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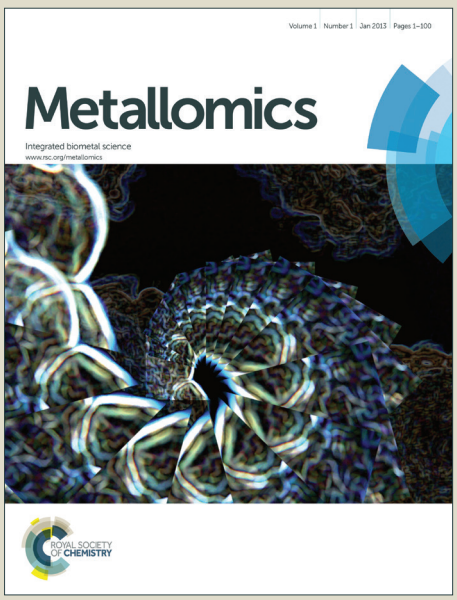

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Contrasting regulation of macrophage iron homeostasis in response to infection with *Listeria monocytogenes* depending on localization of bacteria

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Short Title: Iron controls growth of *Listeria monocytogenes*

Abbreviations: *L.m.*, *Listeria monocytogenes*; LLO, listeriolysin O; SFN, Sulforaphane; Fpn1, ferroportin1; TfR, transferrin receptor; Lcn2, Lipocalin2; IL, interleukin; Dmt1, divalent metal transporter1; CPM, counts per minute; NTBI, non-transferrin bound iron; ROS, reactive oxygen species; DFP, deferiprone; TNF, tumor necrosis factor; wt, wild-type;

Keywords: *Listeria monocytogenes*, iron, ferroportin1, macrophages

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Abstract

Due to its multiple roles for the proliferation and pathogenicity of many microbes on the one hand and via modulation of immune effector functions on the other hand the control over iron homeostasis is thought to play a decisive role in the course of infections. Diversion of cellular iron traffic is considered as an important defense mechanism of macrophages to reduce metal availability for intracellular bacteria residing in the phagosome. However, evidence is lacking whether such alterations of iron homeostasis also become evident upon infection with bacteria gaining access to the cytosol like *Listeria monocytogenes*. Here we show that infection of macrophages with *L. monocytogenes* triggers the expression of the major cellular iron exporter ferroportin1 and induces cellular iron egress. As the growth of *Listeria* within macrophages is promoted by iron, stimulation of ferroportin1 functionality limits the availability of the metal for *Listeria* residing in the cytoplasm, whereas ferroportin1 degradation upon hepcidin treatment increases intracellular bacterial growth. In parallel to an increase of ferroportin1 expression, infected macrophages induce anti-microbial immune effector mechanisms such as TNFα formation or NO expression which are aggravated upon iron deficiency. These adaptive changes of iron homeostasis and immune response pathways are only found in macrophages infected with *Listeria* which express listeriolysin O and are therefore able to escape from the phagosome to the cytoplasm. Listeriolysin O deficient *Listeria* which are restricted to the phagosome are even killed by excess iron which may be based on “iron intoxification” via macrophage radical formation, because iron supplementation in that setting is paralleled by increased ROS formation.

Our results indicate that ferroportin1 mediated iron export is a nutritional immune effector pathway to control infection with *Listeria* residing in the cytoplasm, whereas a different strategy is observed in mutant *Listeria* restricted to the phagosome, where iron remains in the macrophages likewise contributing to ROS mediated intoxification of bacteria.

Introduction

Iron is an indispensable element for nearly all living organisms, predominantly due to its ability to act as an ideal redox catalyst for cellular and enzymatic processes like DNA synthesis, mitochondrial respiration or oxygen transport.[1] In reflection to its importance its availability in mammals is controlled by a hormone, hepcidin.[2] However, due to its central metabolic functions a sufficient availability of iron is also crucial for the proliferation and pathogenicity of many microbes which thus have an essential need for this metal. Therefore, a struggle for this nutrient ensues following an infection[3, 4] and hepcidin expression is induced by inflammatory stimuli and modulated by various infectious challenges.[5] The control over iron homeostasis is thus a central battlefield deciding about the fate of an infection because iron availability also plays important role for immune cell differentiation and proliferation along with specific effects of the metal on innate immune function.[6] Although macrophages depend on small amounts of iron to catalyze the formation of reactive oxygen species (ROS),[7] the reduction of intracellular iron levels in macrophages infected with the intracellular pathogen *Salmonella typhimurium* strengthens anti-microbial innate immune effector pathways and limits pathogen proliferation by withholding iron from bacteria.[8] In line with this, the presence of *Legionella pneumophila*, *Chlamydia* or *Leishmania* species, all of which reside within intracellular vacuoles, influences iron handling of host macrophages and iron depletion restricts the growth of these pathogens.[9]

Macrophages have employed multiple pathways to acquire iron. They can incorporate molecular iron via divalent metal transporter1 (Dmt1), transferrin bound metal through transferrin receptor-1 (TfR1) mediated endocytosis and senescent erythrocytes by phagocytosis followed by subsequent reutilization of iron contained within hemoglobin from these cells.[1] On the other hand, there is only one major cellular iron export mechanism confined to ferroportin1 (Fpn1). Fpn1 expression is posttranslationally controlled by hepcidin, the systemic master regulator hormone of iron metabolism, which exerts its activity upon binding to Fpn1 expressed on the cell surface, resulting in its intracellular degradation and blockade of cellular iron egress.[10]

Listeria monocytogenes is a Gram-positive, intracellular, facultative anaerobic bacterium and one of the most virulent food-borne pathogens responsible for the potentially fatal disease listeriosis.[11] *Listeria* has a characteristic intracellular life cycle following receptor mediated phagocytosis of the bacterium which can be also induced by the pathogen itself. Within the cell *Listeria* is first contained in the phagosome, but via the action of bacterial listeriolysin O (LLO) and two phospholipases, the

pathogen can destabilize the phagosomal vacuole thus gaining access to the cytosol.[12] There, cytosolic bacteria replicate and spread from cell to cell.[13] LLO is a crucial virulence factor for *Listeria*, encoded by the *hly* gene, and bacteria lacking this factor remain in the vacuole and lose most of their virulence.[14] Like most bacteria *Listeria* have an essential need for iron and can acquire the metal by different pathways involving extracellular or surface-bound iron reductases and a ferric citrate uptake system as well as siderophore based uptake mechanisms, although they produce no own siderophores.[15]

The cytosolic stage distinguishes the life cycle of *Listeria* from other intracellular pathogens, like *Salmonella typhimurium*, which remain and replicate in the late phagolysosome.[16] As both bacteria are highly iron dependent, the different life cycles and localization of these pathogens implicates that macrophages may use specific strategies to restrict iron from *L. monocytogenes,* which may partly differ from those observed for *Salmonella* or *Mycobacteria*.

The aim of the present study was therefore to determine the alterations of macrophage iron homeostasis in response to *Listeria* infection and to study the importance of such modifications for host resistance to infection.

Materials and methods

Cell culture

RAW264.7 (murine macrophage) cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified eagles medium (DMEM; purchased from Lonza) containing 10% fetal calf serum (FCS; from Biochrom), 2 mM L-glutamine (Lonza) and 1% penicillin-streptomycin (Lonza) at 37°C in humidified air containing 5% CO_2. Cells were seeded in six centimeter dishes at a density of 0.9 x 10^6 cells per well and grown overnight until 70–80% confluent.

