Macrophage-HFE controls iron metabolism and immune responses in aged mice

More than one in 200 individuals of northern European origin are affected by *HFE*-hemochromatosis, a highly prevalent genetic iron overload disorder.[1] *Hfe* deficiency specifically in hepatocytes causes low expression of the iron regulatory hormone hepcidin, and iron accumulation in organs.[2] Here we report a previously unrecognized role of HFE in macrophages as a regulator of iron and immune responses, which is independent of the hepatocytic role of HFE in maintaining systemic iron homeostasis.

Our previous work solved a long-standing debate by establishing that HFE acted in hepatocytes to control hepcidin levels, thereby preventing systemic iron overload.[2] These studies classified *HFE*-hereditary hemochromatosis as a liver disease. Low hepcidin levels in *HFE*-hereditary hemochromatosis increase the stability of the iron exporter ferroportin (FPN), allowing for excess efflux of iron from macrophages and duodenal enterocytes into the circulation.[3] As a consequence, macrophages from *HFE*-patients and *Hfe*-mutant mice are relatively iron-depleted, despite systemic iron overload.[4,5]

Macrophages play versatile roles in maintaining tissue homeostasis. They are involved in the recycling of aged red blood cells, tissue repair and regeneration, and in immune responses.[6] Alternative activation of macrophages in response to selected sets of pro- and anti-inflammatory stimuli has been linked to changes in intracellular iron levels.[7,8] On the other hand, systemic iron overload increased susceptibility of mice towards infections with *Vibrio vulnificus*[9] and *Yersinia pseudotuberculosis*,[10] while depletion of iron by deferoxamine improved survival of mice receiving lethal doses of lipopolysaccharides (LPS).[11] Interestingly, lack of *Hfe* in mice was beneficial, protecting against invasive *Salmonella enterica* serovar typhimurium infection[12] and macrophages derived from $Hfe^{-/-}$ mice displayed an attenuated inflammatory response to LPS and *Salmonella* challenge.[13] One explanation for these findings may be that low iron levels in *Hfe*-deficient macrophages restrict iron as a nutrient and signal for *Salmonella* growth. Alternatively, *Hfe* may exert a previously unrecognized role in immune cells. Given that iron dyshomeostasis is associated with aging and that aging may undermine the immune responses of macrophages, we tested whether selective lack of *Hfe* in macrophages may affect iron and immune responses in aged mice.

Analyses of iron-related parameters in 45-week old $Hfe^{LysMCre}$ mutant mice, which carry a specific deletion of *Hfe* in myeloid cells,[2] showed significant decreases of non-heme iron levels in the liver, spleen and duodenum (Figure 1A, B). The degree of iron reduction was moderate in comparison to that caused by nutritional iron deficiency (Figure 1A). Consistent with decreased iron levels in the liver and in freshly isolated primary hepatocytes from $Hfe^{LysMCre}$ mice (Figure 1A, C), serum hepcidin levels were decreased, while circulating iron and erythropoietin levels were unchanged (Figure 1D). Hematologic indices and the numbers of macrophages, granulocytes, T cells and B cells in the spleen and bone marrow were not significantly different between $Hfe^{LysMCre}$ mutant and control mice (Figure 1E, *Online Supplementary Figure S1*). In line with low serum hepcidin levels, hepatic expression of hepcidin (*Hamp1*) and genes known to be co-regulated with hepcidin in a BMP/SMAD-dependent manner (such as *Bmp6, Smad7, Id1* and *Pai1*) were decreased (Figure 1F, G). By contrast, the levels of transferrin receptor 1 (TfR1), which is responsible for transferrin-mediated iron uptake, failed to increase in the liver of $Hfe^{LysMCre}$ mice (Figure 1G), likely due to a milder decrease in the liver iron stores in $Hfe^{LysMCre}$ mice than in mice subjected to nutritional iron deficiency (Figure 1A, *Online Supplementary Figure S3A*).

