Heme oxygenase 1 controls early innate immune response of macrophages to *Salmonella* Typhimurium infection

Anna-Maria Mitterstiller[1], **David Haschka**[1], **Stefanie Dichtl**[1], **Manfred Nairz**[1], **Egon Demetz**[1], **Heribert Talasz**[3], **Miguel P. Soares**[5], **Elisa Einwallner**[6], **Harald Esterbauer**[6], **Ferric C. Fang**[4], **Stephan Geley**[2], and **Guenter Weiss**[1,*]

[1]Department of Internal Medicine VI, Infectious Diseases, Immunology, Rheumatology, Pneumology, Medical University of Innsbruck, 6020 Innsbruck, Austria.

[2]Division of Molecular Pathophysiology, Medical University of Innsbruck, 6020 Innsbruck, Austria.

[3]Division of Clinical Biochemistry, Medical University of Innsbruck, 6020 Innsbruck, Austria.

[4]University of Washington School of Medicine, 1959 NE Pacific Street, Seattle, WA, 98195-7735, USA.

[5]Instituto Gulbenkian de Ciência, 2780-156 Oeiras, Portugal.

[6]Department of Laboratory Medicine, Medical University of Vienna, 1090 Vienna, Austria.

Summary

Macrophages are central for the immune control of intracellular microbes. Heme oxygenase 1 (HO-1, *hmox)* is the first and rate limiting enzyme in the breakdown of heme originating from degraded senescent erythrocytes and heme-proteins, yielding equal amounts of iron, carbon monoxide and biliverdin. HO-1 is strongly up-regulated in macrophages in response to inflammatory signals, including bacterial endotoxin. In view of the essential role of iron for the growth and proliferation of intracellular bacteria along with known effects of the metal on innate immune function, we examined whether HO-1 plays a role in the control of infection with the intracellular bacterium *Salmonella* Typhimurium. We studied the course of infection in stably-transfected murine macrophages (RAW264.7) bearing a tetracycline-inducible plasmid producing *hmox* shRNA and in primary HO-1 knockout macrophages. While uptake of bacteria into macrophages was not affected, a significantly reduced survival of intracellular *Salmonella* was observed upon *hmox* knockdown or pharmacological *hmox* inhibition, which was independent of Nramp1 functionality. This could be traced to limitation of iron availability for intramacrophage bacteria along with enhanced stimulation of innate immune effector pathways, including the formation of reactive oxygen and nitrogen species and increased TNF-α expression. Mechanistically, these latter effects result from intracellular iron limitation with subsequent activation of NF-κB and further *inos, tnfa* and *p47phox* transcription along with reduced formation of the anti-inflammatory and radical scavenging molecules, CO and biliverdin as a consequence of HO-1 silencing.

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*For correspondence: guenter.weiss@i-med.ac.at; Tel.: +43(0)512 504 25856; Fax.: +43(0)512 504 25857.

Supporting Information
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Taken together our data provide novel evidence that the infection-driven induction of HO-1 exerts detrimental effects in the early control of *Salmonella* infection, whereas *hmox* inhibition can favourably modulate anti-bacterial immune effector pathways of macrophages and promote bacterial elimination.

Introduction

Macrophages play a central role in innate immune responses towards intracellular pathogens such as *Mycobacteria* and *Salmonella.* Through the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), macrophages eliminate pathogens (MacMicking *et al.,* 1997; Mastroeni *et al.,* 2000; Shiloh and Nathan, 2000; Vazquez-Torres *et al.,* 2000; Bogdan, 2001; Weiss and Schaible, 2015). High output formation of nitric oxide (NO) is due to induction of inducible NO-synthase (iNOS or NOS2, *inos)* by cytokines and bacterial products, and the formation of ROS is mediated by NADPH-oxidase (PHOX or NOX, *phox),* consisting of five subunits. Phox generates bactericidal ROS such as oxygen radicals, H_2O_2 or hydroxyl radicals (Nathan and Shiloh, 2000; Bogdan, 2001; Weiss and Schaible, 2015). While iron can aggregate formation of ROS by the Fenton reaction (Rosen *et al.,* 1995) it also plays a decisive role in host-pathogen interactions (Schaible and Kaufmann, 2004; Ganz, 2009; Nairz *et al.,* 2010; Cassat and Skaar, 2013; Nairz *et al.,* 2014). On one hand, iron is an essential nutrient for many microbes, which have developed multiple pathways to acquire this metal from their environment. Sufficient availability of iron is linked to microbial growth and pathogenicity, whereas iron limitation negatively impacts their proliferation (Kaplan, 2002; Olakanmi *et al.,* 2002; Frawley *et al.,* 2013). On the other hand, iron plays an essential role in the proliferation and differentiation of immune cells and exerts direct effects on innate immune function (Nairz *et al.,* 2014). The latter can be attributed to a negative regulatory effect of iron on interferon-gamma (IFN-γ) -inducible immune effector pathways in macrophages, including iNOS and tumour necrosis factor alpha (TNF-α) formation (Oexle *et al.,* 2003; Nairz *et al.,* 2014). Consequently, competitive interactions between macrophage iron homeostasis and microbial iron acquisition systems form a central frontline that defines the course of infection (Schaible and Kaufmann, 2004; Weinberg, 2009; Nairz *et al.,* 2010).

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a facultative intracellular Gram-negative bacterium that can cause fatal septicemia. *Salmonella* pathogenicity depends on the organism's ability to invade macrophages, thus taking advantage of these cells as a habitat for multiplication and spreading within the host (Leung and Finlay, 1991; Andrews-Polymenis *et al.,* 2010; Lahiri *et al.,* 2010). The intracellular replication of *Salmonella* is highly dependent on a sufficient iron supply (Nairz *et al.,* 2007; Andrews-Polymenis *et al.,* 2010), while a restricted availability of iron in macrophages is associated with impaired proliferation of intracellular bacteria within these cells (Schaible and Kaufmann, 2004; Nairz *et al.,* 2009).

Iron homeostasis within macrophages is regulated at multiple steps and significantly modified upon infection and inflammation (Nairz *et al.,* 2014). Macrophages play a crucial role for body iron homeostasis as they take up and degrade senescent erythrocytes and re-

utilize iron, which accounts for approximately 90% of the daily needs of the metal, mainly used for erythropoiesis (Hentze *et al.,* 2010; Pantopoulos *et al.,* 2012). The enzyme heme oxygenase 1 (HO-1, *hmox)* is of central importance in this process because it degrades heme to yield equal amounts of iron, carbon monoxide and biliverdin (Tenhunen *et al.,* 1968; Ryter and Tyrrell, 2000). Apart from erythrocyte phagocytosis, macrophages can acquire transferrin bound iron via transferrin-receptor 1 (TfR1)-mediated endocytosis or ferrous iron via the divalent metal transporter 1 (Dmt1, Slc11a2). Importantly there is only one well-characterized iron export pathway, involving the transmembrane protein ferroportin (Fpn1, Slc40a1), which transports ferrous iron to the extracellular space where it is oxidized and incorporated into apo-transferrin (Tf) (Hentze *et al.,* 2010; Pantopoulos *et al.,* 2012). The expression of this crucial mediator of iron homeostasis is significantly affected during infection by inflammatory cytokines and their secondary products, both at the transcriptional and the post-transcriptional level (Mulero and Brock, 1999; Ludwiczek *et al.,* 2003). However, the activation of regulatory networks may differ depending on the cellular localization of a microbe (Drakesmith and Prentice, 2012; Fang and Weiss, 2014; Nairz *et al.,* 2014). The metabolic response to extracellular pathogens is considered to cause iron retention in macrophages. This is largely exerted by inflammation-triggered formation of hepcidin and subsequent degradation of Fpn1 on the cell surface, resulting in blockade of macrophage iron egress (Nemeth *et al.,* 2004; Theurl *et al.,* 2008; Ganz, 2009; Armitage *et al.,* 2011; Arezes *et al.,* 2015). In contrast, macrophage challenge with intracellular bacteria, such as *Salmonella,* induces the transcription of *fpn1,* resulting in stimulation of iron export and reduction of intracellular iron concentrations (Nairz *et al.,* 2007; Van Zandt *et al.,* 2008; Ward and Kaplan, 2012; Nairz *et al.,* 2013; Nairz *et al.,* 2015).

Immune modulatory functions have been observed for the three degradation products of heme catabolism, namely iron, carbon monoxide and biliverdin (reviewed by (Ryter *et al.,* 2006; Soares and Weiss, 2015; Wegiel *et al.,* 2015)), thereby linking putative HO-1 cytoprotective properties to the modulation of intracellular iron levels (Ferris *et al.,* 1999). Furthermore, although HO-1 has been implicated in tolerance of malaria and treated bacterial infections with amelioration of tissue damage (Larsen *et al.,* 2010; Gozzelino *et al.,* 2012), the role of HO-1 for the control of intracellular bacterial infections has not been clearly established. We hypothesize, that HO-1 may affect iron availability for intracellular pathogens, thereby influencing the course of infection. Our results provide novel evidence for a direct involvement of HO-1 in host interactions with the intracellular bacterium *S.* Typhimurium.

