CSF1 Restores Innate Immunity After Liver Injury in Mice and Serum Levels Indicate Outcomes of Patients With Acute Liver Failure

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BACKGROUND & AIMS: Liver regeneration requires functional liver macrophages, which provide an immune barrier that is compromised after liver injury. The numbers of liver macrophages are controlled by macrophage colony-stimulating factor (CSF1). We examined the prognostic significance of the serum level of CSF1 in patients with acute liver injury and studied its effects in mice. METHODS: We measured levels of CSF1 in serum samples collected from 55 patients who underwent partial hepatectomy at the Royal Infirmary Edinburgh between December 2012 and October 2013, as well as from 78 patients with acetaminophen-induced acute liver failure admitted to the Royal Infirmary Edinburgh or the University of Kansas Medical Centre. We studied the effects of increased levels of CSF1 in uninjured mice that express wild-type CSF1 receptor or a constitutive or inducible CSF1-receptor reporter, as well as in chemokine receptor 2 (Ccr2)-/- mice; we performed fate-tracing experiments using bone marrow chimeras. We administered CSF1-Fc (fragment, crystallizable) to mice after partial hepatectomy and acetaminophen intoxication, and measured regenerative parameters and innate immunity by clearance of fluorescent microbeads and bacterial particles. RESULTS: Serum levels of CSF1 increased in patients undergoing liver surgery in proportion to the extent of liver resected. In patients with acetaminophen-induced acute liver failure, a low serum level of CSF1 was associated with increased mortality. In mice, administration of CSF1-Fc promoted hepatic macrophage accumulation via proliferation of resident macrophages and recruitment of monocytes. CSF1-Fc also promoted transdifferentiation of infiltrating monocytes into cells with a hepatic macrophage phenotype. CSF1-Fc increased innate immunity in mice after partial hepatectomy or acetaminophen-induced injury, with resident hepatic macrophage as the main effector cells. CONCLUSIONS: Serum CSF1 appears to be a prognostic marker for patients with acute liver injury. CSF1 might be developed as a therapeutic agent to restore innate immune function after liver injury.

Keywords: Drug-Induced Liver Damage; Clearance; Innate Immune Response; M-CSF.

The liver provides an essential immune barrier against gut-derived pathogens entering the portal circulation. Although surgical removal of liver tissue (partial hepatectomy) results in rapid compensatory up-regulation of metabolic function, the liver’s innate immune capacity is markedly impaired. Acute toxic liver injury leads to widespread hepatocyte necrosis and compromises barrier function. Changes in gut wall integrity associated with liver failure facilitate the translocation of gut-derived pathogens. Consequently, sepsis is common in patients with liver failure and is strongly associated with high mortality rates. Liver transplantation is the only effective therapy for life-threatening liver failure but active sepsis is contraindicated in transplantation.

Hepatic macrophages mediate hepatic innate immune defense and promote hepatocyte proliferation after liver injury. Tissue macrophage numbers are controlled during development, and in the steady state, by macrophage colony-stimulating factor (CSF1), which acts through a tyrosine kinase receptor, colony-stimulating factor receptor

Abbreviations used in this paper: ALF, acute liver failure; ALT, alanine aminotransferase; Ccr2, chemokine receptor 2; CSF1, colony-stimulating factor 1; CSF1R, colony-stimulating factor 1 receptor; Fc, fragment, crystallizable; HMGB1, high-mobility group protein 1; mRNA, messenger RNA; PH, partial hepatectomy.

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Hepatic macrophages control circulating CSF1 levels via receptor-mediated endocytosis through CSF1R. In human beings after living donor partial hepatectomy, increased circulating CSF1 is associated with more rapid liver regrowth. In acute toxic liver injury models, monocytederived macrophage recruitment is required for necrotic tissue resorption. In human acute liver injury, hepatic macrophages are implicated in tissue repair and low monocyte counts are associated with mortality. Based on these findings there is a strong rationale for exploring the potential of macrophage-based therapeutics to improve outcomes after acute liver injury.

Here, we show that high serum CSF1 level is associated with survival in patients with acute liver failure (ALF) and outperforms previous markers of outcome in terms of discriminative ability. We show that CSF1 administration in animal disease models promotes rapid recovery of innate immune function and hence has therapeutic potential in human liver failure.

Materials and Methods

Human Work

Ethical approval was obtained from the South East Scotland Research Ethics Committee for patients undergoing partial hepatectomy (PH) at the Hepatobiliary Unit, Royal Infirmary Edinburgh, between December 2012 and October 2013. Liver failure was defined according to Schindl et al. For the acetaminophen-induced ALF cohort, ethical approval was granted by the local human research ethics committee and informed consent was obtained from all patients, or next of kin, before study entry. This study built on previous analysis of this patient cohort by Antoine et al., representing 78 adult patients admitted to the Royal Infirmary Edinburgh (United Kingdom), or the University of Kansas Medical Center (United States), with acute liver injury. Serial patient samples from a second patient cohort were collected at admission to the hospital (as opposed to admission to the specialist liver center with acute liver failure).

Details of serum analyses are provided in the Supplementary Materials and Methods section. Primary hepatocytes were isolated from human liver tissue obtained from liver resection specimens immediately after surgery, with full informed consent and ethical approval from the relevant authorities (National Research Ethics Service reference: 11/NW/0327). See the Supplementary Materials and Methods section for assay details.

Animal Experiments

Animal procedures were approved by the relevant institutional ethics committee (Albert Einstein College of Medicine, United States; University of Edinburgh, United Kingdom; and the University of Glasgow, United Kingdom), and adhered to the Animals (Scientific Procedures) Act of 1986 (United Kingdom) and the National Institutes of Health guide for the Care and Laboratory Animals (United States). Eight- to 12-week-old male mice were used for all experiments. CCR2−/−, C57Bl/6, and MacGreen mice (Tg(Csf1r-Green fluorescent protein)Hume<sup>+</sup>) were bred and maintained under specific pathogen-free conditions. Tg(Csf1r-Mer2(Gre))<sup>pop</sup> were crossed to Rosa floxed stop tomato red and lineage tracing experiments performed as described. Fate tracing bone marrow-derived monocytes was performed using a mouse chimera as previously described. Wild-type C57Bl/6 mice were obtained from Charles River (Margate, Kent, UK). Mice were distributed randomly and maintained on a 12-hour light-dark cycle with feed ad libitum. A two-thirds partial hepatectomy was performed as previously described. Acetaminophen intoxication involved intraperitoneal administration of 350 mg/kg acetaminophen (Sigma-Aldrich, St. Louis, MO). The treatment group received 0.75 mcg/g CSF1-Fc, prepared as described previously (control: phosphate-buffered saline), administered subcutaneously immediately after partial hepatectomy or 12 hours after acetaminophen intoxication and subsequently every 24 hours for up to 3 further doses. Reagents and methodology for immunohistochemistry, flow cytometry, quantification of messenger RNA (mRNA), phagocytosis assay, and serum analyses are provided in the Supplementary Materials and Methods section.

Hepatocyte Toxicity and Metabolic Assays

Details of human and mouse hepatocyte toxicity and metabolic assays are provided in the Supplementary Materials and Methods section.

