On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver

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Iron is an essential component of the erythrocyte protein hemoglobin and is crucial to oxygen transport in vertebrates. In the steady state, erythrocyte production is in equilibrium with erythrocyte removal1. In various pathophysiological conditions, however, erythrocyte life span is compromised severely, which threatens the organism with anemia and iron toxicity2,3. Here we identify an on-demand mechanism that clears erythrocytes and recycles iron. We show that macrophages that express high levels of lymphocyte antigen 6 complex, locus C1 (LY6C1, also known as Ly-6C) ingest stressed and senescent erythrocytes, accumulate in the liver via coordinated chemotactic cues, and differentiate into ferroportin 1 (FPN1, encoded by SLC40A1)-expressing macrophages that can deliver iron to hepatocytes. Monocyte-derived FPN1+Tim-4neg macrophages are transient, reside alongside embryonically derived T cell immunoglobulin and mucin domain containing 4 (Timd4, also known as Tim-4)high Kupffer cells (KCs), and depend on the growth factor Csf1 and the transcription factor Nfκb2 (encoded by Nfe2l2). The spleen, likewise, recruits iron-loaded Ly-6Chigh monocytes, but these do not differentiate into iron-recycling macrophages, owing to the suppressive action of Csf2. The accumulation of a transient macrophage population in the liver also occurs in mouse models of hemolytic anemia, anemia of inflammation, and sickle cell disease. Inhibition of monocyte recruitment to the liver during stressed erythrocyte delivery leads to kidney and liver damage. These observations identify the liver as the primary organ that supports rapid erythrocyte removal and iron recycling, and uncover a mechanism by which the body adapts to fluctuations in erythrocyte integrity.

The physiological 120-day lifespan of human erythrocytes (red blood cells, RBCs)4,5 is shortened in various pathologies6–10. Red blood cell production requires a sufficient supply of iron: when iron stores are low, erythrocytes cannot be produced efficiently, causing anemia and tissue hypoxia. Because iron is acquired poorly through diet, most of the daily amount of iron needed for erythropoiesis is recycled by the reticuloendothelial system in the spleen and liver in a process that depends on the transmembrane iron export protein FPN1 (refs. 1,11,12). Disease-associated erythrocyte damage threatens the organism in several ways: (i) damaged erythrocytes release hemoglobin and heme, which can cause acute kidney injury; (ii) erythrocyte loss leads to anemia; and (iii) the heightened need for erythropagocytosis may overwhelm physiologic erythrocyte-clearing capacities, thereby liberating large quantities of iron, which is toxic in its nontransferrin-bound form1. We therefore hypothesized that vertebrates have evolved mechanisms that can adapt to fluctuations in erythrocyte integrity.

Erythrocytes and leukocytes can be identified as lymphocyte antigen 76 (Ly76, also known as Ter119)+CD45− and Ter119−CD45+ cells, respectively (Supplementary Fig. 1a). Labeling erythrocytes with a membrane-integrating dye (PKH26) ex vivo enabled detection in vivo and the tracking of labeled cells (Fig. 1a). Whereas most ‘young’ erythrocytes circulated in host blood for at least 16 h, aged or stressed erythrocytes (sRBCs)13,14 quickly disappeared (Supplementary Fig. 1b). Stressed erythrocytes co-localized with leukocytes predominantly in the liver (Fig. 1b), but also in the spleen, bone marrow, blood, and lung. Although splenectomized mice had higher percentages of delivered erythrocytes in the circulation then did nonsplenectomized controls, near-complete hepatectomy (95%) resulted in pronounced erythrocyte persistence in the circulation, and splenectomy combined with near-complete hepatectomy had the greatest effect on the persistence of damaged RBCs in the blood (Fig. 1c,d and Supplementary Fig. 2a).

In the steady state, the liver contains abundant F4/80highCD11bint KCs and relatively few CD11bhigh cells (Fig. 1e). The injection of stressed erythrocytes led to dramatic leukocyte fluctuations in the liver. At 3 h after sRBC injection, monocyte numbers peaked (Fig. 1f). At 16 h, a population of CD11bhighF4/80high macrophages (henceforth called transient macrophages (tMΦs)) appeared in the liver (Fig. 1e,f),

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but the number of F4/80$^{\text{high}}$CD11b$^{\text{hi}}$ KCs was diminished. At 72–168 h, the populations returned to near-basal levels (Fig. 1c,f). The spleen, which contains F4/80$^{\text{high}}$CD11b$^{\text{hi}}$ red pulp macrophages (RPMs) and monocytes, accumulated monocytes, but hardly any tMΦs, after stressed erythrocyte challenge (Fig. 1c,f). tMΦ accumulation in the liver was a specific response to the injection of hemoglobin-containing erythrocytes; the injection of hemoglobin-depleted 'ghosts' did not induce the appearance of tMΦs (Fig. 1c).

We used two independent approaches to track sRBC uptake by leukocytes. First, flow cytometry and histology detected PKH26$^+$CD45$^+$ cells in the circulation 0.01 h and 16 h after sRBC delivery. sRBCs (white blood cells, young RBCS (yRBCs), black circles) were injected 1 d after surgery. Sham-operated mice were used as a control. Data are expressed as means ± s.e.m.; n = 4 for the yRBCs, splenectomy, and sham groups; n = 3 for hepatectomy and for hepatectomy combined with splenectomy. * P < 0.05, ** P < 0.001, as compared to yRBCs; ANOVA and Dunnett’s multiple comparison test. (e) Left, representative dot plots (one of three experiments) of gating for F4/80$^+$CD11b$^{\text{int}}$ KCs, F4/80$^+$CD11b$^{\text{hi}}$ monocytes (Mo)/neutrophils (Neu), and F4/80$^+$CD11b$^{\text{hi}}$ tMΦs in the liver, and F4/80$^+$CD11b$^{\text{hi}}$ RPMs, F4/80$^+$CD11b$^{\text{hi}}$ Mo/Neu, and F4/80$^+$CD11b$^{\text{hi}}$ tMΦs in the spleen at steady state (0 h), 16 h, and 72 h after sRBC injection. Right, representative dot plots (one of three experiments) of cells gated as CD45$^+$/Lin$^-$ (i.e., negative for CD3, CD19 and NK1.1) leukocytes in liver and spleen 16 h after erythrocyte ghost injection. (f) Dynamics of the indicated leukocyte populations in liver (left) and spleen (right) at the indicated times after sRBC injection. Data are expressed as means ± s.d.; n = 6 for time-point 0; n = 3 for all other time points. (g) Representative dot plots (one of three experiments) showing uptake of PKH26$^+$ sRBCs in liver (top) and spleen (bottom) by KCs, RPMs, tMΦs and Mo/Neu 16 h after sRBC delivery. (h) Cytoplasmic PKH26 staining in KCs, RPMs, and tMΦs sorted from the liver and spleen 16 h after sRBC delivery. Nuclei with DAPI staining are stained blue, and PKH26-labeled sRBCs are stained red. (i) Dynamics of iron content measured by atomic absorption spectroscopy (AAS) in sorted leukocyte populations in liver (left) and spleen (right) at the indicated times after sRBC injection (each sample pooled from 3–6 mice). (j) Transmission electron microscopy of a liver section 16 h after sRBC injection, showing perihapatocellular leukocyte infiltrates surrounding RBCs (arrows) (one representative image from five different mice).
(Fig. 1i), presumably because of their ingestion of sRBCs. Such ingestion was probably mediated by receptors such as macrophage-scavenging receptor 1 (Msr1, also known in humans as SR-A) and CD36, because mice deficient in those receptors did not scavenge sRBCs as efficiently as WT controls (Supplementary Fig. 5a,b). The spleen was dispensable to the appearance of tMΦs in the liver (Supplementary Fig. 5c), whereas lethal irradiation, which depleted monocytes without affecting KC or RPM numbers at 10 d after irradiation, impaired erythrocyte clearance (Supplementary Fig. 5d–f).