Bacterial strain and *Listeria* infection of macrophages

Wild-type (wt) *Listeria monocytogenes* strain EGDe as well as a mutant form of the same strain lacking the virulence factor listeriolysin O (ΔLLO), were used for all experiments and grown in Brain Heart infusion (BHI, Becton Dickinson) to late-logarithmic phase. Before in vitro infection cells were washed three times with phosphate-buffered saline (PBS; purchased from Lonza) and incubated in complete DMEM without antibiotics. For experiments involving ferrous sulphate, ferrous chloride and deferiprone (DFP; purchased from Sigma), cells were exposed to this substances at a final concentration of 50 µM for 30 min before infection with bacteria was performed (Fig. 1A, B and Fig. 2). After pre-incubation of *L. monocytogenes* in DMEM at 37°C for 10 min, RAW264.7 macrophages were infected at a multiplicity of infection (MOI) of 5 for 1 h at 37°C. After 1 h cells were washed three times with PBS and complete DMEM containing 25 µg ml^{-1} of gentamicin (Life Technologies) was added in order to kill extracellular bacteria. For studies involving Sulforaphane (SFN, LKT Laboratories) and synthetic murine hepcidin (Peptanova), cells were washed three times with PBS, complete DMEM containing 25 µg ml^{-1} of gentamicin was added and cells were treated with SFN or hepcidin to a final concentration of 10 µM or 1 µM, respectively. Thereafter, macrophages were incubated for another 11 h (if not indicated differently), then washed three times in PBS and subjected to protein or RNA isolation. For quantification of intracellular *Listeria* by means of gentamicin protection assay, macrophages were lysed and plated in appropriate dilutions onto BHI agar plates.

Measurement of phagocytic activity

Phagocytic activity was assessed with a short term infection assay, where cells were stimulated with iron and DFP as described above and infected at a MOI of 5 with *Listeria* for only one hour. Afterwards cells were washed extensively six times with PBS to get rid of extracellular bacteria, lysed and plated in appropriate dilutions onto BHI agar plates to determine phagocytic uptake of bacteria.

Phagocytic activity of stimulated cells was also determined with fluorescent zymosan beads (pHrodo®, Life Technologies) according to the manufacturer's protocol. After one hour cells were analyzed by flow cytometry, and phagocytotic capcity was calculated as mean fluorescent intensity and compared to solvent-treated cells.

Isolation of bone marrow – derived macrophages

Tibia and femur from 8 to 12 week-old C57BL/6J mice were collected in ice cold PBS. 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^{-1} 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 6-well plates or 10 cm dishes at 0.5 x 10^6 or 3 x 10^6 cells per well, respectively. One day later cells were stimulated with SFN or hepcidin as described above.

Quantification of iron uptake and release by macrophages

For macrophage iron uptake and release studies, RAW264.7 cells were infected with *L. monocytogenes* as detailed above. Following three washing cycles with serum-free, HEPES-buffered DMEM (2 mM L-glutamine, 25 µg ml^{-1} gentamicin, 25 mM HEPES, pH 7.4) cells were then incubated therein. For determination of non-transferrin bound iron (NTBI) acquisition, 5 µM ^{59}Fe-citrate (Perkin Elmer) was used to investigate the uptake of NTBI exactly as described.[17] Results (counts per minute, CPM) were normalized to protein concentration. For iron release experiments, cells were first incubated with 5 µM ^{59}Fe-citrate for 2 h to allow iron loading and then washed four times with serum-free, HEPES-buffered DMEM and again incubated therein. After an additional incubation of 1 h duration, cellular iron release was measured by means of a γ-counter as described.[17]

Detection of reactive oxygen species (ROS)

RAW264.7 cells were infected with *L. monocytogenes* and stimulated as detailed above. After 12 h cells were harvested and stained intracellularly with a cell permeant dye which gets fluorescent upon oxidation by ROS (CellROX®, Life Technologies) according to manufacturer's protocol as well as with a dead dye (SYTOX® blue dead cell stain, Life Technologies). ROS levels were measured via flow cytometry and values reported as mean fluorescent intensity.

RNA extraction and quantitative real time PCR

RNA was extracted from adherent cells using a guanidinium-isothiocyanate-phenol-chloroform-based protocol (PeqGold Trifast, peqlab) according to the manufacturer's protocol and as described previously[18]. Reverse transcription was performed with 1 µg total RNA, random hexamer primers (200 ng/µl), dNTPs (10 mM), 20 U RNasin (Promega) and 200 U M-MLV reverse transcriptase (Invitrogen) in first strand buffer (Invitrogen). Modulation of the gene expression was investigated by quantitative real-time Taqman PCR (Eurogentec) using the CFX96 PCR System (BioRad) according to the user's manual. Relative gene expression was calculated with the ΔΔCt method by CFX96 Manager. Housekeeping gene hypoxanthine phosphoribosyltransferase (Hprt) was used as reference gene. The following primers and TaqMan probes were used (forward, reverse, probe): muHprt: 5'-GACCGGTCCCGTCATGC-3', 5'-TCATAACCTGGTTCATCATCGC-3', 5'-ACCCGCAGTCCCAGCGTCGTC-3', 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′, muIL10: 5'-CCAGAGCCACATGCTCCTAGA-3', 5'-TGGTCCTTTGTTTGAAAGAAAGTCT-3', 5'-TGCGGACTGCCTTCAGCCAGG-3', muiNOS: 5'-CAGCTGGGCTGTACAAACCTT-3', 5'-CATTGGAAGTGAAGCGTTTCG-3', 5'-CGGGCAGCCTGTGAGACCTTTGA-3', muTNFα: 5'-

TTCTATGGCCCAGACCCTCA-3‘, 5’-TTGCTACGACGTGGGCTACA-3’, 5’-CTCAGATCATCTTCTCAAAATTCGAGTGACAAGC-3‘.

Western blot analysis

Protein extraction and Western blotting were performed exactly as described[19] using a rabbit Fpn1 antibody (1:400; Eurogentec), a rabbit actin antibody (1:1000; Sigma-Aldrich), and appropriate HRP-conjugated secondary antibodies (1:1000; Dako).

Statistical analysis

Statistical analysis war performed using a GraphPad Prism software package. Results were expressed as mean ± SEM. Statistical tests included unpaired 2-tailed Student's t test and 1-way ANOVA followed by Bonferroni-Holmes Multiple Comparison Test. P values of 0.05 or less were considered to denote significance.

Results

Impact of exogenous iron sources on intracellular *Listeria* growth

We first studied the impact of cellular iron supplementation or deprivation on intracellular proliferation of *L. monocytogenes* within macrophages. Non-transferrin bound iron (NTBI), provided as ferrous sulphate or ferrous chloride, administration to RAW264.7 phagocytes resulted in a significant increase of intracellular bacterial load of wild-type (wt) *L. monocytogenes* after 12 hours of infection as compared to solvent treated infected cells (Fig. 1A). In contrast, a significant reduction of intracellular bacterial numbers was observed when infection of iron supplemented macrophages was performed with the *L. monocytogenes* mutant ΔLLO, which is lacking the *hly* gene encoding the toxin LLO.[14] Of interest, deferiprone (DFP), a drug, which is able to bind and chelate free iron, reduced intracellular proliferation of wt *Listeria* but not of the ΔLLO form.