Statistics

Statistical analysis was performed on GraphPad Prism V6.0 (GraphPad Software, Inc, La Jolla, CA), except for logistic regression analyses, which were conducted in R. All data are presented as mean ±SEM unless otherwise stated. Two-tailed Student t test and 1-way and 2-way analysis of variance with Bonferroni adjustment were used for analysis of data. Human serum analyses and development of the logistic regression models were completed by a qualified statistician. The level of significance was set at a P value less than .05 for all analyses.

Results

Serum CSF1 Increases According to Extent of Partial Hepatectomy and Is Associated With Survival in Acute Liver Failure

In a cohort of 55 patients undergoing up to 75% PH (cohort details: Supplementary Figure 1C), serum CSF1 was increased significantly compared with healthy controls. There was a small reduction on day 1 after surgery followed by a marked increase in CSF1 level by postoperative day 3 (Figure 1A). There was no correlation between serum CSF1 level and blood loss (Supplementary Figure 1B). The initial decrease in serum CSF1 level may be owing to removal of tumor cells, which secrete CSF1. We hypothesized that the subsequent increase in serum CSF1 level might be produced by proliferating hepatocytes. Because of the risks associated with liver biopsy in human beings, we examined a mouse model of two-thirds PH. CSF1 mRNA was unchanged after PH (Supplementary Figure 1C). In the patient cohort the CSF1 increase was related to the extent of resection (Figure 1B). Two patients developed postoperative liver failure and both had serum CSF1 levels below the 25th percentile (Figure 1C, and the clinical details are shown in Supplementary Figure 1D).

We sampled serum from a large patient cohort with established acetaminophen-induced ALF on arrival at the
specialist center (cohort details: Supplementary Figure 2A). The assessment of ALF and the requirement for liver transplantation currently is based on the validated modified King’s College Hospital criteria, which reflect poor clinical condition and likelihood of death. Low serum CSF1 was associated significantly with patient deterioration to meet King’s College Hospital criteria (Supplementary Figure 2B and C), and subsequent death or liver transplantation (Figure 2A). Regardless of final outcome, those patients with a Systemic Inflammatory Response Score greater than 2 had a significantly lower CSF1 level (Supplementary Figure 2D). Serial samples in a separate patient cohort (details shown in Supplementary Figure 2E), followed up from first presentation to hospital, showed that serum CSF1 levels continued to increase in patients whose liver regenerated, whereas CSF1 levels decreased in patients who deteriorated (Figure 2B). In the livers removed from transplant recipients, CSF1 was detected in hepatocytes and nonparenchymal cells (Supplementary Figure 2E). Given the risks of liver biopsy, we used the mouse model to assess hepatic CSF1 gene expression. In contrast to PH, hepatic CSF1 mRNA expression increased significantly after acetaminophen intoxication, peaking at day 2 (Supplementary Figure 2F).

The current best available prognostic biomarker in ALF is serum acetyl-high mobility group box-1 (HMGB1). This damage-associated molecular pattern is released from necrotic tissue and by activated immune cells in response to injury. We assessed the discriminative ability of acetyl-HMGB1, alongside CSF1, and also established clinical measures including bilirubin, prothrombin time, and alanine aminotransferase (ALT) level using the receiver operator characteristic curve (Figure 2C). Serum CSF1 and acetyl-
HMGB1 show similar profiles, whereas bilirubin, PT, ALT, and Acute Physiology and Chronic Health Evaluation II (APACHE II) score were of limited value. There was an inverse correlation between CSF1 and acetyl-HMGB1 (Figure 2D). When combined in a logistic regression model, only CSF1 showed significance (Figure 2E and Supplementary Figure 2F), indicating that serum CSF1 level was a better predictor of outcome than acetyl-HMGB1 (Supplementary Figure 2G and H). Figure 2F provides example CSF1 values with risk of death based on the CSF1 alone model.

Sustained CSF1R Stimulation Induces Hepatic Enlargement Involving Macrophage Accumulation in Mice

The association of low serum CSF1 with poor prognosis in ALF provides a rationale for therapeutic use. Some studies have reported that CSF1R is expressed outside the macrophage lineage.29,30 We therefore examined CSF1R expression using MacGreen mice, where enhanced green fluorescent protein (eGFP) is under the control of the Csf1r promoter.19 Multiphoton ex vivo imaging of liver confirmed CSF1R expression limited to cells with tissue macrophage morphology (Figure 3A). To confirm this, we crossed Csf1r-Mer-Cre-Mer to Rosa26-LSL-dTom reporter mice to allow tamoxifen-induced labeling of Csf1r+ cells, as previously described.20 Co-localization of the pan-macrophage marker F4/80 confirmed that all dTomato+ (Csf1r+) cells belonged to the macrophage lineage (Figure 3B). These data are supported by expression profiling from the Functional Annotation of Mammalian Genomes 5 (FANTOM5) consortium, which show no detectable Csf1r mRNA in hepatocytes isolated from control, or regenerating, mouse liver or isolated human hepatocytes.31 To assess the therapeutic potential of CSF1, we used the CSF1-Fc fusion protein, which overcomes the short half-life of CSF1 protein in vivo.24 CSF1-Fc treatment of mice promoted hepatic macrophage accumulation, but the mechanisms were unclear.24,32 Six hours after CSF1-Fc administration to uninjured mice there was a marked up-regulation of hepatic chemokines, particularly CCL2, CCL7, and CCL12, which are ligands for the CCR2 receptor and highly expressed by classic (Ly6C+) blood monocytes (Figure 3C, and array details are shown in Supplementary Figure 3A). After 4 days of CSF1-Fc treatment, 20% of the liver was composed of F4/80+ macrophages compared with 2% in steady state (Figure 3D). This macrophage accumulation initiated hepatocyte proliferation at day 4.24 Mechanisms of hepatocyte proliferation are multifactorial, with an up-regulation of many cytokines and chemokines associated with the inflammatory response.24 Despite the induction of proinflammatory cytokine mRNA in the liver, serum ALT and aspartate aminotransferase levels were reduced with CSF1-Fc treatment, whereas bilirubin level was unchanged (Figure 3F) and there was no hepatocyte apoptosis (Supplementary Figure 3B). The spleen increased in size, although the weights of the other organs did not change (Supplementary Figure 3C and D).

Macrophage Accumulation Involves In Situ Proliferation and CCR2-Related Infiltration

Infiltrating monocyte-derived macrophages and tissue resident macrophages can be distinguished by relative expression of F4/80 and CD11b and may remain distinctly regulated entities in steady-state liver and after acetaminophen-induced injury.20,33,35 After CSF1-Fc treatment, there was a 2-fold increase in cells with a resident hepatic macrophage phenotype (F4/80hiCD11bhi) and more than a 5-fold increase in the monocyte-derived infiltrating macrophages (F4/80loCD11bhi cells), consisting predominantly of Ly6Chi monocytes (Figure 4A). Both F4/80loCD11bhi and F4/80hiCD11blo cells proliferated markedly in situ after CSF1-Fc administration (Figure 4B and Supplementary Figure 4A and B). Liver macrophages in the treated livers were fate-mapped using tissue-protected bone marrow chimeric mice, where only the hind legs of recipient CD45.1+CD45.2+ animals were irradiated before engraftment of congenic CD45.1+ bone marrow. In these animals, F4/80hiCD11bhi macrophages and blood monocytes showed equivalent donor chimerism (Figure 4C). In phosphate-buffered saline–treated animals, F4/80hi/CD11blo cells remained almost exclusively of host origin, consistent with their proposed tissue origin.20,33,34 However, after CSF1-Fc treatment, approximately 20% of the F4/80hi/CD11blo cells were derived from recruited cells. Thus, the increase in liver macrophages resulted from infiltration of monocytes, proliferation of infiltrating and resident cells, as well as a minor role for differentiation of infiltrating macrophages into a resident macrophage phenotype. The only other cell population that increased significantly was eosinophils (Supplementary Figure 4C), which may have responded to eosinophil chemoattractants CCL3, 4, 7, and 12, detected 6 hours after CSF1-Fc administration (Figure 3C).

