Cell-specific expression of Hfe determines the outcome of Salmonella enterica serovar Typhimurium infection in mice


Haematologica 2020 [Epub ahead of print]


Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Cell-specific expression of Hfe determines the outcome of Salmonella enterica serovar Typhimurium infection in mice

Manfred Nairz1,*, Christoph Metzendorf2,3,*, Maja Vujic-Spasic2,3,4,+, Anna-Maria Mitterstiller1,*, Andrea Schroll1, David Haschka1, Alexander Hoffmann1,5, Laura von Raffay1, Richard Sparla2,3, Christian W. Huck6, Heribert Talasz7, Patrizia L. Moser8, Martina U. Muckenthaler2,3,# and Günter Weiss1,5,#

1Department of Internal Medicine II, Infectious Diseases, Immunology, Rheumatology, Pneumology, Medical University of Innsbruck, 6020 Innsbruck, Austria.
2Department of Pediatric Hematology, Oncology and Immunology, University of Heidelberg, INF 350, 69120 Heidelberg, Germany.
3Molecular Medicine Partnership Unit, 69120 Heidelberg, Germany.
4Institute of Comparative Molecular Endocrinology, Ulm University, 89081 Ulm, Germany.
5Christian Doppler Laboratory for Iron Metabolism and Anemia Research, Medical University of Innsbruck, 6020 Innsbruck, Austria.
6Institute for Analytical Chemistry and Radiochemistry, University of Innsbruck, 6020 Innsbruck, Austria.
7Biocenter, Division of Clinical Biochemistry, Medical University of Innsbruck, 6020 Innsbruck, Austria.
8Institute of Pathology, INNPATH, 6020 Innsbruck, Austria.

* M.N. and C.M. contributed equally
+ M.V.-S. and A.-M. M. contributed equally
# M.M. and G.W. contributed equally

Correspondence: martina.muckenthaler@med.uni-heidelberg.de and guenter.weiss@i-med.ac.at

Scientific category: Iron metabolism & its disorders
Running title: Cell-specific role of Hfe in Salmonella infection
**Article Summary:**
The hemochromatosis-associated protein Hfe is expressed both on parenchymal and immune cells; here we investigated a putative cell type-specific function of Hfe in host defense against *Salmonella* Typhimurium.

We show that the lack of Hfe on macrophages limits iron-dependent intracellular *Salmonella* growth, whereas hepatocyte-specific *Hfe* deletion increases serum iron, impairs systemic cytokine output and causes early death from *Salmonella* bacteremia.
Mutations in *HFE* cause hereditary hemochromatosis type I hallmarked by increased iron absorption, iron accumulation in hepatocytes and iron deficiency in myeloid cells. *HFE* encodes an MHC-I like molecule, but its function in immune responses to infection remains incompletely understood.

Here, we investigated putative roles of Hfe in myeloid cells and hepatocytes, separately, upon infection with *Salmonella* Typhimurium, an intracellular bacterium with iron-dependent virulence. We found that constitutive and macrophage-specific deletion of *Hfe* protected infected mice. The propagation of *Salmonella* in macrophages was reduced due to limited intramacrophage iron availability for bacterial growth and increased expression of the anti-microbial enzyme nitric oxide synthase-2. By contrast, mice with hepatocyte-specific deletion of *Hfe* succumbed earlier to *Salmonella* infection because of unrestricted extracellular bacterial replication associated with high iron availability in the serum and impaired expression of essential host defense molecules such as interleukin-6, interferon-γ and nitric oxide synthase-2. *Wild-type* mice subjected to dietary iron overload phenocopied hepatocyte-specific *Hfe* deficiency suggesting that increased iron availability in the serum is deleterious in *Salmonella* infection and underlies impaired host immune responses. Moreover, the macrophage-specific effect is dominant over hepatocyte-specific *Hfe*-depletion, as *Hfe* knock-out mice have increased survival despite the higher parenchymal iron load associated with systemic loss of *Hfe*.

We conclude that cell-specific expression of Hfe in hepatocytes and macrophages differentially affects the course of infections with specific pathogens by determining bacterial iron access and the efficacy of anti-microbial immune effector pathways. This may explain the high frequency and evolutionary conservation of human *HFE* mutations.
INTRODUCTION

Most patients with hereditary hemochromatosis (HH) show homozygous C282Y missense mutations in the gene HFE (1, 2). They are hallmarked by parenchymal iron deposition particularly in hepatocytes, cardiomyocytes and pancreatic acinar cells, leading to organ damage. Conversely, monocytes and macrophages are iron-deficient in type I HH (3-5).

An allelic frequency of approximately 5%-10% renders the HFE C282Y missense mutation the most common genetic defect in individuals of Northwestern European ancestry. It has been hypothesized that the mutation may protect from iron-deficiency and/or infections; thus conferring an evolutionary advantage to healthy heterozygous carriers (6, 7). Several mechanisms by which the HFE protein controls systemic iron balance have been proposed: Early studies have shown that HFE, in association with β2-microglobulin, directly interacts with transferrin receptor-1 (TFR1) on the cell surface (8) and lowers its affinity for transferrin-bound iron (TBI). Once the iron regulatory hormone hepcidin had been discovered, it became apparent that the HFE C282Y mutation causes systemic hepcidin deficiency and its consequences (9). The HFE mutation disrupts iron-inducible BMP/SMAD (for Bone Morphogenetic Protein/Suppressor of Mothers Against Decapentaplegic) signaling and prevents appropriate hepcidin transcription (1, 2). The relative lack of hepcidin then causes unrestricted dietary iron absorption by the duodenum and increased iron export from iron-recycling macrophages due to the stabilization of the iron exporter ferroportin (FPN)-1 (10, 11). As a result, iron accumulates in parenchymal cells where it causes tissue damage by toxic radical formation (12).

HFE is an MHC-I like protein, but so far it remained unclear whether it plays a role in immune function and/or host-pathogen interaction. HFE-deficient monocytes and macrophages are iron poor (3, 4, 13). Possible explanations include reduced TBI uptake, increased iron export via FPN1 as a consequence of decreased hepcidin levels or increased synthesis of the siderophore-iron binding peptide lipocalin (LCN)-2 (3, 5, 13).

For almost all bacteria, iron is essential as it stimulates growth and thus impacts on the course and outcome of many infectious diseases (14). However, iron requirements, iron uptake strategies and proliferation kinetics may greatly vary between bacterial species, possibly explaining species-specific effects on infection
outcomes (15). In the mouse, constitutive Hfe deficiency partially protects from S. enterica Typhimurium (S. Tm.) infection (13). By contrast, Hfe deficient mice are more susceptible to Mycobacterium avium infection (16). Furthermore, human monocyte-derived macrophages from patients with HH limit iron availability for intracellular Mycobacterium tuberculosis, resulting in an improved control of infection (17). On the other hand, individuals with HH type I are highly susceptible to infection with Yersinia species, whose virulence is iron-dependent, as documented by case reports of human subjects and by mouse models (18, 19). These diverse outcomes are counterintuitive given that all three pathogens, Salmonella, Mycobacterium and Yersinia, share a predominately intracellular lifestyle pointing to the importance of cell- and tissue-specific iron distribution for susceptibility to these infections (20).

Because Hfe exerts contrasting effects in different infectious diseases, we asked whether Hfe plays a cell type-specific role during infection and whether this is linked to alterations of tissue iron distribution or associated with iron-independent effects of Hfe. We herein demonstrate that macrophage-specific deletion of Hfe (LysMCre+ Hfefl/fl) recapitulates the antibacterial phenotype of constitutive Hfe−/− mice in response to Salmonella Typhimurium infection. By contrast, exclusive deletion of Hfe in hepatocytes (AlfpCre+ Hfefl/fl) is associated with an adverse outcome of S. Tm. infection. These contrasting cell type-specific effects of Hfe-deficiency correlate with bacterial iron availability and anti-microbial effector immune functions. Our findings support the idea that Hfe controls iron concentrations in the microenvironment thus differentially affecting immune effector mechanisms and bacterial growth in intra- and extracellular compartments.