Results

Intramacrophage bacterial replication is augmented by heme oxygenase 1

We first studied the impact of reduced heme oxygenase activity on intracellular bacterial counts in RAW264.7 cells infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 10. The number of *Salmonella* colony forming units (CFUs) recovered from macrophages after 24 h of infection was significantly reduced following pre-treatment with the HO-1 Inhibitor zinc (II) protoporphyrin IX (ZnPP, 10 μM) as compared to the solvent-treated control cells (Fig. 1A). To rule out off-target effects of ZnPP we generated RAW264.7 cells

stably expressing doxycycline inducible *hmox* or *luciferase* control shRNA (RAW.sh; hmox.sh; ctrl.sh). Doxycycline induction caused an overall reduction of *hmox* mRNA expression of 80 to 90%. Infection of these cells with *S*. Typhimurium resulted in significant and time-dependent decrease of bacterial numbers within macrophages as compared to macrophages stable transfected with control shRNA expressing luciferase (Fig. 1D). To verify these findings in primary cells, we studied bone marrow derived macrophages (BMDM) obtained from macrophage specific HO-1 knockout mice (LysM-Cre$^{+/+}$HO-1$^{fl/fl}$ and control LysM-Cre$^{-/-}$HO-1$^{fl/fl}$) (Fig. 1F). Following infection with S. Typhimurium we observed significantly better control of intracellular bacterial numbers in macrophage specific HO-1 knockout cells (BMDM) compared with control cells, which confirmed the phenotype observed in RAW.sh cells. This observation could not be attributed to differences in *Salmonella* uptake or containment because intramacrophage bacterial numbers were the same in *hmox* knockdown and control macrophages as well as in primary wild type and HO-1 knockout bone marrow derived macrophages at 1 h after infection (Fig. 1B, C and G).

Based on the fact that *hmox* activity increases the intracellular concentration of molecular iron and because *Salmonella* are highly dependent on a sufficient supply of iron, we studied the effects of *hmox* silencing on infection with a *Salmonella* strain (Fig. 1E) that is deficient in enterobactin synthesis, sitABCD- and feo-mediated iron uptake (*S*. mt; *entC sit feo*) (Crouch *et al.,* 2008). Although, the differences in bacterial counts between ctrl.sh and hmox.sh macrophages were less prominent than during infection with wt *Salmonella,* intramacrophage numbers of mutant *Salmonella* were still significantly lower upon *hmox* silencing than in wt macrophages. This indicated that bacterial iron restriction contributes to impaired bacterial proliferation upon *hmox* knockdown but is not exclusively responsible for the observed protective effect.

Effects of hmox silencing on macrophage iron homeostasis and bacterial iron acquisition

We subsequently studied the putative changes in macrophage iron homeostasis and expression of central iron transport genes in the absence/presence of *hmox* and in response to *S*. Typhimurium infection. The iron acquisition genes, transferrin receptor 1 (TfR1, *tfr1)* and divalent metal transporter 1 (Dmt1, *dmt1*), showed no difference in expression between uninfected hmox.sh and ctrl.sh macrophages. However, we observed a significant increase in *tfr1* and *dmt1* expression following infection with *S*. Typhimurium that was significantly higher in hmox. sh than in ctrl.sh cells (Fig. 2A and B). These changes were paralleled by a significant increase in transferrin- bound iron (TBI) and non-transferrin bound iron (NTBI) uptake by uninfected and infected hmox.sh cells as compared to ctrl.sh. (Fig. 2D and E). Increased cellular iron export (Fig. 2C and F) was observed in *Salmonella* infected hmox.sh macrophages 20 h post-infection, although cytoplasmic ferroportin *(fpn1)* mRNA levels were reduced at this time. Importantly, the enhanced iron export corresponded to increased Fpn1 protein levels in macrophages infected with *Salmonella,* as determined by Western Blot analysis (Fig. 2 G). In addition, the increased TfR1 and reduced ferritin protein levels in *hmox* knockdown macrophages at baseline and upon *Salmonella* infection are consistent with reduced intracellular iron concentrations as a consequence of increased iron export (Fig. 2G).

To gain further insights into the dynamics of cellular iron trafficking, we determined total intracellular iron concentrations via atomic absorption spectrometry (Fig. 2H). We found that hmox.sh phagocytes exhibited significantly lower iron contents following infection compared to infected control cells, which was also true when the infection was performed with a *Salmonella* mutant strain deficient in iron acquisition (Fig. 2H). We then assessed the effects of *hmox* knockdown on iron incorporation by engulfed bacteria. Remarkably, the high iron flux observed in *hmox* knockdown cells resulted in a significant reduction in iron acquisition by intracellular *Salmonella* as compared to *Salmonella* in ctrl.sh macrophages. The *Salmonella* triple mutant strain acquired significantly less iron than wt *Salmonella* (Fig. 2I).

Furthermore we wanted to examine whether or not the expression of the phagolysosomal protein Nramp1 (Slc11a1) is critical for the observed *hmox* mediated effects. Nramp1 expression confers resistance to a number of intracellular pathogens and mediates iron efflux into the cytoplasm along with the amelioration of a number of antimicrobial functions (Cellier *et al.,* 2007; Fritsche *et al.,* 2012). To study this issue we used RAW 264.7 macrophages which are stably transfected with a functional or non-functional Nramp1. As described in the literature the intracellular multiplication of *Salmonella* was significantly lower in RAW cells expressing the functional Nramp1 than in macrophages lacking Nramp1 activity (Cellier *et al,* 2007; Fritsche *et al,* 2012). However, blockade of HO-1 activity by ZnPP resulted in comparable reductions of bacterial numbers in both cell types suggesting that the growth promoting effect of HO-1 towards intramacrophage *Salmonella* is independent of Nramp1 functionality (Figure S2 B).

Impact of HO-1 on macrophage effector functions

We then studied whether *hmox* knockdown impacts the efficacy of macrophage immune effector functions in the course of *Salmonella* infection, either via the observed alterations in macrophage iron transport pathways or by other mechanisms. We found that silencing of *hmox* was paralleled by a significant time-dependent induction of *p47* phagocyte oxidase *(p47phoX)* and inducible nitric oxide synthase *(nos2)* mRNA expression as compared to infected control macrophages (Fig. 3A and B). The expression of *p47phox* declined over time, whereas *nos2* expression progressively increased, reflecting the varying importance of these two radical forming genes at early and later stages of infection respectively (Mastroeni *et al.,* 2000). However, at all time points, both genes were significantly higher expressed in hmox.sh than in control. sh cells (Fig. 3A and B). Furthermore, tumour necrosis factor alpha *(tnfa)* and interleukin 10 *(il10)* mRNA expression increased in both infected cell types. However, whereas *tnfa* formation was significantly higher in hmox.sh macrophages at early time points of infection and then declined, no significant differences in *il10* expression were observed between infected hmox.sh and ctrl.sh macrophages (Fig. 3D and E). Importantly, upon infection with *Salmonella, hmox* knockdown macrophages expressed significantly higher levels of the anti-microbial siderophore scavenging peptide lipocalin 2 *(lcn2).* Of note, in comparison to control macrophages an increased expression of *tnfa, il6* and *lcn2* was also seen in *hmox* knockdown cells upon treatment with either heat-inactivated *Salmonella* (Fig. 4) or lipopolysaccharide (data not shown), pointing to a general and early immune de-activating effect of *hmox* in inflammatory macrophages.

Notably, *hmox1* knockdown in the cells was not compensated by an increase of *hmox2* expression (Supplemental Figure S3 B).

In accordance with the transcriptional analyses, the concentrations of TNF-α, IL-6 and Lcn2 were significantly increased in culture supernatants of hmox.sh cells infected with *Salmonella* at 20 h post infection as compared to infected control.sh cells (Fig. 5A–C), whereas IL-10 concentrations did not differ between ctrl. sh and hmox.sh macrophages (Figs 5D and 4).

We then studied whether *hmox* knockdown macrophages exhibit better pathogen control because of increased reactive oxygen (ROS) and RNS production, as both *p47phox* and inducible *nos2* expression were significantly increased in such infected cells.

Salmonella-infected hmox.sh and control.sh macrophages were supplemented with the membrane-permeable compound N-acetyl-L-cysteine (NAC), known to quench ROS (Victor *et al.,* 2003). NAC treatment significantly augmented the bacterial load of hmox.sh and control.sh macrophages to a comparable level (Fig. 6A). This suggested that the effects of *hmox* on macrophage antimicrobial activity are dependent on oxidative stress. To further assess ROS production, cells were assayed 30 min and 3 h post infection for oxyradical generation. *Salmonella-infected* hmox.sh cells produced significantly more ROS in comparison to the analogously treated crtl.sh macrophages (Fig. 6B). Notably, the production of RNS, measured via the concentration of the stable NO end-product nitrite in culture supernatants, was also increased in hmox.sh macrophages (Fig. 5E). To examine the possibility that higher ROS and cytokine production or heme accumulation may cause increased cell death in *hmox* deficient cells, we performed measurements of lactate dehydrogenase activity as a surrogate for cytotoxicity. Of note, we found no significant differences between *hmox* deficient (hmox.sh) and control cells with or without *Salmonella* infection (Supplementary Figure S3 A)

Improved *Salmonella* control by *hmox* macrophages involves NF-κB activation

NF-κB is an important regulator of inflammatory responses, including the production of TNF-α. Moreover, it is linked to *hmox* gene regulation. Quantification of the specific DNA binding activity to the NF-κB subunit p65 revealed significantly increased p65 binding activity in the *hmox* knockdown cells infected with *Salmonella* Typhimurium compared to infected ctrl.sh cells (Fig. 7A). Western blot experiment data demonstrated that hmox.sh macrophages exhibit higher phosphorylation of the NF-κB p65 subunit in comparison to crtl.sh macrophages (Fig. 7B).