Ligands for the CCR2 receptor, which are potent monocyte chemoattractants, were up-regulated early after CSF1-Fc administration (Figure 3A). To examine the role of recruited monocytes, we tested CSF1-Fc administration on Ccr2-/- mice, hypothesizing that the mobilization and recruitment of infiltrating F4/80hi CD11b hi macrophages would be prevented because Ly6C hi monocytes are thought to depend on CCR2 signals for release from the bone marrow and extravasation into inflamed tissues.15,36 Surprisingly, Ccr2-/- mice developed a pronounced Ly6C hi monocytosis after CSF1-Fc administration, indicating that CSF1 can overcome the CCR2 requirement for marrow release (Supplementary Figure 4D). Nevertheless, CSF1-Fc–driven hepatic engraftment by infiltrating macrophages was reduced by CCR2 deficiency (Figure 4D). Accumulation of resident F4/80-CD11blo macrophages was largely unaffected, consistent with local proliferation being the major means of expansion. The increase in eosinophils was unaffected by CCR2 deficiency. CSF1-Fc treatment increased hepatic neutrophils in CCR2 deficiency (Supplementary Figure 4E), probably owing to the deficit in infiltrating monocytes, which regulate neutrophil activity.35 Importantly, CCR2 deficiency prevented the increase in liver-to-body weight ratio observed in wild-type mice after
**A**

Serum CSF1 (ng/mL)

- Healthy volunteer
- Survived
- Died / liver transplantation

Admission to liver unit with ALF

**B**

Serum CSF1 (ng/mL)

- Died/LT (n = 10)
- Survived (n = 10)

Admission to hospital

Days post presentation

**C**

Sensitivity %

- CSF1 (AUC 0.86)
- aHMGB1 (AUC 0.85)
- PT (AUC 0.74)
- ALT (AUC 0.56)
- Bilirubin (AUC 0.62)
- APACHEII (AUC 0.73)

**D**

Log(acetyl-HMGB1)

- Survived
- D/LT

**E**

Components of combined logistic regression model

| Est. | Std. error | Z     | p     | False +ve rate | False -ve rate |
|------|------------|-------|-------|----------------|----------------|
| (Intercept) | 2.65       | 0.71  | 3.8   | <.0001         |                |
| Log(acetyl-HMGB1) | 0.31       | 0.17  | 1.9   | .064           | 0.11           |
| CSF1 | -0.36      | 0.14  | -2.6  | .011           | 0.17           |

**F**

Logistic regression model for CSF1

| CSF1 value | Logit(p) | Chance of death |
|------------|----------|-----------------|
| 1          | 2.11     | 89%             |
| 5          | -0.05    | 49%             |
| 10         | -2.75    | 6%              |

Logit(p) = intercept coeffic. - CSF1 coeffic. x (CSF1 value)

P = exp[logit(p)]/(1 + exp(logit(p)))
Although F4/80lo/CD11bhi macrophages are not completely dependent on CCR2 for mobilization and trafficking to tissues, the data suggest the action of CSF1-Fc on monocyte-derived rather than resident macrophages is the critical step promoting hepatic enlargement.

Figure 2. High serum CSF1 level is associated with survival in acute liver failure in human beings. (A) Serum CSF1 level in healthy volunteers and in patients after acetaminophen intoxication on arrival to a specialist liver unit (survived, n = 47; died/liver transplantation, n = 31). (B) Serial CSF1 samples of patients on first presentation to hospital after acetaminophen intoxication (n = 10/group). (C) Receiver operator characteristic curve for serum CSF1, acetyl-HMGB1 (aHMGB1), prothrombin time (PT), ALT, bilirubin, and APACHE II score with areas under curve (AUC value) for patients who subsequently survived or died/required liver transplantation. (D) Dot plot of serum CSF1 level vs log(serum acetyl-HMGB1) on presentation to the specialist liver center (slope difference: $F = 0.15$, $P = .70$; intercept difference: $F = 8.03$, $P = .006$). (E) Details of the combined logistic regression model. (F) Example of serum values and predicted chance of death based on logistic regression involving CSF1 alone (model 2). $^* P < .05$, $^{**} P < .01$, $^{***} P < .001$, $^{****} P < .0001$.

Figure 3. Hepatic enlargement after CSF1R stimulation. (A) Representative multiphoton image of ex vivo liver (MacGreen mouse Tg [CSF1r-GFP]Hume). (B) Representative immunofluorescence images of Csf1r-Mer2CreJve × Rosa floxed stop tomato red after induction. (C) Cytokine/chemokine array of liver tissue 6 hours after CSF1-Fc treatment vs control (n = 4/group). (D) Quantification of hepatic F4/80 immunohistochemistry in phosphate-buffered saline (PBS) control-treated (n = 8) and mice treated with CSF1-Fc for 2 or 4 days (n = 4/group). (E) Quantification of hepatocyte proliferation in control-treated mice and at day 2 and day 4 after CSF1-Fc administration (20 high-power fields/mouse). (F) Serum parameters after 4 days CSF1-Fc (grey, hollow circles) or PBS control (black, solid circles) treatment once daily (2-way repeated-measures analysis of variance with Bonferroni post hoc). Alk phos, alkaline phosphatase; Creat, creatinine. $^* P < .05$, $^{**} P < .01$, $^{***} P < .001$, $^{****} P < .0001$. 
Figure 4. CSF1-receptor stimulation recruits monocytes and induces macrophage proliferation in uninjured mouse liver. (A) Number of hepatic macrophage populations (F4/80hi/CD11blo and F4/80lo/CD11bhi) day 2 after CSF1-Fc administration (n = 8/group) relative to mean of control group and representative Ly6C profile of F4/80hi (red) and F4/80lo (blue) populations. (B) Percentage of hepatic macrophage populations expressing markers of proliferation (Ki67 and bromodeoxyuridine [BRDU]) on day 2 after CSF1-Fc administration relative to the mean of the control group. (C) Fate tracing bone marrow–derived monocytes using chimeric mice showing conversion of infiltrating cells to resident macrophage phenotype driven by CSF1-Fc administration. Representative dot plots of hepatic macrophage profile and representative Ly6C profile normalized to mode. (D) Hepatic gene expression of phagocytic markers macrophage receptor with collagenous structure (MARCO), macrophage scavenger receptor 1 (MSR1), and mannose receptor (MR) vs relevant control. (E) Multiphoton image of ex vivo Csf1r-eGFP mouse liver after injection of fluorescent microbeads and clearance from the circulation following sham or two-thirds PH with control or CSF1-Fc (n = 6/group/time point). (F) Net fluorescence liver, spleen, lung, kidney, and brain on day 2 after PH and CSF1-Fc or control (n = 6 per group). *P < .05, ** P < .01, *** P < .001, **** P < .0001.