METHODS

Salmonella infection in vivo

All infection experiments were performed according to the guidelines of the Medical University of Innsbruck and the Austrian Ministry for Science and Education based on the Austrian Animal Testing Act of 1988 (approvals BMWFW-66.011/0074-C/GT/2007, 66.011/0154-II/3b/2010 and 66.011/0031-WF/V/3b/2015). Male mice were used at 12-16 wks. of age and infected i.p. with 500 colony forming units (CFU) of S. Tm. diluted in 200 μl of phosphate buffered saline (PBS). Unless
otherwise specified, *S. Tm. Wild-type (WT)*, strain ATCC 14028s was used for the experiments. Where appropriate, mice were fed an iron adequate control diet (C1000 from Altromin containing 180 μg per g) or an iron-enriched diet (C1038 from Altromin supplemented with 25 mg carbonyl iron per g). After 3 weeks, mice were infected i.p. with 500 CFU of *S. Tm.* diluted in 200 μl of PBS as detailed in the Supplementary Methods.

*In vitro* experiments

The isolation of bone marrow-derived macrophages (BMDM) was performed as detailed in the Supplementary Methods.

RNA extraction and quantitative real-time PCR

Preparation of total RNA, reverse transcription and quantification of mRNA expression by quantitative Taqman RT-PCR was performed as described (21). Results were first normalized using the housekeeping gene *Hprt* and then divided by the means of the control group (*WT Hfe*<sup>+/+</sup> or *Cre*<sup>-</sup> mice as appropriate) to obtain expression data that is relative to the respective control group. Sequences of primers and probes are listed in the Supplementary Methods.

Measurement of iron and protein concentrations

Measurement of tissue iron concentrations has been described in detail (22). The serum iron concentration was quantified using the QuantiChrom Iron Assay Kit (BioAssay Systems). Intracellular iron concentrations were determined in adherent bone marrow macrophages by atomic absorption spectrometry as described (23). The quantification of protein levels in sera and tissues is detailed in the Supplementary Methods.

Statistical analysis

Statistical analysis was carried out using a GraphPad Prism statistical package and Microsoft Excel. We determined significance by unpaired two-tailed Student’s *t*-test or Mann-Whitney test to assess data, where only two groups existed. For the comparison of organ bacterial loads and mRNA expression, data were log-transformed prior to Student’s *t*-test. ANOVA with Bonferroni correction was used
when more than two groups existed. Survival was compared by log-rank test. Generally, p-values less than 0.05 were considered significant.

RESULTS

Hepatocyte-specific Hfe deletion stimulates extracellular growth of Salmonella Typhimurium

We previously reported that mice lacking Hfe in all cell types (Hfe\(^{-/-}\) mice) were partially protected from S. Tm. infection (13, 20).

Consistently, we could recapitulate this finding in a different strain of Hfe\(^{-/-}\) mice in which exons 3-5 (24) rather than exons 2-3 (13) of Hfe were deleted. We found that also these Hfe\(^{-/-}\) mice survived significantly longer (Fig. 1A) and carried reduced numbers of bacteria in spleen, liver and serum in response to S. Tm infection when compared to Hfe\(^{+/-}\) littermates (Fig. 1B-1D). To delineate in which cell type the absence of Hfe confers protection from infection, we next analyzed mice with selective Hfe-deficiency in hepatocytes (referred to as AlfpCre\(^{+}\) Hfe\(^{0/-}\)). Previous analyses of the AlfpCre\(^{+}\) Hfe\(^{0/-}\) line showed an iron phenotype comparable to Hfe\(^{-/-}\) mice (25), with elevated iron levels in serum and liver and iron deficiency in the spleen.

AlfpCre\(^{+}\) Hfe\(^{0/-}\) and control mice (AlfpCre\(^{-}\) Hfe\(^{0/-}\)) were infected with S. Tm. and survival time was monitored for 14 days (336 hours). Unexpectedly and in contrast to the previous findings in Hfe\(^{-/-}\) mice, we observed significantly shortened survival in the AlfpCre\(^{+}\) Hfe\(^{0/-}\) mice (Fig. 1E). Bacterial burden in spleen and liver was not substantially altered, when compared to control mice (Fig. 1F and 1G). By contrast, the number of bacteria circulating in the serum was significantly higher in AlfpCre\(^{+}\) Hfe\(^{0/-}\) mice (Fig. 1H). This finding suggested that Hfe-deficiency in hepatocytes does not confer protection against S. Tm. infection related death but even aggravates the infection phenotype.

Macrophage-specific Hfe-deletion phenocopies the protective effect of constitutive Hfe deletion in mice infected with Salmonella Typhimurium

We next tested the response to S. Tm. infection in mice lacking Hfe in myeloid cells (referred to as LysMCre\(^{+}\) Hfe\(^{0/-}\)) (25) in comparison to control mice (LysMCre\(^{-}\) Hfe\(^{0/-}\)). Interestingly, macrophage-specific Hfe depletion fully recapitulated the
protective effect observed in $Hfe^{-/-}$ mice, including prolonged survival (Fig. 1I) and reduced bacterial load in spleen, liver and serum (Fig. 1J-1L). Importantly, the alleles required for tissue-specific recombination to generate the cell type-specific $Hfe$-depletion models, LysMCre (macrophage-specific Cre-recombinase expression) and AlfpCre (hepatocyte-specific Cre-recombinase expression) alone had no effect on survival and bacterial burden in spleen, liver and serum (Supplementary Fig. S1A-S1D and (26)), excluding non-specific effects of the Cre-recombinases. We conclude that the lack of $Hfe$ in myeloid cells is sufficient to protect mice from $S. Tm.$ infection related consequences. This finding demonstrates an important extra-hepatic function of Hfe in vivo.

Salmonella-infection of LysMCre$^+$ $Hfe^{0/0}$ mice causes iron depletion in macrophages

To understand the mechanism underlying divergent disease outcomes of $S. Tm.$ infection in AlfpCre$^+$ $Hfe^{0/0}$ and LysMCre$^+$ $Hfe^{0/0}$ mice, we analyzed iron-related parameters. Iron localization was detected in tissue sections of Salmonella-infected mice by Prussian blue staining and tissue iron levels were quantified by colorimetric measurement. $S. Tm.$-infected $Hfe^{-/-}$ (Fig. 2A and 2B) and LysMCre$^+$ $Hfe^{0/0}$ mice (Fig. 2G and 2H) showed reduced iron levels in the spleen consistent with the protective phenotype observed in these mouse strains. This was not apparent in infected AlfpCre$^+$ $Hfe^{0/0}$ mice (Fig. 2D and 2E). By contrast, infected $Hfe^{-/-}$ (Supplementary Fig. S2A and S2B) and AlfpCre$^+$ $Hfe^{0/0}$ mice (Supplementary Fig. S2C and S2D) showed hepatocellular iron accumulation, while liver iron levels were normal in infected LysMCre$^+$ $Hfe^{0/0}$ mice (Supplementary Fig. S2E and S2F). Importantly, the reduction of splenic iron levels in infected $Hfe^{-/-}$ and LysMCre$^+$ $Hfe^{0/0}$ mice correlated with diminished intracellular iron levels in bone marrow macrophages (Fig. 2C and 2I), while AlfpCre$^+$ $Hfe^{0/0}$ bone marrow macrophages had normal iron content (Fig. 2F). This finding suggests that upon Salmonella infection, macrophages lacking $Hfe$ show reduced iron levels.

High serum iron in AlfpCre$^+$ $Hfe^{0/0}$ mice allows for increased proliferation of Salmonella

$Hfe^{-/-}$ and AlfpCre$^+$ $Hfe^{0/0}$ mice infected with $S. Tm.$ WT for 72 hours showed elevated serum iron levels compared to $Hfe^{+/+}$ or AlfCre$^-$ $Hfe^{0/0}$ mice, respectively
(Fig. 3A and 3B). In contrast, serum iron levels in infected LysMCre+ Hfe0/0 mice were comparable to infected LysMCre− Hfe0/0 mice (Fig. 3C). Notably, in the setting of Salmonella infection, serum levels of hepcidin-1 were not different between the mouse strains (Fig. 3D-F). Moreover, Salmonella-infected Hfe−/− mice presented with increased serum concentrations of the siderophore-capturing peptide Lcn2 (Fig. 3G) while hepatocyte-specific (Fig. 3H) or macrophage-specific (Fig. 3I) Hfe deletion did not affect serum Lcn2 levels. Thus, the presence of Hfe in hepatocytes is necessary and sufficient to limit serum iron levels both in steady state (25) and in response to S. Tm. infection. Moreover, hepcidin-1 induction in response to Salmonella infection is appropriate in mice lacking Hfe. In contrast, the enhanced production of Lcn2 is only observed in the complete absence of Hfe (13) suggesting that different cell-types mediate iron- and immune-regulatory effects of Hfe.