Impact of heme catabolites on bacterial survival and immune regulation

Induction of HO-1 and the degradation products of heme catabolism were shown to exert potent cytoprotective effects and homeostatic actions in preclinical models of inflammatory disorders such as sepsis, colitis and ischaemia-reperfusion injury (Otterbein *et al.,* 2000; Yet *et al.,* 2001; Hegazi *et al.,* 2005; Chung *et al.,* 2008). We questioned whether the lack of one of these products from the HO-1 catalysis might possibly be responsible for the improved bacterial killing found in the *hmox* knockdown macrophages. Therefore, we exposed *Salmonella* infected cells to iron (ferric ammonium citrate (FAC) as iron source), the CO-

releasing agent CORM-2 or bilirubin and examined the bacterial load along with ROS production after this treatment (Supplemental Figure S1). Iron supplementation significantly increased the bacterial load in both, *hmox* knockdown and ctrl.sh macrophages thereby increasing the intramacrophage bacterial numbers in hmox.sh phagocytes to that observed in control cells. Moreover, CORM-2 treatment significantly reduced bacterial numbers in both cells types (Figure S1 A), but the differences between control and *hmox* knockdown cells remained evident. Addition of bilirubin did not change bacterial numbers in ctrl.sh cells but increased those in hmox.sh macrophages resulting in an insignificant change of bacterial numbers (Figure S1 A). ROS levels were significantly higher in *hmox* knockdown than in control cells, which was not significantly altered in the presence of CORM-2 or bilirubin (Fig. S1 B).

Discussion

This study provides evidence that *hmox* gene knockdown results in improved control of infection with intracellular bacteria. These can be attributed to impaired bacterial survival, mainly as a consequence of iron limitation for intramacrophage microbes along with stimulation of antimicrobial immune effector pathways including radical formation.

Specifically, our data show that *hmox* depletion causes an induction of Fpn1-mediated iron efflux (Fig. 2C, E) which limits the availability of iron for *Salmonella*. The efficacy of this Fpn1-mediated pathway to restrict intracellular bacteria growth has been shown in different *in vitro* bacterial models employing *Mycobacteria, Chlamydia, Listeria* and *Salmonella* (Chlosta *et al.,* 2006; Nairz *et al.,* 2007; Paradkar *et al.,* 2008; Van Zandt *et al.,* 2008; Bellmann-Weiler *et al.,* 2010; Velayudhan *et al.,* 2014; Haschka *et al.,* 2015). The increase in Fpn1 expression may be linked to enhanced NO formation as described recently (Nairz *et al.,* 2013) whereas a reduced expression of hepcidin in hmox.sh cells may contribute to Fpn1 stabilization on the cell surface.

In addition, *Salmonella* cannot utilize heme and thus requires the *hmox* breakdown product iron as a growth factor (Andrews-Polymenis *et al.,* 2010). Therefore, *hmox* knockdown limits bacterial iron availability. Further, bacterial iron limitation in *hmox*-silenced macrophages is further promoted via increased production of *Lcn2* which binds and neutralizes bacterial siderophores (Flo *et al.,* 2004; Berger *et al.,* 2006). Moreover, Lcn2 mediates shuttling of iron across cell membranes (Nairz *et al.,* 2009; Liu *et al.,* 2014; Nairz *et al.,* 2015) which is most likely linked to its capacity to bind a mammalian siderophore (Bao *et al.,* 2010; Liu *et al.,* 2014).

Reduction in intracellular iron concentrations has been shown to promote the production of pro-inflammatory molecules such as TNF-α, IL-6, iNOS *(nos2)* or CCL-20 by activated macrophages (Weiss *et al.,* 1994; Melillo *et al.,* 1997; Ludwiczek *et al.,* 2003; Oexle *et al.,* 2003; Varesio *et al.,* 2010), and these pathways are of importance for the control of intracellular pathogens like *Salmonella* (Andrews- Polymenis *et al.,* 2010; Weiss and Schaible, 2015).

Our results, demonstrating increased *p47phox* expression and ROS formation, are in line with a previous observation reporting enhanced ROS formation in HO-1 knock out cells (Orozco *et al.,* 2007). The oxidative burst is a central defence mechanism against intracellular pathogens, including *Salmonella* (Mastroeni *et al.,* 2000; Vazquez-Torres *et al.,* 2000). Thus, increased ROS and RNS production resulting from *hmox* knockdown enhances immune control of *Salmonella.* In addition, TNF-α produced in larger quantities as a result of *hmox* silencing further contributes to RNS and ROS production because of the co-activation of *inos* and *p47phox* expression (Ables *et al.,* 2001). This leads to the question regarding the mechanism underlying increased expression of these immune response genes.

We detected increased NF-κB activity in association with improved anti-microbial immune responses in hmox.sh macrophages, which is consistent with NF-kB as a central regulator of inflammatory responses (Tak and Firestein, 2001). Intracellular free or labile iron has been observed to play an important role in stress signalling by NF-κB and is also associated with the expression of HO-1 (Varesio *et al.,* 2010; Evstatiev and Gasche, 2012).

However, *hmox* knockdown macrophages have low iron content and reduced iron pools at the same time expressing significantly higher levels of NF-κB than the corresponding control cells. It might be speculated that labile iron molecules (Wessling-Resnick, 2010) which become abundant in *hmox* knockdown macrophages before being exported from these cells may activate NF-κB via Fenton chemistry mediated radical production. Alternatively, NF-κB activation may be a consequence of an increased oxidative burst, which is a regulator of NF-κB activation in inflammatory macrophages (Karin and Greten, 2005; Gloire *et al.,* 2006; DiDonato *et al.,* 2012). This is consistent with our findings of increased NADPH oxidase *(p47phox)* expression, the molecular source of ROS in response to LPS treatment (Gloire *et al.,* 2006). In addition, the reduced abundance of CO and bilirubin, two end-products of the HO-1 pathway, further contributes to increased or prolonged oxidative stress, as these molecules have radical scavenging capacities and anti-inflammatory activities (Gozzelino *et al.,* 2010).

Moreover, consistent with our observation that NAC treatment results in increased bacterial numbers and a reduction of TNF-α formation in *hmox* knockdown macrophages during *Salmonella* infection, NAC can prevent LPS-induced NF-κB activation and the production of inflammatory cytokines (Gloire *et al.,* 2006). These observations suggest that NF-κB is activated because of a lack of HO-1 derived anti-inflammatory molecules (CO, bilirubin) and is consistent with previous findings showing that the CO releasing compound, CORM-2, inhibits the DNA binding activity of NF-κB resulting in reduced TNFα production (Sawle *et al.,* 2005; Megias *et al.,* 2007; Motterlini *et al.,* 2012). Moreover, regulation of iron metabolism by HO-1 inhibits NF-κB activation via a mechanism that targets specifically the phosphorylation of its RelA/p65 subunit (Seldon *et al.,* 2007).

Of note, CO was found to promote maturation of myeloid cells needed to sense and kill bacteria (Wegiel *et al.,* 2014). Furthermore CO modulates the response of macrophages to bacterial LPS (Otterbein *et al.,* 2000), thereby decreasing macrophage Toll-like receptor signalling with downstream activation of NF-κB (Wang *et al.,* 2009) and promoting bacterial clearance together with HO-1 (Onyiah *et al.,* 2013). Here we noted that the CO releasing

agent CORM-2 reduced intramacrophage numbers of *Salmonella* Typhimurium in ctrl.sh and *hmox* knockdown cells. This is in a line with previously described bactericidal properties of CORMs against several pathogens including E. *coli, Staphylococcus aureus* and *Pseudomonas aeruginosa (Desmard et al.,* 2009; *Nobre et al.,* 2009; *Tavares et al.,* 2011).

It is important to note, that HO-1 appears to have a dual role in infections. While our observations demonstrate that HO-1 exerts detrimental effects during the early control of intramacrophage bacteria like *Salmonella*, other investigators have found that HO-1 contributes to improved control of *Salmonella* replication within macrophages by exerting cytoprotective effects (Zaki *et al.,* 2009) and prevents macrophages from programmed cell death during *Myco-bacterium tuberculosis* infection (Silva-Gomes *et al.,* 2013). The dual role of HO-1 may thus become most evident in the course of persisting infections. Its downregulation appears to be beneficial for the immediate control of infection with intracellular pathogens, but in non-resolving infection HO-1 expression at later stages may be necessary to reduce inflammation driven tissue damage.

Up-regulation of HO-1 during malaria or sepsis was demonstrated in association with infection tolerance and limitation of tissue damage resulting in an improved survival (Gozzelino *et al.,* 2010; Larsen *et al.,* 2010). On the other side of the coin, HO-1 expression is associated with increased susceptibility to *Salmonella* bacteremia during malaria infection, and increased mortality from *Salmonella* infection in that setting (Cunnington *et al.,* 2012). Furthermore, a recent study of *Mycobacteria abscessus* found that inhibition of HO-1, especially at the early stages of infection, limits intracellular bacterial numbers (Abdalla *et al.,* 2015).

