



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

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

Citation for published version:

Stutchfield, BM, Antoine, DJ, MacKinnon, AC, Gow, DJ, Bain, CC, Hawley, CA, Hughes, M, Francis, B, Wojtacha, D, Man, TY, Dear, JW, Devey, LR, Mowat, A, Pollard, JW, Park, BK, Jenkins, SJ, Simpson, KJ, Hume, DA, Wigmore, SJ & Forbes, SJ 2015, 'CSF1 Restores Innate Immunity Following Liver Injury in Mice and Serum Levels Indicate Outcomes of Patients With Acute Liver Failure', *Gastroenterology*, vol. 149, no. 7, pp. 1896-1909 . <https://doi.org/10.1053/j.gastro.2015.08.053>

Digital Object Identifier (DOI):

[10.1053/j.gastro.2015.08.053](https://doi.org/10.1053/j.gastro.2015.08.053)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Gastroenterology

Publisher Rights Statement:

This is the author's final peer-reviewed manuscript as accepted for publication

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



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

Short Title: CSF1 indicates outcome in liver failure

Authors: Stutchfield B.M.^{1,2}, Antoine D.J.³, Mackinnon A.C.¹, Gow D.J.⁴, Bain C.C.⁵, Hawley C.A.⁶, Hughes M.², Francis B.⁷, Wojtacha D.¹, Man T.Y.¹, Dear J.W.⁸, Devey L.R.⁶, Mowat A.Mcl.⁵, Pollard J.W.⁹, Park B.K.³, Jenkins S.J.⁶, Simpson K.J.², Hume D.A.⁴, Wigmore S.J.², Forbes S.J.^{1*}

Affiliations: ¹MRC Centre for Regenerative Medicine, University of Edinburgh, UK; ²Division of Clinical and Surgical Sciences, University of Edinburgh, UK; ³MRC Centre for Drug Safety Science, Dept. Molecular and Clinical Pharmacology, University of Liverpool, UK; ⁴The Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK; ⁵Institute of Infection, Immunity and Inflammation, University of Glasgow, UK; ⁶MRC Centre for Inflammation Research, University of Edinburgh, UK; ⁷Department of Biostatistics, Institute of Translational Medicine, University of Liverpool, Merseyside, UK; ⁸National Poisons Information Service Edinburgh, Royal Infirmary of Edinburgh, UK; ⁹MRC Centre for Reproductive Health, University of Edinburgh, UK.

Grant Support: Wellcome Trust, Scottish Translational Medicine and Therapeutics Initiative to BMS; MRC Centre for Regenerative Medicine, Edinburgh UK; UK Regenerative Medicine Platform to SJF; BBSRC Institute Strategic Programme Grant to The Roslin Institute and MRC Grant to DAH; MRC New Investigators Research Grant to SJJ.

Abbreviations: CSF1: colony stimulating factor 1; CSF1R: CSF1 receptor; HMGB1: high mobility group protein 1; PH: partial hepatectomy

Correspondence: SJ Forbes, Professor of Transplantation and Regenerative Medicine, Scottish Centre for Regenerative Medicine, 5 Little France Drive, Edinburgh BioQuarter, Edinburgh EH16 4UU. Tel: +44 (0)131 651 9500; Fax: +44 (0)131 651 9501. Email: stuart.forbes@ed.ac.uk

Acknowledgements: Serum collection and collaboration from Hartmut Jaeschke and Matt Sharp at Kansas Medical Centre, USA. Multiphoton imaging support from B McColl and M. Pattison, Roslin Institute, University of Edinburgh. We acknowledge colleagues at Zoetis, Kalamazoo (Graeme Bainbridge, Pamela L. Boner, Greg Fici, David Garcia-Tapia, Roger A. Martin, Theodore Oliphant, John A. Shelly, Raksha Tiwari, Thomas L. Wilson) who collaborated in the development, characterization and production of CSF1-Fc.

Author contributions: All authors have been involved in acquisition of data and critical review of the manuscript; Stutchfield BM, Antoine DJ, Mackinnon AC, Jenkins SJ, Hume DA, Wigmore SJ, Forbes SJ were involved in study design and concept;

Stutchfield BM, Hume DA, Wigmore SJ and Forbes SJ obtained funding; Mackinnon AC, Bain CC, Jenkins SJ, Mowat A.Mcl, Wojtacha D, Man TY produced experimental data and/or key materials

Conflict of interest statement: A patent application has been filed by the University of Edinburgh for CSF1 based therapeutics in the treatment of liver disease. BM Stutchfield, DJ Gow, DA Hume and SJ Forbes are listed as co-inventors. No other author disclosures.

Abstract

Background & Aims: Liver regeneration requires functional liver macrophages, which provide an immune barrier that is compromised following liver injury. Numbers of liver macrophages are controlled by macrophage colony stimulating factor (CSF1). We examined the prognostic significance of 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 *Ccr2*^{-/-} mice; we performed fate-tracing experiments using bone marrow chimeras. We gave CSF1-Fc to mice following 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, 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 trans-differentiation of infiltrating monocytes into cells with a hepatic macrophage phenotype. CSF1-Fc increased innate immunity in mice following partial hepatectomy or acetaminophen-induced injury, with resident hepatic macrophage the main effector cell.

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 following liver injury.

Keywords: drug-induced liver damage; clearance; immune response; M-CSF

Introduction

The liver provides an essential immune barrier against gut-derived pathogens entering the portal circulation¹. While surgical removal of liver tissue (partial hepatectomy) results in rapid compensatory upregulation of metabolic function, the liver's innate immune capacity is markedly impaired^{2, 3}. Acute toxic liver injury leads to widespread hepatocyte necrosis and compromises barrier function⁴. Changes in gut wall integrity associated with liver failure facilitate the translocation of gut-derived pathogens⁵. Consequently, sepsis is common in patients with liver failure and is strongly-associated with high mortality rates^{6, 7}. 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 defence and promote hepatocyte proliferation following liver injury^{8, 9}. 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, CSF1R^{10, 11}. *Csf1*-deficient mice (*op/op*) have few tissue macrophages and impaired liver regeneration following partial hepatectomy¹². Hepatic macrophages control circulating CSF1 levels via receptor-mediated endocytosis through CSF1R¹³. In humans following living donor partial hepatectomy, elevated circulating CSF1 is associated with more rapid liver regrowth¹⁴. In acute toxic liver injury models, monocyte-derived macrophage recruitment is required for necrotic tissue resorption¹⁵. In human acute liver injury, hepatic macrophages are implicated in tissue repair and low monocyte counts are associated with mortality^{16, 17}. Based upon these findings there is a strong rationale for exploring the potential of macrophage-based therapeutics to improve outcomes following acute liver injury.

Here we demonstrate that high serum CSF1 is associated with survival in patients with ALF and outperforms previous markers of outcome in terms of discriminative ability. We demonstrate 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 (2) for patients undergoing PH at the Hepatobiliary Unit, Royal Infirmary Edinburgh, between December 2012 and October 2013. Liver failure was defined according to Schindl et al.⁷. For the acetaminophen induced acute liver failure (ALF) cohort, ethical approval was granted by the local human research ethics committee and informed consent obtained from all patients, or next of kin, before study entry. This study builds on previous analysis of this patient cohort by Antoine et al.¹⁸, representing 78 adult patients admitted to the Royal Infirmary Edinburgh, UK or the University of Kansas Medical Centre, USA with acute liver injury. Serial patient samples from a second patient cohort were collected at admission to hospital (as opposed to admission to the specialist liver centre with acute liver failure)¹⁸. Details of serum analyses are provided in the supplementary methods. Primary hepatocytes were isolated from human liver tissue obtained from liver resection specimens immediately following surgery, with full informed consent and ethical approval from the relevant authorities (National Research Ethics Service REC reference: 11/NW/0327). See Supplementary Methods for assay details.

Animal experiments

Animal procedures were approved by the relevant institutional ethics committee (Albert Einstein College of Medicine, USA, University of Edinburgh, UK, and the University of Glasgow, UK) and adhered to the Animals (Scientific Procedures) Act 1986, UK and NIH guide for the Care of Laboratory Animals, USA. 8 -12 week male mice were used. *CCR2*^{-/-}, C57Bl/6 and MacGreen mice (*Tg(Csf1R-GFP)*_{Hume}¹⁹ were bred and maintained under specific pathogen-free conditions. *Tg(Csf1r-Mer2iCre)*_{jwp} were crossed to Rosa floxed stop tomato red and lineage tracing experiments performed as described²⁰. Fate tracing bone marrow-derived monocytes was performed using a mouse chimera as previously described²¹. Wild type C57BL/6 mice were obtained from Charles River. Mice were randomly distributed and maintained on 12-hr light-dark cycle with feed *ad libitum*. 2/3 partial hepatectomy was performed as previously described²². Acetaminophen intoxication involved intraperitoneal

administration of 350mg/kg acetaminophen (Sigma Aldrich)²³. Treatment group received 0.75mcg/g CSF1-Fc, prepared as described previously²⁴ (control: PBS) administered subcutaneously immediately following partial hepatectomy or 12 hours following acetaminophen intoxication and subsequently every 24 hours for up to three further doses. Reagents and methodology for immunohistochemistry, flow cytometry, quantification of mRNA, phagocytosis assay and serum analyses are provided in the supplementary methods.

Hepatocyte toxicity and metabolic assays

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

Statistics

Statistical analysis was performed on Graphpad Prism V6.0, except logistic regression analyses which were conducted in R^{25} . All data are presented as mean +/- standard error of the mean unless otherwise stated. Two tailed Student's t test are used where appropriate to analyse parametric data. One-way and Two way ANOVA with Bonferroni adjustment are stated when used. Human serum analyses and development of the logistic regression models were completed by a qualified statistician. Level of significance was set at $p<0.05$ for all analyses (figures $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$).

Results

Serum CSF1 rises 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 1A) serum CSF1 was significantly elevated compared to healthy controls. There was a small reduction at day 1 post 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

fall in serum CSF1 level may be due to removal of tumour cells, which secrete CSF1²⁶. We hypothesized that the subsequent rise in serum CSF1 level might be produced by proliferating hepatocytes. Due to the risks associated with liver biopsy in humans, we examined a mouse model of 2/3 PH CSF1 mRNA was unchanged following 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; clinical details in Supplementary Figure 1D).

We sampled serum from a large patient cohort with established acetaminophen-induced ALF on arrival at the specialist centre (cohort details: Supplementary Figure 2A)¹⁸. Assessment of ALF and requirement for liver transplantation is currently based on the validated modified King's College Hospital (KCH) criteria which reflect poor clinical condition and likelihood of death. Low serum CSF1 was significantly associated with patient deterioration to KCH 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 (SIRS²⁷) >2 had a significantly lower CSF1 level (Supplementary Figure 2D). Serial samples in a separate patient cohort (details Supplementary Figure 2E), followed from first presentation to hospital, showed serum CSF1 levels continued to increase in patients whose liver regenerated, whereas CSF1 levels fell in those who deteriorated (Figure 2B). In the livers removed from transplant recipients, CSF1 was detected in hepatocytes and non-parenchymal 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 following acetaminophen intoxication peaking at Day 2 (Supplementary Figure 2F).

The current best available prognostic biomarker in ALF is serum acetyl-HMGB1 (high mobility group box-1)¹⁸. This DAMP (damage associated molecular patterns) is released from necrotic tissue and by activated immune cells in response to injury²⁸. We assessed the discriminative ability of acetyl-

HMGB1, alongside CSF1 and also established clinical measures including bilirubin, prothrombin time and ALT level using the receiver operator characteristic (ROC) curve (Figure 2C). Serum CSF1 and acetyl-HMGB1 demonstrate similar profiles, whereas bilirubin, PT, ALT and APACHEII 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; 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 report that CSF1R is expressed outside the macrophage lineage^{29, 30}. We therefore examined CSF1R expression using MacGreen mice, where eGFP is under the control of the *Csf1r* promoter¹⁹. 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 labelling of CSF1R⁺ cells, as previously described²⁰. Co-localisation of the pan-macrophage marker F4/80 confirmed that all dTomato⁺ cells (CSF1R⁺) cells belonged to the macrophage lineage (Figure 3B). These data are supported by expression profiling from the FANTOM5 consortium that show no detectable CSF1R mRNA in hepatocytes isolated from control, or regenerating, mouse liver or isolated human hepatocytes³¹.

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 following CSF1-Fc administration to uninjured mice there was a marked upregulation of hepatic chemokines, particularly CCL2, CCL7 and CCL12, which are ligands for the CCR2 receptor and highly expressed by classical (Ly6C⁺) blood

monocytes (Figure 3C; array details Supplementary Figure 3A). After 4 days of CSF1-Fc treatment 20% of the liver was composed of F4/80⁺ macrophages compared to 2% in steady state (Fig 3D). This macrophage accumulation initiated hepatocyte proliferation at day 4²⁴. Mechanisms of hepatocyte proliferation are multifactorial with an upregulation of many cytokines and chemokines associated with the inflammatory response²⁴. Despite the induction of pro-inflammatory cytokine mRNA in the liver, serum ALT and AST were reduced with CSF1-Fc treatment, while bilirubin was unchanged (Fig 3F) and there was no hepatocyte apoptosis (Supplementary Fig 3B). The spleen increased in size although weight of other organs did not change (Supplementary Fig 3C and 3D).

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 following acetaminophen-induced injury^{20, 33-35}. Following CSF1-Fc treatment, there was a two-fold increase in cells with a resident hepatic macrophage phenotype (F4/80^{hi}CD11b^{lo}) and over a five-fold increase in the monocyte-derived infiltrating macrophages (F4/80^{lo}CD11b^{hi} cells), consisting predominantly of Ly6C^{hi} monocytes (Figure 4A). Both F4/80^{hi}CD11b^{lo} and F4/80^{lo}CD11b^{hi} cells proliferated markedly *in situ* following CSF1-Fc administration (Figure 4B; 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, F4/80^{lo}CD11b^{hi} macrophages and blood monocytes exhibited equivalent donor chimerism (Figure 4C). In PBS treated animals F4/80^{hi}/CD11b^{lo} cells remained almost exclusively of host origin, consistent with their proposed tissue origin^{20, 33, 34}. However, following CSF1-Fc treatment, approximately 20% of the F4/80^{hi}/CD11b^{lo} 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 following CSF1-Fc administration (Figure 3C).

Ligands for the CCR2 receptor, which are potent monocyte chemoattractants, were upregulated 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 F4/80^{lo} CD11b^{hi} macrophages would be prevented since Ly6C^{hi} monocytes are thought to depend upon CCR2 signals for release from the bone marrow and extravasation into inflamed tissues^{15, 36}. Surprisingly *Ccr2*^{-/-} mice developed a pronounced Ly6C^{hi} monocytosis following 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^{hi}CD11b^{lo} 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 due to the deficit in infiltrating monocytes which regulate neutrophil activity³⁵. Importantly, CCR2 deficiency prevented the increase in liver to body weight ratio observed in wild type mice following CSF1-Fc administration (Figure 4C). Although F4/80^{lo}/CD11b^{hi} macrophages are not completely dependent on CCR2 for mobilisation 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.