Shortly after stressed erythrocyte challenge, we detected erythrophagocytosing macrophages by scanning electron microscopy (Fig. 1j). At 16 h, we observed a punctate pattern of iron stores, consistent with the confinement of iron in macrophages (Supplementary Fig. 6a). By 1 week after challenge, however, iron appeared in a diffuse parenchymal pattern, which is indicative of its accumulation in hepatocytes (Supplementary Fig. 6a). The greatest increase in iron levels at 16 h occurred in the liver (Supplementary Fig. 6b). This increase corresponded to high levels of liver hepcidin (Supplementary Fig. 6b), which is produced by hepatocytes in response to iron accumulation and is therefore a good marker of iron load in the liver. To test whether the appearance of tMΦs was a general response to the increased demand for erythrophagocytosis,
we used three anemia models: a model of hemolytic anemia induced by phenylhydrazine (PHZ) delivery\(^1, 17, 18\); a model of anemia of inflammation induced by heat-inactivated \textit{Brucella abortus}\(^19, 20\); and a model of sickle cell anemia\(^21\). In each model we detected tMΦ activation in the liver, but not in the spleen (Supplementary Fig. 7). In humans, erythrocyte damage resulted in erythrocyte accumulation in circulating CD14\(^{hi}\)CD16\(^{-}\) monocytes, as assessed by detection of the erythrocyte marker CD235a (encoded by GYPA) (Supplementary Fig. 8a, b) and by measurements of iron content inside monocytes (Supplementary Fig. 8c). Monocyte ingestion of erythrocytes was associated with increased serum iron levels and decreased serum haptoglobin levels relative to the steady state (Supplementary Fig. 8d, e). Macrophage-ingested iron is either stored in the cytoplasm bound to ferritin or is exported via FPN1 (ref. 11). Ferritin and FPN1 were expressed in both the spleen and liver in the steady state (Supplementary Fig. 9a–c), as expected\(^17, 18\). Among leukocyte populations, KCs and RPMs expressed FPN1 on their surfaces (Fig. 2a) for iron transport, as assessed by an iron-release assay (Supplementary Fig. 9d). However, the transfection of stressed, iron-containing erythrocytes resulted in increased expression of FPN1 in the liver, but not in the spleen, as compared to steady-state controls (Fig. 2a and Supplementary Fig. 9a–c), and led to increased FPN1 surface expression on tMΦs, specifically in the liver (Fig. 2a). The appearance of tMΦs in the liver required the injection of intact stressed erythrocytes (Supplementary Fig. 9e) and was exacerbated in response to competitive inhibition of the heme-cleaving enzyme heme oxygenase (Hmox) 1 by tin-protoporphyrin IX (SnPP-IX) (Supplementary Fig. 9f). These results suggest that the liver is uniquely mechanistically equipped to respond to an increased demand for erythropagocytosis.

Given the phenotypic similarities between monocytes and tMΦs, we tested whether Ly-6C\(^{hi}\) monocytes give rise to FPN1\(^+\) tMΦs in the liver. We adoptively transferred GFP\(^+\)Ly-6C\(^{hi}\) or Ly-6C\(^{low}\) monocytes to mice receiving stressed erythrocytes. Immediately after transfer, both monocyte subsets circulated in the blood, but neither produced FPN1 (Fig. 2b). 16 h later, Ly-6C\(^{hi}\) monocytes expressing F4/80 appeared in the liver, thus resembling tMΦs (Fig. 2b). By 72 h, these Ly-6C\(^{hi}\) monocyte-derived tMΦs had further differentiated to resemble KCs; they still contained erythrocytes (or their remnants) and still expressed FPN1 and the classical macrophage

**Figure 3** Cs1 and Nrf2 are essential for FPN1\(^+\) macrophage generation. (a–c) Cs1 (a) and Cs2 (b) mRNA expression, and the ratio of Cs1 to Cs2 expression (c) in liver and spleen at steady state (phosphate-buffered saline, PBS) and 6 h after challenge with sRBCs or ghosts. Rps29 was used as a housekeeping gene. Data are expressed as means ± s.e.m. (*n* = 8 PBS and sRBCs; *n* = 6 ghosts). ***P < 0.001 Kruskal–Wallis and Dunn’s multiple comparison test, comparing all groups to controls. (d) Slc40a1 mRNA expression in mouse Ly-6C\(^{hi}\) monocytes cultured with recombinant (r) Cs1 or Cs2, with or without sRBCs. Data are expressed as means ± s.e.m. (*n* = 3 per group). ***P < 0.001 unpaired two-tailed *t* test, comparing rCs1- and rCs2-incubated monocytes for each condition. (e) Slc40a1 mRNA expression on human CD14\(^{hi}\)CD16\(^{-}\) monocytes cultured with rCsf1 or rCsf2, with or without sRBCs. Data are expressed as means ± s.e.m. (*n* = 6 per group). *P < 0.05, ***P < 0.001 ANOVA Bonferroni’s multiple comparison test, comparing rCsf1- and rCsf2-incubated monocytes for each condition. (f) Slc40a1 mRNA expression on Ly-6C\(^{hi}\) monocytes cultured with rCs1 and decreasing amounts of rCs2, together with sRBCs. Data are expressed as means ± s.e.m. (*n* = 5 per group, except rCs2 0.01 ng/ml group, where *n* = 3). ***P < 0.001, **P < 0.01 compared to rCs1-incubated monocytes; ANOVA and Dunnett’s multiple comparison test. (g) Slc40a1 mRNA expression on mouse Ly-6C\(^{hi}\) monocytes sorted from WT or Nrf2\(^−/−\) mice and cultured with rCs1 or rCs2, together with sRBCs. Data are expressed as means ± s.e.m. ***P < 0.001 compared to rCs1-incubated monocytes for the same strain; Mann–Whitney *U* test (*n* = 5 per group, except Nrf2\(^−/−\) Cs2 group, where *n* = 4). (h, i) Representative dot plots (one of three experiments) (h), and quantification of KCs and tMΦs (i) in liver of WT mice at steady state (white bars) and 16 h after sRBC injection in WT mice, WT mice treated with the c-fms tyrosine kinase inhibitor Ki20227 (S1F1 inh.), or Nrf2\(^−/−\) mice (gray bars). Data are expressed as means ± s.e.m. (*n* = 3 for WT control, WT sRBC, and WT sRBC/ Ki20227; *n* = 4 for Nrf2\(^−/−\) sRBC). ***P < 0.001 compared to sRBC-challenged WT mice; ANOVA Bonferroni’s multiple comparison test.
signature proteins \textsuperscript{22} Mertk and CD64 (encoded by \textit{FcgRI}) on their surfaces (Fig. 2b). By contrast, Ly-6C\textsuperscript{low} monocytes infiltrated the liver in lower numbers than did Ly-6C\textsuperscript{high} monocytes, did not differentiate to tMΦs, and remained FPN1\textsuperscript{−}, Mertk\textsuperscript{−}, and CD64\textsuperscript{−} (Fig. 2b and Supplementary Fig. 9g). Moreover, monocyte-to-FPN1\textsuperscript{−} macrophage differentiation was specific to the liver, and only a few of the transferred cells from either subset accumulated in the spleen (Fig. 2b). We observed a similar phenomenon after transferring GFP\textsuperscript{+}Ly-6C\textsuperscript{high} monocytes to sickle-cell-disease model mice (Supplementary Fig. 10a,b).