In the spleen of 45-week old $Hfe^{LysMCre}$ mice, the decrease in iron levels correlated with increased protein levels of Fpn and TfR1, (Figure 1H), contrasting the effect of nutritional iron deficiency which was associated with low Fpn levels (*Online Supplementary Figure S3B*). In the duodenum of 45-week old $Hfe^{LysMCre}$ mice, increased mRNA expression of iron importers such as *Dcytb* and *Dmt1* was observed, whereas the levels of *Fpn* and *TfR1* were not statistically different (Figure 1I).

We conclude that 45-week old $Hfe^{LysMCre}$ mice show significant alterations in iron metabolism. This is in contrast to the iron state of 12-week old $Hfe^{LysMCre}$ mice, which showed no changes in any of the iron-related parameters investigated (Figure 1J-M).[2]

We next monitored systemic responses of 45-week old $Hfe^{LysMCre}$ mice to iron overload and immune challenges in the absence of macrophage-*Hfe*. We subjected 45-week old $Hfe^{LysMCre}$ mutant and control mice to parenteral iron overload. The mice were given a single intra-peritoneal injection of iron-dextran solution. The ability to accumulate iron in tissues and to increase hepcidin was comparable between $Hfe^{LysMCre}$ mutant and control mice (Figure 2A, B), suggesting that macrophage HFE is dispensable for iron accumulation and hepcidin responses in the setting of the parenteral iron overload used in this study.

By contrast, 45-week old $Hfe^{LysMCre}$ mice had a better survival in response to LPS-induced endotoxin shock (10 mg/kg; LPS from *E. coli* 055:B5, L2630) (Figure 1H), compared to 12-week old $Hfe^{LysMCre}$ mice which succumbed to the endotoxin shock at a similar rate as that in the controls (Figure 2C, D). Likewise, constitutive $Hfe^{-/-}$ and hepatocyte-specific *Hfe*-mutant mice ($Hfe^{AlfpCre}$), and mice maintained on an iron-deficient diet prior to LPS challenge, were not protected from endotoxin shock (Figure 2E, F). Based on these data we conclude that a specific lack of *Hfe* in macrophages in 45-week old mice may provide an adaptive mechanism aimed at protecting the host from inflammatory injury.

Given that the release of serum hepcidin, various pro- and anti-inflammatory cytokines and chemokines is considered a central feature of the inflammatory response, we next measured the expression of a set of serum immune mediators in 45-week old, $Hfe^{LysMCre}$ mutant mice during endotoxin shock. The overall expression pattern of hepcidin, pro- and anti-inflammatory cytokines and chemokines was similar between mutant $Hfe^{LysMCre}$ mice and control animals (*Online Supplementary Figure S2*), implying that the endotoxin shock triggered common pathways. Interestingly, $Hfe^{LysMCre}$ mutant mice showed a trend towards a higher degree of induction of granulocyte colony-stimulating factor, interferon γ, interleukin (IL)-1β, IL-17α (*Online Supplementary Figure S2C*) and reduced induction of eotaxin, IL2, IL4, IL5, IL6, IL10, IL13, macrophage inflammatory protein 1α, and tumor necrosis factor α (TNF-α) mediators compared to control mice (*Online Supplementary Figure S2D*), raising the question of whether differential expression of these immune mediators may explain the remarkably higher tolerance of aged $Hfe^{LysMCre}$ mice to LPS-induced shock.

To better understand the role of macrophage-*Hfe* at the cellular level, we next isolated primary macrophages from $Hfe^{LysMCre}$, $Hfe^{AlfpCre}$ (deletion of *Hfe* in hepatocytes) and constitutive *Hfe* knock-out ($Hfe^{-/-}$) mice and com-