CSF1-Fc treatment accelerates recovery of innate immune capacity following partial hepatectomy

Patient survival depends upon the rapid restoration of liver macrophage functions to clear pathogenic material. We therefore tested the effect of CSF1-Fc upon innate immune function in injury models. CSF1-Fc administration increased liver size over controls at 4 days following PH (Figure 5A).

Hepatocyte staining (CYP2D) per unit area (Supplementary Figure 5A) was reduced following CSF1-Fc treatment, indicating the increased size was due 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 to controls. To confirm the role of endogenous CSF1 signalling, implied from studies of *op/op* mice¹², 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 remodelling genes associated with regeneration (Supplementary Figure 5C and 5D).

Following 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 elevation in genes encoding phagocytic receptors^{37, 38}, such as MARCO (macrophage receptor with collagenous structure) and MSR1 (macrophage scavenger receptor 1, with a reciprocal reduction following CSF1 blockade (Figure 5D). To assess the impact on clearance of insoluble material and bacteria-derived particles, we injected fluorescent-labelled 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 *E. coli* bioparticles which fluoresce when taken up into acidified vesicles and injected these into the portal vein (Figure 6A). CSF1-Fc treatment increased both internalisation capacity and the absolute yield of positive cells (Figure 6B). Relatively few infiltrating monocyte-derived cells (F4/80^{lo} CD11b^{hi}) internalised the labelled *E.coli*, but CSF1-Fc treatment again increased the clearance capacity (Figure 6C). The Ly6C^{lo} monocyte population consistently showed a greater propensity for phagocytosis compared to the Ly6C^{hi} population based on percentage of the populations phagocytosing the *E.coli* particles both in the control

and CSF1-Fc treated groups (Figure 6C). The resident F4/80^{hi} CD11b^{lo} 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 following acetaminophen administration to mice^{16, 35}. Enhanced macrophage accumulation could facilitate recovery by rapidly clearing necrotic debris and restoring hepatic immune function. We treated mice with CSF1-Fc 12 hours following acetaminophen intoxication, the point of maximal injury³⁹. CSF1-Fc treatment expanded the macrophage compartment and increased 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 described¹⁵, and both these populations were boosted by CSF1-Fc (Supplementary Figure 6B). Expression of mRNA for clearance receptors MARCO and MSR 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, Alk phos) decreased in CSF1-Fc treated mice, with reciprocal change following CSF1R blockade (Figure 7E; Supplementary 7F and G). These findings most likely reflect changes in the clearance of these enzymes by hepatic macrophages⁴⁰ (Figure 7C). Serum albumin was reduced by CSF1-Fc treatment, likely a reflection of the pro-inflammatory state given hepatic albumin gene expression was unchanged from control (Supplementary Figure 6D). Serum total protein was also unchanged (Supplementary Figure 6E). To further explore potential direct effects of CSF1-Fc on hepatocytes we assessed hepatocyte

viability and performed metabolic assays, which demonstrated no direct effect of CSF1-Fc on either mouse or human hepatocytes (Supplementary Figure 7A and B). Further, following 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 demonstrated a clear association between reduced serum CSF1 level and poor outcome in acute liver failure in humans. 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-renewal³³. Fate mapping of hematopoietic cells indicated that CSF1-Fc can also drive conversion of circulating monocytes to cells of a resident macrophage phenotype. This novel finding demonstrates 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. Following PH, the therapeutic requirement to optimise liver function and boost regeneration must be weighed against the potential to promote cancer recurrence. Malignant tumours can themselves produce CSF1 which mediates macrophage accumulation, supporting tumour growth⁴¹. Indeed, our series of preoperative patients with cancer *in situ* had elevated serum CSF1. However, effective elimination of circulating tumour cells, which are indicative of recurrence, requires hepatic macrophages which depend upon CSF1^{10, 42, 43}. The ability to enhance the innate immune capacity of the liver by increasing hepatic macrophage density may be valuable from an antimicrobial standpoint and may theoretically reduce cancer recurrence rates which can reach 60%⁴⁴.

ALF represents a different clinical challenge to PH. The low serum CSF1 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 sepsis¹⁷. Together with our results, these findings indicate that supplementary CSF1 therapy in the setting of low serum CSF1 might facilitate recovery by increasing monocyte numbers, induce a pro-regenerative macrophage phenotype, increase monocyte HLA-DR expression⁴⁵ and enhance phagocytic capacity. Multiorgan involvement is characteristic of clinical deterioration in ALF and previous reports implicating CSF1 signalling in recovery following both kidney and brain injury highlight potential wider benefits of this strategy^{30, 46}. The ability to predict patient deterioration, using a marker such as CSF1, prior to 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, where innate immunity may be impaired.

There is mounting evidence for a CSF1 – CCR2 axis in monocyte recruitment with the induction of these factors following acute hepatic injury³⁵. The chemokine signalling induced in the liver following 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 deficit, suggesting that CSF1-Fc either mobilises monocytes from other sources (e.g. the spleen) or overcomes the CCR2-dependence on bone marrow release.

In contrast to PH, where the increase in available CSF1 is related to reduced clearance, in acute liver toxicity hepatic CSF1 mRNA increased (Fig Suppl. Fig 2c). Evidence of local production was also seen in the liver of patients (Fig Suppl. Fig 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 might also promote clearance of circulating tumour cells. Some of the earliest studies of CSF1 treatment demonstrated an impact on tumour metastasis²⁹. CSF1-Fc has already

been shown to be safe in pigs²⁴ and the native protein was previously tested by continuous infusion in human phase 1 trials and was well-tolerated⁴⁷.

In summary, we have shown that elevated 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 following liver injury could be used to stratify patients according to severity and to identify candidates for CSF1 therapy.

Figure Legends

Figure 1: Serum CSF1 increases following partial hepatectomy in humans according to extent of resection

(a) Serum CSF1 in healthy volunteers and patients undergoing partial hepatectomy to remove cancer. (b) Mean serum CSF1 categorised according to extent of resection. (c) Box and whisker plots showing minimum to maximum values with patients developing postoperative liver failure overlaid with red dots.

Figure 2: High serum CSF1 level is associated with survival in acute liver failure in humans

(a) Serum CSF1 level in healthy volunteers and in patients following 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 following acetaminophen intoxication (n=10/group). (c) Receiver operator characteristic curve for serum CSF1, acetyl-HMGB1 (aHMGB1), prothrombin time (PT), ALT (alanine aminotransferase), bilirubin and APACHEII score with areas under curve (AUC value) for patients who subsequently survived or died/required liver transplantation. (d) Dot plot of serum CSF1 level versus log(serum acetyl-HMGB1) on presentation to the specialist liver centre (slope difference: $F=0.15$, $p=0.70$; Intercept difference: $F=8.03$, $p=0.006$). (e) Details of the combined logistic regression model. (f) Example serum values and predicted chance of death based on logistic regression involving CSF1 alone (Model 2).

Figure 3: Hepatic enlargement following CSF1R stimulation

(a) Representative multiphoton image of ex vivo liver (MacGreen mouse *Tg(CSF1r-GFP)_{Hume}*) (b) Representative immunofluorescence images of *Csf1r-Mer2iCre_{JWP}* x Rosa floxed stop tomato red following induction (c) Cytokine/Chemokine array of liver tissue 6 hours following CSF1-Fc treatment versus control (n=4/group). (d) Quantification of hepatic F4/80 immunohistochemistry in 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 following CSF1-Fc administration (20 HPF/mouse). (f) Serum parameters following 4 days CSF1-Fc (grey, hollow circles) or PBS control (black, solid circles) treatment once daily (2-way repeated measures ANOVA with Bonferroni post hoc).

Figure 4: CSF1 receptor stimulation recruits monocytes and induces macrophage proliferation in uninjured mouse liver

(a) Number of hepatic macrophage populations (F480^{hi}/CD11b^{lo} and F480^{lo}/CD11b^{hi}) day 2 following CSF1-Fc administration (n=8/group) relative to mean of control group and representative Ly6C profile of F480^{hi} (red) and F480^{lo} (blue) populations. (b) Percentage of hepatic macrophage populations expressing markers of proliferation (Ki67 and BRDU) day 2 following CSF1-Fc administration relative to mean of control group. (c) Fate tracing bone marrow derived monocytes using chimeric mice demonstrating conversion of infiltrating cells to resident macrophage phenotype driven by CSF1-Fc. (% cells derived from blood monocytes is based on the ratio of chimerism in hepatic populations to chimerism in circulating blood monocytes) (d) Number of hepatic macrophage populations Day 2 following CSF1-Fc administration in WT and CCR2^{-/-} mice (n=8/group) with representative Ly6C profile of F480^{hi} (red) and F480^{lo} (blue) populations (Solid = CCR2^{-/-}; dotted line = WT) (e) Liver weight to body weight ratio following 2 days control (black) or CSF1-Fc administration (grey) in wild type and CCR2^{-/-} mice (n=8/group).

Figure 5: CSF1-Fc enhances hepatic phagocytic capacity post partial hepatectomy in mice

(a) Liver weight to body weight ratio following partial hepatectomy (PH) with CSF1-Fc or control (n=8/group). (b) Ki67⁺ hepatocytes per HPF following PH (n=8/timepoint/group). (c) Number of resident (red) or infiltrating (blue) hepatic macrophage populations Day 2 following PH and CSF1-Fc administration. Representative dot plots of hepatic macrophage profile and representative Ly6C profile normalised to mode. (d) Hepatic gene expression of phagocytic markers MARCO (macrophage receptor with collagenous structure), MSR1 (macrophage scavenger receptor 1) and MR (mannose receptor) versus relevant control. (e) Multiphoton image of ex vivo *Csf1r*-eGFP mouse liver following injection of fluorescent microbeads and clearance from the circulation following sham or 2/3 PH with control or CSF1-Fc (n=6/group/timepoint). (f) Net fluorescence liver, spleen, lung, kidney and brain Day 2 following PH and CSF1-Fc or control. (n=6 per group).

Figure 6: Contribution of hepatic phagocytes to clearance of pathogenic material

(a) Immunohistochemistry of hepatic macrophages (F4/80=green) following isolation by adherence and administration of *E. coli* bioparticles (pHrodoTM) (red). (b) Relative number and percentage of resident macrophages phagocytosing *E. coli* bioparticles (Phrodo). Representative density plots of phagocytic cells in the resident macrophage population. (c) Relative number of infiltrating macrophages phagocytosing *E. coli*

bioparticles compared to the mean of the control group with percentage of cells phagocytosing *E. coli* particles according to Ly6C expression status. **(d)** Pie charts illustrating proportion of phagocytic cells in the liver with bar chart showing absolute cell number comparison for resident and infiltrating macrophages, neutrophils and eosinophils.

Figure 7: 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 (DAB) at Day 2 and Day 4 following acetaminophen with PBS control or CSF1-Fc. **(c)** Hepatic expression of phagocytosis associated genes following GW2580 (red), AFS98 (blue) or CSF1-Fc (grey) relative to mean of control group (vehicle, rat IgG2a, PBS respectively). **(d)** Net ex vivo liver fluorescence 15 minutes following injection of fluorescent beads. **(e)** Serum liver associated biochemistry tests at day 3 following acetaminophen intoxication and either GW2580 (red), AFS98 (blue) or CSF1-Fc (grey) compared to control (vehicle, rat IgG2a, PBS respectively).

References

1. Gao B, Jeong WI, Tian Z. Liver: An organ with predominant innate immunity. *Hepatology* 2008;47:729-36.
2. van de Poll MC, Wigmore SJ, Redhead DN, et al. Effect of major liver resection on hepatic ureagenesis in humans. *Am J Physiol Gastrointest Liver Physiol* 2007;293:G956-62.
3. Schindl MJ, Millar AM, Redhead DN, et al. The adaptive response of the reticuloendothelial system to major liver resection in humans. *Ann Surg* 2006;243:507-14.
4. Canalese J, Gove CD, Gimson AE, et al. Reticuloendothelial system and hepatocytic function in fulminant hepatic failure. *Gut* 1982;23:265-9.
5. Wang XD, Soltesz V, Andersson R, et al. Bacterial translocation in acute liver failure induced by 90 per cent hepatectomy in the rat. *Br J Surg* 1993;80:66-71.
6. Arvaniti V, D'Amico G, Fede G, et al. Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis. *Gastroenterology* 2010;139:1246-56, 1256 e1-5.
7. Schindl MJ, Redhead DN, Fearon KC, et al. The value of residual liver volume as a predictor of hepatic dysfunction and infection after major liver resection. *Gut* 2005;54:289-96.
8. Balmer ML, Slack E, de Gottardi A, et al. The liver may act as a firewall mediating mutualism between the host and its gut commensal microbiota. *Sci Transl Med* 2014;6:237ra66.
9. Selzner N, Selzner M, Odermatt B, et al. ICAM-1 triggers liver regeneration through leukocyte recruitment and Kupffer cell-dependent release of TNF-alpha/IL-6 in mice. *Gastroenterology* 2003;124:692-700.
10. **MacDonald KP, Palmer JS**, Cronau S, et al. An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood* 2010;116:3955-63.
11. Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, Jr., et al. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A* 1990;87:4828-32.

12. Amemiya H, Kono H, Fujii H. Liver Regeneration is Impaired in Macrophage Colony Stimulating Factor Deficient Mice After Partial Hepatectomy: The Role of M-CSF-Induced Macrophages. *J Surg Res* 2009.
13. Bartocci A, Mastrogiannis DS, Migliorati G, et al. Macrophages specifically regulate the concentration of their own growth factor in the circulation. *Proc Natl Acad Sci U S A* 1987;84:6179-83.
14. Matsumoto K, Miyake Y, Umeda Y, et al. Serial changes of serum growth factor levels and liver regeneration after partial hepatectomy in healthy humans. *Int J Mol Sci* 2013;14:20877-89.
15. Holt MP, Cheng L, Ju C. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J Leukoc Biol* 2008;84:1410-21.
16. **Antoniades CG, Quaglia A, Taams LS**, et al. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology* 2012;56:735-46.
17. Antoniades CG, Berry PA, Davies ET, et al. Reduced monocyte HLA-DR expression: a novel biomarker of disease severity and outcome in acetaminophen-induced acute liver failure. *Hepatology* 2006;44:34-43.
18. Antoine DJ, Jenkins RE, Dear JW, et al. Molecular forms of HMGB1 and keratin-18 as mechanistic biomarkers for mode of cell death and prognosis during clinical acetaminophen hepatotoxicity. *J Hepatol* 2012;56:1070-9.
19. Sasmono RT, Oceandy D, Pollard JW, et al. A macrophage colony-stimulating factor receptor-green fluorescent protein transgene is expressed throughout the mononuclear phagocyte system of the mouse. *Blood* 2003;101:1155-63.
20. **Schulz C, Gomez Perdiguero E**, Chorro L, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 2012;336:86-90.
21. Jenkins SJ, Ruckerl D, Cook PC, et al. Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science* 2011;332:1284-8.