To test whether Ly-6C\textsuperscript{high} monocytes can support long-term KC renewal or whether tissue-derived KCs eventually outcompete monocyte-derived cells, we joined C57BL/6 and GFP\textsuperscript{+} mice by parabiosis (Supplementary Fig. 10c,d).\textsuperscript{23} In the absence of erythrocyte delivery, Ly-6C\textsuperscript{high} blood monocyte chimerism rose to \textasciitilde25\% in the first 2 weeks and remained unchanged for \textasciitilde100 d, whereas KC chimerism remained low, at \textasciitilde2\%, for the duration of the experiment (Fig. 2c). Erythrocyte delivery 2 weeks after parabiosis began had no effect on blood monocyte chimerism, but it increased chimerism among KCs to \textasciitilde15\% for \textasciitilde28 d (Fig. 2c). By contrast, RPM chimerism did not rise in the spleen as a consequence of erythrocyte challenge. These data show that a high demand for erythropagocytosis mobilizes erythrocyte-ingesting Ly-6C\textsuperscript{high} monocytes to the liver, where they first differentiate to iron-recycling tMΦs and then to iron-recycling KC-like cells. When demand declines, monocyte-derived KC-like cells disappear, and the remaining KC replenish, independently of monocytes or other circulating progenitors.

To dig deeper into how monocyte and tissue-derived macrophages differ, we sorted liver KCs and tMΦs 16 h after erythrocyte challenge into groups that either had or had not ingested erythrocytes. We then profiled gene expression by RT–PCR (Fig. 2d) and surface protein expression by flow cytometry (Supplementary Fig. 10e), detecting numerous differences, including the expression of SpiC, which encodes a transcription factor required for RBC recycling.\textsuperscript{17,18} Next, because increased KC chimerism after erythrocyte challenge (Fig. 2c), as well as a shift of tMΦs to the KC gate (Fig. 2b), indicated that, for a limited time, KCs are a mixed population composed of both tissue- and blood-derived cells, we performed formal lineage-tracing experiments. We mated Cx3cr1\textsuperscript{CreERT2} females with ROSA26-tdTomato\textsuperscript{\textit{CreERT2}} (R26-tdT) males\textsuperscript{24,25}, giving rise to Cx3cr1\textsuperscript{CreERT2}R26-tdT pups with tdT\textsuperscript{+} KCs and tdT\textsuperscript{−} monocytes (Fig. 2e). On postnatal (P) day 70, we transplanted GFP\textsuperscript{+} bone marrow to generate GFP\textsuperscript{+} monocytes in the grown pups (Fig. 2e and Supplementary Fig. 11a). On day P91, we challenged the mice with stressed erythrocytes and, 7 d later, detected embryonically derived tdT\textsuperscript{+} GFP\textsuperscript{−} KCs (eKCs) and monocyte-derived tdT\textsuperscript{−} GFP\textsuperscript{+} KCs (mKCs) in the KC gate. We then sorted these cells for microarray analysis (Fig. 2e). Our data showed numerous differences between the cells (Fig. 2f and Supplementary Fig. 11b), although the expression of iron metabolism mediators was comparable (Supplementary Fig. 11b,c). Among the differentially expressed genes, Timd4, which produces the surface Tim-4 protein, was highly expressed in embryonic but not monocyte-derived KCs (Fig. 2f), a finding that we confirmed by flow cytometry (Fig. 2g).

**Figure 4** The on-demand mechanism of erythrocyte disposal preserves homeostasis. (a) Ccl2 (left) and Ccl3 (right) mRNA levels in the liver and spleen in response to sRBC or PBS injection. Data are expressed as means \pm s.e.m. (\textit{n} = 6 per group). ***\textit{P} < 0.001; ANOVA Bonferroni’s multiple comparison test. (b) Quantification of Ly-6C\textsuperscript{high} monocyte and tMΦ numbers in liver, spleen, blood, and bone marrow 16 h after treatment with Ccr2 and Ccr5 antagonists (Ant) or 16 h after sRBC injection with or without Ant treatment. Data are expressed as means \pm s.e.m. (Ant bone marrow, \textit{n} = 10; sRBC bone marrow, \textit{n} = 9; sRBC/Ant bone marrow, \textit{n} = 10; all other groups (liver, spleen, blood), \textit{n} = 14). ***\textit{P} < 0.0001, \*\textit{P} < 0.05; ANOVA Bonferroni’s multiple comparison test. (c) Quantification of PKH26\textsuperscript{+}Ter119\textsuperscript{+}CD45\textsuperscript{+} cells in the blood, as a fraction of the entire erythrocyte pool in the circulation 16 h after sRBC injection with or without Ant treatment. Data are expressed as means \pm s.e.m. (\textit{n} = 4 per group). \*\textit{P} < 0.05; Mann–Whitney U test. (d) eLPI levels in mice 16 h after treatment with Ant or after injection of sRBC with or without Ant treatment. Data are expressed as means \pm s.e.m. (\textit{n} = 8 Ant, \textit{n} = 9 sRBC, \textit{n} = 7 sRBC/Ant). **\textit{P} < 0.001; ANOVA Bonferroni’s multiple comparison test. (e) Haptoglobin and hemopexin mRNA levels in the liver of mice 16 h after treatment with Ant or after injection of sRBC with or without Ant treatment. Data are expressed as means \pm s.e.m. (\textit{n} = 4 per group). \*\textit{P} < 0.05; ANOVA Bonferroni’s multiple comparison test. (f) Liver ALT, AST, amylase, and blood urea nitrogen (BUN) activities or levels in serum of mice 16 h after treatment with Ant or after injection of sRBC with or without Ant treatment. Data show means \pm s.e.m. (\textit{n} = 4 per group). \*\textit{P} < 0.05, **\textit{P} < 0.01, and ***\textit{P} < 0.001; ANOVA Bonferroni’s multiple comparison test. (g) Prussian blue staining in kidneys from the indicated groups of mice. The two sRBC/Ant micrographs show a glomerulus (left) and proximal tubuli (right). Iron depositions appear blue; hemosiderin appears brown. One of three representative images is shown.
These data show that as tMΦs temporarily shift to the KC gate, they nevertheless differ from their embryonically derived counterparts.

