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# Interferon-γ limits the availability of iron for intramacrophage *Salmonella typhimurium*

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**In stimulating effector functions of mononuclear phagocytes, IFN-γ is of pivotal importance in host defense against intramacrophage pathogens including salmonellae. As the activity of IFN-γ is modulated by iron and since a sufficient availability of iron is essential for the growth of pathogens, we investigated the regulatory effects of IFN-γ on iron homeostasis and immune function in murine macrophages infected with *Salmonella typhimurium*. In *Salmonella*-infected phagocytes, IFN-γ caused a significant reduction of iron uptake *via* transferrin receptor 1 and resulted in an increased iron efflux caused by an enhanced expression of the iron exporter ferroportin 1. Moreover, the expression of haem oxygenase 1 and of the siderophore-capturing antimicrobial peptide lipocalin 2 was markedly elevated following bacterial invasion, with IFN-γ exerting a super-inducing effect. This observed regulatory impact of IFN-γ reduced the intracellular iron pools within infected phagocytes, thus restricting the acquisition of iron by engulfed *Salmonella typhimurium* while concomitantly promoting NO and TNF-α production. Our data suggest that the modulation of crucial pathways of macrophage iron metabolism in response to IFN-γ concordantly aims at withdrawing iron from intracellular *Salmonella* and at strengthening macrophage immune response functions. These regulations are thus consistent with the principles of nutritional immunity.**

**Key words:** IFN-γ · Iron · Macrophage · Nitric oxide · *Salmonella*

See accompanying article by Collins

## Introduction

Host defense against intracellular microbes such as salmonellae or mycobacteria strongly depends on cell-mediated immunity, a major component of which is characterized by interactions between Th1 cells and macrophages [1]. By secreting Th1 cytokines, particularly IFN-γ, antigen-specific Th1 cells activate a plethora of microbicidal mechanisms in infected macrophages. Specifically, in mononuclear phagocytes infected with *Salmonella enterica* serovar *typhimurium* (*S. typhimurium*), IFN-γ promotes the internalization of bacteria and stimulates their elimination by various mechanisms including reactive oxygen and nitrogen species (ROS and RNS), generated *via* NADPH phagocyte oxidase and iNOS, respectively [2–5]. In *Salmonella*-infected mice, treatment with recombinant IFN-γ increases host survival and decreases bacterial numbers in liver and spleen [6]. Conversely, neutralization of murine IFN-γ functions with specific antibodies results in reduced host survival and increased bacterial counts [7].

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In humans, the central importance of IFN-γ for immune response against salmonellae is highlighted by the fact that patients with genetic defects in the IL-12-induced production of IFN-γ or in the IFN-γ receptor 1 selectively suffer from infections with salmonellae and otherwise weakly pathogenic mycobacteria since their phagocytes fail to eliminate these microbes [8, 9].

Iron serves as an essential nutrient for nearly all pathogenic microorganisms, and the expression of iron acquisition systems by infectious agents is associated with their virulence as this trace element is essential for microbial growth and proliferation [10, 11]. In infections with intracellular parasites, including *Salmonella* serovars, the interplay between the activity of IFN-γ and iron homeostasis within macrophages is a central battlefield deciding about the course of the disease [12, 13]. *S. typhimurium* is a facultative intracellular microbe able to invade macrophages and to exploit these cells for multiplication and systemic spreading [14, 15]. Within phagocytes, however, *S. typhimurium* has limited access to mammalian iron resources. Therefore, *Salmonella* has evolved both siderophore-dependent and -independent mechanisms to acquire iron from intracellular host sources; on the one hand, *S. typhimurium* produces enterochelin and salmochelins *via* its enzymes entC and iroB, respectively, the latter encoded within the *iroA* gene cluster [16, 17]. On the other hand, non-siderophore-bound ferrous iron is taken up by Feo, a pathway that may be most relevant in microenvironments with low oxygen tension [18]. Of note, both siderophore-dependent and -independent mechanisms of iron uptake are linked to bacterial virulence [11, 19, 20]. Remarkably, mammals have evolved an innate defense mechanism which specifically interferes with siderophore-mediated iron uptake by microbes; lipocalin 2 (Lcn2) is secreted by neutrophils and macrophages in response to bacterial infections and captures iron-laden microbial siderophores [21, 22]. Thus, Lcn2 withholds siderophore-bound iron from infectious agents and can deliver it to mammalian cells instead, which are able to import the complex *via* lipocalin 2 receptor (LcnR) [23–25]. In line with these observations, iron restriction from invading pathogens has been hypothesized to be an efficient strategy of host defense [13, 26].

Macrophages possess several mechanisms to acquire iron, including the endocytosis of transferrin-bound iron (TBI) *via* transferrin receptor 1 (TfR1), the uptake of ferrous non-transferrin-bound iron (NTBI) *via* divalent metal transporter 1 (Dmt1) and the phagocytosis of senescent red blood cells followed by the mobilization of haem-iron *via* haem oxygenase 1 (Hmox1) [27–29]. By contrast, the sole cellular iron efflux pathway is mediated *via* the transmembrane protein ferroportin 1 (Fpn1), which shifts ferrous iron to the extracellular space [30]. Of interest, the expression of Fpn1 on the cell surface is negatively regulated by the acute phase reactant hepcidin antimicrobial peptide (Hamp). Both hepatocellular iron overload and inflammation stimulate the formation of Hamp, which triggers the internalization of Fpn1, thus blocking iron release [31, 32]

Intriguingly, there are reciprocal interconnections between the activity of IFN-γ and iron homeostasis in mononuclear phagocytes. On the one hand, iron exerts inhibitory effects towards IFN-γ-driven macrophage functions; for instance, iron abrogates the stimulatory impact of IFN-γ on antigen-presentation *via* HLA-DR, on cell-adhesion mediated by ICAM1 and on the production of RNS catalyzed by iNOS [33–36]. On the other hand, IFN-γ modulates the intracellular iron content within phagocytes by regulating the expression of crucial iron genes *via* both transcriptional and post-transcriptional mechanisms; in human monocytes for example, IFN-γ reduces the endocytic uptake of TBI *via* TfR1 [37, 38]. Conversely, the expression of Dmt1, which mediates the acquisition of NTBI, is enhanced upon activation with IFN-γ [38]. Moreover, by stimulating the generation of NO *via* iNOS, IFN-γ promotes the binding of iron regulatory proteins (IRP) to iron responsive elements (IRE) and thus controls the posttranscriptional expression of key proteins involved in cellular iron homeostasis [12, 39].

Because of these mutual interconnections between the efficacy of IFN-γ-driven immune reactions, macrophage iron homeostasis and the pathogenicity of intracellular microbes, we used a well established *in vitro* model employing RAW264.7 macrophages and virulent *S. typhimurium* to investigate the putative impact of IFN-γ on iron homeostasis in *Salmonella*-infected macrophages and to study the consequences of changes in host iron metabolism on innate immune effector functions and on the iron acquisition by the intracellular pathogen.

## Results

### Effects of IFN-γ on iron acquisition and iron release by *Salmonella*-infected macrophages

We first examined the putative changes in iron acquisition by *Salmonella*-infected RAW264.7 macrophages in response to IFN-γ by investigating the regulation of TfR1 and Dmt1 expression and by measuring cellular $^{59}$Fe-uptake. TfR1 mRNA expression was not significantly different between *Salmonella*-infected macrophages and control cells, which is consistent with our previous report [40]. Of interest, the stimulation of cells with IFN-γ significantly reduced the expression of TfR1 mRNA in both control cells and *Salmonella*-infected phagocytes, respectively (Fig. 1). These changes in TfR1 mRNA levels were accompanied by corresponding alterations in TfR1 protein expression (Fig. 2A). Accordingly, the acquisition of TBI by macrophages was not influenced by the invasion of *S. typhimurium* itself but was reduced upon addition of IFN-γ. Compared to control RAW264.7 macrophage-like cells, the intake of TBI was reduced to 55.5 ± 17.7% of the baseline level in phagocytes stimulated with IFN-γ and to 29.0 ± 9.0% in *Salmonella*-infected cells subsequently activated with IFN-γ (Fig. 1). As the atypical MHC class I molecule Hfe affects TfR1-mediated iron uptake [41], we also investigated - based on our results on the modulation of TfR1 levels - putative changes in Hfe expression. IFN-γ significantly induced Hfe expression in both control and *Salmonella*-infected RAW264.7 phagocytes (Fig. 1), which paralleled the reduced uptake of TBI into these cells, accordingly.