22. Mitchell C, Willenbring H. A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. *Nat Protoc* 2008;3:1167-70.
23. Henderson NC, Pollock KJ, Frew J, et al. Critical role of c-jun (NH2) terminal kinase in paracetamol- induced acute liver failure. *Gut* 2007;56:982-90.
24. **Gow DJ, Sauter KA**, Pridans C, et al. Characterisation of a Novel Fc Conjugate of Macrophage Colony-stimulating Factor. *Mol Ther* 2014; 22(9): 1580-92
25. R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, 2010.
26. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature* 2013;496:445-55.
27. Bone RC, Balk RA, Cerra FB, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992;101:1644-55.
28. Bonaldi T, Talamo F, Scaffidi P, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J* 2003;22:5551-60.
29. Menke J, Iwata Y, Rabacal WA, et al. CSF-1 signals directly to renal tubular epithelial cells to mediate repair in mice. *J Clin Invest* 2009;119:2330-42.
30. Alikhan MA, Jones CV, Williams TM, et al. Colony-stimulating factor-1 promotes kidney growth and repair via alteration of macrophage responses. *Am J Pathol* 2011;179:1243-56.
31. **Arner E, Daub CO, Vitting-Seerup K**, et al. Gene regulation. Transcribed enhancers lead waves of coordinated transcription in transitioning mammalian cells. *Science* 2015;347:1010-4.
32. Cecchini MG, Dominguez MG, Mocci S, et al. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. *Development* 1994;120:1357-72.

33. Yona S, Kim KW, Wolf Y, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 2013;38:79-91.
34. Gomez Perdiguero E, Klapproth K, Schulz C, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. *Nature* 2015;518:547-51.
35. **Zigmond E, Samia-Grinberg S**, Pasmanik-Chor M, et al. Infiltrating monocyte-derived macrophages and resident kupffer cells display different ontogeny and functions in acute liver injury. *J Immunol* 2014;193:344-53.
36. Auffray C, Sieweke MH, Geissmann F. Blood monocytes: development, heterogeneity, and relationship with dendritic cells. *Annu Rev Immunol* 2009;27:669-92.
37. van der Laan LJ, Dopp EA, Haworth R, et al. Regulation and functional involvement of macrophage scavenger receptor MARCO in clearance of bacteria in vivo. *J Immunol* 1999;162:939-47.
38. Ling W, Loughheed M, Suzuki H, et al. Oxidized or acetylated low density lipoproteins are rapidly cleared by the liver in mice with disruption of the scavenger receptor class A type I/II gene. *J Clin Invest* 1997;100:244-52.
39. McGill MR, Sharpe MR, Williams CD, et al. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest* 2012;122:1574-83.
40. Radi ZA, Koza-Taylor PH, Bell RR, et al. Increased serum enzyme levels associated with kupffer cell reduction with no signs of hepatic or skeletal muscle injury. *Am J Pathol* 2011;179:240-7.
41. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell* 2010;141:39-51.
42. Bayon LG, Izquierdo MA, Sirovich I, et al. Role of Kupffer cells in arresting circulating tumor cells and controlling metastatic growth in the liver. *Hepatology* 1996;23:1224-31.
43. Uchikura K, Ueno S, Takao S, et al. Perioperative detection of circulating cancer cells in patients with colorectal hepatic metastases. *Hepatogastroenterology* 2002;49:1611-4.

44. Kulaylat AN, Schubart JR, Stokes AL, et al. Overall survival by pattern of recurrence following curative intent surgery for colorectal liver metastasis. *J Surg Oncol* 2014.
45. Becker S, Warren MK, Haskill S. Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *J Immunol* 1987;139:3703-9.
46. Luo J, Elwood F, Britschgi M, et al. Colony-stimulating factor 1 receptor (CSF1R) signaling in injured neurons facilitates protection and survival. *J Exp Med* 2013;210:157-72.
47. VandePol CJ, Garnick MB. Clinical applications of recombinant macrophage-colony stimulating factor (rhM-CSF). *Biotechnol Ther* 1991;2:231-9.

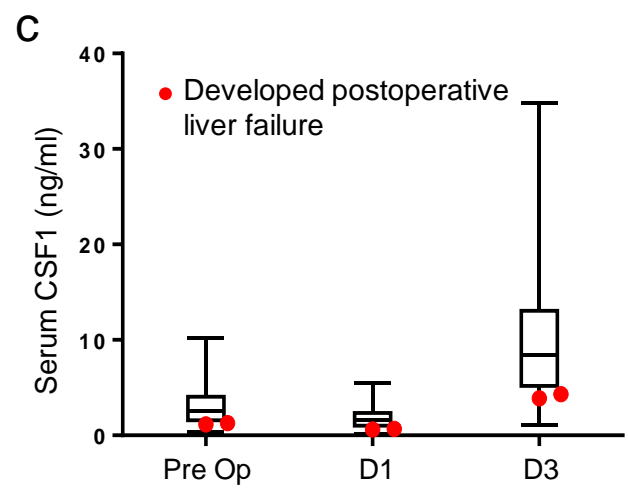
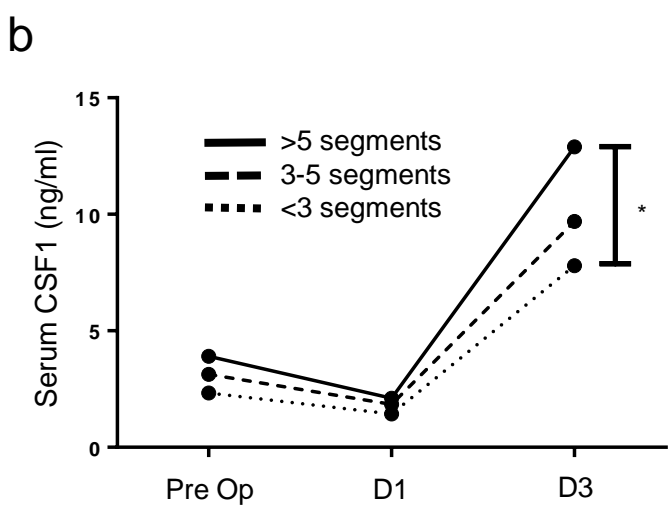
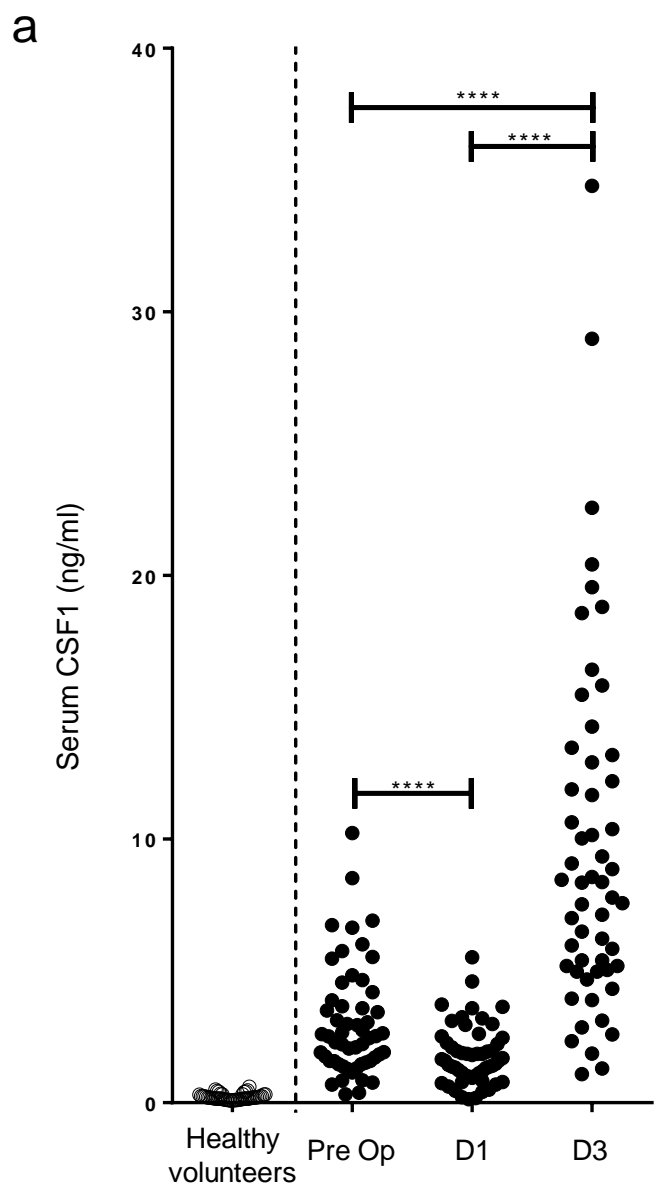


Figure 1: Serum CSF1 increases following partial hepatectomy in humans according to extent of resection

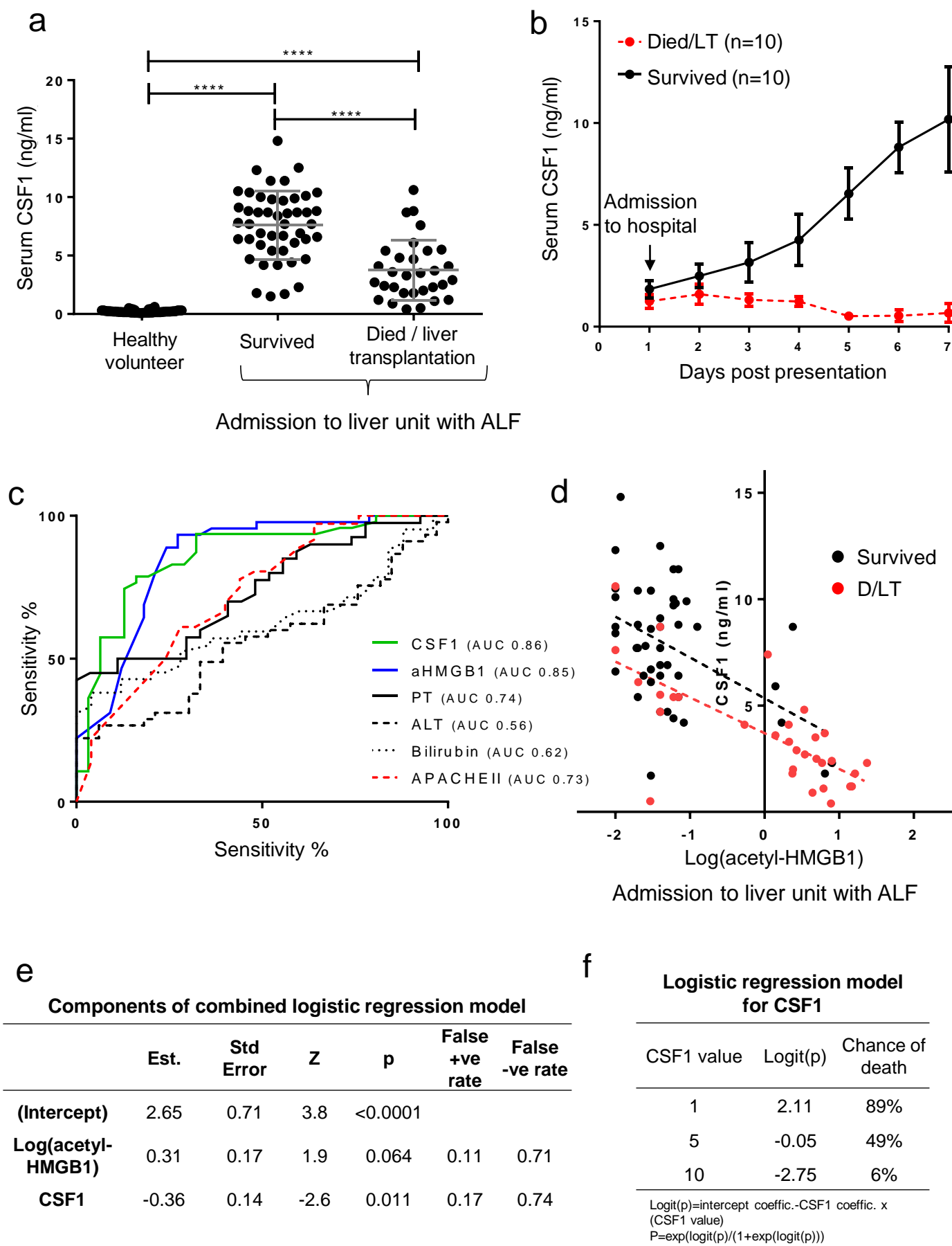


Figure 2: High serum CSF1 level is associated with survival in acute liver failure in humans

