ARTICLE

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

Manfred Nairz,[1*] Christoph Metzendorf,[2,3*] Maja Vujic-Spasic,[2,3,4+] Anna-Maria Mitterstiller,[1+] Andrea Schroll,[1] David Haschka,[1] Alexander Hoffmann,[1,5] Laura von Raffay,[1] Richard Sparla,[2,3] Christian W. Huck,[6] Heribert Talasz,[7] Patrizia L. Moser,[8] Martina U. Muckenthaler[2,3#] and Günter Weiss[1,5#]

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

*MN and CM contributed equally as co-first authors
+MV-S and A-MM contributed equally as co-second authors
#MM and GW contributed equally as co-senior authors

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ABSTRACT

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* knockout 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 antimicrobial 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

Correspondence:

MARTINA U. MUCKENTHALER
martina.muckenthaler@med.uni-heidelberg.de

GÜNTER WEISS
guenter.weiss@i-med.ac.at

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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 has 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 mice, 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*$^{+}$ *Hfe*$^{fl/fl}$) recapitulates the antibacterial phenotype of constitutive *Hfe*$^{-/-}$ mice in response to *S.* Tm. infection. By contrast, exclusive deletion of *Hfe* in hepatocytes (*AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$) 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 weeks of age and infected by intraperitoneal (i.p.) injection 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 by i.p. injection with 500 CFU of *S.* Tm. diluted in 200 μL of PBS as detailed in the *Online Supplementary Methods*.

In vitro experiments

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

RNA extraction and quantitative real-time polymerase chain reaction

Preparation of total RNA, reverse transcription and quantification of mRNA expression by quantitative Taqman real-time polymerase chain reaction (qRT-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*$^{+/+}$ or *Cre*$^{-}$ mice as appropriate) to obtain expression data that is relative to the respective control group. Sequences of primers and probes are listed in the *Online 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 *Online 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 3-524 rather than exons 2-313 of Hfe were deleted. We found that also these $Hfe^{-/-}$ mice survived significantly longer (Figure 1A) and carried reduced numbers of bacteria in spleen, liver and serum in response to *S.* Tm infection when compared to $Hfe^{+/+}$ littermates (Figure 1B to D). In order 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 $AlfpCre^{+}$ $Hfe^{fl/fl}$). Previous analyses of the $AlfpCre^{+}$ Hfefl/fl line showed an iron phenotype comparable to $Hfe^{-/-}$ mice,[25] with elevated iron levels in serum and liver and iron deficiency in the spleen.

$AlfpCre^{+}$ $Hfe^{fl/fl}$ and control mice ($AlfpCre^{-}$ $Hfe^{fl/fl}$) 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 $AlfpCre^{+}$ $Hfe^{-/-}$ mice (Figure 1E). Bacterial burden in spleen and liver was not substantially altered, when compared to control mice (Figure 1F and G). By contrast, the number of bacteria circulating in the serum was significantly higher in $AlfpCre^{+}$ $Hfe^{fl/fl}$ mice (Figure 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 $LysMCre^{+}$ $Hfe^{fl/fl}$)[25] in comparison to control mice ($LysMCre^{-}$ $Hfe^{fl/fl}$). Interestingly, macrophage-specific Hfe depletion fully recapitulated the protective effect observed in Hfe-/- mice, including prolonged survival (Figure 1I) and reduced bacterial load in spleen, liver and serum (Figure 1J to L). 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 the spleen, liver and serum (*Online Supplementary Figure S1A* to D 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 $LysMCre^{+}$ $Hfe^{fl/fl}$ mice causes iron depletion in macrophages

In order to understand the mechanism underlying divergent disease outcomes of S. Tm. infection in $AlfpCre^{+}$ $Hfe^{fl/fl}$ and $LysMCre^{+}$ $Hfe^{fl/fl}$ 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 $Hfe^{-/-}$ (Figure 2A and B) and $LysMCre^{+}$ $Hfe^{fl/fl}$ mice (Figure 2G and H) showed reduced iron levels in the spleen consistent with the protective phenotype observed in these mouse strains. This was not apparent in infected $AlfpCre^{+}$ $Hfe^{fl/fl}$ mice (Figure 2D and E). By contrast, infected $Hfe^{-/-}$ (*Online Supplementary Figure S2A and B*) and $AlfpCre^{+}$ $Hfe^{fl/fl}$ mice (*Online Supplementary Figure S2C and D*) showed hepatocellular iron accumulation, while liver iron levels were normal in infected $LysMCre^{+}$ $Hfe^{fl/fl}$ mice (*Online Supplementary Figure S2E and F*). Importantly, the reduction of splenic iron levels in infected $Hfe^{-/-}$ and $LysMCre^{+}$ $Hfe^{fl/fl}$ mice correlated with diminished intracellular iron levels in bone marrow macrophages (Figure 2C and I), while $AlfpCre^{+}$ $Hfe^{fl/fl}$ bone marrow macrophages had a normal iron content (Figure 2F). This finding suggests that upon *Salmonella* infection, macrophages lacking Hfe show reduced iron levels.