The growth factors colony stimulating factor (Csf)1 and Csf2 instruct myeloid cell fate decisions.26,27 After erythrocyte challenge, the liver and spleen had augmented Csf1 expression (Fig. 3a), the spleen, but not liver, had augmented Csf2 expression (Fig. 3b), the lung had augmented expression of neither Csf1 nor Csf2 (Supplementary Fig. 12a), and the bone marrow had augmented Csf2 expression (Supplementary Fig. 12b). Consequently, the liver had a high Csf1-to-Csf2 ratio, whereas the spleen and bone marrow had a low Csf1-to-Csf2 ratio (Fig. 3c and Supplementary Fig. 12b), after stressed erythrocyte injection. To evaluate whether these two growth factors cultivate KCs was unaffected by Csf1 blockade (Fig. 4d). The number of tMΦs in the liver was inversely correlated with eLPI, AST, and BUN (Supplementary Fig. 15). Moreover, we saw increased iron deposition and nephron damage in the kidney (Fig. 4g and Supplementary Fig. 16a). We observed similar nephron damage after Hmox1 inhibition, which is predicted to result in hem-induced oxidative end organ damage30, as well as in the PHZ-induced hemolysis and sickle cell disease models (Supplementary Fig. 16b). Finally, the clinical manifestations of inadequately cleared, stressed erythrocytes by FPN1+ tMΦs were phenocopied in mice challenged with stressed erythrocytes whose macrophages lacked FPN1 (Supplementary Fig. 17a,b)31, and they were ameliorated in a rescue experiment using LysM-specific, FPN1-deficient mice that received stressed erythrocytes (Supplementary Fig. 17c). These findings indicate that hepatic recruitment of Ly-6Chigh monocytes and their differentiation into iron-recycling macrophages preserves organisal homeostasis during fluctuations of erythrocyte integrity.

In summary, we show that Ly-6Chigh monocytes and their progeny propagate on-demand, high-capacity erythrophagocytosis, and iron recycling. This liver-specific phenomenon is transient, dynamic, requires cell-intrinsic and environmental triggers, and controls iron homeostasis during erythrocyte damage (Supplementary Fig. 18). Understanding and eventually harnessing this mechanism may help to treat persons with abnormally heightened erythrocyte elimination due to sepsis, various forms of inflammation, frequent blood transfusions, or inborn or acquired defects in erythrocyte stability.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: Genome-wide expression data were deposited in the GEO repository under GSE77106.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
I.T., I.H., and M. Nairz conceived the project, designed and performed experiments, analyzed and interpreted data, and wrote the manuscript; P.T., D.H., M.A., S.H., L.M.S.G., T.A.W.H., M.S., S.S., A.M.F., A.A., S.R., C.M., M.T., P.W., Y.I., G.E.W., N.K.H., B.G.C. and M.M. performed experiments; T.L.A., F.W., M.P. and P.W. provided materials and intellectual input; O.M.D.L., J.L.B., M. Nahrendorf, and G.W. provided intellectual input and edited the manuscript; L.B. and E.R. conceived and conducted the clinical trial. R.W. edited the manuscript; H.Y.L. conceived the project, helped interpret data, and edited the manuscript; F.K.S. conceived the project, designed experiments, analyzed and interpreted data, and wrote the manuscript.

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COMPEING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Animals. C57BL/6J (wild-type, WT), B6.SJL-Purp5ec8/BoyJ (CD45.1), C57BL/6J-Tg(UBC-GFP)30Scha1 (GFP), B6.129X1-Nf2tm1Mountain1 (Nf2f2−/−), B6.Cg-Mtr1C9Ck1−/− (Mtr1−/−), B6.129F1-Cd3e128N2S1/2 (CD3ε−/−), B6.129-Hbαm1Tow/Hbbtm2S5H2 (HbA/Bβ), B6.129-Hbαm1Tow/Hbbtm2S5H2 (sickle cell disease, CDC) and B6.129-Hbαm1Tow/Hbbtm2S5H2 (nonsickling transgenic control, tcg) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). GM-CSF-deficient mice (Cd3ε−/−) on a C57BL/6J background were bred in-house. B6.129S6-Tg(Rosa26-tdTomato)7Scha1 (ROSA26-Tdtomato2/2) or B6-Tdtomato2/2 and B6.129P2(Cg)-Cxc3r1tm2.1cre/ERT2Jung (Cxc3r1-CreERT22/2) mice were kindly provided by M.P. Mice carrying the myeloid-specific knockout of the Fnp1 gene (LysMCre+/+;Slc40a1f/f) were kindly provided by F.W., backcrossed on a C57BL/6 background and bred in house. Unless otherwise indicated, age-matched female animals were used at 8–12 weeks of age. When appropriate, animals were randomly assigned to interventions. All protocols were approved by the Animal Review Committee at Massachusetts General Hospital (Protocol No. 2011N000035 and 2015N000044) and by the Federal Ministry of Science, Research and Economy in Austria (Project No. BMWFUW-66.011/0115-WF/3b/2014).

Erythrocyte preparation and PKH26 labeling. RBC preparation. Isolation, storage, and aging of red blood cells (RBCs) from the blood of C57BL/6J WT mice was performed as described previously13. In detail, whole blood collected from C57BL/6J WT mice was pooled, centrifuged at 400g for 10 min, and the buffy coat was removed. In a second step, RBCs were diluted in PBS and used for experiments. RBCs were hemolyzed (with PBS to distilled water (1:15), followed by at least 5 washes with the same buffer and centrifugation at 30g for 5 min, until a white pellet was obtained. The pellet of RBC ghosts was resuspended in PBS and used for experiments in vivo.

Animal models and interventions in vivo. Hepatectomy and spleenectomy. Lobectomy of liver tissue was performed after blood vessel ligation with a 7-0 Ethilon suture (Ethicon, Somerville, NJ). Removal of the left, median, and right lateral lobe (lobes 2–5) represented 95% liver mass. When appropriate, animals were randomly assigned to interventions. All mice were euthanized 16 h later. Serum and organ samples were collected and processed for liver and kidney function parameters and histology.

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Mouse cells. Collection. Peripheral blood was collected by retroorbital bleeding with heparinized capillaries and, unless appropriate, erythrocytes were lysed in RBC lysis buffer (BioLegend, San Jose, CA, USA). Spleen and liver tissues were excised after vascular perfusion with 20 ml sterile PBS. Organs were minced and strained through a 40-µm nylon mesh (BD Biosciences, San Jose, CA, USA). To increase the yield of hepatic macrophages, minced liver tissues were treated with busulfan and transplanted with either LysMCre+/+;Slc40a1f/f mice were treated with busulfan and transplanted with either LysMCre+/+;Slc40a1f/f (WT) or LysMCre+/+;Slc40a1f/f (FPN1−/−) bone marrow cells, as described above for WT mice. 3 weeks after transplantation, mice were infused with sRBCs and euthanized 16 h later. Serum and organ samples were collected and processed for liver and kidney function parameters and histology.