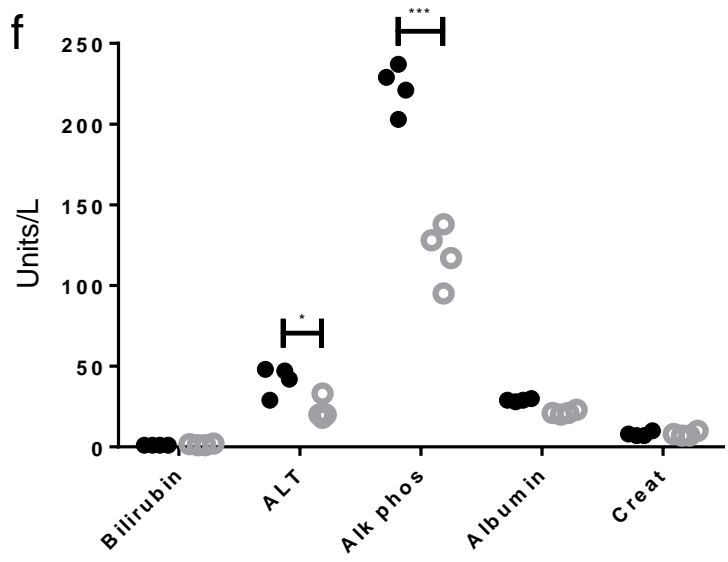
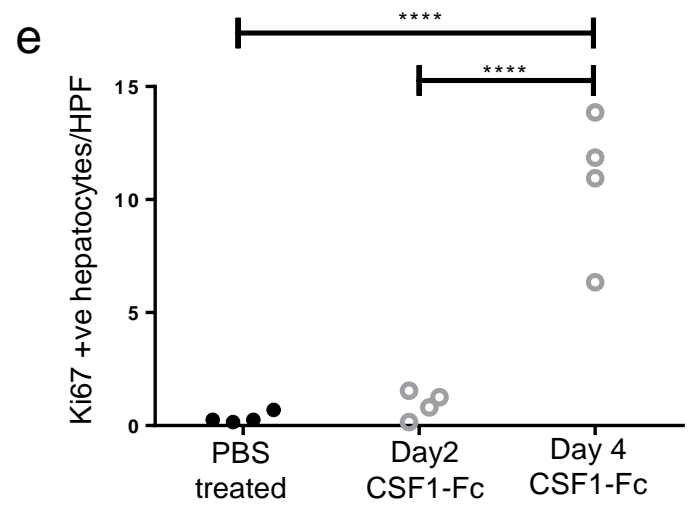
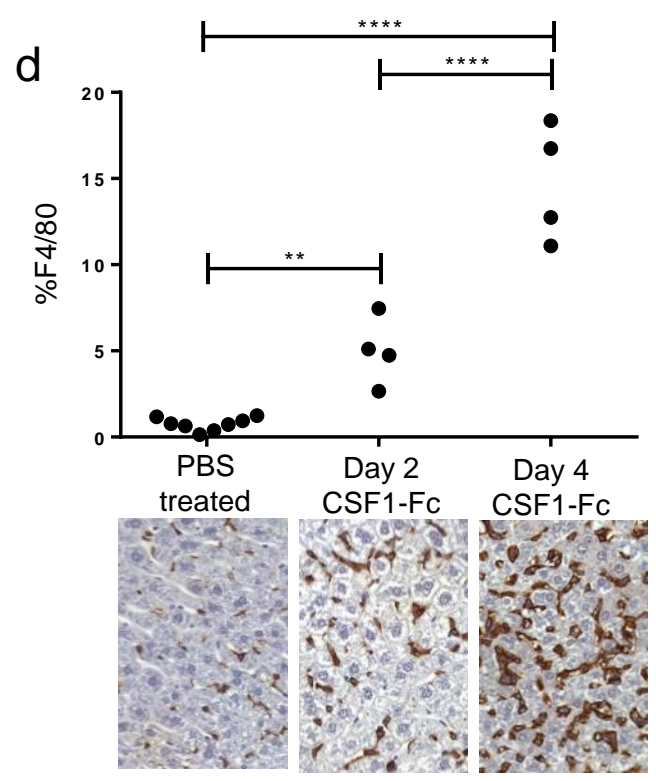
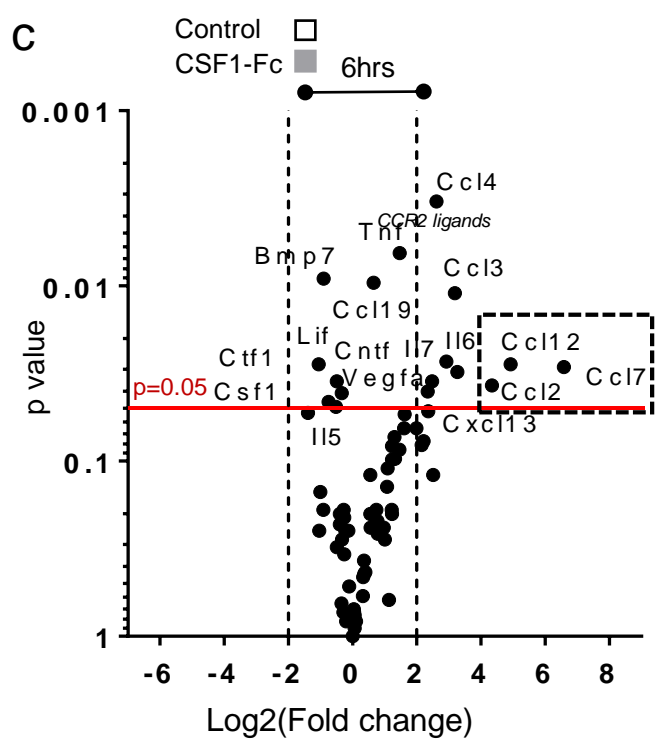
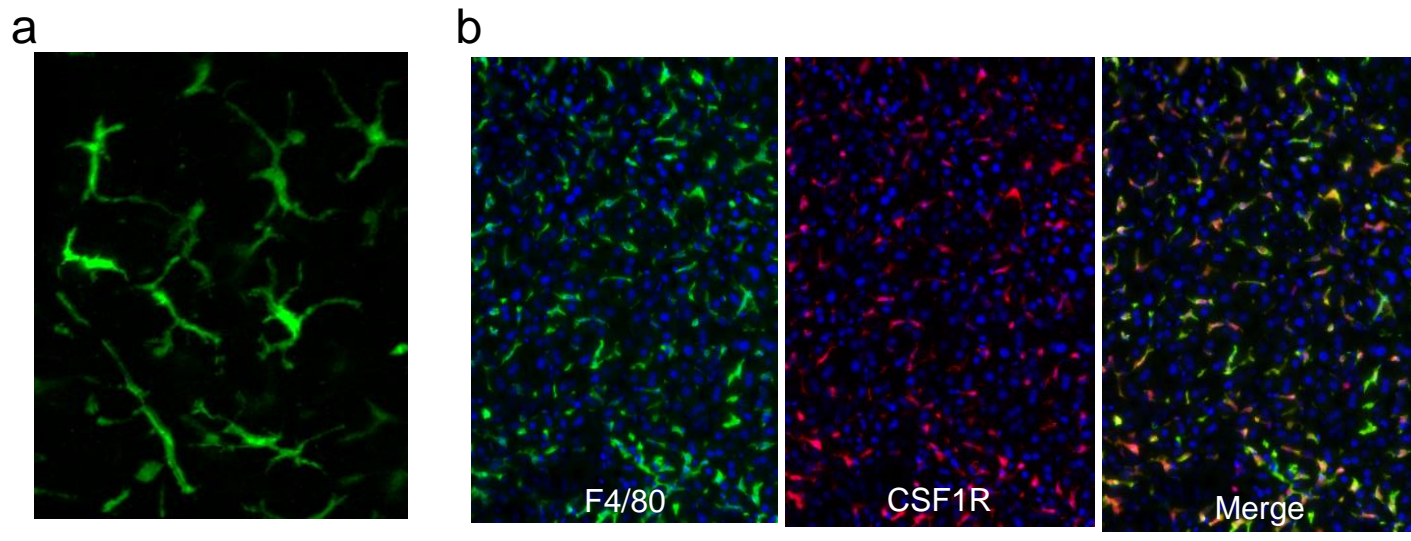


Figure 3: Hepatic macrophage accumulation following CSF1-Fc administration

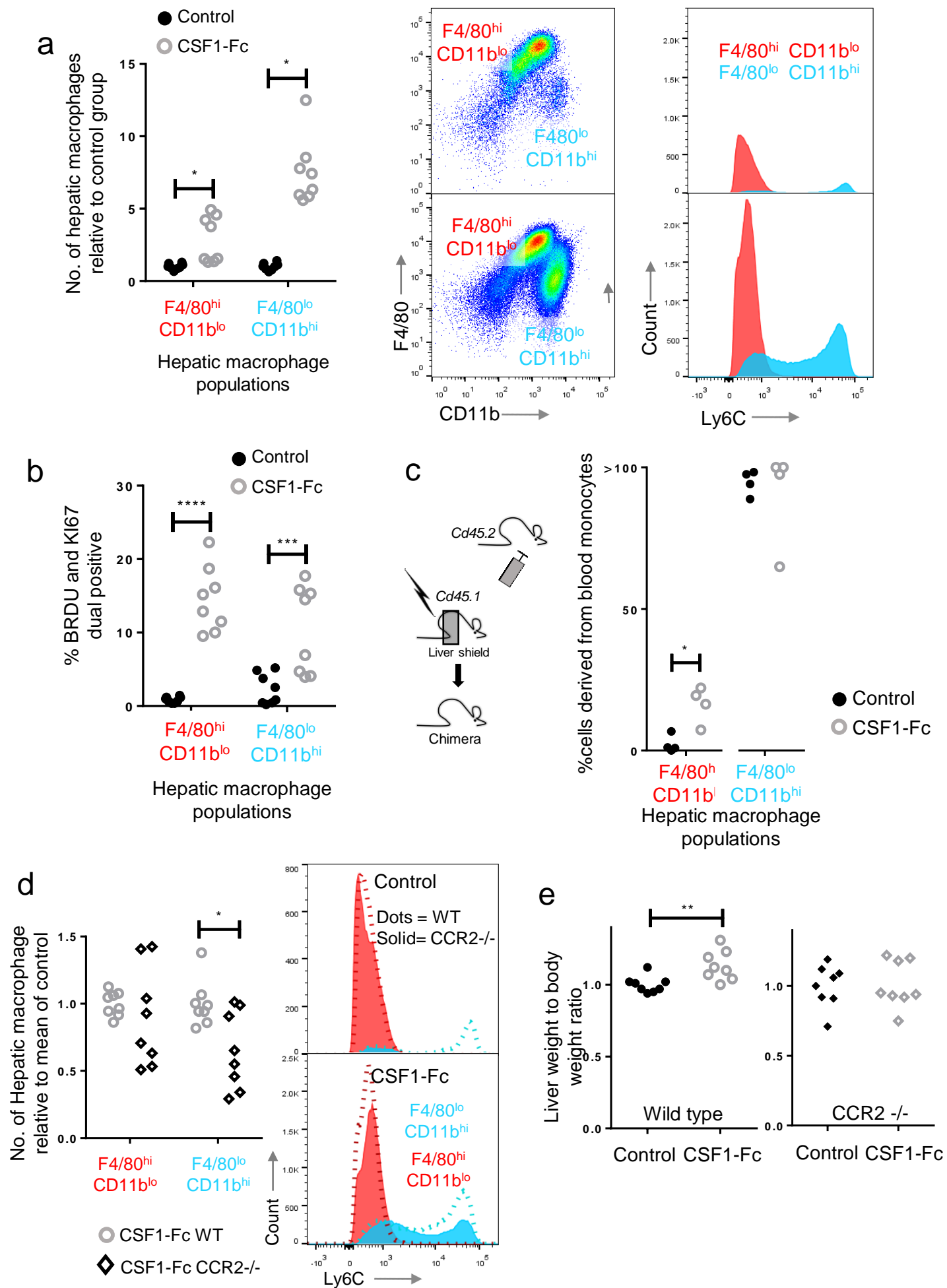


Figure 4: CSF1 receptor stimulation recruits monocytes and induces macrophage proliferation in uninjured mouse liver.

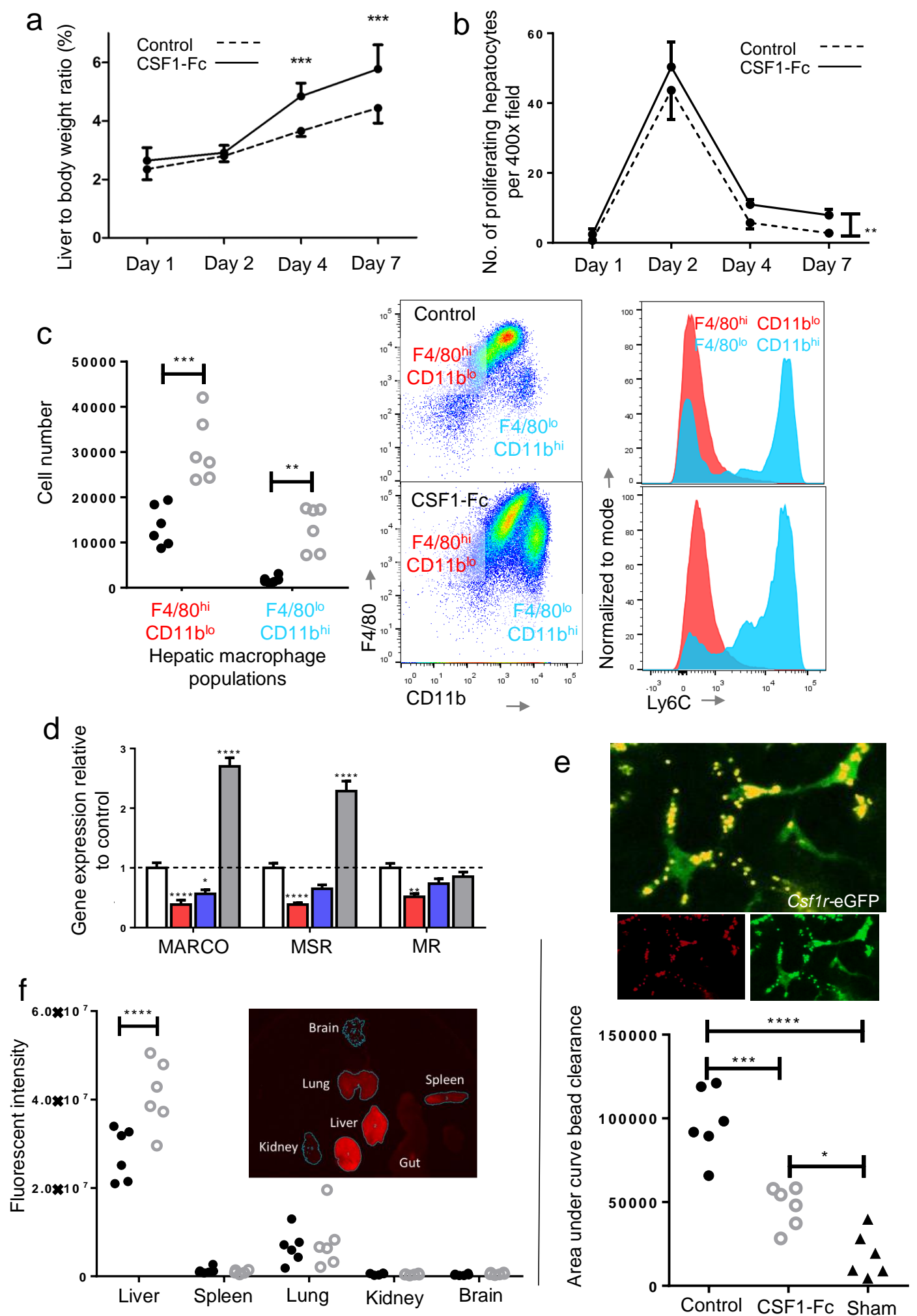


Figure 5: CSF1-Fc enhances hepatic phagocytic capacity post partial hepatectomy in mice