 

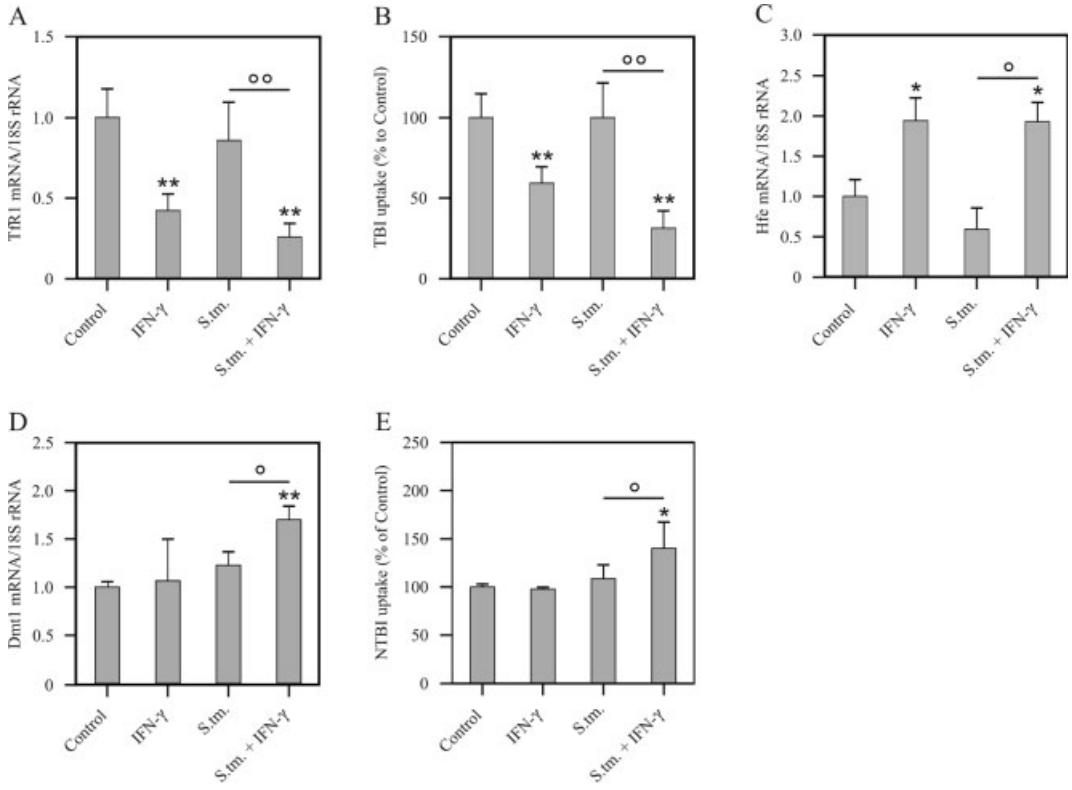


**Figure 1.** Effects of IFN-γ on iron acquisition by *Salmonella*-infected macrophages. RAW264.7 cells were infected with *S. typhimurium* (S. tm.) at a MOI of 10 and stimulated with 50 U/mL IFN-γ for 24 h. TfR1, Hfe and Dmt1 mRNA levels (A, C and D, respectively) were determined by quantitative real-time PCR (Q-PCR). Values were corrected for the amount of 18S ribosomal RNA, which was determined in parallel. Results are shown as relative differences of this ratio as compared to unstimulated control macrophages (= 1.0). Data are expressed as mean ± SD of five independent experiments. Uptake of TBI and of NTBI (depicted in B and E, respectively) was determined as described in *Materials and methods*. Data are shown as mean ± SD of five independent experiments performed in duplicates and are expressed as percentage of relative iron uptake as compared to the control (= 100%). $^{*}p<0.05$, $^{**}p<0.01$ compared with the control; $^{\circ}p<0.05$, $^{\circ\circ}p<0.01$ as compared with cells infected with S. tm.

In contrast to its inhibitory effect on TfR1 expression, we found that IFN-γ increased Dmt1 mRNA expression by 1.7 ± 0.1-fold in *Salmonella*-infected macrophages which were concurrently stimulated with IFN-γ as compared to control cells (Fig. 1). Correspondingly, in comparison to control cells, the uptake of NTBI by infected macrophages was enhanced by 1.6 ± 0.2-fold following their stimulation with IFN-γ (Fig. 1).

To test whether the stimulation of *Salmonella*-infected macrophages with IFN-γ may alter cellular iron release, we investigated the expression of Fpn1 as well as iron export across the cell surface membrane. Fpn1 mRNA levels were not regulated by IFN-γ alone but significantly increased following *Salmonella* infection (Fig. 3). In *Salmonella*-infected phagocytes subsequently stimulated with IFN-γ, the expression of Fpn1 amounted to 3.1 ± 0.7-fold the baseline level as measured in control cells. The observed alterations in Fpn1 mRNA levels resulted in corresponding changes of Fpn1 protein expression as estimated by immunoblot analysis (Fig. 2A). Paralleling the increase in Fpn1 mRNA and protein expression, we observed that in comparison to controls, cellular iron efflux was increased to 316 ± 74% of the baseline level in *Salmonella*-infected phagocytes and further enhanced to 373 ± 68% in infected cells subsequently stimulated with IFN-γ (Fig. 3).

## Regulation of Hmox1, Lcn2 and Hamp expression in response to *Salmonella* infection and IFN-γ stimulation

Following an infection period of 24 h, Hmox1 transcripts were significantly induced in *Salmonella*-invaded phagocytes and further enhanced upon addition of IFN-γ (Fig. 3). These alterations were paralleled by comparable alterations in Hmox1 protein expression (Fig. 2B).

When studying the effects of IFN-γ on the expression of Hamp and Lcn2, two antimicrobial peptides involved in iron homeostasis, we found a modest induction of Hamp expression in response to bacterial invasion whereas IFN-γ did not alter the expression of Hamp mRNA in control nor *Salmonella*-infected macrophages (Fig. 3).

Remarkably, when investigating Lcn2 mRNA levels, we found a strong up-regulation in response to both IFN-γ and to *Salmonella* infection, respectively, and IFN-γ had an additive effect on Lcn2 transcript expression in *Salmonella*-infected RAW264.7 macrophages (Fig. 3), which was confirmed by immunoblot analysis (Fig. 2B).

 

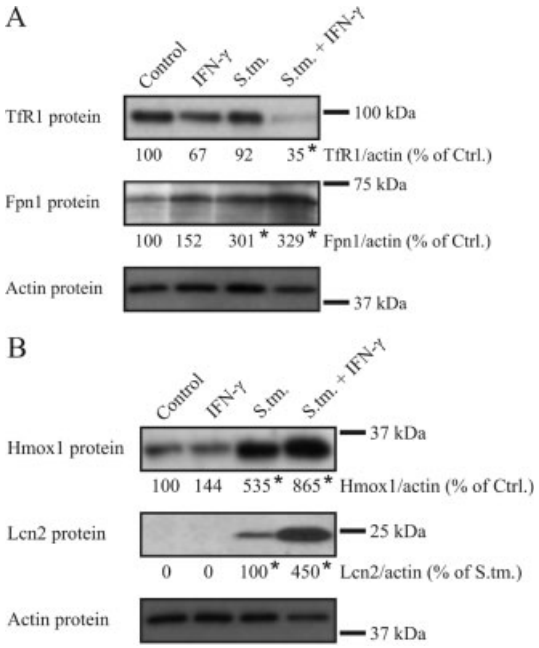


**Figure 2.** Effects of IFN-γ and of *Salmonella* infection on macrophage TfR1, Fpn1, Hmox1 and Lcn2 protein expression. RAW264.7 cells were infected with *Salmonella* and stimulated with IFN-γ exactly as described in the legend to Fig. 1. Whole-cell lysates were analyzed by immunoblotting using specific antibodies to TfR1 and Fpn1 protein (A) as well as Hmox1 and Lcn2 protein (B). Band intensities were quantified by densitometric scanning. One of four representative immunoblot experiments is shown. The numbers below the figures represent the mean arbitrary density units corrected for actin levels and compared with the unstimulated control (= 100%). Lcn2 protein remained undetectable in control macrophages. Therefore, its induction in *Salmonella*-infected phagocytes in response to IFN-γ is compared to infected, solvent-treated RAW264.7 cells (= 100%). Asterisks indicate statistically significant differences between mean arbitrary density units as compared to controls. $^{*}p<0.05$.