Both antagonists were administered again 6 h after sRBC transfer. SnPP-IX injection. In some experiments, RBC transfer was accompanied by i.p injection of the Hmox1 inhibitor SnPP-IX at 0.5 mg/kg body weight (Cayman Chemical, Ann Arbor, MI, USA). SnPP-IX was dissolved in 0.2 M NaOH, and the pH was adjusted to 7.40 with 1 M HCl. SnPP-IX was administered every 8 h, starting 30 min before sRBC transfer. Irradiation. In some experiments, mice were lethally radiated (10 Gy), but not reconstituted, with donor bone marrow cells for 10 d. Adoptive transfer. Ly-6chigh and Ly-6clo monocytes were isolated by flow-assisted cell sorting (FACS) from spleen or bone marrow of GFP+ mice and 1 × 106 cells/mouse were injected i.v., followed by an additional sRBC injection. Parabiosis. Mice were joined in parabiosis, as previously described12. In brief, the opposing lateral sides of mice were shaved, skin incisions made from behind the ear to the tail, and the skin was bluntly detached from the subcutaneous fascia over a width of about 1 cm. At the laterally exposed areas of each parabiotic, a 1-cm long incision of the peritoneum was made, and the corresponding edges were sutured together, joining the two peritoneal cavities. Finally, the parabionts were joined by suturing the corresponding scapulas and the skin with Ethilon 5-0 (Ethicon, Albuquerque, NM, USA). Both parabions were injected in parallel with 400 µl sRBCs or saline as controls. Hemolytic anemia model involving PHZ. C57BL/6J WT mice were administered 2 mg/mouse phenylhydrizine (PHZ; from Sigma-Aldrich, St. Louis, MO, USA) dissolved in 200 µl PBS i.p. 3 d after injection, peripheral blood was drawn from the cheek and measured for RBC content on a VetScan hematology analyzer (Abaxis Veterinary Diagnostics, Union City, CA, USA). Liver was excised after vascular perfusion with 20 ml sterile PBS, minced, and strained through a 40-µm nylon mesh (BD Biosciences, San Jose, CA, USA). The single-cell suspension was then stained with antibodies for flow-cytometric analysis. Brucella abortus-induced anemia model. To induce anemia, animals were injected i.p. with 1 × 106 particles per mouse of a commercially heat-killed B. abortus preparation (strain 1193-3; US Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories, San Francisco, CA, USA). Control mice were injected i.p. with normal saline. During the experiments, mice were studied on days 0, 2, 4 and 9 after injection. Sickle cell anemia model. Unless otherwise indicated, sickle cell disease mice and transgenic controls were studied in steady state. Lineage-tracing experiments: radiation-free bone marrow chimeras. 2–3-month-old WT mice were treated with low-dose busulfan (Busilvex, purchased from Pierre Fabre, Freiburg, Germany; 10 mg/kg, i.p.) and transplanted with 50–70 × 106 bone marrow cells obtained from GFP+ mice 3 d later. Blood leukocyte chimerism (GFP positivity) was tested every week, and typically reached 50–90% among Ly-6chigh monocytes. 3 weeks after bone marrow transplantation, mice were treated with sRBCs or PBS and euthanized 4–7 days later; the chimera of organ-resident myelocytes was checked with flow cytometry with co-staining for lineage and myeloid cell markers. tdTomato mice. Pregnant Cx3cr1-CreERT2 mice (mated with R26-Tdtomato) were injected s.c. with tamoxifen (Sigma-Aldrich, 8 mg/ml in 400 µl corn oil) on days E13.5 and 15.5 to induce tdTomato expression in embryonic macrophages/microphages. Pups were derived by Cesarean cut on day E21 and placed with nursing FVB/N dams. The Cx3cr1-CreERT2;R26-Tdtomato mice were distinguished from the genuine litter by coat color. At the age of 10 weeks (P70), mice were transplanted with GFP bone marrow after busulfan pre-conditioning (see ‘Radiation-free bone marrow chimeras’) and challenged with sRBCs or PBS as described above (P91). Chimerism and tdTomato expression among blood leukocytes and organ-resident myelocytes was determined by flow cytometry. FNP1-deficient rescue experiments. LysMCre+/+;Slc40a1f/f mice were treated with busulfan and transplanted with either LysMCre+/+;Slc40a1f/f (WT) or LysMCre+/+;Slc40a1f/f (FPN1−/−) bone marrow cells, as described above for WT mice. 3 weeks after transplantation, mice were infused with sRBCs and euthanized 16 h later. Serum and organ samples were collected and processed for liver and kidney function parameters and histology.

Mouse cells. Collection. Peripheral blood was collected by retroorbital bleeding with heparinized capillaries and, unless appropriate, erythrocytes were lysed in RBC lysis buffer (BioLegend, San Jose, CA, USA). Spleen and liver tissues were excised after vascular perfusion with 20 ml sterile PBS. Organs were minced and strained through a 40-µm nylon mesh (BD Biosciences, San Jose, CA, USA). To increase the yield of hepatic macrophages, minced liver
tissue was digested in 450 U/ml collagenase I, 60 U/ml DNase I and 60 U/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 20 min at 37 °C while shaking. Cells were counted in a Neubauer chamber. Sorting. Mouse monococytes, macrophages, neutrophils, B cells and T cells from blood, spleen and liver homogenates were isolated by FACS with a FACS Aria II cell sorter. For adoptive transfer and culture experiments, mouse spleen homogenates were enriched for monocytes before FACS. In brief, splenocytes were incubated with 1 μl anti-CD11b (clone M1/70, BD Biosciences, San Jose, CA, USA) or anti-CD115 Ab (clone AF589, ebioscience, San Diego, CA, USA) per 1 x 10^6 cells in sterile 2% FBS, 0.5% BSA in PBS for 30 min on ice, followed by MACS bead incubation and positive selection with a Midi MACS separator and LS column (all from Miltenyi, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. KC sorting in lineage-tracing experiments. To maximize the yield of KCs, animals were perfused with Liberase (Roche, Mannheim, Germany) according to a published protocol\(^3\). The obtained single-cell suspension was centrifuged at 30g for 3 min to pellet hepatocytes, and the supernatants were centrifuged at 5 x 10^4 cells per tube; at least three such tubes were collected and the supernatants were centrifuged at 300 g with the addition of 1:100 Eppendorf tubes containing 350 μl RLT Buffer (Qiagen, Hilden, Germany), with the addition of 1:100 β-mercaptoethanol. The amount of the sorted eKCs and mKCs was 5 x 10^6 cells per tube; at least three such tubes were collected per animal and pooled in the course of RNA isolation.