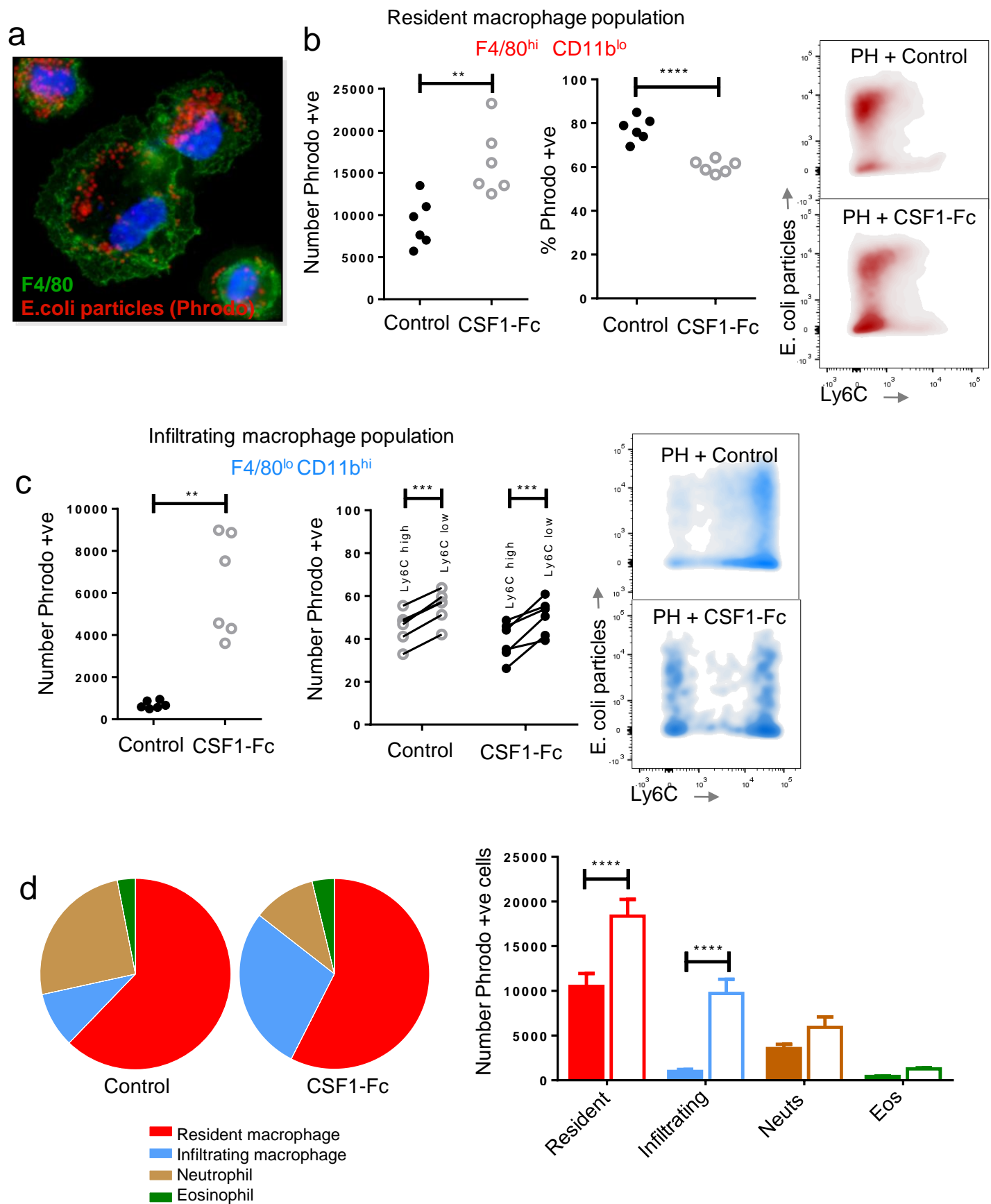


Figure 6: Contribution of hepatic phagocytes to clearance of pathogenic material

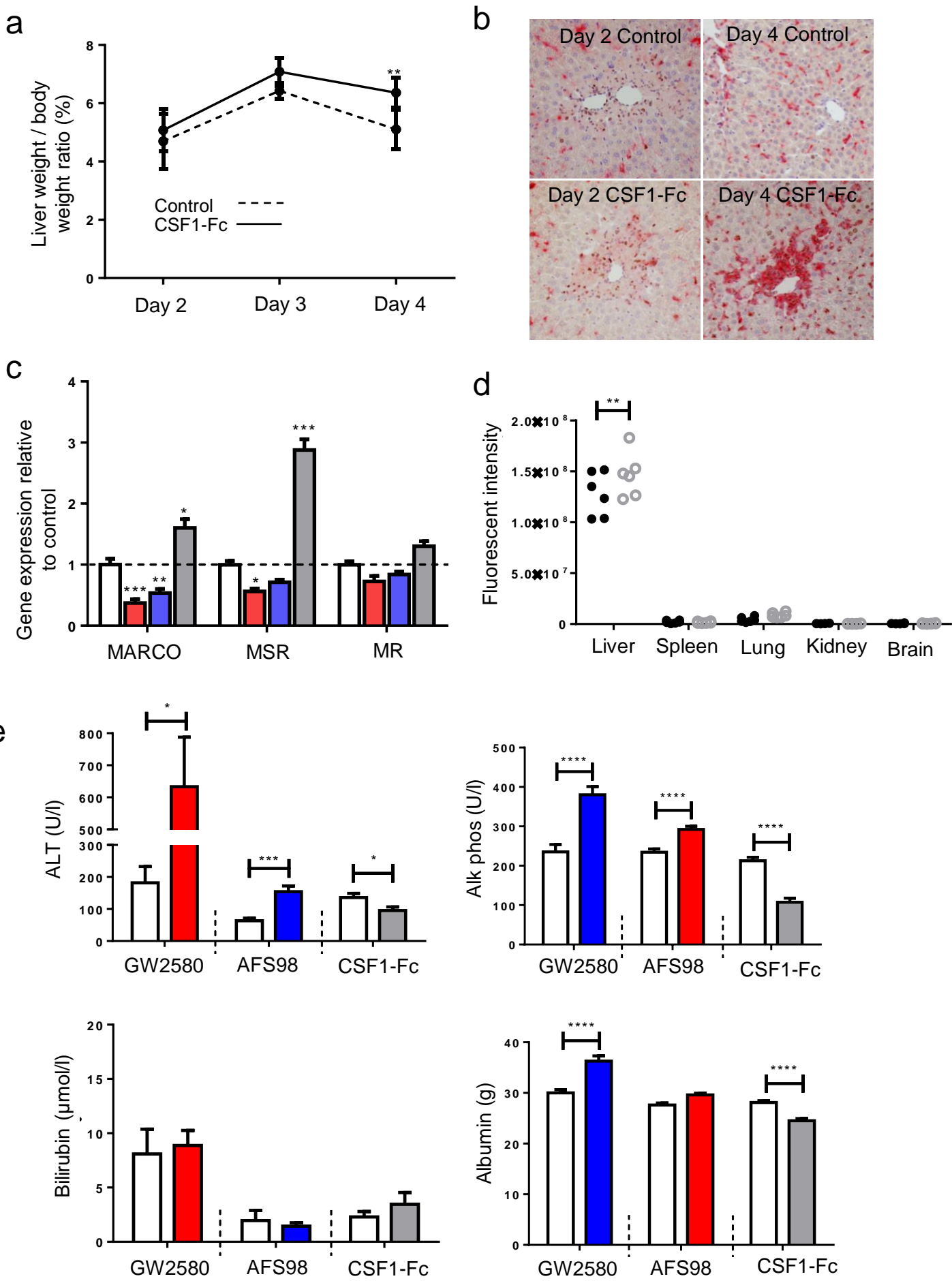
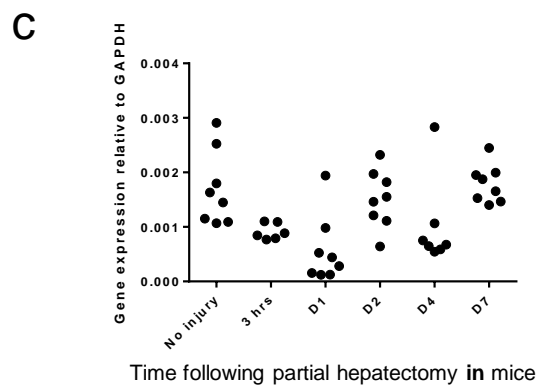
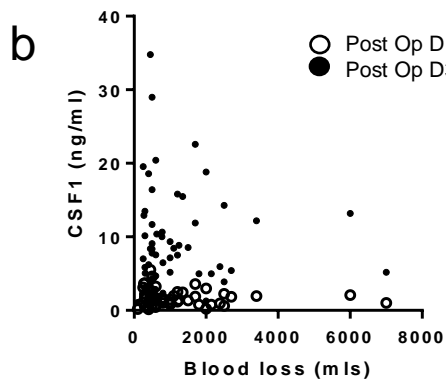


Figure 7: CSF1-Fc and acetaminophen intoxication in mice

a

Extent resection	n	Mean age (SD)	M:F	BMI (SD)	ASA	Diagnosis	Post op hepatic failure	Blood loss Mean (SD)	Mortality
>5 segments	10	60.6 (16.2)	5:5	27.7 (3.9)	2	CLM (6) Carcin (1) HCC (2) Cholangio (1)	1	1915 (1543)	0/10
3-5 segments	28	61.5 (10.4)	14:14	27.4 (5.7)	2	CLM (22) Cyst (3) Carcin. (2) Abscess (1)	1	1063 (1425)	0/28
<3 segments	17	63.9 (10.3)	17:3	29.9 (5.2)	2	CLM (10) HCC (4) Haemang. (2) Mets ?prim (1)	0	1011 (777)	0/17
<i>Overall</i>	55	62.1	18:11	28.3	2	CLM (38) Carcin. (3) HCC (6) Haemang. (2) Cholangio (1) Abscess (1) Mets ?prim (1)	2	1202 (1294)	0/55

For healthy controls see Supplementary Figure 8



d

	Age	Sex	BMI	ASA	Diagnosis	Extent of resection	Blood loss (mls)	Post op hepatic failure	Outcome
1	65	F	30.5	2	Carcinoid	6 segments (1,4,5,6,7,8)	2500	Bilirubin >54 and hepatic encephalopathy (grade 3)	Improved with supportive care
2	63	M	29	2	Colorectal liver metastasis	4 segments (5,6,7,8)	500	Bilirubin >54, ascites and hepatic encephalopathy (grade 1)	Improved with supportive care

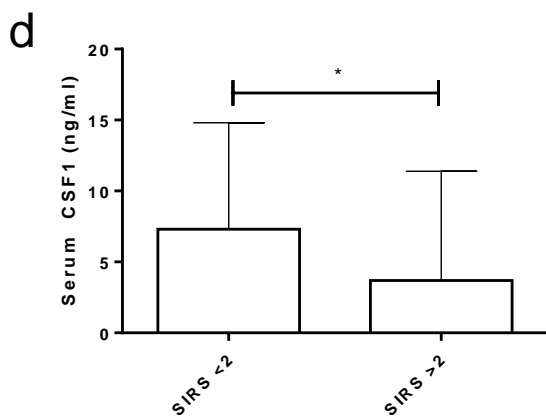
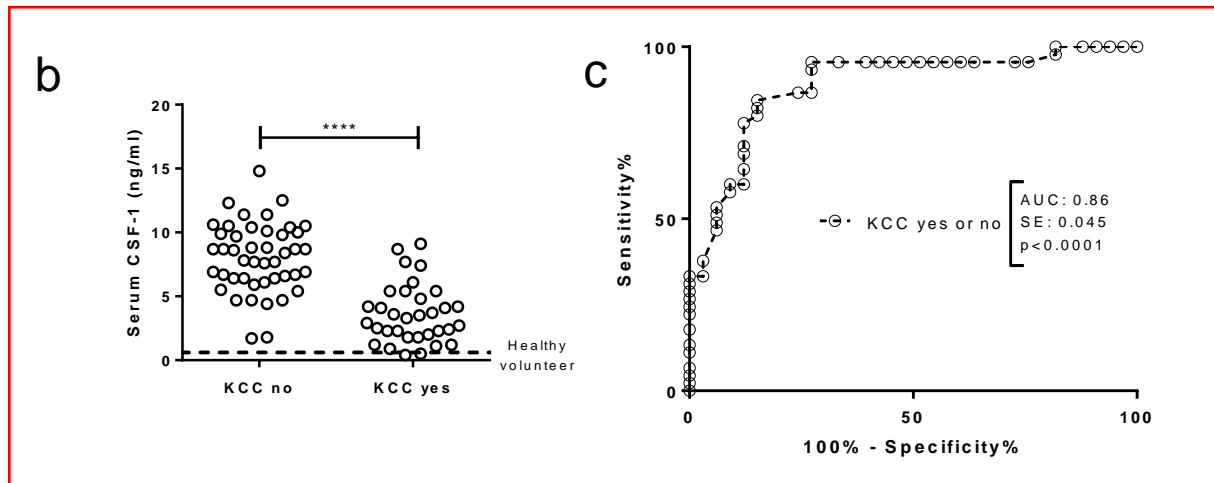
Supplementary Figure 1: Supporting details for partial hepatectomy in humans

Supplementary Figure 1: Supporting details for partial hepatectomy in humans

(a) Details of patients undergoing partial hepatectomy 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 hepatectomy in mice.

Outcome	n	Age	M:F	ALT (U/L)	PT (Sec)	Creatinine ($\mu\text{mol/L}$)	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)



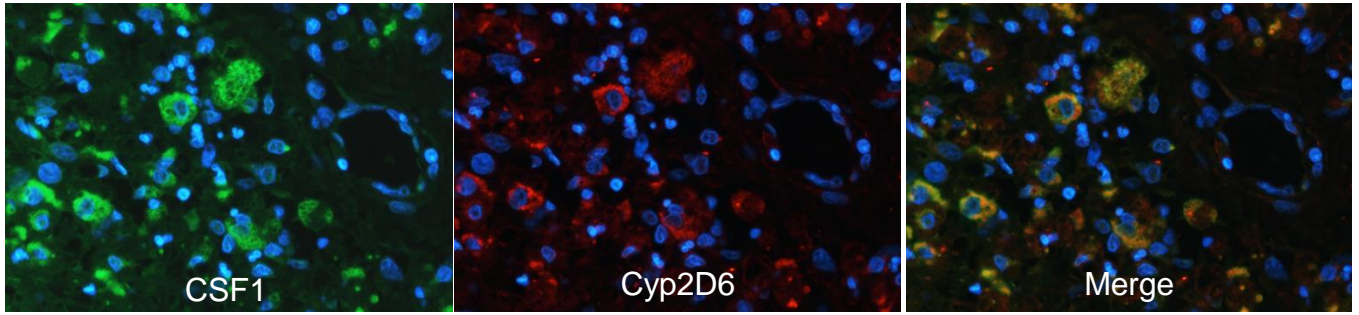
Systemic inflammatory response scoring (SIRS)
One point for each of the following

- Temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$
- Heart rate $>90\text{bpm}$
- Tachypnoea $>20\text{bpm}$
- White cell count $<4000\text{ cells/mm}^3$ or $>12,000\text{ cells/mm}^3$

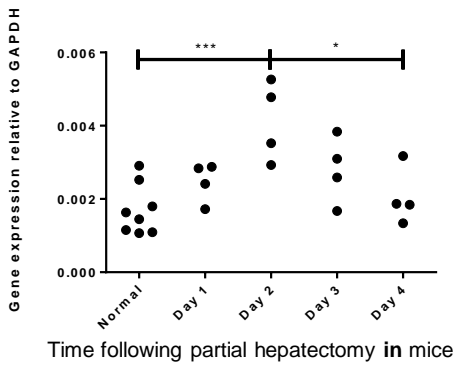
Outcome	n	Age	M:F	ALT (U/L)	Bilirubin (mg/dl)	Acetyl-HMGB1 (ng/ml)	CSF1 (ng/ml)
Survived	10	39 (9)	2:3	1370 (1104)	3.1 (1.8)	0.33 (0.29)	1.84 (1.33)
Died/Liver transplantation	10	41 (14)	2:3	3873 (1902)	3.5 (1.9)	2.06 (2.59)	1.25 (1.08)

Supplementary Figure 2: Supporting details for acetaminophen intoxication in humans.