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

$Hfe^{-/-}$ and $AlfpCre^{+}$ $Hfe^{fl/fl}$ mice infected with *S.* Tm. WT for 72 hours showed elevated serum iron levels compared to $Hfe^{+/+}$ or $AlfpCre^{-}$ $Hfe^{fl/fl}$ mice, respectively (Figure 3A and B). In contrast, serum iron levels in infected $LysMCre^{+}$ $Hfe^{fl/fl}$ mice were comparable to infected $LysMCre^{-}$ $Hfe^{fl/fl}$ mice (Figure 3C). Notably, in the setting of *Salmonella* infection, serum levels of hepcidin-1 were not different between the mouse strains (Figure 3D to F). Moreover, *Salmonella*-infected $Hfe^{-/-}$ mice presented with increased serum concentrations of the siderophore-capturing peptide Lcn2 (Figure 3G) while hepatocyte-specific (Figure 3H) or macrophage-specific (Figure 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 $Hfe^{-/-}$, $AlfpCre^{+}$ $Hfe^{fl/fl}$ and $LysMCre^{+}$ $Hfe^{fl/fl}$ mice may affect bacterial proliferation. We spiked RPMI medium with 10% of serum from uninfected mice of all three 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 includ-

ed serum-spiked RPMI treated with 100 μM desferasirox (DFX) to deplete the medium of chelatable iron. Alternatively, we added 100 μM $FeSO_4$ 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*$^{+}$ *Hfe*$^{fl/fl}$ mice, bacterial growth was strongly enhanced, while it was not affected in serum from *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$ (Figure 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 $FeSO_4$, independent of the *Hfe* status of the mice the sera were derived from (Figure 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 *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice facilitated extracellular growth of *S.* Tm., an effect that was reduced by the lack of all three iron uptake systems (Figure 4B) or abolished by iron chelation (Figure 4A). Notably, the addition of recombinant murine Lcn2 reduced the growth of *S.* Tm. in a dose-dependent fashion, even though it did not abolish the differences between *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$ and *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice (Figure 4C). This suggests that in the presence of high Lcn2 concentrations, iron uptake pathways of *Salmonella* not targeted by Lcn2, such as the feo and sitABCD systems, are able to compensate. Apparently, feo and sitABCD can maintain a sufficient supply of iron for

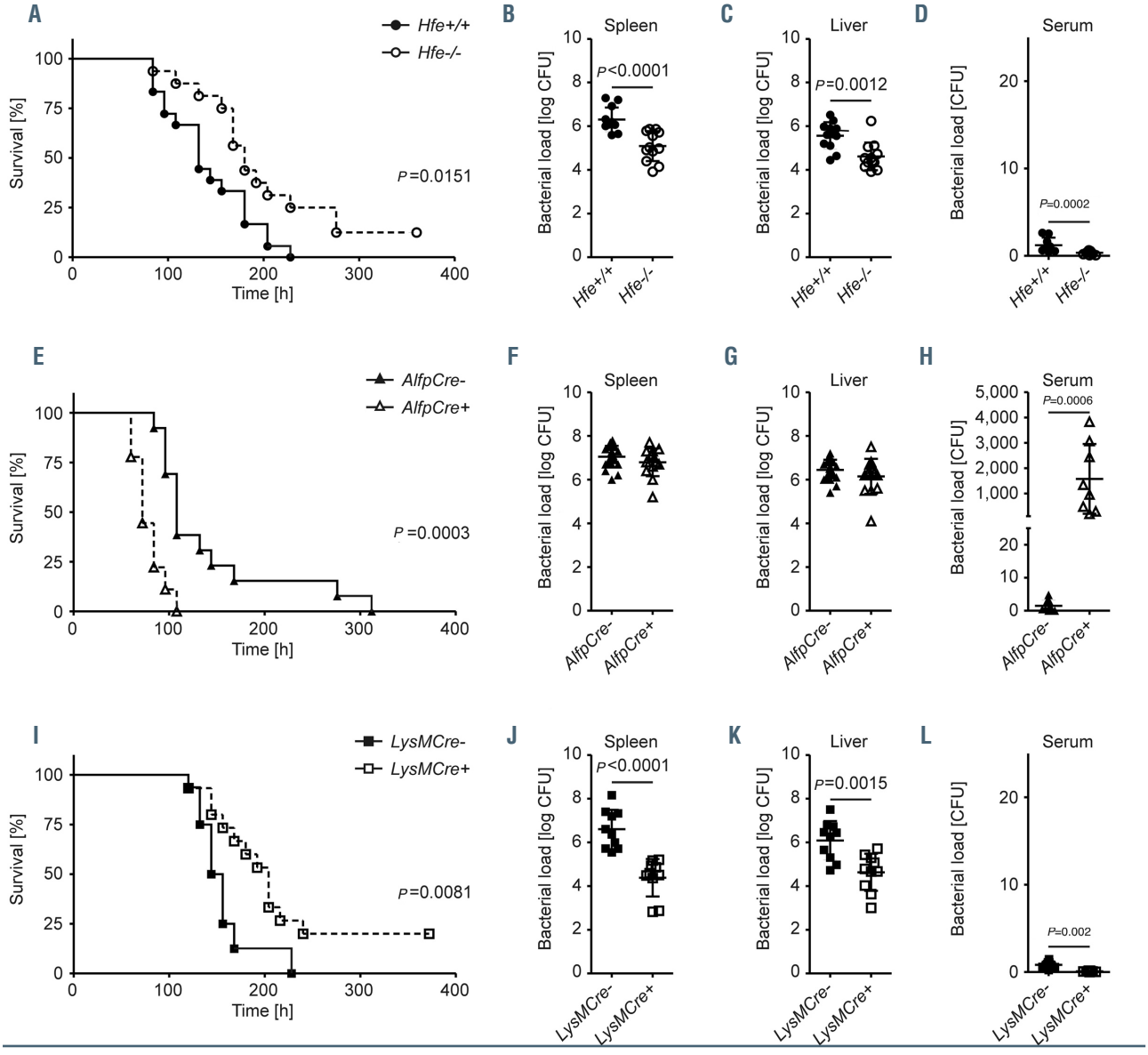

Figure 1. Cell-type specific effect of *Hfe* deletion on the course of systemic *Salmonella* infection. Survival (A, E and I) and bacterial load in spleen (B, F and J), liver (C, G and K) and serum (D, H and L) of *Hfe*$^{-/-}$ (A-D) mice and mice lacking Hfe in hepatocytes (*AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ in E-H) or macrophages (*LysMCre*$^{+}$ *Hfe*$^{fl/fl}$ in I-L), respectively, compared to matched controls. Mice were infected with 500 colony forming units (CFU) of *S. enterica* serovar Typhimurim by intraperitoneal injection and monitored for 14 days (336 hours). 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*$^{+/+}$, n=16 for *Hfe*$^{-/-}$, n=13 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, n=9 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$, n=16 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, n=15 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$. Log CFU data of tissue bacterial load of randomly selected mice euthanized after 72 hours 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*$^{+/+}$, n=12 for *Hfe*$^{-/-}$, n=20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, n=14 for AlfpCre+ Hfefl/fl, n=10 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, n=10 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$.