Figure 5. CSF1-Fc enhances hepatic phagocytic capacity after partial hepatectomy in mice. (A) Liver weight to body weight ratio after PH with CSF1-Fc or control (n = 8/group). (B) Ki67+ hepatocytes per high-power field after PH (n = 8/time point/group). (C) Number of resident (red) or infiltrating (blue) hepatic macrophage populations on day 2 after PH and CSF1-Fc administration. Representative dot plots of hepatic macrophage profile and representative Ly6C profile normalized to mode. (D) Hepatic gene expression of phagocytic markers macrophage receptor with collagenous structure (MARCO), macrophage scavenger receptor 1 (MSR1), and mannose receptor (MR) vs relevant control. (E) Multiphoton image of ex vivo Csf1r-eGFP mouse liver after injection of fluorescent microbeads and clearance from the circulation following sham or two-thirds PH with control or CSF1-Fc (n = 6/group/time point). (F) Net fluorescence liver, spleen, lung, kidney, and brain on day 2 after PH and CSF1-Fc or control (n = 6 per group). *P < .05, ** P < .01, *** P < .001, **** P < .0001.
CSF1-Fc Treatment Accelerates Recovery of Innate Immune Capacity After Partial Hepatectomy

Patient survival depends on the rapid restoration of liver macrophage functions to clear pathogenic material. We therefore tested the effect of CSF1-Fc on innate immune function in injury models. CSF1-Fc administration increased liver size over controls at 4 days after PH (Figure 5A). Hepatocyte staining (CYPD2) per unit area (Supplementary
Figure 5. CSF1-Fc and acetaminophen intoxication in mice. (A) Liver weight to body weight ratio with CSF1-Fc or control. (B) Representative immunohistochemistry F4/80 (red) and Ki67 (3,3′-diaminobenzidine [DAB]) at day 2 and day 4 after acetaminophen with phosphate-buffered saline control or CSF1-Fc. (C) Hepatic expression of phagocytosis-associated genes after GW2580 (red), AFS98 (blue), or CSF1-Fc (grey) relative to the mean of the control group (vehicle, rat IgG2a, phosphate-buffered saline, respectively). (D) Net ex vivo liver fluorescence 15 minutes after injection of fluorescent beads. (E) Serum liver-associated biochemistry tests at day 3 after acetaminophen intoxication and either GW2580 (red), AFS98 (blue), or CSF1-Fc (grey) compared with control (vehicle, rat IgG2a, phosphate-buffered saline, respectively). MARCO, macrophage receptor with collagenous structure; MR, mannose receptor; MSR1, macrophage scavenger receptor 1. *P < .05, **P < .01, ***P < .001, ****P < .0001.

Figure 6. Contribution of hepatic phagocytes to clearance of pathogenic material. (A) Immunohistochemistry of hepatic macrophages (F4/80 = green) after isolation by adherence and administration of E coli bioparticles (pHrodo) (red). (B) Relative number and percentage of resident macrophages phagocytosing E coli bioparticles (pHrodo, ThermoFisher Scientific, Waltham, MA). Representative density plots of phagocytic cells in the resident macrophage population. (C) Relative number of infiltrating macrophages phagocytosing E coli bioparticles compared with the mean of the control group with the percentage of cells phagocytosing E coli particles according to Ly6C expression status. (D) Pie charts illustrating the proportion of phagocytic cells in the liver with a bar chart showing the absolute cell number comparison for resident and infiltrating macrophages, neutrophils, and eosinophils. Eos, eosinophils; Neuts, neutrophils. *P < .05, **P < .01, ***P < .001, ****P < .0001.

Figure 7. CSF1-Fc and acetaminophen intoxication in mice. (A) Liver weight to body weight ratio with CSF1-Fc or control. (B) Day 2 Control Day 4 Control (C) Day 2 CSF1-Fc Day 4 CSF1-Fc Figure 5A) was reduced after CSF1-Fc treatment, indicating the increased size was owing to increased non-parenchymal cell accumulation. Peak hepatocyte proliferation (day 2) was not increased by CSF1-Fc treatment (Figure 5B), although at later time points the macrophage accumulation did promote increased hepatocyte proliferation compared with controls. To confirm the role of endogenous CSF1 signaling implied from studies of op/op mice, we treated...
with a CSF1R kinase inhibitor (GW2580) or a blocking antibody against the CSF1 receptor (AFS98). Both treatments reduced hepatocyte proliferation (Supplementary Figure 5B) and affected the expression of macrophage-related cytokine and matrix remodeling genes associated with regeneration (Supplementary Figure 5C and D).

After PH, macrophages accumulated more rapidly in the liver of CSF1-Fc–treated mice, involving both monocyte-derived infiltration and proliferation (Figure 5C). There was a corresponding increase in genes encoding phagocytic receptors, such as macrophage receptor with collagenous structure and macrophage scavenger receptor 1, with a reciprocal reduction after CSF1 blockade (Figure 5D). To assess the impact on clearance of insoluble material and bacteria-derived particles, we injected fluorescent-labeled latex microbeads intravascularly. These were rapidly and selectively taken up by liver phagocytes. There was minimal uptake by the spleen, lung, kidney, brain, and circulating cellular populations (Supplementary Figure 5E–G). Multiphoton imaging of the Csf1r-eGFP mouse liver confirmed that microbeads were phagocytosed by hepatic macrophages with CSF1-Fc treatment, causing enhanced clearance from the circulation (Figure 5E). Ex vivo whole-organ fluorescence imaging indicated this enhanced clearance capacity was clearly due to liver uptake (Figure 5F). To extend these findings to potential pathogens, we used pH-sensitive Escherichia coli bioparticles that fluoresce when taken up into acidified vesicles and injected these into the portal vein (Figure 6A). CSF1-Fc treatment increased both the internalization capacity and the absolute yield of positive cells (Figure 6B). Relatively few infiltrating monocyte-derived cells (F4/80hi CD11bhi) internalized the labeled E coli, but CSF1-Fc treatment again increased the clearance capacity (Figure 6C). The Ly6Clo monocyte population consistently showed a greater propensity for phagocytosis compared with the Ly6Cni population based on the percentage of the populations phagocytosing the E coli particles both in the control and CSF1-Fc–treated groups (Figure 6C). The resident F4/80hi CD11blo cells remained the dominant phagocyte in the liver (Figure 6D).