## Effects of IFN-γ on ferritin expression and on intracellular iron traffic in *Salmonella*-infected macrophages

To investigate the effects of IFN-γ on the availability of iron within *Salmonella*-infected macrophages, we first studied the expression of ferritin in response to these stimuli. *Salmonella* infection resulted in a marked enhancement of ferritin mRNA and protein levels. In comparison to controls, the mRNA levels of both the ferritin light (L-ferritin) and heavy chains (H-ferritin), respectively, were significantly enhanced upon *S. typhimurium* infection and further, but not significantly, increased by IFN-γ treatment (Fig. 4A). Immunoblot analyses revealed a similar yet less pronounced increment in the protein expression of both ferritin subunits, which, for L-ferritin, amounted to 1.9 ± 0.4-fold the baseline level following sole infection with *Salmonella* and to 2.1 ± 0.5-fold the baseline level upon stimulation of invaded phagocytes with IFN-γ (Fig. 4B, upper panel). In contrast, the H-ferritin subunit was hardly detectable in cytoplasmatic protein extracts derived from control cells but its expression was evident in RAW264.7 cell infected with *S. typhimurium*.

In order to study the effects of these alterations on macrophage iron content, we evaluated the cytoplasmatic labile iron pool and the ferritin storage iron pool in response to IFN-γ and to *Salmonella* infection. When performing gel retardation assays for the determination of IRP binding affinity for IRE, we found that *Salmonella*-infected macrophages had a 3.1 ± 0.4-fold increased IRP1-binding activity, while infected cells subsequently incubated with IFN-γ displayed an IRP1-binding activity, which was 4.4 ± 0.6-fold above the control level (Fig. 4C), indicating a reduction in the cytoplasmatic iron content within infected and/or IFN-γ treated macrophages. Of interest, the binding activity of IRP2 was also markedly increased in RAW 264.7 cell infected with *S. typhimurium* by 2.4 ± 0.5-fold, but was contrastingly affected in infected macrophages concurrently stimulated with IFN-γ, the latter displaying an IRP2-binding activity which was in the range of controls (0.9 ± 0.3-fold of the baseline level; Fig. 4C).

In accordance with the results obtained on cellular iron efflux, the incorporation of $^{59}$Fe into ferritin multimeric shells was significantly reduced upon stimulation with IFN-γ and upon *Salmonella* infection (Fig. 4B, lower panel). Similarly, atomic absorption spectrometry revealed that both IFN-γ and *S. typhimurium* infection resulted in a significant reduction of the total cellular iron content within RAW264.7 macrophages (Fig. 4D).

## Limitation of iron availability for intramacrophage *Salmonella* upon IFN-γ treatment

To test whether this observed induction of iron export as well as of Hmox1 and Lcn2 expression following *Salmonella* infection and IFN-γ activation may serve as a mechanism to withdraw this essential nutrient from internalized bacteria, we directly measured the acquisition of $^{59}$Fe, delivered as either TBI or NTBI, by intramacrophage *Salmonella*. We employed three strains of *S. typhimurium,* namely wild-type *S. typhimurium* C5RP4, wild-type *S. typhimurium* H5542 and strain H5709, the latter characterized by a deficiency in its enzyme entC, which renders it unable to synthesize the siderophores enterochelin and salmochelins, consequently. *Salmonella* wild-type strains C5RP4 and H5542 imported similar amounts of $^{59}$Fe in comparison to each other from both TBI and NTBI sources (Fig. 5A and B). Remarkably, the uptake of radioactive TBI by bacteria residing within RAW264.7 macrophages stimulated with IFN-γ was significantly reduced as compared to microbial TBI uptake within unstimulated macrophages (Fig. 5A). In comparison, IFN-γ did not affect iron uptake by *entC* deficient *Salmonella*. Within RAW264.7 cells incubated with NTBI, applied as $^{59}$Fe-citrate, we observed a similar trend, namely a significant reduction of iron acquisition by intramacrophage *Salmonella* upon IFN-γ treatment (Fig. 5B). Of note, NTBI was more effectively acquired by *Salmonella* than TBI (70.1 ± 11.3 amol/h per $10^3$ H5542 bacteria of NTBI as compared to 22.4 ± 2.3 amol/h per $10^3$ bacteria of TBI, $p<0.01$). In parallel, we studied the iron uptake potential of extracellular *Salmonella* strain H5542 by incubating these bacteria with $^{59}$Fe citrate for

 

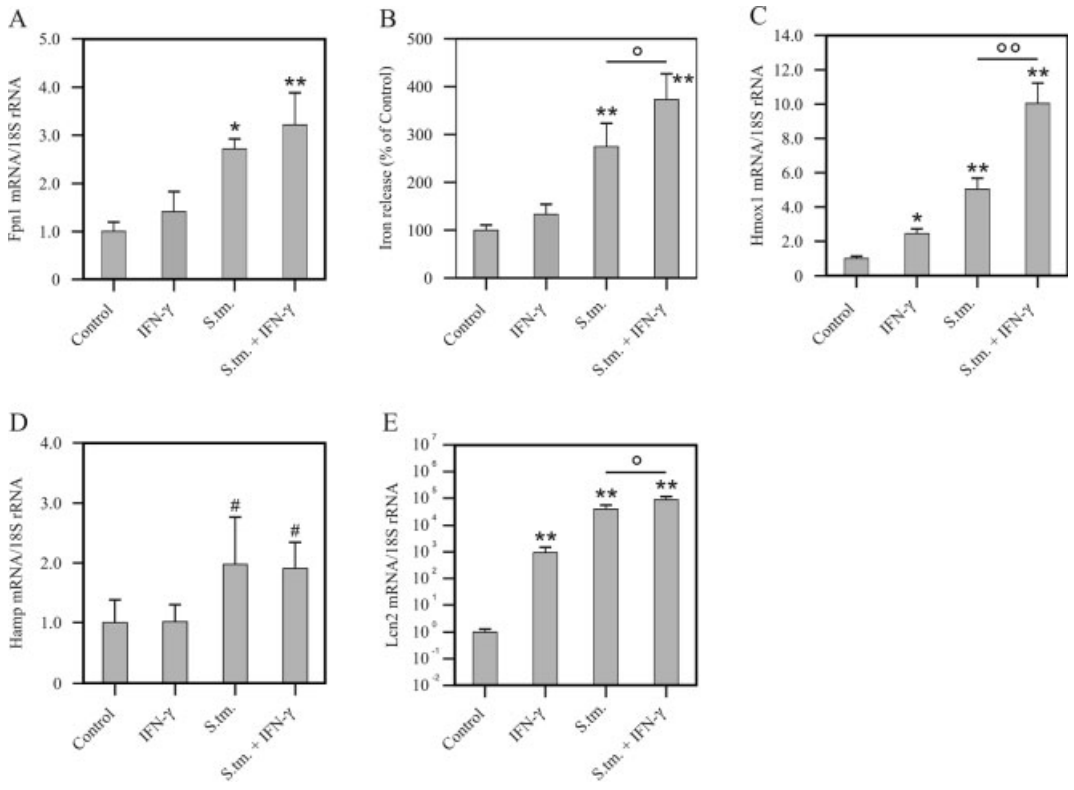


**Figure 3.** Effects of IFN-γ and of *Salmonella* infection on macrophage Fpn1, Hmox1, Hamp and Lcn2 expression as well as on cellular iron export. RAW264.7 cells were infected with *Salmonella* and activated with IFN-γ exactly as described in the legend to Fig. 1. The mRNA levels of Fpn1, Hmox1, Hamp and Lcn2 (A, C, D and E, respectively) were determined by Q-PCR. Values were corrected for the amount of 18S ribosomal RNA, determined in parallel. Results are shown as relative differences of this ratio as compared to unstimulated control macrophages (= 1.0). Data are expressed as means ± SD of six independent experiments. Cellular iron release (B) was determined as described in *Materials and methods*. Data are shown as mean ± SD of six independent experiments performed in duplicates and are expressed as percentage of relative iron release as compared to the control (= 100%). #$p<0.10$, *$p<0.05$, **$p<0.01$ compared with the control; °$p<0.05$, °°$p<0.01$ compared with cells exposed to S. tm.