bacteria when enterobactin incorporation is blocked by Lcn2. Thus, the proliferation advantage of *S.* Tm. in extracellular compartments of *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice is a specific effect of increased iron availability even though it is not linked to a specific bacterial iron uptake pathway.

Hfe does not affect the phagocytic activity of macrophages

Lower bacterial numbers in *Hfe*$^{-/-}$ and *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$ macrophages could theoretically be explained by altered phagocytosis. Therefore, we next compared the phagocytic capacity of bone marrow-derived macrophages isolated from WT and *Hfe*$^{-/-}$ mice. However, differences were not detected, suggesting that *Hfe* in macrophages does not affect the phagocytic capacity of macrophages (*Online Supplementary Figure 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 *Hfe* in macrophages is sufficient to suppress intracellular growth of *S.* Tm., while hepatocyte-specific Hfe depletion supports iron-dependent extracellular growth of *Salmonella*. However, the finding that *Hfe*$^{-/-}$ and *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice show comparable serum iron levels while resulting in

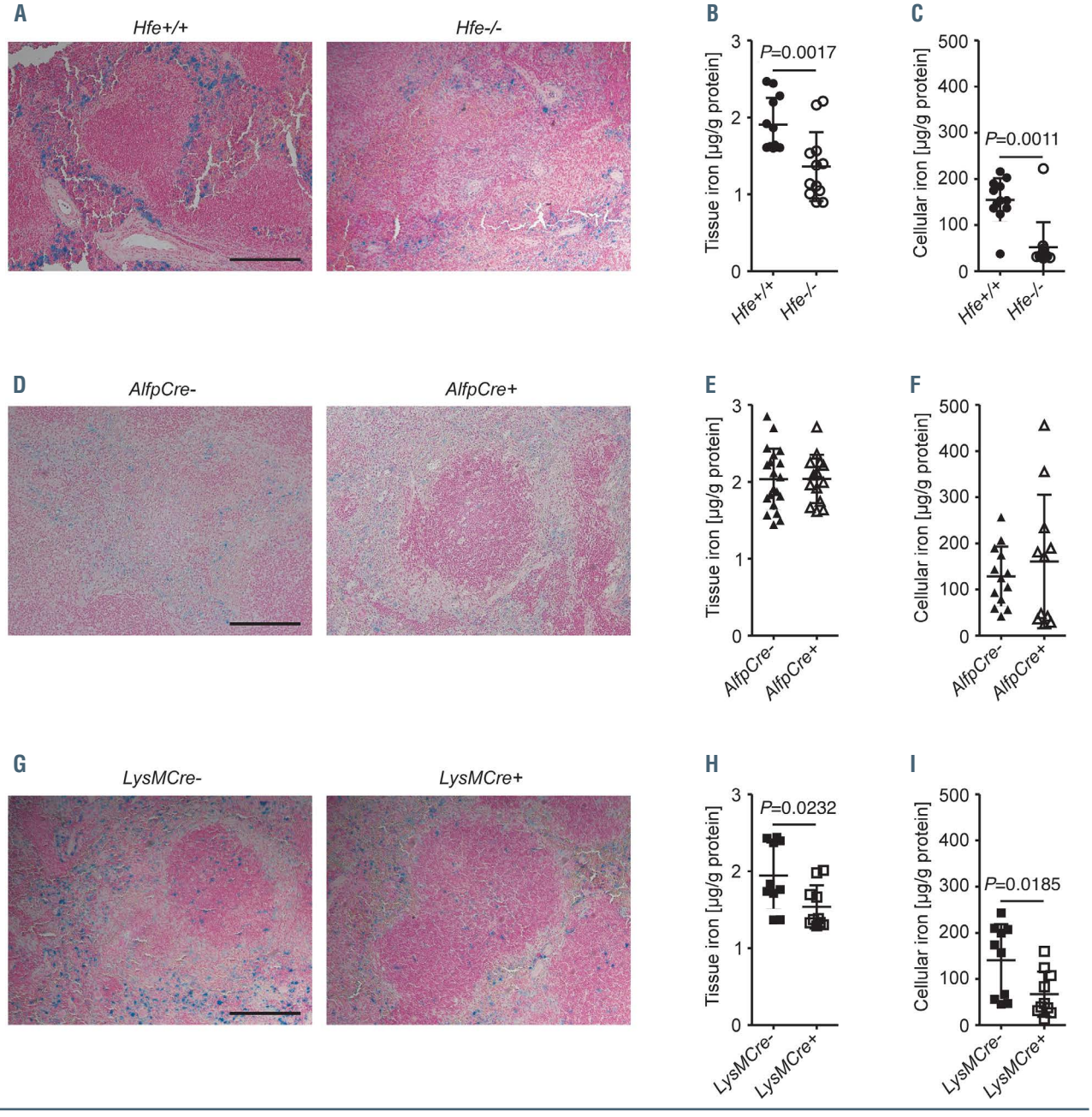

Figure 2. Reduced iron content in spleen and bone marrow macrophages in the absence of Hfe. Spleen sections of *Hfe*$^{-/-}$ mice (A), *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice (D) and *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$ mice (G) infected for 72 hours with *Salmonella* were stained by Prussian blue to analyze iron distribution. Scale bars: 200 µM. 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*$^{+/+}$, n=12 for *Hfe*$^{-/-}$, n=13-20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, n=11-14 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$, n=10 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, n=10 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$.

contrasting infection outcomes suggested dominant effects of *Hfe* in myeloid cells. In order to identify the responsible mechanisms, we monitored gene response patterns of iron and immune genes in spleens and livers of *S.* Tm.-infected mice.

