Nonalcoholic fatty liver disease (NAFLD) encompasses a range of manifestations, including steatosis and cirrhosis. Progressive disease is characterized by hepatic leukocyte accumulation in the form of steatohepatitis. The adhesion molecule vascular adhesion protein-1 (VAP-1) is a membrane-bound amine oxidase that promotes leukocyte recruitment to the liver, and the soluble form (sVAP-1) accounts for most circulating monoamine oxidase activity, has insulin-like effects, and can initiate oxidative stress. Here, we determined that hepatic VAP-1 expression is increased in patients with chronic liver disease and that serum sVAP-1 levels are elevated in patients with NAFLD compared with those in control individuals. In 4 murine hepatic injury models, an absence or blockade of functional VAP-1 reduced inflammatory cell recruitment to the liver and attenuated fibrosis. Moreover, disease was reduced in animals expressing a catalytically inactive form of VAP-1, implicating enzyme activity in the disease pathogenesis. Within the liver, hepatic stromal cells expressed functional VAP-1, and evaluation of cultured cells revealed that sVAP-1 promotes leukocyte migration through catalytic generation of ROS, which depended on VAP-1 enzyme activity. VAP-1 enhanced stromal cell spreading and wound closure and modulated expression of profibrotic genes. Together, these results link the amine oxidase activity of VAP-1 with hepatic inflammation and fibrosis and suggest that targeting VAP-1 has therapeutic potential for NAFLD and other chronic fibrotic liver diseases.
Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis

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Nonalcoholic fatty liver disease (NAFLD) encompasses a range of manifestations, including steatosis and cirrhosis. Progressive disease is characterized by hepatic leukocyte accumulation in the form of steatohepatitis. The adhesion molecule vascular adhesion protein-1 (VAP-1) is a membrane-bound amine oxidase that promotes leukocyte recruitment to the liver, and the soluble form (sVAP-1) accounts for most circulating monoamine oxidase activity, has insulin-like effects, and can initiate oxidative stress. Here, we determined that hepatic VAP-1 expression is increased in patients with chronic liver disease and that serum sVAP-1 levels are elevated in patients with NAFLD compared with those in control individuals. In 4 murine hepatic injury models, an absence or blockade of functional VAP-1 reduced inflammatory cell recruitment to the liver and attenuated fibrosis. Moreover, disease was reduced in animals expressing a catalytically inactive form of VAP-1, implicating enzyme activity in the disease pathogenesis. Within the liver, hepatic stromal cells expressed functional VAP-1, and evaluation of cultured cells revealed that sVAP-1 promotes leukocyte migration through catalytic generation of ROS, which depended on VAP-1 enzyme activity. VAP-1 enhanced stromal cell spreading and wound closure and modulated expression of profibrotic genes. Together, these results link the amine oxidase activity of VAP-1 with hepatic inflammation and fibrosis and suggest that targeting VAP-1 has therapeutic potential for NAFLD and other chronic fibrotic liver diseases.

Introduction

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of liver disease encompassing steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis and is increasingly recognized as the leading cause of liver dysfunction in Western societies. NAFLD is strongly associated with obesity, dyslipidemia, insulin resistance, and diabetes mellitus (1–4) and is considered to be a hepatic manifestation of the metabolic syndrome (5).

Steatosis sensitizes the liver to the induction of inflammation by a second pathogenic insult that promotes oxidative stress and activation of the inflammasome, resulting in steatohepatitis (6, 7). Oxidative stress may occur as a consequence of dietary or environmental factors on the background of the metabolic syndrome (8). Persistent inflammation in response to liver injury is the critical factor that drives progression to fibrosis, cirrhosis, and hepatocellular carcinoma (9). Inflammation at any site, including the liver, is the result of an accumulation of leukocytes organized into an inflammatory infiltrate. For this to occur, leukocytes must be recruited from the circulation with interactions by endothelium and positioned within the tissue (10).

Vascular adhesion protein-1 (VAP-1) is a 170-kDa homodimeric type 2 transmembrane sialoglycoprotein that has a short cytoplasmic tail with no known signal sequence, a single transmembrane segment, and a large extracellular domain (11). VAP-1 is constitutively expressed on human hepatic endothelium and supports lymphocyte adhesion and transendothelial migration across primary hepatic sinusoidal endothelium in vitro and in several models of liver inflammation in vivo (12–15). Cloning of VAP-1 revealed it to be a copper-dependent semicarbazide-sensitive amine oxidase (SSAO) [E.C.1.4.3.6] known as amine oxidase copper-containing 3 (AOC3), which catalyzes the oxidative deamination of exogenous and endogenous primary amines (such as those generated from sarcosine, creatinine, and adrenaline), resulting in the generation of aldehyde, ammonia, and H2O2 (16). These products activate NFκB-dependent chemokine secretion and adhesion molecule expression in liver endothelium (17, 18) and may also initiate and propagate oxidative stress following the conversion of H2O2 to hydroxyl free radicals. VAP-1 is also found in adipocytes and smooth muscle cells, and provision of SSAO substrates to these cells in vitro have insulin-like effects on their metabolism, which is mediated via PI 3-kinase activation by H2O2 (19–21).
A soluble form of VAP-1 (sVAP-1) accounts for nearly all of the circulating amine oxidase activity in humans (22–24). The level of sVAP-1 in serum is regulated in part by changes in plasma insulin (25), and sVAP-1 levels are elevated in a number of inflammatory conditions (23, 26). Our group has previously shown that circulating levels of sVAP-1 are substantially elevated in the serum of patients with several chronic inflammatory liver diseases (alcoholic liver disease, primary biliary cirrhosis, primary sclerosing cholangitis), but not in rheumatoid arthritis or inflammatory bowel disease. VAP-1 is shed from the hepatic vasculature and adipose tissue, and measurement of VAP-1 levels in hepatic and portal veins suggests that the liver is a major source of sVAP-1 (23, 26). Elevated serum sVAP-1 levels have also been reported in patients with diabetes, obesity, and complications of the metabolic syndrome such as atherosclerosis and congestive heart failure (27–31).

These observations led us to propose that VAP-1 may have an important role in the progression of NAFLD and establishment of chronic liver disease (CLD). Here, we show that hepatic expression of VAP-1 is increased in human NAFLD in association with markedly elevated serum levels of sVAP-1 when compared with those in controls matched for age, sex, and metabolic phenotype. We provide evidence that VAP-1 promotes the progression of steatohepatitis, because VAP-1–deficient mice (Aoc3–/–) and WT animals treated with a neutralizing Ab were protected from the development of severe steatohepatitis and onset of fibrosis in 3 murine models of steatohepatitis and a carbon tetrachloride (CCL4) model of fibrosis. In particular, VAP-1 deficiency protects against the accumulation of intrahepatic T cells, NKT cells, and myeloid subsets. Furthermore, through the use of mice expressing catalytically inactive VAP-1 as a consequence of an Aoc3 point mutation knock-in, we demonstrate that the amine oxidase activity of VAP-1 is crucial for the accumulation of these effector immune cells and the establishment of fibrosis. We demonstrate that hepatic stromal cells express VAP-1, which can promote the expression of fibrotic markers and accelerate wound healing. These findings strongly implicate VAP-1 in the pathogenesis of fibrotic liver disease and provide evidence that VAP-1 is a potential therapeutic target in NAFLD and other chronic inflammatory liver diseases.