### The Impact of CSF1-Fc on Acetaminophen Toxicity

The predictive value of serum CSF1 levels in patients with ALF, and the ability of CSF1-Fc to promote regeneration and improve clearance functions, suggests therapeutic potential in acetaminophen toxicity. Macrophage accumulation, proliferation of resident macrophages, and infiltration of monocyte-derived macrophages is essential for recovery and subsequent regeneration after acetaminophen administration to mice. Enhanced macrophage accumulation could facilitate recovery by rapidly clearing necrotic debris and restoring hepatic immune function. We treated mice with CSF1-Fc 12 hours after acetaminophen intoxication, the point of maximal injury. CSF1-Fc treatment expanded the macropage compartment and increased the liver weight to body weight ratio (Figure 7A). CSF1-Fc increased macrophage accumulation at the area of necrosis (Figure 7B), without significantly increasing the affected area (Supplementary Figure 6A). In control-treated animals there was a predominance of infiltrating monocytes relative to resident hepatic macrophages as previously described, and both of these populations were boosted by CSF1-Fc (Supplementary Figure 6B). Expression of mRNA for clearance receptors (macrophage receptor with collagenous structure and macrophage scavenger receptor) was enhanced in the livers of CSF1-Fc–treated animals (Figure 7C), associated with an increase in the phagocytic capacity of the liver detected using injected microbeads (Figure 7D). Despite the profound macrophage changes in the liver, serum cytokines were unaffected by CSF1-Fc treatment (Supplementary Figure 6C). Increased macrophage recruitment did not produce additional injury. Serum injury markers (ALT, alkaline phosphatase) decreased in CSF1-Fc–treated mice, with reciprocal change after CSF1R blockade (Figure 7E and Supplementary 7F and G). These findings most likely reflect changes in the clearance of these enzymes by hepatic macrophages (Figure 7E). Serum albumin level was reduced by CSF1-Fc treatment, likely a reflection of the proinflammatory state given that hepatic albumin gene expression was unchanged from control (Supplementary Figure 6D). Serum total protein also was unchanged (Supplementary Figure 6E). To further explore the potential direct effects of CSF1-Fc on hepatocytes we assessed hepatocyte viability and performed metabolic assays, which showed no direct effect of CSF1-Fc on either mouse or human hepatocytes (Supplementary Figure 7A and B). Furthermore, after acetaminophen intoxication in mice, there was no change in cytochrome p-450 activity assessed by CYP2E1 expression with CSF1-Fc treatment (Supplementary Figure 7C).

### Discussion

We have shown a clear association between reduced serum CSF1 level and poor outcome in acute liver failure in human beings. CSF1-Fc treatment produced hepatic macrophage accumulation through in situ macrophage proliferation and recruitment of monocyte-derived cells in mouse models. Resident macrophages in the mouse are largely maintained through self-renewal. Fate mapping of hematopoietic cells indicated that CSF1-Fc also can drive conversion of circulating monocytes to cells of a resident macrophage phenotype. This novel finding shows the plasticity in the resident and infiltrating macrophage compartments and provides new evidence that bone marrow–derived macrophages can contribute to the resident macrophage population given appropriate stimuli.

CSF1-Fc–driven hepatic macrophage accumulation enhanced innate immune capacity in mouse models of liver injury. After PH, the therapeutic requirement to optimize liver function and boost regeneration must be weighed against the potential to promote cancer recurrence. Malignant tumors themselves can produce CSF1, which mediates macrophage accumulation, supporting tumor growth.
Indeed, our series of preoperative patients with cancer in situ had increased serum CSF1 levels. However, effective elimination of circulating tumor cells, which are indicative of recurrence, requires hepatic macrophages, which depend on CSF1. The ability to enhance the innate immune capacity of the liver by increasing hepatic macrophage density may be valuable from an antimicrobial standpoint and theoretically may reduce cancer recurrence rates, which can reach 60%.44

ALF represents a different clinical challenge to PH. The low serum CSF1 level in those who required liver transplantation or died in our patient cohort is consistent with the monocytopenia described in ALF, particularly given the persistence of monocyte precursors in the bone marrow.16,17 Monocytes express low levels of HLA-DR in ALF, which can impair the response to sepsis.17 Together with our results, these findings indicate that supplementary CSF1 therapy in the setting of low serum CSF1 levels might facilitate recovery by increasing monocyte numbers, induce a proregenerative macrophage phenotype, increase monocyte HLA-DR expression,35 and enhance phagocytic capacity. Multiorgan involvement is characteristic of clinical deterioration, which could highlight potential wider benefits of this strategy. The ability to predict patient deterioration, using a marker such as CSF1, before meeting the current clinical criteria for transplantation (King’s College Hospital criteria) could facilitate the earlier stratification of patients with the greatest need. It would be interesting to study the role of CSF1 in acute-on-chronic liver failure, in which innate immunity may be impaired.

There is mounting evidence for a CSF1–CCR2 axis in monocyte recruitment with the induction of these factors after acute hepatic injury. The chemokine signaling induced in the liver after CSF1-Fc administration was not restricted to the CCR2 receptor, and monocyte extravasation into the liver parenchyma was impaired, but not prevented by the CCR2 defect, suggesting that CSF1-Fc either mobilizes monocytes from other sources (eg, the spleen) or overcomes the CCR2 dependence on bone marrow release.

In contrast to PH, in which the increase in available CSF1 is related to reduced clearance, in acute liver toxicity hepatic CSF1 mRNA increased (Supplementary Figure 2C). Evidence of local production also was seen in the liver of patients (Supplementary Figure 2D). The increased local hepatic CSF1 production may drive macrophage accumulation during the early response to injury, when phagocytosis is essential to clear dying hepatocytes. As well as the improved clearance of insoluble and infective material to reduce the risk of sepsis, macrophages also might promote clearance of circulating tumor cells. Some of the earliest studies of CSF1 treatment showed an impact on tumor metastasis. CSF1-Fc already has been shown to be safe in pigs, and the native protein was tested previously by continuous infusion in human phase 1 trials and was well tolerated.

In summary, we have shown that increased serum CSF1 is an important response to liver injury, and impairment of this response is associated with poor outcome in acute liver failure. Serum CSF1 response after liver injury could be used to stratify patients according to severity and to identify candidates for CSF1 therapy.

Supplementary Material
Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.08.053.

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Conflicts of interest
These authors disclose the following: a patent application has been filed by the University of Edinburgh for CSF1-based therapeutics in the treatment of liver disease and B. M. Stutchfield, D. J. Gow, D. A. Hume, and S. J. Forbes are listed as co-inventors. The remaining authors disclose no conflicts.

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Supplementary Materials and Methods

Human Serum Samples

Serum samples were blinded and cytokine analysis was completed in a random order. Serum CSF1 was analyzed using the Meso Scale Discovery CSF1 immunoassay and analyzed on a Meso QuickPlex SQ120 (Meso Scale Diagnostics, Rockville, MD). Serum acetyl-HMGB1 was analyzed by mass spectrometry.

Clinical Scoring

King’s college criteria in the context of acetaminophen-induced liver failure was defined as arterial pH less than 7.3, international normalized ratio greater than 6.5, serum creatinine level greater than 300, and the presence of encephalopathy. The systemic inflammatory response criteria were met when 2 or more of the following occurred: body temperature higher than 38°C or less than 36°C, heart rate faster than 90 beats per minute, respiratory rate greater than 20 breaths per minute, or white blood cell count greater than 12,000/cf mm or less than 4000/cf mm. The APACHE II score was calculated as previously described.

Reagents

CSF1-Fc is a conjugate of porcine CSF1 with the Fc region of porcine IgG1α (43.82 kilodaltons) produced by Zoetis (Florham Park, NJ) for D. Hume (UK patent application GB1303537.1). Porcine CSF1 is equally active in mice. The Fc conjugate provides increased circulating half-life. CSF1-Fc did not show any endotoxin-like activity in murine bone marrow–derived macrophages. CSF1-receptor blockade was induced by the CSF1R tyrosine kinase inhibitor, GW2580 (160 mg/kg suspended in 0.5% hydroxypropylmethylcellulose and 0.1% Tween 80, LC Laboratories, Woburn, MA), or using the antibody AFS98 produced by Sudo et al and provided by BioServ UK (Sheffield, Yorkshire, UK). CSF1-Fc, GW2580, and AFS98 were administered immediately after two-thirds partial hepatectomy or 12 hours after acetaminophen intoxication (point of maximal injury).