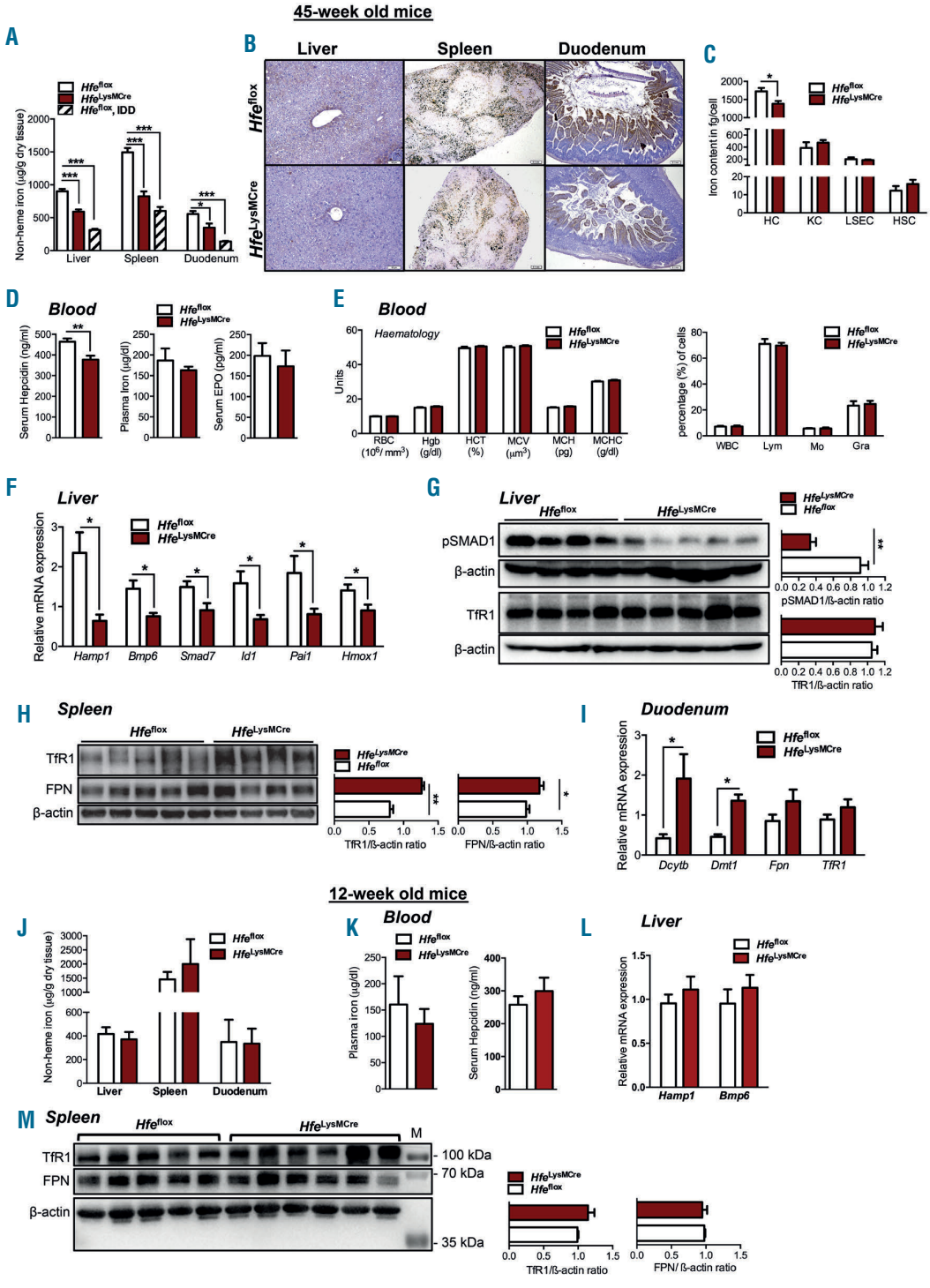

Figure 1. Lack of macrophage-*Hfe* in 45-week old mice contributes to moderate systemic iron deficiency in contrast to physiological iron homeostasis present in 12-week old *Hfe*^{LysMCre} mice. (A) Non-heme iron levels in the liver, spleen and duodenum from 45-week old *Hfe*^{LysMCre} mutant and *Hfe*^{flox} control mice, and from mice kept on an iron-deficient diet (IDD). n indicates the number of livers (n=19, 15, 5), spleens (n=19, 15, 5) and duodenums (n=6, 6, 5) isolated from *Hfe*^{LysMCre}, *Hfe*^{flox} and IDD mice, respectively. (B) Perls staining for iron deposits in the liver, spleen and duodenum of 45-week old *Hfe*^{LysMCre} mutant and control mice. Scale bar 50 µm (liver) and 100 µm (spleen and duodenum). Representative stainings of three sections are shown. (C) Intracellular total iron levels measured by total-reflection x-ray fluorescence in primary hepatocytes (HC), Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), and hepatic stellate cells (HSC) from 45-week old *Hfe*^{LysMCre} and *Hfe*^{flox} mice (n=3-5). (D) Serum hepcidin, iron and erythropoietin (EPO) levels, and (E) hematologic parameters including red blood cell (RBC) count, hemoglobin (Hgb) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and the percentages of white blood cells (WBC) including lymphocytes (Lym), monocytes (Mo), and granulocytes (Gra) in the blood of 45-week old *Hfe*^{LysMCre} mutant (n=6) and *Hfe*^{flox} control mice (n=6). (F) Relative mRNA expression of iron-related genes in the liver of 45-week old *Hfe*^{LysMCre} mutant (n=6) and *Hfe*^{flox} control mice (n=9) determined by real-time polymerase chain reaction (PCR). (G, H) Representative immunoblot analysis of pSMAD1, transferrin receptor 1 (TfR1) and ferroportin (FPN), relative to β-actin levels (shown in histograms on the right), in the livers and spleens of *Hfe*^{LysMCre} mutant (n=8) and