### Results

Circulating serum [sVAP-1] is significantly elevated in human NAFLD and is independently associated with the presence of liver disease. Because circulating levels of sVAP-1 are significantly elevated in the serum of patients with chronic inflammatory liver diseases and in those with complications of the metabolic syndrome (23, 26–30), we investigated whether sVAP-1 levels in patients with the metabolic syndrome are associated with the presence and severity of NAFLD. We compared serum levels of sVAP-1 in 144 patients with biopsy-proven NAFLD and 74 control patients matched for age and metabolic phenotype but without biochemical or radiological evidence of liver disease (Table 1). The cohorts were well matched for age and BMI. A larger proportion of NAFLD patients were male than in the control cohort, but sVAP-1 levels were higher in females when compared with those in males in both cohorts. An increased number of patients in the NAFLD cohort had hypertension and diabetes, which is unsurprising, given the close association between these conditions and NAFLD. A large proportion of both cohorts had impaired glucose tolerance. Cholesterol levels were similar in each cohort, but those with NAFLD had higher triglyceride levels.

We found that soluble VAP-1 levels were significantly increased in the NAFLD group when compared with those in controls matched for age and metabolic phenotype (median, 837 ng/ml [interquartile range [IQR], 655–1,051 ng/ml] versus 256 ng/ml [IQR, 212–308 ng/ml], P < 0.0001; Figure 1A and Table 1). sVAP-1 levels in the NAFLD cohort correlated with the amine oxidase activity of the patients’ serum (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI73722DS1). Univariate analysis showed that the homeostatic model of insulin resistance (HOME-IR) (r = 0.3, P = 0.03) and diabetic status (r = 0.32, P = 0.0002) were correlated with sVAP-1 in the NAFLD cohort (Supplemental Table 1), whereas fibrosis...
The area under the receiver operating curve (AUROC) to predict histological NASH by serum sVAP-1 in our cohort was 0.82 (95% CI, 0.74–0.90), and a cutoff level of greater than 793.5 ng/ml estimated NASH with a sensitivity of 67.7% and specificity of 89.7% (data not shown).

We determined the clinical and biochemical characteristics of our NAFLD cohort that were independently associated with the presence of significant fibrosis (Kleiner classification F ≥2; ref. 32) on liver biopsy (Figure 1F) using univariate descriptive statistics followed by multiple logistic regression analysis with backward elimination (Table 3 and Supplemental Table 2). This revealed sVAP-1 to be a good predictor of the presence of significant fibrosis (F ≥2) (β coefficient, 0.32, \( P = 0.001 \)). The AUROC to predict significant fibrosis (F ≥2) by serum sVAP-1 was 0.71 (95% CI, 0.62–0.79), and a cutoff level of greater than 1,018 ng/ml estimated F ≥2 with a sensitivity of 45.2% and specificity of 91.5% (data not shown).

Hepatic VAP-1 expression is increased in human CLD. We investigated hepatic expression of VAP-1 in the spectrum of NAFLD by immunohistochemistry (Figure 2A). In normal liver and in patients with simple steatosis, we observed that VAP-1 expression was confined to vascular structures, portal vessels, central veins, and sinusoids. In patients with steatohepatitis, we detected more pronounced VAP-1 staining in septal areas when compared with staining in normal liver tissue or simple steatosis, which was partly associated with neovessels in the hepatic scar (Figure 2A). We extended our study to other end-stage CLDs, namely primary biliary cirrhosis, primary sclerosing cholangitis, and alcoholic liver disease. In all 3 diseases, immunohistochemistry demonstrated increased staining of VAP-1 in fibrotic septa (Figure 2B).

Multicolor confocal microscopy showed that rather than having a predominantly endothelial (CD31+) distribution, VAP-1 stained...
a-SMA+ and CD90+ stromal cells within the NASH liver with markedly increased VAP-1 expression throughout the expanded space of Disse (Figure 2D). In contrast, we did not detect VAP-1 in biliary epithelial cells (EpCAM+) or hepatocytes (CK18+) (Figure 2C).

Stromal cells express VAP-1, which promotes amine oxidase–dependent lymphocyte migration in vitro. To determine whether the VAP-1 protein could be synthesized by stromal cells, we isolated activated hepatic stellate cells (HSCs) from nonfibrotic human tissue and in vivo–activated liver myofibroblasts (aLMFs) from end-stage chronically diseased human liver tissue. Both cell types had detectable VAP-1 protein localized to cytoplasmic vesicles with little membranous expression (Figure 3A), and these cells were capable of de novo synthesis of AOC3 mRNA at levels that were comparable to those in hepatic sinusoidal endothelial cells (HSECs). We observed similar levels in the HSC cell line LX2, allowing us to use this cell line to model HSC responses (Figure 3B). Expression of AOC3 mRNA in LX2 cells was upregulated by PDGF-BB and TGF-β1 (Figure 3C), consistent with increased expression of VAP-1 in fibrotic tissues. The addition of recombinant VAP-1 (rVAP-1) had no effect on AOC3 expression in LX2, indicating that transcription of VAP-1 is not modulated by extracellular VAP-1 in this system.

We detected sVAP-1 in conditioned media from in vitro–activated HSC (aHSC) and aLMF cultures and confirmed enzyme activity by detecting H2O2 generation in response to the SSAO substrate benzylamine (Figure 3D). This activity could be blocked by the SSAO inhibitor 2-bromoethylamine (BEA), but not by the lysyl oxidase–specific (LOX-specific) inhibitor β-aminopropionitrile (β-APN). The release of VAP-1 from the stromal compartment in vitro and the increased circulating levels of sVAP-1 in patients with steatohepatitis prompted us to investigate whether sVAP-1 could provide a promigratory signal for leukocytes. Multicolor confocal microscopy of tissue sections taken from the liver of patients with NASH cirrhosis demonstrated a close association between VAP-1+ stromal cells and infiltrating leukocytes (Figure 3E). Conditioned media from in vitro–cultured aHSCs promoted the migration of lymphocytes in Boyden chamber assays, and the addition of a function-blocking anti-VAP-1 Ab (TK8-14) reduced their migratory capacity by more than 50% (Figure 3F). In parallel studies, highly purified human sVAP-1 induced dose-dependent migration of peripheral blood lymphocytes in Boyden chamber assays with a maximal response between 10 and 100 ng/ml (Figure 3G) that was comparable to that seen with 10 ng/ml CXCL12, a potent lymphocyte chemotactic factor. The related amine oxidase LOX has branched expression (Figure 3A), and these cells were capable of de novo synthesis of AOC3 mRNA in LX2, allowing us to use this cell line to model HSC responses (Figure 3B). Expression of AOC3 mRNA in LX2 cells was upregulated by PDGF-BB and TGF-β1 (Figure 3C), consistent with increased expression of VAP-1 in fibrotic tissues. The addition of recombinant VAP-1 (rVAP-1) had no effect on AOC3 expression in LX2, indicating that transcription of VAP-1 is not modulated by extracellular VAP-1 in this system.

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These human data demonstrated that enzymatically active VAP-1 expressed by aHSCs is upregulated in the diseased liver and is capable of modulating leukocyte recruitment. This led us to study the role of VAP-1 in liver fibrogenesis in vivo.

VAP-1 deficiency attenuates CCl4–induced hepatic fibrosis. We used a CCl4 model to induce hepatic injury in WT (C57BL/6) mice, VAP-1–KO (Aoc3−/−) mice, and WT mice that received weekly doses of a monoclonal anti–VAP-1 Ab (BTI-T1029). Control animals received mineral oil (MO) alone. One group of animals was sacrificed after 8 weeks of CCl4 treatment and a second group following an additional 4-week period of resolution during which CCl4 treatment was stopped. We found that untreated Aoc3−/− animals had an overtly normal phenotype with macroscopically and microscopically normal livers, consistent with previous reports (37).