As expected, mRNA expression of ferritin heavy chain (*H-Ft*) was significantly decreased in the spleen (hallmarked by iron deficiency) and increased in the liver (hallmarked by iron overload) of infected $Hfe^{-/-}$ mice. However, *H-Ft* remained unchanged in the other infected Hfe-models (Tables 1 and 2). Likewise, in livers of $Hfe^{-/-}$ and $AlfpCre^{+}$ $Hfe^{fl/fl}$ mice, we found significantly reduced expression of *Tfr1* (2x and 1.3x in $Hfe^{-/-}$ and $AlfpCre^{+}$ $Hfe^{fl/fl}$, respectively), consistent with hepatic iron overload (Table 2).

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 $AlfpCre^{+}$ $Hfe^{fl/fl}$, but neither in $Hfe^{-/-}$ nor $LysMCre^{+}$ $Hfe^{fl/fl}$ mice (Table 1). *Il-10* was decreased in livers of $Hfe^{-/-}$ mice and Tnf was increased in livers of $LysMCre^{+}$ $Hfe^{fl/fl}$ 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 $AlfpCre^{+}$ $Hfe^{fl/fl}$ 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 (Figure 5) and liver (*Online Supplementary Figure S4*). For

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).

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

Statistics: unpaired, two-sided student *t*-test; *P<0.05; **P<0.01; ***P<0.005; ****P<0.0001; WT: wild-type; ns: not significant.

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).

Gene	WT (n = 10)	Hfe-KO (n = 12)	P	$AlfpCre^{-}$ (n = 20)	$AlfpCre^{+}$ (n = 14)	P	$LysMCre^{-}$ (n = 15)	$LysMCre^{+}$ (n = 15)	P
Iron genes									
Dmt1	1 +/- 0.24	1.06 +/- 0.64	ns	1 +/- 0.42	0.98 +/- 0.68	ns	1 +/- 0.57	1.08 +/- 0.68	ns
Fpn1	1 +/- 0.21	1.08 +/- 0.47	ns	1 +/- 0.2	1.61 +/- 0.63	***	1 +/- 0.38	1.08 +/- 0.34	ns
Hamp1	1 +/- 0.59	1.51 +/- 1.05	ns	1 +/- 0.46	0.91 +/- 0.46	ns	1 +/- 0.45	1.01 +/- 0.36	ns
H-Ft	1 +/- 0.24	1.59 +/- 0.35	***	1 +/- 0.26	1 +/- 0.33	ns	1 +/- 0.37	0.83 +/- 0.37	ns
Tfr1	1 +/- 0.23	0.49 +/- 0.19	****	1 +/- 0.26	0.75 +/- 0.36	*	1 +/- 0.49	0.77 +/- 0.46	ns
Hmox1	1 +/- 0.29	0.88 +/- 0.44	ns	1 +/- 0.37	1.32 +/- 0.79	ns	1 +/- 0.49	0.99 +/- 0.46	ns
Immune genes									
Lcn2	1 +/- 0.9	11.8 +/- 6.3	****	1 +/- 1.96	2.36 +/- 3.81	ns	1 +/- 2.32	1.15 +/- 1.7	ns
Nos2	1 +/- 1.34	1.38 +/- 2.4	ns	1 +/- 0.86	0.96 +/- 1.04	ns	1 +/- 1.06	1.17 +/- 1.25	ns
phox-p47	1 +/- 0.56	1.19 +/- 0.79	ns	1 +/- 0.55	0.99 +/- 0.47	ns	1 +/- 0.66	1.05 +/- 0.62	ns
TNF	1 +/- 1.02	1.33 +/- 1.5	ns	1 +/- 0.72	1.14 +/- 1.23	ns	1 +/- 0.51	1.65 +/- 0.93	*
IL-6	1 +/- 0.17	0.94 +/- 0.27	ns	1 +/- 0.56	0.99 +/- 0.86	ns	1 +/- 1.1	1.11 +/- 1.02	ns
IL-10	1 +/- 0.15	0.83 +/- 0.17	*	1 +/- 1.05	1.47 +/- 1.36	ns	1 +/- 0.85	0.76 +/- 0.75	ns
IFN γ	1 +/- 0.24	1.12 +/- 0.25	ns	1 +/- 0.47	0.98 +/- 0.69	ns	1 +/- 0.49	0.97 +/- 0.57	ns

Statistics: unpaired, two-sided student *t*-test; *P<0.05; **P< 0.01; ***P< 0.005; ****P<0.0001; WT: wild-type; ns: not significant.

instance, H-Ft protein expression was lower in the spleen of *Hfe*$^{-/-}$ mice compared to *Hfe*$^{+/+}$ littermates (Figure 5B). Furthermore, splenic Nos2 protein levels were higher in *Hfe*$^{-/-}$ and *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$ mice and lower in *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice as compared to their respective counterparts expressing Hfe (Figure 5F). In addition, H-Ft (*Online Supplementary Figure S4B*) was higher and Tfr1 protein (*Online Supplementary Figure S4C*) was lower in both *Hfe*$^{-/-}$ mice and *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice in comparison to the corresponding controls.