control mice (n=8). (I) Relative mRNA expression of iron transporters in the duodenum of 45-week old *Hfe*^{LysMCre} mutant (n=4) and *Hfe*^{flox} control mice (n=8) measured by real-time PCR. (J-L) Non-heme iron levels (J), serum iron and hepcidin levels (K), and relative mRNA expression of hepcidin (*Hamp1*) and *Bmp6* (L) in the livers of 12-week old *Hfe*^{LysMCre} mutant (n=5) and *Hfe*^{flox} control mice (n=6). (M) Representative immunoblot analysis of TfR1 and FPN, relative to β-actin levels (shown in histograms on the right), in the spleens of 12-week old *Hfe*^{LysMCre} mutant (n=5) and *Hfe*^{flox} control mice (n=6). M: Prestained Protein Marker PageRuler. n indicates the number of mice used in the analysis. Data are shown as mean ± standard error of mean. Statistically significant differences are indicated as **P*<0.05, ***P*<0.005, ****P*<0.0005.

pared iron-related parameters. These mouse models enable the dissociation of the cell-autonomous actions of HFE in macrophages (present in *Hfe*^{LysMCre} and *Hfe*$^{-/-}$ mice) from those that are a consequence of altered systemic hepcidin expression (present in *Hfe*$^{-/-}$ and *Hfe*^{AlfpCre} mutant mice).[2] We showed that lack of *Hfe* in macrophages derived from aged *Hfe*$^{-/-}$ and aged *Hfe* ^{LysMCre} mice correlated with reduced intracellular total iron levels whereas macrophages derived from *Hfe*^{AlfpCre} mice, which carry a wild-type *Hfe* allele in all extra-hepatocytic cell types, including macrophages, showed no statistically significant difference in iron levels compared to their respective controls although there was a tendency towards less iron in *Hfe*^{AlfpCre} cells (Figure 3A).

Consistent with our findings in the spleen of *Hfe*^{LysMCre} mice (Figure 1G), we showed that FPN protein and mRNA levels were significantly increased in *Hfe*-deficient macrophages while *TfR1* expression was unaltered irre-