In line with previous studies, we detected bridging fibrosis by picosirius red staining at 8 weeks in WT animals, with significant fibrosis resolution 4 weeks after stopping CCl4 treatment (Figure 4A). Alanine aminotransferase (ALT) levels were comparable between WT, Aoc3−/−, and Ab-treated animals given CCl4, suggesting that the absence of VAP-1 did not diminish the degree of initial liver injury (Figure 4B). This finding was similar to that seen for blockade of a related amine oxidase, lysyl oxidase–like protein-2 (LOXL2), in a CCl4–induced injury model (38).

We found that Aoc3−/− mice and WT animals treated with anti–VAP-1 Ab had significantly less fibrosis at 8 weeks, as demonstrated by picosirius red staining and reduced staining for α-SMA (Figure 4, C and D). Four weeks after stopping CCl4, we observed that fibrosis was greatly reduced in all animals, although WT animals had significantly more extensive picosirius red staining compared with that seen in MO-treated control mice (Figure 4C). We confirmed an increase in fibrosis in the CCl4–treated WT mice relative to that in Aoc3−/− mice at 8 weeks by real-time quantitative reverse transcription PCR (qRT-PCR) of whole liver for collagen I (Col1a1) and α-SMA (Acta2), and there was a trend toward a

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Table 3. Parameters that independently correlated with significant fibrosis (Kleiner score F ≥2) following multivariate analysis
mine whether leukocyte infiltration preceded fibrosis, we quantified lymphocyte and monocyte subsets in the livers of WT and Aoc3−/− animals prior to CCl4 administration (base), following 2, 4, or 8 weeks of CCl4, and at 12 weeks (resolution). Lymphocyte recruitment occurred within the first 2 weeks, whereas myeloid cells peaked at 8 weeks following injury (Figure 5, A–C). There was a significant reduction in the absolute number of all subsets in the livers of Aoc3−/− mice compared with that seen in WT animals receiving the same dose of CCl4. The numbers of cells detected in the WT livers increased progressively up to the 8-week treatment time point and returned to the levels observed for the Aoc3−/− group 4 weeks after cessation of CCl4 administration (base). Our finding that leukocytes were recruited during the first 2 weeks following CCl4 exposure suggests that the Ab offers protection against CCl4-induced fibrosis (Figure 5, A–C). There was a significant reduction in the absolute number of all subsets in the livers of Aoc3−/− mice compared with that seen in WT animals receiving the same dose of CCl4. The numbers of cells detected in the WT livers increased progressively up to the 8-week treatment time point and returned to the levels observed for the Aoc3−/− group 4 weeks after cessation of CCl4. At the early (2-week) time point, we observed a marked reduction in the numbers of CD8+ T cells and CD19+ B cells in Aoc3−/− animals and significantly fewer CD4+ T cells, and we detected NK1.1+ and CD3+NK1.1+ NKT cells at 4 weeks, 8 weeks, and 4 weeks, respectively (Figure 5, A and B). Infiltration with macrophages and myeloid cells peaked at 8 weeks and was significantly reduced in Aoc3−/− mice at both the 4-week and 8-week time points (Figure 5, A and C). We observed no significant differences in the numbers of cells within the WT and Aoc3−/− livers prior to the administration of CCl4 (Figure 5A, Base). Our finding that leukocytes were recruited during the first 2 weeks following CCl4 exposure suggests that the Ab offers protection against CCl4-induced fibrosis (Figure 5, A–C). There was a significant reduction in the absolute number of all subsets in the livers of Aoc3−/− mice compared with that seen in WT animals receiving the same dose of CCl4. The numbers of cells detected in the WT livers increased progressively up to the 8-week treatment time point and returned to the levels observed for the Aoc3−/− group 4 weeks after cessation of CCl4. At the early (2-week) time point, we observed a marked reduction in the numbers of CD8+ T cells and CD19+ B cells in Aoc3−/− animals and significantly fewer CD4+ T cells, and we detected NK1.1+ and CD3+NK1.1+ NKT cells at 4 weeks, 8 weeks, and 4 weeks, respectively (Figure 5, A and B). Infiltration with macrophages and myeloid cells peaked at 8 weeks and was significantly reduced in Aoc3−/− mice at both the 4-week and 8-week time points (Figure 5, A and C). We observed no significant differences in the numbers of cells within the WT and Aoc3−/− livers prior to the administration of CCl4 (Figure 5A, Base). Our finding that leukocytes were recruited during the first 2 weeks following CCl4 exposure suggests that the Ab offers protection against CCl4-induced fibrosis (Figure 5, A–C). There was a significant reduction in the absolute number of all subsets in the livers of Aoc3−/− mice compared with that seen in WT animals receiving the same dose of CCl4. The numbers of cells detected in the WT livers increased progressively up to the 8-week treatment time point and returned to the levels observed for the Aoc3−/− group 4 weeks after cessation of CCl4. At the early (2-week) time point, we observed a marked reduction in the numbers of CD8+ T cells and CD19+ B cells in Aoc3−/− animals and significantly fewer CD4+ T cells, and we detected NK1.1+ and CD3+NK1.1+ NKT cells at 4 weeks, 8 weeks, and 4 weeks, respectively (Figure 5, A and B). Infiltration with macrophages and myeloid cells peaked at 8 weeks and was significantly reduced in Aoc3−/− mice at both the 4-week and 8-week time points (Figure 5, A and C). We observed no significant differences in the numbers of cells within the WT and Aoc3−/− livers prior to the administration of CCl4 (Figure 5A, Base). Our finding that leukocytes were recruited during the first 2 weeks following CCl4 exposure suggests that the Ab offers protection against CCl4-induced fibrosis (Figure 5, A–C).
Figure 3. Isolated human hepatic stromal cells express functional VAP-1 capable of driving lymphocyte migration in vitro. (A) VAP-1 was detected in isolated human aHSCs (left) and aLMFs (right) (DAPI blue). Scale bars: 50 μm. (B) AOC3 mRNA was expressed by HSECs, aLMFs, aHSCs (4 independent patient isolates each) and by the HSC cell line LX2 (3 repeats) at similar levels (dotted line represents negative controls). (C) Expression of AOC3 could be induced following a 24-hour incubation with PDGF-BB or TGF-β1 (both at 10 ng/ml) but not with rVAP-1 (500 ng/ml). Data represent 2 independent experiments measured in triplicate. (D) Amine oxidase activity of aHSCs (n = 4 isolates) in the absence and presence of VAP-1/LOX inhibitor BEA or the LOX-specific inhibitor 2-APN. (E) CD3+ lymphocytes (left) and CD68+ macrophages (right) were closely associated with VAP-1+ vessels and stroma in cirrhotic livers. Scale bars: 50 μm. (F) Conditioned media from primary cell cultures of aHSCs (n = 3 isolates) promoted VAP-1-dependent migration of peripheral blood lymphocytes in modified Boyden chamber assays. (G) Highly purified sVAP-1 induced the migration of peripheral blood lymphocytes in modified Boyden chamber assays in vitro (n = 3 lymphocyte preparations, 12 random fields/sample). (H) Lymphocyte migration was induced by 100 ng/ml sVAP-1 and is shown compared with the baseline response to media alone (n = 3 lymphocyte preparations, 12 random fields per sample). Data were calculated by 1-way ANOVA with Tukey’s post-hoc test (C, G, and H), paired Student’s t test (D), and Mann-Whitney U test (F). *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
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Figure 4. VAP-1 contributes to scar formation in CCl₄-induced murine fibrosis. (A) Picrosirius red staining of liver from WT mice receiving an 8-week treatment of MO, CCl₄, or CCl₄ plus a function-blocking anti-VAP-1 Ab (WT CCl₄ + Ab) or liver from Aoc3⁻/⁻ mice that received CCl₄ (Aoc3⁻/⁻ CCl₄). Lower panels depict the same experimental groups following an additional 4 weeks of resolution after stopping CCl₄ at 8 weeks. Scale bars: 200 μm. (B) Serum ALT levels and (C and D) Sirius red (C) and α-SMA (D) staining following 8 weeks of vehicle or CCl₄ administration (white bars) and 8 weeks of treatment followed by 4-week resolution (black bars). Fibrosis was also determined by qRT-PCR for Col1a1 and Acta2 (E and F). 3–4 animals per group. 1-way ANOVA with Tukey’s post-hoc test: *P < 0.05, **P < 0.01 and ***P < 0.001 versus WT MO at 8 weeks; †P < 0.05 and #P < 0.001 versus WT MO at 12 weeks.

administration prior to the development of fibrosis (Figure 5B) is consistent with reports implicating them in fibrogenesis (40).