**Salmonella** iron acquisition pathways differently affect extracellular proliferation

S. Tm. is a bacterial pathogen with dual lifestyle. First, S. Tm. is able to persist and replicate extracellularly, e.g. on contaminated food and surfaces, in the gut lumen and in the serum. Early after the invasion of a murine host, S. Tm. preferentially infects macrophages to propagate intracellularly. We therefore investigated how serum iron availability in Hfe−/−, AlfpCre+ Hfe0/0 and LysMCre+ Hfe0/0 mice may affect bacterial proliferation. We spiked RPMI medium with 10% of serum from uninfected mice of all 3 strains and inoculated spiked samples with bacteria. We used S. Tm. WT and isogenic mutants lacking either single or all three major bacterial iron uptake systems (enterobactin, feo and sitABCD) (27). In addition, we included serum-spiked RPMI treated with 100 µM desferasirox (DFX) to deplete the medium of chelatable iron. Alternatively, we added 100 µM FeSO4 to saturate any iron-binding factors (e.g. transferrin, lactoferrin and Lcn2). S. Tm. WT growth was only inhibited by serum from Hfe−/− mice, possibly due to the presence of high Lcn2 (13). In serum from AlfpCre+ Hfe0/0 mice, bacterial growth was strongly enhanced, while it was not affected in serum from LysMCre+ Hfe0/0 (Fig. 4A). In addition, growth of S. Tm. WT was strongly restricted by the presence of the iron chelator DFX and enhanced by the addition of FeSO4, independent of the Hfe status of the mice the sera were derived from (Fig. 4A). In liquid cultures of iron uptake mutant S. Tm. strains, growth was most pronouncedly inhibited in the case of the triple mutant (entC, feo and sitABCD
deletion). Importantly, we saw that the iron-rich serum of \textit{AlfpCre}^+ \textit{Hfe}^{0/0} mice facilitated extracellular growth of \textit{S. Tm.}, an effect that was reduced by the lack of all three iron uptake systems (Fig. 4B) or abolished by iron chelation (Fig. 4A). Notably, the addition of recombinant murine Lcn2 reduced the growth of \textit{S. Tm.} in a dose-dependent fashion, yet it did not abolish the differences between \textit{AlfpCre}^- \textit{Hfe}^{0/0} and \textit{AlfpCre}^+ \textit{Hfe}^{0/0} mice (Fig. 4C). This suggests that in the presence of high Lcn2 concentrations, iron uptake pathways of \textit{Salmonella} not targeted by Lcn2 such as the \textit{feo} and \textit{sitABCD} systems are able to compensate. Apparently, \textit{feo} and \textit{sitABCD} can maintain a sufficient supply of iron for bacteria when enterobactin incorporation is blocked by Lcn2. Thus, the proliferation advantage of \textit{S. Tm.} in extracellular compartments of \textit{AlfpCre}^+ \textit{Hfe}^{0/0} mice is a specific effect of increased iron availability yet not linked to a specific bacterial iron uptake pathway.

**Hfe does not affect the phagocytic activity of macrophages**

Lower bacterial numbers in \textit{Hfe}^- and \textit{LysMCRe}^+ \textit{Hfe}^{0/0} macrophages could theoretically be explained by altered phagocytosis. Therefore, we next compared the phagocytic capacity of bone marrow-derived macrophages isolated from \textit{WT} and \textit{Hfe}^- mice. However, differences were not detected, suggesting that \textit{Hfe} in macrophages does not affect the phagocytic capacity of macrophages (Supplementary Fig. S3).

**Cell type-specific Hfe deletions differentially affect iron homeostasis and anti-microbial immune gene and protein expression in spleen and liver**

So far, our results indicate that lack of \textit{Hfe} in macrophages is sufficient to suppress intracellular growth of \textit{S. Tm.}, while hepatocyte-specific \textit{Hfe} depletion supports iron-dependent extracellular growth of \textit{Salmonella}. However, the finding that \textit{Hfe}^- and \textit{AlfpCre}^+ \textit{Hfe}^{0/0} mice show comparable serum iron levels yet contrasting infection outcomes suggested dominant effects of \textit{Hfe} in myeloid cells. To identify the responsible mechanisms, we monitored gene response patterns of iron and immune genes in spleens and livers of \textit{S. Tm.}-infected mice.

As expected, mRNA expression of ferritin heavy chain (\textit{H-Ft}) was significantly decreased in the spleen (hallmarked by iron deficiency) and increased in the liver (hallmarked by iron overload) of infected \textit{Hfe}^- mice. However, \textit{H-Ft} remained unchanged in the other infected \textit{Hfe}-models (Tables 1 and 2). Likewise, in livers of \textit{Hfe}^- and \textit{AlfpCre}^+ \textit{Hfe}^{0/0} mice, we found significantly reduced expression of
We next studied the expression of central immune genes involved in the control of infection with intramacrophage bacteria. Importantly, mRNA expression of Il-6 and Ifn-γ was decreased in the spleen in AlfpCre+ Hfe0/0 mice, but not in Hfe−/− nor LysMCre+ Hfe0/0 mice (Table 1). Il-10 was decreased in livers of Hfe−/− mice and Tnf was increased in livers of LysMCre+ Hfe0/0 mice (Table 2).

By contrast, Nos2 (for nitric oxide synthase-2, AKA inducible Nos) expression was increased in mice lacking Hfe either globally or in macrophages, specifically, and decreased in AlfpCre+ Hfe0/0 mice (Table 1). Therefore, reduced macrophage iron levels selectively promote the expression of splenic Nos2, whereas high serum iron has a broader inhibitory effect on antimicrobial host responses in the spleen.

Importantly, the protein levels of iron and immune genes mirrored the mRNA expression levels in both spleen (Fig. 5) and liver (Supplementary Fig. S4). For instance, H-Ft protein expression was lower in the spleen of Hfe−/− mice compared to Hfe+/+ littermates (Fig. 5B). Furthermore, splenic Nos2 protein levels were higher in Hfe−/− and LysMCre+ Hfe0/0 mice and lower in AlfpCre+ Hfe0/0 mice as compared to their respective counterparts expressing Hfe (Fig. 5F). In addition, H-Ft (Supplementary Fig. S4B) was higher and Tfr1 protein (Supplementary Fig. S4C) was lower in both Hfe−/− mice and AlfpCre+ Hfe0/0 mice in comparison to the corresponding controls.

**Hepatocyte-specific deletion of Hfe impairs cytokine formation**

Finally, we aimed at better understanding why AlfpCre+ Hfe0/0 mice succumb to early death. Based on the result of organ-specific immune gene analyses (Tables 1 and 2), we assessed the levels of these circulating mediators of immunity as well as markers of liver synthesis and damage: C3 is a complement factor produced by hepatocytes and essential for the activation of the membrane attack complex which then destroys bacterial cell walls. However, C3 levels were not affected by high serum or parenchymal iron (Fig. 6A). IL-6, the key cytokine for the initiation of the acute-phase response, was reduced in the serum (Fig. 6B) but not in the liver (Supplementary Fig. 4E) of AlfpCre+ Hfe0/0 mice (Fig. 6B). This and the unaltered production of hepcidin-1 (Figure 3E and Table 2) suggest that the acute-phase
response is intact in AlfpCre\(^+\) Hfe\(^{0/0}\) mice. Moreover, GPT activity (for glutamate-pyruvate transaminase) was reduced in AlfpCre\(^+\) Hfe\(^{0/0}\) mice, ruling out increased iron-induced hepatic injury (Supplementary Fig. S4A). Rather, a specific defect in the pro-inflammatory cytokine output was associated with the poor outcome of Salmonella-infected AlfpCre\(^+\) Hfe\(^{0/0}\) mice. Specifically, serum IFN-\(\gamma\) concentrations (Fig. 6C) were significantly lower in AlfpCre\(^+\) Hfe\(^{0/0}\) mice and may have contributed to the insufficient induction of cellular effector mechanisms such as Nos2 in the spleen (Table 1).

**Increased serum iron due to dietary iron overload enhances extracellular Salmonella growth and reduces IFN-\(\gamma\) levels**

To further investigate whether the reduced cytokine production in AlfpCre\(^+\) Hfe\(^{0/0}\) mice is linked to increased cellular iron levels, we next maintained WT mice on an iron adequate (IA) or high iron diet for three weeks to induce iron overload (IO) prior to Salmonella infection. We observed increased bacterial load in serum (Fig. 6D), spleen and liver (Supplementary fig. S5B and S5C) along with unaltered IL-6 (Fig. 6E) but reduced IFN-\(\gamma\) concentrations in the serum (Fig. 6F). This finding suggests that reduced levels of IFN-\(\gamma\), the central cytokine orchestrator of immune responses against intracellular bacteria (28), are a direct consequence of increased serum iron.