Our study adds another piece to the puzzle concerning the role of HO-1 in infection. Although HO-1 may be detrimental for the host and beneficial for the pathogen in the early stages of *Salmonella* infection, HO-1 subsequently promotes disease tolerance via cytoprotective effects and the prevention of macrophage apoptosis.

In summary (Fig. 8), the data presented here provide new mechanistic insights into the role of HO-1 in early *Salmonella* infection, emphasising its capacity to modulate anti-bacterial immune effector pathways and cellular iron homeostasis. The current study shows that *hmox* knockdown in macrophages limits bacterial replication by reducing intracellular iron pools and subsequent bacterial iron incorporation along with stimulation of anti-microbial immune effector mechanisms.

Of importance, our data allow us to speculate that during infection, cells with low HO-1 expression may be more resistant to infection with *Salmonella* than macrophages that strongly induce HO-1 as a consequence of iron recycling. This goes along with the observation that hemophagocytic macrophages, which express high levels of HO-1, provide a survival niche for *Salmonella* (Nix *et al.,* 2007). Accordingly, infection with *Salmonella* leads to increased HO-1 expression in host cells, which may be seen as an attempt of the pathogen to weaken host-derived anti-microbial effector pathways and to secure a sufficient supply of iron.

Experimental procedures

Vectors for gene knockdown

Complementary shRNA oligonucleotides directed against either *hmox1* or luciferase and containing *BamHI* and *HindIII* sticky ends (hmox1_1.sh 5′-GATCCCC-caagcagaacccagtctatTTCAAGAGAatagactgggttctgcttgTTTTTG-GAAA-3′ and 5′-AGCTTTTCCAAAAAcaagcagaacccagtctatTTCAAGAGAatagactgggttctgcttgGGG-3′ or hmox1_2.sh 5′-GATCCCCcaacagtggcagtgggaatTTCAAGAGAattcccactgccactgttgTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAcaacagtggcagtgggaat-TTCAAGAGAattcccactgccactgttgGGG-3′ (the sense target sequence is underlined; synthesized by MWG Ebersberg, Germany) were annealed and cloned into *BglII-HindIII* sites of the digested, dephosphorylated pENTR-THT-III. This GATEWAY-compatible vector contains a tetracycline-sensitive H1 promoter (THT) that controls the expression of the shRNA. The THT-shRNA cassette was recombined into the lentiviral RNAi destination vectors for conditional knockdown. The pGLTR-X-FP (encoding TetR-T2A-GFP) and pGLTR-X-S (encoding PuroR) destination vector for constitutive shRNA expression (Sigl *et al.,* 2014) were used.

Generation of stable shRNA cell lines

Confluent 293 T cells were transiently transfected with the lentiviral expression vector pGLTR-X-FP (now including the sequence for *hmox* shRNA expression) in combination with psPAX and the VSV-G encoding vectors using Metafectene™ (Biontex, Martinsried, Germany). After 48 and 72 h viral supernatants were harvested, sterile filtered and supplemented with 4 μg/ml polybrene. Five×10^5 target cells (RAW264.7) were infected with 0.5 ml viral supernatant for 6 h. The newly designated RAW.sh cells were transfected with the lentiviral expression vector pGLTR-X-S together with the packaging vectors and Metafectene™ (Sigl *et al.,* 2014). This allows dual selection for both GFP (via FACS sorting) and puromycin (Puro), before their knockdown efficiency was determined, and the cells were then used for subsequent experiments. Inducible shRNA transduction reached 82% (hmox.sh 2) and 94% (hmox.sh 1) *hmox1* gene knockdown efficacy—after treatment of the RAW.sh macrophages with 1 μg/ml doxycycline 24–48 h prior to experiments as compared to the luciferase control (ctrl.sh). Apart from phenotype control, and unless stated differently, hmox.sh 1 was used for the *hmox* knockdown experiments (hereafter termed hmox.sh).

Cell culture

RAW 264.7 murine macrophage-like cells were maintained in complete DMEM containing 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all purchased from Biochrom AG) at 37°C in humidified air containing 5% CO_2. Cells were stimulated with 50 U/ml recombinant murine IFN-γ (muIFN-γ; purchased from R&D) and 10 ng/ml LPS *(Escherichia coli* 055:B5; obtained from Sigma); or 10 μM of the HO-1 inhibitor zinc (II) protoporphyrin IX (ZnPP; Sigma) dissolved in 0.1 N NaOH, for 24 h. Control samples were treated with PBS. Thereafter, supernatants were harvested and macrophages subjected to RNA preparation.

Atomic absorption spectrometry

Intracellular iron levels were determined by atomic absorption spectrometry as described elsewhere (Theurl *et al.,* 2009).

Quantification of iron uptake and release by macrophages

To evaluate transferrin-bound iron (TBI) and non-transferrin-bound iron (NTBI) uptake or cellular release, RAW264.7 cells were infected with *S.* Typhimurium as detailed below and ^{59}Fe-TBI or NTBI uptake and release were determined as described in Ludwiczek *et al.* (2003) and Nairz *et al.* (2007) using 10 μM of ^{59}FeSO$_4$ (Perkin Elmer®) plus 200 μM Trisodium-Citrate (Sigma) or 12.5 μg/ml ^{59}Fe-bound Transferrin (Tf) respectively.

Bacterial iron acquisition

The iron uptake of intracellular *Salmonella* was assessed according to a modified protocol described in (Nairz *et al.,* 2007). In brief, a standard *in vitro Salmonella* infection of RAW cells at a multiplicity of infection (MOI) of 10 was performed. One hour later, cells were washed 3 times with PBS plus gentamicin (25 μg/ml), then resuspended in serum-free uptake/release Buffer (DMEM; 2 mM L-glutamine; 25 mM Hepes; plus 1 μg/ml doxycycline and 10 μg/ml gentamycin). For the negative control cells were infected with heat-inactivated *Salmonella*. They are used as a defined inflammatory stimulus not compromised by bacterial number. Ten μM of ^{59}FeSO$_4$ (Perkin Elmer®) mixed with 400 μM Trisodium- Citrate (Sigma) were added. After incubation for 18 h under standard culture conditions cells were washed three times with NaCl 0.9% containing 50 μM FeSO$_4$. Buffer was changed to washing buffer (0.01% SDS containing 1 mg/ml Pronase; Roche) and the cells were gently scraped from the dish. The cells were broken open by adding glas-beads (Lenz Laborglas GmbH) and vortexing the cell suspension at the highest setting for 1 min. An aliquot of the suspension was kept for assessment of total iron and bacterial load (CFU) by plating serial dilutions onto agar plates. One thousand U/ml DNAse I (Roche) was added to the remaining lysate for 10–20 min. Thereafter the supernatant was loaded onto 0.22 μm PDVF Filters (Milipore) and centrifuged 5 min at 10 000×g. The filter containing the trapped bacteria was washed 3–5 times with washing buffer, cut into pieces and placed into a γ-counter tube to assess the amount of Salmonella-associated ^{59}Fe.

Isolation of bone marrow-derived macrophages (BMDM)

Tibias and femurs from 8 to 12 week old mice with macrophage specific deletions of HO-1 (LysM-Cre$^{+/+}$HO-1$^{fl/fl}$; HO$^{-/-}$) and controls (LysM-Cre$^{-/-}$HO-1$^{fl/fl}$; wt) were collected in ice-cold PBS. The mice were kept under pathogen-free conditions at the Instituto Gulbenkian de Ciencia (Mamiya *et al.,* 2008; Wegiel *et al.,* 2014) or the KILM Research Labs of the Medical University of Vienna (Jais *et al.,* 2014). Bones were sterilized with 70% ethanol and flushed with a 25-G needle using cold PBS containing 1% penicillin-streptomycin. Cells were seeded onto ⌀ 10 cm dishes in DMEM supplemented with 10% FCS, 1% penicillin-streptomycin and 50 μg/ml macrophage colony-stimulating factor (M-CSF, purchased from Peprotech). Medium was changed every 2 days, and 6 days after preparation cells were harvested and seeded onto six-well plates at 0.5×10^6 cells per well. One day later cells were infected with *Salmonella* as described below.

Salmonella *infection* in vitro

RAW264.7 cells stable transfected with *hmox1* shRNA or BMDM were used for *in vitro* infection assays generating comparable results. Prior to *in vitro* infection, macrophages were incubated in complete medium without antibiotics. Wild-type *Salmonella enterica* serovar Typhimurium (S. Typhimurium; S. Tm wt) strain ATCC14028 (resistant to tetracycline) or a *Salmonella* triple mutant derivative *entC::aph sit::bla feo::Tn10* (Kan^r Ap^r Tet^r) were used for infection. The mutant strain, deficient in enterobactin synthesis, SitABCD- and Feo-mediated iron uptake *(ΔentC Δsit Δfeo)* was constructed and grown as previously described (Crouch *et al.,* 2008). *Salmonella* were grown under sterile conditions in LB broth (Sigma) to late-logarithmic phase. Macrophages were infected with S. Typhimurium at a MOI of 10 and harvested as described (Nairz *et al.,* 2009). For experiments employing heat-inactivated *Salmonella*, bacteria were suspended in complete DMEM, incubated at 70°C for 20 min and cooled to 37°C prior to use. For phagocytosis experiments, infections were terminated after 1 h by extensively washing the cells five times with PBS and subsequent lysis of the cells with Na-deoxycholate (Sigma). Please see Supporting Information for additional details regarding heme catabolism products.