Hepatocyte-specific deletion of *Hfe* impairs cytokine formation

Finally, we aimed at better understanding why *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ 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.

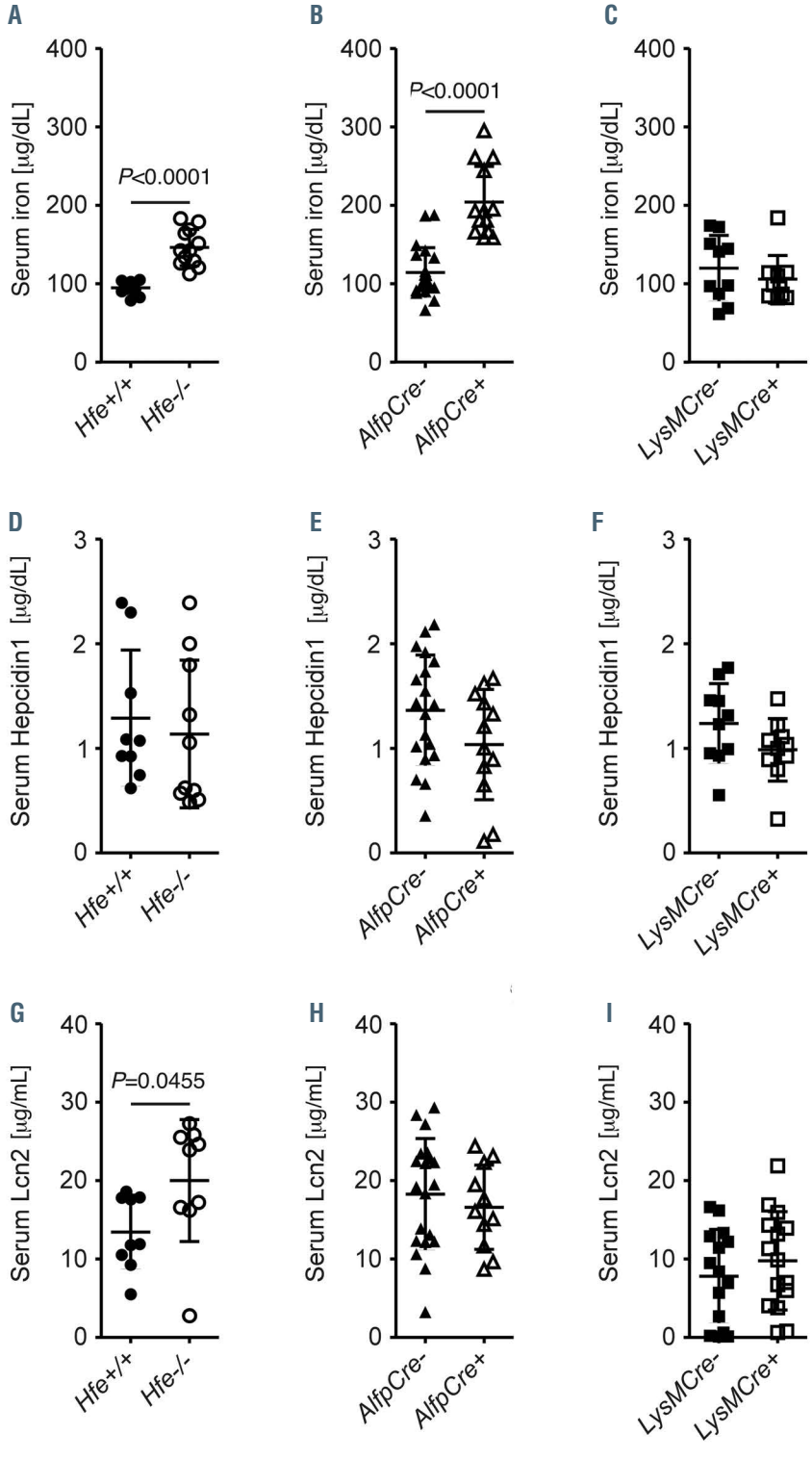

Figure 3. Serum iron and hepcidin-1 levels are differentially affected by Hfe. Serum iron (A to C), hepcidin-1 (D and F) and Lcn2 (G to I) concentrations of the mice infected for 72 hours were compared by Mann-Whitney test. n=9-12 for *Hfe*$^{+/+}$, n=9-12 for *Hfe*$^{-/-}$, 20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, n=11-13 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$, n=10-15 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, n=10-15 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$.

However, C3 levels were not affected by high serum or parenchymal iron (Figure 6A). IL-6, the key cytokine for the initiation of the acute-phase response, was reduced in the serum (Figure 6B) but not in the liver (*Online Supplementary Figure 4E*) of *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice (Figure 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*$^{fl/fl}$ mice. Moreover, glutamate-pyruvate transaminase (GPT) was reduced in *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice, ruling out increased iron-induced hepatic injury (*Online Supplementary Figure S4A*). Rather, a specific defect in the pro-inflammatory cytokine output was associated with the poor outcome of *Salmonella*-infected *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice. Specifically, serum IFN-γ concentrations (Figure 6C) were significantly lower in *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ 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-γ levels

In order to further investigate whether the reduced cytokine production in *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice and may have