**DISCUSSION**

The challenge of mice with the facultative intracellular bacterium S. Tm. uncovered an important extra-hepatic function of Hfe in macrophages and novel cell type-specific roles of Hfe in infection control and immune regulation: Mice lacking \(Hfe\) either in all cell types or selectively in the myeloid compartment were more resistant to Salmonella infection and protected from early death compared to WT littermates expressing \(Hfe\). Conversely, hepatocyte-specific \(Hfe\) deletion was deleterious to the host, triggering early death in response to Salmonella infection. These findings are somewhat unexpected because the primary iron overload patterns of \(Hfe^{-/-}\) mice and hepatocyte-specific \(Hfe\) knock-out mice are alike (25). Mice with myeloid-specific \(Hfe\) deletion by contrast, show no apparent iron-phenotype but are resistant to S. Tm. infection much like \(Hfe^{-/-}\) mice (13). This suggests that the putative
immune-regulatory roles of Hfe in macrophages are partially separated from its iron-regulatory functions or mediated via micro-environmental rather than systemic effects. Further studies using combinations of pathogens and exogenous iron sources will be required to ravel out underlying regulatory networks. However, the lack of Hfe in macrophages is sufficient to explain the improved survival of constitutive Hfe⁻/⁻ mice infected with Salmonella. This fact may directly be related to the profound tropism of Salmonella for myeloid cells and points to an important Hfe function in macrophages (29, 30). We noted that upon Salmonella infection, LysMCre⁺ Hfe⁺/+ and Hfe⁺/+ mice have lower iron levels in the spleen. Indeed, it has been suggested that lower levels of iron in macrophages may be protective against pathogens such as Salmonella that propagate within macrophages (31).

Surprisingly, we found accelerated death of AlfpCre⁺ Hfe⁺/+ mice due to impaired resistance to Salmonella infection, although macrophage iron content was unaffected, and bacterial loads in spleen and liver were not different as compared to AlfpCre⁻ Hfe⁺/+ mice. Our data rather indicate that enhanced extracellular bacterial proliferation in the iron-rich serum is deleterious to AlfpCre⁺ Hfe⁺/+ mice. Apart of iron-induced bacterial growth, the reduced levels of IFN-γ detected in the spleen and serum of infected AlfpCre⁺ Hfe⁺/+ mice and of WT mice maintained on a high iron diet may offer a partial explanation. Unlike AlfpCre⁺ Hfe⁺/+ mice in steady state (25), we herein exclusively report on the setting of Salmonella infection and observed that Salmonella-infected AlfpCre⁺ Hfe⁺/+ mice show normal iron content in the spleen and bone marrow macrophages, suggesting that high serum iron levels impair IFN-γ production and its anti-microbial activity as shown in vitro and in vivo (32, 33).

To a large extent, host defense against intracellular microbes relies on direct antimicrobial effector functions of macrophages (28, 34). Reactive nitrogen (RNS) and oxygen species, generated by Nos2 and phagocyte oxidase (phox), interfere with bacterial metabolism and exert toxic effects to limit Salmonella replication within macrophages and counteract systemic spread in infected mice (35-38). TNF and IFN-γ have partly overlapping functions in the sense that both of them stimulate the expression of Nos2 and the assembly of phox subunits in Salmonella-infected macrophages (36). IL-6 in contrast, is the major cytokine inducer of the acute-phase reaction and centrally involved in the adaptation of iron homeostasis upon inflammatory stress (39). In the setting of Salmonella infection, IL-6 fine-tunes
myeloid cell functions as it promotes bacterial killing but is also associated with alternative macrophage activation (40).

Given unaltered bacterial loads in the mononuclear phagocyte system, the high numbers of bacteria found in the serum of \textit{AlfpCre}\textsuperscript{+} \textit{Hfe}\textsuperscript{fl/fl} mice may result from enhanced extracellular proliferation rather than differential phagocytosis, which is supported by the enhanced bacterial growth we observed in \textit{AlfpCre}\textsuperscript{+} \textit{Hfe}\textsuperscript{fl/fl} serum-spiked medium. \textit{Hfe}\textsuperscript{−/−} mice, in contrast, have reduced numbers of \textit{Salmonella} in the serum, which may in part be attributable to increased serum Lcn2 concentrations (Fig. 3), which are already present in the absence of infection (13, 41). Our findings also support the concept that after invasion into myeloid cells, \textit{Salmonella} has limited access to serum and hepatocellular iron pools. Rather, \textit{Salmonella} may use intramacrophage iron sources such as ferritin (26).

Iron metabolism and immune function have multiple interconnections including effects of iron availability on immune cell differentiation as well as direct effects of iron on cytokine formation and innate immune responses (42, 43). In addition, iron genes and their products modulate the body’s response to inflammation (44). Here we extend on these observations by demonstrating that the expression of the antimicrobial enzyme \textit{Nos2} is partially affected by \textit{Hfe}: \textit{Nos2} expression in the spleen was highest in \textit{Hfe}\textsuperscript{−/−} mice, moderately increased in the setting of myeloid-specific \textit{Hfe} deficiency and markedly reduced in mice with hepatocyte-specific \textit{Hfe} deficiency. This suggests that the transcriptional induction of \textit{Nos2} in macrophages may be affected by a paracrine \textit{Hfe}-dependent pathway. In contrast, when myeloid cells express \textit{Hfe} and serum iron levels are high because of hepatocyte-specific \textit{Hfe} deficiency, the induction of \textit{Nos2} was severely impaired in the spleen. Iron inhibits \textit{Nos2} transcription (45) but this regulation fails to explain the reduced \textit{Nos2} mRNA and protein levels in the spleens of \textit{AlpfCre}\textsuperscript{+} \textit{Hfe}\textsuperscript{fl/fl} mice, which are relatively iron-poor in steady-state and iron-adequate in \textit{S. Tm.} infection (25). We propose that an iron-mediated immune-deregulation secondary to the low levels of \textit{Ifn-γ} mRNA in spleens of these mice is a possible explanation because IFN-γ is a major inducer of \textit{Nos2} (46). In addition, RNS counteract \textit{Salmonella}’s virulence and IFN-γ promotes \textit{Salmonella} degradation in mouse macrophages (47). These mechanisms are also of central importance for immunity in human subjects because monogenetic defects in the \textit{IFN-γ} pathway result in increased susceptibility to non-typhoid \textit{Salmonella} and
atypical mycobacteria (48). However, additional studies are required to characterize the regulatory networks that appear to link Hfe to IFN-γ and Nos2 levels.

Additionally, the observed differences in the expression of immune genes between spleen and liver argue for the involvement of other types of immune, non-parenchymal or stromal cells. In this context, it will be particularly interesting to study the effects of Hfe depletion in lymphocyte subsets in the context of bacteremia because an effect of Hfe on T-cell differentiation has been proposed in different models (49).

*Salmonella* can acquire iron via different pathways. Its major siderophores, enterobactin and salmochelins, bind ferric iron with extremely high affinity, thus initiating its uptake via siderophore receptors. Independently of siderophores, ionic iron is acquired via feo, sitABCD and a less well characterized low affinity iron uptake system (15). It is interesting to note that the *entC sit feo* triple mutant did grow better in medium spiked with sera of hepatocyte-specific *Hfe*-deficient mice than in sera of other mice. The growth of the triple mutant was significantly impaired as compared to *Salmonella* with only single deletions of iron uptake systems, *entC*, *sit* or *feo*, respectively (Fig. 3). This suggests that the growth attenuation of this strain was partially conserved in high iron conditions. Moreover, the addition of high concentrations of recombinant murine Lcn2 to spiked sera of *AlfpCre+ Hfe0/0* mice and corresponding controls inhibited bacterial growth in liquid cultures but did not abolish the differences between the two genotypes of mice whereas the iron chelator DFX did. These findings suggest that *Salmonella* is able to circumvent Lcn2’s growth inhibiting effects by iron uptake mechanisms that act independent of enterobactin and are thus resistant to Lcn2. Salmochelins, which are glycosylated enterobactin derivatives to which Lcn2 cannot bind, may one of the ways by which *Salmonella* resists the immune response (27). However, further studies are required to understand the mechanisms of *Salmonella*’s metabolic adaptation to iron withdrawal by the host’s immune response.