**VAP-1 promotes the development of severe steatohepatitis in a murine methionine choline-deficient diet.** Mice fed a methionine choline-deficient (MCD) diet develop liver injury with histology similar to that of human NASH including steatosis, inflammatory cell infiltration, and early fibrosis (41). To further investigate the role of VAP-1 in the development of liver disease in vivo, we administered an MCD diet for 6 weeks to 3 groups of 6 mice: (a) WT (C57BL/6), (b) Aoc3⁻/⁻, and (c) WT mice treated with weekly monoclonal anti-VAP-1 Ab started 1 week prior to the diet (WT plus BTT-1029). At sacrifice, we found that serum ALT levels were elevated in all mice, with no significant differences between the 3 groups, similar to that seen for the CCl₄ model (Figure 6A). Bilirubin levels were raised in the Ab-treated group compared with those in the WT and KO animals, but we observed no differences in weight loss or blood glucose levels in these groups (Supplemental Figure 2). Low-power microscopy of H&E-stained liver sections demonstrated macrovesicular steatosis in association with inflammatory foci in WT mice (Figure 6B). We found significantly fewer inflammatory foci in the livers of mice treated with anti-VAP-1 Ab and in Aoc3⁻/⁻ mice compared with those found in WT mice (Figure 6C). Steatosis determined by Oil red O staining was present in all mice, but was less extensive in anti-VAP-1-treated mice and particularly reduced in Aoc3⁻/⁻ mice (Figure 6, D and E). This finding was unexpected, because SSAO activity modulates glucose uptake, whereas the MCD diet model does not induce insulin resistance.

**Aoc3⁻/⁻ mice show a reduction in intrahepatic CD4⁺ and NKT cell populations in response to MCD diet-induced steatohepatitis.** Having demonstrated by histology that there was a reduction in inflammatory infiltrates in Aoc3⁻/⁻ or anti-VAP-1-treated
animals on an MCD diet, we characterized changes in the infiltrate in detail at 6 weeks by quantifying and phenotyping liver-infiltrating leukocytes (Figure 7). WT mice fed an MCD diet showed a 2.2-fold increase in intrahepatic CD3+ T cells compared with that seen in control mice that received normal chow (NC) (Figure 7A). The number of intrahepatic CD3+ T cells was significantly reduced in $\text{Aoc3}^{-/-}$ mice compared with that in WT animals due to a reduction in the numbers of intrahepatic CD4+ T cell and CD3+ NK1.1+ NKT cell populations (Figure 7, B and E). In contrast to the CCl4 model, we found no significant differences in the expansion of intrahepatic CD8+ T cells (~4.5-fold increase vs. NC-fed mice) and CD19+ B cells (~1.7-fold increase vs. NC-fed mice) between WT and $\text{Aoc3}^{-/-}$ mice (Figure 7, C and F). MCD diet-fed WT mice demonstrated a 4.4-fold increase in CD11b+F4/80+ macrophages and a 6.8-fold increase in CD11b+GR1+ monocytes compared with NC-fed mice. MCD diet-fed $\text{Aoc3}^{-/-}$ mice had similar numbers of CD11b+F4/80+ macrophages but fewer intrahepatic CD11b+GR1+ cells compared with what was observed in WT animals fed an MCD diet (Figure 7, G and H).

$\text{Aoc3}^{-/-}$ and Ab-treated mice show reduced fibrogenesis in response to an MCD diet. Parenchymal infiltration by leukocytes drives the development of fibrosis in NAFLD (5, 42), leading us to investigate whether $\text{Aoc3}^{-/-}$ and Ab-treated mice are protected against fibrosis following 6 weeks of an MCD diet. Both VAP-1 deficiency and prophylactic anti–VAP-1 Ab blockade resulted in significant and similar reductions in $\alpha$-SMA expression (Figure 8, A and B) and collagen deposition (Figure 8, C and D) compared with that seen in WT mice when assessed by histological morphometric analysis and by qRT-PCR of total $\text{Acta2}$ and $\text{Colla1}$ mRNA from whole mouse liver (Figure 8E). We observed a similar pattern in WT mice that received therapeutic doses of anti–VAP-1 Ab for the last 4 weeks of the diet: the recruitment of CD11b+F4/80+ macrophages was significantly reduced in WT mice receiving anti–VAP-1 Ab, and this was associated with an attenuated fibrotic response (Figure 9, A and B).

Figure 5. Reduced leukocyte infiltration of the liver after CCl4 treatment in $\text{Aoc3}^{-/-}$ mice. The number of liver-infiltrating cells in WT or $\text{Aoc3}^{-/-}$ mice after CCl4, for 2, 4, or 8 weeks or following 8 weeks of CCl4 treatment followed by 4 weeks of resolution (Res) was quantified by flow cytometry. (A) There were significantly more T cells, B cells, NKT cells, and NK cells in WT animals (black bars) treated with CCl4, compared with those in $\text{Aoc3}^{-/-}$ mice (white bars). Data are expressed as the number of infiltrating cells per gram of fresh liver tissue (A) or fold change over baseline (Base) values (B and C: upper panels: WT; lower panels: $\text{Aoc3}^{-/-}$) for 3 to 5 animals per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ by 2-way ANOVA with Bonferroni’s correction.
studied the pathological changes at 6, 9, and 12 months during evolution of NAFLD and compared these outcomes with mice fed NC and standard drinking water and allowed unrestricted activity. Serum ALT levels were elevated in mice exposed to the WLM by 6 months, but we observed no significant differences in ALT levels between WT or Aoc3–/– mice at either 6 or 9 months (Figure 10A). Aoc3–/– mice gained more weight during the first month of the diet compared with that seen in the WT group (Supplemental Figure 7). Aoc3 expression was increased in WT mice following 9 months of the diet when compared with that detected in animals on a NC diet (Figure 10A), and the extent of fibrosis at both the 9- and 12-month time points was reduced in the Aoc3–/– animals, as evaluated by collagen deposition levels (Figure 10, B and C) and Acta2 and Col1a1 expression (Figure 10D). Although steatosis was present in all the WLM diet–fed animals, we found that the extent of steatosis was reduced in Aoc3–/– mice compared with that in WT mice at the 9-month time point (Figure 10, E and F). This was similar to what we observed with the MCD diet–fed mice (compare Figure 6).

We quantified immune cells isolated from the livers of WT and Aoc3–/– mice fed NC or those on the WLM diet at 6 and 9 months. We observed only a modest increase in CD8+ T cells, NK1.1+ cells, CD11b+F4/80+ cells, and Cd11b+GR1+ myeloid cells at 6 months, and there were no significant differences between WT and Aoc3–/– mice in the numbers of any cell type (Figure 11). However, by 9 months, the WLM mice developed an inflammatory infiltrate comparable in profile and absolute cell numbers to what we observed in mice after 6 weeks on an MCD diet (compare Figures 7 and 11).