20 min in the absence of mononuclear phagocytes. We found that these microbes readily incorporated an extrapolated amount of 7.6 ± 0.4 fmol/h per $10^3$ bacteria of ionic iron, which is approximately 100-fold higher than intramacrophage iron acquisition by these bacteria (data not shown). To confirm the inhibitory impact of IFN-γ on the acquisition of iron by intramacrophage *Salmonella*, experiments with primary peritoneal macrophages from C57BL/6 mice were performed. In perfect accordance with the results obtained for RAW264.7 cells, we found that the stimulation of primary peritoneal macrophages with IFN-γ substantially diminished the access of engulfed wild-type *Salmonella* to either TBI or NTBI (Fig. 5C and D). Again, there was no effect of IFN-γ on the incorporation of iron by *entC*-deficient *Salmonella* strain H5709.

## Impact of IFN-γ and of exogenous iron perturbations on intracellular survival of *Salmonella* and on macrophage immune effector functions

We next asked whether the observed changes of macrophage iron homeostasis in response to IFN-γ may impact the intracellular survival of *Salmonella* and/or the efficacy of macrophage immune functions. Therefore, we tested the effects of iron supplementation to RAW264.7 cells infected with *Salmonella* towards bacterial viability and towards the IFN-γ-driven production of RNS, ROS and TNF-α. We found that the delivery of TBI resulted in a dose-dependent increase in the survival of *Salmonella* within unstimulated RAW264.7 mononuclear phagocytes, while IFN-γ efficiently counteracted this growth-promoting effect of iron supplementation (Fig. 6). In parallel, TBI dose-dependently reduced the generation of nitrite by unstimulated *Salmonella*-infected macrophages from 10.65 ± 2.97 μM nitrite in cells grown under control conditions to 3.08 ± 1.21 μM in infected phagocytes supplemented with 50 μg/mL of TBI (Fig. 6). The stimulation of *Salmonella*-infected macrophages with IFN-γ significantly increased the generation of RNS, however, the addition of TBI at a concentration as high as 50 μg/mL reduced nitrite formation as compared to IFN-γ treated/*Salmonella*-infected cells grown under iron-adequate conditions from 23.55 ± 1.13 μM nitrite to 14.93 ± 0.75 μM ($p < 0.05$, Fig. 6).

Similarly, we observed that TBI displayed a dose-dependent inhibitory effect on the production of TNF-α by *Salmonella*-infected macrophages while the addition of IFN-γ not only increased TNF-α formation, but also abolished this metal-mediated inhibitory effect completely (Fig. 6).

 

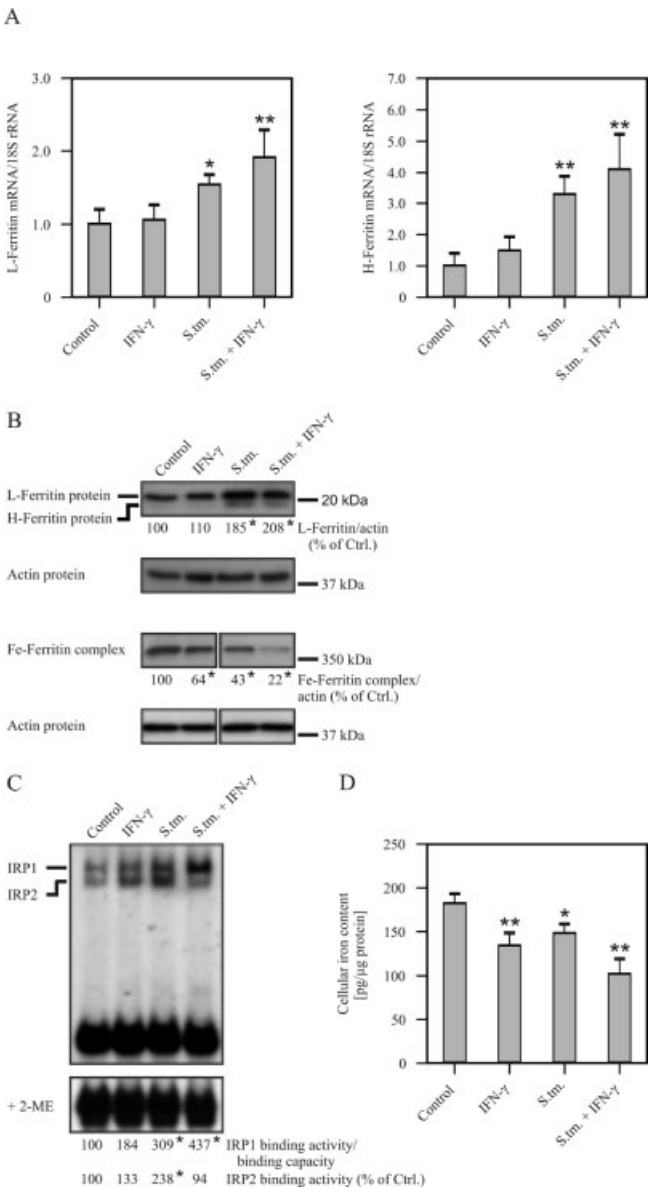


**Figure 4.** Effects of IFN-γ on intracellular iron traffic in *Salmonella*-infected macrophages. RAW264.7 cells were infected with *Salmonella* and activated with IFN-γ exactly as described in the legend to Fig. 1. The mRNA levels of L-Ferritin and H-Ferritin subunits, respectively, were determined by Q-PCR (A). Values were corrected for the amount of 18S ribosomal RNA, determined in parallel. Results are shown as relative differences of this ratio as compared to the unstimulated control macrophages (= 1.0). Data are expressed as means ± SD of five independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ as compared with the control. Ferritin protein levels (B) were determined by immunoblot analysis and quantified by densitometric scanning. The murine L-Ferritin subunit is extensively glycosylated and thus represents the upper band in immunoblots [69]. One of five representative immunoblot experiments is shown. The numbers below the figures represent the mean arbitrary density units of specific L-Ferritin bands corrected for actin and compared to the unstimulated control (= 100%). The incorporation of acquired $^{59}$Fe into ferritin multimers was investigated as detailed in *Materials and methods* (B). Band intensities were quantified by means of densitometric scanning. Endogenous IRP activity was determined by gel retardation assays as described in *Materials and methods*. The 2-mercaptoethanol (2%) was used to fully activate IRP1 binding capacity as a loading control (C). The numbers below the figures represent the mean arbitrary density units of five independent experiments as compared to the unstimulated control (= 100%). Asterisked values denote statistical significance between mean arbitrary density units as calculated by ANOVA. $^{*}p<0.05$ as compared with the control. The total cellular iron content was measured by means of atomic absorption spectrometry as detailed in *Materials and methods* and normalized for protein content (D). Data are expressed as means ± SD of five independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ as compared with the control.

Of note, the supplementation of TBI had only limited effect on the production of ROS by *Salmonella*-infected RAW264.7 cells as measured by the determination of the total oxidative capacity (TOC). While the TOC in cell culture supernatants of infected macrophages increased, though not significantly, with higher supplemented iron concentrations, this was not observed upon addition of IFN-γ, although the TOC in general was significantly higher as compared to *Salmonella*-infected, solvent-treated cells (Fig. 6).