In conclusion, our study exclusively reports on Hfe’s role in infection to demonstrate a pivotal extra-hepatic function of Hfe and to highlight the importance of intracellular iron levels within macrophages for the control of infections with *Salmonella*. We found that the lack of *Hfe* in macrophages increased host resistance to this particular pathogen secondary to reduced intracellular iron availability and
increased Nos2 expression. Importantly, this effect was dominant over possible growth-promoting effects that increased serum iron levels may impose. In contrast, serum iron levels determined both, IFN-γ levels and extracellular bacterial replication and increased bacteremia preceding early mortality in mice lacking Hfe exclusively in hepatocytes. Our data suggest that the high penetrance of HFE mutations may originate from the immune modulatory effects of HFE enabling a better control of infections with intracellular pathogens.

ACKNOWLEDGEMENTS

The authors would like to thank Sylvia Berger, Ines Brosch, Sabine Engl, Ines Glatz and Markus Seifert for excellent technical support.

We also would like to thank Ferric C. Fang, Departments of Laboratory Medicine and Microbiology, University of Washington, for providing Salmonella mutants and intellectual input.

This work was supported by grants from the Austrian Research Fund (FWF sponsored doctoral programme W-1253 HOROS to G.W. and stand-alone project P 33062, to M.N.), the Christian Doppler Society (to G.W.), the Tyrolean Research Fund (TWF, to M.N.) and by the ‘Verein zur Förderung von Forschung und Weiterbildung in Infektiologie und Immunologie an der Medizinischen Universität Innsbruck’.

C.M. was funded through a postdoctoral scholarship from the Medical Faculty of Heidelberg University, Germany and the Virtual Liver Network funding initiative (BMBF). M.U.M. was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 1036).

AUTHOR CONTRIBUTIONS

M.N. and C.M. planned and conducted experiments, acquired and analyzed data and drafted the manuscript, M.V.-S., A.-M.M., A.S., D.H., A.H., L.v.R., R.S., C.W.H., H.T. and P.L.M. performed experiments, M.M. and G.W. conceived and designed the study, obtained funding and wrote the manuscript.

DISCLOSURES
The authors declare that there is no conflict of interest.

REFERENCES


**TABLE LEGENDS**

**Table 1:**
Spleen samples of *Salmonella*-infected mice were analyzed by qRT-PCR 72 h post infection. mRNA expression relative to the house keeping gene *Hprt* was quantified. Data were normalized for the corresponding WT (*Hfe*+/+ or Cre− mice as appropriate) and are shown as means ± standard deviations for 5-12 mice per group. Results were compared using *t*-test. Statistical significant differences relative to the respective control are indicated.

**Table 2:**
Liver samples of *Salmonella*-infected mice were analyzed by qRT-PCR 72 h post infection. mRNA expression relative to the house keeping gene *Hprt* was quantified. Data were normalized for the corresponding WT (*Hfe*+/+ or Cre− mice as appropriate) and are shown as means ± standard deviations for 5-12 mice per group. Results were compared using *t*-test. Statistical significant differences relative to the respective control are indicated.
### Table 1: Gene expression in spleen of *S. Tm.* injected mice, 72 h post infection. Gene expression was normalized to expression of *Hprt* and is relative to the respective control group (means +/- SD).

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT (n = 10)</th>
<th>Hfe-KO (n = 12)</th>
<th>p</th>
<th>AlfCre- (n = 20)</th>
<th>AlfCre+ (n = 14)</th>
<th>p</th>
<th>LysMCre- (n = 15)</th>
<th>LysMCre+ (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Iron genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dmt1</em></td>
<td>1 +/- 1.06</td>
<td>2.85 +/- 3.1</td>
<td>ns</td>
<td>1 +/- 0.42</td>
<td>0.98 +/- 0.5</td>
<td>ns</td>
<td>1 +/- 0.55</td>
<td>0.73 +/- 0.33</td>
<td>ns</td>
</tr>
<tr>
<td><em>Fpn1</em></td>
<td>1 +/- 0.38</td>
<td>1.2 +/- 0.88</td>
<td>ns</td>
<td>1 +/- 0.26</td>
<td>1.28 +/- 0.44</td>
<td>*</td>
<td>1 +/- 0.46</td>
<td>0.87 +/- 0.49</td>
<td>ns</td>
</tr>
<tr>
<td><em>Hamp1</em></td>
<td>1 +/- 1.11</td>
<td>1.4 +/- 1.58</td>
<td>ns</td>
<td>1 +/- 1.02</td>
<td>1.12 +/- 1.15</td>
<td>ns</td>
<td>1 +/- 0.95</td>
<td>1.14 +/- 0.58</td>
<td>ns</td>
</tr>
<tr>
<td><em>H-Ft</em></td>
<td>1 +/- 0.22</td>
<td>0.41 +/- 0.2</td>
<td>****</td>
<td>1 +/- 0.72</td>
<td>0.63 +/- 0.32</td>
<td>ns</td>
<td>1 +/- 0.68</td>
<td>0.9 +/- 0.62</td>
<td>ns</td>
</tr>
<tr>
<td><em>Tfr1</em></td>
<td>1 +/- 0.43</td>
<td>1.23 +/- 1.41</td>
<td>ns</td>
<td>1 +/- 0.57</td>
<td>1.55 +/- 1.05</td>
<td>ns</td>
<td>1 +/- 0.84</td>
<td>1.16 +/- 0.87</td>
<td>ns</td>
</tr>
<tr>
<td><em>Hmox1</em></td>
<td>1 +/- 0.68</td>
<td>0.77 +/- 0.61</td>
<td>ns</td>
<td>1 +/- 0.67</td>
<td>0.9 +/- 0.37</td>
<td>ns</td>
<td>1 +/- 0.65</td>
<td>0.79 +/- 0.53</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Immune genes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lcn2</em></td>
<td>1 +/- 1.69</td>
<td>1.1 +/- 0.89</td>
<td>ns</td>
<td>1 +/- 1.72</td>
<td>0.61 +/- 0.61</td>
<td>ns</td>
<td>1 +/- 0.72</td>
<td>1.43 +/- 0.85</td>
<td>ns</td>
</tr>
<tr>
<td><em>Nos2</em></td>
<td>1 +/- 1.06</td>
<td>4.23 +/- 2.09</td>
<td>***</td>
<td>1 +/- 0.81</td>
<td>0.37 +/- 0.23</td>
<td>**</td>
<td>1 +/- 0.83</td>
<td>1.87 +/- 1.2</td>
<td>*</td>
</tr>
<tr>
<td><em>phox-p47</em></td>
<td>1 +/- 0.96</td>
<td>0.88 +/- 0.49</td>
<td>ns</td>
<td>1 +/- 0.55</td>
<td>0.61 +/- 0.25</td>
<td>*</td>
<td>1 +/- 0.31</td>
<td>1.14 +/- 0.33</td>
<td>ns</td>
</tr>
<tr>
<td><em>TNF</em></td>
<td>1 +/- 0.66</td>
<td>1.29 +/- 2.29</td>
<td>ns</td>
<td>1 +/- 0.62</td>
<td>0.82 +/- 0.71</td>
<td>ns</td>
<td>1 +/- 0.5</td>
<td>1.02 +/- 0.38</td>
<td>ns</td>
</tr>
<tr>
<td><em>IL-6</em></td>
<td>1 +/- 0.73</td>
<td>0.74 +/- 1.04</td>
<td>ns</td>
<td>1 +/- 0.31</td>
<td>0.6 +/- 0.36</td>
<td>***</td>
<td>1 +/- 0.66</td>
<td>1.02 +/- 0.5</td>
<td>ns</td>
</tr>
<tr>
<td><em>IL-10</em></td>
<td>1 +/- 0.81</td>
<td>2.35 +/- 2.93</td>
<td>ns</td>
<td>1 +/- 1.12</td>
<td>0.9 +/- 0.75</td>
<td>ns</td>
<td>1 +/- 0.79</td>
<td>1.2 +/- 0.63</td>
<td>ns</td>
</tr>
<tr>
<td><em>IFN-g</em></td>
<td>1 +/- 0.71</td>
<td>2.74 +/- 3.18</td>
<td>ns</td>
<td>1 +/- 0.28</td>
<td>0.45 +/- 0.29</td>
<td>****</td>
<td>1 +/- 0.72</td>
<td>1.17 +/- 0.66</td>
<td>ns</td>
</tr>
</tbody>
</table>

Statistics: unpaired, two-sided student *t*-test, * p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.0001
Table 2: Gene expression in livers of *S. Tm.* injected mice, 72 h post infection. Gene expression was normalized to expression of *Hprt* and is relative to the respective control group (means +/- SD).