Although the MCD diet model of steatohepatitis recapitulates many of the histological features of human NASH, it is an acute model resulting in weight loss and is not associated with systemic insulin resistance and as such does not mirror the physiological changes that occur during metabolic syndrome–induced human NAFLD. Thus, we felt it important to investigate the effects of VAP-1 deficiency in chronic models of steatohepatitis that are associated with obesity and insulin resistance.

C57BL/6 mice fed a high-fat diet (HFD) for 18 weeks develop obesity, glucose intolerance, hyperinsulinemia, hyperleptinemia, and hypercholesterolemia (43). The main hepatic pathology seen following an HFD is macrovesicular steatosis, with the development of steatohepatitis and modest fibrosis after a prolonged HFD (44). We observed that WT mice fed an HFD for 18 weeks developed severe macrovesicular steatosis, inflammatory foci, and thin, fibrous septa, all of which were greatly reduced in Aoc3–/– animals, together with reduced Acta2 and Colla1 expression (Supplemental Figures 3–6). Although the HFD model more closely resembles the pathophysiology of human NASH, the extent of steatohepatitis is not as severe as that seen with the MCD diet model (45). Tetri et al. described a murine model of steatohepatitis that develops in response to a diet high in trans fats and fructose and interventions to promote sedentary behavior (46). This Western lifestyle model (WLM) is characterized by obesity with impaired glucose tolerance and increased plasma insulin, resistin, and leptin comparable to the pathological changes seen in NAFLD and at 16 weeks, animals develop severe steatosis with significant necroinflammatory activity. We exposed WT and Aoc3–/– mice to this regimen and studied the pathological changes at 6, 9, and 12 months during evolution of NAFLD and compared these outcomes with mice fed NC and standard drinking water and allowed unrestricted activity.

Serum ALT levels were elevated in mice exposed to the WLM by 6 months, but we observed no significant differences in ALT levels between WT or Aoc3–/– mice at either 6 or 9 months (Figure 10A). Aoc3–/– mice gained more weight during the first month of the diet compared with that seen in the WT group (Supplemental Figure 7).

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reductions in hepatic CD4+ and NK1.1+ T cells as those seen in the Aoc3–/– mice (Figure 12), confirming that the amine oxidase activity of VAP-1 is involved in the generation of hepatitis in vivo. While mice with catalytically inactive VAP-1 had levels of injury similar to those seen in the WT mice at 4.5 weeks (Supplemental Figures 9–12), catalytic inactivation of VAP-1 resulted in a significant loss of Acta2 expression at 6 weeks (Figure 12B), suggesting that the reduced infiltrate results in an attenuated fibrotic response.

VAP-1 enhances wound healing by hepatic stromal cells in vitro. The presence of VAP-1 protein within fibrotic septa of chronically diseased human liver tissue suggested that it might contribute to remodeling of the scar via direct effects on the hepatic stroma. Short-term (24-hour) treatment with sVAP-1 at 500 ng/ml had no effect on aHSC activation, proliferation, or apoptosis in vitro based on detachment assays, cell proliferation measurements, and qRT-PCR for the prosurvival factor BCL2 and the associated proapoptotic factor BAX (Supplemental Figure 13). However, we observed increased cell spreading in impedance-based assays upon treatment with either sVAP-1 or a recombinant form of the protein produced in CHO cells (rVAP-1) (Figure 13A). The potent mitogen PDGF-BB had no effect on cell spreading over the same time course. This prompted us to investigate the effect of de novo synthesized VAP-1 on stromal cell function. Overexpression of an N-terminal GFP-tagged form of VAP-1 (GFP-WT-VAP-1, described in ref. 47) in LX2 cells accelerated wound healing in a scratch wound assay when compared with that in the control transfectant plasmid Lifeact-GFP (pLifeact-GFP), which is known to have little-to-no effect on cell motility (ref. 48 and Figure 13B). This effect was not dependent on enzyme activity, as expression of the enzyme-dead mutant of VAP-1 (GFP-Y471F-VAP-1) had a similar effect (Figure 13B). In complementary experiments, siRNA-mediated knockdown of AOC3 in LX2 cells retarded wound closure when compared with that seen in scrambled controls (Figure 13C).

Expression of VAP-1 regulates the transcription of profibrotic factors. To investigate the mechanism by which VAP-1 drives the fibrotic response, we measured the expression levels of genes associated with fibrosis in transfected LX2 cells by qRT-PCR.
Overexpression of AOC3 in cells transfected with GFP-tagged VAP-1 plasmid constructs was demonstrated by a 2,000-fold increase in message when compared with that in control cells transfected with Lifeact-GFP (Figure 14A). We found that expression of PDGFRβ, which is a characteristic of HSC activation, was increased in GFP-WT-VAP-1-transfected cells, and this was dependent on the enzymatic activity of VAP-1, as the enzyme-dead mutant (GFP-Y471F-VAP-1) did not elicit a similar response, suggesting that expression of catalytically active VAP-1 provides a profibrogenic stimulus. This hypothesis was supported by the observation that GFP-WT-VAP-1 induced upregulation of both COL1A1 and LOXL2 (Figure 14A), the latter being a related amine oxidase that has been implicated in the development of fibrosis in a number of disease models (38). We confirmed the close association between VAP-1 and the expression of profibrotic genes in siRNA-mediated knockdown experiments, in which reduced AOC3 expression in LX2 cells downregulated the expression of both COL1A1 and LOXL2 (Figure 14B).

Discussion
In NAFLD, the progression of steatosis to cirrhosis is driven by steatohepatitis, which develops as a consequence of activation of the inflammasome in response to liver injury (49–52). Steatohepatitis is characterized by infiltration of the hepatic parenchyma with T cells, macrophages, and neutrophils (53), and previous findings that VAP-1 plays an important role in the recruitment of lymphocytes and monocytes to the liver prompted us to study its role in NAFLD (12, 15, 39). We have reported that VAP-1 is shed into the circulation as a soluble enzyme that accounts for most of the circulating monoamine oxidase activity in human blood and that levels are increased in chronic inflammatory liver diseases, but not in acute liver injury (23, 26). We now report elevated sVAP-1 levels in a well-defined cohort of NAFLD patients that is associated with increased VAP-1 expression in the liver. Because sVAP-1 levels are elevated in patients with obesity and type 2 diabetes, we compared these levels in NAFLD with those in a group of patients matched for BMI and metabolic phenotype who had no evidence of liver involvement. We found that sVAP-1 levels were elevated in the metabolic syndrome cohort compared with those in healthy controls, but were significantly lower compared with levels in NAFLD patients. Circulating sVAP-1 levels also correlated with histological evidence of steatohepatitis and fibrosis on liver biopsy, and multivariate analysis showed that VAP-1 was independently associated with fibrosis stage. Reliable methods to assess steatohepatitis,
are closely associated with sinusoidal endothelium and act as pericytes, contributing to the regulation of endothelial differentiation and function (54). In response to injury, quiescent HSCs proliferate and transdifferentiate into activated myofibroblasts, which deposit matrix during hepatic fibrogenesis. We found that both in vivo–activated aLMFs and in vitro–activated HSCs expressed and secreted enzymatically active VAP-1, which was a potent leukocyte chemotactic factor at levels comparable to those found in vivo. Thus, VAP-1 can modulate leukocyte migration in both its transmembranous and soluble forms. This property was dependent in part on SSAO activity associated with the generation of H$_2$O$_2$ and, to our knowledge, is the first report of SSAO directly stimulating leukocyte motility. It is likely that VAP-1 production by endothelial cells and myofibroblasts generates a local gradient of sVAP-1/H$_2$O$_2$, which stimulates leukocyte migration and recruitment. Our finding that the chemotactic effect of sVAP-1 was reduced by treatment with pertussis toxin is consistent with a contribution from autocrine activation of chemotactic factors that signal through GPCRs, as has been described for neutrophils migrating in response to H$_2$O$_2$ (55). Autocrine chemoattractant production and direct modification of redox-sensitive intracellular receptors has been shown in monocyte migration in response to lysyl oxidase, another enzyme that generates H$_2$O$_2$ (56, 57). This model is consistent with a study in zebrafish, in which a tissue gradient of H$_2$O$_2$ produced by an unidentified amine oxidase recruits leukocytes to the scar during wound healing (35). Thus VAP-1–generated H$_2$O$_2$ could operate in a similar way, resulting in a local tissue gradient that promotes and sustains the recruitment of leukocytes to the hepatic parenchyma. We suggest that endothelial VAP-1 promotes the recruitment of effector cells to the inflamed liver and that VAP-1 produced by HSCs positions them within the hepatic scar. These findings are consistent with, and provide a potential mechanism to explain, previous observations that VAP-1 expressed by specialized splenic fibroblasts guides lymphocyte migration within the spleen (58).