Collection of Mouse Tissues

Mice were culled via CO₂ inhalation and after a midline laparotomy blood was aspirated from the inferior vena cava for serum analysis. Mice were perfused through the inferior vena cava and viscera were excised and weighed. Viscera were either collected at the point of maximal injury or placed in phosphate-buffered saline for flow cytometry.

Immunohistochemistry

Sections of formalin-fixed tissue (3 μm) were used for immunostains. Ki67, bromodeoxyuridine, and CYPD2 required heat-mediated antigen retrieval with 0.01 mol/L sodium citrate, pH 6.0, for 10 minutes. Primary antibodies were used at the following dilutions: Ki67 (Leica Microsystems, Wetzlar, Germany) 1:500, bromodeoxyuridine (Abcam, Cambridge, UK) 1:100, F4/80 (clone CI:A3-I; Biolegend, San Diego, CA) 1:100, and CYPD2 (Abcam) 1:100. Appropriate secondary antibody was applied at a 1:250 dilution. Dual immunohistochemistry with F4/80 and bromodeoxyuridine or Ki67 was performed by first developing F4/80 using the tyramide signal amplification system (PerkinElmer, Waltham, MA) with subsequent heat-mediated antigen retrieval followed by bromodeoxyuridine or Ki67 staining. Ki67 and F4/80 dual immunohistochemistry also was performed by developing F4/80 with an alkaline phosphatase substrate kit (red, Vector Laboratories, Burlingame, CA), and after heat-mediated antigen retrieval Ki67 was developed with 3,3-diaminobenzidine (Dako, Ely, Cambridgeshire, UK). Stained slides were blinded and images were taken on a Nikon Eclipse E600 (Shinagawa, Tokyo, Japan). For image quantification of F4/80 staining, 20 nonoverlapping images were photographed at ×200. The extent of 3,3’-diaminobenzidine (DAB) staining was quantified using image analysis software (Adobe Photoshop CS6). For CYPD2 quantification, images were quantified using image analysis software (Adobe Photoshop CS6, Adobe, San Jose, CA). For Ki67 quantification, 20 serial nonoverlapping images were photographed at ×400, and then hepatocytes were identified by assessment of morphology.

Flow Cytometry

Liver was digested in 2 mg/mL collagenase D (Sigma-Aldrich) at 37°C for 30 minutes and then passed through a 100-μm filter. A 7-minute 50g spin was performed to remove hepatocytes. Further purification of nonparenchymal cells was performed using 30% Percoll (Sigma) gradient. Cells were stained with fixable viability dye eFluor 780 and then incubated with Fc block (True- affincX; Biolegend), before staining with CD45 (clone:30F11, AF700; Biolegend), F4/80 (clone:BM8, PE/Cy7; Biolegend), CD11b (clone:RM208, fluorescein isothiocyanate; Invitrogen, Waltham, MA), Ly6C (clone:HK1.4, PerCP/Cy5.5; Biolegend), dump gate (PE: CD3 [clone:17A2, PE; Biolegend], CD19 (clone:6D5, PE; Biolegend), Siglec F (clone:E502440, PE; BD Biosciences, Franklin Lakes, NJ), and Ly6G (clone:IA8, PE; BD Biosciences). For the proliferation assay, cells were fixed and permeabilized using the BD Pharmingen bromodeoxyuridine flow kit and then stained with antithromboxyuridine (fluorescein isothiocyanate; BD Pharmingen) and Ki67 (eFluor 600; eBioscience, San Diego, CA). Flow cytometry was performed using the LSR Fortessa (BD Biosciences).

Quantification of mRNA

Quantification of mRNA levels by real-time reverse-transcription polymerase. RNA extraction kits (Qiagen, Hilden, Germany) were used to extract RNA from whole tissue. Predesigned validated primer sets for macrophage receptor with collagenous structure, macrophage scavenging receptor 1, mannose receptor, interleukin 6, oncostatin M, tumor necrosis factor, interferon γ, interleukin 10, and glyceraldehyde-3-phosphate dehydrogenase
were purchased from Qiagen (Qiagen Quantitect Primers). Quantitative real-time polymerase chain reaction was performed using Express SYBR Green (Qiagen, UK). Gene expression was calculated relative to glyceraldehyde-3-phosphate dehydrogenase for each sample. Gene array at 6 hours after CSF1-Fc administration was performed using cytokine and chemokine array RT^2 profiler polymerase chain reaction arrays and analyzed using the online RT^2 profiler polymerase chain reaction array data analysis (version 3.5; Qiagen, UK), and presented by Volcano plot. Affymetrix (Santa Clara, CA) Mouse gene 1.1 ST array data were accessed from the Gene Expression Omnibus website and analyzed using GEO2R, with Benjamini and Hochberg (false-discovery rate) correction applied to the entire data series.

**Phagocytosis Assay**

Under 2% isofluorane anesthesia, the inferior vena cava was cannulated and 100 μL of red fluorescent bead solution (1:5 latex beads 1.0 μm, fluorescent red; Sigma-Aldrich) was infused through the cannula (1:2 solution for assay after paracetamol injury). Ex vivo fluorescent quantification was performed at 1 minute after bead injection and 15 mL 0.9% NaCl flush (Supplementary Figure 5A). For assessment of bead clearance from the circulation 20 μmol/L of blood was removed from the cannula every 2 minutes starting from 1 minute after injection for 15 minutes and immediately fixed (300 μL fluorescence-activated cell sorter-lyse; BD Biosciences). After the 15-minute time point the mice were perfused with 15 mL 0.9% saline through the inferior vena cava cannula with portal vein outflow. Organs then were removed (liver, spleen, lungs, kidney, brain) and imaged using a Kodak (Rochester, NY) In-Vivo Multispectral FX image station (excitation, 550 nm; emission, 600 nm; exposure, 1 s; F-stop, 2.8). Subsequently, blood samples were analyzed using a LSR-Fortessa flow cytometer (BD Biosciences) with fluorescent beads detected on the blue channel (B695/40) by a 1-minute sample collection on the low-flow rate setting. Multiphoton imaging was performed using a Zeiss (Oberkochen, Germany) LSM7 MP with Coherent Chameleon Ti:Sa laser.

**Mouse Serum Analyses**

Serum biochemistry assays were performed using commercially available kits by a biochemist, including ALT (Alpha Laboratories, Eastleigh, Hampshire, UK), alkaline phosphatase (Roche Diagnostics, Risch-Rotkreuz, Switzerland), total bilirubin (Alpha Laboratories), and albumin (Alpha Laboratories). Total serum protein was analyzed using the Bradford assay as previously described. Serum cytokines and chemokines were analyzed using the Milliplex mouse cytokine/chemokine array (Merck-Millipore, Darmstadt, Germany) in collaboration with a Merck-Millipore biomarker specialist.

**Hepatocyte Metabolic and Toxicity Assays**

Human and mouse hepatocytes were isolated from liver tissue as previously described. The glutathione depletion assay, MTS (3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reduction assay, and lactate dehydrogenase (LDH) leakage assay were performed on human and mouse hepatocytes as previously described.