Phagocytosis is not enhanced through iron stimulation of cells

To exclude the possibility that an increased phagocytic capacity of iron stimulated macrophages could be responsible for the differences in bacterial loads, we tested the phagocytic function of macrophages. First, we infected RAW264.7 mononuclear cells with *Listeria* wt and the ΔLLO mutant form for only 1 hour and enumerated the intracellular bacterial load (Fig. 1B). In this assay we even found a significant decrease of bacterial numbers by iron stimulated cells likewise reflecting reduced phagocytosis, which is in line with earlier observations.[20] This effect was less pronounced in *Listeria* ΔLLO infected cells. Of interest, pre-incubation of macrophages with DFP and subsequent infection with wt *Listeria* also resulted in decreased bacterial numbers. As different pathways may have been responsible for these observations, like an increase in phagocytosis-associated killing in iron loaded cells, we wanted to verify the direct effects of iron loading/ withholding on phagocytosis by using inert fluorescent zymosan beads (Fig. 1C). In these experiments we could confirm, that phagocytosis is decreased in iron loaded macrophages, whereas no modulatory effects were observed in DFP stimulated cells.

ROS production differs upon localization of *Listeria*

Based on the observation of distinct effects of iron supplementation on the growth of intramacrophage wt and ΔLLO *Listeria* (Fig. 1A) and the known interaction between iron and radical formation via

Fenton chemistry along with the role of ROS for innate immune responses, we then studied ROS formation in *Listeria* infected macrophages.

We found that in the absence of infection iron supplementation already increased intramacrophage ROS content (Fig. 2). Following infection we observed significantly elevated intracellular ROS levels in macrophages infected with wt but not with ΔLLO *Listeria* (Fig. 2). Of note, iron supplementation resulted in increased ROS formation in both infection scenarios, however, ROS formation was significantly higher in cells infected with ΔLLO than with wt *Listeria*.

Sequence of expression of iron homeostasis and immune effector genes following *Listeria* infection

To gain further insights into the kinetics of iron homeostasis and immune effector responses following *Listeria* infection of macrophages we performed time dependent expression analyses of relevant genes. Following infection with either wt or ΔLLO *Listeria* Dmt1 mRNA was significantly up-regulated in infected cells over time (Fig. 3A), whereas no changes over time were observed for TfR1 mRNA levels as compared to non–infected macrophages (Fig. 3B). Intriguingly, Fpn1 mRNA expression significantly increased over 24 hours post infectionem with wt *Listeria* but remained unchanged following infection with the mutant ΔLLO strain (Fig. 3D). These alterations of mRNA levels were also reflected by increased Fpn1 protein expression after 12 hours of infection with wt but not ΔLLO *Listeria* (Fig. 3E). Of interest, hepcidin mRNA expression by macrophages was increased during the first few hours after infection with *Listeria* wt and then declined toward 24 hours whereas Fpn1 mRNA expression showed the reverse picture (Fig. 3C, D). These data indicated distinct regulatory patters of macrophage iron homeostasis depending on the pathogenicity and localization of *Listeria* (wt versus ΔLLO strain).

As alterations of iron homeostasis also affect innate immune function[6] we studied the expression of cytokines known to be influenced by iron availability and being of importance for host responses towards infection with intracellular pathogens. In general, immune effector function was more pronounced upon infection with *L. monocytogenes* wt than with the mutant strain. We found that tumor necrosis factor alpha (TNFα) mRNA expression rapidly increased after infection and then declined over time, however, always being significantly higher in wt than in ΔLLO infected macrophages, whereas IL-10 mRNA expression increased at later time points (Fig. 3F, H). Inducible nitric oxide synthase (iNOS) mRNA was strongly induced over time in macrophages infected with wt *Listeria* (Fig.

3G), whereas the siderophore capturing molecule lipocalin2 (Lcn2) was not induced following infection (Fig. 3I), which is in line with the fact that *Listeria* do not produce siderophores.[21]

Effects of *Listeria* infection on macrophage iron uptake and release

As our results suggested that the expression of Fpn1 is differently regulated upon infection with wt and ΔLLO *Listeria,* and because Fpn1 has previously been shown to play important roles for the immune control of iron mediated proliferation of intracellular bacteria,[9] we further explored the effects of infection triggered modulation of Fpn1 expression for *Listeria* residing in the cytosol (wt) or being restricted to phagolysosome (ΔLLO). We thus determined cellular iron uptake into infected macrophages using ^{59}Fe, provided as ferrous citrate into macrophages (Fig. 4A). We found no changes in the uptake of iron by this method although increased expression of Dmt1 mRNA was observed (Fig. 3A). On the other hand, we found that cellular iron export is significantly increased in macrophages infected with wt *L. monocytogens* (Fig. 4B), whereas reduced in macrophages infected with the mutant form ΔLLO of *Listeria*. This nicely parallels the expression pattern of Fpn1 in *Listeria* infected macrophages (Fig 3D, E).

Effects of ferroportin1 modulation on intramacrophage *Listeria* survival

Our results obtained so far indicated an important role of Fpn1 in the course of *Listeria* infection. We therefore wanted to study intramacrophage survival of *Listeria* following modulation of Fpn1 expression and the predicted alterations of iron export and cytoplasmic iron levels. To do so we used murine hepcidin, which degrades Fpn1 and blocks cellular iron export. For stimulation of Fpn1 expression we used Sulforaphane (SFN), an Nrf2 activator, as Nrf2 has been shown to be central for Fpn1 expression in macrophages.[8a, 22] Corresponding to reduced Fpn1 levels upon hepcidin treatment (Fig. 4C) we observed a significant increase in intracellular numbers of wt *Listeria* (Fig. 4D). In contrast, SFN treatment resulted in increased Fpn1 expression (Fig. 4C), which was paralleled by significantly decreased bacterial load of macrophages with wt *Listeria*. Of note, we observed the corresponding alterations in bacterial load when using the ΔLLO mutant strain of *Listeria*, although the changes were less pronounced (Fig. 4D). These results were also confirmed by experiments using bone marrow derived macrophages (BMDM) infected with *Listeria* wt or *Listeria* ΔLLO (data not shown).

Discussion

The results of this study highlight the importance of alterations in cellular iron homeostasis of macrophages in the course of infection with *Listeria spp*. We observed different adaptive changes of iron homeostasis and immune response pathways depending on the localization of the bacteria, either in the cytoplasm or in the phagolysosome (Fig. 5).

The iron dependency of *Listeria* has been described previously[23, 24] with the focus being set at that time on the interaction between bacterial iron excess and their pathogenicity in macrophages. Subsequent work then characterized the different iron acquisition systems of *Listeria*.[25, 26] The interplay of macrophages, iron and *Listeria* investigated in our study showed clearly an enhanced intracellular bacterial growth of wt *Listeria* after addition of iron, but surprisingly a decreased load of the mutant form ΔLLO. This raises the question on the underlying mechanisms for these divergent results. Notably, the replication of the mutant form is impaired in the phagolysosome[27] and - in being adapted to the cytoplasm as habitat - there are probably no sufficient bacterial defense strategies of such bacteria to withstand anti-microbial defense mechanism involving radical formation which are central for the host responses against intraphagosomal bacteria.[28] The formation of ROS in the phagosomal environment can be further aggravated by iron via Fenton chemistry, which may underlie our observation of reduced survival of ΔLLO *Listeria* upon iron supplementation, for such a defense strategy mainly observed with copper or zinc the term metal mediated bacterial intoxification has been coined.[29]

In contrast, wt *Listeria* residing in the cytoplasm can use the metal for their growth and proliferation and may be protected from ROS mediated damage by cytoplasmic (host) and likewise bacterial radical detoxifying system, which may be better expressed in the normal habitat of *Listeria.* This is confirmed by our data. We found significantly increased ROS formation in iron loaded macrophages infected with the ΔLLO mutant strain in comparison to cells infected under standard conditions. This is in line with a recent report that LLO can suppress NADPH oxidase to promote *Listeria* infection[30] and NAPDH oxidase mediated ROS formation is aggravated by iron.