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT ( (n = 10) )</th>
<th>Hfe-KO ( (n = 12) )</th>
<th>p</th>
<th>AlfpCre- ( (n = 20) )</th>
<th>AlfpCre+ ( (n = 14) )</th>
<th>p</th>
<th>LysMCre- ( (n = 15) )</th>
<th>LysMCre+ ( (n = 15) )</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dmt1</em></td>
<td>1 +/- 0.24</td>
<td>1.06 +/- 0.64</td>
<td>ns</td>
<td>1 +/- 0.42</td>
<td>0.98 +/- 0.68</td>
<td>ns</td>
<td>1 +/- 0.57</td>
<td>1.08 +/- 0.68</td>
<td>ns</td>
</tr>
<tr>
<td><em>Fpn1</em></td>
<td>1 +/- 0.21</td>
<td>1.08 +/- 0.47</td>
<td>ns</td>
<td>1 +/- 0.2</td>
<td>1.61 +/- 0.63</td>
<td>***</td>
<td>1 +/- 0.38</td>
<td>1.08 +/- 0.34</td>
<td>ns</td>
</tr>
<tr>
<td><em>Hamp1</em></td>
<td>1 +/- 0.59</td>
<td>1.51 +/- 1.05</td>
<td>ns</td>
<td>1 +/- 0.46</td>
<td>0.91 +/- 0.46</td>
<td>ns</td>
<td>1 +/- 0.45</td>
<td>1.01 +/- 0.36</td>
<td>ns</td>
</tr>
<tr>
<td><em>H-Ft</em></td>
<td>1 +/- 0.24</td>
<td>1.59 +/- 0.35</td>
<td>***</td>
<td>1 +/- 0.26</td>
<td>1 +/- 0.33</td>
<td>ns</td>
<td>1 +/- 0.37</td>
<td>0.83 +/- 0.37</td>
<td>ns</td>
</tr>
<tr>
<td><em>Tfr1</em></td>
<td>1 +/- 0.23</td>
<td>0.49 +/- 0.19</td>
<td>****</td>
<td>1 +/- 0.26</td>
<td>0.75 +/- 0.36</td>
<td>*</td>
<td>1 +/- 0.49</td>
<td>0.77 +/- 0.46</td>
<td>ns</td>
</tr>
<tr>
<td><em>Hmx1</em></td>
<td>1 +/- 0.29</td>
<td>0.88 +/- 0.44</td>
<td>ns</td>
<td>1 +/- 0.37</td>
<td>1.32 +/- 0.79</td>
<td>ns</td>
<td>1 +/- 0.49</td>
<td>0.99 +/- 0.46</td>
<td>ns</td>
</tr>
<tr>
<td>Immune genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lcn2</em></td>
<td>1 +/- 0.9</td>
<td>11.8 +/- 6.3</td>
<td>****</td>
<td>1 +/- 1.96</td>
<td>2.36 +/- 3.81</td>
<td>ns</td>
<td>1 +/- 2.32</td>
<td>1.15 +/- 1.7</td>
<td>ns</td>
</tr>
<tr>
<td><em>Nos2</em></td>
<td>1 +/- 1.34</td>
<td>1.38 +/- 2.4</td>
<td>ns</td>
<td>1 +/- 0.86</td>
<td>0.96 +/- 1.04</td>
<td>ns</td>
<td>1 +/- 1.06</td>
<td>1.17 +/- 1.25</td>
<td>ns</td>
</tr>
<tr>
<td><em>phox-p47</em></td>
<td>1 +/- 0.56</td>
<td>1.19 +/- 0.79</td>
<td>ns</td>
<td>1 +/- 0.55</td>
<td>0.99 +/- 0.47</td>
<td>ns</td>
<td>1 +/- 0.66</td>
<td>1.05 +/- 0.62</td>
<td>ns</td>
</tr>
<tr>
<td><em>TNF</em></td>
<td>1 +/- 1.02</td>
<td>1.33 +/- 1.5</td>
<td>ns</td>
<td>1 +/- 0.72</td>
<td>1.14 +/- 1.23</td>
<td>ns</td>
<td>1 +/- 0.51</td>
<td>1.65 +/- 0.93</td>
<td>*</td>
</tr>
<tr>
<td><em>IL-6</em></td>
<td>1 +/- 0.17</td>
<td>0.94 +/- 0.27</td>
<td>ns</td>
<td>1 +/- 0.56</td>
<td>0.99 +/- 0.86</td>
<td>ns</td>
<td>1 +/- 1.1</td>
<td>1.11 +/- 1.02</td>
<td>ns</td>
</tr>
<tr>
<td><em>IL-10</em></td>
<td>1 +/- 0.15</td>
<td>0.83 +/- 0.17</td>
<td>*</td>
<td>1 +/- 1.05</td>
<td>1.47 +/- 1.36</td>
<td>ns</td>
<td>1 +/- 0.85</td>
<td>0.76 +/- 0.75</td>
<td>ns</td>
</tr>
<tr>
<td><em>IFNg</em></td>
<td>1 +/- 0.24</td>
<td>1.12 +/- 0.25</td>
<td>ns</td>
<td>1 +/- 0.47</td>
<td>0.98 +/- 0.69</td>
<td>ns</td>
<td>1 +/- 0.49</td>
<td>0.97 +/- 0.57</td>
<td>ns</td>
</tr>
</tbody>
</table>

Statistics: unpaired, two-sided student t-test, * p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.0001
FIGURE LEGENDS

Figure 1: Cell-type specific effect of Hfe deletion on the course of systemic Salmonella infection

Survival (A, E and I) and bacterial load in spleen (B, F and J), liver (C, G and K) and serum (D, H and L) of Hfe\(^{-/-}\) (A-D) mice and mice lacking Hfe in hepatocytes (AlfpCre\(^{+}\) Hfe\(^{+/+}\) in E-H) or macrophages (LysMCre\(^{+}\) Hfe\(^{+/+}\) in I-L), respectively, compared to matched controls.

Mice were infected with 500 CFU of S. enterica serovar Typhimurim by intraperitoneal injection and monitored for 14 days (336 h). Data represent two independent experiments. Statistics: survival data between control and mutant mice were compared using the Log-rank (Mantel-Cox) Test. \(n = 18\) for Hfe\(^{+/+}\), 16 for Hfe\(^{-/-}\), 13 for AlfpCre\(^{-}\) Hfe\(^{+/+}\), 9 for AlfpCre\(^{+}\) Hfe\(^{+/+}\), 16 for LysMCre\(^{-}\) Hfe\(^{+/+}\), 15 for LysMCre\(^{+}\) Hfe\(^{+/+}\). Log colony-forming unit (CFU) data of tissue bacterial load of randomly selected mice euthanized after 72 h of Salmonella infection were compared using student t-test. CFU data of serum bacterial load were compared by Mann-Whitney test. \(n = 12\) for Hfe\(^{+/+}\), 12 for Hfe\(^{-/-}\), 13-20 for AlfpCre\(^{-}\) Hfe\(^{+/+}\), 11-14 for AlfpCre\(^{+}\) Hfe\(^{+/+}\), 10 for LysMCre\(^{-}\) Hfe\(^{+/+}\), 10 for LysMCre\(^{+}\) Hfe\(^{+/+}\).

Figure 2: Reduced iron content in spleen and bone marrow macrophages in the absence of Hfe

Spleen sections of Hfe\(^{-/-}\) mice (A), AlfpCre\(^{+}\) Hfe\(^{+/+}\) mice (D) and LysMCre\(^{+}\) Hfe\(^{+/+}\) mice (G) infected for 72 h with Salmonella were stained by Prussian blue to analyze iron distribution. Scale bars: 200 \(\mu\)M.

Iron content in infected spleen (B, E and H) and bone marrow macrophages (C, F and I) was measured and normalized for protein content. Data were compared by Mann-Whitney test. \(n = 12\) for Hfe\(^{+/+}\), 12 for Hfe\(^{-/-}\), 13-20 for AlfpCre\(^{-}\) Hfe\(^{+/+}\), 11-14 for AlfpCre\(^{+}\) Hfe\(^{+/+}\), 10 for LysMCre\(^{-}\) Hfe\(^{+/+}\), 10 for LysMCre\(^{+}\) Hfe\(^{+/+}\).