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For healthy controls see Supplementary Figure 8

**Supplementary Figure 1.** Supporting details for partial heptectomy in humans. (A) Details of patients undergoing partial heptectomy categorised according to extent of resection (n=55). (B) Dot plot showing blood loss versus serum CSF1 (no relationship between these variables). (C) Details of patients developing postoperative liver failure (n=2). Blood loss according to extent of resection. (D) Hepatic CSF1 gene expression following partial heptectomy in mice.
Outcome  |  n  | Age  | M:F | ALT (U/L) | PT (Sec) | Creatinine (μmol/L) | Acetyl-HMGB1 (ng/ml) | CSF1 (ng/ml)  
---|---|---|---|---|---|---|---|---
Survived  | 47 | 37 (13) | 21:26 | 4412 (3764) | 38.7 (30.0) | 129.8 (120.1) | 0.46 (1.52) | 7.81 (2.82)  
Died/Liver transplantation  | 31 | 42 (15) | 10:21 | 4814 (3060) | 70.0 (38.8) | 213.8 (112.8) | 4.49 (5.70) | 3.69 (2.49)  

Healthy controls

| n  | Age  | M:F | Acetyl-HMGB1 (ng/ml) | CSF1 (ng/ml)  
---|---|---|---|---
50 | 35 (10) | 4/5 | 0.06 (0.09) | 0.22 (0.12)  

Systemic inflammatory response scoring (SIRS)

One point for each of the following:
- Temperature >38°C or <36°C
- Heart rate > 90bpm
- Tachypnoea >20bpm
- White cell count <4000 cells/mm³ or >12,000 cells/mm³

Outcome  |  n  | Age  | M:F | ALT (U/L) | Bilirubin (mg/dl) | Acetyl-HMGB1 (ng/ml) | CSF1 (ng/ml)  
---|---|---|---|---|---|---|---
Survived  | 10 | 39 (9) | 2:3 | 1370 (1104) | 3.1 (1.8) | 0.33 (0.29) | 1.84 (1.33)  
Died/Liver transplantation  | 10 | 41 (14) | 2:3 | 3873 (1902) | 3.5 (1.9) | 2.06 (2.59) | 1.25 (1.08)  

AUC: 0.86
SE: 0.045
p<0.0001
### Supplementary Figure 2. (continued)

**A** Details of acetaminophen intoxication patients presenting to the specialist liver unit with acute liver failure grouped according to survivors versus those who subsequently required liver transplantation or died (patient cohort and acetyl-HMGB1 values as per Antoine et al.18; healthy control data also shown). **(B)** Serum CSF1 level in healthy volunteers and in patients following paracetamol intoxication on arrival to a specialist liver unit grouped according to whether patients subsequently deteriorated to meet the King’s college criteria or not (KCC no: n=45; KCC yes: n=33). **(C)** Receiver operator characteristic curves based on serum CSF1 level in patients according to King’s College Criteria and also patients who subsequently survived or died/required liver transplantation. **(D)** Serum CSF1 level according to systemic inflammatory response score (SIRS) on admission to the tertiary referral hospital with acute liver failure (NB. SIRS scores available for n=60). **(E)** Details of patients from first presentation to hospital following acetaminophen intoxication (n=10 per group; patients randomly selected from patient cohort as per Antoine et al.18). **(F)** Immunohistochemistry for the CSF1 protein in explant liver following acetaminophen intoxication. **(G)** Hepatic CSF1 gene expression following acetaminophen intoxication in mice (One way ANOVA with Bonferroni post hoc). **(H)** Deviance residuals for logistic regression models. **(I)** Analysis of deviance comparing combined Log(acetyl-HMGB1) + CSF1 model (Model 1) and CSF1 alone (Model 2). *P < .05, **P < .01, ***P < .001, ****P < .0001.
### Table A

| Symbol       | Log$_2$(FC) | p Value | Cntf | 2.34  | 4.0E-02 | Il10 | 1.23  | 9.8E-02 | Il4   | 0.56  | 2.4E-01 | Tnfsf11 | 0.72  | 2.3E-01 |
|--------------|-------------|---------|------|--------|---------|------|-------|---------|-------|-------|---------|---------|-------|---------|
| Adipoq       | 1.22        | 1.9E-01 |      |        |         |      |       |         |       |       |         |         |       |         |
| Bmp2        | 0.04        | 7.0E-01 | Cdf1 | -0.75  | 4.6E-02 | Il11 | -0.23 | 2.5E-01 | Il5   | -0.52 | 4.9E-02 | Tnfsf13b | 0.36  | 4.4E-01 |
| Bmp4        | 0.32        | 5.6E-01 | Csf2 | -0.23  | 2.5E-01 | Il12a| 0.35  | 3.7E-01 | Il6   | 2.92  | 2.7E-02 | Vegfa   | -0.34 | 4.1E-02 |
| Bmp6        | 0.06        | 9.0E-01 | Csf3 | -0.23  | 2.5E-01 | Il12b| 1.62  | 5.4E-02 | II7   | 3.26  | 3.1E-02 | Xcl1    | 1.09  | 1.1E-01 |
| Bmp7        | -0.90       | 9.1E-03 | Ctf1 | -1.06  | 2.8E-02 | Il13 | 0.05  | 7.3E-01 | Il9   | -0.23 | 2.5E-01 |         |       |         |
| Col1        | -0.23       | 2.6E-01 | Cxcl1| -0.29  | 7.3E-01 | Il15 | 1.09  | 1.1E-01 | Ltf   | -0.49 | 3.5E-02 |         |       |         |
| Col11       | 1.32        | 9.7E-02 | Cxcl1| 2.51   | 1.2E-01 | Il16 | 0.06  | 7.6E-01 | Lta   | -0.26 | 2.1E-01 |         |       |         |
| Col12       | 4.92        | 2.8E-02 | Cxcl10| 1.46  | 8.6E-02 | Il17a| -0.23 | 2.5E-01 | Ltb   | -0.91 | 1.9E-01 |         |       |         |
| Col17       | -0.20       | 8.2E-01 | Cxcl11| 1.23  | 2.0E-01 | Il17f| -0.40 | 2.0E-01 | Mlf   | -0.40 | 2.3E-01 |         |       |         |
| Col19       | 0.65        | 9.6E-03 | Cxcl12| -0.33 | 2.8E-01 | Il18 | 1.23  | 8.2E-02 | Mstn  | -0.23 | 2.5E-01 |         |       |         |
| Col2        | 4.34        | 3.7E-02 | Cxcl13| 2.48  | 3.5E-02 | Il1a | 2.21  | 7.7E-01 | Nodal | -0.35 | 6.5E-01 |         |       |         |
| Col20       | -0.11       | 5.2E-01 | Cxcl16| 0.55  | 1.2E-01 | Il1b | 1.99  | 6.5E-02 | Osm   | -0.49 | 3.1E-01 |         |       |         |
| Col22       | -1.39       | 5.3E-02 | Cxcl3 | -0.23 | 2.5E-01 | Il1m | 0.77  | 2.2E-01 | P4    | 1.30  | 7.3E-02 |         |       |         |
| Col24       | 0.78        | 2.6E-01 | Cxcl5 | 0.40  | 4.3E-01 | Il2  | -0.23 | 2.5E-01 | Ppbp  | 1.13  | 6.2E-01 |         |       |         |
| Col3        | 3.19        | 1.1E-02 | Cxcl9 | 2.15  | 8.1E-02 | Il21 | -0.23 | 2.5E-01 | Spp1  | 2.36  | 5.2E-02 |         |       |         |
| Col4        | 2.61        | 3.3E-03 | Fasl  | 1.00  | 2.8E-01 | Il22 | -0.23 | 2.5E-01 | Tgfb2 | 0.11  | 6.2E-01 |         |       |         |
| Col5        | -0.26       | 3.4E-01 | Gpi1  | -1.01 | 1.5E-01 | Il23a| 0.55  | 2.0E-01 | Thpo  | -1.04 | 2.5E-01 |         |       |         |
| Col7        | 6.58        | 2.9E-02 | Hc1   | 1.60  | 6.5E-02 | Il24 | -0.23 | 2.5E-01 | Tnf   | 1.47  | 6.5E-03 |         |       |         |
| Cd40lg      | 0.74        | 1.9E-01 | Ifna2 | -0.28 | 1.9E-01 | Il27 | 0.33  | 4.6E-01 | Tnfsf11b | -0.13 | 2.5E-01 |         |       |         |
| Cd70        | -0.23       | 2.6E-01 | Ilng  | 0.97  | 2.4E-01 | II3  | -0.23 | 2.5E-01 | Tnfsf10 | 1.07  | 1.4E-01 |         |       |         |