Comparable to the data obtained for *Salmonella*-infected macrophages supplemented with TBI, we saw that NTBI, added as ferrous sulfate, promoted the intracellular persistence of *S. typhimurium* in phagocytes not otherwise activated in a dose-dependent fashion. Whereas solvent-treated macrophages contained 94 800 ± 10 200 *S. typhimurium*, the bacterial load of RAW cells incubated with 50 μM ferrous sulfate was significantly higher and amounted to 179 300 ± 17 900 (Fig. 7). Of interest, IFN-γ had the capacity to limit the intracellular bacterial growth to 52 700 ± 6000 pathogens in macrophages incubated under iron-adequate conditions and to 90 300 ± 22 300 in cells exposed to 50 μM ferrous sulfate. In parallel, we found that at a concentration of 50 μM, NTBI significantly inhibited the formation of nitrite and TNF-α by *Salmonella*-infected phagocytes, which were otherwise left unstimulated. Again, the activation of invaded mononuclear phagocytes with IFN-γ resulted in nitrite and TNF-α levels significantly higher than those observed in solvent-treated cells (Fig. 7). Thus, IFN-γ was able to counteract the inhibitory effects of iron, delivered *via* two different pathways, on the production of RNS and TNF-α by macrophages infected with *S. typhimurium*. In contrast, ferrous sulfate resulted in a slight but not significant increase in the TOC of infected macrophages but did not further enhance the production of ROS in *Salmonella*-invaded RAW264.7 cells stimulated with IFN-γ (data not shown).

## Discussion

Herein, we provide evidence that the efficacy of cellular immunity to control *S. typhimurium*, a facultative intracellular microbe exploiting mononuclear phagocytes as one of its preferred habitats [42, 43], is governed in part by the reciprocal relationship between macrophage iron homeostasis and the activity of IFN-γ. Extending the knowledge on the mutual interconnections between iron status and IFN-γ-activity, we report that IFN-γ possesses the capacity to

 

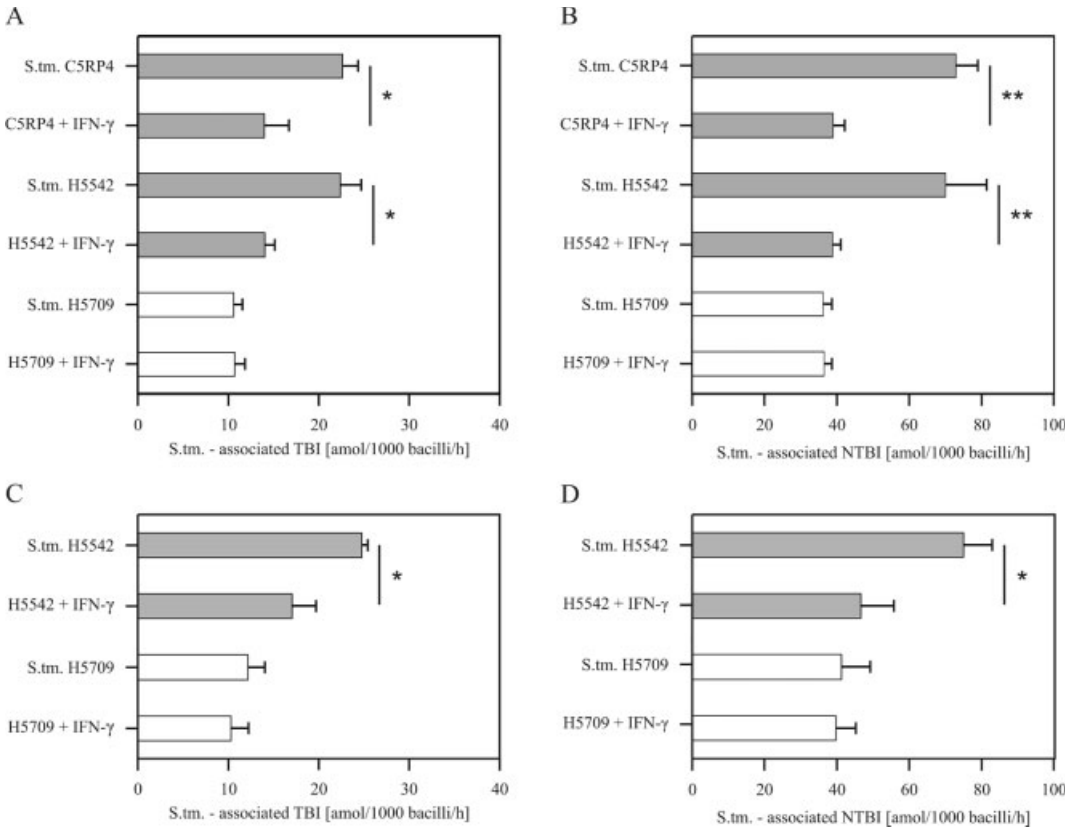


**Figure 5.** Effects of IFN-γ on iron acquisition by intramacrophage *Salmonella*. The acquisition of NTBI and TBI by intramacrophage bacilli was determined as described in *Materials and methods* using three strains of *S. typhimurium*, *i.e.* wild-type *S. typhimurium* strain C5RP4, wild-type *S. typhimurium* strain H5542 and the *entC*-deficient strain *S. typhimurium* H5709, the latter unable to produce any siderophores. RAW264.7 cells (A and B) or primary murine peritoneal macrophages (C and D) were infected with either of these strains as indicated and stimulated with IFN-γ exactly as described in the legend to Fig. 1. Data are shown as means ± SD of five independent experiments and are normalized for the number of bacteria as determined by selective plating. $^{*}p<0.05$, $^{**}p<0.01$ in comparison with the mean $^{59}$Fe uptake by *Salmonella* residing within infected, solvent-treated macrophages as calculated by ANOVA.

reduce the availability of iron for *S. typhimurium* residing within macrophages.

We observed that the infection of phagocytes with *S. typhimurium* stimulated Fpn1-mediated iron efflux as well as the transcriptional expression of Hmox1 and Lcn2. It is noteworthy that the induction of macrophage iron export was strictly dependent on the intracellular presence of viable *S. typhimurium* as neither heat-inactivated bacteria nor lipopolysaccharide, a major component of the Gram-negative bacterial cell wall, were sufficient to trigger this induction observed in response to living microbes (details not shown). Remarkably, the stimulatory effects of *Salmonella* infection on iron release as well as on Hmox1 and Lcn2 levels were enhanced upon addition of IFN-γ to infected cells (Fig. 3), causing a reduction of the cytoplasmatic labile iron pool as well as the ferritin-associated storage iron pool within *Salmonella*-infected phagocytes (Fig. 4). It is highly likely that either of these macrophage iron resources would be potentially accessible to engulfed *Salmonella* and a reduction of these nutritive pools may contribute to the withholding of iron from invaded microbes, consequently. By secreting siderophores, *Salmonella* residing within adapted vacuoles may gain access to cytosolic iron, for instance [13]. Therefore, the reduction of the cytoplasmatic iron pool by the activity of IFN-γ may contribute to the antimicrobial effects of this cytokine. Moreover, the high levels of Lcn2 produced by infected macrophages upon activation with IFN-γ may further contribute to the withdrawal of iron by directly capturing iron-laden enterochelin [21]. Although Lcn2 has primarily been identified as a secreted antimicrobial peptide, we were able to detect Lcn2 in cytoplasmatic protein extracts of *Salmonella*-infected macrophages. We therefore propose that Lcn2 may also fulfill intracellular functions, binding microbial iron-siderophore complexes within invaded host cells, for example. Moreover, Lcn2 may contribute to cellular iron release by transferring iron-laden siderophores to the extracellular space. This idea is consistent with the finding that Lcn2 is able to export iron through the cell surface membrane *via* LcnR [24]. However, even in the presence of Lcn2, iron entering *Salmonella*-containing phagolysosomes may still be available for these microbes by siderophore-independent strategies such as feo-mediated ferrous iron import [11]. This assumption is supported by our data on the preserved capacity to acquire approximately half-maximal amounts of iron by the siderophore-deficient *Salmonella* strain

 