RNA preparation, reverse transcription and polymerase chain reaction

Preparation of total RNA and quantification of mRNA expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed exactly as described in (Nairz *et al.,* 2009) using SsoFast™ qPCR Supermix (BioRad™). The primers and probes used for the experiments are shown in Supporting Information (Table S1).

Western blot analysis

Protein extracts were prepared with cytoplasmic lysis buffer (25mM Tris-HCl [pH7.4], 40mM KCl, and 1% Triton X-100) supplemented with 1 mg/ml aprotinin and 1 mg/ml leupeptin (all obtained from Sigma). Twenty micrograms of total protein was run on 10% −15% SDS-polyacrylamide gels, and Western blotting was performed exactly as described (Theurl *et al.,* 2006) with a mouse anti-human TfR1 antibody (1:1000; Invitrogen), rabbit anti-human Ferritin (1:500; Sigma), rabbit anti-Fpn (1:400; self designed; Eurogentic), rabbit anti-HO-1 (1:1000; Enzo), rabbit anti-iNOS (1:1000; BD), rabbit anti-NFκB p65 or rabbit anti-phosphor NFκB p65 (both 1:1000; Cell Signalling). Blotting with either rabbit anti-TBP (1:1000; Cell Signalling) or rabbit anti-Actin antibody (1:1000; Sigma-Aldrich) was performed as a loading control.

Transcription factor assays

Nuclear protein extracts were prepared with the Nuclear and Cytoplasmic Extraction Reagent (Thermo Scientific). NF-kB p65-binding activity of nuclear extracts was assessed with a commercially available chemi-luminescent transcription factor assay kit in exact accordance with the manufacturer's instructions (Thermo Scientific).

Detection of cytokines, nitrite and reactive oxygen species

Determination of cytokines in culture supernatants was performed with ELISA kits for TNF-α and Lcn2 (from R&D; Wiesbaden, Germany) and IL-6, IL-10 (from BD), following

manufacturer's instructions. Determination of nitrite, the stable oxidation product of NO, was carried out with the Griess-Ilosvay's nitrite reagent (Merck) as described by Nairz *et al.* (2009). Detection of ROS was carried out after a standard infection experiment using CellROX®DeepRed Flow Cytometry Assay Kit (Life Technologies) according to the manufacturer's instructions. In brief, CellROX®DeepRed was added at the concentration of 500 nM to the infected or induced cells and incubated for 30 min at 37°C. SYTOX Blue Dead Cell stain (1 mM) was then added and the cells analyzed by FACS sorting within the next 120 min. The following controls were included: Cells treated with N-acetyl-L-cysteine (NAC) or tert-butyl hydroperoxide (TBHP), and an unstained cell sample.

Statistical analysis

Statistical analysis was done with an SPSS statistical package. Significance was determined by analysis of variance (ANOVA) combined with Bonferroni's correction. Unless otherwise specified, data are depicted as lower quartile, median and upper quartile (boxes) with minimum and maximum ranges. Individual values and means of log transformed values are depicted. Generally, *P* values less than 0.05 were considered significant in any test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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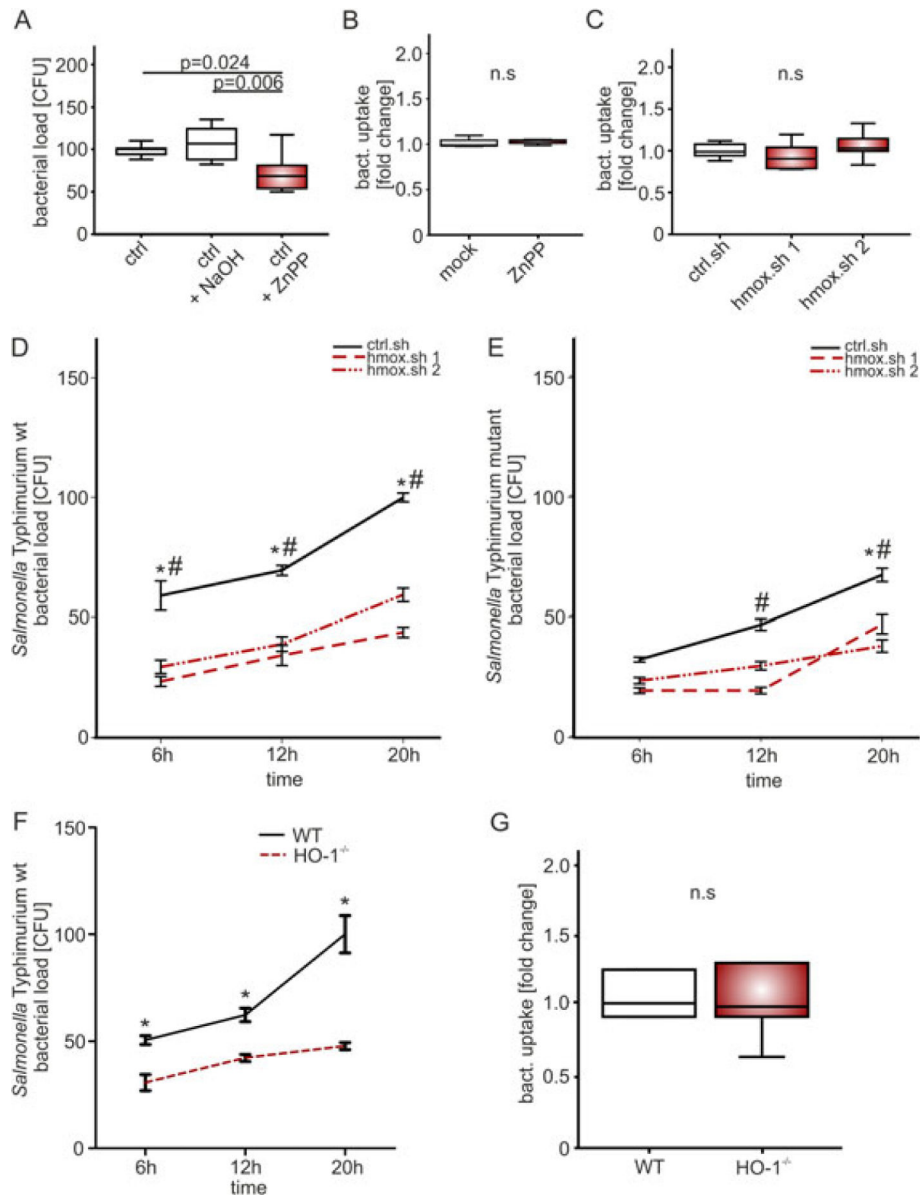
Fig. 1. Heme oxygenase gene knockdown reduces the survival of intracellular *Salmonella* Typhimurium.

(A) RAW 264.7, (B-E) RAW.sh macrophages (ctrl.sh, hmox.sh 1 and 2) and (F-G) BMDM from LysM-Cre$^{+/+}$ HO-1$^{fl/fl}$ (HO-1$^{-/-}$) and LysM-Cre$^{-/-}$ HO-1$^{fl/fl}$ (wt) mice were infected with (A-D, F-G) *Salmonella enterica* serovar Typhimurium (S. Tm) wild-type (wt) or (E) an isogenic *Salmonella* Typhimurium mutant (ΔentC Δsit Δfeo) strain at a MOI of 10 for the indicated period of time.

(A) Bacterial burden (CFU) was determined 24 h post-infection following treatment with 10 μM of HO-1 Inhibitor ZnPP or solvent (NaOH, PBS). (B-C, G) Bacterial uptake relative to mock/control (fold change) was measured one hr post infection to determine phagocytosis. (A-C, G) Data from three independent experiments performed in triplicate are shown ($n =$ 9). Boxes depict lower quartile, mean and upper quartile; whiskers show maximum and minimum range. *P*-values are reported as determined by statistical analysis using ANOVA