In order to test the hypothesis that VAP-1 links inflammation and fibrosis in response to liver injury, we compared the response to CCl$_4$-induced liver injury in WT mice, Aoc3$^{-/-}$ animals, and WT mice treated with a neutralizing anti–VAP-1 Ab. In response to CCl$_4$, WT mice developed septal fibrosis and increased leukocyte infiltration. The absence of VAP-1 in Aoc3$^{-/-}$ mice or its blockade by Ab in WT animals markedly reduced fibrosis and accelerated recovery in response to CCl$_4$, implicating VAP-1 in fibrogenesis.
fructose and trans-fat diet develop NASH over a 9- to 12-month period, we were able to study the kinetics of hepatitis and fibrosis development in a model that recapitulates human NAFLD. Both WT and Aoc3–/– mice developed steatosis and liver injury, as measured by ALT levels after 6 months, before the development of steatohepatitis that developed between 6 and 9 months, and they developed fibrosis that was apparent at 12 months. The severity of steatosis was reduced, though not abolished, in Aoc3–/– animals (similar to our findings with MCD diet– and HFD-fed mice), suggesting that VAP-1 promotes steatosis as well as steatohepatitis. We expected this would be an SSAO-dependent effect of VAP-1, but surprisingly, steatosis was reduced by Ab treatment, which does not inhibit SSAO activity toward small conventional amine substrates, and was unaffected in mice expressing enzyme-dead VAP-1. The reasons for this are currently unclear. VAP-1 substrates have been shown to activate glucose transport (59), and feeding mice the SSAO inhibitor semicarbazide limits weight gain and inhibits fat deposition in adipose tissue (60). However, Bour et al. demonstrated that Aoc3–/– animals have reduced leukocyte infiltration into white adipose tissue associated with increased fat deposition, which could not be rescued by expression of VAP-1 from the human adipocyte–specific aP2 promoter (61). These data suggest that the role of VAP-1 in the development of hepatic steatosis is complex and that the effects of adipocyte-specific VAP-1 on the release of adipokines must also be taken into consideration.

Steatohepatitis in response to the WLM was characterized by a mixed inflammatory cell infiltrate that drives the development of fibrosis. In the absence of VAP-1, the absolute numbers of both lymphoid and myeloid cells were substantially reduced, consistent with a model in which VAP-1 promotes the recruitment of an infiltrate that drives the development of fibrosis. Several properties of VAP-1 SSAO suggest that it could have roles in NAFLD progression in addition to the ability to recruit inflammatory cells. These include the local generation of ROS by SSAO activity and the ability of SSAO to sensitize insulin signaling. Our current data generated in 3 different models of NAFLD suggest that VAP-1 contributes to the establishment of steatohepatitis and progression to fibrosis. This was evidenced by reduced inflammation, steatosis, and fibrosis in the absence of VAP-1 in an HFD, an MCD diet, and in the WLM. Using the WLM of NAFLD, in which mice given a high-
The activity (63), has been associated with a reduction in the risk of fibrosis in NASH patients (64). However, the fact that we were able to effectively prevent fibrosis using a VAP-1–blocking Ab that does not bind to the enzyme active site or inhibit deamination of small soluble substrates in vitro suggests a more complex mechanism. It is possible that the Ab prevents access of endogenous, cell-associated macromolecular substrates to the enzyme in vivo, although until the endogenous ligands of murine VAP-1 are defined, it is difficult to test this hypothesis. An alternative explanation is that recruitment requires sequential interactions involving enzyme-dependent and -independent steps; if either of these is blocked, recruitment fails. A similar mechanism was proposed for the recruitment of granulocytes across endothelial monolayers (65). Both hypotheses are consistent with our murine data and suggest that both VAP-1–blocking Abs and enzyme inhibitors have therapeutic potential.

We used wound-healing and transfection studies to examine the direct effects of VAP-1 expression on the stromal cell population. VAP-1 promoted the wound-healing response, induced spreading of aHSCs, and enhanced expression of profibrotic genes (COL1A1, LOXL2), indicating that VAP-1 has direct effects on stellate cells that could promote scarring. There is a precedent for the involvement of VAP-1 in matrix remodeling, because its overexpression in arterial smooth muscle cells increases elastin deposition in the arterial wall (66). The contribution of SSAO activity to these functions remains unclear, however, since accelerated wound healing was independent of enzyme activity, whereas expression of profibrotic genes was unaffected by the transfection of LX2 cells with a catalytically inactive form of the protein (Figures 13 and 14). A similar effect has been observed for LOXL2, in which enzyme-dead forms of the protein retained function (67, 68).

We suggest that VAP-1 plays a complex role in the pathogenesis of CLD. Its ability to promote the recruitment of leukocytes to the liver in response to initial liver injury and its contribution to the development of fibrosis suggest that targeting VAP-1 therapeutically, through Ab blockade or enzyme inhibitors, could prevent the progression of liver disease.

Methods

Study populations. Serum from NAFLD patients (n = 133) was obtained at the time of the patient’s admission for liver biopsy. All patients included in the study had histologically confirmed NAFLD, with biopsies graded...
Liver tissue from explanted livers was taken from normal organ donor liver tissue that was surplus for surgical requirements or from uninvolved liver removed at hepatic resection for secondary liver tumors; diseased tissue was obtained from patients undergoing liver transplantation for CLD.

Isolation of human HSCs and aLMFs and maintenance of the LX2 cell line. HSCs were isolated from nonfibrotic human liver tissue, and aLMFs were purified from fibrotic tissue as described previously (69). Cell viability was confirmed using trypan blue exclusion, and autofluorescence of HSCs suggested a greater than 90% purity. Cells were plated on plastic in DMEM containing 16% FCS (Invitrogen) and used within 4 passages. HSCs that adhered to plastic after isolation were termed aHSCs to differentiate them from their quiescent phenotype.

The HSC line LX2 (a gift of S. Friedman, Mount Sinai School of Medicine, New York, New York, USA) was maintained in DMEM containing 2% FCS (Invitrogen).

Soluble VAP-1 measurements. Soluble VAP-1 levels in human serum were measured by time-resolved immunofluorometric assay at Biotie Therapies Corp. Briefly, a biotin-conjugated monoclonal anti–VAP-1 Ab (clone TK8-14; eBioscience) was immobilized onto a streptavidin-coated microtiter plate, to which serum samples were added. sVAP-1 that had bound to the plate was then detected with a europium-conjugated anti–VAP-1 Ab (clone TK8-18; eBioscience). VAP-1 levels were measured by time-resolved fluorescence at 615 nm (VICTOR 2 Multilabel Counter; PerkinElmer). Quantification of VAP-1 concentration in serum was based on a reference sample of highly purified human serum VAP-1 (Biovian Ltd.). The calibration range of the method was from 1.5 to 1,500 ng/ml. The linearity ($R^2$) of the calibration (standard) curves was 0.997–1.000.