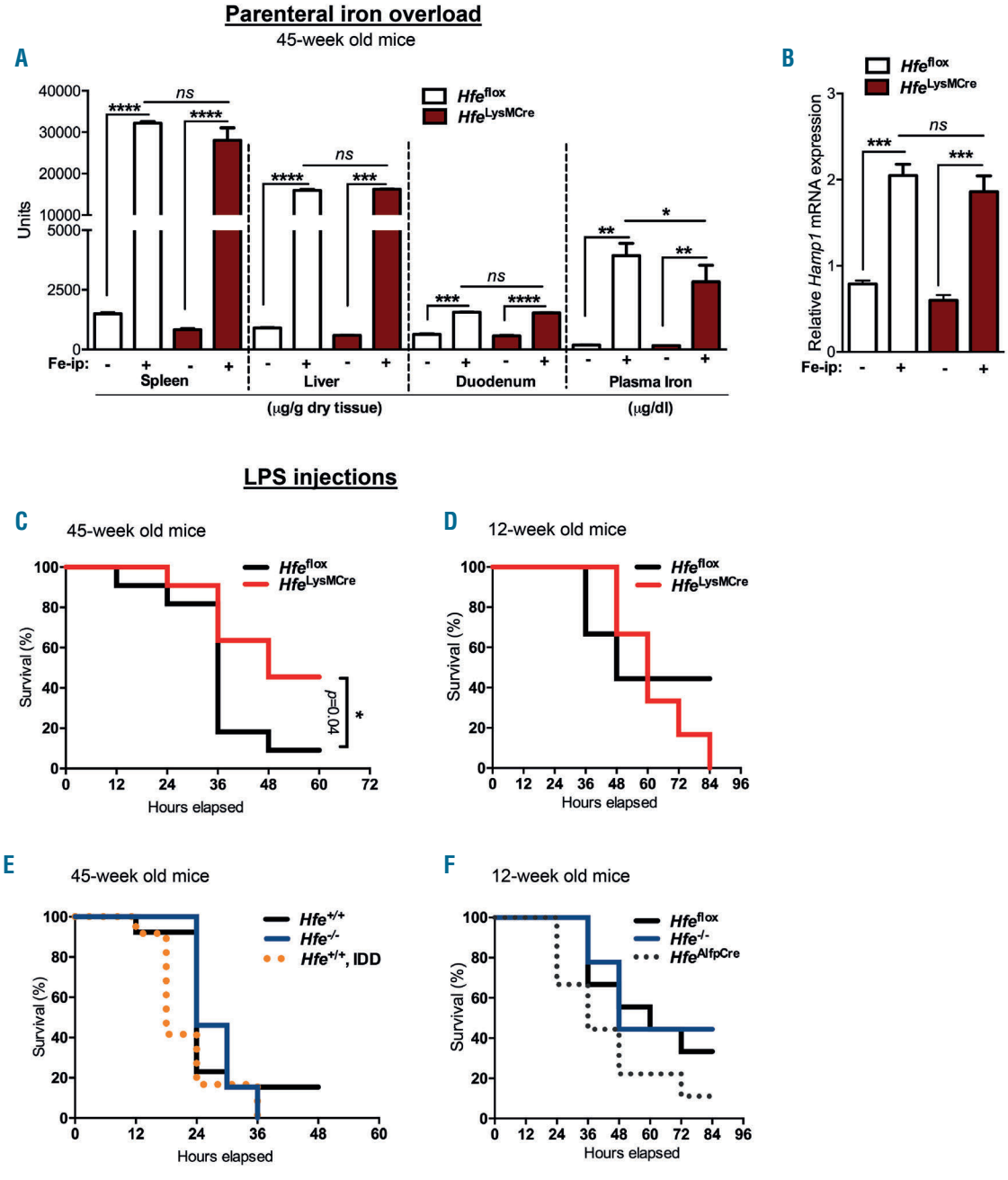

Figure 2. Lack of macrophage-*Hfe* in 45-week old mice is dispensable during parenteral iron overload but contributes to better survival of *Hfe*^{LysMCre} mutant mice during lipopolysaccharide-induced endotoxin shock. (A, B) Non-heme and plasma iron levels (A), and hepcidin mRNA expression (B) in 45-week old *Hfe*^{LysMCre} mutant (n=6) and control mice (n=6) following parenteral iron overload. Data are shown as the mean ± standard error of mean. Statistically significant differences are indicated as **P*<0.05, ***P*<0.005, ****P*<0.0005, *****P*<0.0001. n indicates the number of mice used in the analysis. (C-F) Survival of 45-week old *Hfe*^{LysMCre} mutant and control mice (n=11, 11) (C), 12-week old *Hfe*^{LysMCre} mutant and control mice (n=10, 10) (D), 45-week old *Hfe*$^{-/-}$ and control mice, and mice maintained on an iron-deficient diet for 4 weeks (IDD) (n=13, 13, 12) (E) and 12-week old *Hfe*^{AlfpCre}, *Hfe*$^{-/-}$ and control mice (n=9, 9, 9), which were injected with lipopolysaccharide (10 mg/kg) and monitored every 6 h for up to 4 days. The percentage survival rate was expressed by Kaplan-Meier curves. Statistical analyses were performed using the log-rank test and the results were considered statistically significant when **P*<0.05. ns: not statistically significant; Fe-ip: intraperitoneal iron injections; LPS: lipopolysaccharide.

