* and *B*).]

did not differ between WT and stabilin-1$^{-/-}$ mice after the injury or during resolution (*SI Appendix*, Fig. S9 *B–K*). Collectively, these results demonstrate that the profibrogenic response in stabilin-1 deficiency is associated with a proinflammatory 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 profibrogenic 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, because 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. S10 *A* and *B*).

Fibrosis in ENDO stab-1$^{-/-}$ mice was comparable to WT mice in both CCl_4 liver injury and MCD diet (*SI Appendix*, Fig. S11 *A–C*), whereas fibrogenesis was increased in MACRO stab-1$^{-/-}$ animals (Fig. 6 *A* and *B*) with significantly more accumulation of collagen III associated with increased α-SMA staining in MACRO stab-1$^{-/-}$ mice versus WT after CCl_4 injury (Fig. 6 *C–F*). Fibrosis resolution was delayed in the MACRO stab-1$^{-/-}$ animals, as demonstrated by persistently elevated transcript levels of α-SMA and increased hydroxyproline staining, and critically for our hypothesis, we found persistent elevation of CCL3 compared with WT animals (Fig. 6 *G–I*) and increased serum ALT levels in response to CCl_4 as seen in the full knockout (Fig. 6*J*). We also detected significantly elevated hydroxyproline content in MACRO stab-1$^{-/-}$ mice compared with WT mice after the MCD diet (*SI Appendix*, Fig. S11*D*). 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. S11 *E* and *F*). In contrast, very few ceroid-laden macrophages were seen in the livers of CCl_4-treated MACRO stab-1$^{-/-}$ animals (*SI Appendix*, Fig. S11 *E* and *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-wk model of CCl_4 liver injury performed in MACRO stab-1$^{-/-}$ and WT mice followed by adoptive transfer of WT myeloid cell elements or vehicle control at 24 and 72 h after the final injection of CCl_4 followed by an analysis of fibrogenesis at 120 h. 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. S12 *A*, *D*, and *F*), whereas in the MACRO stab-1$^{-/-}$, there was a trend of reduced transcription in nearly all fibrogenic markers in mice receiving WT myeloid cells (*SI Appendix*, Fig. S12*B*). We confirmed the presence of adoptively transferred Dsred^{+} myeloid cells within liver tissue at sites of collagen deposition (*SI Appendix*, Fig. S12*C*). Finally, we demonstrated a significant reduction in hepatic collagen III expression in MACRO stabilin-1$^{-/-}$ mice receiving WT myeloid cells compared with vehicle control (*SI Appendix*, Fig. S12 *E* and *F*).

Discussion

This study reports a mechanism that 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

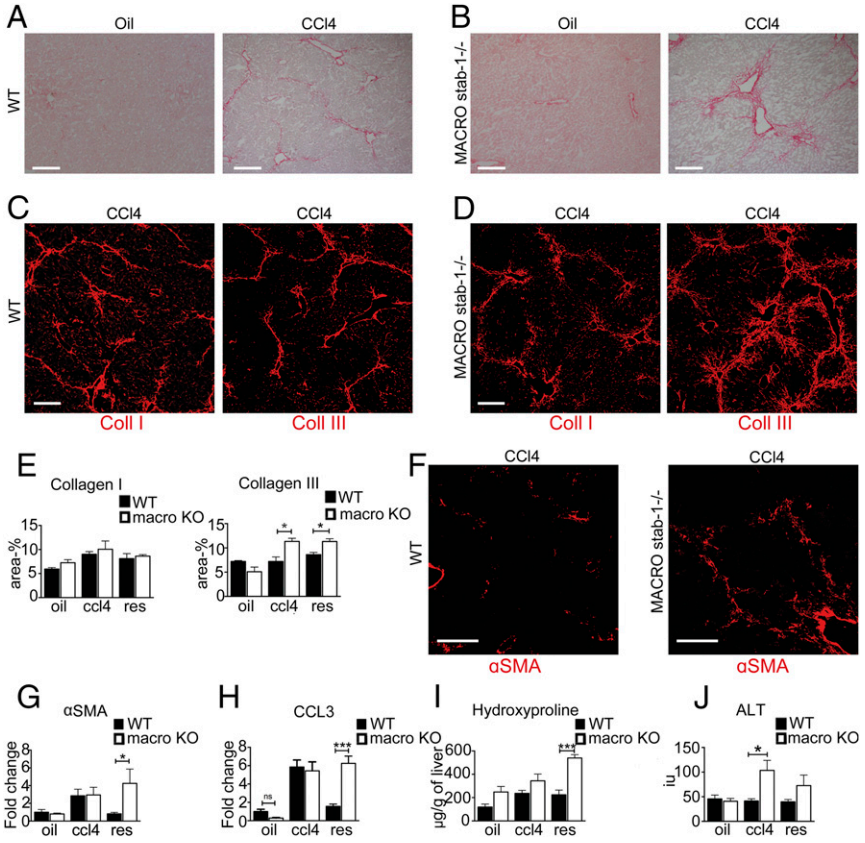

Fig. 6. Stabilin-1 deficiency on macrophages leads to increased ECM deposition. WT and macrophage stabilin-1–deficient (MACRO stab-1$^{-/-}$) mice were subjected to (*A–J*) CCl_4-induced liver injury: control (oil); 8-wk CCl_4 injury (CCl_4), and 4-wk resolution after the CCl_4 injury (Res). (*A* and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

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 oxidized 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-recognized 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 up-regulate 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 profibrogenic 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 up-regulate 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 WT myeloid cells can promote resolution of fibrosis in stabilin-1 deficiency. These results suggest that macrophages that 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 pathway 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. However, in response to liver injury, protection and resolution require the involvement of stabilin-1$^+$ macrophages. These findings describe a previously unidentified pathway 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 license no. 5587/04.10.07/2014).