Cell migration assay. Chemotaxis assays were performed as previously described (70). Briefly, 30 μl aHSC-conditioned medium, highly purified sVAP-1 (Biotie Therapies Corp.), or recombinant CXCL12 (10 ng/ml; Peprotech) was placed in the lower wells of a custom-made 48-well chemotaxis chamber (Neuro Probe) separated from an upper chamber containing cell suspension (peripheral blood leukocytes, and staged according to the Kleiner classification; extensive clinical and biochemical characteristics were recorded at the time of biopsy (Table 1). A control cohort matched for age, BMI, and metabolic phenotype was established and fasted serum samples and anthropometric data collected ($n = 74$, Table 1). Exclusion criteria for all patients were: age of less than 18 years, consumption of greater than 20 g ethanol per day if female and greater than 30 g per day if male, infection with viral hepatitis or HIV, treatment with corticosteroids or immunosuppressive therapy within the past 12 weeks, and treatment with monoamine oxidase inhibitors. Further exclusion criteria for the control cohort were biochemical or radiological evidence of liver disease. Serum was stored at −80°C until later use.

Human tissue. Human tissue and blood samples were collected from patients admitted to the University Hospitals Birmingham NHS Foundation Trust and the Newcastle-Upon-Tyne Hospitals NHS Foundation Trust. Liver tissue from explanted livers was taken from normal organ donor liver tissue that was surplus for surgical requirements or from uninvolved liver removed at hepatic resection for secondary liver tumors; diseased tissue was obtained from patients undergoing liver transplantation for CLD.

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100 U/ml catalase (Sigma-Aldrich) was included in the test wells to assess the contribution of H2O2; in others, the enzyme activity of VAP-1 was inhibited by preincubation with 2.5 mM BEA (Sigma-Aldrich) for 30 minutes prior to the assay.

**Cellular proliferation assays.** A CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) was used to measure the total nucleic acid content of aHSCs after 8 days in culture in the absence and presence of 10 ng/ml PDGF-BB (Peprotech) or 500 ng/ml highly purified serum–derived sVAP-1 (Biotie Therapies Corp.) according to the manufacturer’s instructions.

**Cellular attachment assay.** Activated HSCs were seeded onto plates and allowed to adhere for 24 hours in DMEM containing 16% FCS in the presence of 1.5 mM gliotoxin (Sigma-Aldrich) or sVAP-1 (500 ng/ml) at 37°C and 5% CO2. Adherent cells were counted in 12 high-power fields and normalized to the cells receiving media alone. The data represent 3 separate aHSC isolates measured in triplicate.

1.5 × 10⁶ cells/ml) by a polycarbonate membrane (5-μm pores, Nucleopore Filtration; Neuro Probe). Negative controls containing media alone or media containing inhibitor alone were included in each assay. The assembled chamber was incubated for 2 hours at 37°C in a humidified CO₂-enriched atmosphere. Following incubation, the filter was removed from the chamber and nonmigrated cells on the upper surface were removed by wiping before being air dried, fixed in methanol, and stained with hematoxylin (Diff-Quik; Sigma-Aldrich). The number of stained cells in 4 high-power fields (×40) for each of 6 wells per sample was counted, giving 24 fields of view per condition, and migration was calculated as the median of these 24 counts expressed as the number of migrating cells per high-power field. To assess the contribution of GPCR, leukocytes were pretreated with 100 ng/ml pertussis toxin (PTx; Sigma-Aldrich) for 30 minutes at 37°C, washed, and resuspended in RPMI containing 0.1% BSA prior to the assay. The viability of the leukocytes was not affected by this treatment. In some assays, the enzyme activity of VAP-1 was inhibited by preincubation with 2.5 mM BEA (Sigma-Aldrich) for 30 minutes prior to the assay.

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Ratonic, the contract breeder. Aoc3 point–mutation knock-in mice were backcrossed onto a C57BL/6 genetic background by genOway and supplied by the contract breeder Charles River Laboratories. Mutant allele mice were identified by PCR screening of purified genomic DNA with specific primers.

CCl4 model. Female mice were injected twice weekly for 2, 4, 6, or 8 weeks with either CCl4 (1.0 ml/kg CCl4 diluted 1:4 in MO; Sigma-Aldrich) or MO alone. Animals were sacrificed 96 hours after the final dose of CCl4. In parallel experiments, animals received vehicle or CCl4 for 8 weeks, as above, at which point vehicle or CCl4 treatment was stopped, and the animals were sacrificed following 4 weeks of resolution.

Animal diets. MCD diet: Male mice were fed an MCD diet (MP Biomedicals) ad libitum for 4.5 to 6 weeks. HFD: Male mice were fed an HFD diet that provided 55% of calories from fat (Special Diets Services) ad libitum for 18 weeks. WLM diet: Male mice were fed a diet with 45% of calories obtained from fat and 30% of the fat in the form of partially hydrogenated vegetable oil (Custom Research Diet TD.06303; Harlan Laboratories) in conjunction with fructose-supplemented drinking water (55% fructose, 45% glucose by weight at a concentration of 42 g/l) ad libitum for 6, 9, or 12 months. Control animals received NC and nonsupplemented drinking water.

Animals treated with the VAP-1 function–blocking Ab (mouse anti-mouse BTT-1029, 9 mg/kg; Biotie Therapies Corp.) received a) prophylactic weekly i.v. injections 1 week prior to and weekly throughout the experiment, or b) therapeutic twice-weekly i.v. injections for the final 4 weeks of the 6-week experiment. BTT1029 is a mouse IgG2a mAb generated in Aoc3–/– animals by immunizing with human recombinant VAP-1. The Ab was selected from several hybridomas recognizing recombinant human VAP-1. A secondary screening was made to select antibodies that bound to recombinant mouse VAP-1 (Biotie Therapies Corp.).

Blood samples were withdrawn by cardiac puncture during isoflurane anesthesia, after which the mice were sacrificed by cervical dislocation. Livers were dissected and processed for paraffin embedding, cryosectioning, and RNA extraction (stored in RNAlater; QIAGEN). Bilirubin and ALT were measured at the clinical biochemistry laboratory of the Women’s Hospital Birmingham (Birmingham, United Kingdom), and serum glucose concentrations were obtained using Accu-Chek (Roche).

Cell-spreading assay and xCELLigence impedance measurements. The xCELLigence system was used according to the manufacturer’s instructions (Roche Applied Science) to measure the impedance every minute for 4 hours of a monolayer of 10,000 aHSCs per well in a pre-equilibrated E-Plate using integrated software. Data were normalized at the 40-minute time point to produce a dimensionless normalized cell index (NCI), and cell spreading was calculated using real-time cell analyzer (RTCA) software (Roche Applied Science). Two independent isolates of aHSCs were studied, with each treatment measured in quadruplicate.