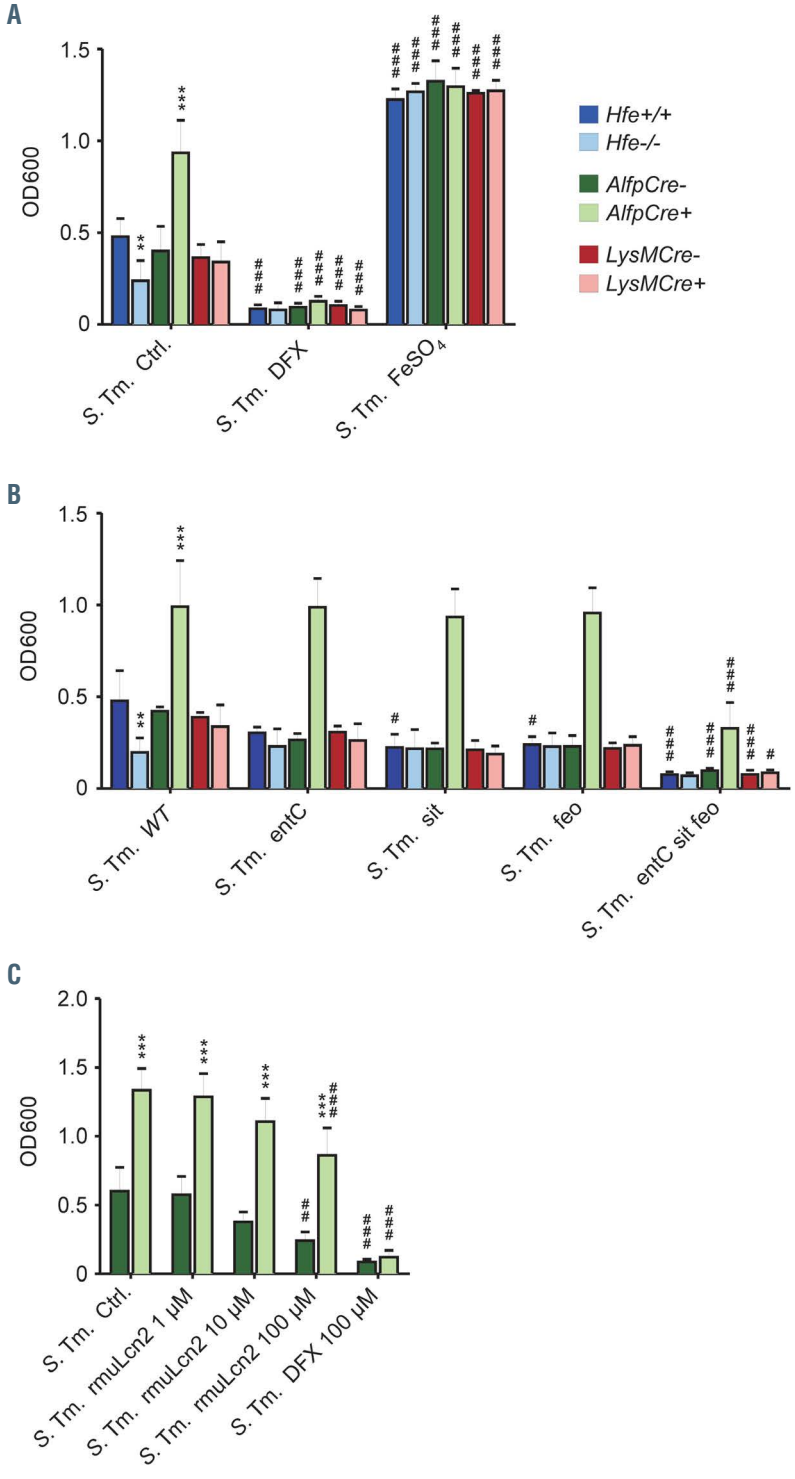

Figure 4. Bacterial proliferation is affected by Hfe. RPMI was spiked with 10% sera of naïve mice of the indicated genotypes. Spiked RPMI was inoculated with wild-type (WT) *S. enterica Typhimurium* (S. Tm.) and its isogenic derivatives mutated in one of three or all three iron uptake systems (*entC, sitABCD, feo*). Where applicable, deferasirox (DFX), ferrous sulfate ($FeSO_4$) and recombinant murine Lcn2 (rmuLcn2) was added. Liquid cultures were assessed for extracellular bacterial proliferation (G to 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.