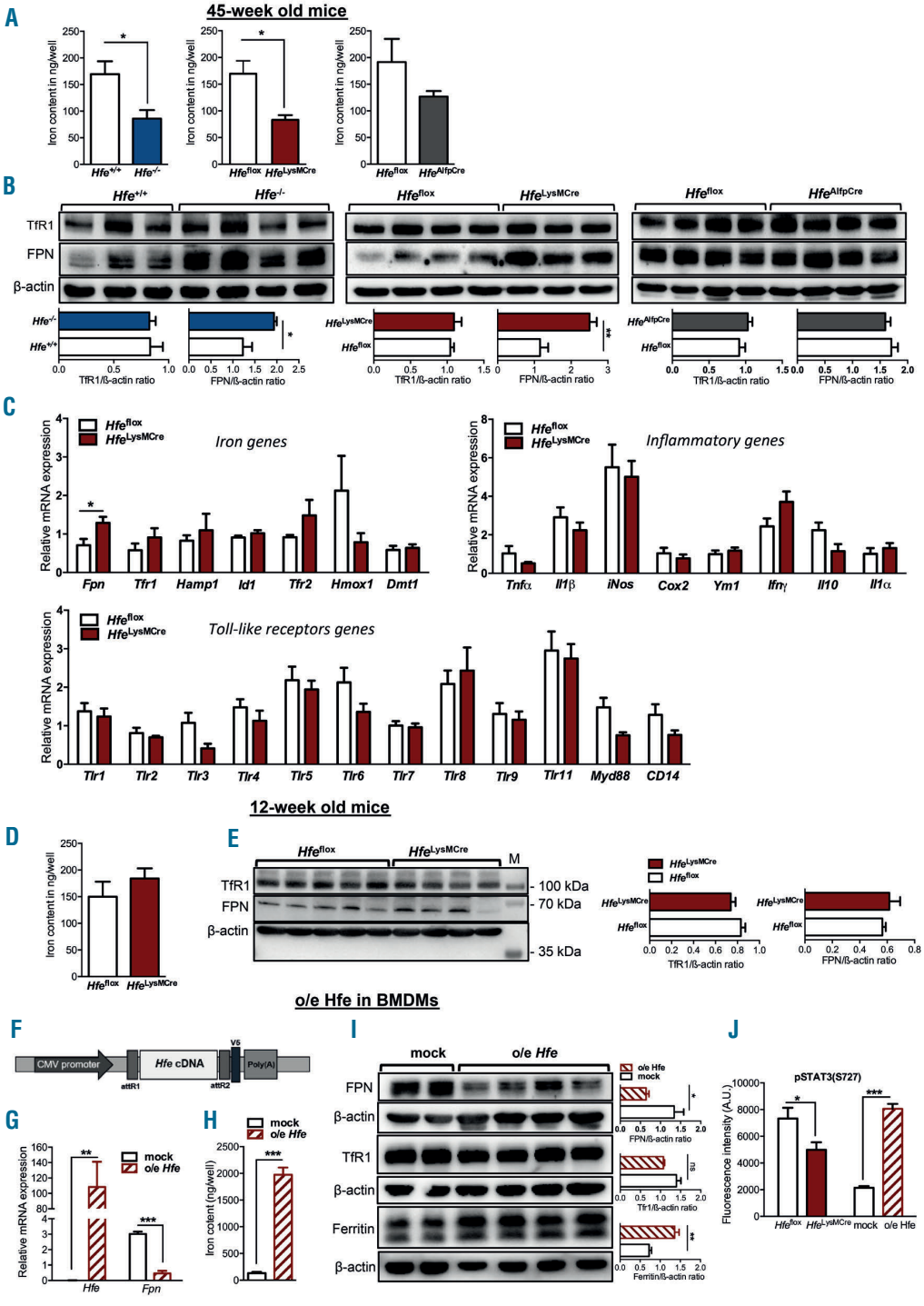

Figure 3. Hfe in macrophages controls cellular iron homeostasis *via* ferroportin. (A) Intracellular total iron levels in bone marrow-derived macrophages (BMDM) from 45-week old *Hfe*$^{-/-}$, *Hfe*^{LysMCre} and *Hfe*^{AlfpCre} mutant mice (n=3, 4). (B) Representative immunoblot analysis of transferrin receptor 1 (TfR1) and ferroportin (FPN) relative to β-actin levels (shown in histograms below) in 45-week old *Hfe*$^{-/-}$, *Hfe*^{LysMCre} and *Hfe*^{AlfpCre} mutant mice. (C) Relative mRNA quantification of iron-related genes, inflammatory cytokines and toll-like receptors genes by real-time polymerase chain reaction in BMDM derived from 45-week old *Hfe*^{LysMCre} mutant and control mice (n=6, 6). (D, E) Intracellular total iron levels (D) and immunoblot analysis (E) of TfR1 and FPN, relative to β-actin levels (shown in histograms on the right), in BMDM from 12-week old *Hfe*^{LysMCre} mutant and *Hfe*^{flox} control mice (n=5, 4). M: Prestained Protein Marker PageRuler (Thermo Scientific). (F) Schematic illustration of the *Hfe* cDNA construct cloned into the pcDNA6.2 vector under the control of the cytomegalovirus (CMV) promoter, tagged with V5 epitope at the C-terminal end and a poly-A tail. (G) Relative mRNA expression of *Hfe* and *Fpn*, and (H) intracellular total iron levels in transiently transfected BMDM with vector carrying the *Hfe* cDNA construct (indicated as 'o/e *Hfe*') or empty vector ('mock') (n=4, 5). (I) Representative immunoblot analysis, from two independent experiments, of FPN, TfR1, ferritin and relative quantification to β-actin levels (shown in histograms on the right) in BMDM transiently transfected with a vector carrying *Hfe* cDNA or an empty vector ('mock') (n=4, 6). (J) Phosphorylation status of STAT3(Ser727) in the BMDM from *Hfe*^{LysMCre} mutant and *Hfe*^{flox} control mice (n=7, 7) and in BMDM transiently transfected with a vector carrying *Hfe* cDNA or an empty vector ('mock') (n=9, 9) measured by Bio-Plex Pro Cell Signaling MAPK Panel. AU: arbitrary units. Data are shown as mean ± standard error of mean. Statistically significant differences are indicated as **P*<0.05, ***P*<0.005. n indicates the number of mice used in the analysis.

spective of the genotypes (Figure 3B). The mRNA expression of hepcidin and other iron-related genes, pro- and anti-inflammatory cytokines, and toll-like receptors was not affected by the lack of macrophage-*Hfe* (Figure 3C).

Importantly, intracellular total iron levels, FPN and TfR1 protein expression, were not changed in macrophages isolated from 12-week old $Hfe^{LysMCre}$ mutant mice (Figure 3D, E), supporting our previous findings.[2]

Conversely, overexpression of *Hfe* in primary wild-type macrophages counteracted FPN induction and intracellular iron deficiency (Figure 3F-I). We observed an interesting correlation between HFE expression in macrophages and the STAT3 signaling pathway, whereby the latter's activity was significantly decreased in *Hfe*-deficient macrophages and 3-fold increased upon *Hfe* overexpression (Figure 3J). Additional phospho-proteins studied, including pp38MAPK, pERK1/2, pMEK1, pJNK, pATF-2, pp90RSK, pHSP27, and p53, were not significantly different between *Hfe*-deficient and *Hfe*-overexpressing macrophages (*data not shown*). The contribution of STAT3 signaling to FPN expression in *Hfe*-deficient macrophages deserves further investigations.