and Bonferroni correction. (D-F) Bacterial burden (CFU) was determined as mean ± SEM of at least three independent experiments performed in triplicate ($n = 9$) and normalized (%) relative to control. Whiskers show maximum and minimum range. *P*-values are reported as determined by statistical analysis using ANOVA and Bonferroni's correction. * $P < 0.001$ for crtl.sh versus hmox.sh 1 or control (wt) BMDM versus HO-1$^{-/-}$ cells; # $P < 0.001$ for ctrl.sh versus hmox.sh 2 macrophages.
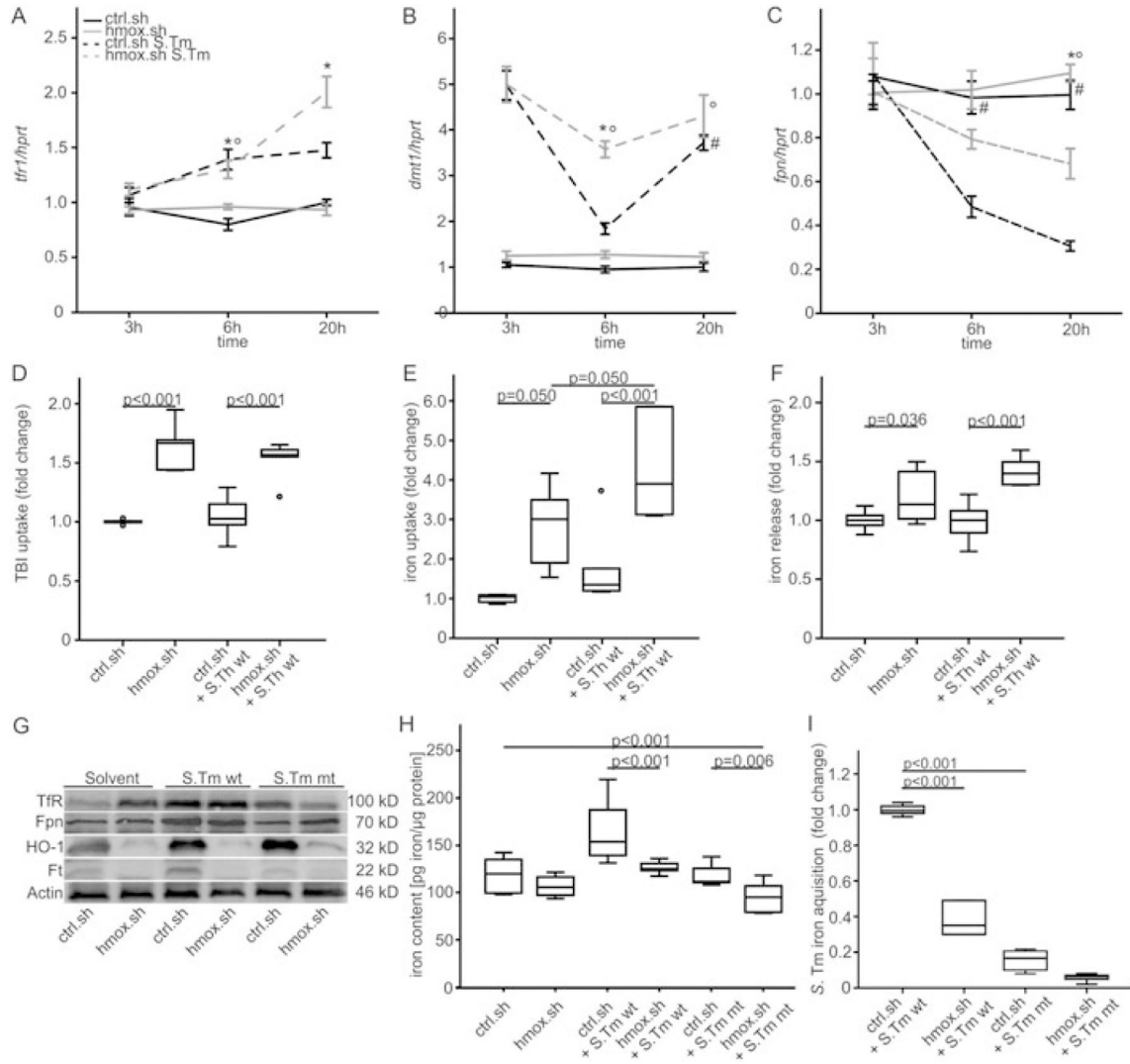
Fig. 2. Effects of *hmox* on iron acquisition and iron release in *Salmonella* infected macrophages.
(A-B, D-E) Iron import. Ctrl.sh cells (black line) and *hmox* knockdown macrophages (hmox.sh; grey line) were infected with *Salmonella* Typhimurium wild-type (S. Tm wt; dashed line) strain at a MOI of 10 for the indicated period of time.

(A) *tfr1* and (B) *dmt1* levels were determined by qRT-PCR. Values were normalized to the housekeeping gene *hprt,* and fold-changes relative to the untreated control (20 h) are shown. (D, E) The uptake of (D) TBI (transferrin loaded with ^{59}Fe) and (E) iron (^{59}Fe) for 4 h following an 18 h infection period was determined as described in Materials and methods. (C, F) Iron export. Macrophages were treated exactly as described above. (C) *fpn1* levels by qRT-PCR and (F) iron (^{59}Fe) release 20 h post infection were determined. (A-C) Data from at least three independent experiments are shown (ctrl.sh n = 10; hmox.sh n = 9) and expressed as mean ± SEM. *P*-values are reported as determined by post hoc with Bonferroni correction. *# ° $P < 0.001$ (* ctrl.sh vs. hmox.sh infected with S. Tm; # ctrl.sh vs. ctrl.sh *S.*Tm; ° hmox.sh vs. hmox.sh *S.*Tm). (D-F) Data were compared and are depicted of three independent experiments (D, E) n = 6, (F) n = 12 ctrl.sh, n = 8 hmox.sh. *P*-values are reported as determined by post hoc analysis with Bonferroni's correction following

ANOVA. (G) Western blot analysis of whole cell lysates using specific antibodies to TfR1, Fpn1, heme oxygenase (HO-1), Ferritin (Ft) and the control β-Actin. One of three representative western blot experiments is shown. (H) Cellular iron content in phagocytes was measured by atomic absorption spectrometry. Macrophages were infected with *Salmonella* wt or mt (*entC sit feo*) for 48 h or left untreated. Results were normalized to protein content. Data from three independent experiments are shown (n = 8 untreated and S. Tm mt infected, *n* = 10 infected with *S*. Tm wt). (I) Bacterial iron acquisition within macrophages was determined after loading of infected macrophages with ^{59}Fe. Data were normalized for *Salmonella* numbers determined by CFU count. Three independent experiments were carried out in duplicate (*n* = 6). Data were compared by ANOVA using Bonferroni's correction (*P*-values). Values (boxes) are depicted as lower quartile, median and upper quartile with maximum and minimum range.
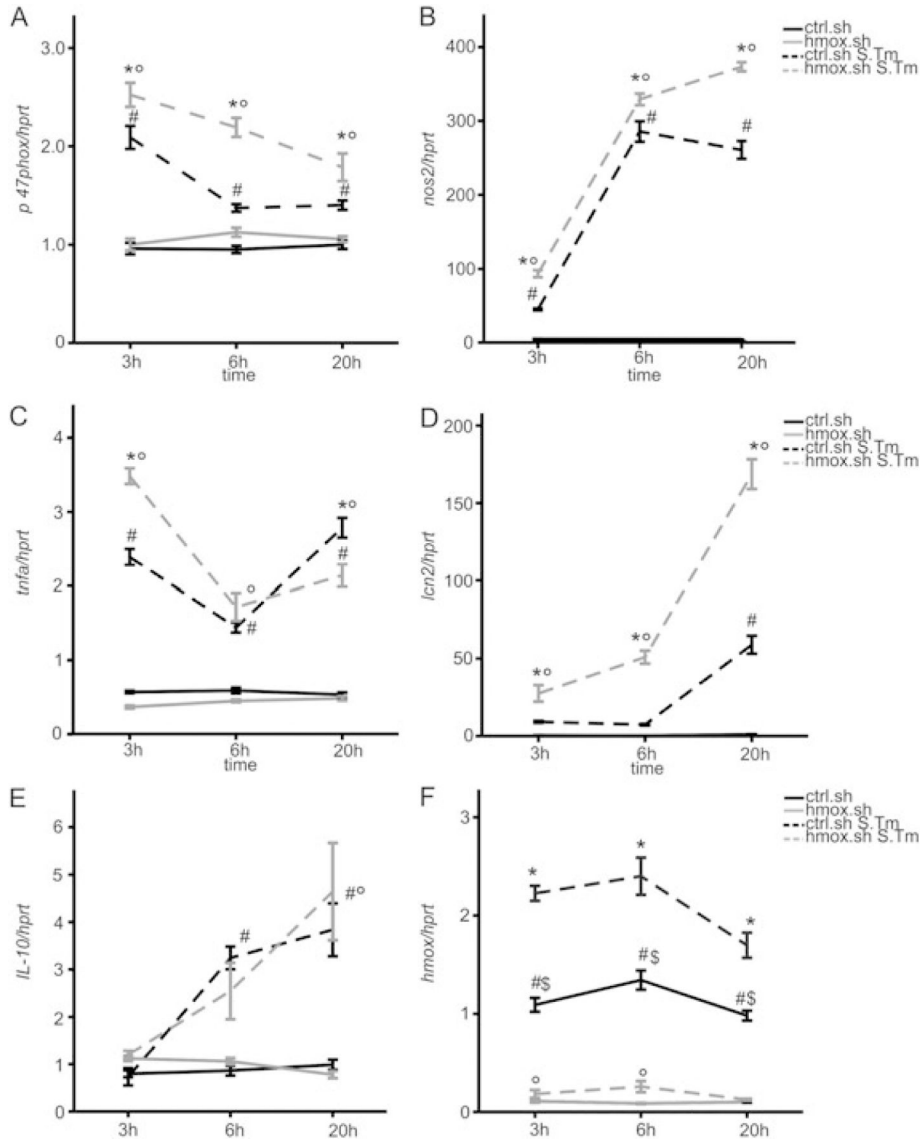
Fig. 3. Effects of *hmox* gene knockdown on *p47phox, nos2, tnfa, Il-10* and *lcn2* transcript expression.