Human cells. Collection. Five people (one female, four males) undergoing cardiac surgery with the necessity for extracorporeal circulation in the form of cardiopulmonary bypass (CPB) involving the Cell Saver autologous blood recovery system (Haemonetics Corporation, Braintree, MA, USA) were enrolled in an observational study approved by Partners Human Research Committee (Protocol No. 2013P002596/MGH). Written informed consent was obtained in accordance with the Declaration of Helsinki from these persons. Indications for cardiac surgery were aortic valve replacement and concurrent coronary artery bypass grafting (n = 1), and repair of an aneurysm of the aortic sinus (n = 1). A mean volume of 755 ml of cell saver, including autologous RBC but no packed RBCs, were transfused in the course of surgeries that had a mean duration of 242 min and a mean CPB time of 105 min. Peripheral blood samples were taken before, 15 min after, and 4 h after extracorporeal circulation. Sorting. Human monocytes were isolated by FACS with a FACS Aria II cell sorter (BD Biosciences). Human peripheral blood mononuclear cells (hPBMCs) were enriched for monocytes before FACS. PBMCs were isolated from heparinized whole blood by the use of Ficol density gradient centrifugation. For further enrichment, hPBMCs were incubated with 2 μl anti-CD11b, anti-CD18, anti-CD28, anti-CD54 (clone ICRF44, BD Biosciences), anti-HLA-DR (clone L243, BD Biosciences), anti-CD11a (clone 6D5, BD Biosciences), anti-CD49b (clone DX5, BD Biosciences), anti-CD11c (clone M1/70, BD Biosciences), anti-CD11b (clone CM1, BD Biosciences), anti-CD14 (clone MP9, BD Biosciences), anti-CD16 (clone 3G8, BD Biosciences), anti-CD14 (clone MP9, BD Biosciences), anti-CD36 (clone NCAM162, BD Biosciences), anti-CD235a (clone RA-G2, BD Biosciences). Cells were first gated using FSC/SSC characteristics, and doublets were excluded by comparing FSC-height and -area signals. Classical monocytes were identified as CD14+, CD11b+, F4/80high, MHCIIlow, SSCint, CD11clow, Ly-6Cint cells. Neutrophils were identified as CD45+, Lin−, CD11b+, MHCIIlow, SSCint, Ly-6Chigh cells. Red pulp macrophages (RPMs) were identified as CD45+, Lin−, CD11b+, F4/80high, MHCIIlow, CD11clow, SSCint, Ly-6Cint cells. The following antibodies were used for human flow cytometric analyses: anti-CD11b (clone ICRF44, BD Biosciences), anti-CD11c (clone 6D5, BD Biosciences), anti-CD36 (clone NCAM162, BD Biosciences), anti-CD235a (clone RA-G2, BD Biosciences). Cells were first gated using FSC/SSC characteristics, and doublets were excluded by comparing FSC-height and -area signals. Classical monocytes were identified as HLA-DR+, CD11b+, CD14+, CD115+, CD16− and non-classical monocytes as HLA-DR−, CD11b+, CD14+, CD16−. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v8.8.6 (Tree Star, Inc., Ashland, USA).

Cell and molecular biology. Cell culture. Human and mouse monocyte subtypes were cultured at 0.7 x 10^6/500 μl in RPMI-1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, in a 24-well plate (Falcon; obtained from Fisher Scientific, Pittsburgh, PA, USA). Recombinant (r) CSF1 or rCSF1 (50 ng/ml, Preprotech, Rocky Hill, NJ, USA), rCSF2 or rCSF2 (100 ng/ml, Preprotech, Rocky Hill, NJ, USA) were added to the cell cultures for 72 h. 6 h before termination of the experiment, a tenfold excess of sRBCs was added. In vitro RBC uptake. 50,000 mouse FAC-sorted cells were seeded onto 24-well plates in 500 μl RPMI-1640 supplemented with 10% autologous serum, l-glutamine and antibiotics. Subsequently, cells were stimulated with PBS or 50,000 PKH26-labeled naïve rYBCs or sRBCs for 1 h. Cells were washed 2 x in 1 x RBC lysis buffer to remove surplus RBCs, and incubated in RPMI-1640 for another 2 h. Thereafter, cells were washed twice with 1 ml of 1 x RBC lysis buffer for 10 min before antibody staining for flow cytometry. To test for the possibility of cell–cell transfer of PKH26, 50,000 FAC-sorted cells per population tested were seeded in monoculture or coculture in 24-well plates for up to 72 h. To identify individual populations after co-culture, KCs were harvested from CD45.2+ GFP+ donors, whereas mKCs and monocytes were of the CD45.1+ genotype (and thus GFP−). To identify the cellular source of Ccl2, Ccl3 and Csf1, individual cell populations were FAC-sorted from liberase-perfused liver of mice under steady-state conditions (KCx) or 16 h after elicitation of mKCs and monocytes with sRBCs. 250,000 cells were seeded onto 6-well plates in 1 ml RPMI-1640 supplemented with 10% FBS, l-glutamine, and antibiotics. After an additional 16 h of culture, supernatants were frozen and chemokine/growth factor concentrations were measured by means of a specific ELISA strictly according to the manufacturer’s (R&D) protocol. 3F-Fe-release assay. The iron release assay was performed with the use of 59Fe-citrate (PerkinElmer, Llanfairisk, UK). Sorted KC and RPM were seeded in 24-well plates, each well with 30,000 cells. The release assay was performed exactly as described previously\(^4\). In brief, seeded KCs/RPMs were washed three times with 1 ml of serum-free, HEPS-buffered DMEM (2 mM l-glutamine, 50 mg/ml ampicillin, 25 mM HEPS (Sigma-Aldrich), pH 7.4) and incubated in 2 ml of this solution. Thereafter, 5μM 59Fe-citrate was added to the samples and incubated for 2 h to allow iron loading. Cells were then washed with 500 μl of serum-free, HEPS-buffered DMEM and incubated for 2 h. After three washing cycles (with 500 μl of 0.9% NaCl plus 50 μM 59FeCl3), cells were lysed with 400 μl 0.1% SDS and quantified by a γ-Counter. Protein concentration was determined by BCA assay (BCA Protein Assay Kit; Pierce Thermo Scientific). Western blotting. Cytosolic protein was extracted from freshly isolated tissue and western blotting was performed with...
as previously described. Anti-Ferritin antibody (2 µg/ml, Dako, Austria), rabbit anti-rat Ferroportin-antibody, rabbit anti-mouse- Hmox1 antibody (ADI-SPA-895-D, Enzo Life Sciences, 1:1,000), rabbit anti-mouse-human TIR1 (H68.4, Invitrogen, 1:1,000) or anti-actin (2 µg/ml, Sigma, Germany) were used as described previously. NIH ImageJ was used to quantify western blot bands densitometrically. ELISA: Cytosolic protein was isolated from liver and spleen by ten consecutive freeze–thaw cycles performed in PBS in the presence of 1x Halt Protease inhibitor cocktail (Pierce Pierce Thermo Scientific, Grand Island, NY, USA). Homogenous extracts were centrifuged at 10,000g for 10 min and supernatants used as input in specific ELISA kits (LifeSpan BioSciences, Seattle, WA, USA), RNA preparation from tissue, reverse transcription, and real-time PCR were performed as previously described. Total RNA preparation from nitrogen-frozen mouse tissue and cultured cells, reverse transcription, and real-time polymerase chain reaction (RT–PCR) were performed as previously described. For total RNA preparation from 3 x 10^6 freshly sorted human and mouse monocytes, cells were lysed in RLT buffer with 1% β-mercaptoethanol. RNA was isolated with the RNeasy Micro Kit (Qiagen, Venlo, Netherlands), followed by cDNA transcription.