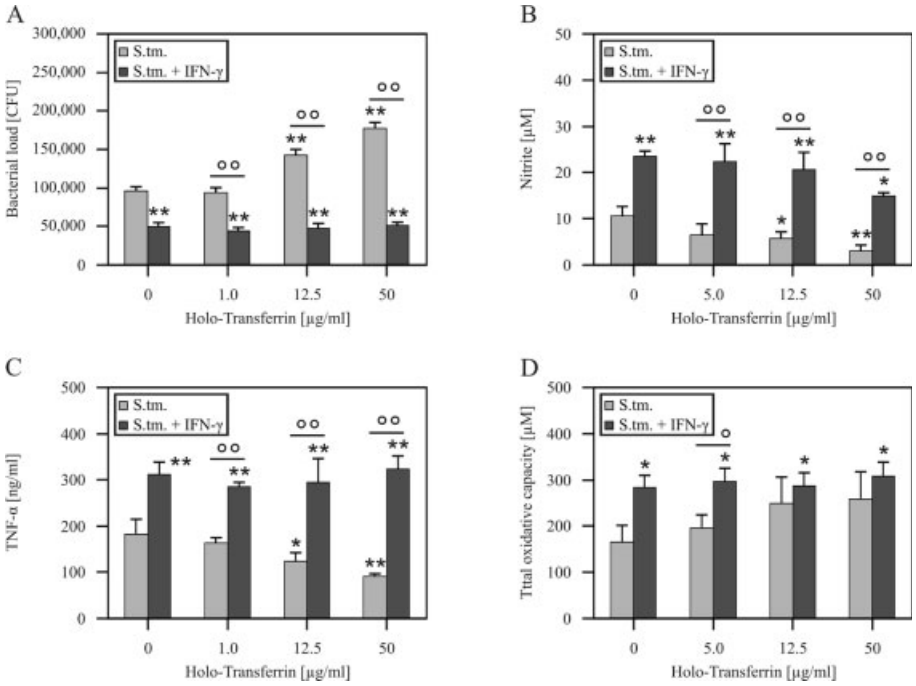


**Figure 6.** Impact of IFN-γ and of exogenous TBI supplementation on intramacrophage survival of *Salmonella* and on macrophage immune effector functions. RAW264.7 macrophages were infected with *Salmonella*, stimulated with IFN-γ and exposed to serial concentrations of human holo-transferrin. The bacterial load was determined by selective plating as described in *Materials and methods* (A). Cell culture supernatants were analyzed for the formation of nitrite (RNS), TNF-α and ROS (measured as total oxidative capacity) as described in *Materials and methods* (B, C and D, respectively). Data are shown as mean ± SD of five independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with solvent-treated, *Salmonella*-infected control macrophages; $^{\circ}p<0.05$, $^{\circ\circ}p<0.01$ for the effect of IFN-γ as calculated by ANOVA.

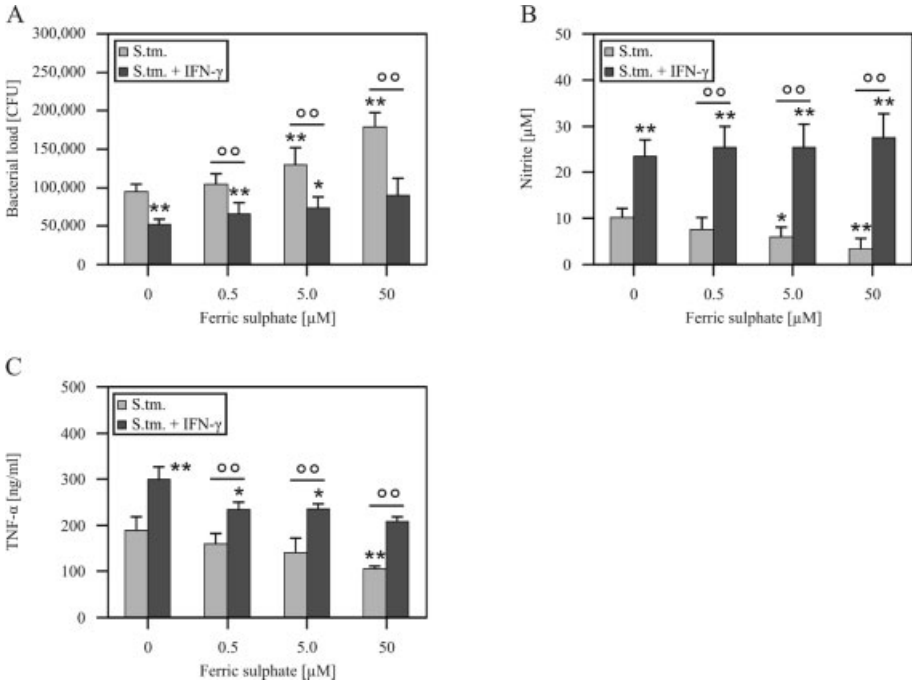


**Figure 7.** Impact of IFN-γ and of exogenous NTBI supplementation on intramacrophage survival of *Salmonella* and on macrophage immune effector functions. RAW264.7 macrophages were infected with *Salmonella*, stimulated with IFN-γ and exposed to serial concentrations of ferrous sulfate. The bacterial load was determined by selective plating as described in *Materials and methods* (A). Cell culture supernatants were analyzed for the formation of RNS, TNF-α and ROS as described in *Materials and methods* (B and C and data not shown, respectively). Data are shown as mean ± SD of five independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with solvent-treated, *Salmonella*-infected control macrophages; $^{\circ\circ}p<0.01$ for the effect of IFN-γ as compared to solvent-treated cells.

 

H5709 (which does not produce aerobactin either) residing within macrophages. Nevertheless, the enhanced iron efflux from *Salmonella*-infected phagocytes may provide a mechanism of microbial iron withholding that can counteract several of the bacterial pathways of iron uptake available.

Hamp is supposed to be the central iron-regulatory hormone in the circulation and primarily acts by binding to Fpn1, the sole iron exporter present within the cell surface membrane. This physical interaction mediates the internalization and degradation of Fpn1, thus reducing cellular iron release [32]. Although a substantial up-regulation of Hamp following infection with *Mycobacterium avium* or *Mycobacterium tuberculosis* and stimulation with IFN-γ has recently been reported [44], we measured a small induction of Hamp transcripts in macrophages infected with *S. typhimurium* without any synergistic effect of IFN-γ (Fig. 3). The reason for the discrepancy of these observations remains unclear. It is possible that the different induction of Hamp in response to various intramacrophage pathogens is strictly dependent on the intracellular localization of the microbes, which is different between mycobacteria and salmonellae. On the other hand, it is also possible that mycobacteria and salmonellae cause different Hamp responses because they activate different sets of intracellular pattern recognition receptors due to differences in their cell surface architecture. Alternatively, *S. typhimurium* may be able to actively suppress the up-regulation of Hamp for instance by effectors translocated *via* one of its two type III secretion systems. Such a capacity could circumvent the antimicrobial properties of Hamp and would probably be beneficial for the pathogen.

Interestingly, TfR1 and Dmt1, which represent two of the most important routes of iron acquisition applied by mononuclear phagocytes, were regulated divergently upon activation of *Salmonella*-infected cells with IFN-γ (Fig. 1). Whereas IFN-γ substantially reduced the uptake of TBI *via* TfR1, the cytokine slightly enhanced the influx of ferrous iron *via* Dmt1, as previously also shown for human monocytes [38]. One could speculate that ionic iron imported *via* Dmt1 may promptly be shifted back to the extracellular space *via* Fpn1, the expression of which was also found to be increased upon activation of macrophages with IFN-γ. The high IRP1 binding activity as well as the low total cellular iron content observed in infected macrophages following their activation with IFN-γ would underscore this hypothesis (Fig. 4). In contrast, iron acquired *via* TfR1-mediated endocytosis may be less accessible for rapid re-export rendering a blocking mechanism for TBI import pivotal to limit the availability of TBI for intramacrophage microbes. It is also possible that, upon activation with IFN-γ, *Salmonella*-infected macrophages aim at taking up a defined amount of ionic iron *via* Dmt1 for the maintenance of metabolic processes such as mitochondrial respiration or for the production of ROS.

We also found that the expression of Hmox1, which catalyzes the rate-limiting step in the degradation of haem, was substantially increased following *Salmonella* infection (Fig. 3). Intriguingly, IFN-γ caused an even more pronounced induction of Hmox1 levels. It is likely that the enhanced expression of Hmox1 influences the redox status within infected phagocytes by decreasing intracellular levels of pro-oxidant haem molecules and by producing the anti-oxidant haem-derivatives biliverdin and bilirubin, subsequently [45]. Thus, Hmox1 may fulfill cytoprotective effects in *Salmonella*-infected phagocytes. Nevertheless, an induction of Hmox1 is also invariably associated with a reduction in the haem-associated iron pool [46], a mechanism which may contribute to the withholding of iron from internalized bacteria, some of which may be able to utilize haem molecules as an iron source *via* specific acquisition systems [47, 48]. Given that the incorporation of freshly imported radioactive iron into ferritin multimers was markedly reduced upon addition of IFN-γ (Fig. 4), we propose that the iron released from haem moieties is exported through the cell surface membrane rather than being stored intracellularly. In fact, iron released from the protoporphyrin ring of haem molecules may contribute to the transcriptional induction of Fpn1 as observed herein and reported in our previous study [40].