When analyzing the regulation of iron genes in macrophages over time in response to infection with wt *Listeria* we observed a significantly increased expression of Fpn1 and a concordant stimulation of cellular iron release. The mechanism by which Fpn1 expression is induced by *Listeria* infection remains elusive but, according to recent evidence arising from studies with the intracellular bacterium

Salmonella typhimurium, it is very likely that the increased formation of NO activates Nrf2 which then induces the transcriptional activation of Fpn1.[8a, 22, 31]

Accordingly, we could demonstrate that stimulation of Nrf2 activity by Sulforaphane (SFN) resulted in increased ferroportin1 expression and impaired the growth of wt *Listeria* whereas blockade of Fpn1 expression by hepcidin restricts macrophage iron export and thus promoted bacterial growth. The latter may be also augmented by negative effects of macrophage iron loading on anti-microbial immune effector pathways such as NO formation or TNFα production.[8a, 32]

Of note, we did not observe such regulations with the ΔLLO mutant strain. This may be due to the fact that innate immune effector pathways have not been activated in macrophages in a comparable fashion as seen for wt *Listeria* and that accordingly NO formation, as a major driver for Nrf2 and Fpn1 expression was almost absent. An enhanced production of TNFα in macrophages infected with a LLO producing strain of *Listeria* in comparison to a nonproducing strain was previously described,[33] which is in nice complementation to our results. Lipocalin-2 on the other side is not induced by *Listeria* wild-type nor by the ΔLLO mutant strain, probably reflecting the fact that *Listeria* is not producing its own siderophores.[21]

It appears that the ΔLLO mutant form can be easily controlled in the phagolysosome,[34] because LLO is acting as the proposed master regulator of virulence of *Listeria* and thus induces innate immune responses in infected cells.[35]

The finding of an up-regulation of Dmt1 on mRNA level in the course of infection with the wild-type as well as with the mutant strain was not expected from a previous study.[8b] Due to a lack of a good anti-Dmt1 antibody for immunofluorescence staining, we performed functional iron uptake experiments to study the relevance of the increase in Dmt1 gene expression upon infection. As these experiments did not show any change of NTBI uptake, we hypothesize that the up-regulation of increased Dmt1 mRNA does not translate into enhanced Dmt1 expression on the cell surface, but may be rather enriched in phagosomal membranes.[36] Armitage *et al.*[37] recently showed IL-6 dependent autocrine inhibition of macrophage iron efflux by small amounts of hepcidin produced by macrophages. In a line with this finding we observed an increased hepcidin mRNA expression in phagocytes infected with *Listeria* wt, most likely in terms of this autocrine regulation of Fpn1.[38]

Our results described herein contribute to a better understanding of adaptive changes of iron homeostasis in response to infections with intracellular bacteria and may be of relevance for tailoring new therapeutic principles to combat such infections.