Ctrl.sh (black line) and hmox.sh (grey line) macrophages infected with *Salmonella* (black or grey dashed line) were subjected to qRT-PCR at indicated time points. Expression of genes of interest was normalized to *hprt* expression and fold-change relative to untreated control is shown. Data from three independent experiments ($n = 10$ ctrl.sh; $n = 8$ hmox. sh) are shown. Error bars represent means ± SEM, values are reported as determined by ANOVA using Bonferroni's correction. * $ $P < 0.001$ for hmox.sh versus ctrl.sh, both infected with *S*. Tm (*) or both uninfected ($); ° $P < 0.001$ for hmox.sh uninfected versus infected with *S*. Tm; # $P < 0.001$ for comparison of ctrl.sh and ctrl.sh infected with *S*. Tm.
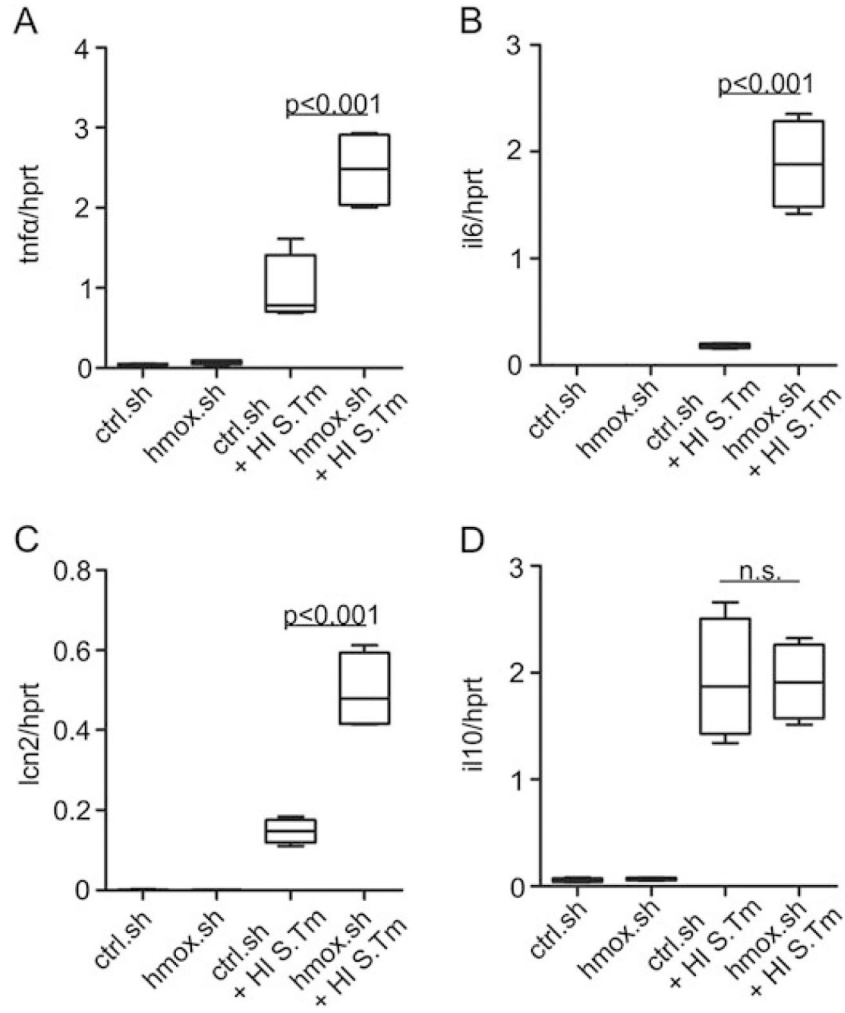
Fig. 4. Early response to *Salmonella* is improved in *hmox* knockdown macrophages.
Ctrl.sh and hmox.sh macrophages supplemented with an identical number of heat-inactivated *Salmonella* (HI *S.* Tm; as a defined inflammatory stimulus not compromised by bacterial number) as described in Fig. 3 were subjected to qRT-PCR. Gene expression of (A) *tnfa,* (B) *il6,* (C) *lcn2* and (D) *il10* was determined 6 h post-infection and normalized to *hprt* expression levels. (A-D) Fold changes relative to untreated controls are shown. Data from three independent experiments are shown. Error bars represent means ± SEM; values are reported as determined by ANOVA using Bonferroni's correction.
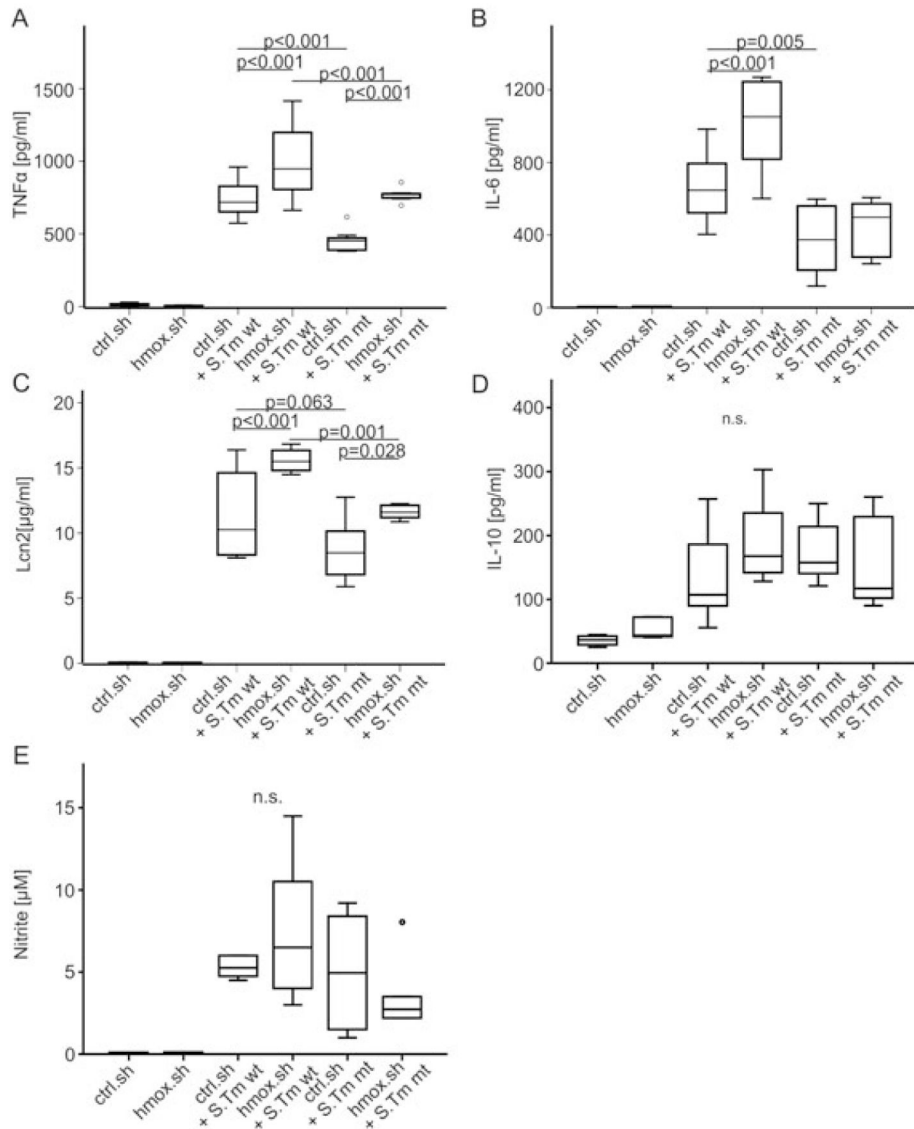
Fig. 5. Production of pro-inflammatory mediators, Lcn2, IL-10 and nitrate during *Salmonella* infection following *hmox* gene knockdown.