### Graph B

**PBS Control** vs. **CSF1-Fc**

(Images of TUNEL +ve control (DNAse treated)

### Graph C

![Graph showing relative organ weight comparison](image)

**Relative organ weight**

- Spleen
- Kidney
- Lung
- Heart
- Brain

**Values**

- 0.0
- 0.5
- 1.0
- 1.5
- 2.0
Supplementary Figure 3. Supporting details for CSF1-Fc treatment of uninjured mice. (A) Array data 6 hours following CSF1-Fc administration in uninjured mice (n=4/group). (B) TUNNEL immunohistochemistry following PBS control or CSF1-Fc administration (positive control DNAse treated section). (C) Organ weight relative to mean of control group following 2 days treatment with PBS control (black solid circles), or CSF1-Fc (grey hollow circles). (D) Representative F4/80 immunohistochemistry following 2 days treatment with PBS control or CSF1-Fc.
Supplementary Figure 4. Supporting details for CSF1-Fc treatment of uninjured mice. (A) Representative flow cytometry dot plot of BRDU and Ki67 expression in resident (red) and infiltrating (blue) macrophages. (B) Representative dual immunohistochemistry F4/80 (green) and BRDU or Ki67 (red) Day 2 following CSF1-Fc administration or control. (C) Number of hepatic dendritic cells (CD11c/MHCII +ve), eosinophils and neutrophils in control (black circles) and CSF1- Fc (grey circles) treated mice relative to mean of control group. (D) Number of Ly6C low, intermediate and high monocytes in wild type and Ccr2-/- mice following 2 days treatment with CSF1-Fc or PBS control (two way ANOVA with Bonferroni post hoc). (E) Number of hepatic dendritic cells (CD11c/MHCII +ve), eosinophils and neutrophils in control (black circles) and CSF1-Fc (grey circles) treated mice relative to mean of control group. *P < .05, ** P < .01, *** P < .001, **** P < .0001.
Supplementary Figure 5. Supporting data for partial hepatectomy model. (A) Quantification CYPD2 immunofluorescence (red) per 20x HPF/mouse (control n=8; CSF1-Fc n=7; t test). (B) Number of mitotic figures and Ki67 positive hepatocytes per high powered field following partial hepatectomy and either GW2580, AFS98 or CSF1-Fc administration versus control (vehicle gavage, rat IgG2a, PBS; n=8/group; 2-way ANOVA comparing intervention with relevant control, Bonferroni post hoc). (C) Hepatic gene cytokine expression at Day 2 following partial hepatectomy and either GW2580, AFS98 or CSF1-Fc administration versus control (vehicle gavage, rat IgG2a, PBS; n=8/group; 2-way ANOVA comparing intervention with relevant control, Bonferroni post hoc). (D) Hepatic MMP and UPAR (urokinase plasminogen activator) gene expression Day 2 following partial hepatectomy and either GW2580, AFS98 or CSF1-Fc administration versus control (vehicle gavage, rat IgG2a, CSF1-Fc; n=8/group; 2-way ANOVA comparing intervention with relevant control, Bonferroni post hoc). (E) Exvivo fluorescent imaging of organs 1 minute following injection of fluorescent micro beads in to the inferior vena cava. (F) Flow cytometry plots demonstrating bead and cell gating of blood samples following fluorescent micro bead injection in to the inferior vena cava. (G) Representative flow plots of blood sampled from the inferior vena cava at 1 minute and 15 minutes following injection of fluorescent microbeads in to the circulation. Gating strategies including total fluorescent bead count (“Total beads”) and bead count with in blood cellular populations (“Cells + beads”). *P < .05, **P < .01, ***P < .001, ****P < .0001.
**Figure a:** Graph showing the percentage of area necrosis or cellular infiltrate over days 2, 3, and 4.

**Figure b:** Scatter plot comparing cell number over various days and conditions.

**Figure c:** Bar graph illustrating gene expression relative to GAPDH.

**Figure d:** Comparison of albumin gene expression relative to GAPDH in controls and CSF1-Fc treated conditions.

**Figure e:** Comparison of total protein levels in controls and CSF1-Fc treated conditions.

**Total cytokines/chemokines analysed:**
- G-CSF
- Eotaxin
- GM-CSF
- IFN-G
- IL-1a
- IL-1b
- IL-2
- IL-4
- IL-3
- IL-5
- IL-6
- IL-7
- IL-9
- IL-10
- IL-12
- p40
- p70
- LIF
- IL-13
- LIX
- IL-15
- IL-17
- IP-10
- KC
- MCP-1
- MIP-1a
- MIP-1b
- M-CSF
- MIP-2
- MIG
- RANTES
- VEGF
- TNF-a (analytes below background not shown on histogram)
Supplementary Figure 6. Supporting details for acetaminophen intoxication in mice, (A) Quantification of area of necrosis and cellular infiltrate at Day 2, 3 and 4 following acetaminophen intoxication. (B) Hepatic macrophage phenotype D3 following acetaminophen administration (F4/80+/CD11b− = resident macrophage population; F4/80−/CD11b+ = infiltrating macrophage population. (C) Serum cytokine array Day 4 following partial hepatectomy and either PBS control or CSF1-Fc treatment (2-way ANOVA and Bonferroni post hoc ns). (D) Hepatic albumin gene expression relative to GAPDH comparing control and CSF1-Fc treated mouse liver (n=8/group; t test ns). (E) Total protein concentration at D3. (F) Serum ALT following acetaminophen intoxication in control (dotted line) and CSF1-Fc treated (solid line) mice. *P < .05, ** P < .01, *** P < .001, **** P < .0001.
Supplementary Figure 7. No evidence of direct hepatocyte effects of CSF1-Fc. (A) MTS reduction assay, LDH leakage assay and GSH content on mouse hepatocytes exposed to APAP and increasing concentration of CSF1-Fc showing no dose related effect. (B) MTS reduction assay, LDH leakage assay and GSH content on human hepatocytes exposed to APAP and increasing concentration of CSF1-Fc showing no dose related effect. (C) Whole liver assessment of CYP2E1 expression relative to actin assessed via Western blot and mRNA day 3 post acetaminophen intoxication with control or CSF1-Fc treatment.