Figures

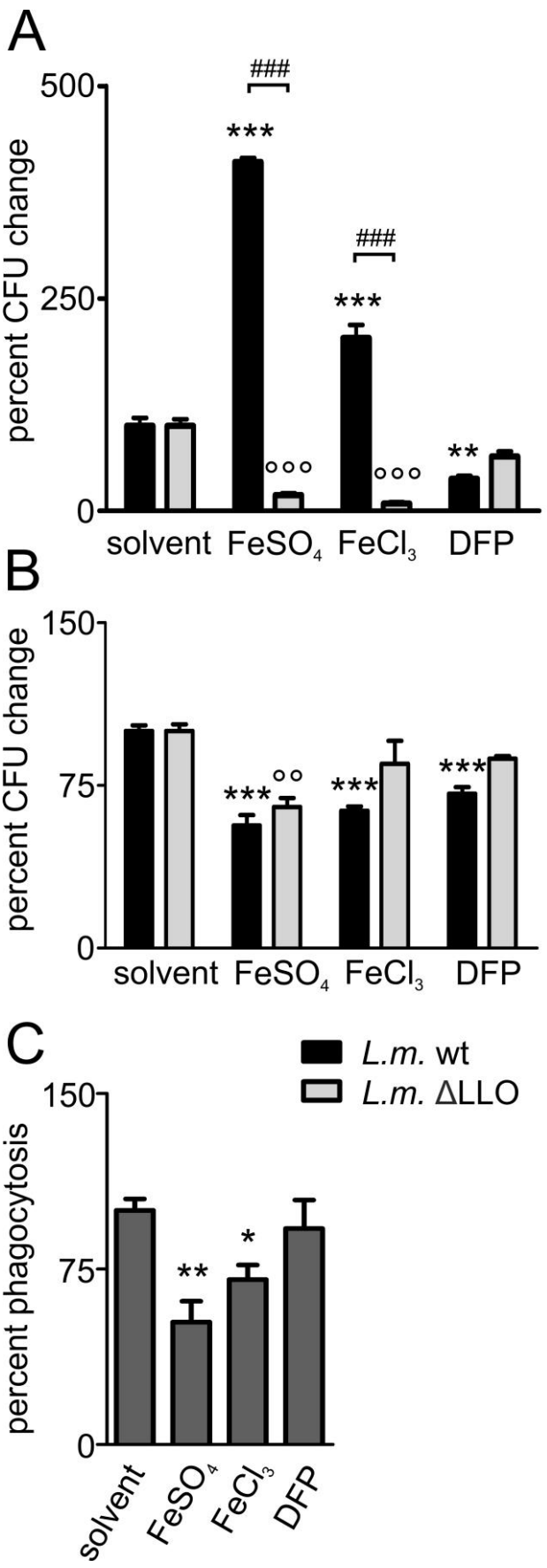

Fig. 1

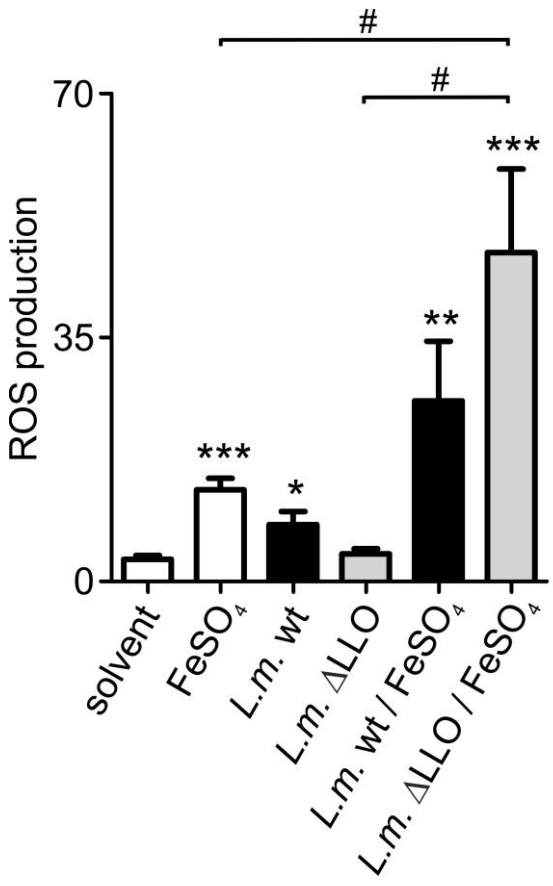

Fig. 2

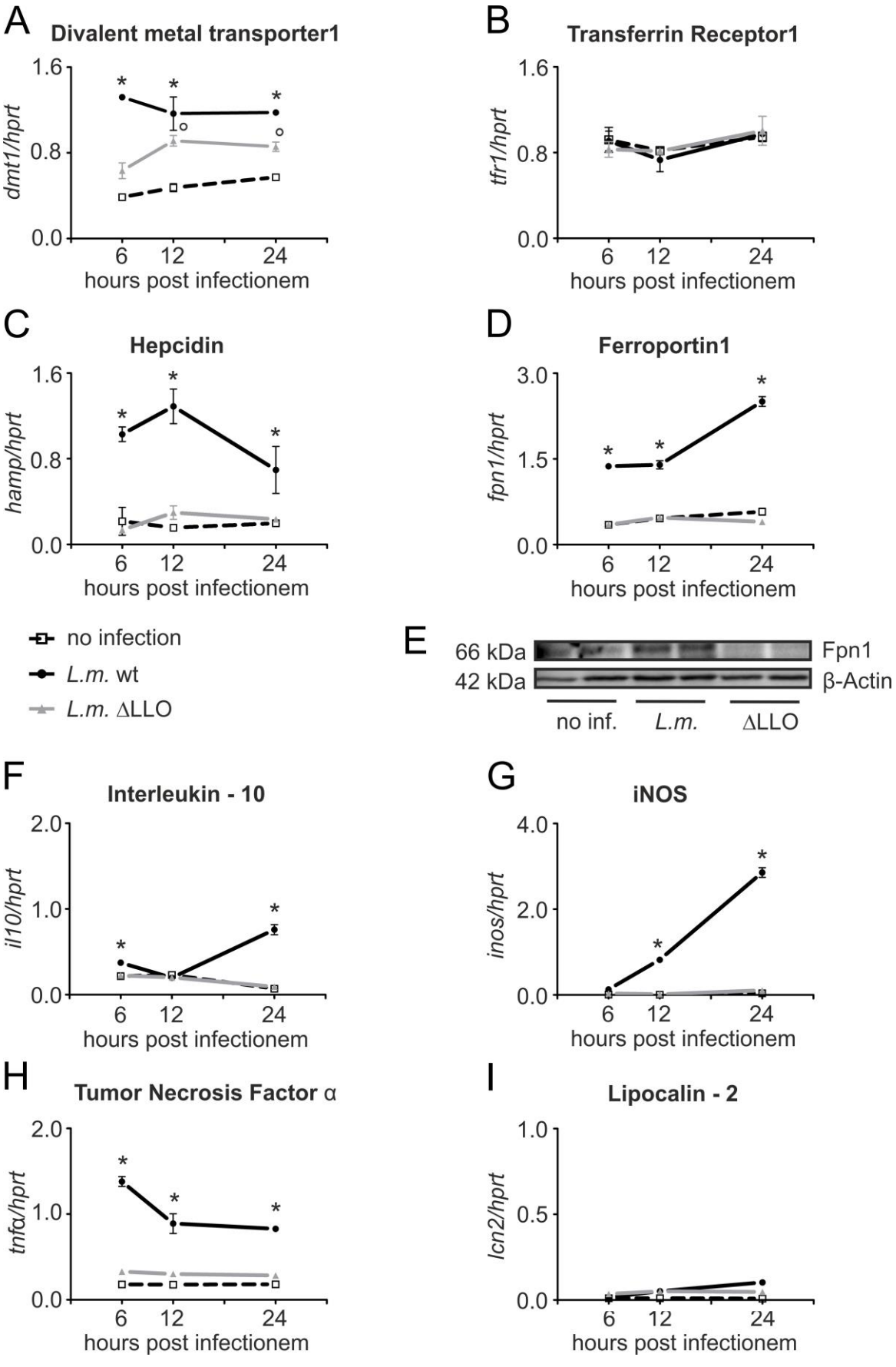

Fig. 3

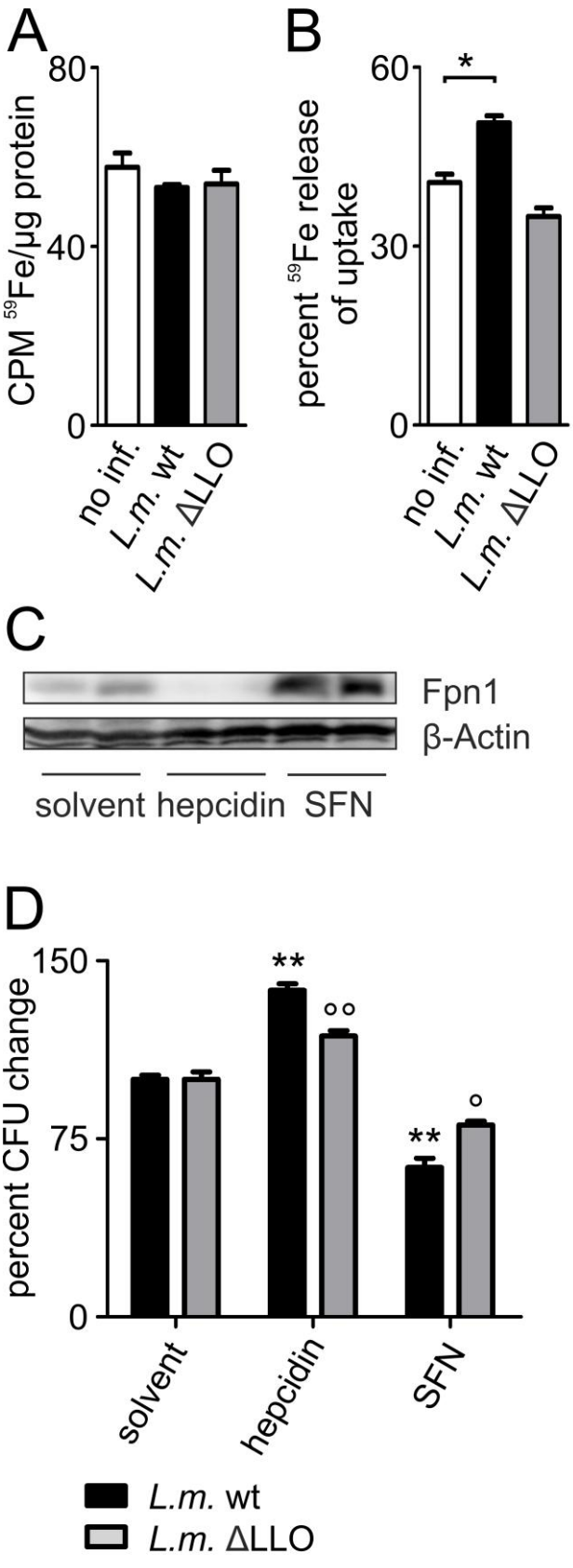

Fig. 4

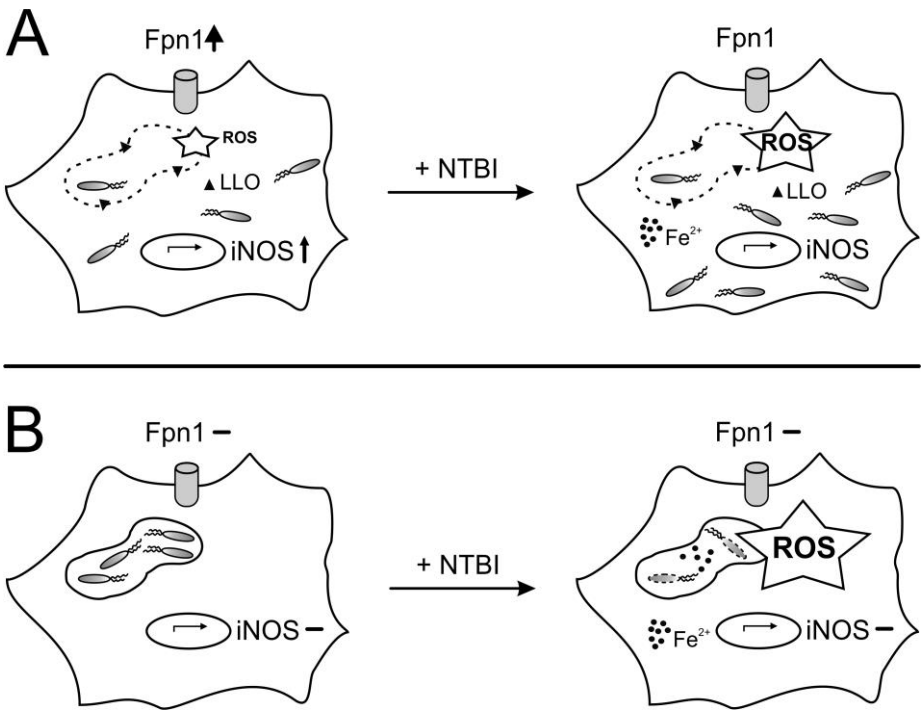

Fig. 5

Figure legends

Figure 1: Influence of exogenous iron sources and the iron chelator deferiprone (DFP) on intramacrophage survival of *Listeria* in macrophages. RAW264.7 murine macrophages were supplemented with ferrous sulphate, ferrous chloride and DFP for 30 min and subsequently infected with a MOI of 5 with *L. monocytogenes* wt and *L. monocytogenes* ΔLLO (A). 12 hours post infectionem macrophages were lysed and colony forming units (CFU) were enumerated. To determine phagocytic capacity of macrophages as a function of iron load, cells were infected with *Listeria* wt and *Listeria* ΔLLO for 1 h, lysed and CFU were enumerated (B). To further investigate phagocytic activity of macrophages, fluorescent zymosan beads were used and cells were analyzed in flow cytometry (C). Data are shown as mean ± SEM of three independent experiments performed in triplicates. Superscripts indicate statistical significance as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with solvent-treated *L.m.* wt infected cells (A,B) and compared to solvent-treated zymosan beads loaded cells (C), °°P < 0.01, °°°P < 0.001 compared with solvent-treated L.m. ΔLLO infected cells (A,B), $^{\#\#\#}P < 0.001$ compared *L.m.* wt infected cells versus *L.m.* ΔLLO infected cells (A).

Figure 2: ROS production following *Listeria* infection. RAW264.7 cells were treated as indicated and subsequently infected with *L.m.* wt and *L.m.* ΔLLO for 12 hours. Afterwards cells were stained with CellRox® to detected intracellular ROS as well as with a dead dye and analyzed with flow cytometry. Data are shown as mean ± SEM of two independent experiments performed in duplicates. Superscripts indicate statistical significance as follows: *P < 0.05, **P < 0.001, $***P < 0.001$ compared with solvent-treated non infected cells and $^{\#}P < 0.05$.

Figure 3: Time dependent modulation of iron homeostasis and immune modulatory genes following *Listeria* infection. RAW264.7 cells were infected with *L.m.* wt and *L.m.* ΔLLO with a MOI of 5 for 6, 12 and 24 hours. Dmt1 (A), TfR1 (B), hamp (C), Fpn1 (D), Il10 (F), Inos (G), Tnfα (H) and Lcn2 (I) mRNA levels were determined by quantitative light cycler PCR. Values were corrected for the amount of the housekeeping gene hprt. Protein levels of Fpn1 were analyzed by Western blotting using specific antibodies to Fpn1 (E). One of two representative blots are shown. Data of the time course are expressed as mean ± SEM of three independent experiments performed in triplicates. Superscripts indicate statistical significance compared with the control of the corresponding time point as follows: *P < 0.05 *L.m.* wt, °P < 0.05 *L.m.* ΔLLO.