Collectively, the results from primary macrophages show that macrophage-*Hfe* expression controls iron accumulation by regulating expression of the iron exporter FPN. However, the LysM-Cre does not target all macrophage types with the same efficacy and its expression varies among tissues. This may explain the lack of an iron-poor phenotype in Küpffer cells in contrast to bone marrow-derived macropahges. Our findings are in line with an earlier study by Drakesmith *et al.*, who showed that HFE expression in THP1 macrophages inversely correlated with FPN levels and iron release, whereas iron uptake by TfR1 was not affected.[14] Other studies using mouse or human *Hfe/HFE*-deficient blood monocyte/macrophage cultures[4,5] also demonstrated increased FPN expression in isolated cells. Similarly, reciprocal bone marrow[15] and liver transplantation[5] studies between wild-type and $Hfe^{-/-}$ mice suggested a diminished capacity of macrophages to store iron, being consistent with a function of HFE in inhibiting iron release.[14] However, under all these conditions, the observed effects could not be dissociated from the systemic effects of hepcidin.

In summary, our findings provide compelling new data on the long-standing question of the role of HFE in macrophages.

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References

1. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G--> A (C282Y) HFE hereditary haemochromatosis mutation in the USA. Lancet. 2002;359(9302):211-218.
2. Vujic Spasic M, Kiss J, Herrmann T, et al. Hfe acts in hepatocytes to prevent hemochromatosis. Cell Metab. 2008;7(2):173-178.
3. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004;306(5704):2090-2093.
4. Jacolot S, Yang Y, Paitry P, Ferec C, Mura C. Iron metabolism in macrophages from HFE hemochromatosis patients. Mol Genet Metab. 2010;101(2-3):258-267.
5. Garuti C, Tian Y, Montosi G, et al. Hepcidin expression does not rescue the iron-poor phenotype of Kupffer cells in Hfe-null mice after liver transplantation. Gastroenterology. 2010;139(1):315-322.e1.
6. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445-455.
7. Vinchi F, Costa da Silva M, Ingoglia G, et al. Hemopexin therapy reverts heme-induced proinflammatory phenotypic switching of macrophages in a mouse model of sickle cell disease. Blood. 2016;127(4):473-486.
8. Tangudu NK, Alan B, Vinchi F, et al. Scavenging Reactive Oxygen Species Production Normalizes Ferroportin Expression and Ameliorates Cellular and Systemic Iron Disbalances in Hemolytic Mouse Model. Antioxid Redox Signal. 2018;29(5):484-499.
9. Arezes J, Jung G, Gabayan V, et al. Hepcidin-induced hypoferremia is a critical host defense mechanism against the siderophilic bacterium Vibrio vulnificus. Cell Host Microbe. 2015;17(1):47-57.
10. Miller HK, Schwiesow L, Au-Yeung W, Auerbuch V. Hereditary Hemochromatosis Predisposes Mice to Yersinia pseudotuberculosis Infection Even in the Absence of the Type III Secretion System. Front Cell Infect Microbiol. 2016;6:69.
11. Wang S, Liu C, Pan S, et al. Deferoxamine attenuates lipopolysaccharide-induced inflammatory responses and protects against endotoxic shock in mice. Biochem Biophys Res Commun. 2015;465(2):305-311.
12. Nairz M, Theurl I, Schroll A, et al. Absence of functional Hfe protects mice from invasive Salmonella enterica serovar Typhimurium infection via induction of lipocalin-2. Blood. 2009;114(17):3642-3651.
13. Wang L, Johnson EE, Shi HN, et al. Attenuated inflammatory responses in hemochromatosis reveal a role for iron in the regulation of macrophage cytokine translation. J Immunol. 2008;181(4):2723-2731.
14. Drakesmith H, Sweetland E, Schimanski L, et al. The hemochromatosis protein HFE inhibits iron export from macrophages. Proc Natl Acad Sci U S A. 2002;99(24):15602-15607.
15. Makui H, Soares RJ, Jiang W, Constante M, Santos MM. Contribution of Hfe expression in macrophages to the regulation of hepatic hepcidin levels and iron loading. Blood. 2005;106(6):2189-2195.