Primer sets without probes used SYBR Green detection. They were designed by Primer3 software or the NCBI Primer Blast. The following primers and probe sets were used:

- **Hamp (hepcidin):** fw 5'-GGC AGA CAT TGG CAT ACC ACC AAT-3', rv 5'-TGG AAC AGA TAC CAC ACT GGG AA-3', probe FAM-CCCA ACT TCC CCA TCT GCA TCT GC-3' BHQ-1.
- **Slc40a1:** fw 5'-CTCA CCA TTA GGA GGA TTG ACC AGC TGC-3', rv 5'-CAA ATG TCA TAA TCT GCC GCA-3', probe FAM-CAAG CAT CCT CAG GCC CAT GCC-3' BHQ-1.
- **Mrc1:** fw 5'-AGG ACG TGA AGC CCC GAC TGC-3', probe FAM-CCCA ACT GGG AAA ATC TG-3', rv 5'-TGG AGG CAA AAT ACT GAG CAG CTA GAT AG-3'.
- **Slc1a2:** fw 5'-GGG GTA TTT CTA CAC CAG CAA-3', rv 5'-CAC ACA CTT GCC GGT TCT TCT-3', probe FAM-CTC AGT CAG TGG AAC ATG GAA GCC-3' BHQ-1.
- **Tfrc:** fw 5'-TGG TGT CTT CGG GTA AAT GGT GTA AGC-3', rv 5'-TGG TGT CTT CGG GTA AAT GGT GTA AGC-3', probe FAM-CTC AGT CAG TGG AAC ATG GAA GCC-3' BHQ-1.
- **Cdc2:** fw 5'-AGT GGA TTT TTA CAA CACT AAT CAG TGT GGA AG-3', rv 5'-TGG GAT GTT CAG GAA TTG GGA AG-3', probe FAM-CTC AGT CAG TGG AAC ATG GAA GCC-3' BHQ-1.
- **Cd43:** fw 5'-AGG TGG TGA TAG CCG GTA TGT-3', probe FAM-AGT AAG AAA CAC AAG GAG TGG GTA AGA AAT GGT-3'.
- **Cd34:** fw 5'-CCA GCA GAG CAT CCT GCT AGA-3', rv 5'-TGG TGC TTT TTT TGT TGA AAG AAA TTC T-3', probe FAM-TGG AGA GGT CTA GGT GCC-3'.
- **Cf:** fw 5'-CGG TTG GTT GGT GTT CTT-3', rv 5'-CGG TCT ATG CGG TAT GAC-3', probe FAM-CCCA ACT GGG AAC TCT GCC GGT TCT CGG-3' BHQ-1.
- **Cf2:** fw 5'-AGG TGG TGT CTT CTC TCC TAC AGC CGG AAG AT-3', rv 5'-TGG TGC TTT TCC TCC TAC AGC CGG AAG AT-3'.
- **Cd21:** fw 5'-TGC TTC TTC TCC TCC TAC AGC CGG AAG AT-3', rv 5'-TGG TGC TTT TCC TCC TAC AGC CGG AAG AT-3'.
- **Cyp2e1:** fw 5'-AGG GTG TTA CAG GGG TGT-3', rv 5'-TGG GTG GTA ATC CAT AGA GCC CAG-3' BHQ-1.
- **Srebf1:** fw 5'-CCG GGA AAT TTT TCC TCA AA-3', rv 5'-TGG TGT TTG TTG ATG AGC TGG AG-3'.
- **Ppara:** fw 5'-CTG TCA TCA CAG ACA CCA CCT TCT C-3', rv 5'-TAT TCA TCG AGA CTC GAT GTT CAG G-3'.
- **Cd163:** fw 5'-CAT GTG GGT AGA TCA GTG GCC AA-3', rv 5'-GAT CAG TAG CTA GCC CAG-3'.
- **Retna:** fw 5'-ATG GTT GGA AGA TCA GTG GCC AA-3', rv 5'-GAT CAG TAG CTA GCC CAG-3'.
- **Chi33:** fw 5'-CCG GCC CAG-3', probe FAM-TGC GGA CTG CCT TCA AG-3'.
- **Spc:** fw 5'-CCA CTA GAG GGG AAA ATC TG-3', rv 5'-TGG AGG CAA AAT ACT GAG CTA GAT AG-3'.
- **Put:** fw 5'-TGG AGA AGC TGA TGG CCT GG-3', rv 5'-CCA GCA GGA ACT GGT ACA AG-3'.
- **Irfl:** fw 5'-ATG TGG TTG TGC TCT GGC GA-3', rv 5'-ATG TGG TTG TGC TCT GGC GA-3', probe FAM-CTG GCT TCT TAC GCC AAC-3'.
- **Clc43:** fw 5'-TGG TGC TCT GGC CTC GCT TAC AGA-3', probe FAM-CCCA ACT GCC GGA ACT-3'.

Melting curve analysis was performed with each run to test specificity in SYBR green assays. Agarose gel electrophoresis of PCR products was performed with first primer tests to ensure a single product. RT–PCR was performed on a Bio-Rad CFX96 light cycler. Sosfot Probes Supermix and Sosfot EvaGreen Supermix (Bio-Rad, Vienna, Austria) were used according to the manufacturer's instructions. Relative quantities were calculated by Bio-Rad's CFX Manager software on the basis of a standard curve calculated from four serial tenfold dilutions of the standard. This curve was pipetted on each individual plate.

**Chemistry.** Blood samples from anesthetized mice were collected. Whole blood was further processed by centrifugation at 10,000 rpm for 5 min for serum isolation, which was then tested by using comprehensive metabolic panels (AST, ALT, BUN, Amylase) and an Abaxis Piccolo chemistry analyzer.