Figure 4: Influence of ferroportin1 modulation on *Listeria* infection. RAW264.7 cells were infected with *Listeria* with a MOI of 5 for 1 h. The uptake of non-transferrin-bound iron (NTBI) was determined over a period of 2 h by quantification of ^{59}Fe in a γ-counter as described in *Materials and methods* (A). Cellular iron release experiments were conducted under the same infection conditions as described in *Materials and methods* (B). Data are expressed as mean ± SEM of three independent experiments performed in quadruplicates. Superscripts indicate statistical significance as follows: *P < 0.05. Fpn1 protein levels after stimulation of RAW267.4 cells were determined by Western blot analysis (C). RAW267.4 cells were infected with *L.m.* wt and *L.m.* ΔLLO for 1 h and afterwards stimulated with exogenous hepcidin and SFN for another 11 h (D). Data are expressed as mean ± SEM of five independent experiments performed in quadruplicates. Superscripts indicate statistical significance as follows: **P < 0.01 compared with solvent-treated *L.m.* wt infected cells, °P < 0.05, °°P < 0.01 compared with solvent-treated *L.m.* ΔLLO infected cells.

Figure 5: Changes of iron homeostasis and immune response pathways depending on the localization of the Listeria. (A) Macrophages infected with wt *Listeria*, which express the pathogenic factor listeriolysin O (LLO) and are thus enabled to penetrate from the phagolysosome to the cytoplasm, increase ferroportin1 (Fpn1) expression resulting in reduction of cytoplasmic iron levels and a limited availability of this growth factor for bacteria. This may be a consequence of increased formation of inducible nitric oxide synthase (iNOS) which can enhance Fpn1 expression, and the subsequent reduction of cytoplasmic iron levels further enhanced iNOS expression and other anti-microbial immune effector pathways. Upon administration of non-transferrin bound iron (NTBI) wt *Listeria* residing in the cytoplasm are able to grow and replicate better (A, right panel). (B) Macrophages infected with the ΔLLO mutant form of *Listeria,* which is restricted to the phagolysosome, do not upregulate ferroportin1 nor increase iNOS expression. Supplementation of NTBI to these cells results in enhanced killing of bacteria which is linked to increased, likewise iron catalyzed, formation of reactive oxygen species (ROS) in the phagolysosome.