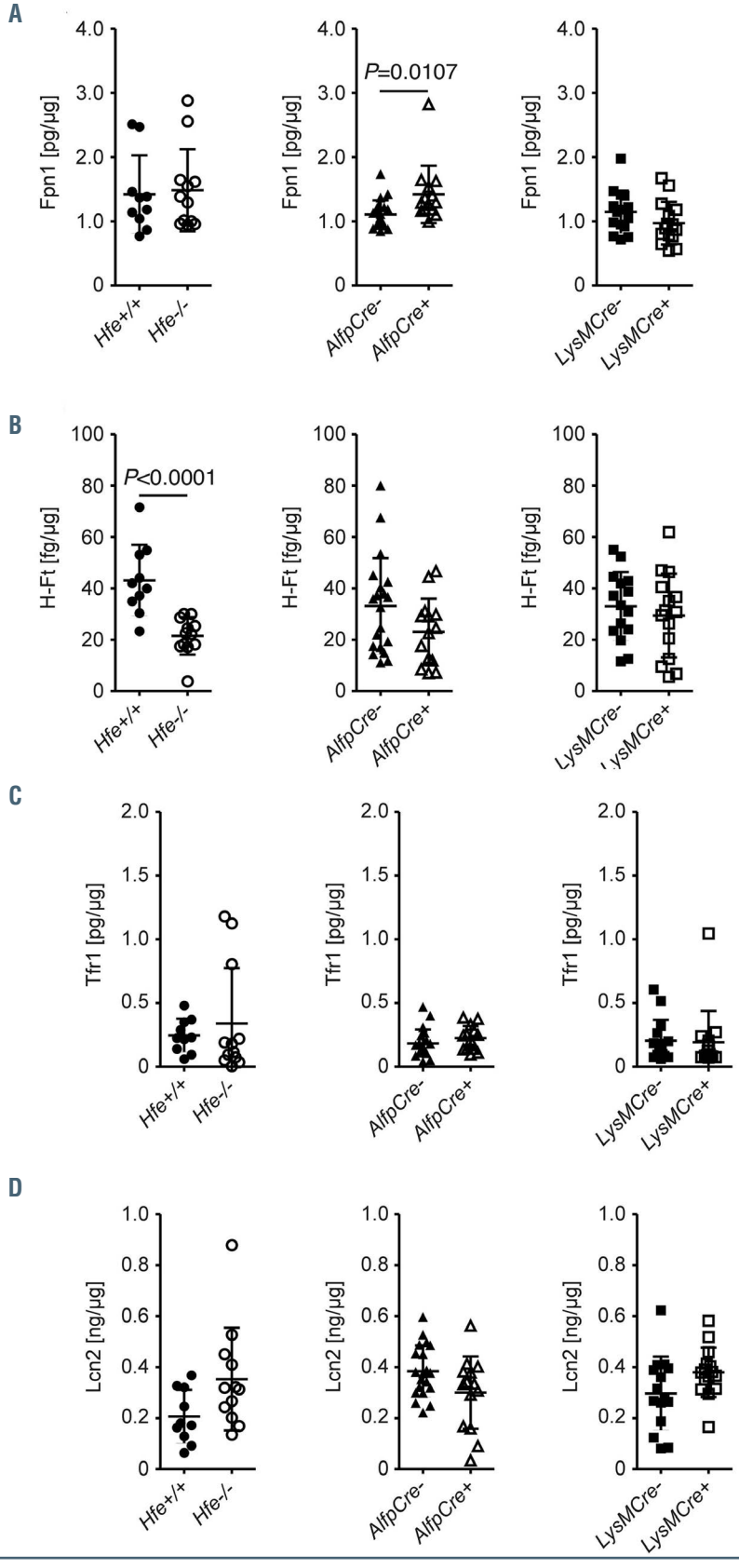

Figure 5. Figure continued on following page.

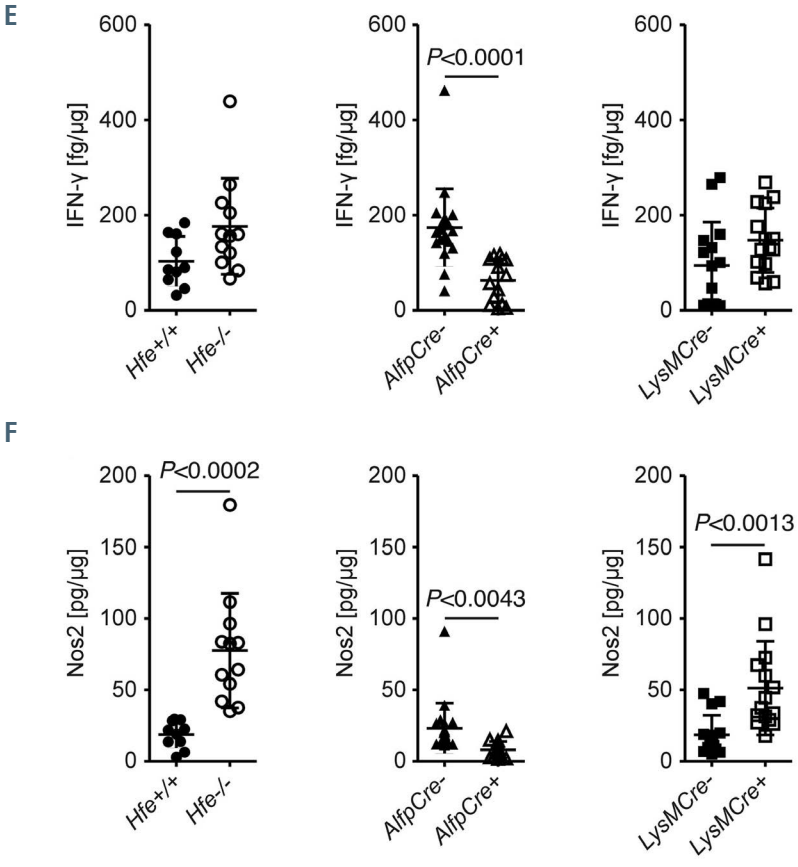

Figure 5. Cell-type specific effects of Hfe on protein expression in the spleen. Spleen homogenates were prepared to quantify the expression of iron and immune relevant proteins by enzyme-linked immunosorbent assay. Protein levels of Fpn1 (A), H-Ft (B), Tfr1 (C), Lcn2 (D), IFN-γ (E) and Nos2 (F), normalized 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*$^{+/+}$, n=12 for *Hfe*$^{-/-}$, n=20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, n=14 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$, n=15 for *LysMCre*$^{-}$ *Hfe*$^{fl/fl}$, n=15 for *LysMCre*$^{+}$ *Hfe*$^{fl/fl}$.

contributed to the insufficient induc 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 an increased bacterial load in the serum (Figure 6D), spleen and liver (*Online Supplementary Figure S5B and C*) along with unaltered IL-6 (Figure 6E) but reduced IFN-γ concentrations in the serum (Figure 6F). This finding suggests that reduced levels of IFN-γ, 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* knockout 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*$^{fl/fl}$ 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*$^{fl/fl}$ mice due to impaired resistance to *Salmonella* infection, although macrophage iron content was unaffected, and bacterial loads in the spleen and liver were not different as compared to *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$ mice. Our data rather indicate that enhanced extracellular bacterial proliferation in the iron-rich serum is deleterious to *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice. Apart from iron-induced bacterial growth, the reduced levels of