(A-E) Ctrl.sh and hmox.sh cells were infected with *Salmonella* wt (*S.* Tm wt) or mutant *entC sit feo* (*S.* Tm mt) for 20 h before culture supernatants were analyzed for formation of (A) TNFα, (B) IL-6, (C) Lcn2, (D) IL-10 and (E) nitrate. (A-E) Boxes depict lower quartile, mean and upper quartile; whiskers show minimum and maximum range. Data are shown as mean ± SEM from at least three independent experiments. *P*-values are reported as determined by ANOVA using Bonferroni's correction.
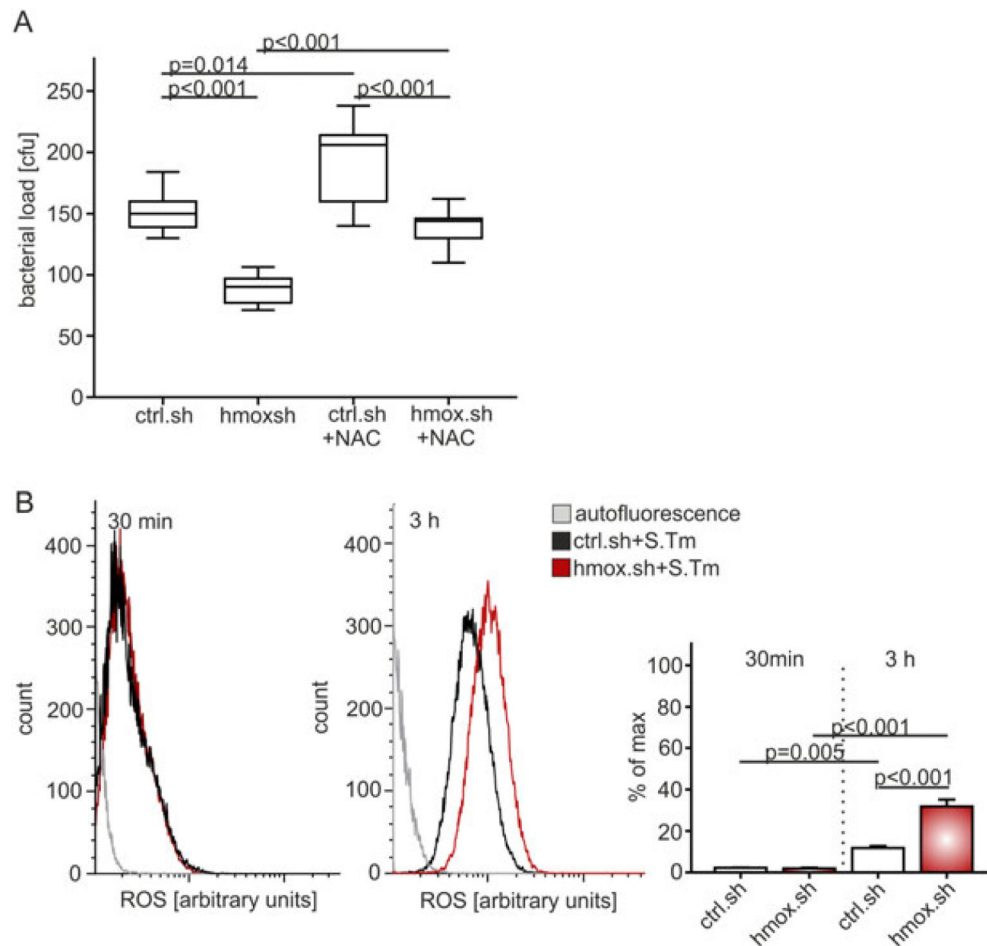
Fig. 6. Increased ROS production by *hmox* knockdown macrophages reduces their bacterial burden.

(A) Macrophages (hmox.sh versus ctrl.sh) were infected with *Salmonella* Typhimurium wt (*S.* Tm) and thereafter treated with the radical scavenger N-acetyl-L-cysteine (NAC) for 20 h. Boxes are depicted as lower quartile, median and upper quartile with minimum and maximum range. Data of at least three independent experiments are shown ($n = 10$). *P*-values are reported as determined by ANOVA using Bonferroni's correction.

(B) ROS levels were measured via FACS analysis in ctrl.sh and hmox.sh cells infected with *Salmonella* at 30 min and 3 h post infection. The histograms represent the average ROS levels measured by CellROX™ fluorescence. Data of three independent experiments performed in triplicate are shown. *P*-values are reported as determined by ANOVA using Bonferroni's correction.
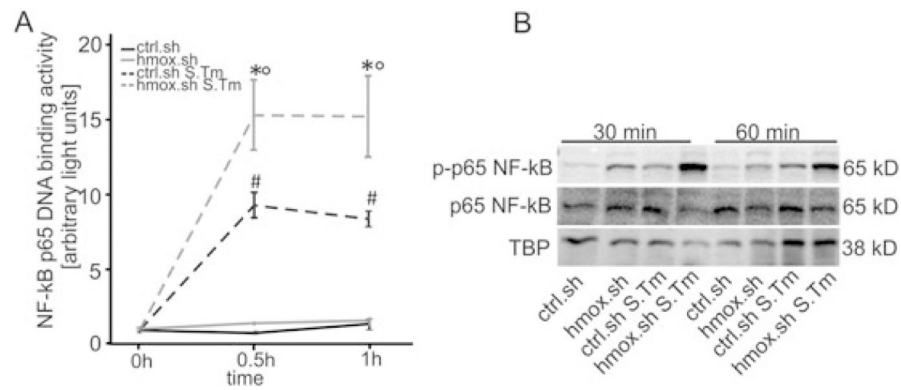
Fig. 7. Knockdown of *hmox* in macrophages show increased NF-κB activation.
RAW.sh macrophage-like cells were infected with *Salmonella* for the indicated periods of time. NF-κB DNA binding activity in nuclear protein extracts was evaluated by means of a specific p-65 NF-κB chemo-luminescence transcription factor assay. Data from three independent experiments are shown and expressed as arbitrary light units. Error bars are depicted as means ± SEM and were compared by ANOVA with Bonferroni's correction. Asterisks indicate statistically significant differences between *Salmonella* infected control and *hmox* knockdown macrophages: * $p < 0.001$ for comparison of ctrl.sh versus hmox.sh infected with *S.* Tm; # $p < 0.001$ ctrl.sh versus ctrl.sh *S.* Tm;°$p < 0.001$ between hmox.sh and hmox.sh *S.* Tm.

(B) Protein extracts from parallel experiments described in (A) were used for evaluation of phosphorylated (p-) p65 NF-κB by means of Western blot analysis as described in Material and methods. Antibodies to p65 NF-κB and TATA-binding protein (as loading control) were used. One of three representative Western blot experiments is shown.
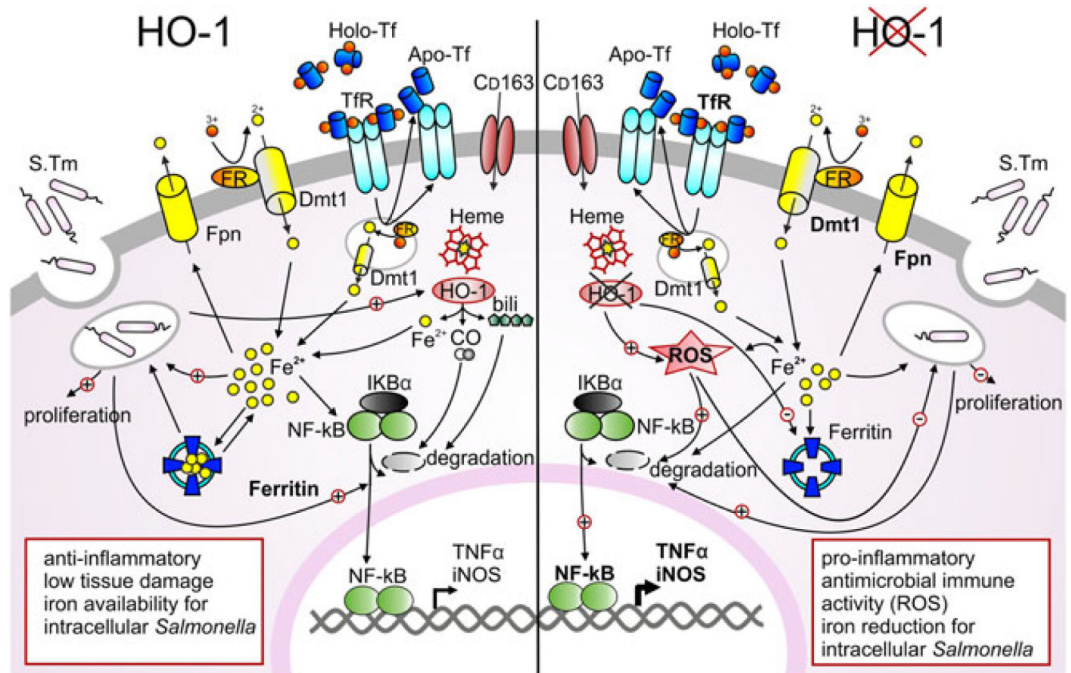
Fig. 8. Effects of heme oxygenase in regulating iron homeostasis and innate immune response of macrophages during *Salmonella* infection.

Macrophages play an important role in infected tissue. Within these cells heme bound iron is degraded via HO-1 to equal amounts of iron, carbon monoxide (CO) and biliverdin (bili). Ferric iron is acquired via transferrin receptor 1 (TfR1) mediated endocytosis of holoTf, reduced within the vesicle by a ferric reductase (FR) and released to the cytoplasmic pool via divalent metal transporter (Dmt1), while Tf and TfR are recycled to the surface. During infection, bacterial pathogens may employ mechanisms to increase the abundance of potential iron sources. Transitory iron accumulation in the cytosol may promote storage into ferritin, ferroportin (Fpn1)-mediated export and reduction of TfR1 expression, thereby limiting iron for the pathogen or lowering intracellular iron levels. Both LPS and iron stimulate NF-κB expression, resulting in an inflammatory response that includes expression of iNOS, TNFα and other cytokines. Macrophages with absent HO-1 function show an increased iron transport activity, strong iron utilization via TfR1 and Dmt1 and increased iron export via Fpn1. Cells may simultaneously restrict iron levels during *Salmonella* infection and increase production of reactive oxygen species (ROS). Restriction of iron and increased ROS production promote NF-κB mediated activation of a pro-inflammatory immune response that results in improved *Salmonella* control and may also result in tissue injury.