References

1. (a) K. Pantopoulos, S. K. Porwal, A. Tartakoff, L. Devireddy, Mechanisms of mammalian iron homeostasis. *Biochemistry* 2012, *51*. 5705-24, DOI: 10.1021/bi300752r; (b) M. W. Hentze, M. U. Muckenthaler, B. Galy, C. Camaschella, Two to tango: regulation of Mammalian iron metabolism. *Cell* 2010, *142*. 24-38, DOI: 10.1016/j.cell.2010.06.028.
2. C. H. Park, E. V. Valore, A. J. Waring, T. Ganz, Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *The Journal of biological chemistry* 2001, *276*. 7806-10, DOI: 10.1074/jbc.M008922200.
3. M. Nairz, A. Schroll, T. Sonnweber, G. Weiss, The struggle for iron - a metal at the host-pathogen interface. *Cellular microbiology* 2010, *12*. 1691-1702, DOI: DOI 10.1111/j.1462-5822.2010.01529.x.
4. J. E. Cassat, E. P. Skaar, Iron in Infection and Immunity. *Cell Host Microbe* 2013, *13*. 510-520, DOI: DOI 10.1016/j.chom.2013.04.010.
5. H. Drakesmith, A. M. Prentice, Hepcidin and the iron-infection axis. *Science* 2012, *338*. 768-72, DOI: 10.1126/science.1224577

338/6108/768 [pii].
6. M. Nairz, D. Haschka, E. Demetz, G. Weiss, Iron at the interface of immunity and infection. *Front Pharmacol* 2014, *5*. 152, DOI: 10.3389/fphar.2014.00152.
7. H. L. Collins, S. H. Kaufmann, U. E. Schaible, Iron chelation via deferoxamine exacerbates experimental salmonellosis via inhibition of the nicotinamide adenine dinucleotide phosphate oxidase-dependent respiratory burst. *Journal of immunology* 2002, *168*. 3458-63.
8. (a) M. Nairz, U. Schleicher, A. Schroll, T. Sonnweber, I. Theurl, S. Ludwiczek, H. Talasz, G. Brandacher, P. L. Moser, M. U. Muckenthaler, F. C. Fang, C. Bogdan, G. Weiss, Nitric oxide-mediated regulation of ferroportin-1 controls macrophage iron homeostasis and immune function in Salmonella infection. *The Journal of experimental medicine* 2013, *210*. 855-73, DOI: 10.1084/jem.20121946; (b) M. Nairz, I. Theurl, S. Ludwiczek, M. Theurl, S. M. Mair, G. Fritsche, G. Weiss, The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular Salmonella typhimurium. *Cellular microbiology* 2007, *9*. 2126-40, DOI: 10.1111/j.1462-5822.2007.00942.x; (c) S. Chlosta, D. S. Fishman, L. Harrington, E. E. Johnson, M. D. Knutson, M. Wessling-Resnick, B. J. Cherayil, The iron efflux protein ferroportin regulates the intracellular growth of Salmonella enterica. *Infection and immunity* 2006, *74*. 3065-7, DOI: 10.1128/IAI.74.5.3065-3067.2006.
9. (a) N. K. Das, S. Biswas, S. Solanki, C. K. Mukhopadhyay, Leishmania donovani depletes labile iron pool to exploit iron uptake capacity of macrophage for its intracellular growth. *Cellular microbiology* 2009, *11*. 83-94, DOI: 10.1111/j.1462-5822.2008.01241.x; (b) R. Ben-Othman, A. R. Flannery, D. C. Miguel, D. M. Ward, J. Kaplan, N. W. Andrews, Leishmania-mediated inhibition of iron export promotes parasite replication in macrophages. *PLoS pathogens* 2014, *10*. e1003901, DOI: 10.1371/journal.ppat.1003901; (c) P. N. Paradkar, I. De Domenico, N. Durchfort, I. Zohn, J. Kaplan, D. M. Ward, Iron depletion limits intracellular bacterial growth in macrophages. *Blood* 2008, *112*. 866-74, DOI: 10.1182/blood-2007-12-126854; (d) A. K. Singh, C. Mukhopadhyay, S. Biswas, V. K. Singh, C. K. Mukhopadhyay, Intracellular pathogen Leishmania donovani activates hypoxia inducible factor-1 by dual mechanism for survival advantage within macrophage. *PloS one* 2012, *7*. e38489, DOI: 10.1371/journal.pone.0038489; (e) S. M. Mair, M. Nairz, R. Bellmann-Weiler, T. Muehlbacher, A. Schroll, I. Theurl, P. L. Moser, H. Talasz, F. C. Fang, G. Weiss, Nifedipine affects the course of Salmonella enterica serovar Typhimurium infection by modulating macrophage iron homeostasis. *The Journal of infectious diseases* 2011, *204*. 685-94, DOI: 10.1093/infdis/jir395; (f) R. Bellmann-Weiler, V. Martinz, K. Kurz, S. Engl, C. Feistritzer, D. Fuchs, J. Rupp, M. Paldanius, G. Weiss, Divergent modulation of Chlamydia pneumoniae infection cycle in human monocytic and endothelial cells by iron, tryptophan availability and interferon gamma. *Immunobiology* 2010, *215*. 842-8, DOI: 10.1016/j.imbio.2010.05.021; (g) R. Bellmann-Weiler, A. Schroll, S. Engl, M. Nairz, H. Talasz, M. Seifert, G. Weiss, Neutrophil gelatinase-associated lipocalin and interleukin-10 regulate