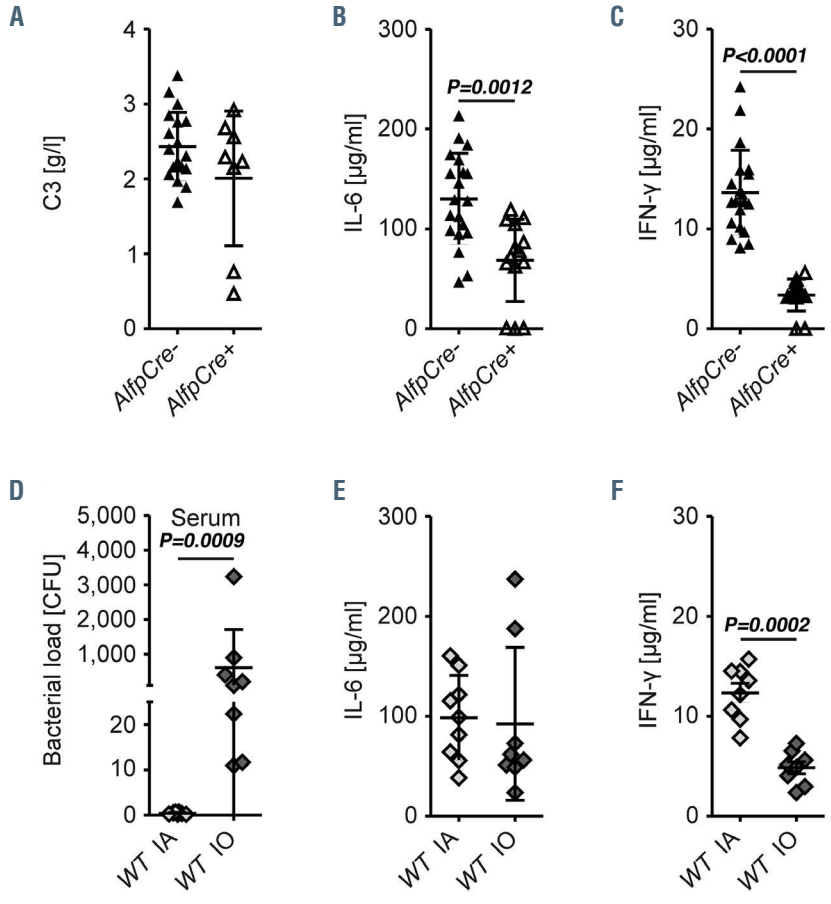

Figure 6. Elevated iron levels correlate with reduced IFN-γ production and increased bacterial numbers in the serum. Serum complement factor C3 (A), IL-6 (B) and IFN-γ (C) concentrations were analyzed in *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice infected for 72 hours with *Salmonella*. n=19-20 for *AlfpCre*$^{-}$ *Hfe*$^{fl/fl}$, n=8-14 for *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$. sIndependently, wild-type (WT) mice were fed an iron-adequate (IA) or iron-enriched diet (IO) 3 weeks prior to and during S. *enterica Typhimurium* (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, n=8 for IO.

IFN-γ detected in the spleen and serum of infected *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice and of WT mice maintained on a high iron diet may offer a partial explanation. Unlike *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice in steady state,[25] we herein exclusively report on the setting of *Salmonella* infection and observed that *Salmonella*-infected *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice show normal iron content in the spleen and bone marrow macrophages, suggesting that high serum iron levels impair IFN-γ production and its antimicrobial 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 *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ mice may result from enhanced extracellular proliferation rather than differential phagocytosis, which is supported by the enhanced bacterial growth we observed in *AlfpCre*$^{+}$ *Hfe*$^{fl/fl}$ serum-spiked medium. *Hfe*$^{-/-}$ mice, in contrast, have reduced numbers of *Salmonella* in the serum, which may in part be attributable to increased serum Lcn2 concentrations (Figure 3), which are already present in the absence of infection.[13,41] Our findings also support the concept that after invasion of myeloid cells, *Salmonella* has limited access to serum and hepatocellular iron pools. Rather, *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 these observations by demonstrating that the expression of the antimicrobial enzyme Nos2 is partially affected by *Hfe*: Nos2 expression in the spleen was highest in *Hfe*$^{-/-}$ mice, moderately increased in the setting of myeloid-specific *Hfe* deficiency and markedly reduced in mice with hepa-

tocyte-specific Hfe deficiency. This suggests that the transcriptional induction of Nos2 in macrophages may be affected by a paracrine *Hfe*-dependent pathway. In contrast, when myeloid cells express *Hfe* and serum iron levels are high because of hepatocyte-specific *Hfe* deficiency, the induction of Nos2 was severely impaired in the spleen. Iron inhibits Nos2 transcription45 but this regulation fails to explain the reduced Nos2 mRNA and protein levels in the spleens of *AlpfCre*$^{+}$ *Hfe*$^{fl/fl}$ mice, which are relatively iron-poor in steady-state and iron-adequate in *S.* Tm. Infection.[25] We propose that an iron-mediated immune-deregulation secondary to the low levels of Ifn-γ mRNA in spleens of these mice is a possible explanation because IFN-γ is a major inducer of Nos2.[46] In addition, RNS counteract *Salmonella*'s virulence and IFN- promotes *Salmonella* degradation in mouse macrophages.[47] These mechanisms are also of central importance for immunity in human subjects because monogenetic defects in the IFN-γ pathway result in increased susceptibility to non-typhoid *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 (Figure 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*$^{+}$ *Hfe*$^{fl/fl}$ 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.

Disclosures

The authors declare that there is no conflict of interest.

Contributions

MN and CM planned and conducted experiments, acquired and analyzed data and drafted the manuscript; MV-S, A-MM, AS, DH, AH, LvR, RS, CWH, HT and PLM performed experiments; MM and GW conceived and designed the study, obtained funding and wrote the manuscript.

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