Of note, the IRE-binding activities of the two IRP were, at least in part, divergently regulated in response to IFN-γ treatment and to microbial infection (Fig. 4). IRP1 binding to IRE was significantly increased in infected cells subsequently treated with IFN-γ, whereas IRP2-binding function under these conditions was not altered in comparison to controls. The high IRP1 affinity may occur as a consequence of the reduced TBI uptake into and increased iron efflux from *Salmonella*-infected IFN-γ-stimulated macrophages and the formation of NO. In contrast, the relatively low IRP2-binding activity may result from the degradation of IRP2 upon production of $NO^+$, hypoxia or activation of ubiquitin- and non-ubiquitin-mediated pathways, and may thus prevent a compensatory induction of TfR1 expression under these conditions [49–52]. Changes in cellular oxygen levels under the inflammatory stimuli applied in our experiments may determine the specific contributions of IRP1 and IRP2, respectively, to the regulation of macrophage iron homeostasis in response to *S. typhimurium* infection and IFN-γ stimulation [51]. Furthermore, macrophage-derived cytokines including IL-1ß, IL-6 and IL-10 were secreted in substantial amounts by *Salmonella*-infected cells (details not shown) and may contribute to the regulation of cellular iron homeostasis by non IRP-mediated transcriptional and translational regulation of TfR1 and/or ferritin expression [53–55].

Expectedly, the exposition of infected macrophages to increasing amounts of iron impaired macrophage effector functions and promoted the intracellular survival of *Salmonella* (Fig. 6 and 7), which can be referred to the negative regulatory effects of iron on macrophage immune effector pathways such as the formation of RNS or TNF-α [34–36, 56, 57]. Importantly, IFN-γ was able to specifically counteract these immune-debilitating effects of iron in macrophages invaded by *Salmonella*. This fact underscores the importance of IFN-γ in immunity against *S. typhimurium*, especially under conditions of elevated iron levels, and highlights its pathophysiological relevance in the control of macrophage iron metabolism.

In summary, the results of this investigation indicate that IFN-γ exerts a fundamental regulatory impact on the iron homeostasis in

 

murine macrophages infected with the intracellular pathogen *S. typhimurium*. Upon activation with IFN-γ, we have observed a reduction in the uptake of TBI, which, in conjunction with an increased iron export, lowered the iron content within *Salmonella*-infected macrophages and the access of engulfed bacteria to iron, consequently. We propose that these regulations, in association with the induction of Hmox1 and Lcn2, may contribute to the antimicrobial effects of IFN-γ, which are consistent with a role of this key Th1 cytokine in nutritional immunity.

## Materials and methods

### Cell culture

Murine macrophage-like RAW264.7 cells were obtained from the American Type Culture Collection and grown in DMEM (Biochrom AG) containing 10% heat-inactivated FCS (PAA), 2 mM L-glutamine (Biochrom) and 50 μg/mL ampicillin (Sigma-Aldrich) at 37°C in humidified air containing 5% $CO_2$. *S. typhimurium* strain C5RP4 (kindly provided by P. Mastroeni, Cambridge University, Cambridge, UK), a virulent wild-type isolate carrying natural resistance against ampicillin, was used for infection experiments and grown in Luria-Bertani (LB) broth (Sigma-Aldrich) containing 50 μg/mL ampicillin to late-logarithmic phase. Thioglycolate-elicited peritoneal macrophages were harvested from 10–14-week-old male C57BL/6 mice (Charles River Laboratories) exactly as described [58]. The peritoneal macrophages ($1 \times 10^6$) were seeded in six-well dishes in DMEM containing 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 10 mM HEPES (Sigma-Aldrich). The next day, cells were washed five times in HEPES-buffered DMEM without antibiotics and then incubated therein for subsequent infection assays with *S. typhimurium* strains H5542 and H5709 performed exactly as detailed below for RAW264.7 cells. In order to measure the uptake of $^{59}Fe$ by intramacrophage bacteria, *S. typhimurium* strains H5542 and H5709 were used for infection experiments. H5542 is identical to *S. typhimurium* strain ATCC14028 and was grown under sterile conditions in LB broth, its *entC*-deficient derivative H5709 is identical to H5542 but *fepA*:Tn*10*dTc *ent*. *S. typhimurium* strain H5709 was generated as described [59] and grown in LB broth containing 50 μg/mL tetracycline (Sigma-Aldrich). For macrophage infection experiments employing *S. typhimurium* strains H5542 and H5709, complete DMEM without antibiotics was used. After preincubation of *S. typhimurium* in complete DMEM at 37°C for 20 min, $1 \times 10^6$ RAW264.7 macrophages, seeded in six-well dishes, were infected with *S. typhimurium* at a MOI of 10 for 1 h exactly as described previously [40]. Control cells were treated with complete DMEM without any microbes. After 1 h, RAW264.7 cells were washed three times with PBS (Invitrogen) and repleted with complete DMEM containing 16 μg/mL of gentamicin (Sandoz) in order to kill extracellular bacteria. Thereafter, macrophages were stimulated with 50 U/mL recombinant murine IFN-γ (R&D Systems) or PBS as solvent, incubated for another 23 h, washed three times in PBS and subjected to protein or RNA isolation. In some experiments, heat-inactivated *S. typhimurium* strain C5RP4, which had been incubated at 70°C for 20 min, and doses of 10–100 ng/mL of lipopolysaccharide, purified from *S. typhimurium* (Sigma-Aldrich), were included in the study as previously described [40]. For quantification of intracellular *Salmonella,* macrophages were stimulated with serial concentrations of ferrous sulfate and human holo-transferrin, lysed in 0.5% deoxycholic acid (all from Sigma-Aldrich) and plated under sterile conditions in appropriate dilutions onto LB agar plates.

### Quantitative real-time PCR (Q-PCR)

Preparation of total RNA from RAW264.7 macrophages was performed by a guanidinium-isothiocyanate-phenol-chloroform-based extraction method exactly as described [60]. Quantification of mRNA expression was carried out by quantitative real-time PCR following reverse transcription exactly as described [61]. Amplifications conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 60 s. The following primers and TaqMan probes were used: muTfR1: 5′-CGCTTTGGGTGCTGGTG-3′, 5′-GGGCAAGTTTCAACAGAAGACC-3′, 5′-CCCACACTGGACTTCGCCGCA-3′, muDmt1: 5′-CCAGCCAGTAAGTTCAAGGATCC-3′, 5′-GCGTAGCAGCTGATCTGGG-3′, 5′-TGGCCTCGCGCCCCAACA-3′, muFpn1: 5′-CTACCATTAGAAGGATTGACCAGCT-3′, 5′-CAAATGTCATAATCTGGCCGA-3′, 5′-CAACATCCTGGCCCCCATGGC-3′, muLcn2: 5′-GCCTCAAGGACGACAACATCA-3′, 5′-TTCTCTGTCCCCACCGACCAATGC-3′, 5′-CACCACCCATTCAGTTGTCAAT-3′, muHamp: 5′-TGTCTCCTGCTTCTCCTCCTTG-3′, 5′-CAGCCTGAGCAGCACCACCTATCTCC-3′, 5′-AGCTCTGTAGTCTGTCTCATCTGTTGA-3′, muHfe: 5′-TCATGAGAGTCGCCGTGCT-3′, 5′-AGCCCAGGGCCCCGTGGAT-3′, 5′-TGGCTTGAGGTTTGCTCCA-3′, muHmox1: 5′-GTGATGGAGCGTCCACAGC-3′, 5′-TGGTGGCCTCCTTCAAGG-3′, 5′-CGACAGCATGCCCCAGGATTTGTC-3′, muH-Ferritin: 5′-GCGAGGTGGCCGAATCT-3′, 5′-CAGCCCGCTCTCCCAGT-3′, 5′-CCTGCAGGATATAAAGAAACCAGACCGTGA-3′, muL-Ferritin: 5′-GAAACTCATCAAGAAGATGGGCA-3′, 5′-TGGTTGTGGCCCCGC-3′, 5′-CCATCTGACCAACCTCCGCAGGGT-3′, 18S rRNA: 5′-CCTGCCCTTTGTACACACCG-3′, 5′-CGATCCGAGGGCCTCAC-3′, 5′-CCGTCGCTACTACCGATTGGATGGTTT-3′.