intramacrophage Chlamydia pneumoniae replication by modulating intracellular iron homeostasis. *Immunobiology* 2013, *218*. 969-78, DOI: 10.1016/j.imbio.2012.11.004.
10. E. Nemeth, M. S. Tuttle, J. Powelson, M. B. Vaughn, A. Donovan, D. M. Ward, T. Ganz, J. Kaplan, Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science* 2004, *306*. 2090-3, DOI: 10.1126/science.1104742.
11. V. Ramaswamy, V. M. Cresence, J. S. Rejitha, M. U. Lekshmi, K. S. Dharsana, S. P. Prasad, H. M. Vijila, Listeria--review of epidemiology and pathogenesis. *Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi* 2007, *40*. 4-13.
12. C. Alberti-Segui, K. R. Goeden, D. E. Higgins, Differential function of Listeria monocytogenes listeriolysin O and phospholipases C in vacuolar dissolution following cell-to-cell spread. *Cellular microbiology* 2007, *9*. 179-95, DOI: 10.1111/j.1462-5822.2006.00780.x.
13. M. M. Gedde, D. E. Higgins, L. G. Tilney, D. A. Portnoy, Role of listeriolysin O in cell-to-cell spread of Listeria monocytogenes. *Infection and immunity* 2000, *68*. 999-1003.
14. M. A. Hamon, D. Ribet, F. Stavru, P. Cossart, Listeriolysin O: the Swiss army knife of Listeria. *Trends in microbiology* 2012, *20*. 360-8, DOI: 10.1016/j.tim.2012.04.006.
15. H. P. McLaughlin, C. Hill, C. G. Gahan, The impact of iron on Listeria monocytogenes; inside and outside the host. *Current opinion in biotechnology* 2011, *22*. 194-9, DOI: 10.1016/j.copbio.2010.10.005.
16. K. Y. Leung, B. B. Finlay, Intracellular replication is essential for the virulence of Salmonella typhimurium. *Proceedings of the National Academy of Sciences of the United States of America* 1991, *88*. 11470-4.
17. S. Ludwiczek, E. Aigner, I. Theurl, G. Weiss, Cytokine-mediated regulation of iron transport in human monocytic cells. *Blood* 2003, *101*. 4148-54, DOI: 10.1182/blood-2002-08-2459.
18. M. Nairz, G. Fritsche, P. Brunner, H. Talasz, K. Hantke, G. Weiss, Interferon-gamma limits the availability of iron for intramacrophage Salmonella typhimurium. *European journal of immunology* 2008, *38*. 1923-36, DOI: 10.1002/eji.200738056.
19. I. Theurl, V. Mattle, M. Seifert, M. Mariani, C. Marth, G. Weiss, Dysregulated monocyte iron homeostasis and erythropoietin formation in patients with anemia of chronic disease. *Blood* 2006, *107*. 4142-8, DOI: 10.1182/blood-2005-08-3364.
20. B. S. van Asbeck, H. A. Verbrugh, B. A. van Oost, J. J. Marx, H. W. Imhof, J. Verhoef, Listeria monocytogenes meningitis and decreased phagocytosis associated with iron overload. *British medical journal* 1982, *284*. 542-4.
21. N. Simon, V. Coulanges, P. Andre, D. J. Vidon, Utilization of exogenous siderophores and natural catechols by Listeria monocytogenes. *Applied and environmental microbiology* 1995, *61*. 1643-5.
22. S. Marro, D. Chiabrando, E. Messana, J. Stolte, E. Turco, E. Tolosano, M. U. Muckenthaler, Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter. *Haematologica* 2010, *95*. 1261-8, DOI: 10.3324/haematol.2009.020123.
23. P. Andre, S. Oberle, V. Specklin, Y. Lombard, D. J. Vidon, Low-level iron-dependent mutants of Listeria monocytogenes and their virulence in macrophages. *Canadian journal of microbiology* 2003, *49*. 78-84, DOI: 10.1139/w03-015.
24. C. E. Alford, T. E. King, Jr., P. A. Campbell, Role of transferrin, transferrin receptors, and iron in macrophage listericidal activity. *The Journal of experimental medicine* 1991, *174*. 459-66.
25. P. E. Klebba, A. Charbit, Q. Xiao, X. Jiang, S. M. Newton, Mechanisms of iron and haem transport by Listeria monocytogenes. *Molecular membrane biology* 2012, *29*. 69-86, DOI: 10.3109/09687688.2012.694485.
26. R. B. Rea, C. G. Gahan, C. Hill, Disruption of putative regulatory loci in Listeria monocytogenes demonstrates a significant role for Fur and PerR in virulence. *Infection and immunity* 2004, *72*. 717-27.
27. A. Camilli, C. R. Paynton, D. A. Portnoy, Intracellular methicillin selection of Listeria monocytogenes mutants unable to replicate in a macrophage cell line. *Proceedings of the National Academy of Sciences of the United States of America* 1989, *86*. 5522-6.

28. F. C. Fang, Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* 2004, *2*. 820-32, DOI: 10.1038/nrmicro1004.
29. (a) V. E. Diaz-Ochoa, S. Jellbauer, S. Klaus, M. Raffatellu, Transition metal ions at the crossroads of mucosal immunity and microbial pathogenesis. *Front Cell Infect Microbiol* 2014, *4*. 2, DOI: 10.3389/fcimb.2014.00002; (b) M. I. Hood, E. P. Skaar, Nutritional immunity: transition metals at the pathogen-host interface. *Nat Rev Microbiol* 2012, *10*. 525-37, DOI: 10.1038/nrmicro2836.
30. G. Y. Lam, R. Fattouh, A. M. Muise, S. Grinstein, D. E. Higgins, J. H. Brumell, Listeriolysin O suppresses phospholipase C-mediated activation of the microbicidal NADPH oxidase to promote Listeria monocytogenes infection. *Cell Host Microbe* 2011, *10*. 627-34, DOI: 10.1016/j.chom.2011.11.005.
31. N. Harada, M. Kanayama, A. Maruyama, A. Yoshida, K. Tazumi, T. Hosoya, J. Mimura, T. Toki, J. M. Maher, M. Yamamoto, K. Itoh, Nrf2 regulates ferroportin 1-mediated iron efflux and counteracts lipopolysaccharide-induced ferroportin 1 mRNA suppression in macrophages. *Archives of biochemistry and biophysics* 2011, *508*. 101-9, DOI: 10.1016/j.abb.2011.02.001.
32. (a) H. Oexle, A. Kaser, J. Most, R. Bellmann-Weiler, E. R. Werner, G. Werner-Felmayer, G. Weiss, Pathways for the regulation of interferon-gamma-inducible genes by iron in human monocytic cells. *J Leukoc Biol* 2003, *74*. 287-94; (b) G. Weiss, G. Werner-Felmayer, E. R. Werner, K. Grunewald, H. Wachter, M. W. Hentze, Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med* 1994, *180*. 969-76; (c) A. Mencacci, E. Cenci, J. R. Boelaert, P. Bucci, P. Mosci, C. Fe d'Ostiani, F. Bistoni, L. Romani, Iron overload alters innate and T helper cell responses to Candida albicans in mice. *J Infect Dis* 1997, *175*. 1467-76.
33. M. A. Vazquez, S. C. Sicher, W. J. Wright, M. L. Proctor, S. R. Schmalzried, K. R. Stallworth, J. C. Crowley, C. Y. Lu, Differential regulation of TNF-alpha production by listeriolysin-producing versus nonproducing strains of Listeria monocytogenes. *Journal of leukocyte biology* 1995, *58*. 556-62.
34. P. Cossart, M. F. Vicente, J. Mengaud, F. Baquero, J. C. Perez-Diaz, P. Berche, Listeriolysin O is essential for virulence of Listeria monocytogenes: direct evidence obtained by gene complementation. *Infection and immunity* 1989, *57*. 3629-36.
35. F. Stavru, C. Archambaud, P. Cossart, Cell biology and immunology of Listeria monocytogenes infections: novel insights. *Immunological reviews* 2011, *240*. 160-84, DOI: 10.1111/j.1600-065X.2010.00993.x.
36. N. Jabado, F. Canonne-Hergaux, S. Gruenheid, V. Picard, P. Gros, Iron transporter Nramp2/DMT-1 is associated with the membrane of phagosomes in macrophages and Sertoli cells. *Blood* 2002, *100*. 2617-22, DOI: 10.1182/blood-2002-04-1182.
37. A. E. Armitage, L. A. Eddowes, U. Gileadi, S. Cole, N. Spottiswoode, T. A. Selvakumar, L. P. Ho, A. R. Townsend, H. Drakesmith, Hepcidin regulation by innate immune and infectious stimuli. *Blood* 2011, *118*. 4129-39, DOI: 10.1182/blood-2011-04-351957.
38. I. Theurl, M. Theurl, M. Seifert, S. Mair, M. Nairz, H. Rumpold, H. Zoller, R. Bellmann-Weiler, H. Niederegger, H. Talasz, G. Weiss, Autocrine formation of hepcidin induces iron retention in human monocytes. *Blood* 2008, *111*. 2392-9, DOI: blood-2007-05-090019 [pii]

10.1182/blood-2007-05-090019.