f



g



h

	Deviance residuals				
	Min	1Q	Median	3Q	Max
CSF1	-	-	-0.3097	0.676	2.516
Log(acetyl-HMGB1)	-	-	-0.5125	0.647	2.138
Combined model	-	-	-0.3428	0.570	2.809
	1.9447	0.7227		7	1
	1.9763	0.6779		0	2
	2.1060	0.6573		1	1

i

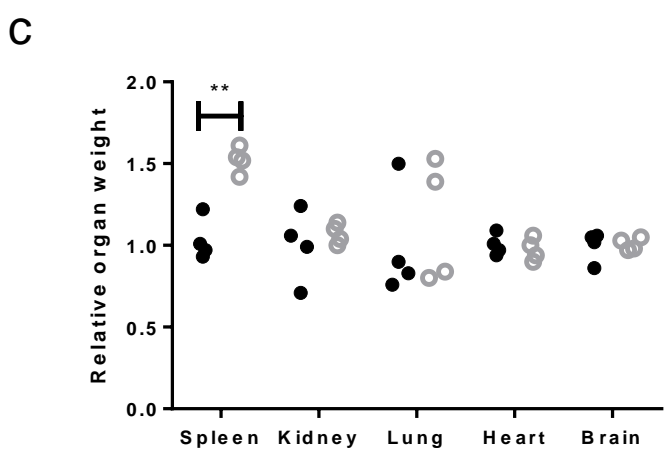
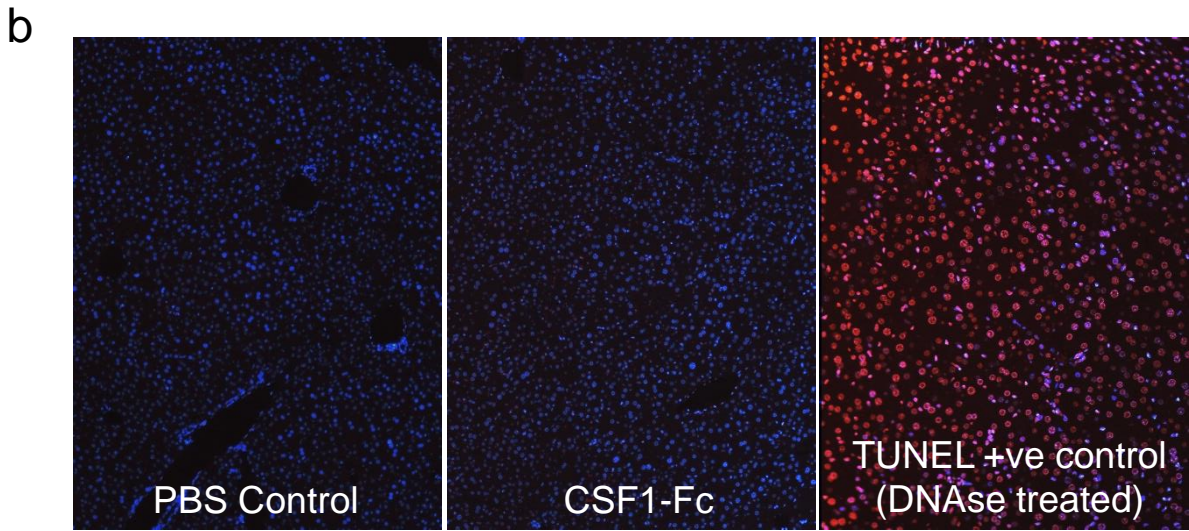
		Analysis of Deviance		
		Degrees of freedom	Residual deviance	p
Model 1	Log(acetyl-HMGB1) + CSF1	75	67.299	0.0657
Model 2	CSF1 alone	76	70.687	

Supplementary Figure 2: Supporting details for acetaminophen intoxication in humans.

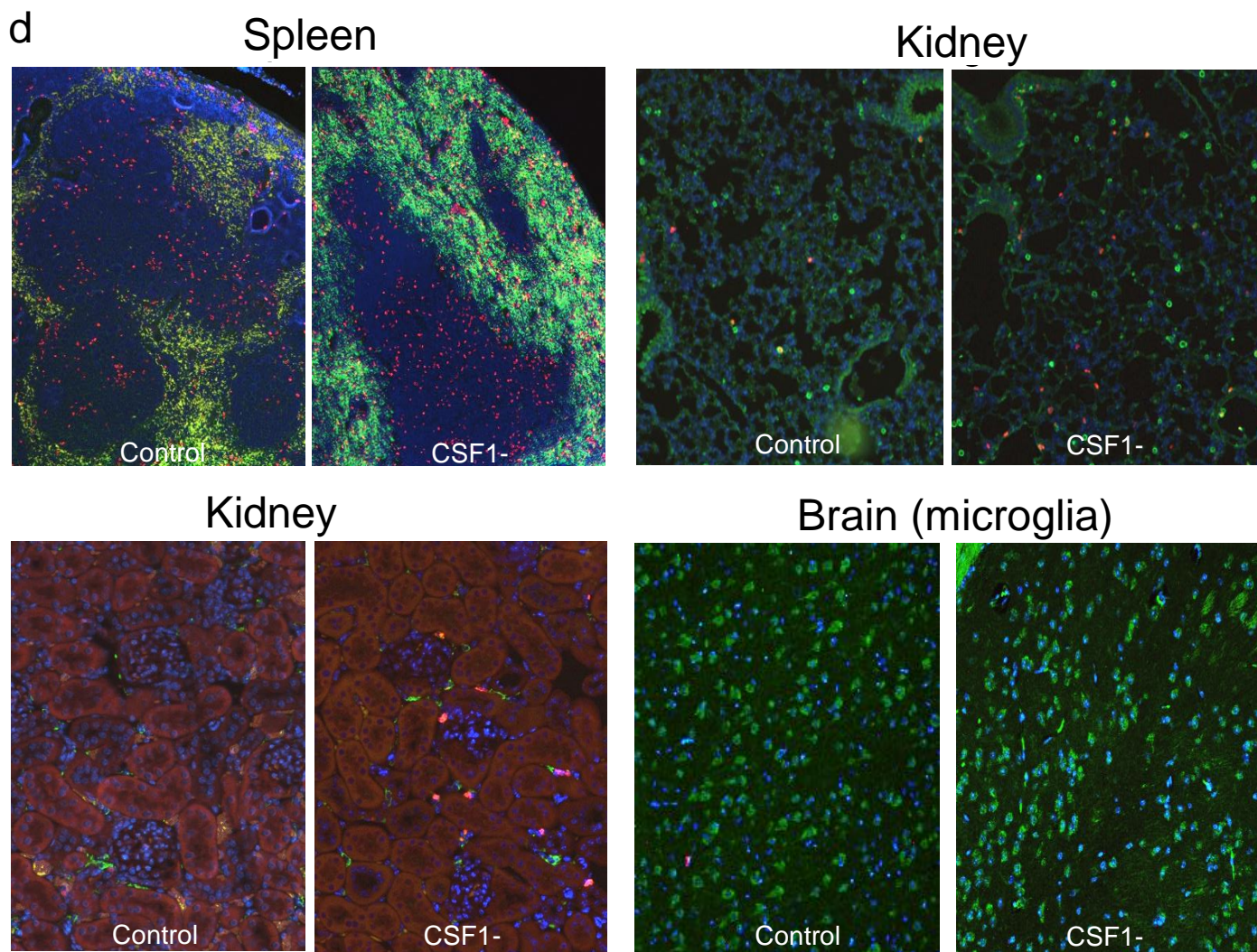
(a) Details of acetaminophen intoxication patients presenting to the specialist liver unit with acute liver failure grouped according to survivors versus those who subsequently required liver transplantation or died (patient cohort and acetyl-HMGB1 values as per Antoine et al.²⁴; healthy control data 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.²⁴). (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).

a

Symbol	Log ₂ (FC)	p Value												
Adipoq	1.22	1.9E-01	Cntf	2.34	4.0E-02	Il10	1.23	9.8E-02	Il4	0.56	2.4E-01	Tnfsf11	0.72	2.3E-01
Bmp2	0.04	7.0E-01	Csf1	-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	5.9E-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	Il7	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			
Ccl1	-0.23	2.5E-01	Cx3cl1	-0.29	7.3E-01	Il15	1.09	1.1E-01	Lif	-0.49	3.5E-02			
Ccl11	1.32	9.7E-02	Cxcl1	2.51	1.2E-01	Il16	0.06	7.6E-01	Lta	-0.26	2.1E-01			
Ccl12	4.92	2.8E-02	Cxcl10	1.46	8.6E-02	Il17a	-0.23	2.5E-01	Ltb	-0.91	1.9E-01			
Ccl17	-0.20	8.2E-01	Cxcl11	1.23	2.0E-01	Il17f	-0.40	2.0E-01	Mif	-0.40	2.3E-01			
Ccl19	0.65	9.6E-03	Cxcl12	-0.33	2.8E-01	Il18	1.23	8.2E-02	Mstn	-0.23	2.5E-01			
Ccl2	4.34	3.7E-02	Cxcl13	2.48	3.5E-02	Il1a	2.21	7.7E-02	Nodal	-0.35	6.5E-01			
Ccl20	-0.11	5.2E-01	Cxcl16	0.55	1.2E-01	Il1b	1.99	6.5E-02	Osm	-0.49	3.1E-01			
Ccl22	-1.39	5.3E-02	Cxcl3	-0.23	2.5E-01	Il1rn	0.77	2.2E-01	Pf4	1.30	7.3E-02			
Ccl24	0.78	2.6E-01	Cxcl5	0.40	4.3E-01	Il2	-0.23	2.5E-01	Pbbp	1.13	6.2E-01			
Ccl3	3.19	1.1E-02	Cxcl9	2.15	8.1E-02	Il21	-0.23	2.5E-01	Spp1	2.36	5.2E-02			
Ccl4	2.61	3.3E-03	Fasl	1.00	2.8E-01	Il22	-0.23	2.5E-01	Tgfb2	0.11	8.2E-01			
Ccl5	-0.26	3.4E-01	Gpi1	-1.01	1.5E-01	Il23a	0.55	2.0E-01	Thpo	-1.04	2.5E-01			
Ccl7	6.58	2.9E-02	Hc	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	Tnfrsf11b	-0.13	2.5E-01			
Cd70	-0.23	2.5E-01	Ifng	0.97	2.4E-01	Il3	-0.23	2.5E-01	Tnfsf10	1.07	1.4E-01			

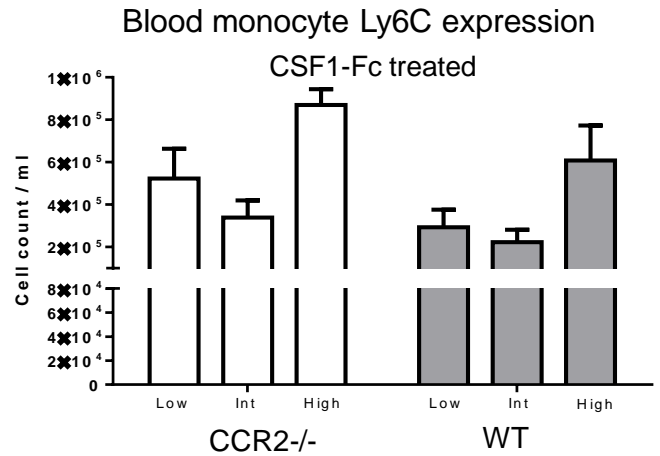
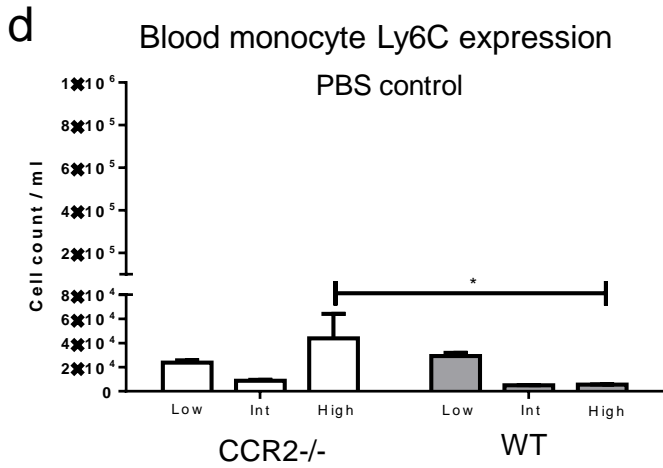
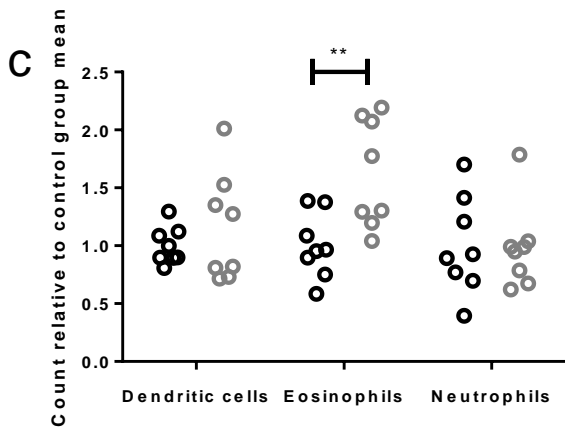
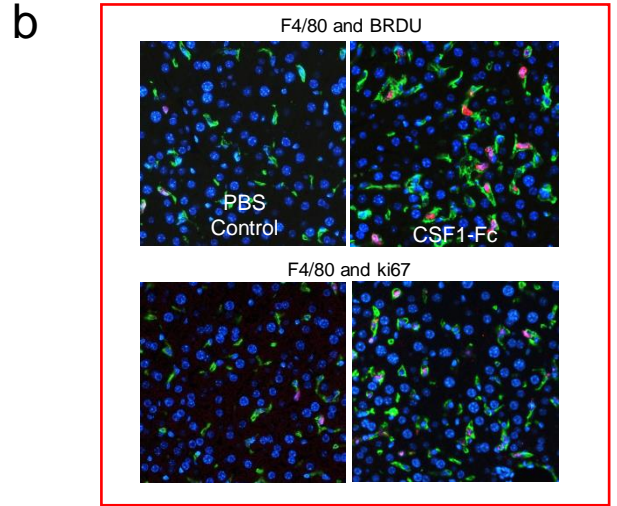
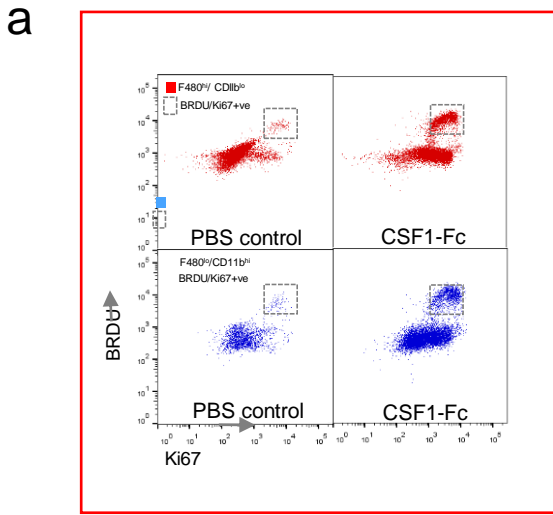


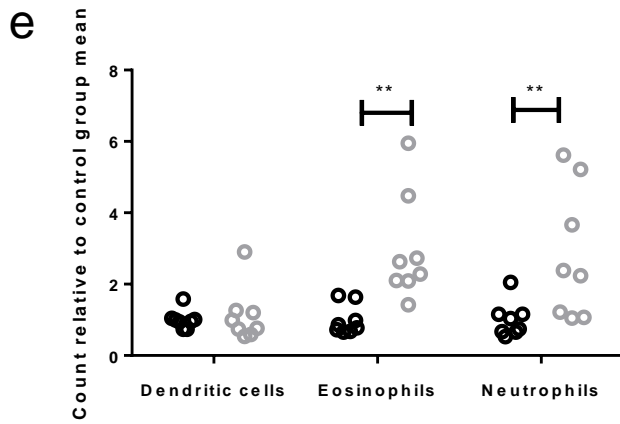
Supplementary Figure 3: Continued.