Figure 3: Serum iron and hepcidin-1 levels are differentially affected by Hfe

Serum iron (A-C), hepcidin-1 (D-F) and Lcn2 (G-I) concentrations of the mice infected for 72 h were compared by Mann-Whitney test. \(n = 9-12\) for Hfe\(^{+/+}\), 9-12 for

**Figure 4: Bacterial proliferation is affected by Hfe**

RPMI was spiked with 10% sera of naïve mice of the indicated genotypes. Spiked RPMI was inoculated with WT S. Tm. and its isogenic derivatives mutated in 1 out of 3 or all 3 iron uptake systems (entC, sitABCD, feo). Where applicable, deferasirox (DFX), ferrous sulfate (FeSO4) and recombinant murine Lcn2 (rmuLcn2) was added. Liquid cultures were assessed for extracellular bacterial proliferation (G-I) using the optical density at 600 nm (OD600). ** p < 0.01, *** p < 0.001 for the comparison between mouse genotypes, # p < 0.05, ## p < 0.01 and ### p < 0.001 for the comparison to solvent (Ctrl.) or the S. Tm. WT strain as applicable. n = 4-6 independent experiments.

**Figure 5: Cell-type specific effects of Hfe on protein expression in the spleen**

Spleen homogenates were prepared to quantify the expression of iron and immune relevant proteins by ELISA. Protein levels of Fpn1 (A), H-Ft (B), Tfr1 (C), Lcn2 (D), IFN-γ (E) and Nos2 (F), normalized for total protein content, are depicted as means ± standard deviations. Statistically significant differences as calculated by unpaired, two-sided student t-test are indicated. n = 10 for Hfe+/+, 12 for Hfe−/−, 20 for AlfpCre− Hfe+/−, 14 for AlfpCre+ Hfe+/−, 15 for LysMCre− Hfe+/−, 15 for LysMCre+ Hfe+/−.

**Figure 6: Elevated iron levels correlate with reduced IFN-γ production and increased bacterial numbers in the serum**

Serum complement factor C3 (A), IL-6 (B) and IFN-γ (C) concentrations were analyzed in AlfpCre+ Hfe+/− mice infected for 72 hrs with Salmonella. n = 19-20 for AlfpCre− Hfe+/−, 8-14 for AlfpCre+ Hfe+/−.

Independently, WT mice were fed an iron-adequate (IA) or iron-enriched diet (IO) 3 weeks prior to and during S. Tm. infection. Serum bacterial load (D), IL-6 (E) and IFN-γ (F) concentrations were determined. Statistically significant differences as calculated by Mann-Whitney test are indicated. n = 8-9 for IA, 8 for IO.
SUPPLEMENTARY APPENDIX

Supplementary Information

Cell-specific expression of Hfe determines the outcome of Salmonella enterica serovar Typhimurium infection in mice

Manfred Nairz1,*, Christoph Metzendorf2,3,*, Maja Vujic-Spasic2,3,4,+, Anna-Maria Mitterstiller1,+, Andrea Schroll1, David Haschka1, Alexander Hoffmann1,5, Laura von Raffay1, Richard Sparla2,3, Christian W. Huck6, Heribert Talasz7, Patrizia L. Moser8, Martina U. Muckenthaler2,3,# and Günter Weiss1,5,#

1Department of Internal Medicine II, Infectious Diseases, Immunology, Rheumatology, Pneumology, Medical University of Innsbruck, 6020 Innsbruck, Austria.
2Department of Pediatric Hematology, Oncology and Immunology, University of Heidelberg, INF 350, 69120 Heidelberg, Germany.
3Molecular Medicine Partnership Unit, 69120 Heidelberg, Germany.
4Institute of Comparative Molecular Endocrinology, Ulm University, 89081 Ulm, Germany.
5Christian Doppler Laboratory for Iron Metabolism and Anemia Research, Medical University of Innsbruck, 6020 Innsbruck, Austria.
6Institute for Analytical Chemistry and Radiochemistry, University of Innsbruck, 6020 Innsbruck, Austria.
7Biocenter, Division of Clinical Biochemistry, Medical University of Innsbruck, 6020 Innsbruck, Austria.
8Institute of Pathology, INNPATH, 6020 Innsbruck, Austria.

* M.N. and C.M. contributed equally
+ M.V.-S. and A.-M. M. contributed equally
# M.M. and G.W. contributed equally

Correspondence:
martina.muckenthaler@med.uni-heidelberg.de and guenter.weiss@i-med.ac.at
Supplementary Methods

Salmonella strains

Unless otherwise specified, we used the S. Tm. WT strain ATCC 14028s for infection experiments. In some experiments, isogenic mutant derivatives deficient for either of the three (entC::aph, Δsit::bla and Δfeo::Tn10 (Tet’), respectively) or all three (entC::aph Δsit::bla Δfeo::Tn10 (Tet’)) iron acquisition pathways were additionally used. The construction of these strains has been described (27).

Salmonella infection in vivo

Mice were back-crossed on a C57BL/6J genetic background for at least 10 generations and housed in the EMBL animal facility under a constant light-dark cycle with ad libitum access to food and water. All mouse breeding were approved by and conducted in compliance with the guidelines of the EMBL Institutional Animal Care and Use Committee. Following infection, mice were monitored twice daily for signs of illness and moribund mice were sacrificed. The bacterial load of organs was determined by plating serial dilutions of organ homogenates on LB agar (Sigma-Aldrich) under sterile conditions and the number of bacteria was calculated per gram of tissue.

Measurement of and protein concentrations

Cytokine (IL-6 and IFN-γ) and Lcn2 levels in sera and tissues were determined by specific ELISA sets (obtained from BD Biosciences for cytokines and R&D, respectively, for Lcn2). Serum levels of hepcidin-1 were measured by a specific ELISA set (Intrinsic Lifesciences), too. Also, levels of other proteins in tissues were quantified by specific ELISA sets (Fpn1 and Tfr1 kits were from Wuhan Fine biotech, the FTH kit was from Blue Gene, the Nos2 kit was purchased from Antikoerper Online) according to the manufacturers’ recommendations. To this end, tissue extracts were prepared in cytoplasmatic lysis buffer (25 mM Tris-HCl, pH 7.4, 40 mM KCl, 1% Triton X-100) containing 1 mg/ml aprotinin and 1 mg/ml leupeptin (all obtained from Sigma-Aldrich). Extracts were diluted 50 to 50,000 fold in PBS before use in the respective ELISA. To ensure accurate comparisons of tissue protein levels, ELISA results were normalized for the total protein content as assessed by the Bradford method.
Histopathology

Histologic examination of spleens and livers was performed on formalin-fixed tissue sections automatically stained with H&E or for Prussian blue according to standard protocols. For image acquisition, a Zeiss Axioskop 2 microscope equipped with a 5x objective with a numerical aperture of 0.12, an AxioCam MRc5 digital camera and the AxioVision Rel. 4.8 software were used.

*In vitro* experiments

For the isolation of bone marrow-derived macrophages (BMDM), age- and sex-matched mice were sacrificed. Bone marrow cells were recovered from tibiae and femora and erythrocytes were lysed by two rounds of incubation for 10 min in RBC Lysis Buffer (Biolegend) and extensive washing in PBS as described (26). BMDM were enriched by plastic adherence and differentiated using 100 ng/ml recombinant murine (rmu) M-CSF (Prepotech). After 6 to 8 days in culture, BMDM were transferred to 6-well plates and grown overnight. Thereafter, BMDM were left untreated, stimulated with LPS and/or exposed to fluorescence-labelled bacteria (pHrodo® Green BioParticles; Life Technologies, ThermoFisher Scientific) and incubated at 4°C (unspecific surface binding) or 37°C (phagocytosis) for 15 min. After extensive washing in PBS, phagocytosis was assessed by flow cytometry.

