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.3 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.4 As a consequence, macrophages from HFE-patients and Hfe-mutant mice are relatively iron-depleted, despite systemic iron overload.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. Alternative activation of macrophages in response to selected sets of pro- and anti-inflammatory stimuli has been linked to changes in intracellular iron levels.5,6 On the other hand, systemic iron overload increased susceptibility of mice towards infections with Vibrio vulnificus7 and Yersinia pseudotuberculosis,7 while depletion of iron by deferoxamine improved survival of mice receiving lethal doses of lipopolysaccharides (LPS).8 Interestingly, lack of Hfe in mice was beneficial, protecting against invasive Salmonella enterica serovar typhimurium infection9 and macrophages derived from Hfe−/− mice displayed an attenuated inflammatory response to LPS and Salmonella challenge.10 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−/−/AlfpCre mutant mice, which carry a specific deletion of Hfe in myeloid cells,5 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−/−/AlfpCre 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 hepavidin (Hamp1) and genes known to be co-regulated with hepavidin in a BMP/SMAD-dependent manner (such as Bmp6, Smad7, Id1 and Pai1) were decreased (Figure 1E, G). By contrast, the levels of transferrin receptor 1 (TIR1), which is responsible for transferrin-mediated iron uptake, failed to increase in the liver of Hfe−/−/AlfpCre mice (Figure 1G), likely due to a milder decrease in the liver iron stores in Hfe−/−/AlfpCre mice than in mice subjected to nutritional iron deficiency (Figure 1A, Online Supplementary Figure S3A).

In the spleen of 45-week old Hfe−/−/AlfpCre mice, the decrease in iron levels correlated with increased protein levels of Fpn and TIR1 (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−/−/AlfpCre mice, increased mRNA expression of iron importers such as Dcytb and Dmt1 was observed, whereas the levels of Fpn and TIR1 were not statistically different (Figure 1I).

We conclude that 45-week old Hfe−/−/AlfpCre mice show significant alterations in iron metabolism. This is in contrast to the iron state of 12-week old Hfe−/−/AlfpCre mice, which showed no changes in any of the iron-related parameters investigated (Figure 1J–M).5

We next monitored systemic responses of 45-week old Hfe−/−/AlfpCre mice to iron overload and immune challenges in the absence of macrophage-Hfe. We subjected 45-week old Hfe−/−/AlfpCre 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−/−/AlfpCre 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−/−/AlfpCre mice had a better survival response in response to LPS-induced endotoxin shock (10 mg/kg; LPS from E. coli O55:B5, L2630) (Figure 1H), compared to 12-week old Hfe−/−/AlfpCre mice which succumbed to the endotoxin shock at a similar rate as that in the controls (Figure 2C, D). Likewise, constitutive Hfe−/− and hepavidin-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 hepavidin, 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−/−/AlfpCre mutant mice during endotoxin shock. The overall expression pattern of hepavidin, pro- and anti-inflammatory cytokines and chemokines was similar between myeloid-specific Hfe−/−/AlfpCre mice and control animals (Online Supplementary Figure S2), implying that the endotoxin shock triggered common pathways. Interestingly, Hfe−/−/AlfpCre 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, IL11, 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−/−/AlfpCre mice to LPS-induced shock.

To better understand the role of macrophage-Hfe at the cellular level, we next isolated primary macrophages from Hfe−/−/AlfpCre, Hfe−/−/LysMCre (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<sup>flk</sup> mice. (A) Non-heme iron levels in the liver, spleen and duodenum from 45-week old Hfe<sup>flk</sup> mutant and Hfe<sup>flk</sup> 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<sup>flk</sup>, Hfe<sup>flk</sup> and IDD mice, respectively. (B) Perls staining for iron deposits in the liver, spleen and duodenum of 45-week old Hfe<sup>flk</sup> 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<sup>flk</sup> mutant and control 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<sup>flk</sup> mutant (n=6) and Hfe<sup>flk</sup> control mice (n=6). (F) Relative mRNA expression of iron-related genes in the liver of 45-week old Hfe<sup>flk</sup> mutant (n=6) and Hfe<sup>flk</sup> 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<sup>flk</sup> mutant (n=8) and control mice (n=8). (I) Relative mRNA expression of iron transporters in the duodenum of 45-week old Hfe<sup>flk</sup> mutant (n=4) and Hfe<sup>flk</sup> 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<sup>flk</sup> mutant (n=5) and Hfe<sup>flk</sup> 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<sup>flk</sup> mutant (n=5) and Hfe<sup>flk</sup> 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\textsuperscript{LysM Cre} and Hfe\textsuperscript{-/-} mice) from those that are a consequence of altered systemic hepcidin expression (present in Hfe\textsuperscript{-/-} and Hfe\textsuperscript{Alfp Cre} mutant mice). We showed that lack of Hfe in macrophages derived from aged Hfe\textsuperscript{-/-} and aged Hfe\textsuperscript{LysM Cre} mice correlated with reduced intracellular total iron levels whereas macrophages derived from Hfe\textsuperscript{Alfp Cre} 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\textsuperscript{Alfp Cre} cells (Figure 3A).

Consistent with our findings in the spleen of Hfe\textsuperscript{LysM Cre} 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 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^lox/lox and Hfe^loxcre mutant mice (n=3, 4). (B) Representative immunoblot analysis of transferrin receptor 1 (TIR1) and ferroportin (FPN) relative to β-actin levels (shown in histograms below) in 45-week old Hfe^+/+ , Hfe^lox/lox and Hfe^loxcre 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^loxcre 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^loxcre mutant and Hfe^lox 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 VS 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, TIR1, 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^loxcre mutant and Hfe^lox 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<sup>−/−</sup> mutant mice (Figure 3D, E), supporting our previous findings.<sup>14</sup>

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, p90RSK, pHSP27, and pS3, 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 Kupffer cells in contrast to bone marrow-derived macrophages. 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.<sup>14</sup> Other studies using mouse or human Hfe/HFE-deficient blood monocyte/macrophage cultures<sup>3,5,10</sup> also demonstrated increased FPN expression in isolated cells. Similarly, reciprocal bone marrow<sup>13</sup> and liver transplantation<sup>14</sup> studies between wild-type and Hfe<sup>−/−</sup> mice suggested a diminished capacity of macrophages to store iron, being consistent with a function of HFE in inhibiting iron release.<sup>14</sup> 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.

Naveen Kumar Tangudu,1 Dilay Yilmaz,1 Katharina Wörle,1 Andreas Gruber,1 Silvia Colucci,3 Kerstin Leopold,1 Martina U. Muckenthaler<sup>1</sup> and Maja Vujić Spasic<sup>4</sup>

1 Institute of Comparative Molecular Endocrinology, Ulm University, Ulm; 2 Institute of Analytical and Bioanalytical Chemistry, Ulm University, Ulm and 3 Department of Pediatric Hematology, Oncology and Immunology, University of Heidelberg, Molecular Medicine Partnership Unit, Heidelberg, Germany

Correspondence: MAJA VUJIČ SPASIC – maja.vujic@uni-ulm.de

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