Murine models of liver injury. All mice were maintained and housed under conventional conditions in the Biomedical Services Unit at the University of Birmingham or at the University of Turku. Up to 5 mice were housed per cage under alternating 12-hour light/12-hour dark cycles at 23°C. WT C57BL/6 (WT), Aoc3–/–, and Aoc3 point–mutation knock-in mice (SSAO KO, harboring a mutation in the sequence for an active site tyrosine to render the protein catalytically inactive) aged 6 to 8 weeks were used in the studies. C57BL/6 mice were obtained from Charles River Laboratories. Aoc3–/– mice were re-derived from the previously described Aoc3–/– line (37) by blastocyst transfer at Tac-
qRT-PCR. Total cellular RNA was isolated using a RNeasy kit (QIAGEN). RNA concentration and purity were measured using a Nano photometer (Geneflow Ltd.) or a Nanodrop 1000 (Thermo Scientific), and 1 μg of total RNA was transcribed to cDNA using the First Strand Synthesis Kit (Roche) or SuperScript III System (Invitrogen). Real-time PCR was carried out on each sample in triplicate using a Roche Lightcycler 480 machine and Probes Master Mix (200 nM probe, 500 nM primers; Roche Diagnostics) or TaqMan Assay Mix (Invitrogen), as detailed in Supplemental Table 3. Cycling conditions were as follows: 95°C for 10 seconds, 55–60°C (target dependent) for 50 seconds, and 72°C for 1 second. For relative quantification analysis, data were normalized to the appropriate housekeeping gene using “E-analysis” (Roche Diagnostics) and the device software (version 1.5.0.39). Relative expression levels are shown as fold change compared with the appropriate unstimulated control, tissue, or animal. For absolute quantification studies, the number of copies of AOC3 mRNA in matched amounts of starting total RNA from each cell type was determined from a calibration curve of known dilutions of a plasmid encoding AOC3.

Histopathology and immunohistochemistry. For morphological analysis, murine liver specimens were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm sections. Slides were deparaffinized according to standard procedures and stained with freshly made H&E. To evaluate fibrosis, the sections were stained with Van Gieson’s solution (Sigma-Aldrich) or Picrosirius Red (Sigma-Aldrich) staining according to standard protocols. For semi-quantitative determination of hepatic infiltration, 5 random fields of H&E-stained sections at 20× objective magnification were assessed per animal. Inflammatory scores were determined as follows: 0 = no foci per field; 1 = 1–2 foci; 2 = 3–4 foci; 3 = >4 foci. For VAP1 staining in murine tissue, acetone-fixed sections were sequentially incubated with an anti–VAP-1 mAb (TK7-88; ref. 71) and HRP-conjugated anti-rat Ig (Dako). Immunoreactivity was visualized using DAB as a chromogen. The sections were counterstained with hematoxylin, and micrographs were acquired using an Olympus BX60 microscope and the Cell D program.

Oil red O staining of freshly cut sections of murine liver tissue (7-μm thickness) was carried out to evaluate the extent of steatosis. Sections were incubated in 60% propan-2-ol for 5 minutes, followed by a 15-minute incubation with Oil red O reagent (0.3% Oil red O in propan-2-ol/water; Sigma-Aldrich). Next, the samples were washed with propan-2-ol, then water, and mounted in Immunomount (Fisher Scientific). A Mouse-on-Mouse kit (Vector Laboratories) was used in conjunction with an anti-α-SMA mAb (clone IA4, murine IgG2a, 5 μg/ml; Sigma-Aldrich) to detect α-SMA in 7-μm-thick tissue slices. The staining was visualized using a VECTASTAIN Elite ABC kit and NovaRed substrate (Vector Laboratories). Determination of the surface area covered by the stain was performed by analysis of 3 to 5 random fields per animal (depending on the size of the section) at ×20 magnification and threshold analysis with ImageJ software version 1.42q (NIH).

Human tissue samples (10 × 10 × 5 mm) were taken from livers exhibiting a normal phenotype, mild steatosis, moderate steatosis, severe steatosis, or cirrhosis (4 individuals per group, based on histology of hematoxylin-stained tissue sections as assessed by a trained pathologist [SGH]). These samples were fixed in formalin and processed through to paraffin wax. Tissues were deparaffinized and rehydrated to water and, after a low temperature retrieval technique (ALTER), were immunostained on a Dako Autostainer using a Prestige rabbit anti-human VAP-1 polyclonal Ab (HPAO000980, 1:200; Sigma-Aldrich), a Vector ImmPRESS secondary reagent kit, and NovaRED as a chromogen (both from Vector Laboratories). Immunohistochemistry on frozen sections taken from patients who underwent transplantation for end-stage CLD (alcoholic liver disease, primary biliary cirrhosis, primary sclerosing cholangitis) was performed in a similar manner using TK8-14 as the anti-VAP-1 Ab (BMSI033; eBioscience).

Immunofluorescence. Fresh-frozen, acetone-fixed tissue or 4% parafomaldehyde-fixed cells were incubated for 1 hour at room temperature with PBS containing 0.1% Triton X-100 and 10% goat serum (serum-PBST). Following overnight incubation with primary Abs against the antigens α-SMA (clone IA4, 5 μg/ml; Sigma-Aldrich); CD31 (clone 9G11, 20 μg/ml; R&D Systems); VAP-1 (clone TK8-14, 2.5 μg/ml; Biotie Therapies Corp.); VAP-1 (clone 174-5, 2 μg/ml; Novus Biologics); EpCAM (clone HEA125, 100 μg/ml; Progen); CK18 (clone DC10, 1 μg/ml; Dako); CD90 (clone SE10, 1 μg/ml; eBioscience); CD3 (clone UCHT1, 1 μg/ml; AbDSerotec); and CD68 (clone KPI, 1 μg/ml; Abcam), samples were washed 3 times in PBST and incubated with Alexa Fluor-conjugated secondary Abs (1:500; Invitrogen) for 1 hour at room temperature. Nuclei were visualized using DAPI nucleic acid stain (Invitrogen). Fluorescence images were acquired using a Zeiss LSM 510 confocal fluorescence microscope (ZEISS).

Quantification of liver-infiltrating immune cells. Mouse livers were harvested and perfused with PBS via cardiac puncture following disconnection of the hepatic portal vein. The organs were then weighed, dissociated in a gentleMACS C Tube (Miltenyi Biotec), and the resulting immune cells were then purified and analyzed by flow cytometry. Inflammatory cells were gated as a CD45+ cell population (anti–CD45-PerCP-Cy5.5, clone 30-F11; BD Biosciences), and non-viable cells were excluded using a LIVE/DEAD fixable stain (Invitrogen). Lymphocytes were characterized based on staining using a cocktail of anti–CD3-Pacific blue (clone 500A2); anti–CD4-PE (clone RM4-5); anti–CD8a-APC (clone 53-6.7); anti–CD19-APC-Cy7 or anti–CD19-BV510 (both clone ID3); and anti–NK1.1-FITC (clone PK136) or DX5-FITC (clone DX5) Abs (all from BD Biosciences), while the monocyte subsets were identified by staining with anti–CD11b-PE (clone M1/70; BD Biosciences); anti–GR1-APC (clone RB6-8C5; BD Biosciences); and anti–F4/80–FITC (clone BM8; eBioscience) Abs. Absolute cell counts were determined with AccuCheck Counting Beads (Invitrogen), and the number of cells was normalized to the total liver weight. Data were analyzed using a CyAn ADP flow cytometer (Beckman Coulter) or a BD LSRII using Summit version 4.3 or Flowjo version 10.0.7 software where appropriate.

Determination of amine oxidase activity in murine tissues and human serum samples. SSAO activity from human sera was measured as previously described (31). Briefly, 2.4 μl serum was incubated in the absence or presence of the SSAO inhibitor hydroxylamine (5 μM) for 30 minutes prior to the addition of SSAO substrate (benzylamine, 0.5 mM), HRP, and Amplex Red reagent (Molecular Probes). The SSAO-dependent production of hydrogen peroxide was followed for 60 minutes using a Tecan Infinite M200 spectrofluorometer. A calibration curve of exogenously added hydrogen peroxide was used to normalize the data. The SSAO activities in sera were reported as nmol hydrogen peroxide produced by 1 ml serum in 1 hour. SSAO activity from mouse fat lysates was measured as previously described (37). Briefly, fat tissue was lysed in PBS containing 0.2% NP-40. Protein concentrations were
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