**Histology and microscopy.** Immunofluorescence. Cell specific uptake of PKH26-labeled RBCs was visualized by immunofluorescence microscopy. Sorted WBC populations from liver, spleen, and blood were prepared to a cell suspension of 2.5 x 10^6 cells/ml. 200 µl of suspension per cuvette was centrifuged at 500 rpm for 3 min. After air-drying, cells were embedded in Vectashield Mounting Medium with DAPI H1200 (Vector Lab, Burlingame, CA) and analyzed using a Nikon 80i upright epifluorescence microscope with Hamamatsu Orca CCD camera and complete image analysis software (IP Lab). Transmission electron microscopy: Tissues were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA) overnight at 4 °C. The tissues were rinsed in buffer, followed by propylene oxide, 100%. They were then infiltrated with Epon resin in buffer and dehydrated through a graded series of ethanol to 100%, followed by propylene oxide, 1%. They were then infiltrated with Epon resin in a 1:1 solution of Epon and propylene oxide overnight on a rocker at RT. The following day, they were placed in fresh Epon for several hours and then embedded in Epon overnight at 60 °C. Thin sections were cut on a Leica EM UC7 ultramicrotome, collected on formvar–coated grids, stained with uranyl acetate and lead citrate and examined using a JEOL JEM 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital-imaging system (Advanced Microscopy Techniques, Danvers, MA).
Iron detection. Prussian blue staining. Liver tissue was embedded in TissueTek O.C.T. compound (Sakura Finetek, Alphen aan den Rijn, Netherlands), frozen and sectioned into 6-µm thick slices. Kidney tissue was fixed overnight in 10% paraformaldehyde, and embedded in paraffin before sectioning. The Iron Stain Kit (Sigma-Aldrich, Saint Louis, MO, USA) was used for Prussian blue staining according to the manufacturer’s instructions. Tissues were stained by a histologist blinded to the study design and treatment group. A Zeiss Axioscope 40 microscope with a 40 x lens and an AxioCam MRc5 (Carl Zeiss AG, Oberkochen, Germany) was used. The slides were scanned by a digital scanner NanoZoomer 2.0RS (Hamamatsu, Japan). Representative fields were photodocumented using a pixellink system. Serum and tissue iron measurement. Serum iron concentrations were measured with a commercially available colorimetric iron quantification kit (QuantiChrom Iron Assay Kit, BioAssay Systems, Hayward, CA, USA). Tissue iron was measured with a colorimetric method, as previously described36, and adapted to a 96-well format, as detailed elsewhere37. Briefly, 20–30 mg tissues were homogenized in cytoplasmic lysis buffer (25 mM Tris-HCl, pH 7.4, 40 mM KCl, 1% Triton X-100) supplemented with 1 µg/ml aprotinin and 1 µg/ml leupeptin (all from Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 20,000g for 15 min. 100 µl of the obtained supernatant was supplemented with 50 µl acid hydrolysate solution (7 ml HCl 37%, 3 g TCA diluted with aqua destillata (a.d.) to 10 ml). The samples were incubated at 65 °C with vertical mixing (100 rpm) for 24 h. Samples were then centrifuged at 3,000g for 15 min. 60 µl of the obtained supernatant from each sample were transferred into a 96-well plate in doubles, and 160 µl of a colorimetric solution (1:1 mixture of 15.6 g sodium acetate diluted to 45 mL with a.d., and 17 mg of bathophenanthroline disulfonic acid and 22 mg l-ascorbic acid diluted to 5 ml with a.d) was added to each sample, and a Fe²⁺ standard curve was prepared via serial dilution of a purchased 10 mg/ml FeSO₄ stock (BioAssay Systems, Hayward, CA, USA). Probes were incubated for 15 min at RT to allow colorimetric reaction, following photometric measurement of optical density at 539 nm. The calculated iron quantity was normalized against the wet tissue weight for each sample. Intracellular iron determination by graphite furnace atomic absorption spectrometry in sorted cells. Intracellular iron measurements were carried out in a blinded manner by using a PerkinElmer Analyst 800 equipped with a transversely heated graphite atomizer (THGA). A Zeeman-effect background correction was realized by a 0.8 T magnetic field, oriented longitudinally with respect to the optical path. A PerkinElmer Lumina single-element iron hollow cathode lamp was driven at a constant current of 30 mA after proper equilibration (i.e., ≥220 min). For the absorption measurements, the 248.3-nm line (spectral bandwidth 0.7 nm) was chosen. The samples were suspended in 200 µl of a 0.1% (v/v) solution of nitric acid (Rotipuran Supra, 69%, Carl Roth GmbH, Karlsruhe, Germany) in high-purity water (Milli-Q, Merck-Millipore, Darmstadt, Germany) by extended periods (i.e., ≥230 min) of vortexing and ultrasonication at 30–40 KHz. After an initial estimation of the sample’s iron quantity, a five-point linear calibration was established in the range between 0 (i.e., <0.004 µM) and 0.106 µM. The calibration standards were prepared by diluting a 0.1 M standard stock solution of (NH₄)₂Fe(SO₄)₂ (Merck-Millipore, Darmstadt, Germany) by 0.1% (v/v) aqueous solution of nitric acid (vide supra). The absence of detectable iron (i.e., <0.004 µM) in the dilution agent, as well as in the sample cups, and the glassware was verified throughout the analyses. A linear fit of the 15 data points (k = 0.978, d = 0.006 µM) yielded a coefficient of determination of 0.992. Samples with iron concentrations exceeding the calibration range (i.e., ≥0.106 µM) were diluted appropriately. The blank solution, the calibration standards, and the samples were supplied to the atomizer in randomized fashion as triplicates, using a PerkinElmer AS-800 autosampler with an injection volume of 20 µl. The solvent was evaporated by a slow temperature gradient to 130 °C, ashing took place at a maximum temperature of 1,000 °C, and the atomization profile was read at 2,000 °C. The graphite tube, which was protected against oxidation by high-purity argon (99.999%), Messer Austria GmbH, Gumpoldskirchen, Austria), was cleaned out after each analysis at 2,450 °C. The integrity of each analysis was verified by a visual inspection of the respective time-dependent atomization profile. Enhanced plasmalab plasma iron detection. eLPI (enhanced plasmalab plasma iron) was assessed by the FeROS eLPI kit, a fluorescence-based assay intended for semiquantitative detection in vitro of both overt and cryptic redox active forms of NTBI (Alferix Tel Aviv, Israel). eLPI of >0.3 units indicated the potential for iron-mediated production of reactive oxygen species in the sample. Microarray measurement and microarray data processing. Total RNA was isolated from eKCs and mKCs sorted from Cx3cr1CreERT2;R26-tdT mice with the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The quality and quantity of RNA was estimated using a Bioanalyzer with Nano total RNA Chips (Agilent, Santa Clara, CA, USA); the RIN values exceeded 7.8 for all samples used for microarray measurements. Total RNA was amplified, fragmented, and labeled with biotin using the GeneChip WT Pico Reagent Kit (Affymetrix, Santa Clara, CA, USA) and samples were hybridized to GeneChip Mouse ST Arrays 2.0, according to the manufacturer’s instructions. Probeset signal intensities were background-corrected and normalized using the RMA algorithm. Significantly regulated genes were identified using paired t tests (pairing by animal, limma package of R/Bioconductor) with Benjamini–Hochberg correction for multiple testing. Genes regulated at least 1.5-fold with P < 0.1 were considered to be significant. The microarray analysis was done with CARMAweb 1.5 software38.

Statistics. Results are expressed as mean ± s.e.m. or mean ± s.d., as indicated. Where appropriate, the sample size required to reach statistical power was calculated assuming an alpha = 0.05 (P value) and a beta = 0.1 (90%). Statistical tests included the Kolmogorov-Smirnov test or D’Agostino & Pearson omnibus normality test (depending on sample number) used to test for normality distribution. t test was used to compare variances. If variances were substantially different, specific corrections were used as stated in detail in each figure legend. Calculations for statistical differences between various groups were carried out by ANOVA technique and Bonferroni’s or Dunnett’s correction for multiple tests. Otherwise, a two-tailed unpaired Student’s t test was used. In case of non-normality in distribution, a Mann–Whitney U test was used. For serial comparisons in human subjects, the two-tailed paired Student’s t test was used. Calculations for statistical differences between various groups were carried out by Kruskal–Wallis Dunn’s multiple comparison test. P < 0.05 was used to determine the statistical significance of parametric and nonparametric data. Gene-expression data clustering (average linkage algorithm, Euclidean distance measure) and heat-map representation were done with Genesis software39.