Supplementary Figure 3: Supporting details for CSF1-Fc treatment of uninjured mice.

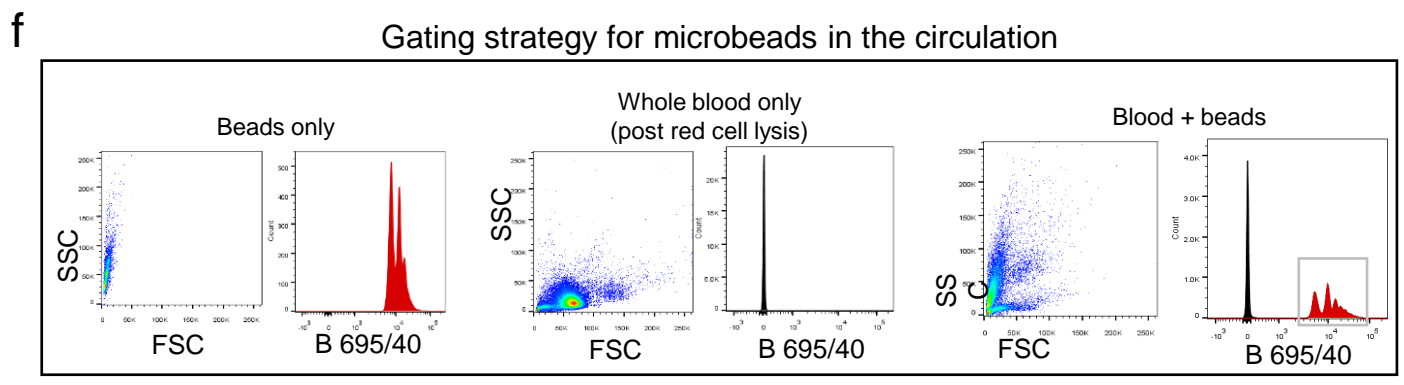
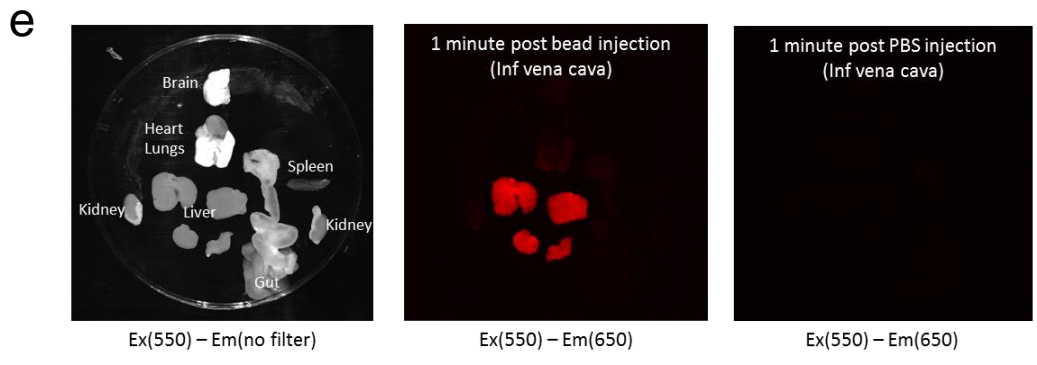
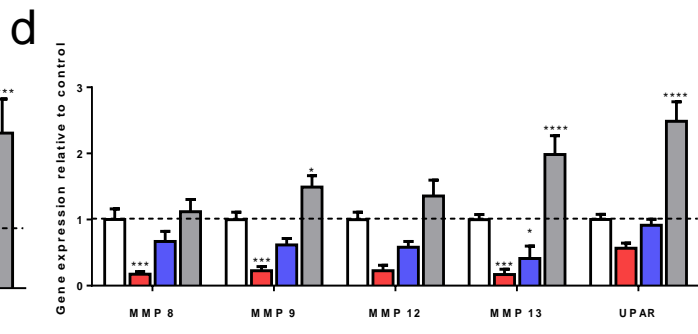
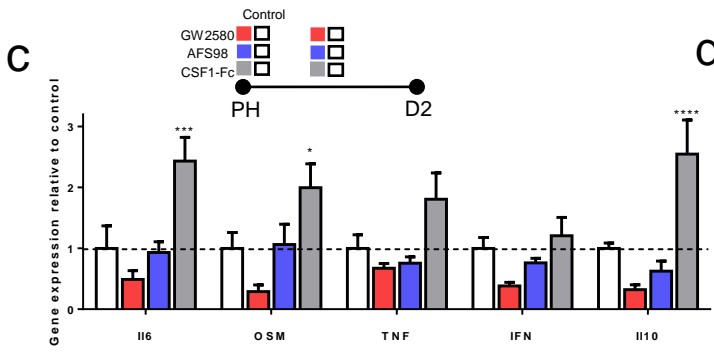
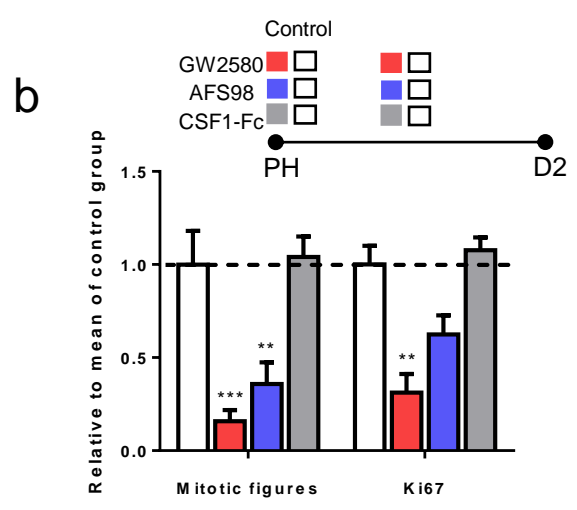
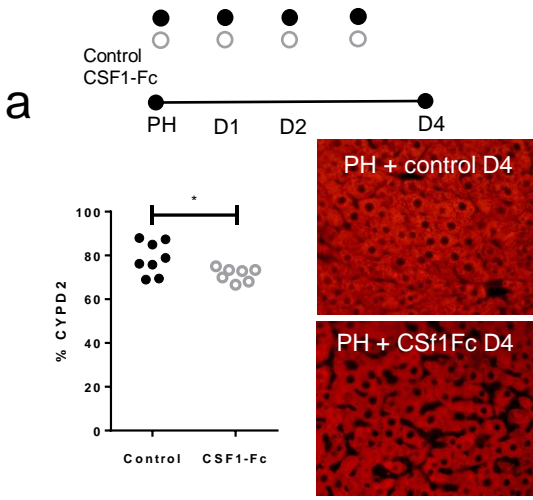
(a) Array data 6 hours following CSF1-Fc administration in uninjured mice (n=4/group). **(b)** TUNNEL immunohistochemistry following PBS control or CSF1-Fc administration (positive control DNase treated section). **(c)** Organ weight relative to mean of control group following 2 days treatment with PBS 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.

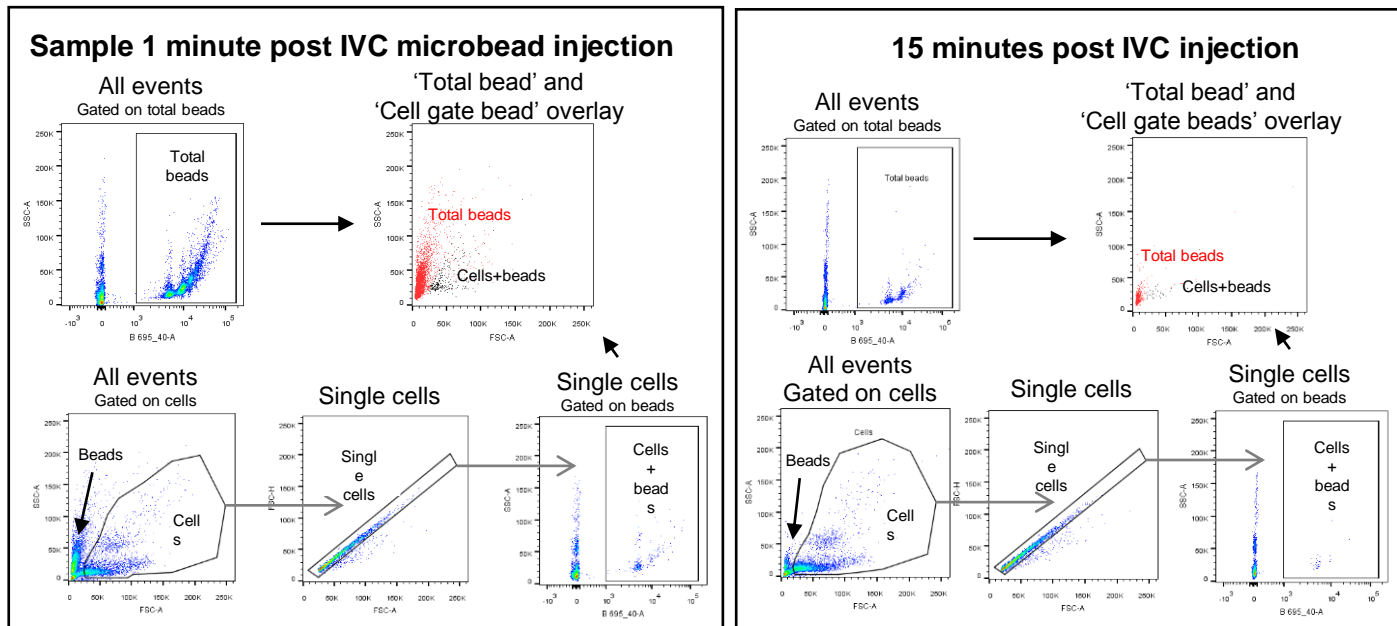
(a) Representative flow cytometry dot plot of BRDU and Ki67 expression in resident (red) and infiltrating (blue) macrophages. (b) Representative dual immunohistochemistry F4/80 (green) and BRDU or Ki67 (red) Day 2 following CSF1-Fc administration or control. (c) Number of hepatic dendritic cells (CD11c/MHCII +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.



Supplementary Figure 5: continued.

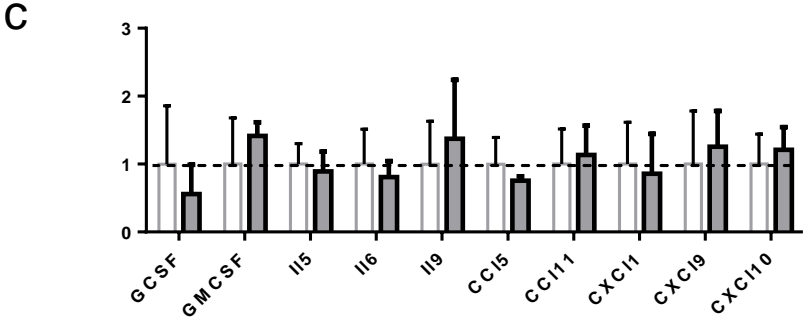
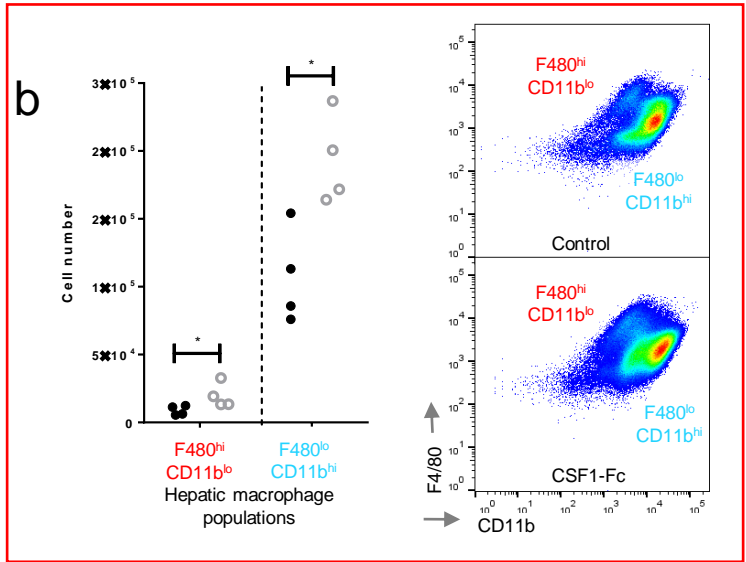
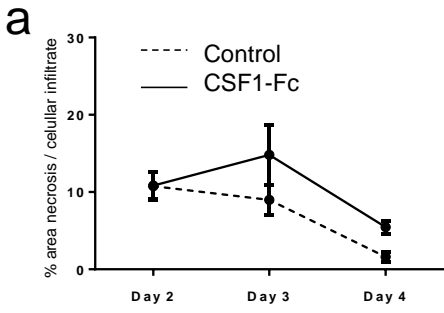
g

Minimal uptake of microbeads by blood phagocytes at 1 and 15 minutes post microbead injection

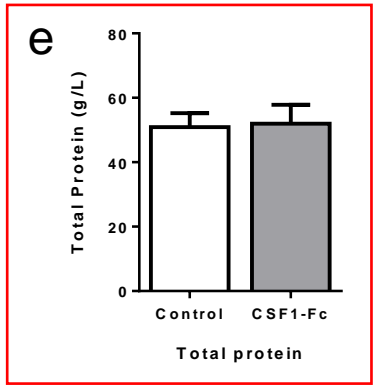
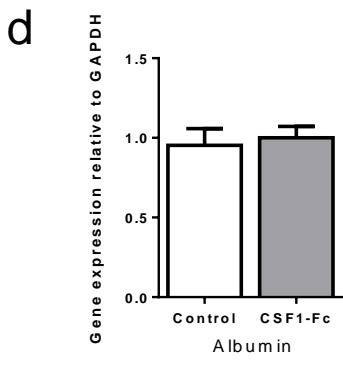


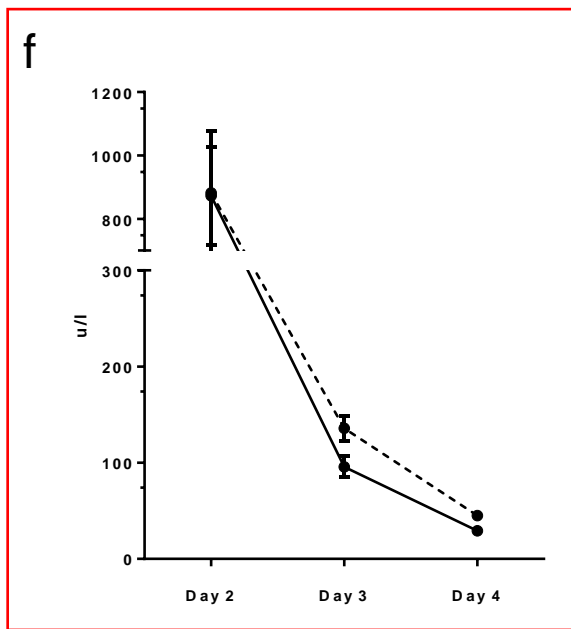
Supplementary Figure 5: Supporting data for partial hepatectomy model

(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 microbeads into the inferior vena cava. (f) Flow cytometry plots demonstrating bead and cell gating of blood samples following fluorescent microbead injection into 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 into the circulation. Gating strategies including total fluorescent bead count (“Total beads”) and bead count within blood cellular populations (“Cells+beads”).