Sequences of primers and probes

Primers and probes, carrying 5’FAM and 3’BHQ1 labels, were obtained from Microsynth. Sequences were as follows (primer forward, primer reverse, probe):

3', 5'-CACCACCCATTCAGTTGCTAA-3', **Hamp1**: 5'-GGCAGACATTTGCGATACCA-3', 5'-TGCAACAGATACCACACTGGGAA-3', 5'-CCAACTTCCCCATCTGATCTTCTGC-3', **H-Fr**: 5'-GCGAGGTGCCGAATCT-3', 5'-CAGCCCGCTTCCCAAGT-3', 5'-CCTGCAAGTATAAAGAAAACCAGACCGTGA-3', **Hmox1**: 5'-GGCAGACATTGCGATACCA-3', 5'-TGAGGCCTTCTTCAAGG-3', 5'-CGACAGCATGCCAGATGTTGTC-3', **Hprt**: 5'-GACCGGTCCCGCTGTAG-3', 5'-TCATAACCTGATTCATCATCGC-3', 5'-ACCAGCGTCCACAGCGTGC-3', **Nos2**: 5'-CAGCTGGGCTGTACCAACCT-3', 5'-CATTGGAAGTGAGGCTTTCG-3', 5'-CGGGAGCACTG-3', **Phox-p47**: 5'-CAGTCAGCAATGGAAGGAGCAGCG-3', 5'-AGTCAGCAATGGAAGGAGCAGCG-3', 5'-CATCACAGGGCCCACCACCACTTCAGA-3', **Tfr1**: 5'-CGCTCTTGTTGCGCTGTG-3', 5'-GGGCAAGGTTTCCAACAGAGACC-3', 5'-CCCACACTGGAGTGCGGCCGCA-3', **Tnf**: 5'-TTCTATGGCCCAGACCCTCA-3', 5'-TTGCTACGACGCTTGTAC-3', 5'-CTCAGATCATCTTTCTCAAAATTGCGAAG-3'.
Legends to Supplementary Figures

Supplementary Figure S1. Alb-Cre\textsuperscript{+} mice not carrying \textit{loxP} (Hfe\textsuperscript{WT/WT}) sites have normal control of \textit{Salmonella} infection.

Survival (A) and bacterial load in spleen (B), liver (C) and serum (D) of mice with insertion of the \textit{Cre}-gene into the hepatocyte-specific albumin (\textit{Alfp}) promoter region (\textit{AlfpCre}\textsuperscript{+} Hfe\textsuperscript{WT/WT}) and their respective controls (\textit{AlfpCre}\textsuperscript{−} Hfe\textsuperscript{WT/WT}). Mice were infected with 500 CFU of \textit{S. enterica} serovar Typhimurium by intraperitoneal injection. Data represent two independent experiments. \(n = 16\) for \textit{AlfpCre}\textsuperscript{−} Hfe\textsuperscript{WT/WT}, \(20\) for \textit{AlfpCre}\textsuperscript{+} Hfe\textsuperscript{WT/WT}. Statistics: survival data (A) between transgenic and control mice were compared using the Log-rank (Mantel-Cox) Test. Log CFU data of tissue bacterial load (B and C) of transgenic and control mice were compared using student \(t\)-test. We used Mann-Whitney testing to compare bacterial numbers in serum. No statistically significant differences were observed. \(n = 10\) for \textit{AlfpCre}\textsuperscript{−} Hfe\textsuperscript{WT/WT}, \(10\) for \textit{AlfpCre}\textsuperscript{+} Hfe\textsuperscript{WT/WT}.

Supplementary Figure S2. Absence of \textit{Hfe} in hepatocytes causes parenchymal iron accumulation in the liver.

Liver sections of \textit{Hfe}\textsuperscript{−/−} mice (A), \textit{AlfpCre}\textsuperscript{+} mice (C) and \textit{LysMCre}\textsuperscript{+} mice (E) infected for 72 hrs were stained by Prussian blue to assess iron distribution. Scale bars: 200 \(\mu\text{M}\).

Hepatic iron content (B, D and F) was measured and normalized for protein content. Data were compared by Mann-Whitney test. \(n = 12\) for \textit{Hfe}\textsuperscript{+/−}, \(12\) for \textit{Hfe}\textsuperscript{−/−}, \(20\) for \textit{AlfpCre}\textsuperscript{−} Hfe\textsuperscript{0/+}, \(14\) for \textit{AlfpCre}\textsuperscript{+} Hfe\textsuperscript{0/+}, \(9\) for \textit{LysMCre}\textsuperscript{−} Hfe\textsuperscript{0/+}, \(9\) for \textit{LysMCre}\textsuperscript{+} Hfe\textsuperscript{0/+}.

Supplementary Figure S3. \textit{Hfe} does not affect phagocytosis.

The phagocytic capacity of \textit{Hfe}\textsuperscript{+/−} and \textit{Hfe}\textsuperscript{−/−} BMDM was assessed following incubation with FITC-labeled bacteria and addition of PBS or LPS at 37°C for 15 min by flow cytometry. Cells not exposed to FITC-labeled bacteria served as blank. The FITC-signal intensity of cells kept on ice (4°C) marks unspecific binding to the cell surface and uptake. One of three representative experiments is shown in histograms depicting fluorescence intensity.
Supplementary Figure S4. Dietary iron overload does increase the bacterial load in spleen and liver.

Serum samples of AlfpCre<sup>+</sup> mice infected with <i>S. Tm.</i> for 72 hrs were used to measure serum GPT activity (A). Data were compared by Mann-Whitney testing.

Bacterial load in spleen (B) and liver (C) of C57BL/6 mice fed an iron-adequate (IA) diet or subjected to oral iron overload (IO) with an iron-enriched diet containing 25 mg/g of iron 3 weeks prior and during infection. Mice were infected with 500 CFU of <i>S. Tm.</i> by intraperitoneal injection. Log CFU data of two independent experiments were compared using student t-test. \( n = 8 \) for IA, \( n = 8 \) for IO.

Supplementary Figure S5. Hepatic protein expression as a function of cell-type specific <i>Hfe</i>-deletion.

Protein levels of Fpn1 (A), H-Ft (B), Tfr1 (C), Lcn2 (D) and IL-6 (E) were measured by ELISA and normalized for protein content. Data are presented and were compared as in fig. 4. \( n = 10 \) for <i>Hfe</i><sup>+/+</sup>, \( 12 \) for <i>Hfe</i><sup>-/-</sup>, \( 20 \) for <i>AlfpCre</i><sup>-</sup> <i>Hfe</i><sup>fl/fl</sup>, \( 14 \) for <i>AlfpCre</i><sup>+</sup> <i>Hfe</i><sup>fl/fl</sup>, \( 15 \) for <i>LysMCre</i><sup>-</sup> <i>Hfe</i><sup>fl/fl</sup>, \( 15 \) for <i>LysMCre</i><sup>+</sup> <i>Hfe</i><sup>fl/fl</sup>.

Supplementary Figure S6. Cell-type specific <i>Hfe</i>-deficiency determines outcome of <i>Salmonella</i> infection.

Compared to WT mice (n denotes normal) expressing <i>Hfe</i> in all cell types (+), <i>Salmonella</i>-infected mice with <i>Hfe</i>-deficiency (-) in macrophages (global or specific) show reduced bacterial numbers (\( \downarrow \)) in spleen and liver, increased <i>Nos2</i> expression and increased survival time. <i>Salmonella</i>-infected mice with hepatocyte-specific <i>Hfe</i>-deficiency or mice with dietary iron overload show elevated serum iron levels and higher bacterial burden (\( \uparrow \)) in the serum as well as reduced IFN-\( \gamma \) production in the spleen and liver.
Supplementary Figure S1.
Supplementary Figure S2.

A  

B  

C  

D  

E  

F  

Hfe+/+  

Hfe+/-  

AlfpCre-  

AlfpCre+  

LysMCre-  

LysMCre+  

Tissue iron [µg/g protein]  

Tissue iron [µg/g protein]  

Tissue iron [µg/g protein]  

Tissue iron [µg/g protein]  

p<0.0001  

p<0.0001  

[Image: liver sections and graphs showing iron content]
Supplementary Figure S3.
Supplementary Figure S4.

A

B

C

D

E
Supplementary Figure S5.
Supplementary Figure S6.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hfe WT</th>
<th>Hfe +/-</th>
<th>Alb-Cre Hfe fl/fl</th>
<th>LysM-Cre Hfe fl/fl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hfe on myeloid cells</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hfe on hepatocytes</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hfe on other cells</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Intracellular S. Tm.</td>
<td>n</td>
<td>↓</td>
<td>n</td>
<td>↓</td>
</tr>
<tr>
<td>Extracellular S. Tm.</td>
<td>n</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Serum iron</td>
<td>n</td>
<td>↑</td>
<td>↑</td>
<td>n</td>
</tr>
</tbody>
</table>