Liver Injury Models.

CCl_4 injury model. The 8-wk-old mice were injected twice weekly for 8 wk 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 killed 72 h after the final dose of CCl_4 (27) or after a 4-wk recovery period.

MCD diet. Mice were fed an MCD diet (Harlan Laboratories, TD90262) for 6 wk ad libitum. Control animals received normal chow for 6 wk (27).

Human Tissue. Tissue and blood samples from patients were obtained with written informed consent and with Local Research Ethics Committee approval (reference nos. 06/Q2702/61 South Birmingham, Birmingham, UK and 04/Q2708/41 South Birmingham).

For statistical analysis, see *SI Appendix, SI Methods*.

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1. Kocabayoglu P, Friedman SL (2013) Cellular basis of hepatic fibrosis and its role in inflammation and cancer. *Front Biosci (Schol Ed)* 5:217–230.
2. Schneiderhan W, et al. (2001) Oxidized low-density lipoproteins bind to the scavenger receptor, CD36, of hepatic stellate cells and stimulate extracellular matrix synthesis. *Hepatology* 34(4 Pt 1):729–737.
3. Kang Q, Chen A (2009) Curcumin suppresses expression of low-density lipoprotein (LDL) receptor, leading to the inhibition of LDL-induced activation of hepatic stellate cells. *Br J Pharmacol* 157(8):1354–1367.
4. Xie G, et al. (2012) Role of differentiation of liver sinusoidal endothelial cells in progression and regression of hepatic fibrosis in rats. *Gastroenterology* 142(4):918–927 e916.
5. Duffield JS, et al. (2005) Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 115(1):56–65.
6. Kzhyshkowska J (2010) Multifunctional receptor stabilin-1 in homeostasis and disease. *ScientificWorldJournal* 10:2039–2053.
7. Li R, et al. (2011) Role of liver sinusoidal endothelial cells and stabilins in elimination of oxidized low-density lipoproteins. *Am J Physiol Gastrointest Liver Physiol* 300(1):G71–G81.
8. Workman G, Sage EH (2011) Identification of a sequence in the matricellular protein SPARC that interacts with the scavenger receptor stabilin-1. *J Cell Biochem* 112(4):1003–1008.
9. Kzhyshkowska J, et al. (2008) Alternatively activated macrophages regulate extracellular levels of the hormone placental lactogen via receptor-mediated uptake and transcytosis. *J Immunol* 180(5):3028–3037.
10. Shetty S, et al. (2011) Common lymphatic endothelial and vascular endothelial receptor-1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal endothelium. *J Immunol* 186(7):4147–4155.
11. Iredale JP (2007) Models of liver fibrosis: Exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 117(3):539–548.
12. Varela-Rey M, et al. (2009) Non-alcoholic steatohepatitis and animal models: Understanding the human disease. *Int J Biochem Cell Biol* 41(5):969–976.
13. Schledzewski K, et al. (2011) Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors. *J Clin Invest* 121(2):703–714.
14. Benyon RC, et al. (1999) Progelatinase A is produced and activated by rat hepatic stellate cells and promotes their proliferation. *Hepatology* 30(4):977–986.
15. Cassiman D, Libbrecht L, Desmet V, Denef C, Roskams T (2002) Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. *J Hepatol* 36(2):200–209.
16. Wynn TA, Barron L (2010) Macrophages: Master regulators of inflammation and fibrosis. *Semin Liver Dis* 30(3):245–257.
17. Fallowfield JA, et al. (2007) Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol* 178(8):5288–5295.
18. Salguero Palacios R, et al. (2008) Activation of hepatic stellate cells is associated with cytokine expression in thioacetamide-induced hepatic fibrosis in mice. *Lab Invest* 88(11):1192–1203.
19. Yamada S, et al. (2001) Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine. *J Lipid Res* 42(8):1187–1196.
20. Seehafer SS, Pearce DA (2006) You say lipofuscin, we say ceroid: Defining autofluorescent storage material. *Neurobiol Aging* 27(4):576–588.
21. Heinrichs D, et al. (2013) The chemokine CCL3 promotes experimental liver fibrosis in mice. *PLoS One* 8(6):e66106.
22. Seki E, et al. (2009) CCR1 and CCR5 promote hepatic fibrosis in mice. *J Clin Invest* 119(7):1858–1870.
23. Esterbauer H, Gebicki J, Puhl H, Jürgens G (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med* 13(4):341–390.
24. Politz O, et al. (2002) Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. *Biochem J* 362(Pt 1):155–164.
25. Ramachandran P, et al. (2012) Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci USA* 109(46):E3186–E3195.
26. Karikoski M, et al. (2014) Clever-1/stabilin-1 controls cancer growth and metastasis. *Clin Cancer Res* 20(24):6452–6464.
27. Weston CJ, et al. (2015) Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis. *J Clin Invest* 125(2):501–520.