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 IL-12 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)

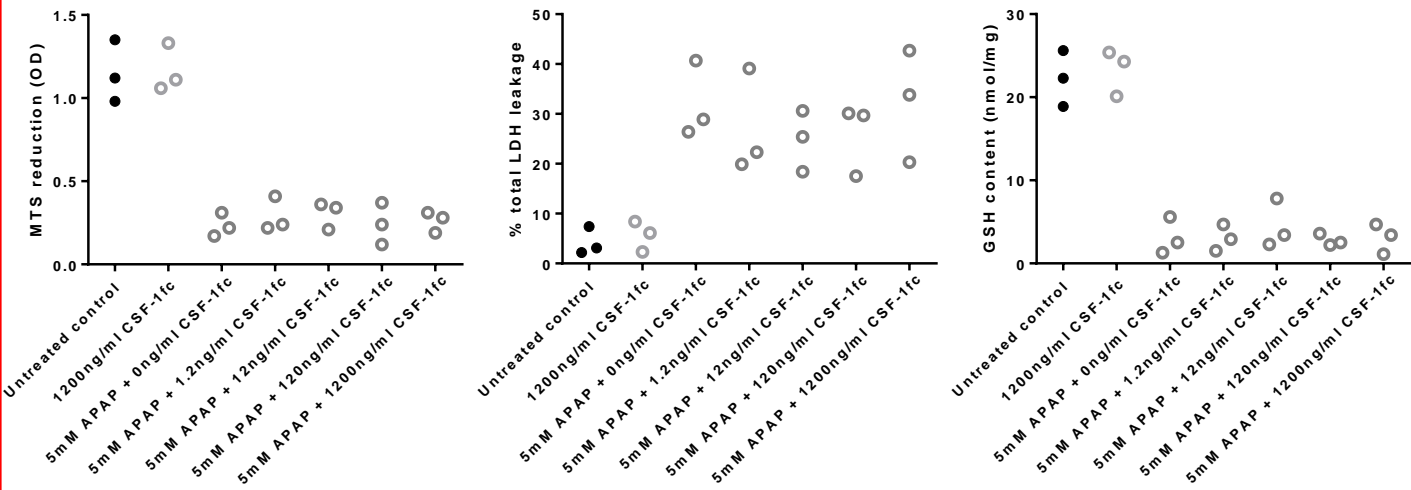




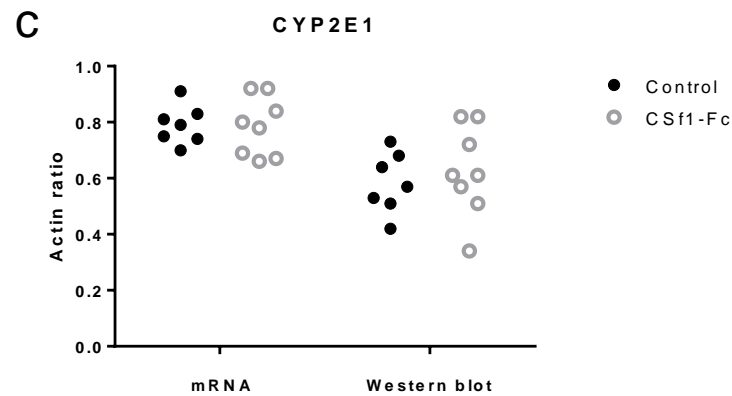
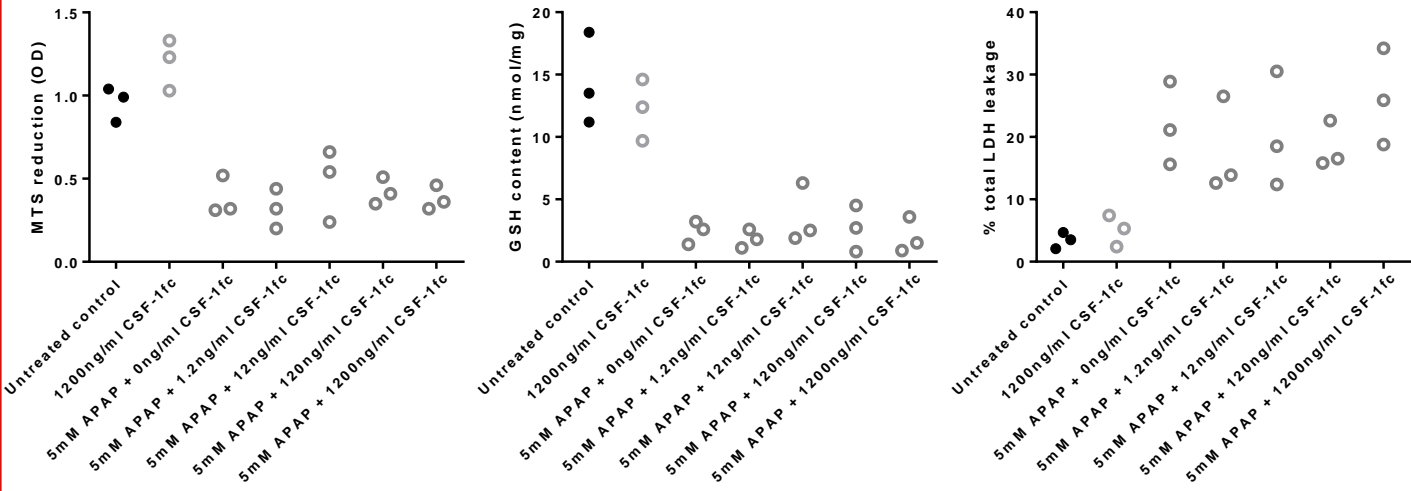
Supplementary Figure 6: Supporting details for acetaminophen intoxication in mice

(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.

a Mouse hepatocyte viability and glutathione depletion assays



b Human hepatocyte viability and glutathione depletion assays



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.

Supplementary Methods

Human serum samples

Serum samples were blinded and cytokine analysis completed in a random order. Serum CSF1 was analysed using the Meso Scale Discovery® CSF1 immunoassay and analysed on a Meso QuickPlex SQ120. Serum acetyl-HMGB1 was analysed by mass spectrometry.

Clinical scoring

King's college criteria in the context of acetaminophen induced liver failure was defined as arterial pH<7.3, international normalized ration (INR) > 6.5, serum creatinine > 300 and the presence of encephalopathy¹. The Systemic Inflammatory Response Criteria (SIRS) were met when 2 or more of the following occurred: body temperature >38°C or < 36°C; heart rate >90 beats per minute; respiratory rate >20 breaths per minute; white blood cell count >12,000/cf mm or <4,000/cf mm². APACHE2 score was calculated as previously described³.

Reagents

CSF1-Fc is a conjugate of porcine CSF1 with the Fc region of porcine IgG1A (43.82kD total) produced by Zoetis for D. Hume (UK patent application GB1303537.1). Porcine CSF1 is equally active in mice⁴. The Fc conjugate provides increased circulating half-life. CSF1-Fc did not exhibit any endotoxin like activity in murine BM-derived macrophages⁵. CSF1 receptor blockade was induced by the CSF1R tyrosine kinase inhibitor, GW2580 (160mg/kg suspended in 0.5% hydroxypropylmethylcellulose and 0.1% Tween 80⁵, LC laboratories), or using the antibody AFS98 produced by Sudo et al. ⁶, and provided by BioServ UK. CSF1-Fc, GW2580 and AFS98 were administered immediately following 2/3 partial hepatectomy or 12 hours following acetaminophen intoxication (point of maximal injury⁷).

Collection of mouse tissues

Mice were culled via CO₂ inhalation and following a midline laparotomy blood was aspirated from the inferior vena cava for serum analysis. Mice were perfused through the inferior vena cava and viscera excised and weighed. Viscera were either fixed in 4% formalin for immunohistochemistry, placed in RNA later® (Life Technologies), or placed in PBS for flow cytometry.

Immunohistochemistry

Three µm sections of formalin-fixed tissue were used for immunostains. Ki67, BRDU and CYPD2 required heat mediated antigen retrieval with 0.01M sodium citrate pH 6.0 for 10 minutes. Primary antibodies were used at the following dilutions: Ki67 (Leica) 1:500, BRDU (Abcam) 1:100, F4/80 (clone CI:A3-I, Biolegend) 1:100, CYPD2 (Abcam) 1:100. Appropriate secondary antibody was applied at a 1:250 dilution. Dual immunohistochemistry with F4/80 and BRDU or Ki67 was performed by first developing F4/80 using the Tyramide signal amplification system (PerkinElmer®) with subsequent heat mediated antigen retrieval followed by BRDU or Ki67 staining. Ki67 and F4/80 dual immunohistochemistry was also performed by developing F4/80 with an alkaline phosphatase substrate kit (red, Vector) and following heat mediated antigen retrieval Ki67 was developed with 3,3'-diaminobenzidine (Dako). Stained slides were blinded and images taken on the Nikon Eclipse E600. For image quantification of F4/80 staining 20 non overlapping images were photographed at x200. The extent of DAB staining quantified using image analysis software (Adobe Photoshop CS6). For CYPD2 quantification images were quantified using image analysis software (Adobe Photoshop CS6). For Ki67 quantification 20 serial non overlapping images were photographed at x400 then hepatocytes identified by assessment of morphology.

Flow cytometry

Liver was digested in 2mg/ml collagenase D (Sigma Aldrich) at 37degC for 30minutes then passed through a 100µm filter. 7 minute 50G spin to remove hepatocytes. Further purification of nonparenchymal cells using 30% percoll® (Sigma) gradient. Cell stained with fixable viability dye eFluor 780® then incubated with Fc block (TrustainfcX™, Biolegend) prior to staining with CD45

(clone:30F11, AF700, Biolegend), F480 (clone:BM8, PECy7, Biolegend), CD11b (clone:RM208, FITC, Invitrogen), 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), Ly6G (clone:IA8, PE, BD Biosciences). For proliferation assay cells were fixed and permeabilised using BD Pharmingen BRDU flow kit then stained with antiBRDU (FITC, BD Pharmingen) and Ki67 (eF660, eBioscience). Flow cytometry performed using the LSR Fortessa.

Quantification of Messenger RNA

Quantification of messenger RNA (mRNA) Levels by Real-Time Reverse-Transcription Polymerase RNA extraction kits (Qiagen) were used to extract RNA from whole tissue. Predesigned validated primer sets for MARCO (macrophage receptor with collagenous structure), MSR1 (macrophage scavenging receptor 1), MR (mannose receptor), Il6, OSM (oncostatin M), TNF (tumour necrosis factor), IFN γ (interferon gamma), Il10 and GAPDH were purchased from Qiagen (Qiagen Quantitect Primers). Quantitative real-time PCR was performed using Express SYBR Green (Qiagen, UK). Gene expression was calculated relative to GAPDH for each sample. Gene array at 6 hours following CSF1-Fc administration was performed using Cytokine and Chemokine array RT² Profiler PCR arrays and analysed using the online RT² profiler PCR Array Data Analysis (Version 3.5, Qiagen, UK) and presented by Volcano plot. Affymetrix Mouse gene 1.1 ST Array data were accessed from the Gene Expression Omnibus website and analysed using GEO2R with Benjamini & Hochberg (False discovery rate) correction applied to the entire data series.

Phagocytosis assay

Under 2% isofluorane anaesthesia the inferior vena cava was cannulated and 100 μ l of red fluorescent bead solution (1:5 Latex beads 1.0 μ m, fluorescent red, SIGMA-ALDRICH®) infused through the cannula (1:2 solution for assay following paracetamol injury). Ex vivo fluorescent quantification was performed at one minute following bead injection and 15mls 0.9% NaCl flush (see Supplementary Figure 5a). For assessment of bead clearance from the circulation 20mcl of blood was removed from the cannula every two minutes starting from 1 minute post injection for 15 minutes and immediately

fixed (300µl FACS-Lyse, BD Biosciences). After the 15 minute timepoint mice were perfused with 15mls 0.9% saline through the IVC cannula with portal vein outflow. Organs were then removed (Liver, spleen, lungs, kidney, brain) and imaged using a Kodak In-Vivo Multispectral FX image station (Excitation: 550nm; Emission: 600nm; Exposure 1 sec; f-stop 2.8). Subsequently blood samples were analysed 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 low flow rate setting. Multiphoton imaging was performed using a Zeiss LSM7 MP with Coherent Chameleon Ti:Sa laser.

Mouse Serum Analyses

Serum biochemistry assays were performed using commercially available kits by a biochemist, including alanine aminotransferase (ALT; Alpha Laboratories), alkaline phosphatase (Alk phos; Roche Diagnostics), total bilirubin (bili; Alpha Laboratories Ltd), albumin (Alb; Alpha Laboratories). **Total serum protein was analysed using the Bradford assay as previously described⁸.** Serum cytokines and chemokines were analysed using MILLIPLEX® mouse cytokine/chemokine array (Merck-Millipore) 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^{9, 10}. Glutathione (GSH) depletion assay, MTS reduction assay and LDH leakage assay were performed on human and mouse hepatocytes as previously described¹⁰.

References

1. O'Grady JG, Alexander GJ, Hayllar KM, et al. Early indicators of prognosis in fulminant hepatic failure. *Gastroenterology* 1989;97:439-45.
2. Bone RC, Balk RA, Cerra FB, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992;101:1644-55.
3. Knaus WA, Draper EA, Wagner DP, et al. APACHE II: a severity of disease classification system. *Crit Care Med* 1985;13:818-29.
4. Gow DJ, Garceau V, Kapetanovic R, et al. Cloning and expression of porcine Colony Stimulating Factor-1 (CSF-1) and Colony Stimulating Factor-1 Receptor (CSF-1R) and analysis of the species specificity of stimulation by CSF-1 and Interleukin 34. *Cytokine* 2012;60:793-805.
5. Jenkins SJ, Ruckerl D, Thomas GD, et al. IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J Exp Med* 2013;210:2477-91.
6. Sudo T, Nishikawa S, Ogawa M, et al. Functional hierarchy of c-kit and c-fms in intramarrow production of CFU-M. *Oncogene* 1995;11:2469-76.
7. McGill MR, Sharpe MR, Williams CD, et al. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *J Clin Invest* 2012;122:1574-83.
8. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
9. Kia R, Kelly L, Sison-Young RL, et al. MicroRNA-122: a novel hepatocyte-enriched in vitro marker of drug-induced cellular toxicity. *Toxicol Sci* 2015;144:173-85.
10. Huebener P, Pradere JP, Hernandez C, et al. The HMGB1/RAGE axis triggers neutrophil-mediated injury amplification following necrosis. *J Clin Invest* 2015;125:539-50.