### Immunoblot analysis

Protein extracts from RAW264.7 cells were prepared using the radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) supplemented with 1 μg/mL aprotinin and 1 μg/mL leupeptin (all from Sigma-Aldrich). 8–30 μg of total protein were run on 10–15% SDS-polyacrylamide gels and immunoblotting was performed exactly as described [62] using a mouse anti-human TfR1 antibody (0.5 mg/mL, Zymed), a rabbit anti-human ferritin

 

antibody (1 μg/mL, Dako), a rabbit anti-human ferroportin 1 antibody (1:250; kindly provided by Andrew McKie, Kings College, London, UK), a rat anti-mouse Lcn2 antibody (1:400, R&D Systems), a rabbit anti-rat Hmox1 (1:2000, Stressgen) or a rabbit anti-Actin antibody (1 μg/mL, Sigma-Aldrich), the latter used as a loading control.

### Gel-retardation assay

Protein extracts for gel retardation assays were prepared using cytoplasmic lysis buffer (25 mM Tris-HCl pH 7.4, 40 mMKCl, 1% Triton X-100) containing 1 μg/mL aprotinin and 1 μg/mL leupeptin (all from Sigma-Aldrich). For investigation of IRP binding activity, a $^{32}$P-labeled IRE probe was prepared by an *in vitro* transcription procedure exactly as described [63] and the analysis of RNA/protein complexes was carried out by non-denaturing gel electrophoresis. The 2-mercaptoethanol (2%) was used for *in vitro* stimulation of IRP1-binding activity to ensure for equal loading of protein extracts [64].

### Quantification of the intracellular iron content

The intracellular iron content of RAW264.7 macrophage-like cells was determined by means of Graphite furnace atomic absorption spectrometry (GFAAS), carried out as described [65] on a Unicam Model Solaar, 939 QZ, atomic-absorption spectrometer using extended lifetime graphite tubes (Thermo Electron Corporation). Cells were treated exactly as described above, washed three times in normal saline and centrifuged at 300 × *g* for 5 min. Cell pellets were suspended by ultrasonication for 40 s in 0.1% ultrapure nitric acid and 0.2% Triton-X100. A pyrolysis temperature of 1000°C and an atomization temperature of 2000°C were used. Values were normalized for total cellular protein content as measured by a bicinchoninic acid-based protein assay kit (Pierce).

### Quantification of iron uptake, storage and release by macrophages

For macrophage iron uptake and release studies, RAW264.7 cells were infected with *S. typhimurium* as detailed above. Following three washing cycles with serum-free, HEPES-buffered DMEM (2 mM L-glutamine, 25 mM HEPES, pH 7.4) cells were then incubated therein. For determination of TBI acquisition, $^{59}$Fe-labeled transferrin was added at a concentration of 12.5 μg/mL, whereas 5 μM $^{59}$Fe-citrate (DuPont New England Nuclear) was used to investigate the uptake of NTBI exactly as described [38]. For iron release experiments, cells were first incubated with 5 μM $^{59}$Fe-citrate for 4 h to allow for iron loading and then washed four times with and repleted with 2 mL of serum-free, HEPES-buffered DMEM. After an additional incubation for 2 h, cellular iron release was measured by means of a γ-counter as described [38]. In parallel to each iron-release study, a trypan-blue-exclusion assay was performed to ensure that neither treatment interfered with the integrity of the RAW264.7 cell surface membrane. For investigation of iron incorporation into the ferritin shell, RAW264.7 cells were infected with *S. typhimurium* as described above and incubated in 5 μM $^{59}$Fe-citrate for additional 23 h. Thereafter, cells were washed three times; protein extracts were prepared using the RIPA buffer and separated on 8% SDS polyacrylamide gels. The $^{59}$Fe incorporation into ferritin multimers was then determined by autoradiography of the gels, on which $^{59}$Fe-ferritin complexes had migrated with an apparent molecular mass of approximately 450 kDa.

### Determination of iron acquisition by *S. typhimurium*

RAW264.7 cells were seeded in complete DMEM and infected with *S. typhimurium* strains C5RP4 (wild-type), H5542 (wild-type) or H5709 (*entC*-deficient) at a MOI of 10 for 24 h as detailed above. In separate experiments, primary peritoneal macrophages were isolated and infected with *S. typhimurium* strains H5542 or H5709 at a MOI of 10 for 24 h. Thereafter, cells were washed three times and repleted with serum-free, HEPES-buffered DMEM. After the addition of either 12.5 μg/mL of $^{59}$Fe-labeled transferrin or 5 μM $^{59}$Fe-citrate, macrophages were incubated for additional 4 h. In parallel, the same number of *S. typhimurium* was suspended in serum-free, HEPES-buffered DMEM and incubated for 20 min with 10 μM $^{59}$Fe-citrate to determine their acquisition of radioactive iron in the absence of macrophages. Intracellular or suspended bacilli were harvested according to a modified protocol as described [66]. Briefly, RAW264.7 cells or primary peritoneal macrophages, respectively, were lysed in 0.1% SDS in the presence of 10 000 U/mL DNase-I (Invitrogen) and EDTA-free protease inhibitor (Roche). Cell lysates were withdrawn into microfuge tubes and incubated at room temperature for 10 min; aliquots of 50-μL volume were transferred into γ-counter tubes for assessment of total iron in the macrophage lysate. The released bacilli were centrifuged at 10 000 × *g* for 10 min at room temperature. The supernatant was removed, and the bacterial pellet was washed three times with 0.01% SDS in DMEM containing 1 mg/mL proteinase K (Roche). The bacilli were finally resuspended in 500 μL of 0.01% SDS in DMEM containing 1 mg/mL proteinase K. Of the bacterial suspension, an aliquot of 50 μL was plated in serial dilutions onto agar plates to quantify released bacteria while the remaining volume was filtered through centrifugal filter devices with a PDVF membrane of 0.22 μm pore size (Millipore). The filters containing the trapped bacilli were washed three times, cut into pieces and placed into a γ-counter tube. The amount of *Salmonella*-associated $^{59}$Fe was assessed using a γ-counter. No association of $^{59}$Fe to *S. typhimurium,* heat-inactivated at 70°C for 20 min, could be detected.

 

### Detection of TNF-α, nitrite and total oxidative capacity

Concentrations of TNF-α, IL-1ß, IL-6 and IL-10 in cell culture supernatants were determined by specific ELISA sets (BD PharMingen). Determination of nitrite, an oxidation product of NO, and thus a surrogate parameter for the production of RNS was carried out with the Griess-Ilosvay's nitrite reagent (Merck) exactly as described [67]. The formation of ROS was studied by measuring the TOC in culture supernatants using the TOC Kit (Tatzber), which is based on the peroxidase-mediated oxidation of tetramethylbenzidine [68].

### Densitometric quantification

For quantification of band intensities, X-ray films (Amersham), exposed to immunoblotting membranes or retardation-assay gels, were scanned using a Fluor-S Multi-Imager and Quantity One software package (Bio-Rad).

### Statistical analysis

Statistical analysis was carried out using a SPSS statistical package. Calculations for statistical differences were carried out by ANOVA test using Bonferroni correction for multiple tests.

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*Abbreviations:* **Dmt:** divalent metal transporter 1 · **Fpn1:** ferroportin 1 · **Hamp:** hepcidin antimicrobial peptide · **Hmox1:** haem oxygenase 1 · **IRE:** iron responsive element · **IRP:** iron regulatory protein · **Lcn2:** lipocalin 2 · **NTBI:** non-transferrin-bound iron · **RNS:** reactive nitrogen species · **TBI:** transferrin-bound iron · **TfR1:** transferrin receptor 1 · **TOC:** total oxidative capacity

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